2,3-*erythro*-Dihydroxyhexacosanoic acid and homologs: isolation from yeast cerebrin phosphate and determination of their structures

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Abstract Homologs of methyl esters of very polar fatty acids were obtained by methanolysis of cerebrin phosphate isolated from baker's yeast. The major ester component was isolated by preparative gas–liquid chromatography and was found to be 2,3-dihydroxyhexacosanoic acid as deduced from the mass spectra of its trimethylsilyl ether and isopropylidene derivative, reaction with periodate, and comparison of its chromatographic behavior with that of synthetic *erythro*- and *threo*-dihydroxy acids. Its infrared spectrum supported the above conclusions. From their retention times by gas–liquid chromatography, the homologs were found to be saturated, unbranched 2,3-dihydroxy fatty acids with 24–27 carbon atoms. The synthesis of the new fatty acids, *erythro*- and *threo*-2,3-dihydroxyhexacosanoic acids, is also reported. A method for separating *trans*-2-hexacosenoic acid, a key intermediate of the above synthesis, and its isomer, *trans*-3-hexacosenoic acid, both formed by dehydrobromination of 2-bromohexacosanoic acid, is also described.

Supplementary key words gas–liquid chromatography · thin-layer chromatography · mass spectrometry · 2-hydroxy fatty acids · phytosphingosine · synthesis of 2,3-dihydroxyhexacosanoic acid · 3,4-dihydroxyhexacosanoic acid · 13,14-dihydroxydocosanoic acid · 2-hexadecenoic acid · 3-hexadecenoic acid

CEREBRINS, ceramide-like compounds that are composed of a sphingosine base and a long-chain fatty acid linked by an amide bond, have been isolated from various sources, including yeasts (1–4), mycelium of a surface-cultivated penicillin-producing fungus (5), and *Aspergillus sydowi* (6). They were found to be acyl amides of phytosphingosine (2-amino-3,4-dihydroxyoctadecanol) or its C_{20} homolog (7). Of the acyl moieties, 2-hydroxyhexacosanoic acid has been identified as the major fatty acid. It is accompanied by its homologs and their corresponding unsubstituted fatty acids (2, 4, 5). All of these cerebrins were, however, products of autolysis of the fungus or of saponification of crude yeast lipids. Oda and Kamiya (8) made an effort to isolate cerebrin from an intact baker's yeast without success. Instead, they obtained cerebrin phosphate in which the phosphate group is attached to position 1 of phytosphingosine. The fact that the yield of the cerebrin phosphates increases after brief autolysis of the yeast suggests that cerebrin phosphates are degradation products of yet a larger lipid complex. A complex sphingoglycolipid containing inositol, phosphorus, and mannose, as well as very long chain unsubstituted and 2-hydroxy fatty acids, was recently isolated from plasma membrane of baker's yeast (9).

In addition to the previously reported 2-hydroxy fatty acids, we have now identified 2,3-dihydroxy fatty acids in cerebrin phosphate. A dihydroxy fatty acid was obtained previously from a penicillin-producing fungus and its structure was determined to be 2,3-dihydroxytetracosanoic acid by Oda (5) from its elemental analysis and its chromic acid oxidation product. Subsequently, the same acid was synthesized by Palameta and Prostenik (10). However, no direct comparison of the synthetic compound with the fungal dihydroxy acid was made, and the synthetic procedure employed by these authors does not exclude the possibility of the formation of 3,4-dihydroxy acids or other isomers.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid partition chromatography; chain length of a fatty acid is expressed by a subscript after C, e.g., C_{26} represents a compound with 26 carbon atoms; OV-1 and SE-30 are nonpolar silicon gums, and DEGS is a polymer of diethylene glycol succinate; GLC–MS, combination of GLC and mass spectrometry; TMS, trimethylsilyl.

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This communication describes the isolation and structure determination of 2,3-dihydroxy fatty acids obtained from yeast cerebrin phosphate and the unequivocal synthesis of *erythro-* and *threo*-2,3-dihydroxyhexacosanoic acids.

EXPERIMENTAL PROCEDURES

Chromatography

Unisil, 100–200 mesh (Clarkson Chemical Co., Williamsport, Pa.), was used for column chromatography throughout this study. Unsubstituted monohydroxy and dihydroxy fatty acid methyl esters derived from yeast cerebrin phosphate by methanolysis were eluted with 44 ml each of hexane–benzene 6:4, benzene, and $CHCl_3$, respectively. TLC was performed using precoated silica gel G plates (0.25 mm thick, 20 × 20 cm or 2.5 × 9 cm; Analtech, Inc., Newark, Del.) or $AgNO_3$-impregnated silica gel G plates. The latter were prepared using essentially the procedure reported by Morris (11). Ninhydrin, bromothymol blue spray, iodine vapor, or charring with 50% H_2SO_4 was used for general lipid detection and 2,4-dinitrophenylhydrazine spray specifically for aldehyde detection.

