

(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. **AU 2007342275 B2**

(54) Title
**Polypeptides and biosynthetic pathways for the production of stereoisomers of mon-
atin and their precursors**

(51) International Patent Classification(s)
C12P 17/10 (2006.01)

(21) Application No: **2007342275** (22) Date of Filing: **2007.10.19**

(87) WIPO No: **WO08/085575**

(30) Priority Data

(31) Number	(32) Date	(33) Country
11/584,016	2006.10.20	US
11/714,279	2007.03.06	US

(43) Publication Date: **2008.07.17**

(44) Accepted Journal Date: **2013.06.20**

(71) Applicant(s)
Cargill, Incorporated

(72) Inventor(s)
**Solheid, Christopher;McFarlan, Sara C.;Brazeau, Brian J.;Gort, Steven J.;Kollmann,
Sherry R.;Sanchez-Riera, Fernando A.;Laplaza, Jose M.;De Souza, Mervyn L.;Hicks,
Paula M.**

(74) Agent / Attorney
Pizzeys Patent and Trade Mark Attorneys, GPO Box 1374, BRISBANE, QLD, 4001

(56) Related Art
US 2005/0221455 A1
WO 2007/133183 A2
US 2005/0282260 A1

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
17 July 2008 (17.07.2008)

PCT

(10) International Publication Number
WO 2008/085575 A3

(51) International Patent Classification:
C12P 17/10 (2006.01)

(21) International Application Number:
PCT/US2007/081932

(22) International Filing Date: 19 October 2007 (19.10.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
11/584,016 20 October 2006 (20.10.2006) US
11/714,279 6 March 2007 (06.03.2007) US

(71) Applicant (for all designated States except US):
CARGILL, INCORPORATED [US/US]; P.O. Box
5624, Minneapolis, Minnesota 55440-5624 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BRAZEAU, Brian, J.** [US/US]; 1873 Munster Avenue, St. Paul, Minnesota 55116 (US). **DE SOUZA, Mervyn, L.** [IN/US]; 10935 38th Avenue North, Plymouth, Minnesota 55441 (US). **GORT, Steven, J.** [US/US]; 3207 Quarles Rd., Brooklyn Park, Minnesota 55429 (US). **HICKS, Paula, M.** [US/US]; 20161 Stonegate Drive, Bend, Oregon 97702 (US). **KOLLMANN, Sherry, R.** [US/US]; 12031 99th Avenue North, Maple Grove, Minnesota 55369 (US).

LAPLAZA, Jose, M. [US/US]; 11283 50th Place North, Plymouth, Minnesota 55442 (US). **MCFARLAN, Sara, C.** [US/US]; 2151 Bayard Ave., St. Paul, Minnesota 55116 (US). **SANCHEZ-RIERA, Fernando, A.** [US/US]; 14028 Vale Court, Eden Prairie, Minnesota 55346 (US). **SOLHEID, Christopher** [US/US]; 4218 Washburn Avenue N, Minneapolis, Minnesota 55412 (US).

(74) Agents: **PARSONS, M., Angela** et al.; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

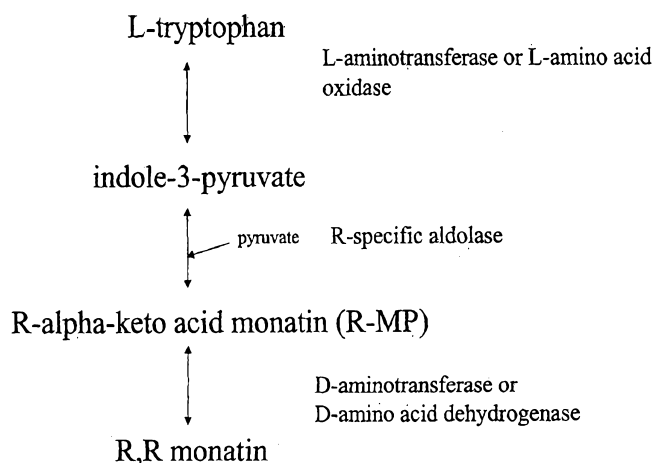
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,

[Continued on next page]

(54) Title: POLYPEPTIDES AND BIOSYNTHETIC PATHWAYS FOR THE PRODUCTION OF STEREOISOMERS OF MONATIN AND THEIR PRECURSORS

FIG. 1



(57) Abstract: Monatin and certain stereoisomers of monatin, such as R,R monatin and S,R monatin, as well as salts thereof, are produced using polypeptides and biosynthetic pathways. These polypeptides and biosynthetic pathways are also useful in the production of R-2-hydroxy-2-(indoly-3-ylmethyl)-4-keto glutaric acid, an intermediate that is formed in certain monatin synthesis pathways, including some biosynthetic pathways.

WO 2008/085575 A3



ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL,
PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

- *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:

19 March 2009

POLYPEPTIDES AND BIOSYNTHETIC PATHWAYS FOR THE PRODUCTION OF STEREOISOMERS OF MONATIN AND THEIR PRECURSORS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application is a continuation-in-part of U.S. Application No. 11/584,016, filed October 20, 2006, which is a continuation-in-part of U.S. Application No. 11/411,229, filed April 26, 2006, which claims the benefit of U.S. Provisional Application No. 60/674,932, filed April 26, 2005.

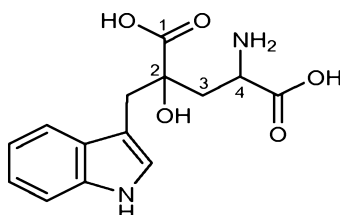
BACKGROUND OF THE INVENTION

Field of the Invention

[0002] This disclosure provides polypeptides and biosynthetic pathways that are useful in the production of D-tryptophan, indole-3-pyruvate, R-2-hydroxy-2-(indol-3-ylmethyl)-4-keto glutaric acid (R-MP) and certain stereoisomers of monatin, such as R,R and S,R monatin, and salts thereof.

Background Art

[0003] Monatin is a high-intensity sweetener having the chemical formula:



Monatin includes two chiral centers leading to four potential stereoisomeric configurations. The R,R configuration (the “R,R stereoisomer” or “R,R monatin”); the S,S configuration (the “S,S stereoisomer” or “S,S monatin”); the R,S configuration (the “R,S stereoisomer” or “R,S monatin”); and the S,R configuration (the “S,R stereoisomer” or “S,R monatin”). As used herein, unless stated otherwise, the term “monatin” is used to refer to compositions including all four stereoisomers of monatin, compositions including any combination of monatin stereoisomers, (e.g., a composition including only the R,R and S,S, stereoisomers of monatin), as well as a single isomeric form.

- [0004] For purposes of this disclosure, the monatin carbon backbone will be numbered as illustrated above, with the carbon directly covalently attached to the alcohol group being identified as the 2-position carbon and the carbon directly covalently attached to the amino group being identified as the 4-position carbon. Consequently, references herein to R,R monatin, S,S monatin, R,S monatin, and S,R monatin mean: 2R,4R monatin, 2S,4S monatin, 2R,4S monatin, and 2S,4R monatin, respectively, unless otherwise indicated.
- [0005] It should be noted that in the literature, the monatin carbon backbone has also been numbered using an alternative convention, with the carbon attached to the alcohol group being the 4-position carbon, and the carbon attached to the amino group being the 2-position carbon. Accordingly, for example, references to 2S,4R monatin in this disclosure would be the same as references to 2R,4S monatin in literature using the alternative numbering convention.
- [0006] Furthermore, because of various naming conventions, monatin is known by a number of alternative chemical names, including: 2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid; 4-amino-2-hydroxy-2-(1H-indol-3-ylmethyl)-pentanedioic acid; 4-hydroxy-4-(3-indolylmethyl)glutamic acid; and, 3-(1-amino-1,3-dicarboxy-3-hydroxy-but-4-yl)indole.
- [0007] Certain isomeric forms of monatin can be found in the bark of roots of the *Schlerochiton ilicifolius* plant located in the Transvaal Region of South Africa. U.S. Patent Application Nos. 10/422,366 ("the '366 application"), 10/979,821 ("the '821 Application"), and 11/114,922 ("the '922 application"), which are hereby incorporated by reference, discloses, *inter alia*, polypeptides, pathways, and microorganisms for *in vitro* and *in vivo* production of monatin.

BRIEF SUMMARY OF THE INVENTION

- [0008] This disclosure provides, among other things, polypeptides and biosynthetic pathways that are useful in the production of D-tryptophan, indole-3-pyruvate, R-2-hydroxy 2-(indol-3ylmethyl)-4-keto glutaric acid (also referred to as R-alpha keto acid monatin, R-monatin precursor, R-MP, and the alpha keto form of monatin) and certain stereoisomers of monatin, such as R,R and S,R monatin, and salts thereof. The methods include the use of one or more polypeptides, and in particular, enzymes, such as racemases (e.g., glutamate racemases, aspartate racemases and alanine racemases), broad

specificity D-aminotransferases (also called D-alanine aminotransferases, D-amino acid aminotransferases and D-aspartate aminotransferases), L-aminotransferases (including L-tryptophan-aminotransferases, L-aromatic aminotransferases, L-aspartate aminotransferases, and L-alanine-aminotransferases), aldolases (e.g., R-specific aldolases), D-phenylglycine aminotransferases (also called D-4-hydroxyphenylglycine aminotransferase), D-methionine aminotransferases, glutamate decarboxylases, aspartate decarboxylases and aspartate-4-decarboxylases to produce monatin compositions enriched with the 4-R isomer forms and/or to produce R,R monatin without having to use stoichiometric amounts of D-amino acid substrate as the amino acid donor for MP amination.

[0009] In an effort to be concise, wherever intermediates/products are identified in the specification and claims (e.g. monatin or monatin precursor) as being formed, the term “and/or salts thereof” should be understood to be included where applicable. In other words, for example, the phrase “indole-3-pyruvate is converted to MP” should be understood to read “indole-3-pyruvic acid is converted to MP and/or salts thereof.” A person of ordinary skill, in fact, would appreciate that under reaction conditions shown the salts of the intermediates/products are in fact present or also present.

[0010] According to some embodiments, the method produces a monatin composition wherein the monatin component of the composition includes only the R,R and S,R form of monatin. The term “only,” when used to indicate that only certain isomers are formed, unless otherwise stated means that the pathway would produce only the identified isomers if racemization did not occur. Consequently, the term “only” should not be taken to mean absence of other isomers, but rather a person of ordinary skill would understand that other isomeric forms may be present in a relatively small amount due to racemization which may occur. According to some embodiments, the method produces a monatin composition wherein the monatin component of the composition includes only the R,R form of monatin (thus meaning except to the extent racemization occurs resulting in other isomeric forms).

[0011] As used herein, the phrase “monatin composition” means a composition including one or more isomers of monatin; the term can also mean a composition including only a single isomeric form of monatin and nothing else, depending on the context.

[0012] In some embodiments, in accordance with the present invention, a process for producing a monatin composition is provided, which includes producing indole-3-pyruvate from L-tryptophan, producing 2-hydroxy 2-(indol-3-ylmethyl)-4-ketoglutaric acid (“monatin precursor” or “MP”) from indole-3-pyruvate, and producing monatin from MP. The reaction of L-tryptophan to produce indole-3-pyruvate is facilitated by an enzyme having greater specificity, greater activity, or both for L-tryptophan as a substrate than for R-MP, R,R monatin, or both. According to certain embodiments, the reaction of indole-3-pyruvate is facilitated by an enzyme having R-specific aldolase activity and consequently produces R-MP. According to certain embodiments, a racemase enzyme is provided that can facilitate epimerization of the amino acid that is formed as a byproduct of the L-tryptophan transamination reaction (or that is formed from another amino acid that is a byproduct of the tryptophan reaction) from one isomeric form to another isomeric form.

[0013] In some embodiments according to the invention, a process for producing a monatin composition is provided, which includes producing indole-3-pyruvate from L-tryptophan, producing 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid (“monatin precursor” or “MP”) from indole-3-pyruvate, and producing monatin from MP. The reaction of L-tryptophan to produce indole-3-pyruvate is facilitated by an enzyme having greater specificity, greater activity, or both for L-tryptophan as a substrate than for R-MP, R,R monatin, or both, and the reaction of MP to form monatin is facilitated by an enzyme, which is stereoselective for R-MP.

[0014] It should be noted that, where references are made to a series of reactions such as in the preceding paragraphs, the invention does not require each step to be explicitly performed; it is sufficient that the steps may be implicitly performed. In other words, for example, the process for producing a monatin composition, which includes producing indole-3-pyruvate from L-tryptophan, producing 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid (“monatin precursor” or “MP”) from indole-3-pyruvate, and producing monatin from MP, wherein each reaction is facilitated by an appropriate enzyme, can be performed by combining L-tryptophan with the enzymes and setting conditions so that the enumerated reactions could occur. In such an instance L-tryptophan could react to produce indole-3-pyruvate, the indole-3-pyruvate produced from the L-tryptophan reaction could react to form MP, and the MP produced from the indole-3-pyruvate

reaction could react to form monatin. The process could also be performed, by way of example, by providing a compound that can produce L-tryptophan, under conditions suitable for L-tryptophan production to occur and combining that compound with enzymes capable of facilitating the series of reactions set forth under conditions which would be suitable for those reactions to occur. As yet another example, the process could be performed by providing a microorganism genetically engineered to produce monatin according to the described pathway, and providing appropriate conditions for the fermentation process to occur. For example, a microorganism, which naturally produces large amounts of L-tryptophan (or D-tryptophan) could be genetically engineered to produce or over-produce one or more of the enzymes used to facilitate reactions in the pathway to monatin, and appropriate conditions could be provided so that the microorganism would thereby produce monatin.

[0015] In other embodiments according to the invention, a process for producing monatin is provided, in which an α -keto acid substrate forms an L-amino acid when L-tryptophan is converted to indole-3-pyruvate, indole-3-pyruvate reacts to form MP (which can include both R-MP and S-MP though preferably includes only or predominately R-MP), and the L-amino acid reacts to regenerate (also referred to as "recycle") the α -keto acid substrate when R-MP is converted to R,R monatin. The reaction of R-MP to form R,R monatin is facilitated by a stereoinverting aminotransferase such as D-methionine aminotransferase (EC 2.6.1.41) or an enzyme derived from a D-phenylglycine aminotransferase.

[0016] In other embodiments according to the invention, a process for producing a monatin composition is provided, which includes producing D-tryptophan from L-tryptophan, producing indole-3-pyruvate from D-tryptophan, producing R-MP from indole-3-pyruvate, and producing R,R monatin from R-MP. The production of the D-tryptophan from the L-tryptophan is facilitated by a tryptophan racemase and functional equivalents thereof. In certain further embodiments, the reactions of D-tryptophan to form indole-3-pyruvate and of MP to form monatin are facilitated by the same enzyme. In yet other further embodiments, the reaction of indole-3-pyruvate is facilitated by an enzyme having R-specific aldolase activity and consequently R-MP is formed, and the reactions of D-tryptophan to form indole-3-pyruvate and of R-MP to form R,R monatin are facilitated by the same enzyme.

[0017] In other embodiments according to the invention, a method for producing R,R-monatin, or a salt thereof, comprising, or consisting essentially of, (a) producing D-tryptophan from L-tryptophan utilizing a tryptophan racemase (the racemase should have limited or no activity on monatin), (b) producing indole-3-pyruvate from D-tryptophan, (c) producing R-monatin precursor from indole-3-pyruvate, and (d) producing R,R-monatin from R-monatin precursor is disclosed herein.

[0018] While multiple embodiments are disclosed, still other embodiments of the present invention may become apparent to those skilled in the art from the specification. As should be realized from the description herein, the invention is capable of modifications in various aspects, all without departing from the spirit and scope of the present invention. Accordingly, the drawings and detailed description are to be regarded as illustrative in nature and not restrictive.

BRIEF DESCRIPTION OF THE FIGURES

[0019] **FIG. 1** is a flow chart that shows an example of an enzymatic process for producing R,R monatin from L-tryptophan in accordance with the invention. In this example, the process includes using an L-aminotransferase (examples of which include an L-tryptophan aminotransferase, an L-aromatic aminotransferase, an L-aspartate aminotransferase, and an L-alanine aminotransferase) in the reaction of L-tryptophan that has greater specificity and/or selectivity for L-tryptophan as a substrate than for R-MP and/or the process includes using an L-amino acid oxidase with limited activity and/or specificity for R,R monatin as a substrate. In the specific example diagrammed in FIG. 1, an L-aminotransferase or L-amino acid oxidase converts L-tryptophan to indole-3-pyruvate, indole-3-pyruvate is reacted with an R-specific aldolase and pyruvate to produce R-alpha-keto acid monatin (R-MP), and R-MP is converted to R,R monatin by a D-aminotransferase or a D-amino acid dehydrogenase. As shown on FIG. 1, the reactions are reversible, but for the purposes of the invention, it is not required that the reactions proceed in the reverse direction.

[0020] **FIG. 2** is a flow chart that shows an example of another process for producing R,R monatin in accordance with the invention. In this example, the process includes using an enzyme to convert R-MP to monatin which is stereoselective for R-MP. In the specific example diagrammed in FIG. 2, tryptophan is shown to be converted to indole-3-

pyruvate in a reversible reaction. The indole-3-pyruvate can be reacted with a non-stereospecific aldolase to reversibly form alpha-keto acid monatin (both R- and S-MP). The R-MP is reversibly converted to R,R monatin by a stereoselective D-aminotransferase or a stereoselective D-amino acid dehydrogenase. Any S-MP that is formed by the non-stereospecific aldolase can be converted back into indole-3-pyruvate if a stereoselective D-aminotransferase or D-amino acid dehydrogenase is utilized. For the purposes of the invention, it is not required that the reactions shown as being reversible proceed in the reverse direction.

[0021] **FIG. 3** is a flow chart that shows an example of yet another process for producing R,R monatin from L-tryptophan in accordance with the invention. In this example, the process includes converting L-tryptophan to D-tryptophan using a tryptophan racemase and using a D-amino acid product in the reaction coupled to the reaction forming indole-3-pyruvate as a substrate in the reaction coupled to the reaction forming R,R monatin. In the specific example diagrammed in FIG. 3, L-tryptophan is converted to D-tryptophan by a tryptophan racemase in a reversible reaction. The D-tryptophan is reacted with alpha-ketoglutarate (α -KG) and a broad specificity D-aminotransferase to produce indole-3-pyruvate and D-glutamate. Indole-3-pyruvate is reacted with pyruvate and an R-specific aldolase and converted to R-alpha-keto acid monatin (R-MP), and R-MP is reacted with a broad specificity D-aminotransferase and D-glutamate to form R,R monatin and alpha-ketoglutarate (α -KG). As shown on FIG. 3, each of the reactions are reversible, but for the purposes of the invention, it is not required that the reactions proceed in the reverse direction.

[0022] **FIG. 4** is a flow chart that shows an example of yet another process for producing R,R monatin from L-tryptophan in accordance with the invention. In this example, the process includes converting the L-amino acid formed in the reaction coupled with the L-tryptophan reaction to a D-amino acid; this D-amino acid acts as an amino donor for the reaction in which R-MP is converted to R,R monatin. In the specific example diagrammed in FIG. 4, L-tryptophan is reacted with an L-aminotransferase and alpha-ketoglutarate to produce indole-3-pyruvate and L-glutamate. Indole-3-pyruvate is reacted with pyruvate and an R-specific aldolase and converted to R-alpha-keto acid monatin (R-MP), and R-MP is reacted with a broad specificity D-aminotransferase and D-glutamate to form R,R monatin and alpha-ketoglutarate. As shown on FIG. 4, the reactions are

reversible, but for the purposes of the invention, it is not required that the reactions proceed in the reverse direction.

[0023] **FIG. 5** is a flow chart that shows an example of yet another process for producing R,R monatin from L-tryptophan in accordance with the invention. In this example, the process includes enzymatically facilitating the conversion of R-MP to R,R monatin using a stereoinverting enzyme so that the L-amino acid formed by the reaction coupled to the L-tryptophan reaction can be used as a substrate for the reaction coupled to the R-MP to R,R monatin reaction. In the specific example diagrammed in FIG. 5, L-tryptophan is reacted with an L-aminotransferase and oxaloacetate, pyruvate or alpha-ketoglutarate (α -KG) to produce indole-3-pyruvate, and L-aspartate (if oxaloacetate is used), L-alanine (if pyruvate is used) or L-glutamate (if α -KG is used). Indole-3-pyruvate is reacted with pyruvate and an R-specific aldolase and converted to R-alpha-keto acid monatin (R-MP), and R-MP is reacted with a stereoinverting aminotransferase and L-aspartate, L-alanine or L-glutamate to form R,R monatin and oxaloacetate (if L-aspartate is used), pyruvate (if L-alanine is used) or alpha-ketoglutarate (α -KG, if L-glutamate is used). As shown on FIG. 5, the reactions are reversible, but for the purposes of the invention, it is not required that the reactions proceed in the reverse direction.

[0024] **FIG. 6** is a flow chart that shows an example of yet another process for producing R,R monatin in accordance with the present invention. In this example, the process includes recycling the L-amino acid produced in the reaction forming indole-3-pyruvate with the D-amino acid used as a reactant with R-MP in the reaction forming R,R monatin through a series of conversion reactions. In the specific example diagrammed in FIG. 6, L-tryptophan is reversibly reacted with an L-aminotransferase and oxaloacetate to produce indole-3-pyruvate and L-aspartate. Indole-3-pyruvate is reacted in a reversible manner with pyruvate and an R-specific aldolase and converted to R-alpha-keto acid monatin (R-MP), and R-MP is reversibly reacted with a D-aminotransferase and D-alanine to form R,R monatin and pyruvate. The L-aspartate is converted to L-alanine and CO₂ using an aspartate 4-decarboxylase. The L-alanine is converted to D-alanine with an alanine racemase. For the purposes of the invention, it is not required that the reactions shown as being reversible proceed in the reverse direction.

[0025] **FIG. 7** is a flow chart that shows an example of yet another process for producing R,R monatin in accordance with the present invention. In this example, the process

includes pushing the L-tryptophan reaction forward (i.e., driving the reaction toward the production of indole-3-pyruvate) by converting the L-amino acid byproduct of that reaction into another product. In this example, the L-amino acid L-aspartate byproduct is converted into L-alanine in an irreversible reaction using a decarboxylase. In the specific example diagrammed in FIG. 7, L-tryptophan is reversibly reacted with an L-aminotransferase and with alpha-ketoglutarate (α -KG) or oxaloacetate to produce indole-3-pyruvate and L-glutamate (if α -KG is used) or L-aspartate (if oxaloacetate is used). Indole-3-pyruvate is reversibly reacted with pyruvate and an R-specific aldolase and converted to R-alpha-keto acid monatin (R-MP). R-MP is reacted in a reversible manner with a D-aminotransferase and a D-amino acid to form R,R monatin and any of oxaloacetate, pyruvate or α -KG. The L-glutamate or L-aspartate that was a product of the L-aminotransferase reaction is converted to either 4-aminobutanoate and CO₂ (if L-glutamate is the substrate) or to β -alanine and CO₂ (if L-aspartate is the substrate) using a glutamic acid or an aspartate decarboxylase. For the purposes of the invention, it is not required that the reactions shown as being reversible proceed in the reverse direction.

[0026] **FIG. 8** is a flow chart that shows an example of yet another process for producing R,R monatin in accordance with the present invention. In this example, the process includes recycling the amino acid byproduct of the L-tryptophan reaction with the amino acid reactant of the R-MP reaction through a series of conversion reactions. In the specific example diagrammed in FIG. 8, L-tryptophan is reacted reversibly with an L-aminotransferase and with alpha-ketoglutarate (α -KG) to produce indole-3-pyruvate and L-glutamate. Indole-3-pyruvate is reversibly reacted with pyruvate and an R-specific aldolase and converted to R-alpha-keto acid monatin (R-MP). R-MP is reacted in a reversible manner with a D-aminotransferase and D-alanine to form R,R monatin and pyruvate. An L-alanine aminotransferase and pyruvate are used to reversibly convert the L-glutamate that was a product of the L-aminotransferase reaction back to α -KG, with L-alanine as a co-product. An alanine racemase reversibly converts the L-alanine to the D-alanine that is useful in the third reaction, (the D-aminotransferase reaction. For the purposes of the invention, it is not required that the reactions shown as being reversible proceed in the reverse direction.

[0027] **FIG. 9(A and B)** show the amino acid sequence alignment of various published *Bacillus* D-amino acid aminotransferases ("DAATs"). Underlined amino acids indicate

the regions of homology. Five PCR primers were designed based on the conserved regions. The PCR primers are as follows: 5'-GAAGACCGTGGTTATCAATTT-3' (SEQ ID NO:65) (forward primer, F1 as indicated in FIG. 9A), 5'-GATGGTATTTACGAAGTAATC-3' (SEQ ID NO:66) (forward primer, F2 as indicated in FIG. 9A), 5'-AGATTTAATATCACAACGTAAC-3' (SEQ ID NO:67) (reverse primer, R1 as indicated in FIG. 9A), 5'-GCCAAGTAAAATTTAAGATTTA-3' (SEQ ID NO:68) (reverse primer, R2 as indicated in FIG. 9A), 5'-ATTTGCTGGGTGCGTATAAAG-3' (SEQ ID NO:69) (reverse primer, R3 as indicated in FIG. 9B). The D-amino acid aminotransferase encoded by the BspHATgene is SEQ ID NO:205, the D-amino acid aminotransferase of *B. halodurans* is SEQ ID NO:206, the D-amino acid aminotransferase encoded by the GsteDATgene is SEQ ID NO:207, the D-amino acid aminotransferase of *B. cereus* 145 is SEQ ID NO:208; the BsubDAT is SEQ ID NO:209, and the D-amino acid aminotransferase of *B. licheniformis* is SEQ ID NO:210.

[0028] **FIG. 10(A and B)** show the amino acid sequence alignment of the two novel DAATs: one from ATCC 4978 (*B. rotans*, Fig. 10A; SEQ ID NO:211) and one from ATCC 7063 (*B. serosididis*, Fig. 10B (SEQ ID NO:212) with the *B. sphaericus* DAAT (SEQ ID NO:205 and SEQ ID NO:213) cloned in Example 18. Non-homologous amino acids are underlined.

[0029] **FIG. 11** shows the tryptophan racemization assay results of the broad specificity amino acid racemase ("BAR") compared with an alanine racemase from *G. stearothermophilus* and a mutant thereof (Y354A). The racemase preparations compared were (#1) BAR-200 µg (#2) BAR crude extract-50 µL; (#3) BAR crude extract-10 µL; (#4) BAR crude extract-5 µL; (#5) Y354A-1000 µg; (6) Y354A-200 µg; (#7) wild-type (WT) alanine racemase-1000 µg; (#8) wild-type (WT) alanine racemase-200 µg; (9) no enzyme.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations and Terms

[0030] The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. As used herein, "including" means "comprising." Wherever the term

"includes" is used, it should be understood that "includes but is not limited to" is meant, whether or not "is limited to" is explicitly stated. In addition, the singular forms "a" or "an" or "the" include plural references unless the context clearly dictates otherwise. For example, reference to "comprising a protein" includes one or a plurality of such proteins, and reference to "comprising the cell" includes reference to one or more cells and equivalents thereof known to those skilled in the art, and so forth. The term "about" encompasses the range of experimental error that occurs in any measurement. Unless otherwise stated, all measurement numbers are presumed to have the word "about" in front of them even if the word "about" is not expressly used.

[0031] Conservative substitution: a substitution of one amino acid for another amino acid in a polypeptide, which substitution has little to no impact on the activity of the polypeptide. The substitution is considered conservative independent of whether the exchanged amino acids appear structurally or functionally similar. For example, ideally, a tryptophan aminotransferase polypeptide including one or more conservative substitutions retains tryptophan aminotransferase activity. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard procedures such as site-directed mutagenesis or PCR or other methods known to those in the art.

[0032] Non-limiting examples of amino acids that may be substituted for an original amino acid in a protein and that may be regarded as conservative substitutions if there is little to no impact on the activity of the polypeptide include: ala substituted with ser or thr; arg substituted with gln, his or lys; asn substituted with glu, gln, lys, his or asp; asp substituted with asn, glu or gln; cys substituted with ser or ala; gln substituted with asn, glu, lys, his, asp or arg; glu substituted with asn, gln lys or asp; gly substituted with pro; his substituted with asn, lys, gln, arg or tyr; ile substituted with leu, met, val or phe; leu substituted with ile, met, val or phe; lys substituted with asn, glu, gln, his or arg; met substituted with ile, leu, val or phe; phe substituted with trp, tyr, met, ile or leu; ser substituted with thr or ala; thr substituted with ser or ala; trp substituted with phe or tyr; tyr substituted with his, phe or trp; and val substituted with met, ile or leu.

[0033] Further information about conservative substitutions can be found in, among other locations, Ben-Bassat *et al.*, *J. Bacteriol.* 169:751-757, (1987); O'Regan *et al.*, *Gene*

77:237-251, (1989); Sahin-Toth *et al.*, *Protein Sci.* 3:240-247, (1994); Hochuli *et al.*, *Bio/Technology* 6:1321-1325, (1988); WO 00/67796 (Curd *et al.*) and in standard textbooks of genetics and molecular biology.

[0034] Derived: For purposes of the specification and claims, a substance is “derived” from an organism or source if any one or more of the following are true: 1) the substance is present in the organism/source; 2) the substance is removed from the native host; or, 3) the substance is removed from the native host and is evolved, for example, by mutagenesis.

[0035] Isolated: The term “isolated” as used herein refers to any substance removed from its native host; the substance need not exhibit any specific degree of purity. For example “isolated nucleic acid” refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5’ end and one on the 3’ end) in the naturally-occurring genome of the organism from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

[0036] The term “isolated” as used herein with reference to nucleic acid also includes any non-naturally-occurring nucleic acid because non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome. For example, non-naturally-occurring nucleic acid such as an engineered nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus

(e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or eukaryote. In addition, a non-naturally-occurring nucleic acid can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence.

[0037] A nucleic acid existing among hundreds to millions of other nucleic acid molecules within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest is not to be considered an isolated nucleic acid.

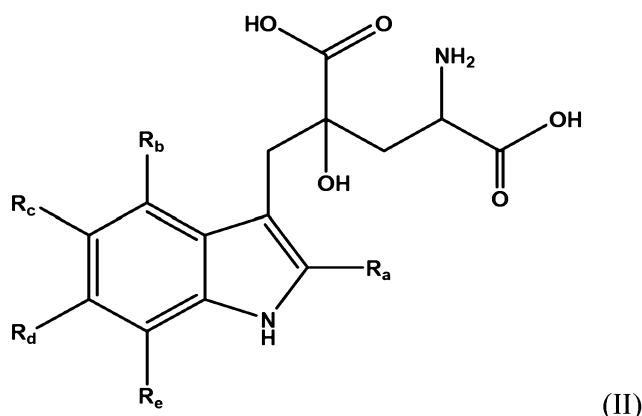
[0038] **Fragment:** A “fragment” as used herein with regard to a protein or polypeptide or nucleic acid is a portion of the protein, polypeptide or nucleic acid, respectively. Fragments can have the same or substantially the same amino acid or nucleic acid sequence as the longer protein, polypeptide or nucleic acid sequence from which the fragment is derived. Fragments which have different three dimensional structures as compared to that of the longer protein, polypeptide or nucleic acid are also included. An example of this, is a “pro-form” molecule, such as a low activity proprotein that can be modified by cleavage to produce a mature enzyme with significantly higher activity. A fragment of a protein or polypeptide can be an enzymatically active portion of a protein or polypeptide.

[0039] **Purified:** The term “purified” as used herein indicates that contaminants have been removed from the sample of interest. The term “purified” does not require absolute purity, but rather is intended as a relative term, unless otherwise indicated by the context. Thus, for example, a purified polypeptide or nucleic acid preparation can be one in which the subject polypeptide or nucleic acid is at a higher concentration than the polypeptide or nucleic acid would be in its natural environment within an organism or at a higher concentration than in the environment from which it was removed.

[0040] **Stereoinverting aminotransferase:** A “stereoinverting aminotransferase” is a polypeptide capable of preferentially or selectively producing a chiral amino acid product (such as monatin) while using an opposite chirality substrate as the amino donor. For example, a stereoinverting aminotransferase may be a D-phenylglycine aminotransferase (also called D-4-hydroxyphenylglycine aminotransferase) that preferentially or selectively uses L-glutamate as a substrate to produce R,R monatin. Non-limiting examples of stereoinverting aminotransferases include D-methionine aminotransferase (EC 2.6.1.41) and enzymes having D-phenylglycine aminotransferase activity or D-4-hydroxyphenylglycine aminotransferase activity.

- [0041] **Complementing Gene:** A “complementing gene” is a gene that, when expressed, nullifies a mutation in an organism. For example, if an organism has a null mutation in one of the genes required for synthesis of tryptophan by the cell, a complementing gene could be one that, when expressed, allows the strain to grow on minimal medium (i.e., without tryptophan).
- [0042] **Stereoselective Enzyme:** A “stereoselective enzyme” is an enzyme that has greater specificity and/or greater activity for one stereoisomer, as compared to the specificity and/or activity for another stereoisomer. For example, a stereoselective enzyme is one that has greater specificity and/or activity for R-MP than for S-MP. In preferred embodiments, a stereoselective enzyme has limited activity for one stereoisomer as compared to another. “Limited” activity means activity that is minimally or not perceptible, for example as determined according to experiments provided herein. Example 6, for example, identifies HEXAspCP9T/R122G as an enzyme with limited activity on S,S monatin. Example 8 identifies the *S. meliloti* TatA as another enzyme with limited activity for S-MP. In Example 18, the *B. halodurans* D-aminotransferase had higher selectivity for R-MP as compared with S-MP, resulting in higher stereopurity of R,R monatin. Also, Example 19 indicates that the hybrid DAT has limited activity on S-MP compared to R-MP.
- [0043] **Homolog:** The term “homolog” as used herein indicates that a protein or a nucleic acid exhibits a relatively high degree of sequence identity to a sequence of another protein or nucleic acid when the two sequences are aligned using standard methods. For example, an R-specific aldolase is a homolog of the aldolase of SEQ ID NO:22 if the R-specific aldolase contains at least about 50% sequence identity to the aldolase of SEQ ID NO:22 when the two sequences are aligned using standard methods.
- [0044] **EC number:** The enzyme classification number as assigned by the International Union of Biochemistry and Molecular Biology.

[0045] **Monatin derivative:** As used herein, the phrase "monatin derivative" has the following structure:



wherein, R_a , R_b , R_c , R_d , and R_e each independently represent any substituent selected from a hydrogen atom, a hydroxyl group, a C_1 - C_3 alkyl group, a C_1 - C_3 alkoxy group, an amino group, or a halogen atom, such as an iodine atom, bromine atom, chlorine atom, or fluorine atom. However, R_a , R_b , R_c , R_d , and R_e cannot simultaneously all be hydrogen. Alternatively, R_b and R_c , and/or R_d and R_e may together form a C_1 - C_4 alkylene group, respectively..

[0046] **Substituted Tryptophan:** As used herein, "substituted tryptophan" means one or more carbon atoms of the indole ring of the tryptophan is independently substituted with one or more of the R_a , R_b , R_c , R_d , and R_e substituent groups defined above. However, R_a , R_b , R_c , R_d , and R_e cannot simultaneously all be hydrogen. In one embodiment, the substituted tryptophan contains the same substituent group(s) on the indole ring as the final monatin derivative.

Biosynthetic Pathways to Produce R,R and Other Stereoisomers of Monatin

[0047] As described, *inter alia*, in WO 03/091396 A2 (see, *e.g.*, Figures 1–3 and 11-13), monatin can be produced from tryptophan through a multi-step pathway involving biological conversions (i.e. facilitating the reaction of a substrate to a product with a polypeptide). A pathway described involves biologically converting tryptophan to indole-3-pyruvate, biologically converting indole-3-pyruvate to 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid ("MP"), and biologically converting MP to monatin. The biosynthesis pathway of the present invention that is used to produce monatin may comprise, or consist essentially of, one or more of the steps, mechanisms and/or pathways

described below. The steps, mechanisms, and/or pathways described below are simply intended to be exemplary.

[0048] One method of producing monatin, or a salt thereof, comprises (a) producing indole-3-pyruvate from L-tryptophan, (b) producing monatin precursor from the indole-3-pyruvate, and (c) producing monatin from the monatin precursor.

[0049] Enzymes useful for converting tryptophan to indole-3-pyruvate include members of the enzyme classifications ("EC") 2.6.1.27, 1.4.1.19, 1.4.99.1, 2.6.1.28, 1.4.3.2, 1.4.3.3, 2.6.1.5, 2.6.1.-, 2.6.1.1, 2.6.1.21 and 3.5.1.-. These classes include polypeptides such as: tryptophan aminotransferase, which converts L-tryptophan and α -KG (i.e., α -ketoglutarate, also called 2-oxoglutarate) to indole-3-pyruvate and L-glutamate; D-tryptophan aminotransferase, which converts D-tryptophan and a 2-oxo acid to indole-3-pyruvate and an amino acid; tryptophan dehydrogenase, which converts L-tryptophan and NAD(P) to indole-3-pyruvate and NH_3 and NAD(P)H; D-amino acid dehydrogenase, which converts D-amino acids and FAD to indole-3-pyruvate and NH_3 and FADH_2 ; tryptophan-phenylpyruvate transaminase, which converts L-tryptophan and phenylpyruvate to indole-3-pyruvate and L-phenylalanine; L-amino acid oxidase, which converts an L-amino acid and H_2O and O_2 to a 2-oxo acid and NH_3 and H_2O_2 ; D-amino acid oxidase, which converts a D-amino acid and H_2O and O_2 to a 2-oxo acid and NH_3 and H_2O_2 ; and tryptophan oxidase, which converts L-tryptophan and H_2O and O_2 to indole-3-pyruvate and NH_3 and H_2O_2 . These classes also contain tyrosine (aromatic) aminotransferase, aspartate aminotransferase, D-amino acid (or D-alanine) aminotransferase, and broad (multiple substrate) aminotransferase which have multiple aminotransferase activities, some of which can convert tryptophan and a 2-oxo acid to indole-3-pyruvate and an amino acid. In addition, these classes include phenylalanine deaminases, which can convert tryptophan to indole-3-pyruvate and ammonium in the presence of water.

[0050] Enzymes useful for converting indole-3-pyruvate to MP include members of enzyme classes EC 4.1.3.-, 4.1.3.16, 4.1.3.17, and 4.1.2.-. These classes include carbon-carbon synthases/lyases, such as aldolases that catalyze the condensation of two carboxylic acid substrates. Enzyme class EC 4.1.3.- are those synthases/lyases that form carbon-carbon bonds utilizing oxo-acid substrates (such as indole-3-pyruvate) as the electrophile, while EC 4.1.2.- are synthases/lyases that form carbon-carbon bonds

utilizing aldehyde substrates (such as benzaldehyde) as the electrophile. For example, KHG aldolase (EC 4.1.3.16) and ProA aldolase (EC 4.1.3.17), are known to convert indole-3-pyruvate and pyruvate to MP. Although ProA aldolase can be thought to identify only the 4-hydroxy-4-methyl-2-oxoglutarate aldolase derived from *Comamonas testosteroni*, herein the term ProA aldolase is used to mean any polypeptide with 4-hydroxy-4-methyl-2-oxoglutarate aldolase activity unless otherwise stated. Suitable examples of Pro aldolases include *Comamonas testosteroni* ProA (SEQ ID NO:1 (nucleic acid sequence), SEQ ID NO:2 (amino acid sequence)) and *Sinorhizobium meliloti* ProA (NCBI Accession No.: CAC46344), or enzymes that display homology to *Comamonas testosteroni* ProA (SEQ ID NO:1 (nucleic acid sequence), SEQ ID NO:2 (amino acid sequence)) and/or *Sinorhizobium meliloti* ProA (NCBI Accession No.: CAC46344). For example, suitable enzymes may have at least about 40%, 50%, 60%, 70%, 80%, 90%, 95%, and/or 99% amino acid sequence identity with *Comamonas testosteroni* ProA (SEQ ID NO: 2) and/or *Sinorhizobium meliloti* ProA (NCBI Accession No.: CAC46344). MP can also be generated using chemical reactions, such as the aldol condensations.

[0051] Enzymes useful for the conversion of MP to monatin include members of the enzyme classes (EC): tryptophan aminotransferases (2.6.1.27), tryptophan dehydrogenases (1.4.1.19), D-amino acid dehydrogenases (1.4.99.1), glutamate dehydrogenases (1.4.1.2-4), phenylalanine dehydrogenase (1.4.1.20), tryptophan-phenylpyruvate transaminases (2.6.1.28), or more generally members of the aminotransferase family (2.6.1.-) such as aspartate aminotransferase (EC 2.6.1.1), tyrosine (aromatic) aminotransferase (2.6.1.5), D-tryptophan aminotransferase, or D-alanine (2.6.1.21) aminotransferase (*see* Figure 2 of WO 03/091396 A2). This reaction can also be performed using chemical reactions. Amination of the keto acid (MP) is performed by reductive amination using ammonia and sodium cyanoborohydride. Figures 11-13 of WO 03/091396 A2 show additional polypeptides that can be used to convert MP to monatin, as well as providing increased yields of monatin from indole-3-pyruvate or tryptophan.

[0052] The taste profile of a monatin composition can be altered by controlling the relative amount of the various stereoisomers of monatin in the composition. The present disclosure provides pathways and substances for producing monatin compositions with a desired percentage of R,R monatin and/or S,R monatin.

[0053] The chirality of the monatin compounds that is produced by pathways such as those exemplified herein can be altered both by pH and by the polypeptides used for the biological conversions. When monatin is formed using a biosynthetic pathway, the following can be considered. In a biocatalytic reaction, the chirality of the monatin carbon-2 (*see* chemical structure above) is determined by the enzyme that converts indole-3-pyruvate to MP. Multiple enzymes (e.g., from EC 4.1.2.-, 4.1.3.-) can convert indole-3-pyruvate to MP. Thus, one can choose the enzyme that forms the desired isomer. Alternatively, the enantiospecificity of the enzyme that converts indole-3-pyruvate to MP can be modified through the use of directed evolution or catalytic antibodies can be engineered to catalyze the desired reaction. Once MP is produced (either enzymatically or by chemical condensation), the amino group can be added stereospecifically. Either the R or S configuration of carbon-4 (*see* previous chemical structure) can be generated depending on whether a D- or L- aromatic acid aminotransferase is used. Many aminotransferases are specific for the L-isomer, however, D-tryptophan aminotransferases exist in certain plants (Kohiba and Mito, Proceedings of the 8th International Symposium on Vitamin B₆ and Carbonyl Catalysis, Osaka, Japan 1990). Moreover, D-alanine aminotransferases (EC 2.6.1.21), D-methionine-pyruvate aminotransferases (EC 2.6.1.41) and both (R)-3-amino-2-methylpropanoate aminotransferase (EC 2.6.1.61), (S)-3-amino-2-methylpropanoate aminotransferase (EC 2.6.1.22), and D-phenylglycine aminotransferase have been identified. Certain aminotransferases may only accept the substrate for this reaction with a particular configuration at the C2 carbon. Therefore, even if the conversion to MP is not stereospecific, the stereochemistry of the final product can be controlled through the appropriate selection of an aminotransferase. Because the reaction is reversible, the unreacted MP (undesired isomer) can be recycled back to its constituents and a racemic mixture of MP can be reformed.

[0054] Referring now to the figures, the following should be noted. The flow charts identify examples of pathways for producing monatin, but the pathways shown on the figures, and the methods of the invention, are not limited to any particular method for practicing the pathways, unless otherwise stated. For example, the pathways may be practiced *in vivo*, *in vitro*, or a combination thereof.

[0055] Furthermore, practice of a method of the invention utilizing one or more of the pathways disclosed herein does not require that each of the identified components (e.g. reactants and enzymes) is explicitly provided by the practitioner; rather, it is sufficient that the components, (or sources of components), and reaction conditions are present in the composition (or host cell) or otherwise available so that the pathway can potentially proceed. In other words, for example, if a figure depicts a process for producing a monatin composition, which includes producing indole-3-pyruvate from L-tryptophan, producing 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid (“monatin precursor” or “MP”) from indole-3-pyruvate, and producing monatin from MP, wherein each reaction is facilitated by an appropriate enzyme, it is contemplated that practice of that pathway includes combining L-tryptophan with α -ketoglutarate and enzymes contemplated for facilitating the identified reactions, and under conditions suitable for each of the reactions to occur without also explicitly providing indole-3-pyruvate or MP. In such an instance L-tryptophan could react with α -ketoglutarate to produce indole-3-pyruvate. Depending upon the conditions and the provided enzyme, the indole-3-pyruvate produced from the L-tryptophan reaction can react to form MP, and then depending upon the conditions and the provided enzyme, the MP produced from the indole-3-pyruvate reaction can react to form monatin.

[0056] It should also be noted that practice of a method of the invention utilizing one or more of the pathways disclosed herein does not require the practitioner to explicitly provide the identified starting materials or enzymes, if such materials or enzymes are otherwise already present or available, or capable of being synthesized from a substance that is already present or available in the reaction milieu. In other words, it is contemplated that practice of any pathways that identify L-tryptophan as a starting material would include providing a compound that can produce L-tryptophan, under conditions suitable for L-tryptophan production to occur and combining that compound with enzymes capable of facilitating the series of reactions set forth under conditions that would be suitable for those reactions to occur. As another example, it is also contemplated that practicing the identified pathway includes providing a microorganism genetically engineered to produce monatin according to the described pathway, and providing appropriate conditions for the fermentation process to occur. For example, a microorganism, which naturally produces large amounts of L-tryptophan or D-tryptophan

(see U.S. Patent No. 5,728,555) be can be genetically engineered to produce or over-produce one or more of the enzymes used to facilitate (catalyze) reactions in the pathway to monatin, and appropriate conditions can be provided so that the microorganism would thereby produce monatin.

[0057] Turning now to FIG. 1, the flow chart shown schematically depicts a process in accordance with the invention for making a monatin composition including R,R monatin. As shown in FIG.1, the overall pathway involves a reaction of tryptophan to form indole-3-pyruvate, a reaction of indole-3-pyruvate to produce MP, and a reaction of MP to produce monatin, including R,R monatin.

[0058] FIG. 1 further illustrates specific permutations of this overall pathway, designed to increase the production of the R,R form of monatin at the expense of the S,S, R,S and S,R forms of monatin. In particular, FIG. 1 illustrates the embodiment wherein: the aminotransferase enzyme utilized in the L-tryptophan reaction has greater activity and/or specificity for that reaction versus the reactions of MP and 4S monatin or the oxidase has greater activity and/or specificity for L-tryptophan than for 4R monatin; the enzyme which facilitates the reaction of indole-3-pyruvate is an R-specific aldolase; and, the enzyme which facilitates the reaction of MP is a broad specificity D-enzyme, preferably evolved to work more efficiently with the R isomer of MP.

[0059] FIG. 1 also illustrates particular permutations designed to make the production of R,R monatin more economical. For example, in FIG. 1 L-tryptophan—as opposed to D-tryptophan or combinations of L- and D-tryptophan—is identified as the starting material. While the choice of the specific form of tryptophan does not impact the chirality of the ultimate monatin compounds in the monatin composition (because the tryptophan reaction forms indole-3-pyruvate, which has no chirality), some may prefer utilizing L-tryptophan as a starting material at least because L-tryptophan is currently less expensive and more easily obtainable than D-tryptophan.

[0060] Focusing now on the first reaction shown in FIG. 1, when tryptophan is converted to indole-3-pyruvate any one or more of alpha-ketoglutarate, oxaloacetate, and/or pyruvate reacts with the tryptophan to form an amino acid (glutamate, aspartate, and alanine respectively) and indole-3-pyruvate. FIG. 1 depicts the embodiment wherein the tryptophan starting material is L-tryptophan, and the alpha-ketoglutarate, oxaloacetate,

and/or pyruvate produce the L-isomer form of the amino acid (e.g. L-glutamate, L-aspartate, and/or L-alanine, respectively).

[0061] As shown in FIG. 1, an approach to enhancing the production of R,R monatin involves facilitating the reaction of L-tryptophan with an enzyme having greater specificity, greater activity, or both for tryptophan as opposed to MP or monatin, and facilitating the reaction of MP with a D-specific enzyme. As is disclosed in WO 03/091396 A2, certain enzymes can facilitate the reaction of tryptophan to produce indole-3-pyruvate, as well as the amination reaction of MP to produce monatin. Use of an L-aminotransferase in the amination step creates an S chiral center at the monatin C-4 position, whereas use of a D-enzyme creates a D chiral center at the monatin C-4 position. Thus, in the instance where an L-aminotransferase, which facilitates the tryptophan reaction, is also active in the MP reaction, R,S and S,S monatin can be formed, depending on the form of MP present. In addition, certain other enzymes—the L-amino acid oxidases—can not only facilitate the reaction of tryptophan to indole-3-pyruvate, but may have a side activity for the degradation of R,R monatin. According to some embodiments, this 4R side activity is minimized or eliminated. An oxidase side activity on 4S forms of monatin would decrease or minimize them from the final product and could be desirable depending on the final composition desired. Consequently, the greater the specificity and/or activity of the L-enzyme chosen for tryptophan versus the MP or monatin, the greater the amount of R,R and S,R produced versus S,S and R,S monatin.

[0062] Suitable enzymes for the tryptophan reaction, in accordance with the embodiment illustrated in FIG. 1, include: L-aminotransferases capable of facilitating a reaction of L-tryptophan to form indole-3-pyruvate, and which have greater specificity for that reaction over the reaction of R-MP to form 4S isomers of monatin; and, L-amino acid oxidases capable of facilitating a reaction of L-tryptophan to form indole-3-pyruvate, and which have greater specificity and/or activity for that reaction versus the reaction of 4R isomers of monatin to form MP, and functional equivalents of any of the foregoing. More specifically, non-limiting examples of suitable enzymes can be chosen from L-tryptophan aminotransferases (EC 2.6.1.27) and tyrosine (aromatic) aminotransferases (EC 2.6.1.5) and L-amino acid oxidases (EC 1.4.3.2), and mutants derived from enzymes having aspartate aminotransferase activity.

- [0063] Example 6 identifies a specific enzyme, a mutant HEXaspC polypeptide which includes a Pro 9 to Tyr substitution and an Arg 122 to Gly substitution useful for facilitating the reactions of L-tryptophan and α -KG, oxaloacetate, pyruvate, or combinations thereof to form indole-3-pyruvate and L-glutamate, L-aspartate, and L-alanine, respectively. Another specific enzyme having "limited" activity is TatA, the L-tryptophan aminotransferase from *S. meliloti*. Other enzymes suitable for the tryptophan reaction in accordance with preferred embodiments of the pathway shown in Fig. 1 include those with the following characteristics: an enzyme that transaminates MP at 1/10 the rate or less than the rate of L-tryptophan as in Example 6 or an enzyme when used with a racemase, as in Example 9, that produces greater than 90% of the 4R isomers of monatin.
- [0064] Examples of enzymes not having a high degree of specificity for the L-tryptophan to indole-3-pyruvate conversion compared to the MP to monatin conversion include: HEXaspC (Example 6), *Leishmania major* broad specificity aminotransferase (WO 03/091396 A2), the Porcine aminotransferase (WO 03/091396 A2) and *Rhodobacter sphaeroides* TatA (Example 9). These enzymes may, however, be evolved, for example through mutagenesis to have limited activity for R-MP and/or R,R monatin versus tryptophan.
- [0065] Focusing now on the second reaction identified in FIG. 1, the choice of enzyme for facilitating (or catalyzing) the reaction of indole-3-pyruvate to MP influences the relative amount of R,R monatin versus S,R monatin produced. In general, the greater the relative amount of R-MP versus S-MP produced, the greater the relative amount of R,R monatin versus S,R monatin produced (when a D-enzyme facilitates the reaction of MP to monatin). Enzymes useful in this regard include any enzymes that produce a higher R-MP:S-MP ratio than that produced by the reaction of indole-3-pyruvate and pyruvate when facilitated by any one of the *E. coli* KHG aldolase (Genbank Accession No. AAC74920.1), the *Bacillus* KHG aldolase (Genbank Accession No. CAB14127.1) or the *Comamonas testosteroni* ProA aldolase (SEQ ID NO:1 (nucleic acid sequence), SEQ ID NO:2 (amino acid sequence)). Thus, if it is desired to preferentially produce R-MP, one or more enzymes capable of producing greater amounts of R-MP relative to S-MP can be used. When a monatin composition having the R,R form of monatin as its only monatin component is desired, an enzyme that selectively produces R-MP as opposed to S-MP (an

“R-specific enzyme”) should be used. Examples of R-specific enzymes that may be used to selectively produce R-MP as opposed to S-MP are the aldolase of SEQ ID NO:22, the aldolase of SEQ ID NO:104 and *Sinorhizobium meliloti* HMG aldolase, as shown in Example 3.

[0066] FIG. 1 identifies the particular embodiment wherein an R-specific aldolase facilitates the reaction of indole-3-pyruvate and pyruvate to form R-MP. Also contemplated, however, is the use of aldolases for the indole-3-pyruvate and pyruvate reaction which preferentially produce R-MP, as well as aldolases that produce a higher R-MP:S-MP ratio than is produced by any one of the *E. coli* KHG aldolase (Genbank Accession No. AAC74920.1), the *Bacillus* KHG aldolase (Genbank Accession No. CAB14127.1) or the *Comamonas testosteroni* ProA aldolase (SEQ ID NO:1 (nucleic acid sequence), SEQ ID NO:2 (amino acid sequence)). In addition, it is also contemplated that indole-3-pyruvate may react with a different C3 source (for example serine or cysteine) to form R-MP and consequently other enzymes (for example other lyases or synthases) may facilitate such a reaction. Other substrates that are readily converted to pyruvate (such as oxaloacetate) may also be used. Example 3 provides sources of aldolase enzymes that may preferentially or selectively produce R-MP or produce a higher R-MP:S-MP ratio than is produced by the reaction of indole-3-pyruvate and pyruvate when facilitated by any one of the *E. coli* KHG aldolase (Genbank Accession No. AAC74920.1), the *Bacillus* KHG aldolase (Genbank Accession No. CAB14127.1), the *Comamonas testosteroni* ProA aldolase (SEQ ID NO:1 (nucleic acid sequence), SEQ ID NO:2 (amino acid sequence)), the aldolase of SEQ ID NO:22 or the aldolase of SEQ ID NO:104. Example 5 also provides screening methods for identifying such enzymes. It is also contemplated that enzymes, which preferentially or selectively produce R-MP or produce more R-MP than any one of the *E. coli* KHG aldolase (Genbank Accession No. AAC74920.1), the *Bacillus* KHG aldolase (Genbank Accession No. CAB14127.1) or the *Comamonas testosteroni* ProA aldolase (SEQ ID NO:1 (nucleic acid sequence), SEQ ID NO:2 (amino acid sequence)) may be evolved from aldolases known or found in nature. Any techniques known in the art for evolving enzymes, for example to improve a desired characteristic—such as to increase the activity of an enzyme for a substrate—as compared to the wild-type enzyme can be used. Examples 4, 5, 6, 7, 9, 10, and 11 provide some techniques for evolving enzymes.

- [0067] Focusing now on the last step of the pathway identified in FIG. 1, the reaction of R-MP to form R,R monatin is shown to be facilitated by a broad specificity D-aminotransferase, for example D-alanine aminotransferase (EC 2.6.1.21, also known as D-amino acid aminotransferase or D-aspartate aminotransferase) or a D-amino acid dehydrogenase. As discussed above, the conversion of MP to monatin is an amination reaction, which creates a chiral center at the monatin C-4 carbon. Where the R-chiral form is desired at the C-4 position, enzymes should be used which produce "R" chiral centers in amino acids. Non-limiting exemplary enzymes include: a D-alanine-aminotransferase derived from *Bacillus* (Examples 15-18), including the D-alanine-aminotransferase derived from *Bacillus halodurans* (Example 18), a mutated branched chain aminotransferase that has modified stereospecificity (Example 7), and mutated D-aminotransferases derived from *Bacillus* (Example 6B).
- [0068] Another exemplary enzyme includes a hybrid D-aminotransferase. The hybrid D-aminotransferase can contain structural elements from two differing amino acid aminotransferases. The hybrid D-aminotransferase can then be further evolved (e.g. via mutagenesis or recombinant engineering) for improved performance in converting MP to monatin. An example of such a hybrid D-aminotransferase is shown in Example 19. The hybrid D-aminotransferase illustrated in Example 19 included elements from a D-aminotransferase from *B. sphaericus* and a D-aminotransferase from *G. stearothermophilus*. R,R-monatin was produced utilizing this D-aminotransferase (Example 19).
- [0069] Example 2 also illustrates the production of R,R monatin utilizing various D-aminotransferases.
- [0070] According to some embodiments, the D-aminotransferase has greater specificity, greater activity, or both for the R-MP as a substrate than for indole-3-pyruvate. In certain other embodiments, the D-aminotransferase has limited activity for the indole-3-pyruvate as a substrate. Enzymes with such characteristics may be evolved or mutated from existing enzymes, for example as shown in Example 6.
- [0071] Also, in some embodiments, the reaction of R-MP to form R,R monatin can be facilitated by a D-amino acid dehydrogenase. Example 20 illustrates the production of R,R monatin from R-MP utilizing a D-amino acid dehydrogenase (D-AADH-101 through

108, BioCatalytics). These D-amino acid dehydrogenases may be further evolved (e.g. via mutagenesis or recombinant engineering) for improved performance.

[0072] FIG. 2 depicts another strategy for targeting production of R,R monatin. Whereas in the embodiment of FIG. 1, the aldolase used in the reaction of indole-3-pyruvate to form R-MP influences the ratio of R,R:S,R formed, in the embodiment of FIG. 2, the D-enzyme that facilitates the conversion of MP to monatin influences the ratio of R,R:S,R formed. According to the embodiment of FIG. 2, a non-stereospecific enzyme may be used to facilitate the conversion of indole-3-pyruvate to MP, and consequently both S-MP and R-MP can be formed. To obtain a desired ratio of R,R monatin to S,R monatin, a D-enzyme is chosen (or evolved) with appropriate stereoselectivity for R-MP versus S-MP. Where a monatin composition having the R,R form of monatin as its only monatin component is desired, an enzyme that selectively facilitates the reaction of R-MP to monatin as opposed to S-MP to monatin would be preferred. For example, the *Bacillus halodurans* D-aminotransferase (Example 18) and the hybrid D-aminotransferase containing structural elements from both *Bacillus sphaericus* and *Geobacillus stearothermophilus* (Example 19) may be utilized as the enzyme that selectively facilitates the reaction of R-MP to monatin.

[0073] FIG. 3 illustrates another alternative pathway for production of compositions enriched in R,R monatin. The pathway of FIG. 3 is a modification of the pathway of FIG. 1. In the pathway shown in FIG. 3, indole-3-pyruvate is produced indirectly, rather than directly, from L-tryptophan. More specifically, L-tryptophan is converted to D-tryptophan, and D-tryptophan is then converted to indole-3-pyruvate. Example 4 illustrates the production of R,R monatin from L-tryptophan using a tryptophan racemase or a broad specificity amino acid racemase.

[0074] The conversion of L-tryptophan to D-tryptophan can be facilitated by a tryptophan racemase or functional equivalent thereof. Example 4 provides potential sources of tryptophan racemases and broad specificity amino acid racemases and screening methods for identifying such enzymes. Example 4 describes examples of tryptophan racemases and broad specificity amino acid racemases that are capable of converting L-tryptophan into D-tryptophan. These tryptophan racemases can be further evolved (e.g. via mutagenesis or recombinant engineering) for improved performance. An example of a racemase having the activity of a tryptophan racemase is an alanine racemase isolated

from *Geobacillus stearothermophilus*. More specifically, examples of racemases having the activity of a tryptophan racemase are alanine racemases derived from SEQ ID NO:41, such as an alanine racemase corresponding to SEQ ID NO:41 with a Y354A mutation.

[0075] Non-limiting examples of tryptophan racemases include homologs or mutants of amino acid racemases (EC 5.1.1.-), for example serine racemase, in which the homologs or mutants are capable of converting L-tryptophan to D-tryptophan. Non-limiting examples of sources from which the amino acid racemase may be derived include microorganisms such as *Salmonella typhimurium*, *Escherichia coli*, *Bacillus subtilis*, *Bacillus sphaericus*, *Bacillus halodurans*, *Geobacillus stearothermophilus*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Schizosaccharomyces pombe*, *Bacillus cereus*, *Enterococcus gallinarum*, *Pediococcus pentosaceus*, *Bacillus pumilus*, *Lactobacillus fermenti*, *Lactobacillus brevis*, *Aquifex pyrophilus*, *Lactobacilli*, *Streptococcus*, *Anabaena* sp., *Pseudomonas striata*, *Lentinus edodes*, *Scapharca brouhtonii*, *Desulfurococcus* sp., *Thermococcus* sp., and *Pseudomonas striata*. Additional non-limiting examples of sources from which the amino acid racemase may be derived include silkworm, rat brain, or mouse brain. These amino acid racemases may be evolved (e.g. via mutagenesis or recombinant engineering) for improved performance in converting L-tryptophan to D-tryptophan.

[0076] Non-limiting examples of potential sources from which suitable tryptophan racemases may be derived include: microorganisms such as *Pseudomonas*, for example *Pseudomonas chlororaphis* (*Pseudomonas aureofaciens*) (ATCC15926), and *Burkholderia pyrrocinia* (ATCC15958). Additional non-limiting examples of potential sources from which suitable tryptophan racemases may be derived include plants, for example tobacco plants, such as *Nicotiana tabacum*, wheat plants, such as *Triticum aestivum*, beets, tomatoes, and *Sclerochiton ilicifolius*.

[0077] Non-limiting examples of suitable broad specificity amino acid racemases include broad specificity amino acid racemase is chosen from a broad specificity amino acid racemase of SEQ ID NO:120, a broad specificity amino acid racemase corresponding to SEQ ID NO:120 with a Y396C mutation, a broad specificity amino acid racemase of SEQ ID NO:128, a broad specificity amino acid racemase corresponding to SEQ ID NO:128 with a I384M mutation, an alanine racemase from *Vibrio fischeri*, an alanine racemase of Genbank Accession No. AAW85230.1, an alanine racemase of Genbank Accession No.

YP_204118, a broad specificity amino acid racemase isolated from *Pseudomonas taetrolens*, a broad specificity amino acid racemase corresponding to the broad specificity amino acid racemase isolated from *Pseudomonas taetrolens* with a I384M mutation, a broad specificity amino acid racemase isolated from *Pseudomonas putida* (also known as *Pseudomonas striata*), a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:116, a broad specificity amino acid racemase of Genbank Accession No. ZP_00898332.1 GI:82735470, a broad specificity amino acid racemase from ATCC 4683, a broad specificity amino acid racemase of SEQ ID NO:204, a broad specificity amino acid racemase corresponding to SEQ ID NO:204 with a I384M mutation, a broad specificity amino acid racemase isolated from *P. putida* KT2440, a broad specificity amino acid racemase isolated from *P. putida* NBRC 12996, a broad specificity amino acid racemase from ATCC 7966, a broad specificity amino acid racemase isolated from *Pseudomonas* strain 2150, a broad specificity amino acid racemase isolated from *P. oleovorans*, a broad specificity amino acid racemase isolated from *P. aureofaciens*, a broad specificity amino acid racemase isolated from *P. putida* 12633, a broad specificity amino acid racemase isolated from *P. fluorescens*, a broad specificity amino acid racemase isolated from *P. putida* SCRC-744, a broad specificity amino acid racemase isolated from *P. graveolens*, a broad specificity amino acid racemase isolated from *P. striata* AKU 083, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Pseudomonas taetrolens* containing amino acids 24-409, an enzyme having the activity of a broad specificity amino acid racemase containing amino acids 24-409 of SEQ ID NO:204, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas jandei*, an enzyme having the activity of a broad specificity amino acid racemase from ATCC 49572, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:194, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas sobria*, an enzyme having the activity of a broad specificity racemase from ATCC 35994, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:192, an enzyme corresponding to the broad specificity amino acid racemase isolated from *Aeromonas caviae* with a L383M mutation, an enzyme having the activity of a broad specificity amino acid racemase from ATCC 14486 with a L383M mutation, an enzyme having the activity of a broad specificity amino acid

racemase encoded by a gene containing a partial sequence of SEQ ID NO:195, an enzyme having the activity of a broad specificity amino acid racemase encoded by a gene containing a partial sequence of SEQ ID NO:196, an enzyme having the activity of a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:201, an enzyme having the activity of a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:202, an enzyme corresponding to the amino acid racemase from *Aeromonas caviae* with a D76N mutation, an enzyme having the activity of a broad specificity amino acid racemase corresponding to SEQ ID NO:179 with a D76N mutation, a broad specificity amino acid racemase isolated from *Aeromonas hydrophila*, a broad specificity amino acid racemase of SEQ ID NO:177, a broad specificity amino acid racemase containing amino acids 22-408 of SEQ ID NO:177, a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:146, a broad specificity amino acid racemase isolated from *Aeromonas caviae*, a broad specificity amino acid racemase of SEQ ID NO:179, a broad specificity amino acid racemase containing amino acids 22-408 of SEQ ID NO:179, a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:155, a broad specificity amino acid racemase of SEQ ID NO:151, and active fragments and homologs thereof.

[0078] Other non-limiting examples of suitable broad specificity amino acid racemases include one or more enzymes comprising a sequence having a percent sequence identity of at least 90% to one or more broad specificity amino acid racemases chosen from a broad specificity amino acid racemase of SEQ ID NO:120, a broad specificity amino acid racemase corresponding to SEQ ID NO:120 with a Y396C mutation, a broad specificity amino acid racemase of SEQ ID NO:128, a broad specificity amino acid racemase corresponding to SEQ ID NO:128 with a I384M mutation, an alanine racemase from *Vibrio fischeri*, an alanine racemase of Genbank Accession No. AAW85230.1, an alanine racemase of Genbank Accession No. YP_204118, a broad specificity amino acid racemase isolated from *Pseudomonas taetrolens*, a broad specificity amino acid racemase corresponding to the broad specificity amino acid racemase isolated from *Pseudomonas taetrolens* with a I384M mutation, a broad specificity amino acid racemase isolated from *Pseudomonas putida* (also known as *Pseudomonas striata*), a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:116, a broad specificity amino acid racemase of Genbank Accession No. ZP_00898332.1 GI:82735470, a broad

specificity amino acid racemase from ATCC 4683, a broad specificity amino acid racemase of SEQ ID NO:204, a broad specificity amino acid racemase corresponding to SEQ ID NO:204 with a I384M mutation, a broad specificity amino acid racemase isolated from *P. putida* KT2440, a broad specificity amino acid racemase isolated from *P. putida* NBRC 12996, a broad specificity amino acid racemase from ATCC 7966, a broad specificity amino acid racemase isolated from *Pseudomonas* strain 2150, a broad specificity amino acid racemase isolated from *P. oleovorans*, a broad specificity amino acid racemase isolated from *P. aureofaciens*, a broad specificity amino acid racemase isolated from *P. putida* 12633, a broad specificity amino acid racemase isolated from *P. fluorescens*, a broad specificity amino acid racemase isolated from *P. putida* SCRC-744, a broad specificity amino acid racemase isolated from *P. graveolens*, a broad specificity amino acid racemase isolated from *P. striata* AKU 083, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Pseudomonas taetrolens* containing amino acids 24-409, an enzyme having the activity of a broad specificity amino acid racemase containing amino acids 24-409 of SEQ ID NO:204, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas jandei*, an enzyme having the activity of a broad specificity amino acid racemase from ATCC 49572, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:194, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas sobria*, an enzyme having the activity of a broad specificity racemase from ATCC 35994, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:192, an enzyme corresponding to the broad specificity amino acid racemase isolated from *Aeromonas caviae* with a L383M mutation, an enzyme having the activity of a broad specificity amino acid racemase from ATCC 14486 with a L383M mutation, an enzyme having the activity of a broad specificity amino acid racemase encoded by a gene containing a partial sequence of SEQ ID NO:195, an enzyme having the activity of a broad specificity amino acid racemase encoded by a gene containing a partial sequence of SEQ ID NO:196, an enzyme having the activity of a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:201, an enzyme having the activity of a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:202, an enzyme corresponding to the amino acid racemase from *Aeromonas caviae* with a D76N mutation, an enzyme having the activity

of a broad specificity amino acid racemase corresponding to SEQ ID NO:179 with a D76N mutation, a broad specificity amino acid racemase isolated from *Aeromonas hydrophila*, a broad specificity amino acid racemase of SEQ ID NO:177, a broad specificity amino acid racemase containing amino acids 22-408 of SEQ ID NO:177, a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:146, a broad specificity amino acid racemase isolated from *Aeromonas caviae*, a broad specificity amino acid racemase of SEQ ID NO:179, a broad specificity amino acid racemase containing amino acids 22-408 of SEQ ID NO:179, a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:155, a broad specificity amino acid racemase of SEQ ID NO:151, and active fragments thereof. In some embodiments, the percent sequence identity is at least 95%.

[0079] Still other non-limiting examples of suitable broad specificity amino acid racemases include a broad specificity amino acid racemase encoded by Genbank Accession No. AB096176, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to Genbank Accession No. AB096176 with a substitution such that the racemase includes a I384M mutation, a broad specificity amino acid racemase encoded by SEQ ID NO:119, a broad specificity amino acid racemase encoded by SEQ ID NO:127, a broad specificity amino acid racemase encoded by CP 000020.1 GI:59478708 region 800842..802053, a broad specificity amino acid racemase encoded by NC_006840, a broad specificity amino acid racemase encoded by KT2440 Bar DNA, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:119 with a substitution such that the racemase includes a Y396C mutation, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:127 with a substitution such that the racemase includes a I384M mutation, a broad specificity amino acid racemase encoded by the partial gene sequence of SEQ ID NO:151, a broad specificity amino acid racemase encoded by the partial gene sequence of SEQ ID NO:154, a broad specificity amino acid racemase encoded by NZ_AALM01000002 nucleotides 53173..54402, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:140, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:140 with a substitution such that the racemase includes a I384M mutation, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID

NO:178, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:178 with a substitution such that the racemase includes a L383M mutation, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:193, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:176, and homologs thereof.

[0080] Other non-limiting examples of suitable broad specificity amino acid racemases include a broad specificity amino acid racemase having a percent sequence identity of at least 90% to one or more broad specificity amino acid racemases chosen from a broad specificity amino acid racemase encoded by Genbank Accession No. AB096176, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to Genbank Accession No. AB096176 with a substitution such that the racemase includes a I384M mutation, a broad specificity amino acid racemase encoded by SEQ ID NO:119, a broad specificity amino acid racemase encoded by SEQ ID NO:127, a broad specificity amino acid racemase encoded by CP 000020.1 GI:59478708 region 800842..802053, a broad specificity amino acid racemase encoded by NC_006840, a broad specificity amino acid racemase encoded by KT2440 Bar DNA, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:119 with a substitution such that the racemase includes a Y396C mutation, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:127 with a substitution such that the racemase includes a I384M mutation, a broad specificity amino acid racemase encoded by the partial gene sequence of SEQ ID NO:151, a broad specificity amino acid racemase encoded by the partial gene sequence of SEQ ID NO:154, a broad specificity amino acid racemase encoded by NZ_AALM01000002 nucleotides 53173..54402, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:140, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:140 with a substitution such that the racemase includes a I384M mutation, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:178, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:178 with a substitution such that the racemase includes a L383M mutation, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:193, and a broad specificity

racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:176,. In some embodiments, said percent sequence identity is at least 95%.

[0081] In some embodiments, D-tryptophan is produced from L-tryptophan or L-tryptophan is produced from D-tryptophan utilizing one or more enzymes chosen from an enzyme having the activity of an alanine racemase from *Vibrio fischeri*, an enzyme having the activity of an alanine racemase of Genbank Accession No. AAW85230.1, an enzyme having the activity of an alanine racemase of Genbank Accession No. YP_204118, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Pseudomonas taetrolens*, an enzyme having the activity of a broad specificity amino acid racemase corresponding to the broad specificity amino acid racemase isolated from *Pseudomonas taetrolens* with a I384M mutation, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:204, an enzyme having the activity of a broad specificity amino acid racemase corresponding to SEQ ID NO:204 with a I384M mutation, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Pseudomonas taetrolens* containing amino acids 24-409 an enzyme having the activity of a broad specificity amino acid racemase containing amino acids 24-409 of SEQ ID NO:204, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas jandei*, an enzyme having the activity of a broad specificity amino acid racemase from ATCC 49572, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:194, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas sobria*, an enzyme having the activity of a broad specificity racemase from ATCC 35994, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:192, an enzyme corresponding to the broad specificity amino acid racemase isolated from *Pseudomonas putida* with a L383M mutation, an enzyme corresponding to the amino acid racemase *Pseudomonas putida* KT2440 BAR with a L383M mutation, an enzyme having the activity of a broad specificity amino acid racemase from ATCC 35994 with a L383M mutation, an enzyme having the activity of a broad specificity amino acid racemase corresponding to SEQ ID NO:120 with a L383M mutation, an enzyme having the activity of a broad specificity amino acid racemase encoded by a gene containing a partial sequence of SEQ ID NO:195, an enzyme having the activity of a broad specificity amino acid racemase encoded by a gene containing a

partial sequence of SEQ ID NO:196, an enzyme having the activity of a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:201, an enzyme having the activity of a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:202, an enzyme corresponding to the amino acid racemase from *Aeromonas caviae* with a D76N mutation, an enzyme having the activity of a broad specificity amino acid racemase corresponding to SEQ ID NO:179 with a D76N mutation, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas hydrophila*, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:177, an enzyme having the activity of a broad specificity amino acid racemase containing amino acids 22-408 of SEQ ID NO:177, an enzyme having the activity of a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:146, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas caviae*, an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:179, an enzyme having the activity of a broad specificity amino acid racemase containing amino acids 22-408 of SEQ ID NO:179, an enzyme having the activity of a broad specificity amino acid racemase containing a partial sequence of SEQ ID NO:155, and an enzyme having the activity of a broad specificity amino acid racemase of SEQ ID NO:151 and active fragments thereof.

[0082] The pathway shown in FIG. 3 has certain benefits, including that even when R,R monatin is the desired product, the same enzyme can be used for the reaction that produces indole-3-pyruvate as for the reaction that produces monatin as a product. That is, in the pathway illustrated in FIG. 1, an L-aminotransferase (or suitable L-enzyme) facilitates the reaction producing indole-3-pyruvate, but a D-aminotransferase facilitates the reaction producing monatin. By contrast in the pathway of FIG. 3, a certain D-aminotransferase that facilitates the reaction producing indole-3-pyruvate, can also facilitate the reaction producing monatin. Consequently, in pathways according to FIG. 3, broad specificity D-aminotransferases may be preferred when there is a desire to use the same enzyme for the reaction forming indole-3-pyruvate as for the reaction forming monatin. By contrast, in pathways according to FIGS. 1, 2, 4, 6, 7, and 8 production of monatin may be more efficient when a D-aminotransferase is chosen that has limited activity and/or specificity for indole-3-pyruvate as compared to R-MP.

[0083] Another benefit of the pathway schematically represented in FIG. 3 is that the amino acid product of the reaction coupled to the reaction producing indole-3-pyruvate can now be used as a substrate in the reaction coupled to the reaction producing monatin. That is, in the pathway illustrated in FIG. 1, L-tryptophan reacts to produce indole-3-pyruvate and at the same time oxaloacetate, alpha-ketoglutarate and/or pyruvate react to produce an L-amino acid. Because the reaction of R-MP to form monatin is coupled with a reaction utilizing a D-amino acid as a substrate, the L-amino acid of the reaction forming indole-3-pyruvate is not, under the conditions shown, recycled for use in the reaction coupled to the R-MP reaction. By contrast, in the pathway illustrated in FIG. 3, the reaction of D-tryptophan to form indole-3-pyruvate is coupled to a reaction forming a D-amino acid product, which D-amino acid can be recycled for use in the reaction coupled to the R-MP reaction. This allows one to use non-stoichiometric amounts of amino acceptor in step one and the amino donor for step 3 is produced in step 1. In some embodiments of the invention, the D-amino acid is D-alanine.

