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Asymmetric synthesis of nonproteinogenic amino acids with L-amino acid transaminase: synthesis of (2*S*)-2-amino-4-oxo-4-phenylbutyric and (3*E*,2*S*)-2-amino-4-phenylbutenoic acids

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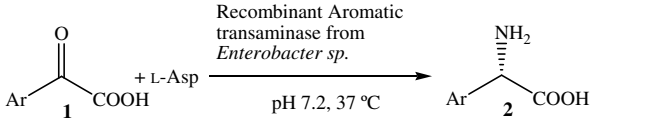
Abstract—2,4-Dioxo-4-phenylbutyric acid and 2-oxo-4-phenylbut-3-enoic acid are converted to the corresponding (*S*)-2-amino acids by recombinant *Escherichia coli* whole cells over-expressing aromatic transaminase from *Enterobacter* sp. BK2K-1 (AroAT*Es*) in high yields (68–78%) and high enantiomeric purity (>99%) using L-aspartic acid as an amino donor.

1. Introduction

Enantiomerically pure nonproteinogenic amino acids are of considerable synthetic interest since they serve as building blocks in asymmetric synthesis and for the preparation of anticancer and antiviral compounds.[1] Also, by incorporating unnatural amino acids into biologically active peptides and proteins, their activity, stability, bioavailability, and binding specificity may be improved.[2] A large number of chemical[1–3] and enzymatic[4] methods for their synthesis have been reported in the literature. While the chemical methods involving asymmetric synthesis require several steps to obtain the final product, the chemo-enzymatic approach is much simpler. Most enzymatic methods are based on reductive amination,[5] transamination,[6] and kinetic resolution with lipase,[7] pronase,[8] penicillin acylase,[9] and hydantoinase coupled with decarbamoylase.[10] Among these, the methodology of using transaminase is by far the most simple and high yielding. It constitutes the transfer of an amino group from a donor molecule (usually a natural amino acid such as L-aspartic or L-glutamic acid) to an accepter possessing a prochiral keto group. Although the reaction requires participation of pyridoxal 5′-phosphate (PLP) as a co-factor, it is usually tightly bound to the enzyme and does not require an external co-factor regeneration system.[4] We have earlier reported a putative aromatic aminotransferase from *Enterobacter* sp. BK2K-1 over-expressed in recombinant *E. coli* BL21 and its use in the synthesis of L-homophenylalanine.[6a] Continuing with our efforts to broaden the scope of its application in the synthesis of nonproteinogenic amino acids, herein, we report the synthesis of (2*S*)-2-amino-4-oxo-4-phenylbutyric acid **2a** and (3*E*,2*S*)-2-amino-4-phenylbutenoic acid **2b** in high isolated yields (>68–78%) and with high enantiomeric purity (ee >99%) (Scheme 1). The unnatural amino acid **2a** and its derivatives are inhibitors of kynurenine-3-hydroxylase[11–14] and have a potential to be useful as neuroprotective agents; while β,γ-unsaturated α-amino acids have been



1a. Ar = Ph-CO-CH$_2$
1b. Ar = Ph-CH=CH

2a. Ar = Ph-CO-CH$_2$
2b. Ar = Ph-CH=CH

e.e.>99%, yield 68-78%

Scheme 1.

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found to be reversible or irreversible inhibitors of a number of enzymes.[15–18]

