

Multistep Enzyme Catalyzed Reactions for Unnatural Amino Acids

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Abstract

The use of unnatural amino acids, particularly synthetic α -amino acids, for modern drug discovery research requires the availability of enantiomerically pure isomers. Starting from a racemate, one single enantiomer can be obtained using a deracemization process. The two more common strategies of deracemization are those obtained by stereoinversion and by dynamic kinetic resolution. Both techniques will be here described using as a substrate the D,L-3-(2-naphthyl)-alanine, a non-natural amino acid: the first one employing a multi-enzymatic redox system, the second one combining an hydrolytic enzyme together with a base-catalyzed substrate racemization. In both cases, the final product, L-3-(2-naphthyl)alanine, is recovered with good yield and excellent enantiomeric excess.

Key words: D-Amino acid oxidase, Amino transferase, Non-natural amino acid, Multi-enzyme reaction, Deracemization, Dynamic kinetic resolution

1. Introduction

Unnatural amino acids, the non-genetically-coded amino acids that either occur naturally or are chemically synthesized, and particularly synthetic α -amino acids, are a growing group of compounds required for a large number of biotechnological applications (from the pharmaceutical and cosmetics to the agrochemicals field) (1–4). Due to their structural diversity and functional versatility, they are widely used as chiral building blocks and molecular scaffolds in constructing chemical combinatorial libraries. Furthermore, they have played a significant role in the area of peptide research (5). Unnatural amino acids are not only of great value for the de novo design of peptides and proteins with a high propensity to fold with predetermined secondary or tertiary structure (6–8), but are also in their own valuable pharmaceuticals (e.g., L-DOPA), as well as

components of numerous therapeutically relevant compounds (e.g., D-3-(2-naphthyl)-alanine is found in the peptide drug Nafarelin) (9). It is thus evident that unnatural amino acids are becoming indispensable tools in drug discovery efforts.

The worldwide increase of the market for enantiopure raw materials largely requires the development of new products by new chiral technologies and improved enantioselective processes. In particular, the use of biocatalysts is largely investigated. In recent times, the term deracemization has been used to describe techniques becoming increasingly important for the preparation of chiral compounds as single enantiomers. The term refers to a process in which starting from a racemate, one single enantiomer is obtained. Deracemization methods are particularly suited for non-racemic amino acid preparation since the synthesis of the racemic forms are well established. Non-natural amino acids are particularly required in preparative scale and high enantiomeric excess for the preparation of peptidomimetics.

Here we present two alternative methodologies for obtaining the same compound, L-3-(2-naphthyl)-alanine (L-2-NpAla), by deracemization: the first method is the deracemization by stereo-inversion and the second one is by dynamic kinetic resolution (DKR). The substrate employed in the two methods has been D,L-3-(2-naphthyl)-alanine (D,L-2-NpAla), a non-natural amino acid of interest for the preparation of peptidomimetics and drugs candidates.

2. Materials

2.1. Deracemization by Stereo-Inversion

1. All reagents and solvents are purchased from Sigma-Aldrich.
2. ^1H NMR Spectra are recorded on Bruker ARX 400 instrument operating at the ^1H resonance frequency of 400 MHz. Chemical shifts (δ , ppm) are reported relative to tetramethylsilane (TMS) as an internal standard. All spectra are recorded in DMSO- d_6 (when not differently indicated) at 305 K.
3. Silica gel 60 F₂₅₄ plates (Merck) are used for analytical TLC. Detection is achieved with UV light followed by I₂, ninhydrin or potassium permanganate staining.
4. HPLC analyses are performed on a Merck-Hitachi apparatus L6200 intelligent pump fitted with a UV detector.
5. The bioconversion is followed on a Zorbax SB-Aq column (150 mm×4.6 mm, 5 μm ; Agilent Technologies) using trifluoroacetic acid (TFA) 0.1%/acetonitrile (70/30), flow rate at 1 mL/min, with benzoic acid as internal standard, detector at 254 nm.

6. The enantiomeric excess of L-2-NpAla is determined with a Crownpak CR+ (250 mm, 4 μ m, Chiral Technologies), using aqueous perchloric acid (pH 1.5): methanol (9:1), flow rate at 1 mL/min, detector at 254 nm.
7. Optical rotations are determined with a Propol Digital Polarimeter Dr Kenchen, and $[\alpha]_D$ values are given in units of deg cm²/g at 25°C.
8. Oxygen consumption assays are performed with a Hansatech oxygen electrode (Hansatech Instruments Ltd, Pentney, UK).
9. UV measurements are carried out with a UV/Vis Jasco V-560 spectrophotometer.

