Marine Sterols. XXII.[1] Occurrence of 3-Oxo-4,6,8(14)-triunsaturated Steroids in the Sponge *Dysidea herbacea*

Masaru KOBAYASHI,*,[a] Madala M. KRISHNA,[b] Keisuke ISHIDA[a] and Vallurupalli ANJANEYULU*,[b]

Faculty of Pharmaceutical Sciences, Hokkaido University,[a] Kita-ku, Sapporo 060, Japan and School of Chemistry, Andhra University,[b] Visakhapatnam 530 003, India. *Received July 29, 1991*

Ten 3-oxo-4,6,8(14)-triunsaturated steroids with cholestane (1,2), ergostane (3,4,5,7) and stigmastane skeletons (6,8,9,10), were isolated from a sponge, *Dysidea herbacea*, collected off the coast of the Lakshadweep Islands, Indian Ocean. Of these, 7 to 10 were obtained as C-24 epimeric mixtures. Compound 2 had previously been reported from a sponge, *Dictyonella insica*. The hydroperoxide 10 was shown to be an artefact formed from the 24-ethylidene derivative 6 during storage. The structures of 1 to 10 were derived from spectroscopic evidence.

Keywords sponge; *Dysidea herbacea*; 3-oxo-4,6,8(14)-triunsaturated steroid; hydroperoxy steroid

Marine invertebrates contain a variety of sterols which have attracted interest from comparative biochemical and phylogenetic points of view.[2] The earlier hypothesis that correlated the diversity of sterol composition of invertebrates with their evolutionary level[3] is now regarded as incorrect, since, for example, many lower animals such as gastropods (mollusk) and crustaceans (arthropod) were shown to contain only cholesterol, as do vertebrates.[4] On the other hand, it is still not known why many marine invertebrates contain biosynthetically unusual sterols, unlike the evolutionarily higher vertebrates.

Examination of the lipid extract of a marine sponge *Dysidea herbacea*, collected off the coast of the Lakshadweep Islands, Indian Ocean, resulted in the isolation of a mixture of steroids which showed lower polarity than the ubiquitous 3β-hydroxy-Δ^5-sterol. High-performance liquid chromatography (HPLC) of the mixture on a reversed-phase system showed that the mixture was composed of at least nine components (**1**—**9**). The same polarity of the components of the mixture, which gave only one spot on silica gel thin-layer chromatography (TLC), indicated them to bear a common steroid nucleus. When the TLC plate was viewed under a 380 nm ultraviolet (UV) lamp, it showed bright fluorescence. The mixture was found, on storage, to give a very small amount of a decomposition product **10**, which shows similar fluorescence. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of the mixture showed a rather simple pattern (δ 0.96 (18-H_3), 1.00 (19-H_3), 5.73 (1H, s, 4-H), 6.03 (1H, d, J=9.5 Hz, 6-H), 6.62 (1H, d, J=9.5 Hz, 7-H)). The carbon-13 NMR (^{13}C-NMR) spectrum showed the signals of C-18 (δ 18.8) and C-19 (16.7), besides those of one keto (δ 199.3) and six olefinic carbons (δ 123.0, 124.5, 134.0, each d, and 124.5, 156.1, 164.2, each s). The mass spectrum (MS) showed an intense fragment ion at m/z 267, which was derived from side chain cleavage. These spectral data, together with the UV absorption (348 nm) indicated the compounds to be the second example of steroids, isolated from a marine source, having a conjugated 3-oxo-4,6,8(14)-triunsaturated moiety. An ergostane derivative **5**, having this steroid nucleus, has been synthesized by Barton and Bruun from 14-dehydroergosterol,[5a] and was later found in fungi.[5b-d] Natural occurrence of such derivatives, other than in fungi, has not been known until the recent discovery, by Ciminiello *et al.*, of cholesta-4,6,8(14),22-tetraen-3-one (**2**), in the sponge *Dictyonella insica* of the Mediterranean Sea.[6] The ^{1}H- and the ^{13}C-NMR signals of the steroid nucleus, reported by Ciminiello *et al.*, and the UV data by Barton and Bruun were identical with those of our sample isolated from *D. herbacea*.

