STUDIES IN THE CHEMISTRY OF THE LIPIDS

OF TUBERCLE BACILLI

A Thesis submitted for the degree of

Doctor of Philosophy

- by -

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CONTENTS

INTRODUCTION 1

DISCUSSION

Section I. The mycolic acids from human tubercle bacilli

a) Previous researches 11

b) Present investigations 20

c) Stereochemical investigations 53

d) Biogenesis of the human mycolic acids 57

e) Nomenclature 64

Section II. Phthiocerol and related diols.

a) Previous researches 66

b) Present investigations 69

c) Stereochemical investigations 90

d) Biogenesis 95

Section III. Methyl-branched long-chain acids of biological interest.

a) Previous researches 98

b) Present investigations 106

EXPERIMENTAL

General notes on techniques employed 113

Section I. The mycolic acids from human tubercle bacilli

Chromatography of the crude mycolic acids 117

The methyl esters of the mycolic acids 118

Chromatography of the mixture of methyl mycolates . . 119

Removal of methyl mycolate -III as its oxime 120

Chromatography of a mixture of methyl mycolate -I and-II 121

Acetylation of a mixture of methyl mycolate -I . . and -II 122

Large scale chromatography of mixtures of methyl acetylmycolate -I and -II 123

Chromatography of fractions consisting mainly of methyl acetylmycolate -I 123

Chromatography of fractions consisting mainly of methyl acetylmycolate -II 124

Further chromatography of mixtures of methyl acetylmycolate -I and -II 125

Methyl acetylmycolate -I 125

Methyl acetylmycolate -II 126

Methyl mycolate -III oxime 127

Chromatographic studies of the oxime of methyl mycolate -III 127

Methyl acetylmycolate -III oxime 128

Chromatographic studies of the oxime of methyl acetylmycolate -III 129

Methyl 13-oxohexacosanoate oxime 131

Methyl acetylmycolate -III 132

Methyl anhydromycolate -III 133

Methyl acetylmycolate -III thioketal 134

Methyl mycolate -I 134

Methyl 2-epimycolate -I 137

Methyl anhydromycolate -I 139

Methyl mycolate -II 139

Methyl anhydromycolate -II 141

Methyl mycolate -III 142

Section II. Phthiocerol and related diols

Thin-layer and unidimensional multiple chromatography of crude phthiocerol 143

O-Isopropylidene derivative of phthiocerol 144

Ethylidene acetals of phthiocerol 145

Chromatography of the ethylidene acetals 145

Ethylidenephthiocerol A 147

Ethylidenephthiocerol B 149

Phthiocerol A 150

Phthiocerol B 151

Chromatography of the ethylidene acetal of phthiodiolone A 152

Diacetylphthiodiolone A oxime 152

Ethylidenephthiodiolone A oxime 154

Phthiodiolone A 155

Ethylidenephthiodiolone A 156

Section III. Methyl-branched long-chain acids

Methyl hydrogen β-methylglutarate 158

Methyl 3-methyl-5-oxo-5-(2-thienyl) pentanoate . . . 158

Methyl 3-methyl-5-(2-thienyl) pentanoate 159

1-Bromoheptane 160

Nonan-2-one 160

Ethyl 2-cyano-3-methyldec-2-enoate 161

Hydrogenation of ethyl 2-cyano-3-methyldec-2-enoate . 162

1-Bromo-3-methyldecane 162

5-Methyldodecan-2-one 164

Ethyl 2-cyano-3,6 -dimethyltridec-2-enoate 165

Methyl 3,12,15 - trimethyldocosanoate 167

3,12,15-Trimethyldocosanoic acid 169

2-Iodothiophen 169

2,2′- Bithienyl 170

Methyl 3-methyl-5-oxo-5-(5-2,2′-bithienyl) pentanoate . 170

Methyl 3-methyl-5-(5-2,2′-bithienyl) pentanoate 171

2-Methylnonanoic acid 172

Methyl 3-methyl-5-(5′-2-methylnonyl-5-2,2′-bithienyl)
-pentanoate 172

Methyl 3,15-dimethyldocosanoate. 174

REFERENCES 175

INTRODUCTION

An important feature of Mycobacterium tuberculosis is its ability to synthesize a large amount of lipids. The latter were claimed[1,2,3], to be responsible for the acid fastness of the bacilli i.e. their resistance to decolourisation by mineral acid after staining with phenol-fuchsin. Moreover the lipids of the human tubercle bacillus on injection into test animals produce tubercular lesions [4,5], and certain constituents have been claimed to have antigenic properties[6,7]. This behaviour has led to extensive investigations of the constituents of the lipids in order to correlate their chemical constitution with their biological activity.

Systematic researches into the nature of the lipids of human tubercle bacilli as well as those of the avian and bovine variety were initiated by Anderson[8,9], and his collaborators. These investigators extracted the living bacilli and separated the lipids into acetone-soluble fats, phosphatides, and waxes[9]. Hydrolysis of the fats and phosphatides gave mainly "phthioic acid" (dextro-rotatory alkyl-branched long-chain acids), tuberculostearic acid (10-methyloctadecanoic acid[10]) and common long-chain acids

(palmitic, stearic and oleic). Prolonged hydrolysis of the wax fraction gave mainly "mycolic acid" (β-hydroxy-acids of very high molecular weight) "phthiocerol" (long-chain diols) and "mycocerosic acid" (laevo-rotatory alkyl-branched long-chain acids). The methods of purification used by these workers were mainly distillation, crystallization and differential solubility. Since it was shown later that most of the above constituents were homologous mixtures, the power of such separation procedures would be limited. Moreover, the then accepted criteria of purity (melting point, boiling point, refractive index, density, optical rotation, microanalysis) are in the case of long-chain compounds, relatively insensitive to the presence of homologues and contaminants of similar structure.

Since about 1945 four main groups of workers have been active in investigating the lipids of tubercule bacilli. Lederer and his school in Paris used the extraction prodedure of Anderson and employed chromatography on alumina as their main purification technique. In particular Asselineau working mainly on the mycolic acids from various strains of human and bovine bacilli developed the idea[11] of"chromatographic homogeneity" i.e. repeated chromatography of fractions until their properties (m.p., rotation, etc.) were constant. By this criterion he was able to describe many apparently

different mycolic acids containing methoxy-, hydroxy- and keto-functions in addition to the β -hydroxy group[12,13]. Other workers in this group fractionated crude phthiocerol by chromatography on alumina into a family of long-chain diols[14]. However, having obtained these purified components the physical techniques available for the investigation of structure (e.g. ultra-violet and infra-red spectra), while being valuable for the identification of functional groups, gave little or no information about their location. Chemical degradations were hampered by the presence of homologues and the difficulty encountered in purifying and characterising the degradation products.

Polgar and his collaborators[15,16,17] in Oxford prepared many synthetic analogues of "phthioic acid", of which 3, 13, 19 - trimethyltricosanoic acid was thought for several years to be identical with the natural product. Some of these synthetic methyl-branched acids were found to be more effective than the natural acid in causing granulomatous lesions in test animals[18].

In the extraction procedure of Anderson[9] (as mentioned above) the fat, phosphatide and wax fractions were separated. Mutual solubility effects result in considerable overlap in the composition of such fractions. With these considerations in mind Chanley and Polgar[19] hydrolysed the total lipid

extracts of steam-killed bacilli by a two-step hydrolysis. Mild alkaline hydrolysis liberated the dextrorotatory acids and vigorous hydrolysis of the remaining material gave "mycolic acid", "phthiocerol" and the laevorotatory acids.

Fractionation of the dextrorotatory acids by crystallization of derivatives of their acetol esters or by the solubility of their potassium salts in ether gave an acid shown, by chemical degradation and ultra-violet spectra to be $\alpha\beta$-unsaturated[19,20,21]. This acid was named mycolipenic acid and further degradation and synthesis showed that it is essentially 2, 4 (L), 6 (L) - trimethyltetracos- - 2 - enoic acid[22,23]. The laevorotatory acids were purified by distillation and the resulting mycoceranic acid was thought to be mainly a 2 (D), 4 (D), 6 (D) - trimethyl - substituted C_{31} acid.[24,25,26]. Later it was shown that mycoceranic acid was a mixture whose main components are 2 (D), 4 (D), 6 (D) - trimethylhexacosanoic and 2 (D), 4 (D), 6 (D), 8 (D) - tetramethyloctacosanoic acids[27,28,29,30].

The above dextrorotatory and laevorotatory acids have been shown respectively to correspond to the phthioic and mycocerosic acids of Anderson[31.29]. Polgar and his workers also studied the mycolic acids[32,33], and phthiocerol[34,35], isolated from their hydrolytic procedure and contributed evidence towards the structures of these compounds.

Cason and his associates in Berkeley have been working solely on the methyl-branched acids from tubercle bacilli using material obtained by Anderson and his collaborators. An $\alpha\beta$-unsaturated acid isolated from phthioic acid was named C_{27}- phthienoic acid[36] and has since been shown[31] to be identical with mycolipenic acid. A saturated laevorotatory acid named mycosanoic acid[37] is probably identical with mycoceranic acid. For the purification of the above compounds Cason used the complexing properties of urea[38] and also differences in their rate of esterification[39]. He developed the method of exhaustive oxidation with chromic acid (used in 1934 by Spielman[10] to determine the structure of tuberculo_stearic acid) using gas chromatography to identify the products[40]. A similar procedure using potassium permanganate as oxidant has been developed independently by Murray[41]. This technique was probably the most direct method available for the determination of the structure of a methyl-branched acid before the advent of mass spectroscopy.

Stenhagen and his co-workers in Sweden pioneered the application of modern physical techniques to the determination of the structure of lipids from tubercle bacilli. Their early work on X-ray analysis[42,43,44], and monolayer properties[43,45], was handicapped by the inhomogeneity of the compounds studied. However more recently they have developed gas chromatography and mass spectroscopy of long-chain compounds

and successfully applied these techniques to many problems in this field. Polgar's structure for mycolipenic acid was confirmed[46] and the structure of the main component of phthiocerol[47,48] was established by mass spectroscopy. The work of Stenhagen and his school has laid the foundation of the modern approach to the determination of the structure of the components of tubercle bacilli.

This thesis is concerned with studies on the mycolic acids and phthiocerol isolated from human tubercle bacilli and also with the synthesis of methyl-branched long-chain acids for biological studies. These three subjects are introduced briefly below.

The mycolic acids may be defined as β-hydroxy acids of high molecular weight with a long alkyl side-chain in α-position[49]. The simplest acid of this type is coryno mycolic acid (I) isolated from Corynebacterium diphtheriae and acids of greater complexity have been isolated from

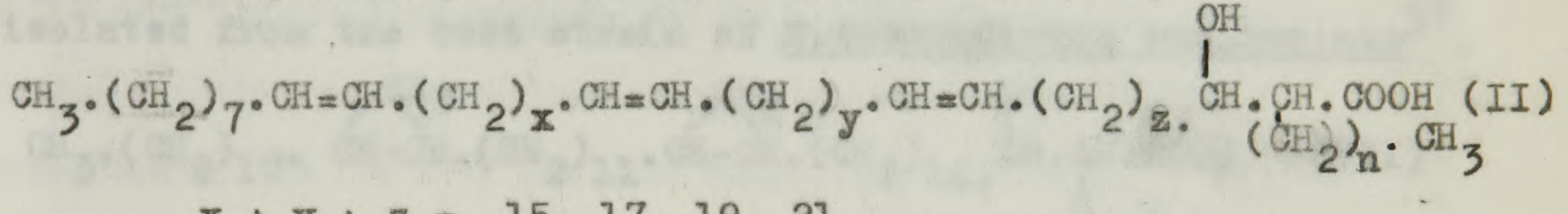
$$C_{15}H_{31}\cdot \underset{|}{\overset{OH}{CH}}\cdot \underset{|}{\overset{C_{14}H_{29}}{CH}}\cdot COOH \quad - \quad (I)$$

mycobacteria and other sources. A comprehensive review of work in this field before 1962 has been given by Asselineau[50].

The first part of this thesis concerns a detailed study of the mycolic acids isolated from a mixed human strain

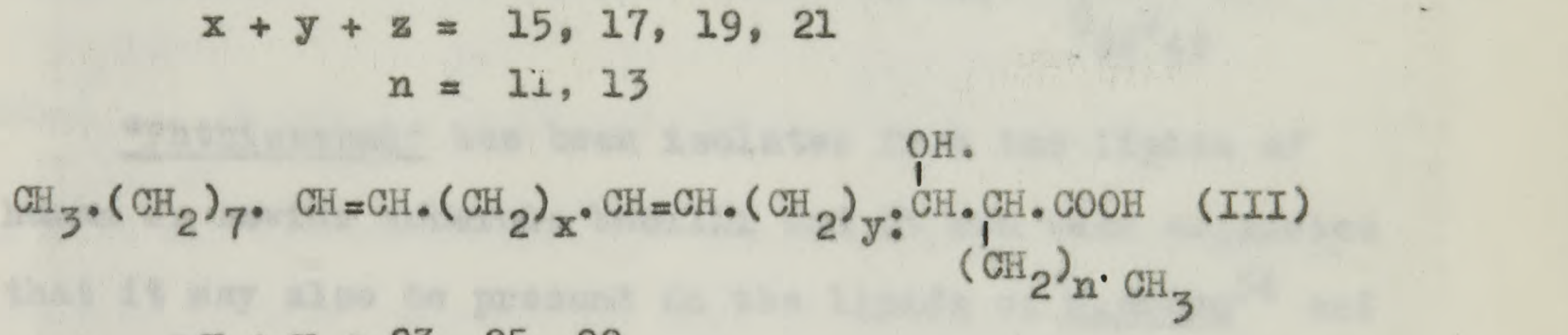
(D.T., P.N. and C.[51]) of M. tuberculosis making use of modern techniques, especially thin-layer chromatography, nuclear magnetic resonance and mass spectrometry. Since this work was started other investigators have proposed structures for mycolic acids from various bacterial lipids.

The mycolic acids from Nocardia asteroides have been shown to be homologous unsaturated acids (containing ca. 50 carbon atoms) having the structures (II) and (III)[52].



$$CH_3.(CH_2)_7.CH{=}CH.(CH_2)_x.CH{=}CH.(CH_2)_y.CH{=}CH.(CH_2)_z.\underset{\displaystyle (CH_2)_n.CH_3}{\overset{\displaystyle OH}{\overset{|}{CH}.CH.COOH}} \quad (II)$$

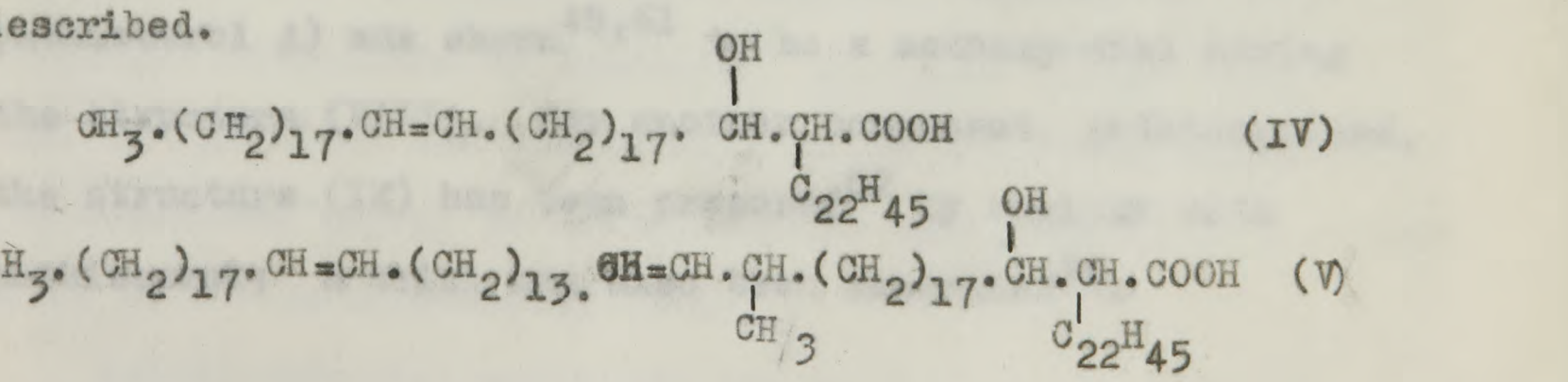
$$x + y + z = 15, 17, 19, 21$$
$$n = 11, 13$$



$$CH_3.(CH_2)_7.CH{=}CH.(CH_2)_x.CH{=}CH.(CH_2)_y.\underset{\displaystyle (CH_2)_n.CH_3}{\overset{\displaystyle OH}{\overset{|}{CH}.CH.COOH}} \quad (III)$$

$$x + y = 23, 25, 29$$
$$n = 11, 13.$$

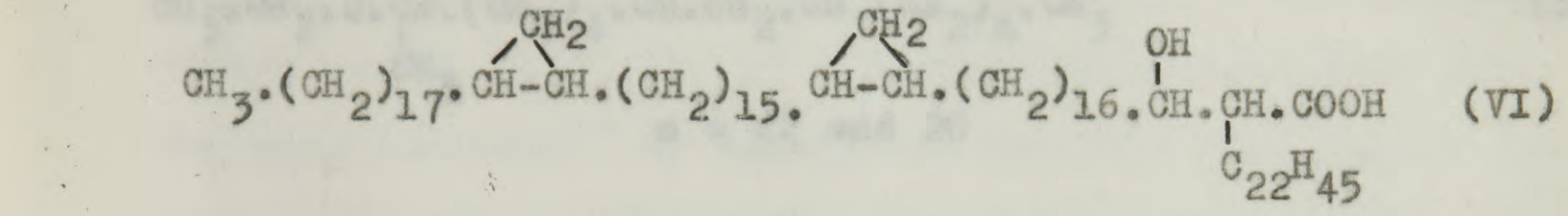
Unsaturated acids of different types have been isolated from M. Smegmatis ; a mono-unsaturated acid[53] (ca. C_{60}) (IV) and a methyl-branched diene acid[54] (ca. C_{80}) (V) have been described.



$$CH_3.(CH_2)_{17}.CH{=}CH.(CH_2)_{17}.\underset{\displaystyle C_{22}H_{45}}{\overset{\displaystyle OH}{\overset{|}{CH}.CH}}.COOH \quad (IV)$$

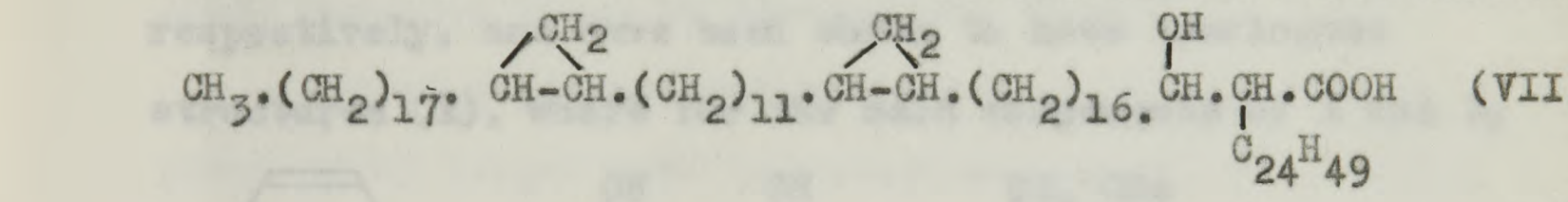
$$CH_3.(CH_2)_{17}.CH{=}CH.(CH_2)_{13}.\underset{\displaystyle CH_3}{CH}{=}CH.\underset{\displaystyle C_{22}H_{45}}{\overset{\displaystyle OH}{\overset{|}{CH}.CH}}.(CH_2)_{17}.COOH \quad (V)$$

A mycolic acid containing two cyclopropane groups has been obtained from the lipids of M. kansasii and the structure (VI) proposed[55]; the same structure has also been suggested for a mycolic acid from M. avium[56].



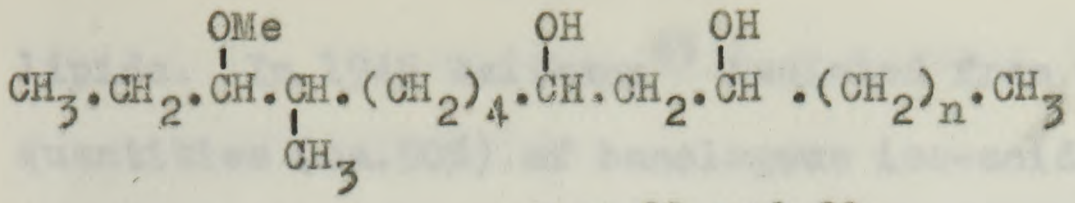
(VI)

An acid reported to have an analogous structure (VII) has been isolated from the Test strain of M.tuberculosis var.hominis[57].



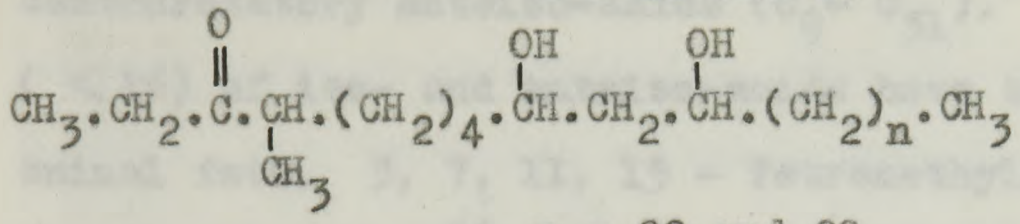
(VII)

"Phthiocerol" has been isolated from the lipids of human or bovine tubercle bacilli and it has been suggested that it may also be present in the lipids of M.avium[34] and M.phlei[59]. The "phtyoglycol" described earlier[60] by Stendal is probably also a specimen of "phthiocerol". Later it was shown that "phthiocerol" contains several homologous β-diols[14]; the main component, which retained the name phthiocerol (redesignated later in this thesis as phthiocerol A) was shown[48,61] to be a methoxy-diol having the structure (VIII). For another component, phthiodiolone, the structure (IX) has been proposed[62] by analogy with phthiocerol; a triol has also been described[14].



(VIII)

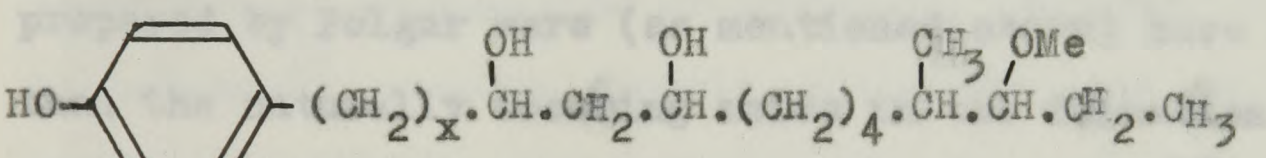
n = 22 and 20



(IX)

n = 22 and 20

Recently, two phenol-glycols, A and B, have been obtained[63] from M.kansasii and M.tuberculosis var.bovis, respectively, and have been shown to have homologous structures (X), where for the main components of A and B,



(X)

x = 18 and 16, respectively. These structures correspond closely in some respects to that of phthiocerol.

The second part of this thesis describes a re-investigation of crude phthiocerol.

Methyl-branched long-chain acids were unknown as natural products until 10-methyloctadecanoic (tuberculostearic) acid was isolated by Anderson[64] from tubercle bacilli and its structure determined by Spielman[10] in 1934. Since that time the phthioic and mycocerosic acids have been shown (as described above) to contain acids which are 2, 4, 6 - and 2, 4, 6, 8 - methyl-substituted.

Methyl-branched acids are not exclusive to bacterial

lipids. In 1945 Weitkamp[65] isolated from wool-fat large quantities (ca.50%) of homologous iso-acids (C_{10}- C_{28}) and dextrorotatory anteiso-acids (C_9- C_{31}). Small quantities ($<1\%$) of iso- and anteiso-acids have been found in many animal fats. 3, 7, 11, 15 - Tetramethylhexadecanoic acid has been isolated[66] by Hansen and Shorland from butter fat, presumably being derived from phytol of green plants. An excellent review of the higher saturated branched-chain acids has been given by Abrahamsson et.al[67].

Some of the synthetic analogues of phthioic acid prepared by Polgar were (as mentioned above) more active than the naturally occuring acids in the formatien of the tissue reaction characteristic of tuberculosis[18]. 3, 12, 15 - Trimethyldocosanoic acid was particularly potent and in order to provide more material for further biological studies syntheses cf this acid and some analogues are described in the final section of this thesis.

DISCUSSION

Section I. The mycolic acids from human tubercle bacilli

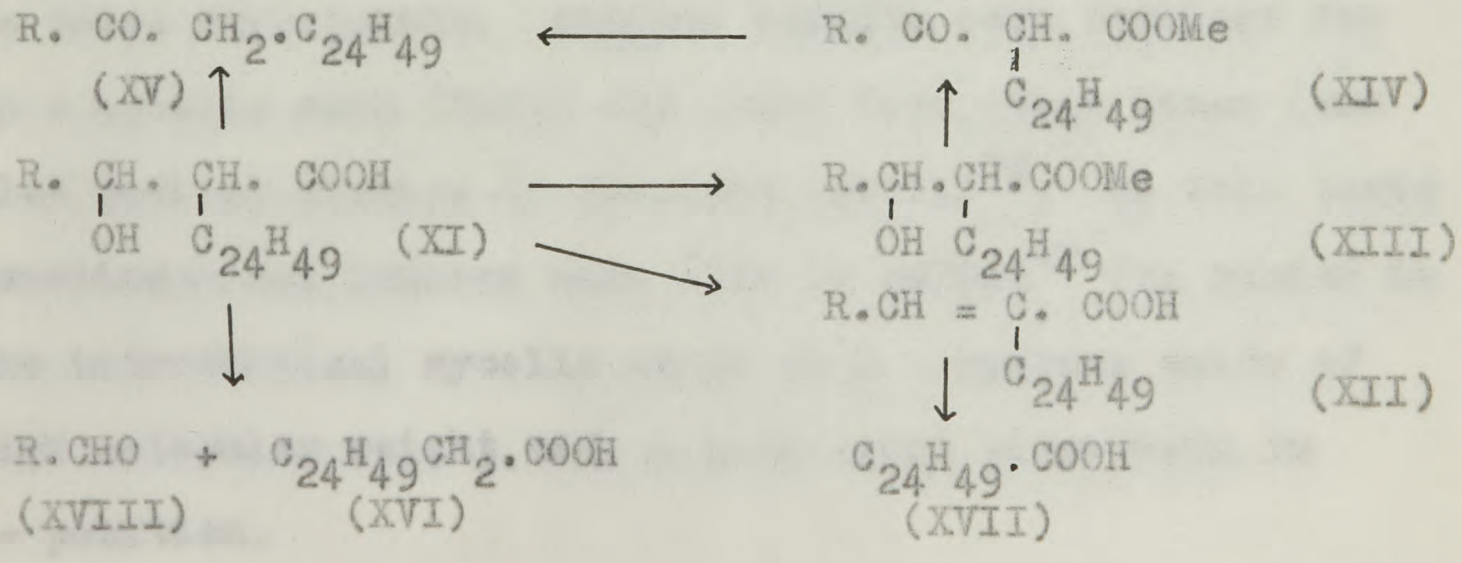
a) Previous researches

The mycolic acids from a human strain (H 37) of tubercle bacilli were first studied by Stodola, Lesuk and Anderson in 1938[68]. The isolation of crude mycolic acid, earlier described by Anderson[69] as an unsaponifiable wax, was relatively straightforward. The living bacillus was extracted with ethanol-ether (1 : 1), followed by chloroform. The chloroform extract was evaporated, the residue hydrolysed, the product taken up in ether and the crude mycolic acid precipitated by addition of methanol. Anderson and his associates[68] gave the formula of mycolic acid as $C_{88}H_{176}O_4$ with one carboxyl, one hydroxyl and one methoxyl group. Attempted distillation[68] at 300° under reduced pressure gave a volatile product identified as hexacosanoic acid. Chromic acid oxidation gave smaller fragments among which stearic, hexacosanoic and hexadecane -1, 16 - dicarboxylic acids were identified[70].

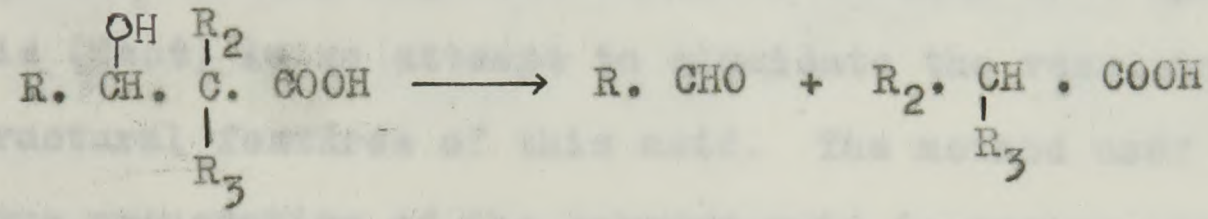
Asselineau and Lederer in 1949[71] applied chromatography on alumina to the crude mycolic acid isolated by Anderson's procedure from the Test strain of human tubercle bacilli. By repeated chromatography they were able to separate mycolic acid into fractions of different melting point which they classified in order of elution as α-, β-, γ- and δ- mycolic acids[71,72]. α-Mycolic acid, m.p. 55-56° ($C_{88}H_{176}O_4$)

constituted about 80% of the crude acids, β -mycolic acid m.p. 71-73°, ($C_{88}H_{176}O_4$) about 15%; γ-mycolic acid, m.p. 56-57°, ($C_{87}H_{174}O_3$) and δ -mycolic acid, m.p. 59-60° ($C_{88}H_{176}O_3$) were obtained as about 3% of the original mixture. Other human strains of tubercle bacilli yielded similar acids, variations in properties being observed in each case. Some of these acids were shown to contain two hydroxyl groups or a keto-group in addition to the hydroxyl. Asselineau[73] has tabulated the properties of many of these acids.

The α - mycolic acid (Test) (XI) has been the acid most throughly investigated by Asselineau. By dehydration[74] he obtained an αβ-unsaturated acid ($\lambda_{max.}$ 218 mμ, ε 13,000) which was named anhydromycolic acid (XII). Oxidation of methyl mycolate (XIII) with chromic acid gave a β - keto - ester ($\lambda_{max.}$ 260 mμ, ε 520) (XIV) which was hydrolysed and decarboxylated to give a neutral ketone (XV). The same ketone was obtained by direct oxidation of the free

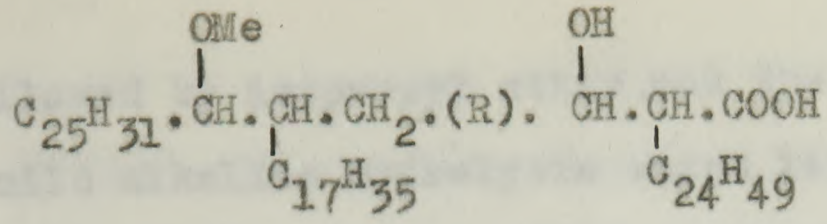


mycolic acid (XI). These reactions proved that the hydroxyl group was in β - position with respect to the carboxyl. It was now possible to explain the pyrolysis of mycolic acid reported by Anderson since it was well-known that β-hydroxy-acids with one or two branches in α -position on pyrolysis undergo cleavage as shown below,



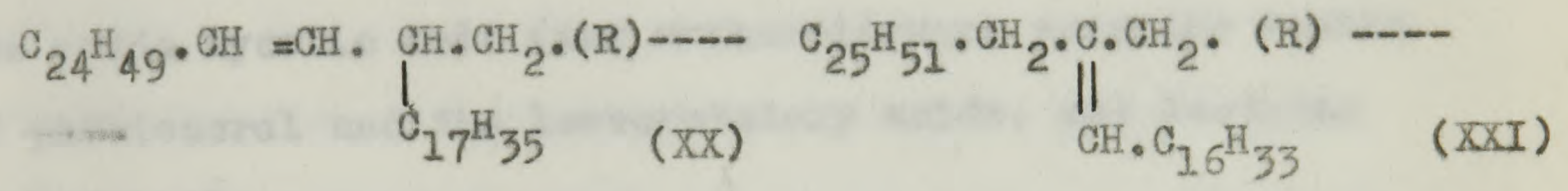
If the β - hydroxyl group was protected, e.g. by acetylation, the pyrolysis no longer occurred. The isolation of hexacosanoic acid (XVI) (or methyl hexacosanoate from methyl α - mycolate) suggested the presence of a tetracosyl chain in α - position. For confirmation, the anhydro-acid (XII) was ozonized and pentacosanoic acid(XVII) was identified among the products; it was assumed that this acid was formed by breakdown of 2 - oxohexacosanoic acid[75]. The aldehyde (XVIII) obtained in the pyrolysis was not studied in these experiments. Similar results were obtained for β - mycolic acid (Test) and acids from other human (and also bovine) strains of tubercle bacilli[72]. On this basis Asselineau and Lederer were able to define[49] (as stated in the introduction) mycolic acids as β - hydroxy acids of high molecular weight with a long alkyl side-chain in α - position.

In the case of α- and β-mycolic acids (Test) the procedure described above did not lead to identical anhydroacids (XII), and neutral ketones (XV)[76]. The difference between these acids was therefore due to the portion, R, shown to contain a methoxyl group in both cases. Asselineau[73] carried out extensive studies on α-mycolic acid (Test) in an attempt to elucidate the remaining structural features of this acid. The method used in the above preparation of the anhydro-acid (reaction with acetic anhydride in the presence of potassium hydrogen sulphate) also gave, by demethylation, a dihydroxy-acid amongst other products. Treatment of this dihydroxy-acid with acetyl chloride resulted in the formation of a mono-unsaturated hydroxy-acid (x - anhydromycolic acid) which on pyrolysis gave hexacosanoic acid. Ozonolysis of this compound yielded pentacosanoic and heptadecanoic acids. Chromic acid oxidation of the above dihydroxy-acid was shown to attack the β - hydroxyl group preferentially. Similar oxidation of the original mycolic acid gave a diketone suggesting that the methoxyl group corresponded to a secondary hydroxyl. C - methyl determination indicated that this acid contained three methyl groups. On this basis the partial structure[73] (XIX) was proposed for α-mycolic acid (Test). The observed ozonisation products were explained by assuming that



(XIX)

elimination of the methoxyl function gave x - anhydromycolic acids having the partial structures (XX and XXI). An



(XX) (XXI)

analogous process has been postulated in the demethoxylation of phthiocerol[77] (see later).

Further studies on other mycolic acids from human strains by these workers gave in each case confusing results. The main contribution of Asselineau, Lederer and their co-workers was the fact that all mycolic acids are α-branched and contain a β-hydroxy-group as defined above. No general pattern for the structural features of the remainder of the molecule emerged except that it was thought that one or more long alkyl branches were present in addition to the α-branch.

Morgan and Polgar[32] in 1957 studied a mycolic acid from a mixed strain (D.T., P.N., and C.) of human tubercle bacilli. This acid was obtained in the course of earlier investigations by Chanley and Polgar[19] whose isolation procedure (already outlined in the Introduction) was as follows. Steam-killed bacilli were extracted with acetone

followed by isopropyl ether, and the extracts subjected to a mild alkaline hydrolysis which liberated the methanol-soluble dextrorotatory acids. The methanol-insoluble residue was vigorously hydrolysed with alkali. Addition of methanol to a benzene solution of the hydrolysis products precipitated the crude mycolic acid (the mother-liquors were the source of phthiocerol and the laevorotatory acids, see Sections II and III).

Morgan and Polgar attempted to fractionate this mycolic acid by repeated precipitation from its benzene solution with methanol and also by chromatography on alumina. In each case, and also after chromatography of the corresponding methyl ester, no change in melting point was observed. Pyrolysis of this mycolic acid gave hexacosanoic acid, which is in agreement with the earlier studies described above, and infra-red spectra showed the presence of a methoxyl group. The corresponding anhydro-acid, prepared by treating the tosylate with alkali, was oxidized with potassium permanganat e in acetone. A straight-chain acid, thought to be pentacosanoic acid, was removed from the products as its urea complex leaving a branched-chain methoxy-acid named O-methylmeromycolic acid. This acid could be distilled unchanged at 300-320°/ 0.06 m.m., and treatment with thionyl chloride did not remove the methoxyl group; these two experiments appeared to

rule out the presence of either an α- or β - methoxyl group (c.f. ref. 78 and 79). Demethylation with acetic anhydride in the presence of toluene - p - sulphonic acid gave an acetoxy-acid, O-acetylmeromycolic acid. Hydrolysis of this acid gave the corresponding hydroxy-acid, meromycolic acid, which did not form a γ-lactone thus eliminating the possibility that meromycolic acid was a γ-hydroxy-acid. Further, since removal of the methoxyl group did not lead to an unsaturated acid the methoxyl group was probably not attached to a tertiary carbon atom.

Pyrolysis of methyl O-acetylmeromycolate followed by oxidation of the resulting unsaturated product gave a straight-chain acid, presumed to be heptadecanoic acid, together with the half-ester of a branched-chain dicarboxylic acid. On this evidence Morgan and Polgar[32] proposed the partial structure (XXII) for this mycolic acid where

$$CH_3.(CH_2)_n.\underset{OMe}{CH}.R.\underset{OH}{CH}.\underset{C_{24}H_{49}}{CH}.COOH. \qquad (XXII)$$

n is probably 16 and R represents a branched-chain alkyl residue.

Malani and Polgar[33] continued the studies on the above sample of mycolic acid applying gas-liquid chromatography (GLC) for the first time to the degradation products. Chromatography of the methyl ester of crude mycolic acid on

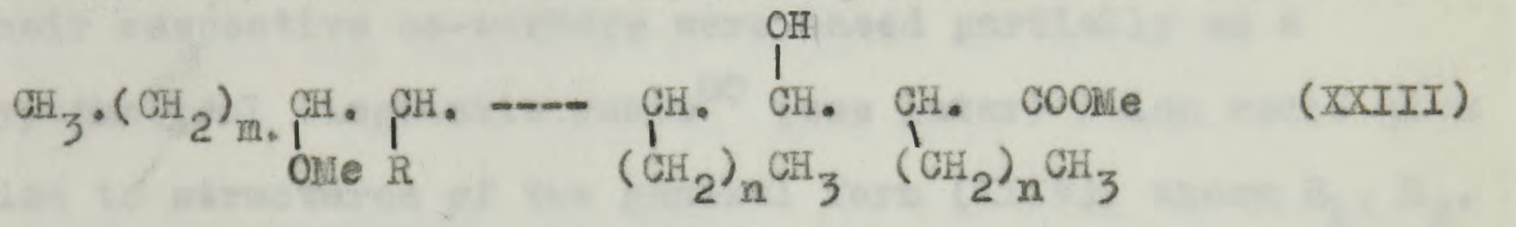
alumina afforded material containing a methoxyl group (ester A) followed by a fraction (ester B) in which no methoxyl group was detected. Extensive degradative studies were carried out on ester A. Pyrolysis of methyl mycolate gave straight-chain esters which were shown by GLC to have as the major constituent (75%) methyl hexacosanoate as expected, but also to contain methyl tetracosanoate in considerable quantity (25%). The products obtained by permanganate oxidation of methyl anhydromycolate A were shown by GLC of the corresponding esters to consist essentially of tetracosanoic and docosanoic acids, in the approximate ratio of 3:1. Comparison with the products obtained by pyrolysis of ester A indicated that the oxidation of the $\alpha\beta$-unsaturated ester proceeded according to the scheme,

$$-CH = \underset{\underset{CH_2R}{|}}{C}.\ COO\ Me \longrightarrow R.COOH.$$

The 'pentacosanoic acid' isolated earlier by Morgan and Polgar[32] was shown to be a mixture (3:1) of C_{24} and C_{22} acids.

In further experiments, oxidation of the unsaturated esters formed by pyrolysis of methyl O-acetylmeromycolate A gave straight chain acids with a preponderance of C_{18} and C_{19} acids. Hydrogenation of the parent acids of the above unsaturated esters gave deoxymeromycolic acid A, which on α-bromination, esterification and dehydrobromination gave an $\alpha\beta$-unsaturated ester. Oxidation of this ester with potassium

permanganate was said to give, amongst other products (possibly including keto-esters), docosanoic and tetracosanoic acids. These results pointed to a partial structure (XXIII) for ester A, where for the main components m is about 17



(XXIII)

and n is 21 and 23.

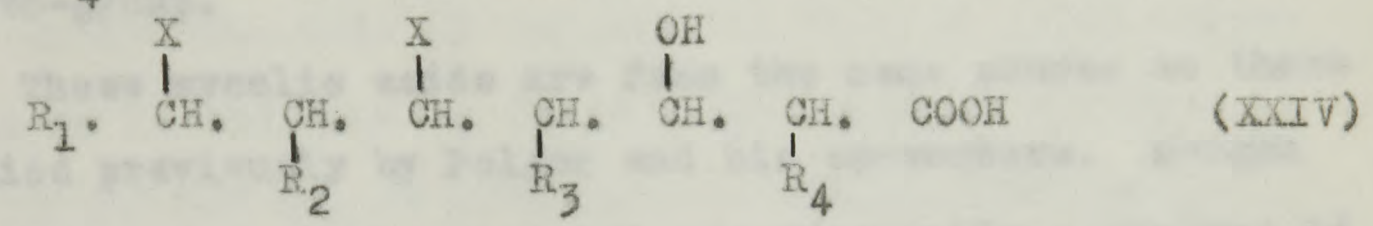
The structures proposed by Polgar and his co-workers are similar in general form to those proposed by Asselineau and Lederer in that they include long alkyl branches in addition to the α - branch.

Ställberg - Stenhagen and Stenhagen[43], in 1945, obtained X-ray diffraction photographs of Anderson's mycolic acid ($C_{88}H_{176}O_4$) and calculated the probably length of the molecule as either 50 or 100 Å. Eighty-eight carbon atoms in a straight chain would be expected to correspond to a chain length of 111 Å. Since the acid studied was microcrystalline and probably inhomogeneous, no further structural details were discerned.

These authors[43] also studied the monolayer properties of this acid. They concluded that "the structure of the molecule probably allows access to the water of at least one other polar group as well as the carboxyl" and that "the cross-section of the molecule at no part of its length

can be larger than that occupied by three parallel hydrocarbon chains."

The structures proposed by Asselinean and Polgar and their respective co-workers were based partially on a hypothetical biogenetic route[80] (see later) which would give rise to structures of the general form (XXIV), where R_1, R_2, R_3 and R_4 are long alkyl chains and X can be H, OMe or OH



(all being derived from carbonyl precursors). It is difficult to reconcile such a structure with the Stenhagens' results which suggested a more linear type of molecule.

b) Present investigations

The mycolic acids used in this work were those obtained from the lipids of the mixed human strain (D.T., P.N. and C[51]) in the course of previous work on phthiocerol[34]. Thin-layer chromatography (TLC) of the free acids showed the presence of one major component, small amounts of less polar material and some phthiocerol which was more polar. Chromatography of the crude mycolic acids on silica gel gave a yellow wax, shown to consist of ketones and other non-acid unsaponifiable material, which has not been studied further, followed by a

mixture of the mycolic acids and phthiocerol. This latter mixture was treated with diazomethane; unidimensional multiple chromatography[81] (UMC) of the product showed the presence of three mycolic esters in comparable amounts. The three esters are subsequently referred to as methyl mycolate - I, -II and -III, respectively, in order of increasing polarity; the ester -III was shown to contain a keto-group.

These mycolic acids are from the same source as those studied previously by Polgar and his co-workers. Morgan and Polgar's 'methyl mycolate' was presumably a mixture of all three esters since no fractionation was achieved[32]. The esters A and B described by Malani and Polgar[33] were now investigated by UMC; 'methyl mycolate A' consisted mainly of ester -I and -II and 'methyl mycolate B' was a mixture of all three esters.

