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Combining Aldolases and Transaminases for the Synthesis of 2-Amino-4-Hydroxybutanoic acid

Karel Hernandez[†], Jordi Bujons[†], Jesús Joglar[†], Simon J. Charnock[‡], Pablo Domínguez de María[‖], Wolf Dieter Fessner[§] and Pere Clapés*[†]

†Instituto de Química Avanzada de Cataluña-IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

‡Prozomix Ltd., Station Court, Haltwhistle, Northumberland, NE49 9HN, UK.

§Institut für Organische Chemie und Biochemie, Technische Universität Darmstadt, Alarich-Weiss-Str. 4, 64287 Darmstadt, Germany.

‖Sustainable Momentum S.L. Ap. Correos 3517. 35004 Las Palmas de Gran Canaria, Spain.

ABSTRACT: Amino acids are of paramount importance as chiral building blocks of life, for drug development in modern medicinal chemistry, and for the manufacture of industrial products. In this work, the stereoselective synthesis of (*S*)- and (*R*)-2-amino-4-hydroxybutanoic acid was accomplished using a *Systems Biocatalysis* approach comprising a biocatalytic one-pot cyclic cascade by coupling of an aldol reaction with an ensuing stereoselective transamination. A Class II pyruvate aldolase from *E. coli*, expressed as a soluble fusion protein, in tandem with either an (*S*)- or (*R*)-selective, pyridoxal phosphate-dependent, transaminase were used as catalysts to realize the conversion, with formaldehyde and alanine being the sole starting materials. Interestingly, the Class II pyruvate aldolase was found to tolerate formaldehyde concentrations of up to 1.4 M. The cascade system was found to reach product concentrations for (*S*)- or (*R*)-2-amino-4-hydroxybutanoic acid of at least 0.4 M, rendering yields between 86% and >95%, respectively, productivities of >80 g L^{-1} d^{-1}, and *ee* >99%. KEYWORDS Biocatalysis • Substrate Cycling • Green Chemistry • Aldolases • Transaminases.

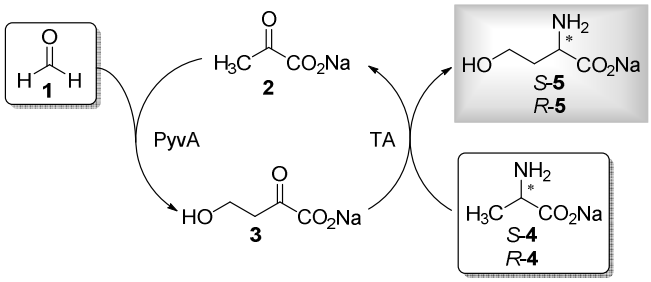
INTRODUCTION

Amino acids are compounds of paramount importance for living organisms, as building blocks for protein and peptide synthesis, chiral intermediates in modern medicinal chemistry and valuable building blocks in the manufacture of industrial products.[1] An interesting example is 2-amino-4-hydroxybutanoic acid (i.e. homoserine), a non-canonical amino acid homologous to L-serine by insertion of an additional methylene unit into the side chain and an important metabolic intermediate.[2] It also constitutes a valuable building block for the synthesis of 3-hydroxypropionic acid and 1,3-propanediol, important starting materials in the manufacture of biocompatible plastic and polytrimethylene terephthalate (PTT), among other industrial products.[3] Furthermore, L-homoserine lactone derivatives are useful compounds for studying the quorum sense signaling (QSS) mechanism (i.e. cell-to-cell communication) and indeed as potential QSS inhibitors in new antimicrobial strategies against pathogenic Gram-negative and Gram-positive bacteria (anti-virulence intervention strategy).[1c,4]

Industrial production of amino acids, including L-homoserine, has to date predominantly been achieved via microbial fermentation (i.e. cell factories).[2c,2e,3b,5] However, drawbacks of this technology include the unpredictable physiological behavior of cellular production systems upon engineering of elusive metabolic pathways and a rather limited flexibility towards the synthesis of structurally diverse non-natural products.[6] By contrast, isolated enzyme systems for *in vitro* production (i.e. *Systems Biocatalysis*)[6] offer simplicity and adaptability, because these are unrestrained from problems arising from the intricate nature of cellular metabolic pathways. In addition, such systems benefit from the ever increasing number and diversity of available native enzymes, as well as their engineered variants, active towards a broad variety of substrate analogues. From a practical point of view, *in vitro* systems are easier to manage and monitor because of their lower complexity.[5b,6-7] Needless to say that the concept of *Systems Biocatalysis* shares all the synthetic and environmental advantages that have become the hallmark of commercial biocatalytic processes.

