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STEROL COMPOSITION OF THE "LIVING FOSSIL" CRINOID *GYMNOCRINUS RICHERI*

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Abstract—1. The composition of sterol mixture from the "living fossil" crinoid *Gymnocrinus richeri* collected off Nouméa (New Caledonia) was investigated.

2. The free 3β-OH sterol mixture was found to contain 14 components, Δ^5 and ring saturated stanols, identified by GC–MS.

3. Cholest-4-en-3-one, cholesta-1, 4-dien-3-one (this latter firstly isolated from a marine source), 5α-8α-epidioxy sterols, and 5α-ergosta-7,22-diene-3β,5,6β-triol were also present, their characterization being accomplished by EI-MS and ^{1}H-NMR. The methanol extract also contained sterol sulphates, which were identified by GC–MS after solvolysis to remove the sulphate group.

INTRODUCTION

Recently we have examined a deep water stalked "living fossil" crinoid *Gymnocrinus richeri* discovered at 520 m depth during the CALSUB oceanographic campaign in 1986 directed toward the exploration of the bathial zone off New Caledonia, which is particularly rich in "living fossils". Examination of the pigments in the methanol extract led us to the isolation of five new violet pigments; gymnochrome A, gymnochrome B, gymnochrome C, gymnochrome D and isogymnochrome D, which constitute a novel group of brominated phenanthroperylenequinones (De Riccardis *et al.*, 1991).

As a continuation of our studies we have now examined the hexane and the dichloromethane extracts of the crinoid *Gymnocrinus richeri*, and we wish to report the composition of the sterol mixture.

Only sporadic papers on sterol composition of crinoids have appeared in literature (Goad, 1978). These animals usually contain a typical complex mixture of sterols found in other phyla, as expected for filter feeder organisms.

In addition to Δ^5 sterols and ring saturated stanols, we have isolated from *Gymnocrinus richeri* a low level of cholest-4-en-3-one, three Δ^6 5α-8α-epidioxy sterols, a rare 9(11)-unsaturated 5α,8α-epidioxy sterol, 5α-ergosta-7,22-diene-3β, 5,6β-triol and cholesta-1,4-diene-3-one. This latter has been isolated for the first time from a marine organism. Sterol sulphates have also been isolated from the methanol extract and they were analyzed after solvolysis to remove the sulphate group.

MATERIALS AND METHODS

Sources and preparation of sterol fractions

The animals were collected off New Caledonia at 520 m depth, during 1986. They were freeze dried and despatched to the laboratory at Naples. Reference specimens were deposited at the Museum National d'Histoire Naturelle in Paris, 57, rue Cuvier.

The freeze dried animals (1.3 kg) were extracted successively with *n*-hexane (3 l, 2.82 g extract) and dichloromethane (4 l, 4.28 g extract) (Soxlhet) and then with methanol (5 l, 15.2 g extract) at room temperature.

