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DETERMINATION OF EPIMERIC PEPTIDES FOR ASSESSING ENANTIOMERIC PURITY OF STARTING MATERIALS AND STUDYING RACEMIZATION IN PEPTIDE SYNTHESIS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

   Chromatography of the epimeric peptide pairs for the series Xxx-Lys, Lys-Yyy, Gly-Xxx-Lys, Gly-Lys-Yyy (Xxx is the activated residue during coupling, Yyy is the residue aminolysing the activated residue forming the peptide bond) and some neutral, acidic and N-methylated peptides on a μBondapak C_{18} column is described. Good resolution was achieved in most cases using isocratic elution with aqueous ammonium acetate, or buffers of lower pH. Observations on factors affecting retention of peptides are made, and the potential use of these model compounds for determining enantiomeric content in amino acid derivatives and studying racemization are discussed.

INTRODUCTION

   Information on the extent of racemization to be expected during peptide synthesis has been acquired using model peptides. Couplings are carried out under selected conditions and the epimer produced is separated from the principal product by gas–liquid[1,2], ion-exchange[3,4] or thin-layer chromatography[5,6] and quantitated by various techniques, or the mixture is analysed using nuclear magnetic resonance (NMR) spectroscopy[7–9]. (For a review see ref. 10.) A variety of N-substituted amino acids and amino acid esters are included in the models, so varying tendencies to racemize are embodied in the test systems. Consequently, it has sometimes been difficult to compare results from different laboratories, and impossible to compare such properties of residues as their tendency to racemize or their influence, as the incoming nucleophile, on racemization at the activated residue. The solution to the difficulty lies in the use of series of several model peptides where two of the three
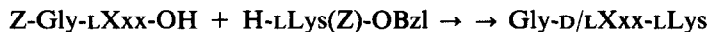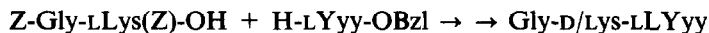
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possible variables (the activated residue, its N-substituent, and the amino containing component) are fixed, and only the residue in question is varied[11]. The first series introduced consisted of

$$\text{Bz-LXxx-OH} + \text{H-LLys(Z)-OMe} \rightarrow \text{Bz-D/LXxx-LLys(Z)-OMe*}$$

the epimeric products being analyzed by ^{1}H NMR spectroscopy by virtue of the separated methyl ester proton peaks[9]. More representative models involving the coupling of dipeptide acids, namely

$$\text{Z-Gly-LXxx-OH} + \text{H-LLys(Z)-OBzl} \rightarrow \rightarrow \text{Gly-D/LXxx-LLys}$$

and

$$\text{Z-Gly-LLys(Z)-OH} + \text{H-LYyy-OBzl} \rightarrow \rightarrow \text{Gly-D/Lys-LLYyy}$$

for Xxx = the difunctional amino acids, the epimeric products being determined with an amino acid analyzer are now also available[4]. However, there is a serious shortcoming to this method of analysis. Quantitation is based on determination of the color produced by reaction of the peptides with ninhydrin, and epimeric peptides give different color yields. Consequently, precise determinations require availability of the pure isomeric peptides as reference compounds. Synthesis of a mixture starting with one enantiomeric and one racemic component, unfortunately, does not give a 1:1 mixture of epimers. Therefore they must be synthesized separately. It is for this and other reasons, and to take advantage of the potential of high-performance liquid chromatography (HPLC) that we have adapted our series of model peptides[4] to analysis by reversed-phase HPLC. The study includes chromatography of the L–L and D–L/L–D isomers of the peptide series Gly-Xxx-Lys, its protected precursors, and Gly-Lys-Yyy, the dipeptide series Xxx-Lys and Lys-Yyy which can be used to determine the enantiomeric purity of amino acid derivatives, and a few non-basic peptides.

EXPERIMENTAL

Apparatus

The equipment was a Waters Scientific high-performance liquid chromatograph consisting of a Model 6000A solvent delivery system, a Model U6K universal injector, a Model 450 variable-wavelength detector, a Model 660 gradient programmer with Model 45 pump, a Houston Instruments Omniscribe single pen recorder and a Model 730 data module. A Waters 30 cm × 4 mm I.D. stainless-steel column with 10-μm μBondapak C$_{18}$ packing, protected by a No. 84550 guard tube containing Bondapak C$_{18}$/corasil was used throughout. The sample was introduced using a 25-μl No. 802 Hamilton high-pressure injection syringe.

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* Abbreviations: in partially protected amino acids and peptides, the ionizable function is expressed as –OH for carboxylic and H– for amino, and ( ) indicates side-chain substitution. For convenience, designation of ionizable functions is dropped for unprotected peptides. Bz = Benzoyl, Bzl = benzyl, Z = benzyloxycarbonyl, Xxx = the activated residue during coupling, Yyy = the residue aminolysing the activated residue forming the peptide bond.

Operation conditions

The flow-rate was between 0.4 and 1.5 ml/min, the temperature was ambient (*ca.* 21°C). Isocratic elution was carried out with 0.01 *M* ammonium acetate (pH 6.6), 0.01 *M* ammonium acetate buffer, pH 4.4, 0.01 *M* potassium dihydrogen phosphate buffer, pH 3.2, and 0.1% phosphoric acid (pH 1.8). A small amount (to 10%) of acetonitrile was added in a few cases. Sixty percent aqueous methanol increased to 70% according to profile 10 (concave curve) of the programmer was used for gradient elution. Peptides were detected by measuring absorbance at 208 nm, protected peptides at 215 nm. Solvents including water were HPLC-grade, from Baker; chemicals for buffer preparation were reagent grade from Fisher Scientific. Peptides were dissolved in water, protected peptides in acetonitrile–methanol (1:1) at concentrations of 1.0 to 10 mmol/l, and 10 to 20 μl of solution were injected into the chromatograph.

