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3-Keto steroids from the marine organisms *Dendrophyllia cornigera* and *Cymodocea nodosa*

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ABSTRACT

The new (20R)-22E-cholesta-4,22-diene-3,6-dione (**1**), along with three known 3-keto steroids were isolated from the deep-water Mediterranean scleractinian coral *Dendrophyllia cornigera* (**2**–**4**). Moreover, four known related 3-keto steroids were isolated from the sea grass *Cymodocea nodosa* (**5**–**8**). The structure elucidation of steroid **1** and the full NMR resonance assignments of all isolated metabolites were based on interpretation of their spectral data. All compounds are reported for the first time as metabolites of the investigated organisms. Compounds **2** and **3** showed significant cytotoxicity against lung cancer NSCLC-N6 cell line.

1. Introduction

Steroids have exhibited an astonishing array of pharmacological activities and continue to be one of the most intriguing classes of compounds for a variety of reasons, including their challenging structural complexity. In the framework of our efforts towards the discovery of bioactive metabolites from marine organisms we focused our attention on the isolation of new steroids from marine organisms found in the Greek Seas. An initial screening of fourteen marine organisms [1], showed that *Cymodocea nodosa* and *Dendrophyllia cornigera* were among the species showing interesting steroidal profiles being at the same time less excessively investigated. From an unidentified species of *Dendrophyllia* has been reported the isolation of leptosamine pigments and aplysinopsin-type alkaloids, while from the deep-water Mediterranean scleractinian *D. cornigera* a highly heterogeneous mixture of glucosylceramides has been isolated [2,3]. From *C. nodosa* have been isolated in the past C-24 sterols [4] and phenolic metabolites [1,5]. Marine phanerogames compose an ecologically important group of plants in the Mediterranean Sea and provide dietary sterols to other organisms. During the course of our chemical investigation, we isolated eight keto steroids from the sea grass *Cymodocea nodosa* and the coral *Dendrophyllia cornigera*. In particular from the hard coral *D. cornigera*, we isolated a new (20R)-22E-cholesta-4,22-diene-3,6-dione steroid (**1**), along with three known keto steroids (**2**–**4**) all possessing a cholestane side chain. Additionally four 3-keto steroids (**5**–**8**) with an 24-ethyl-cholestane side chain were obtained from the sea grass *C. nodosa*. We herein report their isolation, structure elucidation and antiproliferative activity.

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2. Experimental

2.1. General methods

Optical rotations were measured using a Perkin-Elmer model 341 polarimeter and a 10 cm cell. UV spectra were recorded in a spectroscopic grade C_6H_{14} on a Shimadzu UV-160A spectrophotometer. IR spectra were obtained using a Paragon 500 Perkin-Elmer spectrometer. NMR spectra were recorded on a Bruker AC 250.3 MHz and Bruker DRX 400 MHz spectrometer. Chemical shifts are given on a δ (ppm) scale using TMS as internal standard. $CDCl_3$ was used as NMR solvent. EIMS data were obtained on a Hewlett Packard 5973 Mass Selective Detector. Column chromatography was performed with Kieselgel 60 (Merck), while HPLC was conducted using a Pharmacia LKB 2248 model and an GBC LC-1240 refractive index detector, with Techsil SP 44160 25 cm × 7.8 mm, Kromasil 100SIL/5 μm 25 cm × 8 mm, Spherisorb S10W 25 cm × 10 mm, columns. TLC was obtained with Kieselgel 60 F_{254} (Merck aluminum support plates).

2.2. Collection, extraction and isolation

Cymodocea nodosa, was collected from 5 m depth, at Ag. Cosmas gulf near Athens, while the coral *D. cornigera* was collected from 80 m depth near the island of Serifos at the Mediterranean Sea. Both organisms were exhaustively extracted with a mixture of dichloromethane-methanol (3:1) that after evaporation gave 14.1 g and 1.2 g of oil residues, respectively. The extracts were chromatographed on Silica gel columns with step gradient elution (c-hexane to EtOAc). In the case of *D. cornigera*, the fraction which was eluted with 30% EtOAc in c-hexane, was subjected to silica gravity chromatography and the new fraction eluted with 20% EtOAc in c-hexane, was purified by normal phase HPLC (column Techsil), using as a mobile phase c-hexane-EtOAc (88:12), to afford metabolites **1** (2.8 mg) and **2** (1.4 mg). The gravity column fraction eluted with 10% EtOAc in c-hexane was further purified by normal phase HPLC, using c-hexane-EtOAc (95:5) as the mobile phase, to give compounds **3** (1.2 mg) and **4** (1.0 mg) in pure form.

