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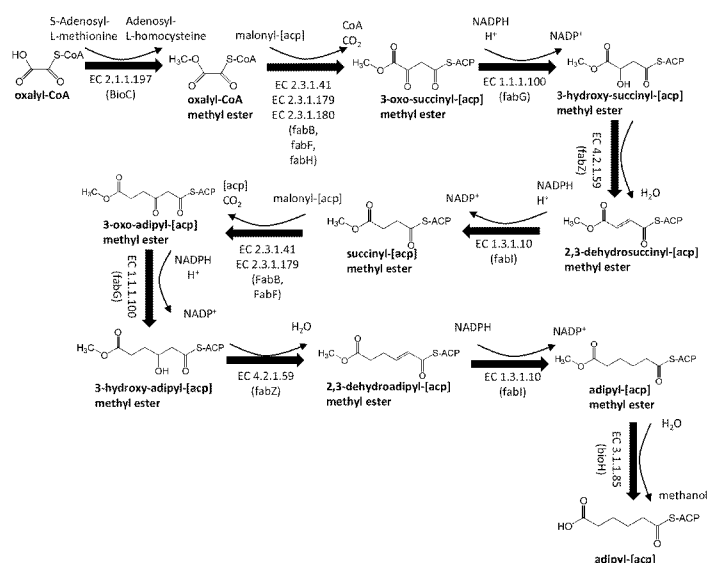
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(54) Title: METHODS OF PRODUCING 6-CARBON CHEMICALS VIA METHYL-ESTER SHIELDED CARBON CHAIN ELONGATION

FIGURE 1A



(57) Abstract: This document describes biochemical pathways for producing adipic acid, 6-aminohexanoic acid, 6-hydroxhexanoic acid, hexamethylenediamine, caprolactam, or 1,6-hexanediol by forming one or two terminal functional groups, comprised of carboxyl, amine or hydroxyl group, in a C6 aliphatic backbone substrate. These pathways, metabolic engineering and cultivation strategies described herein rely on the enzymes or homologs accepting methyl ester shielded dicarboxylic acid substrates.

Methods Of Producing 6-Carbon Chemicals Via Methyl-Ester Shielded Carbon Chain Elongation

TECHNICAL FIELD

This invention relates to methods for biosynthesizing one or more of adipic
5 acid, 6-aminohexanoic acid, 6-hydroxhexanoic acid, hexamethylenediamine,
caprolactam, and 1,6-hexanediol (hereafter “C6 building blocks”) from oxalyl-CoA
and either (i) malonyl-CoA or (ii) malonyl-[acp] and acetyl-CoA using one or more
isolated enzymes such as *methyltransferases*, *β -ketoacyl-[acp] synthases*, *β -*
ketothiolases, *dehydrogenases*, *reductases*, *hydratases*, *thioesterases*, *methylesterases*,
10 *CoA-transferases*, *reversible CoA-ligases* and *transaminases* or using recombinant
host cells expressing one or more such enzymes.

BACKGROUND

Nylons are polyamides that are generally synthesized by the condensation
polymerisation of a diamine with a dicarboxylic acid. Similarly, Nylons may be
15 produced by the condensation polymerization of lactams. A ubiquitous Nylon is
Nylon 6,6, which is produced by reaction of hexamethylenediamine (HMD) and
adipic acid. Nylon 6 can be produced by a ring opening polymerization of
caprolactam. Therefore, adipic acid, hexamethylenediamine and caprolactam are
important intermediates in the production of Nylons (Anton & Baird, Polyamides
20 Fibers, Encyclopedia of Polymer Science and Technology, 2001).

Industrially, adipic acid and caprolactam are produced via air oxidation of
cyclohexane. The air oxidation of cyclohexane produces, in a series of steps, a
mixture of cyclohexanone (K) and cyclohexanol (A), designated as KA oil. Nitric acid
oxidation of KA oil produces adipic acid (Musser, Adipic acid, Ullmann’s
25 Encyclopedia of Industrial Chemistry, 2000). Caprolactam is produced from
cyclohexanone via its oxime and subsequent acid rearrangement (Fuchs, Kieczka and
Moran, Caprolactam, Ullmann’s Encyclopedia of Industrial Chemistry, 2000)

Industrially, hexamethylenediamine (HMD) is produced by hydrocyanation of
C6 building block to adiponitrile, followed by hydrogenation to HMD (Herzog and
30 Smiley, Hexamethylenediamine, Ullmann’s Encyclopedia of Industrial Chemistry,
2012).

Given a reliance on petrochemical feedstocks; biotechnology offers an alternative approach via biocatalysis. Biocatalysis is the use of biological catalysts, such as enzymes, to perform biochemical transformations of organic compounds.

Both bioderived feedstocks and petrochemical feedstocks are viable starting materials for the biocatalysis processes.

Accordingly, against this background, it is clear that there is a need for sustainable methods for producing one or more of adipic acid, caprolactam, 6-aminohexanoic acid, 6-hydroxyhexanoic acid, hexamethylenediamine, and 1,6-hexanediol (hereafter “C6 building blocks”) wherein the methods are biocatalyst based (Jang *et al.*, *Biotechnol. Bioeng.*, 2012, 109(10), 2437 – 2459).

However, no wild-type prokaryote or eukaryote naturally overproduces or excretes C6 building blocks to the extracellular environment. Nevertheless, the metabolism of adipic acid and caprolactam has been reported (Ramsay *et al.*, *Appl. Environ. Microbiol.*, 1986, 52(1), 152 – 156; and Kulkarni and Kanekar, *Current Microbiology*, 1998, 37, 191 – 194).

The dicarboxylic acid, adipic acid, is converted efficiently as a carbon source by a number of bacteria and yeasts via β -oxidation into central metabolites. β -oxidation of Coenzyme A (CoA) activated adipate to CoA activated 3-oxoadipate facilitates further catabolism via, for example, pathways associated with aromatic substrate degradation. The catabolism of 3-oxoadipyl-CoA to acetyl-CoA and succinyl-CoA by several bacteria and fungi has been characterized comprehensively (Harwood and Parales, *Annual Review of Microbiology*, 1996, 50, 553 – 590). Both adipate and 6-aminohexanoate are intermediates in the catabolism of caprolactam, finally degraded via 3-oxoadipyl-CoA to central metabolites.

Potential metabolic pathways have been suggested for producing adipic acid from biomass-sugar: (1) biochemically from glucose to *cis,cis* muconic acid via the ortho-cleavage aromatic degradation pathway, followed by chemical catalysis to adipic acid; (2) a reversible adipic acid degradation pathway via the condensation of succinyl-CoA and acetyl-CoA and (3) combining β -oxidation, a fatty acid synthase and ω -oxidation. However, no information using these strategies has been reported (Jang *et al.*, *Biotechnology & Bioengineering*, 2012, 109(10), 2437 – 2459).

The optimality principle states that microorganisms regulate their biochemical networks to support maximum biomass growth. Beyond the need for expressing heterologous pathways in a host organism, directing carbon flux towards C6 building blocks that serve as carbon sources rather than as biomass growth constituents, contradicts the optimality principle. For example, transferring the 1-butanol pathway from *Clostridium* species into other production strains has often fallen short by an order of magnitude compared to the production performance of native producers (Shen *et al.*, *Appl. Environ. Microbiol.*, 2011, 77(9), 2905 – 2915).

The efficient synthesis of the six carbon aliphatic backbone precursor is a key consideration in synthesizing C6 building blocks prior to forming terminal functional groups, such as carboxyl, amine or hydroxyl groups, on the C6 aliphatic backbone.

SUMMARY

This document is based at least in part on the discovery that it is possible to construct biochemical pathways for producing a six carbon chain aliphatic backbone precursor, in which one or two functional groups, i.e., carboxyl, amine, or hydroxyl, can be formed, leading to the synthesis of one or more of adipic acid, 6-aminohexanoate, 6-hydroxhexanoate, hexamethylenediamine, caprolactam, and 1,6-hexanediol (hereafter “C6 building blocks”). Adipic acid and adipate, 6-hydroxhexanoic acid and 6-hydroxhexanoate, and 6-aminohexanoic and 6-aminohexanoate are used interchangeably herein to refer to the compound in any of its neutral or ionized forms, including any salt forms thereof. It is understood by those skilled in the art that the specific form will depend on pH. These pathways, metabolic engineering and cultivation strategies described herein rely on fatty acid elongation and synthesis enzymes or homologs accepting methyl-ester shielded dicarboxylic acids as substrates.

In the face of the optimality principle, it surprisingly has been discovered that appropriate non-natural pathways, feedstocks, host microorganisms, attenuation strategies to the host’s biochemical network and cultivation strategies may be combined to efficiently produce one or more C6 building blocks.

In some embodiments, the C6 aliphatic backbone for conversion to one or more C6 building blocks is adipyl-[acp] or adipyl-CoA (also known as 6-carboxyhexanoyl-CoA), which can be formed from oxalyl-CoA and malonyl-[acp],

malonyl-CoA, or acetyl-CoA, via two cycles of carbon chain elongation using either NADH or NADPH dependent enzymes. See FIG. 1A, FIG. 1B and FIG. 1C.

In some embodiments, a terminal carboxyl group can be enzymatically formed using a *pimeloyl-[acp] methyl ester methylesterase*, a *thioesterase*, an *aldehyde dehydrogenase*, a *7-oxoheptanoate dehydrogenase*, a *6-oxohexanoate dehydrogenase*, a *reversible CoA ligase* (e.g., reversible *succinyl-CoA ligase*) or a *CoA-transferase* (e.g., a *glutaconate CoA-transferase*). See FIG. 1A, FIG. 1B, FIG. 1C and FIG. 2.

In some embodiments, a terminal amine group can be enzymatically formed using a *ω -transaminase* or a *deacetylase*. See FIG. 3 and FIG. 4.

In some embodiments, a terminal hydroxyl group can be enzymatically formed using a *4-hydroxybutyrate dehydrogenase*, *5-hydroxypentanoate dehydrogenase* or a *6-hydroxyhexanoate dehydrogenase* or an *alcohol dehydrogenase*. See FIG. 5 and FIG. 6.

In one aspect, this document features a method for biosynthesizing a product selected from the group consisting of adipic acid, 6-aminohexanoate, 6-hydroxhexanoate, hexamethylenediamine, caprolactam, and 1,6-hexanediol. The method includes enzymatically synthesizing a six carbon chain aliphatic backbone from oxalyl-CoA and either (i) acetyl-CoA or malonyl-CoA via two cycles of methyl ester shielded carbon chain elongation or (ii) malonyl-[acp] via two cycles of methyl-ester shielded carbon chain elongation, and enzymatically forming one or two terminal functional groups selected from the group consisting of carboxyl, amine, and hydroxyl groups in the backbone, thereby forming the product. The six carbon chain aliphatic backbone can be adipyl-[acp] or adipyl-CoA. A *malonyl-[acp] O-methyltransferase* can convert oxalyl-CoA to oxalyl-CoA methyl ester. Each of the two cycles of carbon chain elongation can include using (i) a *β -ketoacyl-[acp] synthase* or a *β -ketothiolase*, (ii) a *3-oxoacyl-[acp] reductase*, an *acetoacetyl-CoA reductase*, a *3-hydroxyacyl-CoA dehydrogenase* or a *3-hydroxybutyryl-CoA dehydrogenase*, (iii) an *enoyl-CoA hydratase* or a *3-hydroxyacyl-[acp] dehydratase*, and (iv) an *enoyl-[acp] reductase* or a *trans-2-enoyl-CoA reductase* to produce adipyl-[acp] methyl ester or adipyl-[acp] methyl ester. A *pimeloyl-[acp] methyl ester methylesterase* can remove the methyl group from adipyl-CoA methyl ester or adipyl-[acp] methyl ester. The *malonyl-[acp]O-methyltransferase* can have at least 70%

sequence identity to the amino acid sequence set forth in SEQ ID NO: 16. The *pimeloyl-[acp] methyl ester methylesterase* can have at least 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 17.

5 The two terminal functional groups can be the same (e.g., amine or hydroxyl) or can be different (e.g., a terminal amine and a terminal carboxyl group; or a terminal hydroxyl group and a terminal carboxyl group).

A ω -transaminase or a deacetylase can enzymatically form an amine group. The ω -transaminase can have at least 70% sequence identity to any one of the amino acid sequences set forth in SEQ ID NO. 8 – 13.

10 A 6-hydroxyhexanoate dehydrogenase, a 5-hydroxypentanoate dehydrogenase, a 4-hydroxybutyrate dehydratase, or an alcohol dehydrogenase can enzymatically form a hydroxyl group.

A thioesterase, an aldehyde dehydrogenase, a 7-oxoheptanoate dehydrogenase, a 6-oxohexanoate dehydrogenase, a CoA-transferase (e.g. a
15 glutaconate CoA transferase), or a reversible CoA-ligase (e.g., a reversible succinate-CoA ligase) can enzymatically forms a terminal carboxyl group. The thioesterase can have at least 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1.

A carboxylate reductase and a phosphopantetheinyl transferase can form a
20 terminal aldehyde group as an intermediate in forming the product. The carboxylate reductase can have at least 70% sequence identity to any one of the amino acid sequences set forth in SEQ ID NO. 2 – 7.

Any of the methods can be performed in a recombinant host by fermentation. The host can be subjected to a cultivation strategy under aerobic, anaerobic, micro-aerobic or mixed oxygen/denitrification cultivation conditions. The host can be
25 cultured under conditions of nutrient limitation. The host can be retained using a ceramic hollow fiber membrane to maintain a high cell density during fermentation.

In any of the methods, the host's tolerance to high concentrations of a C6 building block can be improved through continuous cultivation in a selective
30 environment.

The principal carbon source fed to the fermentation can derive from biological or non-biological feedstocks. In some embodiments, the biological

feedstock is, includes, or derives from, monosaccharides, disaccharides, lignocellulose, hemicellulose, cellulose, lignin, levulinic acid and formic acid, triglycerides, glycerol, fatty acids, agricultural waste, condensed distillers' solubles, or municipal waste.

5 In some embodiments, the non-biological feedstock is or derives from natural gas, syngas, CO₂/H₂, methanol, ethanol, benzoate, non-volatile residue (NVR) or a caustic wash waste stream from cyclohexane oxidation processes, or a terephthalic acid/isophthalic acid mixture waste stream.

This document also features a recombinant host that includes at least one
10 exogenous nucleic acid encoding (i) a *malonyl-[acp] O-methyltransferase*, (ii) a *β-ketoacyl-[acp] synthase* or a *β-ketothiolase*, (iii) a *3-oxoacyl-[acp] reductase*, *acetoacetyl-CoA reductase*, a *3-hydroxyacyl-CoA dehydrogenase* or a *3-hydroxybutyryl-CoA dehydrogenase*, (iv) an *enoyl-CoA hydratase* or *3-hydroxyacyl-[acp] dehydratase*, (v) an *enoyl-[acp] reductase* or a *trans-2-enoyl-CoA reductase*,
15 and (vi) a *pimeloyl-[acp]methyl ester methylesterase*, the host producing adipyl-[acp] or adipyl-CoA.

A recombinant host producing adipyl-[acp] or adipyl-CoA further can include at least one exogenous nucleic acid encoding one or more of a *thioesterase*, an *aldehyde dehydrogenase*, a *7-oxoheptanoate dehydrogenase*, a *6-oxohexanoate*
20 *dehydrogenase*, a *glutaconate CoA-transferase*, a reversible *succinyl-CoA ligase*, an *acetylating aldehyde dehydrogenase*, or a *carboxylate reductase*, the host producing adipate or adipate semialdehyde.

A recombinant host producing adipate semialdehyde further can include at least one exogenous nucleic acid encoding a *ω-transaminase*, and producing 6-
25 aminohexanoate.

A recombinant host producing adipate semialdehyde further can include at least one exogenous nucleic acid encoding a *4-hydroxybutyrate dehydrogenase*, a *5-hydroxypentanoate dehydrogenase* or a *6-hydroxyhexanoate dehydrogenase*, the host producing 6-hydroxyhexanoic acid.

30 A recombinant host producing adipate semialdehyde, 6-aminohexanoate, or 6-hydroxyhexanoic acid further can include a *carboxylate reductase*, a *ω-*

transaminase, a *deacetylase*, an *N-acetyl transferase*, or an *alcohol dehydrogenase*, the host producing hexamethylenediamine.

A recombinant host producing 6-hydroxyhexanoic acid further can include at least one exogenous nucleic acid encoding a *carboxylate reductase* or an *alcohol*
 5 *dehydrogenase*, the host producing 1,6-hexanediol.

The recombinant host can be a prokaryote, e.g., from the genus *Escherichia* such as *Escherichia coli*; from the genus *Clostridia* such as *Clostridium ljungdahlii*, *Clostridium autoethanogenum* or *Clostridium kluyveri*; from the genus
 10 *Corynebacteria* such as *Corynebacterium glutamicum*; from the genus *Cupriavidus* such as *Cupriavidus necator* or *Cupriavidus metallidurans*; from the genus *Pseudomonas* such as *Pseudomonas fluorescens*, *Pseudomonas putida* or *Pseudomonas oleovorans*; from the genus *Delftia acidovorans*, from the genus *Bacillus* such as *Bacillus subtilis*; from the genus *Lactobacillus* such as *Lactobacillus delbrueckii*; from the genus *Lactococcus* such as *Lactococcus lactis* or from the genus
 15 *Rhodococcus* such as *Rhodococcus equi*.

The recombinant host can be a eukaryote, e.g., a eukaryote from the genus *Aspergillus* such as *Aspergillus niger*; from the genus *Saccharomyces* such as *Saccharomyces cerevisiae*; from the genus *Pichia* such as *Pichia pastoris*; from the genus *Yarrowia* such as *Yarrowia lipolytica*, from the genus *Issatchenkia* such as
 20 *Issatchenkia orientalis*, from the genus *Debaryomyces* such as *Debaryomyces hansenii*, from the genus *Arxula* such as *Arxula adenoinivorans*, or from the genus *Kluyveromyces* such as *Kluyveromyces lactis*.

Any of the recombinant hosts described herein further can include one or more of the following attenuated enzymes: *polyhydroxyalkanoate synthase*, an *acetyl-CoA*
 25 *thioesterase*, a *phosphotransacetylase* forming acetate, an *acetate kinase*, a *lactate dehydrogenase*, a *menaquinol-fumarate oxidoreductase*, a *2-oxoacid decarboxylase* producing isobutanol, an *alcohol dehydrogenase* forming ethanol, a *triose phosphate isomerase*, a *pyruvate decarboxylase*, a *glucose-6-phosphate isomerase*, a *transhydrogenase* dissipating the NADH or NADPH imbalance, an *glutamate*
 30 *dehydrogenase* dissipating the NADH or NADPH imbalance, a NADH/NADPH-utilizing *glutamate dehydrogenase*, a *pimeloyl-CoA dehydrogenase*; an *acyl-CoA*

dehydrogenase accepting C6 building blocks and central precursors as substrates; a *glutaryl-CoA dehydrogenase*; or a *pimeloyl-CoA synthetase*.

Any of the recombinant hosts described herein further can overexpress one or more genes encoding: an *oxaloacetase*, a *PEP carboxylase*, a *PEP carboxykinase*, a
5 *pyruvate carboxylase*, a *PEP synthase*, an *acetyl-CoA synthetase*, a 6-
phosphogluconate dehydrogenase; a *transketolase*; a *puridine nucleotide transhydrogenase*; a *formate dehydrogenase*; a *glyceraldehyde-3P-dehydrogenase*; a *malic enzyme*; a *glucose-6-phosphate dehydrogenase*; a *fructose 1,6 diphosphatase*; a *L-alanine dehydrogenase*; a *L-glutamate dehydrogenase* specific to the NADH or
10 NADPH used to generate a co-factor imbalance; a *methanol dehydrogenase*; a *formaldehyde dehydrogenase*; a *diamine transporter*; a *dicarboxylate transporter*; *S-adenosylmethionine synthetase*; and/or a *multidrug transporter*.

The reactions of the pathways described herein can be performed in one or more cell (e.g., host cell) strains (a) naturally expressing one or more relevant
15 enzymes, (b) genetically engineered to express one or more relevant enzymes, or (c) naturally expressing one or more relevant enzymes and genetically engineered to express one or more relevant enzymes. Alternatively, relevant enzymes can be extracted from any of the above types of host cells and used in a purified or semi-purified form. Extracted enzymes can optionally be immobilized to a solid substrate
20 such as the floors and/or walls of appropriate reaction vessels. Moreover, such extracts include lysates (e.g. cell lysates) that can be used as sources of relevant enzymes. In the methods provided by the document, all the steps can be performed in cells (e.g., host cells), all the steps can be performed using extracted enzymes, or some of the steps can be performed in cells and others can be performed using
25 extracted enzymes.

Many of the enzymes described herein catalyze reversible reactions, and the reaction of interest may be the reverse of the described reaction. The schematic pathways shown in FIGs. 1-6 illustrate the reaction of interest for each of the intermediates.

30 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent

to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims. The word “comprising” in the claims may be replaced by “consisting essentially of” or with “consisting of,” according to standard practice in patent law.

DESCRIPTION OF DRAWINGS

FIG. 1A is a schematic of an exemplary biochemical pathway leading to adipyl-[acp] using NADPH-dependent enzymes and oxalyl-CoA and malonyl-[acp] as central metabolites.

FIG. 1B is a schematic of an exemplary biochemical pathway leading to adipyl-CoA using NADPH-dependent enzymes and oxalyl-CoA and either acetyl-CoA or malonyl-CoA as central metabolites.

FIG. 1C is a schematic of an exemplary biochemical pathway leading to adipyl-CoA using NADH-dependent enzymes and oxalyl-CoA and either acetyl-CoA or malonyl-CoA as central metabolites.

FIG. 2 is a schematic of exemplary biochemical pathways leading to adipate using adipyl-[acp], adipyl-CoA, or adipate semialdehyde as central precursors.

FIG. 3 is a schematic of exemplary biochemical pathways leading to 6-aminohexanoate or caprolactam using adipyl-CoA, adipate, or adipate semialdehyde as central precursors.

FIG. 4 is a schematic of exemplary biochemical pathways leading to hexamethylenediamine using 6-aminohexanoate, 6-hydroxhexanoate or adipate semialdehyde as central precursors.

FIG. 5 is a schematic of exemplary biochemical pathways leading to 6-hydroxhexanoate using adipate, adipyl-CoA, or adipate semialdehyde as central precursors.

FIG. 6 is a schematic of an exemplary biochemical pathway leading to 1,6-hexanediol using 6-hydroxhexanoate as a central precursor.

FIG. 7 contains the amino acid sequences of an *Escherichia coli* thioesterase encoded by *tesB* (see GenBank Accession No. AAA24665.1, SEQ ID NO: 1), an *Escherichia coli* thioesterase encoded by *YciA* (see GenBank Accession No. AAB60068.1, SEQ ID NO: 1), *Mycobacterium marinum* carboxylate reductase (see Genbank Accession No. ACC40567.1, SEQ ID NO: 3), a *Mycobacterium smegmatis* carboxylate reductase (see Genbank Accession No. ABK71854.1, SEQ ID NO: 4), a *Segniliparus rugosus* carboxylate reductase (see Genbank Accession No. EFV11917.1, SEQ ID NO: 5), a *Mycobacterium massiliense* carboxylate reductase (see Genbank Accession No. EIV11143.1, SEQ ID NO: 6), a *Segniliparus rotundus* carboxylate reductase (see Genbank Accession No. ADG98140.1, SEQ ID NO: 7), a *Chromobacterium violaceum* ω -transaminase (see Genbank Accession No. AAQ59697.1, SEQ ID NO: 8), a *Pseudomonas aeruginosa* ω -transaminase (see Genbank Accession No. AAG08191.1, SEQ ID NO: 9), a *Pseudomonas syringae* ω -transaminase (see Genbank Accession No. AAY39893.1, SEQ ID NO: 10), a *Rhodobacter sphaeroides* ω -transaminase (see Genbank Accession No. ABA81135.1, SEQ ID NO: 11), an *Escherichia coli* ω -transaminase (see Genbank Accession No. AAA57874.1, SEQ ID NO: 12), a *Vibrio fluvialis* ω -transaminase (see Genbank Accession No. AEA39183.1, SEQ ID NO: 13), a *Bacillus subtilis* phosphopantetheinyl transferase (see Genbank Accession No. CAA44858.1, SEQ ID NO: 14), a *Nocardia* sp. NRRL 5646 phosphopantetheinyl transferase (see Genbank Accession No. ABI83656.1, SEQ ID NO: 15), a *Bacillus cereus* malonyl-CoA methyltransferase (see GenBank Accession No. AAS43086.1, SEQ ID NO: 16), and an *Escherichia coli* pimeloyl-[*acp*] methyl ester esterase (see GenBank Accession No. AAC76437.1, SEQ ID NO: 17).

FIG. 8 is a bar graph of the relative absorbance at 412 nm after 20 minutes of released CoA as a measure of the activity of a thioesterase for converting adipyl-CoA to adipate relative to the empty vector control.

FIG. 9 is a bar graph summarizing the change in absorbance at 340 nm after 20 minutes, which is a measure of the consumption of NADPH and activity of *carboxylate reductases* relative to the enzyme only controls (no substrate).

FIG. 10 is a bar graph of the change in absorbance at 340 nm after 20 minutes, which is a measure of the consumption of NADPH and the activity of *carboxylate reductases* for converting adipate to adipate semialdehyde relative to the empty vector control.

FIG. 11 is a bar graph of the change in absorbance at 340 nm after 20 minutes, which is a measure of the consumption of NADPH and the activity of *carboxylate reductases* for converting 6-hydroxhexanoate to 6-hydroxhexanal relative to the empty vector control.

FIG. 12 is a bar graph of the change in absorbance at 340 nm after 20 minutes, which is a measure of the consumption of NADPH and the activity of *carboxylate reductases* for converting N6-acetyl-6-aminohexanoate to N6-acetyl-6-aminohexanal relative to the empty vector control.

FIG. 13 is a bar graph of the change in absorbance at 340 nm after 20 minutes, which is a measure of the consumption of NADPH and activity of *carboxylate reductases* for converting adipate semialdehyde to hexanedial relative to the empty vector control.

FIG. 14 is a bar graph summarizing the percent conversion after 4 hours of pyruvate to L-alanine (mol/mol) as a measure of the *ω -transaminase* activity of the enzyme only controls (no substrate).

FIG. 15 is a bar graph of the percent conversion after 24 hours of pyruvate to L-alanine (mol/mol) as a measure of the *ω -transaminase* activity for converting 6-aminohexanoate to adipate semialdehyde relative to the empty vector control.

FIG. 16 is a bar graph of the percent conversion after 4 hours of L-alanine to pyruvate (mol/mol) as a measure of the *ω -transaminase* activity for converting adipate semialdehyde to 6-aminohexanoate relative to the empty vector control.

FIG. 17 is a bar graph of the percent conversion after 4 hours of pyruvate to L-alanine (mol/mol) as a measure of the *ω -transaminase* activity for converting hexamethylenediamine to 6-aminohexanal relative to the empty vector control.

FIG. 18 is a bar graph of the percent conversion after 4 hours of pyruvate to L-alanine (mol/mol) as a measure of the ω -transaminase activity for converting N6-acetyl-1,6-diaminohexane to N6-acetyl-6-aminohexanal relative to the empty vector control.

5 FIG. 19 is a bar graph of the percent conversion after 4 hours of pyruvate to L-alanine (mol/mol) as a measure of the ω -transaminase activity for converting 6-aminohexanol to 6-oxohexanol relative to the empty vector control.

FIG. 20 is a table of the conversion after 1 hour of adipyl-CoA methyl ester to adipyl-CoA by *pimeloyl-[acp] methyl ester methyl esterase*.

10

DETAILED DESCRIPTION

This document provides enzymes, non-natural pathways, cultivation strategies, feedstocks, host microorganisms and attenuations to the host's biochemical network, which generates a six carbon chain aliphatic backbone from central metabolites in which one or two terminal functional groups may be formed leading to the synthesis
15 of adipic acid, 6-aminohexanoic acid, hexamethylenediamine, caprolactam, or 1,6-hexanediol (referred to as "C6 building blocks" herein). As used herein, the term "central precursor" is used to denote any metabolite in any metabolic pathway shown herein leading to the synthesis of a C6 building block. The term "central metabolite" is used herein to denote a metabolite that is produced in all microorganisms to support
20 growth.

