A NEW APPROACH TO THE SYNTHESIS OF N-METHYLAMINO ACIDS AND THEIR PEPTIDES

A thesis submitted to
the
Division of Sciences
School of Graduate Studies
University of Ottawa

by

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in partial fulfillment of the requirements for
the degree of

Doctor of Philosophy
in
Biochemistry

Ottawa April 1970

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To the Memory of

JOHN GARRETT

and

HOWARD FLOREY

"If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts, he shall end in certainties".

Francis Bacon: The Advancement of Learning

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to all the members of our research team, past and present, who gave so generously of their time and knowledge during the course of this work.

My thanks are also due to Mrs. Ruth Demayo for technical assistance, to Mme. Louise Viau for secretarial assistance, to Mrs. Angela Eriksen who undertook the arduous task of typing the manuscript and to the Department of Chemistry, University of Ottawa for making available the n.m.r. spectrometer and the polarimeter.

Finally to Dr. Leo Benoiton, to whom I shall always be indebted, I express my deepest thanks for his patience, understanding and guidance. It was a pleasure to work in his laboratory.

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LIST OF ABBREVIATIONS

A	acetic acid
atm	atmosphere
B	*n*-butanol
b. p.	boiling point
C	chloroform
cm	centimeter
d.	decomposition
dm	decimeter
DMAC	N, N-dimethylacetamide
DMF	N, N-dimethylformamide
g	gram
g. l. c.	gas liquid chromatography
h	hour
i	iso
i. r.	infrared
l	liter
M	methanol
M	molar
mg	milligram
ml	milliliter
min	minute
mm	millimeter
mmol	millimole
mol	mole
m. p.	melting point

n	normal
N	normality (of solution)
nm	nanometer
n. m. r.	nuclear magnetic resonance
o	ortho
p	page
p	para
s	second
t	tertiary
TEA	triethylamine
TFA	trifluoracetic acid
THF	tetrahydrofuran
v	volume
w	weight
W	water
°C	degree Celsius
μmol	micromole
*	denotes new compound.

NOMENCLATURE FOR AMINO ACIDS AND PEPTIDES

The abbreviated designations used for the amino acids and N-methylamino acids were based on those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [European J. Biochem. 1, 375 (1967)]. The symbols were not used to represent the structural formulae of the amino acids which were represented as follows:

H-Gly-OH	Glycine
MePhe-OH	N-Methyl-L-phenylalanine.

Protected amino acids were represented as recommended, e.g.

Ac-MeLeu-OMe	Acetyl-N-methyl-L-leucine methyl ester
H-Ala-OMe.HCl	L-Alanine methyl ester hydrochloride.

Substitutions in the side chains were also represented as recommended, e.g.

H-Lys(Me)-OH	ε-N-Methyl-L-lysine
MeTyr(Me)-OMe.HBr	N,O-Dimethyl-L-tyrosine methyl ester hydrobromide.

The symbols represented the L-amino acids where optical isomerism was possible.

The following abbreviations were used for the protecting groups:

Ac	Acetyl
Boc	t-Butyloxycarbonyl
Bu	Butyl
Bz	Benzoyl
Bzl	Benzyl
Cbz	Carbobenzoxy
Et	Ethyl
Me	Methyl
Ph	Phenyl
Pht	Phthaloyl
Pr	Propyl
Tos	Tosyl = p-Toluenesulfonyl
Trt	Trityl = Triphenylmethyl

Note that for the difunctional amino acids the acyl substituents must occur on the nitrogen and in the text the N's have been omitted. With lysine where ambiguities could arise the N's are included with the appropriate Greek letter(s) to designate which nitrogen is substituted. The N's are included in the names of all the N-alkyl substituted compounds.

I.

INTRODUCTION

At the present time there is considerable biochemical interest in the ε-N-alkyl-derivatives of lysine. Some ε-N-alkyl-lysines are naturally occurring, others have been obtained after the acid hydrolysis of chemically modified enzymes with lysine active centres and several ε-N-alkyllysine derivatives have been used in studies on lysine specific enzymes.

ε -N-Methyllysine was first isolated from a natural source, the flagellar protein of the bacterium Salmonella typhimurium, by Ambler and Rees (1959). Later Murray (1963) found that it was a minor constituent of histones from various higher animals and wheat germ, and very recently Kuehl and Adelstein (1969) have found it in rabbit muscle myosin. Commercial preparations of calf thymus histones have been reported to contain ε -N, ε -N-dimethyllysine [Paik and Kim (1967)] and ε -N, ε -N, ε -N-trimethyllysine [Hempel, Lange and Birkofer (1968)]; the latter derivative has also been found in cytochrome c isolated from Neurospora crassa and wheat germ [Delange, Glazer and Smith (1969)]. In all these cases the ε -N-methylated-lysines were incorporated in the polypeptide chains of proteins. The occurrence of free ε -N-methylated-lysines has also been reported, e.g., ε -N-methyllysine has been found in human plasma [Perry, Diamond and Hansen (1969)] and in bovine brain and blood [Matsuoka et al (1969)] and ε -N, ε -N, ε -N-trimethyllysine has been found in the seeds of the plant Reseda luteola L. [Larsen (1968)] and in two species of algae [Takemoto, Daigo and Takagi (1964, 1965)].

An interesting explanation for the occurrence of ε-N-methyllysine in Salmonella flagella has recently been proposed by Parish and Ada (1969). Certain strains of Salmonella have flagellar protein containing ε-N-methyllysine and others do not; genetic studies on some of these strains by Stocker, McDonough and Ambler (1961) suggested that methylation of the lysine occurred after flagellin synthesis. Since only one part of the flagellin molecule contained methylated lysine residues and this part was exposed in the completed flagella Parish and Ada suggested that methylation occurred after the assembly of the flagellin sub-units into flagella. It has been shown [Benoiton and Deneault (1966), Seely (1969)] that the methylation of lysine derivatives decreases their susceptibility to tryptic action. Parish and Ada (1969) therefore expected and were able to confirm in preliminary experiments that the flagella would be resistant to trypsin. Flagellin resistant to trypsin-like enzymes would be an advantage for Salmonella since these organisms thrive in the mammalian gut, an environment rich in tryptic enzymes.

The methylation of the ε-amino groups of lysine residues in histones takes place after the synthesis of the polypeptide chain has been completed [Allfrey, Faulkner and Mirsky (1964); Tidwell, Allfrey and Mirsky (1968)]; it is enzymatically catalysed by a reaction in which S-adenosylmethionine serves as methyl group donor [see Kim and Paik (1965); Comb, Sarkar and Pinzino (1966); Kaye and Sheratzky (1969)]. Not all histones contain methylated lysine residues and the sequence studies of Delange, Fambrough, Smith and Bonner (1969a, 1969b) on calf thymus histone IV and pea seedling histone IV suggest that

methylation may be highly specific, e.g. in the calf thymus histone only 1 out of the 11 lysine residues is methylated and the methylated residue occurs unambiguously at position 20 in the polypeptide chain; in the homologous pea seedling histone residue 20 is not methylated. The methylated lysine at residue 20 in calf thymus histone IV is a mixture of ε-N-methyllysine and ε-N-dimethyllysine [Delange et al (1969b)].

The functional significance of the ε-N-methyllysines in histones is not known but it has been suggested that methylation [Allfrey et al (1964)] like acetylation [Allfrey et al (1964); Gershey, Vidali and Allfrey (1968)] and phosphorylation [see Gershey, Haslett, Vidali and Allfrey (1969) for references] of histones may alter their binding to DNA and thereby affect chromatin structure and function. Allfrey et al (1964) have specifically suggested that the methylation of histones may play a role in the regulation of gene transcription. If this is the case, the methylation must be reversible [Allfrey et al (1964)]; an enzyme, ε-alkyllysinase, which oxidatively demethylates ε-N-methyllysine and ε-N, ε-N-dimethyllysine has been reported [Kim, Benoiton and Paik (1964)]. This enzyme was found to occur mainly in the mitochondria although there was some activity in the nuclear and soluble fractions. Since the fractionation was crude, the presence of this enzyme in the nucleus has not been definitely established (the activity in the nuclear fraction could be due to mitochondrial contamination). It is therefore possible that ε-alkyl-lysinase may not be the enzyme involved in the reversible methylation of histones in the nucleus; however, the occurrence of an enzyme with similar specificity in the nucleus is essential if Allfrey's hypothesis is correct.

For the sequence studies on histones now in progress for example in the laboratories of Bonner and Smith [see e.g. Delange et al (1969b)] and of Starbuck and Busch [see e.g. Sautiere et al (1968) and Quagliarotti et al (1969)], good standard samples of the ε-N-methylated-lysines are essential. In these studies, the methylated-lysines are identified by amino acid analysis [e.g. Delange et al (1968)], and the ninhydrin colour yield values, which can only be obtained with pure samples, are essential for the quantitative estimation of residues. A detailed knowledge of the effect of trypsin and carboxypeptidase B on peptides containing methylated-lysine residues would also be useful for the sequence studies [see e.g. Delange et al (1969a) who obtained a low yield of ε-N-methyllysine during the carboxypeptidase B digestion of a peptide from calf thymus histone IV]. Furthermore, any search for the enzyme that demethylates histones would be assisted if methylated-lysine containing peptides, preferably containing ^{14}C-labelled methyl groups, were available for use as synthetic substrates. Such substrates would also be useful for studies on ε-alkyllysinase.

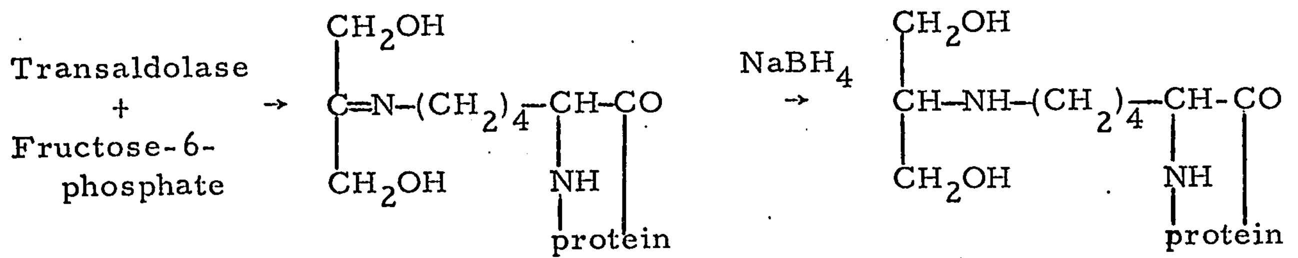
The general method of sodium borohydride reduction of Schiff bases was introduced into enzyme chemistry by Fischer, Kent, Snyder and Krebs (1958) who succeeded in identifying the binding site of pyridoxal phosphate in muscle phosphorylase by reducing the labile intermediate complex formed between the protein and the coenzyme to a stable form which on chymotryptic digestion yielded a dipeptide identified as ε-N-pyridoxalphosphate-lysylphenylalanine [Fischer (1965)]. This result led to similar studies with other enzymes

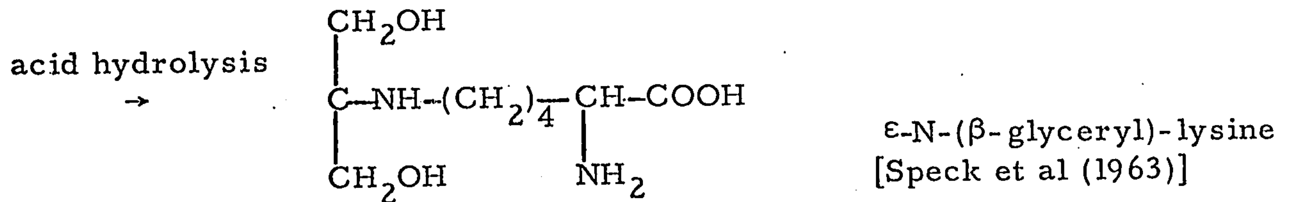
requiring pyridoxal phosphate co-factors [for a list of references see Vallee and Riordan (1969)]. The technique was later employed by Horecker and co-workers [see Speck et al (1963) and Rosen et al (1964)] and by Westheimer and co-workers [see Westheimer (1963) and Warren et al (1966)] to establish that there were lysine residues involved in the activity of aldolase and acetoacetate decarboxylase respectively. The reactions involved in the trapping of the labile enzyme substrate complexes are illustrated on the opposite page (Fig. I. 101). In all cases an ε -N-alkyllysine derivative was identified in the acid hydrolysate of the reduced enzyme substrate complex. The identification of ε-N-alkylated-lysines isolated after the reduction of enzyme-substrate complexes in this manner would be greatly facilitated if these compounds could be synthesised readily by unambiguous routes.

Benoiton and Deneault (1966) reported that ε-N-methyl-L-lysine methyl ester and α-N-benzoyl, ε -N-methyl-L-lysine amide were substrates for trypsin. Recently Seely (1969), in a study directed towards finding out the detailed geometry of the active site of trypsin, has determined kinetic data for the trypsin catalysed hydrolysis of ε-N-methyl-L-lysine methyl ester, α-N-benzoyl, ε-N-methyl-L-lysine methyl ester and α-N-benzoyl, ε -N-methyl-L-lysine amide. All these compounds were substrates for trypsin although the rates of hydrolysis were relatively slow compared with the rates of hydrolysis of the corresponding lysine derivatives, e. g. ε-N-methyl-L-lysine methyl ester was hydrolysed at 1/19 of the rate of L-lysine methyl ester. Seely also found that the methyl esters, the

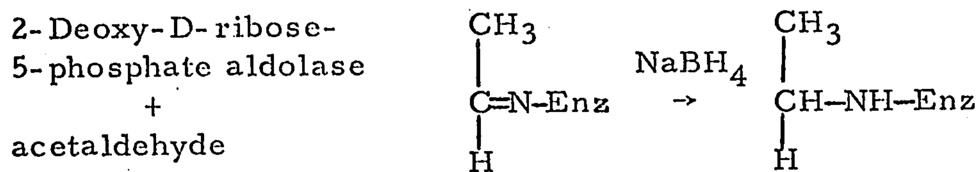
Fig. I. 101

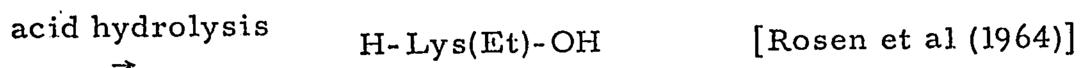
Formation of ε-N-alkyllysines by the sodium borohydride reduction of some enzyme-substrate complexes

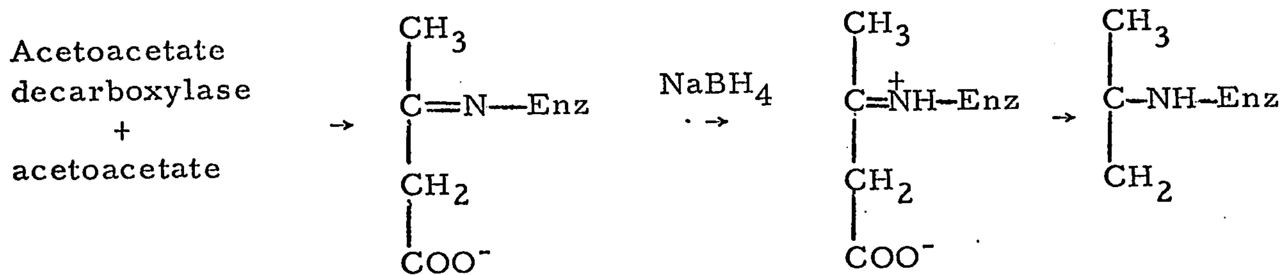


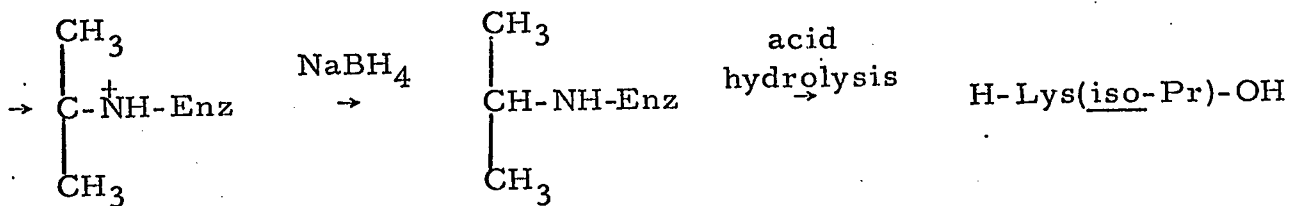


ε-N-(β-glyceryl)-lysine
[Speck et al (1963)]









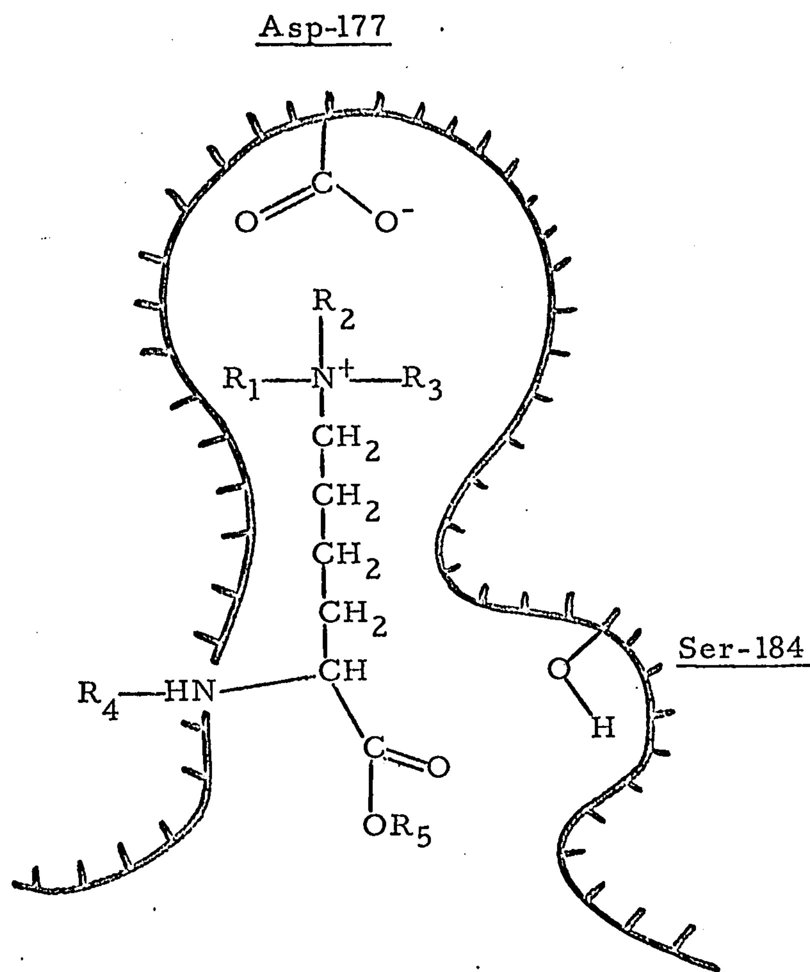
[Warren et al (1966)]

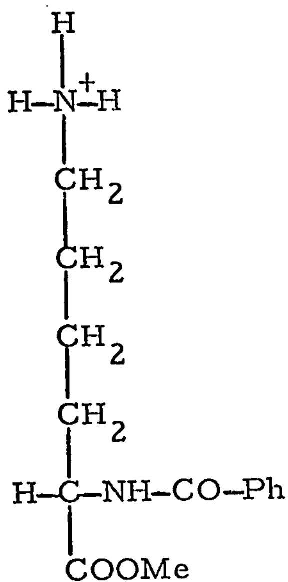
α-N-benzoyl methyl esters and the α-N-benzoyl amides of ε-N,-ε-N-dimethyl-L-lysine and ε-N, ε-N, ε-N-trimethyl-L-lysine were not substrates for trypsin; of particular interest was the fact that neither α-N-benzoyl, ε-N, ε-N-dimethyl-L-lysine methyl ester nor α-N-benzoyl, ε-N, ε-N-trimethyl-L-lysine methyl ester were inhibitors of the hydrolysis of α-N-benzoyl-L-lysine methyl ester. In Fig. I. 102 a schematic representation of the active site of trypsin is given, together with the structural formulae of some of the compounds mentioned above. Serine residue 184 has long been believed to be the catalytic site of trypsin [for references see Singer (1968)] and recently aspartic acid residue 177 has been identified as the site of binding of the positively charged substrates the so called anionic binding site [Smith and Shaw (1969); Eyl and Inagami (1970)]. Seely's results indicated that either a methylamino group is the largest group that can fit into the anionic binding site of trypsin or that two free hydrogen atoms on the ε-nitrogen atom are essential for activity. In order to distinguish between these two explanations kinetic studies on esters and α-N-benzoyl esters of ε-N-ethyl-L-lysine and possible some higher ε-N-alkyl-L-lysines are required.

Experiments with carboxypeptidase B [Seely and Benoiton (1969)] have shown that poly-ε-N-methyl-L-lysine is hydrolysed at a rate comparable with that for poly-L-lysine. It was also found that poly-ε-N-, ε-N-dimethyl-L-lysine was hydrolysed by the enzyme but at a slower rate. Benoiton and his collaborators plan to make a detailed study of kinetics of hydrolysis of a number of small peptides containing C-terminal ε-N-alkylated lysines in order

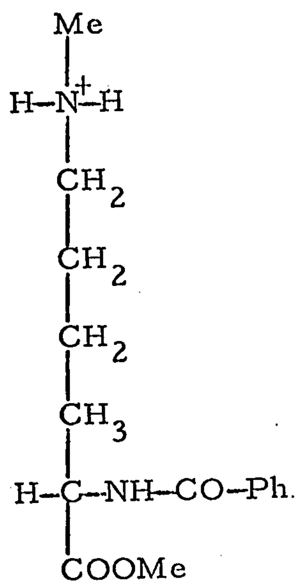
Fig. I. 102

The active site of trypsin and the structure of some of the substrates investigated by Seely (1969).

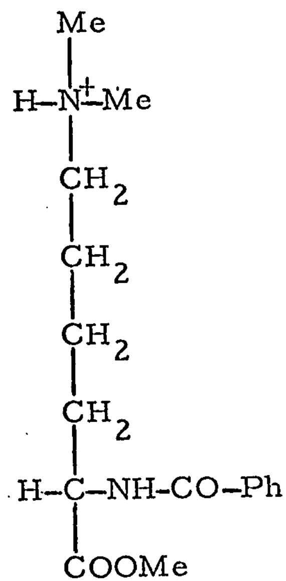




good substrate



substrate



not a substrate

to learn something about the exact specificity and the mechanism of action of carboxypeptidase B; therefore peptides such as α-N-hippuryl, ε-N-methyl-L-lysine and α-N-hippurylglycyl, ε -N-methyl-L-lysine are required.

In summary, the synthesis of ε -N-alkylated-lysines is of interest for three main reasons:

(1) to assist in the identification and analysis of these amino acids when they occur in natural products;

(2) to assist in further studies on the role of ε -N-methylated-lysines in histone biochemistry;

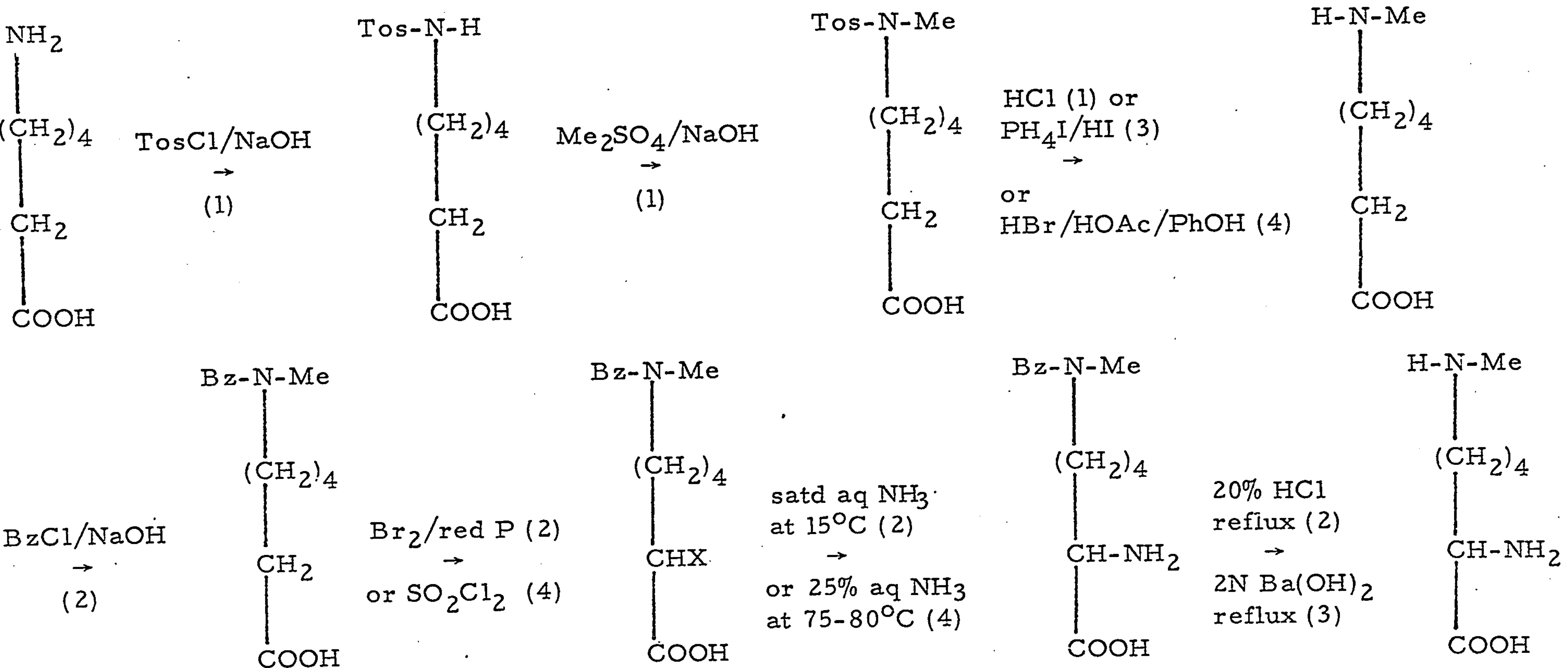
(3) to allow further studies on the lysine specific enzymes trypsin and carboxypeptidase B.

This thesis describes an investigation, the object of which was to devise new or improved methods for synthesising optically active ε-N-alkyllysines. An ideal method would be the one that gave directly intermediates (such as ε -N-carbobenzoxy,-ε -N-methyl-L-lysine methyl ester) useful for the synthesis of the peptides and the other derivatives required for further studies on trypsin, carboxypeptidase B and ε -alkyllysinase.

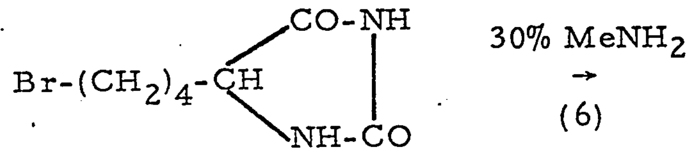
ε -N-Methyllysine is available from ε -aminocaproic acid [Enger and Steib (1930), Neuberger and Sanger (1944) and Poduska (1959)] by route 1 (Fig. I. 103) and from 5-(δ-bromobutyl)-hydantoin [Babineau and Berlinguet (1962)] by route 2 (Fig. I. 103). None of these syntheses is adaptable for the preparatinn of optically active material. Benoiton (1964) has reported the only successful

Fig. I. 103. Synthetic routes to ε-N-methyl-DL-lysine

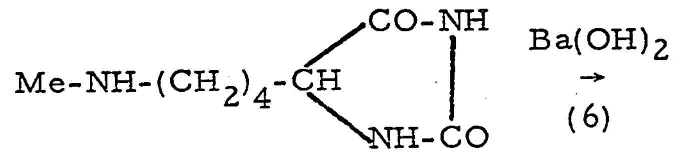
Route 1:



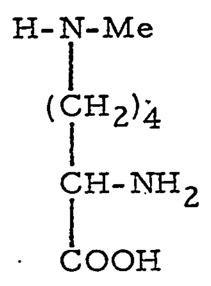
Route 2:



30% $MeNH_2$ → (6)



$Ba(OH)_2$ → (6)



(1) Thomas and Gorne (1918)

(2) Enger and Steib (1930)

(3) Neuberger and Sanger (1944)

(4) Poduska (1959)

(5) Prepared according to Gaudry (1948)

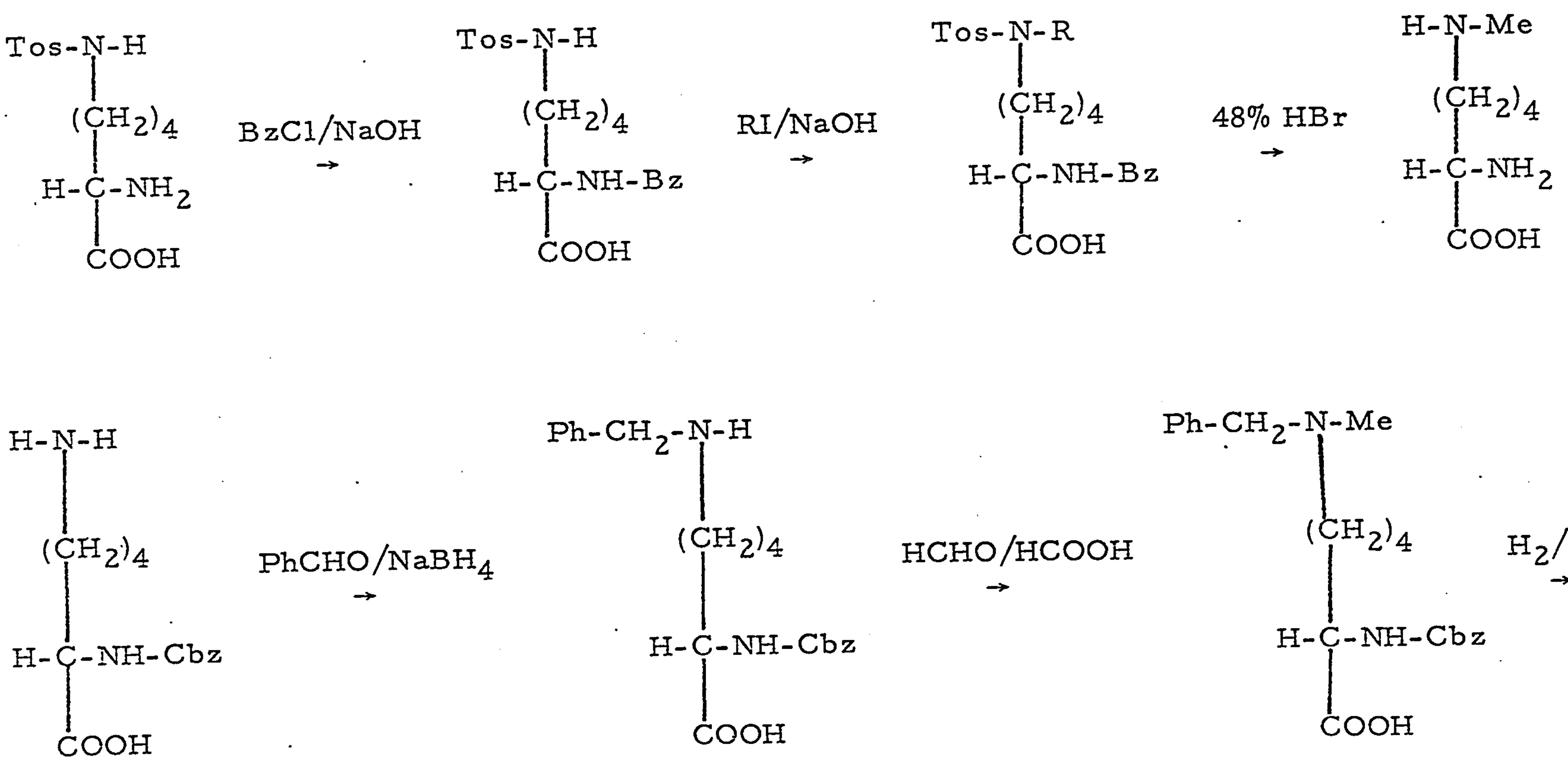
(6) Babineau and Berlinguet (1962)

syntheses of optically active ε-N-methyl-L-lysine. Two different routes were employed (Fig. I. 104). The first involved the methylation of an α-N-protected, ε-N-tosyllysine with 2 N NaOH and methyl iodide at 70°C using the methylation procedure of Fischer and Lipschitz (1915). The second method started from α-N-carbobenzoxylysine and involved the preparation of the ε-N-benzyl derivative which was then methylated with formic acid/formaldehyde. This method had been previously used by Quitt, Hellerbach and Vogler (1963) for the preparation of α-N-methylamino acids.

The Fischer-Lipschitz method gave a product that was contaminated with up to 10% of lysine and purification could only be effected by treatment with the enzyme lysine decarboxylase. The conditions required to remove the benzoyl and tosyl protecting groups were extremely vigorous and could lead to some racemisation. The Quitt-Hellerbach-Vogler method required as starting material α-N-carbobenzoxylysine [Bezas and Zervas (1961)] which is difficult to prepare [Benoiton and Berlinguet (1966)]. It gave a pure product but the method was not generally applicable for other alkylations. No intermediates suitable for use in peptide synthesis were obtained by either procedure.

Besides the Fischer and Quitt methods already referred to, two other methods for the synthesis of optically active mono-N-methylamino acids are described in the literature. The first, due to Fischer and Mechel (1916), involves the methylamination of an optically active haloacid. This method is theoretically of general application, providing the optically active haloacid is available. In practice it has

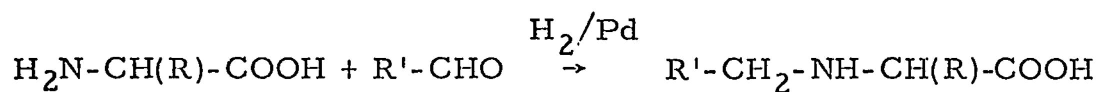
Fig. I. 104. Synthetic routes to ε -N-methyl-L-lysine [Benoiton (1964)].

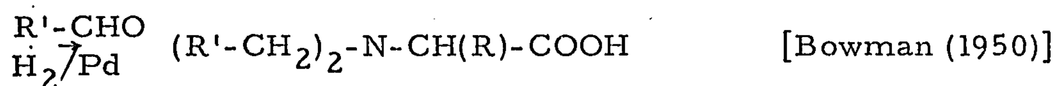


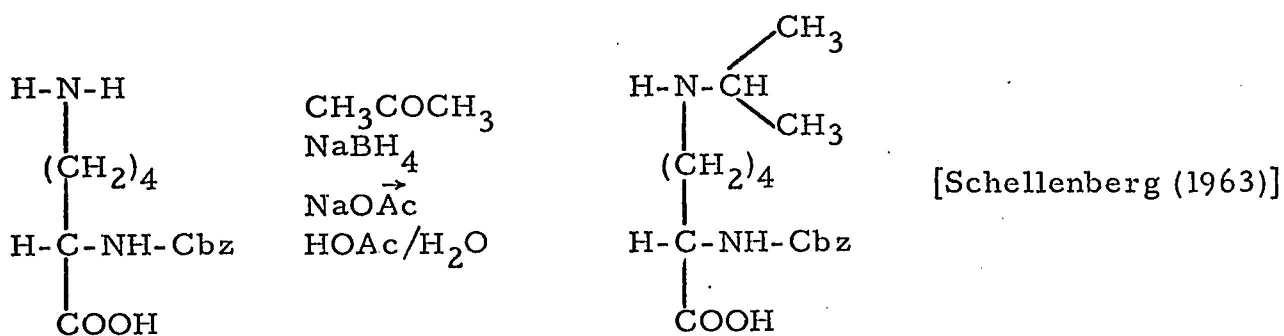
only been applied to the synthesis of α-N-methylamino acids since, for these compounds, the appropriate haloacids may be readily obtained by treating the corresponding amino acid with a nitrosyl halide. At best the method is rather tedious and in the case of ε-N-alkyllysines it appeared that the preparation of the starting material would be particularly difficult. In the second method (see Fig. I. 105), which is due to Bowman (1950), amino acids are condensed with aldehydes in the presence of hydrogen and palladised charcoal. This is not a very satisfactory method since dimethylamino acids are invariably formed with formaldehyde. With the higher aldehydes glycine and alanine give dialkylamino acids and the other amino acids give mixtures of mono- and di-alkylated products. In favourable cases steric factors lead to the formation of a single product, e.g. N-butylvaline [Bowman (1950)] ; however, it seemed unlikely that it would be possible to obtain pure monoalkylated products from α-N-protected-lysines and the separation of a mixture of ε-mono- and ε-di-alkyllysines would be extremely difficult. A modified version of the method has been described by Schellenberg (1963) who reduced the intermediate Schiff base produced by the condensation of acetone and α-N-carbobenzoxylysine with sodium borohydride giving the ε -isopropyl derivative of lysine in good yield. Application of this method to the preparation of ε -N-ethyllysine from acetaldehyde and α-N-carbobenzoxylysine gave a mixture of the mono- and di-ethyl products. None of these methods was very promising. It was therefore decided to look for an entirely new method of synthesising optically active N-alkylamino acids including ε -N-alkyllysines.

Fig. I. 105

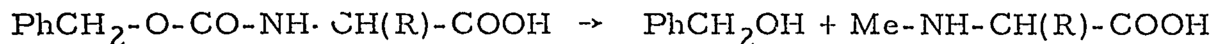
Synthesis of N-alkylamino acids by the reductive condensation of amino acids with aldehydes and ketones



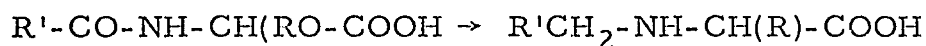




The first approach involved the use of metal hydrides to reduce N-carbobenzoxy- and N-acyl-amino acids to N-methyl- and N-alkyl-amino acids respectively.

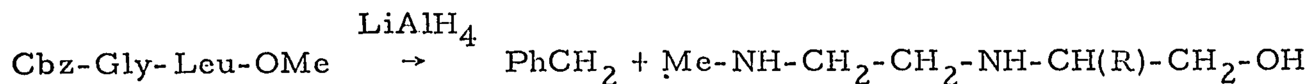


$$PhCH_2\text{-}O\text{-}CO\text{-}NH\cdot CH(R)\text{-}COOH \rightarrow PhCH_2OH + Me\text{-}NH\text{-}CH(R)\text{-}COOH$$



$$R'\text{-}CO\text{-}NH\text{-}CH(RO\text{-}COOH \rightarrow R'CH_2\text{-}NH\text{-}CH(R)\text{-}COOH$$

Karrer and Nicolaus (1952) had shown that carbobenzoxyglycylleucine methyl ester was reduced by lithium aluminium hydride to an N-methylamino alcohol and benzyl alcohol:



$$Cbz\text{-}Gly\text{-}Leu\text{-}OMe \xrightarrow{LiAlH_4} PhCH_2 + Me\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}NH\text{-}CH(R)\text{-}CH_2\text{-}OH$$

Some preliminary studies were therefore carried out on the reduction of carbobenzoxyamino acids (Coggins and Benoiton, unpublished results). The problem was to find a way of selectively reducing carbobenzoxyamino groups to methylamino groups in the presence of carboxyl groups. Lithium aluminium hydride reduced carbobenzoxybenzylamine to N-methylbenzylamine in 95% yield and experiments with carbobenzoxyamines showed that the reaction was a general one. Under the same conditions carbobenzoxyamino acids and their salts were reduced to N-methylamino alcohols. Some milder reducing agents were tried; lithium borohydride failed to reduce carbobenzoxybenzylamine while lithium aluminium tri-t-butoxyhydride gave a satisfactory yield of N-methylbenzylamine. When carbobenzoxyalanine was treated with the latter reagent alanine was recovered

in 52% yield. These results were not very encouraging and since, by this time, another method had begun to give promising results, we did not investigate this method any further.

The second approach was to attempt the direct methylation of N-acyl- and N-carbobenzoxy-amino acids with methyl iodide and a suitable base. Das, Gero and Lederer (1967) had reported the successful permethylation of peptides using methyl iodide and silver oxide with DMF as solvent. They pointed out that this reaction would allow the preparation of monomethyl derivatives of primary amines by the route:

$$R\text{-}NH_2 \rightarrow R\text{-}NH\text{-}Ac \rightarrow R\text{-}N(Me)\text{-}Ac \rightarrow R\text{-}NH\text{-}Me$$

A literature search revealed that much work had been done on the methylation of amides, although modern text-books of organic chemistry rarely discuss it.

The alkylation of acetanilide was first described by Hepp in 1877. Acetanilide was converted to its sodium salt by treatment with sodium in xylene; the sodium derivative, when heated with methyl iodide, gave acetyl-N-methylaniline which, on acid hydrolysis, yielded N-methylaniline. Benzanilide [Hepp (1877)] and formanilide [Norton and Livermore (1887)] were also used in the synthesis of N-methylaniline by analogous routes. Pictet (1887) showed that the reaction could be carried out using a hot solution of KOH in 95% alcohol and methyl iodide. A number of N-alkylanilines were prepared by this route [Pictet and Crepieux (1888)], e.g. ethyl, <u>n</u>-propyl, <u>iso</u>-propyl

and iso-amyl. Later Thielpape and Fulde (1935) found that methylation of acetanilide went equally well when dimethyl sulfate was used instead of methyl iodide. All this early work was concerned with the alkylation of anilides or naphthalides [the first example of the latter had been described by Pschorr and Karo (1906)].

In 1949, Fones reported the use of sodium hydride in boiling xylene to prepare the sodium derivatives of a number of mono-substituted amides which were then alkylated with various alkyl halides. Fones alkylated both aromatic and aliphatic amides; however, he failed to methylate p-nitroacetanilide. Pachter and Kloetzel (1952) succeeded in alkylating the latter compound using solid KOH and methyl iodide in refluxing acetone.

The alkylation of tosylamides has also been widely employed as a route to secondary amines. Hinsburg (1891) reported the methylation of a sulfonamide using dimethyl sulfate and aqueous alkali. Later authors, e.g. Hinsburg and Strupler (1895), sometimes used alkyl halides instead of dimethyl sulfate. Fischer et al (1913, 1915) applied this method to the synthesis of some N-methylamino acids. The methylation of optically active tosylamino acids, using methyl iodide and 2 N alkali, gave tosyl-N-methylamino acids which, on hydrolysis with hot concentrated HCl, yielded optically active N-methylamino acids. One limitation of the method was the very vigorous hydrolytic conditions required to remove the tosyl group [see, e.g. Blanchard et al, (1944)]. Later, du Vigneaud and Behrens (1937) demonstrated that tosyl groups could be cleaved with sodium in liquid ammonia and took advantage of this in the synthesis of N-methyl-L-histidine.

Although the protecting group was readily removed under these conditions, the isolation of the final product was rather troublesome. As has already been mentioned, Benoiton (1964) had shown that the application of this method to the synthesis of ε-N-methyllysine gave an impure product.

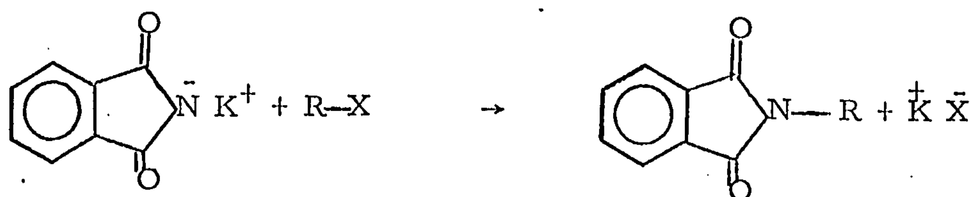
Urethanes were successfully alkylated using the Hepp method [Kraft (1890)]. More recently Dannley and Lukin (1957) have reported the use of sodium hydride and alkyl halides for the alkylation of urethanes.

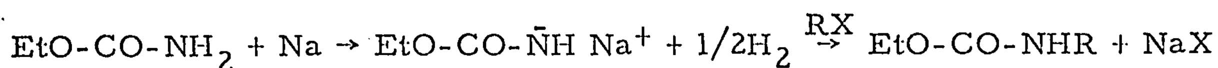
The alkylation of cyclic amides (lactams) has also been reported, e.g. caprolactam on treatment with excess dimethyl sulfate gave N-methyl caprolactam [Benson and Cairns (1948)] and diketopiperazine (which is in effect a cyclic amide) gave, on treatment with methyl iodide and silver oxide, sarcosine anhydride [Karrer et al, (1922)].

The preparation of α-N-methyllysine from α-N-tosyl, ε-N-benzoyllysine by Izumiya and Ota (1951) and the subsequent preparation of ε-N-methyllysine from α-N-benzoyl, ε-N-tosyllysine by Benoiton (1964) demonstrates that tosylamino groups are much easier to methylate than benzoylamino groups. The reactivity of tosylamino groups is due to the nucleophilic character of the nitrogen atom. The tosyl substituent does not completely suppress the nucleophilicity of the nitrogen as, for example, a benzoyl substituent does [Bodanszky and Ondetti (1966), p. 25]. The reactivity of tosyl-protected-amino acids is well known to peptide chemists who have sometimes found that the protection afforded by the tosyl group is insufficient, e.g. Bodanszky and Birkhimer (1960) observed spontaneous lactam formation

with carboxyl activated α-N-carbobenzoxy, δ-N-tosylornithine. These results suggested that the alkylation of acyl- and alkyloxycarbonyl-amino acids would require much more vigorous conditions than the alkylation of tosylamino acids; the use of a strong base such as sodium or sodium hydride would be essential.

In peptide synthesis the most commonly used N-protecting group is the carbobenzoxy group [Bergmann and Zervas (1932); Rydon (1962)]; this group has the great advantage of being easy to remove [see Bodanszky and Ondetti (1966), p. 25]. Carbobenzoxyamino acids are urethanes. It was therefore decided to study the methylation of carbobenzoxyamino acids using methyl iodide and sodium hydride; these reagents had been successfully used by Dannley and Lukin (1957) for the methylation of urethanes. Dannley and Lukin used a variety of solvents including toluene, xylene, excess alkyl halide and DMF. With DMF as solvent the yields were better than with xylene or toluene, although the improvements in yield were not as remarkable as those obtained by Sheehan and Bolhoffer (1950) when DMF was used instead of xylene in the analogous Gabriel synthesis. Both these reactions involve anionic intermediates:

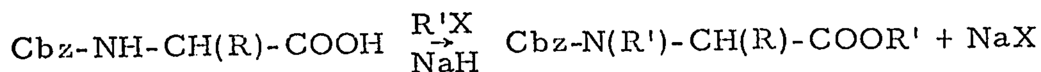




$$EtO\text{-}CO\text{-}NH_2 + Na \rightarrow EtO\text{-}CO\text{-}\bar{N}H\ Na^+ + 1/2H_2 \xrightarrow{RX} EtO\text{-}CO\text{-}NHR + NaX$$

Evidence for the participation of a salt intermediate in the alkylation of urethanes was obtained by Kraft (1890) who isolated the sodium salt of ethyl carbamate after treatment of an ethereal solution of the urethane with sodium. Two factors contribute to the enhanced rates of both reactions in DMF. One is the high solubility of the reagents and intermediates in the solvent [Sheehan and Bolhoffer (1950)]. The other is the high reactivity of the anionic intermediates in DMF solution; this high reactivity is due to poor solvation of the anions in the dipolar aprotic solvent [Parker (1962)]. These results suggested that the use of DMF as a solvent in the alkylation of carbobenzoxyamino acids might be an advantage.

The following reaction was expected between carbobenzoxyamino acids and alkyl halides in the presence of sodium hydride:



$$Cbz\text{-}NH\text{-}CH(R)\text{-}COOH \xrightarrow[NaH]{R'X} Cbz\text{-}N(R')\text{-}CH(R)\text{-}COOR' + NaX$$

Because the reaction product was an ester, the possibility of α-C-alkylation had to be considered. Sodium hydride is a strong base, e.g. it is frequently used as the base in malonic ester syntheses. A reaction between sodium hydride, ethyl acetate and benzyl chloride giving ethyl dibenzylacetate has been reported [Cristol et al (1948)]. Since α-C-alkylation would be a very undesirable side reaction, and, even more important, because any ionisation of the α-CH group would lead to racemisation, it was clear that the use of excess sodium hydride had to

be avoided. Further, as reactions involving anions take place more readily in DMF than in most other solvents [Parker (1962)], the use of DMF as a solvent would promote the side reaction involving α-C-alkylation. Therefore it was decided not to use neat DMF as a solvent for the reaction. THF was chosen as an alternative because carbobenzoxy-amino acids dissolve in it readily and it can be refluxed at moderate temperatures (65 - 70°C). The salt intermediates are not very soluble in THF but this can be partially remedied by adding small amounts of DMF.

It was hoped that this route would provide an efficient method of synthesising N-alkylamino acids in general and could also be adapted for the synthesis of ε-N-alkyllysines. It seemed a particularly attractive route because there was the possibility of obtaining directly intermediates useful for peptide synthesis, e.g. carbobenzoxy-N-methylamino acids and N-methylamino acid methyl esters.

II.

MATERIALS AND METHODS

II. 1. MATERIALS

(a) Preparation and purification of reagents

Acetic anhydride: Reagent grade acetic anhydride (Fisher) was redistilled and the fraction boiling at 135 - 137°C (760 mm) collected.

Benzaldehyde: Reagent grade benzaldehyde (Fisher) was washed with aq. 10% Na_2CO_3 and then water. After drying ($MgSO_4$) it was redistilled under nitrogen; the fraction boiling at 79°C (25 mm) was collected [Vogel (1964), p. 694].

N-Carboethoxyphthalimide: This was prepared by a modified version of the method of Nefkens et al (1960). Phthalimide (29 g; 200 mmol) was stirred overnight with DMF (100 ml) and triethylamine (28 ml; 200 mmol). Solution was incomplete and so more DMF (25 ml) and triethylamine (7 ml; 50 mmol) were added and the mixture warmed to 40°C and stirred until all the solid had dissolved. Then the solution was cooled to 0°C and stirred vigorously while a total of 20 ml (200 mmol) of ethyl chloroformate was added in 10 equal portions over a period of 45 min. Stirring was continued at 0°C for 45 min and then at 25°C for 1 h. The reaction mixture was poured into water (600 ml) with stirring and the white precipitate collected and dried under vacuum over P_2O_5. The crude yield was 37. 0 g (84%); this was recrystallised from 96% ethanol to give white needles (32. 0 g; 73%), m. p. 90°C. A second recrystallisation from 96% ethanol gave

29.2 g (67%), m.p. 90 - 91°C, lit. m.p. 80°C [Nefkens et al (1960)]. N.b.: The product appeared to undergo a change of crystalline form at 82 - 83°C. (Pierce give m.p. 90-91°C in their catalogue).

Chloroform: This was purified according to Fieser (1957), p. 283.

Diazomethane: Ethereal solutions of diazomethane were prepared from N-methyl, N-nitroso-*p*-toluenesulfonamide by the method of Vogel (1964), p. 969.

Dimethylformamide: This was purified by shaking with solid KOH and then CaO, followed by distillation [Leader and Gormley (1951)]. The fraction distilling at 151 - 154°C (753 mm) was collected.

30% Hydrogen bromide in acetic acid: This was prepared by passing dry HBr gas into ice-cold acetic acid in a flask protected with a drying tube until the theoretical weight increase had occurred.

Silver oxide: Reagent grade silver oxide (Fisher) was purified in 10 g batches by washing with water (50 ml), methanol (50 ml) and ether (50 ml). The product was dried under vacuum over P_2O_5 for 16 h [Thomas et al (1968a)].

Tetrahydrofuran: For the early part of this work THF was purified by shaking with solid KOH and then distilling from lithium aluminium hydride [Corey (1966), p. 94]. This purified solvent was found to peroxidise very readily. Later THF supplied by Baker which contained butylated hydroxytoluene was used without purification.

<u>Thionyl chloride:</u> Reagent grade thionyl chloride (Baker) was purified by distilling first with quinoline and then with raw linseed oil as described by Fieser (1957), p. 345.

<u>Triethylamine:</u> This was dried over CaO and then distilled.

<u>Trityl chloride:</u> Reagent grade trityl chloride (Pierce) was purified by recrystallisation from benzene containing 5% by volume of acetylchloride [Vogel (1964), p. 815]. Only product melting in the range 111 - 113°C was used. The recrystallised material was stored in a desiccator and its m. p. checked before use.

(b) Reagents obtained from commercial suppliers

Alumina (adsorption), 80 - 200 mesh [F]; Boron trifluoride etherate [E]; Carbobenzoxy chloride [P]; 2, 2-Dimethoxypropane [E]; Dowex 50W-X8, 20-50 mesh [B]; Ethyl iodide [B]; Methyl iodide [F]; 10% Palladium on powdered charcoal catalyst [MCB]; n-Propyl iodide [B]; Sodium hydride, 50% dispersion in oil [BDH].

All the other materials used were normal reagent grade.

(c) Sources of amino acids and peptides

(i) Amino acids and N-methylamino acids:

L-Alanine [G]; α-Aminobutyric acid [S]; γ-Aminobutyric acid [N]; α-Aminoisobutyric acid [S]; ε-Aminocaproic acid [S]; L-Asparagine [N]; Glycine [B]; L-Isoleucine [G]; L-Leucine [G]; DL-Leucine [G]; L-Lysine monohydrochloride [N]; N-Methyl-DL-alanine [C]; α-C-Methyl-DL-leucine [C]; N-Methyl-L-leucine [C]; ε-N-Methyl-L-lysine monohydrochloride and ε-N-Ethyl-L-lysine monohydrochloride were gifts of Dr. N.L. Benoiton; L-Phenylalanine [G]; Sarcosine [C]; L-Valine [G].

(ii) Amino acid derivatives: L-Alanine N-carboxyanhydride [Mi]; t-Butyloxycarbonyl-L-leucine [Mi]; Carbobenzoxy-L-aspartic acid [Ma]; ε-Carbobenzoxy-L-lysine [P]; Carbobenzoxy-L-tyrosine [Ma]; ε-N-Acetyl-L-lysine and N-Trityl-L-leucine were gifts of Dr. N.L. Benoiton.

(iii) Peptides: Carbobenzoxyglycyl-L-leucine methyl ester [C]; Carbobenzoxy-L-leucylglycine methyl ester [C]; Glycylglycine [P]; Glycyl-L-leucine [Ma] and L-Leucylglycine [Ma] were gifts of Dr. A.T. Matheson; Poly-L-leucine [Mi]; N-Trityl-L-leucyl-L-alanine methyl ester was a gift of Dr. N.L. Benoiton.

(d) List of Manufacturers

[B]	J.T. Baker, Phillipsburg, New Jersey, U.S.A.
[BDH]	British Drug Houses, Poole, Dorset, England.
[C]	Cyclo Chemical Corporation, Los Angeles, California, U.S.A.
[E]	Eastman Organic Chemicals, Rochester, New York, U.S.A.
[F]	Fisher Scientific, Fair Lawn, New Jersey, U.S.A.
[G]	General Biochemicals, Chagrin Falls, Ohio, U.S.A.
[Ma]	Mann Research, New York, U.S.A.
[MCB]	Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.
[Mi]	Miles Laboratories Inc., Elkhart, Indiana, U.S.A.
[N]	Nutritional Biochemicals Corporation, Cleveland, Ohio, U. S. A.
[S]	Sigma Chemical Corporation, Saint Louis, Missouri, U.S.A.

II. 2. METHODS

(a) Chemical

Hydrogenations: All the hydrogenations were carried out at 1 atm pressure in an apparatus similar to that described by Wiberg (1960), p. 228.

Melting points: These were determined using a Hoover Capillary Melting Point Apparatus (supplied by A. H. Thomas, Philadelphia); the values given are uncorrected.

Microanalyses: These were carried out by Dr. C. Daessle, Montreal, Quebec, Canada.[a]

Peptide hydrolyses: The peptide (20 - 100 mg) was placed in a pyrex glass test tube (capacity 20 ml) which carried a spherical glass joint. 6N HCl (5 - 10 ml) was added and the tube closed by clamping in place a vacuum stopcock which was attached to a glass joint matching the joint on the test tube. The sample was frozen solid by immersing the tube in liquid nitrogen; then the tube was evacuated and the stopcock closed. The sample was allowed to thaw out slowly and then placed in an oven at 110°C for 1 - 4 days. At the end of the hydrolysis the sample was transferred to a 100 ml round bottomed flask and evaporated to dryness. The residue was re-evaporated several times with water, then dissolved in water, diluted with pH 2. 2 diluter buffer and analysed for amino acids.

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[a] Samples for analysis were dried in vacuo over P_2O_5 for 2 h. at 65°C.

Pre-drying of THF and DMF: These solvents were pre-dried immediately before use in the alkylation experiments by treatment with 0.2 g of sodium hydride dispersion per 10 ml. After filtratinn the solvent or mixture of solvents was used immediately.

Preparation of a standard solution of carbobenzoxyleucine: Carbobenzoxyleucine (ca. 10.6 g; 40 mmol) was dissolved in THF (50 ml) and the mixture stored in a glass stoppered volumetric flask. The exact concentration of carbobenzoxyleucine was obtained as follows: a 5 ml aliquot was removed, evaporated to an oil and then treated with 30% HBr in acetic acid (10 ml) for 1 h at 25°C; after evaporation the product was dissolved in water (50 ml) and the solution made up to 200 ml exactly; a 1 ml aliquot was diluted to 10 ml with pH 2.2 diluter buffer and a 1 ml sample analysed on the long column of the amino acid analyser eluting with pH 4.25 buffer. For the alkylation experiments, suitable volumes of the solutinn were transferred to the reaction flasks and evaporated to leave the required amount of carbobenzoxyleucine as an oil in the flask. This was a more accurate way of obtaining exact amounts of carbobenzoxyleucine than weighing it out directly.

Refluxing conditions: In all the alkylation experiments and in the boron trifluoride catalysed esterifications the reaction flasks were heated in a wax bath at 70 - 80°C. Under these conditions THF and methanol reflux vigorously. The condensers were always fitted with drying tubes.

