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Biocatalytic Cascade Reaction for the Asymmetric Synthesis of L- and D-Homoalanine

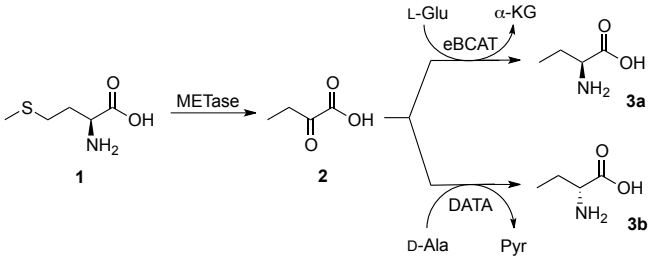
Marcus V. de M. Silva,[a, b] Ingrid C. R. Costa,[a] Rodrigo O. M. A. de Souza,[b] and Uwe T. Bornscheuer*[a]

Abstract: Unnatural amino acids attract growing attention for pharmaceutical applications as they are useful building blocks for the synthesis of a number of chiral drugs. Here, we describe a two-step enzymatic method for the asymmetric synthesis of homoalanine from L-methionine, a cheap and readily available natural amino acid. First, the enzyme L-methionine γ-lyase (METase), from *Fusobacterium nucleatum,* catalyzed the γ-elimination of L-methionine to 2-oxobutyrate. Second, an amino acid aminotransferase catalyzed the asymmetric conversion of 2-oxobutyrate to either L- or D-homoalanine. The L-branched chain amino acid aminotransferase from *Escherichia coli* (eBCAT), using L-glutamate as amino donor, produced L-homoalanine (32.5% conv., 28% y, 99%ee) and the D-amino acid aminotransferase from *Bacillus* sp. (DATA) used D-alanine as amino donor to produce D-homoalanine (87.5% conv., 69% y, 90%ee). Thus, this concept allows for the first time the synthesis of both enantiomers of this important unnatural amino acid.

Unnatural amino acids, in high optical purity, attract growing attention for pharmaceutical application as they are useful building blocks for the synthesis of a number of chiral drugs.[1] For instance, L-homoalanine is a key chiral intermediate in the synthesis of important drugs like antiepileptic (levetiracetam and brivaracetam) and antituberculosis (ethambutol) compounds.[2] The optical purity of these drugs plays an important role in their therapeutic safety and efficacy, where the (*R*)-enantiomer of levetiracetam has no antiepileptic activity and the (2*R*, 2'*R*) stereoisomer of ethambutol can cause blindness.[3] Even though these chiral drugs are now available as generic drugs, their production cost is still very high. For many patients, especially from developing countries, the cost of the treatment is still prohibitive and the use of cheaper and less effective alternative medications is their only option.[4]

Although many methods have been described in the literature, the synthesis of chiral unnatural amino acids remains an important challenge.[3a, 5] Unlike natural amino acids, fermentation and extraction methods are not practical for their production.[6] Different strategies for asymmetric synthesis or chiral resolution are used, but most of them involve multiple steps or are complex and environmentally unfriendly.[7] Alternatively, several approaches using enzymes as catalysts have been described.[8] For example, acylases[9], amidases[10], and hydantoinases[11] have been used for the kinetic resolution of racemic mixtures of *N*-acyl amino acids, amino acid amides, and hydantoins, respectively. Asymmetric syntheses starting from prochiral α-keto acids are also possible using aminotransferases[12] or dehydrogenases.[13] Enzymatic cascades involving combinations of several biocatalytic steps in one pot is another strategy, which has gained attention recently.[14] Galkin and co-workers synthesized different D-amino acids from the corresponding α-keto acids and ammonia by coupling four enzymatic reactions: formate dehydrogenase (FDH), L-alanine dehydrogenase (L-AlaDH), alanine racemase (AlaR) and D-amino acid aminotransferase (DATA).[15] In this process, pyruvate was converted to D-alanine through L-alanine by the reaction of L-AlaDH and AlaR. NADH was regenerated by oxidation of formate by the FDH. The D-enantiomers of glutamate, leucine, norleucine and methionine could be synthesized with high optical purities and conversions. However, this method was not applicable to the synthesis of D-norvaline and D-homoalanine. These compounds were obtained in low optical purity due to the action of L-AlaDH, which reduced α-ketobutyrate and α-ketovalerate more rapidly than pyruvate, and AlaR (that racemized D-homoalanine and D-norvaline). Another elegant method was developed by Park, Kim and Shin.[2b] They synthesized L-homoalanine from L-threonine by a cascade reaction of threonine deaminase and ω-transaminase using benzylamine as amino donor. In this system 91% of L-threonine was converted to L-homoalanine.

