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Simultaneous enzymatic synthesis of (*S*)-3-fluoroalanine and (*R*)-3-fluorolactic acid

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Abstract

A coupled enzymatic system for the simultaneous synthesis of (*S*)-3-fluoroalanine (**1a**) and (*R*)-3-fluorolactic acid (**3**) with L-alanine dehydrogenase (L-AlaDH) from *Bacillus subtilis* and rabbit muscle L-lactate dehydrogenase (L-LDH) using *rac*-**1** and NAD^+ is described. Analysis of isolated products of the laboratory preparative scale process revealed **1a** in 60% yield and 88% ee and **3** in 80% yield and over 99% ee. The compounds **1a** and **3** represent chiral building blocks for the synthesis of several products with pharmacological activity.

Fluorinated α-hydroxyacids and α-aminoacids are considered to be highly versatile chiral building blocks in asymmetric synthesis for the production of several compounds of pharmacological interest.[1]

(*S*)-3-Fluoroalanine or 3-fluoro-D-alanine (**1a**) is recognized as an antibiotic via irreversible inactivation of bacterial alanine racemase.[2] It also acts as an inhibitor of serine palmitoyltransferase[3] and it is a potential precursor for the synthesis of fluoroamino compounds.[1]

(*R*)-3-Fluorolactic acid (**3**), on the other hand, represents an important three carbon, chiral building block, 1,2,3-trisubstituted for the synthesis, for instance, of β-adrenergic blocking agents of the aryloxypropanolamine group such as (*S*)-propranolol and (*S*)-moprolol. It is also an intermediate for the synthesis of other products of pharmaceutical interest such as (*S*)-hydroxypyrrolidine-2-one which in its open form [(*S*)-4-amino-2-hydroxybutanoic acid] is considered to be one of the most potent inhibitors known of the neurotransmitter GABA, also showing anticancer activity. Compound **3** is also a potential intermediate for the production of carnitin and GABOB.[4]

A useful methodology for the synthesis of chiral building blocks is represented by the utilization of enzymes as catalysts as widely reported in the literature.[4,5] The use of dehydrogenases in organic synthesis is limited by the fact that the coenzymes (NAD^+ or $NADP^+$) are very expensive

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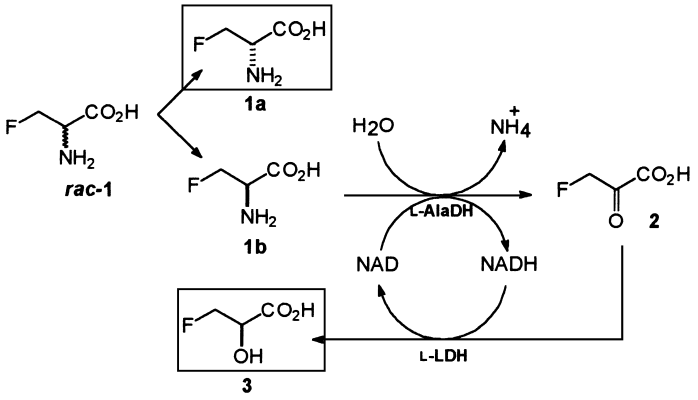
reagents to be used in stoichiometric concentrations. Overwhelmingly, the use of catalytic amounts of coenzymes, that can be achieved with a convenient method for recycling the coenzyme, is an important step for the economical viability of the process.[4]

(*R*)-3-Fluoroalanine (**1b**) was continuously produced by Ohshima et al. in the reductive sense with L-AlaDH from *Bacillus sphaericus* from **2** and ammonium formate in a proper reactor.[6] In that system, the recycle of the coenzyme was made possible by oxidation of the formate to produce CO_2, catalyzed by yeast formate dehydrogenase.

Our group has recently described the enzymatic production of **3**.[4] We envisaged an enzymatic system capable of simultaneously producing **1a** via resolution of *rac*-**1** by oxidation of **1b**, and **3** by reduction of **2**. The overall reaction system devised is shown in Scheme 1.



Scheme 1.

In the proposed system, L-AlaDH and L-LDH were designed to be used in the presence of catalytic concentrations of NAD^+ with *rac*-**1** as starting material. Therefore, L-AlaDH will catalyse the oxidative deamination of the L-enantiomer of *rac*-**1** in the presence of NAD^+, thus producing NADH, NH_4^+ and **2**. This latter compound will then be enantioselectively reduced to **3**, in the presence of NADH, by the action of L-LDH, so allowing the desirable recycling of NADH to NAD^+.

This process presents the advantage that the product of the principal reaction is the appropriate feedstock of the recycling reaction. In addition, as soon as **2** is formed, it is reduced, establishing then a steady state condition that will avoid inhibition of L-AlaDH by either the main reaction product (**2**) or by NADH. Thus, the overall system can only be vulnerable to inhibition by ammonium and/or by the product of the recycling reaction (**3**).

To test the performance of the proposed enzymatic system, preliminary experiments were conducted with *rac*-alanine, in which the L-enantiomer is the natural substrate for L-AlaDH. The results obtained showed that L-alanine was converted into lactic acid salt in 98% yield, therefore confirming the validity of the proposed model under experimental conditions.