RESULTS AND DISCUSSION

A stereoselective biocatalytic one-pot cascade methodology[8] was envisioned for the synthesis of both enantiomeric forms of homoserine (*S*-**5** and *R*-**5**), comprising cyclic aldol addition and subsequent reductive amination reactions, with effective pyruvate (**2**) recycling (Scheme 1): *i*) aldol addition of **2** to formaldehyde (**1**); *ii*) reductive amination of aldol adduct **3**, using alanine (*S*-**4** or *R*-**4**) as the amine donor, to produce *S*-**5** or *R*-**5**, respectively, with concomitant release of **2**, to be recycled by recurrent aldol addition, thus favoring the reductive amination equilibrium towards **5**. Notably, by applying such a synthetic cycle the approach can be implemented with readily available and inexpensive substrates (in this example, alanine and formaldehyde), while requiring just catalytic amounts of pyruvate to trigger the overall pathway.



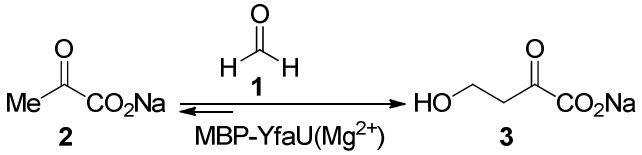
Scheme 1. One-pot biocatalytic cascade synthesis of *S*-5 or *R*-5 with substrate recycling, starting from formaldehyde (1) and *S*-4 or *R*-4, respectively. PyvA: pyruvate aldolase, TA: (*S*)- or (*R*)-selective transaminase.

A viable commercial process requires an aldolase tolerating high concentrations of aldehyde substrates, which are strongly electrophilic, a transaminase that is active and selective under similar reaction conditions, and for the overall biotransformation to be cost-effective.

Biochemical studies have been reported for the *in vitro* enzymatic aldol addition of pyruvate to formaldehyde using a mammalian hydroxyoxobutyrate aldolase (EC 4.1.2.1), which is a Class I aldolase that acts via covalent substrate activation by Schiff base formation at a lysine residue in the active site.[9] However, such enzymes easily become inactivated by nonspecific reactions involving the essential lysine residue in the presence of strong electrophiles, such as formaldehyde, even at low concentrations.[10] In contrast, Class II pyruvate aldolases utilize a divalent metal ion as an essential cofactor to promote the enolization of the pyruvate nucleophile and do not possess a sensitive active-site lysine, and thus appeared potentially more suitable for the target reaction.[11] Among them, YfaU (EC 4.1.2.53) was selected from the genome of *E. coli* K-12.[12] YfaU is known to catalyze the reversible cleavage of pyruvate from 2-keto-3-deoxy-L-rhamnonate, 2-keto-3-deoxy-L-lyxonate and 4-hydroxy-2-ketopentanoic acid to furnish L-lactaldehyde, hydroxyethanal, and ethanal, respectively. Nevertheless, hitherto reported studies regarding this enzyme concern fundamental biochemical studies only with little or no direct connection to any potential synthetic utility.[12] The *rhmA* gene encoding the YfaU protein was initially cloned in the pQE40 expression vector (Qiagen) to generate a fusion protein with very soluble dihydrofolate reductase (DHFR) at the N-terminus.[13] However, under these conditions it was found that DHFR-YfaU was mostly expressed as insoluble inclusion bodies (Figure S2A). This problem was circumvented by expressing YfaU as a fusion protein with maltose binding protein (MBP) from *E. coli* at the N-terminus (MBP-YfaU), along with a His_6-tag for affinity purification (Figure S2B) (yield 180 mg of MBP-YfaU L^{-1} of bacterial growth medium). While the structure of native YfaU is hexameric (a trimer of dimers, the dimer being the minimal repetitive unit),[12a] the MBP-YfaU fusion protein only forms a dimer as determined by gel filtration (see SI). It appears that the MBP fusion prevented the hexameric biological assembly but not functional dimer formation. Recombinant MBD-YfaU apparently exhibited a high affinity for its natural Mg^{2+} cofactor from the bacterial growth medium, which predominantly remained bound in the active site even subsequent to purification via IMAC and dialysis against 10 mM Na^+ phosphate buffer, pH 7.0 (MBP-YfaU(Mg^{2+}) 2.5 U mg^{-1}, see SI Table S1).

