I. INTERACTION BETWEEN ASYMMETRIC SOLUTES AND SOLVENTS: DIPEPTIDES AS SOLVENTS

II. THE SOLID PHASE SYNTHESIS OF AMIDATED PEPTIDES

A Dissertation

Presented to

the Faculty of the Department of Chemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Cheng C. Yang

May, 1971

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. Wolfgang Parr, for his interest, guidance and advice. The assistance of Mrs. J. Novotny in the gas chromatographic work is also appreciated.

This investigation was partially supported by a grant from the Robert A. Welch Foundation, Houston, Texas. (Grant number E-404)

I. INTERACTION BETWEEN ASYMMETRIC SOLUTES AND SOLVENTS: DIPEPTIDES AS SOLVENTS

II. THE SOLID PHASE SYNTHESIS OF AMIDATED PEPTIDES

An Abstract of A Dissertation

Presented to

the Faculty of the Department of Chemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in Chemistry

---

by

Cheng C. Yang

May, 1971

ABSTRACT

The interaction of a dipeptidic phase, N-trifluoroacetyl-L-phenylalanyl-L-leucine cyclohexyl ester (I), with solutes, consisting of N-trifluoroacetyl (N-TFA) and N-pentafluoropropionyl (N-PFP) esters of α-amino acids, has been studied at 100-130°C. The difference in behavior, as judged by the separation factors of enantiomers, between (I) and N-TFA-L-valyl-L-valine cyclohexyl ester (II), depends on the compound used for comparison. The following regularities were observed: (1) Resolution improves as the alkyl residue of the ester function of the solute increases from a primary to a secondary to a tertiary group. (2) The alkyl radical attached to the asymmetric carbon of the solute decreases resolution when substituted in the β-position (with respect to the carboxylic group), while the reverse is true of substitution of the γ-position. (3) Under the same conditions, N-PFP derivatives have about 25% lower retention volumes throughout than corresponding N-TFA esters. Because of the higher volatility of N-PFP derivatives, arginine, cysteine, lysine and tryptophan are resolved in either 100 ft or 400 ft columns. The advantage of the N-PFP derivatives is that they have shorter retention times without a significant loss in the resolution factors.

The influence of the perfluoroacyl,heptafluorobutyl and pentadecaoctanoyl derivatives of amino acid esters, other than trifluoroacetyl group, with respect to retention times and resolution factor has also been studied.

TABLE OF CONTENTS

CHAPTER TWO PAGE

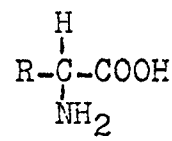
LIST OF TABLES

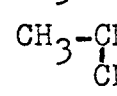
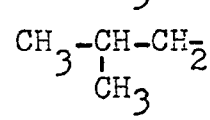
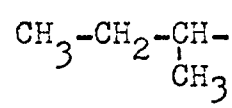
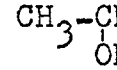
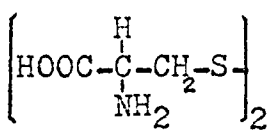
TABLE PAGE

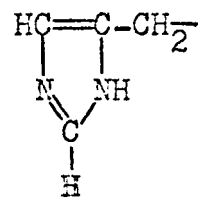
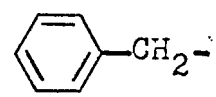
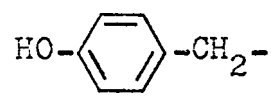
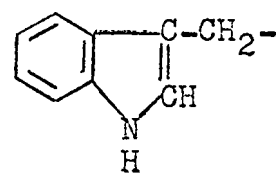
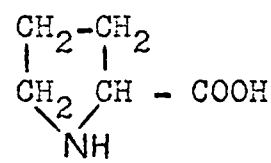
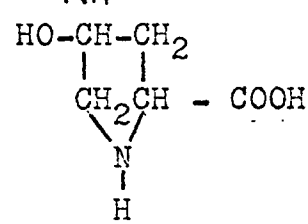
LIST OF FIGURES

ABBREVIATIONS

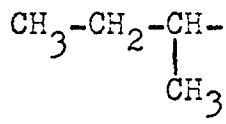
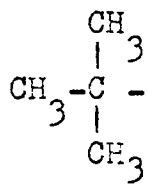
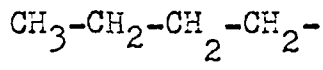
(A) Amino Acids — General Structure



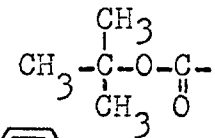
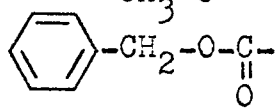
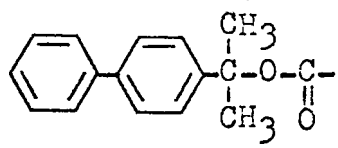
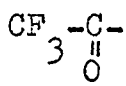
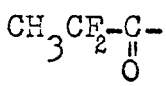
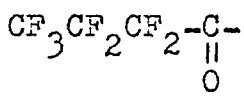
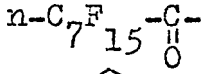
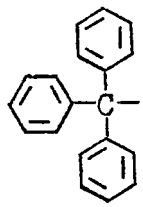
	Amino acids	Abbreviations	Structures of R
1.	Glycine	Gly	H-
2.	Alanine	Ala	CH_3-
3.	Valine	Val	
4.	Leucine	Leu	
5.	Isoleucine	Ileu	
6.	Serine	Ser	$HO-CH_2$-
7.	Threonine	Thr	
8.	Cysteine	Cys	$HS-CH_2$-
9.	Cystine*	$(Cys)_2$	
10.	Methionine	Met	$CH_3-S-CH_2-CH_2$-
11.	Glutamic acid	Glu	$HOOC-CH_2-CH_2$-
12.	Aspartic acid	Asp	$HOOC-CH_2$-

Amino acids	Abbreviations	Structures of R
13. Lysine	Lys	$H_2N-CH_2-CH_2-CH_2-CH_2-$
14. Hydroxylysine	Hylys	$H_2N-CH_2-\underset{\underset{OH}{|}}{CH}-CH_2-CH_2-$
15. Arginine	Arg	$H_2H-\underset{\underset{NH}{\|}}{C}-NH-(CH_2)_3-$
16. Histidine	His	
17. Phenylalanine	Phe	
18. Tyrosine	Tyr	
19. Tryptophan	Try	
20. Proline*	Pro	
21. Hydroxy proline*	Hypro	

* Molecular structures

Amino acids	Abbreviations	Structure of R
Allo-isoleucine	aIleu	
tertiary-Leucine	tLeu	
Norleucine	Nleu	

(B) Protecting Groups of Amino Acids

	Protecting group	Abbreviation	Structures
1.	tert.-Butyloxy-carbonyl	BOC	
2.	Benzyloxycarbonyl	CBO	
3.	2-(p-Diphenyl)-isopropyloxy-carbonyl	DPOC	
4.	Trifluoroacetyl	TFA	
5.	Pentafluoro-propionyl	PFP	
6.	Heptafluorobutyryl	HFB	
7.	Pentadecafluoro-octanoly	PDFO	
8.	Triphenylmethyl	Trit	

(C) Solvent and Reagents

	Solvent or reagent	Abbreviation	Structures
1.	N,N-dimethyl formamide	DMF	$(CH_3)_2-N-C(=O)-H$
2.	Dicyclohexylamine	DCHA	$C_6H_{11}-NH-C_6H_{11}$
3.	Dicyclohexyl-carbondiimide	DCC	$C_6H_{11}-N=C=N-C_6H_{11}$
4.	Polymer (Resin)		Chloromethylated copolystyrene-2% divinylbenzene

CHAPTER ONE

INTERACTION BETWEEN ASYMMETRIC SOLUTES AND SOLVENTS: DIPEPTIDES AS SOLVENTS

INTRODUCTION

Attempts have been undertaken during recent years to apply the method of gas chromatography to the resolution of racemates. The considerable advances made in the separation of various diastereomeric pairs by gas-chromatography (1, 2) on optically inactive phases have generated highly sensitive methods to be used for determining the degree of optical purity of optically active compounds. The method is based on chromatography of the diastereomers formed by coupling these compounds with some other asymmetric compounds. These techniques have proved to be far more accurate than traditional measurements of specific rotation.

A detailed investigation of the possibility of separating fifteen different racemates (carbohydrates, alcohols, alkyl halides, alkenes) by gas chromatography on a series of optically active phases (sucrose octapropionate, heptamethylsucrose, menthyl stearate, and poly-(propylene oxide)) was made by Goeckner (3). The author was unable to observe splitting of the chromatographic peaks, despite varying the temperature and the speed of the carrier gas.

Complete separation of the isomers of racemic esters of the N-trifluoroacetyl derivatives of valine, alanine,

and isoleucine was achieved for the first time in 1966 by Gil-Av (4). A capillary column of 75,000 theoretical plates efficiency coated with an optically active phase (dodecyl ester of N-trifluoroacetyl-L-isoleucine) was used. The degree of separation of the enantiomorphs increased with an increase in the size of the alcoholic part in the ester of the amino acid. However, this increase was accompanied by a corresponding increase in the retention time, which reached 300-400 min, with almost complete blurring of the chromatographic zone. Additionally, the high temperature of the experiment (210°C) casts doubt on the lasting configurational stability either of the optically active phase or of the compounds undergoing separation.

In general, amino acid enantiomers can be separated by gas chromatography if they are converted into suitable derivatives. Two different methods are utilized for this separation: 1) derivatization (esterification or acylation, respectively) of the enantiomeric amino acids with optically active reagents in order to form diastereomers and chromatography on an optically inactive stationary phase (5-10); 2) derivatization with an optically inactive reagent and chromatography on an optically active stationary phase.

The first approach, based on the separation of

diastereomers, causes some difficulties even if the stationary phases are readily available and can be operated at all temperatures necessary to achieve separation (10). The method requires high optical purity of the reagents used for derivatization, which are not always available. Optical impurities lead to more peaks where corrections are not always possible, especially when only small amount of isomers are to be detected. In the second approach (11-13), optically inactive reagents are used to prepare the derivatives. In this system the only correction to be made is for the racemization during the hydrolysis of peptides and proteins. The optically active stationary phases reported thus far to give the best results for separation of amino acid enantiomers are N-TFA-L-valyl-L-valine cyclohexyl ester (11, 12) and N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester (13).

N-TFA-L-valyl-L-valine cyclohexyl ester shows its best separation properties when operated at 110°. N-TFA-L-phenylalanyl-L-valine cyclohexyl ester, which has a higher molecular weight and lower vapor pressure, can be operated upto 140°C, suitable derivatives are TFA-amino acid methyl, isopropyl, and t-butyl ester.

The influence of the alcohol used for esterification has been investigated by Gil-Av et al. (12). It was found

that the resolution increased in the order, primary < secondary < t-butyl alcoholic groups. One of the limitations N-TFA-amino acid isopropyl ester is their low volatility. In particular, N-TFA derivatives of cysteine, lysine, arginine tryptophan, histidine and cystine have not yet been resolved by gas chromatography on optically active stationary phases. There are two principal ways of overcoming these difficulties. More temperature stable optically active stationary dipeptide phases have to be synthesized, or the volatility of the derivatives used for separation has to be increased.

In the present study, the second approach is used, since the method based on the synthesis and use of more temperature stable optically active stationary phases is not always successful. Parr et al. (14) synthesized N-TFA-L-phenylalanyl-L-phenylalanine cyclohexyl ester which, when coated on capillary columns, can be operated up to 170°C, but with very poor resolution.

Resolution of amino acid derivatives by gas chromatography provided a valuable analytical method in the field of protein and amino acid chemistry. The speed of analysis, requirement of limited microgram quantities, relatively low initial cost of equipment, and the clear resolution of

enantiomers are advantages of this technique over ion-exchange and other method. This technique has been used for detecting the racemization in solid phase peptide synthesis (15).

STATEMENT OF THE PROBLEM

In this study, the separation of enantiomeric amino acid derivatives will be carried out on two dipeptide phases, N-trifluoroacetyl-L-phenylalanyl-L-leucine cyclohexyl ester (I) and N-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester (II). The main objective of this work is to investigate the interaction between asymmetric solutes and solvents by the structural variations:

(1) Study the influence of bulkiness of alcoholic groups in order to improve the resolution.

(2) Study the influence of the alkyl at the asymmetric carbon by using leucine, norleucine, alloisoleucine, and t-leucine derivatives.

(3) Investigate the influence of trifluoroacetyl, pentafluoropropinyl, heptafluorobutyl, and pentadecaoctanoyl derivatives of amino acid esters with respect to retention times and resolution factor.

(4) Apply the different acylating reagent to attempt a separation of the enantiomers of arginine, lysine, histidine, cysteine and tryptophan.

EXPERIMENTAL

A) Gas Chromatography

A Varian 1200-1 gas chromatograph equipped with attachments for capillary columns and a flame ionization detector were used to carry out these experiments.

The stainless steel columns were cleaned with 200 ml portions of chloroform, acetone, water, nitric acid (conc.), water, ammonia (conc.), water, acetone, and chloroform. The dried columns were coated with a solution of 0.75 g N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester in 10 ml of ether. The columns were then conditioned at 120°C and 20 psi for 24 hours.

B) Synthesis of N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester

(1) L-leucine cyclohexyl ester hydrochloride

Five grams of L-leucine were suspended in 100 ml cyclohexanol and anhydrous HCl was bubbled through the mixture at room temperature until a clear solution was obtained. After 12 hours at room temperature, excess HCl was removed by bubbling dry N_2 through the solution. The

reaction mixture was transferred to a rotatory evaporator, and cyclohexanol was removed *in vacuo*. The solid residue was suspended in petroleum ether, filtered off, and recrystallized from methylene chloride and ether. Yield: 8.1 g, 95.3% M. P. 205-206 °C.

(2) N-t-BOC-L-phenylalanine

N-t-BOC-L-phenylalanine was prepared from t-butyl carbazate and L-phenylalanine according to the pH-stat method of Schnabel (16).

(3) N-t-BOC-L-phenylalanyl-L-leucine cyclohexyl ester

Ten millimoles(2.5 g) of L-leucine cyclohexyl ester hydrochloride and 2.9 g (11 mmol) N-t-BOC-L-phenylalanine were dissolved in 100 ml methylene chloride. The solution was titrated to pH 7 with triethylamine. After cooling with ice water, 2.25 g dicyclohexylcarbodiimide (11 mmol) dissolved in 50 ml methylene chloride were added, and the mixture was stirred for 30 minutes at 0 °C. After 24 hours at room temperature, the precipitate was filtered off. 1 ml glacial acetic acid was added to the filtrate, and the solution was stirred for an

additional 30 minutes. After filtration, the organic solvents were evaporated under reduced pressure and an oil was obtained, which was not further purified.

(4) L-phenylalanyl-L-leucine cyclohexyl ester

The above oil (3) was treated with 50 ml trifluoroacetic acid for 20 minutes at room temperature to remove the BOC group. After evaporation of the trifluoroacetic acid *in vacuo*, a crystalline residue was obtained. The precipitate was filtered off, washed with ether, and the dried sample showed a melting point of 191°C.

(5) N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester

Product (4) was suspended in 40 ml methylene chloride, cooled in an acetone-dry ice bath and 15 ml trifluoroacetic anhydride were added. After 1 hour at room temperature the solution was evaporated *in vacuo*, and a colorless oil was obtained, which could be crystallized from ether and pentane. Yield: 2.5 g, 55%, M.P. 110°C.

C) Esterification and acylation of amino acids

(1) Esterification of the amino acids with methanol.

Ten milliliters of anhydrous methanol containing 1.25 meq/ml of dry HCl gas were added to 10 mg of amino acid in a heavy-wall 12 ml screw-top Pyrex tube. The tube was capped tightly with a teflon-lined cap and placed in an oven at 100°C for 1 hour. Methanol and HCl were removed with the aid of a rotatory evaporator and a water-bath set at 40°C.

(2) Esterification of the amino acids with isopropanol

Five milligrams of racemic amino acid or amino acids mixture was esterified with 20 ml isopropanol, 3 N with respect to hydrogen chloride, in a teflon-lined, screw-cap Pyrex tube for 1 hr at 100°C. This procedure caused racemization to a certain extent. When dealing with protein hydrolysates, esterification with 1.25N HCl in isopropanol is recommended (13). Excess isopropanol and hydrochloric acid were removed in vacuo as described above.

(3) Esterification of the amino acids with isobutene

Three hundred mg of a mixture of the four amino acids studied was dissolved in 10 ml of absolute dioxane, placed in a 500 ml Parr hydrogenation pressure bottle and 0.7 ml of conc. sulfuric acid was added. Ten milliliters of liquid isobutene at -70° was then introduced and the bottle rapidly assembled in a hydrogenation apparatus. After shaking for 5 hr at room temperature, the pressure was released and the reaction mixture immediately poured into an ice-cold mixture of 50 ml of ethyl ether and 40 ml of 1 N NaOH. The aqueous layer was then extracted three times with ether, the combined ether extracts dried over Na_2SO_4 and the solvent evaporated to dryness <u>in vacuo</u>.