Host microorganisms described herein can include endogenous pathways that can be manipulated such that one or more C6 building blocks can be produced. In an endogenous pathway, the host microorganism naturally expresses all of the enzymes catalyzing the reactions within the pathway. A host microorganism containing an
25 engineered pathway does not naturally express all of the enzymes catalyzing the reactions within the pathway but has been engineered such that all of the enzymes within the pathway are expressed in the host.

The term "exogenous" as used herein with reference to a nucleic acid (or a protein) and a host refers to a nucleic acid that does not occur in (and cannot be
30 obtained from) a cell of that particular type as it is found in nature or a protein encoded by such a nucleic acid. Thus, a non-naturally-occurring nucleic acid is

considered to be exogenous to a host once in the host. It is important to note that non-naturally-occurring nucleic acids can contain nucleic acid subsequences or fragments of nucleic acid sequences that are found in nature provided the nucleic acid as a whole does not exist in nature. For example, a nucleic acid molecule containing a genomic DNA sequence within an expression vector is non-naturally-occurring nucleic acid, and thus is exogenous to a host cell once introduced into the host, since that nucleic acid molecule as a whole (genomic DNA plus vector DNA) does not exist in nature. Thus, any vector, autonomously replicating plasmid, or virus (e.g., retrovirus, adenovirus, or herpes virus) that as a whole does not exist in nature is considered to be non-naturally-occurring nucleic acid. It follows that genomic DNA fragments produced by PCR or restriction endonuclease treatment as well as cDNAs are considered to be non-naturally-occurring nucleic acid since they exist as separate molecules not found in nature. It also follows that any nucleic acid containing a promoter sequence and polypeptide-encoding sequence (e.g., cDNA or genomic DNA) in an arrangement not found in nature is non-naturally-occurring nucleic acid. A nucleic acid that is naturally-occurring can be exogenous to a particular host microorganism. For example, an entire chromosome isolated from a cell of yeast x is an exogenous nucleic acid with respect to a cell of yeast y once that chromosome is introduced into a cell of yeast y.

In contrast, the term “endogenous” as used herein with reference to a nucleic acid (e.g., a gene) (or a protein) and a host refers to a nucleic acid (or protein) that does occur in (and can be obtained from) that particular host as it is found in nature. Moreover, a cell “endogenously expressing” a nucleic acid (or protein) expresses that nucleic acid (or protein) as does a host of the same particular type as it is found in nature. Moreover, a host “endogenously producing” or that “endogenously produces” a nucleic acid, protein, or other compound produces that nucleic acid, protein, or compound as does a host of the same particular type as it is found in nature.

For example, depending on the host and the compounds produced by the host, one or more of the following enzymes may be expressed in the host in addition to a *malonyl-[acp] O-methyltransferase* and a *pimeloyl-[acp] methyl ester methyltransferase*: a β -ketoacyl-[*acp*] synthase, a β -ketothiolase, a 3-oxoacyl-[*acp*] reductase, acetoacetyl-CoA reductase, a 3-hydroxyacyl-CoA dehydrogenase, a 3-hydroxybutyryl-CoA dehydrogenase, an enoyl-CoA hydratase, 3-hydroxyacyl-[*acp*] dehydratase, an enoyl-

[*acp*] reductase, a *trans*-2-enoyl-CoA reductase, a thioesterase, a reversible CoA ligase, a CoA-transferase, an acetylating aldehyde dehydrogenase, a 6-oxohexanoate dehydrogenase, a 7-oxoheptanoate dehydrogenase, an aldehyde dehydrogenase, a carboxylate reductase, a ω -transaminase, a *N*-acetyl transferase, an alcohol
 5 dehydrogenase, a deacetylase, a 6-hydroxyhexanoate dehydrogenase, a 5-hydroxypentanoate dehydrogenase, or a 4-hydroxybutyrate dehydrogenase. In recombinant hosts expressing a carboxylate reductase, a phosphopantetheinyl transferase also can be expressed as it enhances activity of the carboxylate reductase.

For example, a recombinant host can include at least one exogenous nucleic acid encoding (i) a malonyl-[*acp*] O-methyltransferase, (ii) a β -ketoacyl-[*acp*] synthase
 10 or a β -ketothiolase, (iii) a 3-oxoacyl-[*acp*] reductase, acetoacetyl-CoA reductase, a 3-hydroxyacyl-CoA dehydrogenase or a 3-hydroxybutyryl-CoA dehydrogenase, (iv) an enoyl-CoA hydratase or 3-hydroxyacyl-[*acp*] dehydratase, (v) an enoyl-[*acp*] reductase or a *trans*-2-enoyl-CoA reductase and (vi) a pimeloyl-[*acp*] methyl ester methylesterase,
 15 and produce adipyl-[*acp*] or adipyl-CoA.

Such recombinant hosts producing adipyl-[*acp*] or adipyl-CoA further can include at least one exogenous nucleic acid encoding one or more of a thioesterase, an aldehyde dehydrogenase, a 7-oxoheptanoate dehydrogenase, a 6-oxohexanoate dehydrogenase, a glutaconate CoA-transferase, a reversible succinyl-CoA ligase, an
 20 acetylating aldehyde dehydrogenase, or a carboxylate reductase and produce adipic acid or adipate semialdehyde. For example, a recombinant host producing adipyl-[*acp*] or adipyl-CoA further can include a thioesterase, a reversible Co-ligase (e.g., a reversible succinyl-CoA ligase), or a CoA transferase (e.g., a glutaconate CoA-transferase) and produce adipic acid. For example, a recombinant host producing
 25 adipyl-CoA further can include an acetylating aldehyde dehydrogenase and produce adipate semialdehyde. For example, a recombinant host producing adipate further can include a carboxylate reductase and produce adipate semialdehyde.

A recombinant hosts producing adipate semialdehyde further can include at least one exogenous nucleic acid encoding a ω -transaminase and produce 6-
 30 aminohexanoate. In some embodiments, a recombinant host producing adipyl-CoA includes a carboxylate reductase and a ω -transaminase to produce 6-aminohexanoate.

A recombinant host producing adipate or adipate semialdehyde further can include at least one exogenous nucleic acid encoding a 6-hydroxyhexanoate

dehydrogenase, a *5-hydroxypentanoate dehydrogenase* or a *4-hydroxybutyrate dehydrogenase*, and produce 6-hydroxhexanoic acid. In some embodiments, a recombinant host producing adipl-CoA includes an *acetylating aldehyde dehydrogenase*, and a *6-hydroxyhexanoate dehydrogenase*, a *5-hydroxypentanoate dehydrogenase* or a *4-hydroxybutyrate dehydrogenase* to produce 6-hydroxhexanoate. In some embodiments, a recombinant host producing adipate includes a *carboxylate reductase* and a *6-hydroxyhexanoate dehydrogenase*, a *5-hydroxypentanoate dehydrogenase* or a *4-hydroxybutyrate dehydrogenase* to produce 6-hydroxhexanoate.

A recombinant hosts producing 6-aminohexanoate, 6-hydroxhexanoate or adipate semialdehyde further can include at least one exogenous nucleic acid encoding a *ω -transaminase*, a *deacetylase*, a *N-acetyl transferase*, or an *alcohol dehydrogenase*, and produce hexamethylenediamine. For example, a recombinant host producing 6-hydroxhexanoate can include a *carboxylate reductase* with a *phosphopantetheine transferase* enhancer, a *ω -transaminase* and an *alcohol dehydrogenase*.

A recombinant host producing 6-hydroxhexanoic acid further can include one or more of a *carboxylate reductase* with a *phosphopantetheine transferase* enhancer and an *alcohol dehydrogenase*, and produce 1,6-hexanediol.

Within an engineered pathway, the enzymes can be from a single source, i.e., from one species or genus, or can be from multiple sources, i.e., different species or genera. Nucleic acids encoding the enzymes described herein have been identified from various organisms and are readily available in publicly available databases such as GenBank or EMBL.

Any of the enzymes described herein that can be used for production of one or more C6 building blocks can have at least 70% sequence identity (homology) (e.g., at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100%) to the amino acid sequence of the corresponding wild-type enzyme. It will be appreciated that the sequence identity can be determined on the basis of the mature enzyme (e.g., with any signal sequence removed).

For example, a *thioesterase* described herein can have at least 70% sequence identity (homology) (e.g., at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100%) to the amino acid sequence of an *Escherichia coli* *thioesterase* encoded by

tesB (see GenBank Accession No. AAA24665.1, SEQ ID NO: 1) or an *Escherichia coli* thioesterase encoded by *YciA* (see GenBank Accession No. AAB60068.1, SEQ ID NO: 1). See FIG. 7.

For example, a *carboxylate reductase* described herein can have at least 70%
 5 sequence identity (homology) (e.g., at least 75%, 80%, 85%, 90%, 95%, 97%, 98%,
 99%, or 100%) to the amino acid sequence of a *Mycobacterium marinum* (see
 Genbank Accession No. ACC40567.1, SEQ ID NO: 3), a *Mycobacterium smegmatis*
 (see Genbank Accession No. ABK71854.1, SEQ ID NO: 4), a *Segniliparus rugosus*
 (see Genbank Accession No. EFV11917.1, SEQ ID NO: 5), a *Mycobacterium*
 10 *massiliense* (see Genbank Accession No. EIV11143.1, SEQ ID NO: 6), or a
Segniliparus rotundus (see Genbank Accession No. ADG98140.1, SEQ ID NO: 7)
carboxylate reductase. See, FIG. 7.

For example, a ω -transaminase described herein can have at least 70%
 sequence identity (homology) (e.g., at least 75%, 80%, 85%, 90%, 95%, 97%, 98%,
 15 99%, or 100%) to the amino acid sequence of a *Chromobacterium violaceum* (see
 Genbank Accession No. AAQ59697.1, SEQ ID NO: 8), a *Pseudomonas aeruginosa*
 (see Genbank Accession No. AAG08191.1, SEQ ID NO: 9), a *Pseudomonas syringae*
 (see Genbank Accession No. AAY39893.1, SEQ ID NO: 10), a *Rhodobacter*
sphaeroides (see Genbank Accession No. ABA81135.1, SEQ ID NO: 11), an
 20 *Escherichia coli* (see Genbank Accession No. AAA57874.1, SEQ ID NO: 12), or a
Vibrio fluvialis (see Genbank Accession No. AEA39183.1, SEQ ID NO: 13) ω -
transaminase. Some of these ω -transaminases are diamine ω -transaminases. See,
 FIG. 7.

For example, a *phosphopantetheinyl transferase* described herein can have at
 25 least 70% sequence identity (homology) (e.g., at least 75%, 80%, 85%, 90%, 95%,
 97%, 98%, 99%, or 100%) to the amino acid sequence of a *Bacillus subtilis*
phosphopantetheinyl transferase (see Genbank Accession No. CAA44858.1, SEQ ID
 NO:14) or a *Nocardia* sp. NRRL 5646 *phosphopantetheinyl transferase* (see Genbank
 Accession No. ABI83656.1, SEQ ID NO:15). See FIG. 7.

30 For example, a *malonyl-CoA methyltransferase* described herein can have at
 least 70% sequence identity (homology) (e.g., at least 75%, 80%, 85%, 90%, 95%,
 97%, 98%, 99%, or 100%) to the amino acid sequence of a *Bacillus cereus* *malonyl-*

CoA methyltransferase (see GenBank Accession No. AAS43086.1, SEQ ID NO:16).
See, FIG. 7.

For example, a *pimeloyl-[acp] methyl ester esterase* described herein can have at least 70% sequence identity (homology) (e.g., at least 75%, 80%, 85%, 90%, 95%,
5 97%, 98%, 99%, or 100%) to the amino acid sequence of an *Escherichia coli* *pimeloyl-[acp] methyl ester esterase* (see GenBank Accession No. AAC76437.1, SEQ ID NO:17). See, FIG. 7.

The percent identity (homology) between two amino acid sequences can be determined as follows. First, the amino acid sequences are aligned using the BLAST
10 2 Sequences (BI2seq) program from the stand-alone version of BLASTZ containing BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained from Fish & Richardson's web site (e.g., www.fr.com/blast/) or the U.S. government's National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). Instructions explaining how to use the BI2seq program can be found in the readme file
15 accompanying BLASTZ. BI2seq performs a comparison between two amino acid sequences using the BLASTP algorithm. To compare two amino acid sequences, the options of BI2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to
20 any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\BI2seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology (identity), then the designated output file will present those regions of
25 homology as aligned sequences. If the two compared sequences do not share homology (identity), then the designated output file will not present aligned sequences. Similar procedures can be following for nucleic acid sequences except that blastn is used.

Once aligned, the number of matches is determined by counting the number of
30 positions where an identical amino acid residue is presented in both sequences. The percent identity (homology) is determined by dividing the number of matches by the length of the full-length polypeptide amino acid sequence followed by multiplying the

resulting value by 100. It is noted that the percent identity (homology) value is rounded to the nearest tenth. For example, 78.11, 78.12, 78.13, and 78.14 is rounded down to 78.1, while 78.15, 78.16, 78.17, 78.18, and 78.19 is rounded up to 78.2. It also is noted that the length value will always be an integer.

5 It will be appreciated that a number of nucleic acids can encode a polypeptide having a particular amino acid sequence. The degeneracy of the genetic code is well known to the art; i.e., for many amino acids, there is more than one nucleotide triplet that serves as the codon for the amino acid. For example, codons in the coding sequence for a given enzyme can be modified such that optimal expression in a particular species (e.g., bacteria or fungus) is obtained, using appropriate codon bias tables for that species.

 Functional fragments of any of the enzymes described herein can also be used in the methods of the document. The term “functional fragment” as used herein refers to a peptide fragment of a protein that has at least 25% (e.g., at least: 30%; 40%; 15 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 98%; 99%; 100%; or even greater than 100%) of the activity of the corresponding mature, full-length, wild-type protein. The functional fragment can generally, but not always, be comprised of a continuous region of the protein, wherein the region has functional activity.

 This document also provides (i) functional variants of the enzymes used in the methods of the document and (ii) functional variants of the functional fragments 20 described above. Functional variants of the enzymes and functional fragments can contain additions, deletions, or substitutions relative to the corresponding wild-type sequences. Enzymes with substitutions will generally have not more than 50 (e.g., not more than one, two, three, four, five, six, seven, eight, nine, ten, 12, 15, 20, 25, 30, 35, 25 40, or 50) amino acid substitutions (e.g., conservative substitutions). This applies to any of the enzymes described herein and functional fragments. A conservative substitution is a substitution of one amino acid for another with similar characteristics. Conservative substitutions include substitutions within the following groups: valine, alanine and glycine; leucine, valine, and isoleucine; aspartic acid and glutamic acid; 30 asparagine and glutamine; serine, cysteine, and threonine; lysine and arginine; and phenylalanine and tyrosine. The nonpolar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The

polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Any substitution of one member of the above-mentioned polar, basic or acidic groups by another member of the same group can be deemed a conservative substitution. By contrast, a nonconservative substitution is a substitution of one amino acid for another with dissimilar characteristics.

Deletion variants can lack one, two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid segments (of two or more amino acids) or non-contiguous single amino acids. Additions (addition variants) include fusion proteins containing: (a) any of the enzymes described herein or a fragment thereof; and (b) internal or terminal (C or N) irrelevant or heterologous amino acid sequences. In the context of such fusion proteins, the term "heterologous amino acid sequences" refers to an amino acid sequence other than (a). A

heterologous sequence can be, for example a sequence used for purification of the recombinant protein (e.g., FLAG, polyhistidine (e.g., hexahistidine), hemagglutinin (HA), glutathione-S-transferase (GST), or maltosebinding protein (MBP)).

Heterologous sequences also can be proteins useful as detectable markers, for example, luciferase, green fluorescent protein (GFP), or chloramphenicol acetyl transferase (CAT). In some embodiments, the fusion protein contains a signal sequence from another protein. In certain host cells (e.g., yeast host cells), expression and/or secretion of the target protein can be increased through use of a heterologous signal sequence. In some embodiments, the fusion protein can contain a carrier (e.g., KLH) useful, e.g., in eliciting an immune response for antibody generation) or ER or Golgi apparatus retention signals. Heterologous sequences can be of varying length and in some cases can be a longer sequences than the full-length target proteins to which the heterologous sequences are attached.

Engineered hosts can naturally express none or some (e.g., one or more, two or more, three or more, four or more, five or more, or six or more) of the enzymes of the pathways described herein. Thus, a pathway within an engineered host can include all exogenous enzymes, or can include both endogenous and exogenous enzymes. Endogenous genes of the engineered hosts also can be disrupted to prevent

the formation of undesirable metabolites or prevent the loss of intermediates in the pathway through other enzymes acting on such intermediates. Engineered hosts can be referred to as recombinant hosts or recombinant host cells. As described herein recombinant hosts can include nucleic acids encoding one or more of a

5 *methyltransferase*, a *synthase*, *β -ketothiolase*, a *dehydratase*, a *hydratase*, a *dehydrogenase*, a *methylesterase*, a *thioesterase*, a *reversible CoA-ligase*, a *CoA-transferase*, a *reductase*, *deacetylase*, *N-acetyl transferase* or a ω -*transaminase* as described in more detail below.

In addition, the production of one or more C6 building blocks can be

10 performed *in vitro* using the isolated enzymes described herein, using a lysate (e.g., a cell lysate) from a host microorganism as a source of the enzymes, or using a plurality of lysates from different host microorganisms as the source of the enzymes.

Enzymes generating the C6 aliphatic backbone for conversion to C6 Building Blocks

15 As depicted in FIG. 1A, FIG. 1B and FIG. 1C, the C6 aliphatic backbone for conversion to one or more C6 building blocks can be formed from malonyl-[acp], or acetyl-CoA and malonyl-CoA, via two cycles of methyl ester shielded carbon chain elongation associated with biotin biosynthesis using either NADH or NADPH dependent enzymes.

20 In some embodiments, a methyl ester shielded carbon chain elongation route comprises using a *malonyl-[acp] O-methyltransferase* to form an oxalyl-CoA methyl ester, and then performing two cycles of carbon chain elongation using malonyl-[acp] and a *β -ketoacyl-[acp] synthase*, a *3-oxoacyl-[acp] reductase*, a *3-hydroxyacyl-[acp] dehydratase*, and an *enoyl-[acp] reductase*. A *pimeloyl-[acp] methyl ester esterase*

25 can be used to cleave the resulting adipyl-[acp] methyl ester.

In some embodiments, a methyl ester shielded carbon chain elongation route comprises using a *malonyl-[acp] O-methyltransferase* to form an oxalyl-CoA methyl ester, and then performing two cycles of carbon chain elongation using either acetyl-CoA or malonyl-CoA and (i) a *β -ketothiolase* or a *β -ketoacyl-[acp] synthase*, (ii) an

30 *acetoacetyl-CoA reductase*, a *3-oxoacyl-[acp] reductase*, or a *3-hydroxybutyryl-CoA dehydrogenase*, (iii) *enoyl-CoA hydratase*, and (iv) a *trans-2-enoyl-CoA reductase*. A

pimeloyl-[acp] methyl ester esterase can be used to cleave the resulting adipyl-CoA methyl ester.

In some embodiments, a *methyltransferase* can be a *malonyl-[acp] O-methyltransferase* classified, for example, under EC 2.1.1.197 such as the gene
5 product of *bioC* from *Bacillus cereus* (see Genbank Accession No. AAS43086.1, SEQ ID NO:16) (see, for example, Lin, 2012, *Biotin Synthesis in Escherichia coli*, Ph.D. Dissertation, University of Illinois at Urbana-Champaign).

In some embodiments, a β -ketothiolase may be classified, for example, under EC 2.3.1.16, such as the gene product of *bktB*. The β -ketothiolase encoded by *bktB*
10 from *Cupriavidus necator* accepts acetyl-CoA and butanoyl-CoA as substrates, forming the CoA-activated C6 aliphatic backbone (see, e.g., Haywood *et al.*, *FEMS Microbiology Letters*, 1988, 52:91-96; Slater *et al.*, *J. Bacteriol.*, 1998, 180(8):1979 - 1987).

In some embodiments, a β -ketoacyl-[*acp*] synthase may be classified, for
15 example, under EC 2.3.1.- (e.g., EC 2.3.1.41, EC 2.3.1.179 or EC 2.3.1.180) such as the gene product of *fabB*, *fabF*, or *fabH*.

In some embodiments, a *3-hydroxyacyl-CoA dehydrogenase* may be classified under EC 1.1.1.35, such as the gene product of *fadB*, or classified under EC 1.1.1.157, such as the gene product of *hbd* (can be referred to as a *3-hydroxybutyryl-CoA*
20 *dehydrogenase*), or classified under EC 1.1.1.36, such as the gene product of *phaB* (see, for example, Liu & Chen, *Appl. Microbiol. Biotechnol.*, 2007, 76(5), 1153 – 1159; Shen *et al.*, *Appl. Environ. Microbiol.*, 2011, 77(9), 2905 – 2915; or Budde *et al.*, *J. Bacteriol.*, 2010, 192(20), 5319 – 5328).

In some embodiments, a *3-oxoacyl-CoA reductase* may be classified under EC
25 1.1.1.100, such as the gene product of *fabG* (Budde *et al.*, 2010, *supra*; Nomura *et al.*, *Appl. Environ. Microbiol.*, 2005, 71(8), 4297 – 4306) .

In some embodiments, an *enoyl-CoA hydratase* may be classified under EC 4.2.1.17, such as the gene product of *crt*, or classified under EC 4.2.1.119, such as the gene product of *phaJ* (Shen *et al.*, 2011, *supra*; or Fukui *et al.*, *J. Bacteriol.*, 1998,
30 180(3), 667 – 673).

In some embodiments, an *enoyl-[acp] dehydratase* such as a *3-hydroxyacyl-[acp] dehydratase* may be classified under EC 4.2.1.59, such as the gene product of *fabZ*.

5 In some embodiments, a *trans-2-enoyl-CoA reductase* may be classified under EC 1.3.1.- (e.g., EC 1.3.1.38, EC 1.3.1.8, EC 1.3.1.44), such as the gene product of *ter* (Nishimaki *et al.*, *J. Biochem.*, 1984, 95, 1315 – 1321; Shen *et al.*, 2011, *supra*) or *tdter* (Bond-Watts *et al.*, *Biochemistry*, 2012, 51, 6827 – 6837).

In some embodiments, an *enoyl-[acp] reductase* may be classified under EC 1.3.1.10 such as the gene product of *fabI*.

10 In some embodiments, a *pimeloyl-[acp] methyl ester esterase* may be classified, for example, under EC 3.1.1.85 such as the gene product of *bioH* from *E. coli*. See Genbank Accession No. AAC76437.1, SEQ ID NO:17.

Enzymes generating the terminal carboxyl groups in the biosynthesis of C6 Building Blocks

15 As depicted in FIG. 2, a terminal carboxyl group can be enzymatically formed using a *thioesterase*, an *aldehyde dehydrogenase*, a *7-oxoheptanoate dehydrogenase*, a *6-oxohexanoate dehydrogenase*, a *CoA-transferase* or a reversible *CoA-ligase*.

In some embodiments, the second terminal carboxyl group leading to the synthesis of a C6 building block is enzymatically formed by a *thioesterase* classified, for example, under EC 3.1.2.-, such as the gene product of *YciA*, *tesB* (GenBank Accession No. AAB60068.1, SEQ ID NO: 2; Genbank Accession No. AAA24665.1, SEQ ID NO: 1) or *Acot13* (see, for example, Cantu *et al.*, *Protein Science*, 2010, 19, 1281 – 1295; Zhuang *et al.*, *Biochemistry*, 2008, 47(9), 2789 – 2796; or Naggert *et al.*, *J. Biol. Chem.*, 1991, 266(17), 11044 – 11050).

25 In some embodiments, the second terminal carboxyl group leading to the synthesis of a C6 building block is enzymatically formed by an *acyl-[acp] thioesterase* classified under EC 3.1.2.-, such as the gene product of *fatB* or *tesA*. The *acyl-[acp] thioesterases* encoded by Genbank Accession Nos. ABJ63754.1 and CCC78182.1 have C6 – C8 chain length specificity (Jing *et al.*, 2011, *BMC Biochemistry*, 12(44)).

In some embodiments, the second terminal carboxyl group leading to the synthesis of adipic acid is enzymatically formed by an *aldehyde dehydrogenase*

classified, for example, under EC 1.2.1.3 (see, for example, Guerrillot & Vandecasteele, *Eur. J. Biochem.*, 1977, 81, 185 – 192).

In some embodiments, the second terminal carboxyl group leading to the synthesis of adipic acid is enzymatically formed by a *dehydrogenase* classified under EC 1.2.1.- such as a *6-oxohexanoate dehydrogenase* (e.g., the gene product of *ChnE* from *Acinetobacter sp.*) or a *7-oxoheptanoate dehydrogenase* (e.g., such as the gene product of *ThnG* from *Sphingomonas macroglutabida*). See, for example, Iwaki *et al.*, *Appl. Environ. Microbiol.*, 1999, 65(11), 5158 – 5162; or López-Sánchez *et al.*, *Appl. Environ. Microbiol.*, 2010, 76(1), 110 - 118. For example, a *6-oxohexanoate dehydrogenase* can be classified under EC 1.2.1.63. For example, a *7-oxoheptanoate dehydrogenase* can be classified under EC 1.2.1.-.

In some embodiments, the second terminal carboxyl group leading to the synthesis of adipic acid is enzymatically formed by a *CoA-transferase* (e.g., a *glutaconate CoA-transferase*) classified, for example, under EC 2.8.3.12 such as from *Acidaminococcus fermentans*. See, for example, Buckel *et al.*, 1981, *Eur. J. Biochem.*, 118:315 – 321.

In some embodiments, the second terminal carboxyl group leading to the synthesis of adipic acid is enzymatically formed by a reversible *CoA-ligase* (e.g., a *succinate-CoA ligase*) classified, for example, under EC 6.2.1.5 such as from *Thermococcus kodakaraensis*. See, for example, Shikata *et al.*, 2007, *J. Biol. Chem.*, 282(37):26963 – 26970.

Enzymes generating the terminal amine groups in the biosynthesis of C6

Building Blocks

As depicted in FIG. 3 and FIG. 4, terminal amine groups can be enzymatically formed using a *ω-transaminase* or a *deacetylase*.

In some embodiments, the first or second terminal amine group leading to the synthesis of 6-aminohexanoic acid is enzymatically formed by a *ω-transaminase* classified, for example, under EC 2.6.1.18, EC 2.6.1.19, EC 2.6.1.29, EC 2.6.1.48, or EC 2.6.1.82 such as that obtained from *Chromobacterium violaceum* (Genbank Accession No. AAQ59697.1, SEQ ID NO: 8), *Pseudomonas aeruginosa* (Genbank Accession No. AAG08191.1, SEQ ID NO: 9), *Pseudomonas syringae* (Genbank

Accession No. AAY39893.1, SEQ ID NO: 10), *Rhodobacter sphaeroides* (Genbank Accession No. ABA81135.1, SEQ ID NO: 11), *Escherichia coli* (Genbank Accession No. AAA57874.1, SEQ ID NO: 12), *Vibrio Fluvialis* (Genbank Accession No. AAA57874.1, SEQ ID NO: 13), *Streptomyces griseus*, or *Clostridium viride*. Some
5 of these ω -transaminases are diamine ω -transaminases (e.g., SEQ ID NO:12). For example, the ω -transaminases classified, for example, under EC 2.6.1.29 or EC 2.6.1.82 may be diamine ω -transaminases.

The reversible ω -transaminase from *Chromobacterium violaceum* (Genbank Accession No. AAQ59697.1, SEQ ID NO: 8) has demonstrated analogous activity
10 accepting 6-aminohexanoic acid as amino donor, thus forming the first terminal amine group in adipate semialdehyde (Kaulmann *et al.*, *Enzyme and Microbial Technology*, 2007, 41, 628 – 637).

The reversible 4-aminobutyrate:2-oxoglutarate transaminase from *Streptomyces griseus* has demonstrated analogous activity for the conversion of 6-
15 aminohexanoate to adipate semialdehyde (Yonaha *et al.*, *Eur. J. Biochem.*, 1985, 146:101 - 106).

The reversible 5-aminovalerate transaminase from *Clostridium viride* has demonstrated analogous activity for the conversion of 6-aminohexanoate to adipate semialdehyde (Barker *et al.*, *J. Biol. Chem.*, 1987, 262(19), 8994 – 9003).

