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Note

Separation of enantiomeric protected dipeptides by chiral high-performance liquid chromatography

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Diastereomers of protected peptides can be separated by reversed- and normal-phase high-performance liquid chromatography (HPLC)[1–3] and can also be analysed by ^{1}H NMR spectroscopy[4]. These methods have been applied to the study of racemization in peptide synthesis[5–8]. However, there are no powerful or convenient methods for the determination of enantiomers of protected dipeptides, which may be produced in the asymmetric formation of peptide bonds by chemical or enzymatic methods[9,10].

Enantiomers of free dipeptides have been separated by thin-layer chromatography (TLC), gas chromatography (GC) and HPLC. Günther *et al.*[11] developed a direct method for the resolution of enantiomeric free dipeptides on chiral TLC plates, Lindner *et al.*[12] reported the separation of enantiomeric glycine dipeptides after dansylation by reversed-phase HPLC with chiral metal chelate additives and enantiomeric dipeptides with esterification in the carboxyl-terminal position and perfluoroacylation in the N-terminal position can be separated by GC on a chiral stationary phase[13]. Owing to the inaccuracy of quantification in TLC and the possibility of racemization of protected dipeptides in the deprotecting and derivatization steps for analysis by GC, it is imperative to develop a facile means of analysing for protected dipeptides directly and quantitatively in order to study racemization and asymmetric induction in peptide synthesis. In this work, the separation of eight sets of enantiomeric protected dipeptides, (*Z*)-AA_1-AA_2-OBzl (Bzl is benzyl), where AA_1 and AA_2 are Ala and Phe, respectively, were studied by chiral HPLC.

EXPERIMENTAL

L-Amino acids were purchased from Kyowa Fermentation (Tokyo, Japan) and D-amino acids from Sigma (St. Louis, MO, U.S.A.). All solvents were obtained from Alps Chemical (Taipei, Taiwan). A chiral Pirkle type 1-A column (25 cm × 4.6 mm I.D.) and Pirkle-concept Sumipax OA-1100 and OA-2200 chiral columns (15 cm × 4.6 mm I.D., 5 μm) were obtained from Regis Chemical (Morton Grove, IL, U.S.A.) and a Chiralcel OD column (25 cm × 4.6 mm I.D.) from Daicel Chemical Industries (Tokyo, Japan).

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Protected dipeptides were synthesized by the dicyclohexylcarbodiimide coupling method and their purity was checked by TLC on silica gel type 60 (E. Merck, Darmstadt, F.R.G.) with detection by the chlorine–tolidine method[14]. The HPLC system from Waters Assoc. (Milford, MA, U.S.A.) used for the analytical separations consisted of an M6000A solvent delivery unit and a U6K universal injector, coupled to a Model M450 variable-wavelength UV spectrophotometer and an SIC Chromatocorder 12 integrator (System Instruments, Tokyo, Japan). Enantiomers of protected dipeptides were separated on chiral columns using isopropanol (IPA) in *n*-hexane as the mobile phase at room temperature and detected by UV spectrophotometry at 254 nm.

RESULTS AND DISCUSSION

Free D,L-amino acids and enantiomers of free dipeptides are easily converted into diastereomers, which could be separated using general reversed-phase or ion-exchange columns[15]. In recent years, they have also been separated directly by TLC or HPLC with the chiral ligand-exchange method[11,12,16]. Because of the lack of free amino and carboxyl groups, protected amino acids and peptides cannot form diastereomers of ligand complexes with metal ions in the mobile phase and a chiral stationary phase. Therefore, other types of commercially available chiral columns have been introduced to separate enantiomers of protected dipeptides. Among the chiral columns tested, the Pirkle type 1-A chiral column is the best. According to Table I and Fig. 1, four pairs of D–D/L–L protected dipeptides and the D–L/L–D

TABLE I

SEPARATION OF ENANTIOMERIC PROTECTED PEPTIDES USING PIRKLE-TYPE 1-A, CHIRALCEL OD AND SUMIPAX OA 1100 CHIRAL COLUMNS

k' = Capacity factor; α = separation factor.

