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(54) Title: A HOST CELL MODIFIED TO PRODUCE LACTAMS

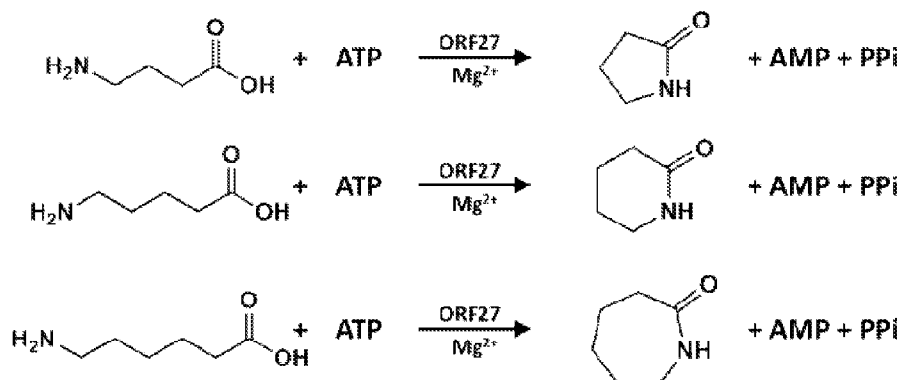


FIG. 1

(57) Abstract: The present invention provides for a genetically modified host cell capable of producing a lactam comprising a 2-pyrrolidone synthase, or an enzymatically active fragment thereof, heterologous to the host cell.



A Host Cell Modified to Produce Lactams

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The application claims priority to U.S. Provisional Patent Application Ser. No. 62/346,294, filed June 6, 2016, which is herein incorporated by reference in its entirety. This application is related to U.S. Patent Application Ser. No. 15/099,488, filed on April 14, 2016, which is hereby incorporated by reference.

STATEMENT OF GOVERNMENTAL SUPPORT

[0002] The invention was made with government support under Contract Nos. DE-AC02-05CH11231 awarded by the U.S. Department of Energy and grant no. NSF EEC 0540879 awarded by the National Science Foundation. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention is in the field of microbial production of lactams.

BACKGROUND OF THE INVENTION

[0004] Lactams are important compounds used in the manufacture of commercial polymers. ϵ -Caprolactam (caprolactam) is used in the production of nylon 6 found in fabrics, coatings, plastics of many compositions, lubricants, etc. The worldwide use of nylons requires the production of approximately four million metric tons of caprolactam annually (1). δ -Valerolactam (valerolactam) has been proposed as a monomer for nylon 5 and nylon 6,5 synthesis, addition of which tunes the properties of the resulting polymers (2-4).

[0005] Currently, both caprolactam and valerolactam are synthesized from starting materials extracted from petroleum. Caprolactam production starts from cyclohexanone, which is first converted to its oxime. Treatment of this oxime with acid induces the Beckmann rearrangement to give caprolactam (5). Such production involves energy intensive processes and harsh acidic reaction conditions and produces large amount of waste salts. On the other hand, due to the lack of raw five-carbon petrochemical feedstocks, valerolactam is still too

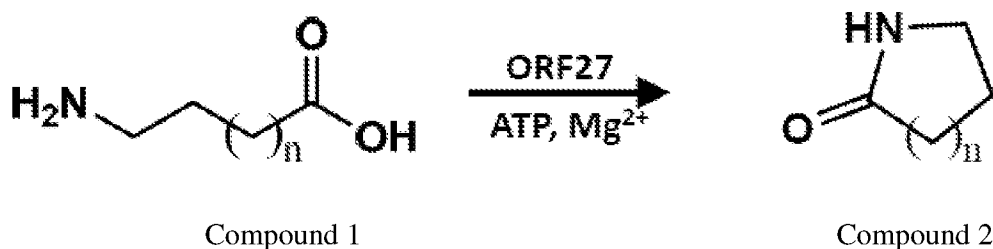
costly to achieve wide adoption for nylon synthesis.

[0006] Unlike chemical dehydration, enzymatic or whole-cell-catalyzed reactions can be performed at lower temperature and pressure. Although several ω -amino fatty acids have been biosynthesized (3, 6, 7), full biosynthetic pathways to produce lactams are largely unknown. This is due to a lack of enzymes capable of performing the last ring closing step. In terms of polymer chemistry, ring open polymerization of these lactam monomers is preferred over condensation of their corresponding ω -amino fatty acids, because condensation chemistry generates water during each monomer addition, and adversely impacts polymer properties such as molecular weight and polydispersity, and leads to undesirable thermal and mechanical properties. To date, only one enzyme, *Candida antarctica* lipase B (CALB, commercially available as N435), was reported to conduct a reversible aminolysis reaction that can be utilized for valerolactam and caprolactam synthesis (8). However, the reported enzymatic reaction occurs under vacuum over P_2O_5 , and requires high temperature and long reaction times to overcome the energy barrier of lactam formation (70% conversion at 90°C and 20% conversion at 55°C over a three-day period). Also, the intermolecular aminolysis reaction results in multiple side products, including macrocyclic dimer and trimer lactams, which are hard to eliminate during product purification. As such, there is no suitable enzyme capable of synthesizing industrially important lactams under microbial fermentation conditions.

[0007] Previously, ORF27 from *Streptomyces aizunensis*, was hypothesized to be either a 4-aminobutyryl-CoA synthetase or a 4-guanidinobutyryl-CoA synthase (9-12), although we demonstrated that expression of ORF27 in vivo resulted in the formation of γ -butyrolactam from 4-aminobutyric acid(13).

SUMMARY OF THE INVENTION

[0008] The present invention provides for a genetically modified host cell comprising an ORF27 (or 2-pyrrolidone synthase), or an enzymatically active fragment thereof, or a fusion protein comprising an ORF27, or an enzymatically active fragment thereof, linked to a saccharide binding protein. In some embodiments, the ORF27 is heterologous to the host cell. In some embodiments, the 2-pyrrolidone synthase is *Streptomyces aizunensis* 2-pyrrolidone synthase or ORF27, or any enzyme capable of catalyzing the following reaction (1):



or reaction (2): Compound 3 $\xrightarrow[\text{ATP, Mg}^{2+}]{\text{ORF27}}$ Compound 4;

wherein Compound 3 is a “Substrate” and Compound 4 is a “Product” as indicated in Table 2 herein.

[0009] The present invention provides for a method of producing a Compound 2 in a genetically modified host cell, comprising: (a) culturing the genetically modified host cell in a medium under a suitable condition such that the culturing results in the genetically modified host cell producing a Compound 2. The host cell comprises an enzyme capable of catalyzing a Compound 1 into the Compound 2. In some embodiments, the method further comprises introducing one or more nucleic acid(s) into the host cell encoding the enzyme operably linked to a suitable promoter capable of transcription in the host cell, and optionally encoding the one or more enzyme(s) of a pathway for synthesizing Compound 1 from an endogenous produced substrate compound, such as produced from a carbon source, or a substrate compound obtained from the medium; wherein the introducing step is prior to the culturing step. In some embodiments, the method further comprises separating Compound 2 from the host cell and/or the medium, wherein the separating step is subsequent, concurrent or partially concurrent with the culturing step.

[0010] In some embodiments, the Compound 1 is aminobutyrate, and Compound 2 is 2-pyrrolidone. In some embodiments, Compound 1 is 5-AVA, and Compound 2 is valerolactam. In some embodiments, Compound 1 is 6-AHA, and Compound 2 is caprolactam.

[0011] In some embodiments, the fusion protein comprises the N-terminal of the ORF27, or an enzymatically active fragment thereof, linked to the C-terminal of the saccharide binding protein. In some embodiments, the fusion protein comprises the C-terminal of the ORF27, or an enzymatically active fragment thereof, linked to the N-terminal of the saccharide binding

protein. In some embodiments, the saccharide binding protein is a disaccharide binding protein, such as a maltose binding protein (MBP), lactose binding protein, or sucrose binding protein. Suitable MBP include, but are not limited to, the MBPs of *Pyrococcus furiosus*, *Pyrobaculum aerophilum*, and *E. coli*. In some embodiments, the saccharide binding protein and ORF27 are linked via a peptide linker. In some embodiments, peptide linker is one to about twenty amino acid residues long. In some embodiments, peptide linker is about five to about fifteen amino acid residues long. In some embodiments, peptide linker is about ten amino acid residues long. A suitable peptide linker comprises: SSGLVPRGSH (SEQ ID NO:14). The peptide linker is any sequence of amino acid residues that does not interfere or reduce the enzymatic activity of the ORF27, or enzymatically active fragment thereof, and does not interfere or reduce the ability of the saccharide binding protein to stabilize the ORF27, or enzymatically active fragment thereof.

[0012] The present invention further provides for an isolated compound 2 produced from the method of the present invention.

[0013] ORF27 is an enzyme involved in the biosynthesis of ECO-02301 in *Streptomyces aizunensis*. ORF27 has a broad substrate spectrum and cyclizes γ -aminobutyric acid into butyrolactam, 5-aminovaleric acid (5-AVA) into δ -valerolactam, and 6-aminohexanoic acid (6-AHA) into ϵ -caprolactam. In one embodiment, the host cell, such as *E. coli*, expressing ORF27 produces valerolactam and/or caprolactam when 5-AVA and/or 6-AHA, respectively, are added to the culture medium. When integrated with a 5-AVA or 6-AHA precursor biosynthetic pathway, the host cell is able to produce valerolactam or caprolactam, respectively, from a substrate compound, such as lysine, which in turn can be synthesized from a carbon source, such as glucose. The carbon source can be a renewable source. U.S. Patent No. 8,404,465 discloses the metabolic pathway, and corresponding enzymes, for synthesizing 6-AHA from lysine.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The foregoing aspects and others will be readily appreciated by the skilled artisan from the following description of illustrative embodiments when read in conjunction with the accompanying drawings.

[0015] Figure 1. Proposed butyrolactam, valerolactam and caprolactam formation from their respective linear substrates catalyzed by ORF27.

[0016] Figure 2. ORF27 catalyzes multiple reaction pathways.

[0017] Figure 3A. Valerolactam production in recombinant *E. coli*. Valerolactam biosynthesis pathway.

[0018] Figure 3B. Valerolactam production in recombinant *E. coli*. Production of valerolactam via fermentation. Titer gain by day 1-3 after induction in strains JZ-440 (ORF27 negative control) and JZ-441 from 0 g/L, 1 g/L, 5 g/L and 10 g/L lysine feeding.

[0019] Figure 4. SDS-PAGE of ORF27 purified from *E. coli*. Ready gel for Tris-Glycine Gel (10% precast, Bio-Rad) was used. For molecular weight determination, PageRuler™ Prestained Protein Ladder (10 to 180 kDa, ThermoFisher Scientific) was used as protein ladder.

[0020] Figure 5A. LC-MS analysis of 4-guanidinobutyryl-CoA formation catalyzed by ORF27. 4-guanidinobutyryl-CoA synthetase reaction.

[0021] Figure 5B. LC-MS analysis of 4-guanidinobutyryl-CoA formation catalyzed by ORF27. LC-MS confirmed that the reaction system requires ORF27, 4-guanidino butyric acid (4-GBA) and CoASH for 4-guanidinobutyryl-CoA (4GB-CoA) product formation.

[0022] Figure 6A. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. ORF27 minus, ATP for activation, CoASH plus..

[0023] Figure 6B. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. ω-amino fatty acid minus, ATP, CoASH plus.

[0024] Figure 6C. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. 6-AHA, ATP, CoASH minus..

[0025] Figure 6D. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. 6-AHA, ATP, CoASH plus.

[0026] Figure 6E. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. 5-AVA, ATP, CoASH minus.

[0027] Figure 6F. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. 5-AVA, ATP, CoASH plus.

[0028] Figure 6G. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. GABA, ATP, CoASH minus. H) GABA, ATP, CoASH plus. I) 6-AHA, ADP, CoASH plus. J) 5-AVA, ADP, CoASH plus. K) GABA, ADP, CoASH plus.

[0029] Figure 6H. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. GABA, ATP, CoASH plus.

[0030] Figure 6I. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. 6-AHA, ADP, CoASH plus.

[0031] Figure 6J. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. 5-AVA, ADP, CoASH plus.

[0032] Figure 6K. Nucleotide and lactam product analysis of ORF27 catalyzed lactam formation. GABA, ADP, CoASH plus.

[0033] Figure 7A. ORF27 catalyzed butyrolactam formation. A reaction mixture containing 57 μ M of ORF27, 5 mM ω -amino fatty acids substrates, 1 mM ATP or ADP, 0.5mM CoASH and 1 mM $\text{Mg}(\text{Cl})_2$ in 100 mM HEPES (pH=8) was incubated at 25°C for 19h and quenched with methanol. The quenched reaction was filtered to get rid of protein aggregates before loading onto LC-MS.

[0034] Figure 7B. ORF27 catalyzed valerolactam formation. A reaction mixture containing 57 μ M of ORF27, 5 mM ω -amino fatty acids substrates, 1 mM ATP or ADP, 0.5mM CoASH and 1 mM $\text{Mg}(\text{Cl})_2$ in 100 mM HEPES (pH=8) was incubated at 25°C for 19h and quenched with methanol. The quenched reaction was filtered to get rid of protein aggregates before loading onto LC-MS.

[0035] Figure 7C. ORF27 catalyzed caprolactam formation. A reaction mixture containing 57 μ M of ORF27, 5 mM ω -amino fatty acids substrates, 1 mM ATP or ADP, 0.5mM CoASH and 1 mM $\text{Mg}(\text{Cl})_2$ in 100 mM HEPES (pH=8) was incubated at 25°C for 19h and quenched with methanol. The quenched reaction was filtered to get rid of protein aggregates before loading onto LC-MS.

[0036] Figure 8. pH profile of ORF27 catalyzed butyrolactam formation. A reaction mixture containing 5 μ M ORF27, 1 mM GABA, 1 mM ATP, 1mM CoASH and 1 mM $\text{Mg}(\text{Cl})_2$ in 100 mM HEPES (pH 7.5) was incubated at 25°C for 30 min. The reaction was quenched by

equal volume of methanol, and the filtered solution was analyzed by analytical method described above.

[0037] Figure 9A. Production of industrial lactam via biosynthesis. LC-MS analysis of valerolactam production from recombinant *E. coli* expressing ORF27 with 1mM 5-AVA feeding.

[0038] Figure 9B. Production of industrial lactam via biosynthesis. LC-MS analysis of caprolactam production from recombinant *E. coli* expressing ORF27 with 1mM 6-AHA feeding.

[0039] Figure 10. Sequence alignment of different maltose-binding periplasmic proteins (MBPs) from *Pyrococcus furiosus*, *Pyrobaculum aerophilum*, and *E. coli* (SEQ ID NOs:2-4, respectively). Amino acid sequences are aligned using ClustalW2 Multiple sequence alignment. Conserved amino acid residues are indicated by “*”, “:”, or “.”

[0040] Figure 11. The biosynthetic pathway from α -ketoglutarate to 6-ACA, AA, and/or AAP, and the respective enzymes which catalyzes each step.

DETAILED DESCRIPTION OF THE INVENTION

[0041] Before the invention is described in detail, it is to be understood that, unless otherwise indicated, this invention is not limited to particular sequences, expression vectors, enzymes, host microorganisms, or processes, as such may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting.

[0042] In order to more fully appreciate the invention the following definitions are provided.

[0043] As used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to an "expression vector" includes a single expression vector as well as a plurality of expression vectors, either the same (e.g., the same operon) or different; reference to "cell" includes a single cell as well as a plurality of cells; and the like.

[0044] The term “about” as used herein when referring to a numerical value means a range of values from one or two less or greater than the value, or a range of values from less than 10%

(or at least one) of the value to greater than 10% (or at least one) of the value.

[0045] The terms "optional" or "optionally" as used herein mean that the subsequently described feature or structure may or may not be present, or that the subsequently described event or circumstance may or may not occur, and that the description includes instances where a particular feature or structure is present and instances where the feature or structure is absent, or instances where the event or circumstance occurs and instances where it does not.

[0046] The term "enzymatically active fragment" is any enzyme that has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95% or 99% identical to the amino acid sequence of the indicated amino acid sequence. The enzymatically active fragment retains amino acids residues that are recognized as conserved for the enzyme. The enzymatically active fragment may have non-conserved amino acid residues replaced or found to be of a different amino acid, or amino acid(s) inserted or deleted, but which does not affect or has insignificant effect on the enzymatic activity of the enzymatically active fragment. The enzymatically active fragment may be found in nature or be an engineered mutant thereof.

[0047] The terms "host cell" and "host microorganism" are used interchangeably herein to refer to a living biological cell that can be transformed via insertion of an expression vector. Thus, a host organism or cell as described herein may be a prokaryotic organism (e.g., an organism of the kingdom Eubacteria) or a eukaryotic cell. As will be appreciated by one of ordinary skill in the art, a prokaryotic cell lacks a membrane-bound nucleus, while a eukaryotic cell has a membrane-bound nucleus.

[0048] The term "heterologous DNA" as used herein refers to a polymer of nucleic acids wherein at least one of the following is true: (a) the sequence of nucleic acids is foreign to (i.e., not naturally found in) a given host microorganism; (b) the sequence may be naturally found in a given host microorganism, but in an unnatural (e.g., greater than expected) amount; or (c) the sequence of nucleic acids comprises two or more subsequences that are not found in the same relationship to each other in nature. For example, regarding instance (c), a heterologous nucleic acid sequence that is recombinantly produced will have two or more sequences from unrelated genes arranged to make a new functional nucleic acid. Specifically, the present invention describes the introduction of an expression vector into a host microorganism, wherein the expression vector contains a nucleic acid sequence coding

for an enzyme that is not normally found in a host microorganism. With reference to the host microorganism's genome, then, the nucleic acid sequence that codes for the enzyme is heterologous.

[0049] The terms "expression vector" or "vector" refer to a compound and/or composition that transduces, transforms, or infects a host microorganism, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. An "expression vector" contains a sequence of nucleic acids (ordinarily RNA or DNA) to be expressed by the host microorganism. Optionally, the expression vector also comprises materials to aid in achieving entry of the nucleic acid into the host microorganism, such as a virus, liposome, protein coating, or the like. The expression vectors contemplated for use in the present invention include those into which a nucleic acid sequence can be inserted, along with any preferred or required operational elements. Further, the expression vector must be one that can be transferred into a host microorganism and replicated therein. Preferred expression vectors are plasmids, particularly those with restriction sites that have been well documented and that contain the operational elements preferred or required for transcription of the nucleic acid sequence. Such plasmids, as well as other expression vectors, are well known to those of ordinary skill in the art.

[0050] The term "transduce" as used herein refers to the transfer of a sequence of nucleic acids into a host microorganism or cell. Only when the sequence of nucleic acids becomes stably replicated by the cell does the host microorganism or cell become "transformed." As will be appreciated by those of ordinary skill in the art, "transformation" may take place either by incorporation of the sequence of nucleic acids into the cellular genome, i.e., chromosomal integration, or by extrachromosomal integration. In contrast, an expression vector, e.g., a virus, is "infective" when it transduces a host microorganism, replicates, and (without the benefit of any complementary virus or vector) spreads progeny expression vectors, e.g., viruses, of the same type as the original transducing expression vector to other microorganisms, wherein the progeny expression vectors possess the same ability to reproduce.

[0051] The terms "isolated" or "biologically pure" refer to material that is substantially or essentially free of components that normally accompany it in its native state.