Based on these results, we turned our attention to the fluorinated feedstock (*rac*-**1**), which was obtained from **2**, via reductive amination with satisfactory yield.[7,8]

The assays were carried out with *rac*-**1** on analytical scale by using the coupled enzymatic system. After 90 h of reaction, over 90% of **1b** was consumed. Once the efficiency of the coupled system on analytical scale was proved, we undertook this reaction on a preparative scale (100 mg) in order

to isolate and characterize the reaction products. After 24 h, about 93% of **1b** was converted into **3**, via reduction of the intermediate **2** by action of L-LDH.

(*S*)-3-Fluoroalanine (**1a**) was isolated with 60% yield and 88% ee.[9] The results of IR, ^{1}H NMR and elemental analysis (C,H,N) of **1a** agreed with the literature.[10] In this system, **3** was obtained in 80% yield and over 99% ee, which is consistent with our previously published results.[4]

In conclusion, in the main reaction of the coupled system, *rac*-**1** was submitted to an enzymatic resolution, thus leaving unreacted **1a** in good yield and high ee. As far as we know, this is the first example in which L-AlaDH was used in a preparative scale reaction in the oxidative sense, which is thermodynamically unfavorable for the isolated reaction. The driving force for the whole system was provided by the recycle system, which also afforded another important chiral building block, **3**.

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9. (a) L-AlaDH from *Bacillus subtilis* (EC 1.4.1.1, suspension in 2.4 M $(NH_4)_2SO_4$, pH 7.0), L-LDH from rabbit muscle (EC 1.1.1.27, solution in 50% glycerol containing 10 mM potassium phosphate buffer, pH 7.5), L-GluDH from bovine liver type III (lyophilized), NAD^+ (grade III-C), NADH (grade III), bovine serum albumin (fraction V),

sodium pyruvate, sodium fluoropyruvate and D,L-alanine were obtained from Sigma. α-Ketoglutarate and $NaBH_4$ were purchased from Aldrich. All other chemicals used were of analytical grade and obtained from Merck. (b) Reaction mixture contained in a total volume of 50 mL: 0.05 M phosphate buffer (pH 7.9), 20 mM *rac*-fluoroalanine, 373 UI of L-AlaDH, 4250 UI of L-LDH and 0.05% (w/v) BSA. (c) Reaction mixture was maintained under magnetic stirring and the temperature was kept at 25°C by circulating water with a thermocirculating bath. The reaction was started by adding 0.25 mM NAD^+. The extent of the reaction was measured by removing at different times aliquots (10–50 μL) of the medium that were immediately diluted with 0.5 mL of 0.05 M phosphate buffer (pH 7.9) and heated at 100°C for 5 min in order to inactivate the enzymes. (d) The concentration of ammonium present in these samples was determined by 'end point' assays in the presence of 0.4 mM NADH, 0.5 mM of α-ketoglutarate and 1 mg of L-GluDH in a total volume of 2 mL at 25°C. The decrease of NADH absorption was followed at 340 nm before and after the addition of the enzyme in silica cuvettes with a 1 cm light path using a Beckman DU 70 recording spectrophotometer until consumption of the substrate. The concentration of ammonium was calculated from the difference of NADH absorption (before and after the addition of L-GluDH) by using a molar absorption coefficient of NADH of 6220 M^{-1} cm^{-1}. (e) The commercial suspension of L-AlaDH was desalinized by ultrafiltration with Centricon-10 concentrators (AMICON), through five consecutives centrifugations at 5°C for 1 h and 2000 G. The original volume was made up with phosphate buffer (10 mM, pH 7.2). (f) Preparative work up was conducted by thermal treatment of reaction medium (100°C, 15 min), HCl (3 M) addition until pH 2, active charcoal addition, stirring during 15 min, filtration and ionic exchange chromatography (40 mL of wet Dowex 50 W-X8 H^+) eluted successively with water (for isolation of **3**) and 1 M NH_4OH (for isolation of **1a**). An overall yield of 80% and over 99% ee were obtained for **3** and 60% yield and 88% ee were obtained for **1a** [^{1}H NMR spectrum (D_2O): δ 4.02 (ddd) $J_{H,F}=29.5$ Hz, $J_{H,H}=4.5$ and 3.1 Hz; δ 4.81 (ddd) $J_{H,F}=47.4$ Hz, $J_{H,H}=4.5$ and 3.0 Hz. IR (KBr) 3428, 3061, 2987, 2923, 1615, 1356 and 1022 cm^{-1}]. The determination of ees for **1a** and **3** were carried out by chiral high performance liquid chromatography, which was performed by using a chiral column (ET 250/8/4 Nucleosil® Chiral-1); eluent: 1 mM copper sulfate, pH 4.6; flow rate: 1 mL/min; temperature 23°C; UV detection at 235 nm. Compound **1a**: $[\alpha]_D^{25}=-8.7$ ($c=0.8$, 1 M HCl), and **3** (as methyl ester):[4] $[\alpha]_D^{25}=-4$ ($c=1$, EtOH).

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