(4) Acylation of amino acid esters

Amino acid methyl esters or isopropyl esters (hydrochloride salt) were suspended in 20 ml of methylene chloride in a teflon-lined screw-cap Pyrex tube; the tube was cooled down to -20°C and 4 ml of trifluoroacetic anhydride (Eastman Kodak) and pentafluoropropionic anhydride (Pierce Chem. Co.), respectively, were added. The reaction mixture was then allowed to warm up to room

temperature. After 1 hr at room temperature, the solvent and the excess reagent were removed *in vacuo*. The residue was dissolved in 1 ml of chloroform. The acylation of isopropyl and t-butyl amino acid esters also followed this procedure.

For acylation of isopropyl esters of arginine, and lysine, the following procedure (17) was used: Four milliliters of methylene chloride and 1 ml of pentafluoropropionic anhydride were added to a test tube containing 10 mg of arginine isopropyl ester. The suspension was shaken for 15 min at room temperature and one ml aliquots of the solution were transferred to a heavy-wall 12 ml screw-top Pyrex tube. The tube was capped tightly with a teflon-lined cap and placed in a 150° C oil bath for 5 min. (This operation was done in a hood behind a safety shield) After acylation the tube was cooled by placing it in a beaker of water. Pentafluoropropionic anhydride and methylene chloride were removed *in vacuo*, and 1 ml spectroscopic grade chloroform was added. The sample was then ready for direct injection into the gas chromatograph

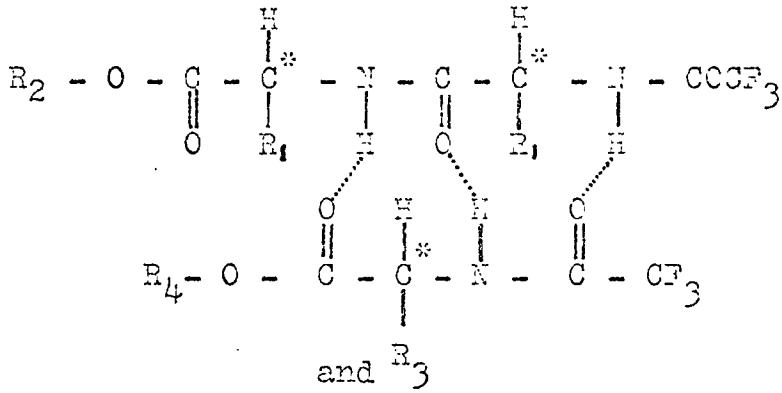
and analysis by GLC. The tri-PFP derivative of arginine rapidly decomposed even when stored in a refrigerator. It was used immediately after being made.

The peaks of the alloisoleucine, leucine and norleucine derivatives were identified by chromatographing mixtures enriched in one of the enantiomers. For t-leucine, however, only the racemate was avilable, and assignment was made by extrapolation.

RESULTS AND DISCUSSION

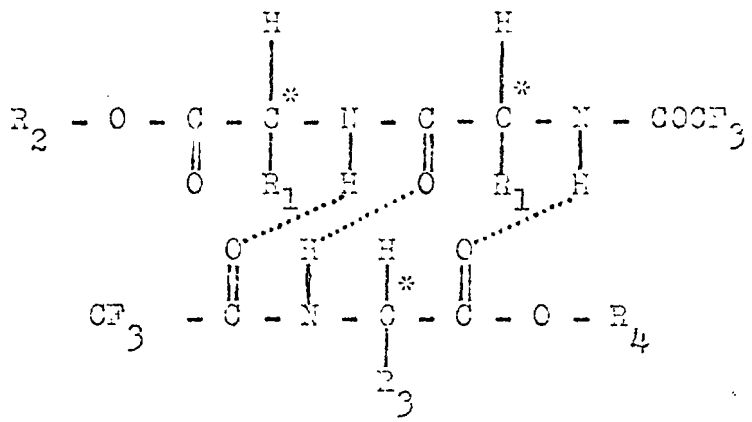
The application of dipeptide derivatives to the gas chromatographic enantiomeric analyses of α-amino acids using N-TFA-L-valyl-L-valine cyclohexyl ester (phase II) as the stationary phase, has been described by Nakaparksin and co-authors (12, 18). In an attempt to extend the method to the resolution of as wide a range of the common protein amino acids as possible, Koening, Parr and co-authors (13) have subsequently introduced the use of N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester (phase I). The present work is concerned mainly with the influence of structural factors on the interaction of solutes, consisting of N-acyl-amino acid ester, with the stationary phase (I) and (II).

The high efficiency of the dipeptide phases for the separation of enantiomeric solutes can be explained (19) by association between the enantiomeric solutes and the chiral solvent through a triply hydrogen bonded complex, such as:



(1)

and R_3



(2)

The possibility of formation of complexes (1) and (2) is corroborated by inspection of Courtauld Models, as in the parallel case of the N-TFA-L-valyl-L-valine cyclohexyl ester (II). Association of this type, occurring in addition to hydrogen bonding at two points, increases the requirement for a good steric fit of the molecules involved, and thus enhances the selective action of the solvent.

The experimental data, given in Tables I - XVIII and Fig. 1, show that the nature of the alcohol linked to the carboxylic acid group was found to have the same influence on the interaction with phase I, as with phase II (12, 19) and other similar solvents (20). Accordingly, the resolution factors increase as the radical of the alcohol changes in the order primary < secondary < tertiary, with the effect being particularly large for the bulky tertiary group. On the other hand, the influence of the substituent of the asymmetric carbon and of the acyl group attached to the

FIGURE 1

Plot of the logarithm of the resolution factor ($r_{L/D}$) versus the inverse of the absolute temperature for N-TFA (o) and N-PFP (o) esters of norleucine.

Figure 1

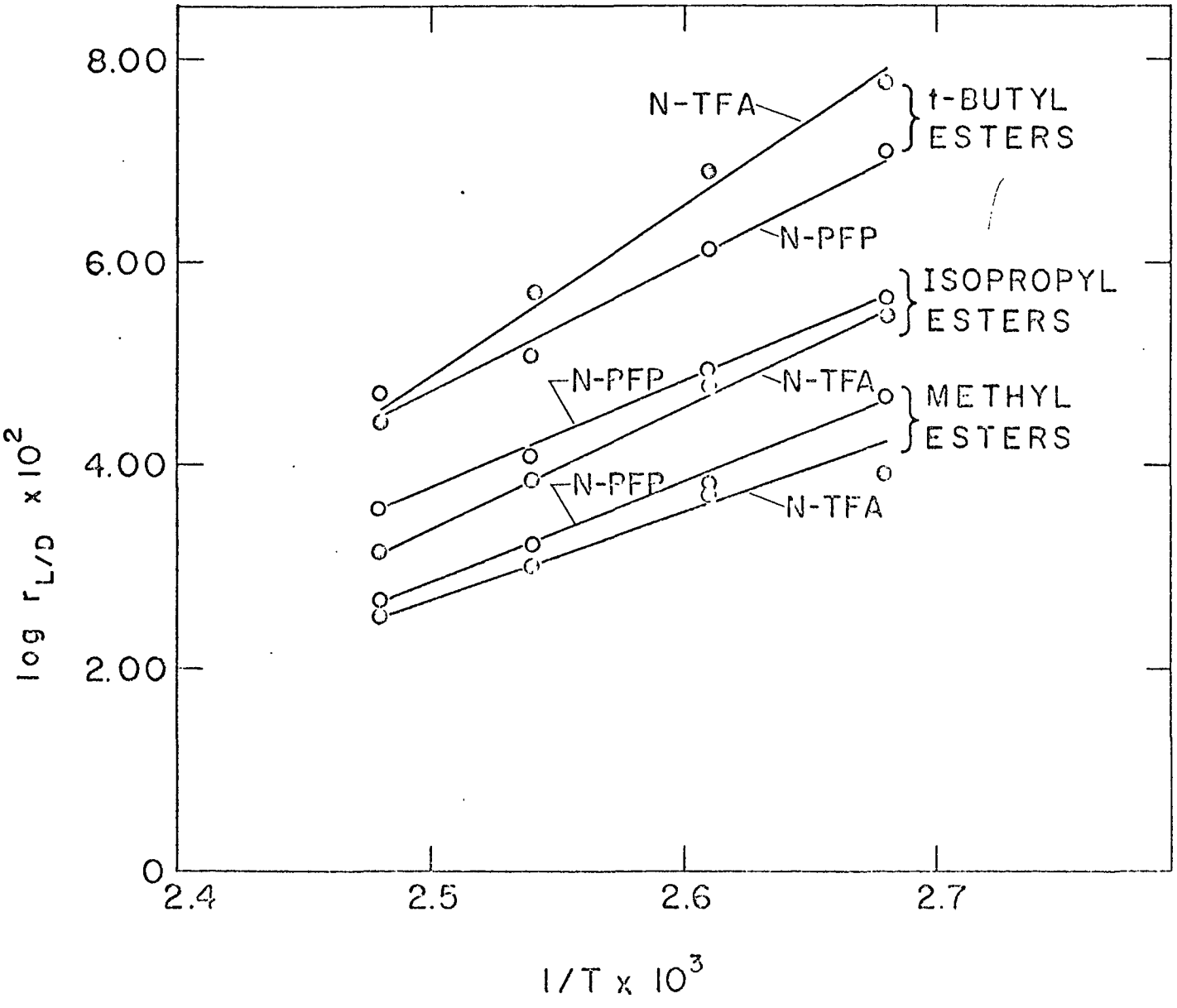

8.00
6.00
4.00
2.00
0
2.4
2.5
2.6
2.7
log $r_{L/D}$ x 10^2
1/T x 10^3
N-TFA
t-BUTYL ESTERS
N-PFP
ISOPROPYL ESTERS
N-PFP
N-TFA
METHYL ESTERS
N-PFP
N-TFA

TABLE I

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 100°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-methyl ester			N-PFP-O-methyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	5.30	0.338	1.028	3.90	0.248	1.038
L-tert.-leucine	5.45	0.347		4.05	0.258	
D-allo-isoleucine	8.45	0.538	1.083	6.15	0.392	1.081
L-allo-isoleucine	9.15	0.583		6.65	0.424	
D-leucine	11.85	0.755	1.110	8.75	0.557	1.120
L-leucine	13.15	0.838		9.80	0.624	
D-norleucine	14.35	0.914	1.094	10.60	0.675	1.113
L-norleucine	15.7	1.000		11.80	0.752	

RT : retention times
RRT : relative retention times
* : N-TFA-L-norleucine methyl ester was was taken as reference.
$r_{L/D}$: $(RRT)_L/(RRT)_D$

TABLE II

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 100°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-isopropyl ester			N-PFP-isopropyl ester		
	RT	RTT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	8.05	0.366	1.050	5.90	0.268	1.059
L-tert.-leucine	8.45	0.384		6.25	0.284	
D-allo-isoleucine	11.85	0.539	1.114	9.05	0.411	1.122
L-allo-isoleucine	13.20	0.600		10.15	0.461	
D-leucine	15.50	0.705	1.151	11.80	0.536	1.144
L-leucine	17.85	0.811		13.50	0.613	
D-norleucine	19.40	0.882	1.134	14.85	0.675	1.138
L-norleucine	22.00	1.000		16.90	0.768	

* N-TFA-L-norleucine isopropyl ester was chosen as reference.

TABLE III

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 100°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-tert.-butyl ester			N-PFP-O-tert.-butyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	7.8	0.356	1.096	5.70	0.260	1.087
L-tert.-leucine	8.55	0.390		6.20	0.283	
D-allo-isoleucine	11.6	0.530	1.168	8.845	0.386	1.142
L-allo-isoleucine	13.55	0.619		9.65	0.441	
D-leucine	14.35	0.655	1.192	10.45	0.477	1.201
L-leucine	17.1	0.781		12.55	0.573	
D-norleucine	18.3	0.836	1.196	13.30	0.607	1.177
L-norleucine	21.9	1.000		15.65	0.715	

* N-TFA-L-norleucine tert.-butyl ester was chosen as reference.

TABLE IV

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 110°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-methyl ester			N-PFP-O-methyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	3.75	0.361	1.000	2.810	0.269	1.000
L-tert.-leucine						
D-allo-isoleucine	5.65	0.543	1.078	4.15	0.399	1.073
L-allo-isoleucine	6.05	0.581		4.45	0.429	
D-leucine	7.85	0.755	1.102	5.90	0.567	1.102
L-leucine	8.65	0.831		6.50	0.625	
D-norleucine	9.55	0.918	1.089	7.15	0.688	1.091
L-norleucine	10.40	1.000		7.80	0.750	

* N-TFA-L-norleucine methyl ester was chosen as reference.

TABLE V

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 110°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-isopropyl ester			N-PFP-O-isopropyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	5.40	0.386	1.037	3.95	0.282	1.038
L-tert.-leucine	5.60	0.400		4.10	0.292	
D-allo-isoleucine	7.80	0.557	1.096	5.90	0.421	1.093
L-allo-isoleucine	8.55	0.611		6.45	0.460	
D-leucine	10.05	0.719	1.129	7.45	0.532	1.128
L-leucine	11.35	0.813		8.45	0.600	
D-norleucine	12.55	0.896	1.116	9.55	0.682	1.120
L-norleucine	14.0	1.000		10.70	0.764	

* N-TFA-L-norleucine isopropyl ester was chosen as reference.

TABLE VI

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 110°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-tert.-butyl ester			N-PFP-O-tert.-butyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	5.15	0.377	1.087	3.95	0.289	1.076
L-tert.-leucine	5.60	0.410		4.25	0.311	
D-allo-isoleucine	7.55	0.553	1.146	5.75	0.431	1.113
L-allo-isoleucine	8.65	0.637		6.40	0.469	
D-leucine	9.25	0.678	1.189	6.90	0.505	1.167
L-leucine	11.0	0.806		8.05	0.590	
D-norleucine	11.65	0.853	1.172	8.65	0.634	1.150
L-norleucine	13.65	1.000		9.95	0.729	

* N-TFA-L-norleucine tert.-butyl ester was chosen as reference.

TABLE VII

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 120°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-methyl Ester			N-PFP-O-methyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	2.15	0.318	1.000	1.95	0.288	1.000
L-tert.-leucine						
D-allo-isoleucine	3.90	0.577	1.056	2.8	0.415	1.071
L-allo-isoleucine	4.12	0.610		3.0	0.444	
D-leucine	5.20	0.770	1.077	3.80	0.563	1.092
L-leucine	5.60	0.830		4.15	0.615	
D-norleucine	6.30	0.933	1.071	4.55	0.674	1.077
L-norleucine	6.75	1.000		4.90	0.726	

* N-TFA-L-norleucine methyl ester was chosen as reference.

TABLE VIII

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 120°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-isopropyl ester			N-PFP-O-isopropyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	3.7	0.418	1.027	2.70	0.305	1.044
L-tert.-leucine	3.8	0.429		2.82	0.317	
D-allo-isoleucine	5.22	0.589	1.082	3.9	0.441	1.090
L-allo-isoleucine	5.65	0.638		4.25	0.480	
D-leucine	6.55	0.740	1.107	4.90	0.554	1.102
L-leucine	7.25	0.819		5.40	0.610	
D-norleucine	8.10	0.915	1.093	6.05	0.684	1.099
L-norleucine	8.85	1.000		6.65	0.751	

* N-TFA-L-norleucine isopropyl ester was chosen as reference.

TABLE IX

400' x 0.02" Stainless steel capillary column coat with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 120°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-tert.-butyl ester			N-PFP-O-tert.-butyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	3.7	0.411	1.081	2.15	0.239	1.070
L-tert.-leucine	4.0	0.444		2.30	0.256	
D-allo-isoleucine	5.3	0.588	1.123	3.8	0.422	1.092
L-allo-isoleucine	5.95	0.661		4.15	0.461	
D-leucine	6.35	0.706	1.150	4.5	0.500	1.122
L-leucine	7.3	0.811		5.05	0.561	
D-norleucine	7.9	0.878	1.140	5.65	0.628	1.115
L-norleucine	9.0	1.000		6.30	0.700	

* N-TFA-L-norleucine tert.-butyl ester was chosen as reference.

TABLE X

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 130°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-methyl ester			N-PFP-O-methyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	1.85	0.411	1.000	1.40	0.311	1.000
L-tert.-leucine						
D-allo-isoleucine	2.70	0.600	1.037	1.95	0.433	1.077
L-allo-isoleucine	2.80	0.622		2.10	0.461	
D-leucine	3.55	0.788	1.070	2.55	0.567	1.078
L-leucine	3.80	0.844		2.75	0.611	
D-norleucine	4.25	0.944	1.059	3.15	0.700	1.063
L-norleucine	4.50	1.000		3.35	0.744	

* N-TFA-L-norleucine methyl ester was chosen as reference.

