Pergamon

Tetrahedron: *Asymmetry* 9 (1998) 1753–1758

TETRAHEDRON: *ASYMMETRY*

Chemoenzymatic synthesis of the two enantiomers of 7-azatryptophan

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Received 24 March 1998; accepted 27 March 1998

Abstract

7-Azatryptophan is an unnatural α-amino acid with a very potent fluorescent activity. It is used as a vehicle for probing the structure and dynamics of proteins and peptides. Diastereoselective alkylation, diastereoselective protonation and enzymatic resolution have been tested for preparing enantiomerically pure 7-azatryptophan.

1. Introduction

In 1955, Robison et al. described the synthesis of racemic 7-azatryptophan via 3-(N-dimethylaminomethyl)-7-azaindole or 7-azagramine.[1] The first known biological activity of 7-azatryptophan was the inhibition of the *Tetrahymena pyriformis* growth, through inhibition of tryptophan metabolism,[2] while replacement of tryptophan by 7-azatryptophan in enzyme sequences resulted in inactivation.[3]

On the other hand, tryptophan and its derivatives are well known for their fluorescent activity[4] due to the indole ring. Negrerie et al. proved the lone (395 nm) and exponential signal of 7-azatryptophan. Rich et al. used N_1-Me-7-azatryptophan as an optical standard.[5] 7-Azatryptophan is now used as a vehicle for probing the structure and dynamics of proteins and peptides.

The first enantioselective synthesis of (−)-(R)-7-azatryptophan was published in 1992[6] by Fallis et al. via alkylation of the (1R,4R)-camphor imine of *tert*-butylglycinate. The diastereoisomeric excess was greater than 98% but the yield of alkylation was only 27%.

One enzymatic synthesis was described in 1989: tryptophan synthase [EC.4.2.1.20], which is a non-commercial recombinant enzyme, catalysed the direct condensation of 7-azaindole with (L)-serine in 8 days and in 88% yield. This enzymatic condensation allowed the preparation of enantiomerically pure 4,

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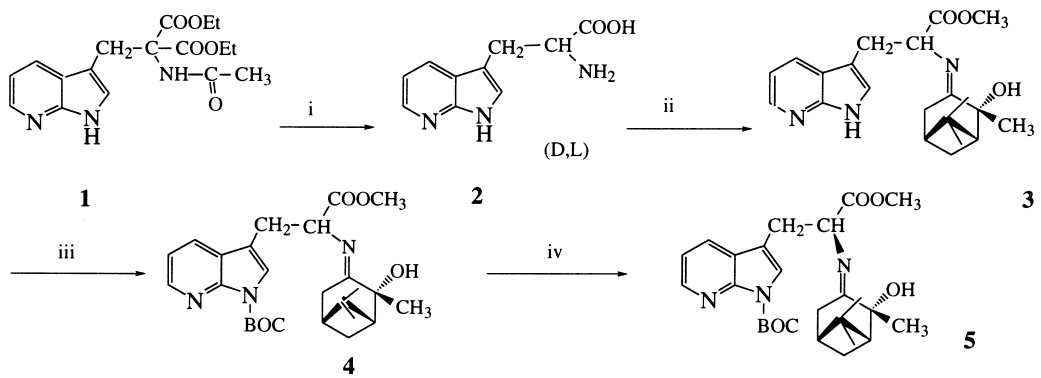
0957-4166/98/$19.00
PII: S0957-4166(98)00130-X

5 and 6-azatryptophans. However, this method is limited by the price of the recombinant enzyme and the necessary use of cofactors (pyridoxal phosphate).[7]

2. Results

Two different diastereoselective methods were tested, diastereoselective alkylation and diastereoselective protonation (deracemization) using 2-hydroxypinan-3-one (abbreviated to H.P.) as the chiral auxiliary (Scheme 1).



i: HCl 12N reflux. ii:1)$ClSiMe_3$, MeOH Yield= 95%, 2) H.P. (S,S,S), BF_3-Et_2O, Toluene Yield= 85%. iii: BOC_2O, DCM Yield=80%. iv 1) 2eqts KHMDS, THF, -80°C, 2) sat NH_4Cl Yield=95%; d.e. = 66%.

Scheme 1.

Diastereoselective alkylation is a well known procedure in our laboratory,[8] however the synthesis of the alkylating reagent (3-bromomethyl-7-azaindole) needed several steps and proceeded with very poor yields.

