it has recently been reported that methanolysis under similar conditions results in the formation of four stereoisomers (Weiss, 1964).

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8,9,13-Triacetoxydocosanoic Acid, an Extracellular Lipid Produced by a Yeast*

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ABSTRACT: An extracellular lipid produced by the yeast NRRL YB-2501 was shown to be the triacetate of 8,9,13-trihydroxydocosanoic acid.

The structure of the trihydroxy acid was established by its conversion to suberic acid and 5-ketotetradecanoic acid.

In previous publications (Wickerham and Stodola, 1960; Stodola and Wickerham, 1960; Stodola *et al.*, 1962; Maister *et al.*, 1962) we described the formation of the extracellular lipids tetraacetylphytosphingosine and triacetyldihydrosphingosine by the yeast *Hansenula ciferrii*. When this work was extended to other species, we found that a new yeast NRRL YB-2501 produced more than a gram of extracellular lipd per liter of culture liquor. An examination of this lipid has shown it to be composed largely of 8,9,13-triacetoxydocosanoic acid (compound I). In this paper we report the production, isolation, characterization, and determination of the structure of this compound.

The species used in this work is a yeast or yeastlike organism of indefinite taxonomic position. It was isolated from frass of white spruce, *Picea glauca* (Moench) Voss. A description of the species will be published elsewhere.

The lipid was produced in a yield of 1.42 g/liter by growing the yeast for 7 days at 25° in shaken flasks on a medium containing 5% malt extract and 1% glucose. The crude product, obtained as an oil by extraction of the culture liquor with hexane, was shown by thin-layer chromatography to consist largely of a single component. Silicic acid chromatography gave a pure liquid having the composition $C_{28}H_{50}O_8$, the infrared absorption spectrum of which showed strong bands at 5.75 (ester or δ-lactone) and 5.85 μ (carboxyl). Further bands at 3.45 and 6.85–6.96 μ corresponded to CH and CH_2 stretching frequencies in saturated aliphatic compounds, and a 7.30 μ band indicated the presence of $C-CH_3$ groups.

Saponification of the pure C_{28} acid yielded a $C_{22}H_{44}O_5$ acid (compound II) (mp 157–158°) showing strong infrared absorption bands at 3.15 (OH) and 5.85 μ (COOH). Hydroxyl analyses of the C_{22} acid and the loss of six carbons on saponification suggested that the

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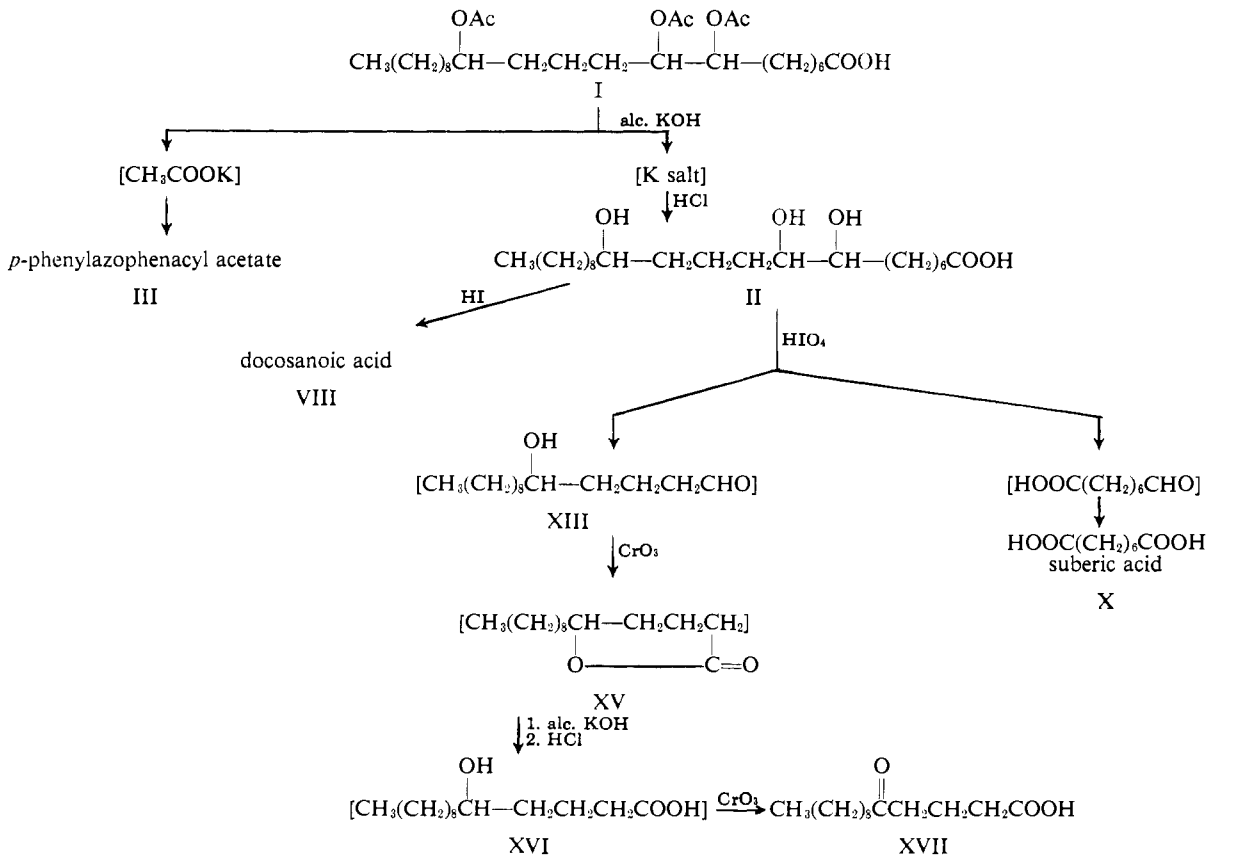