TABLE XI

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 130°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-isopropyl ester			N-PFP-O-isopropyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	2.50	0.439	1.020	1.90	0.333	1.026
L-tert.-leucine	2.55	0.447		1.95	0.342	
D-allo-isoleucine	3.55	0.623	1.070	2.70	0.473	1.055
L-allo-isoleucine	3.80	0.666		2.85	0.500	
D-leucine	4.35	0.763	1.080	3.20	0.561	1.109
L-leucine	4.70	0.824		3.55	0.623	
D-norleucine	5.35	0.938	1.065	4.05	0.710	1.086
L-norleucine	5.70	1.000		4.40	0.772	

* N-TFA-L-norleucine isopropyl ester was chosen as reference.

TABLE XII

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester 130°C isothermal, carrier gas He 9 psi

Amino acid	N-TFA-O-tert.-butyl ester			N-PFP-O-tert.butyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	2.45	0.434	1.061	1.90	0.336	1.026
L-tert.-leucine	2.6	0.460		1.95	0.345	
D-allo-isoleucine	3.45	0.611	1.101	2.55	0.451	1.098
L-allo-isoleucine	3.80	0.673		2.80	0.496	
D-leucine	4.05	0.717	1.136	3.00	0.531	1.116
L-leucine	4.6	0.814		3.35	0.593	
D-norleucine	5.1	0.903	1.108	4.72	0.658	1.115
L-norleucine	5.65	1.000		4.15	0.734	

* N-TFA-L-norleucine tert.-butyl ester was chosen as reference.

TABLE XIII

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-valyl-L-valine cyclohexyl ester 100°C isothermal, carrier gas He 20 psi

Amino acid	N-TFA-O-methyl ester			N-PFP-O-methyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	4.90	0.338	1.000	3.80	0.262	1.000
L-tert.-leucine						
D-allo-isoleucine	7.75	0.534	1.071	6.00	0.441	1.091
L-allo-isoleucine	8.30	0.572		6.55	0.452	
D-leucine	11.60	0.800	1.077	9.30	0.641	1.075
L-leucine	12.50	0.862		10.0	0.690	
D-norleucine	13.5	0.931	1.074	10.7	0.738	1.084
L-norleucine	14.5	1.00		11.5	0.800	

* N-TFA-L-norleucine methyl ester was chosen as reference.

TABLE XIV

400' x 0.02". Stainless steel capillary column coated with N-TFA-L-valyl-L-valine cyclohexyl ester 100°C isothermal, carrier gas He 20 psi.

Amino acid	N-TFA-O-isopropyl ester			N-PFP-O-isopropyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	7.65	0.350	1.048	5.95	0.272	1.059
L-tert.-leucine	8.00	0.366		6.30	0.288	
D-allo-isoleucine	11.35	0.519	1.137	8.90	0.407	1.146
L-allo-isoleucine	12.90	0.590		10.20	0.467	
D-leucine	16.05	0.735	1.150	12.85	0.588	1.155
L-leucine	18.45	0.844		14.85	0.679	
D-norleucine	19.15	0.876	1.141	15.45	0.707	1.149
L-norleucine	21.85	1.00		17.75	0.812	

* N-TFA-L-norleucine isopropyl ester was chosen as reference.

TABLE XV

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-valyl-L-valine cyclohexyl ester 100°C isothermal, carrier gas He 20 psi

Amino acid	N-TFA-O-tert.-butyl ester			N-PFP-O-tert.-butyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	7.70	0.316	1.117	6.00	0.246	1.125
L-tert.-leucine	8.60	0.353		6.75	0.277	
D-allo-isoleucine	11.60	0.476	1.276	9.00	0.370	1.211
L-allo-isoleucine	14.80	0.608		10.90	0.448	
D-leucine	15.90	0.653	1.286	12.05	0.495	1.282
L-leucine	20.45	0.840		15.45	0.635	
D-norleucine	19.25	0.790	1.265	14.75	0.605	1.247
L-norleucine	24.35	1.000		18.40	0.756	

* N-TFA-L-norleucine tert.-butyl ester was chosen as reference.

TABLE XVI

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-valyl-L-valine cyclohexyl ester 110°C isothermal, carrier gas He 20 psi

Amino acid	N-TFA-methyl ester			N-PFP-O-methyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	3.20	0.362	1.000	2.45	0.277	1.000
L-tert.-leucine						
D-allo-isoleucine	4.90	0.554	1.051	3.80	0.429	1.079
L-allo-isoleucine	5.20	0.588		4.10	0.463	
D-leucine	7.15	0.808	1.070	5.65	0.638	1.071
L-leucine	7.65	0.864		6.05	0.683	
D-norleucine	8.30	0.938	1.066	6.55	0.740	1.069
N-norleucine	8.85	1.000		7.00	0.791	

* N-TFA-L-norleucine methyl ester was chosen as reference.

TABLE XVII

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-valyl-L-valine cyclohexyl ester 110°C isothermal, carrier gas He 20 psi

Amino acid	N-TFA-O-isopropyl ester			N-PFP-O-isopropyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	5.20	0.377	1.038	3.75	0.272	1.053
L-tert.-leucine	5.40	0.391		3.95	0.286	
D-allo-isoleucine	7.70	0.558	1.104	5.55	0.402	1.117
L-allo-isoleucine	8.50	0.615		6.20	0.449	
D-leucine	10.3	0.746	1.126	7.70	0.558	1.136
L-leucine	11.60	0.841		8.75	0.634	
D-norleucine	12.30	0.888	1.121	9.20	0.666	1.131
L-norleucine	13.80	1.000		10.40	0.757	

* N-TFA-L-norleucine isopropyl ester was chosen as reference.

TABLE XVIII

400' x 0.02" Stainless steel capillary column coated with N-TFA-L-valyl-L-valine cyclohexyl ester 110°C isothermal, carrier gas He 20 psi

Amino acid	N-TFA-O-tert.-butyl ester			N-PFP-O-tert.-butyl ester		
	RT	RRT	$r_{L/D}$	RT*	RRT	$r_{L/D}$
D-tert.-leucine	5.05	0.348	1.099	3.95	0.272	1.063
L-tert.-leucine	5.55	0.383		4.20	0.290	
D-allo-isoleucine	7.35	0.570	1.197	5.70	0.393	1.184
L-allo-isoleucine	8.80	0.607		6.75	0.465	
D-leucine	9.55	0.658	1.257	7.45	0.514	1.235
L-leucine	12.00	0.828		9.20	0.635	
D-norleucine	11.65	0.803	1.245	9.20	0.635	1.190
L-norleucine	14.50	1.000		10.95	0.755	

* N-TFA-L-norleucine tert.-tutyl ester was chosen as reference.

nitrogen does not follow a simple pattern.

On phase II, which can be operated at a maximum temperature of about 110°C, eleven of the common protein amino acids have been resolved (12). Further extension of the method requires a higher operating temperature, which, in turn, means the need for a less volatile phase and relatively higher selectivity, because of the lowering of resolution with temperature. The choice of phenylalanine and leucine for synthesizing phase I can be justified by the data which were available on the interaction of these two amino acids with asymmetric solvents. Thus, on phase II, phenylalanine showed the highest resolution factor in most ester series examined (12), whereas leucine derivatives had intermediate values, which were still higher than those of valine esters. By assuming that the effects found for solutes should be equally operative for solvents of similar structure and, further, that too much bulkiness in a molecule leads to a depression of the efficiency of resolution (20), the combination of the above two amino acids in phase I seemed commendable.

These expectations were only partially fulfilled, as can be seen in Tables I-XVIII, in which data for the two phases are given. For the methyl esters, with the exception

of the N-PFP -alloisoleucine derivative, the resolution factors are indeed higher. The effect is particularly striking for the t-leucine derivatives, as they are not separated at all on phase II. On the other hand, the isopropyl derivatives show about the same or lower values on phase I, and for the t-butyl esters a considerable drop of the efficiency of separation is registered on the Phe-Leu phase. The highest difference was observed for N-TFA-alloisoleucine t-butyl ester, which at 100°C showed a depression of 0.108 on phase I. This behavior of the t-butyl esters corroborates other observations that too much hydrogen bonding and, thereby, decreases the molar fraction of the association complex in the stationary phase (20).

The influence of the alkyl group attached to the asymmetric carbon of the solute was studied on a series of N-acyl esters of α-amino acids, having six carbon atoms in their molecule. The substituent is primary in norleucine, secondary in allo-isoleucine, and tertiary in t-leucine. In addition, leucine, which has an isobutyl radical attached to the α-carbon, was also investigated.

Contrary to the observations made for the alcohol group, a tertiary substituent (in t-leucine) reduces the resolution factor throughout (Tables I-XVIII)as has also

been found previously, when using N-TFA-L-isoleucine lauryl ester as the solvent (20). No constant trend emerges when comparing allo-isoleucine with norleucine derivatives. In some cases, the secondary substituent leads to a large resolution factor as, for instance, for the N-TFA-t-butyl ester of allo-isoleucine ($r_{L/D}$ 1.276, compared with $r_{L/D}$ 1.265 for norleucine). In other cases the values are about the same, or the norleucine derivatives show higher resolution factors (see t-butyl esters, Tables I-XII). However, the derivatives of leucine, with the exception of the N-PFP-methyl esters, show the highest resolution throughout.

The effect of substitution of the alkyl attached to the α-carbon thus seems to be fairly complex and dependent on the position of branching. Apparently, at the β-carbon (β with respect to the carboxylic group) the effect of bulkiness on resolution is counter-balanced by a weakening of the hydrogen bonds involved in the formation of the association complex. Data obtained previously (12) support this view. Thus, it has been showed (22) that the derivatives of valine have a lower resolution factor than those of α-amino butyric acid; the same is true of allo-isoleucine, as compared with norvaline. On the other hand, the resolution factor increases for the derivatives of leucine

as compared with those of norvaline. In leucine, as in the isopropyl and t-butyl esters discussed above, branching occurs at a position two atoms removed from the group participating in hydrogen bonding (NH, respectively CO, see complexes (1) and (2). In both cases enhancement of resolution by increase of bulkiness is observed. It should be pointed out that allo-isoleucine, compared with norleucine, shows a change at both the β- and α-carbon, which might account for the complexities observed.

A change of the resolution factor on replacing the TFA by a PFP group does not seem to follow any definable pattern, and varies with the amino acid, the phase, and temperature. The effect is small for the methyl and isopropyl esters, but larger for the t-butyl derivatives. A maximum difference of 0.065 has been observed for esters of allo-isoleucine on phase (II) at 100°C. A marked effect is the reduction of the retention volume for all N-PFP derivatives by about 25%.

The influence of the temperature on resolution has been studied previously for the N-TFA-alanine t-butyl ester on phase II (19). A straight line was obtained when one plotted log r versus 1/T. The behavior of solutes on phase I has been investigated between 100°C and 130°C, and typical

results are shown in Figure 1 for the norleucine derivatives (see also Tables I-XVIII). If the experimental error is taken into account, the fit of the data with straight lines appears satisfactory.

In spite of the rapidly decreasing $r_{L/D}$ values at higher temperature (Table IX-XII), very satisfactory peak resolution has been obtained at 130°C, as can be seen in Figures 2 and 3, which show chromatograms of the amino acids studied. In addition, Koenig, Parr and coauthors (13) have reported good peak resolution for a series of amino acids at 140°C on phase I. Since the retention volume is greatly reduced at 130-140°C (it is approximately halved in an interval of 20°C) and bleeding is negligible, phase I is suitable for extending the scope of the method.

N-TFA-L-phenylalanyl-L-valine cyclohexyl ester has thus been found suitable for the purpose for which it was designed, though its selectivity, as measured by the $r_{L/D}$ values, is not much different from that of phase II. Systematic studies of solvents derived from various dipeptides should lead to a better understanding of the combined effect of the two α-substituents on selectivity. Possibly, such work might result in the finding of larger resolution factors at higher temperatures. It should be mentioned, that present experience

FIGURE 2

Chromatogram of N-TFA-α-amino acid isopropyl esters with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester as the stationary phase at 130°C. Chromatographic conditions: 400 ft x 0.02 in. stainless steel capillary; carrier gas He at 9 psi; injector 190°C; detector 280°C.

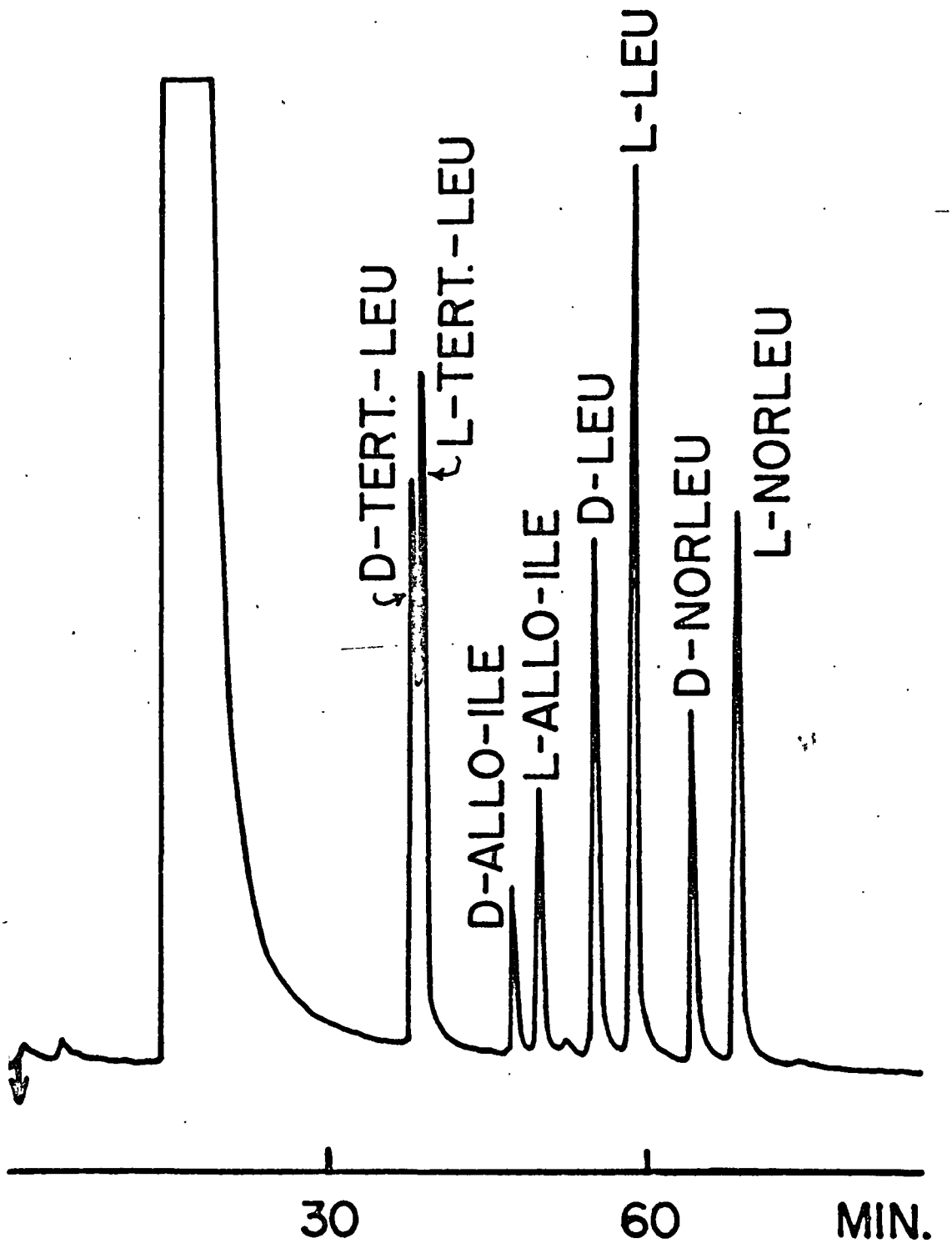
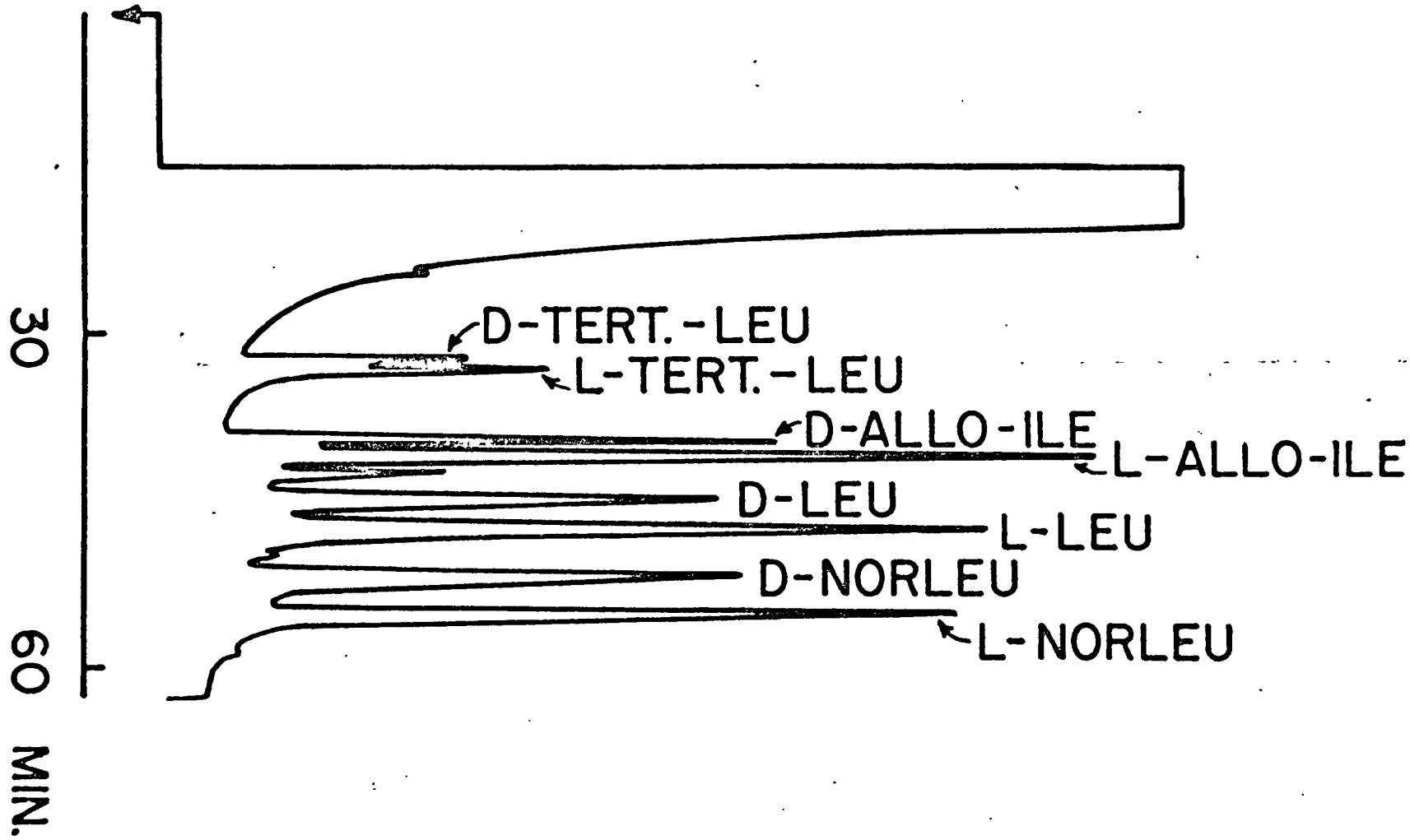

