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Efficient chemoenzymatic synthesis of enantiomerically pure β-heterocyclic amino acid derivatives

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Abstract

Enantiomerically pure (*S*)-pyrazolylalanine and its derivatives are nonproteinogenic amino acids with antidiabetic activity. A short and effective enantioselective synthesis of these compounds is described, using a kinetic resolution by an acylase from *Aspergillus* sp. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

(*S*)-β-Pyrazolylalanine is a naturally occurring amino acid which was first isolated by Shinano and Kayo from the pressed juice and the seeds of watermelon, *Citrullus vulgaris*,[1] and has subsequently been found in other cucurbit plants.[2] Cucurbit seeds contain significant amounts of γ-L-glutamyl-β-pyrazolylalanine together with free β-pyrazolylalanine.[3] Several racemic syntheses have been described,[4,5] a resolution procedure[6] and two enantiospecific syntheses[7] have been reported, the sign of the specific rotation for the (*S*)-enantiomer being different. Monteiro et al. have reported an efficient synthesis of a series of β-heterocyclic amino acids by Michael addition of heterocyclic nucleophiles to commercially available *N*-di-*tert*-butyloxycarbonyl dehydroalanine methylester. After total deprotection they obtained racemic mixtures of these amino acids.[5]

We present herein a short and efficient strategy to obtain enantiomerically pure β-heterocyclic amino acids in nearly quantitative yields.

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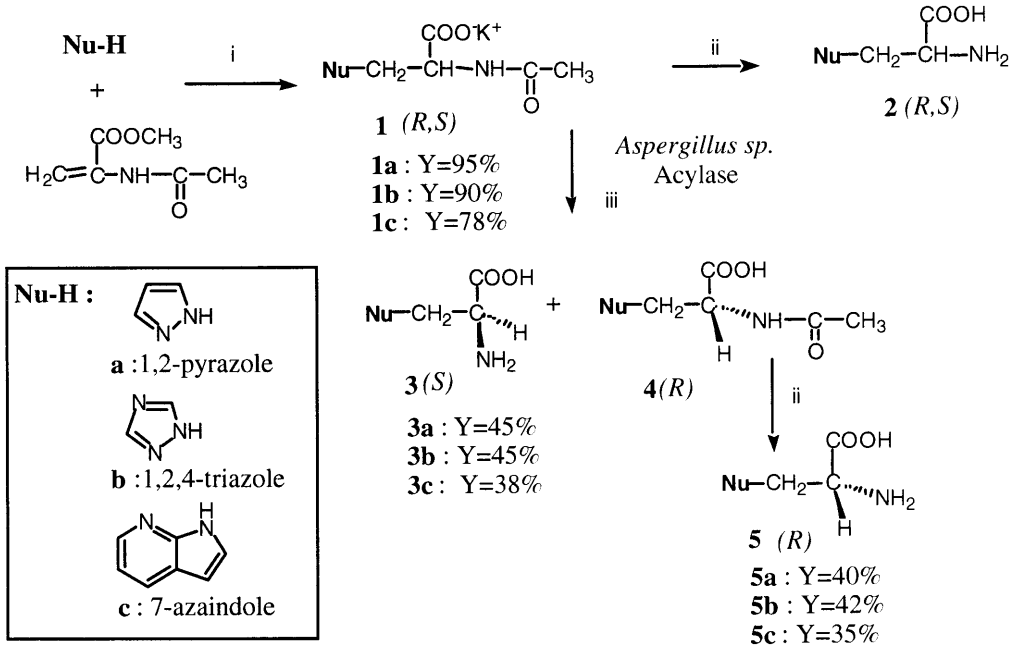
2. Results

Needing appreciable quantities of the two enantiomerically pure isomers for biological activity studies, we describe a short and practical synthesis of either enantiomer of β-pyrazolylalanine **3a**, β-triazolylalanine **3b** and azaindolylalanine **3c** which can be performed on a gram scale (Scheme 1).



Scheme 1. i: K_2CO_3, CH_3CN/0.5% H_2O. ii: HCl 6N. iii: *Aspergillus* sp. acylase, phosphate buffer pH 7.2/5×10^{-4} M $CoCl_2$

Reaction of an excess of pyrazole **a**, 1,2,4-triazole **b** or 7-azaindole **c** on commercially available methyl-2-acetamidoacrylate in CH_3CN at 60°C in the presence of K_2CO_3 led directly to the potassium salt of *N*-acetyl-β-heterocyclic alanines **1a, 1b** and **1c** in 95%, 90% and 78% isolated yields, respectively. The presence of nearly 0.5% water in acetonitrile was sufficient to perform also the saponification under basic conditions. Acidic deprotection (6N HCl) gave racemic β-heterocyclic alanines **2a, 2b** and **2c** in quantitative yields. These compounds were necessary to determine the conditions for a complete separation of the two enantiomers on chiral column chromatography.

