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Feroxosides A-B, two norlanostane tetraglycosides from the Caribbean sponge *Ectyoplasia ferox*

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Abstract—Feroxosides A and B, have been isolated from the polar extract of the Caribbean sponge *Ectyoplasia ferox*. Their structures have been determined to be unusual C-4 nor-lanostane triterpenes glycosylated with a rhamnose containing tetrasaccharide chain, by interpretation of spectral data and chemical degradation. Absolute stereochemistry at C-23 has been determined by application of the modified Mosher method for secondary alcohols. Feroxosides A-B are moderately cytotoxic (IC_{50} 19 μg/mL) against murine monocyte-macrophage cell line.
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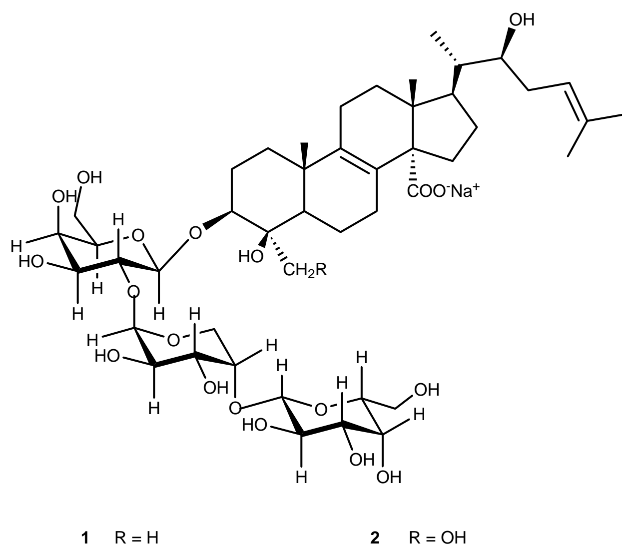
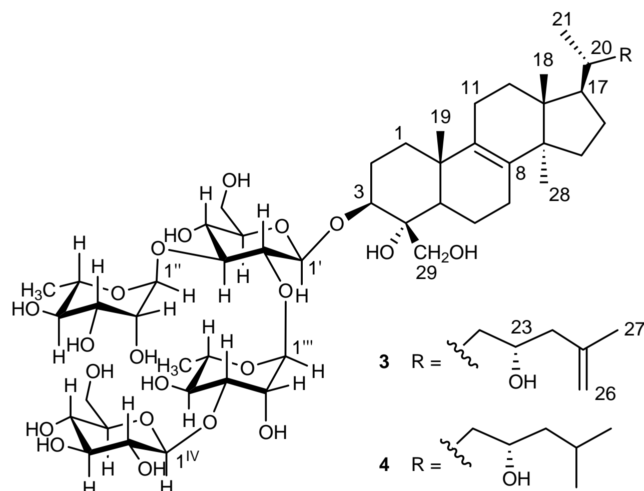
Saponins are among the less well-represented secondary metabolites isolated from marine sponges. To the best of our knowledge, not more than a dozen molecules with this structural framework have been reported to date, e.g. eryl-osides[1] and formoside[2] from *Erylus* spp., sarasinosides[3] from *Asteropus* spp., ulososides[4] from *Ulosa* sp., and wondosterols[5] from a *Poecillastra wondoensis*/*Jaspis wondoensis* association. In addition, in the course of our chemical investigation of the sponge *Ectyoplasia ferox*, Duchassaing and Michelotti, (Demospongiae, family Raspaliidae, order Axinellida), we recently isolated two unique antitumor nortriterpene glycosides, ectyoplaside A (**1**) and B (**2**).[6] Analysis of the methanolic extract obtained by another specimen of the same sponge has now led to the isolation, together with ectyoplasides, of two new terpenoid saponins, named feroxoside A (**3**) and B (**4**). In this paper we describe the isolation and structural elucidation of feroxo-sides, which differ from ectyoplasides for both the aglycone and the sugar moieties.



The MeOH extract of the Caribbean sponge *E. ferox*, collected along the coasts of Grand Bahama Island, was partitioned against *n*-hexane, CCl_4, $CHCl_3$, and *n*-butanol, according to the Kupchan method.[7] The butanol soluble material, most abundant in saponins, was initially separated by MPLC over silica gel (230–400 mesh) eluting with a solvent gradient system of increasing polarity from EtOAc to MeOH. Fractions eluted with MeOH–EtOAc 9:1 were combined and further purified by reversed-phase HPLC (eluent MeOH–H_2O 7:3) to furnish pure feroxosides A (**3**, 16.0 mg) and B (**4**, 8.3 mg) as white amorphous solids.



Keywords: marine metabolites; terpene glycosides; NMR spectroscopy; stereochemistry.

