EXTRACELLULAR GLYCOLIPIDS OF RHODOTORULA SPECIES

THE ISOLATION AND SYNTHESIS OF 3-D-HYDROXYPALMITIC AND 3-D-HYDROXYSTEARIC ACIDS[1]

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ABSTRACT

Several species of the red yeast *Rhodotorula* produce extracellular glycolipids which consist of a mixture of mannitol and pentitol esters of 3-D-hydroxypalmitic and 3-D-hydroxystearic acids. One molecule of the long chain acid is attached to each polyol molecule and most of the remaining hydroxyl groups including the one on the fatty acid are acetylated.

The formation of extracellular lipid by *Rhodotorula graminis* was reported by di Menna in 1958 (1). Later Deinema (2, 3) studied extracellular lipid production in *R. graminis* and in two strains of *R. glutinis*. She also investigated the composition of the lipids and found acetic acid, a number of known and unknown long-chain fatty acids, and polyols among the hydrolysis products. In an investigation of the production of extracellular lipids by yeasts which is being carried out in this laboratory (4), three species of *Rhodotorula*, which form such lipids, were isolated.

The composition of the lipids produced by these species and also that produced by *R. glutinis* CBS 4648, originally studied by Deinema, were investigated. The "oily" extracellular material, obtained when the yeasts were grown in shaken culture, was heavier than water and therefore was easily isolated. It was found to be a mixture of polyol esters, which was soluble in both methanol and hexane. However, on one occasion when *R. glutinis* strain 4648 was grown in fermentors the oil produced was only partly soluble in methanol, and could be separated by solvent distribution into polyol esters and triglycerides. The principal fatty acids of the latter were oleic and palmitic acids. It is probably this extracellular triglyceride which gave rise to the ordinary fatty acids found by Deinema (2, 3).

Acid methanolysis of the polyol esters gave free polyols, the methyl esters of hydroxy acids, and methyl acetate. The relative amounts of the first two groups of components corresponded approximately to a ratio of 1 molecule of fatty acid to 1 molecule of polyol. The polyols were analyzed as the acetates by gas liquid chromatography (GLC) and consisted of a mixture of D-mannitol, D-arabitol, and minor amounts of xylitol. The fatty acid ester portion was almost the same for each strain and was a mixture of 3-D-hydroxypalmitic (85%) and 3-D-hydroxystearic (15%) acids. The evidence for this conclusion is described below.

A mixture of palmitic and stearic acids was obtained by reduction of the hydroxy acids with red phosphorus and hydrogen iodide followed by zinc and hydrochloric acid, showing that the fatty acids contained unbranched carbon chains. This finding and the observation that the carbon numbers, on a silicone column, were 17.7 and 19.7, or almost two units greater than the carbon numbers of palmitic and stearic acids, suggested that

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the fatty components were hydroxy acids (5, 6). The methyl esters were partly separated into C_{16} and C_{18} components by countercurrent distribution. The only isolatable product of chromic acid oxidation of the C_{16} ester was myristic acid, which indicated that the hydroxyl group was at position 3.

Further evidence for this was derived from the action of base on the original polyol esters and on the acetoxy ester, obtained by acetylation of the hydroxy ester. Treatment of the polyol ester with sodium methoxide solution, to effect methanolysis, produced an unsaturated C_{16} ester in addition to the hydroxy ester. The acid corresponding to this ester was found to be *trans*-2-hexadecenoic acid. This acid was also obtained by alkaline hydrolysis of the polyol ester or the C_{16} acetoxy ester. Linstead *et al.* (7) have shown that when β-acetoxy esters are treated with bases β-elimination of acetic acid occurs and an α,β-unsaturated acid is produced. The relative amounts of unsaturated and hydroxy acid obtained by alkali treatment of the polyol ester and of methyl 3-acetoxypalmitate were compared by GLC analysis. The relative amount of unsaturated acid yielded by the polyol ester was slightly lower than that yielded by the pure acetoxy ester indicating that in most, but not all, of the polyol ester molecules the 3-hydroxyl group was acetylated. The results showed that the 3-hydroxystearic acid was also mainly acetylated. Therefore in working with a lipid which contains a 3-acetoxy fatty acid grouping hydrolysis must be carried out in an acid medium. The previous workers (2, 3) employed alkaline hydrolysis and consequently obtained confusing results.