Rotations: Optical rotations were measured with a Perkin Elmer Model 141 Polarimeter equipped with a 1 dm cell.

(b) Spectroscopic

Infrared spectra were obtained using a Unicam Model SP200 Spectrophotometer. The spectra were scanned in the range 650 - 5000 cm^{-1} and the polystyrene bands at 1495 and 1603 cm^{-1} were used as references. Oils were run as liquid films between NaCl plates and solids were run either as nujol mulls or as KBr discs (0.5 - 1.0 mg of sample in 250 mg of KBr).

A Unicam Model SP800 Spectrophotometer was used to measure the visible spectra. The didymium bands at 673 and 686 nm were used as references and the spectra were scanned in the range 300 - 700 nm.

Nuclear magnetic resonance spectra were obtained using a Varian Model T60 Spectrometer. The probe temperature was 34°C and the samples were run as 10% w/v solutions in deuterochloroform, trifluoracetic acid or deuterium oxide. Tetramethylsilane 1% v/v was added to the solvents as an internal standard except in the case of deuterium oxide when sodium 3-(trimethylsilyl)-propane sulfonate 1% w/v was used (the latter compound was a gift of Dr. I.C.P. Smith). Spectra were scanned in the range 0 - 10τ in trifluoracetic acid and deuterium oxide solutions and in the range -2 - 10τ in deuterochloroform solutions.

(c) Chromatographic

(i) Thin layer chromatography: Plates were prepared in the following way. 30 g of silica gel GF_{254} (prepared according to Stahl by E. Merck AG, Darmstadt) was shaken with 60 ml of water in a stoppered flask for 1.5 min to give a slurry which was immediately spread as a 0.25 mm layer on a bed of six 20 x 20 cm glass plates (or a corresponding area of 5 x 20 cm plates) using a Stahl applicator (supplied by C. Desaga GmbH, Heidelberg). The plates were left to dry horizontally at 25°C overnight; they were not activated by heating.

Most of the compounds investigated by this technique were ninhydrin positive; they were detected by spraying the plates with a 0.5% solution of ninhydrin in n-butanol and then heating in an oven at 100°C for 5 min. The colour of the spots varied considerably from compound to compound, being either yellow, brown or red. Ninhydrin negative compounds containing one or more -NH- groups were detected using the chlorine/starch/potassium iodide method of Rydon and Smith (1952). The plates were exposed to chlorine gas in a chromatography tank for 0.5 min, then placed for 1 h in a stream of air (25°C) from a hair dryer and finally sprayed with an aqueous solution containing 0.5% starch and 0.5% potassium iodide. The spots were dark blue and frequently there was a white patch in the middle. Plates which had previously been treated with ninhydrin could still be chlorinated and stained with the starch/KI reagent since chlorine bleached the ninydrin spots.

Two solvent systems were used: n-butanol/acetic acid/water (4/1/1, v/v/v) designated the B4/A1/W1 solvent system and chloroform/methanol (9/1, v/v) designated the C9/M1 solvent system. All the compounds investigated by t. l. c. were derivatives of lysine; in Table II. 201 some representative R_f values are given.

(ii) Amino acid analyses: Amino acid analyses were carried out using a Beckman Model 120B Automatic Amino Acid Analyser essentially according to the method Spackman, Stein and Moore (1958). The instrument was fitted with three columns: a long column (0.9 x 50 cm) containing AA-15 resin (Beckman Instruments, Palo Alto, California), a short column (0.9 x 7 cm) containing AA-27 resin (Beckman) and a third column (0.9 x 15 cm) containing Aminex A-5 resin (Bio Rad Laboratories, Richmond, California). Four different eluting buffers were used: 0.20 N sodium citrate, pH 3.28; 0.20 N sodium citrate, pH 4.25; 0.35 N sodium citrate, pH 5.28, and 0.35 N sodium citrate, pH 6.48. The individual amino acids were determined by the H x W method of Spackman, Stein and Moore (1958) which involves use of the formula

$$\mu mol = \frac{H \times W}{C}$$

Constants for the common amino acids were determined using a calibration mixture (obtained from Bio Rad) and for the other amino acids by using standard solutions. The pH of samples was adjusted to 2.2 before analysis; when necessary the samples were diluted with 0.20 N sodium citrate buffer, pH 2.2 (called sample diluter), so that

Table II. 201

R_f values for some lysine derivatives on silica gel G

Lysine derivative	R_f in B4/A1/W1[a]	R_f in C9/M1[b]
Cbz-Lys(Cbz)-OH	0.84	
Cbz-Lys(Trt)-OMe	0.81	
Trt-Lys(Cbz)-OMe	0.79	runs at solvent front
H-Lys(Cbz)-OBzl	0.62	
Trt-Lys-OMe	0.54	0.09
H-Lys(Cbz)-OMe	0.48	0.66
H-Lys(Cbz)-OH	0.45	0.02
Cbz-Lys-OMe	0.44	
Cbz-Lys-OH	0.40	does not move
H-Lys-OMe	0.09	does not move
H-Lys-OH	0.06	does not move

a n-Butanol/acetic acid/water (4/1/1, v/v/v).

b Chloroform/methanol (9/1, v/v).

the final concentration was in the range 0.1 - 1.0 μmol/ml (1 ml samples were analysed). Analyses carried out on the same day or on successive days were reproducible to ± 1%. The reproducibility from week to week was not quite so good and new constants were determined for each group of analyses. Some representative elution times and constants are given in Tables II. 202 - II. 204.

Table II. 202

Amino acid analyser data for the 50 cm column of AA-15 resin

Amino acid	Elution conditions[a]	Elution time (min)	Constant
Aspartic acid	1	44.5	20.2
Glycine	1	89.0	20.6
Alanine	1	95.5	19.6
α-Aminoisobutyric acid	1	104.0	1.8[b]
α-Aminobutyric acid	1	108.0	21.4
Valine	1	121.5	19.9
α-C-Methylleucine	1	145.0	2.0
Isoleucine	1	146.5	22.1
Leucine	1	150.5	22.1
Tyrosine	1	170.0	21.9
Phenylalanine	1	176.5	21.3
Glycyl-L-leucine	1	211.0	-
γ-Aminobutyric acid	1	304.0	-
α-C-methylleucine	2	46.5	2.0
Leucine	2	51.0	23.9
γ-Aminobutyric acid	3	45.5	24.1
α-N-Methyl, ε-N-methyllysine	3	148.0	-
Lysine	3	165.0	22.1
ε-N-Methyllysine	3	180.0	20.2

a Column temperature 57°C; buffer flow rate 68 ml/h; condition 1, pH 3.28 buffer for 85 min followed by pH 4.25 buffer; condition 2, pH 4.25 buffer only and condition 3, pH 5.28 buffer only.

b Unsymmetrical peak.

Table II. 203

Amino acid analyser data for the 7 cm column of AA-27 resin

Amino acid	Elution conditions[a]	Elution time (min)	Constant
ε-N-carbobenzoxy-lysine	1	40.0	25.9
Lysine	1	86.0	28.3
α-N-Carbobenzoxy-lysine	1	110.0	6.4
ε-N-Acetyllysine	2	10.0	21.9
ε-N-Carbobenzoxy-lysine	2	22.5	21.9
α-N-Carbobenzoxy-lysine	2	23.0	7.7
Lysine	2	28.0	23.8
Ammonia	2	36.0	14.1

a Column temperature 57°C; buffer flow rate 68 ml/h; condition 1, pH 4.25 buffer for 95 min then pH 5.28 buffer; condition 2, pH 5.28 buffer only.

Table II. 204

Amino acid analyser data for the 15 cm column of Aminex A- 5 resin

Amino acid	Elution conditions[a]	Elution time (min)	Constant
α-N-Methyl, ε-N-methyllysine	1	94.0	9.3
Lysine	1	103.0	44.1
ε-N-Methyllysine	1	114.5	40.6
ε-N-Ethyllysine	1	137.0	35.5
ε-N-Propyllysine	1	205.0	-
Lysine[b]	2	84.0	43.5
ε-N-Methyllysine[b]	2	97.5	39.7
ε-N, ε-N-Dimethyllysine[b]	2	105.0	39.2
ε-N, ε-N ε-N-Trimethyllysine[b]	2	111.5	35.5

a Column temperature 25°C; buffer flow rate 34 ml/h; condition 1, pH 5.28 buffer only; condition 2, pH 6.48 buffer only.

b The standard solutions used for determining these constants were donated by Dr. J.H. Seely[(see Seely et al (1969a)].

III.

PREPARATION OF STARTING MATERIALS

III. 1. ACYLAMINO ACIDS

Acetylglycine: This was prepared using the method described by Greenstein and Winitz for the preparation of chloracetyl-DL-alanine [Greenstein and Winitz (1961), procedure 21-7, p. 1834]. 4N Sodium hydroxide was used instead of 2 N and acetic anhydride instead of chloracetyl chloride. After the addition of the reagents was complete the reaction mixture was stirred for 2 h in the cold; then it was acidified to pH 2 and the product collected by filtration, washed with 0.2 N HCl and dried under vacuum over NaOH. On the scale 100 mmol the yield was 7.86 g (67%), m.p. 205 - 206°C d., lit. m.p. 206°C d. [Dakin (1929)].

Acetyl-L-alanine: This was prepared by a modified version of the method used for acetyl-L-leucine. The NaOH used was 4 N and after acidification at the end of the reaction the product was obtained by extraction into ethyl acetate (3 x 50 ml). The combined ethyl acetate extracts were dried ($MgSO_4$), filtered and evaporated to give a crystalline solid which was recrystallised from a small volume of boiling ethyl acetate. On the scale 100 mmol the final yield was 3.46 g (26%), m.p. 126 - 127°C, lit. m.p. 125°C [Greenstein and Winitz (1961), p. 1832].

Acetyl-L-valine: This was prepared by the method used for acetyl-L-leucine. On the scale 50 mmol the yield after recrystallisation from water was 4.98 g (63%), m.p. 169 - 169.5°C, lit. m.p. 168°C [Greenstein and Winitz (1961), p. 2376].

Acetyl-L-isoleucine: This was prepared by the method used for acetyl-L-leucine. On the scale 50 mmol the yield after recrystallisation from water was 4.80 g (55%), m.p. 151°C, lit. m.p. 150°C [Greenstein and Winitz (1961), p. 2065].

Acetyl-L-leucine: L-Leucine (6.55 g; 50 mmol) was dissolved in 2 N NaOH (25 ml) and the solution cooled in an ice-bath. Then a total of 30 ml of 2 N NaOH and 5 g (50 mmol) of acetic anhydride were added in 5 equal portions, alternately over a period of 30 min with vigorous mechanical stirring and cooling in an ice-bath. Stirring was continued in the cold for 1 h with small additions of N NaOH to keep the pH alkaline. Then the pH was adjusted to 2 with 4N HCl and after stirring for 10 min in the cold the solid product was collected by filtration, washed with a little ice-cold water and dried under vacuum over P_2O_5. Recrystallisation from 50% acetic acid gave white needles (5.91 g; 68%), m.p. 183 - 184°C, lit. m.p. 185°C [Greenstein and Winitz (1961), p. 2093].

Acetyl-DL-leucine: This was prepared from L-leucine by the method of Greenstein and Winitz (1961), procedure 10 - 8, p. 834. On the scale 50 mmol the yield was 6.08 g (70%), m.p. 157 - 159°C, lit. m.p. 159°C [Greenstein and Winitz (1961), p. 834].

Acetyl-L-phenylalanine: This was prepared by the method used for acetyl-L-leucine except the NaOH was N. On the scale 12.5 mmol the yield after recrystalliation from water was 1.93 g (74%), m.p. 170 - 171.5°C, lit. m.p. 171°C [Greenstein and Winitz (1961), p. 2174].

Benzoylglycine: This was a gift of Mrs. R. Demayo; the m.p. was 189.5 - 190.5°C, lit. m.p. 187.5°C [Greenstein and Winitz (1961), p. 1270].

Benzoyl-L-alanine: This was a gift of Mr. G.J. Moore; the m.p. was 149.5°C, lit. m.p. 150 - 151°C [Greenstein and Winitz (1961), p. 1269].

Benzoyl-L-leucine: This was prepared in a way similar to that described by Greenstein and Winitz (1961), procedure 10 - 178, p. 1267. L-Leucine (13.1 g; 100 mmol) was dissolved in ice-cold 2 N NaOH (60 ml) and a total of 15 g (110 mmol) of benzoyl chloride and 60 ml of 2 N NaOH added alternately in 10 equal portions over a period of 30 min with vigorous stirring and ice-bath cooling. Stirring was continued for 1 h at room temperature and then the solution extracted with ether (25 ml). The pH of the aqueous layer was adjusted to 2 with concentrated HCl and the oily product which separated out was extracted into ethyl acetate (3 x 100 ml). The combined extracts were dried ($MgSO_4$), the solvent evaporated and the resulting oil triturated with petroleum ether until it began to crystallise. The petroleum ether was poured off and the semi-solid product dried overnight under vacuum. The now completely crystalline mass was recrystallised from boiling carbon tetrachloride giving fine white crystals 21.9 g (93%), m.p. 103 - 104°C, lit. m.p. 105 - 107°C [Greenstein and Winitz (1961), p. 1270].

Formyl-L-leucine: This was prepared according to the method of Greenstein and Winitz (1961), procedure 10 - 46, p. 921. The product was recrystallised from water and then from ethyl acetate. On the scale 50 mmol the yield was 5.90 g (74%), m.p. 140.5 - 142°C, lit. m.p. 142 - 144°C [Heard and Young (1963)].

Phthaloyl-L-leucine: This was prepared in a way similar to that described by Nefkens et al (1960). L-Leucine (2.62 ; 20 mmol) and Na_2CO_3 (2.12 g; 20 mmol) were dissolved in water (60 ml) with vigorous stirring; carboethoxyphthalimide (4.50 g; 20 mmol) was added and stirring continued for 20 min. The pH was adjusted to 2 with concentrated HCl and the resulting white sticky precipitate dissolved by heating the solution. After standing for 2 days at 0°C the crystalline product was collected (3.10 g; 59%), m.p. 112 - 118°C. Recrystallisation from toluene-petroleum ether gave white needles (2.46 g; 47%), m.p. 116 - 119°C, lit. m.p. 118.5 - 119.5°C [Greenstein and Winitz (1961), p. 905].

III. 2. CARBOBENZOXYAMINO ACIDS

Carbobenzoxyglycine: This was prepared according to Greenstein and Winitz (1961), procedure 10 - 28, p. 891. On the scale 100 mmol the yield after recrystallisation from chloroform was 17.68 g (85%), m.p. 119 - 120°C, lit. m.p. 120°C [Greenstein and Winitz (1961), p. 892].

Carbobenzoxy-L-alanine: This was prepared by the method of Greenstein and Winitz (1961), procedure 10 - 28, p. 891. The product was recrystallised from ether-petroleum ether. On the scale 200 mmol the yield was 27.5 g (62%), m.p. 84 - 84.5°C, lit. m.p. 87°C [Greenstein and Winitz (1961), p. 892].

Carbobenzoxy-L-valine: This was prepared by the method of Greenstein and Winitz (1961), procedure 10 - 29, p. 891. The product was a colourless oil which was dried under high vacuum over P_2O_5. Attempts to crystallise the oil from ethyl acetate-petroleum ether failed, c.f. Grassman and Wunsch (1958) who reported a crystalline product m.p. 66 - 67°C.

Carbobenzoxy-L-leucine : This was prepared in the same way as carbobenzoxy-L-valine. The product, which was a colourless oil, was obtained in yields ranging from 50 - 80%.

Carbobenzoxy-DL-leucine: This was prepared in the same way as carbobenzoxy-L-valine. Attempts to crystallise the product, which was a colourless oil, from ethyl acetate-petroleum ether failed; c.f. Grassman and Wunsch (1958) who reported a crystalline product m.p. 52 - 55°C.

Carbobenzoxy-L-isoleucine: This was prepared in the same way as carbobenzoxy-L-valine. The product was a colourless oil.

Carbobenzoxy-L-phenylalanine: This was prepared by a modified version of the method of Greenstein and Winitz (1961), procedure 10 - 28, p. 891. The crude crystalline product, m.p. 127 - 133°C was dissolved in ethyl acetate (100 ml) and extracted with N HCl (2 x 100 ml) and water (100 ml), c.f. Grassman and Wunsch (1958). The ethyl acetate solution was dried ($MgSO_4$) and the solvent evaporated to give an oil which was recrystallised from ethyl acetate-petroleum ether. On the scale 25 mmol the yield was 5.66 g (76%), m.p. 86 - 87°C, lit. m.p. 88 - 89°C [Grassman and Wunsch (1958)].

Carbobenzoxy-L-asparagine: This was prepared according to Greenstein and Winitz (1961), procedure 10 - 30, p. 895. After acidification the crystalline product was collected and recrystallised from a large volume of boiling water. On the scale 100 mmol the yield was 12.3 g (46%), m.p. 162 - 164°C, lit. m.p. 165°C [Greenstein and Winitz (1961), p. 892].

Carbobenzoxy-γ-aminobutyric acid: This was prepared by the method of Greenstein and Winitz (1961), procedure 10 - 28, p. 891. On the scale 100 mmol the crude yield was 17.92 g (76%), m.p. 55 - 62°C. Recrystallisation three times from ethyl acetate-petroleum ether gave 12.28 g (52%), m.p. 65 - 66°C, lit. m.p. 66-67°C [Evans and Irreverre (1959)].

Carbobenzoxy-ε -aminocaproic acid: This was prepared in the same way as carbobenzoxy-γ-aminobutyric acid. On the scale 25 mmol the yield after one recrystallisation from ethyl acetate-petroleum ether was 5.04 g (76%), m.p. 55 - 55.5°C, lit. m.p. 57 - 58°C [Rothe and Kunitz (1957)].

III. 3. PEPTIDES

Acetyl-DL-leucylglycine methyl ester: Glycine methyl ester hydrochloride (1. 26 g; 10 mmol) was suspended in acetonitrile (50 ml) and triethylamine (1. 01 g; 10 mmol) added. The mixture was stirred with a magnet for 10 min at 25°C; then acetyl-DL-leucine (1. 73 g; 10 mmol) was added followed by a solution of dicyclohexylcarbodiimide (2. 27 g; 11 mmol) in acetonitrile (5 ml). Stirring was continued for 24 h. Acetic acid was added to decompose the excess dicyclohexylcarbodiimide and after stirring for a few min the reaction mixture was filtered (Celite) and the filtrate evaporated to give an oil. This was redissolved in chloroform (25 ml) and extracted with N HCl (25 ml), water (25 ml), 5% $NaHCO_3$ (25 ml) and water (25 ml). The chloroform solution was dried (Na_2SO_4) and the solvent removed to give an oil that was redissolved in ether. Addition of n-hexane followed by evaporation gave a mixture of oil and crystals. Some seed crystals were removed and the remainder of the product dissolved in boiling ether (400 ml). Petroleum ether (1500 ml) was added, the solution seeded and left to stand overnight at 4°C. Fine white crystals (0. 61 g; 25%), m. p. 110 - 112°C were obtained. The purity of the product was established by hydrolysing a sample with 6 N HCl in an evacuated, sealed tube for 48 h at 110°C. 35. 6 μmol (8. 70 mg) of the peptide gave 37. 4 μmol (105%) of leucine and 38. 5 μmol (108%) of glycine; the ratio leucine: glycine was 1. 00: 1. 03.

Carbobenzoxyglycylglycine: This was prepared by the method of Greenstein and Winitz (1961), procedure 10 - 28, p. 891, except that after the reagents had been added, stirring was continued at 0°C for 2 h. The white crystalline product was recrystallised from 5% acetic acid. On the scale 20 mmol the yield was 3.48 g (66%), m.p. 178-180°C, lit. m.p. 178 - 179°C [Greenstein and Winitz (1961), p. 1170].

Carbobenzoxyglycylglycyl-L-leucine methyl ester; Carbobenzoxyglycylglycine (1.33 g; 5 mmol) was dissolved in ice-cold THF (15 ml) and triethylamine (0.51 g; 5 mmol) and ethyl chloroformate (0.54 g; 5 mmol) added. This mixture was allowed to stand for 30 min at 0°C. Meanwhile L-leucine methyl ester hydrochloride (0.91 g; 5 mmol) was suspended in ice-cold tetrahydrofuran and triethylamine (0.51 g; 5 mmol) added; this solution was cooled to 0°C and added to the mixed anhydride solution. The reaction mixture was stirred for 1 h at 0°C and then overnight at 25°C. The solvent was evaporated and the residue redissolved in chloroform (50 ml). The chloroform solution was washed with water (50 ml), N HCl (50 ml), water (50 ml), 5% $NaHCO_3$ (50 ml) and water (50 ml) and then dried (Na_2SO_4). The solvent was removed to give an oil (1.56 g; 79%) which was crystallised from ether-petroleum ether giving fine white crystals (1.32 g; 67%), m.p. 86 - 88°C. The purity of the product was established by hydrolysing a sample with 6 N HCl (10 ml) for 72 h in an evacuated sealed tube at 110°C. 95.4 μmol (37.55 mg) of peptide gave 218 μmol (114%) of glycine and 105 μmol (111%) of leucine. The ratio glycine:leucine was 2.07:1.00.

III. 4. DERIVATIVES OF LYSINE

ε-N-Benzylidene-L-lysine: This was prepared by the method of Bezas and Zervas (1961). On the scale 50 mmol the yield of crude product after drying was 9. 2 g (78%).

α-N-Carbobenzoxy-L-lysine: This was prepared from crude ε-N-benzylidene-L-lysine by the method of Bezas and Zervas (1961). On the scale 39 mmol the yield of product after crystallisation from water was 3. 68 g (34%), m. p. 229 - 231°C d., lit. m. p. 232 - 233°C d. [Bezas and Zervas (1961)]. The mother liquor on concentration yielded a further crop of crystals (2. 13 g; 19%), m. p. 226 - 228°C d. Amino acid analysis of both fractions showed that they were contaminated with ε-N-carbobenzoxylysine (see p. 83). Purification was effected by the method of Savrda and Bricas (1968). The fractions were dissolved in 0. 5 N NaOH (10 ml) and put onto a column made of copper carbonate (10 g) and alumina (50 g). The column was eluted with 0. 5 N NaOH and the fractions containing the product (identified by t. l. c.) were pooled and the pH adjusted to 6. 2. On concentration and standing in the cold overnight pure α-N-carbobenzoxy-L-lysine deposited. During the purification ε-N-carbobenzoxylysine was retained by the column and α-N-carbobenzoxylysine was quantitatively recovered in the eluent; however, the amount of crystalline α-N-carbobenzoxylysine that could actually be isolated after the purification was limited by the presence of salt in the neutralised eluent. The column could be used only once for the purification of up to 4 g of material; the recovery of crystalline α-N-carbobenzoxylysine varied from 50 - 80%.

α-N, ε-N-Dicarbobenzoxy-L-lysine: This was prepared by the method of Bergmann et al (1935). On the scale 100 mmol the product was obtained as an oil in 97% yield [Bergmann et al (1935) also obtained an oil].

III. 5. MISCELLANEOUS COMPOUNDS

Glycine methyl ester hydrochloride: This was prepared by a modified version of the method of Brenner and Huber (1953). Thionyl chloride (26 ml, 350 mmol) was cautiously added with stirring to ice-cold methanol (100 ml; 2500 mmol). Then glycine (7.5 g; 100 mmol) was added and the mixture stirred overnight. After 1 h solution was complete, but later the product began to crystallise out. The solvent was evaporated and the crystalline residue re-evaporated with methanol (3 x 50 ml). The solid product was recrystallised twice from methanol by the slow addition of ether. The yield was 11.25 g (90%), m.p. 178 - 180°C, lit. m.p. 175°C [Greenstein and Winitz (1961), p. 929].

L-Leucine methyl ester hydrochloride: This was prepared by the HCl method as described by Greenstein and Winitz (1961), procedure 10 - 48, p. 926. After two recrystallisations from methanol-ether the product had m.p. 151 - 152°C, lit. m.p. 150 - 151°C [Greenstein and Winitz (1961), p. 930]. Yields varied in the range 70 - 80%.

Tosyl-L-leucine: This was prepared in a way similar to that described by Greenstein and Winitz (1961), procedure 10-25, p. 886. p-Toluenesulfonyl chloride (25.7 g; 135 mmol) was added to a solution of L-leucine (13.1 g; 100 mmol) in N NaOH (130 ml) and the mixture stirred vigorously at 25°C for 4 h. The pH was checked every 30 min and N NaOH added when necessary to keep the

pH above 10. The solution was filtered, cooled to 0°C and the pH adjusted to 2 with concentrated HCl. The oily precipitate was extracted into ethyl acetate (3 x 200 ml), the combined extracts dried ($MgSO_4$) and evaporated to give an oil (13.8 g; 48%) which crystallised on prolonged evaporation. Recrystallisation from ethyl acetate-petroleum ether gave 10.2 g (36%), m.p. 114 - 119°C. This was recrystallised from 20% ethanol to give fine white crystals (7.7 g; 27%), m.p. 122 - 124°C, lit. m.p. 124°C [Greenstein and Winitz (1961), p. 888].

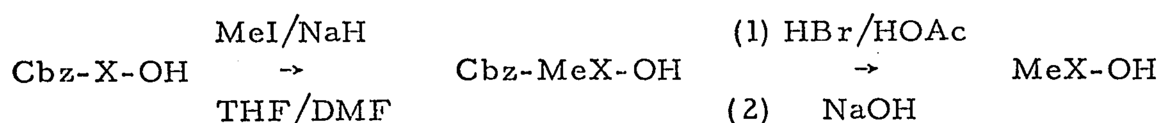
IV.

RESULTS AND DISCUSSION

IV. 1. A NEW ROUTE TO N-METHYLAMINO ACIDS

(a) Establishment of a new route to N-methylamino acids

Our first experiments were designed to find out whether the following route to N-methylamino acids was practical:



A solution of carbobenzoxyleucine in THF/DMF (10/1, v/v) was heated with methyl iodide and sodium hydride at 70 - 80°C for 8 h under reflux. The ratio of reactants, i. e. MeI:NaH:Cbz-Leu-OH was 4:2:1 (this method of expressing the ratio of alkyl halide :base: R-X-OH will be used throughout the thesis). The product of the reaction was deprotected and isolated using Dowex 50(H^+). Analysis of a sample showed that it contained N-methylleucine corresponding to a 46% yield from carbobenzoxyleucine. It also contained leucine corresponding to 39% of unchanged starting material. A second experiment with carbobenzoxyglycine using a reactant ratio of 4:2. 4:1 gave sarcosine corresponding to 63% of the starting material and glycine corresponding to 0. 2% of starting material. The isolation of almost pure sarcosine in reasonable yield demonstrated the feasibility of using this route for the synthesis of N-methylamino acids.

We then set out to find the optimal conditions of reaction using carbobenzoxyleucine as starting material. The results of a series of experiments are shown in Table IV. 101. The most dramatic result was that when the ratio of reactants was 4:2:1 (i. e. the amount of sodium hydride was exactly the amount required for the reaction) the

Table IV. 101

Methylation of carbobenzoxyleucine under various conditions[a]

Me:NaH: Cbz-Leu-OH[b] (equiv)	Reaction time (h)	Yield of N-methyl-leucine[c] (%)	Residual leucine[c] (%)	Starting material accounted for (%)
4:4:1	8	74	1.1	75
4:4:1	24	73	0.2	73
16:4:1	24	73	0.1	73
4:4:1	24	73	0.2	73
4:2:1	8	46	39.0	85
4:4:1	8	74	1.1	75
4:4:1	8	79	2.2	81
4:4:1	8	77	5.2[d]	82

a Mixture heated in THF (20 ml) and DMF (1.5 ml) at 70 - 80°C.

b 5 mmol of Cbz-Leu-OH.

c Determined with the amino acid analyser after deprotection with HBr/HOAc followed by NaOH.

d DMF omitted.

amount of unchanged starting material was very high (39%). Doubling the amount of sodium hydride reduced the residual starting material to 1.1% while increasing the reaction time to 24 h reduced it still further to 0.2%. Increasing the amount of methyl iodide produced a further decrease in residual starting material (to 0.1%). When DMF was omitted the amount of residual starting material increased. The total amount of starting material accounted for in these experiments was always less than 100%. This was because the solution of carbobenzoxyleucine was prepared by weighing it and dissolving it in THF. As a quantitative procedure this proved to be unsatisfactory, since carbobenzoxyleucine is an oil which is difficult to obtain completely free of solvents. In all later experiments, the carbobenzoxyleucine solution was prepared approximately by weighing and the exact concentration determined by removing an aliquot, deprotecting with HBr/HOAc, and analysing for leucine.

Too large an excess of sodium hydride was undesirable, since it might lead to racemisation via base catalysed abstraction of the α-H or even to α-C-methylation. Therefore a further experiment was carried out using a reactant ratio of 8:3:1 (i.e. 50% excess of NaH); the result is given in Table IV. 102. The yield of N-methylleucine was 96% and the amount of residual carbobenzoxyleucine 1%. Within the limits of experimental error all the starting material was accounted for; this is very good evidence that there were no side reactions.

The methylation of other N-protected leucines was investigated (Table IV. 102). Acetyl- and benzoyl-leucine were efficiently methylated and very little unreacted starting material remained. An

Table IV. 102

Methylation of N-protected-leucines[a]

N-protected leucine	MeI:NaH:R-Leu-OH[b] (equiv)	Reaction time (h)	Yield of N-methyl-leucine[c] (%)	Residual leucine[c] (%)	Starting material accounted for (%)
Cbz-Leu-OH	8:3:1	24	96	1.1	97
Boc-Leu-OH	8:3:1	24	86	3.0	89
Bz-Leu-OH	4:4:1	8	96	0.1	96
Ac-Leu-OH	4:4:1	8	88	0.7	89
Ac-Leu-OH	4:4:1	8	32	0.2	32[d]
For-Leu-OH	8:3:1	24	95	4.0	99
Tos-Leu-OH	8:3:1	24	61	4.0	65
Pht-Leu-OH	8:3:1	24	33	11.8	54[e]
	8:3:1	24	46	26.3	81[f]
Trt-Leu-OH	8:3:1	24	11	84.6	96

a Mixture heated in THF (10 ml) and DMF (1 ml) at 70 - 80°C.

b 1 or 2 mmol of R-Leu-OH.

c Determined with the amino acid analyser after suitable deprotection.

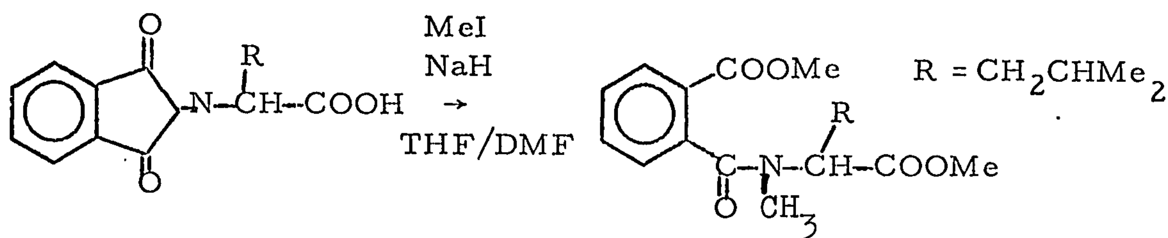
d Deprotection too mild.

e Deprotection with hydrazine; α-C-methylleucine (yield 9%) also present.

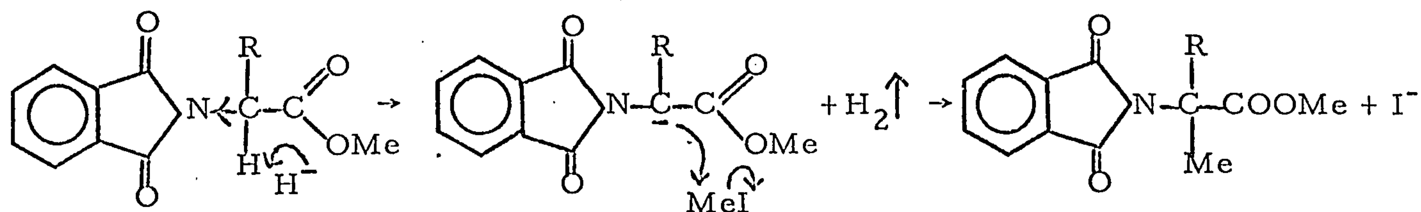
f Deprotection with NaOH followed by HCl; α-C-methylleucine (yield 9%) also present.

interesting point arose out of the experiment with acetylleucine. Deprotection of acetyl-N-methylleucine methyl ester using refluxing 2N HCl for 2 h was incomplete; only 32% of the starting material was accounted for. A control experiment with acetylleucine using the same hydrolytic conditions gave 88% yield of leucine. Efficient deprotection was achieved by using the same conditions as for the benzoyl compound, i. e. refluxing 6N HCl for 12 h. Formyl- and t-butyloxycarbonyl-leucine were also methylated efficiently, although in these two cases relatively large amounts (3 - 4%) of starting material remained unreacted. With tosylleucine there was 4% unchanged starting material and the total amount of starting material accounted for was only 65%. Possibly this was due to inefficient deprotection; in a control experiment the deprotection method gave 96% yield of leucine from tosylleucine; however, as the acetylleucine experiment had indicated, deprotection of the N-methylamino acid derivative may be more difficult than deprotection of the corresponding amino acid derivative.

Since we were ultimately interested in achieving the selective monomethylation of lysine, two groups that might be expected to provide protection against N-methylation were also investigated. Phthaloylleucine was substantially N-methylated presumably because of partial conversion to o-(carboxymethyl)-benzoylleucine methyl ester under the conditions of the reaction. (It was pointed out by one of the examiners that the source of the oxygen required for this reaction must be an impurity, e. g. water or alcohol, in the solvents or alkyl halide used for the reaction).



Some α-C-methylation also occurred; evidence for this was the appearance of a new peak which emerged from the long column of the amino acid analyser ahead of leucine and which was chromatographically indistinguishable from a standard sample of α-C-methylleucine. The strong electron withdrawing properties of the phthaloyl group, which are exemplified most clearly by the acidity of the -NH- group of phthalimide, must lead to labilisation of the α-CH group of phthaloylleucine compared with that of the other acyl-leucines. This accounts for the α-C-methylation:



Clearly the phthaloyl group was unsuitable for our purpose. We were more fortunate in an experiment with N-trityl-leucine. This compound was scarcely methylated (only 11%) and 85% of unchanged N-tritylleucine remained. The trityl group looked extremely promising for protection against N-methylation and was later successfully used in the lysine work [Part IV. 1 (e)]. The protection afforded must be due to steric hindrance.

Another variable of importance was the exact order of addition of reagents. In his work on the N-alkylation of amides Fones (1949) had deliberately prepared amide salts by treating the amides with sodium hydride in xylene; then alkylation was effected by the addition of alkyl halide. On the other hand, Dannley and Lukin (1957) in their studies on the alkylation of urethanes had treated solutions of the urethanes in the alkyl halide diluted with DMF with sodium hydride. We had followed the Dannley-Lukin procedure in our first experiments. A study was now made to find out what happened if we changed the order of mixing the reagents. The results of experiments with carbobenzoxyleucine and t-butyloxycarbonylleucine are given in Table IV. 103. Whenever sodium hydride was added before methyl iodide a substantial amount of starting material (35 - 67%) was not accounted for. Side reactions must occur, but since our 'normal' mode of addition of reagents was successful we did not investigate this point any further.

The applicability of our methylation procedure to some other carbobenzoxyamino acids was investigated (Table IV. 104). Carbobenzoxyglycine and carbobenzoxyalanine were both efficiently methylated and in both cases all the starting material was accounted for.

Table IV. 103

Effect of the order of addition of reagents[a]

Compound methylated	Mode of addition of reagents	Yield of N-methyl-leucine[b] (%)	Residual leucine[b] (%)	Starting material accounted for (%)
Cbz-Leu-OH	'normal'	91	0.3	91
Cbz-Leu-OH	10 min prior reflux with NaH dispersion	54	1.0	55
Cbz-Leu-OH	30 min prior reflux with NaH dispersion	22	28.0	50
Boc-Leu-OH	'normal'.	86	3.0	89
Boc-Leu-OH	prior treatment with NaH dispersion till hydrogen evolution stopped	37	27.9	65
Boc-Leu-OH	20 min prior reflux with NaH dispersion	26	7.2	33

a R-Leu-OH (2 mmol) in THF (10 ml) and DMF (1 ml) mixed as described with NaH (6 mmol) and MeI (16 mmol) and then heated for 24 h at 70 - 80°C.

b Determined with the amino acid analyser after deprotection with HBr/HOAc followed by NaOH.

Table IV. 104

Methylation of carbobenzoxyamino acids[a]

Compound	Yield of N-methylamino acid[b] (%)	Residual amino acid[b] (%)	Starting material accounted for (%)
Cbz-Gly-OH	99	0.3	99
Cbz-Ala-OH	96	0.3	96
Cbz-Leu-OH	96	1.1	97
Cbz-γAbu-OH	-[c]	0.5	-

a Cbz-X-OH (2 mmol) in THF (10 ml) and DMF (1 ml) mixed with MeI (16 mmol) and NaH (6 mmol) and then heated for 24 h at 70 - 80°C.

b Determined with the amino acid analyser after deprotection with HBr/HOAc followed by NaOH.

c No standard sample of γ-(methylamino)butyric acid was available at the time of the experiment.

We concluded from this experiment that our method would be generally applicable to the α-amino acids with alkyl side chains. An experiment with γ-(carbobenzoxyamino)-butyric acid resulted in the almost quantitative consumption of starting material which suggested that methylation went almost to completion in this case also. Since no standard for γ-(methylamino)-butyric acid was available at the time of the experiment, this conclusion could not be verified directly. However, as is shown by later preparative experiments and by the studies on ε-carbobenzoxylysine derivatives, ω-carbobenzoxyamino groups like α-carbobenzoxyamino groups are methylated efficiently by our procedure.

The possibility of using ethyl and n-propyl iodides to prepare N-ethyl- and N-propyl-amino acids from carbobenzoxyamino acids was also investigated. The extent of the reaction was determined by measuring the amount of unchanged starting material since the products were not sufficiently ninhydrin positive to be measured with the amino acid analyser [see Part IV. 2 (a)]. Results for comparative studies of the methylation, ethylation and propylation of carbobenzoxyleucine and carbobenzoxyglycine are given in Table IV. 105. With glycine, methylation and ethylation went extremely well, to 100% and 99% respectively, while propylation went to the extent of 85%. With leucine, although methylation went to the extent of 99%, the other alkylations were quite inefficient, especially the propylation. In the most successful ethylation experiment with leucine the reaction went approximately as well as the propylation of glycine. The much less complete ethylation and propylation of carbobenzoxyleucine compared with carbobenzoxyglycine must be due to the steric effect of the t-butyl

Table IV. 105

Alkylation of carbobenzoxyamino acids with various alkyl iodides[a]

Carbobenzoxyamino acid	Alkyl iodide	RI:NaH:Cbz-X-OH[b] (equiv)	Time of reaction (h)	Residual amino acid[c] (%)
Cbz-Gly-OH	methyl	8:3:1	24	0.3
Cbz-Gly-OH	ethyl	32:3:1	24	0.9
Cbz-Gly-OH	n-propyl	32:3:1	24	1.1
Cbz-Leu-OH	methyl	4:4:1	8	1.1
Cbz-Leu-OH	ethyl	4:4:1	8	52.0
Cbz-Leu-OH	n-propyl	4:4:1	8	80.6
Cbz-Leu-OH	methyl	8:3:1	24	1.1
Cbz-Leu-OH	ethyl	8:3:1	24	12.3

a Mixture heated in THF (10 ml) and DMF (1 ml) at 70 - 80°C.

b 2 mmol of Cbz-X-OH.

c Determined with the amino acid analyser after deprotection with HBr/HOAc followed by NaOH.

side chain of leucine compared with the single hydrogen atom side chain of glycine. We were interested in the possibility of ethylating and propylating ε-carbobenzoxylysine derivatives; these results gave us good reason to believe that we could achieve our objective since the unbranched side chain of lysine would offer less steric hindrance than the branched side chain of leucine.

The methylation and ethylation of polyleucine were attempted (Table IV. 106). Methylation went very well indeed; 91% N-methylleucine was found after hydrolysis and only 2.4% unreacted leucine. Ethylation was much less efficient (36% of unreacted leucine residues). The methylation experiment led us to propose that our method could be successfully used for the permethylation of peptides for mass spectrometric studies [Coggins and Benoiton (1968b)] (see Part IV. 4).

At this stage in our work a new paper appeared from Lederer's laboratory [Vilkas and Lederer (1968)] describing the use of the methylsulfinyl carbanion [Corey and Chaykovsky (1962)] as the base for the permethylation of peptides with methyl iodide. A second paper from the same laboratory [Thomas et al (1968a)] described an improved version of the original methyl iodide/silver oxide/DMF method for the permethylation of peptides [Das, Gero and Lederer (1967)]. A series of experiments were carried out with acetylleucine and carbobenzoxyleucine to compare our 8:3:1 method of methylation with these two methods (Table IV. 107).

Table IV. 106

Alkylation of poly-L-leucine[a]

Alkyl iodide	Yield of N-alkylamino acid[b] (%)	Residual amino acid[b] (%)
methyl	91	2.4
ethyl	-[c]	36.4

a Poly-L-leucine (1 mequiv of peptide bonds) in THF (25 ml) and DMF (1 ml) mixed with RI (20 mmol) and NaH (3 mmol) and then heated for 24 h at 70 - 80°C.

b Determined with the amino acid analyser after hydrolysis with 6 N HCl for 96 h in an evacuated sealed tube at 110°C.

c No N-ethylleucine standard was available at the time of this experiment.

Vilkas and Lederer used approximately 1 equiv of base for each methylatable group [just as Hakomori (1964) had done for the permethylation of polysaccharides]. We tried this and since it was not very successful we also tried using twice the theoretical amount of base. Thomas et al did not describe the exact stoichiometry that they used; however, it was clear that they used very large excesses of both methyl iodide and sodium hydride; we tried various stoichiometries. The results (Table IV. 107) showed that our method gave a more complete reaction than any of the others; this despite the fact that the acetyleucine experiment was less successful than an earlier one (Table IV. 102). With our method the starting material was almost quantitatively accounted for, indicating an absence of side reactions.

The results with the methyl iodide/silver oxide/DMF method showed a marked dependence on the amounts of the reagents used. In the most successful case, with the largest excesses of methyl iodide and silver oxide, the reaction was 85% complete. In all the experiments the starting material was quantitatively accounted for, which indicates that there were no side reactions. Optimisation of the conditions might have given a more complete reaction.

With the methyl iodide/methylsulfinyl carbanion method the yields of methylated products were low (23 - 35%) and the recovery of starting material was poor (35 - 71%). Side reactions obviously occurred; these were possibly of the same nature as those obtained with our method when the base was added before the methyl iodide (see Table IV. 103) because, in this case, the base was also added before the methyl iodide. It is known that the system molecular oxygen/DMSO/base

Table IV. 107

Methylations using various bases

Compound	Base	MeI:base: R-Leu-OH[a] (equiv)	Yield of N-methyl-leucine[b] (%)	Residual leucine[b] (%)	Starting material accounted for (%)
Ac-Leu-OH	sodium hydride[c]	8:3:1	88	2.9	91
Ac-Leu-OH	silver oxide[d]	16:2:1	43	60.0	103
		32:4:1	41	49.5	91
		32:8:1	85	10.7	96
Ac-Leu-OH	methylsulfinyl carbanion[e]	8:2:1	35	28.1	63
		8:4:1	35	0.0	35
Cbz-Leu-OH	sodium hydride[c]	8:3:1	96	1.1	97
Cbz-Leu-OH	silver oxide[d]	16:4:1	66	27.1	93
Cbz-Leu-OH	methylsulfinyl carbanion[e]	8:4:1	23	27.7	71

a 1 or 2 mmol R-Leu-OH.

b Determined with the amino acid analyser after suitable deprotection.

c Conditions as in Table IV. 104.

d Method of Thomas et al. (1968).

e Method of Vilkas and Lederer (1968).

is a potent oxidising agent [Russell et al (1965)]; although we prepared and handled the methylsulfinyl carbanion under nitrogen the total exclusion of oxygen is very difficult and side reactions involving oxidation may have also contributed to the disappearance of starting material.

The effect of substituting DMSO for DMF in the methylation of acetylleucine and carbobenzoxyleucine using the 8:3:1 conditions was investigated (Table IV. 108). With acetylleucine there was 42% methylation and 37% of unchanged starting material. This was a poor result compared with the DMF control experiment in which the methylation was nearly complete. The recovery of starting material was 79%; not as good as with DMF. In two experiments with carbobenzoxyleucine only 18% and 32% respectively of methylation occurred. Almost all of the rest of the starting material was unaccounted for. This is attributed to side reactions possibly involving oxidation. An alternative side reaction involving N-methylation followed by α-C-methylation could not be ruled out since the product of such a reaction would react very poorly with ninhydrin [since it is both an N-methyl and an α-C-methyl-amino acid; see Part IV. 2(a)] and therefore would probably not have been detected.

At a much later stage in our work we decided to investigate the time course of the methylation reaction with carbobenzoxyleucine and carbobenzoxyglycine. The experiments showed (Table IV. 109) that the methylation of carbobenzoxyglycine was essentially complete in 1 h whereas the reaction with carbobenzoxyleucine took 4 h to reach the

Table IV. 108

Methylations using DMSO instead of DMF[a]

Compound	DMSO or DMF	Yield of N-methyl-leucine[b] (%)	Residual leucine[b] (%)	Starting material accounted for (%)
Ac-Leu-OH	DMF	88	2.9	91
Ac-Leu-OH	DMSO	42	36.8	79
Cbz-Leu-OH	DMF	96	1.1	97
Cbz-Leu-OH	DMSO	18	0.9	19
Cbz-Leu-OH	DMSO	32	0.6	33

a R-Leu-OH (2 mmol) in THF (10 ml) and DMF (1 ml) or DMSO (1 ml) mixed with MeI (16 mmol) and NaH (6 mmol) and then heated for 24 h at 70 - 80°C.

b Determined with the amino acid analyser after suitable deprotection.

Table IV. 109

Effect of time on the methylation of carbobenzoxyamino acids[a]

Cbz-X-OH	Reaction time (h)	Yield of sarcosine[b] (%)	Yield of N-methyl-alanine[b] (%)	Residual glycine[b] (%)	Yield of N-methyl-leucine[b] (%)	Residual leucine[b] (%)
Cbz-Gly-OH	1.0	89	1	1	-	-
Cbz-Gly-OH	2.0	84	1	1	-	-
Cbz-Gly-OH	4.0	83	1	1	-	-
Cbz-Gly-OH	8.0	91	1	1	-	-
Cbz-Leu-OH	1.0	-	-	-	75	9
Cbz-Leu-OH	2.0	-	-	-	84	2
Cbz-Leu-OH	4.0	-	-	-	78	1

a Cbz-X-OH (1 mmol) in THF (10 ml) and DMF (1 ml) mixed with MeI (8 mmol) and NaH (3 mmol) and then heated at 70 - 80°C.

b Determined with the amino acid analyser after deprotection with HBr/HOAc followed by NaOH.

same degree of completion. The amount of starting material accounted for in the carbobenzoxyglycine experiments ranged from 86 - 93% and did not change significantly with reaction time. With carbobenzoxyleucine, 79 - 86% of the starting material was accounted for; again, there was no significant change with time. The reason for the slightly lower amounts of starting material accounted for in these experiments compared with earlier experiments is not known. The slower reaction with carbobenzoxyleucine must again be due to steric hindrance from the t-butyl side chain. These experiments were carried out after the development of the improved method for N-methylamino acid analysis had been developed [see Part IV. 2(a)]. This improved analytical technique revealed a small amount of side reaction with carbobenzoxyglycine; the side product was N-methylalanine, i.e. some α-C-methylation had occurred. It is interesting to note that the amount of this side product did not increase with time, although sodium hydride and methyl iodide were present in excess throughout the reaction. This must mean that the side reaction took place only in the early stages. No side products were detected in the experiments with carbobenzoxyleucine.

The methylation of carbobenzoxyglycine was further investigated using different solvents (Table IV. 110). With a 24 h reaction time and the normal 8:3:1 reactant ratio, no side reaction was observed when the solvent was neat THF. The reaction was less complete than usual. There was 4.3% unchanged starting material compared with only 1% when the solvent was THF/DMF (10/1, v/v) and the reaction time was 8 h (Table IV. 109). In the latter case there had been 1% side reaction. When the solvent was THF/DMF (1/1, v/v) there was 2.5% side reaction and only 1.6% of starting material was unchanged. Two experiments in

Table IV. 110

Effect of DMF on the methylation of carbobenzoxyglycine[a]

Solvent	Yield of sarcosine[b] (%)	Yield of N-methyl-alanine[b] (%)	Residual glycine[b] (%)	Starting material accounted for (%)
THF	85	0.0	4.3	89
THF/DMF 1/1 (v/v)	88	2.5	1.6	92
DMF	25	0.0	0.6	26
DMF	43	0.5	5.4	49

a Cbz-Gly-OH (1 mmol) in solvent (10 ml) mixed with MeI (8 mmol) and NaH (3 mmol) and then heated for 24 h at 70 - 80°C.

b Determined with the amino acid analyser after deprotection with HBr/HOAc followed by NaOH.

which the solvent was neat DMF gave erratic results. After deprotection sarcosine was the major product but less than half the starting material was accounted for. The reason for this is unknown.

Our observation of α-C-methylation of glycine is in agreement with the finding by Thomas (1969) of 'artifacts' in the mass spectra of glycine containing peptides after permethylation by our method. The question of glycine side reactions will be discussed in detail in the part of this thesis dealing with peptides (Part IV. 4). Glycine is the only amino acid with which we have encountered side reactions and even with glycine these have not interfered with preparative work [see Part IV. 1(c)].

The following procedures were used for the alkylation experiments described in this section; additional information is given in the footnotes to the tables.

Alkylation using sodium hydride as base:

A solution of the N-protected-amino acid (1 - 2 mmol) in 10 ml of THF/DMF (10/1, v/v) was treated with alkyl iodide (8 - 16 mmol) followed by sodium hydride dispersion (0.14 - 0.29 g; 3 - 6 mmol NaH). The mixture was heated under reflux in an oil bath at 70 - 80°C for 24 h. After cooling the reaction mixture was evaporated and the residue was treated with suitable reagents to remove the protecting groups. The final solution of amino acids was acidified and analysed on the long column of the amino acid analyser.

Deprotection: Carbobenzoxyamino acid esters and t-butyloxycarbonylamino acid esters were deprotected by treatment with 30% HBr in acetic acid (10 ml) for 2 h at 25°C. The reagent was removed by evaporation and the residue dissolved in water (25 ml) and ether (10 ml). The ether layer (which contained the nujol from the sodium hydride dispersion and the unwanted deprotection products) was discarded. The aqueous layer was treated with 50% NaOH to pH 12 and then heated at 100°C for 30 min. After cooling, the pH was adjusted to 2 with 6 N HCl and the solution made up to a suitable volume for analysis.

Acetyl- and benzoyl-N-methylleucine methyl esters were deprotected by refluxing with 6 N HCl (30 ml) for 12 h. The acid was evaporated and the residue redissolved in water and analysed. Formylamino acid esters were deprotected by refluxing with 4 N HCl (25 ml) for 4 h. Polyleucine was hydrolysed with 6 N HCl (10 ml) in an evacuated, sealed tube at 110°C for 96 h as described for peptide hydrolyses in Part II.

Tosyl-N-methylleucine methyl ester was deprotected by heating in a stoppered flask at 70 - 80°C with 30% HBr in acetic acid (20 ml) and phenol (0.2 g) for 4 h. [This was a modified version of the method of Poduska, Rudinger and Sorm (1955)]. A control experiment on tosylleucine gave leucine in 96% yield.

In the first experiment with phthaloylleucine, deprotection was effected by the hydrazine method [Sheehan and Frank (1949)]. The crude residue from the reaction was extracted with boiling ethanol (25 ml) and this extract evaporated to give an oil which was redissolved in ethanol (7.5 ml). M Hydrazine hydrate in ethanol (2.5 ml) was added and the mixture refluxed 1 h. After evaporation to dryness the

product was dissolved in 2 N HCl (25 ml) and heated at 50°C for 30 min. The solution was filtered, extracted with ether (2 x 25 ml), evaporated to dryness and the residue redissolved in water (25 ml). 50% NaOH was added to pH 12, and the solution heated for 30 min at 100°C. After cooling it was acidified to pH 2 with 6 N HCl and made up to a suitable volume for analysis. A control experiment using this method gave 93% leucine from phthaloylleucine. In a second experiment, a simpler method of deprotection was used [Benoiton (1968) , modified from Sheehan and Witney (1962)]. The crude residue from the reaction was shaken with N NaOH (10 ml) and THF (10 ml) for 1 h at 25°C. After evaporation to dryness the residue was refluxed with 4 N HCl (15 ml) for 2 h; the resulting solution was evaporated and the residue redissolved in water for analysis.

Methylations using methylsulfinyl carbanion as base:

The methylsulfinyl carbanion was generated as described by Corey and Chaykovsky (1962). Sodium hydride dispersion (0.1 g; 2 mmol NaH) was stirred magnetically with DMSO (10 ml) under an atmosphere of nitrogen at 70 - 80°C until hydrogen evolution ceased (ca. 45 min). After cooling to room temperature a solution of the N-protected-amino acid (2 mmol) in DMSO (5 ml) was added and the mixture stirred under nitrogen for 1 h at 25°C. Methyl iodide (1 ml; 16 mmol) was added and stirring under nitrogen continued for 12 h at 25°C. Evaporation of the mixture gave a solid product which was deprotected in the usual manner.

Methylations using silver oxide as base:

The N-protected-amino acid (1 mmol) was dissolved in DMF (3 ml) and silver oxide (2 g; 8 mmol) and methyl iodide (1 ml; 16 mmol) were added. The mixture was heated in a sealed tube at 50°C for 4 h ; the silver oxide was filtered off and the solution evaporated to give a solid product which was deprotected in the usual manner.

(b) Preparation of N-methylleucine and some protected N-methylleucines

Having established the best procedure for methylation, we now tested our method on the preparative scale. All the experiments, unless specifically mentioned, were carried out on the L-isomers. First, N-methylleucine was prepared from carbobenzoxyleucine by the following route:

$$\text{Cbz-Leu-OH} \xrightarrow[\text{THF/DMF}]{\text{MeI/NaH}} \text{Cbz-MeLeu-OMe} \xrightarrow[\text{(2) 30\% HBr/HOAc}]{\text{(1) } \underline{\text{N}} \text{ NaOH/THF}} \text{MeLeu-OH.HBr}$$

$$\xrightarrow[\text{(2) aq } NH_3]{\text{(1) Dowex 50 } (H^+)} \text{MeLeu-OH}$$

The product was obtained in 61% yield. After recrystallisation from methanol it had a rotation slightly higher than reported for material prepared by the Quitt-Hellerbach-Vogler method (1963). Its chromatographic properties on the amino acid analyser and its n.m.r. and i.r. spectra were identical with those of an authentic sample of N-methylleucine. Amino acid analysis also showed that there was no contamination with leucine. The integration of the n.m.r. spectrum was in good agreement with theory and the spectrum showed no evidence of any α-C-methylation. The microanalysis also agreed with theory. The optical purity was uniquely established by the chromatographic method

of Manning and Moore (1968b); a method for applying this technique to N-methylleucine is described in Part IV. 2(b). It was possible to detect one part of the D-isomer in the presence of 100 parts of the L-isomer, i.e. samples of N-methyl-L-leucine found to be optically pure by this method contain less than 1% of the D-isomer.

It was concluded that the method gave analytically and optically pure N-methyl-L-leucine from carbobenzoxy-L-leucine in reasonably good yield. N-Methylleucine was also prepared using catalytic hydrogenation to remove the carbobenzoxy group after methylation. The overall yield was comparable with that when HBr/HOAc was used for deprotection; in this case the tedious resin step to remove HBr was avoided.

This method for synthesising N-methylamino acids has one very important advantage over all the earlier methods. It allows the direct preparation of optically pure N-protected N-methylamino acids and N-methylamino acid methyl ester. Some of these derivatives may be used directly in the synthesis of peptides containing N-methylamino acids (for discussion see Part V).

Twice recrystallised N-methylleucine methyl ester hydrobromide was prepared in 54% yield from carbobenzoxyleucine. The microanalysis and the n.m.r. spectrum agreed quantitatively with theory and amino acid analysis after saponification showed that N-methylleucine was liberated in the theoretical amount; no leucine was found.

Recrystallised carbobenzoxy-N-methylleucine was obtained in 37% yield from carbobenzoxyleucine. Again microanalysis, n. m. r. spectroscopy and amino acid analysis established the purity of the product. The rather low yield was due to incomplete saponification. I. r. spectroscopy showed that some unsaponified carbobenzoxy-N-methylleucine methyl ester remained even after refluxing for 2 h with 2 equiv of N NaOH/THF (1/1, v/v). For the preparative experiments the conditions were 2 equiv of reagent for 30 min at room temperature. Similar saponification conditions were used in the preparation of N-methylleucine except the reaction time was 2 h. The relatively low yields in this case may also have been due to incomplete saponification. A detailed study of the conditions required for the efficient saponification of carbobenzoxy-N-methylleucine methyl ester has not been made.

Benzoyl-N-methylleucine and acetyl-N-methylleucine were prepared from benzoyleucine and acetylleucine respectively. The crude yield of benzoyl-N-methylleucine was 92%; this product was pure since the m. p. did not rise on recrystallisation. Saponification was obviously not a problem; complete hydrolysis of the ester took place with N NaOH/THF in 30 min at 25°C. The crude yield of acetyl-N-methylleucine was 70%. The poorer yield in this case probably reflects the greater solubility of the product in water rather than inefficient saponification. Analytical and spectroscopic data for benzoyl-N-methylleucine was good. The acetyl compound was obtained in both an anhydrous and a hydrated form. These forms had distinct melting points and were interconvertible; n. m. r. and microanalysis showed that the hydrated form was the monohydrate.