D-TERT.-LEU
L-TERT.-LEU
D-ALLO-ILE
L-ALLO-ILE
D-LEU
L-LEU
D-NORLEU
L-NORLEU
30
60
MIN.

FIGURE 3

Chromatogram of N-PFP-α-amino acid isopropyl esters with N-TFA-phenylalanyl-L-leucine cyclohexyl ester as the stationary phase at 130°C. Other chromatographic conditions as in Figure 2.


30
60
MIN.
D-TERT.-LEU
L-TERT.-LEU
D-ALLO-ILE
L-ALLO-ILE
D-LEU
L-LEU
D-NORLEU
L-NORLEU

indicates that data for a limited number of amino acids are sufficient to assess the general performance of a phase.

The present study also suggests that a proper choice of the derivatizing agent could further improve separation. It seems of interest to investigate systems in which the TFA group is replaced by other perfluoroacyl groups.

A striking result of replacing the N-TFA by a N-PFP group is the lowering of retention time. A more detailed study on the influence of the N-acyl group and of fluorine substitution on the retention time and resolution factors is given in Table XIX. The derivatives have been prepared by the reaction of heptafluorobutyric or pentadecafluoro-octanoic anhydride, respectively, in chloroform (ratios, 1:4) with the corresponding leucine ester in a sealed tube as described in the experimental part.

The table reveals that N-PFP and N-HFB leucine esters are, in general, more volatile than their corresponding N-TFA derivatives. They require about 20-27% less time, depending on the ester used. N-PFP-leucine isopropyl ester shows the highest reduction in retention time, 21.6%. N-HFB leucine esters are less volatile than N-PFP derivatives, but are still more volatile than N-TFA derivatives. However, N-PDFO derivatives require twice as much time as the

TABLE XIX

COMPARISON OF GAS CHROMATOGRAPHIC DATA OF DIFFERENT N-PERFLUOROACYL LEUCINE ESTERS[c]

Leucine ester	TFA[a]	$r_{L/D}$	PFP[a]	$r_{L/D}$	HFB[a]	$r_{L/D}$	PDFO[a]	$r_{L/D}$	Time reduction[b] (%) N-PFP	N-HFB
Methyl	D 6.10 L 6.60	1.082	4.45 4.85	1.090	4.90 5.35	1.091	12.40 13.65	1.100	26.9	19.3
Isopropyl	D 7.85 L 8.85	1.127	5.70 6.40	1.123	6.20 7.00	1.129	15.30 17.40	1.137	27.6	21.0
tert.-Butyl	D 7.40 L 8.70	1.176	5.40 6.30	1.167	6.00 6.85	1.142	15.35 17.15	1.117	27.1	19.5

a Corrected retention volumes.

b Reference compound is always the corresponding N-TFA-leucine ester, arbitrarily set at 100%.

c Column: 400 ft. x 0.02 in. I.D. stainless-steel capillary coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester, 110°C isothermal, carrier gas He, pressure 10 p.s.i.

corresponding N-TFA derivatives. The highest volatility is reached with the pentafluoroacyl derivatives.

Of greater significance is the fact that the reduction in retention time does not affect the resolution factors of the L - and D-enantiomers $r_{L/D}$. On the contrary, N-PFP-leucine isopropyl esters show the same or a slight increase in the resolution factor. Fig. 4 shows the separation of N-TFA, N-PFP, N-HFB and N-PDFO-L, L-leucine isopropyl esters, injected together on a 400 ft column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester. All isomers are well resolved with high efficiency in short time. These derivatives, especially the PFP amino acid isopropyl esters, have two advantages. Due to their high volatility, the PFP derivatives made the resolution of lower volatile amino acid enantiomers possible. All, but three of the common protein amino acids, have been resolved on N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester. A chromatogram showing the resolution of tyrosine, arginine, and lysine is given in Figure 5.

FIGURE 4

Gas chromatogram of N-perfluoroacyl leucine isopropyl esters. Instrument, Varian-Aerograph 1200-I. Column, 400 ft. x 0.02 in. I.D., stainless-steel capillary, coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester. Temperature 110°C isothermal, injector 100°C, detector FID 280°C. Carrier gas He, pressure 10 psi.

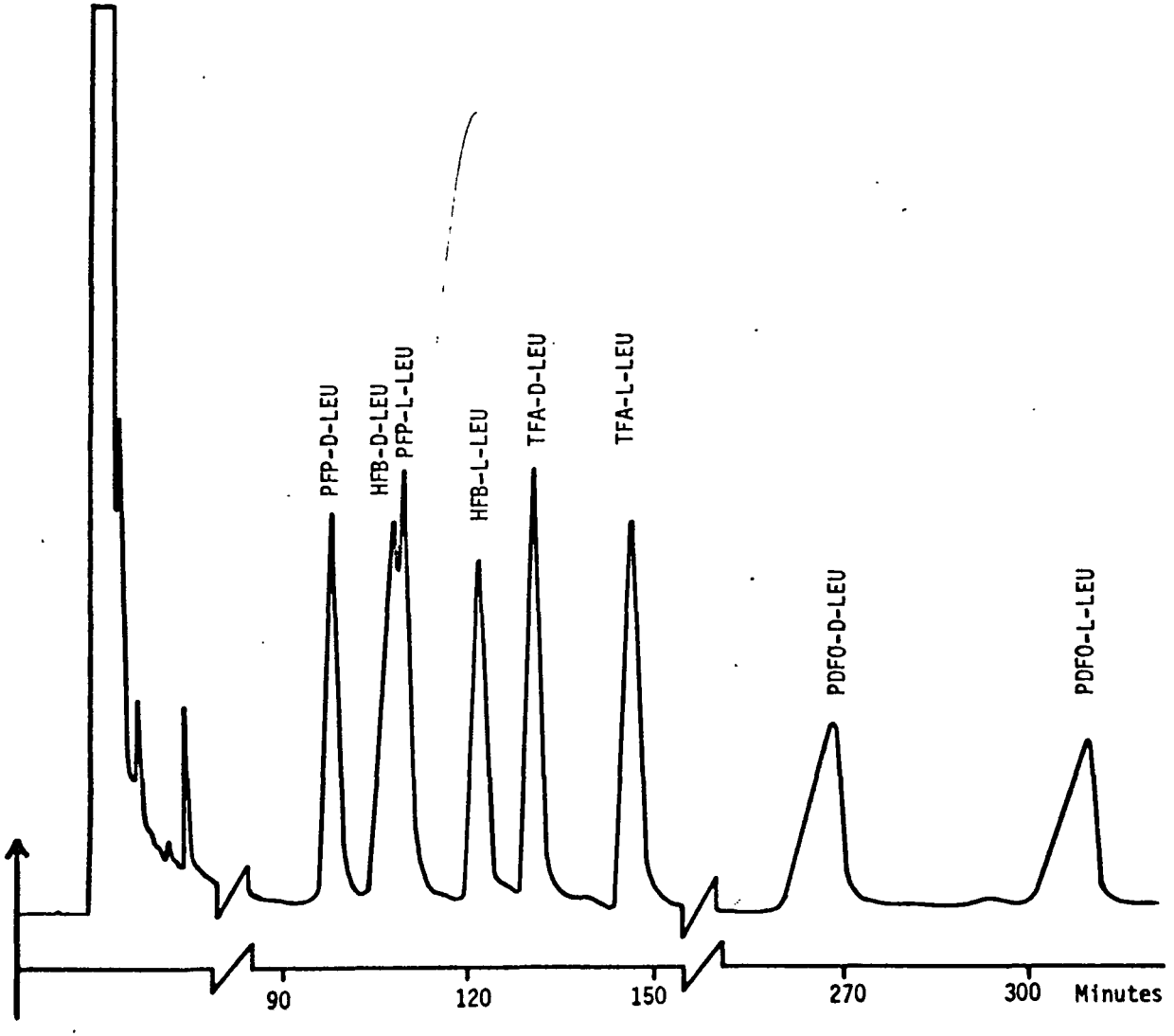
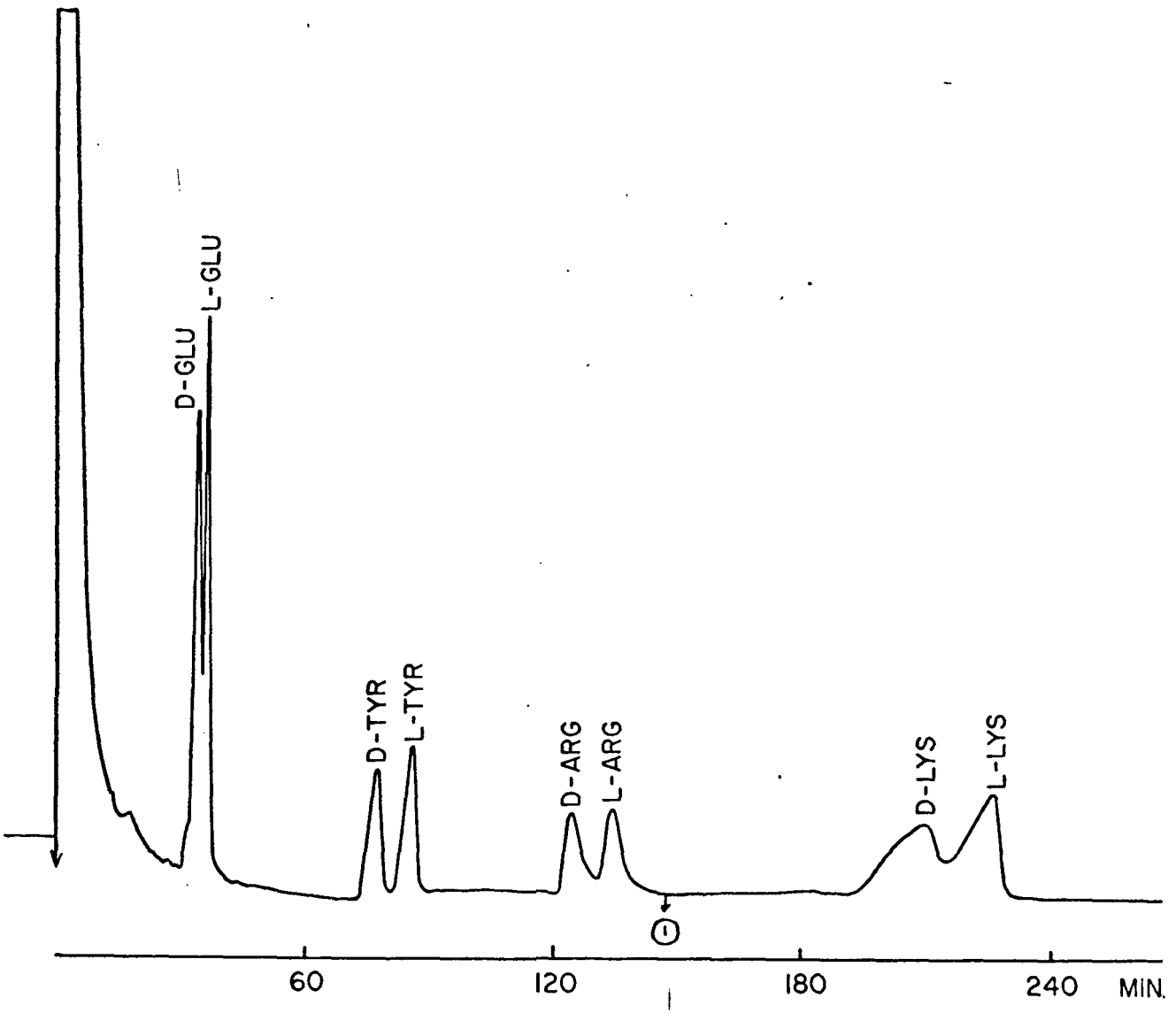

PFP-D-LEU
HFB-D-LEU
PFP-L-LEU
HFB-L-LEU
TFA-D-LEU
TFA-L-LEU
PDFO-D-LEU
PDFO-L-LEU
90
120
150
270
300
Minutes

FIGURE 5

Chromatogram of N-PFP-α-amino acid isopropyl esters with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester as the stationary phase. Chromatographic conditions: 125°C isothermal; injector 180°C; carrier gas He at 16 psi. Φ Manual pressure increase to 27.5 psi.


D-GLU
L-GLU
D-TYR
L-TYR
D-ARG
L-ARG
D-LYS
L-LYS
60
120
180
240
MIN.

CONCLUSION

The difference in behavior, as judged by the separation factors of enantiomers, between N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester (I) and N-TFA-L-valyl-L-valine cyclohexyl ester (II), depends on the compound used for comparison. The following regularities were observed: As for (II), resolution improves as the alkyl residue of the ester function of the solute increases from a primary to a secondary to a tertiary group. The alkyl radical attached to the asymmetric carbon of the solute decreases resolution, when substituted in the β-position (with respect to the carboxylic group), while the reverse is true of substitution in the γ-position. Under the same conditions, N-PFP derivatives have about 25% lower retention volumes throughout than corresponding N-TFA esters. So far, the TFA-amino acid isopropyl esters have been the most suitable derivatives for this purpose, allowing resolution of higher volatile amino acids. PFP-amino acid isopropyl esters yield separation of the lower volatile amino acids without a significant loss in the resolution factors. Because of this fact, it is now (21) possible to resolve seventeen pairs of the common protein amino acids, including arginine, lysine, typtophan, and cystein. Separation of histidine has been tried without success.

