Furan fatty acid steryl esters from the marine sponge *Dictyonella incisa* which show inflammatory activity

P. Ciminiello[a], E. Fattorusso[a]*, S. Magno[a], A. Mangoni[a], A. Ialenti[b] and M. Di Rosa[b]

[a] *Dipartimento di Chimica delle Sostanze Naturali, Via D. Montesano 49, I-80131 Napoli (Italy), and* [b] *Dipartimento di Farmacologia Sperimentale, Via D. Montesano 49, I-80131 Napoli (Italy)*
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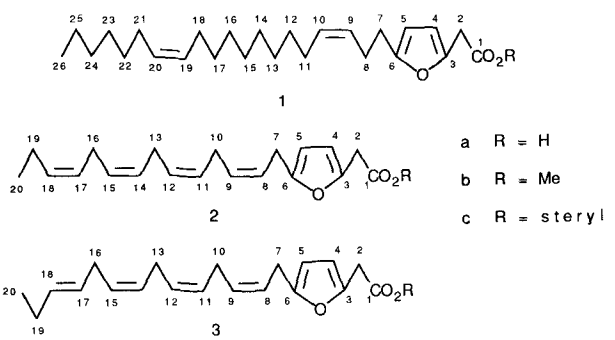
Summary. Three new furan fatty acids, (9*Z*,19*Z*)-3, 6-epoxyhexacosa-3,5,9,19-tetraenoic acid (**1a**), (8*Z*, 11*Z*, 14*Z*, 17*Z*)-3, 6-epoxyeicosa-3,5,8,11,14,17-hexaenoic acid (**2a**), and (8*Z*,11*Z*,14*Z*,17*E*)-3, 6-epoxyeicosa-3,5,8,11,14,17-hexaenoic acid (**3a**), and a new polyunsaturated fatty acid (5*Z*,8*Z*,11*Z*,14*Z*,17*E*)-eicosa-5,8,11,14,17-pentaenoic acid (**6a**), present in the sponge *Dictyonella incisa* as the respective steryl esters, have been isolated as methyl esters and their structures have been determined by spectral and chemical analysis. The furan fatty acid esters have shown a high inflammatory activity, which suggests their potential role as feeding deterrents.
Key words. Dictyonella incisa; furan fatty acids; inflammatory activity, by histamine release.

Several reports have shown that marine sponges are rich sources of $C_{24}-C_{30}$ fatty acids, in contrast to the $C_{14}-C_{22}$ analogs typically found in higher animals. Examples of compounds with straight chain, terminal methyl branched (iso-), or internal methyl branched carbon skeletons have been found among the long chain fatty acids isolated from the sponges *Aplysina fistularis*[1], *Strongylophora durissima*[2], and *Petrosia hebes*[3]. Unusual substituents have been found in the fatty acid chains from some species: cyclopropyl from *Calyx nicaensis*[4], bromo from *Petrosia hebes*[3], methoxy from *Higginsia tethyoides*[5,6], acetoxy from *Polymastia gleneni*[7], and cyclic peroxide from a *Plakortis* sp.[8]. In spite of the numerous interesting structural features of sponge fatty acids, their biological significance, if any, remains undetermined.

During a search for biologically active sponge metabolites, we isolated two cyclopropane-containing fatty acids [*cis*-11,12-methyleneoctadecanoic (lactobacillic) acid and *cis*-9,10-methylenehexadecanoic acid], which have been frequently encountered as constituents of bacterial lipids[9], and three new long-chain furan fatty acids (**1a**, **2a**, and **3a**) from extracts of the Mediterranean sponge *Dictyonella incisa*. Compounds **1a**, **2a**, and **3a** are present in the sponge as the respective steryl esters **1c**, **2c**, and **3c**, and have been isolated as the methyl esters **1b**, **2b**, and **3b**. We recently isolated the incisterols, a new class of highly degraded sterols[10], and (22*E*)-cholesta-4,6,8(14)-tetraen-3-one[11] from the same sponge. Interestingly compounds **1c**–**3c**, as well as compounds **1b**–**3b**, possess a high inflammatory activity, which could enable them to play a role in deterring predators from *D. incisa*.