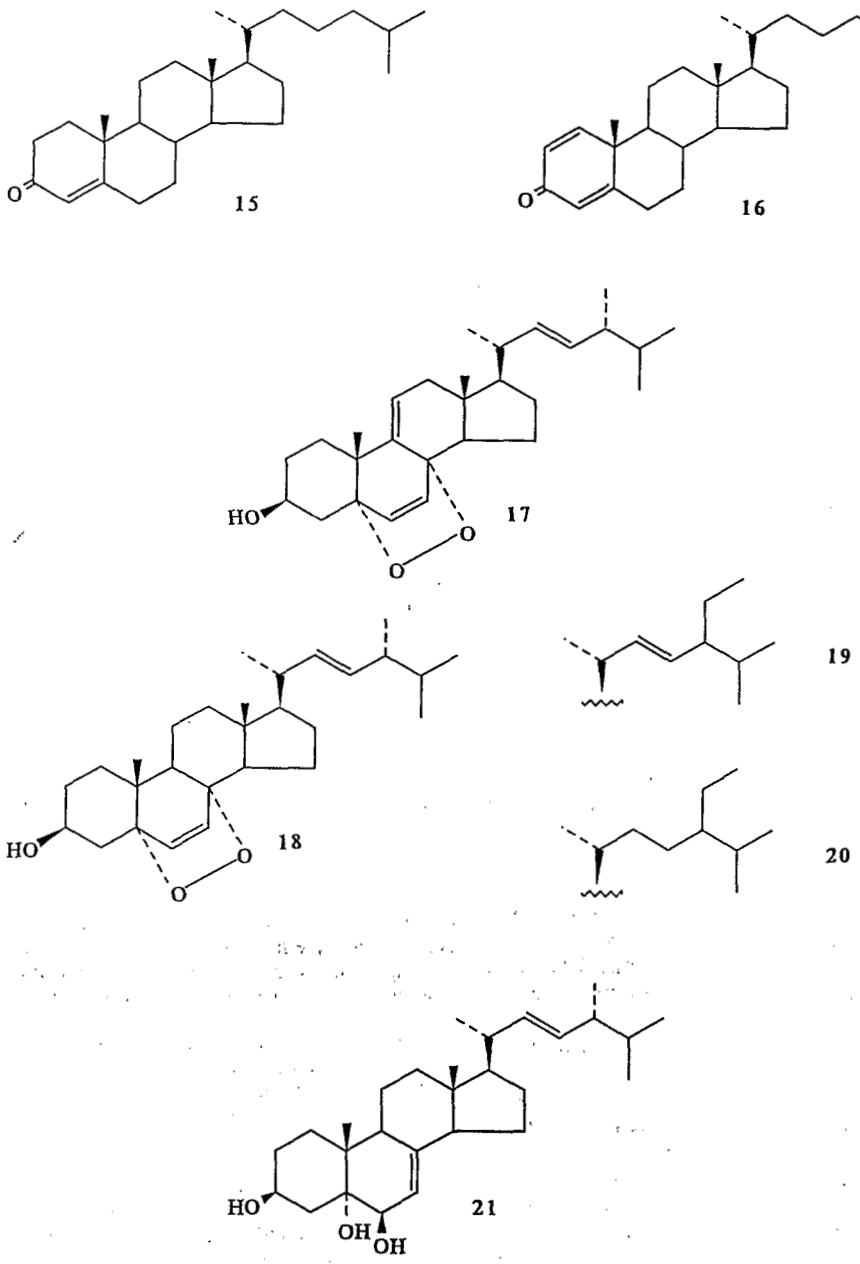
The hexane extract was purified by medium pressure chromatography over a column of Silica gel (Merck Kieselgel 60, 230–400 mesh, 200 g) using *n*-hexane–ethyl acetate ranging from 9:1 to 1:1 as eluant. The first eluted fractions contained the ketones **15** and **16** (2 mg). The ketone **15** was further purified by HPLC by using a column of Whatman Partisil M9 10/50 ODS-2 in methanol to give 0.7 mg of pure material. Subsequent fractions yielded the conventional 3β-hydroxysterols (475 mg) and were followed by the epidioxy sterols mixture (103 mg).

Free sterols: analysis

The crude sterols were purified by crystallization from EtOH and fractionated by reverse phase HPLC (Whatman Partisil M9 10/50 ODS-2 column with methanol as eluant) to give pure **1**, **5**, **7** and **13** (see Table 1). Ten milligrams of the crude sterols were acetylated (pyridine–Ac_2O; room temperature) and the mixed acetates were submitted to GC–MS with a temperature program (Column 25 m × 0.20 mm HP-5, acquisition parameters: initial temperature 150°C, final temperature 280°C, rate 5.00°C min^{-1}, total time 60 min).

Sterols sulphates: isolation and analysis

The methanol soluble material (15.2 g) was chromatographed in two portions on a column of Sephadex LH-20 (4 × 60 cm) by using MeOH as eluant to give, in the first fractions, a mixture of sulphated monohydroxysteroids (235 mg). R_f in SiO_2 0.7, TLC in *n*-butanol–acetic acid–water (60:15:25). Fifty milligrams of this fraction was solvolyzed in dioxane (0.5 ml)–pyridine (0.5 ml) and, after the mixture had cooled, water (5 ml) was added and the solution was extracted with diethyl ether (3 times). The organic layer was washed with water and evaporated under reduced pressure to give the free sterols mixture. This material was acetylated and the mixture acetates submitted to GC–MS as above.



Scheme 1.

5α,8α Epidioxy sterols: isolation

The peroxides mixture (103 mg) was purified by silica gel column chromatography (Merck Kieselgel 60, 230–400 mesh, 10 g) using *n*-hexane/ethyl acetate as eluant, ranging from 9:1 to 8:2. The fractions eluted with *n*-hexane/ethyl acetate 8:2 were further fractionated by reverse phase HPLC (Whatman Partisil M9 10/50 ODS-2 column) with methanol as eluant, to give pure **17** (4.8 mg), **18** (2.7 mg), **19** (1.8 mg) and **20** (0.6 mg) in that order.

