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New cytotoxic steroids from the gorgonian *Isis hippuris*. Structure–activity studies

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Abstract—*Isis hippuris* has proven to be a rich source of new cytotoxic polyoxygenated steroids. Thirteen novel polyoxygenated steroids, along with some known compounds, have been isolated from the Indonesian gorgonian *I. hippuris* and their structures have been elucidated by spectroscopic studies. Most of the compounds were isolated as pairs of the 3-hydroxy/3-keto steroids. Although these systems contain the same steroid nucleus, they differ in their side chains. The high number of steroids isolated and the cytotoxic activity data obtained allowed us to deduce a number of structure–activity relationships. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Marine organisms have proven to be a natural source of polyoxygenated sterols with unprecedented structures that show a variety of biological activities.[1] Among these organisms, gorgonians are distinguished by their high content of these types of compounds.[2] In particular, the gorgonian *I. hippuris* features an interesting group of highly oxygenated sterols that were published under the name of hippurins and hippuristanols and are characterized by a 22*R*- or 22*S*-ketal functionality.[3] Some of these compounds exhibit potent antitumor activities.[4] More recently, hippuristanone A, a 17β,20β-epoxy polyoxygenated steroid, has been isolated from a Taiwanese specimen.[5]

During the course of our study into cytotoxic metabolites from Indonesian gorgonians, we have focused our search on two different collections of *I. hippuris* due to the high cytotoxicity found in their methanolic extracts. Bioassay-guided isolation afforded thirteen new polyoxygenated steroids (**4–11** and **13–17**) along with the known compounds **1–3** and **12**. The structures of these compounds were determined on the basis of spectroscopic data and their cytotoxicities were evaluated against several tumor cells. In this paper, we describe the isolation, structure elucidation, and cytotoxicity of these steroids. Furthermore, some important conclusions regarding the structure–activity relationships of these related compounds can be inferred from the data obtained.

2. Results and discussion

The methanolic extracts of the specimens of two collections of *I. hippuris*, collected along the coast of Sulawesi (Indonesia), were fractionated by the previously described partition procedure.[6] The hexane and CH_2Cl_2 partitions gave a steroid mixture that was repeatedly chromatographed by silica gel flash column chromatography (CH_2Cl_2/MeOH mixtures) and normal and reversed-phase HPLC to give pure compounds **1–17**. All the compounds can be sorted into four series by the nature of the side chain structure: compounds **1–6** incorporate a spiroketal ring structure; compounds **7–9** are characterized by an orthoester ring; compounds **10–15** contain an epoxy group; and finally, compounds **16** and **17** have an acyclic side chain.

2.1. Steroids with a spiroketal ring in the side chain (1–6)

Three known hippuristanol steroids, hippuristanol (**1**), 3-acetyl-2α-hydroxy-22-*epi*-hippuristanol (**2**), and 22-*epi*-hippuristanol (**3**, major component of the steroid mixture) were identified by comparison of their spectral data with those described in the literature.[3] In order to obtain more derivatives of this type of compound for biological assays, we performed several acetylation reactions. Acetylation of **2** gave only the diacetylated compound **18**, but acetylation of **3** gave a mixture of the mono- and diacetylated products **4** and **19**, respectively.

Very important fragments can be deduced from the proton and carbon NMR chemical shifts in the characterisation of the new related derivatives. A spiroketal ring can be easily deduced from the NMR signals corresponding to H-16α and C-22 at δ_H 4.3–4.5 and δ_C 114–119, respectively (22*S* series: δ_H~4.4 and δ_C~118; 22*R* series: δ_H~4.3 and δ_C~115). Proton and carbon signals at δ_H~4.0 (br s) and

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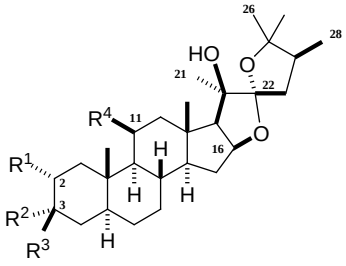
Table 1. ^{13}C NMR and DEPT data for **3–9** ($CDCl_3$)

Position	3	4	5	6	7	8	9
1	32.3 t	32.5 t	32.0 t	38.5 t	38.4 t	37.0 t	32.1 t
2	28.6 t	25.7 t	28.9 t	38.1 t	38.1 t	31.5 t	28.9 t
3	66.3 d	70.0 d	66.5 d	212.0 s	211.9 s	71.3 d	66.5 d
4	35.3 t	32.3 t	35.8 t	44.6 t	44.6 t	38.2 t	36.1 t
5	39.9 d	40.8 d	38.5 d	46.6 s	46.6 d	44.8 d	39.1 d
6	27.8 t	27.7 t	28.4 t	28.8 t	28.7 t	28.5 t	28.4 t
7	31.5 t	31.5 t	31.7 t	31.7 t	31.5 t	31.9 t	31.8 t
8	30.2 d	30.2 d	34.1 d	34.0 d	34.5 d	34.6 d	34.6 d
9	58.1 d	57.9 d	54.3 d	53.7 d	53.8 d	54.5 d	54.4 d
10	36.3 s	35.9 s	36.1 s	35.7 s	35.6 s	35.5 s	35.8 s
11	68.0 d	68.0 d	20.1 t	20.8 t	20.9 t	20.8 t	20.3 t
12	48.8 t	48.8 t	32.1 t	29.7 t	39.9 t	40.1 t	40.1 t
13	42.1 s	42.1 s	42.7 s	42.7 s	42.2 s	42.3 s	42.3 s
14	58.4 d	58.3 d	56.7 d	56.4 d	54.5 d	54.7 d	54.7 d
15	31.8 t	32.2 t	40.5 t	40.4 t	32.3 t	32.3 t	32.2 t
16	79.0 d	79.0 d	79.3 d	79.2 d	71.6 d	71.7 d	71.7 d
17	66.5 d	66.3 d	65.3 d	65.3 d	57.1 d	57.2 d	57.1 d
18	27.0 q	29.7 q	27.0 q	14.1 q	14.3 q	14.3 q	14.4 q
19	13.9 q	13.9 q	11.2 q	11.5 q	11.4 q	12.3 q	11.2 q
20	82.6 s	82.6 s	82.5 s	82.5 s	82.3 s	82.3 s	82.3 s
21	19.5 q	19.5 q	16.7 q	16.6 q	18.4 q	18.4 q	18.5 q
22	118.6 s	118.6 s	118.6 s	118.6 s	85.2 d	85.2 d	85.2 d
23	39.9 t	39.8 t	39.7 t	39.8 t	33.6 t	33.7 t	33.7 t
24	41.0 d	41.0 d	41.0 d	41.0 d	38.7 d	38.8 d	38.8 d
25	84.2 s	84.2 s	84.2 s	84.2 s	85.6 s	85.7 s	85.6 s
26	29.1 q	29.1 q	29.0 q	29.1 q	23.7 q	23.7 q	23.7 q
27	23.0 q	23.0 q	22.0 q	23.0 q	22.2 q	22.1 q	22.0 q
28	14.1 q	14.3 q	14.0 q	14.0 q	16.0 q	16.0 q	16.0 q
29					117.8 s	117.8 s	117.8 s
30					23.2 q	23.2 q	23.2 q
$OCOCH_3$		170.0 s			170.4 s	170.3 s	172.9 s
$OCOCH_3$		21.5 q			22.5 q	22.5 q	22.6 q

δ_C~66 are indicative of an α-OH at C-3 and signals at δ_H~4.3 (br s) and δ_C~68 suggest a β-OH at C-11. Taking into account these key signals, the new hippuristanol steroids **4–6** were assigned to the 22*S* series on the basis of the chemical shift of the C-22 signal at δ_C>118 in the ^{13}C NMR spectrum and the downfield shift of H-16α (δ_H 4.43). The structures of new compounds in this series (**4–6**) were established by direct correlation with 22-*epi*-hippuristanol (**3**), which differs only in the substitution pattern at positions 3 and 11.