Resolution of racemic *N*-acetyl-β-pyrazolylalanine methyl ester with α-chymotrypsin in phosphate buffer was unsuccessful, suggesting that β-pyrazolylalanine bears a close resemblance to histidine.[8] Resolution of *N*-acetyl-β-pyrazolylalanine with acylase from *Aspergillus* sp. gave outstanding results yielding the two enantiomerically pure β-pyrazolylalanine enantiomers **3a** and **5a** with excellent e.e. (99%+1%); the enantiomeric purity was checked on chiral Crown Pack CR^+.

(*S*)-β-Triazolyl alanine **3b** is known as an important metabolite in plants of the fungicide Myclobutanil. A biosynthetic pathway from *O*-acetyl-L-serine and 1,2,4 triazole catalyzed by a cysteine synthase present in higher plants has been reported.[9] *N*-Acetyl-β-triazolylalanine was obtained in 95% yield using the same experimental conditions and the resolution with *Asper-*

gillus sp. acylase gave the two β-triazolylalanine enantiomers **3b** and **5b,** respectively in 45% and 42% yield after recrystallization (e.e. = 99%+1%).

The azaindolylalanine is an isostere of the fluorescent probe, 7-azatryptophan, which was prepared by a chemoenzymatic pathway.[10] The *N*-acetyl-azaindolylalanine **1c** was synthesized in 78% yield and the (*S*)-azaindolylalanine **3c** was obtained in 38% final yield after recrystallization using the same experimental conditions as for the **3a** synthesis (e.e. = 99%+1%).

This very short synthesis (only two steps) is an efficient route to a series of enantiomerically pure unnatural amino acids. In order to use this strategy in combinatorial chemistry, we have performed this synthesis of racemic compounds on solid support.[11]

3. Experimental

Thin-layer chromatography (TLC) was performed on Merck precoated silica gel 60F254 plates and spots were visualized by ultraviolet light or/and iodine vapour. The spots were revealed also by 0.2% ninhydrin solution. HPLC was performed on Waters HPLC system with a 486 UV detector apparatus on a Crown Pack CR^+ column. Melting points were obtained on a Büchi 510 apparatus and were not corrected. Optical rotations were recorded with a Perkin–Elmer 141 polarimeter at the sodium D line. Mass spectra were measured by Electrospray on Micromass apparatus (Manchester, UK), Platform model ESI (eluent H_2O/CH_3CN 0.1% TFA (1/1) (vol/vol)). ^1H NMR Spectra were recorded on a Brucker spectrometer AC 250.

3.1. Potassium salt of (RS)-N-acetylpyrazolylalanine *1a*

Pyrazole (2.1 g, 30 mmol) was dissolved in 35 ml acetonitrile containing 0.1% H_2O and K_2CO_3 (3.4 g, 24.6 mmol) was added. The mixture was stirred for 10 min. Then methyl-2-acetamidoacrylate (2 g, 13 mmol) was added and the mixture was stirred for 48 h at 60°C. The disappearance of the methyl-2-acetamidoacrylate was followed by TLC in ethyl acetate (R_f = 0.85). After filtration, the solvent was evaporated under reduced pressure. The excess of pyrazole was eliminated by precipitation in a mixture of $MeOH/Et_2O$. The potassium salt of (*RS*)-*N*-acetylpyrazolylalanine **1a** was directly obtained in 90% yield.

3.2. Acylase from aspergillus genus (TCI) purification

The commercially available acylase from *Aspergillus* sp. from TCI (10 000 U g^{-1}) was eluted on a Sephadex G50 Gel-filtration column with distilled water and a solution of $CoCl_2$ (5×10^{-4} M). The elution was followed on a Waters refractometer and checked by Bradford test. The enzyme was lyophilized and stored at −18°C. Only 30% in weight of protein was obtained from the crude commercial powder. Preliminary enzyme purification was necessary to facilitate (*S*)-amino acid and (*R*)-*N*-acetyl amino acid separation.

