

## Structural Identification of the $\beta$ -Hydroxy Fatty Acid-Based Diester Preen Gland Waxes of Shorebirds

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The intact C<sub>33</sub>–C<sub>52</sub> diester wax esters of the preen gland of the shorebirds *Limosa lapponica*, *Pluvialis squatarola*, and *Pluvialis fulva* were determined, using synthesized standards, to comprise predominantly C<sub>12</sub>–C<sub>16</sub>  $\beta$ -hydroxy fatty acids esterified with a C<sub>8</sub>–C<sub>18</sub> fatty acid at the  $\beta$ -hydroxy position and with predominantly C<sub>12</sub>–C<sub>20</sub> fatty alcohols esterified at the carboxyl group.

Birds protect their feathers with wax that is produced in a special gland, the preen gland, located on the rump at the bottom of the tail feathers in the dorsal caudal tract. The chemical composition of these preen gland waxes is very complex and varies significantly from order to order.<sup>1</sup> We have shown that the composition of preen waxes not only varies with bird species but for many species of sandpipers also changes during the year.<sup>2,3</sup> Specifically, a shift in the preen wax composition, from lower molecular weight monoesters to high molecular weight diesters, is observed during courtship and incubation, but generally only for the incubating sex.<sup>4</sup> The production of diester waxes appears to reduce the olfactory cues for mammalian predators to find nests.<sup>5</sup> Detailed chemical analysis has shown that the intact C<sub>30</sub>–C<sub>50</sub> diester waxes of the preen gland of the migrating bird *Calidris canutus* (Linnaeus, 1758) comprise predominantly C<sub>12</sub>–C<sub>18</sub> alkane-1,2-diols esterified with octanoic, decanoic, and dodecanoic acid at the C-1 position and also with predominantly even-carbon-numbered fatty acids at the other position (Figure 1a).<sup>6</sup> Screening of preen waxes of many other species of birds produced during incubation by gas chromatography revealed the occurrence of similar high molecular weight waxes (e.g., Figure 1b).<sup>3,4</sup>

Herein we describe in detail the chemical composition of another type of high-molecular-weight diester preen wax occurring in the shorebirds *Limosa lapponica* (Linnaeus, 1758), *Pluvialis squatarola* (Linnaeus, 1758), and *Pluvialis fulva* (Gmelin, 1789). Methanolysis of the high-molecular-weight preen wax of these birds resulted in the formation of only small amounts of C<sub>16</sub>–C<sub>20</sub> alkane-1,2-diols, the characteristic building blocks of the diol wax.<sup>6</sup> Instead, relatively high amounts of  $\beta$ -hydroxy fatty acids and straight-chain alcohols, in addition to fatty acids and 1,2-alkane-diols, were formed (Figure 1c). The  $\beta$ -hydroxy fatty acids were identified as their methyl esters and TMS derivatives and comparison of their mass spectra with those reported in the literature.<sup>7</sup> Their carbon-number distributions exhibit a strong even-over-odd carbon number predominance and is dominated by C<sub>12</sub>, C<sub>14</sub>, and C<sub>16</sub> (Table 1) depending on species. The fatty acids and alkan-1-ols released also show a strong even-over-odd carbon number predominance, dominated by C<sub>12</sub>, C<sub>14</sub>, and C<sub>16</sub> and C<sub>16</sub> and C<sub>18</sub>, respectively. However, it is likely that during workup some of the volatile fatty acids (<C<sub>10</sub>) have been partially lost due to their volatility. The results indicated that the high-molecular-weight waxes are diesters composed of C<sub>10</sub>–C<sub>18</sub>  $\beta$ -hy-

droxy fatty acids, C<sub>8</sub>–C<sub>18</sub> straight-chain fatty acids, and C<sub>12</sub>–C<sub>18</sub> straight-chain alcohols and, in addition, contain some diester waxes based on alkan-1,2-diols previously identified. Earlier indirect evidence for the occurrence of a  $\beta$ -hydroxy fatty acid-based diester wax was obtained by the study of preen waxes of female mallard ducks, *Anas platyrhynchos* (Linnaeus, 1758).<sup>8</sup>

