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New cytotoxic steroids from the soft coral *Clavularia viridis*

Chang-Yih Duh[a,b,*], *I-Wen Lo*[a], *Shang-Kwei Wang*[c], *Chang-Feng Dai*[d]

[a] *Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan*
[b] *Center of Asia-Pacific Marine Researches, National Sun Yat-sen University, Kaohsiung, Taiwan*
[c] *Department of Microbiology, Kaohsiung Medical University, Kaohsiung, Taiwan*
[d] *Institute of Oceanography, National Taiwan University, Taipei, Taiwan*

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ABSTRACT

Ten new cytotoxic steroids, stoloniferones H–Q (**1–10**) were isolated from the methylene chloride solubles of the soft coral *Clavularia viridis*. The structures of the metabolites were elucidated on the basis of spectroscopic (IR, MS, and 1D and 2D NMR) analysis and their cytotoxicity against selected cancer cells was measured *in vitro*.

1. Introduction

The genus *Clavularia* has afforded many types of bioactive prostanoids, terpenoids and steroids [1–11]. As part of our search for bioactive substances from marine organisms, the soft coral *Clavularia viridis* Quoy and Gaimard (class *Anthozoa*, subclass *Octocorallia*, order *Stolonifera*) was studied because the CH_2Cl_2 extracts showed significant cytotoxicity to HT-29 (human colon adenocarcinoma), and P-388 (mouse lymphocytic leukemia) cell cultures as determined by standard procedures [12,13]. Bioassay-guided fractionations of the CH_2Cl_2 extracts resulted in the isolation of ten new cytotoxic steroids, stoloniferones H–Q (**1–10**).

2. Experimental

2.1. General

Optical rotations were determined on a JASCO DIP-181 polarimeter. UV spectra were obtained on a Shimadzu UV-160A spectrophotometer, and IR spectra were recorded on a Hitachi 26–30 spectrophotometer. The NMR spectra were recorded on Bruker Avance 300 NMR spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C, respectively, in $CDCl_3$ using TMS as internal standard. ESIMS spectra were obtained with a Bruker APX II mass spectrometer. Si gel 60 (Merck, 230–400 mesh)

* *Corresponding author*. Tel.: +886 7 5252000x5036; fax: +886 7 5255020.
E-mail address: yihduh@mail.nsysu.edu.tw (C.-Y. Duh).
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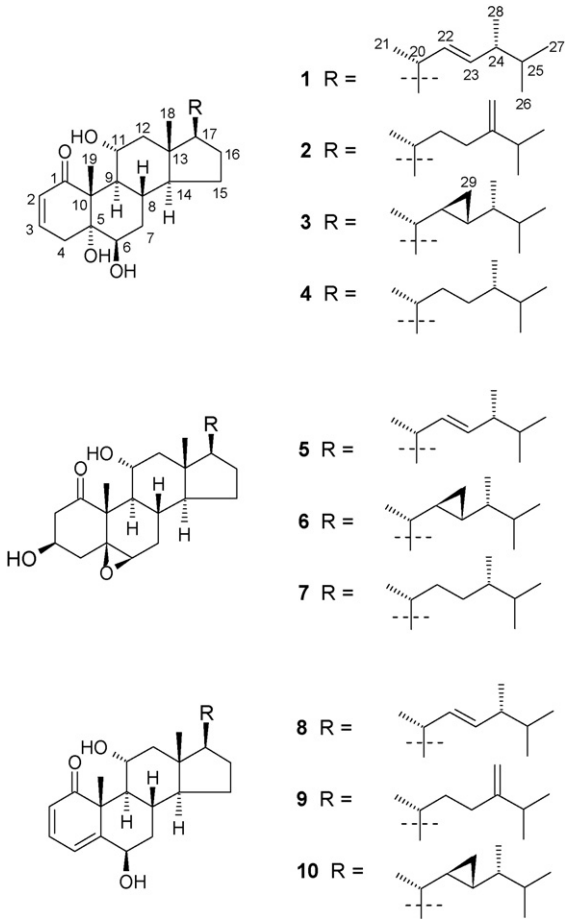
was used for column chromatography; precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analysis.

2.2. Animal material

The soft coral *Clavularia viridis* was collected at Green Island, off Taiwan, in July 2003, at a depth of 2–3 m and was stored for 1 month in a freezer until extraction. A voucher specimen, NSUGN-061, is deposited in the Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Taiwan.

