Formation of a Long-Chain Alcohol Ester of Hydroxy Fatty Acid Sophoroside by Fermentation of Fatty Alcohol by a *Torulopis* Species[1]

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Received March 13, 1972

Fermentation of oleyl alcohol by *Torulopsis bombicola* produces the oleyl alcohol ester of the sophoroside of 17-hydroxyoleic acid (40%) together with lactonic and acidic hydroxy acid sophorosides. The major product was characterized as the octadecyl derivative **3** and the structure was established by degradation and by synthesis of the α anomer of the heptaacetate. The composition of the fermentation product was obtained by gas–liquid chromatographic analysis of the trimethylsilyl ethers of the hydrogenated deacetylated products.

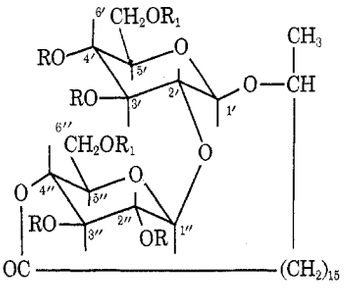
We have previously shown that when long-chain fatty acids are fermented by a species of yeast of the genus *Torulopsis* the acids are hydroxylated at the penultimate or terminal carbon atom and the hydroxy acids produced are converted to sophorosides.[2] The products exist mainly in lactonic (**1**) and acidic forms (**2**) both having acetate groups at the 6′ and 6″ positions.[3] The fermentation of fatty alcohols has now been investigated in the hope that alcohol sophorosides with surface-active properties would be obtained. Another possible result hoped for was that alcohol sophoroside would be formed first, then the terminal methyl group oxidized to carboxyl, thus producing a larger proportion of ω-hydroxy acid sophoroside than is usually obtained from fatty acids.

Oleyl alcohol was used in these experiments since being liquid it was more readily taken up by the yeast cells. There was, however, no appreciable formation of alcohol sophoroside nor was there an increase in the proportion of ω-hydroxy acid sophoroside. About 40% of the product, examined after hydrogenation, consisted of octadecyl ester **3** octadecyl 17-L-[(2′-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]octadecanoate 6′,6″-diacetate.

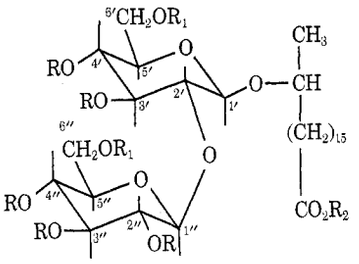
Thin layer chromatography indicated that the crude fermentation product also contained appreciable amounts of lactone and acid. After removal of about one-third of the lactone by crystallization, the remainder of the product was hydrogenated. Subsequently, a combination of column chromatography and crystallization yielded about 15% of the original amount of ester **3** in pure form.

Alkaline hydrolysis gave octadecanol and sophorosyl hydroxy acid isolated as methyl ester **4**,[3] and methanolysis with sodium methoxide gave deacetylated octadecyl ester **5**. Acetylation of **3** gave heptaacetate **6**. This compound was also prepared from crude neutral product (obtained by chromatographic removal of **2**) by acetylation and separation from lactone hexaacetate (**7**).

The nmr spectrum of **3** in dimethyl sulfoxide-d_6 was similar to that of methyl ester **8**,[3] except for additional signals due to the octadecyl portion. In particular, it contained five low-field proton doublets assigned to the five secondary hydroxyl groups of the sophorose portion, showing that the acetate groups are at the 6′ and 6″ positions.