Synthesis of peptides

All peptides were synthesized by conventional procedures in solution using derivatives bearing benzyl-based protecting groups essentially as described in previous work[9,12]. Sources for most of the amino acid derivatives are given in ref. 4. All benzyl esters were hydrochlorides, which were neutralized with N-methylmorpholine. Protected dipeptide acids were prepared by reaction of the amino acid with the N-succinimidyl ester of Z-Gly-OH, except Z-Gly-Lys(Z)-OH, Z-Val-Lys(Z)-OH, Z-MeAla-Lys(Z)-OH, Z-Pro-Lys(Z)-OH, Z-Gly-Met-OH and Z-Gly-Tyr-OH which were obtained by saponification of the methyl esters. Couplings were carried out in dichloromethane using dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole except for cases where racemization during coupling was desired. Epimeric mixtures containing COOH-terminal lysine were obtained from H-DLLys(Z)-OBzl or Z-DLXxx-OH; those containing NH$_2$-terminal lysine from Z-DLLys(Z)-OH. Epimeric tripeptide pairs containing lysine in the central position were obtained by coupling Z-LXxx-LLys(Z)-OH with the L-benzyl ester using N-ethyl-N'-(γ-dimethylaminopropyl)carbodiimide hydrochloride in the presence of N-methylmorpholine. Substantial racemization occurs under these conditions[12]. Peptide mixtures containing no lysine were obtained from racemic Z-Xxx-OH or Z-Gly-Xxx-OH derivatives. Larger peptides were obtained by coupling an activated Z-dipeptide with the corresponding di- or tripeptide ester.

After completion of coupling reactions, mixtures were washed with dilute aqueous hydrochloric acid and then sodium hydrogen carbonate, the solvent was dried and evaporated off, and the products were deprotected by catalytic hydrogenation in 80% aqueous ethanol containing 5% of acetic acid. The catalyst was filtered off, the solvent was removed under reduced pressure and the residue was dried for 30 min on a vacuum pump.

Products consisted of L–L and enantiomeric L–D or D–L isomers depending on the method of synthesis. Because of selectivity during coupling, they were not necessarily 1:1 mixtures. The L–L isomers were identified by appropriate couplings of a representative number of non-racemic derivatives for each series of epimeric mixtures.

Detector response of epimers

Equal amounts of Z-Gly-LXxx-LLys(Z)-OBzl and Z-Gly-DXxx-LLys(Z)-OBzl

for Xxx = Leu and Val, obtained under conditions producing no racemization using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole[12], were mixed and deprotected together by hydrogenolysis. Chromatography was carried out with eluents of pH 1.8, 4.4 and 6.6. Peaks were integrated visually by the height times width at half-height method, and also using the data module.

Expression of results

The emergence of peaks from the column is expressed in terms of capacity factor $k' = (t_R - t_0)/t_0$ where t_R = retention time and t_0 = void time. The separation of peaks is expressed as resolution $R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$ where w = width at baseline, and t_{R2} and t_{R1} are retention times of the two isomers.

RESULTS

Elution and separation

Data on the separation of pairs of epimeric basic peptides, neutral peptides, and fully protected basic peptides appear in Tables I–II, III and IV, respectively. Purely aqueous solvents sufficed to elute all the basic peptides from the C_{18} stationary

TABLE I

CHROMATOGRAPHIC DATA ON THE SEPARATION OF EPIMERIC BASIC DIPEPTIDES

Isocratic elution from a μBondapak C_{18} column using 0.01 M ammonium acetate (pH 6.6); 0.01 M ammonium acetate buffer, pH 4.4; 0.01 M potassium dihydrogen phosphate buffer, pH 3.2; or 0.1% phosphoric acid (pH 1.8) as eluents, at flow-rates of 1 ml/min or 0.5 ml/min.

Peptide	Eluent pH	k'_{L-L}	k'_{D-L}	R_s
Ala-Lys	6.6*	0.18	0.32	0.92
Pro-Lys	6.6*	0.26	0.80	2.27
Val-Lys	6.6*	1.00	2.10	3.39
Leu-Lys	6.6	1.50	4.86	3.25
Ile-Lys	6.6	1.96	3.25**	2.24
Tyr-Lys	6.6	2.75	4.56	2.84
Phe-Lys	6.6	6.80	11.30	1.75
Trp-Lys	3.2***	3.87	6.33	2.96
Phe(4Cl)-Lys	4.4	1.78	5.24	4.42
MeVal-Lys	1.8, 6.6	0	0	
Lys-Ala	1.8, 6.6	0	0	
Lys-Pro	6.6			<0.5
Lys-Val	6.6*	0.5	0.70	1.35
Lys-Ile	6.6*	1.75	2.50	1.30
Lys-Leu	6.6*	1.77	2.09	0.92
Lys-MeVal	6.6	3.63	4.38	2.73
Lys-Phe	1.8§	5.12	7.83	2.26
Lys-Thr	3.2§	1.68	2.12	0.95
Lys-Phe(4Cl)	3.2§	3.42	4.17	1.03
His-Leu	3.2§	0.97	2.12	3.90

* Flow-rate 0.5 ml/min.
** L-Ile-D-Lys.
*** Containing 1% acetonitrile.
§ No resolution using pH 6.6 eluent.

TABLE II

CHROMATOGRAPHIC DATA ON THE SEPARATION OF EPIMERIC BASIC TRIPEPTIDES AND TETRAPEPTIDES

Isocratic elution from a μBondapak C_{18} column using 0.01 M ammonium acetate (pH 6.6); 0.01 M ammonium acetate buffer, pH 4.4; 0.01 M potassium dihydrogen phosphate buffer, pH 3.2; or 0.1% phosphoric acid (pH 1.8) as eluents, at flow-rates of 1 ml/min or 0.5 ml/min.

