BIOORGANIC CHEMISTRY 6, 239–247 (1977)

Racemization Studies in Peptide Synthesis Using High-Pressure Liquid Chromatography

MURRAY GOODMAN, PHILIP KEOGH, AND HARRY ANDERSON

Department of Chemistry, University of California, San Diego
La Jolla, California 92093

Received January 24, 1977

We have synthesized the dipeptides benzoyl-L-phenylalanyl-L-alanyl benzyl ester and benzoyl-L-phenylalanyl-L-alanyl methyl ester by both the solid-phase and solution coupling methods. A variety of coupling reagents and solvents was employed. Each coupling reaction was analyzed by high-pressure liquid chromatography for extent of racemization. Baseline separations were achieved which allowed the direct, rapid, and reproducible determination of dipeptide diastereomers. Our successes in the separation of dipeptide diastereomers indicate the future value of applying high-pressure liquid chromatography to the separation of larger peptide diastereomers.

INTRODUCTION

In modern peptide synthesis, it is essential to obtain optically pure compounds. Stepwise elongation of a peptide chain using urethane-protected amino acids is a method generally free from racemization (*1*), although certain amino acids containing electron-withdrawing functional groups in their side chains may require special precautions (*2, 3*). The coupling of protected peptide fragments presents greater difficulties, however, because the carboxyl terminal amino acids can be racemized more easily.

A convenient approach for the study of racemization involves the synthesis of specific model peptides to yield a product whose optical purity can be measured by some suitable technique. The ideal model system should include the following five requirements: (1) The coupling reactions should involve intermediates which racemize easily. (2) There should be no racemization in any step prior to or following the coupling reaction. (3) The method should involve the fewest possible manipulations following the coupling step to prepare the sample for the analysis of racemization; preferably the crude product should be analyzed directly. (4) The analysis should be sensitive enough to measure small amounts of racemate (0.5% or less). (5) Interference in the analytical procedure from side products of the coupling reaction or other impurities should be minimal.

To date, a system has not been designed which meets all of these requirements. The methods which have been used to study racemization include fractional recrystallization (*4, 5*), isotopic dilution (*6*), gas chromatography (*1, 7*), ion-exchange chromatography (*8, 9*), nuclear magnetic resonance (*10, 11*) and enzymatic digestion followed by ion-exchange chromatography (*12*). Recently there have been reports of the separation of amino acid *N*-d-10-camphorsulfonyl-*p*-nitrobenzyl esters on silica gel (*13*) and of the separation of amino acid enantiomers on optically active supports (*14*).

