# 2,3-Dihydroxy Fatty Acids-Containing Waxes in Storks (*Ciconiidae*)

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Uropygial gland secretions from five out of a total of seven species forming the genus *Ciconia* (family Ciconiidae; order Ciconiiformes) were found to consist of mixtures of monoester waxes, diester waxes, triester waxes, and triglycerides. Monoester waxes were composed of unbranched fatty acids and alcohols, whereas diester waxes derived from both 2- and 3-hydroxy fatty acids esterified with unbranched alcohols and fatty acids. Interestingly, triester waxes were also found deriving from either 2-hydroxy alkylmalonic acids or from *erythro*-2,3-dihydroxy fatty acids the latter of which have not yet been found in vertebrates so far. To compare the typical mass spectrometric fragmentation of this class of compounds *erythro*-2,3-dihydroxyhexadecanoic acid has been synthesized.

## Introduction

Comparative analysis of uropygial gland lipids has been successfully used for chemotaxonomic studies of birds (Jacob and Ziswiler, 1982; Jacob, 1984) since they exhibit a surprisingly constant qualitative composition within distinct taxa, mainly at the order and occasionally also at the family level but significant differences between these taxa. Some ciconiiform birds (storks, ibisses, marabus, herons) have been analysed so far and, taking the data together, it appears that the above correlation does not hold for this order, since significant qualitative differences were found between the genera *Ciconia* (Jacob, 1976), *Nycticorax* (Jacob, 1975), *Ardea* (Poltz and Jacob, 1974), *Leptoptilos* (Jacob and Pomeroy, 1979), *Threskiornis* (Jacob, 1978a), *Theristicus* (Jacob, 1978a), and *Scopus* (Jacob, 1978a), all belonging to the same order (Ciconiiformes). With regard to the numerous positive findings, however, which clearly indicate the validity of the chemotaxonomic hypothesis it appears that the Ciconiiformes are a polyphyletic assemblage of species. Recently, some *Ciconia* species (*Ciconia nigra*, *C.abdimii*, *C.episcopus*, *C.maguari*) became available to us allowing a more detailed chemosystematic analysis of this genus comprising a total of 7 species from which hitherto only *Ciconia ciconia* (white stork) had been investigated (Jacob, 1976).

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## Material and Methods

The uropygial glands were excised from naturally died zoo animals and extracted with 60 ml chloroform/methanol (2:1; v/v) each. After addition of 20 ml water to the extract, the lower layer, which contained all lipid material, was evaporated to dryness. Thin-layer chromatography on silica gel plates (DC-Fertigplatten, Kieselgel 60, E. Merck) indicated the presence of monoester waxes, diester waxes, triglycerides, and a lipid class more polar than diester waxes , but less than triglycerides (triester waxes). Lipids were purified by column chromatography on silica gel (Woelm, 14.5% water content) by elution with 100 ml cyclohexane/benzene (9:1; v/v) for monoester waxes, 75 ml cyclohexane/benzene (3:2; v/v) for diester and triester waxes, and 50 ml benzene/chloroform (9:1; v/v) for triglycerides. Monoester waxes and triglycerides were transesterified with 5% methanolic HCl to give fatty acid methyl ester and alcohols (in case of monoester waxes). After chromatography of the mixture on silica gel (see above) methyl esters were eluted with 100 ml cyclohexane/benzene (3:1; v/v) and alcohols with 50 ml chloroform/methanol (95:5; v/v). The latter were oxidized by treatment with $CrO_3$/acetic acid in cyclohexane for 12 h (Jacob and Zeman, 1970) resulting in the corresponding fatty acids which then were esterified

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as above. The fraction containing di- and triester waxes was saponified with 2*N* methanolic sodium hydroxide (15% water) for 1 h. Extraction yielded alcohols which were treated as above and further analysed as fatty acid methyl esters. After acidification with conc. HCl the methanol/water phase was extracted with chloroform. The extract which contained fatty acids and hydroxy acids was esterified as above and separated into the corresponding fatty acid methyl ester and hydroxy acid methyl esters by chromatography on silica gel; fatty acid methyl esters were eluted with 100 ml cyclohexane/benzene (3:1; v/v), whereas hydroxy acid methyl esters were eluted with 100 ml cyclohexane/benzene (1:1;v/v).