Crystalline N-ethylleucine ethyl ester hydrobromide was prepared in 60% yield using twice as much alkyl halide as in the methylation experiments. Recrystallisation was not very successful, giving a final yield of only 18%. A sample was saponified and the resulting solution found to contain a small amount of leucine (corresponding to 1.4% by weight of starting material if it was assumed that the contamination in the sample was due to leucine ethyl ester hydrobromide). The integration of the n.m.r. spectrum agreed well with theory.

Carbobenzoxy-N-ethylleucine was also prepared; it was obtained as an oil in very low yield (13%) because of incomplete saponification. The n.m.r. spectrum of the product confirmed its identity but also showed that carbobenzoxyleucine was present as an impurity.

In a preparation of tosyl-N-methylleucine similar saponification difficulties were encountered. Using the 'normal' hydrolytic conditions, i.e. N NaOH/THF for 30 min at 25°C, the crude yield of product was 24%. In a second experiment tosyl-N-methylleucine methyl ester was isolated in 50% yield after the attempted saponification. Blanchard et al (1945) have previously noticed the resistance of tosyl-N-methylleucine methyl ester to hydrolysis. After heating the compound with concentrated HCl/acetic acid (4/6, v/v) for 24 h at 100°C in a sealed tube they isolated 20% of unhydrolysed starting material.

In Table IV. 111, data concerning the saponification of N-protected-N-alkylleucine esters is collected together. A striking difference can be seen between the two readily saponifiable acyl-N-methylleucine methyl esters and the carbobenzoxy- and tosyl-N-methylleucine methyl esters which both show marked resistance to

Table IV. 111

Effect of the N-protecting group on the saponification of N-protected-N-methylleucine ester[a]

N-protected-N-methylleucine ester	Yield[b] of N-protected-N-methylleucine (%)
Bz-MeLeu-OMe	92
Ac-MeLeu-OMe	70
Tos-MeLeu-OMe	24
Cbz-MeLeu-OMe	44
Cbz-EtLeu-OEt	13

a Esters shaken with 2 equiv N NaOH/THF (1/1, v/v) for 30 min at 25°C.

b Based on R-Leu-OH starting material; determined by weighing.

saponification. It is well known that steric hindrance has a considerable effect on the rates of hydrolysis of esters [see e. g. Gould (1959), p. 318]. A good example of this in amino acid chemistry is the resistance to saponification shown by N-tritylamino acid methyl esters [Zervas and Theodoropoulos (1956)].

It can be readily seen from the inspection of models that the resistance to saponification shown by carbobenzoxy- and tosyl-N-methylleucine esters is also mainly steric in origin. The carbobenzoxy and tosyl protecting groups are 'flexible' and can themselves provide some hindrance to the attack of the hydroxyl ion on the carbonyl carbon atom of the esters. When this is coupled with the introduction of a methyl group very near the carbonyl carbon atom under attack, saponification is considerably impeded. In contrast the acetyl protecting group is very small and the benzoyl protecting group is not 'flexible' and therefore these groups do not interfere sterically with saponification. [On the contrary, Bender and Turnquest (1955) showed that benzoylphenylalanine ethyl ester was hydrolysed 1. 7 times faster than phenylalanine ethyl ester because of the stronger electron withdrawing properties of the benzoylamino group compared with the amino group]. The introduction of a methyl group near the ester carbonyl carbon atom in these compounds does not impede saponification to the extent that it is noticeable in the preparative experiments. This is significant, because it also shows that the electron donating effect of the methyl group is, by itself, insufficient to account for the large changes in the rates of saponification observed with some of the N-protected-leucine esters. Although the methylation of the nitrogen atom in the amide, tosylamide and urethane groups must cause some decrease in the

saponification rates of the esters by reducing the electron withdrawing effects of these groups, the marked resistance to saponification of the methyl esters of carbobenzoxy- and tosyl-N-methylleucine depends on additional structural features of these molecules. We believe that the crucial feature is the steric hindrance provided by the long 'flexible' nature of the protecting groups. In Fig. IV. 101 diagrams and photographs of Dreiding models are shown to illustrate this theory for carbobenzoxy-N-methylleucine methyl ester and carbobenzoxyleucine ester. For comparison purposes benzoyl-N-methylleucine methyl ester and benzoylleucine methyl ester are also shown. Detailed confirmation of the theory would require a series of kinetic experiments on these and other compounds.

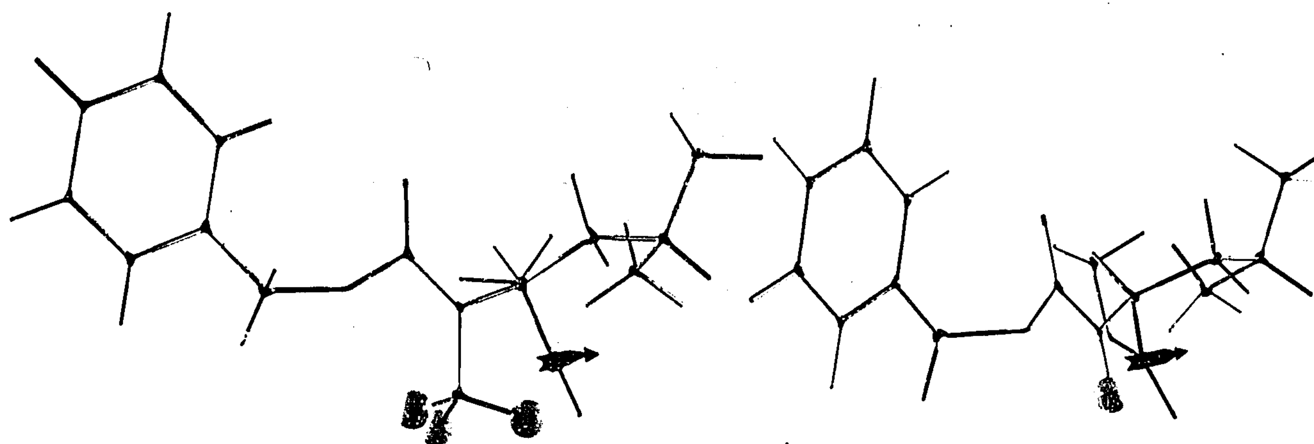
In the following practical section details of the n.m.r. and i.r. spectra of the compounds have been omitted. Full details and a discussion of the spectra are given in Part IV. 3.

N-Methyl-L-leucine:

(i) To a solution of carbobenzoxy-L-leucine (31.7 mmol) in a mixture of THF (100 ml) and DMF (10 ml) was added methyl iodide (15.8 ml; 254 mmol) and sodium hydride dispersion (4.56 g; 95 mmol NaH). The mixture was heated at 70 - 80°C under reflux for 24 h. Evaporation gave a semi-solid residue which was shaken with 2 N NaOH (50 ml) and THF (50 ml) for 1 h at 25°C. The THF was evaporated and the resulting aqueous solution was extracted with ether (2 x 50 ml), cooled to 0°C and treated with 4 N HCl to pH 2. The oil which began to separate out was extracted into ethyl acetate

Fig. IV. 101

Steric hindrance in the hydrolysis of carbobenzoxy-N-methylleucine methyl ester

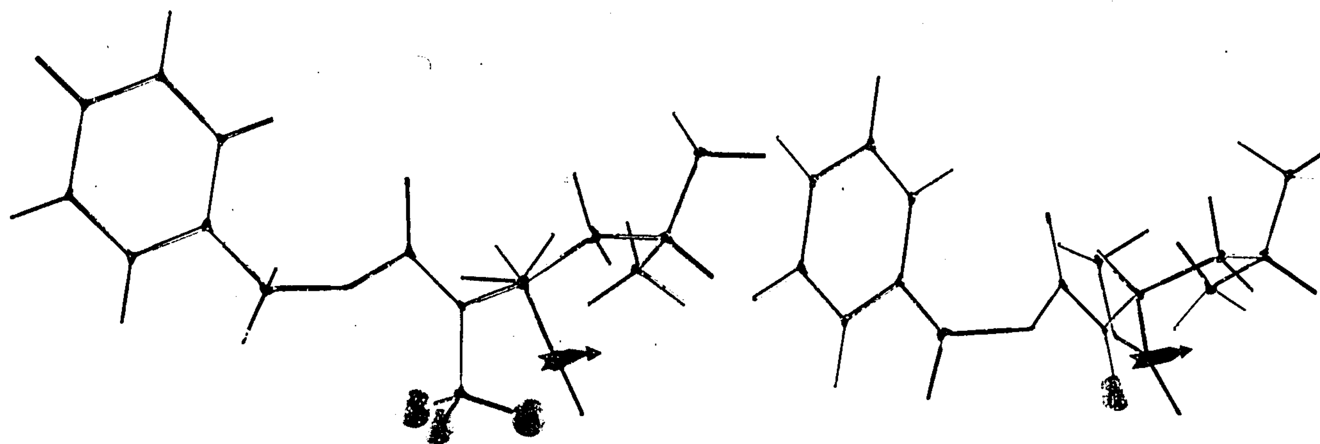


Cbz-MeLeu-OMe Cbz-Leu-OMe

The arrows mark the positions of the ester carbonyl groups. This photograph clearly shows the long flexible nature of the carbobenzoxy group and the close proximity of the N-methyl group in Cbz-MeLeu-OMe to the ester group.

Fig. IV. 101

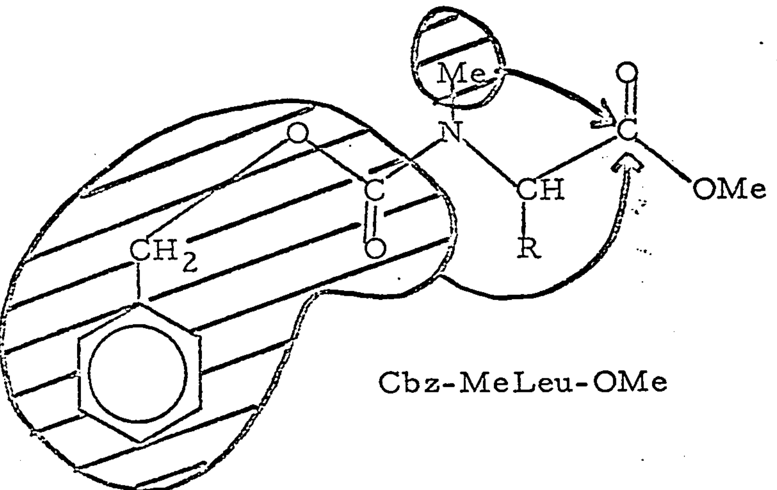
Steric hindrance in the hydrolysis of carbobenzoxy-N-methylleucine methyl ester

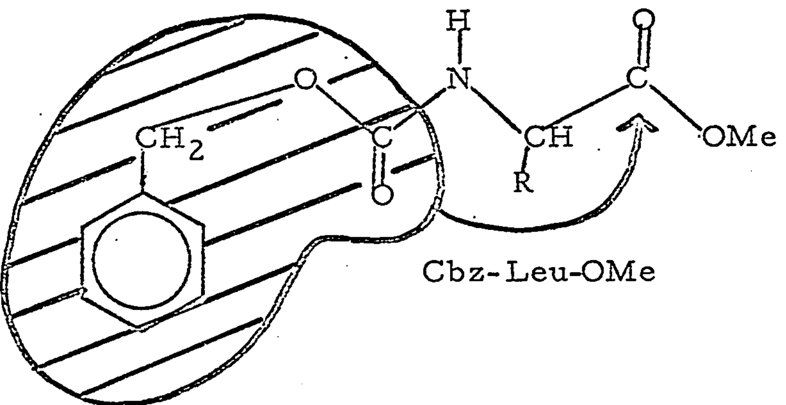


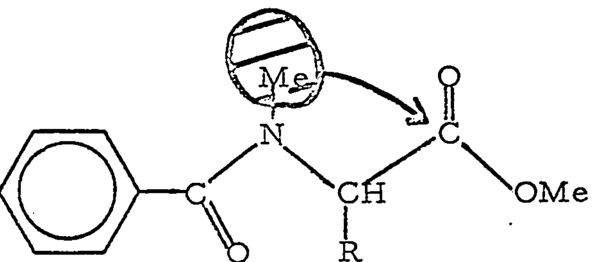
Cbz-MeLeu-OMe Cbz-Leu-OMe

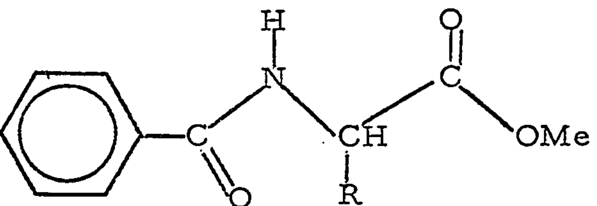
The arrows mark the positions of the ester carbonyl groups. This photograph clearly shows the long flexible nature of the carbobenzoxy group and the close proximity of the N-methyl group in Cbz-MeLeu-OMe to the ester group.

Fig. IV. 101 (continued)

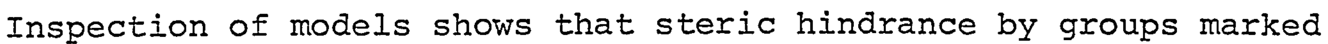








This diagram compares the steric hindrance due to carbobenzoxy and benzoyl side chains.

Inspection of models shows that steric hindrance by groups marked 

at the position shown by the arrows is significant.

(3 x 100 ml); the combined extracts were dried ($MgSO_4$) and the solvent removed to give an oil which was treated with 30% HBr in acetic acid (50 ml) for 2 h at 25°C. Evaporation of the reagent gave an oil which was dissolved in water and extracted with ether (2 x 25 ml). The aqueous layer was stirred with Dowex 50(H^+) (50 ml) for 30 min; after filtering, the solution was treated a second time with resin (50 ml) and the combined batches washed slowly with water (4 l) on a sintered glass funnel. The washed resin was stirred with aqueous 10 N NH_3 (100 ml) for 30 min; the resulting solution was evaporated to dryness and the residue dissolved in water (25 ml) and re-evaporated several times. A light, white crystalline product (2.79 g; 61%) similar in appearance to leucine was obtained. A sample (1 g) was recrystallised from methanol to give hard, white crystals (0.46 g). Amino acid analysis showed that the product contained no leucine and that it was chromatographically indistinguishable from an authentic sample of N-methylleucine; the constant was 15.7 compared with 15.9 for the authentic sample. The optical purity of the product was established by the method described in Part IV. 2(b). Rotation measurements gave $[\alpha]_D^{27}$ = +32.3° (c = 1, 6 N HCl), lit. $[\alpha]_D^{21}$ = +31.8° (c = 1, 6 N HCl) [Quitt, Hellerbach and Vogler (1963)].

Analysis calculated for $C_7H_{15}NO_2$ (145.20); C 57.9; H 10.4; N 9.65%.
Found ; C 56.9; H 9.9; N 9.7%.

The i.r. and n.m.r. spectra of the synthetic and authentic samples were identical; in particular, the integration of the n.m.r. spectrum of the synthetic sample was in very good agreement with theory (see Part IV.3).

(ii) In a second preparation using 10.5 mmol of carbobenzoxy-L-leucine the method was similar except for the final deprotection step. The crude carbobenzoxy-N-methylleucine was dissolved in 90% acetic acid (50 ml) and 10% palladium on charcoal catalyst (1.4 g) added. Hydrogenation was carried out for 24 h at 1 atm. The catalyst was filtered off (Celite), and the solution evaporated to give a white solid which was recrystallised from water-acetone to give a light, white crystalline product (0.70 g; 46%) which was identical with the recrystallised material obtained by method (i).

<u>N-Methyl-DL-leucine:</u> This was prepared from carbobenzoxy-DL-leucine (4 mmol) using method (i) above. The yield was 0.33 g (61%).

<u>N-Methyl-L-leucine methyl ester hydrobromide:</u>* Methyl iodide (11.8 ml; 190 mmol) and sodium hydride dispersion (3.42 g; 71 mmol NaH) were added to a solution of carbobenzoxy-L-leucine (23.8 mmol) in THF (50 ml) and DMF (5 ml). The mixture was heated under reflux at 70 - 80°C for 24 h. Evaporation followed by re-evaporation with ether (25 ml) gave a yellow semi-solid product. This was dissolved in ether (100 ml) and extracted with water (2 x 25 ml). The ether layer was dried ($MgSO_4$) and the solvent removed to give a mixture of two immiscible oils, one of which was pale yellow. The oils were treated with 30% HBr in acetic acid (50 ml) for 2 h at 25°C. Evaporation gave a yellow oil which crystallised on treatment with ether (100 ml). The crude yellow product (4.51 g; 71%) was recrystallised from methanol-ether to give very slightly coloured

crystals (3.70 g; 65%), m.p. 105 - 106°C. These were dissolved in water (25 ml) and extracted with ether (3 x 10 ml). The colourless aqueous layer was evaporated and the white crystalline residue recrystallised from methanol-ether giving fine white crystals (3.08 g.; 54%), m.p. 105 - 107°C, $[\alpha]_D^{25}$ = +17.5° (c = 2, DMF).

Analysis calculated for $C_8H_{18}NO_2Br$ (240.15): C 40.0; H 7.55; N 5.8%.

Found : C 40.25; H 7.7; N 5.8%.

A sample of the product (0.5 g, ca. 2 mmol) was saponified with N NaOH (20 ml) for 2 h at 25°C. Then 4 N HCl was added to pH 2 and the solutinn diluted to 100 ml and stirred with Dowex 50(H^+) (50 ml) for 30 min. The resin was collected on a sintered glass funnel and washed with water (1 l); then it was stirred with aq 10 N NH_3 (50 ml) for 30 min. The solution was evaporated to give crystalline N-methyl-L-leucine. Amino acid analysis showed that this material contained no leucine. It was shown to be optically pure by the method given in Part IV. 2(b).

N-Ethyl-L-leucine ethyl ester hydrobromide:*

A solution of carbobenzoxy-L-leucine (12.4 mmol) in THF (50 ml) and DMF (5 ml) was treated with ethyl iodide (15.8 ml; 198 mmol) and sodium hydride dispersion (1.78 g; 37.2 mmol NaH) and then heated under reflux at 70 - 80°C for 24 h. After evaporation and re-evaporation with ether (25 ml) the residue was dissolved in ether (100 ml) and washed with water (2 x 25 ml). The ether layer was dried ($MgSO_4$) and the solvent removed to give an oil which was treated with 30% HBr in acetic acid (25 ml) for 2 h at 25°C. Evaporation gave an oil which was dissolved in water (25 ml) and extracted with ether (2 x 25 ml);

evaporation of the aqueous layer gave a white crystalline solid (1.97 g; 60%). Recrystallisation from ethanol-ether gave white crystals (0.61 g; 18%), m.p. 142 - 144°C. The n.m.r. spectrum established the identity of the product (see Part IV. 3). A sample of the product (30.55 mg) was saponified with 0.1 N NaOH (5 ml) at 50°C for 30 min; then the pH was adjusted to 2.2 with 4 N HCl and the volume made up to 10 ml with pH 2.2 diluter buffer. Amino acid analysis showed that the solutinn contained 0.183 μmol of leucine. If this were present in the crystalline product as leucine ethyl ester hydrobromide then the contamination represents 1.4% by weight.

Carbobenzoxy-N-methyl-L-leucine:*

Carbobenzoxy-L-leucine (21.3 mmol) was methylated by the same procedure as that used for the synthesis of N-methyl-L-leucine methyl ester hydrobromide. The mixture of oils obtained by evaporation of the ethereal solution of the crude product was shaken with N NaOH (40 ml) and THF (40 ml) for 30 min at 25°C. The THF was evaporated and the aqueous layer extracted with ether (2 x 20 ml), cooled to 0°C and treated with 4 N HCl to pH 2. The product was extracted into ethyl acetate (3 x 40 ml); the combined extracts were dried ($MgSO_4$) and the solvent removed to give a colourless oil (2.64 g; 44%) which crystallised on scratching. Recrystallisation from water gave white needles (1.82 g; 31%), m.p. 73 - 74°C, $[\alpha]^{25}_{D}$ = -26.1 (c = 1, DMF).

Analysis calculated for $C_{16}H_{23}NO_4$ (279.33): C 64.5; H 7.6; N 5.0%

Found : C 64.7; H 7.4; N 4.8%

The ether extracts after saponfication were dried ($MgSO_4$) and the solvent removed to give an oil. I.r. spectroscopy showed that the oil contained a substantial amount of unsaponified carbobenzoxy-N-methyl leucine methyl ester. The oil was refluxed with N NaOH (40 ml) and THF (40 ml) for 1 h and then worked up to give an additional yield of carbobenzoxy-N-methylleucine (0.49 g of oil; 8%) which on crystallisation from water gave white needles (0.35 g; 6%), m.p. 73 - 74°C.

Carbobenzoxy-N-ethyl-L-leucine:* Carbobenzoxy-L-leucine (10.8 mmol) was ethylated by the same procedure as that used in the synthesis of N-ethyl-L-leucine ethyl ester hydrobromide. The oil obtained by evaporation of the ether soluble portion of the crude reaction product was shaken with N NaOH (20 ml) and THF (20 ml) for 30 min at 25°C. The THF was evaporated and the aqueous layer was extracted with ether (2 x 10 ml), cooled to 0°C and treated with 4 N HCl to pH 2. The product was extracted into ethyl acetate (3 x 20 ml) and the solvent removed to give a colourless oil (0.37 g; 13%). Attempted crystallisation from water was unsuccessful. The identity of the product was established by n.m.r. spectroscopy (see Part IV. 3).

Benzoyl-N-methyl-L-leucine*: A solution of benzoyl-L-leucine (2.35 g; 10 mmol) in THF (50 ml) and DMF (5 ml) was heated under reflux at 70 - 80°C for 24 h with methyl iodide (5 ml; 80 mmol) and sodium hydride dispersion (1.44 g; 30 mmol NaH). The reaction mixture was evaporated and then re-evaporated with ether (20 ml) to give a semi-solid residue which was dissolved in ether (100 ml) and washed with water (2 x 25 ml). The ether layer was dried ($MgSO_4$) and evaporated to give an oil which was shaken with N NaOH (20 ml) and THF (20 ml) for 30 min at 25°C. After evaporation of the THF the aqueous layer was extracted with ether (2 x 10 ml), cooled to 0°C and 4N HCl added to pH 1. A cream coloured solid separated out; after 15 min this was collected and dried giving 2.28 g (92%) of crude product., m.p. 135 - 137°C. Recrystallisation from water gave white crystals (1.85 g; 74%), m.p. 135 - 137°C, $[\alpha]_D^{24} = -54.8$ (c = 2; DMF).
Analysis calculated for $C_{14}H_{19}NO_3$ (249.30): C 67.4; H 7.7; N 5.6%.
Found : C 67.7; H 7.6; N 5.7%.

Acetyl-N-methyl-L-leucine*: Acetyl-L-leucine (1.73 g; 10 mmol) was methylated using the same procedure as that used for the methylation of benzoyl-L-leucine. The crude product obtained after acidification was extracted into ethyl acetate (3 x 25 ml). The combined extracts were dried ($MgSO_4$) and evaporated to give a crystalline mass (1.31 g; 70%). Recrystallisation from water gave white needles (1.02 g), m.p. 83-85°C. The n.m.r. spectrum of this material showed that it was hydrated. After drying under vacuum over P_2O_5 for 2 h at 65°C the m.p. rose to 107 - 108°C and the n.m.r. spectrum showed that there was no longer any water of hydration. Microanalysis was carried out on the hydrate.

Analysis calculated for $C_9H_{17}NO_3$ (187.24): C 57.7; H 9.2; N 7.5%.
Calculated for $C_9H_{17}NO_3.H_2O$ (205.25): C 52.7; H. 9.3; N 6.8%.
Found: C 53.4; H 9.5; N 7.1%.
For the monohydrate $[\alpha]_D^{25}$ = -34.5 (c = 1, DMF).

<u>Tosyl-N-methyl-L-leucine and tosyl-N-methyl-L-leucine methyl ester</u>: Tosyl-L-leucine (1.43 g; 5 mmol) was methylated by the same procedure used for the methylation of benzoyl-L-leucine. The product was obtained using a work-up similar to that for benzoyl-N-methyl-L-leucine. Saponification was carried out with <u>N</u> NaOH (10 ml) and THF (10 ml) for 30 min. The product did not crystallise on acidification; the oil which was separated was extracted into ethyl acetate (3 x 20 ml) and after drying ($MgSO_4$) and evaporation the combined extracts yielded a colourless oil (0.35 g; 24%). Crystallisation from ethanol-water gave white crystals (0.25 g; 17%), m.p. 81 - 87°C. N.m.r. spectroscopy established the identity of the product. The preparation was repeated on the scale 2.5 mmol to find out why the yield was so low. In this case the yield of crude tosyl-N-methyl-L-leucine (oil) was 0.15 g (21%). The ether extracts of the aqueous solution obtained after saponification were dried ($MgSO_4$) and evaporated to yield an oil. On trituration with petroleum ether this gave a white crystalline solid (0.40 g; 50%), m.p. 66 - 69°C which was identified by i.r. and n.m.r. spectroscopy as tosyl-N-methyl-L-leucine methyl ester. [Blanchard et al (1944) who also obtained this compound give a m.p. of 75 - 76°C after recrystallisation].

(c) Preparation of N-methylamino acid methyl esters and acyl-N-methylamino acids

The general applicability of our method to the synthesis of some derivatives of the other N-methylamino acids containing simple alkyl side chains was investigated. The method was found to be applicable to the preparation of a representative number of N-methylamino acid methyl ester hydrobromides and acyl-N-methylamino acids. The compounds synthesised and some of their properties are given in Tables IV. 112 - IV. 115. The analyses and the n. m. r. and i. r. spectra of these compounds established their identity and purity. Full details of the spectra are given in Part IV. 3.

No evidence of any side reactions such as α-C-methylation was obtained. The synthetic sarcosine methyl ester hydrobromide and benzoylsarcosine were checked for contamination by the N-methylalanine derivatives by deprotection followed by amino acid analysis. No contamination was found. The yield of acetyl-N-methylalanine was very poor; this is attributed to the high water solubility of acetyl-N-methylalanine. For this reason the synthesis of acetylsarcosine by our route failed. In both cases the products could have been obtained using a modified work-up but no attempt was made to do this. The spectral properties of acetylsarcosine were determined on a sample prepared by the acetylation of sarcosine. The yields for all the other compounds synthesised ranged from satisfactory to good. The lower yields were often due to poor recoveries on recrystalliation. Methylation of two ω-carbobenzoxyamino acids proceeded very efficiently which led us to expect no problems with the alkylation of the ε-N-carbobenzoxyamino groups of ε-N-carbobenzoxylysine derivatives.

N-Methylamino acid methyl ester hydrobromides:

A solution of the carbobenzoxyamino acid (20 mmol) in THF (50 ml) and DMF (5 ml) was treated with methyl iodide (10 ml; 160 mmol) and sodium hydride dispersion (2.88 g; 60 mmol NaH). The mixture was heated under reflux at 70 - 80°C for 24 h. After evaporation the residue was re-evaporated with ether (20 ml) and then dissolved in ether (100 ml) and washed with water (2 x 25 ml). The ether layer was dried ($MgSO_4$) and the solvent removed to give a mixture of two immiscible oils. These were treated with 30% HBr in acetic acid (50 ml) for 2 h at 25°C. The reagent was evaporated and the residual brown oil dissolved in water (25 ml) and extracted with ether (2 x 10 ml). The aqueous layer was evaporated to give a crude crystalline product which was dried overnight under vacuum over P_2O_5. Recrystallisation was effected from methanol-ether.

Benzoyl-N-methylamino acids: To a solution of the benzoylamino acid (10 mmol) in THF (50 ml) and DMF (5 ml) was added methyl iodide (5 ml; 80 mmol) and sodium hydride dispersion (1.44 g; 30 mmol NaH). The mixture was heated under reflux for 24 h at 70 - 80°C. Evaporation gave a semi-solid residue which was re-evaporated with ether (20 ml) and then dissolved in ether (100 ml) and washed with water (2 x 25 ml). The ether layer was dried ($MgSO_4$) and the solvent removed to give a mixture of two immiscible oils. The oils were shaken with N NaOH (20 ml) and THF (20 ml) for 30 min at 25°C. Then the THF was evaporated and the aqueous layer extracted with ether (2 x 10 ml), cooled to 0°C and the pH adjusted to 1 with 4 N HCl. The product was extracted into ethyl acetate (3 x 25 ml), the combined extracts were dried ($MgSO_4$) and evaporated to give a crude crystalline product which was recrystallised from water.

Table IV. 112

Yields, melting points and rotations of N-methylamino acid methyl ester hydrobromides

MeX-OMe.HBr	Yield of once recrystallized product[a] (%)	m.p. (^{o}C)	$[\alpha]_D^{25}$ C = 2, DMF
MeGly-OMe.HBr*	73	93 - 103	-
L-MeAla-OMe.HBr*	82	109 - 109.5	
L-MeVal-OMe.HBr *	61	127 - 128	+ 17.0[b]
L-MeIle -OMe.HBr *	56	126.5-127.5	+ 23.8
L-MeLeu-OMe.HBr*	65	105 - 106	+ 17.5
L-MePhe-OMe.HBr*	81	132 - 133	+ 37.3
MeγAbu-OMe.HBr*	79	64 - 67	-
Meε Acp-OMe.HBr*	63	51 - 53	-

a All compounds recrystallised from methanol-ether.

b Measured at 24°C.

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Table IV. 113

Microanalyses for N-methylamino acid methyl ester hydrobromides

MeX-OMe.HBr	Formula	Molecular weight	Requires			Found		
			C (%)	H (%)	N (%)	C (%)	H (%)	N (%)
MeGly-OMe.HBr*	$C_4H_{10}NO_2Br$	184.05	26.1	5.5	7.6			
L-MeAla-OMe.HBr*	$C_5H_{12}NO_2Br$	198.08	30.3	6.1	7.1	29.6	6.0	6.8
L-MeVal-OMe.HBr*	$C_7H_{16}NO_2Br$	226.13	37.2	7.1	6.2	37.3	7.4	5.8
L-MeIle-OMe.HBr*	$C_8H_{18}NO_2Br$	240.15	40.0	7.6	5.8	40.2	7.6	5.8
L-MeLeu-OMe.HBr*	$C_8H_{18}NO_2Br$	240.15	40.0	7.6	5.8	40.3	7.7	5.8
L-MePhe-OMe.HBr*	$C_{11}H_{16}NO_2Br$	274.17	48.2	5.9	5.1	48.4	6.1	5.3
MeγAbu-OMe.HBr*	$C_6H_{14}NO_2Br$	212.10	34.0	6.7	6.6			
MeεAcp-OMe.HBr*	$C_8H_{18}NO_2Br$	240.15	40.0	7.6	5.8			

Acetyl-N-methylamino acids: These were prepared from the appropriate acetylamino acid (10 mmol) using the same procedure as for the preparation of benzoyl-N-methylamino acids from the corresponding benzoylamino acids. The crude crystalline products were recrystallised either from ethyl acetate-petroleum ether (Ac-MeAla-OH, Ac-MeVal-OH) or water (Ac-MeIle-OH, Ac-MePhe-OH).

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Table IV. 114

Yields, melting points and rotations of acyl-N-methylamino acids

Acyl-MeX-OH	Once re-crystallised yield[a] (%)	m.p. (^{o}C)	$[\alpha]^{25}_{D}$ c = 1, DMF
Bz-MeGly-OH	53	105 - 106[b]	-
Bz-L-MeAla-OH*	57	133[c]	- 31.7
Bz-L-MeLeu-OH*	74	135 - 137	- 54.8
Ac-L-MeAla-OH*	7	110 - 112	- 50.0
Ac-L-MeVal-OH*	73	112 - 114	- 144.3
Ac-L-MeIle-OH*	39	121.5-122.5	- 135.4
Ac-L-MeLeu-OH*	55	107 - 108[d]	- 34.5
Ac-L-MePhe-OH	50	146.5-147.5[e]	- 58.3

a. All compounds recrystallised from water except Ac-L-MeAla-OH and Ac-L-MeVal-OH which were recrystallised from ethyl acetate-petroleum ether.

b. Lit. m.p. 103.5 - 104°C [Cocker and Lapworth (1931)].

c. Lit. m.p. for Bz-DL-MeAla-OH was 129-129.5^{o}C [Cocker (1937)].

d. After drying; the initial product was the mono hydrate m.p. 83 - 85°C.

e. Lit. m.p. 144-144.5^{o}C, $[\alpha]_D$ = -76.5 (EtOH) [du Vigneaud and Mayer (1932-3)].

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Table IV. 115

Microanalyses for acyl-N-methylamino acids

Ac-MeX-OMe	Formula	Molecular Weight	Requires C (%)	Requires H (%)	Requires N (%)	Found C (%)	Found H (%)	Found N (%)
Bz-MeGly-OH	$C_{10}H_{11}NO_3$	193.20	62.2	5.7	7.3	62.1	5.8	7.1
Bz-MeAla-OH	$C_{11}H_{13}NO_3$	207.22	63.8	6.3	6.8	64.1	6.6	6.9
Bz-MeLeu-OH *	$C_{14}H_{19}NO_3$	243.30	67.4	7.7	5.6	67.7	7.6	5.7
Ac-MeAla-OH *	$C_6H_{11}NO_3$	145.16	49.6	7.6	9.7	50.5	7.4	10.2
Ac-MeVal-OH *	$C_8H_{15}NO_3$	173.21	55.5	8.7	8.1	55.4	8.6	8.2
Ac-MeIle-OH *	$C_9H_{17}NO_3$	187.24	57.7	9.2	7.5	58.0	8.7	7.4
Ac-MeLeu-OH. H_2O *	$C_9H_{19}NO_4$	205.25	52.7	9.3	6.8	53.4	9.5	7.1
Ac-MePhe-OH	$C_{12}H_{15}NO_3$	221.25	65.1	6.8	6.3	64.7	7.2	6.5

(d) The reaction of methyl iodide/sodium hydride with some functional groups found in amino acid side chains

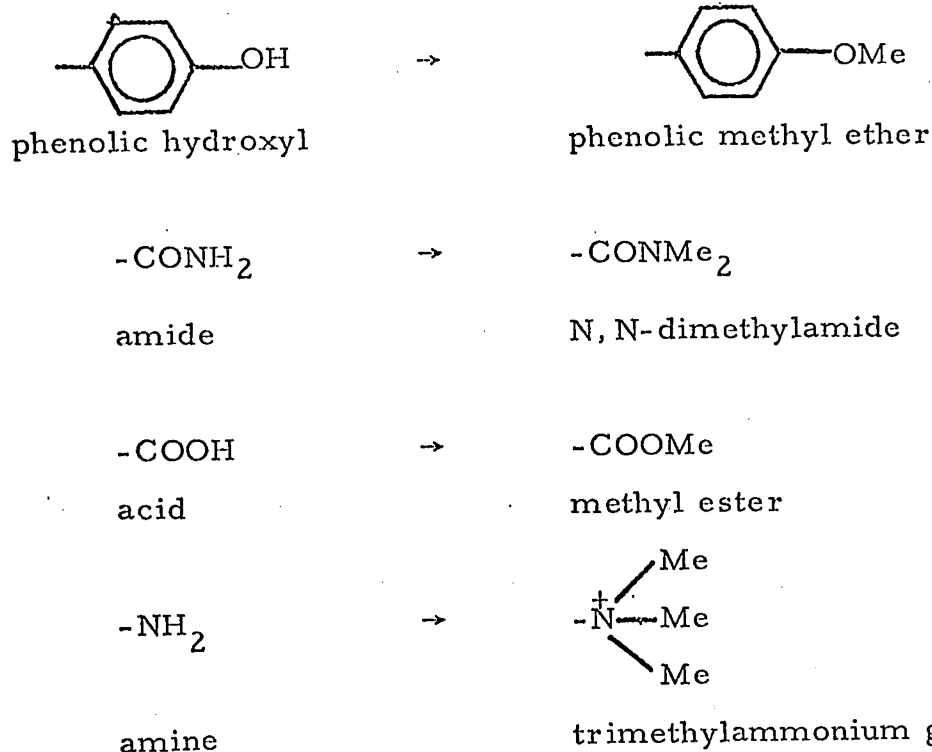
Methylation experiments were carried out on carbobenzoxytyrosine, carbobenzoxyasparagine and α-N-trityllysine methyl ester in order to find out how methyl iodide/sodium hydride reacted with phenolic -OH groups, primary amide groups and amino groups.

With carbobenzoxytyrosine the product after removal of the carbobenzoxy group was N,O-dimethyltyrosine methyl ester hydrobromide; the yield after recrystallisation was 71%. The identity and purity of the product was established by n.m.r. spectroscopy; full details of the spectrum are given in Part IV. 3.

Carbobenzoxyasparagine yielded, after removal of the carbobenzoxy group, β-(N,N-dimethylamido)-N-methylaspartic acid methyl ester; the hydrobromide was an oil and all attempts at crystallisation were unsuccessful. The n.m.r. spectrum of the oil and the i.r. spectra of the intermediate carbobenzoxy compound and the oil established the identity of the product; full details of the spectra are given in Part IV. 3.

α-N-Trityllysine methyl ester on methylation yielded α-N-trityl, ε-N,ε-N, ε-N-trimethyllysine methyl ester which was isolated as the iodide salt; full details of this experiment are given in Part IV. 1(e).

From these experiments we concluded that under our reaction conditions the following groups found in the side chains of the naturally occurring amino acids were methylated:



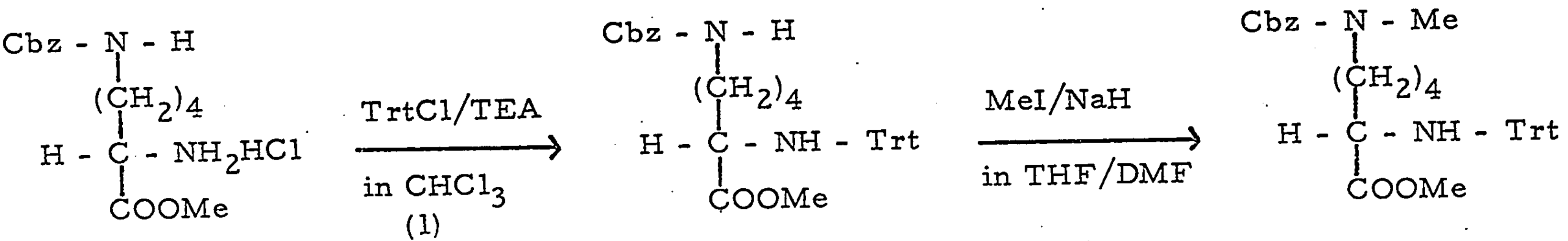
The mass spectral studies of Lederer and co-workers [see, e.g. Franek et al (1969)] have shown that aliphatic hydroxyl groups are also methylated by methyl iodide/sodium hydride. Investigation of the reaction of methyl iodide/sodium hydride with derivatives of the sulfur containing amino acids and with derivatives of arginine, histidine and tryptophan was not undertaken. The side-chains of these amino acids certainly react with methyl iodide/ sodium hydride as they do with methyl iodide/silver oxide [for discussion see Lederer (1968)] : the identification of the products would be a problem in itself and was in any case beyond the scope of this thesis.

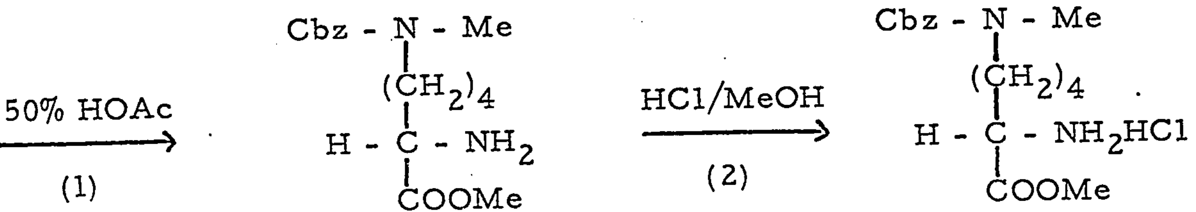
N, O-Dimethyl-L-tyrosine methyl ester:* A solution of carbobenzoxy-L-tyrosine (1.58 g; 5 mmol) in THF (25 ml) and DMF (2.5 ml) was heated at 70 - 80°C with methyl iodide (5 ml; 80 mmol) and sodium hydride dispersion (1.2 g; 25 mmol NaH) for 24 h. The product was obtained using a work-up similar to that described for the synthesis of N-methylamino acid methyl ester hydrobromides [Part IV. 1(c)]. The yield of crude crystalline product was 1.40 g (92%); recrystallisation from methanol-ether gave fine white crystals (1.08 g; 71%), m.p. 138 - 144°C.

β-(N, N-Dimethylamido)-N-methyl-L-aspartic acid methyl ester hydrobromide:* A solution of carbobenzoxy-L-asparagine (1.33 g; 5 mmol) in THF (25 ml) and DMF (5 ml) was heated at 70 - 80°C with methyl iodide (5 ml; 80 mmol) and sodium hydride dispersion (1.2 g; 25 mmol NaH) for 24 h. The product was obtained as a pale yellow oil by means of a work-up similar to that used for the preparation of N-methylamino acid methyl ester hydrobromides [see Part IV. 1(c)]. Attempted crystallisation from methanol-ether failed.

(e) The synthesis of some alkylated lysine derivatives

The main object of our work was to find a new route for the synthesis of ε-N-alkyllysines. In Part IV. 1(a) it was established that the trityl group provided considerable protection against methylation and in Part IV. 1(c) it was shown that ε-N-carbobenzoxyaminocaproic acid could be efficiently methylated. A suitable starting material therefore appeared to be a lysine derivative protected in the α-position with a trityl group and in the ε-position with a carbobenzoxy group. ε-Carbobenzoxy-L-lysine is commercially available and was the obvious starting material. It is too insoluble to tritylate directly and must be converted to ε-N-carbobenzoxy-L-lysine methyl ester hydrochloride and then tritylated. We developed two new procedures for the esterification step: the first involved the use of refluxing methanol and boron trifluoride etherate [see Coggins et al (1970) and Part IV. 5], and the second the use of 2, 2-dimethoxypropane in the presence of a small amount of concentrated HCl (see Part IV. 5). Using ε-N-carbobenzoxy-L-lysine methyl ester as starting material the following route to α-N-carbobenzoxy, ε-N-methyl-L-lysine methyl ester was investigated:





(1) Zervas and Theodoropoulos (1956) : (2) Coggins et al (1970).

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Tritylation of ε-N-carbobenzoxylysine methyl ester was carried out using trityl chloride and triethylamine in chloroform as described by Zervas and Theodoropoulos (1956). T. l. c. showed that the product contained unreacted starting material. A simple purification was devised taking advantage of the fact that tritylamino acid esters are difficult to saponify [Zervas and Theodoropoulos (1956)] whereas the starting material can be readily saponified. The crude product was treated with *N* NaOH/THF for 30 min at 25°C; after evaporation of the THF the product was dissolved in chloroform and washed with base to remove the ε-N-carbobenzoxylysine liberated by the saponification of the unreacted starting material. T. l. c. showed that the product was now completely free of starting material.

A comment on the t. l. c. of α-N-trityl, ε-N-carbobenzoxylysine methyl ester is necessary. This fully protected compound reacts with ninhydrin giving a brown spot if the plate is heated at 100°C for 10 min (twice as long as usual). The colour yield is very high, which suggests that the trityl group is cleaved under these conditions. We found that the most useful solvent system for the t. l. c. of lysine derivatives was *n*-butanol/acetic acid/water (4/1/1, v/v/v) abbreviated B4/A1/W1 (see Table II. 201). An artifact is observed when spots of α-N-trityl, ε-N-carbobenzoxylysine methyl ester are developed with this solvent system. The artifact takes the form of a long, faintly ninhydrin positive, streak stretching from the spot for the compound through the spot for ε-N-carbobenzoxylysine methyl ester towards the origin. There must be a small amount of deprotection during the development of the plate which is presumably caused by the acetic acid

in the solvent. T. l. c. using the chloroform/methanol (9/1, v/v) solvent system, abbreviated C9/M1, gave no artifact. Generally the purity of lysine derivatives was checked with the B4/A1/W1 solvent system which separates many more compounds than the C9/M1 solvent system (see Table II. 201); however, the final purity of α-N-trityl-protected lysines had to be ascertained with the C9/M1 solvent system.

Since α-N-trityl, ε -N-carbobenzoxylysine methyl ester was an oil the yield had to be determined by analysis. The compound was dissolved in methanol and an aliquot removed, deprotected and analysed for lysine using the amino acid analyser. Yields in the range 59-71% were obtained.

To establish the best methylating conditions a series of experiments were carried out using various amounts of methyl iodide and sodium hydride. The results are given in Table IV. 116. The extent of the methylation reaction varied considerably with the amount of sodium hydride used. With 2 and 3 equiv of sodium hydride the amounts of unreacted starting material were 38% and 12% respectively; however, with 5 equiv of sodium hydride, less than 1% of the starting material was not methylated. Doubling the amount of methyl iodide did not improve the yield of methylated product, in fact the yield decreased slightly. In all the experiments the amount of starting material accounted for was in the range 85 - 88%; however, in a later experiment (see Table IV. 117) the starting material was quantitatively accounted for. Side reactions, if there were any, would be expected to increase with the amount of sodium hydride, yet the

Table IV. 116

Methylation of α-N-trityl, ε-N-carbobenzoxy lysine methyl ester[a]

MeI:NaH: Trt-Lys(Cbz)-OMe[b] (equiv)	Yield of H-Lys(Me)-OH[c] (%)	Residual H-Lys-OH[c] (%)	Starting material accounted for (%)	Composition of deprotected products	
				H-Lys(Me)-OH (%)	H-Lys-OH (%)
8:2:1	53	32	85	62.2	37.8
8:3:1	74.5	12	87	85.9	14.1
8:5:1	85	0.6	86	99.2	0.8
16:3:1	73	14.5	88	83.5	16.5

a Mixture heated in THF/DMF (10/1, v/v) (10 ml) at 70 - 80° under reflux for 24 h.

b 0.69 mmol of Trt-Lys(Cbz)-OMe.

c Determined with the amino acid analyser after deprotection with HBr/HOAc followed by NaOH.

amount of starting material accounted for does not vary significantly with the conditions. The incomplete recovery of starting material in these experiments may simply be due to a poor analysis of the α-N-trityl, ε-N-carbobenzoxylysine solution used.

Methylation of α-N-trityl, ε-N-carbobenzoxy-L-lysine methyl ester using a reactant ratio of 8:5:1 was carried out on the preparative scale. Recrystallised ε-N-carbobenzoxy, ε-N-methyl-L-lysine methyl ester hydrochloride was obtained in 57% yield; the analysis and the integral of the n.m.r. spectrum were in excellent agreement with theory. Amino acid analysis after deprotection revealed a small amount of contamination with lysine (which corresponded to less than 1% by weight on the assumption that the impurity was H-Lys(Cbz)-OMe.HCl); ε-N-methyllysine was liberated in 105% yield. This represented the first direct synthesis from an L-lysine derivative of an ε-N-methyl-L-lysine derivative suitable for use in the synthesis of C-terminal ε-N-methyl-L-lysine containing peptides. [The only other synthesis of an ε-N-carbobenzoxy, ε-N-methyl-L-lysine derivative was reported by Benoiton and Deneault (1966) who prepared ε-N-carbobenzoxy, ε-N-methyl-L-lysine in 45% yield by carbobenzoxylating the copper salt of ε-N-methyl-L-lysine. The latter compound had been prepared from α-N-carbobenzoxy-L-lysine, which is itself difficult to prepare (Benoiton and Berlinguet, 1966, in 42% yield (Benoiton, 1964)]. The overall yield from ε-N-carbobenzoxy-L-lysine methyl ester hydrochloride was in the range 34 - 41%. The successful synthesis of this compound by an unambiguous route using steps that involved no risk of racemisation and giving a pure product had been the primary objective of this investigation. All the

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steps (including the preparation of the starting material from the commercially available ε-N-carbobenzoxy-L-lysine, see Part IV. 5) had been carefully studied and the conditions optimised.

The possibility of using similar routes for the synthesis of ε-N-ethyl-L-lysine and ε-N-propyl-L-lysine derivatives was also investigated. Some analytical experiments were carried out first. The results, given in Table IV. 117, showed that with an 8:5:1 reactant ratio ethylation was very efficient; only 1. 2% of α-N-trityllysine methyl ester remained unchanged after the reaction. Propylation was much less efficient and there was 20% of unreacted starting material using the same reactant ratio.

The preparation of ε-N-carbobenzoxy, ε-N-ethyl-L-lysine ethyl ester hydrochloride was therefore attempted using ε-N-carbobenzoxylysine ethyl ester hydrochloride as starting material. The product did not crystallise and so it was treated with HBr/HOAc to give crystalline ε-N-ethyl-L-lysine ethyl ester dihydrobromide. The overall yield of this compound from α-N-carbobenzoxylysine ethyl ester hydrochloride was only 12%; this was partly due to a relatively low yield in the tritylation step (only 49% compared with 69 - 71% for the corresponding methyl ester) and to inefficient crystalliation of the final product (much of the oil that was obtained did not crystallise). The identity and purity of the product was confirmed by n. m. r. spectroscopy. Amino acid analysis after saponification showed that ε-N-ethyllysine was liberated in 105% yield and there was a little lysine contamination which corresponded to 1. 5% by weight on the assumption that the impurity was present as lysine ethyl ester dihydrobromide.

Table IV. 117

Alkylation of α-N-trityl, ε-N-carbobenzoxylysine methyl ester [a]

Alkyl iodide	RI:NaH: Trt-Lys(Cbz)-OMe [b]	Yield of H-Lys(R)-OH (%) [c]	Residual H-Lys-OH (%) [c]	Starting material accounted for (%)
methyl	8:5:1	103	0.6	104
ethyl	8:5:1	90	1.2	91
n-propyl	8:5:1	- [d]	20	- [d]
ethyl	32:5:1	98	2.8	101
ethyl	8:5:1 then 16:10:1 [e]	89	3.9	93

a Mixture heated THF/DMF (10/1, v/v) (10 ml) at 70 - 80° for 24 h.

b 0.98 mmol of Trt-Lys(Cbz)-OMe.

c Determined with the amino acid analyser after deprotection with HBr/HOAc followed by NaOH.

d No constant for H-Lys(Pr)-OH was available at the time of the experiment.

e After 24 h heating with a reactant ratio of 8:5:1 more methyl iodide and sodium hydride was added and the mixture heated for a further period of 24 h.

The method did not look a very promising one for the synthesis of α-N-carbobenzoxy, ε-N-propyl-L-lysine propyl ester. In a trial experiment α-N-trityl, ε-N-carbobenzoxylysine methyl ester was alkylated with n-propyl iodide and sodium hydride using a similar procedure to that used for the methylation of this compound. The crude reaction product was completely deprotected by refluxing with 4 N HCl. Some white crystalline material was isolated as the monohydrochloride and amino acid analysis showed that it contained 23% by weight of lysine hydrochloride. There was another major ninhydrin positive peak emerging from the 15 cm column of the amino acid analyser at 205 min. This was believed to be the desired product ε-N-propyllysine. N.m.r. spectroscopy established that there was some of the propyl derivative in the product (the triplet due to the C-terminal methyl group was well resolved); however, it was extremely crude. This was not a satisfactory route for the synthesis of ε-N-propyllysine derivatives because the propylation reaction was incomplete.

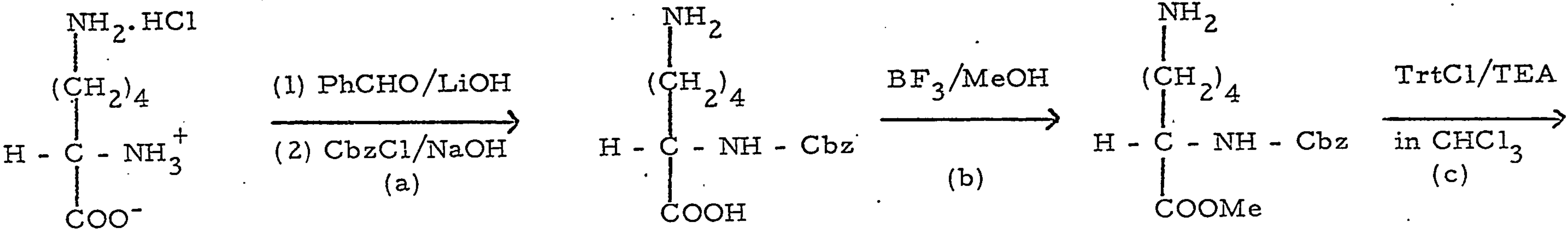
The use of the amino acid analyser in assessing the results of these experiments was invaluable and a comment on the separation conditions employed is necessary. An efficient separation of lysine, all the ε-N-monoalkyllysines, α-N-methyllysine and α-N,-ε-N-dimethyllysine was achieved on the 15 cm column of the amino acid analyser which contained Aminex A-5 resin (see Table II. 205, p. 280). Elution was carried out at 25°C using pH 5.28 buffer at half flow rate. The use of a low column temperature for the separation of ε-N-methylated-lysines was first reported by Paik and Kim (1967)

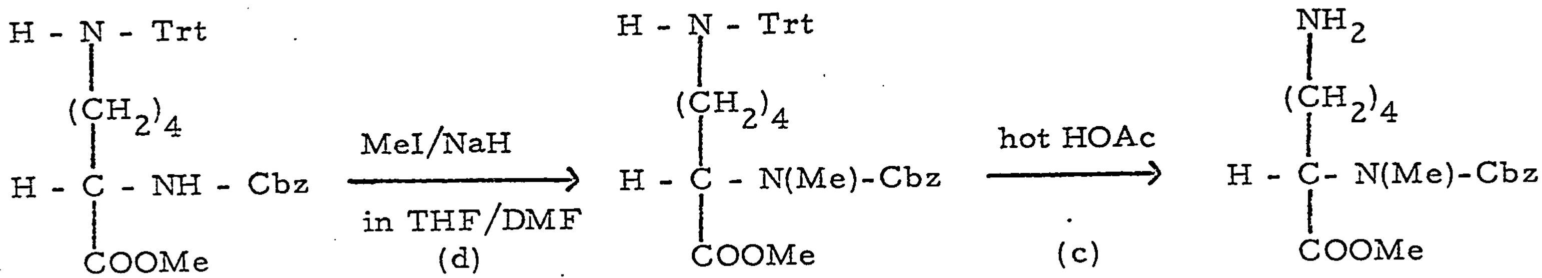
and the usefulness of the Aminex A-5 resin for similar separations was established by Seely et al (1969a). Our conditions were the same as those of Seely et al (1969a) except for the pH of the eluting buffer (Seely et al used pH 6.48 buffer). The separation of the three ε-N-monoalkyllysines was extremely good. The basicities of these compounds must be very similar and the separation therefore depends primarily on differences in the hydrophobic interactions of the N-alkyl groups with the resin; lowering the temperature accentuated these differences. The width of the peaks increased considerably as the size of the alkyl group increased [the widths of the peaks at the half heights were H-Lys-OH 3.5 min, H-Lys(Me)-OH 4 min, H-Lys(Et)-OH 6 min, H-Lys(Pr)-OH 9.5 min].

The possibility of synthesising derivatives of α-N, ε-N-dimethyllysine and α-N-methyllysine by routes analogous to those used for the preparation of ε-N-carbobenzoxy, ε-N-methyllysine methyl ester was also investigated.

Methylation of α-N, ε-N-dicarbobenzoxy-L-lysine gave after deprotection a 48% yield of α-N, ε-N-dimethyl-L-lysine monohydrochloride (attempts to crystallise the intermediate α-N-methyl, ε-N-methyllysine methyl ester dihydrobromide were unsuccessful). Recrystallisation of the product gave a final yield of 24%. N.m.r. spectroscopy established the identity of the product and confirmed its purity.

The following route to α-N-methyllysine derivatives was investigated:





(a) Bezas and Zervas (1961).

(b) Coggins et al (1970) and Part IV. 5 of this thesis.

(c) Zervas and Theodoropoulos (1956).

(d) Part IV. 1(a) of this thesis.

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α-N-Carbobenzoxy-L-lysine was prepared from lysine by the method of Bezas and Zervas (1961). Traces of ε-N-carbobenzoxylysine were removed from the product by passing it through a column of copper carbonate and alumina as described by Savrda and Bricas (1968). The final product was checked for purity on the amino acid analyser; to do this a chromatographic method had to be developed for the separation of α-N-carbobenzoxylysine and ε-N-carbobenzoxylysine. A good separation was achieved on the short column of the analyser; the ε-isomer and lysine were eluted with pH 4.25 buffer and then the α-isomer was eluted with pH 5.28 buffer (see Table II. 203, p. 28B). The marked difference in the elution times of the two carbobenzoxylysines is very interesting. ε-N-Carbobenzoxylysine behaves as a neutral amino acid with a large aromatic side chain; it has no net charge at this pH and is readily eluted [under the same conditions tyrosine is eluted at 18 min (J. Tong, personal communication)]. α-N-Carbobenzoxylysine is, however, much more basic, firstly because the free ε-NH_2 group has a higher pK'_a than a free α-NH_2 group (e.g. the ε-NH_2 group of lysine has a pK'_a of 10.8 and the α-NH_2 group has a pK'_a of 9.2 [Edsall and Wyman (1958), p. 465]) and secondly because the acidity of the carboxyl group is somewhat diminished since it is no longer adjacent to a charged α-NH_3^+ group (e.g. the pK'_a of the -COOH group of lysine is 2.2 and of lysyllysine is 3.0 [Edsall and Wyman (1958), p. 465] and the pK'_a of glycine is 2.4, of acetylglycine 3.6 and of alanylglycine 3.2 [Edsall and Wyman (1958), pp. 452 and 466]). The basicity of α-N-carbobenzoxylysine is such that at pH 4.25 it is eluted more slowly than lysine. This must mean that

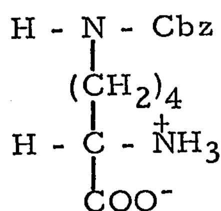
at pH 4.25 α-N-carbobenzoxylysine, like lysine, has a net positive charge; because of the aromatic group in the side chain it is eluted more slowly than lysine. At pH 5.28 α-N-carbobenzoxylysine emerges ahead of lysine and is not resolved from the ε-isomer; at this pH the carboxyl group must be completely ionised so that the molecules carry no net charge and the compound behaves like a neutral amino acid (this explanation is represented diagrammatically in Fig. IV. 102 opposite). The pH of elution is therefore critical for the successful separation of the carbobenzoxylysines. It is interesting to note that the constant for ε-N-carbobenzoxylysine is 21.9 whereas the value for α-N-carbobenzoxylysine is 7.7. A free α-amino group thus appears to give a much higher colour yield than a free ε-amino group; however, it is not always true that α-amino groups give higher colour yields than ω-amino groups, e.g. α-aminobutyric acid has a constant of 21.4 and γ-aminobutyric acid has a constant of 24.1 [see Table II. 202, p. 28A; Spackman, Stein and Moore (1958) give constants for the latter compounds of 24.9 and 27.3].