2.3. Extraction and isolation

The bodies of the soft coral *C. viridis* were freeze dried to give 1.90 kg of a solid, which was extracted with CH_2Cl_2 (5.0 l × 3). After removal of solvent in vacuo, the residue (80 g) was chromatographed over silica gel 60 using *n*-hexane and *n*-hexane/EtOAc mixtures of increasing polarity. Elution by *n*-hexane/EtOAc (60:40 v/v) afforded fractions containing compounds **8–10**. Elution by *n*-hexane/EtOAc (50:50 v/v) afforded fractions containing compounds **1–7**. Compounds **1–7** were further purified by C_{18} HPLC column using MeOH/H_2O (86:14 v/v) as solvent system. Compounds **8–10** were further purified by C_{18} HPLC column using MeOH/H_2O (88:12 v/v) as solvent system.



2.3.1. *Stoloniferone H* (**1**)

Colorless amorphous solid; $[\alpha]_D^{25} = +15.0$ (c = 0.1, $CHCl_3$); UV (MeOH) λ_{max} 224 nm (log ε 3.56); IR ν_{max} 3420, 1653 cm^{-1}; ^1H NMR see Table 1; ^{13}C NMR see Table 2; HRESIMS *m/z* 467.3134 (calcd for $C_{28}H_{44}O_4Na$, 467.3137).

2.3.2. *Stoloniferone I* (**2**)

Colorless amorphous solid; $[\alpha]_D^{25} = +12.0$ (c = 0.1, $CHCl_3$); UV (MeOH) λ_{max} 225 nm (log ε 3.58); IR ν_{max} 3430, 1656 cm^{-1}; ^1H NMR see Table 1; ^{13}C NMR see Table 2; HRESIMS *m/z* 467.3134 (calcd for $C_{28}H_{44}O_4Na$, 467.3137).

2.3.3. *Stoloniferone J* (**3**)

Colorless amorphous solid; $[\alpha]_D^{25} = +26.0$ (c = 0.1, $CHCl_3$); UV (MeOH) λ_{max} 224 nm (log ε 3.46); IR ν_{max} 3400, 1658 cm^{-1}; ^1H NMR see Table 1; ^{13}C NMR see Table 2; HRESIMS *m/z* 459.3476 (calcd for $C_{29}H_{47}O_4$, 459.3474).

2.3.4. *Stoloniferone K* (**4**)

Colorless amorphous solid; $[\alpha]_D^{25} = +18.0$ (c = 0.1, $CHCl_3$); UV (MeOH) λ_{max} 223 nm (log ε 3.38); IR ν_{max} 3480, 1657 cm^{-1}; ^1H NMR see Table 1; ^{13}C NMR see Table 2; HRESIMS *m/z* 469.3291 (calcd for $C_{28}H_{46}O_4Na$, 469.3294).

2.3.5. *Stoloniferone L* (**5**)

Colorless amorphous solid; $[\alpha]_D^{25} = -45.0$ (c = 0.1, $CHCl_3$); IR ν_{max} 3439, 1703 cm^{-1}; ^1H NMR see Table 3; ^{13}C NMR see Table 2; HRESIMS *m/z* 445.3319 (calcd for $C_{28}H_{45}O_4$, 445.3318).

2.3.6. *Stoloniferone M* (**6**)

Colorless amorphous solid; $[\alpha]_D^{25} = -42.0$ (c = 0.1, $CHCl_3$); IR ν_{max} 3441, 1705 cm^{-1}; ^1H NMR see Table 3; ^{13}C NMR see Table 2; HRESIMS *m/z* 481.3292 (calcd for $C_{29}H_{46}O_4Na$, 481.3294).

2.3.7. *Stoloniferone N* (**7**)

Colorless amorphous solid; $[\alpha]_D^{25} = -23.0$ (c = 0.1, $CHCl_3$); IR ν_{max} 3443, 1705 cm^{-1}; ^1H NMR see Table 3; ^{13}C NMR see Table 2; HRESIMS *m/z* 469.3292 (calcd for $C_{28}H_{46}O_4Na$, 469.3294.

2.3.8. *Stoloniferone O* (**8**)

Colorless amorphous solid; $[\alpha]_D^{25} = +10.0$ (c = 0.1, $CHCl_3$); UV (MeOH) λ max nm (log ε) 314 (4.20); IR ν_{max} 3409, 1652 cm^{-1}; ^1H NMR see Table 4; ^{13}C NMR see Table 2; HRESIMS *m/z* 449.3029 (calcd for $C_{28}H_{42}O_3Na$, 449.3031).