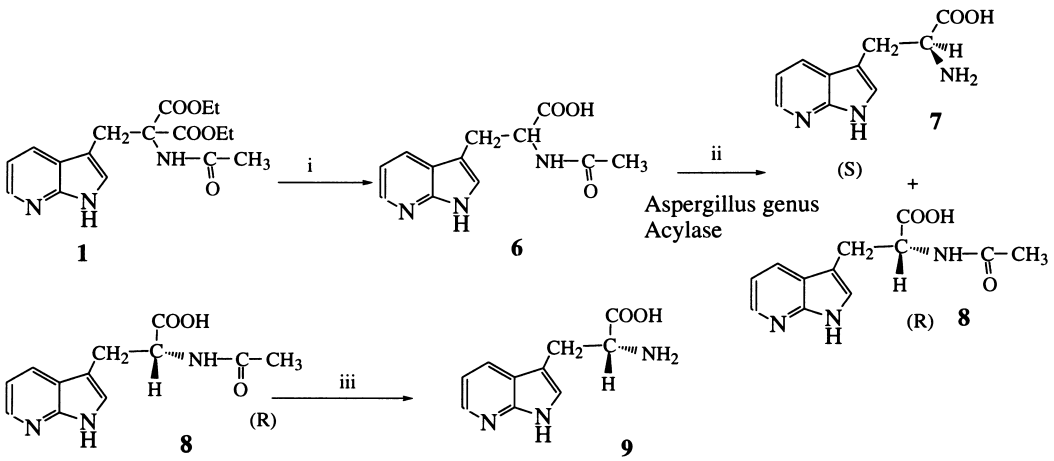
Diastereoselective protonation is a recent method[9] carried out in two steps. 7-Azatryptophan methyl ester was quantitatively obtained from the racemic mixture **2**; the Schiff base was prepared using the chiral auxiliary (S,S,S)-2-hydroxypinan-3-one in toluene with a catalytic amount of $BF_3 \cdot Et_2O$. The diastereoisomers **3** were not separated at this stage, but when the N_1 is protected the separation of both diastereoisomers **4** was easily achieved.

The Schiff base is deprotonated with a base in THF; several protecting groups (Boc, Z) and bases (*tert*-BuOK, LDA, KHMDS) were tested at −80°C (Table 1). The best results were obtained with 2 equivalents of KHMDS. The protonation was carried out with an NH_4Cl saturated solution in quantitative yield. The diastereoisomeric excess was evaluated by ^1H NMR and gave in the best case a d.e. of 66%.

Table 1

Base	Solvent	Base equivalents	Protecting group	d.e. (%)
tert-BuOK	THF	3	-	20
LDA	THF	3	-	20
KHMDS	toluene	3	-	40
KHMDS	THF	2	BOC	66
tert- BuOK	THF	2	Z	50
KHMDS	THF	2	Z	60

We tested as a third method the enzymatic resolution with an efficient acylase, *Aspergillus* genus acylase (Scheme 2).



i: 1) NaOH, 2) HCl pH 3-4, reflux, Yield=87%. ii: *Aspergillus genus* acylase, Phosphate Buffer pH 7.2-5.10^{-4}M $CoCl_2$ Yield (S) = 45%. iii: HCl reflux, Yield (R) = 40%.

Scheme 2.

From compound **1**, we carried out a saponification and a decarboxylation under controlled pH to obtain the racemic mixture of N-Ac-7-azatryptophan **6**. Resolution was achieved with the purified *Aspergillus* genus acylase in 36 hours. The separation step between (S)-7-azatryptophan **7** and (R)-N-Ac-7-azatryptophan **8** was carried out by precipitation of **7** in MeOH at pH 3. (R)-N-Ac-7-Azatryptophan **8** was hydrolysed to yield **9**.

The yields were excellent and for easier manipulation the enzyme was immobilized on different supports:

(i) by copolymerization with long chain amphipatic agents such as PEG;[10]
(ii) by a covalent bond on alumina preactivated with colamine-O-phosphate and glutaraldehyde;[11] and
(iii) by a covalent bond on Eupergit C (Rhom Society).

The first immobilization method produced the same yields as using the soluble enzyme; however the reaction time was longer than with the soluble acylase (48 h) and the enzyme elimination by gel filtration was replaced by a simple filtration. Moreover, this system allowed the automation of the process and the reuse of the immobilised enzyme that was stabilized.

3. Conclusion

In our hands, the best method for preparing the two enantiomerically pure 7-azatryptophan isomers is the enzymatic resolution with *Aspergillus* genus acylase.