Racemic methyl 3-hydroxypalmitate was synthesized and compared with the ester obtained from the glycolipid. The carbon numbers, on the silicone column, were the same and the infrared spectra in carbon tetrachloride solution were indistinguishable. However, the infrared spectra, compared as Nujol mulls, were quite different. The 3-hydroxy esters and acids obtained from the glycolipid were quite strongly laevorotatory in chloroform, but in methanol the specific optical rotation was quite small, which is characteristic of 3-hydroxy fatty acids (8). The negative rotation suggests that the compounds have the D-configuration (9).

To establish the configuration and structure of the acids of the glycolipid 3-D-hydroxypalmitic and 3-D-hydroxystearic acids were synthesized from (+) methyl hydrogen β-acetoxyglutarate by the general method of Serck-Hanssen (8). The products were identical with those obtained from the yeast lipid.

It appears that these two particular 3-hydroxy acids have not been obtained from natural sources previously, but 3-D-hydroxymyristic acid has been isolated from *Escherichia coli* (10), 3-D-hydroxylauric acid from a metabolite of *Isaria cretacea* (11), and 3-D-hydroxycapric acid from a metabolite of *Pseudomonas aeruginosa* (12).

Approximately 3.5 molecules of acetic acid were obtained per molecule of glycolipid but the points of substitution of the acetic acid and fatty acid residues on the polyol were not investigated. The product is probably a complex mixture of isomers. The fatty acids and polyols of the intracellular lipids of three of the four strains were also analyzed. The composition of the polyol fraction is shown in Table I. The two lipids are quite different, the intracellular lipid being mostly triglycerides and the extracellular lipid a mixture of esters of 5 and 6 carbon polyols. Table II shows the composition of the fatty acid fractions. The two lipids obtained from *R. glutinis* strain 4648 closely resemble each other in fatty acid composition and strain 16A8 is also similar. However, the acids from *R. graminis* strain 6CB contain different relative amounts of palmitic and oleic acids. The composition of the cell fat of *R. graminis* di Menna has been reported (13) but differed from that of the strain used here in having a considerably lower oleic acid content.

TABLE I

Polyols of the intra- and extra-cellular lipids

		Glycerol	Arabitol (mol. %)	Xylitol (mol. %)	Mannitol
R. glutinis strain 4648	Intracellular	86.9	8.2	1.2	3.7
	Extracellular triglycerides	94.5	5.5	—	—
	Extracellular polyol esters	—	49.7	—	50.3
R. glutinis strain 16A8	Intracellular	81.3	4.9	1.1	12.7
	Extracellular	—	24.0	3.5	72.5
R. graminis strain 6CB	Intracellular	97.9	0.8	—	1.3
	Extracellular	—	15.6	7.5	76.9

TABLE II

Fatty acid composition of lipids other than polyol esters

Fatty acids, weight %	*R. glutinis* strain 4648		*R. glutinis* strain 16A8	*R. graminis* strain 6CB
	Extracellular	Intracellular		
Myristic	1.2	1.1	1.5	1.3
Palmitic	31.4	32.7	28.7	21.6
Palmitoleic	1.2	1.1	3.2	1.6
Stearic	8.3	8.7	2.4	2.3
Oleic	43.2	40.0	46.3	61.0
Linoleic	12.3	11.4	10.6	11.3
Linolenic	1.9	2.3	5.2	0.7
Unidentified	0.5	2.7	2.1	0.2
Oil content %	—	71	64	74

EXPERIMENTAL

Melting points were determined using a Leitz hot-stage microscope. Optical rotations were measured at 25° C in a 1 dm tube.