The synthesis of 4-oxo-amino acid **2a** in its racemic form has been carried out in a variety of ways. Some of the notable routes are (a) condensation of α-oxo monohalides with acetamidomalonate followed by hydrolysis and decarboxylation;[19–21] (b) addition of β-benzoylacrylic acid and its ethyl ester to ethanolamine;[22] (c) reduction of the isonitroso derivative of β-benzoyl propinic acid;[23] (d) reaction of an electrophilic glycine cation equivalent with neutral carbon nucleophiles;[24] and (e) reaction of *N*-acylamino-2-bromoacetates, via *N*-acylimino acetates, with higher order mixed cuprates, trimethylsilyl enol ethers, and β-dicarbonyl compounds.[25] Enantiomerically pure **2a** has been prepared by classical resolution of *N*-acetyl derivative of the racemic amino acid with α-methyl benzylamine[21] or by enzymatic resolution with hog renal acylase.[25] The *N*-trifluoroacetyl derivative has been resolved by enantioselective hydrolysis with carboxypeptidase A.[26] Asymmetric synthesis via Friedel–Craft reaction with aspartic acid derivatives,[14,28,29] Mannich type reactions of *N*-carbamate-protected α-imino esters using a chiral copper(II)–diamine complex,[30] and catalytic enantioselective alkylation of N,O-acetals[31] have also been reported. In comparison, very few syntheses of β,γ-unsaturated α-amino acids such as **2b** have been reported, probably because of the tendency of the double bond to migrate into conjugation with the carboxyl group under basic conditions.[27,32] Racemic **2b** can be prepared in relatively low yields by the classical Strecker synthesis from cinnamaldehyde[33] or by condensation of phosphorane synthons.[34] The resolution of the racemic form has been carried out by enantioselective hydrolysis of its *N*-acetyl derivative with biodiastase[35] while asymmetric synthesis has been achieved by condensation of benzaldehyde with the dianion of L-aspartic acid followed by decarboxylative dehydration[36] to generate **2** as a mixture of (*E*) and (*Z*) isomers. An interesting approach based on a three-component Mannich type reaction involving the condensation of an organoboronic acid with amine and α-keto acid has been described by Petasis and Zavialov.[37] All the approaches described above suffer from disadvantages such as multi-step synthesis and low yields. Our present methodology involving the aminotransferase catalyzed stereospecific transfer of an amino group from L-aspartic acid to α-keto acids **1** is very simple and provides products with excellent enantiomeric purity in high yields.

2. Results and discussion

2.1. Asymmetric synthesis of 2a and 2b by enzymatic transamination

The transaminase reaction involves transfer of the amino group from the amino donor to the amino acceptor via an intermediate Schiff's base formation with PLP, and is prone to inhibition by concentrations of both donor and the acceptor as well as the products.[6] In our previous studies,[6a] we had observed that both L-aspartic acid and L-glutamic acid can be used as amino donors for AroAT*Es*. Although L-glutamate is preferable due to its low cost, L-aspartate is better suited since the oxaloacetate produced during the deamination of aspartate undergoes subsequent spontaneous and/or enzymatic decarboxylation to pyruvate which is further metabolized to carbon dioxide. This drives the equilibrium to the formation of the desired product and inhibition due to oxaloacetate is minimized. We have thus used L-aspartic acid as an amino donor for the synthesis of **2**. In order to examine the substrate inhibition, the AroAT*Es* activity of the cell extract (75 U/mL) obtained from the recombinant *E. coli* expressing AroAT*Es* was measured at various substrate and aspartate concentrations. We observed that aspartate did not seriously inhibit the reaction up to a concentration of 200 mM. However, the reaction was inhibited by substrate **1a** beyond 50 mM and by substrate **1b** beyond 20 mM. The effect of aspartate and substrate concentration on the reaction was found to be similar in case of both whole cells and cell extract. However, the reaction with whole cells was easier to perform. Thus, the synthesis of amino acids **2** was carried out with whole cells of recombinant *E. coli* BL21(DE3) over-expressing AroAT*Es* from *Enterobacter* sp. BK2K-1 in 0.05 M phosphate buffer (pH 7.2) containing 2 equiv of L-aspartic acid at 37 °C. The optimum substrate concentrations were 50 and 20 mM, respectively, for **1a** and **1b**. The reaction was easily followed by UV–vis spectrophotometry since the keto acids have a strong peak around 310 nm, which decreases as the α-keto group is converted to an amino group, and a new peak around 254 nm appears. When the reaction mixture, after completion of the reaction, was supplemented with more substrate and fresh cells, very little conversion of freshly added substrate took place indicating that the reaction is susceptible to inhibition by both substrate and the product. The inhibition of reaction with **1b** at a lower concentration (>20 mM) than that with **1a** (>50 mM) is not surprising in view of the observations that the β,γ-unsaturated α-amino acids act as inhibitors of PLP-dependent aminotransferases.[15–18]

At the end of the reaction, the reaction mixture contains 1 equiv of unreacted aspartic acid along with the product **2**. The separation was achieved by crystallization from HCl in case of **2a** (yield 78%) and by boiling with 0.05 M phosphate buffer at pH 7.2 in case of **2b** (yield 69%). Enantiomeric purity of both the products was >99% as determined by HPLC analysis on a chiral stationary phase after hydrogenation over 10% Pd–C to obtain (*S*)-homophenylalanine and then conversion to its *N*-benzoyl derivative.