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Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data of the aglycone portion of feroxoside A (**3**) and B (**4**)

Position	**3**		**4**	
	δ_C (mult.)	δ_H (int., mult., J in Hz)	δ_C (mult.)	δ_H (int., mult., J in Hz)
1ax	36.5 (CH_2)	1.76 (1H, dd, 11.8, 6.5)	36.7 (CH_2)	1.75 (1H, br.dd, 11.5, 6.5)
eq		1.24 (1H, m)		1.23 (1H, m)
2ax	27.0 (CH_2)	2.33 (1H, dt, 11.8, 8.5)	27.3 (CH_2)	2.32 (1H, dt, 11.5, 8.5)
eq		1.89[a]		1.90[a]
3	85.2 (CH)	3.83[a]	85.1 (CH)	3.81[a]
4	65.0 (C)		65.0 (C)	
5	49.1 (CH)	1.38[a]	49.1 (CH)	1.37[a]
6ax	23.4 (CH_2)	2.10[a]	23.1 (CH_2)	2.10[a]
eq		1.63 (1H, m)		1.62 (1H, m)
7ax	33.1 (CH_2)	2.20 (1H, dd, 11.5, 8.1)	33.0 (CH_2)	2.22 (1H, dd, 11.5, 8.1)
eq		2.10[a]		2.10[a]
8	126.2 (C)		126.1 (C)	
9	136.0 (C)		136.8 (C)	
10	37.1 (C)		37.3 (C)	
11	27.1 (CH_2)	2.11[a] (2H)	27.1 (CH_2)	2.11[a] (2H)
12ax	38.0 (CH_2)	1.43[a]	38.5 (CH_2)	1.43[a]
eq		2.04[a]		2.03[a]
13	43.0 (C)		43.2 (C)	
14	52.8 (C)		53.0 (C)	
15a	30.0 (CH_2)	1.62[a]	30.0 (CH_2)	1.62[a]
b		1.38[a]		1.33[a]
16a	29.2 (CH_2)	1.95 (1H, m)	25.6 (CH_2)	1.95[a]
b		1.42[a]		1.41[a]
17	56.3 (CH)	1.18 (1H, dd, 7.5, 5.5)	56.9 (CH)	1.18[a]
18	11.9 (CH_3)	0.68 (3H, br.s)	11.4 (CH_3)	0.69 (3H, br.s)
19	20.0 (CH_3)	0.97 (3H, br.s)	20.0 (CH_3)	0.98 (3H, br.s)
20	44.0 (CH)	1.78 (1H, m)	43.9 (CH)	1.77 (1H, m)
21	18.8 (CH_3)	1.00 (3H, d, 6.6)	19.0 (CH_3)	1.00 (3H, d, 6.6)
22a	44.5 (CH_2)	1.50 (1H, bt, 10.5)	45.0 (CH_2)	1.50 (1H, bt, 10.5)
b		1.06 (1H, dt, 10.5, 3.5)		1.05 (1H, dt, 10.5, 3.5)
23	67.5 (CH)	3.86[a]	66.7 (CH)	3.75[a]
24a	47.7 (CH_2)	2.24 (1H, dd, 10.1, 5.1)	48.9 (CH_2)	1.40[a]
b		2.09[a]		1.18[a]
25	143.1 (C)		25.0 (CH)	1.79[a]
26a	113.2 (CH_2)	4.80 (1H, s)	22.3 (CH_3)	0.93 (3H, d, 7.1)
b		4.74 (1H, s)		
27	23.1 (CH_3)	1.76 (3H, br.s)	22.3 (CH_3)	0.93 (3H, d, 7.1)
28	29.6 (CH_3)	1.31 (3H, s)	30.1 (CH_3)	1.31 (3H, s)
29a	63.1 (CH_2)	4.25 (1H, d, 11.8)	62.7 (CH_2)	4.26 (1H, d, 11.8)
b		3.40 (1H, d, 11.8)		3.41 (1H, d, 11.8)

Recorded in CD_3OD.
[a] Overlapped with other signals.

The structure of the more abundant feroxoside A (**3**), $[\alpha]_D=-16$ (c=0.05 in MeOH), was inferred by extensive application of spectroscopic methods, above all 2D NMR techniques. The FAB mass spectrum of **3** exhibited a quasi-molecular ion peak at m/z 1075 $[M-H]^-$, in the negative ion mode, and at m/z 1099 $[M+Na]^+$, in the positive ion mode. The molecular formula of **3** was determined as $C_{53}H_{88}O_{22}$ on the basis of high-resolution FABMS (negative ions) peak at m/z 1075.5698 ($C_{53}H_{88}O_{22}$ requires m/z 1075.5689), and was in accordance with ^{13}C NMR data. The IR (KBr) spectrum of **3** showed absorption bands due to hydroxyl groups (ν_{max} 3410 cm^{-1}) and double bonds (ν_{max} 1635 cm^{-1}).