2.2. Deracemization by Dynamic Kinetic Resolution

1. The subtilisin in CLEA® form is marketed by CLEA Technologies and sold under the name Alcalase CLEA-ST.
2. The analytical equipments, unless otherwise specified, are the same used in Subheading 2.1.
3. The solvents used for HPLC analysis and sample preparation were purchased from Sigma-Aldrich and were all of HPLC grade.
4. The Chiralcel-OD and the Crownpak-CR+ column were bought from Daicel Chemical Industries, Ltd.
5. Chromatographic analysis for substrate conversion: Chiralcel-OD 250 mm, at 25°C, using hexane/isopropanol 997:3 at 1.2 mL/min, detector at 210 nm. Peaks: 6 min (naphthalene), 44.5 min (D-boc-2-NpAla-SEt), 48.5 min (L-boc-2-NpAla-SEt).
6. Chromatographic analysis for final product chiral evaluation: Crownpak-CR+ 250 mm, at 25°C, using [HClO₄(aq.) pH 1.5]/methanol 9:1 at 1.1 mL/min, detector at 210 nm. Peaks: 29.9 min (D-2-NpAla), 38.9 min (L-2-NpAla).
7. The sample taken from the reaction is dissolved in 1 mL of methanol and filtered through a 40- μ m PPE membrane using a plastic syringe. The resulting solution is directly injected in the HPLC system.

3. Methods

3.1. Deracemization by Stereo-Inversion

Deracemization by stereo-inversion is a multistep chemo-enzymatic transformation which starts with a racemic amino acid derivative. While one of the enantiomers (R_f) is not affected by the enzymatic transformation, the other enantiomer (S_f) is transformed into a compound (A_f) which can, in turn, be transformed into the starting compound of opposite configuration (R_f) or into a racemate. In the

first case, the convergent process gives only the enantiomer (R_f); in the second one, in successive cycles the enantiomeric excess increases at each cycle, eventually reaching complete conversion into one single enantiomer. At the end of the process, the enantiomer (S_e) is completely transformed into (R_f) (10). This methodology allows the complete recovery of a single enantiomer from a racemic mixture.

Here we present the deracemization of the amino acid D, L-3-(2-naphthyl)-alanine by stereo-inversion based on a combination of an amino acid oxidase of D-selectivity with an amino transferase with L-specificity.

D-Amino acid oxidase (EC 1.4.3.3, DAAO) is a highly stereoselective flavoenzyme which catalyses the dehydrogenation of the D-isomer of amino acids to give the corresponding α -imino acids and, after subsequent hydrolysis, α -keto acids and ammonia (11). Oxygen, the terminal redox acceptor, reoxidizes the reduced FAD cofactor to give hydrogen peroxide. In particular, DAAO from the yeast *Rhodotorula gracilis* exhibits a very high turnover number, tight binding with the coenzyme FAD, a broad substrate specificity and has an active site large enough to accommodate even substrates of considerable size (12, 13).

Amino transferases (ATs) catalyse the reversible transfer of an amino to a keto group in a reaction between an amino acid (as an amino donor) and a keto acid (as amino acceptor). Many bacterial ATs accept a wide array of keto acids as amino acceptors and are useful as biocatalysts in the preparation of AAs. Among the ATs used in biotransformations, aspartate amino transferase (EC 2.6.1.1, AAT) shows a wide substrate specificity both towards the amino donor and the acceptor. Problems arising from the application of this system for the production of L-amino acids from the corresponding α -keto acid concern the equilibrium constant of the reaction which, being closed to 1, requires a system to shift the equilibrium towards the desired side. Several multiple enzymatic systems have been applied to solve the problem, both with whole cell catalysts and with coupled enzyme strategies. The problem of equilibrium of the transamination reaction has been solved by using L-cysteine sulfinic acid (L-CSA) as the amino donor which furnishes an unstable β -keto sulfinic acid, which is readily decomposed into pyruvate and sulfur dioxide without the need of catalysis (14, 15). Moreover, because of the high acidity of L-CSA, the purification of the amino acid product by ion exchange chromatography is highly simplified.

We report the application of DAAO from *R. gracilis* to generate the 2-naphthylpyruvic acid (2-NpPA) from the racemic mixture of 3-(2-naphthyl)-alanine and the in situ transformation of