Specimens of *D. incisa* were collected in November 1988 along the coast of Portofino Promontory and stored frozen. The Et_2O soluble extract from the lyophilized material was chromatographed on a MPLC silica gel column and fractions containing fatty acid steryl esters, as judged by ^{1}H NMR spectroscopy, were subjected to methanolysis by refluxing in MeOH/MeONa. The resulting methyl esters were purified by repeated reversed-phase HPLC, to give pure **1b**, **2b**, and **3b**, and a sterol mixture. GC-MS analysis of the sterol mixture showed it to contain cholesta-5,7,22-trien-3β-ol, 24-methylcholesta-5,7,22-trien-3β-ol and 24-ethylcholesta-5,7,22-trien-3β-ol. These compounds were recently shown to be the major components of the free sterol fraction from *D. incisa*[10].

Data from HRMS established a molecular formula of $C_{27}H_{44}O_3$ for compound **1b**. The presence of a long unbranched aliphatic hydrocarbon chain was deduced from a large signal at δ 1.26 and a distorted methyl signal at δ 0.90 in the ^{1}H NMR spectrum of **1b**. Additional resonances in the ^{1}H NMR spectrum suggested the presence of two isolated double bonds [δ 5.44 (1H, dt, $J = 11.5$ and 6.5 Hz), δ 5.40 (1H, dt, $J = 11.5$ and 6.5 Hz), and δ 5.52 (2H, t, $J = 6.5$ Hz)], a 2,5 disubstituted furan ring [δ 6.04 (1H, d, $J = 3$ Hz) and δ 5.86 (1H, d, $J = 3$ Hz)] and a carbomethoxy group (δ 3.25, 3H, s), which accounted for all the formal unsaturations implied by the molecular formula. The ^{13}C NMR signals corresponding to the above functionalities were observed at δ 155.6, 145.8, 108.5, 105.9 (furan carbons), δ 131.0, 129.9 (2C), 128.2 (olefinic carbons), and δ 170.0, 51.4 (carbo methoxy group).

All the ^{1}H–^{1}H couplings needed to support the part structure from C-2 to C-11, including the long-range interaction between the β-furan hydrogens and H_2-2 and H_2-7 were evident in the COSY spectrum, and double resonance experiments were used to verify all the scalar

couplings. With the furan ring and one double bond located in the fatty acid chain, we next turned our attention to location of the second disubstituted double bond. We assumed that it must link C-19 and C-20, since permanganate-periodate oxidation of **1b** afforded *n*-heptanoic acid, which was identified by GLC-MS analysis. ^{1}H NMR data provided evidence for the stereochemistry of the C-9/C-10 double bond. A scalar coupling of 11.5 Hz between H-9 and H-10 indicated that the double bond had the *Z* configuration. Proton NMR data were not useful for assigning the configuration of the C-19/C-20 double bond, since the resonances of the relevant olefinic protons were coincidental. Thus, the olefinic geometry was determined on the basis of the ^{13}C chemicals shifts of C-18 and C-21 (δ 27.7 for both carbons; the assignment was based on a selective decoupling experiment), which were appropriate for allylic methylene groups of a *Z* double bond internally located in an unbranched chain (the expected value for the *E* isomer is about 5 ppm downfield) [12]. The mass spectrum (see Experimental), whose base peak at *m/z* 153 results from the preferred cleavage between C-7 and C-8, agreed with the proposed structure of methyl (9*Z*,19*Z*)-3,6-epoxyhexacosa-3,5,9,19-tetraenoate **1b**.

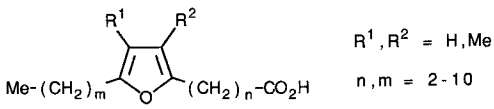
Compound **2b** was analyzed as $C_{21}H_{28}O_3$ by HRMS, which indicated eight degrees of unsaturation. Spectral similarities between **2b** and **1b** (see table) revealed that the part structures from C-1 to C-6 were identical. An eight-proton multiplet centred at δ 5.41, and a six-proton multiplet at δ 2.79 in the ^{1}H NMR spectrum of **2b** indicated the presence of four non-conjugated double bonds and three double allylic methylenes, respectively.