NMR data confirmed that the enzymatic aldol addition of **2** to **1** yielded desired intermediate **3**. Remarkably, while screening the best reaction conditions for the critical carboligation step, MBP-YfaU(Mg^{2+}) retained activity at substrate concentrations of >1 M for both **1** and **2** (IC_{50} [**1**] = 1.4 M) (Figure 1), leading to an outstanding productivity of 140 g L^{-1} d^{-1} of intermediate **3** at equimolar [**1**] = [**2**] = 1 M even under preliminary non-optimized conditions. This observation was very surprising, given that strongly electrophilic aldehydes such as **1** often denature enzymes even at low concentration.

Incubation with other divalent cations showed that MBP-YfaU liganded with Co^{2+} or Ni^{2+} exhibited ~3 or 2-fold higher activity (see SI and Table S1), respectively. As observed for other Class II aldolases, some non-physiological metals, particularly Co^{2+} and Ni^{2+}, can improve the activity and/or even catalyze side reactions by taking advantage of the reactive intermediates generated by the catalytic machinery.[12a,14] No product formation was observed in control experiments performed under the same conditions without enzyme, or in the presence of EDTA. Control reactions using bovine serum albumin as non-catalytic protein in the presence of Mg^{2+} or Ni^{2+}, resulted in a background level of <0.5% conversion after 24 h.



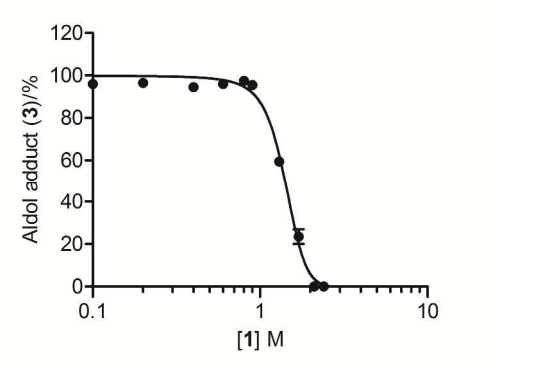


Figure 1. Influence of the concentration of **1** on the yield of **3** in the aldol addition of **2** to **1** catalyzed by MBP-YfaU(Mg^{2+}). Conditions: [**2**] = [**1**], 50 mM Na^+ phosphate buffer, pH 7.0, and 24 h of reaction (experimental details in SI).

In addition, the formation of a by-product **6** was also observed (Scheme 2), which arose from the subsequent addition of product **3** to a second equivalent of **1**. For example, in batch conditions, with a 1:1 molar ratio of substrates and MBP-YfaU(Mg^{2+}) as biocatalyst (specific conditions: [**2**] = [**1**] = 1 M, 50 mM Na^+ phosphate buffer, pH 7, MBP-YfaU(Mg^{2+}) (31 mg, 77 U, 10 U mL^{-1}) and 24 h of reaction at 25°C), a mixture of **3**:**6** in a ratio of 9:1 (by NMR analysis) was observed. Interestingly, it was found that this side reaction was mainly catalyzed by Ni^{2+} and Co^{2+} ions themselves, with no **6** produced with just Mg^{2+} present (Figure S7). However, MBP-YfaU(Mg^{2+}) did catalyze the secondary reaction, but to a much lower extent (<10%) (Figure S6). Thus, MBP-YfaU(Mg^{2+} or Zn^{2+}) were the most selective biocatalysts towards the synthesis of **3** (Figure S6), with the former being selected for the purpose of this work.