REFERENCES

1. Gil-Av, E., and D. Nurok, Proc. Chem. Soc., 146 (1962).

2. Gil-Av, E., and R. Charles-Sigler, G. Fischer, and D. Nurok, J. Gas Chromatog., 4, 51 (1966).

3. Goeckner, N. A., Dissertation Abs., 19, 3127 (1959).

4. Gil-Av, E., Feibusk, B., and R. Charles-Sigler, Tetrahedron Letters 1009 (1966).

5. Charles, R., Fischer, G., and Gil-Av, E., Israel J. Chem. 1, 234 (1963).

6. Gil-Av, E, Charles, R., and Fischer, G., J. Gas Chromatog. 17, 408 (1965)

7. Halpern, B., and Westley, J. W., Biochem. and Biophys. Res. Comm. 19, 361 (1965).

8. Pollock, G.E., Oyama, V. I., and Johnson, R. D., J. Gas Chromatog. 3, 174 (1965).

9. Halpern, B., and Westley, J. W., Chem. Comm. 12, 246 (1965).

10. Pollock, G. E., and Oyama, V. I., J. Gas Chromatog. 4, 126 (1966).

11. Gil-Av, E., and Feibush, B., Tetrahedron Letters, 3345 (1967).

12. Nakaparksin, S., Birrell, P., Oró, J., and Gil-Av, E., J. of Chrom. Sci. 8, 177 (1970).

13. Koenig, W., Parr, W., Lichtenstein, H., Bayer, E., and Oró, J., J. of Chrom. Sci., 8, 183 (1970).

14. Parr, W., Koenig, W., and Bayer, E., Southwest Regional ACS Meeting, Tulsa, Okla. (1969).

15. Bayer, E., E. Gil-Av, W. A. Koenig., S. Nakaparksin, J. Oró, and W. Parr, J. Am. Chem. Soc., 92, 1738 (1970).

16. Schnabel, E., Liebigs Ann. Chem, 702, 188 (1967).

17. Gehrke, C. W., and Stalling, D. L., Sep. Sci. 2(1), (1967).

18. Birrell, P., E. Gil-Av, S. Nakaparksin and J. Oró, Amer. Chem. Soc. Southwest Regional Meeting, Dallas, Dec. 4-6, 1968.

19. Feibush, B., and E. Gil-Av, Tetrahedron, 26, 1361, 1970.

20. Gil-Av, E., B. Feibush, and R. Charles-Sigler, "Chromatography 1966" L. B. Littlewood, Ed., Institute of Petroleum, London, 1967, 9277.

21. Parr, W., J. Pleterski, C. Yang and E. Bayer, J. Chrom. Sci, 9, 141, 1971.

22. Parr, W, C. Yang, E. Bayer and E. Gil-Av, J. Chrom. Sci. 8, 591 (1970).

CHAPTER TWO

THE SOLID PHASE SYNTHESIS OF AMIDATED PEPTIDES

ABSTRACT

The syntheses of some amino acid t-butyl esters were carried out. The stability of the α, β and γ-t-butyl ester and the conditions of cleavages of these t-butyl group were determined in several different basic and acidic media.

The considerably more acid-labile 2-(p-diphenyl)-isopropyloxycarbonyl (DPOC) group was used for protection of the amino group of amino acids. This group can be selectively removed in weak acid in presence of t-butyl ester groups. Some DPOC-derivatives of amino acids were prepared and their cleavage conditions studied.

Various methods were employed to synthesize the C-terminal amidated peptides. If the peptides did not contain aspartic or glutamic acids, a two-step reaction, methanolysis followed by ammonolysis, was a better way to prepare the amidated peptides. But if aspartic or glutamic acid was present in the peptide sequence, the direct ammonolysis with liquid ammonia in DMF was a better choice.

The syntheses of a C-terminal hexapeptide amide of secretin and two tetradecapeptides of scotophobin analogs were achieved in order to show that the method can be applied in synthesis of polypeptides.

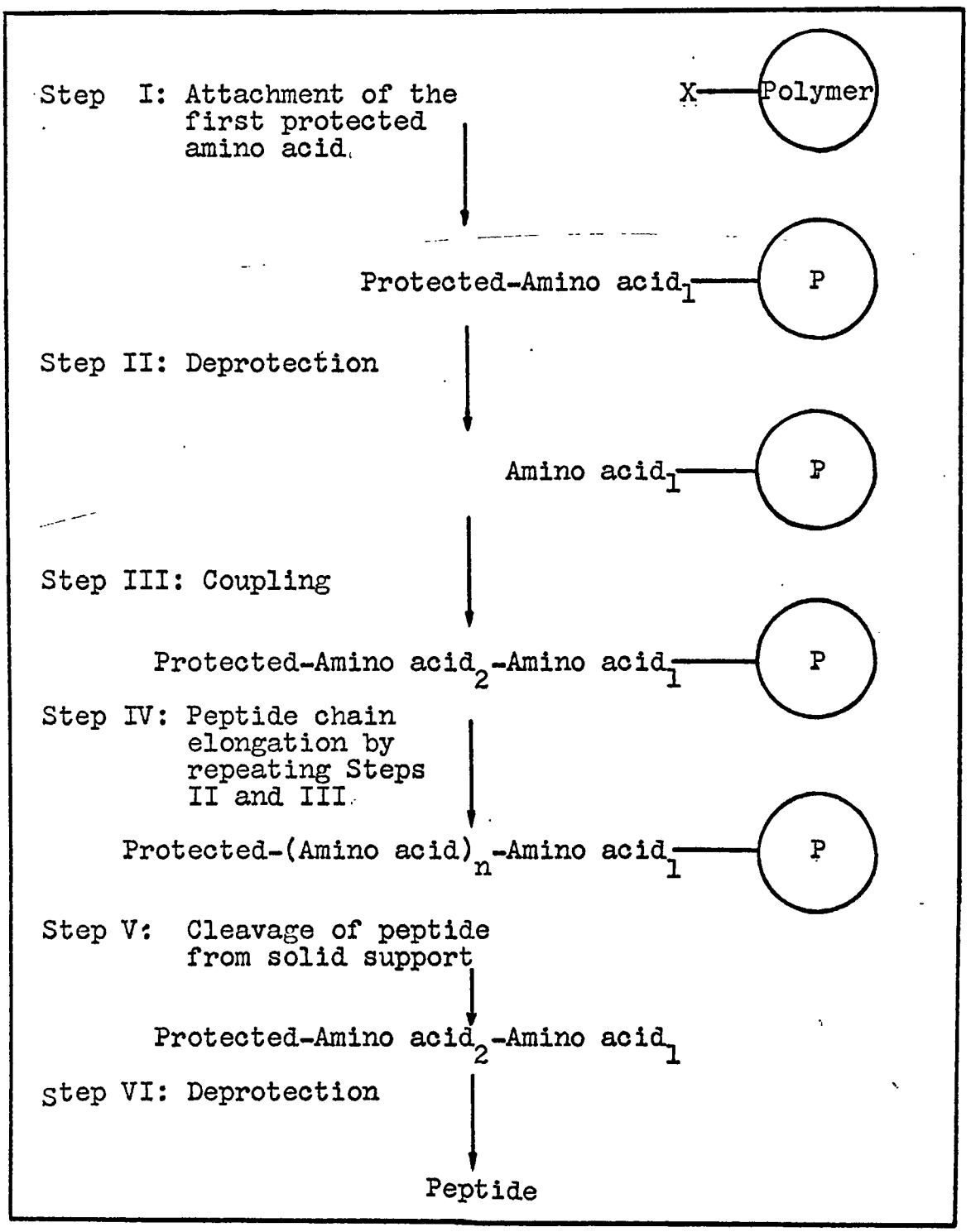
INDRODUCTION

Solid-phase peptide synthesis was based on the idea (1) that a peptide chain could be assembled in a stepwise manner while it was attached at one end to a solid support. With the growing chain covalently anchored to an insoluble particle at all stages of the synthesis, the peptide would also be completely insoluble, and, furthermore, it would be in a suitable physical form to permit rapid filtration and washing. Therefore, after each of the reactions was completed, the mixture could be filtered off and thoroughly washed to remove excess reactants and by-products. The intermediates in the synthesis were thus purified by a very simple and rapid procedure rather than by the usual tedious recrystallization methods. When a multi-step process, such as the preparation of a long polypeptide was attempted, the saving in time and effort and material could be very great.

In solid-phase peptide synthesis the first step was the attaching of a functional group (usually halide or hydroxyl groups) to a suitable solid particle through which the first amino acid of the proposed peptide chain was attached by a stable covalent bond. In principle, either end of the amino acid could be bound (see Fig. 6), while all its other reactive group were suitably protected. Selective

FIGURE 6

THE GENERAL SCHEME FOR SOLID-PHASE PEPTIDE SYNTHESIS



removal of the α-protecting group, followed by coupling with a second protected amino acid, lead to the first peptide bond. The deprotection and coupling reactions were repeated alternately until the desired sequence as outlined in figure 6 had been assembled, and finally, the bond which had been holding the peptide chain to the solid support was cleaved. The free peptide was then in solution and could be isolated and purified by suitable procedures. The fact that all of the steps just described were heterogeneous reactions between a soluble reagent in the liquid phase and the growing peptide chain in the insoluble, solid phase led to the introduction of the term solid-phase peptide synthesis to distinguish this synthetic approach from the classical one in which homogeneous reactions in the liquid phase were employed.

The solid phase method offered three main advantages: it simplified and accelerated the multistep synthesis because it was possible to carry out all the reactions in a single reaction vessel and thereby avoided the manipulations and attendant losses involved in the repeated transfer of materials; it also avoided the large losses which normally were encountered during the isolation and purification of intermediates; and most important it could lead to high yields of final products through the use of excess

reactants to force the individual reactions to completion.

The solid-phase peptide synthesis also had its disadvantages: during the synthesis the peptide can be washed repeatedly but could not be purified by recrystallization, except in the final step; in addition to this, variable shrinking or swelling properties of solid supports in different solvents are observed, and the reactions are diffusion controlled. These problems led to the formation of damaged, optically non-homogeneous, truncated and failure peptide sequence as by-products. The truncated and failure sequences were the result of incompleted coupling or delocking reactions.

The method and various modifications of it have been used for the synthesis of about 200 peptides (2). Among those the longest was synthesized by Merrifield (3) and co-workers (ribonuclease with 124 amino acids) and Li (4) and co-workers (human growth hormone with 188 amino acids). It was recognized (5, 6, 7) that the general solid-phase approach for peptide synthesis could be applied equally well in principle to the stepwise synthesis of other polymers containing repeating units such as polynucleotides or polysaccharides, where products of defined structure and known molecular size were important. Although it was

a very difficult problem, several studies on the preparation of polynucleotides have now been made (8, 9, 10, 11, 12).

The idea of using solid-bound, immobilized reactants found application in several other synthetic and degradative reactions. Although this method represented a new approach to polypeptide synthesis, it depended primarily on the chemistry of conventional peptide synthesis. The protecting groups and coupling reactions were those which had evolved over the past 70 years.

The principal change from the chemistry of classical synthesis was due to the introduction of the solid support, all the reactions associated with its preparation, and with the attachment and release of peptide chain. A second major difference was the severe restriction in the range of reactions that were possible. Because this approach dealed with every amino acid in essentially the same way during the continuous stepwise synthesis, marked limitations in the reaction conditions were imposed. Many of the special combinations of protecting groups that could be introduced and removed in the fragment synthesis of selected sequences could not be used in a general way in the presence of all other amino acids. The goal, therefore, was to find a

completely compatible set of protecting groups, coupling reactions and deprotection conditions.

In recent years naturally occurring peptides with high biological activities in such diverse fields as antibiotics, bacterial growth factors, hormones, smooth muscle stimulants and pain-producing substances have been isolated. Solid-phase peptide synthesis has been shown to be a convenient way for synthesizing those peptides. It has been found that t-butyl esters of amino acids and peptides have advantages over the customarily used methyl, ethyl or benzyl esters. Specifically, they were more stable as the free bases to self-condensation reactions and thus could usually be stored and used as such. This avoided the customary neutralization of the hydrogen halide salts with bases such as triethylamine during a peptide synthesis, a complicating procedure which added to the danger of racemization in sensitive case as well (16, 17).

One method for the synthesis of amino acid and peptide t-butyl esters was using the N-protected amino acids, isobutene saturated solvents (methylene chloride) and conc. sulfuric acid as a catalyst. In spite of the extra steps necessary it seemed likely that the utility of these t-butyl esters would make this worth while. t-Butyl ester

of peptides could be prepared in this manner also. The most convenient N-protecting group was carbobenzoxy (CBO group). CBO derivatives of amino acids could be prepared in very high yield by reacting carbobenzoxyl chloride with amino acids in basic medium. Carbobenzoxyl group could be removed by catalytical hydrogenation without affecting the t-butyl group.

The second method entailed the reaction of silver salt of acylamino acids or acylpeptides with t-butyl iodide. Although this was straightforward, the first method was simpler, gave better yields and was preferable for large-scale reactions.

Roeske (18) reported a procedure for converting free amino acids to their t-butyl esters by reaction with isobutene in a mixture of dioxane and conc. sulfuric acid. The yields reported at that time were around 45%. By using a more dilute reaction mixture, he was able to raise the yields to 60-75% in many cases. The yield depended on the solubility of the amino acid in dioxane-sulfuric acid. Diethylene glycol-sulfuric acid was also reported as a satisfactory solvent for the reaction, but the removal of diethylene glycol from the product was a difficult task.

The free tert.-butyl esters of most amino acids were stable and distillable liquids. This was another advantage of the tert.-butyl esters in addition to their possible acid hydrolysis. They were very stable to hydrazinolysis and aminolysis (19) and much more difficult to hydrolyze by alkali than the methyl or ethyl esters. The variations in the synthesis of peptides with aminodicarboxylic acids have thus been greatly extended. The synthesis of the tert.-butyl esters of arginine, N-substituted arginine, histidine, and tryptophan has not yet been reported. The esterification of the hydroxyl function also took place (20). This did not pose a problem since the tert.-butyl ethers could be cleaved just as readily as the esters.

The cleavage of the tert.-butyl esters could be carried out with hydrogen chloride in ethyl acetate (21) or methylene chloride (22), with toluenesulfonic acid in benzene (19), or with trifluoroacetic acid (23, 24). The latter reagent has been frequently used for the synthesis of sequences of biologically active peptides. One advantage was the simultaneous removal of the tert.-butyloxycarbonyl (BOC) group. The t-butyl ester could be cleaved with HBr in glacial acetic acid as long as the N-protecting groups present were stable to acid (e.g., the phthalyl group) (19).

t-Butyl ester was often used as a side-chain protection for carboxyl groups. If t-butyl was being used, t-butyloxy-carbonyl group could not be used for the α-amino group protection, since there was no way to cleave either of them without affecting the other. So it was important for peptide synthesis if there was some α-amino protecting group that could be selectively cleaved in presence of t-butyl protected side-chain carboxylic acid.

The 2-(p-diphenyl)-isopropyloxycarbonyl (DPOC) residue has been used (25) for the selective protection of α-amino groups in the synthesis of peptides containing additional acid-labile protecting residues. The acidolytic cleavage of the 2-(p-diphenyl)-isopropyloxycarbonyl group proceeded at much the same rate as the trityl residue and up to 6×10^4 times faster than the t-butyloxycarbonyl group (26). It was split off by dilute acetic acid and other weakly acidic reagents at rates which permitted a selective cleavage in the presence of other acid-labile protecting groups, especially those derived from t-butanol.

A number of peptide syntheses have been carried out with the new group either in the conventional manner or by the solid-phase method. No effect due to steric hindrance, as observed previously with the N-trityl residue, was

encountered. The application of the N-DPOC group to solid-phase peptide synthesis permitted the use of a new combination of protecting groups in which the side chains of trifunctional amino-acids were blocked by acid-labile residues that could be easily split in the final step of the synthesis.

STATEMENT OF THE PROBLEM

The most common reaction used to remove the finished peptide from the solid support was the nucleopilic displacement of the peptide by HBr in trifluoroacetic acid or liquid HF, yielding a peptide with a free C-terminus. However, this technique was not applicable when one wished to synthesize amidated peptide hormones, since in these cases the C-terminus was amidated. It was obvious to cleave the peptides by ammonolysis in methanolic ammonia (13). The general approach, however, was still fraught with difficulties. Only in the case of glycine as a C-terminal amino acid did the ammonolysis proceed smoothly (14). Difficulties were encountered when more bulky amino acids such as valine in secretin were present as C-terminal amino acids. More recently, Beyerman (15) succeeded in the oxytocin synthesis by removing the peptide as ester by methanolysis from the resin and subsequent ammonolysis.

The purposes of these investigations were the following:

(1) To search for a general method for ammonolysis which can be applied to all common protein amino acids.

(2) To study the stability of protecting groups and side chain carboxylic group protection under

those cleavage conditions.

(3) To synthesize model peptides in order to demonstrate the suitability of the technique.