Thus, the NMR spectral data of **4** were found to be very similar to those of 22-*epi*-hippuristanol (**3**) (Table 1) except for the signals that can be assigned to an additional acetate group (δ_H 2.04 (s) and δ_C 170.0 (s) and 21.5 (q)), indicating that one of the two hydroxyl groups in **3** is acetylated in **4**. The downfield shifts of the H-3 (by 0.95 ppm) and C-3 (by 3.7 ppm) signals in the NMR spectra of compound **4**, in comparison to those of **3**, allowed the assignment of the acetate group to position 3. The (+)-LRFABMS displayed two pseudomolecular ions, $[M+Na]^+$ at *m/z* 527 and $[M+H]^+$ *m/z* 505, and the (+)-HRFABMS of the $[M+Na]^+$ ion at *m/z* 527.3335 ($C_{30}H_{48}O_6Na$, Δ 1.4 mmu), corresponding to the molecular formula $C_{30}H_{48}O_6$, which confirmed the proposed structure. Therefore, compound **4** was named as 3-acetyl-22-*epi*-hippuristanol.

The structure of the new compound, **5**, was easily deduced as the 11-dehydroxy derivative of 22-*epi*-hippuristanol from its NMR spectra, which lacked the signals at δ_H 4.30 (br s)



1: $R^1 = R^3 = H$, $R^2 = R^4 = OH$ (22*R*

2: $R^1 = R^4 = OH$, $R^2 = OAc$, $R^3 = H$

3: $R^1 = R^3 = H$, $R^2 = R^4 = OH$

4: $R^1 = R^3 = H$, $R^2 = OAc$, $R^4 = OH$

5: $R^1 = R^3 = H$, $R^2 = OH$, $R^4 = H$

6: $R^1 = H$, $R^2 = R^3 = O$, $R^4 = H$

18: $R^1 = R^2 = OAc$, $R^3 = H$, $R^4 = OH$

19: $R^1 = R^3 = H$, $R^2 = R^4 = OAc$

Table 2. ^{1}H NMR data for **7**, **10**, and **14** ($CDCl_3$)

Position	**7** δ (mult., J (Hz))	**10** δ (mult., J (Hz))	**14** δ (mult., J (Hz))
1	2.23 (m), 2.03 (m)	2.29 (m), 2.22 (m)	1.45 (m), 1.22 (m)
2	2.37 (dt, 6.3, 14.5, 14.5)	2.37 (dt, 6.1, 14.6, 14.6)	1.61 (m)
3			4.04 (brs)
4	2.26 (m), 2.08 (m)	2.24 (m), 2.08 (m)	1.55 (m), 1.35 (m)
5	1.51 (m)	1.53 (m)	1.54 (m)
6	1.36 (m)	2.29 (m), 1.36 (m)	1.24 (m)
7	1.69 (m)	2.03 (m), 1.86 (m)	1.71 (m), 1.68 (m)
8	1.53 (m)	1.48 (m)	1.53 (m)
9	0.73 (dt, 4.3, 11.3, 11.3)	0.75 (m)	0.79 (m)
11	2.01 (m), 2.12 (m)	1.59 (m), 1.41 (m)	1.21 (m)
12	2.04 (m), 1.17 (m)	1.75 (m), 1.38 (m)	1.79 (m)
14	0.9 (m)	1.29 (m)	1.25 (m)
15	2.08 (m), 1.33 (m)	1.64 (m), 1.36 (m)	2.32 (m), 1.30 (m)
16	4.52 (dt, 4.2, 7.3, 7.3)	1.79 (m), 1.74 (m)	5.17 (t, 7.7)
17	0.99 (m)		
18	1.07 (s)	0.92 (s)	0.98 (s)
19	1.03 (s)	0.99 (s)	0.77 (s)
21	1.36 (s)	1.53 (s)	1.56 (s)
22	3.98 (dd, 5.0, 8.2)	4.72 (d, 10.8)	4.45 (d, 11.0)
23	1.64 (m), 1.08 (m)	2.24 (m)	2.21 (dd, 6.7, 10.7)
24	2.21 (m)	1.95 (m)	1.88 (q, 7.1)
26	1.43 (s)	1.46 (s)	1.46 (s)
27	1.37 (s)	1.53 (s)	1.53 (s)
28	0.99 (d, 6.9)	0.92 (d, 6.1)	0.99 (d, 7.1)
29		0.84 (d, 6.8)	0.81 (d, 6.6)
30	1.53 (s)		
OAc	1.96 (s)	2.08 (s)	
		1.97 (s)	

and δ_C 68.0 (attributable to the presence of an OH at C-11) and showed the presence of an additional carbon signal at δ_C 20.1 (t). On the other hand, the (+)-LRFABMS showed the pseudomolecular ions $[M+H]^+$ at m/z 447 and $[M+Na]^+$ at m/z 469 along with the fragment $[M-H_2O+H]^+$ at m/z 429. (+)-HRFABMS of the $[M+H]^+$ ion at m/z 447.3467 ($C_{28}H_{47}O_4$, Δ 0.8 mmu) which allowed us to determine the molecular formula of **5** as $C_{28}H_{46}O_4$. On the basis of these data, compound **5** was named as 11-dehydroxy-22-*epi*-hippuristanol.

In the NMR spectrum of compound **6**, the carbon resonance of C-3 at δ_C 212.0 (s) as well as the absence of the signals assigned to the 3-OH group in the NMR spectra of compound **5** were indicative of a ketone functionality at C-3. The molecular formula of **6** was confirmed as $C_{28}H_{44}O_4$ by its (+)-LRFABMS, which shows the pseudomolecular ion $[M+H]^+$ at m/z 445 and the $[M-H_2O+H]^+$ fragment at m/z 427. Consequently, the chemical structure of compound **6** was determined as 11-dehydroxy-22-*epi*-hippuristan-3-one.

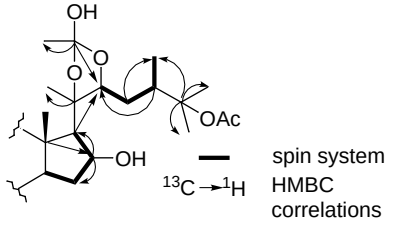


Figure 1. Selected ^{1}H–^{1}H COSY and HMBC correlations of **7**.

2.2. Steroids with an orthoester ring in the side chain (7–9)

Compound **7** was obtained as a colorless solid. The ^{13}C NMR spectrum displayed 32 signals: eight methyl groups, nine methines, eight methylenes, and seven quaternary carbons, as determined by a DEPT analysis.

Comparison of the ^{13}C NMR data from C-1 to C-14 in compound **7** with the corresponding signals in **6** (see Table 1) showed that both compounds share a similar steroid skeleton nucleus (including a ketone functionality at C-3), but differ in the side chain and the substitution in the D ring. The carbonyl signal at δ_C 170.4 (s) along with the methyl group at δ_C 22.5 (q) indicate the presence of an acetyl group; the quaternary carbon at δ_C 85.6 (s) along with two signals at δ_C 23.7 (q) and 22.2 (q), assigned to two methyl groups, suggested the existence of an $-OC(CH_3)_2-$ group in the molecule; in addition, carbon signals at δ_C 85.2 (d), 82.3 (s), and 71.6 (d) indicated the presence of three additional oxygenated carbons. Finally, a ketal carbon at δ_C 117.8 (s) along with a methyl group at δ_C 23.2 suggested the presence of an orthoester group in the side chain of **7**.

In the ^{1}H NMR spectrum of **7** it is possible to distinguish clearly the eight methyl groups: the H_3-18 and H_3-19 methyl groups at δ_H 1.07 (s) and 1.03 (s); one methyl group corresponding to the acetyl group at δ_H 1.96 (s); four methyl singlets: two corresponding to the $-OC(CH_3)_2-$ fragment in the side chain at δ_H 1.43 (s) and 1.37 (s) and another two at δ_H 1.53 (s) and 1.36 (s); and one methyl doublet with a coupling constant of J=6.9 Hz at δ_H 0.99, suggesting the presence of a methyl group in the side chain of **7**. Finally, the signals at δ_H 4.52 (dt, J=4.2, 7.3, and 7.3 Hz, 1H) and

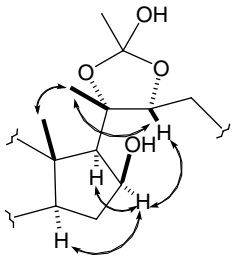


Figure 2. Selected ROESY correlations of **7**.

3.98 (dd, J=5.0 and 8.2 Hz, 1H) indicate the presence of two oxygenated methine carbons (Table 2).

The complete structural identification of compound **7** was based on its 2D NMR spectra. An HMQC experiment allowed us to relate all the carbons to their corresponding protons. The coupled signals observed in the ^{1}H–^{1}H COSY spectrum reveal the presence of the spin system –CHMe–CH_2–CHO– in the side chain (Fig. 1).