Peptide	Eluent pH	k'_{L-L}	k'_{D-L}	R_s
Gly-Ala-Lys	6.6*	0.33	0.53	1.21
Gly-Met-Lys	6.6	0.75	1.79	3.85
Gly-Val-Lys	6.6*	1.08	2.17	4.08
Gly-Ile-Lys	6.6	2.00	4.38**	4.83
Gly-Leu-Lys	6.6	2.15	4.25	3.87
Gly-Pro-Lys	6.6*	2.79	3.94	2.16
Gly-Tyr-Lys	6.6	3.70	5.45	2.70
Gly-Phe-Lys	6.6	6.38	12.25	4.98
Gly-Lys-Ala	1.8*	0.10	0.21	1.39
Gly-Lys-Pro	6.6*	0.83***	1.67***	0***
Gly-Lys-Val	6.6*	1.32	2.07	2.00
Gly-Lys-Ile	6.6	3.05	5.18	2.38
Gly-Lys-Leu	6.6*	3.58	5.65	2.04
Gly-Lys-Phe	2.2	6.18	10.3	3.31
Gly-Lys-Lys	6.6	1.20	1.20	0§
Leu-Gly-Lys	4.4	1.78	3.18	2.93
Val-Lys-Gly	4.4*	0.34	0.95	2.83
Phe(4Cl)-Lys-Gly	4.4	2.35	4.55	2.49
Gly-Leu-Lys-Gly	4.4	2.18	4.34	3.82
Gly-Xxx-Gly-Lys§§				0§
Ala-Val-Gly-Lys	3.2§§§	0.41	1.54†	3.55
Gly-Leu-Lys-Gly-Gly	3.2§§§	1.33	2.13	1.60

    * Flow-rate 0.5 ml/min.
    ** Gly-aIle-Lys.
    *** An additional peak with $k' = 1.27$.
    § No resolution using eluent of any pH.
    §§ Xxx = Leu, Met, Val. Peptides synthesized by Dr. A. Paquet.
    §§§ Containing 1% acetonitrile.
    † L-D-L.

phase. Addition of a small amount of acetonitrile was required to elute some of the neutral peptides, and to shorten the elution times for Trp-Lys and Ala-Val-Gly-Lys. Two peptides, Lys-Ala and MeVal-Lys, were not retained by the support. The doubly charged Gly-Lys-Lys was retained, but the isomers remained coincident over the pH range (1.8–6.6) tested. The two COOH-terminal proline peptides emerged incompletely resolved, the tripeptide showing up as three peaks. And the three tetrapeptide pairs with alternating glycyl residues remained coincident. The other 49 epimeric pairs examined could be separated with an R_s value > 1.20 except three, with an R_s = 0.92–0.95. Aqueous ammonium acetate (pH 6.6) as solvent gave good results for the basic dipeptides and tripeptides except for those containing an aromatic residue which required an acidic pH (1.8 or 3.2) to shorten the retention times. Best results for the basic pentapeptide and tetrapeptides and the neutral di- and tripeptides were achieved using pH 3.2 or 4.4 eluents. Small variations (ca. 0.2) in the pH of the

TABLE III

CHROMATOGRAPHIC DATA ON THE SEPARATION OF EPIMERIC NEUTRAL AND ACIDIC PEPTIDES

Isocratic elution from a μBondapak C_{18} column using 0.01 M ammonium acetate buffer, pH 4.4 or potassium dihydrogen phosphate buffer, pH 3.2 containing ( )% acetonitrile as eluents at flow-rates of 1 ml/min or 0.5 ml/min.

Peptide	Eluent pH	k'_{L-L}	k'_{D-L}	R_s
Gly-Leu-Ala**	4.4 (3)	1.10	3.20	5.14
Gly-Ala-Ala**	3.2*	0.25	0.77	2.11
Gly-Ala-Leu	4.4 (3)	1.48	3.75	7.02
Gly-Ser-Leu	4.4*	2.91	4.53	3.91
Gly-Val-Leu**	4.4 (10)	1.38	4.43	7.75
Gly-Leu-Asp***	3.2	3.54	5.10	2.50
Gly-Leu-Gly-Leu				0
Asp-Leu§	3.2	5.17	7.42	4.96
Phe-Ser	4.4 (1)	1.26	3.27	6.68
Phe-Asp	4.4 (1)	1.53	3.33	5.62
Phe-Glu	4.4 (1)	1.83	6.14	10.77
Phe-Lys	4.4 (1)	1.00	2.83	5.81
MeVal-Leu	4.0 (0.5)	8.95	5.93	4.00

     * Flow-rate 0.5 ml/min.
   ** Corresponding dipeptide pairs not containing glycine also well separated by the same eluent.
 *** No retention using a pH 6.6 eluent.
    § No resolution using a pH 4.4 or 6.6 eluent.

solvent had an insignificant effect on the results. The chromatographic profiles obtained for several representative epimeric pairs of unprotected basic peptides are given in Figs. 1 and 2. The limit of detection of one epimer in the presence of the other obviously depends on the resolution achieved. In most cases, it is below 0.1%. On the other hand, solvent composition was critical for separation of the isomers of the protected peptides series Z-Gly-Xxx-Lys(Z)-OBzl. Aqueous acetonitrile was of no use. Best results were achieved using an aqueous methanol gradient (from 60% to 70%), but only for one peptide was an $R_s > 1.25$ attained (Table IV).