1, R = H; R_1 = Ac
7, R = R_1 = Ac



2, R = R_2 = H; R_1 = Ac
3, R = H; R_1 = Ac; R_2 = $CH_3(CH_2)_{17}$
4, R = R_1 = H; R_2 = CH_3
5, R = R_1 = H; R_2 = $CH_3(CH_2)_{17}$
6, R = R_1 = Ac; R_2 = $CH_3(CH_2)_{17}$
8, R = H; R_1 = Ac; R_2 = CH_3

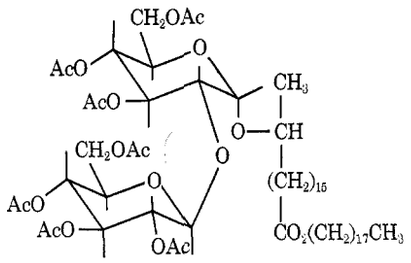
The formation of **3** in the fermentation was surprising, and, to confirm that **3** was in fact an octadecyl ester, synthesis of heptaacetate **6** by the Koenigs–Knorr reaction was attempted. 17-L-Formyloxyoctadecanoyl chloride[4] was allowed to react with octadecanol and the formate group was removed from the product to give octadecyl 17-L-hydroxyoctadecanoate. Reaction of acetobromosophorose[3] with this hydroxy ester, however, yielded a heptaacetate which resembled but was not identical with **6**. The product was dextrorotatory, whereas **6** is levorotatory, indicating that the reaction had produced a mixture of α and β anomers. This was confirmed by the nmr spectrum ($CDCl_3$) of the product, which showed a doublet at δ 4.61, assigned to H-1″ of the α form **9** by analogy with the spectrum of the heptaacetate of the α form of **4**,[3] and a doublet at δ 4.46 assigned to H-1′ of **6**. The relative intensities of these signals showed that about 30% of α anomer was present. The Koenigs–Knorr reaction was repeated, but 30–50% of the α anomer was always produced. Accordingly, both the synthetic

(1) (a) NRCC No. 12527. (b) Part VIII in the series "Fermentation of Long-Chain Compounds by *Torulopsis* sp." Part VII: E. Heinz, A. P. Tulloch, and J. F. T. Spencer, *Biochem. Biophys. Acta*, **202**, 49 (1970).

(2) A. P. Tulloch, J. F. T. Spencer, and P. A. J. Gorin, *Can. J. Chem.*, **40**, 1326 (1962).

(3) A. P. Tulloch, A. Hill, and J. F. T. Spencer, *ibid.*, **46**, 3337 (1968).

(4) A. P. Tulloch, *Chem. Phys. Lipids*, **6**, 235 (1971).



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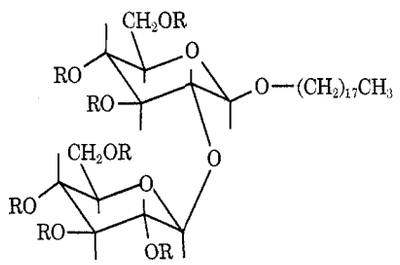
mixture and compound **6** were completely inverted to α-heptaacetate **9** by treatment with 3% hydrogen bromide in acetic acid.[3] The two products were indistinguishable.



10, R = Ac
11, R = H

To make it easier to detect possible minor amounts of oleyl alcohol β-sophoroside in the fermentation product by chromatography, octadecyl β-sophoroside heptaacetate (**10**) was synthesized by the Koenigs–Knorr reaction. About 35% of the product crystallized as pure β anomer, but nmr spectroscopy indicated that the mother liquors contained the α form in quantities corresponding to the formation of 20% of α anomer in the reaction. Deacetylation of **10** gave octadecyl β-sophoroside (**11**).

The possibility of analyzing the fermentation products as trimethylsilyl ethers by gas–liquid chromatography (glc) was investigated. Although trimethylsilyl ethers of pure diacetates **1** or **8** could be separated, the natural product also contained compounds lacking one or both acetate groups which had shorter retention times (as trimethylsilyl ethers), resulting in a complex mixture which could not be analyzed satisfactorily. Trimethylsilyl ethers of the deacetylated compounds sophoroside **11**, methyl ester **4**, and octadecyl ester **5**, however, were well separated by glc. A mixture of these three compounds was used to determine relative response factors for flame ionization detectors, making possible quantitative analysis of the fermentation products.

