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STUDIES ON THE SYNTHESIS AND STEREOCHEMISTRY OF N-METHYLAMINO ACIDS

A thesis submitted to

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF ROUTES

LIST OF ROUTES

LIST OF ABBREVIATIONS

A	acetic acid
atm	atmosphere
B	n-butanol
b.p.	boiling point
C°	degree celsius
C	chloroform
cm	centimeter
DCHA	dicyclohexylammonium salt
dm	decimeter
DMF	N,N-dimethylformamide
g	gram
h	hour
l	litre
lit	literature
MeOH	methanol
M	molar
MeI	methyl iodide
mg	milligram
min	minute
ml	milliliter

mm	millimeter
mmol	millimole
mol	mole
m.p.	melting point
NaH	sodium hydride
N	normality
n	normal
n.m.r.	nuclear magnetic resonance
p	page
pet-ether	30-60°C fraction Petroleum Ether
s	second
t	tertiary
TEA	triethylamine
tfa	trifluoracetic acid
THF	tetrahydrofuran
t.l.c.	thin layer chromatography
v	volume
w	weight
µmol	micromole

NOMENCLATURE FOR AMINO ACIDS AND PEPTIDES

The abbreviations used for amino acids and N-methyl-amino acids are based on those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [J. Biol. Chem. 247, 997 (1972)]. The symbols are not used to represent the structural formulae of amino acids, which were represented as follows:

H-Ala-OH	Alanine
MeLeu-OH	N-methyl-L-leucine

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

Boc-MeLeu-OMe	N-tert-butyloxycarbonyl,N-methyl-L-leucine methyl ester.
Z-MeSer-OH.DCHA	N-benzyloxycarbonyl,N-methyl-L-serine dicyclohexylammonium salt.

Substitutions on the side chains are also represented as recommended e.g.

H-Lys(Z)-OBzl	ε-N-benzyloxycarbonyl-L-lysine benzyl ester.
Boc-Ser(Me)-OH.DCHA	N-tert-butyloxycarbonyl, O-methyl-L-serine dicyclohexylammonium salt.

The symbols represent the L-amino acids where optical isomerism is possible.

The following abbreviations are used for the protecting groups:

Boc	tert-butyloxycarbonyl
Bzl	benzyl
Et	ethyl
Me	methyl
Tos	tosyl = p-toluenesulfonyl
Z	benzyloxycarbonyl

I. INTRODUCTION

N-Methylamino acids have so far not been found as constituents of peptide hormones or proteins. However, they are present in a series of antibiotics which have been discovered and isolated from bacteria, fungi and actinomycetes. Some examples, which were mainly compiled from the monograph of Schroder and Lubke (1966) where detailed references are given, are given in Table I. 101. N-methyl-L-isoleucine and N-methyl-L-valine were first found respectively in Enniatins A and B [Plattner and Nager (1948)]. Since then, L-phenylsarcosine, N, β-dimethyl-L-leucine and sarcosine were found in Etamycin [Sheehan et al (1957)]. Keller-Schierlein et al. (1959) found that N-methyl-L-valine and N-methyl-L-alanine are constituents of Echinomycin. Then Straphymycin was found containing N-methyl-L-phenylalanine by Vanderhaeghe and Parmentier (1960), whereas the structural analogue Ostreogrycin β has N-methyl-L-phenylalanine replaced by its p-dimethylamino derivative, as was shown by Todd et al (1960). During the same year, Brockmann (1960) found N-methyl-L-isoleucine, sarcosine and N-methyl-L-valine in Actinomycins and N-methyl-L-leucine was found in Sporidesmolide I by Russell (1960). A few years later, N-methyl-L-alanine was found in Pithiomycolide [Briggs et al (1964)]. N-Methyl-L-alanine and N-methyl L-valine were found as constituents of Destruxin B

Table I 101

Some naturally occurring peptides containing N-methylamino acids

Peptide	N-methylamino acid present	References[a]
The actinomycins	H-MeVal-OH, H-MeGly-OH, H-MeIle-OH, H-MeAla-OH	1, p. 397-405 and 3
Etamycin	H-MeGly-OH, H-MeGly(Ph)-OH, H-MeLeu(Me)-OH	1, p. 405-6
Staphomycin S factor	H-MePhe-OH	1, p. 406-7
Ostregrycin	H-MePhe(NMe_2)-OH	1, p. 408-9
Echinomycin	H-MeVal-OH, H MeAla-OH	1, p. 409-10
Lateritin	H-MeVal-OH	1, p. 411-2
The enniatins	H-MeVal-OH, H-MeLeu-OH, H-MeIle-OH	1, p. 412-15
The sporidesmolides	H-MeLeu-OH	1, p. 418-20
Pithomycolide	H-MeAla-OH	1, p. 423
Aspochracin	H-MeAla-OH, H-MeVal-OH	2.
The destrxins	H-MeAla-OH, H-MeVal-OH	1, p. 420 and 2
The ilamycins	H-MeLeu, H-MeNorVal(HCO)-OH	1, p. 447

[a] 1. Schroder and Lubke (1966)

2. Myokei et al (1969)

3. Katz et al (1973); (1975)

by Tamura et al (1964) and they were also found as constituents of Aspochracin by Myokei et al (1969). Recently, intensive studies of the Actinomycin Z series by Katz and coworkers has revealed the presence of N-methyl-L-alanine and methyl proline derivatives [Katz et al (1973, 1975)].

Many of these N-methylamino acid containing peptides show strong antibiotic activity, for example the actinomycins and enniatins. Some are toxic for insects such as destruxins and aspochracin and others are toxic for cattle, such as sporides molides. The reasons for the potent biological activity of these peptides are unknown. However, studies on synthetic analogues should allow the identification of the structural features which are necessary for activity. In fact, studies on the enniatins [Studer et al (1965)] and actinomycins. [Bodanszky and Perlman (1969)] have shown that N-methyl groups are essential for their activities. It has also been suggested that the presence of N-methylamino acids in antibiotics probably precludes helix formation and hence facilitates cyclization which also plays an important role for the full activity of these natural products [Bodanszky and Perlman (1969)].

There is little knowledge available concerning the mechanism of the biogenesis of N-methylamino acids by microorganisms. Radioisotopic experiments with S. antibioticus and S. chrysomallus have suggested that glycine and L-valine are the direct precursors of the sarcosine and N-methyl-L-valine

in the actinomycins [Katz and Weissbach (1962)]. The biochemical source of the N-methyl groups in these N-methyl-amino acids has been investigated [Ciferri et al (1964)] and it has been confirmed that the ^{14}C-methyl group of methionine is incorporated into glycine and L-valine possibly via S-adenosyl methionine which serves as the principal methyl donor in many biological systems. Recently, it has been suggested that the N-methylation of amino acids during actinomycin synthesis may occur prior to or after incorporation of the amino acids into a peptide molecule and this reaction seems to require an activated, enzyme-bound form of amino acid [Fukagawa et al (1974)].

Sarcosine was found as the free amino acid in certain animals and plants [Meister (1965)] and in certain microorganisms which metabolize one carbon compound as sole energy source [Ribbons et al (1970)]. In animal systems, sarcosine arises from betaine via a series of demethylation reactions [Meister (1965a)].

N-Methyl-L-glutamate occurs naturally as an intermediate metabolite of Pseudomonas MA, a nonphotosynthetic bacterium, which can grow on methylamine as the sole carbon and nitrogen source [Shaw et al (1966)]. The initial steps for the metabolism of methylamine in this organism involve the condensation of methylamine and L-glutamate to yield N-

methyl-L-glutamate and ammonia [Pollock and Hersh (1971)], followed by the cleavage of N-methylglutamate to yield glutamate and formaldehyde [Hersh et al (1971)]. This reaction is catalyzed by two particular enzymes, namely N-methylglutamate synthetase which catalyzes the reversible formation of N-methyl-L-glutamate from L-glutamate and methylamine [Pollock and Hersh (1973)] and N-methylglutamate dehydrogenase which catalyzes the cleavage of N-methyl-L-glutamate to yield glutamate and formaldehyde [Hersh et al (1972)]. The latter enzyme has also been found capable of catalyzing the cleavage of other authentic N-methylamino acids. Isotope experiments indicate that the carbon-nitrogen bond of glutamate is broken and the entire methylamine molecule incorporated to form N-methyl-L-glutamate [Ribbons et al (1970)].

5-Hydroxy-N-methylpyroglutamate has also been found as a natural product of Pseudomonas MA. It has been found that this compound is synthesized enzymatically from α-ketoglutarate and methylamine [Hersh et al (1969)].

N-Methylalanine dehydrogenase has recently been isolated and purified from Pseudomonas MS which belongs to the same family as Pseudomonas MA and can depend on methylamine as the sole carbon and nitrogen source [Lin and Wagner (1975)]. This enzyme has been found responsible for the synthesis of N-methylalanine in the presence of serine or pyruvate [Kung and Wagner (1972)]. Although N-methylalanine has been identified

unequivocally as the product of N-methylalanine dehydrogenase [Lin and Wagner (1975)], the configuration of the compound has not been determined.

The role of N-methylalanine in the metabolism of methylamine by Pseudomonas MS as well as that of other methylated amino acids, N-methylglutamate and 5-hydroxy, N-methylpyroglutamate by Pseudomonas MA is unknown, but it has been suggested that these N-methylamino acids could eventually generate formaldehyde for entry into the 1-carbon pool by the action of N-methyl-L-glutamate dehydrogenase which was found present in both microorganisms and has a broad substrate specificity for N-methylamino acids [Hersh et al (1972)].

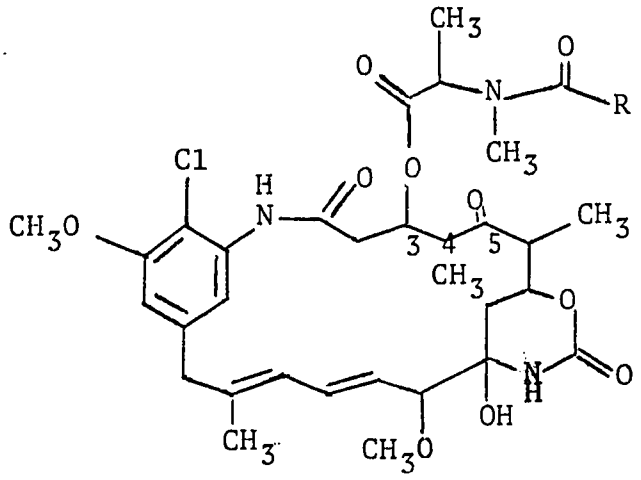
N-Methylamino acids have also been found in higher plants. Two amino acids found among the free amino acids of Gifblaar (Dichapetaleum Cymosum) were isolated and identified as N-methyl-L-alanine and N-methyl-L-serine [Eloff and Grobbelaar (1967), (1969)]. These N-methylamino acids were found primarily in the very young leaves which are very toxic because of the high concentration of fluoroacetate. Therefore, it has been suggested that the metabolism of N-methyl-L-alanine and -serine are coupled with that of fluoroacetate [Eloff and Grobbelaar (1967)]. More recently, antitumor agents, Maytansine and Maytan-valine, which were isolated from Maytenus serata, were found to contain a N-acetyl, N-methylalanine [Kupchan (1972)]

The structures of these natural products are shown in Figure I, 101. It has been shown that the ester moiety of these compounds enables them to act as a potent antileukemic agent whereas maysine, nomaysine, maysenine which have no ester chain at the C-3 position show no antileukemic activity [Kupchan (1974)]. The mechanism of the antileukemic activity of maytansine and maytanvaline is still in speculation, but it was suggested that the ester moiety at C-3 position allows them to form highly selective complexes with growth regulating enzyme [Kupchan (1974)].

Several synthetic N-methylamino acids, namely N-methylaspartic acid, N-methylglutamic acid and N-methylcysteic acid possess neuropharmacological activity, that is, they excite mammalian nerve cells and cause contraction of crustacean muscle [Watkins (1962)]. N-Methyl-aspartic acid was found the most potent excitant comparing with the other amino acids which possess the same effect [Curti et al (1961)]. The D-isomers of these N-methylamino acids had stronger activity than the L-isomers where both enantiomers were available [Curti et al (1963)]. It has been suggested that the pharmacological action of this acidic N-methylamino acids could be due to permeability changes which they evoke in the neuronal membrane rather than to metabolic effect [Harvey and McIlwain (1968)]. However, it has been demonstrated recently that N-methyl-D-aspartic acid could cause an increase in

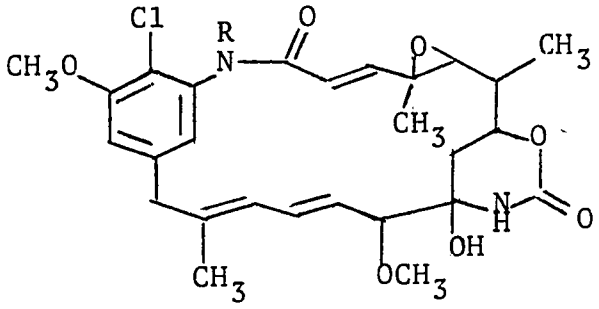
Figure I 101

Structure of Maytansinoids [Kupchan (1974)]



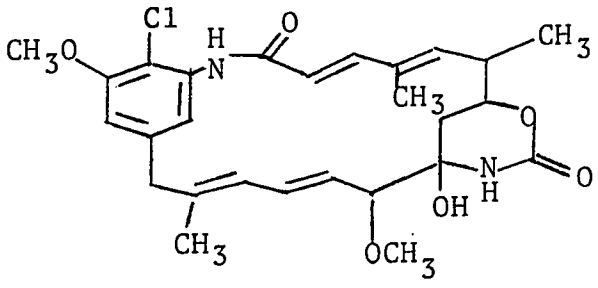
Maytansine R=CH_3

Maytanvaline R=$CH_2\ CH(CH_3)_2$



Maysine R=CH_3

Nomaysine R=H



Maysenine

glucose utilization [Watkins (1971)] and a decrease in acetate utilization in neuronal metabolism [Watkins (1971a)].

Analogues of biologically active peptides, especially the peptide hormones, have been prepared in order to establish structure and function relationships and to find derivatives with modified, protracted or more potent activity. Frequently, these analogues have contained N-methylamino acids or O-methylamino acids. It has been known for a long time that N-methylamino acid containing peptides, especially dipeptides are more resistant to hydrolysis because of the tendency toward the formation of ring compounds [Levene et al (1924)] Studying the specificity of Cathepsin C, Izumiya and Fruton (1955) found that methylation of the terminal α-amino group of a substrate dipeptide diminishes the rate of enzymic hydrolysis. Therefore, it was suggested that analogues of biologically active peptides containing N-methylamino acids might resist enzymic inactivation [Zaoral et al (1959)]. [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 et al (1961), Huguenin and Boissonnas (1961)]. Synthetic [2-N-methyl phenylalanine]-adrenocorticotropin-(1-19) has been shown to be more resistant to exopeptidase digestion despite the fact that this analogue does not increase steroidogenic response [Blake and Li (1972)]. More recently, studies on [1-Sarcosine]-

angiotensin II showed that the introduction of an N-methyl-amino acid into this position not only made the peptide more resistant to degradation by plasma angiotensinase but also increased its binding to the receptor site on aortic muscle [Hall et al (1973)].

Substitution of N-methylamino acids in biologically active peptide analogues profoundly alters the secondary structure (hydrogen bonding capacity) of the peptides and hence influences the biological activity. Replacement of glycine by sarcosine in oxytocin [Cash et al (1962)], lysine vasopressin [Meienhofer and du Vigneaud (1961)], or bradykinin [Schröder (1966)] and the replacement of tyrosine or phenylalanine by their N-methyl derivatives in oxytocin [Huguenin and Boissonnas (1961)] greatly reduced or even abolished their activities. On the other hand, replacement of leucine by its N-methyl derivative in Leuteinizing hormone releasing factor increased its potency by five times [Ling and Vale (1975)].

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 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 N-methylamino acid containing peptides have been employed as model compounds for this study in hope that the barriers to rotation can be estimated by comparing the theoretical values with the data of the model compounds [Bystrov et al (1969)]. N-Methyl-L-alanine derivatives were used for studying the cis and trans isomerization of the polypeptide system using nuclear magnetic resonance spectroscopy [Goodman (1974)].

The alkylation of toluenesulfonylamide has long been employed as a route to secondary amines. Hinsberg (1891) reported the methylation of a sulfonamide using dimethyl sulfate and aqueous alkali. Later, alkyl halides were used instead of dimethylsulfate [Hinsberg and Strupler (1895)]. Fischer et al (1913, 1915) applied this method to the synthesis of some N-methylamino acids. The methylation of optically active N-toluenesulfonylamino acids using methyl iodide and 2N alkali, gave N-toluenesulfonyl, 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 toluenesulfonyl group. Later, du Vigneaud and Behrens (1937)

demonstrated that the toluenesulfonyl group 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.

Condensation of amino acids with aldehydes in the presence of hydrogen and palladised charcoal gives dimethylamino acids [Bowman (1950)]. With the higher aldehydes, glycine and alanine give dialkylamino acids and the other amino acids give mixtures of mono and dialkylated products. Since the separation of mixtures of mono and dimethylamino acids is extremely difficult, this is not a satisfactory method to obtain N-methylamino acids.

Izumiya (1949, 1950) reported the synthesis of N-methylthreonine, its alloisomer and N-methylserine using β-methoxythreonine and propenoic acid as the starting materials, respectively [Figures I, 102, 103]. In his methods β-methoxythreonine was first converted to the N-toluenesulfonyl derivative and then methylated and detosylated using the same procedures as Fischer (1915), whereas propenoic acid was converted to α-bromo-β-methoxypropionic acid and then either methylated via N-toluenesulfonyl derivatives using the method of Fischer (1915) or substituted with methylamine to give N-methylserine. However, it has been suggested that substitution reactions on α-halogenated amino acids with methylamine might

Figure I 102

Synthesis of N-methylthreonine [Izumiya (1949)]

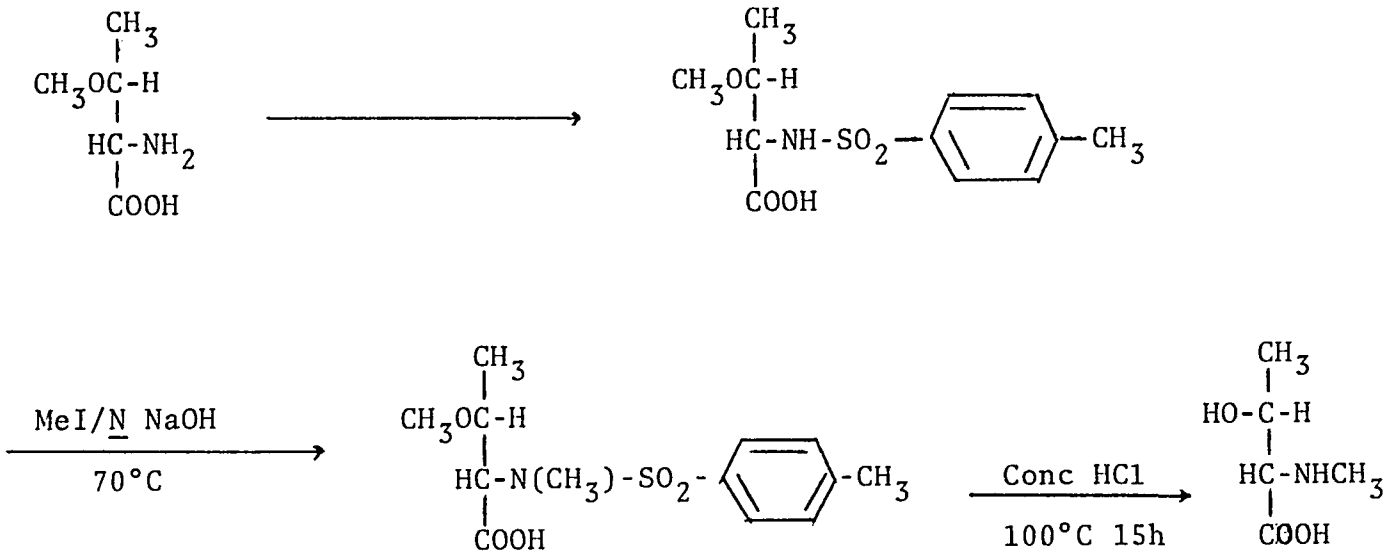
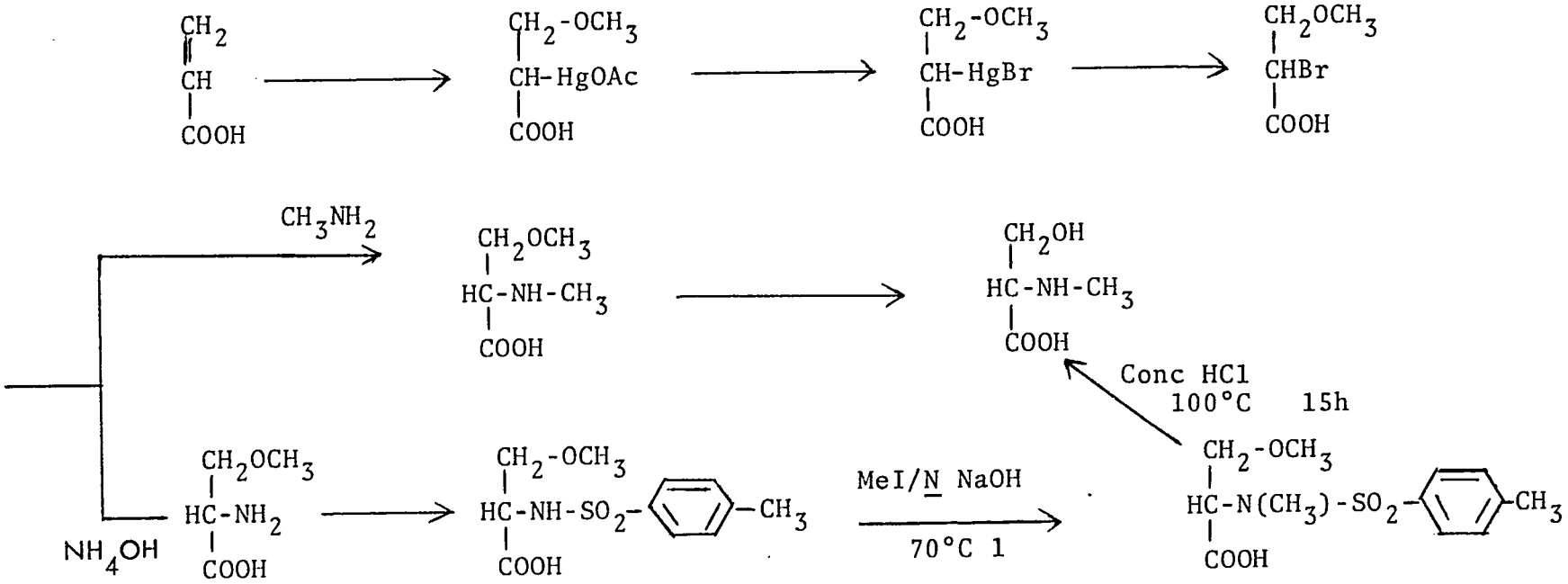


Figure I 103

Synthesis of N-methylserine [Izumiya (1950)]



possibly produce partially racemized compounds [Quitt (1963) p. 165]. It appears that the preparation of the starting materials (β-methoxythreonine and α-bromo-β-methoxypropionic acid) are particularly difficult, hence, the methods have not been popularly used for preparing N-methylthreonine and serine.

Using the principle of reductive alkylation, Quitt and coworkers (1963a) reported the methylation of N-benzylamino acids using formic acid and formaldehyde followed by hydrogenation to give optically active N-methylamino acids. The method was immediately adopted by Ebata et al (1966) to study the properties of N-methylamino acids. In fact, this has been the most common method used for preparing N-methylamino acids during the past decade. However, N-methylamino acids thus obtained must yet be converted to an appropriately protected derivative prior to use in peptide synthesis. It has been known that N-methylamino acids are more sterically hindered than unmethylated amino acids [Rudinger (1963) p.133]. Derivatization of N-methylamino acids often presents problems.

Urethanes were successfully alkylated using the Hepp method [Kraft (1890)]. Dannley and Lukin (1957) have reported the use of sodium hydride and alkyl halides for the alkylation of urethanes. Coggins and Benoiton (1968) then reported a new approach to the synthesis of N-methylamino acid derivatives. Their approach differed from previous procedures in that the derivatives are not prepared from the N-methylamino

acid, but instead a derivative of the parent amino acid is used as starting material and this is converted to the corresponding N-methylamino acid derivative. The reaction involves the methylation of an N-acyl or N-benzyloxycarbonyl-amino acid with sodium hydride and methyl iodide to give the N-protected, N-methylamino acid methyl ester. By selective deprotection, the resultant derivatives can be used directly for peptide synthesis.

Lederer and associates (1967) adopted Kuhn's (1955) methylation procedure to permethylate an N-acyloligopeptide hy treatment with methyl iodide and silver oxide in DMF at room temperature for determination of the amino acid sequence via mass spectrometry of the oligopeptides. This permethylation-reaction also was applied to N-benzyloxycarbonyl [Thomas et al (1968)] and N-tert-butyloxycarbonyl-peptides [Agarwal et al (1968)]. Applying this methylation procedure, Olsen (1970) published a synthetic study on N-protected, N-methylamino acids and found that an unprotected carboxyl group also underwent methylation to give the corresponding methyl ester. A similar study was also reported by Okamoto et al (1974). Generally, saponification is used to selectively remove the methyl ester [Bodanszky and Ondetti (1966) p. 43]. Racemization has generally been assumed to be negligible during saponification, although a recent report [Kenner and Seely (1972)] shows that saponification of a benzy-

loxycarbonyl dipeptide methyl ester can cause a small degree of racemization. An intensive study of the deprotection by saponification and acidolysis of fully protected N-methylamino acids [McDermott and Benoiton (1973b)] shows that appreciable racemization occurs during saponification of N-benzyloxycarbonyl, N-methylamino acid methyl esters by aqueous sodium hydroxide. Hence the optical purity of the N-methylamino acid derivatives obtained by Olsen (1970) and Okamoto et al (1974) is quite questionable.

