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Separation of peptide diastereomers by reversed-phase high-performance liquid chromatography and its applications

IV[a]. New derivatization reagent for the enantiomeric analysis of α- and β-amino acids

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ABSTRACT

A new derivatization reagent, (Z–L-Val–Aib–Gly–ONSu, Z = benzyloxycarbonyl; Aib = α-aminoisobutyric acid; ONSu = N-oxysuccinimide), was developed for the enantiomeric analysis of various amino acids, including unusual α-amino acids having a long or bulky alkyl side-chain or a substituted phenyl or heteroaromatic ring and β-substituted β-amino acids. The diastereomers derived from various amino acids and this chiral reagent were well resolved by reversed-phase high-performance liquid chromatography using aqueous methanol as the eluent. As it is generally considered that in chiral derivatization for the separation of enantiomers the chiral centres in the diastereomeric derivatives should be as close as possible to each other, it is surprising that the above diastereomers, which have two chiral centres separated by nine bonds, can be well separated. This can be explained by the conformational difference between the L–L and L–D isomers.

INTRODUCTION

It is very important to analyze, both qualitatively and quantitatively, enantiomers of amino acids, *e.g.*, for examining the optical purity of unusual amino acids that have been optically resolved or synthesized asymmetrically, or for determining the absolute configuration of constituent amino acids of naturally occurring peptides, especially antibiotics and toxins. For the purpose of finding good derivatives applicable to such enantiomeric analysis, we have investigated the separation behaviour of diastereomers of various peptides by reversed-phase high-performance

[a] For Part III, see ref. 1.

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liquid chromatography (RP-HPLC). Although the separation of amino acid enantiomers without prior derivatization by the use of chiral stationary phases may provide the most promising method, the separation of diastereomers produced by a precolumn derivatization with chiral reagents, by means of ordinary stationary phases, is still useful, particularly when the same instrument must be routinely used for various investigations and, hence, the use of special columns and eluents is not advantageous[2].

Recently we found that diastereomers of protected tetrapeptides (**1**) containing two achiral amino acids (X and Y) between two chiral amino acids exhibit marked separation, particularly when X is an α,α-dialkylated glycine such as α-aminoisobutyric acid (Aib) and Y is glycine (Gly)[4].

Z–(L/D)–Val–X–Y–L–Phe–OCH_3
(1)[a]

It is generally considered that in chiral derivatization for the separation of enantiomers, the two chiral centres in the diastereomeric derivatives should be as close as possible to each other in order to maximize the difference in chromatographic properties; in general, three bonds separate the two centres, and derivatives having distances exceeding four bonds are not often useful[5]. From this point of view, it is surprising that the separation of diastereomers of **1** is excellent in spite of the fact that two chiral centres in **1** are separated by nine bonds.

In this study, taking account of the excellent separation of diastereomers of the tetrapeptide (**1**) in which X–Y = Aib–Gly, a new derivatization reagent (**2**) (where ONSu = N-oxysuccinimide) has been successfully developed for the enantiomeric analysis of various α- and β-amino acids by RP-HPLC.

Z–L-Val–Aib–Gly–ONSu
(2)

EXPERIMENTAL

Apparatus

The HPLC system used was constructed with a Shimadzu (Kyoto, Japan) Model LC-3A pumping system, a Rheodyne (Cotati, CA, U.S.A.) Model 7125 sample injector with a 20-μl loop and a Shimadzu Model SPD-2A UV detector (monitoring at 254 nm). A column (150 mm × 4.6 mm I.D.) packed with Cosmosil $5C_{18}$ (Nacalai Tesque, Kyoto, Japan) was used, the temperature of which was maintained at 30°C by a thermostated bath. HPLC data were processed with a Shimadzu C-R2AX Chromatopac. The mobile phase was 65% aq. methanol at a flow-rate of 1 μl/ml, unless stated otherwise.

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[a] Abbreviations according to the IUPAC–IUB Commission[3] are used: Z = benzyloxycarbonyl; Hep = heptyline (2-aminoheptanoic acid); Tle = *t*-leucine; Mle = γ-methylleucine; ONSu = N-oxysuccinimide; pNA = *p*-nitroanilide; DCC = dicyclohexylcarbodiimide; HOBt = 1-hydroxybenzotriazole.

