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Note

High-performance liquid chromatographic improvement of the Young racemization test

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The Young racemization test, introduced to peptide chemistry in 1964[1], was for many years one of the most important and widely used tests for evaluation of the quality of peptide reagents, optimization of the conditions of peptide synthesis reactions and for studying the mechanism of peptide bond formation. According to the test the degree of racemization for the model reaction (where Bz is benzoyl)

$$2\text{Bz-L-Leu} + 2\text{GlyOEt} \rightarrow \text{Bz-L-Leu-GlyOEt} + \text{Bz-D-Leu-GlyOEt}$$

was determined by measuring the optical rotation of the product; the lower the optical rotation, the higher is the degree of racemization. There are disadvantages in using the Young test, such as low sensitivity (detection limit of racemate ranges from 1 to 3%) and its susceptibility to contamination of side products causing errors in the measurement of the optical rotation. Some improvements decrease the detection limit to 0.001%, by use of isotopically labelled compounds[2], and eliminating the by-product interaction[3]. Despite their potential usefulness, the improvements have not gained general acceptance due to the high cost of the isotope method and the tedious procedures involved. However, the Young test still has great potential because there are only a few racemization tests, including the Young test, which offer the possibility of studying racemization phenomena in peptide synthesis without the interference from asymmetric induction.

In this paper we report an improvement of the Young test by employing high-performance liquid chromatography (HPLC) on a chiral stationary phase for the resolution and quantitation of protected enantiomeric peptides in the reaction mixture. With this simplified procedure the analysis is rapid, sensitive (detection limit 0.01% of racemate) and precise. Furthermore this method may be used for the determination of the degree of racemization not only for the Young model but also for other protected peptides.

EXPERIMENTAL

Apparatus and reagents

An Hewlett-Packard chromatograph Model 1090A equipped with diode array detection (DAD) and controlled by an HP 85B workstation was used for quantitative

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analysis. The chiral column was prepared by passing a solution of 3,5-dinitrobenzoyl-L-leucine (DNB-L-Leu) (Aldrich) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (Fluka) through a stainless-steel column (100 mm × 4.6 mm I.D.) packed with Nucleosil APS 100-5 (J.J.'s Chromatography)[4]. Tetrahydrofuran (THF) was distilled from above Na–K alloy, alcohols were dried over molecular sieves and *n*-hexane (Reachim) was used as obtained.

The coupling reaction

The reactions on a 0.25-mmol scale were carried out in THF at 25.0°C for 24 h in thermostatted vessels with magnetic stirring. Amino acid components, coupling reagents and the additive, except triethylamine, were added as solids. The initial concentration of the reagents was 0.050 *M*.

RESULTS

The recent discoveries of the possibilities of resolution and quantitation of enantiomeric protected dipeptides using commercially available HPLC chiral columns (easy to prepare in any chemical laboratory also)[5–7] prompted us to modify the Young racemization test and to convert it into a general method of studying peptide bond formation. Thus the HPLC modification of the Young test reported here not only simplifies the test and increases its accuracy but makes possible the determination of the degree of racemization in the synthesis of many peptides other than the Young model containing C-terminal glycine, thus providing much more information. DNB-L-Leu grafted on the aminopropyl silica (Pirkle phase) appeared the best of a few stationary phases tested in our laboratory for separations of the ethyl benzoyl-

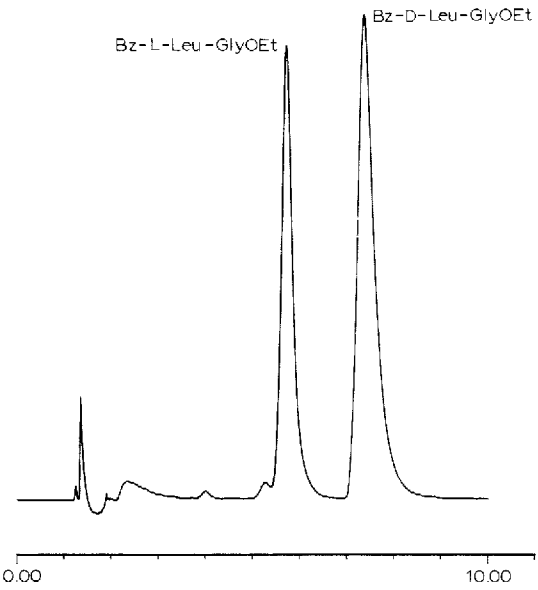

Fig. 1. Chromatogram of the reaction mixture: Bz-LeuOH + HCl · GlyOEt/BPO-Cl. Column: DNB-Leu-silica, 100 mm × 4.6 mm. Mobile phase: ethanol–*n*-hexane (3:97), flow-rate 1.0 ml/min. Detection: 254 nm.

TABLE I

SEPARATION FACTORS, α, AND RESOLUTIONS, R_s, FOR ENANTIOMERS OF DIPEPTIDES Bz-DL-AA-Gly-OEt, AND PARTITION FACTORS, k', FOR THE FIRST ELUTING L-ENANTIOMER

For chromatographic conditions see Fig. 1.

Amino acid	*3% Ethanol*			*5% Ethanol*			*5% Isopropanol*			*35% THF*		
AA	k'	α	R_s	k'	α	R_s	k'	α	R_s	k'	α	R_s
Ala	7.98	1.33	0.40	4.46	1.31	2.25	8.51	1.33	1.43	3.25	1.31	1.14
Val	3.62	1.25	0.72	2.04	1.24	1.59	3.43	1.26	1.17	2.10	1.20	0.79
Leu	3.61	1.41	0.48	2.15	1.38	2.20	3.20	1.49	1.76	1.77	1.35	1.17
Phe	6.91	1.41	0.56	3.99	1.38	2.28	7.23	1.44	1.71	2.46	1.34	1.14

amino acid glycinates (Fig. 1). On the Pirkle phase the separation factor, α, of the protected enantiomeric dipeptides seems to be almost independent of the mobile phase modifier concentration (Table I). Moreover the change of the modifier from a protic (ROH) to an aprotic (THF) solvent does not significantly influence the α value. This is consistent with the charge-transfer interaction model according to which the prevalent enantiomer recognition takes place at the stationary phase. On the other hand, the resolution, R_s, is far better in the mobile phase containing a protic modifier mainly due to the improved peak symmetry. The repeatability of the method was good ($\pm 0.5\%$ for D-enantiomer and $\pm 0.6\%$ for L-enantiomer) and the detection limit was found to be 0.5 nmol (0.01%).

The present method was employed to 1,3-dicyclohexylcarbodiimide–1-hydroxybenzotriazole (DCC–HOBt), EEDQ and bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) mediated peptide synthesis (Table II). The racemization degree and the yield of the products can readily be determined almost instantly (analysis time less than 10 min) using simple isocratic HPLC equipment. The reaction mixture was injected directly on the HPLC column thus avoiding any disadvantages commonly accompanying the Young test.

TABLE II

COMPARISON OF SOME COMMON COUPLING REAGENTS IN THE REACTION Bz-L-Leu + HCl · GlyOEt + n · NEt_3 IN THF AT 25°C

Coupling method	n	*Yield (%)*		*Racemization (%)*
		D-isomer	*L-isomer*	
DCC–HOBt	1	4.8	85.2	10.7
EEDQ	1	2.1	65.6	6.2
BOP-Cl	3	12.8	47.5	42.5

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