The location of the orthoester group in the side chain of **7** between carbons C-20 and C-22 was established with the help of an HMBC experiment, which showed cross-correlations between the following signals: the ketal carbon signal at δ_C 117.8 and protons H-22 (δ_H 3.98) and H_3-30 (δ_H 1.53); the quaternary C-20 at δ_C 82.3 and H_3-21 at δ_H 1.36; and the methine C-17 at δ_C 57.1 and H-22 (Fig. 1). The HMBC experiment was also used to locate the OH group at C-16 and to establish the link between the –CHMe–CH_2–CHO– substructure and the –$OC(CH_3)_2$– fragment, as depicted in Fig. 1.

Basic hydrolysis of compound **7** gave compound **20**, which corresponds to the deacetyl derivative of **7**. The upfield shift of the terminal dimethyl signals H_3-26 and H_3-27 (from δ 1.37 and 1.43 in **7** to δ 1.14 and 1.24 in **20**) established the position of the acetoxy group at C-25.[7] The relative β stereochemistry for the hydroxy group at C-16 was deduced from the ROESY spectrum of **7**, which exhibits a cross correlation between H-16 and H-14. Furthermore, the ROESY correlations showed in Fig. 2 allowed us to establish the relative stereochemistry in the D ring and the C-20/C-22 carbon fragment in the side chain of compound **7**. The determination of the relative stereochemistry of the remaining chiral center, C-24, and the absolute configuration of this compound are underway.

The (+)-LRFABMS of **7** does not exhibit a pseudomolecular ion but displays fragments at *m/z* 531 $[M-H_2O+H]^+$, *m/z* 451 $[M-2AcOH+Na]^+$, *m/z* 471 $[M-H_2O-AcOH+H]^+$, and *m/z* 411 $[M-H_2O-2AcOH+H]^+$. (+)-HRFABMS of the ion $[M-2AcOH+Na]^+$ at *m/z* 451.3184 ($C_{28}H_{44}O_3Na$, Δ 0.4 mmu) allowed us to determine the molecular formula of compound **7** as $C_{32}H_{52}O_7$. On the basis of these data, compound **7** was named as orthohippurinsterone A.

Table 3. ^{13}C NMR and DEPT data for **10**–**17** ($CDCl_3$)

Position	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	38.4 t	32.1 t	38.3 t	32.0 t	32.0 t	38.3 t	32.2 t	38.5 t
2	38.0 t	29.0 t	38.0 t	28.9 t	28.9 t	38.0 t	29.0 t	38.1 t
3	211.7 s	66.5 d	211.7 s	66.5 d	66.4 d	211.7 s	66.6 d	212.1 s
4	44.6 t	35.7 t	44.5 t	35.7 t	35.7 t	44.5 t	35.9 t	44.6 t
5	46.5 d	39.0 d	46.5 d	38.9 d	38.9 d	46.3 d	39.1 d	46.7 d
6	28.7 t	28.3 t	28.5 t	28.2 t	28.2 t	28.6 t	28.5 t	28.8 t
7	31.0 t	30.9 t	30.8 t	31.7 t	31.5 t	31.2 t	31.9 t	31.6 t
8	35.4 d	35.6 d	34.6 d	34.7 d	35.0 d	34.4 d	34.7 d	34.6 d
9	53.3 d	53.9 d	53.2 d	53.9 d	53.7 d	53.0 d	54.3 d	53.8 d
10	35.6 s	36.0 s	35.6 s	36.0 s	36.0 s	35.6 s	36.1 s	35.6 s
11	21.5 t	20.8 t	21.3 t	20.6 t	20.6 t	21.3 t	20.6 t	23.9 t
12	36.2 t	36.5 t	36.3 t	36.6 t	36.4 t	36.1 t	40.6 t	40.4 t
13	43.7 s	43.7 s	43.1 s	43.1 s	43.3 s	43.3 s	43.6 s	43.6 s
14	54.4 d	54.8 d	48.9 d	48.9 d	48.7 d	48.1 d	56.8 d	55.4 d
15	23.5 t	23.4 t	33.3 t	33.2 t	32.0 t	32.0 t	22.1 t	22.1 t
16	31.3 t	31.6 t	70.1 d	70.1 d	70.8 d	70.7 d	23.8 t	29.3 t
17	79.2 s	79.3 s	79.4 s	79.4 s	76.8 s	76.7 s	55.4 d	56.5 d
18	15.3 q	15.3 q	15.5 q	15.5 q	15.2 q	15.1 q	13.6 q	13.6 q
19	11.4 q	11.1 q	11.4 q	11.1 q	11.1 q	11.4 q	11.1 q	11.4 q
20	66.9 s	66.9 s	67.4 s	67.4 s	66.6 s	66.6 s	77.5 s	77.5 s
21	16.7 q	16.7 q	16.5 q	16.5 q	16.4 q	16.5 q	20.2 q	20.2 q
22	78.0 d	78.0 d	77.3 d	77.3 d	77.2 d	77.2 d	79.8 d	79.8 d
23	33.7 d	33.7 d	33.5 d	33.4 d	33.9 d	34.0 d	31.9 t	31.9 t
24	40.7 d	41.0 d	40.2 d	40.8 d	40.7 d	40.1 d	41.0 d	40.9 d
25	85.6 s	85.6 s	85.5 s	85.5 s	85.7 s	85.8 s	85.5 s	85.5 s
26	25.0 q	25.0 q	25.1 q	25.1 q	25.1 q	25.0 q	22.6 q	22.6 q
27	22.8 q	22.7 q	22.6 q	22.6 q	22.6 q	22.7 q	23.9 q	23.9 q
28	10.4 q	10.5 q	10.4 q	10.4 q	10.2 q	10.2 q	15.9 q	16.0 q
29	11.7 q	11.7 q	11.9 q	11.9 q	11.7 q	11.8 q		
O*C*OCH$_3$	170.5 s	170.5 s	171.4 s	171.4 s	171.0 s	171.0 s	172.5 s	172.6 s
	170.0 s	170.0 s	169.8 s	169.9 s	170.0 s	169.9 s	170.3 s	170.3 s
					169.8 s	169.8 s		
OCO*C*H$_3$	22.7 q	22.5 q	22.6 q	22.5 q	21.1 s	21.3 q	21.5 q	21.3 q
	21.0 q	21.0 q	23.0 q	21.0 q	20.9 s	21.1 q	21.3 q	21.5 q
					20.6 s	20.9 q		

Compounds **8** and **9** were found to be the C-3 hydroxy derivatives of compound **7**, as deduced from the absence of the ketone carbon signal at δ_C 212 ppm in compound **7** and the presence of signals between δ_H 3–4 ppm, corresponding to H-3 in **8** and **9**. Interestingly, compound **8** is the 3β-OH derivative (H-3 at δ_H 3.58 (m) and C-3 at 71.3 (d)), as deduced from the NOE cross-peak found between H-3 and H-5 at δ_H 1.10 in the NOESY experiment on this compound, while compound **9** is the 3α-OH derivative (H-3 at δ_H 4.05 (br s) and C-3 at 66.5 (d)). The chemical shift values for the signals for C-1–C-10 in compound **9** are in agreement with those reported for 5α-cholestan-3α-ol[8] and confirmed the α orientation of the hydroxyl group in **9**.

The (+)-LRFABMS of **8** shows a very small pseudomolecular ion peak, $[M+H]^+$, at *m/z* 551 accompanied by the fragment ions $[M-H_2O+H]^+$ at *m/z* 533, $[M-H_2O-AcOH+H]^+$ at *m/z* 473, and $[M-H_2O-2AcOH+H]^+$ at *m/z* 413. While the (+)-LRFABMS of **9** does not show the pseudomolecular ion, it does show intense fragment ions at *m/z* 555 $[M-H_2O+Na]^+$, *m/z* 473 $[M-H_2O-AcOH+H]^+$, and *m/z* 413 $[M-H_2O-2AcOH+H]^+$. (+)-HRFABMS of the fragment ion at *m/z* 555.3656 ($C_{32}H_{52}O_6Na$ Δ 0.6 mmu) of compound **9** showed the molecular formula to be $C_{32}H_{54}O_7$. On the basis of these data, we named compound **8** as orthohippurinsterol A and **9** as orthohippurinsterol B.