The GC separation of all fractions was carried out with a 25m x 0.32mm NB 54 capillary column (Nordion; 0.25 μm) under isothermical conditions (column temperature:160°C or 180°C; injection port and detector temperature:200°C) using a Delsi DI 700 instrument adapted to an electronic integrator system Shimadzu CR 3A. Equivalent chain lengths (ECL values) were obtained from a semi-logarithmic plot of the retention times against chain length with reference standards of unbranched fatty acid methyl esters (n-$C_{10}$-$C_{20}$).

*Erythro-2,3-dihydroxyhexadecanoic methylester*

Methyl *cis*-hexadec-2-enoate (90 mg) prepared from the acid by transmethylation with 5% methanolic HCl was dissolved in 4 ml acetonitrile and added to a solution of $RuCl_3.H_20$ (5 mg) and $NaIO_4$ (105 mg) in water (1 ml) at 0°C. After a reaction time of 30 min a solution of $Na_2S_2O_5$ (500 mg) in water (10 ml) was added and stirred for 30 min at room temperature. The mixture was extracted with 3 x 30 ml ethylacetate, the combined extracts dried over $MgSO_4$ and evaporated. The crude product was then purified by flash chromatography on silica (elution with petroleum ether/ethylacetate 30/70; v/v). After discarding the first fraction (28 mg) colourless crystals of *erythro*-2,3-dihydroxyhexadecanoic methylester were obtained (32.3 mg; 32% yield, F.: 63–64°C; 400 MHz $^1$H-NMR ($CDCl_3/D_2O$) δ 4.21 (d, 1H, -CH(α)-OH, $J$ = 3.6 Hz) 3.84–3.80 (m, 1H, -CH(β)-OH) 3.79 (s, 3H, -$OCH_3$) 1.51–1.22 (m, 24H, -$CH_2$-) 0.85 (t, 3H, -$CH_3$, $J$ = 6.85).

## Results

After extraction and purification of the extracts by chromatography on silica, fractions of monoester, di- and triester waxes, and triglycerides were obtained the quantitative compositions of which are listed in Table I for the five species investigated.

Table I. Composition of the uropygial gland lipids from five *Ciconia* species (in %).

| Species | Monoester waxes | Di-/Triester waxes % | Triglycerides |
|---|---|---|---|
| *Ciconia nigra* | 80.5 | 14.7 | 4.8 |
| *Ciconia abdimii* | 1.6 | 80.3 | 18.1 |
| *Ciconia episcopus* | 1.0 | 77.1 | 21.9 |
| *Ciconia maguari* | 17.1 | 58.5 | 24.4 |
| *Ciconia ciconia** | 81.9 | 7.6 | 10.5 |

* taken from Jacob, 1976.

After transesterification with methanolic HCl (monoester waxes and triglycerides) or alkaline saponification (di- and triester waxes) with metha-

Table II. Constituents (fatty acids and alcohols) of the monoester waxes from five *Ciconia* species (in %).

