Composition of Novel Triesters from the Skin of the Rhino Mutant Mouse[1]

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ABSTRACT

Composition of two novel triesters, derived from the skin of the rhino mutant mouse, is described. Chemical and spectroscopic analysis of the products of pancreatic hydrolysis of the triesters showed that these are comprised predominantly of isomer I (92.7 mole %). The syntheses of two reference compounds, 1-O-hexadecanoyl-2-[(14-hexadecanoyloxy)O-tetradecanoyl] 1,2-hexadecanediol (Ia) and 2-O-hexadecanoyl-1-[(14-hexadecanoyloxy)O-tetradecanoyl]-1,2-hexadecanediol (IIa), corresponding in their structures to isomers I and II of the triester, wax have also been described.

INTRODUCTION

In a previous communication (1), we have reported the occurrence of triesters, a novel neutral lipid, in the skin of the rhino mutant mouse. This lipid was shown to be composed of three structural units involving fatty acids, ω-hydroxy fatty acids and long chain 1,2-alkanediols. Two alternate structures, 1-O-acyl-2-[(ω-acyloxy)O-acyl]-1,2-alkanediol (I) or 1-[(ω-acyloxy)O-acyl]-2-O-acyl-1,2-alkanediol (II), were proposed for this lipid. In the present report, we describe a further distinction between these two structures on the basis of spectroscopic and chemical evidence.

MATERIALS AND METHODS

For this study, male and female homozygous rhino ($hr^{rh}hr^{rh}$) mice on a C57BL10J-related background, 5 mo old or older, were obtained from the Skin and Cancer Hospital Animal Colony. They were fed on a commercial breeder diet ad libitum.

Chromatographic procedures and the method used for the isolation of the triesters were the same as described previously (1).

Nuclear magnetic resonance (NMR) spectra were recorded at 220 MHz on Varian Hr-220 NMR spectrometer in 0.4-0.5 ml distilled carbon tetrachloride using tetramethylsilane as internal standard. Analyses were run at about 25 C on 10-15 μmol samples using Fourier transform. Infrared (IR) spectra were determined as solid or liquid film on AgCl plates on a Perkin-Elmer 237-B spectrophotometer.

CrO_3-pyridine complex (sarett reagent) was prepared by the procedure of Ratcliffe and Rodehorst (2). Jones reagent (chromic acid/acetone) was prepared by a modified procedure of Djerassi et al. (3). Silylation was done with pyridine:hexamethyldisilazane:trimethylchlorosilane 9:3:1. Alkaline hydrolysis was performed as described in (4). 1,2-Hexadecanediol was prepared by lithium aluminum hydride reduction of methyl 2-hydroxy-hexadecanoate. Hexadecanoic anhydride was prepared according to Selinger and Lapidot (5). 1,14-Tetradecanediol was purchased from Eastman Kodak, Rochester, NY.

Preparation of Ia and IIa (Fig. 1)

To distinguish between isomers I and II of triester wax, two model compounds, 1-O-hexadecanoyl-2-[(14-hexadecanoyloxy)O-tetradecanoyl]-1,2-hexadecanediol (Ia) and 2-O-hexadecanoyl-1-[(14]hexadecanoyloxy)O-tetradecanoyl]-1,2-hexadecanediol (IIa), were prepared by the following procedure. 1- or 2-O-Hexadecanoyl-1,2-hexadecanediol (IIIa or IVa,

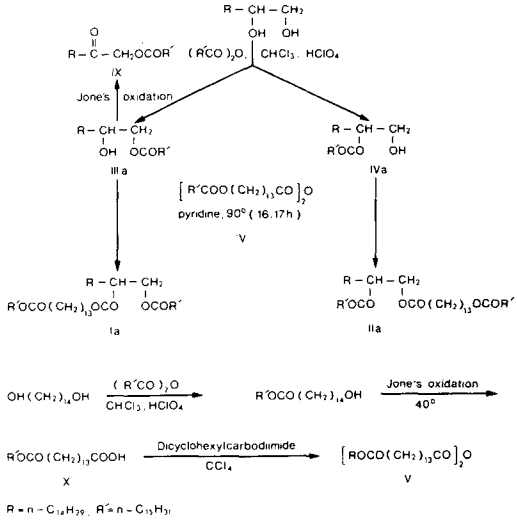

FIG. 1. Preparation of reference triester lipids (Ia and IIa) and of the intermediate materials IIIa, IVa and V.