ISSN 0045-2068

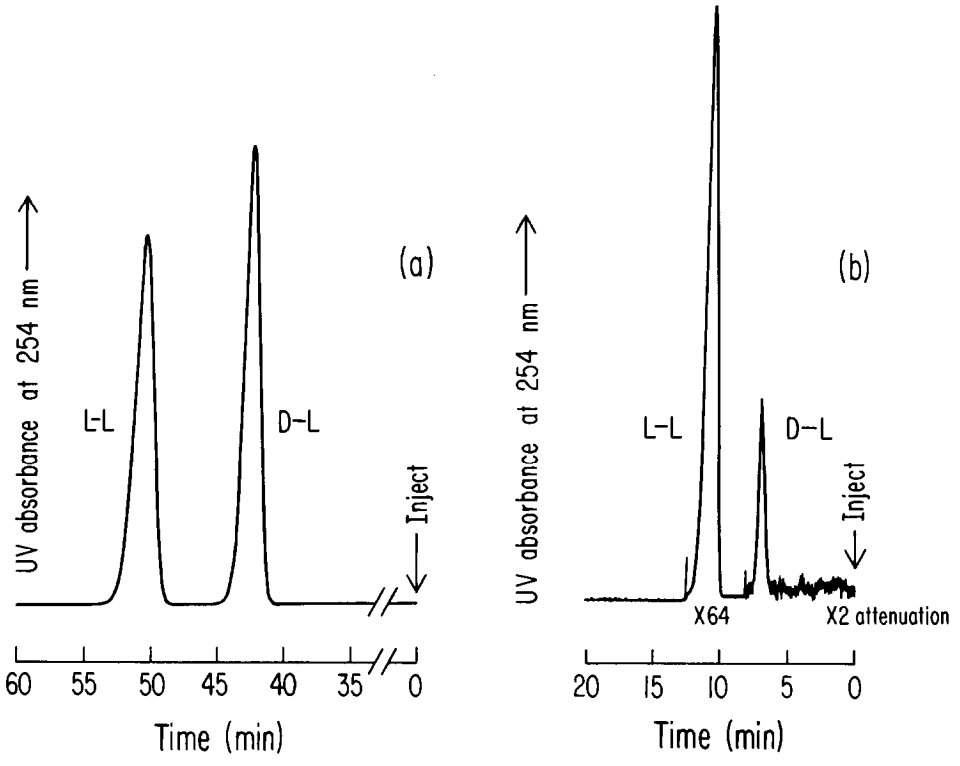


FIG. 1. High-pressure liquid chromatographic separation of diastereomers. (a) Benzoyl-DL-phenylalanyl–L-alanine methyl ester on a 60-cm Microporasil column. The solvent was chloroform that was 0.7% in 95% ethanol. A flow rate of 1 ml/min was employed and the pressure was 1300 psi. (b) Benzoyl phenylalanyl alanine benzyl ester using 99.5% of the L–L diastereomer and 0.5% of the D–L diastereomer on a 60-cm Microporasil column. The solvent was chloroform containing 0.6% ethanol. The flow rate was 4 ml/min, and the pressure was 3600 psi.

We have reported (*15*) some preliminary data on the use of high-pressure liquid chromatography to separate diastereomers of amino acid derivatives and dipeptides. We have improved this technique, broadened its scope, and used it to measure the extent of racemization during the synthesis of model peptides. We used a variety of coupling reagents and solvents in both solution and solid-phase techniques.

Our initial attempt (*15*) at separations of peptide diastereomers was carried out using a Waters Associate Model C 930 single-piston pump. Subsequent investigation revealed that chromatographic separations of diastereomers using chloroform as solvent were very sensitive to trace amounts of polar solvents. We were able to circumvent these polar solvent contamination problems by employing a Waters Model 6000A dual-piston pump with two 30-cm Microporasil columns. Using these columns, we could obtain baseline separations for the following phenylalanine peptides[1] using

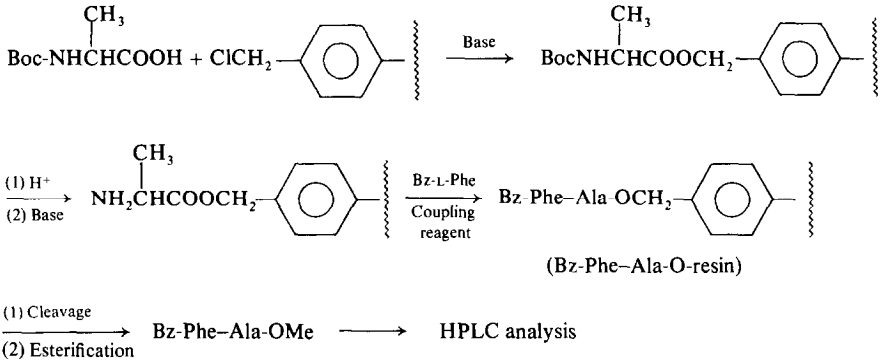
[1] Abbreviations used in this paper are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature. Bz = benzoyl; PEA = α-phenethylamine.

chloroform/ethanol as the solvent: Z-L-Phe–DL-Ala-OMe, Bz-DL-Phe–L-Ala-OBzl, Z-L-Phe–DL-Phe-OMe, Bz-DL-Phe–L-Ala-OMe, Z-L-Phe–DL-Ala-OBzl, Z-DL-Phe-*S*-2-PEA. The order of elution of the diastereomers was established by injecting the individual diastereomers and measuring their retention times. Over 200 separations of Bz-DL-Phe–L-Ala-OMe using a 0.7% solution of 95% ethanol in chloroform and of Bz-DL-Phe–L-Ala-OBzl using a 0.6% solution of 95% ethanol in chloroform were performed on 60-cm Microporasil columns.