The glycoterpene nature of **3** was suggested by a preliminary inspection of its ^{1}H NMR spectrum (in CD_3OD, Tables 1 and 2). It exhibited the signals of: (i) seven methyl groups (four singlets and three doublets) (ii) some overlapping signals from δ 1.0 to 2.4 (iii) a number of signals between δ 3.4 and 5.3, attributable to protons on oxygen-bearing carbons. The ^{13}C NMR spectrum of **3** (in CD_3OD, Tables 1 and 2) indicated the tetrasaccharide nature of the sugar portion, showing the resonances of four anomeric carbons (δ 101.0, 101.6, 101.7, and 104.3). In addition, four sp^2 carbons at δ 113.2 (CH_2), 126.2 (C), 136.0 (C), and 143.1 (C) were present in the ^{13}C NMR spectrum of **3,** suggesting that a *gem*-disubstituted and a tetrasubstituted double bond were part of feroxoside A (**3**). All the proton resonances were unambiguously associated with the relevant carbon atoms by using the 2D ^{1}H-detected HMQC spectrum.

Inspection of HOHAHA (HOmonuclear HArtmann HAhn) spectrum of **3** allowed us to detect eight distinct spin systems (evidenced in Fig. 1), four of them belonging to the aglycone moiety and the remaining to the tetrasaccharide. The proton sequence within each spin system was elucidated by following the series of cross peaks of the COSY spectrum, while data arising from the HMBC experiment (Fig. 1) were used to locate the tetra-substituted carbon atoms (Table 1), and to interconnect the partial substructures. In this regard, the following HMBC cross-peaks were particularly diagnostic: H_2-29 (δ 4.25 and 3.40) and C-4 (δ 65.0); H_3-19 (δ 0.97) and C-10 (δ 37.1)/

Table 2. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data of the sugar portion of feroxosides A (**3**) and B (**4**)

Position	**3**		**4**	
	δ_C (mult.)	δ_H (int., mult., *J* in Hz)	δ_C (mult.)	δ_H (int., mult., *J* in Hz)
1′	101.6 (CH)	4.95 (1H, d, 8.8)	101.8 (CH)	4.96 (1H, d, 8.8)
2′	79.7 (CH)	3.42 (1H, t, 8.8)	79.7 (CH)	3.47 (1H, t, 8.8)
3′	78.7 (CH)	3.64[a]	78.8 (CH)	3.63[a]
4′	78.8 (CH)	3.67 (1H, dd, 9.6, 8.5)	78.8 (CH)	3.67 (1H, dd, 9.5, 8.5)
5′	76.6 (CH)	3.38[a]	76.7 (CH)	3.38[a]
6′a	61.2 (CH_2)	3.91[a]	60.5 (CH_2)	3.90[a]
b		3.79 (1H, dd, 12.5, 3.7)		3.82[a]
1″	101.0 (CH)	5.04 (1H, br.s)	101.1 (CH)	5.04 (1H, br.s)
2″	72.2 (CH)	3.82 (1H, d, 1.8)	72.5 (CH)	3.83 (1H, d, 1.8)
3″	72.0 (CH)	3.89 (1H, dd, 8.5, 1.8)	72.4 (CH)	3.90 (1H, dd, 8.5, 1.8)
4″	76.9 (CH)	3.62[a]	77.0 (CH)	3.60[a]
5″	67.4 (CH_2)	4.20 (1H, dq, 10.5, 6.6)	66.4 (CH_2)	4.22 (1H, dq, 10.5, 6.6)
6″	21.2 (CH_3)	1.42 (3H, d, 6.6)	21.0 (CH_3)	1.42 (3H, d, 6.6)
1‴	101.7 (CH)	5.23 (1H, br.s)	101.7 (CH)	5.23 (1H, br.s)
2‴	72.0 (CH)	3.97 (1H, d, 1.8)	72.0 (CH)	3.98 (1H, d, 1.8)
3‴	73.9 (CH)	3.77 (1H, dd, 8.5, 1.8)	73.1 (CH)	3.78 (1H, dd, 8.5, 1.8)
4‴	74.2 (CH)	3.44[a]	74.5 (CH)	3.45[a]
5‴	69.5 (CH)	4.13 (1H, dq, 11.7, 5.9)	69.5 (CH)	4.11, (1H, dq, 11.7, 5.9)
6‴	17.6 (CH_3)	1.35 (3H, d, 5.9)	17.7 (CH_3)	1.35 (3H, d, 5.9)
1^{IV}	104.5 (CH)	4.55 (1H, d, 8.1)	104.3 (CH)	4.57 (1H, d, 8.0)
2^{IV}	76.7 (CH)	3.59[a]	77.5 (CH)	3.59[a]
3^{IV}	79.1 (CH)	3.62[a]	78.7 (CH)	3.61[a]
4^{IV}	74.3 (CH)	3.47 (1H, dd, 8.8, 7.3)	74.5 (CH)	3.48 (1H, dd, 8.8, 7.3)
5^{IV}	76.9 (CH)	3.61[a]	76.9 (CH)	3.68[a]
6^{IV} a	61.1 (CH_2)	4.10[a]	60.3 (CH_2)	4.12[a]
b		3.71[a]		3.69[a]

Recorded in CD_3OD.
[a] Overlapped with other signals.