EXPERIMENTAL

A) The syntheses of amino acid t-butyl esters*

(1) β-Benzyl L-aspartate

Ten milliliters sulphuric acid was added to 100 ml anhydrous ether followed by 100 ml benzyl alcohol. The ether was removed under vacuum, and 13.4 g finely ground L-aspartic acid was added in several portions while the mixture was magnetically stirred. The ensuing solution was left at room temperature for 24 hours, 200 ml 95% ethanol was added, followed by 50 ml pyridine, which was added dropwise while the solution was vigorously stirred. The mixture was cooled overnight; the deposit was then filtered off and washed by trituration with ether. Recrystallization from water containing a few drops of pyridine afforded pure β-benzyl L-aspartate. Yield: 8.9-9.9 g, 40-45% M.P. 218-220 °C.

(2) β-Benzyl α-t-butyl L-aspartate hydrochloride.

Twenty-five milliliters of liquid isobutene was added to a solution of 3.0 g (0.013 mole) of β-benzyl L-aspartate in a mixture of 25 ml of

*All the amino acids used were in L-form, if not specified.

dioxane and 2.5 ml of conc. sulfuric acid in a 500 ml pressure bottle, and the mixture was mechanically shaken at room temperature for 4 hr. The solution was poured immediately into a cold mixture of 200 ml of 1 N sodium hydroxide, and the aqueous phase was washed three times with ether. The combined layers were dried over sodium sulfate and evaporated under vacuum to about 5 ml. After addition of 25 ml of ether, dry hydrogen chloride wash bubbled through the solution and crystalline hydrochloride. After recrystallization from ethyl acetate. The yield was 3.0 g, 73% M.P. 115-117°C.

(3) α-Butyl L-tyrosinate

Three grams of L-tyrosine was dissolved in a mixture of 25 ml of dioxane and 6.0 g of p-toluenesulfonic acid monohydrate or 2.5 ml of conc. sulfuric acid. Twenty-five milliliters of liquid isobutene was added slowly, and the reaction mixture was shaken for 20 hr. The solution was added to a cold mixture of 100 ml of ethyl acetate; 100 ml of water and 5 ml of 5 N sodium hydroxide were added and, after adjusting pH to 9.1, the product was extracted twice with ethyl acetate.

Evaporation of the sovent left a crystalline product. Yield: 1.8 g, 45% M.P. 140-143°C. The ester was recrystallized for analysis from ethyl acetate-petroleum ether, M.P. 143-145°C.

(4) α-t-Butyl L-aspartate

A suspension of 5.93 g (0.0188 mole) of β-benzyl α-t-butyl L-aspartate hydrochloride in 200 ml of ether was treated with 20 ml of 25% potassium carbonate solution; the liberated ester was immediately extracted into the ether, and the aqueous solution washed again with 50 ml of ether. The ether was dried over sodium sulfate and evaporated in vacuo. The oily residue was dissolved in a mixture of 125 ml of 95% ethanol and 75 ml of water; 0.2 g of 5% palladium on charcoal was added, and the solution was shaken under 3 atm of hydrogen for 4 hr. The catalyst was removed by filtration, and the solution was evaporated in vacuo to 50 ml. When 400 ml of acetone was added, a gel formed which changed to a crystalline precipitate when the mixture was stirred. The yield was 2.69 g, 76% M.P. 178-179°C dec.

The other amino acid t-butyl ester hydrochloride salts had been synthesized by the method described above. Their m.p.'s (uncorrected) and yields were leucine, 167-169°C, 70%; valine, 141-143°C, 65%; isoleucine, 159-160°C, 70%; t-leucine, 166-167, 68%; alanine, 240-241°C, 55%; phenylalanine, 243°C, dec., 45%.

B) The syntheses of 2-(p-diphenyl)-isopropyloxycarbonyl (DPOC) derivatives of amino acids

(1) Dimethyl-p-xenylcarbinol

In a 3 liter three-necked flask equipped with reflux condenser, magnetic stirrer, and a pressure-equalizing separatory funnel were placed 48 g (2 g-atom) of Mg-turnings, and a small crystal of I_2. The flask was heated in an electric mantle until the I_2 vaporized; 500 ml of anhydrous ether was added, and 28 g of methyl iodide in 50 ml of anhydrous ether was then added through the separatory funnel. The reaction started spontaneously, and the remainder of methyl iodide was added at a rate such that the solution boiled gently under reflux.

General, the addition was completed at the end of 1-2 hr and by then all Mg should be dissolved. (if some of Mg was not dissolved, more methyl iodide could be added and warming up was required in order to restart the reaction.)

Three hundred and seventy one grams (1.84 mole) of p-acetylbiphenyl, dissolved in 1.5 l of benzene, was added during the course of 1 hr After standing overnight the mixture was placed on a steam bath and boiled under reflux for 2 hr. The reaction mixture was cooled in an ice-salt bath and then poured slowly, under constant stirring into a mixture 200 g of cracked ice and 8 ml of conc. sulfuric acid. The mixture was stirred at intervals until all the solid which separated at the benzene-water interface had been dissolved. If necessary, 5 g of NH_4Cl was added to facilitate the decomposition of magnesium salt, and additional benzene might be added if the amount present was insufficient to dissolve all the product. When the solids had disappeared, the benzene layer was separated and washed successively with 100 ml of H_2O, 100 ml of 5% $NaHCO_3$, and

finally with 100 ml H_2O. The solvent was removed in a rotatory evaporator to give 330 g (85%) of crude dimethyl-o-xenylcarbinol. Another similar run gave a 78% yield. The material, after recrystallization from a mixture of benzene and hexane, melted at 92-93 °C.

(2) 2-(p-Diphenyl)-isopropyl-phenyl-carbinol (I)

Two hundred and twelve grams (1 mole) of (p-diphenyl)-dimethyl-carbinol, 1 l of methylene chloride, and 120 ml of pyridine are placed in a three necked flask equipped with a mechanical stirrer. The mixture was cooled to -5 °C and kept it under a nitrogen atmosphere. A solution containing 152 ml phenyl chloroformate and 500 ml methylene chloride was added slowly in the course of 30 min (under vigorous stirring and the temperature was kept close to -5 °C). During the addition a precipitate formed. The reaction mixture was stored overnight at 0 °C. Then the reaction mixture was poured into a beaker containing cracked ice and finally diluted with 1 l methylene chloride. The organic phase was separated and washed three times with water, the

organic phase was dried with anhydrous sodium sulfate, and methylene chloride was evaporated in a rotatory evaporator. The remaining residue was dissolved in 1 l of ethyl acetate at 60°C. The solvent was evaporated until about 600 ml were left; then, the solution was kept in the refrigerator overnight. The precipitate formed, was filtered off and dried. Yield: 261 g, M.P. 115-116°C dec. The mother liquid was further concentrated in vacuo until about 100 ml were left, after keeping the mixture in a refrigerator, the precipitate was filtered off. Yield: 31.5 g, 88% M.P. 114-115°C dec.

(3) 2-(p-Diphenyl)-isopropyloxycarbonyl-hydrazide (II)

One hundred and six grams (0.5 mole) of 2-(p-diphenyl)-isopropyl-phenyl-carbinol (I) was suspended in 200 ml DMF. The mixture was cooled in an ice bath, and 125 ml of hydrazine hydrate was added. After being stirred for 6 hr at room temperature, the mixture was cooled in an ice bath, and 1 l of ice water was added in small portions. The mixture was stored in the refrigerator overnight for crystallization. The

precipitate was filtered off, washed with 1 N NaOH (ice cold), then washed with cold water until the washing was neutral. The obtained product was recrystallized from 200 ml CCl_4 and 40 ml petroleum ether. Yield: 103 g, 76% M.P. 106-108°C.

(4) 2-(p-Diphenyl)-isopropyloxycarbonylazide (III)

Twenty seven grams of 2-(p-diphenyl)-isopropyl-oxycarbonyl-hydrazide (II) in 270 ml acetonitrile was treated with a cooled solution of 50 ml 6 N HCl and 100 ml acetonitrile at -25°C. After the temperature was kept at -15°C to -20°C, 22 ml 5 M sodium nitrite was added. After waiting for 10 min at -15°C, the mixture was cooled to -25°C, and 2 N sodium carbonate solution was added until the pH reached 6-8. The solution was poured under cooling (ice salt mixture) into 1 l water and stirred until a complete crystallization was obtained. The precipitate was filtered off, washed with ice water, dissolved in ether, separated from the water, dried over anhydrous sodium sulfate, and the ether was evaporated <u>in vacuo</u> at room temperature. The crystalline residue (28.3 g) was used without further purification. For determination of the

content of azide, exactly 40 mole of the product was dissolved in 3 ml of CH_3OH; 1 ml of H_2O was added and titrated immediately with 0.1 N NaOH. The content of the azide was 84.5%.

The 2-(p-diphenyl)-isopropyloxycarbonyl (DPOC) derivatives of amino acids had been synthesized by reacting either the carbonate I or the azide III with methyl esters or salts of amino acids as described in the following paragraphs (methods A and B).

Method A:

One tenth mole (11.5 g) of L-proline was dissolved in 125 ml of anhydrous methanol and cooled in an ice-salt bath. HCl gas was passed into the solution until saturation. Methanol was then removed in vacuo. The saturation and distillation procedure was repeated with fresh methanol, and the oil was allowed to stand in a desiccator over P_2O_5 and NaOH pellets overnight. The oil crystallized, and the crystals were washed repeatedly with anhydrous ether. L-proline methyl ester hydrochloride formed. Yield: 10.5 g, 64%

M.P. 111°C. 2.85 g (17.2 mmol) of L-proline methyl ester hydrochloride was dissolved in 9.8 ml DMF, and 2.4 ml of triethylamine was added dropwise, and the solution was kept at 0°C under stirring. After two minutes, 6.3 g (18.8 mmol) 2-(p-diphenyl)-isopropyoxylcarbonyl azide was added in small portions; 3.6 ml of triethylamine in 5 ml of DMF was also added slowly in the course of 30 min. (at the end of this addition, the solution should be basic). The reaction mixture was stirred overnight at room temperature. When 100 ml of ice water was added to the solution, the precipitates formed were then dissolved in 300 ml isopropyl alcohol (at 40°C), and treated with 11 ml of 2 N NaOH for 2 hr at 40°C. The solution was evaporated <u>in vacuo</u>, and 100 ml of H_2O was added, washed two times with ether, cooled the solution to 0°C, and the aqueous phase was acidified to pH=3 with solid citric acid. The aqueous solution was extracted three times with equal amount of ether. The ether extractions were combined, neutralized, dried over anhydrous sodium sulfate and filtered. The ether was evaporated to about 20 ml; 3.5 ml of dicyclohexylamine (DCHA)

was added and cooled the solution; DCHA-salt began to precipite. Yield: 7.35 g, 80% M.P. 173-175°C dec.

Method B:

Twenty mmoles (2.62 g) of L-leucine was dissolved in 9.3 ml 2.15 N solution of Triton B (benzyltrimethylammonium hydroxide) in methanol at 50°C and stirred for 15 min. 6 ml of DMF was added and evaporated the solution down to about 5 ml; this procedure was repeated in order to remove the water formed.

The oily residue was dissolved in 10 ml of DMF; 6.66 g (0.02 mole) 2-(p-diphenyl)-isopropyl-phenylcarbonate (I) was added at 50°C and stirred for 3 hr. At the end of the reaction, the solution became dark brown. The solution was extracted with 200 ml ether-water (1:1) mixture; the organic phase was discarded; the aqueous phase was cooled to 0°C and acidified to pH=3 with 1 N citric acid and extracted with ether. The ether extracts were washed and dried, and ether was removed <u>in vacuo</u> at room temperature.

The crystallines were rubbed well with 4 ml of anhydrous ether and 5 ml of petroleum ether (30-60°C). The insoluble crystals were filtered off and gave DPOC-Leucine. Yield: 5.2 g, 70% M.P. 225-230°C dec.

Several other amino acid DPOC-derivatives were also synthesized by either method A or B. Their m.p.'s and yield were as follows: Threonine 190-194°C (dec.), 67%; Glycine, 175-178°C (dec.), 64%; Serine, 184-189°C (dec.), 57%. N-DPOC-r-t-butyl-L-aspartate cyclohexylamine salt used in this experiment was obtained from Mann Research Laboratories, New York.

(5) O-Benzyl-N-2-(p-diphenyl)-isopropyloxycarbonyl-L-serine

Freshly cut sodium metal (920 mg, 40 mg-atoms) was added to freshly distilled anhydrous ammonia (120 ml) at -70°C, and N-2-(p-diphenyl)-isopropyl-oxycarbonyl-L-serine (4.2 g 15 mmol) was added with stirring under nitrogen. The mixture was vigorously stirred until colorless, and then sodium metal (ca. 5 mmol) was added, followed by

benzyl bromide (3.72 ml, 31 mmol). The turbid solution was stirred for 30-60 min at -50 to -30°C to give a clear solution. The ammonia was then removed by slow evaporation and lyophilized. The residue was dissolved in distilled water (20 ml), and the solution was extracted with ether (two 20 ml portions). The aqueous phase was chilled, acidified to pH 3.5 with solid citric acid, saturated with sodium chloride, and extracted with ethyl acetate (four 100 ml portions). The combined organic layers were washed with saturated sodium chloride solution (three 35 ml portions) and dried over anhydrous sodium sulfate. The ethyl acetate was removed in vacuo at room temperature to give a colorless oil. The oil was dissolved in chloroform (8 ml), placed on a 3 x 45 cm column of silicic acid (150 g, Baker Analyzed), and eluted with chloroform (800 ml). The chloroform was evaporated in vacuo to give O-benzyl-N-2-(p-diphenyl)-isopropyl-oxycarbonyl-L-serine as a clear oil (1 g, 15%). Further elutions with methanol yielded 2.7 g of a mixture of N-2-(p-diphenyl)-isopropyl-oxycarbonyl-L-serine and traces of O-benzyl-N-2-(p-diphenyl)-isopropyl-

oxycarbonyl-L-serine as an oil.

(6) The relative stability of β-butyl ester and N-DPOC of aspartic acid in acidic medium.

Five milligrams of t-butyl-DPOC-aspartate cyclohexyl amine salt was put in each of four test tubes. 1 ml of H_2O (containing one drop of acetic acid), 80% acetic acid, glacial acetic acid, and 75% chloroacetic acid was added to the test tubes respectively. The test tubes were shaken to dissolve the solids and were kept for 8 hr at room temperature. The reaction mixtures were then analyzed by TLC and compared with standard aspartic acid.

C) <u>The solid phase syntheses of amidated peptides</u>

(1) The formation of amidated peptides by ammonolyses

a) Two-step cleavage as described by Li (27)

To 500 mg of N-t-butyloxycarbonyl-L-tyrosine resin ester was added 10 ml of DMF, 10 ml of methanol, and 4.4 ml of triethylamine. The mixture was stirred at 42°C for 22 hr and filtered. The resin was washed with warm

(60°C) DMF, and the combined filtrate was evaporated. The resin was retreated by the same procedure for another 22 hr, filtered, and the filtrate was evaporated to a residue which was combined with the residue of the first filtrate. The total residue weighed 185 mg (98%).

The above methyl ester was dissolved in 11 ml of DMF, 8 ml of ethylene glycol, and 5 ml of water. The solution was saturated with ammonia at 0°C and stored in a pressure bottle at room temperature. After 24 hr the solution was resaturated again and stored for 2 more days in a pressure bottle at room temperature. At this time the mixture was evaporated to dryness in vacuo. Yield 180 mg (~98%). The resulting N-BOC-tyrosine-amide was treated with trifluoroacetic acid for 15 min; the trifluoroacetic acid was then removed in vacuo. The residue showed a single spot on the thin layer chromatograph having a R_f=0.76 (tyrosine methyl ester R_f=0.68, standard tyrosine amide R_f=0.77).

b) Direct ammonolysis of tert.-butyloxycarbonyl-L-Val-polymer

One gram of the BOC-L-val-resin ester (capacity 0.31 mmole/g) was dried over P_2O_5 (20 °C, 0.01 torrs, 12 hr) and carefully washed three times each with 10 ml portions of DMF, absolute ethanol, dioxane, absolute ethanol, benzene, methylene chloride, absolute ethanol, DMF. The suspension of resin in 50 ml of DMF was transferred into a 450 ml Parr hydrogenation bottle. The bottle was cooled in an acetone-dry ice bath to -70 °C, and 50 ml of liquid ammonia was added. The bottle was stoppered immediately and wrapped with 2-3 layers of cloths. The mixture was kept at room temperature for 6 days (the bottle was under pressure). After being cooled to -40 °C by dry ice, the pressure was released by opening the stopper slowly. Ammonia was allowed to evaporate through a column filled with calcium chloride, raising the temperature slowly to 20 °C. The traces of ammonia were removed on the rotatory evaporator at 0.1 torr. The resin was filtered off and washed

with two 20 ml portions of warm (60°C) DMF, and successively with warm methylene chloride, absolute ethanol, and DMF. The washing was probably one of the most important steps in this ammonolysis. The filtrates were then combined and evaporated to dryness in vacuo. The crude product BOC-val-NH_2 weighed 47 mg (65%). The crude product was treated with trifluoroacetic acid for 15 min to remove the BOC group. Trifluoroacetic acid was removed in vacuo; the residue showed a single spot R_f=0.73 by thin layer chromatography, (standard Val-amide showed an R_f=0.72).