[0084] FIGS. 4 and 5 illustrate additional modifications of the pathway shown in FIG. 1. These modifications are directed to recycling the amino acid product formed by the reaction coupled with the L-tryptophan transamination reaction with the amino acid reactant of the reaction coupled to the MP to monatin reaction.

[0085] Turning to FIG. 4, the recycling is accomplished by providing an enzyme that can facilitate the conversion of an L-amino acid to a D-amino acid and vice versa. More specifically, where, as is shown in FIG. 4, α -KG reacts to form L-glutamate when L-tryptophan reacts to form indole-3-pyruvate, a glutamate racemase (EC 5.1.1.3) or functional equivalent can be provided that can facilitate the conversion of L-glutamate to D-glutamate and vice versa. In such an instance, the L-glutamate formed as a product along with the production of indole-3-pyruvate is partially removed by virtue of its conversion to D-glutamate, and the D-glutamate formed from the conversion of L-glutamate is then available as a substrate for the reaction coupled with the MP to monatin reaction. Similarly, the α -KG formed in the reaction of D-glutamate is available as a substrate for the reaction coupled to the L-tryptophan to indole-3-pyruvate reaction.

[0086] Non-limited examples of potential sources from which a glutamate racemase may be derived include *Pediococcus pentosaceus*, *Bacillus pumilus*, *Lactobacillus fermenti*, *Lactobacillus brevis*, *E. coli*, *Aquifex pyrophilus*, and *Bacillus subtilis*. More specifically

(also non-limiting), the glutamate racemase may be expressed from a nucleic acid such as *Pediococcus pentosaceus murI* gene (Genbank Accession No. L22789), or *Lactobacillus brevis* glutamate racemase.

[0087] Where oxaloacetate reacts to form L-aspartate when L-tryptophan reacts to form indole-3-pyruvate, an aspartate racemase (EC 5.1.1.13) or functional equivalent can be provided to convert L-aspartate to D-aspartate. In such an instance, the L-aspartate that is formed in the same reaction that produces indole-3-pyruvate is partially removed by virtue of its conversion to D-aspartate, and the D-aspartate is then available to as a substrate for the reaction coupled to the MP to monatin reaction. Similarly, the oxaloacetate formed in the reaction of D-aspartate is available to act as a substrate for the reaction coupled to the L-tryptophan to indole-3-pyruvate reaction.

[0088] Non-limiting examples of suitable enzymes having aspartate racemase activity include ASPR-101 (BioCatalytics, Inc., 129 N. Hill Ave, Suite 103, Pasadena, CA 91106-1955) and homologs or mutants of an amino acid racemase (EC 5.1.1.-) which are capable of facilitating the conversion of L-aspartate to D-aspartate.

[0089] Non-limiting examples of potential sources from which aspartate racemases may be derived include: *Desulfurococcus*, *Thermococcus*, *bivalve mollusk Scapharca brouhtonii*, *Acinetobacter*, *Agrobacterium*, *Archaeoglobus*, *Bacillus*, *Bordetella*, *Bradyrhizobium*, *Brevibacterium*, *Burkholderia*, *Campylobacter*, *Candida*, *Caulobacter*, *Clostridium*, *Desulfitobacterium*, *Desulfotalea*, *Enterococcus*, *Erwinia*, *Escherichia*, *Ferropasma*, *Helicobacter*, *Klebsiella*, *Lactobacillus*, *Mannheimia*, *Medicago*, *Mesorhizobium*, *Methanococcus*, *Methanosarcina*, *Oceanobacillus*, *Oenococcus*, *Pediococcus*, *Polaribacter*, *Pseudomonas*, *Pyrococcus*, *Ralsonia*, *Shigella*, *Sinorhizobium*, *Salmonella*, *Sphingomonas*, *Streptococcus*, *Thermoanaerobacter*, *Vibrio*, *Wolinella*, *Xanthomonas*, *Xanthobacter*, *Yersinia* and *Zymomonas*.

[0090] Where pyruvate reacts to form L-alanine when L-tryptophan reacts to form indole-3-pyruvate, an alanine racemase or functional equivalent can be provided to convert L-alanine to D-alanine. In such an instance, the L-alanine that is formed in the same reaction that produces indole-3-pyruvate is removed by virtue of its conversion to D-alanine, and the D-alanine formed from the conversion of L-alanine is then available to act as a substrate for the reaction coupled to the MP to monatin reaction. Similarly, the

pyruvate formed in the reaction of D-alanine is available to act as a substrate for the reaction couple with the L-tryptophan to indole-3-pyruvate reaction.

[0091] Non-limiting examples of suitable alanine racemases include A8936 (Sigma, PO Box 14508, St. Louis, MO, 63178) and the *Geobacillus stearothermophilus* alanine racemase as described in Example 4.

[0092] Non-limiting examples of potential sources from which the alanine racemase may be derived include: *Brucella abortus*, *Streptococcus faecalis*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Schizosaccharomyces pombe*, *Bacillus cereus*, and *Lentinus edodes*.

[0093] Examples 9 and 12 illustrate the use of the above racemases, their impact on increasing the ratio of the desired monatin product, and provide potential sources for the racemase enzymes.

[0094] Turning to FIG. 5, a stereoinverting aminotransferase is used to facilitate the reaction of R-MP to monatin. Although typically the R-MP (or S-MP) reaction to form R,R monatin (or S,R monatin) is coupled with the reaction of a D-amino acid, a stereoinverting aminotransferase may facilitate the coupled reactions of R-MP (or S-MP) to form R,R monatin (or S,R monatin) using an L-amino acid. In this way, the L-amino acid product of the L-tryptophan aminotransferase reaction may be used as a substrate for the transamination of MP to monatin, and the product (i.e. oxaloacetate, pyruvate, and/or α -KG) of the reaction coupled to the MP to monatin reaction can be used as a starting material for the reaction coupled to the L-tryptophan to indole-3-pyruvate reaction. Non-limiting examples of stereoinverting aminotransferases that may be used include mutants derived from D-phenylglycine aminotransferase (EC 2.6.1.72, also known as D-4-hydroxyphenylglycine aminotransferase), D-methionine aminotransferase (EC 2.6.1.41, also known as D-met-aminotransferase and D-methionine-pyruvate aminotransferase), and homologs thereof. Non-limiting examples of potential sources from which the mutants of D-phenylglycine aminotransferase may be derived include *Pseudomonas*, such as *Pseudomonas putida* LW-4 and *Pseudomonas stutzeri* ST-201. Non-limiting examples of potential sources from which the D-methionine aminotransferase may be derived include cauliflower and peanut.

[0095] Examples 10 and 11 together provide potential sources of stereoinverting enzymes, and methods of making such enzymes. The examples also provide screening

methods for identifying such enzymes. It is also contemplated that such enzymes may be evolved from stereoinverting enzymes known or found in nature. As a non-limiting example, the stereoinverting aminotransferase can be a homolog or mutant of a D-amino acid aminotransferase or a homolog or mutant of an amino acid racemase (EC 5.1.1.-).

[0096] FIGS. 6 and 7 also illustrate modifications to the pathway of FIG. 1. The pathways illustrated in FIGS. 6 and 7 provide methods to push equilibrium reactions forward (i.e., toward the direction of monatin production) by removing the byproduct of the tryptophan reaction with an irreversible reaction and in some cases providing substrate for the MP reaction.

[0097] Turning to FIG. 6, the pathway shown removes the L-amino acid product of the reaction coupled to the tryptophan reaction by converting it to a different L-amino acid and CO₂, and then provides a substrate for the reaction coupled to the MP reaction by converting the newly formed L-amino acid to a D-amino acid. Specifically, L-tryptophan is shown to react alongside oxaloacetate to form indole-3-pyruvate and L-aspartate. An aspartate 4-decarboxylase (EC 4.1.1.12) or functional equivalent is used to facilitate the conversion of L-aspartate to L-alanine and carbon dioxide, and an enzyme with alanine racemase activity is used to facilitate the conversion of L-alanine to D-alanine, which D-alanine can serve as an amino donor for the conversion of R-MP to monatin.

[0098] Turning to FIG. 7, the pathway shown illustrates additional methods for removing the L-amino acid product of the reaction coupled to the tryptophan reaction. Embodiments as presented in the figure produce a byproduct(s) that is unavailable to react in the reverse direction, for example due to volatility (e.g., carbon dioxide) or by spontaneous conversion to an unreactive endproduct. An example of such an approach includes embodiments in which α -KG reacts alongside L-tryptophan to produce L-glutamate, and, if desired, a glutamate decarboxylase (EC 4.1.1.15) or functional equivalent can be provided to facilitate the conversion of L-glutamate to 4-aminobutanoate (with carbon dioxide as a byproduct). Non-limiting examples of potential sources from which the L-glutamate decarboxylase can be derived include: *Clostridium perfringens*, *C. welchii*, or *E. coli*.

[0100] Another example of such an approach for driving the tryptophan reaction forward (in the direction of monatin production) includes reactions in which oxaloacetate is utilized as a co-substrate in the reaction that utilizes L-tryptophan and in which the

oxaloacetate is converted to L-aspartate; if desired, an aspartate decarboxylase (EC 4.1.1.11) or functional equivalent can be provided to facilitate the conversion of L-aspartate to β -alanine (with carbon dioxide as a byproduct).

[0101] Turning to FIG. 8, the pathway shown illustrates yet additional methods for converting the L-amino acid product of the reaction coupled to the tryptophan reaction to a substrate for the reaction coupled to the MP reaction. Specifically, where α -KG is utilized in the same reaction as L-tryptophan, and in which the α -KG forms L-glutamate, an enzyme with L-alanine aminotransferase activity and pyruvate can be provided, wherein the L-alanine aminotransferase enzyme facilitates the reaction of pyruvate and L-glutamate to form L-alanine. An alanine racemase or functional equivalent can also be provided in order to facilitate the conversion of the L-alanine to D-alanine, which D-alanine can be used as a substrate along with MP to form monatin and pyruvate. See Example 12.

[0102] Implicitly described in the biosynthesis pathways above, and in the reactions described in the Examples below, are mixtures containing one or more compounds and/or enzymes required in the biosynthesis pathways for producing monatin, including R,R monatin, or monatin precursor, including R monatin precursor.

[0103] For production *in vitro*, any or all of the biosynthetic pathways described herein or individual steps in the pathways described herein can be conducted in *in vitro* solution or *in vivo*, in a host cell, in series or in parallel. When the method of the invention utilizes one or more reactions that are performed *in vitro*, the biosynthetic reaction that is performed *in vitro* can be performed by combining the desired ingredients for the reaction(s) by admixture in an aqueous reaction medium or solution. The reaction mixture so formed is maintained for a period of time sufficient for the desired product(s) to be synthesized.

[0104] Additionally, the activity of one or more enzymes can be enhanced through the continuous use of cofactors during purification of the one or more enzymes. For example, including pyridoxal-5'-phosphate when purifying *B. sphaericus* D-alanine aminotransferase results in increased activity (Example 14).

[0105] When one or more of the reactions in the pathways of the invention are to be performed *in vitro*, any or all of the enzymes utilized in the biosynthesis pathways described herein can optionally be immobilized onto a solid support. Examples of such

solid supports include those that contain epoxy, aldehyde, chelators, or primary amine groups. Specific examples of suitable solid supports include, but are not limited to, Eupergit[®] C (Rohm and Haas Company, Philadelphia, PA) resin beads and SEPABEADS[®] EC-EP (Resindion). Example 21 illustrates the immobilization of the *B. sphaericus* D-alanine aminotransferase onto Eupergit[®] C resin beads. Example 22 illustrates the immobilization of the *Sinorhizobium meliloti* ProA aldolase onto Eupergit[®] C resin beads. Production of R,R monatin utilizing these immobilized enzymes is shown in Example 23.

[0106] When one or more of the reactions in the pathways of the invention are to be performed *in vivo*, a person of ordinary skill in the art may routinely optimize production of monatin in a microorganism, including R,R monatin, by various methods. Such a microorganism can be one that naturally is better than another in one or more of the following, non-limiting example embodiments, or that (relative to the microorganism before such modification) has been modified to:

- Increase the uptake of tryptophan by the microorganism, such as genetically-engineering a microorganism to express or overexpress a *tnaT* gene;
- Increase the uptake of D-tryptophan by the microorganism, such as by (i) growing a microorganism in a medium that contains only minimal amounts of L-amino acids, (ii) modifying the phenylalanine permease protein, and/or (iii) creating a chimeric protein that has at least that part of the sequence that confers the activity of a general aromatic amino acid permease protein and at least that part of the sequence that confers the activity of a phenylalanine permease protein (Cosgriff AJ, Brasier G, Pi J, Dogovski C, Sarsero JP, Pittard AJ. "A study of AroP-PheP chimeric proteins and identification of a residue involved in tryptophan transport," *J Bacteriol.*, 182(8):2207-17 (2000));
- Prevent or decrease secretion of indole-3-pyruvate by the microorganism;
- Increase the uptake of indole-3-pyruvate by the microorganism;
- Prevent or decrease the available indole-3-pyruvate from being degraded within the microorganism; and
- Alleviate the toxicity of D-tryptophan, such as by (i) overproducing tRNA^{trp} in a microorganism that is not deficient in deacylase, (ii) genetically-engineering a

microorganism to express or overexpress an appropriate D-amino acid deacylase, (iii) genetically-engineering a microorganism to express or overexpress tRNA^{trp} and to express or overexpress an appropriate D-amino acid deacylase, (iv) generating a biomass of a genetically engineered microorganism and then induce the monatin operon, (v) using peptides or proteins rich in phenylalanine, tyrosine, and isoleucine to complement auxotrophs, (vi) genetically-engineering a microorganism to express or overexpress the general aromatic amino acid permease, (vii) not using or minimizing the use of D-amino acids other than D-tryptophan, (viii) providing L-tryptophan as the external substrate and genetically-engineering a microorganism to express or overexpress a racemase that converts the L-tryptophan to D-tryptophan, and/or (ix) providing D-tryptophan at a level below the threshold at which inhibition of D-tryptophan uptake occurs.

[0107] The above embodiments are described in more detail below.

[0108] The relative effect of each optimization method may be determined by comparing the amount of monatin produced by a genetically-engineered microorganism, with an appropriate control, e.g., a microorganism that has not been genetically engineered and/or exposed to the optimization method. The appropriate control to be utilized will be obvious to a person of ordinary skill in the art from the optimization method being tested.

[0109] As indicated above, one method for increasing the amount of monatin produced by a microorganism is to increase the uptake of tryptophan, one of the substrates utilized in the monatin biosynthesis pathways described above, by the microorganism. One way in which the amount of tryptophan that a microorganism uptakes may be increased is to genetically-engineer the microorganism to express or overexpress in monatin production strains a *tnaT* gene that encodes a protein that is homologous to, or in the family of, a sodium-dependent neurotransmitter transporter. In one embodiment, such a *tnaT* gene is that from *Symbiobacterium thermophilum*. Without being bound by theory, it is believed that expression of the *S. thermophilum tnaT* gene in *E. coli* confers on the bacterial host the ability to accumulate tryptophan from the medium and the ability to grow on tryptophan as a sole carbon source. Therefore, genetically-engineering the microorganism to express or overexpress the *tnaT* gene, for example *tnaT* from *S. thermophilum*, may be used to provide increased tryptophan uptake from the medium in which the microorganism is grown. Homologs of the *tnaT* gene from *S. thermophilum*

found in bacteria and Archaea may also be used. Thus, genetically engineering the microorganism to express or overexpress a *tnaT* gene, for example a *tnaT* gene from *S. thermophilum*, or a homolog thereof, may be used to increase the amount of monatin, including R,R monatin, produced by a microorganism.

[0110] In some embodiments, *in vivo* monatin production, including R,R monatin production, may be increased by increasing the uptake of D-tryptophan, a substrate that may be utilized in a monatin biosynthesis pathway. One way in which the amount of D-tryptophan that a microorganism uptakes may be increased is to only include low amounts of L-amino acids in the growth medium of the microorganism. Without being bound by theory, it is believed that upon depletion of L-amino acids in the growth medium, the general aromatic amino acid permease system mediates the uptake of D-tryptophan from the growth medium. Thus, growing the microorganism in a growth medium with low amounts of L-amino acids may be used to increase the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.

[0111] In some embodiments, *in vivo* monatin production, including R,R monatin production, may be increased by modifying the phenylalanine permease protein to increase D-tryptophan uptake. The microorganism may be genetically engineered to express or overexpress such a modified phenylalanine permease protein, as well as to express or overexpress a TnaT transporter, to increase the amount of D-tryptophan available inside the microorganism which may then be converted into monatin. Thus, genetically-engineering the microorganism to express or overexpress a modified phenylalanine permease protein may be used to increase the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.

[0112] In some embodiments, *in vivo* monatin production, including R,R monatin production, may be increased by creating a chimeric protein that has at least that part of the sequence that confers the activity of a general aromatic amino acid permease protein and at least that part of the sequence that confers the activity of a phenylalanine permease protein. A chimeric protein of the general aromatic amino acid permease protein and the phenylalanine permease protein may be more effective in transporting tryptophan. Thus, genetically-engineering the microorganism to express or overexpress a chimeric protein of the general aromatic amino acid permease protein and the phenylalanine permease

protein may be used to help maximize the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.

[0113] In some embodiments, *in vivo* monatin production, including R,R monatin production, may be increased by preventing or decreasing secretion of indole-3-pyruvate from a microorganism. Indole-3-pyruvate is an intermediate in the monatin synthetic pathways described above. One way in which secretion of indole-3-pyruvate from the microorganism may be prevented or decreased is to eliminate the activity of transporters that have the ability to transport indole-3-pyruvate or related compounds, such as auxins, from the microorganisms. For example, these transporters may be "knocked out" in the microorganisms used to produce monatin. Thus, monatin, including R,R monatin, may be produced in a microorganism in which the transporters that have the ability to transport indole-3-pyruvate from the microorganism are "knocked out."

[0114] In some embodiments, *in vivo* monatin production, including R,R monatin production, may be increased by increasing the uptake of indole-3-pyruvate by the microorganism. One way in which the uptake of indole-3-pyruvate by a microorganism may be increased is to increase expression of transporters that transport indole-3-pyruvate into the microorganism. Examples of transporters that may be activated or the activity of which may be increased include, but are not limited to, the amino acid/auxin family of transporters and proton symport permeases that exhibit homology to AUX1. All of these transporters have been reported to exist in fungi, such as *Saccharomyces cerevisiae*. Prusty, R., and Grisafi, P., *Proc. Natl Acad. Sci. USA* 101:4153-4157 (2004). Additionally, homologous transporters from other microorganisms may also be utilized. Thus, genetically-engineering the microorganism to express or overexpress a transporter that increases the uptake of indole-3-pyruvate into the microorganism may be used to increase the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.

[0115] In some embodiments, *in vivo* monatin production, including R,R monatin production, may be increased by preventing or decreasing the available indole-3-pyruvate from being degraded within the microorganism. A high concentration of the monatin precursor, a product of a reaction between indole-3-pyruvate and pyruvate will shift the equilibrium toward production of monatin. One way in which the indole-3-pyruvate may be prevented or decreased from being degraded within the microorganism is to "knock

out" the *ipdC* gene, or homologs thereof. The *ipdC* gene, or homologs thereof, encode indole-3-pyruvate decarboxylase. Indole-3-pyruvate decarboxylase decarboxylates indole-3-pyruvate to yield indole-3-acetaldehyde. The presence of indole-3-acetaldehyde initiates a cascade of additional degradation reactions. By "knocking out" production of indole-3-pyruvate decarboxylase, the available indole-3-pyruvate should not be degraded. The higher concentration of indole-3-pyruvate should shift the equilibrium toward production of monatin. Thus, genetically-engineering the microorganism so that expression or overexpression of the *ipdC* gene, or homologs thereof, is decreased may be used to increase the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.

[0116] In some embodiments, *in vivo* monatin production, including R,R monatin production, may be increased by inactivating the indole-3-acetaldehyde dehydrogenase. Indole-3-acetaldehyde dehydrogenase is involved in the degradation pathway of indole-3-acetaldehyde. By inactivating the indole-3-acetaldehyde dehydrogenase, the concentration of indole-3-acetaldehyde should be increased, shifting the equilibrium back toward production of indole-3-pyruvate. As a result, the higher concentration of indole-3-pyruvate should shift the equilibrium of the reaction toward production of monatin. Thus, genetically-engineering the microorganism so that expression of indole-3-acetaldehyde is decreased may be effective in increasing the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.

[0117] In some embodiments, *in vivo* monatin production, including R,R monatin production, may be increased by alleviating the toxicity of D-tryptophan and/or increasing the tolerance and uptake of D-tryptophan by the microorganism. Although some of the biosynthetic pathways for production of monatin described above utilize D-tryptophan as a starting material, D-tryptophan may be toxic to the microorganism. Therefore, alleviating the toxicity and/or increasing the tolerance and uptake of D-tryptophan by the microorganism may be used to increase the amount of monatin, including R,R monatin, produced by a microorganism.

[0118] One way in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased is to genetically-engineer a microorganism that is not deficient in deacylase to overproduce tRNA^{trp}, (i.e., the tRNA for tryptophan).

- [0119] Another way in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased is to genetically-engineer the microorganism to express or overexpress the appropriate D-amino acid deacylases, including the tyrosine or tryptophan D-amino acid deacylases. By expressing or overexpressing the appropriate D-amino acid deacylase, it is expected that recycling of the tRNA species would occur and that a build up of a pool of tRNAs charged with D-amino acids, such as tRNA^{trp}, would be avoided. Thus, genetically-engineering the microorganism to express or overexpress the appropriate D-amino deacylase may be effective in increasing the amount of monatin, including R,R monatin, produced by the microorganism.
- [0120] Yet another way in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased is to genetically-engineer a microorganism to express or overexpress tRNA^{trp} and to express or overexpress the appropriate D-amino acid deacylases, including tyrosine or tryptophan D-amino acid deacylases. Thus, genetically-engineering the microorganism to express or overexpress the appropriate D-amino deacylase, in combination with expression or overexpression of tRNA^{trp}, may be used to increase the amount of monatin, including R,R monatin, produced by the microorganism.
- [0121] Still yet another way in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased is to generate a biomass of a genetically engineered microorganism, and then, induce the monatin operon. The biomass induced for monatin production may then be transferred to fresh media that is depleted of L-amino acids. The transfer of the biomass to the fresh media depleted of L-amino acids should alleviate the inhibitory effects of L-tyrosine and also promote D-tryptophan uptake. Thus, generating a biomass and transferring it to a medium with low concentrations of L-amino acids may be used to increase the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.
- [0122] Another way in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased in auxotrophic strains, especially tyrosine and phenylalanine auxotrophic strains, is to use peptides or proteins rich in phenylalanine, tyrosine, and isoleucine to complement auxotrophs and alleviate competition for the transport of the D-amino acids, such as D-tryptophan. Thus, growing

the microorganism in a medium containing peptides or protein rich in phenylalanine, tyrosine, and isoleucine may be used to increase the amount of monatin, including R,R monatin, produced by the microorganism.

[0123] Yet another way in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased is to genetically-engineer the microorganism to express or overexpress the general aromatic amino acid permease ("aroP"), which will increase D-tryptophan uptake. The expression or overexpression of the aroP gene may be increased by cloning the aroP gene on a high copy number plasmid. The aroP gene and the monatin operon may be induced concurrently to generate cells that are capable of both uptaking D-tryptophan and then converting the D-tryptophan to monatin. Thus, genetically-engineering the microorganism to express or overexpress the aroP gene and concurrently inducing the monatin operon may be used to increase the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.

[0124] Still yet another way in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased is to avoid the use of D-amino acids, other than D-tryptophan. Thus, growing the microorganism in a medium that does not contain D-amino acids other than D-tryptophan may be used to increase the amount of monatin, including R,R monatin, produced by the microorganism.

[0125] Another mechanism in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased is to provide L-tryptophan as the external substrate, rather than D-tryptophan. A racemase may then be used to convert the L-tryptophan to D-tryptophan. Genetically-engineering the microorganism to express or overexpress a racemase, such as those described above, that converts L-tryptophan to D-tryptophan may be used to increase the amount of monatin, including R,R monatin, produced by the microorganism.

[0126] Yet another mechanism in which the toxicity of D-tryptophan may be alleviated or the tolerance and uptake of D-tryptophan may be increased is to provide D-tryptophan at a level below the threshold at which inhibition of D-tryptophan uptake occurs. Thus, growing the microorganism in a medium that contains D-tryptophan in an amount that is lower than the inhibition amount may be used to increase the amount of monatin, including R,R monatin, produced by the genetically engineered microorganism.

- [0127] Furthermore, the biosynthetic pathways described herein can utilize a substituted tryptophan to yield monatin derivatives that are likely to be sweet. In some embodiments, the substituted tryptophan to be used in the biosynthetic pathways described herein includes chlorinated tryptophan and 5-hydroxytryptophan.
- [0128] For example, chlorinated D-tryptophans, which have structural similarities to R,R-monatin, have been identified as non-nutritive sweeteners (particularly 6-chloro-D-tryptophan). Similarly, halogenated and hydroxy-substituted forms of monatin have been found to be sweet. U.S. Published Patent Application No. 2005/0118317. Halogens and hydroxyl groups should be substitutable for hydrogen, particularly on positions 1-4 of the benzene ring in the indole of tryptophan, without interfering in subsequent conversions to D- or L-tryptophan, indole-3-pyruvate, MP, or monatin. Substituted indoles have been shown in the literature to be suitable substrates for PLP-enzymes and have yielded substituted tryptophans. Fukuda, D.S., *et al.*, "Production of Substituted L-Tryptophans by Fermentation," *Appl. Environ. Microbiol.*, 21:841-43 (1971). The halogen does not appear to sterically hinder the tryptophan synthase beta subunits catalytic mechanism and the enantiospecificity was also intact.
- [0129] The individual reactions shown in the biosynthetic pathways described herein can be facilitated (catalyzed) by a single enzyme or by a mixture of multiple enzymes acting concurrently.
- [0130] The methods of the invention can be used to make a monatin composition that contains a desired percentage of R,R-monatin, or a minimum desired percentage of R,R-monatin. In addition to the reaction steps described above, a specific reaction step can be catalyzed by more than one enzyme, for example, a mixture of enzymes, so that the resulting composition or preparation contains a desired percentage of R,R-monatin, including, for example, a minimum desired percentage of R,R-monatin, or a maximum desired percentage of R,R-monatin. Alternatively, the monatin made by two separate engineered pathways according to the methods of the invention be combined to produce a composition or preparation containing such desired percentage of R,R-monatin.
- [0131] When an enzyme of a designated class of enzymes is utilized as an example, it is expected that an enzyme with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99% homology could also be utilized in that reaction. For example, an R-specific aldolase with at least about

50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99% homology to the aldolase of SEQ ID NO:22 could be utilized in any of the above described pathways to yield R,R monatin. Another non-limiting example of an enzyme that could be utilized in any of the above described pathways to yield R,R monatin includes a D-aminotransferase with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99% homology to the D-aminotransferase corresponding to ATCC 4978 with a T243N mutation. Yet another non-limiting example of an enzyme that could be utilized in any of the above described pathways to yield R,R monatin includes a broad specificity amino acid racemase with at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99% homology to the broad specificity amino acid racemase of SEQ ID NO:120.

[0132] Additionally, when an enzyme of a designated class of enzymes is utilized as an example, it is expected that a fragment of that enzyme that has the same activity could also be utilized in that reaction. For example, a fragment of the aldolase of SEQ ID NO:22 that also functions as an aldolase could be utilized in any of the above described pathways to yield R,R monatin.

[0133] Monatin that is produced utilizing one or more of the polypeptides or biosynthetic pathways disclosed herein, is generally at least about 0.5-30% R,R-monatin, by weight of the total monatin produced. In other embodiments, the monatin produced utilizing one or more of the polypeptides or biosynthetic pathways disclosed herein, is greater than 30% R,R-monatin, by weight of the total monatin produced; for example, the R,R-monatin is 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% of the total monatin produced. Alternatively, various amounts of two or more preparations of monatin can be combined so as to result in a preparation that is a desired percentage of R,R-monatin. For example, a monatin preparation that is 30% R,R-monatin can be combined with a monatin preparation that is 90% R,R-monatin; if equal amounts of 30% and 90% R,R-monatin preparations are combined, the resulting monatin preparation would be 60% R,R-monatin.

[0134] The monatin, or an intermediate (including monatin precursor), produced utilizing one or more of the polypeptides or biosynthetic pathways disclosed herein, may be purified from the components of the reaction. In one embodiment, the monatin or

intermediate, such as monatin precursor, may be purified simply by removing the substance that is to be purified from the enzyme preparation in which it was synthesized.

[0135] In other embodiments, the intermediate, monatin precursor or monatin is purified from a preparation in which it was synthesized so that the resulting "purified" composition or preparation is at least about 5-60% monatin by weight of total organic compounds. In another embodiment, the monatin or intermediate, such as monatin precursor, may be purified to a degree of purity of at least about 70%, 80%, 90%, 95% or 99% by weight of total organic compounds. The monatin, or the intermediate (including monatin precursor), produced utilizing one or more of the polypeptides or biosynthetic pathways disclosed herein, may be purified from the components of the reaction by any method known to a person of ordinary skill in the art. In one embodiment, the monatin or intermediate may be purified as described in Example 13. Optimally, the purified monatin or intermediate may be repeatedly recrystallized until the desired degree of purity is achieved.

[0136] All patents and publications cited herein are fully incorporated by reference herein in their entirety.

EXAMPLES

EXAMPLE 1

Detection of Monatin, Monatin Precursor, Tryptophan, Alanine, Aspartate, and Glutamate

[0137] This example describes methods used to detect the presence of monatin, monatin precursor ("MP"), tryptophan, aspartate, alanine, and glutamate. It also describes a method for the separation and detection of the four stereoisomers of monatin.

LC/MS/MS Multiple Reaction Monitoring ("MRM") Analysis of Monatin and Tryptophan

[0138] Analyses of mixtures for monatin and tryptophan derived from *in vitro* or *in vivo* biochemical reactions were performed using a Waters/Micromass liquid chromatography-tandem mass spectrometry (LC/MS/MS) instrument including a Waters 2795 liquid

chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor placed in series between the chromatograph and a Micromass Quattro Ultima triple quadrupole mass spectrometer. LC separations were made using an Xterra MS C₈ reversed-phase chromatography column, 2.1mm x 250 mm at 40 °C. The LC mobile phase consisted of A) water containing either (i) 0.05% (v/v) trifluoroacetic acid or (ii) 0.3% formic acid and 10 mM ammonium formate and B) methanol containing either (i) 0.05% (v/v) trifluoroacetic acid or (ii) 0.3% formic acid and 10 mM ammonium formate.

[0139] If the LC mobile phase consisted of A) water containing 0.05% (v/v) trifluoroacetic acid and B) methanol containing 0.05% (v/v) trifluoroacetic acid, gradient elution was linear from 5% B to 35% B, 0-4 min, linear from 35% B to 60% B, 4-6.5 min, linear from 60% B to 90% B, 6.5-7 min, isocratic at 90% B 7-11 min, linear from 90% B to 95% B, 11-12 min, linear from 95% B to 5% B, 12-13 min, with a 2 min re-equilibration period between runs. The flow rate was 0.25 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of protonated molecular ions ($[M + H]^+$) of the analytes of interest, and production of characteristic fragment ions. The following instrumental parameters were used for LC/MS/MS Multiple Reaction Monitoring (MRM) analysis of monatin and tryptophan: Capillary: 3.5 kV; Cone: 40 V; Hex 1: 20 V; Aperture: 0 V; Hex 2: 0 V; Source temperature: 100 °C; Desolvation temperature: 350 °C; Desolvation gas: 500 L/h; Cone gas: 50 L/h; Low mass resolution (Q1): 12.0; High mass resolution (Q1): 12.0; Ion energy: 0.2; Entrance: -5 V; Collision Energy: 8; Exit: 1V; Low mass resolution (Q2): 15; High mass resolution (Q2): 15; Ion energy (Q2): 3.5; Multiplier: 650. Five monatin-specific parent-to daughter MRM transitions are used to specifically detect monatin in *in vitro* and *in vivo* reactions. The transitions monitored are 293.1 to 158.3, 293.1 to 168.2, 293.1 to 211.2, 293.1 to 230.2, and 293.1 to 257.2. Tryptophan is monitored with the MRM transition 204.7 to 146.4. For internal standard quantification of monatin and tryptophan, four calibration standards containing four different ratios of each analyte to d5-tryptophan and d5-monatin, are analyzed. These data are subjected to a linear least squares analysis to form a calibration curve for monatin and tryptophan. To each sample is added a fixed amount of d5-tryptophan and d5-monatin (d5-monatin was synthesized from d5-tryptophan according to the methods from WO03/091396 A2), and the response ratios (monatin/d5-monatin; tryptophan/d5-tryptophan) used in conjunction

with the calibration curves described above to calculate the amount of each analyte in the mixtures.

[0140] If the LC mobile phase was A) water containing 0.3% formic acid and 10 mM ammonium formate and B) methanol containing 0.3% formic acid and 10 mM ammonium formate, the gradient elution was linear from 5% B to 45% B, 0-8.5 min, linear from 45% B to 90% B, 8.5-9 min, isocratic from 90% B to 90% B, 9-12.5 min, linear from 95% B to 5% B, 12.5-13 min, with a 4 min re-equilibration period between runs. The flow rate was 0.27 mL/min, and PDA absorbance was monitored from 210 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of protonated molecular ions ($[M + H]^+$) of the analytes of interest, and production of characteristic fragment ions. The instrumental parameters used for this secondary mobile phase are the same as above. Four monatin-specific parent-to daughter MRM transitions and one tryptophan specific parent to daughter transition are used to specifically detect monatin and tryptophan in *in vitro* and *in vivo* reactions. The transitions monitored are 293.1 to 158.0, 293.1 to 168.0, 293.1 to 211.5, and 293.1 to 257.0. Tryptophan is monitored with the MRM transition 205.2 to 146.1. For internal standard quantification of monatin and tryptophan, four calibration standards containing four different ratios of each analyte to d5-tryptophan and d5-monatin, are analyzed. These data are subjected to a linear least squares analysis to form a calibration curve for monatin and tryptophan. To each sample is added a fixed amount of d5-tryptophan and d5-monatin (d5-monatin was synthesized from d5-tryptophan according to the methods from WO03/091396 A2), and the response ratios (monatin/d5-monatin; tryptophan/d5-tryptophan) in conjunction with the calibration curves described above are used to calculate the amount of each analyte in the mixtures. Parent to daughter mass transitions monitored for d5-tryptophan and d5-monatin are 210.2 to 151.1, and 298.1 to 172.0 respectively.

Accurate Mass Measurement of Monatin

[0141] High resolution MS analysis was carried out using an Applied Biosystems-Perkin Elmer Q-Star hybrid quadrupole/time-of-flight mass spectrometer. The measured mass for protonated monatin used tryptophan as an internal mass calibration standard. The calculated mass of protonated monatin, based on the elemental composition $C_{14}H_{17}N_2O_5$

is 293.1137. Monatin produced using the biocatalytic process described in Examples 2 and 3 showed a measured mass of 293.1144. This is a mass measurement error of less than 2 parts per million ("ppm"), providing conclusive evidence of the elemental composition of monatin produced enzymatically.

Chiral LC/MS/MS ("MRM") Measurement of Monatin

- [0142] Determination of the stereoisomer distribution of monatin in *in vitro* and *in vivo* reactions was accomplished by derivitization with 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide ("FDAA"), followed by reversed-phase LC/MS/MS MRM measurement.

Derivatization of Monatin with FDAA

- [0143] To 50 μ L of sample or standard and 10 μ L of internal standard was added either 100 μ L or 200 μ L of a 1% solution of FDAA in acetone. Twenty or forty μ L, respectively, of 1.0 M sodium bicarbonate was added, and the mixture incubated for 1 h at 40 °C with occasional mixing. The sample was removed and cooled, and neutralized with 20 μ L of 2.0 M HCl (more HCl may be required to effect neutralization of a buffered biological mixture). After degassing was complete, samples were ready for analysis by LC/MS/MS.

LC/MS/MS Multiple Reaction Monitoring for the Determination of the Stereoisomer Distribution of Monatin in *in vitro* and *in vivo* Reactions

- [0144] Analyses were performed using the LC/MS/MS instrumentation described above. LC separations capable of separating all four stereoisomers of monatin (specifically FDAA-monatin) were performed on a Phenomenex Luna 2.0 x 250 mm (3 μ m) C18 (2) reversed phase chromatography column at 40 °C. The LC mobile phase consisted of A) water containing 0.05% (mass/volume) ammonium acetate and B) acetonitrile. The elution was isocratic at 13% B, 0-2 min, linear from 13% B to 30% B, 2-15 min, linear from 30% B to 80% B, 15-16 min, isocratic at 80% B 16-21 min, and linear from 80% B to 13% B, 21-22 min, with an 8 min re-equilibration period between runs. The flow rate was 0.23 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of

deprotonated molecular ions ($[M - H]^-$) of FDAA-monatin, and production of characteristic fragment ions.

- [0145] The following instrumental parameters were used for LC/MS analysis of monatin in the negative ion ESI/MS mode: Capillary: 2.0 kV; Cone: 25 V; Hex 1: 10 V; Aperture: 0 V; Hex 2: 0 V; Source temperature: 100°C; Desolvation temperature: 350 °C; Desolvation gas: 500 L/h; Cone gas: 50 L/h; Low mass resolution (Q1): 12.0; High mass resolution (Q1): 12.0; Ion energy: 0.2; Entrance: -5V; Collision Energy: 20; Exit: 1V; Low mass resolution (Q2): 12; High mass resolution (Q2): 12; Ion energy (Q2): 3.0; Multiplier: 650. Three FDAA-monatin-specific parent-to daughter transitions are used to specifically detect FDAA-monatin in *in vitro* and *in vivo* reactions. The transitions monitored for monatin are 543.2 to 268.1, 543.2 to 499.3, and 543.2 to 525.3. Monatin internal standard derivative mass transition monitored was 548.2 to 530.3. Identification of FDAA-monatin stereoisomers is based on chromatographic retention time as compared to purified synthetic monatin stereoisomers, and mass spectral data. An internal standard is used to monitor the progress of the reaction and for confirmation of retention time of the S,S stereoisomer.

Liquid Chromatography-Post Column Fluorescence Detection of Amino Acids Including Glutamate and Alanine

- [0146] Liquid chromatography with post-column fluorescence detection (LC/OPA) for the determination of glutamate and alanine in *in vitro* and *in vivo* reactions was performed on a Waters 2690 LC system or equivalent combined with a Waters 474 scanning fluorescence detector, and a Waters post-column reaction module. Semi-quantitative analyses of monatin and tryptophan were also performed using this method. LC separations were performed on an Interaction-Sodium loaded ion exchange column at 60°C. Mobile phase A was Pickering Na 328 buffer (Pickering Laboratories, Inc.; Mountain View, CA). Mobile phase B was Pickering Na 740 buffer. The gradient elution was from 0% B to 100% B, 0-20 min, isocratic at 100% B, 20-36 min, and linear from 100% B to 0% B, 36-37 min, with at least a 5 min re-equilibration period between runs, depending on sample matrix. The flow rate for the mobile phase was 0.5 mL/min. The flow rate for the OPA post-column derivatization solution was 0.5 mL/min. The fluorescence detector settings were EX 338-340 nm and Em 420-425 nm. Norleucine

was employed as an internal standard for the analysis. Identification of amino acids was based on chromatographic retention time data for purified standards.

Detection of L- and D-Amino Acids by LC/MS/MS

[0147] Samples containing a mixture of L- and D-amino acids such as lysine, alanine, methionine, tyrosine, leucine, phenylalanine, tryptophan, glutamate, and aspartate from biochemical reaction experiments were first treated with formic acid to denature protein. The sample was then centrifuged and filtered through a 0.45 μ m nylon syringe filter prior to LC/MS/MS analysis. Identification of L- and D-amino acids was based on retention time and mass selective detection. LC separation was accomplished by using Waters 2690 liquid chromatography system and an ASTEC 2.1 mm x 250 mm Chirobiotic TAG chromatography column with column temperature set at 45° C. LC mobile phase A and B were 0.25% acetic acid and 0.25% acetic acid in methanol, respectively. Isocratic elution was used for all methods to separate the L and D isomers. Lysine was eluted using 80% mobile phase A, and 20% B. Glutamate, alanine, and methionine were separated with elution of 60% mobile phase A and 40% B and a flow rate of 0.25 mL/min. Aspartate, tryptophan, tyrosine, leucine, and phenylalanine were separated isomerically with 30% mobile phase A and 70% B with a flow rate of 0.3 mL/min for all but phenylalanine, which was run at a flow rate of 0.25 mL/min.

[0148] The detection system for analysis of L- and D-amino acids included a Waters 996 Photo-Diode Array (PDA) detector and a Micromass Quattro Ultima triple quadrupole mass spectrometer. The PDA, scanning from 195 to 350 nm, was placed in series between the chromatography system and the mass spectrometer. Parameters for the Micromass Quattro Ultima triple quadrupole mass spectrometer operating in positive electrospray ionization mode (+ESI) were set as the following: Capillary: 3.0 kV; Cone: 20 V; Hex 1: 15 V; Aperture: 1 V; Hex 2: 0 V; Source temperature: 100 °C; Desolvation temperature: 350 °C; Desolvation gas: 530 L/h; Cone gas: 30 L/h; Low mass Q1 resolution: 12.5; High mass Q1 resolution: 12.5; Ion energy 1: 0.2; Entrance: -5; Collision: 8; Exit 1: 10; Low mass Q2 resolution: 12.5; High mass Q2 resolution: 12.5; Ion energy 2: 0.5; Multiplier: 650 V. MS/MS experiments with Multiple Reaction Monitoring (MRM) mode were set up to selectively monitor reaction transitions of 147.8 to 84.2 and 147.8 to 102.1 for glutamate, 134.00 to 74.30, and 134.00 to 88.2 for aspartate, 147.3 to 85.0 for lysine, 150.3 to 104.8 for methionine, 182.3 to 137.0 for

tyrosine, 132.3 to 87.0 for leucine, and 166.3 to 121.0 for phenylalanine. In the case where two transitions are listed, the latter transitions were used for quantification. For tryptophan, MS/MS experiments with Multiple Reaction Monitoring (MRM) mode were set up to selectively monitor reaction transitions of 205.2 to 118.2, 205.2 to 146.1, and 205.2 to 188.2, and the transition from 212.1 to 151.1 for d8-DL tryptophan. Tryptophan quantification was achieved by determining the ratio of analyte response of transition 205.2 to 146.1 to that of the internal standard, d8-D,L tryptophan. Alternatively, quantification of tryptophan, glutamate, and aspartic acids were based off signal responses of $m/z=146.5$, $m/z=102.1$, and $m/z=88.2$, respectively.

Production of Monatin and Monatin Precursor ("MP") for Standards and for Assays

Production of Monatin

[0149] A racemic mixture of R,R and S,S monatin was synthetically produced as described in U.S. Patent No. 5,128,482.

[0150] The R,R and S,S monatin were separated by a derivatization and hydrolysis step. Briefly, the monatin racemic mixture was esterified, the free amino group was blocked with Cbz, a lactone was formed, and the S,S lactone was selectively hydrolyzed using an immobilized protease enzyme. The monatin can also be separated as described in Bassoli, A. *et al.*, *Eur. J. Org. Chem.*, 8:1652-1658, (2005).

MP Production

[0151] R-MP was produced by the transamination of R,R monatin using AT-103 broad range D-aminotransferase (BioCatalytics) in 0.1 M potassium phosphate buffer, using sodium pyruvate as the amino acceptor. S-MP was produced by the transamination of S,S monatin using AT-102 L-aminotransferase (BioCatalytics) in 0.1 M potassium phosphate buffer, using sodium pyruvate as the amino acceptor. Both reactions were carried out at 30 °C and at a pH of approximately 8.0-8.3, for approximately 20 hours. Both compounds were purified using preparative scale HPLC with a Rohm and Haas (Philadelphia, PA) hydrophobic resin (XADTM1600), eluting in water. Samples containing greater than 90% purity monatin precursor were collected and freeze-dried.

EXAMPLE 2

Production of Monatin from Indole-3-Pyruvate

[0152] AT-103 transaminase was part of a transaminase library purchased from BioCatalytics (Pasadena, CA) and the enzyme was tested for production of monatin in coupled reactions using the ProA aldolase from *C. testosteroni*. The aldolase was prepared as described in WO 03/091396 A2. AT-103 is a broad specificity D-transaminase (EC 2.6.1.21) from a *Bacillus* species that requires a D-amino acid (such as D-glutamate, D-aspartate, or D-alanine) as the amino acid donor. Enzymes and additional components/substrates were added directly to the reaction buffer provided in the kit, which contained 100 mM potassium phosphate buffer pH 7.5, 100 mM amino donor, and 0.1 mM pyridoxal-5'-phosphate ("PLP"). To one mL of reaction buffer were added: 4 mg indole-3-pyruvate, 20 mg pyruvate, approximately 50 µg ProA provided in a cellular extract, 1 µL 2 M MgCl₂, and 2 mg of the aminotransferase enzyme (AT-103). Reactions were performed in duplicate. The reactions were incubated overnight at 30 °C with gentle shaking (100 rpm). The samples were filtered and submitted for reversed-phase LC/MS/MS analysis as described in Example 1. The results indicated that approximately 370 µg/mL monatin were produced using AT-103 enzyme. The results were further analyzed to determine ratios of S,R/R,S versus R,R/S,S monatin, on the basis of the peak areas of the two stereoisomer pools that resolve during the chromatographic separation. Of the total monatin produced by AT-103, 69% was R,R/S,S monatin in comparison to the mixed isomers. This enzyme (AT-103) is homologous to the *Bacillus subtilis* DAT enzyme described in WO 03/091396 A2, which is known to have a broad specificity for D-amino acids. Chiral analysis was performed using the FDAA methodology described in Example 1, which verified that the D-aminotransferase was making predominantly R,R monatin, and some S,R monatin as expected. Further transamination experiments with S,S monatin or R,R monatin and α-ketoglutarate as substrates verified that the BioCatalytics enzyme was highly selective for the D-configuration at carbon 4, as expected. In these experiments, no glutamate was detected in the reaction with S,S monatin and α-ketoglutarate as substrates.

[0153] To decrease the amount of S,S monatin or R,S monatin produced as byproducts in coupled reactions with AT-103 (the broad range D-transaminase) and the ProA aldolase, the aldolase was purified using His-Bind cartridges, following manufacturer's protocols (Novagen, Madison, WI). The purified enzyme preferably should not contain wild-type L-aminotransferase activities that can be present in cellular extracts (such as the native *E. coli* AspC or TyrB activities). The His-Bind eluent was desalted to remove imidazole using PD-10 columns (G25 Sephadex, Amersham-Pharmacia) and was eluted in 50 mM Tris-Cl, pH 7. Experiments were carried out in duplicate in a volume of 1 mL and contained 100 mM Tris-Cl buffer, pH 7.8, 50 µg ProA aldolase, 4 mg indole-3-pyruvate, 1 or 2 mg D-aminotransferase, 200 mM sodium pyruvate, 2 mM MgCl₂, 3 mM potassium phosphate, 0.1 mM PLP, and 14.7 mg of D-glutamate. The tubes were incubated at 30 °C with gentle shaking. Two-hour time points were taken and frozen immediately at -20 °C. The pH was adjusted at two hours from 5 to between 7-8 using NaOH, and the assays were incubated overnight. Samples were filtered and analyzed for monatin as described in Example 1. The two-hour samples did not have detectable amounts of monatin, probably due to the low pH. The overnight samples contained approximately 190 ng/mL monatin when 1 mg of D-aminotransferase was used, and approximately 84% was R,R monatin and 16% was S,R monatin. When 2 mg of D-aminotransferase were used, 540 ng/mL monatin was produced, approximately 71% was R,R monatin.

[0154] Similar experiments were conducted using Biocatalytics Aminotransferase buffer, which contained 100 mM potassium phosphate pH 7.5, 0.1 mM PLP, and 100 mM D-glutamate. Solid indole-3-pyruvate and D-aminotransferase were added as above. ProA aldolase (50 µg), MgCl₂, and 50 mM pyruvate were added from stock solutions. The assays were treated as above, although no pH adjustment was required in this case. A negative control was done with just the BioCatalytics supplied enzyme and buffer, which did not contain monatin. The experimental results are shown in Table 1.

Table 1: Production of Monatin from Indole-3-Pyruvate in Phosphate Buffer

D-Aminotransferase (mg)	Time (hours)	Total Monatin (ng/mL)	%R,R
0	2	0	n/a
1	2	6780	not determined
2	2	13170	55%
0	16	0	n/a
1	16	15000	not determined

D-Aminotransferase (mg)	Time (hours)	Total Monatin (ng/mL)	%R,R
2	16	28930	51%

[0155] The production of monatin in phosphate buffer is clearly higher than that in Tris buffered systems.

[0156] To compare activities of the cloned *B. subtilis* DAT from WO 03/091396 A2 with the BioCatalytics enzyme (AT-103) additional assays were done. The *B. subtilis* *dat* gene was also subcloned into pET30a to remove the His-6 tag. Untagged and tagged enzyme were produced in BL21(DE3), as described in WO 03/091396 A2. Cellular extracts were made and total protein assays were done to estimate protein concentration as described previously. Duplicate one mL reactions were done which contained: 500 µg D-aminotransferase, 50 µg ProA aldolase, 100 mM potassium phosphate pH 7.5, 3 mM MgCl₂, 4 mg indole-3-pyruvate, 200 mM sodium pyruvate, 7.35 mg (50 mM) D-glutamate, and 0.1 mM PLP. Samples were incubated at 30 °C for 1 hour, 2 hours, and overnight, and were filtered for LC/MS/MS analysis. The samples contained only the S,R and R,R stereoisomers of monatin, as determined by the FDAA derivitization protocol described in Example 1. The results are summarized in Table 2 below. The % RR was determined by peak areas that were separated by reversed phase chromatography.

Table 2: Comparison of D-Aminotransferase Enzymes

Enzyme	Time (Hours)	Monatin (ppb)	% RR Monatin
<i>B. sub</i> DAT-HIS	1	512	not determined
<i>B. sub</i> DAT untagged	1	1056	not determined
BioCatalytics AT-103	1	2353	not determined
<i>B. sub</i> DAT-HIS	2	894	~80-90%
<i>B. sub</i> DAT untagged	2	1913	~80%
BioCatalytics AT-103	2	6887	92.5%
<i>B. sub</i> DAT-HIS	16	3014	31
<i>B. sub</i> DAT untagged	16	5612	33
BioCatalytics AT-103	16	16131	66

[0157] The removal of the HIS-6 tag appears to have improved the activity of the *B. subtilis* D-aminotransferase; however, the BioCatalytics D-aminotransferase homolog clearly had the highest activity. It also showed greater substrate preference for the R-monatin precursor. Increased incubation times appear to reduce the enantiomeric excess of R,R monatin that is produced.

[0158] Because the *Bacillus* D-aminotransferase enzymes have a preference for pyruvate as an amino acceptor, and D-alanine as an amino donor, it was expected that D-alanine could be utilized as the amino donor for conversion of MP to monatin with similar or better results. Duplicate one mL reactions were done which contained: 500 μ g D-aminotransferase, 50 μ g purified ProA aldolase, 100 mM potassium phosphate pH 7.5, 3 mM $MgCl_2$, 4 mg indole-3-pyruvate, 100 mM sodium pyruvate, 25 mM D-glutamate or D-alanine, and 0.1 mM PLP. Samples were incubated for 2 hours, and treated as above prior to analysis. When D-alanine was used as the amino donor, slightly higher levels of monatin were produced (23 versus 21 ppm) as expected. Additionally, it is expected that high concentrations of pyruvate may inhibit the transamination step, thus dosing in smaller amounts of pyruvate over time may improve the overall rate of monatin production. One can see from the above data that even though one-half of the pyruvate was used in this case compared to the above table, significantly more monatin was produced. Even though ProA aldolases in the literature were reported to produce primarily S-enantiomers of aldol condensation products, the ProA aldolase used in this study clearly makes a high percentage of R-MP and in coupled assays produces up to 92% R,R monatin. The high percentage of R,R monatin is not due to D-aminotransferase selectivity, as was shown in Example 19.

EXAMPLE 3

3A: Production of R,R Monatin from D-Tryptophan

[0159] The following were added per 1 mL of reaction mixture: approximately 60 μ g *C. testosteroni* ProA aldolase (supplied in cellular extracts, as described in WO 03/091396 A2), 4 mM $MgCl_2$, 50 mM D-tryptophan, 0.5 mg BioCatalytics D-aminotransferase (AT-103), 100 mM sodium pyruvate, 100 mM potassium phosphate buffer pH 7.5 or 100 mM sodium acetate buffer pH 8, 0.05 mM PLP, 3 mM potassium phosphate (only to the acetate reactions), and 10 mM α -ketoglutarate. Experiments were run in duplicate, with negative controls in which no aldolase was added. Samples were incubated overnight (20 hours) at 30 °C with gentle shaking. The actual pH of the sodium acetate samples was approximately 5, while the final pH for the phosphate buffered samples was

approximately 7. None of the aldolases appeared to have significant activity at pH 5; the sample containing ProA aldolase was slightly above the negative control but probably not above experimental error. In potassium phosphate, the ProA aldolase produced 73.4 ppm monatin with a ratio of R,R:S,R of 1.7:1 (~63% R,R from D-tryptophan).

- [0160] Because the *Bacillus* D-aminotransferase enzymes have a preference for pyruvate as an amino acceptor, and D-alanine as an amino donor, it was expected that the addition of alpha-ketoglutarate is unnecessary when producing R,R or S,R monatin from D-tryptophan. The above experiment was repeated (in 100 mM potassium phosphate buffer) using purified ProA aldolase (50-60 µg), and an incubation time of 2.5 hours. Duplicate experiments were run, with and without alpha-ketoglutarate. When 10 mM alpha-ketoglutarate was added, 56.1 ppm monatin was formed using D-tryptophan as the substrate (79.5 % R,R, 20.5% S,R). When alpha-ketoglutarate was omitted, 102.5 ppm monatin was formed (79% R,R, 21% S,R).

Comparison of Total Monatin Production and Isomeric Distribution for HMG Aldolases from Sinorhizobium meliloti, C. testosteroni, and the Aldolase of SEQ ID NO:22.

- [0161] AT-103 transaminase (a broad specificity D-aminotransferase) was purchased from BioCatalytics (Pasadena, CA) and either this enzyme or the *B. sphaericus* recombinant enzyme produced in Example 18 was used in coupled reactions with HMG aldolases to produce monatin from D-tryptophan and pyruvate as described in U.S. Published Application No. 2005282260.
- [0162] The HMG aldolases from *C. testosteroni* (ProA) and *S. meliloti* were prepared and purified as described in U.S. Publication No. 20040063175 and WO 03091396 A2. To produce test quantities of the aldolase of SEQ ID NO:22, a 50 mL culture was grown in Luria-Bertani ("LB") medium containing ampicillin (100 µg/mL), to an OD₆₀₀ of approximately 0.5. The strain containing the SEQ ID NO:21 construct was induced with 200 µg/L anhydrotetracycline. The cells were grown 5 hours post-induction, and cellular extracts were prepared according to manufacturer's protocols (Novagen, Madison, WI, Bugbuster reagent). Benzonuclease and protease inhibitor were also added. The soluble proteins in the cellular extracts were separated on a BioRad Laboratories Experion Automated Electrophoresis Station and analyzed for concentration and percent expression

using the Experion Software version 1.1.98.0. The aldolase of SEQ ID NO:22 was used as a crude (unpurified) enzyme for the reactions below.

[0163] The following were added per 1 mL of reaction mixture: approximately 50 µg aldolase, 4 mM MgCl₂, 50 mM D-tryptophan, 0.5 mg purified *B. sphaericus* D-aminotransferase, 200 mM sodium pyruvate, 100 mM potassium phosphate buffer pH 7.5, and 0.05 mM PLP. Experiments were run in duplicate, with negative controls in which no aldolase was added. Samples were incubated 1 hour and overnight (18 hours) at 30 °C with gentle shaking. Small amounts of monatin (< 0.5 ppm) are produced without aldolase in overnight reactions, due to non-enzymatic reactions catalyzed by magnesium and phosphate. Those values were subtracted from the numbers shown below, and averaged results are shown. The only stereoisomers detected when producing monatin using these methods are R,R and S,R. The percent R,R is listed below in Table 3, and was determined by reversed-phase LC peak area.

Table 3: Total Monatin Produced from D-Tryptophan and % R,R

Enzyme (time point)	Total Monatin (ppm)	% R,R Monatin
<i>C. testosteroni</i> ProA (1 hour)	16.63	86.45
<i>C. testosteroni</i> ProA (18 hours)	86.86	63.1
<i>S. meliloti</i> HMG (1 hour)	20.5	96.7
<i>S. meliloti</i> HMG (18 hours)	88.3	89.9
SEQ ID NO:22 (1 hour)	14.70	100
SEQ ID NO:22 (18 hours)	95.14	97.35

[0164] The 18 hour sample for the aldolase of SEQ ID NO:22 was also analyzed for stereoisomeric distribution by the FDAA derivatization method listed in Example 1, which yielded a result of 94.9% R,R and 5.1% S,R monatin. The aldolase of SEQ ID NO:22 has a higher enantiospecificity for production of R-MP as compared to *C. testosteroni* and *S. meliloti* HMG aldolases.

[0165] The same experiments were done, side by side, using L-tryptophan as the starting substrate and coupling the aldolases with HexAspC broad specificity L-aminotransferase produced and purified as described in U.S. Published Application No. 2005282260. These reactions should yield primarily S,S monatin and R,S monatin. The reactions were also supplemented with 10 mM alpha-ketoglutarate as the amino acceptor for L-

tryptophan transamination. Again, duplicate results are averaged below for total monatin (subtracting background levels without aldolase present), and percent S,S monatin is shown based on reversed phase LC peak area. In some cases, because the aldolases are quite R-specific and produce little total monatin, the reversed phase estimates of stereoisomeric distribution are less accurate due to some tailing of the tryptophan peak that can co-elute with the S,S/R,R monatin peak. The trends are still informative in comparing R-specificity of the aldolases. Results from further analysis using the FDAA derivatization method are shown below in Table 4 in parentheses for several samples, and are more accurate. Total monatin numbers above approximately 400 ppm are higher than the linear range of the scale of the standards used to quantitate the results, so are qualitative results. The *C. testosteroni* ProA aldolase typically produces 95-100% S,S monatin, as shown in U.S. Published Application No. 2005282260.

Table 4: Total Monatin Produced from L-Tryptophan and % S,S

Enzyme (time point)	Total Monatin (ppm)	% S,S Monatin
<i>C. testosteroni</i> ProA (1 hour)	440.35	92.5
<i>C. testosteroni</i> ProA (18 hour)	958.3	92.2
<i>S. meliloti</i> HMG (1 hour)	45.9	66.3
<i>S. meliloti</i> HMG (18 hour)	108.1	61.4
SEQ ID NO:22 (1 hour)	17.85	55.1 (18.9)
SEQ ID NO:22 (18 hour)	135.5	27.3 (19.1)

[0166] One can see that the R-specificity of the aldolase of SEQ ID NO:22 is quite high compared to the benchmark ProA enzyme. This R-specificity is also reflected in the low % S,S monatin produced, despite the high degree of specificity of the HexAspC aminotransferase for S-MP in these reactions. Again the *S. meliloti* HMG aldolase falls between the *C. testosteroni* ProA aldolase and the aldolase of SEQ ID NO:22 in terms of R-specificity, based on the levels of S,S monatin produced. The total monatin numbers, when comparing S,S monatin production versus R,R monatin production, are not indicative of the aldolase activity. The D-aminotransferase is less active than HexAspC for MP transamination reactions, particularly at the concentrations of MP that are present in these reactions.

[0167] For further comparison of the aldolase of SEQ ID NO:22 to the ProA enzyme from *C. testosteroni*, varying ratios of D-aminotransferase to aldolase were utilized in reactions starting with D-tryptophan (no duplicate samples for these experiments). The

reactions were carried out as described above. For the reactions in which the aldolase concentration was kept constant, approximately 50 µg aldolase was used. For reactions in which the amount of D-aminotransferase was kept constant, 0.5 mg was used. For the 2 and 10 mg/mL concentration of D-aminotransferase, lyophilized enzyme was used. For the 2 highest D-aminotransferase concentrations, duplicates were run.

Table 5. Effect of D-Aminotransferase Concentration on R,R Monatin Production

Aldolase	Concentration of D-Aminotransferase	Time	Total Monatin (approximate ppm)	% R,R Monatin
SEQ ID NO:22	0.25 mg/mL	1 hour	2	100
SEQ ID NO:22	0.25 mg/mL	overnight	141	97.1
SEQ ID NO:22	0.5 mg/mL	1 hour	8	100
SEQ ID NO:22	0.5 mg/mL	overnight	273	96.5
SEQ ID NO:22	1 mg/mL	1 hour	34	100
SEQ ID NO:22	1 mg/mL	overnight	638	96.5
SEQ ID NO:22	2 mg/mL	1 hour	979	100
SEQ ID NO:22	2 mg/mL	overnight	1910	97.3
SEQ ID NO:22	10 mg/mL	1 hour	2930	99.1
SEQ ID NO:22	10 mg/mL	overnight	2950	96.5
<i>C. testosteroni</i> ProA	0.25 mg/mL	1 hour	4	78.7
<i>C. testosteroni</i> ProA	0.25 mg/mL	overnight	257	61.1
<i>C. testosteroni</i> ProA	0.5 mg/mL	1 hour	25	79.0
<i>C. testosteroni</i> ProA	0.5 mg/mL	overnight	480	62.5
<i>C. testosteroni</i> ProA	1 mg/mL	1 hour	74	73.8
<i>C. testosteroni</i> ProA	1 mg/mL	overnight	810	68.1
<i>C. testosteroni</i> ProA	2 mg/mL	1 hour	325	73.1
<i>C. testosteroni</i> ProA	2 mg/mL	overnight	2220	71.9
<i>C. testosteroni</i> ProA	10 mg/mL	1 hour	2910	59.7
<i>C. testosteroni</i> ProA	10 mg/mL	overnight	2450	67.5

[0168] For monatin levels above 400 ppm, the results are not in the linear range of the standard curve and are approximate values only. The maximum amount of R,R monatin produced, when diluted appropriately, was approximately 1100 ppm. FDAA stereoisomeric analysis was done for the aldolase of SEQ ID NO:22 with 10 mg/mL D-aminotransferase samples. At two hours, the sample contained 98.5% R,R monatin. At 17 hours, the sample contained 95.9% R,R monatin. The aldolase of SEQ ID NO:22 produced high percentages of R,R monatin, even after long incubation times and using large amounts of aminotransferase. If adequate D-aminotransferase is supplied, the

aldolase of SEQ ID NO:22 produces as much total monatin as *C. testosteroni* ProA aldolase, indicating a similar specific activity.

Table 6. Effect of Aldolase Concentration on R,R Monatin Production

Aldolase	Concentration of Aldolase	Time	Total Monatin (ppm)	% R,R Monatin
SEQ ID NO:22	25 µg/mL	1 hour	7.0	100
SEQ ID NO:22	25 µg/mL	overnight	275	97.4
SEQ ID NO:22	50 µg/mL	1 hour	9.0	97.3
SEQ ID NO:22	50 µg/mL	overnight	334	95.7
SEQ ID NO:22	100 µg/mL	overnight	297	93.3
<i>C. testosteroni</i> ProA	25 µg/mL	1 hour	16	78.2
<i>C. testosteroni</i> ProA	25 µg/mL	overnight	491	73.2
<i>C. testosteroni</i> ProA	50 µg/mL	1 hour	18	64.1
<i>C. testosteroni</i> ProA	50 µg/mL	overnight	437	63.0
<i>C. testosteroni</i> ProA	100 µg/mL	1 hour	26	62.5
<i>C. testosteroni</i> ProA	100 µg/mL	overnight	513	61.5

[0169] When the aldolase concentration is varied, there is not much of an increase in total monatin. The percent R,R decreases with time and also with aldolase concentration, particularly when the D-aminotransferase is limiting.

[0170] To further examine the R-specificity of the aldolases tested, experiments were done starting with L-tryptophan and HexAspC aminotransferase, which was produced and purified as described in U.S. Published Application No. 2005282260. The HexAspC shows a strong selectivity for transamination of S-MP versus R-MP, thus percentages above 50% R,S monatin indicate a highly stereospecific aldolase. Ten mM alpha-ketoglutarate was supplied as an amino acceptor; however, at high concentrations, pyruvate is also utilized by the L-aminotransferase. In these reactions, typically only S,S and R,S monatin are produced within the limits of detection of the FDAA derivatization protocol.

Table 7. Effect of L-Aminotransferase Concentration on S,S Monatin Production

Aldolase	Concentration of L-Aminotransferase	Time	Total Monatin (approximate ppm)	% S,S Monatin
SEQ ID NO:22	0.25 mg/mL	1 hour	13	33.8
SEQ ID NO:22	0.25 mg/mL	overnight	127	34.2
SEQ ID NO:22	0.5 mg/mL	1 hour	31	30.9

Aldolase	Concentration of L-Aminotransferase	Time	Total Monatin (approximate ppm)	% S,S Monatin
SEQ ID NO:22	0.5 mg/mL	overnight	272	26.8
SEQ ID NO:22	1 mg/mL	1 hour	34	20.3
SEQ ID NO:22	1 mg/mL	overnight	385	23.5
<i>C. testosteroni</i> ProA	0.25 mg/mL	1 hour	523	94.2
<i>C. testosteroni</i> ProA	0.25 mg/mL	overnight	1817	93.7
<i>C. testosteroni</i> ProA	0.5 mg/mL	1 hour	602	91.8
<i>C. testosteroni</i> ProA	0.5 mg/mL	overnight	2122	89.9
<i>C. testosteroni</i> ProA	1 mg/mL	1 hour	873	90.2
<i>C. testosteroni</i> ProA	1 mg/mL	overnight	1237	82.6

Table 8. Effect of Aldolase Concentration on S,S Monatin Production

Aldolase	Concentration of Aldolase	Time	Total Monatin (ppm)	% S,S Monatin
SEQ ID NO:22	25 µg/mL	1 hour	11	25.1
SEQ ID NO:22	25 µg/mL	overnight	112	20.0
SEQ ID NO:22	50 µg/mL	1 hour	18	31.8
SEQ ID NO:22	50 µg/mL	overnight	160	27.0
SEQ ID NO:22	100 µg/mL	1 hour	33	33.2
SEQ ID NO:22	100 µg/mL	overnight	238	41.4
<i>C. testosteroni</i> ProA	25 µg/mL	1 hour	305	86.4
<i>C. testosteroni</i> ProA	25 µg/mL	overnight	1094	87.5
<i>C. testosteroni</i> ProA	50 µg/mL	1 hour	575	90.9
<i>C. testosteroni</i> ProA	50 µg/mL	overnight	1449	89.5
<i>C. testosteroni</i> ProA	100 µg/mL	1 hour	817	93.6
<i>C. testosteroni</i> ProA	100 µg/mL	overnight	1360	89.7

[0171] For aldolases that are highly R-specific, such as SEQ ID NO:22, less total monatin is produced and increasing the amount of aldolase does increase total monatin (as well as % S,S). These aldolases produce less S-MP substrate, the preferred substrate for the L-aminotransferase used. For enzymes that are less R-specific, such as ProA, increasing aldolase does not significantly improve total monatin production or % S,S monatin. Increasing the amount of L-aminotransferase added decreases the percentage of S,S monatin produced.

[0172] The activity and specificity of the aldolase of SEQ ID NO:22 was further studied in two buffer systems—100 mM potassium phosphate, as above, and 100 mM 3-(N-morpholino)propanesulfonic acid ("MOPS") (with 3 mM potassium phosphate). The assays were performed as above, using 1 mg/ml AT-103 D-aminotransferase and 50 mM D-tryptophan. Experiments were run in duplicate for 4.5 hours. The aldolase of SEQ ID NO:22 produced 116 ppm monatin and 99.1 % R,R monatin in potassium phosphate

(FDAA derivatization method). In MOPS, the aldolase of SEQ ID NO:22 produced 75.5 ppm monatin, and 96.2% was R,R monatin. The background levels of monatin produced in MOPS, without the aldolase of SEQ ID NO:22, were significantly higher, and the percent R,R was lower with MOPS, even in the controls. It is possible that the D-aminotransferase selectivity and activity are affected by the presence of the MOPS.

Subcloning of SEQ ID NO:21

[0173] The aldolase gene of SEQ ID NO:21 was received from Diversa Corporation, San Diego, California. SEQ ID NO:21 was part of an environmental library which was screened by Diversa Corporation for aldolase genes. However, the aldolase gene of SEQ ID NO:21 may be reconstructed by any method known to a person of ordinary skill in the art. For example, the aldolase gene of SEQ ID NO:21 may be reconstructed utilizing assembly PCR methods, as described in Examples 10, 18 and 19.

[0174] The following primers were used to PCR amplify the aldolase gene (SEQ ID NO:21): 5'-gaggagctcgagtcagacgtatttcagtccttttc-3' (SEQ ID NO:23) and 5'-agaagacatatgatttatcagccggggac-3' (SEQ ID NO:24). The resulting PCR product was digested with *Xho* I and *Nde* I to cut at the sites that had been engineered into the primers. The fragment was gel purified (QIAquick® Gel extraction Kit (Qiagen, Valencia, CA)) and ligated (using T4 DNA ligase) with pET28b (EMD Biosciences/Novagen Madison, WI) that had been digested with *Xho* I and *Nde* I and gel purified. The ligation was transformed into TOP10F' chemically competent cells (Invitrogen, Carlsbad, CA). Colonies growing on the plates were screened for inserts and several isolates with inserts were submitted for DNA sequence analysis (Agencourt, Beverly, MA).

Purification of the Aldolase of SEQ ID NO:22

[0175] Confirmed aldolase clones were transformed into either BL21 (DE3) or BL21 (DE3) pLysS competent cells (Novagen Madison, WI). Overnight cultures grown with the appropriate antibiotic were diluted into fresh media (typically 1:100) and grown to an OD₆₀₀ ~0.6 with aeration at 37 °C. Cultures were then induced with 1 mM isopropyl thiogalactoside ("IPTG") and shifted to 30 °C (with aeration) and incubation was continued overnight. Cells were harvested by centrifugation. The cell pellet was typically subjected to one freeze thaw cycle to assist with cell lysis. The cell pellet was lysed in BugBuster and Benzonase (Novagen, Madison, WI) (according to the

manufacturer's protocol). Cell debris was removed by centrifugation. The crude protein extract was applied to a HisBind column (Novagen, Madison, WI) that had been prepared according to the manufacturer's protocol. The column was washed and protein was eluted according to the manufacturer's protocol. The purified protein was desalted with PD-10 columns (GE Healthcare, Piscataway, NJ). The buffer used for the exchange was 50 mM potassium phosphate pH 7.5, 100 mM NaCl, 4 mM MgCl₂. Purified protein was concentrated with Amicon centrifugal concentrators (Millipore, Billerica, MA).

3B: Cloning of SEQ ID NO:103 and Assay of the Aldolase of SEQ ID NO:104

[0176] The gene encoding the aldolase of SEQ ID NO:104 (the DNA sequence of the gene is shown as SEQ ID NO:103) was subcloned into the pET28b expression vector (EMD Biosciences/Novagen, Madison, WI) with an N-terminal His-tag to allow for purification.

[0177] The primers used for cloning are shown below:

5'-ATAAGACATATGCCTATCGTTGTTACGAAG-3' (*Nde* I restriction site) (SEQ ID NO:105) and

5'-ATAAGAGGATCCTTATTCCTCGGGCAGCCGCTC-3' (*Bam*H I restriction site) (SEQ ID NO:106).

[0178] A clone containing SEQ ID NO:103 was received from Diversa Corporation, San Diego, CA, and used as a template for PCR. However, SEQ ID NO:103 can be reconstructed by other methods known to a person of ordinary skill in the art. For example, SEQ ID NO:103 can be reconstructed utilizing assembly PCR methods, as described in Examples 10, 18 and 19. SEQ ID NO:103 was amplified by PCR, digested with appropriate enzymes (*Nde* I and *Bam*H I), and gel purified (QIAquick[®] Gel extraction Kit (Qiagen, Valencia, CA)). The digest was ligated into pET28b (EMD Biosciences/Novagen Madison, WI) that had been digested with *Nde* I and *Bam*H I and gel purified. The ligation was transformed into TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA). Miniprep DNA from colonies was analyzed for the presence of inserts by size comparison using agarose gel electrophoresis. Isolates with an insert were submitted for DNA sequence analysis (Agencourt, Beverly, MA).

Purification of Aldolases

[0179] The confirmed aldolase clone was transformed into BL21(DE3)pLysS competent cells (Novagen Madison, WI). The aldolase of SEQ ID NO: 22 was also prepared simultaneously for comparison of activity. Induction was overnight in Terrific Broth (Tartoff and Hobbs, Bethesda Research Laboratories Focus 9:12 (1987) at 30 °C with 50 µg/mL kanamycin. Overnight cultures grown with the appropriate antibiotic were diluted into fresh media (typically 1:100) and grown to an OD₆₀₀ ~0.6, with aeration, at 37 °C. The cultures were then induced with 1 mM IPTG and shifted to 30 °C (with aeration) and incubation was continued overnight. The cells were harvested by centrifugation. The cell pellet was typically subjected to one freeze thaw cycle to assist with cell lysis. The cell pellet was lysed in BugBuster and Benzonase Nuclease (EMD Biosciences/Novagen, Madison, WI) (according to the manufacturer's protocol). Cell debris was removed by centrifugation. The crude protein extract was applied to a 10 mg capacity HIS-Bind column (EMD Biosciences/Novagen, Madison, WI) that had been prepared according to the manufacturer's protocol. The column was washed and protein was eluted according to the manufacturer's protocol. The purified protein was desalted with PD-10 columns (GE Healthcare, Piscataway, NJ) and eluted in 50 mM potassium phosphate buffer, pH 7.5 containing 4 mM MgCl₂ and 200 mM NaCl. Purified protein was concentrated with Amicon centrifugal concentrators (5000 MW cutoff) (Millipore, Billerica, MA). After concentration, it was noted that some of the aldolase of SEQ ID NO:104 had precipitated. However, the precipitation did not appear to affect the level of activity of the enzyme. Protein was stored at -80 °C until assayed. The protein assays were done using the Pierce BCA kit (Rockford, IL) and the microtiter plate protocol using Bovine Serum Albumin ("BSA") as the protein standard. The Experion Pro260 electrophoresis system (Bio-Rad, Hercules, CA) was used to calculate the percentage of aldolase in the purified sample, and to evaluate expression levels in the soluble cell extract and in total protein.

Testing of Purified Aldolases

[0180] Purified aldolases were tested for their ability to produce R,R monatin from D-tryptophan. Assays were done in microcentrifuge tubes in duplicate with purified protein, using the same concentration of enzyme per assay (50 µg/mL). Two mg/mL of Biocatalytics AT-103 was used as the D-aminotransferase. The following were added per

1 mL of reaction mixture: aldolase, 4 mM MgCl₂, 50 mM D-tryptophan, D-aminotransferase, 200 mM sodium pyruvate, 100 mM potassium phosphate buffer pH 7.5, and 0.05 mM PLP. The samples were incubated at 30 °C with shaking. Thirty minute, 1 hour, 3 hour, and overnight (19 hour) samples were taken. Table 9 shows the averaged results of total monatin produced at each time point and the % R,R monatin produced, as determined by reversed phase peak areas. Additional FDAA-derivatization LC/MS/MS analysis as described in Example 1 was done for some of the reactions and is shown in parentheses.

