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DERIVATIZATION OF N-PROTECTED AMINO ACIDS FOR CHIRAL SEPARATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new method has been developed for determining the optical purity of N-protected amino acids and their active ester derivatives. The method is based on the formation of diastereomeric dipeptide derivatives with either the D- or the L-form of O-(4-nitrobenzyl)-tyrosine methyl ester. In the case of amino acid derivatives with a carboxyl group, dicyclohexylcarbodiimide was used as the coupling agent. Using a silica column and a mixture of hexane and 2-propanol as the eluent, with a gradient programme, several pairs of enantiomeric amino acid derivatives were successfully resolved. The favourable UV characteristics of the derivatives enabled the contaminating optical antipode to be determined down to the 0.1% level.

INTRODUCTION

As a result of the intensive search for biologically active peptide derivatives, several synthetic peptides have been introduced into the therapy of various diseases in the past two decades. The continuously increasing demand for purity of drugs and the rapid development in chromatographic instrumentation and separation systems have made the investigation of the chromatographic purity of these peptide drugs a very important research field.

In this framework, the most thoroughly investigated problem has been the chromatographic estimation of the optical purity of peptides. After some very important achievements with gas chromatography (GC)[1,2], the possibilities of which are naturally limited by the molecular mass of the peptides, the latest entry into this field is high-performance liquid chromatography (HPLC). A variety of diastereomeric peptides have been separated on various stationary phases, mainly reversed-phase systems[3–6].

Since the optical purity of peptides is largely determined by that of the amino acids used in their syntheses, the determination of the latter is an important task in all laboratories dealing with the analytical support of peptide syntheses.

GC, especially capillary GC with chiral stationary phases, provides an excellent means for this purpose, but the disadvantage is that double derivatization is necessary (usually the formation of 2-propyl ester, N-trifluoroacetyl or pentafluoropropionyl derivatives[7]). Moreover, in the case of N-carbobenzyloxy derivatives, the latter protecting group must be removed before derivatization.

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In the past ten years, HPLC has become the most frequently used method for the determination of the optical purity of amino acids and their derivatives. This problem has been solved by various research groups using three general methods:

(1) Separation of the enantiomers on chemically bonded chiral stationary phases[8–12].

(2) Separation on ordinary stationary phases, with the use of chiral additives in the mobile phase, which form diastereomeric metal complexes or hydrogen-bonded complexes with the enantiomeric amino acids. Typical chiral additives are, *e.g.*, copper(II) complexes[13,14], N-acetyl-L-valine *tert.*-butylamide[15,16], quinine[17], etc.

(3) Separation by means of ordinary stationary phases and eluents, but with a pre-column derivatization with chiral reagents to form covalently bound diastereomeric derivatives. This method is especially useful in those laboratories where various investigations must be routinely carried out on the same instrument and, hence, the use of special columns and eluents is not advantageous.

The chiral reagents used in amino acid analysis include: *tert.*-butoxycarbonyl-L-amino acid-N-hydroxysuccinimide esters[18,19], 2,3,4,6-tetraacetyl-D-glycopyranosyl isothiocyanate[20], (−)-α-methoxy-α-methyl-1-naphthalene-acetic acid[21], N-carboxy-L-leucine anhydride or N-carboxy-L-phenylalanine anhydride[22], 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide[23], etc. A common feature of these reagents is that they all react with the amino group of the amino acids and, therefore, they are not suitable for the derivatization of N-protected amino acid derivatives. This can be considered a major disadvantage, since, in many cases, these derivatives are the commercially available starting materials for the syntheses of various peptides. For this reason, the estimation of their optical purity is often as important as that of the underivatized amino acids.

The aim of this paper is to describe a new HPLC method, in which D- or L-O-(4-nitrobenzyl)-tyrosine methyl esters are used as new chiral reagents, for the derivatization of the carboxyl groups of N-protected amino acids and their active ester derivatives, and for the determination of their optical purity.

EXPERIMENTAL

Apparatus

A Varian 5000 Series high-performance liquid chromatograph (Varian Associates, Palo Alto, CA, U.S.A.) was used, with a Varichrom UV detector (Varian), a Valco loop injector (Valco Instruments, Houston, TX, U.S.A.), a ternary gradient system, and a Hewlett-Packard 3390 integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Solvents and chemicals

The solvents (hexane, 2-propanol and methanol) were purchased from Aldrich (Aldrich, Beerse, Belgium) and were of spectrophotometric grade. Triethylamine (Fluka, Buchs, Switzerland) and dicyclohexylcarbodiimide (Merck, Darmstadt, F.R.G.) were of analytical grade.