The ammonolysis of BOC-L-phenylalamine resin ester and BOC-L-tyrosine resin ester had been carried out according to the procedure described above. Identifications of both products were done by comparing with the authentic compounds by thin layer chromatography. Yields for phenylalanine and tyrosine, 73% and 95% respectively (based on the crude products).

c) Synthesis of C-terminal hexapeptide amide of secretin (L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valine amide).

The synthesis was carried out with three grams of N-t-butyloxycarbonyl (BOC)-L-valine resin ester (capacity: 0.39 mmole /g). The following steps were used to introduce each new acid: cleavage of BOC-group with 1 N HCl in glacial acetic acid for 30 min, neutralization of the hydrochloride with 10% triethylamine in DMF for 10 min, coupling of new BOC-amino acids (5 folds excess, reaction time 2 hr), using dicyclohexylcarbodiimide as condensing agent. L-glutamine was introduced as p-nitrophenyl ester of the BOC-amino acid (reaction time 8 hr) and the coupling reaction of BOC-L-leucine was repeated once. After each step, excess reagents were removed by washing successively with methylene chloride, DMF, ethanol, glacial acetic acid, and DMF.

The protected hexapeptide resin was then transferred into a pressure bottle, suspended in 50 ml of DMF, cooled to -70 ^{o}C, and 50 ml of

liquid ammonia (cooled to -70°C) was added. The bottle was immediately stoppered, transferred to a Parr-Medium-Pressure-Hydrogenation apparatus and kept in room temperature for 6 days. After being cooled to -70°C, the bottle was opened, ammonia was removed *in vacuo*, and the resin was filtered and washed twice with warm DMF, methylenechloride, and ethanol. Solvents were evaporated *in vacuo* at 40°C. The BOC-group was cleaved by treating the crude product with trifluoroacetic acid for 30 min. Trifluoroacetic acid was then removed *in vacuo* and the residue was twice recrystallized from DMF-ether. Yield 482 mg (64%); m.p. 250°C (dec.); Ref. 255-260°C. Amino acid analysis after hydrolysis with 6 N HCl, 24 hr at 100°C, gave the following molar ratio: Leu: 2.92; Glu: 1.04; Gly: 1.00; val: 0.96; NH_3: 2.09 (Gly being taken as 1.00). The amide showed two spots (R_f = 0.68 and a trace at R_f = 0.42) by thin layer chromatography of N-trifluoroacetate on silica gel (F 254 Merk, solvents: pyridine 70; methylethyl ketone 30; water 20; acetic acid 10). The major spot with R_f = 0.68 was identified as the C-terminal

hexapeptide amide of secretin by comparing R_f values with an authentic sample. The other spot was identified as valine amide.

(2) The protection of side-chain carboxylic groups in the formation of amidated C-terminals

a) To 50 mg of γ-t-butyl-carbobenzoxy (CBO)-L-glutamate was added 10 ml of DMF, 10 ml of methanol, and 4.4 ml of triethylamine. The mixture was stirred at 42°C for 22 hr. The CBO-group was removed by hydrogenation (30 psi,4 hr) in 10 ml t-butanol, 1 ml of acetic acid, and 10 mg of palladium on charcoal (10 %). After filtration and evaporation, the residue showed two spots on TLC, R_f: 0.75 and 0.65. These were identified as glutamic acid methyl and t-butyl esters,respectively, with an intensity ratio of 3:8. The methanolysis of t-butyl-L-valinate was also carried by the same procedure as above. Two spots were also found by TLC(R_f: 0.88 and 0.71, valine t-butyl and methyl esters respectively,with intensity ratio of 7:3).

b) Ten ml of DMF, 10 ml of methanol, and 4.4 ml of triethylamine were added to 10 mg of L-glutamic acid. The mixture was stirred at 40°C for 48 hr and evaporated. The residue showed two spots on TLC, R_f= 0.23 and 0.75, identified as glutamic acid and methyl glutamate respectively, with an intensity ratio of 15:1.

c) Twenty mg of t-butyl valinate hydrochloride were neutralized by 1 N NaOH at 0°C, extracted with ether, and the ether dried over anhydrous sodium sulfate. The ether was removed <u>in vacuo</u>, and the resulting residue was then dissolved in 50 ml DMF, transferred into a pressure bottle, cooled to -70°C, and 50 ml of liquid ammonia was added. The bottle was immediately stoppered, transferred to a Parr-Medium-Hydrogenation apparatus and kept at room temperature for 6 days. After cooled the bottle to -70°C, the bottle was opened. Ammonia and solvent were removed <u>in vacuo</u>. The residue showed a single spot on TLC, R_f= 0.88, and was identified as t-butyl valinate.

d) Ten mg of N-CBO-γ-t-butyl-L-glutamate was dissolved in 50 ml of DMF, transferred into a pressure bottle, cooled to -70°C, and 50 ml of liquid

ammonia was added. The bottle was immediately stoppered, transferred to a Parr-Medium-Hydrogenation apparatus and kept at room temperature for 6 days. After cooling the bottle to -70 °C, the bottle was opened, ammonia and solvent were removed in vacuo. The residue was then dissolved in 10 ml t-butanol and 2 ml of glacial acetic acid, and 10 mg of palladium on charcoal (10%) was added. The mixture was subjected to hydrogenation at 30 psi for 3 hr at room temperature to removed the CBO-group. The catalyst was filtered off and the solvent was removed in vacuo. The residue gave a single spot on TLC, R_f= 0.71. It was identified as γ-t-butyl-L-glutamate (standard with R_f= 0.69).

(3) The syntheses of two scotophobin analogs:

a) H-Ser-Asn*-Asn-Asn-Gln-Gln-Gly-Lys-Ser-Ala-Gln-Gln-Gly-Gly-NH_2
(14, 12, 7, 4, 1)

and

b) H-Ser-Asp*-Asn-Asn-Gln-Gln-Gly-Lys-Ser-Ala-Gln-Gln-Gly-Gly-NH_2
(14, 12, 7, 4, 1)

In order to test the above model experiments, two 14-amino acid amidated peptides were synthesized. The model experiments did not exactly simulating the condition in actual peptide synthesis; if the functional groups were stable in a model experiment,

it would have been stable in actual synthesis, but the reverse was not always true. The syntheses were carried out with one gram of N-t-butyoxy-carbonyl (BOC)-glycine resin ester (capacity 0.25 mmol/g). The following steps were used to introduce each new amino acid: (1) cleavage of BOC-group with 1 N HCl in glacial acetic acid for 30 min, (3) neutralization of hydrochloride with 10% triethylamine in DMF for 10 min. (the solvent was collected, and resin washed with once with DMF, solution, titrated for the content of HCl to determine the efficiency of BOC-group cleavage). (3) a coupling of new BOC-amino acids in CH_2Cl_2-DMF (3:1), (4 folds in excess, reaction time 2 hr), using dicyclohexylcarbodiimide (DCC) as condensing agent. This coupling step was repeated once for each amino acid to insure the completion of coupling reaction. DMF was used as co-solvent to prevent the possible complicating effect of tertiary structure of the peptide during the coupling. L-glutamine and L-asparagine were introduced as p-nitrophenyl ester of the BOC-amino acid (reaction time 8 hr, no condensing agent needed). After each step, excess reagents

were removed by washing successively with methylene chloride, DMF, absolute ethanol, glacial acetic acid, benzene, and DMF. The synthesis was carried out as described up to 12th amino acid (asparagine). The resin was divided into two parts. One part continued to couple asparagine, then serine. The protected tetradecapeptide-resin (1.1g) was added 10 ml of DMF, 10 ml of methanol, and 4.4 ml of triethylamine. The mixture was stirred at 42°C for 22 hr, filtered, the resin was washed with warm (50°C) DMF, and the combined filtrate was evaporated. The resin was re-retreated by the same procedure for another 22 hr, filtered, and the filtrate was evaporated to a residue which was combined with the residue of the first filtrate.

The above methyl ester was dissolved in 11 ml of DMF, 8 ml of methanol. The solution was saturated with ammonia at 0°C and stored in a pressure bottle at room temperature. After 20 hr the solution was resaturated with ammonia at 0°C, and stored for 2 more days in a pressure bottle at room temperature. At this time the mixture was evaporated to near-dryness at 40°C. The

residue was then dissolved in 30 ml of methanol, 15 ml of glacial acetic acid, and 50 mg of palladium on charcoal (10%), and hydrogenated a room temperature (hydrogen pressure: 30 psi) for 8 hr to removed O-benzyl group of serine. After filtration and evaporation in vacuo at room temperature, the residue was treated with 1 N HCl in glacial acetic acid for 30 min, but many precipitates formed. The precipitates showed positive ninhydrin reaction, meaning that at least partial cleavage of the BOC-group resulted. Two more cleavages (30 min each), in 1 N HCl acetic acid and in trifluoroacetic acid were carried out. The total crude product weighed 450 mg.

The other part of resin was coupled with γ-t-butyl-DPOC-aspartic acid instead of asparagine, and cleavage was done in glacial acetic acid (7 hr, free amino group titrated). Then O-benzyl serine was coupled. The protected tetradecapeptide resin was then transferred into a pressure bottle, dissolved in 50 ml of DMF, cooled to -70°C, and 50 ml of liquid ammonia (cooled to -70°C) was added. The bottle was immediately stoppered,

transferred to a Parr-Medium-Pressure-Hydrogenation apparatus and kept at room temperature for 6 days. After cooling the bottle to -70°C, the bottle was opened, ammonia was removed *in vacuo*, and the resin was filtered and washed twice with warm (50°C) DMF, methylene chloride, and ethanol. Solvents were removed *in vacuo* at 40°C.

The residue was then dissolved in 30 ml of methanol, 15 ml of glacial acetic acid, added to 50 mg of palladium on charcoal (10%), and hydrogenated at room temperature (30 psi) for 8 hr to remove O-benzyl groups and probably t-butyl group. After filtration and evaporation *in vacuo* at room temperature, the residue was treated with trifluroacetic acid for 30 min. Trifluoroacetic acid was then removed and the product precipitated by ether. The product weighed 310 mg. Further purification and biological test will be carried out by Dr. G. Ungar, Department of Anaesthesiology and Pharmacology, Baylor College of Medicine, Houston, Texas.

RESULTS AND DISCUSSION

In searching for protection of the side chains of carboxylic acids, which will be stable under the ammonolysis condition and additionally can be easily cleaved off, the t-butyl group was the one of the groups to be considered. Since t-butyl esters are stable under hydrazinolysis and aminolysis, they might be stable during ammonolysis too.

In order to investigate the stability of t-butyl ester as a protection of the side chain of the carboxylic acids, several t-butyl esters of amino acids were synthesized (Table XX) by reacting free amino acids with isobutene in a mixture of dioxane and conc. sulfuric acid (18) under pressure.

TABLE XX

Amino Acid t-Butyl Esters Synthesized

t-Butyl Ester Hydrochloride	M. P. (°C)		Yield %
	found	reported	
L-alanine	240-241	a	55
L-valine	141-143	147-149	65
L-leucine	167-169	166-167	70
L-phenylalanine	243 (dec.)	a	45
L-isoleucine	159-160	158-160	70
L-t-leucine	166-167	b	68
L-tyrosine*	143-145	143-145	45
α-L-aspartic acid	178-179	178-179	76
β-benzyl-L-aspartate	115-117	115-117	73

*, a, and b see next page.

* free amino ester
a decomposed without melting
b not available

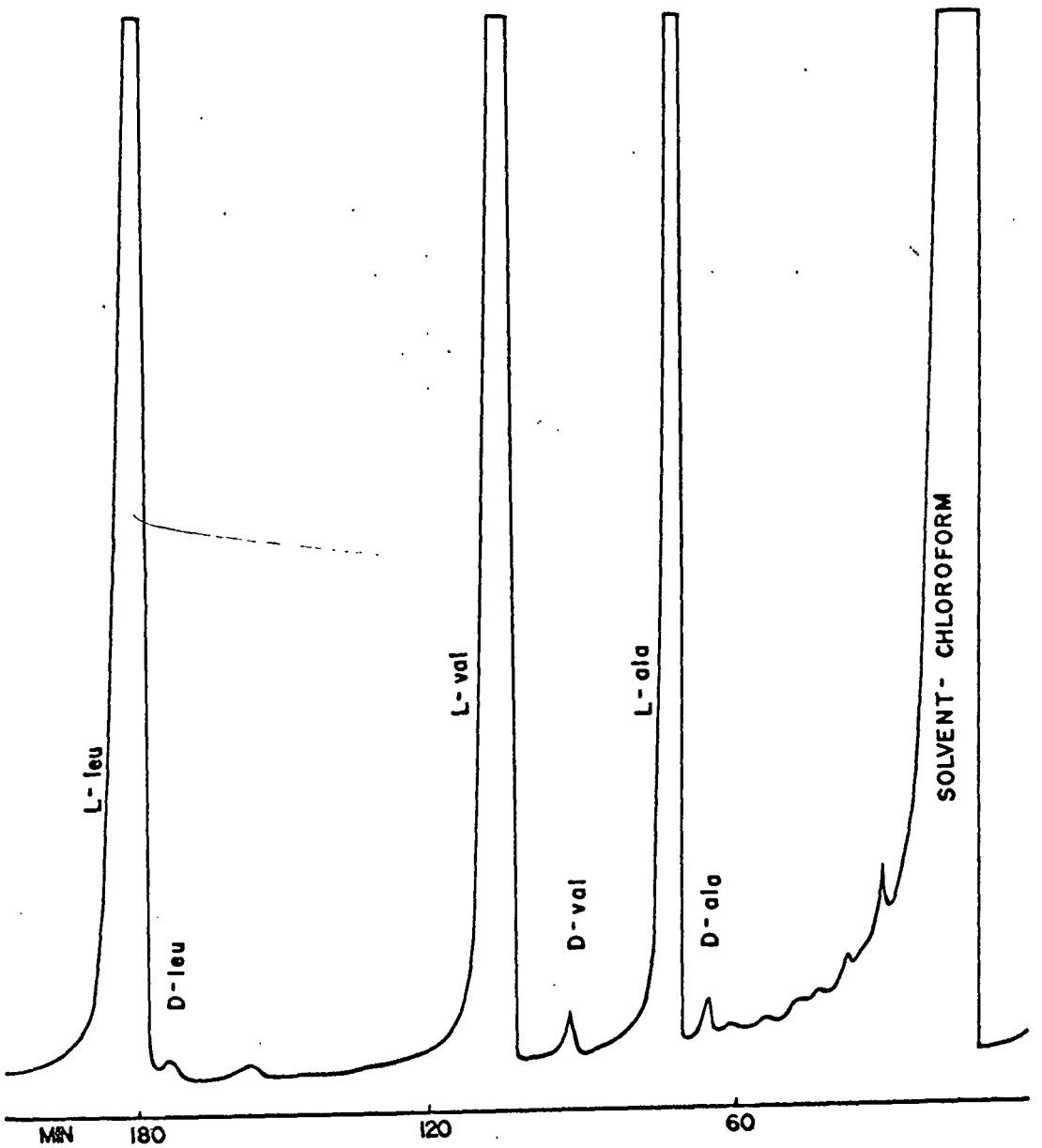
Since reacemization has been an important problem in peptide synthesis, and since during the synthesis of t-butyl ester derivatives of amino acids high concentrations of sulfuric acid had been used, in 4 to 8 hr reactions, and dilute sodium carbonate or sodium hydroxide had been used to neutralize the reaction mixture, it was possible that recemization took place to some extent. Some of the esters, valine, leucine, and alanine t-butyl esters have been acetylated with trifluoroacetic anhydride, and the resulting N-TFA-L-valine-t-butyl esters was injected into a gas chromatograph with a optically active stationary phase, and two peaks appeared (both have been identified by mass spectrophotometry). Their relative size had been measured by an integrator (Fig.7). The extent of racemization was 0.47%, 0.65%, 0.71% for leucine, valine, alanine, respectively. Some D amino acid may have been present in the starting material, and acylation might also have caused racemization. From the results we can conclude that racemization is negligible under the conditions specified in the experimental part.

The relative stability of the t-butyl ester group and

FIGURE 7

Chromatogram of N-TFA-leucine, valine and alanine t-butyl ester with N-TFA-L-phenyl-alanine-L-leucine cyclohexyl ester as the stationary phase at 120°C. Chromatographic conditions: 400 ft x 0.02 in. stainless capillary; carrier gas He at 9 psi; injector 190°C; detector 280°C.