2.3.9. *Stoloniferone P* (**9**)

Colorless amorphous solid; $[\alpha]_D^{25} = +9.2$ (c = 0.1, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε) 313 (4.12); IR ν_{max} 3420, 1656 cm^{-1}; ^1H NMR see Table 4; ^{13}C NMR see Table 2; HRESIMS *m/z* 449.3028 (calcd for $C_{28}H_{42}O_3Na$, 449.3031).

2.3.10. *Stoloniferone Q* (**10**)

Colorless amorphous solid; $[\alpha]_D^{25} = +18.6$ (c = 0.2, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε) 313 (4.22); IR ν_{max} 3380, 1655 cm^{-1}; ^1H NMR see Table 4; ^{13}C NMR see Table 2; HRESIMS *m/z* 463.3192 (calcd for $C_{29}H_{44}O_3Na$, 463.3188).

2.4. Cytotoxicity testing

P-388 cells were kindly supplied by J. M. Pezzuto, formerly of the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago; HT-29 cells were purchased from the American Type Culture Collection. Cytotoxic assays

Table 1 – ^{1}H NMR spectroscopic data[a] (300 MHz) of 1–4 in $CDCl_3$

	1	2	3	4
2	6.02 dd (9.9, 2.4)[b]	6.01 dd (9.9, 2.4)	6.02 dd (9.9, 2.4)	6.02 dd (9.9, 2.4)
3	6.69 dt (9.9, 2.4)	6.69 dt (9.9, 2.4)	6.68 dt (9.9, 2.4)	6.69 dt (9.9, 2.4)
4α	2.22 m	2.16 m	2.08 m	2.15 m
4β	3.43 dt (19.8, 2.4)	3.43 dt (19.8, 2.4)	3.42 dt (19.8, 2.4)	3.44 dt (19.8, 2.4)
6	3.66 br s	3.67 br s	3.67 br s	3.67 br s
7	1.82 m, 1.61 m	1.70 m, 1.62 m	1.82 m, 1.62 m	1.70 m, 1.61 m
8	1.83 m	1.95 m	1.94 m	1.91 m
9	2.00 m	2.01 m	2.02 m	2.01m
11	3.70 dt (3.3, 10.5)	3.70 dt (3.6, 10.5)	3.70 dt (3.6, 10.2)	3.71 dt (3.6, 10.5)
12α	1.43 m	1.40 m	1.44 m	1.42 m
12β	2.34 dd (12.6, 3.3)	2.36 dd (12.3, 3.6)	2.36 dd (12.3, 3.6)	2.37 dd (12.6, 3.6)
14	1.25 m	1.23 m	1.31 m	1.25 m
15	1.52 m, 1.08 m	1.62 m, 1.14 m	1.63 m, 1.12 m	1.62 m, 1.09 m
16	1.75 m, 1.27 m	1.86 m, 1.30 m	1.86 m, 1.42 m	1.61m, 1.35 m
17	1.28 m	1.31 m	1.38 m	1.28 m
18	0.74 s	0.78 s	0.74 s	0.77 s
19	1.48 s	1.48 s	1.48 s	1.49 s
20	2.06 m	1.43 m	0.87 m	1.38 m
21	1.05 d (6.6)	0.99 d (6.3)	0.97 d (6.6)	0.96 d (6.0)
22	5.15 dd (15.6, 7.2)	1.50 m, 1.18 m	0.29 m	1.42 m, 0.97 m
23	5.19 dd (15.6, 7.8)	2.15 m, 1.93 m	0.53 m	1.41 m, 0.96 m
24	1.89 m		0.51 m	1.24 m
25	1.50 m	2.28 m	1.70 m	1.49 m
26	0.82 d (6.3)	1.03 d (6.9)	0.87 d (6.3)	0.78 d (6.9)
27	0.84 d (6.3)	1.03 d (6.9)	0.88 d (6.0)	0.86 d (6.9)
28	0.91 d (6.9)	4.72 s, 4.66 s	0.89 d (6.3)	0.79 d (6.6)
29			0.13 m	
OH-11	5.19 d (5.7)	5.03 d (6.3)	4.99 d (5.7)	4.99 d (5.7)

[a] Assigned by COSY, HSQC, NOESY, and HMBC experiments.
[b] *J* values (in Hz) in parentheses.

were carried out according to the previously described procedures [13].

