DIBROMO- AND TETRABROMOSTEARIC ACIDS IN THE SEED OIL OF *Eremostachys moluccelloides*

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As reported previously [1], the total fatty acids of the seed oil of *Eremostachys moluccelloides*, family Labiatae contained unknown acids. In the present paper we give the methods of isolating and proving the structure of two bromostearic acids present in this oil.

When the combined methyl esters (MEs) of the acids of the oil were fractionated with the aid of urea, six fractions enriched to different extents with saturated and oleic acids (fractions 1-3) and with linoleic acid (fractions 4-6) were obtained. Fraction 4 was separated by preparative chromatography in a thin layer of silica gel (TLC) containing $AgNO_3$ (TLC + $AgNO_3$) in system 1 into the MEs of linoleic ($C_{18:2}$) and oleic ($C_{18:1}$) acids.

When the $C_{18:1}$ ME isolated was then subjected to TLC on silica gel in system 2 two spots were obtained, one of which (R_f 0.78) coincided in its chromatographic mobility with ordinary fatty acid MEs and when the chromatogram was treated with 50% H_2SO_4 and heated it showed a dark brown coloration. The other spot (R_f 0.7), corresponding in position to triglycerides and giving a pinkish brown color after the sulfuric acid treatment was shown by subsequent investigations to consist of the combined MEs of bromostearic acids. The complete separation of the mixture of MEs of the $C_{18:1}$ and bromostearic acids was achieved by chromatographing the mixture by the TLC method on alumina in system 3. This gave three spots with R_f 0.7, 0.51, and 0.36, the first of which (R_f 0.7) corresponded to the marker $C_{18:1}$ ME. Under the same conditions, by preparative TLC we isolated substances with R_f 0.51 and 0.36.

The methyl dibromostearate (I) (R_f 0.51) proved to be an individual substance according to its chromatographic behavior in system 3. It was isolated in the form of an oil and gave a positive Beilstein test. In its mass spectrum, M^+ 454/456/458 ($C_{19}H_{36}Br_2O_2$). The IR spectrum of (I) in the 700-3600 cm^{-1} region was almost identical with that of the $C_{18:2}$ [2], except for the absence from the spectrum of (I) of the absorption band of an olefinic bond (3010, 1650 cm^{-1}). The NMR spectrum of (I) (Fig. 1a) showed a complex signal with its center at 5.92 ppm (τ scale) corresponding to 2 H and relating to the protons on carbon atoms bearing halogen ($-CH_2\underline{C}H\underline{B}rC\underline{H}\underline{B}rCH_2-$). On the basis of these facts, the structure of (I) may be considered to be as follows:

$$CH_3(CH_2)_nCH_2CHBrCHBrCH_2(CH_2)_mCOOCH_3, \quad (I)$$
$$m + n = 12.$$

Methyl tetrabromostearate (R_f 0.36) was additionally purified from more polar impurities by rechromatography (TLC) on alumina, using solvent system 2. The isolated solid methyl tetrabromostearate (II) was chromatographically pure and gave a positive Beilstein test. The molecular weight of (II) (M^+) was 610/612/614/616/618 ($C_{19}H_{34}Br_4O_4$, mass spectrum). The NMR spectrum of (II) (Fig. 1b) had a multiplet in the 5.61-ppm region (2 H), assigned to the protons attached to the "inner" pair of carbon atoms of a $-\underline{C}H\underline{B}rCH_2\underline{C}H\underline{B}r-$ group, and a complex signal with its center at 5.96 ppm (2 H), due to the protons of an "outer" pair of carbon atoms bearing halogen ($-CH_2\underline{C}H\underline{B}rC\underline{H}\underline{B}r-$).

