



US 20240026396A1

(19) **United States**

(12) **Patent Application Publication**

Buller et al.

(10) **Pub. No.: US 2024/0026396 A1**

(43) **Pub. Date: Jan. 25, 2024**

(54) **METHOD FOR PRODUCING TERTIARY BETA-HYDROXY-ALPHA-AMINO ACIDS**

Publication Classification

(71) Applicant: **Wisconsin Alumni Research Foundation**, Madison, WI (US)

(51) **Int. Cl.**
C12P 13/06 (2006.01)
C12P 13/08 (2006.01)

(72) Inventors: **Andrew Buller**, Madison, WI (US);
Tyler Doyon, Eau Claire, WI (US);
Samantha Bruffy, Madison, WI (US);
Anthony Meza, Madison, WI (US)

(52) **U.S. Cl.**
CPC *C12P 13/06* (2013.01); *C12P 13/08* (2013.01)

(73) Assignee: **Wisconsin Alumni Research Foundation**, Madison, WI (US)

(57) **ABSTRACT**

(21) Appl. No.: **18/351,559**

(22) Filed: **Jul. 13, 2023**

Related U.S. Application Data

(60) Provisional application No. 63/388,722, filed on Jul. 13, 2022.

A method to make tertiary β -hydroxy α -amino acids. The method includes reacting a ketone substrate and a primary or secondary β -hydroxy α -amino acid with an L-threonine aldolase and/or an L-threonine transaldolase for a time, at a temperature, and at a pH wherein the reaction yields a tertiary β -hydroxy α -amino acid product.

Specification includes a Sequence Listing.

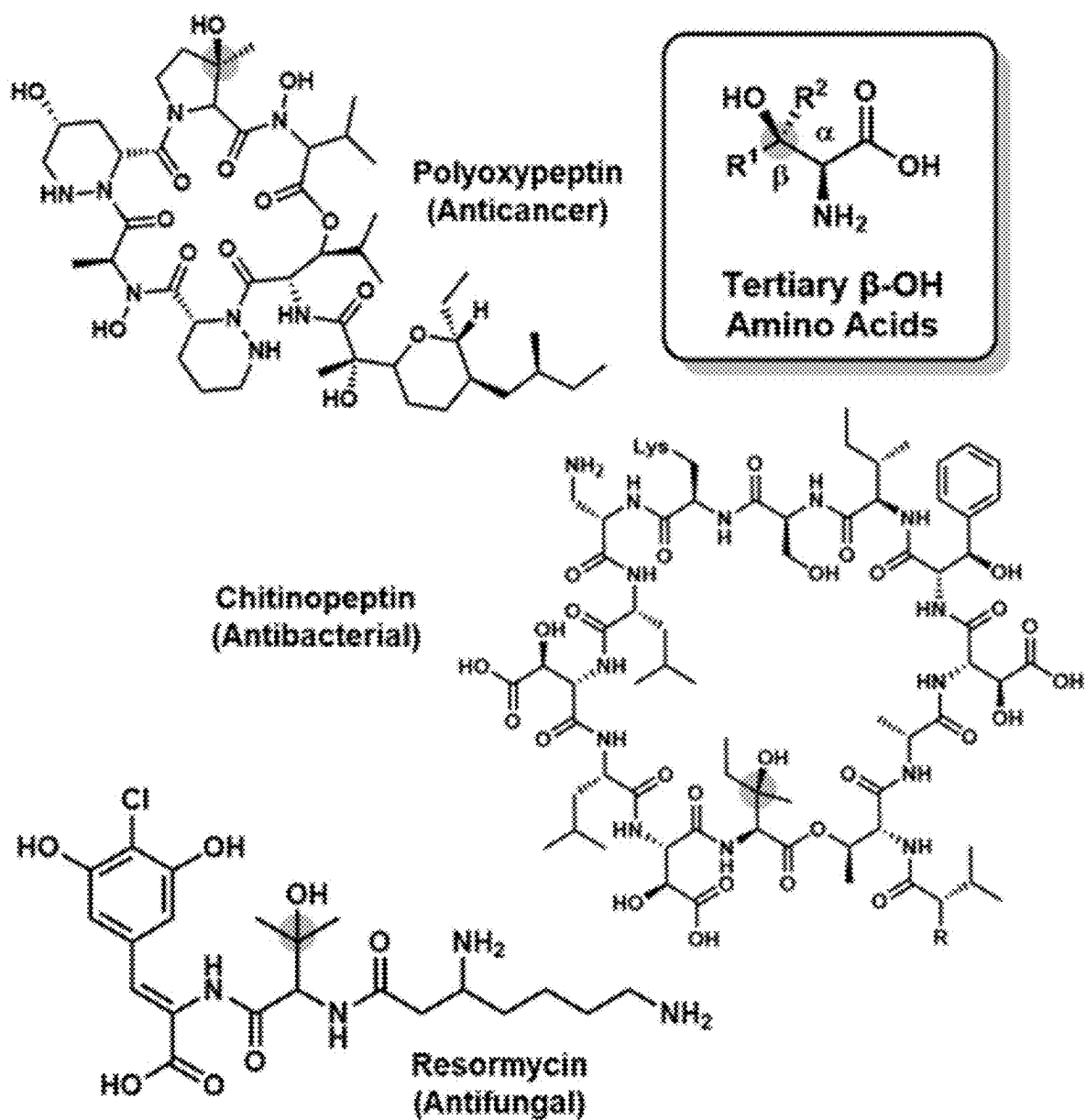


Fig. 1 (PRIOR ART)

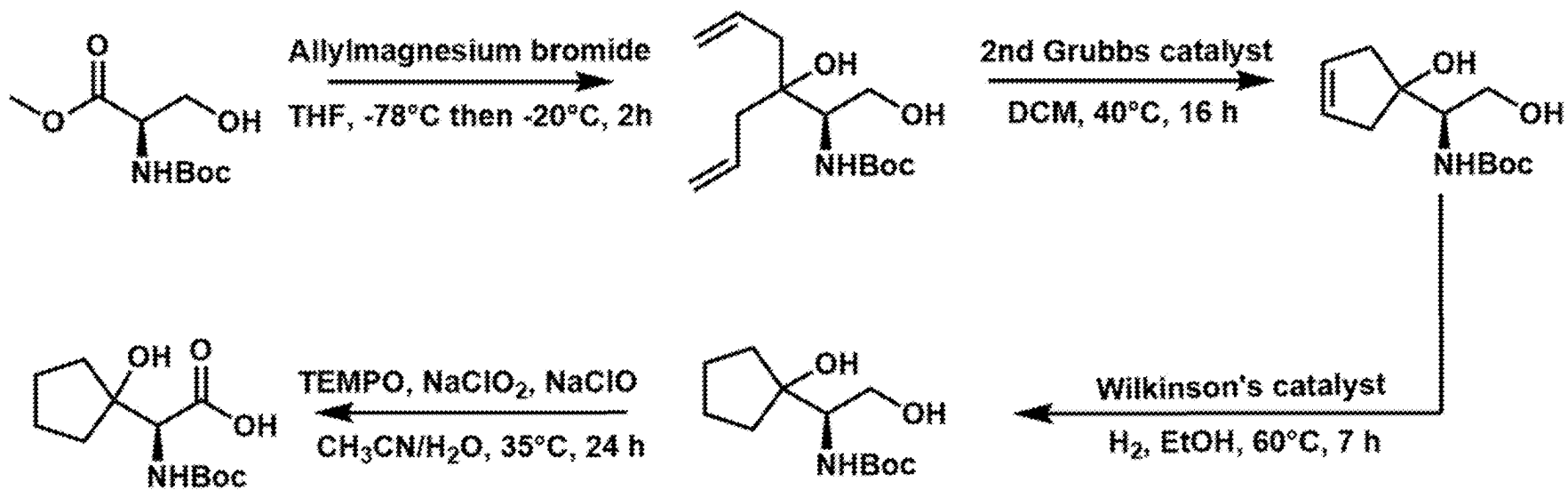
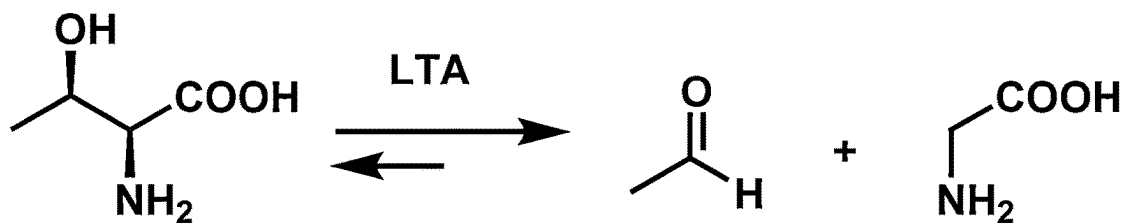
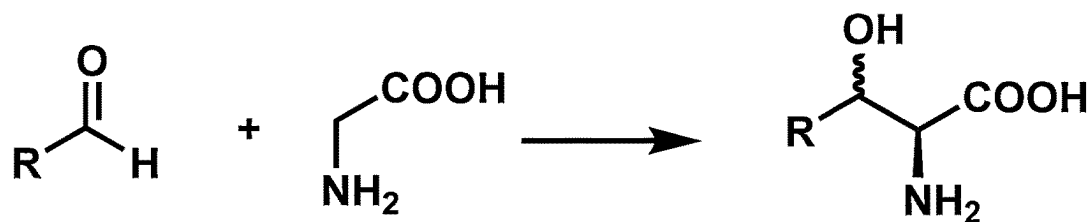


Fig. 2 (PRIOR ART)

Native, reversible reaction



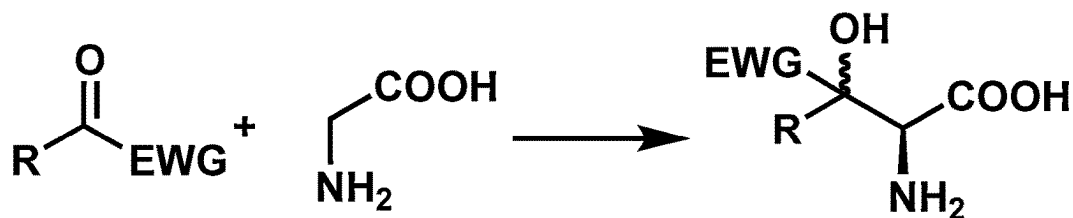
Previously reported synthetic reactions:



Aldehydes

Chiral 2° alcohol sidechain

Claimed here:



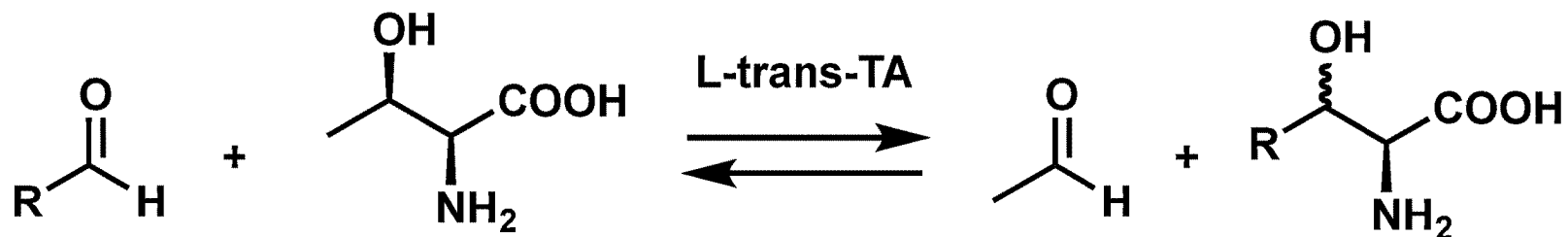
Activated *Ketone*

Chiral 3° alcohol sidechain

EWG = Electron withdrawing group
e.g. -CN; -COOMe; -CF₃

Fig. 3

Native, reversible reaction



Previously shown to work with a wide array of R-groups

Claimed here

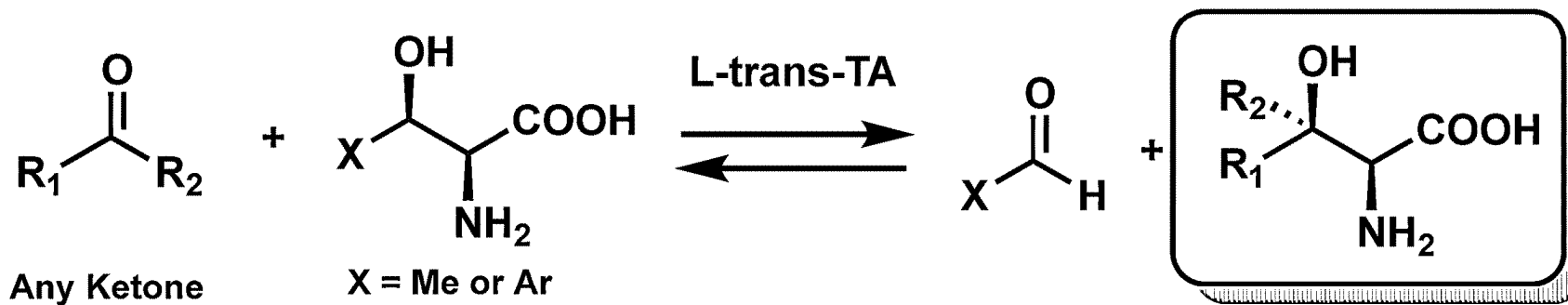


Fig. 4

Claimed here

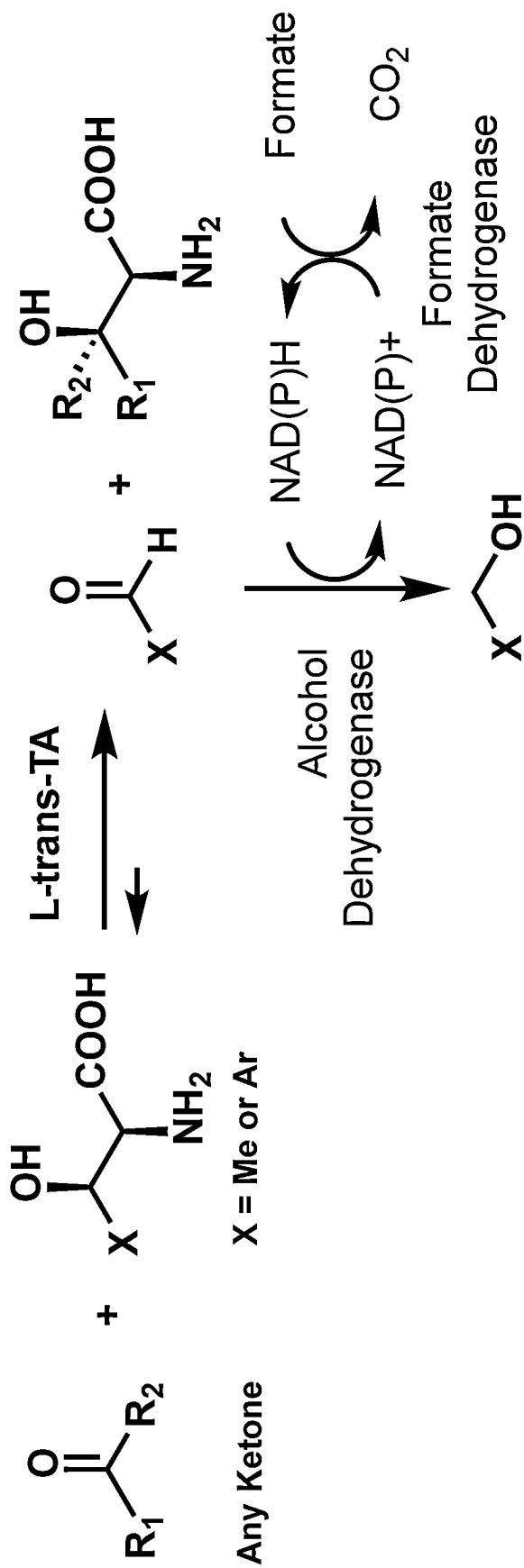


Fig. 5

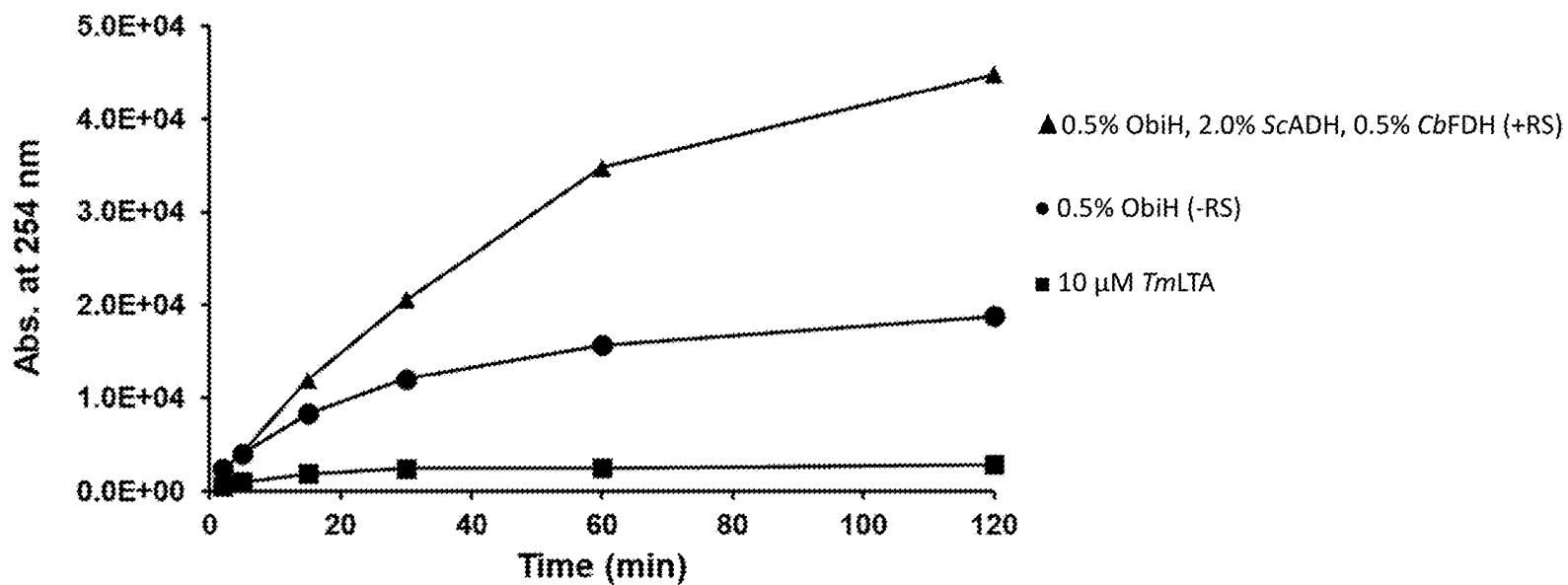
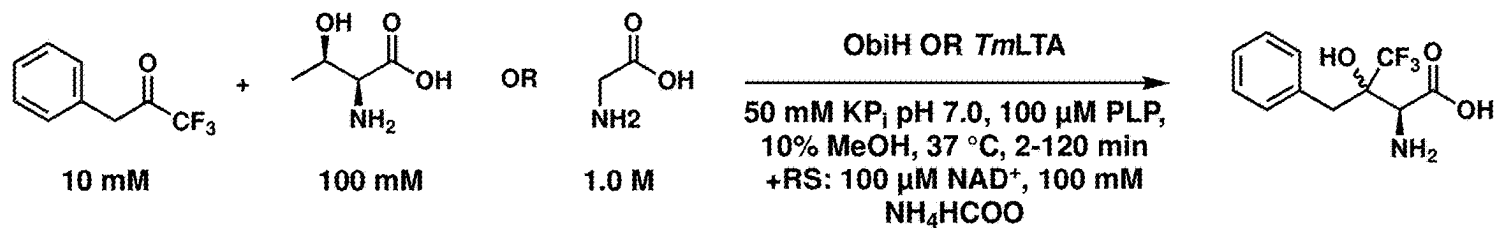


Fig. 6

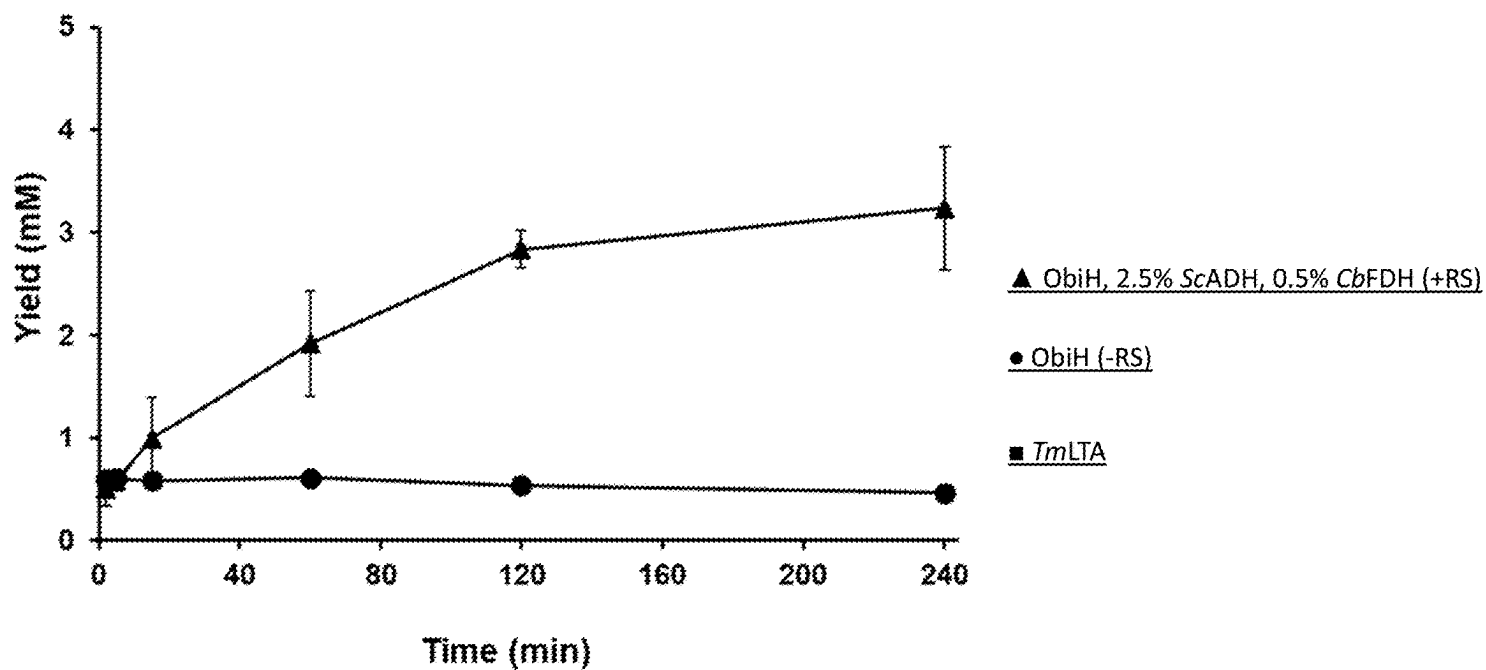
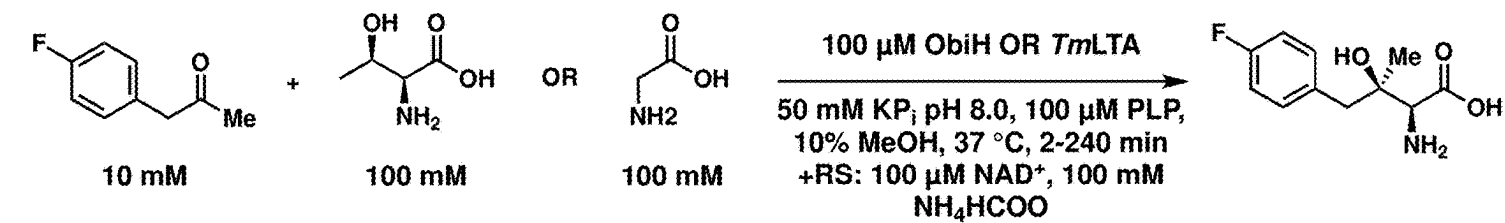


Fig. 7

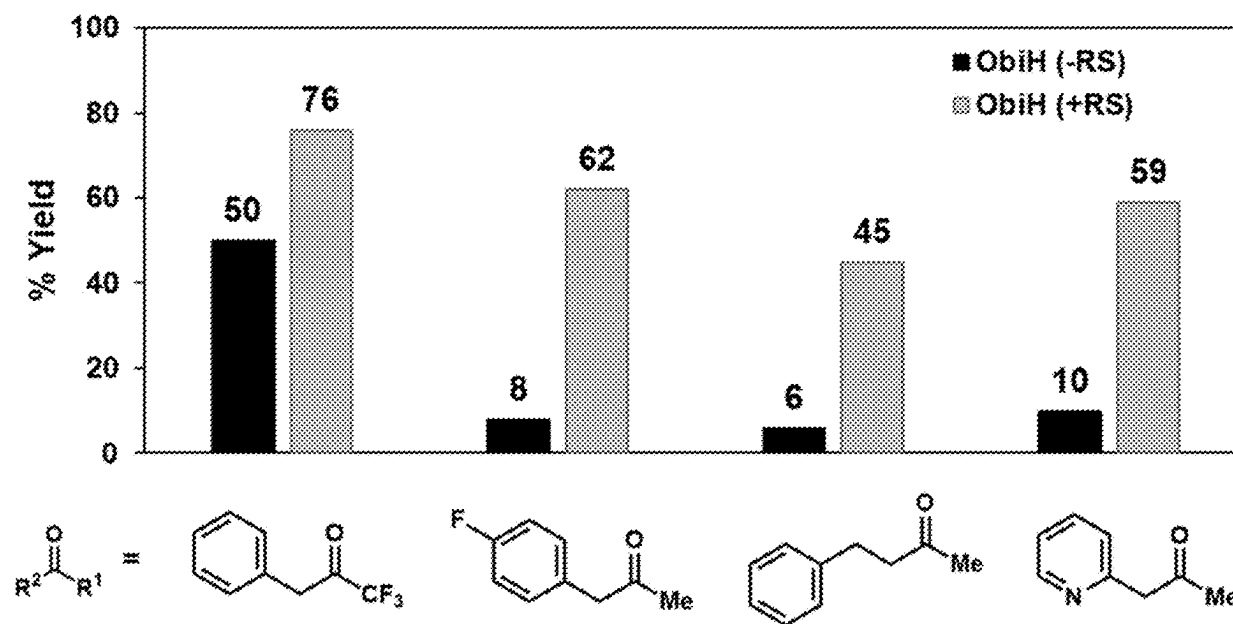
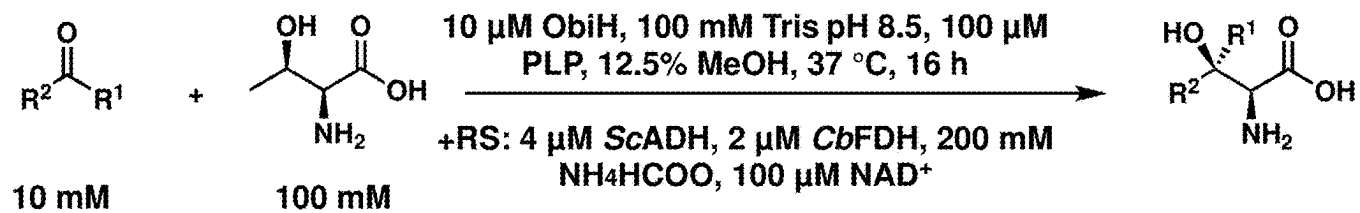


Fig. 8

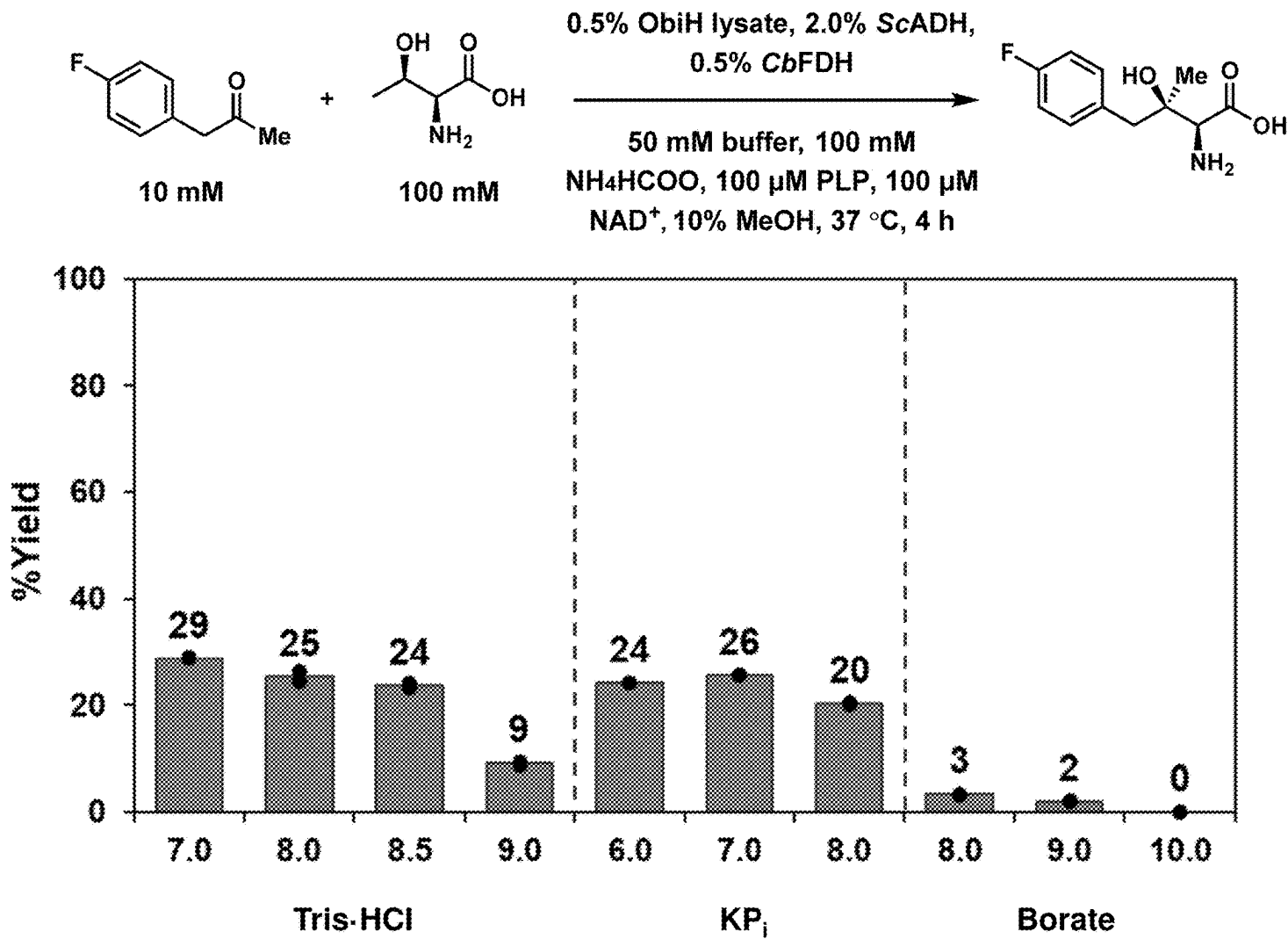
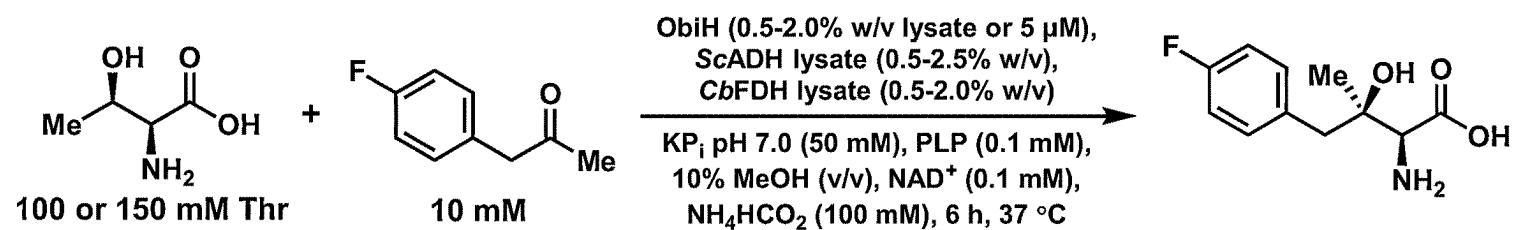


Fig. 9



entry	Thr (mM)	ObiH	ScADH (w/v)	CbFDH (w/v)	d.r.	yield (%)
1	100	0.5%	0.5%	0.5%	10:1	39%
2	100	1.0%	1.0%	1.0%	7:1	43%
3	100	1.5%	1.5%	1.5%	5:1	43%
4	100	2.0%	2.0%	2.0%	4:1	41%
5	100	2.0%	0.5%	0.5%	7:2	29%
6	100	0.5%	2.0%	0.5%	14:1	53%
7	100	0.5%	0.5%	2.0%	12:1	38%
8	100	2.0%	0.5%	2.0%	4:1	30%
9	150	0.5%	2.5%	0.5%	17:1	67%
10	150	5 μM	2.5%	0.5%	14:1	73%