C-9 (δ 136.0); H_2-7 (δ 2.10 and 2.20) and C-8 (δ 126.2); H_3-18 (δ 0.68) and C-13 (δ 43.0)/C-14 (δ 52.8); H_3-27 (δ 1.76) and C-25 (δ 143.1). This analysis allowed us to identify the aglycone moiety of **3** as an unprecedented nor-lanostane triterpene, with a methylene group at C-25 and hydroxyls at C-23, C-29, and C-4, respectively. The last OH group, as in ectyoplasides A (**1**) and B (**2**),[6] replaces the methyl group usually linked at C-4 in lanostane derivatives. To the best of our knowledge, the co-occurrence of hydroxymethyl and hydroxyl groups at C-4 of a lanostane skeleton is a unique feature of *Ectyoplasia* triterpenoids.

Furthermore, some diagnostic spatial couplings evidenced through the ROESY spectrum of **3** (Fig. 2), integrated by $^{3}J_{HH}$ values (Table 1), allowed us to elaborate the relative stereochemistry of the nor-lanostane moiety. Taking these data into account and assuming that our aglycone possesses the absolute configuration invariably found in all the lanostane derivatives isolated to date, the absolute stereochemistry of the chiral centers belonging to the tetracyclic system of **3** can be assigned as reported in figure. Notably, the dipolar coupling between H_2-29 and H_3-19 (Fig. 2) indicates a configuration at C-4 which is opposite of that found in ectyoplaside B (**2**), and, accordingly, ^{1}H and ^{13}C NMR data of the ring A of **3** appear rather different from parallel data of **2**.[6] Finally, the sugar unit has been confidently linked

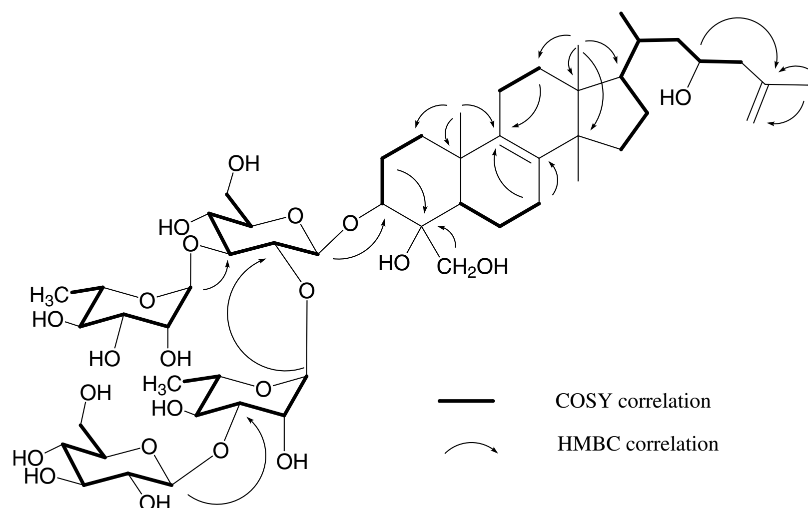


Figure 1. Partial structures by COSY and key HMBC correlations.

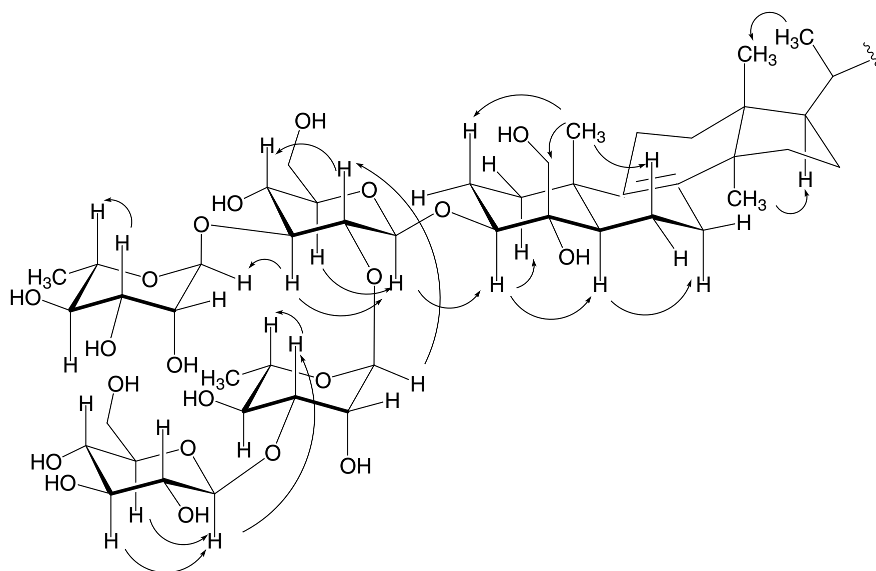


Figure 2. Spatial couplings of feroxoside A, evidenced through the ROESY spectrum.

at C-3 of the aglycone basing on the HMBC correlation between the downfield shifted C-3 (δ 85.2) and H-1′ (δ 4.95), further supported by the ROESY cross-peak between H-1′ and H-3 (δ 3.83).