Chromatography of crude methyl mycolate on neutral Spence alumina resulted in a mixture of methyl mycolate -I and -II followed by a mixture of all three esters. During this separation a considerable proportion (ca. 12%) of the esters were hydrolysed; thus in order to achieve a separation of these three esters on a large scale, chromatography on this adsorbent did not seem to be practicable . Consequently, the ketone component (methyl mycolate -III) of the above mixture of esters was converted into its oxime and isolated by chromatography on silica gel;

this adsorbent did not effect an efficient separation of esters -I and -II. At this stage the nature of the difference between esters -I and -II was unknown. Chromatography of a small amount of a mixture of methyl mycolate -I and -II on Woelm alumina gave samples of essentially pure esters -I and -II. Infra-red and nuclear magnetic resonance (n.m.r.) spectra detected the presence of a methoxyl group in methyl mycolate -II; methyl mycolate -I appeared to have no other oxygen functions in addition to the β-hydroxy-ester system.

It did not appear advisable to modify chemically methyl mycolate -II in order to facilitate its separation from methyl mycolate -I since the obvious approach, demethylation, required vigorous procedures (e.g. boiling with acetic anhydride in the presence of toluene - p - sulphonic acid[32]). Conversion of both esters into acetyl-derivatives seemed to offer advantages for their chromatographic separation; the reduction in overall polarity achieved would emphasize the small difference between the two molecules (assuming that there was no interaction between the methoxyl and hydroxyl groups). A portion of the mixture of methyl mycolate -I and -II was acetylated and in comparative experiments samples of the acetyl-derivatives were chromatographed on silica gel and Woelm alumina. The former adsorbent gave practically no resolution of the two components while the latter gave a

good partial separation. It would have been uneconomic to use Woelm alumina for this separation on a large scale, so, as an alternative, Camag neutral alumina was tried and was found to be equally efficient. The remainder of the mixture of methyl mycolate -I and -II was acetylated and repeated chromatography of these acetyl-derivatives on Camag alumina gave methyl acetylmycolate -I, mp. 39-40°, $[\alpha]_{589}^{20}$ + 4.9°, and methyl acetylmycolate -II, m.p. 31-32°, $[\alpha]_{589}^{20}$ + 0.8°.

The fractions which preceded those containing methyl acetylmycolate -I in the above separations were shown to contain small amounts of a slightly less polar component. This material is probably analogous to the "pre-mycolates" described recently by Etemadi[82] and shown to be present in various bacterial lipids. In this instance, this component was not detectable by UMC in the original mixture and only became apparent after it was concentrated in the early fractions by the above chromatography.

The great utility of UMC in following sequences of partial separations, such as the above, must be emphasized. A compound is not necessarily chromatographically homogeneous if only one spot is detected on the thin-layer plate; small amounts of impurities of a closely similar nature will not be clearly distinguished. In the absence of supplementary

purification techniques (e.g. crystallization, distillation etc.) which will readily remove traces of compounds having very similar structures, it is necessary to rechromatograph a component thus concentrating the impurities in certain fractions where they can be detected by UMC. When, after repeated chromatography, all of the fractions are identical on UMC, then the compound may be regarded as being chromatographically homogenous (in respect of the adsorbents employed). This procedure is an extension of Asselineau's criterion[11] of "chromatographic homogeneity" (see Introduction) which was based on the melting points (or some other physical property) of the fractions.

It should be noted that most derivatives of the individual mycolic acids are found to crystallize readily from ethyl acetate. While this procedure is convenient for a final purification, it is usually of little value as a means of separating the various mycolic acids or their derivatives from each other.

The crude oxime of methyl mycolate -III obtained above was purified by chromatography on silica gel giving methyl mycolate -III oxime, m.p. 41-42°. UMC of this oxime, however, demonstrated the presence of two very similar components in the approximate proportion of 1:5. Two possible explanations were envisaged, positional isomerism of the original keto-group

or geometrical isomerism (*syn* and *anti* forms) of the hydroxyimino group. In both cases the observed difference in polarity might be due, at least partially, to preferential intra-molecular hydrogen bonding of the hydroxyimino groups to the β-hydroxyl group in the case of one isomer. Attempts to separate these components by chromatography on silica gel and alumina columns of varying activity resulted in only partial resolution.

Conversion of this hydroxy-oxime to the corresponding acetoxy-oxime offered the possibility of an improved separation of the two components since the overall polarity would be reduced. Also, if intramolecular hydrogen bonding of the hydroxyimino grouping was an important factor, then in the case of the acetoxy-derivatives the position should be simplified. Accordingly, the oxime of methyl mycolate -IIIwas converted into crude methyl mycolate -III, which after acetylation and formation of the corresponding oxime gave methyl acetylmycolate -III oxime, m.p. 36-37°. UMC again showed the presence of two very similar components in the ratio ca. 1 : 5, suggesting that intra-molecular hydrogen bonding of the hydroxyimino group was not an important factor. Repeated chromatography of this acetoxy-oxime gave fractions in which the ratios of the two components were approximately 2 : 1 and 1 : 10. The infra-red and n.m.r. spectra of these two mixtures were

indistinguishable; the melting points and rotations, although not identical, were very similar. A small amount of the former fraction was hydrolysed to the corresponding ketone and then the oxime was remade. UMC of this product showed that the original proportions (1 : 5) of the two components were restored, suggesting that the difference between the components is due to geometrical isomerism of the hydroximino grouping.

For comparative studies, methyl 13-oxohexacosanoate (available from previous work[83]) was converted into its oxime. UMC of this compound showed no resolution into two components. Thus the original keto-group in mycolic ester -III could not be in such a simple environment.

Methyl acetylmycolate -III, mp. 43.5-44°, $[\alpha]_{589}^{20}$ + 6.2° $\lambda_{max.}$ 213 mμ (ε 309), was prepared by hydrolysis of its oxime and repeated chromatography of the product. During the course of one of these chromatographic separations (on alumina) some elimination of acetic acid occurred, resulting in a sample of methyl anhydromycolate -III, m.p. 52.52.5°, $[\alpha]_{589}^{20}$ + 1.7°, $\lambda_{max.}$ 216 mμ (ε 13,100). Treatment of methyl acetylmycolate -III with ethanedithiol in the presence of boron trifluoride etherate gave methyl acetylmycolate -III thioketal, mp. 31-35°. A consideration of the spectral properties of the compounds described so far will be given later.

Methyl acetylmycolate -I was hydrolysed with benzene-methanolic (4:1) potassium hydroxide. The resulting crude mycolic acid -I was shown by TLC to contain a small amount of a second, less-polar, component found later to be the C-2 epimer. Esterification of this material gave a mixture of methyl mycolate -I and its C-2 epimer (less polar on UMC). This partial inversion due to enolisation involving hydrogen on the α- carbon atom is to be expected (c.f.ref.84) Repeated chromatography of the above mixture gave methyl mycolate -I, m.p. 49-50°, $[\alpha]_{589}^{20}$ + 3.7°, and fractions enriched in its C-2 epimer. Further column chromatography of these fractions and, finally, purification by preparative thin-layer chromatography (PLC) gave a small sample of methyl 2-epimycolate-I, m.p. 56-57°, $[\alpha]_{589}^{20}$ - 0.6°. In the course of one of the above chromatographic separations (on alumina) some dehydration occurred giving an impure sample of the anhydro-ester -I which was purified by further chromatography to give methyl anhydromycolate -I, m.p. 39-41°, $[\alpha]_{589}^{20}$ -0.1°, $\lambda_{max.}$ 216 mμ (ε 13,200).

Methyl acetylmycolate -II was hydrolysed by the same method used for ester-I giving a mixture of mycolic acid -II and its C-2 epimer. In addition the ultra-violet spectrum showed absorption suggesting the presence of some anhydromycolic acid -II. Chromatography of the corresponding methyl esters gave methylmycolate -II, m.p. 48-48.5°, $[\alpha]_{589}^{20}$

+ 0.1°, methyl anhydromycolate -II, m.p.32-35°, $[\alpha]_{589}^{20}$ -2.8°, $\lambda_{max.}$ 216 mμ (ε 7,900) and fractions enriched in methyl 2-epimycolate-II. The ultra-violet absorption of this anhydro-ester was weaker than those of the anhydro-esters -I and -III, suggesting the presence of some βγ-unsaturation which is confirmed by n.m.r. spectrometry (see later). Thus it appears that elimination during chromatography on alumina (esters -I and -III) gave anhydro-esters having mainly αβ-unsaturation but alkaline treatment of the acetyl-derivative gave a mixture containing αβ- and βγ-unsaturated material. It is not clear why no anhydro-acid was formed during the hydrolysis of methyl

acetylmycolate -I since the acetoxy-esters -I and -II were treated essentially in the same manner.

In the light of the above results methyl acetylmycolate -III was hydrolysed with potassium hydroxide in aqueous methanol-benzene solution (1:1:1). The presence of water in the solvent was expected to reduce the amount of inversion of the α-centre (c.f. ref. 84). TLC of the crude mycolic acid -III showed only a trace of a component presumed to be the C-2 epimer. The ultra-violet spectrum showed no absorption attributable to anhydromycolic acid -III. PLC of the corresponding methyl esters removed the trace of epimeric material giving methyl mycolate -III, m.p.52-53°, $[\alpha]_{589}^{20}$ + 5.0°, $\lambda_{max.}$ 215 mμ (ε 256). In the course of the above studies spectral data were obtained for all of the pure compounds described above and the results of these measurements are discussed below.

The infra-red spectra of the mycolic esters and their acetyl - and anhydro-derivatives show the expected carbonyl absorptions and in the case of methyl mycolate -II a band a 1090 $cm.^{-1}$ due to methoxyl. In accordance with the findings of Gastambide-Odier et al.[85] all three esters give a peak at 1020 $cm.^{-1}$ indicating the presence of cyclopropane groups in the molecules. Moreover, a band at 3060 $cm.^{-1}$ indicated in all cases the presence of cyclopropylmethylene groups[86], the band in ester -I having about twice the intensity of

those in ester -II and ester -III. It is of interest to note that Asselineau and Lederer[72] in 1953 suspected that certain bands in the infra-red spectrum of methyl α-mycolate (Test) might be due to cyclopropane groups; unfortunately no chemical confirmation was obtained for this observation.

The n.m.r. spectrum (all spectra in $CDCl_3$ unless otherwise stated) of methyl mycolate -I (Fig.1) shows bands at τ 10.3 (2H) amd τ 9.4 (6H) which are attributable to two 1,2 - disubstituted cyclopropane groups. A band at τ 9.12 (6H) shows the presence of terminal methyl groups. This region of the spectrum is similar to those of the published spectra of authentic cis - 1,2-disubstituted cyclopropane compounds[87,88] (containing one cyclopropane and one terminal methyl group). This suggests that there are only two terminal methyl groups in methyl mycolate -I, and in consequence no other long alkyl chains in addition to that in α-position are present. It should be noted that it is difficult in the case of long-chain compounds to determine directly the number of methyl groups by integration of their n.m.r. spectra because of the proximity of the large $-CH_2-$band. Direct measurement of the area of a $-CH_3$ band is possible if the $-CH_2-$ band is assumed to be symmetrical and allowance made for the portion underlying the $-CH_3$ signal.

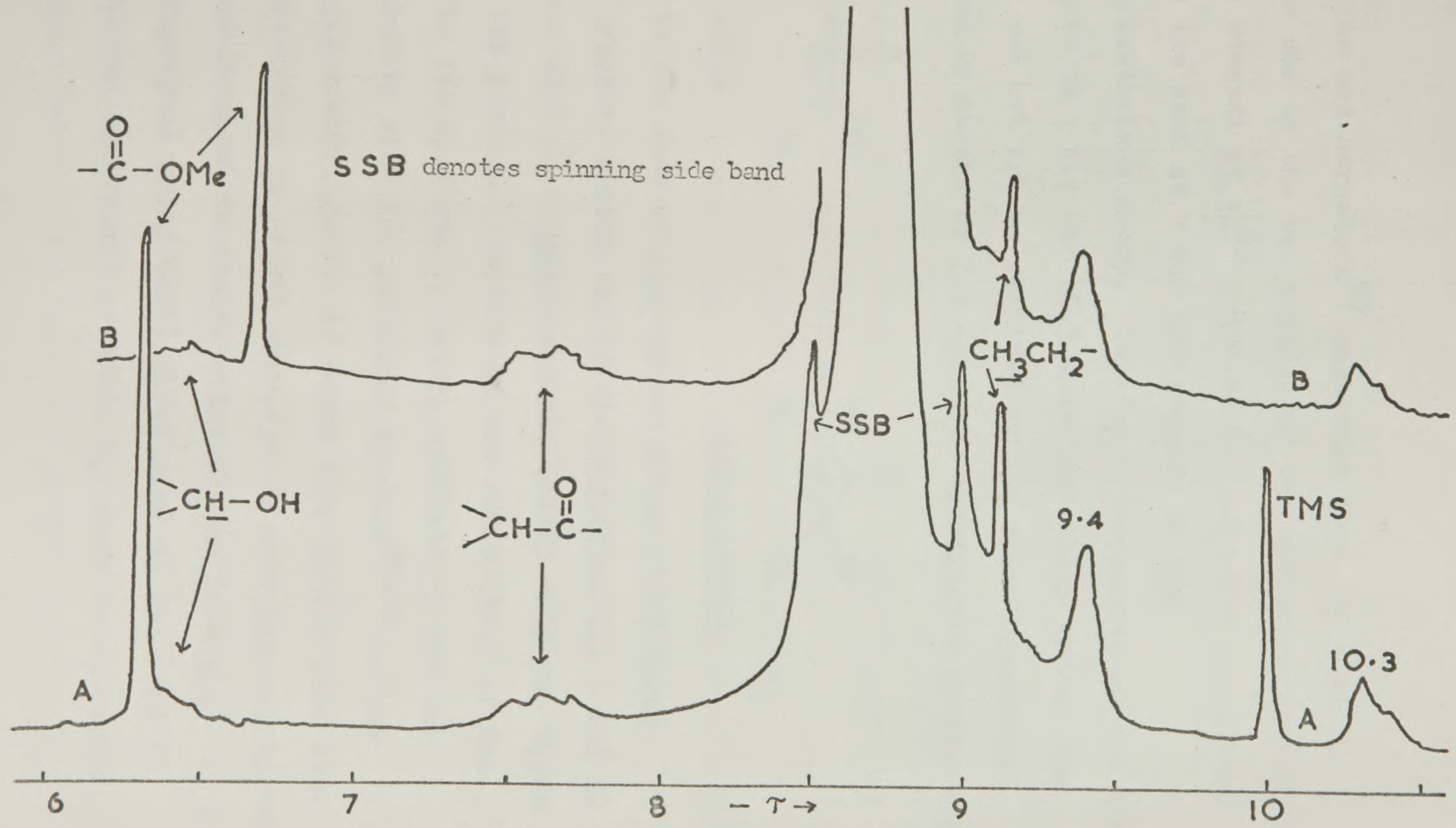
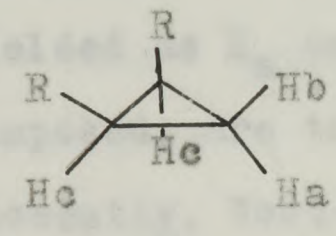


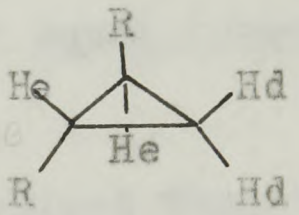
Fig. 1 Methyl mycolate-I, A in $CDCl_3$ (D_2O); B in benzene.

Hopkins and Bernstein[87] suggested that the band at τ10.3 was due to the two protons of the cyclopropylmethylene group but Etemadi et al.[55] proposed the opposite assignment, i.e. that the band at τ 9.4 corresponds to the cyclopropylmethylene group. Careful measurements of the areas of the bands at τ 9.4 and 10.3 show that these are in the ratio 3:1 and not 1:1 as required by the above hypotheses.

Molecular models of 1,2 -dialkyl-substituted cyclopropanes



cis (XXV)



trans (XXVI)

show that in the case of cis- substitution (XXV) three different types of proton may be distinguished (a, b and c) while in the case of a trans-compound (XXVI) only two types (d and e) are present. In view of the ratio (3:1) of the two bands in the spectrum of methyl mycolate -I and the close similarity with the published spectra[87,88] of the authentic cis-cyclopropanes, it seems very likely that the cyclopropane groups in methyl mycolate -I have cis-configuration.

It is of interest to decide which proton in a cis - 1, 2 - dialkylcloropane group (XXV) gives rise to the band at τ10.3. The two equivalent protons, H_c, must be contained

in the band at τ 9.4 and this band must also include either H_a or H_b. H_a is constrained in close proximity to H_b and the two H_c's and is thus heavily shielded. H_b, while being shielded by H_a, is relatively deshielded by the presence of the alkyl groups, R, and therefore is likely to appear downfield with respect to H_a. It then follows that the signal at τ 10.3 is due to H_a, i.e. the cyclopropylmethylene proton trans to the two substituent groups. In the case of the trans - cyclopropane (XXVI) none of the protons are as shielded as H_a and two bands of equal intensity, perhaps superimposed, are to be expected.

Recently, Wood and Reiser[89] showed that in the n.m.r. spectrum of methyl cis - 9, 10 - methyleneoctadecanoate the band at τ 10.3 is equivalent to one proton but they also proposed that the band at τ 9.4 was due to only two protons; the fourth cyclopropane proton was thought to be at τ 9.2. The two cyclopropylmethylene protons (XXV, H_a and H_b) were stated to be the most equivalent, resulting in the signal at τ 9.4; the other two protons (H_c) thus giving rise to widely separated bands at τ 9.2 and 10.3. These workers also described the spectrum of methyl trans - 9, 10 - methyleneoctadecanoate observing a broad band at τ 9.8 to which they attributed only one proton. Both of these interpretations are clearly incompatible with the above argument based on the molecular models (XXV and XXVI),

although the position of the absorption in the case of the trans-compound is interesting (see later).

The proton on the β - carbon atom (>CH-OH group) is not readily apparent in the spectrum of methyl mycolate -I (Fig.1). On close inspection it appears to be under the methyl ester singlet. The spectrum was redetermined in benzene solution[90] (see also Fig.1) and the >CH-OH signal is revealed. The pattern of the methyl group signals is unchanged, confirming the absence of a methyl branch (see later).

The n.m.r. spectrum of methyl mycolate -II (Fig.2) shows a singlet at τ 6.67 confirming the presence of a methoxyl group. A band at τ 10.3 (IH) suggests a single cis - 1, 2 - disubstituted-cyclopropane group, but bands at τ 9.4 (2H) and τ 9.8 (IH), as compared with a single band at τ 9.4 (2 x 3H) in the case of methyl mycolate -I, point to a different environment of the cyclopropane group. The possible presence of an adjacent >CH-OMe group which might shield one of the ring protons and shift its signal to higher field seems to be supported by the appearance of the >CH-OMe proton signal (τ 7.10, ill-defined multiplet). A doublet at τ 9.17, superimposed on triplets (τ 9.12) due to two terminal methyl groups, indicates the possible presence of a methyl branch. In benzene solution (see also Fig.2) the pattern of the methyl group bands is altered, the methyl

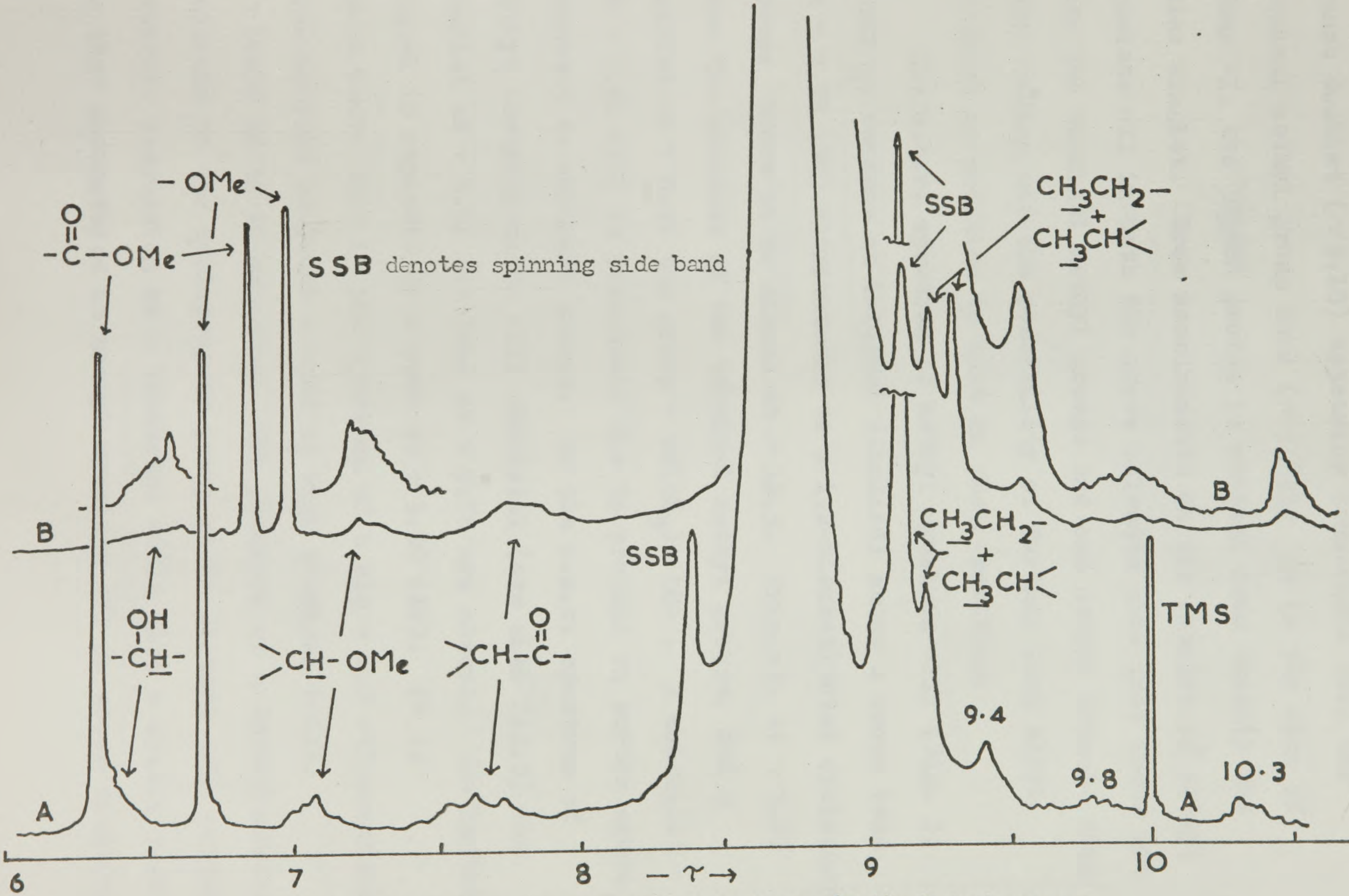


Fig. 2 Methyl mycolate-II, A in $CDCl_3$ (D_2O); B in benzene.

branch doublet (τ 9.18) appearing down-field from the terminal methyl group band (τ 9.22). As in the case of ester -I, the >CH-OH proton is removed from beneath the ester singlet. Area measurements on the spectra of methyl mycolate -II in both the above solvents show that there are only two terminal methyl groups and one methyl branch, thus again ruling out the possibility of further long alkyl branches in addition to that in the α-position.

The n.m.r. spectrum of methyl mycolate -III (Fig. 3) ($CDCl_3$, benzene as internal standard) shows a broad band at τ 9.78 (3H) attributable to a 1,2-disubstituted cyclopropane group; there is no signal at τ 10.3. Triplets at τ 9.07 show the presence of two terminal methyl groups, and a doublet at τ 8.91 the group - $CH(CH_3)$. CO- . A multiplet at τ 7.55 (3H) is presumably due to protons on carbon atoms adjacent to carbonyl groups. In the n.m.r. spectrum of methyl acetylmycolate -III thioketal (see also Fig.3) the doublet at τ 8.91 and band at τ 9.78 are absent; the latter signal is replaced by a peak at τ 9.90 (4H). It is shown above that in the spectrum of a cis - 1,2 -disubstituted cyclopropane compound a band of high chemical shift (τ 10.3) is to be expected. The presence of a keto-function adjacent to the cyclopropane group might disturb this pattern. However, conversion to a thioketal would give a system similar to that encountered in methyl mycolate -II in whose spectrum

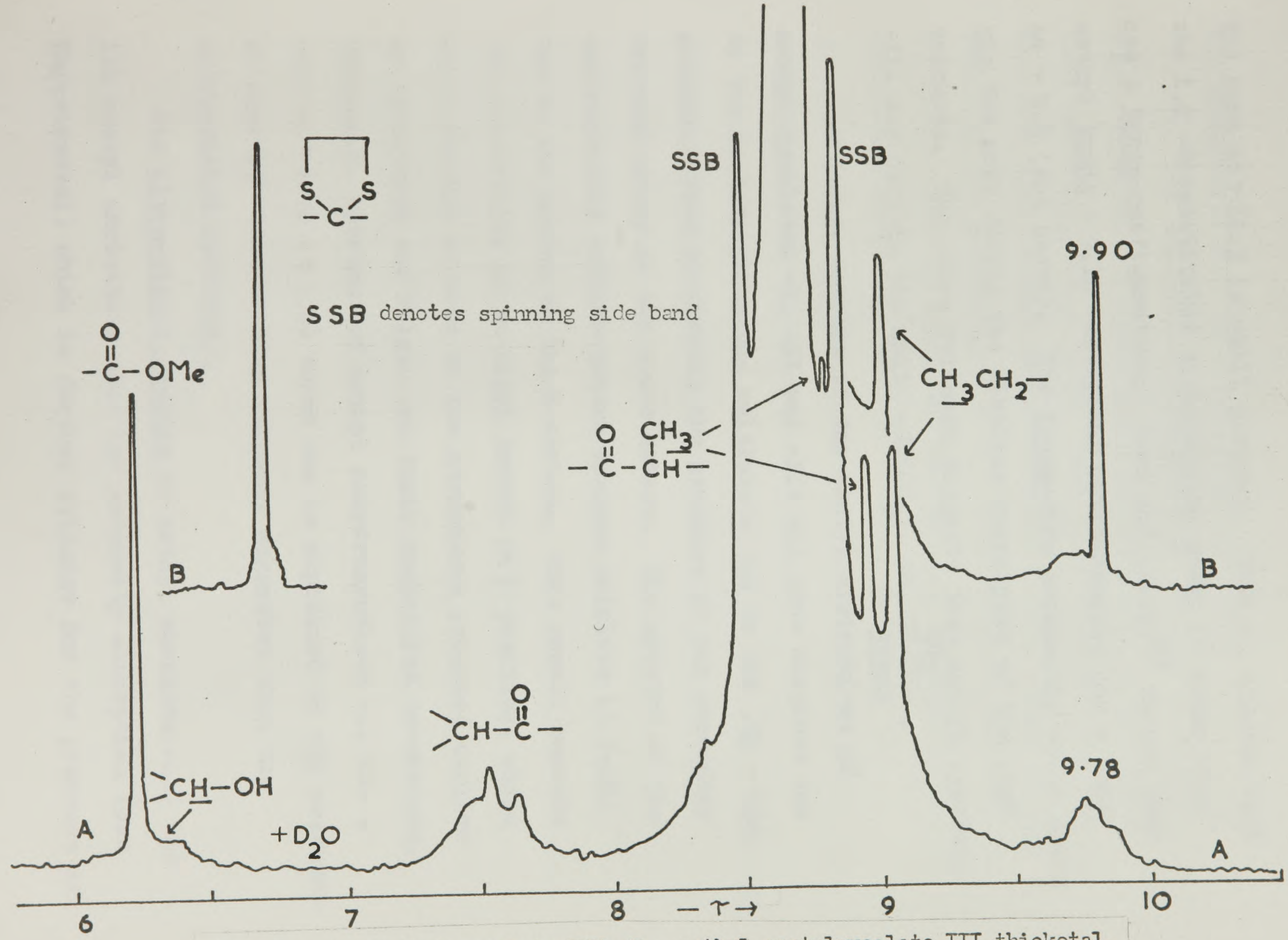


Fig. 3, A - Methyl mycolate-III; B - methyl acetylmycolate-III thioketal.

the band at τ 10.3 is still present. Thus it appears that the 1,2 -disubstituted cyclopropane group in ester -III has a trans-configuration; Wood and Reiser[89] showed that methyl trans - 9,10 -methylene octadecanoate has a band at τ 9.8 (see above). The trans-form presumably could arise via the enol during the alkaline hydrolysis of the lipid extracts. The above evidence suggests that methyl mycolate -III may contain the unit $-CH(CH_3).CO.\underset{\text{trans}}{\overset{CH_2}{\diagup\diagdown}}CH-CH-$.

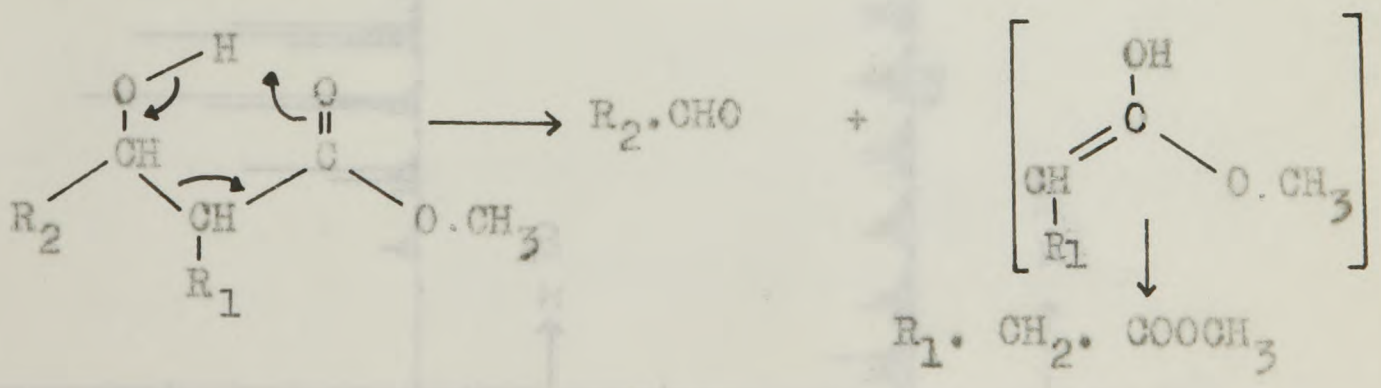
The n.m.r. spectra of the acetyl derivatives of methyl mycolates -I, -II and -III all show singlets due to the acetoxy-group and multiplets due to the >CH - OAc grouping, thus confirming the presence of one secondary hydroxyl group in the above esters. The spectra of the corresponding anhydro-esters contain triplets (τ 3.28) due to the proton on the β-carbon. This result removes the possibility of an alkyl branch in γ-position, which was a feature of some of the structures proposed earlier by Asselineau and Polgar and their respective co-workers. The n.m.r. spectrum of methyl anhydromycolate -II has a band at τ 4.70 (< 1 H) which can be explained by the presence of some βγ -unsaturation (already suggested from the ultra-violet spectrum).

The ultra-violet spectra of methyl mycolate -III and its acetyl derivative show low intensity absorption (see Experimental) which is further evidence for the presence of

the grouping - CO - CH(CH$_2$)CH - (cf. ref. 91).

The low resolution mass spectrum (Fig.4) of methyl anhydromycolate -I shows peaks attributable to molecular ions (M) at m/e 1104, 1132, 1160, 1188 and 1216 (the most abundant peak in any series is underlined). Peaks at m/e 393, 421 are presumably due to 3,4-cleavage adjacent to the $\alpha\beta$-double bond and those at 382, 410 can be explained by a McLafferty rearrangement[92] if a small amount of the $\beta\gamma$-unsaturated isomer is present; this could be formed by rearrangement in the mass spectroneter.

The mass spectrum of methyl mycolate -I (Fig.5) contains peaks at m/e 712, 740, 768, 796, 824 and m/e 382, 410. These are due to fragments formed by pyrolysis of the mycolic ester in the mass spectrometer. Thus the first series of peaks given above are due to meromycolic aldehydes and the second group correspond to straight-chain esters (C_{24} and C_{26}) derived from the α-branch. Etemadi has recently proposed[93] a concerted cyclic mechanism for this pyrolysis as shown below



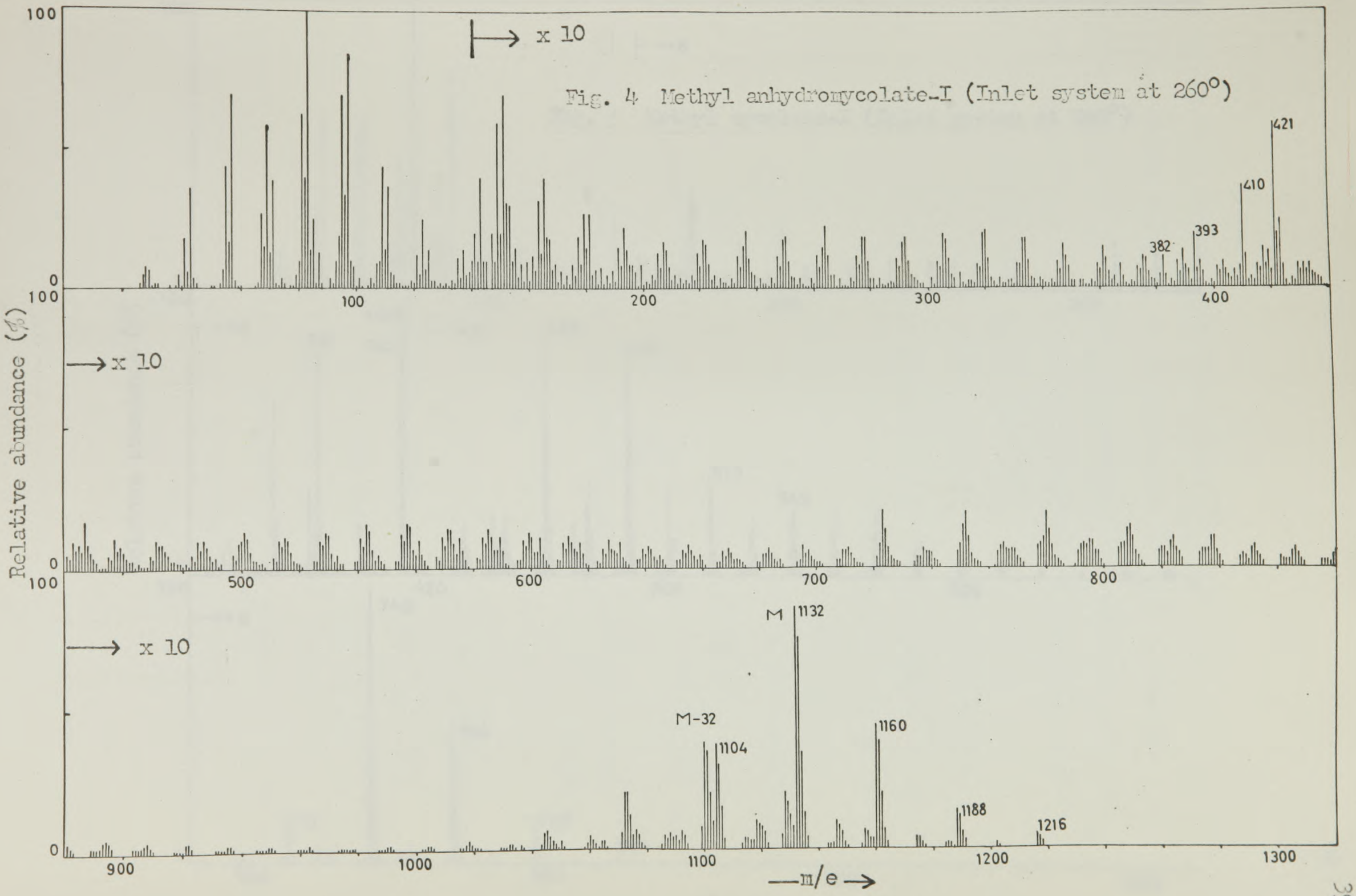


Fig. 4 Methyl anhydromycolate-I (Inlet system at 260°)

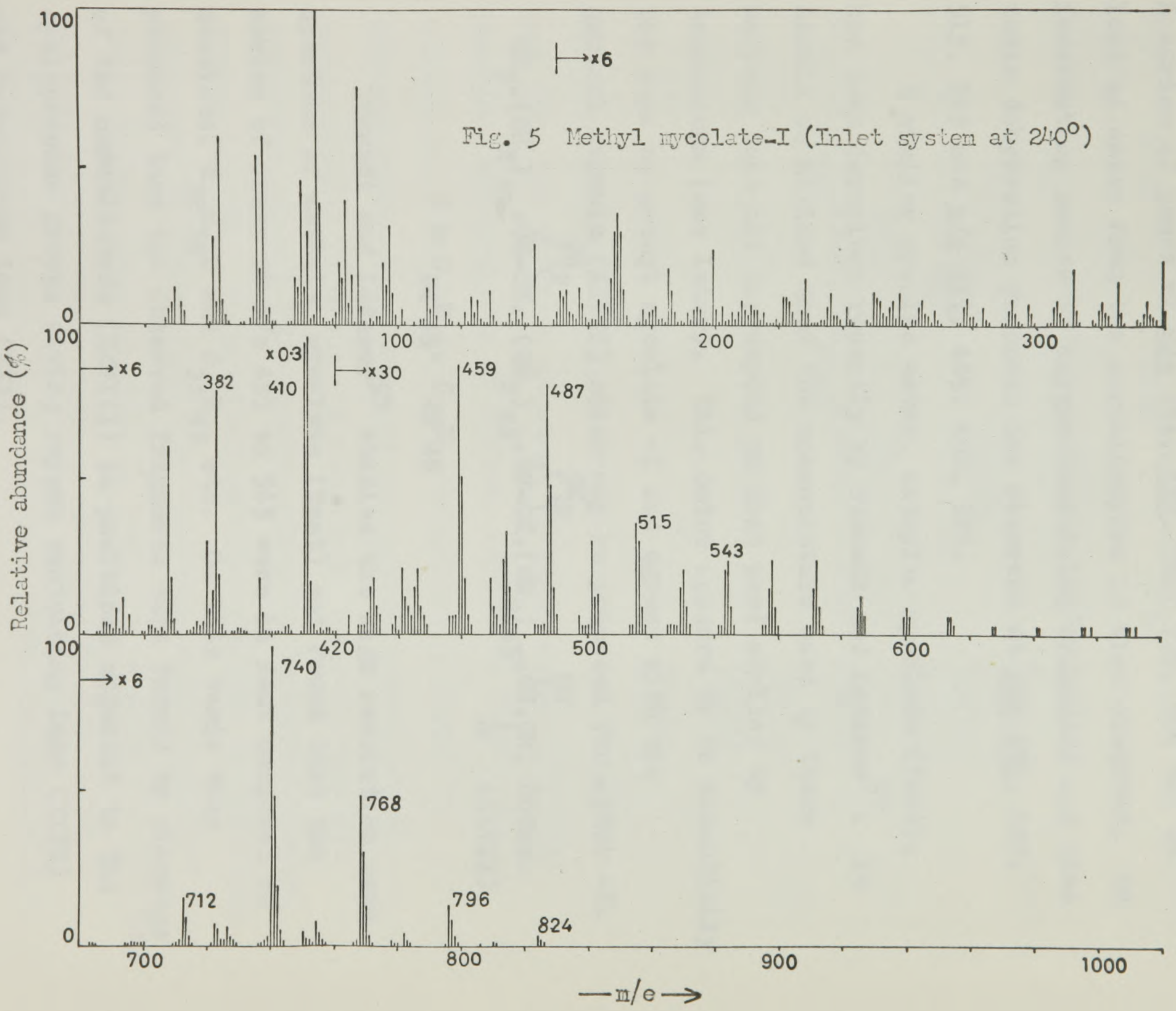
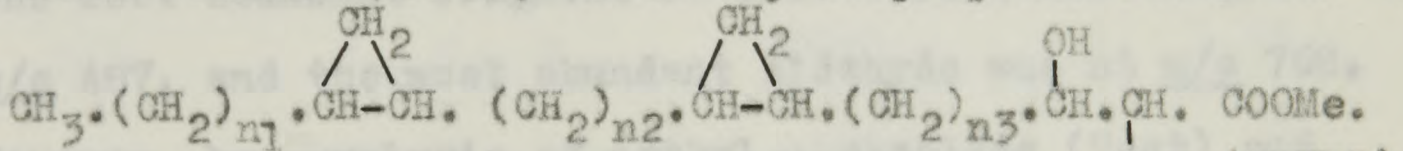


Fig. 5 Methyl mycolate-I (Inlet system at 240°)

A series of peaks at m/e 694, 722, 750, 778, 806 due to loss of water from the meroaldehydes is also observed. An interesting series of oxygen-containing fragments and also their dehydration products are observed at m/e 459, 487, 515, 543 and m/e 441, 469, 497, 525.

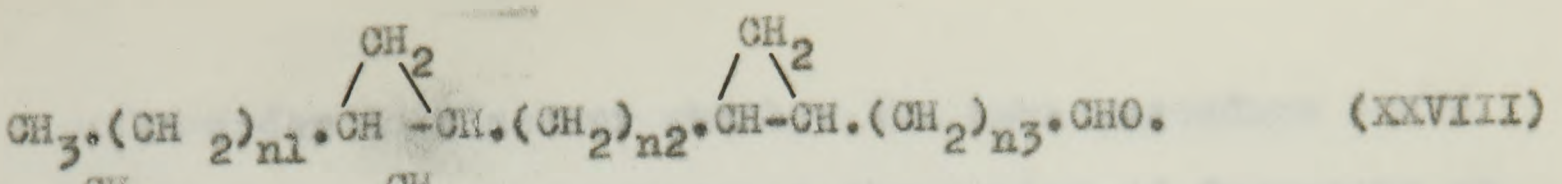
A similar mycolic ester, methyl α-mycolate (Test), has been described recently by Etemadi and Lederer[57]. It should be noticed that the nomenclature used by these workers does not correspond to that used earlier by Asselineau (see later). This ester appears to be essentially the same as methyl mycolate -I and agrees with the general formula (XXVII) which may be proposed for ester -I.



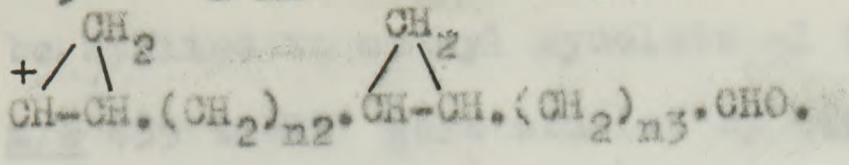
(XXVII)

R = $C_{24}H_{49}$, $C_{22}H_{45}$

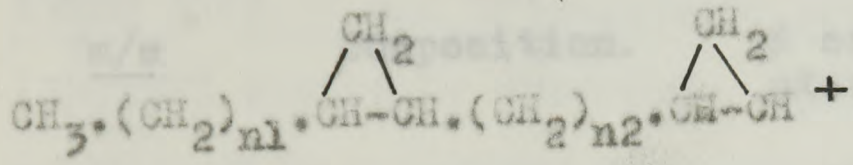
Etemadi and Lederer[57] studied the high resolution mass spectrum of methyl α-mycolate (Test) and found that the series of peaks at m/e 459 to 543 were in fact composed of doublets, $C_{32}H_{39}O$ and $C_{33}H_{63}$ etc. On this basis they proposed that the observed fragments were formed by cleavage of the meroaldehyde (XXVIII) in positions adjacent to the cyclopropane groups giving oxygen containing ions (XXIX) and hydrocarbon ions (XXX).



(XXVIII)



(XXIX)



(XXX)

m/e		
	$C_{32}H_{59}O$	
	$C_{34}H_{63}O$	(487)
	$C_{36}H_{67}O$	
	$C_{38}H_{71}O$	
m/e	$C_{33}H_{63}$	
	$C_{35}H_{67}$	(487)
	$C_{37}H_{71}$	
	$C_{39}H_{75}$	

The most abundant fragment of this series was that at m/e 487, and the most abundant aldehyde was at m/e 768. However, by pyrolysis of methyl α-mycolate (Test) and oxidation of the resulting meroaldehyde followed by esterification they obtained the corresponding methyl meromycolates in whose mass spectrum the most abundant homologue had m/e 770, corresponding to the meroaldehyde having m/e 740. By considering this aldehyde as the major component in conjunction with the fragments at m/e 487 (i.e. in the above formulae $n_1 + n_2 + n_3 = 44$, $n_1 + n_2 = 28$, $n_2 + n_3 = 27$) these authors calculate that $n_1 = 17$, $n_2 = 11$ and $n_3 = 16$. The structure (VII) incorporating these values was then proposed for the main component of methyl α-mycolate (Test).