2.3. Steroids with an epoxy group (10–15)

The absence of signals between 110–120 ppm in the ^{13}C NMR spectra of compounds **10**–**17** indicates that they do not bear either a spiroketal ring or an orthoester ring.

The proton and carbon spectral data of compound **10** indicate that there are a lot of structural similarities between this compound and those discussed above. The spectra show the presence of a similar steroid skeleton nucleus (including a ketone functionality at C-3). However, there are significant differences in the side chain substitution pattern and in the D ring (Table 3).

The ^{13}C NMR spectrum indicates the presence of 33 carbons, of which nine are methyl, nine methine, seven methylene, and eight quaternary carbons, as determined by a DEPT analysis.

The carbonyl signals at δ_C 170.5 (s) and 170.0 (s) along with two methyl groups at δ_C 22.7 (q) and 21.0 (q) indicate the presence of two acetyl groups; the quaternary carbon at δ_C 85.6 (s), along with two signals at δ_C 25.0 (q) and 22.8 (q), which were assigned to two methyl groups, suggest the existence of an $-OC(CH_3)_2-$ group in the molecule; carbon signals at δ_C 79.2 (s) and 66.9 (s) suggest the presence of an epoxy group, and the methine at 78.0 (d) is indicative of the existence of an additional oxygenated carbon.

In the ^1H NMR spectrum of **10** it is possible to distinguish clearly the nine methyl groups: the 18-H_3 and 19-H_3 methyl groups at δ_H 0.92 (s) and 0.99 (s); two methyl groups corresponding to two acetyl groups at δ_H 2.08 (s) and 1.97 (s); two methyl singlets of the $-OC(CH_3)_2-$ fragment in the side chain at δ_H 1.46 (s) and 1.53 (s); and two doublets at δ_H 0.93 (d, *J*=6.8 Hz) and 0.84 (d, *J*=6.1 Hz) suggesting the presence of two methyl groups in the side chain of **10**. Finally, the signal at δ_H 4.72 (d, *J*=10.8 Hz, 1H) confirmed the presence of an oxygenated methine carbon.

The complete structural identification of compound **10** is based on its 2D NMR spectra. Thus, an HMQC experiment allowed us to assign all the carbons to their corresponding protons and the coupled signals observed in the $^1H-^1H$ COSY spectrum revealed the presence of the spin system $-CH(O)-CH(Me)-CH(Me)-$ in the side chain. The link between this substructure and the $-OC(CH_3)_2-$ fragment was established through an HMBC experiment (see Fig. 3). The HMBC correlation between the carbonyl signal at δ_C 170.0 and H-22 at δ_H 4.72 showed the location of one of the acetoxy groups to be at the C-22 position.

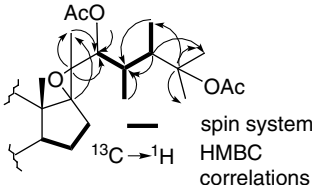
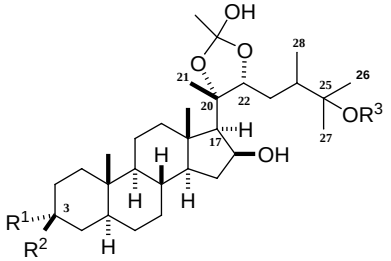


Figure 3. Selected HMBC correlations of **10**.

The location of the epoxy group between carbons C-17 and C-20 in compound **10** was established with the help of an HMBC experiment, which showed cross-correlations between the methyl H_3-21 protons at δ_H 1.53 and the quaternary carbons C-17 (δ_C 79.2) and C-20 (δ_C 66.9), as well as between the H-22 proton at δ_H 4.72 and the quaternary carbon C-20 (see Fig. 3).

Once again, the location of the acetoxy group at C-25 was deduced from the basic hydrolysis of compound **10** to give compound **21**, whose ^1H NMR spectrum showed the upfield shift of the terminal dimethyl signals H_3-26 and H_3-27 (from δ 1.53 and 1.46 in **10** to δ 1.24 and 1.22 in **21**).

The (+)-LRFABMS of compound **10** exhibits the pseudomolecular ion at *m/z* 567 $[M+Na]^+$ and shows two



7: $R^1 = R^2 = O$, R^3= Ac

8: R^1 = H, R^2 = OH, R^3= Ac

9: R^1 = OH, R^2 = H, R^3= Ac

20: $R^1 = R^2 = O$, R^3= H

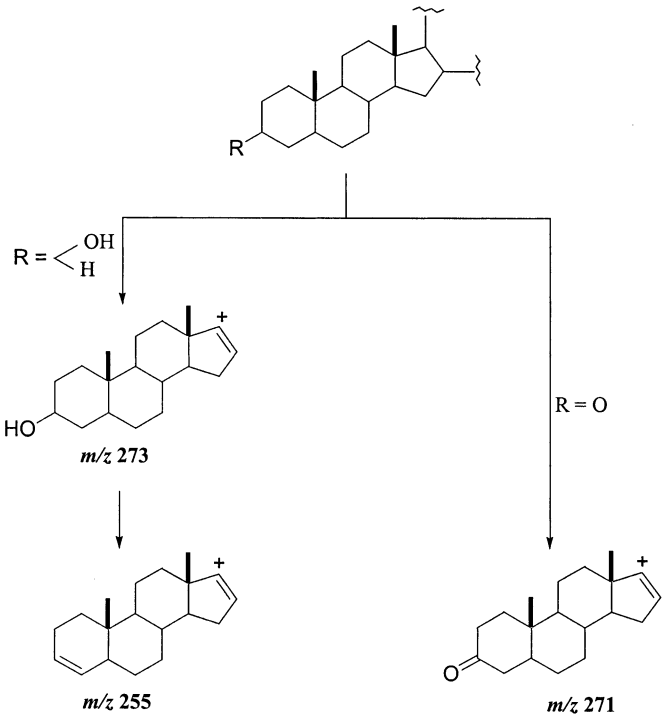
prominent peaks at *m/z* 527 $[M-H_2O+H]^+$ and *m/z* 407 $[M-H_2O-2AcOH+H]^+$. (+)-HRFABMS (+) of the pseudomolecular ion at *m/z* 567.3652 ($C_{33}H_{52}O_6Na$, Δ 0.9 mmu) which allowed us to determine the molecular formula of **7** to be $C_{33}H_{52}O_6$. The base peak at *m/z* 271, corresponding to $[M-H-\text{side chain } (C_{14}H_{24}O_5)]^+$, is in agreement with the proposed structure for the side chain. A model pattern of mass fragmentation for these steroids is shown in Scheme 1.

During the preparation of this manuscript, Sheu et al.[5] reported the isolation of hippuristerone A and the elucidation of its structure by NMR and single-crystal X-ray diffraction. This compound differs from compound **10** in having an additional OH group at C-16 and was also isolated as a minor component (compound **12**) in the work reported here. The relative stereochemistry of compound **10** was determined by a ROESY experiment and by comparison of the NMR chemical shift values with those of hippuristerone A. On the basis of these data the new compound, **10**, was named as hippuristerone B.

Comparison of the ^{13}C NMR and ^1H NMR data of compound **11** with those of **10** show a marked similarity between the two compounds. However, some differences are apparent, such as the absence of the signals assignable to the 3-keto group of **10**, a fact suggesting that compound **11** corresponds to the C-3 hydroxy derivative of compound **10** (Table 3). The α orientation of the hydroxyl group was deduced from the chemical shifts of C-3 at δ 66.5 and H-3 at δ 4.06, which are in agreement with values observed for the proton and carbon shifts from C-1 to C-10 in compound **9**.

The (+)-LRFABMS of **11** displays the fragment ions $[M+Na]^+$ at *m/z* 569, $[M-H_2O+H]^+$ at *m/z* 529, $[M-AcOH+Na]^+$ at *m/z* 509, $[M-AcOH+H]^+$ at *m/z* 487, $[M-H_2O-AcOH+H]^+$ at *m/z* 469, $[M-H_2O-2AcOH+H]^+$ at *m/z* 427, and $[M-2H_2O-2AcOH+H]^+$ at *m/z* 409. The molecular formula of **11** was determined to be $C_{33}H_{54}O_6$ on the basis of the (+)-HRFABMS of the pseudomolecular ion at *m/z* 569.3821 ($C_{33}H_{54}O_6Na$, Δ 0.3 mmu). Once again the peak at *m/z* 273, corresponding to $[M-H-\text{side chain } (C_{14}H_{24}O_5)]^+$, confirmed the proposed structure for the side chain (see Scheme 1). On the basis of this NMR and MS analysis, compound **11** was designated as hippuristerol B.