Method of Oil Production

The yeasts were isolated from flowers obtained from several localities in Canada. On the basis of carbon assimilation tests (14) the yeasts were considered to be *R. glutinis* strain 16A8, *R. mucilaginosa* strain S6A1, and *R. graminis* strain 6CB. The last strain resembled *R. graminis* di Menna C.B.S. 2826 but differed from this culture in not assimilating cellobiose, L-arabinose, dulcitol, and salicin (J. F. T. Spencer and H. Phaff, unpublished work). The medium used consisted of glucose (4%), yeast extract (0.2%), potassium dihydrogen phosphate (0.1%), and magnesium sulphate heptahydrate (0.02%). Flasks containing 50 ml of medium were agitated on a Gump shaker having a radius of 1 in. at 230 r.p.m. for a period of 8–10 days. Three liter fermentors were used at 28° C, with an air flow of 1 liter/minute, a stirring speed of 400 r.p.m., and inoculated with 100 ml of 24-hour shaken culture. The yield of extracellular lipid was 1–2 g/liter. The cells and lipid were allowed to settle and the lipid was washed away from the cells with light petroleum, b.p. 60–80° C.

Gas–Liquid Chromatographic Analysis

The hydroxy fatty acid esters were analyzed by means of a silicone on celite column as previously described (5). Methyl 3-hydroxypalmitate and methyl 3-hydroxystearate had carbon numbers of 17.7 and 19.7 respectively. On this column methyl 2-hexadecenoate has a carbon number of 16.5 and is completely separated from methyl palmitate. On the other hand, methyl 9-hexadecenoate has a carbon number which is very slightly lower than that of methyl palmitate.

The separation of all the pentitol and hexitol acetates except for those of glucitol and galactitol was described by Jones and co-workers (15) using a column packing which consisted of a mixture of polyester and non-polar phases. In the present work an 8 ft × 3/16 in. copper column packed with 1:30 butanediol. succinate (16) on 40–60 mesh acid-washed firebrick was used. The oven temperature was 212° C and the

helium flow rate 60 ml/minute. This column partly separated glucitol and galactitol acetates and the retention times of the acetates relative to arabitol pentaacetate were as follows: ribitol 0.89, arabitol 1.00 (18 minutes), xylitol 1.24, mannitol 2.60, galactitol 2.97, glucitol 3.12. The column used by Jones and co-workers separated the acetates in the same order, except for the last two, and also effected a good separation of allitol, talitol, and iditol acetates from the other three. It is probable that the present column would separate all six hexitol acetates.

To separate and collect larger quantities of arabitol and mannitol acetates and fatty acid esters an 8 ft × 5/16 in. copper column with 1:6 silicone grease on acid-washed celite was used at 240° C, with injector at 290° C, and a flow rate of 60 ml helium/minute.

Composition of the Extracellular Lipids

The lipid had a molecular weight (osmometric in acetone) of 560, a saponification equivalent of 110.5, and gave 0.44 g of volatile acid (calculated as acetic acid) per gram of lipid. The volatile acid was analyzed by gas chromatography as the decyl ester (17) and acids of chain length longer than two carbon atoms were absent. The analyses do not correspond to a definite composition and the lipid is probably quite a complex mixture. It contains approximately 3 to 4 molecules of acetic acid for each lipid molecule.

Acid Methanolysis of the Extracellular Lipid

Lipid (11.56 g) was refluxed for 1 hour with 3% methanolic hydrogen chloride and the acid neutralized with silver carbonate. After filtration the volatile material was removed on a rotary evaporator and collected in a dry-ice trap. This fraction, which contained methyl acetate, was saponified and a portion of the salt obtained treated with S-benzyl thiouronium chloride to give S-benzyl thiouronium acetate with a melting point of 135–138° C, which was not depressed by an authentic sample.