Determination of the nature of each monosaccharide belonging to the tetrasaccharide chain and elucidation of the inter-sugar linkages were achieved as follows. When the anomeric proton at δ 4.95 (H-1′) of the sugar directly linked to the aglycone was used as a starting point, a sequence of four oxymethynes and one oxymethylene could be identified from COSY and HOHAHA spectra. The large coupling constants observed between all the oxymethine protons, typical of axial-axial relationships, and the relatively high-field resonances of H-5′ (δ 3.38) led to the assignment of this sugar as a β-glucopyranoside. In addition, the ROESY cross-peaks of H-1′ with H-3′ and H-5′, and of H-2′ with H-4′ further supported this conclusion. Continuing, the spatial couplings of H-2′ (δ 3.42) with H-1‴ (δ 5.23), and of H-3′ (δ 3.64) with H-1″ (δ 5.04) indicated positions 2 and 3 of the inner glucose as glycosidic linkage sites. Further evidence for the (3′→1″) and (2′→1‴) linkages came from the HMBC spectrum, which evidenced prominent correlation peaks between H-2′ and the anomeric carbon at δ 101.7 (C-1‴) and between H-3′ and the anomeric carbon at δ 101.0 (C-1″).

The spin systems of both monosaccharides linked to the inner glucose comprised four oxymethynes and one methyl group, and they were identified as two rhamnopyranoses due to the axial-axial couplings H-3″-H-4″ (J=8.5 Hz)/ H-3‴-H-4‴ (J=8.5 Hz) and H-4″-H-5″ (J=10.5 Hz)/ H-4‴-H-5‴(J=11.7 Hz), and to the equatorial-axial relationship between H-2″ and H-3″ (J=1.8 Hz)/H-2‴ and H-3‴(J=1.8 Hz). The α-anomeric configuration of both these sugars was judged by the very low $J_{H-1/H-2}$ ($J<1$ Hz), indicative of an equatorial-equatorial relationship, supported by the absence of spatial couplings between H-1″ and H-3″ (and between H-1‴ and H-3‴), and by the resonances of C-5″ (δ 67.4) and C-5‴ (δ 69.5) in the ^{13}C NMR spectrum of **3** (Table 2).[8] The presence of rhamnose in feroxoside A is remarkable since, to the best of our knowledge, this is the first report of a rhamnose containing glycoside from a marine sponge.

Finally, the fourth monosaccharide was identified as a further β-glucopyranose considering that its pattern of proton chemical shifts and coupling constants is very similar to that previously measured for the first hexopyranose. The ROESY cross peak between H-3‴ (δ 3.77) and H-1^{IV} (δ 4.55) and the HMBC cross peak C-3‴/H-1^{IV} were both clearly indicative of the fourth sugar residue being linked at position 3‴. Therefore, if we assume that these monosaccharides belong to the most commonly found stereochemical series (D for glucose and L for rhamnose), the sugar moiety of feroxoside A is completely defined.

In order to establish the absolute configuration at the chiral center C-23, 9 mg of feroxoside A (**3**) were subjected to enzymatic hydrolysis with the use of an excess of glycosidase mixture extracted from *Charonia lampas* in citrate–phosphate buffer (pH 5). After three days at 40°C under stirring, the reaction was stopped and, after neutralization and filtration, the obtained mixture was partitioned between EtOAc and water. Then, the organic phase was purified by HPLC (EtOAc/*n*-hexane 9:1) affording 1.8 mg of the aglycone **5**. Compound **5** was treated with (−)-(*R*)- and (+)-(*S*)-2-methoxy-2-phenyl-2-trifluoromethylacetic acid

$\Delta\delta = \delta$ (*S*)-MTPA ester - δ (*R*)-MTPA ester (in Hz)

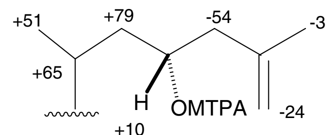
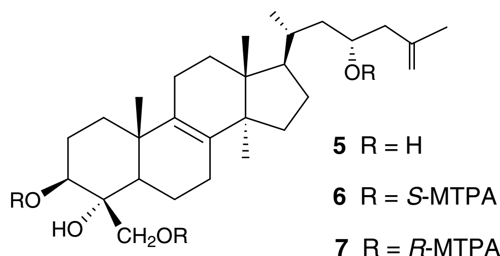


Figure 3. Application of modified Mosher's method for the absolute stereochemistry at C-23.

(MTPA) chloride, *N,N*-dimethylaminopyridine (DMAP) in pyridine to furnish the (*S*)-MTPA ester **6**, and the (*R*)-MTPA ester **7**, respectively. The absolute configuration at C-23 was determined as *R* by analysis of $\Delta\delta$ (δ_S-δ_R) values, in accordance with the modified Mosher's method (Fig. 3).[9]



All the above data indicate feroxoside A (**3**) to be 3β-O-[β-D-glucopyranosyl (1→3) α-L-rhamnopyranosyl (1→2) β-D-glucopyranosyl (3→1) α-L-rhamnopyranosyl]-4α, 23*R*, 29-trihydroxy-30-nor-lanosta-8(9), 25-diene.