3. Conclusion

In this study, we have successfully demonstrated the asymmetric synthesis of two nonproteinogenic amino acids with excellent enantiomeric purity using a recombinant *E. coli* expressing an aromatic aminotransferase. The starting materials can be synthesized in >90% overall yield in only one or two steps with cheap raw materials and the final product is obtained directly in a single step. The reaction can be conveniently performed with an approximately 4:1 (w/w) substrate to biomass ratio in 24 h and substrate load-

ing of 5–10 g/L. In case of substrate **1a**, the enzymatic reaction is completely stereo and regiospecific, converting only the α-carbonyl group to an amino group. In case of substrate **1b**, the reaction is carried out under very mild conditions and hence no migration of the double bond is observed (NMR data). The methodology is very much simpler and gives higher yields than the previously known techniques.

4. Experimental

2,4-Dioxo-4-phenylbutyric acid was prepared by condensation of acetophenone with diethyl oxalate and subsequent acidic hydrolysis as described in the literature.[38] (*E*)-2-oxo-4-phenylbut-3-enoic acid **1a** was prepared as a potassium salt by condensation of benzaldehyde with pyruvic acid in methanolic KOH as per the literature procedure.[39] Isopropyl β-D-thiogalactopyranoside (IPTG), pyridoxal 5′-phosphate (PLP), and kanamycin were obtained from Sigma–Aldrich (MO, USA). UV–vis spectra were recorded on a Hewlett Packard 845× spectrophotometer. NMR spectra were recorded on Brucker Spectrospin 300 spectrometer. Optical rotation was measured on Jasco 1030 polarimeter. HPLC analyses were performed using a Waters HPLC system. MALDI-Mass was determined on Brucker MALDI-TOF MS. IR spectra were recorded on Thermo Nicolet Nexus 670 spectrometer.

4.1. Asymmetric synthesis of (2*S*)-2-amino-4-oxo-4-phenylbutyric acid 2a

The construction of recombinant *E. coli* BL21(DE3) harboring the plasmid pET24ma-AroAT*Es* has been described earlier.[6a] The recombinant *E. coli* was grown at 37 °C in 1 L of Luria–Bertani (LB) medium containing 25 μg/mL of kanamycin. At $OD_{600\,nm}$ value of 0.6, the expression of the enzyme was induced with 1 mM IPTG. After 6 h of induction, the cell pellet was harvested from the culture broth, washed once with cold 50 mM Tris–HCl buffer (pH 7.2), and stored at −70 °C. The biocatalytic reaction was carried out in shake flask on an orbital shaker at 37 °C. The wet cells were added to 100 mL reaction mixture consisting of substrate **1a** (960 mg, 5 mmol) and L-aspartic acid (1.33 g, 10 mmol) in phosphate buffer (0.05 M, pH 7.2). The weight of biocatalyst was estimated to be 380 mg dry cell weight based on the optical density of the reaction mixture at 600 nm (0.3 mg/mL dry cell weight gives absorbance 1.0). The reaction was followed on a Hewlett Packard 845× UV–vis spectrophotometer. At intervals of 2 h, an aliquot (100 μL) was centrifuged in an Eppendorf tube and the supernatant was serially diluted to 10 μM with 80% acetonitrile–water solution containing 0.1% formic acid, and its UV spectrum was recorded. The starting substrate shows a strong peak at 316 nm which disappears as the reaction proceeds and a new peak appears at 254 nm. The reaction was generally complete in 24 h. The pH of the medium was adjusted to 8.5 to dissolve any precipitated amino acid and the cells were then centrifuged in cold. The cold supernatant was transferred to a conical flask, acidified with HCl to pH 2.0, centrifuged to remove precipitated protein, and extracted with ethylacetate (2×10 mL). The aqueous phase was again brought to pH 7.2 with NaOH and lyophilized. The residual powder was suspended in distilled water (10 mL), heated in a boiling water bath for 5 min, and cooled in ice. The precipitated amino acid was filtered, suspended in 6 M HCl (5 mL), and heated on a water bath until all the product dissolved. The solution was then cooled in ice to obtain white crystals of the hydrochloride salt (740 mg, 78%). $[\alpha]_D^{25} = +40.5$ (*c* 0.1, 6 M HCl). Lit.[29] $[\alpha]_D^{22} = +44.3$ (*c* 0.1, 6 M HCl for amino acid). IR (KBr): 3320, 3150, 3050, 2910, 2530, 2080, 1680, 1640, 1590, 1530, 1485, 1400, 1210, 745, 680 cm^{-1}. ^1H NMR (D_2O, 300 MHz): δ 3.79 (2H, m), 4.22 (1H, m), 7.4–7.8 (3H, m), 8.0–8.2 (2H, m). MALDI-TOF MS, *m*/*z* 193.32 ([M^+], exact mass calcd for $C_{10}H_{11}NO_3$ 193.07), 215.3 [$M+Na^+$] calcd for $C_{10}H_{10}NNaO_3$ 215.06.