Table 9: Total Monatin Produced from D-tryptophan and % R,R

Aldolase (hr)	Total monatin (ppm)	% R,R monatin (Reversed Phase LC peak area)
SEQ ID NO:22 (0.5)	16	99.1
SEQ ID NO:22 (1)	53.2	99.2 (99.0)
SEQ ID NO:22 (3)	207.8	98.6 (98.1)
SEQ ID NO:22 (19)	544.9	95.3 (93.2)
SEQ ID NO:104 (0.5)	37.8	98.8
SEQ ID NO:104 (1)	71.2	99.3 (99.5)
SEQ ID NO:104 (3)	245.2	99.0 (99.0)
SEQ ID NO:104 (19)	585.4	96.7 (96.1)
no aldolase (0.5)	0	
no aldolase (1)	0	
no aldolase (3)	0.6	58.3
no aldolase (19)	6.5	61.5

[0181] The aldolase of SEQ ID NO:104 had higher activity than the aldolase of SEQ ID NO:22 and higher stereospecificity for production of R,R monatin.

[0182] The DNA sequence of the gene that encodes the aldolase of SEQ ID NO:104 is shown below:

```
atgcctatcg ttgttacgaa gatcgaccga cccagcgcg cggaacgtcga
aaggatcgcc gcctatggtg tcgagacctt gcatgaagcg caaggacgaa
ccgggttgat ggcgtccaat atgcgcccaa tctatcgccc tgcgcacatt
gccgggcccc cggtgacctg ccttgtggcg cctggcgaca attggatgat
ccatgtcgcc gtcgaacagt gccagccggg agatgtcctg gtcgtggtac
cgaccagccc ctgcgaagac ggctatttcg gcatctgct ggcgacctcg
ctgcggtcgc gcgggggtcaa aggtctgac atcgaggccg gcgtacgcga
```

tatcgcgaca ttgaccgaga tgaaattccc ggtctggtcc aaggcgggtgt
 tcgcgcaagg aacgggtcaag gagaccatcg ccagcgtcaa tgtgccccto
 gtctgcgcgg gcgcccgcac cgtgccgggc gatctgatcg ttgccgacga
 cgacgggggtc gtcgtgattc caagacgttc cgttccggcg gtcctttcca
 gcgccgaggc ccgcgaagag aaggaagccc gcaaccgcgc ccgcttcgaa
 gctggcgagc tgggcctcga cgtctacaac atgcgccagc gcctggccga
 caagggcttg cgctatgtcg agcggctgcc cgaggaatag (SEQ ID
 NO:103).

[0183] The protein sequence of the aldolase of SEQ ID NO:104 is as follows:

Met Pro Ile Val Val Thr Lys Ile Asp Arg Pro Ser Ala Ala Asp Val
 Glu Arg Ile Ala Ala Tyr Gly Val Ala Thr Leu His Glu Ala Gln Gly
 Arg Thr Gly Leu Met Ala Ser Asn Met Arg Pro Ile Tyr Arg Pro
 Ala His Ile Ala Gly Pro Ala Val Thr Cys Leu Val Ala Pro Gly Asp
 Asn Trp Met Ile His Val Ala Val Glu Gln Cys Gln Pro Gly Asp
 Val Leu Val Val Val Pro Thr Ser Pro Cys Glu Asp Gly Tyr Phe
 Gly Asp Leu Leu Ala Thr Ser Leu Arg Ser Arg Gly Val Lys Gly
 Leu Ile Ile Glu Ala Gly Val Arg Asp Ile Ala Thr Leu Thr Glu Met
 Lys Phe Pro Val Trp Ser Lys Ala Val Phe Ala Gln Gly Thr Val Lys
 Glu Thr Ile Ala Ser Val Asn Val Pro Leu Val Cys Ala Gly Ala Arg
 Ile Val Pro Gly Asp Leu Ile Val Ala Asp Asp Asp Gly Val Val Val
 Ile Pro Arg Arg Ser Val Pro Ala Val Leu Ser Ser Ala Glu Ala Arg
 Glu Glu Lys Glu Ala Arg Asn Arg Ala Arg Phe Glu Ala Gly Glu
 Leu Gly Leu Asp Val Tyr Asn Met Arg Gln Arg Leu Ala Asp Lys
 Gly Leu Arg Tyr Val Glu Arg Leu Pro Glu Glu (SEQ ID NO:104).

EXAMPLE 4

4A: (1) Tryptophan Racemase

[0184] R,R-monatin has been produced using D-aminotransferase and an aldolase when D-tryptophan was used as the starting material (Example 3). That notwithstanding, L-tryptophan may be a preferred starting material for several reasons. For example, L-

tryptophan may be less expensive and more readily available than D-tryptophan. This disclosure describes several methods for obtaining an active tryptophan racemase. Yields of R,R monatin are improved by using an R-specific aldolase, i.e., an aldolase that preferentially or selectively produces R-MP. FIGS. 1 and 2 illustrate methods for producing stereoisomerically-enriched R,R monatin from L-tryptophan using a tryptophan racemase, a D-aminotransferase and an R-specific aldolase.

[0185] A selection for a tryptophan racemase was created by constructing a strain that requires an active racemase for growth. A tryptophan auxotroph needs a source of L-tryptophan when grown on minimal medium. Supplementing the medium with D-tryptophan is one way to select for a racemase that converts D-tryptophan to L-tryptophan. The tryptophan auxotrophs were tested for growth on minimal medium supplemented with D-tryptophan. The strains, CAG18455 and CAG18579 from the Coli Genetic Stock Center and NRRL B-12264 (also *lipA*⁻, λ DE3lysogenized, and cured of its plasmid), did not grow when supplemented with D-tryptophan but grew when supplemented with L-tryptophan. *E. coli* may be used as a host organism but other host organisms also may be used, such as yeast, other bacteria, or other eukaryotic organisms. A tryptophan auxotroph (specifically NRRL B-12264 (also *lipA*⁻, λ DE3 lysogenized and cured of its plasmid)) will grow on D-tryptophan when it has been transformed with a D-aminotransferase. This confirms the ability of *E. coli* to transport D-tryptophan into the cell.

[0186] Salcher and Lingens described the presence of a tryptophan racemase in *Pseudomonas aurereofaciens* (ATCC15926). Salcher, O., and Lingens, F., *J. Gen. Microbiol.* 121:465-471 (1980). Tryptophan racemase has also been described in several plants including tobacco, beets, tomato, and wheat and the enzyme appears to be induced by conditions of osmotic stress or drought. Tryptophan racemase may play a role in *Sclerochiton ilicifolius* in the native monatin production pathway. To isolate this racemase activity, an expression library is constructed from ATCC15926 (or another organism with tryptophan racemase activity) and the library is transformed into the tryptophan auxotroph. A strain is selected that will grow using D-tryptophan as the tryptophan source. A similar method is also used to screen many strains with known racemases to look for a racemase with activity on D-tryptophan. Examples of racemases that may have activity on D-tryptophan include alanine, serine, and glutamate racemases.

Yoshimura T., and Esaki, N., "Amino Acid Racemases: Functions and Mechanisms," *Journal of Bioscience and Bioengineering* 96, 103-109, (2003).

- [0187] Alanine racemase is pyridoxal 5'-phosphate (PLP) dependent and has been cloned from *Salmonella typhimurium* (*dadB* gene). Other sources of alanine racemases are *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Schizosaccharomyces pombe*, and *Bacillus cereus*. A basidiomycetous mushroom, *Lentinus edodes*, also contains a broad activity alanine racemase.
- [0188] Serine racemase is also PLP dependent and is found in Eukaryotes (e.g. silkworm, rat brain, mouse brain cDNA), as well as in bacteria (*Enterococcus gallinarum*).
- [0189] Glutamate racemase is PLP-independent and has been cloned from *Pediococcus pentosaceus*, *Bacillus pumilus*, *Lactobacillus fermenti*, *Lactobacillus brevis*, *E. coli*, *Aquifex pyrophilus*, and *Bacillus subtilis*. Some glutamate racemases are very specific and, consequently, even structurally similar amino acids aspartate, asparagine, and glutamine may not be substrates for the enzyme.
- [0190] Aspartate racemases also exist and are PLP independent. Aspartate racemases are found in *Lactobacilli*, *Streptococcus* strains, and some archaea such as *Desulfurococcus* and *Thermococcus* strains. The bivalve mollusk *Scapharca brouhtonii* also contains an aspartate racemase.
- [0191] Other racemases found in the literature include amino acid racemase (EC 5.1.1.10) from *Anabaena* sp. and *Pseudomonas striata*, proline racemase, and multifunctional phenylalanine racemase. Related epimerases or racemases are also being tested. Potential racemases are tested to make sure they are not D-tryptophan aminotransferases. The screening of potential racemases is done by sequence analysis and/or an enzyme assay. This screening method for selection of a tryptophan racemase is also used for other bacteria or archaea for which tryptophan racemase has been described, as well as for eukaryotic cDNA libraries that have been constructed in such a manner as to allow expression.
- [0192] Enzymes that pass the test as a tryptophan racemase are screened for activity on monatin as described in Example 8. Ideally, one obtains an enzyme that is very specific for tryptophan and has little or no racemase activity on monatin.
- [0193] A tryptophan racemase also may be evolved and/or improved (via mutagenesis or recombinant engineering) from an existing racemase, transaminase, or epimerase.

Additionally, because crystal structures for alanine aminotransferases (and other aminotransferases) are known, these may be used as a basis for rational, structure based mutagenesis. The process described above is used as an initial selection for tryptophan racemase activity and as a screen for improved activity.

(2) *Tryptophan Racemase Libraries*

Construction of Libraries:

[0194] *Burkholderia pyrrocina* (ATCC15958) and *Pseudomonas chlororaphis* (ATCC15926) were obtained from the American Type Culture Collection, P.O. Box 1549, Manassas, VA, 20108, USA. They were grown as recommended by ATCC and genomic DNA was prepared according to the method described in Mekalanos, J.J., "Duplication and amplification of toxin genes in *Vibrio cholerae*," *Cell* 35:253-263, (1983). The genomic DNA was partially digested with the *Sau3A* I restriction enzyme. 1-3 Kbp fragments were gel purified using a Qiagen QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). The purified DNA was ligated into pTrc99a (Amersham, Piscataway, NJ) that had been digested with *Bam*H I and purified as above. The ligation was done at room temperature with overnight incubation using a 3:1 molar ratio of insert to vector. The ligated library was transformed into TOP10F' chemically competent cells (Invitrogen, Carlsbad, CA) and plated on LB medium with 100µg/mL ampicillin. After overnight incubation of the transformation plates, colonies were scraped off of the plates, washed with liquid LB medium and an appropriate size cell pellet was mini-prepped using a Qiagen QIAquick® mini-prep kit (Qiagen, Valencia, CA). Approximately 30,000 colonies were pooled and mini-prepped.

[0195] The pooled plasmid was transformed into CAG18455 (*trpC83::Tn10*, *rph-1*) or CAG18579 (*trpC::Tn10kan*, *rph-1*) (from the Coli Genetic Stock Center). Both strains are tryptophan auxotrophs so they will not grow on M9 minimal medium (Difco) unless the medium is supplemented with tryptophan. The transformants were plated on M9 minimal medium supplemented with D-tryptophan. This selects for a strain that can convert D-tryptophan to L-tryptophan.

[0196] Prior to transformation of the library, the strains were tested for growth on minimal medium with L- or D-tryptophan. The strains were tested for growth on minimal

medium supplemented with D-tryptophan and no growth was observed. Both strains grew on identical medium supplemented with L-tryptophan instead of D-tryptophan. Additionally, a derivative of NRRL B-12264 (the strain used had been cured of the tryptophan operon plasmid, lysogenized with λ DE3, and deleted for *lipA*, in addition to the other chromosomally encoded mutations (*serB*, Δ *trpED*, *tnaA2*, *aroP*)) was transformed with a D-specific aminotransferase from *Bacillus subtilis* (WO 03/091396). The NRRL B-12264 strain could not grow on minimal medium supplemented with D-tryptophan, but grew on identical medium supplemented with L-tryptophan instead of D-tryptophan. Expression of the D-aminotransferase was driven by the T7 promoter. The transformed strain was able to grow on M9 minimal medium supplemented with D-tryptophan.

[0197] The colonies that grow on the D-tryptophan medium are screened. The plasmid is isolated and retransformed into the parent strain (CAG18455 or CAG18579) to confirm that growth on D-tryptophan medium is dependent on the plasmid and not on a host mutation. The nucleotide sequence of the plasmids that complement the tryptophan auxotrophy are analyzed. Clones that are determined to contain a tryptophan racemase gene are further analyzed.

[0198] The tryptophan racemase from other tissue sources is isolated in a similar fashion. There are literature reports of tryptophan racemase activity in both tobacco tissue culture cells (*Nicotiana tabacum* L. var. Wisconsin 38) (Miura, G.A., and Mills, S.E., "The conversion of D-tryptophan to L-tryptophan in cell cultures of tobacco," *Plant Physiol.* 47:483-487, (1974)) and in crude protein extracts of wheat (*Triticum aestivum*) (Rekoslavskaya, N.I., *et al.*, "Synthesis and physiological function of D-tryptophan during wheat germination," *Russian J. Plant Physiol.* 44:196-203, (1997)). A cDNA expression library is made from tissue, as described in the literature, and the expression library is used to transform a tryptophan auxotroph as described above.

[0199] It would be expected that if the same strains are used and the same growth conditions are reproduced as described in the literature, the enzyme with tryptophan racemase activity could be isolated or the mRNA could be isolated and a cDNA expression library could be prepared that would contain a coding sequence for an enzyme with tryptophan racemase activity. For instance, certain growth stages or certain medium

components may be required to induce cellular production of an enzyme with tryptophan racemase activity.

(3) *Tryptophan Racemase Assay*

[0200] Clones that are identified as potentially having a tryptophan racemase are transformed into a strain of *E. coli* commonly used for expression of recombinant proteins, such as BL21. The cells are grown in LB broth to an optical density at 600 nm of 0.4-0.6. The promoter driving expression of the racemase is induced with IPTG (isopropyl-beta-D-thiogalactopyranoside) (0.1 mM final concentration). After induction, the cells are allowed to express the protein for 1-3 hours at 37 °C (with aeration). The cells are harvested and lysed by French press, sonication, or by chemical means (such as BugBuster (Novagen, Madison, WI)). The lysed cells are centrifuged to remove the cell debris. The clarified extract is used directly in assays.

[0201] Varying amounts of extract is added to a solution such that the final concentration is 50 mM potassium phosphate (pH 7.0) and 2 mM L-tryptophan. Pyridoxal-5'-phosphate is added at a final concentration of 10 μ M. The samples are incubated and then analyzed by LC/MS. The presence of a D-tryptophan peak when only L-tryptophan is used as a substrate indicates a positive result. D-tryptophan concentration should increase with increasing time until equilibrium is reached, and the rate should also increase with protein concentration until the concentration of enzyme is high enough that it is no longer saturated with substrate. D-tryptophan may also be converted to L-tryptophan as above.

[0202] A complementing gene may code for a D-aminotransferase. This transamination reaction requires an alpha-keto acid such as α -ketoglutarate, oxaloacetate, or pyruvate as an amino acceptor. These compounds will likely be present in a cell extract, usually in small amounts. These compounds may be removed using a PD-10 desalting column and the assay may still be performed in a crude extract. Likewise, a complementing gene may also code for a D-amino acid oxidase or D-amino acid dehydrogenase. These enzymes also require cofactors and co-substrates that can be removed by a PD-10 desalting column. The tryptophan racemase activity is purified using conventional column chromatography. Finally, the open reading frame identified as a potential tryptophan racemase is cloned into an expression vector with an affinity tag. The potential

tryptophan racemase is then purified by affinity chromatography. In either case the purified protein is used in enzyme assays essentially as described above.

(4) Reverse Genetic Engineering of Tryptophan Racemase

[0203] The tryptophan racemase can be purified from either plant or microbial sources by conventional protein purification techniques, including ammonium sulfate fractionation and conventional column chromatography. Once the protein has been purified such that a spot can be isolated on a 2-D gel, peptide microsequencing techniques or conventional Edman type amino acid sequencing are utilized (on the internet, *see* "golgi.harvard.edu/microchem/" for descriptions of the protocols and equipment typically used for this type of work). In some cases, however, the genome sequence of the organism cannot be used as a source of the protein for the protein purification because such sequence has not been determined yet. In that situation, the first set of degenerate primers may be designed based on sequence available from the closest known relative of the protein source. Degenerate PCR and genome walking is then performed according to established protocols to isolate the tryptophan racemase coding sequence.

(5) Cloning of Alanine Racemase from *Geobacillus stearothermophilus*

[0204] The alanine racemase (SEQ ID NO:41) from *Geobacillus stearothermophilus* was cloned. Genomic DNA from *G. stearothermophilus* (ATCC12980D) was purchased from the ATCC (Manassas, VA). The following primers were used to amplify the alanine racemase gene from *G. stearothermophilus*: 5'-atggacgagttcaccgcga-3' (SEQ ID NO:25) and 5'-ttatgcatcgcttcacgcg-3' (SEQ ID NO:26). The PCR product was ligated to pCR-Blunt-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA). Correct clones were confirmed by sequencing (Agencourt, Beverly, MA). A correct clone was used as template in a subsequent PCR reaction.

[0205] The following primers were used to amplify the alanine racemase: 5'-ataatagatcctcatccgcgccaacggcg-3' (SEQ ID NO:27) and 5'-gggaaaggtaccgaggaataataaatggacgagttcaccgcg-3' (SEQ ID NO:28). The PCR product was digested with the restriction enzymes *Kpn* I and *Bam*H I. These enzymes cut at sites that had been engineered into the primers. The digested PCR product was gel purified and ligated to the *E. coli* plasmid vector pTrc99a that had been digested with *Kpn* I and *Bam*H I and subsequently gel purified. The ligation was transformed into TOP10F'

chemically competent cells and plated on LB plated supplemented with 50 µg/mL kanamycin. Isolates were screened for inserts and several isolates with an insert were confirmed to have the correct sequence (SEQ ID NO:40) by sequence analysis (Agencourt, Beverly, MA).

[0206] The pTrc99a/alanine racemase construct was subjected to Site-Directed Mutagenesis ("SDM") using the Stratagene (La Jolla, CA) Quick-Change Multi Site-Directed Mutagenesis kit. The mutagenic primers were as follows:

5'-gccggacgacacgcacattnnkcggtcgtgaaggcgaacgcc-3' (SEQ ID NO:29);

5'-gtgaaggcgaacgcctatggannkggggatgtgcaggtggcaagg-3' (SEQ ID NO:30);

5'-cctcccgccctggcggttgccnnktggatgaggcgctcgttta-3' (SEQ ID NO:31);

5'-caaccaggcgaaaaggtagcnnkgtgacgtacactgcgcag-3' (SEQ ID NO:32);

5'-gatcgggacgattccgatcggnkcgcgacggctgctccgccg-3' (SEQ ID NO:33); and

5'-gccatttggaacgatcaacnnkgaagtgccttgacgatcag-3' (SEQ ID NO:34)

(n = any nucleotide and k = g or t).

[0207] Residues for mutagenesis were selected by analysis of the existing crystal structure of *G. stearothermophilus* alanine racemase. Large amino acid residues located between 5 and 10 Å from the active site were chosen.

[0208] All six primers were used in the SDM reaction as directed in the manufacturer's protocol. The SDM reaction was transformed into XL-10 Gold according to the manufacturer's protocol. The transformation reaction was plated on LB medium supplemented with 100 µg/mL ampicillin. LB broth was added to the plates and the colonies were scraped off the plates. The resuspended cells were allowed to grow at 37 °C for several hours and the plasmids were mini-prepped using the QIAquick[®] mini-prep kit. The resulting mutagenized library was then used to transform the tryptophan auxotroph CAG18455. The transformation was plated on M9 minimal medium that had been supplemented with glucose, trace elements, vitamins, 100 µg/mL ampicillin, 100 µM IPTG, and 3 mM D-tryptophan. After several days of incubation at 37 °C, colonies grew. These colonies were streaked on LB (100 µg/mL ampicillin). The plasmids were isolated from these isolates and were retransformed into CAG18455. The retransformed cells were plated on LB containing 100 µg/mL ampicillin. After isolated colonies formed, they were streaked on M9 D-tryptophan medium as described above. The

colonies all seemed to re-grow, indicating that the growth was because of the mutagenized version of the racemase. No growth of the control cells was observed.

[0209] Several of the isolates were assayed for *in vitro* activity. Cells were grown to an OD₆₀₀ approximately 0.6 and induced with 100 μ M IPTG. Cells were incubated at 37 °C for an additional two hours and were harvested by centrifugation. Cell pellets were stored at –80 °C until use the next day. Cell pellets were thawed on ice. Cells were disrupted with BugBuster (primary amine free) cell lysis reagent and Benzonase (Novagen, Madison, WI). Cell debris was removed by centrifugation (~10,000 x g for 30 minutes at 4 °C). The supernatant was saved as the crude cell extract.

[0210] Assay buffer contained 50 mM potassium phosphate (pH 8.0), 10 μ M pyridoxal phosphate, 0.01% β -mercaptoethanol, and 50 mM D- or L-tryptophan. 200 μ L extract was added per mL of assay. Samples were frozen representing a time 0 time point, as well as, 30 minute and overnight time points. The samples were spun, filtered, and submitted for chiral LC/MS/MS analysis as described in Example 1.

Table 10: Results of Assay Starting from L-Tryptophan

Time (Minutes)	L-Tryptophan (ppm)	D-Tryptophan (ppm)
0	1240	3.6
30	1193	24.5
Overnight	1192	583.2

Table 11: Results of Assay Starting from D-Tryptophan

Time (minutes)	L-Tryptophan (ppm)	D-Tryptophan (ppm)
0	0.5	7506
30	0.5	7519
Overnight	14.9	7463

[0211] The DNA sequence of the racemase gene in this isolate was determined (SEQ ID NO:42) and the isolate was found to have three mutations. The mutations in the corresponding protein isolate are as follows: M35C, F66E, and Y354A (SEQ ID NO:43). An additional mutation (P197L) was found in this mutant. This is a spontaneous mutation and was not part of the site-directed mutagenesis.

[0212] The mutagenized racemase was cloned into pET30 (Novagen, Madison, WI) for expression and purification. The following primers were used to PCR amplify the racemase gene from the pTrc99a construct: 5'-gggaaaggtaccgaggaataataaatggacgagtttcaccgcg-3' (SEQ ID NO:35) and 5'-

gcggcgccatggacgagtttcaccgcg-3' (SEQ ID NO:36). The PCR product was digested with *Nco* I and *Bam*H I, gel purified, and ligated to pET30 that had been digested with *Nco* I and *Bam*H I and subsequently gel purified. The ligation was transformed into TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). Isolates from the transformation were screened for inserts. Plasmids with an insert were submitted for sequencing (Agencourt, Beverly, MA). Isolates with the correct sequence were transformed into BL21 λ DE3 or BL21 λ DE3 pLysS for expression and purification. The new construct is designated pET30Trp racemase.

(6) *Purification of Tryptophan Racemase*

[0213] An overnight culture with the pET30Trp racemase construct was subcultured into fresh LB medium with the appropriate antibiotics (50 μ g/mL kanamycin and 20 μ g/mL chloramphenicol) and grown to an OD₆₀₀ ~0.6 (37 °C with aeration). Expression was induced with 100 μ M IPTG and incubation was continued at 37 °C with aeration for 2 hours. The cells were harvested by centrifugation and stored at -80 °C until use. The cell pellet was thawed on ice and cells were lysed using BugBuster Primary Amine Free Cell Lysis Reagent and Benzonase Nuclease (Novagen, Madison, WI). Cell debris was removed by centrifugation and the supernatant was used as the crude protein extract. The crude protein extract was filtered using a 0.45 μ m syringe filter and applied to a HisBind column (Novagen, Madison, WI) that had been pre-equilibrated according to the manufacturer's instructions. The column was washed and the protein was eluted as directed in the manufacturer's protocol. The purified protein was desalted with a PD-10 column (GE Healthcare, Piscataway, NJ) using 50 mM potassium phosphate pH 8.0, 10 μ M pyridoxal-5'-phosphate ("PLP") as the eluent. The desalted protein was concentrated using Amicon centrifugal concentrators (Millipore, Billerica, MA). Wild-type alanine racemase was purified as described above.

(7) *Assay of Tryptophan Racemase*

[0214] The purified racemase was tested in several assays. In one assay, the production of hydrogen peroxide by a D-amino acid oxidase was used as a detection system. D-tryptophan substrate for the oxidase was produced from L-tryptophan via the racemase enzyme isolated as described in this Example. The assay included 0, 1, 10, 25, 50, 100, 200 μ g of enzyme per assay, 50 mM potassium phosphate pH 8.0, 10 μ M PLP, 50 mM L-

tryptophan. The assays were incubated 1 hour at 37 °C. After incubation, 100 mg/mL D-amino acid oxidase (AOD-101 BioCatalytics, Pasadena, CA) and 0.5 mM FAD was added to the reaction mix. The generation of hydrogen peroxide was measured using the Amplex Red reagent kit (Molecular Probes, Eugene, OR) and a Perkin Elmer HTS 7000 Plus BioAssay Reader Fluorometer (Wellesley, MA). The assay data is summarized in Tables 12 and 13 below:

Table 12: Standard Curve

H ₂ O ₂ Concentration (μM)	Fluorometer Reading
0	485
1	8691
2	16958
3	24719
4	31692
5	38083

Table 13: Results of Assay

Protein Concentration (μg/assay)	Wild-type Racemase (Fluorometer Reading)	Mutant (Trp) Racemase (Fluorometer Reading)
0	5226	5192
1	4272	6215
10	4149	10543
25	4239	21177
50	3141	30465
100	3160	39068
200	2370	35163

[0215] The results of the assay indicate that the mutant racemase is required for production of hydrogen peroxide. The amount of hydrogen peroxide produced increased when the amount of the mutant racemase added was increased.

[0216] The activity of the racemase (wild-type and mutant) on alanine was analyzed. The reaction buffer contained: 100 mM potassium phosphate pH 8.0, 10 μM PLP, 50 mM L-alanine, 12 μg/mL wild-type racemase or 94 μg/ml mutant racemase. The reactions were stopped with 1 volume of 0.5 M formic acid and analyzed by LC/MS/MS using a Chirobiotic column as described in Example 1.

[0217] The assay data is summarized in Table 14 below.

Table 14

Time (minutes)	Wild-type Racemase (ppm D-Alanine Produced)	Mutant Racemase (ppm D-Alanine Produced)
0	65	87
5	334	2430
10	1161	3257
20	1670	4003
30	3075	4621
40	3177	4931
60	3986	5328

[0218] The mutated racemase appears to retain activity on the original substrate, alanine.

[0219] The activity of the mutated racemase was tested using one of L-tryptophan, D-tryptophan, L-alanine, and D-alanine as the substrate. The reaction buffer contained: 100 mM potassium phosphate pH 8.0, 10 μ M PLP, 50 mM substrate, 94 μ g/mL mutant racemase. The reactions were stopped with 1 volume of 0.5 M formic acid and analyzed as described in Example 1. The assays with alanine as the substrate were incubated at room temperature ($\sim$ 22 $^{\circ}$ C) and assays with tryptophan as the substrate were incubated at 37 $^{\circ}$ C. The results are summarized in Table 15 below.

Table 15

Time (minutes)	ppm D-trp Produced from L-trp	ppm L-trp Produced from D-trp	ppm D-ala Produced from L-ala	ppm L-ala Produced from D-ala
0	None detected	0.8	420.5	565.9
5	None detected	1	1268	1874
10	None detected	1.4	1448	1968
20	None detected	2.2	1590	1505
30	0.3	2.8	1840	1923
40	3.1	2.8	1779	1960
60	9	3.7	1295	1070
1080	57.4	66.7	1611	2932

[0220] The racemase enzyme works in both directions and retains wild-type activity.

[0221] The mutant racemase was tested on several substrates. The enzyme used in the assay was purified as previously discussed. The assay conditions are as follows: 50 mM potassium phosphate pH 8.0, 10 μ M PLP, 25 mM substrate, 40 μ g/mL mutant racemase. The reactions were stopped with 1 volume of 2 M formic acid and analyzed as described in Example 1. The assays were incubated at 37 $^{\circ}$ C. The results (in ppm D-isomer produced from the L-isomer) are summarized in Table 16 below (nd = none detected).

Table 16

Time (Minutes)	Lys	Ala	Glu	Met	Tyr	Leu	Trp	Phe
0	12	156	86	104	nd	nd	nd	nd
3	2310	2180	607	1200	nd	37	nd	nd
5	2450	1310	1110	1290	nd	80	nd	14
10	6630	2850	1950	2260	11	139	nd	14
20	9550	1970	4660	2090	30	280	nd	47
30	15500	2090	4860	1750	63	320	nd	22
60	10200	2540	4490	2150	136	710	nd	54
120	18000	2430	6340	1940	224	1050	nd	188
240	13200	1830	6560	1990	515	1170	15	490

[0222] It is likely that this racemase will racemize other amino acids in addition to the ones tested here.

[0223] Although the mutated racemase appears to have activity on a wide variety of amino acids, there does not appear to be any racemase activity on monatin. The enzyme used in the assay was purified as previously discussed. The assay conditions are as follows: 100 mM potassium phosphate pH 8.0, 10 μ M PLP, 50 mM monatin, 1 mg/mL mutant racemase. The assays were incubated at 37 °C. The assays were analyzed by FDAA derivitization as described in Example 1. The results of the assay are shown in Table 17 below.

Table 17

Time (Hours)	S,S Monatin Starting Substrate	R,R Monatin Starting Substrate
0	100% SS	100% RR
1	100% SS	100% RR
18	100% SS	100% RR

[0224] Even after 18 hours there was no apparent conversion of S,S monatin to S,R monatin or of R,R monatin to R,S monatin using the mutant racemase.

[0225] The ideal enzyme has activity on tryptophan, but little or no activity on other amino acids or amino acid like compounds, particularly monatin. If the enzyme has significant activity on monatin, the enzyme may be mutagenized to decrease the activity on monatin and/or glutamate, while keeping the tryptophan activity unchanged or at a level high enough for the enzyme to be useful in monatin production. Techniques that may be used for mutagenesis include, but are not limited to, error prone PCR, site-

directed mutagenesis, modeling to identify site-directed mutagenesis targets (sites that may be involved in substrate binding), passage through mutagenic strains, and DNA shuffling.

(8) *Tryptophan Racemase Monatin Production*

[0226] The following were added per 1 mL of reaction mixture: approximately 50 μ g aldolase of SEQ ID NO:22, 16 mg/mL purified tryptophan racemase, 4 mM MgCl_2 , 50 mM L-tryptophan, 0.5 mg D-aminotransferase (purified from *Bacillus sphaericus* as described in Example 14), 100 mM sodium pyruvate, 100 mM potassium phosphate buffer pH 7.5, and 0.05 mM PLP. Because pyruvate is an acceptable amino acceptor for the broad specificity D-aminotransferase, α -ketoglutarate was not used. A control was included in which D-tryptophan was the starting substrate and no racemase was included. The samples were incubated 2 hours or overnight (20 hours) at 30 °C with gentle shaking. Samples were analyzed as described in Example 1. The results of the assay are shown below in Table 18 (nd = none detected).

Table 18

Time (Hours)	Starting Substrate	ppm Total Monatin	RR/SS% RPLC	RS/SR% RPLC	% RR FDAA	%SR FDAA
2	L-trp	nd	0	0		
18	L-trp	7.4	100	0	96.5	3.5
2	D-trp	12	99.17	0.83		
18	D-trp	170	98.65	1.35	97.5	2.5

[0227] Table 18 shows production of R,R monatin using a tryptophan racemase to convert the L-tryptophan substrate to D-tryptophan. The production of R,R monatin from D-tryptophan, without using the tryptophan racemase, was utilized as a control. The percent R,R monatin produced is nearly the same with either L- or D-tryptophan as the starting material. This result indicates the racemase does not have detectable activity in catalyzing the racemization of R,R monatin.

(9) *Isolation of the Key Amino Acid Changes*

[0228] Several revertants of the mutagenized alanine racemase were created. The revertants were made by site-directed mutagenesis using the QuikChange Multi Site-

Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as previously described using the following primers:

5'-gccatttggaacgatcaactatgaagtccttgacgatcag-3' (SEQ ID NO:37);

5'-ctcccgctggcggttgcccttcttgatgaggcgctcgctttaag-3' (SEQ ID NO:38); and

5'-gccggacgacgcacattatggcggtcgtaaggcgaacgcc-3' (SEQ ID NO:39).

[0229] The primers were used individually, and in combination, in an attempt to make the six possible combinations of the three mutations in positions 35, 66, and 354 (numbering based on the ATCC 12980 derived amino acid sequence). Several combinations of the mutations were created and tested for tryptophan racemase activity. The assay conditions were as follows: 50 mM potassium phosphate pH 8.0, 10 μ M PLP, 30 mM L-tryptophan, 100 μ g/mL enzyme. The assays were incubated at 37 °C for the specified timeperiod. The samples were analyzed as described in Example 1.

[0230] The results of the assays are summarized in Table 19 below (nd = none detected).

Table 19

Time (Minutes)	MF1	MF2	MY1	Mutated Racemase
0	nd	Nd	nd	nd
5	nd	Nd	nd	nd
10	nd	Nd	nd	nd
20	nd	Nd	nd	nd
30	nd	Nd	nd	nd
40	nd	Nd	nd	nd
60	9.8	Nd	nd	12.5
1080	54.8	90.8	nd	92.4

Mutation list:

MF1: N41S (spontaneous mutation), P197L, Y354A

MF2: F66E, P197L, Y354A

MY1: M35C, F66E, P197L

Mutagenized racemase: M35C, F66E, P197L, Y354A

[0231] The results indicate that the Y354A mutation is required for activity on tryptophan. When this mutation was absent there was no detectable activity on tryptophan. See also W.M. Patrick, J. Weisner, and J.M. Blackburn, ChemBioChem

2002 No. 8, 789-792, which also illustrates the importance of this residue in broadening substrate specificity.

- [0232] An alanine racemase may be further converted to a broader specificity racemase by random methods such as mutagenic PCR, passage through mutagenic strains, or other methods to those known in the art. A more focused evolution of the alanine racemase may be focused on active site residues, including Lys129, Met134, and the residues including and between Gly283 and Trp288 (numbering from *Geobacillus stearothermophilus*).

4B: Isolation of the Y354A Single Mutant

- [0233] The wild-type *Geobacillus stearothermophilus* alanine racemase (SEQ ID NO:41) cloned into pET30 in Example 4A was used as a template for site-directed mutagenesis to make the Y354A change. The mutagenesis was performed using the QuickChange-Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following mutagenic primer was used to make the Y354A change, 5'-gccatttggaacgatcaacgcggaagtgccttgacacgatcag-3' (SEQ ID NO:107). The site-directed mutagenesis was done as described in the manufacturer's protocol. Several isolates were sequenced (Agencourt, Beverly, MA) and an isolate with the correct sequence was selected and used for further analysis.
- [0234] The pET30Y354A single mutant was transformed into *E. coli* BL21(DE3)pLysS competent cells. Purified protein was prepared in the following manner. The strain was grown in LB or Terrific Broth (at 37 °C with aeration) to an OD₆₀₀ of 0.4-0.6 and induced with 1 mM IPTG. Incubation was continued at 37 °C with aeration for ~3 hours. The cells were harvested by centrifugation and the cell pellet was stored at -80 °C.
- [0235] The cell pellet was thawed on ice and then resuspended in an appropriate volume of BugBuster (Novagen, Madison, WI) plus Benzonase nuclease (Novagen, Madison, WI). Cell debris was removed by centrifugation, and the cell-free extract was applied to a HIS-Bind column (Novagen, Madison, WI) that had been equilibrated with Binding buffer. The column was washed with Binding buffer and Wash buffer and the protein was eluted with Elution buffer (as directed in the manufacturer's protocol). The purified protein was desalted using a PD-10 column (GE Healthcare, Piscataway, NJ). The protein was desalted into 50 mM potassium phosphate pH 8.0 and 10 µM pyridoxal-5'-phosphate according to the manufacturer's protocol. The protein was concentrated using

an Amicon centrifugal concentrator (Millipore, Billerica, MA). The purified and concentrated protein was divided into small aliquots and stored at -80°C until use.

[0236] The purified Y354A was compared to wild-type alanine racemase (prepared in the manner described above) in both alanine and tryptophan assays. Assays were performed in 50 mM potassium phosphate buffer, pH 8, and 10 μM PLP as described in Example 4A using 400 μL of purified concentrated protein ($> 1 \text{ mg/mL}$ final concentration) and 50 mM substrate. Detection of D-alanine and D-tryptophan was performed using the chiral amino acid methodology described in Example 1. The results are shown in Table 20 below.

Table 20

Enzyme	Substrate	Time (min)	D isomer produced (ppm)
Wild-type	L-tryptophan	0	nd*
		10	nd
		30	nd
		60	nd
		1080	nd
Y354A		0	nd
		10	198
		30	568
		60	1386
		1080	10080
Wild-type	L-alanine	0	5140
		10	5960
		30	6280
		60	6500
		1080	5040
Y354A		0	4760
		10	4980
		30	4980
		60	4200
		1080	5000

*nd= none detected

[0237] These data were analyzed without the use of an internal standard, and are thus semi-quantitative and should be used for comparative purposes. Nonetheless, these results show that the Y354A single mutation is sufficient to broaden the specificity of the alanine racemase so that it can catalyze amino acid racemization using alternative

substrates. Note that the enzyme is so active with alanine as a substrate that at time zero the reaction has essentially reached equilibrium already.

4C: Saturation Mutagenesis of Position 354 of the *Geobacillus stearothermophilus* Alanine Racemase

[0238] Several mutants were isolated in the following manner. A primer was made to conduct “NNK” random mutagenesis at position 354. The primer, 5'-gccatttgaaacgatcaacnnkgaagtccttgacgatcag-3' (SEQ ID NO:108), was used with the QuickChange Multi Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The template used for the reaction was the wild-type *G. stearothermophilus* alanine racemase cloned into pET30. Mutagenesis was conducted according to the manufacturer's protocol. Several mutants were isolated using this method. The following mutants were isolated using this method: Y354D, Y354E, Y354L, Y354H, and Y354K. Other amino acid substitutions were made using the QuickChange Multi Kit and these primers:

Y354N 5'-ccatttgaaacgatcaacaacgaagtccttgacgatcag-3' (SEQ ID NO:109) and
Y354G 5'-gccatttgaaacgatcaacggcgaagtccttgacgatcag-3' (SEQ ID NO:110).

[0239] Additional mutations were made with the standard QuickChange Kit and the following primers:

	Y354C	5'-
TCGCCATTTGGAAACGATCAACTGCGAAGTGCCTTGACAG-3' (SEQ ID NO:111)		
and Y354T 5'-GGAAACGATCAACACGGAAGTGCCTTGACAG-3' (SEQ ID NO:112)		

and their respective reverse complements. The plasmids with the desired sequences (Agencourt, Beverly, MA) were transformed into the expression strain *E. coli* BL21(DE3). Gene induction was carried out as described in Example 4B. The mutants with activity are compared in the table below. The assays were done using L-tryptophan as a substrate as described above, with the Y354A mutant as a positive control. The relative activities of the mutant racemases are all compared to Y354A in the table below. The proteins were purified and assays were performed as described in Example 4B using 100 µg of purified protein. The results are shown in Table 21 below.

Table 21

Mutant	Relative activity
Y354A	1.0
Y354C	0.18
Y354T	0.22
Y354G	0.69
Y354L	0.37
Y354N	0.03
Y354S	0.02

[0240] Y354I, Y354M, and Y354P had trace amounts of activity that were not quantifiable given the limits of detection.

[0241] Y354D, Y354E, Y354H, and Y354K had little or no activity under the conditions in this assay.

[0242] Y354F, Y354Q, Y354R, and Y354W mutants were constructed using similar methods as above, and also appeared to have no activity under the conditions of this assay.

4D: Mutagenesis of Amino Acid Positions 35 and 265 of the *Geobacillus stearothermophilus* Alanine Racemase

[0243] Several mutants were isolated in the following manner. Two primers were made to conduct “NNK” random mutagenesis at amino acid positions 35 and 265 using the Y354A mutant (described in Example 4B) as the template. The gene encoding Y254A was subcloned into pPRONco vector using Nco I and BamH I restriction sites; this construct was used in mutagenesis reactions. The pPRONco plasmid is a derivative of the pPROLar.A122 vector (BD Biosciences Clontech, Palo Alto, CA) in which one of the Nco I sites was removed by mutation T1538C. The following primers (5'-phosphorylated) were used with the QuikChange Multi Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA):

Y265X: 5'-CAA CCA GGC GAA AAG GTG AGC NNK GGT
GCG ACG TAC ACT GCG CAG-3' (SEQ ID NO:113) and

M35X: 5'-GCC GGA CGA CAC GCA CAT TNN KGC GGT
CGT GAA GGC GAA CGC C-3' (SEQ ID NO:114)

wherein N indicates G, C, T, or A and K indicates G or T.

[0244] Mutagenesis was conducted according to the manufacturer's protocol. Plasmids containing mutant genes were transformed into electrocompetent tryptophan auxotrophic strain CY15077 (obtained from the *E. coli* Genetic Stock Center, Yale). Several mutants were isolated after several days of growth on M9 minimal medium-containing Nobel agar plates that had been supplemented with 0.4% glucose, trace elements, vitamins, 50 µg/mL kanamycin, 100 µM IPTG, and 3 mM D-tryptophan. R195H spontaneous mutations occurred in some cases.

[0245] The following mutants were isolated using this method (all also contain Y354A): Y265M, Y265C, M35V/R195H, M35S/Y265S.

[0246] Clones were induced with 1 mM IPTG in LB medium containing kanamycin as described in Example 4B. Assays were conducted using cell free extracts. The level of expression of the target protein was estimated using the ExperionPro260 electrophoresis system (Bio-Rad). One mL assays contained 30 mM L-tryptophan, 50 mM potassium phosphate pH 8, and 50 µM PLP, and approximately 200 µg of mutant enzyme. Samples were incubated at 30 °C overnight. Samples were acidified with 2% formic acid, spun, filtered, and diluted 10-fold for detection of D-tryptophan as described in Example 1. Only the M35V/R195H mutant had detectable activity, and it appears to be on the same order of magnitude as the Y354A alone.

[0247] Additionally, Y265A amino acid substitutions were made in Y354A (from Example 4B), Y354N (from Example 4C), and wild-type backgrounds in pET30 constructs using the QuickChange Multi Kit (Stratagene) and the following primer, using the manufacturer's protocols: 5'-AGG CGA AAA GGT GAG CGC GGG TGC GAC GTA CAC TG-3' (SEQ ID NO:115). Plasmid DNA was isolated, transformed into *E. coli* BL21(DE3), and induced and assayed as above. None of the constructs containing the Y265A mutation appeared to have activity under these conditions.

4E: Cloning of *Pseudomonas putida* KT2440 Broad-specificity Amino acid Racemase ("BAR")

[0248] A BAR (Broad specificity Amino acid Racemase) was identified in *P. putida* KT2440 using information from literature (Roise, D., Soda, K., Yagi, T., Walsch, C.T., *Biochemistry* 23, 5195-5201, (1984)). *P. putida* KT2440 is also known as ATCC 47054. The active site of a BAR enzyme from *P. striata* was sequenced and reported – LTAVLKADAYGXGIGL (SEQ ID NO:116), wherein X indicates any naturally occurring amino acid. This sequence was used to BLAST the *P. putida* KT2440 genome sequence available in NCBI. A protein with a nearly identical consensus sequence was identified. The primers were designed to clone the gene from genomic DNA obtained from the American Type Culture Collection (ATCC, Manassas, VA) and are as follows:

5'-AGAAGACATATGCCCTTTCGCCGTAGGG-3' (SEQ ID NO:117) and

5'-AGAAGAGGATCCTCAGTCGACGAGTATCTTCG-3' (SEQ ID NO:118).

[0249] PCR was conducted under standard conditions and the PCR product was purified (QIAquick® PCR purification kit, Qiagen, Valencia, CA). The purified PCR product was digested with *Nde* I and *Bam*H I. The digested PCR product was gel purified (QIAquick® Gel Extraction Kit, Qiagen, Valencia, CA) and ligated to pET30 and pET28 that had been digested and gel purified in a similar manner. Clones with inserts were sequenced (Agencourt, Beverly, MA) and isolates with the correct sequence were identified (pET30 KT2440 BAR and pET28 KT2440BAR) and used in later studies.

[0250] The KT2440 BAR DNA sequence (SEQ ID NO:119) is as follows:

```
atgccctttcgccgtacccttctggctgcacccctggcacttctgatcaccggacaggccccct
gtatgcggcaccaccgtgtcgatggacaacggcaccaacaccctgaccgtgcaaacagca
atgcctgggtcgaagtcagcgccagcgccctgcagcacaacatccgcacgctgcaggccga
gctggccggcaagtccaagctgtgcgccgtgctcaaggccgatgcctatggccacggtatcg
gcctggtaatgccatcgatcatcgcccaaggcgtgccctgcgtggcggtggccagcaacgag
gaggccccgcgtgggtccgcgcagtggttcaccggggcaactggtgcgggtacgcctggcca
gcctcagcgagctggaagatggcttgagctacgacatggaagagctggtgggcagcgcgga
atttccccgccaggccgatgccatgccgcgcgccatggcaagacctgcgcatcacatggc
```

gctcaactccagcggcatgagccgcaacggggtggagatggccacctggtccggcgtggc
gaagcgctgcagatcaccgaccagaagcacctcaagctggcgcgtgatgaccacttcgc
cgtggaagacaaggacgatgtacgcaaggcctggcggcattcaacgagcagaccgactg
gttgatcaagcacgccaggctggaccgcagcaagctcacctgcacgccccaactcgttcg
ctacgctggaagtgccggaagcgcgccctggacatggtacgaacgggtggcgcgctgttcgg
cgacaccgtgccggcgcgaccgagtacaacgtgcgatgcagttcaaatcgacgtggcg
gcggtgcacagctatccggccggcaacaccgtgggctatgaccgcaccttcacctggccccg
tgattcgcggtggccaacattacggcgggtactccgatggctaccgccgggtattaccaac
aagggccatgtgctgatcaacggccaccgtgtgccggtcgtgggcaaggtgtcgatgaacac
gctgatggtgatgtcaccgacttcctgatgtgaaggggggtaacgaagtgtgctgttcggc
aagcaggccggggcgaaatcaccagccgagatggaagaaatcaacggcgcggttgctc
gccgattgtacaccgtatggggcaattccaaccgaagatactcgtcgactga.

[0251] The KT2440 BAR amino acid sequence (SEQ ID NO:120) is as follows:

MPFRRTLAAASLALLITGQAPLYAAPPLSMDNGTNTLTVQN
SNAWVEVSASALQHNIRTLQAELAGKSKLCAVLKADAYGH
GIGLVMPsIIAQGVPCVAVASNEEARVVRASGFTGQLVRVR
LASLSELEDGLQYDMEELVGSAEFARQADAIAARHGKTLRI
HMA LNSSGMSRNGVEMATWSGRGEALQITDQKHLKLVAL
MTHFAVEDKDDVRKGLAAFNEQTDWLIK HARLDRSKLTLH
AANSFATLEVPEARLDMVRTGGALFGDTVPARTEYKRAMQ
FKSHVAAVHSYPAGNTVGYDRTFTLARDSRLANITVGYS
GYRRVFTNKGHVLINGHRVPVVGKVSMTNLMVDVTD
VTKGGNEVVLFGKQAGGEITQAEMEEINGALLADLYTVWGN
SNPKILVD.

Purification of P. putida KT2440 BAR.

[0252] The pET30 KT2440 BAR plasmid described above was transformed into BL21 DE3 pLysS (Invitrogen, Carlsbad, CA). The resulting strain was grown in LB or Terrific Broth at 37° C with aeration to an OD₆₀₀ of 0.4-0.6 and induced with 1 mM IPTG. Incubation was continued 3-4 hours at 37° C with aeration. The cells were harvested by centrifugation and the cell pellet was stored at -80 °C until use. The cell pellet was thawed on ice. The cells were lysed with BugBuster and Benzonase (Novagen, Madison,

WI). Cell debris was removed by centrifugation and the cell free extract was either used immediately or stored at -80°C . The KT2440 BAR gene was also cloned into the *Nde*I-*Bam*H I sites of pET28 and transformed into BL21 DE3 pLysS competent cells. This construct did not appear to express soluble protein very efficiently so the untagged version (pET30 KT2440 BAR) was used in future studies.

[0253] The extract was applied to an UnoQ column (BioRad, Hercules, CA) that had been equilibrated with at least 5 column volumes buffer A (25 mM potassium phosphate pH 8.0, 10 μM pyridoxal-5'-phosphate (PLP)). The column was washed with 2 column volumes of buffer A. The protein was eluted with a linear gradient of buffer B (buffer A + 1 M NaCl) from 0-100% buffer B over 20 column volumes and 5 ml fractions were collected from the time the gradient started. Fractions were assayed using the Amplex Red method described in Example 4A(7). Briefly, 100 μg D-amino acid oxidase (Sigma, St. Louis, MO), 0.05 mM FAD, 25 mM L-trp, and a small volume of the fraction to be assayed were combined in 50 μL H_2O and added to 50 μL Amplex Red reaction buffer prepared as directed in the manufacturer's protocol. Fractions with activity were desalted with a PD-10 column (GE Healthcare, Piscataway, NJ) and concentrated with Amicon centrifugal concentrators (Millipore, Billerica, MA). Purified protein was stored at -80°C .

Assay of BAR Enzyme

[0254] Amplex Red assays were set up as described in this example. *P. putida* KT2440 BAR was used at 200 μg (purified as described in this example). Wild-type *G. stearothermophilus* alanine racemase and the Y354A were purified as described in Example 4B and used at either 200 μg or 1000 μg . CE is cell-free extract that was prepared as described in this example. The results for the 60 minute time point are shown in Table 22 below. The entire timecourse is shown in Figure 11.

Table 22

Enzyme	Fluorometer reading (at 60 minutes)
BAR (200)	56943
Y354A (200)	7860
Y354A (1000)	13587

WT alanine racemase (200)	3646
WT alanine racemase (1000)	3639
BAR CE (5 μ L)	16228
BAR CE (10 μ L)	26662
BAR CE (50 μ L)	>58000
No Enzyme	1510

[0255] The purified protein was also assayed for tryptophan racemase activity in 50 mM potassium phosphate pH 8, 10 μ M PLP, and 30 mM L-tryptophan as described in Example 4A. Either 200 μ g or 1000 μ g of purified enzyme was used in the assays (indicated in parentheses). D-tryptophan was analyzed using the chiral amino acid method in Example 1 for detection. The results are shown in Table 23 below.

Table 23

Enzyme	Time	ppm of D-tryptophan
BAR (200)	0	0
	5	172
	10	410
	20	844
	30	1318
	60	2362
	120	2594
	240	2762
	1080	2294
Y354A (200)	0	0
	5	0
	10	0
	20	0
	30	12
	60	22
	120	44
	240	56
	1080	368
Y354A (1000)	0	0

	5	0
	10	12
	20	18
	30	40
	60	80
	120	146
	240	218
	1080	1164

[0256] The assays indicate that the *P. putida* KT2440 BAR enzyme is much more active on tryptophan than the *G. stearothermophilus* derived enzymes and mutants thereof. While the BAR enzymes of *P. putida* KT2440 and *P. putida* NBRC 12996 are described in U.S. Published Application No. 2005/0095670 A1, they were not assayed for their ability to racemize tryptophan.

[0257] The KT2440 BAR amino acid sequence was used to search for other potential BAR proteins present in organisms whose whole genome sequences are available in the public domain. The following table presents a list of organisms with enzymes that are homologous to KT2440 BAR and the BLAST P-score. The results are shown in Table 24. The Genbank accession numbers of the corresponding proteins are indicated in the left-hand column.

Table 24

Accession number	P-Score	Organism
YP_070906.1 GI:51596715	1.16^{-127}	<i>Yersinia pseudotuberculosis</i>
NP_669165.1 GI:22125742	1.82^{-127}	<i>Yersinia pestis</i> KIM
NP_937085.1 GI:37676689	3.53^{-127}	<i>Vibrio vulnificus</i> YJ016
YP_131472.1 GI:54310452	2.30^{-125}	<i>Photobacterium profundum</i> SS9
YP_204118.1 GI:59711342	9.84^{-126}	<i>Vibrio fischeri</i> ES114 (IG-30)
NP_230956.1 GI:15641324	3.92^{-124}	<i>Vibrio cholerae</i> El Tor N16961
NP_799227.1 GI:28899622	8.23^{-124}	<i>Vibrio parahaemolyticus</i> RIMD 2210633
YP_133597.1	1.87^{-122}	<i>Photobacterium profundum</i> SS9

GI:54303604		
NP_859997.1 GI:32265965	1.07^{-118}	<i>Helicobacter hepaticus</i> ATCC51449
ABC20456.1 GI:83573904	3.91^{-56}	<i>Moorella thermoacetica</i>
YP_461029.1 GI:85858827	2.19^{-52}	<i>Syntrophus</i> sp.
YP_320911.1 GI:75906615	1.50^{-50}	<i>Anabaena variabilis</i> ATCC29413
YP_388039.1 GI:78356590	1.38^{-45}	<i>Desulfovibrio desulfuricans</i> G20 (JGI)
NP_971703.1 GI:42526605	2.95^{-45}	<i>Treponema denticola</i> 35405

[0258] The BLAST P-score indicates that there are enzymes that are highly homologous to the KT2440 BAR and these highly homologous enzymes may have activity on substrates other than alanine even though they have been annotated as alanine racemases in public databases. One can see that there is a grouping of highly related racemases, and then a drop-off in homology occurs from *Moorella thermoacetica* onward. By blasting NCBI, another closely related homologous gene was found from *P. taetrolens* (Genbank Accession No. AB096176, nucleotide sequence) as well as in *P. putida* F1 (ZP_00898332.1 GI:82735470, protein sequence, coded by NZ_AALM01000002 nucleotides 53173..54402). Based on this information, further cloning work was done to isolate BAR genes in other *Pseudomonas* species, *Vibrio*, *Yersinia*, and *Photobacterium*. See examples 4I, 4J, 4K, 4L, and 4N.

[0259] Additionally a literature review gave other indications of where broad specificity amino acid racemases might exist. The following is a list of organisms which have had publications suggesting the presence of a broad specificity amino acid racemase, along with one reference for each organism:

1. *P. putida* IFO12996, also known as NBRC 12996, *P. putida* 12996, and *P. striata* (Nagata, Shinji; Esaki, Nobuyoshi; Tanizawa, Katsuyuki; Tanaka, Hidehiko; Soda, Kenji, Agricultural and Biological Chemistry (1985), 49(4), 1137-41)
2. *P. putida* SCRC-744 (Asano, Yasuhisa; Endo, Kaori, Applied Microbiology and Biotechnology (1988), 29(6), 523-7)
3. *P. graveolens*, also known as *P. taetrolens* (Soda, Kenji; Yorifuji, Takamitsu; Ogata, Koichi, Journal of Biological Chemistry (1971), 246(16), 5085-92)

4. *Aeromonas caviae*, also known as *Aeromonas punctata* subspecies *caviae* (Inagaki, Kenji; Tanizawa, Katsuyuki; Tanaka, Hidehiko; Soda, Kenji, Agricultural and Biological Chemistry (1987), 51(1), 173-80)
5. *P. striata* AKU 0813 (Soda, Kenji; Osumi, Takaharu, Biochemical and Biophysical Research Communications (1969), 35(3), 363-8)
6. *P. aureofaciens* (Salcher, Olga; Lingens, Franz, Journal of General Microbiology (1980), 121(2), 465-71)
7. *P. putida* 12633 (Wolf, Larissa B.; Sonke, Theo; Tjen, Kim C. M. F.; Kaptein, Bernard; Broxterman, Quirinus B.; Schoemaker, Hans E.; Rutjes, Floris P. J. T., Advanced Synthesis & Catalysis (2001), 343(6+7), 662-674)
8. *P. fluorescens* strains (Ju, Jiansong; Yokoigawa, Kumio; Misono, Haruo; Ohnishi, Kouhei, Journal of Bioscience and Bioengineering (2005), 100(4), 409-417)
9. *P. miyamizu* (Chibata, Ichiro; Tosa, Tetsuya; Sano, Ryujiro, Applied Microbiology (1965), 13(4), 618-24)
10. *P. oleovorans* strains, also known as *P. putida*
11. *Pseudomonas* strain 2150 (Okazaki, Hiroshi, Agricultural and Biological Chemistry (1968), 32(2), 254-6)

[0260] Based upon this literature, further work was done to successfully isolate BAR-encoding genes from *P. putida* 12996 and *Aeromonas* species, as described in Examples 4I and 4O. One skilled in the art would be able to isolate the genes encoding the BAR enzymes from the rest of these organisms (where genomic sequence data is limited) using reverse genetics techniques or genomic/cDNA expression libraries, as described in Example 11 for instance. Because the two *Pseudomonas putida* racemase homologs tested in this example (4E and 4I) were both broad specificity amino acid racemases, as well as the *P. taetrolens* (*graveolens*) enzyme (as described in Example 4L), it is expected that all homologous *Pseudomonas* derived racemases (such as those described in the above literature) would also have broad activity.

4F: Monatin Production with *P. putida* KT2440 BAR

[0261] A monatin production assay was done with the purified *P. putida* KT2440 BAR (as purified in Example 4E) (100 µg) or purified Y354A (as purified in Example 4B) (500 µg), D-aminotransferase (BioCatalytics AT-103 (Pasadena, CA)) (500 µg), and the

aldolase of SEQ ID NO:104 (Example 3B) (50 μ g). In addition to the enzymes above, the following were added per 1 mL of reaction mixture: 4 mM MgCl_2 , 50 mM L-tryptophan, 100 mM sodium pyruvate, 100 mM potassium phosphate buffer pH 7.5, and 0.05 mM PLP. As a control, the experiment was done without racemase and starting with D-tryptophan. A summary of the results is presented in Table 25, below.

Table 25

Substrate	Racemase	Time	Total Monatin	%R,R	%S,S	%R,S	%S,R
L-trp	Y354A	2 hours	None detected				
		18 hours	None detected				
L-trp	BAR	2 hours	None detected				
		18 hours	38.6 ppm	92.1	5		2.9
L-trp	None	2 hours	None detected				
		18 hours	None detected				
D-trp	None	2 hours	19.9 ppm	Not tested	Not tested	Not tested	Not tested
		18 hours	221.25 ppm	97.8	0.2		2

[0262] No monatin was detected using Y354A in this experiment. This racemase has been used in the past to produce monatin, but a much higher level of enzyme was used (at least 2 mg and up to 10 mg to see higher levels of monatin). The *P. putida* KT2440 BAR was used to produce monatin from L-tryptophan. The 100 μ g KT2440 BAR used in this experiment was not enough to see monatin production after two hours, but was sufficient to see monatin production after 18 hours. The stereoisomer distribution indicated that most of the monatin produced is the R,R isomer. There was no R,S isomer produced. This indicates that KT2440 BAR is not able to detectably racemize the R,R isomer of monatin (racemization of the R,R isomer would produce the R,S isomer). There was a significant amount of the S,S isomer produced in this experiment. This is probably due to the fact that the AT-103 used in this experiment is not highly purified and may contain L-aminotransferases from the cellular extract, and that there is a large amount of L-tryptophan present to serve as an amino donor for transamination of S-MP.

4G: Mutagenesis of *P. putida* KT2440 BAR at Position 396

- [0263] The gene encoding the BAR enzyme from *P. putida* KT2440 (described in Example 4E) was modified to produce an enzyme with a Y396C mutation. A similar mutation was described in a poster entitled “Tryptophan Racemase Derived from Broad Specificity Amino Acid Racemase by Directed Evolution” (Sato, M., *et al.*, 10th International Symposium on the Genetics of Industrial Microorganisms, Prague, June 24-28 2006) and K. Kino, M. Sato, M. Yoneyama, and K. Kirimura, *Appl Microbiol Biotechnol* (2007) 73:1299-1305. Mutagenesis was done using the QuickChange-Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA), using the BAR gene in pET30 which results in an untagged protein. The following mutagenic primer was used to make the Y396C change: 5'-TTGCTCGCCGATTTGTGCACCGTATGGGGCAATTC-3' (SEQ ID NO:121).
- [0264] The site-directed mutagenesis was done as described in the manufacturer's protocol. Several isolates were sequenced (Agencourt, Beverly, MA) and an isolate with the correct sequence was selected and used for further analysis.
- [0265] The plasmid was transformed into BL21(DE3) (Novagen, Madison, WI). Recombinant protein was produced in Overnight Express II medium (Novagen, Madison, WI) containing 50 µg/mL kanamycin according to manufacturer's protocols. Cell-free extracts were prepared using BugBuster (Novagen, Madison, WI) according to manufacturer's protocols, and analyzed for percent expression of the target protein using the Experion method described above.
- [0266] Total protein assays were done using a Pierce BCA kit (Rockford, IL). Tryptophan racemase assays with the mutant enzyme were performed in triplicate using the wild-type enzyme prepared in the same manner as a positive control. The assays contained per mL: 30 mM L-tryptophan, 50 mM potassium phosphate pH 8, 10 µM PLP, and approximately 200 µg of racemase protein in a cell free extract. Zero, 20 minute, 1 hour, and overnight samples were collected, treated with 2% formic acid, filtered, and diluted 1:10 for analysis using the chiral amino acid method described in Example 1. At the 20 minute time point, the Y396C mutant produced approximately 264 ppm D-tryptophan, as compared to 761 ppm for the wild-type enzyme. At 1 hour, the mutant produced an average of 444 ppm D-tryptophan, as compared to 1110 ppm for the wild-type enzyme. The overnight samples contained 2300 ppm D-tryptophan, whereas the

wild-type enzyme had reached equilibrium by producing approximately 3000 ppm D-tryptophan.

[0267] The activity of the mutant enzyme is approximately 35-40% of the wild-type under the conditions tested. However, this mutant was expected to have significantly less activity on alanine compared to the wild-type, which may be advantageous in reactions to produce monatin. Less D-alanine would be expected to be converted to L-alanine as a byproduct. Sato *et al.* reported that the BAR enzyme derived from *P. putida* IFO 12996 containing the I384M mutation alone had a specific activity of 26000 nmol/min/mg for alanine, whereas the I384M/Y396C mutant had only 825 nmol/min/mg activity for alanine. The KT2440 enzyme already contains methionine at position 384 inherently, thus is comparable to the I384M mutant described in IFO 12996.

[0268] Further experiments were conducted to assay the alanine racemase activity of the Y396C mutant. The conditions were as described above for L-tryptophan racemase activity, although 40 minute and 2 hour time points were taken. When compared to wild-type BAR from KT2440, the Y396C mutant did in fact have less alanine racemase activity. However, when monatin production experiments were repeated as in Example 4F, this mutant did not provide an advantage in production of total monatin (produced approximately 1/3rd the amount of monatin). The Y396C mutant also produced lower purity of R,R monatin (86.2-87.3% R,R versus 97.6-99.3% R,R monatin for the wild-type enzyme, using the FDAA-derivatization technique described in Example 1).

4H: Cloning of *P. putida* KT2440 BAR with Alternative Sequence Tags

Experimental Overview

[0269] The *Pseudomonas putida* KT2440 BAR described in Example 4E was expressed with a variety of sequence tags to improve solubility and aid in purification because the N-terminal His-tag from the pET28 construct appeared to produce an insoluble protein. The gene was subcloned, expressed, and tested for activity in conversion of L-tryptophan to D- tryptophan.

Polymerase Chain Reaction Protocol

[0270] The gene encoding the *P. putida* KT2440 BAR was subcloned from the pET30 construct described in Example 4E. Polymerase chain reactions were performed using approximately 100 ng of plasmid DNA using primers designed with 5' restriction sites and overhangs for cloning into the pET30a vector (without a stop codon, producing a C-terminal His-tag) (Novagen, Madison, WI), pET22-b(+) (periplasmic leader sequence), and pASK-IBA3 (IBA, Göttingen, Germany), producing a C-terminal Strep-tag. The same N-terminal primer was used for the pET30a cloning as in Example 4E.

The primer sequences used are as follows:

pET30 C term-XhoI: 5'-

AAGTCGCTCGAGGTCGACGAGTATCTTCGGG-3' (SEQ ID NO:122);

pASK N term: 5'-

ACGGTAGGTCTCAAATGCCCTTTCGCCGTACC-3' (SEQ ID NO:123);

pASK C term: 5'-

AACCGTGGTCTCAGCGCTGTCGAGGAGTATCTTCGGG-3' (SEQ ID NO:124);

pET 22 N term: 5'-

GCTCCACATGTCTCCCTTTCGCCGTACCCTTCTGGCTGCA TC-3' (SEQ ID NO:125); and

pET22 C term: 5'-

CCGCCGGATCCTCAGTCGACGAGTATCTTCGGGTTGGAA TTGC-3' (SEQ ID NO:126).

[0271] The following PCR protocol was used for the pET30 and pASK constructs: in a 50 µL reaction 1 µL template, 1 µM of each primer, 0.2 mM each dNTP, 3.5 U Expand High Fidelity Polymerase, and 1X Expand buffer (Roche, Indianapolis, IN) with Mg were used. The thermocycler program used included a hot start at 94 °C for 3 minutes, followed by 8 repetitions of the following steps: 94 °C for 30 seconds, 51 °C for 30

seconds, and 72 °C for 2 minutes. Twenty-two more cycles were carried out using a 55 °C annealing temperature. After 30 cycles, the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. Clean PCR products of the correct size were obtained (approximately 1200 bp). For the pET22 construct, a similar amplification protocol was utilized. However, PfuTurbo (Stratagene) was used as the polymerase in a protocol recommended by the manufacturer.

Cloning

[0272] The PCR products for the pET30 and pASK-IBA3 constructs were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA). The products and vectors were digested with *Nde* I/*Xho* I (pET30a) or *Bsa* I (pASK-IBA3) followed by purification from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The digested vectors and insert were ligated using the Rapid™ DNA Ligation Kit (Roche, Indianapolis, IN). Approximately 50 ng of treated insert, 100 ng of treated vector (3 to 1 molar ratio of insert to vector), 5 U of T4 DNA ligase, and 1X ligation buffer were incubated for 20 minutes at room temperature. The ligation mixtures were transformed into TOP10 chemically competent *E. coli* cells and plated on LB plates containing appropriate antibiotics. The PCR product for the pET22 construct was purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into pCR-Blunt II-TOPO (Invitrogen). The insert sequence was verified and cloned as a *Bam*H I / *Pci* I fragment into the *Nco* I and *Bam*H I restriction sites of pET-22b(+) (Novagen, Madison, WI).

[0273] Plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing (Agencourt, Beverly, MA).

Gene Expression and Assays

[0274] Plasmid DNA was transformed into *E. coli* expression host BL21(DE3) (Novagen, Madison, WI). Induction of the pET30 and pET22 constructs in BL21 (DE3) were performed using the Overnight Express II protocol (Novagen, Madison, WI). Induction of the pASK-IBA3 construct was performed according to manufacturer's protocols. Cell extracts were prepared from the overnight cultures by suspending cell pellets in Novagen

BugBuster™ reagent containing benzonase nuclease according to manufacturer's protocols. The supernatants (cell extracts) were loaded onto 4-15% gradient gels for analysis of the soluble proteins and estimation of percent expression. Total protein assays were conducted using the Bio-Rad kit (Bradford assay method) using BSA as a standard. The C-terminally His-tagged BAR expressed well and was soluble, a marked improvement over the N-terminally His-tagged protein. However, the pASK-IBA3 Strep-tagged protein did not appear to express well in total protein or in the soluble fraction. The C-terminally His-tagged BAR was purified using Novagen His-Bind columns, and still appeared to be soluble after elution. However, upon storage at -80 °C in imidazole overnight, the protein precipitated upon thawing. The protein was therefore assayed in cellular extracts. It is expected that if the protein is removed from imidazole elution buffer more expediently, it would remain soluble.

[0275] Cell extracts were assayed for tryptophan racemase activity using the following protocol. One mL reactions were carried out in 50 mM potassium phosphate (pH 8.0), 10 μM PLP, and 30 mM L tryptophan. The reactions were initiated by the addition of approximately 200 μg of racemase enzyme and were incubated at 30 °C overnight for 5 minutes, 30 minutes, 120 minutes, and overnight. Three percent formic acid was added to each time point to stop the reaction and the precipitated protein was removed by centrifugation. Samples were diluted 1:10 and frozen at -80 °C until they were analyzed for D-tryptophan by the chiral amino acid method described in Example 1. In this assay, an internal standard was not employed, resulting in semi-quantitative analysis. Untagged *P. putida* KT2440 BAR was prepared in the same way and used as a positive control. Purified BAR (from Example 4E) was also used as a positive control. Results are shown in Table 26 below.

Table 26: Production of D-tryptophan by KT2440 BAR constructs

Tag	Time (minutes)	ppm D tryptophan
untagged	5	247
	30	1842
	120	off scale
	Overnight	4671
untagged-purified	5	173
	30	1689
	120	5235
	overnight	4176
C-terminal His tag	5	257

	30	2113
	120	3810
	overnight	4535
Periplasmic	5	397
	30	4243
	120	4686
	overnight	2978

[0276] The pASK-IBA3 cell extracts typically had undetectable activity, or very low activity as expected because expression was so poor. The C-terminally His-tagged and periplasmic tagged BAR enzymes clearly have as much or greater activity than the untagged BAR enzyme from KT2440. Reactions have typically reached equilibrium by 2 hours, 30 minutes for the periplasmic-tagged BAR. Reactions were repeated using different amounts of enzyme. The trend was consistent, that the periplasmic KT2440 BAR has similar or greater activity than untagged BAR. It was noted that activity does not scale linearly with the amount of cellular extracts added to the assays; however, it is possible that there is something inhibitory in the BugBuster buffer or that increased concentrations cause aggregation of the enzyme. Assays were repeated after desalting the protein using a PD-10 column as described elsewhere, and eluting in 50 mM potassium phosphate buffer pH 8 containing 10 μ M PLP. Removal of the detergent and Tris buffer (from Bugbuster) does appear to improve the results when larger volumes of cell extract are utilized in the assay.

[0277] Cellular extracts were prepared again for the C-terminally HIS-tagged KT2440 BAR enzyme. As expected, when the imidazole from the elution buffer was removed immediately using a PD-10 column, the protein remained in solution. Analysis by SDS-PAGE showed a highly purified band at the expected molecular weight. The purified C-terminally HIS tagged protein was re-assayed as above and compared to the wildtype (untagged) BAR enzyme, both before and after freezing. In both cases the C-tagged purified enzyme showed comparable activity to the untagged protein.

[0278] Analysis using the Signal P 3.0 Server at the Technical University of Denmark (www.cbs.dtu.dk/services/SignalP/) predicts a leader sequence of 24 amino acids in length for this racemase.

4I: Cloning of *P. putida* NBRC 12996 BAR

[0279] The *P. putida* NBRC 12996 BAR sequence is 91% identical at the DNA level and 94% identical at the protein level to the BAR protein described above from strain KT2440. The NBRC 12996 strain of *P. putida* was obtained from the NITE (National Institute of Technology and Evaluation) Biological Resource Center in Japan. Primers were the same as the primers used for the cloning of the *P. putida* KT2440 BAR gene described in Example 4E. The strain was grown on medium containing peptone 10 g, yeast extract 2 g, MgSO₄·7H₂O 1 g, distilled water 1 L, agar 15 g, pH 7.0. Cells were scraped off a Petri plate and resuspended in 400 µL deionized and autoclaved H₂O. This cell suspension was used as template for the PCR reaction. The resulting PCR product was purified with the QIAquick[®] PCR purification kit (Qiagen, Valencia, CA) and digested with *Bam*H I and *Nde* I. The digested DNA was run on a 1% agarose gel and the most prominent band was cut out of the gel. The DNA was purified with the QIAquick[®] gel extraction kit (Qiagen, Valencia, CA). The resulting fragment was ligated to pET30 that had been digested with *Nde* I and *Bam*H I and purified as described above. The ligation was transformed in to TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) and plated on LB plates supplemented with 50 µg/mL kanamycin. Isolated colonies were streak purified and used to start cultures for plasmid preps. The plasmids were run on a gel to confirm the presence of an insert. Plasmids with an insert were sent to Agencourt for sequencing (Beverly, MA). An isolate with the correct sequence was identified and used in later studies.

[0280] The NBRC12996 BAR DNA sequence is (SEQ ID NO:127):

```

atgccctttcgccgtaccctcctggctgcatccctcgctctgctgactggccaggcccgct
gtacggcgaccgccccctgtcgatggacaacggcaccaccgcccctgaccgcgagaacag
caacgcctgggtcgaaatcagtgccggcgactgcaacacaacatccgtacctgcaggccg
agttggggcggaagtccaagctgtgcgccgtgctcaaggccgacgcctatggccacggtatc
ggcctggtgatgccgtgatcatcgcccaggggcggtgccctgcgtggcggtggccagcaacg
aggaggcacgcgtggtccgcccagtggttcaccgggcaactggtgcggtgacgcctggc
cagcctcggcgaagtgaagatgccttgacgtacgacatggaagagctggttggcagcgcc
gagttcgcggccagctcgatgccatcgccgaacgccacggcaagaccctgcgcattcacat
ggcgctcaattccagcggcatgagccgcaacggcggtggaatgaccacctggtccggccgg
ggtgaagcgctgcagatcactgaccagaagcacctccagctggtcgcgtgatgactcattc

```

gccgtggaagacaaggacgatgtgcgcaaaggcctggcagcgttaacgaacagaccgact
 ggctgatcaagcacgcgaagcttgatcgagcaagctcacctgcatgccgccaactcctcg
 ctacgctggaagtgccggaagcgacacctggacatggtgcgtaccgggtggcgcgctgttcggc
 gacaccgtgccgacgcgcaccgaataccaacgtgtcatgcagtcaagtcgcacgtggcggc
 ggtgcacagctaccggcgaggcaacaccgtcggctacgaccgcaccttcaccctggcgcg
 gattcgcgcctggccaacatcacctgggttactccgatggctaccgccgggtgttccaac
 aagggccatgtgtgatcaacggccaccgagtgccagtgggtgggcaaggtgtgatgaacac
 cttgatggtcgatgtcaccgatttccccgatgtgaaggggggcaacgaagtgggtgctgttcggc
 aaacaggccgggaggaggatcacccaggccgagatagaagaaatcaacggcgcgctgctc
 gccgacctctacacgtatggggcagttccaacccgaagatactcgtcgactga.

[0281] The NBRC12996 BAR amino acid sequence (SEQ ID NO:128) is:
 MPFRRTLLAASLALLITGQAPLYAAPPLSMDNGTTALTAQN
 SNAWVEISAGALQHNIRTLQAEELGGKSKLCAVLKADAYGH
 GIGLVMPSIIAQGVPCVAVASNEEARVVRASGFTGQLVRVR
 LASLGEVEDALQYDMEELVGSAEFARQLDAIAERHKGTLRI
 HMALNSSGMSRNGVEMTTWSGRGEALQITDQKHLQLVAL
 MTHFAVEDKDDVRKGLAAFNEQTDWLIKHAKLDRSKLTL
 HAANSFATLEVPEAHLDMVRTGGALFGDTVPTREYQVRM
 QFKSHVA AVHSYPAGNTVGYDRTFTLARDSRLANITVGYS
 DGYRRVFTNKGHV LINGHRVPVVGKVSMNTLMVDVTD
 FPDVKGGNEVVLFGKQAGREITQAEIEEINGALLADLYTVWGS
 SNPKILVD.