Chemicals

α-Amino acids were obtained from Wako Jun-yaku (Osaka, Japan), Tanabe Seiyaku (Osaka, Japan), Aldrich (Milwaukee, WI, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). Methanol was distilled and water was deionized and then glass distilled.

β-Amino acids were prepared as follows: optically active L-β-amino acids were prepared from L-α-amino acids by the Arndt–Eistert reaction[6,7]. Racemic β-(substituted phenyl)-β-alanines were prepared by addition of hydroxylamine to the corresponding substituted cinnamic acids[8].

Synthesis of derivatization reagent, Z–L-Val–Aib–Gly–ONSu (2)

Z–Aib and Gly–OCH_3 · HCl were coupled in the presence of triethylamine in dichloromethane by the DCC–HOBt method. The resulting Z–Aib–Gly–OCH_3 was debenzyloxycarbonylated with 25% hydrobromic acid–acetic acid and coupled with Z–L-Val by the method described above. Z–L-Val–Aib–Gly–OCH_3 was hydrolysed in methanol under alkaline conditions and then the N-Z-tripeptide was coupled with N-hydroxysuccinimide by using DCC in dioxane–ethyl acetate (1:1). The crude product (**2**) was recrystallized from ethyl acetate; m.p. 120–121°C, $[\alpha]_D^{25}$ + 3.8° (*c* 1, methanol).

Derivatization

An amino acid (50 μmol) was dissolved into 7% methanolic HCl (1 ml) and the solution was stirred at 80–90°C in a water-bath for 1 h. After the solution had been concentrated under the reduced pressure, the residual methyl ester hydrochloride, the reagent (**2**) (27 mg, 55 μmol) and 0.5 ml of a freshly prepared solution of triethylamine (50 μmol) in dichloromethane were mixed and stirred at room temperature for 2–3 h. An aliquot (0.5–1.0) μl) of the reaction mixture was injected into the liquid chromatograph. A typical chromatogram is shown in Fig. 1.

RESULTS AND DISCUSSION

The separation of diastereomers of tetrapeptides (**3**) containing various amino acids at the C-terminal was examined. Table I summarizes the HPLC data for diastereomers of **3**. HPLC analysis was carried out using the conditions described under Experimental.

Z–L-Val–Aib–Gly–(L/D)-amino acid–OCH_3
(**3**)

Excellent separations of diastereomers were obtained for all peptides (**3**) examined, except when the amino acid is Pro. Interestingly, for amino acids with an alkyl side-chain, the more bulky the side-chain the shorter is the retention time of **3** and the better is the separation of diastereomers. When the amino acid has a polar side-chain, the separation of diastereomers is poor because of rapid elution in 65% methanol, but baseline separation is still exhibited. Every L–D isomer is eluted faster than the corresponding L–L isomer. As shown in Table I, excellent separations are obtained for some unusual amino acids having a long alkyl side-chain (Hep) or a bulky side-chain

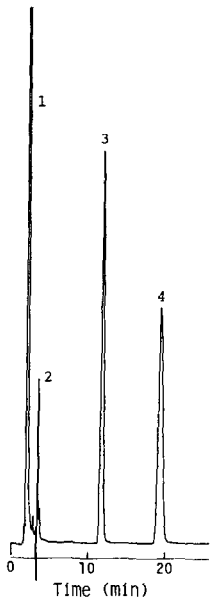


Fig. 1. Chromatogram of the reaction mixture of Z–L-Val–Aib–Gly–ONSu (**2**) and DL-Phe–OCH_3. See Experimental for details of reaction and chromatographic conditions. Peaks: 1 = excess **2** + HOSu + DL-Phe–OCH_3; 2 = CH_2Cl_2; 3 = Z–L-Val–Aib–Gly–D-Phe–OCH_3; 4 = Z–L-Val–Aib–Gly–L-Phe–OCH_3.

TABLE I

SEPARATION OF DIASTEREOMERS OF Z–L-Val–Aib–Gly–(L/D)-AMINO ACID–OCH_3 (**3**)

k' = Capacity factor; α = separation factor.