| | *C.nigra* | *C.abdimii* | *C.episcopus* | *C.maguari* | *C.ciconia** |
|---|---|---|---|---|---|
| **Fatty acids** | | | | | |
| *n*-$C_{10}$ | 27.8 | - | - | 4.1 | 41.3 |
| *n*-$C_{11}$ | 0.2 | - | - | - | - |
| *n*-$C_{12}$ | 64.7 | - | - | 61.0 | 54.5 |
| *n*-$C_{14}$ | 5.3 | 1.0 | 1.0 | 24.2 | 4.2 |
| *n*-$C_{15}$ | 0.2 | 0.4 | 0.1 | tr | - |
| *n*-$C_{16}$ | 1.8 | 22.4 | 25.6 | 8.7 | - |
| *n*-$C_{17}$ | - | 1.0 | 0.5 | - | - |
| *n*-$C_{18}$ | - | 15.1 | 17.4 | 2.0 | - |
| *n*-$C_{16:1}$ | - | 2.8 | 1.5 | - | - |
| *n*-$C_{17:1}$ | - | 1.4 | 0.5 | - | - |
| *n*-$C_{18:1}$ | - | 44.5 | 46.0 | - | - |
| *n*-$C_{20:1}$ | - | 3.7 | 2.2 | - | - |
| *n*-$C_{22:1}$ | - | 6.4 | 4.2 | - | - |
| *n*-$C_{18:2}$ | - | 1.3 | 1.0 | - | - |
| **Alcohols** | | | | | |
| *n*-$C_8$ | 3.7 | - | - | - | - |
| *n*-$C_{10}$ | 77.8 | - | - | 3.1 | 92.6 |
| *n*-$C_{11}$ | 1.0 | - | - | 0.5 | - |
| *n*-$C_{12}$ | 9.8 | - | 1.1 | 15.2 | 7.4 |
| *n*-$C_{13}$ | 0.4 | - | - | 0.2 | - |
| *n*-$C_{14}$ | 0.9 | 5.5 | 7.1 | 7.7 | - |
| *n*-$C_{15}$ | 0.3 | 5.3 | 2.4 | 0.9 | - |
| *n*-$C_{16}$ | 3.0 | 36.7 | 40.7 | 25.5 | - |
| *n*-$C_{17}$ | 0.5 | 3.9 | 3.2 | 1.8 | - |
| *n*-$C_{18}$ | 2.6 | 23.4 | 25.0 | 45.1 | - |
| *n*-$C_{20}$ | - | 5.4 | 1.2 | tr | - |
| *n*-$C_{16:1}$ | - | 7.1 | 9.1 | - | - |
| *n*-$C_{17:1}$ | - | 2.3 | 1.1 | - | - |
| *n*-$C_{18:1}$ | - | 8.7 | 8.0 | - | - |
| *n*-$C_{18:2}$ | - | 1.7 | 1.1 | - | - |

* Taken from Jacob, 1976.

Table III. Constituents (fatty acids, hydroxy acids and alcohols) of the diester and triester waxes from five *Ciconia* species (in %).

| | *C.nigra* | *C.abdimii* | *C.episcopus* | *C.maguari* | *C.ciconia** |
|---|---|---|---|---|---|
| **Fatty acids** | | | | | |
| *n*-$C_{10}$ | tr | 78.7 | 54.9 | 8.1 | 80.3 |
| *n*-$C_{11}$ | - | - | 1.6 | 0.2 | - |
| *n*-$C_{12}$ | 60.0 | 17.0 | 40.5 | 53.1 | 18.2 |
| *n*-$C_{13}$ | - | - | - | 0.2 | - |
| *n*-$C_{14}$ | 16.7 | 2.5 | 2.7 | 26.8 | 1.5 |
| *n*-$C_{15}$ | 4.6 | - | - | 0.4 | - |
| *n*-$C_{16}$ | 18.7 | 1.8 | 0.3 | 8.0 | - |
| *n*-$C_{17}$ | - | - | - | 0.2 | - |
| *n*-$C_{18}$ | - | - | - | 2.2 | - |
| *n*-$C_{18:1}$ | - | - | - | 0.8 | - |
| **Alcohols** | | | | | |
| *n*-$C_{10}$ | 84.5 | 34.6 | - | 1.5 | 77.9 |
| *n*-$C_{11}$ | 0.8 | - | - | 0.2 | - |
| *n*-$C_{12}$ | 14.7 | 6.7 | - | 2.6 | 22.1 |
| *n*-$C_{13}$ | - | - | - | 3.2 | - |
| *n*-$C_{14}$ | - | 3.3 | 15.9 | 2.3 | - |
| *n*-$C_{15}$ | - | - | 3.0 | 3.0 | - |
| *n*-$C_{16}$ | - | 20.6 | 46.3 | 48.2 | - |
| *n*-$C_{17}$ | - | 0.9 | 4.5 | 0.1 | - |
| *n*-$C_{18}$ | - | 10.1 | 27.7 | 38.9 | - |
| *n*-$C_{16:1}$ | - | 1.2 | 0.7 | - | - |
| *n*-$C_{18:1}$ | - | 22.6 | 1.9 | - | - |
| **Hydroxy acids** | | | | | |
| *2-hydroxy fatty acids* | | | | | |
| 2-OH-$C_8$ | tr | - | - | - | - |
| 2-OH-$C_{10}$ | 9.9 | - | 1.6 | - | 22.3 |
| 2-OH-$C_{12}$ | 49.5 | - | 1.4 | 46.6 | 70.2 |
| 2-OH-$C_{14}$ | 2.8 | - | - | 18.1 | 7.5 |
| *3-hydroxy fatty acids* | | | | | |
| 3-OH-$C_{10}$ | 2.2 | 6.4 | 0.6 | - | - |
| 3-OH-$C_{12}$ | 32.4 | - | 0.9 | 12.5 | - |
| 3-OH-$C_{14}$ | 3.2 | - | - | 4.5 | - |
| *erythro-2,3-dihydroxy fatty acids* | | | | | |
| *e*-2,3-diOH-$C_{10}$ | - | 88.5 | 24.1 | - | - |
| *e*-2,3-diOH-$C_{12}$ | - | 5.1 | 71.4 | - | - |
| *2-hydroxy-alkyl-malonic acids* | | | | | |
| 2-OH-$C_9(COOH)_2$ | - | - | - | 10.1 | - |
| 2-OH-$C_{11}(COOH)_2$ | - | - | - | 8.2 | - |

