Tetrahedron, 1962, Vol. 18, pp. 437 to 450. Pergamon Press Ltd. Printed in Northern Ireland

THE PHTHIENOIC ACIDS FROM TUBERCLE BACILLUS

NEW METHOD FOR DEGRADATION OF BRANCHED ALKANE CHAINS*

J. CASON, P. TAVS and A. WEISS
Dept. of Chemistry, University of California

(*Received* 15 *September* 1961)

Abstract—Oxidative degradation of C_{27}-phthienoic acid has established beyond any reasonable doubt the structure, 2,4,6-trimethyl-2-tetracosenoic acid, in agreement with the previous structural assignment by other investigators. One degradative method consisted of allylic bromination of the α,β-unsaturated acid, dehydrohalogenation, ozonolysis, and oxidation with cold permanganate. A new method of degradation of hydrocarbon chains, depending on oxidation with chromyl acetate, was applied as an independent determination of structure and examination for homogeneity. The C_{25}-phthienoic acid has also been isolated, characterized, and assigned the structure, 2,4,6-trimethyl-2-docosenoic acid. Gas chromatographic analysis of previously isolated fractions of acids from the tubercle bacillus indicates the occurrence of the series of phthienoic acids ranging from C_{22} to C_{28}. There are probably present two other C_{27}-α,β-unsaturated acids, in addition to C_{27}-phthienoic acid.

IN almost simultaneous communications, two groups of investigators[1,2] reported that a major constituent of the physiologically active phthioic acid[3] from tubercle bacillus is an α,β-unsaturated acid. Subsequently, Cason and Sumrell[4] described a crystalline polymorphic acid, regarded as relatively homogeneous, which they termed C_{27}-phthienoic acid.[5] The ultra-violet spectrum and other properties indicated that this acid was an α-methyl-α,β-unsaturated acid, with two additional branches in the chain. Subsequent investigations[6–8] assigned the nearly complete structure, *trans*-2,4,x-trimethyl-2-tetracosenoic acid.

Structural investigations at the Oxford laboratories proceeded by way of oxidation of a mixture of the higher acids containing about 70 per cent of α,β-unsaturated acids. On the basis of the products isolated, the complete structure, 2,4,6-trimethyl-2-tetracosenoic acid was assigned;[9] however, the structural investigations at Berkeley

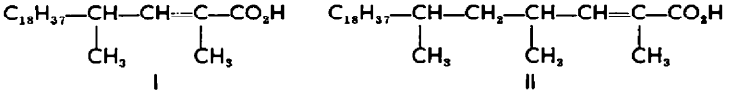
* This investigation was supported in part by a research grant (No. E-86) from the National Institutes of Health, U.S. Public Health Service.

[1] J. D. Chanley and N. Polgar, *Nature, Lond.* **166,** 693 (1950).
[2] J. Cason and G. Sumrell, *J. Amer. Chem. Soc.* **72,** 4837 (1950).
[3] M. A. Spielman and R. J. Anderson, *J. Biol. Chem.* **112,** 759 (1936).
[4] J. Cason and G. Sumrell, *J. Biol. Chem.* **192,** 405 (1951).
[5] The Oxford investigators assigned the name "mycolipenic acid" [cf. N. Polgar and R. Robinson, *Chem. & Ind.* 685 (1951)] to a component acid to which they assigned a structure based on degradation of a mixture of the tubercle acids. The best sample of acid reported at that time had an optical rotation of about 70% of that reported for C_{27}-phthienoic acid, and the ultraviolet absorption indicated about this same content of α,β-unsaturated acid. Since investigations in Sweden, confirmed by the present report, have shown that C_{27}-phthienoic acid has the structure assigned by the Oxford investigators to a component of the mixture which was degraded, and since the name assigned by us retains features of the name used by R. J. Anderson, it seems justified that most investigators in this field have used the name, C_{27}-phthienoic acid. Uniform use of this name would simplify the literature, especially in view of the occurrence of a homologous series of phthienoic acids.
[6] J. Cason, N. K. Freeman, and G. Sumrell, *J. Biol. Chem.* **192,** 415 (1951).
[7] J. Cason and C. F. Allen, *J. Biol. Chem.* **205,** 449 (1953).
[8] J. Cason and M. J. Kalm, *J. Org. Chem.* **19,** 1947 (1954).
[9] N. Polgar, *J. Chem. Soc.* 1008 (1954).

continued, since there appeared to be no other way of determining whether this structure applied to C_{27}-phthienoic acid. The structure assigned by Polgar was accepted by investigators in Sweden[10] as applying to C_{27}-phthienoic acid; however, a later report by us[11] cited several lines of evidence against this structure: (a) ozonolysis and silver oxide oxidation yielded an acid with only about half the optical rotation of 2,4-dimethyldocosanoic acid; (b) neutral material from the ozonolysis contained a C_{16} diketone; (c) data from X-ray diffraction tentatively indicated a maximum chain length of twenty carbons. In spite of this evidence against the third branch being a methyl group in the 6-position, mass spectrometric analysis carried out as a part of the extensive and fruitful investigations by the Stenhagens and R. Ryhage,[12] have subsequently supported the 2,4,6-trimethyl structure. In addition, the Oxford investigators have now reported the isolation,[13] from the lipids of the tubercle bacillus, of an acid whose properties are in agreement with those originally reported by us for C_{27}-phthienoic acid. This acid also agreed in properties with their synthetic (+)-2,4(L), 6(L)-trimethyl-2-tetracosenoic acid.

Our oxidative degradations, which have extended over several years, have now developed in full support of the structure, *trans*-2,4,6-trimethyl-2-tetracosenoic acid, for C_{27}-phthienoic acid. Apparently, the dimethyldocosanoic acid obtained by us from ozonolysis[11] was partly racemized, probably from the long digestion with silver oxide; and the diketone may well have arisen from a trace impurity containing an additional double bond and branch in the structure. Subsequent investigations[14] have shown that rate of ozone attack at a saturated tertiary position is quite slow unless such position is also allylic to a double bond, as in the γ-position in C_{27}-phthienoic acid. In addition, spectral evidence[15] has indicated presence in the acids from tubercle bacillus of structures containing both an α,β-unsaturation and a non-conjugated double bond. In retrospect, it is of interest that the sample of acid which was ozonized had a slightly higher optical rotation than other samples we have isolated, although all other properties were in excellent agreement.

