Ectyoplasides A–B – Unique Triterpene Oligoglycosides from the Caribbean Sponge *Ectyoplasia ferox*

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Dedicated to the memory of Professor Luigi Minale

Keywords: Saponins / Terpenoids / Sponges / *Ectyoplasia ferox*

Two novel norlanostane triglycosides, ectyoplasides A (**1**) and B (**2**), have been isolated from the methanolic extracts of the Caribbean sponge *Ectyoplasia ferox* (Raspaliidae, Axinellida). Their structures have been established by extensive application of high-resolution FABMS and two-dimensional NMR techniques, supported by chemical and enzymatic degradation methods. Ectyoplasides A and B have been shown to possess identical sugar chains (composed of two β-galactose units and one α-arabinose unit), but to differ in their aglycone moieties, which possess nortriterpene skeletons with an unprecedented substitution pattern on ring A. Ectyoplasides A and B show moderate cytotoxic activity when tested in vitro towards various cell lines

Introduction

Ectyoplasia ferox, Duchassaing and Michelotti (Demospongiae, family Raspaliidae, order Axinellida) is a brownish orange sponge commonly found as an encrusting species along the coasts of the Caribbean Sea.[1] In spite of its wide diffusion, very little is known about the secondary metabolism of *E. ferox* and, to date, only one report on the phospholipid fatty acid composition has appeared in the literature.[2] However, during a recent symposium, Pawlik et al.[3] reported that a mixture of nonseparable polar metabolites obtained from this sponge has been found to exhibit strong antifeedant activity.

In the course of our research program aimed at the chemistry of Caribbean marine organisms and focused particularly on the biologically active secondary metabolites, we have recently begun a chemical investigation of this sponge. Our interest in this organism started when we became aware, through preliminary ^{1}H-NMR and chromatographic analyses, that its polar extracts were surprisingly rich in terpenoid saponins, an unusual feature for a marine sponge.

The present paper deals with the isolation and structural elucidation of two major components of this mixture, ectyoplasides A (**1**) and B (**2**). Their chemical structures, determined by extensive application of high-resolution FABMS and 2D-NMR techniques, are characterized by an unprecedented substitution pattern at ring A of their aglycone moieties, which have been identified as nortriterpenes with a tetracyclic skeleton of the lanostane type. In addition, **1** and **2** possess an invariant triglycosidic chain as a sugar portion, linked at C-3.

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Results and Discussion

A specimen of *E. ferox*, collected by hand along the coasts of San Salvador Island, Bahamas, was exhaustively extracted with MeOH at room temperature and the fresh methanolic extracts were successively partitioned against *n*-hexane, CCl_4, $CHCl_3$, and *n*-butanol, following a slightly modified Kupchan method.[4] The butanol-soluble material, which appeared to be the richest in saponins, was initially separated by MPLC on silica gel (230−400 mesh), eluting with a solvent gradient system of increasing polarity from EtOAc to MeOH. Fractions eluted with MeOH/EtOAc (7:3) were pooled and then further purified by reversed-phase HPLC (eluent MeOH/H_2O, 8:2) yielding pure ectyoplasides A (**1**, 13.5 mg) and B (**2**, 4.5 mg) as white, amorphous solids.

Our structural assignment began with the most abundant triglycoside, ectyoplaside A (**1**). It exhibits an intense pseudomolecular peak in the positive-ion HRFAB mass spectrum at *m/z* 953.4734 [$M^- + Na^+ + H^+$], while two diagnostic peaks were detected at *m/z* 951.4560 [$M^- + Na^+ - H^+$] and *m/z* 929.4731 [M^-] in the negative-ion HRFAB mass spectrum. Combination of these data allowed us to assign the molecular formula $C_{46}H_{73}NaO_{19}$ to **1**, which was further confirmed by NMR evidence.

The presence of a number of signals between δ = 3.2 and δ = 5.5 in the ^{1}H-NMR spectrum (CD_3OD) of **1** (Table 1), attributable to protons on carbon atoms bearing heteroatom substitution, accompanied by typical terpene resonances including six methyl signals, led us to presume a terpene-glycosidic nature for **1**. Analysis of the ^{13}C- and ^{1}H-

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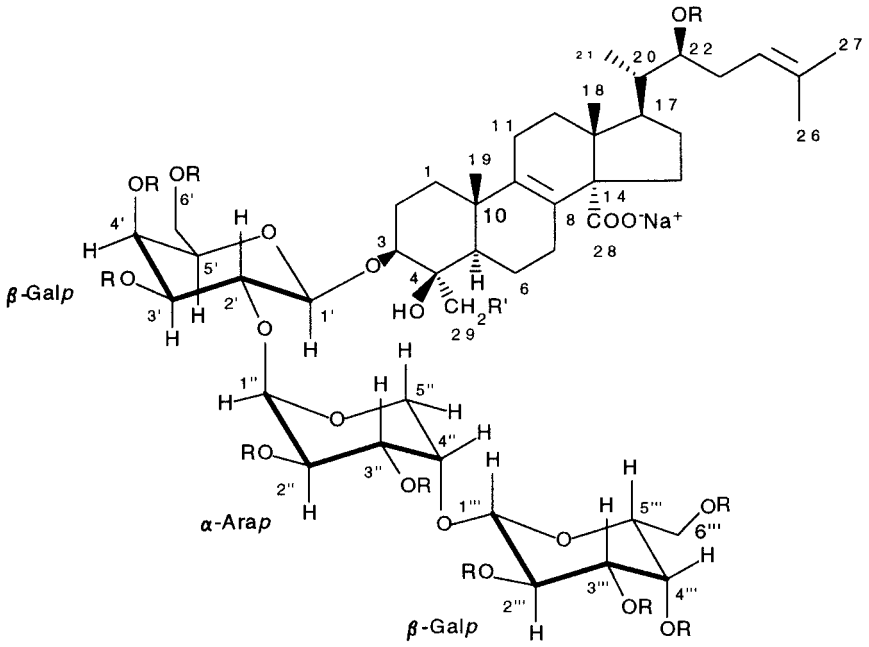


1	R = H	R' = H	**2**	R = H	R' = OH
1a	R = Ac	R' = H	**2a**	R = Ac	R' = OAc

NMR spectra (Tables 1 and 2) of **1** was therefore divided into two phases, the first dedicated to the elucidation of the aglycone structure, which was mainly performed on the native saponin **1**, while the second phase focused on the carbohydrate subunit, the structure of which was deduced from data obtained from the peracetyl derivative **1a**.