TABLE IV

CHROMATOGRAPHIC DATA ON THE SEPARATION OF PROTECTED EPIMERIC TRIPEPTIDES Z-Gly-L/DXxx-LLys(Z)-OBzl

Gradient elution from a μBondapak C_{18} column using 60% aqueous methanol increased to 70% according to profile 10 (concave curve) of the programmer.

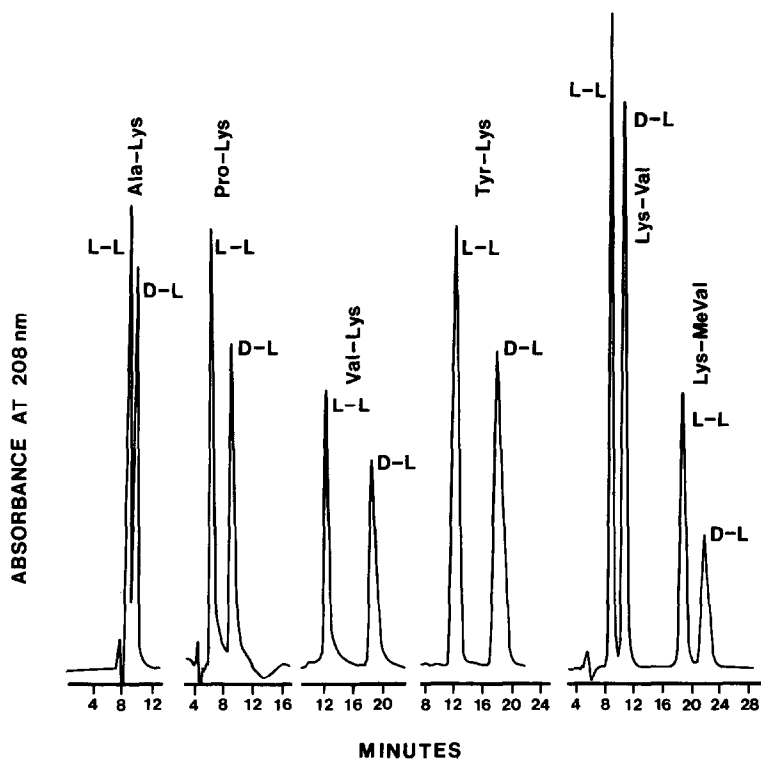| Xxx	k'_{L-L}	k'_{D-L}	R_s
Ala	8.19	8.39	0.62
Val	11.65	12.13	1.17
Pro	11.9	11.9	0
Ile	15.47	16.14	1.35
Phe	16.64	17.11	0.98
Leu	16.72	17.19	1.20

Fig. 1. Chromatography of basic epimeric dipeptides on a μBondapak C_{18} column. Elution with 0.01 M ammonium acetate at 0.5 ml/min; 1 ml/min for Tyr-Lys.

Effect of stereochemistry on retention and UV-absorbance

The peptides examined contain two chiral residues, except Ala-Val-Gly-Lys which contains three. In all cases, the negative isomer or isomer with residues of opposite configuration[10] was retained longer by the C_{18} stationary phase than the positive or L–L isomer, in agreement with expectations. On the other hand, the L–L and D–L isomers generated identical responses, within the sensitivity of the method, in the detector set at 208 nm for the four cases examined.

Effect of amino acid sequence on retention and resolution of isomers

Data on the basic peptides containing leucine and valine are collected in Table V. These show that the sequence generally has a minor influence on k' and R_s. However, some observations can be made. The negative effect on retention of a lysyl residue was greater when it was at the NH_2-terminus (Lys-Yyy) than when in other positions (Xxx-Lys, Gly-Xxx-Lys, Gly-Lys-Yyy). This influence of the ε-amino group was also more pronounced in the D–L/L–D isomers, thus decreasing the R_s for any epimeric pair, with the consequence that the poorest separations were achieved with the Lys-Yyy series. With a few exceptions (Xxx-LLys versus Gly-Xxx-LLys for Xxx = LVal, DVal and LPhe), an NH_2-terminal glycyl residue had a positive effect on retention, the extent sometimes depending on the stereochemistry of the peptide.

TABLE V

CHROMATOGRAPHIC DATA SHOWING THE INFLUENCE OF N-METHYLATION, A GLY-CYL RESIDUE, AND SEQUENCE ON CAPACITY FACTOR AND RESOLUTION

Selected examples from Tables I and II. Isocratic elution using a pH 6.6 eluent.

Peptide	k'_{L-L}	k'_{D-L}	R_s
MeVal-Lys	0	0	
Lys-MeVal	3.63	4.38	2.73
Val-Lys	1.00	2.10	3.39
Lys-Val	0.50	0.70	1.35
Gly-Val-Lys	1.08	2.17	4.08
Gly-Lys-Val	1.32	2.07	2.00
Leu-Lys	1.50	4.86	3.25
Lys-Leu	1.77	2.09	0.92
Gly-Leu-Lys	2.15	4.25	3.87
Gly-Lys-Leu	3.58	5.65	2.04
Leu-Gly-Lys*	2.23	3.85	2.61
Gly-Leu-Lys-Gly*	3.87	8.88	3.74
Gly-Leu-Gly-Lys			0**
Gly-Leu-Lys-Gly-Gly***	1.33	2.13	1.60