An F & M model 7624A (Hewlett-Packard, Avondale, Pa.) or Packard model 700 (Packard Instruments, Downers Grove, Ill.) was used for GLC. For analytical purposes, glass columns (6 ft) packed with 3% OV-1 or with 25% DEGS on Chromosorb W were used with a flame ionization detector. The effluents were collected in glass tubing with a Swinny hypodermic adapter containing a Millipore filter (12). The material collected was eluted and purified by preparative TLC to eliminate the contamination from the silicone gum.

Mass spectrometry

High resolution mass spectra were obtained with a CEC-21-110B high resolution spectrometer (Consolidated Electrodynamic Corp., Monrovia, Calif.). Low resolution mass spectra were obtained with a GLC–MS–computer system described by Hites and Biemann (13) using a 3% SE-30 column.

Infrared and ultraviolet spectrometry

Infrared spectra were taken as KBr disks, as solutions in CCl_4 or CS_2, or as thin films. A Beckman IR-33 infrared spectrometer (Beckman Instruments, Fullerton, Calif.) was used for this purpose. Ultraviolet spectra were obtained with a Unicam SP1800 ultraviolet spectrophotometer (Pye Unicam Ltd., Cambridge, England).

Other analyses

Melting points were determined with a Fisher-Johns melting point apparatus. Phosphorus was determined according to Rouser, Siakotos, and Fleischer (14). Elemental analysis was performed by the microanalytical laboratory at Massachusetts Institute of Technology.

Isolation of cerebrin phosphate from yeast

A pure culture of baker's yeast was purchased from Federal Yeast Corp. (Baltimore, Md.). Crude cerebrin phosphate (135 mg) was isolated from 4.5 kg of the yeast according to the procedure of Oda and Kamiya (8). The contaminating esters were eliminated by mild alkaline methanolysis (15). The cerebrin phosphate thus obtained was washed twice with 2 ml of ether at 0°C and recrystallized repeatedly from glacial acetic acid and finally from ethanol.

Methanolysis of cerebrin phosphate and isolation of fatty acid methyl esters

Cerebrin phosphate (30 mg) was subjected to methanolysis with 3 ml of 5% methanolic HCl at 75°C for 17 hr (16). This reaction mixture was extracted three times with 3 ml each of hexane–ether 99:1. The combined hexane–ether extract was evaporated under nitrogen. The residue was fractionated by chromatography on columns containing 1 g of Unisil as described above.

Isolation of the individual esters

Methyl esters of dihydroxy fatty acids were converted to diacetyl derivatives (see below) and fractionated by preparative GLC on 10% SE-30 at 275°C. An aliquot containing 500 μg of the esters was injected for each experiment.

Only the most abundant homolog, which was found to be 2,3-diacetylhexacosanoic acid methyl ester during the course of this study, was analyzed further. This compound was purified by silica gel TLC with hexane–ether 6:4, and the purity was examined by analytical GLC with an OV-1 column. Diacetyl derivatives were deacetylated by a mild alkaline methanolysis and purified further by preparative TLC with chloroform–methanol 97:3.

Periodate oxidation of dihydroxy fatty acid from yeast

The dihydroxy fatty acid was reacted with periodate according to the procedure of Sweeley and Moscatelli (17). A 0.7-mg aliquot of methyl 2,3-dihydroxyhexacosanoate, purified by preparative TLC, was dissolved in 1.10 ml of $CHCl_3$–CH_3OH 3:8 and mixed with 0.2 ml of freshly prepared 20 mM $NaIO_4$. The mixture was allowed to stand in the dark at room temperature for 48 hr. The excess periodate was determined, and the product was isolated as previously described (18). Identification of the aldehyde produced was achieved by TLC by comparing it with an authentic standard and by