Scheme 2. Sequential catalysis by MBP-YfaU(Mg^{2+}) and Ni^{2+} or Co^{2+} in the aldol addition of 2 to 1 and subsequent aldol addition of 3 to a second equivalent of 1 to yield 6.

The next step was the stereoselective enzymatic reductive amination of **3** (Scheme 1). To this end, we screened a library of 51 individual pyridoxal phosphate (PLP)-dependent transaminases, provided by Prozomix Ltd. as lyophilized crude cell free extracts, using *S*-**4** and *R*-**4** as amine donors (Figure 2). From the best hits showing high conversion rates (i.e. >80% after 24 h, Figure 2), enzyme TA51 from *Vibrio fluvialis* (*ee* >99%) was selected for further studies. The thermodynamic equilibrium constant for the desired reaction was 0.77 (see SI), which compares favorably with other enzymatic reductive aminations.[8a,15]

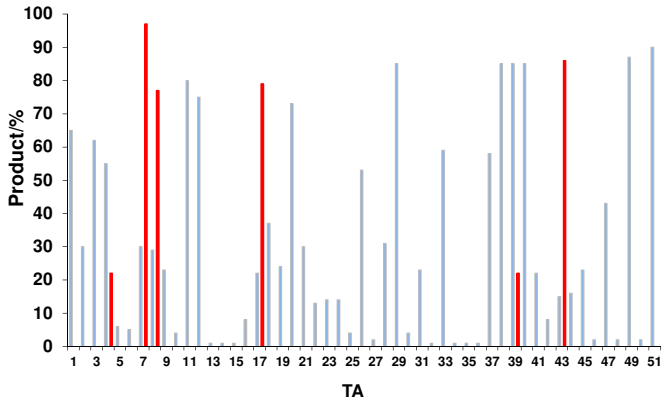


Figure 2. Screening of transaminases (Prozomix) for the reductive amination of **3** to *S*-**5** (blue) and *R*-**5** (red) at 24 h (full conditions in SI). TA51 corresponds to transaminase from *Vibrio fluvialis* (supplied by Prozomix Ltd).

With a suitable transaminase identified, we next investigated the optimal [**2**] required for effective substrate cycling within the biocatalytic cascade (Scheme 1, Figure S10). To trigger the biocatalytic cascade, in principle the initial amount of **2** should be kept low to overcome the thermodynamic limitations of the reductive amination. Indeed, a reaction comprising ≤0.05 mmol of **2**, 0.1 mmol of *S*-**4**, portion wise addition of **1** (0.1 mmol, 0.0125 mmol per hour, 8 additions), MBP-YfaU(Mg^{2+}) (10 U) and TA51 (2.8 U) successfully demonstrated the recycling of **2** as the deaminated component produced in the transamination.

We next observed that the lower the pyruvate:alanine **2**:*S*-**4** ratio (e.g. ≤0.5) was chosen, the lower the amount of **1** was tolerated by the system in each addition (Figure 3). Presumably, inhibitory or inactivation effects are the most plausible explanation, as indicated by the experiments conducted ad hoc (Figure S11). However, reducing the amount of **1** was also found to be detrimental, presumably because of the thermodynamic limitations for the reductive amination that are caused by an excess of unreacted pyruvate.

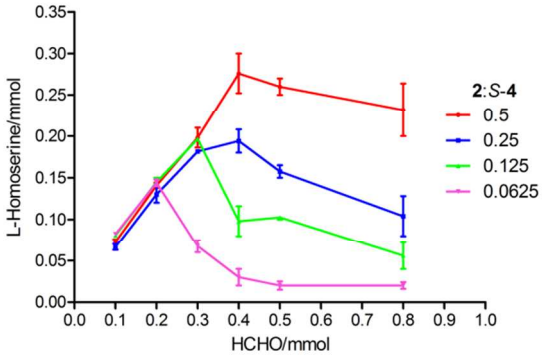


Figure 3. Effect of the total amount of **1** added on the *S*-**5** formed at 24 h and at different **2**:*S*-**4** ratios. Conditions: *S*-**4** (0.1 mmol), **1** added portion wise (0.0125 to 0.1 mmol per

hour, 8 additions), with MBP-YfaU(Mg^{2+}) and TA51 as biocatalysts. V = 1 mL.