    * New data. Elution order of epimers not established.
    ** Eluent pH 6.6 or 4.4.
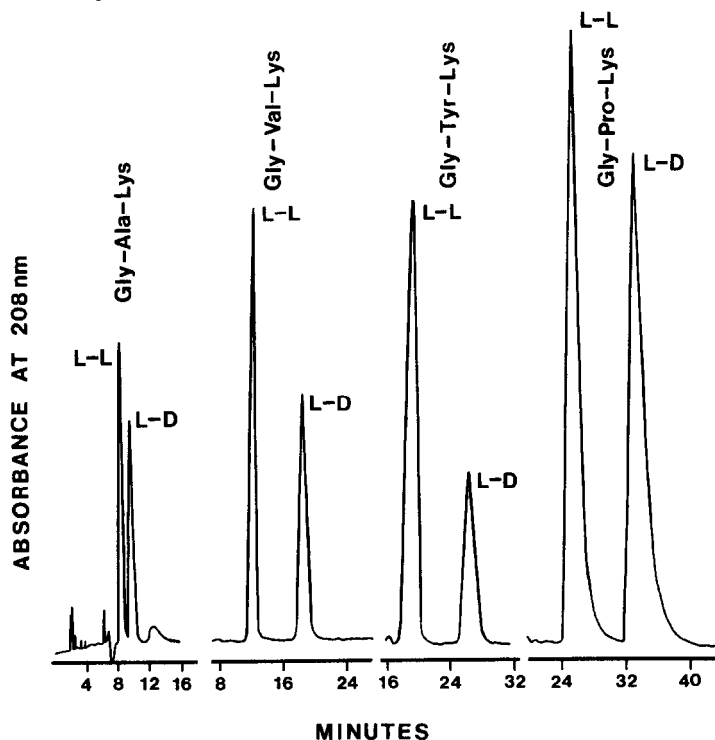   *** pH 3.2 eluent containing 1% acetonitrile.

MINUTES

Fig. 2. Chromatography of basic epimeric tripeptides on a μBondapak C$_{18}$ column. Elution with 0.01 M ammonium acetate at 0.5 ml/min; 1 ml/min for Gly-Tyr-Lys.

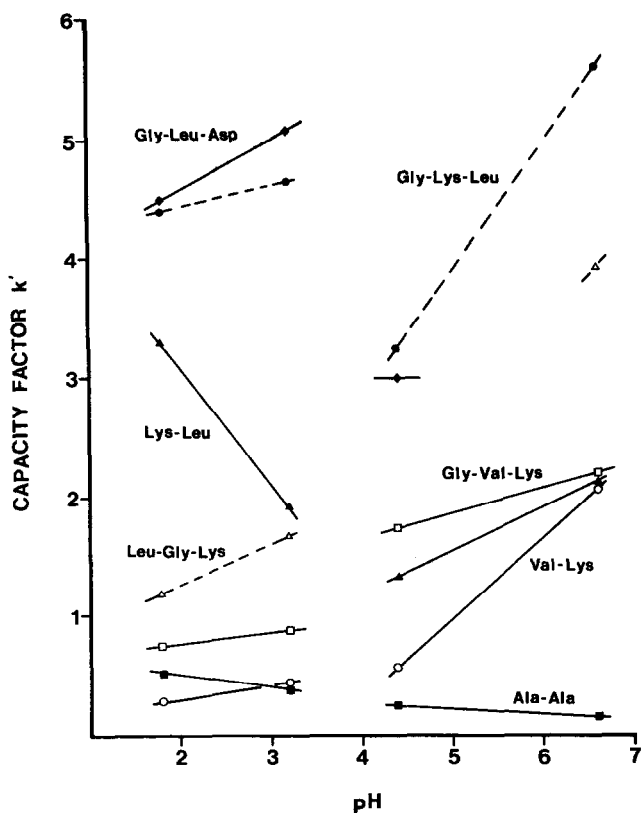Fig. 3. Effect of pH on the capacity factors of peptides (D–L isomers) chromatographed on a μBondapak C₁₈ column. Elution with ammonum acetate buffer at pH 4.4 and 6.6, phosphate buffer at pH 3.2, and phosphoric acid. The lines are interrupted because ion-pairing with the phosphate anion probably occurs at the lower pH's.

Tripeptide isomers containing glycine were well resolved, but epimeric tetrapeptides containing two glycyl residues were resolved only when the two chiral residues were adjacent. Carboxyl-terminal proline peptides (two examples) were not resolved, while other proline-containing peptide isomers were well resolved.

Effect of N-methylation
        N-Methylation of a peptide bond (Lys-Val → Lys-MeVal, Table I) had a pronounced efect on the capacity factors of both isomers, increasing them by 6–7 times, and a favorable effect on resolution. However, N-methylation of an NH₂-terminus (Val-Lys → MeVal-Lys) had the opposite effect. In fact, it had such a strong negative effect on retention that the peptide was not retained at all. In the neutral peptide MeVal-Leu, the N-methyl group had the effect of inverting the usual sequence of elution of the two epimers, the D–L-isomer emerging first (Table III).

Effect of eluent pH on retention
        Fig. 3 shows the effect of pH on the capacity factors of the negative (D–L)

isomer of several peptides chosen as representative of different types of peptides. The capacity factors of the basic peptides decreased substantially with a decrease in eluent pH from 6.6 to 4.4. Those of the COOH-terminal lysyl peptides continued to decrease as the solvent pH was lowered. However, in contrast, the capacity factors of the other lysine-containing peptides increased as the eluent became more acidic below pH 4.4. The acidic peptide had maximum retention at pH 3.2. The retention of the neutral peptide was not affected by a change in pH of the eluent.