O-(4-Nitrobenzyl)-L-tyrosine methyl ester hydrochloride [m.p. 174–176°C; $\alpha_D^{20} = +9.0°$ ($c = 1$, methanol)] and O-(4-nitrobenzyl)-D-tyrosine methyl ester hydrochloride [m.p. 174–175°C; $\alpha_D^{20} = -9.5°$ ($c = 1$, methanol)] were synthesized from L- and D-tyrosine, respectively, as with the O-benzyl-L-tyrosine methyl ester[24].

Reagents

The reagents were prepared using methanol as the solvent: D- and L-O-(4-nitrobenzyl)-tyrosine methyl ester hydrochloride, 15 mmol/dm^3; dicyclohexylcarbodiimide, 30 mmol/dm^3; triethylamine, 30 mmol/dm^3.

Procedure for N-protected amino acids with a free carboxyl group

About 0.01 mmol of the sample is dissolved in a mixture of 1 ml of one of the O-(4-nitrobenzyl)-tyrosine reagents* and 1 ml of the dicyclohexylcarbodiimide reagent. The mixture is allowed to stand at room temperature for 60 min. There, 10–50 μl of the reaction mixture are injected into the chromatograph, depending on the quantity of the enantiomeric impurity.

Procedure for active esters of N-protected amino acids

The procedure is exactly the same as above, the only difference being that instead of the dicyclohexylcarbodiimide reagent, the same volume of triethylamine reagent is used.

Chromatography

The column was a LiChrosorb Si 60-10 column (25 cm × 4.6 mm I.D.) (Chrompack). The flow-rate was 2 ml/min.

The gradient programme was as follows: starting solution 98:2 mixture of hexane and 2-propanol; the proportion of the latter was increased to 5% in the first 20 min and then to 40% within 10 min. The cycle was terminated with a 15-min isocratic section at a ratio of 60:40.

The wavelength of the UV detector was 270 nm.

RESULTS AND DISCUSSION

The advantageous features of the new reagent are as follows.

Simple reaction conditions

Fig. 1 shows the equation of the general reaction between N-protected amino acids and the new regent. Under the very simple experimental conditions described in the preceding section, the reaction takes place within 60 min (Table I). Dicyclohexylcarbodiimide (DCC) is used as the coupling agent, but it also serves as the acid-binding agent. No heating, evaporation or extraction are necessary; the reaction mixture can be injected directly. A typical chromatogram, that of the reaction mixture of Z-D-Ser(OBu^t)-OH, containing 2.6% of the L-antipode and the L-form of the reagent, can be seen in Fig. 2.

These simple reaction conditions for the derivatization of the free carboxyl group could only be achieved in such a way that the completeness of the reaction was to some extent sacrified. For example, in the case of the above-mentioned reaction of the serine derivative, a conversion of *ca.* 60% was calculated from the concentrations of the amino acid and the reagent, as well as from the peak areas of

* As for the selection of the L- or D-form of the reagent (see Results and discussion).

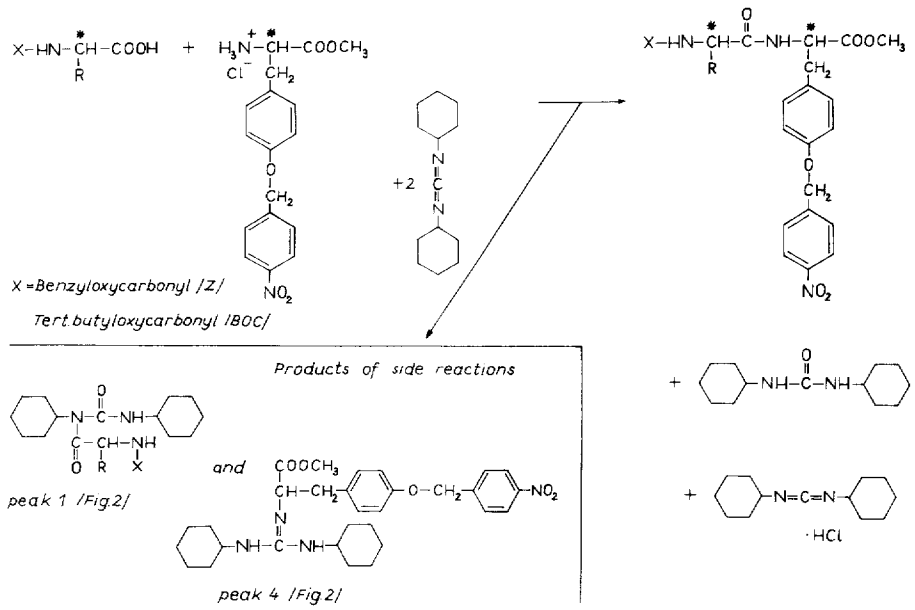

Fig. 1. Reaction scheme of N-protected amino acids with Tyr(O-4-nitrobenzyl)-OCH_3.