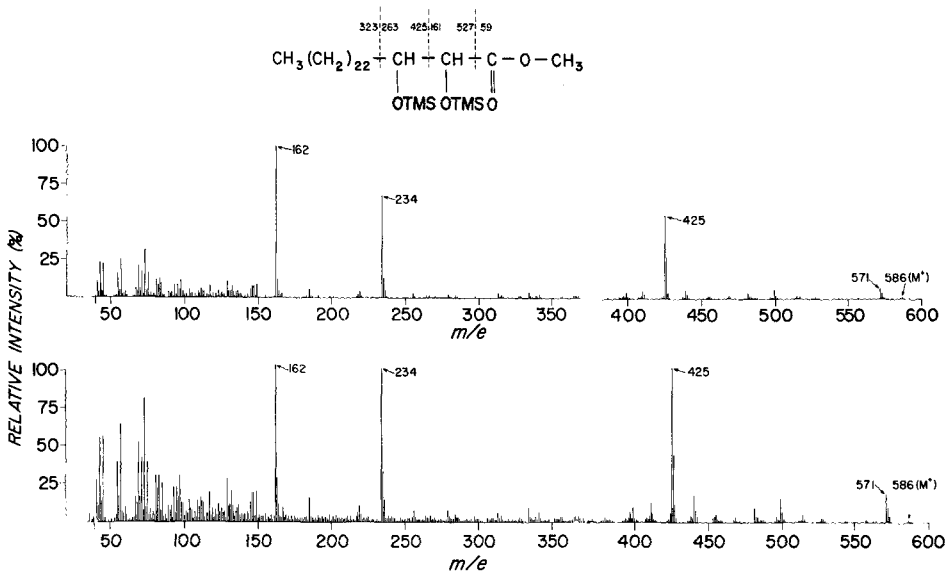


FIG. 1. Mass spectra (70 eV) of methyl 2,3-di-*O*-TMS-hexacosanoate. The top spectrum is of the ester derived from yeast cerebrin phosphate and the bottom one is of the synthetic compound (*erythro* isomer).

spraying the plate with dinitrophenylhydrazine; benzene was the developing solvent.

The fatty aldehyde thus obtained was oxidized with silver oxide (19), and the fatty acid produced was isolated by column chromatography on Unisil. A small amount of unreacted aldehyde was first eluted with hexane–benzene 6:4; chloroform eluted the fatty acid.

Synthesis of 2,3-dihydroxyhexacosanoic acid

Trans-*2-hexacosenoic acid.* This fatty acid was synthesized from hexacosanoic acid (Lachat Chemicals, Inc., Chicago Heights, Ill.) by α-bromination[3] followed by dehydrobromination (20). Briefly, hexacosanoic acid was first converted to the acid bromide and then reacted with bromine in a sealed tube at 95°C for 5 hr. After isolation of the α-bromo acid, it was refluxed for 4 hr with 20 ml of *t*-butanol containing 25 mmoles of potassium *t*-butoxide. The fatty acid was extracted from the acidified reaction mixture with hexane–ether 4:1, recrystallized from hexane, and then further recrystallized from hexane–ether 5:4. After esterification of the hexacosenoic acid by diazomethane, methyl hexacosenoate was purified by Unisil column chromatography with a single elution with hexane–benzene 6:4.

It has been reported that the migration of the double bond from C-2,3 to C-3,4 occurs during the course of dehydrobromination under these conditions (20). In agreement with this observation, we obtained two peaks, tentatively labeled A and B, upon GLC of the methyl esters. The ratio of the amount of A to B was 1:2.7. The relative retention times of the components to methyl *n*-hexacosanoate were 0.95 and 1.14 on the OV-1 column and 1.21 and 1.56 on DEGS. These two isomers were separated by preparative GLC using an SE-30 column. On periodate–permanganate oxidation, tricosanoic acid was obtained from compound A, whereas compound B yielded mainly tetracosanoic acid. These results clearly indicated that A is methyl 3-hexacosenoate and B is methyl 2-hexacosenoate. Furthermore, in agreement with this assignment, the ultraviolet spectrum of compound B in methanol showed an intense *K* band (max 214 nm), whereas A had no such absorption. The infrared spectra of both isomers showed absorption at 965 cm^{-1} due to the *trans* double bond, and hydrogenation of these isomers yielded the same methyl *n*-hexacosanoate. Their mass spectra, which were similar, also confirmed that both were methyl hexacosenoate. Indicative ions were present at M^+ (m/e = 408), $M^+ - 15$, $M^+ - 32$, $M^+ - 74$, m/e = 87 ($CH_3OC(OH)=CH-CH_2^+$), and m/e = 74 ($CH_3OC(OH)=CH_2^+$). Unexpectedly, the ions at m/e = 113 ($CH_3O-C(=O)-CH=CH-CH_2-CH_2^+$) and m/e = 311 ($M - 97$) (assigned as $C_{20}H_{39}O_2$ by high resolution MS), which were reported as diagnostic fragments of methyl

[3] Private communication from Drs. N. S. Radin and A. C. Arora.

esters of 2-unsaturated acids (21), were abundant in both spectra but were more intense in that of the 2-isomer.

2,3-Dihydroxyhexacosanoic acid. Oxidation of the double bond of methyl *trans*-2-hexacosenoate with alkaline $KMnO_4$ under the conditions described by Palameta and Prostenik (10) afforded the corresponding *cis*-2,3-diol; 5 μmoles of the above methyl *trans*-2-hexacosenoate were hydrolyzed, and the resulting acid was dissolved in 2 ml of 0.31 N KOH at 60°C. After the mixture was cooled to 0°C, 0.5 ml of 1.6% $KMnO_4$ was added dropwise while stirring, and the stirring was continued for another 2 hr at the same temperature. The reaction mixture was then acidified with sulfuric acid and decolorized with Na_2SO_3, and the fatty acids were extracted with ether.