The structure of the second saponin, named feroxoside B (**4**), $[\alpha]_D=-25$ (c=0.02 in MeOH), was readily determined basing on the considerable similarities with feroxoside A. The HRFABMS (negative ions) indicated its molecular formula as $C_{53}H_{90}O_{22}$ (*m/z* 1077.5853; $C_{53}H_{90}O_{22}$ requires *m/z* 1077.5846), which differs from that of feroxoside A only in having two more hydrogen atoms. The ^{1}H and ^{13}C NMR profiles obtained for **4** (Tables 1 and 2) showed strict resemblances with corresponding spectra of **3**. In particular, the ^{1}H NMR spectrum of **4** differed from that of **3** only by: (i) lacking the vinylic methylene signals at δ 4.74 and 4.80, and of the methyl singlet at δ 1.76 (ii) the presence of a 6H doublet at δ 0.93 (iii) an upfield shift of H_2-24 (δ 1.40 and 1.18 instead of δ 2.09 and 2.24) and of H-23 (δ 3.75 instead of δ 3.86). On the other hand, the midfield region of the ^{1}H NMR spectrum of **4** appeared practically superimposable to that of feroxoside A, suggesting that these saponins must possess the same sugar portion. Accordingly, the ^{13}C NMR resonances (Tables 1 and 2) of **4** appeared almost identical to those of **3**, with only two exceptions, i.e. the sp^2 signals of the double bond $\Delta^{25,26}$ were replaced by two sp^3 signals at δ 25.0 and 22.3.

All these data led us to propose the structure **4** for feroxoside B, which corresponds to the 25,26-dihydro derivative of feroxoside A. This conclusion was finally proved by catalytic hydrogenation (H_2/10% Pd, on charcoal catalyst) of feroxoside A (**3**) (4 mg). After work-up, 2.5 mg of a compound identical to **4** (by $[\alpha]_D$ and NMR data) was obtained, thus also indicating that the absolute configuration of the chiral centers of feroxoside B (**4**) must be assigned as that of the corresponding carbons in **3**. Feroxoside B (**4**) has thus been unambiguously determined as 3β-O-[β-D-glucopyranosyl (1→3) α-L-rhamnopyranosyl (1→2) β-D-glucopyranosyl (3→1) α-L-rhamnopyranosyl]-4α, 23*S*, 29-trihydroxy-30-nor-lanosta-8(9)-ene.

Feroxosides A-B are partly responsible for the cytotoxic activity exhibited by the methanol extract of *Ectyoplasia ferox*.[6] They are moderately cytotoxic (IC_{50} 19 μg/mL) against J-774, murine monocyte-macrophage cell line.

1. Experimental

1.1. General methods

FABMS spectra (CsI ions) were performed in a glycerol/thioglycerol matrix on a VG Prospec-Autospec (Fisons) mass spectrometer. Optical rotations were measured at 589 nm on a Perkin–Elmer 192 polarimeter using a 10 cm microcell. IR (KBr) spectra were measured on a Bruker IFS-48 spectrophotometer. ^{1}H and ^{13}C NMR spectra were determined on a Bruker AMX-500 spectrometer at 500.13 and 125.77 MHz, respectively; chemical shifts were referenced to the residual solvent signal (CD_3OD: δ_H 3.34, δ_C 49.0; $CDCl_3$ δ_H 7.26). Homonuclear ^{1}H connectivities were determined by the COSY experiment. The 2D HOHAHA experiment was performed in the phase sensitive mode (TPPI) with a MLEV-17 sequence for mixing. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 500 ms. The reverse multiple-quantum heteronuclear correlation (HMQC) spectra were recorded by using a pulse sequence with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bound to ^{13}C; the inter-pulse delays were adjusted for an average $^1J_{CH}$ of 140 Hz. Medium pressure liquid chromatography was performed on a Büchi apparatus with an SiO_2 column (230–400 mesh). High performance liquid chromatographies (HPLC) were achieved on a Beckman apparatus equipped with a refractive index detector and LUNA C18 (2) or SI60 (250×4 mm) columns.