Isolation of 5α-ergosta-7,22-diene-3β,5,6β-triol

The CH_2Cl_2 extract was purified by medium pressure chromatography over a column of silica gel (Merck Kieselgel 60, 230–400 mesh, 200 g) using *n*-hexane/ethyl acetate ranging from 9:1 to 100% ethyl acetate. The fractions eluted with ethyl acetate contained 5α-ergosta-7,22-diene-3β,5,6β-triol **21** (2.5 mg).

Instrumentation

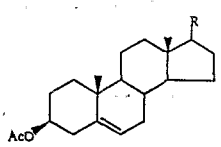
MS spectra were recorded on a Kratos MS-50 instrument. ^{1}H-NMR were taken at 250 MHz on a Bruker WM-250 spectrometer. High performance liquid chromatographies (HPLC) were performed on a Waters apparatus equipped with Waters 6000 A pump, a U6K injector and a differential refractometer model 401.

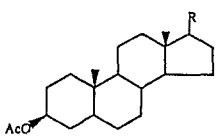
Combined GLC–MS analyses were performed on a Hewlett–Packard 5890 gas chromatograph with a mass selective detector MDS HP 5970. A column 25 m × 0.20 mm HP-5 (cross linked Ph Me Silicone 5%) with a 0.33 μm film thickness was employed.

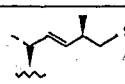
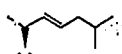
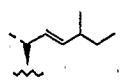
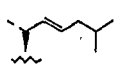
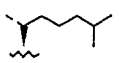
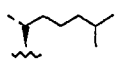
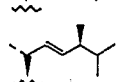
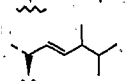
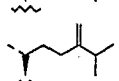
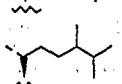
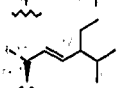
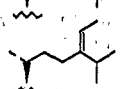
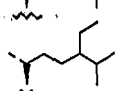
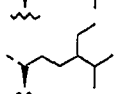
RESULTS AND DISCUSSION

Cholest-4-en-3-one (**15**) has been identified by EI-MS (m/z 384, M^+), ^{1}H-NMR (δ_H 5.75 broad s,

Table 1. Sterol composition of *Gymnochrinus richeri*





	Side chain-R	Nucleus	M^+ (m/z)	Mobility GC–MS*	Mobility HPLC†	Free sterols (%)	Sulphated sterols (%)
1		Δ^5	366 M^+-AcOH	0.75	0.71	9.5	4.0
2		Δ^5	366 M^+-AcOH	0.82	—	18.0	43.3
3		Δ^0	428	0.82	—	1.8	—
4		Δ^0	428	0.87	—	2.0	4.5
5		Δ^5	368 M^+-AcOH	1.00	1.00	12.1	26.4
6		Δ^0	430	1.05	—	2.0	7.0
7		Δ^5	380 M^+-AcOH	1.17	0.82	29.0	10.8
8		Δ^0	442	1.27	—	2.5	1.4
9		Δ^5	380 M^+-AcOH	1.50	—	4.4	1.6
10		Δ^5	382 M^+-AcOH	1.52	—	2.8	1.0
11		Δ^5	394 M^+-AcOH	1.67	—	1.5	—
12		Δ^5	394 M^+-AcOH	1.72	—	6.4	—
13		Δ^5	396 M^+-AcOH	2.10	1.14	6.6	—
14		Δ^0	458	2.17	—	1.4	—

*As acetates, standard cholesteryl acetate.
†As free sterols on Whatman Partisil M9 10/50 ODS-2 column and pure methanol as eluant, standard cholesterol.

H-4), u.v. (λ max 239 nm, CH_3OH) and i.r. (V_{max} 1675 cm^{-1}, $CHCl_3$) data and comparison with literature data (Sheikh and Djerassi, 1974; Hirano and Djerassi, 1982). Steroidal Δ^4-3-ketones have been previously detected from marine organisms in small amounts.

Identification of cholesta-1,4-dien-3-one (**16**) was also based on comparison of EI-MS, ^{1}H-NMR, u.v. and i.r. spectra with published data (Wilds and Djerassi, 1946; Minami *et al.*, 1986).

The ring A dienone gave rise to a u.v. maximum at 243 nm in EtOH (ε = 12,000) and an intense i.r. band at 1662 cm^{-1} ($CHCl_3$) typical of conjugated ketone absorption. Further evidence of this system was provided by the ^{1}H-NMR spectrum which showed signals for three olefinic protons at low field (7.07 d, J = 10 Hz; 6.23 dd, J = 8, 2.5 Hz and 6.07 brs) in a pattern characteristic of steroidal $\Delta^{1,4}$-3-ones.