In a continuing investigation of the conditions of methylation, Stoochnoff and Benoiton (1973) found that N and O methylation of N-benzyloxycarbonyl tyrosine took place at room temperature using sodium hydride and methyl iodide in neat THF. The conditions were then found applicable to the N-methylation of N-benzyloxycarbonylamino acids without ester formation [McDermott and Benoiton (1973a)]. This provides the simplest and most efficient method 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 benzyloxycarbonylamino acids without the isolation of intermediates. No saponification is required since no methyl ester is formed in the reaction and the products are potentially much more useful intermediates than N-p-toluenesulfonyl,

N-methylamino acids because the deprotection of N-benzyloxycarbonyl group is much simpler than the detosylation of N-p-toluenesulfonyl group.

The solid phase method of peptide synthesis was developed by Merrifield (1963) and ever since, the method has been popularly employed to synthesize numerous peptide analogues [Erickson and Merrifield (1976), p..257]. Although the benzyloxycarbonyl protecting group was originally used for α-amino functions in solid phase peptide synthesis [Merrifield (1963)], later the tert-butyloxycarbonyl protecting group was found to be more satisfactory. Therefore, all subsequent solid phase syntheses of peptides have used this protecting group. The major advantage is that tert-butyloxycarbonyl protecting group can be selectively removed without affecting the benzyl esters and methyl esters on the side-chains and also preventing undesired cleavage from the supporting resin. Hence, N-tert-butyloxycarbonyl, N-methylamino acids are useful materials for solid phase peptide synthesis.

This thesis describes the application of the method of McDermott and Benoiton (1973a) for synthesizing N-tert-butyloxycarbonyl, N-methylamino acids which can be used directly for peptide synthesis without any further derivatization or saponification.

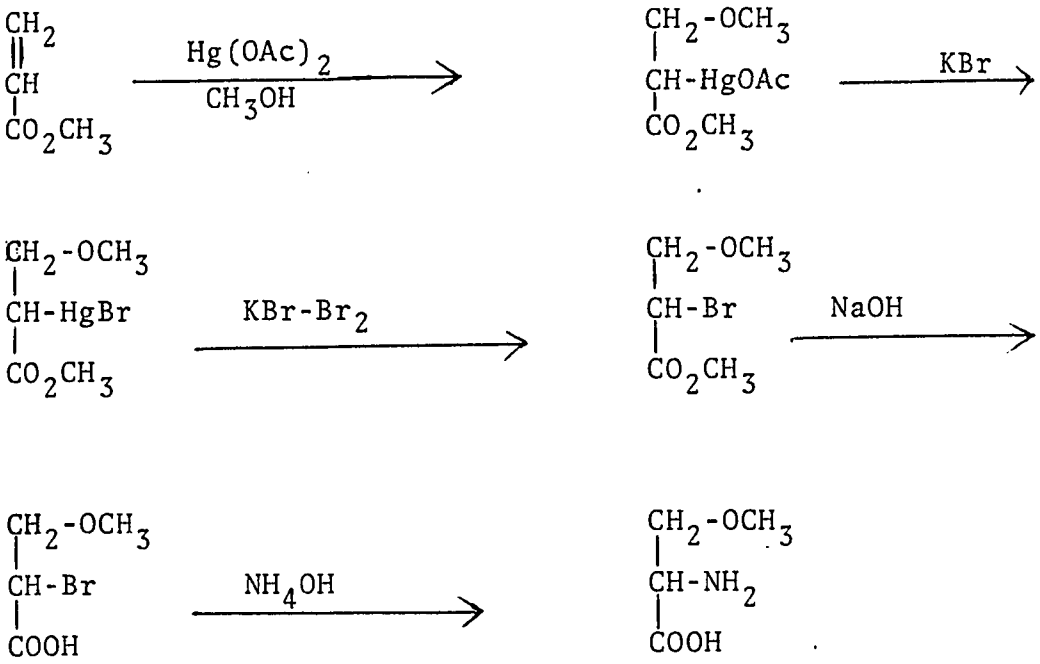
O-Methylated serine and threonine are the commoly used amino acid analogues for isosteric and isofunctional

replacements in peptide synthesis. They are of interest because the hydroxyl groups of threonine and serine can act either as proton donors or acceptors in hydrogen bond formation while the methoxy group of these amino acids can only act as proton acceptors. Hence, substitution of these amino acid analogues in a peptide may not only change the conformation but also affect the hydrogen bonding capacity and lipophilic or hydrophilic properties of the peptides [Rudinger (1971) p. 319]. Replacement of the hydroxyl group with O-methyl function can also furnish information on the role of the hydroxyl groups in a peptide hormone [Khosla et al (1976)].

O-Methyl-DL-serine was first synthesized by Schiltz and Carter (1936) via mercuration of methyl acrylate. The reactions involved is shown in Figure I, 104. Resolution into O-methyl-L-serine and N-acetyl, O-methyl-D-serine was achieved by hydrolysis of the N-acetyl derivative with hog renal acylase [Greenstein and Winitz (1961) p. 2233]. In addition, N-phthaloyl, O-methyl-L-serine was prepared by Fles and Belenovic (1956) by resolution of N-phthaloyl-O-methyl-DL-serine via the brucine salt. Recently, Hodges and Merrifield (1974) published a six-step method of obtaining optically pure N-tert-butyloxycarbonyl, O-methyl-L-serine using L-serine as the starting material. The compound obtained are shown to be optically pure (<0,1% D isomer). A simpler method of obtaining this compound would be very useful.

Figure I 104

Synthesis of O-methyl-DL-serine [Schiltz and Carter(1936)]



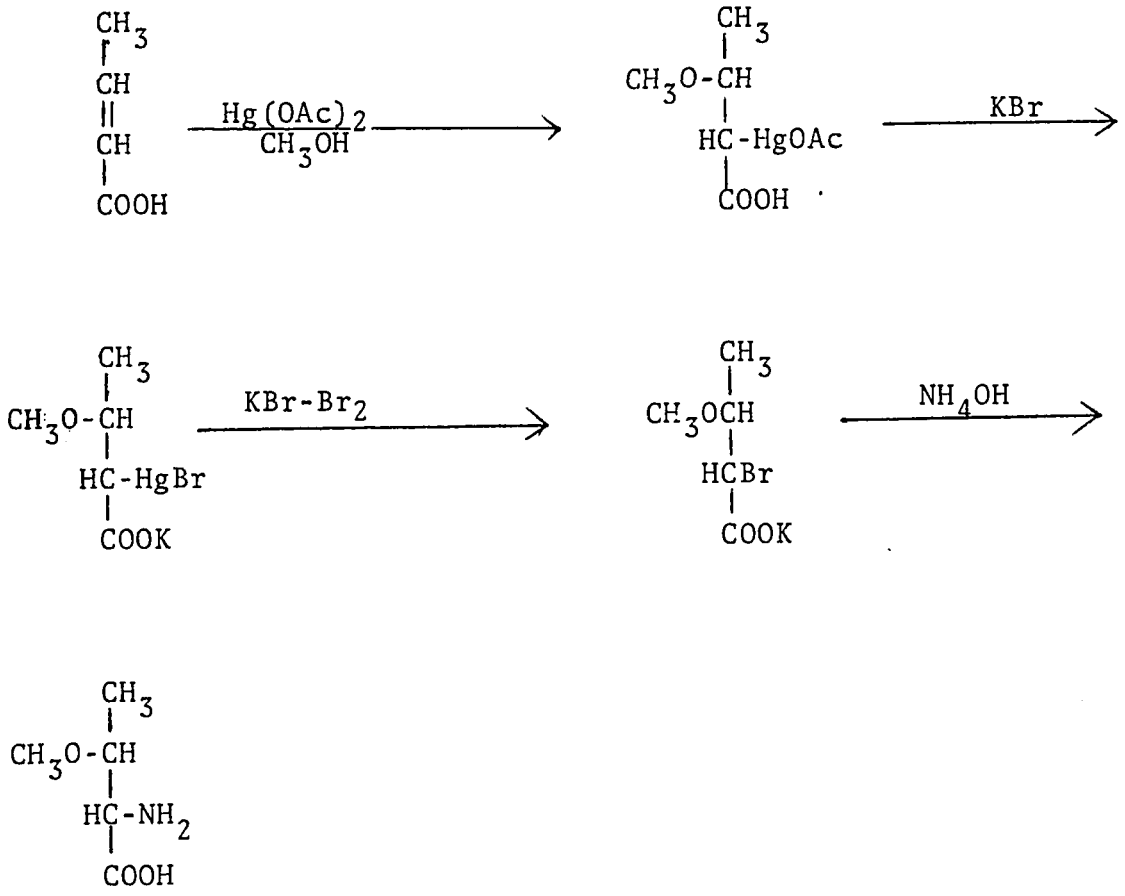
O-Methyl-DL-threonine was first synthesized by West and Carter (1937) via mercuration of crotonic acid. The reactions involved are shown in Figure I, 105. The resolution of O-methyl-DL-threonine and O-methyl-DL-allothreonine was achieved either via the brucine salts on their corresponding N-formyl derivatives [West and Carter (1937a)] or via the action of hog renal acylase I on the corresponding N-chloroacetyl derivatives [Greenstein and Winitz (1961a) p. 2251]. Rudinger (1965) found that reaction of N-benzyloxycarbonyl threonine or its methyl ester with a large excess of silver oxide and methyl iodide afforded N-benzyloxycarbonyl, O-methylthreonine methyl ester. Applying this method and using benzyl ester derivatives as starting material, Smulson and Rabinovitz (1968) reported the synthesis of N-benzyloxycarbonyl, O-methylthreonine benzyl ester. Recently, Jorgenson et al (1973), using the same procedure, published the synthesis of N-tert-butyloxycarbonyl, O-methyl threonine methyl ester. The disadvantage of this methylating procedure is that it is difficult to complete the methylation despite repetition of the methylation using fresh reagents.

This thesis also describes the development of a new, simple and direct method of preparing N-tert-butyloxycarbonyl, O-methylserine and N-benzyloxycarbonyl O-methylthreonine and their related compounds.

In the past, the optical purity of amino acids

Figure I 105

Synthesis of O-methyl-DL-threonine [West and Carter (1937)]



has generally been established using stereo-specific enzymes such as L and D-amino acid oxidase. Blanchard et al (1944) reported the oxidation of N-methyl-L-leucine catalyzed by the L-amino acid oxidase which was isolated from rat kidney. However, the specificity of this enzyme towards N-methylamino acids and their derivatives has not been studied in sufficient detail. Therefore, it is not certain if the optical purity of N-methylamino acids can be established using L-amino acid oxidase. Optical rotation measurements have served as the only criterion of optical purity for N-methylamino acids. These measurements, which require isolation of the pure N-methylamino acid in relatively large amount (10 to 20 mg), do not establish the optical purity, but serve simply as a means of comparison with previously isolated compounds.

Manning and Moore (1968) described a chromatographic method for the determination of amino acid enantiomers. The method consists of reacting the amino acid with L-leucine N-carboxyanhydride, followed by analysis for the leucyl-amino acid dipeptide with an Amino Acid Analyzer. Choice of the appropriate chromatographic system allows complete separation of the diastereomeric L,L and L,D-dipeptides. Attempts to apply this method to N-methylamino acids, using L-alanine N-carboxyanhydride instead of L-leucine N-carboxyanhydride had been made [Coggins and Benoiton (1970)]. They found that the reaction was sluggish and more by-products were formed, but it

was reported that the method of Manning and Moore (1968) is applicable to determine the enantiomeric purity of N-methylamino acids. This conclusion was retracted later, as the diastereomic peaks of H-Ala-MeLeu-OH were found to have been misassigned [McDermott and Benoiton (1973b)]. Since then, two reports on the use of this method for configurational assignments for N-methylalanine and N-methylphenylalanine [Mayer et al (1974)]; and optical purity determination for N-methylleucine [Ling and Vale (1975)] have been published. It is therefore necessary again to reexamine this question.

Despite the many methods available for determining the optical purity of amino acids, there is still no general method available for determining the optical purity of N-methylamino acids or their derivatives at present. N-protected, N-methylamino acids are useful, in peptide synthesis, so a method applicable to the derivatives as well as free N-methylamino acids would be valuable. This thesis describes an investigation, the object of which was to devise a new method for determining the optical purity of N-methylamino acids and their derivatives. An ideal method would be one that gave high sensitivity, unequivocal separation of enantiomers and was simple to apply. The method should also allow investigation of the optical purity of N-methylamino acids and their derivatives obtained by various methods.

II. MATERIALS AND METHODS

II. 1. MATERIALS

(a) Reagents obtained from commercial suppliers

Aminex A-5 resin [B]; Benzyloxycarbonyl chloride [P]; N,N'-dicyclohexylcarbodiimide [P]; Dicyclohexylamine [E]; Dowex 40W-X8, 20-50 mesh [Ba]; Formaldehyde solution, 37.0% by weight [F]; Fomic acid [F]; Methyl iodide [F]; tert-Butyl-azidoformate [P]; 10% Palladium on powdered charcoal catalyst [MCB]; Sodium hydride, 50% dispersion in oil [Ba]; Trifluoro-acetic acid, 99% [P].

(b) Purification and preparation of reagents

Acetonitrile: Reagent grade acetonitrile (Fisher) was distilled from P_2O_5 and the fraction boiling at 79-82°C collected.

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

1, 4- Dioxane: Reagent grade 1, 4- dioxane (Fisher) was poured through a column of aluminum oxide. The column was used until the effluent began to give a positive peroxide test, performed as follows; equal volume of dioxane and 4% aqueous KI were mixed, the dioxane was considered peroxide free if the solution was colourless after one minute. Purified dioxane was stored in a brown bottle under nitrogen in the cold. An alternate procedure for storing the purified solvent was to keep a little alumina in the bottom of the storage bottle and then the dioxane was filtered just before it was used. [Stewart and Young (1969) p. 31].

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

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 (1968)].

Tetrahydrofuran: Reagent grade tetrahydrofuran (Fisher) was refluxed over $LiAlH_4$ for 24 h, and distilled directly into the reaction vessel.

Thionyl chloride: Reagent grade thionyl chloride (Baker) was purified by distilling first over quinoline and then from a raw linseed oil as described by Fieser (1957), p. 345.

Triethylamine: The reagent (Pierce) was let stand over sodium hydroxide and distilled from a mixture with about 2% of phenyl isocyanate [Sauer, (1963)].

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 occured.

(c) Sources of amino acids and derivatives

(i) Amino acids

L-Alanine [G]; DL-Alanine [P]; L-Isoleucine [GIB]; D-Alloisoleucine [I]; L-Leucine [GIB]; DL-Leucine [G]; L-Lysine HCl [A]; DL-Lysine HCl [E]; L-Threonine [S]; L-Val [GIB].

(ii) Amino acid derivatives

N-ε-benzyloxycarbonyl-L-Lysine [P]; N-benzyloxycarbonyl-L-serine [C]; N-benzyloxycarbonyl-L-threonine [C]; N-benzyloxycarbonyl, O-t-butyl-L-serine dicyclohexyl-ammonium salt [Flu]; N-t-butyloxycarbonyl-L-alanine [Be]; N-t-butyloxycarbonyl-L-leucine H_2O [Be]; N-t-butyloxycarbonyl-L-isoleucine $\frac{1}{2}H_2O$ [Be]; N-t-butyloxycarbonyl-L-phenylalanine [Be]; [C], [PEN]; N-t-butyloxycarbonyl L-valine [Be]; N-t-butyloxycarbonyl, O-benzyl-L-serine [BAC]; N-t-butyloxycarbonyl, O-benzyl-L-tyrosine [BAC]; N-t-butyloxycarbonyl, O-benzyl-L-tyrosine [BAC].

(d) Compounds obtained by synthesis

N-benzyloxycarbonyl amino acids: N-benzyloxycarbonyl amino acids were prepared by the method of Greenstein and Winitz (1961), procedure 10-28, p. 891. Amino acids were acylated in 4 N NaOH with benzyloxycarbonyl chloride at 5°C. For acylated of L-threonine, 0.5 mol $NaHCO_3$ was used instead of 4 N NaOH. The yields of N-benzyloxycarbonyl amino acids by this method were 80-86%.

N-benzyloxycarbonyl, N-methyl amino acids: N-benzyloxycarbonyl, N-methyl amino acids were prepared by the method of McDermott and Benoiton (1973a). The benzyloxycarbonyl amino acids were methylated with sodium hydride and methyl iodide in the mol ratio (1:3:8). Tetrahydrofuran was used as solvent. The products were obtained as oil with 82-84% yield. Without crystallization, the products were characterized with Proton n.m.r. and amino acid analyzer.

α-Amino acid N-carboxyanhydride: L-alanine N-carboxyanhydride and DL-Leucine N-carboxyanhydride were prepared by the method of Leuchs et al. (1907) using the carboalkoxy procedure. A mixture of 5 g of the N-benzyloxycarbonyl amino acid in 25 ml purified thionyl chloride was warmed at 60°C for 30 min. Moisture was excluded by using a calcium

chloride drying tube. After removal of excess thionyl chloride under reduced pressure, the residue was triturated with pet-ether until it solidified. L-alanine N-carboxyanhydride was recrystallized from ether/pet-ether, m.p. 92°C; lit m.p. 92°C [Greenstein and Winitz (1961), p. 864]. DL-Leucine N-carboxyanhydride was recrystallized from ethyl acetate/pet-ether, m.p. 49-50°C; lit. m.p. 48-50°C [Greenstein and Winitz (1961) p. 864]. The products were stored in a drying bottle over calcium sulphate to prevent moisture and kept in the refrigerator until it was used.

N-tert-butyloxycarbonyl amino acids: N-tert-butyloxycarbonyl amino acids were prepared using magnesium oxide; the Schwyzer method [Schwyzer et al. (1959)]. A mixture of amino acid (20 mmol), t-butyloxycarbonyl azidoformate (4.3 g, 30 mmol), magnesium oxide (1.6 g, 40 mmol), dioxane (60 ml), and water (30 ml) was stirred at 40-45°C for 20 h. Magnesium oxide was removed by filtration. The filtrate was washed 3 times with ether to remove unreacted azide. The remaining aqueous phase was chilled with ice and acidified with solid citric acid. The N-tert-butyloxycarbonyl amino acid was extracted with ethyl acetate. The ethyl acetate was washed 3 times with small portion of water (saturated with NaCl solution for water soluble derivatives). It was dried over $MgSO_4$ and evaporated under reduced pressure.

All the N-tert-butyloxycarbonyl amino acids were obtained in 86-90% yield. Without attempting to crystallize them the products were characterized using proton n.m.r. and were immediately used for synthesizing the N-tert-butyloxycarbonyl, N-methyl amino acids.

N-p-Toluenesulfonyl amino acids: N-p-toluenesulfonyl-L-alanine and valine were prepared by the general procedure of Fisher and Lipschitz (1915). To a solution of 5 g L-amino acid in 55 ml of N NaOH was added 11 g p-toluenesulfonyl chloride. The mixture was stirred vigorously at room temperature for 3 h; the excess acid chloride removed by filtration and the filtrate acidified to congo red with diluted HCl. N-p-toluenesulfonyl amino acid, which separated as white precipitate, was collected, washed and dried. N-p-toluenesulfonyl-L-alanine was recrystallized from ethyl acetate/pet-ether, 93% yield, m.p. 133-135°C; lit m.p. 134-135°C [Fisher and Lipschitz (1915)]. N-p-toluenesulfonyl-L-valine was recrystallized from ethyl acetate/pet-ether, 72% yield, m.p. 146-147°C; lit m.p. 147°C [Harris and Work (1950)].

(e) List of Manufacturers

[B] Bio-Rad Laboratories, Richmond, California, U.S.A.

[Ba] J.T. Baker, Phillipsburg, New Jersey, U.S.A.

[BAC] Bachem. Inc., Marina Del Rey, California, U.S.A.

[Be] Beckman Inst. Inc., Pala Alto. California, U.S.A.

[C] Chemical Dynamic Cooperation, South Plainfield, New Jersey, U.S.A.

[E] Eastman Kodak Co., Rochester, New York, U.S.A.

[F] Fisher Scientific, Fair Lawn, New Jersey, U.S.A.

[Flu] Fluka AG, Buchs, Switzerland

[GIB] Grand Island Biological Co., Grand Island, New York U.S.A.

[I] ICN Pharmaceuticals, Inc., Cleveland, Ohio, U.S.A.

[MCB] Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.

[P] Pierce Chemical Co., Rockford, Illinois, U.S.A.

[PEN] Peninsula Laboratories, San Carlos, California, U.S.A.

II. 2. METHODS

(a) General methods of deprotection

Hydrogenation: N-benzyloxycarbonyl amino acids and derivatives, N-benzyl amino acids and benzyl esters were deprotected by hydrogenation. All the hydrogenations were carried out at 1 atm pressure in an apparatus similar to that described by Wiberg (1960), p. 228. 80% Acetic acid and Palladium on charcoal were used as solvent and catalyst respectively. After the deprotection, amino acids were recovered in high yields 85-92% by evaporation.

30% hydrogen bromide in acetic acid: Sample was suspended in 30% HBr/AcOH for 2 h at room temperature to remove the N-benzyloxycarbonyl groups. Upon completion, HBr/AcOH were removed by evaporation [Ben-Ishai and Berger (1952)]. The extent of N-methylation was determined by quantitative amino acid analysis of the deprotected sample (p. 36).

70% aqueous trifluoroacetic acid (v/v): N-tert-butyloxycarbonyl amino acids and derivatives were deprotected by suspending the samples in 70% tfa for 2 h at roon temperature [Sieber (1968), p. 236]. The extent of N-methylation was determined by quantitative amino acid analysis of the deprotected sample (p. 36).

(b) General methods

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

Microanalyses: Samples for analysis were dried in vacuo over P_2O_5 for 24 h at 56°C and the analyses were carried out by Organic Microanalyses Montreal, Quebec, Canada.

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

Proton Nuclear Magnetic Resonance Spectroscopy: Proton n.m.r. (Varian Model T60 Spectrometer) was used routinely for monitoring reactions and characterizing products. The spectra were obtained at probe temperature 34°C and the samples were run as 10% (w/v) solutions in deuterochloroform, trifluoroacetic 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. R. R. Fraser). Spectra were scanned in the range 0-10 δ (ppm). Proton n.m.r. (Varian Model HA-100) was used to examine a few protected lysyl dipeptides. The spectra

were obtained at room temperature and the samples were run as 10% w/v solution in deuterochloroform with 1% v/v tetramethylsilane as internal standard. The spectra were scanned in the range of 0-10 δ (ppm).

(c) Amino acid analyses

Amino acid analyzer: Amino acid analyses were carried out using a Beckman Model 120B Automatic Amino Acid Analyzer essentially according to the Method of 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 Aminex A-5 resin (Bio-Rad Laboratories, Richmond, California), and a medium column of (0.9 x 15 cm) containing Aminex A-5 resin. Three different eluting buffers were used generally: 0.20 N sodium citrate, pH 3.28; 0.20 N sodium citrate, pH 4.25; 0.35 N sodium citrate, pH 5.28. The individual amino acid was determined by the HXW method of Spackman, Stein and Moore (1958) which involved the formula $\mu mol = \frac{HXW}{C}$ (H = net height of peak = absorbance; W = width of peak at half net height; C = Constant.

N-methylamino acids were analyzed at half of the normal flow rate (34 ml/h) according to the method of Coggins and Benoiton (1970). Constants for the common amino acids were determined using a calibration mixture (obtained from Bio-Rad) and for N-methylamino 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, so that the final concentration was in the range

1.0-2.0 µmol/ml (0.5 ml samples were analyzed). Analyses carried out on the same day or on successive days were reproducible to ± 1%. The reproducibility from week to week was not so good and new constants were determined for each group of analyses.

Thin layer chromatography: Pre-coated thin layer chromatography plates of silica gel-25 uv 254 (20 x 20 cm) were purchased from Macherey-Nagel and Co., Germany.

The compounds investigated by this technique were ninhydrin positive; they were detected by spraying the plates with 0.5% solution of ninhydrin in n-butanol and then heating in an oven at 100°C for 5 min. The colours of the spots were reddish brown and purple.

Two solvent systems were used: n-butanol/acetic acid/water (4/1/1, v/v/v), and n-butanol pyridine/acetic acid/water (6/1/1/2, v/v/v/v). The compounds investigated by tlc were derivatives of serine and threonine.

III. RESULTS AND DISCUSSION

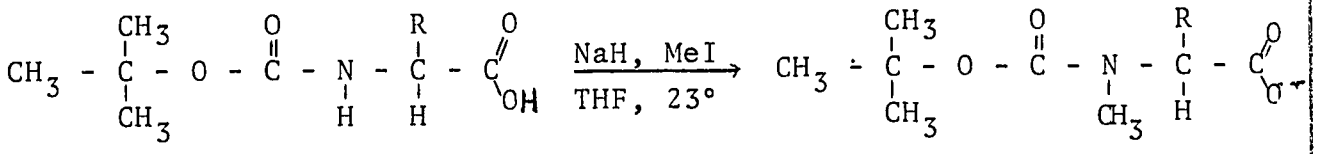
III. 1. The synthesis of N-tert-butyloxycarbonyl, N-methyl amino acids by N-methylation

(a) Preparation of N-tert-butyloxycarbonyl, N-methyl amino acids

A new method of preparing N-benzyloxycarbonyl derivatives of N-methylamino acids by N-methylation of the N-benzyloxycarbonylamino acid using sodium hydride and methyl iodide in THF/DMF (10:1) at 80°C for 24 h has been described [Coggins and Benoiton (1968)]. The method has subsequently been modified by allowing the methylation in neat THF at room temperature [McDermott and Benoiton (1973a)]. The latter method is preferable since the former also esterifies the carboxyl group, and removal of the ester group by saponification is accompanied by some racemization [McDermott and Benoiton (1973b)]. This method for synthesizing N-methylamino acids has one very important advantage over all the earlier methods. It allows direct preparation of N-protected N-methylamino acids which may be used directly in the synthesis of peptides containing N-methylamino acids. N-tert-butyloxycarbonylamino acids are useful in solid phase peptide synthesis because the N-tert-butyloxycarbonyl group can be selectively removed. Our first experiments were designed to find out whether N-tert-butyloxycarbonyl, N-methyl amino acid, which can be used directly for peptide

synthesis, could be prepared by N-methylation with sodium hydride and methyl iodide in THF as shown in the following route [1].

Route [1]. N-methylation of Boc-amino acids.



N-tert-butyloxycarbonyl-L-leucine was chosen as model compound for testing the reaction. It is generally believed that the side-chain of leucine is less sterically hindered and it may be easier to be N-methylated. The results of a series of experiments showed that the product contained less than 0.1% of unmethylated leucine and the method gave analytically and optically pure product in good yield.