* Taken from Jacob, 1976.

nolic NaOH the wax constituents were analysed by GC using the relative retention times and GC/MS for their identification. Results are summarized in Tables II-IV.

Apart from common fatty acids and alcohols four types of hydroxy acids were observed as constituents of the diester and triester waxes three of which have previously been identified in other bird species, e.g. 2-hydroxy fatty acids in *Ciconia ciconia* (Jacob, 1976), 3-hydroxy fatty acids in *Columba palumbus* (Jacob and Zeman, 1972), and 2-hydroxy-alkylmalonic acids in species not closely related to each other such as *Anas strepera, Picus viridis, Turdus merula* (Jacob and Grimmer, 1973). In addition, diester waxes were detected in *C.abdimii* and *C.episcopus* which contained a more polar type of hydroxy acid which turned out to be *erythro*-2,3-dihydroxy fatty acids. A typical mass spectrum exhibiting only poor fragmentation is presented in Fig. 1. A base peak of *m/z* 90 is supposed to originate analogously to the McLafferty rearrangement fragment *m/z* 74 observed in the

Table IV. Composition of the triglyceride fatty acids from five *Ciconia* species (in %).

| | *C.nigra* | *C.abdimii* | *C.episcopus* | *C.maguari* | *C.ciconia** |
|---|---|---|---|---|---|
| $n$-$C_{10}$ | 11.4 | 3.7 | 6.3 | tr | 35.4 |
| $n$-$C_{12}$ | 29.7 | 1.0 | 59.8 | 9.5 | 32.2 |
| $n$-$C_{13}$ | 0.4 | - | - | 1.7 | - |
| $n$-$C_{14}$ | 51.1 | 4.8 | 6.8 | 12.2 | 10.5 |
| $n$-$C_{15}$ | 7.4 | 0.7 | - | 2.5 | - |
| $n$-$C_{16}$ | - | 25.5 | 8.7 | 40.4 | 14.3 |
| $n$-$C_{17}$ | - | 1.1 | - | 2.4 | - |
| $n$-$C_{18}$ | - | 2.4 | 10.1 | 31.3 | 2.7 |
| $n$-$C_{20}$ | - | - | - | - | - |
| $n$-$C_{14:1}$ | - | 1.9 | - | - | - |
| $n$-$C_{15:1}$ | - | 0.6 | - | - | - |
| $n$-$C_{16:1}$ | - | 13.5 | 3.4 | - | - |
| $n$-$C_{17:1}$ | - | 0.7 | - | - | - |
| $n$-$C_{18:1}$ | - | 42.6 | 4.9 | - | 4.9 |
| $n$-$C_{18:2}$ | - | 1.5 | - | - | - |

* Taken from Jacob, 1976.

mass spectra of saturated fatty acid methyl esters (see Fig. 2).

No molecular ion is found, but small fragments with *m/z* 159 (M-59; loss of -$COOCH_3$) indicating a substitution at the C-2-position, *m/z* 119 (+CHOH-CHOH-$COOCH_3$), *m/z* 129 ($CH_3(CH_2)_6$-CHOH+), and *m/z* 141 (M-$COOCH_3$-$H_2O$) can be observed. As shown in Fig. 3 analogous fragments are found in the mass spectrum of the synthetic $C_{16}$-homologue with the identical base peak *m/z* 90 and *m/z* 119, whereas shifts by 84 mass units (corresponding to 6 $CH_2$-groups) are found for *m/z* 129 (becoming *m/z* 213), *m/z* 141 (becoming *m/z* 225), and *m/z* 159 (becoming *m/z* 243).