Comparison of the NMR data (^1H, ^{13}C, DEPT, ^1H–^1H COSY, HMQC, and HMBC) of compound **12** with those of **10** shows that these two compounds have similar



Comp.	*m/z* 273 (%)	*m/z* 255 (%)	Comp.	*m/z* 271 (%)
8	60	21	**7**	100
9	67	35	**10**	95
11	95	62	**15**	40
14	40	20	**17**	28
16	55	27		
17	60	21		

Scheme 1. Mass spectral fragmentations of compounds **7**–**11** and **14**–**17**.

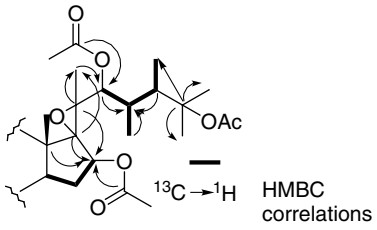


Figure 4. Selected HMBC correlations of **14**.

structures except for the substitution pattern in the D ring. The presence of an additional hydroxy group was deduced by the carbon and proton signals at δ_C 70.1 (d) and δ_H 4.03 (J=8.0 Hz). The (+)-LRFABMS of **12** shows the pseudomolecular ion $[M+Na]^+$ at m/z 583 and the base peak at m/z 481 $[M-AcOH-H_2O-2H+H]^+$. All of the spectral data for compound **12** match those reported by Sheu et al. for hippuristerone A, which was isolated very recently from the same organism.[5]

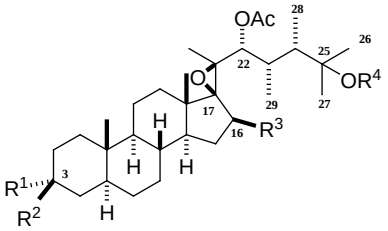
Compound **13** is the C-3 hydroxy derivative of compound **12**, as shown by the presence of the carbon and proton signals corresponding to an OH at C-3 and the absence of the ketone signal seen at δ_C 211.7 (s) in compound **12**. The α orientation of the hydroxyl group was deduced from the chemical shifts of H-3 at δ_H 4.05 and C-3 at δ_C 66.5, which are in agreement with compounds **9** and **11**. The (+)-LRFABMS displays the pseudomolecular ion $[M+Na]^+$ at m/z 585 and also shows significant fragments at m/z 485 $[M-H_2O-AcOH+H]^+$ and at m/z 425 $[M-H_2O-2AcOH+H]^+$. Furthermore, the (+)-HRFABMS of the ion at m/z 585.3763 ($C_{33}H_{54}O_7Na$, Δ 0.4 mmu) allowed us to determine the molecular formula of **13** as $C_{33}H_{54}O_7$. On the basis of these data, the new compound, **13**, was named as hippuristerol A.

Comparison of the NMR data (^1H, ^{13}C, DEPT, $^1H-^1H$ COSY, HMQC, and HMBC) of compound **14** with those of **13** again showed the two compounds to have similar structures. However, one of the hydroxyl groups in **13** is acetylated in **14**. The downfield shift of the signals for H-16 (by 0.55 ppm) and for C-16 (by 0.6 ppm) in the NMR spectra of **14**, in comparison to the corresponding signals in **13**, allowed us to assign the hydroxyl group to the C-16 position and indicated that **14** is the 16-acetyl derivative of **13**. (Table 3). The location of the hydroxyl group at C-16 was confirmed by the HMBC correlations between the proton at δ_H 5.17 (H-16) and the carbons at δ_C 171.0 (s) (OAc-16), 76.8 (C-17), 66.6 (C-20), and 43.3 (C-13) (see Fig. 4). The pseudomolecular ion $[M+Na]^+$ at m/z 627 and the fragment corresponding to the lost of side chain and the acetoxy group (m/z 273) (Scheme 1) shown in the (+)-LRFABMS of **14** agreed with the proposed structure.

Compound **15** is the C-3 oxidised derivative of compound **14**, as revealed by the presence of a signal for the ketone functionality at δ_C 211.7 (s) and the absence of the carbon and proton signals corresponding to an OH at C-3. The (+)-LRFABMS of **15** that showed the pseudomolecular ion $[M+Na]^+$ at m/z 625 and the fragment corresponding to the lost of side chain and the acetoxy group at m/z 271 confirmed the proposed structure. On the other hand, the relative stereochemistry of compounds **14** and **15** was determined by direct comparison of their NMR chemical shift values with those of **10**–**13**. On the basis of these data, compounds **14** and **15** were designated as hippuristerol C and hippuristerone C, respectively.

2.4. Steroids with an acyclic side chain (16 and 17)

NMR analysis (^1H, ^{13}C NMR, and DEPT) showed that **16** contains six quaternary carbons, eight methylenes, 10 methines, and eight methyl groups, two of which correspond to two acetate groups, and suggested a steroid skeleton identical to 24-methylcholestanone. The structural elucidation of the side chain of **16** was based on the following information. The presence in the side chain of the fragment Me_2COAc-, as also found in compounds **10**–**15**, was indicated by the carbon signals in the ^{13}C NMR spectrum at δ_C 85.5 (s, C-25), 22.6 (q, H_3-26), and 23.9 (q, H_3-27). The substructure $-OCH-CH_2-CHMe-$ was deduced from a $^1H-^1H$ COSY experiment, which showed the H_3-28 unit at δ_H 0.90 (d, J=6.6 Hz) to be coupled to the methine H-24 at δ_H 2.00; H-24 is coupled to the methylene H-23 (δ_H 1.24 and 1.08), and these protons are in turn coupled to an oxygenated methine H-22 (δ_H 4.90, dd, J=2.5 and 9.1 Hz). Furthermore, H_3-21 appears as a singlet at δ_H 1.23 (δ_C 20.2) and is linked to the quaternary oxygenated carbon C-20 (δ_C 77.5). All these data indicate the presence of 22,25-diacetoxy-20-hydroxy-24-methyl substitution in the side chain of **16**. The same oxygenated substitution can be found in the side chain of the ecdysone makisterone, although in that case the OH groups at C-22 and C-25 are not acetylated. The ^{13}C NMR spectral data of makisterone are in accordance with those of **16**.[9] The (+)-LRFABMS of **16** exhibits a pseudomolecular ion at m/z 557 $[M+Na]^+$,



10: $R^1 = R^2 = O$, $R^3 = H$, R^4= Ac

11: R^1 = OH, R^2 = H, R^3 = H, R^4= Ac

12: $R^1 = R^2 = O$, R^3 = OH, R^4= Ac

13: R^1 = OH, R^2 = H, R^3 = OH, R^4= Ac

14: R^1 = OH, R^2 = H, R^3 = OAc, R^4= Ac

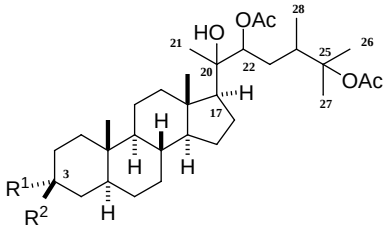
15: $R^1 = R^2 = O$, R^3 = OAc, R^4= Ac

21: $R^1 = R^2 = O$, R^3 = H R^4= H

corresponding to the molecular formula $C_{32}H_{54}O_6$, and fragments at *m/z* 497 $[M-AcOH+Na]^+$ and *m/z* 397 $[M-H_2O-2AcOH+H]^+$ are in agreement with the proposed structure. Thus, compound **16** was named as hippuristerol D.

The last new polyoxygenated steroid isolated, compound **17**, was found to be the C-3 oxidised derivative of compound **16**. The NMR spectral data (^1H NMR, ^{13}C NMR, and DEPT) of **17** are almost identical to those of **16**, but show the characteristic ketone functionality at δ_C 212.1. The (+)-LRFABMS of **17** shows the pseudomolecular ion $[M+Na]^+$ at *m/z* 555 along with fragments at *m/z* 495 $[M-AcOH+Na]^+$, *m/z* 473 $[M-AcOH+H]^+$, *m/z* 455 $[M-AcOH-H_2O+H]^+$, *m/z* 417 $[M-2AcOH-H_2O+Na]^+$, and *m/z* 395 $[M-H_2O-2AcOH+H]^+$. Finally, the molecular formula of compound **17** was determined to be $C_{32}H_{52}O_6$ by (+)-HRFABMS of its pseudomolecular $[M+Na]^+$ ion at *m/z* 555.3661 ($C_{32}H_{52}O_6Na$, Δ 0.1 mmu). Therefore, the name hippuristerone D was assigned for compound **17**.