[0282] Analysis using the Signal P 3.0 program (www.cbs.dtu.dk/services/SignalP/) predicts a signal peptide 24 amino acids in length for this racemase.

Assay of NBRC 12996 BAR

[0283] The pET30 NBRC 12996 BAR was transformed into BL21 DE3 pLysS competent cells and the protein was expressed and a cell-free extract was prepared as described in Example 4E. The purified KT2440 BAR (described in Example 4E) (100 µg) was compared to the 12996 BAR (100 µL of cell-free extract). The results are shown in Table 27 below.

Table 27

Enzyme	Time (min)	ppm L-trp produced
KT2440 BAR	0	0
	30	692
	60	1296
	120	2020
	360	2716
	1080	868
NBRC12996 BAR	0	0
	30	114
	60	254
	120	504
	360	1310
	1080	1312

[0284] The NBRC 12996 BAR has activity for racemization of tryptophan. It is not possible to quantitatively compare activities of the KT2440 BAR with the NBRC 12996 BAR because the NBRC BAR was not purified or quantified for this experiment.

[0285] A poster entitled “Tryptophan Racemase Derived from Broad Specificity Amino Acid Racemase by Directed Evolution” (M. Sato, M. Yoneyama, K. Kirimura, and K. Kino, 10th International Symposium on the Genetics of Industrial Microorganisms, Prague, June 24-28 2006) suggested that I384M mutants of this protein would result in higher tryptophan racemase activity. See also K. Kino, M.Sato, M. Yoneyama, and K. Kirimura, Appl Microbiol Biotechnol (2007) 73:1299-1305.

[0286] Mutagenesis was done using the QuickChange-Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA), using the 12996 BAR gene in pET30 which results in an untagged protein. The following mutagenic primer was used to make the I384M change: 5'-ACCCAGGCCGAGATGGAAGAAATCAACG-3' (SEQ ID NO:129).

[0287] The site-directed mutagenesis was done as described in the manufacturer's protocol. Several isolates were sequenced (Agencourt, Beverly, MA) and an isolate with the correct sequence was selected and used for further analysis. The plasmid was transformed into BL21(DE3) (Novagen, Madison, WI) competent cells. Recombinant protein was produced in Overnight Express II medium (Novagen, Madison, WI)

containing 50 µg/mL kanamycin according to manufacturer's protocols. Cell-free extracts were prepared using BugBuster (Novagen, Madison, WI) according to manufacturer's protocols, desalted, and analyzed for percent expression of the target protein using the Experion method described above.

[0288] Total protein assays were done using a Pierce BCA kit (Rockford, IL). Tryptophan racemase assays with the mutant enzyme were performed using the wild-type enzyme prepared in the same manner as a positive control. Assays contained per mL: 30 mM L-tryptophan, 50 mM potassium phosphate pH 8, 10 µM PLP, and approximately 100 µg of racemase protein in a cell free extract. In the case where 100 µg was not used (based on Experion % expression and Pierce total protein numbers), results were normalized. Zero, 30 minute, 2 hour, and overnight samples were collected, treated with 2% formic acid, filtered, and diluted 1:10 for analysis using the chiral amino acid method described in Example 1.

[0289] Although not completely quantitative since unpurified protein was utilized, the data suggest that the I384M mutation did not appear to significantly impact the activity of the enzyme.

4J: Cloning and Expression of *Vibrio fischeri* Alanine Racemase

Experimental Overview

[0290] The gene encoding the *Vibrio fischeri* putative alanine racemase (Genbank Protein Accession No. AAW85230.1 or YP_204118) was cloned, expressed, and tested for activity in conversion of L-tryptophan to D- tryptophan.

Polymerase Chain Reaction Protocol

[0291] *Vibrio fischeri* genomic DNA (ATCC 700601D) was used as a template for PCR amplification of the alanine racemase gene. Primers were designed based on the published gene sequence from strain ES114 (CP000020.1 GI:59478708 (also listed as NC_006840) region 800842..802053). Polymerase chain reaction was performed on genomic DNA using primers designed with 5' restriction sites and overhangs for cloning into the pET 28 and pET30 vectors (Novagen, Madison, WI).

Primer sequences:

N term:

5'-GCGGCCCATATGAAGTTTACTAAATGTGCAT-3' (SEQ ID NO:130) and

C term:

5'-GGCCGCGGATCCCTATTTGTAGATCTTAGGATTTG-3' (SEQ ID NO:131).

[0292] The gene derived from *V. fischeri* was amplified using the following PCR protocol. 2 μ L of genomic DNA (100 ng/ μ L) was used as template for PCR. In a 100 μ L reaction 2 μ L template (genomic DNA 100 ng/ μ L), 0.5 μ L of each primer (100 μ M stock solution), 0.3 mM each dNTP (Roche dNTP mix (Roche, Indianapolis, IN)), 1 μ L Pfu Turbo Polymerase (Stratagene), and 1X Pfu buffer (Stratagene). The thermocycler program used included a hot start at 95 °C for 3 minutes, 10 repetitions of the following steps: 94 °C for 30 seconds, 52 °C for 45 seconds, and 72 °C for 3 minutes, followed by 20 repetitions of the following steps: 94 °C for 30 seconds, 55 °C for 45 seconds, and 72 °C for 3 minutes. After the 20 repetitions the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a product of 1.2-Kb.

Cloning

[0293] The PCR product was gel purified from 0.8% TAE-agarose gel using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The product was TOPO cloned and transformed into TOP10 cells according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Nde* I and *Bam*H I. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing with universal M13 forward and M13 Reverse primers, and the sequence was found to be identical to the accession number listed above.

[0294] The correct TOPO clone was digested with restriction enzymes *Nde* I and *Bam*H I following the manufacturer's recommended protocols (New England Biolabs, Beverly,

MA) and gel purified from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). Vectors pET28 and pET 30 were prepared by digestion with restriction enzymes *Nde* I and *Bam*H I followed by treatment with shrimp alkaline phosphatase (Roche, Indianapolis, IN) and purification from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA).

- [0295] The digested vectors and insert were ligated using the Rapid TM DNA Ligation Kit (Roche, Indianapolis, IN). Approximately 50 ng of treated insert, 100 ng of treated vector (3 to 1 molar ratio of insert to vector), 5 U of T4 DNA ligase, and 1X ligation buffer were incubated for 5 minutes at room temperature. The ligation reaction was used to transform *E. coli* TOP10 chemical competent cells (Invitrogen, Carlsbad, CA). 3 µL of each ligation reaction was added to 40 µL of TOP10 cells, which were transformed using a 30 second heat shock pulse at 42 °C, followed by incubation on ice for 5 min. The cells were allowed to recover in 250 µL of room temperature SOC medium (Sambrook, J., et al. *Molecular Cloning: A Laboratory Manual* 2nd ed., Plainview, NY, (1989), 1.76-1.81 & A.2)) for 1 hour at 37 °C with shaking at 225 rpm. Cells were plated on LB plates containing kanamycin (50 µg/mL). Plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Nde* I and *Bam*H I.

Gene Expression and Assays

- [0296] Plasmid DNA was subcloned into *E. coli* expression host BL21(DE3) pLysS (Novagen, Madison, WI). The cultures were grown and the plasmids were isolated using Qiagen miniprep kit (Qiagen, Valencia, CA), and analyzed by restriction digest to confirm identity.
- [0297] Induction in BL21DE3 was performed in pET28 (tagged) and pET 30 (untagged) vectors. A time course study was performed with cultures grown at 37 °C in 100 mL LB containing kanamycin (50 mg/L) to an OD₆₀₀ of 0.5 and induced with 100 mM IPTG (isopropyl thiogalactoside) and sampled at 0 and 3 hours post induction. Cells from 0 hour and 4 hour time points were resuspended in 1X sodium dodecyl sulfate buffer containing 2-mercaptoethanol, and heated at 95 °C for 10 minutes, and cooled. Aliquots of these total cellular protein samples were analyzed by SDS-PAGE using a 4-15% gradient gel.

- [0298] Cell extracts were also prepared from the 4 hour cultures by suspending cell pellets from 5 mL of culture in Novagen BugBuster™ reagent containing benzonase nuclease and protease inhibitor cocktail set #3 (Calbiochem - Novabiochem Corp., San Diego, CA) at room temperature for 20 minutes with gentle shaking and centrifuging at 16,000 x g to remove cell debris. The supernatants (cell extracts) were loaded onto 4-15% gradient gels for analysis of the cellular soluble proteins. The 4 hour sample from cloned *V. fischeri* alanine racemase showed a total protein band that corresponded to the correct size (approximately 45 kDa) in the pET 28 (tagged) and pET 30 (untagged) vector.
- [0299] Cells from the induced cultures (100 mL) were centrifuged and washed once with 0.85 % NaCl. Cell pellets were resuspended in 5 mL/g wet cell weight of BugBuster™ (Novagen, Madison, WI) reagent containing 5 µL/mL protease inhibitor cocktail set #3 (Calbiochem-Novabiochem Corp., San Diego, CA) and 1 µL/mL benzonase nuclease. Samples were incubated at room temperature for 20 minutes on an orbital shaker. Insoluble cell debris was removed by centrifugation at 16,000 X g for 20 minutes at 4 °C.
- [0300] Cell extracts were assayed for tryptophan racemase activity using the following protocol. 1 mL reactions were carried out in 50 mM potassium phosphate (pH 8.0), with 0.05 mM PLP and 30 mM L tryptophan. The reactions were initiated by the addition of cell free extracts (or purified positive control racemase) and were incubated at 30 °C overnight. Sample aliquots were taken after overnight incubation (zero minute samples served as control reaction). Concentrated formic acid (5 µL) was added to each 250 µL sample aliquot to stop the reaction and the precipitated protein was removed by centrifugation. Supernatants were removed and frozen at -80 °C until they were analyzed for D-tryptophan by the chiral amino acid method in Example 1.
- [0301] Assay results from cell extracts from pET28 and pET30 induction with 100 mM IPTG (3 hours) demonstrate that *V. fischeri* clones show racemase activity on L-tryptophan, resulting in D-tryptophan production, as did the positive control KT2440 BAR purified racemase produced in Example 4E. All attempts were made to add equivalent amounts of racemase protein to each tube. Under the conditions of the assay we observed higher D-tryptophan production when the *V. fischeri* racemase was cloned in pET30 (untagged) vs pET28 (His-tagged). The results are shown in Table 28. One skilled in the art could improve the enzyme activity of the *V. fischeri* racemase through

ensurance of proper folding, increased soluble expression and stability, and mutagenesis methods as described elsewhere.

Table 28

Enzyme Source	Time Point	substrate	Racemase Volume (200 µg)	D-trp conc (µg/mL)	Average D-Trp (µg/mL)
KT2440 purified racemase	Overnight	L-trp	75 µL	337.2	≥337.2
pET28/ <i>V. fischeri</i> racemase	Overnight	L-trp	93 µL	0.68	
pET28/ <i>V. fischeri</i> racemase	Overnight	L-trp	93 µL	1.08	0.88
pET30/ <i>V. fischeri</i> racemase	Overnight	L-trp	167 µL	4.04	
pET30/ <i>V. fischeri</i> racemase	Overnight	L-trp	167 µL	3.5	3.77

4K: Cloning of the *Photobacterium profundum* BAR Homologs

[0302] The amino acid sequences of two *P. profundum* BAR homologs were obtained from public databases (NCBI). The CR378673 (nucleotides 342444..343658) putative alanine racemase coding sequence was assembled from primers. The amino acid sequence (NCBI accession number CAG21670) was used to design an optimal DNA sequence using the GeneComposer software package (Version 1.0, Emerald Biosciences, Bainbridge Island, WA). The CR378673 optimized DNA sequence (SEQ ID NO:132) is:

```
atgaagctgaagctgagcctggtcgccctggcactgatgggtcagactactgctaatgccga
ccactgctggtggacttcgataacaatgagcgtgaggaacgtgtgcaaagctctaatgcgtgg
ctggagattgataccaagcattcagtggaatattcagttactgcagaaccaactgaaagccg
acaccaagatctgtgcgattatgaaggcggatgcatacggtaatggcattgccggcttgatgcc
tagtatcattgctaaccaagtccttgtgttggtatcaccagcaatgaggaagcgcgggtgggtc
gtaaacaatggctttattgggaagatcatgcgtgtccgtgcagcctcgaagaatgaaattgaggg
tggttgctgagtagcagatggaagaattgatcggtacgaaggctcaagccgatcaaatcatcga
aattgcacgcgcaaattggcacgacgattccggttcatttagccttgaatacaagcggcatgggc
cgcaacgggtcggacctgacgacctacgaaggccaagtgaaggtgtagagattgctggcga
tccaaacctggagattgtcggcatgatgactcattcccgaacgagggactggacgaaatcaa
acggaaagtcaaacgtttcaaagtagaaacgaaatggttaattgattccactgacttgaagcgc
aaagatgtgacgtccacgtcgcaaacagctatatcaccttgaatctgcctgaagcgcactctgg
```

atatggtacgccaggtggcatgctgtatggcgactatccggcgacagcggcgatcagcgta
 tcgtaagcttcaagaccacgttgccctctttgcaccactttccggctggctcaaccattgggtacg
 gatctaccgctgttctggaacgtgattcagttctggctaatctgccgattggctattcggtggctt
 cgcgcgctcgtaggaataaagccgaagtcctgattaacggccagcgtgcgcgcgtcatgg
 gtatggtcagtatgaacacgacgatggtcgatgtaacggatattgtggatgttcagaccaatgaa
 gaagtcgtgatcttggccgccagggttcgaagagattacgggcgaggagacggaagagaa
 gtctaatactgattctccggaacattacactgtgtggggcgccacaaacccgcgtatttatcgcta
 a.

[0303] Primers were designed to cover the entire sequence and both the coding and non-coding strand. The sequence was assembled as in Example 10 using the following primers for the final amplification 5'-agaagacatatgaagctgaagctgagcc-3' (SEQ ID NO:133) and 5'-agaagaggatccttagcgataaatacgcggg-3' (SEQ ID NO:134). The resulting PCR product was cloned into pCR-Blunt II-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA). Plasmids with an insert were sent to Agencourt (Beverly, MA) for sequencing. A plasmid with the correct sequence was identified and used for subsequent cloning steps. The TOPO isolate with the correct sequence was digested with *Nde* I and *Bam*H I, the digest was run on a 1% agarose gel and the correct fragment was cut out of the gel. The DNA was purified using the QIAquick[®] gel extraction kit (Qiagen, Valencia, CA). The resulting DNA fragment was ligated to pET30 that had been digested and purified as described above. Clones with an insert were isolated and sent to Agencourt (Beverly, MA) for sequencing. A clone with the correct sequence was isolated and used in further studies.

[0304] The CR378681 (nucleotides 60191..61408) gene encoding a putative alanine racemase (protein sequence accession number CAG23797) from *P. profundum* was isolated in the same manner as above using the following primers for the final amplification (5'-GGCCTTGGCATATGAACTTTAAGATGACTCTG-3' (SEQ ID NO:135) and 5'-TTCCAATTGGATCCTTACTTCAGGTAGTAACGCGGATTC-3' (SEQ ID NO:136)).

[0305] The DNA sequence of the optimized CR378681 racemase (SEQ ID NO:137) is:

ATGAACTTTAAGATGACTCTGTTAAGCCTGGCCATTACAT
 TCCCGAGCTTCAGCATCTATAGCGCGCCACTGGTCATTGA
 TCAGAACCTGCCAAGCGAACAGTCGATTCAGCAAAGCAA

CAGCTGGCTGGAAGTTAGCCTGGGCCAGTTTAAATCCAA
TATTGAACAATTTAAATCTCATATTAAAGCCGATACTAAG
ATTTGTGCCGTTATGAAAGCCGATGCATACGGCAATGGC
ATCTTCGGTCTGATGCCGACAATTCTGGAACAGCAAATC
CCATGCGTGGCGATTGCAAGTAACGCGGAAGCTCGCGCT
GTGCGTGAAAGCGGGTTTAAGGGCCAGCTGCTGCGTGTC
CGCAGCGCGAGCTTAGGCGAGATTAAACAGTCACTGGAC
CTGAACATTGAAGAACTGATCGGCTCACATCAGCAGGCG
AAGTTCATTGCAGAGCTGGGTGTAGAACGTAATCAGAAG
ATTAACGTTCAATTTAGCTCTGAACGACGGAGGGATGGGT
CGCAATGGGATCGATATGTCTACCGAACAAGGCAAAGCC
GAGGCCCTCGACATCGCGACCCAGGCAAATCTGAACATT
GTTGGTATTATGACTCACTTCCCGAACTATAATGCGGATA
AAGTGCGTGTGAAGCTGAAAGACTTCCAGACAAACTCCA
GCTGGCTGATCAAGCAGGCGGATCTGAAGCGCGATGAAC
TCACGCTCCACGTGGCCAACAGCTATGTGTCCATTAATGT
TCCAGAAGCGCAACTGGATATGGTTCGCCCCGGGCGGCGT
GCTGTATGGCGATCTTCCGACCAATCCGGAATATCCGAG
CATCGTATCGTTCAAGACGCGGATTGCGTCAATTCACCA
GCTGCCAGCATCCCAGACCGTGGGCTACGATTTCGACCTA
TATTACGAAACGTGATAGCGTTCTGGCAAACCTGCCAGT
CGGCTACAGTGATGGCTATCCGCGCCGTATGGGTAAATCA
GGCTGATGTGATTATCAACGGACAACGCGCCAAAGTGGT
GGGTGTGACCAGCATGAATACTAGTATCGTCGATATTAC
CGATATTAAAGGCGTTAAACAGGGTCAAGAAGTTACCCT
GTTTGGCAAGCAGAAGAATGTGCAGATTAGCGTGGCCGA
AATGGAGGATTATTCGAAGTTAATCTTCCCGGAACTGTA
CACCATGTGGGGTCAGGCGAATCCGCGTTACTACCTGAA
GTAA (SEQ ID NO:137).

[0306] The plasmids were transformed into BL21 DE3 pLysS and this strain was used for expression. The strain with the plasmids was grown to an OD₆₀₀ of ~0.6 and expression was induced with 1 mM IPTG. After incubation at 30 °C (CR378681) or 26 °C

(CR378673), with aeration, the cell pellet was harvested by centrifugation and the cell pellet was frozen at -80°C . The cell pellet was thawed on ice and the cells were lysed with an appropriate volume of BugBuster and Benzonase nuclease (see product insert) (Novagen, Madison, WI). The lysate was centrifuged to remove cell debris and the resulting cell-free extract was used in racemase assays. Under these conditions, it was not possible to detect conversion of L-trp to D-trp. The enzymes also did not appear to catalyze the conversion of L-alanine to D-alanine when assayed in similar manner as above for tryptophan, even when the detergent was removed from the cell free extracts. The levels of D-alanine produced in assays were similar as compared to cellular extracts without overexpressed racemase genes, indicating that the background production of D-tryptophan was due to endogenous *E. coli* alanine racemase. The enzymes did not appear to express well and activity may be detected if expression is enhanced or if the enzyme is improved through mutagenesis methods known to those in the art. Protein activity may also be enhanced by methods that can encourage proper folding, as many of the racemase enzymes produced did not appear to be soluble and aggregated or precipitated quite easily. The enzyme activity may be difficult to detect due to improper folding or improper insertion of the pyridoxal-5'-phosphate cofactor. Encouraging proper folding or cofactor insertion may greatly enhance enzyme activity.

4L: Cloning and Expression of *Pseudomonas taetrolens* Arginine Racemase

Experimental Overview

- [0307] *Pseudomonas taetrolens* (also known as *P. graveolens*) arginine racemase (Genbank Accession No. AB096176, nucleic acid sequence) and an I384M mutant thereof, was cloned, expressed, and tested for activity in conversion of L-tryptophan to D-tryptophan. This gene is 72% identical to the *P. putida* BAR gene from KT2440 and 73% identical to the *P. putida* BAR gene from NBRC 12996 described above. The amino acid sequence is 72% identical to both *P. putida* BAR proteins.

Polymerase Chain Reaction Protocol

- [0308] *Pseudomonas taetrolens* (ATCC 4683) was grown in nutrient broth at 28°C with shaking at 225 rpm. Polymerase chain reaction was performed on whole cells using

primers designed with 5' restriction sites and overhangs for cloning into the pET 28 and pET30 vectors (Novagen, Madison, WI).

[0309] The primer sequences were:

N term: 5'-ATAATACATATGCCCTTCTCCCGTACCC -3' (SEQ ID NO:138) and

C term: 5'-GCGGCGGGATCCTTACTGATCTTTCAGGATT-3' (SEQ ID NO:139).

[0310] The gene derived from *P. taetrolens* was amplified using the following PCR protocol. Twenty-five µL of grown cells were lysed at 96 °C for 10 minutes. Cell debris was removed by centrifugation and the supernatant was used as template for PCR. A 100 µL reaction contained 5 µL template (lysed cell supernatant), 1.6 µM of each primer, 0.3 mM each dNTP, 10 U rTth Polymerase XL (Applied Biosystems, Foster City, CA), 1X XL buffer and 1 mM Mg(OAc)₂. The thermocycler program used included a hot start at 94 °C for 3 minutes, 8 repetitions of the following steps: 94 °C for 30 seconds, 52 °C for 30 seconds, and 68 °C for 2 minutes, followed by 22 repetitions of the following steps: 94 °C for 30 seconds, 58 °C for 30 seconds, and 68 °C for 2 minutes. After the 22 repetitions, the sample was maintained at 68 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a product of 1230 bp.

Cloning

[0311] The PCR product was gel purified from 0.8% TAE-agarose gel using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The product was TOPO cloned and transformed into TOP 10 cells according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Nde* I and *Bam*H I. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing with universal M13 forward and M13 Reverse primers.

[0312] The correct TOPO clone was digested with restriction enzymes *Nde* I and *Bam*H I following the manufacturer's recommended protocols (New England Biolabs, Beverly, MA) and gel purified from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). Vectors pET 28 and pET 30 were prepared by digestion with restriction enzymes *Nde* I and *Bam*H I followed by treatment with shrimp alkaline phosphatase (Roche, Indianapolis, IN) and purification from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The digested vectors and

insert were ligated using the RapidTM DNA Ligation Kit (Roche, Indianapolis, IN). Approximately 50 ng of treated insert, 100 ng of treated vector (3 to 1 molar ratio of insert to vector), 5 U of T4 DNA ligase, and 1X ligation buffer were incubated for 5 minutes at room temperature. The ligation reaction was desalted using the High Pure PCR Product Purification Kit (Roche, Indianapolis, IN) and used to transform *E. coli* DH10B electrocompetent cells (Invitrogen, Carlsbad, CA). Ten μ L of each ligation reaction was added to 40 μ L of DH10B cells, which were transformed by electroporation using the BioRad Gene Pulsar II under the following conditions: 2.5 kV, 25 μ F, 200 ohm in a 0.2 cm cuvette. The cells were allowed to recover in 1 mL of room temperature SOC for 1 hour at 37 °C with shaking at 225 rpm. Cells were plated on LB plates containing kanamycin (50 μ g/mL). Plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Nde* I and *Bam*HI.

Gene Expression and Assays

- [0313] Plasmid DNA was transformed into *E. coli* expression host BL21(DE3) pLysS (Novagen, Madison, WI). The cultures were grown and the plasmids were isolated using the Qiagen miniprep kit (Qiagen, Valencia, CA) and analyzed by restriction digest to confirm identity.
- [0314] Induction in BL21DE3 pLysS was initially performed in both pET 28 (histidine-tagged) and pET 30 (untagged) vectors. A time course study was performed with cultures grown at 37 °C in 100 mL LB containing kanamycin (50 mg/L) to an OD₆₀₀ of 0.5 and induced with 100 μ M IPTG (isopropyl thiogalactoside) and sampled at 0 and 3 hours post induction. Cells from 0 hour and 3 hour time points were resuspended in 1X sodium dodecyl sulfate buffer containing 2-mercaptoethanol and heated at 95 °C for 10 minutes, and cooled. Aliquots of these total cellular protein samples were analyzed by SDS-PAGE using a 4-15% gradient gel.
- [0315] Cell extracts were also prepared from the 3 hour cultures by suspending cell pellets from 5 mL of culture in Novagen BugBusterTM reagent containing benzonase nuclease and protease inhibitor cocktail set #3 (Calbiochem-Novabiochem Corp., San Diego, CA) at room temperature for 20 minutes with gentle shaking and centrifuging at 16,000 x g to remove cell debris. The supernatants (cell extracts) were loaded onto 4-15% gradient gels for analysis of the cellular soluble proteins.

[0316] The 3 hour sample from cloned *P. taetrolens* arginine racemase showed a total protein band that corresponded to the correct size (approximately 45 kDa) in the pET 30 (untagged) vector. The *P. taetrolens* pET 30 gene product was over-expressed at a higher level than the *P. taetrolens* pET 28 (histidine-tagged) gene product, but neither of the vectors gave a visible soluble protein band.

[0317] Cells from the induced cultures (100 mL) were centrifuged and washed once with 0.85 % NaCl. Cell pellets were resuspended in 5 mL/g wet cell weight of BugBuster™ (Novagen, Madison, WI) reagent containing 5 µL/mL protease inhibitor cocktail set #3 (Calbiochem-Novabiochem Corp., San Diego, CA) and 1 µL/mL benzonase nuclease. Samples were incubated at room temperature for 20 minutes on an orbital shaker. Insoluble cell debris was removed by centrifugation at 16,000 X g for 20 minutes at 4 °C.

[0318] Cell extracts were assayed for tryptophan racemase activity using the following protocol. One mL reactions were carried out in 50 mM potassium phosphate (pH 8.0), 0.05 mM PLP and 30 mM L tryptophan. The reactions were initiated by the addition of cell free extracts and were incubated at 30 °C overnight. Sample aliquots were taken after overnight incubation (zero minute samples served as control reaction). Concentrated formic acid (5 µL) was added to each 250 µL sample aliquot to stop the reaction and the precipitated protein was removed by centrifugation. Supernatants were removed and frozen at -80 °C until they were analyzed for D-tryptophan by the chiral amino acid method described in Example 1.

[0319] Assay results from cell extracts from pET28 and pET30 induction with 100 µM IPTG (3 hours) demonstrate that *P. taetrolens* clones show racemase activity on L-tryptophan. Again, the tagged version of the BAR appears to be less active and may precipitate or be less soluble than the untagged (pET28). Table 29, below, shows the initial results, although not quantitative as very poor soluble protein was obtained.

Table 29

Treatment	Time Point	Substrate	Racemase extract (200 µg)	D-trp conc (µg/mL)
pET28 / <i>P. taetrolens</i>	0	L-trp	500 µL	nd
pET30 / <i>P. taetrolens</i>	0	L-trp	500 µL	nd
pET28 / <i>P. taetrolens</i>	overnight	L-trp	500 µL	140

pET30 / <i>P. taetrolens</i>	overnight	L-trp	500 μ L	226
------------------------------	-----------	-------	-------------	-----

[0320] Induction of the pET30 (untagged) construct was repeated using same conditions as mentioned above and a visible soluble protein band was observed in SDS-PAGE. The assay was repeated using same the conditions described above and the results, as shown in Table 30, were obtained.

Table 30

Treatment	Time Point	Substrate	Racemase extract (μ L)	D-trp conc (μ g/mL)
<i>P. taetrolens</i> -pET30	0	L-trp	300	Nd
<i>P. taetrolens</i> -pET30	0	L-trp	150	Nd
<i>P. taetrolens</i> -pET30	2 hr	L-trp	300	319
<i>P. taetrolens</i> -pET30	2 hr	L-trp	150	308
<i>P. taetrolens</i> -pET30	Overnight	L-trp	300	1586
<i>P. taetrolens</i> -pET30	Overnight	L-trp	150	1658

[0321] Again, it was noted that doubling of the volumes did not scale to more activity. For future work, it was determined to remove the protein from Bugbuster as quickly as possible after preparation of cell extracts and to store the protein in 50 mM phosphate buffer pH 8 containing 0.01 mM PLP. The detergent in Bugbuster may inhibit the reaction or may cause a loss of activity upon storage.

[0322] Induction of the pET30 construct was carried out again and the cell extract was processed with anion exchange chromatography (as in Example 4E) to give a more pure extract. The assay was repeated with this partially purified prep. The numbers in parenthesis in the Racemase extract column of Table 31 below indicate the approximate amount of partially purified racemase enzyme used in the assay. The results of the assay are shown in Table 31 below.

Table 31

Enzyme Source	Time Point	Substrate	Racemase extract	D-trp conc (μ g/mL)
KT2440	0	L-trp	75 μ L (90 μ g)	nd
NBRC 12996	0	L-trp	42 μ L (200 μ g)	nd
NBRC 12996	0	L-trp	21 μ L (100 μ g)	nd
<i>P. taetrolens</i>	0	L-trp	108 μ L (200 μ g)	nd
<i>P. taetrolens</i>	0	L-trp	54 μ L (100 μ g)	nd
KT2440	2 hr	L-trp	75 μ L (90 μ g)	661

NBRC 12996	2 hr	L-trp	42 μ L (200 μ g)	408
NBRC 12996	2 hr	L-trp	21 μ L (100 μ g)	208
<i>P. taetrolens</i>	2 hr	L-trp	108 μ L (200 μ g)	862
<i>P. taetrolens</i>	2 hr	L-trp	54 μ L (100 μ g)	547
KT2440	overnight	L-trp	75 μ L (90 μ g)	2386
NBRC 12996	overnight	L-trp	42 μ L (200 μ g)	2382
NBRC 12996	overnight	L-trp	21 μ L (100 μ g)	1706
<i>P. taetrolens</i>	overnight	L-trp	108 μ L (200 μ g)	2029
<i>P. taetrolens</i>	overnight	L-trp	54 μ L (100 μ g)	2099

[0323] The non-linearity of the overnight sample in this case is probably due to the fact that the reactions are reaching equilibrium. Clearly, the *P. taetrolens* BAR has significant activity for racemization of tryptophan, as do the 12996 BAR and KT2440 BAR. It appears that the KT2440 BAR and the *P. taetrolens* BAR have similar activity, which is slightly higher than the 12996 BAR.

[0324] The DNA Sequence of the *P. taetrolens* arginine racemase is shown below as SEQ ID NO:140. The PCR sequence gave two changes as compared with the published NCBI sequence. Specifically, the PCR sequence contained an adenosine rather than a guanine at position 902 and a cytosine rather than a guanine at position 921. These DNA changes resulted in one silent mutation as well as one change from glycine to aspartate at amino acid position 301.

```

ATGCCCTTCTCCCGTACCCTGCTCGCCCTTTCCTTGGCAT
GGCATTGCTGCAAAACCCGGCCTTTGCTGCGCCACCCCTG
TCGATGACCGACGGCGTAGCTCAAGTGAATACCCAGGAC
AGCAATGCCTGGGTCGAAATCAATAAAGCCGCGTTTCGAG
CACAACATACGGACTCTGCAAACCGCCCTCGCCGGCAAG
TCGCAGATCTGCGCCGTACTCAAGGCGGATGCCTATGGC
CACGGTATCGGCTTGTTGATGCCCTCGGTGATCGCCATGG
GTGTTCCCTGTGTGCGGTGTCGCCAGCAACGAAGAAGCCC
GCGTCGTGCGCGAGAGCGGTTTCAAGGGTCAACTGATAC
GCGTGCGCACCGCTGCCCTGAGCGAACTGGAAGCTGCAC
TGCCGTACAACATGGAAGAGCTGGTGGGCAACCTGGACT
TCGCGGTCAAGGCCAGCCTGATTGCCGAGGATCACGGTC
GCCCCGCTGGTGGTGCACCTGGGTCTGAATTCCAGCGGCA
TGAGCCGTAACGGAGTGGACATGACCACCGCTCAGGGCC
GTCGTGATGCGGTAGCTATCACCAAGGTGCCAAACCTGG

```

AAGTGC GGGCGATCATGACCCACTTCGCGGT CGAAGATG
CTGCCGACGTGCGTGCCGGGCTCAAGGCCTTCAATCAGC
AAGCCCAATGGCTGATGAACGTGGCCCAGCTTGATCGCA
GCAAGATCACCTGCACGCGGCCAACTCGTTCGCCACAC
TGGAGGTGCCCCGAATCGCATCTGGACATGGTCCGCCCCG
GCGGCGCGCTGTTGCGCGACACCGTACCGTCCCACACCG
AGTACAAGCGGGTCATGCAGTTCAAGTCCCACGTGGCGT
CGGTCAACAGCTACCCCAAGGGCAACACCGTCGGTTATG
ACCGCACGTACACCCTGGGCGCGACTCGCGGTGGCCA
ACATCACCGTCGGCTACTCTGACGGCTACCGCCGCGCGTT
TACCAATAAAGGGATTGTGCTGATCAACGGCCATCGCGT
GCCAGTGGTGGGCAAAGTCTCGATGAACACCCTGATGGT
GGACGTCACTGACGCGCCGGATGTGAAAAGCGGCGATGA
AGTGGTGCTGTTGCGGCACCAGGGCAAGGCCGAGATTAC
CCAGGCTGAGATCGAAGACATCAACGGTGCACTGCTTGC
GGATCTGTATACCGTGTGGGGCAATTCCAACCCTAAAAT
CCTGAAAGATCAGTAA

(SEQ ID NO:140).

[0325] The amino acid sequence of the *P. taetrolens* arginine racemase is shown below as
SEQ ID NO:204.

MPFSRTLLALSLGMALLQNPAFAAPPLSMTDGVAQVNTQD
SNAWVEINKAAFEHNIRTLQTALAGKSQICAVLKADAYGH
GIGLLMPSVIAMGVPCVGVASNEEARVVRESGFKGQLIRVR
TAALSELEAALPYNMEELVGNLDFAVKASLIAEDHGRPLV
VHLGLNSSGMSRNGVDMTTAQGRRDAVAITKVPNLEVR
MTHFAVEDAADVRAGLKAFNQQAQWLMNVAQLDRSKITL
HAANSFATLEVPESHLDMVRPGGALFGDTVPSHTEYKRVM
QFKSHVASVNSYPKGNTVGYDRYTLGRDSRLANITVGYS
DGYRRAFTNKGIVLINGHRVPVVGKVSMTLMVDVTDAP
DVKSGDEVVLFQGHQGAETQAEIEDINGALLADLYTVWG
NSNPKILKDQ

(SEQ ID NO:204).

- [0326] The protein encoded by the gene of SEQ ID NO:140 was analyzed by the signal peptide prediction program Signal P 3.0 (www.cbs.dtu.dk/services/SignalP/) and a leader sequence of 23 amino acids was predicted.
- [0327] The following PCR primer pair was used to clone the *P. taetrolens* gene without amino acids 2-23 of the leader sequence:
- P.taetMinus leader F NdeI: 5'-GGTTAATTCATATGGCGCCACCCCTGTCGAT-3' (SEQ ID NO:180)
- P.taetCtermXho: 5'-AAGTCGCTCGAGCTGATCTTTCAGGATTTTAG-3' (SEQ ID NO:181).
- [0328] The C-terminal primer shown above was also used with SEQ ID NO:138 to produce the wildtype protein in a manner which allowed for purification, as described in Example 4H for the *P. putida* KT2440 BAR. The leaderless *P. taetrolens* racemase, when expressed, was found to lose a significant amount of the activity, as compared with the expression product of the full-length gene. The periplasmic and cytoplasmic protein fractions were isolated for the wildtype expression products, as well as the leaderless constructs, as described in the pET System Manual (Novagen, Madison, WI). The expressed wildtype BAR was present in the periplasmic fraction, while the expression of the leaderless BAR was significantly reduced. The loss in activity of the leaderless *P. taetrolens* BAR may be due to a change in processing and/or folding when expressed in the cytoplasm.

I384M Mutagenesis of *P. taetrolens* BAR

- [0329] Mutagenesis was done using the QuickChange-Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA), using the *P. taetrolens* BAR gene in pET30 which results in an untagged protein. The following mutagenic primer was used to make the I384M change: 5'-TACCCAGGCTGAGATGGAAGACATCAACG-3' (SEQ ID NO:141).
- [0330] The site-directed mutagenesis was done as described in the manufacturer's protocol. Several isolates were sequenced (Agencourt, Beverly, MA) and an isolate with the correct sequence was selected and used for further analysis.
- [0331] The plasmid was transformed into BL21(DE3) cells (Novagen, Madison, WI). Recombinant protein was produced in Overnight Express II medium (Novagen, Madison, WI) containing 50 µg/mL kanamycin according to manufacturer's protocols. Cell-free extracts were prepared using BugBuster (Novagen, Madison, WI) according to

manufacturer's protocols, desalted, and analyzed for percent expression of the target protein using the Experion method described above.

[0332] Total protein assays were done using a Pierce BCA kit (Rockford, IL). Tryptophan racemase assays with the mutant enzyme were performed using the wild-type enzyme prepared in the same manner as a positive control. Assays contained per mL: 30 mM L-tryptophan, 50 mM potassium phosphate pH 8, 10 μ M PLP, and approximately 100 μ g of racemase protein in a cell free extract. In the case where 100 μ g was not used (based on Experion % expression and Pierce total protein numbers), the results were normalized. Zero, 30 minute, 2 hour, and overnight samples were collected, treated with 2% formic acid, filtered, and diluted 1:10 for analysis using the chiral amino acid method described in Example 1.

[0333] Quantification was difficult due to the fact that crude lysates were utilized. The wild-type enzyme appeared to produce similar amounts of D-tryptophan in 30 minutes and in the 2 hour time point, as compared with the I384M mutant. The I384M mutant appeared to have, at most, approximately twice the activity of the wildtype under the conditions tested. When assays were done for monatin production, as in Example 4F, the I384M did not appear to provide any benefit over the wild-type *P. taetrolens* enzyme under the conditions tested.

4M: *Streptomyces coelicolor* Racemase Assays

[0334] *Streptomyces* species are known to produce calcium-dependent antibiotics that contain D-amino acids such as D-tryptophan. It is not known whether the tryptophan is racemized prior to or after incorporation in the antibiotic. Although public *Streptomyces* genomes do not contain a highly homologous alanine racemase to those that have activity from *Pseudomonas* species, cloning work was undertaken to determine if in fact the enzymes had broad specificity as well.

[0335] *Streptomyces violaceoruber* (ATCC BAA-471 deposited as *Streptomyces coelicolor*) was grown in ISP Medium 1 (BD catalog # 276910, Franklin Lakes, NJ). Cells were lysed by ultrasonication and cell debris was removed by centrifugation. Cell-free extracts were assayed for tryptophan racemase activity (starting with L-tryptophan). Under the conditions tested, it was not possible to detect conversion of L-trp to D-trp.

- [0336] *Streptomyces coelicolor* was annotated in ERGO™ as containing 3 alanine racemases (NCBI Accession numbers NP_628903, NP_628089, and NP_629139). The published DNA sequences were amplified from *S. coelicolor* by PCR. Each gene was cloned into vector pET30 using restriction sites *Nde* I and *Bam*H I.
- [0337] The plasmids were transformed into expression host BL21 DE3. Strains with the plasmids encoding proteins NP_628089 and NP_629139 were grown to an OD₆₀₀ of ~0.6 and expression was induced with 100 µM IPTG. After incubation at 37 °C with aeration, the cell pellets were harvested by centrifugation and frozen at –80 °C. Strain with the plasmid encoding protein NP_628903 was grown in Overnight Express medium II at 30 °C and induced per manufacturer's instructions (Novagen, Inc, Madison, WI). The cell pellet was harvested by centrifugation and frozen at –80 °C.
- [0338] The cell pellets were thawed on ice and the cells were lysed with an appropriate volume of BugBuster and Benzonase nuclease (Novagen, Madison, WI). The lysates were centrifuged to remove cell debris and the resulting cell-free extracts were used in racemase assays as described in Example 4E. Under these conditions, it was not possible to detect conversion of L-tryptophan to D-tryptophan, even when the detergent was removed from the cellular extract. The enzymes also did not appear to produce D-alanine from L-alanine when assayed similarly, as compared to cellular extracts of *E. coli* without a recombinant racemase present. It is possible that these enzymes are mis-annotated or have very low activity under the conditions they were assayed. However, it is also possible that the enzymes were not produced correctly in *E. coli*.

4N: Cloning the *Yersinia pseudotuberculosis* BAR Homolog

- [0339] The *Y. pseudotuberculosis* BAR homolog was cloned from genomic DNA obtained from Virginia Miller and Matt Lawrenz at Washington University in St. Louis, MO (strain YPIII). The primers used are as follows: 5'-gcggcgcatatgcacgttcgtttcgtc-3' (SEQ ID NO:142) and 5'-ggcggcgggatcccggtgaaataacttaattctac-3' (SEQ ID NO:143). The gene was amplified from the genomic DNA by PCR and was purified with the QIAquick® PCR purification kit (Qiagen, Valencia, CA). The fragment was digested with *Nde* I and *Bam*H I. The digest was run on a 1% agarose gel and the racemase fragment was cut out of the gel. The DNA was purified from the gel with the QIAquick® gel extraction kit (Qiagen, Valencia, CA). The fragment was ligated to pET30 that had been digested and

purified as described above. The ligation was transformed into TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated and run on a 1% agarose gel to screen for inserts. Several plasmid isolates with an insert were sent to Agencourt (Beverly, MA) for sequencing. An isolate with the correct sequence was selected and transformed into BL21 DE3 pLysS competent cells to make the expression strain.

[0340] The expression strain was grown to an OD₆₀₀ of about 0.6 and then induced with 1 mM IPTG. The culture was grown at 37 °C for 3 hours and the cell pellet was harvested by centrifugation. The cell pellet was stored at -80 °C until use.

[0341] The cell pellet was thawed on ice and resuspended in an appropriate volume of BugBuster plus Benzonase nuclease (see product insert) (Novagen, Madison, WI) to lyse the cells. The cell debris was removed by centrifugation and the resulting cell-free lysate was used in racemase assays as in Example 4E. The extract did not appear to have detectable tryptophan racemase activity.

[0342] The expression strain was again grown to an OD₆₀₀ of 0.6 (3 100 ml cultures) and induced with 1 mM IPTG. One of the cultures was incubated at 37 °C for 6 hours, another was incubated at 30 °C overnight and the third was incubated at 26 °C overnight. Cells were harvested by centrifugation and the cell pellets were stored frozen. The cell pellets were thawed on ice and a cell-free extract was made using BugBuster and Benzonase (Novagen, Madison, WI) as described in this example. An SDS gel (4-15% gradient (BioRad, Hercules, CA)) was run. Induction at 37 °C leads to the expression of very little soluble racemase and the level of soluble racemase was higher when 30 °C was the expression temperature and was even higher when 26 °C was the expression temperature. The cell-free extract made at 26 °C was used in racemase assays and again no tryptophan racemase was detected in this cell-free extract. However, activity assays using L-alanine as the substrate gave very low conversion to D-tryptophan, which can be accounted for by native *E. coli* alanine racemases. The *Y. pseudotuberculosis* BAR homolog may have activity under different conditions and it is possible to improve the activity to the *Y. pseudotuberculosis* BAR homolog by mutagenesis techniques known to those in the art. Protein activity is also enhanced by methods that can encourage proper folding. The enzyme activity may be difficult to detect due to improper folding or

improper insertion of the pyridoxal-5'-phosphate cofactor. Encouraging proper folding or cofactor insertion may greatly enhance enzyme activity.

[0343] The *Y. pseudotuberculosis* YPIII BAR homolog DNA sequence is SEQ ID NO:144 and is shown below:

```
atgcacgttcgtttcatcattattcttattaccattaataactttggtcgctttagccaacccgtat
caaaaaaccatcttagcctgacctcactatctgccaacgccagcaacctgtagtaataatgc
gtggcttgaaatctctcaagggtgcgtggatttcaataactaaaaagatgcttacactgctggataa
taaatccacactttgtgcaatattaaaagggtgatgcctatggacatgacctgaccttagtcacacc
ggatgatgctaaaaacaatgtgcaatgtattgggggtgccagcaatcaggaactaaaaacggta
cgtgatctaggatttacggggcagttgatacgggtcagaagtgaacattaaaagaaatgcaac
aagctatggcttacgatgttgaagaacttattggcgataaaaccgtcgtgagcagttaaataat
attgcaaaactgaatggaaaagttctgcgtatccatctggcactgaactccgcagggatgtctcg
taatgggctggagggtcagtaaggcccgcggtttaaataagacgcaaagacaattgtaggttaaaa
aatctgacaatcgttggcatcatgtgcactaccgggtggaagatgctagcgaaatcaaagca
gacttggctcgattccagcaacaagccaagatgtatcgcggtcacggggctaaaacgtgaa
aagattaagctccacgtcgccaatacattcgcgaccttagcgggtgcctgattcatggttggatat
ggtcctgttgggaggggtgtttatgggtgacaccatcgccagcacagagtataagcgggtcat
gacctcaaatctaacatcgcatcgctgaacaactaccctaaggcggtactgttggctatgacc
ggacctatacattgaaacgtgattccctgctggcgaatatccccgtgggttatgccgatgggtat
cgccgagtatttagtaatgcggggcatgtgattattcaaggtcagcgctgcccgtattaggcaa
aacatcaatgaatacgggtcatggttagacgtcaccgatctgaaaaaagtgaagtttaggtgatgaa
gttgcttcttcgtaagcaaggcaatcggaattcaggcagaagaaattgaagatctcagt
gcgcactctttaccgaaatgtcaattctgtggggcgcaaccaataagcgtattctggtagattaa
(SEQ ID NO:144).
```

[0344] The *Y. pseudotuberculosis* YPIII BAR homolog amino acid sequence is SEQ ID NO:145 and is shown below:

```
MHVRFHHLFLLPLITLVACSQPVSKNHLSTLSLANAQQPV
VNNAWLEISQGALDFNTKKMLTLLDNKSTLCAILKGDYAG
HDLTLVTPVMLKNNVQCIGVASNQELKTVRDLGFTGQLIRV
RSATLKEMQQAMAYDVEELIGDKTVAEQLNNIAKLNGKVL
RIHLALNSAGMSRNGLEVSKARGLNDAKTIVGLKNLTIVGI
MSHYPVEDASEIKADLARFQQQAKDVIAVTGLKREKIKLHV
```

ANTFATLAVPDSWLDMVRVGGVFYGDTIASTEYKRVMTFK
 SNIASLNNYPKGGTVGYDRITYTLKRDSLLANIPVGYADGYR
 RVFSNAGHVIIQQQRLPVLGKTSMTVMVDVTDLKKVSLG
 DEVVLFGKQGNAEIQAEIEIDLSGALFTEMSILWGATNKRIL
 VD (SEQ ID NO:145).

4O: Identification of *Aeromonas* Species BAR Genes

[0345] As mentioned in Example 4E, there is evidence in the literature that the *Aeromonas* species contain a broad specificity amino acid racemase with properties similar to the *Pseudomonas* derived enzymes. This example describes identification and isolation of broad specificity amino acid racemases in *A. hydrophila*, *A. jandei*, *A. sobria* and *A. caviae*, as well as identification of tryptophan racemase activity in *A. caviae* cellular extracts. This example also describes the isolation of the partial nucleic acid sequences of BAR homologs in *A. schubertii* and *A. salmonicida*.

[0346] A blast analysis of the *Aeromonas hydrophila* ATCC7966^T genome on the TIGR website (tigrblast.tigr.org/ufmg/index.cgi?database=a_hydrophila%7Cseq) with the BAR protein sequence from the public *Pseudomonas taetrolens* arginine racemase protein sequence (coded for by accession number AB096176) resulted in the identification of a partial protein sequence with 60% identity at the amino acid level. DNA positions 33912-32746 on contig 1047085923747 corresponded to a region of homology of 390 amino acids. The partial sequence is shown below.

[0347] Partial sequence of Putative BAR from *Aeromonas hydrophila* – Protein 1 (SEQ ID NO:146):

AVAAPYLPLASDHRNGEVQTASNAWLEVDLGAFEHNIQTL
 KDRLGDKGPKICAIMKADAYGHGIDLLVPSVVKAGIPCIGIA
 SNEEARVAREKGFTGRLMRVRAATPAEVEQALPYKMEELI
 GSLVSAQGIADIAQRHHTNIPVHIALNSAGMSRNGIDRLAD
 SKEDALAMLKLGITPVGIMTHFPVEEKEDVKMGLAQFKL
 DSQWLLEAGKLDRSKITIHAANSFATLEVPDAYFDMVRPGG
 LLYGDSIPSYTEYKRVMAFKTQVASVNHYPAGNTVGYDRT
 FTLKRDSWLANLPLGYSDGYRRALS NKAYVLIQQQKVPVV

GKTSMTIMVDVTDLKGVPKPGDEVVLFGRQGAEVKQAD
LEEYNGALLADMYTIWGYTNPCKIK (SEQ ID NO:146).

[0348] Blasting NCBI with the partial putative BAR amino acid sequence from *Aeromonas hydrophila* identifies alanine racemases with highest sequence identity to *Pseudomonas putida* F1 (Accession number ZP_00898332.1 GI:82735470), then *Pseudomonas* KT2440, confirming that this is most likely an amino acid racemase protein. Example 4E demonstrates that the BAR protein from *Pseudomonas* KT2440 is active and converts L-tryptophan to D-tryptophan. Thus, it was thought to be likely that the *A. hydrophila* ATCC7966^T racemase also has broad specificity as well.

[0349] When the *Pseudomonas taetrolens* BAR gene sequence (Accession number AB096176) or the *Pseudomonas* KT2440 BAR gene sequence (Accession number NC_002947.3:6018117..6019190) was used to blast the TIGR website, a DNA sequence with high homology (69%) over 1216 base pairs was observed on contig 1047085923747:a_hydrophila. A P score of 1.5 E-110 was observed. The nucleotide numbers corresponding to the match are 32754-33954. The sequence of the *A. hydrophila* partial gene sequence obtained from these alignments is as follows (SEQ ID NO:147):

```
tcttgggggtt ggtgtagccc cagatggtgt acatgtccgc cagcagggcg
ccgttgtagt cttccagatc cgctgtttc acctcagcct caccctggcg
gccgaacagc accacctcgt caccgggttt gacccctttc agatcggtca
cgtccaccat gatggtgttc atggaggtct tgcccaccac cggcaccttc
tgccctgga tcagcacata ggcttggtg ctacagcgcc gccgatagcc
gtcggagtag ccagcgga ggttggcgag ccaggagtcg cgcttgaggg
tgaaggtgcg gtcataaccg acggtgttgc cggccgggta gtggttgacg
gaggcaacct gggctctgaa cgccatcacc cgcttgtagt cggtgtagga
ggggatggag tcaccgtaca gcaggccgcc cgggcgcacc atgtcgaagt
aggcgtccgg cacttccagg gtggcgaagg agttggcggc gtggatggtg
atcttgctgc gatccagctt gcccgcttcc agcagccact gggagtccag
tttgaactgg gccagcccca tcttgacgtc ctctttctcc tccaccggga
agtgggtcat gatgccgacc ggggtgatcc ccttgagctt gagcatggcc
agcgcgtctt ccttgagtc agccaggcgc agatcgatgc cgttgcggtc
catgccggcg gagttgagcg cgatgtgcac cgggatattg gtgtggtggc
gctgggcgat gtcggcgatg ccctgagcac tcaccaggct gccgatgagc
tcttccatct ttagggcag ggctgttcc acttcggccg ggttgccggc
acgtaccgcg atcaggcggc cgtgaagcc cttctcacgg gccacgcggg
```

cctcttcggtt gctggcgatg ccgatgcagg ggatgccggc cttgaccacc
 gagggcacca gcaggtcgat gccgtggccg taggcgtcgg cttcatgat
 ggcgagatc ttcggccctt tgcaccgag gcgatccttg agggctctgga
 tgttgtgctc gaaggcgccg agatcgactt ccagccaggc attgctggcg
 gtctgcactt cgccgttgcg atgatcgctg gccagcggca ggtaaggggc
 cgcgacggcc tgaccggcca gcaggcccag gatcagcgtg
 gccagcagtg t (SEQ ID NO:147).

- [0350] Pileup analysis suggested that only 5 amino acids from each end of the protein sequence were absent, so a chimeric protein was designed using the known protein sequence fragment above and consensus sequences based on other broad specificity racemases. The following primers were designed to construct this chimera for cloning into pET28 and pET30 using *Nde* I and *Bam*HI sites.

hydrophila FNdeI (SEQ ID NO:148): 5'-
 TTCCAAGGCATATGCCCTTCTCCCGTACACTGCTGGCCACGCTGATCCT 3';
 and

hydrophila RBamHI (SEQ ID NO:149): 5'-
 GGAACCTTGGATCCTCAATCTTTGATTTTCTTGGGGTTGGTGTAGCCCCAGA
 TG 3'.

- [0351] The chimeric gene derived from *A. hydrophila* was amplified using the primers described above and the following PCR protocol. In a 50 μ L reaction, 1 μ L template (genomic DNA ATCC 7965), 1.6 μ M of each primer, 0.3 mM each dNTP, 2.5 U Pfu Polymerase (Stratagene, La Jolla, CA), 1X Pfu buffer, and 2.5 μ L dimethyl sulfoxide were used. The thermocycler program used included a hot start at 94 °C for 3 minutes, 8 repetitions of the following steps: 94 °C for 30 seconds, 53 °C for 45 seconds, and 72 °C for 2.5 minutes, followed by 22 repetitions of the following steps: 94 °C for 30 seconds, 60 °C for 45 seconds, and 72 °C for 2.5 minutes. After the 22 repetitions, the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a product of ~1230 bp.

Cloning

- [0352] The PCR product was gel purified from 0.8% TAE-agarose gel using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The product was TOPO cloned and transformed into TOP 10 cells according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Plasmid DNA was purified from the resulting transformants using the

Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Nde* I and *Bam*H I. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing with universal M13 forward and M13 Reverse primers.

[0353] The consensus sequence obtained below codes for a protein that is 95% homologous at the amino acid level to the published TIGR sequence for *A. hydrophila* (protein 1 above).

[0354] The amino acid sequence for the *A. hydrophila* chimeric protein is shown below as SEQ ID NO:151.

MPFSRTLATLILGLLAGQAVAAPYLPLASDHRNGEVQTAS
NAWLEVDLTAFEQNLQTKTRLGDKGPQICAIMKADAYGHG
IDLLVPSVIKAEIPCIGIASNEEARVAREKGFSGRLMRVRAAT
PIEVEQALPYKLEELVGSLVSAQGISDIALRHHTTIPVHVALN
SAGMSRNGIDLRRLADAKQDALAMLKLKGITPVGIMTHFPVE
EKEDVKLGLAQFKLDSQWLLEAGKLDRSKITIHAANSFATL
AVPDAYFDMVRPGGLLYGDSIPSYTEYKRVMFAKTQVASV
NHYAAGNTVGYDRTFTLKRDSWLANLPLGYSDGYRRALS
NKAYVLIQGQKVPVVGKTSMTIMVDVTDLKGVPKPGDEV
VLFGRQGEAEVKQADLEEYNGALLADMYTIWGYTNPKKIK
D (SEQ ID NO:151).

[0355] The DNA sequence of the *A. hydrophila* chimeric sequence obtained from PCR is shown below as SEQ ID NO:150:

ATGCCCTTCTCCCGTACACTGCTGGCCACGCTGATCCTGG
GCCTGCTGGCCGGTCAAGCCGTCGCAGCCCCCTATCTGCC
TCTGGCAAGCGATCATCGCAACGGCGAAGTACAAACCGC
CAGCAACGCCTGGCTGGAAGTAGATCTGACCGCGTTTGA
ACAGAATCTGCAGACCCTCAAGACCCGCCTCGGCGACAA
GGGCCCCGAGATCTGCGCCATCATGAAGGCGGACGCCTA
CGGTCACGGTATCGATCTGCTGGTTCCTCCGTCATCAAG
GCCGAGATCCCCTGTATCGGCATCGCCAGCAACGAAGAG
GCCCCGCTCGCCCGCGAGAAGGGGTTCAGCGGCCGCCTG
ATGCGGGTACGGGCGCCACACCTATCGAAGTGGAACAG

GCCCTGCCCTACAAGCTGGAAGAGCTGGTTGGCAGCCTG
GTGAGTGCTCAGGGGATCTCCGACATCGCCCTGCGCCAC
CACACCACCATTCGGGTGCATGTCGCCCTCAACTCCGCCG
GTATGAGCCGCAACGGCATCGACCTGCGTCTGGCCGATG
CCAAGCAAGATGCGCTGGCCATGCTCAAGCTCAAGGGGA
TCACCCCGGTCTGGCATCATGACCCACTTCCCGGTGGAGG
AGAAAGAGGACGTCAAGCTGGGGCTGGCTCAGTTCAAGC
TGGACTCCCAGTGGCTGCTGGAAGCAGGCAAGCTGGATC
GCAGCAAGATCACCATCCATGCCGCCAACTCCTTCGCCA
CCCTGGCAGTGCCGGACGCCTACTTTGACATGGTGCGCC
CGGGCGGCCTGCTCTACGGCGACTCCATCCCCTCCTACAC
CGAATACAAGCGGGTGATGGCATTCAAGACCCAGGTTCG
CTCGGTCAACCACTATGCGGCGGGCAACACAGTCGGTTA
TGACCGCACCTTTACTCTCAAACGTGACTCCTGGCTCGCC
AACCTGCCGCTCGGTTACTCCGACGGCTATCGCCGTGCGC
TCAGCAACAAGGCCTATGTGCTGATCCAGGGTCAGAAGG
TGCCGGTGGTCGGCAAGACCTCCATGAACACCATCATGG
TGGACGTGACCGATCTCAAAGGGGTAAAGCCCGGTGATG
AAGTGGTGCTGTTTGGCCGTCAGGGTGAGGCAGAAGTGA
AACAGGCTGATCTGGAGGAGTACAACGGCGCCCTGTTGG
CGGACATGTACACCATCTGGGGCTACACCAACCCCAAGA
AAATCAAAGATTGA (SEQ ID NO:150).

[0356] The correct TOPO clone was digested with restriction enzymes *Nde* I and *Bam*HI following the manufacturer's recommended protocols (New England Biolabs, Beverly, MA) and gel purified from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). Vector pET30a (Novagen, Madison, WI) was prepared by digestion with appropriate restriction enzymes followed by treatment with shrimp alkaline phosphatase (Roche, Indianapolis, IN) and purification from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The digested vector and insert were ligated using the RapidTM DNA Ligation Kit (Roche, Indianapolis, IN). Approximately 65 ng of treated insert, 100 ng of treated vector (3 to 1 molar ratio of insert to vector), 5 U of T4 DNA ligase, and 1X ligation buffer were incubated for

5 minutes at room temperature. The ligation reaction was desalted using the High Pure PCR Product Purification Kit (Roche, Indianapolis, IN) and used to transform *E. coli* DH10B electrocompetent cells (Invitrogen, Carlsbad, CA). Ten μ L of each ligation reaction was added to 40 μ L of DH10B cells, which were transformed by electroporation using the BioRad Gene Pulsar II under the following conditions: 2.5 kV, 25 μ F, 200 Ohm in a 0.2 cm cuvette. The cells were allowed to recover in 1 mL of room temperature SOC for 1 hour at 37 °C with shaking at 225 rpm. Cells were plated on LB plates containing kanamycin (50 μ g/mL). The plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA).

Gene Expression and Assays

- [0357] Plasmid DNA was transformed into *E. coli* expression host BL21(DE3) (Novagen, Madison, WI). The cultures were grown and the plasmids were isolated using Qiagen miniprep kit (Qiagen, Valencia, CA), and analyzed by restriction digest to confirm identity. Induction in BL21DE3 was performed in Overnight Express medium as per manufacturer's instructions (Novagen, Madison, WI).
- [0358] Cells from the induced cultures were centrifuged and washed once with 0.85 % NaCl. Cell pellets were resuspended in 5 mL/g wet cell weight of BugBusterTM (Novagen, Madison, WI) reagent containing 5 μ L/mL protease inhibitor cocktail set #3 (Calbiochem-Novabiochem Corp., San Diego, CA) and 1 μ L/mL benzonase nuclease. Samples were incubated at room temperature for 20 minutes on an orbital shaker. Insoluble cell debris was removed by centrifugation at 16,000 X g for 20 minutes at 4 °C. The extract was desalted using PD10 columns which were equilibrated in 50 mM potassium phosphate, pH 8.0 containing 10 μ M PLP. The cell extract protein was quantified using the Pierce BCA protein assay (Pierce, Rockford, IL) and visualized by SDS-PAGE. SDS-PAGE showed a protein of the expected size of approximately 45 kDa.
- [0359] The cell extracts were assayed for tryptophan racemase activity using the following protocol. One mL reactions were carried out in 50 mM potassium phosphate (pH 8.0), with 0.05 mM PLP and 30 mM L tryptophan. The reactions were initiated by the addition of cell free extracts and were incubated at 30 °C overnight. Sample aliquots were taken at 2 hours and after overnight incubation (zero minute samples served as control reaction). Concentrated formic acid (5 μ L) was added to each 250 μ L sample aliquot to stop the reaction and the precipitated protein was removed by centrifugation.

Supernatants were removed and frozen at -80°C until they were analyzed for D-tryptophan by the chiral amino acid method described in Example 1.

[0360] The assay results from the cell extracts from pET30 induction demonstrate that *A. hydrophila* clones show racemase activity on L-tryptophan. In light of these data it was expected that the full-length TIGR *A. hydrophila* wild-type sequence (Protein 1 above with N and C terminal regions completed) would also give a protein with racemase activity on tryptophan. The full length gene sequence of the *A. hydrophila* BAR was determined using Genome Walker Methods described in Example 15. The gene sequence of the native *A. hydrophila* gene is shown below as SEQ ID NO:176.

```
atgcacaaga agacactgct ggccaccttg atcctggggc tgctggccgg tcaagccgtc
gcagccccct atctgcctct ggcaagcgat catcgcaacg gcgaagtaca aaccgccagc
aacgcctggc tggaagtaga tctgaccgag tttgaacaga atctgcagac cctcaagacc
cgcctcggcg acaaggggccc gcagatctgc gccatcatga aggcggacgc ctacggtcac
ggtatcgatc tgctggttcc ctccgctcgc aaggccgaga tccccgtgat cggcatcgcc
agcaacgaag agggccgcgt cgcccgcgag aaggggttca gcggccgcct gatgcgggta
cgggcccgcc cacctatcga agtggaaacag gccctgccct acaagctgga agagctggtt
ggcagcctgg tgagtgtctc ggggatctcc gacatcgccc tgcgccacca caccaccatt
ccggtgcatg tcgccctcaa ctccgccggt atgagccgca acggcatcga cctgcgtctg
gccgatgcca agcaagatgc gctggccatg ctcaagctca aggggatcac cccggtcggc
atcatgaccc acttcccggg ggaggagaaa gaggacgtca agctgggggt ggctcagttc
aagctggact ccagtggtg gctggaagca ggcaagctgg atcgagcaa gatcaccatc
catgccgcca actccttcgc caccctggca gtgccggacg cctactttga catggtgcgc
ccgggcgggc tgctctacgg cgactccatc ccctcctaca ccgaatacaa cggggtgatg
gcattcaaga ccaggtcgc ctccgtcaac cactatgcgg cgggcaaacac agtcggttat
gaccgcacct ttactctcaa acgtgactcc tggctcgcca acctgccgct cggttactcc
gacggctatc gccgtgcgct cagcaacaag gcctatgtgc tgatccaggg tcagaaggtg
ccggtggtcg gcaagacctc catgaacacc atcatggtg agtgaccga tctcaaaggg
gtaaagcccg gtgatgaagt ggtgctgttt ggccgtcagg gtgaggcaga agtgaaacag
gctgatctgg aggagtacaa cggcgccctg ttggcggaca tgtacacat ctggggctac
accaacccca agaagatcaa acgctga
```

(SEQ ID NO:176).

[0361] The corresponding native protein for the *A. hydrophila* BAR is shown below as SEQ ID NO:177.

```
1  mhkktllatl ilgllagqav aapylplasd hrngevqtas
41  nawlevdlta feqlqtlkt rlgdkgpqic aimkadaygh
81  gidllvpsvi kaeipcigia sneearvare kgfsgrlmr
121 raatpieveq alpykleelv gslvsaggis dialrhhtti
```

161 pvhvalnsag msrngidrl adakqdalam lklkgitpvg
 201 imthfpveek edvklglaqf kldsqwlllea gklrskiti
 241 haansfatla vpdafdmvr pgglllygdsi psyteykrvm
 281 afktqvasvn hyaagntvgy drtftlkrds wlanlplgys
 321 dgrralsnk ayvliqgqkv pvvgktsmnt imvdtldkg
 361 vkpgdevvlf grqgeaevkq adleeyngal ladmytiwg
 401 tnpkkikr

(SEQ ID NO:177).

[0362] The first 21 N-terminal amino acid residues are predicted to be a signal peptide using the program Signal P 3.0 as described in Example 4L.

Table 32

Enzyme preparation	Time Point	Racemase extract	D-trp conc (μg/mL)	D-trp/μg BAR
<i>A. hydrophila</i> - chimera	0	100 μL		
<i>A. hydrophila</i> - chimera	0	600 μL		
KT2440 BAR untagged	0	100 μL		
<i>A. hydrophila</i> - chimera	2 hr	100 μL	846	3.9
<i>A. hydrophila</i> - chimera	2 hr	600 μL	1952	1.5
KT2440 BAR untagged	2 hr	100 μL	1756	10.98
<i>A. hydrophila</i> - chimera	overnight	100 μL	3423	15.6
<i>A. hydrophila</i> - chimera	overnight	600 μL	2652	2
KT2440 BAR untagged	overnight	100 μL	3040	19

[0363] The native *A. hydrophila* gene was cloned and expressed in a manner similar to that described above for the chimera. The same N-terminal primer was utilized for both untagged and C-terminally His-tagged constructs in pET30. The following primers were used:

A. hydroph F NdeI 5'- GGAACCTTCATATGCACAAGAAGACACTGCTGG - 3'
 (SEQ ID NO:182);

A. hydroph R BamHI (untagged) 5' -

GGTTCCAAGGATCCTCAGCGTTTGATCTTCTTGGG - 3' (SEQ ID NO:183); and

A. hydroph R XhoI (C-term tag) 5' -

GGCCAATTCTCGAGGCGTTTGATCTTCTTGGGGT - 3' (SEQ ID NO:184).

[0364] The native *A. hydrophila* BARs (both C-tagged and untagged versions) were found to have comparable activity and had the same order of magnitude of activity as the

chimeric protein. Calculation of specific activities was difficult due to the use of unpurified enzymes.

***A. caviae* Extract Assay**

[0365] *Aeromonas caviae* ATCC 14486 was grown in nutrient broth at 37 °C. Cells from the culture (200 mL) were centrifuged and washed once with 0.85 % NaCl. Cell pellets were resuspended in 5 mL/g wet cell weight of BugBuster™ (Novagen, Madison, WI) reagent containing 5 µL/mL protease inhibitor cocktail set #3 (Calbiochem-Novabiochem Corp., San Diego, CA) and 1 µL/mL benzonase nuclease. Samples were incubated at room temperature for 20 minutes on an orbital shaker. Insoluble cell debris was removed by centrifugation at 16,000 X g for 20 minutes at 4 °C. Cell-free extract was desalted on a PD-10 column (GE Healthcare, Piscataway, NJ).

[0366] Cell-free extract was assayed for tryptophan racemase activity using the following protocol. One mL reactions were carried out in 50 mM potassium phosphate (pH 8.0), 0.05 mM PLP and 30 mM L tryptophan. The reactions were initiated by the addition of cell free extract (either 100 µL or 500 µL) and were incubated at 30 °C overnight. Sample aliquots were taken at 2 hours and after overnight incubation (zero minute samples served as control reactions). Concentrated formic acid (5 µL) was added to each 250 µL sample aliquot to stop the reaction and the precipitated protein was removed by centrifugation. Supernatants were removed and frozen at –80 °C until they were analyzed for D-tryptophan by the chiral amino acid method described in Example 1.

[0367] The assay results from cell extracts of *A. caviae* demonstrated racemase activity on L-tryptophan, as shown in Table 33.

Table 33

Treatment	Time Point	substrate	Racemase extract	D-trp conc (µg/mL)
<i>A. caviae</i>	0	L-trp	100 µL	nd
<i>A. caviae</i>	0	L-trp	500 µL	nd
<i>A. caviae</i>	2 hr	L-trp	100 µL	2
<i>A. caviae</i>	2 hr	L-trp	500 µL	19
<i>A. caviae</i>	overnight	L-trp	100 µL	45
<i>A. caviae</i>	overnight	L-trp	500 µL	130

[0368] After finding activity in the *A. caviae* cell extracts, degenerate primers were designed (based on conserved regions of known BAR homologs) to obtain the BAR gene from this species. Degenerate primer sequences are shown below:

Aer deg F2: 5'-GCCAGCAACGARGARGCMCGCGT-3' (SEQ ID NO:152); and

Aer deg R1: 5'-TGGCCSTKGATCAGCACA-3' (SEQ ID NO:153)

wherein K indicates G or T, R indicates A or G, S indicates C or G, and M indicates A or C.

[0369] The above primers were used to PCR amplify a 715 bp DNA fragment from *A. caviae* (ATCC 14486) genomic DNA. The following PCR protocol was used: A 50 μ L reaction contained 0.5 μ L template (~100 ng of *A. caviae* genomic DNA), 1.6 μ M of each primer, 0.3 mM each dNTP, 10 U rTth Polymerase XL (Applied Biosystems, Foster City, CA), 1X XL buffer, 1 mM Mg(OAc)₂ and 2.5 μ L dimethyl sulfoxide. The thermocycler program used included a hot start at 94 °C for 3 minutes and 30 repetitions of the following steps: 94 °C for 30 seconds, 53 °C for 30 seconds, and 68 °C for 2 minutes. After the 30 repetitions, the sample was maintained at 68 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a product of 715 bp.

Cloning

[0370] The PCR product was gel purified from 0.8% TAE-agarose gel using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The product was TOPO cloned and transformed into TOP 10 cells according to manufacturer's protocol (Invitrogen, Carlsbad, CA). The plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *EcoR* I. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing with universal M13 forward primers.

[0371] The DNA sequence of the *A. caviae* PCR product is shown below as SEQ ID NO:154), with degenerate primer sequence regions underlined:

GCCAGCAACGARGARGCMCGCGTTGCCCGCGAGAAGGG
 CTTCTGAAGGTCGCCTGATGCGGGTACGTGCCGCCACCCC
 GGATGAAGTGGAGCAGGCCCTGCCCTACAAGCTGGAGGA
 GCTCATCGGCAGCCTGGAGAGCGCCAAGGGGATCGCCGA
 CATCGCCCAGCGCCATCACACCAACATCCCGGTGCACAT

CGGCCTGAACTCCGCCGGCATGAGCCGCAACGGCATCGA
TCTGCGCCAGGACGATGCCAAGGCCGATGCCCTGGCCAT
GCTCAAGCTCAAGGGGATCACCCCGGTCTGGCATCATGAC
CCACTTCCCGGTGGAGGAGAAAGAGGACGTCAAGCTGGG
GCTGGCCCAGTTCAAGCTGGACTACCAGTGGCTCATCGA
CGCCGGCAAGCTGGATCGCAGCAAGCTCACCATCCACGC
CGCCAACTCCTTCGCCACCCTGGAAGTACCGGAAGCCTA
CTTTGACATGGTGCGCCCCGGCGGCATCATCTATGGCGA
CACCATTCCCTCCTACACCGAGTACAAGAAGGTGATGGC
GTTCAAGACCCAGGTGCGCTCCGTCAACCACTACCCGGC
GGGCAACACCGTCGGCTATGACCGCACCTTCACCCTCAA
GCGCGACTCCCTGCTGGCCAACCTGCCGATGGGCTACTC
CGACGGCTACCGCCGCGCCATGAGCAACAAGGCCTATGT
GCTGATCMASGGCCA (SEQ ID NO:154), wherein R indicates
A or G, S indicates C or G, and M indicates A or C.

[0372] The amino acid sequence of the partial *A. caviae* BAR enzyme is shown below.

ASNEEARVAREKGFEGRLMRVRAATPDEVEQALPYKLEELI
GSLESAKGIADIAQRHHTNIPVHIGLNSAGMSRNGIDLRQDD
AKADALAMLKLKGITPVGIMTHFPVEEKEDVKLGLAQFKL
DYQWLIDAGKLDRSKLTIHAANSFATLEVPEAYFDMVRPG
GIIYGDTIPSYTEYKKVMAFKTQVASVNHYPAGNTVGYDRT
FTLKRDSLLANLPMGYSDGYRRAMSNKAYVLIXG (SEQ ID
NO:155).

wherein X is H, Q, N, or K.

[0373] The consensus protein sequence fragment of SEQ ID NO:155 above is 89% homologous at the amino acid level to the published TIGR sequence for *A. hydrophila* (protein 1 (SEQ ID NO:146) above). It was expected that because the highly related *Aeromonas hydrophila* protein exhibited broad specificity racemase activity, as well as the *A. caviae* cellular extracts, the full length coding region for *A. caviae*, once obtained, would produce a racemase that also would have a broad specificity with activity on tryptophan. Genome Walker methods were utilized, as described in Example 15, to

obtain the full-length gene sequence of the *A. caviae* BAR gene shown below as SEQ ID NO:178.

```

atgcacaaga aaacactgct cgcgaccctg atctttggcc tgctggccgg ccaggcagtc
gccgccccct atctgccgct cgccgacgac caccgcaacg gtcaggaaca gaccgccgcc
aacgcctggc tggaagtgga tctcggcgcc ttcgagcaca acatccagac cctgaagaat
cgctcggtg acaagggccc gcagatctgc gccatcatga aggcggacgc ctacggtcac
ggcatcgacc tgctggtccc ttccgtggtc aaggcaggca tccccgcat cggcatcgcc
agcaacgaag aagcacgtgt tgcccgcgag aagggttcg aaggctgcct gatgcgggta
cgtgccgcca ccccgatga agtgagcag gccctgccct acaagctgga ggagctcatc
ggcagcctgg agagcgccaa ggggatcgcc gacatcgccc agcgccatca caccaacatc
ccggtgcaca tcggcctgaa ctccgccggc atgagccgca acggcatcga tctgcgccag
gacgatgcca aggccgatgc cctggccatg ctcaagctca aggggatcac cccggtcggc
atcatgaccc acttcccggg ggaggagaaa gaggacgtca agctggggct ggcccagttc
aagctggact accagtggct catcgacgcc ggcaagctgg atcgagcaa gctcaccatc
cacgccgcca actccttcgc caccctggaa gtaccggaag cctactttga catggtgcgc
ccgggcggca tcatttatgg cgacaccatt cctcctaca ccgagtacaa gaaggtgatg
gcgttcaaga cccaggtcgc ctccgtcaac cactaccggc cgggcaacac cgtcggctat
gaccgcacct tcaccctcaa gcgcgactcc ctgctggcca acctgccgat gggctactcc
gacggctacc gccgcgccat gagcaacaag gcctatgtgc tgatccatgg ccagaaggcc
cccgtcgtgg gcaagacttc catgaacacc accatggtgg acgtcaccga catcaagggg
atcaaaccgc gtgacgaggt ggtcctgttc ggacgccagg gtgatgccga ggtgaaacaa
tctgatctgg aggagtacaa cggcgccctc ttggcgga tgtacaccgt ctggggctat
accaacccca agaagatcaa gcgctaa

```

(SEQ ID NO:178).

[0374] The corresponding amino acid sequence for the *A. caviae* native BAR is shown below as SEQ ID NO:179.

```

1  mhkktlatl ifgllagqav aapylpladd hmgqeetaa
41  nawlevdlga fehniqlkn rlgdkgpqic aimkadaygh
81  gidllpsvv kagipcigia sneearvare kgfegrlmrv
121 raatpdeveq alpykleeli gslesakgia diaqrhhtni
161 pvhiglinsag msrngidlrq ddakadalam lklgitpvg
201 imthfpveek edvklglaqf kldyqwlida gkldrsklti
241 haansfatle vpeayfdmvr pggiygdti psyteykkvm
281 afktqvasvn hypagntvgy drtftlkrds llanlpmgys
321 dgyrramsnk ayvlihgqka pvgktsmnt tmvdtvdikg
361 ikpgdevvlf grqgdaevkq sdleeyngal ladmytwggy

```

401 tnpkkikr

(SEQID NO:179).