3.3. Enzymatic resolution by acetyl group hydrolysis: (S)-β-pyrazolylalanine *3a*

The substrate **2a** (2.5 mmol) was dissolved in phosphate buffer with 5×10^{-4} M $CoCl_2$ (15 ml, 0.1 M; pH 7.2). Then the purified acylase (20 mg) dissolved in buffer (0.5 ml) was added. The reaction was stirred at room temperature. When the pH decreased to 6.5, aliquots of 6N NaOH

were added until the optimum pH 7.2 for the best enzyme activity. The reaction could be monitored by a pHstat.

The enantioselective hydrolysis was followed by HPLC chromatography on a chiral column Crown Pack CR$^+$ with a solution of H_2O/perchloric acid pH 1.6 at 0°C as eluent. The mixture was stirred during 32 h without hydrolysis of the (*R*)-*N*-Ac-β-pyrazolylalanine **4a** and the reaction was quenched with 6N HCl until pH 3. The products were separated by elution on ion exchange resin (Dowex 50WX8). The solvent was evaporated under reduced pressure and the residue was dissolved in a solution (10 ml) of distilled water/methanol (4/6) (vol/vol). The ion exchange resin (500 mg) was previously washed 2 times with distilled water and 2 times with methanol. The solution was mechanically stirred with resin for 30 min. The binding of the free (*S*)-amino acid was followed by its disappearance from TLC with ethanol/NH_4OH (4/1) as eluent (R_f=0.4) and ninhydrine. To follow the binding, chiral chromatography could also be used. The resin-bound-amino acid (*S*) was washed with distilled water (15 ml) and filtrated (2 times). The (*R*)-*N*-Ac-β-pyrazolylalanine **4a** and the acylase were in the filtrate. (*S*)-β-Pyrazolylalanine **3a** was eluted with a solution of 2 M NH_4OH (10 ml) and the resin was washed with distilled water. Compound **3a** was obtained in 45% yield after evaporation under reduced pressure and recrystallization in a mixture of water/ethanol (1/9) (vol/vol). The purity of (*S*)-β-pyrazolylalanine **3a** was checked by chiral chromatography (purity >99.5%). The conditions of an efficient separation of the two enantiomers, obtained by complete hydrolysis of (*RS*)-*N*-Ac-β-pyrazolylalanine **2a** with 6N HCl under reflux, had been performed previously. $[\alpha]_D=-68$ (c=9.7 H_2O) (lit. $[\alpha]_D=-72.0$ (c=1.0 H_2O);[7a] mp=239–241°C (lit. mp 241–243°C).[7] ^{1}H NMR (D_2O): δ ppm 4.25 (t, 1H, J=5.05 Hz); 4.75 (d, 2H, $\underline{CH_2}$, J=5.05 Hz); 6.5 (t, 1H, J=2.1 Hz); 7.75 (dd, 2H, J=8.34 Hz and 2.1 Hz). ESI: $[M+H]^+=156$. Anal. calcd for $C_6H_9N_3O_2$, 1/2 H_2O, C, 43.89; H, 6.1; N, 25.5; found: C, 43.88; H, 5.45; N, 25.1.

HPLC on chiral Crown Pack CR$^+$ optimized conditions: wavelengh=200 nm; flow=0.3 ml min^{-1}; pH=1.6 ($HClO_4$/milliQ H_2O); elution at 0°C.

	Retention time
(*RS*)-*N*-Ac-β-Pyrazolylalanine methyl ester	12 min
(*R*)-*N*-Ac-β-Pyrazolylalanine **4a**	14 min
(*S*)-β-Pyrazolylalanine **3a**	10 min
(*R*)-β-Pyrazolylalanine **5a**	7.5 min

3.4. (R)-β-Pyrazolylalanine **5a**

(*R*)-*N*-Ac-β-Pyrazolylalanine **4a** (1.25 mmol) was hydrolyzed in 6N HCl (10 ml) under reflux overnight. Then the mixture was neutralized with 6N NaOH in MeOH (10 ml) and NaCl salts precipitated. After filtration the solution was concentrated to give a white solid (95%). (*R*)-β-Pyrazolylalanine **5a** (1.2 mmol) was bound on Dowex 50WX8 ion exchange resin (500 mg), washed with water and eluted with 2 M NH_4OH (10 ml). After evaporation the residue was crystallized in a mixture of water/ethanol (1/9) (vol/vol) in 90% yield. The purity of (*R*)-β-pyrazolylalanine **5a** was checked by chiral chromatography (purity >99.5%). $[\alpha]_D=+67$ (c=10 H_2O); mp=240–242°C; ESI: $[M+H]^+=156$.

