FACILE PREPARATION OF OPTICALLY PURE [α - 2 H]- α -AMINO ACIDS.

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SUMMARY

Racemic [α - 2 H]- α -amino acids were prepared by heating the corresponding amino acids (Phe, nor-Leu and Dopa) with 0.05 equivalents of benzaldehyde in deuterated-acetic acid. Based on 1 H-nmr measurement, the isotopic purities of these racemized [α - 2 H]- α -amino acids were found to be higher than 99.5%. Methylation of these isotope-labelled amino acids was achieved in methanol/thionyl chloride without affecting isotopic purity. Optically pure [α - 2 H]- α -amino acids were obtained in high yield with high enantiomeric excess via alcalase catalysed resolution.

INTRODUCTION

Isotope labelled compounds are useful for investigation of enzyme-catalyzed stereospecific reactions [Chapman et al., 1986], for metabolism pathway studies [Halliday, 1988] and for tracing the biosynthesis process [Millington et al., 1988]. Methods have been reported for preparation of [α - 2 H]- α -amino acids. Those methods are either complex or require expensive enzymes [Fujihara et al., 1984; Wong et al., 1983; Gout et al., 1978; Yamamoto et al., 1977]. We report here a convenient procedure using an industrial enzyme as a catalyst by which both

enantiomers of [α - ^2H]- α -amino acid can be obtained with high isotopic (>99.5%) and high enantiomeric purity (>90%) and high yield.

Many methods for racemization of optically pure compounds have been developed in order to recycle the unwanted enantiomer [Pugniere et al., 1985; Hongo et al., 1985; Chen et al., 1989]. Racemization of amino acids is generally considered to proceed through the removal

Scheme 1

of the α -methine hydrogen as shown in Scheme I. We have used this racemization method to exchange the deuterium with the α -proton of amino acids in deuterated acetic acid. The deuterated racemic α -amino acid was methylated with thionyl chloride in methanol to afford [α - ^2H]- α -amino acid methyl esters, which were resolved by alcalase catalyzed selective ester hydrolysis in bicarbonate buffer pH 8.2.

Materials and Methods

Alcalase was purchased from NOVO industrial (Denmark) as a brown liquid with a specific activity of 2.5 AU.mL⁻¹. It was used without further purification. The amino acids were purchased from Sigma, USA. Thionyl chloride and benzaldehyde were purchased from E. Merck, Germany. Acetic acid-d (CH_3COOD) was purchased from the Aldrich Chemical Co. USA. Optical rotation was measured on a Universal Polarimeter Schmidt & Haensch, Germany. Tlc was performed on silica gel G pre-coated plates from E Merck Germany. ^1H -nmr spectra was taken on a Bruker amx 300, Germany. Chiral plates were purchased from Macherey-Nagel, Germany. Chiral hplc column Crown-pack-(+) was purchased from Diacel, USA. Common organic solvents, hplc grade and reagent grade, were obtained from a local supplier, the ALPS Chem. Co., Taiwan.

Synthesis of [α - ^2H]-D,L-amino acids ([α - ^2H]-D,L-phenylalanine as example).

To a round-bottom flask (250 mL) were added phenylalanine (1.65 g, 10 mmol), acetic acid-d (CH_3COOD , 50 mL), and benzaldehyde (0.2 mL). The flask was fitted with a reflux condenser and the mixture was thoroughly purged with nitrogen for 10 minutes and then refluxed for one hour. The resulting solution was evaporated under reduced pressure. Methanol (20 mL) was added to the residue and the precipitate was collected by filtration to give [α - ^2H]-D,L-phenylalanine (1.53 g, yield, 92%, Mp: 192-195°C; ^1H -nmr (300 MHz, D_2O): δ : 2.99-3.22 (d,d 2H), δ : 7.15-7.28 (m, 5H).

Synthesis of [α - 2 H]-D,L-amino acid methyl esters ([α - 2 H]-D,L-norleucine methyl ester as example).