other product was acetic acid. This was established by isolation of *p*-phenylazophenacyl acetate (compound III).

Pure acid II was obtained in quantity by saponification of the crude lipid and two crystallizations of the C_{22} acid from acetic acid. Analyses of the methyl (compound IV), *p*-bromophenacyl (compound V), *p*-phenylazophenacyl (compound VI), and *p*-phthalimidophenacyl (compound VII) esters were all in accord with the $C_{22}H_{44}O_5$ formula for compound II. The methyl ester crystallized well, whereas the phenacyl esters showed a tendency to gel.

Acid II was shown to have a straight chain by hydrogen iodide reduction to docosanoic (behenic) acid (compound VIII), which was characterized as the *p*-bromophenacyl ester (compound IX). Cleavage of compound II with periodic acid yielded as one product an aldehyde acid, which on oxidation gave suberic acid (compound X), the identity of which was established by conversion to the bis-*p*-bromophenacyl ester (compound XI) and the bisbenzhydrylammonium salt (compound XII). The other cleavage product was a liquid C_{14} hydroxyaldehyde (compound XIII) which was isolated as the *p*-phenylazobenzohydrazone (compound XIV).

The position of the hydroxyl group on the C_{14} chain was established as follows: The hydroxyaldehyde cleavage product (compound XIII) was oxidized with chromic acid to a neutral product (compound XV) which showed no infrared band for hydroxyl but did have a band at 5.75 μ characteristic of a δ-lactone. Saponification of this neutral product gave an acid, mp 45–48°, presumably 5-hydroxytetradecanoic acid (compound XVI), which was oxidized to a keto acid identical with 5-ketotetradecanoic acid (compound XVII), judging from the properties of the acid itself and those of its *p*-bromophenacyl ester (compound XVIII). The natural 5-ketotetradecanoic acid on KBH_4 reduction yielded the expected racemic 5-hydroxytetradecanoic acid (compound XIX).

These studies establish that the lipid produced by the yeast NRRL YB-2501 is the triacetate of 8,9,13-trihydroxydocosanoic acid. In Table I are shown all the other known naturally occurring higher trihydroxy aliphatic acids of established structure.

Experimental

Production of Crude Lipid. The stock culture of the yeast (YB-2501 in the ARS Culture Collection here) was maintained at 15° on GME agar slants (1% glucose, 5% malt extract, 3% agar, *p*H not adjusted). To prepare inoculum for flasks, transfers were made from the 15° cultures to GME agar slants, which were incubated at 25° and transferred twice serially at 2-day periods. Growth on the second serial slant, when 48 hours old, was suspended in 1 ml of GME liquid, and 0.1 ml of this solution was used to inoculate a 300-ml erlenmeyer flask containing 100 ml of GME medium. The flasks were kept on a Gump shaker at 25°.