[0375] The following PCR primers were utilized to clone the native full-length *A. caviae* BAR in a manner similar to that described above, both tagged and C-terminally his-tagged:

A. caviae F NdeI 5' -

GGAACCTTCATATGCACAAGAAAACACTGCTCGCGACC - 3' (SEQ ID NO:185);

A. caviae R BamHI (untagged) 5' -

GGTTCCAAGGATCCTTAGCGCTTGATCTTCTTGGGGTTG - 3' (SEQ ID NO:186); and

A. caviae R XhoI (C-term tag) 5' -

TTCCAAGGCTCGAGGCGCTTGATCTTCTTGGGGTTGGTA - 3' (SEQ ID NO:187).

[0376] The C-terminally tagged enzyme had comparable activity to the untagged native *A. caviae* BAR. When 200 µg of purified (tagged) racemase enzymes were used in a tryptophan racemase assay as described above, at 30 minutes *A. hydrophila* BAR had produced 249 µg/mL of D-tryptophan, whereas *A. caviae* BAR produced 1034 µg/mL and *P. taetrolens* BAR produced 799 µg/mL.

[0377] The first 21 N-terminal amino acid residues of SEQ ID NO:179 are predicted to be a signal peptide using the program Signal P 3.0, as described in Example 4L. The following N-terminal primer was used to clone the *A. caviae* gene without amino acids 2-21 of the leader sequence:

*A.cav*Minus leader F NdeI 5' CCTTGGAACATATGGCCCCCTATCTGCCGCT 3 (SEQ ID NO:188).'

[0378] The leaderless racemase, when expressed, was found to retain approximately 65% of the activity, as compared with the expression product of the full-length gene. The periplasmic and cytoplasmic protein fractions were isolated for the wildtype expression products, as well as the leaderless constructs, as described in the pET System Manual (Novagen, Madison, WI). The majority of expressed wildtype BAR was found in the periplasm, while the leaderless BAR appeared to remain in the cytoplasm. The reduction

in activity of the leaderless *A. caviae* BAR may be due to a change in processing and/or folding when expressed in the cytoplasm.

[0379] Based on mutations made in the *P. putida* BAR sequences in Examples 4I and 4G, similar modifications were introduced, using the site-directed mutagenesis methods described above, to yield Y395H, Y395C, and L383M mutants in the *A. caviae* wildtype BAR. Mutagenesis at Y395 causes a loss of over 95% of the activity using L-tryptophan as a substrate, even when combined with the L383M mutation. The L383M mutant had approximately 83% of the activity on L-tryptophan at a 2-hour time point. These results suggest that the mutations made in the Kino *et al.* paper are not universally applicable to improvement of BAR enzymes for activity on L-tryptophan. Additional *Aeromonas* BAR sequences were obtained by degenerative PCR methods described above, but using the following primers:

Aer deg F1 mod: 5'- AAGGCSGAYGCCTAYGGYCACGG- 3' (SEQ ID NO:189)

where S is C or G and Y is C or T; and

Aer deg R3: 5' - CGGCGRTAGCCRTCRGAGTA -3' (SEQ ID NO:190), where R is A or G.

[0380] This primer pair gave partial sequences for *A. sobria* (ATCC 35994), *A. jandei* (ATCC 49572), *A. schubertii* (ATCC 43701), and *A. salmonicida* (ATCC 27013 and ATCC 14174). The full-length sequences obtained are as follows:

[0381] The *A. sobria* DNA sequence is shown below as SEQ ID NO:191:

```

ATGCACAAGAAAACGCTATTGGCCACCCTGATCTTCGGCCTGCT
CGCGGGCCAAGCCGTTGCGGCTCCCTATCTGCCCTTGCGACGG
ATCATCGCAACGGTCAGGAGCAAACCGCCAGCAACGCCTGGTT
GGAAGTGGATCTGGGCGCCTTCGAACACAATATCCAGACCCTC
AAGGATCGCCTCGGTGACAAGGGTCCGCAGATCTGCGCCATCA
TGAAGGCCGACGCCTATGGTCATGGCATCGACCTGCTGGTCCCC
TCCGTGGTCAAGGCCAATATCCCCTGCATCGGCATCGCCAGCA
ACGAAGAGGCCCCGCGTCGCCCCGCGAGAAGGGCTTTACCGGCCG
TCTGATGCGGGTGCGTGCCGCCACACCGGCCGAAGTGGAGCAG
GCGCTGCCCTACAAGATGGAAGAGCTGATCGGCAGTCTGGTGA
GTGCTCAGGGGATCGCCGACATCGCCAGCGCCACCACACCAA
TATTCCGGTACACATTGGTCTCAACTCTGCTGGCATGAGCCGCA

```

ACGGTATCGACCTGCGTCTGGCCGATGCCAAGCAGGATGCGCT
 GGCCATGCTCAAGCTCAAGGGGATCACCCCGGTCGGCATCATG
 ACCCACTTCCCGGTGGAGGAGAAAAGAGGACGTCAAGATGGGGC
 TGGCCCAGTTCAAACCTGGACTCTCAGTGGCTGCTGGAAGCGGG
 CAAGCTGGATCGCAGCAAGATCACCATCCACGCCGCCAACTCC
 TTCGCCACCCTGGAAGTGCCGGATGCCTACTTCGACATGGTGCG
 TCCGGGTGGCCTGCTCTACGGCGACTCCATCCCCTCCTACACCG
 AATACAAGCGGGTGATGGCATTCAAGACCCAGGTGCGCTCGGT
 CAACCACTACCCGGCGGGCAATACCGTTGGCTATGACCGTACC
 TTTACCCTCAAGCGTGAATCCTGGCTCGCCAACCTGCCGCTGGG
 CTACTCCGATGGCTACCGCCGTGCGCTCAGCAACAAGGCCTAT
 GTGCTGATCCAGGGTCAGAAGGTGCCGGTGGTCGGCAAGACCT
 CCATGAACACCATCATGGTGGACGTCACTGATCTCAAAGGGGT
 GAAACCCGGTGATGAGGTGGTGCTGTTTGGCCGTCAGGGCGAG
 GCCGAGGTGAAACAGGCTGATCTGGAAGAGTACAACGGCGCCC
 TGTTAGCGGACATGTACACCATCTGGGGCTACACCAACCCCAA
 GAAGATCAAACGCTGA

(SEQ ID NO:191).

[0382] The *A. sobria* protein sequence is shown below as SEQ ID NO:192:

MHKKTLATLIFGLLAGQAVAAPYLPLATDHRNGQEQTASNAWL
 EVDLGA FEHNIQTLKDRLGDKGPQICAIMKADAYGHGIDLLVPSV
 VKANIPCIGIASNEEARVAREKGFTGRLMRVRAATPAEVEQALPY
 KMEELIGSLVSAQGIADIAQRHHTNIPVHIGLNSAGMSRNGIDLRL
 ADAKQDALAMLKLKGITPVGIMTHFPVEEKEDVKMGLAQFKLDS
 QWLLEAGKLDRSKITIHAANSFATLEVPDAYFDMVRPGGLLYGDS
 IPSYTEYKRVMAFKTQVASVNHYPAGNTVGYDRFTLTKRESWLA
 NLPLGYSDGYRRALSNKAYVLIQGQKVPVVGKTSMTIMVDVTD
 LKGVKPGDEVVLFGRQGEAEVKQADLEEYNGALLADMYTIWGY
 TNPKKIKR

(SEQ ID NO:192).

[0383] The *A. jandei* DNA sequence is shown below as SEQ ID NO:193:

ATGCACAAGAAAACACTGCTGGCCACCCTGATCCTCGGCCTGC
TGGCCGGGCAAGCGGTTGCAGCCCCCTACCTGCCGCTGGCCAG
CGATCACCGCAACGGCGAAGTCCAGACCGCCAGCAATGCCTGG
CTGGAAGTCGATCTCGGCGCCTTCGAGCACAATATCCAGACCCT
CAAGGATCGTCTCGGTGACAAGGGGGCCGAAGATCTGCGCCATC
ATGAAGGCGGATGCCTATGGCCACGGTATCGATCTGCTGGTTCC
CTCGGTGGTGAAAGCGGGTATCCCCTGCATCGGTATCGCCAGC
AATGAAGAAGCTCGTGTGCCCCGCGAGAAGGGCTTCACCGGTC
GTCTGATGCGGGTACGTGCTGCCACCCCGGACGAAGTGGAGCA
GGCCCTGCCCTACAAGATGGAGGAGCTGATCGGCAGTCTGGTG
AGTGCTCAGGGCATCGCCGATATCGCCCAGCGCCACCACACCA
CCATTCCGGTGCATATCGCCCTCAACTCCGCCGGCATGAGCCGC
AACGGCATCGATCTGCGGCTGGCCGACTCCAAGCAGGATGCGC
TGGCCATGCTCAAGCTCAAGGGGATCACCCCGGTGCGCATCAT
GACCCACTTCCCGGTGGAGGAGAAAGAGGACGTCAAGATGGGT
CTGGCCCAGTTCAAACCTGGACTCCCAGTGGCTGCTGGAAGCGG
GCAAGCTGGATCGCAGCAAGATCACCATCCACGCCGCCAACTC
CTTCGCAACACTTGAAGTGCCGGATGCCTACTTCGACATGGTGC
GCCCCGGTGGCCTGCTCTACGGTGACTCCATCCCCTCCTACACC
GAGTACAAGCGGGTGATGGCGTTCAAGACCCAGGTTGCCTCCG
TCAACCACTACCCGGCCGGCAACACCGTCGGTTATGACCGCAC
CTTCACCCTCAAGCGCGACTCCTGGCTCGCCAACCTGCCGCTCG
GTTACTCCGATGGCTATCGCCGCTCCCTGAGCAACAAGGCCTAT
GTGCTGATCCAGGGCCAGAAGGTGCCGGTGGTCGGCAAGACCT
CCATGAACACCATCATGGTGGATGTGACCGACCTGAAAGGGGT
GAAACCCGGTGACGAAGTGGTGCTGTTCGGCCGTCAGGGAAAT
GCCGAGGTGAAGCAGGCGGATCTGGAGGAGTACAACGGCGCC
CTGCTGGCGGACATGTACACCATCTGGGGCTACACCAACCCCA
AGAAGATCAAGCACTAA

(SEQ ID NO:193).

[0384] The *A. jandei* protein sequence is shown below as SEQ ID NO:194:

MHKKTLATLILGLLAGQAVAAPYLPLASDHRNGEVQTASNAWL
 EVDLGAFEHNIQTLKDRLGDKGPKICAIMKADAYGHGIDLLVPSV
 VKAGIPCIGIASNEEARVAREKGFTGRLMRVRAATPDEVEQALPY
 KMEELIGSLVSAQGIADIAQRHHTTIPVHIALNSAGMSRNGIDLR
 LADSKQDALAMLKLKGITPVGIMTHFPVEEKEDVKMGLAQFKLDSQ
 WLLEAGKLDRSKITIHAANSFATLEVPDAYFDMVRPGGLLYGDSIP
 SYTEYKRVMAFKTQVASVNHYPAGNTVGYDRTFTLKRDSWLAN
 LPLGYSDGYRRSLSNKAYVLIQGQKVPVVGKTSMTIMVDVTDL
 KGVKPGDEVVLFGRQGNAEVKQADLEEYNGALLADMYTIWGYT
 NPKKIKH

(SEQ ID NO:194).

[0385] The following partial DNA sequences were also obtained:

A. salmonicida (ATCC 27013 and ATCC 14174) (SEQ ID NO:195):

AAGGCSGATGCCTAYGGTCACGGTATCGACCTGCTGGTCCCCTC
 CGTGGTCAAGGCCAATATCCCCTGTATCGGCATCGCCAGCAAC
 GAAGAGGCCCGCGTGGCGCGCGAGAAGGGGTTCAGCGGCCGC
 CTGATGCGGGTACGGGCCGCCACACCGATCGAAGTGGAACAGG
 CCCTGCCCTACAAGCTGGAAGAGCTGGTTGGCAGCCTGGTGAG
 TGCTCAGGGGATCTCCGACATCGCCCTGCGCCACCACACCACC
 ATTCCGGTGATGTCGCCCTCAACTCCGCCGGCATGAGCCGCAA
 CGGCATCGACCTGCGTCTGGCCGATGCCAAGCAAGATGCGCTG
 GCCATGCTCAAGCTCAAGGGGATCACCCCGGTGGCATCATGA
 CCCACTTCCCGGTGGAGGAGAAAGAGGACGTCAAGCTGGGGCT
 GGCCAGTTCAAGCTGGACTCCCAGTGGCTGCTGGAAGCAGGC
 AAGCTGGATCGCAGCAAGATCACCATCCATGCCGCCAACTCCT
 TCGCCACCCTGGCAGTGCCGGACGCCTACTTTGACATGGTGCGC
 CCGGGCGGCCTGCTCTACGGCGACTCCATCCCCTCCTACACCGA
 ATACAAGCGGGTGATGGCATTCAAGACCCAGGTCGCCTCGGTC
 AACCCTATGCGGCGGGCAACACAGTCGGTTATGACCGCACCT
 TTAATCTCAAACGTGACTCCTGGCTCGCCAACCTGCCTCTCGGT
 TACTCCGAYGGCTAYCGCCG

(SEQ ID NO:195) where S is C or G and Y is C or T.

A. schubertii (ATCC 43701) (SEQ ID NO:196):

AAGGCGGATGCCTATGGTCACGGCATCGATCTGCTGGTCCCCTC
 CGTGATCAAGGCCGGCATTCTTGCATCGGCATCGCCAGCAAC
 GAAGAGGCTCGCGTCGCCCCGTGAGAAGGGCTTCGAAGGCCGTC
 TGATGCGGGTGCGCGCCGCCACCCCGCAAGAGGTGGAAGCCGC
 CCTCCCCTACAAGATGGAGGAGCTGGTCGGCAGCCTGGAGAGC
 GCCCGTCTGATGTCTGGAGATTGCCCTGCGTCACCACACCACCAT
 TGCGTACCATCTGGGGCTCAACTCCGCCGGCATGAGCCGCAAC
 GGCCTGGATCTGCGCCTCTCCGACGCCAAGCGCGACGCACTCG
 ACCTGATGAAGCTCAAGGGGCTGCAGGTGGTCGGCATCATGAC
 CCACTTCCCGGTCGAGGAGAAAGAGGACGTGAAGATGGGCTTC
 GCCCAGTTTCAGCTCGACACCCAGTGGCTCATCGAAGCCGCTCG
 TCTGGATCGCAGCAAGTTGACCCTGCACTGTGCCAACTCCTTTA
 CCACCCTGGAGGTGCCCCGAGGCCTATCTGGACATGGTCCGCCC
 GGGCGGCATCATCTATGGCGACACCATTCCTCCTACACCGAAT
 ACAAGAAGGTGATGGCCTTCAAGACCCGGGTGCGCTCGGTCAA
 TCACTACCCGAAGGGGAAATAGCGTCGGCTATGACCGCACCTTC
 ACCCTGGCACGCGACTCCTGGCTCGCCAACTGCCGCTGGGCTA
 CTCCGACGGCTACCGCCGGGCGCTGAGCAACAAGGCCTATGTG
 CTGGTGAATGGCCAGAAGGCCCCCGTGGTGGGCAAGACATCCA
 TGAACACCATCATGGTGGACGTGACCGACATCAAGGGGGTCAA
 ACCGGGTGACGAGGTGGTGCTGTTTGGCCGCCAGGGCAACGCC
 GAGGTGAAGCAGTCCGATCTCGAGGAGTACAACGGCGCCCTCC
 TGGCGGACATGTACACCATCTGGGGCTACACCAATCCACGTAT
 CATCAAGCGCTGA

[0386] It is expected that the full length gene sequences of *A. salmonicida* and *A. schubertii*, obtainable by Genome Walking methods described above, would also encode active BAR enzymes.

[0387] The following PCR primers were designed for cloning the *A. jandei* and *A. sobria* BAR genes:

A. jandei:

Forward NdeI 5' -

CCGGAACCTTCATATGCACAAGAAAACACTGCTGGCCAC-3'

(SEQ ID NO:197) and

Reverse XhoI 5'-

TTCCAAGGCTCGAGGTGCTTGATCTTCTTGGGGTTGGT-3' (SEQ ID NO:198).

A. sobria

Forward NdeI 5'-

CCGGAACCTTCATATGCACAAGAAAACGCTATTGGCCAC-3'

(SEQ ID NO:199) and

Reverse XhoI 5'-

TTCCAAGGCTCGAGGCGTTTGATCTTCTTGGGGTTGGT-3' (SEQ ID NO:200).

[0388] Cloning, expression, and purification methods for the BAR enzymes are described above. Purified C-terminally tagged BARs (100 µg in a 1 mL assay) from *P. taetrolens*, *A. caviae*, *A. jandei*, and *A. sobria* were assayed as described above, except that the initial concentration of L-tryptophan used was approximately 7 mg/mL. At the 60 minute time point, the following amounts of D-tryptophan were produced by each BAR enzyme: *A. caviae*, 1452 µg/mL; *A. jandei*, 407 µg/mL; *A. sobria*, 145 µg/mL; and *P. taetrolens*, 502 µg/mL. The *A. caviae* BAR consistently has a higher reaction rate and reaches equilibrium faster than the other BAR enzymes tested.

[0389] Highly active BAR homologs were analyzed for primary sequence conservation. It was noted that in the motif described in Example 4E (SEQ ID NO:116), the broad specificity racemases of this example all contain the motif KADAYGHGI (SEQ ID NO:201), whereas the alanine racemases with lower activity for tryptophan racemization typically contain the motif KANAYGHGI (SEQ ID NO:202). A D76N mutant of *A. caviae* BAR was made to determine if this position was critical for broad activity. Mutagenesis was done using the QuickChange-Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA), using the C-tagged *A. caviae* BAR gene in pET30. The following mutagenic primer was used to make a D76N change (nucleotide position 226): 5'-CGCCATCATGAAGGCGAACGCCTACGGTCACG-3' (SEQ ID NO:203). The site-directed mutagenesis was done as described in the manufacturer's protocol. The mutant

and the wildtype enzyme were produced as described above and assayed as described above using 200 micrograms of purified protein and approximately 7 mg/mL of L-tryptophan as substrate. At the 30 minute time point, the mutant produced 1929 micrograms per mL of D-tryptophan as compared to 1149 micrograms per mL for the wildtype enzyme. The D76N mutant also reached equilibrium at an earlier time point. The improvement in activity was unexpected. Based on the high homology in this region for *Aeromonas* and *Pseudomonas* BAR enzymes, it is expected that similar mutations in other broad activity racemases would also be beneficial.

4P: Processing of N-Terminal Leader Sequences of BAR Enzymes

[0390] Samples of purified C-terminally His-tagged broad amino acid racemase (BAR) from *P. taetrolens* (SEQ ID NO:204), *A. caviae* (SEQ ID NO:179) and *A. hydrophila* (SEQ ID NO:177) produced in *E. coli* were characterized by reversed-phase liquid chromatography/electrospray ionization-quadrupole-time-of-flight mass spectrometry (LC/ESI-QTOF-MS) to determine at which position the leader sequence of each protein was cleaved. Mass spectra were collected over the range m/z 300 to m/z 1300. Deconvolution (mathematical conversion of the envelope of m/z values resulting from protein molecules of the same mass with different numbers of protons attached, to molecular mass) of mass spectra acquired for each protein was accomplished using the maximum entropy deconvolution software supplied with the MS instrumentation. The molecular mass observed for the *P. taetrolens* BAR was 42651 Da, indicating cleavage of this protein after amino acid A23 (theoretical molecular mass = 42654 Da). The molecular mass observed for the *A. caviae* BAR was 43565 Da, indicating cleavage of this protein after amino acid 21 (theoretical molecular mass = 43568 Da). The molecular mass observed for the *A. hydrophila* BAR was 43450 Da, indicating cleavage of this protein after amino acid 21 (theoretical molecular mass = 43453 Da). When the codons coding for amino acids 2-23 of SEQ ID NO:204 are removed and a partial gene is expressed in *E. coli* in the same manner as described above, the molecular mass observed for the purified expression product is 42483 Da. Based on this result, the mature protein of the leaderless expression product appears to have the first 2 or 3 amino acids removed, making it 1 or 2 amino acids shorter than the mature protein of the wildtype product. When the codons coding for amino acids 2-21 of SEQ ID NO:179 are removed, the

molecular mass observed for the purified expression product is 43500, corresponding to the first two amino acids (MA) being removed (theoretical molecular mass = 43498). The mature protein is therefore missing one additional alanine as compared to the wildtype mature protein, and as shown in Example 4O does not appear to be secreted into the periplasm. If the mature protein from the *P. taetrolens* (SEQ ID NO:204) leaderless expression product is missing a proline in addition to the alanine that is cleaved from both *A. caviae* and *P. taetrolens* leaderless expression products, it may explain the increased reduction in activity. However, the protein may never express and fold appropriately, as evidenced by the dramatic reduction in expression as judged by SDS-PAGE. It is likely that the *P. putida* BAR enzymes described above are processed similarly by *E. coli*, because the N-terminal His-tagged proteins would not purify on a nickel affinity column and the Signal P 3.0 program predicted a leader sequence.

EXAMPLE 5

Selection Method for Screening of Pyruvate Aldolases in Recombinant *E. coli*

[0391] Many of the processes described in Examples 4A(5), 9 and 10(3), and shown in FIGS. 1-8, will work optimally with an aldolase that preferentially produces R-MP from indole-3-pyruvate and pyruvate. Therefore, methods are described to isolate and test clones containing a nucleic acid encoding an aldolase that preferentially produces R-MP. Strains of *Escherichia coli* that require pyruvate supplementation when grown on M9 minimal medium with ribose as the carbon source have been described previously. Ponce, E., *et al.*, "Cloning of the two pyruvate kinase isoenzymes structural genes from *Escherichia coli*.: The relative roles of these enzymes in pyruvate biosynthesis," *J. Bacteriol.* 177:5719-5722, (1995). The relevant genotype of the strain is: $\Delta pykA \Delta pykF$. The double knockout was generated by the method of Datsenko and Wanner, *Proceed. Natl. Acad. Sci. USA* 97:6640-6645, (2000). These strains can form a basis for a pyruvate-generating aldolase screen and to screen for aldolases that are more active on a specific stereoisomer of monatin, a particular stereoisomer of monatin precursor, or an analog of monatin or monatin precursor. An analog of monatin precursor includes compounds that have been identified as substrates for ProA aldolases or KHG aldolases,

such as 4-hydroxy-4-methyl-2-oxoglutarate, 4-carboxy-4-hydroxy-2-oxoadipate, 4-hydroxy-4-methyl-2-oxoadipate, or other carboxyl rich compounds that are converted to pyruvate in an aldol reaction. An example of an analog of monatin that can be used is 4-hydroxy-4-methyl glutamic acid, which can be easily transaminated to 4-hydroxy-4-methyl-2-oxoglutarate (a substrate of ProA) by native aminotransferases in a test cell.

Cloning

[0392] The following primers were used to generate the *pykA* knockout:
 5'-ATGTCCAGAAGGCTTCGCAGAACAAAAATCGTTACCACGTTAGGTGTA
 GGCTGGAGCTGCTTC-3' (SEQ ID NO:3) and
 5'-CTCTACCGTTAAAATACGCGTGGTATTAGTAGAACCCACGGTACCATA
 TGAATATCCTCCTTAG-3' (SEQ ID NO:4).

[0393] The following primers were used to generate the *pykF* knockout:
 5'-AGGACGTGAACAGATGCGGTGTTAGTAGTGCCGCTCGGTACCAGCATA
 TGAATATCCTCCTTAG-3' (SEQ ID NO:5) and
 5'-ATGAAAAAGACCAAAATTGTTTGCACCATCGGACCGAAAACCGGTGTA
 GGCTGGAGCTGCTTC-3' (SEQ ID NO:6).

[0394] A PCR reaction was performed with either pKD3 or pKD4 as template using standard protocols. The PCR product was electroporated into a strain of *E. coli* that expresses the lambda red homologous recombination system. The PCR product had homology to *pykA* or *pykF* and recombined into the chromosome at those sites. When the double crossover occurred, the resulting progeny carried a deleted *pykA* or *pykF* gene and an antibiotic resistance marker. The deleted genes with the antibiotic resistance markers were transduced into an *E. coli* strain (MG1655) using standard P1 transduction techniques.

Strain Analyses

[0395] The double knockout was tested for growth on minimal medium (M9 salts) (Difco) supplemented with Balch's vitamin solution, Balch's modified trace element solution (Balch, W.E., *et al.*, "Methanogens: reevaluation of a unique biological group," *Microbiol. Rev.* 43:260-296, (1979)), and 0.4% D-ribose. No growth was seen for the double mutant unless 5 mM pyruvate was also included in the media. Wild-type MG1655 grew on the above media both in the presence and absence of pyruvate. The

double knockout was tested for growth on the minimal medium described above supplemented with 0.4% glucose rather than ribose. Growth on this medium was similar to that seen with the wild-type strain. With this medium, pyruvate can be generated from glucose *via* the *ptsI* gene product (the enzyme of the phosphotransferase system that makes pyruvate from phosphoenolpyruvate and transfers the phosphate to glucose). The double knockout strain was also tested for growth using the medium as described above supplemented with 0.4% L-arabinose or 0.4% D-xylose rather than ribose. Pyruvate is not generated from growth on these 5-carbon containing (non-PTS) substrates. The double knockout did not grow under these conditions unless it was supplemented with 5 mM pyruvate, while the wild-type strain grew normally both in the presence and absence of pyruvate.

[0396] The *proA* aldolase gene from *Comamonas testosteroni* described in Example 2 of WO 03/091396 A2 (cloned in pET30 Xa/LIC) and the *aspC/proA* gene operon described in Example 3 of WO 03/091396 A2 (cloned in pET30 Xa/LIC and pET32) were sub-cloned into pBAD-TOPO using the pBAD TOPO TA expression kit (Invitrogen).

[0397] Expression of the gene(s), in these constructs, is regulated by the inducible *araBAD* promoter. In the presence of arabinose (for example 0.4%) and IPTG, the gene(s) are expressed. Unless supplemented with pyruvate or a source of pyruvate, the strain will not grow on minimal medium. The medium can be supplemented with monatin, monatin precursor, or an analog of monatin or monatin precursor. Typical ranges of substrate used in the literature are 0.5-5 mM. The ProA aldolase can, for example, convert the monatin precursor into pyruvate and indole-3-pyruvate, thus, providing the strain with a source of pyruvate and allowing growth on minimal medium with 0.4% arabinose. The construct expressing both the *proA* and the *aspC* genes can convert monatin into the monatin precursor and the monatin precursor into pyruvate and indole-3-pyruvate. Additionally, the aminotransferase can convert indole-3-pyruvate to L-tryptophan and complement a tryptophan auxotrophy. This system is used to screen for aldolases and to screen for aldolases that are more active on a specific stereoisomer of monatin, a specific stereoisomer of monatin precursor, or an analog of monatin or monatin precursor. For example, if directed evolution is performed on any of the aldolases disclosed in Example 2 of WO 03/091396 A2, a plate assay utilizing media containing either R or S monatin precursor is used to compare the enantiospecificity of

the resulting mutant enzyme. If growth occurs on the plates containing R-monatin precursor and little or no growth occurs on the plate containing S-monatin precursor, the aldolase has a specificity for substrates containing the R-chirality at the reaction site.

[0398] M9 minimal medium plates were made containing 1x Balch's vitamin solution and Balch's modified trace element solution. Balch, W.E., *et al.*, "Methanogens: reevaluation of a unique biological group." *Microbiol. Rev.* 43:260-296, (1979). Glucose or arabinose was included as the carbon source (0.4% w/v) and plates were supplemented with either 5 mM monatin (R,R; S,S racemic mixture) that had been dissolved in 20 mM potassium phosphate buffer (pH 8.0) or an equal volume of potassium phosphate buffer without monatin. Growth is summarized in Table 34 below.

Table 34

	Glucose	Glucose monatin	Arabinose	Arabinose monatin
MG1655	++++	++++	++++	++++
MG1655 $\Delta pykA \Delta pykF$	++++	++++	+	+
MG1655 $\Delta pykA \Delta pykF$ + <i>aspCproA</i> /pBAD-TOPO	++++	++++	+	++

[0399] It is expected that the screen could be optimized by controlling the levels of ProA and AspC, increasing uptake of monatin, using monatin precursor in the place of monatin (in this case the aminotransferase would not need to be present), or using a less hydrophobic analog of monatin such as those described above. Methods for increasing the uptake of monatin include addition of amino acid mixtures, addition of specific amino acids, the use of detergents, antibiotics, antibiotic analogs, or enzymes that help to permeabilize the cell wall, and the addition of a small amount of pyruvate to allow for growth in case the aldolase cannot provide enough pyruvate to support growth. Polymyxin B nonapeptide (Dixon and Chopra, *Antimicrobial Agents and Chemotherapy* 29:781-788 (1986)) and microcystin RR (Dixon, *et al.*, *FEMS Microbiology Letters* 230:167-170 (2004)) have been described as agents that permeabilize the outer membrane of *E. coli*.

[0400] It is expected that other promoter systems/plasmids can be used in this screening system with equivalent results. Examples include T7 promoter systems and IPTG inducible promoters such as *tac* and *lac*.

- [0401] The *aspC* and the *proA* genes were cloned into the pTrc99a expression vector (Amersham, Piscataway, NJ). The resulting vector was transformed into the tryptophan auxotrophs CAG18455 or CAG18579 (*see* Example 4 for strain descriptions). The transformants were plated on M9 minimal medium with 0.1 mM IPTG and 5 mM monatin. After 3 days at 37 °C, the strains with the operon plasmids formed colonies, while the parent strains did not appear to grow. Additionally, the growth was dependent on the presence of IPTG indicating that expression of the operon was required for growth. In this complementation study, the *aspC/proA* operon formed MP from monatin and indole-3-pyruvate from MP. The indole-3-pyruvate could then be converted to L-tryptophan allowing the tryptophan auxotrophs to grow on M9 minimal medium.
- [0402] Several potential organisms may have the R-specific aldolase and can be tested as described above. The presence of R,R-monatin has been detected in culture supernatants of *Corynebacterium glutamicum*. This suggests the presence of an enzyme that is capable of making the R-monatin precursor. Additionally, the presence of multiple isomers of monatin has been detected in cell free extracts of *Sinorhizobium meloti* using reversed phase chromatography, again indicating the possible presence of an aldolase or aminotransferase capable of making an R stereoisomer of monatin precursor.
- [0403] *Pseudomonas straminea* (*Pseudomonas ochraceae* NGJ1), *Sinorhizobium meliloti*, *Sphingomonas* sp. LB126, *Arthrobacter keyseri* 12B, *Yersinia pestis* strain CO92, *Bradyrhizobium japonicum* str. USDA 110, *Sphingomonas* (*Pseudomonas*) *paucimobilis*, *Yersinia pestis* KIM, *Ralstonia metallidurans* CH34, *Yersinia pseudotuberculosis* IP 32953, *Rhizobium leguminosarum* biovar *viciae* rhiz23g02-p1k_1009_341 (Sanger Institute), *Novosphingobium aromaticivorans* DSM 12444, *Pseudomonas putida* KT2440, *Magnetospirillum magnetotacticum* MS-1, *Rhodopseudomonas palustris* CGA009, *Xanthomonas campestris* ATCC-33913, *Xanthomonas axonopodis* citri 306, and *Streptomyces avermitilis* MA-4680 have homologs that have been discovered by BLAST analysis using *proA* (*Comamonas testosteroni*) as the template. See U.S. Application No. 20050282260. These organisms can be used as a source of DNA and tested in the screen mentioned above.
- [0404] Organisms capable of growth on gallic acid, syringic acid, protocatechuate, phthalate, parahydroxybenzoate, and fluorene may have an aldolase that may make monatin and have potential for the screen mentioned above. The following organisms

metabolize protocatechuate via the 4,5-dioxygenase pathway and may have an aldolase that may be of utility: *Bordetella bronchiseptica* RB50, *Bordetella parapertussis* 12822, *Klebsiella pneumoniae* MGH78578, *Magnetospirillum magnetotacticum* MS-1, *Rhodopseudomonas palustris* CGA009, *Sphingomonas aromaticivorans* F199, *Xanthomonas axonopodis citri* 306, *Xanthomonas campestris* ATCC 33913.

[0405] And the following organisms degrade protocatechuate via the 3,4 dioxygenase pathway and have an aldolase that may be of utility: *Acinetobacter calcoaceticus* ADP1, *Acinetobacter* species ATCC 33305, ADP1, *Agrobacterium tumefaciens* C58, *Azotobacter vinelandii* AvOP, *Bradyrhizobium japonicum* str. USDA 110, *Bradyrhizobium japonicum* tr. USDA 438, *Brucella abortus*, *Brucella melitensis* 16M, *Brucella melitensis* suis 1330, *Burkholderia cepacia* J2315, *Burkholderia fungorum* LB400, *Burkholderia pseudomallei* K96243, *Corynebacterium efficiens* YS-314, *Corynebacterium glutamicum* ATCC-13032, *Mesorhizobium loti* MAFF303099, *Mycobacterium avium* subsp. *paratuberculosis* str. k10, *Pseudomonas aeruginosa* PAO1, *Pseudomonas fluorescens* Pf0-1, *Pseudomonas fluorescens* SBW25, *Pseudomonas putida* KT2440, *Pseudomonas syringae* pv. tomato str. DC3000, *Ralstonia solanacearum*, *Rhodococcus* sp. strain I24 (IG-15), *Sinorhizobium meliloti* 1021, *Streptomyces avermitilis* MA-4680, *Streptomyces coelicolor* A3 (2), and *Xanthomonas axonopodis citri* 306, *Xanthomonas campestris* ATCC-33913.

EXAMPLE 6

6A: Site Directed Mutagenesis of HEXAspC

Experimental Overview

[0406] A hexamutant of *E. coli* AspC (HEXaspC) was found to have better activity as compared to AspC for the production of S,S monatin, as described in Example 6 of WO 03/091396 A2. HEX (accession number: /AHFA gi:127190) contains the following mutations from AspC (*E. coli* numbering): V35L, K37Y, T43I, N64L, T104S, and N285S. Based on structural analysis and literature reports (Rothman, S., and Kirsch, J., *J. Mol. Biol.* 327:593-608, (2003); Rothman, S., *et al.*, *Protein Science* 13:763-772, (2004)), 5 more mutants were created that were expected to increase the kinetic activity toward

substrates utilized in the monatin production pathway: L-tryptophan, S-MP, or both. Two of the mutants increased transamination rates for both tryptophan and S,S monatin. Two of the mutants showed an increased stereoselectivity for the formation of S,S monatin while one was less stereoselective. Based on this, it is expected that a broad specificity D-aminotransferase from *Bacillus sp.* with similar mutations would be useful as the D-aminotransferase in the R,R monatin pathways shown in FIG. 3, and described in Example 4A(4). One of the mutants (HEXaspCP9T/R122G) had increased activity for L-tryptophan transamination, but activity in S,S monatin production or S,S monatin transamination was decreased significantly. Thus, it is expected that this enzyme is useful in the first step of the R,R monatin production pathways shown in FIGS. 1, 2, 4, 5, 6, 7, and 8 and described in Examples 9 and 10(3). In general, an aminotransferase that has activity similar to that of AspC on L-tryptophan, and limited activity on R-MP and S-MP, would be useful for the processes depicted in FIGS. 1, 2, 4, 5, 6, 7, and 8.

Methods and Materials

[0407] The HEX gene cloned in pUC19 was provided by Professor J. F. Kirsch (Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720-3206) and used as the template for the cloning of the gene into pET23a. See Onuffer, J.J., and Kirsch, J.F., "Redesign of the substrate specificity of *Escherichia coli* aspartate aminotransferase to that of *Escherichia coli* tyrosine aminotransferase by homology modeling and site-directed mutagenesis," *Protein Science* 4:1750-1757 (1995). See also NCBI accession number 1AHF_A GI:1127190 (HEX amino acid sequence). The following primers were designed for cloning the HEX gene into the pET23a vector (Novagen, Madison, WI):

HEXaspC primers:

N term: 5'-GCGGAACATATGTTTGAGAACATTACCGCC-3' (SEQ ID NO:7);

C term: 5'-ATAACCGGATCCTTACAGCACTGCCACAATCG-3' (SEQ ID NO:8).

[0408] The following PCR protocol was used for gene amplification: In a 100 μ L reaction, 50 ng DNA template, 1.0 μ M of each primer, 0.2 mM each dNTP, 1 U *Pfu* Turbo Polymerase (Stratagene; LaJolla, CA), and 1X Cloned *Pfu* buffer were added. The thermocycler program utilized a hot start of 94 °C for 5 minutes; followed by 25 cycles of a denaturing step at 94 °C (30 sec), an annealing step at 55 °C (1 min), an extension step

at 72 °C (2 min), and finally a finishing step at 72 °C (7 min). The purified PCR product was digested with *Bam*H I and *Nde*I (New England Biolabs) restriction enzymes. The PCR product was ligated into pET23a that was also digested with *Nde*I and *Bam*H I, using the Roche Rapid DNA Ligation kit (Roche, Indianapolis, IN). The desalted ligations were electroporated into *E. coli* DH10B cells using a Bio-Rad Gene Pulser II system, according to manufacturer's protocols. Miniprep DNA was prepared using a Qiagen Spin Miniprep kit (Qiagen, Valencia, CA) and was used as a template for mutagenesis reactions. The plasmid was transformed into *E. coli* BL21 (DE3) cells according to the manufacturer's protocols (Novagen, Madison, WI).

[0409] The tryptophan residue at position 130 is thought to be important for stacking interactions with the pyridoxyl ring, but also appears to be a source of steric hindrance with the S-monatin precursor ("S-MP") substrate, based on protein modeling observations. Therefore, an amino acid with a smaller hydrophobic side chain (phenylalanine) was used to replace the tryptophan. The rest of the mutations were based on kinetics data in literature, although new combinations of desirable mutations were created. All mutations to HEXaspC, with the exception of W130F, were made using the Stratagene Multi-Change kit by following the manufacturer's instructions. The W130F mutation was made using the Stratagene QuikChange kit according to the manufacturer's instructions with the only exception being that the extension temperature for the PCR reaction was decreased to 66 °C. The primers for the multi-change kit were designed using the QuikChange multi-kit primer design tool on <www.stratagene.com>, except for the W130F single mutation primers.

[0410] The primer sequences are listed in Table 35 below:

Table 35

Primer	Sequence (5' to 3')
aspCW130F_backward	CGCTCTTATGGTTCGGTTTGCTTGGGTTGCTCACCC (SEQ ID NO:9)
aspCW130F_forward	GGGTGAGCAACCCAAGCTTTCCGAACCATAAGAGCG (SEQ ID NO:10)
R122G-1 ^a	CAAAAAATACCAGCGTTAAGGGAGTGTGGGTGAGCA ACC (SEQ ID NO:11)
P9T_4 ^a	CATTACGCGCGCTACTGCCGACCCGATTC (SEQ ID NO:12)

I68V-1 ^a	CACCAAAAATTACCTCGGCGTAGACGGCATCCCTGA ATT (SEQ ID NO:13)
T156A ^a	TGATGCGGAAAATCACGCTCTTGACTTCGATGCAC (SEQ ID NO:14)

^a Denotes a primer that was modified by 5' phosphorylation

Expression of HEXaspC Mutant Genes and Analysis of Enzyme Activity

[0411] Liquid cultures (5 mL) of Novagen Overnight ExpressTM Autoinduction System 2 (Catalog # 71366-3; solutions 1-6) were inoculated from fresh plates or frozen glycerol stocks of the following strains:

E. coli BL21(DE3)::HEXaspCpET23a

E. coli BL21(DE3)::HEXaspCW130FpET23a

E. coli BL21(DE3)::HEXaspCT156ApET23a

E. coli BL21(DE3)::HEXaspCP9T/T156ApET23a

E. coli BL21(DE3)::HEXaspCP9T/R122GpET23a

E. coli BL21(DE3)::HEXaspCR122G/T156ApET23a

[0412] The cultures were incubated at 37 °C at 230 rpm for 6 – 8 h. The OD₆₀₀ of each culture was determined and the volume of culture necessary to obtain an OD₆₀₀ of 0.03-0.05 in 25 mL was calculated. The calculated volumes of each liquid culture were transferred to flasks containing 25 mL of the same medium. The Overnight ExpressTM Autoinduction System 2 is a complete, chemically defined medium for high-level expression with IPTG-inducible expression systems that uses lactose as the inducing agent and does not require monitoring of cell growth. The Overnight Express cultures were incubated at 30 °C with shaking at 230 rpm for 18 h. The cells were harvested by centrifugation and washed once with cold 50 mM MOPS, pH 7.0. The cells were then lysed using BugbusterTM (primary amine free) Extraction Reagent (Novagen Catalog #70923-3, Madison, WI) containing 1 µL/mL benzonase nuclease (Novagen Catalog #70746-3, Madison, WI), 5 µL/mL Protease Inhibitor Cocktail Set II (Novagen Catalog #539132, Madison, WI) and 0.33 µL/10 mL r-Lysozyme (Novagen Catalog #71110-3, Madison, WI) following the Novagen recommended protocol. After incubation at 25 °C for 15 min with gentle shaking, the cell debris from each suspension was pelleted by centrifugation at 21,000g for 15 min at 4 °C. The supernatant was carefully decanted and analyzed as the cell free extract. Inclusion body fractions were isolated by suspending the

cell debris fractions in 30% Bugbuster™ (primary amine free) Extraction Reagent, centrifuging at 21,000 x g for 10 min; suspending the centrifuged pellets in 10% Bugbuster™ (primary amine free) Extraction Reagent, centrifuging again to isolate the washed pellets.

[0413] The cell free extracts and inclusion body fractions were analyzed for protein expression by SDS-PAGE on 4-15% gradient gels (Bio-Rad # 161-1104). For the cell extract samples, twenty micrograms of soluble protein were loaded in each gel lane (premixed with 1X protein loading buffer and heated at 95 °C for 5 min). The inclusion body fractions were dissolved in 1X protein loading buffer (0.2 mL) and heated for 10 minutes at 95 °C. 5 µL of each solution was loaded per gel lane. The amount of each HEX mutant in comparison to the total soluble protein loaded into each lane was calculated by band intensity analysis using Labworks BioImaging 1D-gel tool (UVP, Inc. Upland, CA), and is reported in Table 36 below:

Table 36

Sample	HEXaspC Protein/ Total Soluble Protein
<i>E. coli</i> BL21(DE3)::HEXaspCP9T/T156ApET23a CFE	0.310
<i>E. coli</i> BL21(DE3)::HEXaspCP9T/R122ApET23a CFE	0.145
<i>E. coli</i> BL21(DE3)::HEXaspCpET23a CFE	0.172
<i>E. coli</i> BL21(DE3)::HEXaspCR122A/T156ApET23a CFE	0.174
<i>E. coli</i> BL21(DE3)::HEXaspCW130FpET23a CFE	0.114
<i>E. coli</i> BL21(DE3)::HEXaspCT156ApET23a CFE	0.120

[0414] Analysis of the gels showed that the HEXaspCR122A/T156A mutant was the only protein that was found in substantial quantities as inclusion bodies. The HEXaspCP9T/T156A protein gave the highest level of expression, approximately 90% better than HEXaspC protein. In contrast, the W130F, T156A and P9T/R122G proteins were expressed in lower concentrations than HEXaspC.

[0415] The activity of the HEXaspC mutant proteins for the production of S,S-monatin was measured using the following reaction conditions: Each 1 mL reaction contained 50 mM TAPS, pH 8.2, 4 mM MgCl₂, 3 mM sodium phosphate, pH 8.0, 200 mM sodium pyruvate (pH adjusted to 8), 5 mM α-ketoglutarate (pH adjusted to 8), 50 mM tryptophan, 0.05 mM pyridoxal 3-phosphate, 50 µg/mL ProA aldolase (added as a cell free extract) and varying concentrations (approximately 50 and 500 µg/mL) of aminotransferase

(added as a cell free extract). De-aerated water was used to prepare the stock solutions and to adjust the volume of the reaction mixtures to 1.0 mL. The pyridoxal phosphate was added just prior to the addition of the enzymes. The reaction tubes were incubated at 30 °C with gentle shaking for 4 h. Samples (0.01 mL) were withdrawn at 1, 2, and 4 h after the addition of the enzymes, filtered, and analyzed by LC/MS/MS, as described in Example 1. Monatin production was normalized based on the amount of aminotransferase present in the reactions.

- [0416] Under the conditions of these assays, the HEXaspC and the HEXaspCT156A produced the most total monatin per mg of aminotransferase while the P9T/R122G protein produced the least, followed by HEXaspCW130F. The HEXaspCW130F and P9T/R122G enzymes showed the greatest stereoselectivity for S-MP (greater than 98% S,S-monatin), even when high enzyme concentrations were used (greater than 300 µg/mL). The percentage of S,S-monatin product decreased to less than 90% in the enzymatic reactions containing the P9T/T156A enzyme at high concentration. The other mutants showed a product stereoselectivity very similar to the original HEXaspC mutant (approximately 95% S,S-monatin). Analysis of the product of the reaction containing the HEXaspC enzyme using the FDAA derivitization reagent described in Example 1 showed that the second stereoisomer formed is R,S-monatin.

Assaying of Tryptophan and Monatin Aminotransferase Activity

- [0417] The mutants were tested for transamination activity using S,S monatin and L-tryptophan as substrates. The aminotransferase activity was measured by following the formation of the co-product of the reaction, glutamate, by HPLC with OPA-post-column derivatization as described in Example 1. The reaction mixture contained, in 1.0 mL, 100 mM HEPPS buffer, pH 8.0, 20 mM alpha-ketoglutarate, 0.08 mM pyridoxal phosphate, 25 mM tryptophan or S,S monatin, and enzyme (supplied as 2.5 mg of in cellular extracts protein). All components except the enzyme were mixed together. The enzyme was added to start the reaction and the reaction solution was incubated at 30 °C (gentle shaking) for 90 min. Reactions were done in duplicate, with negative controls in which no enzyme was added. The reaction was stopped by the addition of 10% formic acid (final concentration), the mixture was centrifuged at 21,000 rpm, and the supernatant was carefully removed and filtered. The data were corrected for background levels of glutamate and for the dilution from the addition of acid to precipitate the proteins, then

normalized by amount of mutant aminotransferase added. When tryptophan was utilized as a substrate, HEXaspC produced 13.0 mM glutamate per mg of aminotransferase per hour. The relative activity, expressed as a percentage, of the mutants is as follows: HEXaspCW130F (156%), HEXaspCT156A (151%), HEXaspCP9T/T156A (63.7%), HEXaspCP9T/R122G (116%), and HEXaspCR122G/T156A (107%). When S,S monatin was utilized as a substrate, HEXaspC produced 7.43 mM glutamate per mg of aminotransferase per hour. The relative activity, expressed as a percentage, of the mutants is as follows: HEXaspCW130F (113%), HEXaspCT156A (87.7%), HEXaspCP9T/T156A (67.3%), HEXaspCP9T/R122G (11.2%), and HEXaspCR122G/T156A (114%).

[0418] The HEXaspCP9T/R122G mutant had increased activity for tryptophan to indole-3-pyruvate conversion, but decreased activity for S,S monatin transamination. The ratio of tryptophan to monatin activity was 18.2 in comparison to 1.75 for HEXaspC, making it a desirable candidate for production of R,R monatin using pathways that require an L-aminotransferase, such as those described in Examples 9 and 10(2). As such, the HEXaspCP9T/R122G is an example of an aminotransferase with limited activity on S,S monatin, as well as, MP.

[0419] Most of the mutations improved L-tryptophan activity, but only two mutants increase activity toward both L-tryptophan and S,S monatin (HEXaspCW130F and HEXaspCR122G/T156A). Because 25 mM of substrate was used in these assays, the enzymes were most likely saturated and the activity is a reflection of the k_{cat} of the enzymes. However, under the conditions in which the assays for S,S monatin production were performed, described above, it is unlikely that the concentration of S-MP is sufficient to saturate the enzyme, thus there is no overall increase in S,S monatin production because the increase in k_{cat} is offset by an increase in K_m . It has been reported, for similar substrates, that some of the mutations made increase the k_{cat} but also increase the apparent K_m for the amino acid substrate. If increasing concentrations of substrates were used, it is expected that these two mutants would provide a benefit in production rates of S,S monatin in comparison to HEXaspC. The HEXaspCT156A mutation appears to have increased tryptophan transamination rates without having a significant effect on MP transamination rate under the conditions above for S,S monatin production.

[0420] By comparison of the structures of HEXaspC and one of the *Bacillus* sp. D-aminotransferase enzymes (see, for example, Sugio, S, *et al.*, *Biochemistry* 34:9661-9669, (1995)), the W130F, R122G, T156A, and HEX mutations of AspC could be mapped to corresponding residues in the D-aminotransferase structure. It is expected that similar mutations in the broad specificity D-aminotransferase would improve the overall production of R,R monatin, as described in Example 3A. For example, the functionality provided by tryptophan 130 in AspC is replaced in *Bacillus* D-aminotransferases by hydrogen bonding between the side chains of serines 179-181 and glutamate 166 (YM-1 numbering scheme). To lessen steric hindrance, the glutamate could be mutated to an aspartate residue. Some D-aminotransferases have a threonine residue at position 179, which would increase steric hindrance and should be avoided. The *B. sphaericus* enzyme has an alanine in place of serine at position 181, which may also reduce steric hindrance.

[0421] Additional information from studies of aspartate aminotransferase can be applied to the D-aminotransferase as well. While the AspC enzyme has an arginine in the active site that interacts with the side chain of dicarboxylate substrates, the D-aminotransferase has a loop from Ser240 to Ser243. The side chains of Ser240, Thr242, and Ser243 face the same direction and form a pocket with the hydroxyl group of Ser180 which provides a surface for both nonpolar and polar substrates can interact. Ser180 is involved in PLP binding; however, to improve the activity of a D-aminotransferase with R-MP, one can modify the Ser240, Thr242, or Ser243 residues to accept larger substrates or to favor negatively charged substrates. For instance, Thr242 can be mutated to Ser to reduce the side chain length. One of the residues can be mutated to lysine or arginine, such as Ser243. The residues (YM-1 numbering) Val30-Val36 are located in a beta strand across the active site of the D-aminotransferase and are also important for activity. Tyr31, Val33, Glu32, and Lys35 are thought to face the active site. Tyr31, Glu32, and Val33 are invariant in all the *Bacillus* homologs. Ro, *et al.*, *FEBS Lett* 398:141-145, (1996)) mutagenized Val33 to Ala and found a slightly increased catalytic efficiency for alpha-ketoglutarate transamination and a significantly improved catalytic efficiency for bulkier substrates (less steric hindrance). In some homologs Lys35 is replaced with Arg, but if steric hindrance is a concern, the Lys residue may be preferable. Valines 34 and 36 are also preferable over conservative substitutions such as isoleucine, again due to less steric hindrance for large molecules such as MP. Because the novel D-aminotransferase

("4978") described in Examples 15 and 16 had higher activity than the *B. sphaericus* enzyme and the hybrid DAT described in Example 19, it is the obvious choice for further mutagenesis reactions. The ideas above, based upon crystal structure analysis of YM-1 D-aminotransferase, can be applied to the D-aminotransferase from ATCC strain 4978. The numbering above is one amino acid less than the corresponding amino acid in the 4978 protein sequence.

6B: Characterization of Mutants of D-aminotransferase from ATCC 4978

Experimental Overview

- [0422] The novel D-aminotransferase gene (described in Example 15) from *Bacillus* strain ATCC 4978 was mutagenized using site-directed methods. The mutant genes were expressed and assayed for activities of interest for monatin production pathways.
- [0423] In addition to the ideas listed in Example 6A for site directed mutagenesis targets, other ideas were developed by actual docking of R-MP into the active site of the YM-1 crystal structure and using primary amino acid sequence alignments to determine if the 4978 protein was likely to have similar structural characteristics in that region. It was expected that the following additional mutations would be beneficial (using 4978 amino acid numbering). It was thought that mutagenesis of alanine 153 to arginine would stabilize the second carboxyl group of the substrate (R-MP). This change is likely to increase steric hindrance, so to compensate, the serine residues at positions 181 and 182 were changed to alanine or glycine. It was also hypothesized that one could introduce an arginine at position 180, 181, or 182 and convert one or more of the other serine residues to alanine or glycine to make room for the bulkier side chain of arginine. The phenylalanine at amino acid 200 is spatially close to where R-MP is predicted to dock into the active site and there is a large amount of variability in this residue amongst the D-aminotransferases that catalyze monatin transamination fairly well. It was thought that amino acid modifications at this position could be useful. Mutation of leucine 151 to phenylalanine was predicted to potentially improve interactions with the indole ring of the substrate.
- [0424] Based upon literature, it was hypothesized that mutation of threonine 243 to asparagine may improve R-MP selectivity for transamination reactions. Likewise, it was

thought that mutagenesis of asparagine 100 to alanine may improve the specific activity of the enzyme for monatin transamination reactions (Ro, *et al.*, *FEBS Lett* 398:141-145, (1996); Sugio, S, *et al.*, *Biochemistry* 34:9661-9669, (1995); EP1580268).

[0425] Lee *et al.* characterized mutants of the 141-144 region (loop) and found that D-aminotransferases with the EYcY rather than the LRcD (which is native to the 4978 protein) tend to have a lower K_m for dicarboxylic acid substrates. (Lee SG, Hong SP, Song JJ, Kim SJ, Kwak MS, Sung MH. Functional and structural characterization of thermostable D-amino acid aminotransferases from *Geobacillus* spp. Appl Environ Microbiol. 2006 Feb;72(2):1588-94). Because MP is a dicarboxylic acid substrate, similar to alpha-keto glutarate, and the concentrations of MP are fairly low in a typical monatin production reaction mixture, a decreased K_m could potentially help the activity of a mutant DAT for monatin production.

[0426] The methods below describe creation of the 4978 D-aminotransferase mutants, as well as assay results using these mutants.

Mutagenesis

[0427] The primers for mutagenesis were designed following the suggestions listed in the Stratagene Multi-Change kit (La Jolla, CA). The primers were 5'-phosphorylated. Mutagenesis was done using the Stratagene Multi-Change kit following the manufacturer's instructions. The templates used for mutagenesis were either the pET30 (untagged) or pET28 (tagged) 4978 DAT constructs described in Example 15. The primers are listed below in Table 37:

Table 37

Mutant name	Amino acid change	Primer
4978-22	T243N	5'-GTGATTGTTTCATCAACGAATTCAGAAGTAACGCC-3' (SEQ ID NO:156)
10	T243R	5'-GTGATTGTTTCATCAACGCGTTCAGAAGTAACGCC-3' (SEQ ID NO:157)
7	T243S	5'-GTGATTGTTTCATCAACGAGTTCAGAAGTAACGCC-3' (SEQ ID NO:158)
19	T243A	5'-GTGATTGTTTCATCAACGGCTTCAGAAGTAACGCC-3' (SEQ ID NO:159)
15	N100A	5'-GTGCAGGCCCTCGTGCTCATATTTCCCTGG-3' (SEQ ID NO:160)
B	T243Q	5'-GAAGTGATTGTTTCATCAACGCAGTCAGAAGTAACGC CAATTATC-3' (SEQ ID NO:161)
2	T243N/N100A	above primers used together

[0428] *E. coli* XL10-Gold cells (Stratagene) were transformed and the resultant purified plasmid preparations were sequenced to verify that the correct mutations were incorporated.

Expression and Assay

[0429] Plasmid DNA containing the correct mutations was transformed into *E. coli* BL21(DE3) competent cells and induced as described in Example 16. Cellular extracts were prepared with BugBuster Reagent and Benzonase Nuclease (EMD Biosciences/Novagen, Madison, WI). One mL assays were performed at 30 °C with gentle shaking and contained 10.2 mg D-tryptophan, 0.05 mM PLP, 4 mM MgCl₂, 100 mM potassium phosphate buffer pH 7.5, approximately 50 µg of aldolase, 200 mM pyruvate, and 0.150-0.5 mg/mL D-aminotransferase supplied as cellular extracts. Total protein assays were done using the Bio-Rad (Hercules, CA) total protein kit (Coomassie) or the Pierce (Rockford, IL) BCA kit, and percent expression of the D-aminotransferase was estimated by SDS-PAGE or the Bio-Rad Experion Automated Electrophoresis System. Samples were taken at 3 hours and overnight.

[0430] Using approximately 0.150 mg/mL of D-aminotransferase with the R-specific aldolase of SEQ ID NO:22, the first assays showed that the following mutants (untagged) had transamination activity (in order of highest to lowest): T243N, T243S, T243N/N100A, N100A. It was also noted that the T243N appeared to raise the stereo-purity of the R,R monatin produced. Assays were repeated using purified *Comamonas testosteroni* ProA aldolase (100 µg/mL) and 0.50 mg/mL of D-aminotransferase mutants (untagged, supplied as cellular extract). Samples were taken at 2 hours and overnight. The results for the active proteins are shown below, duplicate results were averaged. The % R,R monatin was determined by peak area on reversed phase HPLC and then measured using the FDAA derivatization method described in Example 1 (results shown in parentheses). The results are shown in Table 38. Only R,R and S,R monatin are produced from D-tryptophan. The T243R mutant did not appear to produce monatin under the conditions tested, and the T243A mutant produced very low levels of monatin.

Table 38

Enzyme - Untagged (time)	Total monatin (ppm)	% R,R
4978 wild-type	4.7	41.6

(2 hours)		
4978 wild-type (overnight)	43.2	35.1 (30.9)
T243S (2 hours)	55.0	37.4 (21.7)
T243S (overnight)	97.7	35.5 (29.8)
T243N (2 hours)	73.2	86.7 (88.3)
T243N (overnight)	120.9	86.3 (86.1)
N100A (2 hours)	12.0	40.8
N100A (overnight)	22.3	41
T243A (2 hours)	0.8	~100
T243A (overnight)	1.3	~100

[0431] Although the assays were performed estimating percent D-aminotransferase using Bio-Rad Experion[®] software, it is clear that the T243S and T243N mutants had increased activity compared to the wild-type enzyme. The T243N mutant also provided an additional benefit of increasing dramatically the % R,R monatin formed. This enzyme has an increased preference for R-MP as compared to S-MP in transamination reactions. The N100A mutant did not increase activity alone or in combination with T243N as suggested in the literature. A V34A site directed mutant of the untagged 4978 DAT was also created using similar methods, as described above. The V34A sited directed mutant was found to have significantly less activity than the wild-type enzyme under the conditions tested.

[0432] Another point of interest in the initial assays was that the wild-type enzyme appeared to have more activity when it was produced with an N-terminal His-tag. Subsequent mutagenesis was done on the tagged version of the gene. Additionally, the most promising mutants above were subcloned into pET28b that has an N-terminal His-tag. These were purified using Novagen HIS-bind columns and the manufacturer's protocol with the recommended buffers (EMD Biosciences/Novagen, Madison, WI). The buffer of the eluent fractions was exchanged, using GE Healthcare PD10 columns (Piscataway, NJ), to the buffer used in the assays.

[0433] One mL assays with purified D-aminotransferase (0.5 mg/mL) and purified R-specific aldolase of SEQ ID NO:104 (50 µg/mL) were conducted at 30 °C with gentle shaking and contained 10.2 mg D-tryptophan, 0.05 mM PLP, 200 mM pyruvate, 4 mM MgCl₂, and 100 mM potassium phosphate buffer pH 7.5. Duplicate samples were incubated for 2 hours and overnight. As a positive control, the *Bacillus sphaericus* DAT (cloned in Example 18) was used in the same assays. The results are shown in Table 39, below (the %R,R as shown by the FDAA assay are indicated in parentheses):

Table 39

Enzyme - Tagged (time)	Total monatin (ppm)	% R,R
4978 wild-type (2 hours)	43	98.4
4978 wild-type (overnight)	96.7	98.3 (95.9)
T243N (2 hours)	197.5	100
T243N overnight	301.2	99.9 (99.6)
<i>B. sphaericus</i> DAT (2 hours)	58.2	99.7
<i>B. sphaericus</i> DAT (overnight)	221.7	98.7 (96.6)
T243Q (2 hours)	7.1	100
T243Q (overnight)	12.4	98.8

[0434] One can see from the data above that the T243N mutant clearly produces the highest amount of monatin at 2 hours. As time increases, the ratio of T243N mutant to *B. sphaericus* DAT positive control is reduced. This result suggests that the T243N mutant is not as stable during the monatin reaction as the *B. sphaericus* DAT. When assayed under similar conditions, the T243S (purified tagged) enzyme had similar levels of activity to the T243N mutant; however, the percent R,R monatin produced was lower (97.2 % at both 2 h and overnight). The T243N/N100A mutant had less activity than the T243N mutant. However, both T243S and T243N/N100A had higher activity than the wild-type 4978 DAT.

[0435] Transamination assays were performed to determine which reaction rates were improved when using the T243N mutant in place of the *B. sphaericus* DAT. One-half mL assays were performed at 30 °C taking time points at 1 hour, 2 hours, and 5 hours. The assays contained 25 mM monatin or D-tryptophan, 25 mM pyruvate, 100 mM potassium phosphate pH 7.5, 50 µM PLP, and 0.1 mg D-aminotransferase (tagged, purified). In the case where less than 100 µg DAT was used, the amount of alanine was normalized to 100 µg of D-aminotransferase. Samples were treated with formic acid and analyzed by LC with post column fluorescence detection (as described in Example 1) for the presence of the co-product, alanine. The results are shown in Tables 40 and 41.

Table 40: Transamination activity with R,R monatin as substrate

Enzyme (assay time)	D-alanine (mM)
wild-type 4978 DAT (2 hr)	0.54
wild-type 4978 DAT (5 hr)	1.11
T243N/N100A (2 hr)	1.32

T243N/N100A (5 hr)	2.78
T243S (2 hr)	1.5
T243S (5 hr)	2.61
T243N (2 hr)	1.26
T243N (5 hr)	2.65
<i>B. sphaericus</i> DAT (2 hr)	0.97
<i>B. sphaericus</i> DAT (5 hr)	2.2

Table 41: Transamination activity with D-tryptophan as substrate

Enzyme (assay time)	D-alanine (mM)
wild-type 4978 DAT (1 hr)	4.55
wild-type 4978 DAT (2 hr)	8.47
T243N/N100A (1 hr)	8.52
T243N/N100A (2 hr)	12.67
T243S (1 hr)	4.89
T243S (2 hr)	8.1
T243N (1 hr)	7.19
T243N (2 hr)	10.83
<i>B. sphaericus</i> DAT (1 hr)	8.7
<i>B. sphaericus</i> DAT (2 hr)	12.54

[0436] For the D-tryptophan reactions, the results show that some of the enzymes had reached equilibrium at 2 hours. The R,R monatin reactions are clearly rate-limiting and improvements to this activity have more of an impact on monatin production rates from D-tryptophan.

[0437] Further assays were done to examine the stability of the T243N 4978 DAT mutant. The wild-type enzyme also loses activity over time. Example 6C describes methods to improve the stability of the T243N D-aminotransferase mutant. When freshly prepared untagged and tagged versions of the T243N mutant are prepared and compared for activity, it was found that the untagged version had a better temporal stability, making it overall a better version of the enzyme to use in monatin production reactions.

[0438] Additional mutants of 4978 DAT were made by methods commonly known to those skilled in the art. However, these mutations all resulted in protein that was

insoluble under the conditions that they were prepared, and thus could not be assayed for activity. The mutations that resulted in insoluble protein were:

S180A/S181A/S182R;
 L151F;
 V34G
 S181R
 A153R/S181A/S182A;
 A153R/S182A;
 A153R/S182G;
 S180R/S181A/S182G;
 S180R/S181A/S182A;
 S180R/S181G/S182G;
 S180G/S181R/S182G; and
 S180A/S181R/S182A.

Additional Mutagenesis

[0439] To create the F200M 4978 DAT mutant, the wild-type 4978 DAT open reading frame from Example 15 (tagged) was amplified with primers 73 and 80 and PfuTurbo[®] DNA Polymerase (Stratagene), cloned into pCRII-Blunt (Invitrogen, Carlsbad, CA) and its sequence was verified (Agencourt, Beverly, MA). The 5' and 3' regions were amplified using primers 80 and 96 and 99 and 103, respectively (Table 42). The amplified DNA was then gel purified using Qiagen QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA). They were combined and subjected again to PCR using primers 80 and 99 (Table 42). The amplified DNA was gel purified as described above and cloned into pCRII Blunt and its sequence verified. The DAT open reading frame was subcloned as an *Nde* I/*Xho* I restriction digest fragment into pET28b.

Table 42

Primer Number	Sequence
73	5'-CATATGAGTTATAGCTTATGGAATGACCAAATTGTGAATG-3' (SEQ ID NO:162)
80	5'-CTCGAGTGCGGCCGCAAGCTTGTCTGACGGAGCTC-3' (SEQ ID NO:163)
96	5'-AATATTTATGGAATTAAAGATGGCGTATTATACACACATCCAG CGAATAACATGATCTTAAATGGTATTACACGTCAAGTAATCATT

	AAATGTGC-3' (SEQ ID NO:164)
99	5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGC-3' (SEQ ID NO:165)
103	5'-CGCCATCTTTAATTCCATAAATATTTGAAGAAGAGCCTTCTG-3' (SEQ ID NO:166)

[0440] The following primers (Table 43) were designed for additional site-directed mutagenesis using the QuikChange[®] Multi Site-Directed Mutagenesis Kit (Stratagene). Mutagenesis was done using the Stratagene Multi-Change kit following the manufacturer's instructions. The template used for mutagenesis was the pET28 (tagged) 4978 DAT construct described in Example 15. A double mutant was also created using the F200Y mutant as the template and doing an additional round of mutagenesis with the T243N (listed above) primer.

Table 43

Mutant	Oligo
141-LRcD-144 -> EYcY	GCAACATTTGTAGAAGACATTCGTTGGAATACTGTTACATTAAATCATT AAATTTACTTGGTGCG (SEQ ID NO:167)
F200Y	GTATTATACACACATCCAGCGAATAACTACATCTTAAATGGTATTACACG TCAAG (SEQ ID NO:168)
S244K	GCAATGGATGAAGTGATTGTTTCATCAACGACTAAAGAAGTAACGCCAA TTATCGACATAGATG (SEQ ID NO:169)
243-TS-244 -> NK	GCAATGGATGAAGTGATTGTTTCATCAACGAATAAAGAAGTAACGCCAA TTATCGACATAGATG (SEQ ID NO:170)
243-TS-244 -> NR	GCAATGGATGAAGTGATTGTTTCATCAACGAATCGTGAAGTAACGCCAAT TATCGACATAGATG (SEQ ID NO:171)

[0441] The mutant coding regions were verified by DNA sequencing (Agencourt). The sequence verified plasmids were transformed into BL21(DE3) cells (Novagen, Madison, WI).

Expression and Assay

[0442] Cultures containing 100 mL LB with 50 µg/mL kanamycin in a 500 mL baffled flask were inoculated with one mL of an overnight culture and grown at 37 °C to an optical density (at 600 nm) of approximately 0.6. Production of the protein was induced by IPTG at a final concentration of 1 mM. Cells were incubated at 30 °C for 4.5 hours after the addition of the IPTG. Cells were centrifuged and frozen at -80 °C. Cells were

disrupted (prepared using Novagen BugBuster reagent containing 1 μ L/mL benzonase nuclease, 5 μ L/mL protease inhibitor cocktail II, and 0.033 μ L/mL rLysozyme following Novagen's recommended protocol) and analyzed by SDS-PAGE. Mutants (141-LRcD-144 -> EYcY) and (243-TS-244 -> NR) resulted in insoluble proteins under the conditions in which they were prepared. Mutant 243-TS-244 -> NK did not have quantifiable activity under the conditions tested and is probably a weak activity enzyme in comparison to wildtype, as is the S244K mutant.

[0443] His-tagged proteins were purified as follows. HIS-bind columns (Novagen, Madison, WI) were equilibrated with 10 mL of 100 mM potassium phosphate, pH 7.8, containing 200 mM NaCl and 50 μ M PLP. Cell-free extracts were loaded on the column. The columns were washed with 10 mL of equilibration buffer, 10 mL equilibration buffer containing 25 mM imidazole, and 10 mL equilibration buffer containing 50 mM imidazole. Proteins were eluted with 5 mL equilibration buffer containing 500 mM imidazole. Proteins were desalted using PD10 columns which were equilibrated in 100 mM potassium phosphate, pH 7.8 containing 50 μ M PLP. The purified proteins were concentrated and quantified using the Bradford Assay (Bio-Rad).

[0444] The D-aminotransferase mutants were assayed using 500 μ g/mL of the D-aminotransferase, 50 μ g/mL of the aldolase of SEQ ID NO:104, 4 mM $MgCl_2$, 50 mM potassium phosphate pH 8, 200 mM sodium pyruvate, 0.05 mM PLP and 20.4 mg/mL D-tryptophan for assay conditions. The final volume was 1.25 mL. Samples (200 μ L) were taken after 0.5, 1, 2 and 14 hours and frozen until the experiment was complete. Samples were filtered, diluted 1 to 10, and analyzed by LC/MS/MS as described in Example 1.

[0445] The wild-type 4978 D-aminotransferase from Example 16 was used as a reference for percent relative activity. Table 44 shows relative activity of each mutant at each time point.

Table 44

D-aminotransferase	Time (hr)	% Activity
4978 wild-type	0.5	100
T243N	0.5	270
F200M	0.5	50
F200Y	0.5	70

F200M/T243N	0.5	183
S244K	0.5	4
4978 wild-type	1	100
T243N	1	289
F200M	1	55
F200Y	1	81
F200M/T243N	1	203
S244K	1	6
4978 wild-type	2	100
T243N	2	266
F200M	2	51
F200Y	2	79
F200M/T243N	2	185
S244K	2	6
4978 wild-type	14	100
T243N	14	254
F200M	14	56
F200Y	14	80
F200M/T243N	14	168
S244K	14	8

[0446] The T243N was the best mutant of all tested for activity in the production of R,R monatin.

6C: Stabilization of the T243N Mutant of the D-Aminotransferase from Strain ATCC 4978

[0447] As shown in Example 6B, the initial activity of the T243N mutant DAT is significantly higher than the *B. sphaericus* DAT, but activity decreases more rapidly. Additional experiments, using the anaerobic protocol described below, indicated that the initial activity of the T243N mutant DAT was up to 8-fold higher than the *B. sphaericus* DAT, however the activity decreased rapidly even under the anaerobic conditions. The following studies were done to try to maintain the higher activity for an extended period of time.

[0448] The T243N mutant of the D-aminotransferase from strain 4978 (described in Example 6B) was purified as the HIS₆-tagged protein as described in Example 26. The aldolase of SEQ ID NO:104 (described in Example 3B) and the *S. meliloti* HMG aldolase were purified as the HIS₆-tagged proteins as described in Examples 22 and 27.

[0449] Conical polypropylene tubes (14 mL) containing 143 mg of D-tryptophan were de-oxygenated in an anaerobic glove box overnight. Stock solutions of 1 M EPPS buffer (pH 8.2), 2 M MgCl₂, 2 M sodium pyruvate and 10 mM PLP were prepared in degassed water and equilibrated in an anaerobic glove box overnight. Stock solutions of 10% (v/v) Tween[®] 80, 1% (v/v) Tween[®] 20, 1% (v/v) Triton X-100, 100% acetone, 100% ethanol and 50% (w/v) glycerol were equilibrated in the anaerobic glove box along with 0.7 g each of trehalose, inositol, sorbitol and erythritol in 2 mL microcentrifuge tubes. Preparations of the purified enzymes were thawed on ice and used immediately in the anaerobic glove box. The stock solutions were added to the 14 mL conical tubes to give a final concentration of 100 mM EPPS, 200 mM pyruvate, 100 mM tryptophan, 1 mM MgCl₂, 0.05 mM PLP, 0.5 mg/mL D-aminotransferase, and 0.01 mg/mL of the aldolase of SEQ ID NO:104 or 0.05 mg/mL of *S. meliloti* HMG aldolase. The proposed enzyme stabilizing components were added at various final concentrations (Tables 45 and 46) to bring the final reaction volume to 7 mL per tube. The reactions were incubated at room temperature in the anaerobic glove box with gentle agitation for up to 24 hours. Samples were removed periodically and analyzed for monatin as described in Example 1 using the LC/MS/MS multiple reaction monitoring method. The initial rates were calculated from the samples withdrawn between 0 and 3 h after the addition of the enzyme.

Table 45 (*S. meliloti* HMG Aldolase)

Additive	Fold Improvement in Initial Rate of Monatin Formation	Fold Improvement in Final Monatin Titer (20 h)
None	1.0	1.0
0.01% (v/v) Tween [®] 80	1.3	1.4
0.1% (v/v) Tween [®] 80	1.3	1.5
0.01% (v/v) Tween [®] 20	1.1	1.5
0.01% (v/v) Triton X-100	1.1	1.2
5% (v/v) Acetone	0.4	0.3
5% (v/v) Ethanol	0.7	0.5
1% (w/v) Glycerol	1.9	1.1
5% (w/v) Glycerol	1.4	1.4

10% (w/v) Glycerol	1.1	1.7
10% (w/v) Trehalose	1.0	1.3
10% (w/v) Inositol	1.3	1.5
10% (w/v) Sorbitol	1.1	1.3
10% (w/v) Erythritol	0.8	1.0

Table 46 (Aldolase of SEQ ID NO:104)

Additive	Fold Improvement in Initial Rate of Monatin Formation	Fold Improvement in Final Monatin Titer at 22 h
0.01% (v/v) Tween [®] 80	1.0	1.0
1% (w/v) Glycerol	1.2	0.9
5% (w/v) Glycerol	1.5	1.5
10% (w/v) Glycerol	1.7	2.1

[0450] The addition of 0.01% - 0.1% (v/v) detergent, such as Triton X-100, Tween[®] 20 or Tween[®] 80, or 1% - 10% (w/v) polyol, such as glycerol, trehalose, inositol or sorbitol, improved the stability of the T243N D-aminotransferase over the lifetime of the experiment.

EXAMPLE 7

Use of Branched Chain Aminotransferases ("BCAT") in the Production of Monatin

[0451] AT-102 and AT-104 are branched chain L-transaminases (EC 2.6.1.42) that were purchased from BioCatalytics (Pasadena, CA). The enzymes were tested for transamination activity using S,S and R,R monatin substrates that were produced chemically. Reactions were performed in a total volume of 0.5 mL, and run in duplicate. The assays contained 50 mM Tris pH 7.8, 0.08 mM PLP, 10 mM α -ketoglutarate (" α -KG"), 5 mM monatin, and 1 mg/mL aminotransferase enzyme. Negative controls did not contain exogenous aminotransferase enzyme. The samples were incubated for 2 hours at 30 °C at 100 rpm shaking. The samples were filtered and LC/MS/MS analysis, as described in Example 1, was run to ascertain glutamate levels. Glutamate levels should correlate stoichiometrically with MP production. When R,R was used as the reaction substrate, very low levels of glutamate were present in the negative controls. AT-104

produced slightly more glutamate than the negative controls, indicating a low level of activity with the R,R monatin substrate (a D-amino acid). Both of the branched chain L-aminotransferases showed activity on S,S monatin. AT-102 produced 102 $\mu\text{g/mL}$ glutamate and AT-104 produced 64 $\mu\text{g/mL}$ glutamate. For comparison, a broad specificity aminotransferase (AT-101, also from BioCatalytics) produced 75 $\mu\text{g/mL}$ under these conditions. The high activity with a branched chain aminotransferase is somewhat unexpected because monatin has more structural similarities to dicarboxylic amino acids and aromatic amino acids that normally serve as substrates for the broad specificity or aspartate aminotransferases. However, due to the glutamic acid backbone of monatin, many of the aminotransferases that can utilize glutamate as an amino donor may also have activity on monatin.