Subsequent analysis of the intact diester wax by GC-MS using EI ionization revealed complex mass spectra in which no molecular ions were encountered. GC-MS with chemical ionization (CI) revealed that the diester waxes are C<sub>32</sub>–C<sub>48</sub> components. Several homologous series of diesters were encountered (e.g., Figure 2a). The major series possess base peaks at  $m/z$  199, 227, or 255 (C<sub>*n*</sub>H<sub>2*n*-1</sub>O<sub>2</sub><sup>+</sup>) (Figure 3b) and fragment ions related to the loss of a C<sub>8</sub>–C<sub>18</sub> fatty acid moiety in their EIMS. These compounds seem to be composed of 3-hydroxy fatty acids esterified with a fatty acid at the 3-hydroxyl group and with an alkan-1-ol at the carboxyl moiety.

In an attempt to fully determine the structure of this series of diesters, three authentic standards, 3-capryloxytetradecanoic acid tetradecyl ester (**1**), 3-capryloxytetradecanoic acid hexadecyl ester (**2**), and 3-palmitoxytetradecanoic acid tetradecyl ester (**3**) were synthesized. These standards are all based on 3-hydroxytetradecanoic acid as a building block but vary in the length of the esterifying groups. The mass spectra of these diester standards **1–3** (Figure 2) are rather similar and do not possess a molecular ion. The molecular ion was established by GC-CI-MS. The mass spectrum of diester **1** is characterized by fragment ions at  $m/z$  127 (C<sub>7</sub>H<sub>15</sub>CO<sup>+</sup>),  $m/z$  422 (M – C<sub>7</sub>H<sub>15</sub>COOH),  $m/z$  439 (M – C<sub>7</sub>H<sub>15</sub>CO),  $m/z$  353 (M – C<sub>14</sub>H<sub>29</sub>O),  $m/z$  227 ( $m/z$  371 – C<sub>7</sub>H<sub>15</sub>COOH), and  $m/z$  209 (C<sub>13</sub>H<sub>25</sub>CO<sup>+</sup>). The mass spectrum of diester **2** revealed fragment ions at  $m/z$  127 (C<sub>7</sub>H<sub>15</sub>CO<sup>+</sup>),  $m/z$  450 (M – C<sub>7</sub>H<sub>15</sub>COOH),  $m/z$  467 (M – C<sub>7</sub>H<sub>15</sub>CO),  $m/z$  353 (M – C<sub>16</sub>H<sub>33</sub>O),  $m/z$  227 ( $m/z$  371 – C<sub>7</sub>H<sub>15</sub>COOH), and  $m/z$  209 (C<sub>13</sub>H<sub>25</sub>CO<sup>+</sup>). The mass spectrum of diester **3** is characterized by fragment ions at  $m/z$  239 (C<sub>15</sub>H<sub>31</sub>CO<sup>+</sup>),  $m/z$  422 (M – C<sub>15</sub>H<sub>31</sub>COOH),  $m/z$  439 (M – C<sub>15</sub>H<sub>31</sub>CO),  $m/z$  465 (M – C<sub>14</sub>H<sub>29</sub>O),  $m/z$  227 ( $m/z$  483 – C<sub>15</sub>H<sub>31</sub>COOH), and  $m/z$  209 (C<sub>13</sub>H<sub>25</sub>CO<sup>+</sup>). The authentic standards co-eluted upon GC analysis with the C<sub>36</sub>, C<sub>38</sub>, and C<sub>44</sub> members of the series of the diester preen waxes of the three bird species investigated.