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TABLE I

Nuclear Magnetic Resonance Spectra of the Products Obtained by Lipase Hydrolysis of the Triesters and Reference Compounds

Proton	(Multiplicity, J, Hz)					
	I or II, Ia and IIa	IVa	IV	VI	VII	VIII
CH_3	0.87 (t)	0.89 (t)	0.87 (t)	0.85 (t)	0.86 (t)	0.83 (t)
$(CH_2)_n$	1.25 (s)	1.26 (s)	1.25 (s)	1.25 (s)	1.25 (s)	1.25 (s)
Allylic methylenes	1.96 (m)[a]		1.93 (m)	1.91 (m)	1.91 (m)	1.92 (m)
alpha-methylenes	2.2 (q)[b]	2.2 (t)	2.2 (t)	2.18 (q)[b]	2.18 (t)	2.11 & 2.19[d] (t)
Position-1-methylene protons of glycol moiety	3.85 & 4.15 (dd, 6.5, 3; 11)[c]	3.43 & 3.52 (dd, 6, 3; 12)[c]	3.43 & 3.52 (dd, 6, 3; 12)[c]	3.46 & 3.54 (dd, 6, 3; 12)[c]		
Position-2-methylene protons of glycol moiety	4.93 (m)	4.76 (m)	4.77 (m)	4.77 (m)		
Methylene protons adjacent to acyloxy group	3.95 (t)			3.94 (t)		3.94 (t)
olefinic protons	5.25 (m)[a]		5.25 (m)	5.25 (m)	5.25 (m)	5.25 (m)
$COOCH_3$					3.58 (s)	3.58 (s)

[a]These signals were not present in the spectra of Ia and IIa.

[b]Appearance of alpha-methylene protons as a quartet apparently results from two partially overlapping triplets.

[c]Two nonequivalent methylene protons of glycol moiety showed different vicinal coupling constants.

[d]Two triplets were observed for methylene protons adjacent to ester carbonyl. Downfield triplet was assigned to methylene protons adjacent to carbomethoxy group by comparison with VII.

49.6 mg, 0.1 mmol was treated with 14-hexadecanoyloxy-tetradecanoic anhydride (V, 88.2 mg, 0.1 mmol) in pyridine (4 ml) at 90 C for 16-17 hr. The reaction mixture was poured over ice and extracted with ether. Combined ether extracts were dried over anhydrous sodium sulfate. Triester lipid (Ia or IIa, 96 mg, yield = 70%) was separated from 14-hexadecanoyloxy-tetradecanoic acid (X) by thin layer chromatography (TLC) (hexane/ether 95:5). NMR (Table I) and IR spectra of both isomers were identical to each other and to that of naturally occurring triester wax (6) except that signals for olefinic and allylic protons were not present in the former spectra. Alkaline hydrolysis of Ia or IIa gave hexadecanediol, 14-hydroxy-tetradecanoic acid, and hexadecanoic acid in the expected proportions.

Preparation of 1-O-Hexadecanoyl-1,2-Hexadecanediol (IIIa), and 2-O-Hexadecanoyl-1,2-Hexadecanediol (IVa)