Effect of eluent pH on resolution

The effect of the pH of the eluent on the resolution of several epimeric peptides selected as representative of different types of peptides is shown in Fig. 4. The COOH-terminal lysyl peptides showed a slight decrease in resolution as the pH was lowered from 6.6 to 4.4, and then a sharper decrease with decreasing pH. On the other hand, the other two lysine-containing peptides showed a slight increase in resolution in going from pH 6.6 to 4.4, and then each showed a much higher resolution at one of the lower pH values examined. The acidic peptide showed maximum resolution at pH 3.2. The neutral peptide showed a moderate increase in resolution as the pH was decreased using either of the two types of buffer.
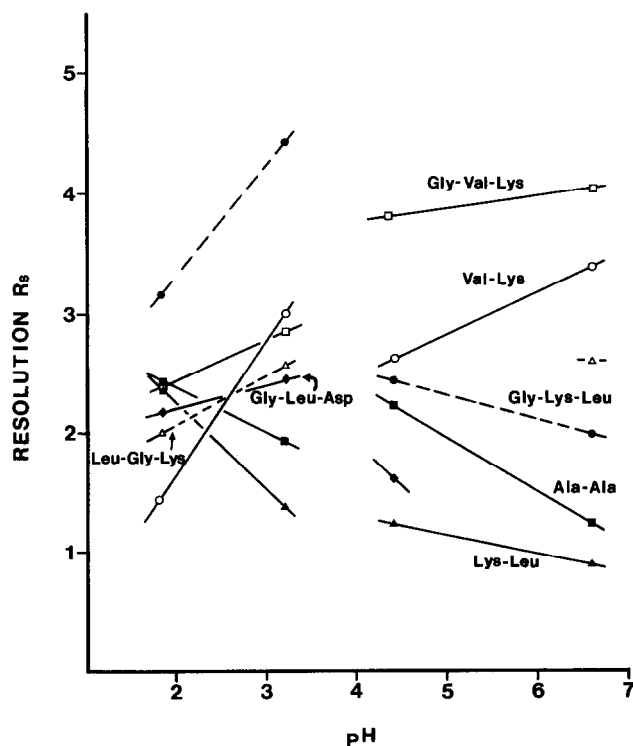

Fig. 4. Effect of pH on the resolution of epimeric peptides chromatographed on a μBondapak C_{18} column. Elution with ammonium acetate buffer at pH 4.4 and 6.6, phosphate buffer at pH 3.2, and phosphoric acid. The lines are interrupted because ion-pairing with the phosphate anion probably occurs at the lower pH's.

DISCUSSION

The methods of detection and quantitation of diastereomeric peptides serve many purposes in peptide research laboratories. Besides being used for their principal function of ascertaining the stereochemical purity of peptides, quantitation of epimeric peptides produced under selected situations provides information on the enantiomeric content of their precursors, on the tendency of these precursors to lose their chiral integrity, and on the factors contributing to this loss in chiral integrity. Various epimeric peptide model systems[1–9] have been designed for the different purposes. Each has its unique attractive features and/or shortcomings. (For a review, see ref. 10). An additional analytical technique for determining stereoisomeric peptides became available with the advent of HPLC. It was to exploit this potential that the present study was initiated. The feasibility of separating epimeric peptides by HPLC had been demonstrated[13,14]. Other reports on the separation of free[15,16] and protected[17–20] epimeric peptides have since appeared.

The method of choice for determining free epimeric peptides has been ion-exchange chromatography followed by spectrophotometric detection after reaction of the isomers with ninhydrin[21]. However, because the epimers do not generate the same amount of color, accurate quantitation cannot be achieved unless the pure isomers are available as reference compounds. HPLC combined with absorbance monitoring at 208 nm, which is a measure of the peptide-bond content of the compounds, allows direct determination of an epimer. That epimeric peptides have the same absorbance has been established for two pairs of tripeptide isomers eluted from the column with aqueous acetate and phosphate buffers at near-neutral and acidic pH's. There is thus immediately a major advantage of HPLC over a conventional amino-acid analyzer for determining diastereoisomers.

The most popular method for determining the enantiomeric purity of an amino acid Yyy involves its reaction with L-Leucine N-carboxyanhydride followed by analysis of the LLeu-L/DYyy isomers with an analyzer[21]. Modifications[22], including isomer analysis by HPLC[15] have been reported. Amino-acid derivatives can be examined similarly after deprotection. We have previously described a method by which the enantiomeric purity of amino-acid derivatives W-Xxx-OH or H-Yyy-OR with appropriate side-chain protecting groups can be established by reacting them with H-LLys(Z)-OBzl or Z-LLys(Z)-OH respectively, followed by deprotection and analysis with an amino-acid analyzer of the corresponding epimeric lysyl dipeptides L/DXxx-LLys and LLys-L/DYyy[4]. The lysyl residue had originally been chosen because of its side-chain so that a dipeptide containing an NH_2-terminal N-methyl-amino acid could be quantitated using ninhydrin[23]. No color is produced by the reaction of MeLeu-Ala with ninhydrin[24]. It is evident from Table I and Fig. 1 that when Xxx and Yyy are neutral residues, their lysyl dipeptide diastereomers are readily and well resolved by purely aqueous buffers, most with a pH 6.6 eluent. When Xxx and Yyy are acidic or hydrophilic (Ser, Thr, His), their combination with phenylalanine or leucine (Table III) produces epimeric pairs resolvable by HPLC. Consequently, with the exception of proline esters, just about any amino acid derivative with cleavable substituent groups can be assessed for enantiomeric purity by this approach. The failure of a COOH-terminal proline dipeptide to emerge as a discrete peak has been studied and demonstrated to be the result of the presence of both the *cis*- and *trans*-

isomeric forms[25]. The conclusion that this phenomenon obtains if proline is not at the NH$_2$-terminus[25], however, should be modified to indicate, according to present evidence (Gly-Pro-Lys, Fig. 2), that it obtains only when proline is at the COOH-terminus of a peptide. Successful HPLC of the Lys-MeVal isomers (Table I) indicates that the lysyl series can probably serve for verifying the chiral integrity of N-methylamino acid esters. The N-protected N-methylamino acid gave a satisfactory separation of epimeric products when combined with a neutral residue (Table III). And since the N-alkoxycarbonyl-N-methylamino acid is the precursor of N-methylamino acids obtained by chemical synthesis[26], this approach, if generally applicable, will serve as a convenient test for estimating the enantiomeric purity of synthetic N-methylamino acids.