TABLE I: Naturally Occurring Higher Trihydroxy Fatty Acids.

Acid	Source	Length of Carbon Chain	Position of OH Groups	References
Aleuritic	Insect lac (*Laccifer lacca*)	16	9, 10, 16	Nagel (1927)
Phloionolic	Cork oak bark (*Quercus suber*)	18	9, 10, 18	Zetsche and Weber (1938)
	Birch bark (*Betula verrucosa*)			Jensen and Rinne (1954)
	Leaf cuticle (*Agave americana*)			Matic (1956)
	Olive leaves (*Olea europaea*)			Meakins and Swindells (1959)
Artemisic	Shrub (*Artemisia monogyna*)	18	9, 10, 14	Kariyone *et al.* (1948)
Ustilic acid B	Smut (*Ustilago zeae*)	16	2, 15, 16	Lemieux (1953)

At intervals the contents of three flasks were combined and extracted with 60 ml of hexane. The yields of almost colorless oils were as follows: 3 days, 196 mg; 5 days, 354 mg; 7 days, 427 mg; 9 days, 425 mg; and 11 days, 410 mg. The maximum yield at 7 days corresponded to a yield of 1.42 g/liter.

8,9,13-Triacetoxydocosanoic Acid (Compound I). Crude lipid (197 mg) was chromatographed on a silicic acid column (Mallinckrodt[1] No. 2847; 50 g), the eluting solvent being petroleum ether (bp 66–69°)–ethyl ether–acetic acid (80:19:1). The pure 8,9,13-triacetoxydocosanoic acid (compound I) from the middle portion of the main peak was a liquid having a specific rotation of $[\alpha]_D^{20}$ −3° (*c* 2.9%, hexane). It gave a positive hydroxamic acid test. The infrared spectrum (AgCl melt) showed strong bands at 5.75, 5.85, 7.30, 8.07, and 9.80 μ.

Anal. Calcd for $C_{28}H_{50}O_8$ (514.7): C, 65.34; H, 9.79. Found: C, 64.98; H, 9.76.

Identification of Acetic Acid. The pure C_{28} acid (30.3 mg) was heated for 2 hours at 80° with 3.20 ml of 0.0736 N alcoholic KOH (4 eq). The reaction mixture was concentrated to dryness and the residue heated 10 minutes at 120° with 0.5 ml of dimethylformamide and 71.3 mg of *p*-phenylazophenacyl bromide (4 eq) to yield 84.5 mg of mixed derivatives, mp 118–140°. Extraction with boiling petroleum ether (bp 92–97°) left a residue (28 mg) of the *p*-phenylazophenacyl derivative of 8,9,13-trihydroxydocosanoic acid (VI), mp 160–161.5°. The petroleum ether extracts yielded an orange crystalline product (56.5 mg), mp 110–123°, which was purified by Florisil chromatography (30.6 mg; mp 124–125.5°). Recrystallization from 90% alcohol gave *p*-phenylazophenacyl acetate (compound III) with the same melting point (127.5–128.5°) and infrared spectrum as an authentic sample (Sugiyama *et al.*, 1951; mp 125–125.5°).

8,9,13-Trihydroxydocosanoic Acid (Compound II). The crude lipid (2.077 g) was refluxed for 1 hour with alcoholic KOH. Acidification gave a quantitative yield (1.57 g) of crude II, mp 135–145°. Two crystallizations from acetic acid yielded pure trihydroxy acid, mp 157–158°, in the form of fine needles, $[\alpha]_D^{20}$ +4° (*c* 3%, pyridine). The acid is almost insoluble at room temperature in the usual solvents but does dissolve in dimethylformamide, dimethyl sulfoxide, and tetramethylurea on warming. A Kuhn-Roth determination showed 1.0 C-methyl group.

Anal. Calcd for $C_{22}H_{44}O_5$ (388.6): C, 68.00; H, 11.41. Found: C, 67.80; H, 11.44.

Methyl 8,9,13-Trihydroxydocosanoate (Compound IV). Acid II (50 mg) was methylated in methanol with 20.3 mg of dimethyl sulfate and 0.059 ml of dicyclohexylethylamine by the DICE procedure (Stodola, 1964), giving 50.7 mg (98%) of crude ester IV melting at 134–135°. A small amount of free acid was removed by alkaline treatment and the product was crystallized from methanol (39.4 mg; mp 135–136°) in the form of fine needles.