The fermentation mixture was hydrogenated, treated with diazomethane to produce esters from acidic products, deacetylated, and converted to trimethylsilyl ethers. Proportions of lactonic and acidic products were estimated by first separating **1** and **3** together from **2** (and compounds lacking acetate groups) by silicic acid column chromatography and analyzing these fractions separately by glc. A peak (2%) with the same emergence temperature as the trimethylsilyl ether of **11** was detected, and, although the presence of **11** was not confirmed further, it clearly did not form more than 2% of the total product. The approximate composition of the product was thus shown to be **11**, 2%; **1**, 20%; **2** (also acids lacking acetates), 25%; **3**, 40%; octadecyl esters lacking acetates, 5%; and unidentified, 8%. Compound **11** was probably originally present as the 6′,6″-diacetate.

Fermentation of long-chain fatty acids and hydrocarbons previously[3] gave mixtures containing about 60% lactones and 40% acids. It appears that, when oleyl alcohol is fermented, part of the alcohol is first oxidized to oleic acid, which is then hydroxylated mainly at the penultimate carbon atom;[2] sophorosidic acid is then formed; about half is enzymatically converted to oleyl alcohol ester; and the rest either is converted to lactone or remains as free acid. Further investigations are being carried out to find out if other alcohols are also converted to esters of type **3** by *Torulopsis* species.

Experimental Section[5]

Fermentation of Oleyl Alcohol.—Strain 319-67 of *Torulopsis bombicola*[6] was used and the medium (3 l. in 5 l. of fermentor) was glucose (10%), yeast extract (1%), and urea (0.1%). The medium was inoculated and 2 days later oleyl alcohol[7] was added (50 g on each of 4 successive days). The medium was agitated at 400 rpm at 23° with air flow of 1 l./min. One day after the last oleyl alcohol addition the clear medium was decanted from the sludge of cells and heated to 70°, when a mixture of product and water (1:1) separated as a heavy, amber-colored "oil" (470 ml). Product (220 g) was obtained by extraction of the "oil" with ethyl acetate.

Thin Layer Chromatography.—All products were examined by tlc. R_f values were as follows: in diethyl ether, **6** and **9**, 0.57; **7**, 0.51; **10**, 0.50; in chloroform–acetone–acetic acid (50:50:1), **1**, 0.26; **3**, 0.20; **2**, 0.11; in chloroform–methanol (3:1), **1**, 0.71; **3**, 0.72; **5**, 0.48; **4**, 0.37; **11**, 0.31.

Octadecyl 17-L-[(2′-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)-oxy]octadecanoate 6′,6″-Diacetate (3).—A solution of 55 g of fermentation product in 220 ml of ethanol was kept at 2° for 1 week and then filtered from 5 g of crystalline lactone (similar to compound **1** but with a 9,10-double bond in the fatty acid chain). The filtrate was made up to 500 ml with ethanol and hydrogenated at 45° over 1 g of 10% palladium on charcoal. After removal of the catalyst, the solution deposited 16.3 g of amorphous lumps. This material was applied to a column of 200 g of silicic acid, and elution with 2 l. of chloroform–methanol (50:1) gave 8.5 g of crude **3**. Rechromatography on a further 200 g of silicic acid and elution with 5 l. of chloroform–acetone (9:1) gave 2.55 g of **3** free from **1**. Recrystallization from ethanol gave 2.30 g of pure **3**: mp 109–111°; $[\alpha]^{25}D$ −15.5° (*c* 2.3, $CHCl_3$); nmr (dimethyl sulfoxide-d_6, 55°) δ 0.84 (t), 1.08 (d), 1.24 (s), 1.98 (s, CH_3CO–), 2.22 (t, $-CH_2CO_2$–), 3.98 (t, $-CH_2OCO$–), 4.37, 4.41 (both d, J = 7.5 Hz, H-1′ and H-1″), 4.86, 4.94, 5.10, 5.20, 5.40 (all d, OH)

Anal. Calcd for $C_{52}H_{96}O_{15}$: C, 64.97; H, 10.07. Found: C, 64.72; H, 9.85. Saponification equivalent calcd, 320.43; found, 323.9; mol acetic acid/mol calcd, 2.0; found, 1.97.