Much of our information on racemization has been obtained using model systems involving activation and coupling of an N-acylamino or N-protected peptide acid with the amino group of a second chiral residue whose carboxylic function is bound in an ester or peptide linkage. The activated peptide is more representative of the real situation. The classical example involves formation of Z-Gly-L/DAla-LLeu-OBzl from the coupling of Z-Gly-LAla-OH with H-LLeu-OBzl[3]. The epimeric products are determined with an amino-acid analyzer after deprotection. We have described a procedure by which the amount of racemization attending peptide-bond formation at an activated residue Xxx can be established by coupling Z-Gly-LXxx-OH with H-LLys(Z)-OBzl followed by analysis for the deprotected peptide epimers Gly-L/DXxx-LLys with an analyzer[4]. In adapting this series to analysis by HPLC we first examined the separation of the protected products since this would avoid the need for a deprotection step. Some success was achieved, (Table IV), but the need for a gradient, the moderate resolutions obtained, and the nearly identical elution times for three of the pairs of peptides left us dissatisfied with this approach. On the other hand, it was found that except for Xxx = Ser, all the unprotected COOH-terminal lysyl tripeptide pairs examined could be well resolved using aqueous ammonium acetate as eluent (Table II). It thus emerged that use of this series, Fig. 2, provides an excellent approach for comparing the tendencies of residues Xxx to racemize during activation and coupling. In fact, one can compare the three different types of residues, β-methyl or hindered (Val), aromatic (Phe) and ordinary aliphatic (Leu) in one experiment if one uses a pH 4.4 eluent which separates the mixture of the three epimeric pairs (Fig. 5). The more hydrophilic seryl residue can be coupled with leucine, a neutral amino acid, in order to compare it with other residues (Table III, Fig. 6). The analogous series, Gly-L/DLys-LYyy, was also well resolved by a purely aqueous solvent except for the COOH-terminal proline peptide even though a pH 1.8 eluent had to be used for Yyy = Ala and Phe. Use of this series allows one to compare the effect of the nature of Yyy on the racemization at an activated residue coupling with Yyy, in this case the activated moiety being Z-Gly-LLys(Z)-OH.

As indicated above, the popular Izumiya and Muraoka test for racemization involves analysis of the Gly-L/DAla-LLeu isomers by ion-exchange chromatography[3]. The separation allows detection of 0.1% D–L in L–L. This limit of detection has been lowered 10–30 times by a prior partition chromatography of the epimers[27]. We have found (Fig. 6, Table III) that HPLC using a pH 4.4 eluent containing 3% of acetonitrile gives a splendid separation of these epimers, with a limit of detection of one isomer in the presence of the other of well below 0.1%.
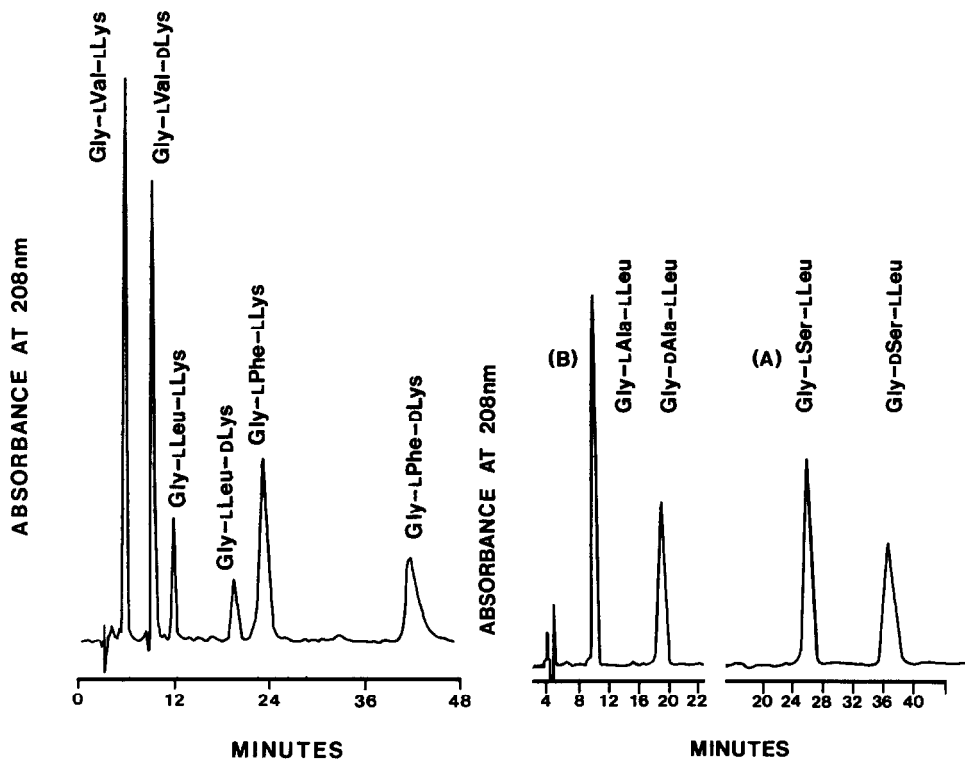
Fig. 5. Chromatography of a mixture of three epimeric peptide pairs on a μBondapak C_{18} column. Elution with 0.1 M ammonium acetate buffer, pH 4.4 at 1 ml/min for 11 min, followed by 1.4 ml/min.

Fig. 6. Chromatography of neutral epimeric tripeptides on a μBondapak C_{18} column. Elution with (A) 0.01 M ammonium acetate buffer, pH 4.4 at 0.5 ml/min and (B) the same buffer containing 3% acetonitrile at 1 ml/min.