Fig. 10

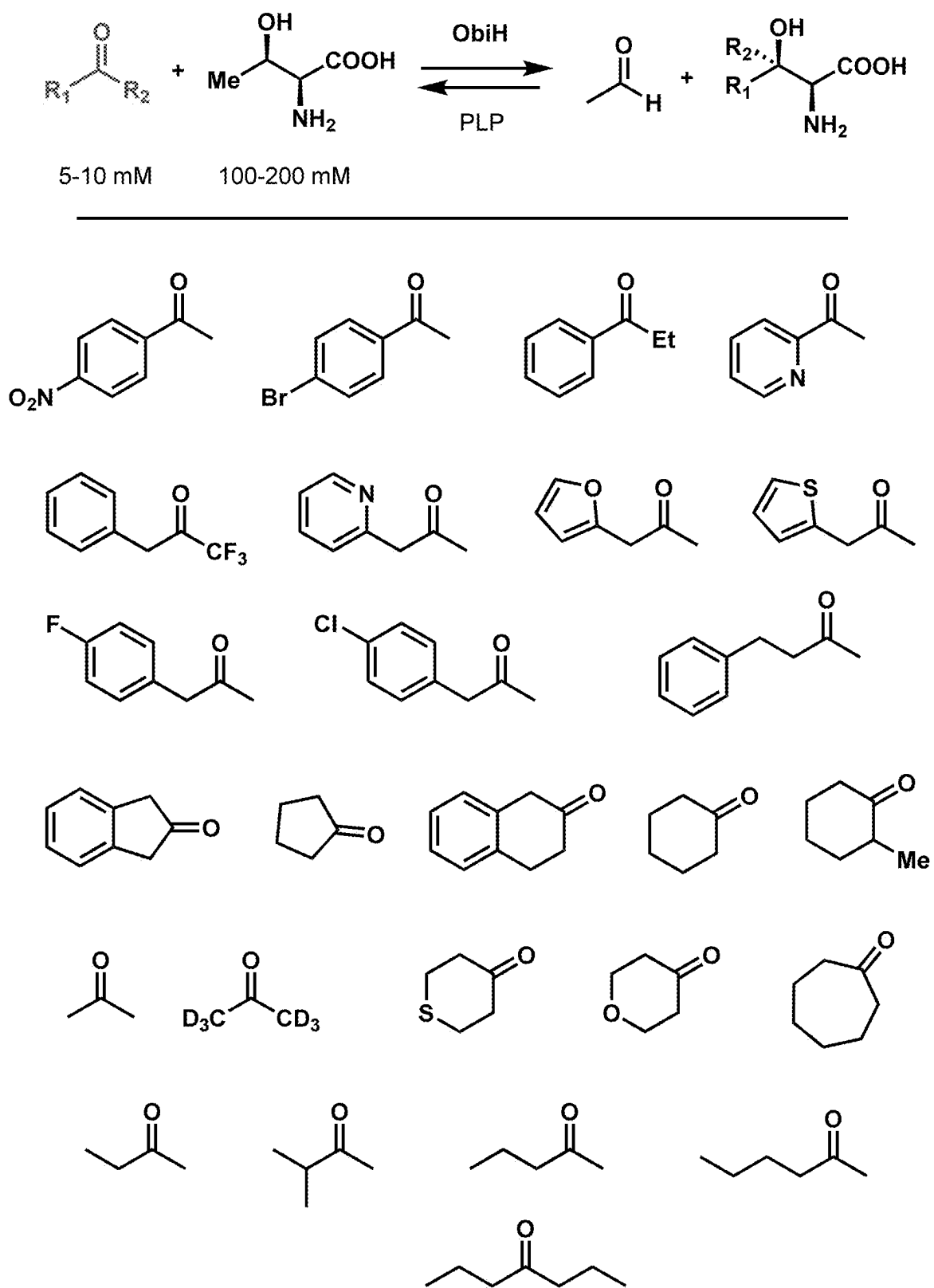


Fig. 11

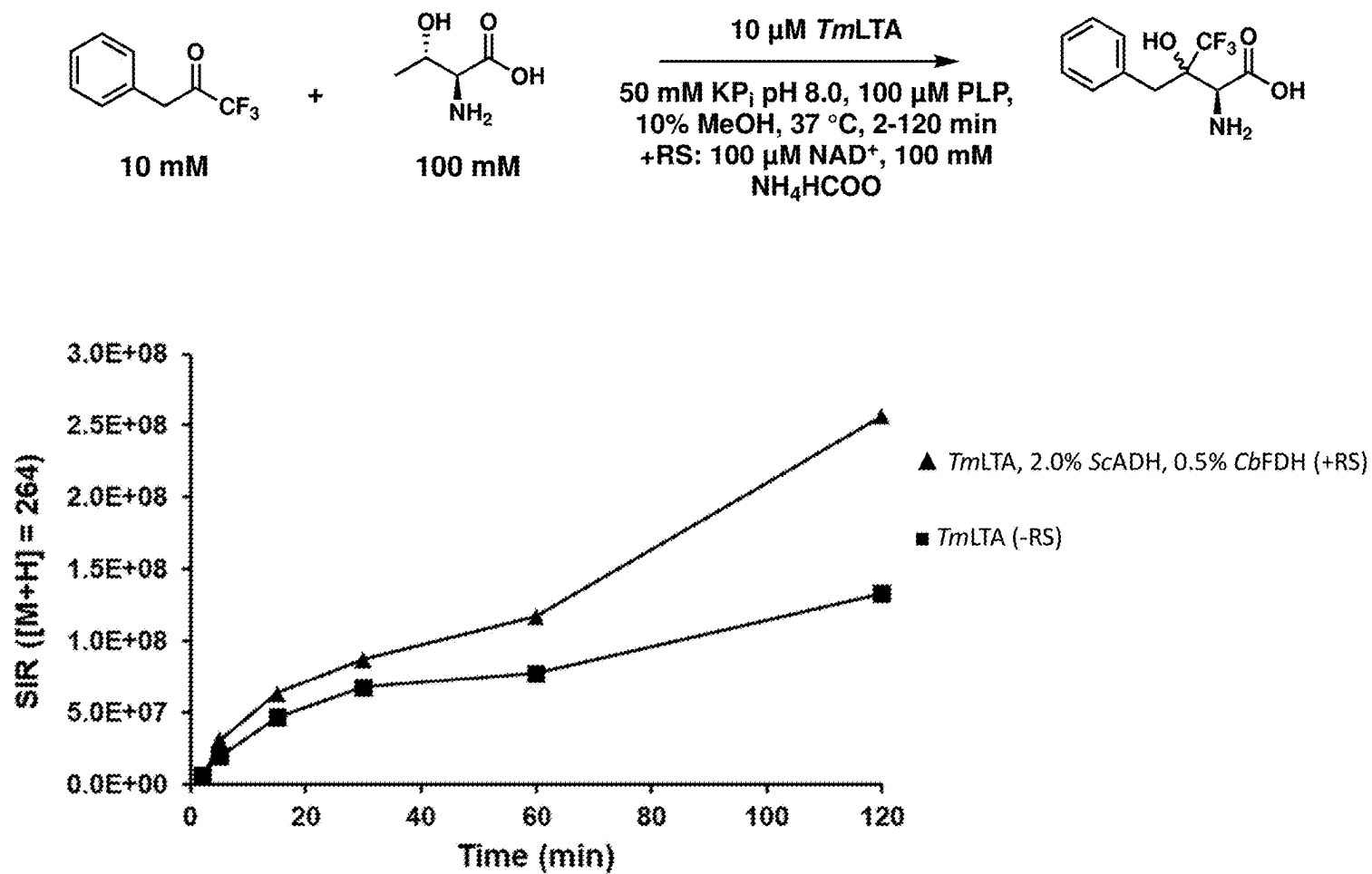


Fig. 12

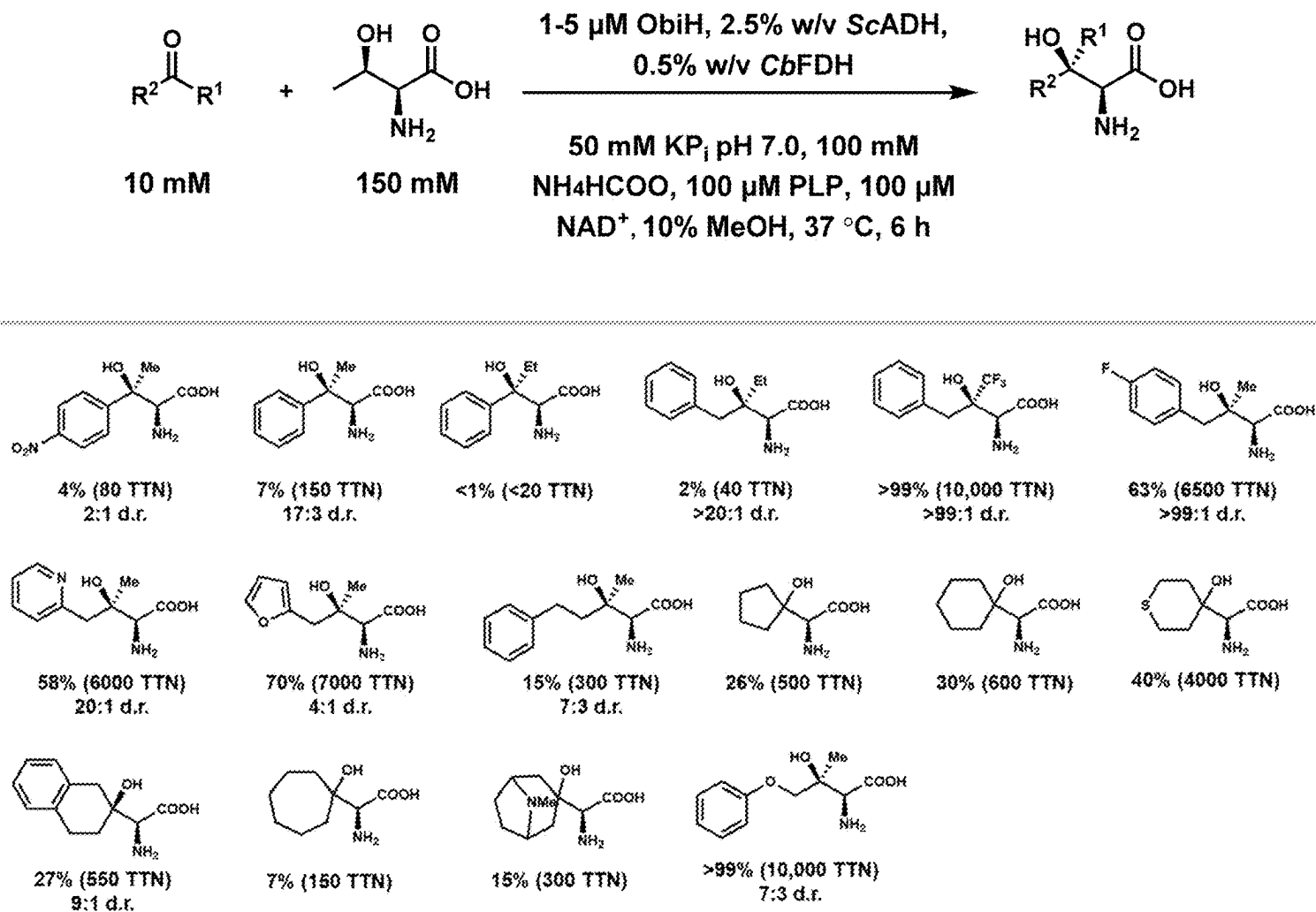


Fig. 13

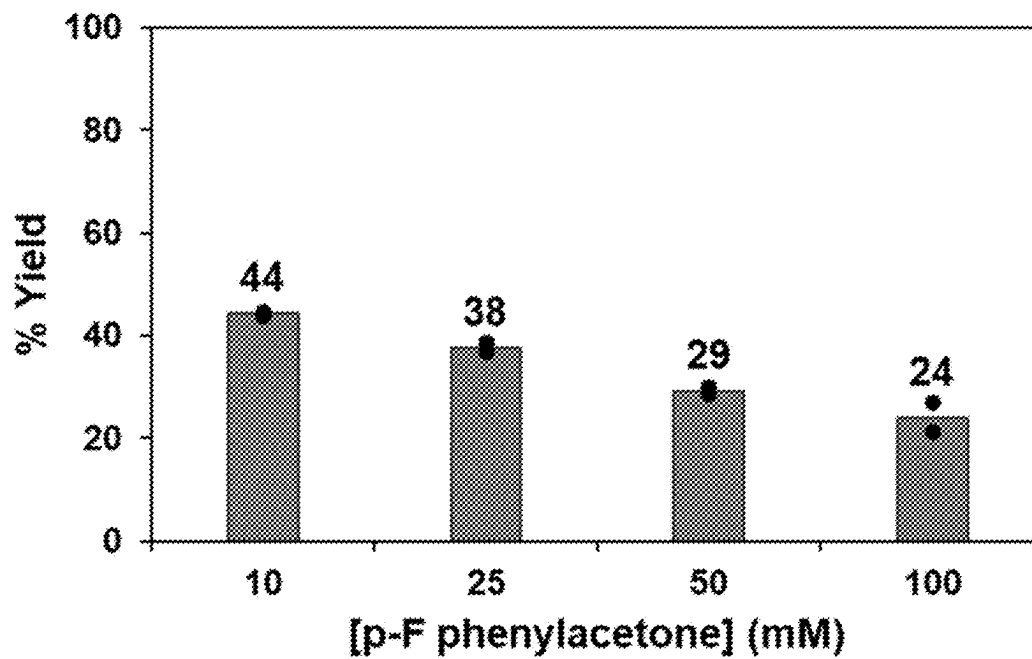
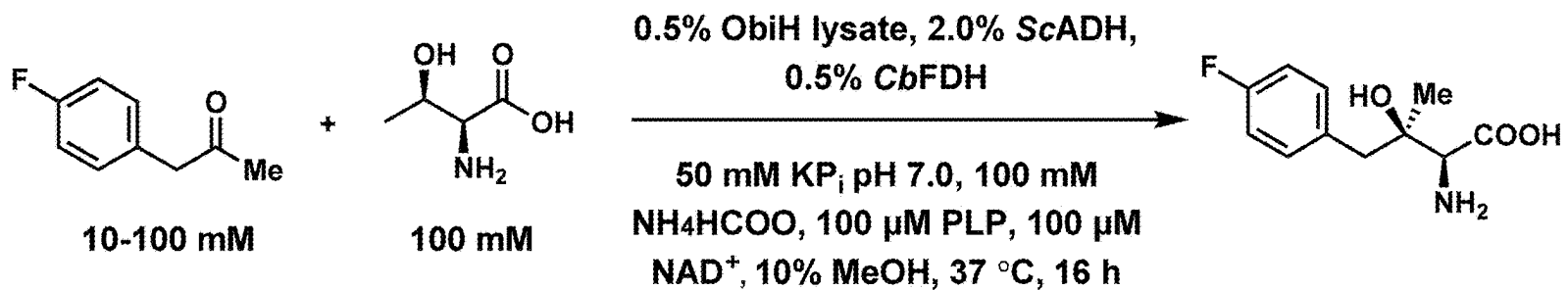


Fig. 14

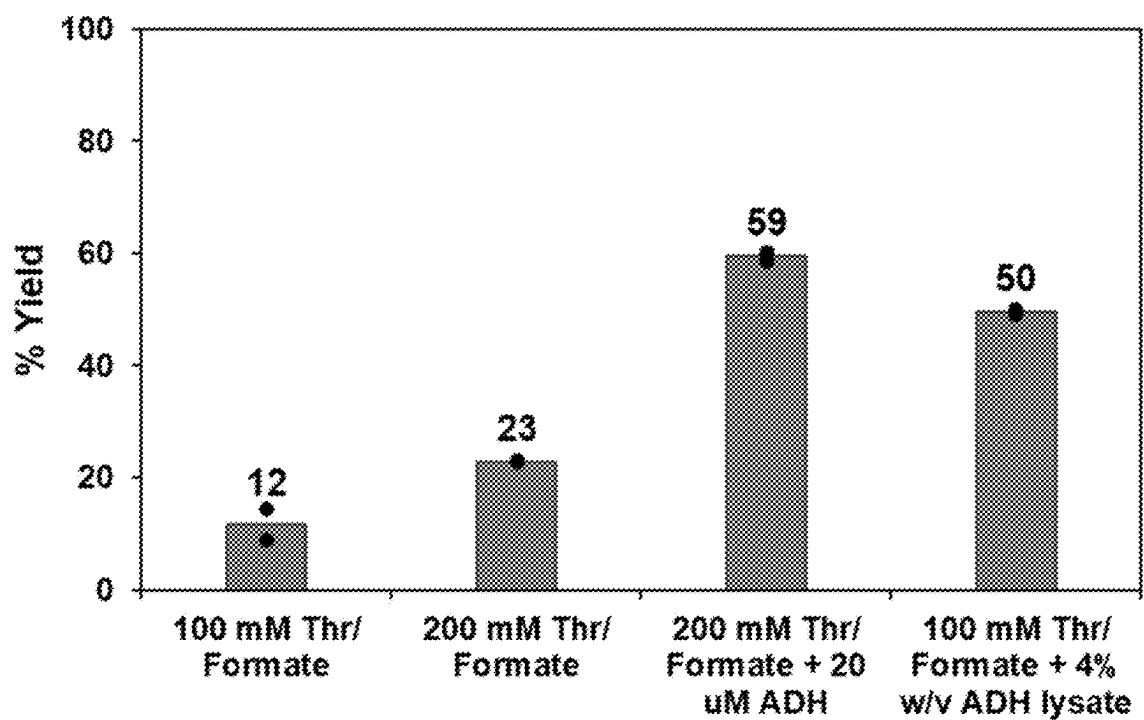
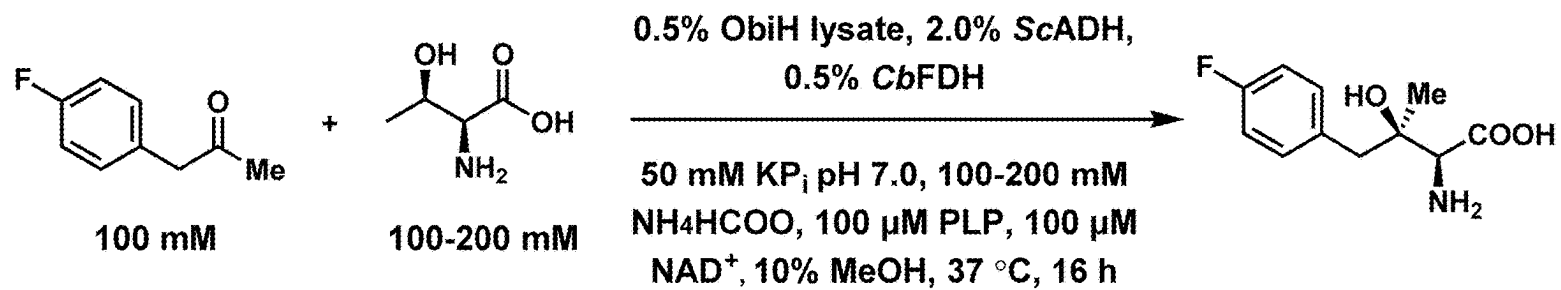


Fig. 15

METHOD FOR PRODUCING TERTIARY BETA-HYDROXY-ALPHA-AMINO ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] Priority is hereby claimed to provisional application Ser. No. 63/388,722, filed Jul. 13, 2022, which is incorporated herein by reference.