In order to discover whether the same procedure could be applied to methyl mycolate -I the series of fragments at m/e 459 to 543 were studied by high resolution mass spectrometry and the results are shown in Table 1.

Table 1.

m/e	Composition.	% of peak studied	% of base peak X 30
459	$C_{32} H_{59} O$	96.1	84
	$C_{33} H_{63}$	3.9	3
487	$C_{34} H_{63} O$	97.1	79
	$C_{35} H_{67}$	2.9	2
515	$C_{36} H_{67} O$	90.9	33
	$C_{37} H_{71}$	9.1	3
543	$C_{38} H_{71} O$	76.9	16
	$C_{39} H_{75}$	23.1	5

It is thus apparent that while the oxygen containing fragments are very prominent, the hydrocarbon ions are no larger than the general background of the mass spectrum (see Fig.5). The hydrocarbon fragments, therefore, appear to have no unusual significance. Etemadi and Lederer[57] did not publish the relative intensities of the doublets observed in their spectrum, nor did they produce a complete

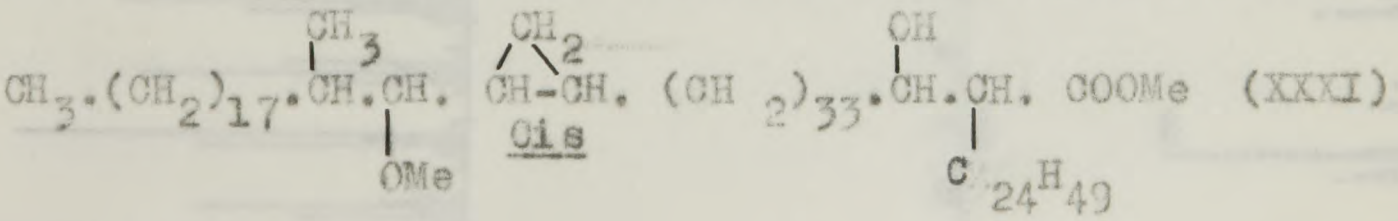
spectrum of methyl α-meromycolate (Test) which might be expected to show similar behaviour to that of the meroaldehyde. It would also be expected that in the spectra of methyl mycolate -I and α-mycolate (Test) a corresponding series of doublets due to cleavage on the other side of the cyclopropane groups of the meroaldehyde (XXVIII) should be observed. According to the calculations of Etemadi and Lederer the main component of this series should be a doublet at m/e 293; no such series is readily apparent in the spectrum of methyl mycolate -I (Fig.5).

The fragments of m/e 740 and 459 in the spectrum of methyl mycolate -I are the most abundant of their respective series and their difference is 281 mass units. Therefore, the corresponding anhydro-ester might also be expected to lose a hydrocarbon fragment of this mass (m/e 1132 ⟶ 851) but no significant peaks are observed in this region. The mass spectra of methyl cis and trans - 9,10 - methylene-octadecanoate, reported by Wood and Reiser[89], do not show large peaks due to cleavage adjacent to the cyclopropane group (see also ref. 91). It would thus be very surprising if such fragments were apparent in the spectrum of methyl mycolate -I and the corresponding anhydro-ester.

The position of the cyclopropane rings in methyl mycolate -I is thus still uncertain. The n.m.r. spectra

suggest two independent cis - 1,2 -disubstituted cyclopropane rings; any interaction between two adjacent cyclopropane rings would probably be reflected in the n.m.r. spectrum. However, the series of peaks at m/e 459 to 543 in the mass spectrum of methyl mycolate -I suggest that there is a point of considerable weakness in the molecule of the meroaldehyde (XXVIII); the possibility that the cyclopropane groups are separated by a single - CH_2- group cannot be ruled out (c.f. ref. 94).

The mass spectrum of methyl anhydromycolate - II (Fig.6) shows ten homologous peaks at m/e 1188 to 1314, that at 1216 (M-32) being the most prominent. Another series of peaks (m/e 1156 ⟶) indicates further loss of 32 mass units (methanol). Cleavage α to the $\rangle$CH-OMe grouping gives peaks at m/e 297, 325 and homologous peaks at m/e 907 to 1033, that at m/e 935 predominating. This latter series is seen to lose a molecule of methanol (m/e 875 ⟶) In the light of the n.m.r. evidence these fragments point to the structure (XXXI) for the main component of methyl



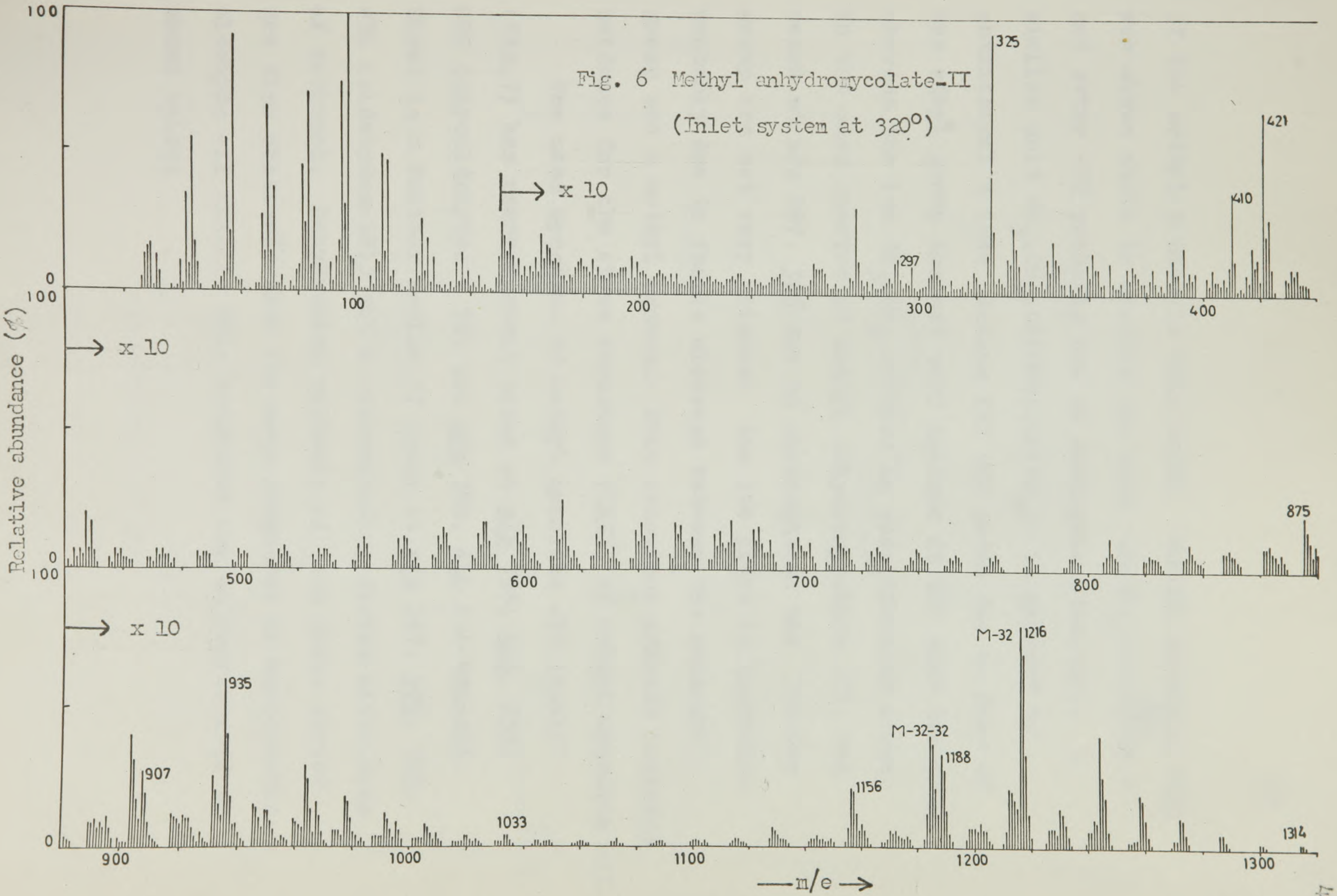
$CH_3.(CH_2)_{17}.CH.CH.CH{-}CH.(CH_2)_{33}.CH.CH.COOMe$ (XXXI)

mycolate -II.

There is at present no direct evidence for the position

Fig. 6 Methyl anhydromycolate-II

(Inlet system at 320°)



of the methyl branch in this ester. Methyl mycolate -III was shown above to contain the unit - $CH(CH_3).CO.\overset{CH_2}{\diagup\diagdown}CH-CH$ - and ester -II probably has an analogous structure. A similar unit $CH_3.CH_2.CH(OMe).CH(CH_3)$- is present in phthiocerol A (See Section II) and peaks due to loss of the ethyl group are not very intense in the mass spectrum, whereas the ion $CH_3.CH_2.CH(OMe)^+$ is exceptionally large. In the mass spectra of methyl anhydromycolate -II, the peaks at m/e 297, 325 due to cleavage α to the $\diagup\diagdown$CH-OMe group are not very intense; the low yield is therefore probably due to facile cleavage between the methoxyl group and a methyl branch. This behaviour affords indirect evidence for the above structure (XXXI) of methyl mycolate -II.

The mass spectrum of methyl mycolate -II itself (Fig.7) has rearrangement peaks at m/e 796, 824, 852, 880 (meroaldehydes - 32) and m/e 382, 410 (α-branch). There is a further series of peaks at m/e 547, 575, 603, 631 (aldehydes-281) and a corresponding series after loss of methanol. Dehydration products of both these series are also observed. For the main component of meromycolic aldehyde -II (XXXII) this breakdown may be represented as shown below;

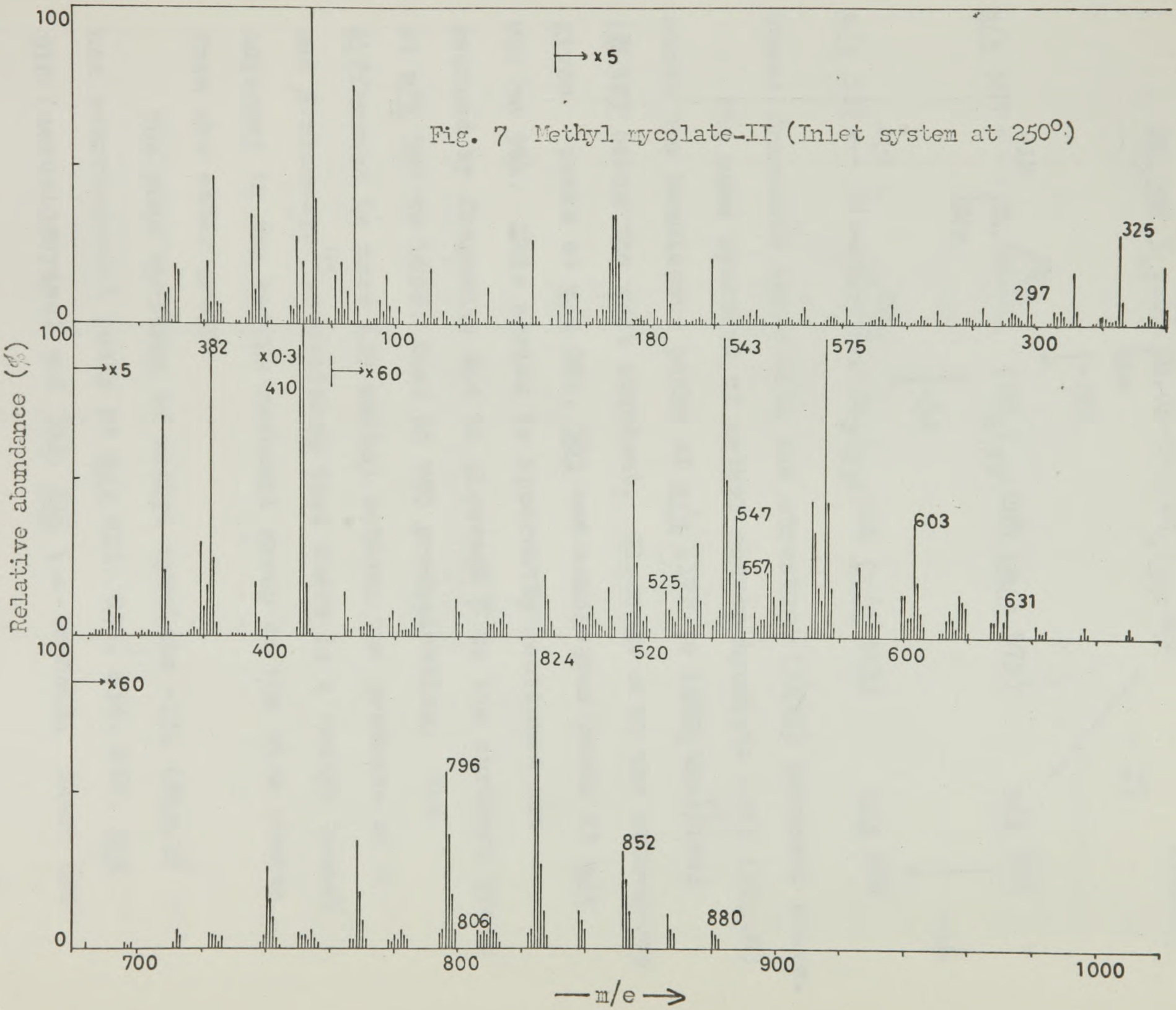
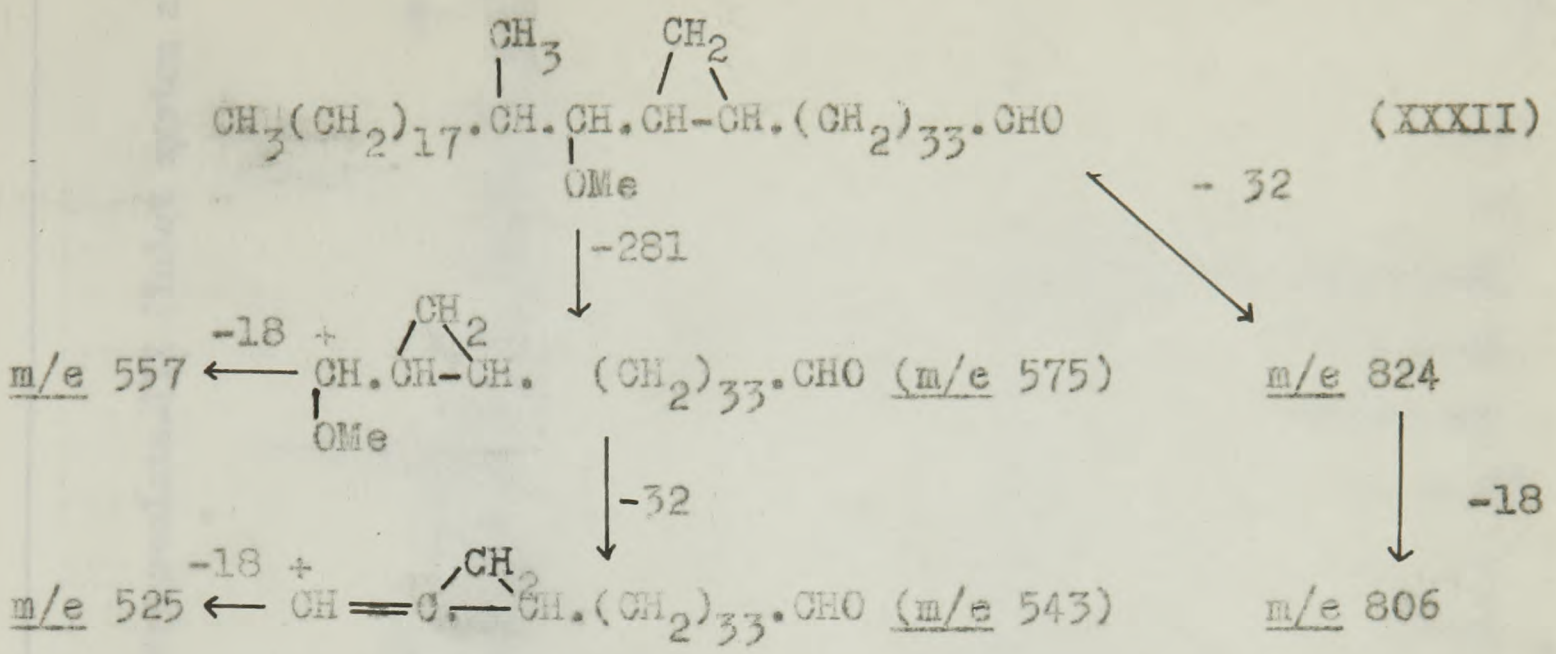


Fig. 7 Methyl mycolate-II (Inlet system at 250°)



These fragments agree with the structure (XXXI) proposed above.

The mass spectrum of methyl anhydromycolate -III (Fig.8) shows ten homologous peaks at m/e 1172 to 1298, that at 1242 (M-32) being the most abundant. Cleavage α to the keto-group gives peaks at m/e 281, 309 and homologous peaks at m/e 891 to 961. This series is apparently continued but swamped by fragments due to cleavage β to the carbonyl group at m/e 920 to 1046, that at 990 predominating. The difference in mass (29 units) between the products of α- and β-cleavage[95] establishes that there is a methyl branch adjacent to the ketone carbonyl group on the side remote from the ester group.

The mass spectrum of methyl mycolate -III (Fig.9) has rearrangement peaks at m/e 812, 840, 854, 868, 882 910 (meroaldehydes) and 382, 410 (α-branch). There are

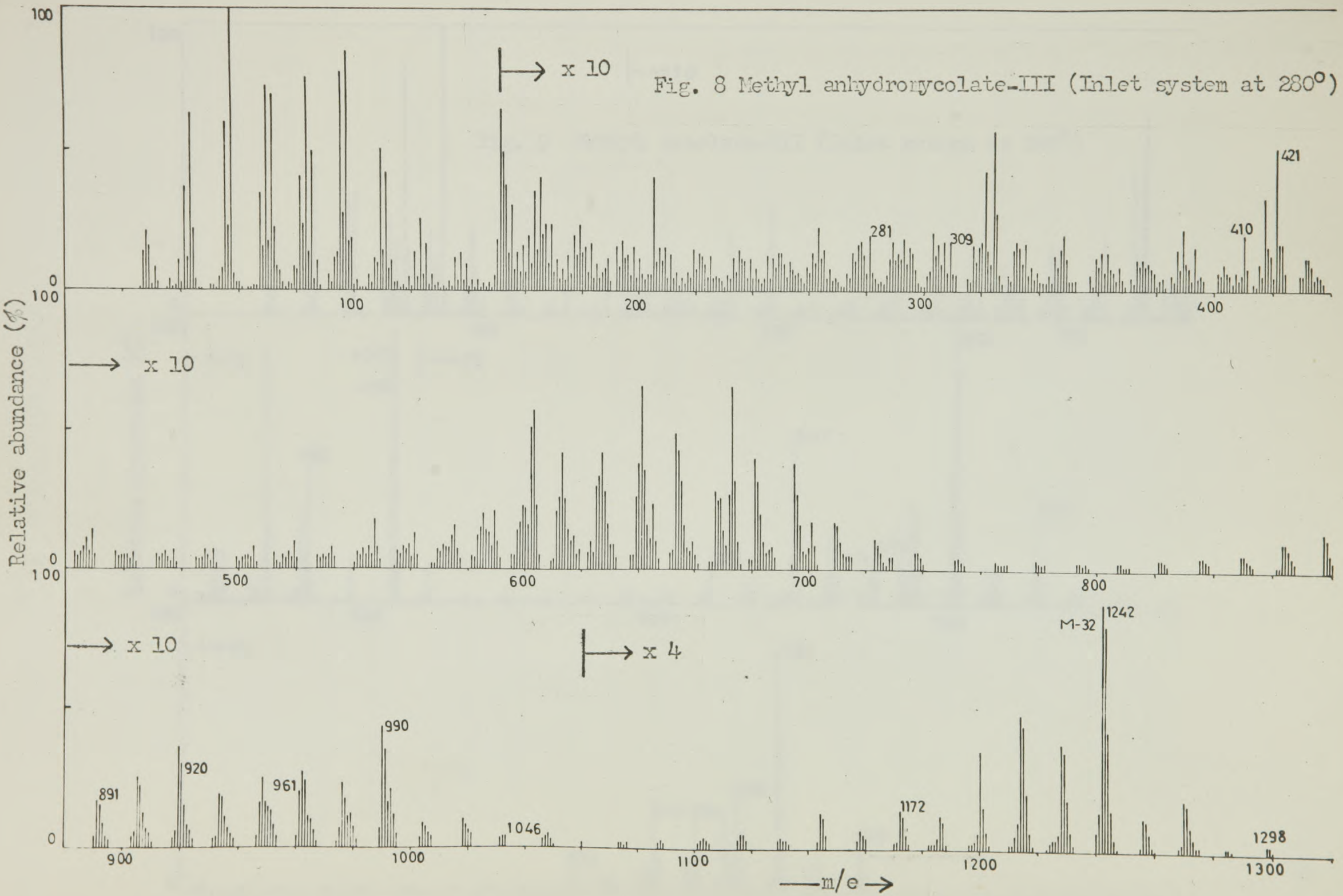


Fig. 8 Methyl anhydromycolate-III (Inlet system at 280°)

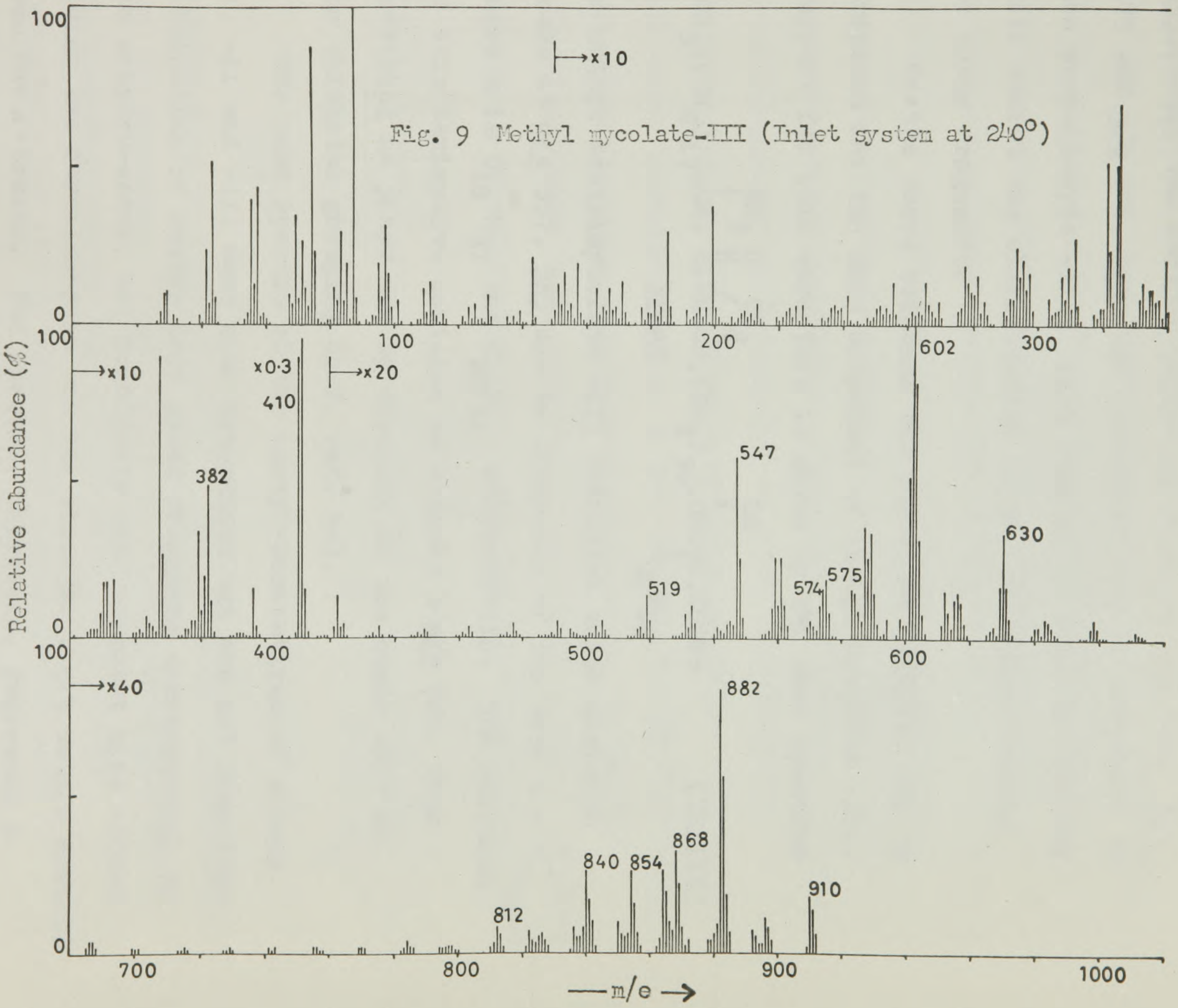
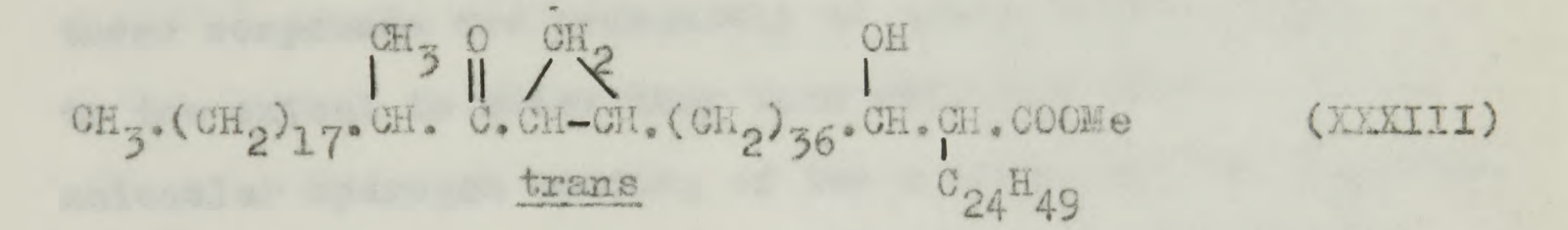


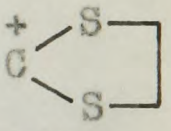
Fig. 9 Methyl mycolate-III (Inlet system at 240°)

apparently two further series of peaks at m/e 519, 547, 575 and m/e 574, 602, 630 presumably due to breakdown of the meroaldehyde -III; high resolution mass spectrometry will assist the clarification of the processes leading to these fragments.

On the above evidence the structure (XXXIII) may be proposed for the main component of methyl mycolate -III. Support for this structure is given by the mass spectrum



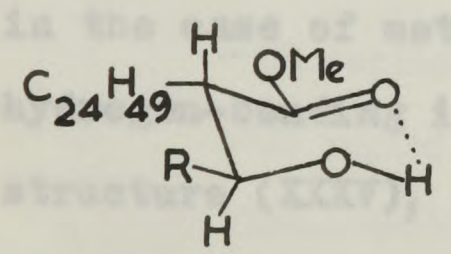
(XXXIII)

of methyl acetylmycolate -III thioketal which contains peaks at m/e 357, 385 due to fragments of the type R -  where R is $C_{18}H_{37}$ and $C_{20}H_{41}$, respectively. The spectrum of this derivative contains no signals > m/e 600, thus providing no peaks due to cleavage on the other side of the thioketal grouping (c.f. ref. 96).

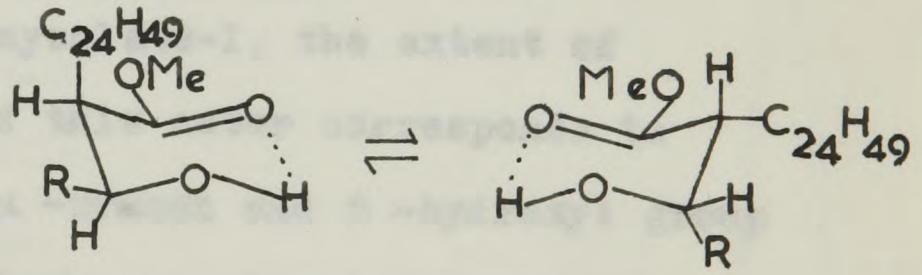
The mass spectra of the acetyl-derivatives of esters -I, -II and -III were also determined but are not described. Elimination of acetic acid gives fragments corresponding to the anhydro-ester, but McLafferty rearrangement also occurs giving the meroaldehydes and the straight chain esters derived from the α-branch. The spectra, therefore, represent a combination of those of the mycolic esters and the anhydro-compounds and less precise information can be obtained from them.

c) Stereochemical investigations.

The mycolic esters described above contain asymmetric centres (at C-2 and C-3) bearing a long alkyl-chain and a hydroxyl group, respectively; two diastereo-isomers in respect of these centres can therefore exist. Methyl mycolate -I and its C-2 epimer correspond to these two isomers and have been studied in order to establish their respective stereochemistry. Observable differences between these compounds are presumably at least partially due to the extent to which they form hydrogen bonds. Intramolecular hydrogen bonding of the α-branched, β-hydroxy-ester system can be represented as shown in the models (XXXIV and XXXV, R represents the remainder of the molecule), assuming that the chair form of the six-membered



(XXXIV)



(XXXV)

bonded ring is the most stable. The compound represented by the structure (XXXIV) is likely to form hydrogen bonds to a greater extent than (XXXV) since in the later structure one of the substituent chains must occupy an axial position thus leading to destabilisation of the ring.

The extent of intra-molecular hydrogen-bonding of hydroxy-esters can be conveniently studied by measuring the absorption in the hydroxyl and carbonyl stretching regions of the infra-red spectrum, determined as a dilute solution in carbon tetrachloride. In the spectrum of methyl 2-epimycolate-I (Fig.10 a) the ratio (0.99) of the free hydroxyl band to that of the bonded hydroxyl is greater than the corresponding ratio (0.56) in the spectrum of methyl mycolate -I (Fig. 10b). Accordingly, the ratios of the free to bonded ester carbonyl absorptions are 1.02 and 0.39, respectively. The spectra of methyl mycolate -II (Fig. 10c) and -III (Fig.10d) correspond to that of methyl mycolate -I ; the spectrum of ester-III includes the ketone carbonyl absorption. These comparisons show that in the case of methyl 2-epimycolate-I, the extent of hydrogen-bonding is less and this ester corresponds to structure (XXXV), i.e. the α -branch and β -hydroxyl group are threo to one another (Fischer projection formula). Methyl mycolate -I, -II and -III therefore have erythro configuration in respect of these centres.

This assignment conflicts with the views, expressed earlier by Asselineau and Lederer[97], which were based on the order of elution of synthetic diastereo-isomers corresponding to corynomycolic acid (I). The acid eluted

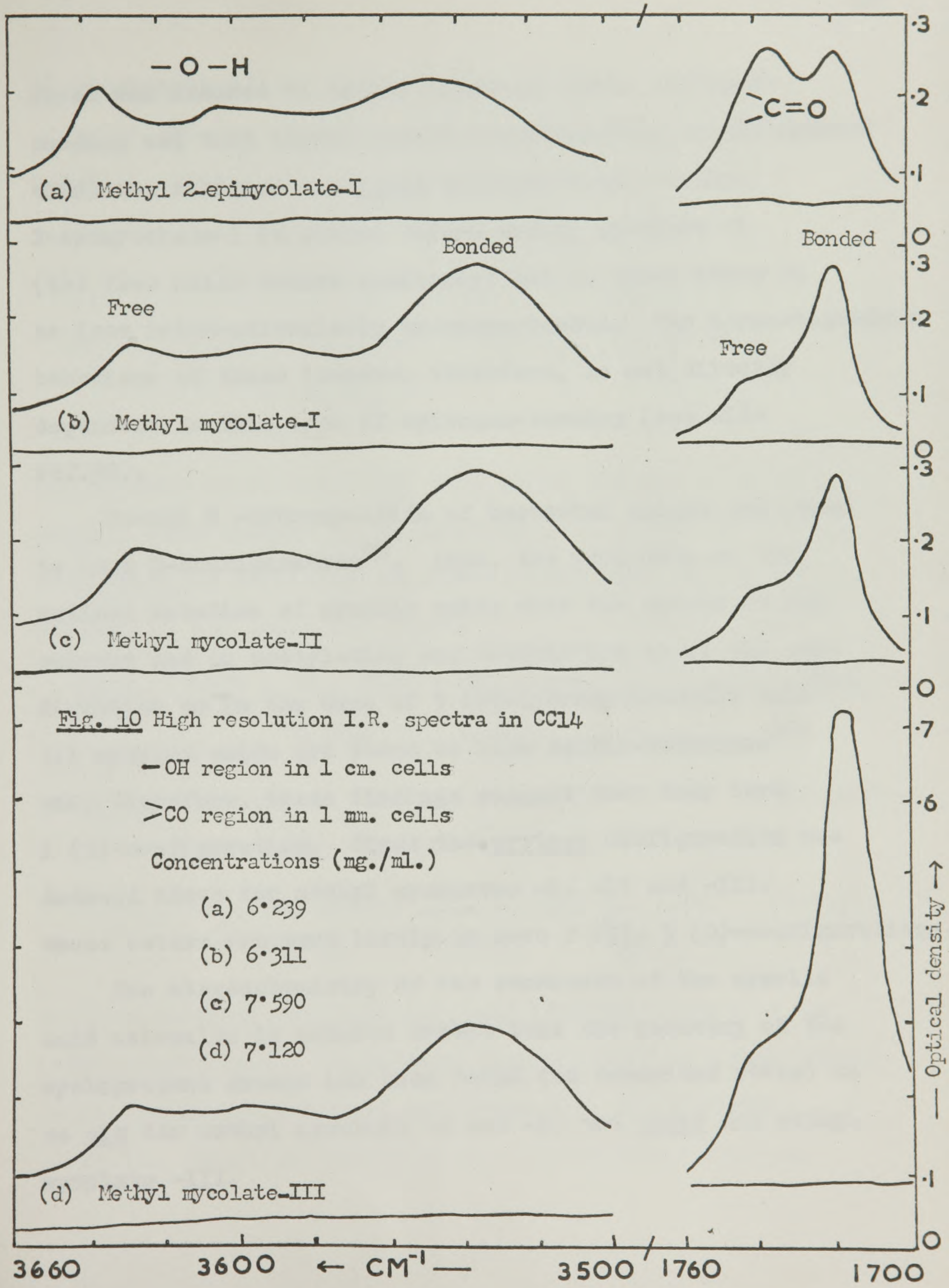


Fig. 10 High resolution I.R. spectra in CCl4

—OH region in 1 cm. cells

>CO region in 1 mm. cells

Concentrations (mg./ml.)

(a) 6·239

(b) 6·311

(c) 7·590

(d) 7·120

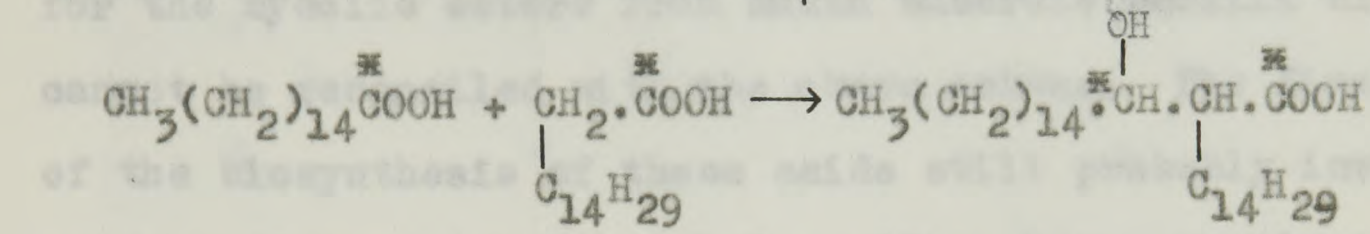
first was assumed to favour intra-molecular hydrogen-bonding and that eluted second (corresponding to the natural acid) was assigned the threo configuration. Methyl 2-epimycolate-I is eluted before methyl mycolate -I (the free acids behave similarly) but is shown above to be less intra-molecularly hydrogen-bonded. The chromatographic behaviour of these isomers, therefore, is not directly dependent on this type of hydrogen-bonding (see also ref.98).

Normal β -hydroxy-acids of bacterial origin are found to have D-configuration[99]. Also, the variation of the optical rotation of mycolic acids with the nature of the solvent and on acetylation and methylation is in the same direction as in the case of 3 (D)-hydroxy-hexanoic acid[100]. All mycolic acids are found to have dextro-rotations[100] and, therefore, these findings suggest that they have 3 (D)-configuration. Since the erythro configuration was deduced above for methyl mycolates -I, -II and -III, these esters are most likely to have 2 (D), 3 (D)-configurations.

The stereochemistry of the remainder of the mycolic acid molecules is unknown except that the geometry of the cyclopropane groups has been found (as described above) to be cis for methyl mycolate -I and -II and trans for methyl mycolate -III.

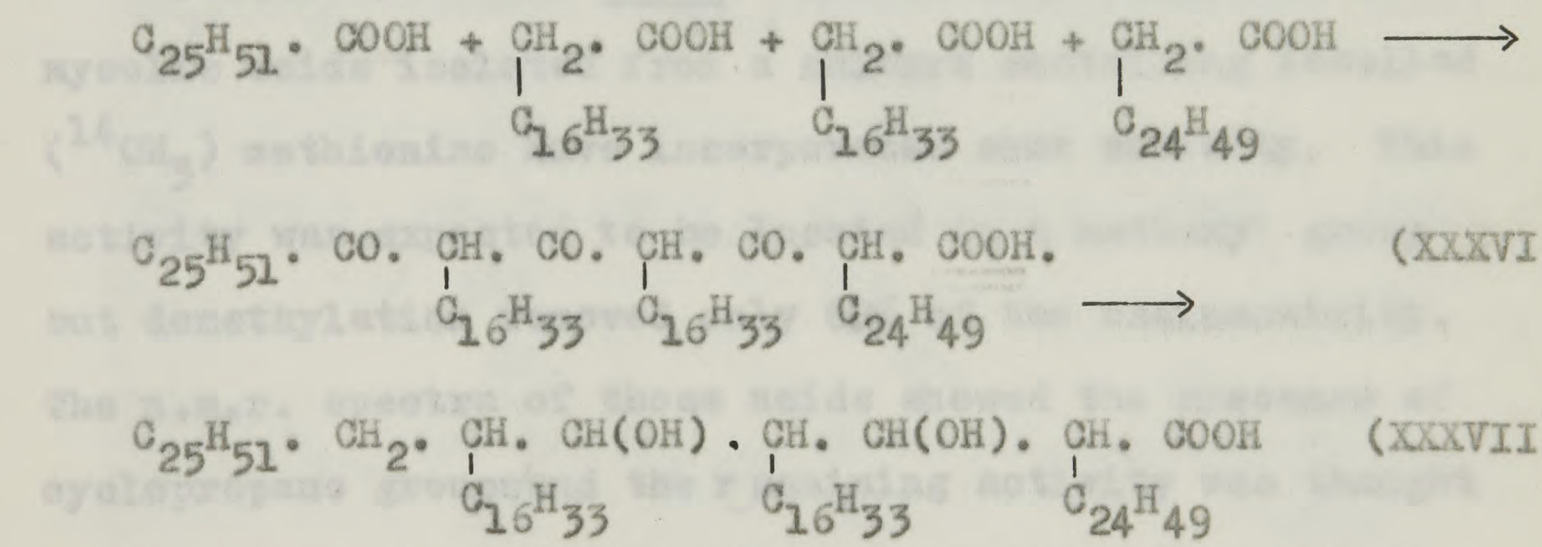
d) Biogenesis of the human mycolic acids

The simplest mycolic acid, corynomycolic acid (I), has been shown[101,102] to be formed *in vivo* by the condensation of two molecules of palmitic acid and reduction of the intermediate β-keto-acid as shown.



(I)

The mycolic acids (ca. C_{88}) from human strains of tubercle bacilli were thought to be built up by a series of similar condensations involving straight chain acids (C_{26} and C_{18}) present in the bacterial lipids (2 x 26 + 2 x 18 = 88)[80,103].



The hypothetical tri-keto-compound (XXXVI), formed as an intermediate, could be reduced appropriately to leave oxygen functions in the required positions, e.g. the structure (XXXVII). Chemical degradations (as mentioned earlier)

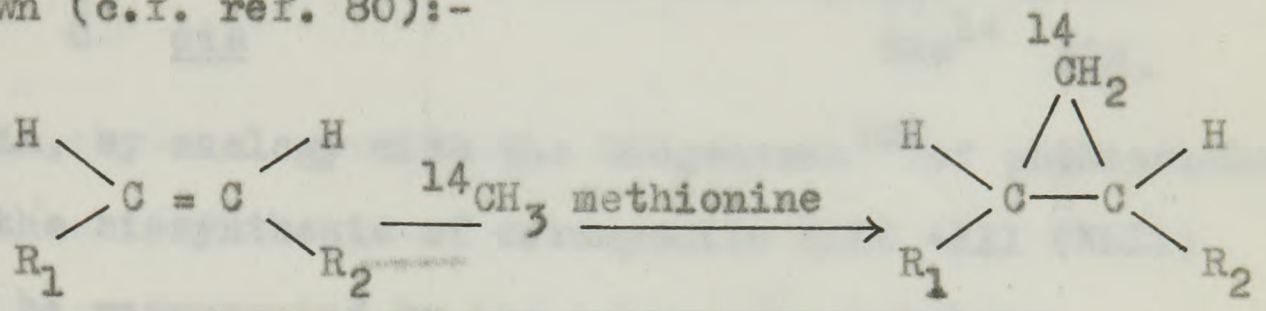
were interpreted with this type of structure in mind and many partial formulae were presented[50] incorporating these structural features.

The structures (XXVII, XXXI and XXXIII) proposed above for the mycolic esters from human tubercle bacilli clearly cannot be reconciled with the above scheme. The final stages of the biosynthesis of these acids still probably involve condensation of two long-chain acids, hexacosanoic acid and the corresponding meromycolic acid (XXXVIII) (c.f.ref.80,104).