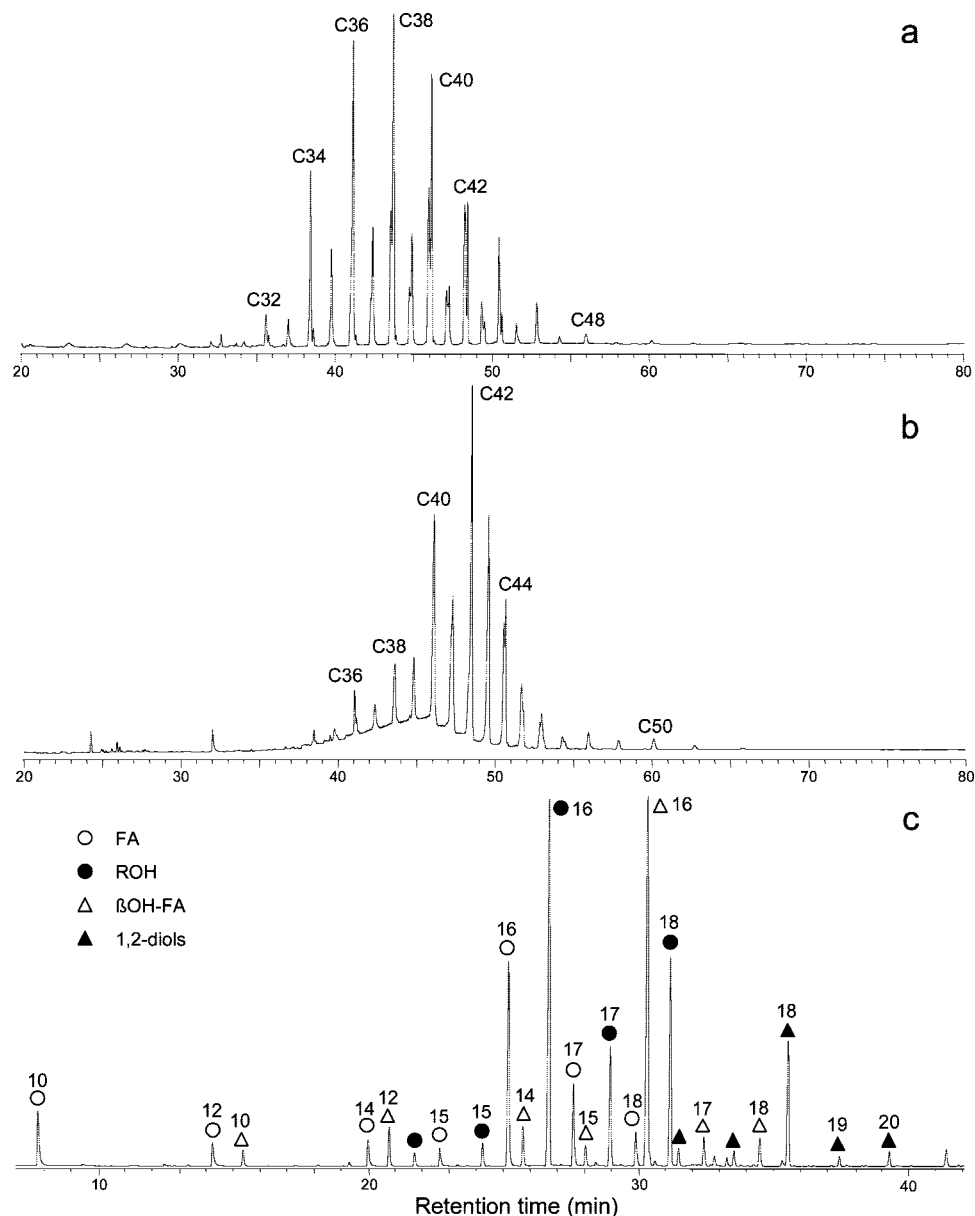
Comparison of the mass spectrum of the C<sub>38</sub> member of *P. squatarola* (Figure 2a) with those of diesters **1–3** (Figures 2b, c, and d) indicates that additional co-eluting C<sub>38</sub> diesters containing a  $\beta$ -hydroxy fatty acid moiety other than C<sub>14</sub> must be present as revealed by the characteristic  $m/z$  171, 199, and 255 fragment ions. Various acyl fragment ions (i.e.,  $m/z$  127, 141, and 155) are also observed in the mass spectrum and are accompanied by corre-