Having succeeded in N-methylation of N-tert-butyloxycarbonylleucine, we now tested the method on the preparative scale as well as with other N-tert-butyloxycarbonyl amino acids. All the experiments unless specifically mentioned were carried out on the L-isomers. The optical purity of the product was established by the ion-exchange chromatography method which is described in Part III. 4 and 5. The method allowed detection of one part of D-isomer in the presence of 1000 parts of the L-isomer, that is, the samples of N-tert-butyl-

oxycarbonyl, N-methylleucine found to be optically pure by this method contained less than 0.1% of the D-isomer. All the N-tert-butyloxycarbonylamino acids used for N-methylation were synthesized by the Schwyzer method (1959) as described in Part II, 1 d. The physical properties of the N-tert-butyloxycarbonyl, N-methylamino acids are given in Tables III. 301-303. It was found that the derivatives of N-methylalanine, valine and phenylalanine had melting points higher than the literature values. This is the first time that N-tert-butyloxycarbonyl, N-methyl valine has been crystallized.

General procedure of N-methylation: N-tert-butyloxycarbonylamino acid (10 mmol) and methyl iodide (5 ml, 80 mmol) were dissolved in THF (30 ml). The solution was cooled to 0°C in a flask protected from moisture by a drying tube containing indicating anhydrous $CaSO_4$. Sodium hydride dispersion (1.32 g, 30 mmol) was added cautiously with gentle stirring. The suspension was stirred at room temperature for 24 h. Ethyl acetate (50 ml) was then added (to consume the sodium hydroxide formed after the addition of water), followed by water, dropwise, to destroy the excess sodium hydride. The solution was evaporated to dryness, and the oily residue partitioned between ether (30 ml) and water (100 ml). The ether layer was washed with aqueous 5% $NaHCO_3$ (50 ml), and the combined aqueous extracts acidified to pH 3 with solid citric acid. The product was extracted into ethyl acetate

(50 ml x 2). The extract was washed with water (50 ml x 2), 5% aqueous sodium thiosulfate (50 ml x 2) to remove iodine, and water (50 ml), dried over $MgSO_4$ and evaporated.

N-tert-butyloxycarbonyl, N-methylleucine: N-tert-butyloxycarbonylleucine (10 mmol, 2.31 g) was N-methylated using the general procedure. The product was crystallized from hot water. After recrystallization from the same solvent, it was obtained in 83% (2.03 g) yield. The compound had the same rotation as reported by Ling and Vale (1975), however it was reported as an oil by Okamoto (1975) who used the silver oxide and methyl iodide in DMF method for N-methylation. The chromatographic properties of the deprotected N-tert-butyloxycarbonyl, N-methylleucine on the amino acid analyzer were identical with those of the authentic compound and there was no contamination with leucine. The n.m.r. spectrum showed no evidence of any α-C-methylation and the integration of the n.m.r. spectrum and microanalysis were in good agreement with theory. Lit. m.p. 57-58°C, $[\alpha]_D^{23}$ -25.3° (c, 1 in DMF) [Ling and Vale (1975)].

N-tert-butyloxycarbonyl, N-methylalanine: The methylation of the N-tert-butyloxycarbonylalanine requires twice the volume of THF. N-Benzyloxycarbonylalanine was methylated in parallel with N-tert-butyloxycarbonylalanine in the normal amount of the THF as described in general method. The sodium salt of N-tert-butyloxycarbonylalanine precipitated after 24 h.

whereas N-benzyloxycarbonylalanine was completely N-methylated in 86% yield (1.6 g) after adding an equal amount of THF and stirring for another 8 h. Long needle-shaped crystals were obtained from ethyl acetate/pet-ether with m.p. 93-94°C which is higher than that for the compound obtained by detosylation of p-toluenesulfonyl, N-methylalanine using 2 N HBr in acetic acid at room temperature for 24 h, and then bocylation [Andreatta and Scheraga (1971)]. Lit. m.p. 91-92 C, $[\alpha]_D^{20}$ -31.8°C (c, 1 in ethanol).

N-tert-butyloxycarbonyl, N-methylisoleucine: N-tert-butyloxycarbonylisoleucine (10 mmol, 2.31 g) was N-methylated according to the general method. The product was obtained as an oil in 90% (2.20 g) yield. The product was converted to the dicyclohexylammonium salt by adding excess amine to the product dissolved in ether, followed by the addition of pet-ether and storage at 4°C. Recrystallization from the same solvent mixture gave yield of 75% (3.19 g). m.p. 117°C and $[\alpha]_D^{26}$ -45.6° (c, 0.5 methanol) lit. oil [Khosla et al (1974)].

N-tert-butyloxycarbonyl, N-methyl-D-alloisoleucine: N-tert-butyloxycarbonyl-D-alloisoleucine (10 mmol, 2.31 g) was N-methylated according to the general procedure. The product could not be crystallized. The dicyclohexylammonium salt was obtained as described for N-tert-butyloxycarbonyl, N-methyl-isoleucine. Recrystallization from ether / pet-ether gave 63% (2.68 g) with m.p. 94°C, $[\alpha]_D^{26}$ +42.6° (c, 0.5 methanol). Lit.

oil for L-isomer [Khosla et al (1974a)].

N-tert-butyloxycarbonyl, N-methylphenylalanine: N-tert-butyloxycarbonylphenylalanine (10 mmol, 2.65 g) was N-methylated using the general procedure above. The product was an oil in 90% (2.5 g) yield. The dicyclohexylammonium salt was obtained as described for the above compounds. Recrystallization from the same solvent mixture (ether/pet-ether) gave 82% (3.55 g) yield. The melting point of the product, 176°C, was found to be higher than that reported by Blake and Li (1972) and Okamoto et al (1974) using silver oxide and methyl iodide in DMF for N-methylation. Lit. m.p. 165-170°C, $[\alpha]_D$ -25° (c, 1 methanol) [Blake and Li (1972)]. m.p. 174 C, $[\alpha]_D^{15}$ -28.1°C (c, 1 ethanol) [Okamoto et al (1974)].

N-tert-butyloxycarbonyl, N-methyl-DL-phenylalanine: N-tert-butyloxycarbonyl-DL-phenylalanine (10 mmol, 2.65 g) was N-methylated using the general procedure above. The product was obtained as crystals from ethyl acetate and pet-ether. Recrystallization from the same solvent mixture gave 85% yield (2.37 g) with m.p. 136°C.

N-tert-butyloxycarbonyl, N-methylvaline: N-tert-butyloxycarbonylvaline was N-methylated using the general procedure. However, the methylation of this compound was extremely sensitive to the quality of THF. Freshly purified THF was required for the methylation. It was found that the product crystallized from ethanol and water after being kept

at 4°C for 2 years in 85% (1.98 g) yield with m.p. 58-59°C. It has been reported that N-tert-butyloxycarbonyl N-methyl-valine was an oil [Meienhofer and Patel (1971), Okamoto et al (1974)]. The recrystallization from the same solvent mixture was normal when a seed crystal was used. The dicyclohexyl-ammonium salt was obtained by the same procedure as described above. The melting point of the dicyclohexylammonium salt recrystallized from ether/pet-ether was 113-114°C and was significantly higher than those reported by Meienhofer and Patel (1971). m.p. 107-110°C; $[\alpha]_D^{20}$ -54.1° (c, 1 methanol) and Okamoto et al (1974), m.p. 109-110°C, $[\alpha]_D^{15}$ -51.9° (c, 1 methanol).

Table III 301

Physical properties of N-tert-butyloxycarbonyl N-methylamino acids

Amino acid	Yield (%)	M.P. (°C)	$[\alpha]_D^{26b}$ (°)
Boc-MeAla-OH	86	93-94[c]	-30.4
Boc-MeLeu-OH	83	56-57[d]	-24.6
Boc-MeVal-OH	85	58-59[e]	-90.0
Boc-MeVal-OH.DCHA	80[a]	113-114	-49.3
Boc-MeIle-OH.DCHA	75[a]	117	-45.6
Boc-D-MeaIle-OH.DCHA	63[a]	94	+42.8
Boc-MePhe-OH.DCHA	82[a]	176	+25.5
Boc-DL-MePhe-OH	85	136[c]	

[a] Yield of dicyclohexylammonium salt which is recrystallized from ether/pet-ether.

[b] (c, 0.5 in methanol) for dicyclohexylammonium salts; (c, 0.5 in ethanol) for acids.

[c] Crystallized from ethyl acetate/pet-ether.

[d] Crystallized for hot water.

[e] Crystallized from ethanol/water.

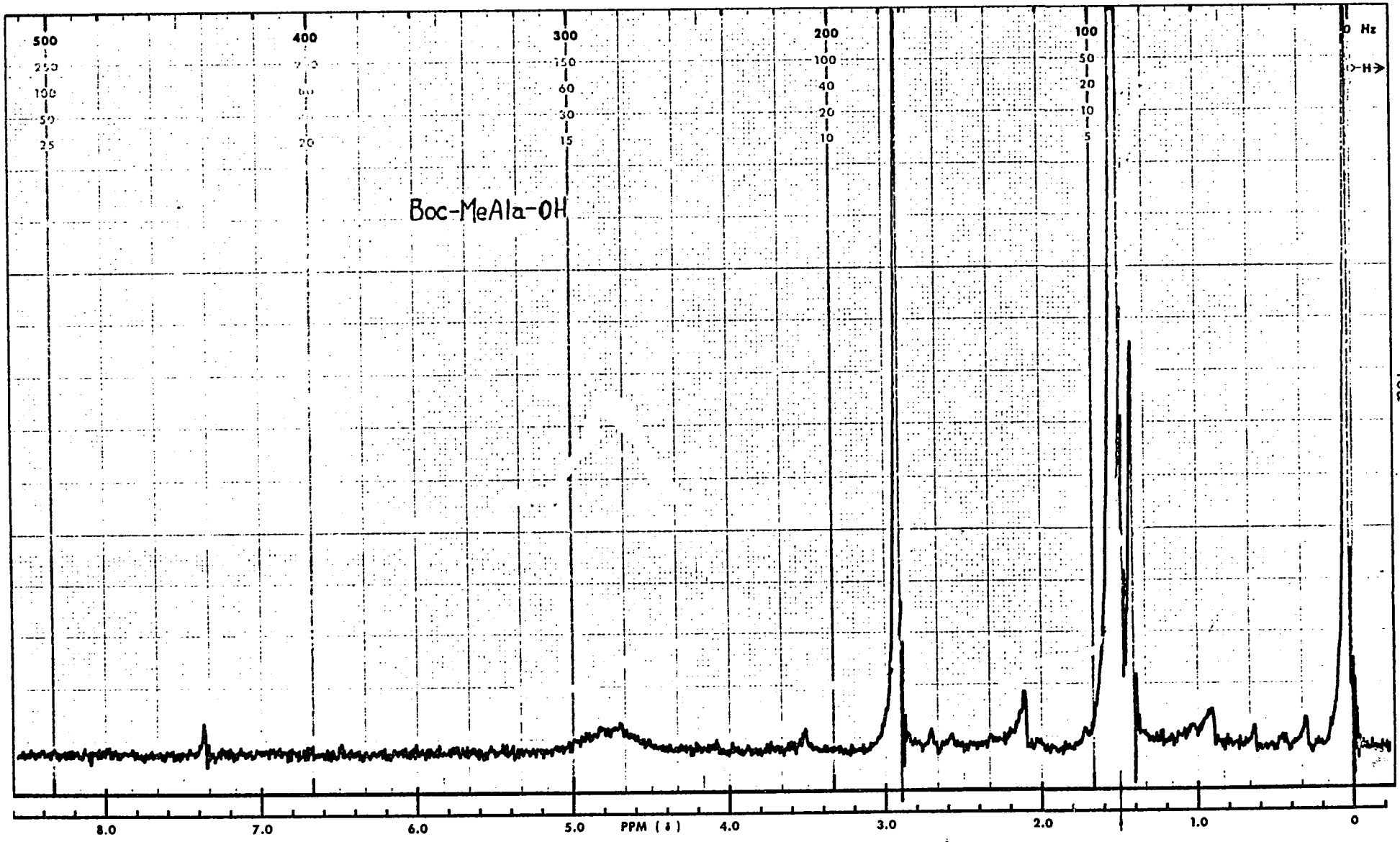
Table III 302

^{1}H-n.m.r. data of N-tert-butyloxycarbonyl, N-methyl-amino acids[a] (ppm).

Amino Acid	δ_{N-CH_3}	δ_{Bu^t}
Boc-MeAla-OH	2.84	1.40
Boc-MeLeu-OH	2.80	1.40
Boc-MeVal-OH	2.84	1.50
Boc-MeIle-OH	2.85	1.50
Boc-D-MeaIle-OH	2.85	1.50
Boc-MePhe-OH	3.00	1.50
Boc-DL-MePhe-OH	3.00	1.50

[a] The signals for $N-CH_3$ and Bu^t were integrated for 3H and 9H respectively.

Nuclear magnetic resonance spectrum of Boc-MeAla-OH in $CDCl_3$



Nuclear magnetic resonance spectrum of Boc-MeVal-OH in $CDCl_3$

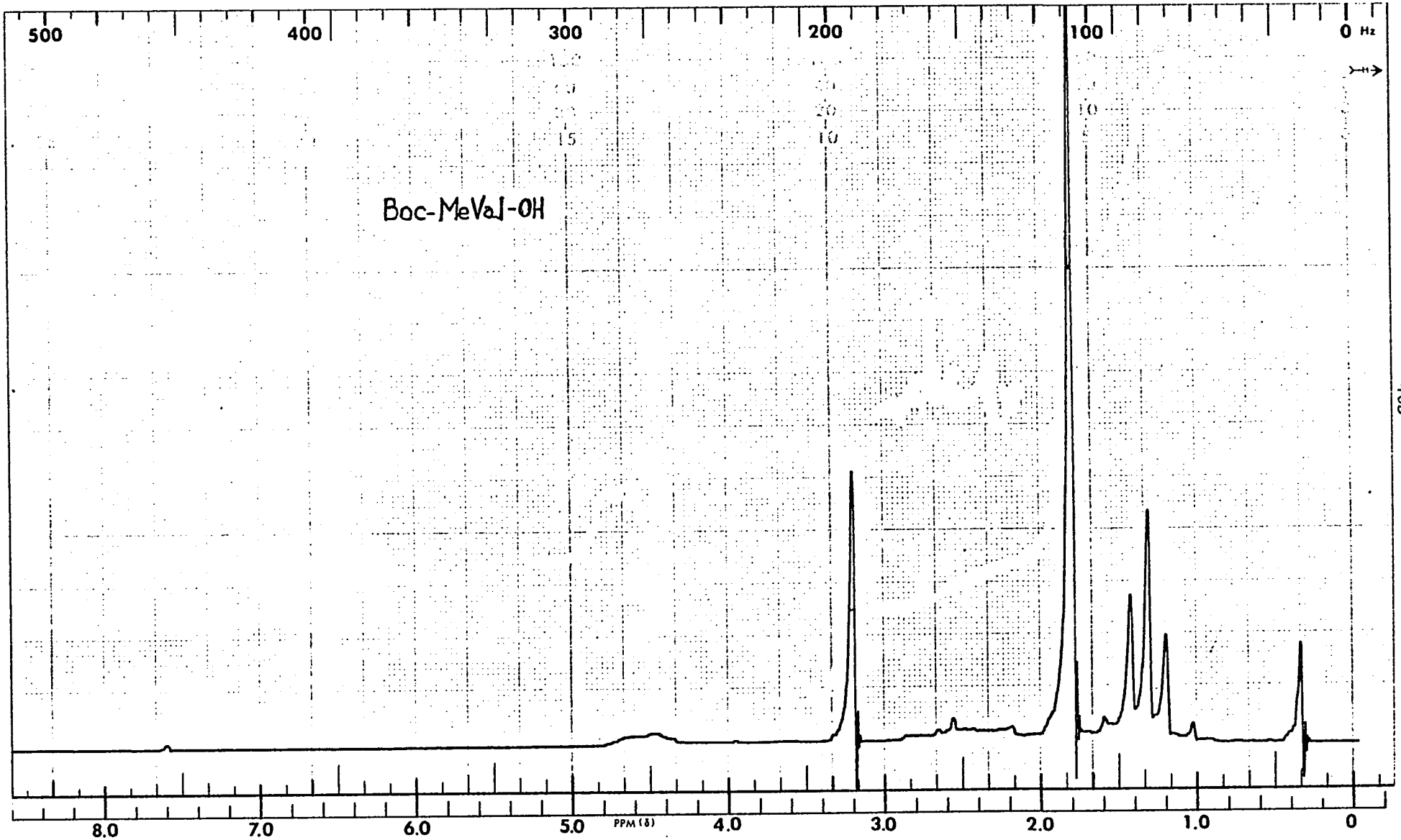


Table III 303

Analytical data for N-tert-butyloxycarbonyl N-methylamino acids

Amino acid	Fomula	C,H,N, Calculated C,H,N, Found		
Boc-MeAla-OH	$C_9H_{17}NO_4$	53.2 53.4	8.4 8.6	6.9 7.1
Boc-MeLeu-OH	$C_{12}H_{23}NO_4$	59.7 59.7	9.45 9.50	5.7 5.5
Boc-MeVal-OH	$C_{11}H_{21}NO_4$	57.1 57.3	9.2 9.4	6.1 6.2
Boc-MeVal-OH.DCHA	$C_{23}H_{44}N_2O_4$	66.9 66.7	10.75 10.6	6.8 6.8
Boc-MeIle-OH.DCHA	$C_{24}H_{46}N_2O_4$	67.6 67.8	10.9 11.0	6.6 6.6
Boc-D-MeaIle-OH.DCHA	$C_{24}H_{46}N_2O_4$	67.6 67.8	10.9 11.0	6.6 6.7
Boc-MePhe-OH.DCHA	$C_{27}H_{44}N_2O_4$	70.4 70.1	9.6 9.5	6.1 5.9

(b) Preparation of N,O-protected N-methylamino acids

The general applicability of the N-methylation method using sodium hydride and methyl iodide in THF at room temperature to the synthesis of some derivatives of the other N-methylamino acids containing anhydroxyl group on their side-chains was investigated. The method was found applicable to the preparation of N-tert-butyloxycarbonyl, O-benzyl, N-methyl-L-threonine and L-tyrosine. The O-benzyl groups of these two compounds were found to be stable to these reaction conditions, allowing the preparation of the appropriate N-methyl derivatives in good yields. The ^{1}H n.m.r. spectra of these products show no evidence of any β-elimination, methyl ester formation nor α-C-methylation. The purity of the products was established with the Amino Acid analyzer and there was no contamination by threonine or tyrosine. Thus this method furnishes a easy way to obtain O-benzyl, N-methyl tyrosine derivative which was thought to be difficult to synthesize [Blake and Li (1972)].

N-Methylation of N-tert-butyloxycarbonyl serine using silver oxide and methyl iodide in DMF gave N-methyldehydroalanine methyl ester [Olsen (1970)], as β-elimination occurred. N-Methylation of N-benzyloxycarbonyl, O-benzyl serine using a similar procedure resulted in a mixture N-benzyloxycarbonyl, N-methyldehydroalanine methyl ester and N-benzyloxycarbonyl, N-methyl, O-benzylserine methyl ester, as β-elimination

had partly occurred in this reaction [Okamoto et al (1974)]. Using sodium hydride and methyl iodide in THF, N-tert-butyloxycarbonyl, O-benzyl serine underwent β-elimination simultaneously with N-methylation to give an excellent yield of N-tert-butyloxycarbonyl, N-methyl dehydroalanine. In fact this provides the easiest method to obtain N-methyl dehydroalanine derivatives despite the suggestion that this derivative could be accessible by methylation of N-tert-butyloxycarbonyl dehydroalanine methyl ester [Grant et al (1975)].

It had been observed in this laboratory that N-benzyloxycarbonyl, O-tert-butyl threonine gave oily N-methyl derivative in good yield using sodium hydride and methyl iodide for N-methylation [McDermott and Benoiton (1973a)]. An attempt to N-methylate N-benzyloxycarbonyl, O-tert-butylserine using the same procedure resulted in a mixture containing about 75% of the desired product, N-benzyloxycarbonyl, N-methyl, O-tert-butylserine, and 25% N-methyldehydroalanine derivative. The latter could be removed easily by crystallizing it out as the dicyclohexylammonium salt, thus allowing a practical synthesis of the desired compound which did not form an insoluble salt with dicyclohexylamine in ether/pet-ether.

It was thought that β-elimination might be

reduced if the temperature of the reaction were lowered. It was found that at 5°C N-benzyloxycarbonyl, O-tert-butylserine was completely N-methylated without any β-elimination, although the reaction was slower. In fact, this temperature allowed the preparation of N-tert-butyloxycarbonyl, N-methyl, O-benzyl-serine in 80% yield as well1. Hence the methylation at 5°C provides us two routes to obtain optically pure N-methylserine which is not readily accessible. The detailed discussions of the preparation of N-methylserine derivatives are in Part III, 2 c,p. 73.

All the starting materials for this section were obtained from commercial suppliers as shown in Part II p. 28. All the experiments unless specifically mentioned were carried out on the L-isomer. Amino Acid analyzer and ^{1}H n.m.r. were used to identify and characterize the products. The physical properties of the products are given in Tables III 304-305. The optical purity of N-benzyloxycarbonyl, N-methyl, O-tert-butyl serine and N-tert-butyloxycarbonyl, N-methyl, O-benzyl threonine was established using the method described in Part III. 4. It was found that they contained less than <.1% D-isomer.

N-tert-butyloxycarbonyl, N-methyl, O-benzyl-threonine: N-tert-butyloxycarbonyl, O-benzylthreonine (5 mmol 1.54 g) was N-methylated according to the general procedure (p. 40). The product was obtained as an oil in 75% (1.2 g) yield.

N-tert-butyloxycarbonyl, N-methyl, O-benzyl-tyrosine: N-tert-butyloxycarbonyl, O-benzyltyrosine (5 mmol 1.40 g) was N-methylated according to the general procedure (p. 40). The product was obtained as an oil in 70% (1.02 g) yield.

N-tert-butyloxycarbonyl, N-methyldehydroalanine: N-tert-butyloxycarbonyl, O-benzyl serine (5 mmol, 1.56 g) was N-methylated according to the general procedure (p. 40). After isolation and purification, the product was obtained as crystals. Recrystallization from ethyl acetate/pet-ether gave 86% (2.06 g) yield with m.p. 80°C. Analysis: calculated for $C_9H_{15}NO_4$ (201.9): C, 53.7; H, 75; N, 6.95. Found: C, 53.8; H, 7.5; N, 6.70.

N-tert-butyloxycarbonyl, N-methyl, O-benzyl-serine: N-tert-butyloxycarbonyl, O-benzylserine (5 mmol, 1.56 g) was N-methylated according to the general procedure (p. 40) except that the reaction vessel was kept in an ice-bath for 15 min after addition of sodium hydride and then left at 5°C for 72 h. The product was isolated and purified as described in the general procedure. The product was obtained as an oil

in 80% (1.31 g) yield.

N-benzyloxycarbonyl, N-methyl, O-tert-butyl-serine: N-benzyloxycarbonyl, O-tert-butylserine (5 mmol, 1.48 g) was N-methylated using the same procedure as N-methylation of N-tert-butyloxycarbonyl, O-benzylserine. The reaction vessel was kept in an ice-bath for 15 min after the addition of sodium hydride and left at 5°C for 48 h. The product was obtained as an oil in 82% (1.27 g) yield.

Table III 304

Properties of N, O-protected, N-methylamino acids[a]

Amino acid	Yield (%)	M.P. (°C)
Boc-MeThr(Bzl)-OH	75	oil
Boc-MeTyr(Bzl)-OH	70	oil
Boc-MeΔAla-OH	86	80[b]
Boc-MeSer(Bzl)-OH[c]	80	oil
Z-MeSer(Bu^t)-OH[d]	82	oil

[a] Compounds obtained for the first time.

[b] Crystallized from ethyl acetate/pet-ether.

[c] O-Methylation at 5°C for 72h

[d] O-Methylation at 5°C for 48h.

Table III 305

^{1}H-n.m.r. data for N-protected , N - methyl , hydroxy amino acids[d] [ppm].

Amino acid	δ_{N-CH_3}	δ_{Bu^t}
Boc-MeThr(Bzl)-OH	3.00	1.50
Boc-MeTyr(Bzl)-OH	2.73	1.37
Boc-MeΔAla-OH[a]	3.18	1.50
Boc-MeSer(Bzl)-OH[b]	2.90	1.40
Z-MeSer(Bu^t)-OH[c]	3.00	1.40

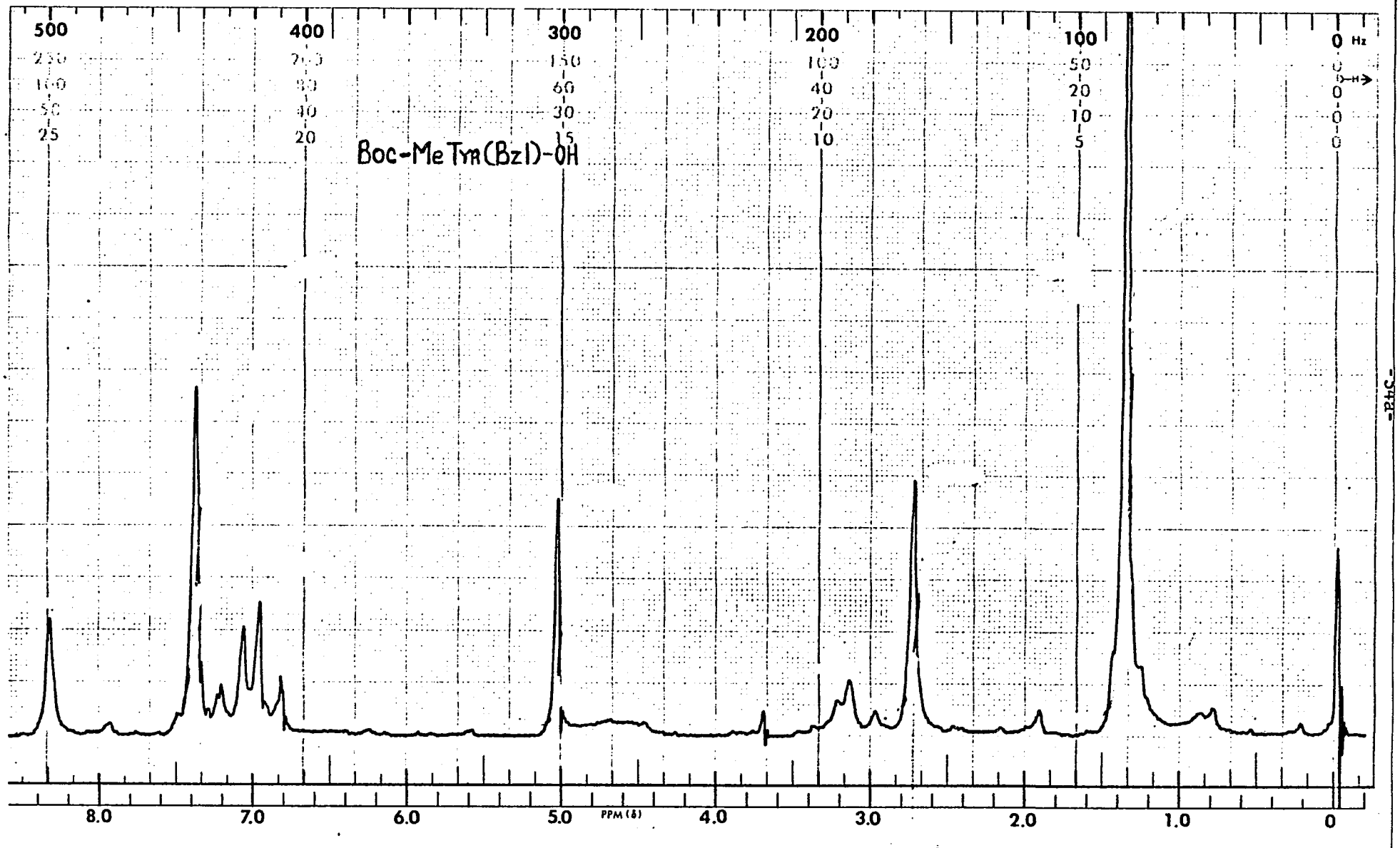
[a]Obtained from N-methylation of Boc-Ser(Bzl)-OH, $\delta_{\beta-H}$, 5.42, $\delta_{\alpha-H}$, 6.00 [ppm].