Further analysis of the ^{1}H and ^{13}C NMR spectra showed that the above polyunsaturated eleven-carbon chain in structure **2b** was situated between an ethyl group (δ_H 2.00 and 0.90; δ_C 20.6 and 14.2) and a methylene group (δ_H 3.28 and δ_C 26.4), which was in turn linked to one of the α-carbon atoms of the furan (i.e. C-6). The *Z* nature of all olefinic double bonds was indicated by the chemical shifts observed in the ^{13}C NMR spectrum for the signals assigned to the allylic methylenes (see table). The structure of **2b** [methyl (8*Z*,11*Z*,14*Z*,17*Z*)-3,6-epoxyeicosa-3,5,8,11,14,17-hexaenoate] was further substantiated by extensive ^{1}H–^{1}H decoupling experiments, which demonstrated the expected long-range couplings between the β-furan protons and H_2-2 and H_2-7.

The NMR spectral data of compound **3b** were very similar to those of **2b**, and the minor differences were attributable to a change in the stereochemistry of the C-17 double bond. The mass spectra were identical. In the ^{1}H NMR spectrum, the chemicals shifts of the protons of the terminal ethyl group were slightly shifted, and a significant modification in the shape of the complex multiplets due to the olefinic and doubly allylic protons (δ 5.35–5.55 and δ 2.72–2.88, respectively) was also observed. The features of the ^{13}C NMR spectrum of **3b** were also analogous to that of **2b**, except that the chemical shifts of the C-16 and C-19 methylene carbons were shifted considerably downfield (see table), in accordance with the change in the stereochemistry of the C-17 double bond from *Z* to *E*. Thus, compound **3b** was formulated as methyl (8*Z*,11*Z*,14*Z*,17*E*)-3,6-epoxyeicosa-3,5,8,11,14,17-hexaenoate.

Furanoid fatty acids have been detected in small quantities in a variety of animals, particularly in fishes, and, to a lesser extent, in plant sources. All the members of this family can be represented by the general formula **4**, where *m* and *n* can vary from 2 to 10 and where R^1 and R^2 are either hydrogen atoms or methyl groups. Their biogenesis has received very little attention, while their possible biological role is completely obscure.

Structurally, compounds **1a–3a** are quite different from furanoid acids **4**. A plausible biosynthetic scheme, starting from the appropriate polyunsaturated fatty acids,



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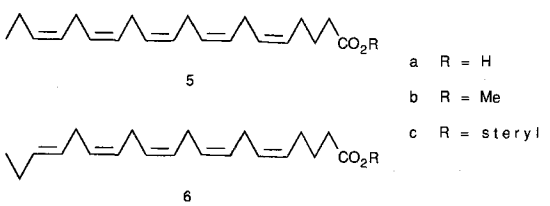
may be hypothesized. As depicted in figure 1, compound **2a** could be generated from (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-eicosa-5,8,11,14,17-pentaenoic acid, a biologically important metabolite of several marine animals, through a process similar to that occurring in prostaglandin biosynthesis. The initial step involves a lipooxygenase-type reaction, followed by ring closure and oxidation.

Support for this hypothesis came from a further examination of the fatty acid steryl ester fraction from *D. incisa*, which forms, together with the free sterol fraction, by far the most abundant portion of the lipid extract. After methanolysis and reversed-phase HPLC of the reaction mixture, small quantities of methyl (5*Z*, 8*Z*,11*Z*, 14*Z*, 17*Z*)-eicosa-5,8,11,14,17-pentaenoate **5b** were iso-



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lated and identified by comparison of its spectral and chromatographic properties with those of an authentic sample.

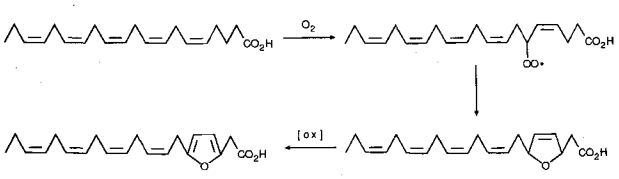

Figure 1. Proposed biosynthesis of the furan fatty acid **2a**.

Interestingly, comparable quantities of methyl (5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*E*)-eicosa-5,8,11,14,17-pentaenoate **6b**, which has not been previously reported as a naturally occurring or synthetic product, were also obtained. Its structure was assigned on the basis of the mass spectrum, which was identical with that of **5b**, and the ^{13}CNMR spectrum, whose resonances were in very good agreement with those calculated by Bus, Sies, and Lie Ken Jie [13, 14], using the fatty acid additivity rules.