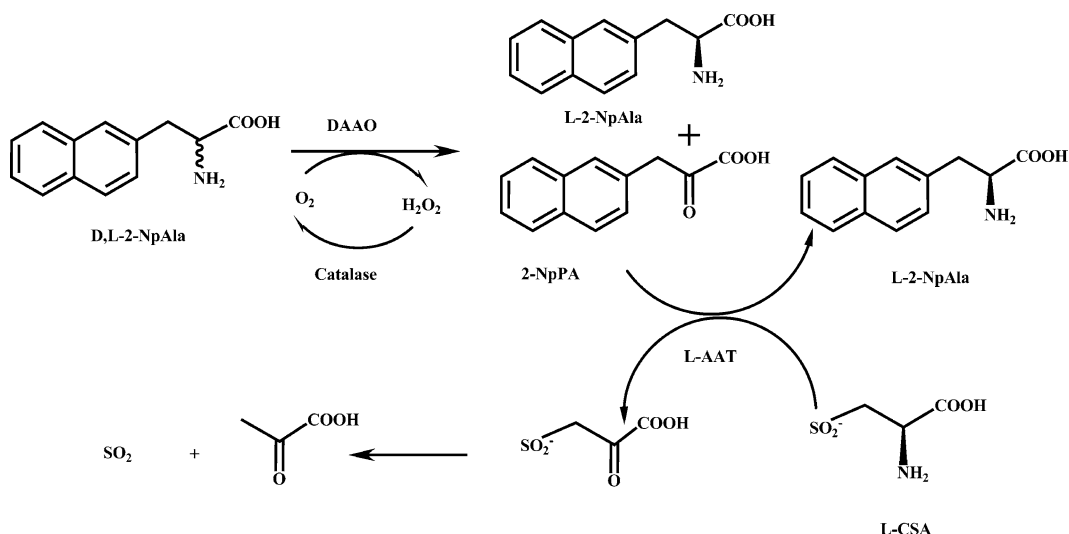


Fig. 1. Multistep enzyme catalysed deracemization of D,L-3-(2-naphthyl)-alanine (D,L-2-NpAla).

the 2-NpPA into L-3-(2-naphthyl)-alanine catalysed by L-AAT using L-cystein sulphinic acid as irreversible amino donor (see Fig. 1) (16).

3.1.1. Enzyme Expression and Purification

1. Recombinant DAAO from *R. gracilis* is expressed and purified from BL21(DE3)pLysS *Escherichia coli* cells as described previously (17).
2. The pure enzyme has a specific activity on D-alanine of 110 U/mg protein.
3. L-Aspartate amino transferase (L-AAT) from *E. coli* is produced and purified following a described procedure from an over-expressing *E. coli* strain TY103 transformed with pUC19-aspC (18–20).

3.1.2. Activity Assay and Kinetic Measurements

1. DAAO activity is assayed with an oxygen electrode at air saturation (0.253 mM O₂) and 25°C, using 28 mM D-alanine as substrate in 75 mM sodium pyrophosphate buffer, pH 8.5. One DAAO unit is defined as the amount of enzyme that converts 1 μmol of D-alanine per minute at 25°C (12, 13, 17).
2. L-AAT is assayed using 2-naphthyl pyruvic acid (2-NpPA) in the following conditions: 1 mL solution of aspartate (40 mM), L-AAT (0.05 U), NADH (0.14 mM), malic dehydrogenase (0.15 U), 2-NpPA (0.2–4 mM) in potassium phosphate buffer 100 mM at pH 8.0; the reaction time course is followed at 340 nm and 25°C.

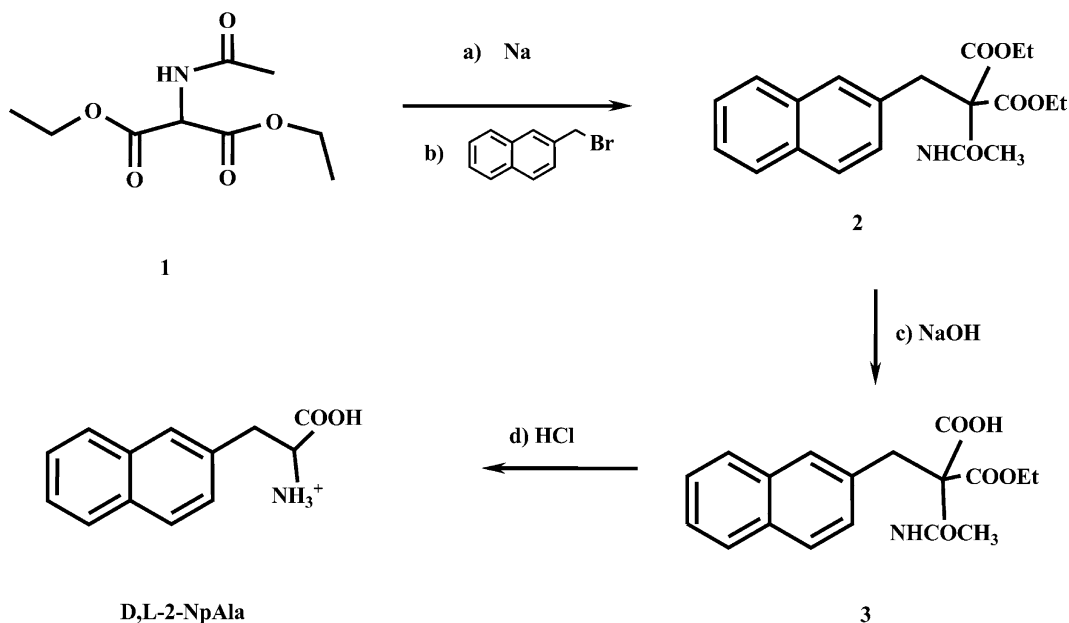


Fig. 2. Synthesis of D,L-2-naphthyl alanine (D,L-2-NpAla).