In this study, we describe a new biocatalytic cascade providing access to both the L- and D-enantiomers of homoalanine. To achieve this goal, we combined two enzymatic reactions (Scheme 1).



Scheme 1. Schematic cascade reaction for the asymmetric synthesis of D- or L-homoalanine by choosing the right combination of biocatalysts. α-KG: α-ketoglutaric acid (**5**), Pyr: (**7**).

The first reaction is catalyzed by L-methionine γ-lyase (METase, EC 4.4.1.11), a fold type I pyridoxal-5´-phosphate (PLP)-dependent enzyme. METase catalyzes the γ-elimination of L-methionine (**1**) to form α-ketobutyrate (**2**), ammonium, and methanethiol.[16] The second reaction involves a transamination catalyzed by branched-chain amino acid aminotransferase from

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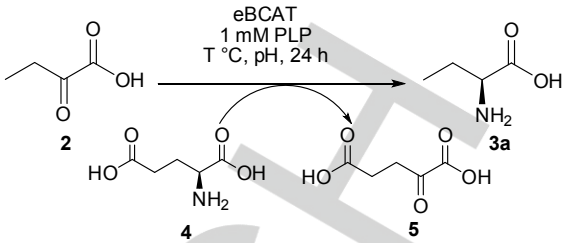
Escherichia coli (eBCAT, EC 2.6.1.42) or D-amino acid aminotransferase (DATA, EC 2.6.1.21) from *Bacillus* sp. Both of these transaminases are fold type IV PLP-dependent enzymes. The eBCAT catalyzes the transamination between L-glutamate (4) and α-ketobutyrate **2** to produce L-homoalanine (**3a**), see Scheme in Table 1. On the other hand, DATA catalyzes the same transamination by using D-alanine (**6**) as amine donor leading to the desired product with opposite configuration (**3b**), see Scheme in Table 2. Together these enzymes display a perfect match for production of these unnatural amino acids. Methionine, a natural amino acid produced in large quantities at low cost,[3a, 17] is converted to **2** "*in situ*" by the METase. In such a way, this approach overcomes the use of this expensive α-keto acid as a starting material.[12a] Furthermore, compared to other cascades that use amino acid dehydrogenases,[14b] there is no requirement for an expensive cofactor such as NADH when aminotransferases are used.[18]

The first step of the cascade presents a γ-elimination catalyzed by METase. For studying this step, we cloned and expressed a METase from *Fusobacterium nucleatum* subsp. *nucleatum* in *E. coli* BL21 (DE3) with vector pET24A.[19] The expression conditions were optimized (see Supporting Information) and the crude lysate produced, after being lyophilized, was used as enzyme source. To better understand the METase reaction, a design of experiment (DOE) with three levels and three variables (3^3) was proposed. For the selection of the variables and the levels used in the DOE we based our decision on the work developed by Suganya and coworkers.[16] These authors demonstrated that the physical properties of METase from different organism are relative similar. For example, pH optimum is in the range from 7.0-8.0 and the optimum temperature around 25-37°C. The reaction time was set to 20 min and it was possible to verify that variables which influenced most the reaction course was temperature and pH, where reactions at 40°C and pH 7.0 lead to highest conversions (37-46%). PLP concentration did not show a significant influence on the final conversion (see Supporting Information for details). This results are corroborated by previous studies in literature where METases with optimal activities at 37ºC[20-25] and pH 7[20-23] were described.

In the second step the reactions were approached from two different strategies since the intermediate α-ketobutyrate is a prochiral molecule, the reaction can occur in a way to obtain both stereoisomers by using eBCAT or DATA.[26] In this way, the transamination reaction was evaluated by using L-glutamic acid (L-Glu) as amine donor in the eBCAT catalyzed reaction and D-alanine (D-Ala) in the DATA catalyzed reaction (Tables 1 and 2).

PLP concentration was fixed to 1 mM, since previous studies have already correlated the higher concentration of PLP with greater activity of the aminotransferases[27-28] and it had no significant influence on previous step of the proposed cascade reaction. Temperature and pH were varied around the values, which were already known as good for the METase reaction. Since for a cascade we wish to perform everything in a one-pot reaction, both enzymes must accept matching reaction conditions.