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**Figure 1.** Gas chromatograms of diester waxes produced by (a) *C. canutus* and (b) *P. squatarola* and (c) the product mixture after methanolysis of the diester wax of the *P. squatarola*. Transesterification products were analyzed as methyl esters and TMS ethers after appropriate derivatization. Key: FA = fatty acids, ROH = alkan-1-ols,  $\beta$ OH-FA =  $\beta$ -hydroxy fatty acids, 1,2-diols = 1,2-alkanediols. Total number of carbon atoms of intact waxes (a and b) and methanolysis products (c) are indicated. Note that the chromatogram of the diesters from *P. squatarola* (b) contains an unresolved complex mixture. This is generated by thermal degradation during GC analysis of the  $\beta$ -hydroxy fatty acid-based diester waxes by elimination of fatty acids, resulting in the formation of monounsaturated wax esters. A similar phenomenon was observed with the authentic diesters 1–3. This process results in a visual under-representation of the  $\beta$ -hydroxy fatty acid-based diester waxes relative to the alkan-1,2-diol-based waxes.

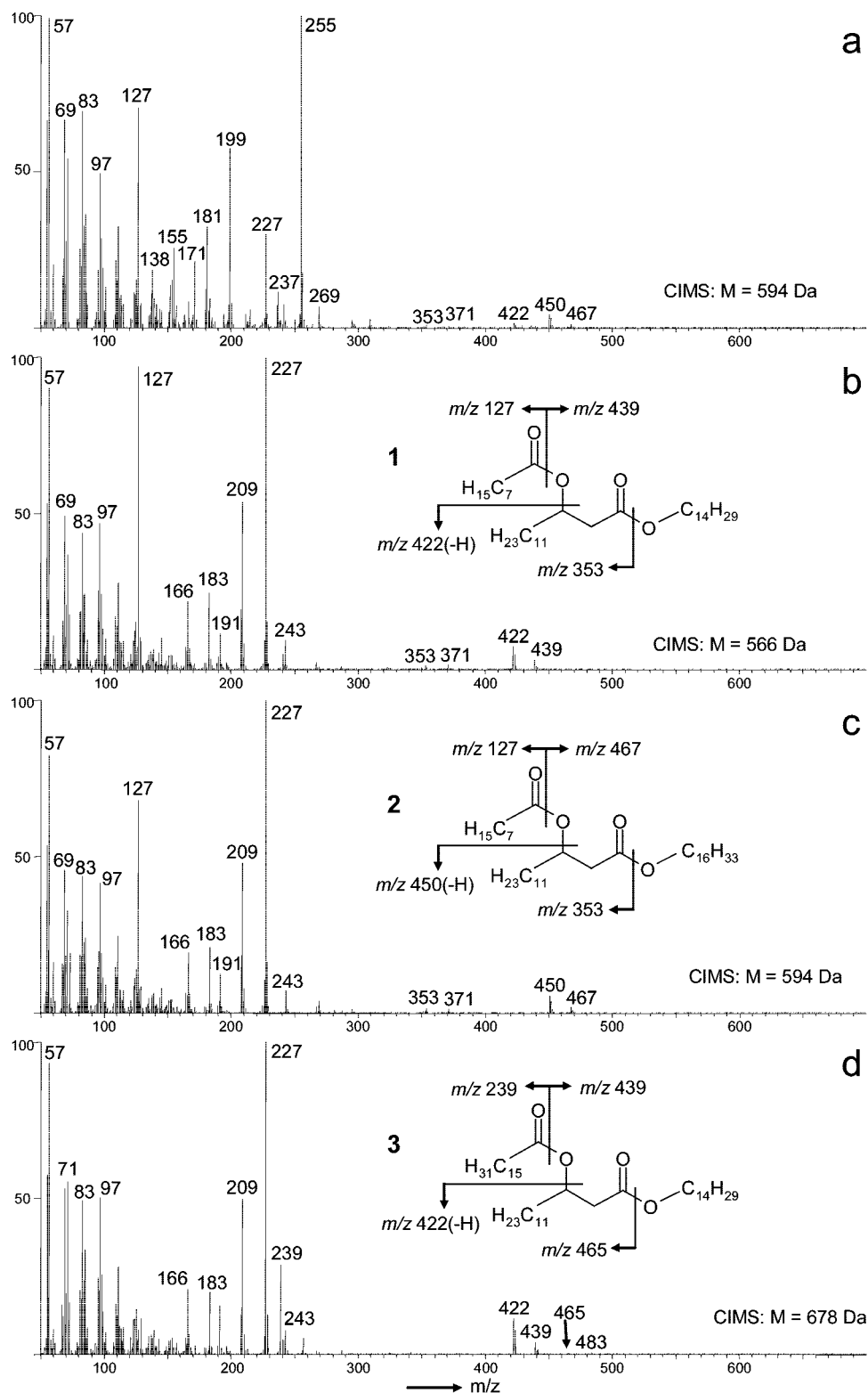
**Table 1.** Relative Composition (mol %) of  $\beta$ -Hydroxy Fatty Acids after Methanolysis of the Diester Preen Waxes of the Three Shorebirds Investigated