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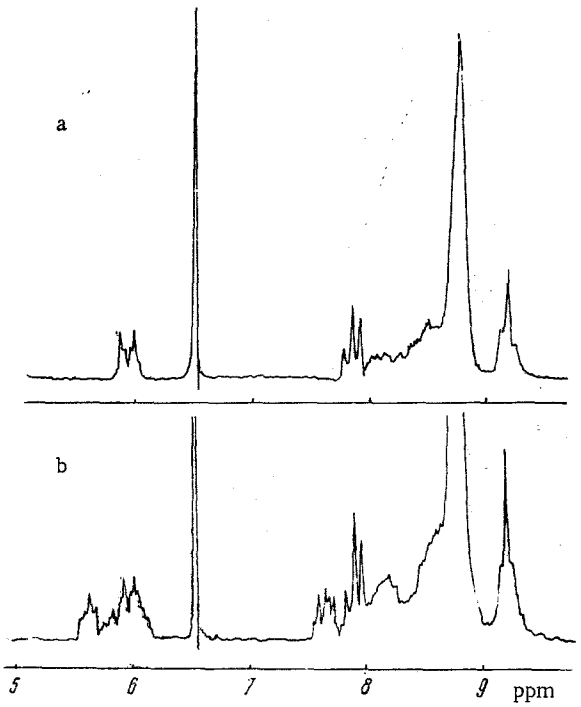

Fig. 1. NMR spectra of methyl 9,10-dibromostearate (a) and methyl 9,10,12,13-tetrabromostearate (b) from the oil of *Eremostachys moluccelloides*.

Thus, the structure of (II) is as follows:

$$CH_3(CH_2)_nCH_2CHBrCHBrCH_2CHBrCHBrCH_2(CH_2)_mCOOCH_3, \quad (II)$$
$$m + n = 9.$$

The structures of compounds (I) and (II) were established definitively by comparing the IR, NMR, and mass spectra of the methyl bromostearates isolated and their synthetic analogs obtained by brominating the MEs of the $C_{18:1}$ (octadec-9-enoic) and $C_{18:2}$ (octadec-9,12-dienoic) acids. In order to determine more accurately the laws of the decomposition of the MEs of brominated fatty acids on electron impact we also recorded the mass spectra of the ME of synthetic 10,11-dibromoundecanoic acid, $CH_2BrCHBr(CH_2)_8COOCH_3$, obtained by the bromination of methyl undec-10-enoate.

The ions present in the region of high mass numbers of the spectra of the MEs of the bromostearic acids are shown in Table 1.

On the basis of an identity of melting points (for compound (II)) and of the NMR, IR, and mass spectra of the natural and synthetic methyl bromostearates the structure of (I) was established as methyl 9,10-dibromostearate and that of (II) as methyl 9,10,12,13-tetrabromostearate.

In the rechromatography of compound (II) under the conditions given above one more compound was isolated in the form of a light yellow oil (III, R_f 0.66) more polar than the methyl tetrabromostearate (II, R_f 0.78). Compound (III) also gave a positive Beilstein test.

The IR spectrum of (III) (film) contained an absorption band at 2830 cm^{-1} and an absorption band at 1100 cm^{-1} (OCH_3) more pronounced than in the spectrum of the ME of an ordinary saturated fatty acid. The NMR spectrum of (III) (in CCl_4) showed the signals of protons at the following τ values: complex signal with its center at 6.08 (1 H, —CHBr), 6.45 (singlet, 3 H, —$COOCH_3$), 6.73 (singlet, 3 H, —OCH_3), 6.88 (multiplet, 1 H, —CH—O—), 7.86 (triplet, 2 H, —CH_2COO), 8.24-8.78 [broad singlet, 26 H, —$(CH_2)_{13}$—], and 9.18 ppm (triplet, 3 H, CH_3—).

The mass spectrum of (III) lacked the peak of the molecular ion but had the peaks of fragments with m/e 375/377 ($M^+ - OCH_3$) (5%), 327 ($M^+ - Br$) (2%), 293/295 [$M^+ - (CH_2)_7CH_3$] (12%), and 249/251 [$M^+ - (CH_2)_7COOCH_3$] (7%).