The fraction of *C. nodosa*, eluted with 20% EtOAc in c-hexane, was purified by normal phase HPLC, using as mobile phase c-hexane-EtOAc (90:10) to afford metabolites **5** (3.5 mg) and **6** (1.0 mg). Moreover, the fraction eluted with 25% EtOAc in c-hexane, was further subjected to normal phase chromatography using a 2 % EtOAc step gradient elution (c-hexane to EtOAc). Fraction eluted with 18% EtOAc in c-hexane, was purified by HPLC, using c-hexane—EtOAc (78:22) as the mobile phase, to afford pure metabolites **7** (3.6 mg), and **8** (2.4 mg).

2.3. Cytotoxicity assays

All compounds isolated from *D. cornigera* and *C. nodosa* were tested against NSCLC-N6 and A549 human tumour cell lines. The NSCLC-N6 cell line [6], derived from a human non-small-cell bronchopulmonary carcinoma (moderately differentiated, rarely keratinising, classified as T2N0M0) was used for all experiments. The cells were cultured in RPMI 1640 medium with 5% fetal calf serum, to which were added 100 IU penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 2 mM glutamine, at 37 °C in an air/carbon dioxide (95:5, v/v) atmosphere. In these conditions, cell doubling time was 48 h. Cells used in all experiments never exceeded 35 passages. Experiments were performed in 96 wells microtiter plates (2×10^5 cells ml^{-1}). Cell growth was estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product by live mitochondria [7]. Eight repeats were performed for each concentration. Control growth was estimated from 16 determinations. Optical density at 570 nm corresponding to solubilized formazan was read for each well on a Titertek Multiskan MKII.

2.4. (20R)-22E-cholesta-4, 22-diene-3,6-dione (1)

Yellow oil $[\alpha]_D^{25} = -25$ (*c* 0.1, CH_2Cl_2); UV (CH_2Cl_2): λ_{max} (nm) (log ε) 204.5 (2.14), 250.0 (2.38); IR (KBr): ν_{max} 1690 cm^{-1}; HRFAB-MS *m/z* 397.3102 (calcd for $C_{27}H_{41}O_2$ $[M+H]^+$ 397.3108); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (50.3 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

3. Results and discussion

Dendrophyllia cornigera was collected from the island of Serifos at the Aegean Sea. The coral was extracted with dichloromethane: methanol (3:1) and the oily extract (1.2 g) was subsequently fractionated with a combination of chromatographic techniques to allow, after final HPLC purification the isolation of metabolites **1** (2.8 mg), **2** (1.4 mg), **3** (1.2 mg), **4** (1.0 mg) (Fig. 1).

Compound **1**, obtained as a colourless oil, had a molecular formula deduced from FAB-MS (*m/z* 397.3102 $[M+H]^+$) and the ^{13}C NMR data as $C_{27}H_{40}O_2$. The mass spectrum included diagnostic fragment ions at *m/z* 285 $[M\text{-}C_8H_{15}]^+$ and 243 $[M\text{-}CH_2CO\text{-}C_8H_{15}]^+$, indicating the presence of a C_8H_{15} monounsaturated side chain. The ^{13}C NMR spectrum showed the presence of a carbon–carbon double bond (δ 162.8, 125.4) and indicated the presence of two conjugated carbonyls (δ 199.5, 202.3). These observations were supported by the UV spectrum, which showed absorption at λ_{max} 250 nm, characteristic of the presence of a Δ^4-3, 6 dione [8]. The side chain double bond was indicated by the multiplets at δ 5.17 and 5.28 ppm in the ^1H NMR spectrum, as well as by the olefinic carbon resonances at δ 137.6 and 127.9 ppm. The chemical shifts of the side chain are in agreement with reported values for steroids containing the (22*E*)-cholestane-chain [9]. The geometry of Δ^{22} double bond, was assigned as *E*, on the basis of the observed vicinal coupling ($J = 15.4$). The H_3-19 protons at δ 1.15 ppm showed correlations with C-1, C-5, C-9 and C-10, while H-2a showed correlation with C-4 in the HMBC spectrum. Additionally in the ^1H-^1H COSY spectrum H-1 and H-2 were coupled, confirming the total assignment of ring A. A doublet of doublets at 2.66 ppm that was assigned to H-7a, showed cross peaks with protons H-7b and H-8 at δ 2.06 and 1.90 ppm, respectively. On the basis of the HMBC correlation between H-4 and C-6 and the COSY correlations between H-7 and H-8, H-8 and H-9, the assignments of ring B were confirmed. In