carbon number	<i>P. squatarola</i>	<i>P. fulva</i>	<i>L. lapponica</i>
C <sub>10</sub>	3.5	2.0	0.5
C <sub>12</sub>	7.2	6.8	35.2
C <sub>14</sub>	6.7	8.5	45.1
C <sub>15</sub>	3.6	5.8	3.4
C <sub>16</sub>	70.0	60.0	13.7
C <sub>17</sub>	4.6	8.5	1.0
C <sub>18</sub>	4.4	7.2	1.0

sponding  $M - C_xH_{2x+1}CO$  and  $M - C_xH_{2x+1}COOH$  fragment ions. This indicates that the  $\beta$ -hydroxy fatty acid moiety is esterified with alkanic acids and alkan-1-ols with variable chain lengths. Members of this homologous series with an even number of carbon atoms

are predominantly composed of C<sub>10</sub>–C<sub>16</sub> even-carbon-numbered  $\beta$ -hydroxy fatty acids esterified with C<sub>8</sub>–C<sub>10</sub> fatty acids at the 3-hydroxyl position and with C<sub>16</sub>–C<sub>18</sub> fatty alcohols at the carboxyl group.

This is the second time intact diester preen wax esters of birds have been identified unambiguously. Our studies<sup>2–5</sup> show that the “biological clock” of migrating birds, in addition to many other physiological parameters, also affects the chemical and, thus, physical characteristics of the preen wax. However, some birds switch to an alkane-1,2-diol-based diester wax,<sup>6</sup> whereas others switch to a diester wax that has even-carbon  $\beta$ -hydroxy fatty acids as the main building blocks. In both cases it is likely that this switch in wax composition is to reduce the olfactory cues for mammalian predators to find nests,<sup>5</sup> but it is uncertain at this stage why different bird species use different chemicals for this purpose.