Monatin Production from Indole-3-Pyruvate using BCAT

[0452] AT-102 and AT-104 were tested for production of monatin in coupled reactions using the ProA aldolase from *C. testosteroni* (produced as described in WO 03091396 A2). Enzymes and additional components/substrates were added directly to the reaction buffer provided in the kit, which contained 100 mM potassium phosphate buffer pH 7.5, 100 mM L-glutamate, and 0.1 mM PLP. To one mL of reaction buffer were added 4 mg indole-3-pyruvate, 20 mg pyruvate, approximately 50 μg ProA provided in a cellular extract, 1 μL 2 M MgCl_2 , and 2 mg of the aminotransferase enzyme to be tested. All reactions were performed in duplicate, and a negative control reaction was done with no additional aminotransferase added. A positive control (AT-101) was utilized for comparison; this enzyme is a broad specificity L-aminotransferase. Background production of monatin is due to native *E. coli* aminotransferases present in the cellular extract of the recombinant ProA enzyme. The reactions were incubated overnight at 30 $^{\circ}\text{C}$ with gentle shaking (100 rpm). The samples were filtered and submitted for reverse phase LC/MS/MS analysis as described in Example 1. The results are presented in Table 47 below.

Table 47

Enzyme	$\mu\text{g/mL}$ Monatin Produced
AT-101	173.05
AT-102	122.05
AT-104	133.05

negative	73.25
----------	-------

[0453] AT-102 and AT-104 aminotransferases clearly produced more monatin than the negative control and were about 50-60% as active as the positive control.

[0454] The branched chain aminotransferase enzyme from *E. coli* has been well studied and crystal structures have been analyzed in detail. Okada, K., *et al.*, (1997) *J. Biochem (Tokyo)* 121:637-641, (1997). The enzyme has a similar overall fold and significant sequence homology to *Bacillus* D-aminotransferase enzymes such as those mentioned in Examples 2, 3A, and 6A. In addition, the BCAT enzymes and the D-aminotransferases from *Bacillus* are the only two types of PLP-dependent aminotransferases to show stereospecificity for *re* face addition of hydrogen to PLP. Yoshimura, T., *et al.*, *J. Am. Chem. Soc.* 115:3897-3900, (1993). BCAT is thought to be the only enzyme in which the alpha-amino acid substrate is bound with its carboxyl group on the same side as the phosphate group, allowing the enzyme to have a similar fold and mechanism to the D-aminotransferases while still retaining specificity for L-amino acids. Peisach, D., *et al.*, *Biochemistry* 37:4958-4967, (1998). It is thought that the L-specificity of BCAT comes from the fact that the polar amino acid side chains of the D-aminotransferase that position the alpha-carboxyl group of the substrate are replaced by nonpolar residues in BCAT. It is expected that if all, or some, of these residues are mutated to the corresponding amino acids of the *Bacillus* D-aminotransferase, one could convert the BCAT into a D-specific aminotransferase. The following mutations can be made to the *E. coli* BCAT (numbering based on accession number gi:14719463): Phe37 to Tyr, Val110 to His, Met108 to Arg. Other polar amino acid substitutions could be made at these sites as well, to tailor the enzyme active site to accept large dicarboxylic acid substrates as described in Example 6A. Tyr165 may need to be converted to Leu as well, to mirror the PLP interaction of the D-aminotransferase; Tyr96 (to Phe), Arg41, and Arg98 may also need to be mutated to prevent binding of the alpha carboxyl group in the incorrect orientation in the BCAT enzyme. Trp127 can also be mutated to Tyr to decrease the likelihood of the hydrophobic side chains binding in a pro-S configuration; Tyr32 and Tyr130 may interact with L-glutamate in the active site of BCAT and can be mutated to negatively charged amino acids to minimize this interaction. Goto, M., *et al.*, *Biochemistry* 42:3725-3733, (2003); Okada, K., *Biochemistry* 40:7453-7463, (2001).

- [0455] Because both the D-aminotransferase enzymes and the branched-chain aminotransferase have activity in production of monatin, it is expected that the BCAT can be converted to a D-aminotransferase with activity in R,R monatin production, while providing another possible D-aminotransferase enzyme to be utilized in the reaction schemes described in many of the Examples. Based on the above results, it is possible that the AT-104 enzyme already shows some activity toward D-amino configurations of monatin.

Bacillus Branched-Chain Aminotransferase Cloning and Mutagenesis

- [0456] *Bacillus licheniformis* contains a putative branched-chain aminotransferase that is more closely related to D-aminotransferases than the *E. coli* branched chain aminotransferase is. It was assayed for D-transamination activity and mutagenized based on predicted active site residues mentioned above for the *E. coli* BCAT.

Strain

- [0457] *B. licheniformis* (ATCC number 14580) was grown on Nutrient Agar at 30 °C overnight. Groups of colonies were placed in 100 µL of sterile water and heated for 10 minutes at 95 °C to disrupt the cells. Three µL were used in subsequent Polymerase Chain Reaction (PCR) amplifications.

Polymerase Chain Reaction Protocol

- [0458] Primers were designed for the *B. licheniformis* gene (915 bp) for cloning into pET 28b and pET 30a vectors (Novagen, Madison, WI) and pTRC99a (GE Healthcare Life Sciences), using the *Nco* I and *Sal* I sites. The pET30 construct contains an N-terminal His-tag and S-tag, whereas the pET 28 construct is untagged.
- [0459] The *B. licheniformis* *bcat* primers were:
N term 5'-GGTTAAGGCCATGGGGGACCAGAAAGACCA-3' (SEQ ID NO:44); and C term: 5'-GGCCTTCCGTCGACTCAGCTGACACTTAAGCT-3' (SEQ ID NO:45).
- [0460] The coding region was amplified using the following PCR protocol. In a 50 µL reaction, 3 µL template, 1 µM of each primer, 0.4 mM each dNTP, 3.5 U Expand High Fidelity Polymerase, and 1X Expand™ buffer (Roche, Indianapolis, IN) with Mg were used. The thermocycler program used included a hot start at 96 °C for 5 minutes, followed by 30 repetitions of the following steps: 94 °C for 30 seconds, 50 °C for 1

minute 45 seconds, and 72 °C for 2 minutes 15 seconds. After 30 cycles, the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. Clean PCR products of the correct size were obtained (approximately 900 bp).

Cloning

[0461] The PCR product was purified and digested with *Sal* I and *Nco* I in *Sal* I buffer (New England Biolabs, Ipswich, MA). The digested vectors (pET28, pET30, and pTRC99a) and the insert were purified using the Qiagen QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA). The ligations were done using the Roche Rapid DNA Ligation Kit (Roche, Indianapolis, IN) and purified. The ligations were transformed into *Escherichia coli* DH10B using a 0.2 cm cuvette and a Bio-Rad Gene Pulser[®] II system, as described in the Bio-Rad electroporation manual. The cells were allowed to recover in 900 µL SOC medium for 30 minutes at 37 °C with shaking at 225 rpm. The cells were plated on LB-agar plates containing kanamycin (25 µg/mL). The plasmid DNA was purified using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Sal* I and *Nco* I. The sequences of plasmids that appeared to have the correct insert were verified by dideoxy chain termination DNA sequencing at Agencourt BioScience Corporation (Beverly, MA). Sequencing verified the coding sequence found in NCBI accession number CP000002 GI 56160984 2851268..2850354, which produces a protein with amino acid sequence as listed in accession number AAU24468 GI:52004526.

Gene Expression and Assays

[0462] Plasmid DNA (pET vectors) was transformed into *E. coli* expression host BL21(DE3) cells (Novagen, Madison, WI) for constructs in pET vectors. The cultures were grown and the plasmids were isolated using Qiagen miniprep kit (Qiagen, Valencia, CA) and analyzed by restriction digest to confirm identity. Induction was typically performed in LB medium containing kanamycin (50 µg/mL). The cells were grown to an OD₆₀₀ of 0.4-0.8 at 37 °C, induced with 0.1 mM IPTG (isopropyl thiogalactoside) and sampled at 3-4 hours post induction. Cell extracts were prepared according to the protocol accompanying the Novagen BugBuster[™] reagent (with benzonase nuclease and Roche complete protease inhibitor cocktail added). High levels of soluble protein were

obtained at the predicted molecular weight, as judged by SDS-PAGE. The soluble proteins in the cellular extracts were separated by SDS-PAGE.

[0463] Cell extracts were analyzed for D-aminotransferase activity by following production of alanine from pyruvate (or glutamate from alpha-ketoglutarate) and D-tryptophan using the following protocol. Duplicate one mL reactions were typically carried out in 100 mM potassium phosphate buffer (pH 7.5), 50 μ M pyridoxal phosphate, 25 mM sodium pyruvate, and 50 mM D-tryptophan. The reactions were initiated by the addition of cell free extracts or purified enzyme and were incubated 15 minutes overnight at 30 °C, with mild shaking. Approximately the same level of D-aminotransferase was added (typically around 0.5 mg) in each assay for comparative purposes and AT-103 (BioCatalytics) was often used as a benchmark enzyme. Formic acid was added to a final concentration of two percent to stop the reaction and the precipitated protein was removed by centrifugation. Control reactions without added protein were also performed. Zero time points were also used as negative controls. Alanine and glutamate were detected using OPA post-column derivatization as described in Example 1. The branched-chain aminotransferase had low levels of D-aminotransferase activity in comparison to the AT-103 and *B. sphaericus* enzymes.

[0464] The branched chain aminotransferase was also tested for the ability to produce monatin from D-tryptophan (as in Example 3A), but did not appear to have activity under the conditions tested.

[0465] The pTRC99a construct was transformed into electrocompetent *E. coli* CAG18455 cells, which are auxotrophic for tryptophan production. Cells were grown in M9 minimal medium with Balch's vitamins with 100 mg/L L-tryptophan, 0.4 % glucose, and calcium chloride. Cells were not able to grow without L-tryptophan. Induction was tested at 10, 100 and 1000 μ M IPTG, at an OD600 of 0.4 for 4.5 hours. Bands at the correct MW were visible on SDS-PAGE. The plasmid was mutagenized using the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene). The primers in Table 48 below were designed as described by the manufacturer.

Table 48

Amino Acid Mutation (<i>E. coli</i> Numbering)	Nucleotide Mutation (<i>B. lich</i> Numbering)	Primer Sequence
--	--	-----------------

Y32F	tac 96-->ttc	ATCACGGATTTTTATTTCGGGGACGGC GTG (SEQ ID NO:46)
Y32D	tac 96-->gac	ATCACGGATTTTTAGACGGGGACGG CGTG (SEQ ID NO:47)
F37Y	ttt 111 --> tat	GGACGGCGTGTATGAAGGGATCAGG G (SEQ ID NO:48)
R41K	agg 123 -->aag	TGTTTGAAGGGATCAAGGTATACGA CGCAAC (SEQ ID NO:49)
F37Y + R41K		GACGGCGTGTATGAAGGGATCAAGG TATACGACG (SEQ ID NO:50)
Y96F	tac276-->ttc	GCTGAAAGACGCTTTCATCCGCTTGG TCG (SEQ ID NO:51)
Y96H	tac276 --> cac	GCTGAAAGACGCTCACATCCGCTTG GTC (SEQ ID NO:52)
R98Y	cgc282 -->tac	CTGAAAGACGCTTACATCTACTTGGT CGTTTCAAGAGG (SEQ IDNO:53)
Y96F + R98Y		GGCTGAAAGACGCTTTCATCTACTTG GTCGTTTCAAGAGG (SEQ ID NO:54)
Y96H + R98Y		GCTGAAAGACGCTCACATCTACTTG GTCGTTTCAAGAGG (SEQ ID NO:55)
L108R	ctc 312 --> cgc	GCAGGTGACCGCGGACTCGATCCAA AC (SEQ ID NO:56)
L110H	ctc318 --> cac	GCAGGTGACCTCGGACACGATCCAA AC (SEQ ID NO:57)
L108R + L110H		GCAGGTGACCGCGGACACGATCCAA ACAATTG (SEQ ID NO:58)
L127Y	ttg369 --> tac	GTCATCATAATTGTGCGAACCATACGC AATATTCCCGAAAC (SEQ ID NO:59)
L127K	ttg369-->aag	GTCATCATAATTGTGCGAACCAGG CAATATTCCCGAAAC (SEQ ID NO:60)
I130E	ata375-->gaa	GTCATCATAATTGTGCGAACCATTGGC AGAATTCCCGAAAC (SEQ ID NO:61)
L127Y + I130E		CGAGTGTGTCATCATAATTGTGCGAACC ATACGCAGAATTCCCGAAAC (SEQ ID NO:62)
L127K + I130E		CCGAGTGTGTCATCATAATTGTGCGAACC AAAGGCAGAATTCCCGAAAC (SEQ ID NO:63)
Y165L	tac477 --> ttg	AATCGCTGAACTTGTTAAACAATATT CTTGTCCGGATCGAGG (SEQ ID NO:64)

[0466] Amino acid mutations were based on the *E. coli* BCAT crystal structure and the numbering in the above table is for the *E. coli* protein. The numbering for the DNA mutations is based on the *B. licheniformis* *bcat* gene.

[0467] The primers were diluted to 0.1 mg/mL and approximately 100 ng of each oligonucleotide primer was typically used in a 50 μ L mutagenesis reaction, proportionately higher concentrations were used for larger primers. For oligonucleotide

primers that were essentially competing for annealing to the same template region, sometimes a sum of 100 ng was used for the whole pool of primers in that region. Two hundred nanograms of template (*B. lich beat* in pTRC99a) were used in the reaction, with 5 μ L of 10X QuikChange[®] buffer, 2 μ L dNTPs, and 2 μ L of the enzyme blend. The amplification products were treated with *Dpn* I restriction endonuclease (Stratagene) (2 μ L) for 2 hours at 37 °C and transferred to a thick wall 1.5 mL tube for ethanol precipitation. The resuspended (concentrated) reaction mix was transformed (2.5 μ L) into XL10-Gold Ultracomp cells included in the QuikChange[®] kit. Several colonies were mini-prepped and sequenced to ensure that mutations were random and to estimate the level of mutagenesis achieved. Colonies were resuspended from the plate and bulk minipreps were done. The miniprep DNA was then transformed into the tryptophan auxotroph strain and plated on minimal medium (with IPTG) described above or using minimal medium containing D-tryptophan as the sole nitrogen source. A second and third round of mutagenesis was done on the bulk minipreps using primers that did not appear to incorporate well in the previous rounds. At each stage, colonies that grew quickly on the minimal medium (larger colonies) were retained for further analysis. The mutants shown in Table 49 below were isolated from the selection plates. In some cases these same mutants appeared on the selection medium more than one time.

Table 49

Clone	Mutations
4	F37Y, Y96F
6	Y96F
28	F37Y, Y165L
32	Y96F, L127K
5-1	F37Y, Y96F, R98Y, L108R, L110H, L127Y
5-2	F37Y, R41K, Y96F, R98Y, L108R, L110H, L127Y

[0468] The mutant constructs were induced to make recombinant protein in LB media; cell extracts were prepared as above. The soluble proteins in the cellular extracts were separated on a BioRad Laboratories Experion[®] Automated Electrophoresis Station and analyzed for concentration and percent expression using the Experion[®] Software version 1.1.98.0. Very low levels of soluble recombinant protein were observed; thus quantitation of the band of interest was not possible. Assays were done to test D-

tryptophan transamination as above using 50-250 μL of cellular extracts. Clones 4, 6, 28, and 32 were assayed using both alpha-ketoglutarate and pyruvate as the amino acceptor and incubated for 2 hours and overnight at 30 °C. The background levels of alanine/glutamate present from the cellular extracts was subtracted. For the assays with 5-1 and 5-2, the protein concentrations estimated by the Experion[®] software for the BCATs were 275.1 ng/ μL for the wild-type enzyme, 409.3 ng/ μL for BCAT 5-1, and 148.2 ng/ μL for BCAT 5-2. The results of the assays are shown in Tables 50-52 below.

Table 50

BCAT	Glutamate (mM) 2 Hours	Glutamate (mM) Overnight
wild-type (100 μL)	0.0912	0.2304
wild-type (250 μL)	0.251	0.521
4 (100 μL)	0.0642	0.1202
4 (250 μL)	0.154	0.295
6 (100 μL)	0.053	0.112
6 (250 μL)	0.141	0.289
28 (100 μL)	0.0586	0.1402
28 (250 μL)	0.155	0.367
32 (100 μL)	0.0616	0.1236
32 (250 μL)	0.167	0.339

Table 51

BCAT	Alanine (mM) 2 Hours	Alanine (mM) Overnight
wild-type (250 μL)	0.199	0.438
4 (250 μL)	0.093	0.249
6 (250 μL)	0.097	0.249
28 (250 μL)	0.117	0.325
32 (250 μL)	0.102	0.285

Table 52

BCAT	Glutamate (mM) 1 Hour	Glutamate (mM) Overnight
wild-type (50 μL)	0.018	0.075
wild-type (100 μL)	0.037	0.152
5-1 (50 μL)	0.005	0.017
5-1 (100 μL)	0.01	0.045
5-2 (50 μL)	0.001	0.011
5-2 (100 μL)	0.003	0.031

- [0469] It is evident that like most L-aminotransferases, the enzymes have a preference for alpha-ketoglutarate compared to pyruvate for the amino acceptor. All the mutants did have D-aminotransferase activity, as did the wild-type parent. It is not clear whether the wild-type enzyme had more or less D-aminotransferase activity, because exact quantitation of the BCAT protein from cellular extracts was not possible. However, it is expected that the mutant enzymes have less L-aminotransferase activity than the wild-type; thus the ratio of D- to L-transamination rate is being improved. Continued mutagenesis could provide an alternative enzyme in pathways to monatin.

EXAMPLE 8

Cloning, Expression, and Testing of Glutamate and Aspartate Racemases

- [0470] This example describes methods used to clone and test amino acid racemase enzymes, which can be used to interconvert between L-glutamate and D-glutamate (or L- and D-aspartate or L- and D-alanine). Glutamate, aspartate, or alanine racemases are useful in a biosynthetic pathway to produce R,R monatin when a step in that pathway produces an L-amino acid (e.g., L-glutamate, L-aspartate, or L-alanine) and another step in the pathway consumes a D-amino acid (e.g., D-glutamate, D-aspartate, or D-alanine). FIG. 4 illustrates a biosynthetic pathway for producing R,R monatin from L-tryptophan using an L-tryptophan-specific aminotransferase, an R-specific aldolase, a D-aminotransferase and a glutamate (or aspartate or alanine) racemase.
- [0471] Genes were cloned into the pET28 and pET30 vectors to generate both non-tagged proteins and fusion proteins with cleavable N-terminal HIS₆-Tag/T7-Tags. The resulting proteins were purified using immobilized metal affinity chromatography.

Experimental Overview

- [0472] Genes encoding glutamate racemases (EC 5.1.1.3) from *Lactobacillus brevis* (Genbank Accession No. D29627, nucleic acid sequence), and *Pediococcus pentosaceus* (*murI* gene) (Genbank Accession No. L22789) were cloned and expressed in *E. coli*. The extracts were tested for activity in conversion of L-glutamate to D-glutamate and D-glutamate to L-glutamate. BioCatalytics aspartate racemase enzyme (EC 5.1.1.13) was also tested for interconversion between L- and D-aspartate.

Isolation of Genomic DNA for Cloning

- [0473] *L. brevis* genomic DNA (ATCC 8287D) was obtained from the American Type Culture Collection. *P. pentosaceus* (ATCC 25745) was grown at 37 °C in *lactobacilli* MRS broth and 2 mL was used for genomic DNA isolation using the method of Mekalanos, J.J., "Duplication and amplification of toxin genes in *Vibrio cholerae*," *Cell* 35:253-263, (1983).

Polymerase Chain Reaction Protocol

- [0474] Primers were designed with 5' restriction sites and overhangs for cloning into the pET 28 and pET30 vectors (Novagen, Madison, WI).
- [0475] *L. brevis* glutamate racemase primers:
N term: 5'-GCGGCGCCATGGAAAATGATCCGATTGGTCTAATG-3' (SEQ ID NO:15), and
C term: 5'-GCGGCGGTTCGACGCAATTACAATTGTGTTTGTC-3' (SEQ ID NO:16).
- [0476] *P. pentosaceus* glutamate racemase primers:
N term: 5'-GCGGCGCCATGGATGTATGTATAATTTTATTAG-3' (SEQ ID NO:17), and
C term: 5'-GCGGCGGTTCGACAAATTTTCATTATTCATTCTAATTT-3' (SEQ ID NO:18).
- [0477] The gene derived from *L. brevis* was amplified using the following PCR protocol. In a 50 µL reaction, 0.150 µg template, 1.6 µM of each primer, 0.4 mM each dNTP, 2.8 U Expand High Fidelity™ Polymerase (Roche, Indianapolis, IN), 0.5 U Pfu polymerase (Stratagene, La Jolla, CA) and 1X Expand™ buffer with Mg were used. The thermocycler program used included a hot start at 96 °C for 3 minutes, 8 repetitions of the following steps: 94 °C for 30 seconds, 52 °C for 45 seconds, and 72 °C for 2 minutes, followed by 22 repetitions of the following steps: 94 °C for 30 seconds, 60 °C for 45 seconds, and 72 °C for 2 minutes. After the 22 repetitions, the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a product of ~830 bp, as judged by comparison to DNA size markers.
- [0478] The gene derived from *P. pentosaceus* was amplified using the following PCR protocol. In a 50 µL reaction, 0.15 µg template, 1.6 µM of each primer, 0.4 mM each dNTP, 2.8 U Expand High Fidelity™ Polymerase, 0.5 U Pfu polymerase and 1X

ExpandTM buffer with Mg were used. The thermocycler program used included a hot start at 96 °C for 3 minutes, followed by 8 repetitions of the following steps: 94 °C for 30 seconds, 37 °C for 45 seconds, and 72 °C for 2 minutes, followed by 8 repetitions of the following steps: 94 °C for 30 seconds, 45 °C for 45 seconds, and 72 °C for 2 minutes, followed by 14 repetitions of the following steps: 94 °C for 30 seconds, 55 °C for 45 seconds, and 72 °C for 2 minutes. After the 14 repetitions, the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a product of ~840 bp, as judged by comparison to DNA size markers.

Cloning

[0479] The PCR products were gel purified from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The PCR products were quantified using a SmartSpec 3000TM spectrophotometer. The products were digested with restriction enzymes *Nco* I and *Sal* I following the manufacturer's recommended protocols (New England Biolabs, Beverly, MA) and gel purified from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). Vectors pET28 and pET30 were prepared by digestion with restriction enzymes *Nco* I and *Sal* I, followed by treatment with shrimp alkaline phosphatase and purification from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA).

[0480] The digested vectors and inserts were ligated using the RapidTM DNA Ligation Kit (Roche, Indianapolis, IN). Approximately 50 ng of treated insert, 100 ng of treated vector (3 to 1 molar ratio of insert to vector), 5 U of T4 DNA ligase (included with the RapidTM DNA Ligation Kit, and 1X ligation buffer were incubated for 5 minutes at room temperature. The ligation reactions were purified using the High Pure PCR Product Purification Kit (Roche, Indianapolis, IN) and were used to transform *E. coli* DH10B electrocompetent cells (Invitrogen, Carlsbad, CA). Ten µL of each ligation reaction was added to 40 µL of DH10B cells and were transformed by electroporation using the BioRad Gene Pulser[®] II under the following conditions: 2.5 kV, 25 µF, 200 ohm in a 0.2 cm cuvette. The cells were allowed to recover in 1 mL of room temperature SOC for 1 hour at 37 °C with shaking at 225 rpm. The cells were plated on LB plates containing kanamycin (50 µg/mL).

[0481] Plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction

digest with *Nco* I and *Sal* I. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing.

Gene Expression and Assays

- [0482] Plasmid DNA, verified by sequence analysis, was subcloned into *E. coli* expression host BL21(DE3) (Novagen, Madison, WI). The cultures were grown and the plasmids were isolated using Qiagen miniprep kit (Qiagen, Valencia, CA), and analyzed by restriction digest to confirm identity.
- [0483] Induction in BL21(DE3) was initially performed with *L. brevis* and *P. pentosaceus* glutamate racemases in both pET28 (untagged) and pET 30 (histidine-tagged) vectors. A time course study was performed with cultures grown in 250 mL LB containing kanamycin (50 mg/L) to an OD₆₀₀ of 0.5 – 0.6 and induced with 100 mM IPTG (isopropyl thiogalactoside) and sampled at 0 and 3 hours post induction. Cells from 600 μ L (0 hour) and 275 μ L (3 hour) were resuspended in 40 μ L sodium dodecyl sulfate buffer containing 2-mercaptoethanol, heated at 95 °C for 10 minutes, and cooled. Aliquots of these total cellular protein samples were analyzed by SDS-PAGE using a 4-15% gradient gel.
- [0484] Cell extracts were also prepared from the 3 hour cultures by suspending cell pellets from 5 mL of culture in 0.625 mL Novagen BugBuster™ reagent containing 0.625 μ L benzonase nuclease and 3 μ L protease inhibitor cocktail set #3 (Calbiochem-Novabiochem Corp., San Diego, CA) at room temperature for 20 minutes with gentle shaking and centrifuging at 16,000 x g to remove cell debris. The supernatants (cell extracts) were loaded onto 4-15% gradient gels for analysis of the cellular soluble proteins.
- [0485] The 3 hour samples from cloned *L. brevis* glutamate racemase and *P. pentosaceus* glutamate racemase showed both total and soluble protein that corresponded to the correct size (approximately 31 kDa). The *L. brevis* pET30 (histidine-tagged) gene product was over-expressed at a higher level than, and was also more soluble (>20% of soluble protein) than, the *L. brevis* pET 28 (untagged) gene product, as well as the *P. pentosaceus* gene products in both vectors. The *P. pentosaceus* gene product showed equal overexpression and solubility in the pET28 and pET30 vectors, which was significantly less than that observed for the *L. brevis* pET30 gene product.

- [0486] Cells from the induced cultures (250 mL) were centrifuged and washed once with 0.85 % NaCl. Cell pellets were resuspended in 5 mL/g wet cell weight of BugBuster™ (Novagen, Madison, WI) reagent containing 5 µL/mL protease inhibitor cocktail set #3 (Calbiochem-Novabiochem Corp., San Diego, CA) and 1 µL/mL benzonase nuclease. Samples were incubated at room temperature for 20 minutes on an orbital shaker. Insoluble cell debris was removed by centrifugation at 16,000 X g for 20 minutes at 4 °C.
- [0487] Cell extracts were assayed for glutamate racemase activity using the following protocol. 400 µL reactions were carried out in 10 mM potassium phosphate (pH 8.0), 0.2 mM dithiothreitol ("DTT"), and 10 mM L-glutamate or D-glutamate. The reactions were initiated by the addition of 20-100 µL of cell free extracts and were incubated at room temperature. Sample aliquots were taken over a time course of 1 minute, 5 minutes, 10 minutes, 20 minutes and 1 hour (zero minute samples served as control reactions). 2 M formic acid (25 µL) was added to each 40 µL sample aliquot to stop the reaction and the precipitated protein was removed by centrifugation. Supernatants were removed and frozen at -80 °C until they were analyzed by LC/MS/MS as described in Example 1.
- [0488] Assay results from cell extracts from pET30 induction with 100 mM IPTG (3 hours) demonstrate that *L. brevis* (Genbank Accession No. BAA06106.1 GI:468450) and *P. pentosaceus* (Genbank Accession No. AAA16761.1 GI:349029) enzymes have significant levels of racemase activity on both glutamate isomers. The *P. pentosaceus* racemase (20 µL of cellular extracts) reached equilibrium between L-and D-glutamate in 10-20 minutes starting with either substrate. The *L. brevis* enzyme (20 µL of cellular extracts) also reached equilibrium in approximately 20 minutes.
- [0489] A partially purified aspartate racemase enzyme (catalog # ASPR-101) purchased from BioCatalytics, Inc. (Pasadena, CA) was assayed for activity on L-aspartate and D-aspartate using a protocol similar to the one above. The commercial enzyme showed racemase activity on both isomers. Using 0.5-1 mg of enzyme, equilibrium was achieved in 20-60 minutes.
- [0490] All three racemases (*L. brevis* glutamate racemase, *P. pentosaceus* glutamate racemase and BioCatalytics aspartate racemase) were also assayed for activity on S,S monatin using the following protocol. 400 µL reactions were carried out in 10 mM potassium phosphate (pH 8.0), 0.2 mM DTT, and 10 mM S,S monatin. The reactions were initiated by the addition of cell free extracts (*L. brevis* and *P. pentosaceus*) or

purified enzyme (BioCatalytics aspartate racemase) and were incubated at room temperature. Sample aliquots were taken over a time course of 1 minute, 5 minutes, 10 minutes, 20 minutes and 1 hour (zero minute samples served as control reactions as well as samples without enzyme). 2 M formic acid (25 μ L) was added to each 40 μ L sample aliquot to stop the reaction and the precipitated protein was removed by centrifugation. Supernatants were removed and frozen at -80°C until they were analyzed by LC/MS/MS (Example 1). No decrease in S,S monatin concentration was noted over time, nor was there any increase in S,R monatin (present initially as <5% contaminating byproduct, even in the no enzyme control). Therefore, none of the racemases assayed showed activity towards monatin.

EXAMPLE 9

Production of R,R Monatin from L-Tryptophan Using Alanine, Glutamate, or Aspartate Racemases

[0491] This example describes methods of producing stereoisomerically-enriched R,R monatin from L-tryptophan using an L-tryptophan (L-tyrosine, or aromatic) aminotransferase, ProA aldolase, alanine, glutamate or aspartate racemase, and a broad specificity D-amino acid aminotransferase. FIG. 5 is a diagram that illustrates the pathway. This approach to production of stereoisomerically enriched R,R monatin requires an enzyme for step 1 that has low activity in the production of monatin from monatin precursor (MP). Based upon earlier results, we used the *Sinorhizobium meliloti* and *Rhodobacter sphaeroides tatA* gene products described in Example 1 from WO 03/091396 A2.

Materials and Methods

[0492] Glutamate racemases from *L. brevis* and *P. pentosaceus* were produced in *E. coli* as described in Example 8. In some cases, the His₆-tagged version of these enzymes were purified using His-Bind 900 cartridges according to manufacturer's protocols (Novagen, Madison, WI) and were desalted to remove imidazole using PD-10 columns (G25 Sephadex, Amersham-Pharmacia). The enzymes were eluted in 25 mM potassium phosphate pH 8.0. Aspartate racemase (ASPR-101) and D-aminotransferase (AT-103)

were purchased from BioCatalytics, Inc., alanine racemase was purchased from Sigma (St. Louis, MO) (catalog number A8936). *S. meliloti* and *R. sphaeroides* tyrosine (aromatic) aminotransferases were prepared as described in Example 1 from WO 03/091396 A2. *Comamonas testosteroni* ProA aldolase was prepared as described in Example 4 from WO 03/091396 A2. Total protein assays were done utilizing the Bio-Rad Protein Assay according to manufacturer's protocols (Hercules, CA).

Reduction in Amount of S,S Monatin Produced using Racemases

[0493] Reaction mixtures (1 mL volume, run in duplicate) contained 100 mM potassium phosphate buffer (pH 8), 2 mM MgCl₂, 0.05 mM pyridoxal 5'-phosphate ("PLP"), 200 mM sodium pyruvate, 5 mM sodium α -ketoglutarate or oxaloacetate, approximately 280 μ g/mL *S. meliloti* TatA supplied in a cellular extract, 1 mg/mL BioCatalytics D-aminotransferase (AT-103), 100 μ L/mL of glutamate racemase cellular extract or 1 mg/mL aspartate racemase, and approximately 100 μ g/mL of ProA aldolase provided as a cellular extract. Solid tryptophan was added at a concentration of 10.2 mg/mL. Negative controls did not contain racemase. Samples were incubated at 30 °C (shaking at 250 rpm) for 1 hour, 2 hours, or overnight. Samples were centrifuged to remove precipitate, syringe filtered, and stored at -80 °C prior to analysis for monatin using the LC/MS/MS method described in Example 1.

[0494] Most of the samples contained >95% S,S monatin, due to the amounts of native L-aminotransferase present in the cellular extracts. However, the samples that contained racemase had a reduced amount of total monatin as a result of the racemase enzymes making L-glutamate less available for transamination of MP. Without racemase, 1545-2355 ppm monatin (predominantly S,S) was produced during the timecourse. With the racemases present, only 340-879 ppm (*L. brevis* enzyme), 444-531 ppm (*P. pentosaceus* enzyme), and 506-1460 ppm monatin (aspartate racemase) were produced. These data indicate that the racemases are active in the reaction conditions required to produce monatin. To minimize formation of S,S monatin from cellular extract enzymes, such as aspartate aminotransferases, further experiments were done with purified enzymes and a higher ratio of D-aminotransferase to L-aminotransferase enzymes.

Conversion of L-tryptophan to 4-R Containing Isomers of Monatin

[0495] The above experiments were repeated using approximately 54 µg of purified L-aminotransferase (either *S. meliloti* or *R. sphaeroides* TatA), 1 mg aspartate aminotransferase (BioCatalytics), 1 mg D-aminotransferase, 5 mM oxaloacetate as the amino acceptor, and 75 µg purified aldolase. The reactions were run in duplicate with a 2 hour sampling time and an overnight incubation time. Negative controls were done with *S. meliloti* L-aminotransferase, but with no racemase. In addition to quantification of R,R/S,S and S,R/R,S monatin peak quantification based on reversed phase chromatography, the percentage of each stereoisomer was determined using the FDAA derivitization technique described in Example 1. The results are shown in Table 53 below.

Table 53

L-Aminotransferase	Incubation Time	Total Monatin (ppm)	% S,S	% R,R	% R,S	% S,R
<i>S. meliloti</i> TatA	2 h	17.1	10.2	58.1	0.8	31.0
<i>S. meliloti</i> TatA	2 h	15.8	13.3	55.3	1.0	30.4
<i>S. meliloti</i> TatA	overnight	77.7	25.8	40.0	1.3	32.9
<i>S. meliloti</i> TatA	overnight	67.9	29.4	37.3	1.5	31.8
<i>R. sphaeroides</i> TatA	2 h	241.2	96.3	2.3	0.8	0.6
<i>R. sphaeroides</i> TatA	2 h	223.2	95.7	2.7	1.0	0.6
<i>R. sphaeroides</i> TatA	overnight	600.4	96.6	1.8	0.5	1.1
<i>R. sphaeroides</i> TatA	overnight	618.5	96.1	2.1	0.5	1.3
no racemase control	2 h	7.1	92.0	1.4	6.6	0.0
no racemase control	2 h	5.7	94.0	1.2	4.8	0.0
no racemase control	overnight	44.6	93.5	1.3	4.7	0.5
no racemase control	overnight	37.5	95.4	0.9	3.7	0.0

[0496] Clearly, the presence of the racemase increased the total amount of monatin produced when *S. meliloti* TatA was used as the enzyme for L-tryptophan transamination. Monatin levels increased from an average of 6.4 to 16.5 ppm in the two hour assay and from 41-73 ppm in the overnight assay. Additionally, the percent of R,R formed increased from about 1% up to as much as 58% by utilizing the racemase enzyme. The S,R stereoisomer of monatin, another potent sweetener, was the other major component, increasing from nearly 0 in the negative controls to 31%. The *R. sphaeroides* TatA

clearly had more activity on S-MP than the *S. meliloti* L-transaminase, demonstrating the importance of having an enzyme that has a high substrate specificity for L-tryptophan as compared to MP when 4-R isomers of monatin are the desired products. With about 10% of the total monatin being 4S at the two-hour time point, the *S. meliloti* TatA could be considered as having limited activity on MP.

[0497] The experiments were repeated with the purified *S. meliloti* TatA (54 µg) and the *L. brevis* glutamate racemase. When purified glutamate racemase was used, approximately 64 µg was used per 1 mL reaction. Cellular extracts containing the glutamate racemase were also tested and 1.4 mg of soluble protein was used. A no racemase negative control was utilized again and all samples were run in duplicate. The results are shown in Table 54 below.

Table 54

Glutamate racemase	Incubation Time	Total Monatin (ppm)	% S,S	% R,R	% R,S	% S,R
<i>L. brevis</i> (purified)	2 h	3.3	49.1	34.2	3.7	13.0
<i>L. brevis</i> (purified)	2 h	3.6	47.9	35.2	3.5	13.4
<i>L. brevis</i> (purified)	overnight	29.3	58.9	24.7	3.2	13.2
<i>L. brevis</i> (purified)	overnight	40.2	55.1	25.0	4.7	15.3
<i>L. brevis</i> (cell extract)	2 h	10.5	45.8	35.9	1.1	17.2
<i>L. brevis</i> (cell extract)	2 h	10.5	47.4	33.9	1.1	17.6
<i>L. brevis</i> (cell extract)	overnight	79.4	70.3	17.9	1.3	10.5
<i>L. brevis</i> (cell extract)	overnight	80.1	69.1	19.1	1.1	10.7
none	2 h	2.7	84.1	7.1	6.3	2.4
none	2 h	3.2	84.9	6.0	6.8	2.2
none	overnight	36.5	92.3	2.3	4.2	1.2
none	overnight	30.5	92.7	2.0	4.1	1.3

[0498] Again, it is clear that the addition of the racemase increases the total monatin produced from L-tryptophan, as well as increases the relative amounts of 4R-containing isomers of monatin as compared to S,S monatin. The use of purified aldolase, racemase, and L-aminotransferase greatly improves the ability to control the desired stereoisomer formation. The ratio of L to D aminotransferase is also a way to manipulate stereochemistry of the final product.

[0499] When comparing results shown in Tables 1 and 2 in Example 2, to results with reaction conditions similar to the conditions above, one can see that approximately 7-29 ppm of monatin were formed from indole-3-pyruvate and the percentages of R,R monatin formed were approximately 51-90%. Using the aspartate racemase increased the total amount of monatin produced to 16-78 ppm monatin, with % R,R of approximately 40-58%. Additionally, a more stable and less expensive raw material (L-tryptophan) was utilized. In Example 3A, approximately 73 ppm monatin was produced from D-tryptophan at a ratio of R,R:S,R of approximately 1.7:1. The total amount of 4R isomers was >80% of the total monatin. Because both R,R-monatin and R,S-monatin are potent sweeteners (>1000 times sweeter than sucrose), the ability to enrich for these isomers, without the need for expensive D-amino acid substrates, is critical.

[0500] It is expected that the availability of a non-specific or R-specific aldolase would increase the reaction rate as well as increasing the percentage of R,R monatin formed. See Example 5. Although the ProA aldolase from *C. testosteroni* used in these assays is reported to predominantly favor substrates in the S-configuration for fission reactions, this Pro A aldolase clearly does produce R-MP. Thus, aldolases that more preferentially produce MP in the R-configuration can help generate even greater percentages of R,R monatin. Additionally, it is expected that finding an L-tryptophan aminotransferase with even lower activity for monatin production would also decrease the amount of S,S and R,S monatin formed. Lastly, improvements can be made to the D-aminotransferase enzyme, or alternative D-aminotransferase enzymes can be used, that would have increased substrate specificity for R-MP versus S-MP. This would also increase formation of the R,R product, if so desired.

[0501] The aspartate racemase experiments were repeated to compare the activity of R-selective aldolase of SEQ ID NO:22 with the activity of the ProA aldolase from *C. testosteroni*. Approximately 50 µg of purified L-aminotransferase (*S. meliloti* TatA), 1 mg aspartate racemase (BioCatalytics), 1 mg D-aminotransferase (AT-103, BioCatalytics), 5 mM oxaloacetate as the amino acceptor, and 50 µg of the appropriate purified aldolase. The reactions were run in duplicate and incubated overnight at 30 °C. The percentage of each stereoisomer was determined using the FDAA derivitization technique described in Example 1. The results are shown below in Table 55.

Table 55

Aldolase	Total Monatin (ppm)	% S,S	% R,R	% R,S	% S,R
SEQ ID NO:22	211		72.7		27.3
<i>C. testosteroni</i>	422	30.2	38.5		31.3

[0502] The *C. testosteroni* ProA distribution of isomers is consistent with the earlier experiments above, whereas when the R-selective aldolase of SEQ ID NO:22 is used, the percent R,R is much higher, undetectable amounts of S,S are formed, and the amount of S,R monatin is lower.

[0503] As described in Examples 2 and 3A, D-alanine can serve as the amino donor for transamination of MP to monatin. Many L-aminotransferases have the ability to utilize pyruvate as an amino acceptor to some extent and produce L-alanine. Because the above-mentioned reactions use high concentrations of pyruvate, it is likely that some of the pyruvate is converted to L-alanine. For example, during transamination of L-tryptophan, the HexAspC enzyme described in Example 6A has been found to convert 10-18% of pyruvate (50-200 mM initial concentrations) to L-alanine in 2 hours if alpha-ketoglutarate is absent. The enzyme showed a 10-fold preference for alpha-ketoglutarate when both amino acceptors were present at high (>50 mM) concentrations. AspC (described in WO 03/091396 A2) also produced some L-alanine from pyruvate. Therefore, it was expected that one can omit the addition of alpha-ketoglutarate or oxaloacetate in the above reactions and utilize an alanine racemase (EC 5.1.1.1) in place of glutamate or aspartate racemase.

[0504] Alanine racemase enzymes were first identified in *Brucella abortus* and *Streptococcus faecalis*. Marr, A.G., and Wilson, P.W., *Arch. Biochem. Biophys.*, 49:424-433, (1954); Wood, W.A., and Gunsalus, I.C., *J. Biol. Chem.* 190:403-416, (1951). The *dadB* gene in *Salmonella typhimurium* was identified as the source of alanine racemase activity and several hundred homologs can be found in genomics databases. Other known sources of alanine racemase activity are *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Schizosaccharomyces pombe*, and *Bacillus cereus*. A basidiomycetous mushroom, *Lentinus edodes*, also contains a broad activity alanine racemase. A thermostable homolog from *Bacillus stearothermophilus* is available for

purchase from Sigma-Aldrich (catalog # A8936) and has been immobilized for commercial applications. Inagaki, K., *Biochemistry* 25: 3268 (1986).

Monatin Production with Alanine Racemase

[0505] Monatin production was tested using ProA aldolase from *C. testosteroni*. Approximately 50 µg of purified L-aminotransferase (*S. meliloti* TatA), 1 mg D-aminotransferase (AT-103, BioCatalytics), pyruvate as the amino acceptor, 50 µg purified aldolase, and 70 µg alanine racemase purchased from Sigma (St. Louis, MO) (catalog number A8936). The reactions were run in duplicate and incubated overnight. The percentage of each stereoisomer was determined using the FDAA derivitization technique described in Example 1. Controls with no racemase were included. The results are shown in Table 56 below.

Table 56

Conditions	Total Monatin	% SS	% RS	% RR	% SR
Ala racemase (1 hour)	4	66	21	12	1
No ala racemase (1 hour)	2.7	69	26	5	0
Ala racemase (24 hours)	82.9	90	5	4	2
No ala racemase (24 hours)	170.3	89	5	4	2

[0506] There was three-fold more R,R monatin in the one hour time point when alanine racemase was present compared to the sample with no alanine racemase. This result shows that it is possible to produce R,R monatin using alanine racemase. The percentage of R,R monatin produced could be improved using an aldolase that selectively produces R-monatin precursor, an L-aminotransferase that does not work or has limited activity on R-monatin precursor and a D-aminotransferase that does not work or has limited activity on indole-3-pyruvate.

EXAMPLE 10

D-phenylglycine Aminotransferase (D-4-Hydroxyphenylglycine Aminotransferase)

[0507] As shown in FIG. 3, a stereoinverting aminotransferase is useful in a biosynthetic pathway for the production of monatin. For example, a D-phenylglycine

aminotransferase or mutant thereof could produce R,R monatin from R-MP with L-glutamate as the amino donor.

(1) *PCR Synthesis of P. stutzeri 4 D-hydroxyphenylglycine Aminotransferase from Oligonucleotide Primers*

[0508] This example describes methods that were used to synthesize 4 D-hydroxyphenylglycine aminotransferase, a stereoinverting enzyme that can be used to convert R monatin precursor to R,R monatin using L-glutamate as the amino donor.

Primer Design

[0509] The published sequence (Genbank Accession No. AY319935, nucleic acid sequence; Genbank Accession No. AAQ8290, protein sequence) for *Pseudomonas stutzeri* 4 D-hydroxyphenylglycine aminotransferase (4 D-HPG AT) was used as a template for PCR primer design. Alternatively, the 4-D-hydroxyphenylglycine aminotransferase from *Pseudomonas putida*, (CAD42450 (protein), AX467211 (nucleotide)) is used as a sequence template. A total of 34 forward primers and 35 reverse primers were designed; forward and reverse primers were 40-mers sharing 20 overlapping base pairs. In addition, 2 outer primers were designed with 5' restriction sites and overhangs for cloning into the pET 28 and pET30 vectors (Novagen, Madison, WI).

[0510] *P. stutzeri* 4 D-HPG AT outer primers: N term (with *Nde*I Site):
5'-GGCCGGCATATGTCGATCCTTAACGACTACAAACGT-3' (SEQ ID NO:19), and
C term (with *Xho*I site):
5'-GGAAGGCTCGAGTCATGATTGGTTTCCAGACAAATT-3' (SEQ ID NO:20).

Polymerase Chain Reaction Protocol

[0511] The gene sequence from *P. stutzeri* was amplified using the following protocols. The primary 100 µL PCR reaction included 0.05 µM of each of the internal 69 primers, 0.4 mM each dNTP, 10 U *rTth* Polymerase XL (Roche, Indianapolis, IN), 0.625 U *Pfu* polymerase (Stratagene, La Jolla, CA), 1X XL buffer and 1 mM Mg(OAc)₂. The thermocycler program used included a hot start at 94 °C for 3 minutes, 15 repetitions of the following steps: 94 °C for 30 seconds, 42 °C for 30 seconds, and 68 °C for 15 seconds, followed by 10 repetitions of the following steps: 94 °C for 30 seconds, 52 °C for 30 seconds, and 68 °C for 30 seconds, followed by 10 repetitions of the following

steps: 94 °C for 30 seconds, 60 °C for 30 seconds, and 68 °C for 1 minute and 15 seconds. After the final 10 cycles, the sample was maintained at 68 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a smear of product at ~0.5 kb on a 0.8% TAE-agarose gel.

[0512] The secondary PCR reaction was set up using the primary PCR reaction as template. The secondary 100 µL PCR reaction included 2.5 µL of the primary PCR reaction, 0.5 µM of each of the 2 outer primers (with *Nde* I and *Xho* I restriction sites), 0.4 mM each dNTP, 10 U *rTth* Polymerase XL, 0.625 U Pfu polymerase, 1X XL buffer and 1 mM Mg(OAc)₂. The thermocycler program used included a hot start at 94 °C for 3 minutes, 10 repetitions of the following steps: 94 °C for 30 seconds, 52 °C for 30 seconds, and 68 °C for 1 minute 30 seconds, followed by 15 repetitions of the following steps: 94 °C for 30 seconds, 60 °C for 30 seconds, and 68 °C for 1 minute 30 seconds. After the 15 repetitions, the sample was maintained at 68 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a distinctive product band at ~1.4 kb on a 0.8% TAE- agarose gel.

[0513] The PCR product was gel purified from 0.8% TAE-agarose gel using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The product was TOPO cloned and transformed into TOP 10 cells according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Nde* I and *Xho* I. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing with universal M13 forward and M13 Reverse primers. Of the 10 clones sequenced, all had at least one mutation from the desired sequence. The best clone had a single base-pair mutation that resulted in an amino acid change. The sequence of this clone was corrected using the QuickChange Mutagenesis protocol according to manufacturer recommendations (Stratagene, La Jolla, CA).

[0514] The corrected TOPO clone was digested with restriction enzymes *Nde* I and *Xho* I following the manufacturer's recommended protocols (New England Biolabs, Beverly, MA) and gel purified from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA). Vectors pET 28 and pET 30 were prepared by digestion with restriction enzymes *Nde* I and *Xho* I, followed by treatment with shrimp alkaline

phosphatase and purification from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Qiagen, Valencia, CA).

- [0515] The digested vectors and insert were ligated using the NEB Quick Ligation Kit (Beverly, MA). Approximately 50 ng of treated insert, 100 ng of treated vector (3 to 1 molar ratio of insert to vector), 5 U of T4 DNA ligase, and 1X ligation buffer were incubated for 5 minutes at room temperature. The ligation mixture was transformed into TOP10F' chemically competent cells (Invitrogen). The cells were allowed to recover in 0.25 mL of room temperature SOC for 1 hour at 37 °C with shaking at 225 rpm. The cells were plated on LB plates containing kanamycin (50 µg/mL). The plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *NdeI* and *XhoI*.

Gene Expression and Assays

- [0516] Plasmid DNA was transformed into *E. coli* expression host BL21(DE3) (Novagen, Madison, WI). The cultures were grown and the plasmids were isolated using Qiagen miniprep kit (Qiagen, Valencia, CA) and analyzed by restriction digest to confirm identity.
- [0517] Induction in BL21(DE3) was performed with *P. stutzeri* D-4-hydroxyphenylglycine aminotransferase in both pET 28 (histidine-tagged) and pET 30 (untagged) vectors. A time course study was performed with cultures grown in 250 mL LB containing kanamycin (50 mg/L) to an OD₆₀₀ of 0.5 – 0.6, induced with 100 mM isopropyl thiogalactoside ("IPTG") and sampled at 0 and 3 hours post induction. An appropriate volume of cells from 0 hours and 3 hours was resuspended in 40 µL sodium dodecyl sulfate buffer containing 2-mercaptoethanol, heated at 95 °C for 10 minutes, and cooled. Aliquots of these total cellular protein samples were analyzed by SDS-PAGE using a 4-15% gradient gel.
- [0518] The cell extracts were also prepared from the 3 hour cultures by suspending cell pellets from 5 mL of culture in 0.625 mL Novagen BugBuster™ reagent containing 0.625 µL benzonase nuclease and 3 µL protease inhibitor cocktail set #3 (Calbiochem-Novabiochem Corp., San Diego, CA) at room temperature for 20 minutes with gentle shaking and centrifuging at 16,000 x g to remove cell debris. The supernatants (cell extracts) were loaded onto 4-15% gradient gels for analysis of the cellular soluble

proteins. When noted, the protein was purified using His-Bind 900 cartridges according to manufacturer's protocols (Novagen, Madison, WI) and were desalted to remove imidazole using PD-10 columns (G25 Sephadex, Amersham-Pharmacia).

(2) Isolation of Organisms with D-Phenylglycine Aminotransferase ("DPGAT")

[0519] Organisms of the genus *Pseudomonas* and like genera, with a stereoinverting D-phenylglycine aminotransferase (also called D-4-hydroxyphenylglycine aminotransferase) are isolated in the following manner. Soil samples are incubated on petri plates with the following medium: (per liter) 15 g agar, 3.4 g KH_2PO_4 , 3.55 g Na_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 8 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg yeast extract, 1 ml 1000x trace elements solution (Balch, W.E., *et al.*, "Methanogens: reevaluation of a unique biological group," *Microbiol. Rev.* 43:260-296, (1979)), and 1 g D-phenylglycine (D-4-hydroxyphenylglycine).

[0520] Isolates are tested by PCR for the presence of a stereoinverting aminotransferase (primers are designed from known D-phenylglycine aminotransferases) or are further enriched for the presence of a stereoinverting aminotransferase as follows: isolates from the plates could be grown in liquid medium as above, without the agar, at 30 °C with shaking to an OD_{600} of about 1.0. The cells are harvested by centrifugation and washed twice with 0.85% NaCl. A 10 mg (wet weight) sample is suspended in 1 ml potassium phosphate buffer (pH 7.0) and 5 mM D-phenylglycine or D-4-hydroxyphenylglycine. Neutralized 15 mM (aminooxy)acetic acid is added to duplicate samples prepared as described above. Consumption of D-phenylglycine (or D-4-hydroxyglycine) is measured by HPLC.

[0521] Isolates capable of degrading D-phenylglycine (or D-4-hydroxyphenylglycine), but do so at a slower rate in the presence of (aminooxy)acetic acid, are selected for further analysis. Isolates are tested, by PCR, for the presence of a stereoinverting aminotransferase (primers are designed from known D-phenylglycine aminotransferases).

[0522] The presence of the stereoinverting aminotransferase is confirmed by growing a culture in a liquid medium as described above, harvesting the cells and making a cell-free crude extract ("CFE") and testing for D-phenylglycine aminotransferase or D-4-hydroxyphenylglycine aminotransferase enzyme activity. CFE is added to a reaction mixture with the following final concentrations: 0.1 M 3-(cyclohexylamino)-1-

propanesulfonic acid ("CAPS") (pH 9.5), 60 mM L-glutamate (sodium salt), 5 mM benzoylformate or 4-hydroxybenzoate and 50 μ M PLP.

[0523] The reverse reaction is measured by adding CFE to a reaction mixture with the following concentrations: 50 mM potassium phosphate (pH 7.0), 60 mM D-phenylglycine or D-4-hydroxyphenylglycine, 5 mM α -ketoglutarate, and 50 μ M PLP. The assays are incubated at 35 °C and aliquots are taken at time points and stopped by boiling for 2 minutes. The product will be quantitated by the HPLC method of Gil-Av, E., *et al.*, "Resolution of underivatized amino acids by reversed phase chromatography," *J. Am. Chem. Soc.*, 102:5115-5117, (1980), or by the methods described in Example 1 directed to the measurement of glutamate formation.

[0524] As an alternative to PCR based methods, the stereoinverting D-phenylglycine aminotransferase is purified from the isolated bacteria by conventional protein purification techniques, including ammonium sulfate fractionation, and conventional column chromatography. Once the protein has been purified to a reasonable degree, peptide microsequencing techniques or conventional Edman type amino acid sequencing are utilized (*see* <http://golgi.harvard.edu/microchem/> for descriptions of the protocols and equipment used for this type of work). Degenerate primers are designed based on the sequence available from the closest known relative of the protein source. Degenerate PCR and genome walking is then performed according to established protocols to isolate the stereoinverting D-phenylglycine aminotransferase coding sequence.

(3) **DPGAT Monatin Production**

[0525] D-hydroxyphenylglycine aminotransferases, as described in (1) and (2) above, are used in crude cell free protein extracts, or purified as described in (1) above. *S. meliloti* and *R. sphaeroides* tyrosine (aromatic) aminotransferases are prepared as described in Example 1 from WO 03/091396 A2. *Comamonas testosteroni* ProA aldolase is prepared as described in Example 4 from WO 03/091396 A2. Total protein assays are done utilizing the Bio-Rad Protein Assay according to manufacturer's protocols (Hercules, CA).

[0526] Reaction mixtures (1 mL volume, run in duplicate) contain 100 mM potassium phosphate buffer (pH 8), 2 mM MgCl₂, 0.05 mM pyridoxal 5'-phosphate ("PLP"), 200 mM sodium pyruvate, 5 mM sodium α -ketoglutarate, approximately 280 μ g/mL *S. meliloti* TatA supplied in a cellular extract, 100 μ L/mL of D-hydroxyphenylglycine

aminotransferase cellular extract or 1 mg/mL purified D-hydroxyphenylglycine aminotransferase, and approximately 100 µg/mL of ProA aldolase provided as a cellular extract. Solid tryptophan is added at a concentration of 10.2 mg/mL. Negative controls are set up without D-hydroxyphenylglycine aminotransferase. The samples are incubated at 30 °C with gentle shaking for ~1 hour or overnight. The samples are centrifuged to remove precipitate, syringe filtered, and stored at –80 °C prior to analysis for monatin using the LC/MS/MS method described in Example 1.

[0527] D-hydroxyphenylglycine aminotransferases with improved activity for monatin production are made using mutagenesis techniques known to those in the art, including: mutagenic PCR, passage through mutagenic strains, site-directed mutagenesis, error-prone PCR, or by methods such as DNA shuffling or other directed evolution technologies. The improved D-hydroxyphenylglycine aminotransferases are selected by growth on minimal medium with R,R-monatin as the source of nitrogen. Initially, the selection is based on growth, but as improved aminotransferases are selected, the screen is growth rate based. That is, cells with mutated versions of the gene are grown and the gene is expressed in minimal medium with R,R-monatin as the nitrogen source. The growth rates of the cells with the mutated versions of the gene are compared to the unmutated version. Those cells with a faster growth rate are selected and the aminotransferase is analyzed further. The D-hydroxyphenylglycine aminotransferase may be further mutagenized until the desired activity is obtained.

(4) *DPGAT Assay*

[0528] The un-His-tagged version of the DPGAT was expressed as described in (1) above and extracts were used in assays. Assays were set up and included 100 mM potassium phosphate pH 7.0, 60 mM D-phenylglycine, 5 mM α -ketoglutarate, and 50 µM pyridoxal-5'-phosphate. The assays were started by adding 100 µL of extract, prepared as described above in this example, per mL of assay volume. Samples were taken at several time points (0, 1, 2, 5, 10, 30, 60, and 120 minutes) and were stopped with an equal volume of 2 M formic acid. A sample was also taken after overnight incubation (~1200 minutes). The samples were analyzed for glutamate production by the LC/post-column fluorescence detection (OPA) method described in Example 1. The results are summarized in Table 57 below.

Table 57

Condition	Time (Minutes)	μmole/mL L-Glutamate
No substrate	0	0.033
	1	0.033
	2	0.033
	5	0.035
	10	0.034
	30	0.036
	60	0.044
	120	0.038
	~1200	0.058
D-phenylglycine	0	0.055
	1	0.112
	2	0.169
	5	0.315
	10	0.387
	30	0.892
	60	1.304
	120	1.514
	~1200	1.056

[0529] The enzyme clearly has some activity on the D-phenylglycine. The enzyme activity was also tested on R,R monatin. The assay was set up as described above and R,R monatin was included at a concentration of 60 mM. The results are indicated below in Table 58.

Table 58

Condition	Time (Minutes)	μmole/mL L-Glutamate
R,R monatin	0	0.041
	1	0.040
	2	0.041
	5	0.041
	10	0.041
	30	0.042
	60	0.041
	120	0.040
	~1200	0.045

[0530] There did not appear to be any detectable activity on R,R monatin. However, it is expected that the random or SDM methods described in this part (3) of this Example could be utilized to improve the transamination activity on R,R monatin or R-MP. For instance, the crystallization and preliminary analysis of the *P. stutzeri* enzyme has been done. Kongsaree, P., *et al.*, *Acta Cryst. D59*:953-954, (2003). Once the structure is

published, docking experiments can be done using software such as Accelrys, to determine where steric hindrances or ionic repulsion may be prohibiting the R,R monatin from binding to the D-hydroxyphenylglycine substrate binding site. D-hydroxyphenylglycine is a somewhat large amino acid, as is R,R monatin. Both compounds have hydrophobic regions and hydroxyl groups. Modifications can be done to the binding pocket, as described in Example 6A, to make the enzyme more amenable to dicarboxylic acid substrates. For instance, a residue near the second carboxyl group may be modified to a base such as arginine. Additionally, The *P. putida* gene described in part (1) and the additional genes that may be isolated as described in (2) can be used as templates for gene shuffling. Additionally, the *P. stutzeri* gene assembled in this Example can be mutagenized using oligonucleotide shuffling or other random mutagenesis methods, and screened as described in (3) above.

EXAMPLE 11

Discovery of a D-Methionine Aminotransferase Gene

Background

- [0531] D-methionine—pyruvate aminotransferase (EC 2.6.1.41) is thought to be another example, although rare, of a stereoinverting transaminase. This enzyme catalyzes the reversible conversion of D-methionine and pyruvate to L-alanine and 4-methylthio-2-oxobutanoate. Oxaloacetate, phenylpyruvate, 2-oxobutyrate, 2-oxovalerate, 2-oxoheptanoate, glyoxylate, and oxoglutarate can also serve as amino acceptors.
- [0532] Transamination of D or L methionine is thought to be part of a pathway to ethylene production in higher plants (cauliflower, tomato, apple, pea stem, banana, peanut), as well as in soil microorganisms (*Escherichia coli*, *Pseudomonas pisi*, *Pseudomonas aeruginosa*, *Bacillus mycoides*, *Acinetobacter calcoaceticus*, *Aeromonas hydrophila* B12E, *Rhizobium trifolii* N2P7, *Penicillium digitatum*, *Saccharomyces cerevisiae*, *Corynebacterium* D7F). Billington, D.C., *et al.*, *Biochem J.* 182:827-836, (1978). In bacteria, L-methionine is typically used as the substrate in the ethylene production studies and broad specificity enzymes such as TyrB or AspC from *E. coli* are thought to be responsible for the transamination. However, Primrose, S.B., *J. Gen.*

Microbiol. 95:159-65, (1976) and Primrose, S.B., *J. Gen. Microbiol.* 98:519-528, (1977) showed that *E. coli* strain SPA O (University of Warwick culture collection) produced nearly as much ethylene from D-methionine as from L-methionine in batch cultures. Because no broad specificity D-aminotransferase has been identified in *E. coli*, one possible explanation could be that the *E. coli* D-amino acid dehydrogenase (encoded by the *dadA* gene) converts the D-methionine to 4-methylthio-2-oxobutanoate. It is also possible that there is a methionine racemase in *E. coli*; however, no such enzyme has been described in the literature.

- [0533] In contrast to *E. coli*, in cauliflower florets (mitochondrial extract preparations) and germinating peanut seeds production of ethylene was higher when D-methionine and pyruvate were supplied to the enzyme extract as compared to L-methionine and pyruvate. Mapson, L.W., *et al.*, *Biochem J.* 115:653-661, (1969); Durham, J.I., *et al.*, *Phytochemistry* 12:2123-2126, (1973). Therefore, the possibility of a combination of methionine racemase and an L-aminotransferase is not supported by the data. Dehydrogenase activity was ruled out by dialysis of cellular extracts of cauliflower; no NAD was present in the assay mixtures. Oxidase activity was ruled out as no consumption of oxygen was noted and there was no requirement for FAD. The D-methionine aminotransferase from peanut tissues was purified, shown to be dependent on PLP, and shown to be independent of L-methionine aminotransferase activity. There is a possibility that these D-methionine—pyruvate aminotransferases actually produce D-alanine as a byproduct (similar to the *Bacillus* enzymes described in Examples 2 and 3A) and that the cells contain alanine racemase to recycle the D-alanine back to L-alanine (or an analogous amino donor). In either case, discovery of the broad specificity D-aminotransferase from higher plants is advantageous for development of processes that produce R,R monatin or S,R monatin.

Experimental Overview

- [0534] D-methionine aminotransferase is partially purified from cauliflower florets and germinating peanut embryos using standard chromatography protocols and a Pharmacia AKTA Explorer system. The protein sequences of homologous proteins are determined by LC/MS/MS fingerprinting techniques and database searching performed by Harvard Microchemistry facility. The coding regions of the plant genes are cloned from a cDNA

library using standard PCR protocols or by synthesis of the gene as described in Example 10(1).

[0535] Alternatively, cDNA expression libraries are constructed (Stratagene, La Jolla, CA) from cauliflower tissue or peanut seeds grown in the presence of D-methionine (and producing ethylene). The libraries are transformed into *E. coli* methionine auxotrophs from the *E. coli* Genetic Stock Center (Yale) such as strains RC519 or AB1931. Plasmids of strains capable of growth on minimal media containing D-methionine contain the coding region of interest (see Example 4A(1), an analogous screening technique).

[0536] Once the coding regions of interest are obtained and are expressed in a standard *E. coli* laboratory strain, the resulting gene products can be used in assays to produce R,R monatin, as described in Example 10(3), in place of the D-hydroxyphenylglycine aminotransferase, with the exception of the pH being 7.5 (the optimal pH for the aminotransferase). If the D-methionine aminotransferase has a strict requirement for D-amino acid donor substrates, the enzyme can be used to make R,R monatin as described in Example 2 and 3. The gene can be mutagenized and screened for increased activity as described in Example 10(3).

Methods

Isolation from Cauliflower

[0537] Four hundred grams of freshly picked cauliflower florets are extracted with 400 mL of a 4 °C sucrose/buffer solution (0.4 M sucrose and 0.1 M sodium phosphate buffer pH 7.4) by alternating soaking and mixing using a blender. Cell debris is removed by filtration with cheesecloth and the resulting solution is centrifuged at 40,000 x g for 30 minutes at 4 °C. The solid material (containing mitochondrial organelles) is resuspended in 20 mL 10 mM sodium phosphate buffer pH 7.4 and enzymes are extracted with 200 mL cold (-30 °C) acetone. The suspension is re-centrifuged and the precipitate is dried using a Savant Speed Vac. The solid material is dissolved in 10 mM sodium phosphate buffer pH 7.4 and residual acetone is removed using a PD-10 column.

[0538] Aminotransferase activity is assayed by incubation of the enzyme preparation with 5 mM D-methionine, 1 mM pyruvate, 0.05 mM PLP and 2 mM EDTA in 0.1 M sodium phosphate buffer pH 7.4. Assays are performed at 25 °C for 16 hours. The 4-methylthio-2-oxobutanoate is measured by formation of the 2,4-dinitrophenylhydrazone derivative,

using LC/MS (m/z of 328) and similar methodology described in Example 1. A 0.4% (w/v) solution of 2,4-dinitrophenylhydrazine in 2M sulfuric acid is prepared and a half volume is added to the assay mixture after incubation. The mixture is mixed with gentle shaking at 30 °C for 30 minutes and the precipitate is collected by centrifugation and analyzed by LC/MS. Protein fractions separated by standard chromatographic techniques are assayed for activity in a similar manner, but the co-product alanine is measured by the OPA post-column derivatization technique described in Example 1.

Isolation from Peanut (Arachia hypogea L. cv. Starr)

- [0539] The D-methionine aminotransferase enzyme from germinating peanut embryo homogenate (minus the cotyledons) is purified according to the method of Durham, J.I., *et al.*, *Phytochemistry* 12:2123-2126, (1973). Reducing agents are used during the preparation of crude extracts to stabilize the enzymes and the cell debris is removed by centrifugation at 33,000 x g. A 35-50% ammonium sulfate fraction is further purified by incubation at low temperature and by removal of the proteins in the precipitate. The supernatant is further fractionated using acetone. The active pools are then further purified by gel filtration chromatography (Sephadex 200 G.E. Healthcare, Piscataway, NJ).
- [0540] As protein fractions become enriched with the transaminase protein, 2D-gel electrophoresis is utilized to separate the enzyme of interest for microsequencing. After elucidation of homologous coding regions in plant sequences deposited at NCBI, the D-aminotransferase protein is produced recombinantly in *Escherichia coli* using standard molecular biology techniques. It is expected that the cellular extracts from cauliflower florets or peanut seeds or recombinantly produced homologous enzymes can be used in production of R,R monatin as described in Example 10(3) (if a stereoinverting transaminase) or Examples 2 and 3A (if a broad specificity D-aminotransferase).

EXAMPLE 12

L-Alanine Aminotransferase/Alanine Racemase/D-Alanine Aminotransferase

- [0541] Figure 8 illustrates the biosynthetic pathway for producing stereoisomerically-enriched R,R monatin from L-tryptophan using L-amino acid aminotransferases (such as

L-aromatic aminotransferases, L-alanine-aminotransferases and/or L-tryptophan-aminotransferases), an R-specific aldolase, an alanine racemase and a D-alanine aminotransferase.

[0542] A tryptophan-specific aminotransferase is described in Example 6A. Alternatively, *S. meliloti* and *R. sphaeroides* tyrosine (aromatic) aminotransferases are prepared as described in Example 1 from WO 03/091396 A2. *Comamonas testosteroni* ProA aldolase is prepared as described in Example 4 from WO 03/091396 A2. Total protein assays are done utilizing the Bio-Rad Protein Assay according to manufacturer's protocols (Hercules, CA). Alanine racemase is purchased from Sigma (St. Louis, MO) (catalog number A8936). D-alanine aminotransferase is purchased from BioCatalytics (Pasadena, CA) (catalog number AT-103).

[0543] L-alanine aminotransferases are widely distributed in eukaryotes, bacteria, and archaea. The following organisms have been identified (based on sequence homology) as containing an L-alanine aminotransferase (EC 2.6.1.2): *Arabidopsis thaliana*, *Ashbya gossypii*, *Azotobacter vinelandii*, *Bifidobacterium longum*, *Caenorhabditis elegans*, *Candida albicans*, *Candida glabrata*, *Chlamydomonas reinhardtii*, *Cryptococcus neoformans*, *Debaryomyces hansenii*, *Homo sapiens*, *Hordeum vulgare*, *Kluyveromyces lactis*, *Magnaporthe grisea*, *Medicago truncatula*, *Mus musculus*, *Neurospora crassa*, *Oryza sativa*, *Phanerochaete chrysosporium*, *Pinus taeda*, *Pseudomonas putida*, *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Rattus norvegicus*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Takifugu rubripes*, *Trypanosoma cruzi*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Yarrowia lipolytica*, and *Zea mays*. Additionally, many aminotransferases have low-level alanine aminotransferase activity and given high levels of L-glutamate and pyruvate can convert it to L-alanine and α -ketoglutarate. An enzyme with low activity is improved with standard mutagenesis techniques, such as error-prone PCR and passage through mutagenic strains, or by directed evolution techniques. The gene for an L-alanine aminotransferase is cloned using publicly available sequences to design primers and using standard techniques to amplify, clone, express and purify the gene/enzyme.

[0544] The reaction mixtures (1 mL volume, run in duplicate) contain 100 mM potassium phosphate buffer (pH 8), 2 mM $MgCl_2$, 0.05 mM pyridoxal 5'-phosphate ("PLP"), 200 mM sodium pyruvate, 5 mM sodium α -ketoglutarate, approximately 280 μ g/mL *S.*

meliloti TatA supplied in a cellular extract (or other L-tryptophan specific aminotransferase) (as in Example 4A(5), 100 µg of an L-alanine aminotransferase, 100 µL/mL of alanine racemase cellular extract or 1 mg/mL purified alanine racemase (Sigma), approximately 280 µg/mL of a broad specificity D-alanine aminotransferase supplied in a cellular extract (Examples 15 and 18 have examples of D-aminotransferases that could work for this reaction) and approximately 100 µg/mL of ProA aldolase provided as a cellular extract. Solid tryptophan is added at a concentration of 10.2 mg/mL. Negative controls are set up without alanine racemase. The samples are incubated at 30 °C with gentle shaking for ~1 hour or overnight. The samples are centrifuged to remove precipitate, syringe filtered, and stored at -80 °C prior to analysis for monatin using the LC/MS/MS method described in Example 1.

EXAMPLE 13

Purification of R,R-Monatin from an Enzymatic Reaction Mixture

[0545] The product, R,R-monatin, was purified from the following reaction mixture. In 0.33 liter, 50 mM ammonium bicarbonate, pH 8.2, 4 mM MgCl₂, 0.05 mM pyridoxal phosphate ("PLP"), 200 mM sodium pyruvate, and 50 mM D-tryptophan were mixed at room temperature in a 500 mL glass bottle until the tryptophan dissolved. The liquid was flushed with nitrogen for several minutes and then 3.0 mg/mL Biocatalytics, Inc. (Pasadena, CA) Broad range D-transaminase (catalog # AT-103) and 0.1 mg/mL purified aldolase of SEQ ID NO:22 were added. The reaction mixture was stirred gently at room temperature. The aldolase was purified as described in Example 3A. Additional aliquots of 50 mM D-tryptophan were added as a solid 15 hours and 22 hours after the mixture was initially prepared. The head space was flushed with nitrogen after each addition. All of the added tryptophan did not dissolve, but the concentration was maintained at about 50 mM. After 40 hours, the remaining solid tryptophan was filtered off. Analysis of the reaction mixture by post column fluorescence detection liquid chromatography (see Example 1) showed that the concentration of tryptophan in the solution was 49 mM and the concentration of monatin was 3.9 mM.

- [0546] The product monatin was purified utilizing two ion exchange chromatography steps. The filtered reaction solution was first applied to a column of BioRad AG50W-X8 resin (140 mL; binding capacity of 1.7 meq/mL). The column was washed with 2 x 150 mL H₂O and then eluted with 1 M NH₄OH (1 x 450 mL, followed by 3 x 150 mL). The NH₄OH fractions were combined, neutralized with HCl and filtered successively through Whatman (Maidstone, England) glass microfibre filters and Gelman Sciences (Ann Arbor, MI) 0.45 µm filters. The clarified solution was then ultrafiltered using an Amicon ultrafiltration stirred cell (Model 8200) with a YM100 (MWCO 100 kDa) (Millipore; Billerica, MA). The filtrate from the ultrafiltration was evaporated to approximately 160 mL using a roto-evaporator with a tepid water bath. The liquid was again clarified by filtering through glass microfibre filters.
- [0547] The resulting solution was applied to a 1 L Fast Flow DEAE Sepharose (Amersham Biosciences) column previously converted to the bicarbonate form by washing with 0.5 L 1 M NaOH, H₂O, and 1.0 M ammonium bicarbonate, pH 8.3, followed by an additional washing using H₂O. The solution was loaded at <2 mL/min and the column was washed with water at 3-4 mL/min until the absorbance at 280 nm was <1. The R,R-monatin was eluted with 50 mM ammonium bicarbonate, pH 8.3 (2.5 L). This fraction was evaporated using a roto-evaporator with a tepid water bath. The resulting syrup was incubated at 4 °C for several days until crystals formed. The crystals were collected, washed with cold 100% ethanol and dried in a vacuum dessicator (0.38 g).
- [0548] Analysis of the solid product for isomeric purity using FDAA derivitization, followed by LC/MS/MS multiple reaction monitoring, (see Example 1) showed that the sample was 96.3% R,R monatin and 3.7% S,R-monatin.
- [0549] The sample was also analyzed for purity with respect to other organic compounds using the total monatin method (see Example 1). The UV absorbance was scanned from 200-500 nm using a Photodiode Array detector. Based upon the integrated peak areas, monatin accounted for 96.1% of the area (including both R,R and S,R peaks).
- [0550] Analysis of the sample by post column fluorescence detection liquid chromatography showed that the amino acid composition of the sample was 98.8% monatin with trace amounts of tryptophan (1.2%) and alanine (0.02%).

- [0551] Elemental analysis was performed at Midwest Microlab, LLC (Indianapolis, IN). This analysis indicated that the sample contained 1% non-combustible (inorganic) material by weight, and ammonium and bicarbonate residuals.