The *trans*-diol was prepared with performic acid oxidation using a modification of the procedure of Swern et al. (22). A mixture of *trans*-2-hexacosenoic acid (2–50 μmoles), 0.5 ml of 90% formic acid, and 0.1 ml of 30% H_2O_2 was heated to 100°C for 2 hr and then evaporated to dryness under a stream of nitrogen. The residue was saponified with 1 ml of 2 N NaOH at 100°C for 30 min. The mixture was then acidified with sulfuric acid, and the fatty acid was extracted with ether. Both *erythro*- and *threo*-2,3-dihydroxyhexacosanoic acids were converted to their methyl esters and purified by Unisil column chromatography. The addition of two hydroxyl groups to the double bond of 2-hexacosenoic acid proceeded in poor yields.

The poor yields in this reaction were not due to migration of the double bond from the C-2,3 position to the C-3,4 position, a process which easily occurs in fatty esters under certain conditions (20). That this was not the case is established because (*a*) the oxidation of both *erythro* and *threo* isomers of synthetic 2,3-dihydroxyhexacosanoic acid by periodate–permanganate yielded tetracosanoic acid with only a trace of tricosanoic acid and (*b*) the mass spectra of the trimethylsilyl derivatives of the synthetic methyl 2,3-dihydroxyhexacosanoates (Fig. 1) showed very abundant ions at *m/e* 425, 234, and 162 (see Results for the assignment of these fragments). These three fragments may be obtained only from the 2,3-dihydroxy derivative and could not be derived from the 3,4-dihydroxy derivative.

No reaction of *trans*-2-hexacosenoic acid was observed upon treatment with OsO_4. The greater resistance of α,β-unsaturated carbonyl compounds to cationoid oxidants than isolated double bonds is due to electron withdrawal by the carbonyl group and has been reported by others (23).

Other fatty acids

2,3-Dihydroxytetracosanoic acid, isolated from the mycelium of penicillin-producing mold Q176, was a gift from the late Dr. T. Oda. *Erythro*- and *threo*-3,4-dihydroxyhexacosanoic acids were synthesized from *trans*-3-hexacosenoic acid (the byproduct of synthesis of the *trans*-2 isomer) by the procedure described for the synthesis of the 2,3-dihydroxy isomers. Both *erythro* and *threo* isomers of 13,14-dihydroxydocosanoic acid were also prepared from erucic acid (Lachat Chemicals, Inc., Chicago Heights, Ill.). In addition, 3-hexadecenoate and erucate were also converted to the corresponding *cis*-diols by treatment with osmium tetroxide according to the procedure of Polito, Naworal, and Sweeley (24).

Derivatization of fatty acids

Methyl esters of fatty acids were prepared either by treatment with 5% methanolic HCl (16) or by reaction with diazomethane (25). Methyl esters were saponified with a mixture of 95% ethanol–40% KOH 19:1 at 80°C for 2 hr (26). Acetylation of dihydroxy fatty acid methyl esters essentially followed the procedure of Carter et al. (27). Isopropylidene derivatives of dihydroxy fatty acids were prepared by the procedure of McCloskey and McClelland (28). The TMS ether of the dihydroxy fatty acid methyl ester was prepared by gently warming the ester with a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane 9:3:1. Hydrogenation of unsaturated fatty acids was accomplished according to the procedure of Brown and Brown (29) as modified by Kishimoto and Radin (30).

Oxidative cleavage of dihydroxy fatty acid and unsaturated fatty acid methyl esters

For the oxidative cleavage of dihydroxy fatty acid or monounsaturated fatty acid methyl esters with $NaIO_4$–$KMnO_4$, the method of Scheuerbrandt and Block (31) as modified by Spence (32) was followed.

RESULTS

Cerebrin phosphate

The overall yield of this lipid from fresh yeast was 0.024%. The melting point of cerebrin phosphate was 170–172°C (dec). Its infrared spectrum (Fig. 2) showed strong amide bands (1650 cm^{-1} and 1540 cm^{-1}) and absorption by the phosphorous group at 1240 cm^{-1} and 1040–1100 cm^{-1} (33) but no absorption at 1750 cm^{-1}, which indicated the absence of any fatty acid ester in this preparation. Elemental analysis of this compound gave the following percentage compositions: C, 65.70; H, 11.15; N, 1.48; P, 3.01.

