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ISOLATION, STRUCTURAL STUDIES AND CHEMICAL SYNTHESIS OF A 'PALMITONE LIPID' FROM *CORYNEBACTERIUM DIPHTHERIAE*

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The isolation of a 'palmitone lipid' from *Corynebacterium diphtheriae* is described. The use of a temporary hydrophobic protecting group allows the obtaining of the lipid in free and pure form. Structural studies by chemical degradation and mass spectrometry allow one to propose structure Ic for this compound, namely 6-(2-tetradecyl 3-keto octadecanoyl)-α-D-trehalose. This structure was confirmed by chemical synthesis.

I. Introduction

Pulse labelling experiments of the incorporation of palmitic acid by *Corynebacterium diphtheriae* cells have shown that a self-condensation product is detected in the first seconds of incubation time [1]. This product, called 'palmitone lipid', was extracted from the total lipids of the bacteria and isolated as a peracetyl derivative.

A preliminary study allowed to propose a partial structure: the palmitone lipid is a C_{32}-β-keto acid ester of trehalose, as it gives palmitone and α-trehalose upon alkaline hydrolysis.

This communication describes the isolation of the 'palmitone lipid' in the free form, the determination of its structure and the description of its chemical synthesis.

II. Materials and Methods

A. Instrumentation

Mass spectra were obtained on a Varian MAT 311 A instrument equipped with a combined ei/fi/fd ion source. For ei measurements, the sample was introduced via the direct probe system at 200°C. The temperature of the ion source was 320°C, the electron energy was settled to 70 eV. In the fd mode, the sample was deposited by the syringe technique on the carbon emitter previously coated with cesium iodide and activated, as described [3]. The source temperature was 100°C, the anode voltage +3kV, the cathode was settled to −6 kV. The emitter current was controlled by a

linear programmer from 0–50 mA, and the spectra were recorded at the best anode temperature.

The IR spectra were measured on a Perkin-Elmer 177 spectrophotometer.

The TLC radioscanner was a RTLS-1A, PANAX instrument.

B. Growth of microorganisms, labelling and extraction procedures.

Corynebacterium diphtheriae C 8 r $(-)^{tox-}$, a non-lysogenic and non-toxinogenic strain obtained from Dr Barksdale (N.Y.) was grown as previously described [7] in shaken 250 ml flasks. The cells were labelled by pouring 2 ml of a water solution of ammonium [-1-^{14}C]-palmitate (5 μCi, specific activity 50 Ci/M) into a vigorously stirred 100 ml suspension of *C. diphtheriae* cells in the late logarithmic growth phase in their culture medium (at 37°C). After 15 s incubation time, this mixture was quickly poured into 375 ml of a boiling chloroform-methanol solution 1 : 2 (v/v), and boiling was continued for 2 min. This one phase mixture was then demixed by addition of 100 ml chloroform and 100 ml water and the lower layer was evaporated under vacuum yielding labelled lipids.

C. Isolation of the 'palmitone lipid'

The labelled lipids were first fractionated on a DEAE-cellulose column, acetate form, according to Rouser et al. [8]. The different fractions were analysed by TLC (Silica Gel H, unactivated plates, solvent $CHCl_3/CH_3OH/H_2O$ 70 : 25 : 2, v/v/v, detection by radiochromatography and anthrone spray). The glycolipids were eluted from the DEAE-column by a chloroform/methanol mixture, 7 : 3, v/v.

The purification of the glycolipid fractions was performed by preparative TLC (Silica Gel H, solvent C/M/W, 70 : 25 : 2, v/v/v). Two main fractions were obtained. Fraction A, R_F = 0.55, yielded labelled palmitone after saponification; fraction B, R_F = 0.80, did not contain any labelled constituent.