Although infra-red evidence[16] had suggested that the group in the 4-position in C_{27}-phthienoic acid is methyl, more rigorous support for this conclusion was sought. Of the several degradative schemes investigated, the most fruitful consisted of allylic bromination of the ester with N-bromosuccinimide, dehydrohalogenation, ozonolysis, and oxidation with permanganate. The procedures were developed by degradation of the ester of 2,4-dimethyl-2-docosenoic acid (I). Various methods that were applied



I II

for bromination and dehydrobromination yielded, in addition to octadecanoic acid, substantial amounts of nonadecanoic acid; and under conditions of optimal yield

[10] C. Collin-Asselineau, J. Asselineau, S. Ställberg-Stenhagen, and E. Stenhagen, *Acta Chem. Scand.* **10**, 478 (1956).
[11] J. Cason, H.-R. Urschler and C. F. Allen, *J. Org. Chem.* **22**, 1284 (1957).
[12] L. Ahlquist, C. Asselineau, J. Asselineau, Ställberg-Stenhagen and E. Stenhagen, *Ark. Kemi* **13**, 549 (1958); R. Ryhage, S. Ställberg-Stenhagen and E. Stenhagen, *Ark. Kemi* **18**, 179 (1961).
[13] D. J. Millin and N. Polgar, *J. Chem. Soc.* 1902 (1958).
[14] J. Cason, J. Searing Fessenden and C. L. Agre, *Tetrahedron* **7**, 289 (1959).
[15] J. Cason, G. Sumrell, C. F. Allen, G. A. Gillies and S. Elberg, *J. Biol. Chem.* **205**, 435 (1953).
[16] J. Cason and K. L. Rinehart, Jr., *J. Org. Chem.* **20**, 1591 (1955).

of degradation products, the amounts (ca. 9 per cent yields) of the two acids were similar. There were also trace amounts of lower homologs, resulting from random oxidation, and about 3 per cent yield of heptadecanoic acid, which results from ozone attack at the position allylic to the double bond introduced by dehydrohalogenation. Presumably, nonadecanoic acid, which contains the carbon at the position of branching in structure I, results from oxidation of the component of the diene which contains methylene at the 4-position. The methylene isomer is actually the principal product of dehydrohalogenation with quinoline,[17] and the chain isomer results from rearrangement of the methylene isomer during heating with the solution of quinoline hydrobromide. In a run in which the rearrangement was carried to completion by boiling for 4 hours in picoline at the dehydrohalogenation step, there was obtained a 10 per cent yield of the C_{18} acid and no detectable amount of the C_{19} homolog (cf. Experimental).

When the above-discussed degradative scheme was applied to C_{27}-phthienoic acid, the branch at the 4-position was established as methyl; and, indeed, the complete structure of the acid was strongly indicated as that in formula II. Results of gas chromatography of the esters of the acids obtained from degradation, as presented (Fig. 1), are consistent only with this structure. There may be noted the regular sequence of minor bands which are due to acids resulting from secondary degradation. There is a somewhat larger amount of the C_{18} acid resulting from cleavage at the branch, which is also allylic to a double bond in one isomer of the dienoic acid. Conspicuous absence of the normal C_{19} acid, and presence of the lower homologs, indicates that the first branch is on carbon 19 from the tail end of the molecule. Eicosanone was also detected in the gas chromatography of neutral material from the degradation; however, presence of several unidentified bands detracted from the value of this aspect of the evidence. The large band, in Fig. 1, with retention time of 10 minutes is that for the expected 2-methyleicosanoic acid,[18] and the remaining large bands have retention times agreeing with those expected for the branched C_{22} acid and for 2,4,-dimethyldocosanoic acid, from oxidation of unbrominated starting material. If the group at the 4-position were larger than methyl, the entire degradative pattern would no longer fit expectations; in particular, there could not be obtained in major amount a degradation acid with 22 carbons (longer retention time than n-C_{21}). Similar arguments apply to the group at position 6; therefore, evidence from this degradation gives support for the entire structure.

In view of the error subsequently revealed in earlier evidence[11] that seemed convincing, an independent degradative scheme has been developed and applied to C_{27}-phthienoic acid. Oxidation with chromic acid in acetic acid had been developed as a general method[14] for location of a branch in a saturated carbon chain, and has proved of great utility,[19] although attack does not occur at positions alpha, beta or gamma to carboxyl. If C_{27}-phthienoic acid has structure II, then chromic acid oxidation

[17] J. Cason, N. L. Allinger, and D. E. Williams, *J. Org. Chem.* **18**, 842 (1953).

[18] On polyester partitioning agents, such as Reoplex-400, an α-methyl ester has a retention time identical with that for the normal isomer with one less carbon atom. Branching methyl in other positions causes retention times to fall near the midway point between the normal isomer and the next lower homolog. This pattern of retention times was established by chromatography of the esters of the complete series of methyloctadecanoic acids prepared previously in these laboratories.

[19] Of the several investigations in this laboratory in which this degradative method has been applied, one has been published thus far: C. L. Agre and J. Cason, *J. Biol. Chem.* **234**, 2555 (1959).

of the hydrogenated acid would be expected to give eicosanone from attack at the branched 6-position. Unfortunately, the method failed completely on both phthienoic acid and the dihydro acid; only traces of neutral degradation products could be detected by gas chromatography. The method also failed on a sample of synthetic 2,4,6-trimethyltetracosanoic acid.[20] Since this failure was attributed to low solubility

