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NEUTRAL LIPIDS OF THE SEEDS OF Eremostachys moluccelloides

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We have previously reported on individual components of the fatty oil of the seeds of Eremostachys moluccelloides, family Labiatae [1-3]. In the present paper we give the results of a further study of the total neutral lipids isolated from the seeds of plants collected in 1976 (in the Tashkent oblast).

The neutral lipids (NL's) of the ripe seeds of E. moluccelloides were chromatographed on a column of silica gel. Elution with hexane yielded the total hydrocarbons, high-molecular-weight alcohols, pigments, esters, and sterols (fraction I, 1% of the weight of the initial extract) [1]; hexane–diethyl ether (95:5) eluted the triglycerides (TG's) (II, 89.6%); hexane–diethyl ether (92:8) eluted the total free fatty acids (FFA's) and the sterols and diglycerides (DG's) (III, 7.2%); diethyl ether eluted the monoglycerides (MG's) (IV, 0.8%); and methanol eluted the phospholipids (V, 1.4%).

Assignments to the appropriate classes were made on the basis of the results of TLC [4] and spectral (IR and NMR) characteristics. The fact that fraction (V) consisted of phospholipids was confirmed by the qualitative reaction with the Vas'kovskii reagent. The phospholipid fraction was not studied.

Since halogen-containing acids have previously been isolated from the oil of E. moluccelloides [3], each of the fractions obtained was subjected to the Beilstein test for the presence of halogen. Fractions II, III, and V gave a positive result. The presence of a helogen in fractions II and III was confirmed by the results of neutron-activation analysis (performed by R. Khamidova of the Institute of Nuclear Physics of the Academy of Sciences of the Uzbek SSR). This showed that fraction II contained 4 ppm of Br and 160 ppm of Cl, and fraction III contained 90 ppm of Br and 1100 ppm of Cl.

The composition of fraction I has been described previously [1]. The fatty-acid composition of the TG fraction (GLC) is given in Table 1. The composition of the acids of the TG's differs from that of the TG's of the seeds of E. moluccelloides collected in 1974 by a lower content of the $C_{20:1}$ acid and by the appearance of small amounts of the $C_{16:1}$ and $C_{18:3}$ acids [1]. The glyceride composition of the TG's calculated from the results of enzymatic hydrolysis is represented by the following types (%):

GlS_3 – none	GlS_2U – 0.2	GlSUS – 0.3
GlUSU – 1.6	GlSUU – 10.0	GlU_3 – 87.9 [5].

By chromatographing fraction III again on a column of silica gel, we isolated di-2-ethylhexyl phthalate identified by its NMR spectrum [2] and FFA's [hexane–diethyl ether (92:8)], free sterols and DG's [hexane–diethyl ether (1:1)]. The Beilstein test was positive for the FAA fraction and the DG fraction (weak).

In all cases the separation was monitored by TLC on Silufol plates in system 1. According to TLC, the DG fraction represented the sum of the α, β-(R_f 0.2) and of the α,α'-(R_f 0.26) isomers with a predominance of the latter. The compositions of the fatty acids of the total DG's (GLC) are given in Table 1. The IR spectrum

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TABLE 1. Fatty-Acid Composition of the Glyceride Fractions of the Seed Oil of Eremostachys moluccelloides (%, GLC)

Acid	Σ nat. MGs	Σ nat. DGs	β-MGs from the nat. DGs	Nat. TGs	β-Mgs from the nat. TGs	Synthetic TGs from the DGs	
						specific hydrolysis	non-specific hydrolysis
$C_{9:0}$	—	—	0,6	—	—	—	—
$C_{16:0}$	13,7	2,9	4,2	3,1	1,8	3,0	14,9
$C_{16:1}$	—	0,5	0,5	0,5	—	—	2,8
$C_{17:0}$	—	0,2	—	—	—	—	—
$C_{18:0}$	9,7	1,1	0,7	1,1	—	—	5,4
$C_{18:1}$	51,2	57,5	58,7	71,9	77,5	74,7	60,6
$C_{18:2}$	25,4	34,7	33,6	22,8	18,4	22,3	16,3
$C_{18:3}$	—	1,2	1,7	0,6	2,3	—	—
$C_{20:1}$	—	1,9	—	Tr.	—	—	—

of the methyl esters of the acids of the total DG's showed the band of an allene group (1965 cm^{-1}), from which it followed that the DG fraction contains laballenic acid (5,6-$C_{18:2}$) [1].