3. Results and discussion

Compound **1** had a molecular formula of $C_{28}H_{44}O_4$ as indicated by HRESIMS and ^{13}C NMR spectroscopic data. ^{13}C NMR and DEPT spectra of **1** exhibited the presence of six methyls, five sp^3 methylenes, nine sp^3 methines, four sp^2 methines, three sp^3 quaternary carbons and one sp^2 quaternary carbon. The IR spectrum of **1** showed absorption due to a hydrogen bonded α,β-unsaturated ketone (1653 cm^{-1}). The presence of a conjugated enone system in **1** was also indicated by UV absorption at 224 nm (log ε 3.56) as well as ^{1}H NMR [δ 6.02 (1H, dd, *J* = 9.9, 2.4 Hz), 6.69 (1H, dd, *J* = 9.9, 2.4 Hz)] (Table 1) and ^{13}C NMR [δ 128.9 (CH), 142.2 (CH), 208.7 (qC)] spectra (Table 2). IR absorption at 3420 cm^{-1} and NMR signals at δ_H 3.66 (1H, br s) and 3.70 (1H, dt, *J* = 3.3, 10.5 Hz) as well as at δ_C 74.7 (CH) and 68.4 (CH) indicated the presence of two secondary hydroxyl groups. The ^{13}C NMR data of **1** indicated some similarities to those of yonarasterol A [14], except for the absence of a secondary acetate at C-6. All C–H correlations of **1** were detected in the HSQC experiment. The ^{1}H–^{1}H COSY spectrum exhibited partial structures **a**, **b**, and **c** (Fig. 1). In the HMBC spectrum, partial structure **a** could be connected to **b** through three quaternary carbons (C-1, C-5 and C-10) and H_3-19 (Fig. 1). Partial structure **b** could be connected to **c** through the remaining quaternary carbon (C-13) and H_3-18. Based on these findings, the planar structure of **1** was concluded as in Fig. 1. The configuration at C-20 and C-24 was determined by comparison of ^{13}C NMR data with those of yonarasterol A and epimeric steroidal side chains [14,15]. The coupling constant between H-22 and H-23 (*J* = 15.6 Hz) suggested the double bond to have *E* configuration. The NOESY correlations (Fig. 2) observed between H-11 and H-8, H-11 and H_3-18, H-11 and H_3-19, H-3 and H-2, H-9 and H-14, H_3-18 and H-8, H_3-19 and H-8, H_3-18 and H-20, H-4β and H_3-19, H-4α and H-6, H_3-21 and H-12β, H-9 and H-12α indicated all trans configurations for ring fusions.

The spectroscopic data of **2**–**4** were analogous to those of **1**, except for NMR signals due to the side chains. As shown

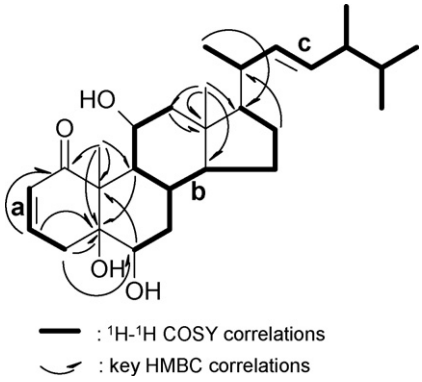


Fig. 1 – ^{1}H–^{1}H COSY and key HMBC correlations of 1.