The presence in the sponge *D. incisa* of appreciable quantities of **1c**, **2c** and **3c** stimulated an investigation to ascertain whether they have a biological function. The assays were performed on the methyl esters **1b**, **2b** and **3b**, and on a mixture of the three steryl esters, since we were not able to obtain pure **1c**, **2c** and **3c**. Injection of **1b** into the rat paw induced an edema with a peak response occurring 1 h after the injection (fig. 2a). Similar inflammatory profiles were exhibited by **2b**, **3b**, and the mixture of the steryl esters **1**–**3c**.

Information on the mechanism of the above inflammatory activity was obtained from in vivo and in vitro experiments (see 'materials and methods'). Injection of **1b** into rats pre-treated with mepyramine and methysergide resulted in a greatly decreased edema (fig. 2a), suggesting that the inflammatory response is brought about by release of histamine and 5-hydroxytryptamine. This was confirmed by the fact that **1b** induced a concentration-related release of histamine from rat peritoneal cells (fig. 2b). When the experiments were carried out in the presence of phosphatidylserine (50 µg/ml), the observed histamine release was increased by about three times at each tested concentration of **1b** (6%, 21%, and 62%, respectively, compared to 2%, 8%, and 26%). The ability to release histamine from rat peritoneal cells was also exhibited by compound **2b**, and so was the enhanced release observed in the presence of phosphatidylserine (fig. 2b). In our experiments the histamine release induced by 5 µg/ml concanavalin A was 13% and 23%, respectively, in the absence or in the presence of phosphatidylserine.

These experiments suggest that **1b** and **2b** are able to induce the release of histamine by a mechanism which is potentiated by phosphatidylserine and possibly depends on extracellular calcium. It is conceivable that the biological activity of compound **3b**, closely related to **2b**, and of the steryl esters **1c**–**3c**, involves the same mechanism. The biological activity exhibited by furan fatty esters from *D. incisa* points to a potential role of these compounds as natural feeding deterrents. The similarity of biological response of all the furan fatty esters assayed suggests that the part of the structure involved belongs to the acyl moiety, particularly to the furanacetyl residue.

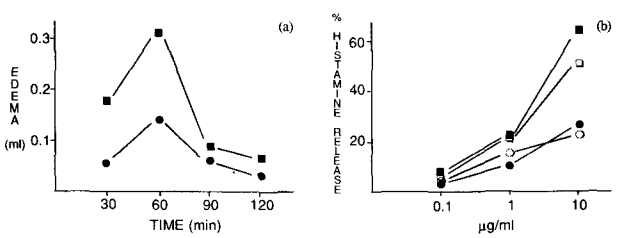

Figure 2. *a* Rat paw edema induced by 10 µg of **1b** (■) and its inhibition by mepyramine (2.5 mg/kg) and methysergide (3 mg/kg) (●). Each point represents the mean of the values from at least 5 rats. *b* Effect of various concentrations of **1b** and **2b** on the histamine release from rat peritoneal cells in the absence (■ and □ respectively) or in the presence (● and ○, respectively) of phospatidylerine (50 µg/ml). Each point is an average of two duplicate experiments.

Materials and methods

General methods. EIMSs were obtained at 70 eV on a Kratos MS50 mass spectrometer. FT-IR spectra were recorded on a Bruker IFS-48 spectrophotometer in $CHCl_3$ solution. ^{1}HNMR spectra were determined on a Bruker AC-400 spectrometer in C_6D_6 solution. Proton chemical shifts were referenced to the residual benzene signal (δ 7.15). ^{13}CNMR spectra were recorded on a Bruker AC-400 spectrometer in $CDCl_3$. The chemical shifts were referenced to the center peak of $CDCl_3$ at 77.0 ppm. The multiplicities of ^{13}C resonances were determined by DEPT experiments which were performed using polarization transfer pulses of 90° and 135°, obtaining in the first case only signals for −CH groups and in the other case positive signals for −CH and $-CH_3$ and negative ones for $-CH_2$ groups. Polarization transfer delays were adjusted to an average C−H coupling of 135 Hz.