The separations of the Bz-DL-Phe–L-Ala-methyl and benzyl esters are shown in Fig. 1. The peaks differ sufficiently in retention times so that 0.5% of Bz-D-Phe–L-Ala-OMe or Bz-D-Phe-L-Ala-OBzl (the D–L diastereomer) is clearly separated from 99.5% of Bz-L-Phe–L-Ala-OMe or Bz-L-Phe–L-Ala-OBzl (the L–L diastereometer). As little as 0.1% of the D–L diastereomer can be detected easily, providing a very sensitive means for the detection of racemization in coupling reactions. Each analysis is rapid, requiring at most 60 min per run. Our choice of Bz-L-Phe-OH as a model system was based on its proven susceptibility to oxazolinone formation during coupling, an established mechanism for racemization (*16*).

COUPLING REACTIONS ON SOLID-PHASE RESINS

As a model system for solid-phase fragment condensations, Bz-L-Phe-OH was coupled to alanine attached to a 1% cross-linked polystyrene resin. Any racemization which occurs produces Bz-D-Phe–L-Ala esters, which are diastereomers of the nonracemized products. The extent of racemization occurring during a given coupling can be measured by carrying out the following series of reactions, and analyzing the cleavage product by high-pressure liquid chromatography.



The Boc–Ala must be esterified to the resin without racemization and without the quaternization side reaction which is known to occur (*17*) when trialkyl amines are used as the bases. Quaternization causes a salt to be formed on the resin; these salts may increase the amount of racemization observed, by analogy with similar reactions in solution (*18*). We combined the reaction conditions used in the Loffet (*19*) and Monahan–Gilon (*20*) esterification procedures and showed this reaction to be free from both racemization and quaternization.

The lack of racemization or asymmetric selection in any of the steps other than the coupling reaction was demonstrated as follows: The compounds Bz-D-Phe–L-Ala and Boc-L-Ala were esterified to resins. The Boc group was removed from the latter with TFA and the resulting salt was neutralized with diisopropylethyl amine. The compound Bz-L-Phe was then coupled to the Ala-O-resin to give the L–L diastereomer using isobutyl chloroformate in THF under conditions which minimize racemization (*21*). Both peptides were cleaved from the resins using anhydrous hydrogen fluoride and esterified in the presence of excess diazomethane. The extent of cleavage was monitored by infrared spectroscopy of the resin residues before and after HF treatment; both showed greater than 90% removal of the peptides. The diazomethane esterification of both dipeptides was quantitative. Most importantly, the HPLC analyses indicated that less than 0.2% of the racemized compounds was present in the crude products of the D–L and L–L diastereomers. These results indicate that the racemization observed for the solid-phase couplings can be compared to the results obtained in solution, because racemization does not occur prior to or following the coupling reaction and asymmetric selection is not observed.

COUPLING REACTIONS IN SOLUTION

In a similar manner, the model condensations in solution were studied by coupling Bz-L-Phe-OH with L-Ala-OBzl; the benzyl ester was chosen for alanine protection since it results in a product which is similar to the peptide derivative attached to the solid phase. Again, as with solid-phase synthesis the diastereomeric dipeptide, Bz-D-Phe–L-Ala-OBzl, in the crude product was separated by HPLC. The couplings in solution used the free base of Ala-OBzl instead of its hydrochloride salt in order to avoid the salt effects on racemization (*18*).

The coupling reactions in solution were performed under two sets of conditions using either equimolar quantities of reactants or a fourfold excess of the activated amino acid component. The latter conditions are more analogous to those used in typical solid-phase synthesis. The procedures employed for the solid-phase couplings were followed, with the exception that Ala-OBzl was substituted for Ala-O-resin. The reactants were used at concentrations of 0.1 *M* for all components of the couplings which represent typical synthetic conditions. For analytical purposes, the solution systems proved more convenient than their solid-phase counterparts because the crude dipeptides could be analyzed directly by HPLC. The results of the couplings under both solid-phase and solution conditions are tabulated in Table 1. All reactions were carried out under the standard conditions for the methods noted. The results, therefore, provide a relative placement of each method from a standpoint of extent of racemization.