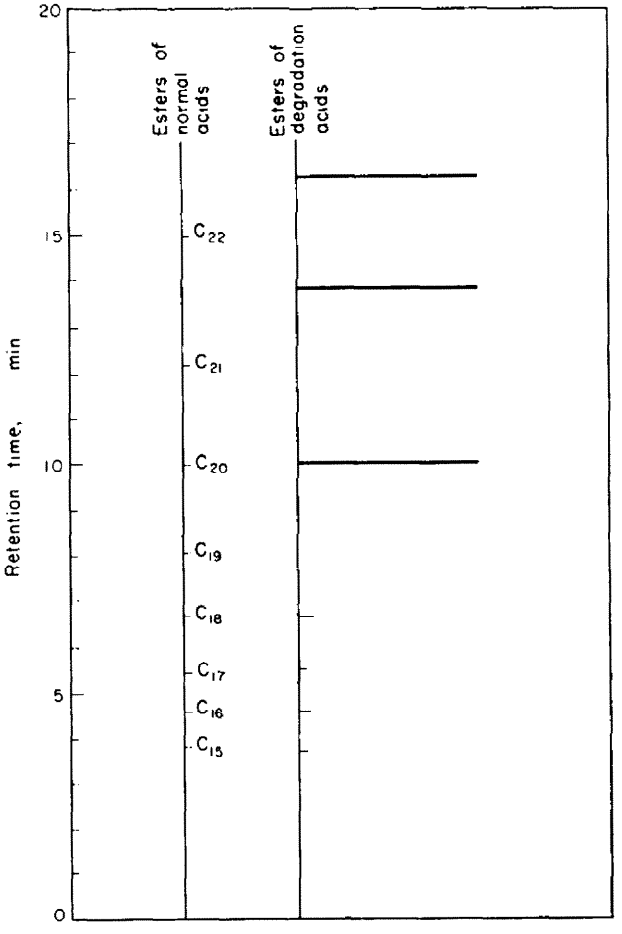


FIG. 1. Gas chromatography on Reoplex 400 (2 m column, 250°) of the methyl esters of the acids obtained from degradation of C_{27}-phthienoic acid by use of allylic bromination, dehydrohalogenation, and oxidation (cf. Experimental). Relative areas of the bands from esters of the degradation acids are indicated by the lengths of the lines designating location of the bands. Small bands representing esters of acids with fourteen or less carbons were obscured by the benzene used as solvent for the material injected.

in acetic acid of the high molecular weight acid, experimentation with other solvents led to the discovery that oxidation takes a different course when carbon tetrachloride is added to the acetic acid. There precipitated during the oxidation chromium-containing complexes with carbonyl compounds, in the manner that has been described[21]

[20] We are grateful for the generous gift by Dr. Stina Ställberg-Stenhagen of a sample of *cis*-2,4,6-trimethyl-2-tetracosenoic acid, which was hydrogenated for use in these oxidations.

[21] B. Houston and C. C. Hobbs, *J. Amer. Chem. Soc.* **76**, 1254 (1954), and the earlier reference cited therein.

when hydrocarbons are oxidized with chromyl chloride. When the reagent was varied further by utilization of the recently-described[22] chromyl acetate, $(CH_3CO_2)_2CrO_2$, there resulted a method which is successful with C_{27}-phthianoic acid (dihydrophthienoic acid); in fact, this reagent will make a somewhat selective attack on a tertiary hydrogen at the γ-position (not selectively attacked in the chromic acid oxidation). In common with chromic acid, chromyl acetate does not selectively attack a tertiary hydrogen at the α- or β-position. The acidic oxidation products from this degradation have not proved informative; however, the carbonyl compounds obtained by decomposing the chromium-containing complex include the ketones which locate the position of branching. Although numerous branched-chain acids have been oxidized with the chromyl acetate reagent, no information has been gathered concerning the mechanism of the reaction, or the reason why this agent attacks the 4-position more selectively than does chromic acid in acetic acid. Experiments designed to determine conditions of optimal convenience and yield are described in the Experimental.

It seemed of particular interest to learn from the chromyl acetate oxidations if the material termed C_{27}-phthienoic acid might contain significant amounts of more than one branched-chain acid.[23] Several degradations confirmed structure II for C_{27}-phthienoic acid and failed to reveal presence of any acid with a branch more remote than position-6. Such a branch would yield a ketone of lower molecular weight than eicosanone. Recorder tracings representative of the evidence appear in Fig. 2. In curve II, in which are shown the bands from the neutral degradation products from methyl C_{27}-phthianoate, there appears the band at 18·4 min, the retention time for 2-eicosanone under the conditions employed. The only other significant band, as determined by reference to the blank run on reagents and solvents (Curve I), is that at 2·8 min, ascribed to methyl 2,4-dimethyl-6-oxoheptanoate, which is the degradation fragment from the front end of the molecule. This band, at a slightly longer retention time than observed for a known sample of methyl 5-oxoheptanoate, is at the expected position for the 2,4-dimethyl ester (cf. 2,4-dimethyldocosanoate, Fig. 1). The assignment of the 2·8 min band is best supported by the observation that it disappears when phthianoic acid is oxidized instead of the ester (cf. Curves III, IV). In this instance, the keto acid is formed; hence, this fragment does not appear in the neutral degradation products. In order to allow detection of lower molecular weight ketones, if present, Curve III was recorded at a lower temperature (under the conditions employed in Curve II, 2-tetradecanone appears at a slightly longer retention time than the keto ester).

A sample of the first specimen of C_{27}-phthienoic acid to be isolated[4] was hydrogenated and used for the degradation represented by Curves III and IV (Fig. 2); so this sample, as well as those recently isolated, appear free of significant contamination with other acids. All of the specimens used for these degradations showed a single highly symmetrical band in gas phase chromatography. In addition, the C_{24} acid obtained previously by ozonolysis of C_{27}-phthienoic acid[11] was subjected to chromyl acetate degradation. The only significant band observed from chromatography of the neutral degradation products was at the retention time of 2-eicosanone.

[22] H. L. Krauss, *Angew. Chem.* **70**, 502 (1958).

[23] Great care was taken to insure pure samples of C_{27}-phthienoic acid for the presently-described degradations (cf. Experimental); however, in another fraction of acids which, from gas chromatographic analysis, appeared to be homogeneous, three components were identified by oxidative degradation (cf. ref. 19).