The trienone mixture was subjected to preparative reversed-phase HPLC eluting with 90% MeOH. The elution sequence of sterols is known to be related to the side chain structure, if they bear a common steroid nucleus. The formula numbers in this report, except for the predominant component **1** (50%), are arranged in the order of their elution, which was in accord with the literature.[7] The hydroperoxide **10** was separated from other compounds by silica gel column chromatography.

Compound **1**, which eluted next to compound **5**, was a trienone having a cholestane-type side chain. The ^{13}C-NMR data (Experimental) for the side-chain were identical

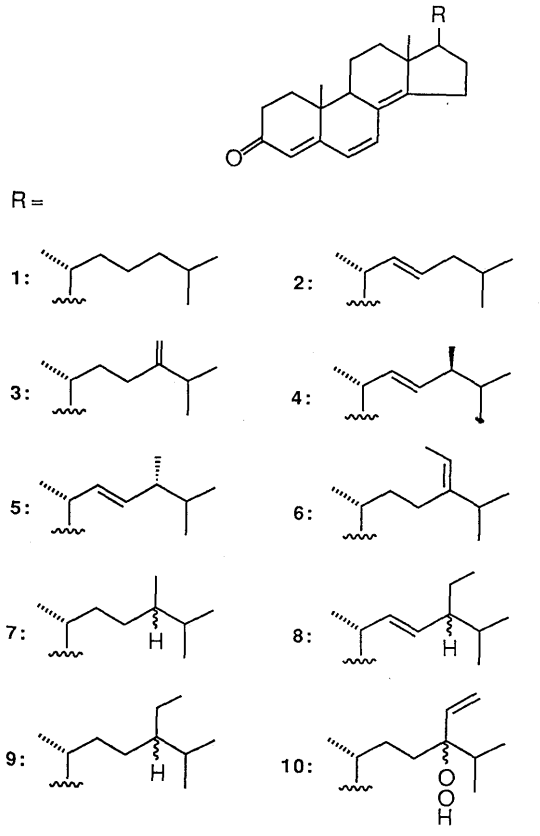

Chart 1

with those of cholestane.[8] The ^{1}H-NMR chemical shifts (21-H_3, δ 0.964, 26, 27-H_3, 0.874, 0.870) were also consistent with the literature values,[9] with minor deviations. The trienone moiety in **1** to **9** was found to exert a small but constant deshielding effect on 21-H_3 (+0.055 ppm) and 26-, 27- and 28-H_3 (+0.015 ppm). Addition of these paramagnetic effects to the literature values reported for the side chain chemical shifts of Δ^5-sterols[9] resulted in a good agreement with the values found in **1** to **9**.

Compound **2**, cholesta-4,6,8(14),22-tetraen-3-one, showed identical ^{1}H-NMR chemical shifts with those reported, *e.g.* three secondary methyl signals at δ 0.876 (6H, d, J=7.0 Hz, 26, 27-H_3) and 1.057 (3H, d, J=7.0 Hz, 21-H_3), and a *trans*-disubstituted C-22 double bond (δ 5.25, dd, J=15.0, 8.5 Hz, 5.34, dt, J=15.0, 6.5 Hz).[6]

Compound **3** was a trienone having a 24-methylenecholestane-type side chain. The terminal methylene proton signals appeared at δ 4.74 and 4.67 as broad singlets. The signals of 21-H_3 was seen at δ 1.001 (d, J=7.0 Hz) while those of the terminal isopropyl group, influenced by the slight obstruction of free rotation about the C-24, 25-bond, appeared at δ 1.031 and 1.037, each as a doublet (J=7.0 Hz). The MS showed the ion at m/z 308 derived by McLafferty-type cleavage at C-22 and C-23 with 1H transfer, a characteristic of Δ^{24} and $\Delta^{24(28)}$ steroids.[10]