Two groups of fatty acid methyl esters were obtained by methanolysis of cerebrin phosphate. When examined by TLC, one group had an R_F value corresponding to 2-hydroxy fatty acid methyl esters and the other to that

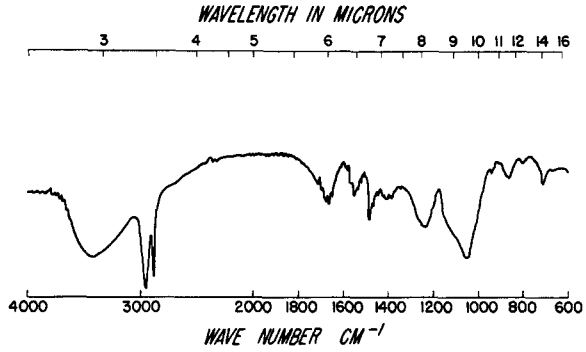


FIG. 2. Infrared spectrum of yeast cerebrin phosphate in KBr.

of authentic 2,3-dihydroxy fatty acid methyl esters. Only traces of unsubstituted acids were detected. Methyl esters of monohydroxy fatty acids were recovered in the benzene eluate from a Unisil column, and the chloroform fraction consisted of only methyl esters of dihydroxy fatty acids. The ratio of the amount of monohydroxy fatty acid esters to that of dihydroxy fatty acid esters was 2.1:1. The gas–liquid chromatogram of the dihydroxy fatty acid ester fraction, analyzed after trimethylsilylation, exhibited one major component which was isolated by preparative GLC. The structure of this compound was identified as methyl 2,3-*erythro*-di-*O*-TMS hexacosanoate as described below.

The sphingosine base was recovered from the methanol layer after methanolysis; TLC of this base revealed two ninhydrin-positive spots. One of these was phytosphingosine, in agreement with the previous finding (8), and the other had a much higher R_F value than phytosphingosine.

Structure determination of 2,3-dihydroxy fatty acids

In Fig. 3 are shown infrared spectra of the dihydroxy fatty acid ester (spectrum 3) and methyl 2-hydroxyhexacosanoate (spectrum 2) isolated from yeast cerebrin phosphate. The spectra were run under identical conditions and both exhibit a strong band at 1750 cm^{-1}, which confirms the presence of an ester group. Spectrum 2 exhibits a single O—H stretching vibration at 3560 cm^{-1} in the higher frequency region compared with two, at 3310 and 3470 cm^{-1}, in spectrum 3. The presence of two peaks, both shifted to lower frequency due to hydrogen bonding, and increased absorption in spectrum 3 confirmed the identity of the compound as a dihydroxy ester.

When the dihydroxy ester was oxidized by periodate, 1 nmole of the ester consumed 1.08 nmoles of the reagent during 48 hr. The fatty aldehyde formed in this reaction was oxidized to a fatty acid by silver oxide. GLC of the methyl ester of this acid fraction revealed that approximately 95% was *n*-tetracosanoic acid.

Further evidence concerning the structure of the dihydroxy ester was provided by mass spectra of its TMS ether and isopropylidene derivative. The spectrum of the TMS ether exhibited ions characteristic of this class of compounds (34), and, as indicated in Fig. 1, a molecular ion (*m/e* 586) and M − 15 (*m/e* 571) ion were clearly observed. These ions and an abundant ion at *m/e* 467 (M − 15) in the mass spectrum of the isopropylidene derivative are all in agreement with the structure of the original molecule being a dihydroxyhexacosanoic acid. Three very abundant ions, which were observed in the spectrum of the TMS ether at *m/e* 162, 234, and 425, confirmed the location of the hydroxyl groups at C-2 and C-3. The ions at *m/e* 162, derived from the McLafferty rearrangement (35) of a hydrogen atom from C-4, and *m/e* 234, also derived from a McLafferty rearrangement but involving the migration of a trimethylsilyl group from the second TMS ether group, establish the presence of one hydroxyl group at C-2. The rearrangement of a TMS group to form the ion at *m/e* 234 is not unexpected since the propensity of the