Table 2 – ^{13}C NMR spectroscopic data[a] (75 MHz) of 1–10 in $CDCl_3$

	1	2	3	4	5	6	7	8	9	10
1	208.7	208.6	208.7	208.7	215.3	215.2	215.4	212.5	212.5	212.5
2	128.9	128.9	128.9	128.9	47.1	47.1	47.1	126.9	126.9	126.9
3	142.2	142.2	142.1	142.2	64.2	64.2	64.2	140.9	140.8	140.8
4	36.8	36.8	36.3	36.8	40.9	40.9	40.9	119.0	119.0	119.0
5	77.9	77.5	77.9	77.9	61.3	61.3	61.3	157.8	157.7	157.7
6	74.7	74.7	74.7	74.7	60.9	61.0	61.0	73.7	73.7	73.7
7	32.9	33.0	32.9	33.0	31.6	31.6	31.6	40.4	40.4	40.4
8	28.5	28.4	28.4	28.4	28.0	28.5	28.0	29.5	29.5	29.5
9	47.5	47.5	47.2	47.5	49.9	49.8	49.8	58.3	58.1	58.2
10	54.0	54.0	54.3	54.0	51.8	51.7	51.7	55.4	55.4	55.4
11	68.4	68.4	68.4	68.4	67.6	67.6	67.6	66.9	66.9	66.9
12	51.0	51.2	51.2	51.2	49.2	49.2	49.3	49.4	49.6	49.5
13	43.1	43.3	43.6	43.2	42.8	43.2	42.9	42.7	42.9	43.2
14	56.0	55.9	55.6	55.9	55.5	55.2	55.4	55.0	54.9	54.6
15	24.0	24.0	24.0	24.0	24.0	24.4	24.1	24.6	24.6	24.9
16	28.8	28.5	28.5	28.5	28.6	29.8	28.2	28.7	28.3	28.6
17	55.9	56.1	57.5	56.1	55.8	57.4	55.9	55.9	56.0	57.5
18	13.8	13.6	13.1	13.6	13.0	12.6	12.8	13.2	13.1	12.9
19	15.1	15.1	15.1	15.1	13.6	13.6	13.6	19.6	19.6	19.6
20	40.3	35.9	39.8	36.3	40.1	40.0	36.1	40.2	35.8	40.1
21	20.8	18.5	19.1	18.7	20.9	19.1	18.8	20.9	18.6	19.2
22	135.6	34.6	25.2	33.6	135.4	25.2	33.6	135.5	34.6	25.2
23	132.0	31.1	24.0	30.7	132.2	24.1	30.6	132.1	31.1	24.1
24	42.8	156.9	45.0	39.1	42.8	45.0	39.1	42.8	156.8	45.0
25	33.1	33.9	33.0	31.5	33.1	32.9	31.5	33.1	33.9	32.9
26	19.7	21.9	18.6	17.7	19.7	18.6	17.7	19.7	21.9	18.6
27	20.0	22.1	20.7	20.6	20.0	20.7	20.5	20.0	22.1	20.8
28	17.7	106.0	15.9	15.5	17.6	15.8	15.5	17.7	106.1	15.9
29			10.5			10.5				10.5

[a] Assigned by DEPT, COSY, HSQC, and HMBC experiments.

in Tables 1 and 2, signals due to an *E* disubstituted double bond were absent in **2**, but those due to an exo double bond [δ_H 4.72 (1H, s) and 4.66 (1H, s); δ_C 156.9 (qC) and 106.0 (CH_2)] appeared in **2**, similar to those observed for stoloniferone G..[11] Thus, the structure of **2** was concluded as a 22,23-dihydro-24,28-dehydro analog of **1**. NMR spectra of **3** suggested the *E* disubstituted olefin in the side chain of **1** to be replaced by a 1,2-disubstituted cyclopropane [δ_H 0.13 (2H, m), 0.29 (1H, m), and 0.53 (1H, m); δ_C 10.5 (CH_2), 25.2 (CH) and 24.0 (CH)]. HMBC analysis indicated that **3** has a cyclopropane at C-22 and C-23. The configuration of four chiral centers (C-20, C-22, C-23, and C-24) in the side chain was determined by comparison of ^{13}C NMR spectral data with those of stoloniferone-d, the stereostructure of which was established by X-ray crystallographic analysis [16]. NMR signals assigned to a –CH_2CH_2– group at C-22 and C-23 [δ_C 33.6 (CH_2) and 30.7 (CH_2)] in **4** were observed instead of the *E* disubstituted double bond in **1** as shown in

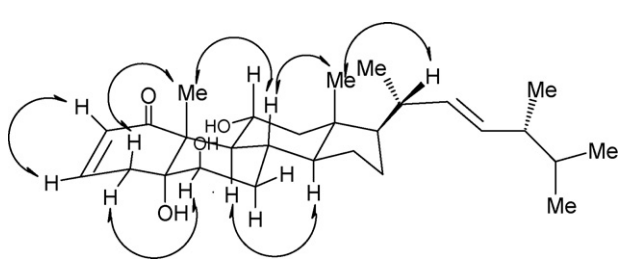


Fig. 2 – Selective NOESY correlations of 1.

Tables 1 and 2. Assignment of the configuration at C-20 and C-24 of **4** was made by comparison of ^{13}C NMR data with those of yonarasterol B and epimeric steroidal side chains [14,15].