20 In some embodiments, a terminal amine group leading to the synthesis of 6-aminohexanoate or hexamethylenediamine is enzymatically formed by a diamine ω -transaminase. For example, the second terminal amino group can be enzymatically formed by a diamine ω -transaminase classified, for example, under EC 2.6.1.29 or classified, for example, under EC 2.6.1.82, such as the gene product of *YgjG* from *E.*
25 *coli* (Genbank Accession No. AAA57874.1, SEQ ID NO: 12).

The gene product of *yjgG* accepts a broad range of diamine carbon chain length substrates, such as putrescine, cadaverine and spermidine (see, for example, Samsonova *et al.*, *BMC Microbiology*, 2003, 3:2).

The diamine ω -transaminase from *E.coli* strain B has demonstrated activity
30 for 1,7 diaminooheptane (Kim, *The Journal of Chemistry*, 1964, 239(3), 783 – 786).

In some embodiments, the second terminal amine group leading to the synthesis of hexamethylenediamine is enzymatically formed by a *deacetylase* such as

acetylputrescine deacetylase classified, for example, under EC 3.5.1.62. The *acetylputrescine deacetylase* from *Micrococcus luteus* K-11 accepts a broad range of carbon chain length substrates, such as acetylputrescine, acetylcadaverine and N⁸-acetylspermidine (see, for example, Suzuki *et al.*, 1986, *BBA – General Subjects*, 882(1):140 – 142).

Enzymes generating the terminal hydroxyl groups in the biosynthesis of C6 Building Blocks

As depicted in FIG. 5 and FIG. 6, a terminal hydroxyl group can be enzymatically formed using an *alcohol dehydrogenase*.

In some embodiments, a terminal hydroxyl group leading to the synthesis of 1,6 hexanediol is enzymatically formed by an *alcohol dehydrogenase* classified, for example, under EC 1.1.1.- (e.g., 1, 2, 21, or 184) such as the gene product of *YMR318C* (classified, for example, under EC 1.1.1.2, see Genbank Accession No. CAA90836.1) (Larroy *et al.*, 2002, *Biochem J.*, 361(Pt 1), 163 – 172), the gene product of the gene product of *YghD*, the gene product of *cpnD* (Iwaki *et al.*, 2002, *Appl. Environ. Microbiol.*, 68(11):5671 – 5684), the gene product of *gabD* (Lütke-Eversloh & Steinbüchel, 1999, *FEMS Microbiology Letters*, 181(1):63 – 71), or a 6-hydroxyhexanoate dehydrogenase classified, for example, under EC 1.1.1.258 such as the gene product of *ChnD* (Iwaki *et al.*, *Appl. Environ. Microbiol.*, 1999, *supra*).

Biochemical pathways

Pathways using NADPH-specific enzymes to adipyl-[acp] as central precursor leading to C6 Building Blocks

In some embodiments, adipyl-[acp] is synthesized from the central metabolite oxalyl-CoA, by conversion of oxalyl-CoA to oxalyl-CoA methyl ester by a *malonyl-CoA O-methyltransferase* classified, for example, under EC 2.1.1.197 such as the gene product of *bioC*; followed by conversion with malonyl-[acp] to 3-oxosuccinyl-[acp] methyl ester by a β -ketoacyl-[acp] synthase classified, for example, under EC 2.3.1.- (e.g., EC 2.3.1.41, EC 2.3.1.179 or EC 2.3.1.180) such as the gene product of *fabB*, *fabF* or *fabH*; followed by conversion to 3-hydroxy-succinyl-[acp] methyl ester by a 3-oxoacyl-[acp] reductase classified, for example, under EC 1.1.1.100 such as

the gene product of *fabG*; followed by conversion to 2,3-dehydrosuccinyl-[acp] methyl ester by a 3-hydroxyacyl-[acp] dehydratase classified, for example, under EC 4.2.1.59 such as the gene product of *fabZ*; followed by conversion to succinyl-[acp] methyl ester by an enoyl-[acp] reductase classified, for example, under EC 1.3.1.10
 5 such as the gene product of *fabI*; followed by conversion to 3-oxoadipyl-[acp] methyl ester by a β -ketoacyl-[acp] synthase classified, for example, under EC 2.3.1. – (e.g., EC 2.3.1.41 or EC 2.3.1.179) such as the gene product of *fabB* or *fabF*; followed by conversion to 3-hydroxy-adipyl-[acp] methyl ester by a 3-oxoacyl-[acp] reductase classified, for example, under EC 1.1.1.100 such as the gene product of *fabG*;
 10 followed by conversion to 2,3-dehydroadipyl-[acp] methyl ester by a 3-hydroxyacyl-[acp] dehydratase classified, for example, under EC 4.2.1.59 such as the gene product of *fabZ*; followed by conversion to adipyl-[acp] methyl ester by an enoyl-[acp] reductase classified, for example, under EC 1.3.1.10 such as the gene product of *fabI*; followed by conversion to adipyl-[acp] by a pimeloyl-[acp] methyl ester esterase
 15 classified, for example, under EC 3.1.1.85 such as the gene product of *bioH*. See FIG. 1A.

Pathways using NADPH-specific enzymes to adipyl-CoA as central precursor leading to C6 Building Blocks

In some embodiments, adipyl-CoA is synthesized from the central metabolite oxalyl-CoA, by conversion of oxalyl-CoA to oxalyl-CoA methyl ester by a malonyl-CoA O-methyltransferase classified, for example, under EC 2.1.1.197 such as the
 20 gene product of *bioC*; followed by conversion with acetyl-CoA to 3-oxosuccinyl-CoA methyl ester by a β -ketothiolase classified, for example, under EC 2.3.1.16 such as the gene product of *bktB* or by conversion with malonyl-CoA by a β -ketoacyl-[acp] synthase classified, for example, under EC 2.3.1.180 such as the gene product of
 25 *fabH*; followed by conversion to 3-hydroxy-succinyl-CoA methyl ester by a 3-oxoacyl-[acp] reductase classified, for example, under EC 1.1.1.100 such as the gene product of *fabG* or an acetoacetyl-CoA reductase classified, for example, under EC 1.1.1.36 such as the gene product of *phaB*; followed by conversion to 2,3-
 30 dehydrosuccinyl-CoA methyl ester by an enoyl-CoA hydratase classified, for example, under EC 4.2.1.119 such as the gene product of *phaJ*; followed by conversion to succinyl-CoA methyl ester by a reductase classified, for example, under

EC 1.3.1.- such as an *enoyl-[acp] reductase* (classified under EC 1.3.1.10) such as the gene product of *fabI* or a *trans-2-enoyl-CoA reductase* (classified under EC 1.3.1.38, EC 1.3.1.8, or EC 1.3.1.44) such as the gene product of *ter* or *tdter*; followed by conversion to 3-oxoadipyl-CoA methyl ester by a *β -ketoacyl-[acp] synthase* classified, for example, under EC 2.3.1.- (e.g., EC 2.3.1.41 or EC 2.3.1.179) such as the gene product of *fabB* or *fabF*, or a *β -ketothiolase* classified, for example, under EC 2.3.1.16 such as the gene product of *bktB*; followed by conversion to 3-hydroxy-adipyl-CoA methyl ester by a *3-oxoacyl-[acp] reductase* classified, for example, under EC 1.1.1.100 such as the gene product of *fabG* or an *acetoacetyl-CoA reductase* classified, for example, under EC 1.1.1.36 such as the gene product of *phaB*; followed by conversion to 2,3-dehydroadipyl-CoA methyl ester by an *enoyl-CoA hydratase* classified, for example, under EC 4.2.1.119 such as the gene product of *phaJ*; followed by conversion to adipyl-CoA methyl ester by a *reductase* classified, for example, under EC 1.3.1.- such as an *enoyl-[acp] reductase* (EC 1.3.1.10) such as the gene product of *fabI* or a *trans-2-enoyl-CoA reductase* (EC 1.3.1.38, EC 1.3.1.8, or EC 1.3.1.44) such as the gene product of *ter* or *tdter*; followed by conversion to adipyl-CoA by a *pimeloyl-[acp] methyl ester esterase* classified, for example, under EC 3.1.1.85 such as the gene product of *bioH*. See FIG. 1B.

Pathways using NADH-specific enzymes to adipyl-CoA as central precursor leading to C6 Building Blocks

In some embodiments, adipyl-CoA is synthesized from the central metabolite, oxalyl-CoA, by conversion of oxalyl-CoA to oxalyl-CoA methyl ester by a *malonyl-CoA O-methyltransferase* classified, for example, under EC 2.1.1.197 such as the gene product of *bioC*; followed by conversion with acetyl-CoA to 3-oxosuccinyl-CoA methyl ester by a *β -ketothiolase* classified, for example, under EC 2.3.1.16 such as the gene product of *bktB* or by conversion with malonyl-CoA by a *β -ketoacyl-[acp] synthase* classified, for example, under EC 2.3.1.180 such as the gene product of *fabH*; followed by conversion to 3-hydroxy-succinyl-CoA methyl ester by a *3-hydroxyacyl-CoA dehydrogenase* classified, for example, under EC 1.1.1.- (e.g., EC 1.1.1.35 or EC 1.1.1.157) such as the gene product of *fadB* or *hbd*; followed by conversion to 2,3-dehydrosuccinyl-CoA methyl ester by an *enoyl-CoA hydratase* classified, for example, under EC 4.2.1.17 such as the gene product of *crt*; followed

by conversion to succinyl-CoA methyl ester by a *trans-2-enoyl-CoA reductase* classified, for example, under EC 1.3.1.44 such as the gene product of *ter* or *tdter*; followed by conversion to 3-oxoadipyl-CoA methyl ester by a β -ketoacyl-[*acp*] synthase classified, for example, under EC 2.3.1.- (e.g., EC 2.3.1.41 or EC 2.3.1.179) such as the gene product of *fabB* or *fabF* or a β -ketothiolase classified, for example, under EC 2.3.1.16 such as the gene product of *bktB*; followed by conversion to 3-hydroxy-adipyl-CoA methyl ester by a *3-hydroxyacyl-CoA dehydrogenase* classified, for example, under EC 1.1.1.35 such as the gene product of *fadB* or under EC 1.1.1.157 such as the gene product of *hbd*; followed by conversion to 2,3-dehydroadipyl-CoA methyl ester by an *enoyl-CoA hydratase* classified, for example, under EC 4.2.1.17 such as the gene product of *crt*; followed by conversion to adipyl-CoA methyl ester by a *trans-2-enoyl-CoA reductase* classified, for example, under EC 1.3.1.44 such as the gene product of *ter* or *tdter*; followed by conversion to adipyl-CoA by a *pimeloyl-[acp] methyl ester esterase* classified under EC 3.1.1.85 such as the gene product of *bioH*. See FIG. 1C.

Pathways using adipyl-CoA or adipyl-[*acp*] as central precursors to adipate

In some embodiments, adipic acid is synthesized from the central precursor, adipyl-CoA, by conversion of adipyl-CoA to adipate semialdehyde by an *acetylating aldehyde dehydrogenase* classified, for example, under EC 1.2.1.10 such as the gene product of *PduB* or *PduP* (see, for example, Lan *et al.*, 2013, *Energy Environ. Sci.*, 6:2672 – 2681); followed by conversion to adipic acid by a *7-oxoheptanoate dehydrogenase* classified, for example, under EC 1.2.1.- such as the gene product of *ThnG*, a *6-oxohexanoate dehydrogenase* classified, for example, under EC 1.2.1.- such as the gene product of *ChnE*, or an *aldehyde dehydrogenase* classified, for example, under C 1.2.1.3. See FIG. 2.

In some embodiments, adipic acid is synthesized from the central precursor, adipyl-CoA, by conversion of adipyl-CoA to adipate by a *thioesterase* classified, for example, under EC 3.1.2.- such as the gene products of *YciA*, *tesB* (GenBank Accession No. AAB60068.1, SEQ ID NO: 2; Genbank Accession No. AAA24665.1, SEQ ID NO: 1) or *Acot13*. See FIG. 2.

In some embodiments, adipic acid is synthesized from the central precursor, adipyl-[*acp*], by conversion of adipyl-[*acp*] to adipate by a *thioesterase* classified, for

example, under EC 3.1.2.- such as the gene products encoded by Genbank Accession No. ABJ63754.1, Genbank Accession No. CCC78182.1, *tesA* or *fatB*. See FIG. 2.

In some embodiments, adipate is synthesized from the central precursor, adipyl-CoA, by conversion of adipyl-CoA to adipate by a *CoA-transferase* such as a
 5 *glutaconate CoA-transferase* classified, for example, under EC 2.8.3.12. See FIG. 2.

In some embodiments, adipate is synthesized from the central precursor, adipyl-CoA, by conversion of adipyl-CoA to adipate by a reversible *CoA-ligase* such as a reversible *succinate-CoA ligase* classified, for example, under EC 6.2.1.5. See
 FIG. 2.

10 In some embodiments, adipate is synthesized from the central precursor, adipate semialdehyde, by conversion of adipate semialdehyde to adipate by a 6-oxohexanoate dehydrogenase or a 7-oxoheptanoate dehydrogenase (classified, for example, under EC 1.2.1.-) such as the gene product of *ThnG* or *ChnE*, or an aldehyde dehydrogenase classified, for example, under EC 1.2.1.3. See FIG. 2.

15

Pathways using adipyl-CoA, adipate semialdehyde, or ϵ -caprolactam as central precursor to 6-aminohexanoate

In some embodiments, 6-aminohexanoate is synthesized from the central precursor, adipyl-CoA, by conversion of adipyl-CoA to adipate semialdehyde by an
 20 *acetylating aldehyde dehydrogenase* classified, for example, EC 1.2.1.10, such as the gene product of *PduB* or *PduP*; followed by conversion of adipate semialdehyde to 6-aminohexanoate by a ω -transaminase classified, for example, under EC 2.6.1.18, EC 2.6.1.19, EC 2.6.1.29, EC 2.6.1.48 or EC 2.6.1.82. See FIG. 3.

In some embodiments, 6-aminohexanoate is synthesized from the central
 25 precursor, adipate semialdehyde, by conversion of adipate semialdehyde to 6-aminohexanoate by a ω -transaminase (e.g., EC 2.6.1.18, EC 2.6.1.19, or EC 2.6.1.48). See FIG. 3.

In some embodiments, 6-aminohexanoate is synthesized from the central precursor, adipate, by conversion of adipate to adipate semialdehyde by a *carboxylate*
 30 *reductase* classified, for example, under EC 1.2.99.6 such as the gene product of *car* in combination with a *phosphopantetheine transferase* enhancer (e.g., encoded by a *sfp* (Genbank Accession No. CAA44858.1, SEQ ID NO:14) gene from *Bacillus*

subtilis or *npt* (Genbank Accession No. ABI83656.1, SEQ ID NO:15) gene from *Nocardia*) or the gene products of *GriC* and *GriD* from *Streptomyces griseus* (Suzuki *et al.*, *J. Antibiot.*, 2007, 60(6), 380 – 387); followed by conversion of adipate semialdehyde to 6-aminohexanoate by a ω -transaminase (e.g., EC 2.6.1.18, EC 2.6.1.19, EC 2.6.1.48, EC 2.6.1.29, EC 2.6.1.82 such as SEQ ID NOs:8-13). The *carboxylate reductase* can be obtained, for example, from *Mycobacterium marinum* (Genbank Accession No. ACC40567.1, SEQ ID NO: 3), *Mycobacterium smegmatis* (Genbank Accession No. ABK71854.1, SEQ ID NO: 4), *Segniliparus rugosus* (Genbank Accession No. EFV11917.1, SEQ ID NO: 5), *Mycobacterium massiliense* (Genbank Accession No. EIV11143.1, SEQ ID NO: 6), or *Segniliparus rotundus* (Genbank Accession No. ADG98140.1, SEQ ID NO: 7). See FIG. 3.

In some embodiments, ϵ -caprolactam is synthesized from 6-aminohexanoate by a hydrolase classified, for example, under EC 3.5.2.-. See FIG. 3.

Pathway using 6-aminohexanoate, 6-hydroxhexanoate or adipate semialdehyde as central precursor to hexamethylenediamine

In some embodiments, hexamethylenediamine is synthesized from the central precursor, 6-aminohexanoate, by conversion of 6-aminohexanoate to 6-aminohexanal by a *carboxylate reductase* classified, for example, under EC 1.2.99.6 such as the gene product of *car* (see above) in combination with a *phosphopantetheine transferase* enhancer (e.g., encoded by a *sfp* (Genbank Accession No. CAA44858.1, SEQ ID NO:14) gene from *Bacillus subtilis* or *npt* (Genbank Accession No. ABI83656.1, SEQ ID NO:15) gene from *Nocardia*) or the gene product of *GriC* & *GriD*; followed by conversion of 6-aminohexanal to hexamethylenediamine by a ω -transaminase (e.g., classified, for example, under EC 2.6.1.18, EC 2.6.1.19, EC 2.6.1.29, EC 2.6.1.48, or EC 2.6.1.82 such as SEQ ID NOs:8-13, see above). See FIG. 4.

The *carboxylate reductase* encoded by the gene product of *car* and the *phosphopantetheine transferase* enhancer *npt* or *sfp* has broad substrate specificity, including terminal difunctional C4 and C5 carboxylic acids (Venkitasubramanian *et al.*, *Enzyme and Microbial Technology*, 2008, 42, 130 – 137).

In some embodiments, hexamethylenediamine is synthesized from the central precursor, 6-hydroxhexanoate (which can be produced as described in FIG. 5), by

conversion of 6-hydroxhexanoate to 6-hydroxhexanal by a *carboxylate reductase* classified, for example, under EC 1.2.99.6 such as the gene product of *car* (see above) in combination with a *phosphopantetheine transferase* enhancer (e.g., encoded by a *sfp* gene from *Bacillus subtilis* or *npt* gene from *Nocardia*) or the gene product of *GriC* & *GriD* (Suzuki *et al.*, *J. Antibiot.*, 2007, 60(6), 380 – 387); followed by conversion of 6-aminohexanal to 6-aminohexanol by a *ω-transaminase* classified, for example, under EC 2.6.1.18, EC 2.6.1.19, EC 2.6.1.29, EC 2.6.1.48, or EC 2.6.1.82 such as SEQ ID NOs:8-13, see above; followed by conversion to 6-aminohexanal by an *alcohol dehydrogenase* classified, for example, under EC 1.1.1.- (e.g., EC 1.1.1.1, EC 1.1.1.2, EC 1.1.1.21, or EC 1.1.1.184) such as the gene product of *YMR318C* (classified, for example, under EC 1.1.1.2, see Genbank Accession No. CAA90836.1) or *YqhD* (from *E. coli*, GenBank Accession No. AAA69178.1) (Liu *et al.*, *Microbiology*, 2009, 155, 2078 – 2085; Larroy *et al.*, 2002, *Biochem J.*, 361(Pt 1), 163 – 172; Jarboe, 2011, *Appl. Microbiol. Biotechnol.*, 89(2), 249 - 257) or the protein having GenBank Accession No. CAA81612.1 (from *Geobacillus stearothermophilus*); followed by conversion to hexamethylenediamine by a *ω-transaminase* classified, for example, under EC 2.6.1.18, EC 2.6.1.19, EC 2.6.1.29, EC 2.6.1.48, or EC 2.6.1.82 such as SEQ ID NOs:8-13, see above. See FIG. 4.

In some embodiments, hexamethylenediamine is synthesized from the central precursor, 6-aminohexanoate, by conversion of 6-aminohexanoate to N6-acetyl-6-aminohexanoate by a *N-acetyltransferase* such as a *lysine N-acetyltransferase* classified, for example, under EC 2.3.1.32; followed by conversion to N6-acetyl-6-aminohexanal by a *carboxylate reductase* classified, for example, under EC 1.2.99.6 such as the gene product of *car* (see above) in combination with a *phosphopantetheine transferase* enhancer (e.g., encoded by a *sfp* gene from *Bacillus subtilis* or *npt* gene from *Nocardia*) or the gene product of *GriC* & *GriD*; followed by conversion to N6-acetyl-1,6-diaminohexane by a *ω-transaminase* classified, for example, under EC 2.6.1.18, EC 2.6.1.19, EC 2.6.1.29, EC 2.6.1.48, EC 2.6.1.46, or EC 2.6.1.82 such as SEQ ID NOs:8-13, see above; followed by conversion to hexamethylenediamine by an *acetylputrescine deacylase* classified, for example, under EC 3.5.1.62. See, FIG. 4.

In some embodiments, hexamethylenediamine is synthesized from the central precursor, adipate semialdehyde, by conversion of adipate semialdehyde to hexanedial by a *carboxylate reductase* classified, for example, under EC 1.2.99.6 such as the gene product of *car* (see above) in combination with a *phosphopantetheine transferase* enhancer (e.g., encoded by a *sfp* gene from *Bacillus subtilis* or *npt* gene from *Nocardia*) or the gene product of *GriC* & *GriD*; followed by conversion to 6-aminohexanal by a ω -*transaminase* classified, for example, under EC 2.6.1.18, EC 2.6.1.19, or EC 2.6.1.48; followed by conversion to hexamethylenediamine by a ω -*transaminase* classified, for example, under EC 2.6.1.18, EC 2.6.1.19, EC 2.6.1.29, EC 2.6.1.48, EC 2.6.1.46, or EC 2.6.1.82 such as SEQ ID NOs:8-13, see above. See FIG. 4.

Pathways using adipate or adipate semialdehyde as central precursor to 1,6-hexanediol

In some embodiments, 6-hydroxhexanoate is synthesized from the central precursor, adipate, by conversion of adipate to adipate semialdehyde by a *carboxylate reductase* classified, for example, under EC 1.2.99.6 such as the gene product of *car* (see above) in combination with a *phosphopantetheine transferase* enhancer (e.g., encoded by a *sfp* gene from *Bacillus subtilis* or *npt* gene from *Nocardia*) or the gene product of *GriC* & *GriD*; followed by conversion to 6-hydroxhexanoate by a *dehydrogenase* classified, for example, under EC 1.1.1.- such as a 6-hydroxyhexanoate *dehydrogenase* classified, for example, under EC 1.1.1.258 such as the gene from of *ChnD* or a 5-hydroxypentanoate *dehydrogenase* classified, for example, under EC 1.1.1.- such as the gene product of *CpnD* (see, for example, Iwaki et al., 2002, *Appl. Environ. Microbiol.*, 68(11):5671 – 5684) or a 4-hydroxybutyrate *dehydrogenase* such as *gabD* (see, for example, Lütke-Eversloh & Steinbüchel, 1999, *FEMS Microbiology Letters*, 181(1):63 – 71). See FIG. 5. Adipate semialdehyde also can be produced from adipyl-CoA using an *acetylating aldehyde dehydrogenase* as described above. See, also FIG. 5.

In some embodiments, 1,6 hexanediol is synthesized from the central precursor, 6-hydroxhexanoate, by conversion of 6-hydroxhexanoate to 6-hydroxhexanal by a *carboxylate reductase* classified, for example, under EC 1.2.99.6

such as the gene product of *car* (see above) in combination with a
phosphopantetheine transferase enhancer (e.g., encoded by a *sfp* gene from *Bacillus subtilis* or *npt* gene from *Nocardia*) or the gene product of *GriC* & *GriD*; followed by
conversion of 6-hydroxhexanal to 1,6 hexanediol by an *alcohol dehydrogenase*
classified, for example, under EC 1.1.1.- such as EC 1.1.1.1, EC 1.1.1.2, EC 1.1.1.21,
or EC 1.1.1.184) such as the gene product of *YMR318C* or *YqhD* (see, e.g., Liu *et al.*,
Microbiology, 2009, 155, 2078 – 2085; Larroy *et al.*, 2002, *Biochem J.*, 361(Pt 1),
163 – 172; or Jarboe, 2011, *Appl. Microbiol. Biotechnol.*, 89(2), 249 - 257) or the
protein having GenBank Accession No. CAA81612.1 (from *Geobacillus*
stearothermophilus). See, FIG. 6.

Cultivation strategy

In some embodiments, the cultivation strategy entails achieving an aerobic,
anaerobic or micro-aerobic cultivation condition.

In some embodiments, the cultivation strategy entails nutrient limitation such
as nitrogen, phosphate or oxygen limitation.

In some embodiments, a cell retention strategy using, for example, ceramic
hollow fiber membranes can be employed to achieve and maintain a high cell density
during either fed-batch or continuous fermentation.

In some embodiments, the principal carbon source fed to the fermentation in
the synthesis of one or more C6 building blocks can derive from biological or non-
biological feedstocks.

In some embodiments, the biological feedstock can be, can include, or can
derive from, monosaccharides, disaccharides, lignocellulose, hemicellulose, cellulose,
lignin, levulinic acid and formic acid, triglycerides, glycerol, fatty acids, agricultural
waste, condensed distillers' solubles, or municipal waste.

The efficient catabolism of crude glycerol stemming from the production of
biodiesel has been demonstrated in several microorganisms such as *Escherichia coli*,
Cupriavidus necator, *Pseudomonas oleovorans*, *Pseudomonas putida* and *Yarrowia*
lipolytica (Lee *et al.*, *Appl. Biochem. Biotechnol.*, 2012, 166, 1801 – 1813; Yang *et al.*,
Biotechnology for Biofuels, 2012, 5:13; Meijnen *et al.*, *Appl. Microbiol.*
Biotechnol., 2011, 90, 885 - 893).

The efficient catabolism of lignocellulosic-derived levulinic acid has been demonstrated in several organisms such as *Cupriavidus necator* and *Pseudomonas putida* in the synthesis of 3-hydroxyvalerate via the precursor propanoyl-CoA (Jaremko and Yu, *Journal of Biotechnology*, 2011, 155, 2011, 293 – 298; Martin and Prather, *Journal of Biotechnology*, 2009, 139, 61 – 67).

The efficient catabolism of lignin-derived aromatic compounds such as benzoate analogues has been demonstrated in several microorganisms such as *Pseudomonas putida*, *Cupriavidus necator* (Bugg *et al.*, *Current Opinion in Biotechnology*, 2011, 22, 394 – 400; Pérez-Pantoja *et al.*, *FEMS Microbiol. Rev.*, 2008, 32, 736 – 794).

The efficient utilization of agricultural waste, such as olive mill waste water has been demonstrated in several microorganisms, including *Yarrowia lipolytica* (Papanikolaou *et al.*, *Bioresour. Technol.*, 2008, 99(7), 2419 - 2428).

The efficient utilization of fermentable sugars such as monosaccharides and disaccharides derived from cellulosic, hemicellulosic, cane and beet molasses, cassava, corn and other agricultural sources has been demonstrated for several microorganism such as *Escherichia coli*, *Corynebacterium glutamicum* and *Lactobacillus delbrueckii* and *Lactococcus lactis* (see, e.g., Hermann *et al.*, *Journal of Biotechnology*, 2003, 104, 155 – 172; Wee *et al.*, *Food Technol. Biotechnol.*, 2006, 44(2), 163 – 172; Ohashi *et al.*, *Journal of Bioscience and Bioengineering*, 1999, 87(5), 647 - 654).

The efficient utilization of furfural, derived from a variety of agricultural lignocellulosic sources, has been demonstrated for *Cupriavidus necator* (Li *et al.*, *Biodegradation*, 2011, 22, 1215 – 1225).

In some embodiments, the non-biological feedstock can be or can derive from natural gas, syngas, CO₂/H₂, methanol, ethanol, benzoic acid, non-volatile residue (NVR), a caustic wash waste stream from cyclohexane oxidation processes, or terephthalic acid / isophthalic acid mixture waste streams.

The efficient catabolism of methanol has been demonstrated for the methylotrophic yeast *Pichia pastoris*.

The efficient catabolism of ethanol has been demonstrated for *Clostridium kluyveri* (Seedorf *et al.*, *Proc. Natl. Acad. Sci. USA*, 2008, 105(6) 2128 - 2133).

The efficient catabolism of CO₂ and H₂, which may be derived from natural gas and other chemical and petrochemical sources, has been demonstrated for *Cupriavidus necator* (Prybylski *et al.*, *Energy, Sustainability and Society*, 2012, 2:11).

5 The efficient catabolism of syngas has been demonstrated for numerous microorganisms, such as *Clostridium ljungdahlii* and *Clostridium autoethanogenum* (Köpke *et al.*, *Applied and Environmental Microbiology*, 2011, 77(15), 5467 – 5475).

The efficient catabolism of the non-volatile residue waste stream from cyclohexane processes has been demonstrated for numerous microorganisms, such as *Delftia acidovorans* and *Cupriavidus necator* (Ramsay *et al.*, *Applied and*
10 *Environmental Microbiology*, 1986, 52(1), 152 – 156).