Hydroxy fatty acid esters (5.30 g) were extracted from the residue, obtained after removing the methyl acetate, by stirring with ether. The polyols (3.18 g) remained and when analyzed by paper chromatography (*n*-butanol–ethanol–water 40:11:19 v/v) only pentitols and hexitols were found. When the polyols from *R. glutinis* strain 4648 were examined a portion was acetylated and separated in 35 mg batches by means of the preparative silicone column. D-Mannitol hexaacetate, so obtained, had $[\alpha]_D$ +32.0° (*c*, 1.0, $CHCl_3$) and melting point 120–123° C, after crystallization from ethanol. The mixed melting point with an authentic sample of D-mannitol hexaacetate was 122–124° C. D-Arabitol pentaacetate was crystallized from ethanol–hexane and had $[\alpha]_D$ +36.8° (*c*, 1.5, $CHCl_3$) and melting point 71–73° C. The mixed melting point was not depressed by an authentic sample.

Structure of the Hydroxy Fatty Acids

The composition of the hydroxy fatty acid methyl ester portion, as analyzed by GLC, was almost the same for the lipids from all the strains examined. Palmitic and stearic acids were very minor components (0.3% or less), the approximate composition was 3-hydroxypalmitate (85%), and 3-hydroxystearate (15%).

Mixed esters (10 mg) were refluxed overnight with 47% aqueous hydrogen iodide (5 ml) and red phosphorus (100 mg), the mixture poured into water, and the acids isolated. The saturated acids obtained by refluxing for 5 hours with concentrated hydrochloric acid (1 ml) and zinc dust (100 mg) were identified, by GLC, as palmitic and stearic acids. Hydroxy methyl esters (3 g) were distributed between 85% ethanol and light petroleum b.p. 40–60° C by 125 cycles of a modified E.C. Apparatus Co. fractionater with 100 tubes and 20 ml capacity each phase. Only a partial separation of the C_{16} and C_{18} esters was obtained. Tubes 52–70 contained methyl 3-hydroxypalmitate (1.3 g) and tubes 84–88 contained methyl 3-hydroxystearate (0.075 g). After crystallization from acetone, methyl 3-D-hydroxypalmitate had melting point 48–49° C and $[\alpha]_D$ −13.8° (*c*, 5.6, $CHCl_3$) and −0.3° (*c*, 7.1, MeOH). Calculated for $C_{17}H_{34}O_3$: C, 71.3%; H, 12.0%. Found: C, 71.2%; H, 11.9%. The peaks in the OH and carbonyl regions of the infrared spectra were as follows: 3380 (s), 3300 (s), 1735 (s), 1690 (s), (Nujol mull); 3550 (w), 1727 (s) cm^{-1}, (0.2% in carbon tetrachloride). Saponification of the methyl ester with potassium hydroxide (5% in 90% ethanol) at 60° C for 1 hour yielded 3-D-hydroxypalmitic acid which had melting point 78–79° C, after crystallization from acetone, and $[\alpha]_D$ −12.9° (*c*, 1.3, $CHCl_3$) and +2.1° (*c*, 6.8, MeOH). Calculated for $C_{16}H_{32}O_3$: C, 70.5%; H, 11.8%; neutral equivalent, 272.5. Found: C, 70.8%; H, 11.7%; neutral equivalent, 275.5.

The C_{18} fraction was crystallized from acetone and yielded methyl 3-D-hydroxystearate, m.p. 55.5–56.5° C; $[\alpha]_D$ −15.0° (*c*, 1.9, $CHCl_3$). Calculated for $C_{19}H_{38}O_3$: C, 72.6%; H, 12.2%. Found: C, 72.5%; H, 12.0%. The peaks in the infrared spectrum (Nujol mull) in the OH and carbonyl regions were 3380 (s), 3300 (s), 1735 (s), 1690 (s) cm^{-1}. Saponification yielded 3-D-hydroxystearic acid which had melting point 84–86° C, after crystallization from acetone and $[\alpha]_D$ −12.0° (*c*, 1.2, $CHCl_3$). Calculated for $C_{18}H_{36}O_3$: C, 71.95%; H, 12.1%. Found: C, 71.8%; H, 12.0%.