A kinetic analysis of the compounds that participate in the cascade starting from **2**, *S*-**4** and **1** (Figure 4) indicates that: a) the first additions of **1** were rapidly converted to **3** consuming the starting quantity of pyruvate; b) *S*-**5** was first detected when the aldol adduct reached a level of ~50 mM (0.05 mmol); c) after that stage, there is an effective recycling of **2**, which favors conversion towards the thermodynamic equilibrium and minimizes TA inactivation by **1**; d) **3** accumulated to a final concentration of ~200 mM (0.2 mmol); and e) under these conditions a 75% yield of *S*-**5** was achieved. Lower yields were obtained (Figures S12A-B) when less **1** was employed; when adding a cumulative 0.2 mmol of **1** (i.e. effectively performing a one-pot, two-step process with no pyruvate recycling) during a 4 h reaction, the yield fell to just 30%, whereas with a cumulative 0.4 mmol of **1** over 8 h, the yield was reduced to 56%.

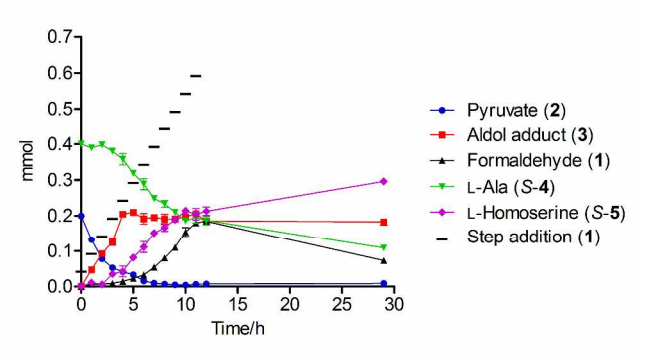


Figure 4. Progress of the biocatalytic cyclic cascade synthesis of *S*-**5** with **1** added portion wise. Conditions: **2** (0.2 mmol), *S*-**4** (0.4 mmol), **1** (12 additions of 0.05 mmol at one hour intervals, total 0.6 mmol), MBP-YfaU(Mg^{2+}) (4 mg, 10 U) and TA51 (20 mg, 2.8 U) in 50 mM Na^+ phosphate buffer, pH 7.0, V = 1 mL. Samples were taken immediately before the addition of **1**. Dashes: cumulative addition of **1**.

We further tried to optimize the system by investigating the effect of continuous feeding of **1**, using a syringe pump, on the yield of *S*-**5** when using transaminases TA20, 26, 39 and 51 (Table 1). Remarkably, when using TA39 the yield of *S*-**5** increased to 86% (*ee* >99% by chiral HPLC) with a cumulative formaldehyde addition of 8.4 mmol (Figure 5), implying a net quantity of 3 mmol of pyruvate recycled, given the final amount of **2** was just 0.06 mmol. To further demonstrate the utility of this system, the synthesis of *R*-**5** was also accomplished in near quantitative yields (*ee* >99%) when employing TA7, 17 or 43 in the presence of *R*-**4** as amine donor.

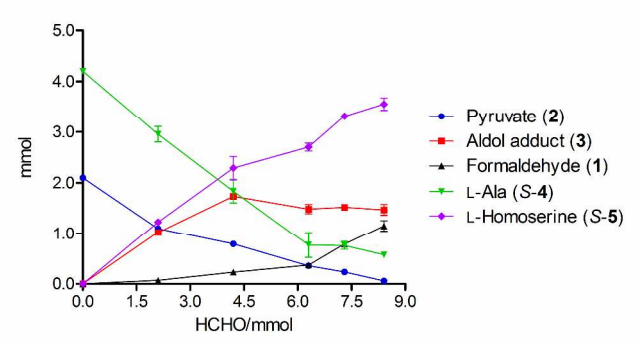


Figure 5. Biocatalytic cyclic cascade system for *S*-**5** synthesis with continuous **1** addition. Effect of total **1** added on the reaction mixture composition after 24 h. Conditions: **2** (2.1 mmol), *S*-**4** (4.2 mmol), PLP (2.1 μmol), MBP-YfaU(Mg^{2+}) (40 mg, 100 U), TA39 (210 mg, 7.4 U), **1** continuously added with a syringe pump (0.57 mmol h^{-1}, 0.18 mL h^{-1}, and duration 4-15 h depending on the experiment), V = 10.5 mL (complete experimental details in SI).