In some embodiments, the host microorganism is a prokaryote. For example, the prokaryote can be a bacterium from the genus *Escherichia* such as *Escherichia coli*; from the genus *Clostridia* such as *Clostridium ljungdahlii*, *Clostridium autoethanogenum* or *Clostridium kluyveri*; from the genus *Corynebacteria* such as
15 *Corynebacterium glutamicum*; from the genus *Cupriavidus* such as *Cupriavidus necator* or *Cupriavidus metallidurans*; from the genus *Pseudomonas* such as *Pseudomonas fluorescens*, *Pseudomonas putida* or *Pseudomonas oleovorans*; from the genus *Delftia* such as *Delftia acidovorans*; from the genus *Bacillus* such as *Bacillus subtilis*; from the genus *Lactobacillus* such as *Lactobacillus delbrueckii*; or
20 from the genus *Lactococcus* such as *Lactococcus lactis*. Such prokaryotes also can be a source of genes to construct recombinant host cells described herein that are capable of producing one or more C6 building blocks.

In some embodiments, the host microorganism is a eukaryote. For example, the eukaryote can be a filamentous fungus, e.g., one from the genus *Aspergillus* such
25 as *Aspergillus niger*. Alternatively, the eukaryote can be a yeast, e.g., one from the genus *Saccharomyces* such as *Saccharomyces cerevisiae*; from the genus *Pichia* such as *Pichia pastoris*; or from the genus *Yarrowia* such as *Yarrowia lipolytica*; from the genus *Issatchenkia* such as *Issatchenkia orientalis*; from the genus *Debaryomyces* such as *Debaryomyces hansenii*; from the genus *Arxula* such as *Arxula adenoinivorans*; or
30 from the genus *Kluyveromyces* such as *Kluyveromyces lactis*. Such eukaryotes also can be a source of genes to construct recombinant host cells described herein that are capable of producing one or more C6 building blocks.

Metabolic engineering

The present document provides methods involving less than all the steps described for all the above pathways. Such methods can involve, for example, one,
5 two, three, four, five, six, seven, eight, nine, ten, eleven, twelve or more of such steps. Where less than all the steps are included in such a method, the first, and in some embodiments the only, step can be any one of the steps listed.

Furthermore, recombinant hosts described herein can include any combination of the above enzymes such that one or more of the steps, e.g., one, two, three, four,
10 five, six, seven, eight, nine, ten, or more of such steps, can be performed within a recombinant host. This document provides host cells of any of the genera and species listed and genetically engineered to express one or more (e.g., two, three, four, five, six, seven, eight, nine, 10, 11, 12 or more) recombinant forms of any of the enzymes recited in the document. Thus, for example, the host cells can contain exogenous
15 nucleic acids encoding enzymes catalyzing one or more of the steps of any of the pathways described herein.

In addition, this document recognizes that where enzymes have been described as accepting CoA-activated substrates, analogous enzyme activities associated with [acp]-bound substrates exist that are not necessarily in the same enzyme class.

20 Also, this document recognizes that where enzymes have been described accepting (R)-enantiomers of substrate, analogous enzyme activities associated with (S)-enantiomer substrates exist that are not necessarily in the same enzyme class.

This document also recognizes that where an enzyme is shown to accept a particular co-factor, such as NADPH, or a co-substrate, such as acetyl-CoA, many
25 enzymes are promiscuous in terms of accepting a number of different co-factors or co-substrates in catalyzing a particular enzyme activity. Also, this document recognizes that where enzymes have high specificity for e.g., a particular co-factor such as NADH, an enzyme with similar or identical activity that has high specificity for the co-factor NADPH may be in a different enzyme class.

30 In some embodiments, the enzymes in the pathways outlined herein are the result of enzyme engineering via non-direct or rational enzyme design approaches with aims of improving activity, improving specificity, reducing feedback inhibition,

reducing repression, improving enzyme solubility, changing stereo-specificity, or changing co-factor specificity.

In some embodiments, the enzymes in the pathways outlined herein can be gene dosed (i.e., overexpressed by having a plurality of copies of the gene in the host
5 organism), into the resulting genetically modified organism via episomal or chromosomal integration approaches.

In some embodiments, genome-scale system biology techniques such as Flux Balance Analysis can be utilized to devise genome scale attenuation or knockout strategies for directing carbon flux to a C6 building block.

10 Attenuation strategies include, but are not limited to; the use of transposons, homologous recombination (double cross-over approach), mutagenesis, enzyme inhibitors and RNA interference (RNAi).

In some embodiments, fluxomic, metabolomic and transcriptomal data can be utilized to inform or support genome-scale system biology techniques, thereby
15 devising genome scale attenuation or knockout strategies in directing carbon flux to a C6 building block.

In some embodiments, the host microorganism's tolerance to high concentrations of a C6 building block can be improved through continuous cultivation in a selective environment.

20 In some embodiments, the host microorganism's endogenous biochemical network can be attenuated or augmented to (1) ensure the intracellular availability of oxalyl-CoA, acetyl-CoA, and/or malonyl-CoA, (2) create an NADH or NADPH imbalance that may only be balanced via the formation of one or more C6 building blocks, (3) prevent degradation of central metabolites, central precursors leading to
25 and including one or more C6 building blocks and/or (4) ensure efficient efflux from the cell.

In some embodiments requiring intracellular availability of oxaloyl-CoA for C6 building block synthesis, an *oxaloacetase* can be overexpressed in the host (Han *et al.*, 2007, *J. Biol. Chem.*, 282, 9581 – 9590).

30 In some embodiments requiring the intracellular availability of oxalyl-CoA, a *PEP carboxykinase* or *PEP carboxylase* can be overexpressed in the host to generate

anaplerotic carbon flux into the Krebs cycle towards oxaloacetate (Schwartz *et al.*, 2009, *Proteomics*, 9, 5132 – 5142).

In some embodiments requiring the intracellular availability of oxalyl-CoA, a *pyruvate carboxylase* can be overexpressed in the host to generated anaplerotic
5 carbon flux into the Krebs cycle towards oxaloacetate (Schwartz *et al.*, 2009, *supra*).

In some embodiments requiring the intracellular availability of oxalyl-CoA, a *PEP synthase* can be overexpressed in the host to enhance the flux from pyruvate to PEP, thus increasing the carbon flux into the Krebs cycle via *PEP carboxykinase* or *PEP carboxylase* (Schwartz *et al.*, 2009, *supra*).

10 In some embodiments requiring intracellular availability of acetyl-CoA for C6 building block synthesis, endogenous enzymes catalyzing the hydrolysis acetyl-CoA such as short-chain length *thioesterases* can be attenuated in the host organism.

In some embodiments requiring the intracellular availability of acetyl-CoA for C6 building block synthesis, an endogenous *phosphotransacetylase* generating acetate
15 such as *pta* can be attenuated (Shen *et al.*, *Appl. Environ. Microbiol.*, 2011, 77(9):2905–2915).

In some embodiments requiring the intracellular availability of acetyl-CoA for C6 building block synthesis, an endogenous gene in an acetate synthesis pathway encoding an *acetate kinase*, such as *ack*, can be attenuated.

20 In some embodiments requiring the intracellular availability of acetyl-CoA and NADH for C6 building block synthesis, an endogenous gene encoding an enzyme that catalyzes the degradation of pyruvate to lactate such as a *lactate dehydrogenase* encoded by *ldhA* can be attenuated (Shen *et al.*, 2011, *supra*).

In some embodiments requiring the intracellular availability of acetyl-CoA
25 and NADH for C6 building block synthesis, endogenous genes encoding enzymes, such as *menaquinol-fumarate oxidoreductase*, that catalyze the degradation of phophoenolpyruvate to succinate such as *frdBC* can be attenuated (see, e.g., Shen *et al.*, 2011, *supra*).

In some embodiments requiring the intracellular availability of acetyl-CoA
30 and NADH for C6 building block synthesis, an endogenous gene encoding an enzyme that catalyzes the degradation of acetyl-CoA to ethanol such as the *alcohol dehydrogenase* encoded by *adhE* can be attenuated (Shen *et al.*, 2011, *supra*).

In some embodiments, where pathways require excess NADH co-factor for C6 building block synthesis, a recombinant *formate dehydrogenase* gene can be overexpressed in the host organism (Shen *et al.*, 2011, *supra*).

5 In some embodiments, where pathways require excess NADH or NADPH co-factor for C6 building block synthesis, an endogenous *transhydrogenase* dissipating the co-factor imbalance can be attenuated.

In some embodiments, an endogenous gene encoding an enzyme that catalyzes the degradation of pyruvate to ethanol such as *pyruvate decarboxylase* can be attenuated.

10 In some embodiments, an endogenous gene encoding an enzyme that catalyzes the generation of isobutanol such as a *2-oxoacid decarboxylase* can be attenuated.

In some embodiments requiring the intracellular availability of acetyl-CoA for C6 building block synthesis, a recombinant *acetyl-CoA synthetase* such as the gene product of *acs* can be overexpressed in the microorganism (Sato *et al.*, *J. Bioscience and Bioengineering*, 2003, 95(4):335 – 341).

15 In some embodiments, carbon flux can be directed into the pentose phosphate cycle to increase the supply of NADPH by attenuating an endogenous *glucose-6-phosphate isomerase* (EC 5.3.1.9).

In some embodiments, carbon flux can be redirected into the pentose phosphate cycle to increase the supply of NADPH by overexpression a *6-phosphogluconate dehydrogenase* and/or a *transketolase* (Lee *et al.*, 2003, *Biotechnology Progress*, 19(5), 1444 – 1449).

20 In some embodiments, where pathways require excess NADPH co-factor in the synthesis of a C6 building block, a gene such as *UdhA* encoding a *puridine nucleotide transhydrogenase* can be overexpressed in the host organisms (Brigham *et al.*, *Advanced Biofuels and Bioproducts*, 2012, Chapter 39, 1065 - 1090).

In some embodiments, where pathways require excess NADPH co-factor in the synthesis of a C6 Building Block, a recombinant *glyceraldehyde-3-phosphate-dehydrogenase* gene such as *GapN* can be overexpressed in the host organisms
30 (Brigham *et al.*, 2012, *supra*).

In some embodiments, where pathways require excess NADPH co-factor in the synthesis of a C6 building block, a recombinant *malic enzyme* gene such as *maeA* or *maeB* can be overexpressed in the host organisms (Brigham *et al.*, 2012, *supra*).

5 In some embodiments, where pathways require excess NADPH co-factor in the synthesis of a C6 building block, a recombinant *glucose-6-phosphate dehydrogenase* gene such as *zwf* can be overexpressed in the host organisms (Lim *et al.*, *J. Bioscience and Bioengineering*, 2002, 93(6), 543 - 549).

10 In some embodiments, where pathways require excess NADPH co-factor in the synthesis of a C6 building block, a recombinant *fructose 1,6 diphosphatase* gene such as *fbp* can be overexpressed in the host organisms (Becker *et al.*, *J. Biotechnol.*, 2007, 132:99 - 109).

In some embodiments, where pathways require excess NADPH co-factor in the synthesis of a C6 building block, endogenous *triose phosphate isomerase* (EC 5.3.1.1) can be attenuated.

15 In some embodiments, where pathways require excess NADPH co-factor in the synthesis of a C6 building block, a recombinant *glucose dehydrogenase* such as the gene product of *gdh* can be overexpressed in the host organism (Sato *et al.*, *J. Bioscience and Bioengineering*, 2003, 95(4):335 – 341).

20 In some embodiments, endogenous enzymes facilitating the conversion of NADPH to NADH can be attenuated, such as the NADH generation cycle that may be generated via inter-conversion of *glutamate dehydrogenases* classified under EC 1.4.1.2 (NADH-specific) and EC 1.4.1.4 (NADPH-specific). For example, avoiding dissipation of an NADH imbalance towards C6 building blocks, a NADPH-specific *glutamate dehydrogenase* can be attenuated.

25 In some embodiments, an endogenous *glutamate dehydrogenase* (EC 1.4.1.3) that utilizes both NADH and NADPH as co-factors can be attenuated.

In some embodiments, a membrane-bound *enoyl-CoA reductases* can be solubilized via expression as a fusion protein to a small soluble protein such as a *maltose binding protein* (Gloerich *et al.*, *FEBS Letters*, 2006, 580, 2092 – 2096).

30 In some embodiments using hosts that naturally accumulate polyhydroxyalkanoates, the endogenous *polymer synthase* enzymes can be attenuated in the host strain.

In some embodiments, a *L-alanine dehydrogenase* can be overexpressed in the host to regenerate L-alanine from pyruvate as an amino donor for ω -transaminase reactions.

5 In some embodiments, a *L-glutamate dehydrogenase* specific for the co-factor used to achieve co-factor imbalance can be overexpressed in the host to regenerate L-glutamate from 2-oxoglutarate as an amino donor for ω -transaminase reactions. For example, promoting dissipation of the NADH imbalance towards C6 building blocks, a NADH-specific *glutamate dehydrogenase* can be overexpressed.

10 In some embodiments, enzymes such as *pimeloyl-CoA dehydrogenase* classified under, EC 1.3.1.62; an *acyl-CoA dehydrogenase* classified, for example, under EC 1.3.8.7 or EC 1.3.8.1; and/or a *glutaryl-CoA dehydrogenase* classified, for example, under EC 1.3.8.6 that degrade central metabolites and central precursors leading to and including C6 building blocks can be attenuated.

15 In some embodiments, endogenous enzymes activating C6 building blocks via Coenzyme A esterification such as *CoA-ligases* (e.g., a *pimeloyl-CoA synthetase*) classified under, for example, EC 6.2.1.14 can be attenuated.

In some embodiments, a *methanol dehydrogenase* and a *formaldehyde dehydrogenase* can be overexpressed in the host to allow methanol catabolism via formate.

20 In some embodiments, an *S-adenosylmethionine synthetase* can be overexpressed in the host to generate S-Adenosyl-L-methionine as a co-factor for *malonyl-[acp] O-methyltransferase*.

25 In some embodiments, the efflux of a C6 building block across the cell membrane to the extracellular media can be enhanced or amplified by genetically engineering structural modifications to the cell membrane or increasing any associated transporter activity for a C6 building block.

30 The efflux of hexamethylenediamine can be enhanced or amplified by overexpressing broad substrate range multidrug transporters such as *Blt* from *Bacillus subtilis* (Woolridge *et al.*, 1997, *J. Biol. Chem.*, 272(14):8864 – 8866); *AcrB* and *AcrD* from *Escherichia coli* (Elkins & Nikaido, 2002, *J. Bacteriol.*, 184(23), 6490 – 6499), *NorA* from *Staphylococcus aureus* (Ng *et al.*, 1994, *Antimicrob Agents*

Chemother, 38(6), 1345 – 1355), or *Bmr* from *Bacillus subtilis* (Neyfakh, 1992, *Antimicrob Agents Chemother*, 36(2), 484 – 485).

The efflux of 6-aminohexanoate and hexamethylenediamine can be enhanced or amplified by overexpressing the solute transporters such as the *lysE* transporter
5 from *Corynebacterium glutamicum* (Bellmann et al., 2001, *Microbiology*, 147, 1765 – 1774).

The efflux of adipic acid can be enhanced or amplified by overexpressing a dicarboxylate transporter such as the *SucE* transporter from *Corynebacterium glutamicum* (Huhn et al., *Appl. Microbiol. & Biotech.*, 89(2), 327 – 335).

10

Producing C6 Building Blocks Using a Recombinant Host

Typically, one or more C6 building blocks can be produced by providing a host microorganism and culturing the provided microorganism with a culture medium containing a suitable carbon source as described above. In general, the culture media
15 and/or culture conditions can be such that the microorganisms grow to an adequate density and produce a C6 building block efficiently. For large-scale production processes, any method can be used such as those described elsewhere (Manual of Industrial Microbiology and Biotechnology, 2nd Edition, Editors: A. L. Demain and J. E. Davies, ASM Press; and Principles of Fermentation Technology, P. F. Stanbury
20 and A. Whitaker, Pergamon). Briefly, a large tank (e.g., a 100 gallon, 200 gallon, 500 gallon, or more tank) containing an appropriate culture medium is inoculated with a particular microorganism. After inoculation, the microorganism is incubated to allow biomass to be produced. Once a desired biomass is reached, the broth containing the microorganisms can be transferred to a second tank. This second tank can be any
25 size. For example, the second tank can be larger, smaller, or the same size as the first tank. Typically, the second tank is larger than the first such that additional culture medium can be added to the broth from the first tank. In addition, the culture medium within this second tank can be the same as, or different from, that used in the first tank.

30 Once transferred, the microorganisms can be incubated to allow for the production of a C6 building block. Once produced, any method can be used to isolate C6 building blocks. For example, C6 building blocks can be recovered selectively

from the fermentation broth via adsorption processes. In the case of adipic acid and 6-aminohexanoic acid, the resulting eluate can be further concentrated via evaporation, crystallized via evaporative and/or cooling crystallization, and the crystals recovered via centrifugation. In the case of hexamethylenediamine and 1,6-hexanediol, distillation may be employed to achieve the desired product purity.

The invention is further described in the following example, which does not limit the scope of the invention described in the claims.

EXAMPLES

EXAMPLE 1

Enzyme activity of *thioesterases* using adipyI-CoA as a substrate and forming adipate

A sequence encoding an N-terminal His-tag was added to the genes from *Escherichia coli* encoding the *thioesterases* of SEQ ID NOs: 1 and 2 respectively (see FIG. 7), such that an N-terminal HIS tagged *thioesterase* could be produced. Each of the resulting modified genes was cloned into a pET15b expression vector under control of the T7 promoter. Each expression vector was transformed into a BL21[DE3] *E. coli* host. The resulting recombinant *E. coli* strains were cultivated at 37°C in a 500mL shake flask culture containing 50 mL Luria Broth (LB) media and antibiotic selection pressure, with shaking at 230 rpm. The culture was induced overnight at 17°C using 0.5 mM IPTG.

The pellet from the induced shake flask culture was harvested via centrifugation. The pellet was resuspended and lysed in Y-per™ solution (ThermoScientific, Rockford, IL). The cell debris was separated from the supernatant via centrifugation. The *thioesterases* were purified from the supernatant using Ni-affinity chromatography and the eluate was buffer exchanged and concentrated via ultrafiltration.

The enzyme activity assay was performed in triplicate in a buffer composed of 50 mM phosphate buffer (pH = 7.4), 0.1 mM Ellman's reagent, and 667 μM of adipyI-CoA (as substrate). Each enzyme activity assay reaction was initiated by adding 0.8 μM of the gene product of SEQ ID NO 1 and 4.1 μM of the gene product of SEQ ID NO 2 to the assay buffer containing the adipyI-CoA and incubating at 37

°C for 20 min. The release of Coenzyme A was monitored by absorbance at 412 nm. The absorbance associated with the substrate only control, which contained boiled enzyme, was subtracted from the active enzyme assay absorbance and compared to the empty vector control. The gene product of SEQ ID NO 1 and SEQ ID NO 2
5 accepted adipyl-CoA as substrate as confirmed via relative spectrophotometry (see FIG. 8) and synthesized adipate as a reaction product.

EXAMPLE 2

Enzyme activity of ω -transaminase using adipate semialdehyde as substrate and forming 6-aminohexanoate

10 A nucleotide sequence encoding a His-tag was added to the genes from *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Rhodobacter sphaeroides*, and *Vibrio fluvialis* encoding the ω -transaminases of SEQ ID NOs: 7, 8, 9, 10 and 12, respectively (see FIG. 7) such that N-terminal HIS tagged ω -transaminases could be produced. Each of the resulting modified genes was cloned
15 into a pET21a expression vector under control of the T7 promoter and each expression vector was transformed into a BL21[DE3] *E. coli* host. The resulting recombinant *E. coli* strains were cultivated at 37°C in a 250mL shake flask culture containing 50 mL LB media and antibiotic selection pressure, with shaking at 230 rpm. Each culture was induced overnight at 16 °C using 1 mM IPTG.

20 The pellet from each induced shake flask culture was harvested via centrifugation. Each pellet was resuspended and lysed via sonication. The cell debris was separated from the supernatant via centrifugation and the cell free extract was used immediately in enzyme activity assays.

Enzyme activity assays in the reverse direction (i.e., 6-aminohexanoate to
25 adipate semialdehyde) were performed in a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 10 mM 6-aminohexanoate, 10 mM pyruvate and 100 μ M pyridoxyl 5' phosphate. Each enzyme activity assay reaction was initiated by adding cell free extract of the ω -transaminase gene product or the empty vector control to the assay buffer containing the 6-aminohexanoate and incubated at 25°C for
30 24 h, with shaking at 250 rpm. The formation of L-alanine from pyruvate was quantified via RP-HPLC.

Each enzyme only control without 6-aminohexanoate demonstrated low base line conversion of pyruvate to L-alanine. See FIG. 14. The gene product of SEQ ID NO 7, SEQ ID NO 9, SEQ ID NO 10 and SEQ ID NO 12 accepted 6-aminohexanoate as substrate as confirmed against the empty vector control. See FIG. 15.

5 Enzyme activity in the forward direction (i.e., adipate semialdehyde to 6-aminohexanoate) was confirmed for the *transaminases* of SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10 and SEQ ID NO 12. Enzyme activity assays were performed in a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 10 mM adipate semialdehyde, 10 mM L-alanine and 100 μ M pyridoxyl 5' phosphate. Each enzyme activity assay reaction was initiated by adding a cell free
10 extract of the ω -*transaminase* gene product or the empty vector control to the assay buffer containing the adipate semialdehyde and incubated at 25°C for 4 h, with shaking at 250 rpm. The formation of pyruvate was quantified via RP-HPLC.

The gene product of SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10 and SEQ ID NO 12 accepted adipate semialdehyde as substrate as confirmed
15 against the empty vector control. See FIG. 16. The reversibility of the ω -*transaminase* activity was confirmed, demonstrating that the ω -*transaminases* of SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10 and SEQ ID NO 12 accepted adipate semialdehyde as substrate and synthesized 6-aminohexanoate as a reaction
20 product.

EXAMPLE 3

Enzyme activity of *carboxylate reductase* using adipate as substrate and forming adipate semialdehyde

25 A nucleotide equence encoding a HIS-tag was added to the genes from *Segniliparus rugosus* and *Segniliparus rotundus* that encode the *carboxylate reductases* of SEQ ID NOs: 5 and 7, respectively (see FIG. 7), such that N-terminal HIS tagged *carboxylate reductases* could be produced. Each of the modified genes was cloned into a pET Duet expression vector along with a *sfp* gene encoding a HIS-
30 tagged *phosphopantetheine transferase* from *Bacillus subtilis*, both under the T7 promoter. Each expression vector was transformed into a BL21[DE3] *E. coli* host and the resulting recombinant *E. coli* strains were cultivated at 37°C in a 250mL shake

flask culture containing 50 mL LB media and antibiotic selection pressure, with shaking at 230 rpm. Each culture was induced overnight at 37 °C using an auto-induction media.

The pellet from each induced shake flask culture was harvested via centrifugation. Each pellet was resuspended and lysed via sonication, and the cell debris was separated from the supernatant via centrifugation. The *carboxylate reductases* and *phosphopantetheine transferases* were purified from the supernatant using Ni-affinity chromatography, diluted 10-fold into 50mM HEPES buffer (pH = 7.5), and concentrated via ultrafiltration.

Enzyme activity assays (i.e., from adipate to adipate semialdehyde) were performed in triplicate in a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 2 mM adipate, 10 mM MgCl₂, 1 mM ATP and 1 mM NADPH. Each enzyme activity assay reaction was initiated by adding purified *carboxylate reductase* and *phosphopantetheine transferase* gene products or the empty vector control to the assay buffer containing the adipate and then incubated at room temperature for 20 min. The consumption of NADPH was monitored by absorbance at 340 nm. Each enzyme only control without adipate demonstrated low base line consumption of NADPH. See FIG. 9.

The gene products of SEQ ID NO 5 and SEQ ID NO 7, enhanced by the gene product of *sfp*, accepted adipate as substrate, as confirmed against the empty vector control (see FIG. 10), and synthesized adipate semialdehyde.

EXAMPLE 4

Enzyme activity of *carboxylate reductase* using 6-hydroxyhexanoate as substrate and forming 6-hydroxyhexanal

A nucleotide sequence encoding a His-tag was added to the genes from *Mycobacterium marinum*, *Mycobacterium smegmatis*, *Mycobacterium smegmatis*, *Segniliparus rugosus*, *Mycobacterium massiliense*, and *Segniliparus rotundus* that encode the *carboxylate reductases* of SEQ ID NOs: 3 - 7, respectively (see FIG. 7) such that N-terminal HIS tagged *carboxylate reductases* could be produced. Each of the modified genes was cloned into a pET Duet expression vector alongside a *sfp* gene encoding a His-tagged *phosphopantetheine transferase* from *Bacillus subtilis*, both

under control of the T7 promoter. Each expression vector was transformed into a BL21[DE3] *E. coli* host. Each resulting recombinant *E. coli* strain was cultivated at 37°C in a 250mL shake flask culture containing 50 mL LB media and antibiotic selection pressure, with shaking at 230 rpm. Each culture was induced overnight at 37°C using an auto-induction media.

The pellet from each induced shake flask culture was harvested via centrifugation. Each pellet was resuspended and lysed via sonication. The cell debris was separated from the supernatant via centrifugation. The *carboxylate reductases* and *phosphopantetheine transferase* were purified from the supernatant using Ni-affinity chromatography, diluted 10-fold into 50 mM HEPES buffer (pH = 7.5) and concentrated via ultrafiltration.

Enzyme activity (i.e., 6-hydroxyhexanoate to 6-hydroxyhexanal) assays were performed in triplicate in a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 2 mM 6-hydroxyhexanal, 10 mM MgCl₂, 1 mM ATP, and 1 mM NADPH. Each enzyme activity assay reaction was initiated by adding purified *carboxylate reductase* and *phosphopantetheine transferase* or the empty vector control to the assay buffer containing the 6-hydroxyhexanoate and then incubated at room temperature for 20 min. The consumption of NADPH was monitored by absorbance at 340 nm. Each enzyme only control without 6-hydroxyhexanoate demonstrated low base line consumption of NADPH. See FIG. 9.

The gene products of SEQ ID NO 3 - 7, enhanced by the gene product of *sfp*, accepted 6-hydroxyhexanoate as substrate as confirmed against the empty vector control (see FIG. 11), and synthesized 6-hydroxyhexanal.

EXAMPLE 5

Enzyme activity of ω -transaminase for 6-aminohexanol, forming 6-oxohexanol

A nucleotide sequence encoding an N-terminal His-tag was added to the *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Rhodobacter sphaeroides*, *Escherichia coli*, and *Vibrio fluvialis* genes encoding the ω -transaminases of SEQ ID NOS: 8 - 13, respectively (see FIG. 7) such that N-terminal HIS tagged ω -transaminases could be produced. The modified genes were cloned into a pET21a expression vector under the T7 promoter. Each expression

vector was transformed into a BL21[DE3] *E. coli* host. Each resulting recombinant *E. coli* strain were cultivated at 37°C in a 250mL shake flask culture containing 50 mL LB media and antibiotic selection pressure, with shaking at 230 rpm. Each culture was induced overnight at 16°C using 1 mM IPTG.

5 The pellet from each induced shake flask culture was harvested via centrifugation. Each pellet was resuspended and lysed via sonication. The cell debris was separated from the supernatant via centrifugation and the cell free extract was used immediately in enzyme activity assays.

10 Enzyme activity assays in the reverse direction (i.e., 6-aminohexanol to 6-oxohexanol) were performed in a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 10 mM 6-aminohexanol, 10 mM pyruvate, and 100 μ M pyridoxyl 5' phosphate. Each enzyme activity assay reaction was initiated by adding cell free extract of the ω -transaminase gene product or the empty vector control to the assay buffer containing the 6-aminohexanol and then incubated at 25 °C for 4 h, with
15 shaking at 250 rpm. The formation of L-alanine was quantified via RP-HPLC.

Each enzyme only control without 6-aminohexanol had low base line conversion of pyruvate to L-alanine. See FIG. 14.

20 The gene products of SEQ ID NO 8 - 13 accepted 6-aminohexanol as substrate as confirmed against the empty vector control (see FIG. 19) and synthesized 6-oxohexanol as reaction product. Given the reversibility of the ω -transaminase activity (see Example 2), it can be concluded that the gene products of SEQ ID 8 – 13 accept 6-aminohexanol as substrate and form 6-oxohexanol.