Compound	*Enantiomers*	*Pirkle-type 1-A*			*Chiralcel OD*		*Sumipax OA 1100*	
		k'	α	*Isopropanol–n-hexane eluent*	k'	α	k'	α
(*Z*)-Phe-Phe-OBzl	L–L	5.10	1.16	1:6	3.64	1.29	1.26	1.00
	D–D	6.64			4.70		1.26	
	L–D	7.41	1.15	1:6	4.70	1.08	1.59	1.08
	D–L	8.53			5.10		1.47	
(*Z*)-Phe-Ala-OBzl	L–L	7.29	1.18	14:100	2.90	1.03	2.18	1.06
	D–D	8.59			3.00		2.06	
	L–D	7.76	1.17	14:100	3.00	1.20	2.18	1.00
	D–L	9.12			3.60		2.18	
(*Z*)-Ala-Phe-OBzl	L–L	6.72	1.18	1:6	3.60	1.44	1.59	1.35
	D–D	7.94			5.20		1.18	
	L–D	8.53	1.08	1:6	4.00	1.13	1.94	1.22
	D–L	9.18			4.50		1.59	
(*Z*)-Ala-Ala-OBzl	L–L	4.76	1.14	1:6	2.80	1.34	1.71	1.16
	D–D	5.43			3.76		1.47	
	L–D	6.18	1.09	1:6	3.04	1.05	1.94	1.13
	D–L	6.67			3.20		1.71	

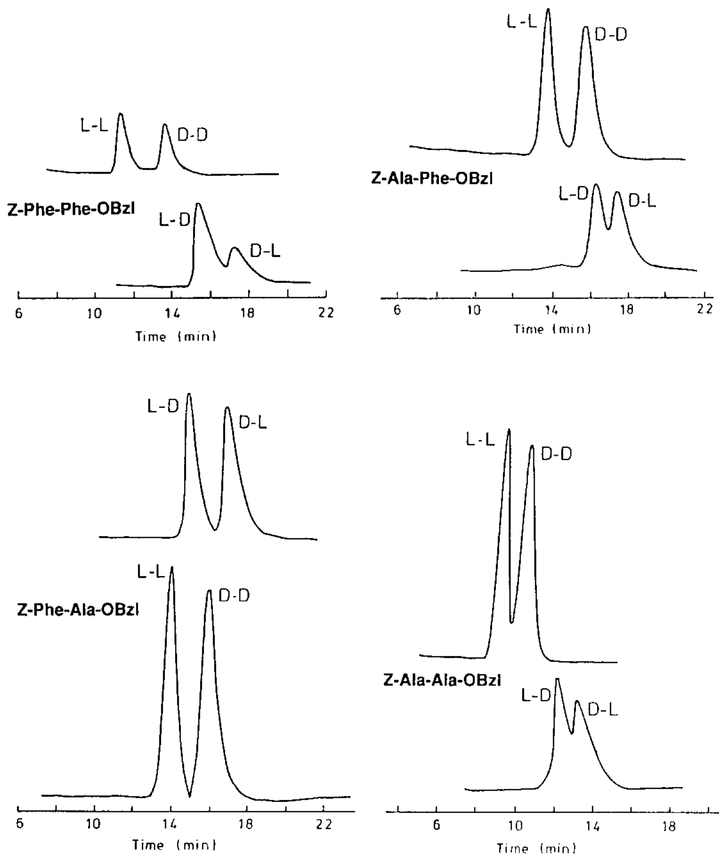

Fig. 1. Separation of stereoisomers of protected dipeptides on a chiral Pirkle type 1-A column. Mobile phase, as in Table I; flow-rate, 1.0 ml/min; detection, 254 nm at room temperature.

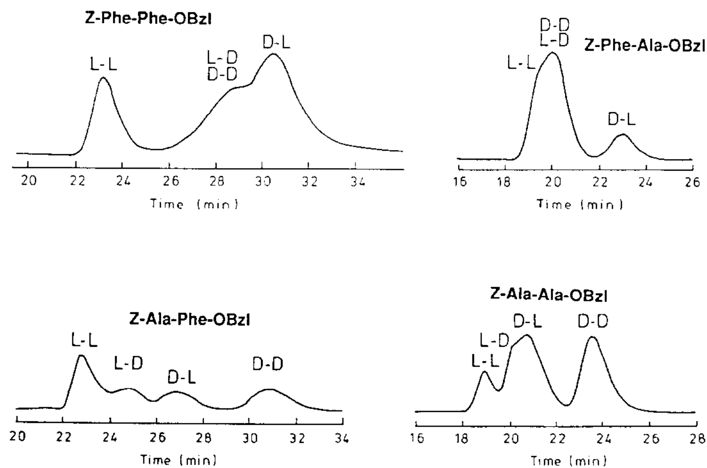

Fig. 2. Separation of stereoisomers of protected dipeptides on a Chiralcel OD column. Mobile phase, *n*-hexane–isopropanol (5:1); flow-rate, 0.45 ml/min; detection, 254 nm at room temperature.

enantiomers of (Z)-Phe-Ala-OBzl are separated well by the Pirkle type 1-A chiral column, and three other D–L/L–D pairs are partially separated with tailing peaks. The elution order of the four protected dipeptides for each set is L–L, D–D, L–D and D–L, except the reverse order of L–D and D–D in the case of (Z)-Ala-Phe-OBzl.