[b]N-methylation at 5°C for 72h.

[c]N-methylation at 5°C for 48h.

[d] The signals for $N-CH_3$ and Bu^t were integrated for 3H and 9H respectively.

Nuclear magnetic resonance spectrum of Boc-MeTyr(Bzl)-OH in $CDCl_3$



Nuclear magnetic resonance spectrum of Boc-MeThr(Bzl)-OH in $CDCl_3$

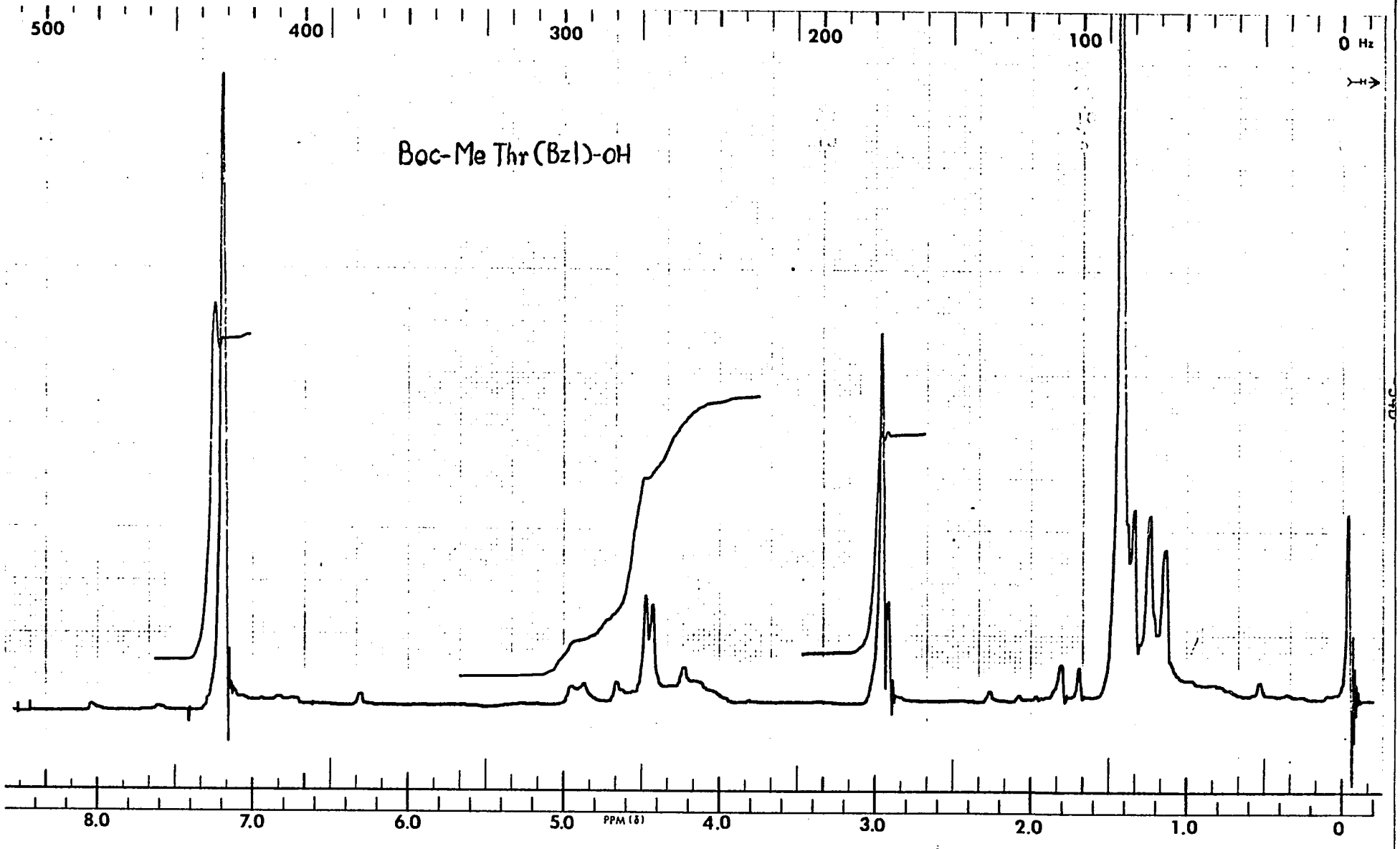


Table 111 306

O-Methylation of N-tert-butyloxycarbonyl-L-serine under various condition[a].

Boc-Ser-OH: NaH: MeI (equivalent)	% of $-OCH_3$ integral (3.35ppm)[b]	% of $N-CH_3$ integral (2.9ppm)[b]
1 : 3 : 8	100	32
1 : 3 : 4	100	35
1 : 2 : 4	100	20
1 : 1.5: 4	100	--
1 : 1.3: 4	90	--
1 : 1 : 4	56	--

[a] mixture containing 2 mmol of Boc-Ser-OH was stirred in THF (20 ml) at 5°C, 24h.

[b] determined with ^1H n.m.r. The % integration of $O-CH_3$ and $N-CH_3$ are standardized to the integration of the N-tert-butyl (1.43 ppm) in the ratio of 1 : 1 : 3.

(c) The problems of using commercially obtained N-tert-butyloxycarbonylamino acids for N-methylation.

Commercially available N-tert-butyloxycarbonyl-amino acids obtained from various sources are described in Part II c. p 28. We were unable to achieve complete N-methylation of these samples except N-tert-butyloxycarbonylleucine and alanine (Beckman). 30-50% unmethylated product were found for N-tert-butyloxycarbonylisoleucine, phenylalanine and valine. The latter two compounds were purified before N-methylation by recrystallization from ethyl acetate/pet-ether and pet-ether respectively.[Anderson (1957)]. Similar results of unmethylation were obtained. A 50:50 mixture of the commercial N-tert-butyloxycarbonylphenylalanine and N-tert-butyloxycarbonylphenylalanine synthesized by us gave the same incomplete reaction despite the fact that an experiment with the synthetic N-tert-butyloxycarbonylphenylalanine N-methylated run in parallel was successful. N-Methylation of N-tert-butyloxycarbonyl, O-benzyl threonine, and tyrosine were incomplete when carried out on a 2 mmol scale, but complete on a 1 mmol scale. It was then suggested by Dr. Chaturvedi of Chemical Dynamic Corp.(a personal communication to Dr. Benoiton) that our difficulties might be due to the presence of hydrazoic acid, which could be removed by using reduced pressure. It was found that there were no difficulties with the methylations when starting materials

(N-tert-butyloxycarbonylphenylalanine and valine) were left overnight under vacuo at 56° before they were used. Other commercial amino acids were dried either under vacuo at 56° or at room temperature overnight.

N-Benzyloxycarbonyl, O-tert-butylserine (Fluka) is commercially available in the form of its dicyclohexylammonium salt. The dicyclohexylamine was removed before N-methylation by dissolving the salt in 10% citric acid, extracting the O-tert-butylserine derivative into ethyl acetate and washing the extract with water. The extract was then dried over $MgSO_4$ and evaporated to dryness. N-Benzyloxycarbonyl, O-tert-butylserine was obtained as clear oil.

Some commercially available N-tert-butyloxycarbonyl-amino acids contain water of hydration, namely N-tert-butyloxycarbonylleucine and isoleucine. Sodium hydride is very sensitive to moisture, as it can be destroyed to form sodium hydroxide. Therefore, the water of hydration was removed by drying a solution of the compound in chloroform (10 ml/mmol) over $MgSO_4$ for 30 min. Chloroform was removed by evaporation. Final traces of chloroform were removed by re-evaporating after having added THF several times.

III. 2. Synthesis of O-methylserine and O-methylthreonine derivatives and related compounds

(a) A new route to O-methylserine and O-methylthreonine derivatives

It has been observed in this laboratory that methylation of N-benzyloxycarbonyl-L-threonine at room temperature using sodium hydride and methyl iodide gave a mixture of 60% N-methylated and 25% O-methylated products together with some unchanged material [McDermott and Benoiton (1973a)]. We now found that O-tert-butyl and O-benzyl serine derivatives could be N-methlated at 5°C without any β-elimination. It was therefore of interest to find if N-protected-L-serine and threonine could be N-methylated at this temperature to provide a one-step method to obtain these compounds which have not yet been accessible. In preliminary experiments, it was observed that O-methylation of these hydroxy amino acids took place at 5°C using sodium hydride and methyl iodide. In fact, it was found that 78% of the N-benzyloxycarbonyl-L-threonine was O-methylated and 80% of the N-benzyloxycarbonyl-L-serine was O-methylated together with 6% N-methylation after 72 h. No β-elimination was observed in either case. This result furnished a new insight that optically pure O-methyl-L-serine and O-methylthreonine which are much desired for peptide analogue synthesis might be obtained by this simple one-step method. The results

also suggested that serine derivatives are more easily O-methylated than threonine derivatives. On further investigation, N-tert-butyloxycarbonyl-L-serine was found to be a better starting material for obtaining the O-methyl serine derivative, because O-methylation of N-tert-butyloxycarbonyl-L-serine was found much less hindered than the N-benzyloxycarbonyl-L-serine.

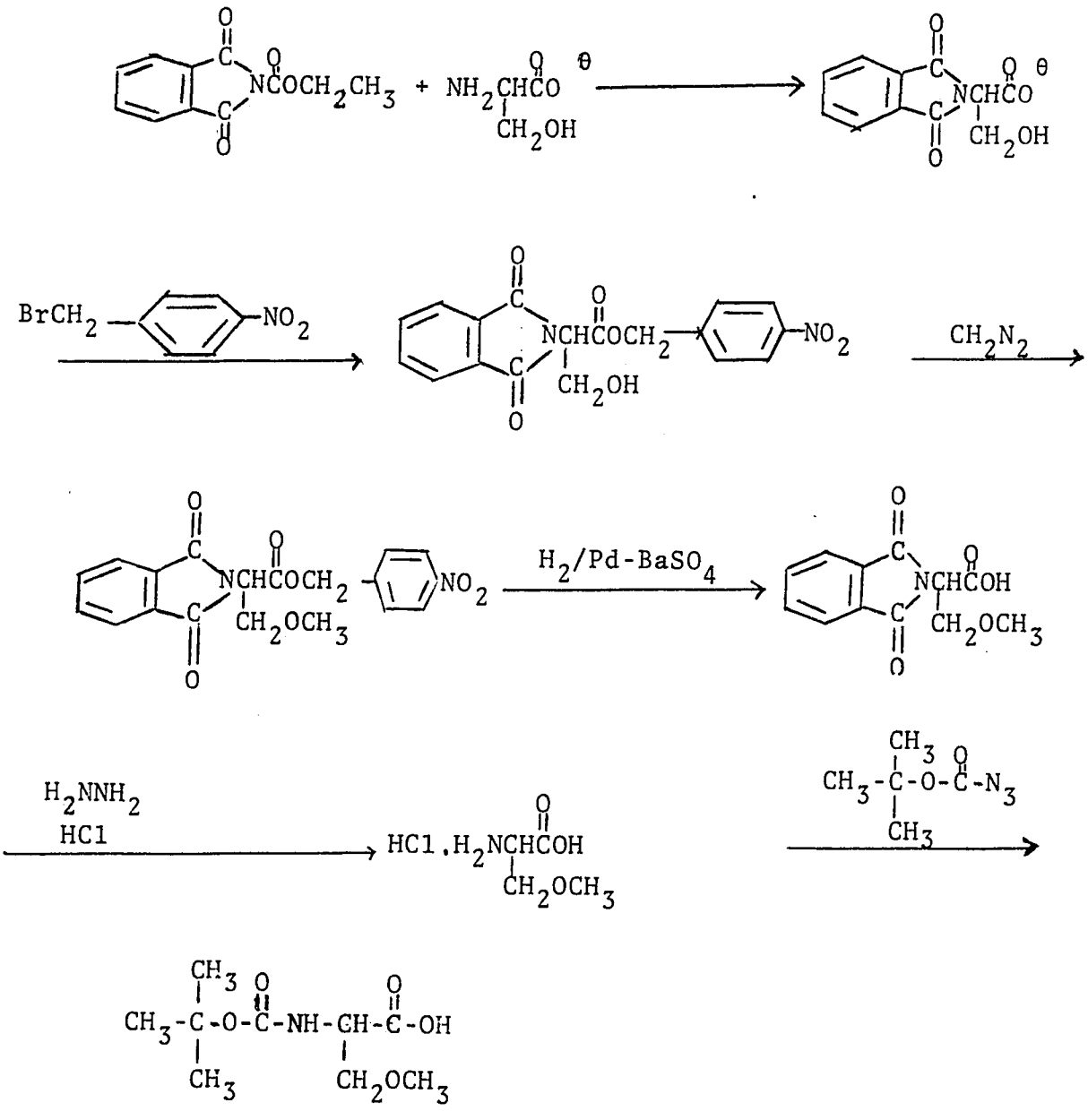
We then set out to find the optimal reaction conditions using N-tert-butyloxycarbonyl-L-serine as starting material. The results of a series of experiments are shown in Table III. 306. When the ratio of reactants was 1:3:8 (the normal ratio used for N-methylation), the amount of N-methylation present was high (32%). Reducing the amount of methyl iodide used did not have any effect on the reaction. When the amount of sodium hydride was reduced to two equivalents, there was 20% of N-methylation. O-methylation was complete with no N-methylated product present when 1.5 equivalents of sodium hydride were used. Using an amount of sodium hydride less than that, O-methylation was found to be incomplete. However, by suspending the product in dichloromethane, the unreacted starting material precipitated and could be removed by filtering through Celite. Amino acid analysis of these samples after deprotection with 70% trifluoroacetic acid in water showed that they contained less than 0.1% of serine and less than 0.1% N,O-dimethyl serine.

Erratic results were obtained under a few circumstances. When a cold room constantly used by others was used; 5-10% N-methylation was found. This seemed to suggest that higher temperatures (>5°C) favours N-methylation. When the temperature was kept constant at 0°C, no methylation occurred as the solution solidified after a few hours. The same observation was found when an inadequate amount of THF was used. In fact the amount of THF required was three times that used for normal N-methylations. The solution became clear when the reaction was complete. The N-tert-butyloxycarbonyl, O-methyl-L-serine obtained by this method was shown to be enantiomerically pure and was obtained in good yield. Therefore it provides a better alternative to the six-step method reported by Hodges and Merrifield (1974) as shown in route [2].

Attempts to O-methylate N-benzyloxycarbonyl-L-serine using the same conditions resulted in 5-10% N-methylated derivatives besides the desired product. It was found that N-methylation of N-benzyloxycarbonyl derivatives at 5°C occurred much faster than that of N-tert-butyloxycarbonyl derivatives [Part III. 3, p. 77].

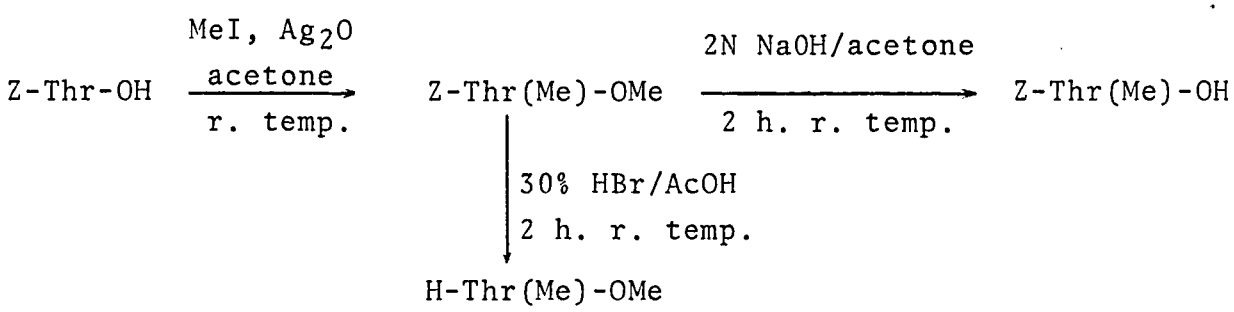
The methylation of N-benzyloxycarbonyl threonine using a large excess of methyl iodide andsilver oxide in acetone at room temperature gave N-benzyloxycarbonyl, O-methyl-threonine methyl ester [Chimiak and Rudinger (1965)]. In order to obtain a derivative suitable for peptide synthesis, it is

Route [2] Synthesis of N-tert-butyloxycarbonyl, O-methyl -L-serine. [Hodges and Merrifield (1974)]



necessary either to remove the methyl ester by saponification or to remove the amino protecting group by acidolysis as shown in route [3].

Route [3]. Synthesis of O-methylthreonine derivative [Chimiak and Rudinger (1965)].



Furthermore, it has been found in this laboratory that the O-methylation using silver oxide and methyl iodide in acetone was not complete. 1-3% of threonine was found in the product after deprotection. N-tert-butyloxycarbonyl, O-methylthreonine methyl ester had been prepared by a similar method [Jorgenson et al (1973)]. An examination of this reaction in this laboratory showed that etherification was only 48-65%. In spite of the fact that unreacted N-tert-butyloxycarbonyl threonine could be removed by washing with ether, the yield of O-methyl derivative obtained by this method was low.

A mixture of O-methylated product (78-85%) and starting material were found when N-benzyloxycarbonyl threonine

was methylated at 5°C for 72 h using 3 equivalents sodium hydride and 8 equivalents methyl iodide. Prolonging the reaction time to 96 h , 6-10% N-methylation was found. Using the same molar equivalent of sodium hydride and methyl iodide as for the O-methylation of N-tert-butyloxycarbonyl serine (1:1.5:4), less than 30% O-methylation was found. Despite the incomplete methylation, unreacted N-benzyloxycarbonyl threonine could be precipitated in dichloro methane, which provides an excellent method to purify N-benzyloxycarbonyl, O-methylthreonine. The purified product was found to be enantiomerically pure using the method as described in Part III. 4. p.89. The main advantage of this method is that the product can be directly used for peptide synthesis without saponification. It has been observed in this laboratory that saponification of methyl esters of N-benzyloxycarbonyl, N-methyl amino acids can cause racemization, whereas in a N-monosubstituted amino acid ester, the >N-H group next to the α-C-H appears to inhibit racemization [McDermott and Benoiton (1973a)]. Thus, the O-methyl threonine derivatives obtained by using silver oxide and methyl iodide are likely to be optically pure, however this has not been proven yet.

Methylation of N-tert-butyloxycarbonyl threonine at 5°C using the normal methylation ratio of sodium hydride and methyl iodide (1:3:8) resulted in 35% N-methylation and complete O-methylation. Reducing the amount of sodium hydride and methyl

iodide to that for the O-methylation of N-tert-butyloxycarbonyl serine (1:1.5:4), 5-10% N-methylation and incomplete O-methylation was found. When the reaction was carried out at 0°C for 96 h, similar results were obtained i.e. incomplete O-methylation together with 5-10% N-methylation. Since the separation of O-methylated and N,O-dimethylated threonine derivatives is difficult, the attempt to obtain N-tert-butyloxycarbonyl, O-methyl threonine was abandoned.

Preparation of N-tert-butyloxycarbonyl, O-methyl-L-serine N-tert-butyloxycarbonyl-L-serine (10 mmol 2.05 g) and methyl iodide (40 mmol 2.5 ml) were dissolved in purified THF (100 ml), and sodium hydride dispersion (0.66 g; 15 mmol) was added cautiously with gentle stirring to the solution at 0°C. The suspension was protected from the atmosphere by a drying tube. The reaction vessel was kept in the ice bath for 15 min after addition of sodium hydride, and left stirring at 5°C. The solution became clear after 24 h and the reaction was complete. N-tert-butyloxycarbonyl, O-methyl-L-serine was isolated and purified by the same procedure as described in the general methylation procedure (Part III. 1. p. 40). Solid citric acid was used for acidification. The product was obtained as an oil in 91% yield (2.0 g.). The dicyclohexylammonium salt was prepared by adding excess amine to the product dissolved in ether, followed by the addition of pet-ether and storage at 4°C. Recrystallization from the same solvent mixture gave

85% (3.1 g) yield with m.p. 116-117°, $[\alpha]_D^{26}$ + 18.2° (c. 1, in methanol). Lit. m.p. 153-154° (monocyclohexylamine salt), $[\alpha]_D^{25}$+ 19.8° [Hodges and Merrifield (1974)]. The optical purity of N-tert-butyloxycarbonyl, O-methyl-L-serine was established by using the method described in Part III. 4, p. 89.

Preparation of N-benzyloxycarbonyl, O-methyl-L-threonine. N-benzyloxycarbonyl-L-threonine (5 mmol 1.26 g) and methyl iodide (40 mmol, 2.5 ml) were dissolved in purified THF (50 ml), and sodium hydride dispersion (0.66 g, 15 mmol) was added cautiously with gentle stirring to the solution at 0°C. The suspension was protected from atmospheric moisture by a drying tube. The reaction vessel was kept in the ice bath for 15 min. after the addition of sodium hydride, and left stirring at 5°C. The reaction was stopped after 72 h by adding ethyl acetate (25 ml), water was then added dropwise to destroy the excess sodium hydride. The product was ioslated and purified as described in the general methylation prodcedure (Part III. 1 p. 40); 5 N HCl was used for acidification. The product obtained as an oil was suspended in dichloromethane (20 ml) and left at 4°C for 2 h and the solution was then filtered through celite. The filter was washed with dichloromethane (2 x 20 ml). The combined filtrates was evaporated to dryness. A clear oil of N-benzyloxycarbonyl, O-methyl-L-threonine was obtained in 75% (0.99 g) yield. The dicyclohexylammonium salt was obtained by adding excess amine to the product dissolved in methanol and

ether (1:1), followed by the addition of pet-ether and storage at 4°C. Recrystallization from the same solvent mixture gave 61% (1.10 g) yield with m.p. 140°C $[\alpha]_D^{23}$ +10.4 (c. 0.5, in methanol) lit. m.p. 138-139°C [Chimiak and Rudinger (1965)].

(b) Preparation of the N-benzyloxycarbonyl, N, O-dimethyl derivatives of L-serine and L-threonine.

In a continuing investigation of the conditions for methylation of serine and threonine derivatives, it was found that N-benzyloxycarbonyl, N, O-dimethyl derivatives could be obtained by methylation of the parent amino acids at 5°C using 6 equivalents of sodium hydride and 16 equivalents methyl iodide in purified THF. Proton n.m.r. studies revealed that there was no β-elimination, α-C-methylation nor methyl ester formation. Amino acid analysis of the products showed that they contained less than 0.1% of unreacted starting material. Attempts to methylate the N-tert-butyloxycarbonyl serine under the same conditions was not successful. A mixture of N, O-dimethyl derivatives (25-30%) and N-methyl dehydroalanine derivative (70-75%) were found for the serine derivative. A similar result was obtained when N-tert-butyloxycarbonyl, O-methyl-L-serine was N-methylated at 5°C using the normal amount of sodium hydride and methyl iodide used for general N-methylation. The results seem to suggest that N-tert-butyloxycarbonyl derivatives are much less stable than N-benzyloxycarbonyl derivatives in the large excess amount of sodium hydride to undergo β-elimination to form dehydroalanine derivative.

N-benzyloxycarbonyl, N, O-dimethyl-L-serine and L-threonine. N-benzyloxycarbonyl derivatives (5 mmol) and methyl iodide (80 mmol, 5 ml) were dissolved in purified THF (50 ml),

and sodium hydride dispersion (30 mmol, 1.32 g) was added cautiously with gentle stirring to the solution at 0°C. The suspension was protected from atmospheric moisture by a drying-tube. The reaction vessel was kept in the ice bath for 15 min after addition of sodium hydride, and left stirring at 5°C for 3 days for serine derivative and 5 days for threonine derivative. The products were isolated and purified using the same procedure as described above. The dicyclohexylammonium salts were prepared by adding excess amine to the products dissolved in ether, followed by the addition of pet-ether and storage at 4°C. Recrystallization from the same solvent mixture gave 72% yield for serine derivative and 81% yield for threonine derivative. The physical properties of these compounds are shown in Tables 307-310. The enantiomeric purity of N-benzyloxycarbonyl, N,O-dimethyl serine was established using the method described in Part III. 4. p. 89.

Table III 307

Physical properties of dicyclohexylammonium salts of N-protected methylated derivatives of serine and threonine.

Compound	Yield %	M.p. °C	$[\alpha]_D^{23}$ [a] (°)
Boc-Ser-(Me)-OH	85	116-117	+18.2
Z-MeSer(Me)-OH	72	130-131	-31.0
Z-MeSer-OH[b]	74	153-154	-6.8
Z-Thr(Me)-OH	61	140	+10.4
Z-MeThr(Me)-OH	81	115	-20.0
Boc-MeThr-OH	72	150	-12.2

[a] (c. 1, methanol)

[b] Based on all criteria, this compound is pure. However, the CHN analysis, though close, was outside the normal acceptable limits.