Anal. Calcd for $C_{23}H_{46}O_5$ (402.7): C, 68.60; H, 11.52. Found: C, 68.30; H, 11.58.

Quantitative acetylation (acetic anhydride, pyridine, sealed tube, 65°, 2 hours) showed 2.99 hydroxyl groups.

p-Bromophenacyl 8,9,13-Trihydroxydocosanoate (Compound V). Acid II (19.94 mg) was converted to the *p*-bromophenacyl derivative in acetone-dimethylformamide (7 minutes at 100°) by the DICE procedure (Stodola, 1963) to yield 28.3 mg (94%) of crude product (mp 151.5–153°). Recrystallization from dimethyl-

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

formamide gave 24 mg of pure derivative melting at 155–156°.

Anal. Calcd for $C_{30}H_{49}BrO_6$ (585.6): C, 61.53; H, 8.43. Found: C, 61.07; H, 8.35.

p-Phenylazophenacyl 8,9,13-Trihydroxydocosanoate (Compound VI). Acid II (19.20 mg) gave a 98% yield of crude derivative (mp 158–159°) by the DICE method. Crystallization from 1-butanol yielded pure material (pale yellow needles, mp 161–162°).

Anal. Calcd for $C_{36}H_{54}N_2O_6$ (610.8): C, 70.79; H, 8.91. Found: C, 70.28; H, 8.94.

p-Phthalimidophenacyl 8,9,13-Trihydroxydocosanoate (Compound VII). The crude derivative (mp 168–170°) prepared from compound II in 98% yield by the DICE method was crystallized from dimethylformamide; needles; mp 170–171°.

Anal. Calcd for $C_{38}H_{53}NO_8$ (651.9): C, 70.02; H, 8.19. Found: C, 70.00; H, 8.52.

Conversion of 8,9,13-Trihydroxydocosanoic Acid (Compound II) to Behenic Acid (Compound VIII). Acid II (100 mg) was refluxed for 22 hours with 20 ml of 47% hydriodic acid, and the resulting iodo compounds were heated for 8 hours with granular zinc and 10% HCl. The product was reduced catalytically (Adams catalyst in acetic acid) to yield 73 mg of crude behenic acid. Crystallization from acetic acid gave almost pure acid, mp 78–79.5°. Its identity was established by infrared spectra, X-ray diffraction patterns, and a mixture melting point with authentic behenic acid of mp 79.5–80.5° (Francis and Piper, 1939; mp 79.95°).

p-Bromophenacyl Behenate (Compound IX). Behenic acid (68.12 mg; mp 79.5–80.5°), *p*-bromophenacyl bromide (55.60 mg), and DICE (0.07 ml) were dissolved in a few drops of acetone and heated in an open test tube at 80° for 15 minutes. The crude product (104.2 mg; 97%; mp 91–92°) was crystallized from hexane (needles; mp 94–95°).

Anal. Calcd for $C_{30}H_{49}BrO_3$ (537.6): C, 67.03; H, 9.19. Found: C, 67.14; H, 9.44.

Behenic acid (3.295 mg; mp 78–79.5°) prepared from compound II by HI reduction was converted to compound IX by the procedure described above. The crude product (4.0 mg; 77%; mp 92–93°) was recrystallized from hexane to give 2.80 mg of pure derivative (mp 94–95°) having an infrared spectrum identical with that of the product described above.

Periodic Acid Cleavage of 8,9,13-Trihydroxydocosanoic Acid (Compound II). A suspension of compound II (38.8 mg) and periodic acid (27.4 mg; 20% excess) in 0.25 ml of dimethylformamide was stirred with a glass rod. The clear solution which resulted in 3–4 minutes was allowed to stand in the dark for 20 minutes. A solution of 50 mg of sodium bicarbonate in 0.5 ml of water was added, and the resulting oily suspension was extracted with ether. After being washed with water the ether solution was evaporated to an oil which was reserved for the preparation of the *p*-phenylazobenzohydrazone.