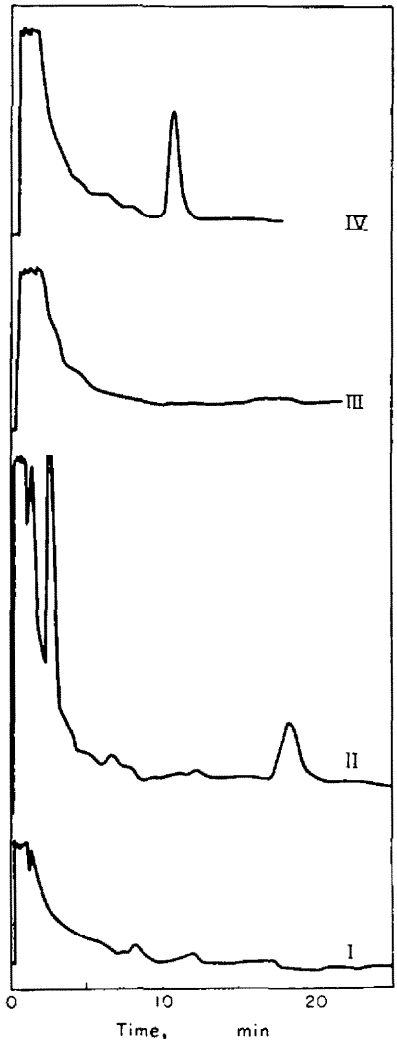


FIG. 2. Gas chromatography of degradation products from chromyl acetate oxidations. *Curve* I: 1·5 m column of diethyleneglycol succinate (DEGS), 185°. Blank run on reagents and solvents, processed as in actual degradation. The peak at about 1·5 min was present in all runs, although sometimes it was larger or smaller, unless it was obscured by a broader solvent peak. Variable minor peaks, usually not corresponding to retention times for normal ketones, are characteristic. *Curve* II: 1·5 m column of DEGS, 180°. Neutral products from oxidation of methyl C_{27}-phthianoate, prepared from acid isolated and hydrogenated as described in Experimental. *Curve* III: 1·5 m column of silicone grease (10% on Chromosorb), 155°. Neutral products from oxidation of C_{27}-phthianoic acid, sample first isolated.[4] *Curve* IV: same as for curve III, except at 225°.

Furthermore, the tracings from this degradation acid and from a sample of synthetic 2,4-dimethyldocosanoic acid were identical. It is concluded, therefore, that the acid from ozonolysis is 2,4-dimethyldocosanoic acid, and its low rotation must be ascribed to partial racemization at the α-position during the oxidation with silver oxide and isolation.

Although the investigators at Oxford have presented the view[24] that C_{27}-phthienoic acid (mycolipenic acid) is the only α,β-unsaturated acid present in significant amounts in the lipids from tubercle bacillus, mass spectrometric investigations[12] have indicated otherwise, as have investigations by us. Examination[15] of several strains of the organism, grown on diverse media, always revealed presence of several α,β-unsaturated acids. Later,[25] there were obtained fractions containing relatively high percentages of α,β-unsaturated acids which appeared to constitute a homologous series of such compounds. Gas chromatographic analysis has now been applied to these fractions, and there is indicated the presence of a complete series of phthienoic acids ranging from C_{22} to C_{28}.

Results of gas chromatography of various fractions containing methyl phthienoates are presented in Table 1, and representative data on which were based the assignments of chromatography bands are recorded in Fig. 3. It may be noted that a line through the retention times assigned to the phthienoates is parallel to the line through the times for normal esters. This suggests the occurrence of a homologous series of acids, whose esters have retention times corresponding to neither normal isomers nor the singly-branched saturated acids which have been previously identified.[19] In addition, the esters assigned as phthienoates occurred chiefly in fractions which were highly dextrorotatory, and contained a high percentage of α,β-unsaturated ester (judged from UV spectra). Retention times of the esters of saturated acids with the trimethyl substitution fall very close to the times for normal esters with two less carbons. Methyl C_{27}-phthianoate (dihydrophthienoate) has a retention time nearly identical with that for methyl pentacosanoate. For this reason, some of the bands corresponding to normal isomers in the fractions examined may be due entirely or in part to levorotatory trimethylalkanoates. A later publication will be concerned with this type of acid, which is also present in the tubercle bacillus.

In order to add support to the interpretation of the gas chromatography data in Fig. 3, material in the band corresponding to the ester of C_{25}-phthienoic acid was isolated, the ester was saponified, and the resultant acid was purified by crystallization. The ultraviolet absorption (λ_{max} 217 mμ, ε 14,800) of this product shows it to consist entirely of a *trans*-α,β-unsaturated acid with one substituent on the double bond; and this substituent is alpha rather than beta, otherwise some equilibration of the double bond with the β,γ-position would have occurred on alkaline hydrolysis. There must be an asymmetric carbon in the γ-position, hence a substituent, for hydrogenation of the double bond reduces the specific rotation from $+20{\cdot}6°$ to $+1{\cdot}1°$. Finally, chromyl acetate oxidation of the hydrogenated acid yielded 2-octadecanone as the only neutral degradation product. Gas chromatography at two temperatures (similarly to Fig. 1, Curves III, IV) failed to reveal lower molecular weight ketones; so presence of isomers with branches more remote than the 6-position is contraindicated. From these data, C_{25}-phthienoic acid may be assigned the predicted structure, *trans*-2,4,6-trimethyl-2-docosenoic acid. Thus, the occurrence of C_{25} and C_{27}-phthienoic acids has been demonstrated. On the basis of the data in Fig. 3 and Table 1, there seems no reasonable doubt of the occurrence of seven members of the homologous series.

Although most of the material reported in Table I is included in one of the two

[24] A. S. Bailey, N. Polgar, and R. Robinson, *J. Chem. Soc.* 3031 (1953).
[25] J. Cason and G. J. Fonken, *J. Biol. Chem.* **220**, 391 (1956).

TABLE 1. GAS CHROMATOGRAPHIC ANALYSIS OF ESTER FRACTIONS FROM THE LIPIDS OF TUBERCLE BACILLUS

Fractions Chromatographed[a]	Acids assigned from chromatography bands[b]	
	Normal (%)[c]	Phthienoic (%)
B.P. 214–214·5°/2 mm[d]	C_{20} (2) C_{22} (10)	C_{22} (2)[(1)] C_{23} (3)[(2)] C_{25} (46)[e]
B.P. 198–215°/2 mm; Chr. A, Fr. 5; $[\alpha]_D^{20}$ +7.8°; 65% α,β-unsat.	C_{21} (2·5) C_{22} (9·5) C_{23} (7) C_{25} (7·5)	C_{24} (28) C_{27} (trace) C_{28} (30)
B.P. 215–232°/2 mm; Chr. B, Fr. 4; $[\alpha]_D^{3}$ +12·4°; 80% α,β-unsat.	C_{23} (5.5)	C_{24} (8) C_{27} (55)[f]
B.P. 233–244°/2 mm; Chr. C, Fr. 3R; $[\alpha]_D^{20}$ −14·4°; 80% α,β-unsat.	C_{25} (?)[g]	C_{28} (7)[(5)]
B.P. 233–244°/2 mm; Chr. C, Fr. 10, 11; $[\alpha]_D^{20}$ +8·2°; 30% α,β-unsat.	C_{23} (34) C_{24} (9) C_{25} (12·5)	C_{24} (16·5)[(3)] C_{26} (18·5)[(4)] C_{28} (9·5)
B.P. 231–248°/2 mm; Chr. E, Fr. 4; $[\alpha]_D^{20}$ −5·4°; 30% α,β-unsat.[h]	C_{23} (20·5) C_{24} (6) C_{25} (22)	C_{24} (23) C_{26} (29)
B.P. 231–248°/2 mm; Chr. E, Fr. 8; $[\alpha]_D^{20}$ −3·7°; 25% α,β-unsat.	C_{23} (14·5) C_{24} (3) C_{25} (11)	C_{24} (11) C_{26} (20)