Table III 308

The chromatographic data of N-methyl and O-methyl-L-serine and threonine

Compound	Rf (T.l.c.) a	Rf (T.l.c.) b	Amino acid analysis[c] Normal; 68 ml/h Constant	Normal; 68 ml/h Time (min)	Half-normal; 34 ml/h Constant	Half-normal; 34 ml/h Time (min)
H-Ser-OH	0.30	0.18	20.2	58.0	39.0	95.0
H-Ser(Me)-OH	0.36	0.26	14.9	53.0	30.0	91.5
H-MeSer-OH	0.25	0.17	0.13	45.0	3.76	79.0
H-MeSer(Me)-OH	0.26	0.23	0.61	54.0	7.50	80.0
H-Thr-OH	0.22	0.25	20.8	55.0	35	92
H-Thr(Me)-OH	0.23	0.29	22.4	52.2	42.8	90.0
H-MeThr-OH	0.17	0.30	0.71	35.5	18.9	58.0
H-MeThr(Me)-OH	0.15	0.27	0.1	35	4.0	68.0

[a] n-BuOH/Acetic acid/water 4 1 1 v/v/v.

[b] n-BuOH/pyridine/Acetic acid/water 6 1 1 2 v/v/v/v.

[c] Beckman model 120B Amino Analyzer AA-15 (50 cm) resin, eluted with pH 3.28 buffer.

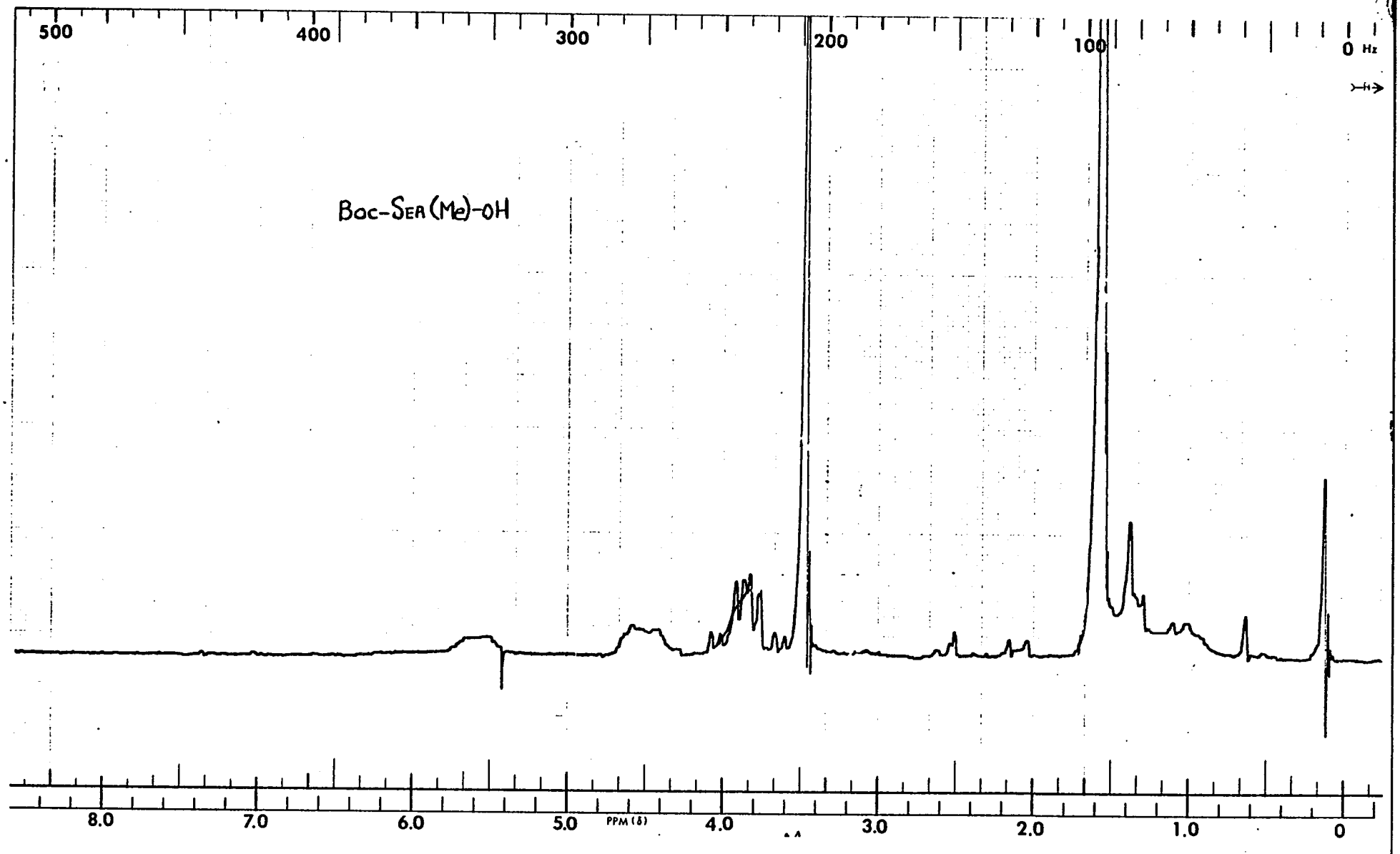
Table III 309

^{1}H- n. m. r. data of methylated derivatives of serine and threonine (ppm)[a]

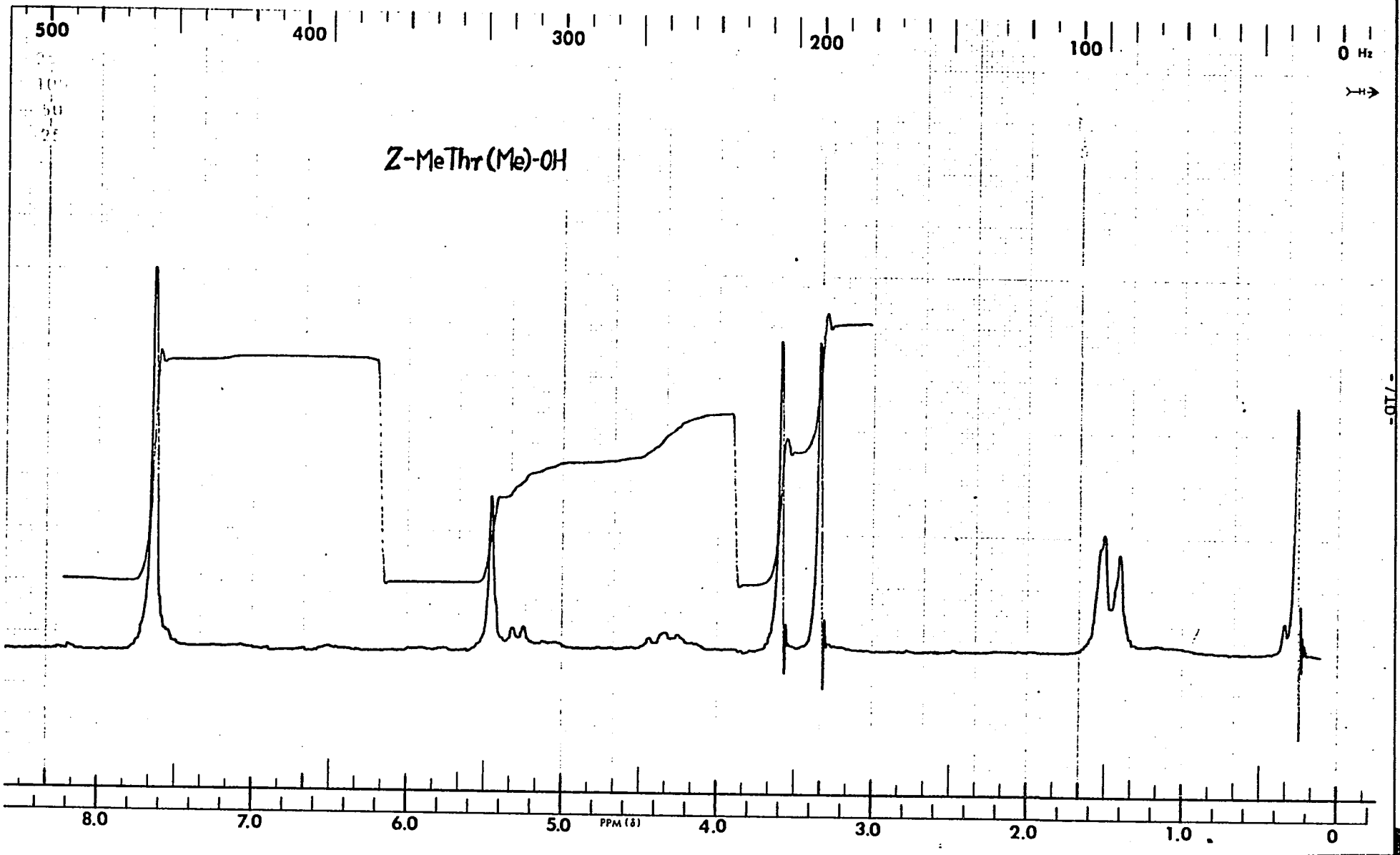
Amino acid	δ_{N-CH_3}	δ_{O-CH_3}
Boc-Ser(Me)-OH		3.34
Z-MeSer-OH	3.02	
Z-MeSer(Me)-OH	3.0	3.20
Boc-MeThr-OH	2.95	
Z-Thr(Me)-OH		3.30
Z-MeThr(Me)-OH	2.95	3.38

[a]Boc (1.43 ppm), Z (7.12 ppm)

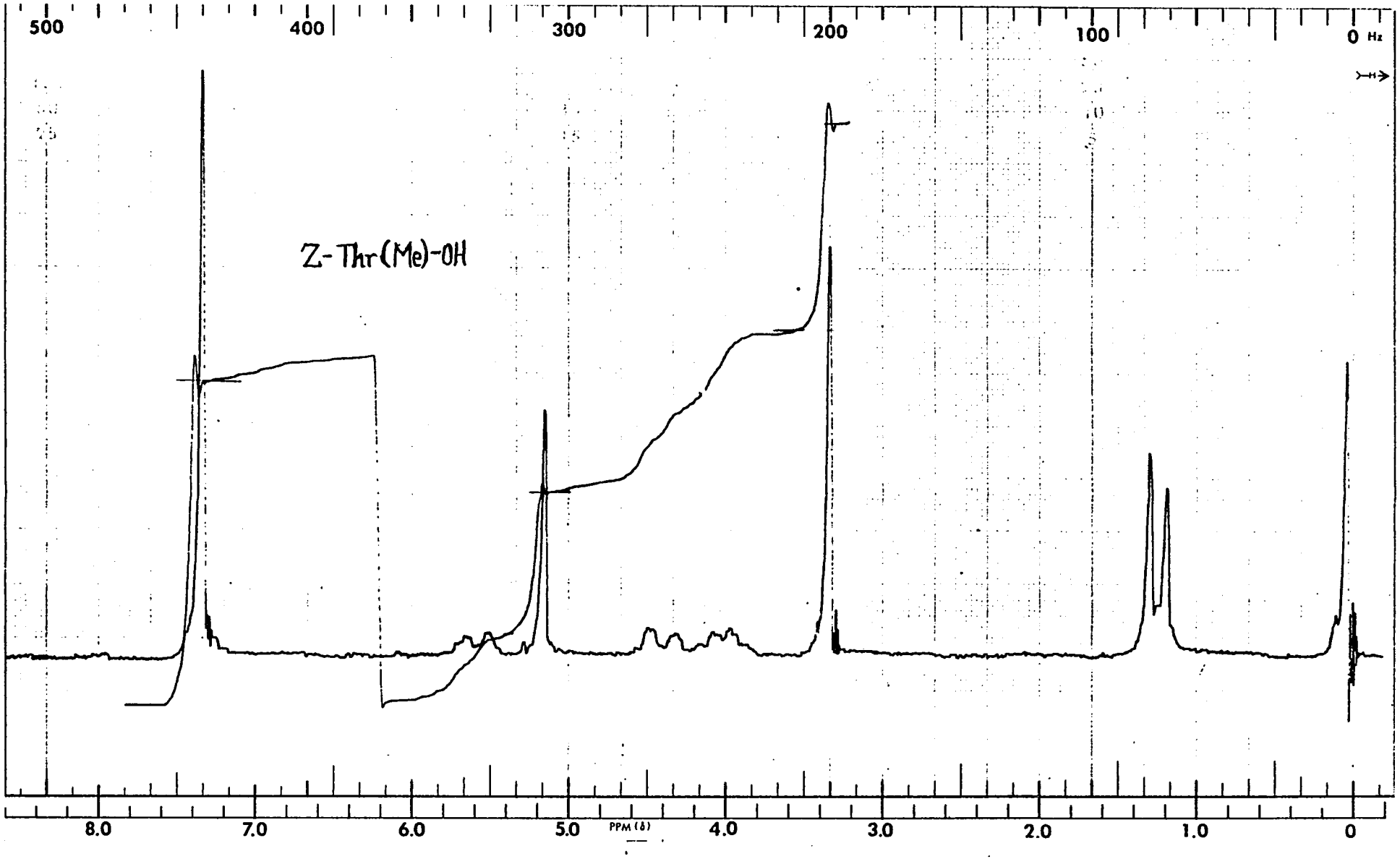
Nuclear magetic resonance spectrum of Boc-Ser(Me)-OH in $CDCl_3$



Nuclear magnetic resonance spectrum of Z-MeThr(Me)-OH in $CDCl_3$



Nuclear magnetic resonance spectrum of Z-Thr(Me)-OH in $CDCl_3$



Nuclear magnetic resonance spectrum of Z-MeSer)OBu^t)-OH in $CDCl_3$

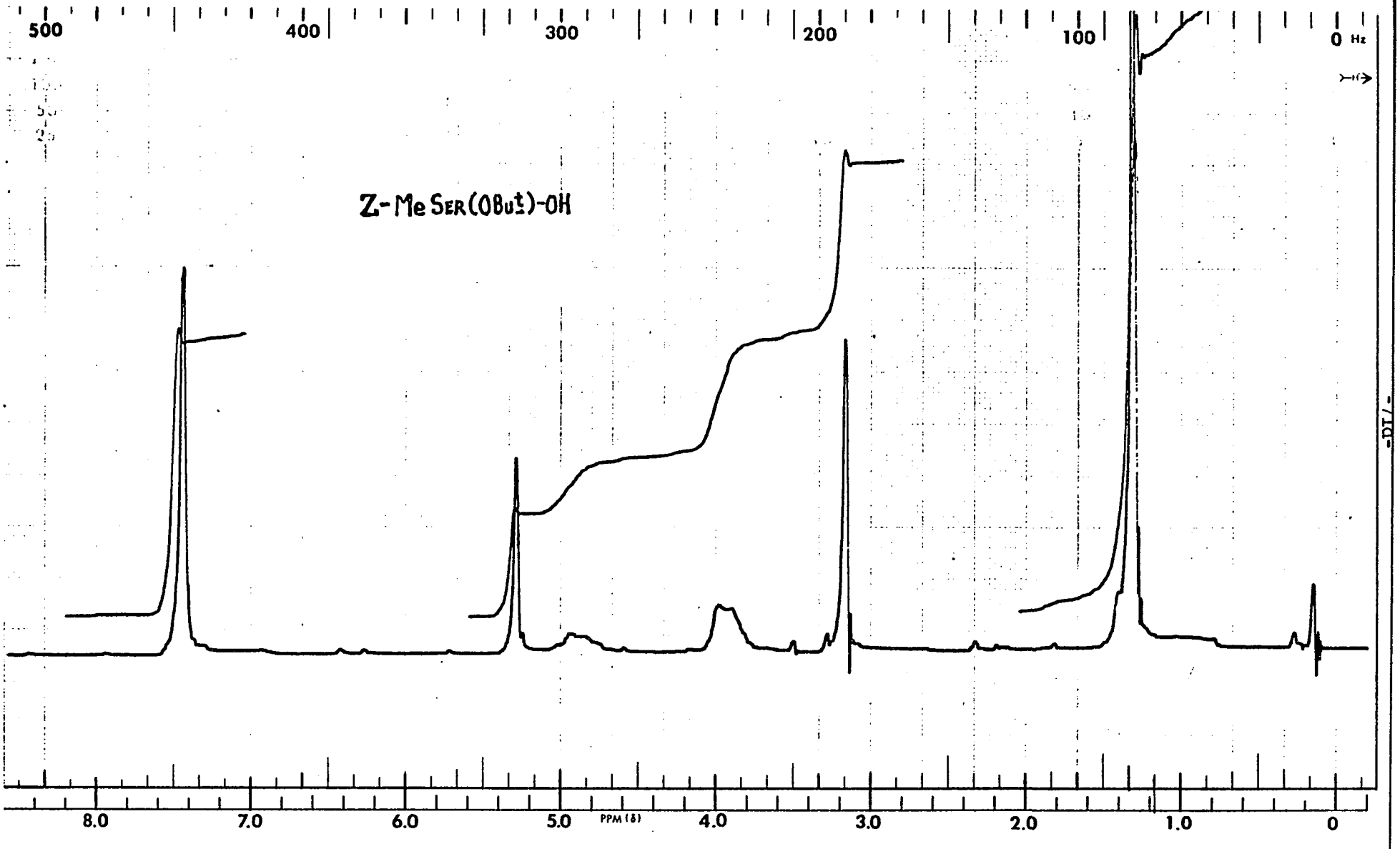


Table III 310

Analytical data of dicyclohexylammonium salts of methylated serine and threonine derivatives

Amino acid	Formula	C,H,N, Calculated / C,H,N Found		
Boc-Ser(Me)-OH	$C_{22}H_{41}N_2O_5$	63.89 63.60	9.99 10.26	6.78 6.96
Z-MeSer(Me)-OH	$C_{25}H_{41}N_2O_5$	66.78 66.62	9.19 9.46	6.23 6.38
Boc-MeThr-OH	$C_{23}H_{43}N_2O_5$	64.60 64.31	10.14 10.20	6.55 6.58
Z-Thr(Me)-OH	$C_{25}H_{40}N_2O_5$	66.78 66.54	9.19 9.39	6.23 6.39
Z-MeThr(Me)-OH	$C_{26}H_{43}N_2O_5$	67.35 67.15	9.34 9.27	6.0 6.14

(c) Preparation of N-protected, N-methyl-L-threonine and serine

Reductive alkylation of the benzylserine using formic acid and formaldehyde [Quitt et al (1963)] is the most commonly used method to prepare N-methylserine. However, N-methylamino acids obtained by this method have been found to contain some D-isomer [Part III, 5. p.124]. Hence the enantiomeric purity of N-methylserine obtained by reductive alkylation is questionable. More recently, Okamoto et al (1974) have reported the synthesis of N-methyl-L-serine by the following route [4].

Route [4] Synthesis of N-methylserine [Okamoto et al (1974)].

$$\text{Z-Ser(Bzl)-OH} \xrightarrow[\text{DMF}]{\text{MeI}\quad Ag_2O} \text{Z-MeSer(Bzl)-OMe}$$

$$\xrightarrow{\text{NaOH}} \text{Z-MeSer(Bzl)-OH} \xrightarrow{H_2/Pd(C)} \text{H-MeSer-OH}$$

N-methylation using methyl iodide and silver oxide in DMF resulted in a methyl ester. It had been observed in this laboratory that saponification of methyl ester using NaOH could cause racemization [McDermott and Benoiton (1973c)]. Furthermore, N-methylation of serine derivatives using silver oxide and methyl iodide in DMF at room temperature resulted in certain amount of β-elimination [Okamoto et al (1974)]. β-elimination

was also found in N-methylation of N-tert-butyloxycarbonyl-L-serine using a similar procedure [Olsen (1970)]. The specific rotation of N-methyl-L-serine reported by Okamoto was lower than that reported by Quitt et al (1963). Thus optically pure N-methylserine derivatives have not been accessible.

Enantiomerically pure N-benzyloxycarbonyl, N-methyl, O-tert-butyl-L-serine has been obtained by N-methylation of the O-tert-butyl-L-serine derivative using sodium hydride and methyl iodide at 5°C. No β-elimination was found in this reaction, hence providing a new route to obtain N-protected, N-methylserine by selective removal of the O-tert-butyl group using 50% trifluoroacetic acid in dichloromethane as shown in the following route [5].

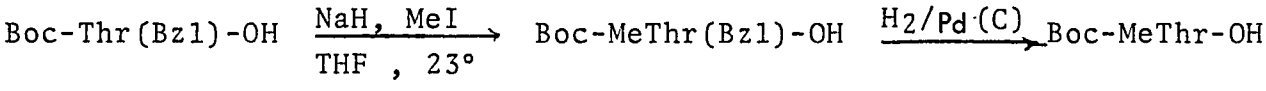
Route [5] Synthesis of N-benzyloxycarbonyl, N-methyl-L-serine

$$\text{Z-Ser(Bu}^t\text{)-OH} \xrightarrow[\text{THF}\quad 5^\circ]{\text{NaH, MeI}} \text{Z-MeSer(Bu}^t\text{)-OH} \xrightarrow{50\%\ \text{TFA / } CH_2Cl_2} \text{Z-MeSer-OH}$$

N-benzyloxycarbonyl, N-methyl, O-tert-butyl-L-threonine was obtained in this laboratory some years ago [McDermott and Benoiton (1973a)]. However, the compound had not been thoroughly characterized. Optically pure N-tert-butyloxycarbonyl, N-methyl, O-benzyl-L-threonine was obtained by N-methylation of the O-benzyl-L-threonine derivative at room

temperature using sodium hydride and methyl iodide in THF. The methylation was complete after 24 h. N-tert-butyloxycarbonyl, N-methyl-L-threonine can be obtained by selective removal of the O-benzyl group using hydrogenation as shown in the following route [6].

Route [6]. Synthesis of N-tert-butyloxycarbonyl, N-methyl-L-threonine



Again the compounds obtained by this method have the important advantage that they can be immediately used for peptide synthesis.

N-benzyloxycarbonyl, N-methyl-L-serine : N-benzyloxycarbonyl, N-methyl, O-tert-butyl-L-serine (2 mmol, 0.62 g)(p. 52). was treated with 50% trifluoroacetic acid-dichloromethane (20mml) for 30 min. The deprotecting agent was removed by evaporation with addition of water several times. The oily residue was resuspended in water (50 ml) and the product was extracted with ethyl acetate (20 ml x 2). The combined extract was washed with water (20 ml x 2), dried over $MgSO_4$ and evaporated to dryness. N-benzyloxycarbonyl, N-methyl-L-serine was obtained as oil. Dicyclohexylammonium salt was prepared by adding excess amine to

the product dissolved in ether, followed by the addition of pet-ether and storage at 4°C. Recrystallization from the same solvent mixture gave 74% (.73 g) yield with melting point 153-154°C. The physical properties and chromatography data are shown in Tables III. 307-310.

N-tert-butyloxycarbonyl, N-methyl-L-threonine: N-tert-butyloxycarbonyl, N-methyl, O-benzyl-L-threonine (2 mmol 0.82 g) was dissolved in 80% acetic acid (10 ml). 100 mg of palladium charcoal catalyst were added to the solution which was hydrogenated in the usual manner for 24 h. After hydrogenation acetic acid was removed by evaporation with addition of water several times. The residue was an oil and was resuspended in water. The product was then extracted with ethyl acetate (20 ml x 2). The combined extracts were washed with water (20 ml x 2), dried over $MgSO_4$ and evaporated. N-tert-butyloxycarbonyl, N-methyl-L-threonine was obtained as an oil in 80% yield (0.65 g). Dicyclohexylammonium salt was obtained by adding excess amine to the product dissolved in ether, followed by the addition of pet-ether and storage at 4°C. Recrystallization with the same solvent gave 72% yield (0.83 g) with m.p. 150°C. The physical properties and chromatography data are shown in Tables III. 307-310.

III. 3. Some chemical and physical properties of N-tert-butyloxycarbonyl, N-methylamino acids.

(a) Comparison of the rate of methylation of N-benzyloxycarbonyl and N-tert-butyloxycarbonyl amino acids at 5°C.

N-methylation of both N-benzyloxycarbonyl and N-tert-butyloxycarbonyl amino acids at room temperature were virtually complete in 24 h. Amino acid analysis of the products showed that they contained less than 0.1% of the unreacted amino acid. Even with the sterically hindered N-benzyloxycarbonyl alloisoleucine only 0.7% of the unreacted amino acid was detected [McDermott and Benoiton (1973a)]. It was found that at 5°C the N-methylation of the two types of derivatives still occurred but more slowly. N-benzyloxycarbonyl, O-tert-butyl-L-serine was completely N-methylated in 48 h whereas the complete N-methylation of N-tert-butyloxycarbonyl, O-benzyl-L-serine took 72 h. In view of the more sluggish reaction at 5°C, the extent of N-methylation for the two types of derivative was compared.

Amino acid analysis of the products obtained by N-methylation of the parent amino acid N-benzyloxycarbonyl and N-tert-butyloxycarbonyl derivatives using sodium hydride and methyliodide at 5°C showed that N-benzyloxycarbonyl-L-alanine underwent 91% N-methylation, and that N-tert-butyloxycarbonyl-L-alanine was 56% N-methylated after 15 h. Hence the methylation

of a N-benzyloxycarbonyl derivative proceeds more quickly. The results may be due to the different solubility of the sodium salt of the two types of derivatives at 5°C. It had been observed that larger amount of THF was required for the methylation of N-tert-butyloxycarbonyl derivatives [Part III. 2. p.58].

N-methylation of N-benzyloxycarbonyl and N-tert-butyloxycarbonyl-L-alanine at 5°C: N-benzyloxycarbonyl and N-tert-butyloxycarbonyl-L-alanine (5 mmol) were N-methylated separately at 5°C by the same procedure as that described in Part III, 2 p. The reactions were stopped after 15 h by adding ethyl acetate to the reaction mixture, followed by the addition of water dropwise to destroy excess of sodium hydride. The product was isolated and purified as previously described [McDermott and Benoiton (1973a)]. 5N HCl was used for acidification of the N-benzyloxycarbonyl derivative and solid citric acid was used for the N-tert-butyloxycarbonyl derivative. The acidified solution was extracted with ethyl acetate (20 ml x 2). The extracts were washed with sodium thiosulfate (20 ml x 2) and with water (20 ml x 2). The extract was then dried ($MgSO_4$) and the solvent was removed to give a colourless oil for both types of derivatives.

