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MODIFIED METHOD FOR THE STEREOSPECIFIC ANALYSIS OF TRIACYLGLYCEROLS

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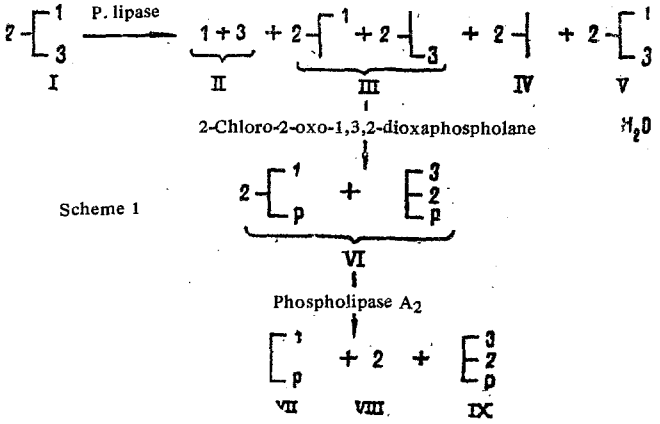
A modification of the well-known method of determining the structures of triacylglycerols that permits the time of analysis to be shortened is proposed. 2-Chloro-2-oxo-1,3,2-dioxaphospholane is used as phosphorylating agent in place of phenyl phosphorodichloridate. A description of the method is given and the results of the analysis of the triacylglycerides of cottonseed oil are presented.

The stereospecific analysis of triacylglycerols proposed by Brockerhoff [1] and consisting of three stages — the hydrolysis of the triacylglycerols with pancreatic lipase, the phosphorylation of the diacylglycerols with phenyl phosphorodichloridate, and the splitting with snake venom phospholipase A_2 of the phosphatidylphenols synthesized — permits the distribution of fatty acid acyl radicals between the sn-1, sn-2, and sn-3 positions of the triacylglycerols to be established.

Because of the length and laboriousness of the analysis (particularly the phosphorylation and phospholipiase hydrolysis stages), the method has not found wide application and is not used to determine the structures of triacylglycerols of modified fats.

To shorten the time of analysis we have proposed to use 2-chloro-2-oxo-1,3,2-dioxaphospholane as the phosphorylating agent in place of phenyl phosphorodichloridate (scheme 1).

Scheme 1



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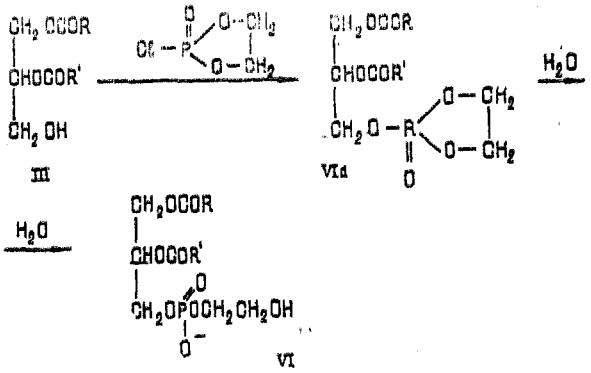
As has been shown previously, the phosphorylation of synthetic diacylglycerols with 2-chloro-2-oxo-1,3,2-dioxaphospholane takes place under mild conditions, rapidly, and with high yields, and is readily performed under laboratory conditions [2, 3]. At the present time, cyclophosphates are the most promising phosphorylating agents and are finding ever wider use in biochemical investigations [3, 4].

We proposed to perform the phosphorylation of the diacylglycerides (III), obtained as the result of the hydrolysis of the triacylglycerols (I) with pancreatic lipase, at 40°C for 30 min in the presence of an excess of the phosphorylating agent (4:1). The opening of the cyclophosphates of the diacylglycerols (VIa, scheme 2) was carried out without their isolation by treatment with moist benzene.



Scheme 2

The selected phosphorylation conditions permit the β-phosphatidylethanol (VI) to be obtained with a yield of 70-75% and do not cause the peroxide oxidation of the lipids, as has been established with the aid of UV spectroscopy.

The use of 2-chloro-2-oxo-1,3,2-dioxaphospholane permits the time not only of the phosphorylation stage but also of the stage of phospholipase hydrolysis to be shortened, since the β-phosphatidylethanols (VI) synthesized are hydrolyzed by snake venom phospholipase A_2 considerably faster than phosphatidylphenols (15 min in place of 4 h).