[a] All fractions except the first had been initially separated by fractional distillation,[4] as a part of the investigation which yielded C_{27}-phthienoic acid, then further fractionated by adsorption chromatography on charcoal.[25] The boiling points[4] of the fractions and the properties[25] of the fractions from charcoal chromatography are those reported. Content of α,β-unsaturated ester is calculated from the previously determined ultra-violet absorption and the average molecular weight estimated from the results of gas chromatography. Several of the molecular weights determined previously by titration were too high, probably on account of small amounts of neutral material in the samples titrated.

[b] Assignment is based on retention time, as illustrated in Fig. 3. The superscripts in parenthesis identify the components whose retention times are plotted in Fig. 3. In instances where the sum of the acids assigned is significantly less than 100%, there was a series of small bands of short retention times.

[c] The content of trimethylalkanoate in the bands with retention times for normal esters has not been determined; however, it is probable that such esters are not present in large amount except in those fractions with a levo rotation.

[d] This narrow cut from fractional distillation was that reported[15] Fig. 1, Fr. 14.

[e] Between the band for the ester of the n-C_{22} acid (at 8·4 min) and the band for C_{25}-phthienoate (at 11·8 min) was an almost completely resolved band (at 9·9 min) amounting to 36% of the total area. Any small amounts of the n-C_{23} ester (at 10·8 min) or C_{24}-phthienoate (at 9·1 min) would probably be masked by the large adjacent bands.

[f] The content of C_{27}-phthienoic acid is rather grossly estimated, for there is an immediately following overlapping band estimated to represent about 30% of the total area in the tracings. Retention times of the two bands were 30·1 and 32·4 min. In the isolations of C_{27}-phthienoate this unidentified ester could not be completely separated by gas chromatography, but was separated by crystallization of the acid. Previous work in these laboratories, as well as mass spectrometric data,[12] have

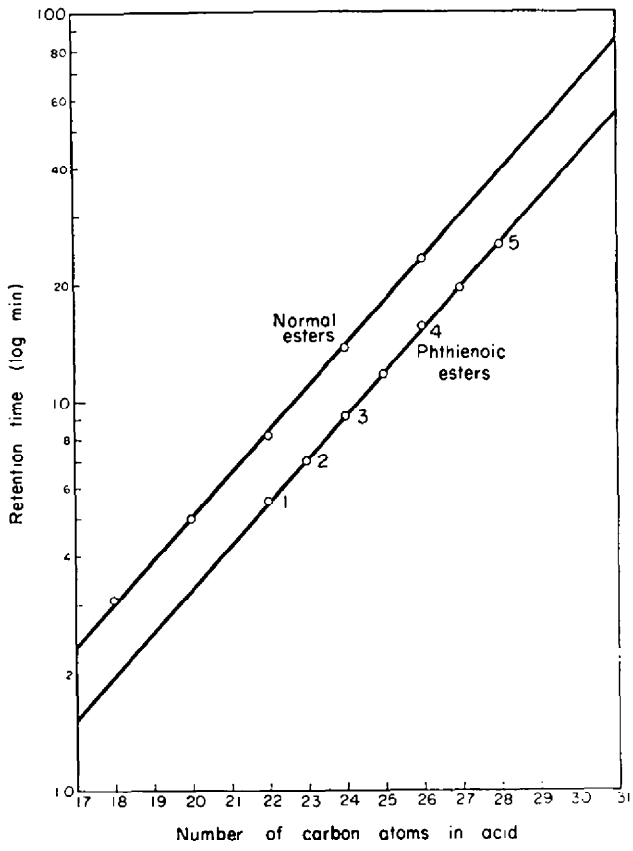


FIG. 3. Retention times recorded in gas chromatography of methyl esters of acids from tubercle bacillus. A 1·5 m column was used at 253°, with 10% high vacuum silicone grease on 40-80 mesh Chromosorb as partitioning agent. The points for normal esters were determined by injection of a mixture of methyl esters of authentic samples of normal acids. The normal esters recorded in Table 1 exhibited retention times in excellent agreement with the values here recorded. The line for the phthienoic esters was drawn through the points plotted for the retention times recorded for the C_{25}- and C_{27}-isomers (samples used for degradation). Points plotted for the other phthienoic esters are numbered to correspond to the numbered components in Table 1. Retention times recorded for the phthienoic esters in other fractions were in good agreement with those selected for plotting.

Footnotes to Table 1. (*Continued*)

indicated that this is an α,β-unsaturated acid of different structure than the phthienoic acids. The occurrence of C_{27}-phthienoate in only this fraction of those examined should not be regarded as unexpected, for fractions rich in C_{27}-phthienoate had been previously used for isolation of this component.

[g] About 90% of the band area in this tracing is at the retention time of the ester of the normal C_{25} acid, but the band is obviously broadened on the trailing side. The data show that this acid with a retention time near that of the normal isomer is α,β-unsaturated, and it probably has one more branching methyl than the phthienoic acids.[25]

[h] In this fraction the percentage of the total area in the phthienoate bands is almost double the content of α,β-unsaturated ester indicated by the ultra-violet absorption. It follows that other components, not α,β-unsaturated, must be present with the same retention times as the phthienoates. It now becomes very doubtful if levorotatory α,β-unsaturated acids are present, contrary to an earlier suggestion.[25]

indicated categories, certain additional types of esters were shown to be present. In a previously examined[15] lot of lipids from a human strain of tubercle bacillus grown on Long's medium, distillation showed a fraction of ester of narrow boiling range (214–214·5°/2 mm), and there was very little material with a boiling point near this range. Since the boiling point is in good agreement with that expected for methyl C_{25}-phthienoate, this fraction was examined for homogeneity by use of gas chromatography (first entry in Table 1). As another illustration of the complexity of the fatty acid mixture from tubercle bacillus, this fraction of one-half degree boiling range proved to contain six components. The major component (46 per cent) does have the retention time of C_{25}-phthienoate, but a second component, present to the extent of 36 per cent, has a retention time corresponding to no type of acid in tubercle bacillus that has been characterized. This may be a C_{24}-α,β-unsaturated acid of the type occurring with C_{27}-phthienoic acid (cf. Table 1, footnote *f*). This fraction also proved to be the only one in which the C_{22}- and C_{23}-phthienoates were detected, and they were very minor components in this fraction. In all lots of lipids of tubercle bacillus that have been examined, there has been a paucity of acids in the range from twenty to twenty-three carbon atoms.

