Lipase hydrolysis of mammalian long-chain 1,2-alkanediol diesters. Nonrandom distribution of fatty acids

Patricia C. Schmid and Harald H. O. Schmid[1]

The Hormel Institute, University of Minnesota, Austin, MN 55912

Abstract Long-chain 1,2-alkanediol diesters were isolated from the total surface lipids of golden Syrian hamsters and Swiss albino mice. Hydrolysis of the diol diester waxes with exocellular lipase from *Rhizopus arrhizus* delemar or with purified porcine pancreatic lipase produced free fatty acids and 2-acyl diols in about 60–80% yield. Nonrandom distribution of the constituent fatty acids at positions 1 and 2 of the alkanediols was observed. In the diester waxes from the hamster, both straight-chain and branched-chain fatty acids of 14 to 20 carbon atoms predominated at position 1 and those of 22 to 26 carbon atoms at position 2. In contrast, the diester waxes of the mouse contained mainly fatty acids of less than 19 carbon atoms, both saturated and monounsaturated, at position 2 and those of greater chain length (20 to 24 carbon atoms) at position 1. The results of the lipase hydrolysis were confirmed by degradation of the diester waxes with Grignard reagent.

Supplementary key words surface lipids · hamster · mice · *Rhizopus arrhizus* delemar lipase · purified pancreatic lipase · deacylation with Grignard reagent

Diesters (1–3) of long-chain 1,2-alkanediols (4, 5) are major constituents of the skin lipids of most mammals (6, 7). The diols usually range in chain length from C_{16} to C_{22} (1–3) and are of the D-configuration (8). The constituent fatty acids are highly complex mixtures and include even and odd numbered, straight and branched chains ranging from C_{14} to C_{30} (1, 5–7). It is not known why the mammalian sebaceous glands produce relatively large amounts of unusual fatty acids nor why diol diesters rather than triglycerides are the major lipid class. However, certain branched-chain fatty acids have fungistatic properties (9) and it has been speculated that the presence of diol diesters may make the surface wax more resistant to degradation by bacterial lipases (6).

Unlike the lipids of internal organs, which are quite similar among different species, the products of the mammalian sebaceous glands show a high degree of species variation with respect to lipid class composition and fatty acid profiles. In general, the most pronounced differences between species are observed in the constituent fatty acids of the 1,2-alkanediol diesters (6). So far it has not been established whether or not these fatty acids are distributed randomly at positions 1 and 2 of the diols.

We now report that the fatty acids of mammalian 1,2-alkanediol diesters are not only distributed nonrandomly, but that the preference for certain types of fatty acids at positions 1 or 2 of the diols appears to be species specific.

MATERIALS AND METHODS

Synthetic standards

Methyl esters of α-hydroxy fatty acids and of standard mixtures of straight-chain and branched-chain fatty acids were obtained from Applied Science Laboratories, Inc., State College, PA. 1,2-Alkanediols were prepared by $LiAlH_4$ reduction of the hydroxy fatty acid methyl esters. 1-*O*-Tetradecanoyl and 2-*O*-tetradecanoyl 1,2-hexadecanediols were prepared via the cyclic acetal and ring cleavage by ozonolysis (10, 11). The isomers were separated by preparative TLC using hexane–diethyl ether 1:1 as described (11), and they were characterized by NMR spectrometry.

Collection of surface lipids

Adult male Swiss albino mice or golden Syrian hamsters (Sprague-Dawley, Madison, WI) were anesthetized with ether and dipped to the neck in 1.5 l of distilled acetone. The solution was filtered, the acetone was removed under reduced pressure, and

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance.

[1] Address correspondence to Dr. Harald H. O. Schmid, The Hormel Institute, University of Minnesota, 801 16th Avenue, N.E., Austin, MN 55912.

the lipids were taken up in a small amount of chloroform.