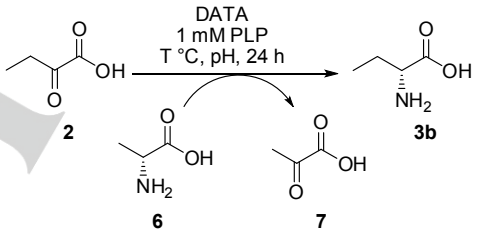
Table 1: Asymmetric synthesis of L-homoalanine catalysed by eBCAT.



Entry	Temperature (°C)	pH	Conversion [%]
1	30	7.0	10
2	30	8.0	11
3	40	7.0	17
4	40	8.0	20

Reaction conditions: 40 mL of a solution containing 50 mM sodium α-ketobutyrate, 50 mM L-Glu in sodium phosphate buffer (50 mM) with 1 mM PLP. The concentration of lyophilized crude lysates was 1 mg/mL. Conversion was determined by HPLC analysis using a Luna Omega PS C18 column with OPA-MCE derivatization.

Table 2: Asymmetric synthesis of D-homoalanine catalyzed by DATA.



Entry	Temperature (°C)	pH	Conversion [%]
1	30	7	53
2	30	8	48
3	40	7	66
4	40	8	47

Reaction conditions: See Table 1 except that 50 mM D-Ala was used instead of L-Glu.

Under optimal conditions for the METase reaction (pH 7 and 40ºC) both amino acid aminotransferases showed activity. In the case of DATA, around 65% conversion could be achieved while eBCAT lead to only 20% of the desired product. The low conversion observed for eBCAT could be related to an unfavorable reaction equilibrium often observed in transaminases-catalyzed reactions. In the case of DATA, the reaction stopped at 65% conversion probably because DATA can use the reaction product homoalanine as amine donor for regenerating alanine in the reaction system. After optimization of the two steps separately, the cascade reaction was performed in batch reactors containing METase/eBCAT or METase/DATA biocatalysts at 40ºC, pH 7 with 1 mM PLP. After the one-pot reaction studies, it was verified that within 24 h the best conversion results for the combination METase-eBCAT were

obtained (Figure 1) whereas for the combination METase-DATA longer reaction time were needed (Figure 2).

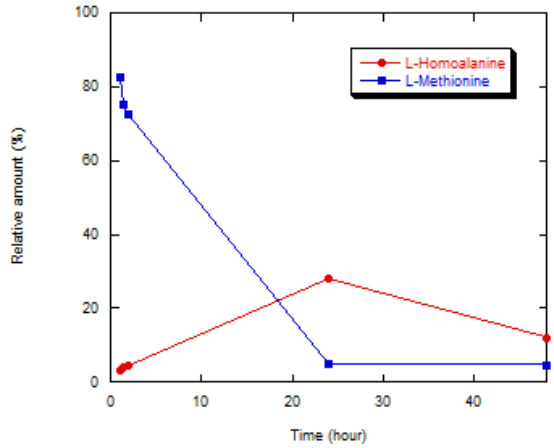


Figure 1. Time course of the cascade reaction by employing METase and eBCAT in the conversion of L-methionine to L-homoalanine.

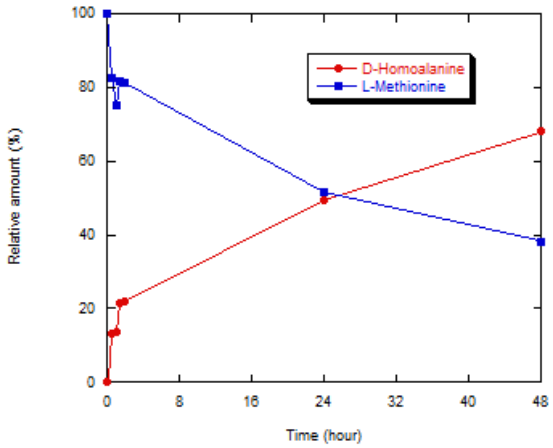


Figure 2. Time course of the cascade reaction by employing METase and DATA in the conversion of L-methionine to D-homoalanine.

Analysis of samples taken later revealed the formation of precipitate, presumably denatured enzyme. The enantiomeric excess of L-homoalanine was >99%ee from the METase-eBCAT reaction and 90%ee for the METase-DATA reaction product of D-homoalanine.