The molecular formula $C_{29}H_{45}NaO_5$ was deduced for the aglycone moiety of ectyoplaside A (**1**) on the basis of ^{13}C-NMR data, and was corroborated by a fragmentation peak detected at *m/z* 497 in the positive-ion FABMS of **1**. Interpretation of COSY- and HOHAHA-NMR spectra allowed us to assign all the ^{1}H signals to five spin systems (essentially one for each ring, and the other for the side chain) and to elucidate the proton sequence within each spin system, aided by comparisons with published data.[5] All the proton resonances were unambiguously associated with relevant carbon atoms on the basis of a 2D ^{1}H-detected HMQC spectrum, whereas an HMBC experiment, showing ^3J cross-peaks, was used to determine the resonances of tetrasubstituted carbon atoms (complete assignment given in Table 2).

This analysis allowed us to identify the aglycone moiety of **1** as a unique nor derivative of penasterol, a lanostane-related triterpene with a rare 14-carboxy group, first isolated from the sponge *Penares sp.*[6] and previously found as the terpene moiety in other spongal saponins (see below). In particular, in this case the aglycone actually possesses a penasterol skeleton with two additional hydroxy groups, linked to C-22 in the side chain and to C-4 (ring A) the latter replacing the usual methyl group. Moreover, the carboxy group linked at C-14 (as indicated by the resonance of C-14 at δ = 62.3 in the ^{13}C-NMR spectrum, which matched very well that reported for penasterol,[6] and by the HMBC correlation peaks of C-28 with 15-H_2) must in this case be a carboxylate on the basis of the following evidence: (i) FABMS data; (ii) absorption bands at ν_{max} = 1635, 1573, and 1454 cm^{-1} in the IR spectrum (KBr) of **1**, which are typical stretching values of the carboxylate group; (iii) the ^{13}C-NMR signal of C-28 at δ = 186.5, attributable to a carboxylate rather than to a carboxylic group because of its relatively low-field chemical shift,[6,7] and (iv) the signal at δ = 1.43, long-range-coupled with 18-H_3 (δ = 0.66) and assigned to 12ax-H, whose signal appears upfield-shifted relative to the data of other penasterol saponins,[5] because it resides in spatial proximity to the C-28 carboxylate rather than a carboxyl group.

With regard to the structure of ring A, its ^{1}H- and ^{13}C-NMR resonances appear quite different from those usually reported for lanostane nuclei.[5–7] These variations can be explained by considering that in this case only one methyl group is linked to C-4, the second being replaced by a hydroxy group. This was suggested by the molecular formula and by the resonance at δ = 72.0 of C-4, inferred from its HMBC cross-peaks with 2-H_2 (δ = 2.70 and 2.00), 5-H (δ = 1.35) and 6-H_2 (δ = 1.92 and 1.63). The presence of this OH group at C-4 leads to dramatic deshielding effects on the adjacent protons. In particular, 29-H_3 resonates at δ = 1.40, with a downfield shift of almost 0.5 ppm; the 5-H signal is shifted to δ = 1.35; the 19-H_3 signal, long-range-coupled with 1ax-H, is shifted to δ = 1.10; and 2ax-

Table 1. ^{1}H-NMR data (500 MHz) of ectyoplasides A (**1**) and B (**2**)[a]

Position	**1** δ_H (int., mult., *J* in Hz)	**2** δ_H (int., mult., *J* in Hz)
1ax	1.84 (1 H, br. dd, 12.5, 6.7)	1.83 (1 H, br. dd, 12.3, 6.7)
1eq	1.26 (1 H, m)	1.26 (1 H, m)
2ax	2.70 (1 H, dt, 12.5, 9.7)	2.78 (1 H, dt, 12.3, 9.7)
2eq	2.00[b]	2.00[b]
3	3.27 (1 H, br. t, 9.7)	3.70 (1 H, br. t, 9.7)
5	1.35[b]	1.57 (1 H, m)
6ax	1.92 (1 H, m)	2.05[b]
6eq	1.63 (1 H, m)	2.15[b]
7ax	2.05[b]	2.16[b]
7eq	2.10[b]	2.16[b]
11	2.16 (2 H, m)	2.16 (2 H, m)
12ax	1.43[b]	1.44[b]
12eq	2.00[b]	2.00[b]
15a	2.12[b]	2.13[b]
15b	1.66[b]	1.66[b]
16a	2.03[b]	2.03[b]
16b	1.36[b]	1.35[b]
17	1.56 (1 H, m)	1.56[b]
18	0.66 (3 H, br. s)	0.65 (3 H, br. s)
19	1.10 (3 H, br. s)	1.13 (3 H, br. s)
20	1.47 (1 H, m)	1.46 (1 H, m)
21	0.94 (3 H, d, 4.9)	0.95 (3 H, d, 4.6)
22	3.66 (1 H, br. t, 7.2)	3.65 (1 H, br. t, 7.2)
23a	2.18 (1 H, dt, 12.5, 7.1)	2.19 (1 H, dt, 12.2, 7.1)
23b	2.08[b]	2.11[b]
24	5.15 (1 H, br. t, 7.1)	5.15 (1 H, br. t, 7.1)
26	1.66 (3 H, br. s)	1.67 (3 H, br. s)
27	1.73 (3 H, br. s)	1.75 (3 H, br. s)
29a	1.40 (3 H, br. s)	4.03 (1 H, d, 13.2)
29b		3.85 (1 H, d, 13.2)
1′	4.48 (1 H, d, 7.7)	4.54 (1 H, d, 7.7)
2′	3.81 (1 H, t, 7.7)	3.81 (1 H, t, 7.7)
3′	3.71[b]	3.74[b]
4′	3.78[b]	3.78[b]
5′	3.75[b]	3.75[b]
6′a,b	3.52[b]	3.52[b]
1′′	5.48 (1 H, d, 3.1)	5.48 (1 H, d, 3.1)
2′′	3.80 (1 H, dd, 6.8, 3.1)	3.80 (1 H, dd, 6.8, 3.1)
3′′	3.88 (1 H, br. d, 6.8)	3.90 (1 H, br. d, 6.8)
4′′	4.11 (1 H, br. s)	4.14 (1 H, br. s)
5′′a	3.77[b]	3.77[b]
5′′b	3.75[b]	3.76[b]
1′′′	4.38 (1 H, d, 7.7)	4.39 (1 H, d, 7.5)
2′′′	3.61 (1 H, dd, 8.2, 7.7)	3.60 (1 H, dd, 8.2, 7.5)
3′′′	3.52[b]	3.52[b]
4′′′	3.85[b]	3.85[b]
5′′′	3.75[b]	3.75[b]
6′′′a,b	4.14 (2 H, m)	4.14 (2 H, m)

[a] Measured in CD_3OD. − [b] Overlapped with other signals.