Compounds **4** and **5** were C-24 diastereomers of ergosta-4,6,8(14),22-tetraen-3-one. Compound **5** was contaminated with the predominant component **1** as a persistent impurity. Reversed-phase HPLC separated the 24*R*- and 24*S*-24-methyl-Δ^{22}-sterols, the 24*S*-isomer eluting before the 24*R*-isomer.[7] Both compounds showed the signals of a *trans*-disubstituted double bond at C-22 (δ 5.20 and 5.25, each dd, J=15.0, 7.5 Hz) and four secondary methyl groups (**4**, δ 0.838 (26-H_3), 0.852 (27-H_3), 0.927 (28-H_3), 1.051 (21-H_3); **5**, δ 0.833 (26-H_3), 0.849 (27-H_3), 0.935 (28-H_3), 1.060 (21-H_3)). The configuration at C-24 of 24-isomeric sterols is distinguishable from the difference of the side-chain methyl signals. In $\Delta^{5,22}$-C_{28} sterol, the $\Delta\delta$'s observed between 24*S*-isomer (24-epibrassicasterol) and 24*R*-isomer (brassicasterol), are −0.002 (21-H_3), +0.001 (26- and 27-H_3) and −0.002 (28-H_3),[9] while those observed between **4** and **5** were −0.009 (21-H_3), +0.005 and +0.003 (26- and 27-H_3) and −0.008 ppm (28-H_3), respectively. The differences are more pronounced in the present case, measured at 400 MHz, than those of the original report based on 220 MHz spectra.[9] The elution pattern in reversed-phase HPLC, and the discrepancies found in the ^{1}H-NMR, indicate clearly that **4** is the 24*S*-isomer and **5** is the 24*R*-isomer.

Compound **6** was obtained at first as a single compound but later it was contaminated with **10**. It showed a secondary vinyl methyl signal at δ 1.58 (d, J=7.0 Hz) and an olefinic proton quartet (δ 5.23, J=7.0 Hz), indicating it to be a 24-ethylidene derivative. The absence of the septet signal due to 25-H of isofucosterol (24(28)*Z*)[11] indicated that the geometry at C-24(28) is the fucosterol-type (24(28)*E*). Weak ions due to the McLafferty-type cleavage (m/z 308, 293 (308-Me)) were observed in the MS.[10]

Compound **7** was a 24-epimeric mixture of a 24-methylcholestane derivative. Generally, 24-methyl- and 24-ethylcholestane isomers are not resolved by gas chromtography or HPLC. The ^{1}H-NMR spectrum (Experimental) showed the composite signals of both isomers, of which the 24*R*-isomer predominated (60%), as judged from the intensities of the slightly shielded 21-H_3 (δ 0.966) and 27-H_3 (0.861), over the 24S-isomer (21-H_3, δ, 0.972, 27-H_3, 0.863).[9]

Compound **8** was a 24-ethyl-Δ^{22}-derivative and showed an apparently homogeneous ^{1}H-NMR spectrum, *e.g.* 22, 23-H (δ 5.09 and 5.21, each dd, J=15.0, 8.5 Hz), one primary methyl (δ 0.816, t, J=7.0 Hz) and three secondary methyl groups (δ 0.817, 0.865 and 1.070, each 3H, d, J=7.0 Hz). However, occurrence of a trace amount of 24-isomer was suggested by a small shoulder associated with the signals at δ 5.21 and 1.070 (3H, d, J=7.0 Hz, 21-H_3). The major difference of the 29-methyl signal ($\Delta\delta$ 0.009)[9] known to appear between Δ^5-sterols, stigmasterol (24*S*) and poriferasterol (24*R*), was not observed. The long-range effect of the trienone moiety on this primary methyl is not clear, so that the stereochemistry at C-24 remains uncertain. In contrast, the ^{1}H-NMR spectrum of the last eluate **9**, having a saturated 24-ethylcholestane-type side chain, showed the signals of the two C-24 epimers with equal intensities, at δ 0.820 (26-H_3), 0.850 (27-H_3) and 0.975 (21-H_3) corresponding to the 24*S*-isomer, and at δ 0.820 (26-H_3), 0.840 (27-H_3), and 0.980 (21-H_3) corresponding to the 24*R* isomer.[9] A single primary methyl signal was observed at δ 0.855 (3H, d, J=7.0 Hz). The $\Delta\delta$'s reported, in the Δ^5-steroid series (sitosterol (24*R*) and clionasterol (24*S*)) were 0.00 (26-H_3), +0.006 (27-H_3) and −0.002 ppm (21-H_3),[9] while the observed values were 0.00 (26-H_3), +0.010 (27-H_3) and −0.005 (21-H_3) ppm, respectively.