TABLE 1. Mass Numbers and Relative Intensities of Some Ions in the Mass Spectra of the Methyl Esters of Bromine-Containing Acids

Ion	Mass number, m/e (relative intensities, %)		
	10,11-diBr-$C_{11:0}$ methyl ester	I	II
M^+	356/358/360 (4)*	454/456/458 (5)	610/612/614/616/618 (<0.1)
$M-OCH_3$	325/327/329 (9)	423/425/427 (7)	579/581/583/585/587 (1)
$M-Br$	277/279 (46)	375/377 (74)	531/533/535/537 (5)
$M-OCH_3-HBr$ or $M-CH_3OH-Br$	245/247 (46)	343/345 (13)	499/501/503/505 (0.3)
$M-Br-HBr$	197 (21)	295 (100)	451/453/455 (5)
$M-OCH_3-2HBr$ or $M-CH_3OH-Br-HBr$	165 (18)	263 (65)	419/421/423 (3)
$M-Br-2HBr$	—	—	371/373 (8)
$M-OCH_3-3HBr$ or $M-CH_3OH-Br-2HBr$	—	—	339/341 (3)
$M-Br-3HBr$	—	—	291 (100)
$M-OCH_3-4HBr$ or $M-CH_3OH-Br-3HBr$	—	—	259 (10)

*Here and below the total intensities of all the polyisotopic peaks are given.

On the basis of the results obtained, the following structure has been established for (III):

$$CH_3(CH_2)_7CHBrCHOCH_3(CH_2)_7COOCH_3. \quad \text{(III)}$$

The position of the methoxy group at C-9 was determined from the mass-spectral characteristics on the basis of fragments with m/e 201 $[CH_3\overset{+}{O}=CH(CH_2)_7COOCH_3]$ (100%), 169 (201 − 32) (14%), 157 $[\overset{+}{(CH_2)}_7COOCH_3]$ (78%), and 137 (169 − 32) (18%).

Similar fragments have been observed in the mass spectra of the MEs of synthetic 9-methoxy-10-halogenostearic acids [3]. We assumed that the 10-bromo-9-methoxystearic acid isolated is a byproduct formed from the natural 9,10-dibromostearic acid in the process of isolation.

Since no method suitable for isolating halogeno acids from the total mixture have been developed at the present time, we have attempted to evaluate the methods existing in lipid chemistry for the isolation and separation of fatty acids from the point of view of the retention of the primary structure of halogen derivatives present in the mixture being separated in very small amounts.

The usual method of isolating the total acids from an oil is alkaline hydrolysis. Under the conditions of severe saponification (6-10% aqueous methanolic KOH, boiling for two hours), halogen is split off from vicinal dibromo acids with the formation of acetylene derivatives [4] or with its replacement and the formation of hydroxy, methoxy [5], or epoxy [6] derivatives. We have checked the possibility of the loss of halogen by bromostearic acids under conditions of cold saponification (10% methanolic KOH, room temperature) both on the oil of *E. moluccelloides* itself and also on synthetic dibromostearic acid. At the end of saponification, Br^- ions were found in the reaction mixture by their positive reaction with a 10% solution of $AgNO_3$.

The results showed that under the conditions of the cold saponification both of the *E. moluccelloides* oil and of dibromostearic acid the degree of splitting out of halogen is extremely small, but it increases if the evaporation of the methanol at the end of hydrolysis is performed at a temperature above 40°C. Apparently, to isolate natural halogen-containing acids from glyceride oils the method of enzymatic hydrolysis may prove to be most suitable.

The generally accepted method of separating MEs according to their degree of unsaturation is TLC on silica gel containing various amounts of $AgNO_3$. It is known that the separation of acids by this method is based on the formation of a labile coordination complex between the π electrons of the double bonds of the unsaturated acids and the Ag^+ ions which is readily decomposed in the desorption of the substance by organic solvents. In this process, neither the positions of the olefinic bonds in the acid nor their configuration changes [7].