Table 2. ^{13}C-NMR data (125 MHz) of compounds **1** and **2** in CD_3OD

Position	**1** δ_C (mult.)	**2** δ_C (mult.)
1	41.2 (CH_2)	41.3 (CH_2)
2	33.4 (CH_2)	33.5 (CH_2)
3	93.2 (CH)	95.0 (CH)
4	72.0 (C)	76.5 (C)
5	56.3 (CH)	50.1 (CH)
6	24.8 (CH_2)	27.4 (CH_2)
7	30.2 (CH_2)	31.1 (CH_2)
8	138.4 (C)	140.3 (C)
9	140.0 (C)	140.0 (C)
10	36.1 (C)	36.6 (C)
11	25.4 (CH_2)	25.4 (CH_2)
12	30.1 (CH_2)	30.2 (CH_2)
13	46.4 (C)	46.4 (C)
14	62.3 (C)	62.1 (C)
15	32.2 (CH_2)	32.2 (CH_2)
16	28.0 (CH_2)	28.4 (CH_2)
17	55.2 (CH)	55.2 (CH)
18	14.5 (CH_3)	14.6 (CH_3)
19	21.9 (CH_3)	22.1 (CH_3)
20	44.3 (CH)	44.3 (CH)
21	15.2 (CH_3)	15.1 (CH_3)
22	76.1 (CH)	76.1 (CH)
23	38.4 (CH_2)	38.4 (CH_2)
24	126.2 (CH)	126.2 (CH)
25	136.5 (C)	136.4 (C)
26	21.3 (CH_3)	21.3 (CH_3)
27	28.2 (CH_3)	28.2 (CH_3)
28	186.5 (C)	186.5 (C)
29	29.1 (CH_3)	60.2 (CH_2)
1′	109.3 (CH)	109.2 (CH)
2′	81.1 (CH)	79.6 (CH)
3′	74.7 (CH)	74.4 (CH)
4′	73.7 (CH)	73.9 (CH)
5′	77.9 (CH)	77.9 (CH)
6′	67.0 (CH_2)	66.2 (CH_2)
1′′	103.5 (CH)	103.4 (CH)
2′′	73.3 (CH)	71.0 (CH)
3′′	72.9 (CH)	72.8 (CH)
4′′	81.4 (CH)	81.2 (CH)
5′′	66.0 (CH_2)	66.2 (CH_2)
1′′′	107.2 (CH)	106.6 (CH)
2′′′	79.0 (CH)	79.0 (CH)
3′′′	78.8 (CH)	78.9 (CH)
4′′′	73.3 (CH)	73.1 (CH)
5′′′	77.4 (CH)	77.4 (CH)
6′′′	67.5 (CH_2)	67.5 (CH_2)

H, because of its spatial proximity to the OH group, resonates at δ = 2.70. These are very low-field resonances relative to the values usually reported for normal lanostane skeletons.[5] Finally, the sugar unit could be confidently placed at C-3 of ring A by interpretation of the key HMBC correlation peak between 1′-H (δ = 4.48) and the significantly downfield-shifted C-3 signal (δ = 93.2). This was further confirmed by the strong ROESY peak between 1′-H and 3-H (δ = 3.27). The above data, together with other diagnostic spatial couplings revealed by the ROESY spectrum of **1** (outlined in Figure 1), also allowed us to deduce the relative configuration of ring A, in particular the location of the OH group in the β (axial) position, and to assign all its proton resonances.

The simultaneous presence of methyl and hydroxy groups at C-4 of ectyoplaside A is a unique feature and, to the best of our knowledge, has not previously been reported for tetracyclic triterpenoids.[8] The biosynthetic origin of this moiety, as well as of the carboxylate group on C-14, is not obvious at first sight. However, they are probably introduced by oxidation steps not normally present in the typical pathway from lanosterol to steroids.

Information derived from COSY and HOHAHA spectra was also used to follow the scalar couplings within the side chain of **1**, in which the presence of a hydroxy group attached at C-22 was deduced. In particular, we used as starting points the two methyl signals of 26-H_3 (δ = 1.66) and 27-H_3 (δ = 1.73) (the relevant ^{1}H-NMR resonances of which were assigned based on the ROESY cross-peak between 27-H_3 and 24-H), which show long-range couplings with 24-H (δ = 5.15) and with 23-H_2 (δ = 2.18 and 2.08).

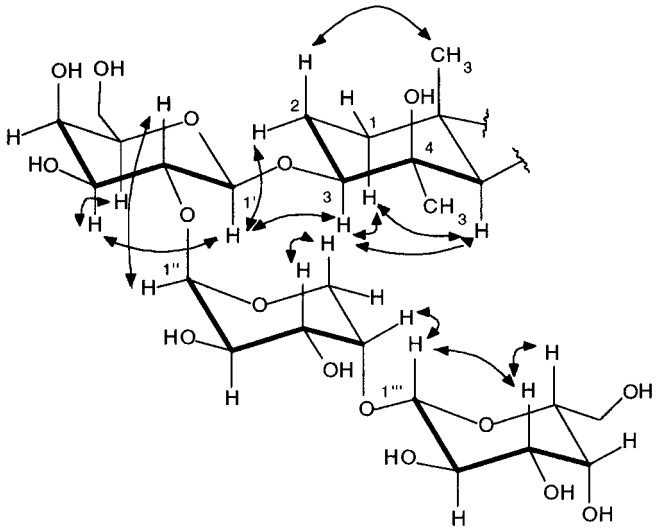


Figure 1. Key ROESY correlation peaks detected for compound **1**

The latter signals show couplings with 24-H and with a low-field proton resonating at δ = 3.66 (22-H), which correlates in the HMQC spectrum with a carbon signal at δ = 76.1, while it shows no other correlation peaks in the COSY spectrum.