Fraction A was trimethylsilylated by the pyridine/hexamethyldisilazan/trimethylchlorosilan 4 : 2 : 1, v/v/v mixture during 15 min and the solvents were evaporated under a nitrogen stream. The residue was taken up in petroleum ether and washed with cold water. The petroleum ether phase was then separated on TLC plates (Silica Gel G, solvent, petroleum ether/diethyl ether, 95 : 5, v/v). The bands were detected both by radioscanning (only one band was detected) and by spraying a 1% Rhodamine B in water solution (3 bands were observed).

The radioactive band was eluted with ether and the staining eliminated by filtration on a small column of florisil.

The recovery of the 'palmitone lipid' was achieved by selective hydrolysis of the TMS groups, as described (methanol/HCl method) [2].

Saponification/acidification of this lipid only gave labelled palmitone (identified by TLC, solvent: petroleum ether/diethyl ether 95 : 5, v/v and by GLC on a SE30 column) and trehalose (paper chromatography, solvent butanol/pyridine/water, 6 : 4 : 3, v/v/v and GLC of the TMS derivatives on a SE 52 column).

D. Synthesis of 6-(2-tetradecyl-3-keto-octadecanoyl)-α-D-trehalose

1. Transesterification method

A mixture of methyl 2-tetradecyl-3-keto-octadecanoate Ia (300 mg), anhydrous potassium carbonate (12 mg) and the hydroxylic derivative (2 mM) in 2 ml of anhydrous *N*-methyl pyrrolidone was heated at 90–100°C under vacuum of 100 Torr. After 6 h reaction time, the mixture was extracted with chloroform and washed.

2. Control analysis by the deacylation/methylation method

The chemical method for the location of acyl groups on a sugar molecule [2] cannot be used with sugar β-keto esters. As previously pointed out, the action of dimsyl sodium on β-keto esters only produced the removal of the acidic proton on carbon-2 and does not cleave the ester group [9].

A modification of the method was thus employed. After protecting all the hydroxyl groups of the free glycolipid by dihydropyrane, a 0.5 M methanolic solution of sodium methanolate was added (3 × 0.1 ml) over a period of 1 hr. The solvents were first evaporated under a stream of dry nitrogen then under vacuum (10^{-2} Torr). The residue was suspended in 0.5 ml of anhydrous diethyl ether, the flask was flushed with nitrogen and a 0.1 ml of a 1 M solution of dimsyl sodium in DMSO was added under vigorous stirring. Methylation, hydrolysis and analysis of methylated monosaccharides were worked up as described [2].

3. 2,3,4,2',3',4',6' Hepta O-trimethylsilyl-α-D-trehalose II

Five g of anhydrous α-trehalose were trimethylsilylated according to Sweeley [10]. After washings and elimination of solvents, the pertrimethylsilyl compound was obtained (9.3 g). Recrystallisation in dry acetonitrile afforded a product (m.p. 80°–82°C) lit: m.p. 80–82°C [11].

This compound was submitted to a selective alkaline hydrolysis as follows. After dissolution in 30 ml of anhydrous methanol and cooling to 0°C, 3.6 ml of a 0.3% potassium carbonate solution in anhydrous methanol were added and the mixture was stirred at 0°C. At regular time intervals, samples were analysed by TLC (benzene/ethyl acetate, 9 : 1 v/v). The R_F of pertrimethylsilyl trehalose was 0.85, and after some hours, a second spot appeared corresponding to a monohydroxylated compound with R_F = 0.57. When a third spot appeared (R_F = 0.09), (6,6' dihydroxylated compound) [11] the reaction was stopped by addition of the calculated amount of acetic acid, and the mixture was extracted by petroleum ether and cold water. The organic layer was concentrated and separated on a neutralized silicic acid column using petroleum ether/diethyl ether mixtures as solvent. 5.6 g of starting material were recovered, and 2.7 g of compound II (R_F = 0.57) were obtained. A second hydrolysis of the recovered starting material afforded 1.8 g of compound II.

The amorphous compound (m.p. 76°–78° $[\alpha]_D^{18}$ = +115°) (C = 0.7, petroleum ether) possesses the expected characteristic spectroscopic properties, hydroxylic band at 3400 cm^{-1} in the IR spectrum, M-15 peak in the mass spectrum at *m/e* 831, oxonium ions at *m/e* 451 and 381.