4. Experimental

Thin-layer chromatography (TLC) was performed on Merck precoated silica gel 60F254 plates and spots were visualised by ultraviolet light and/or iodine vapour. Column chromatography was performed using Geduran silica gel. Chiral TLC was performed on Macherey–Nagel plates and spots were revealed

by 0.2% ninhydrin solution. Chiral HPLC was performed on a Waters HPLC system with a 486 detector apparatus on a Crownpack 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 on Jeol JMS DX 100 and DX 300 apparatus. ^{1}H NMR spectra were recorded on a Brucker spectrometer AC 250.

4.1. Literature preparation[1] of racemic 7-azatryptophan from the commercially available 7-azaindole

The intermediate azagramine synthesis (yield=87%), the alkylation of diethyl acetamidomalonate with azagramine (yield=75%) and the acidic total hydrolysis gave racemic 7-azatryptophan **2** in 50% overall yield. The racemic mixture crystallized with one molecule of water. Mp[1]=262–264°C; mp=260–262°C.

4.2. 7-Azatryptophan methyl ester hydrochloride: 7-azaTrpOMe·HCl

7-azaTrpOH **2** (11 mmol) was dissolved in ethanol (100 ml) under argon and $ClSiMe_3$ (88 mmol, 11.2 ml) was added dropwise. The mixture was stirred at 40°C over 36 hours. The solvent was concentrated under vacuum (yield=95%). Mp=222–225°C.

*4.3. Schiff base of 7-azatryptophan methyl ester: **3***

7-Azatryptophan methyl ester (5.25 mmol, 1.16 g) was neutralized by $NH_{3(g)}$ bubbling in Et_2O. The solvent was evaporated under vacuum. The ester and (S,S,S)-2-hydroxypinan-3-one (3.5 mmol, 0.59 g) were dissolved in toluene (75 ml) and a catalytic amount of $BF_3 \cdot Et_2O$ was added and the mixture was refluxed for 2 hours with elimination of water (Dean–Stark apparatus). The solvent was evaporated and the orange oil was purified by chromatography on silica gel (petroleum ether/Et_2O gradient); the diastereoisomers were not separated (yield=80%). R_f=0.35 (Et_2O) for both diastereoisomers.

*4.4. Protection of the Schiff base: **4***

Schiff base **3** (5.3 mmol, 1.95 g) was dissolved in DCM (100 ml) and Boc_2O (7.7 mmol, 1.68 g) was added. The reaction was stirred at room temperature for 12 hours. The solvent was concentrated under vacuum and the diastereoisomers were separated on silica gel. After purification, **A** and **B** were obtained in 33 and 37% yield, respectively: R_f **A**=0.66 (Et_2O); R_f **B**=0.46 (Et_2O).

A: ^{1}H NMR C_6D_6 δ ppm: 0.68 (s, 3H); 1.03 (s, 3H); 1.47 (s, 9H); 1.58 (s, 3H); 1.80–2.40 (8H); 3.15 (dd, 1H, J^1=8.04 Hz, J^2=10.989 Hz); 3.26 (s, 3H); 3.30 (dd, 1H, J^1=4.85 Hz, J^2=11 Hz); 4.30 (dd, 1H, J^1=4.81 Hz, J^2=8.04 Hz); 6.77 (dd, 1H, J^1=4.74 Hz, J^2=7.84 Hz); 7.58 (dd, 1H, J^1=1.63 Hz, J^2=7.84 Hz); 7.75 (s, 1H); 8.42 (dd, 1H, J^1=1.62 Hz, J^2=4.73 Hz).

B: ^{1}H NMR C_6D_6 δ ppm: 0.72 (s, 3H); 1.08 (s, 3H); 1.5 (s, 9H); 1.66 (s, 3H); 1.80–2.40 (6H); 3.25 (dd, 1H, J^1=4.17 Hz, J^2=13.00 Hz); 3.3 (s, 3H); 3.37 (dd, 1H, J^1=8.24 Hz, J^2=12.6 Hz); 4.35 (dd, 1H, J^1=4.6 Hz, J^2=8.08 Hz); 6.8 (dd, 1H, J^1=4.70 Hz, J^2=7.86 Hz); 7.6 (dd, 1H, J^1=1.60 Hz, J^2=7.20 Hz); 7.8 (s, 1H); 8.5 (dd, 1H, J^1=1.50 Hz, J^2=4.70 Hz).

*4.5. Diastereoselective protonation (deracemization): **5***

2.5 N KHMDS in toluene (4.2 ml, 2.1 equiv.) was diluted in anhydrous THF (20 ml) at −80°C. Schiff base **4** (5 mmol, 1 equiv.) was diluted in anhydrous THF (20 ml) and added dropwise to the

KHMDS solution. The mixture was stirred for 30 min at −80°C and the protonation was carried out with a saturated solution of NH_4Cl. The temperature was allowed to reach room temperature. The aqueous phase was washed several times with Et_2O. The organic phase was dried on $MgSO_4$ and concentrated under vacuum: yield=95%, d.e.=66%.