[0052] As used herein, the terms "nucleic acid sequence," "sequence of nucleic acids," and

variations thereof shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), to any other type of polynucleotide that is an N-glycoside of a purine or pyrimidine base, and to other polymers containing nonnucleotidic backbones, provided that the polymers contain nucleobases in a configuration that allows for base pairing and base stacking, as found in DNA and RNA. Thus, these terms include known types of nucleic acid sequence modifications, for example, substitution of one or more of the naturally occurring nucleotides with an analog; internucleotide modifications, such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), and with positively charged linkages (e.g., aminoalkylphosphoramidates, aminoalkylphosphotriesters); those containing pendant moieties, such as, for example, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.); those with intercalators (e.g., acridine, psoralen, etc.); and those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.). As used herein, the symbols for nucleotides and polynucleotides are those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature (*Biochem.* 9:4022, 1970).

[0053] The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

[0054] The amino acid sequence of *Streptomyces aizunensis* ORF27 is:

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1  MRPMTAKIFA VDSVRPIDEF EQDALRVADV IRERGVCLGD RVMLKAGNSA SYVCVLYALM
61  HIGASIVLVD QQEHKEETRR IALRTGVKVT FVDDETPIDQ DADPIHLYEL MVATQNRPPM
121 DSALSFDWAG ELSDGLIMWT SGSTGSPKGV VKSGGKFLAN LRRNAHQVGH RPDDVLMPLL
181 PFAHQYGLSM VLIAWLTRCS LVIAPYRRLD RALRMARDSG TTVIDATPSS YRSILGLVTR
241 KPALRAHLAG TRMFCVGAAP LDAPLVESYV QEFGLPLLDS YGSTELNNIA FATLDNPVSC
301 GRAMEGIGLR IVDEDGREVA AGQPGEIEVD TPDALLEGQIA EDGSIIPAPT GWQRTGDLGH
361 LDADGNLYVL GRKFAVHRMG YTLYPELIER KVAAEGCPTR IVPLPDELRG SQLVFFVEDD
421 EQRDAGYWRE RLCGLLPFAFE QPNKVVVLEQ FPLNRNGKPD KKELTRMAAE
(SEQ ID NO:1)

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[0055] The enzymatically active fragment is any polypeptide capable of catalyzing reaction (1). The enzymatically active fragment is an enzyme that has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95% or 99% identical to the amino acid sequence of

SEQ ID NO:1. The enzymatically active fragment retains amino acids residues that are recognized as conserved for the enzyme. The enzymatically active fragment may have non-conserved amino acid residues replaced or found to be of a different amino acid, or amino acid(s) inserted or deleted, but which does not affect or has insignificant effect on the enzymatic activity of the enzymatically active fragment. The enzymatically active fragment may be found in nature or be an engineered mutant thereof. The enzymatically active fragment can comprise one or more of the following conserved amino acid sites/residues: acyl-activating enzyme (AAE) consensus motif (residues 137, 140-145, 147-148), acyl-activating enzyme (AAE) consensus motif (residues 140, 257-258, 279-284, 357, 369, 372, 382, 458), AMP binding site (residues 140, 180-181, 227, 229-230, 233, 257-258, 279-284, 357, 369, 372, 379-382, 439), and CoA binding site (residues 180, 229-230, 233, 257, 379-381, 433, 439).

[0056] Suitable saccharide binding protein include any polypeptide when linked to ORF27 stabilizes ORF27, and having an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95% or 99% identical to the amino acid sequence of SEQ ID NO:2, 3, or 4. The polypeptide when linked to ORF27 stabilizes ORF2 retains amino acids residues that are recognized as conserved for the enzyme, such as one or more conserved amino acid residues indicated in Figure 10. In some embodiments, the polypeptide comprises all of the conserved amino acid residues indicated by “*”, indicated by “*” and “:”, or indicated by “*”, “:”, and “.” (as shown in Figure 10); wherein for the conserved amino acid residues indicated by “:” and “.” the amino acid residue can be the corresponding amino acid residue for any one of SEQ ID NOs: 2-4. The polypeptide when linked to ORF27 stabilizes ORF2 may have non-conserved amino acid residues replaced or found to be of a different amino acid, or amino acid(s) inserted or deleted, but which does not affect or has insignificant effect on the enzymatic activity of the enzymatically active fragment. The polypeptide when linked to ORF27 stabilizes ORF2 may be found in nature or be an engineered mutant thereof.

[0057] The amino acid sequence of a fusion MBP-ORF27 (the linker peptide is underlined and in bold) is the following:

MGSSHHHHHH SSGKIEEGKL VIWINGDKGY NGLAEVGKKF EKDTGIKVTV
 EHPDKLEEFK PQVAATGDGP DIIFWAHDFR GGYAQSGLLA EITPDKAFQD
 KLYPFTWDAV RYNGKLIAYP IAVEALSLIY NKDLLPNPPK TWEEIPALDK
 ELKAKGKSAL MFNLQEPYFT WPLIAADGGY AFKYENGKYD IKDVGVDNAG

AKAGLTFLVD LIKNKHMNAD TDYSIAEAAF NKGETAMTIN GPWAWSNIDT
 SKVNYGVTVL PTFKGQPSKP FVGVL SAGIN AASPNKELAK EFLENYLLTD
 EGLEAVNKDK PLGAVALKSY EEELAKDPRI AATMENAQKG EIMPNIPQMS
 AFWYAVRTAV INAASGRQTV DEALKDAQTS SGLVPRGSHM RPMTAKIFAV
 DSVRPIDFEF QDALRVADVI RERGVCLGDR VMLKAGNSAS YVCVLYALMH
 IGASIVLVDQ QEHKEETRRI ALRTGVKVT F VDDETPIDQD ADPIHLYELM
 VATQNRPPMD SALSFDWGE LSDGLIMWTS GSTGSPKGVV KSGGKFLANL
 RRNAHQVGHR PDDVLMPLLP FAHQYGLSMV LIAWLTRCSL VIAPYRRLDR
 ALRMARDSGT TVIDATPSSY RSILGLVTRK PALRAHLAGT RMFCVGAAPL
 DAPLVESYVQ EFGLPLLD SY GSTELNNIAF ATLDNPVSCG RAMEGIGLRI
 VDEDEGREVAA GQPGEIEVDT PDALEGQIAE DGSII PAPTG WQRTGDLGHL
 DADGNLYVLG RKFAVHRMGY TLYPELIERK VAAEGCPTRI VPLPDEL RGS
 QLVFFVEDDE QRDAGYWRER LCGLLP AF EQ PNKV VVLEQF PLNRNGKPKD
 KELTRMAAE* (SEQ ID NO:13)

[0058] The nucleotide sequence of gBlock *davB* is the following:

tataggggaattgtgagcggataacaatttcagaattcaaaagatcttttaagaaggagata
 tacatatgaacaagaagaaccgccaccccgccgacggcaagaagccgatcaccatttttcggc
 ccggacttcccttttgctttcgacgactggctggaacacccggcaggcctgggcagcattcc
 ggctgagcgccatggggaagaggtggccattgtcggtgccggtatcgccggcctggtagcgg
 cctacgagctgatgaagctgggcctcaagccggtggtgtacgaggcttccaagctgggcggc
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 tactacgcccagagaagcccaccgacctgccgcaactgtttcatgaggtagccgacgcttgggc
 cgatgctctggagagcggtgcgagttcgccgatatccagcaggccatccgcgaccgtgatg
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gatgtggatggccctggaccgtaccgcgtacatgcagtcgtcgaaaaccttcgtcatgggtcg
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 tgggttgaaggcgcggtgcagacgtcgtgaatgcgggtgtggggatcatgaaccactttgg
 tggccacacccacccccgacaacccccggcccgatgtgttcaacgaaatcgcccgatcg
 ccctggcggttgaggatcttttaagaaggagatatacatatgcgcatcgctct (SEA ID
 NO: 5)

[0059] The nucleotide sequence of gBlock *davA* is the following:

Cccgatcgccctggcggttgaggatcttttaagaaggagatatacatatgcgcatcgctct
 gtaccagggcgacccaagccactggatgtgcccggaacctgcaacggctgcgccaccagg
 cgagttggcagccgaccgcggcgacagttgctgggtgtgcccgagatgttctgtccggc
 tacaacatcgccctggcccaggctcagcgccctggccgaggcccgatggcccggcagccat
 gacggtgggtggagattgccagggcgacccgtatcgccattgtctatggctaccggagcgcg
 gcgatgacggggcgatctacaacagcgtgcagctgatcgatgcgcatggccgcagcctgagc
 aattaccgcaagaccacctgttcggtgaactggaccgctcgatgttcagccctggtgcgga
 ccacttcccgggtgggtggaactggaaggctggaagggttgccctgctgatctgctacgacatcg
 agttcccggagaacgcccagccctggcgctggacggcgccgagctgatcctggtgccgacg
 gcgaacatgacgccgtacgactttacctgccaggtgaccgtgagggcacgggcgaggaaaa
 ccagtgtacctggtatatgccaaactactgcggcgcggaagacgagatcgagtattgcgggc
 agagcagcatcatcgcccgatggcagcttgcctggccatggccggggcggtgatgagtgccag
 ttgttggcagagctcgagcatgagcgggtggtgcagggcgagggcggtttccctacctgac
 cgatttgcgccaggagctgcacctgcgtaaaggctgaggatccaaactcgagtaaggatctc
 caggcatcaaataaaacgaaaggctcagtc (SEQ ID NO: 6)

[0060] The nucleotide sequence of plasmid pBbA7a-DavB-DavA is the following:

gacgtcctactgcccgtttccagtcgggaaacctgtcgtgccagctgcattaatgaatcgccaacgcgcggggagaggcg
 gtttgcgtattggcgccagggtggtttttttaccagtgagacgggcaacagctgattgcccttcaccgcctggccctgaga
 gagttgcagcaagcgtccacgtggtttgccccagcaggcgaaaatcctgtttgatggtggttaacggcgggatataacatga

gctgtcttcggtatcgctgatatccactaccgagatgtccgcaccaacgcgcagcccggactcggtaatggcgcgattgcgcc
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ct (SEQ ID NO:7)

[0061] The nucleotide sequence of plasmid pET28a-MBP-ORF27 is the following:

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gcgtgaccgctacacttgccagcgccttagcgcgcgcgtcctttcgctttcttcccttccttt
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CCTGGTCACGCGTAAACCGGCCCTGCGTGACACCTGGCCGGCACCCGCATGTTCTGTGTGG
GCGCAGCGCCGTTGGATGCGCCGCTGGTCGAAAGCTACGTTCAAGAGTTTGGTCTGCCGCTG
TTGGACAGCTATGGTTCTACCGAGCTGAACAATATCGCTTTCGCGACCCTGGATAATCCGGT
TTCCTGTGGTCGCGCAATGGAAGGTATCGGTCTGCGTATTGTTGACGAAGATGGTCGTGAAG
TTGCGGCAGGCCAACCGGGCGAAATCGAGGTTGACACTCCGGATGCCCTGGAGGGTCAAATC
GCCGAGGATGGTAGCATTATTCCGGCACCGACCGGCTGGCAGCGTACGGGCGATCTGGGTCA
CTTGGACGCCGACGGCAACCTGTATGTCCTGGGTGCTAAGTTTTCGGTCCACCGCATGGGTT
ATACTTTGTACCCAGAGCTGATTGAGCGCAAAGTGGCCGCTGAGGGCTGCCCGACCCGCATT
GTTCCGCTGCCGGACGAGCTGCGTGGTAGCCAACTGGTCTTTTTTCGTGGAAGATGATGAACA
GCGTGACGCAGGTTACTGGCGTGAACGTCTGTGCGGTTTGCTGCCGGCGTTCGAGCAGCCGA
ACAAGGTGGTCGTTCTGGAGCAGTTTCTCTGAATCGCAATGGCAAGCCGGACAAGAAAGAG
CTGACCCGTATGGCGGCAGAATGATAAGGATCCgaattcgagctccgtcgacaagcttgcg
ccgcactcgagcaccaccaccaccactgagatccggctgctaacaaagcccgaaggaa
gctgagttggctgctgccaccgctgagcaataactagcataaacccttggggcctctaaacg
ggtcttgaggggttttttctgctgaaaggaggaactatatccggat (SEQ ID NO:8)

[0062] In some embodiments, the host cell is capable of synthesizing Compound 1 or

uptaking Compound 1 from the environment or culture. In some embodiments, the host cell further comprises one or more enzymes of a pathway for synthesizing Compound 1 from a carbon source. In some embodiments, the pathway for synthesizing Compound 1 from a carbon source is native to the host cell. In some embodiments, the pathway for synthesizing Compound 1 from a carbon source is heterologous to the host cell. In some embodiments, the carbon source is a carbon source the host cell in the wild-type form is capable of uptaking.

[0063] In some embodiments, the host cell comprises a first one or more nucleic acids encoding the 2-pyrrolidone synthase, or an enzymatically active fragment thereof, operably linked to a promoter capable of expressing the 2-pyrrolidone synthase, or an enzymatically active fragment thereof, in the host cell. In some embodiments, the host cell comprises a second one or more nucleic acids encoding one or more enzymes of a pathway for synthesizing Compound 1 from a carbon source, operably linked to a promoter capable of expressing the one or more enzymes of a pathway for synthesizing Compound 1 from a carbon source in the host cell. In some embodiments, the first and/or second nucleic acids are stably integrated into a chromosome of the host cell. In some embodiments, the first and/or second nucleic acids are capable of stable introduction into the host cell. In some embodiments, the first and/or second nucleic acids are vectors, or expression vectors. In some embodiments, the first and/or second nucleic acids are the same nucleic acid. In some embodiments, the first and/or second nucleic acids are separate nucleic acids.

[0064] In some embodiments, the host cell lacks, or is disrupted for, an endogenous gene encoding betaine-CoA ligase. In some embodiments, the host cell lacks, or is disrupted for, an endogenous gene encoding a GABA transaminase, such as a *gabT* gene. In some embodiments, the host cell comprises an endogenous gene encoding a GadB, or the host cell expresses a heterologous GadB. In some embodiments, the host cell expresses a GadB mutant lacking amino acid residues H465 and T466, GadB_{ΔHT} (13).

[0065] In some embodiments, the host cell comprises endogenous or heterologous genes encoding a L-lysine monooxygenase, such as *Pseudomonas putida davB* gene, or enzymatically active fragment thereof, and a 5-aminovaleramide amidohydrolase, such as *Pseudomonas putida davA* gene, or enzymatically active fragment thereof.

[0066] The amino acid sequence of *Pseudomonas putida* DavA is:

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      10           20           30           40           50
MRIALYQGAP KPLDVPGNLQ RLRHQAQLAA ERGAQLLVCP EMFLTGYNIG
      60           70           80           90          100
LAQVERLAEEA ADGPAAMTVV EIAQAHRIAI VYGYPERGDD GAIYNSVQLI
     110          120          130          140          150
DAHGRSLSNY RKTHLFGELD RSMFSPGADH FPVVELEGWK VGLLICYPDIE
     160          170          180          190          200
FPENARRLAL DGAELILVPT ANMTPYDFTC QVTVRARAQE NQCYLVYANY
     210          220          230          240          250
CGAEDEIEYC GQSSIIGPDG SLLAMAGRDE CQLLAELEHE RVVQGRTAFF
     260
YLTDLRQELH LRKG (SEQ ID NO:9)

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[0067] The amino acid sequence of *Pseudomonas putida* DavB is:

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      10           20           30           40           50
MNKKNRHPAD GKKPITIFGP DFPFAFDDWL EHPAGLGSIP AERHGEEVAI
      60           70           80           90          100
VGAGIAGLVA AYELMKLGLK PVVYEASKLG GRLRSQAFNG TDGIVAELGG
     110          120          130          140          150
MRFPVSSTAF YHYVDKLGLE TKPFPNPLTP ASGSTVIDLE GQTYIAEKPT
     160          170          180          190          200
DLPQLFHEVA DAWADALESG AQFADIQQAI RDRDVPRLKE LWNKLVPLWD
     210          220          230          240          250
DRTFYDFVAT SRSFAKLSFQ HREVFQGVGF GTGGWDSDFP NSMLEIFRVV
     260          270          280          290          300
MTNCDDHQHL VVGGEVQVPQ GIWRHVPERC VHWPEGTSLS TLHGGAPRTG
     310          320          330          340          350
VKRIARASDG RLAVTDNWGD TRHYS AVLAT CQTWLLTTQI DCEESLFSQK
     360          370          380          390          400
MWMALDRTRY MQSSKTFVMV DRPFWKDKDP ETGRDLLSMT LTDRLTRGTY
     410          420          430          440          450
LFDNGNDKPG VICLSYSWMS DALKMLPHPV EKRVLQALDA LKKIYPKTDI
     460          470          480          490          500
AGHIIIGDPIT VSWEADPYFL GAFKGALPGH YRYNQRMAYH FMQQDMPAEQ

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510          520          530          540          550
RGIFIAGDDV SWTPAWVEGA VQTSLNAVWG IMNHFGGHTH PDNPGPGDVF
560
NEIGPIALAD (SEQ ID NO:10)

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[0068] In some embodiments, *n* is an integer from 1 to 20. In some embodiments, *n* is an integer from 1 to 10. In some embodiments, *n* is an integer from 1 to 9. In some embodiments, *n* is an integer from 1 to 8. In some embodiments, *n* is an integer from 1 to 7. In some embodiments, *n* is an integer from 1 to 6. In some embodiments, *n* is an integer from 1 to 7. In some embodiments, *n* is an integer from 1 to 6. In some embodiments, *n* is an integer from 1 to 5. In some embodiments, *n* is an integer from 1 to 4. In some embodiments, *n* is an integer from 1 to 3. When *n* is 1, compound 2 is butyrolactam (2-pyrrolidone). When *n* is 2, compound 2 is valerolactam (2-piperidinone). When *n* is 3, compound 2 is caprolactam.

[0069] The host cell can be any eukaryotic cell, such as a yeast, or prokaryotic cell, such as a bacterium. In some embodiments, the host cell is yeast. Yeast host cells suitable for practice of the methods of the invention include, but are not limited to, *Yarrowia*, *Candida*, *Bebaromyces*, *Saccharomyces*, *Schizosaccharomyces* and *Pichia*, including engineered strains provided by the invention. In one embodiment, *Saccharomyces cerevisiae* is the host cell. In one embodiment, the yeast host cell is a species of *Candida*, including but not limited to *C. tropicalis*, *C. maltosa*, *C. apicola*, *C. paratropicalis*, *C. albicans*, *C. cloacae*, *C. guilliermondii*, *C. intermedia*, *C. lipolytica*, *C. panapsilosis*, *C. zeylenoides*, and *C. tropicalis*. In some embodiments the host cell is a bacterium, such as a Gram-positive or Gram-negative cell. Bacterial host cells suitable for practice of the methods of the invention include, but are not limited to, *Escherichia*, *Bacillus*, *Salmonella*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Streptomyces*, *Cynechocystis*, *Cynechococcus*, *Sinorhizobium*, and *Caulobacter*, including engineered strains provided by the invention. In some embodiments, the 2-pyrrolidone synthase is heterologous to the host cell.

[0070] In some embodiments, the host cell further comprises one or more, or all, of the biosynthetic enzymes, or enzymatically active fragments thereof, to convert α -ketoglutarate into 6-ACA, AA, and/or AAP, or any product in the pathway, shown in Fig. 11. The enzymes are taught in Zhou et al., "Algorithmic co-optimization of genetic constructs and growth conditions: application to 6-ACA, a potential nylon-6 precursor," *Nucleic Acids Res* 43(21):

10560 (2015).

[0071] In some embodiments, the host cell further comprises one or more endogenous or heterologous genes encoding a glutamate dehydrogenase, such as *Escherichia coli glutamate dehydrogenase* gene, or enzymatically active fragments thereof. The host cell is capable of expressing or overexpressing a glutamate dehydrogenase for conversion of glutamate to α -ketoglutarate.