In conclusion, we have developed a new method for the asymmetric synthesis of **3a** and **3b** starting from L-methionine. The key point was the use of a biocascade that coupled the enzymes METase with an amino acid aminotransferase (eBCAT or DATA). Further attempts related to the equilibrium limitation of transaminases need to be addressed in future work to improve the overall yield of the reactions. We are convinced that this biocatalytic cascade reaction represents an elegant strategy for the production of highly significant chiral unnatural amino acids.

Experimental Section

Chemicals

All chemicals were purchased from Sigma–Aldrich (Darmstadt, Germany) or Acros/Thermofisher Scientific (Waltham, USA) in analytical grade and used without further purification. Biocatalysis reactions were performed on an Eppendorf® Comfort Thermomixer shaker.

Expression and production of METase

The synthetic codon optimized gene of the enzyme METase, from *Fusobacterium nucleatum* subsp. *nucleatum* present in the vector pET24A was transformed into *E. coli* BL21 (DE3). Cells were grown in Terrific Broth (TB) media supplemented with kanamycin (50 µg/mL) at 37ºC and 180 rpm. When the cell OD_{600} reached approximately 0.4-0.6, enzyme expression was induced by IPTG (final concentration at 1.0 mM) and the cells were allowed for overnight cultivation at 20°C and 180 rpm. Cells were harvest by centrifugation and resuspended in phosphate buffer (50 mM, PLP 0.1 mM, pH 7.4). The cell suspension was submitted to ultrasonic cell disruption and the supernatant obtained after centrifugation (4500 x g, 30 min, 4°C) was frozen, lyophilized and used as catalyst in the enzymatic reactions (see also Supporting Information).

METase assay

Determination of the activity of the METase was performed as described by Foo.[29] After 20 min reaction, 10% v/v of 50% trichloroacetic acid was added in order to precipitate the enzyme to stop the reaction. Samples were centrifuged and then 50 µL was transferred to a 96-well plate with 150 µl of acetic buffer (1 M, pH 5). The concentration was determined by monitoring the α-ketobutyrate formation at 320 nm. The molar extinction coefficient for α-ketobutyrate was determined (ε_{320} =3.83x10^2 M^{-1} cm^{-1}) with a standard curve using sodium 2-oxobutyrate. One unit of activity was defined as the amount of enzyme that produces 1 µmol α-ketobutyrate per minute.[27] Reaction optimization was performed using selected variables: pH (6.0, 7.0 and 8.0), temperature (25, 32.5 and 40ºC) and PLP concentration (0.10, 0.55 and 1.0 mM) (the DOE matrix with the real values can be found in the Supporting Information). All statistical treatments were performed using STATISTIC 6.0 software.

Expression of eBCAT and DATA

The synthetic codon optimized genes of the enzymes eBCAT and DATA were present in the vectors pET24A and pGASTON respectively. Both constructs were transformed into *E. coli* BL21 (DE3). The cells were grown in Terrific Broth (TB) media supplemented with kanamycin (50 µg/mL) for eBCAT and ampicillin (100 µg/mL) for DATA at 37ºC and 180 rpm. When the cell OD_{600} reached approximately 0.4-0.6, enzyme expression was induced by IPTG (1.0 mM final concentration) for eBCAT or by rhamnose (0.2%) for DATA. The cells were allowed for overnight cultivation at 20°C. Cells were harvested by centrifugation and resuspended in phosphate buffer (50 mM with PLP 0.1 mM, pH 7.4). The cell suspension was submitted to ultrasonic cell disruption and the supernatant obtained after centrifugation (4500 x g, 30 min, 4°C) was frozen, lyophilized and used as catalyst in the enzymatic reactions.[26]

Biocatalysis with amino acid aminotransferases

Solutions containing 50 mM sodium α-ketobutyrate, 50 mM L-Glu (for eBCAT) or 50 mM D-Ala (for DATA) in sodium phosphate buffer (50 mM with 1 mM of PLP) either pH 7.0 or 8.0 were prepared.[26] To this reaction lyophilized enzymatic extracts were added so that the final concentration was 1 mg/mL eBCAT or DATA. Samples were taken after 0, 30, 90, 120 min, 24 and 48 h (Tables 1 and 2). All analyzes were performed by HPLC with OPA-MCE derivatization.

Cascade reactions with METase–eBCAT or METase–DATA

For this, a solution containing 50 mM L-Met, 50 mM L-Glu or D-Ala in phosphate buffer (50 mM pH 7 with 1 mM PLP) was prepared. This solution was adjusted to pH 7 with 10 N NaOH. To this reaction solution lyophilized enzymatic extracts were added (1 mg/mL METase and 1 mg/mL eBCAT or DATA). Samples were taken after 0, 5, 10, 15, 20, 30, 60, 90, 120, 1440 and 2880 min. All analyzes were performed by HPLC OPA-MCE derivatization.