Since on separating the total DG's on silica gel with the addition of 10% H_3BO_3 we were unable to isolate the individual α, β- and α,α'-isomers their glyceride types were determined by using the total DG's. For this purpose, the sum of the natural DG's was also hydrolyzed with pancreatic lipase. In the hydrolysis products, by analytical TC on Silufol plates in system 1, we detected β-MG's (R_f 0.03), residues of unsaturated DG's (R_f 0.2 and 0.26), FFA's, and synthetic TG's (R_f 0.86). The hydrolysis products were separated by preparative TLC in system 2. On the basis of the Beilstein test, the absence of halogen from the β-MG's and its presence in the FFA's was established, from which it was assumed that the halogen-containing acids are esterified mainly in the α,α' positions of the DG's. On the basis of the composition of the acids isolated from the total DG's and β-MG's we calculated the types of DG's for the α,β- and α,α'-isomers (%):

Type of Diglyceride	α,β-DG	α,α'-DG
PP	0,2	0,1
OP	2,9	2,0
LP	1,9	1,4
PO	2,1	2,0
OO	35,8	34,0
LO	23,5	22,3
PL	1,2	1,3
OL	19,6	22,3
LL	12,8	14,6

The results of a chemical analysis of the DG's confirmed those of mass spectroscopy [6], in which the main molecular ions were M^+ 620 and 618, corresponding to the types OO and OL.

It can be seen from an analysis of the facts given above and of Table 1 that the DG's contain acids not detected in the TG's ($C_{9:0}$, $C_{17:0}$), and a greater amount of $C_{18:2}$ and $C_{20:1}$ acids than in the TG's. Furthermore, with an amount of saturated acids nearly equal to that in the TG's, the amount of these acids esterified in the β positions of the DG's was somewhat higher.

The synthetic TG's were isolated from the hydrolysis products and identified on the basis of their IR and NMR spectra. Besides TG's, in this fraction we identified traces of a phthalic acid ester (NMR spectrum; τ scale, 2.6 ppm, multiplet). The fatty-acid composition of the synthetic DG's is given in Table 1.

The capacity of pancreatic and some bacterial lipases for catalyzing the synthesis of TG's is well known [7]. In our experiments, under the conditions of the enzymatic hydrolysis of the DG's that were used, the fatty-acid composition of the synthetic TG's (see Table 1) almost corresponded to that of the natural TG's. These results show that in the esterification reaction catalyzed by pancreatic lipase all the acids present in the reaction mixture take place in accordance with their quantitative amounts.

As mentioned above, in the TG and DG fractions we found a very small amount of halogen-containing compounds. In order to select methods for their isolation excluding loss of halogen [3], we hydrolyzed these fractions with a nonspecific lipase isolated from the culture liquid of the fungus Oospora lactis [8]. In parallel we set up a blank experiment with all the reagents used in the hydrolysis by the nonspecific lipase with the exception of the sample of glycerides. The products that appeared as the results of a blank experiment were analyzed by TLC and the absence of neutral fat from them was shown. From the hydrolysis of the DG's by the nonspecific lipase, by means of preparative TLC we isolated the MG's, the sum of the unhydrolyzed DG's, the FFA's, and the synthetic DG's, and from the hydrolyzate of the TG's we isolated the total FFA's. A quantitative test revealed the presence of halogen in all the fractions isolated from the hydrolyzates. According to NMR spectroscopy, the fraction of synthetic TG's in this case, also, contained a phthalic ester.

Thus, the nonspecific lipase, just like the pancreatic lipase, is capable of catalyzing the back-reaction. However, the fatty-acid composition of the synthetic TG's obtained as the result of nonspecific hydrolysis differed from the composition of the acids of the natural TG's by a considerably higher (almost five times) amount of saturated ($C_{16:0}$, $C_{18:0}$) acids (see Table 1). Consequently, the nonspecific lipase catalyzes the esterification reaction with a definite preference in relation to the saturated acids.

The fraction of natural MG's (IV) having R_f 0.03 in system 1 was identified by comparison of its spectral characteristics with those given in the literature. According to analytical TLC in system 3 the fraction consisted of the combined α- and β-isomers. The fatty-acid composition of the natural MG's is given in Table 1. It can be seen from this that the MG fraction of the oil of E. moluccelloides is rich in saturated acids (23%).

When the initial combined NL's of the seeds of E. moluccelloides was separated on neutral Al_2O_3, a higher amount of FFA's, DG's, and MG's was obtained than when they were separated on silica gel, which confirms the ability of Al_2O_3 to hydrolyze the ester groups that was detected previously [10].

In the separation of the combined NL's on a column of Sephadex LH-20 [11], TG and DG fractions enriched with the halogen-containing components and also with the phthalic acid ester were obtained, which confirms the presence of phthalates in the natural combined NL's.

EXPERIMENTAL

The conditions for recording the IR, NMR, and mass spectra were similar to those described previously [1-3].

The total NL's were isolated by extracting the dry comminuted seeds by steeping them with hexane.