FEDERAL FUNDING STATEMENT

[0002] This invention was made with government support under GM 137417 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted in an XML file with the USPTO and is incorporated herein by reference in its entirety. The Sequence Listing was created on Apr. 5, 2023, is named "SEQ_LIST-P220362US02.xml," and is 14,199 bytes in size.

BACKGROUND

[0004] Modifying the structure of amino acids is an important strategy for tuning the properties of bioactive compounds. Hydroxylating the β -position of amino acids to produce a secondary alcohol is a common modification. There are many conventional synthetic routes to make such amino acids. However, there are very few strategies, synthetic or biological, that are capable of producing α -amino acids that have a tertiary alcohol at the β position. Examples of bioactive compounds bearing tertiary β -hydroxy amino acid sidechains are shown in FIG. 1. Disclosed herein is a non-natural, enzymatic method for producing tertiary β -hydroxy α -amino acids.

[0005] In the patent literature, see, for example, U.S. Pat. No. 2,744,141, issued May 1, 1956, to Hayes and Drake. As noted there, in the past it was conventional to make saturated, linear amino alcohols from the corresponding amino aldehyde using the Mannich reaction. See also U.S. Pat. Nos. 1,824,676 and 1,889,678. However, reducing the aldehyde reactant to the corresponding beta-hydroxy amino acid via this route is quite cumbersome. The Hayes and Drake route yields an aliphatic, saturated secondary or tertiary β -hydroxy α -amino acid by reducing the corresponding amino aldehyde in isopropanol using aluminum isopropoxide as the reductant.

[0006] More recently, see U.S. Pat. Appl. Publ. US 2006/0263861, published Nov. 23, 2006, to Nozaki et al. This reference describes a method for producing optically active β hydroxy amino acids reacting a D- α -amino acid and 5,10-methylene tetrahydrofolic acid in the presence of an enzyme derived from a microorganism belonging to the genera *Paracoccus*, *Aminobacter*, or *Ensifer*.

[0007] The native reaction of L-threonine transaldolases ("L-T-transA") such as ObiH is to catalyze the formation of non-standard amino acids ("nsAA") having a secondary alcohol. See, for example, Scott, T. A.; Heine, D.; Qin, Z.; Wilkinson, B. *Nat. Commun.* 2017, 8 (May), 1 and Schaffer, J. E.; Reck, M. R.; Prasad, N. K.; Wenciewicz, T. A. *Nat. Chem. Biol.* 2017, 13 (7), 737. ObiH has been used to produce a variety of β -hydroxy amino acids via reactions with aldehyde substrates. See Doyon, T. J.; Kumar, P.; Thein,

S.; Kim, M.; Stitgen, A.; Grieger, A. M.; Madigan, C.; Willoughby, P. H.; Buller, A. R.; Thien, S.; Kim, M.; Stitgen, A.; Greiger, A.; Madigan, C.; Willoughby, P. H.; Buller, A. R. *ChemBioChem* 2022, 23 (2), 1; Kumar, P.; Meza, A.; Ellis, J. M.; Carlson, G. A.; Bingman, C. A.; Buller, A. R. *ACS Chem. Biol.* 2021, 16, 95; Xu, L.; Wang, L. C.; and Xu, X. Q.; Lin, J. *Catal. Sci. Technol.* 2019, 9 (21), 5943.

[0008] It has also been previously shown that an L-T-transA can be coupled with a dehydrogenase to improve yield of reactions with aldehyde substrates. Xu, L.; Wang, L. C.; Su, B. M.; Xu, X. Q.; Lin, J. *Bioorg. Chem.* 2021, 110, 104766.

[0009] Reactions with ketone electrophiles are significantly more challenging than with aldehydes. See, for example, Li, Z.; Jangra, H.; Chen, Q.; Mayer, P.; Ofial, A. R.; Zipse, H.; Mayr, H. *J. Am. Chem. Soc.* 2018, 140 (16), 5500. Notably, amino acids with tertiary alcohol sidechains are cumbersome to produce through traditional methodology. See FIG. 2 and Kou, Q.; Wang, T.; Zou, F.; Zhang, S.; Chen, Q.; Yang, Y. "Design, synthesis and biological evaluation of C(4) substituted monobactams as antibacterial agents against multidrug-resistant Gram-negative bacteria," *Eur. J. Med. Chem.* 2018, 151, 98.

[0010] Thus, there remains a long-felt and unmet need for a simplified, streamlined method to make tertiary β -hydroxy α -amino acids cheaply and in high yield.

SUMMARY

[0011] Disclosed herein is a method to make an amino acid having a tertiary alcohol sidechain, wherein the method comprises reacting a pyridoxal-phosphate (PLP)-dependent enzyme with a ketone substrate and a primary or secondary β -hydroxy amino acid for a time, and at a temperature, wherein the reaction yields the desired amino acid with a tertiary alcohol sidechain. The preferred enzymes for use in the method are selected from the group consisting of L-threonine aldolases ("L-TA," EC 4.1.2.5;) and L-threonine transaldolases ("L-T-transA," no general EC number is recognized, but EC 2.2.1.4 is representative; ObiH is a preferred L-T-transA). Natively, all known L-TA and L-T-transA enzymes react with aldehydes and yield chiral secondary alcohol sidechains. No members have been reported previously to react with a ketone, which is an historically distinct chemical challenge. There has been no previous report of using L-TA or L-T-TransA enzymes to yield amino acids having a tertiary alcohol sidechain.

[0012] Thus, disclosed herein is a method to make amino acid having a tertiary alcohol sidechain, the method comprising reacting a pyridoxal-phosphate (PLP)-dependent enzyme selected from the group consisting of an L-threonine aldolase, an L-threonine transaldolase, or a combination thereof, with a ketone substrate and a primary or secondary β -hydroxy α -amino acid for a time, at a temperature, and at a pH wherein the reaction yields a tertiary β -hydroxy α -amino acid. Preferred primary or secondary β -hydroxy α -amino acid reactants include serine, threonine, and 3-phenyl serine.

[0013] In one version of the method, the PLP-dependent enzyme is an L-threonine aldolase.

[0014] In another version of the method, the PLP-dependent enzyme is an L-threonine transaldolase. A preferred PLP-dependent L-threonine transaldolase enzyme for use in the method is ObiH.

[0015] The reaction conditions are very tolerant, with preferred reaction times generally running from about 1 hour to about 12 hours, preferred temperatures from about 30° C. to about 50° C., and preferred pH from about 6 to about 8. These are preferred ranges. Values above and below these stated ranges are explicitly within the scope of the attached claims.

[0016] The method may further comprise reacting the PLP-dependent enzyme and the ketone substrate in the presence of a reducing system. The reducing system is dimensioned, configured, and functions, to reduce aldehyde by-products formed in the reaction. In one version of the method, the method further comprises the step of reducing the aldehyde by products by contacting them with an alcohol dehydrogenase in the presence of NAD(P)H, wherein the alcohol dehydrogenase reduces at least a portion of the aldehyde by-products, and NAD(P)⁺ is generated. The reaction may further comprise regenerating NAD(P)H from the NAD(P)⁺ by contacting the NAD(P)⁺ with a formate dehydrogenase. This can be accomplished by contacting the NAD(P)⁺ with a formate dehydrogenase.

[0017] In a preferred implementation of the method, the ketone substrate further comprises an electron-withdrawing group.

[0018] The method will work with any secondary β-hydroxy α-amino acid as a co-reactant.

[0019] The objects and advantages of the disclosure will appear more fully from the following detailed description of the preferred embodiment of the disclosure made in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] FIG. 1 depicts examples of bioactive compounds bearing tertiary β-hydroxy amino acid sidechains. The panel in the upper-right corner depicts the conventional Greek letter identifiers employed—the α-carbon being the carbon atom bearing the amino moiety.

[0021] FIG. 2 depicts a prior art for producing tertiary alcohol-containing non-standard amino acids. See Kou, Q.; Wang, T.; Zou, F.; Zhang, S.; Chen, Q.; Yang, Y. “Design, synthesis and biological evaluation of C(4) substituted monobactams as antibacterial agents against multidrug-resistant Gram-negative bacteria,” *Eur. J. Med. Chem.* 2018, 151, 98.

[0022] FIG. 3 depicts a generic reaction scheme according to the disclosed method in which an L-TA enzyme (EC 4.1.2.5) is used to synthesize amino acids containing tertiary alcohol sidechains.

[0023] FIG. 4 depicts a generic reaction scheme according to the disclosed method in which an L-T-transA enzyme is used to synthesize amino acids containing tertiary alcohol sidechains.

[0024] FIG. 5 depicts a cascade reaction according to the present method. The reaction overcomes the well-known thermodynamic limitations encountered when using ketone substrates.

[0025] FIG. 6 shows an exemplary reaction of TmLTA and ObiH with an activated, trifluoromethylketone substrate. The time course shows product formation over time. L-TA (square, 1.0 M Gly) produces the least product, corresponding to <10% yield. ObiH forms significantly more product (circle, 100 mM Thr) than TmLTA; that activity is increased further by adding a reducing system (triangle, 100 mM Thr) to provide a thermodynamic driving force.

[0026] FIG. 7 depicts an exemplary reaction of TmLTA and ObiH with a non-activated, a phenyl acetone substrate. No product was observed with L-TA under these conditions (100 mM Gly). ObiH yields detectable product (bottom trace, 100 mM Thr). A major enhancement in yield is observed by adding a reducing system (top trace, 100 mM Thr) to provide a thermodynamic driving force.

[0027] FIG. 8 depicts the increased product yields when using a reducing system in the disclosed method. As shown in the histogram, a reduction system provides a significant increase in activity for diverse ketone substrates.

[0028] FIG. 9 depicts the results of optimizing the buffer and pH in the ObiH-acetaldehyde reductase cascade. Buffer and pH are shown in the X-axis; present product yield on the Y-axis. The general reaction conditions are shown above the histogram. (“KPi” refers to inorganic potassium phosphate buffer comprising KH₂PO₄ (monopotassium phosphate) and K₂HPO₄ (dipotassium phosphate).)

[0029] FIG. 10 depicts the results for catalyst ratio optimization of the ObiH-acetaldehyde reductase cascade.

[0030] FIG. 11 shows a non-limiting, exemplary group of substrates that have been successfully converted to tertiary β-hydroxy α-amino acids using method disclosed herein. Tertiary β-hydroxy amino acids corresponding to each of the illustrated substrates below have been observed through the ObiH-reductase cascade. The reactions were run using the optimized conditions found in FIG. 9, entry 6 (KPi buffer, pH 7.0).

[0031] FIG. 12 presents a reaction and a corresponding yield curve showing that TmLTA can catalyze a transaldolase reaction using alloThr as the substrate (a diastereomer of the standard L-Thr). Top trace: 10 μM TmLTA, 2.0% ScADH, 0.5% CbFDH (+RS); bottom trace: 10 μM TmLTA (−RS).

[0032] FIG. 13 shows representative analytical scope for ObiH, for compounds made according to the present method, including diastereomeric ratio and total turnover number (TTN).

[0033] FIG. 14 depicts an exemplary reaction and corresponding yield histogram showing that the method gives good yields at high substrate concentrations.

[0034] FIG. 15 is an exemplary reaction using 100 mM of ketone substrate and showing that yield was improved by adding more Thr/formate and ADH to the reaction.

[0035] FIG. 16 is an exemplary reaction showing stand-alone ScADH is sufficient for providing increase in activity, which can be improved by adding more ADH and NADH.

DETAILED DESCRIPTION

Abbreviations and Definitions

[0036] “ADH” means “alcohol dehydrogenase” from any source.

[0037] “BmGDH means glucose dehydrogenase from *Bacillus megaterium*. See, for example, Xiu et al. (2022) “Multi-enzyme cascade for sustainable synthesis of L-threo-phenylserine by modulating aldehydes inhibition and kinetic/thermodynamic controls,” *Systems Microbiology and Biomanufacturing* 2:705-715.

[0038] “EWG” means “electron-withdrawing group.” An electron-withdrawing group is an atom or functional group capable of withdrawing electron density from a conjugated system. Electron density can be withdrawn through a bonds (inductive) or through it bonds (resonance).

[0039] Some functional groups are donating groups by one mechanism and withdrawing groups through the other mechanism. Exemplary electron withdrawing groups include, but are not limited to, halo, haloalkyl, $-\text{NH}_3^+$, $-\text{NO}_2$, $-\text{CH}=\text{CH}_2$, $-\text{CN}$, $-\text{SO}_3\text{H}$, $-\text{C}(=\text{O})\text{OH}$, $-\text{C}(=\text{O})\text{H}$, $-\text{C}(=\text{O})\text{R}$, $-\text{C}(=\text{O})\text{OR}$, $-\text{NR}_3^+$, where R is alkyl, such as lower alkyl (e.g., methyl, ethyl, etc.).

[0040] “FDH” means “formate dehydrogenase” from any source. “CbFDH” means a formate dehydrogenase from *Candida boidinii*.

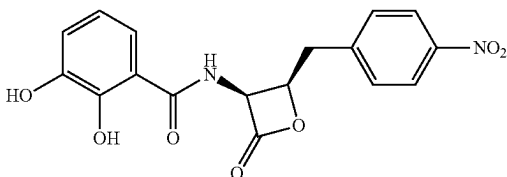
[0041] The term “L-threonine aldolase” (“L-TA”) is used generically herein to refer to any enzyme falling within enzyme classification EC 4.1.2.5. In its native milieu, L-TAs catalyze the cleavage of L-threonine to yield acetaldehyde and glycine. See the top panel of FIG. 3.

[0042] The term “L-threonine transaldolase” (“L-T-transA”) is used generically herein to refer to any enzyme that in its native milieu catalyzes the formation of secondary β -hydroxy α -amino acids. See the middle panel of FIG. 3.

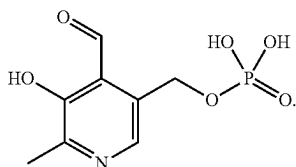
[0043] “NAD(P)+” and “NAD(P)H” nicotinamide adenine dinucleotide phosphate and its reduced form, respectively.

[0044] “nsAA” means “non-standard amino acid.”

[0045] “ObiH” refers to an L-threonine transaldolase isolated from *Pseudomonas fluorescens*. In its native milieu, ObiH is involved in the biosynthesis of the beta-lactam antibiotic obaflourin:



[0046] “PLP” refers to the co-enzyme “pyridoxal-5'-phosphate” (also known as Vitamin B6):



[0047] “RS” means “reducing system.”

[0048] “ScADH” means alcohol dehydrogenase from *Saccharomyces cerevisiae*. Proteins and/or protein sequences are “homologous” when they are derived, naturally or artificially, from a common ancestral protein or protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Nucleic acid or protein sequences of any known gene, including the genes or proteins described herein, can be determined by searching any sequence databases known in the art using the gene name or accession number as a search term. Common sequence databases include GenBank (www.ncbi.nlm.nih.gov), ExPASy (expasy.org), KEGG (www.genome.jp), among others.