Esterification of α-N-carbobenzoxylysine was achieved using refluxing methanol-boron trifluoride, a procedure that had been successfully developed for the synthesis of ε-N-carbobenzoxylysine methyl ester (see Part IV. 5). Crystallisation of the product as its hydrochloride was unsuccessful; consequently the crude free ester, which was isolated in 79% yield, was used directly for the next step.

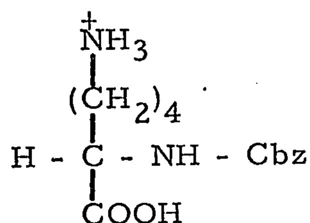
Fig. IV. 102

Schematic representation of the chromatographic properties of the monocarbobenzoxylysines on the amino acid analyser

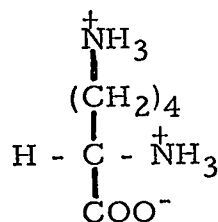
pH 4.25



neutral
rapidly eluted

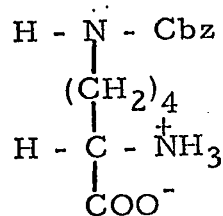


positively charged
slowly eluted

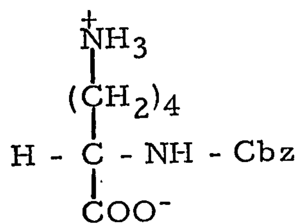


positively charged
slowly eluted

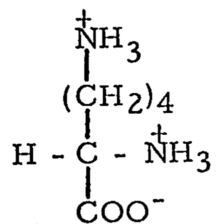
pH 5.28



neutral
rapidly eluted



neutral
rapidly eluted



positively charged
slowly eluted

Tritylation was carried out by the normal Zervas-Theodoropoulos (1956) method. T.l.c. of the product (an oil) using the B4/A1/W1 solvent system gave a single ninhydrin positive spot and revealed no unchanged starting material. This was fortunate, since purification by the method employed for the α-N-trityl-isomer would have been impossible. There was no streak on the plate as there had been with the α-N-trityl-isomer; this reflects the greater resistance to acid of the ε -N-trityl group [see e.g. Amiard and Goffinet (1957) and Bezas and Zervas (1961)]. Better evidence of this resistance to acid of ε -N-trityl groups was obtained when attempts were made to analyse α-N-carbobenzoxy, ε -N-trityllysine methyl ester for lysine in order that the yield of the tritylation step could be determined. The oil was dissolved in methanol and an aliquot deprotected and analysed for lysine using the procedure which had been used successfully for the analysis of α-N-trityl, ε -N-carbobenzoxylysine methyl ester. The result (Table IV. 118) indicated a yield of 7% for the tritylation step. This was hard to believe and so several aliquots were taken and analysed after deprotection using more vigorous conditions; the results and the conditions are given in Table IV. 118. All the conditions would have given complete deprotection of α-N-carbobenzoxylysine methyl ester and so the poor yields obtained in two of the four cases must be due to inefficient removal of the ε-N-trityl group.

Table IV. 118

Deprotection of α-N-carbobenzoxy, ε-N-trityllysine methyl ester under various conditions [a]

Deprotection Conditions	Estimated lysine content of sample[b] (mmol)	Estimated yield of Cbz-Lys(Trt)-OMe from Cbz-Lys-OMe (%)
30% HBr/HOAc (2 ml) for 2 h at 25°C then N NaOH (5 ml)/THF (5 ml) for 0.5 h at 25°C	0.28	7
HOAc (2 ml) for 2 h at 100°C then 30% HBr/HOAc (2 ml) for 2 h at 25°C then N NaOH (5 ml)/THF (5 ml)for 0.5 h at 25°C	0.90	23
6 N HCl (5 ml) for 12 h in evacuated, sealed tube at 100°C	1.90	50
HOAc (2 ml), 30% HBr/HOAc (2 ml), 90% phenol (0.5 ml) for 24 h in evaucated sealed tube at 100°C	1.80	47

a Aliquots consisting of 1/100 of sample deprotected in each case.

b Determined with the amino acid analyser.

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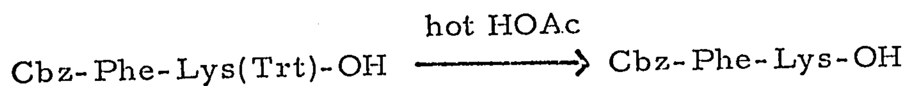
HBr in acetic acid was least effective in cleaving the ε -N-trityl group. This result is in accord with those of Bezas and Zervas (1961) who prepared ε-N-trityllysine methyl ester dihydrobromide by treating α-N-carbobenzoxy, ε-N-trityllysine methyl ester dihydrobromide with dry HBr in acetic acid.

Boiling acetic acid followed by HBr in acetic acid at 25°C was also comparatively inefficient for the cleavage of the ε-N-trityl group. This seemed at first to contrast with statements in the literature by Bezas and Zervas (1961) and Amiard and Goffinet (1957). Bezas and Zervas (1961), for example, in the discussion of their paper do not give any indication that special conditions are necessary for the efficient cleavage of ε-N-trityl groups. However, the yield of the deprotection step



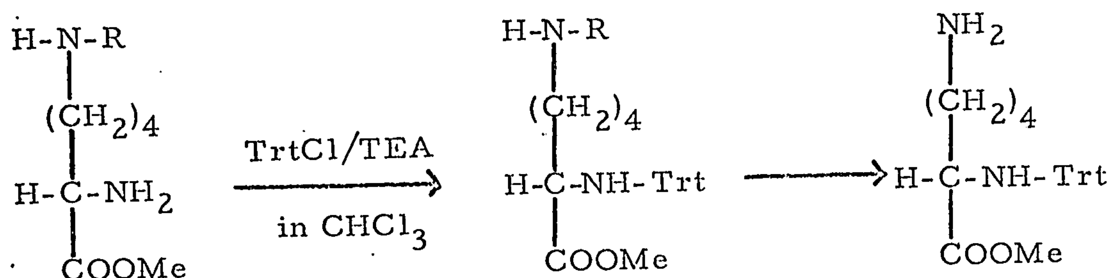
was only 45% whereas deprotection of tritylglycine using similar conditions gave glycine in 93% yield [Zervas and Theodoropoulos (1956)]. Similarly Amiard and Goffinet (1957), who prepared several lysine containing peptides using the trityl group for ε -NH_2 group protection state that: "On peut, en fin de synthese ..... eliminer facilement tous les groupes trityles par hydrolyse en acide acetique dilue." However, in most of their syntheses these authors used boiling 50% acetic acid containing several equivalents of HCl. The latter was included to allow isolation of the product as the hydrochloride although the results in Table IV. 118 suggest this acid may be essential for efficient deprotection.

Hot HBr in acetic acid and hot 6 N HCl gave twice as much deprotection as boiling acetic acid followed by HBr in acetic acid at 25°C. It was assumed for the purpose of calculating a yield of α-N-carbobenzoxy, ε-N-trityllysine methyl ester that deprotection was quantitative with these two reagents. The yield of this compound, the starting material for the methylation step, was therefore about 50%.

The methylation of α-N-carbobenzoxy, ε-N-trityllysine methyl ester using a reactant ratio of 8:5:1 appeared to take place quite efficiently as judged by the disappearance of the amide II band in the i.r. spectrum. The crude product was refluxed with 6 N HCl/acetone (1/1, v/v) for 4 h in order to liberate α-N-methyllysine. An unsuccessful attempt was made to crystallise the product as the mono-hydrochloride. When this had failed a solution of the oil was analysed on the amino acid analyser. It contained one major ninhydrin positive peak which emerged from the 15 cm column (at 25°C with pH 5.28 buffer at half flow rate) at 86 min; this was 8 min ahead of α-N, ε-N-dimethyllysine and 17 min ahead of lysine. The solution also contained a small amount of all three possible impurities, i.e. lysine, ε-N-methyllysine and α-N, ε-N-dimethyllysine. This was clearly an unsatisfactory route for the preparation of α-N-methyllysine.

A study was also made of the effect of methyl iodide and sodium hydride on free amino groups. The compound used for the investigation was α-N-trityllysine methyl ester. This compound was chosen for study since all the possible products of methylation of the ε-amino group could be determined on the amino acid analyser after removal of the α-N-trityl group by using the elution conditions of Seely et al (1969a).

The synthesis of α-N-trityllysine methyl ester was in itself an interesting synthetic problem. The obvious route was as follows:



The problem was what protecting group could be readily introduced into the ε-position and then selectively removed after tritylation ? The trifluoracetyl group was an obvious choice since it is cleaved readily by alkali [for a general discussion of this protecting group see Bodanszky and Ondetti (1966), p. 32] using conditions that would not affect the α-N-trityl ester part of the molecule. However, α-N-trityl, ε-N-carbobenzoxylysine methyl ester had already been prepared as an intermediate in the synthesis of ε-N-carbobenzoxy, ε-N-methyllysine methyl ester (see p. 76) and it was therefore decided to investigate first whether this compound could be selectively deprotected to give the desired product. It was found that catalytic hydrogenation of a methanolic solution of α-N-trityl, ε-N-carbobenzoxylysine methyl ester for 2 h using hydrogen at 1 atm and 10% palladium on charcoal catalyst gave α-N-trityllysine methyl ester in 81% yield. Purification of the product was simply achieved by the selective saponification technique already employed to purify the starting material (see p. 76). Any detritylated material was saponifiable whereas α-N-trityllysine methyl ester resists saponification. Although in our experiments the starting material was purified before hydrogenation, this is unnecessary. One selective saponification at the end is all that is required to obtain pure α-N-trityllysine methyl ester.

This represents the first example of the selective removal of an N-carbobenzoxy group in the presence of an α-N-trityl group. Both protecting groups are acid labile and are quantitatively cleaved by HBr/HOAc at 25°C (see p.77A). The α-N-trityl group is so acid labile that it can be removed by hot acetic acid whereas the carbobenzoxy group is not affected under these conditions [Zervas and Theodoropoulos (1956)]. Selective removal of an α-N-trityl group in the presence of an N-carbobenzoxy group is therefore possible and had been used earlier in the synthesis of ε-N-carbobenzoxy, ε-N-methyllysine methyl ester (see p. 75). The alternative mild method of removing these two protecting groups is catalytic hydrogenation. The result presented here shows that under mild hydrogenation conditions the complete hydrogenolysis of an N-carbobenzoxy group can be achieved while an α-N-trityl group is scarcely affected. The comparative resistance to hydrogenolysis of α-N-trityl groups has been reported by Stelakatos et al (1959) who showed that α-N-tritylamino acid benzyl esters can be converted by selective hydrogenation to the α-N-tritylamino acids in good yields. The selective removal of an N-carbobenzoxy group in the presence of an ε-N-trityl group by means of HBr/HOAc [Bezas and Zervas (1961)] was referred to on p. 86.

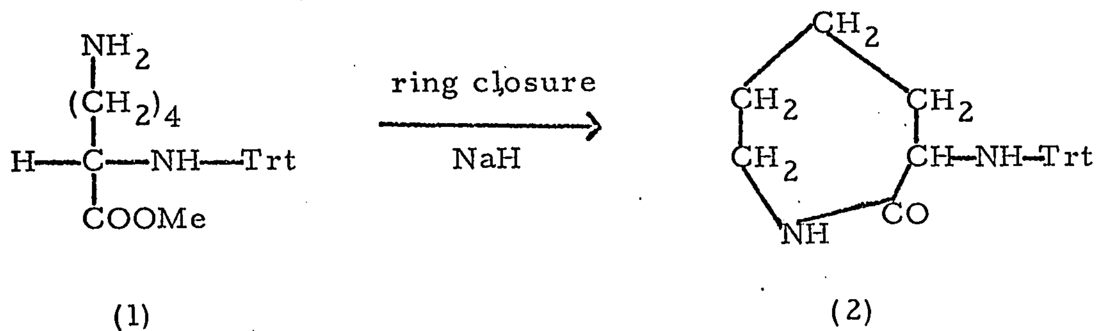
In a preliminary experiment α-N-trityllysine methyl ester was methylated with methyl iodide and sodium hydride in the usual manner using a reactant ratio of 16:6:1. Two products were obtained. The first was a water and ether insoluble cream solid which was isolated directly from the crude reaction mixture. On recrystallisation from chloroform-ether this gave a yellow crystalline solid which was shown by i. r. spectroscopy to be an ester. Hydrolysis with boiling 6 N HCl for 1 h gave a solution containing ε-N, ε-N, ε-N-trimethyllysine and no other amino acids. This compound was tentatively

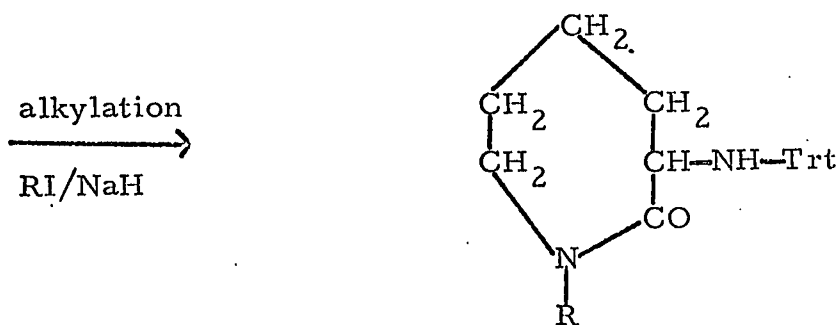
identified as a salt of α-N-trityl, ε-N, ε-N, ε-N-trimethyllysine methyl ester. The second product was obtained as an oil mixed with nujol from the ethereal extract of the crude reaction mixture. Its i. r. spectrum contained no ester band; it had a strong amide I band but no amide II band. Treatment with boiling acetic acid followed by the addition of water gave a white crystalline precipitate which was identified by i. r. spectroscopy as trityl carbinol. The aqueous solution obtained after detritylation was evaporated and the residue refluxed with 6 N HCl for 12 h. Amino acid analysis of the final solution revealed only minute traces of the basic amino acids. These results suggested that the second product was 1-methyl, 3-tritylamino-homopiperidone (formula 3a in Fig. IV. 103 opposite). This compound would have a single amide I band since it is a cyclic amide [Nakanishi (1964), p. 47]; it is a tertiary amide and this explains its resistance to acid hydrolysis. 3-Amino-homopiperidone has been previously prepared in 40% yield by heating a methanolic solution of lysine methyl ester hydrochloride with sodium [Adamson (1943)]. In order to confirm the identity of the two products some further experiments were necessary.

Firstly a deliberate attempt was made to obtain the non cyclic product. The methylation of α-N-trityllysine methyl ester was repeated using a reactant ratio of 8:5:1. Again two products were obtained; however, this time the yield of the ether soluble amide was very small and this compound was discarded. The ether and water insoluble product was isolated and a sample was hydrolysed with 6N HCl. ε-N, ε-N, ε-N-Trimethyllysine was liberated in an amount

Fig. IV. 103

The formation and alkylation of 3-tritylaminohomopiperidone





(3a) R = Me

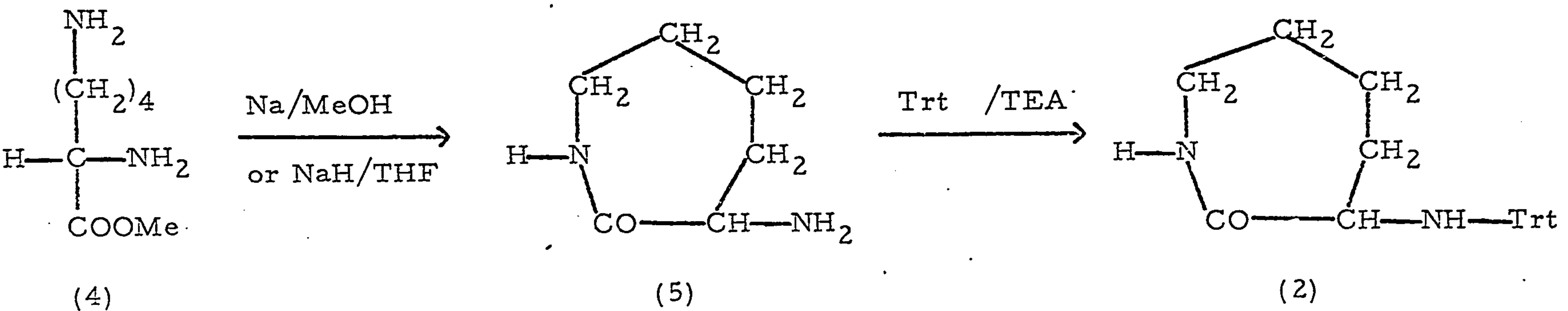
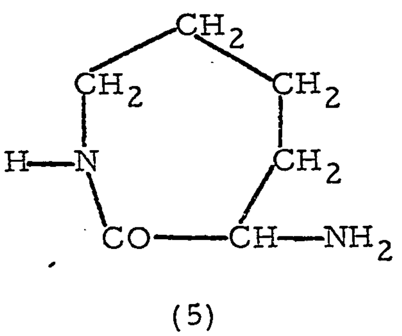
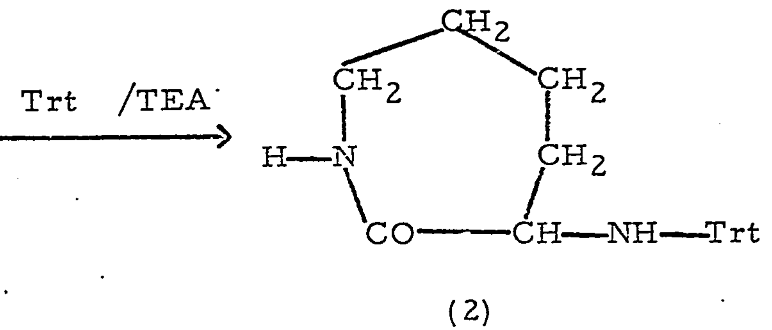
(3b) R = Et

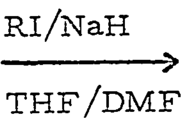
(3c) R = nPr

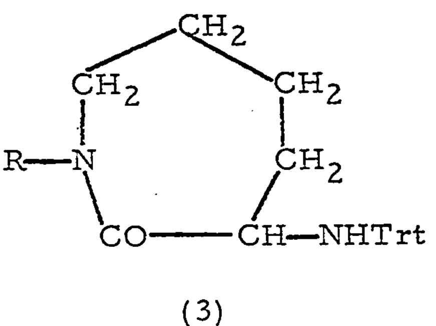
corresponding to 105% yield from the starting material. It is possible that the standard solution used to determine the amino acid analyser constant in this experiment had deteriorated since it was stored for several weeks before use. This would mean that the constant determined at the time of the experiment was too low and this probably means that the yield was overestimated. Nonetheless it is clear that methylation of α-N-trityllysine methyl ester gives a derivative of ε-N, ε-N, ε-N-trimethyllysine in nearly quantitative yield. The i.r. and n.m.r. spectrum of the crude product were consistent with the structure of α-N-trityl, ε-N, ε-N, ε-N-trimethyllysine methyl ester iodide. The integral of the n.m.r. spectrum was a little too high in the region between 7.5 and 9τ where the lysine side chain resonances occur. This was probably due to a little nujol contamination. Recrystallisation from chloroform-ether and the methanol-ether gave pale yellow crystals in 49% yield. The n.m.r. spectrum of the final product showed acetone contamination corresponding to 0.5 molecules per molecule of α-N-trityl, ε-N, ε-N, ε-N-trimethyllysine methyl ester. The acetone was completely removed by heating in vacuo at 65°C. This experiment showed that free amino groups are quantitatively converted to trimethylammonium groups by methyl iodide-sodium hydride. The very high yield of the trimethylated product shows that the quaternisation reaction goes much more readily than the competing cyclisation reaction. Also the experiment establishes a new method of preparing ε-N, ε-N, ε-N-trimethyllysine.

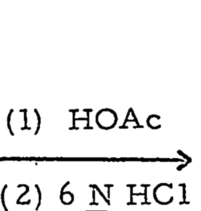
Total synthesis was used to establish that the ether soluble product of the preliminary experiment was 1-methyl, 3-tritylaminohomopiperidone. First 3-tritylaminohomopiperidone was prepared by heating a solution of α-N-trityllysine methyl ester with sodium hydride. The crude reaction product was an oil which was crystallised in 79% yield from petroleum ether. The i.r. spectrum showed two -NH- stretch bands and an amide I band as expected for a cyclic secondary amide [Nakanishi (1964), p. 47] with an amino substituent. The n.m.r. spectrum showed that two protons were exchangeable and this, together with the integral of the spectrum, was fully consistent with the structure. Prolonged hydrolysis with 6 N HCl gave lysine in 83% yield. Alkylation of this authentic 3-tritylaminohomopiperidone with methyl iodide-sodium hydride in the usual manner gave a crude product which had an i.r. spectrum identical with that of the ether soluble product of the preliminary experiment. The cyclisation of α-N-trityllysine methyl ester provides an unambiguous route to the intramolecular anhydride of lysine and may be compared with the unambiguous synthesis of the intermolecular anhydride of lysine (i.e. the diketopiperazine) from ε-N-carbobenzoxylysine methyl ester by Katchalski et al (1946).

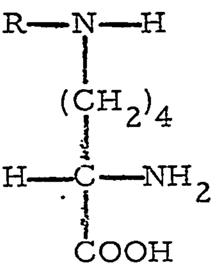
The difficulties encountered in the propylation of α-N-trityl, ε-N-carbobenzoxylysine methyl ester (see p. 80) must have been steric in origin. Examination of a model of 3-tritylaminohomopiperidone suggested that this compound might be relatively easy to alkylate since all the bulky groups are held back from the amide -NH- group. The following route for preparing ε-N-alkyllysines was therefore investigated.









3 a R = Me

b R = Et

c R = nPr

The starting material, lysine methyl ester dihydrochloride, is readily available and Adamson (1943) had shown that it can be converted into 3-aminohomopiperidone in 40% yield. This crystalline compound should be easy to tritylate and it had been shown above (p. 92) that the product 3-tritylaminohomopiperidone was crystalline. There were two uncertain steps, the alkylation and the subsequent hydrolysis to an ε-N-alkyllysine. These two steps were investigated.

Samples of 3-tritylaminohomopiperidone were alkylated in the usual manner with methyl, ethyl and n-propyl iodides respectively. The products were hydrolysed with 6 N HCl for 24 h at 110°C and the yields of basic amino acids determined on the amino acid analyser. The amount of lysine liberated was approximately the same in each case, which suggested that alkylation had occurred to the same extent with all three alkyl halides. Thus the initial assumption that alkylation would occur readily even with a relatively large alkyl group was correct. However, the yields of the ε-N-alkyllysines were poor. This was due to inefficient hydrolysis, since in a control experiment the starting material gave a reasonable yield of lysine. If this route is to have any practical value, conditions for the efficient hydrolysis of the N-alkylated homopiperidones (which can be crystallised) must be found. The method could then be very useful for the preparation of ε-N-alkyllysines for use as chromatographic standards. Ion exchange resins would probably be necessary for the isolation of the final products and there would undoubtedly be a little lysine contamination; however, the amount of this could be measured and allowance made for it in the estimation of amino acid analyser constants.

Table IV. 119

Alkylation of 3-tritylaminohomopiperidone [a]

Alkyl iodide	Lysine liberated on hydrolysis[c] (%)	ε-N-Alkyllysine liberated on hydrolysis[c] (%)
- [b]	62	0
methyl	2.8	32
ethyl	3.4	17
n-propyl	3.0	35 [d]

a 3-Tritylaminohomopiperidone (0.56 mmol) heated with RI (4.5 mmol) and NaH (2.8 mmol) in THF/DMF (10/1, v/v) (7 ml) for 24 h at 70 - 80°C.

b Control experiment for the acid hydrolysis step.

c Determined with the amino acid analyser after hydrolysing an aliquot with 6 N HCl (5 ml) in an evacuated sealed tube for 24 h at 110°C.

d Estimated using the constant for ε-N-ethyllysine.

α-N-Trityl, ε-N-carbobenzoxy-L-lysine methyl ester:* ε-N-Carbobenzoxy-L-lysine methyl ester hydrochloride (3.31 g; 10 mmol) was dissolved in chloroform (30 ml) and triethylamine (2.22 g; 22 mmol) and trityl chloride (2.78 g; 10 mmol) were added. The solution was allowed to stand at 25°C for 8 h. It was then extracted with water (2 x 30 ml) and the chloroform layer evaporated to give an oil. The oil was shaken with N NaOH (20 ml) and THF (20 ml) for 30 min at 25°C. The THF was evaporated and the resulting oil and aqueous solution shaken with chloroform (100 ml). The chloroform layer was collected, washed with water (2 x 25 ml) and dried (Na_2SO_4). The purity of the product was checked by t.l.c. using both the B4/A1/W1 and the C9/M1 solvent systems. Then the chloroform was evaporated and the oil redissolved in methanol (100 ml). The solution was analysed as follows. A 1 ml aliquot was removed, evaporated and the residue treated with 30% HBr in acetic acid (2 ml) for 1 h at 25°C. The reagent was evaporated and the solid residue shaken with N NaOH (5 ml) and THF (5 ml) for 30 min at 25°C; then the THF was evaporated and water (20 ml) and ether (5 ml) added. The ether layer was discarded, the aqueous layer acidified with 4 N HCl and analysed for lysine on the amino acid analyser. Yields in the range 59 - 71% were obtained. The rest of the oil was recovered from the methanolic solution by evaporation and dried over night in vacuo over P_2O_5.

ε-N-Carbobenzoxy, ε-N-methyl-L-lysine methyl ester hydrochloride:* A solution of α-N-trityl, ε-N-carbobenzoxy-L-lysine methyl ester (6.11 mmol) in THF (25 ml) and DMF (2.5 ml) was heated with methyl iodide (3.1 ml; 49 mmol) and sodium hydride dispersion (1.47 g; 31 mmol NaH) at 70 - 80°C for 24 h. The reaction mixture was evaporated, re-evaporated with ether (25 ml), the semi-solid residue dissolved in ether (100 ml) and the resulting solution washed with water (2 x 25 ml). The ether layer was dried ($MgSO_4$) and the solvent removed to give an oil. This was dissolved in acetic acid (20 ml), then water (20 ml) was added and the mixture heated at 100°C for 30 min. More water (25 ml) was added and after cooling the mixture was filtered. The filtrate, which was milky in appearance, was extracted with ether (25 ml) and the clear aqueous solution cooled to 0°C. The pH was adjusted to 9.0 with 4 N NaOH and the solution extracted with ethyl acetate (3 x 50 ml). The combined extracts were dried ($MgSO_4$) and the solvent removed to give a colourless oil. The oil was dissolved in methanol (10 ml), re-evaporated and then redissolved in methanol (20 ml) and methanolic 8 N HCl (0.8 ml) added. Cooling to 0°C followed by the slow addition of ether (400 ml) gave fine white crystals (1.32 g; 63%), m.p. 113.5 - 114.5°C. Recrystallisation from methanol-ether gave 1.21 g (57%), m.p. 113 - 115°C, $[\alpha]_D^{25} = + 15.6^\circ$ (c = 1, H_2O).

Analysis calculated for $C_{16}H_{25}N_2O_4Cl$ (344.84): C 55.7; H 7.3; N 8.1%.
Found : C 55.6; H 6.9; N 8.0%.

A sample was deprotected completely and analysed for amino acids; ε-N-methyllysine was liberated in 105% yield together with a small amount of lysine corresponding to 1% by weight contamination of the product with ε-N-carbobenzoxylysine methyl ester hydrochloride.

α-N-Trityl, ε-N-carbobenzoxy-L-lysine ethyl ester*:

This was prepared from ε-N-carbobenzoxy-L-lysine ethyl ester hydrochloride (3.45 g; 10 mmol) using a procedure similar to that used for the synthesis of the corresponding methyl ester. The yield of the purified product was 49%.

ε-N-Ethyl-L-lysine ethyl ester dihydrobromide*:

α-N-Trityl, ε-N-carbobenzoxy-L-lysine ethyl ester (4.89 mmol) was ethylated using a procedure similar to that described for the methylation of the methyl ester except that ethyl iodide was used instead of methyl iodide. The reactant ratio was 8:5:1 as for the methylation of the methyl ester. The trityl group was removed and α-N-carbobenzoxy, ε-N-ethyl-L-lysine ethyl ester was isolated as for the methyl analogue. Attempted crystallisation of the hydrochloride failed and so the free ethyl ester was recovered and treated with 30% HBr in acetic acid (20 ml) overnight at 25°C. The reagent was evaporated and the residual oil dissolved in water (25 ml) and extracted with ether (2 x 10 ml). Evaporation of the aqueous layer gave a pale yellow oil which was dried overnight in vacuo over P_2O_5. Crystallisation from ethanol-ether gave white needles (0.43 g; 24%), m.p. 134 - 137°C. (The mother liquor yielded a pale yellow oil on evaporation but no further crop of crystals could be obtained). The n.m.r. spectrum of the product confirmed its identity and established its purity. Amino acid analysis of a sample (26.2 mg; 72 μmol) after saponification showed that it contained ε-N-ethyllysine (76 μmol; 105%) and lysine (1.2 μmol which corresponded to 1.6% by weight contamination with lysine ethyl ester dihydrobromide).

ε-N-Propyl-L-lysine: α-N-Trityl, ε-N-carbobenzoxy-L-lysine methyl ester (6.63 mmol) was propylated using the same procedure as for the methylation of this compound, except that n-propyl iodide was used instead of methyl iodide. The product was partially deprotected and worked-up to give the ε-N-carbobenzoxy, ε-N-alkyl-L-lysine ester hydrochloride (an oil) which was refluxed with 4 N HCl for 4 h. The resulting solution was evaporated to give an oil which was twice redissolved in water (20 ml) and re-evaporated. The final oil was dissolved in water (20 ml) and 2 N LiOH added to pH 5.8. The solution was evaporated and the solid residue extracted with boiling ethanol (20 ml). A white crystalline solid (300 mg) was obtained by filtration. Amino acid analysis showed that this contained 23% by weight of lysine monohydrochloride. The major ninhydrin positive peak emerged from the 15 cm column at 205 min (pH 5.28 buffer, half normal flow rate, column temperature 25°C) and this was assumed to be the desired product. The yield estimated on the assumption that the product consisted of only lysine monohydrochloride and ε-N-propyllysine monohydrochloride was 16%. The n.m.r. spectrum of the product established that it did contain some propylated material.

α-N, ε-N-Dimethyl-L-lysine monohydrochloride: A solution of α-N, ε-N-dicarbobenzoxy-L-lysine (2.9 g; 7 mmol) in THF (50 ml) and DMF (5 ml) was heated with methyl iodide (7 ml; 112 mmol) and sodium hydride dispersion (2.0 g; 42 mmol NaH) for 24 h at 70 - 80°C. The reaction mixture was evaporated, re-evaporated with ether (20 ml) and the residue dissolved in ether (100 ml) and washed

with water (2 x 25 ml). The ethereal solution was dried ($MgSO_4$) and the solvent removed to give an oil which was treated with 30% HBr in acetic acid (25 ml) for 2 h at 25°C. After evaporation of the reagent the product was dissolved in water and extracted with ether (2 x 25 ml). The aqueous layer was evaporated to give an oil (2.29 g; 93%) which was exhaustively dried in vacuo over P_2O_5. Crystallisation from methanol-ether failed and so α-N, ε-N-dimethyl-L-lysine methyl ester was recovered as an oil and then refluxed with 2 N HCl (50 ml) for 2 h. Evaporation followed by re-evaporation with water (2 x 25 ml) gave a pale yellow oil. This was dissolved in water (20 ml) and the pH adjusted to 5.8 with 2 N LiOH. The solution was evaporated to dryness and the solid residue extracted with boiling ethanol (20 ml). The crude product (0.71 g; 48%) was collected by filtration and recrystallised from water-ethanol to give white needles (0.34 g; 24%). The n.m.r. spectrum established the identity and purity of the product. Amino acid analysis showed that it contained no lysine and a trace of ε-N-methyllysine corresponding to 0.3% by weight on the assumption that the impurity was present as ε-N-methyllysine monohydrochloride.

<u>α-N-Carbobenzoxy-L-lysine methyl ester:</u>

α-N-Carbobenzoxy-L-lysine (1.40 g; 5 mmol) was esterified in exactly the same manner as ε-N-carbobenzoxy-L-lysine (see p. 158) using methanol (25 ml) and boron trifluoride etherate (3.1 ml; 25 mmol). When esterification was complete the methanol was evaporated and the resulting oil dissolved in water (50 ml) and extracted with ether (2 x 10ml). The aqueous solution was cooled to 0°C, saturatedwith NaCl and then 4 N NaOH added to pH 10 and the solution extracted with ethyl acetate (50 ml); the pH of the aqueous layer was then raised to 11

and two further extractions with ethyl acetate (50 ml) carried out. The combined extracts were dried ($MgSO_4$) and the solvent removed to give an oil which was twice re-evaporated with methanol (20 ml). In a previous experiment attempted crystallisation of the product as its hydrochloride failed and therefore the yield of the oil was determined by amino acid analysis. The product was dissolved in methanol (100 ml) and a 1 ml aliquot removed, evaporated and treated with 30% HBr in acetic acid (2 ml) for 1 h at 25°C. After evaporation of the reagent the resulting oil was treated with N NaOH (10 ml) for 1 h at 25°C and then acidified, made up to 100 ml and an aliquot analysed for lysine. The yield of the reaction was found to be 79%. The bulk of the product was recovered from the methanolic solution by evaporation and dried exhaustively in vacuo over P_2O_5. T.l.c. of the product using the B4/A1/W1 solvent system gave a single ninhydrin positive spot.

α-N-Carbobenzoxy, ε-N-trityl-L-lysine methyl ester*:

α-N-Carbobenzoxy-L-lysine methyl ester (3.86 mmol) was dissolved in chloroform (10 ml) and triethylamine (0.43 g; 4.25 mmol) and trityl chloride (1.08 g; 3.86 mmol) were added. After standing for 16 h the solution was extracted with water (2 x 20 ml) and dried (Na_2SO_4). T.l.c. using the B4/A1/W1 solvent system showed a single spot. Evaporation of the chloroform gave an oil which was dissolved in methanol (100 ml) and several 1 ml aliquots were removed and deprotected using various conditions (for details see Table IV. 118, p.85A). The final solutions were analysed for lysine; the yield as determined by deprotection with 6 N HCl (5 ml) for 12 h in an evacuated sealed tube at 100°C followed by amino acid analysis was 50%. The bulk of the product was recovered from the methanolic solution by evaporation and the oil was exhaustively dried in vacuo over P_2O_5.

α-N-Methyl-L-lysine: A solution of α-N-carbobenzoxy, ε-N-trityl-L-lysine methyl ester (1.9 mmol) in THF (10 ml) and DMF (1 ml) was heated with methyl iodide (0.9 ml; 15.2 mmol) and sodium hydride dispersion (0.46 g; 9.5 mmol NaH) for 24 h at 70 - 80°C under reflux. The reaction mixture was evaporated, re-evaporated with ether and the residue dissolved in ether and washed with water (2 x 25 ml). The ethereal solution was dried ($MgSO_4$) and the ether evaporated to give a mixture of immiscible oils. The i.r. spectrum indicated that methylation had occurred; the amide II band had disappeared. The oils were dissolved in acetone (10 ml), 6N HCl (10 ml) was added and the mixture refluxed for 4 h. The acetone was evaporated and a solid precipitated; this was removed by extraction into ether (2 x 10 ml). The aqueous layer was evaporated to give a pale yellow oil which was twice re-evaporated with water (20 ml), redissolved in water (20 ml) and 2 N LiOH added to pH 5.8. Evaporation gave an oil which was left standing overnight in the presence of ethanol (10 ml). The ethanol was discarded and the residual oil dissolved in water, acidified and analysed for basic amino acids on the 15 cm column of the amino acid analyser (pH 5.28 buffer, half normal flow rate, 25°C). There was one major ninhydrin positive peak which emerged from the column at 86 min and was presumbly the desired product; in addition there were 3 other ninhydrin positive peaks corresponding to significant contamination with lysine, ε-N-methyllysine and α-N,ε-N-dimethyllysine.

α-N-Trityl-L-lysine methyl ester:* A solution of α-N-trityl, ε-N-carbobenzoxy-L-lysine methyl ester (1.59 g; 6.87 mmol) in methanol (50 ml) was hydrogenated for 2 h at 1 atm in the presence of 10% palladium on charcoal catalyst (0.8 g). T.l.c. using the B4/A1/W1 solvent system showed that all the starting material had disappeared and in its place a new slower moving substance had appeared together with a very small amount of lysine methyl ester. After filtration (Celite) and evaporation of the methanol the residue was shaken with N NaOH (10 ml) and THF (10 ml) for 30 min at 25°C. The THF was evaporated and water (20 ml) and chloroform (30 ml) added. The chloroform layer was collected, washed with water (20 ml) and dried (Na_2SO_4). T.l.c. using the B4/A1/W1 solvent system gave a single ninhydrin positive spot, together with a faint ninhydrin positive streak leading back from the spot towards the origin. (This artifact is very similar to that observed with α-N-trityl, ε-N-carbobenzoxylysine methyl ester, see p. 76). T.l.c. with the C9/M1 solvent system gave a single ninhydrin positive spot. The chloroform was evaporated to give an oil; the i.r. spectrum of the oil contained no amide bands which confirmed the loss of the carbobenzoxy group. The oil was dissolved in methanol (100 ml) and a 1 ml aliquot was removed, evaporated and deprotected with 30% HBr in acetic acid and N NaOH as described for α-N-trityl, ε-N-carbobenzoxylysine methyl ester (see p. 95). Amino acid analysis of the final solution showed that the lysine liberated corresponded to a final yield of the methyl ester of 81%. The bulk of the product was recovered as an oil, by evaporating the methanolic solution, and dried exhaustively in vacuo over P_2O_5.

α-N-Trityl, ε-N, ε-N, ε-N-trimethyl-L-lysine methyl ester iodide:* A solution of α-N-trityl-L-lysine methyl ester (0.76 mmol) in THF (10 ml) and DMF (1 ml) was heated with methyl iodide (0.4 ml; 6.1 mmol) and sodium hydride dispersion (0.18 ml; 3.8 mmol) for 18 h at 70 - 80°C. The reaction mixture was evaporated and then shaken with ether (50 ml) and water (25 ml) for 15 min. Filtration gave a cream coloured solid (0.49 g). The i.r. and the n.m.r. spectra of this solid suggested that it was the desired product. A sample (31.0 mg) was refluxed with 6 N HCl (10 ml) for 1 h; the acid was evaporated and the product dissolved in a mixture of water (10 ml) and ether (5 ml). The ether layer was discarded and the aqueous layer analysed for basic amino acids. It was found to contain ε-N, ε-N, ε-N-trimethyllysine (51 μmol corresponding to a yield of 105%) and no other basic amino acids. The remainder of the solid product was recrystallised from chloroform-ether to give yellow crystals (0.39 g; 91%) m.p. 168 - 172°C. A second recrystallisation from acetone-ether gave pale yellow crystals (0.21 g; 49%), m.p. 201 - 202°C. The n.m.r. spectrum of this compound showed that it contained approximately half a molecule of acetone per molecule of product. Drying overnight in vacuo, over P_2O_5 at 65°C removed the acetone; the m.p. was unchanged and the n.m.r. spectrum was now satisfactory.

3-Tritylamino-L-homopiperidone:* A solution of α-N-trityl-L-lysine methyl ester (5.92 mmol) in THF (10 ml) and DMF (10 ml) was heated with sodium hydride dispersion (1.44 g; 30 mmol NaH) for 24 h at 70 - 80°C. The residue from evaporation of the reaction mixture was dissolved in ether (50 ml) and washed with water (2 x 20 ml). The ether layer was dried ($MgSO_4$) and evaporated to give an oil which was dissolved in THF (50 ml) and a 1 ml aliquot removed, evaporated and then hydrolysed with 6 N HCl (5 ml) in an evacuated sealed tube for 72 h at 110°C. Lysine was liberated in 83% yield based on starting material. The bulk of the product was obtained as an oil by evaporation of the THF. Crystallisation from petroleum ether gave a cream coloured product (1.74 g; 79%), m.p. 183 - 185°C.

1-Alkyl-3-tritylamino-L-homopiperidones: A solution of 3-tritylamino-L-homopiperidone (0.56 mmol) in THF/DMF (10/1, v/v) (7 ml) was heated with the appropriate alkyl iodide (4.5 mmol) and sodium hydride dispersion (0.14 g; 2.8 mmol NaH) for 24 h at 70 - 80°C. The residue obtained by evaporation of the reaction mixture was re-evaporated with ether (20 ml) and then dissolved in ether (50 ml) and washed with water (2 x 20 ml). The ether layer was dried ($MgSO_4$) and the solvent removed to give an oil which was redissolved in THF (50 ml). 5 ml aliquots were evaporated and then hydrolysed with 6 N HCl (5 ml) in an evacuated, sealed tube at 110°C for 24 h. A sample of 3-tritylamino-L-homopiperidone (0.16 mmol) was hydrolysed in the same manner as a control. After the hydrolyses the acid was evaporated and the residues redissolved in water (50 ml) and ether (10 ml). The ether layer was discarded and the aqueous layer was

analysed for basic amino acids. The results are given in Table IV. 119 (see p.94A). The remaining 45 ml of the THF solutions of the 1-alkyl-3-tritylaminohomopiperidones were evaporated and the resulting oils were crystallised with difficulty from petroleum ether. The final yields of crystalline material were:

1-methyl-3-tritylamino-L-homopiperidone*	38 mg (20%)	m.p. 115-117°C
1-ethyl-3-tritylamino-L-homopiperidone*	71 mg (34%)	m.p. 124-125°C
1-*n*-propyl-3-tritylamino-L-homopiperidone*	84 mg (40%)	m.p. 136-138°C

IV. 2. METHODS FOR ANALYSING N-METHYLAMINO ACIDS

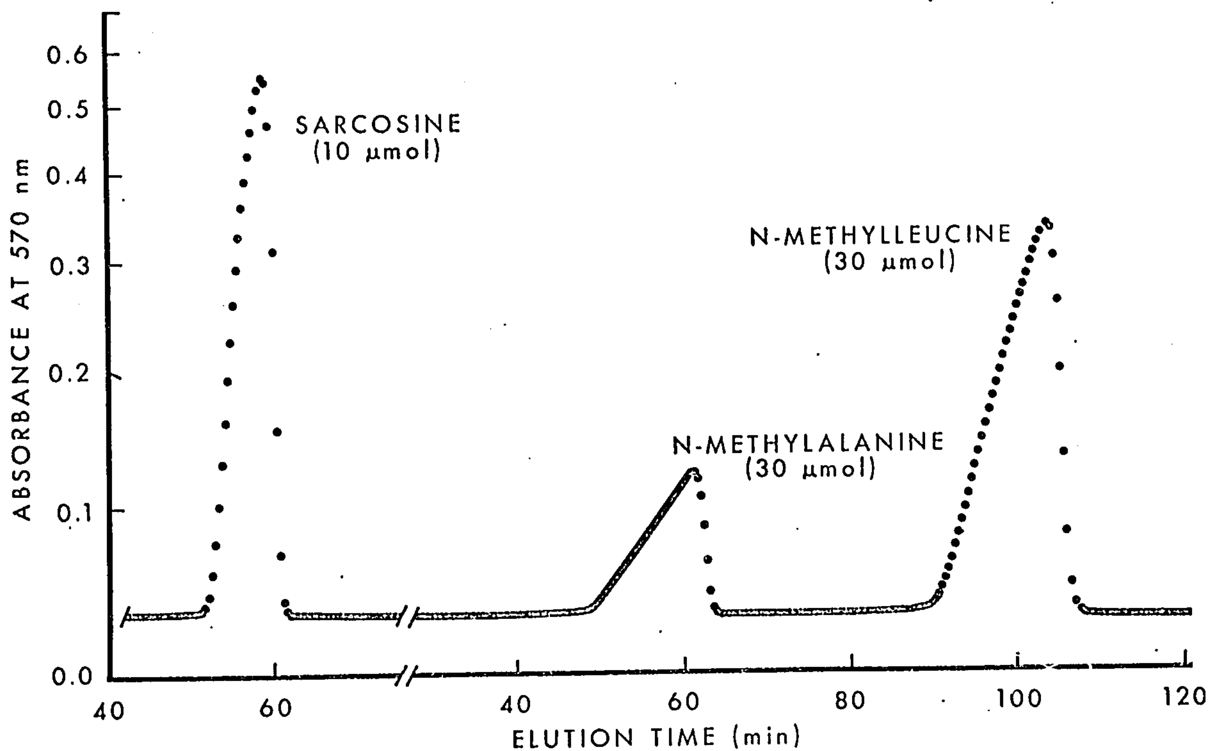
(a) Analysis of N-methylamino acids on the automatic amino acid analyser

N-Methylamino acids like amino acids react with ninhydrin [Moore and Stein (1948), Dalgleish et al (1950), Russell (1960)]. Recently Ebata and co-workers (1966a, 1966b) have shown that solutions of N-methylamino acids can be analysed with the automatic amino acid analyser. However, under the conditions of the analyses the colour yields ranged from 1 - 20% of the colour yields for the corresponding amino acids. Control experiments showed that the colour yields were poor because the time in the reaction coil was insufficient for complete colour development. Also a number of the N-methylamino acids gave very unsymmetrical peaks and the H x W method [Spackman, Stein and Moore (1958)] could not be used to determine these compounds. Instead the much more tedious addition of absorbances method [Spackman, Stein and Moore (1958)] had to be employed. The reproducibility of the analyses was poorer than for amino acids; in groups of four analyses the maximum deviation from the mean was about 6% compared with 3% for amino acids.

In preliminary experiments with sarcosine, N-methylalanine and N-methylleucine on our amino acid analyser we found colour yields in the range 1. 5 - 10% of those for the corresponding amino acids. The compounds were all eluted with pH 3. 28 buffer but gave unsymmetrical peaks (see Fig. IV. 201); the peaks for N-methylalanine and N-methylleucine were particularly unsymmetrical. Elution with pH 4. 25 buffer gave normal symmetrical peaks.

Fig. IV. 201

Chromatograms of N-methylamino acids obtained using pH 3.28 buffer at normal flow rate.



For much of the work described in this thesis N-methylamino acids were analysed on the long column of the amino acid analyser using the normal column temperature (57°C) and buffer flow rate (68 ml/h). Sarcosine was eluted with pH 3.28 buffer and N-methylalanine and N-methylleucine with pH 4.25 buffer. The use of a constant for determining the amount of N-methylamino acid by substitution in the formula

$$\mu mol = \frac{H \times W^*}{C}$$ [Spackman, Stein and Moore (1958)]

was found to be unsatisfactory. The constant varied with the concentration of the samples, as is shown in Fig. IV. 202 for sarcosine. However, plots of H x W versus concentration gave straight lines (Fig. IV. 203). [Seely et al (1969) obtained similar results with ammonia]. Sarcosine also gave a straight line despite the fact that the peaks were unsymmetrical. Reproducibility was poorer than with amino acids and the standard curves were rarely usable for more than two or three weeks. A standard was run with each group of analyses and if the amount of N-methylamino acid estimated from the standard curve differed repeatedly by more than 5% from the actual amount, a new standard curve was determined. Generally analyses carried out on the same day or on two successive days were reproducible to ± 2% (c.f. analyses of amino acids which were reproducible to ± 1%).

'Constants' and elution times for the N-methylamino acids and the corresponding amino acids are given in Table IV. 201. Data are also given for α-C-methylamino acids. These amino acids, like N-methylamino acids, give poor colour yields on the amino acid analyser.

* H = net height of peak = absorbance; W = width of peak at half net height; C is a constant.

Table IV. 201

Chromatographic data for N-methyl- and α-C-methyl-amino acids at normal flow rates

Amino acid	pH of eluting buffer[a]	Concentration of sample (μmol/ml)[b]	Constant	Elution time (min)
sarcosine	3.28	10	2.20	59.0
glycine	3.28	1	20.6	89.0
alanine	3.28	1	19.6	95.5
N-methylalanine	4.25	10	0.31	28.0
alanine	4.25	1	24.0	32.5
α-C-methylalanine	4.25	5	1.82	33.0
N-methylleucine	4.25	10	0.69	35.0
N-ethylleucine	4.25	10	0.03	32.0
leucine	4.25	1	23.9	51.0
α-C-methylleucine	4.25	5	1.95	46.5

a Buffer flow rate 68 ml/h.

b 1 ml samples used.

Fig. IV. 202

Variation of the amino acid analyser constant for sarcosine with concentration at normal flow rate.

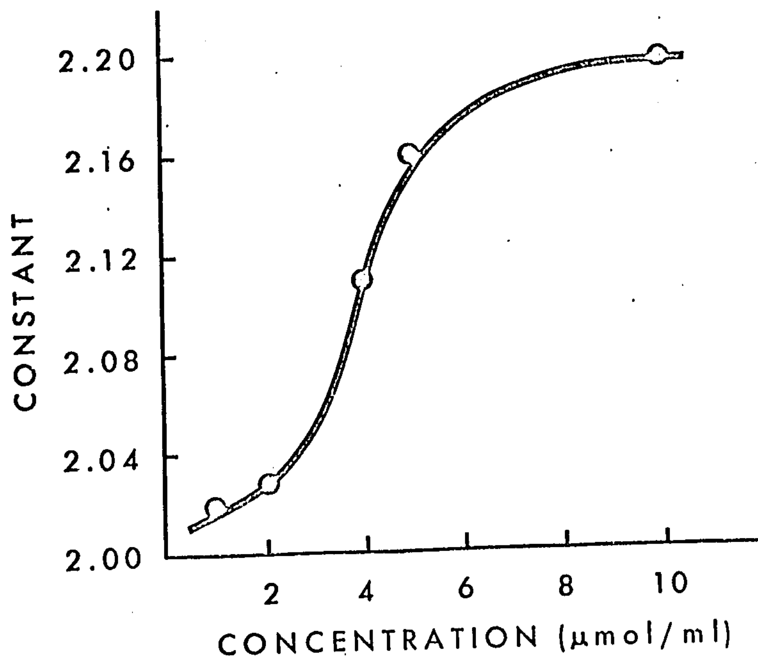
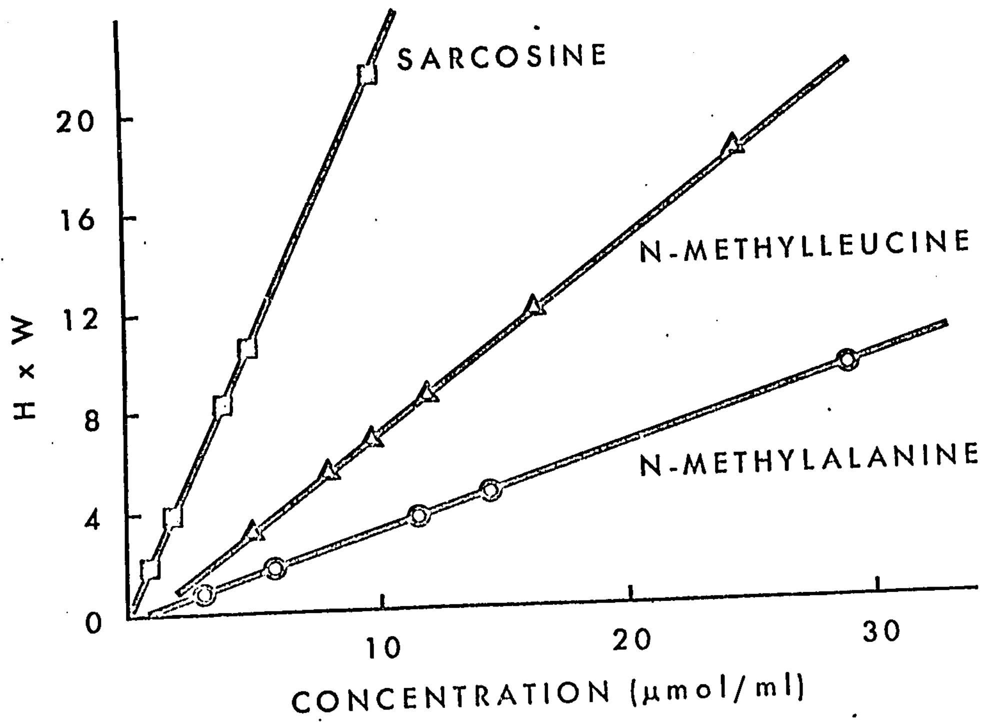


Fig. IV. 203

Standard curves for the analysis of N-methylamino acids at normal buffer flow rates.



After the greater part of the work described in this thesis had been completed an attempt was made to improve the method for analysing N-methylamino acids. First two preliminary experiments were carried out.

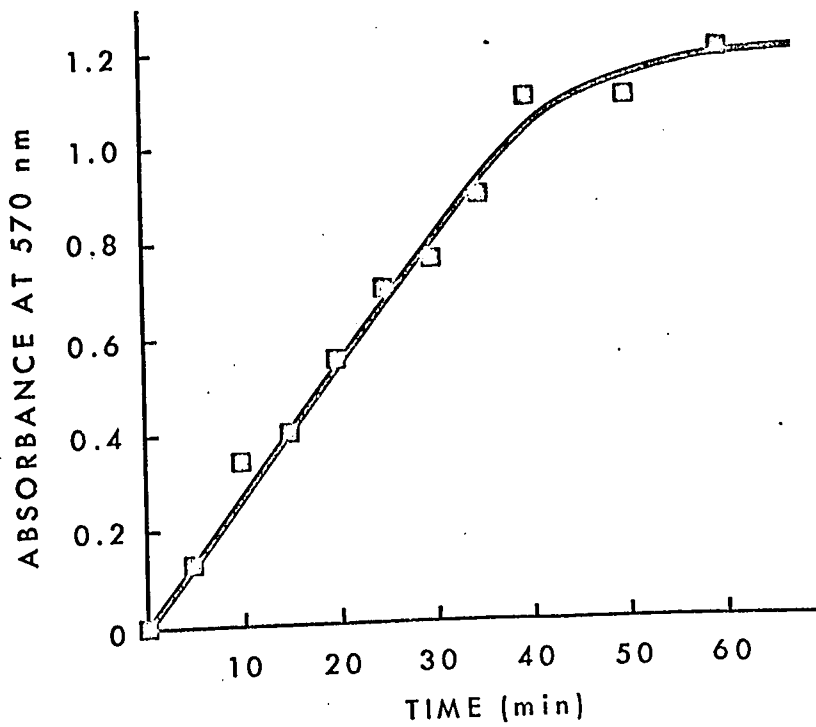
The method of Rosen (1957) was used to investigate the rate of colour development during the sarcosine-ninhydrin reaction. Samples containing 0.5 μmol of sarcosine were mixed with cyanide-acetate buffer (pH 5.5) and ninhydrin reagent and then heated on a boiling water bath for periods ranging from 5 min to 1 h. After appropriate dilution with isopropanol-water (1/1, v/v) the absorbance at 570 nm was read against a blank. The results, illustrated in Fig. IV. 204, showed that the reaction was not complete until after 45 min. This contrasts with glycine for which the reaction was complete in 10 - 12 min [Rosen (1957)].

The absorption spectra of the coloured solutions produced by heating samples of leucine, N-methylleucine and α-C-methylleucine with buffer and ninhydrin reagent [Rosen's method (1957)] for 30 min were observed in the range 350 - 700 nm. The spectra in all three cases were identical having absorption maxima at 408 and 570 nm and minima at 458 nm.

These two experiments showed that the colour reaction is the same for amino acids and N-methylamino acids; however, the rate of reaction is much slower for N-methylamino acids. The time in the reaction coil of the amino acid analyser is approximately 10 min, which is clearly too short for complete reaction between ninhydrin and N-methylamino acids. Ebata et al (1966a, 1966b), who reached the same conclusion, suggested using a longer reaction coil. In our case this

Fig. IV. 204

Variation of the absorbance at 570 nm with time for the sarcosine ninhydrin reaction.



required modification of the instrument. Instead we decided to reduce the buffer flow rate to one half the normal value; this has the effect of increasing the time in the reaction coil by 50% (the ninhydrin flow rate was not changed). A dramatic increase in colour yields was observed (see Table IV. 202). The constants for the N-methylamino acids increased from 10- to 20-fold, compared with increases of about 2-fold for the amino acids (these last changes are due to the increased width of the peaks). The constant for α-C-methylleucine increased 5-fold and that of N-ethylleucine 30-fold. The constants for N-methylamino acids were now really constant and the reproducibility was comparable with that for amino acids. The values of the constants for N-methylamino acids varied from 18 - 93% of the values for the corresponding amino acids. All subsequent analyses for N-methylamino acids were carried out using half normal buffer flow rate.