Alkaline Hydrolysis of 3.—A solution of 0.156 g of compound **3** in 20 ml of ethanol, 20 ml of water, and 10 ml of 0.1 *N* aqueous sodium hydroxide was refluxed for 18 hr and then neutralized with 0.1 *N* hydrochloric acid. After addition of 50 ml of methanol and 50 ml of water the solution was extracted twice with

(5) Nmr spectra were measured at 100 MHz using a Varian HA-100 spectrometer; the temperature was 32° except where otherwise stated; chemical shifts are in parts per million from internal tetramethylsilane. Specific rotations were measured in a 1-dm cell using a Perkin-Elmer Model 141 polarimeter. Silica gel G was used for tlc; compounds were detected by spraying with 50% sulfuric acid and heating with an infrared lamp. Bio-Sil A silicic acid, from Bio-Rad Laboratories, Richmond, Calif., was used for column chromatography. Glc was carried out using an F & M Model 402 gas chromatograph with flame ionization detectors; the column was 3 ft × 0.125 in. stainless steel packed with 80–100 mesh, silanized, acid-washed Chromosorb W coated with 2% silicone SE-30.

(6) J. F. T. Spencer, P. A. J. Gorin, and A. P. Tulloch, *Antonie van Leeuwenhoek*; *J. Microbiol. Serol.*, **36**, 129 (1970).

(7) Oleyl alcohol was prepared by lithium aluminum hydride reduction of pure methyl oleate.

hexane. Evaporation of the hexane extract gave 0.042 g of octadecanol, after crystallization from methanol, mp and mmp 58–59°. The aqueous alcohol solution was treated with Dowex 50, 2 ml of pyridine was added, and the solution was evaporated to dryness. The product was dissolved in methanol and treated with diazomethane, and the solvent removed. Crystallization of the residue from methanol–water (1:2) gave 0.100 g of **4**, mp and mmp with authentic 4[3] 145–148°.

Octadecyl 17-L-[(2′-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)-oxy]octadecanoate (5).—Compound **3** (0.151 g) was dissolved in 5 ml of chloroform, 5 ml of 0.022 *N* methanolic sodium methoxide was added, and the mixture was kept at room temperature for 30 min and then neutralized with 15 μl of acetic acid. Solvents were evaporated and the residue was crystallized from ethanol, giving 0.139 g of **5**: mp 159–162°; $[\alpha]^{25}D$ −13.8° (*c* 1.1, pyridine); nmr (pyridine) δ 4.48 (d, *J* = 7.5 Hz, H-1′), 5.14 (d, *J* = 7.5 Hz, H-1″).

Anal. Calcd for $C_{48}H_{92}O_{13}$: C, 65.72; H, 10.57. Found: C, 65.72; H, 10.76.

Octadecyl 17-L-[2′-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)-oxy]octadecanoate 2″,3′,3″,4′,4″,6′,6″-Heptaacetate (6).—A solution of 1.30 g of **3** in 5 ml of pyridine and 5 ml of acetic anhydride was kept at 25° for 18 hr. Removal of the reagents at 70° and crystallization from methanol gave 1.53 g of **6**: mp 63–65°; $[\alpha]^{25}D$ −6.9° (*c* 2.4, $CHCl_3$); nmr ($CDCl_3$) δ 4.46 (d, *J* = 7.5 Hz, H-1′, assigned as before[3]).

Anal. Calcd for $C_{62}H_{106}O_{20}$: C, 63.56; H, 9.12. Found: C, 63.27; H, 8.91.

Compound **6** was also prepared directly from crude fermentation product. Chromatography of 2.65 g of product on silicic acid gave 2.07 g of neutral material on elution with chloroform–methanol (50:1). After hydrogenation, neutral material was acetylated as above and chromatographed on 200 g of silicic acid; elution with 1500 ml of chloroform–hexane (1:1) gave 0.63 g of **6**. Further elution with the same solvent gave mixtures of **6** and **7**.