As already mentioned, in the separation of the combined MEs of the acids of *E. moluccelloides* by the TLC + 3-5% $AgNO_3$ method the total MEs of the bromostearic acids migrated without separating distinctly but somewhat outstripping the $C_{18:1}$ ME (R_f 0.74). With silica gel containing 30% of $AgNO_3$ the mobility of the total MEs of the bromo acids became equal to the mobility of methyl laballenate (octadeca-5,6-dienoate) (R_f 0.86) [1]. In all the fractions obtained after the preparative separation of the MEs of the acids of the oil by this method (TLC + 3-5% $AgNO_3$), in addition to the corresponding unsaturated acid we detected a similar total of products with a different polarity (TLC in systems 2 and 3). Assuming that the more polar byproducts can be formed from bromostearic acids not only during the alkaline hydrolysis of the oil but also during the chromatography of the total MEs in a thin layer as the result of reactions catalyzed by Ag^+ [5], we separated a model mixture of the MEs of the acids of cottonseed oil ($C_{16:1}$ 1.5%, $C_{18:1}$ 17.5%, $C_{18:2}$ 52%, total saturateds 29%) containing 5% of a mixture of methyl dibromo- and tetrabromostearates by the TLC + 5% $AgNO_3$ method.

In all the fractions isolated, including the starting-zone and diene fractions, halogen was found to be present by a positive Beilstein test, and again by TLC in systems 2 and 3 a similar total amount of byproducts of different polarities basically identical qualitatively with the byproducts of separation of the MEs of the acids of *E. moluccelloides* under similar conditions was also found. The total amount of byproducts rose in all the fractions with an increase in the amount of $AgNO_3$ introduced into the sorbent (5, 15, 30%). Consequently, the TLC + $AgNO_3$ method, like the method of alkaline hydrolysis, is suitable for the separation of mixtures of MEs including halogen-containing esters to only a limited extent and the 10-bromo-9-methoxystearic acid isolated is a byproduct. It is not excluded that the unidentified compounds observed previously by other authors in the separation of mixtures of the MEs of the acids of the oils of the family Labiatae by the TLC + 30% $AgNO_3$ method [8] are either halogen-containing acids present in the oil or their derivatives formed in the process of isolating the combined acids and separating them into their individual components.

We have investigated the seed oils of the species *Nepeta cataria, Salvia virgata, Mentha asiatica, Origanum tyttanthum,* and *Lagochilus occultiflorus,* family Labiatae, for the presence of bromine-containing acids. The presence of halogen was established by the argentometric method after severe alkaline hydrolysis of a sample of the oil. Because of the presence in the caustic potash used for hydrolysis (ch. ["pure"], "Chemapol") of 0.008% of potassium chlorides, the determination of Br^- ions was performed after the separation of the precipitates of silver halides of the Cl^- ions in the form of the silver ammoniate. As controls we subjected to the same treatment samples of cottonseed oil (family Malvaceae) and the seed oil of *Onopordon acantium* (family Compositae), and standard methyl oleate. All the samples of oils of the family Labiatae gave a positive reaction for Br^- ions. This reaction was negative for the control samples.

The only halogen-containing acids that have hitherto been detected in fatty oils isolated from various organs of higher plants are five ω-fluorine-containing acids (seed oil of *Dichapetalum toxicarium*) with a predominance of ω-fluorooleic [9]. This is the first time that bromine-containing fatty acids have been detected in natural seed oils of higher plants.

EXPERIMENTAL

The mass spectra were taken on an MKh-1303 mass spectrometer with a system for the direct introduction of the sample at an ionizing voltage of 40 V and temperatures of the inlet system in the range of 115-150°C; the NMR spectra were taken on an XL-100 spectrometer (Varian) (CCl_4, internal standard TMS, τ scale); and the IR spectra on a UR-10 spectrometer. Conditions for GLC analysis: UKh-2 chromatograph, column 3000 × 4 mm containing 15% of Reoplex-400 on Chromaton N-AW-DMCS, temperature 205°C, carrier gas helium.

The isolation of the total fatty acids from the seed oil, their basic indices, and their partial composition have been described previously [1].