The chromatography of other peptides was examined with other objectives in mind. Nearly all racemization tests involve coupling with an amino acid ester as the nucleophile. Separations of Gly-L/DLeu-LLys-Gly and Gly-L/DLeu-LLys-Gly-Gly will allow us to compare the effect of the nature of the nucleophile, i.e. H-Lys(Z)-OBzl, H-Lys(Z)-Gly-OBzl and H-Lys(Z)-Gly-Gly-OBzl, on the racemization at an activated residue, leucyl in Z-Gly-LLeu-OH in this case, during coupling. The Gly-L/DXxx-Gly-LLys peptides (Table II) were examined as potential test systems for studying racemization at the penultimate residue in a coupling, i.e. at Xxx in a coupling of Z-Gly-LXxx-Gly-OH with H-LLys(Z)-OBzl. Unfortunately, no resolution of epimers could be achieved using any eluent. A satisfactory resolution of epimers of another peptide appropriate for such a study, namely Ala-Val-Gly-Lys, (Table II), was achieved, however, when a third chiral residue was incorporated in the molecule. The tendency of Gly-Leu-Gly$_n$-Leu epimers with n = 1–3 to resist separation on a polystyrene–divinyl benzene column, as opposed to what occurs when n = 0, has been reported[16], and rationalized on the basis that the achiral residue(s) alter the preferred conformations of the isomers and thus the hydrophobic concentration of the sidechains. This phenomenon may obtain here with our Gly-Xxx-Gly-Lys

isomers, but the NH_2-terminal glycyl residue in the tetrapeptides must also be critical to their behavior on the C_{18} column because the tripeptide LLeu-Gly-L/DLys isomers emerged well separated (Table II). It also follows that adjacent chiral residues in epimeric peptides are not essential to their separation by reversed-phase HPLC.

The above discussion has focused on the design and separation of series of epimeric peptides useful for determinations of enantiomeric purity and studies on racemization. A second aspect of this study which warrants discussion concerns the chromatographic behavior of basic peptides on a μBondapak C_{18} column. Considerable information on the chromatography of small, mostly neutral peptides, is available, a detailed analysis of the correlation between composition and retention characteristics having recently appeared[16]. According to the literature, the order of elution of peptides can be predicted on the basis of the hydrophobic effects which are additive, of the side-chains of the constituent amino acids[28,29], the hydrophobicity scale being Trp > Phe > Leu $\simeq$ Ile > Tyr > Val > Met > Pro > Ala[30]. Our results with the basic di- and tripeptides are in agreement with this scale, except that Tyr $\approx$ Ile/Leu, when the non-lysyl residue was at either terminus of the peptide. However, there are two discrepancies when the pertinent residue was in the central position of a tripeptide, *i.e.*, in the Gly-Xxx-Lys series. Here, Tyr and Pro moved upwards in the scale, giving the order Phe > Tyr > Pro > Leu... etc. Thus the hydrophilic properties of Tyr and Pro are manifested less when they are not at the terminus of a peptide. This is in accord with the concept that the side-chain effect of a residue is less when it is separated from charged end-groups[16,31]. With regard to side-chain effects, it is interesting to compare Leu with Ile (Tables I and II), which reveals that it is impossible to generalize, because the apparent hydrophobicity order depends both on the stereochemistry and the sequence of the isomers. Stereochemistry was also critical in determining the positive effect on retention of the extra methylene group in Phe-Glu compared with Phe-Asp (Table III). The change increased the capacity factor of the L–L isomer by 20%, but nearly doubled that of the L–D isomer.

The capacity factors of negative (L–D/D–L) isomers have consistently been found to be greater than those for positive (L–L/D–D) isomers[16]. This has been explained on the basis that the side-chains of adjacent chiral residues in negative isomers are closer together, thus creating a stronger hydrophobic interaction with the support[14,16]. Longer retention times for negative isomers was found for all our unmethylated peptides, which contained a hydrophobic and a basic, acidic, or hydrophilic side-chain. Our results confirm the generality of the conclusion[16] that negative isomers are more strongly bound to the support. In this regard, however, the apparently anomalous behavior of the MeVal-Leu epimers where the negative isomer had the shorter retention time must be noted. Further discussion on this point, as well as on the apparently hydrophilic effect of the amino-terminal methyl group in MeVal-Lys, is deferred pending acquisition of more examples.

It is apparent when comparing the chromatography of the Xxx-Lys with the Lys-Yyy series, and the Gly-Xxx-Lys with the Gly-Lys-Yyy series, and also the dipeptide with the corresponding tripeptide series as exemplified by the data in Table V, that the position of the variable residue in the peptide has a marked effect on the retention of the peptide, a conclusion arrived at by others[16]. Moreover, the effect of the glycyl residue depends on its position in the sequence. Pietrzyk and co-work-

ers[16,31] have concluded that the hydrophobic effect of a side-chain is greater when the residue is further away from the charge at the NH_2-terminus. In this work, the hydrophilic effect of the ε-amino group of lysine is greater when the residue is at the NH_2-terminus. This is consistent. It places the hydrophobic side-chain of the other residue away from the α-amino group, and the two plus charges in the basic peptide closer together.

The effect of pH on the retention and resolution of epimers, Figs. 3 and 4, was examined with the objective of finding good conditions for separating the isomers. Rationalization of the effect of pH on retention has been made on the basis that a decrease in charge in a peptide increases its retention by the apolar support[14,29]. Our results are in agreement with this, and are not discussed further, bearing in mind that an additional factor, hydrophilic ion-pairing by the phosphate counter-ion[28,32] is probably implicated in the behavior of the peptides at lower pH values.

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