Compound **5** was assigned a molecular formula of $C_{28}H_{44}O_4$ as indicated by HRESIMS and ^{13}C NMR spectroscopic data. ^{13}C NMR and DEPT spectra of **5** showed the presence of six methyls, six sp^3 methylenes, ten sp^3 methines, two sp^2 methines, three sp^3 quaternary carbons and one sp^2 quaternary carbon. The IR absorption at 1703 cm^{-1} and ^{13}C NMR signal (Table 2) at δ 215.3 exhibited the presence of a ketone. IR absorption at 3439 cm^{-1} and NMR signals at δ_H 4.19 (1H, m) and 3.80 (1H, dt, J = 4.5, 10.5 Hz) (Table 3) as well as at δ_C 67.6 (CH) and 64.2 (CH) indicated the presence of two secondary hydroxyl groups. The presence of a trisubstituted epoxy was shown by NMR signals at δ_H 3.15 (1H, br s) as well as at δ_C 60.9 (CH) and 61.3 (qC). The spectroscopic data of **5** were analogous to those of stoliferone b [3], except for the data due to C-2 and C-3. The ^{1}H–^{1}H COSY spectrum showed partial structures **d**, **e**, and **f** (Fig. 3). In the HMBC spectrum, partial structure **d** could be connected to **e** through three quaternary carbons (C-1, C-5 and C-10) and H_3-19 (Fig. 3). Partial structure **e** could be connected to **c** through the remaining quaternary carbons (C-13) and H_3-18. Based on this evidence, the planar structure of **5** was concluded as in Fig. 3. Configuration at C-20 and C-24 was determined by comparison of ^{13}C NMR data with those of **1** and epimeric steroidal side chains [14,15]. The coupling constant between H-22 and H-23 (J = 15.6 Hz) defined the double bond to have *E* configuration. The NOESY correlations (Fig. 4)

Table 3 – ^{1}H NMR spectroscopic data[a] (300 MHz) of 5–7 in $CDCl_3$

	5	6	7
2α	3.05 dd (13.5, 6.9)[b]	3.06 dd (12.9, 6.6)	3.04 dd (13.1, 6.6)
2β	2.61 dd (13.5, 5.7)	2.61 dd (12.9, 5.7)	2.60 dd (13.1, 6.0)
3	4.19 m	4.19 m	4.18 m
4α	1.68 m	1.67 m	1.68 m
4β	2.21 m	2.20 m	2.18 m
6	3.15 br s	3.15 br s	3.15 br s
7	2.20 m, 1.31 m	2.20 m, 1.50 m	2.21 m, 1.39 m
8	1.30 m	1.28 m	1.30 m
9	1.23 m	1.39 m	1.38 m
11	3.80 dt (4.5, 10.5)	3.77 dt (4.5, 10.5)	3.79 dt (4.8, 10.5)
12α	1.20 m	1.18 m	1.19 m
12β	2.26 dd (12.0, 4.5)	2.27 dd (12.0, 4.5)	2.27 dd (12.3, 4.8)
14	1.39 m	1.05 m	1.07 m
15	1.58 m, 1.07 m	1.62 m, 1.08 m	1.63 m, 1.05 m
16	1.76 m, 1.28 m	2.09 m, 1.46 m	1.90 m, 1.30 m
17	1.09 m	1.29 m	1.20 m
18	0.67 s	0.62 s	0.66 s
19	1.45 s	1.41 s	1.41 s
20	2.01 m	0.72 m	1.34 m
21	1.03 d (6.6)	0.93 d (6.9)	0.92 d (6.3)
22	5.14 dd (15.6, 7.5)	0.27 m	1.38 m, 0.95 m
23	5.20 dd (15.6, 8.1)	0.52 m	1.30 m, 0.95 m
24	1.85 m	0.50 m	1.23 m
25	1.49 m	1.68 m	1.56 m
26	0.82 d (6.6)	0.86 d (6.6)	0.78 d (6.6)
27	0.82 d (6.6)	0.86 d (6.6)	0.85 d (6.6)
28	0.91 d (6.6)	0.89 d (6.9)	0.77 d (6.6)
29		0.12 m	
OH-11	2.64 br s	2.65 br s	2.66 br s

[a] Assigned by COSY, HSQC, NOESY, and HMBC experiments.
[b] *J* values (in Hz) in parentheses.