The structure of compound II was controlled as follows: a sample was acylated by palmitoyl chloride in pyridine, and the trimethylsilyl groups were removed. The recovered product was identical with 6-*O*-palmitoyl-α-trehalose already described [11] and its analysis by the desacylation/methylation hydrolysis method afforded an equimolar mixture of 6-*O*-methyl-glucose and glucose.

4. 2,3,4,2',3',4',6' Hepta-O-tetrahydropyranyl-α-D-trehalose III

Compound II was acetylated by acetic anhydride in pyridine (0°C overnight) and the TMS groups were removed by heating 6 hr. under reflux with a mixture of 65 ml methanol and 22 ml water. The crude 6-*O*-acetyl-α-D-trehalose was then added to a mixture of anhydrous diethyl ether (20 ml), dihydropyran (3 ml) and p. toluene sulfonic acid (3 mg). After complete dissolution, a methanolate solution was added (100 mg sodium in 50 ml methanol), and the mixture was left to react for 2 hr. The solvents were removed under vacuum and the residue was partitioned between water and ether. The ethereal solution was then evaporated and the crude yellow residue (III) was used without purification in the transesterification reaction.

5. Transesterification of Ia by II

The reaction was carried out as described above. The crude transesterification mixture was submitted to the hydrolysis of TMS groups (0.1 N HCl in chloroform/methanol 9 : 1, v/v, 5 min at 0°C) and fractionated on silicic acid. A fraction eluted with chloroform/methanol, 8 : 2, v/v, gave the β-keto esters of trehalose Ib (R_F = 0.62–0.68, solvent chloroform/methanol/water 65 : 25 : 4, v/v/v). The IR spectrum exhibited two C=O bands at 1700 cm^{-1} and 1740 cm^{-1}. The mass spectrum of the per TMS derivative showed the expected molecular ion (weak intensity) at *m/e* 1322. A TLC separation of the TMS derivatives of this fraction showed two major spots, and two minor ones. Chemical analysis by the above described desacylation/methylation method indicated that two major isomers Ib were present, the 6-*O* acylated (70%) and the 2-*O* acylated one (25%). The 3- and 4-*O*-acylated isomers represented less than 5% of the mixture.

6. Transesterification of Ia by III

The product obtained after transesterification as described above was submitted to partial hydrolysis (10% HCl in methanol, 0°C, 15 min) which effected the cleavage of the protective groups. Chromatography on neutralized silicic acid (silicar CC7) afforded the expected compound Ic in a 1.3% yield.

m.p. 108–110°C, $[\alpha]_D^{20}$ + 75° (C = 0.3, $CHCl_3$)

IR spectra: 2 CO bands 1700 and 1740 cm^{-1}

Mass spectrum: see fig. 1 B

Only one spot was observed by TLC analysis of the TMS derivatives and chemical analysis indicated that only the 6-O-acylated isomer was present.

III. Results

A. Isolation of the 'palmitone lipid'

Labelled cells of *Corynebacterium diphtheriae* are obtained by short time incubation of living cells with ammonium [1-^{14}C] palmitate. The total lipid extract is fractionated by DEAE-cellulose chromatography. The detection of the 'palmitone lipid' in the different fractions is realized by checking the radioactivity of palmitone in the hydrolysis products. A neutral fraction thus obtained consists essentially of a mixture of glycolipids and contains the radioactive 'palmitone lipid' as the only labelled glycolipidic constituent. The purification of the radioactive fraction is carried out by preparative TLC and a labelled product, called fraction A, is isolated. Fraction A behaves as a single spot on TLC. Its alkaline hydrolysis yields radioactive palmitone and unlabelled fatty acids as liposoluble components.