Chromic Acid Oxidation

Methyl 3-D-hydroxypalmitate (0.12 g) was oxidized overnight with 0.1 *N* chromium trioxide in acetic acid (50 ml); water was added, the excess oxidant reduced with sulphur dioxide and the product (0.1 g) extracted with ether. The product was converted to the methyl ester and purified by gas chromatography using the preparative silicone column at 200° C. Saponification and crystallization from hexane yielded myristic acid with melting point 53–54° C, which was not depressed by admixture with pure myristic acid.

Synthesis of the Hydroxy Esters

Racemic Methyl 3-Hydroxypalmitate

Methyl 3-oxopalmitate was prepared from myristoyl chloride (18) and hydrogenated over Raney nickel (19) to give methyl 3-hydroxypalmitate, m.p. 41–43° C. Calculated for $C_{17}H_{34}O_3$: C, 71.3%; H, 12.0%. Found: C, 71.4%; H, 11.8%. Infrared maxima in the OH and carbonyl regions were 3390 (m), 3570 (sh), 1730 (s), (Nujol mull); 3550 (w), 1727 (s) cm^{-1}, (0.2% in carbon tetrachloride.)

3-D-Hydroxypalmitic Acid

(+) Methyl hydrogen β-acetoxyglutarate (8) (1 g) and tridecanoic acid (3 g) were electrolyzed in 0.022 *N* sodium methoxide in methanol (30 ml) until the solution was just alkaline. After the solution was acidified with acetic acid, it was filtered from hydrocarbon by-products, part of the methanol was taken off, 3% methanolic hydrogen chloride added, and the mixture refluxed for 3 hours. Water was added and the product extracted with ether. The crude product (1.1 g) was chromatographed on silicic acid (60–200 mesh); elution with light petroleum (b.p. 60–80° C) containing 2% acetone gave a small amount of methyl tridecanoate followed by the desired hydroxy ester (0.45 g). Crystallization from acetone yielded methyl 3-D-hydroxypalmitate, m.p. 49–50° C, which was not depressed when mixed with the natural ester, $[\alpha]_D$ was −14.3° (*c*, 2.5, $CHCl_3$). Calculated for $C_{17}H_{34}O_3$: C, 71.3%; H, 12.0%. Found: C, 71.1%; H, 12.0%. The infrared spectrum (Nujol mull) and the X-ray powder photograph were indistinguishable from those of the natural product. 3-D-Hydroxypalmitic acid was obtained by saponification, and after crystallization from acetone had melting point 77–79° C and $[\alpha]_D$ −13.8° (*c*, 2.4, $CHCl_3$); the melting point was not depressed by admixture with natural acid. Calculated for $C_{16}H_{32}O_3$: C, 70.5%; H, 11.8%. Found: C, 70.5%; H, 12.0%.

3-D-Hydroxystearic Acid

Pentadecanoic acid was prepared by stepwise oxidation of 1-hexadecene in the same way as the preparation of heptadecanoic acid (5). (+) Methyl hydrogen β-acetoxyglutarate (1 g) was electrolyzed with pentadecanoic acid (3 g) and worked up as in the preparation of the C_{16} ester. After silicic acid chromatography methyl 3-D-hydroxystearate (0.47 g) was obtained and crystallized from light petroleum (b.p. 60–80° C). The melting point was 56.5–57.5° C, undepressed by admixture with the natural ester, and $[\alpha]_D$ was −12.9° (*c*, 4.1, $CHCl_3$). The infrared spectrum (Nujol mull) and the X-ray powder photograph were indistinguishable from those of the natural ester. Calculated for $C_{19}H_{38}O_3$: C, 71.6%; H, 12.2%. Found: C, 72.3%; H, 12.2%. Hydrolysis yielded 3-D-hydroxystearic acid which was crystallized from acetone and had melting point 83–85° C, which was not depressed by the natural acid; $[\alpha]_D$ was −12.2° (*c*, 1.4, $CHCl_3$). Calculated for $C_{18}H_{36}O_3$: C, 71.95%; H, 12.1%. Found: C, 71.9%; H, 12.1%.