[0049] Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity (e.g., identity) over 50, 100, 150 or more residues (nucleotides or amino acids) is routinely used to establish homology (e.g., over the full length of the two sequences to be compared). Higher levels of sequence similarity (e.g., identity), e.g., 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% or more, can also be used to establish homology. Accordingly, homologs of the genes or proteins described herein include genes or proteins having at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity to the genes or proteins described herein.

[0050] Methods for determining sequence similarity percentages (e.g., BLASTP and BLASTN using default parameters) are described herein and are generally available. The homologous proteins should demonstrate comparable activities and (if an enzyme) participate in the same or analogous pathways. Homologs include orthologs and paralogs. “Orthologs” are genes and products thereof in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same or similar function in the course of evolution. Paralogs are genes and products thereof related by duplication within a genome. As used herein, “orthologs” and “paralogs” are included in the term “homologs.”

[0051] For sequence comparison and homology determination, one sequence typically acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence based on the designated program parameters. A typical reference sequence is a nucleic acid or amino acid sequence corresponding to the genes or proteins described herein.

[0052] Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman (1981) *Adv. Appl. Math.* 2:482, by the homology alignment algorithm of Needleman & Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson & Lipman (1988) *Proc. Nat'l. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see Current Protocols in Molecular Biology, F. M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2008)).

[0053] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity for purposes of defining homologs is the BLAST algorithm, which is described in Altschul et al. (1990) *J. Mol. Biol.* 215:403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identify-

ing short words of length “W” in the query sequence, which either match or satisfy some positive-valued threshold score “T” when aligned with a word of the same length in a database sequence. “T” is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters “M” (reward score for a pair of matching residues; always >0) and “N” (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity “X” from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters “W,” “T,” and “X” determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (“W”) of 11, an expectation (“E”) of 10, a cutoff of 100, “M”=5, “N”=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (“W”) of 3, an expectation (“E”) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

[0054] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. The above-described techniques are useful in identifying homologous sequences for use in the methods described herein.

[0055] The terms “identical” or “percent identity”, in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described above (or other algorithms available to persons of skill) or by visual inspection.

[0056] The term “alkyl” refers to a branched or unbranched carbon chain having, for example, about 1-20 carbon atoms, and often 1-12, 1-10, 1-8, 1-6, or 1-4 carbons. Examples include, but are not limited to, methyl, ethyl, 1-propyl, 2-propyl, 1-butyl, 2-methyl-1-propyl, 2-butyl, 2-methyl-2-propyl (t-butyl), 1-pentyl, 2-pentyl, 3-pentyl, 2-methyl-2-butyl, 3-methyl-2-butyl, 3-methyl-1-butyl, 2-methyl-1-butyl, 1-hexyl, 2-hexyl, 3-hexyl, 2-methyl-2-pentyl, 3-methyl-2-pentyl, 4-methyl-2-pentyl, 3-methyl-3-pentyl, 2-methyl-3-pentyl, 2,3-dimethyl-2-butyl, 3,3-dimethyl-2-butyl, hexyl, octyl, decyl, dodecyl, and the like. The

alkyl can be unsubstituted or substituted. The alkyl can also be optionally partially or fully unsaturated in certain embodiments. As such, the recitation of an alkyl group optionally includes both alkenyl and alkynyl groups. The alkyl can be a monovalent hydrocarbon radical, as described and exemplified above, or it can be a divalent hydrocarbon radical (i.e., an alkylene). In some embodiments, certain alkyl groups can be excluded from a definition. For example, in some embodiments, methyl, ethyl, propyl, butyl, or a combination thereof, can be excluded from a specific definition of alkyl in an embodiment.

[0057] The term “cycloalkyl” refers to cyclic alkyl groups of, for example, 3 to about 12, 3 to about 10, 3 to about 8, about 4 to about 8, or 5-6, carbon atoms having a single cyclic ring or multiple condensed rings. Cycloalkyl groups include, by way of example, single ring structures such as cyclopropyl, cyclobutyl, cyclopentyl, cyclooctyl, and the like, or multiple ring structures such as adamantyl, and the like. The cycloalkyl can be unsubstituted or substituted. The cycloalkyl group can be monovalent or divalent, and can be optionally substituted as described for alkyl groups. The cycloalkyl group can optionally include one or more sites of unsaturation, for example, the cycloalkyl group can include one or more carbon-carbon double bonds, such as, for example, 1-cyclopent-1-enyl, 1-cyclopent-2-enyl, 1-cyclopent-3-enyl, cyclohexyl, 1-cyclohex-1-enyl, 1-cyclohex-2-enyl, 1-cyclohex-3-enyl, and the like.

[0058] As used herein, “aryl” refers to an aromatic hydrocarbon group derived from the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. The radical attachment site can be at a saturated or unsaturated carbon atom of the parent ring system. The aryl group can have from 6 to about 20 carbon atoms. The aryl group can have a single ring (e.g., phenyl) or multiple condensed (fused) rings, wherein at least one ring is aromatic (e.g., naphthyl, dihydrophenanthrenyl, fluorenyl, or anthryl). Typical aryl groups include, but are not limited to, radicals derived from benzene, naphthalene, anthracene, biphenyl, and the like. The aryl can be unsubstituted or optionally substituted, as described for alkyl groups.

[0059] The term “heteroaryl” refers to a monocyclic, bicyclic, or tricyclic ring system containing one, two, or three aromatic rings and containing at least one nitrogen, oxygen, or sulfur atom in an aromatic ring, and that can be unsubstituted or substituted, for example, with one or more, and in particular one to three, substituents, as described in the definition of “substituted”. Typical heteroaryl groups contain 2-20 carbon atoms in addition to the one or more heteroatoms. Examples of heteroaryl groups include, but are not limited to, 2H-pyrrolyl, 3H-indolyl, 4H-quinoliziny, acridinyl, benzo[b]thienyl, benzothiazolyl, β -carboline, carbazolyl, chromenyl, cinnolinyl, dibenzo[b,d]furan, furazanyl, furyl, imidazolyl, imidazolyl, indazolyl, indolisinyl, indolyl, isobenzofuran, isoindolyl, isoquinolyl, isothiazolyl, isoxazolyl, naphthyridinyl, oxazolyl, perimidinyl, phenanthridinyl, phenanthrolinyl, phenarsazinyl, phenazinyl, phenothiazinyl, phenoxathiinyl, phenoxazinyl, phthalazinyl, pteridinyl, purinyl, pyran, pyrazinyl, pyrazolyl, pyridazinyl, pyridyl, pyrimidinyl, pyrimidinyl, pyrrolyl, quinazolinyl, quinolyl, quinoxalinyl, thiazolyl, thianthrenyl, thiazolyl, thienyl, triazolyl, tetrazolyl, and xanthenyl. In one embodiment the term “heteroaryl” denotes a monocyclic aromatic ring containing five or six ring atoms containing carbon and 1, 2, 3, or 4 heteroatoms independently selected

from non-peroxide oxygen, sulfur, and N(Z) wherein Z is absent or is H, O, alkyl, aryl, or $-(C_1-C_6)$ alkylaryl. In some embodiments, heteroaryl denotes an ortho-fused bicyclic heterocycle of about eight to ten ring atoms derived therefrom, particularly a benz-derivative or one derived by fusing a propylene, trimethylene, or tetramethylene diradical thereto.

[0060] The term “substituted” indicates that one or more hydrogen atoms on the group indicated in the expression using “substituted” is replaced with a “substituent.” The number referred to by “one or more” can be apparent from the moiety one which the substituents reside. For example, “one or more” can refer to, e.g., 1, 2, 3, 4, 5, or 6; in some embodiments 1, 2, or 3; and in other embodiments 1 or 2. The substituent can be one of a selection of indicated groups, or it can be a suitable group known to those of skill in the art, provided that the substituted atom’s normal valency is not exceeded, and that the substitution results in a stable compound. Suitable substituent groups include, i., alkyl, alkenyl, alkynyl, alkoxy, halo, haloalkyl, hydroxy, hydroxyalkyl, aryl, aroyl, (aryl)alkyl (e.g., benzyl or phenylethyl), heteroaryl, heterocycle, cycloalkyl, alkanoyl, alkoxy-carbonyl, amino, alkylamino, dialkylamino, trifluoromethyl, trifluoromethoxy, trifluoromethylthio, difluoromethyl, acylamino, nitro, carboxy, carboxyalkyl, keto, thioxo, alkylthio, alkylsulfinyl, alkylsulfonyl, arylsulfinyl, arylsulfonyl, heteroarylsulfinyl, heteroarylsulfonyl, heterocyclesulfinyl, heterocyclesulfonyl, phosphate, sulfate, hydroxyl amine, hydroxyl(alkyl)amine, and cyano. Additionally, suitable substituent groups can be, e.g., $-X$, $-R$, $-O-$, $-OR$, $-SR$, $-S-$, $-NR_2$, $-NR_3$, $=NR$, $-CX_3$, $-CN$, $-OCN$, $-SCN$, $-N=C=O$, $-NCS$, $-NO$, $-NO_2$, N_2 , $-N_3$, $-NC(=O)R$, $-C(=O)R$, $-C(=O)NRR$, $-S(=O)_2O-$, $-S(=O)_2OH$, $-S(=O)_2R$, $-OS(=O)OR$, $-S(=O)_2NR$, $-S(C)R$, $-OP(=O)O_2RR$, $-P(=O)O_2RR$, $-P(=O)(OH)_2$, $-C(=O)R$, $-C(=O)X$, $-C(S)R$, $-C(O)OR$, $-C(O)O-$, $-C(S)OR$, $-C(O)SR$, $-C(S)SR$, $-C(O)NRR$, $-C(S)NRR$, or $-C(NR)NRR$, where each X is independently a halogen (“halo”): F, Cl, Br, or I; and each R is independently H, alkyl, aryl, (aryl)alkyl (e.g., benzyl), heteroaryl, (heteroaryl)alkyl, heterocycle, heterocycle(alkyl), or a protecting group. As would be readily understood by one skilled in the art, when a substituent is keto ($=O$) or thioxo ($=S$), or the like, then two hydrogen atoms on the substituted atom are replaced.

[0061] Numerical ranges as used herein are intended to include every number and subset of numbers contained within that range, whether specifically disclosed or not. Further, these numerical ranges should be construed as providing support for a claim directed to any number or subset of numbers in that range. For example, a disclosure of from 1 to 10 should be construed as supporting a range of from 2 to 8, from 3 to 7, from 1 to 9, from 3.6 to 4.6, from 3.5 to 9.9, and so forth.

[0062] All references to singular characteristics or limitations of the disclosed method shall include the corresponding plural characteristic or limitation, and vice-versa, unless otherwise specified or clearly implied to the contrary by the

context in which the reference is made. The indefinite articles “a” and “an” mean “one or more.”

[0063] All combinations of method steps disclosed herein can be performed in any order, unless otherwise specified or clearly implied to the contrary by the context in which the referenced combination is made.

[0064] The method disclosed herein can comprise, consist of, or consist essentially of the essential elements and steps described herein, as well as any additional or optional ingredients, components, or limitations described herein or otherwise useful in synthetic/enzymatic organic chemistry. The method disclosed herein may be practiced in the absence of any element or step which is not specifically disclosed herein.

Description of the Method

[0065] Newly developed and disclosed herein is a method of reacting an L-TA enzyme (EC 4.1.2.5) and/or an L-T-transA enzyme with a ketone substrate to yield a tertiary β -hydroxy α -amino acid product. The basic approach is illustrated in the bottom panel of FIG. 3, wherein “EWG” is an electron-withdrawing group of any description (for example, a halogen, a halo-alkyl group, a cyanate group, and alkyl carboxylate group, and the like; see the definition of EWG hereinabove). As noted above, the core of the method is to react a pyridoxal-phosphate (PLP)-dependent enzyme, either a L-threonine aldolase (L-TA; EC 4.1.2.5) or a L-threonine transaldolase, (such as C-His-TmLTA (SEQ ID NO: 1) or ObiH (SEQ ID NO: 3), respectively) with a ketone substrate and in the presence of an amino acid (glycine is shown in the bottom panel of FIG. 3) to produce an amino acid with a tertiary alcohol sidechain. It is much preferred, but not required, that the ketone substrate includes an EWG to speed the reaction. The EWG functions to activate the ketone group itself, thereby accelerating the reaction. The reaction functions with unactivated ketones, but yields are quite small without a strong driving force. This is because the reaction is reversible and subject to thermodynamic limitations. Thus, while including an EWG in the ketone substrate is not strictly required, it is strongly preferred. The reaction is very flexible in that the required enzyme(s) function to catalyze the reaction in the form of whole cells, lysates, or purified enzyme.

[0066] As shown in FIG. 4, the reaction may also be conducted using an L-T-transA enzyme (such as, but not limited to, ObiH). Here, the L-T-transA catalyzes the formation of C—C bond with threonine to yield an amino acid having a tertiary β -hydroxy sidechain. See FIG. 4, bottom panel. The native, reversible reaction of the L-T-transA enzyme is shown in FIG. 4, top panel. The bottom panel of FIG. 4 illustrates an exemplary implementation of the present method in which a ketone (any ketone) is reacted with a secondary β -hydroxy aryl serine or a secondary β -hydroxy alkyl serine to yield a tertiary β -hydroxy α -amino acid. An aldehyde is formed as a by-product.

[0067] Yield of the reaction is improved by coupling the L-T-transA reaction with an appropriate alcohol dehydrogenase (ADH), that uses NAD(P)H to reduce the aldehyde

byproduct. This provides increased thermodynamic driving force to the forward reaction and significantly enhances yields.

[0068] Yield of the reaction is further improved by using a co-factor regeneration reaction that replenishes the NAD(P)⁺ that is formed when NAD(P)H reduces the aldehyde. That is, in reducing the aldehyde, the NAD(P)H is oxidized to NAD(P)⁺. Regenerating the NAD(P)H provides still greater thermodynamic driving force for the forward reaction. This is shown in FIG. 5. Across the top of FIG. 5 is shown the same reaction as in the bottom panel of FIG. 4. In the lower-right section of FIG. 5 is shown a cascading co-factor regeneration reaction that regenerates the NAD(P)H to provide additional thermodynamic driving force for the forward reaction shown at the top of FIG. 5. Here, as noted above, an ADH and NAD(P)H are used to reduce the aldehyde by-product of the forward L-T-transA reaction, thereby generating an alcohol and NAD(P)⁺. As shown in the bottom-right of FIG. 5, a formate dehydrogenase enzyme is used to regenerate the NAD(P)H. In this fashion, the aldehyde by-product is continuously removed by the action of ADH, and the NAD(P)H reductant is regenerated by the action of the formate dehydrogenase. The result is a massive increase in thermodynamic driving force to the forward reaction. Yields are thus increased accordingly.

[0069] The results of an exemplary implementation of the method are shown in FIG. 6. As shown across the top of the figure, 10 mM of benzyl-trifluoromethyl ketone were reacted with either threonine or glycine, in the presence of 0.5% ObiH (a L-T-transA enzyme) or TmLTA (L-TA

(bottom trace, 1.0 M Gly) produces the least product, corresponding to <10% yield. ObiH forms significantly more product (middle trace, 100 mM Thr) than TmLTA. That activity is increased further by adding the alcohol dehydrogenase/formate dehydrogenase cascade reaction to provide additional thermodynamic driving force.

[0070] FIG. 7 depicts an exemplary implementation of the method using ObiH or TmLTA without the alcohol dehydrogenase/formate dehydrogenase cascade reaction and without an activated ketone substrate. The substrate was 4-fluorobenzyl methyl ketone and the amino acid was again threonine or glycine. The remaining reaction conditions were as noted above for FIG. 6. Yields were significantly higher for ObiH than for TmLTA. This indicates that the thermodynamics of the L-T-transA-catalyzed reaction (ObiH) are much more favorable than the thermodynamics of the L-TA catalyzed reaction (TmLTA). Under these conditions, the unassisted reaction with TmLTA did not yield any observable product. The ObiH catalyzed reaction did yield product, even with an unactivated ketone and without the alcohol dehydrogenase/formate dehydrogenase cascade reaction (bottom trace). The best yield was realized using an L-T-transA (ObiH) as the enzyme and including in the reaction the alcohol dehydrogenase/formate dehydrogenase cascade reaction (top trace).

[0071] Table 1 below presents a comparison of the yields of an exemplary ObiH-catalyzed reaction, with and without the alcohol dehydrogenase/formate dehydrogenase cascade reaction.