The fractionation of the total MEs (20 g) with the aid of urea was performed at a ratio of sample to urea to absolute MeOH of 1:1:6. The mixture of reactants was heated until the urea had dissolved completely and was allowed to stand first at room temperature until it had cooled, and then in the refrigerator for two hours. The further treatment of the precipitates and filtrates was similar to that described by Iverson et al. [10].

Composition of fraction 4 (GLC): $C_{18:2}$ – 75; $C_{18:1}$ – 25%.

Thin-layer chromatography was performed on silica gel L 5/40 ("Chemapol") with the addition of 10% of gypsum and 3-5% of $AgNO_3$ (on the weight of the silica gel) in benzene (system 1); and on the same adsorbent but without the addition of $AgNO_3$ in the hexane–diethyl ether–98% CH_3COOH (70:30:1) system (system 2). The substances were revealed with 50% H_2SO_4 followed by heating, and the spots were identified by comparison with markers.

The separation of the combined methyl bromostearates was performed by preparative TLC on Al_2O_3 (basic, "Woelm") with the addition of 1% of gypsum in the petroleum ether (up to 50°C)–hexane–diethyl ether (4:5:1) system (system 3). The substances were revealed with a solution of I_2 in MeOH. The esters were eluted from the adsorbent with chloroform, the solvent was evaporated off, hexane was added to the residue and this was evaporated off until the last traces of solvent had been eliminated.

Methyl 9,10-dibromostearate (I) (175 mg) – light yellow oil; M^+ 454/456/458; IR spectrum (film), cm^{-1}: 2960, 2940, 2870, 1750, 1470, 1442, 1370, 1255, 1205, 1180, 1127, 1105, 1027, 892, 730; NMR spectrum, τ, ppm: 5.92 (2H, –CHBrCHBr–), 6.52 (3H, $-OCH_3$), 7.87 (2H, $-\underline{CH_2}CO_2CH_3$), 7.96-8.6 (6H, $-\underline{CH_2}CH_2CO_2CH_3$ and $\underline{CH_2}CHBr-$), 8.8 (20H, $-(CH_2)_{10}-$), 9.2 (3H, $\underline{CH_3}-$).

Methyl 9,10,12,13-tetrabromostearate (II) (100 mg) – white lamellar crystals with mp 57-57.5°C (from hexane); M^+ 610/612/614/616/618; IR spectrum (KBr), cm^{-1}: 2930, 2870, 1745, 1470, 1440, 1380, 1260, 1220, 1200, 1180, 1130, 1110, 1030, 990, 960, 920, 905, 890, 850, 775, 730. NMR spectrum, τ, ppm: 5.61 (2H, $-\underline{CH}BrCH_2\underline{CH}Br-$), 5.96 (2H, $CH_2\underline{CH}BrCHBr-$), 6.53 (3H, $-OCH_3$), 7.67 (2H, $-CHBr\underline{CH_2}CHBr-$), 7.89 (2H, $-\underline{CH_2}CO_2CH_3$), 8.17 (4H, $-\underline{CH_2}CHBr-$), 8.4-8.78 (16H, $-(CH_2)_8-$), 9.12 (3H, CH_3-).

The synthetic analogs of the natural $C_{18:0}$ bromides were obtained by brominating the total MEs of the acids of cottonseed oil by a known method [2], and the reaction products were isolated as described by Sgoutas and Kummerow [11]. The total bromine-containing MEs were separated by TLC on alumina in system 3. Methyl 10,11-dibromoundecanoate was obtained from methyl undec-10-enoate similarly.