EXAMPLE 6

25 Enzyme activity of ω -transaminase using hexamethylenediamine as substrate and forming 6-aminohexanal

30 A nucleotide sequence encoding an N-terminal His-tag was added to the *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Rhodobacter sphaeroides*, *Escherichia coli*, and *Vibrio fluvialis* genes encoding the ω -transaminases of SEQ ID NOS: 8 - 13, respectively (see FIG. 7) such that N-terminal HIS tagged ω -transaminases could be produced. The modified genes were cloned into a pET21a expression vector under the T7 promoter. Each expression

vector was transformed into a BL21[DE3] *E. coli* host. Each resulting recombinant *E. coli* strain were cultivated at 37°C in a 250mL shake flask culture containing 50 mL LB media and antibiotic selection pressure, with shaking at 230 rpm. Each culture was induced overnight at 16°C using 1 mM IPTG.

5 The pellet from each induced shake flask culture was harvested via centrifugation. Each pellet was resuspended and lysed via sonication. The cell debris was separated from the supernatant via centrifugation and the cell free extract was used immediately in enzyme activity assays.

10 Enzyme activity assays in the reverse direction (i.e., hexamethylenediamine to 6-aminohexanal) were performed in a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 10 mM hexamethylenediamine, 10 mM pyruvate, and 100 µM pyridoxyl 5' phosphate. Each enzyme activity assay reaction was initiated by adding cell free extract of the *ω*-transaminase gene product or the empty vector control to the assay buffer containing the hexamethylenediamine and then incubated
15 at 25 °C for 4 h, with shaking at 250 rpm. The formation of L-alanine was quantified via RP-HPLC.

Each enzyme only control without hexamethylenediamine had low base line conversion of pyruvate to L-alanine. See FIG. 14.

20 The gene products of SEQ ID NO 8 - 13 accepted hexamethylenediamine as substrate as confirmed against the empty vector control (see FIG. 17) and synthesized 6-aminohexanal as reaction product. Given the reversibility of the *ω*-transaminase activity (see Example 2), it can be concluded that the gene products of SEQ ID NOs: 8 – 13 accept 6-aminohexanal as substrate and form hexamethylenediamine.

25 EXAMPLE 7

Enzyme activity of *carboxylate reductase* for N6-acetyl-6-aminohexanoate, forming N6-acetyl-6-aminohexanal

30 The activity of each of the N-terminal His-tagged *carboxylate reductases* of SEQ ID NOs: 5 - 7 (see Example 4, and FIG. 7) for converting N6-acetyl-6-aminohexanoate to N6-acetyl-6-aminohexanal was assayed in triplicate in a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 2 mM N6-acetyl-6-aminohexanoate, 10 mM MgCl₂, 1 mM ATP, and 1 mM NADPH. The

assays were initiated by adding purified *carboxylate reductase* and *phosphopantetheine transferase* or the empty vector control to the assay buffer containing the N6-acetyl-6-aminohexanoate then incubated at room temperature for 20 min. The consumption of NADPH was monitored by absorbance at 340 nm. Each enzyme only control without N6-acetyl-6-aminohexanoate demonstrated low base line consumption of NADPH. See FIG. 9.

The gene products of SEQ ID NO 5 - 7, enhanced by the gene product of *sfp*, accepted N6-acetyl-6-aminohexanoate as substrate as confirmed against the empty vector control (see FIG. 12), and synthesized N6-acetyl-6-aminohexanal.

EXAMPLE 8

Enzyme activity of ω -transaminase using N6-acetyl-1,6-diaminohexane, and forming N6-acetyl-6-aminohexanal

The activity of the N-terminal His-tagged ω -transaminases of SEQ ID NOs: 8 – 13 (see Example 6, and FIG. 7) for converting N6-acetyl-1,6-diaminohexane to N6-acetyl-6-aminohexanal was assayed using a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 10 mM N6-acetyl-1,6-diaminohexane, 10 mM pyruvate and 100 μ M pyridoxyl 5' phosphate. Each enzyme activity assay reaction was initiated by adding a cell free extract of the ω -transaminase or the empty vector control to the assay buffer containing the N6-acetyl-1,6-diaminohexane then incubated at 25 °C for 4 h, with shaking at 250 rpm. The formation of L-alanine was quantified via RP-HPLC.

Each enzyme only control without N6-acetyl-1,6-diaminohexane demonstrated low base line conversion of pyruvate to L-alanine. See FIG. 14.

The gene product of SEQ ID NO 8 - 13 accepted N6-acetyl-1,6-diaminohexane as substrate as confirmed against the empty vector control (see FIG. 18) and synthesized N6-acetyl-6-aminohexanal as reaction product.

Given the reversibility of the ω -transaminase activity (see example 2), the gene products of SEQ ID 8 – 13 accept N6-acetyl-6-aminohexanal as substrate forming N6-acetyl-1,6-diaminohexane.

EXAMPLE 9

Enzyme activity of *carboxylate reductase* using adipate semialdehyde as substrate and forming hexanedial

The N-terminal His-tagged *carboxylate reductase* of SEQ ID NO 7 (see Example 4 and FIG. 7) was assayed using adipate semialdehyde as substrate. The enzyme activity assay was performed in triplicate in a buffer composed of a final concentration of 50 mM HEPES buffer (pH = 7.5), 2 mM adipate semialdehyde, 10 mM MgCl₂, 1 mM ATP and 1 mM NADPH. The enzyme activity assay reaction was initiated by adding purified *carboxylate reductase* and *phosphopantetheine transferase* or the empty vector control to the assay buffer containing the adipate semialdehyde and then incubated at room temperature for 20 min. The consumption of NADPH was monitored by absorbance at 340 nm. The enzyme only control without adipate semialdehyde demonstrated low base line consumption of NADPH. See FIG. 9.

The gene product of SEQ ID NO 7, enhanced by the gene product of *sfp*, accepted adipate semialdehyde as substrate as confirmed against the empty vector control (see FIG. 13) and synthesized hexanedial.

EXAMPLE 10

Enzyme activity of *pimeloyl-[acp] methyl ester methylesterase* using adipyl-CoA methyl ester as substrate and forming adipyl-CoA

A sequence encoding an C-terminal His-tag was added to the gene from *Escherichia coli* encoding the *pimeloyl-[acp] methyl ester methylesterase* of SEQ ID NO: 17 (see FIG. 7) such that C-terminal HIS tagged *pimeloyl-[acp] methyl ester methylesterase* could be produced. The resulting modified gene was cloned into a pET28b+ expression vector under control of the T7 promoter and the expression vector was transformed into a BL21[DE3] *E. coli* host. The resulting recombinant *E. coli* strain was cultivated at 37°C in a 500mL shake flask culture containing 100 mL LB media and antibiotic selection pressure, with shaking at 230 rpm. Each culture was induced overnight at 18 °C using 0.3 mM IPTG.

The pellet from each induced shake flask culture was harvested via centrifugation. Each pellet was resuspended and lysed via sonication. The cell debris was separated from the supernatant via centrifugation. The *pimeloyl-[acp] methyl*

ester methylesterase was purified from the supernatant using Ni-affinity chromatography, buffer exchanged and concentrated into 20 mM HEPES buffer (pH = 7.5) via ultrafiltration and stored at 4 °C.

5 Enzyme activity assays converting adipyl-CoA methyl ester to adipyl-CoA were performed in triplicate in a buffer composed of a final concentration of 25 mM Tris·HCl buffer (pH = 7.0) and 5 [mM] adipyl-CoA methyl ester. The enzyme activity assay reaction was initiated by adding *pimeloyl-[acp] methyl ester methylesterase* to a final concentration of 10 [μM] to the assay buffer containing the adipyl-CoA methyl ester and incubated at 30°C for 1 h, with shaking at 250 rpm. The formation of adipyl-
10 CoA was quantified via LC-MS.

The substrate only control without enzyme showed trace quantities of the substrate adipyl-CoA. See FIG. 20. The *pimeloyl-[acp] methyl ester methylesterase* of SEQ ID NO. 17 accepted adipyl-CoA methyl ester as substrate and synthesized adipyl-CoA as reaction product as confirmed via LC-MS. See FIG. 20.

15

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the
20 scope of the following claims.

1 **WHAT IS CLAIMED IS:**

- 2 1. A method for biosynthesizing a product selected from the group consisting of
3 adipic acid, 6-aminohexanoate, 6-hydroxhexanoate, hexamethylenediamine,
4 caprolactam and 1,6-hexanediol, said method comprising enzymatically
5 synthesizing a six carbon chain aliphatic backbone from oxalyl-CoA and either (i)
6 acetyl-CoA or malonyl-CoA via two cycles of methyl ester shielded carbon chain
7 elongation or (ii) malonyl-[acp] via two cycles of methyl-ester shielded carbon
8 chain elongation, and enzymatically forming two terminal functional groups
9 selected from the group consisting of carboxyl, amine, and hydroxyl groups in
10 said backbone, thereby forming the product.
- 11 2. The method of claim 1, wherein the six carbon chain aliphatic backbone is adipyl-
12 [acp] or adipyl-CoA.
- 13 3. The method of claim 1 or 2, wherein a *malonyl-[acp] O-methyltransferase*
14 converts oxalyl-CoA to oxalyl-CoA methyl ester.
- 15 4. The method of claim 3, wherein each of said two cycles of carbon chain
16 elongation comprises using (i) a β -ketoacyl-[acp] synthase or a β -ketothiolase, (ii)
17 a 3-oxoacyl-[acp] reductase, an acetoacetyl-CoA reductase, a 3-hydroxyacyl-CoA
18 dehydrogenase or a 3-hydroxybutyryl-CoA dehydrogenase, (iii) an enoyl-CoA
19 hydratase or a 3-hydroxyacyl-[acp] dehydratase, and (iv) an enoyl-[acp]
20 reductase or a *trans*-2-enoyl-CoA reductase to produce adipyl-[acp] methyl ester
21 or adipyl-[acp] methyl ester.
- 22 5. The method of claim 4, wherein a *pimeloyl-[acp] methyl ester methylesterase*
23 removes the methyl group from adipyl-CoA methyl ester or adipyl-[acp] methyl
24 ester.
- 25 6. The method of any one of claims 3-5, wherein the *malonyl-[acp]O-*
26 *methyltransferase* has at least 70% sequence identity to the amino acid sequence
27 set forth in SEQ ID NO: 16.
- 28 7. The method of claim 5 or claim 6, wherein the *pimeloyl-[acp] methyl ester*
29 *methylesterase* has at least 70% sequence identity to the amino acid sequence set
30 forth in SEQ ID NO: 17.
- 31 8. The method of any one of claims 1-7, wherein said two terminal functional groups
32 are the same.

- 33 9. The method of any one of claims 1-7, wherein said two terminal functional groups
34 are different.
- 35 10. The method of claim 9, wherein said product comprises a terminal amine and a
36 terminal carboxyl group.
- 37 11. The method of claim 9, wherein said product comprises a terminal hydroxyl group
38 and a terminal carboxyl group.
- 39 12. The method of any one of claims 1-8, wherein said two terminal functional groups
40 are amine.
- 41 13. The method of any one of claims 1-8, wherein said two terminal functional groups
42 are hydroxyl groups.
- 43 14. The method of claim 11 or claim 13, wherein a *6-hydroxyhexanoate*
44 *dehydrogenase*, a *5-hydroxypentanoate dehydrogenase*, a *4-hydroxybutyrate*
45 *dehydratase*, or an *alcohol dehydrogenase* enzymatically forms a hydroxyl group.
- 46 15. The method of any one of claims 1-10, wherein a *thioesterase*, an *aldehyde*
47 *dehydrogenase*, a *7-oxoheptanoate dehydrogenase*, a *6-oxohexanoate*
48 *dehydrogenase*, a *glutaconate CoA-transferase*, or a reversible *succinyl-CoA*
49 *ligase* enzymatically forms a terminal carboxyl group.
- 50 16. The method of claim 15, wherein said *thioesterase* has at least 70% sequence
51 identity to the amino acid sequence set forth in SEQ ID NO: 1.
- 52 17. The method of claim 10 or 12, wherein a *ω-transaminase* or a *deacetylase*
53 enzymatically forms the amine group.
- 54 18. The method of claim 17, wherein said *ω-transaminase* has at least 70% sequence
55 identity to any one of the amino acid sequences set forth in SEQ ID NOs: 8 – 13.
- 56 19. The method of any one of claims 1 - 11, wherein a *carboxylate reductase*,
57 enhanced by a *phosphopantetheinyl transferase*, forms a terminal aldehyde group
58 as an intermediate in forming the product.
- 59 20. The method of claim 19, wherein said *carboxylate reductase* has at least 70%
60 sequence identity to any one of the amino acid sequences set forth in SEQ ID NO.
61 3 – 7.
- 62 21. The method of any of the preceding claims, wherein said method is performed in a
63 recombinant host by fermentation.

- 64 22. The method of claim 21, wherein said host is subjected to a cultivation strategy
65 under aerobic, anaerobic, micro-aerobic or mixed oxygen/denitrification
66 cultivation conditions.
- 67 23. The method of claim 21 or claim 22, wherein said host is cultured under
68 conditions of nutrient limitation.
- 69 24. The method according to any one of claims 21-23, wherein said host is retained
70 using a ceramic hollow fiber membrane to maintain a high cell density during
71 fermentation.
- 72 25. The method of any one of claims 21-24, wherein the principal carbon source fed
73 to the fermentation derives from biological or non-biological feedstocks.
- 74 26. The method of claim 25, wherein the biological feedstock is, or derives from,
75 monosaccharides, disaccharides, lignocellulose, hemicellulose, cellulose, lignin,
76 levulinic acid, formic acid, triglycerides, glycerol, fatty acids, agricultural waste,
77 condensed distillers' solubles, or municipal waste.
- 78 27. The method of claim 25, wherein the non-biological feedstock is, or derives from,
79 natural gas, syngas, CO₂/H₂, methanol, ethanol, benzoate, non-volatile residue
80 (NVR) caustic wash waste stream from cyclohexane oxidation processes, or
81 terephthalic acid / isophthalic acid mixture waste streams.
- 82 28. The method of any one of claims 21-27, wherein the host is a prokaryote.
- 83 29. The method of claim 28, wherein said prokaryote is from the genus *Escherichia*
84 such as *Escherichia coli*; from the genus *Clostridia* such as *Clostridium*
85 *ljungdahlii*, *Clostridium autoethanogenum* or *Clostridium kluyveri*; from the
86 genus *Corynebacteria* such as *Corynebacterium glutamicum*; from the genus
87 *Cupriavidus* such as *Cupriavidus necator* or *Cupriavidus metallidurans*; from the
88 genus *Pseudomonas* such as *Pseudomonas fluorescens*, *Pseudomonas putida* or
89 *Pseudomonas oleovorans*; from the genus *Delftia acidovorans*, from the genus
90 *Bacillus* such as *Bacillus subtilis*; from the genus *Lactobacillus* such as
91 *Lactobacillus delbrueckii*; from the genus *Lactococcus* such as *Lactococcus lactis*
92 or from the genus *Rhodococcus* such as *Rhodococcus equi*.
- 93 30. The method of any one of claims 21-27, wherein the host is a eukaryote.
- 94 31. The method of claim 30, wherein said eukaryote is from the genus *Aspergillus*
95 such as *Aspergillus niger*; from the genus *Saccharomyces* such as *Saccharomyces*

- 196 *cerevisiae*; from the genus *Pichia* such as *Pichia pastoris*; from the genus
 197 *Yarrowia* such as *Yarrowia lipolytica*, from the genus *Issatchenkia* such as
 198 *Issatchenkia orientalis*, from the genus *Debaryomyces* such as *Debaryomyces*
 199 *hansenii*, from the genus *Arxula* such as *Arxula adenoinivorans*, or from the genus
 200 *Kluyveromyces* such as *Kluyveromyces lactis*.
- 201 32. The method of claim 21, wherein the host's tolerance to high concentrations of a
 202 C6 building block is improved through continuous cultivation in a selective
 203 environment.
- 204 33. The method of any one of claims 21 - 32, wherein said host comprises one or
 205 more of the following attenuated enzymes: *polyhydroxyalkanoate synthase*, an
 206 *acetyl-CoA thioesterase*, a *phosphotransacetylase forming acetate*, an *acetate*
 207 *kinase*, a *lactate dehydrogenase*, a *menaquinol-fumarate oxidoreductase*, a 2-
 208 *oxoacid decarboxylase* producing isobutanol, an *alcohol dehydrogenase* forming
 209 ethanol, a *triose phosphate isomerase*, a *pyruvate decarboxylase*, a *glucose-6-*
 210 *phosphate isomerase*, a *transhydrogenase* dissipating the NADH or NADPH
 211 imbalance, an *glutamate dehydrogenase* dissipating the NADH or NADPH
 212 imbalance, a NADH/NADPH-utilizing *glutamate dehydrogenase*, a *pimeloyl-CoA*
 213 *dehydrogenase*; an *acyl-CoA dehydrogenase* accepting C6 building blocks and
 214 central precursors as substrates; a *glutaryl-CoA dehydrogenase*; or a *pimeloyl-CoA*
 215 *synthetase*.
- 216 34. The method of any one of claims 21, 32 and 33, wherein said host overexpresses
 217 one or more genes encoding: an *oxaloacetase*, a *PEP carboxylase*, a *PEP*
 218 *carboxykinase*, a *pyruvate carboxylase*, a *PEP synthase*, an *acetyl-CoA synthetase*,
 219 a *6-phosphogluconate dehydrogenase*; a *transketolase*; a *puridine nucleotide*
 220 *transhydrogenase*; a *formate dehydrogenase*; a *glyceraldehyde-3P-*
 221 *dehydrogenase*; a *malic enzyme*; a *glucose-6-phosphate dehydrogenase*; a *fructose*
 222 *1,6 diphosphatase*; a *L-alanine dehydrogenase*; a *L-glutamate dehydrogenase*
 223 specific to the NADH or NADPH used to generate a co-factor imbalance; a
 224 *methanol dehydrogenase*; a *formaldehyde dehydrogenase*; a *diamine transporter*;
 225 a *dicarboxylate transporter*; *S-adenosylmethionine synthetase*; and/or a *multidrug*
 226 *transporter*.

- 127 35. A recombinant host comprising at least one exogenous nucleic acid encoding (i) a
128 *malonyl-[acp] O-methyltransferase*, (ii) a β -*ketoacyl-[acp] synthase* or a β -
129 *ketothiolase*, (iii) a *3-oxoacyl-[acp] reductase*, *acetoacetyl-CoA reductase*, a *3-*
130 *hydroxyacyl-CoA dehydrogenase* or a *3-hydroxybutyryl-CoA dehydrogenase*, (iv)
131 an *enoyl-CoA hydratase* or *3-hydroxyacyl-[acp] dehydratase*, (v) an *enoyl-[acp]*
132 *reductase* or a *trans-2-enoyl-CoA reductase*, and (vi) a *pimeloyl-[acp] methyl*
133 *ester methylesterase*, said host producing adipyl-[acp] or adipyl-CoA.
- 134 36. The recombinant host of any one of claim 35, said host comprising at least one
135 exogenous nucleic acid encoding one or more of a *thioesterase*, an *aldehyde*
136 *dehydrogenase*, a *7-oxoheptanoate dehydrogenase*, a *6-oxohexanoate*
137 *dehydrogenase*, a *glutaconate CoA-transferase*, a reversible *succinyl-CoA ligase*,
138 an *acetylating aldehyde dehydrogenase*, or a *carboxylate reductase*, said host
139 producing adipic acid or adipate semialdehyde.
- 140 37. The recombinant host of claim 36, said host comprising at least one exogenous
141 nucleic acid encoding a ω -*transaminase*, said host producing 6-aminohexanoate.
- 142 38. The recombinant host of claim 36, said host further comprising one or more of a
143 *4-hydroxybutyrate dehydrogenase*, a *5-hydroxypentanoate dehydrogenase* or a *6-*
144 *hydroxyhexanoate dehydrogenase*, said host producing 6-hydroxhexanoic acid.
- 145 39. The recombinant host of any one of claims 35-38, said host further comprising at
146 least one exogenous nucleic acid encoding a ω -*transaminase*, a *deacetylase*, an *N-*
147 *acetyl transferase* or an *alcohol dehydrogenase*, said host producing
148 hexamethylenediamine.
- 149 40. The recombinant host of claim 38, said host further comprising at least one
150 exogenous nucleic acid encoding a (i) *carboxylate reductase* enhanced by a
151 *phosphopantetheinyl transferase* or (ii) an *alcohol dehydrogenase*, said host
152 producing 1,6-hexanediol.
- 153 41. The recombinant host of claim 37, said host further comprising a *lactamase*, said
154 host producing caprolactam.