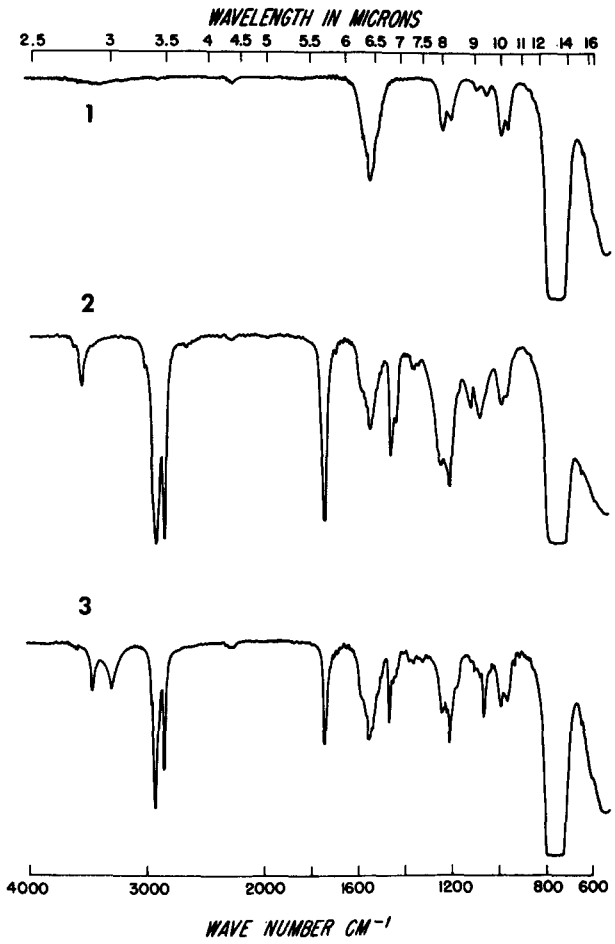
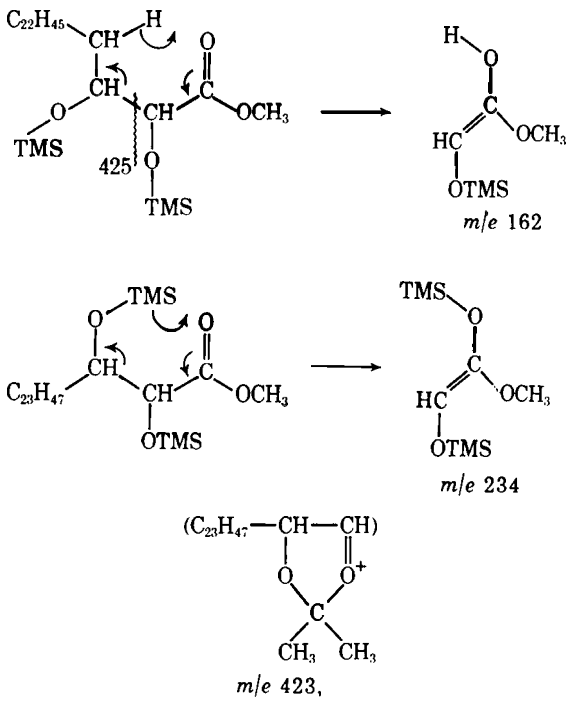


FIG. 3. Infrared spectra of solutions of methyl 2-hydroxyhexacosanoate (spectrum *2*) and methyl 2,3-dihydroxyhexacosanoate (spectrum *3*) from yeast cerebrin phosphate in CCl_4. Spectrum *1* shows the spectrum of CCl_4.

TMS group to migrate upon electron impact is well established (36).

The ion at 425, which involves cleavage of the bond between carbon atoms 2 and 3 and contains 24 of the 26 carbon atoms in the original compound, places the second hydroxy group on C-3. The fragmentation processes leading to these three ions are shown below.



An abundant ion at *m/e* 423, shown above, which was observed in the mass spectra of the isopropylidene derivatives of both the natural and synthetic dihydroxy esters, further confirmed that the two hydroxyl groups in these acids are located on carbon atoms 2 and 3.

The above results unequivocally established that the major component of dihydroxy fatty acids obtained from yeast cerebrin phosphate is 2,3-dihydroxyhexacosanoic acid.

Assignment of configuration

The configurations of the two hydroxyl groups of synthetic 2,3-dihydroxyhexacosanoic acid obtained by *cis* and *trans* addition of hydroxyl groups to *trans*-2-hexacosenoic acid were confirmed as *threo* and *erythro*, respectively, by their R_F values on TLC (Fig. 4). Wood, Bever, and Snyder (37) reported that methyl *threo*-9,10-dihydroxyoctadecanoate prepared by the addition of two hydroxyl groups to oleate moves faster than its *erythro* isomer on silica gel G TLC. Accordingly, we prepared *erythro*- and *threo*-13,14-dihydroxydocosanoic acid from erucic acid by oxidation with OsO_4 and with performic acid, respectively, and confirmed that the R_F value of the *threo* isomer is higher than that of the *erythro* isomer on