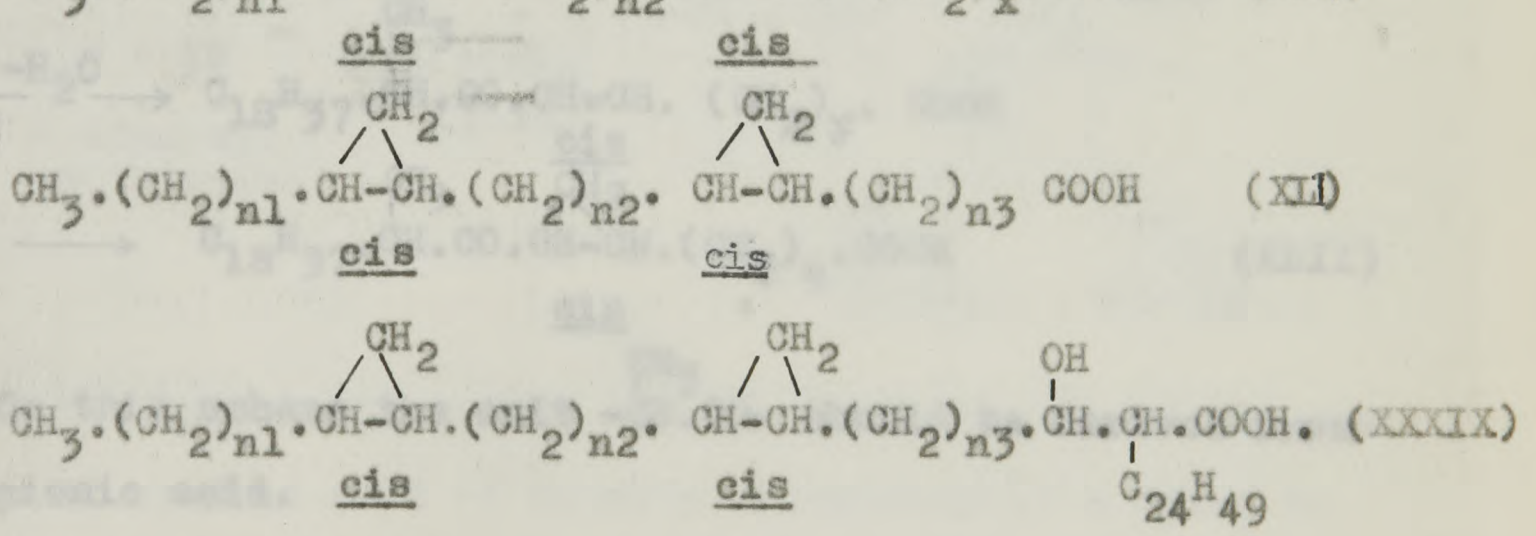
$$\underset{\text{(XXXVIII)}}{\text{R.COOH}} + \underset{C_{24}H_{49}}{CH_2.COOH} \longrightarrow \text{R.CH(OH).} \underset{C_{24}H_{49}}{CH.COOH}$$

Gastambide-Odier et al.[85] showed that crude human mycolic acids isolated from a culture containing labelled ($^{14}CH_3$) methionine have incorporated some activity. This activity was expected to be located in a methoxyl group but demethylation removed only 68% of the radioactivity. The n.m.r. spectra of these acids showed the presence of cyclopropane groups and the remaining activity was thought to be present in these functions or perhaps in a methyl branch. In the case of dihydrosterculic acid, the biosynthesis has been shown recently[105] to take place by direct addition of a $-CH_2-$ unit derived from the $-CH_3$ of

methionine to the double bond of oleic acid. The cyclopropane groups in the mycolic acids are therefore probably formed in a similar manner from olefinic precursors as shown (c.f. ref. 80):-

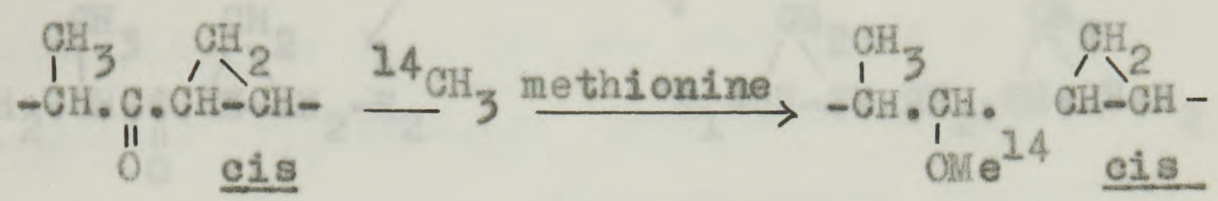


Thus in the case of mycolic acid -I (XXXIX) the biosynthesis may be envisaged as involving formation of a cis-diene (XL), conversion to meromycolic acid -I (XLI) and condensation of this with hexacosanoic acid, followed by reduction.

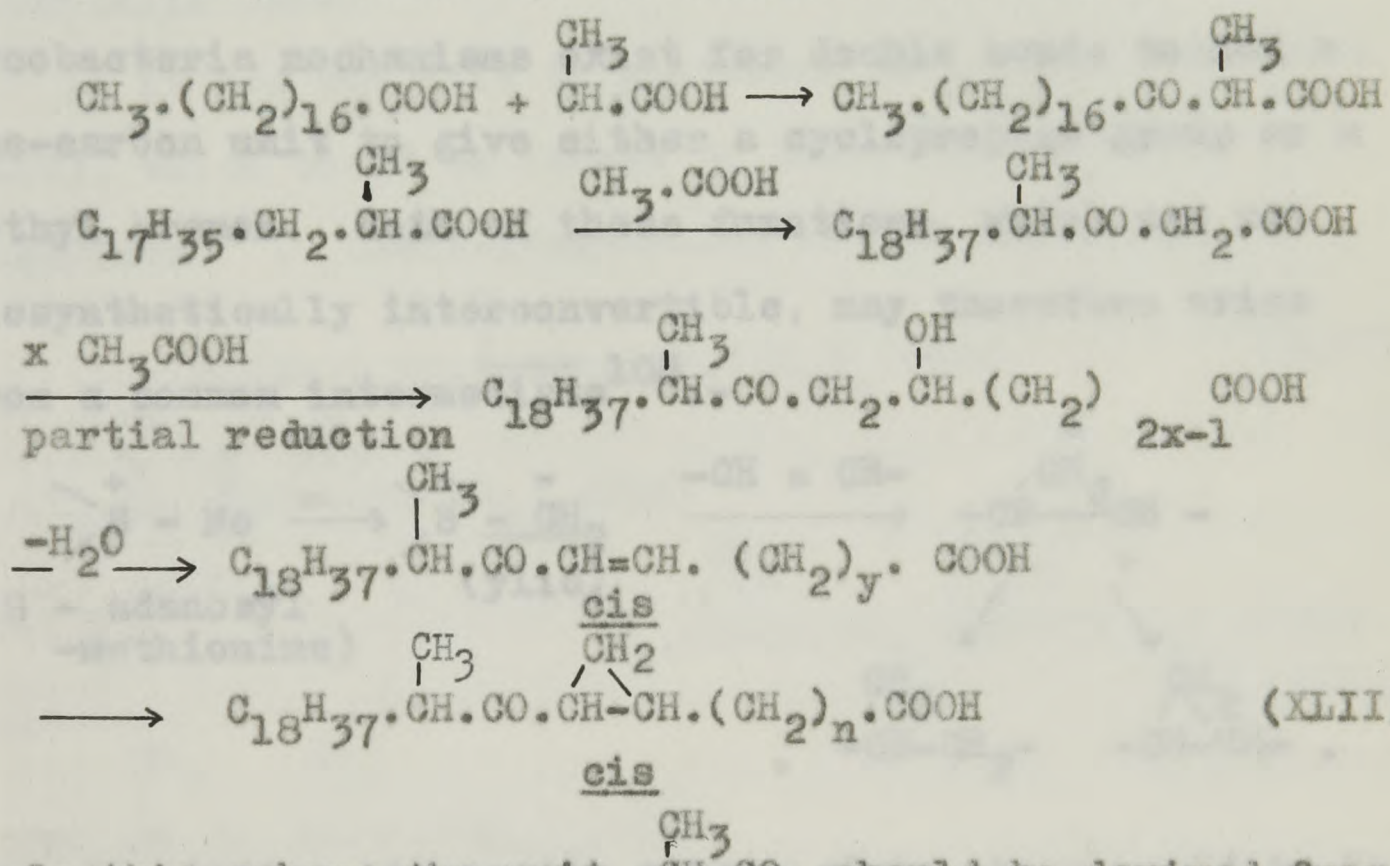


Mycolic acids -II and -III offer more complex problems. By analogy with phthiodiolone A and phthiocerol A[106] (see Section II) the acid -III (with a cis-cyclopropane

group) is probably the precursor of acid -II, as shown partially below:-

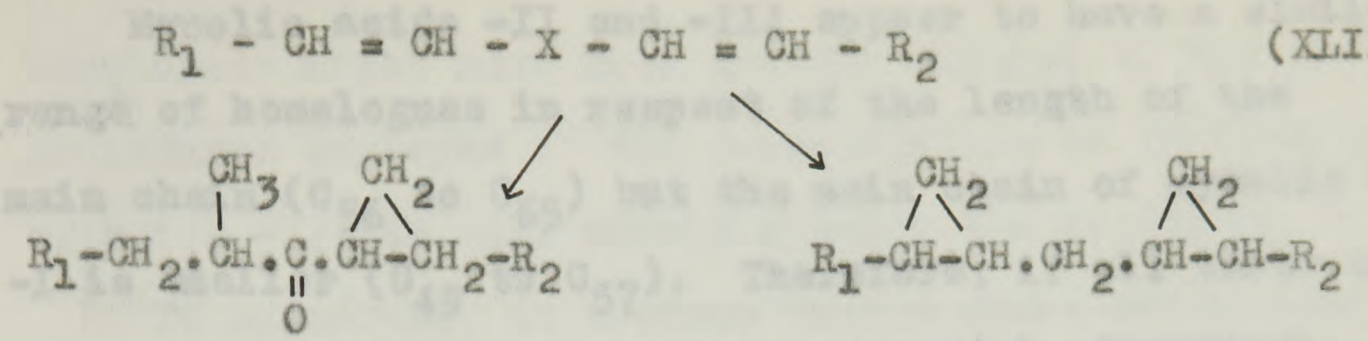


Again, by analogy with the biogenesis[106] of phthiodiolone A, the biosynthesis of meromycolic acid -III (XLII) may be represented by the scheme shown below:-



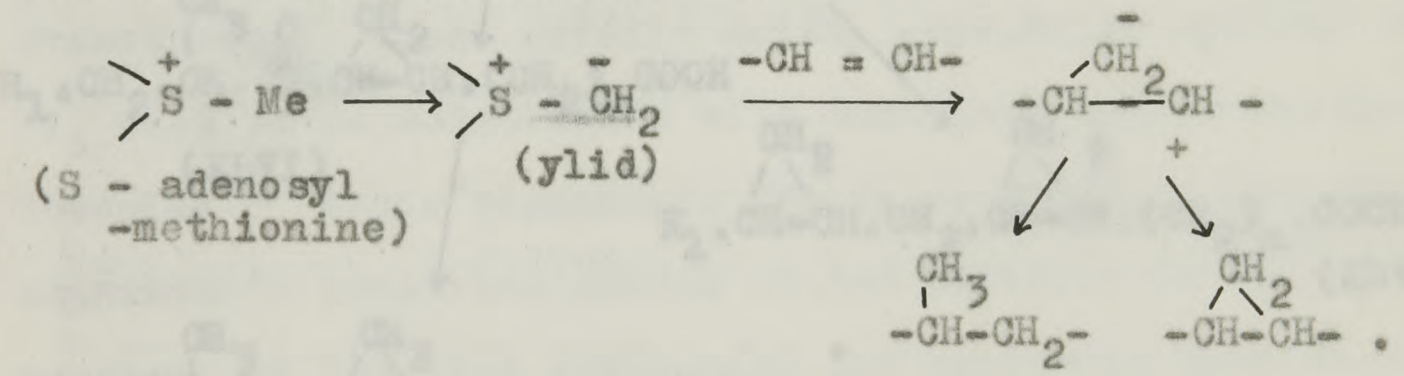
On this scheme the unit -CH.CO- should be derived from propionic acid.

An alternative scheme which would give a common route to all three mycolic acids would involve an intermediate diene shown partially below (XLIII), where X is a single carbon unit.



(XLIII)

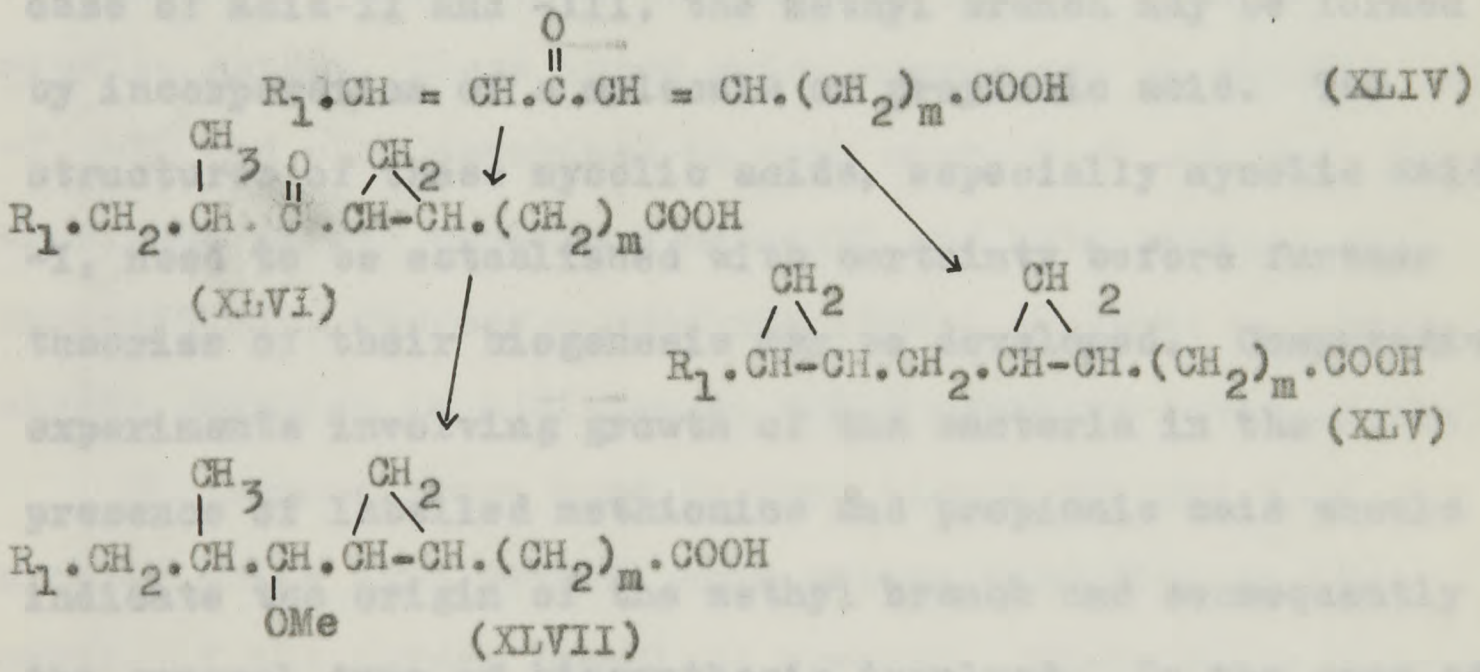
The biosynthesis of tuberculostearic acid has been shown to take place by C-methylation of oleic acid, the methyl group being derived from methionine[107]. Thus in mycobacteria mechanisms exist for double bonds to add a one-carbon unit to give either a cyclopropane group or a methyl branch. Both of these functions, which are not biosynthetically interconvertible, may therefore arise from a common intermediate[108]:-



Thus the latter biogenetic scheme given above would in the case of mycolic acid -I involve conversion of a diene to two cyclopropane rings and in the case of mycolic acid -III conversion to a methyl branch and one cyclopropane group.

Mycolic acids -II and -III appear to have a similar range of homologues in respect of the length of the main chain (C_{56} to C_{65}) but the main chain of mycolic acid -I is smaller (C_{49} to C_{57}). Therefore, if all three acids have a common biogenesis, their essential structural features must be incorporated at an intermediate stage, followed by chain-lengthening to give the corresponding meromycolic acid.

It is now possible to postulate an intermediate acid (XLIV), which could be formed by known biosynthetic procedures[108]. Chain-lengthening of the subsequent



intermediates (XLV), XLVI and XLVII) would lead to the corresponding meromycolic acids. The fact that the main components of methyl mycolate -II and -III differ by several $-CH_2-$ units points to such a chain-lengthening procedure. It is possible, but unlikely, that the proportions of the

homologues might have been greatly altered by the purification procedures employed ; the investigation of further samples of acids -II and -III should clarify the position.

To summarize, it appears that a diene of structure (XLIV) is a possible intermediate leading to all three mycolic acids, assuming that mycolic acid -I has two methylene-interrupted cyclopropane groups. Another alternative is that the biogenesis of acid -I is unrelated to those of acid -II and -III, e.g. these acids may be derived from different olefinic precursors or, in the case of acid-II and -III, the methyl branch may be formed by incorporation of a molecule of propionic acid. The structures of these mycolic acids, especially mycolic acid -I, need to be established with certainty before further theories of their biogenesis may be developed. Comparative experiments involving growth of the bacteria in the presence of labelled methionine and propionic acid should indicate the origin of the methyl branch and consequently the general type of biosynthesis involved. In the case of α-smegmamycolic acid (V) the methyl branch has been shown to be derived from methionine[82]; it thus appears that the methyl branch in mycolic acid -II and -III is quite likely to arise by this process.

e) Nomenclature.

Asselineau and Lederer[71,72] named their chromatographically homogeneous fractions α-, β-, γ-, δ-, etc. mycolic acids, in order of their elution from alumina columns. Since the advent of thin-layer chromatographic analysis, it has become easier to decide which fractions are homogeneous. Consequently, Etemadi and Lederer[57,82] and their co-workers have proposed a modification of the same prefixes used by the above workers. For instance, the mycolic acids from the Test strain have been designated, in order of elution from alumina, as α-, α′- and β- mycolic acids. These three acids correspond to mycolic esters -I, -II and -III described in this work, i.e. α′- and β- mycolates (Test) contain a methoxyl and keto group, respectively, whereas α-mycolate (Test) contains no other oxygen function in addition to the β-hydroxy-ester system.

The nomenclature used in this thesis divides the mycolic acids into several main groups according to the nature of the oxygen functions present. Since these groups are now easily detectable by physical measurements, the chromatographic order of elution need no longer be rigorously adhered to, but is still used as a valuable guide. Differences in the carbon skeleton or the presence of multiple bonds could be expressed by appropriate

subdivisions. It is thought that this new system of nomenclature will enable mycolic acids from different sources to be described in a clearer fashion than is possible with the system of Etemadi and Lederer whose modified prefixes are easily confused with the original system of Asselineau and Lederer.

Section II - Phthiocerol and related diols.

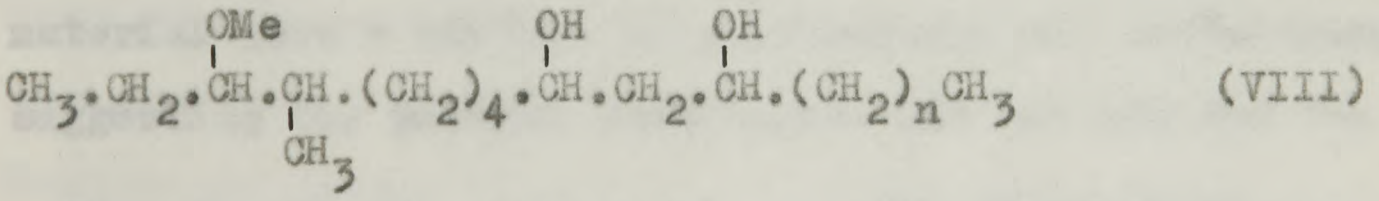
a) Previous researches

The name phthiocerol was given by Stodola and Anderson[109] in 1936 to a methoxy-glycol, $C_{35}H_{72}O_3$, m.p. 73-74°, $[\alpha]_{589}$ - 4.8°, isolated from the chloroform extract of human tubercle bacilli. The hydrocarbon corresponding to phthiocerol, named phthiocerane, was prepared by Ginger and Anderson[110]; treatment of phthiocerol with hydriodic acid was followed by reduction with zinc amalgam in acetic acid and catalytic hydrogenation of the product. X-ray crystallography of phthiocerane[111] and mono-layer studies[45] on phthiocerol itself enabled Ställberg-Stenhagen and Stenhagen to suggest that the molecule of phthiocerol was a long hydrocarbon chain carrying short branches, and having polar groups near one end of the molecule. Infra-red spectra (band at 740 $cm.^{-1}$ due to a propyl group) led to the conclusion[112] that phthiocerane contained a methyl group in the 4-position and was therefore probably 4-methyltritriacontane.

Ställberg-Stenhagen and Stenhagen[113] synthesized many analogous hydrocarbons, and by comparison of their X-ray diffraction patterns with that of phthiocerane confirmed the position of the methyl branch on C-4. A comparison of 4(L)- and 4(DL)-methyltritriacontane with natural phthiocerane[114]

showed that the latter was at least partially racemized and also probably contained a small amount of a higher homologue. The racemization during the preparation of phthiocerane suggested that at least one of the functional groups was at, or was in α-position to, the carbon atom carrying the methyl side-chain[114].

Demarteau-Ginsburg et.al.[14] in 1953 showed that crude phthiocerol contained components in addition to the methoxy-glycol. A triol (phthiotriol), a keto-diol (phthiodiolone) and a methoxy-keto-alcohol (phthiocerolone) were described. The main investigations, however, were concentrated on the methoxy-glycol, which retained the name phthiocerol (redesignated later in this thesis as phthiocerol A). Three groups of workers, Polgar in Oxford, Demarteau-Ginsburg and Lederer in Paris and Ställberg-Stenhagen and Stenhagen in Uppsala, simultaneously produced evidence leading to the structure (VIII) for phthiocerol.

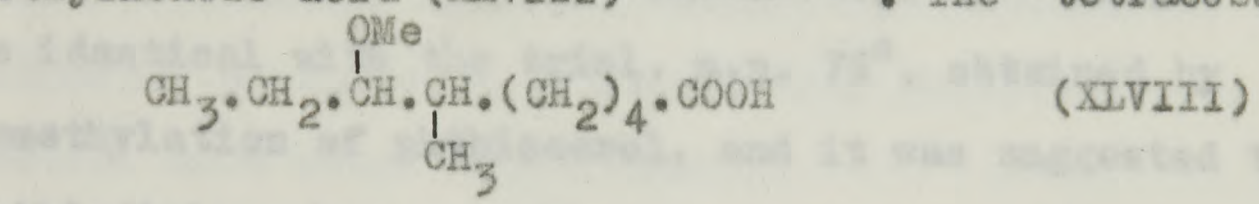


(VIII)

n = 22 and 20.

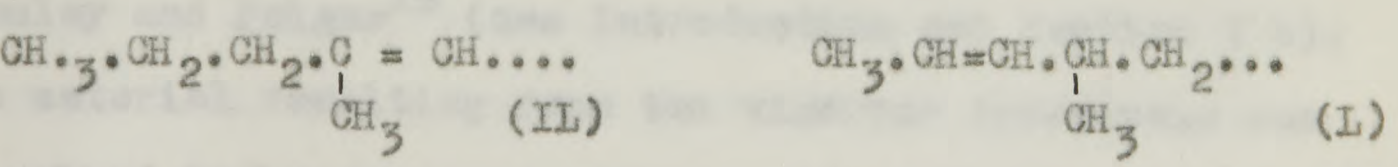
Formation of a benzylidene[34] of isopropylidene[77] derivative of phthiocerol showed that an α- or β-glycol system was present. The existence of a β-glycol system

was demonstrated by mild oxidation to a β-diketone[34,115] and by mass spectrometry[48,116] of phthiocerol. Vigorous oxidation[117] gave products intially described as 'tetracosanoic acid' and a 'C_{11}-methoxy-acid! The latter compound was converted into the corresponding keto-acid and comparison of this with synthetic analogues showed that this oxidation product was essentially 7-methoxy-6-methylnonoic acid (XLVIII)[35,118,119]. The 'tetracosanoic



(XLVIII)

acid' obtained as the other oxidation product was shown later to be a mixture of C_{24} and C_{22} straight-chain acids[48]. The mass spectrum of phthiocerol[120] confirmed that the methoxyl group was in 3-position. Demethylation of phthiocerol[77] (using acetic anhydride in the presence of toluene-p-sulphonic acid) led to a triol, m.p. 75°, and an unsaturated diol, m.p. 72°. Ozonolysis of the latter material gave a mixture of pentan-2-one and acetaldehyde suggesting the partial structures (IL) and (L) for the

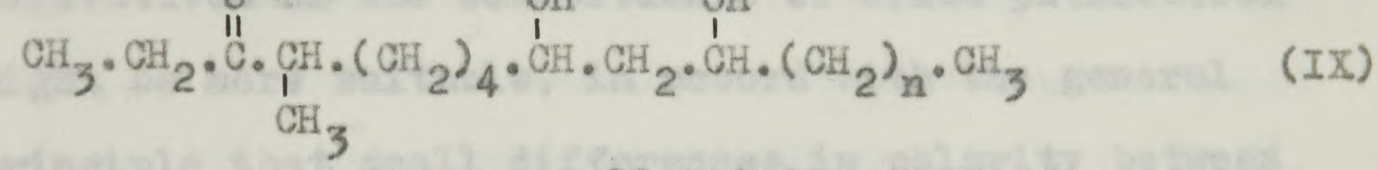


(IL) (L)

unsaturated diol. Gas chromatography and mass spectrometry showed that phthiocerane was in fact a mixture of 4-methyltetratriacontane and 4-methyldotriacontane[47,121]. The

above results showed that phthiocerol is a mixture of homologous diols having the structure (VIII).

As mentioned above, Demarteau-Ginsburg et al.[14] obtained from crude phthiocerol, among other products, a dihydroxyketone, m.p.80°, which they named phthiodiolone. This substance formed an isopropylidene-derivative[62]; moreover, reduction with sodium borohydride or lithium aluminium hydride gave a triol of m.p. 74° thought to be identical with the triol, m.p. 75°, obtained by demethylation of phthiocerol, and it was suggested that phthiodiolone has probably the structure (IX).



$$CH_3.CH_2.\overset{O}{\overset{\|}{C}}.\underset{CH_3}{CH}.(CH_2)_4.CH(OH).CH_2.CH(OH).(CH_2)_n.CH_3 \qquad (IX)$$

n = 22 and 20.

b) Present investigations

The crude phthiocerol used in these investigations was a sample remaining from previous studies[34,35]. It had been isolated essentially according to the procedure of Chanley and Polgar[19] (see Introduction and Section I b). The material resulting from the vigorous hydrolysis was dissolved in benzene and addition of methanol precipitated the crude mycolic acids. The mother-liquors were evaporated,

the residue taken up in benzene and acidic material (the laevorotatory acids) removed, on addition of methanolic barium hydroxide, as the insoluble barium salts. Evaporation of the remaining solution and crystallization of the residue from light petroleum gave crude phthiocerol.

Undimensional multiple chromatography (UMC) of crude phthiocerol showed the presence of the diols (VIII) now named phthiocerol A, diols designed phthiocerol B, ketonic material, presumed to be essentially phthiodiolone, and several more polar constituents.

For further studies it appeared that non-polar derivatives of the constituents of crude phthiocerol might be more suitable, in accord with the general principle that small differences in polarity between components of a mixture would be more marked on reducing the overall polarity of these components. In considering non-polar derivatives attention was turned to cyclic acetals and ketals. Such derivatives would have the additional advantage of distinguishing any hydroxy-compounds present in crude phthiocerol which cannot form cyclic acetals or ketals. The O-isopropylidene derivatives prepared first were found to be unstable to chromatographic procedures. The ethylidene acetals, obtained from crude phthiocerol by the action of acetaldehyde in the presence

of toluene-p-sulphonic acid, proved to be satisfactory. These cyclic acetals showed on UMC the same sequence of polarity as crude phthiocerol, thus indicating that phthiocerol A and B, as well as the ketonic and more polar constituents (see above) are diols capable of forming cyclic acetals.

Column chromatography of the ethylidene acetals on neutral alumina gave a mixture of the acetals of phthiocerol A and phthiocerol B, followed by the acetal of the ketonic material and other more polar products (see Experimental). Repeated chromatography of the mixture of the acetals derived from phthiocerol A and B gave the homogeneous acetals : ethylidenephthiocerol A, m.p. 40°, and ethylidenephthiocerol B, m.p. 35.5°. Hydrolysis of these acetals, with ethanol in the presence of toluene-p-sulphonic acid, gave the parent diols, phthiocerol A m.p. 73-73.5°, $[\alpha]_{589}^{20}$ - 4.5°, and phthiocerol B m.p. 71-71.5°, $[\alpha]_{589}^{20}$ - 8.2°.

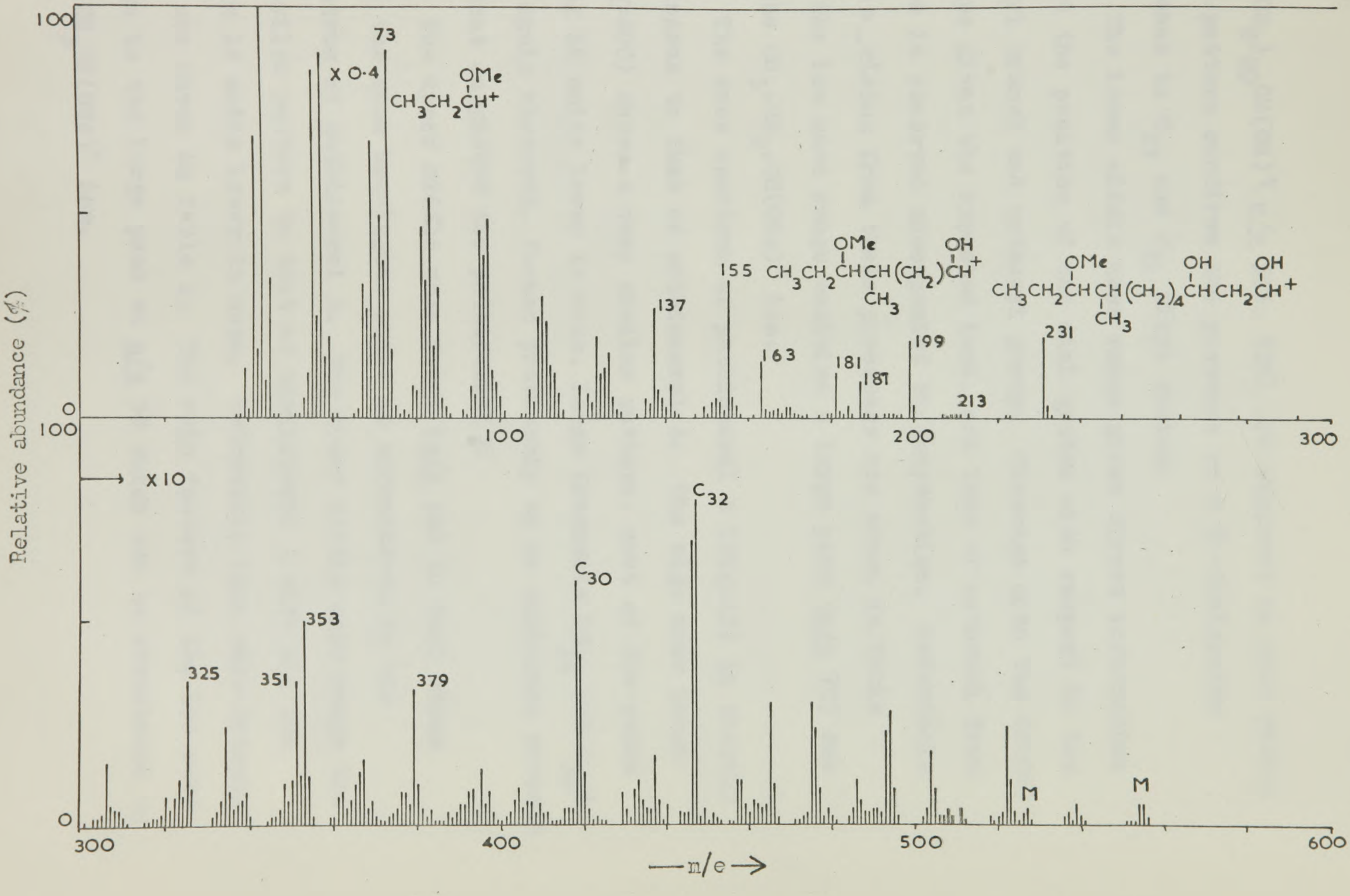
The fractions from the above chromatography, containing the acetal of ketonic material, were repeatedly chromatographed on alumina to give the acetal of the ketone component (subsequently referred to as phthiodolone A) slightly contaminated with chromatographically similar impurities. In order to eliminate these impurities, it was

decided to convert this acetal to the corresponding di-acetoxy-oxime. The acetal was treated with ethanol in the presence of toluene-p-sulphonic acid and the product acetylated. Chromatography of the corresponding oxime resulted in the isolation of ethylidenephthiodiolone A oxime, m.p. 44-45°, an d diacetylphthiodiolone A oxime, m.p.38°. The time allowed for hydrolysis of the acetal of phthiodiolone A was shorter than that used in the decomposition of ethylidenephthiocerol A and B ; this probably explains the presence of the oxime containing an intact ethylidene acetal grouping. A two-stage hydrolysis of the diacetoxy-oxime gave phthiodiolone A, m.p. 76.5°, $[\alpha]_{589}^{20}$ - 2.7°; treatment of this with acetaldehyde in the presence of toluene-p-sulphonic acid gave a pure sample of ethylidene phthiodiolone A, m.p. 42.5-43°.

The mass spectrum of phthiocerol A (Fig.11) determined in these studies differs in some respects from that published by Ahlquist et al.[48]. It shows homologous peaks in the high mass range (m/e >400) due to the molecular ions M (m/e 554, 526), the ions M + 1 (m/e 555, 527) (peaks at M + 1 are possible when hydroxyl groups are present[122]), and simple decomposition products. Loss of methyl groups or water molecules give rise to small peaks at m/e 539, 511 and m/e 536, 508,and loss of methanol gives larger peaks (m/e 522, 494,). Consequent peaks are M - MeOH-H_2O (m/e 504, 476 and M - MeOH-$2H_2O$ (m/e 486, 458); these complete the high mass range with the carbon skeleton intact. It is notable that loss of methanol from the molecular ion is in this case more favourable than loss of water. Larger fragments (C_{32} and C_{30}) are seen at m/e 447, 446 and m/e 419, 418, due presumably to loss of 73 mass units (see below) in addition to the oxygen functions.

The upper middle mass range (m/e 240 to 400) is characterised by homologous fragments formed by cleavage α to the >CH(OH) groups. The ion $CH_3(CH_2)_{22}CH(OH)CH_2CH(OH)^+$ and its homologue are detectable as very small peaks (m/e 397, 369), but their dehydration products m/e 379, 351 and the ions $CH_3(CH_2)_{22}CH(OH)^+$ and

Fig. 11 Phthiocerol A (Inlet system at 110°)



$CH_3(CH_2)_{20}CH(OH)^+$ (m/e 353, 325) are observed in good yield. This pattern confirms the presence of a β-diolsystem attached to C_{23} and C_{21} alkyl chains.

The lower middle mass range gives direct information about the position of the diol system with respect to the methyl branch and methoxyl group. Cleavage α to the CH(OH) groups gives the expected ions, and loss of methanol from these is observed accompanied by dehydration. Metastable ions arising from these processes are shown in Table 2. The low mass range contains a large peak (m/e 73) due to the $CH_3.CH_2.CH(OMe)^+$ ion.

The mass spectrum of phthiocerol B (Fig.12) is closely analogous to that of phthiocerol A. The high mass range (m/e>400) shows a very similar pattern, most of the peaks being 14 units lower in mass. Large fragments (C_{32} and C_{30}) are again observed, formed presumably by an analogous process to that suggested for phthiocerol A.

The upper middle mass range (m/e 240 to 400) shows the same homologous peaks as encountered in the spectrum of phthiocerol A. The lower middle mass range has a similar pattern to that of phthiocerol A with all the peaks 14 units lower in mass. Metastable ions were detected and are shown in Table 2. The main feature of the low mass range is the large peak at m/e 59 which can be attributed to the $CH_3CH(OMe)^+$ ion.

Fig. 12 Phthiocerol B (Inlet system at 150°)

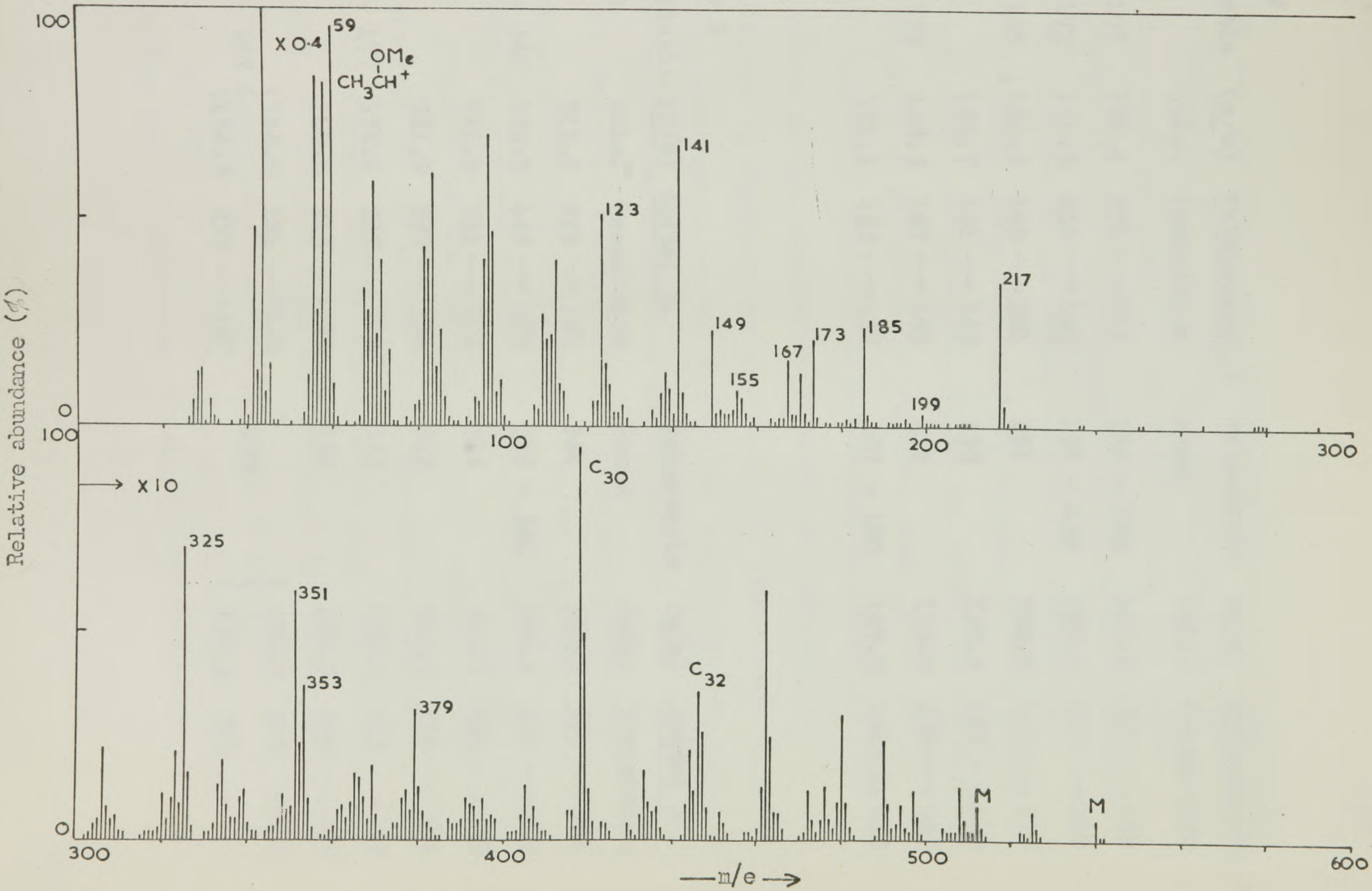


Table 2.

Metastable (m/e) Found	Calc.	Phthiocerol A Transition	Metastable (m/e) Found	Calc.	Phthiocerol B Transition
196 - 197	196.4	231 ⟶ 213	182 - 183	182.6	217 ⟶ 199
171 - 172	171.5	231 ⟶ 199	157 - 158	157.6	217 ⟶ 185
164 - 165	164.6	199 ⟶ 181	151	150.8	185 ⟶ 167
147	146.7	181 ⟶ 163	153	132.9	167 ⟶ 149
128 - 129	128.5	187 ⟶ 155	115	115.0	173 ⟶ 141
121	121.1	155 ⟶ 137	107 - 108	107.3	141 ⟶ 123

Table 3

Metastable (m/e) Found	Calc.	Acetal A. Transition	Metastable (m/e) Found	Calc.	Acetal B Transition
344	343.9	379 ⟶ 361	344	343.9	379 ⟶ 361
339 - 340	339.5	423 ⟶ 379	339 - 340	339.5	423 ⟶ 379
316	315.9	351 ⟶ 333	316	315.9	351 ⟶ 333
312	311.9	395 ⟶ 351	312	311.9	395 ⟶ 351
176 - 177	176.4	257 ⟶ 213	163	163.0	243 ⟶ 199
154	153.8	213 ⟶ 181	140	140.1	199 ⟶ 167
146 - 147	(146.8	181 ⟶ 163	133	(132.9	167 ⟶ 149
	(146.3	239 ⟶ 187		(132.9	225 ⟶ 173

The mass spectra of the ethylidene acetals of phthiocerol A and B (shown in Fig. 13 and 14, respectively) proved to be even more informative than those of the free diols. Mass spectra of 4,6-dialkyl-2-methyl-1,3-dioxane derivatives are characterised by the facile loss of the ethylidene proton or methyl group (cf.ref.123) to give good yields of ions from which the molecular weight can easily be determined. This property is particularly valuable when, as in the present examples, mixtures of homologues are present. The composition of the homologous portions can usually be ascertained by studying the nature of the ions formed in good yield by cleavage α to the ethylidene acetal grouping and the decomposition products of these ions.