To a round-bottom flask (100 mL) was added [α - 2 H]-D,L-norleucine (1.13 g, 10 mmol) and methanol (30 mL). The mixture was cooled to -10°C with a dry-ice bath and thionyl chloride (1 mL) was slowly added. After the addition, the resulting mixture was heated on a heating plate overnight. After cooling, the solvent was evaporated and ether (50 mL) was added to the oily residue to precipitate the [α - 2 H]-D,L-norleucine methyl ester hydrochloride. The product was collected by filtration and recrystallized from methanol/ether 5:1, 60 mL) to give pure [α - 2 H]-D,L-norleucine methyl ester.hydrochloride (1.35 g, 86% yield) mp: $132\text{--}134^{\circ}\text{C}$, ^1H -nmr (300 MHz, D_2O): δ : 0.85-0.89 (m, 3H), δ : 1.25-1.34 (m, 4H), δ : 1.91-2.04 (m, 2H), δ : 3.809 (s, 3H).

Chirally selective hydrolysis of [α - 2 H]-D,L-amino acid methyl ester by alcalase.

[α - 2 H]-D,L-amino acid methyl ester (5 mmol.) was dissolved in sodium bicarbonate (30 mL, 0.2 M, pH 8.2). Alcalase (200 μL) was added to the solution, and the pH of the reaction solution was kept at 8.2 with carbonate-bicarbonate buffer. Hplc (Nucelosil PR-18, eluent: acetonitrile/0.1% TFA in water 15:85) was used to monitor the process of hydrolysis. After half the amount of the ester was hydrolyzed, the pH of the reaction solution was adjusted to 2.0 with 2N HCl. The resolved L-amino acid and D-amino acid ester were isolated by a preparative hplc column using the same condition as the monitor condition. The optical purity of the isolated product was further analyzed by a chiral crown-pack hplc column. The results of yield and optical purity are shown in table 1.

Results and discussion

Many different aldehydes have been used for racemization of amino acids [Yamada et al., 1983; Smith et al., 1989, Chen et al., 1989]. We used benzaldehyde as a catalyst because it is volatile and easy to remove under a vacuum. In a typical reaction, optically pure amino acids were dissolved in acetic acid-d, and 0.05 equivalents of benzaldehyde were added to the amino acid solution. The reaction solution was purged thoroughly with nitrogen and heated to reflux. Small samples were taken at each time interval to check the optical purity of the reactant by hplc using a Crown-pack column or by measuring the optical rotation. After the optical purity was decreased to zero, the reaction mixture was evaporated to dryness under reduced pressure. The [α - 2 H]-D,L-amino acid was obtained after the addition of methanol to precipitate it. Figure 1 shows the time course for optical purity decrease of the phenylalanine, norleucine and 3,4-dihydroxyphenylalanine. The isotopic purity of the [α - 2 H]-D,L-amino acids thus obtained was measured by comparing the ^1H -nmr signal intensities of the α -proton. Figure 2 shows a typical ^1H -nmr spectra of L-phenylalanine (2-c), [α - 2 H]-D,L-phenylalanine, and [α - 2 H]-D,L-phenylalanine methyl ester (2-a). While the α -proton of L-phenylalanine resonates at a chemical shift of $\delta(\text{ppm})$: 3.79-3.86(m) (figure 2-c), that of [α - 2 H]-D,L-phenylalanine for all the signals

Figure 1. Time course for the optical purity decrease of phenylalanine, norleucine, and 3,4-dihydroxyphenylalanine catalysed by benzaldehyde in acetic acid-d.

at the same chemical shift region completely disappeared. The β -CH₂ signals of the L-phenylalanine located at δ (ppm): 2.89-3.18 (m) of figure 2-c also changed from multiplet to simple doublet-doublet of figure 2-b, indicating a more than 99.5% deuterium substitution at α -position of [α -²H]-D,L-phenylalanine. Using the same

method, isotopically pure norleucine and 3,4-di-hydroxyphenylalanine were obtained. Yield and isotopic purity of each compound are shown in table 1.

Figure 2. ¹H-nmr spectra of [α -²H]-D,L-Phenylalanine methyl ester (2-a), [α -²H]-D-Phenylalanine (2-b), and L-phenylalanine (2-c).