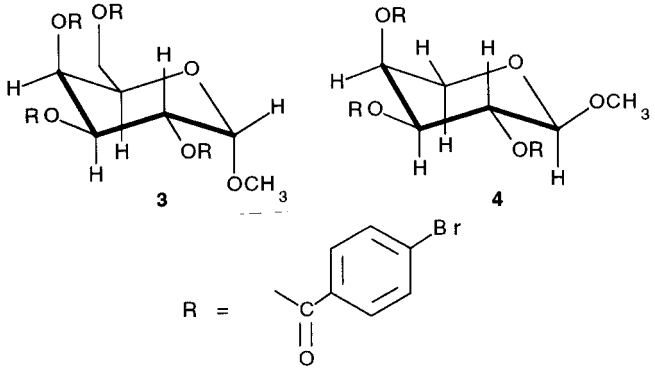
The relative configuration of the two chiral centers C-20 and C-22 of the side chain was deduced by considering a specific feature of the ^{1}H-NMR spectrum, namely the very small (J < 1 Hz) coupling constant between 22-H and 20-H. This unusual value points to a preferential conformation in which the dihedral angle between these two atoms is near to 90°. To assess this, the two alternative 20*R*-22*R*/20*S*-22*S* and 20*R*-22*S*/20*S*-22*R* structures were subjected to a molecular dynamic analysis in the CHARMm force field. The conformational behavior of the molecules was explored with high-temperature (1000 K) molecular dynamics simulation, generating a set of 250 conformations for each molecule. The energies of these structures were minimized and analysis of the obtained results indicates that for both isomers a set of low-energy conformations of the side chain exists at room temperature. Interestingly, in these preferential conformations, the 20-H/22-H angle ranges from 78 to 90° in the *RR*/*SS* isomer, and from 45 to 55° for the *RS*/*SR* isomer. Consequently, we can confidently assign the *RR*/*SS* configuration at centers C-20/C-22 from the above NMR data. Then, if we assume that the aglycone part of our saponin possesses the absolute configuration invariably found in all lanostane derivatives isolated to date,[8] we can assign the *S* configuration to C-20, and hence to C-22 as well. This configuration has previously been found in C-22-hydroxylated triterpenes isolated from various sources.[9−11] Moreover, very small values for the coupling constant of 20-H with 22-H were also measured in these cases.

In the sugar portion of the molecule, the existence of a triglycosidic chain was initially deduced from the ^{13}C-NMR spectrum of **1**, which shows the resonances of three anomeric carbon atoms (δ = 109.3, 103.5, and 107.2, respectively). These were correlated to the relevant anomeric protons through an HMQC experiment. Unfortunately, the remaining ^{1}H-NMR sugar signals are in an excessively crowded region and the superimposition of several key signals prevented us from measuring coupling constants and thus from deducing directly the relative stereochemistry of the monosaccharides. To overcome this difficulty, a derivatization of the saponin **1** appeared indispensable, and, to this end, 3 mg of **1** was acetylated with acetic anhydride in pyridine under standard conditions, to afford the peracetyl derivative **1a**. We repeated the COSY, HOHAHA and ROESY spectra on this molecule (^{1}H-NMR assignments reported in the Experimental Section), and detailed analysis of these data permitted the unambiguous identification of the sugar portion of the molecule.

The signal at δ = 4.60 in the ^{1}H-NMR spectrum ($CDCl_3$) of **1a** exhibits a ROESY coupling (see Figure 1) with 3-H (δ = 3.33) of the aglycone moiety, and thus could be attributed to 1′-H. Starting from this signal, we could identify, on the basis of COSY and HOHAHA evidence, the sequence of a hexose unit, in which the relatively high-field resonances of 5′-H (δ = 3.85) pointed to the pyranose form. Within this spin system, the axial-axial 1′-H/2′-H and 2′-H/3′-H and the axial-equatorial 3′-H/4′-H relationships were deduced by measurement of the coupling constants, whereas the axial position of 5′-H is indicated by the strong ROESY correlation peak with 3′-H (δ = 5.05). On the basis of this evidence, the inner monosaccharide could be identified as a β-galactopyranose. Furthermore, the relatively high-field chemical shift of 2′-H (δ = 4.05) and its ROESY correlation peak with 1′′-H (δ = 5.45) clearly prove the 2′-position to be the glycosidic linkage site. Following the same type of analysis, the second sugar unit was identified as a pentose in the pyranose form, in which the axial-axial coupling between 2′′-H and 3′′-H and the axial-equatorial relationships between 1′′-H and 2′′-H and between 3′′-H and 4′′-H led to an assignment as an α-arabinopyranose. Finally, the third sugar must be linked at the 4′′-position on the basis of a ROESY cross-peak between 4′′-H (δ = 4.35) and 1′′′-H (δ = 4.48), and was easily identified as a second β-galactopyranose because it exhibits a pattern of coupling constants identical to that previously measured for the first monosaccharide. Thus, we succeeded in completely defining the sugar chain of saponin **1**.

In order to confirm the proposed structure of ectyoplaside A (**1**) and to deduce the absolute configuration of the monosaccharides, 4 mg of the saponin **1** was subjected to acidic methanolysis with 1 N HCl in 85% MeOH for 2 h at 70°C. The resulting mixture was neutralized with Ag_2CO_3, centrifuged, and the supernatant was concentrated to dryness under nitrogen. The residue was then partitioned between H_2O and $CHCl_3$. The polar layer (fraction A, 2.2 mg) was found by ^{1}H-NMR analysis to be composed of a mixture of monosaccharide methyl glycosides. Fraction A was then divided into two portions and the first of these (0.8 mg) was subjected to a persilylation reaction with TRI-SIL-Z. The persilylated products were analyzed by GC MS, giving peaks with the same retention times and the same mass spectra as the persilylated galactopyranose methyl gly-

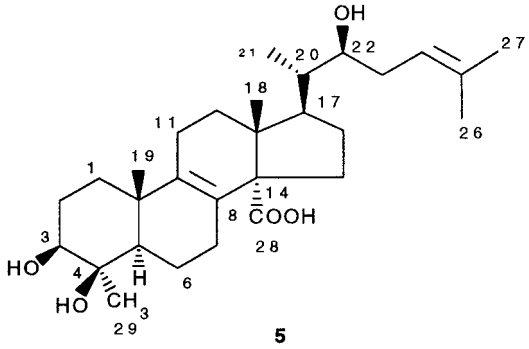
coside and arabinopyranose methyl glycoside prepared as above. A second portion (1.4 mg) of fraction A was per-*p*-bromobenzoylated under standard conditions and the resulting mixture, after work-up, was separated by HPLC on a Whatman Partisil column (eluent *n*-hexane/diethyl ether, 85:15). Only two major UV-absorbing peaks were collected and comparison of their ^{1}H-NMR spectra with those reported in the literature[12] allowed their identification as methyl 2,3,4,6-tetra-*O*-(*p*-bromobenzoyl)-α-galactopyranoside (**3**) and methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-β-arabinopyranoside (**4**). The D configuration of the hexose (**3**; CD: $\Delta\varepsilon_{236} = -30.0$, $\Delta\varepsilon_{254} = +65.2$; A = +90.5) and the L configuration of the pentose (**4**; CD: $\Delta\varepsilon_{236} = -33.1$, $\Delta\varepsilon_{254} = +100.2$; A = +135.5) monosaccharide derivatives were deduced by comparing their exciton split CD curves with those reported by Liu and Nakanishi.[12]



Next, ^{1}H-NMR analysis of the apolar layer (fraction B, 2.0 mg) obtained from the acid methanolysis showed this to be composed of a complex mixture of degradation products derived from the aglycone moiety of the saponin **1**, probably due to facile dehydration at C-22 (giving a conjugated diene), and possibly also at C-4, as well as various products resulting from migrations of the double bond $\Delta^{8(9)}$. This mixture was analyzed no further.