In comparing the spectra of the cyclic acetals the following points are of interest. In the spectrum of phthiocerol A acetal fission of the homologous C_{23} and C_{21} alkyl chains gives the ion m/e 257 (LI). Decomposition of this ion appears to take place via several routes involving initial loss of acetaldehyde (m/e 213), water (m/e 239), or methanol (m/e 225) as shown in the scheme below. Loss of acetaldehyde can be followed by elimination of either water (m/e 195) or methanol (m/e 181), the latter process predominating. Elimination of water from the ion m/e 181 or

Fig. 13 Ethylidenephthiocerol A (Inlet system at 140°)

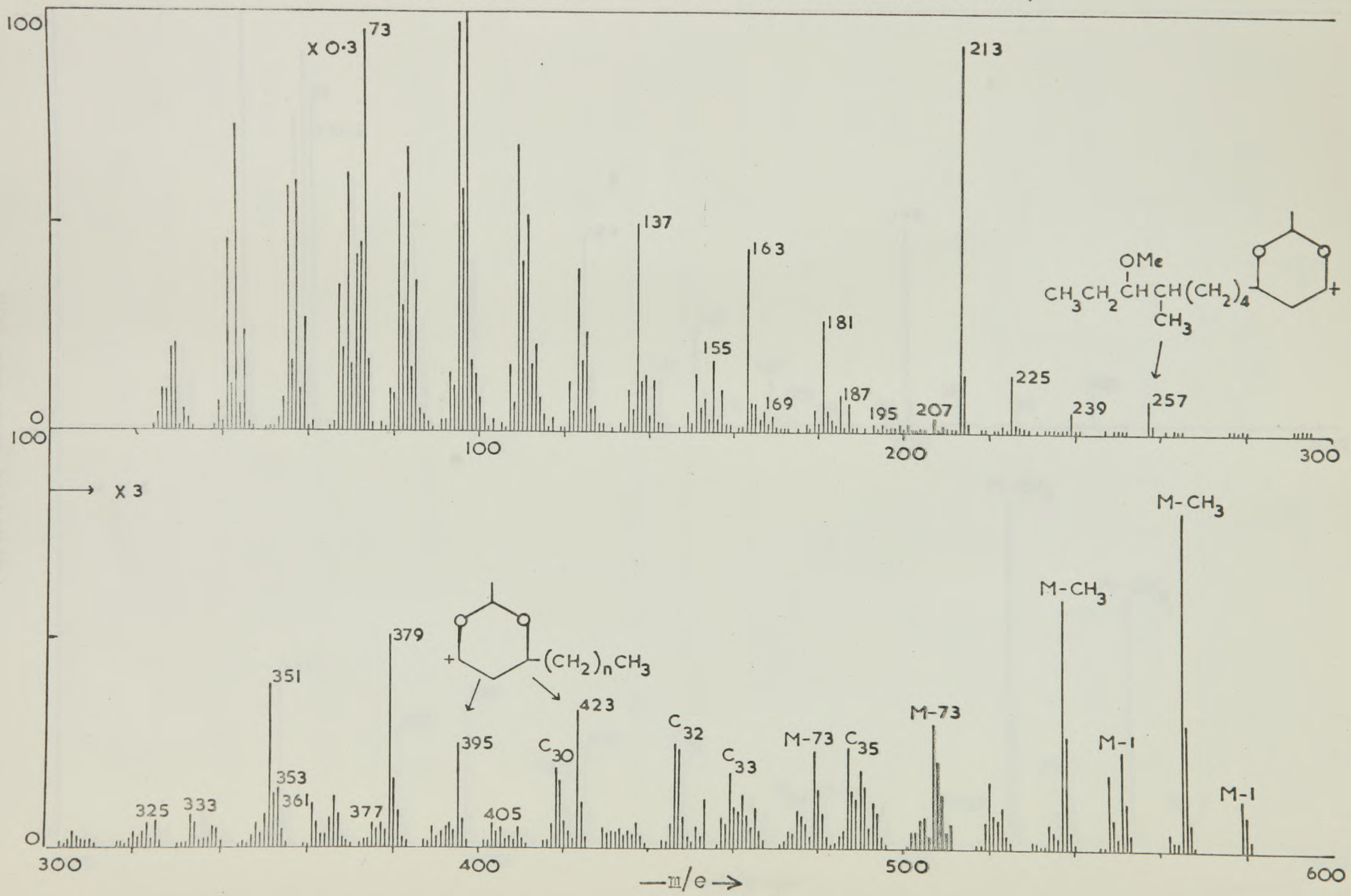
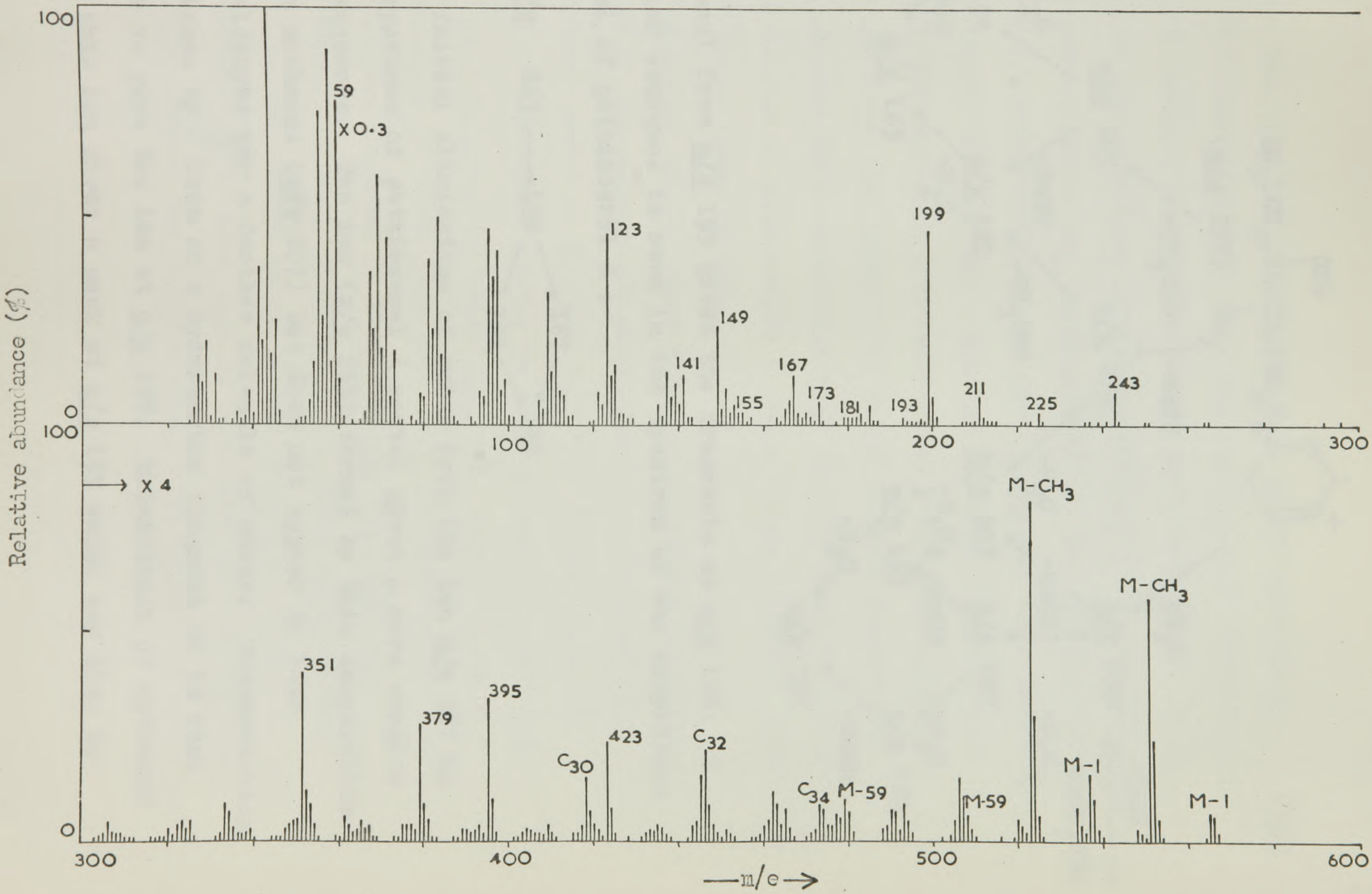
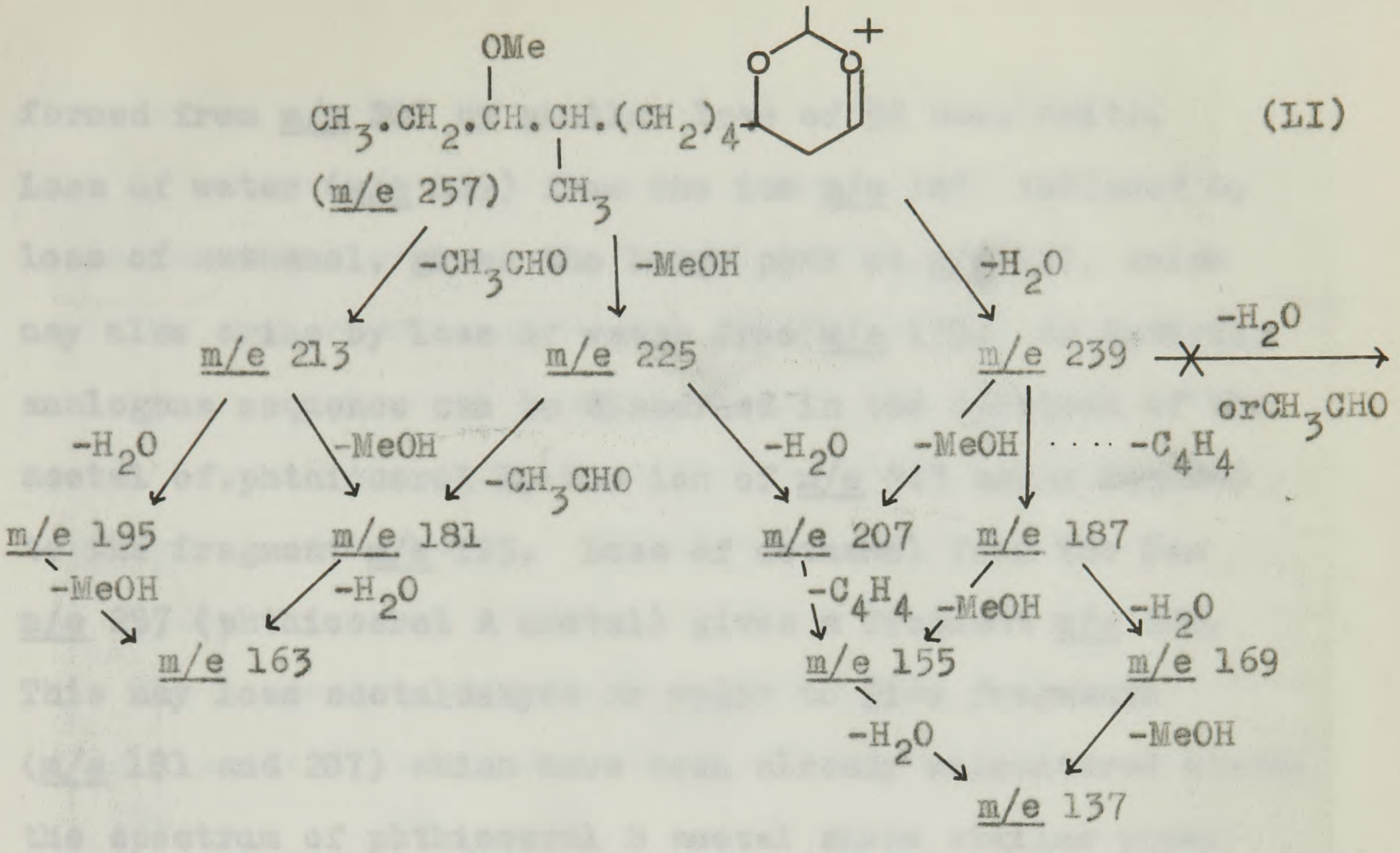


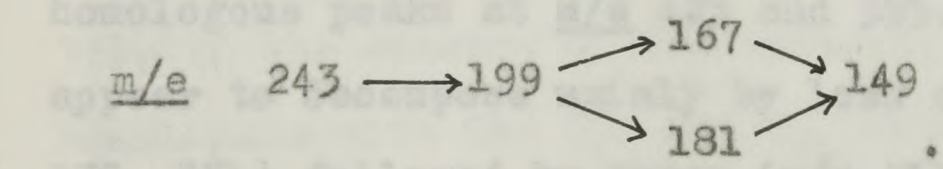
Fig. 14 Ethylidenephthiocerol B (Inlet system at 140°)





(LI)

methanol from m/e 195 gives the fragments at m/e 163. A similar sequence is seen in the spectrum of the ethylidene acetal of phthiocerol B :-



Initial elimination of water from the ion m/e 257 in the spectrum of phthiocerol A acetal gives a more complex ion sequence. The ion (m/e 239) formed by this dehydration loses methanol (m/e 207) but does not appear to lose acetaldehyde nor a further molecule of water. Fragmentation continues by loss of a hydrocarbon fragment of 52 mass units to give the ion at m/e 187. Elimination of methanol from this ion gives a peak at m/e 155 which may also be

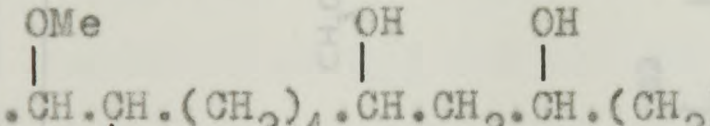
formed from m/e 207 by similar loss of 52 mass units. Loss of water (m/e 169) from the ion m/e 187, followed by loss of methanol, gives the large peak at m/e 137, which may also arise by loss of water from m/e 155. An entirely analogous sequence can be discerned in the spectrum of the acetal of phthiocerol B, the ion of m/e 243 being degaded to the fragment m/e 123. Loss of methanol from the ion m/e 257 (phthiocerol A acetal) gives a fragment m/e 225. This may lose acetaldehyde or water to give fragments (m/e 181 and 207) which have been already encountered above; the spectrum of phthiocerol B acetal shows similar peaks at 14 units lower in mass.

Cleavage on the other side of the acetal grouping gives, in the spectra of phthiocerol A and B acetals, homologous peaks at m/e 423 and 395. These fragments appear to decompose mainly by loss of acetaldehyde (m/e 379, 351) followed by water (m/e 361, 333). Initial elimination of water (m/e 405, 377) followed by loss of a hydrocarbon fragment of mass 52 (m/e 353, 325) and dehydration of these ions (m/e 335, 307), also appears totake place. These modes of decomposition appear to be characteristic of this type of ion, loss of acetaldehyde predominating as the initial step. The extent to which the

alternative process (elimination of water followed by loss of a hydrocarbon of 52 mass units) occurs seems to decrease as the size of the parent ion increases.

The high mass range of the spectra of the ethylidene acetals of phthiocerol A and B contain the expected peaks due to loss of the ethylidene proton and methyl group or elimination of methanol. Metastable ions observed in the spectra of these acetals are given in Table 3.

These spectra suggest that phthiocerol B is a mixture of homologous diols of structure (LII). The ratios of



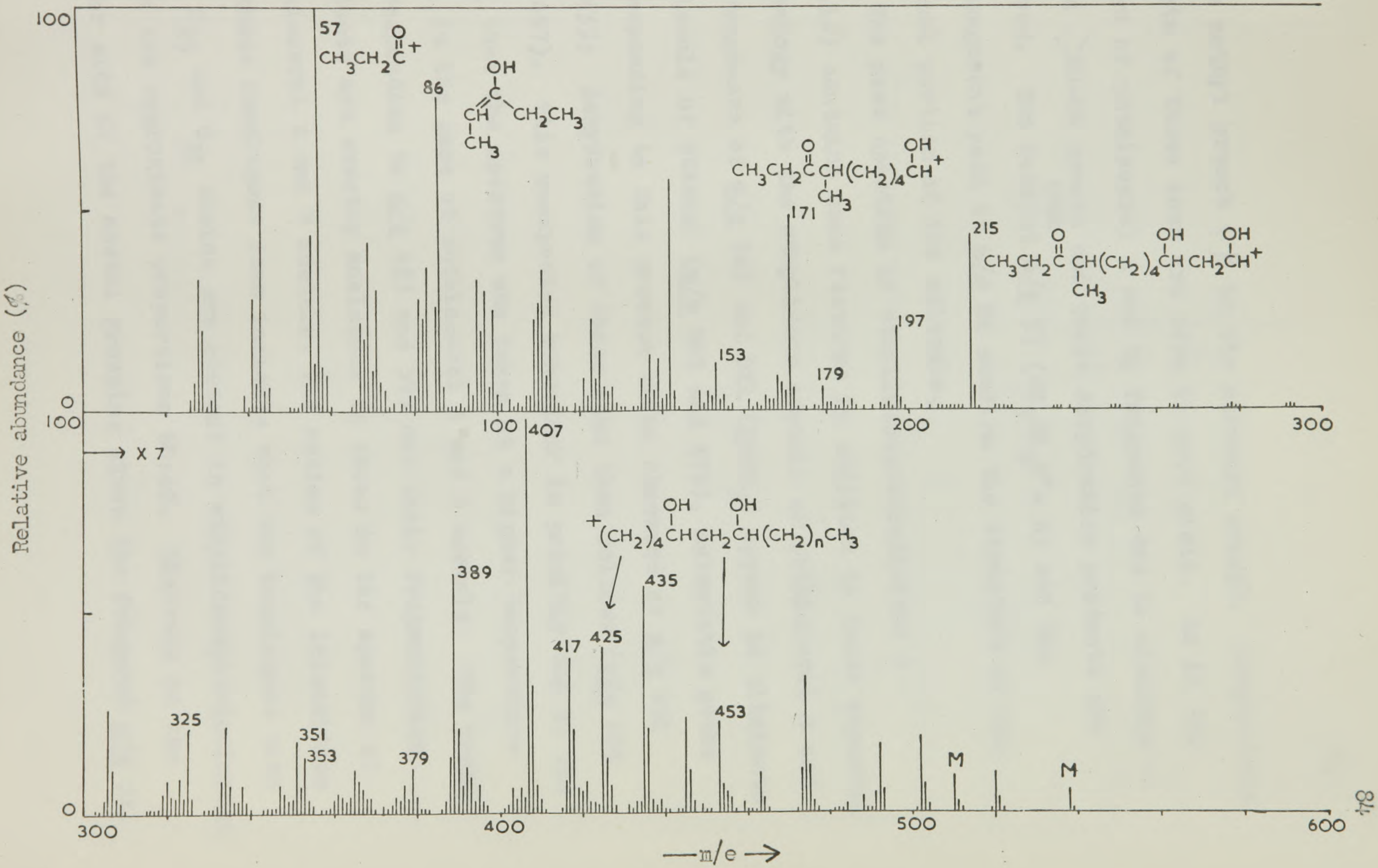
(LII)

n = 22 and 20

the intensities of the peaks at m/e 423, 395, and m/e 379, 351, in the spectra of the acetals, indicate that the homologues with the C_{23} and C_{21} chains are present in phthiocerol A and B in the proportions 57:43 and 41:59, respectively.

The mass spectrum of phthiodiolone A (Fig.15) shows that it has the structure (IX) proposed by previous workers for "phthiodiolone". The high mass range contains peaks due to the loss of water from the molecular ion. Homologous peaks at m/e 425 and 453 presumably represent cleavage α

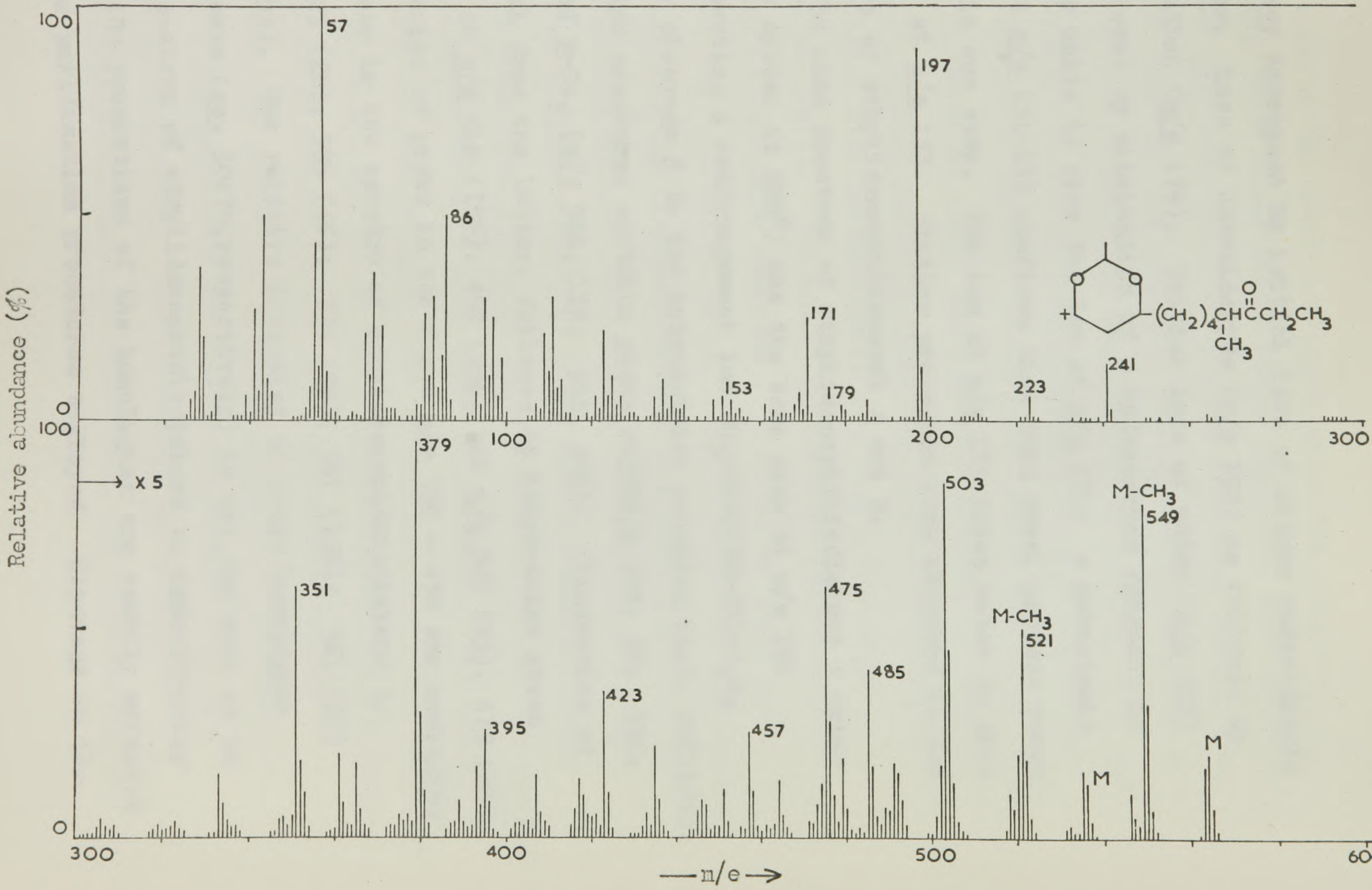
Fig. 15 Phthiodiolone A (Inlet system at 175°)



to the methyl branch (β to the carbonyl group). Dehydration products of these ions are seen in good yield. As in the spectra of phthiocerol A and B, fragments due to cleavage α to the >CH-OH groups and their dehydration products are observed. The base peak at m/e 57 ($CH_3CH_2C^+=O$) and the rearrangement peak at m/e 86 confirm the structure of the terminal portion of the molecule.

The mass spectrum of ethylidenephthiodiolone A (Fig.16) contains some features in addition to those expected by analogy with the ethylidene acetals of phthiocerol A and B. Fragments of m/e 549 and 521 ($M-CH_3$) appear to eliminate a molecule of ethanol (m/e 503 and 475), metastable peaks corresponding to this process being observed at m/e 461 and 433; dehydration of these ions then follows (m/e 485 and 457). This unexpected behaviour is possibly due to the fact that the spectrum was taken at a higher temperature than in the case of phthiocerol A and B acetals. The peaks corresponding to m/e 423 and 395 and their fragmentation products are exactly analogous to those in the spectra of phthiocerol A and B acetals. The ratios of the intensities of these homologous ions indicate that the homologues with the C_{23} and C_{21} chains are present in ethylidenephthiodiolone A in the approximate proportions 60:40. Cleavage on the other side of the acetal grouping gives the fragment m/e 241

Fig. 16 Ethylidenephthiodiolone A (Inlet system at 220°)



which may decompose by initial loss of either acetaldehyde or water. Loss of acetaldehyde (m/e 197) is followed by dehydration (m/e 179). Initial loss of water (m/e 223) is followed by elimination of a hydrocarbon fragment of 52 mass units to give the ion at m/e 171; a metastable peak at m/e 131-132 confirms that this novel process takes place in one step. The ion at m/e 171 loses water to give a peak at m/e 153. Similar processes were observed in the spectra of ethylidenephthiocerol A and B.

The mass spectrum of ethylidenephthiodiolone A oxime (inlet system at 200°) has the base peak at m/e 101 representing a rearrangement ion CH_3-CH=C(NH-OH)-C_2H_5 due to cleavage β to the hydroxyimino grouping (c.f. ref.124). The high mass range contains peaks at M-1 (m/e 578, 2%; 550, 6%) and M-CH_3 (m/e 564, 11%; 536, 30%). Elimination of ethanol from the latter, followed by dehydration gives peaks at m/e 518 (15%), 490 (26%) and m/e 500 (3%), 472 (8%). The series of peaks in the range m/e 330 - 430 are analogous to those in the spectra of ethylidenephthiodiolone A: m/e 423 (2%), 395 (4%); 379 (7%), 351 (16%); 361 (2%) 333 (5%). The relative intensity of these homologous fragments (ca. 30:70, respectively) is not the same as in the spectrum of ethylidenephthiodiolone A, demonstrating that the proportions of the homologues are readily affected by the purification procedures employed. Cleavage on the

other side of the acetal grouping gives the fragment m/e 256 (13%) which loses acetaldehyde (m/e 212, 25%) followed by water (m/e 194, 5%). The process involving initial loss of water from the ion m/e 256, followed by elimination of a fragment of 52 mass units (as expected by analogy with the behaviour of ethylidenephthiodiolone (A) is not observed to a significant extent. Metastable ions observed in the above spectra and their assignments are shown in Table 4.

The nuclear magnetic resonance (n.mr.) spectrum of phthiocerol A is in accordance with the established structure (VIII). The n.m.r. spectrum of phthiocerol B differs in two major respects. It shows an additional doublet due to a methyl group, and the proton at the carbon atom bearing the methoxyl group is a quintet, overlapping the methoxyl singlet, as compared with a quartet in the spectrum of phthiocerol.A. These results are in harmony with the terminal structure $CH_3CH(OMe)CH(CH_3)$ - for phthiocerol B, suggested above on mass spectral evidence. The n.m.r. spectrum of phthiodiolone A is in agreement with the structure IX. The n.m.r.spectra of the ethylidene acetals of the above diols are discussed in the following section (IIc).

The low resolution infra-red spectra of phthiocerol A and B and phthiodiolone A are in agreement with the proposed structures. The spectra of the corresponding ethylidene acetals contain bands which are characteristic

Table 4.

Metastable (m/e) Found	Calc.	Phthiodiolone A Transition (m/e)
180-181	180.5	215 ⟶ 197
163	162.7	197 ⟶ 179
137	136.9	171 ⟶ 153
		Ethylidenephthiodiolone A
467-468	467.6	503 ⟶ 485
461	460.9	549 ⟶ 503
440	439.6	475 ⟶ 457
433	433.1	521 ⟶ 475
344	344.0	379 ⟶ 361
339-340	339.5	423 ⟶ 379
316	315.9	351 ⟶ 333
312	311.9	398 ⟶ 351
206	206.4	241 ⟶ 223
162-163	162.7	197 ⟶ 179
161	161.1	241 ⟶ 197
137	136.9	171 ⟶ 153
131-132	131.1	223 ⟶ 171

of 2-methyl-1,3-dioxane derivatives, especially the strong band at 1410 cm.$^{-1}$ (c.f. ref.125).

c) Stereochemical investigations

The structures (VIII and LII) proposed for phthiocerol A and B contain four asymmetric centres and that (IX) for phthiodiolone contains three similar centres, the carbonyl group replacing the centre at C-3 present in phthiocerol A. For convenience the β-diol system and the asymmetric carbon atoms in the terminal portion of the molecule may be considered in turn since they are separated by a chain of four $-CH_2-$ groups.

In order to establish the relative stereochemistry (threo or erythro) of the β-diol system, threo- and erythro-decan-4,6-diol have been recently prepared as models (work in conjunction with K. Maskens[126]). High-resolution infra-red studies of these diols showed that the degree of intra-molecular hydrogen bonding was greater (ratio of free to bonded hydroxyl absorption 0.70) in the case of the erythro-compound than for the threo-compound (ratio 1.16). The high-resolution spectra of phthiocerol A and B were determined (see Experimental, Section II and ref. 126 and 127) and the corresponding ratios (1.19 in both cases) point to a threo configuration of the diol

system present in these compounds. A similar result (ratio 1.09) is found in the case of phthiodiolone A (see Experimental, Section II). The difference in wave numbers (Δ = 90cm.$^{-1}$) between the absorptions due to bonded and free hydroxyl is indicative of a β-glycol system (c.f. ref.128).

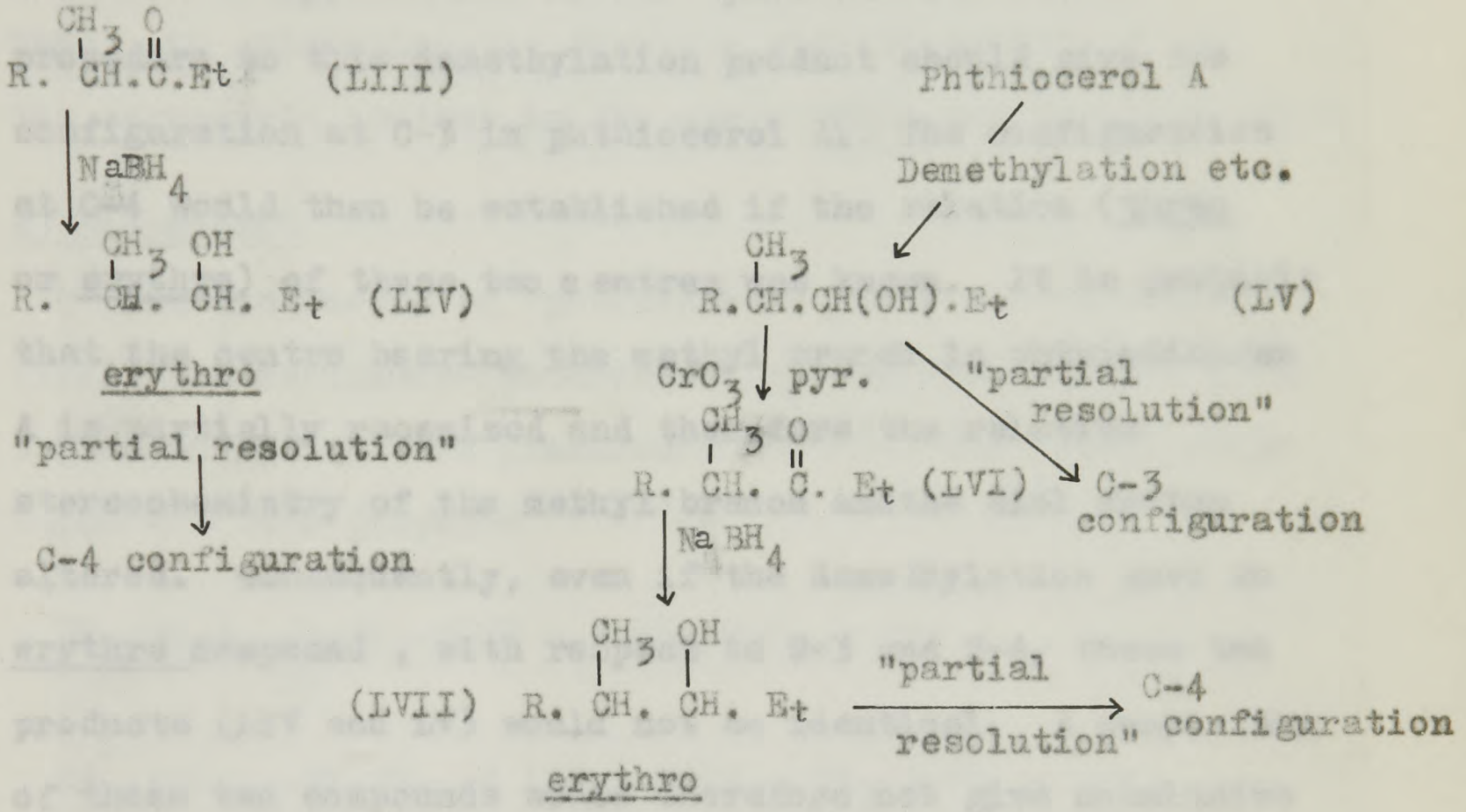
Confirmation of these assignments was provided by a study of the n.m.r. spectra of the corresponding ethylidene acetals. The ethylidene proton signal for the acetal of threo-decan-4,6-diol (τ 5.15) appeared at lower field than that for the erythro-isomer (τ 5.43); a similar difference had been reported earlier for the benzylidene derivatives of the isomeric pentan-2,4-diols[129]. The n.m.r. spectra of the acetals of phthiocerol A and B and phthiodiolone A (see Experimental, Section II and ref.127) contain signals due to the ethylidene proton at τ 5.21 which support the assignment of the threo-configuration to the diol system of the above compounds. A comparison of other features of the n.m.r. spectra was in agreement with these results.

The relative stereochemistry of the methyl branch and methoxyl group in phthiocerol A and B is uncertain. Demarteau-Ginsburg[62] (as mentioned earlier) reduced

'phthiodiolone' with sodium borohydride to a triol, m.p. 74°, which was thought to be identical with the triol, m.p. 75°, obtained by demethylation of 'phthiocerol'. Cram's rule[130] would predict that the reduction of the keto-group would give a hydroxy-group erythro with respect to the methyl branch. The demethylation (acetic anhydride/ toluene-p-sulphonic acid) involves an inversion at C-3 and therefore the methoxyl group and methyl branch in "phthiocerol" were suggested to have threo configuration with respect to one another[62].

The absolute stereo-chemistry of any of these centres is unknown. The "partial resolution" method of Horeau[131] offers a direct and interesting approach. This involves preferential esterification of a secondary alcohol with one of the antipodes of racemic α-phenylbutyric acid. The method depends on the stereochemical hindrance encountered at the asymmetric centre involved. Models show that in an erythro - β - diol system, flanked by similar groups, each centre should cancelthe effect of the other. However, for a threo-diol, as in the case of phthiocerol A, B and phthiodiolone A, a similar effect should be caused by each centre, thus indicating the absolute configuration of these centres.

Sodium borohydride reduction of phthiodiolone A acetal (LIII, R = the homologous portion of the molecule,



including the acetal grouping) would give a hydroxy-group erythro to the methyl branch (LIV) (see also above). Application of the 'partial resolution' procedure should consequently enable to absolute configuration of the centre at C-4 in phthiodiolone A to be determined, assuming that this centre has not been completely racemized by the alkaline hydrolysis of the lipid extracts. Demethylation of phthiocerol A and formation of a cyclic derivative of

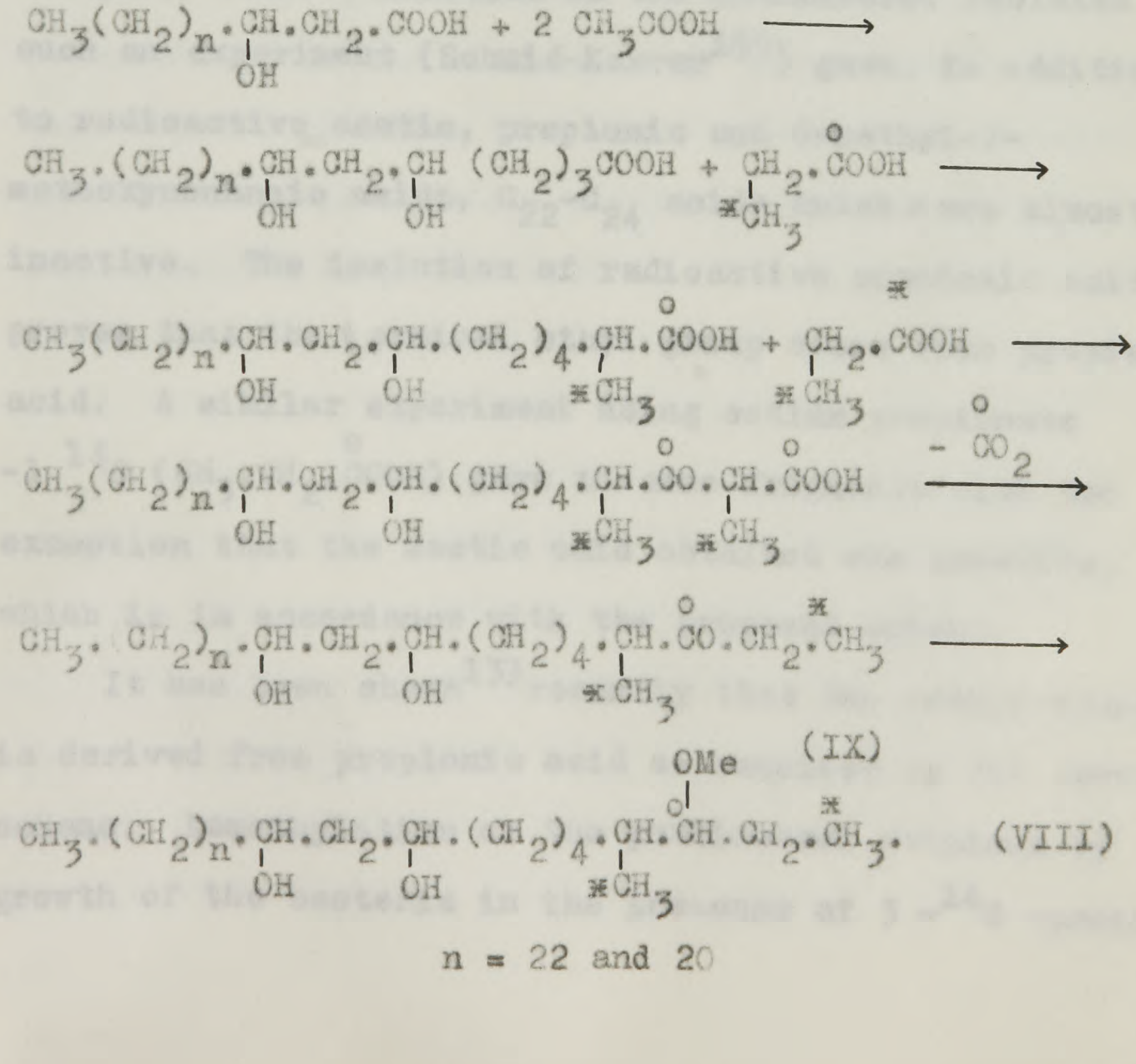
the β- diol system would give a compound (LV) possibly identical with that (LIV) obtained from phthiodiolone A acetal. An application of the 'partial resolution' procedure to this demethylation product should give the configuration at C-3 in phthiocerol A1. The configuration at C-4 would then be established if the relation (threo or erythro) of these two c entres was known. It is probable that the centre bearing the methyl branch in phthiodiolone A is partially racemized and therefore the relative stereochemistry of the methyl branch andthe diol system altered. Consequently, even if the demethylation gave an erythro compound , with respect to C-3 and C-4, these two products (LIV and LV) would not be identical. A comparison of these two compounds would therefore not give conclusive proof of the relative configuration at C-3 and C-4 in phthiocerol.

An alternative approach would involve mild, non-acidic oxidation (e.g. chromium trioxide in pyridine) of the demethylation product (LV) to give the ketone (LVI) without complete racemization of the C-4 centre. Sodium borohydride reduction of this ketone would give an erythro-compound (LVII) and the 'partial resolution' procedure on this alcohol should give the absolute configuration at C-4.

This approach could also be applied to phthiocerol B without requiring the corresponding ketone (phthiodiolone B) which has not yet been found in crude phthiocerol. Thus , in principle, the absolute stereochemistry of all the asymmetric centres in the above diols may be established.

d) Biogenesis

The biogenesis of "phthiocerol" [i.e. the structure (VIII) now designated phthiocerol A] has been studied using synthetic radioactive precursors[132,133]. The scheme shown below has been proposed[132,134]:-



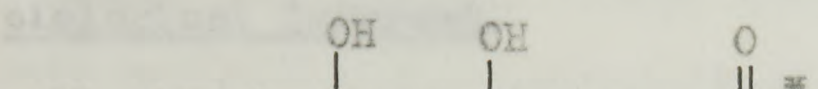
n = 22 and 20

Growth[132] of the $H_{37}Ra$ strain of M. tuberculosis inthe presence of L-methionine methyl - ^{14}C and demethoxylation of the phthiocerol isdated (Zeisel) removed all of the radioactivity. This result showed that methionine is a precursor of the methoxyl group, but that the methyl side-chain does not originate from methionine.

Growth[132] in the presence of sodium propionate -3 ^{14}C ($^{*}CH_3.CH_2.COOH$) would be expected, according to the above scheme, to lead to labelling at C-1 and also the methyl branch. Oxidation of the phthiocerol isolated from such an experiment (Schmid-Karrer[135]) gave, in addition to radioactive acetic, propionic and 6-methyl-7-methoxynonanoic acids, C_{22}-C_{24} acids which were almost inactive. The isolation of radioactive propionic acid proves that the terminal ethyl group comes from propionic acid. A similar experiment using sodium propionate -1 ^{14}C ($CH_3.CH_2.^{\circ}COOH$) gave the same fragments with the exception that the acetic acid obtained was inactive, which is in accordance with the proposed scheme.

It has been shown[133] recently that the methyl branch is derived from propionic acid as required by the above scheme. Demethylation of the phthiocerol obtained by growth of the bacteria in the presence of 3 -^{14}C -propionate,

followed by dehydration and ozonolysis of the product gave a methyl ketone (LVIII). Mass spectrometry of this



$$CH_3.(CH_2)_n.CH(OH).CH_2CH(OH).(CH_2)_4.C(=O).CH_3. \qquad \text{(LVIII)}$$

n = 22 and 20

ketone and of the original phthiocerol showed that a radioactive carbon atom was contained in the methyl branch.

The biosynthesis of phthiocerol B may be explained by a modification of the above scheme involving the final incorporation of acetate rather than propionate. A ketone (IX) (phthiodiolone A) is an essential intermediate in the biogenesis of phthiocerol A ; it thus appears probable that crude phthiocerol contains small amounts of a ketone having a terminal portion corresponding to phthiocerol B.

Section III - Methyl-branched long-chain acids of biological interest.

a) Previous researches.

As mentioned in the Introduction, injection of the lipids of human tubercle bacilli into test animals produced tubercular lesions[4,5,136]. Further studies of the lipids by Anderson and his co-workers showed that this type of activity was geatest in the dextrorotatory "phthioic acid" fraction[137]. The nature of these dextrorotatory acids was uncertain until various methyl-branched long-chain acids were synthesized by Polgar and his co-workers[15,16,17] and their properties, including their biological activity[138] (see later), found to be similar to those of the naturally occuring acids. "Phthioic acid" was later shown (as stated in the Introduction) to consist of methyl-branched long-chain acids some of which were $\alpha\beta$-unsaturated. The biological activity of these long-chain compounds was therefore thought to be associated with the presence of methyl branches.

The simplest methyl-branched acid occuring in bacterial lipids is tuberculostearic acid[10] (10 - methyloctadecanoic acid) and this does not form tubercles when injected into animals[4]. However, synthetic 10-methyltetracosanoic acid was stated to cause

intraperitoneal lesions closely related to tubercles.[139] Husseini and Elberg[140] found that the tissue response to pure synthetic mono-methyl-branched acids depends on the position of the methyl group ; the irritating power was increasingly marked as the methyl group was moved from C - 2 to C - 10. The response to such acids therefore appears to depend on the chain length and the position of the side-chain with respect to the carboxyl group.

The importance of testing acids at the same time and under comparable conditions must be stressed ; for this reason it is difficult to compare the results of different groups of workers. Ungar et al.[18,138] (Glaxo Laboratories Ltd., Greenford) tested many of the synthetic analogues of "phthioic acid" prepared by Polgar and his collaborators [15,16,17] and in a later series of experiments[18] investigated further acids[141] including purified samples of the dextrorotatory (mycolipenic) and laevorotatory (myoceranic) acids[19], prepared by the same workers. The results of these studies are shown in Table 5 and 6; since the tests were carried out using the same procedure, the results are comparable, but further studies would be desirable to confirm them.

Table 5. Acids tested in 1948[138,18].	Minimum dose (mg). for activity.
3,12,15 - trimethyldocosanoic	25
2,12,15 - trimethyldocosanoic	50
3,13,16 - trimethyldocosanoic	50
4,13,16 - trimethyltricosanoic	50
3,13 - dimethyltricosanoic	50
3,12,15 - trimethyldocos-12-enoic	100
3,13, 19 - trimethyltricosanoic	100
3,13,19 - trimethyltricos-13,19-dienoic	100
Crude d-rotatory acid from t.b. bacilli	100
2,2 - dimethyloctadecanoic	inactive at 100 mg.
3-methyltricosanoic	
13,16 - dimethyltricosanoic	
13,17,21 - trimethyldocosanoic	inactive at 200 mg.
2,13,17,21 - tetramethyl docosanoic	
2,13 - dimethylpentacosanoic	

Table 6. Acids tested between 1948 & 1955[18]	Minimum dose (mg.)
Purified l-rotatory acid from t.b. bacilli	5 to 10
2,4 - dimethyldocosanoic	10
Purified d-rotatory acid from t.b. bacilli	20
2-methyloctadec-2-enoic	25
2,4,6,-trimethyltetracos-2-enoic	50
2,4 - dimethyldocos-2-enoic	100
2,4 - dimethyleicos-2-enoic	100

Ungar[18] regarded the lesions produced by synthetic fatty acids as 'foreign-body reaction' and he stated that any resemblance to a specific granuloma at certain stages of development should be interpreted accordingly. Also with regard to the natural dextrorotatory acids the lesions produced closely resemble those seen in tuberculosis but the similarity is associated with the non-specific aspects of tuberculosis pathology. The naturally occuring acids tested have been shown to contain many components which are stereochemically homogeneous, but the synthetic acids are mixtures of stereoisomers ; therefore, no direct comparison can be drawn between these two groups of compounds. From the results of Ungar et al.[18] some conclusions can be drawn regarding the structural features necessary for biological activity of the synthetic acids. It is not established that 2,4-dimethyldocosanoic acid (Table 6) is more active than 3,12,15-trimethyldocosanoic acid (Table 5) since these acids were tested independently, but the exceptional activity of these compounds points to the necessity of having at least one methyl group near the carboxyl ; moreover it appears that substitution at C-2 is inferior to C-3 and C-4 (see Table 5). The high activity of 2-methyloctadec-2-enoic acid shows that an acid with only one branch can be very active. 3-Methyltricosanoic

acid, however, is relatively inactive, but introduction of additional methyl groups further along the chain gives rise to activity. The activity is increased if the two methyl groups remote from the carboxyl are brought closer together in the centre of the chain. The presence of unsaturation in the centre of the chain does not necessarily have a significant effect. An $\alpha\beta$-double bond appears to reduce the activity (e.g. 2,4 -dimethyldocosanoic and 2,4 -dimethyldocos-2-enoic acids). However, independent work[140] has shown that in rabbits the cellular response is greater for the $\alpha\beta$-unsaturated acid (2,4,6-trimethyl-tetracos-2-enoic acid) than for the corresponding saturated acid.