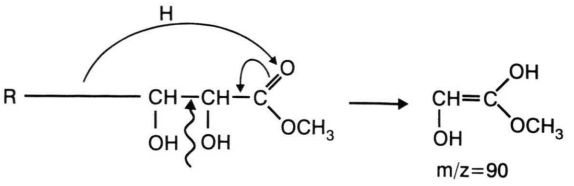


Fig. 2. Formation of the fragment *m/z* = 90 from *erythro*-2,3-dihydroxyalkanoic acid methyl esters by McLafferty rearrangement.

More readily to interpret are the mass spectra of the TMS-derivatives. Those recorded from the silylated dihydroxy acids found in *C.abdimii* and *C.episcopus* as well as from the synthetic *erythro*-2,3-dihydroxyhexadecanoic acid methyl ester are presented in Fig. 4–6. Though no molecular ions are found, fragmentation is more pronounced in the TMS-ethers than in the base compounds. The rearrangement ion *m/z* 90 becomes *m/z* 234 (90 + 2 x 72). The alkyl rest may readily be recognized from the fragment R – CHOTMS with *m/z* 201 (R = $C_7H_{15}$), *m/z* 229 (R = $C_9H_{19}$) and *m/z* 285 (R = $C_{13}H_{27}$), respectively. Common to all 3 compounds are (M-15)-fragments becoming parent peaks, (M-59)-fragments (M – $COOCH_3$), *m/z* 219 ($CH_3$(CHOTMS)$_2$) and the (M-105)-fragment (M – HOTMS – $CH_3$), respectively. These data confirm previous findings by Schmitz *et al.*(1975).

The advantage of the additivity of ECL-increments in GC for the identification of lipid constituents hardly can be overestimated (Jacob,

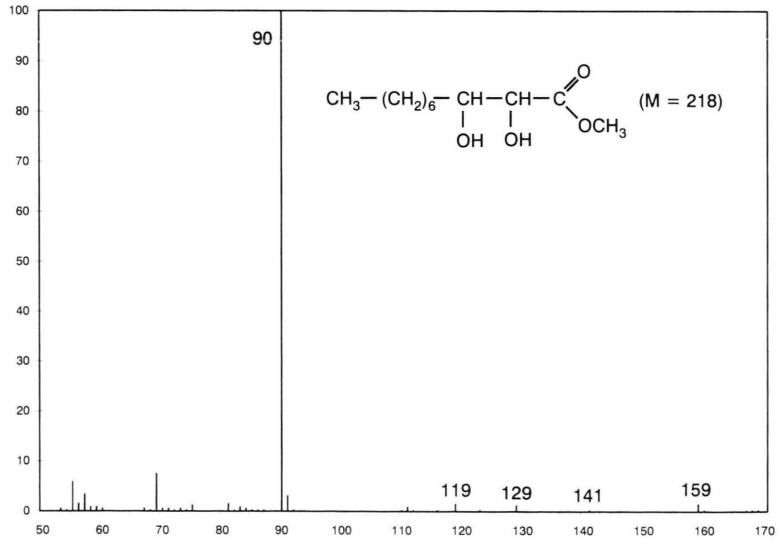


Fig. 1. Mass spectrum of methyl *erythro*-2,3-dihydroxydecanoate derived from the triester waxes of *C.abdimii*.

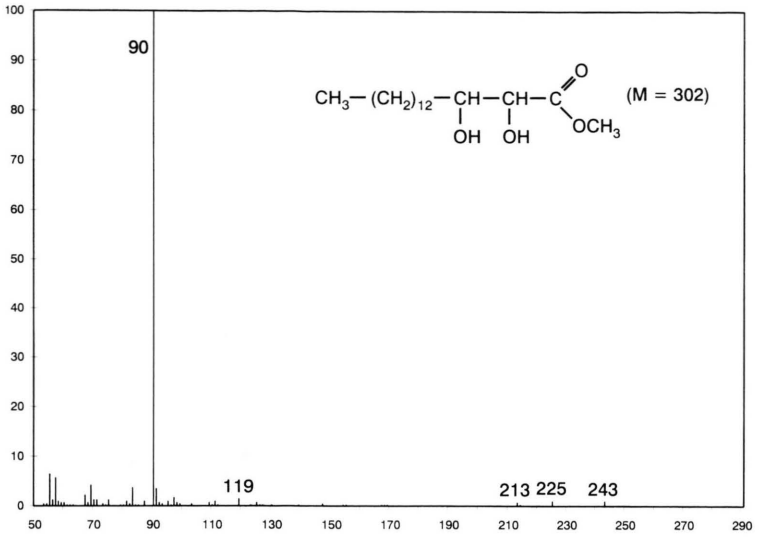


Fig. 3. Mass spectrum of synthetic methyl *erythro*-2,3-dihydroxyhexadecanoate.

