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PEPTIDE RACEMIZATION STUDIES VIA GAS CHROMATOGRAPHY OF L-N-α-CHLOROPROPIONYL AMINO ACIDS—I

EFFECT OF ADJACENT RESIDUES IN THE COUPLING OF PROTECTED DIPEPTIDES WITH AMINO ACID ESTERS[1]

S. LANDE and R. A. LANDOWNE[2]
Departments of Biochemistry and Internal Medicine
Yale University School of Medicine, New Haven, Conn.

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Abstract—The use of an optically active acyl group with known configuration, namely L-α-chloropropionyl moiety, as derivatizing agent for the gas chromatographic analysis of amino acids is introduced as a quantitative and sensitive means of determining racemization of amino acids, regardless of the complexity of the system from which they may originate. This technique has been applied to determine what effect the penultimate amino acid may have on the racemization of L-phenylalanine when protected aminoacyl-L-phenylalanine dipeptides are coupled to form protected tripeptides with carboxyl terminal glycine. After hydrolysis, gas chromatographic analysis for diastereoisomeric L-α-chloropropionylphenylalanine methyl ester showed that the effect on racemization of adjacent blocked acidic or basic amino acids was no greater than that of glycine in the cases studied so far.

INTRODUCTION

THE detection and assay of racemization is of great importance to the peptide chemist and the broad interest in this field in the past few years has resulted in the development of various techniques toward this end. The evaluation of stereochemical purity by optical rotation is a method that lacks specificity and sensitivity. Certain enzymes notably trypsin, chymotrypsin and leucine aminopeptidase are now routinely employed by peptide chemists to determine the stereochemical homogeneity of synthetic peptides. These enzymes and also the D- and L-amino acid oxidases were used to detect the racemization of amino acid residues of the melanotropin peptides following their exposure to hot dilute alkali.[3] Racemization of certain residues within the intact peptide chain is now considered the cause for the curious prolonged hormonal action that is exhibited by these pituitary factors after alkali treatment. In this enzyme study it was found that certain peptide bonds containing fully racemized acyl members were completely resistant to enzyme catalysed hydrolysis although theoretically 50% degradation could be expected. This phenomenon had been observed previously[4] and indicates that under certain circumstances the results of an enzymatic assay can be erroneous both qualitatively and quantitatively.

A method independent of enzymology and polarimetry was developed by Anderson *et al.*[5] in which fractional crystallization techniques allowed the detection of about 1%

[1] Presented in part at the 3*rd Symposium on Biological Applications of Gas Chromatography* Ecole Polytechnique, Paris, September 13 (1965).

[2] The authors are both recipients of Research Career Development Awards from the National Institutes of Health.

[3] T. H. Lee and V. Buettner-Janusch, *J. Biol. Chem.* **238**, 2012 (1963).

[4] M. Bergmann and J. S. Fruton, *J. Biol. Chem.* **124**, 321 (1938).

[5] G. W. Anderson and R. W. Young, *J. Amer. Chem. Soc.* **74**, 5307 (1952); G. W. Anderson, J. Blodinger and A. D. Welcher, *Ibid.* **74**, 5309 (1952).

diastereoisomer in carbobenzoxyglycylphenylalanylglycine ethyl ester. The "Anderson test" is the reaction of carbobenzoxyglycylphenylalanine with glycine ethyl ester followed by fractional crystallization of the neutral product. This test may be used to compare the extent of racemization caused by varying either the peptide forming reagent or the conditions of the reaction. A similar technique was also developed by Williams and Young.[6]

Recently Weygand *et al.*[7] demonstrated that diastereoisomers of certain trifluoracetyl dipeptide methyl esters could be resolved by capillary column gas chromatography. This represented a significant improvement in resolving power, sensitivity and convenience of methodology for the study of racemization. By this technique as well as other physical methods, the resolution of diastereoisomers but not enantiomers is possible. Thus, dipeptides which theoretically may be composed of four stereoisomers—a pair of diastereoisomers and their enantiomers—can be resolved into two peaks. For tripeptides the difficulties inherent in the absolute identification of any of the 8 possible stereoisomers, coupled with their low volatility, indicates that the direct gas chromatographic study of peptides will be limited primarily to dipeptides. However, in general, methods incorporating gas chromatography for the study of racemization appear superior to any other presently available to the peptide chemist.