The accuracy of the proposed method was checked by analyzing the triacylglycerols of cottonseed oil. The results obtained are given in Table 1 and show good agreement between two independent calculations of the amounts of acyl groups in the sn-3 positions of the triacylglycerols, which confirms the correctness of the experimental determination of the sn-1 positions. The results obtained permit the accuracy of our proposed method to be compared with that of Brockerhoff's method [6].

EXPERIMENTAL

UV spectra were recorded on a Hitachi spectrophotometer, and IR spectra on a Perkin-Elmer 257 spectrometer, using thin films.

Preparative TLC was carried out on silica gel LS-5/40 (Chemapol, Czechoslovakia) in the following systems: 1) hexane–diethyl ether–acetic acid (50:50:1); 2) chloroform–methanol–acetone–concentrated ammonia (15:7:5:1); and 3) chloroform–methanol–water (65:25:4). Analytical TLC was performed on Silufol UV-254 in systems 4) diethyl ether–petroleum ether (4:1) and 5) chloroform–methanol (4:1).

The 2-chloro-2-oxo-1,3,2-dioxaphospholane was synthesized by a known method [7, 8]. Refined cottonseed oil from the Tashkent oil and fats combine was studied.

1,2(2,3)-Diacyl-sn-glycerol (III). A mixture of 30 ml of 1 N sodium chloride, 20 ml of 1 M Tris-HCl buffer (pH 8.0), and 5 ml of 0.11 M solution of calcium chloride, previously kept at 40°C, was added to 0.4 g of the oil under investigation. Then, with vigorous stirring, 0.08 g of pancreatic lipase (activity 47.3, grade A, Olaine chemical reagents factory) dissolved in 3 ml of 0.01 m L-histidine was added. Hydrolysis was performed at 40°C for 2.5 min, and was stopped by the addition of 15 ml of 4 N hydrochloric acid. The hydrolysis products were extracted with diethyl ether. The combined ethereal extract was washed with water and dried over calcined sodium sulfate. The solvent was driven off in vacuum. The hydrolysis products were separated by preparative TLC in system 1. The

TABLE 1. Results and Accuracy of the Stereospecific Analysis of the Triacylglycerols of Cottonseed Oil

Compound	Positions	Calculation	Amount, mole %				
			14:0	16:0	18:0	18:1	18:2
I	1,2,3		1,1	28,5	2,1	17,8	50,5
III	1,2;2,3		0,8	21,0	1,7	19,9	56,6
III	1,2;2,3	$\frac{3\times1+IV}{4}$	0,8	22,3	1,6	19,5	55,8
IV	2		Tr.	3,6	Tr.	24,7	71,7
V	1,2,3		1,1	28,7	2,1	18,4	49,7
VII	1		1,4	42,2	3,8	16,6	36,0
VIII	2		Tr.	6,0	1,4	21,6	71,0
IX	2,3		0,9	20,3	1,7	19,0	58,1
	3	3×I–IV–VII	1,9	39,7	2,5	12,1	43,8
	3	2×IX–IV	1,8	37,0	3,4	13,3	44,5

fractions were detected in iodine vapor and were fixed. The monoacylglycerols (R_f 0.09) and the diacylglycerols (R_f 0.43) were extracted from the silica gel with diethyl ether. The fatty acid composition of the initial triacylglycerols, of the unhydrolyzed triacylglycerols (V), of the monoacylglycerols (IV), and of the diacylglycerols (III) were determined by the GLC method.

β-[1,2(2,3)-Diacyl-sn-glycero-3(1)-phosphoryloxy]ethanol (VI). At 20°C, with stirring, a solution of 0.08 g of 2-chloro-2-oxo-1,3,2-dioxaphospholane in 5 ml of benzene was added to a solution of 0.09 g of a diacylglycerol in 2 ml of anhydrous benzene and 0.4 ml of dry triethylamine. The reaction was performed at 40°C for 30 min. The formation of the product was monitored by analytical TLC in systems 4 and 5. The precipitate of triethylamine hydrochloride that deposited was filtered off, and then moist benzene was added to the filtrate and the solvent was driven off in vacuum. Isolation was performed by preparative TLC in system 2, the bands being detected by spraying the edges of the plates with the Vaskovsky reagent. Extraction was carried out with a mixture of chloroform and methanol (2:1). The solvent was eliminated in vacuum. Yield 0.08 g, R_f 0.45 (system 2). IR spectrum, cm^{-1}: 3150 (OH), 3010, 2930, 2860, 1460, 1410 (CH, CH_2, CH_3), 1740 (C = O), 1235 (P = O), 1075 (P–O–C).