Table 1. Biocatalytic cyclic cascade system for *S*-5 and *R*-5 synthesis[a]

Prozomix TA	TA/U[d]	**5** /%	Isolated Yield[b]/%	*ee*/%[c]
20	12	npd[e]	–	–
26	34	11	ni[f]	nd[g]
39	7	86	56	>99(*S*)
51	28	64	29	>99(*S*)
7	480	>95	64	>99(*R*)
17	1160	>95	59	>99(*R*)
43	460	>95	63	>99(*R*)

[a]Conditions: **2** (2.1 mmol) and *S*-**4** (4.2 mmol) in 50 mM Na^+ phosphate buffer, pH 7.0 (7.8 mL), PLP (2.1 μmol), MBP-YfaU(Mg^{2+}) (40 mg, 100 U), TA (as in table), and **1** continuously added with a syringe pump (0.57 mmol h^{-1}, 0.18 mL h^{-1}, total added **1**: 8.4 mmol during 15 h). Reaction time 24 h. V = 10.5 mL (detailed conditions in SI). [b]The products were isolated as Cbz-L- and D-homoserine lactone (*S*-**7** and *R*-**7**, respectively, see SI). [c]Determined by chiral HPLC (see SI). [d]For Unit definition of TA see SI. [e]npd: No product detected. [f]ni: not isolated. [g]nd: not determined.

CONCLUSION

In summary, we have developed a biocatalytic one-pot cyclic cascade system for the complementary, stereoselective synthesis of both L- and D-homoserine in 86% and >95% yield, respectively, and with >99% *ee* each. Differences in the product yields observed could be due to distinct inhibition/deactivation of the transaminases involved in the reaction. Readily available inexpensive starting materials such as formaldehyde and alanine can be used at high concentrations (i.e. 0.8 M), leading to homoserine productivities of >80 g L^{-1} d^{-1} under proof-of-concept conditions. This compares favorably with report-

ed fermentation processes that provide only between 2.9 to 6.4 g L^{-1} d^{-1} of product.[16] The system comprises a simple combination of biocatalytic aldol addition and reductive amination reactions working in tandem, by using the Class II pyruvate aldolase MBP-YfaU(Mg^{2+}) from *E. coli* K12 and an appropriate *S*- or *R*-selective PLP-dependent transaminase available from Prozomix. Plausibly, this *Systems Biocatalysis* concept should be applicable to related pyruvate derived products by using other aldehyde acceptors for the synthesis of a large variety of other amino acids and related useful building blocks. Research towards this end is currently underway in our laboratories.

EXPERIMENTAL SECTION

Biocatalytic cyclic cascade synthesis of L-homoserine. The reaction was carried out in a Falcon tube (50 mL). Initial amount of pyruvate (2.1 mmol) and L-Ala (4.2 mmol) were dissolved in 50 mM Na^+ phosphate buffer, pH 7.0, containing PLP (1 mM). Then, MBP-YfaU(Mg^{2+}) (105 U, 10 U mL^{-1} in the final reaction) and Prozomix TA39 (74 U, 7 U mL^{-1} in the final reaction) were added. After that, formaldehyde was constantly added with a syringe pump at 0.57 mmol h^{-1} (0.18 mL h^{-1}) over 15 h (representing a cumulative addition of 8.4 mmol). The starting reaction volume was 7.8 mL, the final volume was 10.5 mL. Transformation to Cbz-L-homoserine lactone, purification and characterization procedures are described in SI.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI....Materials, General procedures, protein expression and purification, enzymatic activity determination, enzymatic aldol reactions, compound characterization, NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: pere.clapes@iqac.csic.es

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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