The neutral lipids were separately passing 10 g of the total material through a column (d = 19 mm, V = 25 cm^3) containing silica gel ("Woelm"). The volumes of solvents used for eluting the fractions were: hexane – 120 ml (fraction I); hexane – diethyl ether (95 : 5) – 150 ml (II); hexane – diethyl ether (92 : 8) – 200 ml (III); diethyl ether – 150 ml (IV); and methanol – 200 ml (V).

The analytical chromatography of the total NL's according to classes was carried out on Silufol plates in the hexane – methyl ethyl ketone – acetic acid (41 : 9 : 0.5) system (1) [12]. The spots were revealed with I_2 vapor. The total DG's were freed from accompanying substances by preparative TLC in a thin layer of KSK silica gel with 1% of $CaSO_4$ in the petroleum ether – diethyl ether (8 : 7) system (2).

The separation of the total DG derivatives into the α,β- and α,α'-isomers was carried out by preparative TLC on plates (24 × 18 cm) of KSK silica gel with the addition of 1% of gypsum and 10% of H_3BO_3 in the chloroform – acetone (96 : 4) system (3) [9]. The chromogenic agent was 50% H_2SO_4 followed by heating. The substances were eluted from the zones with chloroform – methanol (9 : 1).

Sum of the DG's. IR spectrum, cm^{-1}: 3480, 3010, 2970, 2930, 2860, 1965, 1740, 1655, 1465, 1420, 1380, 1320, 1280, 1240, 1170, 1120, 1100, 1060, 1030, 950, 880, 730 [13]. NMR spectrum (CCl_4; TMS, τ scale, ppm): 9.3 (CH_3, triplet), 8.7 [$(CH_2)_n$, singlet], 8.4 ($CH_2-C-C=$, multiplet), 8.0 ($CH_2C=$, apparent doublet), 7.7 (CH_2COO, triplet), 7.4 (C–OH, singlet), 7.29 ($=CCH_2C=$, triplet), 6.4 (CH_2-OH, doublet), 5.74-6.12 (CH–OH of a glycerol ester not esterified in the β position, multiplet), 5.98 (CH_2-O-C, doublet), 4.9-6.14 (CH–O–C of a glycerol ester esterified in the β position, multiplet), 4.74 (CH=CH, multiplet) [14].

The main ions in the mass spectrum were, m/e: 620, 618 (M^+), 602, 600 ($M - H_2O$), 339 ($M - RCOO$), 325, 323 ($M - RCO$) [6].

The pancreatic lipase was obtained by the method of Zeman and Scharmann [6].

The pancreatic lipase hydrolysis of the combined DG's and TG's were performed by using 0.5 g of sample, 1.2 ml of a 1% solution of poly(vinyl alcohol) (PVA), 6.5 ml of phosphate–citrate buffer (pH 8.0), and 0.4 g of lipase.

The complex lipase preparation from the fungus O. lactis UzLM-2 was obtained by the method described previously [8] and had an activity of 2500 units/g.

Hydrolysis with the nonspecific lipase was performed by using a mixture of 0.5 g of DG's (TG's), 1 ml of a 1% solution of PVA, 6.5 ml of phosphate–citrate buffer (pH 7.5), and 0.03 g of lipase. In both cases, hydrolysis was performed at 37°C for 2 h with constant stirring.

The preparative TLC of the hydrolyzates was performed on KSK silica gel with 1% of gypsum in system 2 (for the isolation of the β-MG's and the FFA's) and with petroleum ether–diethyl ether (8.5:1.5) (for the isolation of the synthetic TG's). The spots were revealed with 50% H_2SO_4 followed by heating. The substances were eluted from the zones with chloroform–methanol (9:1).

In separation on a column of Sephadex LH-20 [11] (36 g of adsorbent and 300 mg of oil), chloroform eluted 11 fractions. The volumes of solvent eluting the various fractions were: I-VI – 5 ml each (TG's); VII – 15 ml (phthalic ester + TG's); VIII-IX – 15 ml each (di-2-ethylhexyl phthalate + FFA's + DG's); X-XI – 250 ml each (DG's + phospholipids). After the separation, the column was regenerated with methanol and was used for a new separation. Fractions III (TG's) and X (DG's) gave a positive Beilstein test.

In all cases, when the eluent was chloroform, the solvent was driven off completely and then benzene was added and this was distilled off until the odor of the solvents had disappeared completely.

SUMMARY

From the sum of the neutral lipids of the seeds of E. moluccelloides we have isolated the natural di- and monoglycerides, have determined their structure, and have established that these classes of lipids contain a higher amount of saturated acids in the β position than the triglycerides of the seeds.

It has also been established that in the hydrolysis of natural diglycerides with the nonspecific lipase of the fungus O. lactis synthetic triglycerides are formed that differ from natural triglycerides by an increased amount of saturated acids.

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