Reaction of the Lipid and of Methyl 3-Acetoxypalmitate with Alkali

The glycolipid was treated with 0.022 *N* sodium methoxide in methanol both at room temperature and under reflux. The relative amounts of methyl 2-hexadecenoate, methyl 3-hydroxypalmitate, and methyl 3-acetoxypalmitate, found in the product by GLC, varied with the time and temperature of the reaction. Similar results were obtained starting with methyl 3-acetoxypalmitate. The latter was prepared by refluxing synthetic racemic methyl 3-hydroxypalmitate with 20 times its weight of acetic anhydride for 1 hour and removing the excess reagent. The acetate ester was distilled with boiling point 110–115° C (bath) at 0.05 mm. The following conditions were found to give the best yield of 2-hexadecenoic acid. Acetoxy ester (0.3 g) was added to boiling *M*/50 potassium hydroxide in methanol (100 ml) and refluxed for 1 hour, potassium hydroxide (0.5 g) in water (5 ml) was then added and the mixture refluxed for a further 30 minutes. Water was added followed by dilute hydrochloric acid and the acids extracted with ether. The product was extracted with light petroleum (b.p. 40–60° C) leaving insoluble 3-hydroxypalmitic acid (0.065 g). The extract was concentrated to 4 ml and cooled to −10° C and *trans*-2-hexadecenoic acid (0.080 g) was obtained with melting point 47–48° C. Reported melting point is 48.7–49° C (20).

Methyl 3-D-acetoxypalmitate (0.3 g) was prepared from the natural hydroxy acid and treated in the same way. GLC analysis of the methyl esters of the crude product showed that the ratio of 2-hexadecenoic acid to 3-hydroxypalmitic acid was 3.44:1. 2-Hexadecenoic acid (0.052 g) was isolated and the melting point and mixed melting point with the 2-hexadecenoic acid, prepared as above from synthetic racemic acetoxy ester was 46.5–47.5° C. The polyol ester from yeast was also treated with *M*/50 potassium hydroxide as described above and the ratio of 2-hexadecenoic acid to 3-hydroxypalmitic acid was 2.82:1.

The Polyols of the Extra- and Intra-cellular Lipids

The intracellular lipids were extracted from the dried yeast cells by the method used for rust spores (21). The lipids were treated with methanolic hydrogen chloride (3%), the acid neutralized with silver carbonate, the mixture filtered, and the methanol taken off. The fatty esters were extracted with ether and the remaining polyols were acetylated by heating at 100° C with a mixture of acetic anhydride and pyridine (1:1). The excess reagent was removed and the acetates were analyzed by GLC. The results are shown in Table I.

The Fatty Acid Composition of the Lipids Other than Polyol Esters

The methanol-insoluble extracellular lipid produced by *R. glutinis* CBS 4648 when grown in fermentors was separated from polar polyol esters by distribution between 90% methanol and light petroleum (b.p. 40–60° C) in four separatory funnels. Methyl esters were prepared from this lipid as before. The fatty acid

methyl esters were analyzed on silicone and polyester columns as previously described (21). The methyl esters from the above-mentioned extracellular triglycerides produced by *R. glutinis* CBS 4648 were oxidized with the permanganate–periodate reagent and the oxidation fragments analyzed (22). The results confirmed the assumption that the unsaturated fatty acids had the usual structures, and no evidence for the presence of isomeric acids was found. The esters of the other lipids were also assumed to have the usual structures. The results are summarized in Table II.

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