The acids having the greatest activity correspond to two main types, the 2,4,- or 2,4,6 - substituted type, corresponding to the naturally occuring mycolipenic and mycoceranic acids (see Introduction) and the 3- or 4-substituted acids with one or more methyl groups in the centre portion of the chain (e.g. 3,12,15-trimethyldocosanoic acid). No acids which combine these features (e.g. 2,4,12,15-substituted) have been tested. In order to investigate further the structural features necessary for activity the following general lines of research

might be followed:-

1) Examination of the effect of chain length.

2) Determination of the optimum number and spacing of methyl groups for an acid of given chain length.

3) A preliminary investigation of the effect of stereochemistry by preparation of threo and erythro pairs of compounds, in the cases where methyl groups are close together.

4) Preparation of optically pure isomers incorporating the best features from the preceding studies.

Renewed interest in the properties of 3,12,15-trimethyldocosanoic acid has arisen in connection with studies (by Dr. W. Hartston, in conjunction with Glaxo Laboratories) on sarcoidosis.[142] This is a fairly benign condition which manifests itself in various forms. It occurs mainly in the lung (but also other organs), where it presents symptoms closely related to tuberculosis, and is often encountered as a skin disease. The condition often disappears spontaneously and no micro-organism is associated with it. Diverse agents such as metals (e.g. Be and Zr) and talcum powder have been shown to trigger off the disease; a lipid extract from pine pollen has been

shown to cause an identical type of granuloma. The tubercle bacillus has often been linked with sarcoidosis but no definite connection has ever been established[142].

The classical method of detecting sarcoidosis is the Kveim test[142]; intradermal injection of a suspension of sarcoid lymph node or spleen results in the appearance of a nodule with the histological pattern of sarcoid. Recent work by Dr. Hartston (private communication) has shown that 3,12,15-trimethyldocosanoic acid injected intradermally in very small quantities into patients suffering from sarcoidosis gives a reaction which is histologically indistinguishable from that given by the Kveim antigen. Other samples remaining from the previous tests (i.e. those in Tables 5 and 6) were also tested but gave no specific reaction. Some of the methyl-branched compounds, prepared more recently by Polgar and Smith[28], were screened for activity in guinea pigs, as before, and the most active of these, methyl 2,4,6,8 - tetramethyloctacosanoate, again gave no specific reaction in sarcoid patients. Thus 3,12,15 - trimethyldocosanoic acid appeared to be unique as a synthetic alternative for the Kveim antigen. In order to provide material for the continuation of these tests a new synthesis of 3,12,15 - trimethyldocosanoic acid was carried out.

The original synthesis of 3,12,15 - trimethyl-docosanoic acid (LIX, R = H) by Polgar and his co-workers[17]

$$CH_3.(CH_2)_6.\underset{\underset{CH_3}{|}}{CH}.(CH_2)_2.\underset{\underset{CH_3}{|}}{CH}.(CH_2)_8.\underset{\underset{CH_3}{|}}{CH}.CH_2.COOR \qquad (LIX)$$

was as follows. Reaction of undec-11-enoyl chloride with methylzinc iodide gave 2-oxododec-11-ene (LX).

3-Methyldecanoic acid (LXI) was prepared from nonan-2-ol

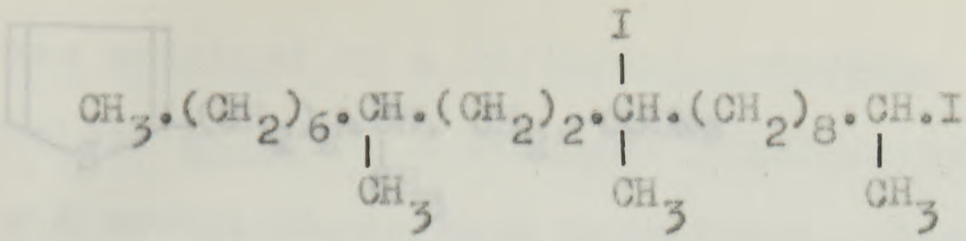
$$CH_3.CO.(CH_2)_8.CH = CH_2 \qquad (LX)$$

$$CH_3.(CH_2)_6.\underset{\underset{CH_3}{|}}{CH}.CH_2.COOH \qquad (LXI)$$

(obtained by a Grignard reaction between 1- bromoheptane and acetaldehyde) by conversion to the iodide and reaction of the latter with diethyl sodiomalonate, followed by hydrolysis and decarboxylation. Reduction (sodium in butanol) of the butyl ester of this acid gave 3 - methyldecan-1-ol. A Grignard reaction of the corresponding bromide (LXII) with the above ketone (LX) afforded the carbinol (LXIII) which on dehydration gave a mixture of 11,14-dimethylheneicos - 1,11 and -1, 10 - dienes.

$$CH_3.(CH_2)_6.\underset{\underset{CH_3}{|}}{CH}.(CH_2)_2.Br \qquad (LXII)$$

$$CH_3.(CH_2)_6.\underset{\underset{CH_3}{|}}{CH}.(CH_2)_2.\overset{\overset{OH}{|}}{\underset{\underset{CH_3}{|}}{C}}.(CH_2)_8.CH = CH_2 \qquad (LXIII)$$



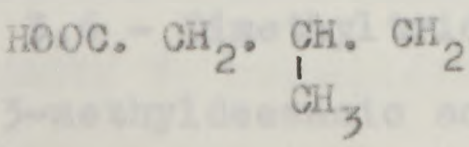
(LXIV)

Addition of hydrogen iodide gave the di-iodide (LXIV) which on treatment with diethyl sodiomalonate resulted in reformation of the double bond in positions adjacent to C-11 and substitution of the iodine atom at C-2. Catalytic hydrogenation of this compound and hydrolysis followed by decarboxylation gave 3,12,15-trimethyldocosanoic acid (LIX, R = H).

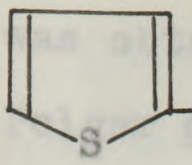
The above synthesis of 3,12,15-trimethyldocosanoic acid had the disadvantage that it involves two malonic ester condensations with secondary iodo-compounds; doubts, probably unjustifiable, have been expressed regarding the purity of compounds prepared by such methods. Also the addition of hydrogen iodide could possibly lead to isomeric iodo-derivatives.

b) Present investigations

A convenient route to 3-methyl-substituted long-chain acids starts with methyl hydrogen β-methylglutarate (LXV) which is readily available[143,144,145]. The desired



(LXV)

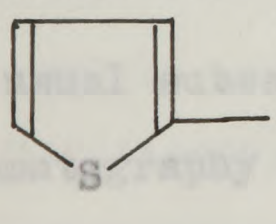


$-(CH_2)_2.CH(CH_3).CH_2.COOMe$ (LXVI)

$CH_3.(CH_2)_6.CH(CH_3).CH_2.CH_2.CH(CH_3).CH_2.COOH$ (LXVII)

hydrocarbon chain may be added to the side carrying the free carboxyl group through use of one of the standard chain-lengthening methods[67,146,147]. The use of thiophen as a chain extender[28,148,149] in conjunction with the above half-ester (LXV) would lead to the intermediate (LXVI). A synthesis of 3,12,15 - trimethyldocosanoic acid (LIX, R = H) using this compound would then require the preparation of 3,6 -dimethyltridecanoic acid (LXVII) as the other intermediate.

Methyl hydrogen β- methylglutarate (LXV) was converted by a Friedel-Crafts reaction of the corresponding acid chloride and thiophen into the thienyl keto-ester (LXVIII) which by Huang-Minlon reduction and subsequent esterification afforded the thienyl ester (LXVI).



$C.CH_2.CH(CH_3).CH_2.COOMe$ (LXVIII)

3,6 - Dimethyltridecanoic acid (LXVII) was prepared *via* 3-methyldecanoic acid (LXI) as an intermediate. The latter

was obtained by a different procedure to that used by Polgar et al.[17] A Grignard reaction between 1-bromoheptane and acetaldehyde gave nonan-2-ol, as before, but this was oxidized to nonan-2-one. This latter compound was condensed with ethyl cyanoacetate[150] to give ethyl 2-cyano-3-methyldec-2-enoate (LXIX), which after hydrogenation,

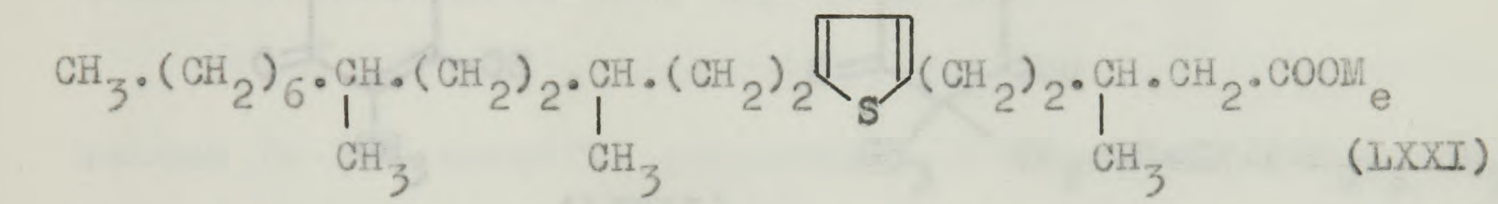
$$CH_3.(CH_2)_6.\underset{\underset{CH_3}{|}}{C} = \underset{\underset{CN}{|}}{C}.COO\,Et \qquad (LXIX)$$

hydrolysis and decarboxylation, followed by hydrolysis of the resulting nitrile gave crude 3-methyldecanoic acid (LXI). On lithium aluminium hydride reduction of the ester of this acid, crude 3-methyldecan-1-ol was obtained; the latter was converted with aqueous hydrogen bromide into 1-bromo-3-methyldecane (LXII). A Grignard reaction between this compound and acetaldehyde, oxidation of the product, followed by a cyanoacetic ester condensation (as above) gave the cyano ester (LXX).

$$CH_3.(CH_2)_6.\underset{\underset{CH_3}{|}}{CH}.(CH_2)_2\underset{\underset{CH_3}{|}}{C} = \underset{\underset{CN}{|}}{C}.COOEt \qquad (LXX)$$

The usual subsequent stages (see above)followed by chromatography afforded 3,6 - dimethyltridecanoic acid (LXVII) and also a sample of the corresponding amide due presumably to incomplete hydrolysis of the intermediate nitrile.

A Friedel-Crafts reaction of the acid chloride of 3,6,-dimethyltridecanoic acid with the thienyl ester (LXVI), and Huang-Minlon reduction ofthe resulting thienyl keto-ester followed by esterification of the acid so obtained, gave the crude ester (LXXI).

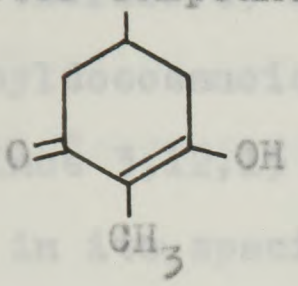


(LXXI)

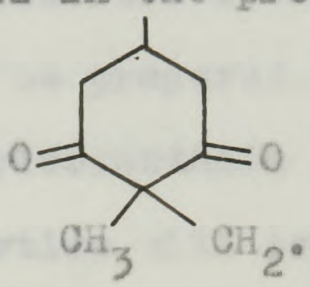
Desulphurisation of this compound with Raney nickel gave methyl 3,12,15-trimethyldocosanoate (LIX, R = Me). A portion of this ester was hydrolysed to the acid (LIX, R=H) and submitted for biological testing (Glaxo Laboratories Ltd.); the results of these tests have not been obtained at the time of writing.

The synthesis of 3,12,15-trimethyldocosanoic acid carried out by Polgar et al.[17] as described above is not easily adaptable for the preparation of pure stereoisomers of this acid. Methyl hydrogen β-methylglutarate can be resolved conveniently[144]; thus the synthesis carried out in the present work can be modified to obtain 3(D),12,15--trimethyl - or 3(L), 12,15-trimethyldocosanoic acid. The 3,6-dimethyltridecanoic acid necessary for this scheme has been synthesized more recently in this laboratory[151] by a more convenient route. This involved the successive

alkylation of 5-methylcyclohexane -1,3 - dione[152] with methyl iodide[153] and 1-bromohept-2-ene[154] to give the diones (LXXII) and (LXXIII), respectively. Treatment of the latter compound withalkali in the presence of hydrazine



(LXXII)



(LXXIII)

resulted in simultaneous ring opening and reduction[153] of the keto-group (of the resulting intermediate keto-acid), leading to the unsaturated acid (LXXIV), which on hydrogenation

$$CH_3.(CH_2)_3.CH=CH.CH_2.\underset{\displaystyle CH_3}{\underset{|}{CH}}.(CH_2)_2.\underset{\displaystyle CH_3}{\underset{|}{CH}}.CH_2.COOH \qquad \text{(LXXIV)}$$

gave 3,6-dimethyltridecanoic acid (LXVII).

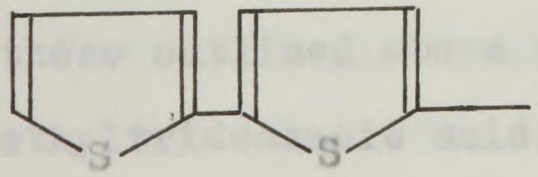
A synthesis of optically pure isomers of 3,12,15-trimethyldocosanoic acid could be effected by using methyl hydrogen β - methylglutarate as the source of all the asymmetric carbon atoms. Anodic coupling of the D - or L - form of methyl hydrogen β- methylglutarate with heptanoic acid would give the corresponding diastereoisomers of 3-methyldecanoic acid (LXI). Further electrolysis of these diastereoisomers with methyl hydrogen β-methylglutarate (D - or L - form) would lead to the individual stereoisomers of 3,6 - dimethyltridecanoic acid (LXVII), depending on the

pairs chosen. This acid could then be combined with the D - or L - form of the thiophen intermediate (LXVI) prepared from methyl hydrogen β-methylglutarate. By this route, therefore, all eight possible isomers of 3,12,15 - trimethyldocosanoic acid may be prepared.

Since 3,12,15 - trimethyldocosanoic acid appears to be unique in its specific properties with regard to sarcoidosis, it was decided to prepare closely analogous compounds for comparative biological studies. The effect of removal of either of the methyl groups at C-12 or C-15 would be interesting and thus methyl 3,15-dimethyldocosanoate (LXXV) was prepared by the following procedure.

$$CH_3\cdot(CH_2)_6\cdot\underset{\displaystyle CH_3}{\overset{|}{CH}}\cdot(CH_2)_{11}\cdot\underset{\displaystyle CH_3}{\overset{|}{CH}}\cdot CH_2\cdot COOMe \qquad \text{(LXXV)}$$

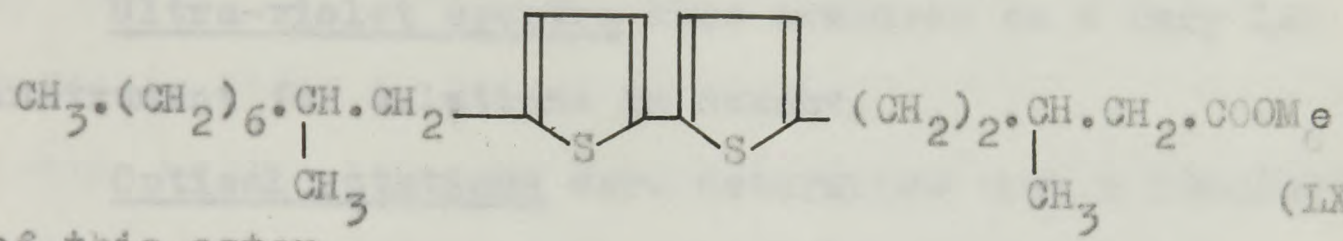
2-Iodothiophen was subjected to an Ullman reaction to give 2,2′-bithienyl.[155] Friedel-Crafts reaction of the latter with the acid chloride of methyl hydrogen β-methylglutarate (LXV), followed by Huang-Minlon reduction and esterification of the product, gave the bithienyl intermediate (LXXVI).



$$(CH_2)_2\cdot\underset{\displaystyle CH_3}{\overset{|}{CH}}\cdot CH_2\cdot COOMe \qquad \text{(LXXVI)}$$

2-Methylnonoic acid was prepared by condensing 1-bromoheptane

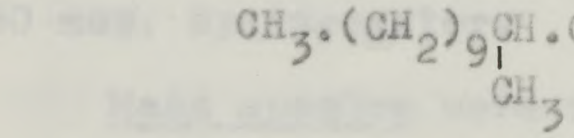
with diethyl methylmalonate, followed by hydrolysis and decarboxylation. A Friedel-Crafts reaction, of the acid chloride derived from this acid, with the above bithienyl-ester (LXXVI), Huang-Minlon reduction and subsequent esterification gave the ester (LXXVII). Methyl 3,15 - dimethyldocosanoate (LXXV) was obtained by desulphurisation



(LXXVII)

of this ester.

Recent work in this laboratory[151] has led to the synthesis of methyl 3-methyltridecanoate (LXXVIII) which,



(LXXVIII)

in conjunction with the thienyl-ester (LXVI) is an intermediate for the preparation of methyl 3,12-dimethyl-docosanoate (required for comparison of its biological properties with those of the above acids). This intermediate (LXXVIII) was prepared by alkylation of 5-methylcyclohexane-1,3-dione with 1-bromohept-2-ene, followed by similar stages to those outlined above for the preparation of 3,6,-dimethyltridecanoic acid.

EXPERIMENTAL

General notes on techniques employed

High-resolution infra-red spectra were measured (by A.P.Sherman and K.D.Bingham) on a Perkin-Elmer 521 spectrophotometer ; other infra-red spectra refer to chloroform solutions, determined on a Perkin-Elmer 237 instrument.

Ultra-violet spectra were measured on a Cary 14M instrument for solutions in hexane.

Optical rotations were determined with a Perkin-Elmer 141 polarimeter for chloroform solutions.

Nuclear magnetic resonance (n.m.r.)spectra were determined (by Mrs. E.E. Richards) using a Perkin-Elmer 60 mcs. spectrometer.

Mass spectra were recorded(by Dr. R.T. Aplin) on an A.E.I. M.S. 9 spectrometer.

Melting points were determined on a Kofler block.

Microanalyses were carried out by Drs. Weiler and Strauss.

Refractive indices were determined using an Abbé refractometer.

Gas-liquid chromatography (GLC) was performed on a Pye Argon Chromatograph fitted with a column (4 feet long, internal diameter 5 mm.) packed with 15% silicone oil (M and B, Embaphase) on Embacel (gas pressure at

the head of the column 10 p.s.i.).

Thin-layer (TLC) and unidimensional multiple chromatography[81] (UMC) were carried out on layers (0.25mm.) of Kieselgel G, unless otherwise stated, and the spots were detected by spraying with a saturated solution of sodium bichromate in concentrated sulphuric acid, followed by heating at 100-150°. The letters w, m, and s in brackets refer to the relative intensities of the spots (weak, medium and strong, respectively). Ketones were detected by spraying with a 0.5% solution of 2,4-dinitrophenylhydrazine in 2N hydrochloric acid[156]. Esters were detected by spraying with a solution containing equal volumes of 12½% sodium hydroxide and 5% hydroxylamine in water, standing for 10 min., and then spraying with glacial acetic acid followed by 10% aqueous ferric chloride[157].

Preparative thin-layer chromatography (PLC) was carried out on layers (1 mm.) of Kieselgel $HF_{254 + 366}$ (prepared by R.J.F. Prior) ; illumination with light of wavelength 366 mμ. showed the position of the separated components. The bands were removed and the required material eluted with ether.

Adsorbents used for column chromatography and their treatment prior to use were as follows :-

Whatman Chromedia S.G.31 silica gel was activated by heating at 110 - 120° for 12 hr. and deactivated reproducibly by addition of fixed amounts (% by weight) of distilled water (0 - 15%).

Camag and Woelm neutral aluminas (activity I) were deactivated by addition of water and standardized by the method of Hermanek et al.[158]

Spence Type H alkaline (pH 10) alumina was acid washed (according to the manufacturers instructions), activated at 300° and deactivated and standardised as above.

In devising eluant systems for efficient chromatographic separations (on plates and columns) attention was paid to previous observations that mixtures of light petroleum with ether, ethyl acetate, or acetone as eluent solvents are markedly superior to the mixtures of light petroleum with benzene often employed[159]. This is in accord with the theoretical considerations by Thoma[81] who showed that the resolving power of solvent mixtures increases as the mole fraction of the minor component of the mixture decreases; in order to elute a given mixture from a given adsorbent benzene as the minor component would have to be added to light petroleum in larger amounts than ether, ethyl acetate, or acetone. Consequently, unless solubility difficulties dictated otherwise, mixtures were adsorbed from petrol onto

chromatographic columns whose activity had been adjusted so that the main components of the mixture could be eluted by mixtures of petrol and a small amount of ether. In the descriptions of chromatographic separations given below, it should be assumed that solvent mixtures of gradually increasing polarity were passed through the column in order to separate the components into the respective zones; however, for the sake of brevity, the only solvent systems recorded are those which actually elute the fractions which are of interest.

Raney nickel for desulphurisation was prepared as described in previous work[28] and was regarded as having activity W-5.

Solutions in organic solvents were dried over anhydrous magnesium sulphate unless otherwise stated.

Petrol refers to light petroleum of b.p. 60-80°.

Solvents and reagents were purified according to standard procedures.

Section I - The mycolic acids from human tubercle bacilli

Chromatography of the crude mycolic acids

The mycolic acids studied in this investigation were those isolated in previous researches on phthiocerol[34]. Thin-layer chromatography (TLC) of this material (petrol/ether/acetic acid, 70/30/2) showed the presence of the mycolic acids (R_F 0.33), small amounts of less polar material and phthiocerol (R_F 0.15).

The crude mycolic acids (78.49 g.) were adsorbed from petrol on activated silica gel (1,500 g.). Petrol (5 l.) and petrol/ether (98/2, 21.; 95/5, 3 l.) eluted a small amount of material (293 mg.) (R_F0.73, petrol/ether, 97/3). Further elution with petrol/ether (95/5, 3 l.; 90/10, 2 l.) gave a yellow wax (4.578 g.), described in detail below. A mixture of the mycolic acids and phthiocerol (68.82 g.) was eluted using the following mixtures in succession; petrol/ether (90/10, 2 l.), petrol/ether/acetic acid (80/20/1, 3 l.; 70/30/1. 1 l.) and ether/acetic acid (100/1, 3 l.; 100/2, 1 l.; 100/5, 24 l.)

The yellow wax, obtained above, was examined by undimensional multiple chromatography (UMC) (petrol/ether, 99/1, 3 passes) which showed the presence of eight components, R_F values 0.13 (s), 0.19 (w), 0.26 (s), 0.31 (w), 0.45 (s) 0.51 (w), 0.60 (m), 0.80 (w). The components having

R_F 0.13, 0.26, 0.60 appeared to be ketones (2, 4, dinitrophenylhydrazine spray reagent) and that having R_F 0.45 to be an ester (hydroxamic acid reagent). Under similar conditions, an authentic sample of methyl mycocerate[27] had the same R_F value (0.45) as this latter material. A small amount (87 mg.) of this wax was hydrolysed with 10% benzene/methanolic potassium hydroxide and UMC of the product showed the removal of the component corresponding to methyl mycocerate, the other components being unchanged.

The methyl esters of the mycolic acids.

The mixture (66.6 g.) of mycolic acids and phthiocerol was dissolved in ether (1 l.) and esterified with an ethereal solution of diazomethane. The solution was allowed to stand for 2 hr. and then dilute hydrochloric acid was added cautiously until no more gas was evolved. The ethereal layer was separated and washed with dilute hydrochloric acid, water and dried. Removal of the ether gave crude methyl mycolate (66.0 g.)

UMC of crude methyl mycolate (petrol/ether 95/5, 4 passes) showed the presence of three mycolic esters in comparable amounts (R_F 0.49, 0.38, 0.26) phthiocerol (R_F 0.0) and traces of less polar material. The three esters are subsequently referred to as methyl mycolates -I, -II and III, respectively ; the ester-III appeared to contain a

keto-function. The products named mycolic esters A and B by Malani and Polgar[33] were investigated by UMC; 'methyl mycolate A' consisted mainly of ester -I and -II and 'methyl mycolate B' was a mixture of all three esters.

Chromatography of the mixture of methyl mycolates.

Crude methyl mycolate (64 g.) was adsorbed from petrol on alumina (Spence, activity II, 1700 g). Petrol 4 l.) and petrol/ether (98/2, 4 l.; 95/5, 4 l.; 90/10 4 l.; 80/20, 3 l.) eluted a pale wax (1.92 g.) described in detail below. Petrol/ether (70/30, 3 l.; 50/50, 4.5 l.) eluted mixtures (MI) (30.91 g.) of methyl mycolate -I and -II. Continued elution with petrol/ether (50/50; 30/70, 1 l.), ether (8 l.) and ether/acetic acid (100/5, 6 l.) gave mixtures (M 2)(17.06 g.) of all three mycolic esters. Ether/acetic acid (100/5, 6 l.) eluted mixtures (8.99 g.) of mycolic acids and phthiocerol. This hydrolysed material was re-esterified with diazomethane as described earlier. The product (8.98 g.) was shown to be a mixture (M3) of all three mycolic esters and phthiocerol.

The pale wax obtained above was shown by UMC (petrol/ether, 99/1, 3 passes) to contain four components [R_F 0.21(m), 0.30 (s), 0.45(s), 0.71(w)] which appeared to be esters,

that having R_F 0.45 corresponding to methyl mycoceranate[27]. On saponification (10% benzene/methanolic potassium hydroxide) of a portion (75 mg.) of this material these spots corresponding to esters disappeared leaving three components (R_F 0.13, 0.26, 0.60) corresponding to the ketones isolated from the chromatography of the crude mycolic acids.

Removal of methyl mycolate -III as its oxime.

The combined mixtures (M2 and M3, 26.0 g.) from the above chromatography, consisting of the three mycolic esters and phthiocerol, were dissolved in benzene (600 ml.) and heated under reflux for 40 hr. with a solution of hydroxylamine hydrochloride (10 g.) and sodium acetate (15 g.) in ethanol (1.5 l.). The mixture was cooled and poured into dilute hydrochloric acid, extracted with ether, the extract washed with water and dried. Removal of the ether gave a pale yellow wax (24.93 g.). UMC (petrol/ether, 95/5, 4 passes) of this material showed unchanged mycolic ester -I (R_F0.49) and -II (R_F0.38), phthiocerol (R_F0.0) and the oxime of methyl mycolate -III (R_F0.10).

The above mixture (24.9g.) was chromatographed in petrol on activated silica gel (800 g.). Elution with petrol/ether (90/10, 2 l.; 80/20, 1.5 l.) gave a mixture (M4) (10.11 g.) of methyl mycolate -I and -II. Continued

elution with petrol/ether (80/20 1 l.) gave a mixture (M5) (542 mg.) of methyl mycolate -I and -II and the oxime of methyl mycolate -III. Petrol/ether 70/30, 1 l.; 50/50, 1 l.) and ether 2 l.) eluted the oxime of methyl mycolate -III (0 l) (8.59 g.). Further elution with ether (5.5 l.) and ether/acetic acid (100/5, 7 l.) gave mixtures (M6) (4.12 g.) of the oxime of methyl mycolate -III and phthiocerol.

Chromatography of a mixture of methyl mycolate -I and -II.

A portion (5.65 g.) of the mixture (M4) of methyl mycolate -I and -II was adsorbed from petrol on alumina (Woelm, activity II, 300 g.). Petrol/ether (90/10, 600 ml.) eluted fractions (M7) (551 mg.) consisting of methyl mycolate -I contaminated with a small amount of methyl mycolate -II. Further elution with this solvent (1.2 l.) and with petrol/ether (85/15, 1.2 l.; 80/20, 400 ml.) gave mixtures (M8) (3.45 g.) of ester -I and -II. Continued elution with petrol/ether (80/20, 1.2 l.) gave a sample (M9) (582 mg.) of methyl mycolate -II contaminated with a trace of methyl mycolate -I.

The infra-red spectrum ($\nu_{max.}$ 1090 cm^{-1}) and n.m.r. spectrum (τ 6.67) of this sample (M9) of methyl mycolate -II showed the presence of a methoxyl group. These signals were absent in the spectra of methyl mycolate -I (M7).

Acetylation of a mixture of methyl mycolate -I and -II.

A portion (4.44 g.) of the mixture (M4) of methyl mycolate -I and -II was dissolved in pyridine (50 ml.) and acetic anhydride (20 ml.) was added. The solution was stirred at 65° for 18 hr., cooled and poured into dilute hydrochloric acid. The mixture was extracted with ether, the extract washed with water and dried. Removal of the ether gave a white solid (4.94 g.) which on UMC (petrol/ether, 95/5, 3 passes) showed the presence of methyl acetylmycolate -I (R_F0.49) and methyl/acetyl mycolate -II (R_F0.37).

A portion (4.8 g.) of the crude acetoxy-esters -I and -II was adsorbed from petrol on activated silica gel (350g.) Elution with petrol/ether mixtures gave practically no resolution of the two components of the mixture. The material from the above chromatography was combined (4.2g.) and adsorbed from petrol on alumina (Woelm, activity II/III, 270 g.). Petrol/ether (98/2, 200 ml.) eluted a sample (M.10) (1.27 g.) of methyl acetylmycolate -I. Further elution with this solvent (400 ml.) gave a mixture (M.11) (1.66 g.) of acetoxy-ester-I and -II. Continued elution with petrol/ether (98/2, 1 l.; 97/3, 400 ml.) gave a sample (M12) (1.13g.) of methyl acetylmycolate -II.

Large scale chromatography of mixtures of methyl acetylmycolate -I and -II.

A mixture (MI, M4) of methylmycolate -I and -II was acetylated as described previously. The hydroxy-esters (35.1g.) with pyridine (200 ml.) and acetic anhydride (100ml.) gade the crude acetoxy-esters (37.3g.)

This material was adsorbed from petrol on alumina (Camag, activity II/III, 1 kg.). Petrol 5 l.) eluted fractions (M 13) (13.64g.) consisting of methyl acetyl-mycolate -I (R_F0.49, as above) contaminated with acetoxy-ester -II (R_F0.37) and a trace of less polar material (R_F0.59). Further elution with petrol (3 l.) and petrol/ether (99/1, 2 l.) gave mixtures (M.14) (8.66g.) of acetoxy-ester -I and -II. Continued elution with petrol/ether (99/1, 2 l; 98/2, 4 l.; 95/5, 2 l.) gave a sample (M15)(11.33g.) of methyl acetylmycolate -II. Petrol/ether (90/10, 2 l.; 80/20, 3 l.) eluted mixtures (1.54g.) of methyl mycolate -I and -II.

Chromatography of fractions consisting mainly of methyl acetylmycolate -I .

Crude methyl acetylmycolate -I (M 10 and M 13) (14.53g.) was chromatographed in petrol on alumina (Camag, activity II, 500 g.) Elution with petrol/ether (99/1,

1.2 l.) gave a mixture (M 16) (4.34g.) of acetoxy-ester-I and material of lower polarity (R_F0.59, as above). Petrol/ether (98/2, 2.4 l.) eluted a sample (IA, 5.24g.) of purified methyl acetylmycolate -I. Elution with petrol/ether (95/5, 1.2 l.; 93/7, 800 ml.; 90/10, 400 ml.) gave mixtures (M 17) (3.89g.) of acetoxy-ester-I and -II.

The fractions (M 16) (4.34g.) from above, containing acetoxy-ester-I and less polar material, were adsorbed from petrol on alumina (Camag, activity II, 190g.). Petrol/ether (99/1, 800 ml.) eluted mixtures (M 18) (1.04g.) of acetoxy-ester-I and the less polar component (R_F0.59). Petrol/ether (97/3, 600 ml.; 90/10, 400 ml.) eluted purified methyl acetylmycolate -I (IB) (2.45 g.).

Chromatography of fractions consisting mainly of methyl acetylmycolate -II.

Crude methyl acetylmycolate -II (M 15) (11.33g.) was chromatographed in petrol on alumina (Camag, activity II, 500g.). Elution with petrol/ether (98/2, 800 ml.) gave mixtures (M 19) (959 mg.) of acetoxy-ester-I and -II. Petrol/ether (95/5, 1.6 l.) eluted purified methyl acetylmycolate -II (IIA) (8.45 g.) Petrol/ether (93/7, 1.2 l.; 90/10, 800 ml.; 50/50, 2 l.) eluted mixtures (1.27g.) of acetoxy-ester-II and methyl mycolate -I and -II.

Further chromatography of mixtures of methyl acetylmycolate -I and -II.

The mixtures (M 11, M 14, M 17, M 19) (16.0g.), containing acetoxy-ester-I and -II, were adsorbed from petrol on alumina (Camag. activity II, 600g.) Petrol/ether (99/1, 2.4 l.) eluted purified methyl acetylmycolate -I (IC) (5.94g.). Elution with petrol/ether (98/2, 1.2 l.; 96/4, 1.2 l.) gave mixtures (M 20)(4.66g.) of acetoxy-ester -I and -II. Purified methyl acetylmycolate -II (II B) (3.79g.) was eluted by petrol/ether (94/6, 2 l.; 92/8, 800 ml.).

Chromatography of the above mixture (M 20) (4.65g.) of acetoxy-ester-I and -II on alumina (Camag, activity II, 220g.), under similar conditions, gave purified acetoxy-ester-I (ID) (1.82g.), mixtures (M 21)(903 mg.), and purified acetoxy-ester-II (II-C) (1.03g.). The above mixture (M 21) (903 mg.) of acetoxy-ester-I and -II was similarly chromatographed on alumina (Camag, activity -II, 85g.) giving purified methyl acetylmycolate-I (I E) (148 mg.), mixtures of acetoxy-ester-I and -II (133 mg.) and purified methyl acetylmycolate -II (II D, 397 mg.).

Methyl acetylmycolate -I,

The collected samples (IA, IB, IC, ID, IE) (15.16g.)

of methyl acetylmycolate -I were chromatographed on alumina (Camag. activity II, 500g.), followed by a final purification on activated silica gel (250g.) yielding methyl acetylmycolate -I (12.90g.), m.p. 39-40° (from ethyl acetate), $[\alpha]_{589}^{20}$ + 4.9°, $[\alpha]_{578}^{20}$ + 5.1°, $[\alpha]_{546}^{20}$ + 5.7°, $[\alpha]_{436}^{20}$ + 9.3°; $\nu_{max.}$ 1740 (ester carbonyl), 1020 and 3060 $cm.^{-1}$ (cyclopropane) (Found: C, 81.9; H, 12.9. $C_{81}H_{156}O_4$ requires C, 81.5; H, 13.1%). The n.m.r. spectrum ($CDCl_3$) showed singlets at τ 6.34 (3H, ester-OMe) and τ 8.00 (3H, $CH_3.CO.$) triplets at τ 9.12 (ca. 6H, terminal CH_3), multiplets at τ 4.85 (1H, $>CH.OAc$) and τ 7.40 ($>CH.COOMe$), and bands at τ 9.40, 10.30 (6H and 2H, cyclopropane).

Methyl acetylmycolate -II

The samples (IIA, IIB, IIC, IID) (14.42 g.) of methyl acetylmycolate -II were purified further as described for methyl acetylmycolate -I, giving methyl acetylmycolate - II (10.61 g.), m.p. 31-32° (from ethyl acetate), $[\alpha]_{589}^{20}$ + 0.8°, $[\alpha]_{578}^{20}$ + 1.7°, $[\alpha]_{546}^{20}$ + 1.9°, $[\alpha]_{436}^{20}$ + 2.9°; $\nu_{max.}$ 1740 (ester carbonyl), 1020 and 3060 (cyclopropane), 1090 $cm.^{-1}$ (methoxyl) (Found: C,80.6; H , 12.8. $C_{88}H_{172}O_5$ requires C, 80.7; H, 13.2%). The n.m.r. spectrum ($CDCl_3$) showed singlets at τ 6.34 (3H, ester-OMe), 8.00 (3H, $CH_3.CO.$) and 6.67 (3H, methoxyl), triplets at τ 9.12

(ca. 6H , terminal CH_3), a doublet ($\underline{J}$ = 5.4 c./sec.) at τ 9.17 (ca. 3H , methyl branch), multiplets at τ 4.85 (1H, >C<u>H</u>. OAc), 7.40 (1H, >C<u>H</u>. COOMe) and 7.10 (1H, >C<u>H</u> OMe), and bands at τ 9.40, 9.80 and 10.3 (2H , 1H, 1H, cyclopropane).

<u>Methyl mycolate -III oxime</u>

The material (OI) (8.59g.) from the above chromatography, consisting essentially of the oxime of methyl mycolate -III, was adsorbed from petrol on deactivated silica gel (5% water, 450g.). Petrol/ether (90/10, 1.2 l.; 85/15, 600 ml.) eluted mixtures (345 mg.) of methyl mycolate -I and -II. Petrol/ether (80/20, 1 l.) eluted <u>methyl mycolate -III oxime</u> (7.52g.), m.p. 41-42° (Found: C, 81.1; H, 13.2; N, 1.0. $C_{88}H_{173}O_4N$ requires C, 80.8; H, 13.2 ; N, 1.1%).

<u>Chromatographic studies of the oxime of methyl mycolate -III.</u>

UMC of methyl mycolate -III oxime (petrol/acetone, 96/4 5 passes) demonstrated the presence of two components (R_F 0.31 and 0.25) in the approximate proportions of 1:5. A portion (4.79g.) of this material was adsorbed from petrol on deactivated silica gel (10% water, 190g.) Elution with petrol/ether mixtures achieved a partial separation of the two components. Chromatography of a portion (3.99g.) on alumina (Woelm, activity IV, 278g.), under similar conditions, gave again only partial resolution of the

component oximes. Further attempts to separate the components using silica gel and alumina columns of varying activity gave unimproved resolution.

Methyl acetylmycolate -III oxime

The samples of the oxime of methyl mycolate -III from the above chromatographic procedures were combined (5.73g.) and heated under reflux with acetone (100 ml.), benzene (40 ml.) and toluene-p-sulphonic acid (100 mg.) The progress of the hydrolysis was followed by TLC and the reaction was found to be complete after 5 days. Two-thirds of the solvents were evaporated and the mixture poured into water and extracted with ether. The extract was washed with aqueous sodium hydrogen carbonate, water and dried. Removal of the solvents gave the crude mycolic ester-III. This material was acetylated as described previously for the mixture of mycolic esters -I and -II; reaction with acetic anhydride (10 ml.) in pyridine (30 ml.) gave crude acetyl-mycolic ester -III (5.65g.) This product was converted into its oxime as described previously for the mixture of the three mycolic esters. Treatment with hydroxylamine hydrochloride (2g.) andsodium acetate (3g.) in ethanol (300 ml.) during 30 hr. gave methyl acetylmycolate -III oxime (5.52g.), m.p. 36-37° (from ethyl acetate) (Found:

C, 80.2; H,13.4; N, 0.8. $C_{90}H_{175}O_5N$ requires C, 80.1; H, 13.0; N, 1.0 %). The infra-red spectrum showed the presence of cyclopropane bands ($\nu_{max.}$ 1020 and 3060 $cm.^{-1}$). The n.m.r. spectrum ($CDCl_3$) showed singlets at τ 6.34 (ester-OMe) and τ 8.00 (acetoxy group), triplets at τ 9.12 (ca. 6H, terminal CH_3), a doublet (J = 7.2c/sec.) at τ 9.05 (ca. 3H, $CH_3.\underset{|}{C}H - \underset{|}{C} =$), multiplets at τ 4.85 (1H, $>C\underline{H}.OAc$), τ 7.70 (3H, $>C\underline{H}.\underset{|}{C}=$) and τ 9.78 (3H, cyclopropane).

Chromatographic studies of the oxime of methyl acetylmycolate -III.

UMC of methyl acetylmycolate -III oxime (petrol/acetone, 96/4, 5 passes) showed that it contained two components in the ratio ca.1 : 5 (R_F 0.55 and 0.49, respectively). This oxime (5.50g.) was chromatographed in petrol on deactivated silica gel (10% water, 230g.). Petrol/ether (95/5, 1 l.) eluted mixtures (M 22) (2.36g.) of the acetoxy-oximes shown by UMC (as above) to contain the two components in the ratio 1:1. Petrol/ether (93/7, 1 l.; 90/10, 800 ml.) eluted material (M23) (2.91g.) containing the above components in the ratio 1 : 7.

The mixture (M 22) (2.35g.) from above was adsorbed from petrol on deactivated silica gel (7% water, 120g.).

Petrol/ether (96/4, 400 ml.) eluted a mixture (M24) (517 mg.) of the component oximes (ratio 2:1) of methyl acetylmycolate -III, m.p. 37-38°, $[\alpha]_{589}^{20}$ + 5.5°, $[\alpha]_{578}^{20}$ + 5.7°, $[\alpha]_{546}^{20}$ + 6.7°, $[\alpha]_{436}^{20}$ + 11.0°, $[\alpha]_{365}^{20}$ + 15.8°. The n.m.r. and infra-red spectra of this fraction showed identical patterns to those of the original mixture of oximes. Continued elution with petrol/ether (96/4, 400 ml.; 95/5, 600 ml.) gave a mixture (M25) (1.039g, ratio of components 1:5). Elution with petrol/ether (94/6, 1 l.; 92/8, 600 ml.) gave material (M26) (877 mg., ratio of components 1:7).

A portion (2.44g.) of the material (M23, M26) enriched in the more polar component was adsorbed from petrol on deactivated silica gel (7% water, 120g.) Petrol/ether (97/3, 400 ml.; 96/4, 1 l.) eluted a mixture (M27) (1.273g., ratio of components 1:7). Petrol/ether (95/5, 600 ml.; 93/7, 800 ml.; 90/10, 800 ml.) eluted a mixture (M28) (1.144g.) of the components (ratio 1:10) of methyl acetylmycolate -IIIoxime, mp. 34-35°, $[\alpha]_{589}^{20}$ + 5.5°, $[\alpha]_{578}^{20}$ + 5.9°, $[\alpha]_{546}^{20}$ + 6.9°, $[\alpha]_{436}^{20}$ + 11.2°, $[\alpha]_{365}^{20}$ + 16.2°. The n.m.r. and infra-red spectra were very similar to those of the other mixture (M24) studied above.

A portion (109 mg.) of the mixture of oximes (M24, ratio of components 2: 1) was converted into the corresponding ketone by the method described above for the hydrolysis of methyl mycolate -III oxime. Treatment with acetone (2 ml.) and toluene-p-sulphonic acid (10 mg.) in benzene (3 ml.) during 5 days gave the crude acetyl-mycolic ester-III. This material (123 mg.) was converted into its oxime by reaction with hydroxylamine hydrochloride (75 mg.), sodium acetate (110 mg.) in ethanol (15 ml.) and benzene (3 ml.) as described previously. The sample (104 mg.) of methyl acetylmycolate -III exime obtained was shown by UMC to contain the two components in the original ratio, 1:5.

Methyl 13-oxohexacosanoate oxime

13-Oxohexacosanoic acid (1.0g.)(available from a previous study[83]) was esterified with 5% methanolic sulphuric acid (50 ml.) in the usual manner giving methyl 13-oxohexacosanoate (980 mg.) m.p. 69-70° (from methanol) (Found: C, 76.2; H, 12.0. $C_{27}H_{52}O_3$ requires C, 76.4, H. 12.3%).