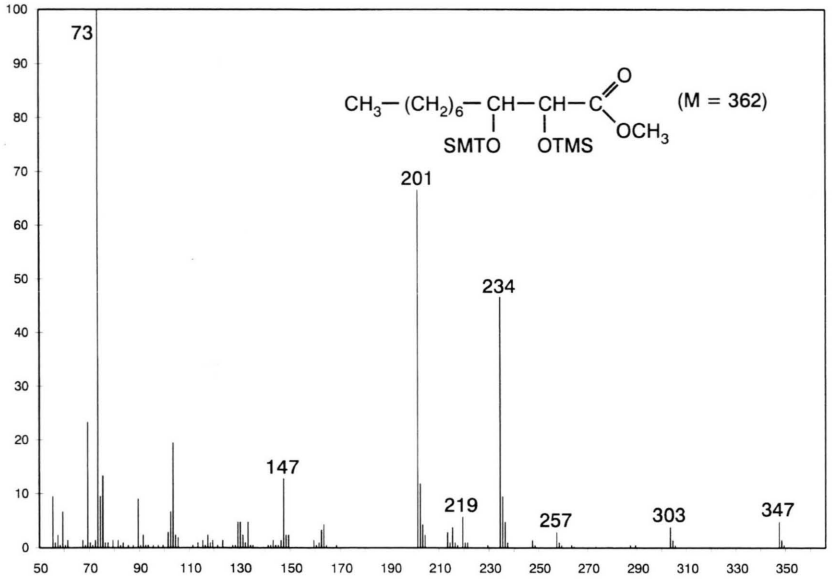


Fig. 4. Mass spectrum of the trimethylsilyl ether of methyl *erythro*-2,3-dihydroxydecanoate derived from the triester waxes of *C.abdimii*.

Table V. Relative GC retention times of *erythro*-2,3-dihydroxy fatty acid methyl esters.

| | Predicted* | Found |
|---|---|---|
| Methyl *e*-2,3-dihydroxydecanoate | 10.00 + 1.15 + 1.40 = 12.55 | 12.55 |
| Methyl *e*-2,3-dihydroxydodecanoate | 12.00 + 1.15 + 1.40 = 14.55 | 14.55 |
| Methyl *e*-2,3-dihydroxyhexadecanoate | 16.00 + 1.15 + 1.40 = 18.55 | 18.55 |

* Predicted from the increment for a 2-OH-substitution (1.15) and a 3-OH-substitution (1.40).

1978b) as illustrated in Table V in which the predicted and the actual relative GC retention times found for *erythro*-2,3-dihydroxy fatty acid methyl esters are compared.

## Discussion

The data presented exhibit great similarities between the five *Ciconia* species investigated, all

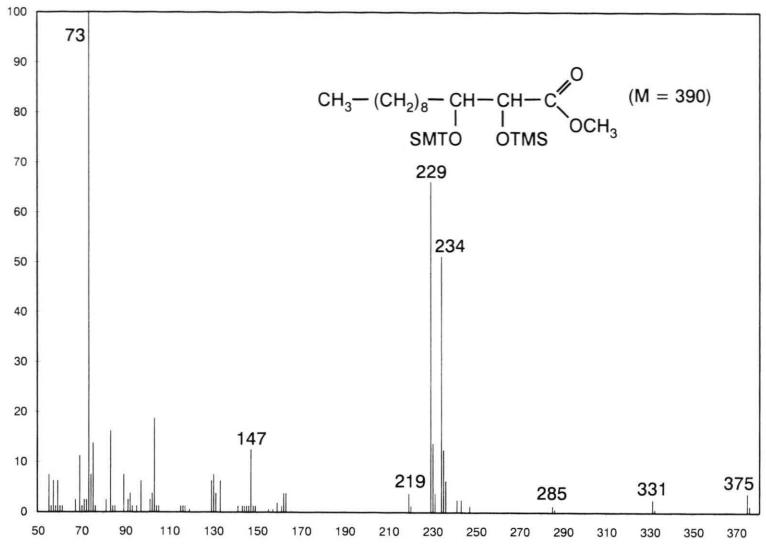


Fig. 5. Mass spectrum of the trimethylsilyl ether of methyl *erythro*-2,3-dihydroxydodecanoate derived from the triester waxes of *C.episcopus*.