The only difficulties we encountered in the analysis of the solution couplings were with the azide procedure which contained substantial amounts of impurities when equimolar amounts of reactants were used. Similarly, many coupling reactions in which a fourfold excess of the activated amino acid was used also resulted in mixtures which were difficult to analyze because of the large excess of acylating reagent used. On the other hand, all solid-phase coupling reactions were easily purified since impurities could be removed simply by washing the resins before cleavage of the dipeptides. In all of our

TABLE 1

RACEMIZATION RESULTS FOR VARIOUS COUPLING REACTIONS

Reaction No.	Coupling reagent[a]	Solvent[a]	Percentage of Bz-D-Phe–L-Ala-OBzl[c]		
			Solid phase[b]	Equimolar solution	4× Excess Bz-Phe in solution
1	DCC	CH_2Cl_2	30 ± 2	50 ± 2[d]	5 ± 1[e]
2	DCC/NHS	CH_2Cl_2	0.5 ± 0.1	0.8 ± 0.1	NA[f]
3	DCC	DMF	40 ± 10	30 ± 2	NA
4	DCC/NHS	DMF	0.8 ± 0.1	1.0 ± 0.1	NA
5	MA	CH_2Cl_2	1.2 ± 0.8	25 ± 5	0.0
6	MA	DMF	37 ± 4	24 ± 9	3[g]
7	MA	THF	0.2 ± 0.1	0.8 ± 0.1	0.1
8	EEDQ	CH_2Cl_2	0.3 ± 0.1	0.3 ± 0.1	0.1[h]
9	EEDQ	DMF	19 ± 5	1.0 ± 0.1	1[h]
10	EEDQ	THF	0.8 ± 0.2	0.6 ± 0.1	NA
11	WRK	DMF	10 ± 2	0.3 ± 0.1	1 ± 0.1
12	Azide	EtOAc		<0.5[i]	

[a] Abbreviations for coupling reagents and solvents: DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; MA, mixed carbonic-carboxylic anhydride; EEDQ, *N*-ethyoxycarbonyl-2-ethoxydihydroquinoline; WRK, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's Reagent K); CH_2Cl_2, dichloromethane; DMF, dimethylformamide; THF, tetrahydrofuran; EtOAc, ethyl acetate; Bz, benzoyl; OBzl, benzyl ester; OMe, methyl ester.

[b] A fourfold excess of Bz-Phe-OH and coupling reagent was used.

[c] Percentage of D–L = D–L diastereomer/(D–L diastereomer + L–L diastereomer).

[d] The averages of two couplings. The magnitudes of the uncertainties represent the range of the two measurements, or the uncertainty of the measurement, whichever is larger.

[e] One reaction.

[f] Not analyzable.

[g] Represents an upper limit, due to impurities in the D–L region of the chromatographic trace.

[h] Estimate of percentage D–L, due to interfering impurities in the L–L region of the chromatographic trace.

[i] See text for explanation.

analyses there exists the unlikely possibility of detecting a uv-absorbing impurity with the same retention time as the D–L diastereomer. If this were suspected, then Bz-D-Phe could be coupled with L-Ala-OBzl. Racemization would produce Bz-L-Phe–L-Ala-OBzl. If this peak were as large as the peak for the D–L diastereomer from the Bz-L-Phe and L-Ala-OBzl coupling reaction, the peak could be considered to be due exclusively to racemization and not to an impurity. It is highly unlikely that there would be two different impurities of equal peak area having the same retention times as the D–L and L–L diastereomers.