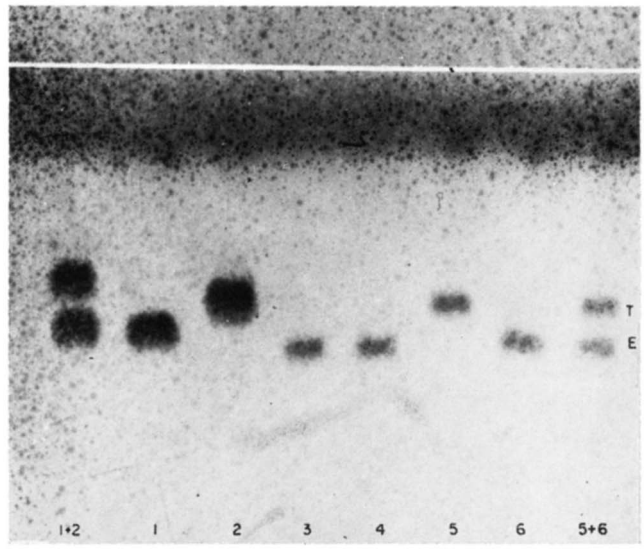


FIG. 4. TLC of 2,3-dihydroxy fatty acid methyl esters on silica gel G ($CHCl_3$–CH_3OH 97:3). Lane *1*, methyl *erythro*-13,14-dihydroxydocosanoate; *2*, methyl *threo*-13,14-dihydroxydocosanoate; *3*, dihydroxy fatty acid methyl esters from a fungus; *4*, dihydroxy fatty acid methyl esters from yeast cerebrin phosphate; *5*, synthetic methyl *threo*-2,3-dihydroxyhexacosanoate; *6*, synthetic methyl *erythro*-2,3-dihydroxyhexacosanoate. The spots were visualized by charring after spraying the plate with 50% H_2SO_4.

TLC as their methyl esters. When R_F values of these isomers were compared with those of *erythro*- and *threo*-2,3-dihydroxyhexacosanoates, it was apparent that the synthetic isomer obtained by performic acid oxidation of hexacos-*trans*-2-enoic acid was *threo*, and the one formed by treatment with alkaline $KMnO_4$ was *erythro*.

The mass spectra of the isopropylidene derivatives of synthetic *threo* and *erythro* methyl 2,3-dihydroxyhexacosanoate were found to agree very closely with the natural compound. The differences in the mass spectra of *threo* and *erythro* derivatives, shown to be adequate for assignment of configuration of diols which are located in the central part of long-chain esters (34), did not allow assignment of configuration in this case in which the diol grouping is located near the carboxyl terminus of the chain. However, further confirmation of configuration of the natural dihydroxy acid as *erythro* was obtained by comparing retention times of the isopropylidene derivative with those of synthetic derivatives by GLC using an OV-1 column. As shown in Fig. 5, the retention time of the natural acid derivative agreed well with that of the synthetic *erythro* derivative and was longer than that of the *threo* derivative.

Homologs of 2,3-dihydroxy, 2-hydroxy, and unsubstituted fatty acids

As shown in Fig. 6, the presence of C_{22}–C_{27} saturated dihydroxy fatty acids, C_{22}–C_{27} saturated and monounsaturated 2-hydroxy fatty acids, and C_{22}–C_{26} saturated and monounsaturated unsubstituted fatty acids were

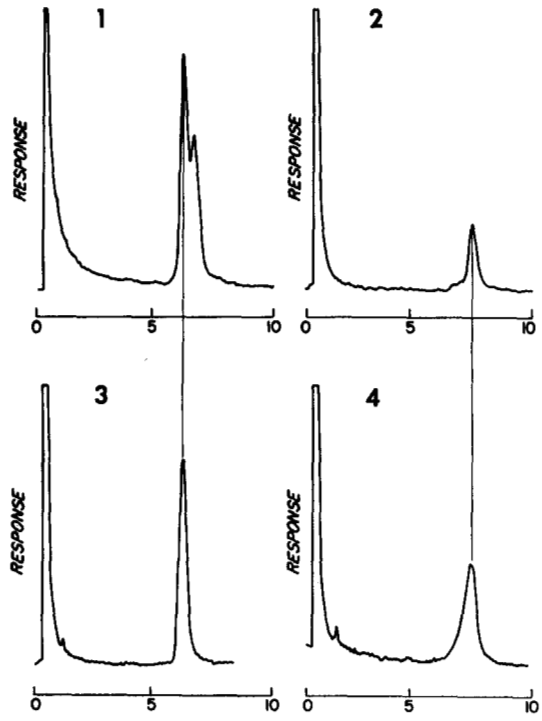


FIG. 5. Gas–liquid chromatograms of the *O*-isopropylidene derivatives of methyl 2,3-dihydroxyhexacosanoates on 3% OV-1 at 250°C. Chromatogram *1* is a mixture of synthetic *erythro* and *threo* isomers; *2*, synthetic *erythro* isomer; *3*, synthetic *threo* isomer; and *4*, the product from yeast cerebrin phosphate.