If, in the formation of diastereoisomers, one of the centers of asymmetry is sterically pure and of known configuration, it follows that both the configuration and the steric homogeneity of the second asymmetric center can be determined. This paper is based on such an approach and demonstrates that by gas chromatography the configuration and steric purity of amino acids can be determined with a high level of accuracy and sensitivity through the formation of derivatives with an optically pure acylating agent. The method is a general one and is completely independent of the complexity of the system from which the amino acids are derived. Thus studies can be made of the racemization accompanying the synthesis of complex peptides, or resulting from the treatment of peptides with hot alkali, as well as the contribution to these effects of other residues in the peptide chain.

The technique requires three steps: (1) the degradation of peptides to constituent amino acids, (2) the separation of the amino acids formed and (3) the synthesis of suitably volatile diastereoisomeric derivatives for gas chromatographic resolution. Acid hydrolysis followed by paper or TLC or the more facile automated ion exchange chromatography[8] amply fulfill the requirements of steps (1) and (2). In view of the limited success thus far in the application of gas chromatography to total amino acid analysis in a single run, the prospects of finding conditions for the simultaneous resolution of amino acid derivatives *and* their diastereomers were considered poor.

L-α-Chloropropionic acid satisfies the requirements of step (3) and introduces the second asymmetric center as an N-acyl moiety. The halo-acid can be prepared from L-alanine in a stable state of high optical purity by the method of Fu *et al.*[9] Chloropropionyl amino acid methyl esters are reasonably volatile; they can be detected by the highly sensitive electron capture system in addition to other more generally employed means such as the argon ionization detector. A combination of both systems

[6] M. W. Williams and G. T. Young, *J. Chem. Soc.* 881 (1963).
[7] F. Weygand, A. Prox, L. Schmidhammer and W. Konig, *Angew. Chem.* (English Ed.) **2**, 183 (1963).
[8] D. H. Spackman, W. H. Stein and S. Moore, *Analyt. Chem.* **30**, 1190 (1958).
[9] S.-C. J. Fu, S. M. Birnbaum and J. P. Greenstein, *J. Amer. Chem. Soc.* **76**, 6054 (1954).

is particularly useful when the relative concentrations of diastereoisomers is greater than 50:1. Although α-bromopropionic acid was previously suggested as a reagent for step (3) and its applicability for the resolution of racemic amino acids was demonstrated,[10] the difficulties in obtaining quantities of the optically pure acid limited its practicability.

Some techniques, wherein the second asymmetric center is introduced as an ester derivative of the amino acid have been reported recently. Trifluoroacetylaminoacyl secondary butyl,[11] secondary octyl[12] and L-methyl esters[13] have been used but none gave separation factors comparable to that achieved for N-α-halopropionyl derivatives without resorting to impractical temperature ranges. Furthermore, secondary butyl and octyl esters of a high degree of steric purity are difficult to obtain. Most recently Halpern *et al.*[14] have reported the resolution of diastereoisomeric L-α-chloroisovaleryl amino acid methyl esters. These results showed the highest separation factors to date with only a slight temperature increase required.

RESULTS AND DISCUSSION

As a first demonstration of the utility of the technique, a study was made of the contribution of penultimate amino acids to racemization when protected dipeptides were coupled to amino acid esters.

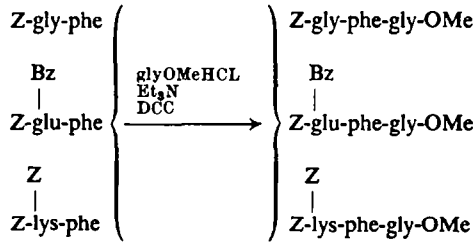

FIG. 1. Reactions studied for phenylalanine racemization.

Three protected dipeptides containing carboxyl terminal phenylalanine were coupled to glycine methyl ester to form the protected tripeptides (Fig. 1). All peptide forming reactions were carried out under identical conditions; dicyclohexylcarbodiimide was the coupling agent used. Although means are available for the reduction of diimide-caused racemization, these were not used. The object of the experiments was to determine the effect of the structure of the starting material on the extent of racemization of the product, not to study the efficacy of the coupling agent in the formation of optically pure peptides. The protected peptides were isolated as the neutral fraction under conditions where no resolution of optical isomers could occur. Further purification was obviated by the need to prevent possible fractionation of isomers and hydrolysis with 6N hydrochloric acid therefore followed.