The [α -²H]-D,L-amino acid was further methylated with thionyl chloride at -10°C in methanol. No change in isotopic purity was observed. These results are also shown in table 1. The ¹H-nmr spectra of D,L-phenylalanine methyl ester (figure 2-a) had one strong resonance at δ (ppm): 3.68 (s, 3H) corresponding to the methyl group absorption.

No peak was observed within the chemical shift of α -proton region. The isotopic purity of the [α -²H]-D,L-phenylalanine methyl ester was the same as [α -²H]-D,L-phenylalanine and the reaction in methanol/thionyl chloride did not affect the isotopic purity of [α -²H]- α -amino acids.

The resolution of the N-unprotected amino acid methyl esters was carried out in carbonate buffer. In an exploratory experiment, 0.1 mmol of [α -²H]- α -phenylalanine methyl ester dissolved in 10 mL of bicarbonate buffer (0.5 M, pH 8.2) was selectively hydrolyzed by alcalase. The time course of the alcalase catalyzed hydrolysis of [α -²H]-D,L-phenylalanine methyl ester was

Figure 1 is a line graph showing the percentage of unhydrolyzed amino acid ester versus hydrolysis time (minutes) for three amino acids: 3,4-dihydroxyphenylalanine, norleucine, and phenylalanine. The y-axis represents the percentage of unhydrolyzed amino acid ester, ranging from 50 to 100. The x-axis represents hydrolysis time in minutes, ranging from 0 to 180. All three amino acids show a decrease in the percentage of unhydrolyzed ester over time, with 3,4-dihydroxyphenylalanine showing the slowest rate of hydrolysis.

Hydrolysis Time (minutes)	3,4-dihydroxyphenylalanine (%)	norleucine (%)	phenylalanine (%)
0	100	100	100
15	95	90	85
30	85	75	65
45	75	65	58
60	68	58	55
75	62	53	50
90	58	50	50
120	55	50	50
180	50	50	50

a preparative scale reaction, each of the [α - 2 H]- α -amino acid methyl esters was selectively hydrolyzed on g-scale (4-5 mmol) reaction. After the L-isomer was completely hydrolyzed (check by hplc), the reaction was quenched by the addition of 1N HCl. The resolved products were isolated by preparative hplc using a RP-18 column. The optical purity of the isolated amino acid and amino acid methyl ester was determined by hplc analysis using a crown-pack chiral column. Figure 4 shows a typical hplc analysis of the [α - 2 H]-D-phenylalanine methyl ester. The enantiomeric excess and yield of each isolated product are shown on table 1.

FILE	1.	METHOD	Q.	RUN	2	INDEX	2	AREA	BC
PEAKE		AREA	RT						
1	0.833	0.99	2131.01						
2	0.225	1.49	14484.03						
3	0.829	2.3	53196.01						
4	0.829	2.3	53196.01						
5	95.173	15.16	618750.01						
TOTAL	104.		641847						

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In summary, the procedure illustrated here describes a practical application of alcalase in isotopical labelled amino acid synthesis, particularly in the preparation of unnatural amino acids. The methyl esters of the amino acids are synthesized in acidic conditions and resolved in aqueous solution, and the isotopic purity is not affected under these conditions. The alcalase has very broad specificity at its s_1' -subsite [Philipp et al., 1983]. This resolution procedure should be applicable to most other amino acids.

Table 1. Yield, optical purity and isotopical purity of $[\alpha\text{-}^2\text{H}]\text{-}\alpha$ -amino acids.

amino acid	$[\alpha\text{-}^2\text{H}]\text{-D,L-}$ amino acid		$[\alpha\text{-}^2\text{H}]\text{-D,L-amino}$ acid methyl ester		$[\alpha\text{-}^2\text{H}]\text{-L-}$ amino acid		$[\alpha\text{-}^2\text{H}]\text{-D-amino}$ acid methyl ester	
	yield,	I.P*.	yield,	I.P.	yield,	e. e.	yield,	e.e.
phenylalanine	95	>99.5	98	>99.5	96	92	99	91
norleucine	99	>99.5	99	>99.5	97	82	99	90
DOPA	99	>99.5	97	>99.5	96	88	99	97

*I.P. : isotopic purity.

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