Deprotection of N-benzyloxycarbonyl and N-tert-butyloxycarbonyl derivatives. The product of N-benzyloxycarbonyl derivatives obtained from the above procedure was treated with 30% HBr in acetic acid and the product of N-tert-butyloxycarbonyl derivative was treated with 70% trifluoroacetic acid

in water for 2 h at 25°C. Evaporation of the reagents gave an oil which was then dissolved in water and extracted with ether (20 ml x 2). The aqueous layer was stirred with Dowex 50 (H^+) (20 ml batch volume) for 30 min. After filtering, the solution was treated a second time with resin (20 ml batch volume) and the combined batches washed slowly with water ($\frac{1}{2}$ l) on a sintered glass funnel. The washed resin was stirred with aqueous 10 N NH_4OH (40 ml) for 30 min; and the extracted solution was evaporated several times. Light, white crystalline products were obtained from both derivatives. Weighed amounts of the products were analyzed using the Amino Acid analyzer.

The Chromatographic data for alanine and N- methylalanine are referred to p. 84.

(b) Comparison of the rate of cleavage of N-tert-butyloxycarbonyl N-methylamino acids and amino acids

N-tert-butyloxycarbonylamino acids are useful in peptide synthesis because the N-tert-butyloxycarbonyl group satisfies the requirements of a selectively removable protecting group. Since N-methylamino acids and their derivatives often behave differently from the corresponding unmethylated compounds under various reaction conditions [Rudinger (1963) p. 133], it was thought useful to compare the rate of cleavage of N-tert-butyloxycarbonyl group from N-methylated and unmethylated amino acids under the same conditions. The methylated and unmethylated derivatives in a solution of trifluoroacetic acid 2% in dichloromethane were quantitatively measured during specified time periods using an Amino Acid analyzer. This dilution of trifluoroacetic acid was used because it was found to give reactions rates slow enough to be measured.

The results of the kinetic studies showed that the acidolysis of N-tert-butyloxycarbonyl, N-methylamino acid and amino acid in 2% trifluoroacetic acid-dichloromethane follows the rate law.

$$kt = \ln \left(\frac{a}{a-x} \right) \qquad [1]$$

k is the apparent first order rate constant, a is the protected amino acid present initially, X is a quantity directly proportional to the mole of free amino acid released at time t,

and (a-x) is a quantity directly proportional to the moles of protected amino acid remaining at time t. The apparent first-order rate constants for deprotection of the four N-tert-butyl-oxycarbonyl derivatives (Table III. 311) were determined from the least-squares lines of best fit through data points plotted on a grid of $\ln \frac{a}{a-x}$ vs time (Figure III, 301). Equation [1] was used to calculate the rate constants of the four derivatives. It is seen that for both L-alanine and L-leucine, the N-tert-butyloxycarbonyl group was cleaved from the N-methylamino acid at least twice as fast (2.5 and 2.1) as from the corresponding amino acid. It had been suggested that acidolytic cleavage of urethane protecting groups was initiated by protonation of the oxygen atom of the urethane carbonyl group [Blaha and Rudinger (1965)]. The results seem to agree with this postulation that the protonation would be favoured by the positive inductive effect of the N-methyl group. However, protonation may also possibly take place at the nitrogen atom which is also favoured by the inductive effect of the N-methyl group.

Kinetic cleavage experiments: The N-tert-butyloxycarbonyl derivative (0.5 - 2.0 μ mol) was dissolved in 2% trifluoroacetic acid-dichloromethane (5.0 ml); 0.5 ml portions of this solution were kept at 23 ± 1°C in oven-dried tubes sealed with teflon-lined screw caps. At selected time periods, the solution within a single tube was freed of dichloromethane and most of the trifluoroacetic acid by evaporation under a

gentle stream of dry nitrogen and the residue was dissolved in pH 7.0 citrate buffer. This quenching procedure required less than 1 min. The mixture was kept frozen until analyzed. An aliquot of this solution was analyzed for the liberated N-methylamino acid and unmethylated amino acid with the Amino Acid analyzer using 1 x 15 cm column of Aminex A-5 resin eluted with 0.2 N sodium citrate buffer at 23°C at half-normal flow (34 ml/h). Ninhydrin color constants were determined for each compound. The integrated peak areas were corrected for the relative ninhydrin response of a standard solution of these compounds. The constants and elution times are given in Table III. 312. N-tert-butyloxycarbonyl derivatives are partially cleaved by this buffer if elution is carried out at 57°C.

Table 111 311

Deprotection of N-tert-butyloxycarbonyl derivative in 2% tfa-dichloromethane

Boc-derivatives	k^a, 10^{-6} sec^{-1}
Boc-MeAla-OH	30,9
Boc-MeLeu-OH	22.1
Boc-Ala-OH	14.7
Boc-Leu-OH	8.9

[a] k = Apparent first - order rate constant determined from the slopeof the lines in Figure 301

Table III. 312

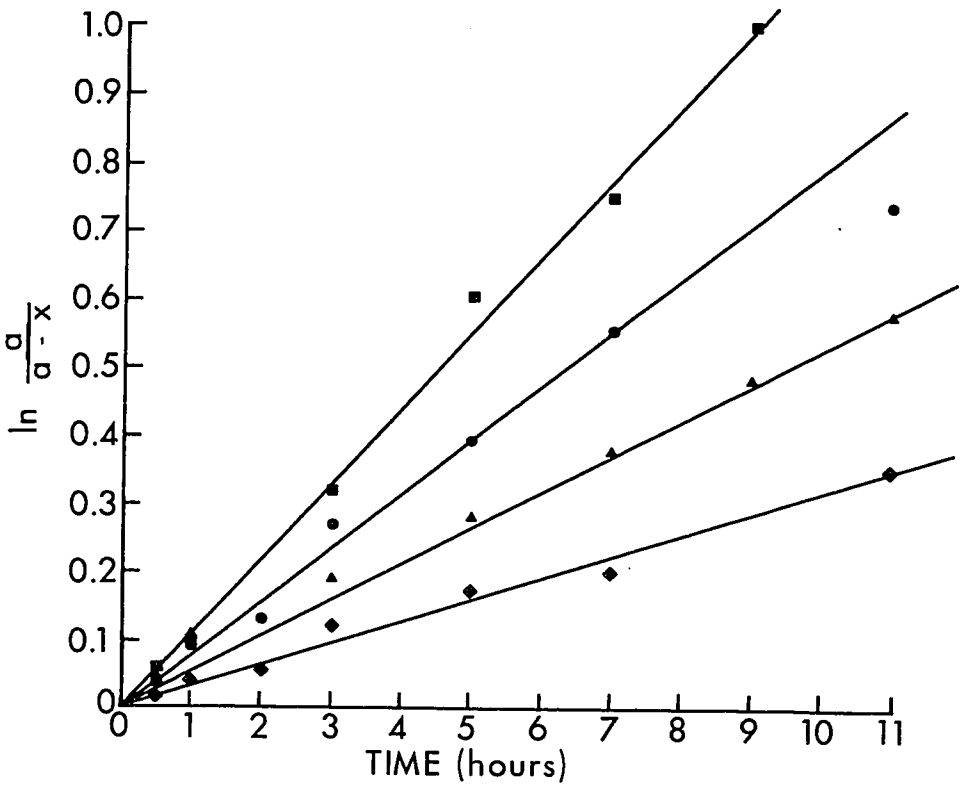
Condition for Amino Acid Analysis and ninhydrin constant of the Amino acids.[a]

Amino acid	Elution buffer pH	Elution time	Constant
H-MeAla-OH	3.28	28.0	2.6
H-Ala-OH	3.28	40.0	38.3
H-MeLeu-OH	4.25	78.5	7.5
H-Leu-OH	4.25	87.5	42.7

[a] 1 x 15 cm column of Aminex A-5 resin eluted with 0.2 N sodium citrate buffer at 23°C at half normal flow rate (34 ml/h), using Beckman B-120 Amino Acid Analyzer.

Figure III 301

Calculated least-square lines of best fit for the apparent first-order cleavage of Boc-derivatives by 2% trifluoroacetic acid in dichloromethane at 23°.



■ Boc - MeAla - OH
▲ Boc - Ala - OH
● Boc - MeLeu - OH
◆ Boc - Leu - OH

(C) Dicyclohexylammonium salts of N-methylamino acid derivatives

Dicyclohexylamine forms salts of outstanding crystallizing properties with a large number of N-protected amino acids [Klieger et al (1196])] furnishing a useful way to prepare crystals of amino acid derivatives, which otherwise exist as oils, for characterization. For example, urethane protected N-methylamino acids generally have lower melting points than the corresponding N-benzyl and N-Acetyl derivatives [Coggins and Benoiton (1972)] and a number of them were, in fact,obtained as oils. They were able to form insoluble dicyclohexylammonium salts in pet-ether and their physical properties were determined as shown in Table III 3]3. Contrarily, N-methylamino acid derivatives protected on the side-chain by a benzyl or tert-butyl group do not form insoluble dicyclohexylammonium salts in pet-ether. They are exemplified by Z-MeAsp-(OBu^t)-OH, Z-MeGlu(OBu^t)-OH, Z-MeThr(Bu^t)-OH, Z-MeSer(Bu^t)-OH, Boc-MeThr(Bzl)-OH, and Boc-MeSer(Bzl)-OH. This property allowed the removal of small amounts of unreacted starting material from the reaction products as the dicyclohexylammonium salts which are insoluble in this solvent [McDermott and Benoiton (1973a)].

Preparation of N-methylamino acid derivative dicyclohexylammoniu m salts: N-methylamino acid derivative

(2 m mol) was dissolved in minimum amount of ether. Excess amount (5 m mol) of dicyclohexylamine was added followed by the addition of pet-ether. The solution was then stored at 4°C. The white precipitate formed was filtered and washed with pet-ether (10 ml x 2). Recrystallization from the same solvent with addition of small amount of dicyclohexylamine afforded 61 - 85% yield .

Table III 313

Physical properties of dicyclohexylammonium salts of N-methyl-amino acid derivatives.

Amino acid	Solvent	M.P. (°C)	$[\alpha]^{23}$[a]
Boc-MeIle-OH	Ether/pet-ether	117	-45.6
Boc-D-MeaIle-OH	Ether/pet-ether	94	+42.8
Boc-MePhe-OH	Ether/pet-ether	176	-25.5
Boc-MeSer(Bzl)-OH	soluble		
Boc-Ser(Me)-OH	Ether/pet-ether	116-117	+18.3
Boc-MeThr-OH	Ether/pet-ether	150	-12.2
Boc-MeThr(Bzl)-OH	soluble		
Boc-MeTyr(Bzl)-OH	soluble		
Boc-MeVal-OH	Ether/pet-ether	113-114	-49.3
Z-MeAsp(OBu^t)-OH[b]	soluble		
Z-MeGlu(OBu^t)-OH[b]	soluble		
Z-MeSer-OH	Ether/pet-ether	153-154	-6.8
Z-MeSer(Me)-OH	Ether/pet-ether	130-131	-31.0
Z-MeSer(Bu^t)-OH	soluble		
Z-Thr(Me)-OH	Ether/methanol/pet-ether	140	+10.4
Z-MeThr(Me)-OH	Ether/pet-ether	115	-20.0
Z-MeThr(Bu^t)-OH[b]	soluble		

[a] (c. 1, methanol)

[b] [McDermott and Benoiton (1973a)]

III. 4. A new method for determining the enantiomeric purity of N-methyl-L-amino Acids and their derivatives

(a) Reexamination of the application of the method of Manning and Moore in determining the enantiomeric purity of N-methylamino acids

In 1968, Manning and Moore described a simple, and elegant chromatographic method for the determination of amino acid enantiomers [Manning and Moore (1968)]. 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 Hirshmann et al (1967). Optically pure L-amino acids gave L,L-dipeptides, whereas partially racemic amino acids gave mixtures of the diastereomeric L,L and L,D-dipeptides. The pairs of diastereomers were separated and analyzed on an automatic amino acid analyzer using appropriate chromatographic systems. One part of the D-isomer could be detected in the presence of 1000 parts of the L-isomer when 2 μ mol samples were analyzed.

An attempt had been made to apply this method to N-methylamino acids in this laboratory, using L-alanine N-carboxyanhydride instead of L-leucine N-carboxyanhydride because the N-methylation experiments had been carried out with leucine derivatives, This reaction was found to be sluggish and more by-products were found, but it was reported that this

method was applicable to determine the optical purity of N-methylleucine [Coggins and Benoiton (1970)]. It was found later that the peptide H-Ala-MeLeu-OH had an unusually low ninhydrin colour-yield and that the peaks had been misassigned [McDermott and Benoiton (1973a)]. Since then, three reports on the use of this method for configuration assigments for N-methylalanine and N-methylphenylalanine [Woodhead et al (1974)], optical purity determination for N-methylleucine [Ling and Vale (1975) and N-methylalanine [Dr. Rich personal comminication with Dr. Benoiton] have come to the attention of this laboratory. Therefore this question was again reexamined.

The reaction was carried out with the procedure described by Manning and Moore (1968), and the peptides were analyzed as described by McDermott and Benoiton (1973b), but also by pumping the elution buffer at half-normal flowrate, which allows definite distinction between N-methylamino acids and amino acids or peptides [Coggins and Benoiton (1970)]. In fact the colour yield increases more than twice if the compound is not an ordinary amino acid or peptide. DL-alanine and DL-Leucine were used as controls (Table III, 314). It was found that DL-leucine N-carboxyanhydride coupled only with DL-N-methylalanine but not with other N-methylamino acids. On the other hand L-alanine N-carboxyanhydride failed to couple with any N-methylamino acid. The results are in agreement with the two reports for N-methylalanine [Woodhead et al (1974) and Dr. Rich's communication]. The results also are consistent

with the previous conclusion for N-methylleucine in this laboratory [MeDermott and Benoiton (1973b)]. There is no obvious explanation for the different behaviour of N-methylalanine towards leucine N-carboxyanhydride and alanine N-carboxyanhydride. Therefore the method of Manning and Moore cannot be generally used for determining the enantiomeric purity of N-methylamino acids.

Preparation of L-alanyl and DL-leucyl dipeptides

The N-methylamino acid was weighed in a 100 x 10 mm pyrex test tube. 1 ml of ice-cold 0.45 M borate buffer, pH 10.4 and one drop of octanoic acid were added. The tube was taken into a cold room at 4°C, L-alanine N-carboxyanhydride or DL-leucine N-carboxyanhydride (100 μ m) was quickly added and the tube was shaken vigorously on a "Vortex genie" stirrer for 2 min. The solution was brought to pH 2 with HCl, diluted to 10 ml with water, filtered (Celite) and suitable aliquots were analyzed on the analyzer.

Table III. 314

Chromatographic data for analysis of diastereomers.[a]

Compound	Elution time (min)	
	D, L	L, L
H-Ala-Ala-OH[b]	20.5	22.5
H-Ala-Leu-OH[b]	36.5	41.5
H-Leu-Ala-OH[c]	29.5	35.0
H-Leu-Leu-OH[c]	56.5	68.0
H-Leu-MeAla-OH[c]	30.5	35.5

[a] Beckman model 120B Amino Acid Analyzer, Aminex A-5 (15 cm) resin, eluted with 0.20 N sodium citrate, pH 4.25, at 68 ml/h; 57°C.

[b] L-alanine N-carboxyanhydride was used to couple with DL-alanine and leucine.

[c] D,L-leucine N-carboxyanhydride was used.

(b) A new method to detemine the enantiomeric purity of N-methylamino acids and their derivatives

Despite the impossibility of using the method of Manning and Moore to determine the optical purity of N-methylamino acids, the optical purity of one of the synthetic derivatives, N-benzyloxycarbonyl, N-methylleucine had been established in this laboratory. This was accomplished by incorporating the N-methylamino acid, through the intermediate benzyl ester, into the dipeptide H-Ala-MeLeu-OH, whose diastereomers could be separated with the amino acid analyzer [McDermott and Benoiton (1973b)]. However, this peptide had an unusually low ninhydrin colour yield (6% of that for H-Ala-Leu-OH), and the colour yield of its retroisomer H-MeLeu-Ala-OH was negligible [McDermott and Benoiton (1973b)]. Since lysine has a ε-amino group at its side-chain, it occurred to us that a lysyl residue should impart a normal colour yield to a dipeptide containing an N-methylamino acid regardless of its position in the peptide, and that the diastereomeric peptide might be separable on a short column of the analyzer which is used to separate basic amino acids. In order to achieve this objective, synthetic N-tert-butyloxycarbonyl and N-benzyloxycarbonyl, N-methylamino acids from this laboratory were coupled directly with a lysine derivative. The reactions are shown in the following route.

Route [7] Synthesis of lysyl dipeptide

$$\text{*Boc-MeX-OH + H-Lys(Z)-OBzl} \xrightarrow[CH_3CN \quad 5°C]{\text{N,N'-Dicycloheylcarbodiimide}}$$

$$\text{*Boc-MeX-Lys(Z)-OBzl} \xrightarrow[\text{(ii) TFA}]{\text{(i) } H_2\text{, Pd(C)}} \text{H-MeX-Lys-OH}$$

L,L(and D,L)

(only for Boc deprotection)

X = amino acid residue

*Z can be used for N-protection

N^{ε}-benzyloxycarbonyl-L-lysine benzyl ester was chosen as the lysine derivative for the coupling, as its protecting groups can be removed by catalytic hydrogenation, which also deprotects the N-benzyloxycarbonyl, N-methylamino acids. N-tert-butyloxycarbonyl derivatives are deprotected by an additional treatment, with trifluoroacetic acid. N^{ε}-benzyloxycarbonyl-L-lysine methyl ester can be used to prepare diastereomers for reference, but not for the analysis of the optical purity because saponification of the dipeptide to remove the methyl ester would partially racemize the peptide [McDermott and Benoiton (]973c)]. For determination of the optical purity of a free N-methylamino acid, it is converted to its N-benzyloxycarbonyl derivative which is obtained as an oil and used as such.

Several coupling agents were examined for the coupling namely; N-ethoxycarbonyl, 2-ethoxy-1, 2-dihydroquinoline

[Belleau and Malek (1968)], and N,N'-dicyclohexylcarbodiimide with N-hydroxysuccinimide as additive [Weygand et al.(1966)] and N,N-dicyclohexylcarbodiimide alone. [Sheehan and Hess (1955)]. The cleanest products were obtained by using the N,N'-dicyclohexylcarbodiimide. Acetonitrile was chosen as solvent to minimize acyl-urea formation [Guttmann and Boissonnas (1958)].

N^{ε}-Benzyloxycarbonyl-L-lysine benzyl ester was prepared as the hydrochloride by the azeotropic method as shown in the following route.

Route [8] Synthesis of N^{ε}-benzyloxycarbonyl-L-lysine benzyl ester

$$\text{H-Lys(Z)-OH} \xrightarrow[\text{Benzene } \Delta]{\text{Bzl-OH, Tos-OH}} \text{H-Lys(Z)-OBzl.Tos}$$

$$\xrightarrow[\text{ii HCl/ethanol}]{\text{i pH 9, Ethylacetate}} \text{H-Lys(Z)-OBzl.HCl}$$

The product was purified by extraction from a solution at pH 9 as has been done for the methyl ester [Benoiton et al (1970)] followed by crystallization. Without the purification [Izumiya et al (1967)], the p-toluenesulfonyl salt always contained some N^{ε}-benzyloxycarbonyl lysine and/or lysine benzyl ester.

Attempts were made to analyze the diastereomeric peptide pairs on the short (basic) column of Amino Acid analyzer using pH 5.28 as eluent. Single broad peaks of diastereomeric dipeptide pairs of H-MeAla-Lys-OH and H-MeVal-Lys-OH were obtained. Buffers of higher pH gave sharper peaks

which emerged sooner but did not separate the diastereomeric dipeptide pairs. Good separation was achieved on the intermediate length (15 cm) column of Aminex A-5 resin, using pH 6.50 as eluting buffer. This additional column has been part of the instrument in this laboratory and has proven useful on several other occasions[McDermott and Benoiton (1973b), Benoiton et al (1970)]. The chromatographic data for several dipeptides are shown in Table III 315. The data were obtained by chromatography of the products obtained by coupling the N-protected, N-methylamino acids with N^{ε}-benzyloxycarbonyl-DL-lysine benzyl ester. An alternative would be to couple theracemic N-methylamino acid derivative with the optically active ester. The D,L-peptide obtained would then be the enantiomorph of the L,D-peptide obtained above. The identities of the peaks were established by synthesis and chromatography of the L,L-peptides. Integration of the peaks gave the relative ninhydrin colour-yield for each pair of diastereomers. Higher colour-yields were observed for an L,D-peptide than for its L,L-isomer. This contrasts with the lower colour-yields for L,D-peptides not containing N-methylamino acids [Manning and Moore (1968)]. These colour-yield ratios are correct provided the mixture from which they were obtained was 50:50 mixture.

All the diastereomers are completely separated by the system described, allowing detection of as little as one part in one thousand of one isomer in diastereomeric mixtures. This was confirmed by analyzing mixtures containing

decreasing amounts of an L,D-peptide, prepared by the addition of a 50:50 diasteromeric mixture to a solution of the L,L-peptide. Since all the synthetic L,L-peptides did give a single peak, this proves that the synthesis of $\underline{N}^{\varepsilon}$-benzyloxycarbonyl lysine benzyl ester (Route [8]) and the coupling reaction (Route [7]) involved in the method do not cause racemization. This establishes the validity of its use for demonstrating that an N-methylamino acid and its derivatives are enantiomerically pure. The accuracy of the method was verified by analyzing samples of N-benzyloxycarbonyl, N-methyl-L-alanine containing 2.5 and 10% D-isomer, prepared by adding racemate to the enantiomer. The results were within 12% of the theoretical value. Higher accuracy could likely be obtained but this was not explored further.

Recently, n.m.r. spectroscopy has been used to distinguish dipeptide diastereomers. Weinstein and Pritchard (1972) have reported different chemical shifts for the alanine methyl group in some protected alanyl dipeptide methyl esters, and Davis et al (1975) have reported different chemical shifts for the methoxy methyl group in the N-benzoyl dipeptide methyl esters containing alanine and valine. Examination of the n.m.r. spectra (100 MHz) of a few protected lysyl dipeptides containing N-methylamino acids revealed that the diastereomers of Z-MeLeu-Lys(Z)-OMe and Z-MeVal-Lys(Z)-OMe could be readily distinguished by virtue of the different chemical shifts of

the methoxyl methyl singlets. (Table III, 316). Integration of the pertinent peaks allowed the demonstration that the products formed by coupling equimolar amounts of N-benzyloxycarbonyl, N-methyl-DL-valine or N-methyl-DL-leucine with N^{ε}-benzyloxycarbonyl-L-lysine benzyl ester were 50:50 mixtures of L,L and D,L isomers. When these protected racemic N-methylamino acids were coupled with 0.1 molar equivalents of ester, the products contained the peptides in the L,L:D,L ratios of 57:43 and 58:42 respectively. It thus transpires that some kinetic resolution obtains, the L,L-isomer being favoured, but not when there is enough ester present to react with all of the N-methylamino acid derivatives. This means that should a N-protected N-methylamino acid containing a small percentage of D-isomer be used in peptide synthesis, where a large excess of this component is used for adding a residue, the resulting peptide would contain a smaller percentage of contaminating isomer. From these n.m.r. data, the colour yield ratios described above are considered as true values.

Use of n.m.r. in fact provides a second method for determining the enantiomeric purity of N-methylleucine and N-methylvaline, and their derivatives, or for establishing the configuration of samples of these N-methylamino acids, although it is less sensitive. The method cannot be used for N-methyl-L-alanine, since the methoxyl singlets of Z-DL-MeAla-Lys(Z)-OMe coincide in $CDCl_3$. It was also observed that

the diastereomers of Boc-MePhe-Lys(Z)-OMe and Z-MeLeu-Lys-OH gave separated N-methyl singlets as shown in Table III 316.

Preparation of N^{ε}-benzyloxycarbonyl-L-lysine benzyl ester hydrochloride . A mixture of N^{ε}-benzyloxycarbonyl-L-lysine (5.60 g;20 mmol), p-toluene sulfonic acid H_2O (4.56 g; 25 mmol), benzyl alcohol (25 ml) and benzene (35 ml) was heated (oil bath) under reflux for 7 h, the water formed being collected in a Dean-Stark receiver. Ether (100 ml) and pet-ether (100 ml) were added with shaking to the cooled mixture. The precipitate was collected and washed well with pet-ether. Four grams of the air-dried product (10.4 g) were suspended in water (100 ml), the pH of the solution was adjusted to 9.4, (a few drops of ethyl acetate solubilized the ester), and the ester was extracted into ethyl acetate (50 ml). The aqueous layer was washed with ethyl acetate (25 ml), the combined extracts were dried ($MgSO_4$) and the solvent evaporated off. Evaporation was repeated after the addition of ethanol (10 ml), and ethanolic HCl (20 ml) was added. After cooling overnight, the crystals were collected and washed by trituration in ether. Yield: 1.5-1.6 g (45-50%), m. p. 140-141°C, $[\alpha]_D^{27}$ -12.4° (C, 0.5 in 0.1 NHCl) lit. m.p. 139°C, $[\alpha]_D$ -9.9° [Erlanger and Brand (1951)] and 138-140°C, $[\alpha]_D$ -7.0 [Wünsch et al (1964)]. Thin layer chromatography on silica gel GF 254 in 1-butanol-acetic acid-water (4:1:1) gave Rf. 0.80. N^{ε}-benzyloxycarbonyl-L-lysine; 0.60. L-lysine benzyl ester; 0.23. N^{ε}-benzyloxycarbonyl-DL-lysine

benzyl ester hydrochloride, and the m. p. of the later compound is 135-135.5. Substantial amounts of L-lysine benzyl ester were formed when the reaction was carried out for a longer period of time.

Derivatization of N-methylamino acid: The N-methylamino acid was converted to its N-benzyloxycarbonyl derivative by the following procedure. The crystals or residue (1 mmol; 0.5 mmmol was occasionally used) remaining after evaporation of a solution were dissolved in 3 ml of 4 N NaOH, 0.2 ml of benzyl choloroformate was added, and the mixture was stirred vigorously at 5°C for 4 h. The solution was extracted with ether (5 ml), acidified to congo red with 5 N HCl while still cold, and the product was extracted into ethyl acetate (10 ml x 2). The combined extracts were washed with water (10 ml x 2), dried ($MgSO_4$), and evaporated to give an oil, which was used as such to avoid fractionation. The yield for N-methyl-L-valine was lower than for N-methyl-L-alanine and N-methyl-L-Leucine.