Compound **10** was an equal mixture of the two 24-epimeric 24-vinyl-24-hydroperoxides. The high-resolution MS showed the molecular formula $C_{29}H_{42}O_3$. The MS showed a series of ions at m/z 422 (M^+-O), 420 ($-H_2O$), 405 ($-OOH$), and 404 ($-HOOH$). It showed the ^{1}H-NMR signals, besides those of the trienone moiety, due to a quaternary vinyl group (δ 5.16, br d, J=17.5 Hz; 5.29, dt, J=11.5, 1.5 Hz; 5.75, ddd, J=17.5, 11.5, 1.5 Hz) and 21-H_3 (1.010, d, J=7.0 Hz), but exhibited four secondary methyl signals (total 6H, δ 0.868, 0.875, 0.890 and 0.892). Two D_2O-exchangeable deshielded hydroperoxide singals, total intensity 1H, were observed at δ 7.02 and 7.03. The side chain of **6** is significantly unstable, as in the case of fucosterol. When freshly harvested and dried brown alga *Ascophyllum nodosum* was exposed to air for 4 weeks, its predominant sterol fucosterol was converted, by aerial oxidation, to 24-ketocholesterol and 24ξ-hydroxy-24-vinylcholesterol.[12] Apparently **10** is an artefact, derived by ene-reaction from **6** and singlet oxygen, during storage. The 3β-hydroxy-Δ^5 derivative of **10**, having cytotoxicity against murine L1210 leukemia and normal lymphoid cells, was previously reported from tunicates, *Phallusia mamillata* and *Ciona intestinalis*.[13] It was claimed that they are products of *de novo* synthesis in those organisms.

Experimental

Optical rotations were determined on a JASCO DIP-370 digital polarimeter. NMR spectra were determined on a JEOL JNM GX-400 spectrometer at 400 MHz (^{1}H) and on a JEOL JNM FX-90Q spectrometer at 22.5 MHz (^{13}C) in $CDCl_3$ with tetramethylsilane (^{1}H, δ 0.00) and $CDCl_3$ (^{13}C, center peak δ 77.1) as internal standards. MS were determined on a JEOL JMS D 300 mass spectrometer. HPLC was carried

out using Senshu pak ODS-4201 (200 × 10 mm i.d.) with 90% MeOH.

Isolation of the Trienones Mixture The sponge *Dysidea herbacea* was collected in January 1990 on the coast of the Lakshadweep Islands (Minicoy Island, 8°17′N, 73°03′E), India. The organism was washed with fresh water, and air-dried (800 g). It was extracted continuously with methylene chloride, and the extract was passed through anhydrous $MgSO_4$. The dark-colored residue (40 g) was chromatographed over a column of silica gel (500 g, 80 × 800 mm, Acme, 100—200 mesh) and eluted with solvent mixtures of hexane–ethyl acetate of increasing polarity. The hexane eluate gave an unidentified compound, code name MF-VA-05-1 (180 mg). The fractions eluted with hexane–ethyl acetate (19 : 1) gave a complex mixture which, on repeated chromatography, afforded a trienone mixture (75 mg) and two unidentified compounds, code names MF-VA-05-2 (70 mg) and MF-VA-05-5 (20 mg). The fractions eluted with hexane–ethyl acetate, 9 : 1 and 4 : 1, gave unidentified compounds, code names MF-VA-05-03 (50 mg) and MF-VA-05-04 (26 mg), respectively. The trienone mixture was partly decomposed on storage. It (65 mg) was repurified by chromatography with hexane–ethyl acetate (19 : 1) to give the trienone mixture (24 mg) and **10** (0.3 mg). The trienone mixture was dissolved in 0.25 ml of MeOH, and charged on an HPLC column in 30 portions. The retention times of the components, relative to that of **1** (41 min), were as follows: **2** (0.81), **3** (0.85), **4** (0.94), **5** (0.97), **6** (1.05), **7** (1.17), **8** (1.19), **9** (1.36), **10** (0.31). The chromatogram was monitored at 300 nm with a UV detector and the eluates were combined accordingly, giving **1** (4.2 mg), **2** (1.0 mg), **3** (0.5 mg), **4**, (1.2 mg), **5** (0.5 mg), **6** (0.6 mg), **7** (0.4 mg, **8** (0.6 mg), and **9** (0.8 mg). Except for **1**, complete removal of the solvent was not possible, and the specific rotations and the molecular extinction coefficients of UV spectra were not recorded.