Medium pressure liquid chromatography (MPLC) was performed on a Büchi 861 apparatus using a SiO_2 (230–400 mesh) column. High performance liquid chromatographies (HPLC) were performed on a Varian 2510 apparatus equipped with an RI-3 refractive index detector, using Hibar columns.

Combined GLC-MS analysis was performed on a Hewlett-Packard 5890 gas chromatograph with a mass selective detector MSD HP 5970 MS and a split/splitness injector for capillary columns, using a fused-silica column, 25 m × 0.20 mm HP-5 (cross-linked 25% Ph Me silicone, 0.33 µm film thickness).

Collection and extraction. Specimens of *D. incisa* were collected from the rocky areas of the Portofino Promontory (Eastern Liguria Coast) at around 10 meters' depth, during November 1988. They were frozen when still alive at −18 °C and then dispatched to the laboratory. Reference specimens are deposited at the Istituto di Zoologia dell'Università di Genova. Freshly collected animals (60 g, dry weight after extraction) were homogenized, lyophilized, and extracted with Et_2O (5 × 500 ml). Evaporation of the combined Et_2O extracts afforded 2.2 g of an oily residue, which was chromatographed by MPLC on an SiO_2 column using a solvent gradient system from petroleum ether to Et_2O. The fractions eluted with petroleum ether/Et_2O 9:1 afforded a mixture (410 mg) containing furan fatty acids steryl esters, as shown by ^{1}HNMR analysis.

Methanolysis of steryl esters. A portion of the above mixture (280 mg) was refluxed with 0.5 M NaOMe (5 ml) in MeOH (10 ml) for 15 min. H_2O was added and the reaction mixture extracted with petroleum ether (6 × 20 ml). The extract was washed with H_2O (2 × 20 ml) and dried on Na_2SO_4. Evaporation of the solvent furnished a mixture of fatty acid methyl esters and free sterols (273 mg). The crude reaction mixture was subjected to preparative reversed-phase HPLC on a RP-18 column (250 × 10 mm) with methanol/tetrahydrofuran 9:1 as the mobile phase, thus obtaining, in decreasing order of polarity, two fractions containing furanoid compounds (fractions A and B), a free sterol fraction (fraction C), and a fatty acid methyl ester fraction (fraction D).

Isolation of ***1b.*** Fraction B (41 mg) was further purified by HPLC using a reversed-phase Hibar RP-18 column (4 × 250 mm) with a mobile phase of methanol/H_2O 95:5, thus obtaining 13 mg of pure **1b**: IR: ν_{max} 1738, 1603 cm^{-1}; EIMS: *m/z* 416.3266 (M^+, 9%; $C_{27}H_{44}O_3$ requires 416.3279), 357 (M^+-$COOCH_3$, 3%), 153 (100), 111 (23); ^1H and ^{13}CNMR data are reported in the table.

Oxidation of ***1b.*** To **1b** (1 mg) in *tert*-butyl alcohol (2 ml), K_2CO_3 0.04 M (0.3 ml) and an aqueous solution (1.8 ml) with 0.023 M $KMnO_4$ and 0.09 M $NaIO_4$ were added. The reaction was allowed to proceed at 37 °C for 18 h. After acidification with H_2SO_4 5 N, the solution was decolorized with aqueous $NaHSO_3$ 1 M and extracted with Et_2O (4 ml in 2 portions). After drying over $CaSO_4$, the combined ethereal extracts were concentrated to 0.5 ml. The resulting solution analyzed by GLC-MS, was found to contain *n*-heptanoic acid.

Isolation of ***2b, 3b, 5b,*** *and* ***6b.*** Further purification of the more polar fraction A (52 mg) was achieved by HPLC on a RP-18 column (4 × 250 mm) with methanol/H_2O 8:2 as the mobile phase to separate the furans **2b** (2.5 mg) and **3b** (3.5 mg) and the fatty acid methyl esters **5b** (0.8 mg) and **6b** (2.0 mg).

2b: IR: ν_{max} 1738, 1601 cm^{-1}; EIMS: *m/z* 328.2057 (M^+, 10%, $C_{21}H_{28}O_3$ requires 328.2031), 269 (M^+-$COOCH_3$, 4%), 259 (10), 255 (7), 193 (36), 192 (36), 153 (38), 124 (68), 118 (100), 79 (99); ^1H and ^{13}CNMR data are reported in the table.