Amino acid		k'	α	*Amino acid*		k'	α
Ala	(D)	1.69	1.34	Pro	(D)	1.70	1.00
	(L)	2.26			(L)	1.70	
Abu	(D)	2.49	1.47	Phg	(D)	4.56	1.22
	(L)	3.66			(L)	5.57	
Nva	(D)	4.20	1.54	Phe	(D)	6.09	1.77
	(L)	6.47			(L)	10.79	
Val	(D)	3.82	1.65	Ser	(D)	1.07	1.16
	(L)	6.32			(L)	1.24	
Nle	(D)	6.92	1.63	Thr	(D)	1.44	1.22
	(L)	11.28			(L)	1.76	
Leu	(D)	6.52	1.66	Asp[a]	(D)	1.85	1.16
	(L)	10.80			(L)	2.14	
Ile	(D)	6.12	1.68	Glu[a]	(D)	2.30	1.19
	(L)	10.35			(L)	2.73	
Tle	(D)	5.61	1.75	Tyr	(D)	1.87	1.74
	(L)	9.81			(L)	3.26	
Hep	(D)	11.48	1.66	Met	(D)	3.68	1.42
	(L)	19.03			(L)	5.24	
Mle	(D)	9.06	1.72	Trp	(D)	4.72	1.61
	(L)	15.58			(L)	7.58	

[a] Dimethyl ester.

TABLE II

FORMATION OF THE DIASTEREOMERS OF TETRAPEPTIDES (3) IN THE REACTIONS BETWEEN Z-L-Val-Aib-Gly-ONSu (2) AND DL-Phe-OCH_3

For reactions conditions, see Experimental.

Parameter	*Reaction time*					
	15 min	*45 min*	*90 min*	*3 h*	*6 h*	*22 h*
Ratio of the peak area of the L-D isomer to that of the internal standard[a]	1.341	1.369	1.410	1.342	1.377	1.400
Ratio of the peak area of the L-L isomer to that of the internal standard[a]	1.316	1.357	1.416	1.368	1.368	1.386
L-D/L-L	1.02	1.01	1.00	0.98	1.01	1.01
L-L (%)	49.5	49.8	50.1	50.5	49.9	49.8

[a] Z-L-Leu-pNA (1.0 mg) was used as an internal standard.

TABLE III

CHIRAL SEPARATION OF UNUSUAL AROMATIC AMINO ACIDS AFTER DERIVATIZATION WITH Z-L-Val-Aib-Gly-ONSu (2)

Amino acid: NH–CH(R)–COO.

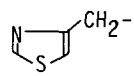
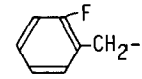
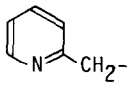
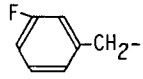
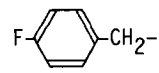
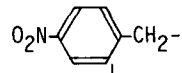
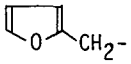
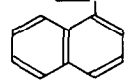
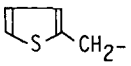
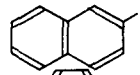
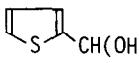
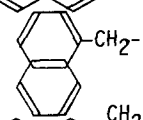
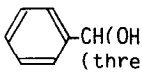
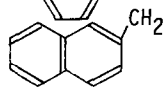
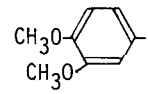
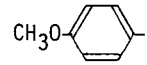
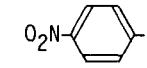
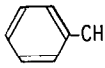
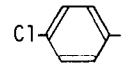
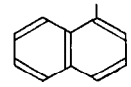
R		*k'*	α	*R*		*k'*	α
		1.99 2.78	1.40			5.79 10.63	1.83
		2.33 3.31	1.42			6.21 11.09	1.79
		3.48 4.19	1.20			6.17 11.30	1.83
		3.74 4.72	1.26			5.18 9.56	1.84
		3.31 4.95	1.50			9.18 12.42	1.35
	(D) (L)	4.63 7.32	1.58		(D) (L)	10.13 12.91	1.27
		2.52 3.16	1.26		(D) (L)	13.26 25.75	1.94
		2.91 3.67	1.26		(D) (L)	13.15 26.37	2.00