The orthoester functionality present in the side chain of compounds **7**–**9** is very rare in steroid structures. As far as we know, the only precedents are orthosterol disulfates A and B, isolated from the marine sponge *Petrosia weinbergi*.[10] A plausible biogenetic origin of these compounds could be a nucleophilic attack by the hydroxyl group at C-20 on the acetoxy group at C-22 and a hydroxylation at the C-16 position from compounds **16** and **17**. Furthermore, compounds **10**–**15**, bearing two methyl groups at C-23 and C-24 and a 17,20-epoxy group are also very unusual within the steroid family. Indeed, two epoxy steroids isolated form the Indian Soft Coral *Sarcophyton crassocaule*[11] and more recently hippuristerone A[5] represent some of the few examples reported in the literature.



16: R^1 = OH, R^2 = H

17: $R^1 = R^2$ = O

2.5. Biological activity

All of the compounds were submitted to cytotoxic activity evaluation studies. These investigations showed that most of the compounds have a range of cytotoxic activities, as shown in Table 4. Furthermore, the high number of steroids isolated allowed us to deduce a number of structure–activity relationships.

The steroids bearing a spiroketal ring are more active than those without this feature, with compounds **1** and **3** being the most active. Among the spiroketal steroids, several conclusions can be reached. Firstly, we can divide these compounds into three categories depending the oxygenated positions in rings A and C: (1) class A contains compounds **1**, **3**, **4**, and **19** with two oxygenated positions at C-3 and C-11, (2) class B consists of compounds **2** and **18** with three oxygenated positions at C-2, C-3, and C-11, and (3) class C covers compounds **5** and **6** with one oxygenated position at C-3. In class A, the C-22 epimerization does not influence the cytotoxic activity (epimers **1** and **3** have the same level of activity). Acetylation of the OH at C-3 (compound **4**) decreases the activity but increases the cytotoxic selectivity and, finally, diacetylation leads to complete loss of activity. Compounds belonging to class B are inactive as either the monoacetylated (**2**) or the diacetylated (**18**) derivatives. On the other hand, for compounds in class C the oxidation level at C-3, i.e. the hydroxyl group (**5**) or ketone (**6**), does not influence the activity. It is worth noting that epihippuristanol is being patented as a cytotoxic compound.[12] The data on the structure–biological activity relationships in these compounds could be very valuable in future synthetic studies.

Table 4. Cytotoxicity assays of **1**–**13** and **16**–**19** (IC_{50} μg/mL)

Compound	P-388	A549	HT29	MEL28
1	0.1	0.1	0.1	0.1
2	>10	>10	>10	>10
3	0.1	0.1	0.1	0.1
4	1	0.125	0.5	0.125
5	5	5	5	5
6	5	5	5	5
7	2.5	5	5	5
8	2.5	5	5	5
9	>10	5	1	>10
10	>10	>10	>10	>10
11	1.25	1.25	1.25	1.25
12	>10	>10	>10	>10
13	1	1	1	1
16	1	1	1	1
17	1	1	1	1
18	>10	>10	>10	>10
19	>10	>10	>10	>10

Compounds **7**–**9**, with an orthoester group in the side chain, have much lower activity than the spiroketal compounds **1**, **3**, and **4**, but have similar activity to **5** and **6**. The C-3 ketone derivative, compound **7**, and the 3β OH derivative, compound **8**, have equal levels of activity, although the 3α OH derivative, compound **9**, was found to be more active and selective against HT29 tumor cells.

The oxidation of the hydroxyl group at C-3 in the steroids bearing an epoxy group and with methyl groups at C-23 and C-24 resulted in the loss of cytotoxic activity (**10/11** and **12/13**). Unfortunately, the decomposition of compounds **14** and **15** prevented us from evaluating their pharmacological activity. Finally, the activity of the steroids with an acyclic side chain, compounds **16** and **17**, is similar to that of compounds **11** and **13**, but in this case the activity does not decrease with oxidation of the hydroxyl group at C-3.

3. Experimental

3.1. General methods

NMR spectra were recorded at 500/125 MHz ($^1H/^{13}C$), AMX-Bruker; 200/50 MHz ($^1H/^{13}C$), Bruker AC-200

NMR spectrometer in $CDCl_3$. Carbon multiplicities were determined using DEPT-135 and DEPT-90 sequences. Atom connectivities were determined using HMQC, HMBC and COSY data. NOESY experiments were carried out using a mixing time of 0.8 s. (+)-LRFABMS were measured on VG-Quattro spectrometer while (+)-HRFABMS were measured on Trisector EBE spectrometer of Micromass Instruments using thioglycerol with 1% of NaI as matrix. Optical rotations were determined on a JASCO DIP-1000 with an Hg lamp at 590 nm. Semi-preparative HPLC was performed using (μ-Porasil and μ-Bondapak C_{18} columns (250×10 mm) with IR detection.

3.2. Collection, extraction, and isolation

Specimens of the gorgonian *I. hippuris* were collected in October 1996 in Indonesia, at a depth of 25 m. Voucher specimens were deposited at the Departamento de Química Fundamental, Universidade de A Coruña, under references UDC 96011 and UDC 96012. A picture can be obtained from the authors.

I. hippuris (UDC 96011): Specimens of the gorgonian (1 kg) were soaked in MeOH at room temperature for 48 h (3×500 mL). The extracts were combined and partitioned between CH_2Cl_2 and H_2O (1:1). The fraction that was soluble in CH_2Cl_2 was evaporated under reduced pressure and the residue partitioned between 10% aqueous MeOH and hexane. Water was added to the polar fraction until the mixture became 50% aqueous MeOH, and this was then extracted with CH_2Cl_2. After evaporation, the combined organic layers yielded 5.7 g of product from the hexane fraction and 2.0 g of product from the CH_2Cl_2 fraction. The fraction that was soluble in CH_2Cl_2 was fractionated by flash column chromatography (silica gel 230–400 mesh, eluting with CH_2Cl_2/EtOAc/MeOH mixtures of increasing polarity) to give several fractions. Fraction C4 (50 mg) was fractionated by HPLC (with 15% EtOAc in hexane) to give pure compound **7** (3.7 mg, 3.7×10^{-4}% wet weight), **10** (2.2 mg, 2.2×10^{-4}% wet weight) and another fraction (11 mg) that was also purified by HPLC (with MeOH/H_2O 92:8) to give pure compound **6** (8.6 mg, 8.6×10^{-4}% wet weight). Purification of fraction C5 (53 mg) was achieved by normal-phase HPLC using EtOAc/hexane (25:75) to give compounds **16** (10.8 mg, 1.08×10^{-3}% wet weight) and **11** (6.2 mg, 0.62×10^{-3}% wet weight). Fraction C6 (59 mg) was fractionated by HPLC (with 25% EtOAc in hexane) to give pure compound **5** (13.2 mg) as well as three fractions that were rechromatographed by HPLC (eluent MeOH/H_2O 85:15) to afford compounds **12** (1.5 mg, 1.5×10^{-4}% wet weight), **17** (3.5 mg) and **9** (3.5 mg, 0.35×10^{-3}% wet weight). Fraction C7 (90 mg) was purified by normal-phase (hexane/EtOAc 6:4) HPLC to give compounds **5** (3.7 mg, 1.69×10^{-3}% wet weight), **17** (3.5 mg, 0.73×10^{-3}% wet weight) and **13** (5.2 mg, 5.2×10^{-4}% wet weight). Fraction C8 (68 mg) was purified by HPLC (eluent hexane/EtOAc 65:35) to give compound **4** (10 mg, 1×10^{-3}% wet weight). Final purification of fraction C11 (37 mg) was achieved by reversed-phase HPLC (with MeOH/H_2O 7:3) to give compound **3** (133 mg, 13.3×10^{-3}% wet weight) and **1** (5.1 mg, 5.1×10^{-4}% wet weight). Rechromatography of fraction C13 (115 mg) on silica gel with EtOAc/hexane (9:1) followed by HPLC (with MeOH/H_2O 9:1) gave compound **2** (2.8 mg, 2.8×10^{-4}% wet weight).