Preparative-scale synthesis of L- and D-homoalanine

For the cascade reaction with METase and eBCAT or METase and DATA on a larger scale, 0.3 g (2 mmol) of L-Met and 0.296 g (2 mmol) of L-Glu (in the case of eBCAT) and 0.1782 g (2 mmol) of D-Ala (in the case of DATA) were used. A final concentration of 1 mg/mL of each enzyme was used. After the reaction, the product was derivatized with di-*tert*-butyl dicarbonate (Boc). For the derivatization step, 1.2 eq. of Boc and 2 eq. of K_2CO_3 were added for each eq. of amino acids in the reaction. The reaction mixture was solubilized in THF:H_2O (1:1) and monitored for 24 h. After the end of the reaction, the supernatant was evaporated and the remaining solid was resuspended in ethanol and subjected to a silica column using a butanol:H_2O:acetic acid (3:1:1) mixture as the mobile phase. The fractions were monitored by TLC (ninhydrin staining), relevant fractions were pooled and the solvent was evaporated. This gave 0.1347 g (32.5% conversion) in the case of eBCAT reactions and 0.3577 g (87.5% conversion) in the case of DATA. Product identity was confirmed by NMR spectroscopy.

NMR spectroscopy

^{1}H- and ^{13}C-NMR spectroscopy were performed on a Varian Gemini-200 (400 MHz and 500 MHz) and Bruker Avance DRX 400 (300 MHz and 400 MHz) device using D_2O as solvent. Chemical shifts are reported in ppm with TMS (δ = 0.00) as the internal standard for ^{1}H-NMR and MeOD (δ= 49.86) for ^{13}C-NMR spectra.

HPLC analyses

For derivatization of the amino acids the methodology described by Turnell & Cooper[30] was used. For this a derivatization solution containing 40 mM *o*-phthalaldehyde (OPA) and 1% (v/v) 2-mercaptoethanol (MCE) in a saturated solution of $Na_2B_2O_4$ (pH 10) with 20% methanol, was used. The sample for analysis was prepared with 250 µl from the reaction, 250 µL of a 0.1N HCl solution and 750 µL of the derivatization solution. 10 µL of the derivatized sample was directly injected into the HPLC. The chromatography was performed at room temperature using the HPLC column Luna Omega PS C18 150 mm. Phase A was composed of phosphate buffer (40 mM, pH 7.8) and phase B was composed of AcCN:MeOH:H_2O (45:45:10). The analysis was performed at 1 ml/min at 338 nm. Further information can be found in the Supporting Information.

Chiral HPLC analyses

For the determination of enantiomeric excess the D,L-homoalanine was derivatized using the methodology described by Nimura and coworkers.[31] For analysis a derivatization solution containing 20 µL 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (100 mM), 20 µL triethylamine (100 mM), 90 µL water/trifluoracetic acid 1 % and 70 µL sample. After 90 min at room temperature the chromatography was performed using the HPLC column Luna Omega C18 150mm, with 0.5 mL/min isocratic elution of methanol:water (45:55), both containing 0.1 % trifluoroacetic acid. Detection was done with a UV detector at 254 nm. The retention times were initially checked with the standards and the retention time of D,L-enantiomers of homoalanine were 7.6 and 9.2 min, respectively.

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Keywords: asymmetric synthesis, cascade reaction, L-methionine γ-lyase; amino acid aminotransferase, L- and D-homoalanine

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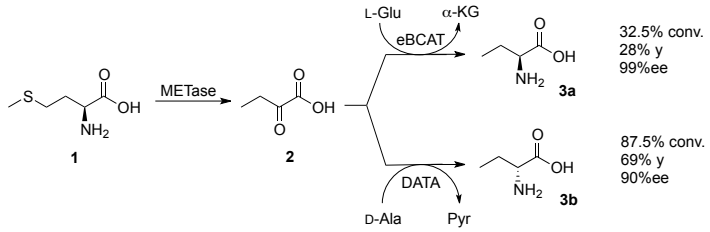
COMMUNICATION



Unnatural amino acids have important applications in pharmaceutical chemistry. We describe a biocatalytic cascade providing access to both, the L- and D-homoalanine using a combination of a L-methionine γ-lyase (METase) and two amino acid transaminases with complementary enantiopreference.

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