FIGURE 1A

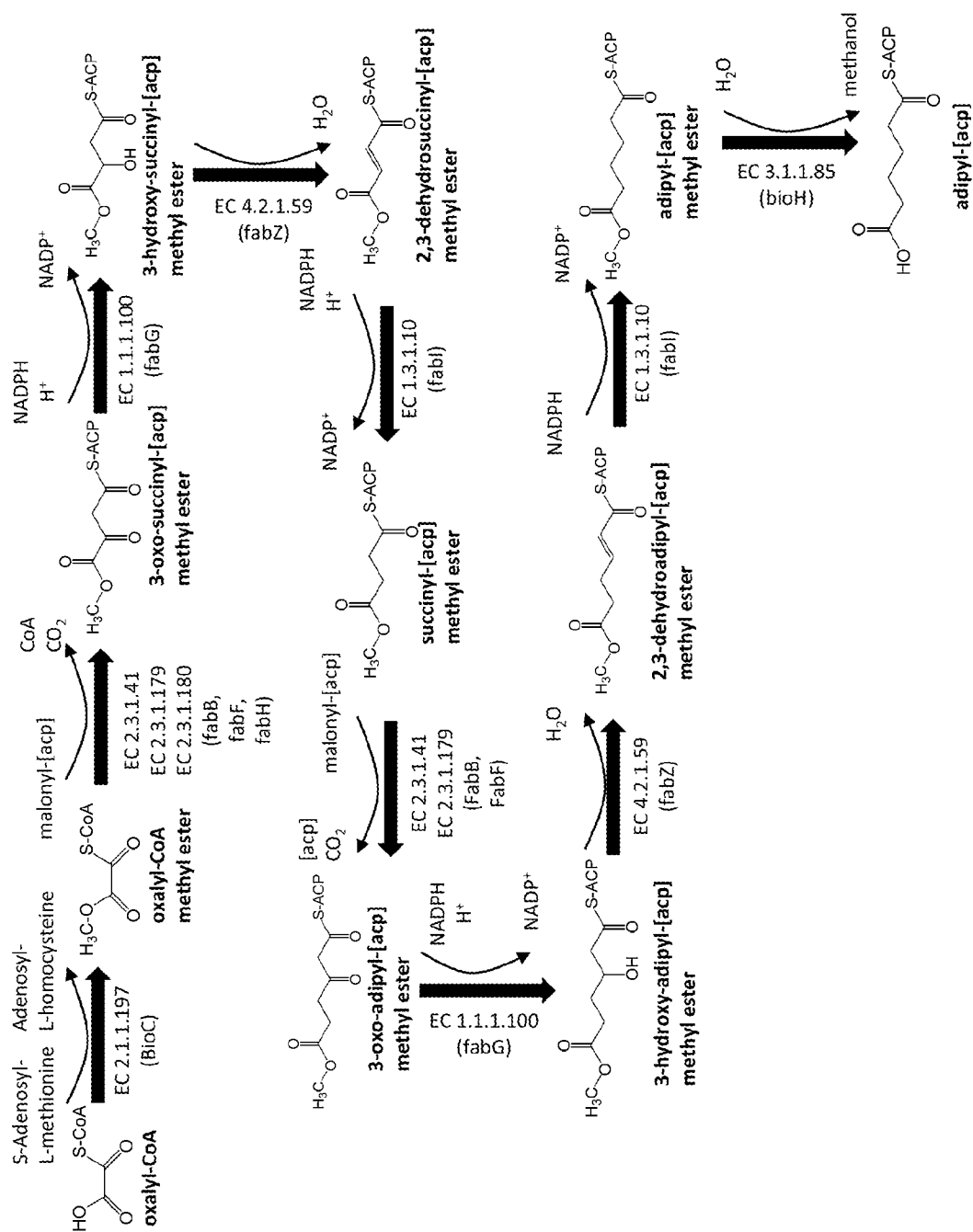


FIGURE 1B

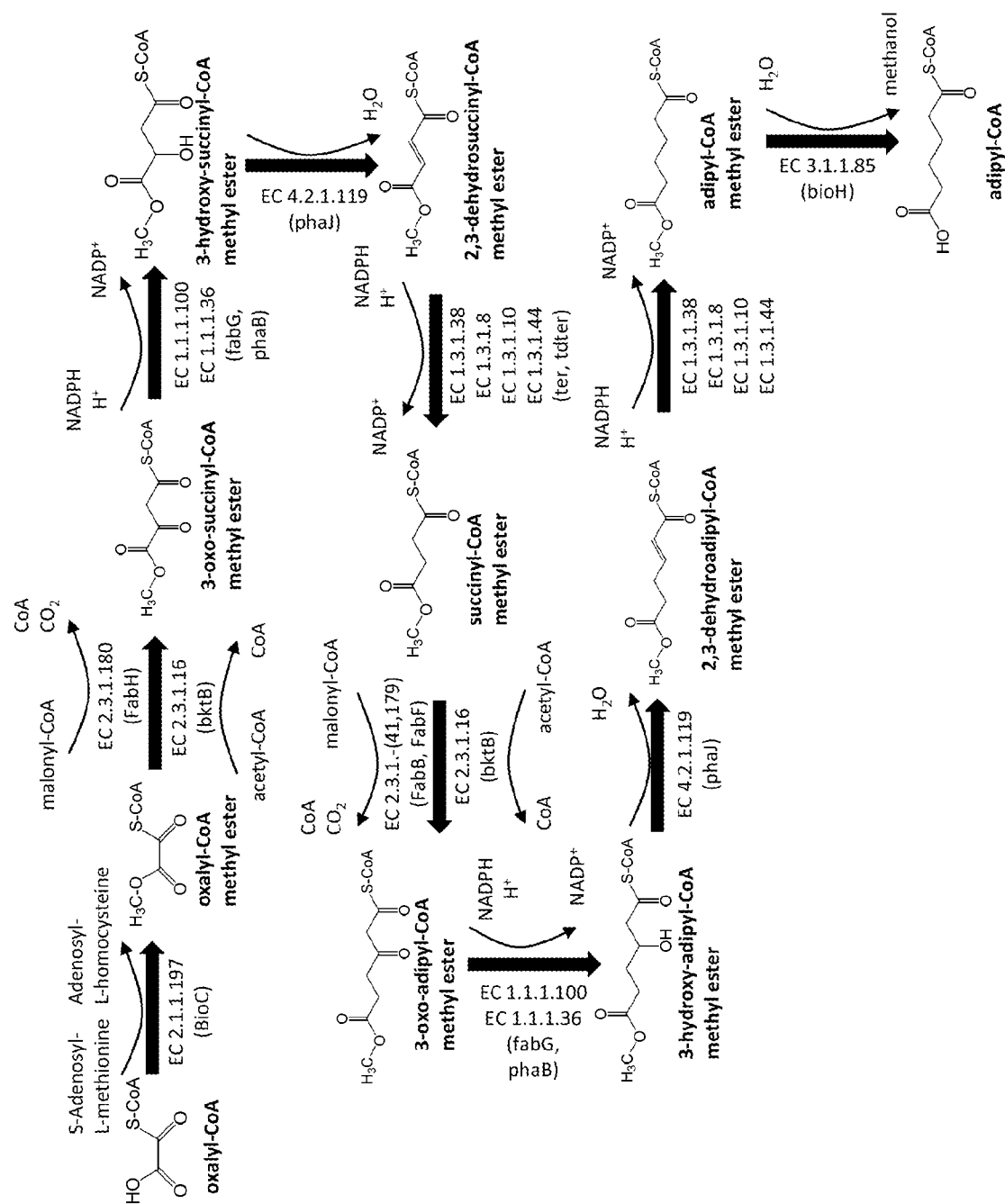


FIGURE 1C

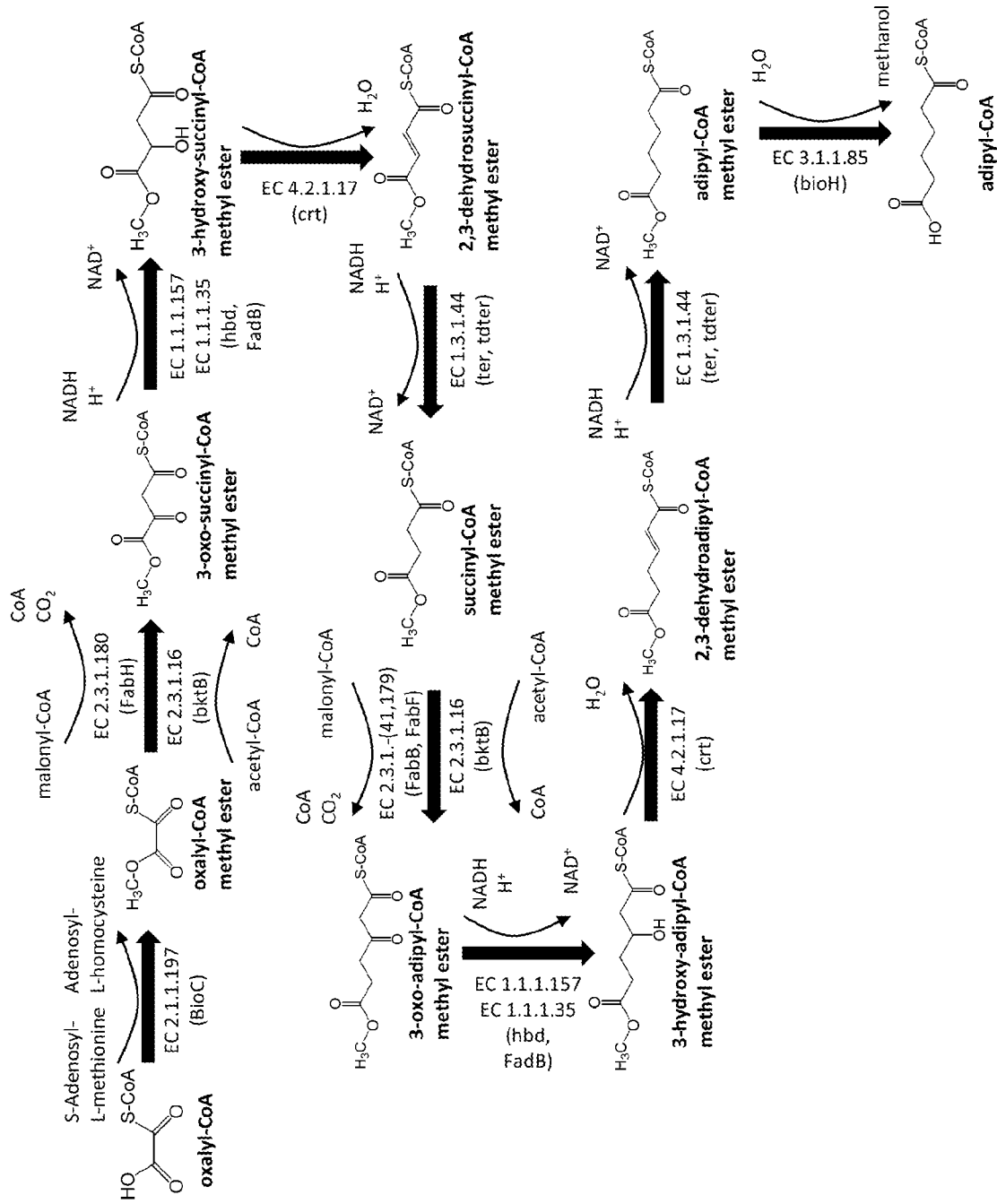


FIGURE 2

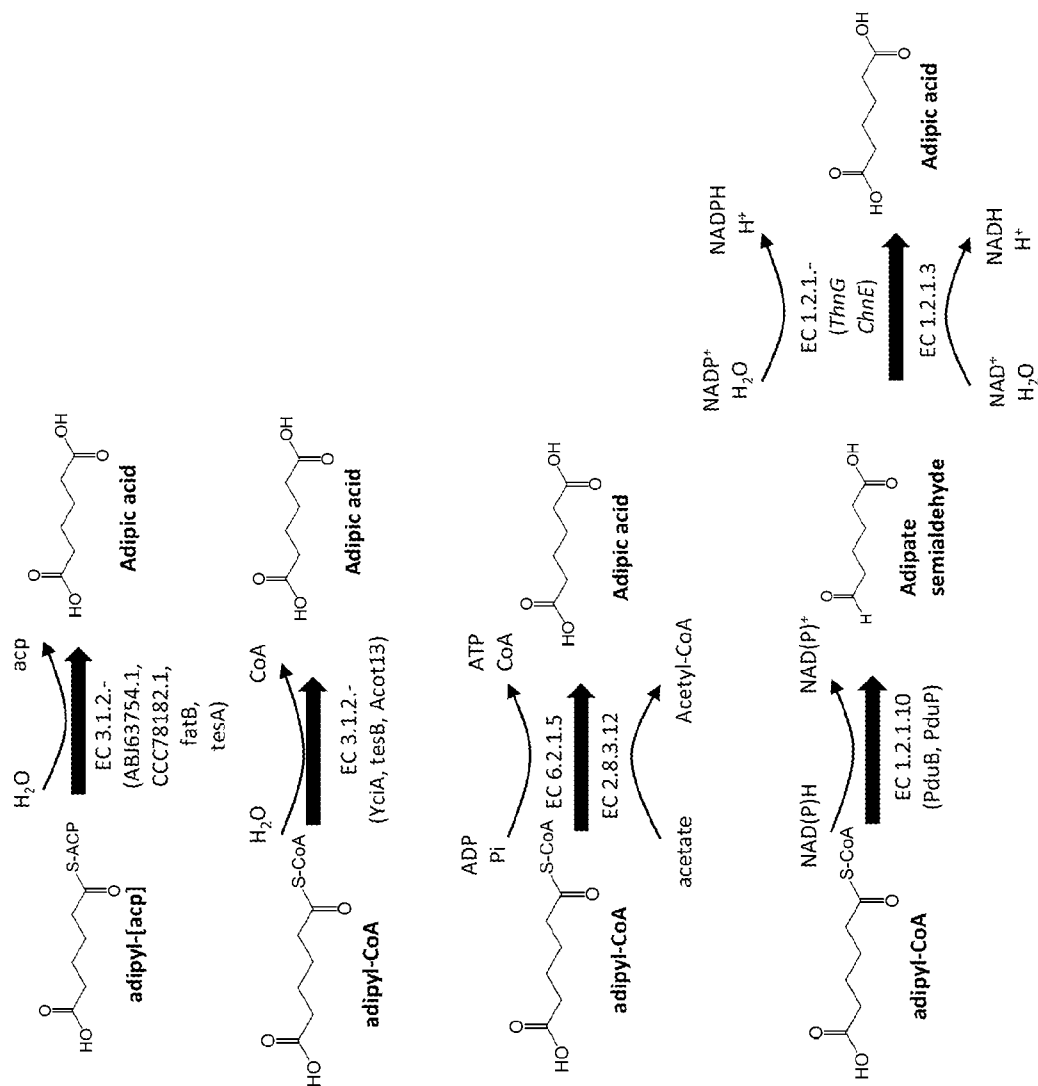
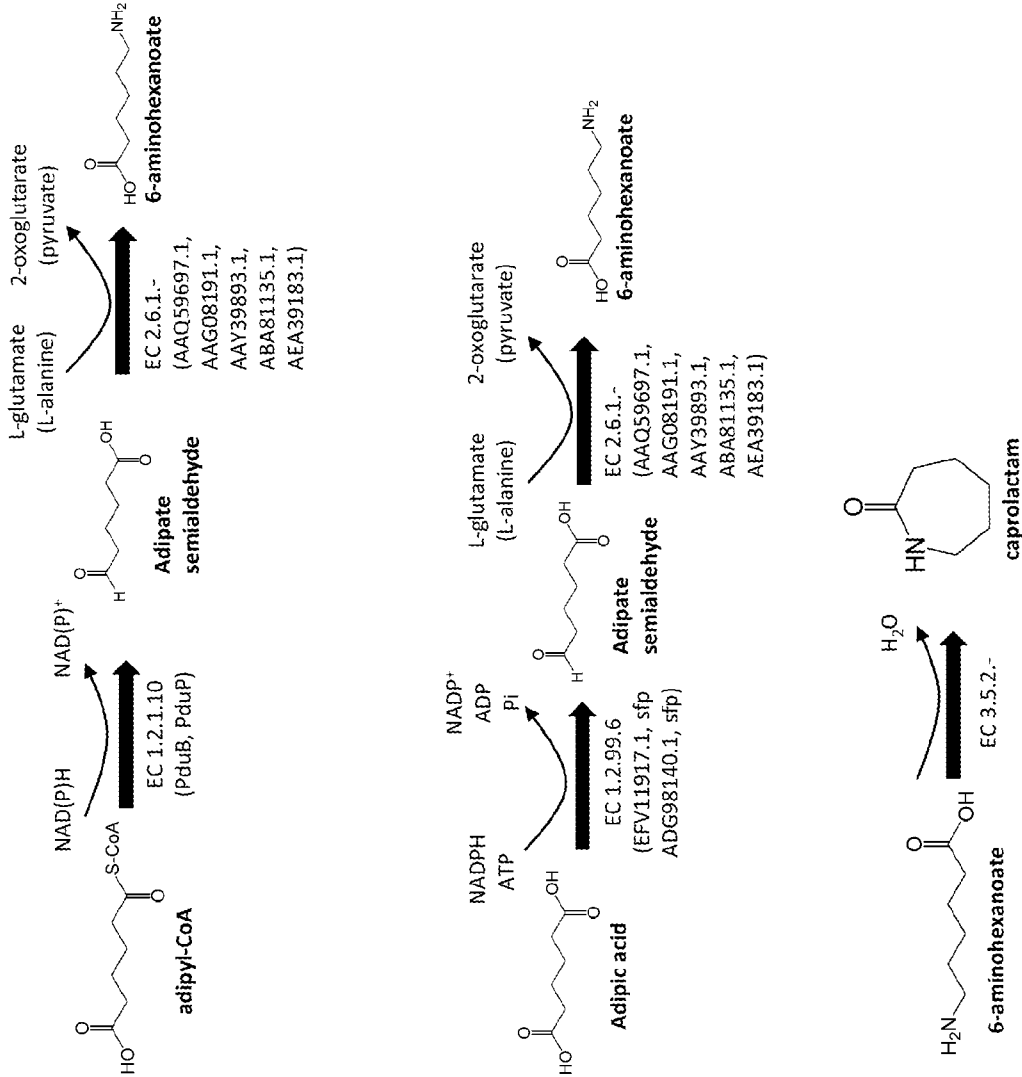


FIGURE 3



6/28

FIGURE 4

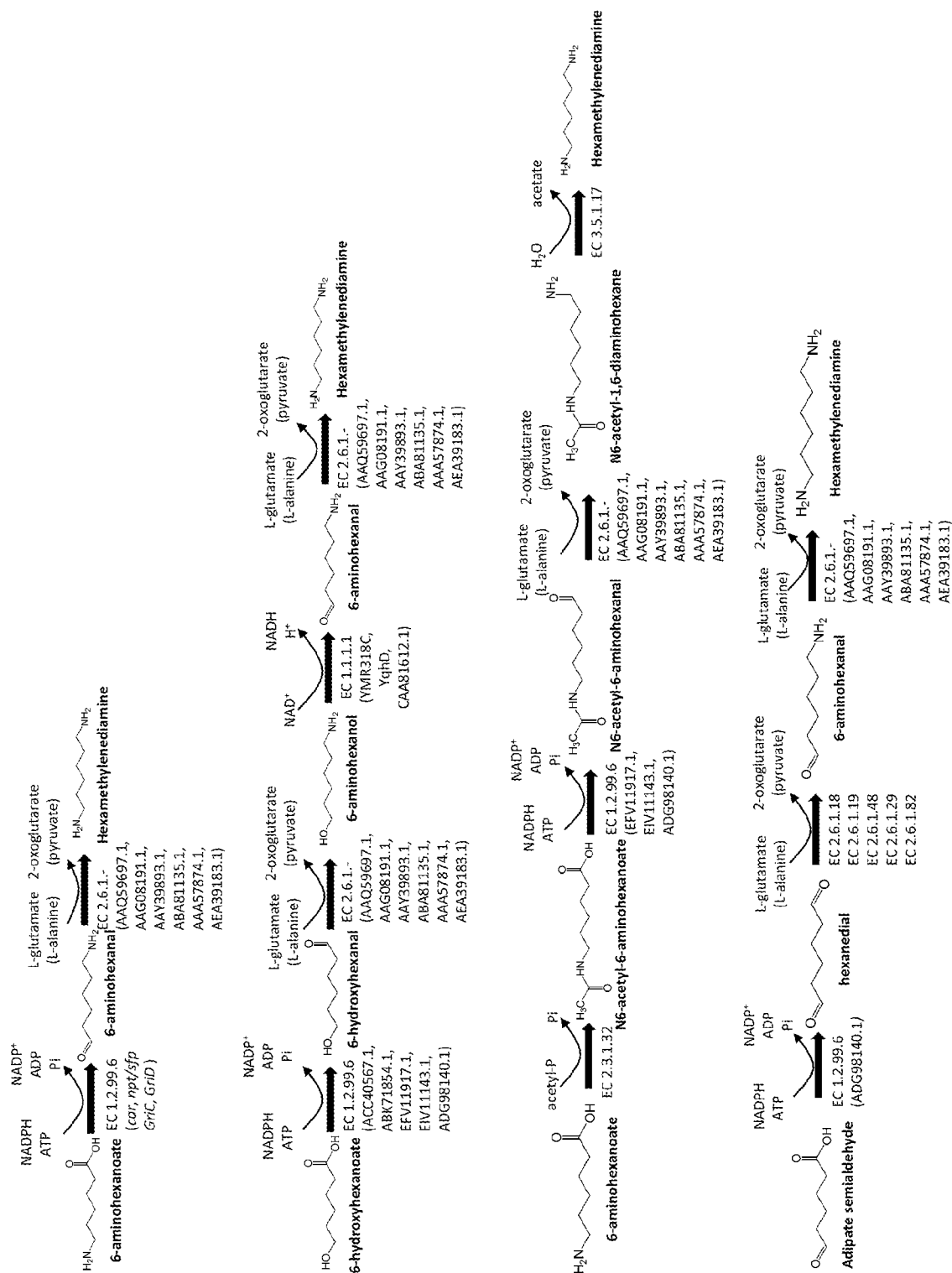


FIGURE 5

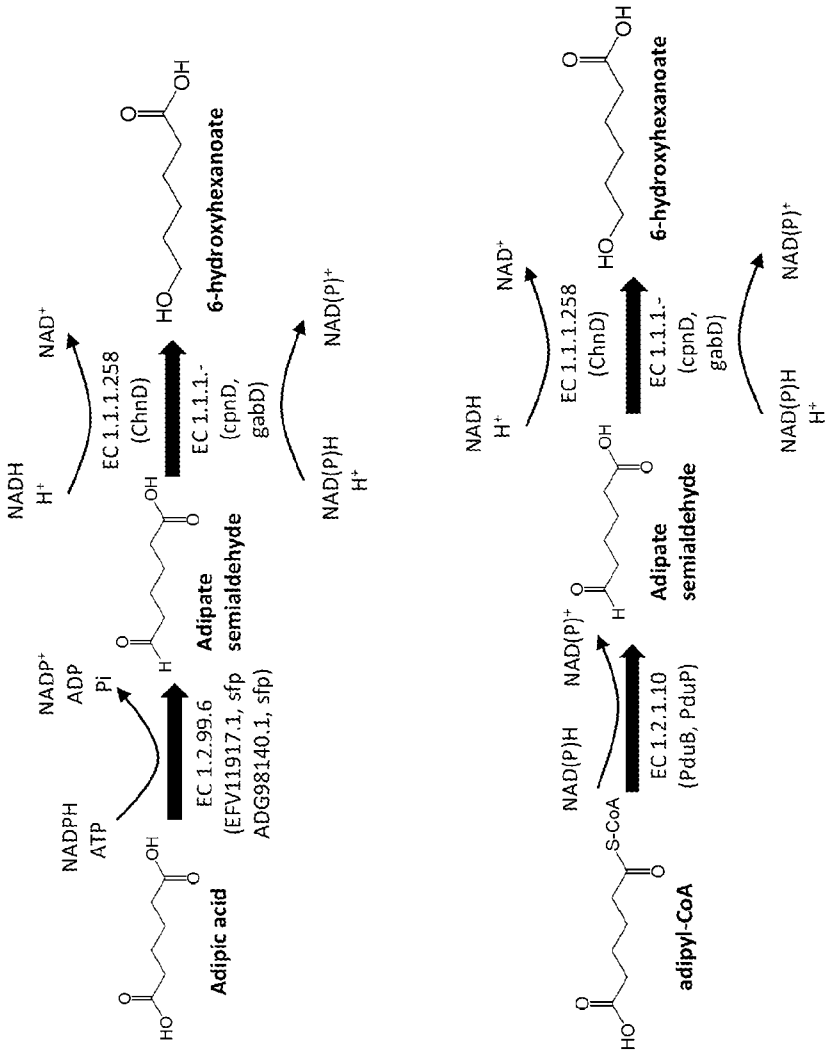
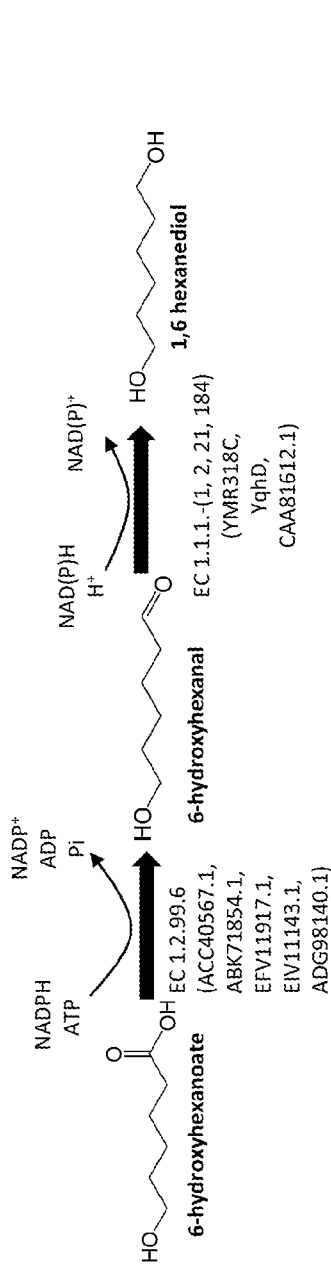


FIGURE 6



9/28

FIGURE 7

SEQ ID NO	Organism	GENBANK reference	Amino acid sequence
1	<i>Escherichia coli</i>	AAA24665.1	MSQALKNLLTLLNLEKIEGLFRGQSEDLGIRQVFGQVVGQALYAAKETVPEERLVHSF HSYFLRPGDSKPIIYDVETLRDGNFSARRVAAIQNGKPIFYMITASFQAPAEAGFEHQKT MPSAPADGSLPSETQIAQSLAHLPPVLKDKFCRPLEVRPVEFHNPILKGHVAEPHRQV WIRANGSVDDLRVHQYLLGYASDLNLFVPALQPHGIGLEPGIQIATIDHSMWFFHRPFN LNEWLLYSVESTSASSARGFVRGEFTQDGVLVASTVQEGVMRNHN
2	<i>Escherichia coli</i>	AAB60068.1	MSTTHNVPPQGDVLRLTLAMPADTNANGDIFGGWLMQSDIGGAILAKEIAHGRVTVRVVE GMTFLRPVAVGDVVCCYARCVQKGTTSVSINIEVWVKVASEPIGORYKATEALFKYVAV DPEGKPRALPVE
3	<i>Mycobacterium marinum</i>	ACC40567.1	MSPTREERLERRIQDLYANDPQFAAKPATATAIAIERPGLPLPQIETVMTGYADRP LAQRSEFVTDAGTGHTTILRLPHFETISYGEIWDRIALADVLSTEQTKPGDRVCLLG FNSVDYATIDMTLARLGAVAVPLQTSAAITQLQPIVAETQPTMIAASVDALADATELALS GQTATRVLVFDHHRQVDAHRAAVESARERLAGSAVETLAEAIARGDVPRGASAGSAPGT DVSDDSLALIYTSGSTGAPKGAMYPRRNVAATFWRKRTWFEFGYEPSITLNFMPMSHVMG RQILYGTLCNGGTAVFVAKSDLSLTFEDLALVRPTELTFRVWDMVFDEFQSEVDRRLV DGADRVALEAQVKAERNDVLGGRYTSALTGSAPISDEMKAWVEELDMHLVEGYGSTE GMILIDGAIRRPVAVLDYKLVDPDLGYFLTRPHRPGELLVKTDLSLFPGYQRAEVTADV FDADGFYRTGDIMAEVGPQFVYLDRRNNVLKLSQGEFTVSKLEAVFGDSPLVRQYIY GNSARAYLLAVVPTQEALDAVPEELKARLGDLSQEVAKAAGLQSYEIPRDFIETTPW TLENGLLTGIRKLARPQLKKHYGELLEQIYTDLAHGQADELSLRQSGADAPVLVTVCR AAALLGGSASDVQPDHFTDLGGDSLALSFTNLLHEIFDIEVPVGVIVSPANDLQALAD YVEAARKPGSSRPTFASVHGASNGQVTEVHAGDLSLDKFIDAATLAEAPRLPAANTQVRT VLLTGATGFLGRYLALEWLERMDLVGKLCIVRAKSDTEARARLKTDFDSDGPELLAHY RALAGDHLEVLAGDKGEADLGLDRQTWQRLADTVLDVDPAAALVNHVLPYSQLFGPNALG TAEELLRLALTSKIKPYSYSTIGVADQIPPSAFTEDADIRVISATRAVDDSYANGYSNSK WAGEVLLREAHDLCLGLPVAVFRCDMILADTTWAGQLNVPMFTRMILSLAATGIAPGSFY ELAADGARQRAHYDGLPVEFIAEAI STLGAQSQDGFHTYHVMNPYDDGIGLDEFVDWLINE SGCPIQRIADYGDWILQRFETALRALPDQRHSSLLPLLLHNVRQPERPVRGSIAPTRDFRA AVQEA KIGPKDIPHV GAPIV KYVS DLRLLGLL

10/28

SEQ. ID NO	Organism	GENBANK reference	Amino acid sequence
4	<i>Mycobacterium smegmatis</i>	ABK71854.1	MTSDVHDATDGVTTETALDDEQSTRRIAEIYATDPEFAAAAPLPAVVDAAHKPGRLAEIL QTLFTGYGDRPALGYRARELATDEGGRTVTRLLPRFDLTLYAQVWSRVQAVAAALRHNFA QPIVPGDAVATIGFASPDYLTLDLCAYILGLSVPLQHNAAPVSRILAEVEPRILTVS AEYLDLVESVRDVSNSQLVFDHHPVEDDHRDALARAREQLAGKGIATTTLDIADEG AGLPAEPIYTAHDQORLAMILYTSGSTGAPKGAMYTEAMVARLWTMSFITGDPPTVINVN FMPLNHLGGRIPISTAVQNGGTSYFVPESDMSTLFDLALVRPTELGLVPRVADMILYQHH LATVDRLVTQGADELTAEQAGAEELREQVLGGRVITGFVSTAPLAAEMRAFLDITLGAHI VDGYGLTETGAVTRDGVIVRPPVIDYKLIIDVPELGVFSTDKPYPRGELLVRSQTLTPGY KRPEVTASVFDNRDGYHTGDVMAETAPDHLVVDNRNVLKLAQGEFVAVANLEAVFSGA ALVRQIFVYGNSESFLLAVVPTPEALEQYDPAALKAALADSLQRTARDAELQSYEVPA DFIVETEPFSAANGLLSGVGKLLRPNLKDRYGQRLEQMYADIAATQANQLRELRAAATQ PVIDTLTQAAATILGTGSEVASDAHFTDLGGDSLSALTLSNLLSDFGFEVPVGTIVNPA TNLAQLAQHIEAQRTAGDRPSFTTVHGADATEIRASELTLDKFIDAEITLRAAPGLPKVT TEPRTLSSGANGWLGKFLTLQWLERLAPVGGTLITIVRGDDAAARALTQAYDTPDEL SRFAELADRLRHLRVAGDIGDPNLGLTPEIWHRLAAEVDLWHPAALVNHVLPYRQLFGP NVVGTAEVIKALTERIKPVTYLSTVSAMGIPDFEEDGDIRTVSPVRPLDGGYANGYGN SKWAGEVLLREAHDLGCLPVATFSDMILAHPRYRGQVNVPMFTRLLSLITGVAPRS FYIGDGERPRAHYPGLTVDFVAEAVTTLGAQQREGVYSDVMNPHDDGISLDVFVDWLIR AGHPIDRVDDYDDWVRRFETALTALPEKRRRAQTVLPILLHAFRAPOAPLRGAPEPEVFHA AVRTAKVGPGDIPHLDEALIDKYIRDREFGLI

11/28

SEQ ID NO	Organism	GENBANK reference	Amino acid sequence
5	<i>Segniliparus rugosus</i>	EFV11917.1	MGDGEERAKRFFQRIGELSATDPQFAAAAPDPVAVVEASDPSSLFTRYLDLTMRGYAERP ALAHRVGAGYETISYGELWARVGAIAAAWQADGLAPGDFVATVGFTSPDVAVVDLAAARS GLVSPLQAGASLAQLVGILEETEPKVLAASASSLEGAVACALAAPSVQRLLVFDLRGPD ASESAADERRGALADAEQEQLARAGRVVWETLADLAARGEALPEAPLFEPAEGEDPLALL IYTSGSTGAPKGAMYSQRLVSQLWGRTPVPGMPNISLHYMPLSHSYGRAVLAGALSAGG TAHFTANSDSLTLFEDIALARPTFLALVPRVCEMLFQESQRGQDVAELRERVLGGRLLVA VCGSAPLSPEMRAFMEEVLGFPLLDGYGSTEALGYMRNGIIQRPPVIDYKLVDPVPELGYR TTDKPYPRGELCIRSTSLISGYKRPETAEVFDAGQYKTDGMVAE/APDHLVVDRSK NVLKLSSQGEFVAVAKLEAAYGTSPPVKQIFVYGNRSERFLLAVVPNAEVLGARDQEEAK PLIAASLQKIAKEAGLSYEVPRDFLIETEPFTTQNGLLSEVGKLLRPKLKARYGEALEA RYDEIAHGQADELRALRDGAGQRPVTVVRAAVAISGSEGAEVGPEANFADLGDSLSA LSLANLLHDVFEVEVPVRIIHGPTASLAGIAKHIEAERAGASAPTAASVHGAGATRIRAS ELTLEKFLPEDLLAAAKGLPAADQVRTVLLTGANGWLGRFLALEQLERLARSGQDGGKLI CLVRGKDAAAARRRIEETLGTDPALAARFAELAEGRLEVPGDVGEPKFGLDDAAWDRLA EEVDVIVHPAALVNHVLPYHQLFGPNVWGTAIEIRLAIATAKRKPVITYLSTVAVAAAGVEPS SFEEGDIRAVVPERPLGDGYANGYGNKSWAGEVLLREAHVGLPVAVFRSDMILAHTR YTGQLNVDPDQFTRLVLSLATGIAPKSFYQQGAAGERQRAHYDGPVDFTEAETTLGAE PSWFDDGGAGFRSDFVFNPHHDGVGLDFVDWVLEAGHPISRIDDHKEWFARFETAVRGLP EAQRQHSLLPLLRAYSFPHPPVDGSSVPTGKFGQAVKAAQVGSDDHDPVHLGKALIVKYAD DLKALGILL