[10] R. A. Landowne, *Chim. Anal., Paris* **47**, 589 (1965).
[11] G. E. Pollock, V. I. Oyama and R. D. Johnson, *J. Gas. Chromatog.* **3**, 174 (1965).
[12] E. Gil-Av, R. Charles and G. Fisher, *J. Chromatog.* **17**, 408 (1965).
[13] S. V. Vitt, M. B. Saporowskaya, I. P. Gudkova and V. M. Belikov, *Tetrahedron Letters* 2575 (1965).
[14] B. Halpern and J. W. Westley, *Chem. Comm.* 246 (1965).

A portion of the hydrolysate was submitted for amino acid analysis; in all cases the experimentally determined molar ratios of amino acids corresponded to the expected theoretical values, indicating that the peptides were homogeneous and were of the desired structure. The remainder of the hydrolysed product was acylated via a mixed anhydride prepared from L-α-chloropropionic acid and pivaloyl chloride[15] and esterified with diazomethane. Without further purification the resulting neutral fraction was subjected to gas chromatography to determine the amounts of diastereoisomeric chloropropionyl phenylalanine methyl esters. In these experiments, fractionation of amino acids prior to acylation was unnecessary as the retention times of α-chloropropionyl methyl esters of glutamic acid, lysine and glycine are widely different from that of phenylalanine. A typical chromatogram is illustrated in Fig. 2; Table 1 lists the results of the analyses.

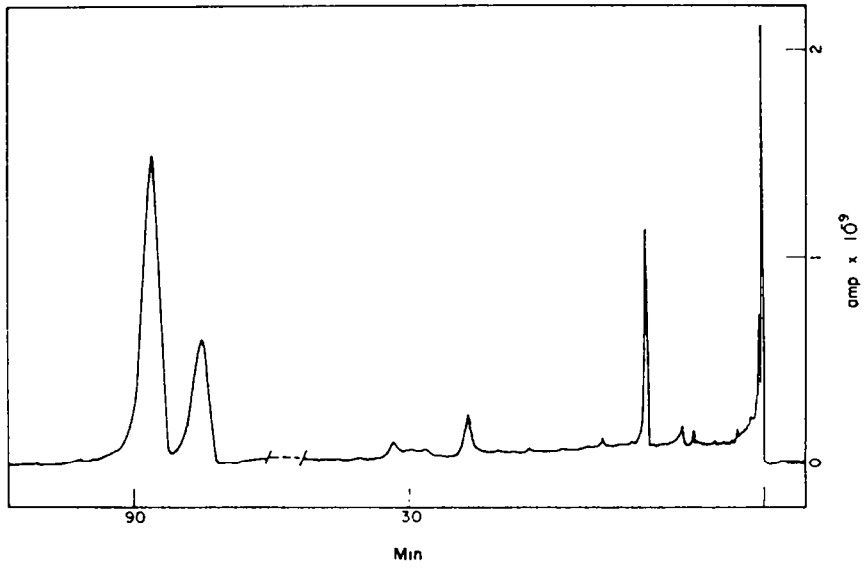

FIG. 2. Gas chromatogram showing resolution of L-α-chloropropionylphenylalanine diastereoisomers derived from glutamic acid-containing tripeptide. Column: 150′ × 0·01″ i.d. capillary with XE-60 liquid phase. Operating conditions given in Table 2.

Control experiments included the preparation of L-α-chloropropionylphenylalanine methyl ester from free L-phenylalanine and from the phenylalanine present in acid hydrolysates of the protected dipeptide starting materials. The results of these experiments are also in Table 1. The 1·7% diastereoisomer in the authentic L,L-product may have been formed as follows: (1) racemization accompanying the transformation of L-alanine into L-α-chloropropionic acid (or some D-alanine in the starting material), (2) racemization of the L-α-chloropropionyl moiety during the acylation process, (3) some D-phenylalanine in the starting material that was acylated and/or (4) racemization induced by the gas chromatographic process. Thus the minor peak may contain the D-α-chloropropionyl-L-phenylalanine methyl ester or its L,D-enantiomorph or both. However, it is this peak that is enlarged when D,L-phenylalanine is acylated instead of L-phenylalanine.

[15] M. Zaoral, *Coll. Czech. Chem. Comm.* **27**, 1273 (1962).

TABLE 1. L-PHENYLALANINE RACEMIZATION

Procedure	Percent of Diastereoisomer found[a]	Net percent due to coupling
Derivative formation	1·7 ± 0·6	—
Hydrolysis of dipeptide Z-R-Phe		
R = glycyl	3·6 ± 0·7	—
R = ε-Z-lysyl	4·0 ± 0·1	—
R = γ-Bz-glutamyl	5·0 ± 0·3	—
Formation of tripeptide Z-R-Phe-Gly-OMe		
R = glycyl	30·4 ± 0·2	26·8 ± 0·7
R = ε-Z-lysyl	29·0 ± 0·3	25·0 ± 0·3
R = γ-Bz-glutamyl	28·4 ± 1·0	23·4 ± 1·0

[a] Measured as the L-α-chloropropionyl derivative of phenylalanine methyl ester.