Methyl 13-oxohexacosanoate (700 mg.) was dissolved in benzene (15 ml.) and heated under reflux with hydroxylamine hydrochloride (600 mg.) and sodium acetate (900 mg.) in ethanol (50 ml.) for 7 hr. The solution was poured into dilute hydrochloric acid, the mixture extracted with ether and the

extract dried. Removal of the solvents gave methyl 13-oxohexacosanoate oxime (678 mg.), m.p. 54-55° (from methanol) (Found: C, 73.5; H, 11.9; N, 3.0. $C_{27}H_{53}O_3N$ requires C, 73.8; H. 12.2; N, 3.2%). UMC (petrol/acetone, 95/5, 6 passes) showed the presence of only one component (R_F 0.40).

Methyl acetylmycolate - III

The samples of methyl acetylmycolate -III oxime from the above investigations were combined (3.86g.) and hydrolysed to methyl acetylmycolate -III as described previously; treatment with acetone (5 ml.) and toluene-p-sulphonic acid (100 mg.) in benzene (50 ml.) during I week gave the crude acetoxy-keto-ester (3.59 g.) This product was adsorbed from petrol on activated silica gel (200g.). Petrol/ether (96/4, 400 ml.; 95/5, 600 ml. 93/7, 400 ml.) eluted a sample of methyl acetylmycolate -III (2.77g.) contaminated with small amounts of more polar substances. This impure ester (2.77 g.) was chromatographed in petrol on alumina (Camag, activity II, 214g.) (Petrol/ether (96/4, 600 ml.; 95/5, 600 ml.) eluted material (A) (228 mg.) shown below to be methyl anhydromycolate -III. Petrol/ether (90/10, 1.4 l.; 85/15, 400 ml.) eluted methyl acetylmycolate -III (M 29) (2.460g.) contaminated with traces of the anhydro-ester. This latter material (M29) was

adsorbed from petrol on activated silica gel (190 g.). Petrol/ether (97/3, 200 ml.) eluted a mixture of methyl anhydromycolate -III and acetylmycolate -III. Petrol/ether (95/5, 1 l.; 90/10, 800 ml.) eluted methyl acetylmycolate -III (1.770 g.), m.p. 43.5-44° (from ethyl acetate), $[\alpha]_{589}^{20}$ + 5.2°, $[\alpha]_{578}^{20}$ + 6.4°, $[\alpha]_{546}^{20}$ + 7.3°, $[\alpha]_{436}^{20}$ + 11.8° $[\alpha]_{365}^{20}$ + 17.2°; $\lambda_{max.}$ 213 mμ (ε 309), 285 mμ (ε 65); $\nu_{max.}$ 1740 (carbonyl), 1020, 3060 $cm.^{-1}$ (cyclopropane) (Found: C, 80.7; H, 13.1. $C_{90}H_{174}O_5$ requires C, 81.0; H, 13.0%). The n.m.r. spectrum ($CDCl_3$, benzene as internal standard) showed singlets at τ 6.39 (3H, ester - OMe) and τ 7.95 (3H, $CH_3.CO$), triplets at τ 9.07 (ca. 6H, terminal CH_3), a doublet (J 6.6 c./sec.) at τ 8.91 (ca. 3H, $CH_3.\overset{|}{C}H\text{-}\overset{|}{C}=$), multiplets at τ 4.85 (1H, $>CH.OAc$) and τ 7.55 (3H, $>CH.CO$) and a band at τ 9.78 (3H, cyclopropane).

Methyl anhydromycolate -III.

The sample (A) of the anhydro-ester-III obtained in the preceding section was recrystallized from ethyl acetate to give methyl anhydromycolate -III (198 mg.), m.p. 52-52.5°, $[\alpha]_{589}^{20}$ + 1.7°, $[\alpha]_{578}^{20}$ + 2.1°, $[\alpha]_{546}^{20}$ + 2.3°, $[\alpha]_{436}^{20}$ + 3.9° $[\alpha]_{365}^{20}$ + 5.4°; $\lambda_{max.}$ 216 mμ (ε 13,100) (Found: C, 82.6; H, 13.0. $C_{88}H_{170}O_3$ requires C, 82.9; H, 13.3%). The n.m.r. spectrum ($CDCl_3$)

contained a triplet (J 7.2 c./sec.) at τ 3.28
(1 H , $-CH_2.\underline{C}H = \overset{|}{C}.COOMe$).

Methyl acetylmycolate -III thioketal.

Methyl acetylmycolate -III (179 mg.) was dissolved in a mixture of acetic acid (5 ml.) and benzene (5 ml.) Ethanedithiol (0.3 ml.) was added, followed by boron trifluoride etherate (0.5 ml.) and the mixture stirred at 50° for 24 hr. The cooled mixture was poured into aqueous sodium hydrogen carbonate, extracted with ether, the extract washed with water and dried. The solvents were removed and the residue (190 mg.) was adsorbed from petrol on silica gel (80 g.). Petrol/ether (97.3, 300 ml.; 95/5, 200 ml.) eluted methyl acetylmycolate -III thioketal (125 mg.) mp 31-35°. The n.m.r. spectrum ($CDCl_3$, benzene as internal standard) showed a singlet at τ 6.78 (4H, thioketal CH_2) and a sharp peak at τ 9.90 (ca. 4H, cyclopropane). The mass spectrum (inlet system at 285°) showed peaks at m/e 357 (1.4%) and 385 (1.8%) (base peak at m/e 43).

Methyl mycolate-I

Methyl acetylmycolate -I (12.28g.) was heated under reflux with potassium hydroxide (10g.) and benzene (160 ml.) and methanol (40 ml.) for 4 hr. The cooled mixture was acidified with dilute hydrochloric acid and extracted with ether, the extract washed and dried. Removal of the solvents

gave crude mycolic acid -I (12.21g.) TLC (petrol/ether/ acetic acid, 70/30/2) of this material showed the presence of two components, R_F0.33(s), 0.45(w). This acid was esterified with an ethereal solution of diazomethane to give crude methyl mycolate -I (11.14g.) UMC (petrol/ether, 95/5, 4 passes) showed the presence of methyl mycolate -I (R_F0.49) containing a component (R_F0.56) shown later to be methyl 2 -epimycolate -I.

Crude methyl mycolate -I (11.1g.) was adsorbed from petrol on silica gel (475g.). Petrol/ether (93/7, 800 ml.) eluted a mixture (M30) 7.91g.) of methyl mycolate -I and its epimer. Elution with petrol/ether (90/10, 2 l.) gave a sample (M31) (2.80g.) of methyl mycolate -I containing a small amount of its epimer. The latter sample (M31) (2.77g.) was adsorbed from petrol on alumina (Camag, activity II/III, 156g.) Petrol/ether (93/7, 600 ml.) eluted a mixture (M32) (667 mg.) of methyl mycolate -I and its epimer. Elution with petrol/ether (90/10, 1 l.; 80/20, 800 ml.) gave a sample (IF) (2.081g.) of methyl mycolate -I.

The mixtures (M30, M32) (8.57g.) obtained above were chromatographed in petrol on alumina (Camag, activity II/III, 450g.) Petrol/ether (95/5, 400 ml.) eluted impure material (M33)(782 mg.) shown later to consist essentially of methyl

anhydromycolate -I. Further elution with petrol/ether (95/5, 400 ml.) gave material (M34) (497 mg.) consisting of similar quantities of methyl mycolate -I and its epimer. Elution with petrol/ether (93/7, 400 ml.; 90/10, 600 ml.) gave methyl mycolate -I (M35) (4.545g.) containing a small amount of its epimer. Petrol/ether (85/15, 600 ml.; 80/20 600 ml.) eluted a sample (IG) (2.262g.) of methyl mycolate -I. The above mixture (M35) (4.330g.) was adsorbed from petrol on alumina (Camag, activity II/III, 143g.) Elution with petrol/ether (94/6, 600 ml.) gave material (M36) 1.092g.) consisting of similar quantities of methyl mycolate -I and its epimer. Continued elution with petrol/ether (94/6, 600 ml.) gave a sample (M37) (1.688g.) of methyl mycolate -I containing a small amount of its epimer. Petrol/ether (90/10, 600 ml.; 80/20, 800 ml.) eluted a specimen (IH) (1.684g.) of methyl mycolate -I.

The samples of methyl mycolate -I (IF, IG, IH) (5.771g.) were adsorbed from petrol on alumina (Camag, activity II/III, 250g.). Petrol/ether (96/4, 400 ml.) eluted a sample (M38) (1.184g.) of methyl mycolate -I containing a trace of its epimer. Continued elution with petrol/ether (96/4, 400 ml.; 95/5, 800 ml.; 93/7, 400 ml.; 90/10,

400 ml.) gave <u>methyl mycolate</u> -I (4.69g.), m.p. 49-50° from ethyl acetate), $[\alpha]_{589}^{20}$ + 3.7°, $[\alpha]_{578}^{20}$ + 3.7°; $[\alpha]_{546}^{20}$ + 4.3° $[\alpha]_{436}^{20}$ + 6.8°, $[\alpha]_{365}^{20}$ + 10.1°; $\nu_{max.}$ 1020, 3060 cm^{-1} (cyclopropane) (Found: C, 82.8; H, 13.2. $C_{79}H_{154}O_3$ requires C, 82.4; H, 13.4%). The n.m.r. spectrum (Fig.1) and high-resolution infra-red spectrum (Fig.10b) are shown in the main section.

<u>Methyl 2-epimycolate -I.</u>

The mixtures (M37, M38) (2.79g.), containing small amounts of methyl 2-epimycolate -I, were adsorbed from petrol on alumina (Camag, activity III, 200g.). Petrol/ether (96/4, 200 ml.) eluted a mixture (M39) (561 mg.) of similar amounts of methyl mycolate -I and its epimer. Petrol/ether (95/5, 1 l.; 90/10, 400 ml.; 80/20, 400 ml.) eluted a sample of methyl mycolate -I (2.125g.). The samples from the previous chromatographic separations, containing similar amounts of methyl mycolate -I and its epimer (M34, M36) were combined with the above sample (M39) and the product (2.07g.) was chromatographed in petrol on alumina (Camag. activity III, 160g.) Petrol/ether (97/3, 300 ml.) eluted a sample (E I) (189 mg.) of methyl 2-epimycolate-I; continued elution with this solvent (100 ml.)

gave a slightly impure sample (M40) (177 mg.) of methyl 2-epimycolate-I. Further elution with petrol/ether (97/3, 400 ml.; 95/5, 200 ml.) gave mixtures (M41)(970 mg.) of methyl mycolate -I and its epimer. Elution with petrol/ether (93/7, 800 ml.) gave methyl mycolate -I (711 mg.). The mixtures (M41) (970 mg.) from the preceding chromatography were adsorbed from petrol on alumina (Camag, activity III, 100g.). Petrol/ether (97/3, 100 ml.) eluted a sample (E2) (47 mg.) of methyl-2-epimycolate -I; continued elution with this solvent (600 ml.) gave mixtures (M42) (707 mg.) of methyl mycolate -I and its epimer. Petrol/ether (95/5, 200 ml.; 93/7, 200 ml.) eluted methyl mycolate -I (190 mg.).

The samples (E1, E2) of methyl-2-epimycolate-I from above (233 mg.) were adsorbed on five 200 x 200 x 1 mm. layers of Kieselgel $HF_{254 + 366}$; the slightly impure sample (M40) (177 mg.) was adsorbed on three similar plates. The plates were irrigated with petrol/ether (90/10, 6 passes) and the bands containing the required epimer removed and extracted with ether. Removal of the ether gave methyl 2-epimycolate-I (370 mg.), m.p. 56-57° (from ethyl acetate), $[\alpha]_{589}^{20}$ -0.6°, $[\alpha]_{578}^{20}$ - 0.7°, $[\alpha]_{546}^{20}$ - 0.8°, $[\alpha]_{436}^{20}$ - 1.2°, $[\alpha]_{365}^{20}$ - 1.5° (Found: C,82.4; H, 13.7. $C_{79}H_{154}O_3$ requires C,82.4; H, 13.4%). The n.m.r.,

low-resolution infra-red and mass spectra were very similar to those of methyl mycolate -I. The high-resolution infra-red spectrum is shown in the main section (Fig.10a).

Methyl anhydromycolate -I.

The fractions (M33) (780 mg.) from the above isolation of methyl mycolate -I were adsorbed from petrol on alumina (Camag, activity II, 100g.) Elution with petrol/ether (99/1, 200 ml.) gave methyl anhydromycolate-I (381 mg.), m.p. 39-41° (from ethyl acetate), $[\alpha]_{589}^{20}$ -0.1°, $[\alpha]_{578}^{20}$ -0.1°, $[\alpha]_{546}^{20}$ -0.1°, $[\alpha]_{436}^{20}$ -0.1°, $[\alpha]_{365}^{20}$ -0.2°; $\lambda_{max.}$ 216 mμ (ε 13,200) (Found: C, 83.4; H, 13.2. $C_{79}H_{152}O_2$ requires C, 83.7; H, 13.4 %). The n.m.r. spectrum ($CDCl_3$) contained a triplet (J 7.2 c./sec.) at τ 3.28 (1H, $-CH_2.\ \underline{CH} = \overset{|}{C}-COOMe$).

Methyl mycolate -II.

Methyl acetylmycolate -II (10.65g.) was hydrolysed as described for acetoxy-ester-I giving crude mycolic acid -II (9.85g.). TLC (petrol/ether/acetic acid, 70/30/2) again showed the presence of two components, R_F 0.33(s), 0.45(w). The ultra-violet spectrum showed absorption at 218 mμ. suggesting the presence of αβ-unsaturated

acid. The crude mycolic acid -II was esterified with an ethereal solution of diazomethane to give crude methyl mycolate -II (9.29g.) UMC (petrol/ether, 95/5, 4 passes) showed the presence of methyl mycolate -II (R_F0.38), a component presumed to be methyl 2-epimycolate-II (R_F0.45) and material shown later to be methyl anhydromycolate -II (R_F0.95).

Crude methyl mycolate -II (9.20g.) was chromatographed in petrol on alumina (Camag, activity III, 450 g.) Petrol/ether (97/3, 200 ml.) eluted a sample (B) (346 mg.) shown below to consist of methyl anhydromycolate -II. Petrol/ether (96/4, 600 ml.) eluted mixtures (M43) (1.469g.) of similar amounts of methyl mycolate -II and its epimer. Elution with petrol/ether (95/5, 800 ml.; 93/7, 600 ml.) gave a sample (M44) (4.765g.) of methyl mycolate -II containing small amounts of its epimer. Petrol/ether (90/10, 400 ml.; 80/20, 400 ml.) eluted a sample (II E) (2.530g.) of methyl mycolate -II. The material (M44) (4.7 1g.) from above was adsorbed from petrol on alumina (Camag, activity III, 200 g.) Elution with petrol/ether (96/4, 800 ml.; 95/5,600 ml.) gave mixtures (M.45) (2.661 g.) of methyl mycolate -II and its epimer. Petrol/ether (93/7, 600 ml.; 90/10, I l.) eluted a sample (II F) (2.088g.) of methyl mycolate -II.

The above samples of methyl mycolate -II (II E, II F) were chromatographed in petrol on alumina (Camag, activity III, 250g.). Petrol/ether (96/4, 400 ml.) eluted methyl mycolate -II (334 mg.) containing a trace of its epimer. Elution with petrol/ether (96/4, 400 ml.; 95/5, 800 ml.; 90/10, 1 l.) gave methyl mycolate -II (4.09g.), m.p. 48-48.5° (from ethyl acetate,) $[\alpha]_{589}^{20}$ + 0.1°, $[\alpha]_{578}^{20}$ + 0.1°, $[\alpha]_{546}^{20}$ + 0.1°, $[\alpha]_{436}^{20}$ + 0.2°, $[\alpha]_{365}^{20}$ + 0.3°; $\nu_{max.}$ 1020, 3060 (cyclopropane), 1090 cm^{-1} (methoxyl) (Found: C, 81.3; H, 13.3. $C_{86}H_{170}O_4$ requires C, 81.5; H, 13.4 %). The n.m.r. spectrum (Fig.2) and high-resolution infra-red spectrum (Fig. 10c.) are shown in the main section.

Methyl anhydromycolate -II.

The sample (B) of the anhydro-ester (346 mg.) obtained in the preceding section was recrystallized from ethyl acetate to give methyl anhydromycolate -II, m.p. 32-35°, $[\alpha]_{589}^{20}$ -2.8°, $[\alpha]_{578}^{20}$ -3.1°, $[\alpha]_{546}^{20}$ -3.4°, $[\alpha]_{436}^{20}$ -5.5°, $[\alpha]_{365}^{20}$ -8.4°; $\lambda_{max.}$ 216 mμ (ε 7,900) (Found: C, 82.9; H, 13.5. $C_{86}H_{168}O_3$ requires C, 82.7; H, 13.5 %). The n.m.r. spectrum contained a triplet (J 7.2 c./sec.) at τ 3.28 (< 1H, $-CH_2 - \underset{|}{C}H = C.$ COOMe) and a multiplet at τ 4.70 (<1 H, -CH = CH-).

Methyl mycolate -III.

Methyl acetylmycolate -III (258 mg.) was heated under reflux in 10% benzene-aqueous-methanolic (1:1:1) potassium hydroxide solution (10 ml.) for 26 hr. The cooled solution was acidified with dilute hydrochloric acid, extracted with ether, the extract dried and the solvents removed. TLC (petrol/ether/acetic acid, 70/30/2) of the residue (260 mg.) showed the presence of mycolic acid -III (R_F 0.33) and a trace of less polar material (R_F 0.45). The above acid was esterified with an ethereal solution of diazomethane and the product (286 mg.) adsorbed on six 200x 200x1 mm. layers of Kieselgel $HF_{254 + 366}$. Three irrigations with petrol/ether (90/10) gave a large band containing the required ester preceded by a trace of material presumed to be its epimer. Removal of the main band and extraction with ether gave methyl mycolate -III (190 mg.), m.p. 52-53° (from ethyl acetate), $[\alpha]_{589}^{20}$ + 5.0°, $[\alpha]_{578}^{20}$ + 5.3°, $[\alpha]_{546}^{20}$ + 6.0°, $[\alpha]_{436}^{20}$ + 10.0°, $[\alpha]_{365}^{20}$ + 14.8°; $\lambda_{max.}$ 215 mμ (ε 256), 285 mμ (ε 52); $\nu_{max.}$ 1020, 3060 $cm.^{-1}$ (cyclopropane) (Found: C, 82.0; H, 13.7. $C_{88}H_{170}O_4$ requires C, 81.7; H, 13.3 %). The n.m.r. spectrum (Fig.3) and high-resolution infra-red spectrum (Fig. 10d) are shown in the main section.

Section II - Phthiocerol and related diols.

Thin-Layer and Undimensional Multiple Chromatography of Crude Phthiocerol. - The crude phthiocerol used in the present investigation was isolated in an earlier study[35]. Thin-layer chromatography with petrol/acetone (70/30) as solvents showed three spots, R_F 0.44(w), 0.51(m), 0.60(s). The second spot (R_F 0.51) was also detectable by spraying with a solution of 2,4-dinitrophenylhydrazine, indicating the presence of ketonic material. The infrared spectrum of crude phthiocerol showed a ketone carbonyl band, $\nu_{max.}$ 1710 $cm.^{-1}$ UMC (petrol/acetone, 85/15, three passes) gave better resolution, revealing the presence of components with R_F 0.25 (w), 0.30(w), 0.38(w), 0.45(m), 0.55(w), 0.61(s). The material with R_F 0.45 was shown, as above, to be the ketonic component. The main constituent, with R_F 0.61, is now named phthiocerol A and the constituent with R_F 0.55 designated phthiocerol B.

A small amount of crude phthiocerol (250 mg.) was recrystallised from petrol, and the product compared with the material remaining in the mother-liquors by UMC (petrol/acetone, 85/15, three passes). The material which crystallised from petrol gave a chromatogram as described above, whereas the material from the mother-liquors showed

traces of material with R_F's > 0.61. The mother-liquors from a second crystallisation from petrol contained no such material.

Recrystallisation from ethyl acetate did not result in removal of this less polar material from crude phthiocerol.

O-Isopropylidene Derivative of Phthiocerol - Crude phthiocerol (259 mg.) was recrystallised once from petrol, and the product kept with acetone (50 c.c.) containing toluene-p-sulphonic acid at room temperature for 18 hr. The solution was poured into water (100 c.c.), extracted with ether, and the extract washed with aqueous sodium hydrogen carbonate, water, and dried. Removal of the ether gave a pale solid (273 mg.) whose infrared spectrum showed no hydroxyl band. UMC (petrol/ether, 95/5, three passes) of the crude O - isopropylidene derivative showed a similar sequence to that of the free diols, with R_F 0.06 (w), 0.18(w), 0.28(w), 0.35(m), 0.51(w), 0.63(s). The spot corresponding to R_F 0.35 showed a positive reaction with 2,4-dinitrophenylhydrazine.

The O-isopropylidene derivative was slightly unstable to chromatography on silica gel and alumina plates, and chromatography on a column of alumina (activity I-II) resulted in almost complete decomposition of the ketals. The O-isopropylidene derivative was, therefore, not studied further.

Ethylidene Acetals of Phthiocerol. - Crude phthiocerol (415 mg.) was recrystallised once from petrol, and the product shaken with acetaldehyde (75 c.c.) and toluene-p-sulphonic acid (100 mg.) at 0° until the mixture became homogeneous; it was then left at the room temperature for 24 hr. The solution was diluted with ether, washed, successively, with saturated aqueous sodium carbonate and water, and dried. Removal of the ether gave the crude ethylidene acetal of phthiocerol (473 mg.).

UMC (petrol/ether, 90/10, three passes) gave a pattern very similar to that of the free diols and O-isopropylidene derivatives, showing R_F 0.06 (w), 0.14(w), 0.22(w), 0.38(m), 0.50(w), 0.60(s).

No decomposition of the cyclic acetals on chromatography on silica gel or alumina plates was observed.

Chromatography of the Ethylidene Acetals.-(i) In a preliminary experiment, the crude acetals (444 mg.) were chromatographed in petrol on alumina(Spence , activity II, 35 g.). Elution in succession with petrol, mixtures of petrol and ether, ether, and mixtures of ether and acetic acid gave an almost full recovery (99.5%) of material with practically no decomposition of the acetals; a partial separation of the various components was observed.

(ii) A larger amount of the acetals, prepared from crude phthiocerol (17.4 g.), acetaldehyde (500 c.c.), and toluene-p-sulphonic acid (500 mg.) as described above, was chromatographed in petrol on alumina(Spence, activity II, 641 g.). Petrol (1.2 l.) eluted a wax (69 mg.) which was shown by UMC (petrol/ether, 90/10, three passes) to be the main component, phthiocerol A acetal (R_F 0.60) contaminated with a trace of other material(R_F 0.95). Petrol/ether (98/2, 800 ml.; 97/3, 1.8 l.) eluted material (a) consisting essentially of the cyclic acetal of phthiocerol A (8.55 g.). Further elution with petrol/ether (97/3, 800 ml.; 96/4, 1.2 l.; 95/5, 1.6 l.; 93/7, 1.4 l.; 92/8, 400 ml.) gave mixtures (MI) (5.47 g.) of the acetals of phthiocerol A (R_F 0.60) and phthiocerol B (R_F 0.50). Petrol/ether (90/10, 400 ml.) eluted mixtures (23 mg.) of the acetals of phthiocerol B and of ketonic material (R_F 0.38), presumed to be phthiodiolone[14]. Further elution with this solvent (1.6 l.), followed by petrol/ether (85/15, 800 ml.; 80/20, 400 ml.) gave an impure sample of the acetal derived from phthiodiolone (2.41 g.) (P I) (subsequently r eferred to as ethylidenephthiodiolone A).

The material remaining on the column was separated partially into many further fractions by elution with petrol/ether

(80/20, 600 ml.), ether (4.4 l.) and ether/acetic acid (100/5, 3 l.) These fractions were recombined (1.15 g.) and examined by UMC (Kieselgel HF_{254} plates). A system of petrol/acetone (96/4, three passes) revealed the presence of components with the following R_F values: 0.13(w), 0.19(w), 0.28(m), 0.35(w), 0.43(w), 0.52(s), 0.54(m), 0.64(s), 0.74(w). Phthiocerol A, B and phthiodiolone acetals showed under these conditions R_F values 0.83, 0.77 and 0.65, respectively. UMC on the same plates but with petrol/ether (90/10, six passes) showed components with R_F values: 0.04(w), 0.08(w), 0.16(m), 0.23(w), 0.29(w), 0.40(s), 0.55(w), 0.65(m), 0.78(s) (the acetals of phthiocerol A, B and phthiodiolone had R_F values 0.79, 0.71 and 0.56, respectively). The components with R_F 0.54, 0.64 (petrol/acetone) and 0.65, 0.78 (petrol/ether) were detectable under an ultraviolet lamp (254 m u), and the component with R_F 0.52 (petrol/acetone) and 0.40 (petrol/ether) gave a positive reaction with 2,4-dinitrophenylhydrazine.

Ethylidenephthiocerol A - The mixture (MI) (5.46 g.) ofthe acetals of phthiocerol A and phthiocerol B was chromatographed in petrol on alumina (Spence, activity II-III, 305 g.). Elution with petrol/ether (98/2, 600 ml.) gave material ($^{a}2$) (1.71 g.) consisting essentially of the acetal of phthiocerol A; continued elution with the same

solvent (1 l.) followed by petrol/ether (97/3, 400 ml.; 95/5, 600 ml.) gave mixtures (MII) (2.46 g.) of the acetals of phthiocerol A and B. Further elution with petrol/ether (95/5, 1 l. ; 90/10, 400 ml.) gave impure phthiocerol B acetal (b_1) (281 mg.).

The above mixtures (MII) were chromatographed in petrol on alumina (Spence, activity II-III, 89g.). Petrol (200 ml.) followed by petrol/ether (99/1, 400 ml.) eluted a sample (a_3) of phthiocerol A acetal (803 mg.). Continued elutionwith petrol/ether (99/1, 800 ml.; 98/2, 700 ml.; 97/3, 500 ml.) gave mixtures (MIII) (720 mg.) of the two acetals. Further elution with petrol/ether (95/5, 300 ml.; 90/10, 400 ml.) afforded impure phthiocerol B acetal (b_2) (65 mg.).

The mixtures (MIII) from above were chromatographed in petrol on alumina (Spence, activity II, 43 g.). Petrol/ether (99/1, 400 ml.) eluted the acetal of phthiocerol A (a_4) (169 mg.). Further elution with this solvent (400 ml.), followed by petrol/ether (98/2, 400 ml.) gave a mixture (130 mg.) of the acetals of phthiocerol A and B. Petrol/ether (95/5, 500 ml.; 90/10, 400 ml.) eluted slightly impure phthiocerol B acetal (b_3) (98 mg.).

The above fractions (a_1) (a_2), (a_3) and(a_4) were combined

(11.1 g.) and chromatographed in petrol on alumina (Spence, activity II, 350 g.). Petrol/ether (98/2, 2.4 l; 97/3, 800 ml.; 96/4, 400 ml.) eluted ethylidenephthiocerol A (7.4 g.), m.p. 40° (from ethanol), $[\alpha]^{20}_{589}$ + 14.4°, $[\alpha]^{20}_{578}$ + 14.8, $[\alpha]^{20}_{546}$ + 16.8°, $[\alpha]^{20}_{436}$ + 26.9°, $[\alpha]^{20}_{365}$ + 38.0° (Found: C, 78.4; H, 12.8. $C_{36}H_{72}O_3$ requires C. 78.2; H , 13.1. $C_{38}H_{76}O_3$ requires C, 78.6; H 13.2%). Further elution with petrol/ether (95/5, 800 ml.; 93/7, 800 ml.; 90/10, 800 ml.; 80/20, 600 ml.) gave material (3.37 g.) consisting mainly of ethylidenephthiocerol A, containing small amounts of the acetal of phthiocerol B.

The infra-red spectrum of ethylidenephthiocerol A showed a methoxyl band at 1090 cm.$^{-1}$ and ethylidene acetal bands at 1410, 1140 and 1100 cm.$^{-1}$. The n.m.r. spectrum (CCl_4) showed two triplets (J 6.6 c./sec) at τ 9.14 (terminal Me), a doublet (J 6c./sec.) at τ 9.21 (Me branch), a doublet (J 4.8 c./sec.) at τ 8.86 (ethylidene Me), a singlet at τ 6.75 (-OMe), a quartet (J 6c./sec.) at τ 7.20 (>CH - OMe), a multiplet at τ 6.24 (-O-CH CH_2 CH-O-) and a quartet (J 4.8 c./sec.) at τ 5.21 (ethylidene CH).

Ethylidenephthiocerol B - The combined fractions, (b_1), (b_2) and (b_3), (444 mg.) from the above chromatographic procedures were adsorbed from petrol on alumina (Spence, activity II, 50 g.). Petrol/ether (99/1, 300 ml.) eluted

a mixture (99 mg.) of the acetals of phthiocerol A and phthiocerol B. Further elution with the same solvent (400 c.c.), followed by petrol/ether (95/5, 200 ml.) afforded ethylidenephthiocerol B as a solid (165 mg.), m.p.35.5° after crystallisation from ethanol at 0°.

The infra-red spectrum showed a methoxyl band at 1090 cm.$^{-1}$ and ethylidene acetal bands at 1410, 1140 and 1100 cm.$^{-1}$. The n.m.r. spectrum (CCl_4) showed a triplet (J 6.6 c./sec.) at τ 9.14 (terminal Me), a doublet (J 6c./sec.) at 9.21 (Me branch), a doublet (J 6.6 c./sec.) at τ 9.03 (Me), a doublet (J 4.8 c./sec.) at τ 8.86 (ethylidene Me), a multiplet at τ 6.25 (-O-CH CH_2CH-O-) and a quartet (J 4.8 c./sec.) at τ 5.21 (ethylidene CH); a quintet (J 6 c./sec.) at τ 6.94 (>CH-OMe) was partially obscured by the methoxyl singlet at τ 6.77.

Phthiocerol A - Ethylidenephthiocerol A (6.29 g.) was heated under reflux in ethanol (100 c.c.) with toluene-p-sulphonic acid (100 mg.) for 4 hr. The mixture was poured into water, extracted with ether and the extract washed with saturated aqueous sodium hydrogen carbonate. Evaporation of the dried extract gave a pale-yellow wax (5.99 g.) which on crystallization from petrol afforded phthiocerol A, m.p. 73-73.5°, $[\alpha]_{589}^{20}$ - 4.5°, $[\alpha]_{578}^{20}$ -4.7°, $[\alpha]_{546}^{20}$ - 5.4°, $[\alpha]_{436}^{20}$ - 9.5° (Found: C, 77.8; H 13.5.Calc.

for $C_{34}H_{70}O_3$: C, 77.5; H, 13.4. Calc. for $C_{36}H_{74}O_3$: C.77.9; H, 13.4%).

The infrared spectrum showed a methoxyl band at 1090 $cm.^{-1}$. A high-resolution infrared spectrum (CCl_4; ca. 0.005 M) showed $\nu_{max.}$ 3536 (ε = 58) and 3626 $cm.^{-1}$ (ε = 69). The n.m.r. spectrum ($CDCl_3$) showed two triplets (J 7.2 c./sec.) at τ 9.10 (terminal Me), a doublet (J 6.6 c./sec.) at τ 9.18 (Me branch), a quartet (J 6 c./sec.) at τ 7.10 ($>$CH -OMe), a multiplet at τ 6.06 (-O-CH-CH_2-CH-O-) and a singlet at τ 6.66 (OMe).

Phthiocerol B - Ethylidenephthiocerol B (125 mg.) was heated under reflux in ethanol (10 c.c.) with toluene-p-sulphonic acid (10 mg.) for 4 hr. The mixture was worked up as described for phthiocerol A to give phthiocerol B (103 mg.), m.p. 71-71.5° (from petrol), $[\alpha]_{589}^{20}$ - 8.2°, $[\alpha]_{578}^{20}$ -8.2°, $[\alpha]_{546}^{20}$ - 9.5°, $[\alpha]_{436}^{20}$ - 16.4° (Found: C.77.4; H,13.5. $C_{33}H_{68}O_3$ requires C, 77.3; H, 13.4 . $C_{35}H_{72}O_3$ requires, C, 77.7; H, 13.4%). A band due to methoxyl (1090 $cm.^{-1}$) was present in the infrared spectrum. A high-resolution infrared spectrum showed $\nu_{max.}$ 3536 (ε =52) and 3626 $cm.^{-1}$ (ε =62). The n.m.r. spectrum ($CDCl_3$) showed a triplet (J 7.2 c./sec.) at τ 9.10 (terminal Me), a doublet (J 6.6 c./sec.) at τ 9.18 (Me branch), a doublet (J 6.6 c./sec.) at τ 8.97 (Me), a multiplet at τ 6.06 (-O-CH-CH_2CH-O-) and

a singlet at τ 6.68 (OMe) overlapping a quintet (J 6 c./sec.) at τ 6.84 (>CH-OMe).

Chromatography of the Ethylidene Acetal of Phthiodiolone A. - A portion (2.26g.) of the impure sample (PI) of ethylidenephthiodiolone A, obtained above in the chromatography of the crude ethylidene acetals, was chromatographed in petrol on alumina (Spence, activity III/IV, 156g.). Petrol/ether (95/5, 1.1 l.) eluted a product (1.89 g.) consisting of ethylidenephthiodiolone A, contamined with small amounts of ethylidenephthiocerol B and ethylidenephthiocerol A (R_F values 0.38, 0.50, 0.60, respectively; petrol/ether, 90/10, three passes), and a constituent of intermediate polarity (R_F 0.45). This material (1.86 g.) was chromatographed in petrol on alumina (Spence, activity III/IV, 150 g.); elution with petrol/ether (97/3, 300 ml.; 95/5, 200 ml.; 90/10, 400 ml.) gave a sample (1.59 g.) of ethylidenephthiodiolone A still containing small amounts of the above contaminants.

Diacetylphthiodiolone A Oxime.- The above sample containing ethylidenephthiodiolone A (1.4 g.) was heated under reflux in ethanol (40 ml.) containing toluene-p-sulphonic acid (50 mg.) for 3 hr. The solution was poured into water and the mixture extracted with ether, the extract washed with 10% aqueous sodium hydrogen carbonate, and dried. Removal of ether gave the crude dihydroxyketone (1.33 g.). This material was dissolved in a mixture of

pyridine (10 ml.) and acetic anhydride (4 ml.) and the solution stirred at 65° for 12 hr. The cooled mixture was poured into dilute hydrochloric acid, extracted with ether, the extract washed with dilute hydrochloric acid, and the ether removed. The crude diacetoxyketone so obtained was dissolved in ethanol (100 ml.), and hydroxylamine hydrochloride (0.9 g.) and sodium acetate (1.35 g.) were added. The mixture was heated under reflux for 18 hr., cooled and poured into dilute hydrochloric acid. Extration with ether and removal of the ether from the dried extract gave the crude oxime (1.45 g.) which was chromatographed in petrol on deactivated (15% water, 100g.) silica gel. Petrol/ether (90/10, 800 ml.) eluted mixtures (MIV)(138 mg.) of diacetoxyketone (R_F0.45; petrol/acetone 85/15) and a component (R_F0.33) shown below to be the oxime of ethylidenephthiodiolone A. Petrol/ether (85/15, 200 ml.) eluted a sample (O) (65 mg.) of this latter material; continued elution with the same solvent (200 ml.) gave a mixture (MV) (84 mg.) of this component and the oxime of diacetylphthiodiolone A (R_F 0.21). Elution with petrol/ether (85:15, 400 ml.; 80:20, 800 ml.) gave diacetylphthiodiolone A oxime (737 mg.), m.p. 38° (from methanol, $[\alpha]_{589}^{20}$ -8.1°, $[\alpha]_{578}^{20}$ -7.9°, $[\alpha]_{546}^{20}$ -8.5°,

$[\alpha]_{436}^{20}$ -13.3°, $[\alpha]_{365}^{20}$ -18.0° (Found: C, 73.0; H, 11.9; N, 2.4. $C_{37}H_{71}O_5N$ requires C, 72.9; H, 11.7; N, 2.3. $C_{39}H_{75}O_5N$ requires C, 73.4; H, 11.8; N. 2.2%). UMC of this oxime (petrol/acetone, 93/7, 4 passes) showed one spot (R_F 0.33). The n.m.r. spectrum ($CDCl_3$) contained singlets at τ 8.01 (acetoxy groups) and quintets (J 6.6.c./sec.) at τ 5.08 (>CHOAc).

Ethylidenephthiodiolone A Oxime. - The mixtures (M IV) and (MV) from the above chromatography, containing the material having R_F 0.33, were combined, and this material (221 mg.) was adsorbed from petrol on three 200 x 200 x 1 mm. layers of Kieselgel $HF_{254+366}$. On irrigation with petrol/acetone (97/3, four passes) three bands were observed. Extraction (ether) of the least and most polar bands gave samples of the diacetoxyketone (40 mg.) (K), and its oxime (64 mg.), respectively. Extraction of the intermediate band gave material (115 mg.) which was combined with the sample (O) (65 mg.) from the column chromatography described in the preceding section; crystallisation from methanol gave ethylidenephthiodiolone A oxime, m.p. 44-45° (Found: C, 76.5; H, 12.8; N.2.3. $C_{35}H_{69}O_3N$ requires C, 76.2; H, 12.6; N, 2.5. $C_{37}H_{73}O_3N$ requires C, 76.7; H, 12.7; N,2.4%) UMC of this oxime (petrol/acetone, 96/4, six passes) showed that it contained

two components (R_F 0.27, 0.34), possibly geometrical isomers in respect of the hydroxyimino grouping. The infrared spectrum showed bands at 3260, 3580 cm.$^{-1}$ (hydroxyimino grouping) and 1410 cm.$^{-1}$ (ethylidene CH). The n.m.r. spectrum ($CDCl_3$) showed a quartet (J 4.8 c./sec) at τ 5.06 (ethylidene CH).

Phthiodiolone A.- Diacetylphthiodiolone A oxime (800 mg.) was dissolved in a mixture of benzene (10 ml.) and acetone (5 ml.) containing toluene-p-sulphonic acid (10 mg.), and the solution heated under reflux for four days. The mixture was poured into water, extracted with ether, the extract washed with 10% aqueous sodium hydrogen carbonate, and dried. The ether was removed and the residue combined with the above sample (K) of diacetoxyketone. This material (831 mg.) was heated under reflux with a solution of potassium hydroxide (1 g.) in benzene (5 ml.) and methanol 5 ml.) for 2 hr. The mixture was poured into dilute hydrochloric acid, extracted with ether, and the extract washed with water. Evaporation of the dried extract gave phthiodiolone A (757 mg.), m.p. 76.5° (from light petroleum, b.p. 40-60°), $[\alpha]^{20}_{589}$ -2.7° $[\alpha]^{20}_{578}$ -2.5°, $[\alpha]^{20}_{546}$ -2.6°, $[\alpha]^{20}_{436}$ -4.5°, $[\alpha]^{20}_{365}$ -7.35° (Found: C, 77.7; H, 12.9. $C_{33}H_{66}O_3$ requires C, 77.6; H, 13.0. $C_{35}H_{70}O_3$ requires C, 78.0; H.13.1%); Demarteau-

Ginsburg *et al.*[14] record m.p. 80° and $[\alpha]_{578}$ -80° ($CHCl_3$) for phthiodiolone. The infrared spectrum showed ketone carbonyl absorption ($\nu_{max.}$ 1710 cm.$^{-1}$); a high-resolution spectrum (*ca* 0.005 M in CCl_4) showed absorption at 3536 cm.$^{-1}$ (ε = 55) and 3626 cm.$^{-1}$ (ε = 60). The n.m.r. spectrum ($CDCl_3$) showed triplets at τ 9.12 (terminal Me) and τ 8.97 (*J* 7.2 c./sec., $CH_3-CH_2-\overset{|}{C}=O$), a doublet (*J* 7.2 c./sec.) at τ 8.94 ($CH_3-\overset{|}{C}H-\overset{|}{C}=O$), a quartet(*J*.7.2 c./sec.) at 7.55 ($CH_3-CH_2-\overset{|}{C}=O$), and a multiplet at τ 6.06 ($H-O-\overset{|}{C}H-CH_2-\overset{|}{C}H-O-H$).

Ethylidenephthiodiolone A. Phthiodolone A (310 mg.) was suspended in acetaldehyde (10 ml.) at 0°, and toluene-p-sulphonic acid (10 mg.) was added. The mixture was stirred at room temperature overnight. The resulting solution was diluted with ether and poured into ice-cold water, the ethereal layer was separated, washed with 10% aqueous sodium hydrogen carbonate, and dried. Removal of the ether gave ethylidenephthiodiolone A (299 mg.), m.p. 42.5-43° (from ethanol) (Found: C, 78.6; H, 12.7. $C_{35}H_{68}O_3$ requires C, 78.3; H, 12.8. $C_{37}H_{72}O_3$ requires C, 78.7; H. 12.9%). The infrared spectrum showed bands at 1710 cm.$^{-1}$ ($>C=O$) and 1410 cm.$^{-1}$ (ethylidene CH). The n.m.r. spectrum (CCl_4) showed a quartet (*J* 4.8 c./sec.) at τ 5.21 (ethylidene C*H*), a doublet (*J* 4.8 c./sec.) at

τ 8.86 (ethylidene Me), and a multiplet at τ 6.24 (-O-C<u>H</u>-CH_2-C<u>H</u>-O-).

Section III - Methyl-branched long-chain acids

Methyl hydrogen β-methylglutarate

β-Methylglutaric anhydride was prepared as described in Organic Syntheses, Coll. Vol. IV, 1963, 630, giving material (173.5 g., 72%) having m.p. 42-43° and b.p. 159-161°/17 m.m. (Organic Syntheses gives m.p. 46°, b.p. 118-122°/3.5 mm., 60-76%). β-Methylglutaric anhydride (172.9g., 1.35 mole) and anhydrous methanol (43.2g., 1.35 mole) were heated under reflux for 5 hr. Distillation gave methyl hydrogen β-methylglutarate (175.7g., 81.5%), b.p. 102-104°/0.22 mm., n_D^{20} 1.4379. (Linstead et al.[144] gave b.p. 107-108°/0.5 mm., n_D^{19} 1.4388).

Methyl 3-methyl-5-oxo-5-(2-thienyl) pentanoate

Methyl hydrogen β-methylglutarate (50 g., 0.31 mole) was heated under reflux with thionyl chloride (75 ml.) for 4 hr. Thionyl chloride was removed by distillation, last traces being removed by azeotropic distillation with dry benzene under reduced pressure.

Thiophen (33.6 g., 0.40 mole) and benzene (100 ml.) were added to the above acid chloride. A solution of stannic chloride (81.5g., 0.31 mole) in benzene (20 ml.) was run in with stirring at 0°. Stirring was continued at room temperature for 2½ hr. An excess of 10% hydrochloric

acid was added and the mixture stirred vigorously for ½ hr. The organic layer was separated and washed with dilute hydrochloric acid, water, and dried. Removal of the solvents gave a dark brown oil (63g.) which was chromatographed in benzene on alumina (Spence, activity IV, 380 g.). Benzene (1 l.) eluted neutral material (48.4g.) ; distillation of a small amount gave methyl 3-methyl-5-oxo-5-(2-thienyl) pentanoate), b.p. 117-119°/0.2mm. (Found: C,58.0; H,6.4; S,14.0. $C_{11}H_{14}O_3S$ requires C,58.4; H,6.2; S,14.2%).

Methyl 3-methyl-5-(2-thienyl) pentanoate

Methyl 3-methyl-5-oxo-5-(2-thienyl) pentanoate (48g., 0.214 mole), diethylene glycol (400 ml) and hydrazine hydrate (46 ml.) were heated with stirring to 190°, and this temperature was maintained until water and excess hydrazine had boiled off. After cooling to 80°, potassium hydroxide (46g.) was added and the mixture heated at 180° for 2 hr. The cooled solution was poured into dilute hydrochloric acid, extracted with ether, the extract dried and the ether removed giving the crude thienyl acid. This acid (36g.) was esterified with 5% methanolic sulphuric acid (100 ml.) giving the crude thienyl ester (35.3g.) Distillation gave methyl 3-methyl-5- (2-thienyl)

pentanoate (29.8 g., 45% from methyl hydrogen β-methylglutarate), b.p. 83-84°/0.15 mm., n_D^{25} 1.5000 (Found: C,62.4; H, 7.6; S, 15.0. Calculated for $C_{11}H_{16}O_2S$: C,62.2; H, 7.6; S, 15.1%) (Shalavina and Gol'dfarb[160] give b.p. 141.5 - 142°/14.5 mm., n_D^{20} 1.5037).