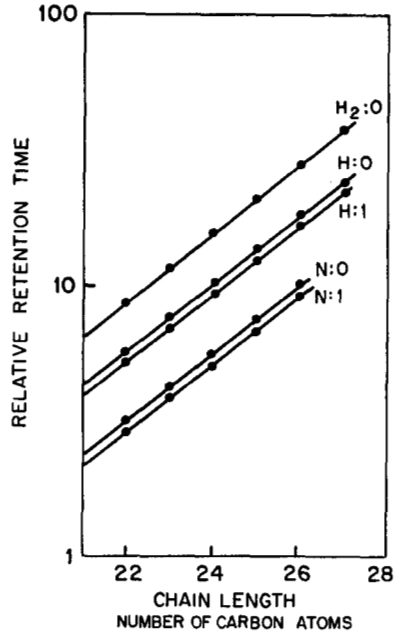


FIG. 6. Plot of logarithms of relative retention times of long-chain fatty acid methyl esters obtained from yeast cerebrin phosphates against chain length. Relative retention times are normalized to that of methyl stearate as 1.0. GLC was performed on 3% OV-1 at 230°C. Homologous groups of fatty acids are expressed as follows: H_2, TMS derivative of 2,3-dihydroxy fatty acid methyl ester; H, TMS derivative of 2-hydroxy fatty acid methyl ester; N, methyl ester of unsubstituted fatty acid. The number after the colon indicates the number of double bonds.

detected by GLC. The C_{26} saturated acid was the predominant homolog in each of the different fatty acid groups.

DISCUSSION

The results of this study as presented above unequivocally identified the dihydroxy fatty acids isolated from the yeast cerebrin phosphate as *erythro*-2,3-dihydroxyhexacosanoic acid and its homologs. To our knowledge, this dihydroxyhexacosanoic acid is the first example of this compound obtained and identified from either a natural source or a synthetic procedure. This also represents the second example of a 2,3-dihydroxy acid of natural origin, the first one being the 2,3-dihydroxytetracosanoic acid isolated from a mold cerebrin by Oda (5). The configuration of that mold cerebrin dihydroxy acid was not established, however, and this study has proved it also to be *erythro* (Fig. 5).

We briefly reexamined the basic structure of the yeast cerebrin phosphate examined by Oda and Kamiya (8) for comparison with the one isolated here and noted some differences in their physical properties. One of these differences was the melting point (they reported 143–144°C while ours was 170–172°C), and another was the result of the elemental analysis (they reported C, 66.59; H, 11.26; N, 1.95; and P, 4.20; our preparation gave C, 65.70; H, 11.15; N, 1.48; and P, 3.01). These differences are probably due to the different strains of yeast used for the isolation and consequently would account for the difference in fatty acid and sphingosine composition. Oda and Kamiya (8) also did not indicate the presence of dihydroxy fatty acids in their preparation.

Conclusive evidence that the dihydroxy fatty acids were components of the yeast cerebrin phosphate examined here is provided by the following observations. First, the infrared spectrum and elemental analysis of our yeast cerebrin phosphate preparation were in agreement with its anticipated structure. The infrared spectrum (Fig. 2) was very similar to that of sphingomyelin, with the exception of a few absorptions, such as the one at 980 cm^{-1}, which is due to both the choline moiety and the *trans* double bond of sphingosine. The similarity of the spectra of cerebrin phosphate and sphingomyelin supports the structure of the former as ceramide-1-phosphate. Finally, upon methanolysis of this lipid, equimolar amounts of fatty acids, which included dihydroxy acids, and sphingosine base were obtained. This observation confirmed the fact that all fatty acids obtained by the methanolysis are from the sphingosine amide, namely

cerebrin phosphate. The TLC of the yeast cerebrin phosphate on silica gel G with $CHCl_3$–CH_3OH–2.5 N NH_4OH 60:35:8 showed two close but separate phosphorus-containing bands. Scraping these bands separately and methanolysis of the powders with methanolic HCl at 110°C (38) yielded similar fatty acids, including dihydroxy acids, and sphingosine bases. This observation indicated that the dihydroxy acids were an integral part of cerebrin phosphate and had not originated from contaminating lipids. We cannot offer an explanation of the appearance of two separate bands with similar compositions. But, it is possible that they result from various combinations of mono- or dihydroxy fatty acids with phytosphingosine or an unknown nonpolar base.

It is interesting to note that the yeast cerebrin phosphate, of which these 2,3-dihydroxy acids are components, also contains phytosphingosine as the major sphingosine base. Both the fatty acid identified in this communication and phytosphingosine contain *vic*-dihydroxyl groups in the *erythro* configuration (19). Phytosphingosine in yeast was shown to be derived from direct hydroxylation of dihydrosphingosine (39, 40). Whether this 2,3-dihydroxyhexacosanoic acid is formed from 2-hydroxyhexacosanoic acid in a similar fashion awaits further study. Whether the 2-hydroxyl group of this acid has the D or L configuration could not be determined due to the lack of material.

If these dihydroxy fatty acids are produced from 2-hydroxy fatty acids, there is a possibility that the mammalian brain, which contains large amounts of 2-hydroxy fatty acids as components of cerebrosides or sulfatides, might also contain 2,3-dihydroxy fatty acids. There is an even greater possibility of the presence of 2,3-dihydroxy acids in kidney, since it is one of the mammalian tissues known to contain phytosphingosine (41). However, our search for dihydroxy acids in calf brain and kidney, as well as in rat brain, has thus far been negative.

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