3.1.3. D,L-2-Naphthyl Alanine Preparation

1. The D,L-2-naphthyl alanine is prepared by a malonic ester synthesis starting from diethyl acetamidomalonate **1** alkylated by 2-bromomethylnaphthalene (**21**).
2. After partial hydrolysis of the diester **2** and subsequent decarboxylation of the monoethylmalonate derivative **3**, the desired product D,L-2-NpAla is obtained (see Fig. 2).

3.1.4. Preparation of Diethyl (2-Naphthylmethyl) Acetamidomalonate 2

1. Prepare a solution of sodium (1.1 g, 46 mmol) in dry ethanol (100 mL).
2. Dissolve diethyl acetamidomalonate **1** (10 g, 46 mmol) in this solution.
3. Add 2-bromomethylnaphthalene (16.3 g, 73.4 mmol) at room temperature.
4. Reflux the reaction mixture for 7 h.
5. Evaporate ethanol and treat the residue with water (200 mL) and extract the product with ethyl acetate (AcOEt).
6. Wash the organic phase twice with water.
7. Dry the organic phase by addition of Na_2SO_4 , filter the suspension and evaporate to dryness to obtain diethyl (2-naphthylmethyl) acetamidomalonate **2** (15.5 g, 94.4% yield).
8. The ^1H NMR data of compound **2** are: δ 7.83–7.70 (m, 3H), 7.41–7.5 (m, 3H), 7.14–7.1 (m, 1H), 4.296 (q, 7.347Hz, 6H), 3.830 (s, 2H), 2.034 (s, 3H), 1.325 (t, 7.347Hz, 6H).

3.1.5. Preparation
of Monoethyl
(2-Naphtylmethyl)
Acetamidomalonate **4**

1. Prepare a solution of diethyl (2-naphtylmethyl)acetamidomalonate **2** (14.15 g, 39.6 mmol) in ethanol (50 mL).
2. Add 200 mL of water and NaOH 10 N (4 mL).
3. Stir the reaction at room temperature and analyse by TLC (eluent: toluene/CH₃CN/CH₃COOH: 6/3/1).
4. After 4 h, the mixture is evaporated.
5. Treat with water (200 mL) and extract with AcOEt (200 mL) at pH 8.
6. Acidify the aqueous phase with 6 N HCl to about pH 2 and extract twice with AcOEt (2 × 150 mL).
7. Collect the organic phases.
8. Dry the organic phases by addition of Na₂SO₄; filter the suspension and evaporate to dryness.
9. The obtained white solid residue (12.5 g) is crystallized from a mixture of ethanol/hexane to give product **3** (6.3 g, 48% yield).
10. The ¹H NMR data of compound **3** are: δ 7.9–7.70 (m, 3H), 7.60–7.46 (m, 3H), 7.204 (m, 1H), 4.20 (q, 6.72, 2H), 3.65 (s, 2H), 2.034 (s, 3H), 1.228 (t, 6.72Hz, 3H).

3.1.6. Preparation of
D,L-3-(2-Naphthyl)-Alanine

1. Prepare a suspension of monoethyl (2-naphthylmethyl) acetamidomalonate **3** (5 g, 15.2 mmol) ([21](#)) in 100 mL water-ethanol (1:1).
2. Add 1 mL of 12 N HCl.
3. Reflux the suspension under magnetic stirring.
4. Monitor the reaction by TLC (AcOEt:*i*-PrOH:AcOH, 2:2:1).
5. After 12 h, add dropwise 10 N NaOH under stirring until a pH of about 6.2 is reached.
6. Collect the white precipitate by filtration.
7. Dry the white solid under vacuum.
8. Recrystallize the solid from AcOEt to obtain D,L-2-NpAla (2.3 g, 70% yield).
9. The ¹H NMR data of D,L-2-NpAla are: δ 3.30 (m, 2H), 4.23 (s, 1H), 7.4–7.56 (m, 3H), 7.75–7.92 (m, 4H), 8.37 (br s, 2H) (see Note 1).

3.1.7. Preparation
of 2-Naphthyl Pyruvic
Acid (2-NpPA) **6**

2-NpPA **6** is prepared (see Fig. [3](#)) from the corresponding azalactone **5** ([22](#)) which in turn is obtained from the commercial 2-naphtaldehyde **4** as described in the literature ([23](#)).