4.6. Racemic N-Ac-7-azatryptophan **6**

Compound **1** (4 mmol, 1.796 g) was dissolved in a mixture of dioxane/2N NaOH (9/1, v/v; 50 ml), and stirred for one day at 40°C. The saponification was followed by TLC in a DCM/20% MeOH solvent system. The mixture was neutralized until pH 8 with a concentrated HCl solution; the solvent was evaporated and NaCl removed by precipitation in MeOH (yield=95%). The salt was dissolved in dioxane (40 ml), concentrated HCl solution was added until pH 4 and the solution was refluxed for 15 hours. The mixture was neutralized until pH 7 and the solvent was evaporated. NaCl was removed with the same treatment in MeOH as for the saponification. Compound **6** was obtained in 90% yield. ^{1}H NMR DMSO-d_6 δ ppm: 1.76 (s, 3H); 3.15 (d, 2H, J=6.02 Hz); 4.3 (t, 1H, J=6.04 Hz); 7.3 (dd, 2H, J=6.03 and 7.99 Hz); 7.7 (d, 1H, J=6 Hz); 7.8 (s, 1H); 8.3 (dd, 1H, J=6.04 and 1.04 Hz); 11.9 (s, 1H).

4.7. Enzymatic resolution with Aspergillus genus acylase (TCI)

The commercially available enzyme from TCI contains only 30% of protein and has to be purified by gel filtration on Sephadex G50 with water and a solution of $CoCl_2$ (5×10^{-4} M) as eluent.

N-Ac-7-Azatryptophan **6** (4 mmol) was dissolved in phosphate buffer (0.1 M, pH 7.2) with $CoCl_2$ solution (5×10^{-4} M, 25 ml). Then acylase (12 mg) was added and the pH was maintained by adding 6 N NaOH. The reaction was vigourously stirred at 40°C and can be monitored with a pH stat. After one day of agitation, enzyme (6 mg) was added and stirred overnight. When the pH was stable, the kinetic enzymatic hydrolysis of the (S) isomer was quenched with HCl until pH 4. The enzyme was removed by gel filtration on Sephadex G50 with water as eluent.

The reaction was followed by chiral TLC on Macherey–Nagel plates using as the eluent a mixture of MeOH (12 ml), CH_3CN (10 ml), H_2O (5.5 ml), EtOH/NH_4OH (4/1, 2.5 ml) and the plates were revealed with ninhydrin. Chiral HPLC was carried out on a Crownpack CR+ column with aqueous $HClO_4$ as an eluent at 0°C.[12]

As described in the literature,[13] derivatization with Marfey reagent was tested to analyse diastereoisomers. The derivatization did not succeed.

(R)-N-Ac-7-Azatryptophan **8** was less soluble at pH 4 than the (S)-7-azatryptophan **7**; it precipitated at 0°C and was separated from **7** by filtration. This operation was repeated until obtention of one spot by TLC. (S)-7-Azatryptophan **7** was crystallized in a water/ethanol system in 45% yield.

R_f (R)=0.60; R_f (S)=0.69; ^{1}H NMR (D_2O) δ ppm: 3.6 (d, 2H, J=6.14 Hz); 4.3 (t, 1H, J=6.10 Hz); 7.66 (dd, 1H, J^1=7.50, J^2=6.20 Hz); 7.75 (s, 1H); 8.5 (dd, 1H, J^1=6.04, J^2=1.05 Hz); 8.7 (dd, 1H, J^1=7.99, J^2=1.05 Hz). Mp=261–262°C, $[\alpha]_D$=+11 (c=10 mg/ml in H_2O/HCl, pH 3).

4.8. (R)-N-Ac-7-Azatryptophan **8** hydrolysis: (R)-7-azatryptophan **9**

(R)-N-Ac-7-Azatryptophan (1.5 mmol) was refluxed in 6 N HCl for 4 hours. The mixture was neutralized and concentrated under vacuum. NaCl was removed by precipitation in MeOH.

(R)-7-Azatryptophan **9** was crystallized from a water/ethanol system in 40% yield. Mp=262–263°C, $[\alpha]_D$=−11 (c=10 mg/ml in H_2O/HCl, pH 3).

4.9. Table of chiral analysis results on Crownpack CR^+ column

Product	**6**	**7**	**9**
t_R (min)	12.5	3	4

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