3b: IR: ν_{max} 1738, 1600 cm^{-1}; EIMS: *m/z* 328.2044 (M^+, 9%; $C_{27}H_{44}O_3$ requires 328.2031), 269 (M^+-$COOCH_3$, 4%), 259 (10), 255 (7), 193 (34), 192 (35), 153 (38), 124 (65), 118 (100), 79 (97); ^1H and ^{13}CNMR data are reported in the table.

5b was identified by comparison of its spectral and chromatographic data with those of an authentic sample.

6b: EIMS: *m/z* 316 (M^+, 0.5%), 287 (2), 247 (2), 205 (6), 201 (9), 161 (10), 133 (23), 118 (39), 105 (37), 93 (49), 91 (74), 79 (100); ^1H and ^{13}CNMR data are reported in the table.

Isolation of cyclopropane fatty acid methyl esters. Fraction D (37 mg) was chromatographed on a Hibar RP-18 column (4 × 250 mm) using MeOH as eluent. Methyl *cis*-11,12-methyleneoctadecanoate (3.2 mg) and methyl *cis*-9,10-methylenehexadecanoate (2.5 mg) were isolated, and identified by comparison of their spectral (^1HNMR

NMR spectral data of compounds **1b**, **2b**, **3b**, and **6b**[a]

pos	**1b** δ_H (mult., *J* in Hz)	δ_C	**2b** δ_H (mult., *J* in Hz)	δ_C	**3b** δ_H (mult., *J* in Hz)	δ_C	**6b** δ_H (mult., *J* in Hz)	δ_C
1		170.0		169.9		169.9		174.2
2	3.39 (s)	34.1	3.37 (s)	34.0	3.37 (s)	34.0	2.10 (t, 7.5)	33.4
3		155.6		155.7		155.7	1.60 (quintet, 7.5)	24.8
4	6.04 (d, 3)	108.5	6.02 (d, 3)	108.6	6.02 (d, 3)	108.6	1.97[b]	26.5
5	5.86 (d, 3)	105.9	5.87 (d, 3)	106.0	5.87 (d, 3)	106.0	5.35–5.55[b]	128.8[d]
6		145.8		146.1		146.1	5.35–5.55[b]	128.9[d]
7	2.54 (t, 7.5)	28.5	3.28 (d, 6.5)	26.4	3.28 (d, 6.5)	26.4	2.75–2.90[b]	25.6
8	2.34 (dt, 6.5, 7.5)	26.3	5.35–5.55[b]	124.9	5.35–5.55[b]	124.9	5.35–5.55[b]	127.9–128.3
9	5.40 (dt, 11.5, 6.5)	128.2[c]	5.35–5.55[b]	130.1	5.35–5.55[b]	130.9	5.35–5.55[b]	127.9–128.3
10	5.44 (dt, 11.5, 6.5)	131.0[c]	2.72–2.85[b]	25.7	2.69–2.82[b]	25.7	2.75–2.90[b]	25.6
11	1.98 (dt, 6.5, 7.5)	27.7	5.35–5.55[b]	128.0–128.7	5.35–5.55[b]	127.8–128.7	5.35–5.55[b]	127.9–128.3
12	1.30[b]	29.3–30.2	5.35–5.55[b]	128.0–128.7	5.35–5.55[b]	127.8–128.7	5.35–5.55[b]	127.9–128.3
13	1.30[b]	29.3–30.2	2.72–2.85[b]	25.7	2.69–2.82[b]	25.7	2.75–2.90[b]	25.6
14	1.30[b]	29.3–30.2	5.35–5.55[b]	128.0–128.7	5.35–5.55[b]	127.8–128.7	5.35–5.55[b]	127.9–128.3
15	1.30[b]	29.4–30.2	5.35–5.55[b]	128.0–128.7	5.35–5.55[b]	127.8–128.7	5.35–5.55[b]	127.9–128.3
16	1.30[b]	29.3–30.2	2.72–2.85[b]	25.7	2.69–2.82[b]	30.4	2.75–2.90[b]	30.4
17	1.30[b]	29.3–30.2	5.35–5.55[b]	127.1	5.35–5.55[b]	127.8–128.7	5.35–5.55[b]	127.9–128.3
18	2.10[b]	27.7	5.35–5.55[b]	132.1	5.35–5.55[b]	132.6	5.35–5.55[b]	132.4
19	5.52 (t, 6.5)	129.9	2.00 (dq, 6.5, 7.5)	20.6	1.95 (dq, 6.5, 7.5)	25.6	1.97[b]	25.5
20	5.52 (t, 6.5)	129.9	0.90 (t, 7.5)	14.2	0.92 (t, 7.5)	13.8	0.92 (t, 7.5)	13.8
21	2.10[b]	27.7						
22	1.30[b]	29.3–30.2						
23	1.30[b]	29.3–30.2						
24	1.30[b]	32.1						
25	1.30[b]	23.0						
26	0.90 (t, 7.5)	14.2						
OMe	3.25 (s)	51.4	3.24 (s)	52.1	3.24 (s)	52.1	3.34 (s)	51.5