Chromatography

Both analytical and preparative TLC were done on layers of Silica Gel H (Merck), 0.5 mm thick, in tanks lined with filter paper. Fractions were made visible by charring after spraying the chromatoplate with 50% sulfuric acid. In preparative TLC, fractions were made visible under ultraviolet light by spraying with 2′,7′-dichlorofluorescein; they were scraped off and eluted with diethyl ether.

GLC was performed on a Packard 428 gas chromatograph equipped with flame ionization detector and a Spectra-Physics computing integrator. A 6′ × ⅛″ aluminum column packed with 10% EGSS-X on 100–120 mesh Gas-Chrom P was operated at 185°C and an 8′ × ⅛″ column packed with 3% OV-101 on 100–120 mesh Gas-Chrom Q was operated at 210°C. All samples were analyzed before and after hydrogenation. Hydrogenation of methyl esters was accomplished in benzene solution at room temperature with PtO_2 catalyst at 2.5 atm H_2 with vigorous shaking for 3 hr.

Purification of diol diesters

Total surface lipids were fractionated by preparative TLC (40 mg per 20 × 20 cm plate) using hexane–benzene 1:1. The diol diester fraction, usually visible without the use of an indicator, was scraped off, eluted, and repurified with hexane–benzene 3:2, developed twice. Approximately one-half of the total lipids obtained from mouse skin consisted of 1,2-diol diesters; of the hamster lipids about 15–20% were diol diesters.

Lipase hydrolysis

Purified diol diesters (3 mg/ml) were emulsified in 0.1 M Tris buffer, pH 7.75, containing 3 mM $CaCl_2$, 0.1 M NaCl, 0.4 mg of sodium deoxycholate, and 0.1 mg of fatty acid-poor bovine serum albumin per ml. The mixture was first warmed to about 40°C to melt the diesters, then it was sonicated with a Branson sonifier (Branson Instruments, Inc., Danbury, CT) for three 30-sec bursts, with a 30–60 sec cooling period between bursts. A cloudy white emulsion was obtained. Lipase hydrolysis was carried out at 37–39°C to maintain the emulsion.

Three ml of emulsion (9 mg of diol diesters) was placed in a test tube with a Teflon-lined screw cap and stirred vigorously with a small magnetic bar. Lipase solution (25 μl, 10 mg/ml of *Rhizopus arrhizus* delemar lipase, Boehringer-Mannheim) was added and stirring was continued for 1 hr. The mouse diol diesters were also hydrolyzed with 0.1 ml of a purified[2] porcine pancreatic lipase (0.18 mg/ml) under the same conditions. The reaction was stopped by adding four volumes of chloroform–methanol 2:1. After centrifugation, the lower phase was removed and the upper phase was acidified with 0.1 ml of 2 N H_2SO_4 and reextracted with chloroform–methanol–water 86:14:1, in a volume equal to the lower phase. The combined lower phases were blown to dryness under N_2. Hydrolyses were done in duplicate or triplicate and the products from each reaction were analyzed separately. A control sample was treated in the same manner, but no lipase was added. Blanks containing lipase but no substrate did not contain detectable amounts of lipid contaminants.

Separation and analysis of hydrolysis products

The products of lipase hydrolysis were analyzed, and unhydrolyzed diesters ($R_f \sim 0.8$), free fatty acids ($R_f \sim 0.4$), and 2-acyl diols ($R_f \sim 0.2$) were isolated by TLC on Silica Gel H in the solvent system hexane–diethyl ether–acetic acid 80:20:1. 1-Acyl diols ($R_f \sim 0.3$) and free diols (near the origin) were not detected. Hydrolysis was usually 60–80% complete as estimated by weighing the unhydrolyzed diesters after purification.

The structure of the 2-acyl diols was confirmed by NMR spectrometry (Varian CFT-20, 79.54 MHz, $CDCl_3$). The proton spectrum of the 2-acyl diol fraction was compared to those of authentic 1-tetradecanoyl-1,2-hexadecanediol and 2-tetradecanoyl-1,2-hexadecanediol. Both the synthetic 2-acyl diol and that produced by lipase hydrolysis gave characteristic signals at 4.91 δ (HC–O–CO–R) and at 3.65 δ (H_2C–OH). The 1-acyl diol exhibited a complex multiplet in the region 3.7–4.2 δ, representing the primary ester methylene and the secondary hydroxy methine protons. No signal was present in the 4.9 δ region.