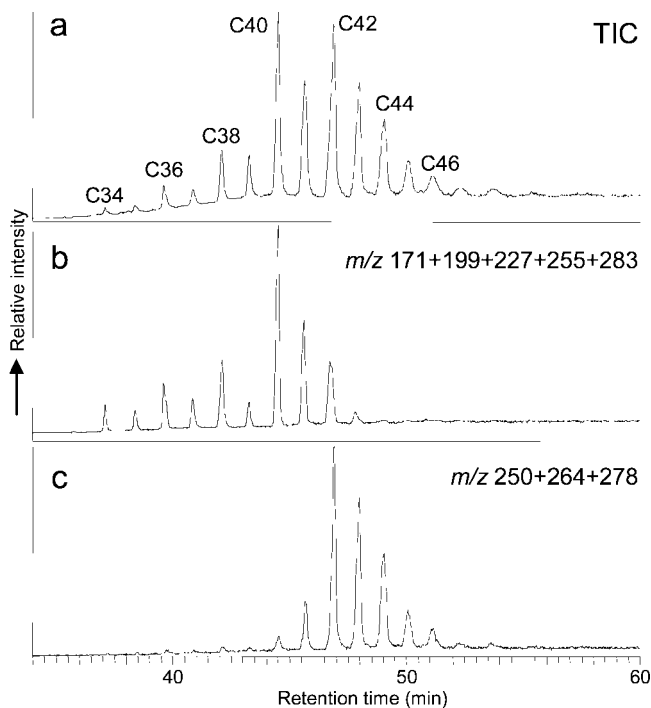


**Figure 2.** Mass spectra (subtracted for background) of (a) the  $C_{38}$  member of diester waxes of *P. squatarola*, (b) synthetic 3-capryloxytetradecanoic acid tetradecyl ester (**1**), (c) synthetic 3-capryloxytetradecanoic acid hexadecyl ester (**2**), and (d) synthetic 3-palmitoxytetradecanoic acid tetradecyl ester (**3**). Tentative fragmentation patterns are indicated. Molecular ions were not observed in the EIMS but were established by CIMS and are given for reference.

### Experimental Section

**General Experimental Procedures.** GC was performed on a Hewlett-Packard 5890 series II chromatograph equipped with an on-column injector and fitted with a  $25 \times 0.32$  mm fused silica capillary column coated with CP-Sil 5 (film thickness  $0.12 \mu\text{m}$ ). Helium was used as carrier gas. For the intact waxes the oven was programmed from 70 to 130 at  $20^\circ\text{C}/\text{min}$ , followed by an increase of  $4^\circ\text{C}/\text{min}$  to

$320^\circ\text{C}$  and held at  $320^\circ\text{C}$  for 35 min. For the methanolized fractions, the oven was held at  $70^\circ\text{C}$  for 3 min and programmed to  $320^\circ\text{C}$  at  $4^\circ\text{C}/\text{min}$ . Compounds were detected using a flame ionization detector (FID). GC-MS was performed on a Thermo Finnigan Trace gas chromatograph using the same column and conditions as described for GC. The column was directly inserted into the electron impact ion source of a Thermo Finnigan Trace DSQ quadrupole mass spectrometer,



**Figure 3.** Total ion current (TIC) of diester wax of *P. squatarola* (a) and mass chromatograms of characteristic ions of C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>, C<sub>16</sub>, and C<sub>18</sub>  $\beta$ -hydroxy fatty acid-based diester waxes (b) and C<sub>18</sub>, C<sub>19</sub>, and C<sub>20</sub> 1,2-alkandiol-based diester waxes (c), revealing the chemical complexity of diester preen waxes.

scanning a mass range of  $m/z$  50–800 at 3 scans  $s^{-1}$  and an ionization energy of 70 eV. GC-MS with chemical ionization using methane as the reagent gas was performed on an HP6890 gas chromatograph coupled to a HP 5973 mass selective detector with a mass range  $m/z$  500–800 and a cycle time of 0.2 s. GC conditions were the same as described above. <sup>1</sup>H and <sup>13</sup>C NMR measurements were performed on a Bruker DMX-600 spectrometer.

**Preen Wax Sources.** Preen wax samples were obtained from a godwit (*Limosa*; bar-tailed godwit, *L. lapponica*) and two plovers (*Pluvialis*; black-belted plover, *P. squatarola*, and Pacific golden plover, *P. fulva*). All birds were caught on their nests during incubation. Preen wax samples of the bar-tailed godwit (June 26, 2002), Pacific golden plover (July 23, 2002), and black-belted plover (July 20, 2003) were obtained on the tundra of the Taimyr Peninsula, Siberia, Russia. The biological material was authenticated by Dr. M. Soloviev and Dr. P. S. Tomkovich. A reference collection of preen waxes is maintained at the Royal Netherlands Institute for Sea Research.