TABLE 1

Comparison of ObiH alone to ObiH with acetaldehyde reductase system			
Entry	ObiH lysate concentration (% w/v)	Reduction system	Product yield (%)
1	0.1% (1 mg/mL)	None	4%
2	0.5% (5 mg/mL)	None	3%
3	1.0% (10 mg/mL)	None	1%
4	0.5% (5 mg/mL)	0.5% CbFDH + ScADH	24%

enzyme), in the presence of KPi buffer (pH 7), PLP, 10% v/v methanol ("MeOH"), 0.1 mM NAD*, and 100 mM ammonium formate, at 37° C., and for times ranging from 2 minutes to 120 minutes. The graph shows the formation of the tertiary β-hydroxy α-amino acid product over time. As shown by the top trace, the yield is greatly improved by including the alcohol dehydrogenase/formate dehydrogenase cascade reaction. The middle trace shows the yield over time without the alcohol dehydrogenase/formate dehydrogenase cascade reaction. The bottom trace shows a very small yield when only 10 μM L-TA enzyme is used. LTA

[0072] The data shows that yield is not improved simply by adding a higher concentration of the L-TA or L-T-transA enzyme; see the first two entries in Table 1. Entry 1 used 1 mg/mL ObiH lysate and yield was 4%. Entry 2 used 5 mg/mL ObiH lysate and product yield dropped to 3%. All other reaction conditions were the same.

[0073] Further efforts to optimize yields resulted in the data shown in Table 2. Here, the concentrations of Thr, ObiH, ScADH, and CbFDH were varied. Entries 10 (73%), 9 (67%), and 6 (53%) had the best yields. The abbreviation

“d.r.” stands for “diastereomeric ratio” of the diastereomer shown in the header of Table 2.

amino acids corresponding to the ketone substrate have been made and observed using the method disclosed herein.

TABLE 2

Yield Optimization						
entry	Thr (mM)	ObiH	ScADH (w/v)	CbFDH (w/v)	d.r.	yield (%)
1	100	0.5%	0.5%	0.5%	10:1	39%
2	100	1.0%	1.0%	1.0%	7:1	43%
3	100	1.5%	1.5%	1.5%	5:1	43%
4	100	2.0%	2.0%	2.0%	4:1	41%
5	100	2.0%	0.5%	0.5%	7:2	29%
6	100	0.5%	2.0%	0.5%	14:1	53%
7	100	0.5%	0.5%	2.0%	12:1	38%
8	100	2.0%	0.5%	2.0%	4:1	30%
9	150	0.5%	2.5%	0.5%	17:1	67%
10	150	5 μ M	2.5%	0.5%	14:1	73%

[0074] FIG. 8 shows the marked improvement in yield when an L-T-transA enzyme is used in conjunction with the alcohol dehydrogenase/formate dehydrogenase cascade reaction (which is referred to as the “reduction system” in FIG. 8). The histogram clearly shows that the reduction system provides an increase in activity for diverse ketone substrates. The ketone substrates tested, from left to right in FIG. 8, were benzyl trifluoromethyl ketone, 4-fluorobenzyl methyl ketone, 2-phenyl-methyl methyl ketone, and 2-pyridinyl methyl ketone. Across the board, the yield was vastly improved by using the alcohol dehydrogenase/formate dehydrogenase cascade reaction.

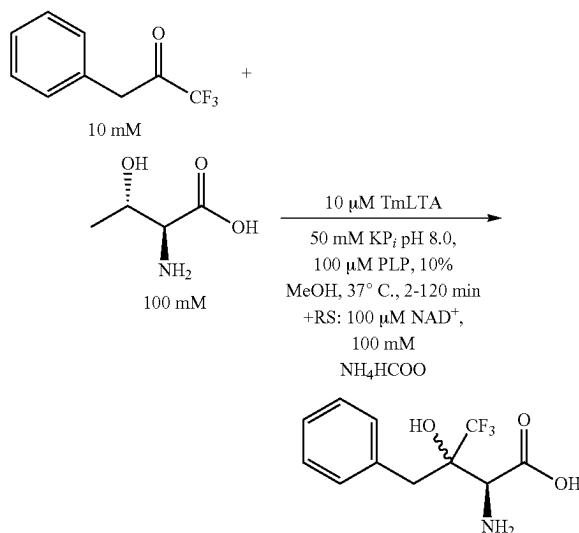
[0075] FIG. 9 is a table showing the effect of buffer composition and pH on an exemplary reaction according to the present method. The reaction is shown at the top of FIG. 9. 100 mM threonine was reacted with 10 mM of 4-fluorobenzyl methyl ketone. The remaining reaction conditions were as noted in FIG. 9 itself. Yields suffered at higher pHs. At pH 8, 9, and 10, using a borate buffer, the highest yields as only 3% (borate, pH 8.0). Using “Tris” (tris(hydroxymethyl)aminomethane) buffer at pH 9.0 only bumped the yield to 9%. Yields ranged from 20% to 29% using Tris or KPi buffers, at pHs ranging from 6.0 to 8.5.

[0076] FIG. 10 is a table analogous to FIG. 9, but showing the effect of optimizing enzyme concentration. The reaction and reaction conditions are the same as shown for FIG. 9. The maximum yield, 49% was achieved using the conditions shown in Entry 6: 0.5% w/v ObiH lysate, 2.0% w/v ScADH lysate, 0.5% w/v CbFDH, and 10:1 diastereomeric ratio (“dr”).

[0077] FIG. 11 presents a selection of the ketone substrates that have been shown to function to yield a tertiary β -hydroxy α -amino acid product using the present method. The ketones include purely aliphatic, symmetric and asymmetric ketones (e.g., methyl ethyl ketone, diethyl ketones), cyclic ketones, heterocyclic ketones, aryl ketones, heteroaryl ketones, etc. For each entry in FIG. 11, tertiary β -hydroxy

These successful reactions were run using the optimized conditions found in FIG. 9, entry 6.

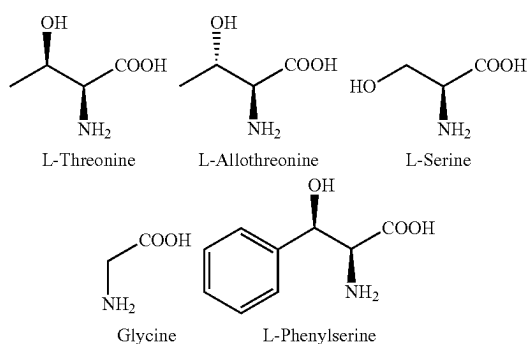
[0078] FIG. 12 presents a reaction and a corresponding yield curve showing that TmLTA can catalyze a transaldolase reaction using alloThr as the substrate (a diastereomer of the standard L-Thr). Yields are boosted by using the ScADH and CbFDH cascade. Here, the reaction was as follows:



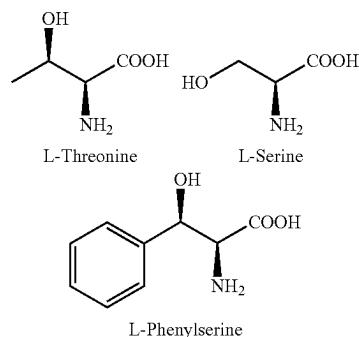
[0079] In this particular implementation of the method, any β -hydroxy-amino acid or glycine can be used for the L-TA reaction. Any β -hydroxy-amino acid can be used for the ObiH reaction, but glycine is not preferred.

[0080] Substrates that have been tested and shown to yield corresponding product include:

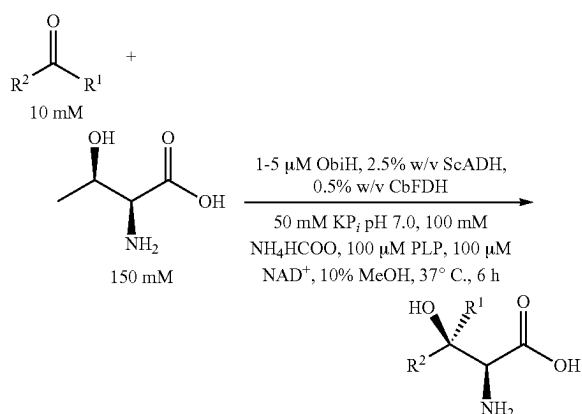
[0081] TmLTA



[0082] ObiH



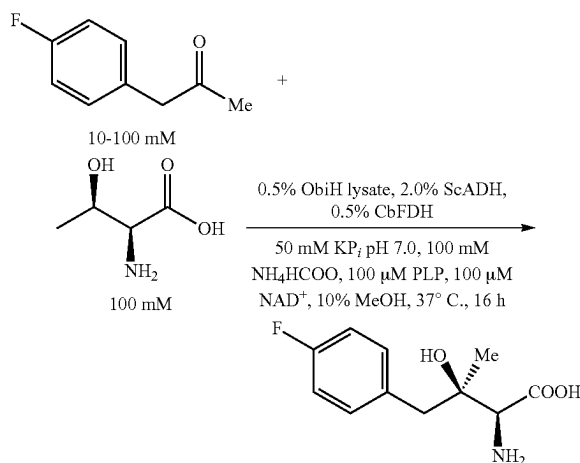
[0083] FIG. 13 shows representative analytical scope for ObiH, for compounds made according to the present method, including diastereomeric ratio and total turnover number (TTN). The test reaction here was:



[0084] As shown from the wide range of products formed, the reaction is exceedingly flexible and give acceptable yields using a host of different ketone substrates.

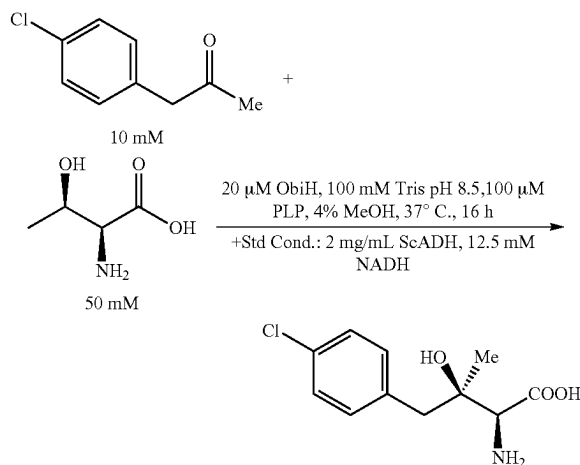
[0085] FIG. 14 depicts the results of an exemplary reaction to investigate the impact of substrate concentration on

product yield. The corresponding yield histogram shows that the method gives good yields at high substrate concentrations. The reaction here was:



[0086] FIG. 15 is an exemplary reaction showing that using 100 mM of ketone substrate, yields were improved by adding more Thr and formate to the reaction. Here, the reaction was the same as shown in FIG. 14. As shown in the histogram, the percent yield was very significantly improved by adding additional threonine and/or additional ADH.

[0087] FIG. 16 is an exemplary reaction showing stand-alone ScADH is sufficient for providing increase in activity, which can be improved by adding more ADH and NADH. The reaction is as follows:



Experimental Procedure

Cloning and Expression of ObiH and ScADH:

[0088] A codon-optimized copy of each was inserted into a pET-28b(+) vector (Millipore Sigma, Burlington, Massachusetts, USA) by the Gibson Assembly method. Gibson D G, Young L, Chuang R Y, Venter J C, Hutchison C A 3rd, Smith H O (2009). "Enzymatic assembly of DNA molecules

up to several hundred kilobases,” *Nature Methods* 6 (5): 343-345. BL21 (DE3) *E. coli* cells (New England Biolabs Inc. Ipswich, Massachusetts, USA) were subsequently transformed with the resulting cyclized DNA product via electroporation. After 45 min of recovery in Luria-Bertani (LB) media at 37° C., cells were plated onto LB plates with 50 µg/mL kanamycin (Kan) and incubated overnight. Single colonies were used to inoculate 5 mL LB+50 µg/mL Kan, which were grown overnight at 37° C., 200 rpm. Expression cultures, typically 1 L of Terrific Broth (TB)+50 µg/mL Kan (TB-Kan), were inoculated from these starter cultures and shaken (200 rpm) at 37° C. After 3 hours (OD₆₀₀~0.6), the expression cultures were chilled on ice. After 30 min on ice, expression is induced with 0.5 mM IPTG, and the cultures were expressed for 16 hours at 20° C. with shaking at 200 rpm. Cells were then harvested by centrifugation at 4,300×g at 4° C. for 15 min. Cell pellets were frozen and stored at -20° C. until purification.

Cloning and Expression of TmLTA and CbFDH:

[0089] A codon-optimized copy of each was inserted into a pET-22b(+) vector (Millipore Sigma, Burlington, Massachusetts, USA) by the Gibson Assembly method. BL21 (DE3) *E. coli* cells were subsequently transformed with the resulting cyclized DNA product via electroporation. After 45 min of recovery in Luria-Bertani (LB) media at 37° C., cells were plated onto LB plates with either 100 µg/mL ampicillin (Amp) and incubated overnight. Single colonies were used to inoculate 5 mL LB+100 µg/mL Amp, which were grown overnight at 37° C., 200 rpm. Expression cultures, typically 1 L of Terrific Broth (TB)+50 µg/mL Amp (TB-Amp), were inoculated from these starter cultures and shaken (200 rpm) at 37° C. After 3 hours (OD₆₀₀~0.6), the expression cultures were chilled on ice. After 30 min on ice, expression is induced with 0.5 mM IPTG, and the cultures were expressed for 16 hours at 20° C. with shaking at 200 rpm. Cells were then harvested by centrifugation at 4,300×g at 4° C. for 15 min. Cell pellets were frozen and stored at -20° C. until purification.

Purification of ObiH, TmLTA, ScADH and CbFDH:

[0090] To purify each protein, cell pellets were thawed on ice and then resuspended in lysis buffer (50 mM potassium phosphate buffer (pH=8.0), 150 mM NaCl, and (for ObiH and TmLTA only) 400 µM pyridoxal 5'-phosphate (PLP)). A volume of 4 mL of lysis buffer per gram of wet cell pellet was used. After resuspension, the cell suspension was placed on ice in a metal container and subjected to lysis using a sonication device at 50% power for 5 sec on and 10 sec off for a total time of 5 min. The resulting lysate was then spun down at 50,000×g to pellet cell debris. Ni/NTA beads (Gold Biotechnology, Inc. (doing business as “GoldBio”) St. Louis, Missouri, USA) were added to the supernatant and incubated on ice for 45 min prior to purification by Ni-affinity chromatography with a gravity column. The column was washed with 5 column volumes of 20 mM imidazole, 150 mM NaCl, 50 mM potassium phosphate buffer (pH=8.0). Washing with higher concentrations of imidazole resulted in slow protein elution. Each protein was eluted with 250 mM imidazole, 150 mM NaCl, 50 mM potassium phosphate buffer, pH 8.0. For ObiH, elution of the desired protein product was monitored by the disappearance of its bright red color (resulting from the release of ObiH) from

the column. FDH and ADH are colorless and their elution was monitored by the addition of 1 µL of eluent to 50 µL of Bradford reagent and evaluating the presence of protein by color change in the reagent (brown to blue). The protein products were dialyzed to <1 µM imidazole in 50 mM potassium phosphate buffer (pH 8.0) with 150 mM NaCl. Purified enzyme was flash frozen in pellet form by pipetting enzyme dropwise into a crystallization dish filled with liquid nitrogen. The enzyme was transferred to a plastic conical and stored at -80° C. until further use. Frozen pellets were thawed at room temperature and centrifuged before use. The concentration of protein was determined by Bradford assay using bovine serum albumin for a standard concentration curve. Generally, this procedure yielded 200-250 mg per L culture for ObiH, 500-600 mg per L culture for TmLTA, 100-120 mg per L culture for FDH and 30-60 mg per L culture for ADH. Protein purity was analyzed by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis using 12% polyacrylamide gels.