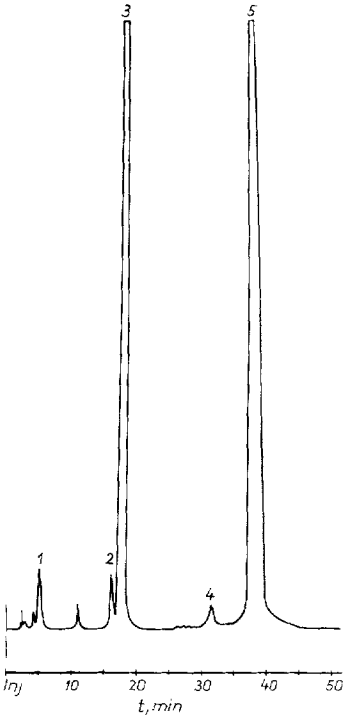

Fig. 2. Chromatogram of the reaction mixture of Z-D-Ser(OBu^t)-OH, containing 2.6% of the L-antipode, and L-Tyr(O-4-nitrobenzyl)-OCH_3. Peaks: (1) N-[Z-Ser(OBu^t)]-dicyclohexylurea; (2) L-Z-Ser(OBu^t)-L-Tyr-(O-4-nitrobenzyl)-OCH_3; (3) D-Z-Ser(OBu^t)-L-Tyr(O-4-nitrobenzyl)-OCH_3; (4) dicyclohexyl guanidine derivative of L-Tyr(O-4-nitrobenzyl)-OCH_3; (5) excess of L-Tyr(O-4-nitrobenzyl)-OCH_3. See Experimental for details of reaction and chromatographic conditions.

TABLE I

FORMATION OF THE DIASTEREOMERIC DIPEPTIDES IN THE REACTION BETWEEN Z-SER(OBu^t)-OH, CONTAINING 2.6% OF THE L-ANTIPODE, AND THE L-FORM OF THE REAGENT AS A FUNCTION OF REACTION TIME

	Time (min)				
	5	*10*	*30*	*60*	*120*
Integrator count (sum of the peak areas of the two diastereomers × 10^{-4})	12.1	15.5	20.1	20.9	20.8
Ratio of the peak areas of the DL- and LL-derivatives	38	36	37	37.5	38

the derivatives (peaks 2 and 3) and the unreacted reagent (peak 5). A competitive side-reaction takes place between the free carboxyl group of the amino acid and DCC, leading to the N-acyl urea derivative seen in Fig. 1 (peak 1 in Fig. 2). Fortunately, the ratio of the rates of the main and side-reactions does not show remarkable enantioselectivity: the ratio of the peak areas of the diastereomeric derivatives does not depend on the extent of the side-reaction, and it is constant as a function of reaction time (see Table I).

Another side-reaction takes place between DCC and the excess reagent, leading to the corresponding guanidine derivative (see Fig. 1 and peak 4 in Fig. 2). As the relative peak areas indicate, this reaction is slow and, hence, it has no influence on the results within the reaction time of 60 min.

Our finding that the side-reactions do not interfere with the determination of the optical purity applies only to simple N-protected amino acid derivatives. The application of this method to more complicated systems requires further study.

Naturally, even simpler reaction conditions are applicable to active ester derivatives. As seen in Fig. 3, in this case the coupling agent DCC can be omitted; only the free base form of the reagent must be liberated by triethylamine from the hydro-

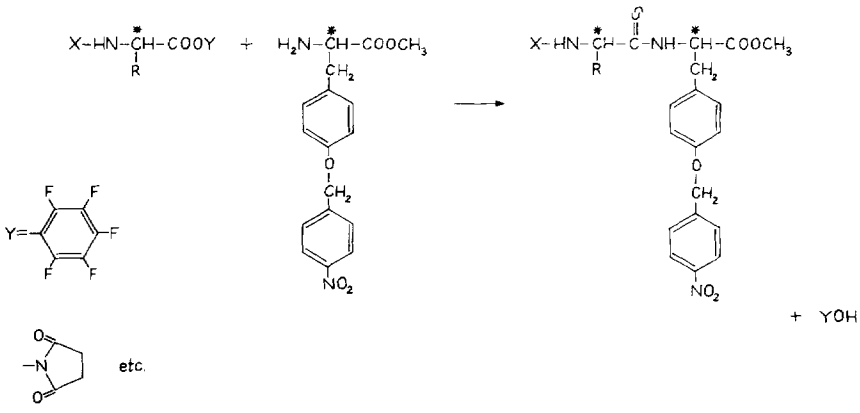

Fig. 3. Scheme of the reaction of active esters of N-protected amino acids with Tyr(O-4-nitrobenzyl)-OCH_3.