A complete explanation of these results in terms of what is known about the reaction of ninhydrin with amino acids is impossible. McAldin (1960) has proposed schemes for the reaction of ninhydrin with amino acids and with primary amines (see Fig. IV. 205). We have shown that the coloured product produced with N-methylamino acids is the same as that produced with amino acids (Ruhemann's purple). De-alkylation of the methylamino group must take place to allow the formation of Ruhemann's purple (4) in Fig. IV. 205) from N-methylamino acids. There are two possible pathways for de-alkylation. An initial oxidation may give formaldehyde and the corresponding amino acid which would then react in the normal way for amino acids. Alternatively the initial oxidation might yield a keto-acid and methylamine. The methylamine would then react in the normal way for primary amines. Formaldehyde would be liberated in both cases and

Table IV. 202

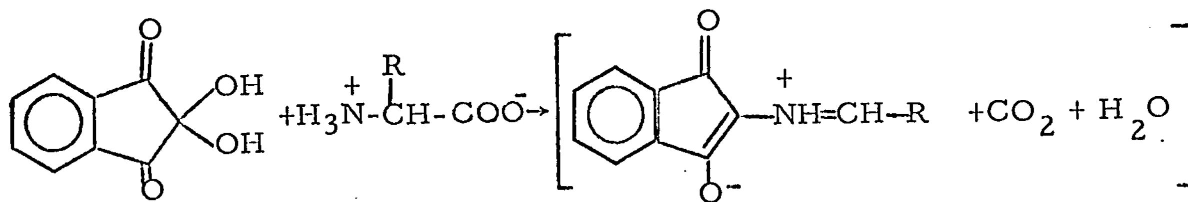
Chromatographic data for N-methylamino acids at normal and half normal buffer flow rates

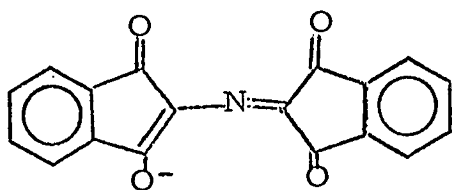
Amino acid	68 ml/h[a]		34 ml/h[a]	
	constant	time(min)	constant	time(min)
sarcosine	3.04	30.0	36.3	59.5
glycine	23.4	33.0	38.9	67.0
N-methylalanine	0.36	28.0	7.5	54.0
alanine	24.1	32.5	42.7	61.0
N-methylleucine	0.74	34.0	15.7	63.0
N-ethylleucine	0.03	32.0	1.0	60.5
α-C-methylleucine	1.95	46.5	11.2	92.5
leucine	23.9	51.0	40.5	102.0
N-methylphenylalanine	0.66	61.0	23.4	107.0
phenylalanine	21.3	77.0	-	-

a Buffer pH 4.25

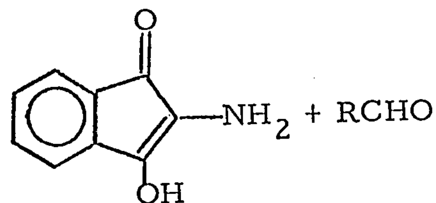
Fig. IV. 205

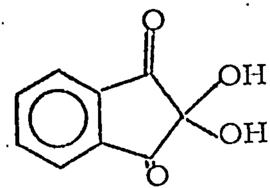
The reaction of ninhydrin with amino acids and amines



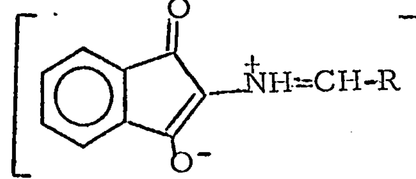


+(1)





+ R-CH2-NH2 → primary amine



this has been reported for sarcosine [McAldin (1960)]. McAldin (1960) favours the initial formation of methylamine; however, his argument is inconclusive. All that can be definitely stated is that the de-alkylation step must be rate limiting to explain the slow rate of reaction of N-methylamino acids with ninhydrin.

The explanation of the poor colour yields obtained with α-C-methylamino acids is not obvious. Once again our results indicate that the final coloured product is the same. Presumably a combination of steric and electronic effects leads to a slower rate of formation of the intermediate (3) in the reaction scheme. It appears that the α-H atom of amino acids is necessary for rapid colour development with ninhydrin.

Our method of using reduced buffer flow rate for the analysis of N-methylamino acids on the automatic amino acid analyser allowed the accurate analysis of samples containing 0.1 μmol of N-methylamino acid. This represented a 20-fold improvement in sensitivity on the method of Ebata et al (1966b). In the most favourable case the detection limit was approximately the same as for the corresponding amino acid, i.e. 0.005 μmol and in the worst case about 6 times larger than this.

The fact that amino acids can be detected in smaller quantities than N-methylamino acids may cause problems in the analysis of mixtures obtained by the hydrolysis of naturally occurring peptides containing N-methylamino acids. The N-methylamino acids may simply be missed.[a] In an attempt to overcome this problem, two groups [Ebata et al (1966b) and Ward et al (1967)] have described the use of g.l.c. for the analysis of hydrolysates obtained from peptide antibiotics. Samples containing 0.01 μmol of amino acid could be

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[a] This is probably what happened with fortuitine [Barber et al (1965)] where amino acid analysis failed to reveal the presence of 2 moles of MeLeu-OH.

analysed after suitable modification (acylation and esterification); the amino acids and the N-methylamino acids gave peaks that were of comparable size [Ward et al (1967)]. Ebata et al (1966b) made a careful comparison of the g.l.c. method with the amino acid analyser method and found that g.l.c. had a major disadvantage. G.l.c. did not give as true an analysis as the amino acid analyser. This was probably due to inefficient preparation of the volatile derivatives required for the g.l.c. analyses. With g.l.c. the 'recoveries' of N-methylamino acids were poor (54 - 74%) in comparison with the 'recoveries' obtained with the amino acid analyser method (75 - 104%).

Our amino acid analyser method gave true analyses which were more reproducible than those obtained by Ebata et al. using an amino acid analyser. No tedious, and possibly inefficient, preparation of volatile derivatives was required and a normal amino acid analyser could be used without modification.

It is less sensitive than g.l.c. and the colour yields of some of the N-methylamino acids are still less than one third of the colour yields of the corresponding amino acids. However, by using high sensitivity cuvettes (longer path length) and a longer reaction coil the amount of sample required could be reduced; increased coil length should also reduce the differences in colour yields between amino acids and N-methylamino acids.

(b) A method for measuring the optical purity of N-methyl-L-leucine

In the past, the optical purity of amino acids has generally been established using stereospecific enzymes such as L- and D-amino acid oxidase. Such methods are not applicable to N-methylamino acids either because the specificity of enzymes towards these compounds and their derivatives has not been studied in sufficient detail, or because no suitable enzymes are available. Rotation measurements have served as the only reliable criterion of optical purity for N-methylamino acids. These measurements which require isolation of the pure N-methylamino acid in relatively large amounts (10 to 20 mg) do not establish the optical purity, but serve simply as a means of comparison with previously isolated compounds.

While our work was in progress, a new method was described for establishing the optical purity of amino acids [Manning and Moore (1968a)]. The method involved coupling an L-amino acid N-carboxyanhydride to the amino acid under test in aqueous solution at pH 10.4 using a small scale modification of the method of Hirschmann et al (1967). Optically pure L-amino acids gave L-L dipeptides, whereas partially racemic amino acids gave mixtures of the diastereoisomeric L-L and L-D dipeptides. The pairs of diastereoisomers were separated and analysed on an automatic amino acid analyser. One part of the D-amino acid could be detected in the presence of 1000 parts of the L-isomer when 2 μmol samples were analysed.

We decided to use this method to establish the optical purity of our synthetic N-methyl-L-leucine. At the time we wished to try the method, the details [Manning and Moore (1968b)] were not available and so experiments were necessary to establish a suitable procedure. L-Alanine N-carboxyanhydride was employed for the couplings and trial experiments were carried out with L-leucine, DL-leucine and DL-alanine. In these experiments a modified version of the synthetic procedure of Hirschmann et al (1967) was employed. Chromatographic results are given in Table IV. 203. In all cases the reaction went to more than 90% completion (estimated by measuring the amount of residual amino acid in the final solution).

The coupling procedure was applied to a sample of synthetic N-methyl-L-leucine. Side reactions occurred, e.g. the amount of L-alanyl-L-alanine formed was nine times greater than in the DL-leucine experiment and there were several unidentified peaks. Further comparison with the DL-leucine experiment showed that the amount of residual alanine was seven times greater, and the area of the L-alanyl-N-methyl-L-leucine peak was only one sixth of the total area of the two alanylleucine peaks. The coupling reaction was obviously inefficient under these conditions. However, the L-alanyl-N-methyl-L-leucine peak was well separated from all the other peaks and the method looked as though it might be practical if relatively large samples were used for the chromatographic analysis.

Table IV. 203

Chromatographic data for some L-alanyl-dipeptides[a]

Dipeptide	Elution time (min)		Colour yield ratio L-D:L-L
	pH 3.28 buffer, 85 min; then pH 4.25 buffer	pH 4.25 buffer	
L-Ala-D-Ala	137	55	0.91
L-Ala-L-Ala	144	64	
L-Ala-D-Leu	196	113	0.85
L-Ala-L-Leu	207	126	
L-Ala-D-MeLeu	180	b	106
L-Ala-L-MeLeu	170	95	
Ala	89	32	
Leu	141	52	

a Long column, normal buffer flow rate.

b Not separated from the L-L isomer under these conditions.

In a second experiment, L-alanine-N-carboxyanhydride was coupled to a mixture of seven parts N-methyl-L-leucine and one part of L-leucine. The area of the L-alanyl-N-methyl-L-leucine peak was still less than half of the area of the L-alanyl-L-leucine peak. This provided direct evidence that N-methylleucine is less reactive towards L-alanine N-carboxyanhydride than leucine. This is presumably due to steric hindrance afforded by the methyl group (see p. 164).

Our coupling procedure required large samples (2000 μmol; 150 to 300 mg): this was rather extravagant if the method was to be used simply to monitor the optical purity of synthetic samples and so a small scale modification of the procedure requiring only 100 μmol (7 to 15 mg) of sample was developed. Fortunately, the coupling reaction worked more efficiently on the reduced scale, e.g. there was only 13% residual alanine as compared with 29% on the 2000 μmol scale. This small scale procedure was adopted as the standard way of preparing L-alanyl dipeptides for checking the optical purity of amino acids and N-methylamino acids in our laboratory.

To determine the composition of a given mixture of D- and L- isomers it is necessary to know the ratio of colour yields for the pure L-D and L-L dipeptides. This can be most easily determined by carrying out an experiment with a racemic mixture. Conditions for separating the peptides completely must be found. Then the colour yield ratio is obtained as follows:

$$\text{colour yield ratio} = \frac{H \times W \text{ for L-D dipeptide}}{H \times W \text{ for L-L dipeptide}}$$

To identify the peaks, one experiment with a pure or nearly pure sample of either the L- or D- isomer is necessary. This is because on some occasions the L-D dipeptide emerges from the amino acid analyser column first and on others the L-L dipeptide emerges first; a priori identifications cannot be made. Chromatographic data for N-methylleucine is shown in Table IV. 203.

With amino acids Manning and Moore (1968b) obtained 90% yields in the coupling reactions. The pairs of diastereoisomeric dipeptides were completely separated and the peaks were narrow like amino acid peaks. We obtained similar results. However, for N-methylleucine the coupling was not efficient and the separation of the peaks was incomplete. This was due to the broadness of the L-alanyl-N-methylleucine peaks which were three times broader at the half height than the alanylleucine peaks (6 min compared with 2 min). As a result we were not able to obtain the sensitivity of Manning and Moore (1968b), i.e. we could not detect one part of the D-isomer in 1000 parts of the L-isomer. An experiment which was carried out on a sample of pure N-methyl-L-leucine that had been deliberately contaminated with 2% of N-methyl-DL-leucine showed that one part of the D-isomer could be detected in the presence of 100 parts of the L-isomer. This appeared to be the limit of the method, since in a second experiment in which there was 1% contamination with the DL-isomer, no difference from the chromatogram for pure N-methyl-L-leucine was observed.

The broadness of the L-alanyl-N-methylleucine peaks made them strikingly different from all the other L-alanyl dipeptide peaks. N.m.r. studies on acetyl-N-methylleucine suggested a possible explanation. At 34°C this compound exists as a mixture of cis and trans isomers because of restricted rotation about the C-N bond of the amide group (see part IV.3). We predict that the L-alanyl-N-methylleucines also exist as equilibrium mixtures of cis and trans isomers at temperatures near to 34°C. It is often the case that the chromatographic mobilities of pairs of geometrical isomers are slightly different. If this were the case for the L-alanyl-N-methylleucines it would lead to peak broadening and thus explain the widths of the peaks.

Our method of measuring the optical purity of N-methyl-L-leucine has a number of advantages over optical rotation measurements. First and most important the optical purity can be completely and unambiguously established since direct measurements of the contamination with the D-isomer are made. Secondly the exact concentration of N-methylleucine is not required. This means that complete purification of the N-methylleucine sample is unnecessary, providing that the contaminants do not interfere with the coupling reaction nor with the chromatographic analysis. As an example, contamination with inorganic salts presents no problems; the optical purity of N-methylleucine obtained from the saponification of an N-methylleucine ester can be determined directly on the solution from the reaction after a suitable adjustment of pH. Third, the amount of sample required is less than for rotation measurements. For the couplings 100 μmol (15 mg) samples were used; however, only one fifth of the product was required for the chromatographic analysis. A coupling reaction using 20 μmol

(3 mg) would have given sufficient dipeptide to allow the detection of one part of the D-isomer in 100 parts of the L. In contrast 10 to 20 mg of purified sample would be necessary for rotation measurements.

The method should be generally applicable to N-methylamino acids and ought to prove very useful in studies on N-methylamino acids isolated from natural products. The optical composition of samples of only 20 μmol will be measurable. Standard samples of the racemate and of one of the pure isomers are required to allow establishment of the separation conditions and the colour yield and to identify the peaks.

Preparation of L-alanyl dipeptides : The small scale procedure adopted for testing the optical purity of amino acids and N-methylamino acids was as follows. The sample (100 μmol) was weighed into a 100 x 10 mm pyrex test tube and 1 ml of ice-cold 0.45 M borate buffer pH 10.4 (prepared by adding 5 N KOH to 0.45 M boric acid at $0^{o}C$) and 1 drop of octanoic acid were added. The tube was taken into the cold room (temperature $4^{o}C$) and L-alanine-N-carboxyanhydride (12.7 mg; 110 μmol) was added rapidly and the tube shaken vigorously on a 'Vortex Genie' stirrer for 2 min. The solution was acidified to pH 2 with N HCl, diluted to 10 ml, filtered (Celite) and then suitable aliquots analysed with the amino acid analyser.

In the larger scale procedure used in the trial experiments 2000 μmol of sample in 50 ml of buffer were stirred under nitrogen at $0^{o}C$ in a small 'Waring Blendor' vessel fitted with a combination electrode. The L-alanine N-carboxyanhydride was added rapidly and stirring continued while 5 N KOH was added drop-wise to maintain the pH at 10.4. After 2 min the solution was acidified, filtered and diluted for analysis.

IV. 3. THE I. R. AND N.M.R. SPECTRA OF N-METHYLAMINO ACID DERIVATIVES

(a) Infrared spectra

In this section infrared spectral data is given for all the compounds synthesised in Part IV. 1 and for a number of the starting materials. The changes in the spectra that accompany methylation are discussed in detail.

The spectra of carbobenzoxyamino acids were run as nujol mulls; the positions of the bands in the -NH stretch region and in the carbonyl region are given in Table IV. 301 and a typical spectrum (Cbz-Leu-OH) is shown in Fig. IV. 301a. In most of the spectra the urethane amide I band and the carboxylic acid carbonyl band occurred as a single broad peak between 1680 and 1720 cm^{-1}. Only for carbobenzoxytyrosine and carbobenzoxyglycine was this peak resolved into two separate bands. The higher frequency side of the bands was assigned to the carboxylic acid group [Cross (1961), p. 67 gives the range 1695 - 1725 cm^{-1} for the acid groups of α-amido acids] and the lower frequency side to the urethane group [the amide I urethane band of carbobenzoxybenzylamine occurs at 1678 cm^{-1} (Coggins and Benoiton, unpublished results). The amide II bands for the carbobenzoxyamino acids occurred at 1520 - 1540 cm^{-1} (cf. 1552 cm^{-1} for carbobenzoxybenzylamine) and the -NH groups gave a single peak near 3300 cm^{-1} (in nujol mulls and liquid films carboxylic acids are associated as dimers and the -OH stretch band

Table IV. 301

Infrared spectra of carbobenzoxyamino acids[a]

	Amide II		Amide I		Acid		
Cbz-X-OH	main band	shoulders	main band	shoulders	main band	shoulders	-NH stretch
Cbz-Gly-OH	1542	1550	1679 1691		1707 1722		3300
Cbz-Ala-OH	1541		1691		b	1705,1716	3250
Cbz-Ile-OH	1517	1529,1535	b	1675	1703 1715		3280
Cbz-Leu-OH	1533	1522,1539	b	1691	1707 1719		3250
Cbz-Phe-OH	1534	1540	1690		b	1704,1717	3240
Cbz-γAbu-OH	1553	1540	1689		b		3250
Cbz-Tyr-OH	1522	1530	1690		1725	1705	3270[c]
Cbz-Asn-OH	1540[d]	1536	1692[d]	1678	b	1716	3250[d]
Cbz-MeLeu-OH	e		1644		1742	1722[f]	e

a. Spectra run as nujol mulls; band frequencies given in cm^{-1}; amide and acid bands strong, -NH stretch bands medium intensity.

b. Amide I and acid bands not resolved.

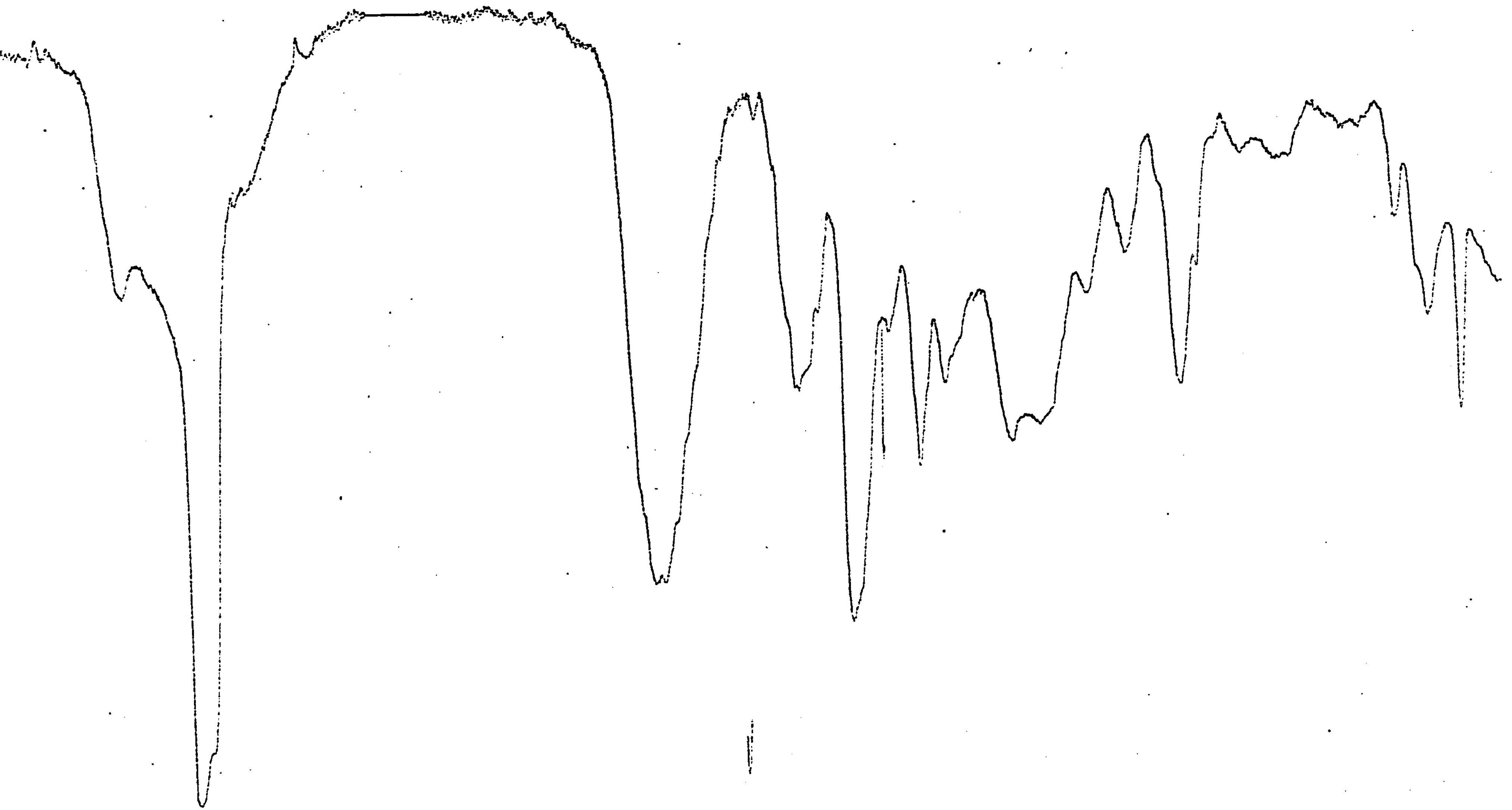
c. Shoulder at 3370 cm^{-1}.

d. Amide I band 1584 cm^{-1}, amide II band 1640 cm^{-1} (shoulder 1630 cm^{-1}) additional -NH stretch bands 3150 cm^{-1} and 3330 cm^{-1}.

e. Bands absent; $N-CH_3$ band occurred as shoulder at 1410 cm^{-1}.

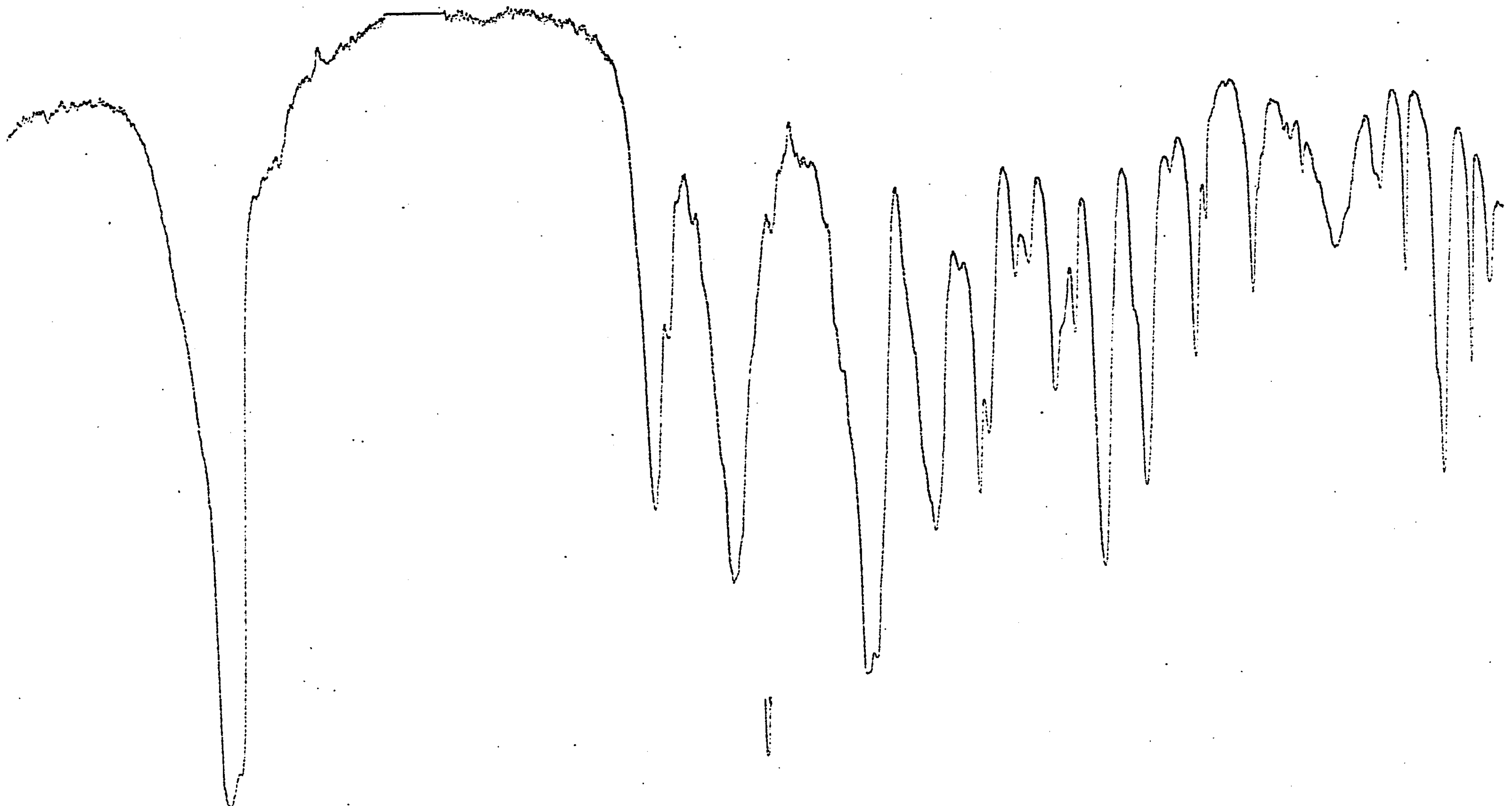
f. Band resolved but relatively weak.

Infrared spectrum of carbobenzoxyleucine



Cbz-Leu-OH (nujol)
Fig. IV . 301a

Infrared spectrum of carbobenzoxy-N-methylleucine



Cbz-MeLeu-OH (nujol)
Fig. IV . 301b

is absent [Dyer (1965), p. 46]). Carbobenzoxytyrosine and carbobenzoxyasparagine had additional absorption bands near 3300 cm^{-1} due to the -OH and -$CONH_2$ groups in the side-chains.

The spectrum of carbobenzoxy-N-methylleucine (see Fig. IV. 301b) was strikingly different from that of carbobenzoxyleucine. The amide II band (characteristic of the -CO-NH- group) and the -NH stretch bands were absent. The amide I band was shifted to lower frequency (from 1692 to 1644 cm^{-1}); the reason for this is not known. Also the carboxyl group bands were shifted to higher frequency (from 1707 and 1719 to 1722 and 1742 cm^{-1}); this may simply reflect a difference in the strength of the hydrogen bonds involved in the association of the acids into dimers, particularly since one of the compounds is an oil and the other a crystalline solid. Many of the spectra of compounds containing N-CH_3 groups that are described on the following pages contain a band near 1400 cm^{-1} which is assigned to the N-CH_3 group. [Pakrashi and Bhattacharyya (1968) have assigned a similar band in the spectra of quinazoline alkaloids to N-CH_3 groups]. In the spectrum of carbobenzoxy-N-methylleucine this band is seen as a shoulder at 1410 cm^{-1}.

On methylation the carbobenzoxyamino acids gave carbobenzoxy-N-methylamino acid methyl esters. Although these compounds were not isolated in the pure form they were obtained from the reaction mixtures as crude oils with nujol contamination (see p. 69). Details of the spectra of these compounds in the carbonyl region are given in Table IV. 302 and a typical spectrum (Cbz-MeLeu-OMe) is shown in Fig. IV. 302b; for comparison, details of the

Table IV. 302

Infrared spectra of carbobenzoxy-N-methylamino acid methyl esters[a]

Cbz-MeX-OMe	Amide I main band	Amide I shoulders	Ester main band	Ester shoulders	N-CH_3
Cbz-MeGly-OMe	1706	1688, 1716	1747	1737, 1757	1412
Cbz-MeAla-OMe	1705		1740		1407
Cbz-MeVal-OMe	1704		1735		1407
Cbz-MeIle-OMe	1704		1737		1409
Cbz-MeLeu-OMe	1704		1736		1410
Cbz-EtLeu-OEt[b]	1708	1691[c]	1724	1738[c]	d
Cbz-Phe-OMe	1707	1695	1738	1409	
Cbz-MeγAbu-OMe	1704	1691	1733	1408	
Cbz-MeTyr(OMe)-OMe	1707	1690[c]	1738	1723[c], 1746	1407
Cbz-MeAsn(Me_2)-OMe	1705	1690	1737		1410
Cbz-Leu-OMe[e]	f	1690, 1708	1728		d
Cbz-Leu-OEt[g]	f	1692, 1708	1728		d

a. Spectra run as nujol mulls; band frequencies given in cm^{-1}; amide and ester bands strong, N-CH_3 bands medium intensity; amide II and -NH stretch bands absent unless specifically mentioned.

b. Cbz-EtLeu-OEt had a weak band at 3300 cm^{-1} presumably due to Cbz-Leu-OEt contamination.

c. Resolved band (rather than shoulder).

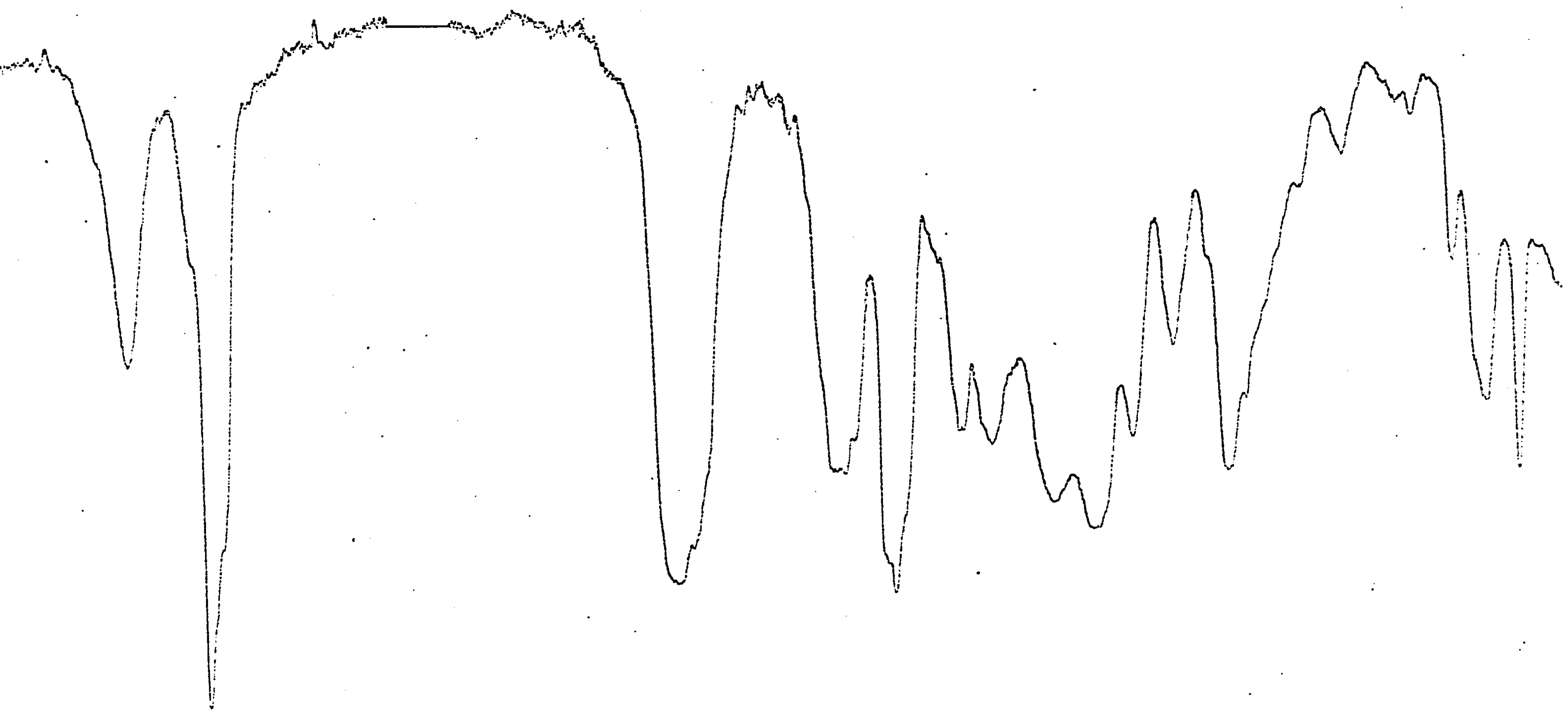
d. Band absent.

e. Amide II band 1521 cm^{-1} (shoulders 1510, 1536 cm^{-1}); -NH stretch band 3280 cm^{-1}.

f. Amide I band and ester band not resolved.

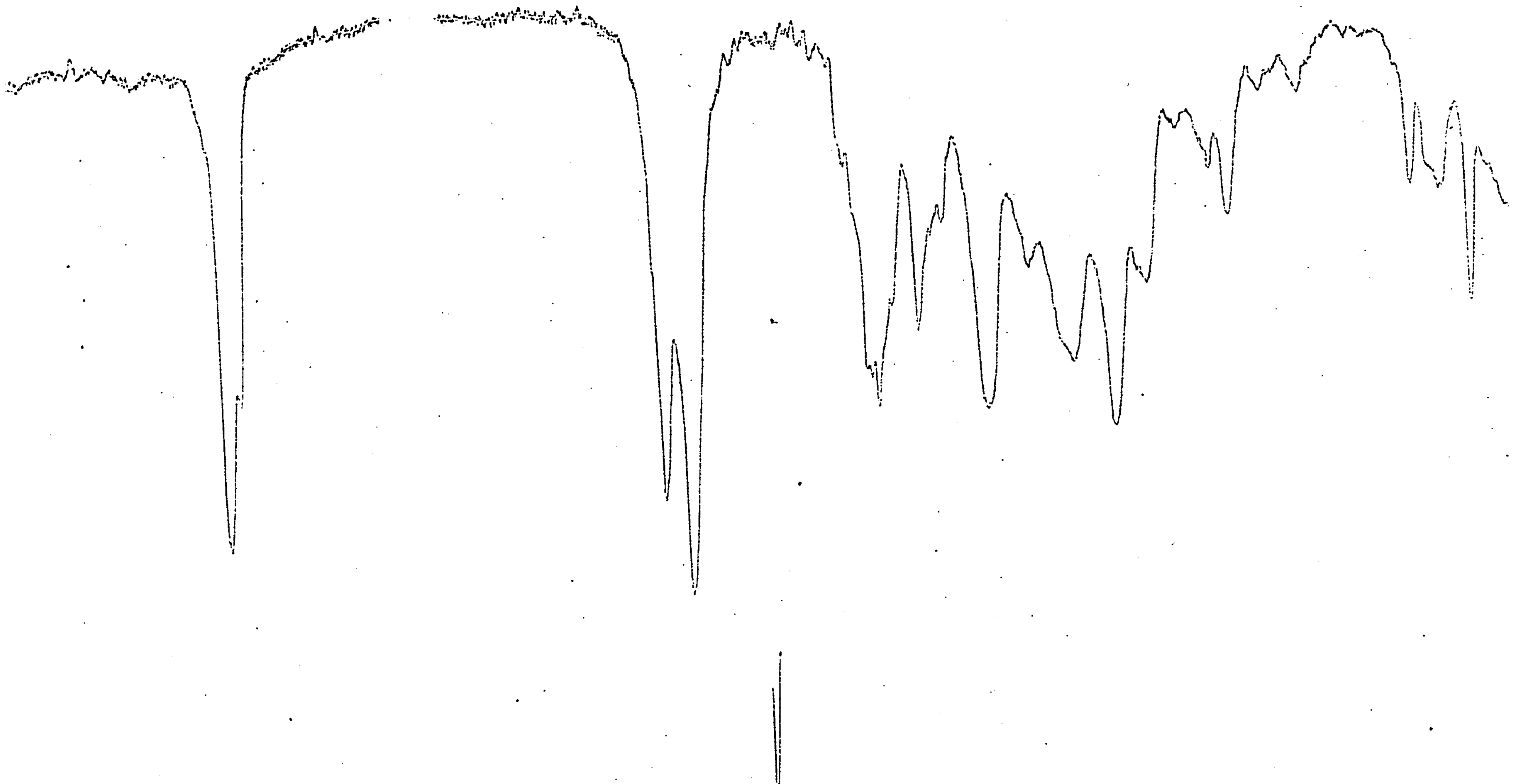
g. Amide II band 1522 cm^{-1} (shoulders 1512, 1534 cm^{-1}); -NH stretch band 3280 cm^{-1}.

Infrared spectrum of carbobenzoxyleucine methyl ester



Cbz-Leu-OMe (nujol)
Fig. IV . 302a

Infrared spectrum of carbobenzoxy-N-methylleucine methyl ester



Cbz-MeLeu-OMe (nujol)
Fig. IV . 302b

spectra of carbobenzoxyleucine methyl ester and carbobenzoxyleucine ethyl ester are also included in the Table and the spectrum of carbobenzoxyleucine methyl ester is shown in Fig. IV. 302a. The principal differences between the spectra of the methylated and non-methylated carbobenzoxyamino acid esters is the absence of bands due to -NH- groups in the spectra of the former compounds. In addition both the ester bands and the amide I bands occur at slightly higher frequency in the methylated compounds. The spectrum of carbobenzoxy-N-ethylleucine ethyl ester has a weak band at 3300 cm^{-1} and several weak bands near 1520 cm^{-1}; these bands are due to a small amount of contamination with carbobenzoxyleucine ethyl ester (n.m.r. showed that Cbz-EtLeu-OH derived from Cbz-EtLeu-OEt contained some Cbz-Leu-OH, see p.131). The spectra of all the carbobenzoxy-N-methylamino acid methyl esters had a medium intensity band between 1407 and 1412 cm^{-1} which is assigned to the $N-CH_3$ group. This band was not present in the spectra of the esters of carbobenzoxyleucine nor in the spectrum of carbobenzoxy-N-ethylleucine ethyl ester.

Spectra of the crystalline N-methylamino acid methyl ester hydrobromides were obtained as KBr discs. Details of the carbonyl and the amine salt bands are given in Table IV. 303 and a typical spectrum (MePhe-OMe.HBr) is shown in Fig. IV. 303b; data for some amino acid methyl ester hydrochlorides are also included in the Table and the spectrum of phenylalanine methyl ester hydrochloride is shown in Fig. IV. 303a. Methylation had no significant

Table IV. 303

Infrared spectra of N-methylamino acid methyl ester hydrobromides[a]

MeX-OMe.HBr	$N-CH_3$	Amine salt -NH de-formation	Ester	Amine salt -NH stretch	Aliphatic -CH stretch
MeGly-OMe.HBr	1411w	1579w	1740s	2420m, 2580s, 2640s	2910s
MeAla-OMe.HBr	1416w	1561m	1741s	2200w, 2380m, 2410m, 2430m, 2660s, 2730s, 2800s	2920s
MeVal-OMe.HBr	1399w	1553m	1733s	2390w, 2460w, 2660s, 2720s, 2800s	2920s
MeIle-OMe.HBr	1397m	1576m	1740 s	2440w, 2480w, 2690s, 2740s	2930s
MeLeu-OMe.HBr	1426m	1548m	1733s	2200w, 2380m, 2460m, 2640s, 2720s, 2790s	2920s
EtLeu-OEt.HBr	b	1561m	1733s	2240w, 2370w, 2440m, 2510m, 2670s, 2760s	2930s
MePhe-OMe.HBr	1410w	1570m	1733s	2220w, 2400m, 2420m, 2580s, 2700s, 2780s	2900s
MeγAbu-OMe.HBr	1405m	1577w	1734s	2370w, 24203, 2740w	2920s
Me Acp-OMe.HBr	1416m	1559w	1715s	2400w, 2720m	2890s
MeTyr(Me)-OMe.HBr	1396w	1521m	1735s	2420w 2710s	2910s
MeAsn(Me_2)-OMe.HBr[c]	1424m	b	1739s	2330w, 2450w, 2680m	2910s
H-Gly-OMe.HCl	b	1500m, 1577m	1752s	2600w, 2650w, 3000vs	d
H-Ala-OMe.HCl	b	1516m, 1582m	1736s	2490w, 2560w, 2600w, 2670m, 2910vs	d
H-Leu-OMe.HCl	b	1513m, 1588m	1734s	2600w, 2880vs	d
H-Phe-OMe.HCl	b	1500m, 1580m	1738s	2600w, 2830vs	d

a. Spectra run as KBr discs; band frequencies given in cm^{-1}; intensities: vs = very strong; s = strong; m = medium; w = weak: shoulders not recorded.

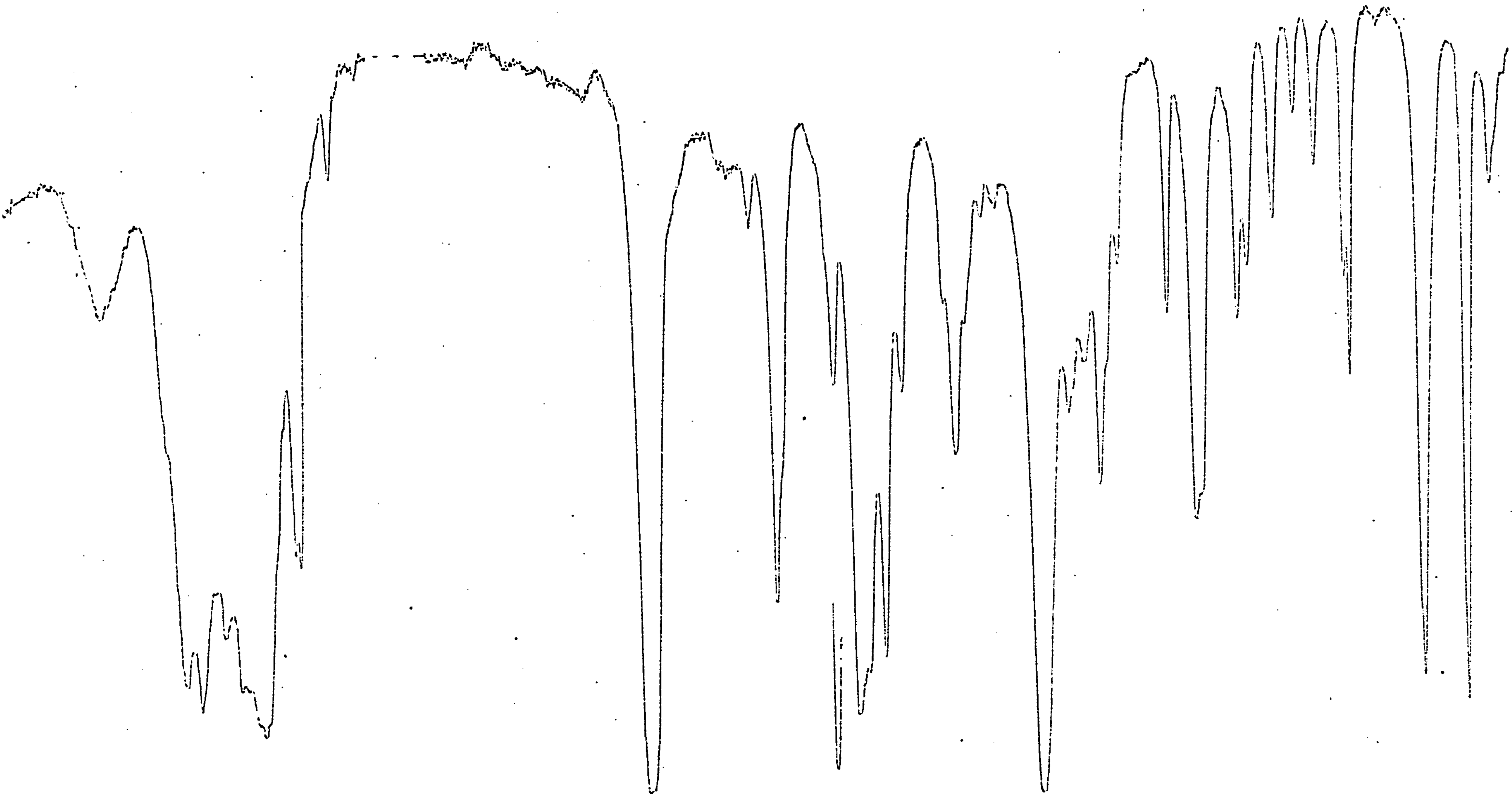
b. Band absent.

c. MeAsn(Me_2)-OMe.HBr was an oil and was run as a liquid film; amide I band 1643 cm^{-1}.

d. -CH stretch band not resolved from 'ammonium' band.

effect on the position of the ester carbonyl absorption which occurred as a single strong band near 1740 cm^{-1} in all the spectra. The pattern of the amine salt bands, however, was quite different for the amino acid methyl ester salts and the N-methylamino acid methyl ester salts. In the unmethylated compounds there were two medium intensity bands in the regions 1500 - 1520 cm^{-1} and 1570 - 1590 cm^{-1} which are assigned to the $-\overset{+}{N}H_3$ deformation vibrations [Nakanishi (1962), p. 39]. The methylated compounds had a single band in the region 1520 - 1580 cm^{-1} which is assigned to the $-\overset{+}{N}H_2$ deformation vibrations [Nakanishi (1962), p. 39]. There were also differences in the region of the spectrum associated with the $-\overset{+}{N}H$ stretching vibrations (2000 - 3000 cm^{-1}). The methylated compounds had a series of bands of varying intensities in the region 2200 - 2800 cm^{-1} which are assigned to the $-\overset{+}{N}H_2$ stretching vibrations [Nakanishi (1962), p. 39] but these bands were in every case resolved from the aliphatic -CH stretching bands near 2900 cm^{-1}. In the unmethylated compounds the strong, broad $-\overset{+}{N}H_3$ stretching band (the 'ammonium' band) obscured the -CH stretching bands completely and in general there were less bands in the region from 2200 - 2800 cm^{-1}. The spectrum of β-(N,N-dimethylamido)-N-methylaspartic acid methyl ester hydrobromide (see p. 73) did not have an $-\overset{+}{N}H_2$ deformation band; this may have been due to the fact that the compound was an oil and was run as a liquid film. The spectra of the N-methylamino acid methyl ester hydrobromides all contained the band near 1400 cm^{-1} which is assigned to the $N-CH_3$ group.

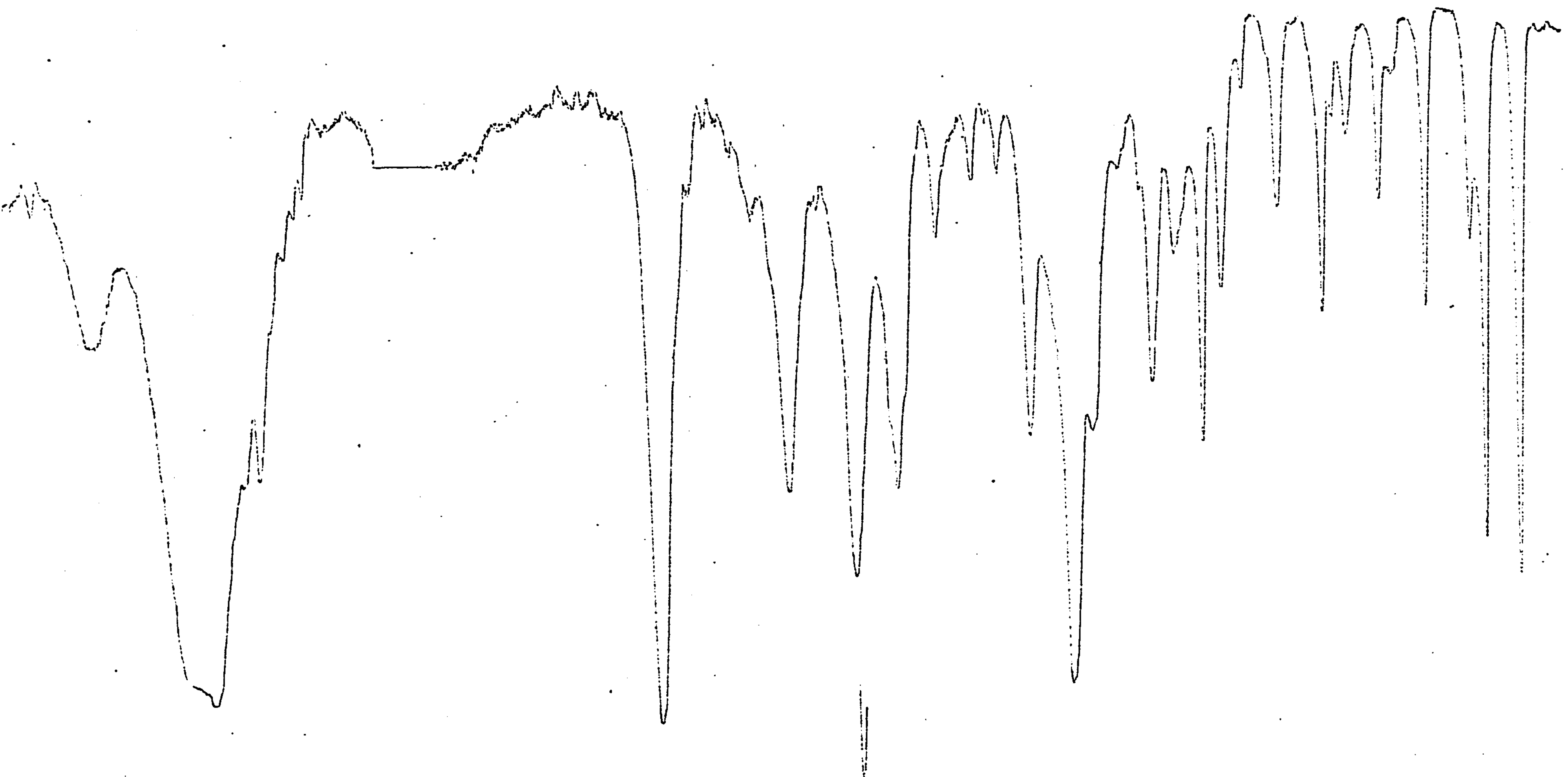
Infrared spectrum of phenylalanine methyl ester hydrochloride



MePhe-OMe.HBr (KBr disc)

Fig. IV . 303b

Infrared spectrum of N-methylphenylalanine methyl ester hydrobromide



solid

H-Phe-OMe.HCl (KBr disc)

Fig. IV . 303a

Tables IV. 304 and IV. 305 contain spectral data for acetyl- and benzoyl-amino acids, acetyl- and benzoyl-N-methylamino acids and acetyl- and benzoyl-N-methylamino acid methyl esters. Representative spectra are shown in Fig. IV. 304a, b, c (Ac-Ile-OH, Ac-MeIle-OH, Ac-MeIle-OMe). The acylamino acids had amide I (1592 - 1644 cm^{-1}), amide II (1543 - 1565 cm^{-1}), acid (1688 - 1738 cm^{-1}) and -NH stretch (3240 - 3330 cm^{-1}) bands. After conversion to the acyl-N-methylamino acid the amide II and -NH stretch bands disappeared, the amide I bands (acyl-N-methylglycines excepted) were shifted to lower frequency (a similar effect was observed with Cbz-MeLeu-OH) and the acid bands (except for Ac-MeVal-OH) were shifted to higher frequency (a similar effect was also observed with Cbz-MeLeu-OH). The spectra of the acyl-N-methylamino acid methyl esters lacked amide II and -NH stretch bands and the amide I bands were shifted considerably (about 40 cm^{-1}) towards higher frequency (Bz-MeLeu-OMe was an exception). This shift is approximately twice as large as the similar shift observed with carbobenzoxy-N-methylamino acid methyl esters. All the N-methyl derivatives of the acylamino acids contained a band in the region 1406 - 1426 cm^{-1} which is assigned to the $N\text{-}CH_3$ groups.

The spectra of leucine, N-methylleucine and α-C-methylleucine are shown in Fig. IV. 305a, b, c. The finger print regions of these spectra (900 - 1350 cm^{-1}) were readily distinguishable; there were also some differences in the amino acid I bands (due to the $-COO^-$ group), the amino acid II bands (due to $-\overset{+}{N}H$ deformation) and in the $-\overset{+}{N}H$ stretch region (2200 - 3000 cm^{-1}). [For a discussion

Table IV. 304

Infrared spectra of acetylamino acids, acetyl-N-methylamino acids and acetyl-N-methylamino esters[a]

Ac-RX-OR	N-CH_3	Amide II	Amide I	Acid	Ester	-NH Stretch
Ac-Gly-OH	b	1555	1592	1716	b	3330
Ac-Ala-OH	b	1559	1621	1701	b	3260
Ac-Val-OH	b	1561	1610	1727	b	3240
Ac-Ile-OH	b	1547	1619	1689	b	3260
Ac-Leu-OH	b	1565	1624	1699	b	3290
Ac-Phe-OH	b	1552	1622	1688	b	3260
Ac-MeGly-OH	1412m	b	1607	1726	b	b
Ac-MeAla-OH	1416m	b	1601	1727	b	b
Ac-MeVal-OH	1407m	b	1588	1705	b	b
Ac-MeIle-OH	1410m	b	1608	1721	b	b
Ac-MeLeu-OH	1426m	b	1595	1713	b	b
Ac-MePhe-OH	1413m	b	1592	1709	b	b
Ac-MeAla-OMe[c]	1407w	b	1665	b	1740	b
Ac-MeIle-OMe[c]	1406m	b	1662	b	1736	b
Ac-MeLeu-OMe[c]	1407m	b	1660	b	1736	b
Ac-MePhe-OMe[c]	1408m	b	1658	b	1735	b

a. Spectra run as KBr discs except where indicated; band frequencies given in cm^{-1}; all the bands were strong unless marked w (weak) or m (medium).

b. Band absent.

c. Spectrum run as nujol mull.

Table IV. 305

Infrared spectra of benzoylamino acids, benzoyl-N-methylamino acids and benzoyl-N-methylamino acid methyl esters

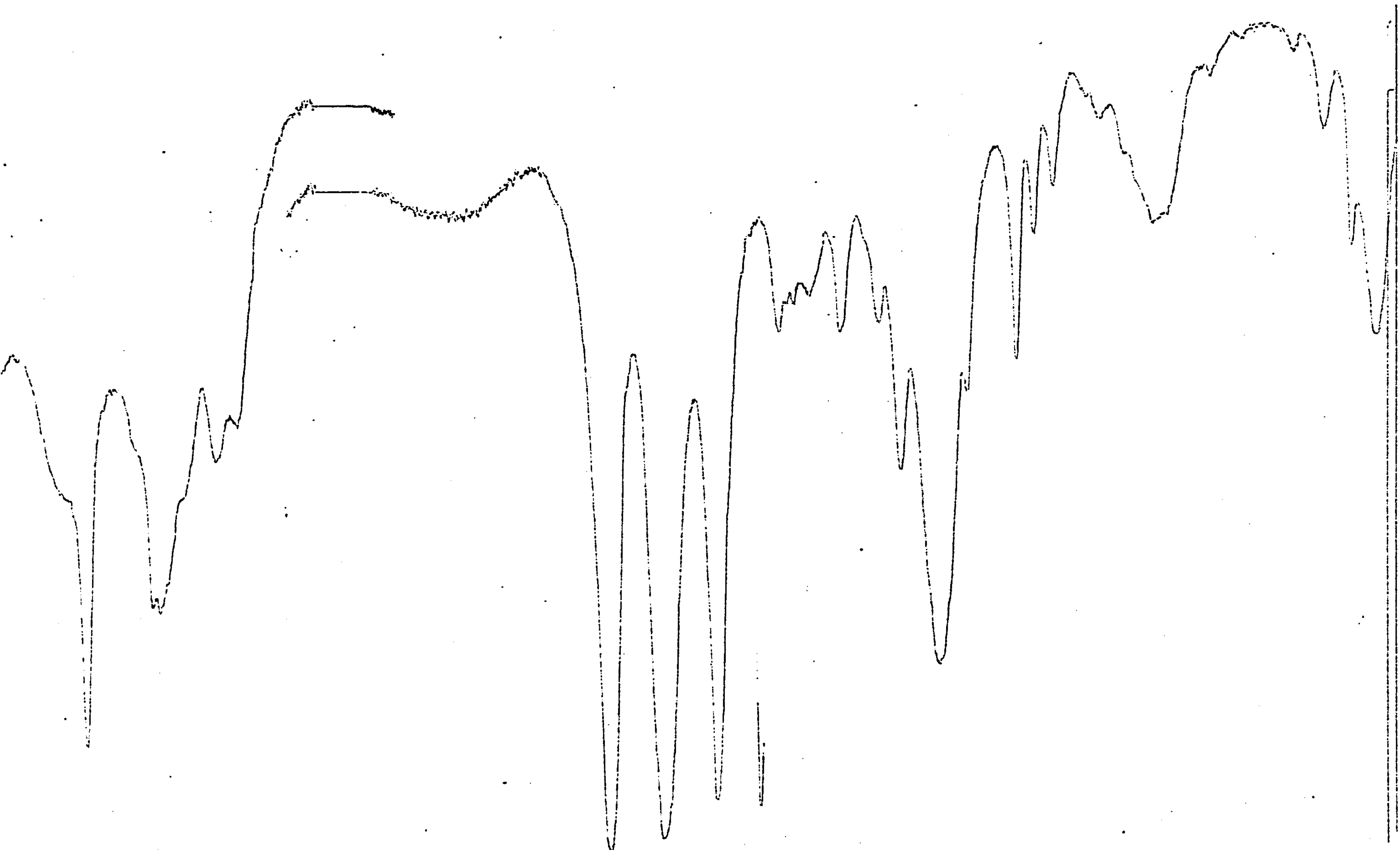
Bz-RX-OR		Amide II		Amide I				
		main band	shoulders	main band	shoulders	Acid	Ester	-NH Stretch
Bz-Gly-OH	b	1553	1568	1602	1614	1738	b	3280
Bz-Ala-OH	b	1547	1567[c]	1603	1637	1740	b	3310
Bz-Leu-OH	b	1543	1564, 1580	1644	1627[c]	1729	b	3310
Bz-MeGly-OH	1409m	b		1605	1572[c]	1735	b	b
Bz-MeAla-OH	1403m	b		1592	1570[c]	1731	b	b
Bz-MeLeu-OH	1409m	b		1578	1552, 1605[c]	1727	b	b
Bz-MeGly-OMe[c]	1402m	b		1643		b	1743	b
Bz-MeAla-OMe[c]	1402m	b		1642		b	1739	b
Bz-MeLeu-OMe[c]	1402m	b		1642		b	1738	b

a. Spectra run as KBr discs except where indicated; band frequencies given in cm^{-1}; all the bands were strong unless marked m (medium).

b. Band absent.

c. Spectrum run as nujol mull.