Enzymes

L-Threonine Aldolase

[0091] L-threonine aldolase (L-TA) includes enzymes falling under Enzyme Commission (EC) number 4.1.2.5. In its native milieu, L-TAs catalyze the cleavage of L-threonine to yield acetaldehyde and glycine. Exemplary L-TA may have an amino acid sequence of SEQ ID NO: 1 or a sequence at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% identical thereto. The coding sequence of SEQ ID NO: 1 is shown as SEQ ID NO: 2.

```

SEQ ID NO: 1 TmLTA (Thermotoga maritima)
                                     (SEQ ID NO: 1)
MIDLRSDTVTKPTEEMRKAMAQAEVGGDDVYGEDPTINELERLAAETFGKE
AALFVPSGTMGNQVSIHAHTORGDEVILEADSHIFWYEVGAMAVLSGVMP
HPVPGKNGAMPDDVRKAI RPRNIHFPR TSLIAI ENTHNRSGGRVVPLEN
I KEICTIAKEHGINVHIDGARIFNASTIASGVVPKEYAGYADSVMFCLSKG
LCAPVGSVVVGD RDFIERARKARKMLGGMRQAGVLAAGI IALTKMVDR
LKEDHENARFLALKLKEIGYSVNPEDVKTNMVLRTDNLKVN AHGFIEAL
RNSGVLNAVSDTEIRLVTHKDVSRNDIEEALNIFEKLERKFSLEHHHHH
H
SEQ ID NO: 2 TmLTA (Thermotoga maritima)
                                     (SEQ ID NO: 2)
ATGATCGATCTCAGGTCACACCGTTACAAAACCAACAGAGAGATGAG
AAAAGCCATGGCACAGGCTGAGGTGGGAGACGATGTGTACGGAGAAGATC
CAACCATCAACGAAC TCGAAAGGCTCGCCG CAGAGACCTTTGGAAAGGAA
GCGGCTCTCTTTGTACCTCCGGCACCATGGGAATCAAGTGAGCATAAT
GGCTCACACCCAGAGGGGCGATGAAGTGATACTGGAGGCAGACAGCCACA
TCTTCTGGTACGAGGTGGAGCCATGGCGGTTCTCTCGGAGTCATGCC
CATCTGTACCTGGAAAAATGGAGCCATGGACCCGATGATGTGAGGAA
GGCCATAAGACCCAGAAACATACACTTCCCAGAACTTCGCTCATTGCCA

```

-continued

TCGAAAACACACACACACCGTTCCGGTGGAGAGTGGTCCCGCTTGAAAAAC
ATAAAAAGAGATTTGCACGATAGCCAAAAGAACACGGCATAAACGTTTACAT
AGATGGTGCAGGAGTCTTCAACGCCTCAATCGCTTCAGGTGTTCCCGTGA
AGGAGTACGCCGGGTACGCCGATTCCGTGATGTTCTGTCTTTCAAAGGT
CTCTGCCACCCGTCGGTTCGGTGGTTGTAGGAGACAGGGACTTCATAGA
AAGAGCGAGAAAAGGCGAGAAAAGATGCTCGGTGGAGGGATGAGACAGGCAG
GTGTTCTCGCTGCCGCTGGAATAATCGCCTTGACAAAAGATGGTAGATCGA
TTGAAAAGAAGATCATGAAAACGCCAGATTTCTCGCCCTGAAGTTGAAAAGA
AATAGGGTACTCCGTGAATCCCGAAGATGTGAAAACCAACATGGTGATTCT
TGAGGACCGACAACTGAAGGTGAACGCGCACGGGTTTATAGAAGCGCTC
AGAAACAGCGGGGTGCTCGCGAACGCCGTATCCGACACGGAGATCAGACT
GGTAACCCACAAAGACGTTTCAAGAAAACGACATAGAAGAGGCTCTGAACA
TCTTCGAAAAACTCTTCAGAAAATTCTCCCTCGAGCACCATCACCATCAC
CATTGA

L-Threonine Transaldolase

[0092] L-threonine transaldolase (L-T-transA) includes enzymes that in their native milieu catalyze the formation of secondary beta-hydroxy alpha-amino acids. One representative group of L T-transA includes enzymes falling under EC number 2.2.1.4. Exemplary L-T-transA may have an amino acid sequence of SEQ ID NO: 3 or a sequence at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% identical thereto. The coding sequence of SEQ ID NO: 3 is shown as SEQ ID NO: 4.

SEQ ID NO: 3 ObiH (Pseudomonas fluorescens ATCC 39502)
(MSEQ ID NO: 3)
MGSSHHHHHSSMSNVKQQTAAQIVDWLSTLKGDKHQRREDSLSLTANENY
PSALVRLTSGSTAGAFYHCSFPFEPVAGEWHFPEPGHMNAIADQVRDLGK
TLIGAQAFDWRPNGGSTAEQALMLAACKPGEFVHFPHRDGGHFALESLE
QKMGIEIFHLPVNPTSLLDIVAKLDEMVRNPHIRIVILDQSFKLWQPL
AEIRSVLPDSCTLTIDMSHDGGLIMGGVEDSPLSCGADIVHGNTHTKTIPIG
PQKGYIGFKAQHPPLVDTSLVVCPHLQSNCHAEQLPPMWWAFKEMELFG
RDYAAQIVSNAKTLARHLHELGLDVTGESFGFTQTHQVHFVAGDLQKALD
LCVNSLHAGGIRSTNIEIPKPGVHGIIRLGVQAMTRRGMKEKDFEVVARF
IADLYPKKTEPAKVAQQIKFELQAFPLAFLAYSFDNYLDEELLAAVYQGA
QR

SEQ ID NO: 4 ObiH (Pseudomonas fluorescens ATCC 39502)
(MSEQ ID NO: 4)
ATGGGCAGCAGCCATCATCATCATCACAGCAGCATGTCAAACGTGAA
GCAGCAGACGGCGCAAATCGTAGATTGGTTGTCAACCTTGGGGAAG
ATCACCATAACCGCAGGACTCCCTTCACTTACCCTAACGAGAACTAC

-continued

CCGTCGGCATTAGTTCGTTTGACTTCAGGTTTCAGCCCGCGGCATTCTA
CCATTGTTCTTTCCCTTCGAGGTTCTCCCGGGGAGTGGCACTTCCCGG
AGCCCGGTCATATGAATGCAATTGCTGACCAGGTTCTGTGATTTAGGTAAA
ACCTTGATTGGTGCCCGAGGATTGCGACTGGCGTCCAAATGGCGGATCAAC
CGCAGAACAGGCACCTTATGCTGGCAGCATGTAACCCGGGAGAGGGGTTCG
TCCATTTTGCTCACCGCAGCGAGGCCATTTTCGCTTTAGAATCTCTTGCG
CAAAAGATGGGCATCGAAATTTCCACTTGCTGTTAATCCGACCTCTCT
GTTAATCGATGTCGCCAAATTGGATGAAATGGTCCGCCGCAACCCGCATA
TTCGCAATTGTCATTCTTGATCAGAGCTTTAAGTGCCTGGCAACCCCTG
GCCGAGATTCTGTTCAAGTTTACCAGACTCATGCACGTTGACTTATGATAT
GAGTCATGATGGGGATTAATATGGGAGGTGCTTCGATTCCCCCTTA
GCTGTGGAGCTGACATCGTCCACGGCAATACTCACAAGACGATTCTGGA
CCGCAAAAGGGGTATATCGGTTTCAAGTCCGCGCAACATCCTTTATTAGT
CGATACAAGTTTATGGGTATGCCCTCACCTTCAAAGTAACTGCCACGCCG
AGCAGCTGCCCGCATGTGGGTTGCCTTCAAGGAAATGGAATTATTGGAA
CGCGATTACGCTGCCCAAATGTTTCAAACGCAAAAACCTTGGCTCGCCA
TCTGCATGAACTGGGATTGGACGTGACCCGAGAATCCTTTGGATTACACAC
AGACACATCAGGTCCATTTTGCTGTAGGAGATTTACAGAAAGCGCTTGAT
CTTTGTGTGAATTCATTACATGCTGGAGGTATCCGTTCCGACCAATATTGA
AATCCAGGGAAACCAGGAGTACATGGCATTCCGTTAGGCGTCCAAGCGA
TGACTCGTTCGTCATGAAGGAGAAAGACTTTGAGGTGGTCCGCCGTTTT
ATCGCCGATCTGTACTTTAAAAAACGGAACTGCCAAGGTCCGACAGCA
AATTAAGGAATTTTACAGGCGTTTCCGCTTGACCTTTAGCCTACTCAT
TTGATAACTATCTTGATGAAGAATTATTGGCAGCCGTTTACCAGGTTGCG
CAGCGCTGA

Alcohol Dehydrogenase

[0093] Alcohol dehydrogenase (ADH) catalyzes reversible oxidation of alcohol to aldehyde with the simultaneous reduction of NAD(P) to NAD(P)H. ADH includes enzymes falling under EC number 1.1.1.1 and 1.1.1.2. Exemplary ADH may have an amino acid sequence of SEQ ID NO: 5 or a sequence at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% identical thereto. The coding sequence of SEQ ID NO: 5 is shown as SEQ ID NO: 6.

SEQ ID NO: 5 ScADH (Saccharomyces cerevisiae)
(MSEQ ID NO: 5)
MGSSHHHHHSSGLVPRGSHMASMTGGQMGSGMSIPETQKGVIFYESH
GKLEYKDI PVPKPKANELLINVKYSGVCHTDLHAWHGDWPLVPLVGG
HEGAGVVGMGENVKGWIKGDIYAGIKWLNGSCMACEYCELGNESNCPHAD
LSGYTHDGSFQQYATADAVOAAHIPPQGTDLAQVAPILCAGITVYKALKSA
NLMAGHWVAISGAGGLGSLAVQYAKAMGYRVLGIDGGEKKEELFRS IGG

-continued

EVFIDFTKEKDIVGAVLTKATDGGAHGVINVSVEAAIEASTRYVRANGTT
 VLVGMPAGAKCCSDVFNQVVKISISIVGSYVGNRADTREALDFFARGLVKS
 PIKVVGLSTLPEIYEKMEKQIVGRYVVDTSK
 SEQ ID NO: 6 ScADH (*Saccharomyces cerevisiae*)
 (SEQ ID NO: 6)
 ATGGGCAGCAGCCATCATCATCATCACAGCAGCGCCTGGTCCGCGG
 CGGCAGCCATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGCGGAT
 CCATGAGTATTCGGAAACACAGAAGGGTGTATCTTTTATGAGTCACAT
 GGCAAATTGGAGTATAAGGATATTCGGTGCCTAAGCCAAAGGCTAATGA
 ACTGCTCATCAATGTTAAATATTCGGTGTGTGTCACTGATTTGCATG
 CGTGGCATGGCGATTGGCCTCTCCGGTTAAGCTCCCTCTTGTGGGGGG
 CATGAAGGGGCTGGCGTTGTTGTTGGAATGGGCGAAAATGTAAAAGTTG
 GAAAATCGGAGACTATGCAGGAATAAAATGGCTTAACGGGTCAATGATGG
 CCTGCGAATATTGCGAATGGGCAATGAAAGTAACTGCCCGCACGCTGAC
 CTCAGTGGATATACACACGATGGCTCCTTTCCAGCAGTATGCCACGGCTGA
 TGCCGTGCAGCGGCACATATTCCTCAGGGGACTGACCTGGCGCAAGTAG
 CACCAATTCTTTGTGACGGCATCACTGTTTATAAAGCTTTGAAGTCAGCT
 AATTTGATGGCCGCCACTGGGTCCGATTTCCAGGAGCCGCTGGCGGGTT
 GGGGTCAATTAGCGTGCATATATGCAGAAAGCAATGGGCTATCGTGTACTTG
 GGATTGATGGAGGCGAAGGCAAGAGGAATATTCCGGAGCATTTGGCGGT
 GAAGTTTTTATTGACTTTACGAAAGAAAAGATATCGTGGGGCAGTTCT
 GAAAGCAACCGACGGTGGCGCTCATGGCGTGATTAACGTATCCGTGAGTG
 AAGCCGCCATCGAAGCATCTACACGTTACGTACGTGCCAATGGTACAAC
 GTACTTGTGGGATGCCCGCCGGCGCAAGTGTGACGTGACGTTTTTAA
 TCAGTTGTGAAGAGTATTAGCATTTGTTGGTAGCTATGTGGGTAACCGTG
 CCGACACGCGCAGGCACTGGACTTCTTTGACCGGACTGGTAAAAAGC
 CCAATCAAGGTAGTCGGTCTGAGTACGCTGCCAGAGATCTACGAAAAAT
 GGAGAAAGGTGAGATTGTTGGTCCGATGTTGTCGATACATCAAATAA

Formate Dehydrogenase

[0094] Formate dehydrogenase (FDH) catalyzes reversible oxidation of formate to carbon dioxide with the simultaneous reduction of NAD(P) to NAD(P)H. FDH includes enzymes falling under EC number 1.17.1.9 and 1.17.1.10. Exemplary FDH may have an amino acid sequence of SEQ ID NO: 7 or a sequence at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% identical thereto. The coding sequence of SEQ ID NO: 7 is shown as SEQ ID NO: 8.

SEQ ID NO: 7 CbFDH (*Candida boidinii*)
 (SEQ ID NO: 7)
 MKIVLVLYDAGKHAADDEEKLYGCTENKLGIANWLKDOGHELITTSDEKEG
 TSELDKHIPDADIIITPPHPYAITKERLDKAKNLKLVVAVGSGDHDIL

-continued

DYINQTKKKISVLEVTGSNNVSVAEHVVMTMLVLRNFPVPAHEQI INHDW
 EVAAIKDAYDIEGKTIATIGAGRIGYRVLERLLPFNPKELLYDYQALP
 KEAEEKVGARRVENIEELVAQADIVTVNAPLHAGTKGLINKELLSKFKKG
 AWLVNTARGAICVAEDVAAALESGOLRGYGGDVVFPQPAKDPWRDMRN
 KYGAGNAMTPHYSGTTLDAQTRYAEGTKNILESFFTKFDYRPODIILLN
 GEYVTKAYGKHKKLEHHHHHH
 SEQ ID NO: 8 CbFDH (*Candida boidinii*)
 (SEQ ID NO: 8)
 ATGAAGATCGTGTAGTTTTGTACGATGCCGAAAACACCGCGCAGATGA
 GGAGAAGTGTACGGATGTACCGAAAATAAGTTGGGTATCGCGAATGGT
 TAAAGGACCAAGGTCATGAGCTGATCACAACATCAGATAAAGAAGGTGAG
 ACTTCGGAGCTGGATAAGCATATTCAGATGCCGACATTATTATTACAAC
 ACCTTTTACCCTGCGTACATCACGAAAGAGCGCCTTGATAAGGCTAAAA
 ATCTGAAGCTTGGTGGTGGCTGGAGTCGGATCGGATCACATTGACTTA
 GATTACATCAATCAAACGGGAAAGAAGATTTCCGTGTTGGAAGTAACGGG
 AAGTAACGTCGTGTCGGTTGCGGAGCACGTCGTGATGACAAATGTTAGTTT
 TAGTCCGTAACCTCGTCCCGCGCACGAAATAATCAACCATGACTGG
 GAGGTGGCCGCTATTGCCAAGGACGCATACGACATTGAAGGGAAGACTAT
 CGCGACGATTGGTGTGGTCCGATCGGTTACCGTGTCTGGAGCGCTTAT
 TGCCGTTTAAATCCAAAAGAGCTGTATACTATGATTACCAAGCACTGCCA
 AAAGAGGCTGAGGAGAAAGTCGGCGCCCGTCGTGTAGAGAATATTGAAGA
 ATTGGTCGCCCAAGCTGACATCGTAACAGTAAACGCGCCATTGCACGCCG
 GCACAAAGGGTTTGATTAACAAGGAGTTACTGAGCAAATCAAAAAAGGA
 GCTTGGCTGTAAACACGTCTCGCGCGCAATTTGCGTAGCAGAAGACGT
 CGCTGCTGCCCTGGAGTCAGGACAGTGTGCGCGGTATGGAGGTGACGTAT
 GGTTCCACAGCCAGCTCCTAAGGATCACCTTGGCGCGATATCGCTAAC
 AAGTACGGTGTGAAACGCAATGACGCCTCATTATTCCGGTACGACGTT
 GGATGCACAAACTCGTTATGCAGAGGGCACAAAAAATATTTTGGAACTCT
 TTTTACAGGCAAGTTCGACTATCGTCTCAAGATATCATTCTGCTTAAT
 GGGGAGTATGTGACAAAGGCATACGGTAAGCATGATAAGAACTCGAGCA
 CCATCACCATCACCATTGA

[0095] Other enzymes may also be used to catalyze the reversible reduction of NAD(P) to NAD(P)H, including glucose dehydrogenases (“GDH’s”, E.C. 1.1.1.47; CAS No. CAS No. 9028-53-9) such as the glucose dehydrogenase from *Bacillus megaterium* (“BmGDH”). See Xiu et al. (2022) “Multi-enzyme cascade for sustainable synthesis of L-threo-phenylserine by modulating aldehydes inhibition and kinetic/thermodynamic controls,” *Systems Microbiology and Biomanufacturing* 2:705-715. GDH’s in general and BmGDH in particular are commercially available from several national and international sources. For example, BmGDH is commercially available from Sigma-Aldrich (St. Louis, Missouri, USA) as a lyophilized powder, catalog no. G7653. Likewise, glucose dehydrogenase from *Pseudomonas* sp. is also commercially available from Sigma-Aldrich, catalog no. 19359.