In addition to the α,β-unsaturated ester whose retention time is quite near that for the C_{27}-phthienoate (Table 1, footnote *f*), a third component of apparently novel structure occurs in the fourth fraction reported in Table 1 (cf. footnote *g*). Present data reinforce the previous suggestion that this fraction contains an α,β-unsaturated acid with four branches in the chain. Retention time in gas chromatography suggests that the acid contains twenty-seven carbons. Thus, there are probably present three different C_{27} α,β-unsaturated acids. Further investigation of this interesting probability will be undertaken.

EXPERIMENTAL

Separation of methyl phthienoates. The α,β-unsaturated acids were separated from the mixture of higher molecular weight acids[26] by taking advantage of their relatively slow rate of esterification. The typical procedure which is described has been modified somewhat from that previously used so that there may be obtained the methyl esters, which are more satisfactory for gas chromatography than the ethyl esters.

To 150 ml 0·26 N methanolic hydrogen chloride was added 1·815 g phthioic acid, and the mixture was stirred for 2·5 hr in a bath at 32–33°. After the first 15 min of stirring, the initially heterogeneous mixture had formed an opalescent solution. Work-up of the reaction by dilution with water and extraction with ether yielded 1·770 g of a mixture of esters and acids. A solution of this mixture in 80 ml of benzene was passed through a three-stage Kies extraction.[27] Each of the first two stages was charged with 0·05 N KOH in aqueous ethanol (30% ethanol), while the third stage contained water. The solution was followed by 200 ml benzene. Evaporation of the total benzene from the extraction yielded 618 mg of esters.

Acids were recovered from the aqueous phases in the Kies extraction by acidification and extraction with ether. The acids obtained from the washed and dried ether solution were esterified by heating under reflux for 4 hr with 20 ml methanol containing 1·6 g conc H_2SO_4. The esterification mixture was worked up as described for the partial esterification to yield 0·974 g of predominantly α,β-unsaturated esters.

For separation of the α,β-unsaturated esters by gas chromatography on high vacuum silicone grease in a 3 m × 15 mm o.d. column, there were injected 0.1 ml samples of a 40% solution of esters in benzene. A typical tracing recorded during a separation is shown in Curve II, Fig. 4. For comparison, there is included (Curve I) a tracing of the esters formed in the partial esterification

[26] This investigation was made possible by the generous gift from Prof. R. J. Anderson of two samples from which the isolations were made. There was used one sample marked "Phthioic acid from 2nd lot of tubercle bacillus, Dec. 1929," and one sample marked "Methyl phthioate, $[\alpha]_D$ +11·6°." The two samples were of similar composition, and yielded identical samples of C_{27}-phthienoic acid.

[27] M. W. Kies and P. L. Davis, *J. Biol. Chem.* **189**, 637 (1951).

procedure. Comparison of Curves I and II reveals the effectiveness of the partial esterification procedure. Collection of the 24·4 min band yielded the C_{25}-phthienoate, while the 40 min band yielded the C_{27}-phthienoate. Correlation of the bands with molecular weight was accomplished by gas chromatography of a sample of the original[4] methyl C_{27}-phthienoate, comparison of properties of the phthienoic acid obtained by saponification (see below), and by equivalent weight determination of the C_{27}-phthienoic acid. For the C_{27}-phthienoate, $[\alpha]_D^{20}$ +14·9°; previously reported[4] +14·7°.

The samples were collected in small tubes whose outlets were packed loosely with solvent-extracted cotton. Various other systems, including use of cigarette filters and passage through a cold

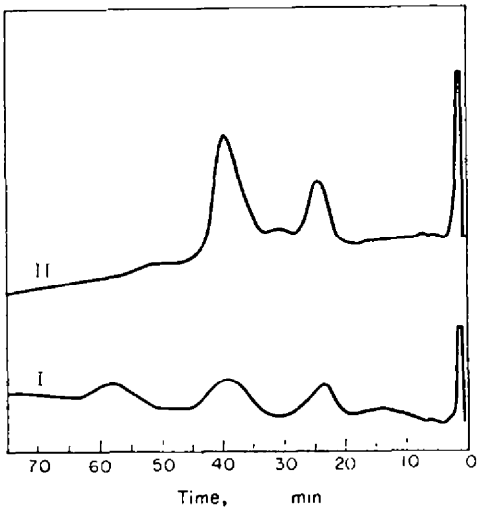


FIG. 4. Gas chromatography of fractions separated from phthioic acid by partial esterification. Chromatography was at 300°, in a 3 m × 15 mm o.d. Pyrex glass column packed with 30% high vacuum silicone grease on Chromosorb, with a helium flow rate of 200 ml/min. *Curve* I: esters formed by esterification for 2·5 hr at 32° (cf. Experimental). *Curve* II: esters of acids not esterified by the partial esterification procedure. A semi-log plot (e.g., cf. Fig. 3) of the retention times from Curve II gives a straight line. Collection of material in the bands at 24.4 and 40.0 min yielded fractions from which were isolated the C_{25}- and C_{27}-phthienoic acids. The small bands at 30·5 and 50·5 min correspond in retention times to C_{26}- and C_{28}-phthienoates.

solvent, retained the effluent aerosol less effectively. In a typical run, injection of a total of 520 mg of the mixed esters yielded 121 mg of C_{25}-phthienoate, 169 mg of C_{27}-phthienoate, and 80 mg total from all other bands (recovery, 71%).

When a sample of the ester from the 40 min band in Curve II, Fig. 4, was chromatographed on an analytical column (4 mm i.d.) containing 10% silicone grease as partitioning agent, there was revealed a shoulder on the trailing side of the band. The presence of this additional component has been noted in all isolations of the C_{27}-phthienoate (also cf. Table 1). Ester formed from acid purified by crystallization, whether presently reported samples or the original samples,[4] did not show this shoulder on the gas chromatography band, on either silicone grease or DEGS (diethyleneglycol succinate), even at temperatures low enough to give retention times up to 110 min. Collection of the first and last halves of the xand separately, followed by rechromatography, also failed to reveal more than one component in the band for the purified ester.