The resolution of fraction A into its components was first obtained by TLC of peracetylated derivatives. A peracetylated 'palmitone lipid' was isolated, and a preliminary study of its structure was already described [1]. However, the recovery of the free lipid from this derivative is not possible, and the determination of the position of the β-keto acid ester linkage on the trehalose moiety could not be easily done on this kind of derivatives.

To obtain the 'palmitone lipid' in the free form fraction A is pertrimethylsilylated and fractionated by TLC, according to a method described for the separation of acylated sugars [2]. Three major glycolipid spots are observed only one of them being labelled. The radioactive lipid is isolated and the free glycolipid is recovered by selective hydrolysis of the TMS groups. The purity and composition of the 'palmitone lipid' is determined by analysis of the alkaline hydrolysis products. α-Trehalose and labelled palmitone are the only constituents that could be detected.

The 'palmitone lipid' is a minor constituent of *C. diphtheriae*. Due to the low amount of the isolated material, a structural study by chemical means only would be extremely difficult. Therefore mass spectrometry methods together with radioactive measurements have been used in this study.

B. Mass spectrometry study of the 'palmitone lipid'

A field desorption mass spectrum obtained by the cesium cationizing method [3], shows an intense cationized ion $(M + Cs)^+$ at *m/e* 951, which indicates a mol wt. of 818 for the free lipid. A low intensity peak at 28 mass units lower shows the presence of an homologous compound containing two carbon atoms less. At least one other peak of low intensity is present at *m/e* 949, that could suggest the presence of an unsaturated compound. However, we have shown that loss of hydrogen from the cationized molecules can occur to a low extent under the fd conditions, and the relative intensity of the corresponding peak in the spectra is consistent with occurrence of such a fragment [3]. The mol wt. determination of this compound, which liberates palmi-

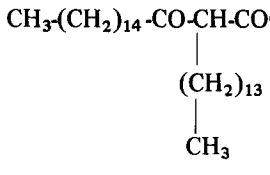
tone (16-keto hentriacontane) and trehalose by alkaline hydrolysis, indicates that the 'palmitone lipid' is a trehalose monoester of 2-tetradecyl-3-keto octadecanoic acid. Moreover, this compound possesses 7 functional groups that could be acetylated, as is shown by the mol. wt. of 1112 found by f.d.m.s. of the peracetyl derivative [1].

The location of the ketoacyl group is tentatively assigned by examination of the ei mass spectrum of the TMS derivative [4]. Characteristic features for the unacylated glucose moiety or a 6-acylated one are observed with the presence of *m/e* 204 and *m/e* 217, the low abundance of *m/e* 132 and the low intensity of *m/e* 218. To assign more precisely the position of acylation, the acyl containing fragments are searched for, and a characteristic ion for a 6-acylation is found at the expected value *m/e* 609 (structure *a*, see later). However, an analogous ion containing two TMS groups is found at *m/e* 711. This ion may result from a more extensively silylated compound in which the 3-keto group has been transformed into the TMS ether of the enol form. Moreover, the expected characteristic ions for other positions of acylation on the trehalose molecule are not present in the spectrum: *m/e* 694 for a 2- or 4- acylation and *m/e* 608 for a 2- or 3-acylation).

In conclusion, if the presence of a 3-keto group on the acyl part of the molecule does not induce any dramatic changes in the fragmentation pathways of the glucosyl moieties of the acyl trehalose, this spectrum is consistent with an acylation on the 6-position. To verify these assumptions, we have synthesized the corresponding compound, namely, 6-(3-keto 2-tetradecyl octadecanoyl) α-D-trehalose and compared the synthetic with the natural sample.