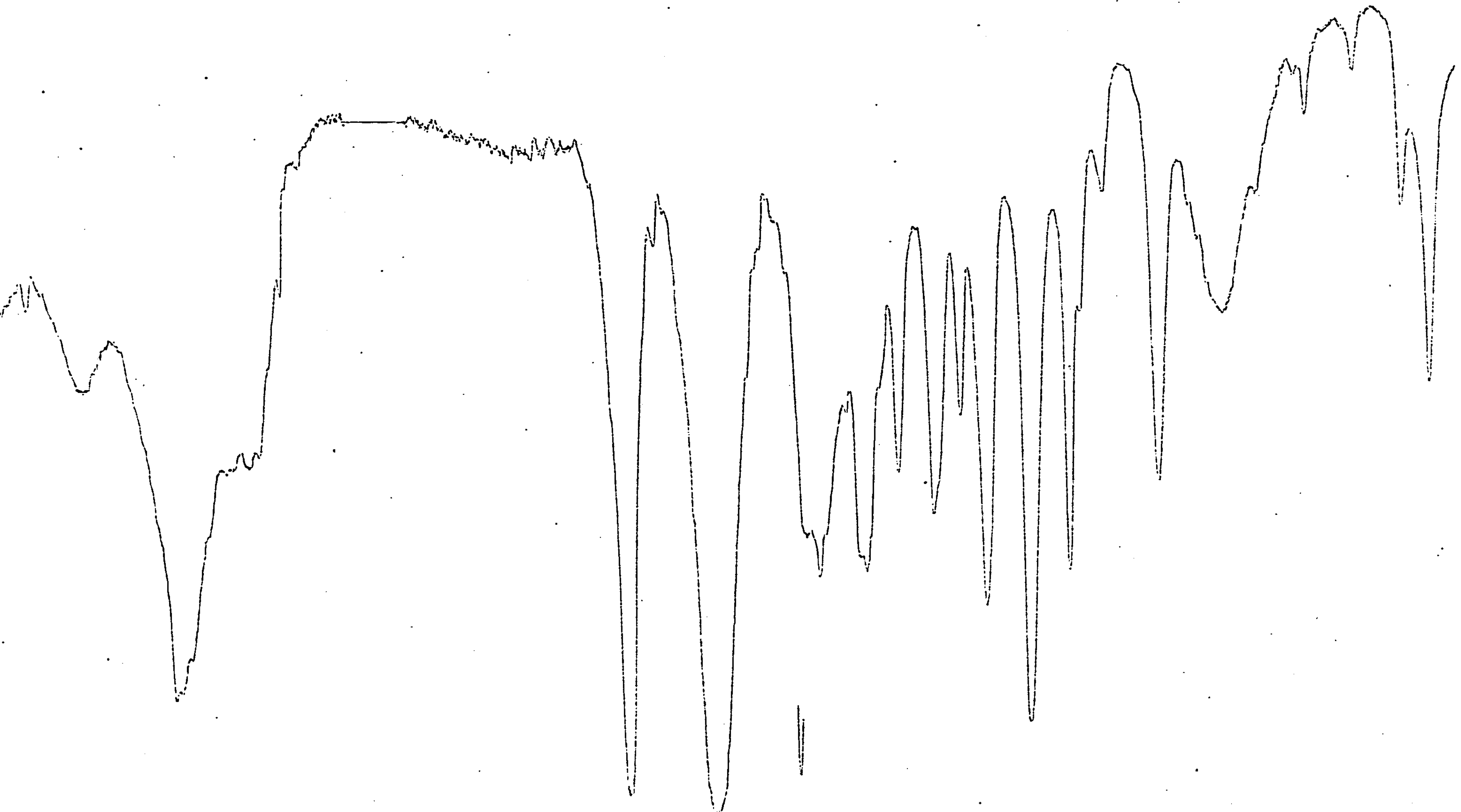
Infrared spectrum of acetylisoleucine



Ac-Ile-OH (KBr disc)

Fig. IV . 304a

Infrared spectrum of acetyl-N-methylisoleucine

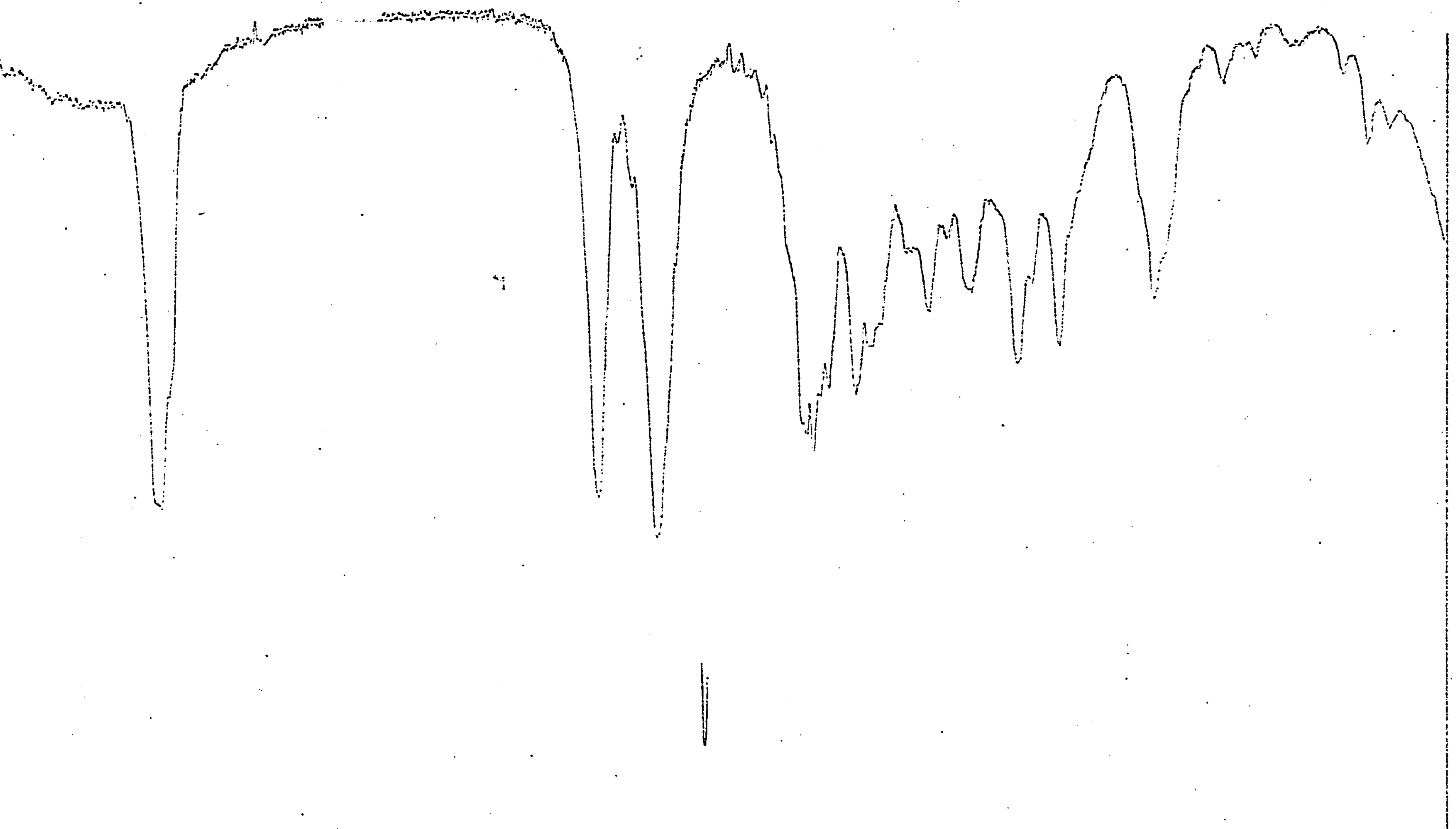


solid

Ac-MeIle-OH (KBr disc)

Fig. IV . 304b

Infrared spectrum of acetyl-N-methylisoleucine methyl ester



Ac-MeIle-OMe + nujol

Fig. IV . 304c

lqd. film

of the i.r. spectra of amino acids see Greenstein and Winitz (1961), p. 1695]. Leucine had well defined amino acid I (1582 cm^{-1}) and amino acid II (1520 cm^{-1}) bands and a broad 'ammonium' band (2900 cm^{-1}) which obscured all other bands in its vicinity; α-C-methylleucine had a much broader amino acid I band (1609 cm^{-1}), a relatively weak amino acid II band (1521 cm^{-1}) and a broad 'ammonium' band (2920 cm^{-1}). [The distortion of the amino acid bands in α-C-methylleucine is probably due to steric factors; see Greenstein and Winitz (1961), p. 1702]. In contrast to the amino acid spectra, N-methylleucine had no amino acid II band and the $-\overset{+}{N}H$ stretch region was considerably modified, e.g. the -CH stretch band was resolved (2890 cm^{-1}) and there were 4 amine salt bands (2390, 2580, 2810, 2960 cm^{-1}). The spectra of sarcosine and glycine and of N-methylalanine and alanine showed differences parallel to those observed in the spectra of N-methylleucine and leucine, e.g. the two N-methylamino acids had no amino acid II bands. The absence of the amino acid II band and the characteristic secondary amine salt pattern in the $-\overset{+}{N}H$ stretch region seems to be characteristic of N-methylamino acids.

In Table IV. 306 some spectral data are given for ε-N-carbobenzoxy, ε-N-methyllysine methyl ester hydrochloride and for some of the intermediates used in its preparation; data are also given for some of the intermediates used in the preparation of ε-N-ethyllysine ethyl ester dihydrobromide, α-N, ε-N-dimethyllysine monohydrochloride and in the attempted preparation of α-N-methyllysine. Two representative spectra are given in Fig. IV. 306a and b

Table IV. 306

Infrared spectra of some lysine derivatives

Lysine derivative	Ester	Amide I	Amide II	N-CH_3	-NH stretch
H-Lys(Cbz)-OMe.HCl	1730s	1690 s	1542 s	b	3300 m
H-Lys(Cbz, Me)-OMe.HCl	1732s	1696 s	b	1409 m	b
H-Lys(Cbz, Et)-OEt[c]	1724s	1692 s	b	b	3330 w
Trt-Lys(Cbz)-OMe[c]	1718s	1705 s	1539 m	b	3280 s
Trt-Lys(Cbz, Me)-OMe	1728s	1708 s	b	1411 m	3270 w
Trt-Lys(Cbz, Et)-OMe	1720s	1692 s	b	b	b
Fbz-Lys(Cbz)-OH	1715m[d] 1736m[d]	1687 s 1647 m	1541 s	(1408 w)	3280 s
Cbz-MeLys(Cbz, Me)-OMe	1721s 1736s	1690 s 1705 s	b	1410 m	b
Cbz-Lys(Trt)-OMe[c]	1718s	1705 s	1540 m	b	3320 a
Cbz-MeLys(Trt)-OMe	1717s	1702 s	b	1409 m	3400 w

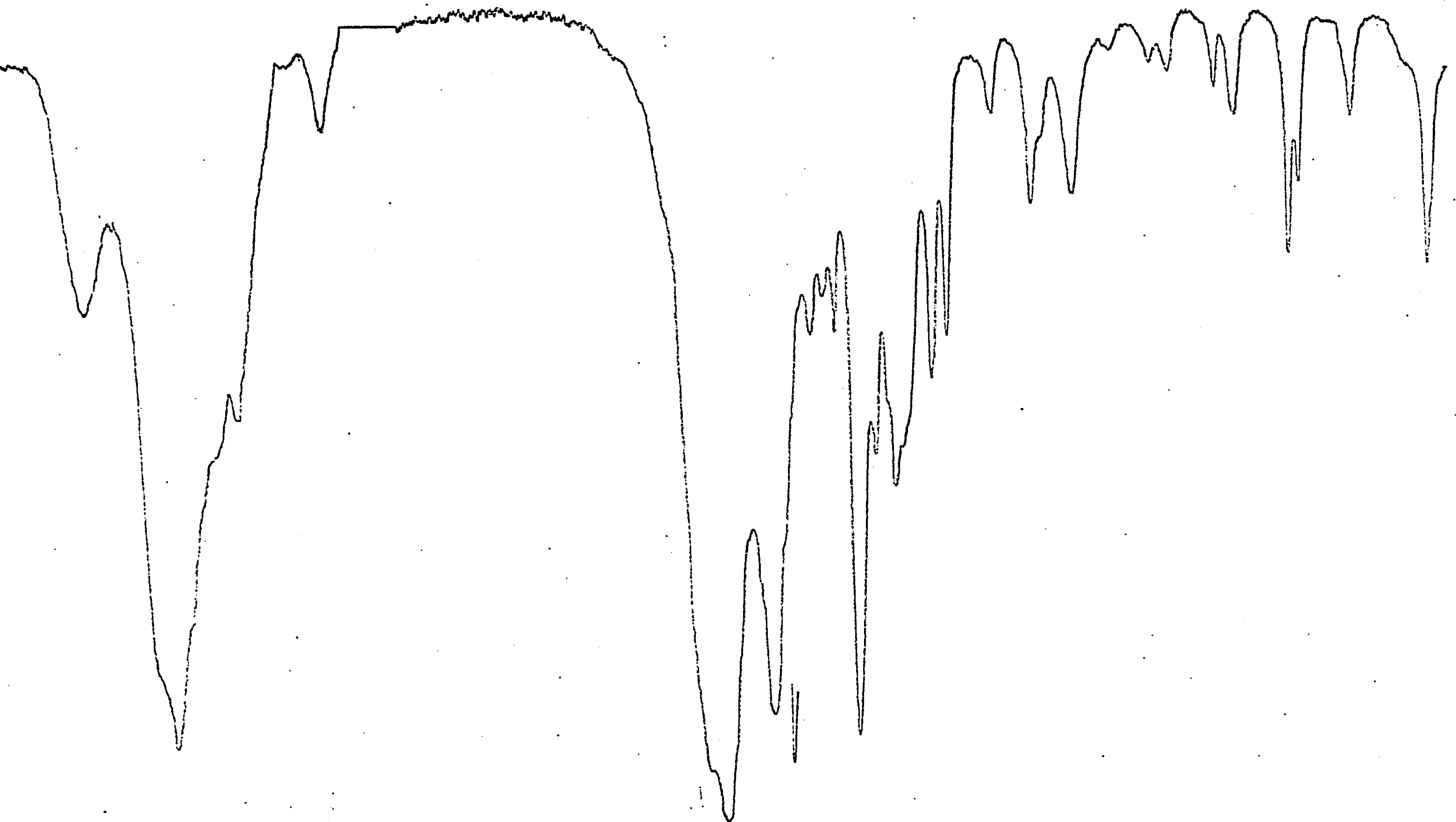
a. Spectra run as nujol mulls except where indicated; band frequencies given in cm^{-1}; intensities - s = strong, m = medium, w = weak.

b. Band absent.

c. Spectrum run as liquid film.

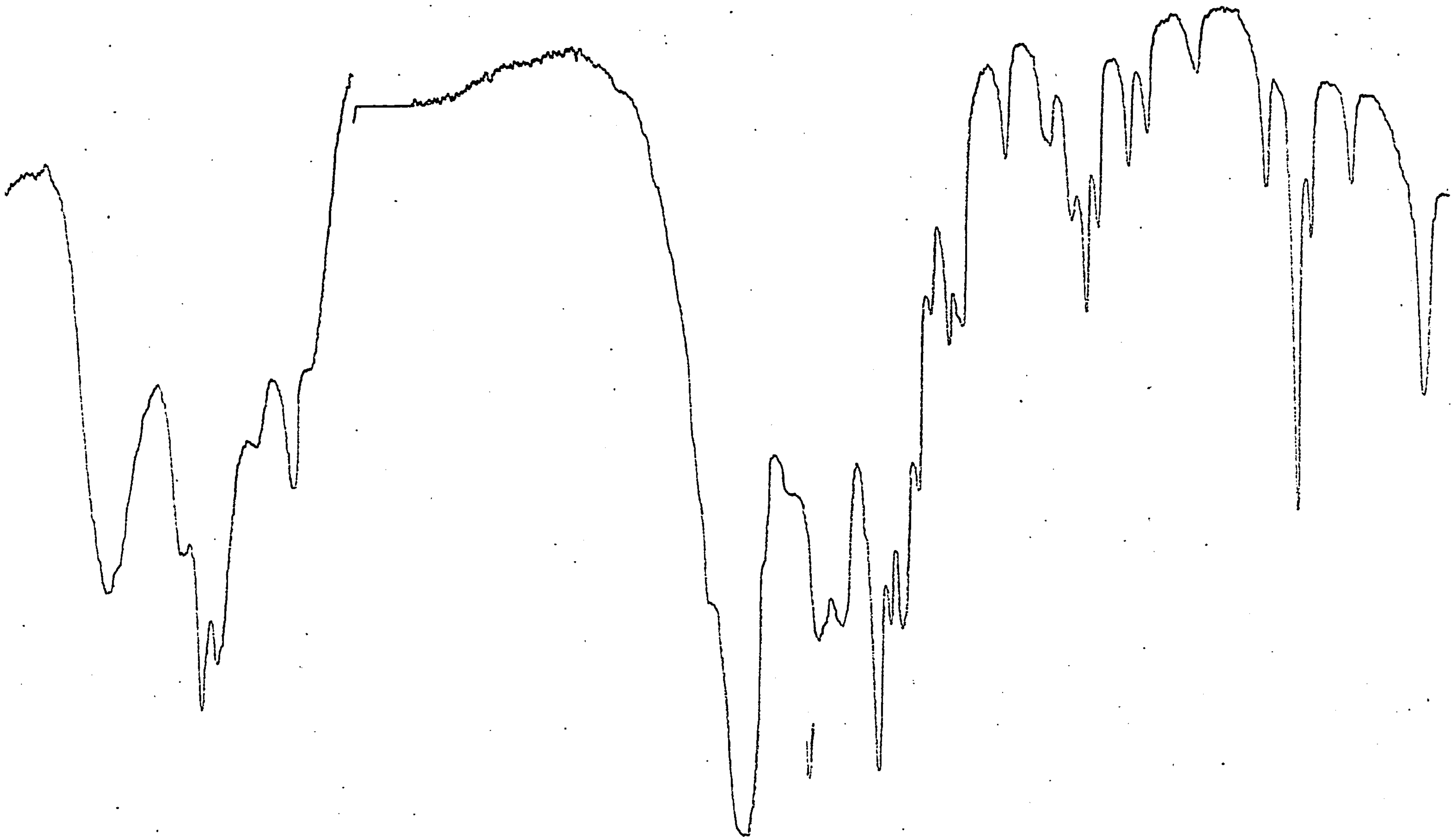
d. Acid bands not ester bands.

Infrared spectrum of leucine



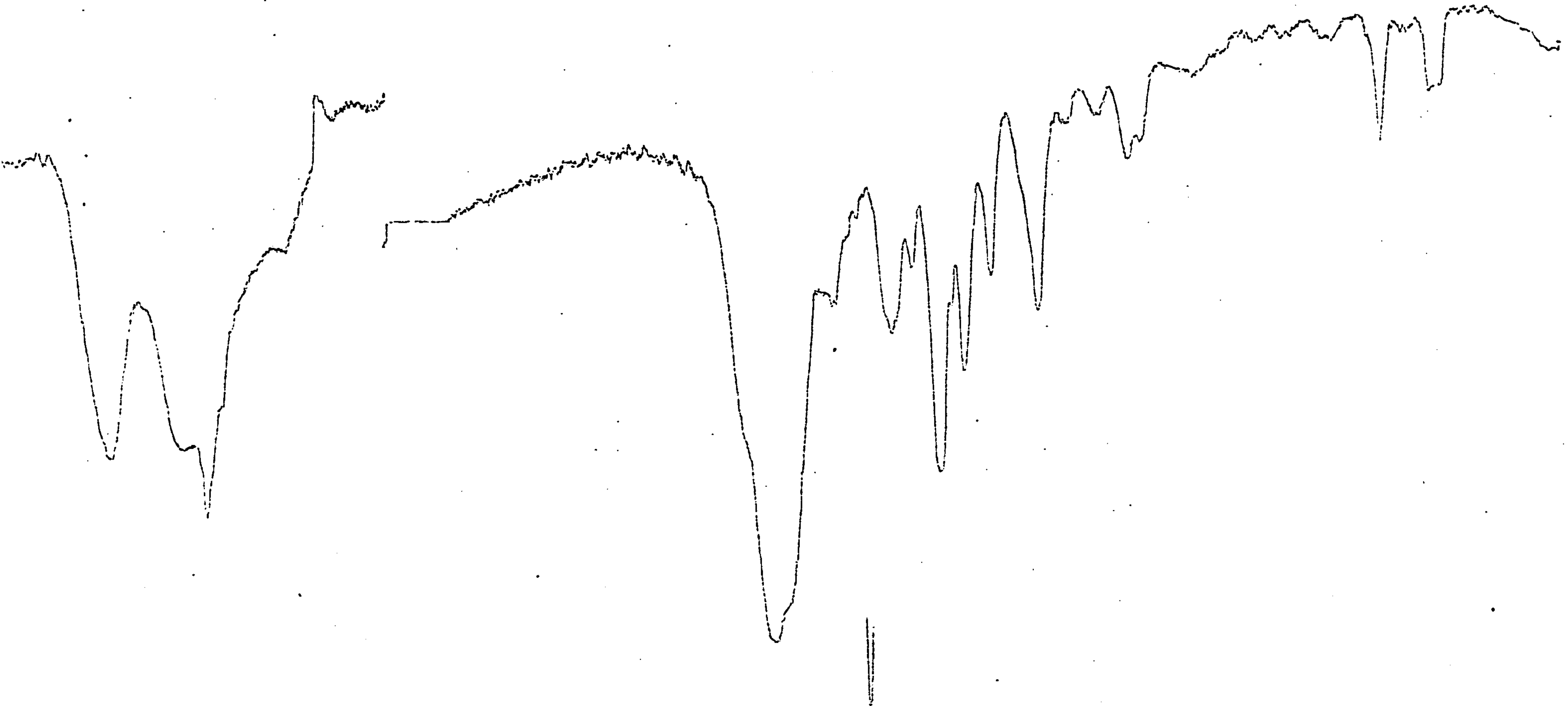
L-Leucine
Fig.IV. 305a

Infrared spectrum of N-methylleucine



N-Methyl-L-leucine(synthesised)
Fig. IV. 305b

Infrared spectrum of α-C-methylleucine



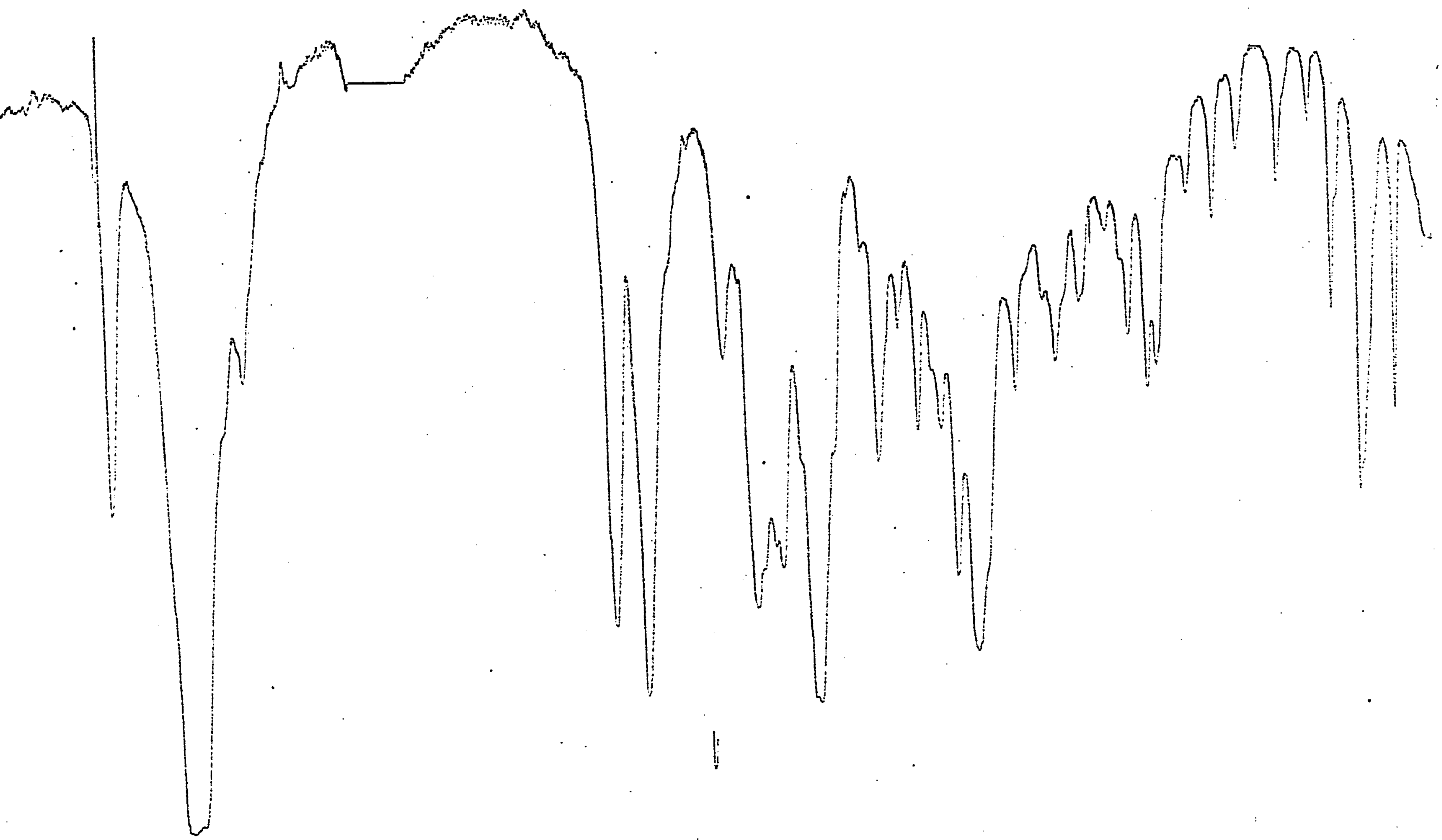
KBr disc

α-C-Methyl-DL-leucine
Fig. IV. 305c

[H-Lys(Cbz)-OMe.HCl and H-Lys(Cbz,Me)-OMe.HCl]. The spectra showed changes accompanying methylation similar to those observed with the carbobenzoxyamino acids (Tables IV. 301, 302). The amide II bands disappeared as did the strong -NH stretch bands. Since several of the compounds contained Trt-NH groups a weak -NH stretch band remained after methylation (e.g. in Trt-Lys(Cbz,Me)-OMe) although with α-N-trityl, ε-N-carbobenzoxy, ε-N-ethyllysine ethyl ester the band was too weak to be observed. The hydrochlorides of ε-N-carbobenzoxylysine methyl ester and ε-N-carbobenzoxy, ε-N-methyllysine methyl ester had strong ammonium bands overlapping the -CH stretch band near 2900 cm^{-1} and two primary amine salt bands ($-\overset{+}{N}H$ deformation) at 1590 cm^{-1} and 1508 cm^{-1} and at 1590 cm^{-1} and 1500 - 1510 cm^{-1} respectively. All the N-methylated derivatives had the $N-CH_3$ band between 1409 and 1411 cm^{-1} (α-N, ε-N-dicarbobenzoxylysine also had a band at 1408 cm^{-1}; the origin of this band is unknown).

The spectrum of α-N, ε-N-dimethyllysine monohydrochloride is shown in Fig. IV. 307a and for comparison the spectra of lysine monohydrochloride and ε-N-methyllysine monohydrochloride are shown in Fig. IV. 307b and c. The finger print regions(900 - 1350 cm^{-1}) were very different for these compounds and the identification of pure samples by infrared analysis would therefore be very simple. There were interesting differences in the spectra in the vicinity of the amino acid I and II bands and near 3000 cm^{-1} which can be correlated with the different functional groups present in these molecules. Lysine monohydrochloride is both an amino acid and a primary amine salt; it has a broad 'ammonium' band (near 2900 cm^{-1}) which is not resolved from the -CH stretch band, two amino acid I bands (1632 and 1607 cm^{-1}) and

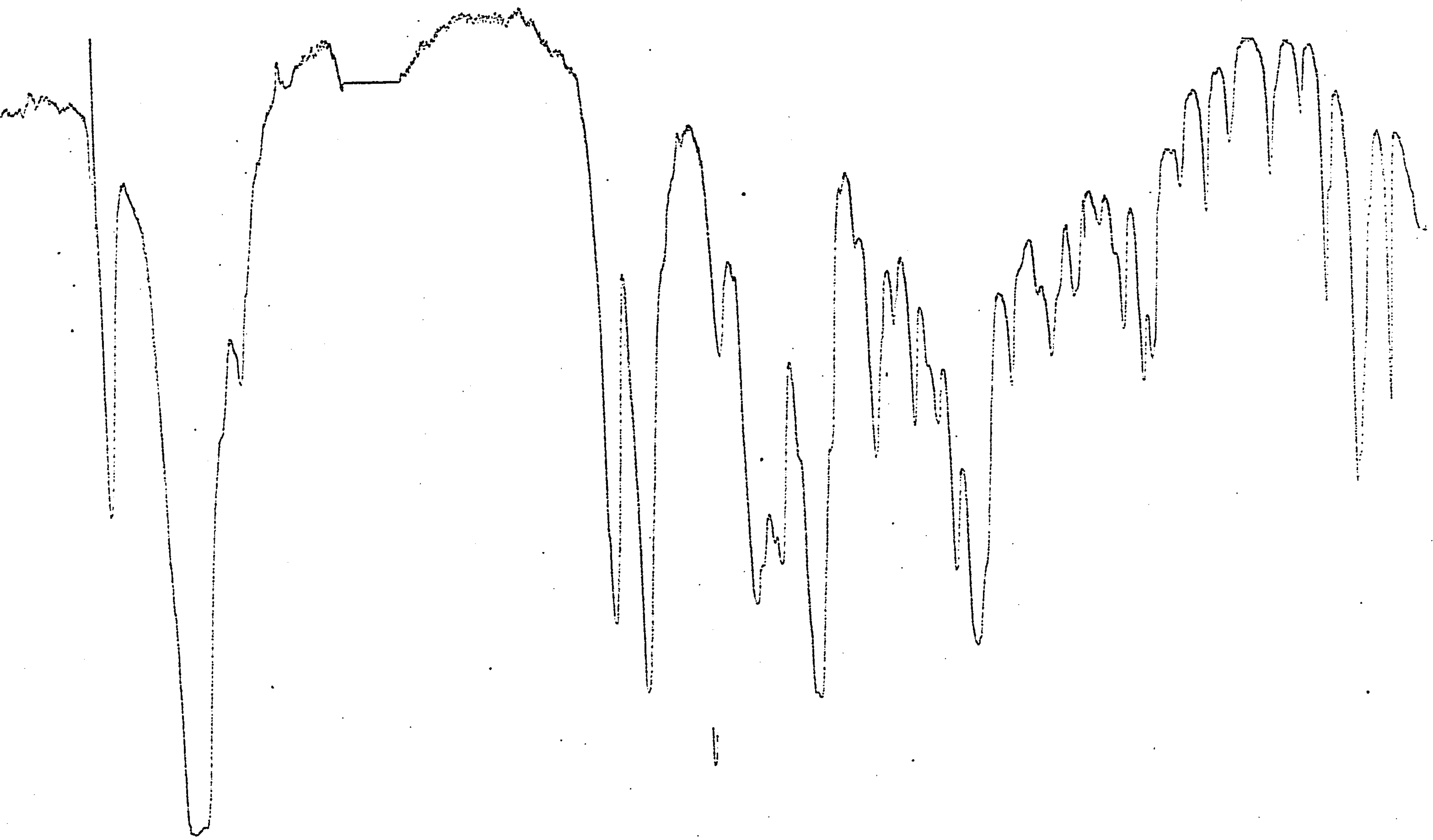
Infrared spectrum of ε -N-carbobenzoxylysine methyl ester



H-Lys(Cbz)-OMe.HCl nujol mull

Fig IV. 30[illegible]a

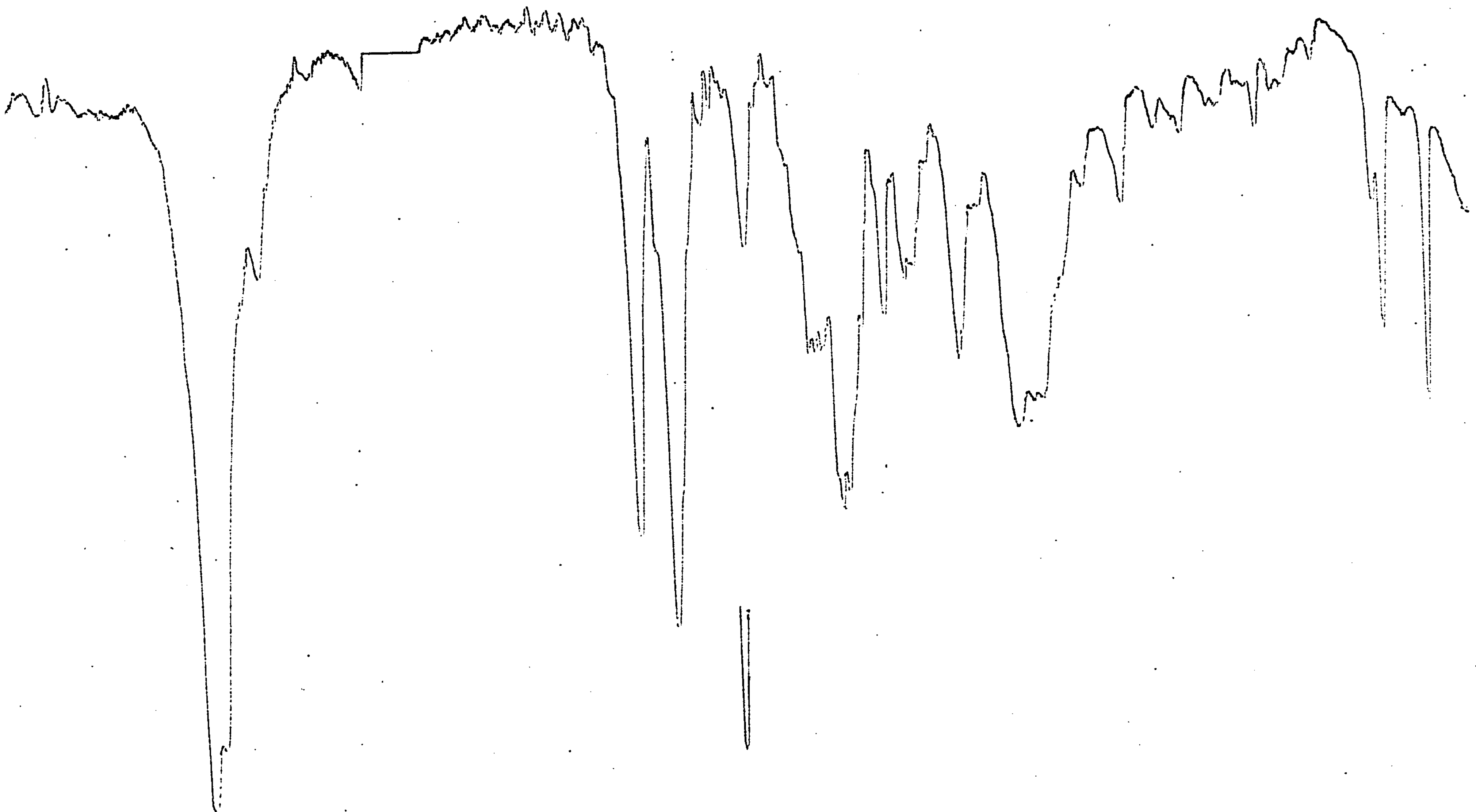
Infrared spectrum of ε -N-carbobenzoxylysine methyl ester



H-Lys(Cbz)-OMe.HCl nujol mull

Fig IV. 306a

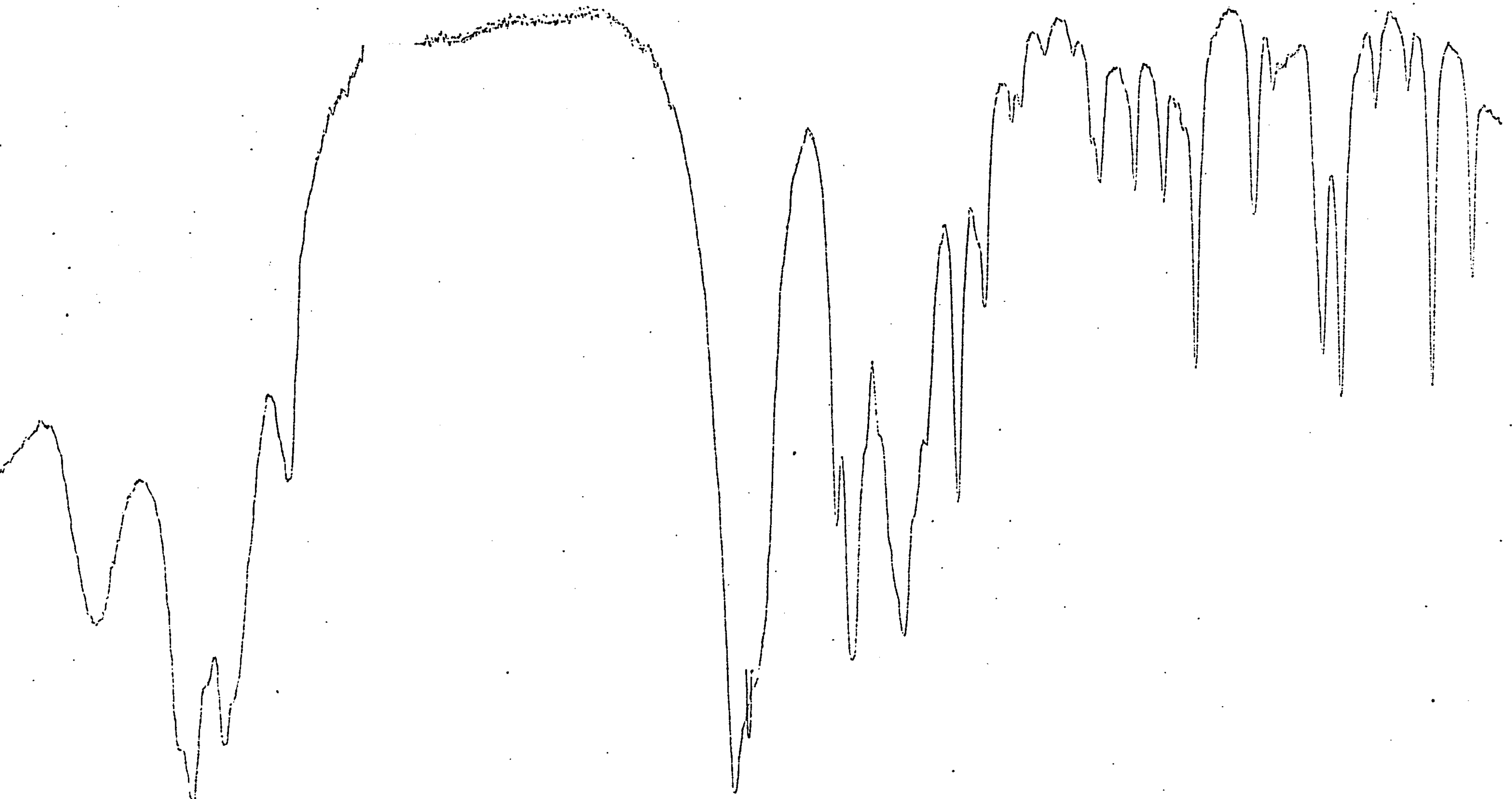
Infrared spectrum of ε-N-carbobenzoxy, ε-N-methyllysine methyl



H-Lys(Me,Cbz)-OMe.HCl nujol mull

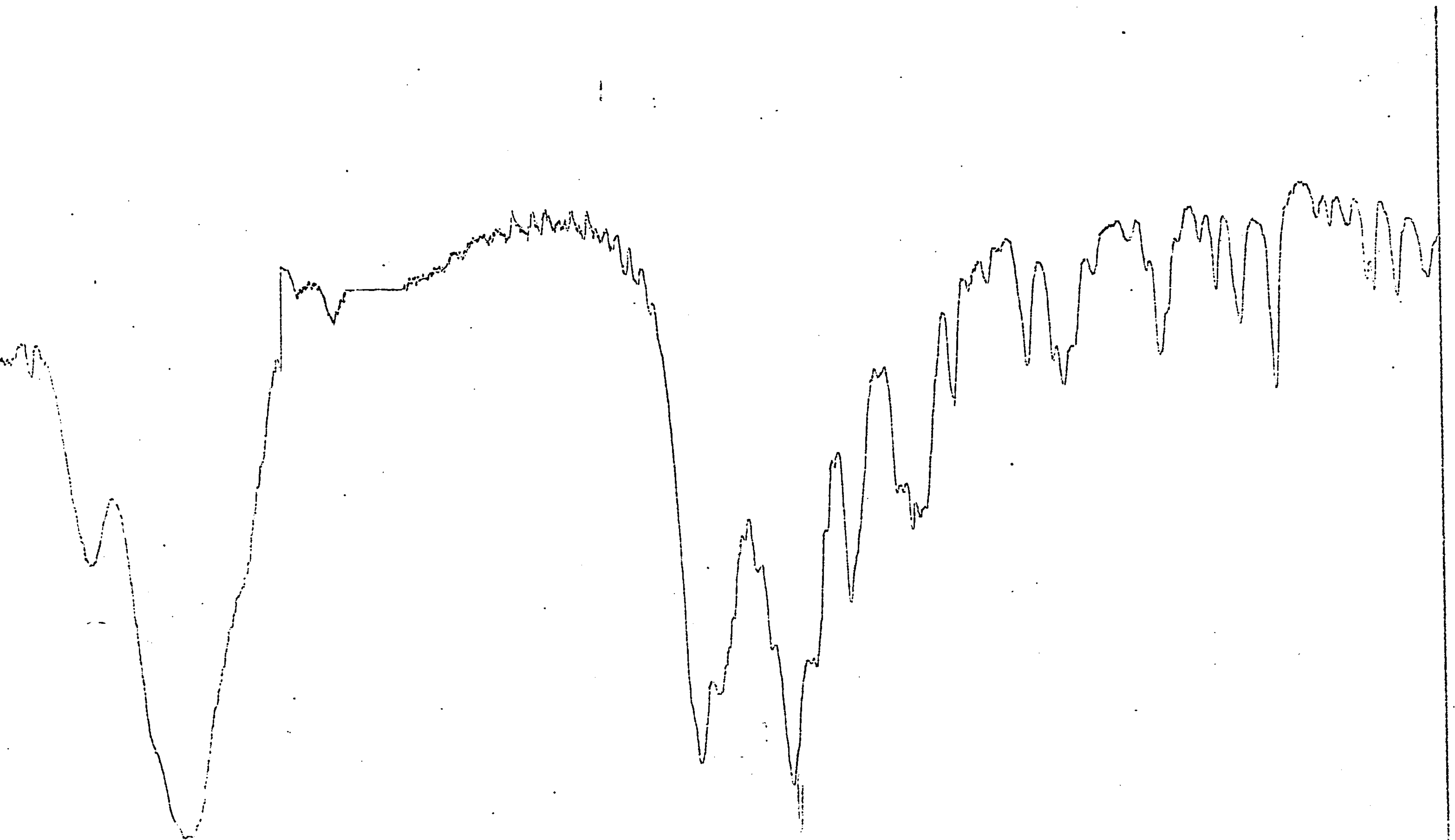
Fig.IV. 306b

Infrared spectrum of α-N, ε -N-dimethyllysine monohydrochloride



MeLys(Me)-OH.HCl KBr disc
Fig. IV . 307a

Infrared spectrum of lysine monohydrochloride

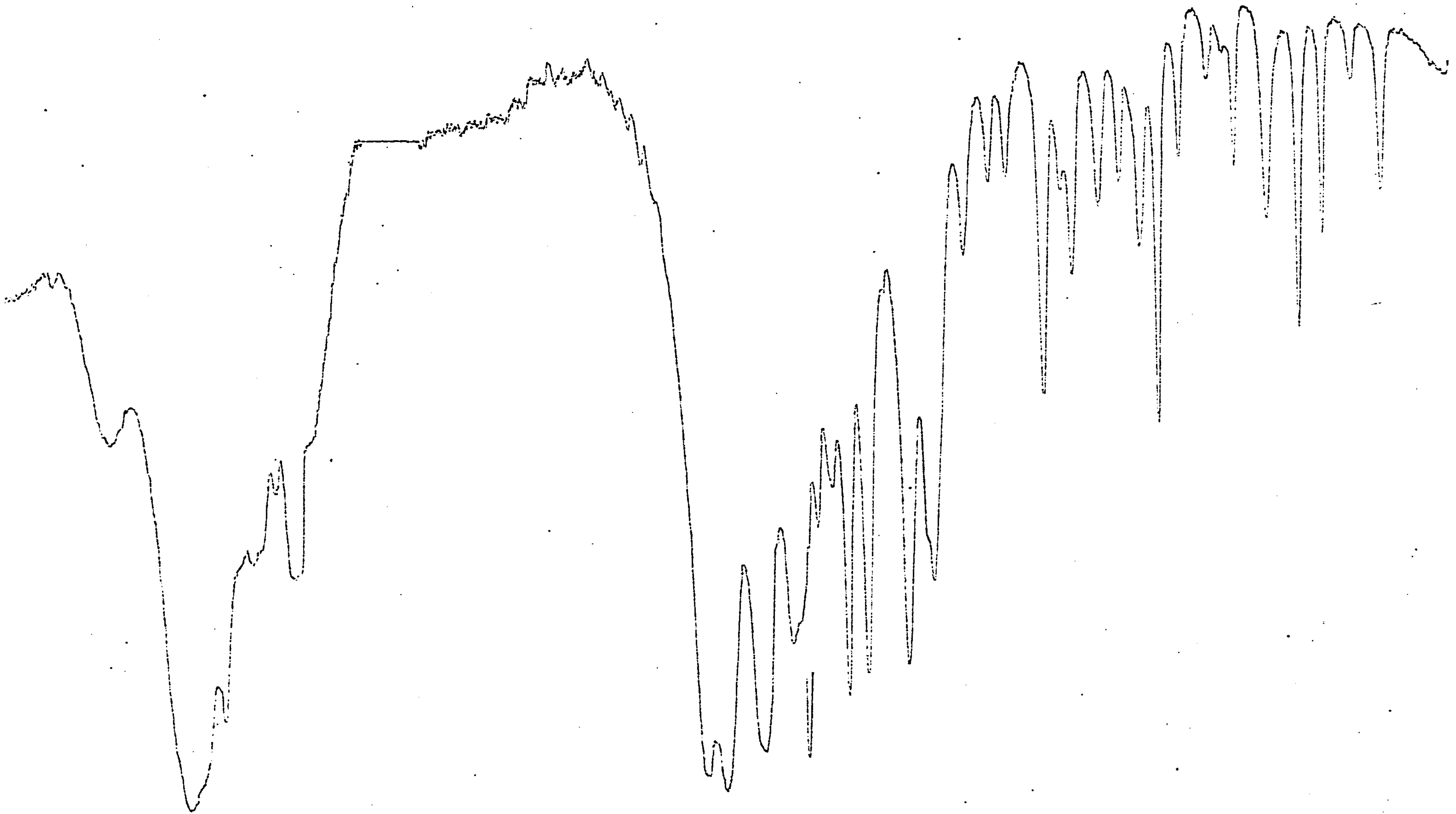


H-Lys-OH.HCl

KBr disc

Fig. IV . 307b

Infrared spectrum of ε-N-methyllysine monohydrochloride



H-Lys(Me)-OH.HCl KBr disc

Fig. IV. 307c

an amino acid II band (1512 cm^{-1}). Both the theoretical primary amine salt bands must coincide in the broad amino acid II band. ε-N-Methyllysine monohydrochloride is both an amino acid and a secondary amine salt; like lysine it has a broad 'ammonium' band (near 3000 cm^{-1}) and two amino acid I bands (1635 and 1613 cm^{-1}); however, the amino acid II band is relatively weak, and there is a secondary amine salt band at 1559 cm^{-1}.

Finally, α-N, ε-N-dimethyllysine monohydrochloride is an N-methylamino acid and a secondary amine salt; it has an N-methylamino acid like spectrum (see p. 123), e. g. the -CH stretch band (2920 cm^{-1}) is resolved from the $-\overset{+}{N}H$ stretch bands and the amino acid II band is absent. The amino acid I band occurs as a single broad band at 1620 cm^{-1} and the secondary amine salt band is not resolved from it.

The results given in this section show clearly that N-methylation leads to substantial changes in the infrared spectra of all the compounds described and that the changes are sufficiently great to allow N-methylation reactions to be followed by infrared spectroscopy. The information provided should also be useful in structure determination studies on compounds containing N-methyl groups.

(b) Nuclear magnetic resonance spectra

In this section data is given for the nuclear magnetic resonance spectra of the compounds synthesised in Part IV. 1. N.m.r. spectroscopy provided an excellent quantitative method of establishing whether or not N-methylation had occurred. The N-methyl signals were readily identifiable and by comparing the integrals of the spectra with the theoretical values the degree of purity of the products could be determined.

Details of the spectra of the N-methylamino acid methyl ester hydrobromides are given in Table IV. 307. Most of the spectra were run in deuterochloroform solution and a typical spectrum (MeLeu-OMe.HBr) is shown in Fig. IV. 308. The N-methyl groups which occurred as triplets near 7.1 τ collapsed to singlets on the addition of D_2O. The methyl ester groups occurred as singlets near 6.1 τ except with the aromatic amino acid esters and the ω-N-methylamino acid esters where there was a small shift to higher field due presumably in the former case to the aromatic ring, and in the latter case to the absence of the α-$\overset{+}{N}H_x$ group. With the α-N-methylamino acids the α-CH resonances were broad and occurred in the range 5.7 - 6.2 τ, frequently overlapping the O-methyl peak. In comparison the ω-N-methylamino acids had α-CH_2 resonances at much higher fields (near 7.9 τ); this is undoubtedly due to the absence of the charged α-$\overset{+}{N}H_x$ group in these compounds. On addition of D_2O the α-CH peaks, when they were sufficiently well resolved to be observed (e.g. MeAla-OMe.HBr) became much sharper. In deuterochloroform solution the α-$\overset{+}{N}H_x$ groups

Table IV. 307

magnetic resonance spectra of N-methylamino acid methyl ester hydrobromides[a]

	N-CH_3		O-CH_3		α-CH_x		-$\overset{+}{N}H_x$		Side chain p
	shift	integral	shift	integral	shift	integral	shift	integral	shift
	6.97t	3.0	6.05s	4.7[d]	5.83m	2.0	2.03bs	2.1	-
	7.13t	3.0	6.07s	4.1[d]	5.82q	d	0.57bs	2.0	8.22d
	7.13t	4.0[e]	6.07s	3.9d	6.17m	d	0.60bd	1.9	8.75d 8.87d
	7.17t	3.0	6.12s	4.0[d]	6.17m	d	0.67bd	1.8	7.5-9.3 cpl
	7.17t	3.0	6.07s	4.1[d]	6.17m	d	0.37bs	2.0	8.00m 9.00d
	6.78q	2.1	5.65q	2.0	6.07m	0.9	0.43bs	1.8	7.93m 8.65 8.42t 9.00d
	7.17t	3.0	6.47s	5.1[f]	5.73m	1.1	2.00bd	1.8	6.67s
	7.17t	3.0	6.27s 6.22s	6.0	5.80m	1.1	0.33bd	2.0	2.95q 6.47t
r[c]	7.20s 7.10s 6.97s	9.2	6.17s	3.0	5.68t	0.9	g	-	6.70d
	7.17s	3.0	6.28s	2.9	7.90t	2.1	g	-	7.47t 7.98q
	7.18s	3.0	6.30s	3.0	7.95t	2.1	g	-	7.92t 8.50m
	-	-	6.10s	5.0[d]	5.92q	d	2.53bs	3.1	-
	-	-	6.17s	4.1[d]	5.77m	d	1.07bs	3.3	8.00m 8.98 d

Footnotes to Table IV. 307

a. Spectra run in deuterochloroform solution except where indicated; chemical shifts given in τ units; Abbreviations:
s (singlet), d (doublet), t (triplet), q (quartet), cplx (complex), b (broad).

b. Spectrum run in deuterochloroform-trifluoroacetic acid (1/1, v/v).

c. Spectrum run in D_2O.

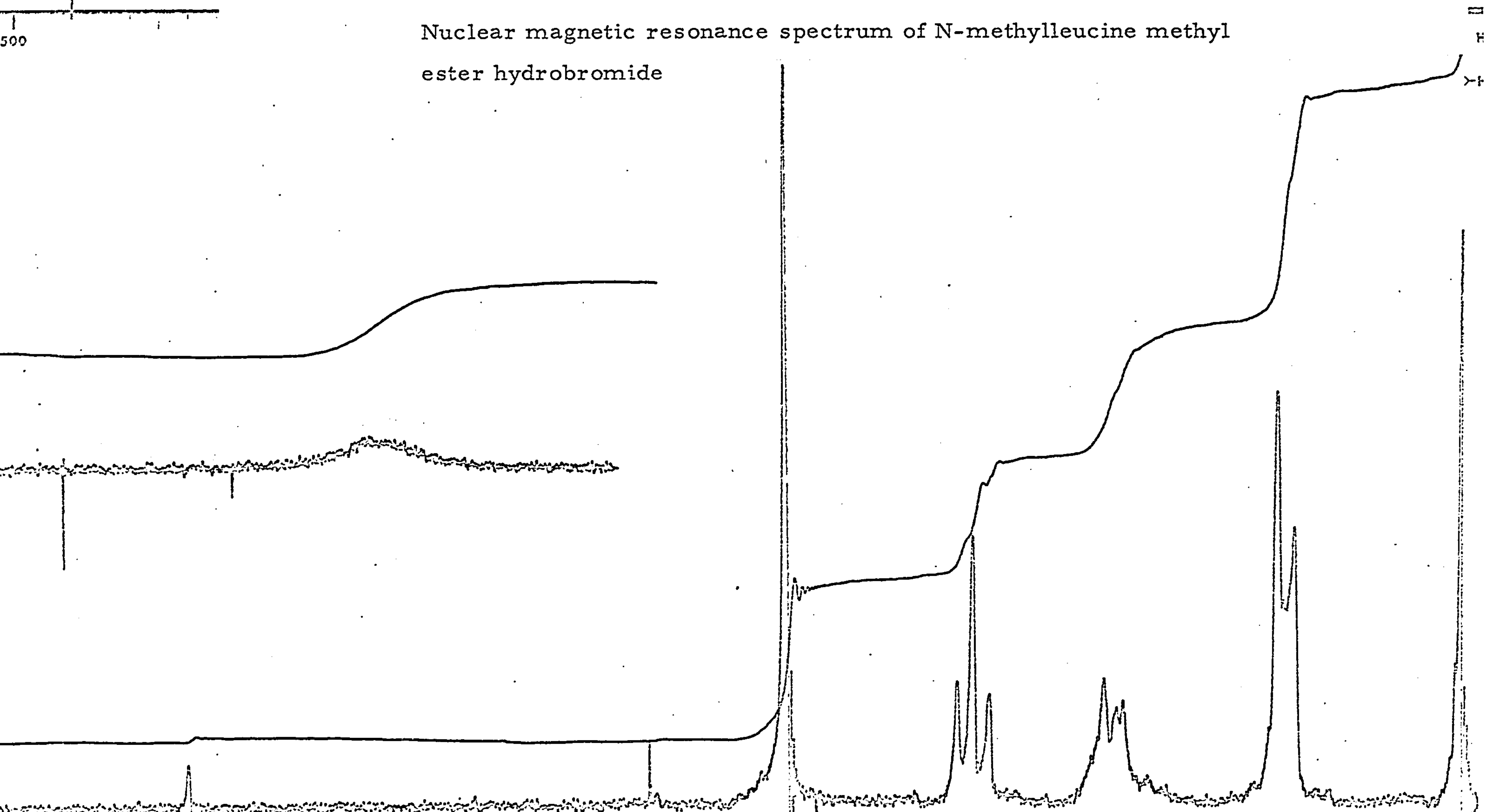
d. α-CH_x not separated from O-CH_3 and so separate integration of -CH_x impossible.

e. β-CH not separated from N-CH_3.

f. β-CH_2 not separated from O-CH_3.

g. -$\overset{+}{N}H_x$ exchanged with D_2O.

Nuclear magnetic resonance spectrum of N-methylleucine methyl ester hydrobromide



occurred as broad peaks at low field (0.3 - 2.0 τ). The two unmethylated amino acid esters shown in the table had α-$\overset{+}{N}H_x$ resonances at higher fields than the corresponding N-methylated derivatives, i.e. methylation of the amino groups caused a small decrease in shielding of the protons bound to the charged nitrogen. N-Ethylleucine ethyl ester hydrobromide had O-CH_2 and N-CH_2 resonances at lower field than the O-CH_3 and N-CH_3 resonances of N-methylleucine methyl ester hydrobromide. This is a general characteristic of such homologues [see Jackman (1959), p. 55-56]. For all the spectra the integrations were in good agreement with theory.