1: Oil. $[\alpha]_D^{28}$ +480° (c=0.84, $CHCl_3$). ^{1}H-NMR δ: 0.870, 0.874 (each 3H, d, J=7.0 Hz, 26, 27-H_3). Other signals, see text. ^{13}C-NMR δ: C-1,2 (34.2), C-3 (199.3), C-4 (123.0), C-5 (164.2), C-6, 8 (124.5), C-7 (134.0), C-9 (44.4), C-10 (36.8), C-11 (19.1), C-12, 22 (35.8, 35.9), C-13 (44.2), C-14 (156.1), C-15 (25.4), C-16, (27.2), C-18, 21 (18.8), C-19 (16.7), C-20 (34.6), C-23 (23.8), C-24 (39.6), C-25 (28.0), C-26, 27 (22.6, 22.8). UV λ_{max}^{EtOH} nm (ε): 348 (21400). MS m/z: 380, 365, 267, 253, 242, 214. High-resolution MS [Found (Calcd)] m/z: $C_{27}H_{40}O$ (M^+), 380.3088 (380.3079).

2: Oil. ^{1}H-NMR, see text. UV λ_{max}^{EtOH} nm: 348. MS m/z: 378, 268, 267, 253, 240, 214. High-resolution MS [Found (Calcd)] m/z: $C_{27}H_{38}O$ (M^+), 378.2903 (378.2920).

3: Oil. ^{1}H-NMR, see text. UV λ_{max}^{EtOH} nm: 348. MS m/z: 392, 377, 308, 293, 279, 267, 253, 242, 214. High-resolution MS [Found (Cacld)] m/z: $C_{28}H_{40}O$ (M^+), 392.3091 (392.3079).

4: Oil. ^{1}H-NMR, see text. UV λ_{max}^{EtOH} nm: 348. MS m/z: 392, 349, 299, 268, 267, 253, 240, 214. High-resolution MS [Found (Calcd)] m/z: $C_{28}H_{40}O$ (M^+), 392.3061 (392.3080).

5: Oil. ^{1}H-NMR, see text. UV λ_{max}^{EtOH} nm: 348. MS, identical with that of compound **4**. Hith-resolution MS [Found (Calcd)] m/z: $C_{28}H_{40}O$ (M^+), 392.3089 (392.3080).

6: Oil. ^{1}H-NMR, δ: 0.988, 0.992 (each 3H, d, partially overlapped by 19-H_3 signal), 1.040 (3H, d, J=7.0 Hz, 21-H_3). Other signals, see text. UV λ_{max}^{EtOH} nm: 348. MS m/z: 406, 391, 309, 308, 267, 253, 240, 214. High-resolution MS [Found (Calcd)] m/z: $C_{29}H_{42}O$ (M^+), 406.3233 (406.3236).

7: Oil. ^{1}H-NMR, δ: 0.792 (3H, d, J=7.0 Hz, 28-H_3), 0.812 (3H, d, J=7.0 Hz, 26-H_3). Other signals, see text. UV λ_{max}^{EtOH} nm: 348. MS m/z: 394, 379, 295, 267, 253, 242, 214. High-resolution MS [Found (Calcd)] m/z: $C_{28}H_{42}O$ (M^+), 394.3222 (394.3235).

8: Oil. ^{1}H-NMR, see text. UV λ_{max}^{EtOH} nm: 348. MS m/z: 406, 268, 267, 253, 240, 214. High-resolution MS [Found (Calcd)] m/z: $C_{29}H_{42}O$ (M^+), 406.3260 (406.3236).

9: Oil. ^{1}H-NMR, see text. UV λ_{max}^{EtOH} nm: 348. MS m/z: 408, 283, 267, 253, 242, 214. High-resolution MS [Found (Calcd)] m/z: $C_{29}H_{44}O$ (M^+), 408.3373 (408.3392).

10: Oil. ^{1}H-NMR, see text. UV λ_{max}^{EtOH} nm: 348. MS m/z: 438, 422, 420, 405, 404, 378, 363, 345, 267, 240, 213. High-resolution MS [Found (Calcd)] m/z: $C_{29}H_{42}O_3$ (M^+), 438.3154 (438.3134).

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