For methanolysis, the free fatty acids or 2-acyl diols were dissolved in 0.5 ml of benzene in a screw-cap tube, 1 ml of 5% w/v HCl/CH_3OH was added, and the tubes were heated at 80°C for 3 hr. After cooling, 0.5 ml of H_2O was added and the aqueous phase was extracted 3 times with 5 ml of hexane–diethyl ether 1:1. Methyl esters were purified by TLC in hexane–diethyl ether 4:1 before analysis by GLC. The free diols were also recovered from the appropriate fractions and analyzed by GLC as their isopropylidene derivatives.

Deacylation with Grignard reagent

The reaction was essentially that described for triglycerides (12, 13) scaled down for 3–5 mg samples.

[2] Momsen, W., and Brockman, H. L. Manuscript in preparation.

TABLE 1. Constituent 1,2-alkanediols (mol %) of hamster and mouse diol diester waxes

No. of C-atoms: No. of Double Bonds	Hamster	Mouse
*iso*15	1.0	
*n*15	2.9	
*iso*16	2.3	
*n*16	16.2	4.4
*n*16:1		0.7
*iso*17	57.4	
*n*17	4.4	1.3
*n*17:1		0.4
*iso*18	1.3	1.4
*n*18	2.0	5.6
*n*18:1		5.9
*iso*19	0.7	1.1
*n*19	1.4	2.1
*n*19:1		1.2
*iso*20	0.4	4.0
*n*20	4.7	45.3
*n*20:1		2.0
*iso*21	3.4	8.3
*n*21	1.8	3.2
*iso*22		5.7
*n*22		3.9
*n*22:1		3.5

Three mmol of ethyl magnesium bromide (EtMgBr) was prepared in 10 ml of anhydrous ether. Diol diesters (3–5 mg) were dissolved in 2 ml of anhydrous ether in a screw-cap test tube and 0.5 ml of EtMgBr solution was added. The reaction was stirred magnetically for 1.5 min, then 0.2 ml of glacial acetic acid followed by 1 ml of H_2O was added. The reaction products were extracted twice with 8 ml of ether. Analysis by TLC on Silica Gel H in hexane–diethyl ether 4:1 indicated 40–50% hydrolysis, the main products being tertiary alcohols and 2-acyl diols. Only small amounts of 1-acyl diols and traces of free diols were formed. The 2-acyl diols were purified, subjected to methanolysis, and the fatty acid methyl esters were analyzed by GLC.

RESULTS AND DISCUSSION

We have found that the exocellular lipase from *Rhizopus arrhizus*, which is known to hydrolyze acyl groups at the 1-position of triglycerides (14) and phospholipids (15), also cleaves the 1-acyl moieties of 1,2-alkanediol diesters. We have applied lipase hydrolysis to the positional analysis of the constituent fatty acids of diester waxes obtained from the skin of golden Syrian hamsters and Swiss albino mice. The alkanediol compositions of the diol diesters are listed in **Table 1.** The major alkanediol of the hamster diol diesters was 15-methyl-1,2-hexadecanediol,[3] whereas that from the mouse was 1,2-eicosanediol.

[3] Schmid, P. C., Wedmid, Y., and Schmid, H. H. O. Manuscript in preparation.

The diester waxes from the hamster contained straight-chain, saturated fatty acids (54%) and branched-chain isomers of both *iso* (38%) and *anteiso* (9%) structure (**Table 2**); those from the mouse contained only 32% straight chain, 27% *iso*, traces of *anteiso*, and 43% monounsaturated fatty acids (**Table 3**).