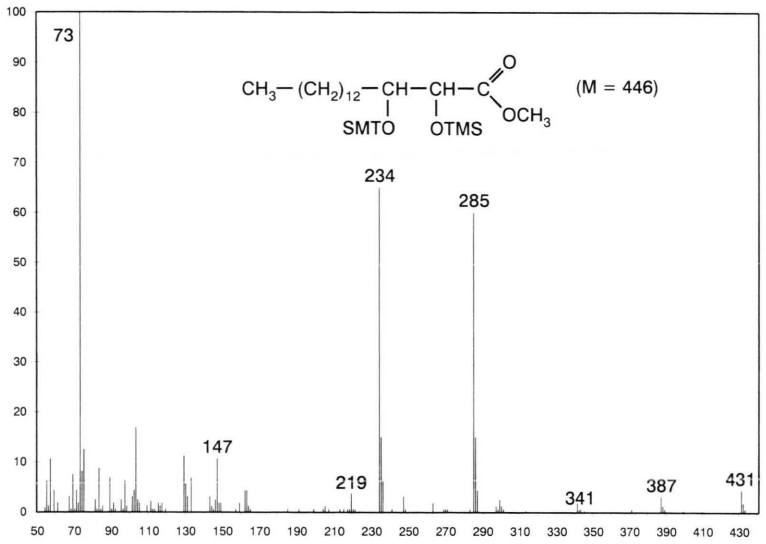


Fig. 6. Mass spectrum of the trimethylsilyl ether of synthetic methyl *erythro*-2,3-dihydroxyhexadecanoate.

possessing uropygial gland lipids which contain mono- and diester waxes as well as triglycerides. Most typical are 2- and 3-hydroxy fatty acids-containing diester waxes and/or *erythro*-2,3-dihydroxy fatty acids-containing triester waxes the biogenesis of which is still unclear. In those species (*C.abdimii* and *C.episcopus*) where high concentrations of the latter were found, 2- and 3-hydroxy acids occur only in minor concentrations. Accordingly, *Ciconia* appears to be a fairly homogenous group of birds with *C.abdimii* and *C.episcopus* more closely related to each other than to *C.nigra*, *C.ciconia* and *C.maguari* as may be deduced from both the composition of the monoester waxes (Table II) and the diester waxes (Table III). This confirms the dendrograms presented by Peters (1931) and Kahl (1979) as well as more recent results based on a multi-dimensional scaling analysis of morphological characters published by Wood (1984).

Although not yet been found in vertebrates, 2,3-dihydroxy fatty acids have previously been detected in plants linked to ceramides and associated with 2-hydroxy fatty acids, e.g. in rice bran (Fujino and Ohnishi, 1976), in bean species such as

*Phaseolus angularis* (Ohnishi and Fujino, 1981), and *P.vulgarus* (Kojima *et al.,* 1991), in fungi such as *Polyporus officinalis* (Cosovic and Prostenik, 1979), *Fomitopsis pinicola* (Asawa and Yoshimoto, 1980) and *Lactarius vellereus* (Cosovic and Prostenik, 1981). They have also been found in bakers' yeast (Hoshi *et al.,*1973) and more recently, linked to lipopolysaccharides and along with 3-hydroxy fatty acids, in a variety of microorganisms from the family *Legionella* (Mayberry, 1981; Sonesson et al, 1989, Moll *et al.,* 1992; Sonesson *et al.,* 1994). The biological significance of these acids is not understood; antimicrobial and fungistatic properties, however, have been reported for 2-hydroxy- and 3-hydroxy fatty acids (Schildknecht and Koob, 1971) and similar, more species-specific interactions may play a role in the protection of the above species against competing organisms. This aspect requires further basical investigations.

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