The spectra of the acetyl-N-methylamino acids were run in deuterochloroform solution and are shown in Table IV. 308. A very conspicuous feature of the spectra, which is clearly shown in the spectrum of acetyl-N-methylvaline (Fig. IV. 309), was the occurrence of two resonances for the α-CH and the N-methyl groups. In three of the compounds two resonances were also observed for the acetyl groups and for the side chain protons. Acetylsarcosine which was insoluble in deuterochloroform gave two N-methyl peaks in trifluoracetic acid solution; in this solvent acetyl-N-methylleucine also gave two resonances for the α-CH, the N-methyl and the acetyl groups. A similar kind of peak splitting was observed with the benzoyl-N-methylamino acids in trifluoracetic acid solution (Table IV. 310) but there was no splitting of either the acetyl, benzoyl or α-CH groups of the acetylamino acids (Table IV. 309 and Fig. IV. 310) or the benzoylamino acids (Table IV. 310). In

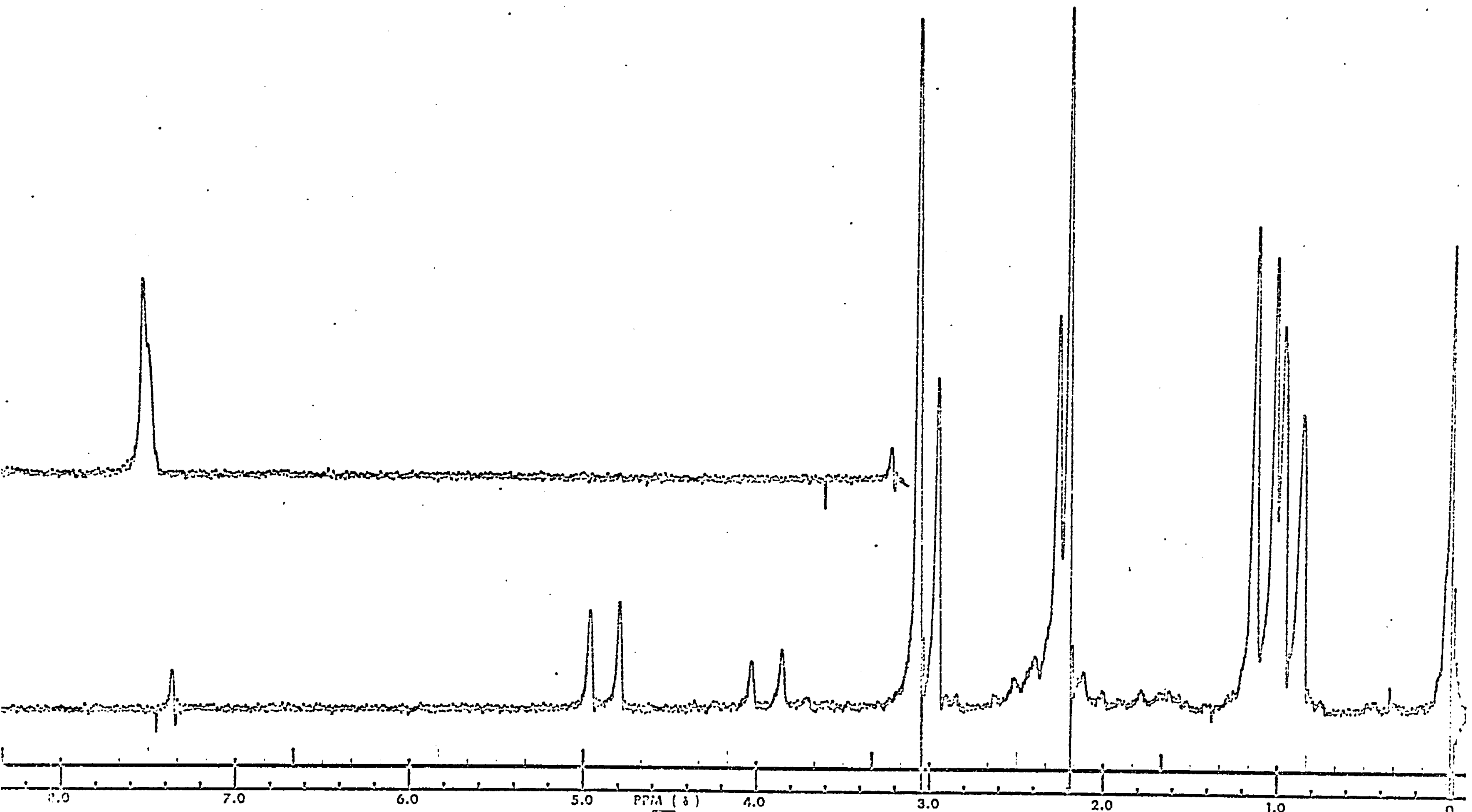

7.0
6.0
5.0
PPM (δ)
4.0
3.0
2.0
1.0
0
MANUAL
AUTO
SAMPLE: Ac-MeVal-OH
REMARKS:
OFFSET (Hz): 0(250)
20
ca 40
SWEEP TIME (SEC):
SWEEP WIDTH (Hz):
FILTER:
RF POWER LEVEL: 0.05
(250)
(500)
(2)
(.05)
SOLVENT: CDCl3
analytical instrument division
DATE:
OPERATOR:
60 MHz NMR
SPECTRUM NO.

Table IV. 309

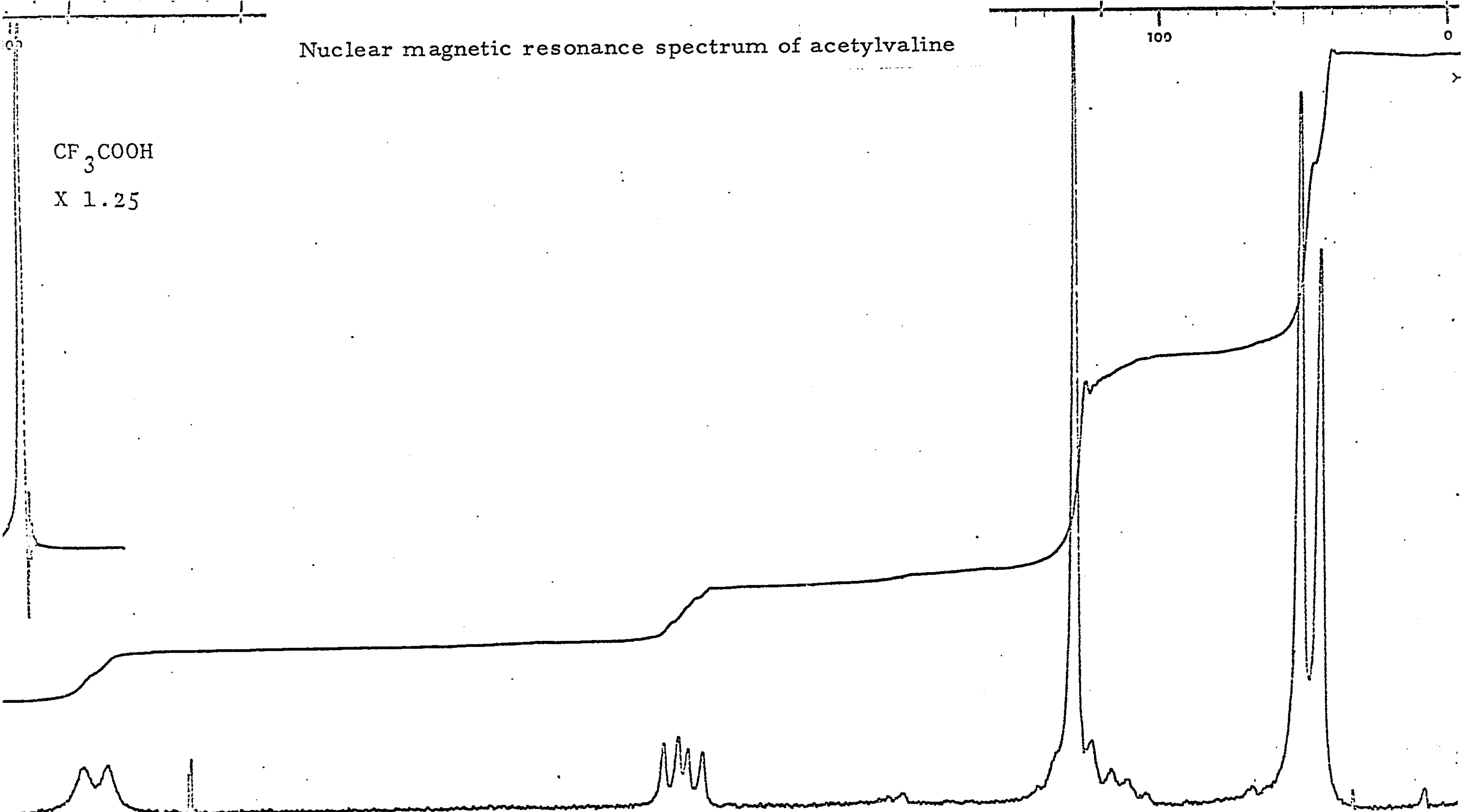
Nuclear magnetic resonance spectra of acetylamino acids[a]

	CH_3CO		CH		-NH		Side chain protons	
Ac-X-OH	shift	integral	shift	integral	shift	integral	shift	integral
Ac-Gly-OH	7.53s	3.0	5.57d	2.0	1.73bs	0.9	-	-
Ac-Ala-OH	7.53s	3.0	5.10qi	0.9	1.73bd	1.0	8.30d	3.0
Ac-Val-OH	7,48s	4.0[b]	5.20qa	1.0	1.75bd	0.9	8.85d 8.0 m	5.8
Ac-Ile-OH	7.50s	3.0	5.11qa	1.0	1.72bd	0.8	7.7- 9.0m	9.0
Ac-Leu-OH	7.53s	3.0	5.07qa	1.0	1.82bd	0.9	8.0- 9.0 m	8.9
Ac-Phe-OH	7.67s	3.0	5.78qa	0.9	1.93bd	1.1	6.62qa 2.60 s	2.1 5.0

a) Spectra run in trifluoracetic acid solution; chemical shifts given in τ units;

abbreviations: s (singlet), d (doublet), qa (quartet), qi (quintet), b (broad).

b) Signal overlapped multiplet due to β-CH.



Nuclear magnetic resonance spectrum of acetylvaline
CF$_3$COOH
X 1.25
100
0
8.0
7.0
6.0
5.0
PPM (δ)
4.0
3.0
2.0
1.0
0
MANUAL
AUTO
SAMPLE: Ac-Val-OH
REMARKS: (TMS off chart
0 cps line 27 cps
from TMS)
OFFSET (Hz): 0(200)
AMPLITUDE: 12.5
AMPLITUDE: ca 30
RATE (RPS):
SWEEP TIME (SEC): 50 250 (250)
SWEEP WIDTH (Hz): 25 50 100 250 500 (500)
FILTER: 1 2 3 4 5 6 7 8 (2)
RF POWER LEVEL: 0.05 (.05)
SOLVENT: CF$_3$COOH
varian
analytical instrument division
DATE:
OPERATOR:
60 MHz NMR
SPECTRUM NO.

Table IV. 310

pectra of benzoyl- and benzoyl-N-methylamino acids [a]

COOH	$N-CH_3$			α-CH			Side chain protons	
integral	shift main	shift minor	integral	shift main	shift minor	integral	shift	integral
-	-	-	-	5.42s	-	2.0	-	-
-	-	-	-	4.92m	-	1.0	8.22d	3.0
-	-	-	-	4.92m	-	1.0	7.7-9.2 cplx	9.5
1.0	-	-	-	5.08m	-	1.0	8.15 d 9.02d	6.2 3.0
-	6.72	6.58	3.0	5.42s	5.60s	2.0	-	-
1.0	6.90	-	3.0	5.67bs	5.78bs	2.0	-	-
-	6.77	6.66	3.0	4.68q	5.07q	1.0	8.25t	3.2
1.1	7.03	-	3.0	4.70vb	5.50vb	1.0	8.50d	3.2
-	6.80	6.63	3.0	4.50t	5.18t	0.9	7.7-9.7 cplx	9.6
1.0	7.03		3.0	4.60m	5.58m	0.9	7.7-9.7 cplx	10.0

; cplx (complex), m (multiplet), s (singlet), t (triplet), q (quartet), b (broad), vb (very broad).

d as doublet at 3.15 τ , integral 1.3.

Table IV. 310

Nuclear magnetic resonance spectra of benzoyl- and benzoyl-N-m

	phenyl group		-COOH		$N\text{-}CH_3$		
	shift	integral	shift	integral	shift main	shift minor	integ
Bz-Gly-OH[b]	1.7-2.7 cplx	6.0[e]	d	-	-	-	-
Bz-Ala-OH[b]	1.7-2.7 cplx	6.0[e]	d	-	-	-	-
Bz-Leu-OH[b]	1.8-2.7 cplx	6.0[e]	d	-	-	-	-
Bz-Leu-OH[c]	1.8-2.8 cplx	5.0[f]	-1.37	1.0	-	-	-
Bz-MeGly-OH[b]	2.12m	5.0	-	-	6.72	6.58	3.0
Bz-MeGly-OH[c]	2.63m	4.9	-1.03	1.0	6.90	-	3.0
Bz-MeAla-OH[b]	2.33m	5.2	d	-	6.77	6.66	3.0
Bz-MeAla-OH[c]	2.63m	5.1	-1.35	1.1	7.03	-	3.0
Bz-MeLeu-OH[b]	2.33m	5.1	d	-	6.80	6.63	3.0
Bz-MeLeu-OH[c]	2.52m	5.0	-1.67	1.0	7.03		3.0

a. Chemical shifts given in τ units; abbreviations: cplx (complex), m (multiplet), s (s

b. Spectrum run in trifluoracetic acid solution.

c. Spectrum run in deuterochloroform solution.

d. Obscured by solvent band.

e. Phenyl protons and -NH overlapped.

f. Phenyl protons and -NH resolved; -NH occurred as doublet at 3.15 τ , integral 1.3.

deuterochloroform solution only the α-CH resonances of the benzoyl-N-methylamino acids were split. The integrals for all the spectra of the acyl-N-methylamino acids agreed well with theory and this, together with the fact that each spectrum appeared to consist of two overlapping spectra with identical coupling patterns, suggested that the double peaks were due to restricted rotation isomerism. Amides can exist in <u>cis</u> and <u>trans</u> forms because of the partial double bond character of the C-N bond which leads to a significant barrier to free rotation about this bond [Dyer (1965), p. 113] (see Fig. IV. 310). The studies of Phillips (1955) on DMF provided the first n.m.r. evidence for the occurrence of restricted rotation isomerism in amides. Subsequently the existence of two 'isomers' has been reported for many mono- and di-N-substituted amides [for a review see Reeves (1965)]. Goodman and Fried (1967) have reported the n.m.r. spectrum of acetyl-N-methylalanine methyl ester which is very similar to the spectrum of acetyl-N-methyl-alanine given in Table IV. 308. The authors drew attention to the splitting of the N-methyl resonance and the occurrence of two side chain C-methyl doublets [see Goodman and Choi (1968)] which they attributed to cis-trans isomerism about the amide C-N bond. No mention was made of any splitting of the α-CH resonance and the appropriate region of the spectrum was not reported. The occurrence of cis-trans isomerism has also been reported recently for acetyl-D-alanyl-N-methyl-D-alanine methyl ester [Bystrov et al (1969)].

The assignment of a "set of signals" in the spectrum of an amide which exhibits restricted rotation "to particular rotamer is a difficult task" [Cooper (1969)] although it has been done successfully for a number of N, N-disubstituted amides [see e. g. Laplanche and Rogers (1963)] and N-monosubstituted amides [Laplanche and Rogers (1964)]. Our data are not sufficient to allow the assignment of the individual peaks in the spectra of the acyl-N-methylamino acids to either the cis or trans isomers. However the α-CH resonances of the acetyl- and benzoyl-N-methylamino acids are completely resolved and a precise estimate of the relative amounts of the two isomers can be made from the integrals of the spectra; the values are given in Table IV. 311. The N-methyl resonances are less well resolved but still estimates of the relative amounts of the two isomers can be made; these values are also given in Table IV. 311. The numerical order of both sets of ratios agrees well but the absolute values differ. Whether any significance can be attached to this difference is not certain. Better resolution of the N-methyl integrals would be required in order to establish that the effect was real and not, as seems possible, merely an artifact. The separation of the sets of resonances seems to be solvent dependent, e.g. the N-methyl signals of the benzoyl-N-methylamino acids are separated in trifluoracetic acid but not in deuterochloroform solutions. Possibly a solvent could be found which would give better separations; alternatively, a higher resolution spectrometer could be used. It is interesting to note that the relative amounts of the two isomers change considerably as the amino acid side chain becomes larger and more branched. Benzoyl-N-methylleucine

Table IV. 311

Restricted rotation in acetyl- and benzoyl-N-methylamino acids[a]

R-MeX-OH	-CH resonances major peak	-CH resonances minor peak	-CH resonances ratio of integrals	$N-CH_3$ resonances major peak	$N-CH_3$ resonances minor peak	$N-CH_3$ resonances ratio of integrals
Ac-MeGly-OH[b]	5.55	-	-	6.67	6.73	5.4
Ac-MeAla-OH	4.70	5.43	2.4	7.00	7.10	3.7
Ac-MeVal-OH	5.17	6.08	1.7	6.95	7.05	2.3
Ac-MeIle-OH	5.07	6.00	1.5	6.97	7.07	2.0
Ac-MeLeu-OH	4.80	5.57	2.8	7.03	7.10	4.9
Ac-MeLeu-OH[b]	4.40	5.07	2.8	6.67	6.73	c
Ac-MePhe-OH	4.73	5.53	3.0	7.15	7.02	d
Bz-MeGly-OH[b]	5.42	5.60	1.5	6.72	6.58	2.1
Bz-MeAla-OH[b]	4.68	5.07	1.3	6.77	6.66	1.6
Bz-MeLeu-OH[b]	4.50	5.18	1.0	6.80	6.63	1.2

a. Spectra run in deuterochloroform solution except where indicated; shifts given in τ units; integrals measured with sweep width of 250 cycles.

b. Spectrum run in trifluoraceticacid solution.

c. Separate integration was impossible.

d. Separate integration was impossible as benzyl CH_2 resonance was very near the minor peak.

occurs as an equimolar mixture of the cis and trans isomers whereas with benzoylsarcosine there is a 2 to 1 preponderance of one of the isomers. Acetyl-N-methylvaline and acetyl-N-methylisoleucine have isomer ratios of 1.7:1 and 1.5:1 whereas acetyl-N-methylalanine, acetyl-N-methylleucine and acetyl-N-methylphenylalanine which do not have a branch at the β-carbon atom have isomer ratios of 2.4:1 and 2.8:1 and 3.0:1 respectively.

A more detailed study of the n.m.r. spectra of the acyl-N-methylamino acids should provide interesting information. Once the resonances have been assigned it should be possible to deduce both thermodynamic and kinetic data for the two isomers. The fact that the isomer ratio was generally not 1:1 implies that there is a difference in the standard free energies between the isomers. From measurements of the equilibrium isomer ratios at particular temperatures the standard free energy difference (ΔF^{o}) between the two isomers can be calculated, e.g. an isomer ratio of 2:1 at 25°C corresponds to a ΔF^{o} difference of approximately 370 cal/mole [Elliel (1962) gives a table relating the % of more stable isomer at equilibrium to ΔF^{o} at various temperatures]. A study of the temperature dependence of the chemical shift difference of e.g. the α-CH resonances of the two isomers should provide information about the rates of interconversion of the two isomers and hence the height of the energy barrier to rotation. Rotation barriers were first estimated in this way by Gutowsky and Holm (1956) and the methods have been reviewed by Phillips (1958) and Reeves (1965). A discussion of the value of such data in theoretical peptide and protein chemistry is given in Part V (see p. 171).

In Table IV. 312 the spectra of some protected N-methylleucines are given. The integrals of the spectra of carbobenzoxy-N-methylleucine, tosyl-N-methylleucine methyl ester and tosyl-N-methylleucine confirmed that these samples were pure. On the other hand the integral of the spectrum of carbobenzoxy-N-ethylleucine was poor. The peaks associated with the N-ethyl group were too small which suggested that the compound was not completely ethylated, i.e. it was contaminated with carbobenzoxyleucine. This was consistent with the infrared data (see p. 120) which showed that the carbobenzoxy-N-ethylleucine ethyl ester used for the preparation of carbobenzoxy-N-ethylleucine was contaminated with carbobenzoxyleucine ethyl ester. The N-methyl resonance in the spectrum of tosyl-N-methylleucine methyl ester was split into two peaks. This is attributed to the occurrence of some kind of restricted rotation isomerism.

The spectra of N-methylleucine, leucine and α-C-methylleucine are shown in Figs. IV. 311a, b and c and details of the chemical shifts and the integrals are shown in Table IV. 313. These spectra are included to demonstrate where the C-methyl singlet of α-C-methylleucine occurs and to show that although it overlaps the leucine side chain protons, it can be readily identified. The singlet occurred at relatively low field for a C-methyl group because of the strong electron withdrawing effect of the α-$\overset{+}{N}H_3$ group. No α-C-methyl singlet is detectable in the spectrum of the sample of synthetic N-methylleucine which eliminates the possibility of any contamination of this sample with N-methyl, α-C-methylleucine, an

Table IV. 312

Nuclear magnetic spectra of some protected N-methylleucines[a]

	aromatic protons		α-CH		CH_x-phenyl		N-CH_x		side chain protons	
	shift	integral	shift	integral	shift	integral	shift	integral	shift	integral
Cbz-MeLeu-OH[b]	2.62s	5.0	5.07m	1.1	4.77s	2.1	7.08s	2.9	8.25d 9.03d	3.3 6.3
Cbz-EtLeu-OH[c]	2.73s	5.0	f	-	4.93s	2.0	5.83q	2.4[f]	8.35d 9.10d	11.2[g]
Tos-MeLeu-OMe[d]	7.55q	4.1	4.73t	0.8	7.55s	3.0	6.93s 7.13s	3.0[h]	8.37d 9.02d	2.9 5.6
Tos-MeLeu-OH	7.55q	3.8	4.73t	0.9	7.55s	3.0	7.15s	3.0	8.37d 9.03d	3.4 6.6

a. Spectra run in deuterochloroform solution; chemical shifts given in τ units; abbreviations: - s (singlet), t (triplet), q (quartet), m (multiplet).

b. Acid proton occurred as singlet at -0.68τ , integral 1.0.

c. Acid proton occurred as singlet at 0.08 τ , integral 0.9.

d. Methyl ester group occurred as singlet at 6.50 τ , integral 2.7.

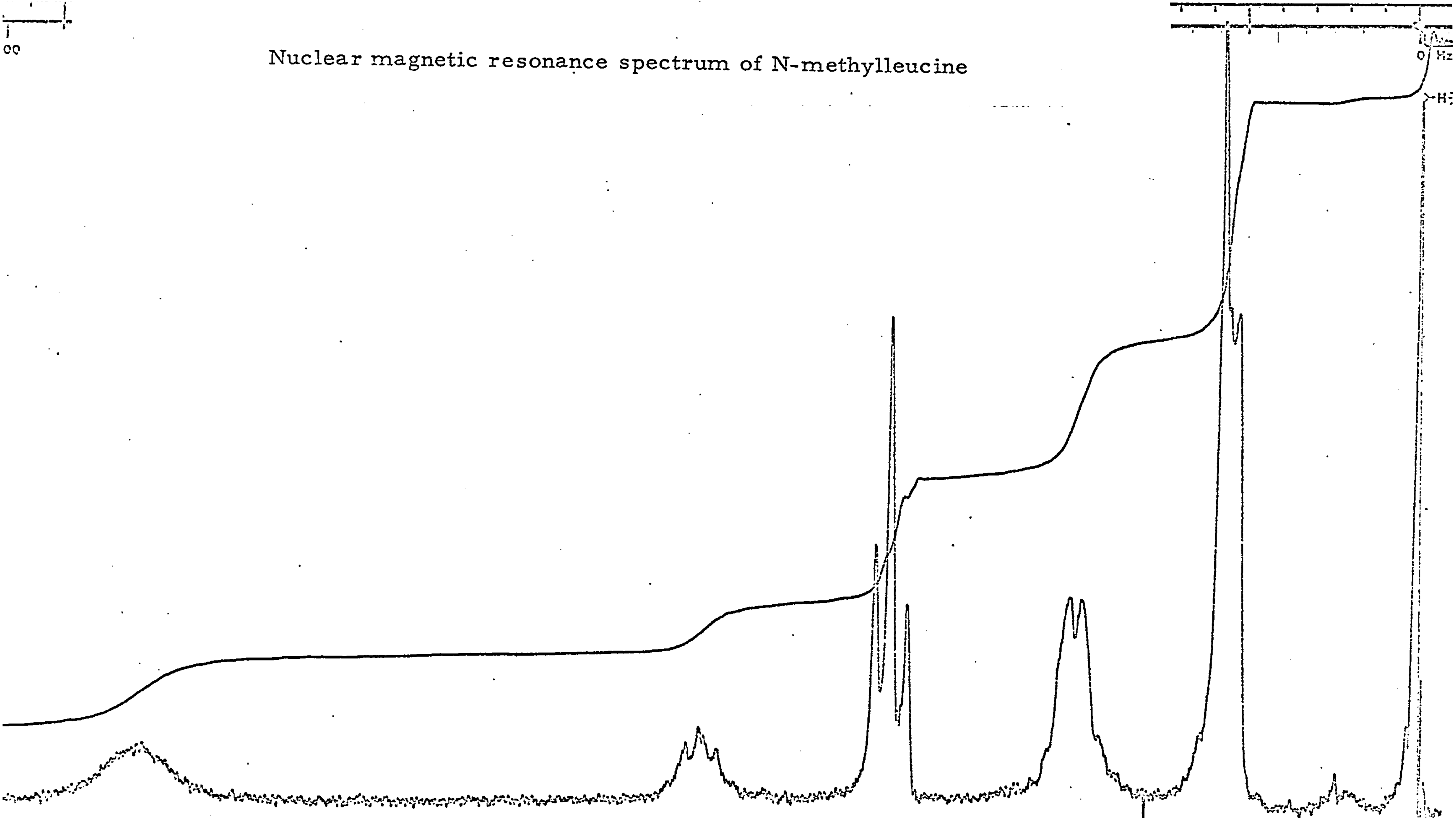
e. Acid proton occurred as singlet at -0.28 τ , integral 1.0.

f. α-CH not resolved from N-CH_2.

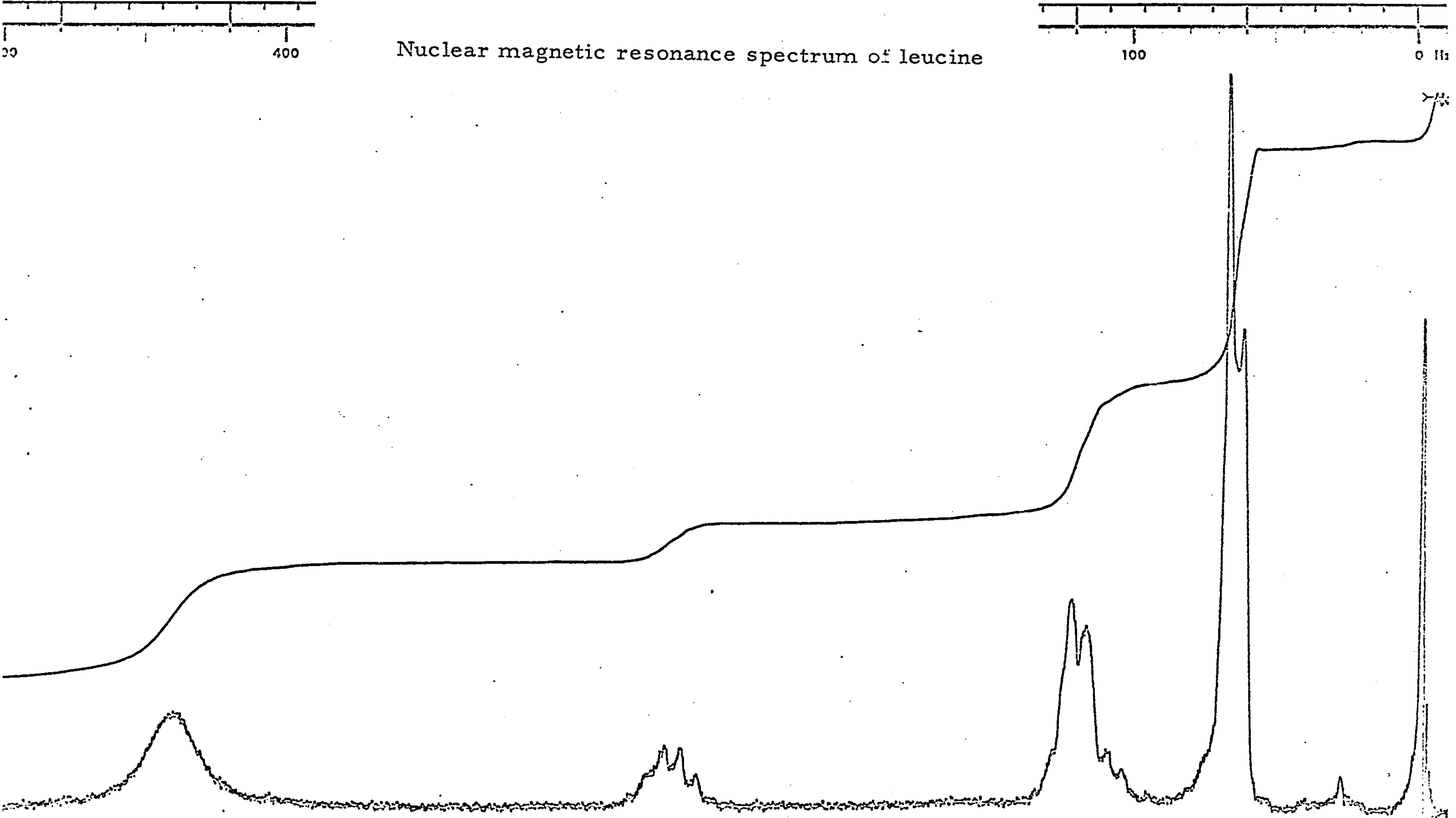
g. CH_3 of N-Et group occurred as triplet at 1.27 τ in between the two side chain peaks; separate integration in this region was impossible.

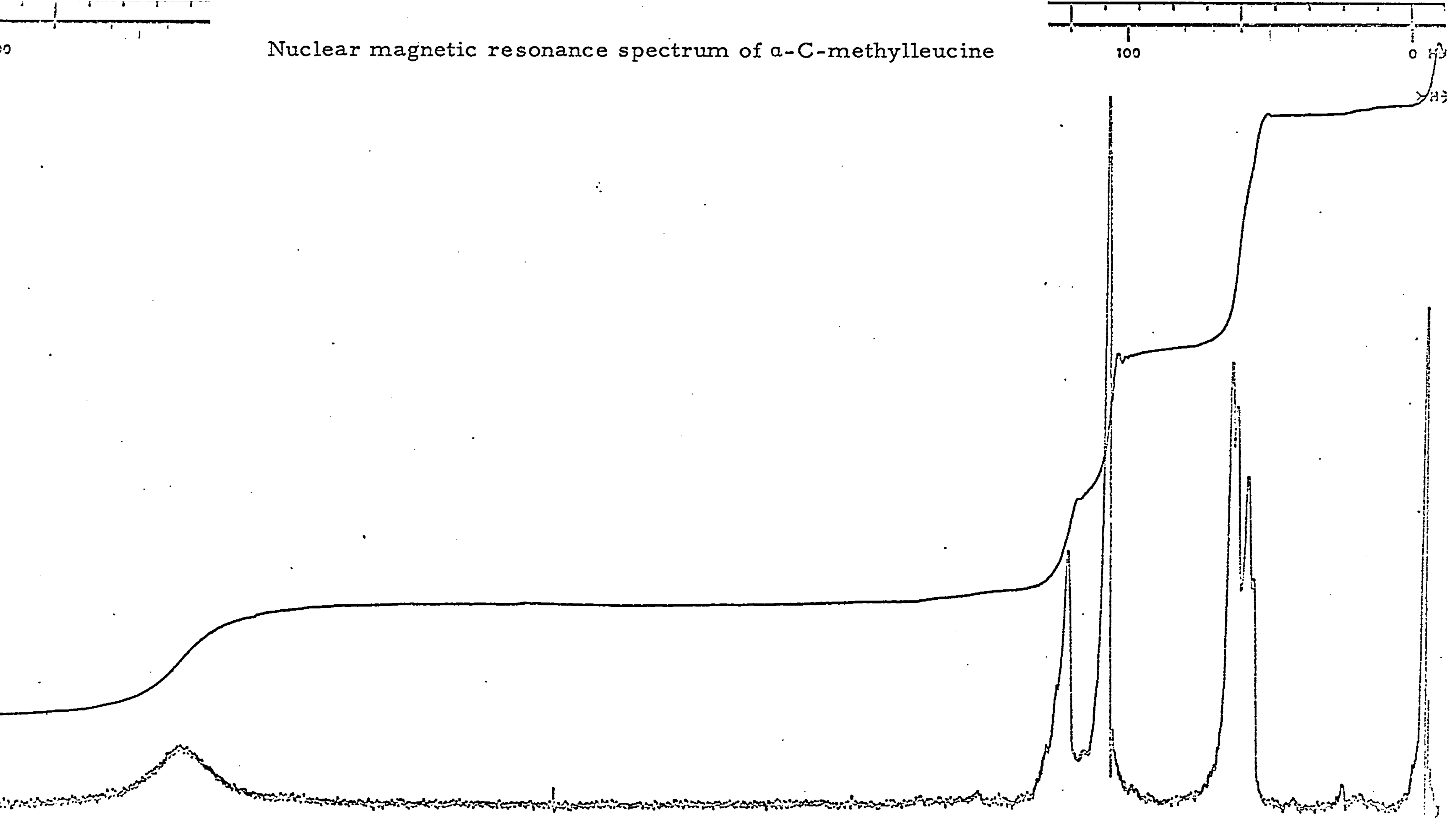
h. The ratio of integrals for the two peaks at 6.93 and 7.13 τ was 1:4.8.

Nuclear magnetic resonance spectrum of N-methylleucine



Nuclear magnetic resonance spectrum of leucine





Nuclear magnetic resonance spectrum of α-C-methylleucine

Table IV. 313

Nuclear magnetic resonance spectra of leucine, N-methylleucine and α-C-methylleucine [a]

	$-\overset{+}{N}H_x$		-CH		side chain protons	
	shift	integral	shift	integral	shift	integral
Leucine	2.58b	2.9	5.53m	1.0	7.97d 8.57d	3.3 6.0
α-C-Methylleucine[b]	2.62b	2.8	-	-	7.90m 8.92m	6.1[c] 6.0
N-Methylleucine[d]	2.40b	1.6	5.70m	1.1	7.95d 8.88d	3.3 5.8

a. Spectra run in trifluoracetic acid solution; chemical shifts given in τ units; abbreviations: b (broad), d (doublet), m (multiplet).

b. C-Methyl group occurred as a singlet at 8.13 τ ; separate integration from the side chain protons was impossible.

c. Includes C-methyl group.

d. N-Methyl group occurred as a triplet at 6.87 τ , integral 3.0.

impurity which might not be separated on the amino acid analyser and would in any case react very poorly with ninhydrin (see p. 107). The resonance of the α-$\overset{+}{N}H_x$ group in N-methylleucine occurred at lower field than the resonances of the α-$\overset{+}{N}H_x$ groups of both of the amino acids, i.e. as with leucine methyl ester (see p.127) methylation caused a small decrease in the shielding of the protons. The integration of the α-$\overset{+}{N}H_2$ group for N-methylleucine was too small whereas the α-$\overset{+}{N}H_3$ integrals for the amino acids were good. This may be a characteristic for N-methylamino acids in trifluoracetic acid solution since the -$\overset{+}{N}H_2$ integrals for ε -N-methyllysine and α-N, ε-N-dimethyllysine were also too low (see Table IV. 316).

The spectra of ε-N-carbobenzoxy, ε -N-methyllysine methyl ester hydrochloride, the methyl and ethyl esters of ε -N-carbobenzoxylysine hydrochloride, ε -N-ethyllysine ethyl ester di-hydrobromide and α-N-trityl, ε-N, ε -N, ε-N-trimethyllysine methyl ester iodide are given in Tables IV. 314 and IV. 315. The integrals of all these spectra confirmed the purity of the products.

The spectra of α-N, ε-N-dimethyllysine monohydrochloride, lysine monohydrochloride and ε -N-methyllysine monohydrochloride are given in Table IV. 316. In D_2O solution the singlet for the N-methyl resonances of the α-N- and the ε-N-methyl groups of α-N, ε -N-di-methyllysine overlapped at 7.28 τ ; in trifluoracetic acid solution the N-methyl resonances were shifted downfield to 6.87 τ and appeared as a quartet. This shift of the N-methyl resonance to lower field in the presence of trifluoracetic acid solution was also observed with ε-N-methyllysine and is a general characteristic of N-methyl groups [Davis (1968)].

Table IV. 314

Nuclear magnetic resonance spectra of some ε -N-carbobenzoxylysine esters

	$-\overset{+}{N}H_3$	phenyl protons	-NH	benzyl CH_2	-CH	$-OCH_x$	$-NCH_2$	$-(CH_2)_3$
H-Lys(Cbz)-OMe.HCl	1.18b (2.6)	2.70s (5.0)	4.42vb (0.7)	4.93s (2.0)	6.17b (0.9)	6.25s (3.0)	6.92b (2.1)	8.00b 8.45b (6.0)
H-Lys(Cbz)-OEt.HCl	1.17b (3.0)	2.65s (5.0)	4.33vb (1.0)	4.88s (2.0)	5.8[b] -	5.73q (3.2)	6.88b (2.1)	7.93b 8.42b (9.8)[c]
H-Lys(Cbz,Me)-OMe.HCl[d]	1.03b (2.9)	2.60s (5.0)	- -	4.83s (1.8)	5.80b (1.1)	6.20s (3.0)	6.67b (2.2)	7.93b 8.57b (5.2)

a. Spectra run in deuterochloroform solution; chemical shifts given in τ units; integrals shown in brackets under the shift values;
abbreviations: b (broad), vb (very broad), s (singlet), q (quartet).

b. Overlapped by $-OCH_2$ quartet, included in $-OCH_2$ integral.

c. Integral includes CH_3 of -OEt group which occurred as triplet at 8.73τ .

d. $N-CH_3$ group occurred as a singlet at 7.07 τ , integral 3.2.

Table IV. 315

Nuclear magnetic resonance spectra of ε-N-ethyllysine ethyl ester and α-N-trityl,ε -N ε -N, ε-N-trimethyllysine methyl ester [a]

H-Lys(Et)-OEt. 2HBr	Chemical shifts (τ units)	Integral
$-\overset{+}{N}H_3$ and $-\overset{+}{N}H_2$	broad band at 2.33 with shoulder at 2.67	5.0
OCH_2 and -CH	quartet at 6.10 overlapping α-CH	2.9
$(\overset{+}{N}CH_2)_2$	multiplet at 6.78	4.0
$-(CH_2)_3-$	complex pattern, main peak at 8.08	6.0
$(CH_3)_2$	2 overlapping triplets, all six peaks resolved, centre of multiplet at 8.62	6.0
Trt-Lys(Me_3)-OMe. I		
$(Ph)_3$	complex pattern between 2.3 and 3.0	15.0
-NH	very broad peak at 3.33	1.0
$\overset{+}{N}-(CH_3)_3$, $\overset{+}{N}-CH_2$ and -CH	singlet with broad base at 6.60	15.0
OCH_3	singlet at 6.80	
$-(CH_2)_3-$	broad peak at 8.33	6.4

a. Spectra run in deuterochloroform solution.

Dropwise addition of NaOD in D_2O to an aqueous solution of α-N, ε-N-dimethyllysine monohydrochloride led to a separation of the N-methyl resonances into two peaks. After the addition of 1 mole there were two peaks of equal area: one was a sharp singlet at 7.47τ due to the still charged $-\overset{+}{N}D_2-CH_3$ group and the other was a slightly broadened singlet at 7.73τ due to the uncharged α-ND-CH_3 group. Addition of a further mole gave two sharp singlets at 7.73 and 7.78τ. The addition of NaOD produced a similar upfield shift of the N-methyl resonance of ε-N-methyllysine monohydrochloride; however, the change only began after the addition of the first mole of NaOD was complete. This is consistent with the fact that the methyl group is attached to the more basic of the two amino groups. The discharging of the α-amino groups of ε-N-methyllysine and α-N, ε-N-dimethyllysine on the addition of 1 mole of NaOD also led to a large upfield shift of the α-CH resonances because of the replacement of the very strongly electron withdrawing α-$\overset{+}{N}D_x$ group by an uncharged α-ND_{x-1} group. On the addition of a second mole of NaOD the ε-CH_2 resonances also shifted upfield as the ε-$\overset{+}{N}D_x$ group was discharged. This technique of titrating the two amino groups by the gradual addition of NaOD provides a simple way of establishing whether the N-methyl groups are attached to the more or less basic amino group.

Comparison of the spectra of the two methyllysines and lysine in trifluoracetic acid solution suggested that the α-$\overset{+}{N}H_x$ resonances occurred at lower field than the ε-$\overset{+}{N}H_x$ resonances. Methylation caused a small downfield shift of these resonances. Similar shifts were observed to accompany the methylation of other α-$\overset{+}{N}H_x$ groups (see p. 132). The data for the -$\overset{+}{N}H_x$ groups of the lysines suggested that this downfield shift cannot be simply attributed to a change in basicity of the amino group.

Table IV. 316

Nuclear magnetic resonance spectra of lysine, ε -N-methyllysine and α-N, ε-N-dimethyllysine

	$\overset{+}{N}H_x$		-CH		$-CH_2$		$N-CH_3$		$-(CH_2)_3-$	
	shift	integral	shift	integral	shift	integral	shift	integral	shift	integral
H-Lys-OH.HCl[b]	2.37bs) 3.00bs)	6.1	5.50m	1.0	6.60m	2.2	-	-	1.3-2.7cplx	6.0
H-Lys-OH.HCl[c]	g		6.22t	1.0	6.93t	1.9	-	-	1.0-2.7cplx	6.0
H-Lys(Me)-OH.HCl[b]	2.32bs) 2.75bs)	4.2	5.47m	1.0	6.58m	5.0[e]	6.98t	e	1.3-2.7cplx	5.3
H-Lys(Me)-OH.HCl[c]	g		6.22t	1.0	6.73t	2.2	7.27s	3.0	1.2-2.3cplx	5.7
H-Lys(Me)-O^-Na^+ [d]	g		6.78t	1.1	7.50t	5.0[e]	7.73s	e	1.43m	6.4
MeLys(Me)-OH.HCl[b]	2.07bs) 2.70bs)	3.4	5.68m	1.0	6.83m	8.0[e]	6.87q	e	1.5-2.7cplx	5.9
MeLys(Me)-OH.HCl[c]	g		6.38t	1.2	6.92t	8.0[e]	7.28s	e	1.2-2.3cplx	6.0
MeLys(Me)-O^-Na^+ [d]	g		7.08t	f	7.50	9.0[f]	7.73s 7.78s	f	1.42m	5.8

a. Chemical shifts given in τ units; abbreviations; s(singlet), t (triplet), q (quartet), m (multiplet), b (broad).

b. Spectrum run in trifluoracetic acid solution.

c. Spectrum run in D_2O solution.

d. Prepared by treating a solution of the monohydrochloride in D_2O with the theoretical amount of NaOD.

e. Separate integration of $-CH_2$ and $N-CH_3$ groups was impossible.

f. Separate integration of -CH, $-CH_2$ and $N-CH_3$ groups was impossible.

g. Not observed because of hydrogen-deuterium exchange.

The integral of the spectrum of α-N,ε -N-dimethyl-lysine was good except for the $-\overset{+}{N}H_2$ region where it was too small. This may be a characteristic of N-methylamino acids (see p.132).

IV. 4. SOME EXPERIMENTS ON THE METHYLATION OF PEPTIDES

Recently there has been much interest in the permethylation of peptides. This has originated from studies by Lederer and his collaborators on the determination of the amino acid sequence of oligopeptides by mass spectrometry [Lederer and Das (1966), Lederer (1968)]. Peptides, even when fully protected, are not very volatile and as a consequence it is often impossible to observe their mass spectra. When mass spectra can be obtained they are invariably complicated and it is quite difficult to conclude anything about the sequence of the peptide. Van Heijenoort (1966) recognised that the low volatility of peptides was due to the presence of -CO-NH- groups which may be involved in intermolecular hydrogen bonds. Peptides which contain few or no peptide hydrogens, e.g. fortuitine [Barber et al (1965)] and H-Ile-Pro-MeGly-MeVal-OMe [Lederer (1968)] are volatile enough to give good mass spectra, which are relatively uncomplicated, and the sequence may be deduced simply. This led Das, Gero and Lederer (1967) to permethylate peptides using methyl iodide/silver oxide/DMF; this method had been used earlier by Kuhn et al (1957) for the permethylation of oligosaccharides. The resulting permethylated peptides were sufficiently volatile to give satisfactory mass spectra at quite low temperatures (150°C). The fragmentation pattern was much simpler than for the corresponding non-methylated derivatives and consisted almost exclusively of sequence determining peaks [Thomas et al (1968a)]. This was probably due to the decrease of pyrolytic reactions as a result of the lower temperatures at which the spectra could be obtained [Lederer (1968)].

Our success with the permethylation of polyleucine had led us to propose the use of our methylation procedure for the preparation of permethylated peptides for mass spectrometry [Coggins and Benoiton (1968b)]. Our method gave a shorter reaction time and the work-up was more convenient than in the Das-Gero-Lederer method.

Improved permethylation techniques were subsequently published by Vilkas and Lederer (1968) and Thomas et al (1968a). We showed in Part IV. 1(a) (Table IV. 107) that our methylation procedure gave 'cleaner' and more efficient methylation of model compounds (Ac-Leu-OH and Cbz-Leu-OH) than both of these methods. It was therefore of interest to find out whether our method could be used for the efficient permethylation of small peptides. We also wished to find out whether it was possible to methylate selectively one of the amino acids in a dipeptide. This might prove to be a useful and novel way of preparing dipeptides containing an N-methylamino acid.

The first experiments were carried out with leucylglycine and glycylleucine. These peptides were acetylated with hot acetic anhydride and acetic acid [Greenstein and Winitz (1961), p. 834] and then esterified with diazomethane. Acetylleucylglycine methyl ester obtained in this way was methylated with 40 equiv of methyl iodide and 2 equiv of sodium hydride per equiv of -NH- groups. The i. r. spectrum of the product (Fig. IV. 401) showed that complete methylation of the -NH- groups had occurred; the amide II band (due to -CO-NH- groups) and the -NH- stretch band had disappeared. In a second experiment the product was hydrolysed with 6 N HCl at 110°C for 72 h. Subsequent amino acid analysis confirmed that extensive

Infrared spectra of acetylleucylglycine methyl ester and acetyl-N-methylleucylsarcosine methyl ester

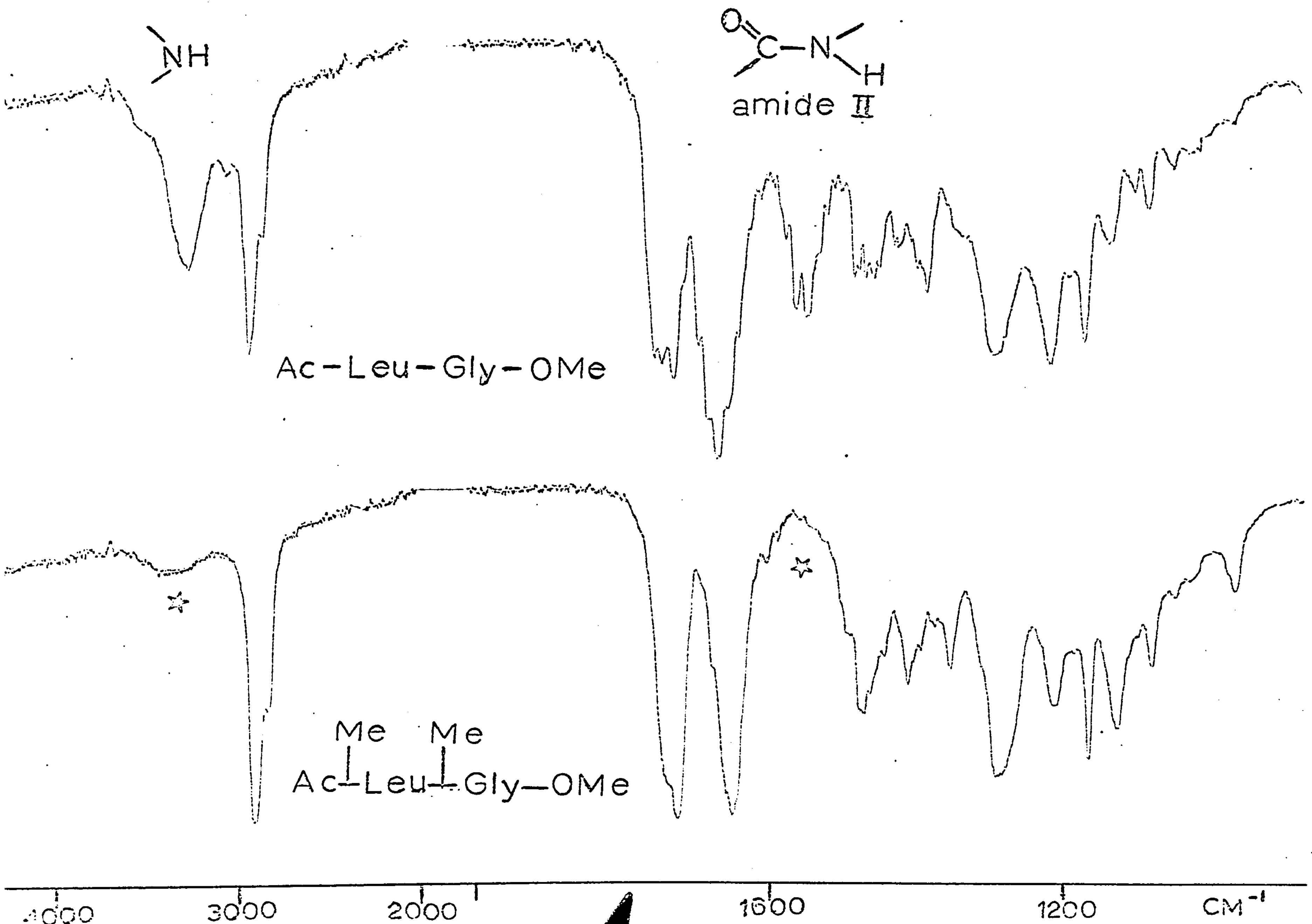


Table IV. 401

Methylation of N-protected dipeptide methyl esters[a]

R-X-Y-OMe	MeI:NaH:peptide NH (equiv)	Yields of amino acids[b] (%)				Starting material accounted for (%)	
		MeGly	Gly	MeLeu	Leu	-Gly-	-Leu-
Ac-Leu-Gly-OMe[c]	40:2:1	72	4	78	8	76	86
Ac-Gly-Leu-OMe[c]	10:1.5:1	83	2	91	3	85	94
Cbz-Leu-Gly-OMe[d]	8:2:1	20	3	30	1	23	31
Cbz-Gly-Leu-OMe[d]	8:2:1	42	3	38	1	45	39
Cbz-Gly-Leu-OMe[d]		0	107	0	101	107	101
Trt-Leu-Ala-OMe[e]	8:3:1	79[f]	7[g]	0	90	86[g]	90

a R-X-Y-OMe in THF (10 ml) and DMF (1 ml) heated with MeI and NaH at 70 - 80°C for 24 h.

b Determined with the amino acid analyser after hydrolysis of the product with 6 N HCl in an evacuated, sealed tube at 110°C for 72 h.

c Prepared from X-Y (0.25 mmol) by treatment with (1) hot HOAc/Ac_2O and (2) CH_2N_2; yield assumed to be quantitative.

d 0.5 mmol R-X-Y-OMe.

e 0.2 mmol Trt-Leu-Ala-OMe.

f MeAla.

g Ala.

methylation had occurred (Table IV. 401). In a similar experiment with acetylglycylleucine methyl ester, in which methylation was carried out with 10 equiv of methyl iodide and 1.5 equiv of sodium hydride per equiv of -NH- groups, the extent of methylation was even greater (Table IV. 401). The amount of starting material accounted for in both cases was high (76 - 94%) although not quite quantitative. In both cases the recovery of leucine was better than the recovery of glycine. In the light of later results we believe that this may have been due to a side reaction in which some of the glycine was converted to N-methylalanine. The normal flow rate method for analysing N-methylamino acids was used in the experiments and under these conditions N-methylalanine is not separated from sarcosine. N-methylalanine has a poor colour yield compared to sarcosine ('constants' are 0.3 and 3.2 respectively) and so would not compensate for the 'missing' sarcosine.

Some experiments were also carried out with carbobenzoxyglycylleucine methyl ester and carbobenzoxyleucylglycine methyl ester; 8 equiv of methyl iodide and 3 equiv of sodium hydride were used per equiv of -NH- groups. I.r. spectroscopy established that all the -NH- groups had been methylated. The products were hydrolysed with 6 N HCl in the usual manner and the final solution analysed using the normal buffer flow rate method. The results of two typical experiments are given in Table IV. 401. These confirmed that methylation had occurred but the amount of starting material accounted for was less than 50%. Each of the experiments was repeated twice and similar results were obtained. Side reactions have not been observed with carbobenzoxyleucine nor with polyleucine and so the poor recovery of leucine cannot be attributed to this. A control

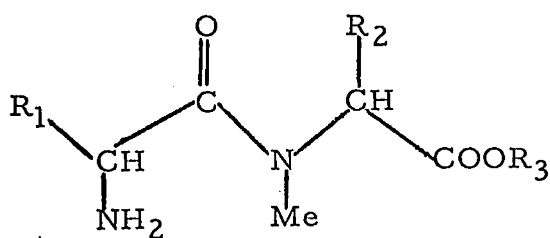
experiment on the hydrolysis of carbobenzoxyglycylleucine methyl ester showed that the hydrolytic method was efficient in this case and that the compound, which had been used as one of the starting materials, was good. Another experiment established that the destruction of sarcosine and N-methylleucine on treatment with 6 N HCl for 96 h at 110°C under the same conditions as for peptide hydrolyses was 0.4% and 0.8% respectively. These results led us to consider two alternative explanations for the poor recoveries of starting material. These were diketopiperazine formation and the resistance to hydrolysis of methylated peptide bonds.

Peptides containing N-methylamino acids show a marked tendency to cyclise [Rudinger (1963)]. This we believe is because the methylated peptide bond exists partially in the cis form. Recently, Bystrov et al (1969) using n.m.r. spectroscopy have shown that acetyl-D-alanyl-N-methyl-D-alanine methyl ester exists 20% in the cis form in aqueous and chloroform solutions at room temperature. Our own studies on acetyl-N-methylamino acids (see Part IV. 3) and those of Goodman and Fried (1967) on acetyl-N-methylalanine methyl ester have shown that these compounds, which contain a 'model' methylated peptide bond, also exist as mixtures of cis and trans forms. The presence of some of the molecules in the cis form in dipeptides will undoubtedly favour cyclisation, as is illustrated in Fig. IV. 402. It is well known that diketopiperazines can be formed in acid solutions [Greenstein and Winitz (1961) chapter 16].[a] Also it seems very likely on steric grounds that methylated diketopiperazines would be highly resistant to acid hydrolysis. The first steps in the acid hydrolysis of the permethylated carbobenzoxy-dipeptide esters would be the rapid removal of the acid

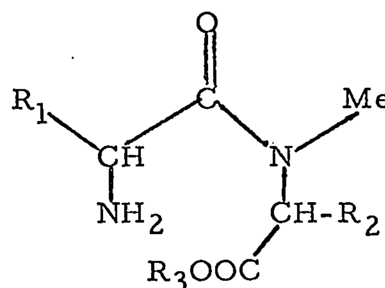
[a] In studies on the acid hydrolysis of sarcosylsarcosine, Levene et al (1924) obtained anomalous results which they also attributed to diketopiperazine formation.

Fig. IV. 402

Mechanism for the cyclisation of dipeptides containing methylated peptide bonds

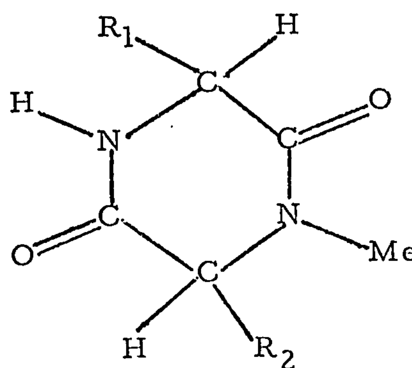


trans



cis

cyclises readily



diketopiperazine

labile carbobenzoxy group followed by hydrolysis of the ester (these two steps could occur simultaneously). Cyclisation of the free dipeptide ester may then occur as readily as hydrolysis of the peptide bond. No hydrolytic problems were encountered with the permethylated acetyl-dipeptide esters. This was probably because the acetyl-N-methyl group and the methylated peptide bond were cleaved at approximately equal rates and so the amount of free dipeptide present during the reaction was less than in the experiments with the carbobenzoxy-dipeptide esters.

Steric factors undoubtedly play a role in hindering the acid hydrolysis of peptides, e.g. dipeptides like valylvaline, in which both amino acids have large branched side chains, are notoriously difficult to hydrolyse [Greenstein and Winitz (1961) p. 1603]. No detailed studies have been undertaken on the rates of hydrolysis of peptides containing methylated peptide bonds. Our own studies on acetyl-N-methylleucine and acetylsarcosine have shown that these compounds are more difficult to hydrolyse with acid than acetylleucine and acetylglycine respectively. Steric factors also play an important role in determining the rates of hydrolysis of amides, especially tertiary amides [see e.g. Gould (1959) p. 328]. Permethylated dipeptides would therefore be expected to be more resistant to acid hydrolysis than the corresponding non-methylated dipeptides. However, this does not explain why there was such a difference in the extent of hydrolysis of the permethylated carbobenzoxy-dipeptide esters and the permethylated acetyl-dipeptide esters. For this reason we favour the explanation involving diketopiperazine formation. The arguments presented do not constitute a proof, but rather a working hypothesis. Further experiments are necessary to prove or disprove this theory.

We concluded from the acetyl-dipeptide ester experiments that our method gave good yields of permethylated product. The poor results with the carbobenzoxy-dipeptide esters were attributed to incomplete hydrolysis. The i.r. spectra of the permethylated products in these cases suggested that efficient methylation had occurred.

Since it is relatively difficult to couple N-methylamino acids [Schroder and Lubke (1965) p. 144] and because side reactions such as diketopiperazine formation frequently compete effectively with dipeptide formation [Rudinger (1963)], we decided to try and prepare a dipeptide with a methylated peptide bond by selective methylation of a suitably protected dipeptide.