1 - Bromoheptane

1-Bromoheptane was prepared as described in Organic Syntheses, Coll. Vol. II, 1943, 246. Heptan-1-ol (350g.), 47% aqueous hydrogen bromide (875 ml.) and concentrated sulphuric acid (184 ml.), after heating under reflux for 7 hr., gave 1-bromoheptane (478g., 89%), b.p. 64.5-65°/14 mm., n_D^{25} 1.4474 (Organic Syntheses gives b.p. 70.6°/19 mm., n_D^{25} 1.4480, yield 87-90%).

Nonan-2-one

1-Bromoheptane (400 g., 2.235 mole) was dissolved in dry ether (1.5 l.). A portion of this solution (150 ml). was added to dried magnesium (54g., 2.22 mole) and the mixture heated to initiate the reaction. The remainder of the solution was added slowly with stirring during 2½ hr. and the mixture stirred and heated under reflux for a further 1 hr. After cooling to room temperature acetaldehyde (111g., 2.53 mole) in ether (350 ml.) was added dropwise with stirring so that the solution refluxed gently.

The mixture was stirred and heated under reflux for 1 hr. The cooled mixture was poured onto crushed ice (2 kg.) and concentrated sulphuric acid (120 ml.). The ethereal layer was separated and the aqueous phase extracted with ether. The combined organic phases were washed with water, 10% aqueous sodium carbonate and dried. Removal of the ether gave crude nonan-2-ol (264g.).

The above alcohol was added dropwise during 1½ hr. to a stirred solution of potassium bichromate (177g.) and concentrated sulphuric acid (240g.) in water (1.2 l.). The mixture was heated on a steam-bath for 2 hr., cooled and extracted with ether. The extract was washed with 10% aqueous sodium hydroxide, water and dried. Distillation gave nonan-2-one (206.5g., 65%), b.p. 78-80°/15 mm., n_D^{20} 1.4213 (Ceuterick[161] gave b.p. 73.8°/10 mm.; Vogel[162] gave n_D^{20} 1.4210).

Ethyl 2-cyano-3-methyldec-2-enoate

A mixture of nonan-2-one (206.5g., 1.45 mole), ethyl cyanoacetate (164g., 1.45 mole), ammonium acetate (11.2g., 0.145 mole), acetic acid (17.4g., 0.29 mole) and dry benzene (200 ml.) was heated to 135° under a water separator. Heating was continued until separation of water ceased (5 hr.) and this temperature maintained for a further 1 hr. After cooling, ether (100 ml.) was added and the solution

washed with water, dried and the solvents removed. Distillation of the residue gave ethyl 2-cyano-3-methyldec-2-enoate (227.5g., 66%) b.p. 174-175°/15 mm., n_D^{25} 1.4626 (Found: C, 70.7; H, 9.8; N, 5.9. $C_{14}H_{23}O_2N$ requires C, 70.9; H, 9.8; N, 5.9%).

Hydrogenation of ethyl-2-cyano-3-methyldec-2-enoate

Ethyl 2-cyano-3-methyldec-2-enoate (225.6g.) was dissolved in ethanol (260 ml.) and the solution divided into three equal portions. To each batch in turn was added Adam's catalyst (platinic oxide) (100 mg.) and the mixtures were shaken under an initial pressure of 4 atmospheres of hydrogen. As soon as the pressure fell to 3 atmospheres, more hydrogen was admitted to restore the initial pressure; this procedure was repeated until the hydrogenation was complete (ca. 12 hr. in each case). The catalyst was removed by filtration, the solutions combined and most of the ethanol distilled. The residue was taken up in ether, washed with dilute hydrochloric acid, water and dried. Removal of the ether gave the saturated ester (209.9g.); TLC (benzene) showed one spot, R_F 0.65 (unhydrogenated ester, R_F 0.63).

1-Bromo-3-methyldecane

The cyano-ester from the previous experiment was converted into 3-methyldecanoic acid by the procedure given by

Polgar and Robinson[15] for the preparation, from the corresponding cyano-ester, of 3-methyloctanoic acid. The cyano-ester (198.7g.) was hydrolysed with 20% aqueous-ethanolic potassium hydroxide (700 ml.) and the cyano-acid obtained was decarboxylated by heating at 180° with copper-bronze (3 hr.). Hydrolysis of the resulting nitrile by heating under reflux with a mixture of acetic acid (200 ml.), water (200 ml.) and concentrated sulphuric acid (200 ml.) for 3 hr. gave crude 3-methyldecanoic acid (151.5g.).

Esterification of this acid with 5% methanolic sulphuric acid (300 ml.) gave crude methyl 3-methyldecanoate (163.0g). A solution of this ester in dry ether (600 ml.) was added to a stirred slurry of lithium aluminium hydride (20g.) in dry ether (850ml.) during 1½ hr. The mixture was stirred and heated under reflux for 3 hr. A 50% ethereal solution of ethyl acetate (150 ml.) was added slowly to the cooled mixture, followed by dilute sulphuric acid (200 ml.). The ethereal layer was separated, washed with water and dried ; removal of the ether gave crude 3-methyldecan-1-ol (150.0g.).

The above alcohol was converted to the corresponding bromide by the procedure already followed for the preparation of 1-bromoheptane. Crude 3-methyldecan-1-ol

(150g.),47% aqueous hydrogen bromide (250 ml.) and concentrated sulphuric acid (50 ml.), after heating under reflux for 5 hr., gave 1-bromo-3-methyldecane (96.9g., 43% from ethyl 2-cyano-3-methyldec-2-enoate), b.p. 115-116°/9 mm., n_D^{25} 1.4473 (Found: C,56.3 ; H, 10.1; Br, 33.9. $C_{11}H_{23}Br$ requires C,56.6; H, 9.9; Br. 34.0%).

5-Methyldodecan-2-one

1-Bromo-3-methyldecane (96.8 g., 0.41 mole) was dissolved in dry ether (60 ml.) and added to dry magnesium (9.4g., 0.41 mole) and the mixture stirred until the reaction commenced. More ether (400 ml.) was added and the flask cooled in ice ; when the reaction became less vigorous, the mixture was heated under reflux for 2 hr. and cooled. Acetaldehyde (25 ml.) in dry ether (75 ml.) was added dropwise with stirring so that the solution refluxed gently (½ hr.) The resulting mixture was stirred and heated under reflux for 1 hr. The cooled mixture was poured onto crushed ice (500 g.) and concentrated sulphuric acid (30 ml.), and crude 5-methyldodecan-2-ol (76.0g.) was isolated as described for nonan-2-ol.

The above alcohol, without further purification, was oxidized as described for the preparation of nonan-2-one

using a solution of potassium bichromate (36.8g.) and concentrated sulphuric acid (27 ml.) in water (250 ml.) and heating for 2 hr. Distillation of the product (63.0g.) gave 5-methyldodecan-2-one (23.7g., 29%) b.p. 125-126°/11mm. (Found: C,78.5 ; H, 13.0. $C_{13}H_{26}O$ requires C,78.7; H , 13.2%).

Ethyl 2-cyano-3,6-dimethyltridec-2-enoate

A mixture of 5-methyldodecan-2-one (22.6g., 0.114 mole) ethyl cyanoacetate (12.8g., 0.113 mole), ammonium acetate (0.87g., 0.113 mole), acetic acid (1.36 g., 0.023 mole) and dry benzene (40 ml.) was heated to 135° under a water separator. Separation of water was complete after 4 hr. and the product was isolated as described in the previous cyanoacetic ester condensation. Distillation of this material gave ethyl 2-cyano-3,6-dimethyltridec-2-enoate (13.0g., 39%) b.p. 130-132°/0.18 mm., n_D^{25} 1.4630 (Found: C, 73.5; H, 10.6; N,4.5. $C_{18}H_{31}O_2N$ requires C,73.7 ; H,10.7 ; N, 4.8%).

3,6-Dimethyltridecanoic acid

Ethyl 2-cyano-3,6-dimethyltridec-2-enoate (12.7g.) was hydrogenated in ethanol (20 ml.) in the presence of platinum oxide (100 mg.) as described previously. The hydrogenated ester (12.5g) was hydrolysed by heating with

10% aqueous-ethanolic potassium hydroxide (50 ml.) and the product (11.6g.), after isolation in the usual manner, was decarboxylated by heating at 180° with copper-bronze (1½ hr.). The resulting crude nitrile was heated under reflux with a mixture of acetic acid (25 ml.), water (25 ml.) and concentrated sulphuric acid (25 ml.) for 2 hr. The mixture was poured into water, extracted with ether, the extract washed with water and dried. Distillation gave a colourless liquid (6.43 g.), b.p. 120-132°/0.2 mm. TLC (petrol/ether, 60/40) showed a main component at R_F0.21, but also components at R_F0.0 (m), 0.62 (w), 0.72(w) and 0.82 (m).

A portion of the distilled material (1.01g.) was adsorbed from petrol on 15% deactivated silica gel (32g.). Petrol-ether (90/10, 600 ml.) eluted 3,6 -dimethyltridecanoic acid (689 mg.), n_D^{25} 1.4460 (Found: C, 74.4; H, 12.5. $C_{15}H_{30}O_2$ requires C,74.3; H, 12.5%. Elution with ether (300 ml.) gave a pale wax (133 mg.), m.p. 45°, whose infra-red spectrum (CCl_4) showed the presence of amide bands, $\nu_{max.}$ 1690, 3430 and 3530 $cm.^{-1}$. The remainder of the distilled acid (4.99 g.) was chromatographed in petrol on deactivated silica gel (15% water, 160g.). Petrol/ether (95/5, 1 l.; 90/10, 600 ml. ; 80/20, 800 ml.) eluted 3,6 -dimethyltridecanoic acid (3.30 g.). Ether

(1 l.) eluted 3,6-dimethyltridecanamide (692 mg.) m.p. 47-48° (Found: C,74.7 ; H, 12.7 ; N, 5.7. $C_{15}H_{31}ON$ requires C,74.6; H,12.9; N,5.8%). The above amide (840 mg.) was hydrolysed by heating under reflux with potassium hydroxide (0.48g.) in water (0.5 ml.) and ethylene glycol (2 ml.) for 5½ hr. The solution was cooled and poured into dilute hydrochloric acid and the mixture extracted with ether, the extract washed with water and dried. Removal of the ether gave a further sample (810 mg.) of 3,6-dimethyltridecanoic acid (the total yield 4.80 g., 40%).

Methyl 3,12,15-trimethyldocosanoate

3, 6-Dimethyltridecanoic acid (4.45 g., 0.018 mole) was heated under reflux with thionyl chloride (20 ml.) for 3 hr. Most of the excess thionyl chloride was distilled and final traces were removed by azeotropic distillation with dry benzene under reduced pressure.

Under anhydrous conditions at 0°, stannic chloride (4.8 g., 0.018 mole) was slowly added (½ hr.) with stirring to the above acid chloride and methyl 3-methyl-5-(2-thienyl) pentanoate (3.91 g., 0.018 mole) in benzene (30 ml.) The solution was stirred at room temperature for 3½ hr.; 10% hydrochloric acid (60 ml.) was added and the mixture

stirred until the purple colour disappeared (½ hr.). The organic layer was separated, washed with dilute hydrochloric acid, water and dried. Removal of the benzene gave an oil (6.95 g.) which was chromatographed in petrol on deactivated silica gel (10% water, 100 g.) Mixtures of petrol and increasing amounts of ether eluted the crude thienyl keto-ester (6.69g.), R_F 0.25 (petrol/ether, 80/20).

The above ester, diethylene glycol (75 ml.) and hydrazine hydrate (5 ml.) were heated to 190° and this temperature maintained until the excess of water and hydrazine was removed. The mixture was cooled to 80°, potassium hydroxide (5g.) added, and reheated to 180° for 2 hr. The cooled solution was poured into dilute hydrochloric acid, extracted with ether and dried. Removal of the ether gave the crude thienyl-acid (6.07g.) which was esterified with 5% methanolic sulphuric acid (30 ml.) giving the corresponding thienyl-ester (5.76 g.), R_F 0.57 (as above).

The above thienyl-ester was heated under reflux and stirred with Raney nickel (prepared from 129g. nickel-aluminium alloy) and ethanol (350 ml.) for 60 hr. The mixture was filtered through a Soxhlet thimble and the nickel extracted with ethanol in a Soxhlet apparatus for 24 hr. The combined ethanolic filtrate and washings were

combined and evaporated ; the pale-green residue was taken up in ether and passed through a small silica gel column (20g.) Removal of the ether gave a colourless oil (4.11g.) which on distillation gave methyl (3,12,15 - trimethyldocosanoate (3.94g.), b.p.210°/1 mm., n_D^{25} 1.4502 (Found: C.78.6; H, 12.9. $C_{26}H_{52}O_2$ requires C,78.7; H,13.2%). GLC (15% silicone oil on Embacel) at 225° showed the presence of only one component having a retention time of 56 min.(methyl docosanoate had a retention time of 40min. under these conditions).

3,12,15-Trimethyldocosanoic acid

A portion (1.4g.) of the ester, obtained in the previous section, was hydrolysed with 10% aqueous-ethanolic potassium hydroxide (15 ml.) during 4 hr. and the product, after isolation in the usual manner, was taken up in ether and passed through a small column of silica gel (20g.). Removal of the ether gave 3,12,15-trimethyldocosanoic acid (1.24g.), n_D^{15} 1.4640 (Found: C,78.3; H, 13.2. Calculated for $C_{25}H_{50}O_2$: C,78.5; H, 13.1%) Polgar et al.[17] gave n_D^{15} 1.4616.

2-Iodothiophen

2-Iodothiophen was prepared as described in Organic Syntheses, Coll. Vol. II, 1943, 357. Thiophen (73g., 0.87 mole). yellow mercuric oxide (156g., 0.72 mole) and iodine (227g., 0.89 mole) gave a 2-iodothiophen

(114.1g., 63%), b.p. 72-73°/14 mm. (Organic Syntheses gives b.p. 73°/15 mm., 72-75%).

2,2′-Bithienyl

A mixture of 2-iodothiophen (42.0g., 0.20 mole), copper bronze (16.0g., 0.25 g. atom) and dimethylformamide (100 ml.) was heated under reflux for 22 hr. The mixture was cooled, diluted with water (500 ml.) and steam distilled. The distillate (2 l.) was cooled to 0° and 2,2′-bithienyl (9.9g., 60%), m.p. 30-32°, collected. In a further similar preparation, 2-iodothiophen (69.8g., 0.332 mole), copper bronze (26.6g., 0.41 g.atom) and dimethylformamide (150 ml.) gave 2,2′-bithienyl (17.0g., 62%), m.p. 30-33° (Wynberg and Logothetis[155] gave m.p. 31-33°, 67%).

Methyl 3-methyl-5-oxo-5-(5-2,2′-bithienyl) pentanoate

Methyl hydrogen β-methylglutarate (24.0g., 0.15 mole) was heated under reflux with thionyl chloride (40 ml.) for 4 hr. Thionyl chloride was removed by distillation, last traces being removed by azeotropic distillationunder reduced pressure with dry benzene.

2,2′-Bithienyl (26.2g., 0.158 mole) in benzene (50 ml.) was added to the above acid chloride. A solution of stannic chloride (39g., 0.15 mole) in benzene (10 ml.) was run in with stirring at 0°. Stirring was continued at

room temperature for 3 hr. An excess of 10% hydrochloric acid was added and the mixture stirred overnight. The precipitated solids were dissolved by addition of chloroform (100 ml.) and the organic layer separated, washed with dilute hydrochloric acid until the washings were colourless, then with water, and dried. Removal of the solvents gave a dark-green solid (40.2g.) which was chromatographed in ether on alumina (Spence, activity II, 400g.) Ether (3.5 l.) eluted neutral material (36.44g.) which crystallized from methanol to give methyl 3-methyl--5-oxo-5-(5-2,2′—bithienyl) pentanoate (27.2g., 59%) as green plates, m.p. 99-99.5° (Found: C,58.5 ; H, 5.5 ; S,20.7. $C_{15}H_{16}O_3S_2$ requires C, 58.4; H, 5.2 ; S, 20.8%).

Methyl 3-methyl-5-(5-2,2′-bithienyl) pentanoate.

Methyl 3-methyl-5-oxo-5-(5-2,2′-bithienyl) pentanoate (26.1g., 0.085 mole), diethylene glycol (400 ml.) and hydrazine hydrate (20 ml.) were heated with stirring to 190° and this temperature maintained until water and the excess of hydrazine were removed. After cooling to 80°, potassium hydroxide (20g.) was added and the mixture heated at 180° for 2 hr. The cooled solution was poured into dilute hydrochloric acid, extracted with chloroform, the extract dried and evaporated giving the crude bithienyl acid. This

acid (26g.) was esterified with 5% methanolic sulphuric acid (100 ml.) giving a dark oil (25.0g.) which was adsorbed from benzene on alumina (Spence, activity II-III, 193g.) Benzene (1.2 l.) eluted a brown oil (19.65g.) which on distillation gave methyl 3-methyl-5-(5-2,2′-bithienyl) pentanoate (15.4g., 62%), b.p. 163-164°/0.18 mm. (Found: C,60.8; H, 6.0; S,21.6. $C_{15}H_{18}O_2S_2$ requires C,61.2 ; H,6.2; S,21.8%). This bithienyl ester darkened rapidly on keeping.

2-Methylnonanoic acid

2-methylnonanoic acid was prepared by the procedure given in Organic Syntheses, Coll.Vol.IV, 1963, 618, for 2-methyldodecanoic acid. Sodium (4.83g., 0.21 g.atom) in ethanol (140 ml.), diethyl methylmalonate (38.3g., 0.22 mole, available from a previous study) and 1-bromoheptane (35.8g., 0.2 mole) gave 2-methylnonanoic acid (27.1g., 79%), b.p. 156-157°/14 mm. (Fieser et al.[163] gave b.p. 115-117°/ 3mm.)

Methyl 3-methyl-5-(5′-2-methylnonyl-5-2,2′-bithienyl) pentanoate.

2-methylnonanoic acid (3.07g., 0.018 mole) was heated under reflux with thionyl chloride (20 ml.) for 3 hr. and the excess thionyl chloride removed as described previously. Methyl 3-methyl-5(5-2,2′-bithienyl) pentanoate

(4.97g., 0.018 mole) and benzene (30 ml.) were added to the above acid chloride. The solution was cooled to 0° and stannic chloride (4.64g., 0.018 mole) was added slowly (½ hr.) The mixture was stirred at room temperature for 3 hr. An excess of 10% hydrochloric acid was added followed by chloroform (50 ml.) to dissolve the precipitate. The mixture was stirred until the blue-green colour was discharged (1 hr.). The organic layer was separated, washed with dilute hydrochloric acid, water and dried. The solvents were removed and the residue adsorbed from ether on alumina (Spence activity I/II, 100g.). Ether (200 ml.) eluted a brown oil (6.88g.).

The above crude bithienyl keto-ester (6.08g.), diethylene glycol (75 ml.) and hydrazine hydrate (10 ml.) were heated with stirring to 190° and this temperature maintained until the excess of hydrazine and water were removed. The mixture was cooled to 80° and potassium hydroxide (5g.) added and the mixture reheated to 180° for 2 hr. The cooled solution was poured into dilute sulphuric acid, extracted with ether and the extract dried. The ether solution was filtered and an excess of an ethereal solution of diazomethane added. Removal of the ether and excess diazomethane gave a dark-brown oil

(5.30g.) which was adsorbed from petrol on alumina (Camag, activity II/III, 200g.). Petrol (400 ml.) and petrol/ether (99/1, 400 ml.; 98/2, 400 ml.; 97/3, 400 ml.) eluted methyl 3-methyl-5-(5′-2-methylnonyl-5-2,2′-bithienyl) pentanoate (3.98g., 51%), n_D^{25} 1.5431 (Found: C, 69.2; H , 8.9; S,14.6. $C_{25}H_{38}O_2S_2$ requires C,69.1 ; H, 8.8; S,14.7%).

Methyl 3,15-dimethyldocosanoate.

The bithienyl ester (3.31g.) from the previous experiment was heated under reflux and stirred with Raney nickel (from 129g. nickel-aluminium alloy) in ethanol (250 ml.) for 30 hr. The mixture was filtered through a Soxhlet thimble and the nickel extracted with ethanol in a Soxhlet apparatus for 24 hr. The ethanol was removed and the residue taken up in ether, washed with dilute hydrochloric acid, and the ethereal solution dried. Distillation gave methyl 3,15 - dimethyldocosanoate (2.76g., 95%) b.p. 195°/0.7 mm., n_D^{25} 1.4483 (Found: C,78.1 ; H ,13.2. $C_{25}H_{50}O_2$ requires C,78.5; H, 13.2%). GLC (as for methyl 3,12,15-trimethyldocosanoate) showed the presence of one component having a retention time of 51 min.

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ABSTRACT

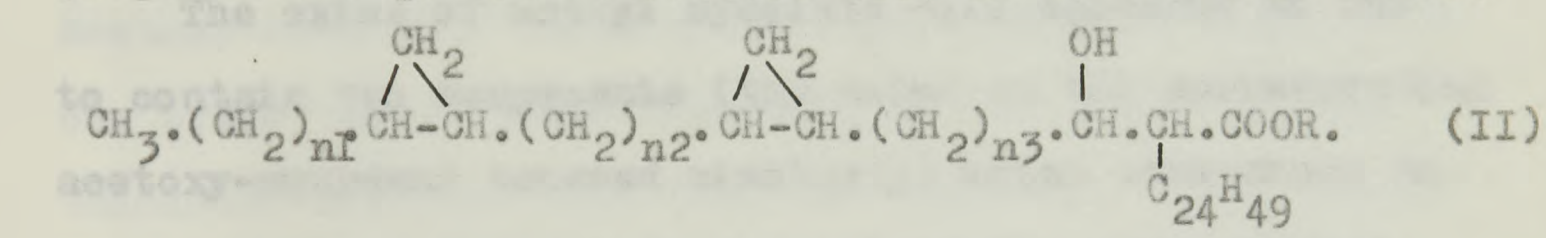
The lipids of human tubercle bacilli on mild alkaline hydrolysis give a methanol-insoluble product, which on prolonged hydrolysis yields three main fractions, the mycolic acids, crude phthiocerol, and the mycoceranic acids[1]. In the course of this work a re-investigation of the first two groups of compounds has been carried out, and synthetic analogues of methyl-branched long-chain acids occuring in tubercle bacilli have been prepared for the purpose of biological testing.

The mycolic acids - These acids have been demonstrated to be β-hydroxy acids of high molecular weight with a long alkyl side-chain in α-position. Acids containing oxygen functions in addition to the β-hydroxyl group have been isolated from various strains of bacilli and a detailed description of the work up to 1961 has been given by Asselineau[2]. More recently, Malani and Polgar[3] have described a mycolic acid containing a methoxyl group, and by oxidative degradations and vapour phase chromatography of the products were able to assign to it a partial structure (I), where n = 21 and 23, m = about 17,

$$CH_3.(CH_2)_m.\underset{OMe}{CH}.\underset{R}{CH} - .....\underset{(CH_2)_nCH_3}{CH}.\overset{OH}{\underset{|}{CH}}.\underset{(CH_2)_nCH_3}{CH}.COOH \qquad (I)$$

and R is an alkyl radical.

Gastambide - Odier et al.[4] investigated mycolic acids from many sources by nuclear magnetic resonance (n.m.r.) spectroscopy, and showed that all those examined from human strains contain cyclopropane rings. Etemadi and Lederer[5] applied mass spectrometry to methyl α-mycolate (Test), and in conjunction with the n.m.r. evidence proposed the general structure (II, R=Me) for this ester.



$$CH_3.(CH_2)_{n1}.\underset{\diagdown\,\diagup}{CH_2}\!\!-\!\!CH.(CH_2)_{n2}.\underset{\diagdown\,\diagup}{CH_2}\!\!-\!\!CH.(CH_2)_{n3}.\overset{OH}{\overset{|}{CH}}.\overset{C_{24}H_{49}}{\overset{|}{CH}}.COOR. \qquad (II)$$

In the present studies unidimensional multiple chromatography[6] (UMC) of crude methyl mycolate from human strains (D.T., P.N. and C.) showed the presence of three mycolic esters which were named methyl mycolates -I, -II and -III, in order of increasing polarity. Ester-III was shown to contain a keto-group and was isolated chromatographically as its oxime. The mixture of the other two esters was acetylated, and repeated chromatography of the product gave methyl acetylmycolate -I and -II. Hydrolysis gave the corresponding hydroxy-esters which were shown to contain small amounts of the C-2 epimers. Removal of this material by chromatography on alumina gave methyl mycolate -I, m.p. 49-50°, $[\alpha]_{589}^{20}$ + 3.7°, and methyl mycolate -II, m.p. 48-48.5°, $[\alpha]_{589}^{20}$ + 0.1°. During the

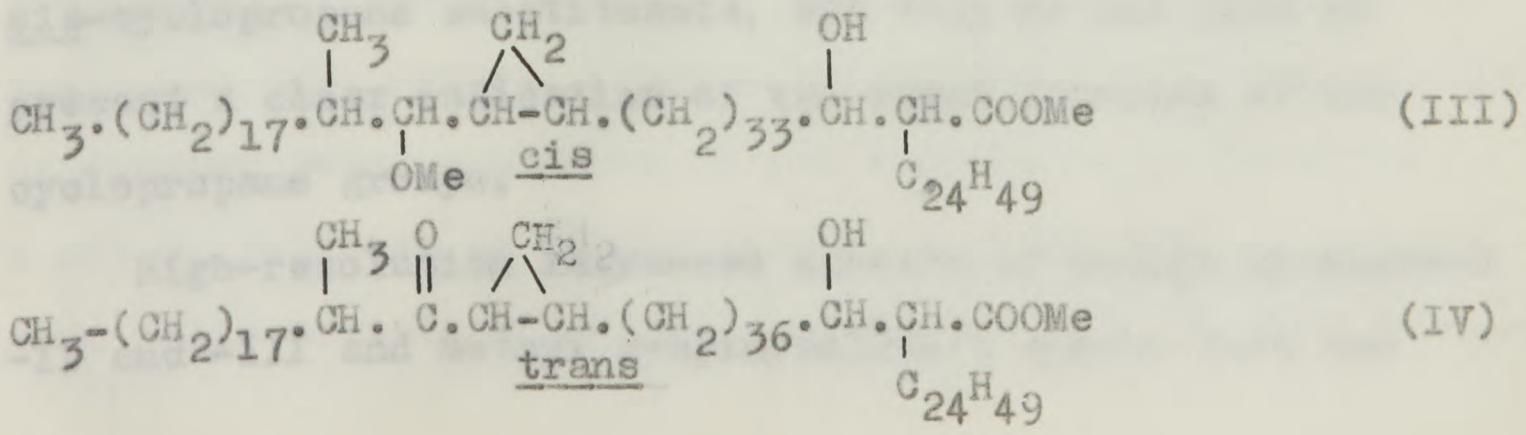
above chromatography some dehydration occurred giving methyl anhydromycolate -I, m.p. 39-41°, and methyl anhydromycolate -II, m.p. 32-35°. The material from the purification of methyl mycolate -I which contained its epimer was chromatographed repeatedly to give methyl 2-epimycolate-I, m.p. 56-57°, $[\alpha]_{589}^{20}$ -0.6°.

The oxime of methyl mycolate -III appeared on UMC to contain two components (the oxime of the corresponding acetoxy-compound behaved similarly) which were shown to be geometrical isomers in respect of the hydroxyimino grouping. Hydrolysis of the oxime of acetoxy-ester-III, and chromatography on alumina gave methyl acetylmycolate -III, m.p. 43.5-44°, $[\alpha]_{589}^{20}$ + 6.2°. This chromatography also resulted in some anhydromycolate-III, m.p. 52-52.5°. Methyl mycolate -III, m.p. 52-53°, $[\alpha]_{589}^{20}$ + 5.0°, was obtained by hydrolysis of methyl acetylmycolate -III; a thioketal derivative of the latter was also prepared.

Infra-red spectra showed that cyclopropylmethylene groups[7] were present in all three esters, and ester-II contained a band due to methoxyl. The n.m.r. spectrum of methyl mycolate -I, showing two bands(6H and 2H) for the cyclopropane groups, was very similar to that reported for methyl cis-9,10-methylenehexadecanoate[8] and indicated

the presence of two non-adjacent cis - 1,2 - disubstituted cyclopropane groups and only two terminal methyl groups. The n.m.r. spectrum of methyl mycolate -II showed that the single cis-cyclopropane group present was in a different environment, possibly adjacent to the >CH-OMe grouping ; a methyl branch was also detected. In the spectrum of acetoxy-ester-III three cyclopropane protons appeared in one broad band; after formation of the corresponding thioketal a single band (4H) was observed. This result suggested that the molecule contained a trans -1, 2 - disubstituted cyclopropane group adjacent to a carbonyl group ; the ultra-violet spectrum was in agreement. The grouping - $CH(CH_3).CO$- was also shown to be present.

Low resolution mass spectra of all the above compounds were determined, the anhydro-esters giving the most informative spectra. The results of these mass spectrometric studies in conjunction with the other evidence pointed to structures (III and IV) for the main components of methyl mycolate -II and methyl mycolate -III.

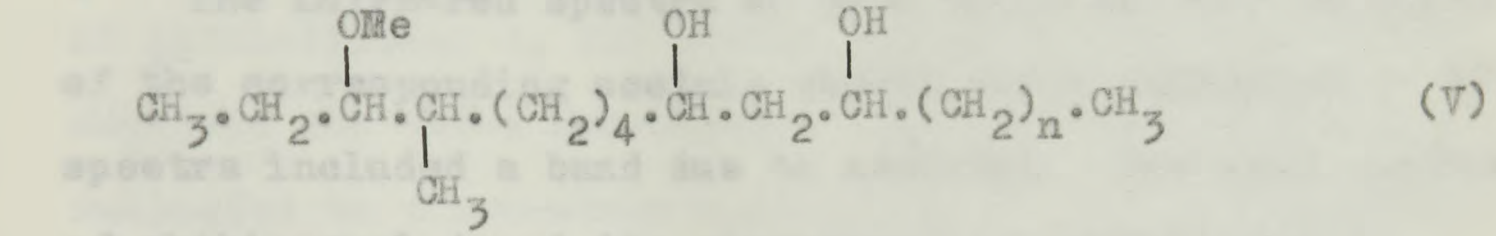


In each case the presence of ten corresponding homologues was demonstrated suggesting a biogenetic relationship between these compounds; the trans- cyclopropane group in ester -III may have been formed via the enol during the alkaline hydrolysis of the crude waxes. The mass spectrum of methyl mycolate -I, together with the other data described above, indicated that is essentially the same as methyl α-mycolate (Test) described by Etemadi and Lederer[5]. These workers supposed that a series of oxygen containing peaks at m/e 459 to 543 were due to cleavage, adjacent to cyclopropane groups, of an aldehyde fragment formed by pyrolysis in the mass spectrometer and gave the structure (II, n1 = 17, n2 = 11, n3 = 16) for the main component of this ester. However, high resolution studies of methyl mycolate -I showed that the basis for the above assignments was insecure ; moreover, recent work[9] has shown that peaks due to cleavage adjacent to cyclopropane groups are not usually observed. The results available suggest that the general structure (II) is correct, with cis-cyclopropane substituents, but they do not give at present a clear indication of the exact location of the cyclopropane groups.

High-resolution infra-red spectra of methyl mycolate-I -II and -III and methyl 2-epimycolate-I showed that the

epimer forms an intra-molecular hydrogen bond to a smaller extent; studies of molecular models showed that esters -I, -II and -III consequently have <u>erythro</u> configuration in respect of C-2 and C-3.

<u>The phthiocerol group</u> - Previous investigations[10] indicated that phthiocerol is a mixture of two homologous β-diols having the structure (V) where n is 22 and 20,

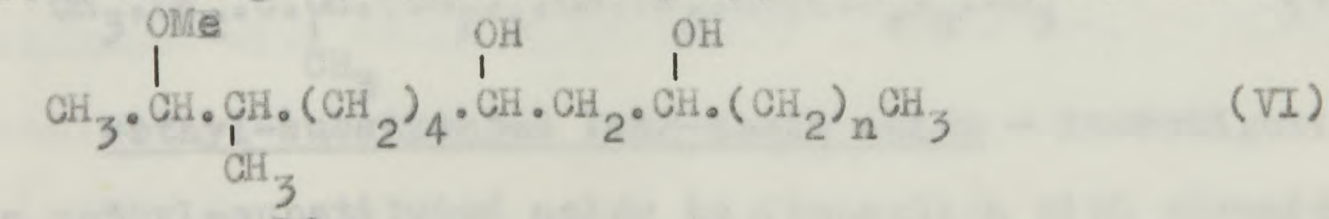


(V)

respectively. In the present studies UMC of crude phthiocerol showed (in order of increasing polarity) the presence of the diols (V) in the following named phthiocerol A, material now designated phthiocerol B and ketonic material presumed to be essentially phthiodiolone.[11] The presence of several more-polar constituents was also observed. The mixture of cyclic acetals formed by reaction of crude phthiocerol with acetaldehyde and toluene-p-sulphonic acid showed on UMC the same sequence of polarity, thus indicating that phthiocerol A and B as well as phthiodiolone contain an analogous diol system. Column chromatography of the acetals on alumina gave a mixture of the acetals of phthiocerol A and B, followed by impure acetal of phthiodiolone and by more polar acetals which latter have not been studied further.

The mixture of acetals of phthiocerol A and B was chromatographed repeatedly to give phthiocerol A ethylidene acetal, m.p. 40°, and phthiocerol B ethylidene acetal m.p. 35·5°. Decomposition of the acetals gave the corresponding diols, phthiocerol A, m.p. 73-73.5°, $[\alpha]_{589}^{20}$ -4.5° and phthiocerol B, m.p. 71-71.5°, $[\alpha]_{589}^{20}$ -8.2°.

The infra-red spectra of both diols as well as those of the corresponding acetals showed close agreement ; all spectra included a band due to methoxyl. The mass spectra of phthiocerol A and its corresponding acetal were in agreement with the structure (V) for phthiocerol A. The mass spectra of phthiocerol B and its acetal were analogous to those of phthiocerol A but the molecular ions were fourteen units lower in mass. A detailed comparison of the intermediate fragments in the above spectra led to the conclusion that phthiocerol B is a mixture of diols having the structure (VI), where n is 22 and 20



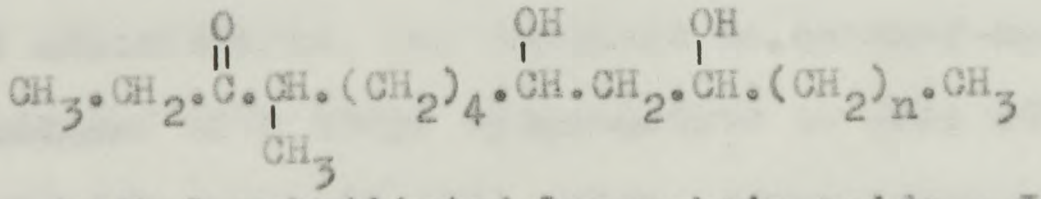
(VI)

respectively[12]. The n.m.r. spectrum of phthiocerol B confirmed that a terminal grouping $-CH(CH_3).CH(OMe)CH_3$ was present.

The impure sample of phthiodiolone ethylidene acetal contained a chromatographically similar compound, and in

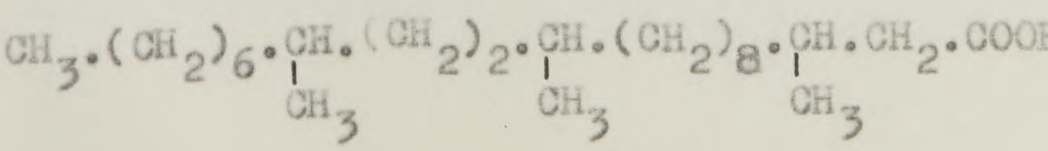
order to remove this impurity the acetal was heated with ethanol containing toluene-p-sulphonic acid and converted essentially to the diol. This was acetylated and the oxime prepared and purified by chromatography giving the oxime of diacetyl-phthiodiolone A, m.p. 38° (named by analogy with phthiocerol A). This chromatography also gave a small amount of the oxime of the ethylidene acetal of phthiodiolone A, the acetal grouping having partially survived the above hydrolysis. The diacetoxy-oxime was subjected to a two-stage hydrolysis to give phthiodiolone A, m.p. 76.5°, $[\alpha]_{589}^{20}$ -2.7° ; a portion was re-converted into phthiodiolone A ethylidene acetal, m.p. 41-42°.

Mass spectra of phthiodiolone A, its acetal and the oxime of this acetal, in conjunetion with n.m.r. evidence, confirmed that phthiodiolone A is a mixture of diols having the structure (VII) where n is 22 and 20, respectively.



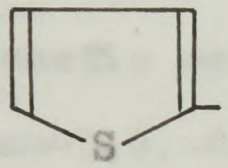
(VII)

Methyl-substituted long-chain acids - Investigations on methyl-substituted acids in connection with sarcoidosis have shown (Glaxo Laboratories Ltd., private communication) that 3,12,15 - trimethyldocosanoic acid (VIII, R = H) is the only acid of those tested to give a specific response



(VIII)

when injected into sarcoid patients. In order to provide material for further biological studies, a new synthesis of this acid has been carried out. The scheme involved the preparation of methyl 3-methyl-5-(2-thienyl) pentanoate (IX) and 3,6-dimethyltridecanoic acid (X) as intermediates.



$$(CH_2)_2 \cdot \underset{\displaystyle CH_3}{\underset{|}{CH}} \cdot CH_2 \cdot COOMe \qquad (IX)$$

$$CH_3 \cdot (CH_2)_6 \cdot \underset{\displaystyle CH_3}{\underset{|}{CH}} \cdot (CH_2)_2 \cdot \underset{\displaystyle CH_3}{\underset{|}{CH}} \cdot CH_2 \cdot COOH \qquad (X)$$

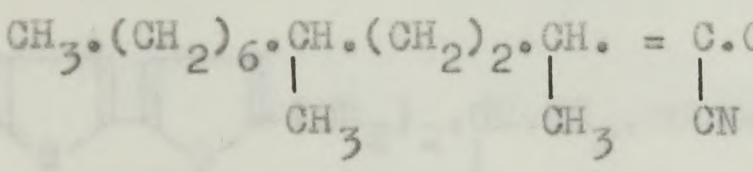
Methyl 3-methyl-5-(2-thienyl) pentanoate (IX) was prepared by a Friedel-Crafts reaction of the acid chloride of methyl hydrogen β-methylglutarate with thiophen, followed by Huang-Minlon reduction of the product.

For the preparation of the intermediate (X), nonan-2-ol, obtained by a Grignard reaction between 1-bromoheptane and acetaldehyde, was oxidised to nonan-2-one. This was condensed with ethyl cyanoacetate to give ethyl 2-cyano-3-methyldec-2-enoate (XI) which, after hydrogenation,

$$CH_3\text{-}(CH_2)_6\text{-}\underset{\displaystyle CH_3}{\underset{|}{C}} = \underset{\displaystyle CN}{\underset{|}{C}} - COOEt \qquad (XI)$$

hydrolysis, decarboxylation, followed by hydrolysis of the resulting nitrile, gave crude 3-methyl decanoic acid.

Lithium aluminium hydride reduction of the ester of this acid gave crude 3-methyldecan-1-ol; treatment of the latter with aqueous hydrogen bromide gave 1-bromo-3-methyldecane. A Grignard reaction between this compound and acetaldehyde, oxidation of the product, followed by a cyanoacetic ester condensation (as above) gave ethyl 2-cyano-3,6,-dimethyltridec-2-enoate (XII). The usual subsequent stages (as above) followed by chromatography

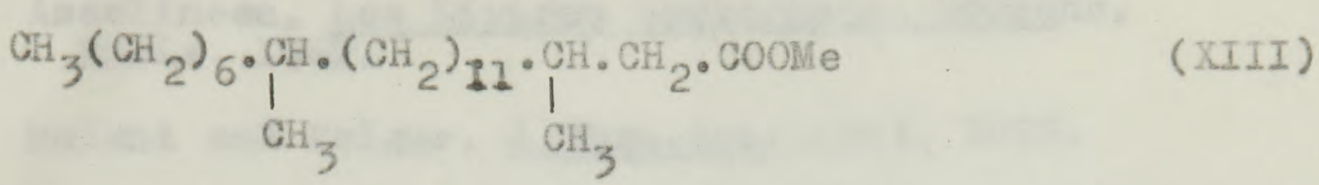


(XII)

gave 3,6 -dimethyltridecanoic acid (X).

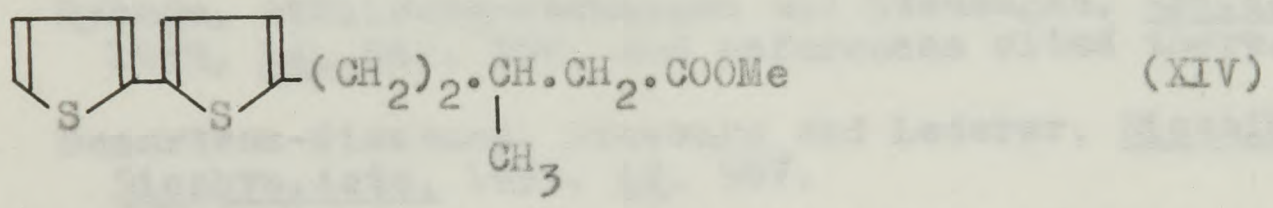
A Friedel-Crafts reaction of the acid chloride of 3,6 -dimethyltridecanoic acid with the above thienyl-ester (IX), Huang-Minlon reduction of the resulting thienyl-keto-ester, desulphurisation with Raney nickel and purification of the methyl ester gave methyl 3,12,15 - trimethyldocosanoate (VIII, R=Me). A portion of this ester was hydrolysed to the acid (VIII,R=H) and submitted for biological testing (Glaxo Laboratories Limited).

To obtain further information on the structural features necessary for biological activity, methyl 3,15-dimethyldocosanoate (XIII) was prepared by the following



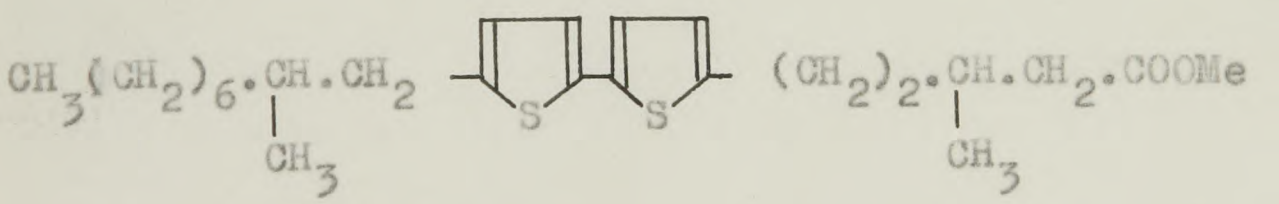
(XIII)

procedure. 2-Iodothiophen was subjected to an Ullmann reaction to give 2,2′-bithienyl[13]. Friedel-Crafts reaction of the latter with the acid chloride of methyl hydrogen β-methylglutarate, followed by Huang-Minlon reduction, gave methyl 3-methyl-5-(5-2,2′-bithienyl) pentanoate (XIV). 2-Methylnonoic acid was prepared by



(XIV)

condensing 1-bromoheptane with diethyl methylmalonate, followed by hydrolysis and decarboxylation. A Friedel-Crafts reaction of the acid chloride derived from this acid with the above bithienyl ester, and Huang-Minlon reduction of the product, followed by esterification, gave the ester (XV).



(XV)

Raney nickel desulphurisation of this bithienyl ester gave methyl 3,15-dimethyldocosanoate (XIII).

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