Octadecyl 17-L-Formyloxyoctadecanoate.—A solution of 5.0 g of 17-L-formyloxyoctadecanoyl chloride[4] in 10 ml of methylene chloride was added to 6.4 g of octadecanol dissolved in 25 ml of methylene chloride and 1.5 ml of pyridine, and the mixture was refluxed for 48 hr. The solution was washed with 2 *N* hydrochloric acid and solvent was evaporated, giving 10.65 g of crude product. This material, chromatographed on 200 g of silicic acid, gave on elution with 3 l. of hexane–chloroform (9:1) 1.53 g of octadecyl formate. Further elution with 4 l. of hexane–chloroform (4:1) gave 2.95 g of formate ester, and elution with 3 l. of hexane–chloroform (3:2) gave 2.45 g of hydroxy ester (see below). Crystallization of formate ester from ethyl acetate gave 1.45 g of pure product, mp 59.5–62.5°, $[\alpha]^{25}D$ −2.6° (*c* 2.1, $CHCl_3$).

Anal. Calcd for $C_{37}H_{72}O_4$: C, 76.49; H, 12.49. Found: C, 76.50; H, 12.52.

Octadecyl 17-L-Hydroxyoctadecanoate.—It had been intended to selectively hydrolyze the formate group (as was done previously[4] in a similar synthesis), but since some formate ester was hydrolyzed on the silicic acid column (above) the hydroxy ester so obtained was used. Crystallization from ethyl acetate gave 1.95 g of pure hydroxy ester, mp 70–72°, $[\alpha]^{25}D$ +2.0° (*c* 2.4, $CHCl_3$).

Anal. Calcd for $C_{36}H_{72}O_3$: C, 78.19; H, 13.13. Found: C, 78.03; H, 13.25.

Octadecyl 17-L-[(2′-*O*-β-D-Glucopyranosyl-α,β-D-glucopyranosyl)oxy]octadecanoate 2″,3′,3″,4′,4″,6′,6″-Heptaacetate (6 + 9).—A solution of 0.33 g of octadecyl 17-L-hydroxyoctadecanoate in 5 ml of methylene chloride was shaken for 1 hr with 0.5 g of silver carbonate and 0.5 g of Drierite, a solution of 0.42 g of acetobromosophorose[3] was then added, and shaking was continued for 3 days. The reaction mixture was applied to a silicic acid column, and elution with chloroform–hexane (1:1) gave 0.35 g of **6** + **9**. Crystallization from ethanol gave 0.12 g of **6** + **9**: mp 55–56°; $[\alpha]^{25}D$ +5.6° (*c* 3.2, $CHCl_3$); nmr ($CDCl_3$) δ 4.46 (d, *J* = 7.5 Hz, H-1′ of **6**), 4.61 (d, *J* = 7.5 Hz, H-1″ of **9**) (the ratio of the first signal to the second was about 2:1), 5.36 (t, H-3′ of **9**).

Anal. Calcd for $C_{62}H_{106}O_{20}$: C, 63.56; H, 9.12. Found: C, 63.42; H, 9.23.

Octadecyl 17-L-[(2′-*O*-β-D-glucopyranosyl-α-D-glucopyranosyl)-oxy]octadecanoate 2″,3′,3″,4′,4″,6′,6″-Heptaacetate (9).—A solution of 1.53 g of **6** in 20 ml of glacial acetic acid was prepared, 2 ml of 33% hydrogen bromide in acetic acid was added, and the mixture was kept for 2 hr. The mixture was poured into ice–water and extracted three times with chloroform. Solvent was removed, giving 1.28 g of crude **9**, which was purified by silicic acid chromatography (elution with hexane–chloroform, 1:1) and two crystallizations from methanol. The yield of pure **9** was 0.68 g: mp 61–64°; $[\alpha]^{25}D$ +33.7° (*c* 2.8, $CHCl_3$); nmr ($CDCl_3$) δ 4.61 (d, *J* = 7.5 Hz, H-1″), 5.36 (t, H-3′).