SEQUENCE LISTING

Sequence total quantity: 8

SEQ ID NO: 1 moltype = AA length = 351
FEATURE Location/Qualifiers
source 1..351
mol_type = protein
organism = Thermotoga maritima

SEQUENCE: 1
MIDLRSDTVT KPTEEMRKAM AQAIEVGDVV GEDPTINELE RLAETFGKE AALFVPSGTM 60
GNQVSIIMHT QRGDEVILEA DSHIFWYEVG AMAVLSGVMP HPVPGKNGAM DPDDVRKAIR 120
PRNIHFPRTS LIAIENTHNR SGRVVPLEN IKEICTIAKE HGINVHIDGA RIFNASIASG 180
VPVKEYAGYA DSVMPCLSKG LCAPVGSVVV GDRDFIERAR KARKMLGGGM RQAGVLAAG 240
IIALTKMVDL LKEDHENARF LALKLKEIGY SVNPEVKTN MVILRTDNLK VNAHGFIKAL 300
RNSGVLNAV SDTEIRLVTH KDVSRRNDIEE ALNIFEKLEFR KFSLEHHHHH H 351

SEQ ID NO: 2 moltype = DNA length = 1056
FEATURE Location/Qualifiers
source 1..1056
mol_type = genomic DNA
organism = Thermotoga maritima

SEQUENCE: 2
atgatcgatc tcagggtccga caccggtaca aaaccaacag aagagatgag aaaagccatg 60
gcacaggctg aggtgggaga cgatgtgtac ggagaagatc caaccatcaa cgaactcgaa 120
aggctcgccg cagagacctt tggaaaggaa gcggctctct ttgtaccctc cggcaccatg 180
ggaaatcaag tgagcataat ggctcacacc cagagggcg atgaagtgat actggaggca 240
gacagccaca tctctcggtg cgaggtcggg gccatggcgg tctctcctcg agtcatgccc 300
catcctgtac ctggaaaaaa tggagccatg gaccocgatg atgtgaggaa ggccataaga 360
cccagaaaca tacacttccc cagaacttcg ctcatgcca tcgaaaaaac acacaacctg 420
tccggtggaa gagtgggtccc gcttgaaaac ataaaagaga tttgacatg agccaagaa 480
cacggcataa acgttcacat agatgggtgc aggatcttca acgctcaat cgcttcaggt 540
gttcccgatg aggagtagcg cgggtacgcc gattccgtga tgttctgtct ttcaaaagg 600
ctctcgccac ccgctcggtt ggtggttga ggagacagg acttcataga aagagcgaga 660
aaggcgagaa agatgctcgg tggagggatg agacaggcag gtgttctcgc tgcccgctga 720
ataatcgctc tgacaaagat ggtagatcga ttgaaagaag atcatgaaaa cgccagatt 780
ctcgccctga agttgaaaga aatagggtac tccgtgaatc ccgaagatgt gaaaaccaac 840
atgggtgattc tgaggaccga caacctgaag gtgaacgcgc acgggttcat agaagcgctc 900
agaaacagcg ggggtctcgc gaacgcccga tccgacacgg agatcagact ggtaaccocac 960
aaagacgctt caagaaaacga catagaagag gctctgaaca tcttcgaaaa actcttcaga 1020
aaattctccc tcgagcacca tcaccatcac cattga 1056

SEQ ID NO: 3 moltype = AA length = 452
FEATURE Location/Qualifiers
source 1..452
mol_type = protein
organism = Pseudomonas fluorescens

SEQUENCE: 3
MGSSHHHHHH SSMNVKQQT AQIVDWLSS LKGDHQYRED SLSLTANENY PSALVRLTSG 60
STAGAFYHCS FPFVVPAGEW HFPEPGHMNA IADQVRDLGK TLIGAQAQFDW RPNNGGSTAEQ 120
ALMLAACKPG EGFVHFARLD GGHFALES LA QKMGIIEIHL PVNPTSLIID VAKLDEMVR 180
NPHIRIVILD QSKFLRWQL AEIRSVLPDS CTLTYDMSHD GGLIMGGVFD SPLSCGADIV 240
HGNTHTKIPG PQKGYIFGKS AQHPLLVDTS LWVCPHLQSN CHABQLPPMW VAFKEMELFG 300
RDYAAQIVSN AKTLARLHE LGLDVTGESF GFTQTHQVHF AVGLDLQKALD LCVNSLHAGG 360
IRSTNIEIPG KPGVHGIRLG VQAMTRRGMK EKDFEVVARF IADLYFKKTE PAKVAQQIKE 420
FLQAFPLAPL AYSFDNYLDE ELLAAVYQGA QR 452

SEQ ID NO: 4 moltype = DNA length = 1359
FEATURE Location/Qualifiers
source 1..1359
mol_type = genomic DNA
organism = Pseudomonas fluorescens

SEQUENCE: 4
atgggcagca gccatcatca tcatcatcac agcagcatgt caaacgtgaa gcagcagacg 60
gcgcaaatcg tagattgggt gtcatacaacc ttgggggaaag atcaccaata ccgagaggac 120
tccctttcac ttaccgctaa cgagaactac ccgtcggcat tagttcgttt gacttcaggt 180
tcgaccgccc gcgcattcta ccattgttct ttccccttcg aggttctctgc cggggagtg 240
cacttcccgg agcccgtca tatgaatgca attgctgacc aggttctgta tttaggtaaa 300
accttgattg gtgcccagcg attcgactgg cgtccaatg cgggatcaac cgagaaacag 360
gcacttatgc ttggcagcatg taaaccggga gagggttctg tccattttgc tcaccgcgac 420
ggaggccatt tcgctttaga atctcttgcg caaaaagatgg gcatacgaat ttccacttg 480
cctgttaatc cgacctctct gttaatcgat gtcgcaaat tggatgaaat ggtccgccc 540
aacccgcata ttcgatttgt cattcttgat cagagcttta agctgcgctg gcaaccocctg 600
gccagatc gttcagtttt accagactca tgcacgttga cttatgatag gagtcatgat 660
gggggattaa ttatgggagg tgccttcgat tccccctta gctgtggagc tgacatcgtc 720
cacggcaata ctccacaagc gatctctgga ccgcaaaaagg ggtatatacgg tttcaagtcc 780
gcgcaacatc cttattatag cgatacaagt ttatgggtat gccctcacct tcaaaagtaac 840
tgccacgccc agcagctgcc gccgatgtgg gttgccttca aggaaatgga attatttga 900

-continued

```

cgcgattacg ctgccc aaat tgtttcaaac gcaaaaacct tggctcgcca tctgcatgaa 960
ctgggattgg acgtgaccgg agaatccttt ggattcacac agacacatca ggtccathtt 1020
gctgtaggag atttacagaa agcgtttgat ctttgtgtga attcattaca tgctggaggt 1080
atccgttcga ccaatattga aatcccaggg aaaccaggag tacatggcat tcgcttaggc 1140
gtccaagcga tgactcgtcg tggcatgaag gagaaagact ttgaggtggt cgcccgtttt 1200
atcgccgacg tgtactttaa aaaaacggaa cctgccaagg tcgcacagca aattaaggaa 1260
tttttacagg cgtttccgct tgcaccttta gcctactcat ttgataacta tcttgatgaa 1320
gaattattgg cagccgttta ccagggtgcg cagcgctga 1359

```

```

SEQ ID NO: 5          moltype = AA length = 382
FEATURE              Location/Qualifiers
source                1..382
                     mol_type = protein
                     organism = Saccharomyces cerevisiae

```

```

SEQUENCE: 5
MGSSHHHHH  SSSLVPRGSH MASMTGGQQM GRGSM SIPET QKGVI FYESH GKLEYKDIPV 60
PKPKANELLI NVKYSV GCHT DLHAWHGDWP LPVKLPLVGG HEGAGVVVGM GENVKGWKIG 120
DYAGIKWLNG  SCMA CEYCEL GNESNCPHAD LSGYTHDGSF QQYATADAVQ AAHIPQGTDL 180
AQVAPILCAG ITVYKALKSA NLMAGHWVAI SGAAGGLGSL AVQYAKAMY RVLGIDGEGE 240
KEELFRSIGG EVFIDFTKEK DIVGAVLKAT DGGAHGVINV SVSEAAIEAS TRYVRANGTT 300
VLVGM PAGAK CCSDFV NQV KSISIVGSYV GNRADTREAL DFFARGLVKS PIKVVGLSTL 360
PEIYKMEKRG QIVGRYVVDV SK 382

```

```

SEQ ID NO: 6          moltype = DNA length = 1149
FEATURE              Location/Qualifiers
source                1..1149
                     mol_type = genomic DNA
                     organism = Saccharomyces cerevisiae

```

```

SEQUENCE: 6
atgggcagca gccatcatca tcatcatcac agcagcggcc tgggtgccgc cggcagccat 60
atggctagca tgactggtgg acagcaaatg ggtcgccgat ccatgagtat tccggaaaca 120
cagaagggtg ttatctttta tgagtcacat ggcaaatgg agtataagga tattccggtg 180
cctaagccaa aggtaatga actgctcatc aatgttaaat attccggtgt gtgtcacact 240
gatttgcatg cgtggcatgg cgattggcct cttccggtta agctccctct tgtggggggg 300
catgaagggg ctggcgttgt tgttgaatg ggcgaaaatg taaaagggtg gaaaaatcgg 360
gactatgcag gaataaaatg gcttaacggg tcatgtatgg cctgcgaata ttgcgaattg 420
ggcaatgaaa gtaactgccg gcaagcgtgc ctcagtggtg atacacacga tggctcctt 480
cagcagtatg ccacggctga tgcctgcaa gcggcacata ttccctcagg gactgacctg 540
gcgcaagt ag caccaattct ttgtgcaggc atcaactgtt ataaagcttt gaagttagct 600
aatttgatgg ccggccactg ggtcgcgatt tcaggagccg ctggcgggtt ggggtcatta 660
gccgtgcaat atgcgaaagc aatgggctat cgtgtacttg ggattgatgg aggcgaaggc 720
aaagaggaat tattccggag cattggcggg gaagttttta ttgactttac gaaagaaaaa 780
gatatcgtgg gggcagttct gaaagcaacc gacggtggcg ctcatggcgt gattaacgta 840
tccgtgagtg aagccgccat cgaagcatct acacgttacg tacgtgcaa tgggtacaact 900
tactttgtt ggatgcccgc cggcgcgaag tgttcagtg acgtttttaa tcaggttggtg 960
aagagtatta gcattgttgg tagctatgtg ggtaaccctg ccgacacgcg cgaggcactg 1020
gacttctttg cagcgggact ggtaaaaaag ccaatcaagg tagtcggtct gactacgctg 1080
ccagagatct acgaaaaaat ggagaaaggt cagattgttg gtcggtatgt tgcgataca 1140
tcaaaataa 1149

```

```

SEQ ID NO: 7          moltype = AA length = 372
FEATURE              Location/Qualifiers
source                1..372
                     mol_type = protein
                     organism = Candida boidinii

```

```

SEQUENCE: 7
MKIVLVLYDA GKHAAD EKL YGCTENK LGI ANWLK DQGHE LITTS DKEGE TSELDKHIPD 60
ADIIITTPFH PAYITKERLD KARNLKL VVV AGVGS DHIDL DYINQ TGKKI SVLEVTGSNV 120
VSVAEHV VMT MLVLRN FVP AHEQI INHDW EVAAI AKDAY DIEGK TIATI GAGRIGYRVL 180
ERLLPFNPKE LLYDYQALP KEAEEKV GAR RVENIEELVA QADIVTVNAP LHAGTKGLIN 240
KELLSKFKKG AWLVNTARGA ICVAEDVAAA LESGQLRGYV GDVWFPPQAP KDHFWRDMRN 300
KYGAGNAMTP HYSGTTLDAQ TRYAEGTKNI LESFPTGKFD YRPQDIILLN GEYVTKAYGK 360
HDKKLEHHHH HH 372

```

```

SEQ ID NO: 8          moltype = DNA length = 1119
FEATURE              Location/Qualifiers
source                1..1119
                     mol_type = genomic DNA
                     organism = Candida boidinii

```

```

SEQUENCE: 8
atgaagatcg tggtagtttt gtacgatgcc ggaaaacacg cggcagatga ggagaagttg 60
tacggatgta ccgaaaataa gttgggtatc gcgaattggt taaaggacca aggtcatgag 120
ctgacacaaa catcagataa agaaggtgag acttcggagc tggataagca tattccagat 180
gccgacatta ttattacaac accttttcac cctgcgtaca tcacgaaaga gcgccttgat 240
aaggctaaaa atctgaagct ttgggtgggt gctggagtcg gatcggatca cattgactta 300
gattacatca atcaaacggg aaagaagatt tccgtggttg aagtaacggg aagtaacgctc 360

```

- continued

```

gtgtccgttg cggagcacgt cgtgatgaca atgttagttt tagtgcgtaa cttegtgccc 420
gcgcacgaac aaattatcaa ccatgactgg gaggtggccg ctattgcaa ggacgcatac 480
gacattgaag ggaagactat cgcgacgatt ggtgctggtc gcatcggtta ccgtgtcctg 540
gagcgcttat tgccgtttaa tccaaaagag ctgttatact atgattacca agcaactgcca 600
aaagaggctg aggagaaagt cggcgccctg cgtgtagaga atattgaaga attggtcgcc 660
caagctgaca tcgtaacagt aaacgcgcca ttgcacgccc gcacaaaaggg ttgtattaac 720
aaggagtac tgagcaaat caaaaagga gcttggttg taaacactgc tcgcgggcga 780
atgtgcgtag cagaagact cgtgctgccc ctggagtcag gacagttgcg cgggtatgga 840
ggtgacgtat ggttcccaca gccagctcct aaggatcacc cttggcgcca tatgctgaac 900
aagtaaggctg ctgaaaacgc aatgacgcct cattattccg gtacgacgtt ggatgcacaa 960
actcgttatg cagagggcac aaaaaatatt ttggaatcct ttttcacagg caagttcgac 1020
tatcgtcctc aagatatcat tctgcttaat ggggagtatg tgacaaaaggc atacggtaag 1080
catgataaga aactcgagca ccatcaccat caccattga 1119

```

What is claimed is:

1. A method to make an amino acid having a tertiary alcohol sidechain, the method comprising reacting a pyridoxal-phosphate (Nil-dependent enzyme selected from the group consisting of an L-threonine aldolase, an L-threonine transaldolase, or a combination thereof, with a ketone substrate and a primary or secondary β -hydroxy α -amino for a time, at a temperature, and at a pH wherein the reaction yields a tertiary β -hydroxy α -amino acid.

2. The method of claim 1, wherein the PLP-dependent enzyme is an L-threonine aldolase.

3. The method of claim 2, wherein the L-threonine aldolase has an amino acid sequence at least 80% identical to SEQ. ID. NO: 1.

4. The method of claim 2, wherein the L-threonine aldolase has an amino acid sequence at least 90% identical to SEQ. ID. NO: 1.

5. The method of claim 2, wherein the L-threonine aldolase has an amino acid sequence at least 95% identical to SEQ. ID. NO: 1.

6. The method of claim 1, wherein the PLP-dependent enzyme is an L-threonine transaldolase.

7. The method of claim 6, wherein the L-threonine transaldolase has an amino acid sequence at least 80% identical to SEQ. ID. NO: 3.

8. The method of claim 6, wherein the L-threonine transaldolase has an amino acid sequence at least 90% identical to SEQ. ID. NO: 3.

9. The method of claim 6, wherein the L-threonine transaldolase has an amino acid sequence at least 95% identical to SEQ. ID. NO: 3.

10. The method of claim 3, wherein the PLP-dependent enzyme is ObiH.

11. The method of claim 1, wherein the time is from about 1 hour to about 12 hours, the temperature is from about 30° C. to about 50° C., and the pH is from about 6 to about 8.

12. The method of claim 1, further comprising reacting the PLP-dependent enzyme and the ketone substrate in the presence of a reducing system.

13. The method of claim 1, wherein the ketone substrate further comprises an electron-withdrawing group.

14. The method of claim 13, wherein the electron-withdrawing group is selected from the group consisting of halo,

haloalkyl, $-\text{NH}_3^+$, $-\text{NO}_2$, $-\text{CH}=\text{CH}_2$, $-\text{CN}$, $-\text{SO}_3\text{H}$, $-\text{C}(=\text{O})\text{OH}$, $-\text{C}(=\text{O})\text{H}$, $-\text{C}(=\text{O})\text{R}$, $-\text{C}(=\text{O})\text{OR}$, and $-\text{NR}_3^+$, where R is alkyl.

15. The method of claim 1, wherein the primary or secondary β -hydroxy α -amino acid is selected from the group consisting of serine, threonine, and 3-phenyl serine.

16. The method of claim 1, further comprising reducing aldehyde by-products formed in the reaction.

17. The method of claim 16, comprising reducing the aldehyde by-products by contacting them with an alcohol dehydrogenase in the presence of NAD(P)H, wherein the alcohol dehydrogenase reduces at least a portion of the aldehyde by-products, and NAD(P)+ is generated.

18. The method of claim 17, wherein the alcohol dehydrogenase has an amino acid sequence at least 80% identical to SEQ. ID. NO: 5.

19. The method of claim 17, wherein the alcohol dehydrogenase has an amino acid sequence at least 90% identical to SEQ. ID. NO: 5.

20. The method of claim 17, wherein the alcohol dehydrogenase has an amino acid sequence at least 95% identical to SEQ. ID. NO: 5.

21. The method of claim 17, further comprising regenerating NAD(P)H from the NAD(P)+ by contacting the NAD(P)+ with a formate dehydrogenase or a glucose dehydrogenase.

22. The method of claim 21, wherein the formate dehydrogenase has an amino acid sequence at least 80% identical to SEQ. ID. NO: 7.

23. The method of claim 21, wherein the formate dehydrogenase has an amino acid sequence at least 90% identical to SEQ. ID. NO: 7.

24. The method of claim 21, wherein the formate dehydrogenase has an amino acid sequence at least 95% identical to SEQ. ID. NO: 7.

25. The method of claim 17, wherein the NAD(P)+ is contacted with a glucose dehydrogenase.

26. The method of claim 25, wherein the glucose dehydrogenase is derived from *Bacillus megaterium* or *Pseudomonas* sp.

* * * * *