[0072] The amino acid sequence of *Escherichia coli* glutamate dehydrogenase is:

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MDQTSLSLESLNHVQKRDPNQTEFAQAVREVMITLWPFLEQNPKYRQMSLLERLVEPERVVIQFRVWVDD
RNQIQVNRARVQFSSAIGPYKGGMRFHPSVNLKILKFLGFEQTFKNALTTLPMGGGKGGSDFDPKGKSE
GEVMRFCQALMTELYRHLGADTDVPAGDIGVGGREVGFMAGMMKKLSNNTACVFTGKGLSFGGSLIRPEA
TGYGLVYFTEAMLKRHGMGFEGMRVSVSGSGNVAQYAEKAMEFGARVITASDSSGTVVDESFGTKEKLA
RLIEIKASRDGRVADYAKEFGLVYLEGQQPWSLPVDIALPCATQNELDVDASHQLIANGVKAVAEGANMP
TTIEATELFQQAGVLFAPGKAANAGGVATSGLEMAQNAARLGWKAEKVDARLHHIMLDIHHACVEHGGE
EQTNVQGANIAGFVKVADAMLAQGV (SEQ ID NO:15)
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[0073] In some embodiments, the host cell further comprises endogenous and/or heterologous genes encoding a D-xylose dehydrogenase, such as *Haloferax volcanii Hv-xdh* gene, or enzymatically active fragments thereof, a D-xylonate dehydratase, such as *Haloferax volcanii Hv-xad* gene, or enzymatically active fragments thereof, a 2-keto-3-deoxyxylonate dehydratase, such as *Haloferax volcanii Hv-HVO-B0027* gene, or enzymatically active fragments thereof, and an α -ketoglutarate semialdehyde dehydrogenase, such as *Haloferax volcanii Hv-HVO-B0039* gene, or enzymatically active fragments thereof. The host cell is capable of expressing or overexpressing of the four-enzymes pathway from *Haloferax volcanii* for conversion of xylose to α -ketoglutarate production.

[0074] The amino acid sequence of *Haloferax volcanii* Hv-xdh is:

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MSPAPTDIVEEFTRRDWQGDDVTGTVRVAMIGLGWWTRDEAIPAVEASEFCETTIVVSSS
KEKAEGATALTESITHGLTYDEFHEGVAADAYDAVYVVTNGLHLPLYVETAAELGKAVLC
EKPLEASVERAEKLVAACDRADVPLMVAYRMQTEPAVRRARELVEAGVIGEPVFVHGHMS
```

QRLLDEVVPDPDQWRLDPELSGGATVMDIGLYPLNTARFVLDADPVRVRATARVDDEAFE
AVGDEHVSFGVDFDDGTLAVCTASQSAYQLSHLRVTGTEGELEIEPAFYNRQKRGFRLSW
GDQSADYDFEQVNQMTEEFDYFASRLSDSDPAPDGDHALVDMRAMDAIYAAAERGTDVA
VDAADSDSADSDSADAAAAANHDADPDS DGT (SEQ ID NO:16)

[0075] The amino acid sequence of *Haloferax volcanii* Hv-xad is:

MVEQAKLSDPNAEYTMRDLSAETIDITNPRGGVRDAEITDVQTTMVDGNYPWILVRVYTD
AGVVGTEAYWGGGDTAIIERMKPFVLGENPLDIDRLYEHLVQKMSGEGSVSGKVIS AIS
GIEIALHDVAGKLLDVPAYQLVGGKYRDEV RVYCDLHTEDEANPQACAEEGVRVVEELGY
DAIKFDLDVPSGHEKDRANRHLRNPEIDHKVEIVEAVTEAVGDRADVAFDCHWSFTGGSA
KRLASELEDYDVWWLEDVPVPPENHDVQKLVTQSTTTPIAVGENVYRKFGQRTLLEPQAVD
IIAPDLPRVGGMRETRKIADLADMYIIPVAMHNVSSPIGTMASAQVAAAIPNSLALEYHS
YQLGWWEDLVEEDDLIQNGHMEIPEKPGGLGLTLDLDAVEAHMVEGETLFDEE (SEQ ID
NO:17)

[0076] The amino acid sequence of *Haloferax volcanii* Hv-HVO-B0027 is:

MHYHQLAVSGERRLTASRDSTTYDLTSADADLRFTGDLARVASIARTSVDR LAAELTEDA
DVVD DAFVDRHATVPVDAEEIWAAGVTYQISEQAREEESSMPDMYFDVYDADRPEVFFKA
TPSRTVEPGDAIGVRGDSEWDVPEPELGIVLRRGEIVGYTVGNDVSSRSIEGENPLYLPQ
AKVYDRCCSIGPCVVTPEDVEDPHELEMSMTIERDGEVIYDDATNTSEMVRSCDELVS YF
TRHNTVPELAVILTGTSLVPEQPFDLQEGDHVDITIEGIGTLSNSVTTV (SEQ ID
NO:18)

[0077] The amino acid sequence of *Haloferax volcanii* Hv-HVO-B0039 is:

MTDP SKNYVNGEWTSETGETTEVTNPANPSEVVAAYQHSNENDAAA VDAVA AEDEWR

NTPGPERGRILREAGTLLAQRKDELTEILTAEEGKARPEAAGEVQRAIDIFHYFSSKAAD
 LGGTKKGASGPNTNLYTRQEPVGVAALITPWNYP IAIPAWKLAPALAAGNTVVLKPASIA
 PGVVIEIARALDEAGLPDGVNLNVVTGPGSSVGSEFIGNEGTDLVSFTGSSQVGEMVYEQA
 TDAGKRVQTELGGKNPTLVADSANPAEAADIVANGGF GTTGQSCTACSRAIVHEDVYDDF
 VAELVDRAESLDVGPGTDHEMGPQVSESELSSTLEYIDIAEAEGATLVAGGGVPEGEAVE
 TGHFVEPTVFTDVPDPMRIAQEEVF GPVVAVIEVSDFDEGLAVANDVDYGLSASIVTDDH
 TEANRFVDEVEAGVVKVNDKTTGLELHVPFGGFKRSSSETWREQDAGLDFYTIEKTVYD
 SY (SEQ ID NO:19)

[0078] In some embodiments, the host cell further comprises endogenous or heterologous genes encoding a indole-3-pyruvate decarboxylase, such as *Lactococcus lactis* *KdcA* gene, or enzymatically active fragments thereof, a pyruvate transaminase, such as *Vibrio fluvialis* *Vfl* gene, or enzymatically active fragments thereof, a homocitrate synthase, such as *Azotobacter vinelandii* *NifV* gene, or enzymatically active fragments thereof, a 3-isopropylmalate dehydratase large subunit, such as *Methanococcus aeolicus* *AksD* gene, or enzymatically active fragments thereof, a 3-isopropylmalate dehydratase small subunit, such as *Methanococcus aeolicus* *AksE* gene, or enzymatically active fragments thereof, a isopropylmalate/isohomocitrate dehydrogenase, such as *Methanococcus aeolicus* *AksF* gene, or enzymatically active fragments thereof. The host cell is capable of expressing or overexpressing of the six-enzyme pathway for 6-aminocaproic acid production.

[0079] The amino acid sequence of *Lactococcus lactis* *KdcA* is:

MYTVGDYLLDRLHELGIIEIFGVPGDYNLQFLDQIIISREDMKWIGNANELNASYMADGYA
 RTKKAAAFLLTFGVGELSAINGLAGSYAENLPVVEIVGSPTSKVQNDGKFVHHTLADGDF
 KHFMKMHEPVTAARTLLTAENATYEIDRVLSQLLKERPVIINLPVDVAAAKAEKPALSL
 EKESSTTNTTEQVILSKIEESLKNQKPVVIAGHEVISFGLEKTVTQFVSETKLPITTLN
 FGKSAVDESLSFLGIYNGKLSEISLKNFVESADFILMLGVKLTDSSSTGAFTHHLDENKM

ISLNIDEGIIFNKVVEDFDFRAVVSSLSSELKGIEYEGQYIDKQYEEFIPSSAPLSQDRLW
QAVESLTQSNETIVAEQGTSTFFGASTIFLKSNRFIGQPLWGSIGYTFFPAALGSQIADKE
SRHLLFIGDGSLLQTLTVQELGLSIREKLNPICFIINNDGYTVEREIHGPTQSYNDIPMWN
SKLPETFGATEDRVVSKIIVRTENEFVSVMKEAQADVNRMYWIELVLEKEDAPKLLKKMGK
LFAEQNK (SEQ ID NO:20)

[0080] The amino acid sequence of *Vibrio fluvialis* Vfl is:

MNKPQSWEARAETYSLYGFTDMPSLHQRGTVVTHGEGPYIVDVNGRRYLDANSGLWNMV
AGFDHKGLIDAAKAQYERFPGYHAFFGRMSDQTVMLSEKLVEVSPFDSGRVFYTNSGSEA
NDTMVKMLWFLHAAEGKPQKRKILTRWNAYHGVTAVSASMTGKPYNSVFGPLPGFVHLT
CPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGAGGVIPPAKGYFQ
AILPILRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDALISSKNLTAGFFPMGAVILG
PELSKRLETAIEAIEEFPHGFTASGHPVGCAIALKAIDVVMNEGLAENVRR LAPRFEERL
KHIAERPNI GEYRGIGFMWALEAVKDKASKTPFDGNLSVSERIANTCTDLGLICRPLGQS
VVLCPFILTEAQMDDEMFDKLEKALDKVFAEVA (SEQ ID NO:21)

[0081] The amino acid sequence of *Azotobacter vinelandii* NifV is:

MASVIIDDTTLRDGEQSAGVAFNADEKIAIARALAE LGVPELEIGIPSMGEEEREVMHAI
AGLGLSSRLLAWCRLCDVDLAAARSTGVTMVDLSLPVSDMLHKKLNDRDRDWALREVARL
VGEARMAGLEVCLGCEDASRADLEFVVQVGEVAQAAGARRLRFADTVGVMEPF GMLDRFR
FLSRRLDMELEVHAHDDFGLATANTLAAVMGGATHINTTVNGLGERAGNAALEECVLALK
NLHGIDTGIDTRGIP AISALVERASGRQVAWQKSVVGAGVF THEAGIHVDGLLKHRRNYE
GLNPDELGRSHSLVLGKHSGAHMVRNTYRDLGIELADWQSQALLGRIRAFSTRTRKSPQP
AELQDFYRQLCEQGNPELAAGGMA (SEQ ID NO:22)

[0082] The amino acid sequence of *Methanococcus aeolicus* AksD is:

MTLAEELSKKVGKKVKAGDVVEIDIDLAMTHDGTTPLSAKAFKQITDKVWDNKKIVIVF
 DHNVPANTLKAANMQKITREFIKEQNIINHYLDGEGVCHQVLPENGHIQPNMVIAGGDSH
 TCTYGAFGAFATGFGATDMGNIYATGKTWLKVPKTIRINVNGENDKITGKDIILKICKEV
 GRSGATYMALEYGGEAIAKKLSMDERMVLSNMAIEMGGKVGLIEADETTYNYLRNVGISEE
 KILELKKNQITIDENNIDNDNYYKIIINIDITDMEEQVACPHHPDNVKNISEVKGAPINQV
 FIGSCTNGRLNDLRIASKYLGKKVHNDVRLIVIPASKSIFKQALKEGLIDIFVDAGALI
 CTPGCGPCLGAHQVGLGDGEVCLATTNRNFKGRMGNTTAEIYLSSPAIAAKSAIKGYITN
 E (SEQ ID NO:23)

[0083] The amino acid sequence of *Methanococcus aeolicus* AksE is:

MIIKGNHILFGDDIDTDAIIPGAYLKTTDPKELASHCMAGIDEKFSTKVKGDIIVAGEN
 FGCGSSREQAPISIKHTGIKAVVAESFARIFYRNCINIGLIPITCEGINEQIQNLKDGDT
 IEIDLQNETIKINSMMLNCGAPKGIEKEILDAGGLVQYTKNKLKK (SEQ ID NO:24)

[0084] The amino acid sequence of *Methanococcus aeolicus* AksF is:

MKIPKICVIEGDGIGKEVIPETVRILKEIGDFEFIEHAGYECFKRCGDALPEKTLKTAK
 ECDAILFGAVSTPKLDETERKPYKSPILTLRKELDLYANVRPIHKLDNSDSSNNIDFIII
 RENTEGLYSGVEYYDEEKELAISERHISKKGSKRIIKFAFEYAVKHHRKKVSCIHKSNIL
 RITDGLFLNIFNEFKEKYKNEYNIEGNDYLV DATAMYILKSPQMFVIVTTNLFGDILSD
 EASGLLGGLGLAPSANIGDNYGLFEPVHGSAPDIAGKGVANPIAAVLSASMMLYILDMKE
 KSRLKDAVKQVLAHKDITPDLGGNLKTKEVSDKIIIEELRKIS (SEQ ID NO:25)

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[0086] It is to be understood that, while the invention has been described in conjunction with the preferred specific embodiments thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

[0087] All patents, patent applications, and publications mentioned herein are hereby incorporated by reference in their entireties.

[0088] The invention having been described, the following examples are offered to illustrate the subject invention by way of illustration, not by way of limitation.

EXAMPLE 1

Application of an Acyl-CoA Synthetase from *Streptomyces aizunensis* for Lactam Biosynthesis

[0089] ϵ -caprolactam and δ -valerolactam are important commodity chemicals used in the manufacture of nylons, with millions of tons produced every year. Biological production of these high valued chemicals has not been possible due to a lack of enzymes that will cyclize the ω -amino fatty acid precursors to the corresponding lactams under ambient conditions. In this study, we demonstrated proof of these bioconversions by in vitro enzyme assays. We found that ORF27, an enzyme involved in the biosynthesis of ECO-02301 in *Streptomyces aizunensis*, has a broad substrate spectrum and can not only cyclize γ -aminobutyric acid into butyrolactam, but also 5-aminovaleric acid (5-AVA) into δ -valerolactam and 6-aminohexanoic acid (6-AHA) into ϵ -caprolactam. The ORF27 lactam formation reaction was characterized by product analysis, and ORF27's activity on the three ω -amino fatty acids were compared. Recombinant *E. coli* expressing ORF27 produced valerolactam and caprolactam when 5-AVA and 6-AHA, respectively, were added to the culture medium. Upon co-expressing ORF27 with a metabolic pathway that produced 5-aminovaleric acid from lysine, we were able to demonstrate production of δ -valerolactam from lysine or directly from glucose.

[0090] In this study, we overexpressed ORF27 in *Escherichia coli* and purified it by affinity chromatography. This enabled us to explore the catalytic properties of this enzyme. ORF27 was confirmed to possess 4-guanidinybutyryl-CoA ligase (4GBL) activity. ORF27 had broad substrate specificity: it could act on linear or branched acid substrates with positively charged or neutral functional groups on the ω -terminal end, yet the enzyme was selective against negatively charged groups on the substrate's ω -terminal end. Intriguingly, ORF27's activity for ω -amino fatty acids activation led to its application as a general lactam synthase, enabling biosynthesis of caprolactam, valerolactam and butyrolactam. Comparative study of ORF27's reaction products for different ω -amino fatty acid precursors was performed to better understand this enzyme's activity as a lactam synthase.