An additional amount of diastereoisomer can be observed in the acyl derivative prepared from the dipeptide hydrolysates (Table 1) and represents the limited amount of racemization induced by the hydrolytic procedure. Glutamic acid seems to enhance the level of acid catalysed racemization relative to the effect of glycine and lysine. Although certain assumptions must be made, this quantity may be applied as a correction factor for hydrolytically induced racemization. Thus, the amount of diastereoisomer formed in the peptide coupling experiments, greater than that obtained from the control experiments, is attributed to racemization resulting from the coupling reaction itself.

The very close similarity in the amount of phenylalanine diastereoisomer found in the tripeptide hydrolysates indicates that, relative to the effect of glycine, racemization of activated carboxyl terminal phenylalanine in protected dipeptides is neither further enhanced nor markedly inhibited by adjacent residues containing blocked carboxyl or amino moieties. However, a slight racemization-inhibiting effect is exhibited by glutamic acid. It is possible that other functional groups may be more active in this respect and they are now under investigation.

EXPERIMENTAL

Amino acid analysis. Di- and tripeptides were hydrolysed by treatment for 48 hr at 110° with constant boiling HCl in sealed, evacuated tubes. Water and HCl were removed by repeated addition of water and evaporation to dryness *in vacuo* at 45°. Analyses were performed automatically with a Spinco Model 120B analyzer.

TABLE 2. OPERATING CONDITIONS FOR α-CHLOROPROPIONYLPHENYLALANINE METHYL ESTER RESOLUTION

	XE-60	50-HB-2000
Column temp	167°	162°
Injection temp	225°	225°
Detector temp	190°	190°
Inlet press	35 p.s.i.	20 p.s.i.
Outlet flow	1·2 ml/min	2·1 ml/min
Scavanger flow (R.T.)	100 ml/min	65 ml/min
Sample split	—	1/50

Gas chromatographic analysis. The sample of α-chloropropionylphenylalanine methyl ester was taken up in abs MeOH so as to give a concentration of 10^{-7} M/μl. Then volumes of 0·1 μl to a few μl could be injected to obtain the proper response according to the column and detection system in use.

TABLE 3. COLUMN PERFORMANCE FOR α-CHLOROPROPIONYLPHENYLALANINE METHYL ESTER DIASTEREOISOMER ANALYSIS

	XE-60	50-HB-2000
Diastereoisomer resolution	1·054	1·055
Total plates obtained	13,000	20,000
HETP[a]	3·5 mm	1·5 mm
Optimum sample size	1×10^{-7} moles	1×10^{-8} moles
Column lifetime	3 months	3 weeks
Analysis time	1½ hr	1 hr

[a] Column lengths given in experimental section.

In some cases dilution of the sample tenfold was required because of the even higher sensitivity available. Analyses were performed on either one or both of the following capillary columns: (a) 150′ × 0·01″ i.d., (⅛″ o.d.) 316 stainless steel tubing[16] coated with XE-60 (General Electric Co.), a polycyanosilicone grease, as liquid phase (b) 100′ of the same capillary tubing but using UCon 50-HB-2000 (Union Carbide Corp), a polyalkylene glycol, as liquid phase. Both liquid phases were applied to the columns as 10% solutions in acetone by the usual method.[17] Operating conditions and column performance are summarized in Tables 2 and 3. The detectors used were either the capillary version of the argon ionization detector with 100 mc of tritiated titanium foil as radiation source or the 1 cm parallel plate electron affinity device with 250 mc of tritium. Argon was the carrier gas in the latter mode as well.