1.2. Collection, extraction and isolation

Specimens of *Ectyoplasia ferox* were collected along the coast of Grand Bahama Island, Bahamas, and identified by Prof M. Pansini (Università di Genova). They were frozen immediately after collection and kept frozen until extraction. Voucher samples were deposited at the Istituto di Zoologia, Università di Genova (Ref. No. 98-01). The sponge (61 g dry weight after extraction) was homogenized and extracted with methanol (4×500 mL). The obtained extract was dissolved in MeOH–H_2O 9:1 and then partitioned against *n*-hexane (3×500 mL) to yield an apolar extract weighing 1.4 g. Then, the water content of the hydromethanolic phase was adjusted to 20% (v/v) and 40% (v/v) and the solutions partitioned against CCl_4 (3×500 mL) and $CHCl_3$ (3×500 mL), respectively, affording a carbon tetrachloride (0.7 g) and a chloroform (2.1 g) extract. Finally, all the MeOH was evaporated from the hydromethanolic layer, and the water solution thus obtained was partitioned against *n*-BuOH. The butanol-soluble material (5.5 g), was subjected to MPLC purification over silica gel (230–400 mesh), eluting with a solvent gradient system of increasing polarity from EtOAc to MeOH. Fractions eluted with MeOH–EtOAc 9:1 were combined and then further purified by reversed phase HPLC (eluent MeOH–H_2O 7:3, flow 0.7 mL/min) yielding pure feroxosides A (**3**, 16.0 mg) and B (**4**, 8.3 mg).

1.2.1. Feroxoside A (3). White amorphous solid. $[\alpha]_D^{25}=-16$ (c=0.05 in MeOH); IR (KBr) ν_{max}=3410, 2930, 1635, 1579 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD): see Tables 1 and 2. FABMS (positive ions, glycerol/thioglycerol matrix) *m/z*

1099. FABMS (negative ions, thioglycerol matrix) *m/z* 1075. HRFABMS (negative ions) *m/z* 1075.5698 $[M-H]^-$, calcd. for $C_{53}H_{88}O_{22}$, *m/z* 1075.5689.

1.2.2. Feroxoside B (4). White amorphous solid. $[\alpha]_D^{25}=-25$ (c=0.02 in MeOH); IR (KBr) ν_{max}=3408, 2928, 1680, 1579 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD): see Tables 1 and 2. FABMS (negative ions, thioglycerol matrix) *m/z* 1077. HRFABMS (negative ions) *m/z* 1077.5853 $[M-H]^-$, calcd. for $C_{53}H_{90}O_{22}$, *m/z* 1077.5846.

1.3. Enzymatic Hydrolysis

A solution of feroxoside A (**3**) (9 mg) in phosphate–citrate buffer (5 mL) at pH 5.0 was incubated with an excess of glycosidase mixture from *Charonia lampas* (Scikagaku Kogyo) at 40°C for 72 hours, under stirring. The mixture was neutralized, filtered and then partitioned between H_2O and EtOAc. The aqueous layer was then evaporated to dryness and the obtained fraction contained salts, the unreacted saponin and a mixture of partial glycosides. The organic extract was dried over Na_2SO_4, filtered, concentrated in vacuo, and then purified by HPLC (LUNA SI60, eluant EtOAc/*n*-hexane 9:1) to afford compound **5** (1.8 mg).

1.3.1. Compound 5. Colorless amorphous oil. $[\alpha]_D^{25}=-4$ (c=0.01 in MeOH). HRFABMS (positive ions, glycerol matrix) *m/z*: 461.3574 [M + H^+], calcd. for $C_{29}H_{48}O_4$, 461.3561. ^1H NMR (CD_3OD): δ 4.80 (H-27a, br.s), 4.75 (H-27b, br.s), 4.25 (H-29a, d, *J*=11.8), 3.88 (H-23, q, *J*=6.0 Hz), 3.75 (H-3, dd, *J*=8.5, 2.5 Hz), 3.38 (H-29b, d, *J*=11.8), 2.25 (H-24a, overlapped), 2.24 (H-2ax, overlapped), 2.20 (H-7eq, dd, *J*=11.5, 8.1), 2.10 (H_2-11, overlapped), 2.10 (H-24b, overlapped), 2.09 (H-7ax, overlapped), 2.06 (H-12eq, overlapped), 2.04 (H-6ax, overlapped), 1.95 (H-16a, m), 1.82 (H-2eq, dd, *J*=11.8, 2.5), 1.79 (H-20, overlapped), 1.78 (H_3-26, br.s), 1.75 (H-1ax, dd, *J*=11.8, 6.2), 1.62 (H-15a, m), 1.58 (H-6eq, m), 1.49 (H-22a, dd, *J*=10.5, 6.0) 1.43 (H-16b, overlapped), 1.42 (H-12ax, overlapped), 1.36 (H-15b, overlapped), 1.34 (H-5, overlapped), 1.30 (H_3-28, s), 1.22 (H-1eq, overlapped), 1.20 (H-17, overlapped), 1.05 (H-22b, ddd, 10.5, 6.0, 3.6), 1.00 (H_3-21, d, *J*=6.5), 0.99 (H_3-19, br.s), 0.67 (H_3-18, br.s).