[0091] To apply ORF27 for renewable chemical production, ORF27 was overexpressed in *E. coli*, and both valerolactam and caprolactam were formed in vivo by feeding their respective precursors, 5-aminovaleric acid (5-AVA) and 6-aminohexanoic acid (6-AHA). To achieve renewable production of valerolactam from sugar, we introduced a two-gene pathway into *E. coli* that converts lysine to 5-AVA. The pathway contains an L-lysine monooxygenase (*davB* from *Pseudomonas putida* KT2440) and a 5-aminovaleramide amidohydrolase (*davA* from *Pseudomonas putida* KT2440). Introducing the genes encoding the 5-AVA biosynthetic pathway and a gene encoding a fusion of ORF27 with maltose binding protein, MBP-ORF27, enabled *E. coli* to produce valerolactam from lysine or directly from glucose.

Materials and Methods

Strains and plasmids.

[0092] All the strains and plasmids utilized in this study are listed in Table 1. The sequences for the plasmids are listed herein.

[0093] Table 1. *E. coli* strains, plasmids and oligonucleotides used.

Name	Relevant genotype	Reference
Host Strains		
DH10B	F ⁻ mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZAM15 Δ lacX74 recA1 endA1 araD139 Δ (ara, leu)7697 galU galK λ -rpsL nupG	Life Technologies (Carlsbad, CA)
BL21 Star (DE3)	F ⁻ ompT hsdSB (rB ⁻ mB ⁻) gal dcm rne131 (DE3)	Life Technologies (Carlsbad, CA)

Name	Relevant genotype	Reference
JW2637-4	F ⁻ , Δ(araD-araB)567, ΔlacZ4787(::rmB-3), λ ⁻ , ΔgabT743::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514	CGSC #11775

Engineered Strains	plasmids	Host
JZ-171	pBbE2C-ORF27	JW2637-4
JZ-172	pBbE2C-RFP	JW2637-4
JZ-440	pBbA7a-DavB-DavA	BL21(DE3) star
JZ-441	pBbA7a-DavB-DavA + pET28a-MBP-ORF27	BL21(DE3) star

Constructed Plasmid	Backbone Source (restriction site)	Gene Source (direct digestion or PCR)	PCR primers
pBbE2C-ORF27	pBbE2C-RFP (BglII, XhoI)	pDNA2.0-ORF27 (BglII, XhoI)	N/A
pET28b-N-ORF27	pET28b (NdeI, XhoI)	pDNA2.0-ORF27 (NdeI, XhoI)	N/A
pET28b-C-ORF27	pET28b (NcoI, XhoI)	pDNA2.0-ORF27 PCR	JZ_27_C6xHis_f, JZ_27_C6xHis_r
pET28a-MBP-ORF27	pET28a-MBP (NdeI, XhoI)	pDNA2.0-ORF27 (NdeI, XhoI)	N/A
pBbA7a-DavB-DavA	pBbA7a-RFP (BglII, XhoI)	DavB, DavA gBlock	N/A

Oligonucleotides	5' → 3' Sequence, restriction site underlined, synthesized by Integrated DNA Technologies, Inc (Coralville, IA)	Target Gene
JZ_27_C6xHis_f	GCGCG <u>ccatgg</u> _gc ATGCGCCCAATGACCGCTAAAATCTTCG (SEQ ID NO:11)	C-6xHis-ORF27
JZ_27_C6xHis_r	GCGCG <u>ctcgag</u> TTCTGCCGCCATACGGGTCAGC (SEQ ID NO:12)	C-6xHis-ORF27

ORF27 Protein Expression and Purification.

[0094] The gene encoding ORF27 (GenBank: AAX98201.1) was purchased from DNA 2.0 (Menlo Park, CA). The synthetic gene was optimized with *E. coli* codon usage and delivered

in pDNA2.0-ORF27. For expression and purification of ORF27, the pDNA2.0-ORF27 plasmid was digested with NdeI and XhoI and cloned into pET28b in order to produce ORF27 with an N-terminal 6xHis tag. The resulting plasmid, pET28b-N-ORF27, was transformed into *E. coli* BL21 Star (DE3) for ORF27 overexpression. To place a C-terminal His-tag on ORF27, primers JZ_27_C6xHis_f and JZ_27_C6xHis_r were used to amplify ORF27 from pDNA2.0-ORF27. The resulting PCR product was digested with NcoI and XhoI and cloned into pET28b.

[0095] For N-terminal 6xHis ORF27 expression, the overnight culture was inoculated (1:100 v/v) into 1 L LB medium containing 5 µg/ml kanamycin. The culture was grown at 37°C until the O.D. reached 0.6 and cooled on ice for 20 min. 0.5 mM IPTG was added to induce N-6xHis ORF27 overexpression for 16 h at 18°C. The cells were harvested by centrifugation (8000 × g, 6 min, 4°C), resuspended in 30 mL of lysis buffer (50 mM HEPES, pH 8.0, 0.5 M NaCl, and 10 mM imidazole), and lysed by sonication on ice. Cellular debris was removed by centrifugation (20,000 × g, 30 min, 4°C). Ni-NTA agarose resin was added to the supernatant (1 mL/L of culture), and the solution was rocked at 4°C for 1 h. The protein resin mixture was loaded onto a gravity flow column, and proteins were washed with washing buffer (50 mM HEPES, pH 8.0, 0.5 M NaCl, and 20 mM imidazole) and eluted with elution buffer (50 mM HEPES, pH 8.0, 0.5 M NaCl, and 250 mM imidazole). Purified proteins (60 mg from 1 L culture) were concentrated to 280 mg/mL and buffer exchanged into storage buffer (50 mM HEPES, pH 8.0, 8% glycerol). The final proteins were aliquoted and flash frozen in liquid nitrogen and stored at -80°C. C-terminal 6xHis ORF27 (65 mg/L LB culture) was produced using BL21 Star (DE3) transformed with pET28b-C-ORF27, purified by Ni-NTA agarose resin and stocked at 220 mg/mL in storage buffer (50 mM HEPES, pH 8.0, 8% glycerol).

[0096] Nickel nitrilotriacetic acid agarose (Ni-NTA) resin and SDS-PAGE gels were purchased from Qiagen and Biorad, respectively. Protein samples were concentrated using 10 KDa MMCO Amicon Ultra filters (Millipore). DNA and protein concentrations were determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific). The purified ORF27 was checked on SDS-PAGE gel for purity (Fig. 4).

ATP-PPi release Assays for ORF27.

[0097] The substrate range of ORF27 was determined by ATP-PPi release assays as previously described (12). Without prior knowledge about the reaction pathways, the ATP-

PPi assay served a semi-quantitative method to compare substrate induced acceleration of ATP consumption. For kinetic investigation of ORF27 activity with different substrates, the inorganic pyrophosphate released by enzymatic reaction was measured continuously using the EnzChek Pyrophosphate Assay Kit (Invitrogen). A typical assay contained in a total volume of 150 μ L: 5 μ M of ORF27, 0-20 mM substrates, 1 mM ATP, 1 mM CoASH and 1 mM $\text{Mg}(\text{Cl})_2$ in 100 mM HEPES, pH 7.5. 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) substrate, purine nucleoside phosphorylase and inorganic pyrophosphatase were added according to the protocol. Reactions were initiated by the addition of ATP and monitored at 360 nm with SpectraMax M2 (Molecular Devices, Sunnyvale, CA). Initial velocities were calculated using the standard curve for inorganic pyrophosphate. For each concentration, control reactions were carried out without enzyme or without ATP. The rates of PPi release were converted to observed rates, and the Michaelis-Menten kinetic parameters were obtained.

[0098] The acids assayed were glutamic acid, γ -aminobutyrate (GABA), 4-guanidinobutyric acid, (S)-3-hydroxyl-butyric acid, valeric acid, 4-methyl-hexanoic acid, 3-aminobutyric acid, 6-aminocaproic acid, 6-guanidinoheptanoic acid, glutaric acid, adipic acid, 2-aminobutyric acid and (Sigma-Aldrich, St. Louis, Missouri). During preparation of substrate stock solution, 6-guanidinoheptanoic acid has low solubility under neutral pH, and HCl was added to obtain 100 mM stock solution in pH ~2.0.

4-Guanidinobutyryl CoA product identification.

[0099] To confirm ORF27's native activity as a 4-guanidinobutyryl CoA synthetase, the quenched reaction with 4-guanidinobutyric acid was analyzed for CoA products as described previously (14). The mass measurements were carried out in the TOF-Scan monitoring mode for the detection of $[\text{M} - \text{H}]^-$ ions (4-guanidinobutyryl-CoA, $m/z = 893.1825$). Negative controls were carried out using no enzyme, no ATP, no substrate or no CoASH.

Product analysis of in vitro ORF27 lactam formation.

[00100] To compare formation of various lactams by ORF27, a reaction mixture containing 57 μ M of ORF27, 5 mM ω -amino fatty acids substrates, 1 mM ATP or ADP, 0.5mM CoASH and 1 mM $\text{Mg}(\text{Cl})_2$ in 100 mM HEPES (pH=8) was incubated at 25°C. The reactions were quenched by addition of methanol to a final concentration of 50% (v/v) at multiple time points (0 min, 15 min, 1 h, 2 h, 4 h and 19 h). The resulting quenched reactions

were kept at 4°C and filtered through 10 K Amicon Ultra-0.5 mL Centrifugal Filters (Millipore) at 8000 ×g for 30min. The filtered solutions were analyzed for lactams and nucleotides using HPLC-MS. Control reactions were carried out without enzyme, without substrate, without ATP, without CoASH or without MgCl₂ (supplying additional 0.2 mM EDTA to chelate Mg²⁺ ions from purified protein stock). Caprolactam, valerolactam and butyrolactam, AMP, ADP and ATP were purchased as standards from Sigma-Aldrich. The pH dependence of ORF27-catalyzed butyrolactam formation was determined using an end-point assay, and the amount of butyrolactam was determined using the HPLC-MS method described above.