C_{25}-Phthienoic acid. A 200 mg sample of the ester from the 24·4 min band (Curve II, Fig. 4) was heated under reflux for 4 hr with a solution of 1 g of KOH in 10 ml 95% ethanol and 1·2 ml water. The cooled reaction mixture was poured into a mixture of 50 ml ice-water and 3 ml conc H_2SO_4, then the reaction products were extracted with 3 portions of ether. After the ether extracts had been washed and dried, solvent was removed at red press, and the residue was dissolved in 15 ml benzene for Kies extraction in the manner described above in connection with partial esterification. This procedure yielded 16 mg neutral material and 165 mg acid as a yellow oil. After crystallization of the acid had been induced by cooling and other usual techniques, the total was crystallized twice from acetone, to yield 40 mg of colourless crystalline acid, m.p. 29–30°; $[\alpha]_D^{20}$ +20·6° ($CHCl_3$); λ_{max} 218 mμ, ϵ 14,900.

Hydrogenation of 13·4 mg C_{25}-phthienoic acid in 3 ml glacial acetic acid was accomplished at atm press in presence of 50 mg pre-hydrogenated platinum catalyst. Essentially complete hydrogenation was indicated by ϵ of 150–350 at 218 mμ for the resultant oil, $[\alpha]_D^{20}$ +1·1° ($CHCl_3$).

C_{27}-*Phthienoic acid* was isolated after saponification of the material from the 40 min band (Curve II, Fig. 4), in a manner analogous to that described for the C_{25}-isomer, except that it was found advantageous to sublime the acid at a pressure of 0·2 mm and at a bath temp. of 185°, prior to crystallization from acetone. For the crystalline acid, m.p. 39·5–41°, $[\alpha]_D^{20}$ +17·3° ±0·3° ($CHCl_3$), λ_{max} 218 mμ, ϵ 14,000 in heptane; previously reported[4,25] m.p. 39–41°, $[\alpha]_D^{20}$ +17·7° ±0·2°, λ_{max} 218 mμ, ϵ 13,100. Titration gave an equivalent weight of 411 (calc. for $C_{27}H_{52}O_2$, 409).

Hydrogenation of this acid was accomplished in the manner reported for the C_{25} isomer.

Degradation of methyl C_{27}-phthienoate via allylic bromination. Best conditions for the degradative sequence were determined in numerous runs on methyl 2,4-dimethyl-2-docosenoate. Principal variable was the method of dehydrohalogenation, and the results of the more informative runs are included in Table 2. It will be noted that longer time and higher temp. give a lower yield of the C_{19} acid, which includes the carbon bearing the methyl branch at the 4-position. This acid no doubt results from oxidation of the methylene isomer. Best conditions are described for the C_{27}-phthienoate in the following paragraphs; however, use of less N-bromosuccinimide and carrying out the bromination reaction for somewhat longer periods gave similar results.

A solution of 57 mg methyl C_{27}-phthienoate in 10 ml carbon tetrachloride was heated under reflux for 80 min with 57 mg N-bromosuccinimide and 3 mg dibenzoyl peroxide. Half the peroxide was added at the beginning of reflux and the remainder after 40 min. After removal of succinimide by filtration, solvent was removed at red press, 8 ml quinoline (synthetic) was added, and the flask was immersed for 10 min in an oil bath pre-heated to 185°. The deep red reaction mixture was cooled and poured into ice-water containing 20 ml conc HCl, then the product was extracted with three 100 ml portions ether. The ether extracts were washed with two 20 ml portions 10%HCl, then with water, and finally dried over magnesium sulfate. Removal of solvent left 91 mg of a dark brown residue containing the unsaturated esters. This was ozonized immediately.

The dark residue was dissolved in 80 ml distilled methylene dichloride, the solution was cooled to −30°, and ozone was passed in at the rate of 0·19 mmole/min for 5 min. About 0·9 mmole of ozone was absorbed. The solution, which had become colourless during the ozonolysis, was allowed to stand for 40 min at −30°, then the solvent was removed under red press at room temp. To the residue were added 500 mg $KMnO_4$, 10 ml water and 10 ml acetone (distilled from $KMnO_4$), then this mixture was stirred for 16 hr at 30°. Excess permanganate was destroyed with bisulfite, the solution was made alkaline with 10% KOH, and neutral material was extracted with five 50 ml portions ether. The neutral material was chromatographed in some runs, and eicosanone was detected, but this analysis was rather unsatisfying on account of the presence of unidentified bands.

The alkaline solution remaining after extraction of neutral material was evaporated to dryness, and the residue was dried for 1 hr at 100° and 30 mm press. Esterification of the residue by heating for 2 hr under reflux with 80 ml methanol containing 6·4 g conc H_2SO_4, followed by the work-up described in earlier sections, yielded 20 mg esters which were subjected to analysis by gas chromatography. Results of this chromatography are summarized in Fig. 1. As a precaution in assigning bands, the chromatography was also carried out on high vacuum silicone grease.

Oxidation with chromyl acetate. The procedure regarded as most satisfactory for this oxidation is described in the following paragraph, while applications and modifications of the procedure are described thereafter. Analytical grade reagents were used.

To a solution of 30 mg of a branched-chain acid or ester in 2 ml carbon tetrachloride were added 60 mg CrO_3 and 0·2 ml acetic anhydride. This mixture was stirred with a magnetic bar in a stoppered flask at room temp. for 30 min. During this period the color of the mixture changed from yellow-brown to a brown-black, and a resinous dark-colored precipitate was formed. At the end of the reaction period, the solvent was removed at red press and room temp., the dark residue was dissolved in 15 ml water, and the resultant solution was stirred for 30 min at room temp. The water solution was next made alkaline by addition of 2·5 ml 10%NaOH, and neutral components were separated by continuous extraction with ether for 4–5 hr. In case of difficulty with emulsions, the water solution was diluted with a few ml saturated NaCl solution. Acids recovered from the alkaline solution represented relatively random attack on the chain and were not examined in most runs. The ether solution of neutral materials was concentrated essentially to dryness by distillation through a half-meter column. The residue was transferred to a 0·3 ml volumetric flask, and volume was made up with washings of the distillation tube. The resultant solution was used for either qualitative or quantitative gas chromatographic analysis.