Table 4 – ^{1}H NMR spectroscopic data[a] (300 MHz) of 8–10 in $CDCl_3$

	8	9	10
2	6.16 d (9.3)[b]	6.16 d (9.3)	6.17 d (9.3)
3	6.99 dd (9.3, 6.0)	6.99 dd (9.3, 6.0)	6.99 dd (9.3, 6.0)
4	6.18 d (6.0)	6.19 d (6.0)	6.19 d (6.0)
6	4.56 br s	4.56 br s	4.57 br s
7	2.08 m, 1.31 m	2.03 m, 1.31 m	2.03 m, 1.30 m
8	2.16 m	2.15 m	2.16 m
9	1.45 m	1.43 m	1.42 m
11	4.20 dt (4.5, 10.5)	4.19 dt (4.8, 10.2)	4.19 dt (4.8, 10.5)
12α	1.26 m	1.25 m	1.23 m
12β	2.36 dd (12.3, 4.5)	2.41 dd (12.6, 4.8)	2.37 dd (12.6, 4.8)
14	1.15 m	1.17 m	1.13 m
15	1.58 m, 1.17 m	1.62 m, 1.17 m	1.62 m, 1.17 m
16	1.76 m, 1.21 m	1.74 m, 1.34 m	1.44 m
17	1.24 m	1.23 m	1.32 m
18	0.79 s	0.79 s	0.74 s
19	1.69 s	1.70 s	1.69 s
20	2.08 m	1.45 m	0.86 m
21	1.03 d (6.6)	0.98 d (6.3)	0.95 d (6.3)
22	5.15 dd (15.6, 7.2)	1.55 m, 1.17 m	0.28 m
23	5.21 dd (15.6, 7.8)	2.09 m, 1.92 m	0.52 m
24	1.89 m	1.89 m	0.51 m
25	1.49 m	2.23 m	1.68 m
26	0.83 d (6.6)	1.02 d (6.3)	0.86 d (6.6)
27	0.83 d (6.6)	1.02 d (6.3)	0.88 d (6.6)
28	0.90 d (6.9)	4.71 s, 4.65 s	0.91 d (6.3)
29			0.12 m
OH-11	4.12 br s	4.15 br s	4.15 br s

[a] Assigned by COSY, HSQC, NOESY, and HMBC experiments.
[b] *J* values (in Hz) in parentheses.

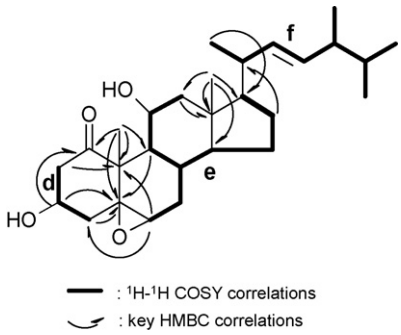


Fig. 3 – ^{1}H–^{1}H COSY and key HMBC correlations of 5.

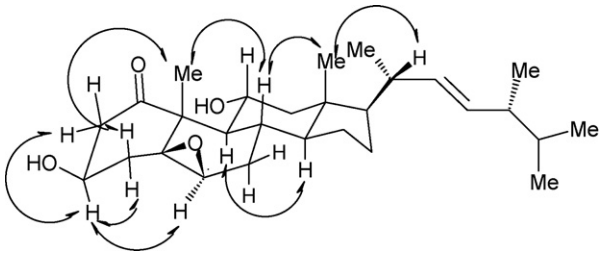


Fig. 4 – Selective NOESY correlations of 5.

observed between H-11 and H-8, H-11 and H_3-18, H-11 and H_3-19, H-3 and H-6, H-9 and H-14, H_3-18 and H-8, H_3-19 and H-8, H_3-18 and H-20, H-4β and H_3-19, H-4α and H-3, H_3-21 and H-12β, H-9 and H-12α proved the relative configuration for each ring junction and chiral center.

The spectroscopic data of **6** and **7** resembled those of **5**, except for NMR signals due to the side chains. NMR spectra of **6** indicated that the *E* disubstituted olefin in the side chain of **5** was replaced by a 1,2-disubstituted cyclopropane [δ_H 0.12 (2H, m), 0.27 (1H, m), and 0.52 (1H, m); δ_C 10.5 (CH_2), 25.2 (CH) and 24.1 (CH)]. An HMBC experiment helped ascertain **6** to have a cyclopropane at C-22 and C-23. The stereochemistry of the four chiral centers (C-20, C-22, C-23, and C-24) in the side chain was confirmed by comparison of ^{13}C NMR data with those of **3**. NMR signals assigned to a $-CH_2CH_2-$ group at C-22 and C-23 [δ_C 33.6 (CH_2) and 30.6 (CH_2)] in **7** replaced the *E* disubstituted double bond signals in **5** as shown in Tables 1 and 2. The configuration at C-20 and C-24 of **7** was determined by comparison of ^{13}C NMR data with those of **4** and epimeric steroidal side chains [14,15].