Lactam Production in Vivo.

[00101] *E. coli* JW2637-4 contains a knockout of *gabT*, which encodes a GABA transaminase (15). This host was initially used to confirm production of valerolactam and caprolactam in vivo. *E. coli* JZ-171 (ORF27) and JZ-172 (RFP negative control) were grown in LB medium containing 1 mM 5-AVA and 6-AHA. Cultures were propagated in Luria-Bertani (LB) medium, which was prepared from dehydrated powder according to the manufacturer's instructions (BD Biosciences, San Jose, CA). To analyze caprolactam production, the culture was pelleted and supernatant was filtered before mixing with 1 volume of MeOH.

In vivo valerolactam biosynthesis.

[00102] The *davA* and *davB* genes were ordered as gBlocks (Integrated Dna Technologies, Coralville, IA) and cloned into the BglII and XhoI sites on pBbA7, a biobrick vector, to generate plasmid pBbA7a-DavB-DavA(16). To circumvent ORF27's limited solubility during incubation, MBP-ORF27 was utilized (13). For high-density shake flask cultures, Studier's autoinduction ZYM-5052 medium was prepared according to the published protocol (17). Lysine at various concentrations (0 g/L, 1 g/L, 5 g/L and 10 g/L) was included in the ZYM-5052 medium. Kanamycin (20 µg/ml) and ampicillin (100 µg/ml) were added where desired to provide selective pressure for plasmid maintenance.

[00103] *E. coli* strains (JZ-441) harboring plasmids containing genes encoding *davA*, *davB* and *MBP-ORF27* were inoculated into 10 mL of LB overnight. On day 2, the overnight culture was inoculated 1:100 (v/v) into 25 mL Studier's autoinduction ZYM-5052 medium with various concentrations of lysine (0 g/L to 10 g/L) and appropriate antibiotics (17). The

culture was incubated at 37°C. When the O.D. reached around 0.6, the culture was cooled to 25°C. The culture was then placed at 25°C incubator and the valerolactam titer was analyzed at 24 h, 48 h and 72 h. JZ-440, which contains only *davA* and *davB*, served as a negative control.

Caprolactam, valerolactam and butyrolactam analytical method

[00104] Liquid chromatography (LC) separation of lactams were conducted at 55°C with an Inertsil ODS-3 reverse-phase C18 column (250 mm length, 2.1 mm internal diameter, 3 µM particle size; GL Sciences) using a 1100 series high-performance LC system (Agilent Technologies). The mobile phase was composed of 0.1% formic acid in H₂O (solvent A) and 0.1% formic acid in MeOH (solvent B). Butyrolactam was separated with the following gradient: 40% to 60% B for 4.5 min, 60% to 100% B for 0.5 min, 100% to 40% B for 0.5 min, held at 10% B for 8.5 min. A flow rate of 0.18 mL/min was used throughout.

Time-of-Flight Mass Spectrometry method for lactam accurate mass monitoring

[00105] The LC system was coupled to an Agilent Technologies 6210 electrospray time-of-flight (TOF) mass spectrometer. Nitrogen gas was used as both the nebulizing and drying gas to facilitate the production of gas-phase ions. The drying and nebulizing gases were set to 11 L/min and 25 psig, respectively, and a drying gas temperature of 320°C was used throughout. ESI was conducted in the positive-ion mode with a capillary voltage of 3.5 kV. Mass measurements were carried out in the TOF-Scan monitoring mode for the detection of [M + H]⁺ ions (2-pyrrolidone, m/z=86.0600; 2-piperidinone, m/z= 100.07569; caprolactam, m/z= 114.09134). The instrument was tuned for a range of m/z 70 to 300. Data acquisition and processing were performed using MassHunter Workstation (Agilent Technologies).

ATP, ADP, AMP analytical method

[00106] Nucleotide product analysis studies was analyzed using an expedited modification of the HILIC method previously described (20). Liquid chromatography (LC) separation of lactams were conducted at 40°C with an SeQuant Zic-pHILIC column (150 mm length, 2.1 mm internal diameter, 5 µM particle size; GL Sciences) using a 1100 series high-performance LC system (Agilent Technologies). The mobile phase was composed of 50mM (NH₄)₂CO₃ in H₂O (solvent A) and acetonitrile (solvent B). ATP, ADP, AMP and CoAs were

separated with the following gradient: 73% to 43% B for 6 min (flow rate 0.25mL/min), 43% to 73% B for 0.2 min (flow rate 0.25mL/min), hold at 73% B for 1 min (flow rate 0.3mL/min), hold at 73% 0.2 min (flow rate 0.3mL/min), held at 73% B for 5.1 min (flow rate of 0.38mL/min). The HPLC system was coupled to TOF MS. ESI was conducted in the negative ion mode, and a capillary voltage of 3.5 kV was utilized. Fragmentor, skimmer, and OCT1 RF voltages were set to 200 V, 65 V, and 300 V, respectively. Mass measurements were carried out in the TOF-Scan monitoring mode for the detection of $[M - H]^-$ ions (ATP, $m/z=505.9885$; ADP, $m/z=426.0221$; AMP, $m/z=346.0558$; CoASH, $m/z=766.1079$). MS experiments were carried out in the full-scan mode (m/z 100 to 1000).

ORF27 Biochemistry and Implications on ECO-02301 Loading Mechanism

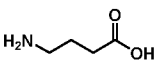
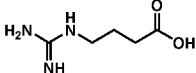
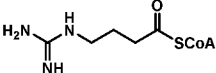
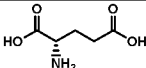
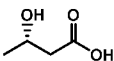
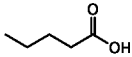
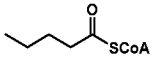
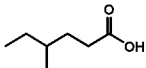
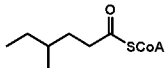
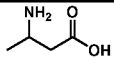
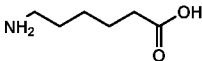
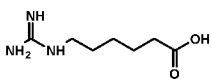
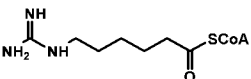
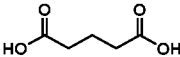
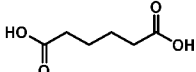
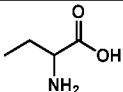
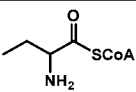
[00107] By exploring its substrate specificity, 4-guanidinobutyric acid is confirmed to be its natural substrate. This is consistent with previous hypothesis that ECO-02301 biosynthesis uses 4-guanidinobutyric acid as a starter unit. The inability to isolate activated 4-aminobutyryl esters in the enzymatic reaction showed the transient nature of the intermediate. This suggests that the proposed amidinohydrolase (ORF33) in the gene cluster may hydrolyze the ureido group later during ECO-02301 biosynthesis rather than before loading onto the first ACP domain.

RESULTS

ORF27 Biochemistry.

[00108] The sensitivity of the ATP-PPi release assay (5 μ M Pi) allowed the initial velocity to be determined within the first 2min of reaction incubation. During this time period, ATP hydrolysis activity by ORF27 was not observed according to the ATP-PPi assay (ATP hydrolysis was observed over longer period of time though, see discussion below). The ATP-PPi release assay revealed that ORF27 has a broad substrate specificity. The K_m and k_{cat} values of various substrates activation are shown in Table 2.

[00109] Table 2. Steady State Parameters of ORF27^a

Substrate	Structure	Product ^b	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (M ⁻¹ min ⁻¹)
γ -Aminobutyrate			17.2	0.35	20.26
4-Guanidinobutyric acid			0.013	0.12	9434
Glutamate ^c		ND	ND	ND	ND
(S)-3-hydroxyl-butyric acid		ND	ND	ND	ND
Valeric acid			52.2	1.70	32.6
4-Methyl-hexanoic acid			12.1	1.3	107.8
3-Aminobutyric acid		ND	ND	ND	ND
6-Aminocaproic acid			0.056	0.17	2962.6
6-Guanidino-hexanoic acid			0.11	0.33	2939.7
Glutaric acid		ND	ND	ND	ND
Adipic acid		ND	ND	ND	ND
2-Aminobutyric acid			1.63	0.036	22.2

a The kinetic parameters of ORF27 were determined in a 100 mM HEPES, pH 7.5 at 25°C using the ATP-PPi release Assays

b Other non-product forming pathways could occur such as ATP hydrolysis or acyl-OAMP intermediate hydrolysis.

c ND not determined.

[00110] HPLC-MS confirmed that ORF27 catalyzes 4-guanidinobutyryl-CoA formation (Figs. 5A and 5B). The ability of ORF27 to accept a broad range of substrates, especially ω -amino fatty acids, implies an interesting biotechnology application. Once the acid group of ω -amino fatty acids has been activated, amide bond formation through intramolecular cyclization is theoretically thermodynamically favored, suggesting that ORF27 might be utilized as a lactam synthase to produce industrially important chemicals, such as caprolactam, valerolactam and butyrolactam (Fig. 1).