I. hippuris (UDC 96012): Specimens of the gorgonian (1 kg) were soaked in MeOH at room temperature for 48 h (3×500 mL). The extracts were combined after removal of solvents under reduced pressure and subjected to solvent partitioning, as described previously, to give hexane (10 g), CH_2Cl_2 (8 g), and *n*-BuOH (4 g) soluble residues. A 7.6 g portion of the product from the CH_2Cl_2 fraction was purified by flash column chromatography (silica gel 230–400 mesh, eluting with hexane/EtOAc/MeOH mixtures of increasing polarity) to give several fractions. Fraction C10 (493 mg) was purified by HPLC (with 20% EtOAc in hexane) to give two fractions, which were rechromatographed by HPLC using acetonitrile/THF (95:5) and MeOH/H_2O (93:7), respectively, to afford compound **7** (43.8 mg, 5.76×10^{-3}%) and compound **10** (26.4 mg, 3.47×10^{-3}%). Fraction C11 (378 mg) was purified by reversed-phase HPLC (15% H_2O/acetonitrile) to give compound **11** (9.1 mg). Rechromatography of fraction C12 (341 mg) on silica gel with hexane/EtOAc (8:2) followed by HPLC (with MeOH/H_2O 9:1) gave two new compounds: **15** (7.6 mg, 1×10^{-3}%) and **8** (4.7 mg, 6.18×10^{-4}%). Purification of fraction C13 (176 mg) was achieved by normal-phase (hexane/EtOAc 8:2) and reversed-phase (MeOH/H_2O 9:1) HPLC to give compound **14** (5.4 mg). The fraction that was soluble in CH_2Cl_2 was fractionated by flash column chromatography (silica gel 230–400 mesh, eluting with CH_2Cl_2/EtOAc/MeOH mixtures of increasing polarity) to give several fractions. One of them directly led to the isolation of compound **3** (75 mg). The other fractions were purified by reversed-phase HPLC eluting with mixtures of MeOH/H_2O (8:2) to give compounds **11** (3.4 mg, 1×10^{-3}%) and **14** (52.3 mg, 3.96×10^{-3}%).

3.2.1. 3-Acetyl-22-*epi*-hippuristanol (4). White powder; $[\alpha]^{27}_D=-23.9$ (c=0.14, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 527 (4, $[M+Na]^+$), 505 (7, $[M+H]^+$), 503 (7, $[M-H]^+$), 487 (39, $[M-H_2O+H]^+$), 485 (20, $[M-H_2O-H]^+$), 469 (7, $[M-2H_2O+H]^+$), 467 (4, $[M-2H_2O-H]^+$, $[M-AcOH+Na]^+$), 427 (7, $[M-AcOH-H_2O+H]^+$), 255 (100); (+)-HRFABMS *m/z* 527.3335 $[M+Na]^+$ (calcd for $C_{30}H_{48}O_6Na$, 527.3348); ^1H NMR ($CDCl_3$, 200 MHz): δ 5.0 (1H, br s, H-3α), 4.44 (1H, m, H-16), 4.30 (1H, br s, H-11), 2.04 (3H, s, OAc), 1.31 (3H, s, H-18), 1.28 (3H, s, H-27), 1.26 (3H, s, H-21), 1.04 (3H, s, H-19), 0.98 (3H, s, H-26), 0.94 (3H, d, J=7.3 Hz, H-28); ^{13}C NMR: see Table 1.

3.2.2. 11-Dehydroxy-22-*epi*-hippuristanol (5). White powder; $[\alpha]^{26}_D=-23.1$ (c=0.625, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 447 (70, $[M+H]^+$), 429 (100, $[M-H_2O+H]^+$), 411 (11, $[M-2H_2O+H]^+$); (+)-HRFABMS *m/z* 447.3467 $[M+H]^+$ (calcd for $C_{28}H_{47}O_4$, 447.3474); ^1H NMR ($CDCl_3$, 200 MHz): δ 4.05(1H, br s, H-3α), 4.43 (1H, m, H-16), 1.31 (3H, s, H-18), 1.29 (3H, s, H-19), 1.28 (3H, s, H-27), 1.26 (3H, s, H-21), 0.99 (3H, s, H-26), 0.95 (3H, d, J=7.3 Hz, H-28); ^{13}C NMR: see Table 1.

3.2.3. 11-Dehydroxy-22-*epi*-hippuristan-3-one (6). White powder; $[\alpha]^{26}_D=-41.5$ (c=0.03, $CHCl_3$); (+)-LRFABMS

m/z (relative intensity): 445 (7, $[M+H]^+$), 467 (1, $[M+Na]^+$), 427 (13, $[M-H_2O+H]^+$), 181 (100). ^1H NMR ($CDCl_3$, 200 MHz): δ 4.43 (1H, m, H-16), 1.31 (3H, s, H-18), 1.28 (3H, s, H-27), 1.14 (3H, s, H-21), 1.14 (3H, s, H-19), 0.99 (3H, s, H-26), 0.95 (3H, d, *J*=6.8 Hz, H-28); ^{13}C NMR: see Table 1.

3.2.4. Orthohippurinsterone A (7). White powder; $[\alpha]^{27}_D=-58.5$ (*c*=0.035, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 531 (9, $[M-H_2O+H]^+$), 471 (31, $[M-H_2O-AcOH+H]^+$), 411 (31, $[M-H_2O-2AcOH+H]^+$), 271 (100); (+)-HRFABMS *m/z* 451.3184 [M−2AcOH+Na] (calcd for $C_{28}H_{44}O_3Na$, 451.3188); ^1H NMR: see Table 2; ^{13}C NMR: see Table 1.

3.2.5. Orthohippurinsterol A (8). $[\alpha]^{23}_D=+2.67$ (*c*=0.07, $CHCl_3$); (+) LRFABMS *m/z* (relative intensity): 551 (3, $[M+H]^+$), 533 (5, $[M-H_2O+H]^+$), 473 (32, $[M-H_2O-AcOH+H]^+$, 167 (100); ^1H NMR ($CDCl_3$, 500 MHz): (4.51 (1H, dt, *J*=4.2, 7.2, 7.2 Hz, H-16), 3.98 (1H, dd, *J*=5.1, 8.1 Hz, H-22), 3.58 (1H, m, H-3), 2.21 (1H, t, *J*=6.4 Hz, H-24), 1.97 (3H, s, OAc), 1.66 (1H, m, H-23), 1.53 (3H, s, H-30), 1.43 (3H, s, H-26), 1.37 (3H, s, H-27), 1.35 (3H, s, H-21), 1.10 (1H, m, H-5), 1.05 (3H, s, H-18), 0.99 (3H, d, *J*=6.8 Hz, H-28), 0.82 (3H, s, H-19); ^{13}C NMR: see Table 1.

3.2.6. Orthohippurinsterol B (9). White powder; $[\alpha]^{27}_D=+11.7$ (*c*=0.07, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 473 (27, $[M-H_2O-AcOH+H]^+$), 413 (85, $[M-H_2O-2AcOH+H]^+$), 161 (100); (+)-HRFABMS *m/z* 555.3656 $[M-H_2O+Na]^+$ (calcd for $C_{32}H_{52}O_6Na$, 555.3662); ^1H NMR ($CDCl_3$, 200 MHz): δ 4.52 (1H, m, H-16), 4.05 (1H, br s, H-3), 3.98 (1H, dd, *J*=5.0, 8.2 Hz, H-22), 1.98 (3H, s, OAc), 1.55 (3H, s, H-30), 1.45 (3H, s, H-26), 1.39 (3H, s, H-27), 1.37 (3H, s, H-21), 1.06 (3H, s, H-18), 0.99 (3H, d, *J*=6.8 Hz, H-28), 0.81 (3H, s, H-19); ^{13}C NMR: see Table 1.

3.2.7. Hippuristerone B (10). White powder; $[\alpha]^{26}_D=+8.7$ (*c*=0.765, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 567 (2, $[M+Na]^+$), 527 (2, $[M-H_2O+H]^+$), 467 (2, $[M-H_2O-AcOH+H]^+$), 407 (6, $[M-H_2O-2AcOH+H]^+$), 271 (100); (+)-HRFABMS *m/z* 567.3652 $[M+Na]^+$ (calcd for $C_{33}H_{52}O_6Na$, 567.3662); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3.