1.4. Preparation of MTPA esters of compound 5

Compound **5** (0.7 mg) was dissolved in 0.5 mL of dry pyridine, treated with (−)-MTPA chloride (15 μL), *N,N*-dimethylaminopyridine (DMAP, a spatula tip), and then maintained at room temperature, with stirring, overnight. After removal of the solvent, the reaction mixture was purified by HPLC on SI60 column (eluent *n*-hexane/EtOAc 1:1), affording (*S*)-MTPA ester **6** (ca. 0.6 mg). Using (+)-MTPA chloride, the same procedure afforded the (*R*)-MTPA ester **7** (ca. 0.6 mg).

1.4.1. Compound 6. [(*S*)-MTPA ester]. Amorphous solid. FABMS (glycerol matrix, positive ions) *m/z* 1109 $[M+H]^+$. ^1H NMR ($CDCl_3$): δ 7.36 and 7.42 (MTPA phenyl protons, m), 5.10 (H-23, m), 4.97 (H-3, dd, *J*=8.5, 3.5 Hz), 4.62 (H-26a, bs), 4.57 (H-29a, d, *J*=11.8 Hz), 4.55 (H-26b, bs), 4.35 (H-29b, d, *J*=11.8 Hz), 3.60 (MTPA OCH_3, s), 2.20 (H_2-7, m), 2.18 (H-2a, dt, *J*=11.5, 8.5 Hz), 2.15 (H-24a, overlapped), 2.12 (H_2-11, H_2-6, H-24b, overlapped), 1.95 (H-12a, overlapped), 1.92 (H-16a, overlapped), 1.85 (H-2b, dd, *J*=11.7, 3.5 Hz), 1.65 (H_3-27, bs), 1.55 (H-1a, H-15a, overlapped), 1.48 (H-22a, bt, *J*=10.5 Hz), 1.33 (H-15b, H-16b, H-1b, H-5, overlapped), 1.26 (H-12b, overlapped), 1.25 (H_3-28, bs), 1.20 (H-17, m), 1.08 (H-20, m), 1.00 (H-22b, *J*=dt, 10.5, 3.5 Hz), 0.96 (H_3-21, d, *J*=6.5 Hz), 0.89 (H_3-19, s), 0.58 (H_3-18, s).

1.4.2. Compound 7. [(*R*)-MTPA ester]. Amorphous solid. FABMS (glycerol matrix, positive ions) *m/z* 1109 $[M+H]^+$. ^1H NMR ($CDCl_3$): δ 7.33 and 7.55 (MTPA phenyl protons, m), 5.09 (H-23, m), 4.94 (H-3, dd, *J*=8.5, 3.5 Hz), 4.63 (H-26a, bs), 4.56 (H-26b, bs), 4.48 (H-29a, d, *J*=11.8 Hz), 4.37 (H-29b, d, *J*=11.8 Hz), 3.65 (MTPA OCH_3, s), 2.20 (H_2-7, m), 2.17 (H-2a, dt, *J*=11.5, 8.5 Hz), 2.17 (H-24a, overlapped), 2.12 (H_2-11, H_2-6, H-24b, overlapped), 1.95 (H-12a, overlapped), 1.92 (H-16a, overlapped), 1.86 (H-2b, dd, *J*=11.7, 3.5 Hz), 1.66 (H_3-27, bs), 1.55 (H-1a, H-15a, overlapped), 1.45 (H-22a, bt, *J*=10.5 Hz), 1.33 (H-15b, H-1b, H-16b, H-5, overlapped), 1.26 (H-12b, overlapped), 1.25 (H_3-28, bs), 1.19 (H-17, m), 1.06 (H-20, m), 0.97 (H-22b, *J*=dt, 10.5, 3.5 Hz), 0.94 (H_3-21, d, *J*=6.5 Hz), 0.89 (H_3-19, s), 0.58 (H_3-18, s).

1.5. Catalytic hydrogenation of feroxoside A

Palladium on charcoal catalyst (10%, a spatula tip) was added to 4 mg of feroxoside A (**3**) in dry EtOH. The solution was stirred at room temperature under an atmosphere of H_2 for 2 h. The catalyst was then removed by filtration and the solvent evaporated to obtain a mixture, which, purified by HPLC on C18 column (eluent MeOH–H_2O 7:3), yielded 2.5 mg of a compound identical to feroxoside B (**4**) in the pure state.

1.6. Cytotoxic activity

J-774 (murine monocyte/macrophage) cells 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). J-774 (4×10^3 cells) were plated on 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 **3** and **4** were added and the cells incubated for 72 h. After 72 h, 25 μL of MTT (5 mg/mL) was added and the cells were incubated for an additional 3 hours. Following this time the cells were lysed and the dark blue crystals solubilized with 100 μL of a solution containing 50% (v:v) *N,N*-dimethylformamide, 20% (w:v) SDS with adjusted 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 **3** and **4** was calculated as: % dead cells=100−(OD treated/OD control)×100. The results of cytotoxic activity are expressed as IC_{50} (the concentration that inhibited the cell growth by 50%): feroxoside A (**3**): 18.5 μg/mL; feroxoside B (**4**): 19.5 μg/mL. All the measurements were repeated on triplicate samples; the data reported are the mean of them.

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