12/28

SEQ ID NO	Organism	GENBANK reference	Amino acid sequence
6	<i>Mycobacterium massiliense</i>	E1V11143.1	MTNETNPQEQLSRRISLRESDPQFRAAQDPVAEQVLRPGHLHSEAIAALMTGYAER PALGERARELVIDQDGRITLRLPRFDTTYYGELWSRTTSVAAAHHHDATHPVKAGDLVA TLGFTSIDYTVLDLAIMILGGVAVPLQTSAPASQWTTILAEAPNTILAVSIELIGAAMES VRATPSIKQVVVFDTPEVDDQREAFEAASQTLAGTGIALETLDVIAARGAALPAAPLYA PSAGDDPLALIYTSGSTGAPKGAMHSENIIVRRWWIREDDVMAGTENLPMIGLNFMPMSHI MGRGTLTSTLSTGGTGYFAASSDMSTLFEDMELIRPTALALVPRVCDMVQRFQTEVDRR LASGDTASAEAAEVAEKADIRDNLFGGGRVSAVMVGSAPLSEELGEFIESCFELNLTGGYG STEAGMVFRRGIVQRPVIDYKLVDPVPELGYFSTDKPHPRGELLKTDGMFLGYKRPEV TASVFDADGFMGTGDIVAEIAHDNIEIIDRRNNVLKLSQGEFVAVATLEAEYANSPVVHQ IYVYGSSERSYLLAVVPTPEAVAAAKGDAALKTTIADSLQDIAKEIQLQSYEVPRDFI IEPQPFTQGNGLTGIAKLARPNIKAHYGPRLEQMYAEIEQQAELRALHGVDPDKPAL ETVLKAAQALLGVSSAEAAADAHFTDLGGDSLALSFSDLIRDFAVEVPVGVVSAAND LGGVAKFVDEQRHSGGTRPTAETVHGAGHTEIRAADLTLDKFEIDEATLHAAPSLPKAAGI PHTVLLTGSNGYLGHYLALEWLERIDKTDGKLIVIRGKNAAEAYGRLEEFADTGDTELL AHFRSLADKHLEVLGADIGDPNLGLDADTWQRLADTVDVIVHPAALVNHVLPYNQLFGPN WGTAEIKLAITTKIKPVTYLSTVAVAADVPTTTFDEESDIRLISAVRPIDDGYANGYG NAKWAGEVLLREAHDLCLGLPVAVFRSDMILAHISRYTGQLNVDPQFTRLILSLIATGIAPG SFYQAQTTGERPLAHYDGLPGDFTAEAITTLGTQVPEGSEGFVYDCVNPHADGISLDNF VDWUEAGYPPIARIDNVTWFTFRDFAIRGLSEKQKQHSLLPLLHAFEQPSAAENHGVVP AKRFQHAVQAAAGIPVGGQDGTIDPHLSRRLIVKYAKOLEQLGLL

SEQ ID NO	Organism	GENBANK reference	Amino acid sequence
7	<i>Segniliparus rotundus</i>	ADG98140.1	MTQSHQTGGQASAAHSRLARRAAELLATDPQAAATLPDPEVVRQATRPGLRLAERVDAIL SGYADRPALGQRSFQTVKDPITGRSSVELLPFTDTITYRELRRERATAIASDLAHHPQAPA KPGDFLASIGFISVDYVAIDVAGVFAGLTAVPLQTGATLATLTAITAETAPTLLFAASIEH LPTAVDAVLATPSVRRLLVFDYRAGSDEDEAVEAAKKRIADAGSSVLVDVDEVARGK SAPKAPLPATDAGDSSLSLIYTSGSTGTPKGMYPERNVAHFVGGVWAAAFDEDAAPP VPAINITFLPSHVASRLSLMPTLARGGLMHFVAKSDLSLTFEDLKLARPTNLFVPRVV EMLYQHYQSELDRRGVQDGTREAEAVKDDLRITGLLGRILTAGFGSAPLSAELAGFIESL LQIHLVDGYGSTEAGPVWRDGYLVKPPVTDYKLIIDVPELGYFSTDSPPHPRGELAICTQTI LPGYKRPETTAEVEFDEDEGFYLTGDVVAQIGPEQFAYVDRRKNVLLKSQGEFVTLAKLEA AYSSPLVRQLFVYSSERSYLLAVVPTPDALKKFGVGEAAKAAIGESLQKIARDEGLQ SYEVPDRFIETDPTFVENGLSDARKSLRPKLKEHYGERLEAMYKELADGQANELRDIR RGVQQRPTLETVRRAAAAAMLGASAAEIKPDHFTDLGGDSLSALTFSNFLHDLFEVDVPV GVVSAANTLGSVAEHIDAQLAGGRARPTFATVHGKGSTTIKASDLTLDKFIDEQTEAA KHLPKPADPPRTVLLTGANGWLGRFLALEWLERLAPAGGKLTIVRGKDAQAQAKARLDA YESGPKLAGHYQDLAAATLLEVLADGDFSEPRGLDEATWNRLEADVDFFSHPGALVNHVL PYNQLGPNVAGVAEIIKAITTRIKPVTYLTSTVAAGAAGVPEPSALDEDEDGDIRTVSAERSV DEGYANGYNSKWGGEVLLREAHDRGTGLPVRFRSDMILAHQKYTGQV/NATDQFTRLVQS LLATGLAPKSYELDAQNRQRAHYDGPVDFTAESITTLGGDGLGEGYRSVNFNPHRDG VGLDEEVDWLEAGHPITRIDDYDQWLSRFETSLRGLPESKRQASVLPLLHAFARPGPAV DGSFERNVFTDQKAKAGAEHDIPHILGKALVLYADDIKQLGLL
8	<i>Chromobacterium violaceum</i>	AAQ59697.1	MQKQRTTSQWRELDAAHHLHPFTDTASLNQAGARVMTREGVYVLDSEGNKIIDGMAGLW CVNVGYGRKDFAEAAARRQMEELPFYNTFFKTTTHPAVVVELSLLAEVTPAGFDRVFTNSG SESVDTMIRMVRRYWDVQKPEKKTIGRWNGYHGSTIGGASLGGMKYMHFQGDLPPIGM AHIEQPWWYKHGKDMTPDEFGVVAARWLEEKILEIGADKVAAFVGEPIQGAGGVVPPAT YWPEIERICRYDVLVLADEVICGFGRTGEWFGHQHFQPDFTAAKGLSSGYLPIGAV FVGKRVAEGLIAGGDFNHGFTSYGHPVCAAAHANVAALRDEGIVQRVKDDIGPYMQKRW RETFSREEHVDDVRGVMVQAFTLVKNKAKRELFPDFGEIGTLCRDIFFRNNLIMRACGD HIVSAPPLVMTAEVDEMLAVAEERCLFEFEQTLKARGLA
9	<i>Pseudomonas aeruginosa</i>	AAG08191.1	MNARLHATSPILGDADLVADQAHYMHGVHVFDDHVRVNGSLNIAAGDGAYYDTAGNRYLD AVGGMWCTNIGLGRREEMARTVAEQTRLLAYSNPFCOMANPRAIELCRKLAELAPGDLDHV FLTTGGSTAVDTRILMHYYQNCRGKRAKHVITRINAYHGSTGLMSLGGKSADRPAEF DFLDERIHHLACPYYYRAPEGLGEAEFDGLVDEFERKILELGADRVGAFISEPVFGSGG VIVPPAGYHRRMWELCQRYDVLVISDEVTSFGRGLGHFFASQAVFGVQPDHILTAKGLTS GYQPLGACIFSRRIWEVIAEPDKGRCSHGFTSYGHPVACAAAKNIEIEREGLLAHAD EVGRYFEERLQSLRDLPIVGDVRGMRFMACVEFVADKASKALFPESLNIGEWVHLRAQKR GLLVRPVHNLNVMSPPILLTREQVDTVVRVLRRESIETVEDLVRAGHR

SEQ ID NO	Organism	GENBANK reference	Amino acid sequence
10	<i>Pseudomonas syringae</i>	AA39893.1	MSANNPQTLEWQALSEHHLAPFSDYKQLKEGPRITRAEGVYLWDSEGNKILDGMISGL WCVAIGYGREELADAASKQMRLEPPYNNLFQTAHPVLELAKAISDAIEPGMNHVFTGS GSEGNMTLMRVRYWALKGQPNKKTISRNVGYHGSTVAGASLGMVYMHQEGDLPIPG VHIPQPYWFGEGDMTPDEFIWAEEQLEKKILEGVENVGAGFAIEPIQAGAGGVVPPD SYWPKIEILSYDILFAADEVICGFGRTSEWFGSDFYGLRPDMMTIAKGLTSGVYPMGG LIVREIVAVLNEGDFNHGFTYSGHPVAAAVALENIRILREEKIVRSETAPYLQKR LRELSHPLVGEVRGVLGALVGLKKTTRERTYDKGAGMICRTFCFNDGLIMRAVGDT MIIAPPLVISAQIDELVEKARTCLDLTLAVLQG
11	<i>Rhodobacter sphaeroides</i>	ABA81135.1	MTRNDATNAAGAVGAAMRDHILLPAQEMAKLKGSAQPVLTAEIGYVHFTEDGRRLLDGPA GMWCAQGYGRREIVDAMAHQAMVLPYASPWYMATSPAARLAEKIATLTGDLNRIFFT GGSTAVDSALRFSEFYNNVLRPQKKRIIVRYDGYHGSTALTAACTGRTGNWPNFDIAQD RISFLSPNPRHAGNRSQEAFLDLVQEFEDRIESLGPDTIAAFLAEPILASGGVLIIPPA GYHAREKAICEKHDIYISDEVVTGFGRCGEWFASEKVFGVVPDIITFAKGVTSGYVPLG GLAISEAVLARISGENAKGSWFTNGYTSNQPVACAAALANIELMEREGIVDQAREMADY FAALASLRDLPGVAETRSVGLVGCVCQLDPTRADGTAEDKAFTLKIDERCFCFELGLVR PLGDLCSIPPLIISRAQIDEMVAIMRQAITEVSAAHGLTAKEPAAV
12	<i>Escherichia coli</i>	AAA57874.1	MNRLPSASALACSAHALNLEKRTLDHEEMKALNREVIEWFKEHVNPGFLEYRKSVTAG GDYGAVEWQAGSLNTLVDTOGGEFIDCLGGGFINVGHNPVVSVAQNQLAKQLPHSQE LLDPLRAMLAKTLAALTGPKLYSFFCNGSTESVEALKAKAYQSPRGKFTFIATSGAF HGKSLGALSATAKSTFRKPFMPLLPGRHV/PFGNIEAMRTALNECKTGGDDVAIVLEPI QGEQGVILPPGYLTAVRKLCDEFALMILDEVQTMGRTGKMFACEHENVQPDILCLAK ALGGVMPIGATIAETEEVSVLFDNPFLHTTTGGNPLACAAALATINVLLEQLNPAQAE QKGMILLDGRQLAREYDVLVQEARKGKMLMAIEFVDNEIGYNFASEMFRQRLVAGTLN NAKTRIEPPLTLTIEQCELVKAARKAALAMRVSEEA
13	<i>Vibrio fluvialis</i>	AEA39183.1	MNKPQSWEARAETSLYGFDTMPSLHQRTGVVVTGHGEPYIVDVNGRRYLDANSGLWNMV AGFDHKLGLIDAAKAQYERFPGYHAFGRMSDQTVMLSEKLVESPFDSGRVFTNSGSEA NDTMVKMLWFLHAAEGKPKRILTRWNAYHGVTAVSASMTGKPYNSVFGLP/LPGFVHLT CPHYWRYGEEGETEEQFVARLARELEETIQREGADTIAGFFAEPVMGAGGVIPPAKGYFQ AILPLIRKYDIPVISDEVICGFGRTGNTWGCVTYDFTPDALISSKNLTAGFFPMGAVILG PELSKRLTAIEAIEEFPHGFTASGHPV/GCAIALKAIDVVMNEGLAENVRRLAPRFEERL KHIAERPNIYEGYRIGFMWALEAVKDKASKATPFDGNLSVSERIANTCTDLGLICRPLGQS VVLCPPELTAQMDIDEMFIDKLEKALDKVFAEVA
14	<i>Bacillus subtilis</i>	CAA44858.1	MKIYGYMDRPLSQEENEFMSFISPEKRCRRFYHKEDAHRTLLGDVLVRSVISRQYQ LDKSDIRFSTQYEGKPCIPDLPAHFNISHSGRWVICAQFSDQPIGIDIEKTKPISLEIAK RFFSKTEVSOLLAKDKDEQTDYFYHLWMSMKESF/KQEGKGLSLPLDSFSVRLHQDQGVSI ELPDSHSPCYIKTYEVDPGYKMAV/CAAHDPDFPEDITMVSVEELL

SEQ ID NO	Organism	GENBANK reference	Amino acid sequence
15	<i>Nocardia</i> sp. NRRL 5646	AB183656.1	MIETILPAGVESAEELLEYPEDLKAHPAEHEHIAKSVEKRRRDFIGARHCARLALAEIGEP PVAIGKGERGAPWPRGVVGSILTHCDGYRAAAVAHKMRFRSIGIDAEPHATLPEGVLDV SLPPERWLTDTDSALHLDRLFLCAKEATYKAWWPLTARWLGFEAAHITFEIEDGSADSG NGTFHSELLVPGQTNDGGTPLLSEFGRWLIADGGFILTAIAYA
16	<i>Bacillus cereus</i>	AAS43086.1	MINKTLQKRFRNVAAVSYDQYANVQKKMAHSLSTLNRRYSTNSSRILELGCCTGVVTE QLSNLFPKAAQJTAIDFAESMIAVAKTRQNNVNVTFYCEDIERLRLEETYDVVISNATFQW LNDLKQVITNLFRHLSIEGILLFTFGQETFQELHASFQRAKEEKNIQNQNETSIGQRFYSK NQLRHICEIETGDVHVSETCYIERFTEVREFLHSIRKVGATNSNEESYCQSPSLFRAMLRL IVERDFTGNEGIMATYHALFVHITKEGKR
17	<i>Escherichia coli</i>	AAC76437.1	MINNIWWQTKGQGNVHLVLLHGWGLNAEVVWRCIDEELSSHFTLHLVDLPFGGRSRGFGALS LADMAEAVLQQAPDKAIWLGWSLGGVLVASQIALTHPERVQALVTVASSPCFSARDEWPGL KPDVLAGFQQQLSDDFQRTVERFLALQTMGTETARQDARALKKTV/LALPMPEVDVINGGL EILKTVDLRQPLQNVSMPEFLRYGYLDGLVPRKVVPMLDKLWPHSESYIFAKAAHAPFIS HPAEFCHLLVALKQRV