3.2.8. Hippuristerol B (11). White powder; $[\alpha]^{24}_D=+2.3$ (*c*=0.225, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 569 (5, $[M+Na]^+$), 529 (3, $[M-H_2O+H]^+$), 509 (5, $[M-AcOH+Na]^+$), 487 (5, $[M-AcOH+H]^+$), 469 (8, $[M-H_2O-AcOH+H]^+$), 427 (10, $[M-2AcOH+H]^+$), 409 (8, $[M-H_2O-2AcOH+H]^+$), 213 (100); (+)-HRFABMS *m/z* 569.3821 $[M+Na]^+$ (calcd for $C_{33}H_{54}O_6Na$, 569.3818); ^1H NMR ($CDCl_3$, 200 MHz): δ 4.74 (1H, d, *J*=10.7 Hz, H-22), 4.06 (1H, br s, H-3), 2.10 (3H, s, OAc), 2.00 (3H, s, OAc), 1.56 (3H, s, H-21), 1.55 (3H, s, H-27), 1.51 (3H, s, H-26), 0.95 (3H, d, *J*=7.3 Hz, H-28), 0.91 (3H, s, H-18), 0.86 (3H, d, *J*=6.8 Hz, H-29), 0.78 (3H, s, H-19); ^{13}C NMR: see Table 3.

3.2.9. Hippuristerone A (12). White powder; $[\alpha]^{24}_D=+1.36$ (*c*=0.66, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 583 (18, $[M+Na]^+$), 445 (2, $[M-H_2O-2AcOH+Na]^+$), 207 (100); ^1H NMR ($CDCl_3$, 200 MHz): δ 4.62 (1H, d, *J*=10.8 Hz, H-22), δ 4.03 (1H, t, *J*=8.0 Hz, H-16), 2.12 (3H, s, OAc), 1.99 (3H, s, OAc), 1.58 (3H, s, H-21), 1.56 (3H, s, H-27), 1.45 (3H, s, H-26), 1.02 (3H, s, H-19), 0.97 (3H, s, H-18), 0.91 (3H, d, *J*=6.8 Hz, H-28), 0.88 (3H, d, *J*=6.3 Hz, H-29); ^{13}C NMR: see Table 3.

3.2.10. Hippuristerol A (13). White powder; $[\alpha]^{25}_D=+6.1$ (*c*=0.4, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 485 (2, $[M-H_2O-AcOH+H]^+$), 425 (2, $[M-H_2O-2AcOH+H]^+$), 282 (100); (+)-HRFABMS *m/z* 585.3763 $[M+Na]^+$ (calcd for $C_{33}H_{54}O_7Na$, 585.3767); ^1H NMR ($CDCl_3$, 200 MHz): δ 4.62 (1H, d, *J*=10.7 Hz, H-22), 4.05 (1H, br s, H-3), δ 4.03 (1H, t, *J*=8.0 Hz, H-16), 2.10 (3H, s, OAc), 1.99 (3H, s, OAc), 1.58 (3H, s, H-21), 1.55 (3H, s, H-27), 1.50 (3H, s, H-26), 0.95 (3H, s, H-18), 0.92 (3H, d, *J*=7.0 Hz, H-28), 0.88 (3H, d, *J*=6.3 Hz, H-29), 0.78 (3H, s, H-19); ^{13}C NMR: see Table 3.

3.2.11. Hippuristerol C (14). $[\alpha]^{30}_D=+10.4$ (*c*=0.195, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 627 (28, $[M+Na]^+$), 191 (100); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3.

3.2.12. Hippuristerone C (15). $[\alpha]^{28}_D=+48.4$ (*c*=0.015, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 625 (8, $[M+Na]^+$), 217 (100); ^1H NMR ($CDCl_3$, 200 MHz): δ 5.19 (1H, t, *J*=7.6 Hz, H-16), 4.46 (1H, d, *J*=10.7 Hz, H-22), 2.14 (3H, s, OAc), 2.07 (3H, s, OAc), 1.98 (3H, s, OAc), 1.57 (3H, s, H-21), 1.55 (3H, s, H-26), 1.44 (3H, s, H-27), 1.01 (6H, s, H-18, H-19), 1.00 (3H, d, *J*=7.3 Hz, H-28), 0.82 (3H, d, *J*=6.3 Hz, H-29); ^{13}C NMR: see Table 3.

3.2.13. Hippuristerol D (16). White powder; $[\alpha]^{27}_D=+3.7$ (*c*=0.175, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 557 (1, $[M+Na]^+$), 497 (1, $[M-AcOH+Na]^+$), 397 (6, $[M-H_2O-2AcOH+H]^+$), 299 (100). ^1H NMR ($CDCl_3$, 200 MHz): δ 4.90 (1H, dd, *J*=2.0, 9.4 Hz, H-22), 4.05 (1H, br s, H-3), 2.10 (3H, s, OAc), 1.99 (3H, s, OAc), 1.47 (3H, s, H-27), 1.37 (3H, s, H-26), 1.23 (3H, s, H-21), 0.90 (3H, d, *J*=6.6 Hz, H-28), 0.86 (3H, s, H-18), 0.79 (3H, s, H-19); ^{13}C NMR: see Table 3.

3.2.14. Hippuristerone D (17). White powder; $[\alpha]^{23}_D=+7.38$ (*c*=0.38, $CHCl_3$); (+)-LRFABMS *m/z* (relative intensity): 555 (1, $[M+Na]^+$), 495 (1, $[M-AcOH+Na]^+$), 473 (1, $[M-AcOH+H]^+$), 455 (1, $[M-AcOH-H_2O+H]^+$), 417 (2, $[M-2AcOH-'''H_2O+Na]^+$), 395 (4, $[M-H_2O-2AcOH+H]^+$), 377 (3, $[M-2H_2O-2AcOH+H]^+$), 181 (100); (+)-HRFABMS *m/z* 555.3661 $[M+Na]^+$ (calcd for $C_{32}H_{52}O_6Na$, 555.3662); ^1H NMR ($CDCl_3$, 200 MHz): δ 4.89 (1H, dd, *J*=2.0, 9.4 Hz, H-22), 2.11 (3H, s, OAc), 1.98 (3H, s, OAc), 1.46 (3H, s, H-27), 1.36 (3H, s, H-26), 1.23 (3H, s, H-21) 1.01 (3H, s, H-19), 0.90 (3H, d, *J*=6.8 Hz, H-28), 0.87 (3H, s, H-18); ^{13}C NMR: see Table 3.

3.2.15. Acetylation of 2 and 3. Each compound (5 mg) was treated with 1 mL of Ac_2O/pyridine (1:1) at room temperature and the mixture was stirred for 21 h. After the usual work up, the acetylated derivatives were purified by flash

chromatography (silica gel). Thus, acetylation of **2** gave the diacetylated compound **18** (4 mg) and acetylation of **3** gave the mono- and diacetylated products **4** (2 mg) and **19** (1.5 mg), respectively. The products were identified by ^{1}H NMR spectroscopy as 3-acetyl-22-*epi*-hippurin-1 compound **18** and 3,11-diacetyl-22-epi-hippuristanol compound **19**.[3]

3.2.16. Basic hydrolysis of compounds 7 and 10. Each compound (3.1 mg) was treated with 5% KOH in MeOH and stirred at room temperature. After 17 h the solvent was removed and saturated aqueous NaCl (3 mL) was added. The aqueous layer was extracted with CH_2Cl_2 (4×3 mL) and the solvent was removed in vacuo. Thus, basic hydrolysis of compound **7** afforded compound **20** (1.5 mg) and compound **10** gave compound **21** (1.0 mg). The products were identified by ^{1}H NMR spectroscopy.

Compound **20**: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 4.54 (1H, m, H-16), 4.12 (1H, m, H-22), 1.56 (3H, s, H-30), 1.37 (3H, s, H-21), 1.24 (3H, s, H-26), 1.14 (3H, s, H-27), 1.09 (3H, s, H-18), 1.04 (3H, s, H-19), 1.02 (3H, d, J=6.8 Hz, H-28).

Compound **21**: ^{1}H NMR ($CDCl_3$, 200 MHz): (3.18 (1H, d, J=10.3 Hz, H-22), 1.53 (3H, s, H-21), 1.24 (3H, s, H-27), 1.22 (3H, s, H-26), 1.02 (3H, s, H-19), 0.99 (3H, d, J=6.8 Hz, H-28), 0.94 (3H, s, H-18), 0.90 (3H, d, J=6.8 Hz, H-29).

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