Our results provide a direct comparison between solid-phase coupling reactions and the corresponding reactions carried out in solution. Obviously the nature of the solid support is important in determining the rates of reaction and possibly rates of racemization for the solid-phase couplings. At this stage of our work we have not carried out investigations on this aspect of peptide synthesis. Hancock and co-workers

have shown, however, that the characteristics of the polymerization and chloromethylation of the polystyrene influence the nature of the reaction sites on the resin (*22*). In the future we will examine the influence of such factors as accessibility of reaction sites, hydrophobicity of the polymer support, and structure of the growing peptide chain on racemization.

High-pressure liquid chromatography has thus proven its ability to provide direct, rapid, and reproducible detection of racemization in dipeptides in a variety of peptide coupling reactions. The reproducible baseline separations of the diastereomers employed in our study encourage us to extend our program to include the analysis and separation of larger synthetic peptides.

EXPERIMENTAL

Materials

Amino acids were purchased from Ajinomoto Company or from Fluka. Benzyloxycarbonyl-L and D-phenylalanine and t-butoxycarbonyl-L-alanine were obtained from Bachem Inc. The compound (−)2-phenethyl amine was purchased from Norse Laboratories. Chloromethylated polystyrene (1% cross-linked; 0.75 mmol of Cl/g) was purchased from Bio-Rad. Other more common reagents were purchased from Eastman, Aldrich, or Baker Chemical Companies. Solvents (Mallinckrodt) were analytical reagent grade and were purified by standard methods.

All diastereomeric standards used in this investigation were prepared and purified according to previously published procedures. Characterization of the diastereomers included infrared analysis, optical rotations, and NMR proton assignments which were carried out on the following instruments: Perkin Elmer Model 180 infrared spectrophotometer, Perkin Elmer Model 141 polarimeter, and Varian HR 220 nuclear magnetic resonance spectrometer.

Cleavages of the dipeptides synthesized on solid-phase support resins employed the HF methodology described by Robinson (*23*).

Coupling Reactions in Solution

All reactions were carried out under the standard conditions for the methods employed (as generally described below for the solid-phase coupling reactions) using 0.2 mmol of the analine benzyl ester component. Solution concentrations of reactants were generally 0.1 *M*. Two sets of conditions were utilized: In the first of these, equimolar quantities of the alanine benzyl ester and acylating agent, Bz-L-Phe-OX were used; and in the second, a fourfold excess of acylating agent was used. Solvents and coupling reagents used are summarized in Table 1. Reactions were generally worked up in the following manner: Precipitates (such as dicyclohexyl urea) were filtered and the filtrate was diluted with ethyl acetate. Any further precipitates were removed by filtration and the solution was then extracted with 1 *M* aqueous $NaHCO_3$ and with 1 *M* HCl. The organic layer was dried over $MgSO_4$ and evaporated to dryness before HPLC analysis.

Esterification of Boc-L-Alanine to Chloromethylated Polystyrene Resin (20)

A solution of t-butoxycarbonyl-L-alanine (4.26 g, 22.5 mmol) in 20 ml of methanol was treated with potassium hydroxide (13.74 g, 21.38 mmol) in methanol and the

solution was stirred for 15 min at room temperature. The solvent was removed *in vacuo*, yielding a crystalline salt which was dried overnight. The salt was dissolved in dimethylsulfoxide (100 ml) and chloromethylated polystyrene resin (1% cross-linking, 30.0 g, 22.5 mmol) suspended in the solution. This mixture was stirred for 45 min at 80°C, cooled to near-room temperature (10 min), and filtered. The resin was washed with methanol, 50% methanol/dichloromethane, dichloromethane, then dried overnight *in vacuo*. Duplicate Gisin (*24*) analyses of the product resin indicated 0.408 Meq and 0.420 Meq of t-butoxycarbonyl-L-alanine/g of resin. This resin was used for all of the solid-phase racemization tests. Typical solid-phase procedures were employed for all coupling reactions, which generally used 500-mg samples of deprotected L-Ala-O-resin (0.207 mmol of alanine).