3.1.8. Preparation
of Azalactone **5**

1. Prepare a solution of 2-naphtaldehyde **4** (10 g, 64 mmol) in acetic anhydride (25 mL).
2. Add *N*-acetylglycine (NAc-gly-OH, 9.75 g, 83 mmol).
3. Add sodium acetate (3.52 g, 70.4 mmol).

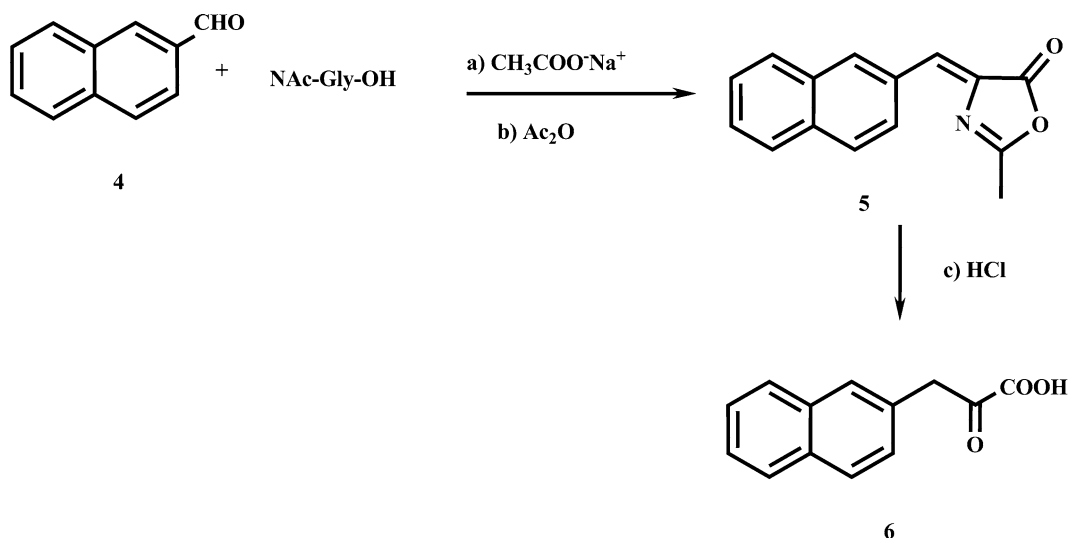


Fig. 3. Synthesis of 2-naphthyl pyruvic acid (2-NpPA) **6**.

4. Heat the mixture to 100°C in an oil bath for 2 h.
5. Stir the reaction mixture at room temperature overnight to allow the corresponding azalactone **5** to precipitate as a yellow solid.
6. Add 25 mL of water.
7. Filter the solid, wash three times with acetone and dry under reduced pressure to give product **5** (8 g, 52% yield) of suitable purity.
8. The ^1H NMR spectrum data of compound **5** are: δ 12.5 (1H, broad), 9.5 (1H, s), 7.8–8.65 (7H, m); 2.05 (3H, s).

3.1.9. Preparation of 2-Naphthyl Pyruvic Acid **6**

1. Prepare 100 mL of a solution of HCl 3 N: dioxane/1:1.
2. Add azalactone **5** (5 g, 21.1 mmol).
3. Stir the suspension to reflux for 4 h.
4. Cool the solution and isolate the product acid by filtration.
5. Wash the residue with water, dry under reduced pressure and crystallize from ethanol/hexane to obtain product **6** (3.59 g, 80% yield).
6. The ^1H NMR spectrum data of compound **6** are: δ 9.2 (1H, broad), 7.5–8.5 (7H, m), 2.5 (2H, s).

3.1.10. Analytical and Preparative Deracemization of *D,L*-2-NpAla with the DAAO-L-AAT System and L-CSA as Amino Donor

1. Prepare 1 mL of reaction mixture by mixing the substrate *D,L*-2-NpAla (from 0.2 to 1 mM) and L-cysteine sulfinic acid CSA (40 mM) in 1 mL of water by sonication at 40°C.
2. Add L-AAT (1 U) and catalase (740 U).
3. Perform the enzymatic reaction at pH 8.0 (by addition of 1 M KOH) and 25°C starting by addition of DAAO (0.16 U).