Lipase hydrolysis of diol diesters

Results of the lipase hydrolysis of the hamster diester waxes are given in Table 2 and are compared to data obtained by chemical degradation. The data show nonrandom distribution of fatty acids between positions 1 and 2 of the 1,2-alkanediols. The shorter chain acids ($\leq C_{20}$) predominate in the number 1 position and the longer chain acids are more prevalent in position 2. Since lipase has not been used before on these compounds or on very long-chain and branched acyl moieties, the possibility that the enzyme preferentially removes short-chain acids was ruled out by the following criteria. *1*) Chemical deacylation

TABLE 2. Constituent fatty acids (mol %) of hamster diol diester waxes

No. of C-atoms	Total	Position 1[a]	Position 2[a]	Position 2[b]
*n*14	3.4	2.1	1.9	2.7
*iso*15	9.4	8.5	2.7	3.3
*n*15	1.5	2.0	1.4	1.5
*iso*16	0.2	0.4		tr
*n*16	1.7	2.5	1.9	1.8
*iso*17	6.2	5.9	2.2	3.9
*n*17	0.6	1.3	0.7	0.7
*iso*18	0.4	1.7	0.1	0.4
*n*18	3.7	5.5	2.2	2.1
*iso*19	9.1	14.8	1.7	2.2
*anteiso*19	4.2	7.8	1.1	1.1
*n*19	3.9	6.6	2.5	2.8
*iso*20	1.1	2.8	0.7	1.0
*n*20	9.3	10.4	9.4	8.9
*iso*21	6.2	3.8	7.4	7.3
*anteiso*21	2.7	6.0	1.2	0.2
*n*21	4.6	4.2	6.7	6.3
*iso*22	0.2	0.6	1.4	1.1
*n*22	6.1	3.2	11.4	10.6
*iso*23	3.3	1.3	7.2	7.0
*anteiso*23	1.4	1.1	2.8	2.1
*n*23	5.7	2.3	10.4	9.8
*iso*24	0.5	0.1	1.0	0.7
*n*24	8.6	2.7	13.0	13.8
*iso*25	1.0	0.3	2.1	2.2
*anteiso*25	0.4	0.7	0.6	tr
*n*25	3.0	0.9	4.4	5.1
*n*26	1.4	0.5	1.9	1.5
*n*28	0.2	tr	tr	tr
$\leq C_{20}$	54.7	72.3	28.5	32.4
$> C_{20}$	45.3	27.7	71.5	67.6

[a] *Rhizopus arrhizus* lipase hydrolysis. Each value is the mean of two incubations (average deviation from the mean was ±10%).
[b] Chemical hydrolysis (Grignard reagent); mean of two determinations.

with a Grignard reagent produced 2-acyl diols with the same fatty acid composition as the 2-acyl diols obtained by lipase hydrolysis. *2*) The diol diesters that were not hydrolyzed by the lipase had the same fatty acid composition as the original sample. *3*) The constituent 1,2-diols from the original sample, from the 2-acyl diol fraction, and from the unhydrolyzed fraction all had the same composition.

Indirect evidence against a selective hydrolysis by the lipase of certain acyl groups is the fact that hydrolysis of the mouse diol diesters gave the opposite pattern of fatty acid distribution (Table 3). The shorter chain acids ($\leq C_{18}$) were found almost exclusively at position 2 of the diols. Throughout this investigation, acyl migration from 2-acyl diol to 1-acyl diol or vice versa was not observed, as checked by TLC.

The mouse diol diester waxes also were readily hydrolyzed by *Rhizopus* lipase and the fatty acid composition of the 2-acyl diols produced by the enzymic hydrolysis was in reasonably good agreement with that of the 2-acyl diols from the Grignard hydrolysis (Table 3). However, there was a deficiency of C_{20} and C_{22} monounsaturated acids in the products of lipase hydrolysis, and enrichment of these two acids in the unhydrolyzed fraction compared to the total fatty acids in the original sample. Hydrolysis under the same conditions with a purified porcine pancreatic lipase gave somewhat better results. Although discrimination against $C_{20:1}$ and $C_{22:1}$ was still apparent, this was not investigated further. This and the other minor inconsistencies between the composition of hydrolysis products and total acyl composition indicate that certain molecular species were attacked more readily than others. In general, the 1,2-diols in the original sample, in the 2-acyl diols, and in the unhydrolyzed fraction had the same composition.