The ^{1}H-NMR values of side chains are identical to those reported for cholesterol. The mass spectrum depicted a molecular ion peak at m/z 382 with an important fragment ion at m/z 269 corresponding to the loss of an entire side chain (C_8H_{17}) with transfer of two hydrogens and m/z 122 (100%) typical for the ring A cleavage (Shapiro and Djerassi, 1964). This compound was previously described as a synthetic

intermediate, while as a natural compound it has been isolated for some microorganisms as a metabolic product of cholesterol (Arima *et al.*, 1969). A pregnane derivative, with this structural feature, has been reported from marine organisms in the telestecean octocoral *Telesto riisei* (Ross and Scheuer, 1979) and in the sea raspberry *Gersemia rubiformis* (Kingston *et al.*, 1977).

The co-occurrence of these ketones with Δ^5-sterols and stanols indicates the presence in this lower organism of some of the enzymes which are essential for the conversion of sterols into steroidal hormones.

GC–MS analysis of the *Gymnocrinus richeri* free sterols (as acetates) revealed that the mixture was complex, with 14 peaks (Table 1). All are known compounds and their identification was achieved with the aid of relative retention time (r.r.t.) in HPLC, GC and MS spectrometry. Analysis of the mass spectral fragmentation in term of known processes was mainly based on the following reviews or papers: Knights (1967), Wyllie and Djerassi (1968), Djerassi (1970), and Budzikiewicz (1972). The major fraction of the free sterols, after crystallization from ethanol, was chromatographed by semi-preparative HPLC using an ODS-2 column (Whatman Partisil M9 10/50) to obtain four pure compounds, **1**, **5**, **7** and **13**, which were submitted to EI-MS and 250 MHz ^{1}H-NMR analyses, and fractions which revealed to be still a mixture.

The (22E, 24R)-24 methylcholesta-5,22-dien-3β-ol (**7**) is the major constituent of the mixture and the stereochemistry at C-24 was deduced by comparison of the ^{1}H-NMR spectral data with those reported for the two C-24 epimers. The spectra are very similar, but the C-21 methyl doublet occurs at higher field (δ_H 0.995) in the spectrum of the more polar component (24S) than in the spectrum of the less polar component (δ_H 1.02; 24R). In **7** the C-21 methyl doublet appears at δ_H 1.02 (Rubistein *et al.*, 1976). In the 24-ethylsteroids the differences in the NMR spectra of the two epimers are too slight; therefore we have preferred to leave the stereochemistry at C-24 in **13** unassigned.

The ^{1}H-NMR spectrum of the more polar sterols fraction obtained from the methanol extracts clearly indicated a mixture of the sterol sulphates (7-line multiplet at δ_H 4.20) (Goodfellow and Goad, 1983). Solvolysis of this material to remove the sulphate group gave a mixture of sterols which were acetylated and the mixed acetates were analyzed by GC–MS as above (Table 1). In the case of the sterol sulphates the major component of the mixture was the (22E)-cholesta-5,22-dien-3β-ol (**2**), followed by the common cholesteryl sulphate.

The 5α,8α-epidioxy Δ^6 sterols have been isolated from marine organisms, such as sponges, tunicates and sea anemones by several groups of investigators (Sheikh and Djerassi, 1974; Fattorusso *et al.*, 1974; Malorni *et al.*, 1978; Guyot and Durgeat, 1981; Findlay and Patil, 1984). We have now isolated three known, more common, epidioxy Δ^6 sterols, **18**, **19** and **20**, along with the rare (22E)-5,8-epidioxy-5α,8α-ergosta-6,9(11), 22-trien-3β-ol (**17**) previously described in a tunicate, *Phallusia mammillata* (Guyot and Durgeat, 1981), and a sea anemone *Metridium senile* (Findlay and Patil, 1984). The structures were deduced from 250 MHz ^{1}H-NMR spectra, electron impact (EI-MS) and comparison with literature data. Particularly significative for the identification of **17** was the deshielding values for the H-6 and H-7 olefinic protons, δ 6.25 and 6.60 (each doublet with J = 8 Hz), compared to the values, δ 6.20 and 6.55, observed in the NMR spectra of the Δ^6 derivatives **18–20**, and the presence of a further olefinic quartet at δ 5.41 ppm.

Finally from the dichloromethane extracts we have also isolated the polyhydroxylated sterol 5α-ergosta-7,22E-diene-3β,5,6β-triol (**21**) previously found in marine organisms such as *Patinopecten yessoensis* (Iorizzi *et al.*, 1988) and sponges (Piccialli and Sica, 1987; Ciminiello *et al.*, 1990).

The identification was based on direct comparison (^{1}H-NMR, HPLC, TLC) with a synthetic sample obtained from ergosterol (Iorizzi *et al.*, 1988).

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