Dipeptide Synthesis: To the N-tert-butyloxycarbonyl or N-benzyloxycarbonyl methylamino acid (0.2 mmol) in 2 ml of acetonitrile at 5°C were added N^{ε}-benzyloxycarbonyl-L-lysine benzyl ester hydrochloride (81.4 mg; 0.2 mmol) and triethylamine (20.3 mg; 0.2 mmol). The mixture was stirred for 15 min, and N,N'-dicyclohexylcarbodiimide (41.2 mg; 0.2 mmol) was added, and the mixture was stirred at 5°C for 18 h.

One drop of acetic acid was added, followed by acetone (5 ml) and the mixture was filtered after cooling for 1 h. The solvents were evaporated off, the residue was taken up in chloroform (35 ml), and the solution was washed successively with 10% aqueous citric acid (x2), water, aqueous $NaHCO_3$ (x2) and water and the solvent was dried and evaporated off. The protected peptides which were analyzed by n.m.r. were prepared in the same manner using N^{ε}-benzyloxycarbonyl lysine methyl ester hydrochloride. The residue was taken up in 80% aqueous acetic acid and the solution was hydrogenated over 10% palladium-on-charcoal catalyst (100 mg) for 18 h after filtration through celite. The catalyst was removed by filtration, the solution was evaporated to dryness, and the residue was left for 1 h in 70% aqueous triflouroacetic acid if a N-tert-butyloxycarbonyl group was present. After evaporation, 10 ml of water were added, and the last traces of N,N'-dicyclohexylurea were allowed to crystallize by leaving the solution at 5°C for several hours.

Analysis of Diastereomers: A 1-ml aliquot of the above solution which had been filtered through celite was diluted to 5 ml with 0.2 N sodium citrate pH 2.2 and 0.5 ml of this was analyzed using the conditions described in Table 315. The relative amounts of the two isomers in the sample are obtained by comparison of the surface area of the two peaks after having divided the surface area of the D.L-

peak by the colour-yield ratio given in Table III 315. These ratios could be obtained by couplings in which either one or both of the components is racemic.

Table 111 315

Chromatographic data for analysis of lysyl dipeptide diasteromers[a]

Lysyl dipeptides	Elution time (min)[b] L,L	D,L	Ninhydrin colour-yield ratio D,L/L,L
H-MeAla-Lys-OH	31	36	1.18
H-MeVal-Lys-OH	31	46	1.38
H-MeLeu-Lys-OH	39	63	1.44
H-MePhe-Lys-OH[c]	32	80	1.10
H-MeSer(Bu^t)-Lys-OH[d]	36	42	1.00
H-MeThr-Lys-OH[d]	34	41	1.01

[a] Beckman model 120B Amino Acid Analyzer, Aminex A-5 (0.9 x 15cm) resin, eluted with 0.35 N sodium citrate buffer, pH 6.50, at 68 ml/h 57°C.

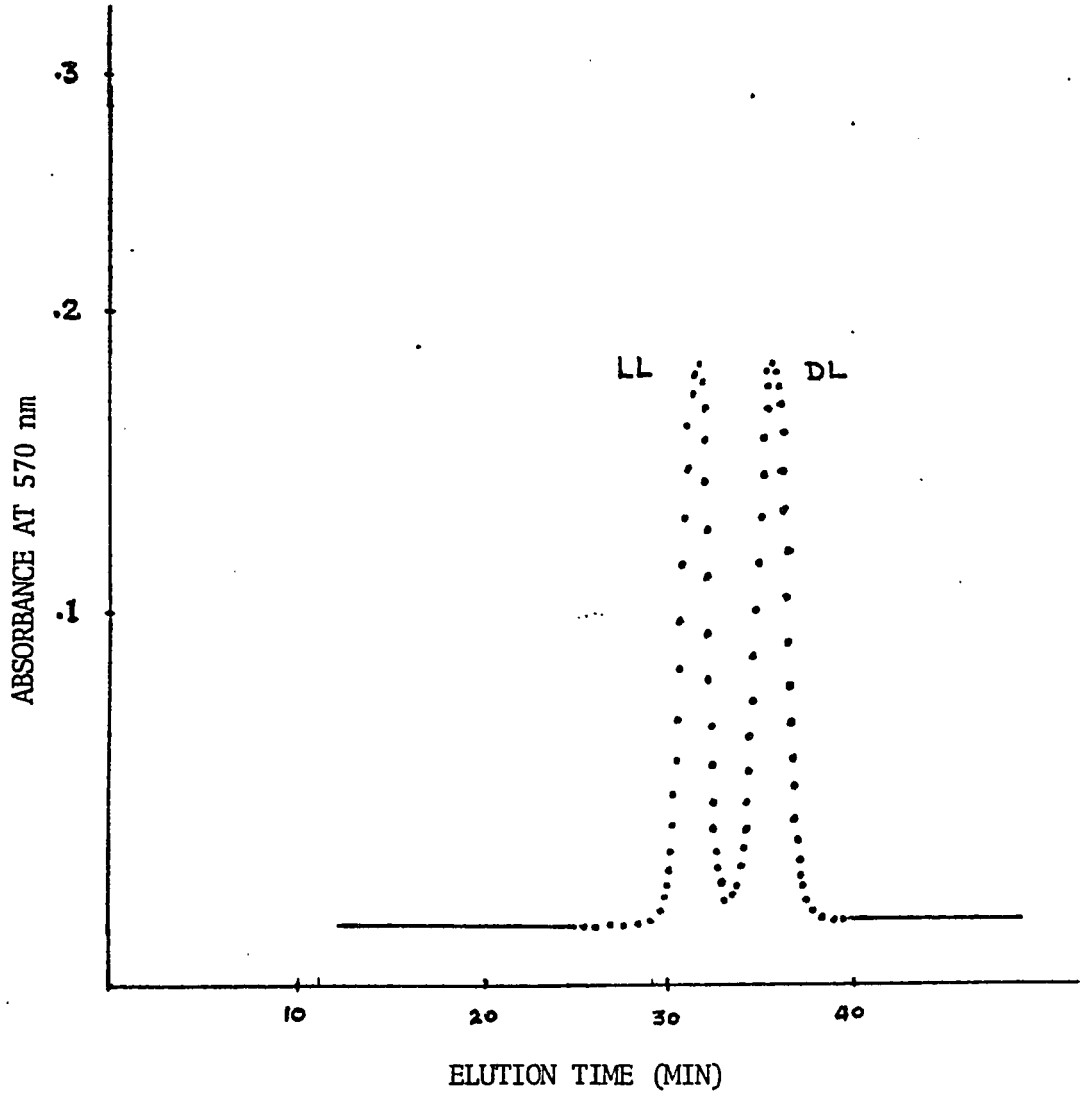
[b] Other elution times: Lysine, 38; Ammonia, 50; N,N-dicyclohexylurea, 53.

[c] pH 7.50 eluting buffer.

[d] pH 5.50 eluting buffer.

CHROMATOGRAMS OF N-METHYLALANYL LYSINE DIPEPTIDES.

THE ANALYTICAL CONDITION ARE SHOWN IN TABLE III 315 p 103



CHROMATOGRAM OF N-METHYLVALYL LYSINE DIASTEROMERIC PEPTIDE (1000=1).

THE ANALYTICAL CONDITION ARE DESCRIBED IN TABLE III 315 p 103.

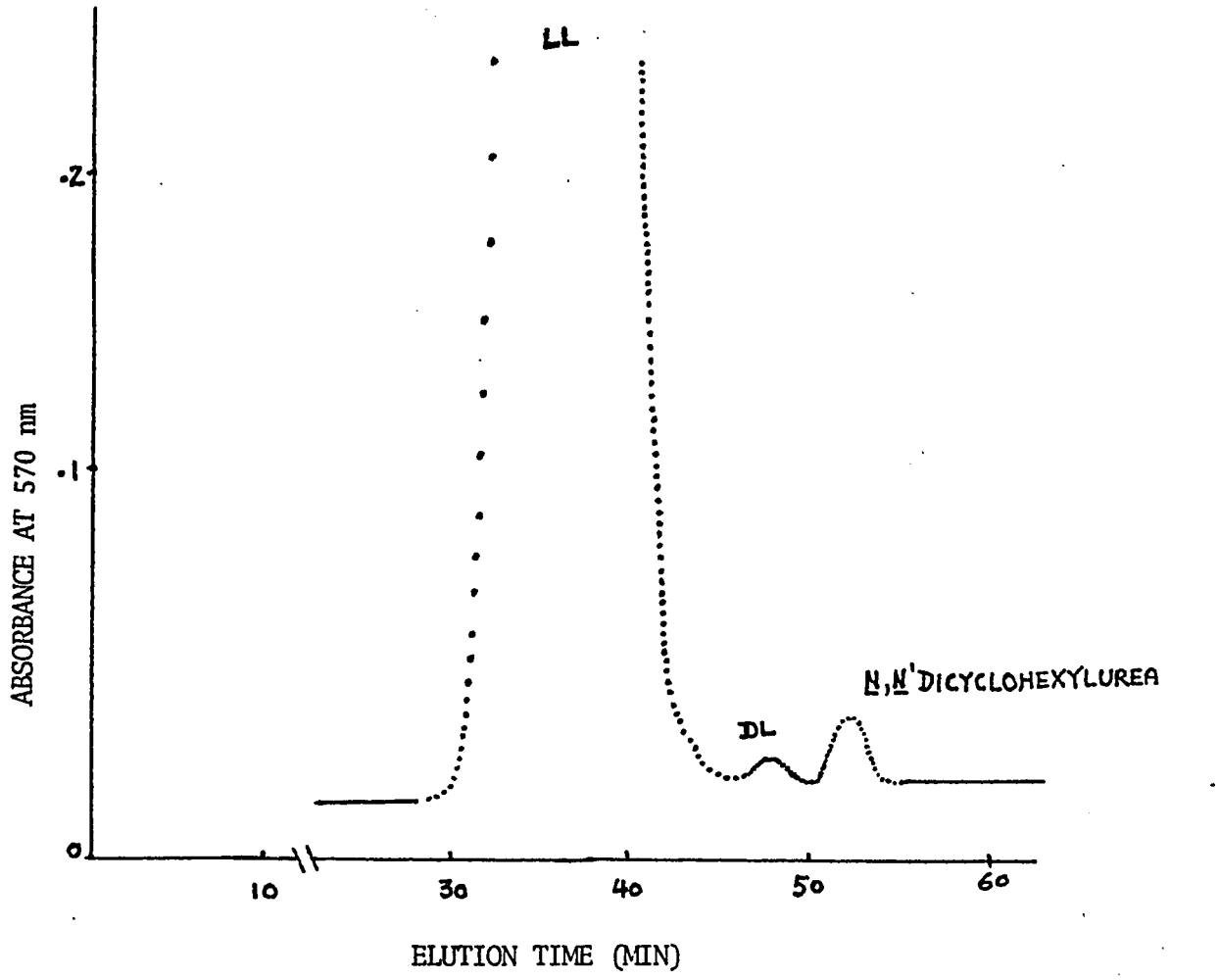


Table 111 316

Chemical shifts of methoxy methyl singlet and N-methyl singlet of protected lysyl dipeptide diastereomers[a].

Peptides	O-CH_3(Cps) L,L	O-CH_3(Cps) D,L	N-CH_3(Cps) L,L	N-CH_3(Cps) D,L
Z-MeLeu-Lys(Z)-OMe	283[b]		371	368
Z-MeVal-Lys(Z)-OMe	287[b]		371.5	366.5
Z-MeLeu-Lys(Z)-OH	282	278		
Boc-MePhe-Lys(Z)-OMe	278	273	374[b]	

[a] ^1H n.m.r. (100 MHZ)

[b] peaks coincide

(c). The general application of the method

It has been observed that the method described above can be applied to a compound in the presence of some of the corresponding unmethylated compound. In fact, the unmethylated peptides were found emerging shortly before the corresponding methylated peptide (Table III, 317), and the unmethylated diastereomeric peptide pairs emerged separately. This provides a new general method for the determination of the enantiomeric purity of amino acids and their derivatives as an alternative to the method of Manning and Moore (1968). The applicability of the method to other amino acid derivatives was shown by the separation of the diastereomeric dipeptide pairs of H-Ser(Me)-Lys-OH, H-Thr(Me)-Lys-OH, H-MeSer-Lys-OH and H-MeThr(Me)-Lys-OH using pH 5.5 buffer (Table III, 317). This suggests that the method can be applied to determine the enantiomeric purity of amino acids with their side-chain modified. Furthermore, it was found that regardless of the position of lysine, the diastereomeric lysyl dipeptide pairs, which contain either N-methylated or unmethylated amino acid, could be separated chromatographically using the Amino Acid analyzer. This was shown by the separation of the diastereomeric pairs of H-Lys-MeVal-OH, H-Lys-MeAla-OH, H-Lys-Ala-OH and H-Lys-Val-OH as shown in Table III, 317.

Table III 317

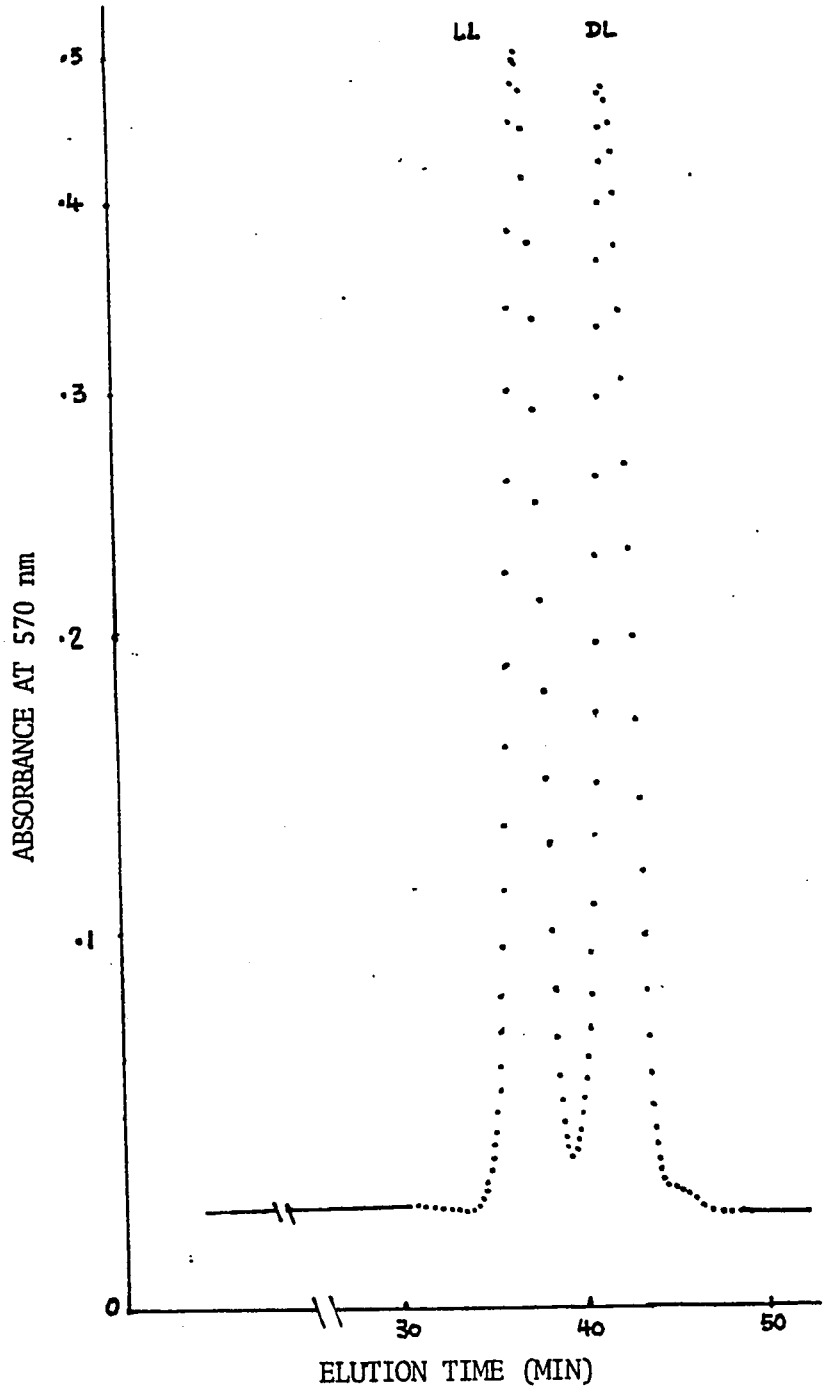
Chromatographic data for lysyl dipeptides[a]

Peptide	Eluting buffer pH	Elution time (min) L,L	D,L	Relative Color ratio D,L/L,L
H-Ala-Lys-OH	6.5	28	31	0.98
H-Val-Lys-OH	6.5	28	42	1.25
H-Leu-Lys-OH	6.5	35	58	1.02
H-Phe-Lys-OH	7.5	28	55.5	1.10
H-Ser(Me)-Lys-OH	5.5	39	44.5	1.01
H-Thr(Me)-Lys-OH	5.5	37.5	45	1.00
H-MeSer(Me)-Lys-OH	5.5	35	40.5	1.00
H-MeThr(Me)-Lys-OH	5.5	32	39.5	1.01
H-Lys-Ala-OH	6.5	37	44.5	1.02
H-Lys-Val-OH	6.5	28	46.5	1.01
H-Lys-MeAla-OH	6.5	39	46.5	1.00
H-Lys-MeVal-OH	6.5	40	56.5	1.01

[a] Beckman model 120B Amino Acid Analyzer, Aminex A-5 (0.9 x 15 cm) resin, eluted with 0.35 N sodium citrate buffer at 57°C at 68 ml/h.

CHROMATOGRAM OF O-METHYLSERYL LYSINE DIASTEROMERIC PEPTIDE

THE ANALYTICAL CONDITION ARE DESCRIBED IN TABLE III 317 p 106.



III. 5. Studies on the enantiomeric purity of N-methylamino acids prepared by various procedures.

(a) Methylation of urethane derivatives using sodium hydride

Having established the method for determining the enantiomeric purity of N-methylamino acids and their derivatives, we were able to determine the enantiomeric purity of all the synthetic N-methylamino acid derivatives which were prepared in this laboratory by methylation of urethane derivatives using sodium hydride. The more recent version of this method involves the methylation of the N-benzyloxycarbonyl and N-tert-butyloxycarbonyl derivatives in THF using sodium hydride and methyl iodide at room temperature to give directly the N-protected, N-methylamino acid [McDermott and Benoiton (1973a), Part III, 1 and 2] as shown in route [9].

Route [9] Synthesis of N-methylamino acid derivatives using sodium hydride and methyl iodide

$$RCONH - CHR^1 - COOH \xrightarrow[THF,\ 23°]{NaH,\ CH_3I} RCON(CH_3) - CHR^1 - COOH$$

R = OCH_2Ph, OBu^t; 5°C for R^1 = CH_2OBzl, CH_2OBu^t

All the derivatives tested were optically pure, except for N-benzyloxycarbonyl, N-methyl-L-isoleucine, which contained 0.9%

of diastereomer. Since procedures are available for deprotecting these derivatives without affecting their chirality, this method provides a source for optically pure N-methylamino acids. The results are shown in Table III. 317.

The earlier version of this method included some DMF as well as THF as solvent, with heating [Coggins and Benoiton (1971)]. The product is the corresponding methyl ester as shown in the following route [10]

Route [10] Synthesis of N-methylamino acid derivative via methyl ester

$$RCONH - CHR^1 - COOH \xrightarrow[THF\text{-}DMF\ (10:1),\ 80°C]{NaH,\ CH_3I} RCON(CH_3) - CHR^1 - COOCH_3$$

$$RCON(CH_3) - CHR^1 - COOCH_3 \begin{cases} \xrightarrow{(a)\ 2N\ NaOH\text{-}MeOH,\ 35°C\ R = OCH_2Ph} RCON(CH_3) - CHR^1 - COOH \\ (b)\ N\ NaOH\text{-}THF;\ R = Ph,\ CH_3 \\ \xrightarrow[R = OCH_2]{5.6\ N\ HBr/AcOH} HN(CH_3) - CHR^1 - COOCH_3 \end{cases}$$

The N-protected, N-methylamino acid is then obtained by saponification. But it has been shown that fully protected N-methylamino acids can undergo some inversion in aqueous base [McDermott and Benoiton (1973c)]. Therefore, products obtained in this manner may not be enantiomerically pure. Because of the success with the recent version of this method, none of the N-methyl-

amino acid derivatives obtained via methyl esters were examined. It should nevertheless be noted that N-methylamino acid derivatives with a free methylamino group are not racemized by base. Therefore optically pure N-methylamino acids are still accessible through this procedure as well as using silver oxide and methyl iodide in DMF if the saponification is carried out after removal of the N-protecting group. Removal of N-protected groups by hydrogenation occurs without change in chirality. However, inversion may occur if hydrogen bromide in anhydrous acetic acid is used on a derivative containing a free carboxyl group. [McDermott and Benoiton (1973c)].

Preparation of N-benzyloxycarbonyl, N-methylamino acids using sodium hydride and methyl iodide: The N-methylation procedure was the same as described in Part III 1. p.38 except that 5 N HCl was used for acidification.

Table III 318a

Enantiomeric purity of N-methylamino acids prepared by methylation of urethane derivatives using sodium hydride and methyliodide in THF at room temperature[a]

Amino Acid	Derivative	
	Boc	Z
H-MeAla-OH	<0.1	<0.1
H-MeLeu-OH	<0.1	<0.1
H-MeIle-OH	<0.1	0.9
H-MeaIle-OH	<0.1	<0.1
H-MePhe-OH	<0.1	<0.1
H-MeSer-OH[b]	<0.1	
H-MeThr-OH[b]	<0.1	
H-MeVal-OH	<0.1	<0.1

[a] % D-isomer

[b] side chain was protected by a benzyl or tert-butyl group; methylation at 5°C.

Table III ~318b

Enantiomeric purity of methylated hydroxyamino acid derivatives prepared by methylation of urethane derivatives using sodium hydride and methyl iodide in THF at 5°C

Amino Acid	D-isomer %
Boc-Ser(Me)-OH	<0.1
Z-MeSer(Me)-OH	<0.1
Z-Thr(Me)-OH	<0.1
Z-MeThr(Me)-OH	<0.1

(b) Methylation of p-toluenesulfonyl derivatives

N-Methylamino acids were first obtained by Fischer and Lipschitz (1915) by methylation of the p-toluenesulfonylamino acid in basic solution with methyl iodide at 65°C and the product was detosylated by one of the various procedures as shown in the following route [11].

Route [11] Synthesis of N-methylamino acid via p-toluenesulfonyl derivative.

$$P\text{-}CH_3\text{-}C_6H_4SO_2NH\text{-}CHR'\text{-}COOH \xrightarrow[65°C,\ 20\text{-}60\ min.]{2N\ NaOH,\ CH_3I} P\text{-}CH_3\text{-}C_6H_4SO_2N(CH_3)\text{-}CHR'\text{-}COOH$$

a) 12N HCl, 100°C, 8 h [Fischer and Lipschitz (1915)]

b) Na/NH_3 [du Vigneaud and Behrens (1937)]

$$\longrightarrow NH(CH_3) - CHR^1 - COOH$$

c) 5.6 N HBr/AcOH, 70°C, 2 h [Ovchinnikov (1962)]

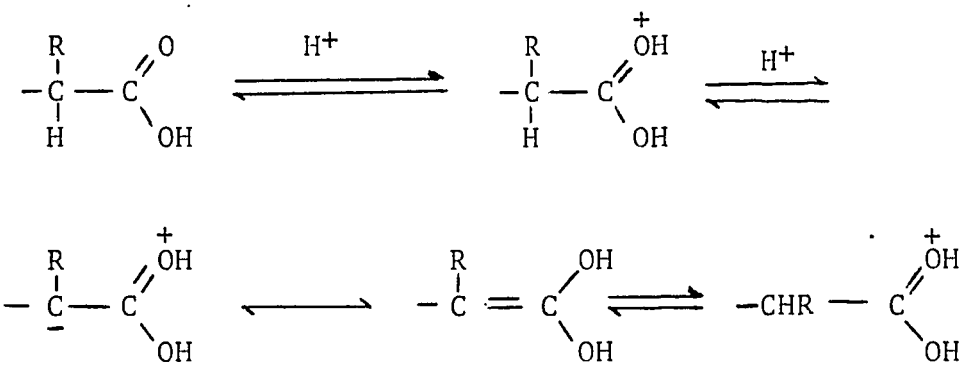
d) 2 N HBr/AcOH, 24 h, room temperature [Weisblat et al (1953)]

Phosphonium iodide in hydriodic acid was also used for p-toluenesulfonylamino cleavage [Fischer and Lipschitz (1915)]. It has generally been accepted that this method gives optically pure products. However, the only criterion of purity available has been specific rotation measurements. Since the method for determining the enantiomeric purity of N-methylamino acid has been developed, the enantiomeric purity of N-methylamino acids

obtained via p-toluenesulfonyl derivatives were determined. p-Toluenesulfonyl N-methyl-L-alanine and valine were synthesized by the procedure of Fischer and Lipschitz (1915). The products were then detosylated by the methods as shown in route [11]. The enantiomeric purities of the N-methylalanine and N-methylvaline were determined using the procedure as described in Part III, 4, p.93 . The results are shown in Table III 319. All cleavage methods using acid led to significant racemization. Only the use of sodium in liquid ammonia for cleavage [du Vigneaud and Behrens (1937)] gave optically pure products. The original acid hydrolysis method of Fischer and Lipschitz (1915) gave less racemization than did acidolytic cleavage by hydrogen bromide in acetic acid [Weisblat et al (1953)], which gave about the same results whether the reactions were carried out at room temperature or at 70°C. The fact that at least one of these methods gave optically pure products proves that the racemization occurs during cleavage and not during methylation. The methylation of N-p-toluenesulfonyl-L-phenylalanine may be exception to this because in their work, Fischer and Lipschitz isolated two fractions of N-p-toluenesulfonyl, N-methyl-L-phenylalanine, one with a specific rotation 15% lower than that of the other [Fischer and Lipschitz (1915)].

The acid-catalyzed racemization of amino acids in 6N hydrochloric acid is well established [Manning and Moore

(1968)]. Ionization of the α-C-H bond has been proposed as the mechanism by Neuberger (1948), as follows.



Heating amino acids in tritiated hydrochloric acid resulted in some incorporation of tritium into the α-position [Manning (1970)]. The racemization of N-methylamino acids during detosylation using the original acid hydrolysis method of Fischer and Lipschitz (1915) can therefore be explained by this mechanism.