Previous investigations on the structure of mammalian 1,2-alkanediol diesters have not been concerned with the positional distribution of the constituent fatty acids, although lipase hydrolysis was used in the identification of triester waxes from skin of the rhino mutant mouse (16). Since diol diesters are major constituents of the sebum of many mammals, the nonrandom distribution of fatty acids in this lipid class is of interest from a biochemical as well as functional point of view.

Although it is difficult to distinguish between the surface lipids produced by the epidermis and those produced by the sebaceous glands, there is general agreement (7) that much of the neutral lipid, and particularly the diester waxes, are constituents of the sebum. The pathways of lipid biosynthesis in the sebaceous glands are not well investigated, but it appears that most of the lipids are synthesized de novo by the gland (7). The reason for the presence of unusual lipids in the sebum, whose biosynthesis undoubtedly requires a rather complex metabolic machinery, remains obscure. Species specificity seems to be a general characteristic which could be related to species recognition and pheromone effects.

We observed that repeated collections of skin surface lipids from the same groups of hamsters or mice yielded diol diesters of virtually identical composition. Preliminary analysis of the diol diesters from the skin of a different strain of mouse (C57BL) showed a similar pattern of fatty acid distribution at positions 1 and 2 as that reported here for the Swiss albino mouse. Systematic comparisons of molecular structures such as those reported here may add new details to the concept of species specificity of mammalian diester waxes.

TABLE 3. Constituent fatty acids (mol %) of mouse diol diester waxes

No. of C-atoms: No. of Double Bonds	Total	Position 1[a]	Position 2[a]	Position 2[b]
*n*14	0.4	0.1 ± 0.02	2.2 ± 0.7	0.7
*iso*15	0.8		1.5 ± 0.5	1.0
*n*15	0.2	tr	1.0 ± 0.3	0.7
*iso*16	3.4	tr	6.3 ± 0.1	5.3
*n*16	7.9	0.6 ± 0.1	16.3 ± 1.2	12.3
*n*16:1	22.0		44.8 ± 1.8	42.1
*iso*18	0.8	1.4 ± 0.1		
*n*18	0.5	1.8 ± 0.1	0.7 ± 0.2	1.5
*n*18:1	6.9		14.8 ± 1.7	12.1
*iso*19	0.1	2.5 ± 0.2	0.1 ± 0.1	
*n*19	0.1	2.0 ± 0.1		0.5
*iso*20	6.1	15.0 ± 0.7	0.6 ± 0.1	2.4
*n*20	10.1	22.4 ± 1.3	1.6 ± 0.8	3.3
*n*20:1	6.7	7.1 ± 0.9		3.2
*iso*21	4.5	4.8 ± 0.1	2.1 ± 2.7	0.9
*n*21	3.1	5.1 ± 0.3	0.7 ± 0.5	1.5
*iso*22	4.1	6.5 ± 0.3	0.6 ± 0.2	2.3
*n*22	6.2	7.9 ± 0.3	3.5 ± 0.3	3.5
*n*22:1	6.5	7.8 ± 0.7	0.8 ± 0.2	3.5
*iso*23	1.4	0.5 ± 0.1		0.9
*n*23	0.4	0.2 ± 0.1	0.4 ± 0.3	0.6
*iso*24	0.2	0.2 ± 0.1	0.3 ± 0.3	0.5
*n*24	1.8	2.8 ± 0.4	1.2 ± 0.2	1.3
*n*24:1	1.3	2.2 ± 0.6		
*iso*25	0.8	0.4 ± 0.02	0.3 ± 0.4	
*iso*26	0.5	0.1 ± 0.01	0.1 ± 0.1	
*iso*27	0.8	0.5 ± 0.2		
*iso*28	1.3	2.2 ± 0.8		
*iso*29	0.8	0.6 ± 0.2		
*n*29	0.8	2.3 ± 1.2		
*iso*30	1.0	2.3 ± 0.6		
$\leq C_{18}$	42.9	4.1	87.6	75.7
$> C_{18}$	58.6	95.4	12.3	24.4

[a] Porcine pancreatic lipase hydrolysis. Three incubations, mean ± SD.