4.2. Asymmetric synthesis of (3*E*,2*S*)-2-amino-4-phenyl-3-butenoic acid 2b

The reaction was carried out with potassium salt of **1b** (428 mg, 2 mmol) and aspartic acid (532 mg, 4 mmol) in phosphate buffer (0.05 M, pH 7.2, 100 mL) and the biocatalyst (100 mg dry cell wt) essentially as described above. The reaction was followed by monitoring the disappearance of the peak at 302 nm. The reaction was complete in 24 h. The recovered crude amino acid was dissolved in 0.1 M HCl (25 mL). The solution was centrifuged to remove small amounts of protein and then solvent was removed on a rotavapor to obtain **2b** as a hydrochloride salt. The residue was boiled with 10 mL of 0.05 M phosphate buffer (pH 7.2), cooled and filtered to obtain the amino acid **2b** (245 mg, 69% isolated yield, ee >99%). IR (KBr): 3446, 1636, 1523, 1452, 1408, 1353, 1321, 1085, 991, 870, 697, 529 cm^{-1}. ^1H NMR (D_2O, 300 MHz): δ 6.6–6.8 (m, 5H), 6.26 (d, 1H, $J = 15.9$ Hz), 5.61 (dd, 1H, $J = 15.9$ and 8.5 Hz), 4.15 (d, 1H, $J = 8.5$ Hz). ^{13}C NMR (300 MHz, D_2O): δ 170.2, 138.6, 134.8, 129.2, 128.9, 127.0, 118.0, 54.7. MALDI-TOF MS, *m*/*z* 177.10 ([$M+H^+$]), exact mass calcd for $C_{10}H_{11}NO_2$ 177.08; 199.1 [{$M+Na$}$^+$] exact mass calcd for $C_{10}H_{10}NaNO_2$ 199.06. $[\alpha]_D^{25} = +38.4$ (*c* 1, 0.1 M HCl); lit.[34] $[\alpha]_D^{20} = -20$ (*c* 1, 0.1 M HCl) for (*R*)-enantiomer of the amino acid.

4.3. Transformation of 2a and 2b to (*S*)-*N*-benzoyl homophenylalanine

The hydrochloride of **2a** or**2b** (100 mg, 0.5 mmol) was dissolved in 10% acetic acid in water (25 mL), 10% Pd–C (50 mg) was added and the solution was hydrogenated for 6 h at room temperature with a hydrogen filled balloon. The catalyst was then filtered off, the solution was evaporated to dryness on a rotavapor, the residue was suspended in ice cold 1 M NaOH (5 mL) and treated with benzoyl chloride (60 μL, 0.5 mmol) under vigorous stirring for 30 min. The solution was then extracted with ethylacetate (1×5 mL) to remove unreacted benzoyl chloride, acidified with HCl, and the product was extracted with ethylacetate. The solvent was then evaporated to dryness to obtain crude *N*-benzoyl derivative which was directly analyzed by HPLC

on chiral stationary phase. The racemic sample was prepared from commercially available homophenylalanine.

4.4. Analysis of enantiomeric purity

Enantiomeric purity of **2a** and **2b** was determined (after hydrogenation to homophenylalanine and conversion to *N*-benzoyl derivative) by HPLC analysis on Chiralpak® AD-H column (250 × 5 mm), Daicel Chemical Industries, Japan. Mobile phase 10% isopropyl alcohol in hexane containing 0.1% trifluoroacetic acid; flow rate 1 mL/min; retention times: (*S*)-benzoyl homophenylalanine 16.6 min, (*R*)-benzoyl homophenylalanine 21.2 min.

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