[a] ^1H assignment based on spin-spin decoupling experiments; ^{13}C assignment based on DEPT and selective decoupling experiments, and comparison with known compounds[11–13]. [b] Submerged by other signals. [c–d] The resonances with the same superscript may be reversed.

and EIMS) and gas-chromatographic properties with those of authentic samples.

Identification of the sterol mixture. The identification of the free sterols obtained from the methanolysis (fraction C, 135 mg) was based upon their GLC-Rt and comparison of their GLC-MS spectra with those of authentic specimens. The quantitation of the sterols was performed by a programmable integrator using 5α-cholestane as an internal standard: (22*E*)-cholesta-5,7,22-trien-3β-ol (42 mg), (22*E*,24ξ)-24-methylcholesta-5,7,22-trien-3β-ol (28 mg), (22*E*,24ξ)-24-ethylcholesta-5,7,22-trien-3β-ol (62 mg).

Purification of ***1c****,* ***2c****, and* ***3c*** *mixture.* The remaining part (130 mg) of the crude fatty acid steryl ester mixture, obtained as described above, was chromatographed by HPLC on a SiO_2 column (10 × 250 mm). Elution with *n*-hexane/EtOAc 95:5 afforded a fraction (32 mg), exclusively composed of furan fatty acid steryl esters **1c**, **2c**, and **3c**, as judged by ^{1}H NMR data, which was used for the biological assays.

Rat paw edema: Edema of the hind paw of male Wistar rats (140–160 g) was produced by injecting 0.1 ml of saline containing 100 μg of **1b**, **2b**, **3b**, and a mixture of the steryl esters **1c**, **2c**, and **3c**. Before the injection the solutions were sonicated in order to obtain homogeneous dispersion of the compounds under investigation. Groups of at least five rats were used and the volume of the paw was determined by a plethismometer immediately after the injection, as previously described[15]. Subsequent readings on the same paw were carried out every 30 min for 2 h and compared to the initial one. In some experiments **1b** was injected in rats pre-treated with mepyramine (2.5 mg/kg) and methysergide (3 mg/kg) both given intraperitoneally 30 min before the paw injection.

Release of histamine. Mixed peritoneal cells (about 5% mast cells) were recovered from male Wistar rats (200–250 g) as previously described[16]. Cells were washed and suspended in Tyrode solution (pH 7.2) having the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 12, NaH_2PO_4 0.4, and glucose 5.6. Aliquots of cell suspension (to a final volume of 1 ml) were allowed to equilibrate at 37 °C in a metabolic shaker with gentle agitation, and histamine release initiated by the addition of 0.1–1–10 μg/ml of **1b** or **2b**, or 5 μg/ml of concanavalin A (final concentration). Some experiments were carried out in the presence of phosphatidylserine (50 μg/ml). The release was terminated after 10 min by addition of 2 ml ice-cold Tyrode solution. Cells and supernatants were recovered by centrifugation (10 min, 150 g, 4 °C), and histamine concentrations in solution and cells were quantified fluorometrically[17]. Histamine release was calculated as a percentage of the total cellular content of the amine. All values were corrected for spontaneous release occurring in the absence of the inducer (about 5%).

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