Anal. Calcd for $C_{62}H_{106}O_{20}$: C, 63.56; H, 9.12. Found: C, 63.26; H, 9.22.

Compound **9** was also prepared in the same way from the synthetic mixture of **6** + **9**, it did not depress the melting point of the above product, and the nmr spectra of the two compounds were indistinguishable; $[\alpha]^{25}D$ was +31.6° (*c* 0.9, $CHCl_3$).

Octadecyl 2-*O*-(2′,3′,4′,6′-Tetra-*O*-acetyl-β-D-glucopyranosyl)-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (10).—Octadecanol (0.16 g) in 5 ml of methylene chloride was shaken with 0.5 g of silver carbonate, 0.5 g of Drierite, and 0.40 g of acetobromosophorose for 2 days. The mixture was filtered, and solvents were removed and applied to a silicic acid column in hexane–chloroform (3:1). Hexane–chloroform (1:1) eluted 0.33 g of crude **10**, which on crystallization from ethanol gave 0.13 g of pure **10** as long needles: mp 85–87°; $[\alpha]^{25}D$ −6.1° (*c* 1.8, $CHCl_3$); nmr ($CDCl_3$) δ 4.44 (d, *J* = 7.5 Hz, H-1).

Anal. Calcd for $C_{44}H_{72}O_{18}$: C, 59.44; H, 8.16. Found: C, 59.32; H, 8.07.

Deacetylation of 0.09 g of **10** with methanolic sodium methoxide and crystallization from methanol gave 0.05 g of octadecyl 2-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**11**): mp 195–197°; $[\alpha]^{25}D$ −19.5° (*c* 0.4, pyridine); nmr (pyridine) δ 4.85 (d, *J* = 7.5 Hz, H-1), 5.26 (d, *J* = 7.5 Hz, H-1′).

Anal. Calcd for $C_{30}H_{58}O_{11}$: C, 60.58; H, 9.83. Found: C, 60.54; H, 9.83.

Deacetylation of the mother liquors left after isolation of crystalline **10** gave an amorphous product, nmr (pyridine) δ 5.22 (d, *J* = 7.5 Hz, H-1′ of **11**), 5.42 (d, *J* = 4 Hz, presumably H-1 of α form of **11**); the ratio of the first signal to the second was about 2:1.

Glc Analysis of Trimethylsilyl Ethers of Fermentation Products.—Trimethylsilyl ethers were prepared as described by Sweeley, *et al.*[8] Isothermal analysis at 280° gave relative emergence times for trimethylsilyl ethers: **11**, 1.00; **1**, 1.26; **4**, 1.44; **8**, 2.29. Temperature programming from 250° to 385° at 5°/min gave emergence temperatures: **11**, 300°; **4**, 310°; **5**, 360°. Correction factors required for quantitative analysis were: **11**, 1.00; **4**, 1.33; **5**, 1.96. Before preparation of trimethylsilyl ethers 10-mg samples were dissolved in 100 μl of methanol and treated with ethereal diazomethane, solvents were removed, the samples were warmed with 100 μl of 0.022 *N* methanolic sodium methoxide for 10 min, the solution was neutralized with acetic acid in methanol, solvents were removed, and the residue was taken up in 100 μl of pyridine.

Composition of Hydroxy Acid Portion of Crude Product.—The percentage of ω-hydroxy acid in the crude product, determined as previously described,[2] was 12%, which is very similar to that formed when oleic acid was fermented.[2]

Registry No.—**3**, 34991-59-8; **4**, 23071-19-4; **5**, 34991-60-1; **6**, 34991-61-2; **9**, 34991-62-3; **10**, 34991-63-4; **11**, 34991-64-5; oleyl alcohol, 143-28-2; octadecyl 17-L-formyloxyoctadecanoate, 34991-65-6; octadecyl 17-L-hydroxyoctadecanoate, 34991-66-7.

(8) C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Amer. Chem. Soc.*, **85**, 2497 (1963).