N-Tritylleucylalanine methyl ester was methylated using 8 equiv of methyl iodide and 3 equiv of sodium hydride per equiv of -NH- groups. Hydrolysis of the product followed by amino acid analysis showed that the trityl group afforded complete protection of the leucine against methylation; on the other hand, the alanine was almost completely methylated. We concluded that it would be possible to prepare peptides of the type H-X-MeY-OMe by methylation of Trt-X-Y-OMe followed by removal of the trityl group. This could have very important synthetic applications. The amount of starting material accounted for in this experiment was satisfactory; there were no problems at the hydrolytic step. This observation cannot be reconciled with the incomplete hydrolysis observed for the carbobenzoxy-dipeptide esters.

After these experiments had been carried out we learned that Lederer's group [Lederer (1968)] had begun to use our method [Coggins and Benoiton (1968a)] for the preparation of permethylated peptides for mass spectrometry.

The first successful application was in studies on an octadecapeptide obtained from a tryptic hydrolysate of pig immunoglobulin λ-chains [Franek et al (1969)]. Permethylation followed by mass spectrometry enabled the sequence of the first 10 amino acids to be determined. The position of a glutamine residue was definitely established and the authors point out that the permethylation followed by mass spectrometry method of sequencing permits acidic amino acids to be readily distinguished from their amides. This may prove to be a very important factor in protein sequence studies, since the 'wet' methods have more often than not proved to be unreliable for distinguishing these residues, e. g. chymotrypsin [Blow, Birktoft and Hartley (1969)], trypsin [Smith and Shaw (1969)], and papain [Lowe (1970)].

In a second paper the partial sequences of a heptapeptide from the zymogen of phospholipase A and a docosapeptide from the λ-chain of pig immunoglobulin were determined by permethylation followed by mass spectrometry. In both cases the N-terminal amino acid was pyroglutamic acid; this had proved difficult to establish by conventional methods. Whenever the N-terminal amino acid of a peptide is blocked, sequential degradation by the normal chemical methods is impossible. The mass spectrometric method shouldprove to be very useful for sequence studies on N-protected peptides of both natural and synthetic origin [de Haas et al (1969)].

In the most recent paper from Lederer's laboratory [Thomas (1969)] the author states: "The Coggins-Benoiton procedure was adopted in our laboratory as the preferential method for N-permethylation, and was found to be useful for most of our problems. However, with these reaction conditions, we encountered C-methylation of some amino acid residues under certain circumstances, particularly when working with sub-milligram peptide quantities". The side reactions referred to were obtained with glycine and aspartic acid and occasionally glutamic acid. Glycyl residues should have been converted to sarcosyl residues with one extra methyl group; instead two and sometimes three extra methyl groups were introduced. Aspartic acid residues should have been converted to N-methyl-aspartyl β-methyl ester residues with two extra methyl groups, but often a third methyl group was introduced. A similar result was observed with glutamic acid.

Since by this time we had developed a more sensitive technique for analysing N-methylamino acids and more specifically since we now had conditions for the separation of sarcosine and N-methylalanine it was decided to repeat the earlier peptide methylation experiments to find out whether any side products could be observed with glycine containing peptides. A re-investigation of the methylation of carbobenzoxyglycine (see p. 50) had already shown that when a reactant ratio of 8:3:1 was used a small amount of N-methylalanine (1%) could be detected after deprotection. No side reaction was observed in the absence of DMF but with DMF/THF (1/1, v/v) the amount of C-methylation was slightly greater than with the normal DMF/THF ratio (i.e. 1/10, v/v).

The repeated peptide methylation experiments were carried out with acetylleucylglycine methyl ester and carbobenzoxyglycylglycylleucine methyl ester. The results (Table IV. 402) showed that some C-methylation of glycine did occur. The extent of the side reaction could not be determined precisely since in both cases the starting material was not quantitatively accounted for. After hydrolysis of permethylated acetylleucylglycine methyl ester the amount of sarcosine liberated was approximately twice as great as the amount of N-methylalanine. With permethylated carbobenzoxyglycylglycylleucine the ratio of sarcosine to N-methylalanine liberated was 5:1.

These results established that one of Thomas's artifacts was N-methylalanine. The nature of the other artifact is not known. It is hard to believe that it is N-methyl, α-C-methylalanine since Thomas has reported no side reactions with alanine containing peptides.

In both of our experiments the amount of starting material accounted for was poor. The results of one and four day hydrolyses were very similar, which showed that it is not simply a time factor which accounts for the poor hydrolyses. The permethylated carbobenzoxy-tripeptide ester gave the worst result; this is again attributed to diketopiperazine formation, this time from methylated-dipeptides formed as intermediates in the hydrolysis. The amount of starting material accounted for in the acetyl-dipeptide ester experiment varied from 39 - 60% compared with 76 - 86% for the same dipeptide in the earlier experiments. The reason for this inconsistency is not known. It appears that the hydrolytic step was less efficient in this later case.

Table IV. 402

Methylation of N-protected-peptide methyl esters [a]

Peptide	MeI:NaH: peptide NH (equiv)	Hydrolysis time (h)	Yields of amino acids[b] (%)					Starting material accounted for (%)	
			MeGly	MeAla	Gly	MeLeu	Leu	-Gly-	-Leu-
Ac-Leu-Gly-OMe	8:3:1	24	26	13	0.2	57	0.6	39	58
Ac-Leu-Gly-OMe	8:3:1	96	26	14	0.2	59	0.7	40	60
Ac-Leu-Gly-OMe	c	48	0	0	108	0	105	108	105
Cbz-Gly-Gly-Leu-OMe	8:3:1	24	21	4	0.2	45	0.4	25	45
Cbz-Gly-Gly-Leu-OMe	8:3:1	96	22	4	0.2	48	0.3	26	48
Cbz-Gly-Gly-Leu-OMe	c	72	0	0	114	0	111	114	111

a N-protected-peptide methyl ester (0.4 mmol) in THF (10 ml) and DMF (1 ml) heated with MeI and NaH at 70 - 80°C for 24 h.

b Determined with the amino acid analyser after hydrolysis with 6 N HCl in an evacuated, sealed tube at 110°C.

c Control hydrolysis of starting material.

The fact that the ratio of sarcosine to N-methylalanine in the carbobenzoxydipeptide experiment was much greater than in the acetyl dipeptide experiment requires comment. Presumably most of the sarcosine liberated comes from the carbobenzoxyglycine part of the molecule since on steric grounds one would expect that the sarcosyl sarcosine peptide bond would be the one most susceptible to hydrolysis. Once the N-terminal sarcosine had been cleaved diketopiperazine formation would occur readily. It appears that the side reaction involving N-methylalanine formation occurs less readily at the carbobenzoxyglycyl group (α-C-methylation of Cbz-Gly-OH occurs only to the extent of 1% under these conditions) than at the leucylglycyl and the glycylglycyl groups, i. e. glycine involved in peptide links is more susceptible to α-C-methylation than glycine in carbobenzoxyglycine.

The inertness of the α-CH group of amino acids towards methyl iodide/sodium hydride is clearly established by our preparative results and by the mass spectral work of Lederer and his collaborators. The fact that optically pure N-methyl-L-leucine (see p. 56) can be obtained after methylation using these reagents demonstrates that the tendency of the α-CH to ionise is negligible. Therefore the occurrence of α-C-methylation with glycine must mean that its α-CH_2 group is much more reactive than the α-CH groups of the other amino acids. One would expect on electronic grounds that carbanion formation would occur more readily with glycine, since there is no inductive effect from the side chain to oppose it; however,

it is hard to believe that this is the entire explanation. Steric factors may also be involved as the α-CH_2 group in glycine is very much more exposed to reagents than the α-CH group of the other amino acids.

We have not carried out any studies on aspartic acid containing peptides; however, methylation of carbobenzoxyaspartic acid followed by removal of the carbobenzoxy group gave N-methyl-aspartic acid dimethyl ester hydrobromide (which could not be crystallised). The n.m.r. spectrum did not reveal any C-methyl groups. Further studies will be necessary on aspartic acid containing peptides to reveal the exact nature of the artifact. The most likely side reaction is alkylation of the β-C atom. We favour this reaction because it would explain why artifacts are observed with both aspartic and glutamic acids (the side reaction would be at the γ-C in the latter case). There is no reason why α-C-methylation should occur in this case when it has not been observed with any other amino acid except glycine. One would expect the two protons on the β-carbon atom to be moderately labile, especially after the carboxyl group is esterified during permethylation.

It is worth reflecting on why Thomas observed extensive side reactions with glycyl and aspartyl residues while in our preparative work with carbobenzoxyglycine, benzoylglycine and carbobenzoxyaspartic acid we experienced no problems of this kind. The major reason must be the large excesses of sodium hydride Thomas used in the peptide permethylations [approximately 1000 μmol of NaH for 1 - 10 mg (4 - 40 μmol at most) of peptide]. Also he added the sodium

hydride before the methyl iodide, a procedure which we have shown leads to side reactions (Table IV. 103, p. 45 A). The use of neat DMF (which is necessary to dissolve most large peptides) may also increase the C-methylation reactions (Table IV. 110, p. 50 A). Moderation of the reaction conditions should reduce or even eliminate the side reactions although in all fairness it should be pointed out that scaling down the reaction to the sub-milligram scale presents special problems, e.g. it is difficult to handle small amounts of sodium hydride.

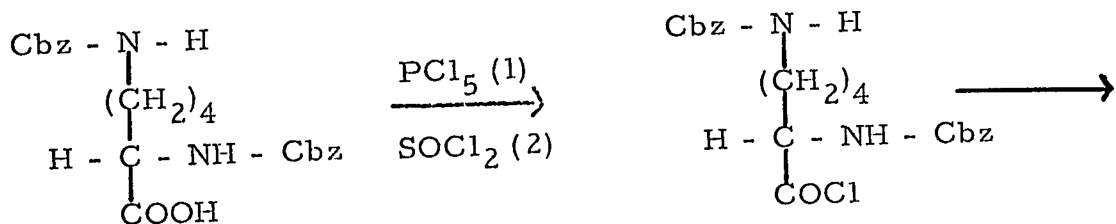
Fortunately the side reactions do not seriously interfere with the usefulness of methyl iodide/sodium hydride/DMF for preparing permethylated peptides for mass spectrometry. Thomas (1969) points out that the use of deuteromethyl iodide instead of methyl iodide in the appropriate cases allows the occurrence of artifacts to be diagnosed. In this way the possibility of mistaking a glycyl residue for an alanyl residue or an aspartyl residue for a glutamyl residue can be eliminated.

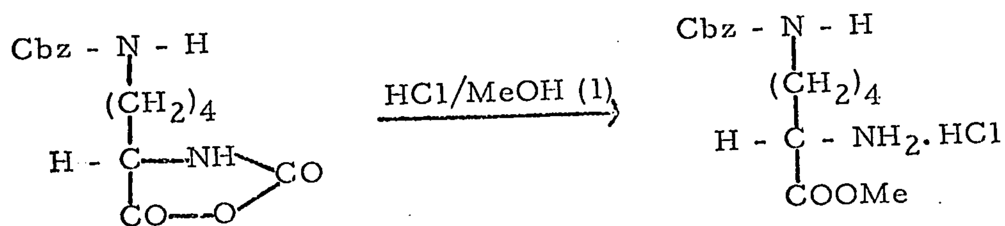
The alternative methods of permethylating peptides also have disadvantages. The methyl iodide/silver oxide/DMF method [Das et al (1967), Thomas et al (1968a)] give some C-methylation artifacts [Thomas (1969)]. Both the 'Hakomori' method [Vilkas and Lederer (1968)] and the method of Kenner's group [Agarwal et al (1969)] (in which a base is generated from N,N-dimethylacetamide (DMAC) and sodium hydride) require the prior preparation of a reagent. The 'Hakomori' method leads to some destruction of the permethylated peptide if the reaction time is too long [Thomas (1969)]. No artifacts have yet been reported for the NaH/DMAC method; however, there is only one report of its use in the literature [Agarwal et al (1969)]. At the present time, artifacts notwithstanding, the 'Coggins-Benoiton method' seems to be the most convenient one for peptide permethylation

IV. 5. ESTERIFICATION OF MONO- ε -N-PROTECTED-LYSINES

(a) A study of the reaction between alcohol-boron trifluoride etherate mixtures and ε -N-substituted-lysines

For studies on the synthesis of ε -N-alkyl-L-lysines large quantities of ε -N-carbobenzoxy-L-lysine methyl ester hydrochloride were required. Two routes to this compound are described in the literature. The first, devised by Bergmann et al (1935) and later used in a modified form by Boissonnas et al (1958), starts from α-N, ε-N-dicarbobenzoxy-L-lysine:





(1) Bergmann et al (1935)

(2) Boissonnas et al (1958)

The second involves the direct esterification of ε-N-carbobenzoxy-L-lysine using thionyl chloride and methanol [Shiba and Kaneko (1960)]. The product obtained by the second route had a low m.p. and was of doubtful purity. An attempt was therefore made to prepare ε-N-carbobenzoxy-L-lysine methyl ester hydrochloride by the method of Bergmann et al (1935). However, neither the starting material, α-N, ε-N-dicarbobenzoxy-L-lysine, nor the intermediate, ε-N-carbobenzoxy-L-lysine N-carboxyanhydride, could be crystallised. The final product proved to be quite difficult to crystallise; eventually crystalline material m.p. 70 - 80°C, lit. m.p. 117°C [Bergmann et al (1935)] was obtained in 55% yield. After three recrystallisations from acetone the m.p. had increased to 111 - 113°C, still 4° below the lit. value, but the yield had fallen to 10%. T.l.c., using the B4/A1/W1 solvent system, revealed that the contaminating material which was being steadily, although not completely, removed by recrystallisation was ε-N-carbobenzoxy-lysine.

Dissatisfaction with this method of preparing ε-N-carbobenzoxy-L-lysine methyl ester hydrochloride prompted us to investigate the feasibility of obtaining the ester directly from ε-N-carbobenzoxy-L-lysine, which is commercially available. The traditional HCl method [Greenstein and Winitz (1961), p. 925] is not applicable because of the lability of the carbobenzoxy group to methanolic HCl [Barkdoll and Ross (1944)]. The thionyl chloride method [Brenner and Huber (1953)], as already mentioned, gave a crude product. It was therefore decided to try two other methods of preparing the required ester: firstly, refluxing methanol in the presence of boron

trifluoride etherate and secondly 2, 2-dimethoxypropane in the presence of catalytic amounts of concentrated HCl. The former method has been successfully used for the synthesis of aliphatic [Hinton and Nieuwland (1932)] and aromatic [Hallas,(1965)] carboxylic acid methyl esters while the latter method has been used to prepare aliphatic carboxylic acid methyl esters [Lorette and Brown (1959)] and amino acid methyl esters [Rachele (1963) and Oelofsen and Li (1968)]. This part of the thesis is concerned with the boron trifluoride-methanol method and the dimethoxypropane method will be described in Part IV. 5(c).

In a preliminary experiment ε-N-carbobenzoxylysine was stirred in methanol with 2 equiv of boron trifluoride etherate for 16 h at 25°C; after a suitable work-up 89% of the starting material was recovered. Since esterification clearly did not occur at 25°C, refluxing conditions were tried; the results of a series of experiments with different amounts of boron trifluoride etherate are given in Table IV. 501. The yields of the various products were determined using the amino acid analyser. Unchanged starting material and free lysine, obtained by cleavage of the carbobenzoxy group during the reaction, were determined on the short column of the analyser after dilution with pH 2. 2 diluter buffer; then the two possible ester products ε-N-carbobenzoxylysine methyl ester and lysine methyl ester were simultaneously saponified and eluted from the column with 0.1 N NaOH (the reagent normally used for column regeneration). The eluted material was collected and after acidification and dilution an aliquot was analysed again on the short column;

Table IV. 501

The effect of the amount of boron trifluoride etherate on the boron trifluoride catalysed methyl esterification of ε-N-carbobenzoxylysine[a]

$BF_3.Et_2O$: H-Lys(Cbz)-OH[b]	Reaction time (h)	Yields of products[c] (%)				Total starting material accounted for (%)
		H-Lys(Cbz)-OMe	H-Lys-OMe	H-Lys(Cbz)-OH	H-Lys-OH	
1:1	16	9.6	0.0	87.8	0.1	98
2:1	16	41.8	1.0	60.2	1.1	104
5:1	16	77.0	3.8	14.0	0.7	96
2:1	32	81.0	6.0	12.9	1.0	101
5:1	32	95.0	4.8	4.6	0.3	105

a H-Lys(Cbz)-OH, $BF_3.Et_2O$ and MeOH (25 ml) heated at 70 - 80° under reflux.

b 2.5 mmol of H-Lys(Cbz)-OH.

c Determined with the amino acid analyser (full details in the text).

the amounts of ε-N-carbobenzoxylysine and lysine found corresponded to the amounts of ε-N-carbobenzoxylysine methyl ester and lysine methyl ester in the original sample. The experiments showed that with a reaction time of 16 h the extent of the esterification reaction varied from 10% to 81% as the amount of boron trifluoride was increased from 1 to 5 equiv. Two experiments in which 2 equiv of boron trifluoride were used demonstrated that the extent of the esterification reaction was also strongly time dependent; doubling the reaction time from 16 to 32 h led to approximately double the amount of esterification. The extent of carbobenzoxy group cleavage also varied with the amount of boron trifluoride; for a reaction time of 16 h it increased from 0.1% to 4.5% as the amount of boron trifluoride was increased from 1 to 5 equiv. Doubling the reaction time also led to an increase in the amount of deprotection but for the two experiments with 5 equiv of boron trifluoride the effect was quite small (a change from 4.5 to 5.1% deprotection). A more detailed investigation of the effect of reaction time on the esterification reaction was made. The results, given in Table IV. 502, are illustrated in Fig. IV. 501. In the presence of 5 equiv of boron trifluoride, the extent of the esterification reaction reaches a plateau at approximately 24 h. There is about 6% deprotection under these conditions, and the reaction is first order with respect to ε-N-carbobenzoxylysine.

The possibility of obtaining esterification with alcohols other than methanol was investigated. Experiments were carried out with ethyl, *n*-propyl, *n*-butyl, benzyl, *iso*-propyl and *t*-butyl alcohols and the results are shown in Table IV. 503. The products of

Table IV. 502

The effect of reaction time on the boron trifluoride catalysed methyl esterification of ε-N-carbobenzoxylysine [a]

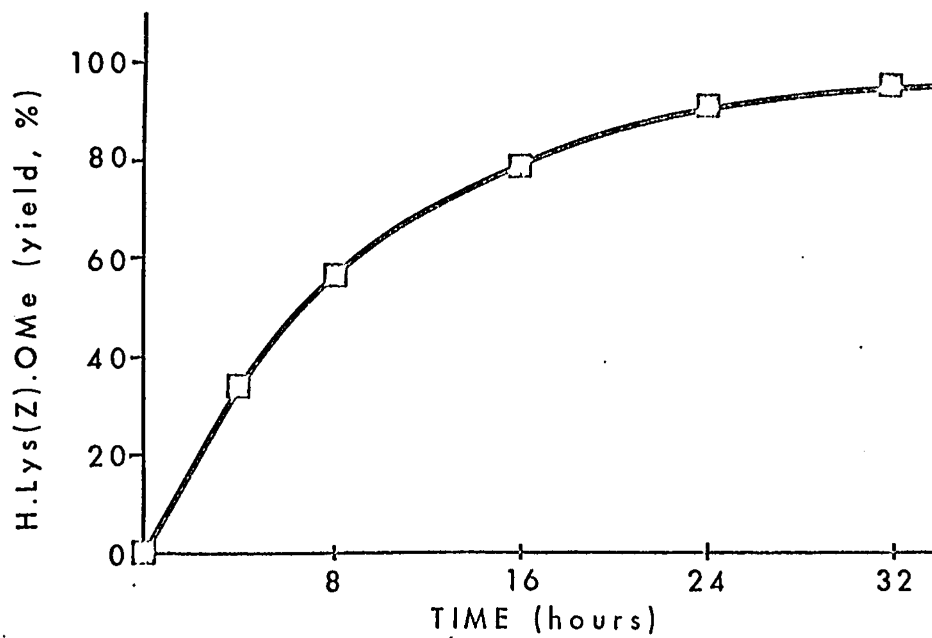
Reaction time (h)	Yields of products[b] (%)				Total starting material accounted for (%)
	H-Lys(Cbz)-OMe	H-Lys-OMe	H-Lys(Cbz)-OH	H-Lys-OH	
4	34.2	0.0	68.4	0.5	103
8	56.5	1.1	41.4	0.8	100
16	77.0	3.8	14.0	0.7	96
24	92.0	5.8	4.8	0.4	103
32	95.0	4.8	4.6	0.3	105

a H-Lys(Cbz)-OH (2.5 mmol), $BF_3.Et_2O$ (12.5 mmol) and methanol (25 ml) heated at 70 - 80°C under reflux.

b Determined with the amino acid analyser (full details in the text).

Fig. IV. 501

Time course of the boron trifluoride catalysed methyl esterification of ε -N-carbobenzoxylysine



the reactions were analysed in essentially the same manner as in the experiments on methyl esterification except the saponification conditions were more vigorous and the t-butyl esters were hydrolysed with acid (in this latter case ε-N-benzoyllysine was used as the starting material to avoid the posssibility of any cleavage of the ε-N-protecting group during the ester hydrolysis). The yields of esters of ε-N-carbobenzoxylysine decreased from 92 to 32% in going from the methyl to n-butyl and then benzyl esters. This was accompanied by a parallel increase in the extent of cleavage of the carbobenzoxy group from about 6 to 40%. Since the same amount of starting material (5 - 15%) remained after the reaction regardless of the primary alcohol used, except for ethyl alcohol, and the extent of esterification was over 70% in all cases except for the benzyl esters, the yields obtained reflect a difference in the susceptibility to cleavage of the urethane group by the various boron trifluoride-alcohol mixtures rather than a difference in the reactivity of the alcohols. The apparently anomalous result with ethyl alcohol, when more starting material was recovered than in the other cases, was confirmed by a duplicate experiment. The lack of esterification observed when iso-propyl and t-butyl alcohols were used was not very surprising since it has been shown [Mooney and Qaseem (1967)] that boron trifluoride etherate reacts with these alcohols to give the water-boron trifluoride complex and a polyolefin.

Table IV. 503

Boron trifluoride catalysed esterification of ε -N-carbobenzoxylysine[a]

ROH	Yields of products[b] (%)				Total starting material accounted for (%)	Total esteri-fication	Total de-protection
	H-Lys(Cbz)-OR	H-Lys-OR	H-Lys(Cbz)-OH	H-Lys-OH			
MeOH	92.0	5.8	4.8	0.4	103	98	6
EtOH	66.5	5.3	28.8	2.4	103	72	8
n-PrOH	67.5	15.0	13.9	3.2	100	83	18
n-BuOH	48.4	27.0	6.7	5.9	88	75	33
BzlOH	32.3	16.0	15.7	24.6	89	48	41
iso-PrOH	12.7	1.2	81.8	6.8	103	14	8
t-BuOH[c]	5.9[d]	0.0	83.9[c]	0.2	90	6	0.2

a H-Lys(Cbz)-OH (2.5 mmol), $BF_3 \cdot Et_2O$ (12.5 mmol) and the alcohol (25 ml) heated at 70 - 80°C under reflux for 24 h.

b Determined with the amino acid analyser (full details in the text).

c H-Lys(Bz)-OH was used instead of H-Lys(Cbz)-OH.

d H-Lys(Bz)-OR.

e H-Lys(Bz)-OH.

The methyl esterification of some other ε-N-substituted-lysines was also investigated. Under conditions which caused 6% cleavage of the ε-N-carbobenzoxy group the ε-N-formyl group was completely cleaved and the ε-N-acetyl group was 12% cleaved (see Table IV. 504). Attempted esterification of ε-N-benzoyllysine with t-butyl alcohol caused only 6.2% cleavage of the benzoyl group. This resistance to cleavage of the N-acetyl, N-benzoyl and N-carbobenzoxy groups stands in striking contrast to the reported cleavage of amides to methyl esters by hot boron trifluoride-methanol [Hamilton and Price (1969) and Toole and Sowa (1937)]. It was demonstrated that the difference was not due to the use of boron trifluoride etherate instead of gaseous boron trifluoride by repeating the esterification of ε-N-carbobenzoxylysine with a methanolic solution of boron trifluoride gas; a result identical with that obtained in Table IV. 503 was obtained.

In all of these esterification experiments the alcohols were predried over $MgSO_4$ and $CaSO_4$ before use and the reflux condensers were fitted with drying tubes. To establish whether the complete exclusion of water was essential the methyl esterification of ε-N-carbobenzoxylysine was repeated with 0.1% and 1.0% of added water. The results which are given in Table IV. 505 showed that the addition of water had no significant effect on the extent of the reaction and the amount of deprotection which occurred.

Table IV. 504

Boron trifluoride catalysed methyl esterification of ε-N-substituted-lysines[a]

ε-N-substituted-lysine	Yields of products[b] (%)				Total starting material accounted for (%)	Total de-protection (%)
	H-Lys(R)-OMe	H-Lys-OMe	H-Lys(R)-OH	H-Lys-OH		
H-Lys(Cbz)-OH	92.0	5.8	4.8	0.4	103	6
H-Lys(For)-OH	0.0	93.3	0.0	1.6	96	95
H-Lys(Ac)-OH	76.3	9.1	12.4	2.7	99	12
H-Lys(Bz)-OH[c]	12.7[c]	0.0[c]	83.9	0.2	90	0.2
Cbz-Lys-OH	94.3[d]	1.2	1.4[e]	0.1	97	1

a H-Lys(R)-OH (2.5 mmol), $BF_3.Et_2O$ (12.5 mmol) and MeOH(25 ml) heated at 70 - 80°C under reflux for 24 h.

b Determined with the amino acid analyser (full details in text).

c *t*-BuOH was used instead of MeOH; esters were *t*-butyl esters and not methyl esters.

d Cbz-Lys-OMe

e Cbz-Lys-OH

Table IV. 505

The effect of added water on the boron trifluoride catalysed methyl esterification of ε-N-carbobenzoxylysine[a]

Added water (% of weight of MeOH)	Yields of products[b] (%)				Total starting material accounted for (%)
	H-Lys(Cbz)-OMe	H-Lys-OMe	H-Lys(Cbz)-OH	H-Lys-OH	
0	77.0	3.8	14.0	0.7	96
0.1	84.0	2.0	21.4	0.6	108
1.0	79.4	2.4	19.6	0.7	102

a H-Lys(Cbz)-OMe (2.5 mmol), $BF_3 \cdot Et_2O$ (12.5 mmol), methanol (25 ml) and water heated at 70 - 80°C under reflux for 16 h.

b Determined with the amino acid analyser (full details in the text).

<u>Method for studying the boron trifluoride catalysed esterification of ε-N-substituted lysines</u>: A mixture of the ε-N-substituted-lysine (2.5 mmol) and boron trifluoride etherate (1.52 ml; 12.5 mmol) in the alcohol (25 ml) was heated at 70 - 80°C under reflux for 24 h. The solution was made up to 50 ml with the alcohol (with ethanol for the experiments with butyl and benzyl alcohols) and a sample removed and diluted with pH 2.2 diluter buffer. An aliquot was put on the short column of the amino acid analyser and the column was eluted with pH 5.28 buffer for 40 min for the determination of the lysine and starting material. The esters which remained on the column were then saponified by removing the buffer from the column head and forcing onto the column 1 ml of 0.1 N NaOH. After 20 min at 57°C (the column temperature) the column head was filled with 0.2 N NaOH (8.5 ml), the 5.28 buffer line was attached to the column, buffer was pumped through and the effluent collected for 20 min. The effluent was acidified, made up to a known volume with pH 2.2 diluter buffer and analysed for the lysine and ε-N-substituted-lysine liberated by saponification of the esters. Control experiments had shown that saponification of the methyl esters was complete without the delay period but that it was required for the complete saponification of the other esters. The *t*-butyl esters which are not saponifiable were determined by difference after analysing for the amino acid before and after cleavage of the ester with hydrogen bromide in acetic acid.

(b) The synthesis of esters of ε-N-carbobenzoxy-L-lysine using hot alcohol-boron trifluoride mixtures

Having established that efficient methyl esterification occurred, accompanied by only a small amount of deprotection, when ε-N-carbobenzoxy-L-lysine was refluxed with 5 equiv of boron trifluoride etherate in excess methanol for 24 h, it was now necessary to develop a procedure for isolating the ε-N-carbobenzoxylysine methyl ester in pure form. The crude reaction mixture contained small amounts of lysine, lysine methyl ester and ε-N-carbobenzoxylysine as well as the required product. A simple and efficient purification was achieved by extracting the ε-N-carbobenzoxylysine methyl ester into an organic solvent at a pH (9.0) intermediate between the pK'_a of the α-amino group of the ester [the pK'_1 of lysine ethyl ester is 7.54 (J. E. Purdie, unpublished data)] and the much higher pK'_a of the -amino group of lysine methyl ester. At this pH lysine, lysine methyl ester and ε-N-carbobenzoxylysine remain in the aqueous phase, with most of the latter precipitating out. The pure L-ester obtained in this way was crystallised as the hydrochloride in a final yield of 58%; the m. p. and rotation agreed well with the lit. values and the purity of the product was further confirmed by microanalysis and n. m. r. spectroscopy. Finally amino acid analysis showed that the compound contained 0.1% of starting material but no detectable amount of lysine nor lysine methyl ester.

The ethyl and n-propyl esters of ε-N-carbobenzoxy-L-lysine were also prepared by this method; the yields were much lower but amino acid analysis showed that the products were pure. An attempt to prepare ε-N-carbobenzoxylysine benzyl ester was unsuccessful, which was not very surprising in the light of the result of the analytical experiment reported in Table IV. 501; the organic layer obtained by extraction of the aqueous solution of reaction products at pH 9 was found to contain dibenzyl ether (identified by i.r. and n.m.r. spectroscopy) and not the expected ester nor benzyl alcohol. The ether must be formed by acid catalysed dehydration; the stability of the intermediate benzyl carbonium ion probably promotes this side reaction.

ε-N-Carbobenzoxy-L-lysine methyl ester hydrochloride:

ε-N-Carbobenzoxy-L-lysine (7.0 g; 25 mmol), boron trifluoride etherate (15.2 ml; 125 mmol) and methanol (100 ml) were heated at 70 - 80°C under reflux for 24 h. After cooling the solution was filtered (Celite) and evaporated to give an oil which was dissolved in water (50 ml) and extracted with ether (2 x 25 ml). The aqueous layer was cooled to 0°C and 4 N NaOH added until the pH was 6.2. After standing for 15 min in the cold the solution was filtered to remove any precipitated ε-N-carbobenzoxylysine, saturated with NaCl, the pH adjusted to 9.0 with 4 N NaOH and the product extracted into ethyl acetate (3 x 100 ml). The combined extracts were dried ($MgSO_4$) and the solvent removed to give an oil which was re-evaporated twice with methanol (10 ml). The oil was dissolved in methanol (20 ml) and

8N methanolic HCl (3 ml) added. Then ether (300 ml) was added slowly with cooling and gentle scratching until crystallisation commenced. After standing overnight at $4^{o}C$ filtration gave a white crystalline product (4.9 g; 58%), m.p. 117 - $118^{o}C$; lit. m.p. $117^{o}C$ [Bergmann et al (1935) and Boissonnas et al (1958)], $[\alpha]_D^{25} = +15.5^{o}$ (c = 2, H_2O), $[\alpha]_D^{25} = +13.3$ (c = 2, DMF) ; lit. $[\alpha]_D^{21} = +15.0$ (c = 2, H_2O) [Boissonnas et al (1958)].

Analysis calculated for $C_{15}H_{23}N_2O_4Cl$ (330.83): C 54.5; H 7.0; N 8.5%.
Found : C 54.7; H 7.2; N 8.5%.

Amino acid analysis showed that the product contained no detectable amounts of lysine nor lysine methyl ester but it did contain 0.1% of ε-N-carbobenzoxylysine.

<u>ε-N-Carbobenzoxy-L-lysine ethyl ester hydrochloride</u>:

This compound was prepared on a 20 mmol scale using a procedure similar to that used for the preparation of ε-N-carbobenzoxy-L-lysine methyl ester except ethanol (100 ml) was used instead of methanol. The final product, white needles (4.21 g; 61%), m.p. 103 - $104^{o}C$ was obtained by passing dry HCl gas through an ethereal solution of the free ester. Recrystallisation from ethyl acetate gave 3.79 g (55%), m.p. 112 - $113^{o}C$. Amino acid analysis showed that the product contained no detectable amount of lysine and 0.1% of ε-N-carbobenzoxylysine but no lysine. Waley and Watson (1954) reported a m.p. of 115.5 - $116.5^{o}C$ for H-Lys(Cbz)-OEt.HCl.H_2O. N.m.r. spectroscopy established that the product obtained above was not hydrated.

ε-N-Carbobenzoxy-L-lysine n-propyl ester hydrochloride:* This compound was prepared on a 5 mmol scale using a procedure similar to that used for the preparation of ε -N-carbobenzoxy-L-lysine methyl ester except n-propanol (25 ml) was used instead of methanol. The final product, white needles (0.51 g; 28%), m.p. 117 - 118°C, was obtained by passing dry HCl gas through an ethereal solution of the free ester. Amino acid analysis showed that the product contained no detectable amount of lysine nor ε -N-carbobenzoxylysine. Saponification liberated the theoretical amount of ε -N-carbobenzoxylysine and a trace of lysine too small to measure ($<0.1\%$).

(c) The synthesis of ε -N-carbobenzoxy-L-lysine methyl ester using 2, 2-dimethoxypropane

A short study was also made of the possibility of preparing ε-N-carbobenzoxy-L-lysine methyl ester by a procedure similar to that described by Oelolfsen and Li (1968) for the synthesis of δ-aminovaleric acid methyl ester. Two preliminary experiments were carried out on the 5 mmol scale using 35 ml of 2, 2-dimethoxypropane and 2. 5 ml and 1 ml respectively of concentrated HCl. After stirring overnight at 25°C the reaction mixtures were worked up to give an aqueous solution of products. T. l. c. using the B4/A1/W1 solvent system showed that the desired ester was formed in good yield in the experiment with 2. 5 ml of concentrated HCl but in the experiment with 1 ml of concentrated HCl there was very little esterification (most of the starting material remained unchanged). Some lysine methyl ester was also formed in the experiment with 2. 5 ml of concentrated HCl. A preparative experiment was then attempted using the same purification procedure as that described for the preparation of ε-N-carbobenzoxy-L-lysine methyl ester when boron trifluoride-methanol was used for the esterification. Pure ε -N-carbobenzoxy-L-lysine methyl ester hydrochloride was isolated in 62% yield. This method is as simple and efficient as the boron trifluoride-methanol method; both methods offer very convenient routes to ε -N-carbobenzoxy-L-lysine methyl ester. The purity of the final product, which in both cases does not require recrystallisation, must be due mainly to the efficiency of the work-up. The boron trifluoride method is

readily adaptable to the synthesis of other simple esters of ε-N-carbobenzoxylysine since the alcohols required as starting materials are readily available; this is not true for the 2, 2-dimethoxy-propane method since the higher 2, 2-dialkoxypropanes are not readily available.

<u>ε-N-Carbobenzoxy-L-lysine methyl ester hydrochloride:</u> ε-N-Carbobenzoxy-L-lysine (2.8 g; 10 mmol), 2, 2-dimethoxypropane (100 ml) and concentrated HCl (5 ml) were stirred together for 16 h at 25°C. Evaporation gave a dark brown oil which was dissolved in water (50 ml) and extracted with ether (2 x 25 ml). The aqueous layer was worked-up to give ε-N-carbobenzoxy-L-lysine methyl ester hydrochloride (2.04 g; 62%) in exactly the same way as described for the preparation of ε-N-carbobenzoxylysine methyl ester hydrochloride using boron trifluoride-methanol. The product had m.p. 117 - 118°C which was identical with the m.p. obtained for material prepared using the boron trifluoride-methanol method [see Part IV. 5(b), p. 159].

V.

GENERAL DISCUSSION

There is considerable biological interest in naturally occurring and synthetic peptides containing N-methylamino acids. A number of N-methylamino acid containing peptides have been isolated from bacteria, fungi and actinomycetes; some examples are given in Table V. 101 which was mainly compiled from the monograph of Schroder and Lubke (1966) where detailed references are given. Many of these peptides show strong antibiotic activity, e.g. the actinomycins and the enniatins; others are toxic for insects, e.g. the destruxins and aspochracin and others for cattle, e.g. the sporidesmolides. The reasons for the potent biological activity of these peptides are not known; however, studies on synthetic analogues should allow the structural features necessary for activity to be identified, e.g. preliminary studies on the actinomycins [Bodanszky and Perlman (1969)] and the enniatins [Studer et al (1965)] have shown that the N-methyl groups are essential for activity. Analogues of other biologically active peptides, especially the peptide hormones, have been prepared in order to establish structure-activity relationships and to find derivatives with modified, longer lived or more potent activities. Frequently these analogues have contained N-methylamino acids, e.g. 2-N-methyltyrosine oxytocin was prepared in the hope that it would resist proteolytic destruction by oxytocinase; however, the compound showed very little oxytocic activity [Jost, Rudinger and Sorm (1961), Huguenin and Boissonnas (1961)].

Table V. 101

Some naturally occurring peptides containing N-methylamino acids

Peptide	N-Methylamino acid present	Reference[a]
The actinomycins	MeVal-OH, MeGly-OH, MeIle-OH	1, pp. 397-405
Etamycin	MeGly-OH, MeGly(Ph)-OH, MeLeu(Me)-OH	1, pp. 405 - 6
Staphlomycin Factor S	MePhe-OH	1, pp. 406 - 7
Ostreogrycin and related substances	MePhe(NMe_2)-OH	1, pp. 408-9
Echinomycin	MeVal-OH, MeAla-OH	1, pp. 409 - 10
Lateritiin	MeVal-OH	1, pp. 411 -2
The enniatins	MeVal-OH, MeLeu-OH, MeIle-OH	1, pp. 412 - 15
The sporidesmolides	MeLeu-OH	1, pp. 418 - 20
Pithomycolide	MeAla-OH	1, pp. 423
Aspochracin	MeAla-OH, MeVal-OH	2
The destruxins	MeAla-OH, MeVal-OH	1, pp. 420 and 2
The ilamycins	MeLeu-OH, MeNorVal(HCO)-OH	1, p. 447

a) 1. Schroder and Lubke (1966).

2. Myokei et al (1969).

The synthesis of N-methylamino acid-containing peptides for studies of the type described has not always proved to be straight forward. Rudinger (1963) refers to two properties of N-methylamino acids which lead to special problems in peptide synthesis. These are steric hindrance and the marked tendency of dipeptides to cyclise.

The first property is graphically illustrated by the difficulties encountered in the carbobenzoylation of N-methylamino acids, e.g. preparation of carbobenzoxy-N-methylphenylalanine by the same procedure as that used for the synthesis of carbobenzoxy-phenylalanine on p. 34 gave only 6% yield of an oily product compared with the 76% yield obtained with phenylalanine [J. R.Coggins, R. Demayo and N. L. Benoiton, unpublished results].

Rather vigorous conditions are required for the esterification of N-methylamino acids, especially those with branched side chains [Schroder and Lubke (1965), p. 144]. Even the methyl esterification of sarcosine is not entirely straight forward, e.g. the method described on p. 39 for the preparation of glycine methyl ester hydrochloride gave sarcosine methyl ester hydrochloride in 86% yield but amino acid analysis and titration experiments revealed the presence after re-crystallisation of 11% of sarcosine hydrochloride [R. Demayo, J. E. Purdie, J. R. Coggins and N. L. Benoiton, unpublished results].

There are many examples in the literature of coupling problems with N-methylamino acids, e.g. the reaction of N-methyl-leucine with alanine N-carboxyanhydride is much less efficient than the corresponding reaction between leucine and alanine N-carboxyanhydride (p. 114); under standard conditions N-methylleucine methyl ester is not

acylated by carbobenzoxyvaline p-nitrophenyl ester [private communication of D. W. Russell, quoted by Rudinger (1963)], an octapeptide containing N-terminal N-methyltyrosine is acylated by an active ester at the unprotected phenolic hydroxyl group and not as expected at the methylamino group and even when the phenolic group is protected there is hardly any reaction at the secondary amino group [Huguenin and Boissonnas (1961)].

The tendency of dipeptides containing a methylated peptide bond to cyclise is shown by the isolation of diketopiperazines after attempts to methylaminate α-bromoacylsarcosine derivatives [Levene et al (1926)]; cyclisation can even occur in acid solution - see, e.g. p. 141 and Levene et al (1924). Another example of the same tendency is the formation of N-methyl-N'-tosyldiketopiperazine as a by product in the treatment of tosylglycylsarcosine with HBr in acetic acid; no corresponding product is formed with tosylglycylglycine under similar conditions [J. Rudinger and V. Gut, unpublished results quoted by Rudinger (1963)].

The new routes for the preparation of N-methylamino acid methyl esters and carbobenzoxy-N-methylamino acids described in this thesis provide simple and efficient methods of preparing the necessary starting materials for the synthesis of N-methylamino acid-containing peptides. The inefficient reactions involved in the protection of N-methylamino acids are avoided and the products are obtained directly from the readily available carbobenzoxyamino acids without the isolation of intermediates. The preparation of the carbobenzoxy derivatives is particularly useful since the carbobenzoxy group is by far the most

extensively used N-protecting group for the N-methylamino acids [Schroder and Lubke (1965), p. 143]. The direct synthesis of tosyl-N-methylamino acids from tosylamino acids and their subsequent use in peptide synthesis has been reported by Leplawy, Jones, Kenner and Sheppard (1960) and by Jost, Rudinger and Sorm (1961). The carbobenzoxy-N-methylamino acids are, however, potentially much more useful intermediates than the tosyl-N-methylamino acids.

For the preparation of acylamino acid hydrazides hydrazinolysis of the corresponding methyl ester is the most practical route [Bodanszky and Ondetti (1961), p. 76]. The products of the methylation reactions are the carbobenzoxy-N-methylamino acid methyl esters; if these esters could be efficiently converted to the hydrazides then the crucial intermediates of the N-methylamino acids required for the azide coupling method will be available in only two steps from the corresponding carbobenzoxyamino acids.

The selective N-methylation of N-tritylleucylalanine methyl ester to give N-tritylleucyl-N-methylalanine methyl ester opens up an entirely new method for the preparation of peptides containing N-methylamino acids. The hindered coupling reaction is completely avoided and the cyclisation dangers could also be avoided if the peptide chain were elongated before removal of the trityl protecting group. Where several successive N-methylamino acids are required in a peptide chain they could be introduced simultaneously by methylation of the appropriate N-trityl-protected peptide. The selective methylation of a tosyldipeptide has been reported by Jost, Rudinger and Sorm (1961) who converted N-tosyl, S-benzylcysteinyl-O-benzyltyrosine methyl

ester to the N-tosyl, N-methyl, S-benzylcysteinyl derivative by treatment with dimethyl sulphate in aqueous alkali. This method is applicable only to the introduction of N-terminal N-methylamino acids which is not, in itself, difficult; if further coupling to the N-terminus is required the hindered acylation problem remains.

Methylation of poly-L-leucine went virtually to completion (see p. 47); the methylation procedure therefore appears to offer an efficient route for the preparation of poly-N-methylamino acids. Poly-N-methyl-L-alanine has been extensively studied by Goodman and co-workers [for references see Goodman and Choi (1968)] because although lacking hydrogen bonds it is helical. The polymer was prepared by the polymerisation of N-methyl-L-alanine N-carboxyanhydride in dioxane solution for 3 weeks [Goodman and Fried (1967)]. This is a rather tedious and slow procedure compared with the 24 h methylation of a poly-L-amino acid.

The permethylation of oligopeptides by our method and its application in mass spectrometric studies was extensively discussed in Part IV. 4.

The successful preparation of ε-N-carbobenzoxy, ε-N-methyl-L-lysine methyl ester will allow for the first time the synthesis of peptides containing ε-N-methyl-L-lysine. Our reasons for wishing to synthesise such peptides are given in the Introduction. In addition the synthesis of analogues of peptide hormones (e. g. lysine vasopressin) containing ε-N-methyl-L-lysine instead of L-lysine would be of some interest; methylation in this case should not interfere with the secondary

structure of the peptide since there would be no changes in hydrogen bonding involving the peptide backbone. The modified peptide would, however, be more resistant to trypsin like enzymes in the blood (e.g. plasmin) and if it had biological activity this might well be longer lived than normal.

The alkylation procedure was applicable to both methylation and ethylation although in the latter case the purified products contained 1 - 2% of unethylated material. No higher N-alkyl derivatives were prepared since the alkylation reactions were generally incomplete presumably because of steric hindrance. Johnstone et al (1969) found that propylation of trifluoracetylamines was also very difficult. When an attempt was made to reduce steric hindrance in the propylation of α-N-trityl, ε-N-carbobenzoxylysine by incorporating the ε-amino group into a cyclic amide linkage alkylation was more complete. However, steric hindrance remained a problem since opening the ring of the N-alkylated lactam to give the required ε-N-propyllysine proved to be extremely difficult.

Alkylation of the carbobenzoxy derivatives of primary amines offers a very attractive route for the preparation of secondary amines, e.g. carbobenzoxybenzylamine on treatment with methyl iodide/sodium hydride followed by deprotection and an appropriate work-up gave N-methylbenzylamine hydrochloride in high yield [J. R. Coggins and N. L. Benoiton, unpublished results]. Johnstone et al (1969) have recently described a method of preparing secondary amines by the alkylation of trifluoracetylamines using the conditions of Pachter and Kloetzel (1952) (see p. 12). These authors imply that the

presence of a strong electron withdrawing group on the nitrogen is necessary for efficient N-methylation. Our results do not support this hypothesis. The preparative application of the method of Johnstone et al (1969) was complicated by the fact that the removal of the trifluoracetyl was not straight forward. Unless excess alkyl halide was very carefully removed there was a base catalysed bimolecular displacement of the trifluoracetyl group by alkyl halide leading to the formation of some dialkylated product. In contrast, the removal of the carbobenzoxy group from carbobenzoxy-N-methylbenzylamine proceeded without difficulty.

Rudinger (1963) has drawn attention to the fact that the m. p.'s of N-methylamino acid derivatives were lower than the m. p.'s of the corresponding amino acid derivatives. This, like the volatility of permethylated peptides [Lederer (1968)] is due to the reduced possibility of inter-molecular hydrogen bonding. Reference to Table V. 102 shows that the m. p.'s of most of the acyl-N-methylamino acids prepared confirmed this general rule. However, there were two exceptions: both carbobenzoxy-N-methyl-L-leucine and benzoyl-N-methyl-L-leucine had higher m. p.'s than the corresponding un-methylated derivatives. The acyl-N-methylamino acids were generally more soluble in organic solvents than the unmethylated compounds, e.g. all the acyl-N-methylamino acids were readily soluble in chloroform whereas the acetyl- and benzoyl-amino acids were so insoluble that it was impossible to obtain their n. m. r. spectra in deuterochloroform solution. The acyl-N-methylamino acids, even those with large hydrophobic side chains were, like the corresponding acylamino acids, appreciably soluble in hot water, e. g. carbobenzoxy-N-methyl-L-leucine, benzoyl-N-methyl-L-leucine and acetyl-N-methyl-L-isoleucine were all successfully recrystallised from boiling water.

Table V. 102

The melting points of acylamino acids and acyl-N-methylamino acids [a]

Acylamino acid	m. p. (°C)	Acyl-N-methylamino acid	m. p. (°C)
Ac-Gly-OH	205 - 6	Ac-MeGly-OH	135 - 7
Ac-Ala-OH	126 - 7	Ac-MeAla-OH	110 - 2
Ac-Val-OH	169 - 169.5	AcMeVal-OH	112 - 4
Ac-Ile-OH	151	Ac-MeIle-OH	121.5 - 122.5
Ac-Leu-OH	183 - 4	Ac-MeLeu-OH	107 - 8
Ac-Phe-OH	170 - 171.5	Ac-MePhe-OH	145 - 6
Bz-Gly-OH	189.5 - 190.5	Bz-MeGly-OH	105 - 6
Bz-Ala-OH	149.5	Bz-MeAla-OH	133
Bz-Leu-OH	105 - 7	Bz-MeLeu-OH	135 - 7
Cbz-Leu-OH	oil	Cbz-MeLeu-OH	73 - 74

a. Except for the glycine derivatives all the melting points are for the L-compounds.

The fact that acyl-N-methyl-L-amino acids and their methyl esters can now be readily prepared opens up some interesting possibilities for further studies on proteolytic enzymes. Elmore and Smyth (1968) have shown that α-N-methylation of α-N-tosyl-L-lysine β-naphthyl ester interferes with the deacylation step of its trypsin catalysed hydrolysis [these authors were led to investigate the effect of α-N-methylation because of the observation of Hein and Niemann (1961) that acetyl-N-methyl-L-tyrosine methyl ester was, in comparison with acetyl-L-tyrosine ethyl ester, a very poor substrate of α-chymotrypsin]. α-N-Methyl, α-N-tosyl-L-lysine β-naphthyl ester (MTLNE) proved to be an excellent reagent for determining the absolute molarity (i.e. 'titrating') of solutions of trypsin. The preparation of analogous 'titrants' [which are extremely useful in kinetic studies - for discussion see Chase and Shaw (1969)] for other proteolytic enzymes would be of interest, e.g. benzoyl-N-methyl-L-alanine p-nitrophenyl ester might be a good 'titrant' for elastase and/or the α-lytic protease of Sorangium [benzoyl-L-alanine methyl ester is an excellent substrate for these enzymes (H. Kaplan and D.R. Whittaker, personal communication)]. Another possible application of acyl-N-methylamino acid derivatives and also of peptides with one or more of the peptide bonds selectively methylated is in the X-ray diffraction studies of the 3-dimensional structures of the active sites of proteolytic enzymes. Studies on a crystalline enzyme substrate complex formed from chemically modified α-chymotrypsin and an acetyl-L-tryptophan ester were limited because the ester was hydrolysed too rapidly to allow the collection of enough data to determine the structure of the complex

[D.M. Blow at CIC-CBC Symposium on Protein Structure and Function, Ste. Marguerite, Quebec, March 1969]. Studies on the acyl-N-methyl esters of the aromatic amino acids which would be hydrolysed much more slowly might prove to be more successful. Even more interesting is the possibility of studying the complex formed between a proteolytic enzyme and a peptide substrate modified by methylation of the peptide bond which is normally cleaved. Providing the peptide is bound normally (kinetic studies should allow this to be determined) and not hydrolysed, it should be possible to locate the binding sites for the peptide on the enzyme. The elegant studies of Phillips and his colleagues [see Blake et al (1967)] on the enzyme-inhibitor complexes of lysosyme have already demonstrated the power of this approach in active site studies.

Conformational analysis of polypeptides and proteins, particularly from the theoretical point of view, is a subject of great topical interest. The problems involved in the calculation of the conformations of polypeptides have been reviewed by Scheraga (1968). The first step in computing the conformation of a polypeptide chain of known sequence is to obtain an expression for the energy of the system as a function of the coordinates of the atoms in space. One of the terms contributing to the energy expression is due to internal rotation about the C-N bonds of the peptide backbone and to calculate this term the heights of the rotation barriers about these bonds must be known. Recently Scheraga and his collaborators [Yan et al (1970)] have described a method for calculating the barriers to rotation and also the *cis-trans* energy differences of a number of model compounds

containing peptide bonds. The models included formamide, acetamide and their N-methyl and N,N-dimethyl derivatives and acetyl-L-prolineamide. Calculations on additional model compounds preferably containing groups analogous with amino acid side chains would be of great value providing experimental data were available for the energy barriers and the cis-trans energy differences. As is mentioned on p. 130, it should be possible to obtain such data from the n.m.r. spectra of the acyl-N-methylamino acids; these compounds are especially interesting because of the variable effect of the side chains on the cis-trans energy differences (see pp. 129 - 30). The barriers to rotation about the C-N bonds in polypeptides cannot be determined experimentally [Scheraga (1968)]. The values have to be estimated and it is only by comparison of the theoretical values with data for good model compounds that the estimates can be improved.

In a 1968 review article Lederer, looking back to the early attempts in his laboratory to prepare volatile peptide derivatives for mass spectrometric studies, makes the following statement: "The methylation of the peptide bond had not yet been described and was considered to be a difficult enterprise (personal communications of several peptide specialists)", [Lederer (1968)]. The successful per-methylation of peptides by Lederer's group [Das et al (1967)] disproved the specialists. The results described in this thesis further establish that the N-methylation of N-protected amino acids and peptides under appropriate conditions proceeds very efficiently and can be used as the basis of a new method for the synthesis of N-methylamino acids and their peptides.

VI.

SUMMARY

1. A detailed study of the methylation of acyl- and alkyloxycarbonyl-leucines with methyl iodide/sodium hydride showed that these derivatives could be quantitatively converted to the corresponding N-methylleucine methyl esters in very high yield.

2. Protection against N-methylation could be achieved using the trityl group. The phthaloyl group was unsuitable for protection because it resulted in the occurrence of some α-C-methylation and because of partial cleavage of the group.

3. The methylation procedure was successfully used for the synthesis of MeLeu-OH, MeLeu-OMe.HBr and Cbz-MeLeu-OH from Cbz-Leu-OH. These products were optically pure and there was no α-C-methylation. Bz-MeLeu-OH, Ac-MeLeu-OH, Tos-MeLeu-OH and Tos-MeLeu-OMe were also prepared from the appropriate starting materials. In no case was α-C-methylation detected.

4. The general applicability of the method to the synthesis of derivatives of the difunctional N-methylamino acids was demonstrated by the synthesis of 16 compounds including the methyl esters of all the common naturally occurring N-methylamino acids. Only in the case of glycine was α-C-methylation shown to occur, in which case 1% of the corresponding N-methylalanine derivative was formed.

5. N-Ethylation was achieved using the same general procedure, EtLeu-OEt.HBr being obtained in good yield from Cbz-Leu-OH. Propylation was, however, incomplete.

6. It was shown that the methylation procedure converted phenolic hydroxyl groups to methyl ethers, primary amino groups to trimethylammonium groups and primary amide groups to N,N-dimethylamido groups.

7. A comparison of the action of the methylating reagents used for the permethylation of peptides prior to sequencing by mass spectrometry showed that with Cbz-Leu-OH and Ac-Leu-OH, methyl iodide/sodium hydride led to more than 90% N-methylation leaving 1 - 3% of starting material unreacted; methyl iodide/silver oxide led to 41 - 85% N-methylation leaving 10 - 60% of starting material unreacted and methyl iodide/methyl sulfinyl carbanion led to 23 - 35% N-methylation with only 28% of residual starting material.

8. Methylation of polyleucine proceeded efficiently as did the methylation of a number of di- and tri-peptides. Methylation of Trt-Leu-Ala-OMe gave Trt-Leu-MeAla-OMe.

9. Serious problems, attributed to steric hindrance and diketopiperazine formation, were encountered in the acid hydrolysis of permethylated di- and tri-peptides.

10. The saponification of Cbz-MeLeu-OMe and Tos-MeLeu-OMe was found to be slow enough to result in relatively poor yields in the synthesis of the corresponding acids.

11. A new synthesis of H-Lys(Me)-OH derivatives was developed as follows:
H-Lys(Cbz)-OMe.HCl → Trt-Lys(Cbz)-OMe → Trt-Lys(Cbz,Me)-OMe
→ H-Lys(Cbz, Me)-OMe.HCl.

12. A convenient synthesis of H-Lys(Et)-OH derivatives was developed along similar lines but the attempted synthesis of an ε-N-propyl-lysine derivative was unsuccessful.

13. MeLys(Me)-OH.HCl was prepared from Cbz-Lys(Cbz)-OH.

14. Synthesis of MeLys-OH derivatives by a route analogous to that in 11 was unsuccessful, but several of the intermediates were obtained.

15. Evidence is presented that ε-N-trityl groups are difficult to remove.

16. A convenient synthesis of Trt-Lys-OMe by selective hydrogenation of Trt-Lys(Cbz)-OMe was developed.

17. Trt-Lys-OMe on treatment with the methylating reagent gave Trt-Lys(Me_3)-OMe.I in almost quantitative yield, thus providing a new synthesis of H-Lys(Me_3)-OH.

18. A method for cyclising Trt-Lys-OMe to 3-tritylhomopiperidone in high yield was discovered; this compounds could be efficiently alkylated but ring opening of the product to give the corresponding ε-N-alkyllysine derivative proved to be difficult.

19. A method is described for the separation of H-Lys(Cbz)-OH, H-Lys-OH and Cbz-Lys-OH on the amino acid analyser.

20. A method is described for the separation of MeLys-OH, MeLys(Me)-OH, H-Lys-OH, H-Lys(Me)-OH, H-Lys(Et)-OH and H-Lys(Pr)-OH on the amino acid analyser.

21. An improved method of analysing N-methylamino and α-C-methylamino acids on the amino acid analyser was developed.

22. A very sensitive procedure for determining the optical purity of N-methylamino acids was developed from the method described by Manning and Moore (1968a).

24. A new method was developed for the analysis of mixtures of amino acids and their esters using the amino acid analyser.

25. It was shown that fully protected lysine derivatives containing an N-trityl group reacted with ninhydrin on prolonged heating at 100°C.

26. Details of the changes in i. r. spectra accompanying the N-methylation of acylamino acids are reported.

27. The i. r. spectra of amino acid ester salts and N-methylamino acid ester salts are compared.

28. The i. r. spectra of amino acids and N-methylamino acids are compared.

29. The n. m. r. spectra of acyl-N-methylamino acids showed evidence of _cis-trans_ isomerism about the amide C-N bond.

30. An n. m. r. method for establishing the position of the alkyl group in mono-N-alkyllysines is described.

31. Difficulties previously encountered in the synthesis of peptides containing N-methylamino acids are reviewed and some specific proposals are made on how the methylation procedure described can be used with advantage in the synthesis of such peptides.

VII.

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