Compound **8** gave a molecular formula of $C_{28}H_{42}O_3$ as shown by HRESIMS and ^{13}C NMR spectroscopic data. The ^{13}C NMR and DEPT spectrum of **8** exhibited the presence of six methyls, four sp^3 methylenes, nine sp^3 methines, five sp^2 methines, two sp^3 quaternary carbons and two sp^2 quaternary carbons. The IR spectrum of **8** showed absorption due to an α, β, γ, δ-unsaturated ketone (1652 cm^{-1}). The presence of a conjugated enone system in **8** was also indicated by UV

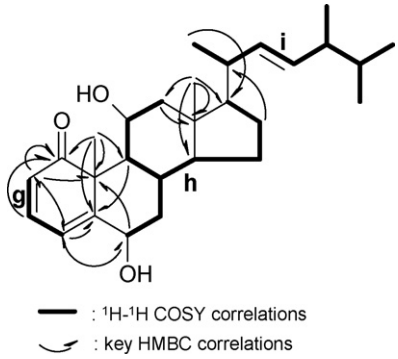


Fig. 5 – ^{1}H–^{1}H COSY and key HMBC correlations of 8.

absorptions at 314 (log ε 4.20) nm as well as ^{1}H NMR [δ 6.16 (1H, d, J = 9.3 Hz), 6.18 (1H, d, J = 6.0 Hz), 6.99 (1H, dd, J = 9.3, 6.0 Hz)] (Table 4) and ^{13}C NMR [δ 119.0 (CH), 126.9 (CH), 140.9 (CH), 157.8 (C)] spectra (Table 2). IR absorption at 3409 cm^{-1} and NMR signals at δ_H 4.56 (1H, brs) and 4.20 (1H, dt, J = 10.5, 4.5 Hz) as well as at δ_C 73.7 (CH) and 66.9 (CH) indicated the presence of two secondary hydroxyl groups. These data were analogous to those of stoloniferone E except for the side chain [11]. The ^{1}H–^{1}H COSY spectrum allowed construction of structures **g**, **h**, and **i** (Fig. 5). In the HMBC spectrum, partial structure **g** could be connected to **h** through three quaternary carbons (C-1, C-5 and C-10) and H_3-19 (Fig. 5). Partial structure **h** could be connected to **i** through the remaining quaternary carbons (C-13) and H_3-18. Based on these results, the planar structure of **8** was concluded as in Fig. 5. Configuration at C-20 and C-24 was determined by comparison of ^{13}C NMR data with those of **1** and epimeric steroidal side chains [14,15]. The coupling constant between H-22 and H-23 (J = 15.6 Hz) defined the double bond to have *E* configuration. The NOESY correlations observed between H-11 and H-8, H-11 and H_3-18, H-11 and H_3-19, H-4 and H-6, H-9 and H-14, H_3-18 and H-8, H_3-19 and H-8, H_3-18 and H-20, H_3-21 and H-12β, H-9 and H-12α indicated the relative configuration for each ring junction and chiral center.

The spectroscopic data of **9** and **10** were similar to those of **8**, except for NMR signals due to the side chains. In the NMR data of **9** (Tables 2 and 4), signals assignable to an exo double bond [δ_H 4.71 (1H, s) and 4.65 (1H, s); δ_C 156.8 (qC) and 106.1 (CH_2)] replaced the *E* disubstituted double bond in **8**. Therefore, the structure of **9** was concluded to be the 22,23-dihydro-24,28-dehydro analog of **8**. NMR spectra of **10** indicated that the *E* disubstituted double bond in the side chain of **8** was replaced by a 1,2-disubstituted cyclopropane [δ_H 0.12 (2H, m), 0.28 (1H, m), and 0.52 (1H, m); δ_C 10.5 (CH_2), 25.2 (CH) and 24.1 (CH)]. HMBC analysis proved **10** to have a cyclopropane at C-22 and C-23. Assignment of the configuration of four chiral centers (C-20, C-22, C-23, and C-24) in the side chain was made by comparison of ^{13}C NMR data with those of **3**.

The cytotoxicity of compounds **1–10** is shown in Table 5. Compounds **1–3**, **5**, **6**, and **8–10** exhibited cytotoxicity against HT-29 cell line. Compounds **1–10** exhibited cytotoxicity against P-388 cell line. All compounds showed stronger cytotoxicity than compounds **1–7** may be due to the presence of α, β, γ, δ-unsaturated carbonyl moiety in the molecule. Compounds **4** and **7** possessing cholestane side chain were less cytotoxic to HT-29 cell line.

Table 5 – Cytotoxicity[a] of 1–10

Compounds	Cell lines IC_{50} (μg/ml)	
	HT-29	P-388
1	1.5	1.2
2	1.8	1.5
3	2.6	2.2
4	4.8	3.6
5	2.9	1.8
6	3.2	2.4
7	6.8	3.9
8	0.2	0.1
9	0.3	0.2
10	0.5	0.3

[a] For significant activity of pure compounds, an IC_{50} of ≤4.0 μg/ml is required.

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