Determination of Br^- Ions. A sample of 1.5-2 g of oil (or 50 mg of a bromo acid) was saponified with 25 ml (or 1 ml in the case of a bromo acid) of 20% methanolic KOH in the boiling water bath for 1.2-2 h. The methanol was evaporated off and, after the addition of 25-30 ml of water (2-3 ml in the case of a bromo acid) the soap was decomposed with 20% HNO_3, and the organic acids were separated by extraction with diethyl ether. The acidic aqueous layer was treated with several milliliters (or a drop) of 10-15% $AgNO_3$ solution [12] until a white flocculant precipitate had formed. The precipitate of silver halides was separated by filtration, washed with water to neutrality, and treated with 10-15 ml of buffer mixture (0.17 g of $AgNO_3$, 2.6 g of KNO_3, and 3.6 ml of 25% NH_4OH in 100 ml of water) to eliminate Cl^- ions. The precipitate of silver bromide was brought into solution by treatment with 27% NH_4OH and the AgBr was reprecipitated by the addition of concentrated HNO_3.

SUMMARY

9,10-Dibromo- and 9,10,12,13-tetrabromostearic acids have been isolated from the seed oil of *Eremostachys moluccelloides* and their structures have been established by IR, NMR, and mass spectroscopy and also by comparison with the spectral characteristics of their synthetic analogs. It has been established that byproducts can be formed from halogen-containing acids present in mixtures of acids under the conditions of generally adopted methods of isolation and separation.

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N-ACYLATED PHOSPHOLIPIDS AND LYSOPHOSPHOLIPIDS OF KENAF SEEDS

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In the separation of the total phospholipids (PLs) of kenaf seeds of the variety Kuban'-333 (*Hibiscus cannabinus*, family Malvaceae) [1, 2] in a column of silica gel, the chloroform–methanol (9:1) fraction yielded a mixture of two PLs — X_1 and X_2 — amounting to 12.3% of the total. These minor components of the PLs were separated preparatively in system 1. The R_f value of the X_1-PL in this system was 0.9 and that of the X_2-PL 0.65. Both compounds were revealed with the Vas'kovskii reagent and gave a negative ninhydrin reaction. In chromatographically homogeneous X_1- and X_2-PLs the N:P ratio was ∿1. Their IR spectra contained, in addition to the absorption bands characteristic for glycerophospholipids [3, 4] the bands of an amide carbonyl at 1650 and 1540 cm^{-1}. The fatty-acid compositions of the X_1- and X_2-PLs were determined by alkaline hydrolysis and methylation of the fatty acids followed by GLC analysis (Table 1). The acid hydrolysis of the X_1- and X_2-PLs showed that their aqueous hydrolyzates contained fatty acids, glycerol (system 2), and ethanolamines (system 3). The spectral characteristics, and also the results of analyses of the products of acid hydrolysis permit the assumption that the X_1- and X_2-PLs are possibly N-acylated derivatives of phosphatidylethanolamine. On mild alkaline hydrolysis the X_1-PL gave fatty acids (see Table 1, O-acyl) and a partially deacylated product which gave a positive reaction for phospholipids (Vas'kovskii reagent), was not revealed by ninhydrin, and was more polar (R_f 0.34, system 1) than X_2-PL. The fatty acids of the de-O-acylated PL were isolated by 10% methanolic alkali (see Table 1, N-acyls) [5]. Like the initial X_1-PL, the product of its partial deacylation on acid hydrolysis gave fatty acids, glycerol, and ethanolamine. When homogeneous X_1-PL was adsorbed from $CHCl_3$ on to alkaline alumina (room temperature, 24 h) in addition to the initial spot a spot of X_2-PL was observed. The facts given confirm the structure of a N-acylphosphatidylethanolamine for X_1-PL. On the basis of the nonidentity of X_2-PL with partially deacylated X_1-PL and of the formation of X_2-PL from X_1 (Al_2O_3), the structure of a N-acyllysophosphatidylethanolamine can be suggested for X_2-PL. However, the L_1-PL did not undergo enzymatic hydrolysis with phospholipase A and we have therefore been unable to make a direct comparison of X_2-PL with 1-acylglycerylphosphoryl-N-acylethanolamine.

The reaction of acetyl chloride with phosphatidylethanolamine gave N-acetylphosphatidylethanolamine, the chromatographic behavior of which (system 1) was identical with that of X_1-PL. The IR spectrum of the product obtained also showed absorption bands of an amide carbonyl at 1660 and 1560 cm^{-1}.

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