The L–L/D–D isomers of (Z)-Ala-Ala-OBzl, (Z)-Ala-Phe-OBzl and (Z)-Phe-Phe-OBzl and the D–L/L–D enantiomers of (Z)-Phe-Ala-OBzl show good separations on the Chiralcel OD column. However, other enantiomers are only partially separated (Fig. 2). The elution order on the Chiralcel OD column for each set is L–L, L–D, D–L and D–D. Apparently, D-amino acid residues undergo greater interaction with the stationary phase of the Chiralcel OD column, which contains cellulose tris-3,5-dimethylphenyl carbamate coated on silica gel.

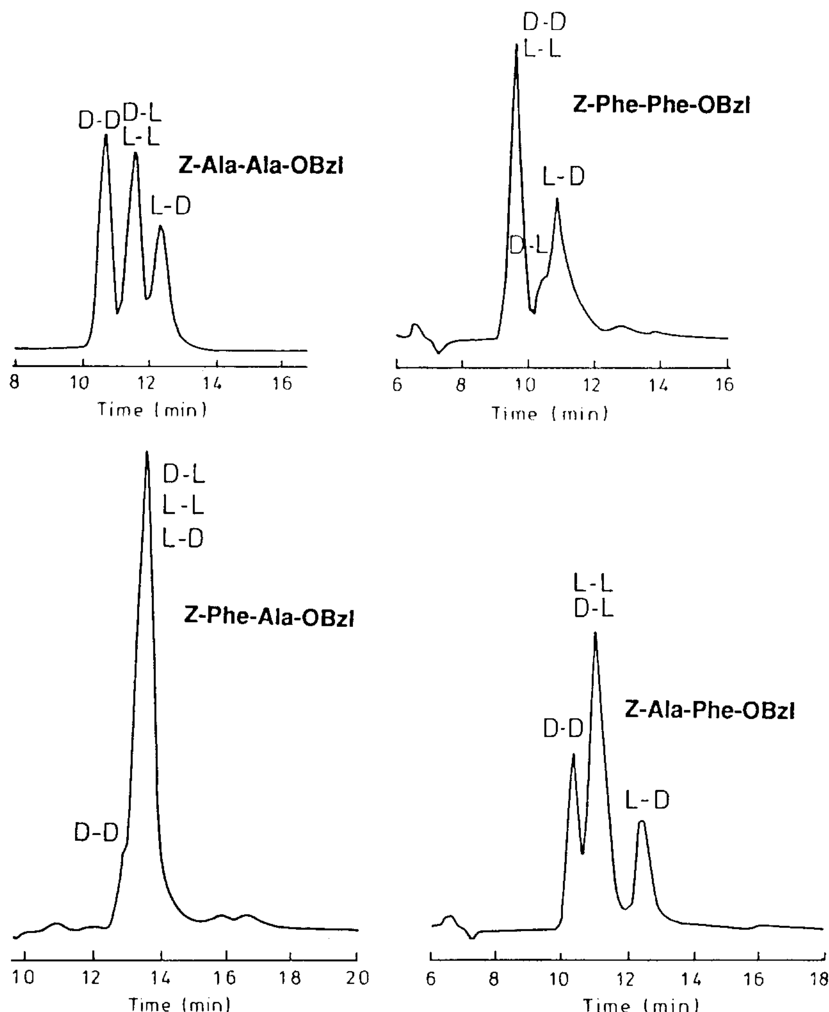

Fig. 3. Separation of stereoisomers of protected dipeptides on a Regis Sumipax OA 1100 column. Mobile phase, n-hexane–isopropanol (5:1); flow-rate, 0.4 ml/min; detection, 254 nm at room temperature.

Four sets of enantiomers, D–D/L–L and D–L/L–D of (Z)-Ala-Ala-OBzl and D–D/L–L and D–L/L–D of (Z)-Ala-Phe-OBzl, can be separated on the Regis Sumipax OA 1100 chiral column (Fig. 3), but none of the sets of enantiomers could be separated on the Regis Sumipax OA 2200 column.

It is believed that Pirkle-concept chiral columns have a similar theoretical basis of separation in which the mechanism of separation of small molecules is explained by three-point interactions involving hydrogen bonding, π–π hydrophobic forces and dipole stacking[17]. The chiral interactions between protected dipeptides and chiral stationary phases are possibly multi-point and more complicated than three-point interactions. The explanation of these interactions by molecular modelling is in progress. Although the four stereoisomers (L–L, L–D, D–L and D–D) are not well separated simultaneously, this enantiomeric separation, combined with diastereomeric separation, will provide an analytical method for studying the asymmetric reactions in peptide synthesis.

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