[b] Chemical hydrolysis (Grignard reagent); mean of two determinations.

This investigation was supported in part by U.S. Public Health Service Research Grant HL 08214 from the Program Project Branch, Extramural Programs, National Heart, Lung and Blood Institute, and by the Hormel Foundation. We wish to thank Dr. Y. Wedmid for recording the NMR spectra and Dr. H. L. Brockman for a sample of purified porcine pancreatic lipase.

Manuscript received 28 October 1977; accepted 27 February 1978.

REFERENCES

1. Nikkari, T., and E. Haahti. 1968. Isolation and analysis of two types of diester waxes from the skin surface lipids of the rat. *Biochim. Biophys. Acta* **164:** 294–305.
2. Nicolaides, N., H. C. Fu, and M. N. A. Ansari. 1969. Diester waxes in surface lipids of animal skin. *Lipids* **5:** 299–307.
3. Downing, D. T., and D. M. Sharaf. 1976. Skin surface lipids of the guinea pig. *Biochim. Biophys. Acta* **431:** 378–389.
4. Wheatley, V. R., and A. T. James. 1957. Studies of sebum. 7. The composition of the sebum of some common rodents. *Biochem. J.* **65:** 36–42.
5. Wilkinson, D. E., and M. A. Karasek. 1966. Skin lipids of a normal and a mutant (asebic) mouse strain. *J. Invest. Dermatol.* **47:** 449–455.
6. Nikkari, T. 1974. Comparative chemistry of sebum. *J. Invest. Dermatol.* **62:** 257–267.
7. Downing, D. T. 1976. Mammalian waxes. *In* Chemistry and Biochemistry of Natural Waxes. P. E. Kolattukudy, editor. Elsevier, Amsterdam, London, New York. 17–48.
8. Bandi, P. C., and H. H. O. Schmid. 1976. Configurational analysis of long-chain alkanediols. *Chem. Phys. Lipids* **17:** 267–274.
9. Noren, B., and G. Odham. 1973. Antagonistic effects of *Myxococcus xanthus* on fungi: II. Isolation and characterization of inhibitory lipid factors. *Lipids* **8:** 573–583.
10. Baumann, W. J., and T. H. Madson. 1974. Ozonolysis of long-chain cyclic acetals: formation of monoesters. *J. Lipid Res.* **15:** 528–529.
11. Chang, N., and H. H. O. Schmid. 1973. Evidence for the incorporation of a long-chain 1,2-alkanediol into diol phospholipids by mammalian brain. *Biochem. Biophys. Res. Commun.* **54:** 648–654.
12. Yurkowski, M., and H. Brockerhoff. 1966. Fatty acid distribution of triglycerides determined by deacylation with methyl magnesium bromide. *Biochim. Biophys. Acta* **125:** 55–59.
13. Christie, W. W., and J. H. Moore. 1969. A semimicro method for the stereospecific analysis of triglycerides. *Biochim. Biophys. Acta* **176:** 445–452.
14. Semeriva, M., G. Benzonana, and P. Desnuelle. 1967. *Rhizopus arrhizus* lipase. I. Positional specificity. *Bull. Soc. Chim. Biol.* **49:** 71–79.
15. Slotboom, A. J., G. H. De Haas, P. P. M. Bonsen, G. J. Burbach-Westerhuis, and L. L. M. Van Deenen. 1970. *Chem. Phys. Lipids* **4:** 15–29.
16. Logani, M. K., D. B. Nhari, and R. E. Davies. 1977. Composition of novel triesters from the skin of the rhino mutant mouse. *Lipids* **12:** 283–287.