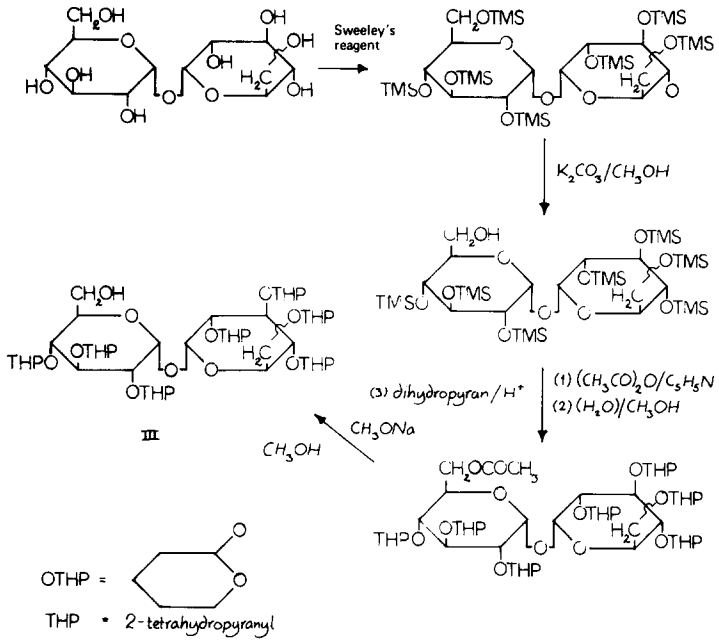
C. Synthesis of 6-(2-tetradecyl-3-keto octadecanoyl)-α-D-trehalose

Previous attempts to obtain β-ketoacylated-α-D-trehalose by direct alkaline transesterification of β-keto acid methyl esters with trehalose were unsuccessful. An intramolecular nucleophilic interaction between an alcoholate anion and the 3-keto group, in an intermediary formed trehalose β-keto acyl ester, induces a rapid retro-Claisen reaction giving rise to the cleavage of the β-keto acyl chain [5]. However, such an interaction would not occur with a selectively protected trehalose molecule, and in this way we have synthesized trehalose derivatives protected on 7 hydroxyl groups, and tried to react the remaining free hydroxyl group with the methyl β-keto acid ester (I_a) in an alkaline transesterification.



I_a ROH = methanol

I_b R-OH = trehalose (without assignment of the acyl location).

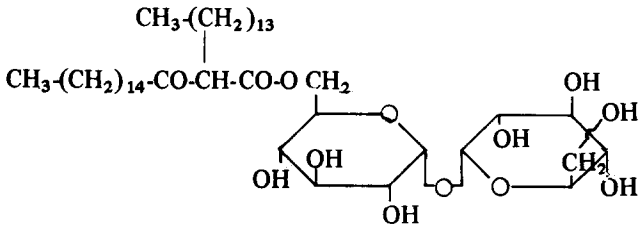


Scheme 1

The synthesis of two trehalose derivatives possessing a free 6-hydroxyl group is presented in Scheme 1.

Transesterification of I_a with 2,3,4,2′,4′,6′-hepta-*O*-trimethyl silyl-α-D-trehalose (II) and subsequent removal of the TMS groups affords I_b in a 15% yield. However, analysis of this product indicates that it contains a mixture of isomers, the 6- and 2-*O* β-ketoacyl isomers being the main components. This mixture could be the result of an isomerisation of compound II, prior to transesterification, in the alkaline reaction medium.

Transesterification of I_a with the 2,3,4,2′,3′,4′,6′ hepta-(*O*-2-tetrahydropyranyl)-α-D-trehalose (III), followed by removal of the protective groups, gives I_b in low yield (1.5%). Analysis of this compound both by TLC separation of the TMS derivatives and by chemical ways indicates that only one isomer is present in which the 6-hydroxyl group of trehalose is esterified. Besides, all spectrometric determinations are consistent with structure I_c.



I_c

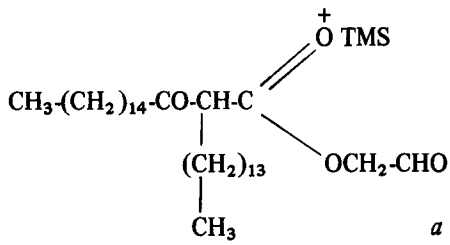
The low yield may be the result of a steric hindrance in the transition state of the transesterification reaction, as it was already mentioned [6]. However, the replacement of the tetrahydro pyranyl groups by *O*-(1-methoxy ethyl) groups does not increase the yield.