To 1,2-hexadecanediol (258 mg, 1 mmol) in chloroform (20 ml) containing perchloric acid (70%, 0.2 ml) was added hexadecanoic anhydride (494 mg, 1 mmol) in small portions over a period of 45 min under stirring. After stirring for 16-17 hr at room temperature, the chloroform was stripped off under a stream of N_2. The residue was treated with 5% aqueous KOH and extracted with ether. Ether extracts were washed free of alkali and dried over anhydrous sodium sulfate. TLC (hexane/ether 70:30) of the reaction product showed the presence of four components characterized as 1,2-O-dihexadecanoyl-1,2-hexadecanediol (42%); a mixture of 1- and 2-hexadecanoyl-hexadecanediols (IIIa and IVa, 45.8%) and 1,2-hexadecanediol (11.8%). These components were separated from each other by column chromatography. The reaction product (620 mg) was applied to a column packed with 30 g silicic acid (100 mesh, Mallinckrodt). 1,2-O-Dihexadecanoyl-1,2-hexadecanediol (258 mg) was eluted with benzene (275 ml); partially acylated 1,2-hexadecanediols (IIIa & IVa 279 mg) with chloroform (600 ml) and the residual 1,2-hexadecanediol (72 mg) was eluted with methanol (100 ml). 1- and 2-O-Hexadecanoyl-1,2-hexadecanediols were finally separated from each other by TLC (hexane/ether 70:30). Position 1-isomer showed a slightly higher Rf (0.49) value than position 2-isomer (Rf 0.38). IR and NMR spectral data for these two isomers given below are in agreement with the proposed structures.

1-O-Hexadecanoyl-1,2-hexadecanediol (IIIa): IR 3447 (OH) and 1709 cm^{-1} (C=O); NMR: δ 0.89 (CH_3, t), 1.25 (CH_2 chain, apparent singlet), 2.2 ($COC\underline{H}_2$, t) 3.60 (CHOH, m), 3.82 (HCHOCOR, dd, Jvic = 7 Hz, Jgem = 11 Hz), 4.0 (HCHOCOR, dd, Jvic = 3.5 Hz, Jgem = 11 Hz). Jones' oxidation of the compound afforded a keto-ester (IX), thus further confirming the proposed structure. IR and NMR spectral data for the keto-ester (IX) are given as follows. IR: 1732 cm^{-1} (C=O); NMR: δ 0.87 (CH_3, t), 1.23 (CH_2 chain, apparent singlet), 2.3 (q, two overlapping triplets, $COCH_2$), 4.47 ($OCCH_2OCO$, S).

2-O-hexadecanoyl-1,2-hexadecanediol (IVa): IR 3447 (OH) and 1739 cm^{-1} (C=O). NMR data are given in Table I.

Preparation of 14-Hexadecanoyloxy-Tetradecanoic Anhydride (V)

1,14-Tetradecanediol (0.53 g, 2.3 mmol) was treated with 1 equivalent of hexadecanoic anhydride (1.23 g, 2.48 mmol) in chloroform (20 ml) containing 1% perchloric acid (70%) as catalyst. Hexadecanoic anhydride was added in small portions during 1 hr under stirring at room temperature and the stirring was continued overnight (18 hr). Chloroform was removed in a stream of N_2. The reaction product was taken up in ether, washed with 5% KOH and with water, and dried over anhydrous sodium sulfate. TLC [hexane/ether 70:30 of the reaction product (1.54 g)] revealed the presence of three components corresponding to residual 1,14-tetradecanediol and its mono- (Rf 0.38) and diacylated (Rf 0.8) products. The monoacylated product was isolated by column chromatography on silicic acid (30 g, 2 x 22 cm). Diacylated (1.1 g) and monoacylated (0.352 g; yield 22%) products were eluted with benzene (230 ml) and chloroform (500 ml), respectively. Residual 1,14-tetradecanediol (0.08 g) was finally eluted with methanol (100 ml).

IR of the monoacylated product exhibited absorption for hydroxyl at 3458 and 3277 cm^{-1} and for ester carbonyl at 1734 cm^{-1}. NMR spectrum displayed signals at: δ 0.87 (CH_3, t), 1.3 (CH_2 chain, apparent singlet), 2.2 ($COCH_2$, t), 3.54 ($C\underline{H}_2OH$, t), 4.00 (CH_2OCOR, t).