This extreme instability of the saponin **1** under acidic conditions convinced us that the intact aglycone of **1** could be obtained only by an enzymatic method. To this end, 5 mg of the saponin **1** was incubated for 72 h at 38°C in phosphate/citrate buffer (pH = 5) with the glycosidase mixture extracted from *Charonia lampas*. After neutralization and filtration, the mixture obtained was dried and partitioned between water and $CHCl_3$. Chromatographic and spectroscopic evidence showed that the polar layer contained the unreacted saponin and a mixture of partial glycosides, while small amounts of the nortriterpene **5** were present in the apolar fraction. The structure of compound **5**, deduced by HRFABMS and ^{1}H-NMR spectroscopy, is in full agreement with that previously determined for the aglycone moiety of the saponin **1**. The ^{1}H-NMR spectrum of **5** has been completely assigned by detailed inspection of COSY and HOHAHA spectra and is reported in the Experimental Section.

The above data show that the structure of ectyoplaside A (**1**) is sodium 3β-*O*-[β-D-galactopyranosyl-(1→4)-α-L-arabinopyranosyl-(1→2)-β-D-galactopyranosyl]-4β,22β-dihydroxy-30-norlanosta-8(9),24-diene-14-carboxylate. A second saponin, also obtained from the butanolic extract of *E. ferox*, was named ectyoplaside B (**2**). The pseudomolecular ion peak at *m/z* 969.4590 [$M^- + Na^+ + H^+$] in the positive-ion HRFABMS and peaks at *m/z* 967.4500 [$M^- + Na^+ - H^+$] and at *m/z* 945.4691 [M^-] in the negative-ion HRFABMS indicated the molecular formula $C_{46}H_{73}NaO_{20}$ for **2**.



The chemical structure of ectyoplaside B (**2**) was mainly deduced by comparing its spectroscopic data with those of compound **1**. In particular, ^{1}H- and ^{13}C-NMR spectra of **2** (complete assignment shown in Tables 1 and 2) reveal significant differences only in some protonic resonances of the first two rings of the aglycone moiety. Indeed, in **2** also the second methyl group at C-4 is replaced by a hydroxymethyl group [29-H_2: δ = 4.03 and 3.85 (d, *J* = 13.2 Hz); C-29: δ = 60.2 (^3J coupled with 3-H and 5-H in the HMBC spectrum)], in accordance with the molecular formula, which indicates only one more oxygen atom than **1**. This CH_2OH group causes marked deshielding effects, above all on 3-H (δ = 3.70 in **2**, δ = 3.27 in **1**), 5-H (δ = 1.57 in **2**, δ = 1.35 in **1**) and 6eq-H (δ = 2.15 in **2**, δ = 1.63 in **1**).

A small amount of ectyoplaside B was then subjected to acetylation, affording the peracetyl derivative **2a**. Detailed analysis of the ^{1}H-NMR spectrum of **2a** (reported in the Experimental Section), performed with the aid of 2D-NMR experiments, revealed that the sugar portion of **2** is identical to that of **1**, thus allowing a complete description of the structure of ectyoplaside B (**2**) as sodium 3β-*O*-[β-D-galactopyranosyl-(1→4)-α-L-arabinopyranosyl-(1→2)-β-D-galactopyranosyl]-4β,22β,29-trihydroxy-30-norlanosta-8(9),24-diene-14-carboxylate.

Ectyoplasides A (**1**) and B (**2**) were tested in vitro for cytotoxic activity with J774 (murine monocyte-macrophage), WEHI164 (murine fibrosarcoma), and P388 (murine leukemia) cell lines. Both compounds were mildly cytotoxic, exhibiting IC_{50} values ranging from 8.5 to 11.0 μg/mL.

Saponins are widely encountered as secondary metabolites among the higher plants. From the marine environment, saponins have mainly been isolated from echinoderms,[13] such as starfish (steroidal glycosides) and sea cucumbers (lanostane glycosides), although some examples of pregnane- or cholestane-type steroidal monoglycosides have also been reported from gorgonians,[14] fish,[15] and soft co-

rals.[16] In contrast, spongal saponins are extremely rare, and although hundreds of Porifera species have been analyzed with regard to their chemical compositions, only two or three saponin skeletons have yet been found, namely in erylosides.[17−19] formoside[5] (mostly glycosides of penasterol derivatives, from *Erylus* spp.) and sarasinosides[20−23] (mostly glycosides of norlanostane derivatives, from *Asteropus* spp.).

It should be noted that all these triterpene saponins have been isolated from sponges of the order Choristida (also known as Astrophorida) and so the isolation of ectyoplasides is especially remarkable because they represent, along with the very recently reported ulososides,[24] the first saponins found in a non-Choristida sponge (the Axinellida order of *E. ferox* is far from the Choristida order from a phylogenetic point of view). In addition, this paper must be considered as the first report on the chemical composition of an *Ectyoplasia* sponge.

Experimental Section

General: Optical rotations (MeOH or $CHCl_3$): Perkin−Elmer 192 polarimeter equipped with a sodium lamp (λ = 589 nm) and a 10-cm microcell. − IR (KBr): Bruker IFS-48 spectrophotometer. − UV spectra (CH_3CN): Beckman DU70 spectrophotometer. − CD spectra (CH_3CN): JASCO 500A polarimeter. − MS: Low- and high-resolution FAB mass spectra (CsI ions, glycerol/thioglycerol matrix): VG Prospec (Fisons) mass spectrometer equipped with an FAB source. EI mass spectra (70 eV): VG Prospec (Fisons) mass spectrometer equipped with an EI source. − ^{1}H- (500.1 MHz) and ^{13}C- (125.0 MHz) NMR spectra: Bruker AMX-500 spectrometer; chemical shifts are referenced to the residual solvent signal (CD_3OD: δ_H = 3.34, δ_C = 49.0; $CDCl_3$: δ_H = 7.26). Methyl, methylene, and methine carbon atoms were distinguished by DEPT experiments. Homonuclear ^{1}H connectivities were determined by performing COSY experiments. The 2D-HOHAHA experiment was performed in the phase-sensitive mode (TPPI) with an MLEV-17 sequence for mixing.[25] One-bond heteronuclear ^{1}H-^{13}C connectivities were determined with the Bax-Subramanian[26] HMQC pulse sequence on using a BIRD pulse 0.50 s before each scan in order to suppress the signals originating from protons not directly bound to ^{13}C (interpulse delay set for $^1J_{CH}$ = 135 Hz). During the acquisition time, ^{13}C broad-band decoupling was performed with the GARP sequence. Two- and three-bond ^{1}H-^{13}C connectivities were determined by HMBC[27] experiments optimized for a $^{2,3}J$ of 9 Hz. − Medium-pressure liquid chromatography (MPLC): Büchi 861 apparatus with an SiO_2 column (230−400 mesh). − HPLC separations: Waters 501 apparatus equipped with a refractometer detector and with Hibar RP-18 LiChrospher (250 × 4 mm) columns. Other separations were achieved using a Whatman Partisil PXS M9 column with a UV detector (λ = 260 nm). − GC-MS analyses: Hewlett-Packard 5890 gas chromatograph with a mass-selective detector MSD HP 5790 MS. A fused-silica column (25 m × 0.20 mm HP-5; cross-linked 25% Ph-Me silicone, 0.33 mm film thickness) was used. Carrier gas: hydrogen, 10 mL/min; temperature 135°C.