4. The reaction is followed at 254 nm by HPLC analysis.
5. Perform the multienzymatic deracemization of D,L-2-NpAla on a preparative scale employing the three enzymes (DAAO, L-AAT, and catalase), 50 mg of D,L-2-NpAla (0.23 mmol, 1 mM) in 240 mL of water and the other reagents used according to the proportion of the analytical conversion previously described (see Note 2).
6. The reaction is complete in 2 h giving as the only product L-2-NpAla, which is recovered by cationic exchange chromatography in 98% yield and 99.5% ee (see Note 3).
7. Importantly, when the (virtual) concentration is increased tenfold, biotransformation of the insoluble substrate is still possible. In this case, and in order to reach completion of the reaction, add fresh AAT (120 U) and DAAO (20 U) four times at 2 h intervals: the rate limiting step of the reaction appears to be the dissolution process of amino acid.
8. Under optimised conditions, conversion of 1.2 mM of D,L-2-NpAla requires 40 U/L of DAAO per hour and the reaction is complete in 220 min. The enantiomeric excess of the obtained L-2-NpAla is >99.9% and the overall yield >90%.

3.2. Deracemization by Dynamic Kinetic Resolution

Hydrolytic enzymes are the most readily available biocatalysts for synthetic applications, particularly for the kinetic resolution of racemic mixtures (24–26). Like in any resolution method, a maximum of 50% yield of enantiomerically pure product can be obtained, based on racemic starting material. However, when kinetic resolution is coupled with an in situ racemization of the substrate (either chemical or enzymatic), the yield limitation can be overcome leading to a much more efficient process: a deracemization based on a dynamic kinetic resolution (DKR) (10, 27, 28).

Requisites for a successful DKR are an enzyme selective for a single enantiomer, a racemizing system acting on the substrate but not on the product, and a rate of racemization of the substrate higher than the rate of conversion to product. These conditions often require the design of suitable substrates. In thioesters, the acidity of the hydrogen in the α -position is higher in comparison to the corresponding oxo esters, amides or acids. The enzymatic transformation of a thioester into the corresponding carboxylate with a higher pK_a of the α -proton is the basis for a successful DKR, provided that the enzymatic systems resist the basic conditions required for substrate racemization. This concept has been applied in the DKR of α -alkyl thioesters (29).

The L-forms of racemic-*N*-*boc*-aryl- α -amino acid thioesters were found to be substrates for the subtilisin-catalysed hydrolysis to the corresponding acids. Moreover, the unreacted D-enantiomer was found to be continuously racemized in the presence of an organic base. The combined reactions in a biphasic system allowed

the deracemization of the amino acid derivatives based on a dynamic kinetic resolution. Excellent yields and enantioselectivities were achieved (30).

Successively, we made some modifications in the system in order to attain an efficient DKR of α -alkyl-amino acid thioesters too which are not racemized under the previous conditions. In this case, a stronger base is needed and it is necessary to run the reaction in an hydrophilic organic solvent (e.g. *tert*-butanol). The enzyme must be of course compatible with these unusual conditions: we found that subtilisin in CLEA[®] preparation is particularly effective in carrying out the resolution with proper activity and stability, could be separated at the end of the reaction and potentially reused.

This technology has been applied to a large number of amino acid thioesters, among which the 2-naphthylalanine derivative, shown here as an example, is included.

3.2.1. Saturated Hydrochloric Acid Solution in 1,4-Dioxane

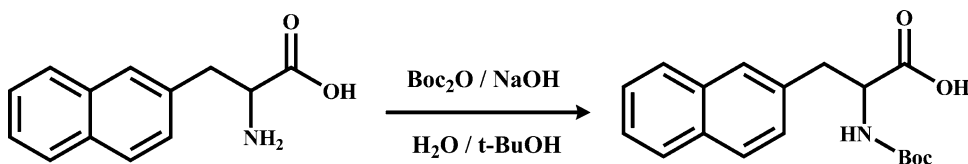
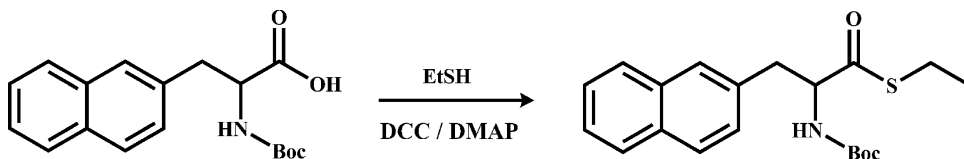
1. Pour 300 mL of 1,4-dioxane in a 500 mL, two necked round-bottomed flask equipped with a water bath and magnetic stirring.
2. The side neck, by a quickfit adapter, is equipped with a sparger immersed in the solvent and connected by PVC tubing to an HCl (g) cylinder (see Note 4), whereas the central neck is connected to an oil bubbler. Set the water bath temperature at 15°C (see Note 5), and switch a vigorous stirring on.
3. Carefully open the cylinder valve in order to assure a small but constant flow of gas into the solution (see Note 6).
4. Once in a while, close the valve, disconnect the cylinder, stopper and weigh the flask. If the weight is not constant, resume the saturation (see Note 7).
5. Once saturated, the dioxane solution is kept in a tightly closed glass bottle stored in a ventilated shelf at room temperature. It is possible to determine the HCl content by dissolving 1 mL of solution in 50 mL of distilled water and titrating with 0.1 N NaOH using phenolphthalein as an indicator (see Note 8).