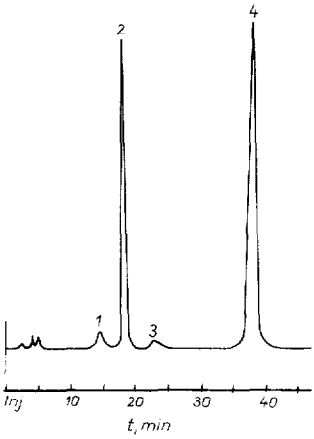

Fig. 4. Chromatogram of the reaction mixture of L-Z-Phe-OPFP, containing 3% of the D-antipode, and D-Tyr(O-4-nitrobenzyl)-OCH_3. Peaks: (1) D-Z-Phe-D-Tyr(O-4-nitrobenzyl)-OCH_3; (2) L-Z-Phe-D-Tyr(O-4-nitrobenzyl)-OCH_3; (3) pentafluorophenol; (4) excess of D-Tyr(O-4-nitrobenzyl)-OCH_3. See Experimental for details of reaction and chromatographic conditions.

chloride before the reaction can take place. It is evident that none of the above-described side-reactions have to be taken into account when active esters are investigated. A typical chromatogram is shown in Fig. 4.

No racemization takes place

An important feature of the new method is that no racemization takes place during the derivatization reaction. This was proved by model experiments (see Table II) and by an investigation of optically pure amino acid derivatives, where no peak of the enantiomeric impurity could be detected (or at least its relative quantity was $<0.1\%$).

Good separation of the enantiomers

As is evident from Figs. 2 and 4 and Table III, the resolution of the diastereomeric derivatives is good, the separation factors ranging between 1.12 and 1.45.

TABLE II

RESULTS OF MODEL EXPERIMENTS: DETERMINATION OF THE L-ANTIPODE IN Z-D-Ser-(OBu^t)-OH

L-Antipode	
Taken (%)	*Found (%)*
0.5	0.60
1.0	1.08
2.0	2.18 ± 0.18 (n = 5)
5.0	5.20 ± 0.24 (n = 5)
10.0	10.31

TABLE III

SEPARATION OF N-PROTECTED AMINO ACIDS AFTER DERIVATIZATION WITH L-Tyr(O-4-NITROBENZYL)-OCH_3

Amino acid derivative	*k'*		
	L	*D*	α
BOC-Phe-OSu	4.0	5.8	1.45
Z-Phe-OPFP	5.4	7.0	1.30
Z-Ser(OBu^t)-OH	6.6	7.4	1.12
Z-Thr(OBu^t)-OH	4.3	4.8	1.12
BOC-Cys(Bz)OPFP	4.0	4.9	1.23
Z-Asp(OBu^t)-OH	6.6	7.4	1.12
Z-Glu(OBu^t)-OH	7.2	8.3	1.15

Because the apolar substituents of the amino acids are bulky, it was found to be advantageous to use silica gel as the sorbent rather than the generally used reversed-phase systems.

Gradient elution was used partly to optimize the separation of the diastereomeric derivatives but mainly because, in this way, the peak of the excess reagent could be eluted within a reasonable time (peak 5 in Fig. 2 and peak 4 in Fig. 4).

Flexibility

With proper selection of the reagent, it is possible to achieve elution of the impurity peak before the main peak, thus facilitating quantitative evaluation of the ratio of the peak areas. This means that if the analytical task is the determination of the D-amino acid impurity in an L-amino acid, the D-form of the reagent can be used, while for the determination of the L-impurity in D-amino acids, the use of the L-form of the reagent is advisable.

Favourable UV characteristics of the derivative

The absorption maximum of the regent bearing the nitrobenzyl moiety is at 270 nm, and the molar absorptivity is 12 400. The former value serves as the basis for a fairly selective detection. The lowest detectable quantity of, for example, Z-D-Ser(OBu^t)-OH was found to be 20 ng. The rather long wavelength of the detection is very useful, because this obviates the difficulties of the short-wavelength detection of peptides.

Good accuracy and precision

Table II demonstrates by means of model experiments that no racemization had taken place in the course of the derivatization reaction. The dates in this table also demonstrate the good accuracy of the method. Also, the relative standard deviations in the table characterize good precision.

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