D. Comparisons between the 'palmitone lipid' and compound I_c

The chromatographic behaviour of the natural and synthetic compound are identical. The two compounds have the same R_F value, either in the free form, or as TMS derivative.

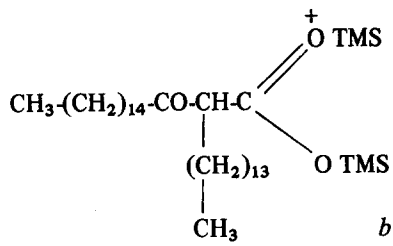
The mass spectra are also comparable. With the f.d.m.s./CsI method, a molecular cationized peak is observed at the same value (*m/e* 951) together with a low intensity peak at *m/e* 949 which agrees with the above assumption for a desaturation reaction on the emitter.

The e.i. mass spectra of the two compounds are nearly identical (fig. 1). High resolution measurement on the characteristic fragment ion *m/e* 609, and the shift of 9 mass units of the corresponding ion in the spectra of trideutero TMS derivatives are consistent with the postulated structure *a* for this fragment.



a

Other intense peaks in these spectra could be easily interpretated by analogy with the spectra of palmitoyl methyl glucopyranosides [4]. The ion that only contains the β-keto acyl chain (structure b) is present at m/e 639.



b

Ions resulting from the splitting of the glycosidic bond possess a low intensity. However, the loss of a neutral molecule from these ions gives an intense peak at *m/e*