Collection, Extraction and Isolation: A specimen of *Ectyoplasia ferox* was collected by hand along the coasts of San Salvador Island, and identified by Prof. M. Pansini (Università di Genova). A voucher sample (no. SS 1305) has been deposited at the Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli, Italy. The sponge (55 g dry weight after extraction), frozen immediately after collection and kept frozen until extraction, was first homogenized and then exhaustively extracted with methanol (4 × 500 mL). The obtained extract was diluted with MeOH/H_2O (9:1) and then partitioned against *n*-hexane (3 × 500 mL) to yield an apolar extract weighing 1.4 g. Successively, the water content of the hydromethanolic phase was adjusted to 20% (v/v) and 40% (v/v) and the solution was partitioned against CCl_4 (3 × 500 mL) and $CHCl_3$ (3 × 500 mL), respectively, to afford a carbon tetrachloride (0.8 g) and a chloroform (2.5 g) extract. Finally, all the MeOH was evaporated from the methanolic layer, and the aqueous solution thus obtained was partitioned against *n*BuOH. The butanol-soluble material (5.7 g), which was the richest in saponins, was subjected to MPLC purification on silica gel (230−400 mesh), eluting with a solvent gradient system of increasing polarity from EtOAc to MeOH. Fractions eluted with MeOH/EtOAc (7:3) were combined and then further purified by reversed-phase HPLC (eluent: MeOH/H_2O, 8:2; flow rate 0.7 mL/min), yielding pure ectyoplasides A (**1**, 13.5 mg) and B (**2**, 4.5 mg).

Ectyoplaside A (1): White, amorphous solid. − $[\alpha]_D^{25}$ = +3 (*c* = 0.002 in MeOH). − IR (KBr): $\tilde{\nu}$ = 3406, 2924, 1635, 1573, 1454, 1348, 1261 cm^{-1}. − ^{1}H- and ^{13}C-NMR (CD_3OD): See Tables 1 and 2. − FABMS (positive ions, glycerol/thioglycerol matrix); *m/z*: 953, 497. − FABMS (negative ions, thioglycerol matrix); *m/z*: 951, 929. − HRFABMS (positive ions); *m/z*: 953.4734 [$M^- + Na^+ + H^+$]; calcd. for $C_{46}H_{74}NaO_{19}$: 953.4721. − HRFABMS (negative ions); *m/z*: 951.4560 [$M^- + Na^+ - H^+$]; calcd. for $C_{46}H_{72}NaO_{19}$: 951.4573; *m/z*: 929.4731 [M^-]; calcd. for $C_{46}H_{73}O_{19}$: 929.4751.

Ectyoplaside A Decaacetate (1a): 3.5 mg of ectyoplaside A (**1**) was peracetylated with Ac_2O/pyridine at room temperature for 12 h yielding 4 mg of ectyoplaside A decaacetate (**1a**) as a colorless, amorphous oil. − $[\alpha]_D^{25}$ = +11 (*c* = 0.002 in $CHCl_3$). − ^{1}H NMR ($CDCl_3$, *J* in Hz): δ = 5.45 (d, *J* = 3.2, 1′′-H), 5.40 (d, *J* = 3.1, 4′′′-H), 5.28 (br. d, *J* = 2.8, 4′-H), 5.22 (t, *J* = 8.1, 2′′′-H), 5.07 (dd, *J* = 7.1, 2.8, 3′-H), 5.05 (24-H, overlapped), 4.95 (dd, *J* = 8.1, 3.1, 3′′′-H), 4.93 (22-H, overlapped), 4.85 (dd, *J* = 9.5, 3.2, 2′′-H), 4.83 (br. d, *J* = 9.5, 3′′-H), 4.59 (d, *J* = 7.9, 1′-H), 4.48 (d, *J* = 8.1, 1′′′-H), 4.33 (br. s, 4′′-H), 4.20 (6′a-H and 6′b-H, overlapped), 4.18 (6′′′a-H and 6′′′b-H, overlapped), 4.05 (dd, *J* = 7.9, 7.1, 2′-H), 3.85 (t, *J* = 6.6, 5′-H and 5′′′-H), 3.50 (br. s, 5′′-H_2), 3.33 (br. d, *J* = 11.5, 3-H), 2.50 (br. dd, *J* = 11.5, 11.2, 2ax-H), 2.30 (m, 23a-H), 2.15 (11-H_2, overlapped), 2.15−1.95 (10 CH_3CO signals, each s), 2.14 (23b-H, overlapped), 2.11 (7ax-H, overlapped), 2.05 (7eq-H, overlapped), 1.96 (12eq-H, overlapped), 1.95 (15a-H, overlapped), 1.88 (2eq-H, overlapped), 1.85 (1ax-H, overlapped), 1.85 (16a-H, overlapped), 1.83 (6ax-H, overlapped), 1.69 (br. s, 27-H_3), 1.62 (br. s, 26-H_3), 1.60 (m, 15b-H), 1.55 (dd, *J* = 7.3, 5.5, 20-H), 1.48 (br. s, 29-H_3), 1.37 (6eq-H, overlapped), 1.35 (12ax-H, overlapped), 1.35 (5-H, overlapped), 1.32 (16b-H, overlapped), 1.25 (1eq-H, overlapped), 1.25 (17-H, overlapped), 1.02 (br. s, 19-H_3), 0.95 (d, *J* = 7.3, 21-H_3), 0.58 (br. s, 18-H_3). − FABMS (positive ions, CsI ions, glycerol matrix); *m/z*: 1373 [$M^- + Na^+ + H^+$].

Molecular Modeling: Computer modeling studies were carried out using the Quanta/CHARMm 4.0 program (Molecular Simulations Inc., 200 Fifth Avenue, Waltham, MA 02154) with a Silicon Graphics Personal Iris 4D-35G computer. No explicit solvent molecules were included in these calculations, but a distance-dependent dielectric constant (RDIE) was used to partially compensate for the absence of solvent. Molecular dynamics (MD) simulations involved a heating period of 5.0 ps, followed by a 5.0 ps equilibration period and then 100 ps of dynamics simulation. The time step of integration was 1 fs. During MD calculations, bond lengths involving

hydrogen atoms were kept fixed using the SHAKE algorithm. The coordinates produced by the simulation were saved every 0.5 ps, giving 250 structures. Each of these was subjected to energy minimization using the conjugated gradient protocol.