The effects of variations in the procedure were established by degradations of 6-methyloctadecanoate. Continuation of the oxidation for longer than 30 min had no effect on the yield of tetradecanone, as did hydrolysis of the complex for longer periods. The yields of both tetradecanone and methyl 6-oxooctadecanoate were affected very little as the CrO_3 was varied from 25 to 80 mg,

TABLE 2. DEGRADATION OF METHYL 2,4-DIMETHYL-2-DOCOSENOATE[a]

Dehydrohalogenation		Yield of acids (%)[c]		
Base	Heating period[b] (hr:min)	C_{17}	C_{18}	C_{19}
$(C_2H_5)_3N$	4:00	—	3·5	5
pyridine	2:00	trace	5·5	3
α-picoline	2:00	2	11·5	2·8
α-picoline	4:00	4	10	—
diphenylamine	0:10[d]	—	7·5	5·5
quinoline	0:10[d]	3·5	9	8·2

[a] The procedure (allylic bromination, dehydrohalogenation, ozonolysis, and permanganate oxidation) was similar to that described for methyl C_{27}-phthienoate, except for the recorded variations in the dehydrohalogenation procedure.

[b] Unless otherwise indicated, heating was at the boiling point of the dehydrohalogenating agent.

[c] Yields of degradation acids were determined by gas chromatography of their esters, and comparison of the areas under the respective bands with the areas resulting from chromatography of known weights of the same esters. Yields are percent of theory for the acid in question. In instances where no yield is given, that ester was not present in reliably detectable amount. At the temperature used for chromatography, small bands for esters of acids below C_{18} were obscured by the large solvent band. A trace of hexadecanoate was usually observable.

[d] Heating was at 150°.

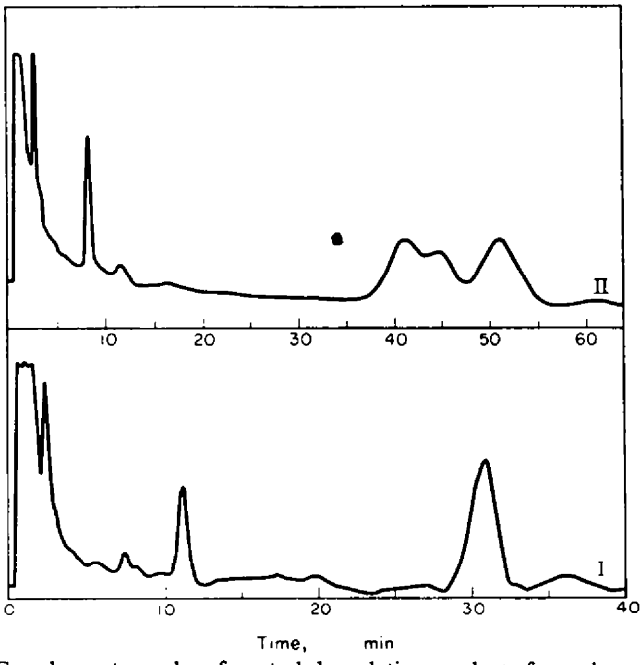


FIG. 5. Gas chromatography of neutral degradation products from chromyl acetate oxidations of compounds of known structure; 1·5 m DEGS column at 175°. The two curves were on different columns giving somewhat different retention times (methyl ester of C_{19} acid used for the oxidations appears at 30·9 min in Curve I, at 41·3 min in Curve II). *Curve* I: degradation of methyl 4-methyloctadecanoate; 2-hexadecanone at 11·3 min; band appearing in blank runs at 2·3 min. *Curve* II: degradation of methyl 6-methyloctadecanoate; 2-tetradecanone at 8·3 min; band appearing in blank runs at 2·5 min; bands from unknown by-products after the starting ester at 41·3 min.

although the amount of recovered 6-methyloctadecanoate decreased rapidly as the amount of CrO_3 was increased. With low ratios of CrO_3, the small band following tetradecanone (Fig. 5) was absent. Increase of CrO_3 to 120 mg reduced the yield of tetradecanone to zero.

Known compounds oxidized by the above-described procedure, and yielding the expected degradation products included 4-, 6-, and 10-methyloctadecanoic acids, 10-methyltetracosanoic acid, and 2,4-dimethyldocosanoic acid. In many cases, the esters were used for the oxidation. In all instances except the 4-methyl acids, injection into chromatography of 10% of the degradation products gave very large bands for the ketones and keto esters resulting from cleavage of the chain on either side of the branch. Yields were considerably lower for the 4-methyl isomers, still lower for the 2,4-dimethyl acid; so injection of about 30% of the total oxidation products is advisable. From these observations, it follows that oxidation of 10 mg samples is satisfactory. The 2- and 3-methyloctadecanoic acids gave no useful degradation products, as was the case in chromic acid oxidation.[14]

Recorder tracings included in Fig. 5 are illustrative of the results obtained, except that these tracings were chosen to show particularly large bands from the reagents ("blank" bands) and from by-products of unknown identity. The prominent band at about 2.5 min in both tracings is an unusually large "blank" band; this band is usually smaller, as shown in Fig. 2. The several small bands appearing in blank runs are also abnormally large in Fig. 5; but do not interfere with identifying the prime degradation product. The relatively larger "blank" bands in Curve I result from the lower yield of ketone from degradation of a 4-methyl acid. The chromatography shown in Curve II was carried to sufficiently long times to show the by-product bands appearing after the band for starting material. Such bands are variable for different compounds, but are always at such long retention times as to present no interference with identification of ketones. Methyl 4- and 6-oxooctadecanoates were also observed when these degradation products were chromatographed on silicone grease at 250°. On DEGS, these compounds appear at very long retention times. Although the keto esters from chain cleavage do not appear in Fig. 5 (the blank interferes with 4-oxopentanoate), in other runs on 6-methyloctadecanoate a prominent band was observed at 3·5 min for 6-oxoheptanoate when chromatography was on silicone grease at 155°. Best information is usually obtained from the ketone, unless the branch is remote from carboxyl; so oxidation of the acid, which gives only the ketone on degradation, is frequently preferable to oxidation of the ester.

The tracings from degradation products of C_{27}-phthianoic acid (hydrogenated phthienoic acid), and its ester, as shown in Fig. 2, are representative ones; however, a total of 8 samples was degraded. In many runs, chromatography was on both DEGS and silicone grease, and at different temperatures; and both blanks and degradations of known compounds were carried through in parallel procedures. Retention times were established by sequential chromatography of known compounds.