14-Hexadecanoyloxy-1-tetradecanol, the monoacylated product, was oxidized by Jones' method (3). The oxidation was carried out at 40 C because of the insolubility of the starting material in acetone at room temperature. After stirring for ½ hr, the reaction mixture was diluted with water and the reaction product was extracted with ether. Ether extracts were washed free of acid and dried over anhydrous sodium sulfate. TLC (hexane/ether 50:50) of the reaction product confirmed the completion of oxidation. IR: 3534 and 3279 (OH), 1731

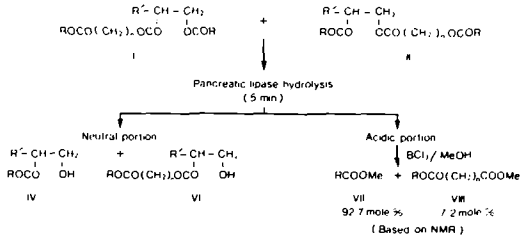

FIG. 2. Pancreatic lipase hydrolysis of triester wax derived from the skin of the rhino mutant mouse.

(OCOR) and 1711 cm^{-1} (COOH).

14-Hexadecanoyloxy-tetradecanoic acid (340 mg, 0.75 mmol) in dry CCl_4 (7.5 ml) was treated with dicyclohexylcarbodiimide (128 mg, 0.62 mmol in 2.5 ml CCl_4) and the reaction mixture was allowed to stand at room temperature for 24 hr (4). The precipitated material (77 mg) was filtered out and the filtrate was evaporated in a stream of nitrogen to recover 14-hexadecanoyloxy-tetradecanoic anhydride (V). The anhydride was purified by repeated crystallizations from acetone (191 mg). IR: 1813 and 1731 cm^{-1}. In contrast to fatty acid anhydrides, absorption at lower frequency was relatively stronger than that at higher frequency because of its overlapping with ester carbonyl absorption.

Lipase Hydrolysis of Triesters

Since IR and NMR spectra of both isomeric model compounds Ia and IIa were found identical to that of naturally occurring triesters, a distinction between two possible structures I and II could not be achieved by comparison of IR and NMR spectra. An attempt was therefore made to partially hydrolyze the sample using pancreatic lipase (Fig. 2). In a typical experiment, 10.5 mg of triesters was treated with 11.2 mg of crude pancreatic lipase (Sigma, St. Louis, MO), 1 ml of tris buffer (pH 8), 2.5 ml $CaCl_2$ (2.2%) and 1 ml of deoxycholic acid (0.05%) and the reaction mixture was shaken for 5 min (8). The reaction mixture was immediately transferred to ice bath and extracted three times with 5 ml of diethyl ether. The combined ether extracts were washed with water and dried over anhydrous sodium sulfate. The hydrolyzate (9.8 mg) was freed from unhydrolyzed triesters by TLC (hexane/ether 95:5) and the partially hydrolyzed sample (3.4 mg) was divided into acidic (1.3 mg) and neutral (2.0 mg) portions on alkaline silicic acid column (9). This procedure was repeated several times. The neutral portion (16.6 mg) was resolved into two components after silylation. These were separated by TLC (hexane/ether 95:5, Rf 0.4 and 0.34, respectively). Individual fractions were hydrolyzed to regenerate hydroxy compounds. These two components from neutral portion were characterized as 2-O-acyl-1,2-alkanediols (IV, 5.4 mg) and 2-[(ω-acyloxy)O-acyl]-1,2-alkanediols (VI, 11.0 mg), respectively. IR (IV): 3574 (OH) and 1734 cm^{-1} (C=O); IR (VI): 3496, 3471 (OH) and 1738, 1691 (C=O). NMR spectral data for IV and VI are given in Table I. The acidic part (22 mg) obtained by lipase hydrolysis was methylated with BCl_3/MeOH (10) and was separated into two components by TLC (hexane/ether 95:5). These two components were characterized as fatty methyl esters (VII, 14.5 mg) and ω-acyloxy fatty methyl esters (VIII, 7.1 mg). NMR spectral data for VII and VIII are shown in Table I.

RESULTS AND DISCUSSION

Neutral Portion—Identification of 2-O-Acyl- and 2-[(ω-Acyloxy)O-Acyl] 1,2-Alkanediols (IV and VI)

TLC of the neutral portion (hexane/ether 60:40) showed a diffused spot moving essentially in the region of monohydric fatty alcohols. No 1,2-alkanediols were observed indicating that no portion of triester wax had undergone complete hydrolysis. Chromic acid/pyridine (2) oxidation of the sample did not yield any ketoester (based on NMR) showing that only primary ester linkage was cleaved by lipase hydrolysis. This observation agrees with the behavior of pancreatic lipase on triacylglycerols.