EXAMPLE 14

Improvement of D-Aminotransferase Activity Retention During Purification

Standard Procedure for the Purification of B. sphaericus HIS₆-D-alanine Aminotransferase

- [0552] Starting from a fresh culture plate (LB agar with 50 µg/mL kanamycin) of BL21(DE3)::*B. sphaericus* *dat* pET30a (Example 18), the cells were grown in 5 mL of Luria-Bertani broth ("LB") with 50 µg/mL kanamycin, at 37 °C with shaking at 225 rpm for 3 - 5 hours. Subsequently, the culture was transferred at 0.25% (v/v) into flasks containing Novagen Overnight Express System II solutions 1-6 (EMD Bioscience, Madison, WI) plus 50 µg/mL kanamycin. The cells were grown at 37 °C and 225 rpm overnight (16-18 hours). When the OD₆₀₀ was approximately 8.0, the cells were harvested by centrifugation in a Beckman (Fullerton, CA) J25II centrifuge with a JS-16.25 rotor at 10,000 rpm for 10 minutes. The cell pellet was washed once with cold 50 mM EPPS buffer (pH 8.2), and the cells were centrifuged again. The washed cell pellet was harvested and used immediately or frozen at -80 °C until needed for purification.
- [0553] To prepare cell-free extract containing the *B. sphaericus* HIS₆-D-alanine aminotransferase (HIS₆-BsphDAT) protein, the cells were suspended in 3-4 volumes of 50 mM EPPS, pH 8.2 and then disrupted using a Microfluidics homogenizer (Newton, MA) (3 passes at 20,000 psi), maintaining the temperature of the suspension below 15 °C. All subsequent purification steps were carried out at 4 °C. The cell extract was centrifuged for 15 minutes at 15,000 x g to remove the cell debris. The supernatant was decanted and used immediately or frozen at -80 °C. An aliquot of the cell free extract was applied either to Novagen HIS-Bind columns (catalog # 70971-4) or to a column of GE Healthcare Chelating SepharoseTM Fast Flow resin (nickel(II) form) (Piscataway, NJ) (at a ratio of 1.2 - 1.5 v/v) that had been previously equilibrated with 50 mM EPPS, pH 8.2, containing 200 mM sodium chloride. After loading the sample, the column was

washed/eluted successively with 3 - 5 volumes of the equilibration buffer, 3 - 5 volumes of the equilibration buffer containing 25 mM imidazole, 3 - 5 volumes of the equilibration buffer containing 50 or 100 mM imidazole and 3 - 5 volumes of the equilibration buffer containing 500 mM imidazole. The HIS₆-BsphDAT protein eluted in the last wash. The 500 mM imidazole wash was concentrated 2 - 10X with Amicon Centricon-70 or Ultra-15 centrifugal filter devices (MWCO 5-10 kDa) (Billerica, MA). The imidazole and sodium chloride were removed by passage through disposable GE Healthcare PD10 desalting columns previously equilibrated with 50 mM EPPS, pH 8.2, containing 50 μ M PLP.

- [0554] The protein concentration of the desalted solution was determined using the Pierce BCA assay kit (Rockford, IL). The purity of each fraction and the level of expression in the cell free extract fraction were determined using a Bio-Rad Experion Pro260 microcapillary chip system (Hercules, CA) or by SDS-PAGE with 4-15% gradient gels. Typically, this procedure produces more than 300 mg of enzyme (from 600 mL of Overnight Express II culture) that is ~90% pure as judged by the Experion software. Aliquots (1-5 mL) of the purified enzyme were stored at -80 °C until use.

Improved Procedure

- [0555] Cell-free extract was prepared as described above. His₆-BsphDAT protein was similarly purified with the following changes: all buffers used for cell disruption and protein purification contained 100 mM potassium phosphate, pH 7.8, with 50 μ M PLP. The protein was purified exclusively with GE Healthcare Chelating SepharoseTM Fast Flow resin (nickel(II) form).

Activity Assay

- [0556] The formation of indole-3-pyruvate and alanine from tryptophan and pyruvate was assayed using the enzyme prepared by both purification procedures. The reaction mixtures contained 100 mM potassium phosphate, pH 7.8, 0.05 mM pyridoxal phosphate (PLP), 100 mM sodium pyruvate, 40 mM D-tryptophan, and 0.03 - 0.1 mg/mL of purified enzyme. The tryptophan was added as a solid. All components except the enzyme were mixed together and incubated at 30 °C until the tryptophan dissolved. The enzyme was then added and the reaction solution was incubated at room temperature. At predetermined time points, the reactions were sampled and the samples immediately

stored on ice and diluted for alanine analysis by the post-column fluorescence detection liquid chromatography method described in Example 1. Table 59 below lists the specific activity of the enzyme preparations as the concentration of alanine formed per mg of enzyme per minute.

Table 59: Effect of Improved Purification Procedure on Enzyme Activity

Enzyme Preparation	Specific Activity ($\mu\text{mole Alanine}(\text{mg})^{-1}(\text{min})^{-1}$)
HIS ₆ -BsphDAT purified without 50 μM PLP	2.9
HIS ₆ -BsphDAT purified with 50 μM PLP	14.2

[0557] The results shown in Table 59 indicate that the use of pyridoxal phosphate (PLP) during the purification process resulted in an enhanced activity.

EXAMPLE 15

Cloning of Two Novel *Bacillus* D-Amino Acid Aminotransferases

[0558] Several *Bacillus* D-amino acid aminotransferases (EC 2.6.1.21, also known as D-alanine aminotransferase or D-aspartate aminotransferase) were produced recombinantly for use in coupled assays for production of R,R monatin, as described in Example 18. These enzymes are homologous to D-aminotransferases described previously for production of monatin (U.S. Publication No. 20040063175 and U.S. Publication No. 2005282260). An approach used for the selection of strains that could be candidates containing novel D-amino acid aminotransferases ("DAATs") was to review the list of *B. sphaericus* strains deposited in ATCC and analyze some that were previously deposited under different species names. The following organisms were ordered from the ATCC: ATCC 4978 -- *Bacillus sphaericus* originally deposited as *Bacillus rotans* and ATCC 7063 -- *Bacillus sphaericus* originally deposited as *Bacillus serositidis* and ATCC 21538 -- *Bacillus sphaericus* originally deposited as *Bacillus circulans*. Known DAAT protein sequences from *Bacillus sphaericus*, *Bacillus halodurans*, *Geobacillus stearothermophilus*, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis* were aligned to obtain sequence regions that were conserved in the various DAAT proteins. Primers were designed in the regions of protein sequence conservation and used for

polymerase chain reactions ("PCR") amplification of DAAT gene sequences from the ATCC strains mentioned above.

- [0559] Five PCR primers were designed based on conserved regions in alignment of published *Bacillus* DAAT sequences (see alignment in FIG. 9).

Polymerase Chain Reaction Protocol

- [0560] Primers were designed as mentioned above based on conserved regions in an alignment of DAATs. Oligonucleotide Primer Sequences are indicated below: 5'-GAAGACCGTGGTTATCAATTT-3' (SEQ ID NO:65) (forward primer), 5'-GATGGTATTTACGAAGTAATC-3' (SEQ ID NO:66) (forward primer), 5'-AGATTTAATATCACAAACGTAAC-3' (SEQ ID NO:67) (reverse primer), 5'-GCCAAGTAAAATTTAAGATTTA-3' (SEQ ID NO:68) (reverse primer), 5'-ATTTGCTGGGTGCGTATAAAG-3' (SEQ ID NO:69) (reverse primer). Expected sizes of PCR fragments based on primer combinations alignment with known DAATs: SEQ ID NO:65 and SEQ ID NO:67 – approximately 380 bp; SEQ ID NO:65 and SEQ ID NO:68 – approximately 395 bp; SEQ ID NO:65 and SEQ ID NO:69 – approximately 534 bp; SEQ ID NO:66 and SEQ ID NO:67 – approximately 336 bp; SEQ ID NO:66 and SEQ ID NO:68 – approximately 346 bp; SEQ ID NO:66 and SEQ ID NO:69 – approximately 510 bp.

- [0561] Combinations of the above primers were used for colony PCR from the following ATCC strains: ATCC 4978 -- *Bacillus sphaericus*, originally deposited as *Bacillus rotans*; ATCC 7063 -- *Bacillus sphaericus*, originally deposited as *Bacillus serositidis*; and ATCC 21538 – *Bacillus sphaericus*, originally deposited as *Bacillus circulans*.

- [0562] The three above mentioned strains were grown on nutrient agar at 30 °C. A single colony was scraped from plates and resuspended in 25 µL sterile distilled water. The cells were lysed at 96 °C for 10 minutes. PCR was carried out as follows: per 50 µL reaction, 5 µL lysed cells, 0.8 µL of each primer, 2 µL dNTPs, 0.8 µL Expand High Fidelity Polymerase (Roche, Indianapolis, IN) and 1X ExpandTM buffer were added. A 3 minute hot start was done at 94 °C, followed by 15 cycles of 94 °C for 30 seconds, 40 °C for 45 seconds, and 72 °C for 2 minutes. Fifteen more cycles were done with an increased annealing temperature of 45 °C. Lastly, a chain extension step was done for seven minutes at 72 °C. Several primer combinations gave expected PCR product sizes

for the above strains. PCR products were cloned using the Zero Blunt TOPO[®] cloning kit as per manufacturers' protocols (Invitrogen) and sequenced by dideoxy chain termination DNA sequencing at Agencourt BioScience Corporation (Beverly, MA). Sequences at both the DNA and amino acid level were aligned with the *B. sphaericus* DAAT sequence. Valid DAAT/DAT sequences were obtained from all three strains, ATCC 4978, ATCC 7063 and ATCC 21538. Two specific strains, ATCC 4978 and ATCC 7063 gave PCR products which when translated yielded protein sequences with distinct amino acid residue changes when compared to the *B. sphaericus* D-aminotransferase sequence.

[0563] Genome walking was carried out to obtain the complete gene sequences for the ATCC 4978 and ATCC 7063 strains. Strain ATCC 4978 was grown up in nutrient broth at 30 °C. Strain ATCC 7063 was grown up on nutrient agar. Genomic DNA was prepared from each strain using the Gentra Kit (Gentra Systems, Minneapolis, MN) as per manufacturer's protocols. Four libraries were constructed for each strain as per manufacturer's protocols (BD GenomeWalker[™] Universal Kit, Clontech, www.Clontech.com). Gene-specific primers were designed as per GenomeWalker[™] manufacturer's protocols based on sequences obtained using conserved primer combinations (see above), allowing for a few hundred homologous base pair overlap with original product. These gene-specific primers were subsequently used with GenomeWalker[™] adaptor primers for PCR of upstream and downstream sequences to complete DAT ORFS.

[0564] The gene-specific oligonucleotide primer sequences are indicated below:

4978 DAT GSP1 Upstrm 5'-GACATGCTCCTCCGCTGTAAATAATTCACC-3' (SEQ ID NO:70);

4978 DAT GSP1 Dwnstr 5'-CCCTGGTGATGAAGTGAAGCCAGTATTAAC-3' (SEQ ID NO:71);

4978 DAT GSP2 Upstrm 5'-ATCGCCAAATTGATAACCACGGTCTTC-3' (SEQ ID NO:72);

4978 DAT GSP2 Dwnstr 5'-ACGTCCCGTAGCAAACCTTTGAAAAAGGTGT-3' (SEQ ID NO:73);

7063 DAT GSP1 Upstrm 5'-TGCATAGAATCGGTGATATGTTTCAGTAGC-3' (SEQ ID NO:74);

7063 DAT GSP1 Dwnstr 5'-GCGGAGAAACGATTACAGAAGGTTCTTCAA-3' (SEQ ID NO:75);

7063 DAT GSP2 Upstrm 5'-GTCACCAAATTGATAACCACGGTCTTC-3' (SEQ ID NO:76); and

7063 DAT GSP2 Dwnstr 5'-GGTGTACTTTATACGCACCCAGCAAAT-3' (SEQ ID NO:77).

[0565] Adaptor oligonucleotide primer sequences:

AP1 5'-GTAATACGACTCACTATAGGGC-3' (SEQ ID NO:78) and

AP2 5'-ACTATAGGGCACGCGTGGT-3' (SEQ ID NO:79).

[0566] Primary GenomeWalkerTM PCRs were carried out as follows: per 50 μ L reaction, 2.5 μ L of DNA library, 2 μ L of each primer (AP1 (SEQ ID NO:78) and the appropriate GSP1), 1.5 μ L dNTPs, 1X XL PCR buffer, 1 mM magnesium acetate, and 1 μ L RTTH polymerase (Roche, Indianapolis, IN) were added. A 3 minute hot start was done at 94 °C, followed by 10 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 68 °C for 1 minute. Twenty more cycles were done with a decreased annealing temperature of 48 °C. Lastly, a chain extension step was done for seven minutes at 68 °C. Secondary Genome WalkerTM PCRs were carried out as follows: per 50 μ L reaction, 1.0 μ L (of a 1:50 dilution) of the primary PCR reaction, 2 μ L of each primer (AP2 (SEQ ID NO:79) and the appropriate GSP2), 1.5 μ L dNTPs, 1X XL PCR buffer, 1 mM magnesium acetate, and 1 μ L RTTH polymerase were added. A 3 minute hot start was done at 94 °C, followed by 10 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 68 °C for 1 minute. Fifteen more cycles were done with a decreased annealing temperature of 48 °C. Lastly, a chain extension step was done for seven minutes at 68 °C.

[0567] Several libraries gave PCR products ranging in size from ~200 bp to ~1.5 Kb. PCR products were TOPO cloned (as above) and sequenced by dideoxy chain termination DNA sequencing at Agencourt BioScience Corporation (Beverly, MA); these new sequences were aligned with initial sequences obtained using conserved primer combinations and start and stop codons were identified. In this way the DAAT complete ORFs were obtained. New primer pairs were designed (with restriction sites for cloning) based on the specific complete DAAT sequences to PCR the entire DAAT gene from ATCC strains 4978 and 7063 individually.

[0568] Oligonucleotide Primer Sequences are indicated below:

ATCC4978DAATNde1F

5'-GGCCTTGGCATATGAGTTATAGCTTATGGAATGACC-3' (SEQ ID NO:80);

ATCC4978DAATBamH1R

5'-GGCCTTAAGGATCCTTATGCGCGAATACCTTTTGGG-3' (SEQ ID NO:81);

ATCC7063DAATNde1F 5'-GGCCTTGGCATATGAGCTACACTTTATGGAATGA-3' (SEQ ID NO:82); and

ATCC7063DAATBamH1R2a 5'-GGCCAAGGATCCGCTACCCACTAATCATTAGA-3' (SEQ ID NO:83).

[0569] The coding regions of the ATCC 4978 and ATCC 7063 DAAT genes were amplified using the following PCR protocol. In a 50 μ L reaction, 3 μ L genomic DNA, 0.8 μ L of each primer, 2 μ L dNTPs, 0.8 μ L Expand High Fidelity Polymerase (Roche, Indianapolis, IN), 1X ExpandTM buffer with Mg, and 0.2 μ L Pfu polymerase (Stratagene, La Jolla, CA) were added. The thermocycler program used included a hot start at 94 °C for 3 minutes, followed by 8 repetitions of the following steps: 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 90 seconds. Twenty-two subsequent cycles were done with an annealing temperature of 58 °C. Lastly, a chain extension step was done for seven minutes at 72 °C. Clean PCR products of the correct size (approximately 850 bp) were obtained for both strains.

[0570] The PCR products for ATCC 4978 and ATCC 7063 DAAT genes were purified using the Qiagen QIAquick[®] PCR purification kit (Qiagen, Valencia, CA), and digested with *Nde* I and *Bam*H I in *Bam*H I buffer (New England Biolabs, Ipswich, MA). *Nde* I and *Bam*H I digested vectors (pET28 and pET30) and insert were purified using the Qiagen QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA). The ligations were done using the Roche Rapid DNA Ligation Kit (Roche, Indianapolis, IN) and purified using the QIAquick[®] PCR purification kit. The ligations were transformed into *Escherichia coli* DH10B using a 0.2 cm cuvette and a Bio-Rad Gene Pulser[®] II system as described in the Bio-Rad electroporation manual. The cells were allowed to recover in 900 μ L SOC medium for 30 minutes at 37 °C with shaking at 225 rpm. The cells were plated on LB-agar plates containing kanamycin (50 μ g/mL). The plasmid DNA was purified using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by PCR and restriction digestion with *Nde* I and *Bam*H I. The sequences of plasmids that

appeared to have the correct insert were verified by dideoxy chain termination DNA sequencing at Agencourt BioScience Corporation (Beverly, MA). Sequence analyses verified the coding sequence for DAAT genes from ATCC 4978 and ATCC 7063, which produced the DNA sequences of SEQ ID NO:84 (ATCC 4978 DAAT DNA sequence) and SEQ ID NO:85 (ATCC 7063 DAAT DNA sequence) and the amino acid sequence of SEQ ID NO:86 (ATCC 4978 DAAT amino acid sequence) and SEQ ID NO:87 (ATCC 7063 DAAT amino acid sequence).

[0571] The alignments of the two novel DAATs from ATCC 4978 and ATCC 7063 with the *B. sphaericus* DAAT (cloned in Example 18) shown in FIG. 10.

[0572] We obtained novel D-aminotransferases from strains ATCC 4978 and ATCC 7063 with protein sequences that have distinct amino acid residue changes when compared to the *B. sphaericus* D-aminotransferase. The DAATs from ATCC 4978 and ATCC 7063 have only 72% and 67% identity with the DAAT from *B. sphaericus* (ATCC 10208). While both of these strains are currently listed as *B. sphaericus* in the ATCC, they were deposited as *B. rotans* and *B. serositidis*. Based on the sequence alignments and the highlighted differences between these two novel DAATs and the DAAT from *B. sphaericus*, a number of candidate residues are identified that can be evaluated for their role (individually or in combination) in increasing DAAT activity for R,R monatin biosynthesis, in these, as well as other DAAT sequences.

EXAMPLE 16

Gene Expression and Assays for ATCC 4978 and ATCC 7063 DAAT Proteins

[0573] The novel DAATs from ATCC 4987 and ATCC 7063, as described in Example 15, (in pET vectors) were transformed into the *E. coli* expression host BL21(DE3) (Novagen, Madison, WI). The cultures were grown using the protocols described above and the plasmids were isolated using Qiagen miniprep kit (Qiagen, Valencia, CA) and analyzed by restriction digestion, as described above, to confirm plasmid identity.

[0574] Induction of the DAAT gene was typically performed in LB medium containing kanamycin (50 µg/mL). The cells were grown to an OD₆₀₀ of 0.4-0.8 at 37 °C, induced with 0.1 mM IPTG (isopropyl thiogalactoside) and sampled at 3-4 hours post induction.

Cell extracts were prepared according to the protocol accompanying the Novagen BugBuster™ reagent (with benzonase nuclease and Roche complete protease inhibitor cocktail added). Soluble proteins were obtained at the predicted molecular weight, as judged by SDS-PAGE, for both ATCC 4978 and ATCC 7063 gene products in pET vectors. Higher levels of soluble protein were observed using constructs without His tags (pET 30). Soluble proteins in the cellular extracts were separated on a BioRad Laboratories Experion Automated Electrophoresis Station (Hercules, CA) and analyzed for concentration and percent expression using the Experion Software version 1.1.98.0.

[0575] Protein extracts from cells with untagged (pET30) constructs were analyzed for D-aminotransferase activity by following production of alanine from pyruvate and D-tryptophan (or R,R monatin) using the following protocol. Duplicate 500 µL reactions were, unless specified below, carried out in 100 mM potassium phosphate buffer (pH 7.5), 80 µM pyridoxal phosphate, 25 mM sodium pyruvate, and 50 mM D-tryptophan or R,R monatin. The reactions were initiated by the addition of cell free extracts (4978 or 7063) or purified enzyme (*B. sphaericus*) and were incubated 15 minutes-2 hours at 30 °C, with mild shaking. Approximately the same level of total protein was added (1.0 mg), unless specified below, in each assay for comparative purposes. Purified *B. sphaericus* (ATCC number 10208) aminotransferase was used as a benchmark enzyme. Formic acid was added to a final concentration of two percent to stop the reaction and the precipitated protein was removed by centrifugation. Control reactions without added protein were also performed. Alanine was detected using LC/OPA post-column derivatization as described in Example 1. The averaged results of the duplicate reactions are shown in Tables 60 and 61 below.

**Table 60: Transamination Activity from ATCC 4978 and ATCC 7063
D-Aminotransferases (15 min)**

D-aminotransferase	Alanine (mM) D-Tryptophan as Substrate	Alanine (mM) R,R Monatin as Substrate
ATCC 4978	7.78	0.32
ATCC 7063	0.28	0.025
<i>B. sphaericus</i> (untagged)	11.93	3.57

Table 61: Transamination Activity from ATCC 4978 and ATCC 7063 D-Aminotransferases (2 Hours)

D-aminotransferase	Alanine (mM) D-Tryptophan as Substrate	Alanine (mM) R,R Monatin as Substrate
ATCC 4978	16.46	2.33
ATCC 7063	2.51	0.21
<i>B. sphaericus</i> (untagged)	13.73	12.23

[0576] Thus, we demonstrated that the D-amino acid aminotransferases from ATCC 4978 and ATCC 7063 indeed possessed D-aminotransferase activity and have the capability to make R,R monatin. The activity of the ATCC 4978 DAAT was higher than that observed for the ATCC 7063 DAAT. Quantitative comparison between 4978 and *B. sphaericus* could not be made since 4978 was unpurified.

EXAMPLE 17

Production of R,R Monatin using the DAAT from ATCC 4978

[0577] The aminotransferase from ATCC 4978 was also tested for the ability to produce monatin from D-tryptophan (as in Example 3A). The following were added per 1 mL of reaction mixture: approximately 50 µg aldolase (*C. testosteroni* ProA aldolase or the aldolase of SEQ ID NO:22, purified), 4 mM MgCl₂, 50 mM D-tryptophan (supplied as solid), 1.0 mg D-aminotransferase, 100 mM sodium pyruvate, 100 mM potassium phosphate buffer pH 7.5, and 0.05 mM PLP. Experiments were run in duplicate, with negative controls in which no aminotransferase was added. Samples were incubated for various lengths of time at 30 °C with gentle shaking. The only stereoisomers detected when producing monatin using these methods are R,R and S,R. The total monatin and percent R,R monatin were detected as described in Example 1 are listed in Tables 62-64 below. The results shown in each of Tables 62-64 is the average value from duplicate reactions.

Table 62: Comparison of *B. sphaericus* and ATCC 4978 D-Aminotransferases for Production of Monatin using Approximately 50 µg *C. testosteroni* ProA

D-Aminotransferase	Total Monatin (mg per g DAT protein) 15 Minutes	Total Monatin (mg per g DAT protein) 30 Minutes	Total Monatin (mg per g DAT protein) 1 Hour	Total Monatin (mg per g DAT protein) 2 Hours
ATCC 4978	419.3	598	1017	1348
<i>B. sphaericus</i> (tagged)	46.5	128	232	241

Table 63: Comparison of *B. sphaericus* and ATCC 4978 D-Aminotransferases for Production of Monatin using Approximately 50 µg *C. testosteroni* ProA

D-Aminotransferase	% R,R Monatin 15 Minutes	% R,R Monatin 30 Minutes	% R,R Monatin 1 Hour	% R,R Monatin 2 Hours
ATCC 4978	48.9	38.4	34.4	33.25
<i>B. sphaericus</i> (tagged)	72.3	63.4	56.1	53.5

Table 64: Comparison of *B. sphaericus* and ATCC 4978 D-Aminotransferases for Production of Monatin using Approximately 50 µg of the Aldolase of SEQ ID NO:22

D-Aminotransferase	Total Monatin (mg per g DAT Protein) 2 Hours	% R,R Monatin 2 Hours
ATCC 4978	501	92.1
<i>B. sphaericus</i> (tagged)	201	95.6

[0578] Thus, we demonstrated that the D-amino acid aminotransferase from ATCC 4978 has the capability to make R,R monatin. The activity of the ATCC 4978 DAAT, when comparing total monatin production in terms of mg monatin per gram protein, was higher than that observed for the *B. sphaericus* DAAT. The use of an R-specific aldolase of SEQ ID NO:22 clearly made an improvement in percentage of R,R monatin formed in comparison to the amount of total monatin produced.

Example 18

Cloning of Published *Bacillus* D-Amino Acid Aminotransferases

- [0579] Several *Bacillus* D-amino acid aminotransferases (EC 2.6.1.21, also known as D-alanine aminotransferase or D-aspartate aminotransferase) were produced recombinantly for use in coupled assays for production of R,R monatin. These enzymes are homologous to D-aminotransferases described previously for production of monatin (U.S. Publication No. 20040063175 and U.S. Publication No. 2005282260).

Strains

- [0580] *B. sphaericus* (ATCC number 10208) and *B. licheniformis* (ATCC 10716) were grown on Nutrient Agar at 30 °C overnight. Groups of colonies were placed in 100 µL of sterile water and heated for 5 minutes at 95 °C to disrupt the cells. Three µL was used in subsequent Polymerase Chain Reaction ("PCR") amplifications. Genomic DNA was ordered for *B. halodurans* (ATCC number BAA-125D) and resuspended in water to a concentration of 100 ng/µL. *Bacillus cereus* genomic DNA (ATCC numbers 1-987D and 14579D) was ordered for cloning as well.

Polymerase Chain Reaction Protocol

- [0581] Primers were designed for the *B. sphaericus* *dat* gene for cloning into pET 28b and pET 30a vectors (Novagen, Madison, WI), using the *Nco* I and *Bam*H I sites. The pET30 construct contains an N-terminal His-tag and S-tag, whereas the pET 28 construct is untagged.

Bacillus sphaericus dat primers:

N term 5'-GATATACCATGGCATACTCATTATGGAATG-3' (SEQ ID NO:88) and C term 5'-GTTATCGGATCCTTAGGCATTAATTGAAATTG-3' (SEQ ID NO:89).

- [0582] The *B. licheniformis* primers and *B. halodurans* primers were designed for cloning into pET 28b and pET 30a vectors using *Nde* I and *Bam*H I sites. The pET30 constructs were untagged in this case, whereas the pET 28 constructs contain a small N-terminal his-tag.

B. licheniformis dat primers:

N term 5'-GGCCGGTTCATATGAAAGTTCTTTTAAACGGC-3' (SEQ ID NO:90) and
C term 5'-CCTTCCGGATCCTTAAACCGTTTGGCTGTCT-3' (SEQ ID NO:91).

B. halodurans primers:

N term 5'-GATATACATATGGATTATTGCCTTTACCAA-3' (SEQ ID NO:92) and C
term 5'-GAATCCGGATCCTCACTGCTTCATCGCTGTTTG-3' (SEQ ID NO:93).

[0583] Primers were designed for the *B. cereus* coding sequences. One set of primers yielded the sequence listed in NCBI as accession AE016877 gi:29899096 5138634... 5139506 (873 bp). One set of primers yielded a product with an additional 12 bp upstream, similar to the *B. thuringiensis* predicted *dat* NCBI accession number AE017355 gi:49328240 4965653... 4966537 (885 bp). Both sets of primers were designed with *Nde* I for the N-terminal region and *Bam*H I restriction site for the C-terminal region. The primers were designed for cloning into pBAD-TOPO TA cloning.

B. cereus primers:

N term 5'-TAAGAGGAATAACATATGGCATACGAAAGATTT-3' (SEQ ID NO:94)
and C-term 5'-GAATTCGGATCCTTAAGAAGATGACATATTGG-3' (shorter PCR
product) (SEQ ID NO:95).

N term 5'-TAAGAGGAATAACATATGGGATCGAAATTGGCA-3' (longer PCR
product) (SEQ ID NO:96).

[0584] The coding regions of the *B. sphaericus*, *B. halodurans*, and *B. licheniformis dat* genes were amplified using the following PCR protocol. In a 50 µL reaction, 3 µL template (2 µL for genomic DNA), 1.6 µM of each primer, 0.25 mM each dNTP, 3.5 U Expand High Fidelity Polymerase (Roche, Indianapolis, IN), and 1X ExpandTM buffer with Mg were used. The thermocycler program used included a hot start at 94 °C for 3 minutes, followed by 8 repetitions of the following steps: 94 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 2 minutes. Twenty-two subsequent cycles were done with an annealing temperature of 58 °C. After 30 cycles the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. Clean PCR products of the correct size were obtained (approximately 850 bp for the *dat* gene).

[0585] *Geobacillus stearothermophilus dat* (accession number J04460 gi:142541), which codes for protein accession number AAA22252 (gi:142542) was constructed using

assembly PCR techniques. The source of this gene/protein is often described as *Bacillus* sp., thermostable *Bacillus* species, or *Bacillus* YM-1. The assembly process is as follows: 43 oligonucleotides (40 mers) were ordered from IDT based on the gene sequence above and its complementary DNA sequence with 20 basepair overlaps between the sense and antisense strands. The primers were diluted to 250 μ M in water and 5 μ L of each primer was mixed together in a microfuge tube. PCR was carried out as follows: per 100 μ L reaction, 1.5 μ L of the primer pool, 4 μ L dNTPs, 1X XL PCR buffer, 1 mM magnesium acetate, 2 μ L *rTth* polymerase (Roche, Indianapolis, IN), and 0.25 μ L Pfu polymerase (Stratagene, La Jolla, CA) were added. A 3 minute hot start was done at 94 °C, followed by 15 cycles of 94 °C for 30 seconds, 40 °C for 30 seconds, and 68 °C for 15 seconds. Ten more cycles were done with an increased annealing temperature of 44 °C and an extension time of 30 seconds (at 68 °C). Ten more cycles were performed at an annealing temperature of 48 °C and an extension time of 75 seconds. Lastly, a chain extension step was done for seven minutes at 68 °C. A secondary PCR was done using the following primers, designed for cloning with *Nde* I (N-term) and *Bam*H I (C-term):

N-term 5'-GGCCTTGGCATATGGGATACACTTTATGGAATGACC-3' (SEQ ID NO:97) and C-term 5'-TTGGAACCGGATCCTTATATATGAAGCGGTTTTGG-3' (SEQ ID NO:98).

- [0586] The second PCR reaction contained per 100 μ L, 2.5 μ L of the primary reaction, 0.4 μ L of each primer, 3 μ L dNTPs, 1X XL PCR buffer, 1 mM magnesium acetate, 2 μ L *rTth* polymerase, and 0.25 μ L Pfu polymerase. A 3 minute hot start was done at 94 °C, followed by 10 cycles of 94 °C for 30 seconds, 42 °C for 30 seconds, and 68 °C for 90 seconds. Fifteen more cycles were done with an increased annealing temperature of 48 °C. Lastly, a chain extension step was done for seven minutes at 68 °C. A third PCR reaction was done using template from the second PCR using the same conditions as the second PCR reaction. A product of approximately 900 bp was visible on an agarose gel.

Cloning

- [0587] The PCR product for *B. sphaericus* DAT was purified using the Qiagen QIAquick® PCR purification kit (Qiagen, Valencia, CA) and digested with *Bam*H I and *Nco* I in *Bam*H I buffer (New England Biolabs, Ipswich, MA). The digested vectors (pET28 and pET30) and insert were purified using the Qiagen QIAquick® Gel Extraction

Kit (Qiagen, Valencia, CA). The ligations were done using the Roche Rapid DNA Ligation Kit (Roche, Indianapolis, IN) and purified using the QIAquick® PCR purification kit. The ligations were transformed into *Escherichia coli* DH10B using a 0.2 cm cuvette and a Bio-Rad Gene Pulser® II system as described in the Bio-Rad electroporation manual. The cells were allowed to recover in 900 µL SOC medium for 30 minutes at 37 °C with shaking at 225 rpm. The cells were plated on LB-agar plates containing kanamycin (25 µg/mL). The plasmid DNA was purified using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Bam*H I and *Nco* I. The sequences of plasmids that appeared to have the correct insert were verified by dideoxy chain termination DNA sequencing at Agencourt BioScience Corporation (Beverly, MA). Sequencing verified the coding sequence found in NCBI accession number AF081278 Region: 134..985 (gi: 3513754), which produces a protein with amino acid sequence as listed in accession number AAC33964 (gi: 3513755).

[0588] The PCR products for *B. licheniformis* DAT (~850 bp) and *G. stearothermophilus* were gel purified and cloned using the Zero Blunt TOPO® cloning kit as per manufacturers' protocols (Invitrogen). The plasmids were transformed into TOP10 chemically competent cells for initial screening. The plasmid DNA was screened by restriction digest and the sequences were verified to match the coding sequence found in NCBI. For *B. licheniformis*, the sequence matched accession number U26947 region 247..1098 (gi:857560), which produces a protein with an amino acid sequence as listed in accession number P54692 (gi:1706292), with the exception of one silent mutation at position 429 from A to G. For *G. stearothermophilus*, the sequence matched the accession number listed above. The coding regions were subcloned by restriction digest (*Nde* I/*Bam*H I), ligated into the pET vectors, and transformed into electrocompetent DH10B cells for amplification.

[0589] The PCR product for *B. halodurans* DAT was gel purified, digested with *Nde* I and *Bam*H I and ligated into pET 28 and pET 30 vectors as above. Amplification of the vector was done in DH10B cells. The miniprep DNA was screened by PCR and the sequence was verified. The gene sequence can be found in accession number NC_002570 (gi:57596592) 2934903..2935754 coding for a protein with amino acid sequence listed in accession number NP_243677 (gi:15615374).

- [0590] The *B. cereus* coding sequences were amplified using a typical PCR protocol and cloned according to manufacturer's protocols (Invitrogen).

Gene Expression and Assays

- [0591] Plasmid DNA was subcloned into *E. coli* expression host BL21(DE3) (Novagen, Madison, WI) for constructs in pET vectors. The cultures were grown and the plasmids were isolated using Qiagen miniprep kit (Qiagen, Valencia, CA), and analyzed by restriction digest to confirm identity. Induction was typically performed in LB medium containing kanamycin (50 µg/mL). The cells were grown to an OD₆₀₀ of 0.4-0.8 at 37 °C, induced with 0.1 mM IPTG (isopropyl thiogalactoside) and sampled at 3-4 hours post induction. The cell extracts were prepared according to the protocol accompanying the Novagen BugBusterTM reagent (with benzonase nuclease and Roche complete protease inhibitor cocktail added). High levels of soluble protein were obtained at the predicted molecular weight, as judged by SDS-PAGE, for both *B. halodurans* gene products, both *B. sphaericus* gene products, both *G. stearothermophilus* gene products, and the untagged *B. licheniformis* gene product. For reactions in which purified protein was used, the His-tagged gene products were purified using His-Bind cartridges following manufacturer's protocols (Novagen, Madison, WI). The eluent fractions were desalted on PD-10 (Amersham Biosciences, Piscataway, NJ) columns and eluted in 25-100 mM potassium phosphate buffer, pH 7.5. Total protein assays were done using the Pierce BCA kit (Rockford, IL) and percent expression was estimated from SDS-PAGE. Alternatively, the soluble proteins in the cellular extracts were separated on a BioRad Laboratories Experion Automated Electrophoresis Station and analyzed for concentration and percent expression using the Experion Software version 1.1.98.0. The pBAD-TOPO constructs containing the *B. cereus* genes were expressed as recommended by Invitrogen, but the levels of expression of the DAATs was such that the recombinant protein could not be distinguished from the other proteins during SDS-PAGE analysis.

- [0592] The cell extracts were analyzed for D-aminotransferase activity by following production of alanine from pyruvate and D-tryptophan (or R,R monatin) using the following protocol. Duplicate one mL reactions were typically carried out in 100 mM potassium phosphate buffer (pH 7.5), 50 µM pyridoxal phosphate, 25 mM sodium pyruvate, and 50 mM D-tryptophan or R,R monatin. The reactions were initiated by the addition of cell free extracts or purified enzyme and were incubated 15 minutes-overnight

at 30 °C, with mild shaking. Approximately the same level of D-aminotransferase was added (typically around 0.5 mg) in each assay for comparative purposes. AT-103 (BioCatalytics) was used as a positive control (or benchmark). Formic acid was added to a final concentration of two percent to stop the reaction and the precipitated protein was removed by centrifugation. Control reactions, without added protein, were also performed. Zero time points were also used as negative controls. Alanine was detected using LC/post-column OPA derivatization as described in Example 1.

[0593] The aminotransferases were also tested for their ability to produce monatin from D-tryptophan (as in Example 3A). The following were added per 1 mL of reaction mixture: approximately 50-100 µg aldolase (typically *C. testosteroni* ProA aldolase, purified), 4 mM MgCl₂, 50 mM D-tryptophan (supplied as solid), 0.5-2 mg D-aminotransferase, 200 mM sodium pyruvate, 100 mM potassium phosphate buffer pH 7.5, and 0.05 mM PLP. Experiments were run in duplicate, with negative controls in which no aminotransferase was added. The samples were incubated 1 hour, 2 hours, and overnight (17-20 hours) at 30 °C with gentle shaking. The only stereoisomers detected when producing monatin using these methods are R,R and S,R. The percent R,R is listed below and was determined by reversed-phase LC peak area. The results of the transamination activity of *B. sphaericus*, *B. licheniformis*, and *B. halodurans* D-aminotransferases after 1 hour is shown in Table 65 below. The data was normalized to 0.5 mg of the D-aminotransferase per mL.

Table 65: Transamination Activity of *B. sphaericus*, *B. licheniformis*, and *B. halodurans* D-Aminotransferases

D-Aminotransferase	Alanine (mM) D-Tryptophan as Substrate	Alanine (mM) R,R Monatin as Substrate
<i>B. halodurans</i> (tagged)	15.5	1.3
<i>B. halodurans</i> (untagged)	17.5	1.4
<i>B. licheniformis</i> (untagged)	28.4	0.21
<i>B. sphaericus</i> (untagged)	29.0	22.5
<i>B. sphaericus</i> (tagged)	17.1	12.0

[0594] The production of monatin using *B. sphaericus*, *B. licheniformis*, and *B. halodurans* D-aminotransferases is shown in Table 66 below. Each reaction contained approximately 90 µg *C. testosteroni* Pro A. The data for the total monatin produced was normalized to the use of 0.5 mg of the D-aminotransferase.

Table 66: Comparison of *B. sphaericus*, *B. licheniformis*, and *B. halodurans* D-Aminotransferases for Production of Monatin

D-Aminotransferase	Total Monatin (ppm) 3 Hours	Total Monatin (ppm) Overnight	% R,R 3 Hours	% R,R Overnight
<i>B. halodurans</i> (tagged)	3.2	13.7	100	99.3
<i>B. halodurans</i> (untagged)	4	15.5	100	99.3
<i>B. licheniformis</i> (untagged)	0.6	8.1	100	29.3
<i>B. sphaericus</i> (untagged)	279.6	577.6	61.55	65.7
<i>B. sphaericus</i> (tagged)	111.2	246	61.0	63.1

[0595] The *B. sphaericus* D-aminotransferase (untagged) had the highest activity for production of monatin from D-tryptophan, but the *B. halodurans* enzyme had much higher selectivity for R-MP versus S-MP than the other enzymes, resulting in higher stereopurity of R,R monatin. The *B. cereus* cell extracts did not have detectable amounts of activity under the conditions tested, although the genes may not have been expressed in the hosts chosen.

[0596] The *G. stearothermophilus* DAT (untagged, which expressed better) was assayed as above and compared to the purified *B. sphaericus* DAT and AT-103 (BioCatalytics). The results are shown in Tables 67 and 68 below. The transamination activity of *G. stearothermophilus*, AT-103, and *B. sphaericus* D-aminotransferase was tested using 0.5 mg of D-aminotransferase per mL (Table 67).

Table 67: Transamination Activity of *G. stearothermophilus*, AT-103, and *B. sphaericus* (Purified) D-Aminotransferases

D-Aminotransferase	Alanine (mM)-15 Minutes D-Tryptophan as Substrate	Alanine (mM)-15 Minutes R,R Monatin as Substrate	Alanine (mM)-2 Hours D-Tryptophan as Substrate	Alanine (mM)-2 Hours R,R Monatin as Substrate
AT-103	8.91	1.21	9.47	6.13
<i>B. sphaericus</i> (tagged)	8.91	1.65	9.53	7.17
<i>G. stearothermophilus</i> (untagged)	2.05	0.053	8.10	0.78

Table 68: Comparison of *G. stearothermophilus*, AT-103, and *B. sphaericus* (purified) for Total Monatin Production

D-Aminotransferase	Total Monatin (ppm) 2 Hours	Total Monatin (ppm) Overnight	% R,R 2 Hours	% R,R Overnight
AT-103	450	645	65.5	60.6
<i>B. sphaericus</i> (tagged)	110	175	64	54
<i>G. stearothermophilus</i> (untagged)	nd	10	n/a	27

[0597] The native *G. stearothermophilus* enzyme is clearly less active for monatin transamination than the AT-103 and *B. sphaericus* enzymes.

EXAMPLE 19

Creation of a Hybrid D-Aminotransferase

[0598] Several *Bacillus* D-amino acid aminotransferases were described in Examples 18 and 15. Although the *G. stearothermophilus* enzyme had low transamination activity on monatin, causing less total monatin to be produced from D-tryptophan, it still had structural elements of interest and it is a thermostable enzyme. Therefore, a hybrid protein was created between the higher activity enzyme (*B. sphaericus*) and the *Geobacillus* enzyme.

Assembly of Hybrid DAT Coding Sequence

[0599] The target protein sequence that was designed is SEQ ID NO:99. SEQ ID NO:100, the coding sequence corresponding to SEQ ID NO:99, was designed based on *E. coli* codon usage.

[0600] The hybrid DAT was constructed using assembly PCR techniques. The assembly process is as follows: 43 oligonucleotides (40 mers) were ordered from IDT based on the gene sequence above and its complementary DNA sequence, with 20 basepair overlaps between the sense and antisense strands. The primers were diluted to 250 μ M in water and 5 μ L of each primer was mixed together in a microfuge tube. PCR was carried out as follows: per 100 μ L reaction, 1.5 μ L of the primer pool, 4 μ L dNTPs, 1X XL PCR buffer,

1 mM magnesium acetate, 2 μ L *rTth* polymerase (Roche, Indianapolis, IN), and 0.25 μ L Pfu polymerase (Stratagene, La Jolla, CA) were added. A 3 minute hot start was done at 94 °C, followed by 15 cycles of 94 °C for 30 seconds, 40 °C for 15 seconds, and 68 °C for 30 seconds. Ten more cycles were done with an increased annealing temperature of 44 °C and an increased annealing time of 30 seconds. Ten more cycles were performed at an annealing temperature of 48 °C and an extension time of 75 seconds. Lastly, a chain extension step was done for seven minutes at 68 °C. A secondary PCR was done using the following primers, designed for cloning with *Nde* I (N-term) and *Bam*H I (C-term):
N-term 5'-GGCCTTGGCATATGGGATACACTTTATGGAATGACCA-3' (SEQ ID NO:101) and C-term 5'-TTGGAACCGGATCCTTAGCTGTTAAGGCTCAGTGGAA-3' (SEQ ID NO:102)

- [0601] The PCR contained per 100 μ L, 2.5 μ L of the primary reaction, 3 μ L dNTPs, 1X XL PCR buffer, 1mM magnesium acetate, 2 μ L *rTth*, and 0.25 μ L Pfu polymerase. A 3 minute hot start was done at 94 °C, followed by 10 cycles of 94 °C for 30 seconds, 42 °C for 30 seconds, and 68 °C for 75 seconds. Fifteen more cycles were done with an increased annealing temperature of 48 °C. Lastly, a chain extension step was done for seven minutes at 68 °C. A product of approximately 850 bp was visible on an agarose gel.

Cloning

- [0602] The PCR product was gel purified using the Qiagen QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA), and cloned using the Zero Blunt TOPO[®] cloning kit as per manufacturers' protocols (Invitrogen). The plasmids were transformed into TOP10 chemically competent cells for initial screening by PCR. The plasmid DNA was screened by restriction digest and the DNA sequence was verified.
- [0603] The plasmid minipreps were digested with *Bam*H I and *Nde* I (New England Biolabs, Ipswich, MA). The digested vectors (pET28 and pET30) and insert were ligated using the Roche Rapid DNA Ligation Kit (Roche, Indianapolis, IN) and purified using the Roche High-Pure PCR Product Purification Kit (Roche, Indianapolis, IN). The ligations were transformed into *Escherichia coli* DH10B cells using a 0.2 cm cuvette and a Bio-Rad Gene Pulser[®] II system as described in the Bio-Rad electroporation manual. The cells were allowed to recover in 900 μ L SOC medium for 30 minutes at 37 °C with

shaking at 225 rpm. The cells were plated on LB-agar plates containing kanamycin (25 µg/mL). The plasmid DNA was purified using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by restriction digest with *Bam*H I and *Nde* I.

Gene Expression and Assays

- [0604] Plasmid DNA was transformed into *E. coli* expression host BL21(DE3) according to manufacturers' protocols (Novagen, Madison, WI). The cultures were grown and the plasmids were isolated using Qiagen miniprep kit (Qiagen, Valencia, CA) and analyzed by PCR to confirm identity. The induction was performed in LB medium containing kanamycin (50 µg/mL). The cells were grown to an OD₆₀₀ of 0.5 at 37 °C, induced with 0.1 mM IPTG (isopropyl thiogalactoside) and sampled at 3 hours post induction. The cell extracts were prepared according to the protocol accompanying the Novagen BugBuster™ reagent (with benzonase nuclease and Roche complete protease inhibitor cocktail added). High levels of total protein were obtained at the predicted molecular weight, as judged by SDS-PAGE, for both gene products. However, the soluble levels of protein were lower. The untagged version of the gene product expressed better and was assayed as a cellular extract. The soluble proteins in the cellular extracts were separated on a BioRad Laboratories Experion Automated Electrophoresis Station and analyzed for concentration and percent expression using the Experion Software version 1.1.98.0, to normalize the amount of D-aminotransferase used in comparative assays.
- [0605] The cell extracts were analyzed for D-aminotransferase activity by following production of alanine from pyruvate and D-tryptophan (or R,R monatin) using the following protocol. Duplicate one mL reactions were carried out in 100 mM potassium phosphate buffer (pH 7.5), 50 µM pyridoxal phosphate, 25 mM sodium pyruvate, and 50 mM D-tryptophan or R,R monatin (unless otherwise noted). The reactions were initiated by the addition of cell free extracts or purified enzyme and were incubated 15 minutes-overnight at 30 °C, with mild shaking. Approximately the same level of D-aminotransferase was added (0.5 mg) in each assay for comparative purposes (unless otherwise noted). AT-103 (BioCatalytics) or *B. sphaericus* D-aminotransferase (Example 18) was used as a benchmark enzyme. Formic acid was added to a final concentration of two percent to stop the reaction and the precipitated protein was removed by centrifugation. Control reactions without added protein were also performed. Zero time

points were also used as negative controls. Alanine was detected using LC/OPA post-column derivatization as described in Example 1. The results of the reactions using 0.5 mg D-aminotransferase per 1 mL reaction volume are shown in Table 69 below.

Table 69: Transamination Activity of *B. sphaericus* (purified), *G. stearothermophilus*, and Hybrid D-Aminotransferases

D-Aminotransferase	Alanine (mM)-15 Minutes D-Tryptophan as Substrate	Alanine (mM)-15 Minutes R,R Monatin as Substrate	Alanine (mM)-2 Hours D-Tryptophan as Substrate	Alanine (mM)-2 Hours R,R Monatin as Substrate
Hybrid DAT (untagged)	13.5	0.084	14.2	0.54
<i>B. sphaericus</i> (tagged)	13.6	4.60	13.9	10.6
<i>G. stearothermophilus</i> (untagged)	6.6	0.18	13.5	2.2

[0606] The aminotransferases were also tested for their ability to produce monatin from D-tryptophan (as in Example 3A). The following were added per 1 mL of reaction mixture: approximately 50-100 µg purified *C. testosteroni* ProA aldolase, 4 mM MgCl₂, 50 mM D-tryptophan (supplied as a solid), 0.5-2 mg D-aminotransferase, 200 mM sodium pyruvate, 100 mM potassium phosphate buffer pH 7.5, and 0.05 mM PLP. Experiments were run in duplicate, with negative controls in which no aminotransferase was added. The samples were incubated 1 hour, 2 hours, and overnight (17-20 hours) at 30 °C with gentle shaking. The only stereoisomers detected when producing monatin using these methods were R,R and S,R. The percent R,R is listed in Table 70 below and was determined by reversed-phase LC peak area. At low monatin concentrations, the percent R,R is not as accurate as judged by RPLC peak area. Therefore, some of the samples were further analyzed by the FDAA derivatization method described in Example 1. The numbers from those results are shown in Table 70 in parentheses.

Table 70: Comparison of *G. stearothermophilus*, Hybrid DAT, and *B. sphaericus* (purified) for Total Monatin Production

D-Aminotransferase	Total Monatin (ppm) 2 Hours	Total Monatin (ppm) Overnight	% R,R 2 Hours	% R,R Overnight
Hybrid DAT (untagged)	9.5	42.5	84.1 (79.8)	81.1 (69.6)
<i>B. sphaericus</i> (tagged)	68.5	182.5	62.7 (53.8)	55.1 (53.5)

<i>G. stearothermophilus</i> (untagged)	4.5	15.0	34.1 (20.7)	32.1 (21.7)
--	-----	------	-------------	-------------

[0607] The Hybrid DAT makes more monatin than the *G. stearothermophilus* enzyme, although the monatin transamination rate of the Hybrid DAT is lower. It is possible that under the conditions for monatin production (where there are low MP concentrations), the Hybrid DAT performs better possibly due to a lower K_m . Also, the Hybrid DAT makes a higher percentage of R,R than either of the parent enzymes. This enzyme appears to have a higher enantioselectivity for R-MP than the parent enzymes. The same assays were done (4 hour incubation time) using the *Sinorhizobium* aldolase described in Example 3A with the Hybrid DAT. The Hybrid DAT produced similar amounts of monatin as above, but using the alternative aldolase, produced 95% R,R (according to FDAA derivatization), as opposed to 80% with the *C. testosteroni* ProA aldolase.

[0608] The Hybrid DAT was also tested for transamination activity of R-MP versus S-MP (produced as described in Example 1). Two hour and overnight assays were conducted at 30 °C using 10 mM R-MP or S-MP, 50 mM D-alanine, 100 mM potassium phosphate pH 7.5, 0.5 mg/mL D-aminotransferase, and 50 μ M PLP. Experiments were run in duplicate and the background levels of monatin from the MP samples was subtracted. The ratios of monatin produced from each substrate are reported for both D-aminotransferases in Table 71 below. Similar trends were observed when pyruvate (produced) ratios were plotted. It is clear that the Hybrid DAT is more selective for R-MP than the AT-103 D-aminotransferase, which does not appear to be selective.

Table 71: Comparison of Hybrid DAT and AT-103 for S-MP and R-MP Transamination

D-Aminotransferase	R-Activity/S-Activity 2 Hours	R-Activity/S-Activity Overnight
Hybrid DAT (untagged)	8.6	2.2
AT-103	0.68	1.68

[0609] In an effort to further improve the Hybrid DAT activity, site directed mutagenesis was done. Primers were designed as suggested in the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). Two different mutants were created: Hybrid DAT 2 and Hybrid DAT 3. The Hybrid DAT 2 includes a mutation at amino acid position 153 from alanine to arginine and a deletion of serine 181. The alanine to arginine mutation was

designed to help coordinate the second carboxyl group in the monatin precursor substrate, as has been shown to be present in the AspC L-aminotransferase. The serine deletion was an attempt to remove some steric hindrance such that the larger monatin precursor molecule can get to the active site more easily. The Hybrid DAT 3 contains a deletion of serines 180-182, replaced by one arginine. Two additional mutants were created, having only the 153 ala to arg mutation or the serine deletion, respectively. All three of the mutants that contained deletions did not make soluble protein, although they overexpressed at very high concentrations. Clearly it is important structurally not to remove amino acids in this region. The ala153arg mutant did not produce monatin under the conditions tested (as above). There is a fair amount of steric hindrance near the 153 position which would make it more difficult to fit the monatin precursor substrate in without the deletions in the 180-182 region. It is expected that mutating the serines to smaller amino acids, such as glycine or alanine, would improve activity toward monatin precursor, particularly when combined with the ala153arg mutation.

EXAMPLE 20

Use of Commercially Available D-Amino acid Dehydrogenase Enzymes

[0610] D-amino acid dehydrogenases were part of a library purchased from BioCatalytics (Pasadena, CA).

Interconversion between MP and Monatin

[0611] The amination of MP to form monatin can be catalyzed by aminotransferases or by dehydrogenases that require a reducing cofactor such as NADH or NADPH. These reactions are reversible and can be measured in either direction. The directionality when using a dehydrogenase enzyme can be largely controlled by the concentration of ammonium salts.

Conversion of Monatin to MP (monatin precursor) using Commercially Available Dehydrogenases

[0612] The oxidative deamination of monatin was monitored by following the increase in absorbance at 340 nm as NAD^+ was converted to the more chromophoric NADH.

- [0613] The assay mixture contained 100 mM sodium bicarbonate, pH 9, 10 mM NAD⁺, 20 mg/mL of D-amino acid dehydrogenase (D-AADH-101 through 108, BioCatalytics), and 50 mM R,R monatin (monopotassium salt) in 0.2 mL. The assay was performed, in duplicate, in a UV-transparent microtiter plate, with incubation at 30 °C. Endpoint absorbances were measured using a Molecular Devices SpectraMax Plus plate_reader. Negative controls were carried out without the addition of enzyme. The change in absorbance for overnight reactions was as follows: no enzyme control, 0.05; D-AADH-101, 0.865; D-AADH-102, 1.075; D-AADH-103, 0.94; D-AADH-104, 0.335; D-AADH-105, 0.78; D-AADH-106, 0.745; D-AADH-107, 0.925; and D-AADH-108, 1.06.

Production of Monatin from MP Using Dehydrogenases

- [0614] R-MP used as a substrate for this assay was produced by the transamination of R,R monatin using AT-103 broad range D-aminotransferase (BioCatalytics) in potassium phosphate buffer, using pyruvate as the amino acceptor. S-MP was produced by the transamination of S,S monatin using AT-102 L-aminotransferase (BioCatalytics) in potassium phosphate buffer, using 2-oxoglutarate as the amino acceptor. Both compounds were purified using preparative scale HPLC.
- [0615] The assay mixture contained 200 mM ammonium formate, 50 mM potassium phosphate pH 7.5, 5 mM NADH, 20 mg/mL D-amino acid dehydrogenase (D-AADH-101 through 108, BioCatalytics), and 10 mM MP (potassium salt) in 0.25 mL. To half of the assays, 2 mg/mL formate dehydrogenase ("FDH") was added (FDH-101, BioCatalytics, 4.8 U/mg). The samples were incubated for 16 hours at 30 °C. The samples were analyzed for monatin using LC/MS/MS and the isomeric distribution was determined using the FDAA method described in Example 1. The background levels of the no D-amino acid dehydrogenase control were subtracted to account for the monatin contamination present in the MP.
- [0616] For production of R,R monatin from R-MP, the enzyme activity was as follows: D-AADH-103 > D-AADH-101 > D-AADH-107 > D-AADH 106 > D-AADH-108 > D-AADH-105. The amount of monatin generated from D-AADH 102 was quite low and D-AADH-104 did not appear to produce monatin from R-MP. Approximately 43 ppm of R,R monatin was produced by D-AADH-103 during the reaction in the absence of formate dehydrogenase. The addition of FDH improved the production of monatin for all the enzymes that had activity. The improvements ranged from 2.4 fold higher monatin to

10.1 fold higher monatin (D-AADH-103). D-AADH-103 produced approximately 434 ppm R,R monatin.

- [0617] When S-MP was used as the reaction substrate and production of S,R monatin was followed, the enzyme activity was as follows: D-AADH-106 > D-AADH-107 > D-AADH-105 > D-AADH-101 > D-AADH-102 > D-AADH-103 > D-AADH-108. D-AADH-104 did not appear to produce S,R monatin in the assays. Approximately 15 ppm S,R monatin was generated by D-AADH-106, 26 ppm when FDH enzyme was also used.

Production of Monatin from Indole-3-Pyruvate

- [0618] Production of monatin from indole-3-pyruvate and pyruvate, using BioCatalytics amino acid dehydrogenase enzymes coupled with the aldolase of SEQ ID NO:22, was assayed under the following conditions: 1 mg/mL dehydrogenase enzyme, 10 mM NADH, 500 µg/mL aldolase (purified), 50 mM potassium phosphate buffer pH 7.5, 4 mM MgCl₂, 20 mg/mL indole-3-pyruvate, 200 mM ammonium formate, and 200 mM pyruvate were incubated at 30 °C at 100 rpm for 20 hours. Negative controls contained no amino acid dehydrogenase enzyme. The experiments were performed in duplicate. None of the dehydrogenases appeared to produce quantifiable amounts of monatin from indole pyruvate and pyruvate (as measured by LC/MS/MS as described in Example 1) in comparison to the negative controls. However, large amounts of alanine and tryptophan were produced. It is expected that increasing the ratio of aldolase to dehydrogenase would improve monatin production. It is also expected that directed evolution approaches can be used to improve the ratio of reductive amination activity on MP versus pyruvate and indole-3-pyruvate.

EXAMPLE 21

Immobilization of *B. sphaericus* D-Alanine Aminotransferase

- [0619] The *Bacillus sphaericus* D-alanine aminotransferase was purified as the HIS₆-tagged protein as described in Example 14.
- [0620] The enzyme was immobilized onto Eupergit[®] C resin beads according to the procedure of Mateo, C, *et al.*, *Biotechnology Progress* 18:629-634, (2002). The purified enzyme (4 mL at 6.0 mg/mL) was dialyzed in 0.4 L of 0.5 M potassium phosphate, pH

7.8 using a Pierce Slide-A-Lyzer Dialysis Cassette (7K MWCO; catalog # 66370; Rockford, IL) for 1 hour at ambient temperature. The buffer was changed and the dialysis was continued for 1 hour. Pyridoxal phosphate ("PLP") was added to a final concentration of 0.05 mM and the resulting solution was mixed with 0.2 g of Eupergit[®] C resin purchased from Sigma-Aldrich (Fluka catalog #46115; St. Louis, MO). The enzyme-resin suspension was incubated at ambient temperature with gentle mixing overnight. The resin beads were separated from the enzyme solution by centrifugation at 4000 x g for 5 minutes. The supernatant was removed and the resin was washed with 3 x 3 mL of 100 mM potassium phosphate, pH 7.8 containing 0.05 mM PLP. The mixture was centrifuged at 3000 x g for 5 minutes between washes. The amount of protein bound to the resin was determined by measuring the amount of protein in each supernatant and subtracting the sum from the original amount of protein to be immobilized. The protein concentrations were measured using a Pierce BCA[™] Protein Assay Kit with bovine serum albumin as the standard (catalog #23225; Rockford, IL). The washed immobilized-enzyme beads were finally suspended in 4 mL of 100 mM potassium phosphate, pH 7.8 containing 0.05 mM PLP. The unreacted epoxy groups of the immobilized-enzyme beads were blocked by incubation with 1.9 M alanine at ambient temperature with gentle mixing. After 24 hours, the beads were washed, as described above, to remove the excess alanine and finally resuspended in 100 mM potassium phosphate, pH 7.8 containing 0.05 mM PLP. The final concentration of immobilized enzyme was 118 mg protein per g resin bead.

EXAMPLE 22

Immobilization of *S. meliloti* ProA Aldolase

- [0621] The *Sinorhizobium meliloti* HMG aldolase ("proA") was purified as the HIS₆-tagged protein using a procedure similar to the one described in Example 14 for the HIS₆-tagged *B. sphaericus* D-alanine aminotransferase.
- [0622] Starting from a fresh culture plate (LB agar with 50 µg/mL kanamycin) of BL21(DE3)::*S. meliloti* proA pET30(Xa/LIC), cells were grown in 5 mL of Luria-Bertani broth ("LB") with 50 µg/mL kanamycin at 37 °C with shaking at 225 rpm overnight.

Subsequently, the culture was transferred at 0.5-0.6% (v/v) into flasks containing 800 mL of LB broth with 50 µg/mL kanamycin. The cells were grown at 37 °C with shaking at 225 rpm until the OD₆₀₀ reached 0.6-0.7. The gene expression was induced by the addition of 0.2 mM IPTG. The cultures were further incubated at 30 °C for 4 hours with shaking at 225 rpm and then harvested by centrifugation in a Beckman J25II centrifuge (Fullerton, CA) with a JS-16.25 rotor at 10,000 rpm for 10 minutes. The cell pellet was washed once with cold 50 mM EPPS buffer, pH 8.2, and the cells were centrifuged again. The washed cell pellet was harvested and used immediately. To prepare cell-free extract containing the *S. meliloti* HIS₆-proA aldolase (HIS₆-SmelproA) protein, the cells were suspended in 3-4 volumes of 50 mM EPPS, pH 8.2, containing 100 mM NaCl, and then disrupted using a Microfluidics homogenizer (Newton, MA) (3 passes at 20,000 psi) while maintaining the temperature of the suspension below 15 °C. All subsequent purification steps were carried out at 4 °C. The cell extract was centrifuged for 15 minutes at 15,000 x g to remove the cell debris. Aliquots of the cell free extract, each containing between 15 and 20 mg of soluble protein, were applied to Novagen HIS-Bind columns (Novagen catalog # 70971-4, Madison, WI) that had been previously equilibrated with the Novagen Bind buffer. The columns were washed with 2 x 10 mL of the Novagen Bind buffer and 1 x 10 mL of the Novagen Wash buffer diluted 1:1 with the Bind buffer. The HIS₆-SmelproA was eluted with 5 mL of the Novagen Elute buffer from each column. The elution fractions from each column were combined and concentrated 2X with Amicon Ultra-15 centrifugal filter devices (MWCO 10 kDa) (Billerica, MA). The buffer was exchanged by passage through disposable GE Healthcare PD10 desalting columns (catalog #17-0851-01) previously equilibrated with 50 mM EPPS, pH 8.2, containing 100 mM NaCl.

[0623] The protein concentration of the desalted solution was determined using the Pierce BCATM Protein Assay Kit (catalog #23225; Rockford, IL). The purity of each fraction and the level of expression in the cell free extract fraction were determined by SDS-PAGE with a Bio-Rad Protean II minigel system (Hercules, CA) and 4-15% gradient gels. Typically, this procedure produced about 60-70 mg of enzyme from 3200 mL of LB culture with a purity of ~90%. Aliquots (1-5 mL) of the purified enzyme were stored at -80 °C until use.

- [0624] The enzyme was immobilized onto Eupergit® C resin beads according to the procedure of Mateo, C., *et al.*, (2002) *Biotechnology Progress* 18:629-634, (2002) and as described in Example 21 for the *B. sphaericus* D-alanine aminotransferase, except that 4 mM magnesium chloride was present in the buffer during immobilization instead of 0.05 mM PLP. After blocking with glycine, the washed immobilized enzyme was suspended in 100 mM potassium phosphate, pH 7.8 containing 4 mM magnesium chloride. The final concentration of *S. meliloti* proA aldolase was 52 mg protein per gram resin bead.

EXAMPLE 23

Production of R,R-Monatin Using Immobilized Enzymes

- [0625] The *B. sphaericus* HIS₆-tagged D-alanine aminotransferase and the *R. meliloti* HIS₆-tagged proA aldolase were purified and immobilized as described in Examples 21 and 22.
- [0626] Solutions of 50 mM sodium pyruvate, 40 mM D-tryptophan, 4 mM MgCl₂, and 50 μM PLP in 100 mM potassium phosphate, pH 7.8 were prepared in 15-mL polypropylene tubes with screw caps. To each of these solutions was added both of the immobilized enzymes to a final volume of 4 mL. The resulting suspensions were incubated at room temperature with gentle mixing for up to 24 hours. The progress of each reactions was followed by HPLC and/or LC-MS analyses, measuring D-tryptophan, D-alanine, R,R-monatin, and pyruvic acid. The isomeric purity of the product monatin was determined using chiral LC/MS/MS. All analytical methods are described in Example 1. Typical results from experiments using immobilized enzymes are shown in Table 72 below. Analysis of the isomeric purity of the monatin formed during the reaction showed that the product of the enzymatic reactions was between 74 and 80% R,R.

Table 72: Production of R,R-Monatin Using Immobilized Enzymes

proA Aldolase Concentration (μg/mL)	D-Alanine Aminotransferase Concentration (μg/mL)	Monatin Concentration (mM) (4 Hour Time point)	Tryptophan Concentration (mM) (4 Hour Time point)	Alanine Concentration (mM) (4 Hour Time point)
50	500	0.06	17.75	20.51
50	1000	0.29	15.03	24.71
100	1000	0.33	15.17	24.73

100	2000	0.54	14.40	29.45
-----	------	------	-------	-------

EXAMPLE 24

In vivo R,R Monatin Production and Transport by *E. coli*

[0627] Operons were constructed to demonstrate *in vivo* production of R,R monatin in *E. coli* using a D-aminotransferase ("DAT") and an R-specific aldolase. The R,R monatin was made from D-tryptophan using the pathway described in U.S. Patent Application Publication No. US 2005/0282260 A1 (Figures 1 and 2 and Example 11). Briefly, D-tryptophan is converted, *in vivo*, to indole-3-pyruvate by a transamination reaction in which an alpha-keto acid is also converted into a D-amino acid. Pyruvate is reacted with indole-3-pyruvate *in vivo* in an aldol condensation using an R-specific aldolase to produce predominantly R-MP. Lastly, the R-MP and the D-amino acid from the first reaction (or any D-amino acid in the cell) are converted *in vivo* to R,R monatin and the corresponding alpha-keto acid.

Construction of Plasmid pCEC-Nde

[0628] Plasmid pCEC-Nde was constructed by replacing the p15A origin of replication of the pPRONde plasmid (pProLAR from Clontech, modified as described in U.S. Published Application No. 20040235123) with the Col E1 origin of replication from pPROTet.E133 (Clontech Laboratories, Inc., Mountain View, CA). Both plasmids were treated with restriction enzymes *Avr* II and *Aat* II (New England Biolabs, Beverly, MA) and the appropriate fragments (1731-bp fragment from pPROTet.E133 carrying the Col E1 origin and chloramphenicol-resistance gene and 760-bp from pPRONde carrying the $P_{lac/ara}$ promoter region and multiple cloning site) were purified by agarose gel electrophoresis followed by extraction and recovery using the QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA). The purified DNA fragments were ligated together using the Quick Ligation Kit (New England Biolabs, Beverly, MA) and the ligation mixture transformed into chemically-competent *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA). Clones were isolated on LB agar containing 50 µg/mL chloramphenicol and confirmed by restriction digests of the plasmid.

[0629] Vector pCEC-Nde was digested with *Nde* I and *Bam*H I in *Bam*H I buffer (New England Biolabs, Ipswich, MA) and treated with shrimp alkaline-phosphatase as per

manufacturer's protocols (Roche, Indianapolis, IN). ATCC 4978 DAT (SEQ ID NO. 84) was digested from vector pET28 (Novagen, Madison, WI) (cloned in Example 15) with *Nde* I and *Bam*H I in *Bam*H I buffer. The *Nde* I and *Bam*H I digested vector and insert were purified using the Qiagen QIAquick[®] Gel Extraction Kit (Qiagen, Valencia, CA). Ligations were done using the Roche Rapid DNA Ligation Kit (Roche, Indianapolis, IN) and purified using the Roche High-Pure PCR purification kit (Roche, Indianapolis, IN). The ligations were transformed into *Escherichia coli* DH10B cells (Invitrogen, Carlsbad, CA) using a 0.2 cm cuvette and a Bio-Rad Gene Pulser[®] II system as described in the Bio-Rad electroporation manual. The cells were allowed to recover in 1.0 mL SOC medium (Sambrook, J., et al. *Molecular Cloning: A Laboratory Manual* 2nd ed., Plainview, NY, (1989), 1.76-1.81 & A.2)) for 1 hour at 37 °C at 250 rpm. Cells were plated on LB-agar plates containing chloramphenicol (25 µg/mL). Plasmid DNA was purified using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and screened for the correct inserts by PCR and restriction digestion with *Nde* I and *Bam*H I. The amino acid sequence from ATCC 4978 DAT is shown as SEQ ID NO. 86.

PCR of the Aldolase Gene of SEQ ID NO:172 from Diversa

[0630] Primers were designed based on the DNA sequence of SEQ ID NO:172, which encodes an enzyme having R-selective aldolase activity. The aldolase of SEQ ID NO:173 and the plasmid containing the nucleic acid sequence encoding that aldolase (the gene sequence of the specific aldolase is shown as SEQ ID NO:172, were obtained from Diversa Corporation, San Diego, California. SEQ ID NO:172 was part of a library which was screened by Diversa Corp. for aldolase genes. However, the aldolase gene of SEQ ID NO:172 may be reconstructed by any method known to a person of ordinary skill in the art. For example, the aldolase gene of SEQ ID NO:172 may be reconstructed utilizing assembly PCR methods known to one skilled in the art. The primers were designed to contain restriction sites and a ribosomal binding site in front of the aldolase gene for cloning and expression. The oligonucleotide primer sequences that were used were:

[0631] AldolaseFpstIrb:

5'-GGCCGGAAGTGCAGAAGAAGGAGATATATAATGAAGCCGGTGGTGGTG-3'
(SEQ ID NO:174) and

[0632] AldolaseRxbal

5'-GGCCAAGGTCTAGATTAGACATAGGTGAGCCC-3' (SEQ ID NO:175).

- [0633] PCR was done using the above primers, with template pET28/SEQ ID NO:172. PCR was carried out as follows: per 50 μ L reaction, 0.5 μ L template, 0.8 μ L of each primer, 2 μ L dNTPs, 0.8 μ L Expand High Fidelity Polymerase (Roche, Indianapolis, IN), 1X Expand™ buffer, and 0.2 μ L Pfu polymerase (Stratagene, La Jolla, CA) were added. A 3 minute hot start was done at 94 °C, followed by 8 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 90 seconds. Twenty-two more cycles were done with an increased annealing temperature of 55 °C. Lastly, a chain extension step was done for seven minutes at 72 °C. The PCR product was purified using the Qiagen QIAquick® PCR purification kit (Qiagen, Valencia, CA), and digested with *Pst* I and *Xba* I in Buffer 3 (New England Biolabs, Ipswich, MA). *Pst* I and *Xba* I digested vector pCECNde/4978 DAT and insert were ligated using the Roche Rapid DNA Ligation Kit (Roche, Indianapolis, IN) and purified using the QIAquick® PCR purification kit. The ligation was transformed into *Escherichia coli* DH10B cells using a 0.2 cm cuvette and a Bio-Rad Gene Pulser® II system as described in the Bio-Rad electroporation manual. The cells were allowed to recover in 1.0 mL SOC medium for 1 hour at 37 °C with shaking at 250 rpm. Cells were plated on LB-agar plates containing chloramphenicol (25 μ g/mL).
- [0634] Transformants were grown in LB broth containing chloramphenicol (25 μ g/mL) at 37 °C with shaking at 250 rpm. Plasmid DNA was purified using the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and inserts were verified by restriction digestion with *Pst* I and *Xba* I. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing (Agencourt; Beverly, MA). The resulting plasmid, pCECNde/4978DAT/SEQ ID NO:172, designated RR2 (vector RR2), was used to transform strain *Escherichia coli* MG1655 as described below.
- [0635] *E. coli* MG1655 strain was grown in LB broth at 37 °C with shaking at 250 rpm. Electrocompetent *E. coli* MG1655 were prepared by subculturing a 1% inoculum of cells to an OD 600 of ~0.6. The bacteria were pelleted by centrifugation (10 minutes at 10,000 x g) and washed in an equal volume of 10% glycerol. The wash was repeated twice in half volume of 10% glycerol. Finally, the cells were washed in one-fourth volume of 10% glycerol. Following centrifugation, the cells were resuspended in 500 μ L of 10% glycerol. Thirty μ L aliquots were frozen and kept at -80 °C until use.
- [0636] Vector RR2 (i.e., plasmid pCECNde/4978DAT/SEQ ID NO:172, prepared above), was transformed into electrocompetent *E. coli* MG1655 cells using a 0.2 cm

cuvette and a Bio-Rad Gene Pulser[®] II system as described in the Bio-Rad electroporation manual. The cells were allowed to recover in 1 mL SOC medium for 1 hour at 37 °C with shaking at 250 rpm. Cells were plated on LB-agar plates containing chloramphenicol (25 µg/mL).

[0637] For inoculum preparation, the *E. coli* MG1655 : : pCECNde/4978 DAT/SEQ ID NO:172 were grown overnight at 37 °C with shaking at 250 rpm in Luria-Bertani ("LB") medium with 25 µg/mL chloramphenicol. For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman *et al. Folia Microbiol.* 35:200-4, 1990), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g (NH₄)₂SO₄, 13.6 g KH₂PO₄. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g MgSO₄·7H₂O, 0.01 g CaCl₂·2H₂O, and 0.5 mg FeSO₄·7H₂O were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution (Neidhardt F.C., Bloch P.L., and Smith D.F., 1974. Culture medium for *Enterobacteria*. *J. Bacteriol.* 119: 736-746). Neidhardt's medium includes (per liter): 0.18 g (NH₄)₆(MO₇)₂₄·4H₂O, 1.24 g H₃BO₃, 0.36 g CoCl₂·6H₂O, 0.12 g CuSO₄ (anhydrous), 0.8 g MnCl₂·4H₂O, and 0.14 g ZnSO₄·7H₂O. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0638] For treatments, 3.1 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 25 µg/mL chloramphenicol. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then 30 °C following induction. At 0.50-0.526 OD₆₀₀, induction of the plasmid genes was initiated. At induction, 1.0 mM IPTG, 0.5% L-arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mL of Balch's vitamins (Balch, W. E., *et al.*, 1979, *Microbiol. Rev.* 43:260-296) were added. Additions of 10 g/L D-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") were made at 3 hours following induction, while 0.2% Tween[®] 20 (polyoxyethylene 20-sorbitan monolaurate) and 10 µg/mL ampicillin were added 6 hours following induction. Either 5 mM D-glutamate or D-alanine was also added at 3 hours following induction. A second addition of 10 g/L sodium pyruvate was made at 24 hours

following inoculation. Samples for monatin and dry cell weight determination were taken at 48 hours.

[0639] The monatin from the 48 hour samples was concentrated via a solid phase extraction ("SPE") column using the methodology described below. The R,R and S,S stereoisomers were analyzed by the FDAA derivatization method of Example 1. Results are shown in Table 73 below.

Method For Concentration Of Monatin Utilizing Solid Phase Extraction

[0640] An Oasis[®] HLB 3cc (60 mg) Extraction Cartridge (Waters Corp., Milford, Massachusetts) was used to concentrate the samples. The fermentation samples were first centrifuged to remove cell material. Formic acid was added to the fermentation sample for a final concentration of about 1%. The extraction cartridge was conditioned with at least 2 mL methanol, followed by 2 mL 1% formic acid prior to the addition of the fermentation sample. Each solution was drawn through the extraction cartridge separately. Then, up to 5 mL of the undiluted, centrifuged fermentation sample containing 1% formic acid was added to the extraction cartridge. The fermentation solution was allowed to draw through the cartridge slowly. The cartridge was rinsed with at least 2 mL of 1% formic acid. 1 mL of either acetonitrile or methanol was added to the cartridge and was allowed to draw through the cartridge slowly. This eluent was placed under a gentle stream of nitrogen until it was dry. The dry sample was reconstituted into 150-200 μ L water or mobile phase. 150 μ L was placed into a 300 μ L HPLC plastic vial and was injected into the liquid chromatograph. Table 73 shows the total monatin per dry cell weight and the portion of the total that was the R,R or S,S stereoisomer.

Table 73: R,R Monatin excreted by *E. coli*

Strain	D-amino acid treatment	48 hour Monatin / dry cell wt. (mg/g)	Portion as:	
		Total Monatin	R,R Monatin	S,S Monatin
<i>E. coli</i> MG1655 : : pCECNde/4978 DAT/SEQ ID NO:172	D-glutamate	0.47	~50%	~50%
<i>E. coli</i> MG1655 : : pCECNde/4978 DAT/SEQ ID NO:172	D-alanine	0.33	nd	nd

[0641] R,R Monatin production and transport was conclusively demonstrated using the strain *E. coli* MG165 : : pCECNde/4978 DAT/SEQ ID NO:172 and D-glutamate. Some S,S monatin was produced, probably as a result of using the *E. coli* MG1655 strain that normally expresses endogenous L-aminotransferases that are capable of making S,S monatin from S-MP. The total monatin produced with D-alanine was not enough to conduct an analysis to determine the stereoisomeric ratio of monatin produced. However, it is expected that the same ratio of R,R monatin would be formed, even though the amount produced was below the threshold of analysis. It is also expected that the same transporters capable of transporting S,S monatin should be capable of transporting R,R monatin as well.