Figure 7


L- leu
D- leu
L- val
D- val
L- ala
D- ala
SOLVENT- CHLOROFORM
MIN
180
120
60

the t-butyloxycarbonyl (BOC) group in the acidic medium was so close, that there was no way to cleave either of them selectively by acids. The relative stability of the t-butyl ester group and 2-(p-diphenyl)-isopropyloxycarbonyl (DPOC) in acidic media will be discussed later.

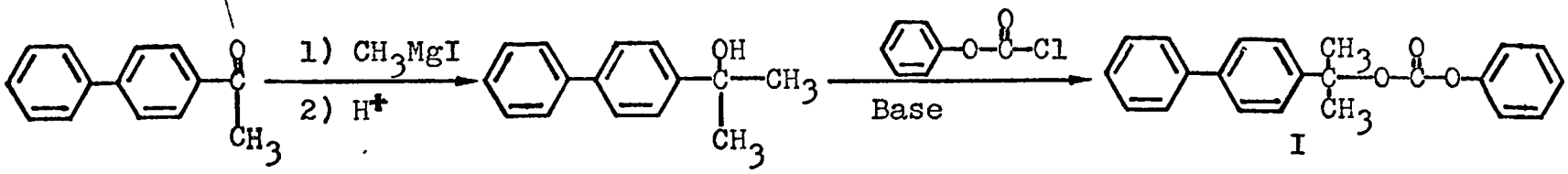
In order to study the cleavage conditions of DPOC group, some of the DPOC derivatives of amino acids were synthesized by the following procedure‡ (26). These physical data are given in Table XXI.

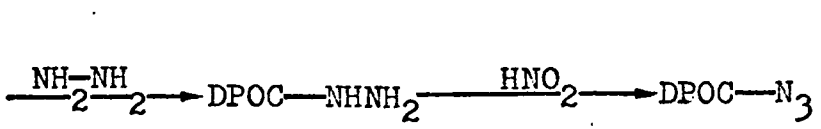
TABLE XXI

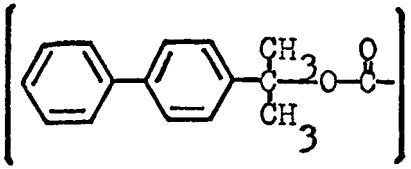
2-(p-Diphenyl)-isopropyloxycarbonyl (DPOC) derivatives of amino acids

DPOC-amino acid-salt	M.P. (°C) found	M.P. (°C) reported	yield %
L-proline DCHA*	173-175 (dec.)	173-175 (dec.)	80 (A)
L-threonine	190-194 (dec.)	a	67 (A)
L-leucine *	225-230 (dec.)	227-230 (dec.)	70 (B)
Glycine DCHA*	175-178 (dec.)	192-193 (dec.)	64 (B)
L-serine	184-189 (dec.)	a	57 (A)

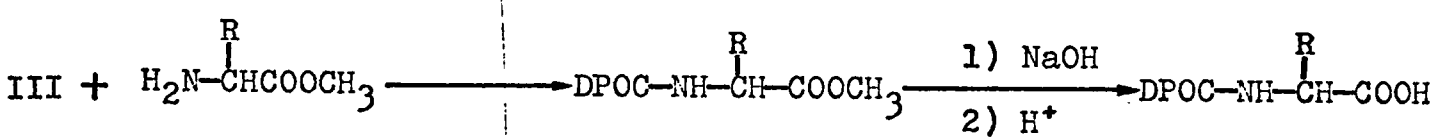
* dicyclohexylamine salt
a not available
A by method A (see next page)
B by method B (see next page)
‡ on next page



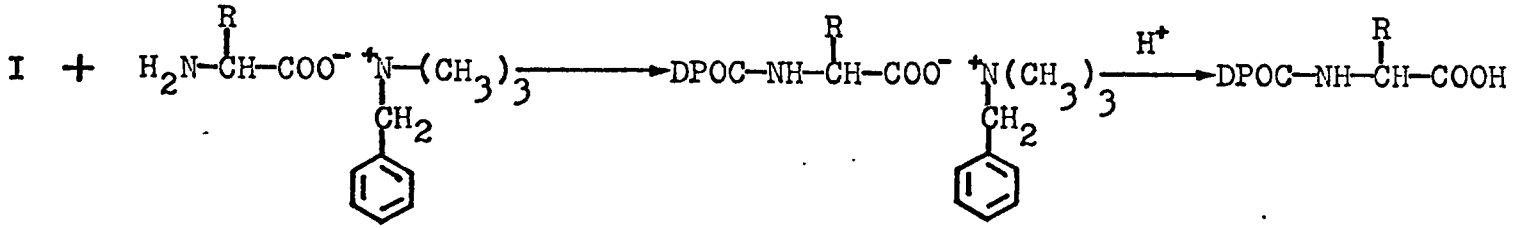


DPOC = 

Method A:



Method B:



O-benzyl-N-DPOC-L-serine was synthesized from N-DPOC-L-serine by reacting benzyl bromide in Na and liquid ammonia mixture (28). However, only very poor yield was obtain (15%). In the study of the relative stability of β-butyl ester and N-DPOC of aspartic acid in media of different acidic strengths (e.g, 80% acetic acid, glacial acetic acid, 75% chloroacetic acid), t-butyl ester was more stable than N-DPOC in acidic media. If the reaction were 8 hr, in all cases, t-butyl ester was cleaved to some extent in the order of 75% chloracetic acid$\gg$80% acetic acid$>$glacial acetic acid. The t-butyl ester group was more stable in glacial acetic acid than in 80% acetic acid, although the former is stronger in acidity. It might be acid-catalyzed hydrolysis also took place. If the reaction time were reduced to 7 hr, t-butyl ester was stable and N-DPOC was completely cleaved off. These results may also be true in the case of glutamic acid.

In searching for a feasible method to synthesize amidated peptides by solid phase method Beyerman et al, (29,15) were able to synthesize C-terminal peptide amides, such as oxytocin, and N-substituted derivatives by ammonolysis and aminolysis, respectively, with the aid of ammonia, methylamine, hydroxylamine, and hydrazine in methanol. In only one such case have esters been isolated; this was looked

upon as a special phenomenon caused by steric hindrance of valine, and which was not observed with glycine (14). However, when treating N-benzyloxycarbonyl-L-prolyl-L-leucyl-glycine-polymer (30) with ethanolic ammonia for 4 hr. Beyerman (31) was able to isolate the ethyl ester as well as the amide. With methanolic ammonia the methyl ester could be detected after 1 hr, but it had disappeared after 3 hr (T.L.C.) From this they concluded that these ammonolysis compete with a base-catalyzed transesterification, followed by ammonolysis.

The tridecapeptide (5-glutamine)-α-melanotropin (N-acetyl-ser$_{1}$-tyr-ser-met-gln$^{*}_{5}$-his-phe-arg-try-gly-lys-pro-val$_{13}$-NH_2), analog of the melanocyte-stimulating hormone (MSH), had been synthesized by the solid-phase method (27). The fully protected tridecapeptide was cleaved from the resin by methanolysis in N,N-dimethylformamide-methanol-triethylamine. Ammonolysis of the methyl ester was effected in DMF-ethylene glycol-water; the final peptide was formed in high yield (81% in methanolysis, $\sim$ 100% in ammonolysis.).

N-BOC-tyrosine resin ester was subjected to the two-step cleavage as described by Li (27), and a quantitative nearly yield (97%) of tyrosine amide was obtained. This experiment demonstrated that the two-step cleavage, methanolysis followed

by ammonolysis, could be used to form tyrosine amide. This technique was suitable for the synthesis of amidated peptides with tyrosine as C-terminal amino acid.

Judged from the hormone (5-[glutamine] -α -MSH, an analog of α-MSH) Li and co-workers synthesized, this method could cause difficulty if the peptide contained aspartic or glutamic acids. Although this natural hormone had been synthesized by two group (32,33) by the classic method, no attempt to synthesize natural α-MSH (5-[glutamic acid]-α-MSH) by the solid phase method had been made.

One question has to be asked, "Is the same method applicable, if peptides having side-chain carboxylic groups are to be synthesized? Is it possible to leave the groups unprotected, and if not, what protecting groups have to be used?"

In order to find a feasible method for the solid-phase synthesis of C-terminal aspartic and glutamic acid containing peptides, several model experiments were carried out under the different ammonolysis conditions to check the stability of the protected and free side chain carboxylic group.

These model experiments were qualitative in nature. The purpose of carrying out these experiments was to check the stability of the functional groups. If the side reactions took place, only rough estimates of the extents of the side products were determined. All the separations and identifications were done on the thin layer chromatographs (TLC, solvent, pyridine 35; methylethyl ketone 15; water 10; glacial acetic acid 5) with ninhydrin as detecting agent. (Table XXII)

From the result it was obvious that neither β nor γ-t-butyl ester of amino acids were stable in the methanolysis. It was understandable because this reaction was carried out in basic medium, and methyl and t-butyl esters existed in an equilibrium, and just a typical base catalyzed transesterification reaction took place.

If the side-chain carboxylic groups were left unprotected during the methanolysis, about 5% of methyl ester was detectable on TLC. Although the esterification in the basic condition was not observed, it was possible that the methyl glutamate was formed by a typical S_{N2} reaction between conjugate base of glutamic acid and methanol. The equilibrium constant for this S_{N2} reaction was small, since a conjugate base of glutamic acid is a much weaker nucleophile than

TABLE XXII

Thin Layer Chromatographic Results

Method: a) methanolysis (first step of the two-step ammonolysis)

b) direct ammonolysis

TLC plates : 5x20 cm, coated with cellulose

Solvent system : pyridine 35; methylethyl ketone 15; water 10; glacial acetic acid 5

Amino acid derivatives	Method a		Method b	
	Products found	Identified[c] as:	Products found	Identified[c] as:
N-BOC-Tyr-polymer	R_f= 0.76	Tyrosine amide	R_f= 0.75	Tyrosine amide
Val-polymer	see ref. (14)		R_f= 0.73	Valine amide
Val* — O+	R_f= 0.88 R_f= 0.71	Val-O+ Val-OCH_3	R_f= 0.88	Val - O+
* O+ | N—CBO-Glu	R_f= 0.75 R_f= 0.65	γ-methyl glutamate γ-t-Butyl glutamate	R_f= 0.71	γ-t-Butyl-glutamate
Glu	R_f= 0.25 R_f= 0.75	Glutamic acid γ-Methyl glutamate	R_f= 0.22	Glutamic acid

c comparing with standards

* Val-O+ : α-t-butyl valinate

N-CBO-Glu (O+) : N-carbobenzoxy-γ-t-butyl-glutamate

hydroxide anion. But since the methanol was present in large amounts, the esterification product might have been detectable.

Neither the free nor the t-butyl-protected side-chain carboxylic group was stable in the methanolysis. So two-step cleavage, methanolysis and ammonolysis, can not be applied in synthesizing the amidated peptides containing aspartic and glutamic acid by solid phase method.

The direct ammonolysis of β-t-butyl-CBO-L-glutamate, glutamic acid and valine in liquid ammonia were carried out by the same procedure as described in the experimental section. Only a single spot was obtained on TLC, and the products were identified as t-butyl-L-glutamate, glutamic acid, and valine, respectively. No glutamine, or valine amide was found on the TLC (see Table XXII). From the these results, side-chain carboxylic groups can be protected with t-butyl groups or left free, without undergoing side reaction, during direct ammonolysis in liquid ammonia.

Under these conditions, BOC-valine, BOC-phenylalanine, BOC-tyrosine, and BOC-proline amide were cleaved from the resin in high yields (55 to 97% based on crude product).

The hexapeptide amide of C-terminal sequence of secretin was synthesized by using direct ammonolysis in order to discover the influence of the peptide length on the cleavage step. From the result, peptide length did not show any effect on the cleavage. Also it showed that the peptide bonds were stable in liquid ammonia for at least 6 days.

Two tetradecapeptide peptides were also synthesized. One had a asparagine residue in the 13th position; another had a aspartic acid residue instead. Both peptides were analogs of scotophobin, but with tyrosine missing from the C-terminal. The purpose of synthesizing these two peptides was to use the t-butyl as side chain carboxylic protection and DPOC as amino group protection in a solid phase synthesis of amidated peptides. From the biological activity of these peptides (a biological test will be carried out), the involvement of C-terminal tyrosine amide in the biological activity could be determined.

CONCLUSION

Several BOC-amino acids were successfully cleaved from the resin support by direct ammonolysis with liquid ammonia in DMF, yielding ranges from 55% to more than 95%. In an attempt to find a generally applicable way of synthizing C-terminal amidated peptides by the solid phase method, t-butyl ester was used as a side-chain carboxylic group protection without noting other side reaction on β- or γ-carboxylic groups. After β or γ-t-butyl group protected aspartic or glutamic acids were attached to the peptides, DPOC-derivatives of amino acids had to be used. DPOC groups were selectively cleaved in glacial acetid acid without affecting the side-chain β- or γ-t-butyl esters. After the whole peptide was synthesized on the resin, direct ammonolysis with liquid ammonia in DMF at room temperature was used to cleave the peptide from the resin, simultaneously forming the C-terminal amide.

The O-benzyl ether and S-benzyl bonds were stable under direct ammonolysis conditions. The C-terminal hexapeptide amide of secretin and two tetradecapeptides of scotophobin analogs were also synthesized.

REFERENCES

1. Merrifield, R. B., Federation Proc., 21, 412 (1962).
2. Merrifield, R. B., Advances in Enzymology, 32, (1969).
3. Gutte, B., and Merrifield, R. B., J. Am. Soc., 91 501, 1969.
4. Li. C. H., and Yamashiro, D., J. Am. Chem. Soc. 92, 7608, 1970.
5. Merrifield, R. B., Endeavour, 24, 3 (1965).
6. Merrifield, R. B., Science, 150, 178 (1965).
7. Marshall, G. R., Doctoral Dissertation, Rockefeller Uni. 1966.
8. Kusch, P., Angew. Chem. 78, 611 (1966).
9. Kusch, P., Kolloid-Z., Z. Polymere, 208, 138 (1966).
10. Rother, M. and Dunkel, W., J. Polymer Sci. B., (Polymer Letter) 5, 589 (1967).
11. Rothe, M., and Schneider, H., Angew. Chem., Itern. Ed., 5, 417 (1966).
12. Zahn, H., Kusch, P., and Shah, J., Kolloid Z., Z. Polymere, 216, 298 (1967).
13. Gordon, M., J. G. Miller and A. R. Day, J. Am. Chem. Soc. 71, 1245 (1949).
14. Bodanszky, M., and J. T. Sheehan, Chem. and Ind., 1966, 1597.
15. Beyerman, H. C., and H. Maassen Van den Brink-Zimmermannova Rec. Trav. chim., 1968, 87, 1196.
16. Anderson, G. W., J. Blodinger and A. D. Welcher, J. Am. Chem. Soc., 74, 5309 (1952)

17. Penneman, H. J., A. F. Marx, and J. F. Arens, Rec. Trav. chim., 78 488 (1959).

18. Roeske, R. W., J. Org. Chem. 28 1251 (1963).

19. Anderson, G. W., and F. M. Callahan J. Am. Chem. Soc. 82, 3359 (1960).

20. Beyerman, H. C., and J. S. Bontekoe, Proc. Chem. Soc. 249 (1961).

21. Schroeder, E., and K. Luebke, Ann. Chem. Liebigs 655, 211 (1962).

22. Taschner, E., C. Wasielewski, T. Sokolowska, and J. F. Biernat, Ann. Chem. Liebigs, 646, 119 (1961).

23. Kappeler, H., and R. Schwyzer, Helv. Chim. Acta, 44 1136 (1961).

24. Schwyzer, R. W. Rittel, H. Kappeler, and B. Iselin, Angew. Chem. 72, 915 (1960).

25. Sieber, P., and B. Iselin, Helv. Chim. Acta, 51 (4), 622 (1968).

26. Sieber, P., and B. Iselin, Helv. Chim. Acta, 51 (4), 614 (1968).

27. Blake, J. J., R. W. Crooks, and C. H. Li, Biochemistry 9, 2071 (1970).

28. Hruby, V. J. & K. W. Ehler, J. Org. Chem. 35 (5) 1690 (1970).

29. Beyerman, H. C., and C. A. M. Boers-Boonekamp, Rec. Trav. chim., 87, 257 (1968).

30. Halpern, B., L. Chew, V. Close, and W. Patton, Tetrahedron Letters, 1968, 5163.

31. Beyerman, H. C., H. Hindriks, and E. W. B. DE Leer, Chem. Comm. 1968, 1668.

32. Guttmann, S., and R. A. Boissonnas, Helv. Chim. Acta, 42, 1257 (1959).

33. Schwyzer, R., A. Costopanagiotis, and P. Sieber, Helv. Chim. Acta, 46, 870 (1963).