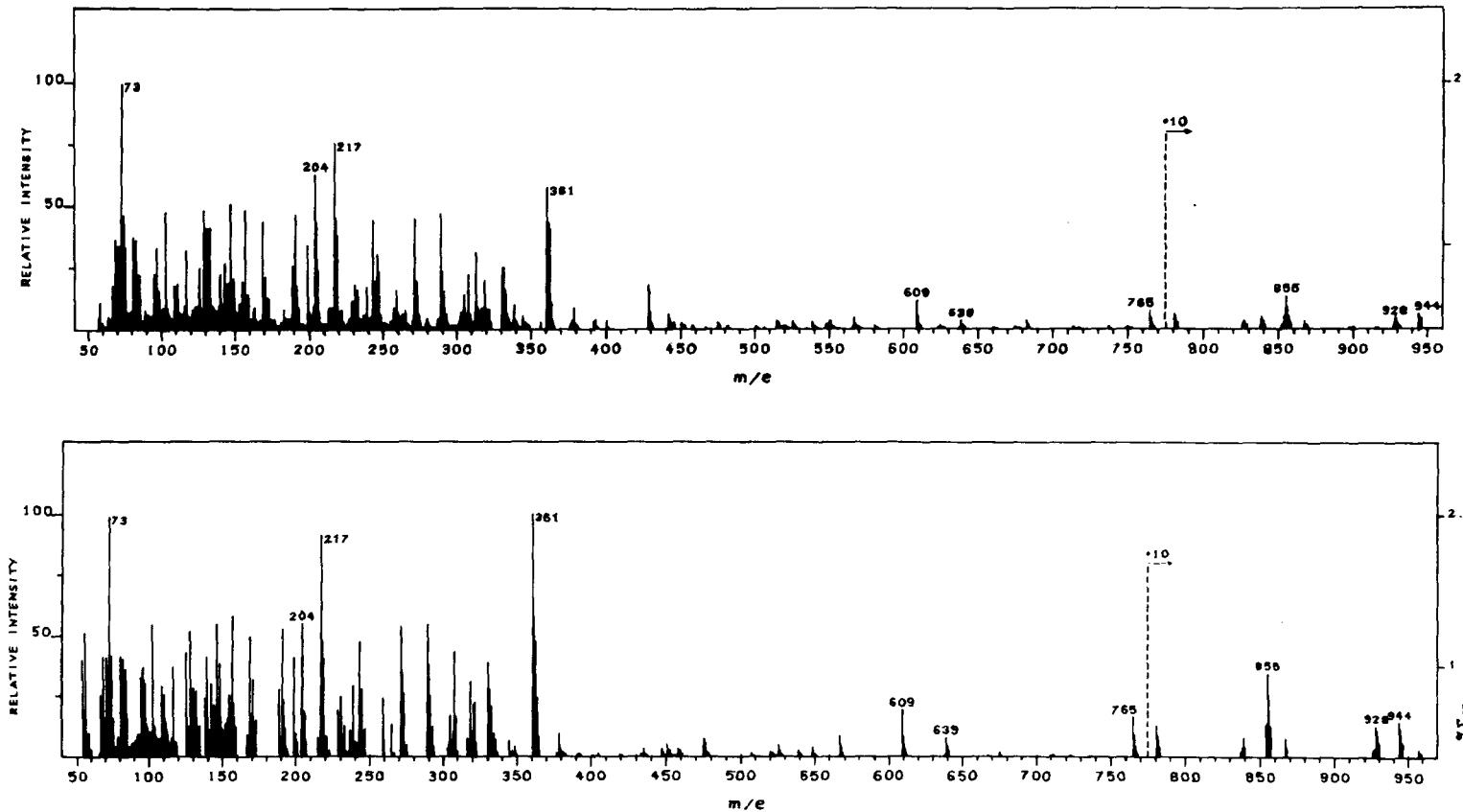


Fig. 1. Comparison between the ei mass spectra of the pertrimethyl silylated 'palmitone lipid' (A) and the synthetic compound I_c (B).

765 (loss of TMSiOH) from the ketoacylated oxonium) and *m/e* 361 (loss of TMSiOH from the unacylated oxonium or loss of the β-keto acid from the acylated one). The presence of an homologous compound in the spectra of the 'palmitone lipid' is shown by a low intensity peak at *m/e* 737 (765–28) absent in the spectrum of the synthetic compound which corresponds to the homolog (mol. wt. 790) already seen in the f.d. spectra of the natural compound.

V. Discussion

The results of our experiments show that the 'palmitone lipid' does possess formula I_c.

However, two questions remain: Firstly, does the isolation procedure of the 'palmitone lipid' produce any change in the structure of this compound and secondly, what is the configuration of the asymmetric center on the 2-position of the acyl chain?

We have already checked that the purification of acylated sugars by means of temporary trimethyl silyl groups is a reliable method towards isomerisation reactions [2]. However, as the 'palmitone lipid' possesses a keto group that could be positioned near glycol groups, one can assume that the lipid could exist in a cyclic form (internal ketal or hydroxyketal forms) that might be destroyed during the mild hydrolysis of the TMS groups. We have confirmed that the labelled 'native' lipid–before the last purification–and the synthetic one have the same chromatographic behaviour, either in the free form or as TMS derivative. However, the existence of cyclic forms that could be very unstable cannot be ruled out.

The configuration of the asymmetric center on the 2-position of the acyl chain cannot be determined by comparison with synthetic samples as the two diastereoisomers that exist in the synthetic product are neither distinguished by chromatography nor by analysis of their spectroscopic properties. This question will be studied further.

The chemical properties of the 'palmitone lipid' (easy decomposition into palmitate esters in a slightly alkaline medium) and the pulse labelling studies lead one to think that this compound may be a key derivative in the metabolism of corynomycolic acid. We propose, as a hypothesis, that either in the biosynthetic way or in the catabolic way, the trehalose molecule could serve as a matrix which would place at the correct interacting distance the functional groups fixed on it, and thus permits the degradation of the C_{32}-β-keto ester I_c into dipalmitate esters (catabolic way), or promotes the condensation of two palmitic esters (or carboxyl-activated one) into C_{32} -β-keto acyl compounds (biosynthetic way) that might be further reduced.

Acknowledgments

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