The racemization observed for acidolytic cleavage agrees with the previous demonstration that hydrogen bromide in acetic acid racemizes N-substituted, N-methylamino acids [McDermott and Benoiton (1973c)]. They found that the L,D-isomer contained 2.6 times as much radioactivity as the L,L-isomer when a peptide was incubated in a tritiated solution of hydrogen bromide in acetic acid and the extent of racemization was determined on the Amino Acid analyzer [McDermott and Benoiton (1973c)]. Hence they proved that ionization and protonation

had taken place during acidolytic cleavage using hydrogen bromide in acetic acid. It is suspected that the inversion observed during cleavage by acid hydrolysis is probably higher than that which would occur for the corresponding unmethylated derivatives, as it has been observed that the presence of an >N-H group next to the α-C-H inhibits racemization [McDermott and Benoiton (1973c)]

Preparation of N-p-toluenesulfonyl, N-methyl-amino acids: N-p-toluenesulfonyl amino acid (1 mol) and methyl-iodide (2 mol, 18 g) were added to 125 ml 2N NaOH (4 mol). The mixture was stirred at 65°C for 20 min in a closed pressure flask. The mixture were then stirred at room temperature for another 20 min. The solution was acidified with 5N HCl and extracted with ethyl acetate (50 ml x 2). The combined extract was washed with water (50 ml x 2), dried over $MgSO_4$ and evaporated to dryness. The products were used as such without recrystallization to avoid fractionation.

Cleavage of p-toluenesulfonyl-amido group using 12N HCl: 2 g of N-p-toluenesulfonyl, N-methylamino acid was suspended in 7.6 ml 12N HCl in a sealed tube and incubated at 100°C for 8 h. The tube was then cooled and opened and toluenesulfonic acid was filtered off. The filter was washed several times with water. The combined filtrate was evaporated to dryness with addition of water several times. The residue was immediately derivatized and coupled with N^{ε}-benzyloxycarbonyl-

L-lysine-benzyl ester for enantiomeric determination.

Cleavage of p-toluenesulfonyl-amido group using sodium in liquid ammonia: 1 g N-p-toluenesulfonyl, N-methyl-amino acid was placed in 6 ml of dry liquid ammonia and reduced by adding 0.5 g of sodium to produce a permanent blue colour. The excess sodium was discharged with NH_4Cl and the ammonia was removed by evaporation. The white residue which remained was dissolved in 20 ml of water and the resulting solution was thoroughly aerated to remove the ammonia. The solution was then evaporated to dryness with addition of water several times. N-methylamino acid recovered was again derivatized for enantiomeric determination using the method as described in Part III, 4. p. 93.

Cleavage of p-toluenesulfonyl-amido group using hydrogen bromide in acetic acid: 1 g of N-p-toluenesulfonyl, N-methylamino acid was treated in 6 N HBr in acetic acid at 70°C for 2 h. Evaporation of the reagent gave an oil which was then dissolved in water and extracted with ether (20 ml). The aqueous layer was stirred with Dowex 50(H^+) (20 ml batch volume) for 30 min ; after filtering, the solution was treated a second time with resin (20 ml batch volume) and the combined resin batches were washed slowly with water ($\frac{1}{2}$ l) on a sintered glass funnel. The washed resin was stirred with 10N NH_4OH (40 ml) for 30 min ; the resulting solution was evaporated several times. The product was derivatized for enantiomeric

purity determination.

Cleavage of p-toluenesulfonyl-amido group using hydrogen bromide in acetic acid at room temperature: 1 g of N-p-toluenesulfonyl, N-methylamino acid was treated with 2N HBr in acetic acid at room temperature for 24 h. N-methyl-amino acid was recovered with the same procedure as described above. The enantiomeric purity was determined using the method described in Part III, 4, p.93.

Table III 319

Enantiomeric purity of N-methylamino acids prepared from the N-p-toluenesulfonyl derivatives[a]

Method of detosylation	H-MeAla-OH	H-MeVal-OH
a) 12NHCl, 100°C 8h	4.4	2.3
b) Na/NH_3	<0.1	<0.1
c) 6N HBr/AcOH, 70°C, 2h	13.6	5.1
d) 2NHBr/AcOH, 24h	11.3	

[a] % D-isomer.

(c) Methylation of urethane derivatives using silver oxide

The methylation of N-benzyloxycarbonyl or N-tert-butyloxycarbonyl derivatives using silver oxide and methyl iodide in DMF at room temperature (sometimes aided by heating) gives the N-methylated, methyl ester [Olsen (1970) ; Okamoto et al (1974)]. In order to obtain a derivative suitable for peptide synthesis by this approach, it is necessary either to saponify the methyl ester [Olsen (1970)] or to remove the amino protecting group [Coggins and Benoiton (1972) p. 145] as shown in route [12]

Route [12] Synthesis of N-methylamino acid derivatives using silver oxide and methyl iodide

$$RCONH - CHR^1 - COOH \xrightarrow[DMF,\ 23 \text{ or } 45°C]{Ag_2O,\ MeI} RCON(CH_3) - CHR^1 - COOCH_3$$

either

$$\xrightarrow{N\ NaOH/EtOH(1:3)} RCON(CH_3) - CHR^1 - COOH$$

or

$$\xrightarrow[70\%\ tfa \quad R = OBu^t]{H_2/Pd(C)\ R = OCH_2Ph} HN(CH_3) - CHR^1 - COOH_3$$

It has been shown in this laboratory that saponification of methyl esters of N-benzyloxycarbonyl, N-methylamino acids can cause racemization [McDermott and Benoiton (1973c)]. It is

generally assumed that racemization occured as a result of simple ionization at the α-carbon-hydrogen bond of the N-methyl-amino acid. However, isoracemization in which the asymmetry is lost by ionization without dissociation [Cram and Gosser (1964), Kovacs et al (1971)] cannot be ruled out. There are two theories to explain why N-methylamino acid methyl ester racemize more readily than corresponding amino acid derivatives during saponification. McDermott and Benoiton suggested that in a N-monosubstituted amino acid ester, the >N-H would generally be more acidic than the α-C-H. Thus in base, this group would be ionized first protecting the neighbouring C-H bond from ionization. In a N-substituted, N-methylamino acid ester, no such effect obtains , and the α-C-H would be ionized more readily. Similarly, ionization of the free carboxyl group of an N-substituted, N-methylamino acid would prevent further ionization at the α-carbon atom. Alternatively, Neuberger (1948) proposed that the electron-donating methyl group favours the polar immonium form of the amide bond which would encourage the ionization of the neighbouring C-H bond. Using the method described in Part III, 4 to study the enantiomeric purity of the N-methylamino acid derivatives obtained by N-methylation of the urethane derivative using silver oxide, we found that all products contained either about 1% or else substantially more of D-isomer. The results are shown in Table III, 320. Generally, all N-tert-butyloxycarbonyl derivatives

were about 2% racemized except for N-methylalanine which was higher. N-Benzyloxycarbonyl derivatives were 8-28% racemized except for N-methylleucine. The high figures were for those which had been more difficult to saponify. These results confirm the previous demonstration in this laboratory that fully protected N-methylamino acids are racemized by base [McDermott and Benoiton (1973c)]. The N-tert-butyloxycarbonyl derivatives were more easy to saponify than the N-benzyloxycarbonyl derivatives, therefore having the higher degree of optical purity. The amino acid side-chains had no effect on the results for the N-tert-butyloxycarbonyl derivatives, which contrasts with the pronounced effect they had on the results for the N-benzyloxycarbonyl derivatives. It would seem that the method of methylation itself does not cause racemization although it has not been verified.

Preparation of N-benzyloxycarbonyl and N-tert-butyloxycarbonyl, N-methylamino acid derivatives using silver oxide and methyl iodide in DMF: The N-benzyloxycarbonyl or N-tert-butyloxycarbonylamino acid was dissolved in anhydrous DMF. To the resulting solution was added methyl iodide (4 to 8 fold molar excess) and silver oxide (3 to 4 fold molar excess). The reaction mixture was stirred for 5-8 h, at room temperature, for the N-benzyloxycarbonyl derivatives and at 45°C for the N-tert-butyloxycarbonyl derivatives. The mixture was filtered and the solid washed with a small volume of DMF. To the filtrate was added approximately 4 volumes of chloroform. The

chloroform phase, in which a precipitate had formed, was washed twice with 5% aqueous potassium cyanide, several times with water, and was dried over $MgSO_4$. The drying agent was removed by filtration and the solvent evaporated in vacuo. The last traces of DMF usually present were removed in vacuo with an oil vacuum pump at a bath temperature below 40°C.

Saponification of N-benzyloxycarbonyl and N-tert-butyloxycarbonyl, N-methyl amino acid derivatives: A solution of 6.0 mmol N-benzyloxycarbonyl or N-tert-butyloxycarbonyl N-methylamino acid methyl ester which was prepared by the above method and 6.2 ml of 1 N sodium hydroxide in 20 ml of 95% ethanol was allowed to stir at room temperature for 1 h. The major portion of the solvent was evaporated in vacuo, after which 15 ml of water was added. The resulting solution was cooled and acidified to pH 3 with 1 N HCl for N-benzyloxycarbonyl derivatives and solid citric acid for N-tert-butyloxycarbonyl derivatives. The aqueous phase was extracted 3 times with ethyl acetate, following which the organic phase was washed several times with water and dried over $MgSO_4$. The drying agent was filtered off and the solvent removed in vacuo.

Table III 320

Enantiomeric purity of N-methylamino acids prepared by methylation of urethane derivatives using silver oxide and methyl iodide in DMF[a]

Amino acid	Derivatives[b] Boc	Z	
H-MeAla-OH	6.3	8.7	
H-MeLeu-OH		0.9	
H-MeIle-OH	1.2[d]	6.0[e]	14.2[c]
H-MePhe-OH	0.8	4.1	
H-MeVal-OH	0.9	4.5	8.7[c]

[a] % D-isomer.

[b] Methylation at 23°C for Z, and 45°C for Boc, followed by saponification for 1h.

[c] 4h required to complete the saponification.

[d] 25% of the ester remained after 1h saponification.

[e] 55% of the ester remained after 1h saponification.

(d) Methylation by successive reductive alkylations

A more common method to prepare N-methylamino acids was introduced by Quitt, Hellerbach and Vogler (1963). This method involved reductive alkylation of the amino acid using benzaldehyde and either of two reducing agents, sodium borohydride, or hydrogen and palladium-on-charcoal catalyst, to give the N-benzylamino acids, followed by methylation by the procedure of Clarke-Eschweiler [Eschweiler (1905) and Clarke et al (1933)] using aqueous formaldehyde in hot formic acid [Quitt et al (1963)]. The reaction is shown in route [13].

Route [13]. Synthesis of N-methylamino acids by reductive alkylation.

$$NH_2 - CHR^1 - COOH \xrightarrow[\text{(a) } OH^-,\ NaBH_4 \quad \text{(b) } OH^-,\ H_2 - Pd(c)]{\text{aqueous PhCHO}} PhCH_2NH - CHR^1 - COOH$$

$$\xrightarrow[HCOOH\ \Delta]{\text{aqueous HCHO}} PhCH_2N(CH_3) - CHR^1 - COOH \xrightarrow[Pd(c)]{H_2} HN(CH_3) - CHR^1 - COOH$$

The first alkylation is carried out on the sodium salt of the amino acid. This promotes Schiff base formation by deprotona-

ting the amino group, and also solubilizes the amino acid. The benzyl group is finally removed by catalytic hydrogenation. N-Methylamino acids obtained by this method have been generally accepted as optically pure which is only based on the criterion of specific rotation measurements. Using our enantiomeric purity determination method, we found that the products prepared using sodium borohydride all contained 2.7-13.9% of D-isomer, except for N-methylleucine which contained 0.3% of D-isomer. Particularly striking are the results for two "hindered" compounds, N-methylisoleucine and N-methylvaline, which had undergone 25% inversion. The results are given in Table III. 321.

In order to identify the origin of the racemization, N-benzylamino acids were prepared by both procedures, and examined for optical purity. All the products, except N-benzyl-L-leucine, were found to be racemized, but generally to a lesser extent than had been found for the corresponding N-methylamino acids. This suggested that racemization might also be occurring during the second step of the synthesis, namely the methylation. This was proven in the case of alanine where an optically pure sample of N-benzyl-L-alanine was synthesized by another procedure (p. 130). Methylation of this N-benzyl-L-alanine by the Clarke-Eschweiler method gave a product containing 5.5% of the D-isomer.

The reductive alkylation of an amino acid to the

benzylamino acid involves the reduction of the Schiff base in the presence of base. It is known that tautomerism of a Schiff base can occur under these conditions [Sollenberger and Martin (1968) p. 349], and this would be promoted by the adjacent carboxylate anion. The partial racemization observed for the N-benzylamino acids can therefore be accounted for on this basis, in the following route.

$$PhCH = N - CHR^1 - COO^{\theta} \rightleftharpoons PhCH_2 - N = CR^1 - COO^{\theta}$$

These results should be taken into account if N-benzylamino acid derivatives are used for purposes, such as the synthesis of optically active α-hydrazino acids [Achiwa and Yamada (1975)].

The mechanism of the Clarke-Eschweiler reaction has been investigated in several laboratories [Cope et al (1960), Pine and Sanchez (1971)]. A common by-product of the reaction is the carbonyl compound, attributed to hydrolysis of the isomerized Schiff base, exemplified as in the following route for a primary amine.

$$RCH_2 - N = CH_2 \longrightarrow RCH = N - CH_3 \xrightarrow{H_2O} RCHO + CH_3NH_2$$

The methylation of a few chiral primary amines including a benzylamine has been examined [Pine and Sanchez (1971), McKenna and Slinger (1958)] and despite formation of some carbonyl product, no evidence for epimerization was obtained, leading to

the conclusion that the Clarke-Eschweiler reaction proceeds without epimerization, and they postulated that any isomerized Schiff base is neither further methylated nor reisomerized but converted completely to the carbonyl compound [Pine and Sanchez (1971)]. However, benzylamino acids incorporate an additional factor besides the benzyl group, the carboxyl group, which would also favour migration of unsaturation. The latter is substantiated by the nature (methylamine and carbon dioxide) and extent of by-product formation during the methylation of amino acids [Clarke et al (1933)]. The combination of these two groups must be sufficient to cause a small but significant reversal of the isomerization as shown in the following route.

$$PhCH_2 - \overset{+}{N}(=CH_2) - CHR^1 - COOH \rightleftharpoons PhCH_2 - \overset{+}{N}(CH_3) = CR^1 - COOH$$

This seems to be the first demonstration of an epimerization occurring during methylation by the Clarke-Eschweiler procedure.

Preparation of N-benzylamino acids using sodium borohydride as reducing agent: Amino acid (0.1 mol) was dissolved in 50 ml 2N NaOH with stirring. Freshly distilled benzaldehyde (10.1 ml, 0.1 mol) was added to the mixture which was stirred for 15-20 min. until homogeneous. Sodium borohydride (1.14 g, 0.03 mol) was then added to the mixture with cooling below 15°C. The mixture was stirred for 30 min. and the addition

of benzaldehyde and sodium borohydride were repeated. The mixture was left stirring for another 2 h. The solution was washed with ether (20 ml x 2) to remove excess benzaldehyde. The aqueous phase was neutralized with 1N HCl to pH 6-7. The benzylamino acid precipitated out, was filtered, washed with water several times and dried in air.

Preparation of N-benzylamino acids using hydrogen and palladium charcoal catalyst: 0.1 mol of amino acid was dissolved in 1N NaOH (100 ml) with stirring. Freshly distilled benzaldehyde (0.1 mol, 10.1 ml) was added to the mixture which was stirred for 15-20 until homogeneous. 3 g 5% palladium charcoal was added to the solution and it was hydrogenated under normal condition. After 24 h, 1 equivalent of hydrogen was taken up. The solution was filtered to remove the palladium charcoal catalyst. The filtrate was then neutralized with 1N HCl to pH 6-7. The benzylamino acid precipitated out, was filtered, washed with water several times and dried in air.

Preparation of N-benzyl, N-methylamino acid: N-benzylamino acid (0.05 mol) was heated with formic acid (5.6 ml, 0.15 mol) and formaldehyde (5 ml, 0.06 mol) 38-40% in a water bath. The N-benzylamino acid gradually went into solution. For N-benzyl, N-methylalanine, leucine and valine, the reaction time was 1½ h. For N-benzyl, N-methylphenylalanine, the reaction time was 4 h. The reagent was removed by evaporation. N-Benzyl, N-methylamino acid was crystallized with the aid of acetone.

Table 312. Enantiomeric purity of N-alkylamino acids prepared by reductive alkylation [2][a]

Amino acid / Reducing agent	N-Methylamino acid	N-Benzylamino acid		
	$NaBH_4$[b]	$NaBH_4$	H_2,Pd(C)	$NaCNBH_3$
Ala	2.7, 4.3	2.6, 2.3	4.5	0.1[c]
Leu	0.3, 0.3	0		
Val	13.9	6.0, 5.1	5.1	d
Phe	3.1	1.6, 1.6		
Ile	12.0			
D-Ala	5.2			

[a] % D-isomer. Each value represents a different experiment.

[b] Followed by reductive alkylation of the N-benzylamino acid using HCHO/HCOOH.

[c] A sample showed 5.5% D-isomer after methylation using HCHO/HCOOH.

[d] Could not be prepared due to insolubility of valine at pH 6.5

(e). A method of preparing optically pure N-benzyl-L-alanine

Having demonstrated that reductive alkylation carried out at alkaline pH gave partially racemized N-benzyl-amino acids (Table III, 321), the use of sodium cyanoborohydrides as reducing agent was examined. These reagents introduced by Borch et al (1972) are stable down to pH 3, and effective at acidic pH. It was found that by carrying out the reduction of L-alanine at pH 6.5, optically pure N-benzyl-L-alanine was obtained in good yield. However, success was not obtained until a pH-stat was used. Addition of sodium cyanoborohydride to an aqueous amino acid solution causes an immediate rise pH of about 3-4 units, and a sample of N-benzyl-L-alanine containing as much as 20% D-isomer was once obtained. This served to demonstrate dramatically the effect of pH on the optical integrity of an amino acid which is undergoing reductive alkylation, and confirmed the suspicion that benzylamino acids prepared by the method of Quitt et al (1963) are not optically pure because base is added to the reaction mixture. However, it is sometimes necessary for practical reasons, to keep the amino acid in solution. N-benzyl-L valine could not be prepared using sodium cyanoborohydride at pH 6.5 because valine is not soluble enough at this pH. However amino acids which are soluble in water at acidic pH, namely aspartic and glutamic acid could be benzylated using sodium

cyanoborohydride to obtain optically pure products

In view of the demonstration that racemization had also occurred during the methylation of N-benzylamino acid by the Clarke-Eashweiler procedure, attempts were made to use sodium cyanoborohydride instead of formic acid as reducing agent for the reductive methylation of N-benzyl-L-alanine. The same solubility problem arose and the attempt to obtained optically pure N-benzyl, N-methyl-L-alanine using sodium cyanoborohydride as the reducting agent was abandoned without further investigation.

Preparation of optically pure N-benzyl-L-alanine: A mixture containing L-alanine (8.9 g; 0.1 mol), 10 ml of benzaldehyde and 50 ml of water was stirred until it became homogeneous (30 min). The pH of the reaction was kept constant at pH 6.5 by titration with N HCl using a pH stat. Sodium cyanoborohydride (1.89 g; 0.03 mol) was added and after stirring for 2 h, the addition of aldehyde and reducing agent were repeated. Stirring was continued until HCl consumption had ceased (24 h). The copious precipitate was brought into solution by adjusting the pH to 8.5, excess benzaldehyde was removed by extraction with ether, and the product was crystallized by bringing the pH back to 6.5. The air-dried product, after washing with ether (yield, 90%) was recrystallized from water-ethanol and was shown to contain <0.1% of the D-isomer using our method for enantiomeric determination.

(f). General discussion of the studies on the enantiomeric purity of N-methylamino acids obtained by various procedures

The results obtained with difunctional amino acids can be summarized as follows: Optically pure N-methylamino acids can be obtained (a) by the methylation of p-toluene sulfonyl amino acids using Na/liquid ammonia for detosylation and (b) N-methylation of urethane derivatives using sodium hydride and methyliodide. For the latter, N-benzyloxycarbonyl groups can be cleaved by catalytic hydrogeration or hydrogen bromide in aqueous acetic acid [McDermott and Benoiton (1973a)], and N-tert-butyloxylcarbonyl groups, by any acidolytic reagent other than hydrogen bromide in anhydrous acetic acid [McDermott and Benoiton (1973a)]. Though it has not been proven definitely, the methods introduced by Olsen (1970) and Coggins and Benoiton (1968) probably give optically pure N-methylamino acids provided that saponification is not used before N-deprotection to obtain the final product. The two alternatives are saponification subsequent to N-deprotection, or O-deprotection by acid hydrolysis. Optically pure N-protected, N-methylamino acids can be obtained by methylation of the corresponding amino acid derivative using the methods of N-methylation for N-benzyloxycarbonyl and N-tert-butyloxycarbonyl and for p-toluenesulfonyl derivatives. N-methylleucine is exceptional in that, in addition

to the above, essentially optically pure products are obtainable also using other methods such as N-methylation using silver oxide and methyl iodide and the reductive alkylation method of Quitt et al (1963).

With respect to the relative sensitivities to racemization of the different amino acids under synthetic conditions, the side-chain of leucine consistently allowed the least amount of recemization. The side-chain of valine and isoleucine allowed surprisingly high amounts of racemization during reductive alkylation (Table III 312) and caused more racemization than others during saponification. This is because their derivatives are more difficult to saponify (Table III 320). When all methods are taken into consideration, alanine is the most sensitive to racemization, since both products obtained by the method of N-methylation using silver oxide and methyl iodide (Table III 320) were substantially racemized. N-methyalanine also racemized more than N-methylvaline during acidolytic removal of the p-toluenesulfonyl group (Table III 319). The demonstration that racemization occurred in the many cases examined provides an explanation for the discrepancies in physical data (melting points and specific rotations) which are recorded in the literature particularly for N-methylalanine and N-methylvaline and their derivatives prepared by various methods.

IV. SUMMARY

1. Studies on methylation of N-tert-butyloxycarbonyl amino acids using methyl iodide and sodium hydride in tetrahydrofuran at room temperature showed that N-tert-butyloxycarbonyl N-methylamino acids could be obtained in excellent yield. This is the best available method of obtaining N-tert-butyloxycarbonyl, N-methylamino acids, and provides an alternative to the N-benzyloxycarbonyl derivatives for N-methylamino acid synthesis.

2. The method also allowed preparation of Boc-MeThr(Bzl)-OH and Boc-MeTyr(Bzl)-OH from the parent amino acid derivatives in high yield.

3. Methylation of Boc-Ser(Bzl)-OH under these conditions gave Boc-MeΔAla through β-elimination.

4. β-elimination was completely suppressed at 5°C, allowing a good synthesis of Boc-MeSer(Bzl)-OH and Z-MeSer(Bu^t)-OH.

5. A new one-step synthesis of pure Boc-Ser(Me)-OH and Z-Thr(Me)-OH has been derived using limited amounts of sodium hydride and at 5°C. The method is much superior to available methods of synthesising these amino acids or their derivatives.

6. A convenient synthesis of N,O-dimethyl derivatives of serine and threonine was developed along similar line by increasing the amount of sodium hydride and methyl iodide used at 5°C. No β-elimination nor side reaction was found.

7. Z-MeSer-OH and Boc-MeThr-OH have been prepared from the appropriate O-protected derivatives

8. A comparison of the rate of N-methylation using alanine derivatives as model, showed that at 5°C N-benzyloxylcarbonyl alanine was methylated two times faster than that of N-tert-butyloxycarbonyl alanine after 15 h.

9. A comparison of the rate of acidolytic cleavage of derivatives showed that a N-tert-butyloxycarbonyl, N-methylamino acid is cleaved at least twice as fast as N-tert-butyloxycarbonyl amino acid.

10. A detailed study of the formation of dicyclohexylammonium salt of N-methyl and O-methylamino acid derivatives showed that all tested N-methylamino acid derivatives formed insoluble dicyclohexylammonium salt except those protected on the side chain by a benzyl or tert-butyl group.

11. The applicability of the method of Manning and Moore for determining the enantiomeric purity of N-methylamino acids has been reexamined. Leucine N-carboxyanhydride was found to couple with only N-methylalanine, showing the limit of the method.

12. A general method capable of determining one part of one thousand of the other isomer for determining the enantiomeric purity of N-methyl amino acids has been derived. The method consists in converting the N-methylamino acid to its N-benzyloxycarbonyl derivative and coupling the derivative with L-Lys(Z)-OBzl using N,N'-dicyclohexylcarbodiimide, followed by removal of protecting groups. The resulting diastereomeric lysyl peptides are analyzed by ion exchanged chromatography on a 15 cm column of Aminex A-5 resin using Amino acid analyzer. No other method is available.

13. This method was found applicable to determine the enantiomeric purity of unmethylated amino acid and their derivatives providing an alternative to the method of Manning and Moore.

14. Proton n.m.r. (100 MHz) studies of a few protected lysyl

dipeptides containing N-methyl amino acid showed that the diastereomers could be distinguished by different chemical shifts of the methoxyl methyl singlets providing a second, albeit less sensitive, method for determining the enantiomeric purity of some N-methyl amino acids.

15. It has been established that all N-methyl amino acid derivatives prepared using methyl iodide and sodium hydride at room temperature are stereochemically pure (<0.1% D-isomer).

16. It has been established that N-methyl amino acids obtained by methylation of p-toluenesulfonyl amino acids are stereochemically pure, provided that sodium in liquid ammonia and not hydrogen bromide in acetic acid or hydrochloric acid is used for detosylation.

17. N-benzyloxycarbonyl, N-methyl amino acids obtained by methylation using silver oxide and method iodide in DMF followed by saponification of methyl ester contained 4-14% D-isomer; N-tert-butyloxycarbonyl, N-methyl amino acid obtained in the same way were about 2% racemized.

18. Partial racemization was found in N-benzyl-amino acids obtained by alkylation of the amino acid sodium salt using benzaldehyde and either sodium borohydride or hydrogen and

palladium on charcoal catalyst.

19. N-methylation of N-benzylamino acids using formic acid and formaldehyde were also partially racemized.

20. It is found that optically pure N-benzylalanine could be obtained if sodium cyanoborohydride at acidic pH is used as reducing agent.

21. Methylation of this optically pure N-benzylalanine using formic acid and formaldehyde resulted in partial racemization. This is the first demonstration of inversion during Clarke- Eschweiler reaction.

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