TABLE IV

CHIRAL SEPARATION OF β-SUBSTITUTED β-AMINO ACIDS AFTER DERIVATIZATION WITH Z-L-Val-Aib-Gly-ONSu (**2**)

β-Amino acid: $NHCHCH_2COO$ (R on the CH)

R		*k'*	α	*R*		*k'*	α
CH_3–	(D)	2.05	1.15			3.22	1.12
	(L)	2.35				3.59	
$(CH_3)_2CH$–	(D)	5.00	1.36			4.98	1.14
	(L)	6.79				5.65	
$(CH_3)_2CHCH_2$–	(D)	8.41	1.22			5.04	1.28
	(L)	10.29				6.44	
$CH_3CH_2(CH_3)CH$–	(D)	8.23	1.44		(D)	5.67	1.11
	(L)	11.86			(L)	6.31	
	(D)	7.89	1.32			10.39	1.22
	(L)	10.41				12.72	
		3.55	1.06			14.48	1.26
		3.77				18.29	
		4.74	1.07				
		5.09					

(Tle, Mle). These results stimulated us to investigate the utility of the tripeptide N-hydroxysuccinimide ester (**2**) as a chiral derivatization reagent for the enantiomeric analysis of unusual amino acids. This reagent can be easily prepared by the conventional method for peptide synthesis, easily purified by recrystallization and stored in the dry state.

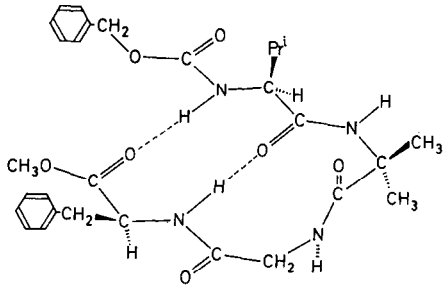


Fig. 2. Proposed conformation (A) for the L-L isomer of **3**.

The derivatization reaction of amino acid methyl ester and the reagent (**2**) takes place almost quantitatively within 1–2 h, and the ratio of the peak areas of the diastereomeric derivatives does not depend on the reaction time (Table II). Satisfactory chromatograms can be obtained in most instances by direct injection of the reaction mixture into the liquid chromatograph, because of the rapid elution of all of the unreacted reagents and by-products (Fig. 1), although better chromatograms can be obtained after the usual washing of the reaction mixture.

This method was applied to the chiral separation of various unusual aromatic amino acids. The results are given in Table III. Every diastereomeric pair of **3** is well separated.

Investigation by NMR and circular dichroism measurements revealed that the marked separation of diastereomers of **3** could be explained by the conformational difference between the L–L and L–D isomers: the L–L isomer of **3** prefers the β-turn conformation (A) with two parallel intramolecular hydrogen bonds in less polar aprotic solvents, as shown in Fig. 2, whereas the corresponding L–D isomer adopts a different β-turn conformation (B) with only an intramolecular hydrogen bond between the Val C=O and amino acid NH groups[4,9]. This is also the case under HPLC conditions, particularly at the instant of contact with the hydrophobic surface of octadecylated stationary phase. Conformation A of the L–L isomer can interact more strongly with the stationary phase than conformation B of the L–D isomer, because hydrophilic groups in conformation A are less exposed outside than those in conformation B and the two hydrophobic side-chains in the L–L isomer are closer than those in the D–L isomer.

We tried to apply the reagent (**2**) to the enantiomeric analysis of β-amino acids. Various β-substituted β-amino acids were converted into the peptides (**4**) and analysed by the same procedure as described above.

Z–L-Val–Aib–Gly–(L/D)-β-amino acid–OCH_3
(**4**)

The results are summarized in Table IV. The separation of diastereomers of **4** is sufficient to be used for enantiomeric analysis, although the separation is less than that for α-amino acids. In this case too, every L–D isomer is eluted in advance of the corresponding L–L isomer. Recently, Griffith *et al.*[10] reported a good method for the HPLC separation of enantiomers of β-amino acids using a chiral stationary phase, but convenient methods using precolumn derivatization for the enantiomeric analysis of β-amino acids have rarely been reported. We therefore believe that our method will be useful for the enantiomeric analysis of not only α but also β-amino acids.

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