**Chemical Analysis of Waxes.** Wax samples were taken from the breeding birds by softly massaging the preen gland with a cotton bud. The intact waxes were extracted with ethyl acetate and analyzed by GC and GC-MS. For methanolysis of the diester waxes<sup>8</sup> about 1 mg was dissolved in 300  $\mu$ L of toluene, 1.5 mL of 14% BF<sub>3</sub> in methanol was added, and the mixture was refluxed for 3 h. After cooling, 2 mL of double-distilled water was added and the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> and dried over Na<sub>2</sub>SO<sub>4</sub>. The fatty alcohols and hydroxy fatty acid methyl esters produced were converted to their trimethylsilyl

derivatives by heating with *N,O*-bis(trimethylsilyl)trifluoroacetamide/pyridine at 60 °C for 20 min, and the mixture was diluted with ethyl acetate and analyzed by GC and GC-MS. The relative amounts of the  $\beta$ -hydroxy fatty acids are based on the integration of the GC peak areas, corrected for the trimethyl silyl group and the methyl group, taking into account the molecular weights of the underivatized compounds.

**Synthesis.** 3-Capryloxytetradecanoic acid tetradecyl ester (**1**) was synthesized in the following manner. 3-Hydroxytetradecanoic acid was converted to 3-hydroxytetradecanoic acid tetradecyl ester by heating (80 °C, 2 h) 3-hydroxytetradecanoic acid (3 mg) with an excess of tetradecanol (50 mg) and *p*-toluenesulfonic acid (2 mg) and isolation of the product (yield 96%, 92% pure by GC) by SiO<sub>2</sub> column chromatography using hexane/diethyl ether (90:10, v/v) as the eluent. The resulting 3-hydroxy wax ester (3.5 mg) was heated (80 °C, 2 h) with an excess of octanoic acid (70 mg) and *p*-toluenesulfonic acid (2 mg), and the diester **1** (3.7 mg; yield 56%, 96% pure by GC) was isolated by Al<sub>2</sub>O<sub>3</sub> column chromatography using CH<sub>2</sub>Cl<sub>2</sub> as the eluent: <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz)  $\delta$  5.22 (1H, m, H-3), 4.06 (2H, t,  $J$  = 6.8 Hz, H-1'), 2.55 (2H, m, H-2), 2.27 (2H, t,  $J$  = 7.6 Hz, H-2''), 1.61 (6H, m, H-4, H-2', H-3''), 1.27 (~48H, m), 0.89 (9H, t,  $J$  = 6.9 Hz, H-14, H-14', H-8''); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz)  $\delta$  173.1 (C, C-1''), 170.6 (C, C-1), 70.3 (CH, C-3), 64.8 (CH<sub>2</sub>, C-1'), 39.4 (CH<sub>2</sub>, C-2), 34.5 (CH<sub>2</sub>, C-2''), 34.0 (CH<sub>2</sub>, C-4), 31.9, 31.7 (CH<sub>2</sub>, C-12, C-12', C-6''), 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 29.0, (CH<sub>2</sub>, C-6–C-11, C-4'–C-11', C-4'', C-5''), 28.6 (CH<sub>2</sub>, C-2'), 25.9 (CH<sub>2</sub>, C-3'), 25.1, 25.0 (CH<sub>2</sub>, C-5, C-3''), 22.7, 22.6 (CH<sub>2</sub>, C-13, C-13', C-7''), 14.1 (CH<sub>3</sub>, C-14, C-14', C-8'').

3-Capryloxytetradecanoic acid hexadecyl ester (**2**) was synthesized in the same way as **1** using hexadecanol instead of tetradecanol in the first step (yield 85%, 60% pure by GC). 3-Palmitoxytetradecanoic acid tetradecyl ester (**3**) was synthesized in the same way as **1** using hexadecanoic acid instead of octanoic acid in the second step (yield 74%, 46% pure by GC).

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