β-(1-Monoacyl-sn-glycero-3-phosphoryloxy)ethanol (VI). A solution of 0.08 g of β-(phosphatidyloxy)ethanol in 5 ml of diethyl ether was treated with 0.016 g of lyophilized kufi venom dissolved in a mixture consisting of 1 ml of 0.01 M borate buffer (pH 7.2) and 0.1 ml of 0.11 M calcium chloride. Hydrolysis was carried out with vigorous stirring at 35-37°C for 15 min. The solvent was driven off in vacuum and the water by distillation with benzene. The dry residue was dissolved in methanol, the solution was filtered, and the methanol was distilled off in vacuum. The hydrolysis products were separated by preparative TLC in system 3 and were detected by the Vaskovsky reagent (R_f 0.31). Extraction was carried out with methanol. The fatty acid composition was determined by the GLC method.

SUMMARY

1. A modification of the well-known method of determining the structures of triacylglycerols is proposed.

2. The use of 2-chloro-2-oxo-1,3,2-dioxaphospholane has permitted a considerable shortening of the time of stereospecific analysis of triacylglycerols.

The method is promising for the analysis of triacylglycerols of plant oils and of hydrotransesterified fats.

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STRUCTURES OF DAUROSIDES A AND B — NEW ACYLATED COUMARIN GLYCOSIDES FROM *Haplophyllum dauricum*

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From the roots and epigeal parts of *Haplophyllum dauricum* (L.) G. Don., new acylated courmarin glycosides have been isolated: dauroside A, $C_{23}H_{28}O_{13}$, mp 145-147°C (ethanol), $[\alpha]_D^{22}$ — 72.7° (methanol), and dauroside B, $C_{30}H_{32}O_{14}$, mp 145-150°C (methanol) $[\alpha]_D^{22}$ 0° (pyridine). Their structures have been established on the basis of chemical transformations and IR, UV, mass, ^1H NMR and ^{13}C NMR spectra.

We have previously [1] reported the isolation from the epigeal part of *Haplophyllum dauricum* (L.) G. Don of umbelliferone and umbelliferone 7-O-β-D-glucopyranoside. Continuing this investigation, from the butanolic fraction of the ethanolic extract [2] we have isolated a new coumarin glycoside, dauroside A (I). Its UV spectrum is very close to that of umbelliferone 7-O-β-glucoside. The PMR spectrum of (I) (DMSO-d_6) contains the signals of protons due to the presence in the molecule of residues of unbelliferone, β-glucose, and α-rhamnose (see Table 1). In actual fact, the acid hydrolysis of (I) led to umbelliferone, D-glucose, and L-rhamnose. The presence of a three-proton singlet at 2.01 ppm (CH_3CO) and of the signal of a gem-acyl proton at 4.77 pmm in the PMR spectrum, and also of a $\nu_{C=O}$ absorption band of an ester grouping (1723 cm^{-1}) in the IR spectrum showed that (I) was an acylated coumarin glucoside. This was confirmed by the formation of acetic acid and of the deacetyl product (II) with mp 186-187°C on mild alkaline hydrolysis of (I) with 0.5% KOH solution at room temperature. The dauroside (I) formed a hexaacetyl derivative with the composition $C_{33}H_{38}O_{18}$ (III), M^+ 722. The PMR spectrum of (III) ($CDCl_3$) showed the signals of the protons of the methyls of six aliphatic acetyl groups at (ppm) 1.90 (3 H), 1.95 (6 H), and 1.98 (9 H). Consequently, (I) is an acylated bioside of umbelliferone. The mass spectrum of (III) contains, in addition to the peak of the molecular ion with m/z 722, strong peaks of ions with m/z 273 (100%), 213, 184, 153, 111, and others, corresponding to fragments of the ($M - CH_3COOH$) ion of tetraacetylrhamnopyranose [3]. The strong peak of an ion with m/z 560 is formed by the splitting out of umbelliferone from the molecular ion. Consequently, in the dauroside A molecule rhamnose is the terminal sugar.

In the PMR spectrum of (III) the ratio of the intensities of the signals of the protons in the 4.5-5.6 and 3.4-4.4 ppm intervals is 8:4, and the signal of the anomeric proton of the rhamnose residue appears at δ 4.62 ppm. These facts permit (I) to be characterized as an acylated rutinoside [4]. The position of attachment of the acetyl group and the definitive structure of dauroside A were established by a study of its ^{13}C NMR spectrum (Table 2). The signals in the ^{13}C NMR spectrum were identified on the basis of an

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