The aqueous solution was lyophilized to a powder which was dissolved in 3 drops of water. Addition of a slight excess of saturated $KMnO_4$ solution, acidification, and addition of $NaHSO_3$ gave a colorless solution which, on cooling in ice, yielded colorless crystals of suberic acid (compound X) (10.4 mg; 60%; mp 138–140°). Its identity was established by mixture melting point and comparison of its infrared spectrum with that of an authentic sample of suberic acid (mp 140–142°). A portion (1.43 mg) of the natural suberic acid yielded 2.37 mg of recrystallized bis-*p*-bromophenacyl derivative (compound XI), mp 145–146° (Kananiwa and Isono, 1952; mp 146°). Its infrared spectrum was indistinguishable from that of an authentic sample. Another portion (1.88 mg) of the suberic acid from the cleavage was heated in 2 drops of tetramethylurea for 5 minutes at 125° with 6.52 mg of the carbamate of benzhydrylamine. Cooling and filtration gave 2.8 mg of the bisbenzhydrylamine salt of suberic acid (compound XII) in the form of bars melting at 174–175°. The infrared spectrum was the same as that of an authentic sample.

Bisbenzhydrylammonium Suberate (Compound XII). Suberic acid (50 mg) and 170 mg of benzhydrylamine were refluxed in ethanol for 5 minutes. Cooling and filtration gave 146 mg (mp 174–176°) of crude salt. Recrystallization from tetramethylurea yielded pure compound XII, mp 175–176°.

Anal. Calcd for $C_{34}H_{40}N_2O_4$ (540.7): C, 75.53; H, 7.46. Found: C, 75.27; H, 7.50.

5-Hydroxymyristaldehyde p-Phenylazobenzohydrazone (Compound XIV). The oil from the periodic acid cleavage, refluxed in ethanol for 30 minutes with 24 mg of *p*-phenylazobenzohydrazide, yielded 37 mg of crude derivative, mp 135–145°. Crystallization from aqueous alcohol gave 27 mg of orange needles of XIV; mp 156.5–157.5°.

Anal. Calcd for $C_{27}H_{38}N_2O_4$ (450.6): C, 71.97; H, 8.50. Found: C, 71.88; H, 8.55.

Conversion of C_{14}-Hydroxyaldehyde (Compound XIII) to 5-Ketotetradecanoic Acid (Compound XVII). Acid II (114 mg) suspended in 0.7 ml of dimethylformamide was allowed to react with a 50% excess of periodic acid (100 mg) for 30 minutes. After addition of an aqueous solution of sodium bicarbonate (74 mg) the reaction mixture was extracted with ether. The ether solution was concentrated to an oil which was dissolved in 0.2 ml of acetic acid and treated with 2 eq of 1.50 N CrO_3 in acetic acid. The oil isolated from this reaction mixture showed no hydroxyl infrared band but did have a strong carbonyl band at 5.75 μ (δ-lactone). Saponification gave 28 mg of an acid melting at 45–48°, presumably an hydroxy acid judging from its infrared spectrum. Oxidation with CrO_3 in acetic acid yielded 16.9 mg of crude keto acid which after two crystallizations from hexane gave 7.7 mg of 5-ketotetradecanoic acid (mp 77–78°). Its melting point was not depressed on admixture with authentic 5-ketotetradecanoic acid, mp 78–79°, prepared by the method of Robinson (1930). Ames and Bowman (1952) reported mp 80–81° for this acid. Infrared spectra of the natural and synthetic acids were the same.

A portion of the 7.7-mg sample of natural 5-ketotetradecanoic acid on reduction with potassium borohy-

dride gave 5-hydroxytetradecanoic acid (compound XIX), mp 65–66°. An admixture with authentic material of the same melting point also melted at 65–66°. Fuqua (1957) reported mp 61.2–62.4° for 5-hydroxytetradecanoic acid prepared by borohydride reduction. This acid is readily converted to the lactone, even by boiling in petroleum ether.

p-Bromophenacyl 5-Ketotetradecanoate (Compound XVIII). A portion (2.440 mg) of the natural 5-ketotetradecanoic acid (compound XIX) (mp 77–78°) was converted to 3.80 mg of *p*-bromophenacyl ester (mp 82–83.5°). Crystallization from hexane gave 2.40 mg of pure compound XVIII having the same mp (84–85°) and infrared spectrum as material prepared from synthetic 5-ketotetradecanoic acid.

Anal. Calcd for $C_{22}H_{31}BrO_4$ (439.4): C, 60.15; H, 7.11. Found: C, 59.90; H, 7.25.

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