Methanolysis and Silylation: 4 mg of ectyoplaside A (**1**) was dissolved in 1 N HCl in 85% MeOH and the solution was heated at 70°C in a stoppered reaction vial for 2 h. After cooling, the reaction mixture was neutralized with Ag_2CO_3, centrifuged, and the supernatant was concentrated to dryness under N_2. The residue was then partitioned between water (2 × 5 mL) and $CHCl_3$ (2 × 5 mL) yielding a polar layer (fraction A, 2.2 mg) and an apolar layer (fraction B, 2.0 mg). Fraction B was not analyzed further, whereas fraction A was divided into two portions. The first of these (0.8 mg) was dissolved in TRISIL Z (0.15 mL, *N*-trimethylsilylimidazole in pyridine, Pierce Chemical Co.), the solution was left at 35°C for 15 min, and then analyzed by GC MS. Peaks obtained for this mixture exhibited the same retention times and the same mass spectra as those obtained from the standard persilylated methyl glycosides of galactose and arabinose, prepared in the same manner.

***p*-Bromobenzoylation and CD:** A second portion of fraction A (1.4 mg) was dissolved in dry pyridine (1.5 mL) and treated with *p*-bromobenzoyl chloride (20 mg) and a catalytic amount of 4-(dimethylamino)pyridine. The mixture was stirred overnight at 60°C, cold water was then added, and after 30 min, the mixture was extracted with $CHCl_3$. The obtained extract was washed successively with saturated aqueous $NaHCO_3$ and water, and then the solvent was evaporated under reduced pressure. The benzoate mixture thus obtained was separated by HPLC: Whatman Partisil PXS M9 column, eluent *n*-hexane/diethyl ether (85:15), UV detector (λ = 260 nm). Only two major peaks were collected.

Methyl 2,3,4,6-Tetra-*O*-(*p*-bromobenzoyl)-α-D-galactopyranoside (3): EIMS; *m/z*: 926. − ^{1}H NMR ($CDCl_3$): δ = 7.92, 7.85, 7.82, 7.63, 7.61, 7.57, 7.53, 7.39 (8 d, *J* = 8.8 Hz, each 2 H, ArH), 5.97 (d, *J* = 3.5 Hz, 1 H, 4-H), 5.90 (dd, *J* = 10.5 and 3.5 Hz, 1 H, 3-H), 5.60 (dd, *J* = 10.5 and 3.5 Hz, 1 H, 2-H), 5.28 (d, *J* = 3.5 Hz, 1 H, 1-H), 4.58 (m, 2 H, 5-H and 6a-H), 4.37 (m, 1 H, 6b-H). − CD (CH_3CN): $\Delta\varepsilon_{236}$ = −30.0, $\Delta\varepsilon_{254}$ = +65.2; A = +90.5.

Methyl 2,3,4-Tri-*O*-(*p*-bromobenzoyl)-β-L-arabinopyranoside (4): EIMS; *m/z*: 713. − ^{1}H NMR ($CDCl_3$): δ = 8.03, 7.88, 7.82, 7.69, 7.59, 7.57, 7.19 (each 2 H, ArH), 5.93 (dd, *J* = 10.5 and 3.5 Hz, 1 H, 3-H), 5.77 (d, *J* = 3.5 Hz, 1 H, 4-H), 5.70 (dd, *J* = 10.5 and 3.5 Hz, 1 H, 2-H), 5.21 (d, *J* = 6.5 Hz, 1 H, 1-H), 4.18 (d, *J* = 12.5 Hz, 1 H, 5a-H), 3.97 (d, *J* = 12.5 Hz, 1 H, 5b-H). − CD (CH_3CN): $\Delta\varepsilon_{236}$ = −33.1, $\Delta\varepsilon_{254}$ = +100.2; A = +135.5.

Enzymatic Hydrolysis: A solution of ectyoplaside A (**1**, 5 mg) in phosphate/citrate buffer (1.5 mL) at pH = 5.0 was incubated with 10 mg of glycosidase mixture from *Charonia lampas* (Scikagaku kogyo) at 38°C for ca. 72 h. The mixture was then neutralized, filtered, and the filtrate was partitioned between H_2O and $CHCl_3$. The aqueous layer was concentrated to dryness and the residue was found to contain salts, the unreacted saponin, and a mixture of partial glycosides. The organic extract was dried with Na_2SO_4, filtered, and concentrated in vacuo, to afford a fraction which was shown to contain compound **5** (ca. 0.3 mg).

Compound 5: Colorless, amorphous oil. HRFABMS (positive ions, glycerol matrix); *m/z*: 475.3430 [M + H$^+$]; calcd. for $C_{29}H_{47}O_5$: 475.3421. − ^{1}H NMR (CD_3OD): δ = 5.15 (br. t, *J* = 9.5, 24-H), 3.67 (dd, *J* = 7.8, 5.6, 22-H), 3.21 (br. d, *J* = 11.0, 3-H), 2.61 (br. dd, *J* = 10.1, 9.2, 2ax-H), 2.20 (11ax-H, overlapped), 2.17 (23a-H, overlapped), 2.16 (11eq-H, overlapped), 2.12 (23b-H, overlapped), 2.12 (7ax-H, overlapped), 2.11 (15a-H, overlapped), 2.05 (7eq-H, overlapped), 2.03 (16a-H, overlapped), 2.00 (12eq-H, overlapped), 1.92 (2eq-H, overlapped), 1.90 (dd, *J* = 10.5, 8.2, 12ax-H), 1.85 (1ax-H, overlapped), 1.85 (6ax-H, overlapped), 1.79 (d, *J* = 8.9, 6eq-H), 1.73 (br. s, 27-H_3), 1.66 (br. s, 26-H_3), 1.66 (15b-H, overlapped), 1.56 (dd, *J* = 7.2, 5.0, 17-H), 1.47 (dd, *J* = 7.0, 5.0, 20-H), 1.38 (br. s, 29-H_3), 1.34 (5-H, overlapped), 1.33 (16b-H, overlapped), 1.26 (1eq-H, overlapped), 1.12 (br. s, 19-H_3), 0.93 (d, *J* = 7.0, 21-H_3), 0.67 (br. s, 18-H_3).