NMR spectrum (Table I) of the relatively faster moving component (IV) showed methylene protons of glycol moiety at δ 3.43 and 3.52 (dd). This represents an upfield shift of these protons relative to triester wax in which the corresponding protons resonate at δ 3.85 and 4.15 (dd). Thus, it was apparent that primary ester linkage cleaved by pancreatic lipase was a part of 1,2-diol moiety. Alkaline hydrolysis of this fraction afforded fatty acids and 1,2-alkanediols. This fraction of the neutral portion was thus comprised of 2-O-acyl-1,2-alkanediols.

NMR spectrum (Table I) of the relatively slower moving fraction (VI) of the neutral portion was essentially similar to that of faster moving fraction except that an additional triplet was observed at 3.94 ppm. Alkaline hydrolysis of the sample yielded ω-hydroxy fatty acids, fatty acids and 1,2-alkanediols. This fraction of the neutral portion was thus comprised of 2-[(ω-acyloxy)O-acyl]-1,2-alkanediols.

Acidic Portion—Identification of Fatty Acids and ω-Acyloxy-Fatty Acids

Acidic portion, after methylation (10), was resolved into two components by TLC. Relatively faster moving component showed NMR spectrum (Table I, VII) and Rf value identical to those of fatty acid methyl esters. The relatively slower moving component yielded fatty acids and ω-hydroxy fatty acids on alkaline hydrolysis suggesting that this component was composed of ω-acyloxy fatty acids. NMR spectrum (Table I, VIII) was in complete agreement with ω-acyloxy methyl esters. A triplet at 3.94 ppm is assigned to methylene protons adjacent to acyloxy group. Final confirmation of the structure was achieved by comparing NMR spectrum of the relatively slower moving component with that of methyl-14-hexadecanoyloxy-tetradecanoate (X). The latter was prepared by partial acylation of 1,14-tetradecanediol with hexadecanoic anhydride followed by Jones' oxidation and methylation.

It has been shown that triester wax on lipase hydrolysis affords 2-O-acyl-1,2-alkanediols, 2-[(ω-acyloxy)O-acyl]-1,2-alkanediols, fatty acids and ω-acyloxy fatty acids. Formation of all these products, assuming that no acyl migration occurs, indicates an isomeric mixture of both possible structures I and II for triesters. Using our model compounds III and IV, it was established that no acyl migration occurs and the reaction is specific for primary ester grouping of 1,2-diol moiety under the experimental conditions used. This specificity of pancreatic lipase was also observed with 1,2-alkane diol diesters. However, when a longer reaction time of ½ hr-12 hr was given, acyl migration to varying degrees was observed. Assuming that enzyme has no particular preference for fatty acid chain lengths, the proportions of isomers I and II can be computed from the molar ratios of fatty acids and ω-acyloxy fatty acids. The molar percentage of ω-acyloxy fatty acids in a mixture with fatty acids, originating from isomer II and I, respectively, in pancreatic lipase hydrolysis, was determined by NMR as 7.2 mole %. ω-acyloxy methyl esters display a triplet at 3.94 δ for methylene protons adjacent to acyloxy group (Table I, VIII). Let x represent the area at 3.94 δ associated with OCH_2 protons, and y represent the total area associated with α-methylene protons of fatty acids and acyloxy fatty acids combined. Since acyloxy fatty acids contain two pairs of α-methylene protons, while fatty acids contain only one pair, the contributions to y of the two types of compounds are equal to 2x and to y-2x, respectively. Thus, the molar concentrations of the two classes are represented by x and by y-2x, respectively, and the relative (percent) concentration of acyloxy acids equals $100x/(y-2x+x) = 100x/(y-x)$. Weights of lipase hydrolysis products were not used to calculate proportions of isomers because of ambiguity involved in estimating average molecular weight of a mixture having wide range of carbon chain lengths (1).

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