Solid-phase dicyclohexylcarbodiimide, dicyclohexylcarbodiimide/N-hydroxysuccinimide, and N-ethoxycarbonyl-2-ethoxydihydroquinoline couplings. The substituted resin (500 mg) was added to a solution of benzoyl-L-phenylalanine (0.223 g, 0.828 mmol) and *N*-hydroxysuccinimide (0.095 g, 0.828 mmol) in 8.0 ml of the appropriate solvent. The coupling reagent (0.828 mmol) was added to the stirred mixture as a solid and and the coupling was allowed to proceed for 12 to 14 hr. The resin was filtered, washed twice with the reaction solvent, alternatively with dichloromethane and methanol (three times), then twice with dichloromethane, and finally dried *in vacuo*. Aliquots of resin (200 mg) were treated with HF and diazomethane according to standard procedures. The crude products of these reactions were used for the racemization analyses without further purification.

Mixed carbonic carboxylic anhydride couplings. For the coupling in dichloromethane, *N*-methylmorpholine (92 μl, 0.828 mmol) was added to a solution of benzoyl-L-phenylalanine (0.223 g, 0.828 mmol) in 8.0 ml of the reaction solvent before cooling to −15°C; for the tetrahydrofuran coupling, the *N*-methylmorpholine was added to the cold solution immediately before the addition of isobutylchloroformate in order to prevent the formation of a precipitate at −15°C. With dimethylformamide, the sequence of operations proved unimportant.

After the benzoyl-L-phenylalanine solution was cooled to −15°C, and the *N*-methylmorpholine was added, isobutylchloroformate (108 μl, 0.828 mmol) was introduced through a rubber septum stopper. The solution was stirred for 30 sec, and transferred under a stream of nitrogen to the dry resin which had been precooled to −15°C. The resin suspension was stirred at −15°C for 15 min, then at room temperature for 12–14 hr. HF and diazomethane treatments preceded analysis for racemization.

N-Ethyl-5-phenylisoxazolium-3'-sulfonate couplings. Triethylamine (116 μl, 0.828 mmol) was added to a solution of benzoyl-L-phenylalanine (0.234 g, 0.869 mmol) in 8.0 ml of dimethylformamide and the mixture was cooled to 0°C in an ice bath. The isoxazolium salt (0.209 g, 0.828 mmol) was added, and stirring was continued for 2 hr, after which time no precipitate remained. Dry resin (500 mg) was added and the mixture was stirred at 0°C for an additional hour, then at room temperature for an additional 12 hr before work-up.

High-Pressure Liquid Chromatography (HPLC)

Equipment. Our initial experiments were carried out with a single-piston Milton Roy Model C930 1000-psi pump, equipped with a Waters 254-nm uv detector and septum

injector and a Texas Instruments Servo Riter II dual-pen recorder. The columns used were 2-ft, 37 to 50-μm Corasil II, Porasil A, Corasil C-18, or neutral alumina columns from Waters Associates or a 50-cm 10-μm silica gel column (the Micropak) from Varian Associates).

Later experiments were carried out with the Waters Model 6000 dual-piston pump, capable of developing 6000 psi at a 9.9 ml/min flow rate in conjunction with a Model U6K loop injector. In these experiments a 60-cm column of 10-μm silica gel, the Microporasil, from Waters Associates was used.

Calibration of the separation of the Diastereomers of Benzoylphenylalanylalanine Benzyl Ester

Three samples were prepared containing different percentages of D–L diastereomers: 30, 10, and 0.5% D–L. These were prepared by mixing appropriate amounts of the L–L and D–L isomers. All weighings were performed on a Cahn gram electrobalance, Model G.

Chromatographic Analysis of Racemization from the Coupling Reactions

The diastereomers from the coupling reactions in solution, benzoly-L-phenylalanyl–L-alanine benzyl ester (L–L diastereomer) and benzoyl-D-phenylalanyl–L-alanine benzyl ester (D–L diastereomer) were separated on a 60-cm column of Porasil using a solvent mixture containing 0.6% ethanol (95%) in distilled chloroform at a flow rate of 2.0 ml/min. The reactions were analyzed by dissolving the product in 20 ml of 5% methanol/chloroform solution, and injecting 20 to 40 μl of this solution. Each measurement was performed at least twice.