3.2.2. Enzyme Activation

1. Suspend 1 g of the enzymatic powder in 10 mL of 100 mM sodium borate buffer (pH 9.0) under magnetic stirring (see Note 9).
2. Check the pH using a glass electrode and bring it to 9.0 by a dropwise addition of a 0.1 M sodium hydroxide solution.
3. Then, filter the mixture through a Buchner funnel, wash it twice with 5 mL of ethanol, once with 5 mL of *tert*-butanol and finally vacuum-dry using a water pump (see Note 10).

3.2.3. Synthesis of D,L-Boc-2-NpAla (see Fig. 4)

1. Pour 15 mL of water together with 0.45 g (11.2 mmol) of sodium hydroxide in a 50 mL round-bottomed flask under magnetic stirring.

Fig. 4. Synthesis of D,L -*boc*-2-NpAla.Fig. 5. Synthesis of D,L -*boc*-2-NpAla-SEt.

2. Then, add 1.5 g (7.5 mmol) of D,L -2-NpAla and 6 mL of *tert*-butanol, obtaining a clear solution.
3. Separately, dissolve 3.32 g (14.9 mmol) of di-*tert*-butyl dicarbonate in 10 mL of *tert*-butanol and slowly drop it into the reaction mixture along 1 h (see Note 11).
4. Let the mixture react overnight.
5. Then (see Note 12), pour the clear solution (see Note 13) in a separatory funnel and extract twice with 50 mL of *n*-hexane.
6. Extract the organic phases twice with 20 mL of a saturated aqueous sodium bicarbonate solution. Bring together the aqueous layers, under magnetic stirring, to pH 2 by careful addition of 6 N HCl (see Note 14). Extract the resulting milky mixture three times with 100 mL of diethylether.
7. Combine the organic phases together, dry over anhydrous sodium sulfate and filter through a cotton plug.
8. Remove the solvent by means of a rotary evaporator, dissolve the oily residue in 50 mL of *n*-hexane and leave overnight in a refrigerator.
9. Collect the white, crystalline precipitate by suction-filtration using a Buchner funnel, wash with cold hexane and dry under reduced pressure to give the final product as a white, crystalline powder (2.00 g, 88% yield).

3.2.4. D,L -*Boc*-2-NpAla-SEt (see Fig. 5)

1. Pour 800 mg (2.5 mmol) of D,L -*boc*-2-NpAla in a 10-mL flask and dissolve in 4 mL of dichloromethane.
2. Then, add 315 mg (5.1 mmol) of ethanethiol and 31 mg (0.3 mmol) of dimethylaminopyridine (DMAP) (see Note 15).
3. Immerse the flask in an ice bath, and keep under magnetic stirring for 10 min.

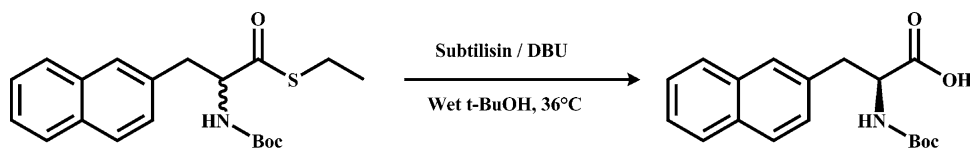


Fig. 6. Dynamic kinetic resolution on D,L -boc-2-NpAla-SEt.

- At this point, add dropwise a solution of dicyclohexylcarbodiimide (DCC, see Note 16) (1.05 g, 5.1 mmol) in 1 mL of dichloromethane using a Pasteur pipette.
- Upon completion of the addition, remove the ice bath and leave the reaction mixture overnight at room temperature under stirring.
- The following day, filter the white mixture through a porous glass in order to eliminate most of the dicyclohexylurea, and evaporate the solvent.
- Purify the residue by flash chromatography (eluent: hexane/ethyl acetate 95/5), obtaining a white, crystalline solid (802 mg, 88% yield). $^1\text{H-NMR}$: δ = 1.169 (t, J = 7.279 Hz, 3H), 1.321 (s, 9H), 2.826 (q, J = 7.279, 2H), 3.160 (br s, 1H) 3.259 (br s, 1H), 4.674 (br s, 1H), 4.941 (br s, 1H), 7.225–7.744 (m, 7H).