L-α-*Chloropropionyl*-L-*phenylalanine methyl ester.* At −5° a mixed anhydride was prepared from 0·13 ml pivaloyl chloride and 0·10 ml L-α-chloropropionic acid in 1·8 ml pyridine. The α-halo-acid was prepared as described by Fu *et al.*[9] After 5 min 0·2 ml of the supernatant was added to a chilled solution of 4 mg L-phenylalanine in 0·2 ml pyridine–water–triethylamine (10:9:1). After 10 min in the cold and 2 hr at room temp the reaction mixture was evaporated *in vacuo* at 40° taken up in water, acidified to pH 2 with 6N HCl and partitioned between water and AcOEt. The organic phase was then washed two times with 1N HCl and 3 times with water, dried over $MgSO_4$, filtered and the filtrate evaporated to dryness *in vacuo* at 40°. On standing, crystallization could be observed but no fractionation was allowed and the entire product was taken up in MeOH and esterified with an ethereal solution of diazomethane. The reaction mixture was evaporated *in vacuo* at 40°, dissolved in AcOEt, extracted 3 times with sat $NaHCO_3$aq and water, dried over $MgSO_4$, filtered and evaporated to dryness. The product was then subjected to gas chromatography and showed 1·7% of diastereoisomer.

L-α-*Chloropropionyl*-D,L-*phenylalanine methyl ester.* This was prepared as above from D,L-phenylalanine. Gas chromatography showed 48·3% of D,L + L,D enantiomers.

Carbobenzoxyglycyl-L-*phenylalanine.* At 0° a mixed anhydride was prepared from 105 mg carbobenzoxy glycine and 0·07 ml isobutylchloroformate in the presence of 0·07 ml triethylamine in 5 ml tetrahydrofuran. After 5 min in the cold the mixed anhydride was added to a solution of 125 mg L-phenylalanine and 160 mg $NaHCO_3$ in 5 ml water. The mixture was stirred for ½ hr at 0° and overnight at room temp, evaporated *in vacuo* at 40°, taken up in 20 ml water and acidified to pH 2 with 6N HCl. The resultant precipitate was triturated with water until solid and then air dried. Molar ratio of an acid hydrolysate: $gly_{1.01}phe_{0.99}$; gas chromatographic analysis of chloropropionyl-phenylalanine methyl ester: 3·6% diastereoisomer.

Carbobenzoxy-γ-benzylglutamylphenylalanine. This compound was prepared as described above from 185 mg carbobenzoxy-γ-benzylglutamic acid. Molar ratio of an acid hydrolysate: $glu_{1.03}phe_{0.94}$; gas chromatographic analysis: 5·0% diastereoisomer.

[16] Especially prepared for gas chromatography by Posen & Kline Tube Co., Inc., Norristown, Pa.
[17] R. A. Landowne and S. R. Lipsky, *Biochim. Biophys. Acta* **46**, 1 (1961).

Bis-carbobenzoxylysylphenylalanine. This was prepared as above from 207 mg of bis-carbobenzoxylysine. Molar ratio of an acid hydrolysate: $lys_{0.96}phe_{1.03}$; gas chromatographic analysis: 4·0% diastereoisomer.

Carbobenozoxyglycylphenylalanylglycine methyl ester. To 16 mg carbobenzoxyglycylphenylalanine in 1 ml $CHCl_3$ was added 0·5 ml solution "A" prepared from 63 mg glycine methyl ester hydrochloride and 0·07 ml triethylamine in 5 ml $CHCl_3$. To the resultant mixture at room temp was added 0·5 ml solution "B" prepared from 52 mg dicylohexylcarbodiimide in 2·5 ml $CHCl_3$. The mixture was stirred overnight at room temp, evaporated in a stream of N_2 and the residue partitioned between AcOEt and 1N HCl. The suspension was filtered and the organic phase was washed twice with 1N HCl, 3 times with water, 3 times with sat $NaHCO_3$aq and finally 3 times with water. The organic phase was then dried over $MgSO_4$, filtered and the filtrate evaporated *in vacuo* at 40°. Molar ratio of an acid hydrolysate: $gly_{2.00}phe_{1.00}$; gas chromatographic analysis: 30·4% diastereoisomer.

Carbobenzoxy-γ-benzylglutamylphenylalanylglycine methyl ester. Prepared as above from 40 mg carbobenzoxy-γ-benzylglutamylphenylalanine, 0·5 ml solution "A" and 0·5 ml solution "B". Molar ratio of an acid hydrolysate: $glu_{1.07}phe_{1.00}gly_{0.93}$; gas chromatographic analysis: 28·4% diastereoisomer.

Bis-carbobenzoxylysylphenylalanylglycine methyl ester. Prepared as above from 73 mg of biscarbobenzoxylysylphenylalanine, 1·5 ml solution "A" and 1·5 ml solution "B". Molar ratio of an acid hydrolysate: $lys_{1.00}phe_{1.03}gly_{0.96}$; gas chromatographic analysis: 29·0% diastereoisomer.

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