[0642] The DNA sequence of SEQ ID NO:172 is shown below.

```
gaagccgg tgggtggtgca gactatcgag cgggccgacc gagcgatcat
cgagggtctg gccgcgtgtg gcgttgccac cgtccatgag gcgcaggggc
gccgggggct gcttgcgctcc tacatgcgcc cgatctattc gggcgctgcg
gttgcgggcct cggccgtcac catcctctct ccaccctgcg acaactggat
gctgcacgtc gccatcgagc agatccagcc gggcgacatt ctggttctcg
gcacgacctc tccgtccgat gccggctatt tcggtgatct gctggcgact
tcggccaagg cgcgcgggtg cgtcggggtg gtcacgatg ccggcgtaag
cgatatccgc gacctgacag cgatgcagtt tccggtctgg tccaaggccg
tttcggccca gggcacgata aaggagacgc tgggttcggt caacgtcccc
gtcgtctgcg ccggtgctct ggtcaatccc ggcgacgtcg tcgtggccga
tgacgacggt gtctgcgtgg tgcgcgcgca ggaagccgcg gaaacgctgg
aaaaggcccc ggcgcggtat gccaatgagg aggaaaagcg ccagcgcttt
gccgctggcg aactcgggct cgacatctac aagatgcgcg aacgcctcgc
tgccctgggg ctcacctatg tctga (SEQ ID NO:172).
```

[0643] The amino acid sequence of the aldolase of SEQ ID NO:173 is shown below.

Met Lys Pro Val Val Val Gln Thr Ile Glu Arg Ala Asp Arg Ala Ile
 Ile Glu Gly Leu Ala Ala Cys Gly Val Ala Thr Val His Glu Ala Gln
 Gly Arg Arg Gly Leu Leu Ala Ser Tyr Met Arg Pro Ile Tyr Ser Gly
 Ala Ala Val Ala Ala Ser Ala Val Thr Ile Leu Ser Pro Pro Cys Asp
 Asn Trp Met Leu His Val Ala Ile Glu Gln Ile Gln Pro Gly Asp Ile
 Leu Val Leu Gly Thr Thr Ser Pro Ser Asp Ala Gly Tyr Phe Gly
 Asp Leu Leu Ala Thr Ser Ala Lys Ala Arg Gly Cys Val Gly Leu
 Val Ile Asp Ala Gly Val Arg Asp Ile Arg Asp Leu Thr Ala Met

Gln Phe Pro Val Trp Ser Lys Ala Val Ser Ala Gln Gly Thr Ile Lys
Glu Thr Leu Gly Ser Val Asn Val Pro Val Val Cys Ala Gly Ala
Leu Val Asn Pro Gly Asp Val Val Val Ala Asp Asp Asp Gly Val
Cys Val Val Arg Arg Glu Glu Ala Ala Glu Thr Leu Glu Lys Ala
Arg Ala Arg Ile Ala Asn Glu Glu Glu Lys Arg Gln Arg Phe Ala
Ala Gly Glu Leu Gly Leu Asp Ile Tyr Lys Met Arg Glu Arg Leu
Ala Ala Leu Gly Leu Thr Tyr Val (SEQ ID NO:173).

EXAMPLE 25

Production of R,R-Monatin Using the Aldolase of SEQ ID NO:104: Optimization of Reaction Conditions

[0644] The *Bacillus sphaericus* (ATCC strain 10208) D-alanine aminotransferase cloned in Example 18 was purified as the HIS₆-tagged protein as described in Example 14 using the improved method, with potassium phosphate buffers containing PLP). The aldolase of SEQ ID NO:104 (cloned in Example 3B) was purified as the HIS₆-tagged protein as described in Example 27.

[0645] The preferred metal cofactor for the aldolase of SEQ ID NO:104 was determined by screening a variety of divalent metals. The reactions were set up anaerobically in 10 mL serum bottles with 7 mL final volumes. A bulk solution consisting of 100 mM potassium phosphate (pH 7.8), 200 mM sodium pyruvate, 0.05 mM PLP and 0.01% (v/v) Tween 80 was prepared to a final volume of 48.8 mL and sparged with nitrogen for 30 minutes. D-Tryptophan (143 mg; final concentration of 100 mM) was dispensed into seven 10 mL serum vials. To each of the vials was added 0.014 mL of a 2 M stock solution of a divalent metal cation, prepared from the chloride salt (final concentration of 4 mM). For the negative control, 0.014 mL of dH₂O was added. The serum vials were capped with rubber septa and sparged with nitrogen via 16-18 gauge needles. Under anaerobic conditions, 5.625 mL of the anaerobic bulk solution was added to each anaerobic serum bottle. Subsequently, the *B. sphaericus* D-alanine aminotransferase and the aldolase of SEQ ID NO:104 were added anaerobically to each serum bottle to a final concentration of 2 mg/mL and 0.05 mg/mL, respectively. The solutions were incubated

at room temperature with gentle mixing for 18 hours. Final monatin was analyzed according to the methods described in Example 1 using the Liquid Chromatography-Post Column Fluorescence Detection of Amino Acids method. (Table 74).

Table 74

Metal Cofactor	Final Monatin (mM) at 18 h
None (negative control)	1.7
Magnesium	10.6
Manganese	10.0
Cobalt	6.7
Zinc	4.9
Nickel	1.5
Calcium	0.7

[0646] The reaction conditions for the aldolase of SEQ ID NO:104 were further investigated with a two-level fractional factorial experiment designed using the statistical software Design Expert 7.0.0 (Stat-Ease, Inc.; Minneapolis, MN). The screening design consisted of a single block of five factors at two levels with four centerpoints (20 runs total). The five factors to be optimized were the metal cofactor concentration, reaction pH, Tween[®] 80 concentration, pyruvate to tryptophan ratio, and the aldolase concentration (Table 75).

[0647] Conical polypropylene tubes (14 mL) containing 143 mg of D-tryptophan were de-oxygenated in an anaerobic glove box (Coy Laboratory Products, Inc; Grass Lake, MI) overnight. Stock solutions of 2 M MgCl₂; 1 M potassium phosphate at pH 7.0, 7.75, and 8.5; 10% (v/v) Tween 80; 2 M sodium pyruvate, and 10 mM PLP were prepared in degassed water and equilibrated in the anaerobic glove box overnight. Stock solutions of purified *B. sphaericus* D-alanine aminotransferase and the aldolase of SEQ ID NO:104 were thawed on ice and used in the anaerobic glove box immediately. Stock solutions were added to the 14 mL conical tubes containing the D-tryptophan to obtain the concentrations determined by the statistical design (Table 75). Degassed water was added to each tube to bring the final volume, along with the enzyme additions, to 7.0 mL. The tubes were incubated at room temperature in the anaerobic glove box with gentle mixing for up to 24 hours. Monatin concentration and isomeric purity were analyzed

according to the methods described in Example 1 using the Liquid Chromatography-Post Column Fluorescence Detection of Amino Acids method and the LC/MS/MS Multiple Reaction Monitoring for the Determination of the Stereoisomer Distribution of Monatin in *in vitro* and *in vivo* Reactions method (FDAA derivatization method).

Table 75

Run #	std #	Block	Mg (mM)	pH	Tween [®] (%)	Pyr:Trp	Aldolase of SEQ ID NO:104 (mg/mL)
20	1	Block 1	5.00	7.75	0.01	2.00	0.05
8	2	Block 1	9.00	8.50	0.02	1.00	0.01
3	3	Block 1	1.00	8.50	0.00	1.00	0.01
16	4	Block 1	9.00	8.50	0.02	3.00	0.09
7	5	Block 1	1.00	8.50	0.02	1.00	0.09
12	6	Block 1	9.00	8.50	0.00	3.00	0.01
6	7	Block 1	9.00	7.00	0.02	1.00	0.09
2	8	Block 1	9.00	7.00	0.00	1.00	0.01
15	9	Block 1	1.00	8.50	0.02	3.00	0.01
4	10	Block 1	9.00	8.50	0.00	1.00	0.09
5	11	Block 1	1.00	7.00	0.02	1.00	0.01
1	12	Block 1	1.00	7.00	0.00	1.00	0.09
13	13	Block 1	1.00	7.00	0.02	3.00	0.09
14	14	Block 1	9.00	7.00	0.02	3.00	0.01
17	15	Block 1	5.00	7.75	0.01	2.00	0.05
11	16	Block 1	1.00	8.50	0.00	3.00	0.09
18	17	Block 1	5.00	7.75	0.01	2.00	0.05
9	18	Block 1	1.00	7.00	0.00	3.00	0.01
19	19	Block 1	5.00	7.75	0.01	2.00	0.05
10	20	Block 1	9.00	7.00	0.00	3.00	0.09

[0648] Statistical analysis of the data indicated that reaction pH, pyruvate:tryptophan ratio and aldolase concentration were the significant factors affecting monatin titer, isomeric purity and carbon yield. A desirability graph was generated using the Design Expert software in which the factors were varied in order to maximize the goals of highest monatin titer and highest isomeric purity under conditions of excess pyruvate. The reactions conditions indicated as optimum were 1 mM MgCl₂, pH >8.0, 0.01% (v/v) Tween[®] 80, and 0.01 mg/mL aldolase of SEQ ID NO:104. This is a 5-fold reduction in the typical amount of aldolase utilized, as well as a 4-fold reduction in the amount of divalent metal typically used.

[0649] Additional experiments were performed to determine the optimum pH range for the reaction process. Stock solutions of 1 M EPPS buffer were prepared at increments of 0.2 pH units between pH 7.0 and 9.0. These solutions were degassed and equilibrated in

the anaerobic glove box overnight. Polypropylene tubes (14 mL) containing 143 mg of D-tryptophan were de-oxygenated in an anaerobic glove box overnight. Stock solutions of 2 M MgCl_2 , 10% (v/v) Tween[®] 80, 2 M sodium pyruvate and 10 mM PLP were prepared in degassed water and equilibrated in the anaerobic glove box. Preparations of purified *B. sphaericus* D-alanine aminotransferase and the aldolase of SEQ ID NO:104 were thawed on ice and used immediately in the anaerobic glove box. The stock solutions were added to the 14 mL conical tubes to give a final concentration of 100 mM EPPS, 200 mM pyruvate, 100 mM tryptophan, 1 mM MgCl_2 , 0.01% (v/v) Tween[®] 80, 0.05 mM PLP, 2 mg/mL *B. sphaericus* D-alanine aminotransferase, and 0.01 mg/mL aldolase of SEQ ID NO:104 in a total volume of 7 mL per tube. The reactions were incubated at room temperature in the anaerobic glove box with gentle agitation for 22 hours. Samples were removed and analyzed for monatin as described in Example 1 using the LC/MS/MS multiple reaction monitoring method (Table 76).

Table 76

Reaction pH	Monatin (mM) at 22 h
7.0	5.8
7.2	9.9
7.4	7.8
7.6	10.6
7.8	14.0
8.0	14.2
8.2	14.3
8.4	12.6
8.6	12.3
8.8	10.8
9.0	11.1

[0650] The results indicated that monatin formation increased with increasing pH between 7.0 – 8.0. Monatin formation reached a maximum in the range of pH 8.0 – 8.2 and decreased above pH 8.4. Additionally, the isomeric purity of monatin decreased above pH 8.4.

EXAMPLE 26

Purification of the T243N Mutant of the D-Aminotransferase
from ATCC Strain 4978

- [0651] The T243N mutant of the D-aminotransferase from ATCC strain 4978 with an amino-terminal HIS₆-purification tag (described in Example 6B) was produced using the EMD Biosciences Overnight Express System II (solutions 1-6) (La Jolla, CA) containing 50 µg/mL kanamycin in shake flasks. This expression system induces the expression of IPTG-inducible systems without the need to monitor cell growth. After inoculation of 200 mL aliquots of the medium (in 1 L flasks) from either liquid cultures or plates of the *E. coli* BL21(DE3) host cells carrying the gene for the T243N mutant D-aminotransferase from ATCC strain 4978 on the plasmid pET28b, the cultures were incubated at 30 °C overnight with shaking at 225 rpm. When the OD₆₀₀ had reached a minimum of 6, the cells were harvested by centrifugation and washed once with buffer.
- [0652] Cell free extract was prepared using EMD Biosciences BugBuster[®] (primary amine-free) Extraction Reagent (La Jolla, CA) containing 1 µL/mL Benzonase[®] Nuclease, 5 µL/mL Protease Inhibitor Cocktail Set II, and 0.033 µL/mL rLysozyme[™] according to the manufacturer's protocol. All subsequent purification steps were carried out at 4 °C. The cell extract was centrifuged for 20-30 minutes at 15,000 x g to remove the cell debris. A 20-25 mL aliquot of the cell free extract was applied to a 45 mL column of GE Healthcare Chelating Sepharose[™] Fast Flow resin (nickel (II) form) (Piscataway, NJ) that had been previously equilibrated with 100 mM potassium phosphate containing 200 mM sodium chloride and 50 µM PLP. To generate the nickel form of the resin, the resin was washed with 150 mL of 200 mM nickel (II) sulfate hexahydrate and then with 150 mL of distilled water. After loading the sample, the column was washed/eluted with 150 mL of the equilibration buffer containing 25 mM imidazole, 150 mL of the equilibration buffer containing 50 mM imidazole and 150 mL of the equilibration buffer containing 500 mM imidazole. The HIS₆-tagged protein eluted in the last wash. The 500 mM imidazole wash was concentrated with Millipore/Amicon Centricon Plus-70 centrifugal filter devices (MWCO 10 kDa) (Billerica, MA) to 15-20 mL according to the manufacturer's instructions. The imidazole and sodium chloride

were removed by passage through disposable GE Healthcare PD10 columns (2.5 mL sample per column) previously equilibrated with 100 mM potassium phosphate, pH 7.8 containing 50 μ M PLP. The purified aminotransferase was eluted with 3.5 mL per column of the same buffer. The protein concentration of each fraction was determined using the Pierce BCA assay kit (Rockford, IL) with BSA as the protein standard. The purity of each fraction and the level of expression in the cell free extract fraction were determined using a Bio-Rad Experion microcapillary chip system (Hercules, CA) or using Bio-Rad 4-15% SDS-polyacrylamide gradient gels run in a Mini PROTEAN[®] 3 cell apparatus. The protein was visualized in the polyacrylamide gels using BioRad Bio-Safe G-250 Coomassie stain and destained with water. Typically this procedure produces ~ 20 mg of enzyme from 200 mL of overnight culture that is 85-90% pure as judged by the Experion software or from analysis of the SDS-PAGE gels. Aliquots (1-5 mL) of the purified enzyme were stored at -80 °C until use.

EXAMPLE 27

Expression and Purification of the Aldolase of SEQ ID NO:104

- [0653] The cloning of the *E. coli* BL21(DE3)pLysS host cells carrying the gene for the aldolase of SEQ ID NO:104 (the gene is shown as SEQ ID NO:103) on the pET28b plasmid is described in Example 3B.
- [0654] The aldolase of SEQ ID NO:104 with an amino-terminal HIS₆-purification tag was produced using the EMD Biosciences Overnight Express System II (solutions 1-6) (La Jolla, CA) containing 50 μ g/mL kanamycin in shake flasks. This expression system induces the expression of IPTG-inducible systems without the need to monitor cell growth. After inoculation of 200 mL aliquots of the medium (in 1 L flasks) from either liquid cultures or plates of the aldolase construct, the cultures were incubated at 30 °C overnight with shaking at 225 rpm. When the OD₆₀₀ had reached a minimum of 6, the cells were harvested by centrifugation and washed once with buffer.
- [0655] To prepare cell free extract containing the aldolase, the cells were suspended in 3-4 volumes of 100 mM potassium phosphate, pH 7.8 and then disrupted using a Microfluidics homogenizer (Newton, MA) (3 passes at 18,000 psi), maintaining the

temperature of the suspension at less than 15 °C. Alternatively, cell free extract was prepared using EMD Biosciences BugBuster[®] (primary amine-free) Extraction Reagent (La Jolla, CA) containing 1 µL/mL Benzonase[®] Nuclease, 5 µL/mL Protease Inhibitor Cocktail Set II, and 0.033 µL/mL rLysozyme[™] according to the manufacturer's protocol. All subsequent purification steps were carried out at 4 °C. The cell suspension was centrifuged for 20-30 minutes at 15,000-20,000 x g to remove the cell debris. A 20-25 mL aliquot of the cell free extract was applied to a 45 mL column of GE Healthcare Chelating Sepharose[™] Fast Flow resin (nickel (II) form) (Piscataway, NJ) that had been previously equilibrated with 100 mM potassium phosphate containing 200 mM sodium chloride. To generate the nickel form of the resin, the resin was washed with 150 mL of 200 mM nickel (II) sulfate hexahydrate and then with 150 mL of distilled water. After loading the sample, the column was washed/eluted with 150 mL of the equilibration buffer containing 25 mM imidazole, 150 mL of the equilibration buffer containing 50 mM imidazole and 150 mL of the equilibration buffer containing 500 mM imidazole. The HIS₆-tagged protein eluted in the last wash. The 500 mM imidazole wash was concentrated with Millipore/Amicon Centricon Plus-70 centrifugal filter devices (MWCO 10 kDa) (Billerica, MA) to 15-20 mL according to the manufacturer's instructions. The imidazole and sodium chloride were removed by passage through disposable GE Healthcare PD10 columns (2.5 mL sample per column) previously equilibrated with 100 mM potassium phosphate, pH 7.8. The purified aldolase was eluted with 3.5 mL per column of the same buffer. The protein concentration of each fraction was determined using the Pierce BCA assay kit (Rockford, IL) using BSA as the protein standard. The purity of each fraction and the level of expression in the cell free extract fraction were determined using a Bio-Rad Experion microcapillary chip system (Hercules, CA) or using Bio-Rad 4-15% SDS-polyacrylamide gradient gels run in a Mini PROTEAN[®] 3 cell apparatus. The protein was visualized in the polyacrylamide gels using BioRad Bio-Safe G-250 Coomassie stain and destained with water. Typically this procedure produces ~ 50 mg of enzyme from 400 mL of overnight culture that is 85-95% pure, as judged by the Experion software. Aliquots (1-5 mL) of the purified enzyme were stored at -80 °C until use. Preparation of the enzyme in this manner reduced the level of precipitation of the enzyme previously noted. The presence of magnesium in the storage buffer had no effect on the level of precipitation.

5 In the specification and the claims the term “comprising” shall be understood to have a broad meaning similar to the term “including” and will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. This definition also applies to variations on the term “comprising” such as “comprise” and “comprises”.

10 The reference to any prior art in this specification is not, and should not be taken as an acknowledgement or any form of suggestion that the referenced prior art forms part of the common general knowledge in Australia.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of producing R,R monatin or salt thereof from D-tryptophan comprising:

- (a) converting L-tryptophan to D-tryptophan;
- (b) converting the D-tryptophan to indole-3-pyruvate (I3P);
- (c) converting the I3P to R-MP; and
- (d) converting the R-MP to R,R monatin;

wherein the conversion of L-tryptophan to D-tryptophan is facilitated by one or more enzymes selected from the group consisting of an alanine racemase comprising the amino acid sequence of SEQ ID NO:41 with a mutation chosen from a Y354C mutation, a Y354T mutation, a Y354G mutation, a Y354L mutation, a Y354N mutation, a Y354S mutation, a Y354I mutation, a Y354M mutation, a Y354P mutation, and a combination of a Y354A, M35V, and R195H mutation, a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:120 or the amino acid sequence of SEQ ID NO:120 with a Y396C mutation, a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:128 or the amino acid sequence of SEQ ID NO:128 with a I384M mutation, a *Vibrio fischeri* alanine racemase of Genbank Accession No. AAW85230.1 or Genbank Accession No. YP_204118, a broad specificity amino acid racemase isolated from *Pseudomonas taetrolens*, a broad specificity amino acid racemase isolated from *Pseudomonas taetrolens* with a I384M mutation, a broad specificity amino acid racemase isolated from *Pseudomonas putida* (also known as *Pseudomonas striata*), a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:116, a broad specificity amino acid racemase of Genbank Accession No. ZP_00898332.1 GI:82735470, a broad specificity amino acid racemase from ATCC 4683, a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:204 or the amino acid sequence of SEQ ID NO:204 with a I384M mutation, a broad specificity amino acid racemase isolated from *P. putida* KT2440, a broad specificity amino acid racemase isolated from *P. putida* NBRC 12996, a broad specificity amino acid racemase from ATCC 7966, a broad specificity amino acid racemase isolated from *Pseudomonas strain* 2150, a broad specificity amino acid

racemase isolated from *P. oleovorans*, a broad specificity amino acid racemase isolated from *P. aureofaciens*, a broad specificity amino acid racemase isolated from *P. putida* 12633, a broad specificity amino acid racemase isolated from *P. fluorescens*, a broad specificity amino acid racemase isolated from *P. putida* SCRC 744, a broad specificity amino acid racemase isolated from *P. graveolens*, a broad specificity amino acid racemase isolated from *P. striata* AKU 083, a broad specificity amino acid racemase comprising amino acids 24-409 of SEQ ID NO:204, an enzyme having the activity of a broad specificity amino acid racemase isolated from *Aeromonas jandei*, a broad specificity amino acid racemase from ATCC 49572, a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:194, a broad specificity amino acid racemase isolated from *Aeromonas sobria*, a broad specificity racemase from ATCC 35994, a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:192, an enzyme corresponding to the broad specificity amino acid racemase isolated from *Aeromonas caviae* with a L383M mutation, an enzyme having the activity of a broad specificity amino acid racemase from ATCC 14486 with a L383M mutation, an enzyme having the activity of a broad specificity amino acid racemase encoded by a gene comprising SEQ ID NO:195, an enzyme having the activity of a broad specificity amino acid racemase encoded by a gene comprising SEQ ID NO:196, an enzyme having the activity of a broad specificity amino acid racemase comprising SEQ ID NO:201, an enzyme having the activity of a broad specificity amino acid racemase comprising SEQ ID NO:202, an enzyme corresponding to the amino acid racemase from *Aeromonas caviae* with a D76N mutation, an enzyme having the activity of a broad specificity amino acid racemase corresponding to SEQ ID NO:179 with a D76N mutation, a broad specificity amino acid racemase isolated from *Aeromonas hydrophila*, a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:177, a broad specificity amino acid racemase comprising amino acids 22-408 of SEQ ID NO:177, a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:146, a broad specificity amino acid racemase isolated from *Aeromonas caviae*, a broad specificity amino acid racemase comprising the amino acid sequence of SEQ ID NO:179, a broad specificity amino acid racemase comprising amino acids 22-408 of SEQ ID NO:179, a broad specificity amino acid racemase comprising a partial sequence of SEQ ID NO:155, a broad specificity amino acid racemase comprising the

amino acid sequence of SEQ ID NO:151, a broad specificity amino acid racemase encoded by Genbank Accession No. AB096176, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to Genbank Accession No. AB096176 with a substitution such that the racemase includes a I384M mutation, a broad specificity amino acid racemase encoded by SEQ ID NO:119, a broad specificity amino acid racemase encoded by SEQ ID NO:127, a broad specificity amino acid racemase encoded by CP 000020.1 GI:59478708 region 800842..802053, a broad specificity amino acid racemase encoded by NC_006840, a broad specificity amino acid racemase encoded by KT2440 Bar DNA, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:119 with a substitution such that the racemase includes a Y396C mutation, a broad specificity amino acid racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:127 with a substitution such that the racemase includes a I384M mutation, a broad specificity amino acid racemase comprising SEQ ID NO:151, a broad specificity amino acid racemase encoded by a sequence comprising SEQ ID NO:154, a broad specificity amino acid racemase encoded by NZ_AALMOI000002 nucleotides 53173..54402, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:140, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO: 140 with a substitution such that the racemase includes a I384M mutation, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO: 178, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:178 with a substitution such that the racemase includes a L383M mutation, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:193, a broad specificity racemase encoded by a nucleic acid sequence corresponding to SEQ ID NO:176, active fragments thereof having racemase activity, and enzymes comprising a sequence with at least 90% sequence identity to any of the above.

2. The method of claim 1, wherein the conversion of R-MP to R,R monatin is facilitated by a broad specificity D-aminotransferase selected from the group consisting of:

a) an enzyme having the activity of a D-aminotransferase chosen from one or more of: a D-aminotransferase corresponding to SEQ ID NO:86 having a T243S mutation, a T243N

mutation, a N100A mutation, a T243Q mutation, T243N and N100A mutations, a F200M mutation, a F200Y mutation, and F200M and T243N mutations;

b) enzymes comprising a sequence having a percent sequence identity of at least 90% to any of the enzymes in a);

c) a *Bacillus halodurans* D-aminotransferase, a hybrid D-aminotransferase that is a hybrid between *Geobacillus stearothermophilus* D-aminotransferase and *Bacillus sphaericus* D-aminotransferase, a *Bacillus sphaericus* D-aminotransferase, a *Geobacillus stearothermophilus* D-aminotransferase, a *Bacillus licheniformis* D-aminotransferase, a D-aminotransferase from ATCC 7063, a *Bacillus licheniformis* branched chain aminotransferase having D-aminotransferase activity; and

d) combinations of a-c.

3. The method of claim 1, wherein the conversion of I3P to R-MP is facilitated by an aldolase enzyme that preferentially produces R-MP to S-MP in a ratio greater than 1:1.

4. The method of claim 3, wherein the aldolase enzyme is an R-specific aldolase enzyme.

5. The method of claim 4, wherein the aldolase enzyme is selected from the group consisting of an aldolase enzyme comprising the amino acid sequence of SEQ ID NO:22, an aldolase enzyme comprising the amino acid sequence set forth in SEQ ID NO:104, and *S. meliloti* HMG aldolase.

6. A method for producing R,R monatin or a salt thereof from D-tryptophan, substantially as hereinbefore described with reference to any one of the examples or figures.

FIG. 1

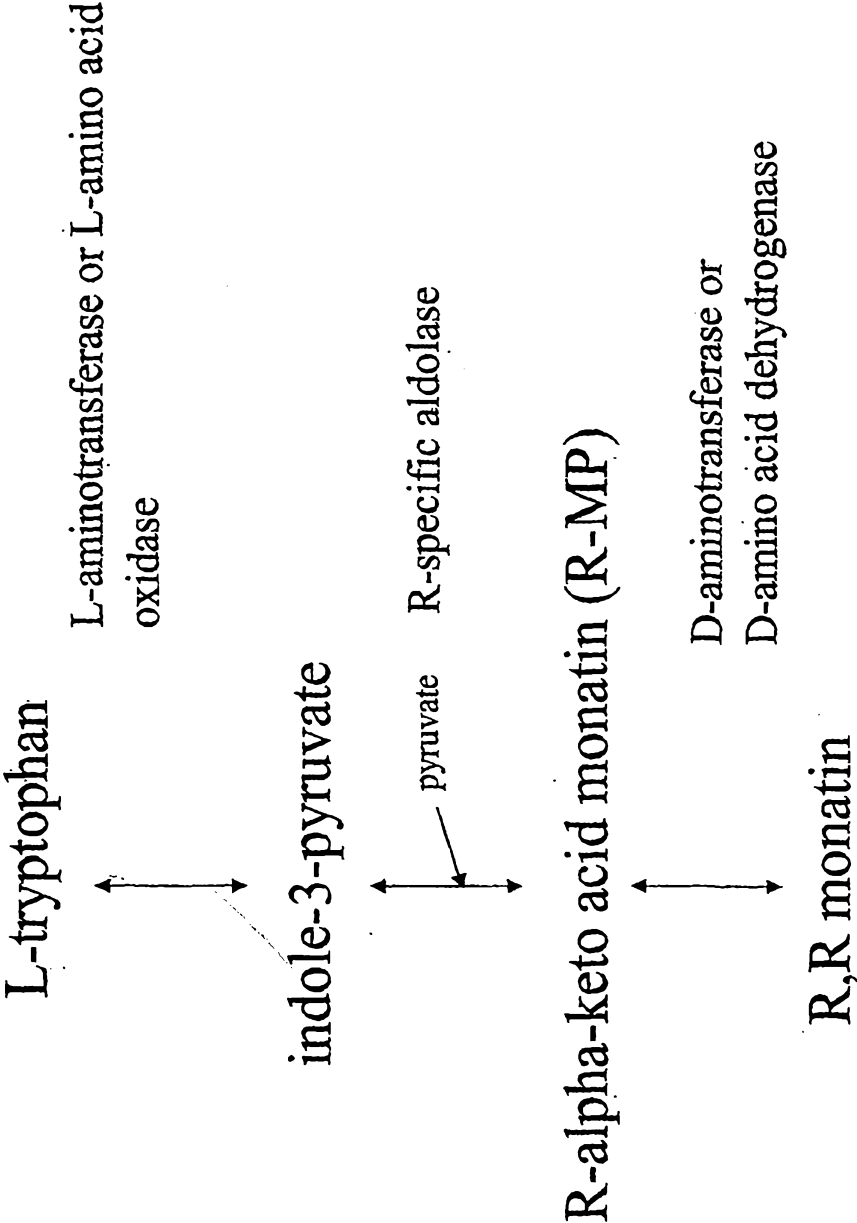


FIG. 2

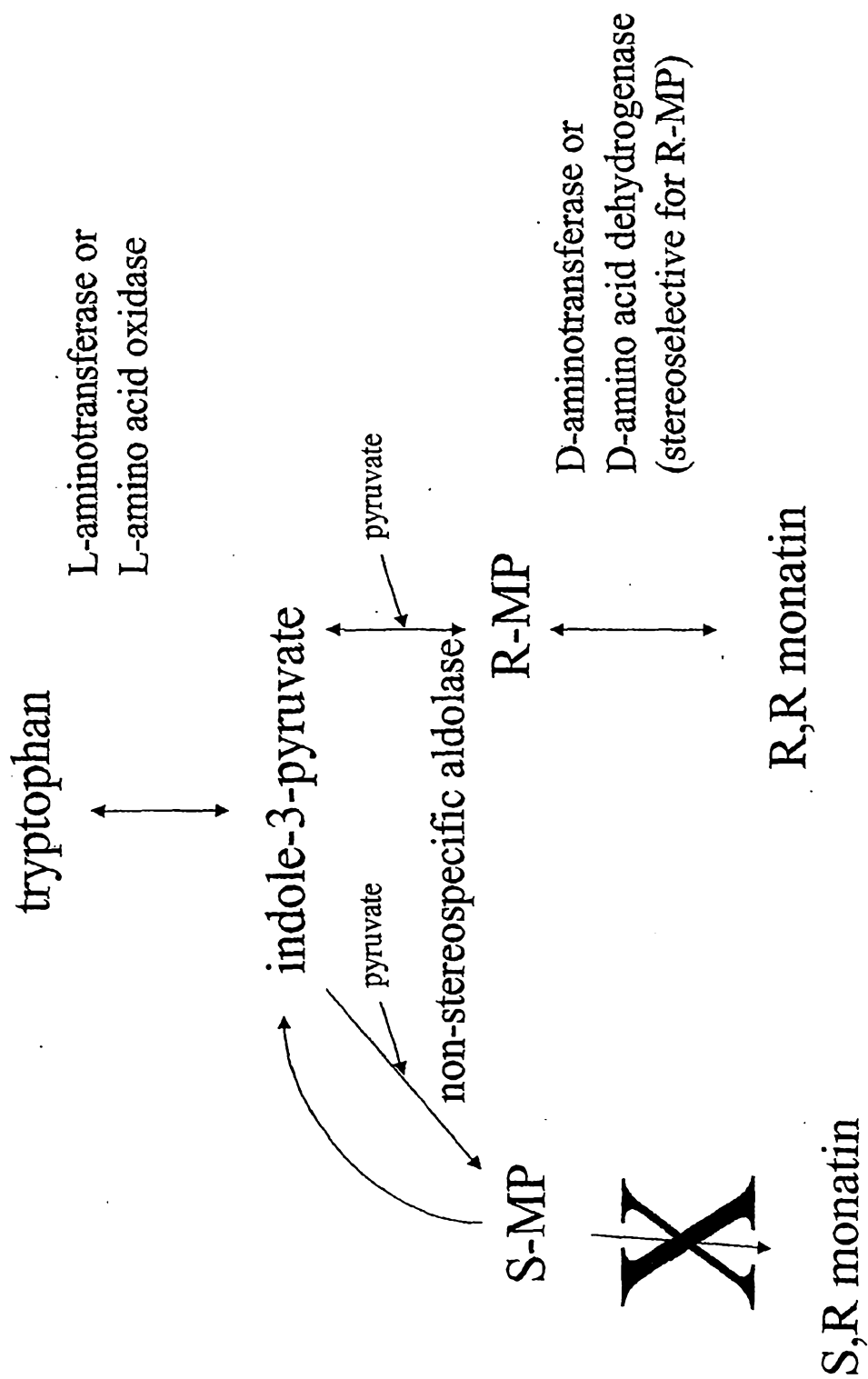


FIG. 3

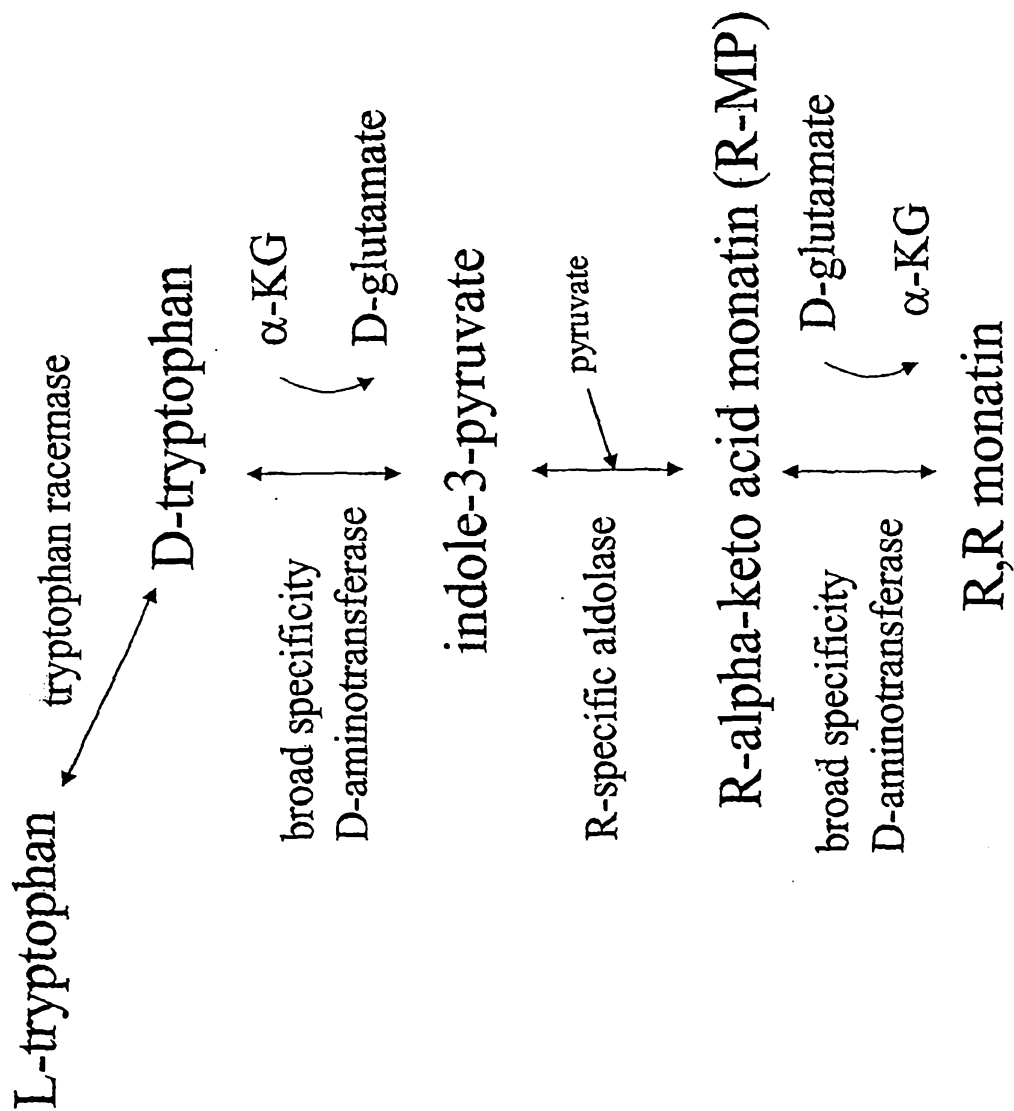


FIG. 4

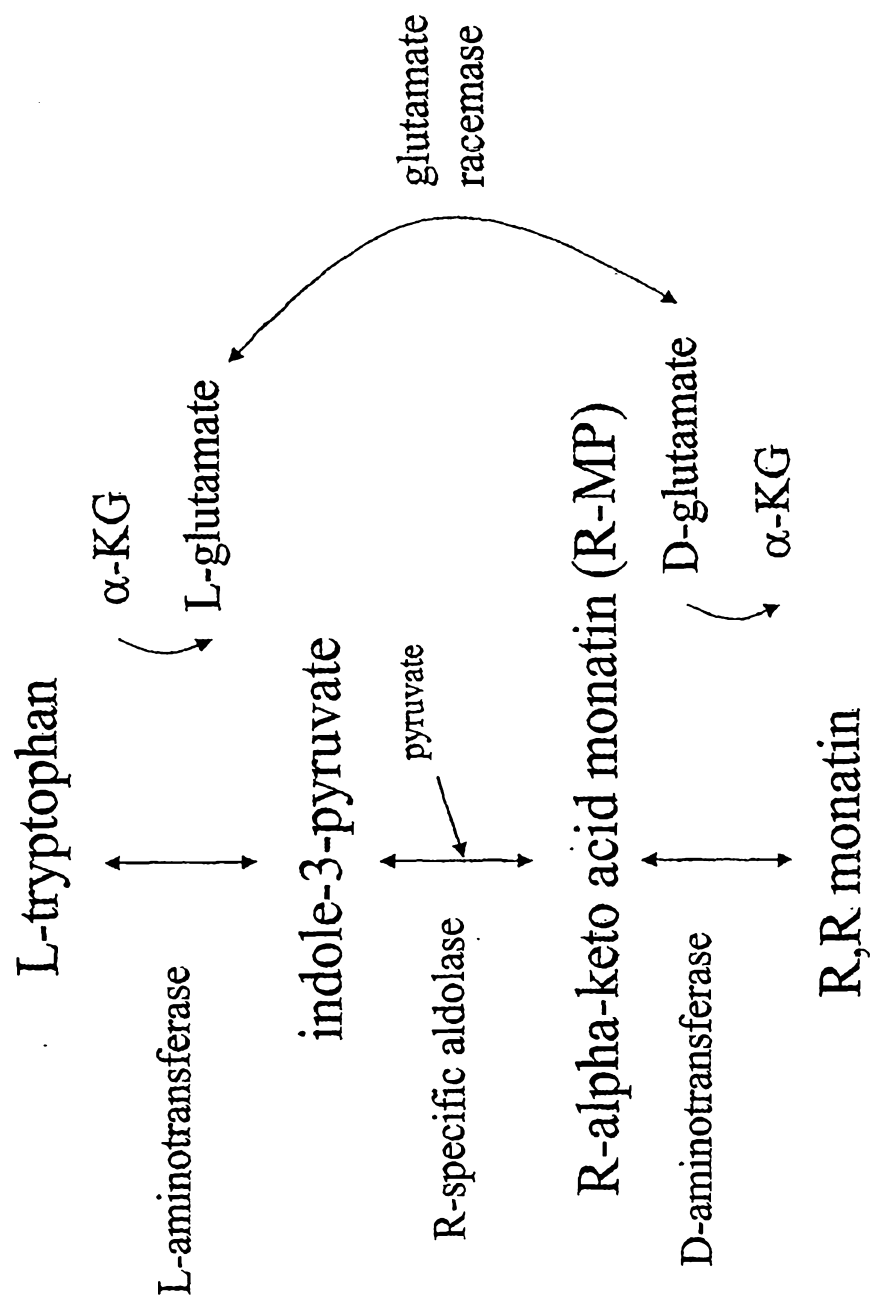


FIG. 5

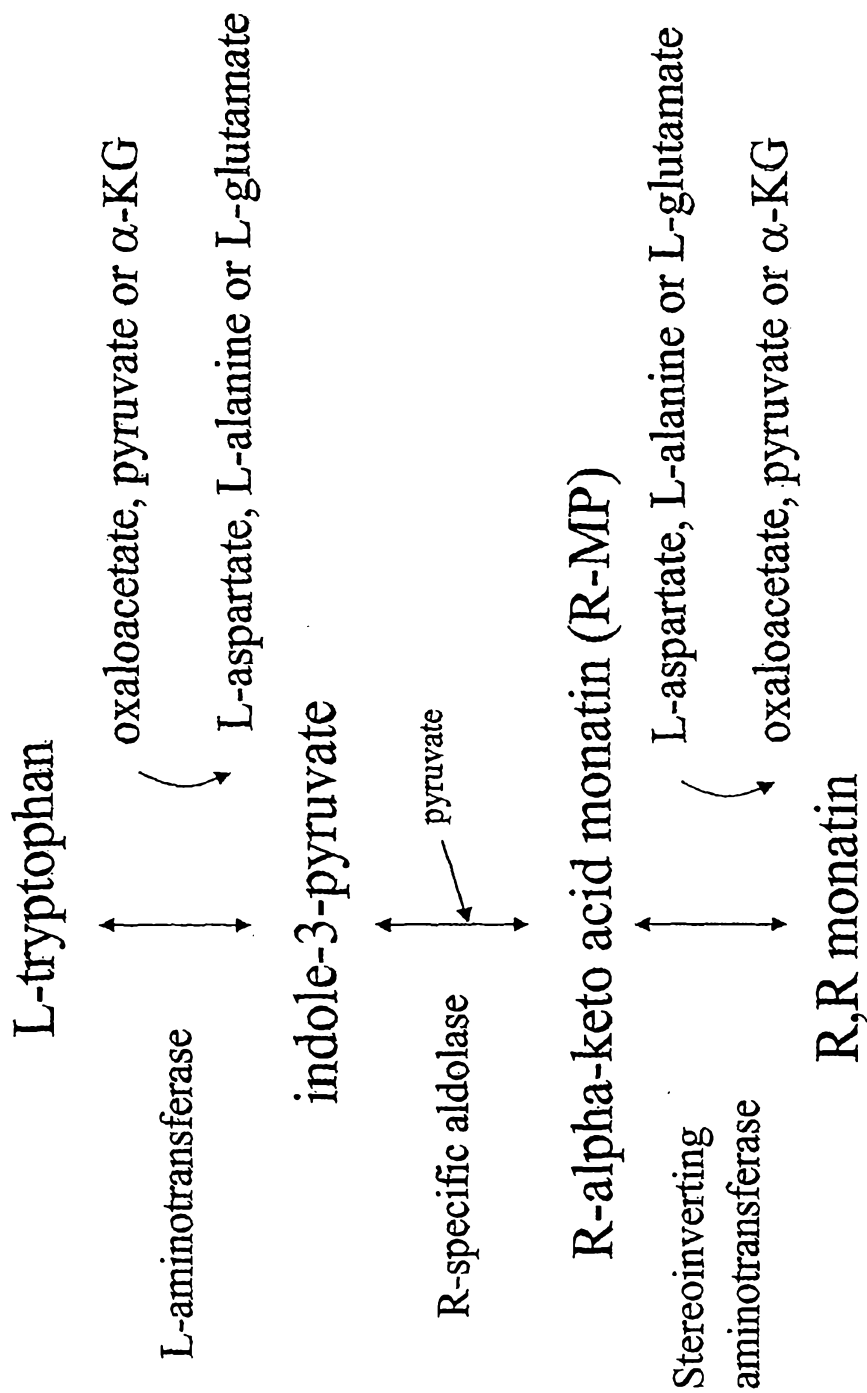


FIG. 6

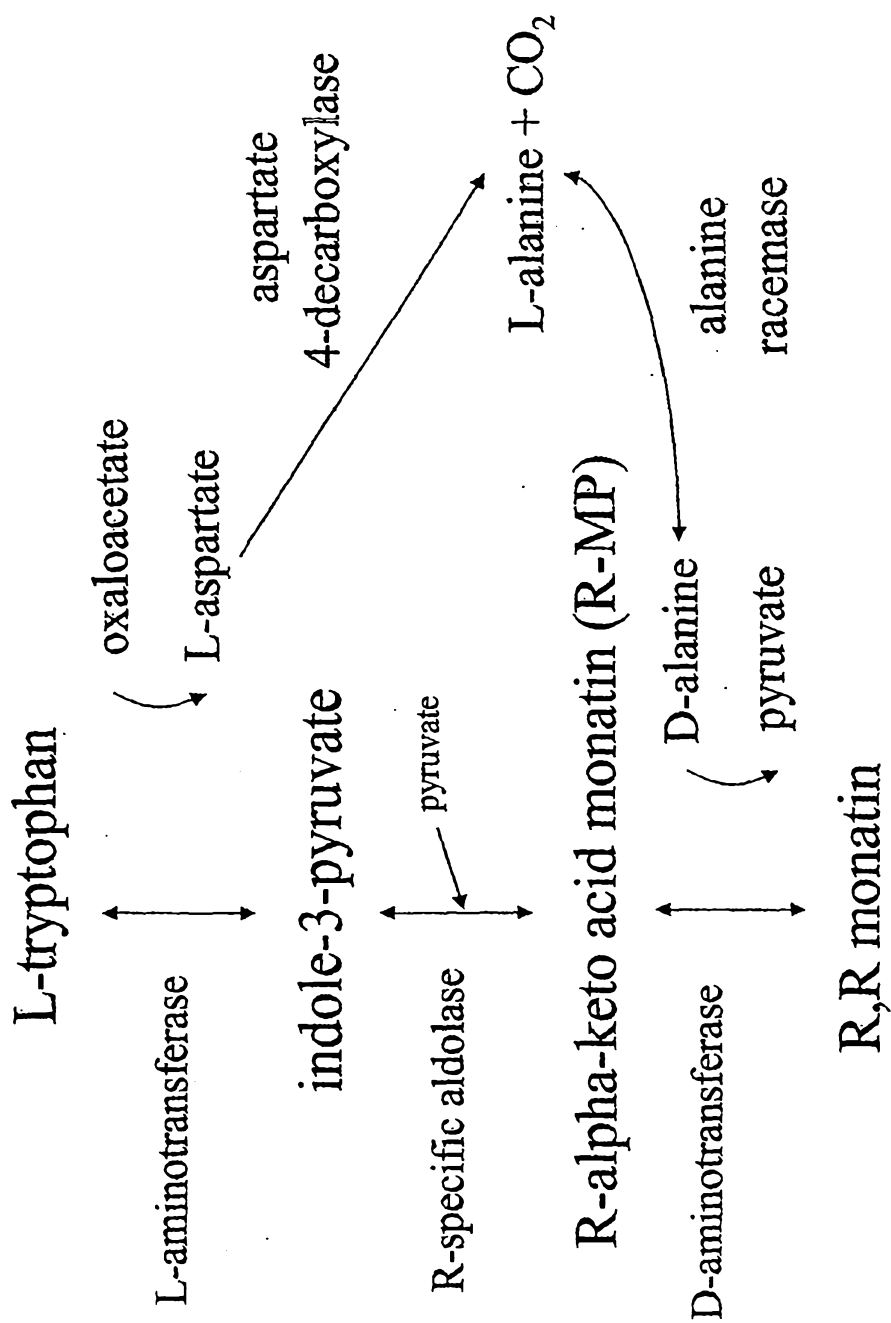


FIG. 7

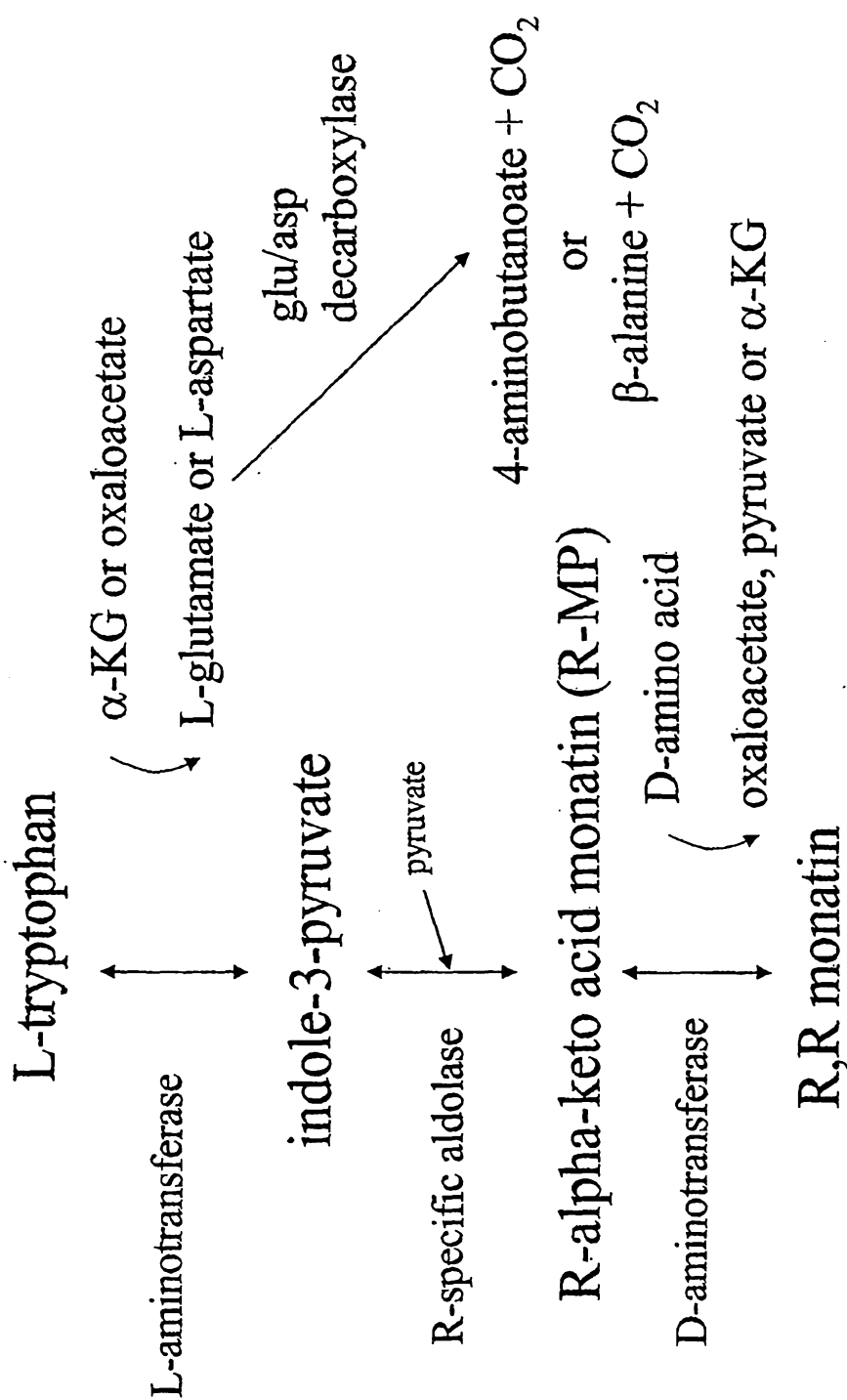


FIG. 8

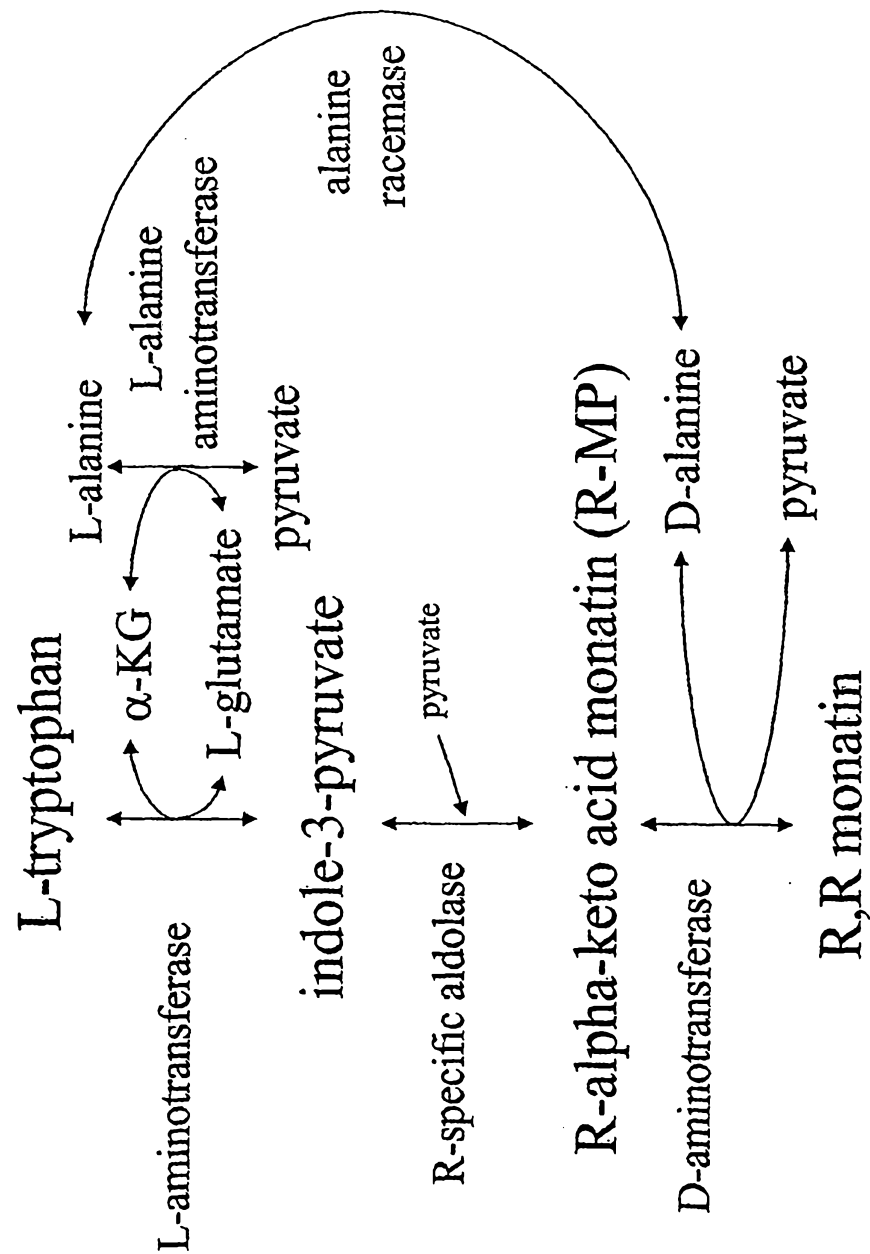


FIG. 9A

		F1	F2
		----->----->	
BsphDATgene	1	mays---lwndqive--egsitisp <u>edrgyqfgdgiyevik</u> vynghmfta	
B. halodurans	1	mdyc---lyqdqlvp--reqlkidp <u>edrgyhfgdgiyevvhv</u> hgkafal	
GsteDATgene	1	mgyt---lwndqivk--deevkidk <u>edrgyqfgdgvye</u> vvkvngemftv	
B.cereus 145	1	layekfvlwndevidttkqqtyiel <u>ergsqfgdgvye</u> virlykgnfhll	
BsubDAT	1	-mkv---lvngrlig--rseasid <u>ledrgyqfgdgiyevir</u> vykgvlfgl	
B.lichenifomis	1	-mkv---lfngrlme--rsecavdi <u>edrgyqfgdgvye</u> viriyngilftl	
BsphDATgene	136	gehidrfyasaekirlvipytkdvlhklldlieknnlnt-ghvyfqitr	
B. halodurans	136	sdhltrfkesaekldlplystdklgelvqqliedknkkeh-gmvyfqmtr	
GsteDATgene	136	nehidrlyasaekiritipytkdkfhqllhelveknelnt-ghiyfqvtr	
B.cereus 145	151	dphitrlyrsmeevelslpfskaelitllykliernhfhedgtiylqvsr	
BsubDAT	45	rehaerffrsaaeigislpsiedlewdlqklvqenavse-gavyiqttr	
B.lichenifomis	133	dehiarlyksaaeigidlsfseaelksqkelvdingrrd-gglylqvtr	
			←
BsphDATgene	283	gttsrnhifpdasvpavl--tgnvktgersienfekgvkatlvedvrwlr	
B. halodurans	283	gisprnhlytrnetp-vl--tgfskp---lpdekresvrlyltddirwlr	
GsteDATgene	283	gtsprahqfpentvkvpi--igytkenprplenlekgvkatfvedirwlr	
B.cereus 145	301	gvqarthvf-sydtptti--yayitkkerpalwieygiraisepdtrwlr	
BsubDAT	94	gvaprkhqy-eaglepqt--taytftvkkpeqeqaygvaaitdedlrwlr	
B.lichenifomis	280	gkaprkhqygagltpqvtaytftpigkpekeqqn---gvsaitaddmrwlr	
			←
		R1	
		----- R2	
		←-----	←
BsphDATgene	427	<u>cdikslnllgav</u> lakqeasekgcyeaailhrgdiiitecssanvygikdgkl	
B. halodurans	415	<u>cdiktinllgnv</u> lakreatdhqcdeallhrdgtvttegsssnvfliknetl	
GsteDATgene	427	<u>cdikslnllgav</u> lakqeaehekgcyeaailhrnntvttegsssnvfikdgil	
B.cereus 145	442	<u>cdikslnllpnv</u> laatkaerkgckeallvrngivtegtshsnfflikngtl	
BsubDAT	141	<u>cdikslnllnvm</u> tkqrayeagafeaillrdgvttegtsssnvyavingtv	
B.lichenifomis	421	<u>cdikslnllnvm</u> ikqkaqeasafeailirdglvttegtsssnvyvakgnvi	

FIG. 9B

R3

```

-----
BsphDATgene      577 ythpannyilngitrqvilkcaaeinlpvieepmtkgdlltmdeiivssv
B. halodurans    565 ythpatnlilngitrqitirlakakgytvveepfpkevikdadeafitst
GsteDATgene      577 ythpannmilkgitrdrvviacaneinmpvkeipftthealkmdelfvtst
B.cereus 145     592 ythpanhlilngiirqyvlslantlhipvqeelfsvrdvyqadecfftgt
BsubDAT          191 rthpanrlilngitrmmilglienkngikldetpvseeelkqaeeifisst
B.lichenifomis  571 ythpvttlilngitrmkvlqlceenglnyeekavtkdellnadevfitst
BsphDATgene      727 ssevtpvldvdgqqigagvpgeptrklqkafeaklp--isina*----
B., halodurans   715 iheitpvtevigdetahfpvgpvtkmlqqafaeia--khsqtamkq*
GsteDATgene      727 tseitpvieidgklirdgkvgeptrklqkqfetkip--kplhi*----
B.cereus 145     742 tieilpmthldgtaiqdggvgaitkklqksfn-kil--lqsnmsss*-
BsubDAT          241 taeiipvvtldggsigsgkpgpvtkqlqaafqesiqqaasis-----
B.lichenifomis  721 taevipvtsidgqtigsgapgltknvqtalqnsi---lsetaktv*-

```

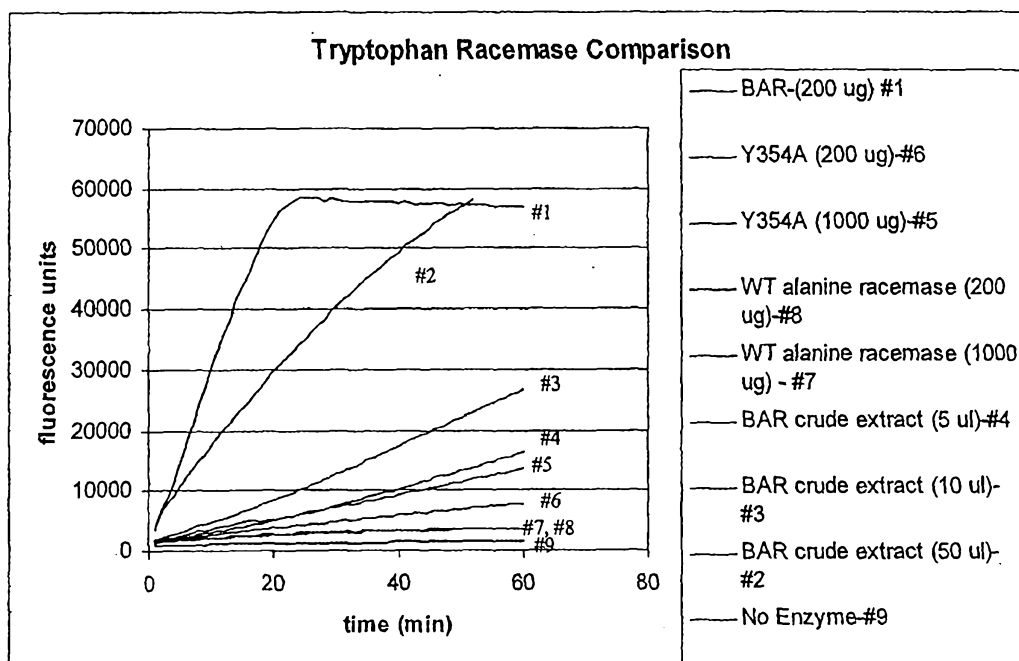
FIG. 10A**Alignment of *B. sphaericus* with ATCC 4978 (*B. rotans*) sequence (72% homology)**

BsphDATgene	1	<u>m</u> ay <u>s</u> l <u>w</u> ndq <u>i</u> ve <u>e</u> gs <u>i</u> t <u>i</u> s <u>p</u> ed <u>r</u> gy <u>q</u> fg <u>d</u> gi <u>e</u> vi <u>k</u> vy <u>n</u> gh <u>m</u> fta <u>g</u> eh <u>i</u> d
ATCC4978DAAT	1	<u>m</u> s <u>y</u> sl <u>w</u> ndq <u>i</u> v <u>n</u> de <u>e</u> v <u>v</u> dk <u>e</u> dr <u>g</u> y <u>q</u> fg <u>d</u> g <u>v</u> y <u>e</u> v <u>v</u> k <u>v</u> yng <u>e</u> l <u>f</u> ta <u>e</u> eh <u>v</u> d
BsphDATgene	151	r <u>f</u> ya <u>s</u> ae <u>k</u> ir <u>l</u> v <u>i</u> py <u>t</u> k <u>d</u> vl <u>h</u> k <u>l</u> lh <u>d</u> l <u>e</u> knnl <u>n</u> t <u>g</u> h <u>v</u> y <u>f</u> q <u>i</u> tr <u>g</u> tt <u>s</u> rn
ATCC4978DAAT	151	r <u>f</u> ya <u>s</u> ae <u>k</u> ir <u>v</u> t <u>i</u> py <u>t</u> k <u>d</u> kl <u>h</u> q <u>l</u> lh <u>q</u> lv <u>e</u> mn <u>k</u> v <u>q</u> t <u>g</u> hi <u>y</u> f <u>q</u> it <u>r</u> g <u>a</u> g <u>p</u> rn
BsphDATgene	301	hi <u>f</u> p <u>d</u> as <u>v</u> p <u>a</u> vl <u>t</u> gn <u>v</u> kt <u>g</u> ers <u>i</u> en <u>f</u> ek <u>g</u> vk <u>a</u> tl <u>v</u> ed <u>v</u> rw <u>l</u> rc <u>d</u> ik <u>s</u> lnl
ATCC4978DAAT	301	hi <u>f</u> p <u>g</u> de <u>v</u> k <u>p</u> vl <u>t</u> g <u>n</u> t <u>k</u> en <u>p</u> rp <u>v</u> an <u>f</u> ek <u>g</u> vk <u>a</u> tf <u>v</u> ed <u>i</u> rw <u>l</u> rc <u>d</u> ik <u>s</u> lnl
BsphDATgene	451	lg <u>a</u> vlak <u>q</u> ea <u>s</u> ek <u>g</u> cy <u>e</u> ai <u>l</u> hr <u>g</u> di <u>i</u> te <u>c</u> ss <u>a</u> ny <u>y</u> gi <u>k</u> dg <u>k</u> ly <u>t</u> h <u>p</u> ann <u>y</u>
ATCC4978DAAT	451	lg <u>a</u> vlak <u>q</u> ea <u>h</u> ek <u>g</u> cy <u>e</u> av <u>l</u> hr <u>d</u> ei <u>v</u> te <u>g</u> ss <u>s</u> ni <u>y</u> gi <u>k</u> dg <u>v</u> ly <u>t</u> h <u>p</u> ann <u>f</u>
BsphDATgene	601	il <u>n</u> git <u>r</u> qv <u>i</u> lk <u>a</u> ae <u>i</u> nl <u>p</u> vie <u>p</u> mt <u>k</u> gd <u>l</u> lt <u>m</u> de <u>i</u> iv <u>s</u> sv <u>s</u> se <u>v</u> tp <u>v</u> i
ATCC4978DAAT	601	il <u>n</u> git <u>r</u> qv <u>i</u> ik <u>a</u> ae <u>i</u> gl <u>p</u> v <u>k</u> ee <u>a</u> mt <u>k</u> t <u>q</u> ll <u>a</u> m <u>d</u> ev <u>i</u> vs <u>t</u> t <u>s</u> ev <u>t</u> pi <u>i</u>
BsphDATgene	751	dv <u>d</u> g <u>q</u> gi <u>g</u> ag <u>v</u> pg <u>e</u> wtr <u>k</u> l <u>q</u> ka <u>f</u> ea <u>k</u> l <u>p</u> i <u>s</u> ina*
ATCC4978DAAT	751	di <u>d</u> gt <u>v</u> ig <u>a</u> g <u>k</u> pg <u>d</u> wtr <u>k</u> l <u>q</u> a <u>q</u> fd <u>t</u> k <u>i</u> p <u>k</u> gi <u>r</u> a*

FIG. 10B**Alignment of *B. sphaericus* with ATCC 7063 (*B. serositidis*) sequence (67% homology)**

BsphDAATpep	1	<u>m</u> <u>a</u> <u>y</u> <u>s</u> <u>l</u> <u>w</u> <u>n</u> <u>d</u> <u>q</u> <u>i</u> <u>v</u> <u>e</u> <u>e</u> <u>g</u> <u>s</u> <u>i</u> <u>t</u> <u>i</u> <u>s</u> <u>p</u> <u>e</u> <u>d</u> <u>r</u> <u>g</u> <u>y</u> <u>q</u> <u>f</u> <u>g</u> <u>d</u> <u>g</u> <u>i</u> <u>y</u> <u>e</u> <u>v</u> <u>i</u> <u>k</u> <u>v</u> <u>y</u> <u>n</u> <u>g</u> <u>h</u> <u>m</u> <u>f</u> <u>t</u> <u>a</u> <u>g</u> <u>e</u> <u>h</u> <u>i</u> <u>d</u>
ATCC7063DAAT	1	<u>m</u> <u>s</u> <u>y</u> <u>t</u> <u>l</u> <u>w</u> <u>n</u> <u>d</u> <u>k</u> <u>i</u> <u>v</u> <u>d</u> <u>d</u> <u>n</u> <u>q</u> <u>v</u> <u>f</u> <u>i</u> <u>n</u> <u>k</u> <u>e</u> <u>d</u> <u>r</u> <u>g</u> <u>y</u> <u>q</u> <u>f</u> <u>g</u> <u>d</u> <u>g</u> <u>v</u> <u>y</u> <u>e</u> <u>v</u> <u>i</u> <u>k</u> <u>v</u> <u>y</u> <u>d</u> <u>g</u> <u>e</u> <u>m</u> <u>f</u> <u>t</u> <u>a</u> <u>t</u> <u>e</u> <u>h</u> <u>i</u> <u>d</u>
BsphDAATpep	51	<u>r</u> <u>f</u> <u>y</u> <u>a</u> <u>s</u> <u>a</u> <u>e</u> <u>k</u> <u>i</u> <u>r</u> <u>l</u> <u>v</u> <u>i</u> <u>p</u> <u>y</u> <u>t</u> <u>k</u> <u>d</u> <u>v</u> <u>l</u> <u>h</u> <u>k</u> <u>l</u> <u>l</u> <u>h</u> <u>d</u> <u>l</u> <u>e</u> <u>k</u> <u>n</u> <u>n</u> <u>l</u> <u>n</u> <u>t</u> <u>g</u> <u>h</u> <u>v</u> <u>y</u> <u>f</u> <u>q</u> <u>i</u> <u>t</u> <u>r</u> <u>g</u> <u>t</u> <u>t</u> <u>s</u> <u>r</u> <u>n</u>
ATCC7063DAAT	151	<u>r</u> <u>f</u> <u>y</u> <u>a</u> <u>s</u> <u>a</u> <u>e</u> <u>k</u> <u>i</u> <u>k</u> <u>l</u> <u>t</u> <u>v</u> <u>p</u> <u>y</u> <u>t</u> <u>k</u> <u>h</u> <u>k</u> <u>l</u> <u>h</u> <u>g</u> <u>l</u> <u>l</u> <u>h</u> <u>e</u> <u>v</u> <u>a</u> <u>n</u> <u>e</u> <u>l</u> <u>k</u> <u>t</u> <u>g</u> <u>n</u> <u>l</u> <u>y</u> <u>f</u> <u>q</u> <u>i</u> <u>t</u> <u>r</u> <u>g</u> <u>a</u> <u>s</u> <u>p</u> <u>r</u> <u>n</u>
BsphDAATpep	101	<u>h</u> <u>i</u> <u>f</u> <u>p</u> <u>d</u> <u>a</u> <u>s</u> <u>v</u> <u>p</u> <u>a</u> <u>v</u> <u>l</u> <u>t</u> <u>g</u> <u>n</u> <u>v</u> <u>k</u> <u>t</u> <u>g</u> <u>e</u> <u>r</u> <u>s</u> <u>i</u> <u>e</u> <u>n</u> <u>f</u> <u>e</u> <u>k</u> <u>g</u> <u>v</u> <u>k</u> <u>a</u> <u>t</u> <u>l</u> <u>v</u> <u>e</u> <u>d</u> <u>v</u> <u>r</u> <u>w</u> <u>l</u> <u>r</u> <u>c</u> <u>d</u> <u>i</u> <u>k</u> <u>s</u> <u>l</u> <u>n</u> <u>l</u>
ATCC7063DAAT	301	<u>h</u> <u>l</u> <u>f</u> <u>p</u> <u>g</u> <u>d</u> <u>d</u> <u>v</u> <u>l</u> <u>p</u> <u>v</u> <u>l</u> <u>t</u> <u>g</u> <u>n</u> <u>v</u> <u>k</u> <u>e</u> <u>a</u> <u>p</u> <u>r</u> <u>s</u> <u>i</u> <u>e</u> <u>n</u> <u>a</u> <u>q</u> <u>k</u> <u>g</u> <u>v</u> <u>k</u> <u>a</u> <u>t</u> <u>f</u> <u>a</u> <u>e</u> <u>d</u> <u>i</u> <u>r</u> <u>w</u> <u>l</u> <u>r</u> <u>c</u> <u>d</u> <u>i</u> <u>k</u> <u>s</u> <u>l</u> <u>n</u> <u>l</u>
BsphDAATpep	151	<u>l</u> <u>g</u> <u>a</u> <u>v</u> <u>l</u> <u>a</u> <u>k</u> <u>q</u> <u>e</u> <u>a</u> <u>s</u> <u>e</u> <u>k</u> <u>g</u> <u>c</u> <u>y</u> <u>e</u> <u>a</u> <u>i</u> <u>l</u> <u>h</u> <u>r</u> <u>g</u> <u>d</u> <u>i</u> <u>i</u> <u>t</u> <u>e</u> <u>c</u> <u>s</u> <u>s</u> <u>a</u> <u>n</u> <u>v</u> <u>y</u> <u>g</u> <u>i</u> <u>k</u> <u>d</u> <u>g</u> <u>k</u> <u>l</u> <u>y</u> <u>t</u> <u>h</u> <u>p</u> <u>a</u> <u>n</u> <u>n</u> <u>y</u>
ATCC7063DAAT	451	<u>l</u> <u>g</u> <u>a</u> <u>v</u> <u>l</u> <u>a</u> <u>k</u> <u>q</u> <u>e</u> <u>a</u> <u>h</u> <u>e</u> <u>k</u> <u>g</u> <u>c</u> <u>y</u> <u>e</u> <u>a</u> <u>i</u> <u>l</u> <u>h</u> <u>r</u> <u>g</u> <u>e</u> <u>t</u> <u>i</u> <u>t</u> <u>e</u> <u>g</u> <u>s</u> <u>s</u> <u>t</u> <u>n</u> <u>v</u> <u>f</u> <u>g</u> <u>i</u> <u>k</u> <u>n</u> <u>g</u> <u>v</u> <u>l</u> <u>y</u> <u>t</u> <u>h</u> <u>p</u> <u>a</u> <u>d</u> <u>n</u> <u>f</u>
BsphDAATpep	201	<u>i</u> <u>l</u> <u>n</u> <u>g</u> <u>i</u> <u>t</u> <u>r</u> <u>q</u> <u>v</u> <u>i</u> <u>l</u> <u>k</u> <u>c</u> <u>a</u> <u>a</u> <u>e</u> <u>i</u> <u>n</u> <u>l</u> <u>p</u> <u>v</u> <u>i</u> <u>e</u> <u>p</u> <u>m</u> <u>t</u> <u>k</u> <u>g</u> <u>d</u> <u>l</u> <u>t</u> <u>m</u> <u>d</u> <u>e</u> <u>i</u> <u>i</u> <u>v</u> <u>s</u> <u>s</u> <u>v</u> <u>s</u> <u>e</u> <u>v</u> <u>t</u> <u>p</u> <u>v</u> <u>i</u>
ATCC7063DAAT	601	<u>i</u> <u>l</u> <u>s</u> <u>g</u> <u>i</u> <u>t</u> <u>r</u> <u>g</u> <u>v</u> <u>l</u> <u>a</u> <u>c</u> <u>a</u> <u>n</u> <u>e</u> <u>i</u> <u>g</u> <u>l</u> <u>p</u> <u>v</u> <u>k</u> <u>q</u> <u>e</u> <u>a</u> <u>f</u> <u>t</u> <u>k</u> <u>d</u> <u>k</u> <u>a</u> <u>l</u> <u>q</u> <u>m</u> <u>d</u> <u>e</u> <u>m</u> <u>f</u> <u>v</u> <u>s</u> <u>s</u> <u>t</u> <u>t</u> <u>s</u> <u>e</u> <u>i</u> <u>t</u> <u>p</u> <u>v</u> <u>i</u>
BsphDAATpep	251	<u>d</u> <u>v</u> <u>d</u> <u>g</u> <u>g</u> <u>g</u> <u>i</u> <u>g</u> <u>a</u> <u>g</u> <u>v</u> <u>p</u> <u>g</u> <u>e</u> <u>w</u> <u>t</u> <u>r</u> <u>k</u> <u>l</u> <u>q</u> <u>k</u> <u>a</u> <u>f</u> <u>e</u> <u>a</u> <u>k</u> <u>p</u> <u>i</u> <u>s</u> <u>--i</u> <u>n</u> <u>a</u> <u>-----</u>
ATCC7063DAAT	751	<u>d</u> <u>l</u> <u>d</u> <u>g</u> <u>v</u> <u>a</u> <u>i</u> <u>n</u> <u>g</u> <u>g</u> <u>e</u> <u>i</u> <u>g</u> <u>e</u> <u>w</u> <u>t</u> <u>r</u> <u>k</u> <u>l</u> <u>q</u> <u>k</u> <u>g</u> <u>f</u> <u>a</u> <u>t</u> <u>k</u> <u>l</u> <u>p</u> <u>g</u> <u>s</u> <u>p</u> <u>a</u> <u>y</u> <u>n</u> <u>l</u> <u>t</u> <u>e</u> <u>y</u> <u>k</u> <u>*</u>

FIG. 11



The Sequence Listing for this application has been submitted to WIPO in electronic format and can be obtained upon request from the International Bureau or found on the WIPO website at <http://www.wipo.int/pctdb/en/sequences/>