3.2.5. Dynamic Kinetic Resolution (see Fig. 6)

- Dissolve the D,L -boc-2-NpAla-SEt (200 mg, 0.56 mmol) in 4 mL of *tert*-butanol, and pour the resulting solution in a 10 mL vial, placed in an orbital shaker thermostated at 36°C.
- At this point, add 170 μL of DBU (170 mg, 1.12 mmol) and 8 mg of naphthalene. Immediately, withdraw an aliquot from the solution, constituting the t_0 sample for the HPLC analysis.
- Add the reactivated enzyme Alcalase CLEA® (200 mg) and switch the shaker on.
- Withdraw aliquots at predetermined intervals in order to monitor the conversion (see Note 17).
- At the end of the resolution process (t_n = 28 h), centrifuge the reaction mixture (4,000 rpm, 5 min) in order to separate the enzyme from the solution.
- Carefully, pour the latter in a separate flask, and wash the solid residue (by resuspending and centrifuging again) twice with 2 mL of *tert*-butanol. Keep it in a fridge (4°C) for successive use.
- Bring the *tert*-butanol solutions together and eliminate most of the solvent using a rotary evaporator.
- Suspend the residue in 10 mL of water and extract with ethyl ether (5 mL, three times).
- Separate the aqueous layer and bring it to pH 3 by a dropwise addition of a 6 N HCl solution.

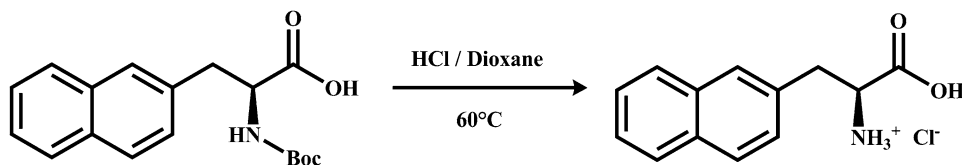


Fig. 7. Deprotection of L-boc-2-NpAla.

10. Extract the resulting dispersion with ethyl acetate (10 mL, three times).
11. Put the organic phases together, dry with sodium sulfate and bring to a rotary evaporator for the complete evaporation of the solvent.
12. A white solid is obtained (165 mg, 93% yield).

**3.2.6. Deprotection of
L-Boc-2-NpAla (see Fig. 7)**

1. Dissolve the obtained L-boc-2-NpAla in 5 mL of a solution of HCl in dioxane (0.8 M).
2. Keep the resulting mixture at 60°C under stirring for 30 min, and then bring to dryness using a rotary evaporator.
3. Resuspend the resulting white solid in 5 mL of diethyl ether and filter through a Buchner funnel, obtaining the final product L-2-NpAla whose enantiomeric excess is measured by HPLC.

4. Notes

1. The ¹H NMR spectrum of D,L-2-NpAla has been recorded in DMSO-d₆/TFA-d₄ because of the low solubility of the compound.
2. The large amount of pyruvic acid produced clearly indicates that the use of cysteine sulphinic acid as amino donor was very effective in completely shifting the equilibrium.
3. The main limit of this reaction system is the extremely low solubility of 2-NpAla and thus the low space/time yield of the bioconversion.
4. It is not necessary to obtain a strictly anhydrous solution. For this reason, no on-line devices (like a concentrate sulphuric acid washing bottle) to completely eliminate water are included in this setup.
5. A rigorous temperature control is not required because the saturation process is not strongly exothermic. Nevertheless, the chosen temperature ensures a good saturation level while keeping the solvent, whose melting point is 12°C, in liquid state.

6. During the saturation phase, an excessive flow of HCl can be detected through a steep colour change of an indicator strip placed after the bubbler.
7. While closing the valves and disconnecting the tubings, pay attention to the possible pressure drops which can give rise to leaks or back-suctions.
8. Usually, the final HCl concentration is around 0.8 M.
9. It is advisable to employ a stirring bar with a central ring together with a moderate rotation speed in order to avoid grinding the enzyme.
10. It is not advisable to proceed to complete dehydration of the preparation because some water is necessary to keep active the enzyme. For this reason, water pump is a mild and effective drying device.
11. If the room temperature is too low to keep the *tert*-butanol liquid, it is possible to slightly warm the solution around 30°C or, alternatively, to use acetone or THF in substitution.
12. The solution should be feebly alkaline.
13. Sometimes, little amounts of a white precipitate (a secondary product) are obtained. In this case, it is convenient to filter the mixture before the extraction.
14. A copious development of carbon dioxide occurs. An efficient stirring is crucial in order to prevent the formation of “acidic spots” where the product would decompose.
15. Due to the strongly disagreeable odour of the ethanethiol, the reaction must be run under an efficient hood, and every piece of glassware having been in contact with the thiol should be treated with an oxidizing solution (e.g., a potassium permanganate solution acidified with a few drops of concentrated sulphuric acid).
16. DCC is a potent allergen and sensitizer. Melting the product could be convenient for easy handling without the risk connected to accidental spillages.
17. Sampling must be carried out under an efficient hood, due to the ethanethiol developing from the reaction.

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