The diastereomers from the solid-phase coupling reactions, benzoyl-L-phenylalanyl–L-alanine methyl ester (L–L diastereomer) and benzoyl-D-phenylalanyl–L-alanine methyl ester (D–L diastereomer), were similarly analyzed, using 0.7% ethanol (95%) in chloroform at a flow rate of 2.0 ml/min. The sample was dissolved in 4 ml of analytical reagent chloroform. Between 40 and 90 μl were injected for each analysis. Measurements were performed at least twice.

ACKNOWLEDGMENT

The authors gratefully acknowledge financial support for this work by Grant AM 15410 from the National Institutes of Health.

REFERENCES

1. F. WEYGAND, A. PROX, L. SCHMIDTHAMMER, AND W. KONIG, *Angew. Chem. Int. Ed. Engl.* **2**, 183 (1963).
2. G. C. WINDRIDGE AND E. C. JORGENSEN, *J. Amer. Chem. Soc.* **93**, 6318 (1971).
3. J. KOVACS, G. L. MAYERS, R. H. JOHNSON, R. E. COVER, AND U. R. GHATAK, *J. Org. Chem.* **35**, 1810 (1970).
4. G. W. ANDERSON AND F. M. CALLAHAN, *J. Amer. Chem. Soc.* **80**, 2902 (1958).
5. M. W. WILLIAMS AND G. T. YOUNG, *J. Chem. Soc.* 881 (1963).
6. D. S. KEMP, S. W. WANG, G. BUSBY, III, AND G. HUGEL, *J. Amer. Chem. Soc.* **92**, 1043 (1970).

7. S. LANDE AND R. A. LANDOWNE, *Tetrahedron* **22**, 3085 (1966).
8. M. BODANSZKY AND L. E. CONKLIN, *Chem. Commun.* 773 (1967).
9. N. IZUMIYA, M. MURAOKA, AND H. AOYAGI, *Bull. Chem. Soc. Japan* **44**, 3391 (1971).
10. B. HALPERN, L. F. CHEW, AND B. WEINSTEIN, *J. Amer. Chem. Soc.* **89**, 5051 (1967).
11. J. C. DAVIES, R. J. THOMAS, AND M. K. WILLIAMS, *Chem. Commun.* **76** (1975).
12. H. R. BOSSHARD, I. SCHECHTER, AND A. BERGER, *Helv. Chim. Acta* **56**, 717 (1973).
13. H. FURUKAWA, E. SAKAKIBARA, A. KAMEI, AND K. ITO, *Chem. Pharmacol. Bull.* **23**, 1625 (1975).
14. D. J. CRAM, G. DOTSEVI, AND Y. SOGAH, *J. Amer. Chem. Soc.* **97**, 1259 (1975).
15. M. GOODMAN AND P. KEOGH, "Separation of dipeptide diastereomers using high pressure liquid chromatography," Proceedings of the 13th European Peptide Symposium, p. 247, 1974.
16. M. GOODMAN AND L. LEVINE, *J. Amer. Chem. Soc.* **86**, 2918 (1964).
17. J. MEIENHOFER, "Hormonal Proteins and Peptides" (C. H. Li, Ed.), Vol. 2, p. 45. Wiley, New York, 1973.
18. M. W. WILLIAMS AND G. T. YOUNG, *J. Chem. Soc.* 3701 (1964).
19. A. LOFFET, *Int. J. Prot. Res.* **3**, 297 (1971).
20. M. W. MONAHAN AND C. GILON, *Biopolymers* **12**, 2513 (1973).
21. G. W. ANDERSON, J. E. ZIMMERMAN, AND F. M. CALLAHAN, *J. Amer. Chem. Soc.* **89**, 5012 (1967).
22. W. S. HANCOCK, D. J. PRESCOTT, P. R. VAGELOS, AND G. R. MARSHALL, *J. Org. Chem.* **38**, 774 (1973).
23. A. B. ROBINSON, "Solid Phase Peptide Synthesis" (J. M. Stewart and J. D. Young, Eds.), p. 41. W. H. Freeman, San Francisco, 1969.
24. B. F. GISIN, *Anal. Chim. Acta* **58**, 248 (1972).