Ectyoplaside B (2): White, amorphous solid. − $[\alpha]_D^{25}$ = −12 (*c* = 0.002 in MeOH). − IR (KBr): $\tilde{\nu}$ = 3500, 2914, 1635, 1573, 1454, 1348, 1261 cm^{-1}. − ^{1}H and ^{13}C NMR (CD_3OD): See Tables 1 and 2. − FABMS (positive ions, glycerol/thioglycerol matrix); *m/z*: 969. − FABMS (negative ions, thioglycerol matrix); *m/z*: 967, 945. − HRFABMS (positive ions); *m/z*: 969.4590 [M$^-$ + Na$^+$ + H$^+$]; calcd. for $C_{46}H_{74}O_{20}Na$: 969.4670. − HRFABMS (negative ions); *m/z*: 967.4500 [M$^-$ + Na$^+$ − H$^+$]; calcd. for $C_{46}H_{72}O_{20}Na$: 967.4512; *m/z*: 945.4691 [M$^-$]; calcd. for $C_{46}H_{73}O_{20}$: 945.4701.

Ectyoplaside B Undecaacetate (2a): 2.0 mg of ectyoplaside B (**2**) was peracetylated with Ac_2O/pyridine at room temperature for 12 h, yielding 2.4 mg of ectyoplaside B undecaacetate (**2a**) as a colorless, amorphous oil. − $[\alpha]_D^{25}$ = −15 (*c* = 0.002 in $CHCl_3$). − ^{1}H NMR ($CDCl_3$, *J* in Hz): δ = 5.45 (d, *J* = 3.2, 1′′-H), 5.40 (d, *J* = 3.1, 4′′′-H), 5.28 (br. s, 4′-H), 5.22 (t, *J* = 8.1, 2′′′-H), 5.05 (24-H, overlapped), 4.95 (d, *J* = 7.3, 3′-H), 4.94 (dd, *J* = 8.1, 3.1, 3′′′-H), 4.93 (22-H, overlapped), 4.85 (dd, *J* = 9.8, 3.2, 2′′-H), 4.82 (d, *J* = 12.9, 29a-H), 4.71 (br. d, *J* = 9.8, 3′′-H), 4.46 (d, *J* = 8.1, 1′-H), 4.46 (d, *J* = 8.1, 1′′′-H), 4.38 (br. s, 4′′-H), 4.22 (dd, *J* = 10.7, 6.0, 6′a-H), 4.16 (d, *J* = 12.9, 29b-H), 4.08 (d, *J* = 6.3, 6′′′a-H and 6′′′b-H), 4.05 (dd, *J* = 10.7, 6.0, 6′b-H), 4.02 (dd, *J* = 8.1, 7.3, 2′-H), 3.89 (3-H, overlapped), 3.87 (t, *J* = 6.3, 5′′′-H), 3.78 (t, *J* = 6.0, 5′-H), 3.50 (br. s, 5′′-H_2), 2.52 (br. dd, *J* = 11.4, 11.0, 2ax-H), 2.30 (m, 23a-H), 2.15−1.95 (11 s, 11 CH_3CO signals), 2.15 (23b-H, overlapped), 2.11 (7ax-H, overlapped), 2.10 (11-H_2, overlapped), 2.05 (7eq-H, overlapped), 1.96 (15a-H, overlapped), 1.95 (12eq-H, overlapped), 1.88 (2eq-H, overlapped), 1.88 (6ax-H, overlapped), 1.85 (16a-H, overlapped), 1.80 (1ax-H, overlapped), 1.69 (br. s, 27-H_3), 1.60 (br. s, 26-H_3), 1.58 (6eq-H, overlapped), 1.58 (15b-H, overlapped), 1.55 (dd, *J* = 6.6, 5.7, 20-H), 1.53 (5-H, overlapped), 1.38 (dd, *J* = 10.3, 8.2, 12ax-H), 1.31 (16b-H, overlapped), 1.25 (17-H, overlapped), 1.22 (1eq-H, overlapped), 1.04 (br. s, 19-H_3), 0.98 (d, *J* = 6.6, 21-H_3), 0.58 (br. s, 18-H_3). − FABMS (positive ions, CsI ions, glycerol matrix); *m/z*: 1431 [M$^-$ + Na$^+$ + H$^+$].

Bioassays, Cells and Materials: WEHI164 cells (murine fibrosarcoma cell line) were maintained in adhesion on Petri dishes with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 U/mL) and streptomycin (100 μg/mL). J774 cells (murine monocyte/macrophage cell line) were grown in suspension culture, in Techne stirrer bottles spun at 25 rpm and incubated at 37°C, in DMEM medium supplemented with 10% FBS, 25 mM Hepes, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL). P388 cells (murine leukemia cell line) were grown in adhesion on Petri dishes with L-15 (Leibovitz) medium supplemented with 10% FBS, 25 mM HEPES, penicillin (100 U/mL) and streptomycin (100 μg/mL). All reagents for cell culture were purchased from Cellbio; MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide] and 6-mercaptopurine were obtained from Sigma.

Cytotoxic Activity: WEHI164, J774, P388 (4 × 10^3 cells) were placed in 96-well plates and allowed to adhere at 37°C in 5% CO_2/95% air for 2 h. Thereafter, the medium was replaced with 50 μL

of fresh medium and then 75 μL aliquots of 1:2 (v/v) serial dilution of test compounds **1** and **2** were added and the cells were incubated for 72 h. The cells viability was assessed through an MTT conversion assay[28]. After 72 h, 25 μL of MTT (5 mg/mL) was added and the cells were incubated for a further 3 h. Subsequently, the cells were lyzed and the dark-blue crystals were solubilized with 100 μL of a solution containing 50% (v/v) *N,N*-dimethylformamide and 20% (w/v) SDS, adjusted to pH = 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620-nm filter. The viability of each cell line in response to treatment with compounds **1** and **2** was calculated as:% dead cells = 100 − (OD treated/OD control) × 100. The cytotoxic activities are expressed as IC_{50} values (μg/mL): ectyoplaside A (**1**): WEHI164: 9.5, J774: 10.5, P388: 8.5; ectyoplaside B (**2**): WEHI164: 10.2, J774: 10.5, P388: 9.5. All measurements were performed in triplicate and the data reported are mean values.

Acknowledgments

This work was supported by M.U.R.S.T., PRIN "Chimica dei Composti Organici di Interesse Biologico", Rome, Italy. We thank Prof. W. Fenical for giving us the opportunity to participate in an expedition to the Caribbean Sea, during which the sponge E. ferox was collected, and Prof. M. Pansini (Istituto di Zoologia, Università di Genova, Italy) for identifying the organism. Mass, UV, IR, and NMR experiments were performed at the "Centro di Ricerca Interdipartimentale di Analisi Strumentale", Università di Napoli "Federico II". We also thank Dr. A. Ianaro for carrying out cytotoxicity assays.

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Received July 28, 1998
[O98354]