[00111] The ORF27-catalyzed lactam formation reaction in vitro was monitored for both nucleotide and lactam products, using either ATP or ADP as the energy source. LC-MS analysis of nucleotides (ATP, ADP, AMP) showed that ORF27 catalyzed a series of reactions (Fig. 2). In the absence of ORF27, ATP remained stable throughout the reaction (Fig. 6A). In the absence of ω -amino fatty acids, ORF27 predominantly hydrolyzed ATP over long term incubation, forming mainly ADP plus Pi and slightly AMP and PPi (Fig. 6B). This seeming contradiction with previous negative control ATP-PPi assay result was because: due to the slow kinetics of the in vitro reaction, ATP-PPi assay was not sensitive enough to detect Pi released caused by enzymatic ATP hydrolysis into ADP and Pi during the first 2 minutes (Fig. 6B). When ω -amino fatty acids were added into the reaction mixture, they facilitate ATP hydrolysis, as well as routing hydrolysis pathway towards forming AMP and PPi rather than ADP plus Pi. Therefore, the observed signal increase in the presence of substrate for the ATP-PPi assay was due to both increased ATP consumption, and having more PPi release instead of Pi release without ω -amino fatty acid substrates.

[00112] Although ORF27 was predicted to be an acyl-CoA synthetase, CoASH was not required for lactam formation (Figs. 6C to 6H). The minimal lactam formation system constituted ω -amino fatty acids, ORF27, ATP and Mg^{2+} (Figs. 7A to 7C). Lactam formation is most rapid for valerolactam, followed by butyrolactam and caprolactam. ADP appeared earlier in reactions containing ω -amino fatty acids but not in control reactions without the substrates, suggesting ω -amino fatty acids dependent ATP hydrolysis into ADP and Pi also

occurred during the reaction.

[00113] When CoASH was added to the corresponding reactions, no 4-aminobutyryl-CoA or 5-aminovaleryl-CoA was detected and barely distinguishable 6-aminohexanoyl-CoA mass ions, corresponding to 0.6% of CoASH signal abundance, were detected (data not shown). Mass ions corresponding to other off-pathway products, such as the ω -amino fatty acids dimers or trimers, were not observed.

[00114] In addition to using ATP, ORF27 could also utilize ADP to activate ω -amino fatty acids and catalyze lactam formation, although the reaction occurred at only 20-50% of the rate of the similar reaction when ATP was the substrate (Figs. 6I to 6K).

[00115] The pH profile of ORF27 activity was determined for butyrolactam, and the enzyme had a pH optimum of 8.0. The enzyme precipitated and became inactive when the pH dropped below 6.0 (Fig. 8).

Valerolactam and caprolactam production in vivo.

[00116] To demonstrate that valerolactam or caprolactam can be produced using whole-cell catalysts, we fed the respective precursors, 5-AVA or 6-AHA, to *E. coli* JZ-171, which overexpressed ORF27. Valerolactam (5-AVA fed) or caprolactam (6-AHA fed) was observed in the medium upon ORF27 expression (Figs. 9A and 9B).

[00117] To demonstrate that valerolactam could be produced directly from glucose with no ω -amino fatty acid feeding, we introduced a pathway for 5-AVA biosynthesis into *E. coli* BL21(DE3)star, resulting in strain JZ-441(2-4). Valerolactam was produced directly from lysine when *davA* and *davB* were introduced into *E. coli* (Fig. 3A). Cells growing in medium supplemented with 5 g/L of lysine produced a maximum of 195 mg/L (~2 mM) valerolactam. Increasing lysine supplementation to 10 g/L caused a reduction in bacterial growth and caused a delayed and decreased production of valerolactam. At 0 g/L lysine feeding, JZ-441 produced 89 mg/L of valerolactam (Fig. 3B). JZ-440, which lacks MBP-ORF27, produced no measurable valerolactam.

DISCUSSION

[00118] Natural product biosynthesis continues to be a rich source of enzyme candidates with novel activities. ORF27, an enzyme in the *Streptomyces aizunensis* ECO-

02301 biosynthetic cluster, was identified to be a 4-guanidinobutyryl-CoA synthetase, which has interesting implications for the ECO-02301 loading mechanism (12, 18, 19).

[00119] ORF27 accepted a wide range of short chain fatty acid substrates and their functionalized analogs. Both linear and branched fatty acid substrates were accepted as substrates. However, the enzyme seems to have little tolerance of polar groups at C3 position. Positively charged substrates, such as the ω -amino fatty acids or the ω -guanidino fatty acids are well tolerated, despite the fact that some substrates such as 6-guanidinohexanoic acid have two more carbons in their backbone than the native substrate. However, enzyme activity on substrates that have a negatively charged group on the ω -terminal end, such as a carboxylic acid group, was not observed, even though glutaric acid and adipic acid have similar steric hindrance as 6-guanidinohexanoic acid. This suggests that ORF27 has strict substrate selection residues around its binding pocket and preferentially favors substrates with a positively charged group on the ω -terminal end.

[00120] The ability of ORF27 to accept various ω -amino fatty acids was explored for lactam biosynthesis. For lactam formation, ORF27 does not require CoASH as substrate and utilizes multiple reaction pathways. The activation of ω -amino fatty acids by ATP facilitates cyclization. This enables five-membered, six-membered and even seven-membered ring formation at mild temperatures, resulting in the production of important industrial lactams such as valerolactam and caprolactam via fermentation. Unlike the reversible aminolysis enzyme CALB that was previously described, ORF27 performs the reaction under milder conditions in an irreversible fashion. The lactam product observed is exclusively cyclized monomers, without dimer or trimer contaminants. These novel features make ORF27 an ideal candidate for lactam biosynthesis. However, the enzyme catalyzed significant ATP and ADP hydrolysis during the reaction. Directed evolution of ORF27 towards less futile ATP and ADP hydrolysis could potentially improve ORF27 as a lactam synthase.

[00121] By introducing *davB* and *davA* into *E. coli*, we demonstrated valerolactam biosynthesis from lysine. Valerolactam production in autoinduction medium was lysine dependent. At concentrations of lysine below 5 g/L, valerolactam production correlated with lysine concentration. However, high concentrations of lysine (e.g., 10 g/L) inhibited bacterial growth and resulted in decreased valerolactam production. Even without lysine feeding, direct lysine production from glucose was sufficient to produce 50% of the valerolactam produced when 5 g/L lysine was fed to the culture. Interestingly, in the absence of ORF27, a

slight amount of valerolactam was observed. Since 5-AVA does not cyclize when incubated in medium at 25°C, the observed valerolactam could result from catalysis by innate *E. coli* enzymes.

[00122] Caprolactam was also biosynthesized by feeding its precursor 6-aminohexanoic acid. With the recent publication of 6-aminohexanoic acid biosynthetic pathway in *E. coli*, ORF27 holds the promise to enable full caprolactam biosynthesis in a microbial host (7). This opens the door to substitute the current petrochemical process with a renewable bioprocess for a bulk chemical with four million ton annual production, potentially could make huge positive impact on our environment.

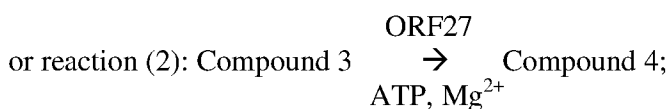
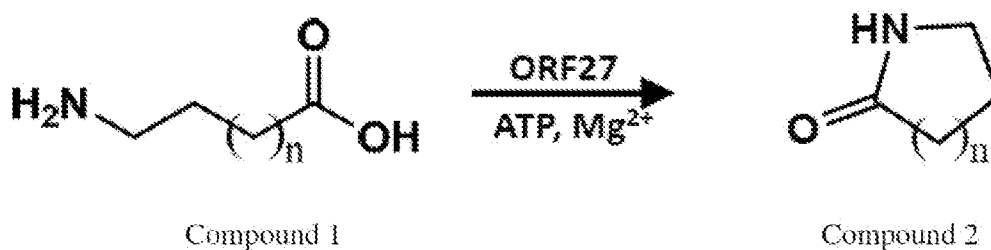
CONCLUSION

[00123] ORF27 is an enzyme in the ECO-02301 biosynthetic cluster. It was identified to catalyze 4-guanidinobutyryl-CoA formation, and was able to activate a wide range of substrates. While acting on ω -amino fatty acids, ORF27 can catalyze the ring closing reaction to produce lactams. Biosynthesis of polymer precursors such as caprolactam, valerolactam and butyrolactam were demonstrated from their respective ω -amino fatty acids. For in vivo production of valerolactam from lysine or glucose, the lysine to 5-AVA pathway was introduced into an *E. coli* strain harboring the gene encoding ORF27.

[00124] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

What is claimed is:

1. A genetically modified host cell comprising a 2-pyrrolidone synthase heterologous to the host cell, an enzymatically active fragment thereof, or an enzymatically active fragment thereof linked to a saccharide binding protein, wherein the 2-pyrrolidone synthase catalyzes the following reaction:



wherein Compound 3 is a “Substrate” and Compound 4 is a “Product” as indicated in Table 2 herein.

2. The genetically modified host cell of claim 1, wherein the 2-pyrrolidone synthase comprises an amino acid sequence having at least 70% identity with SEQ ID NO:1, wherein the amino acid sequence comprises one or more of the following conserved amino acid motifs or sites: acyl-activating enzyme (AAE) consensus motif (residues 137, 140-145, and 147-148), acyl-activating enzyme (AAE) consensus motif (residues 140, 257-258, 279-284, 357, 369, 372, 382, and 458), AMP binding site (residues 140, 180-181, 227, 229-230, 233, 257-258, 279-284, 357, 369, 372, 379-382, and 439), and CoA binding site (residues 180, 229-230, 233, 257, 379-381, 433, and 439).
3. The genetically modified host cell of claim 2, wherein host cell is capable of synthesizing Compound 1 or uptaking Compound 1 from the environment or culture.
4. The genetically modified host cell of claim 3, wherein host cell further comprises one or more enzymes of a pathway for synthesizing Compound 1 from a carbon source.
5. The genetically modified host cell of claim 4, wherein pathway for synthesizing

- Compound 1 from a carbon source that is native to the host cell.
6. The genetically modified host cell of claim 4, wherein pathway for synthesizing Compound 1 from a carbon source that is heterologous to the host cell.
 7. The genetically modified host cell of claim 1, wherein host cell lacks betaine-CoA ligase.
 8. The genetically modified host cell of claim 2, wherein n is an integer from 1 to 20.
 9. The genetically modified host cell of claim 8, wherein n is an integer from 1 to 10.
 10. The genetically modified host cell of claim 9, wherein n is an integer from 1 to 7.
 11. The genetically modified host cell of claim 10, wherein n is an integer from 1 to 3.
 12. A method of producing a Compound 2 in a genetically modified host cell, comprising:
 - (a) providing the genetically modified host cell of claim 1,
 - (b) culturing the genetically modified host cell in a medium under a suitable condition such that the culturing results in the genetically modified host cell producing a Compound 2.
 13. The method of claim 12, further comprising introducing one or more nucleic acid(s) into the host cell encoding the enzyme operably linked to a suitable promoter capable of transcription in the host cell, and optionally encoding the one or more enzyme(s) of a pathway for synthesizing Compound 1 from a carbon source; wherein the introducing step is prior to the culturing step.
 14. The method of claim 12, further comprising separating Compound 2 from the host cell and/or the medium, wherein the separating step is subsequent, concurrent or partially concurrent with the culturing step.

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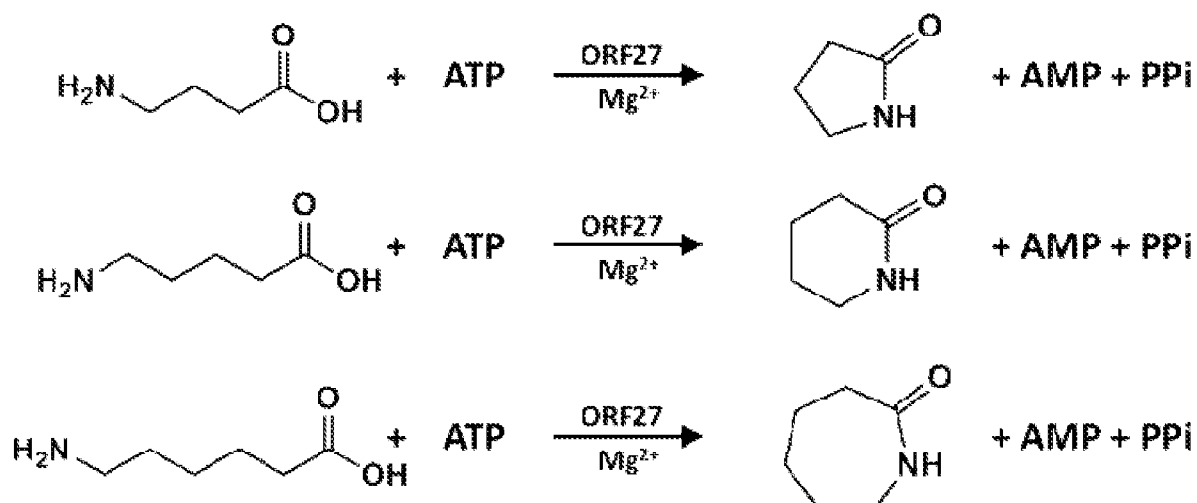


FIG. 1

Fig. 2.

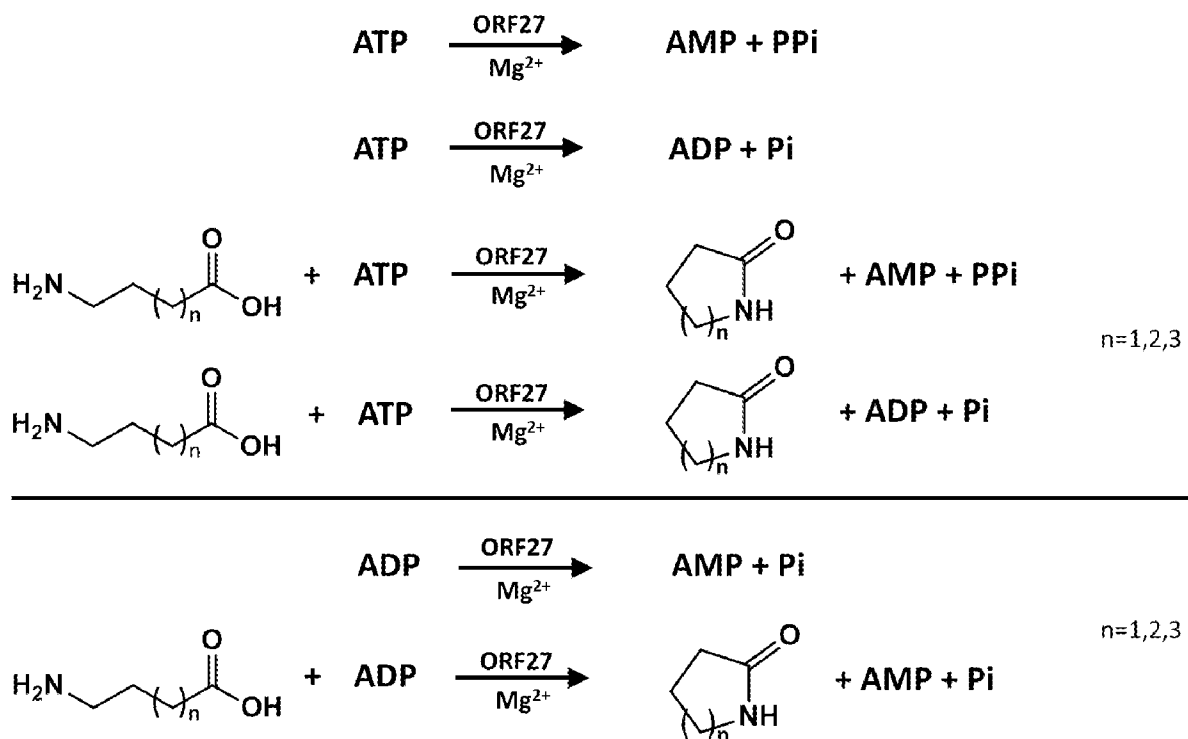


FIG. 2

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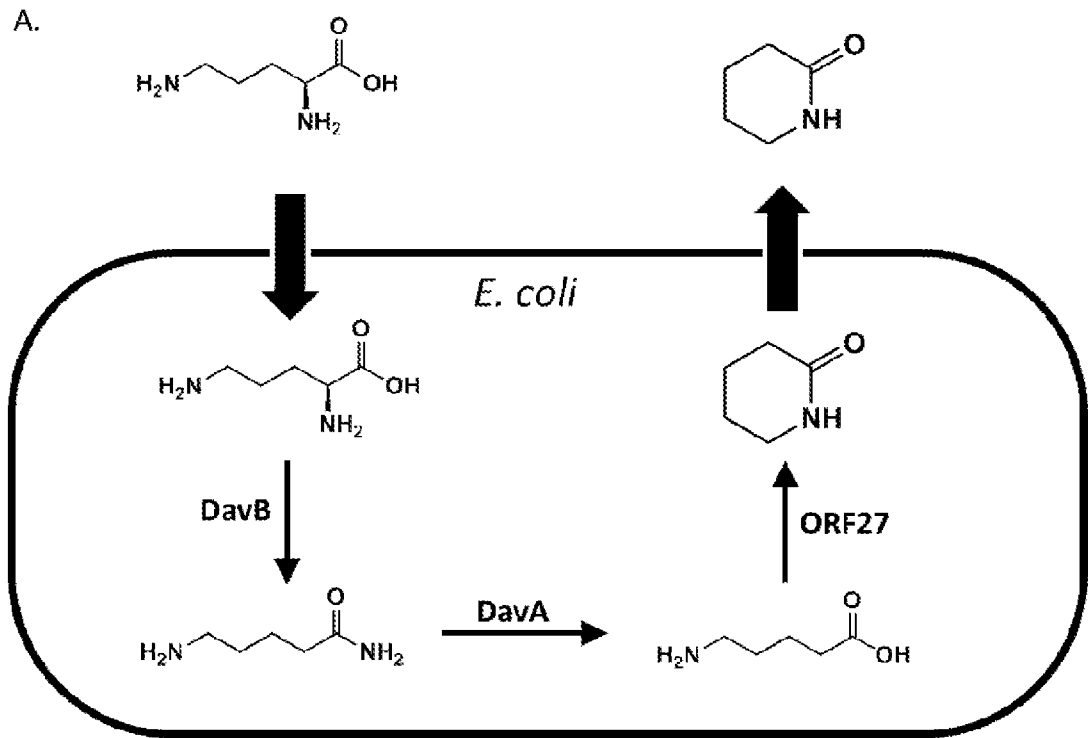


FIG. 3A

B.

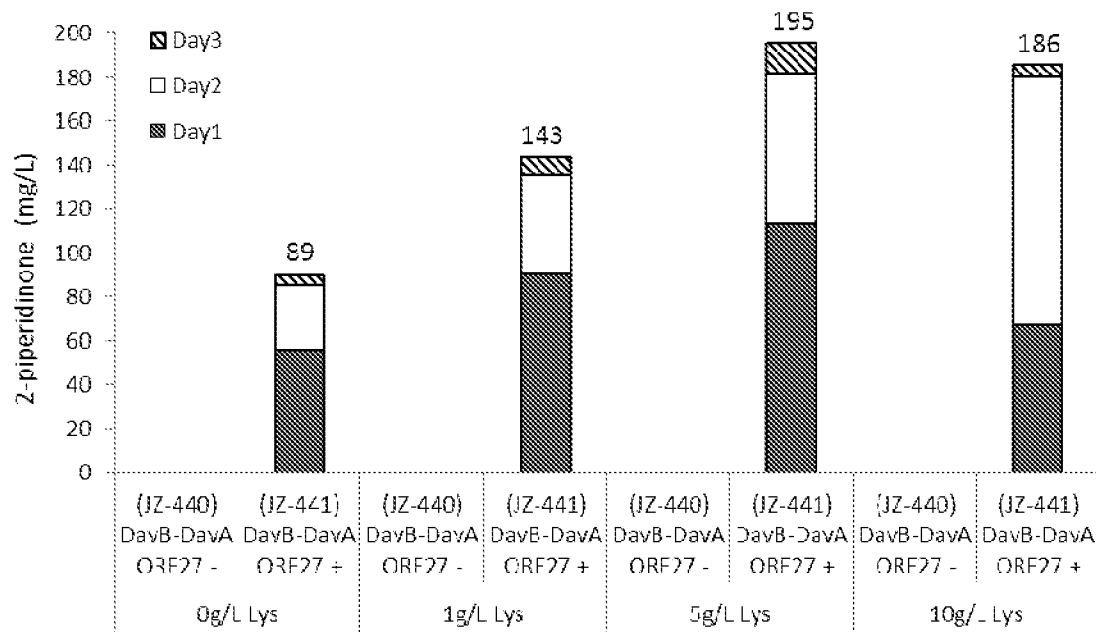


FIG. 3B

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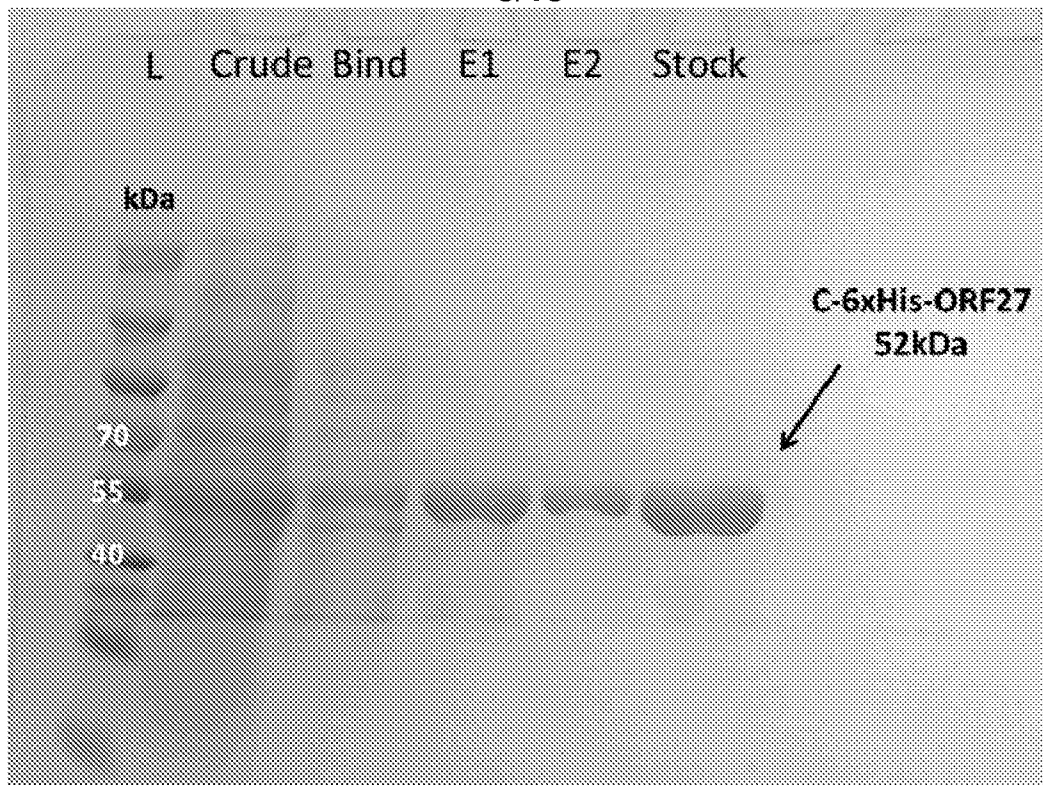


FIG. 4

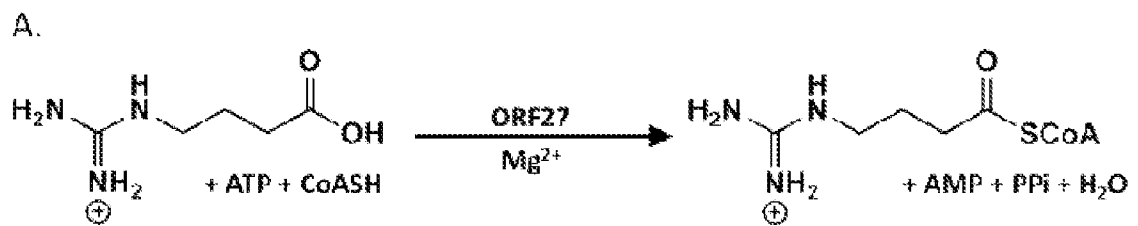


FIG. 5A

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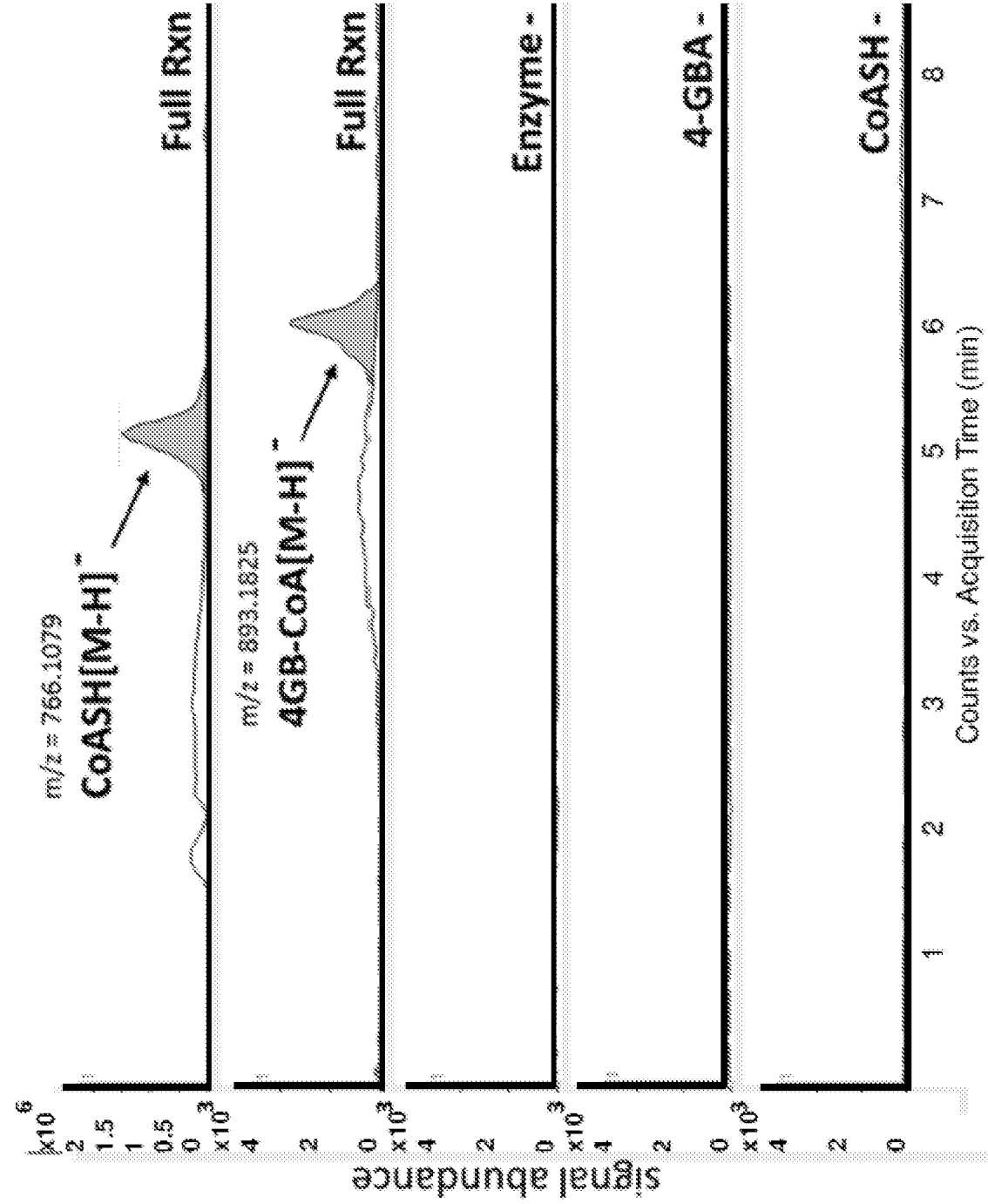


FIG. 5B

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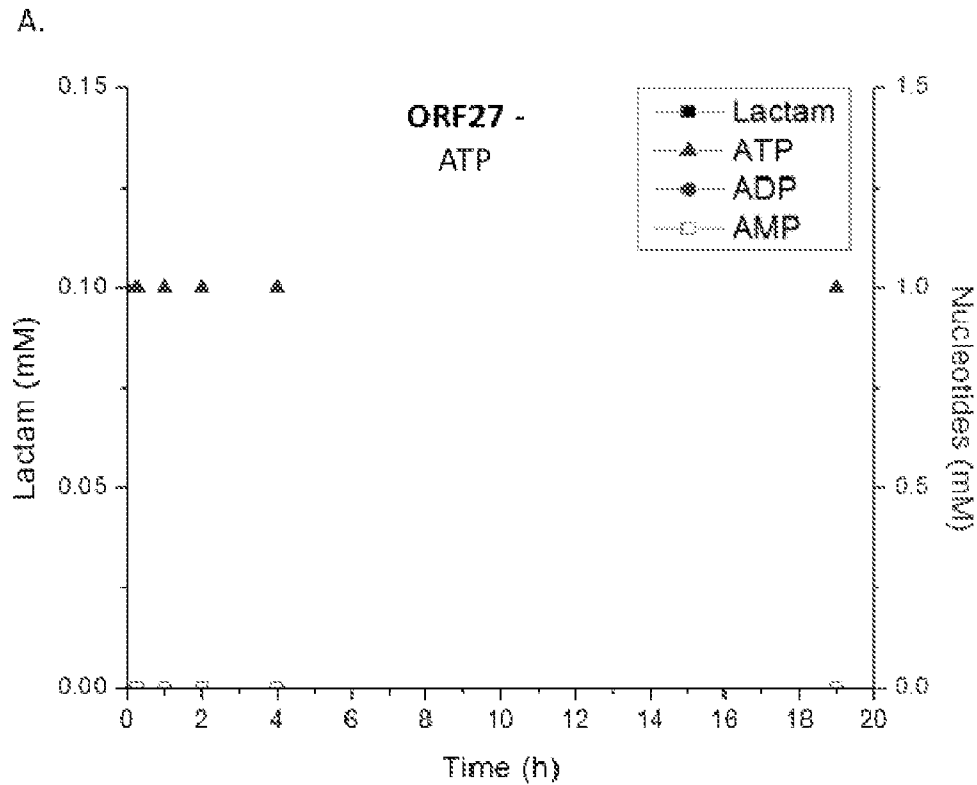


FIG. 6A

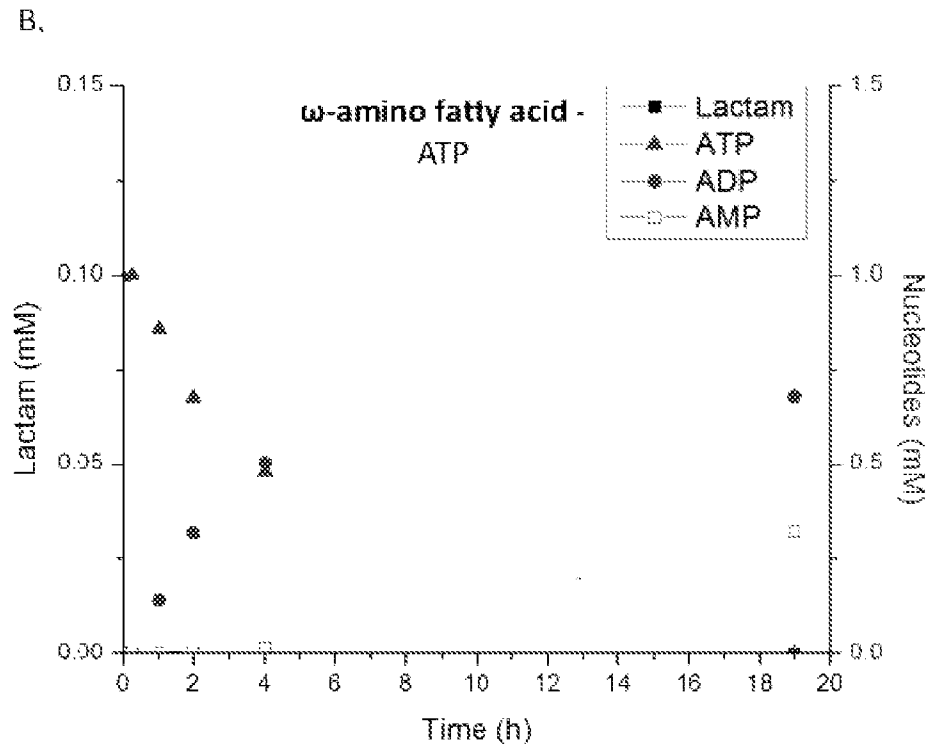


FIG. 6B

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C.

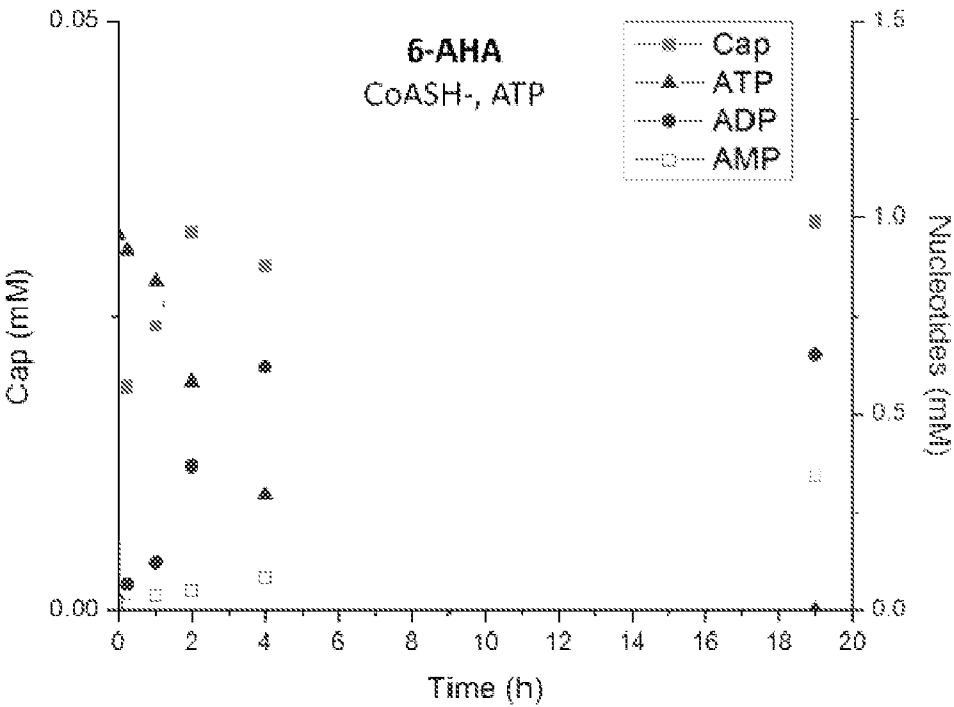


FIG. 6C

D.

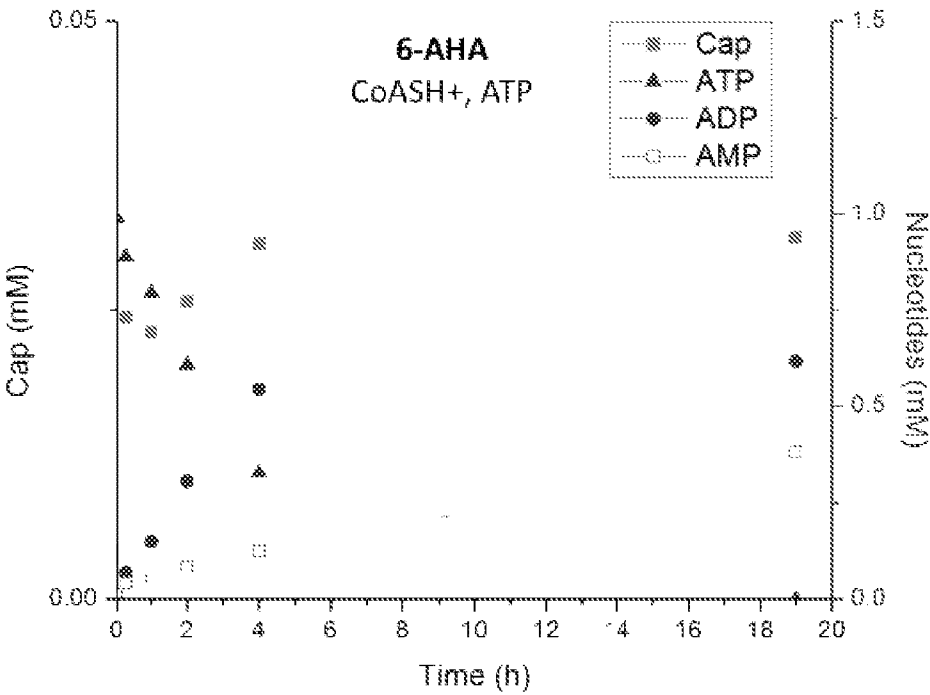


FIG. 6D

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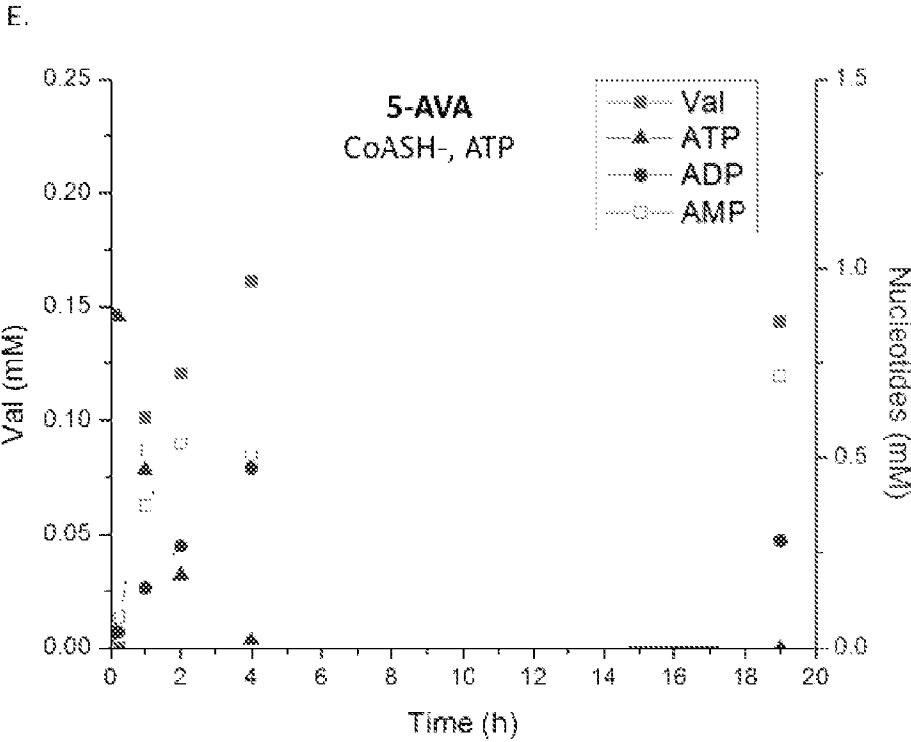


FIG. 6E

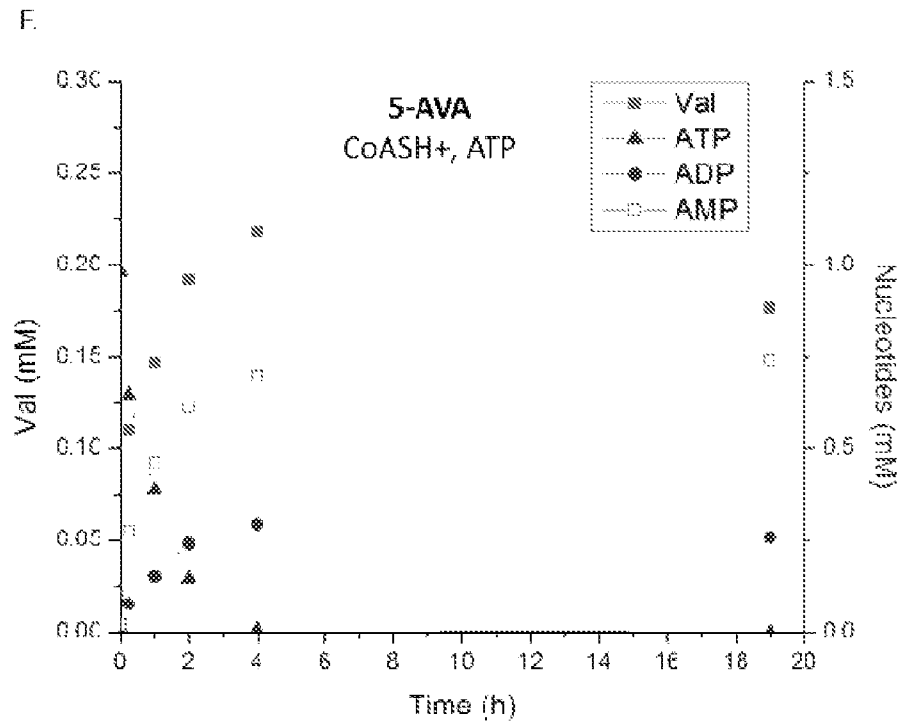


FIG. 6F

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G.

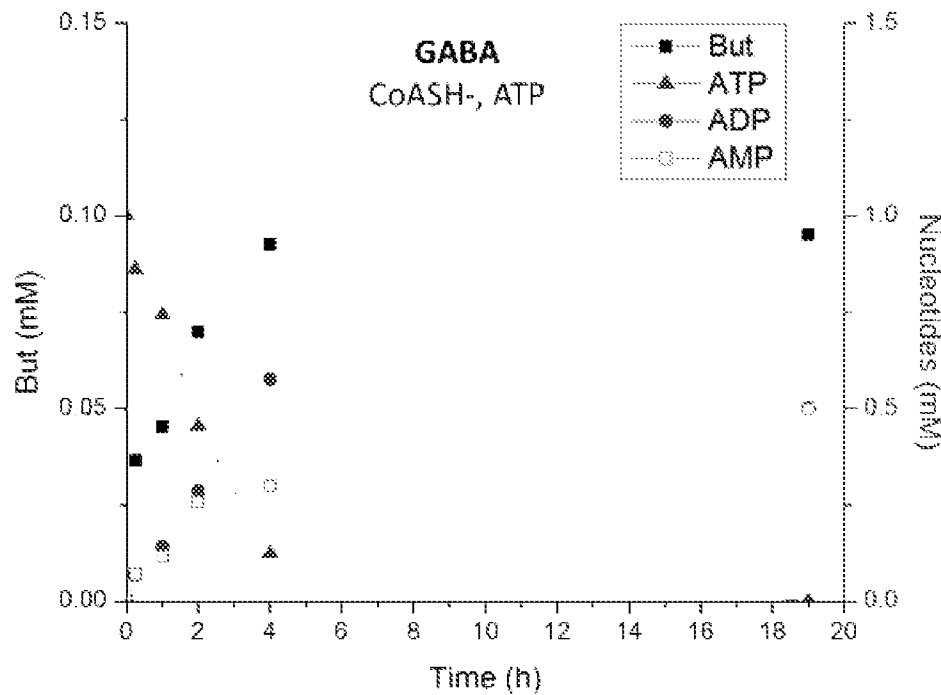


FIG. 6G

H.

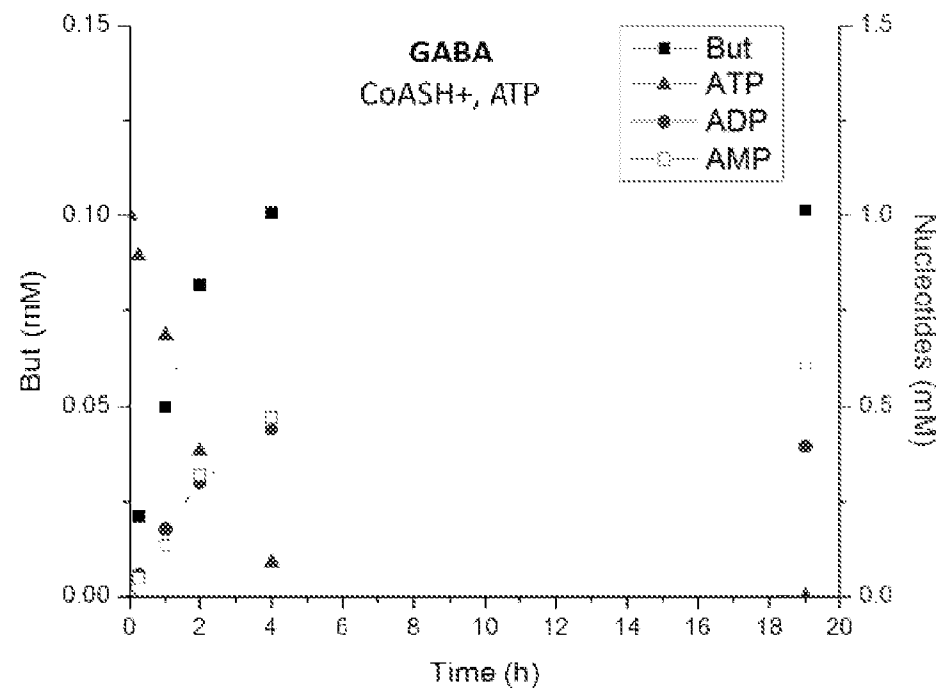


FIG. 6H

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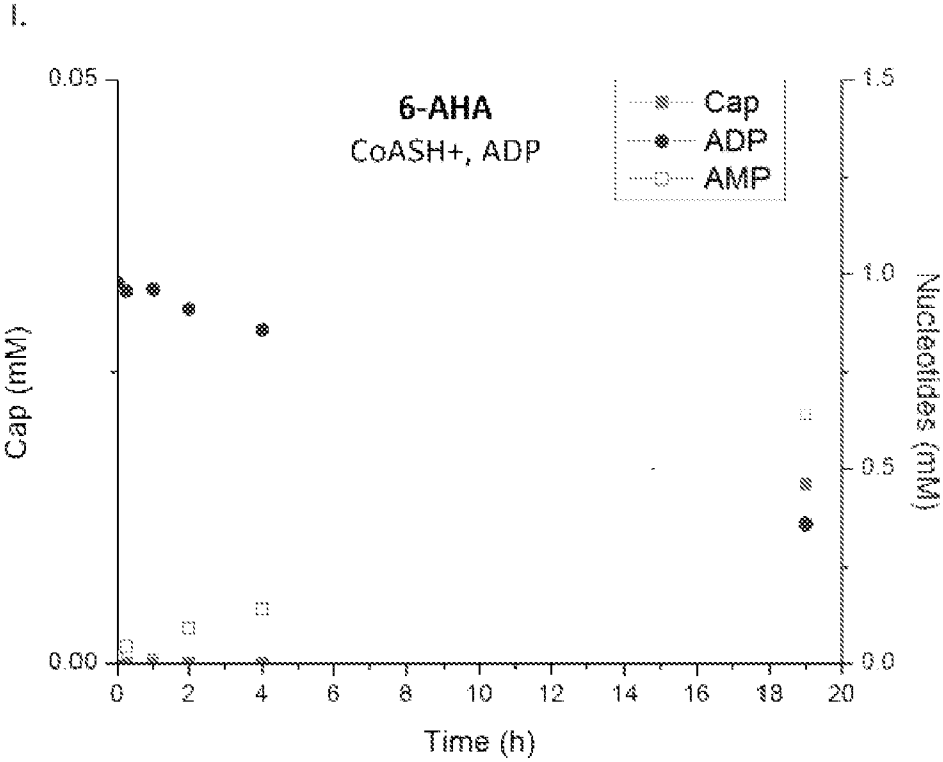


FIG. 6I

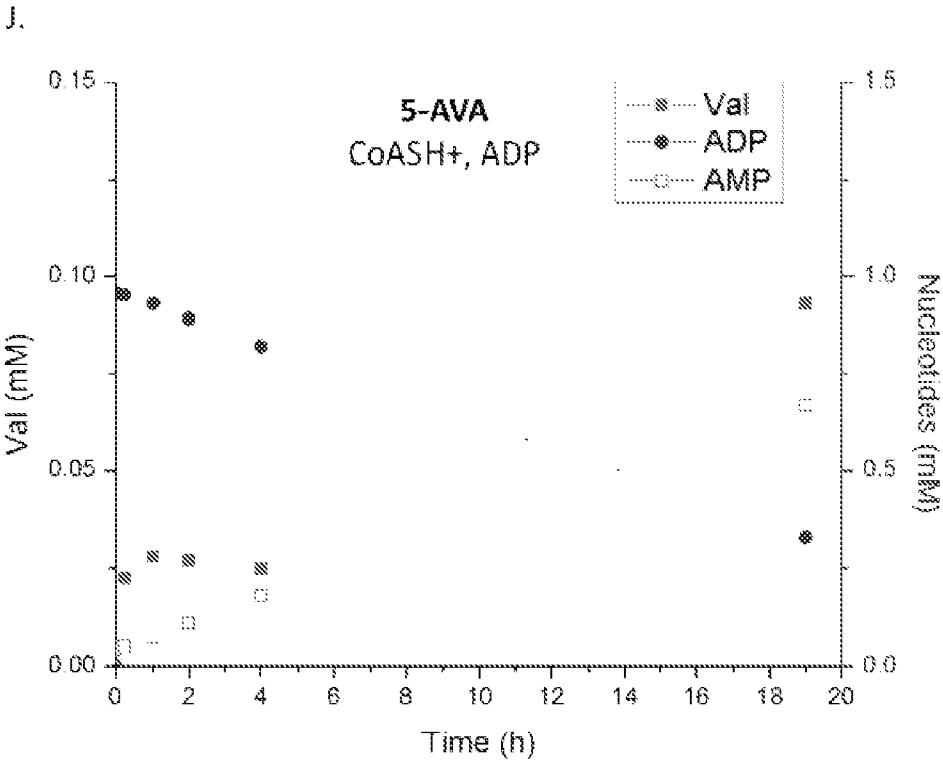


FIG. 6J

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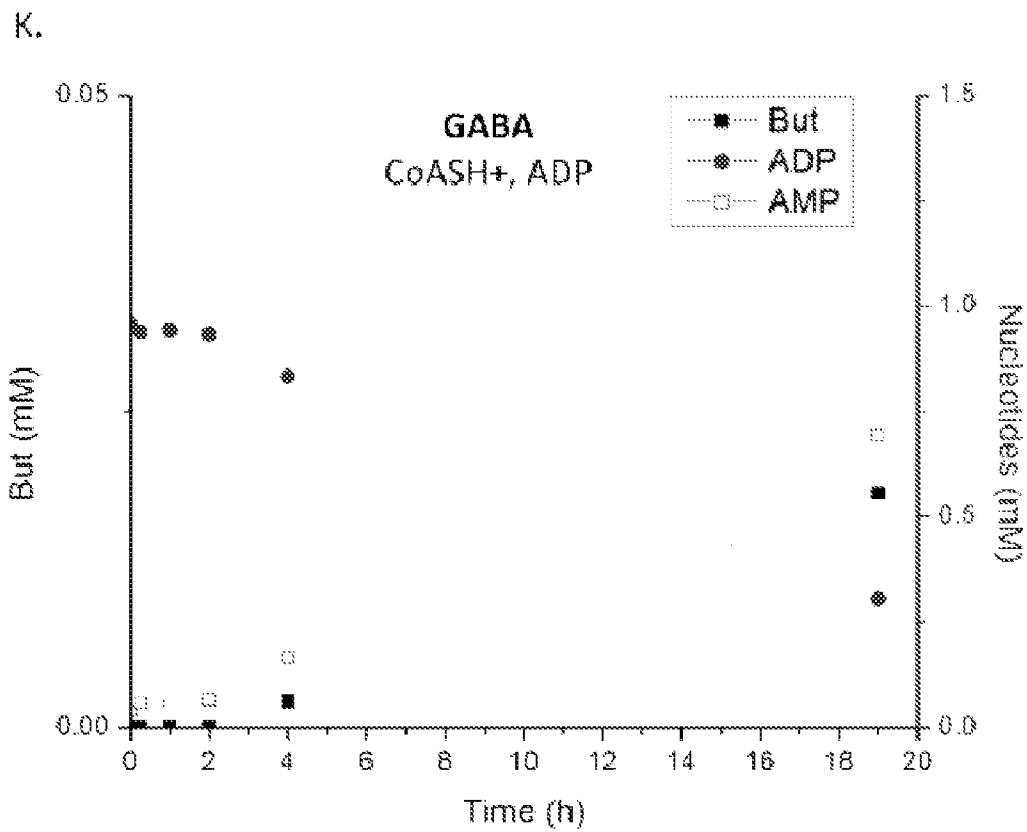
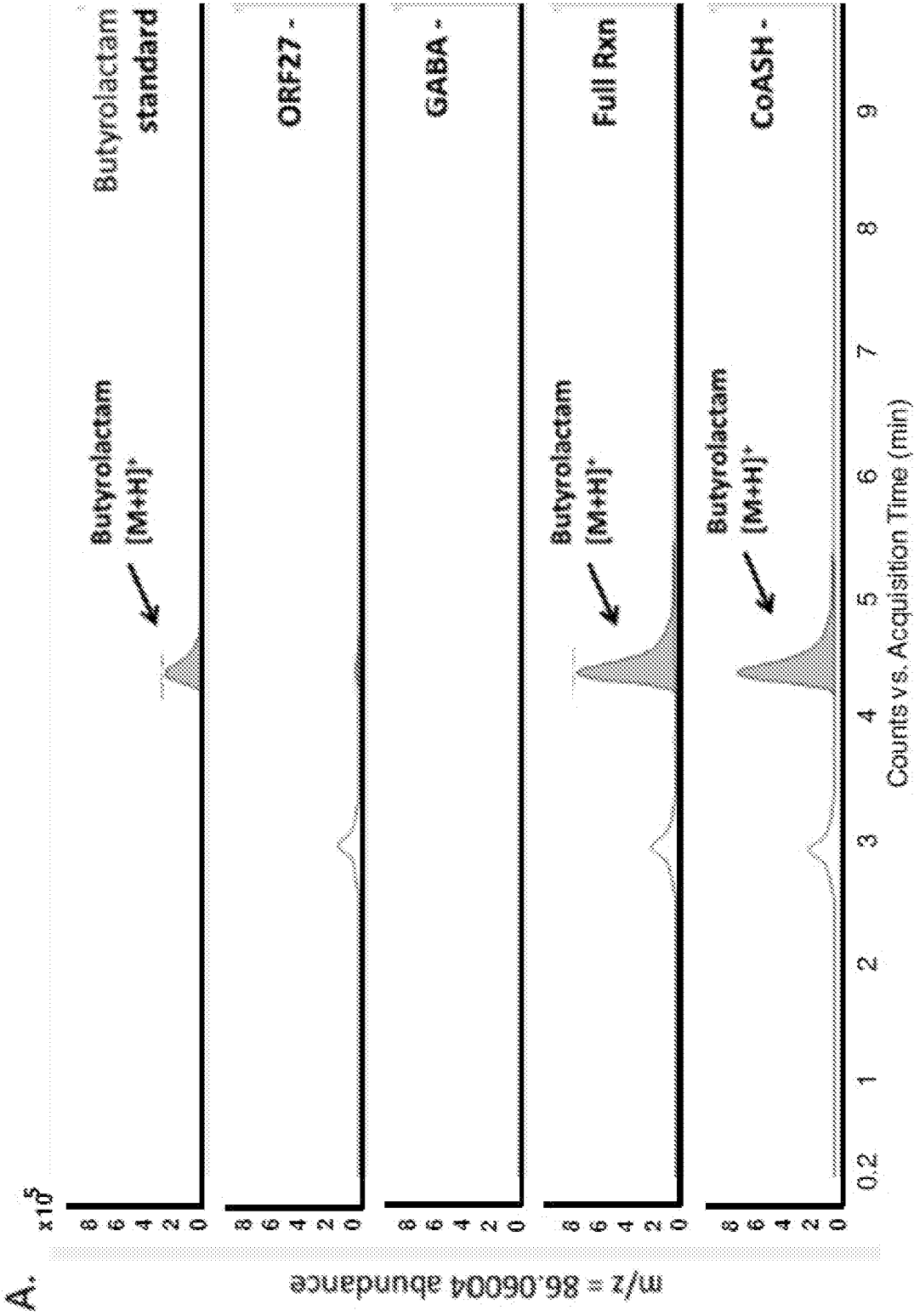
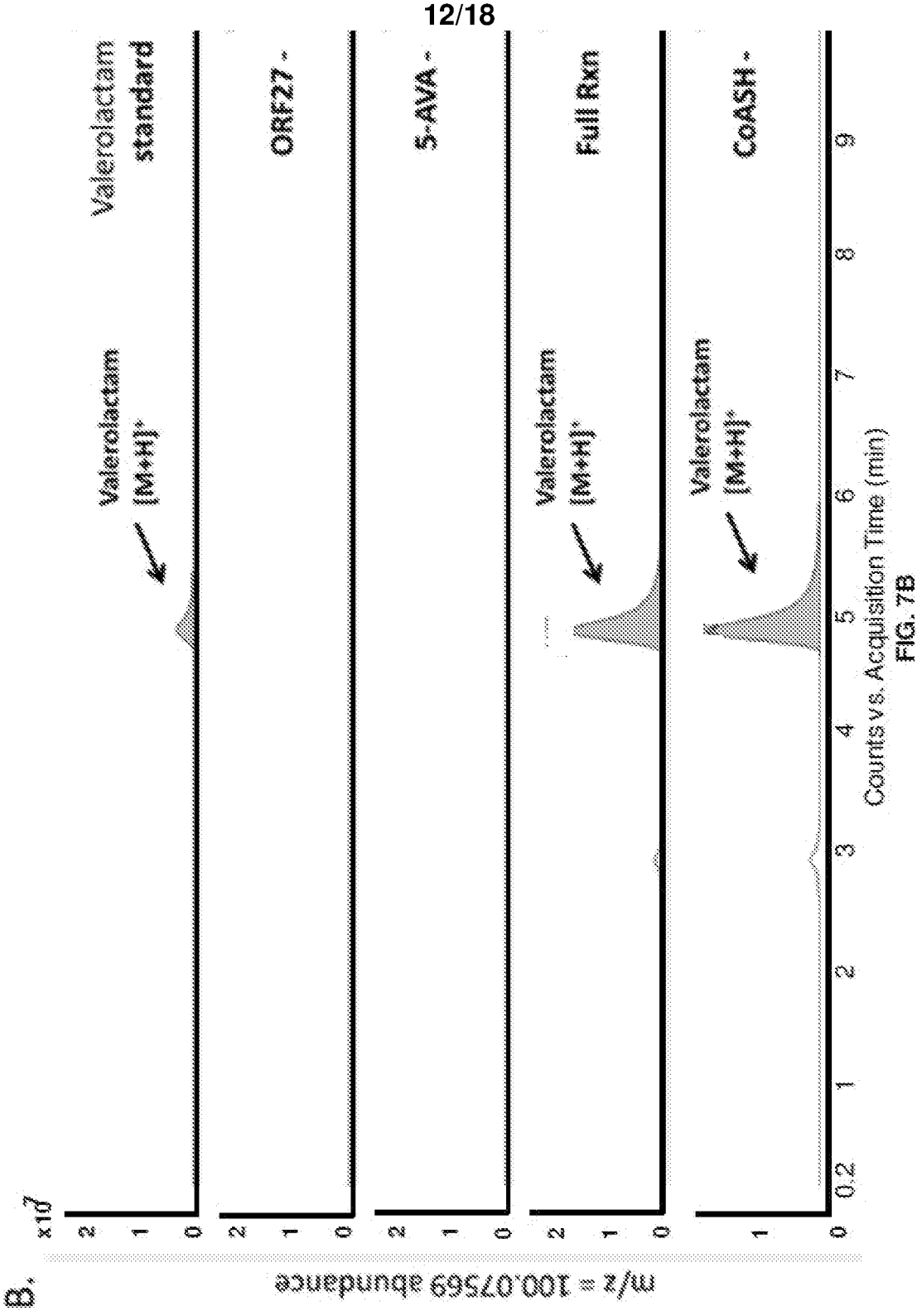
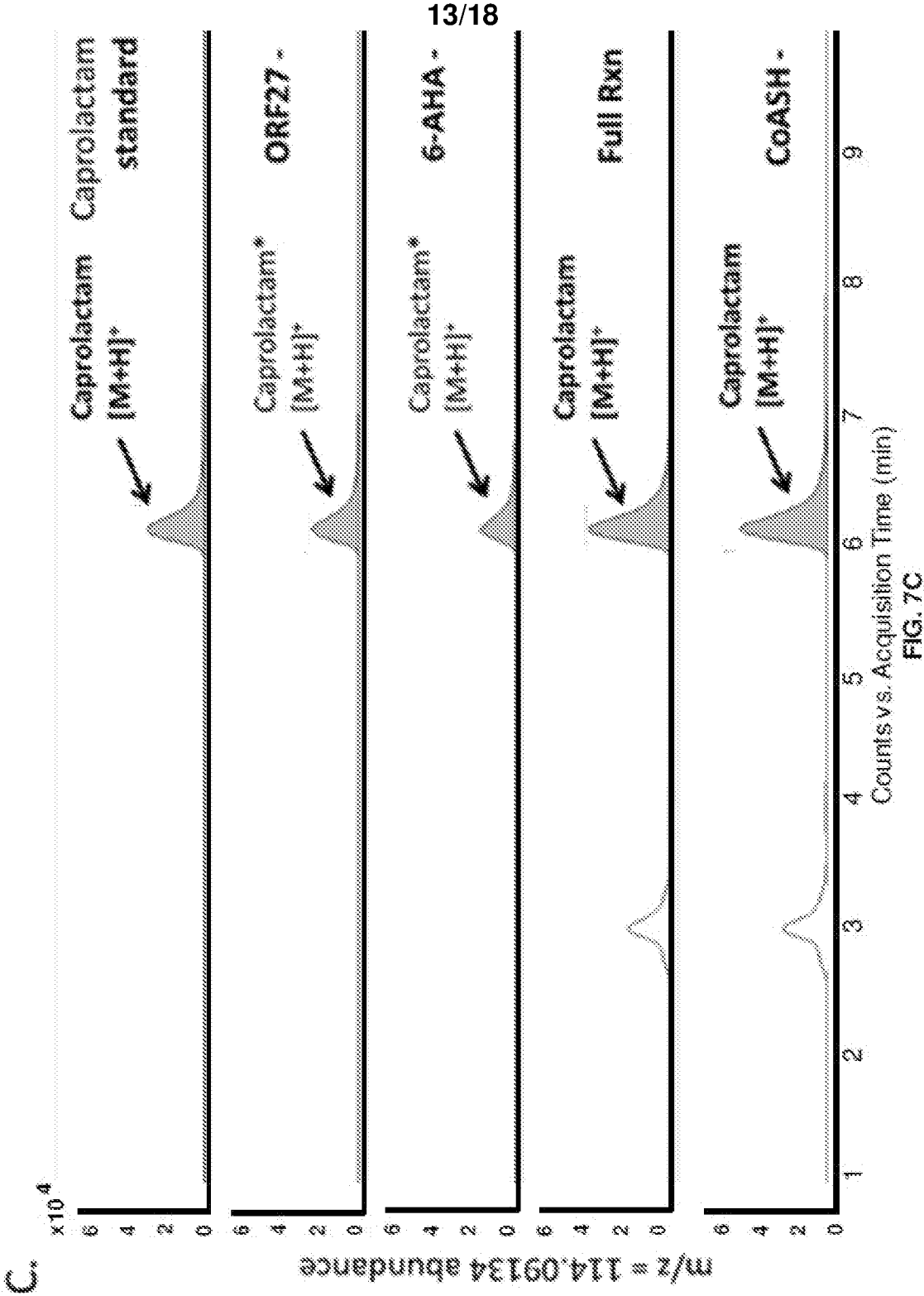


FIG. 6K







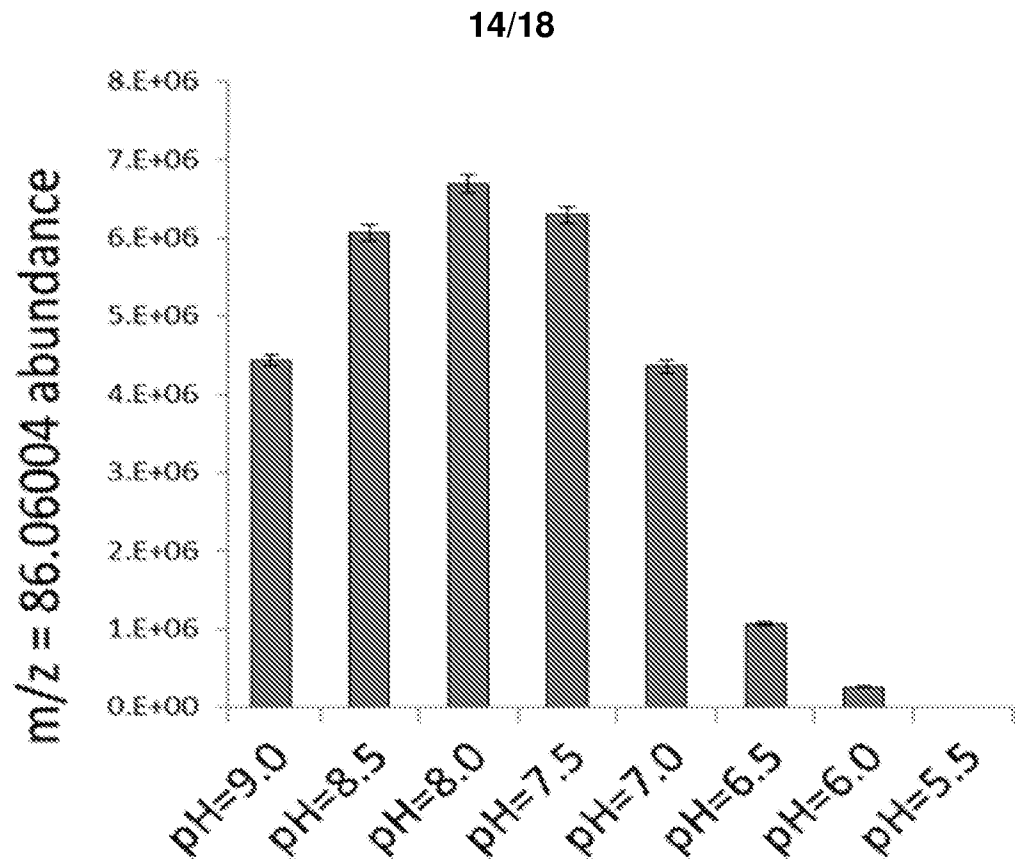


FIG. 8

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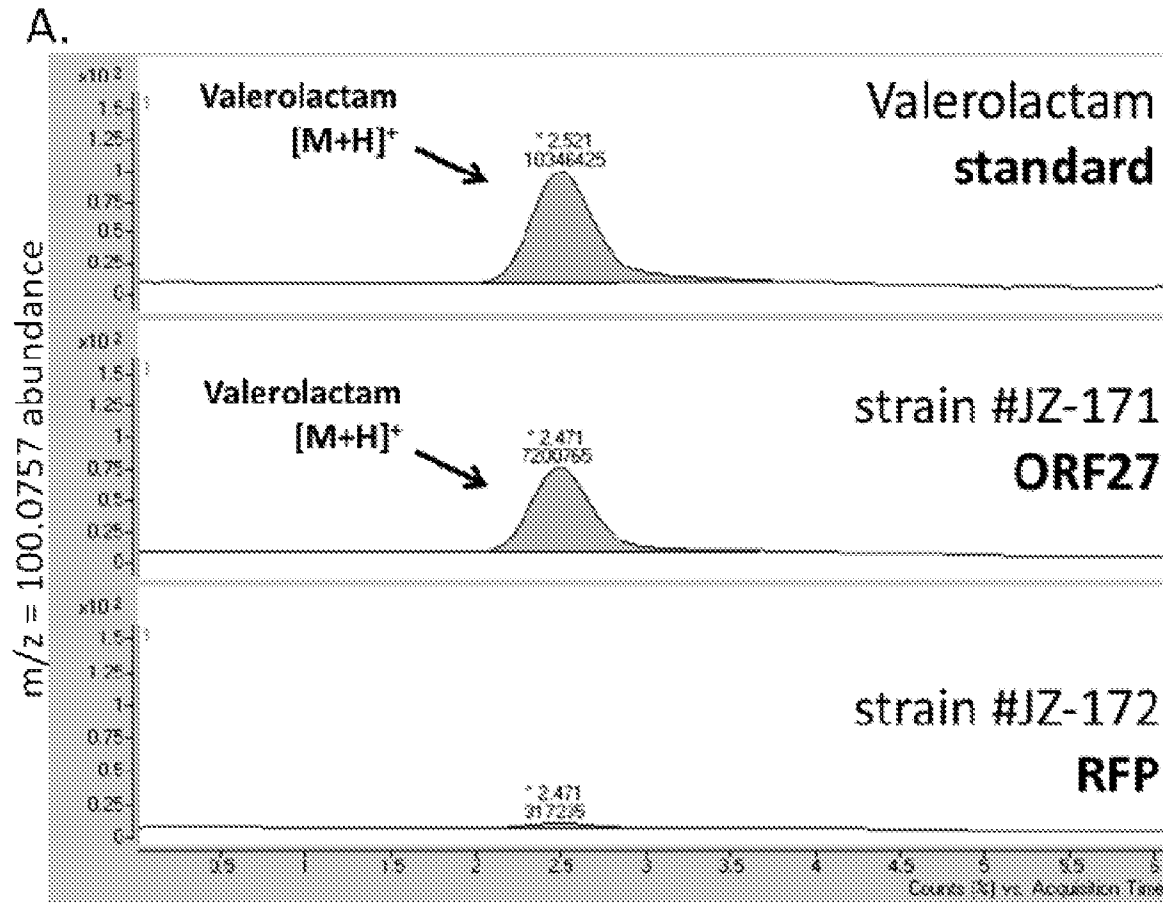


FIG. 9A

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B.

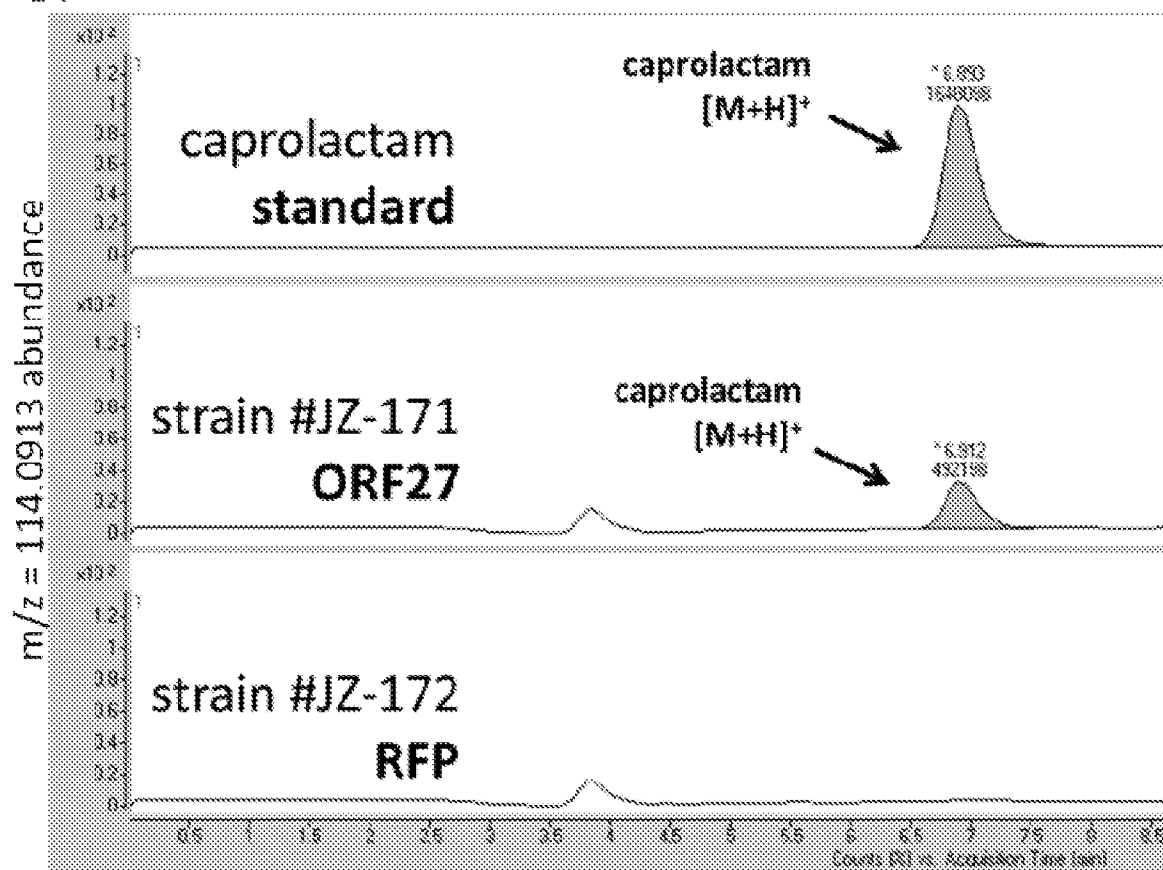


FIG. 9B

P. furiosus VVTHAMQPNLEVFQSLAESYHACPEVSEVFECK~PHELEDALAAAIPTGCGPDLITWA 59
P. aerophilum IRIWHALNPSEESVTKQIAAMITCTHENVQIVFENHAPDLCTAVLAAISGCEKFDLITWA 60
E. coli LVTVINGD~KQVGLASVCKKFFNDT~GKKVVEHPP~DKLEEFKVAATGCGPDLITWA 57

E. furiosus HDWISKPAEAGLLLEPIDESYVTEDELNEFAHQAQDAQKCHHYALPFAAEVATLYNNEN 119
P. aerophilum HDWICLNVEAGVLLKPVNKEVDVLSRFSAPLE-----QYNKHYYGLPFAAEVATLYNNQM 116
E. coli HDSEKSYAQSGLLAEITP-----DNAAFQDELVEPTWDVAVRYNCKLLIAYPIAEVSLIYNDL 115

<i>E. furiosus</i>	VSEPPKTFDETRAINESFYIDENEXIGIAWPIINAYFISAIQAQFGGYVDDKT.....EQ	174
<i>E. aerophilum</i>	VSQPPTTFADLLAIRGCFNKKP--QTGIAIVVPVFETSAWIACGGYVDDET.....EK	170
<i>E. coli</i>	LSPPKATWEETIADLDELKANG--KSMAMENLQEPFTTFLIAADSGYAFYENSKYIND	174
	3..##### *? ? ? . ? ? ? * . * * * . ?.	

E. furiosus POLDKPPTTEGCTFFFTETETWYMAP-TSDINTQGSITFEZGAPFANWNGWSTINDVNNAGT 233
E. aerophilum COLTPDKSTAGCTFFTSXYTHPPVGFETDINTGVNLTGSGAPFANWNGWSTISAVVNGGT 230
E. coli VVDNACAGACACTFFLVLLIKNNHNNADTVLSIAEAAFNKCEATNTTNCPPWASVDPISKV 234

E. furiosus ATGTVLEPPIINDCKEITWEPFGGVLLIYTAAGIINKDAAKKFAKKLITSEESIKTLALG 293
E. aerophilum DUTVAPLEPYN---ATITKPFPGGHHNIVT---IYASKEALDFNHWFTTDFQVAKIMDQ 285
E. coli NYGVTVLEPTK-----GQPSKEPFGVLSAGINAASENKELAELENYLLITDEGLEAVND 290

E. furiosus 352
E. aerophilum 340
E. coli 349

<i>E. furiosus</i>	Q.....NADIEGILKNOQ	366
<i>E. aerophilum</i>	KTISLAUNDVAVDOLL-	355
<i>E. coli</i>	T.....VDEALKDACT	360

FIG. 10

VVWHAQPNELVVFUCLAEETNALCPVEIVFEK-FULEDALAAIPTUCOPDLFWA	59
IRIHALNPESVVKQIAAMTCTHENVVIVENKAPDLQIAYLAAISTGCFDLFWA	60
LVVINGD-KGKNGLAIVGKKFENDK-SIKVTVENP-DKLEENFQAAATCGCDLFWA	57

HDWICKTAEACLEFFIDEVYTEDLNEFAHQAQDAMQAGHYYALPFAAEVVALTYNNEN 119
 HDWICLNVESEVLKPVQNEVADVLRSAPIP-----QYNGSHYGLPFAAEVVALTYNNQM 116
 HDRFCSYAQSCLLAEITP---DKAFQDKLYPFTWDVRYNGKLLIAYPIAVEALISLIYNDL 115

VSQPEPTFDEKKAINEKHYDPANENKIGIAPPINAYFISATACAFGGYVDDAY	EQ 174
VSQPEPTFADLLAINKSGENPPP--XTXGIGIAYVONPYFISAWINACGGYVDDAY	EQ 170
LENPPTWEETIPALDDELKANG--KCALMFNLOQFFYFTWELIADGGYAFKYNCKYDND	174

PGLDPTTIEGTTTTTETIMPTAP TQDINTQCSITLESAPFAMNCSMSINDVKNAGI 233
 QGLDPTXSIAGETTTSTYINPTVCSNFTDINTVNLTLSCAPFCHNCSMSISAVKNGI 230
 VGVDAAGAKAGLTFLVOLIINNNADIDISIAEAFNKGETANTINCFWASUHDISKV 234

NEGVALPPIINDASETWESPFGVGLIYFAAGSINKNDAAWFAKWLTTSEESTKIALB 293
 EVVAPLEPFW---ATYIERPFGHGHVFT---IYASNEAIDFWWFTTDFQVAKIND 285
 NYGVTVVPIYK.....GQSEPFVGLSAGINAASENKEIAKEFLENVLLTDEGLEAVND 290
 *

LGXIEFLTAVLDDEIKNDFVIYGFQDAVGHAYLMPKSPKMSAVWGVDSAIN-SILQDP 333
 LGIVFVVIDNQ-----IQDFVQGFYEAANNIYLMFVSPFNQFVGTVDLIONSIVSQ 340
 RPLGAVALKSY-EFEELADRIATHEMACKGEINPNIQKMSAFVIAVAVIAAAGCGQ 349

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FIG. 10

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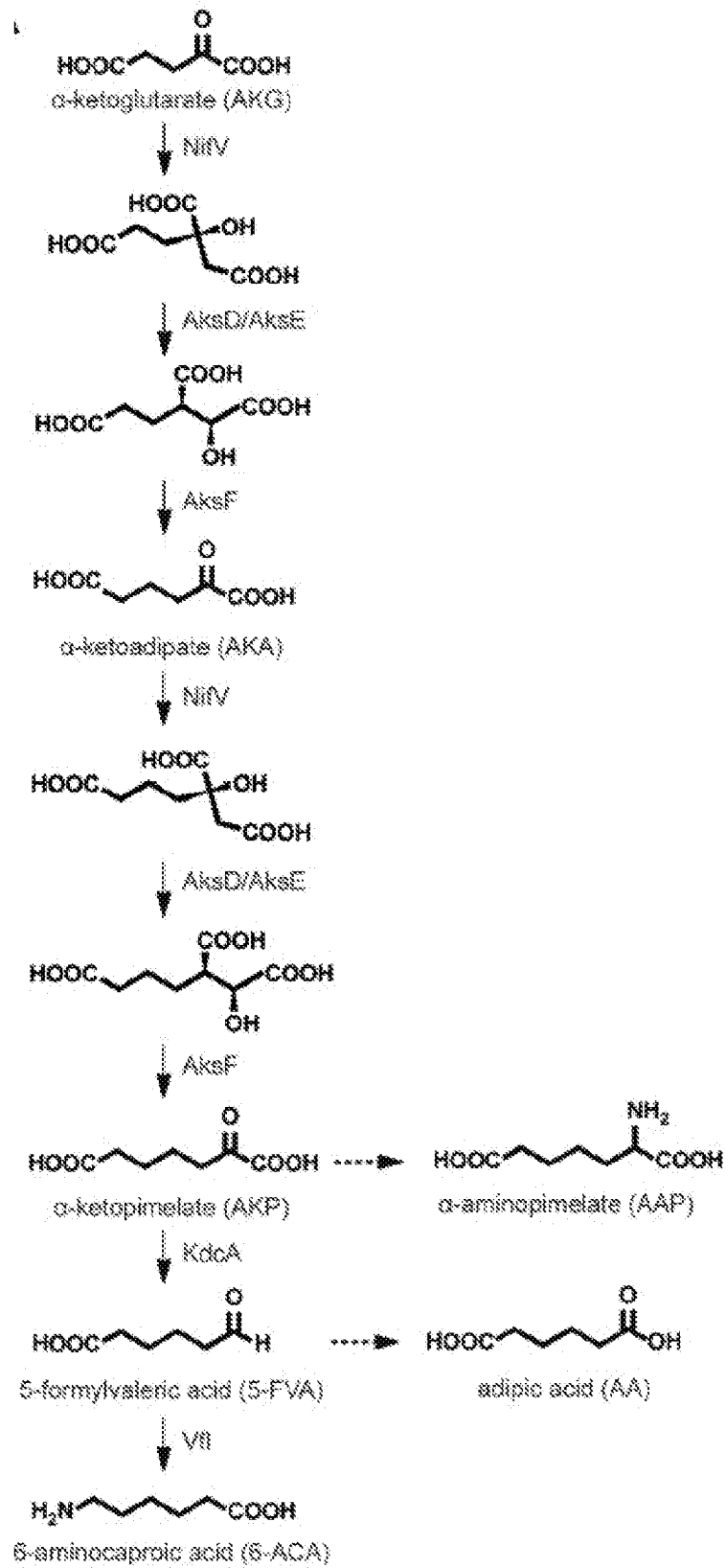


FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/36168

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 15/11, 15/52; C12P 17/10 (2017.01)

CPC - C12N 9/0083, 9/1029, 15/11, 15/52; C12P 17/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2008/0233623 A1 (CHANG, MCY et al.) September 25, 2008; abstract; figure 8; paragraph [0038]	1-14
A	STEVILA, E et al. 'Synthesis Of Lactams Using Enzyme-Catalyzed Aminolysis' Tetrahedron Letters, 2013, Vol. 54, No. 5, pp 370-372; abstract; page 371, scheme 1. http://dx.doi.org/10.1016/j.tetlet.2012.10.133	1-14
A	US 2009/0081673 A1 (SHEN, B et al.) March 26, 2009; paragraph [0162]	1-14
A	US 2004/0087654 A1 (BOX, D et al.) January 8, 2008; paragraph [0054]	1-14

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

25 August 2017 (25.08.2017)

Date of mailing of the international search report

08 SEP 2017

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