3.5. (S)-β-Triazolylalanine **3b** synthesis

(*R*)-*N*-Acetyl-β-triazolylalanine **4b** precipitated and (*S*)-β-triazolylalanine **3b** was soluble at pH 4 and 0°C in a 0.2N HCl solution. The concentration of products was around 50 g l^{-1}. The separation of both products was carried out by simple filtration. The (*S*)-β-triazolylalanine solution was concentrated under reduced pressure. The (*R*)-*N*-acetyl-β-triazolylalanine **4b** precipitated again and this procedure was repeated until one spot appeared by TLC or one peak in chiral chromatography. $[\alpha]_D = -25$ ($c = 10$ H_2O); mp = 233–234°C. ^1H NMR (D_2O): δ ppm 4.25 (t, 1H, $J = 4.30$ Hz); 4.8 (d, 2H, $\underline{CH_2}$, $J = 4.25$ Hz); 8.12 (s, 1H); 8.45 (s, 1H). ESI: $[M+H]^+ = 157$. Anal. calcd for $C_5H_8N_4O_2$, 1.5 H_2O, C, 32.8; H, 6.0; N, 30.6; found: C, 33.06; H, 5.6; N, 31.0.

The purity of (*S*)-β-triazolylalanine was checked by chiral HPLC on Crown Pack CR^+ column optimized conditions: wavelengh = 200 nm; flow = 0.2 ml min^{-1}; pH = 1.7 ($HClO_4$/milliQ H_2O); elution at 0°C.

	Retention time
(*R*)-*N*-Ac-β-Triazolylalanine **1b**	7.0 min
(*S*)-β-Triazolylalanine **3b**	5.0 min
(*R*)-β-Triazolylalanine **5b**	6.0 min

3.6. (S)-7-Azaindolylalanine **3c**

The Michael addition was slower than with pyrazole **a** or triazole **b** and during the addition the aminoester intermediate appears clearly on TLC [$R_f = 0.35$ in ethyl acetate/diethyl ether (1/1)]. The reaction was stopped after 48 h at 60°C with the disappearance of the methyl-2-acetamidoacrylate followed by TLC in ethyl acetate ($R_f = 0.85$). The solvent was evaporated under reduced pressure and the residue was chromatographed on silica gel in ethyl acetate/diethyl ether (1/1) as eluent. The (*RS*)-*N*-acetylazaindolylalanine methyl ester (15%) and (*RS*)-*N*-Ac-7-azaindolylalanine **1c** (78%) were characterized by ^1H NMR. The enzymatic hydrolysis of **1c** by *Aspergillus* sp. acylase was quantitative and the (*S*)-amino acid was easily obtained in 37% yield. $[\alpha]_D = -10$ [$c = 10$ H_2O/HCl (3N)]; ESI: $[M+H]^+ = 206$. Anal. calcd for $C_{10}H_{11}N_3O_2$, C, 58.5; H, 5.4; N, 20.5; found: C, 57.9; H, 5.2; N, 21.0; mp = 278–280°C. ^1H NMR (DMSO-d_6) δ ppm: 3.75 (dd, 1H, $J = 6.5$ Hz and 3.25 Hz); 4.4 (dd, 1H, $J = 13.8$ Hz and 6.5 Hz); 4.85 (dd, 1H, $J = 13.8$ Hz and 3.25 Hz); 6.5 (d, 1H, $J = 3.4$ Hz); 7.15 (dd, 1H, $J = 7.9$ Hz and 4.7 Hz); 7.6 (d, 1H, $J = 3.25$ Hz); 8 (d, 1H, $J = 7.9$ Hz); 8.3 (d, 1H, $J = 3.25$); the purity of (*S*)-7-azaindolylalanine was checked by chiral HPLC on Crown Pack CR^+ column optimized conditions: wavelengh = 200 nm; flow = 0.5 ml min^{-1}; pH = 2 ($HClO_4$/milliQ H_2O); elution at 0°C.

	Retention time
(*R*)-*N*-Ac-β-Azaindolylalanine **4c**	15 min
(*S*)-β-Azaindolylalanine **3c**	9.9 min
(*R*)-β-Azaindolylalanine **5c**	12 min

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