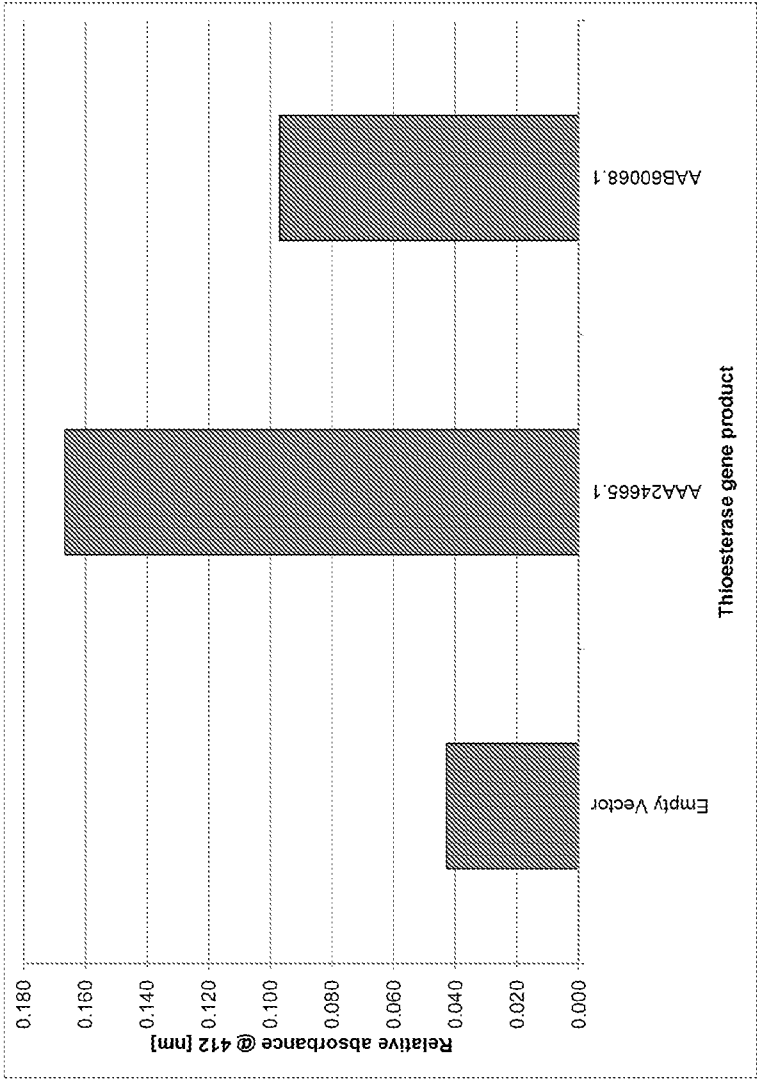


FIGURE 8

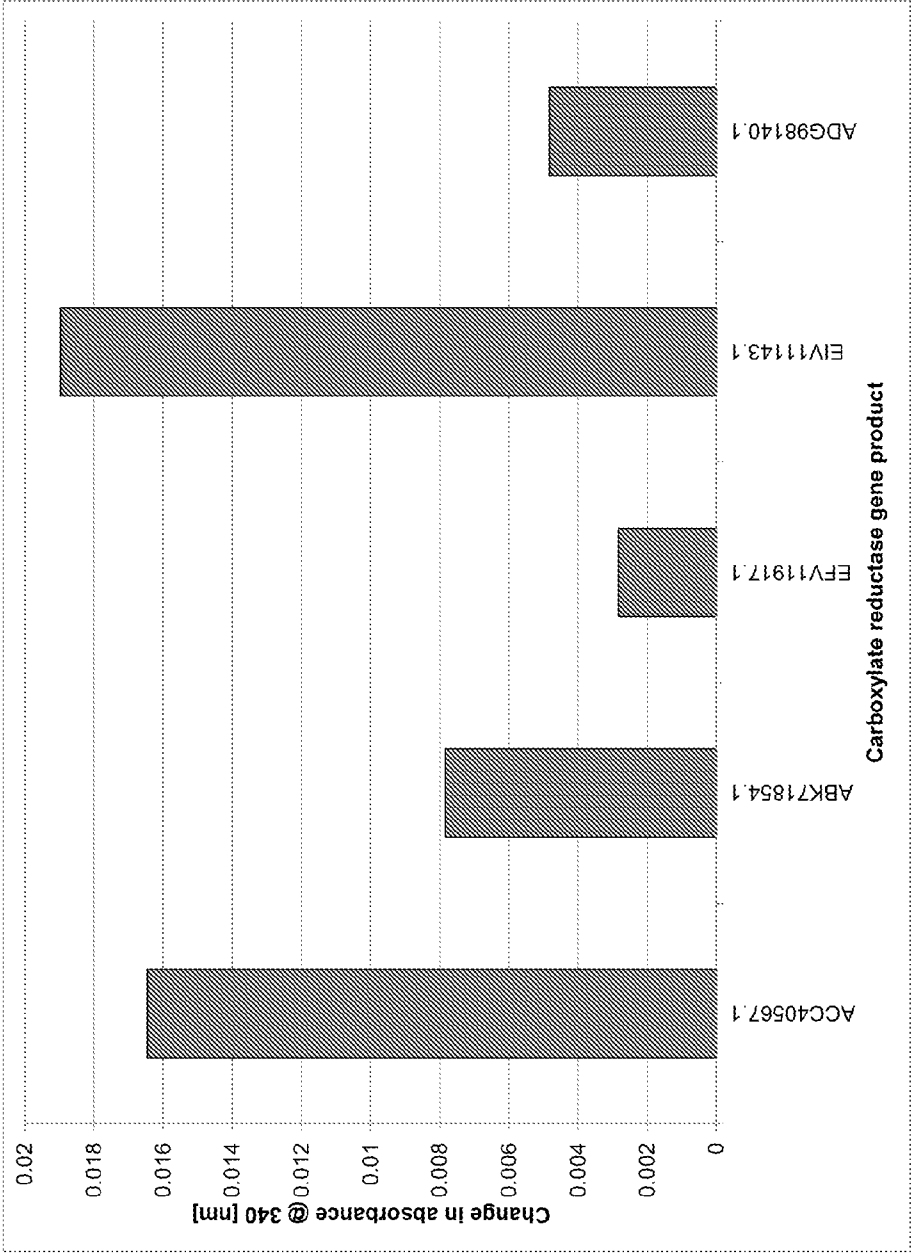


FIGURE 9

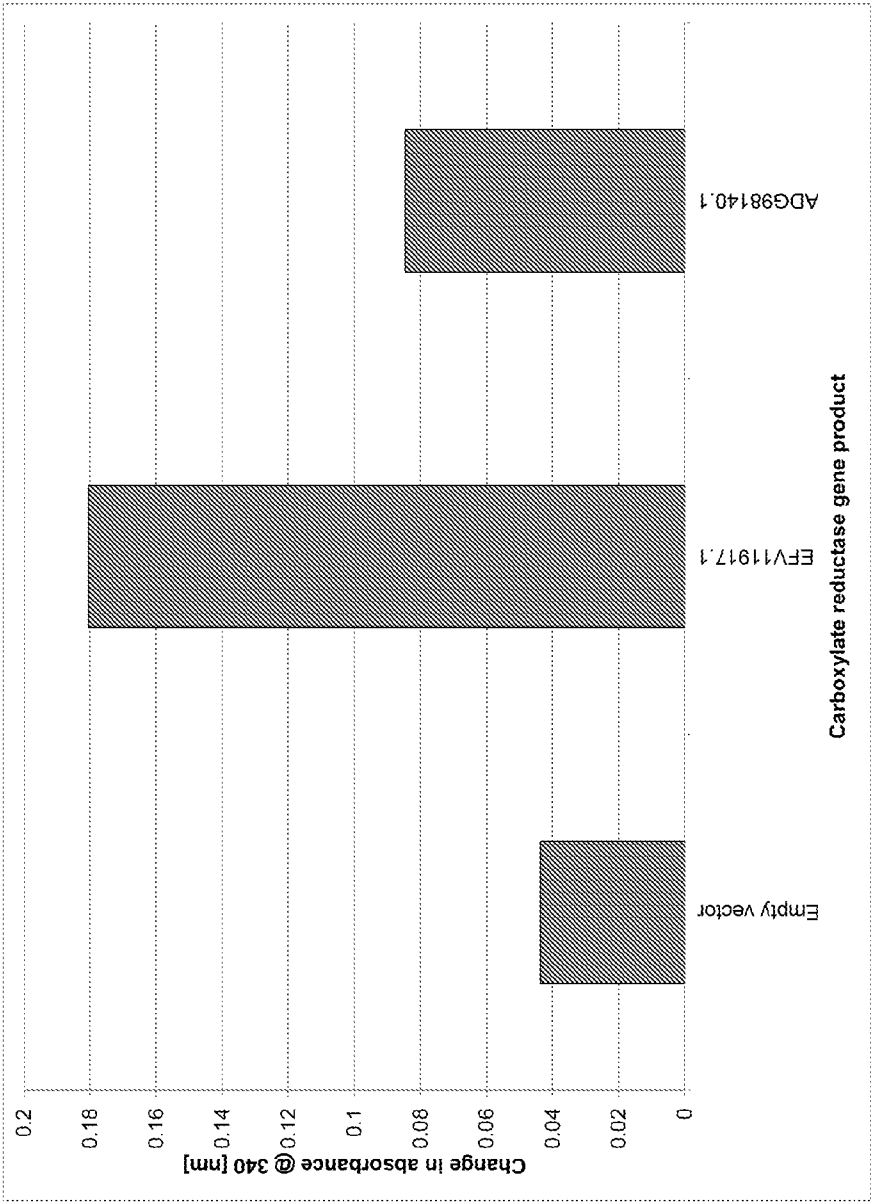


FIGURE 10

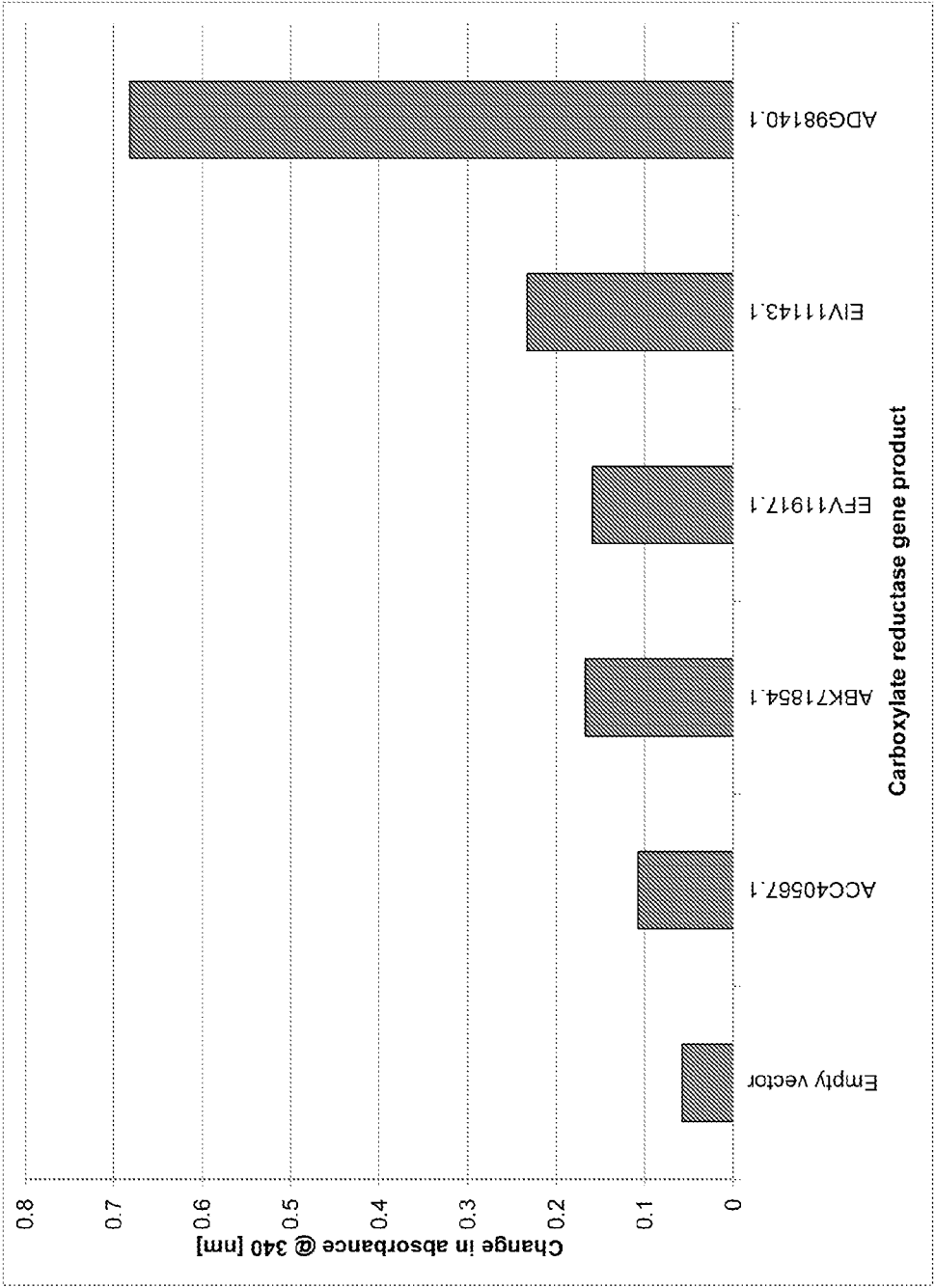


FIGURE 11

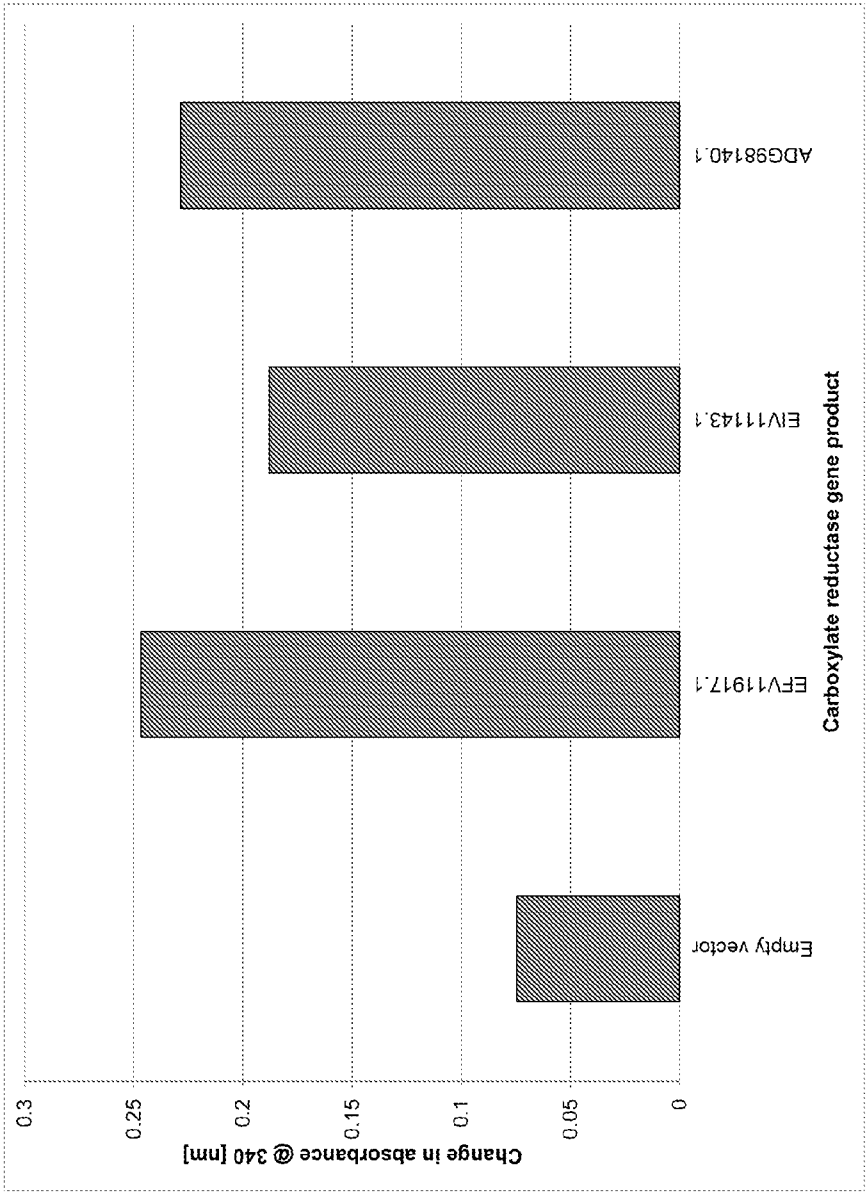


FIGURE 12

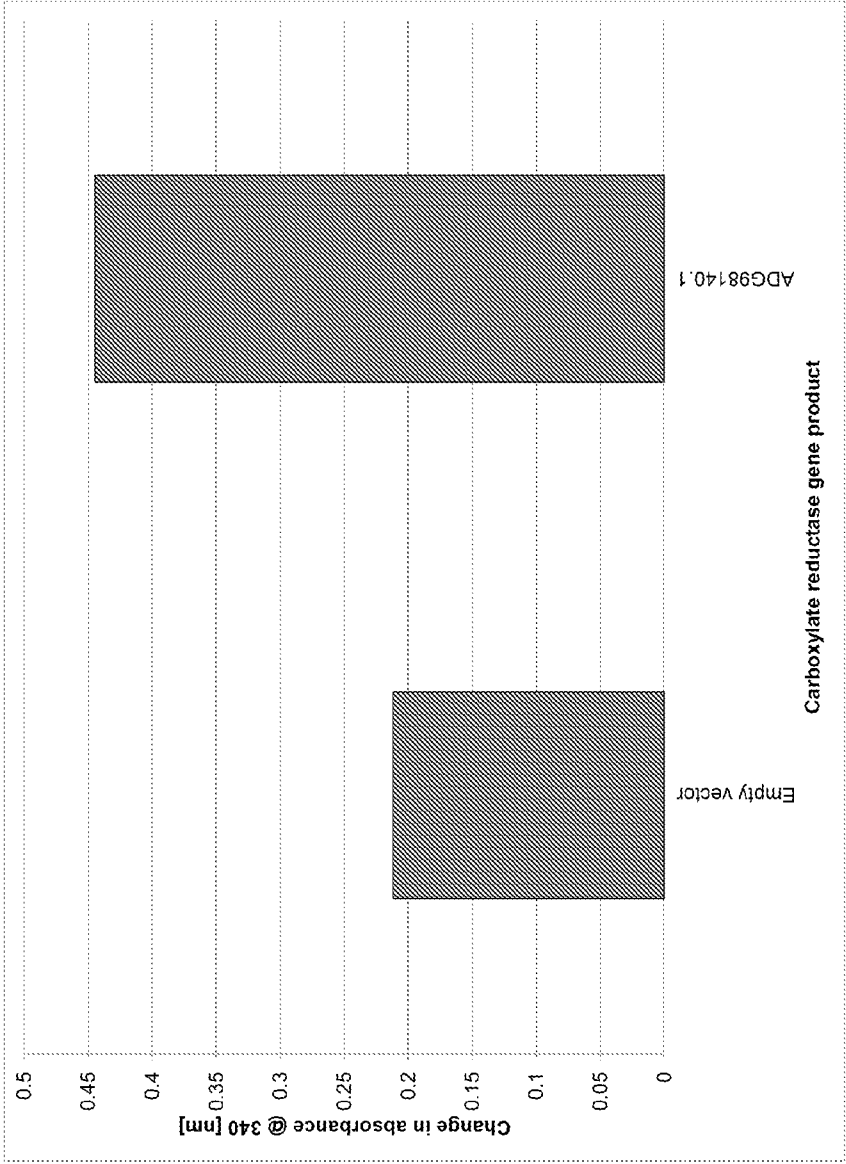


FIGURE 13

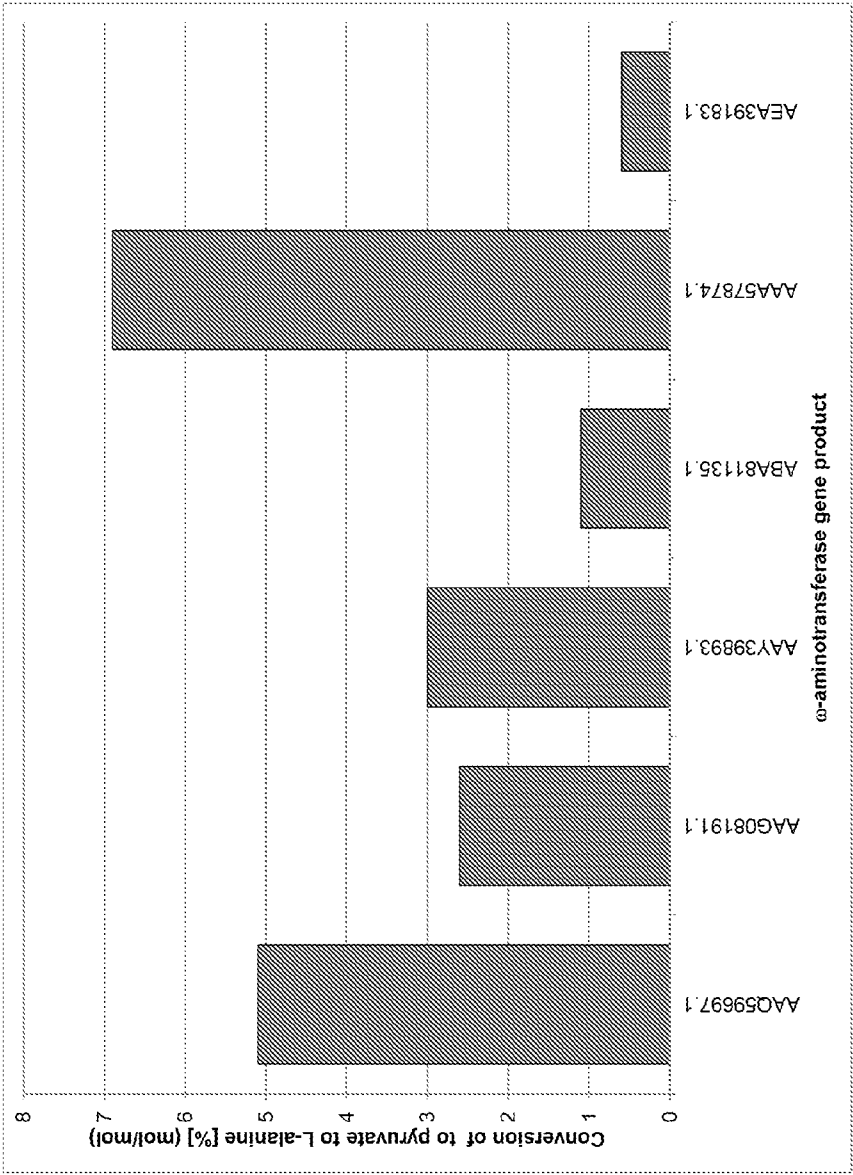


FIGURE 14

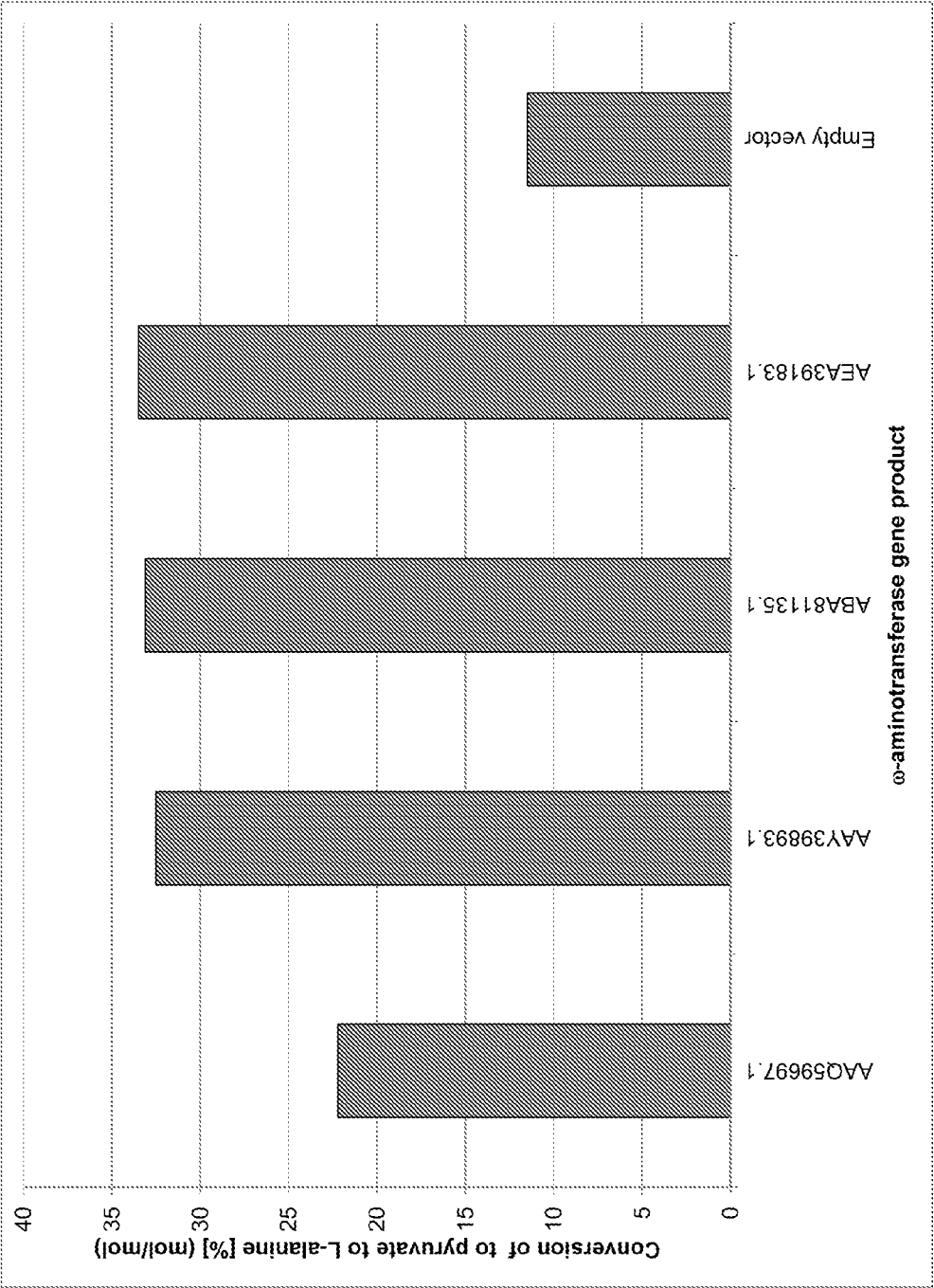


FIGURE 15

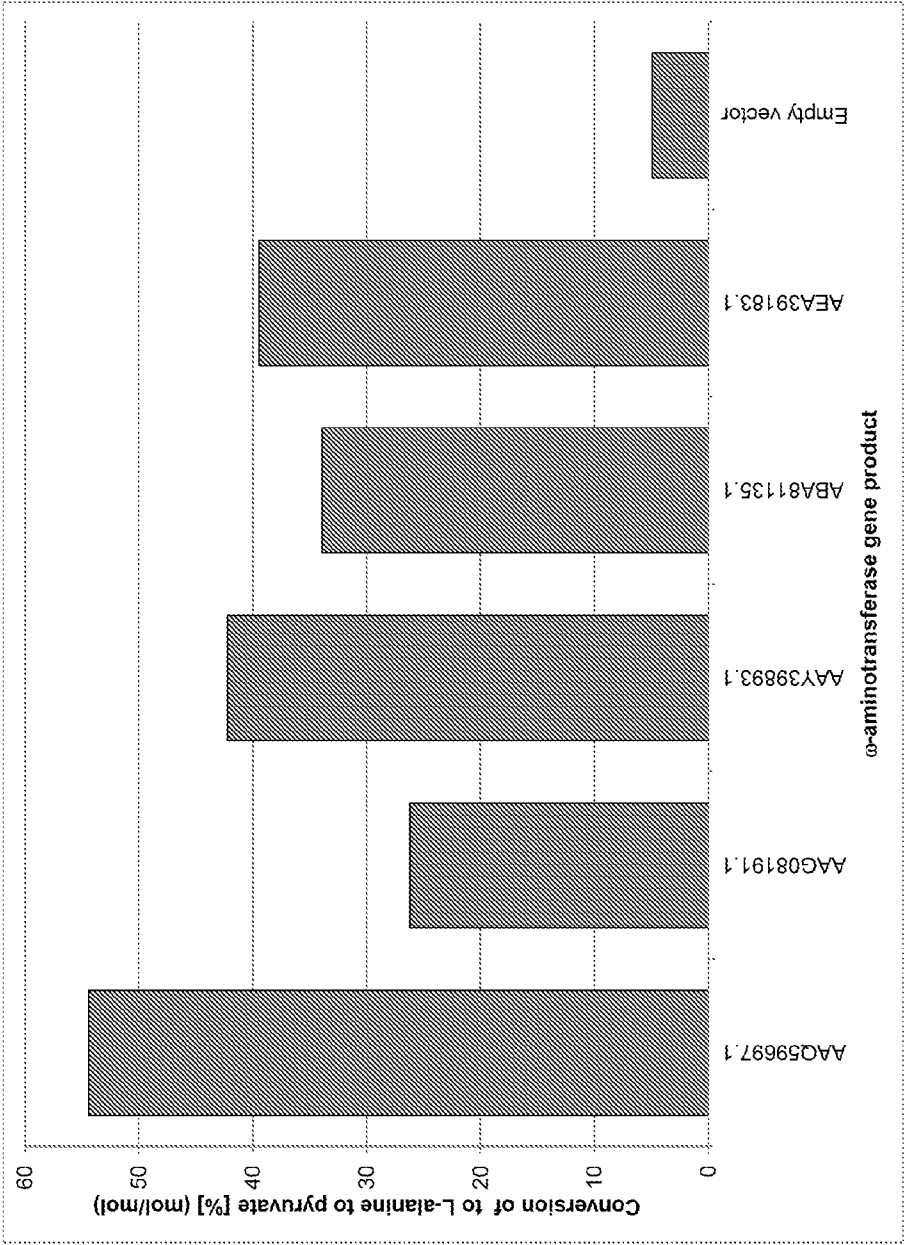


FIGURE 16

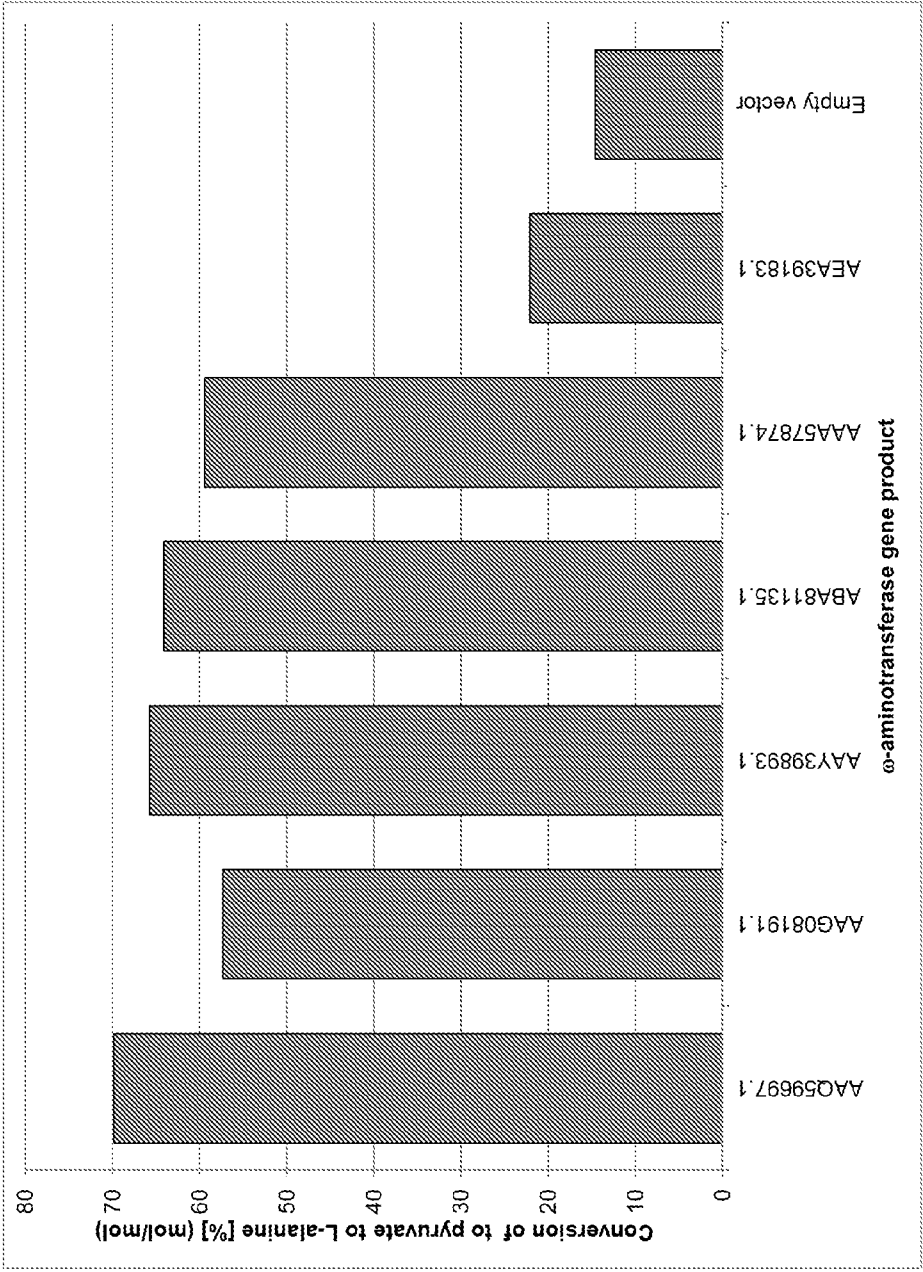


FIGURE 17

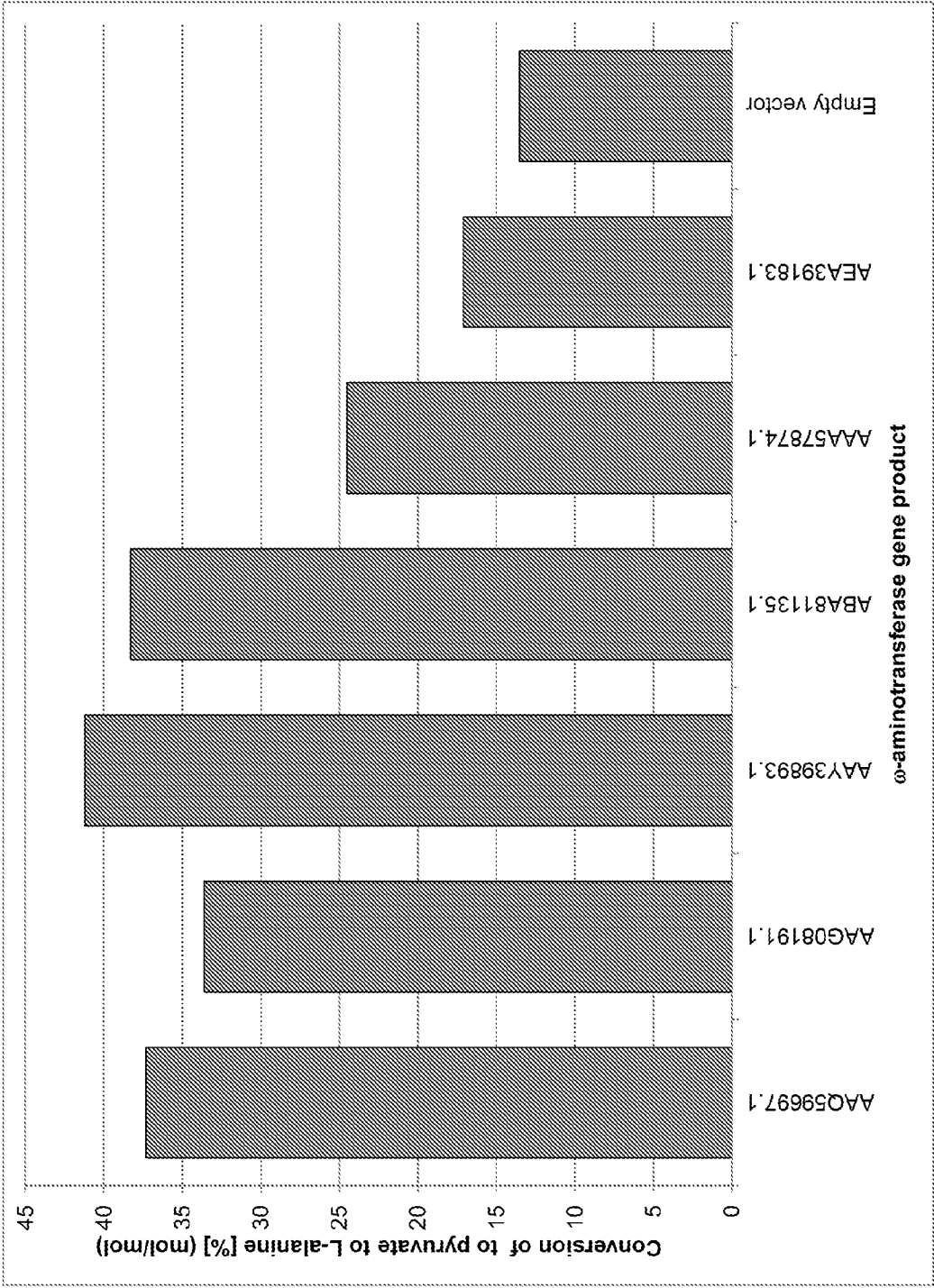


FIGURE 18

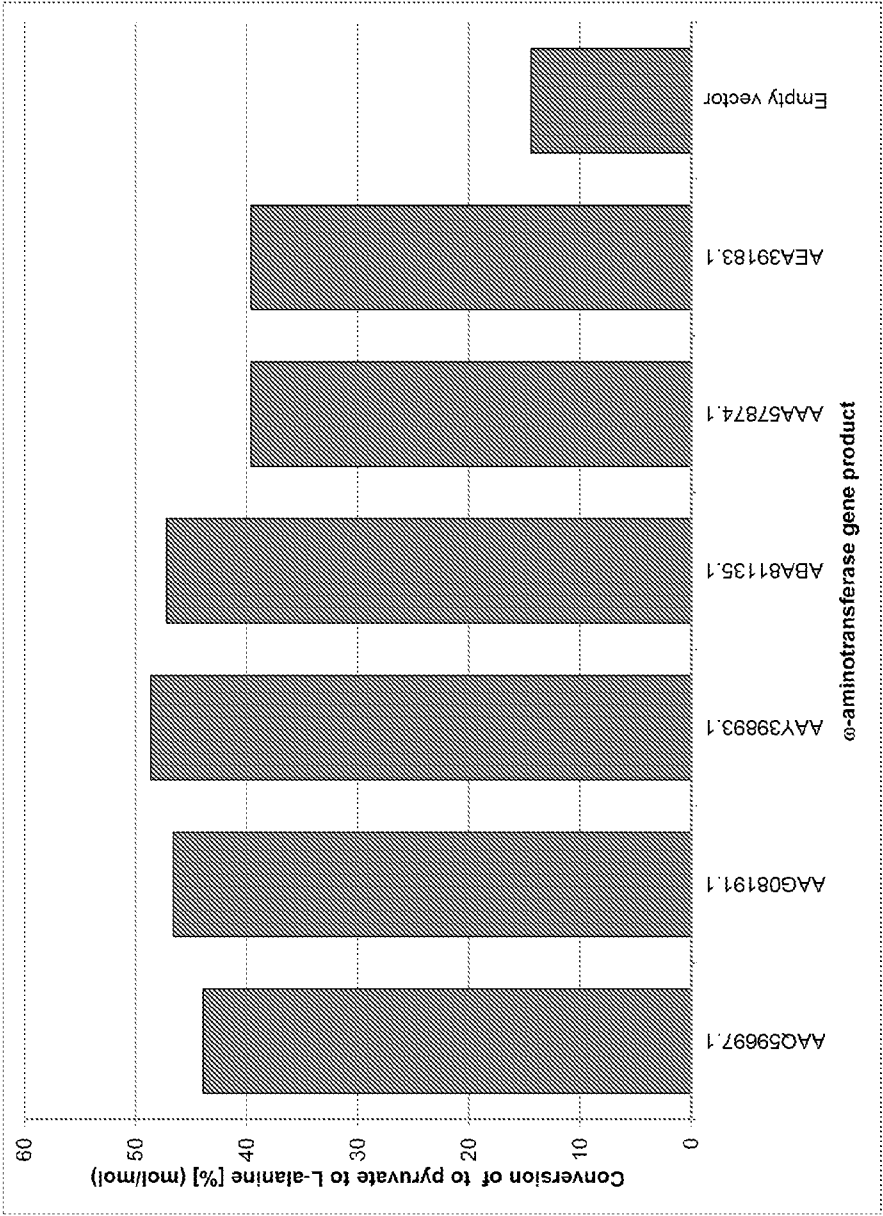


FIGURE 19

FIGURE 20

Sample ID	Analyte	Mwt [g/mol]	Peak Retention Time [min]	Peak Area @ 260nm [mAu]	Observed Mass (m/z)		Comments
					Negative mode (M-H)	Positive mode (M+H)	
Reference Standard	adipyl-CoA methyl ester	909	5.835	4755	908	910	
Biotransformation at 1 [h] time point #1	adipyl-CoA methyl ester	909	nd	nd	nd	nd	No substrate detected after 1 [h]
	adipyl-CoA	896	5.172	2023.3	894	896	
Biotransformation at 1 [h] time point #2	adipyl-CoA methyl ester	909	nd	nd	nd	nd	No substrate detected after 1 [h]
	adipyl-CoA	896	5.18	1499.7	894	896	
Biotransformation at 1 [h] time point #3	adipyl-CoA methyl ester	909	nd	nd	nd	nd	No substrate detected after 1 [h]
	adipyl-CoA	896	5.17	2095.4	894	896	
Substrate only control (no enzyme) at 1 [h]	adipyl-CoA methyl ester	909	5.893	1511.2	n/a	n/a	
time point	adipyl-CoA	896	5.214	22.3	894	896	Trace amount of product detected.