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Cortistatins E, F, G, and H, four novel steroidal alkaloids from marine sponge *Corticium simplex*

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Abstract—Four novel steroidal alkaloids named cortistatins E (**1**), F (**2**), G (**3**), and H (**4**) have been isolated from the marine sponge *Corticium simplex*. The chemical structures of these four cortistatins, which are unique abeo-9(10–19)-stigmastane-type steroidal alkaloids having oxabicyclo[3.2.1]octene and *N*-methyl piperidine or 3-methylpyridine units in the side chain, were elucidated by the detailed 2D-NMR analysis. These four compounds showed only weak anti-proliferative activity against human umbilical vein endothelial cells (HUVECs) at 0.35–1.9 μM concentrations in contrast to cortistatin A (**5**), which was isolated as a highly selective inhibitor of proliferation of HUVECs from the same marine sponge.

1. Introduction

Angiogenesis is the process of generating new capillary blood vessels and disorderly initiated in response to certain pathological conditions, such as solid tumor growth, diabetic retinopathy, psoriasis, and rheumatoid arthritis.[1] Angiogenesis is responsible for the progression of such diseases. Especially, tumor growth and metastasis are highly dependent on angiogenesis. Therefore, specific inhibitors of angiogenesis are expected as promising antitumor agents.[2]

In the course of our study of bioactive substances from marine organisms, we focused on a search for selective inhibitors of proliferation of human umbilical vein endothelial cells (HUVECs), as anti-angiogenic substances.[3,4] On the basis of bioassay-guided separation, we isolated four novel steroidal alkaloids named cortistatins A (**5**), B, C, and D, which were unique abeo-9(10–19)-androstane-type steroidal alkaloids having oxabicyclo[3.2.1]octene and isoquinoline units, from the Indonesian marine sponge *Corticium simplex*.[5] Cortistatin A (**5**) showed highly selective anti-proliferative activity against HUVECs and also inhibited migration and tubular formation of HUVECs induced by VEGF or bFGF at 2–200 nM concentration. Whereas, cortistatins B, C, and D, which were oxidized analogues at C-16 and/or C-17 positions of cortistatin A (**5**), showed weaker activity than that of **5**. In order to elucidate the pharmacophore of cortistatin A (**5**), we further examined the extract of the same marine sponge to isolate four novel derivatives of cortistatin A (**5**), named cortistatins E (**1**), F (**2**), G (**3**), and H (**4**). Details of the structure elucidation of these steroidal alkaloids are presented here (Fig. 1).

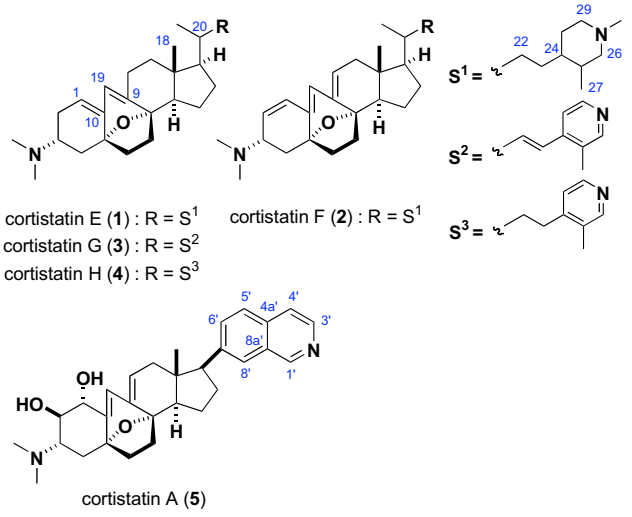

Figure 1. Chemical structures of cortistatins.

2. Results and discussion

The MeOH extract of the titled dried sponge (560 g), which showed selective anti-proliferative activity against

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HUVECs, was subjected to bioassay-guided separation. After solvent partition, the active alkaloids fraction was subjected to LH-20 column chromatography (eluted with MeOH), silica gel column chromatography ($CHCl_3$–MeOH–H_2O containing 0.1% Et_2NH), and HPLC (5-NH_2, CH_3CN–$CHCl_3$–H_2O and ODS, MeOH–H_2O containing 0.1% Et_3N) to isolate four steroidal alkaloids named cortistatins A (**5**)–D (7–28 mg) as major active substances.[5] Further detailed separation of other fractions by HPLC led us to isolate cortistatins E (**1**, 18 mg), F (**2**, 2 mg), G (**3**, 9 mg), and H (**4**, 4 mg).

Cortistatin E (**1**) was obtained as a colorless powder. The ESI-TOF MS of **1** gave a molecular ion [$(M+H)^+$] peak at m/z 481 and the molecular formula was determined as $C_{32}H_{52}N_2O$ by HR ESI-TOF MS in conjunction with NMR analysis. The ^1H and ^{13}C NMR spectra of **1** (Tables 1–3) showed similar signals [δ 5.68 (d-like, J=2.5 Hz), 5.23 (dd-like, J=2.5, 5.0 Hz), 2.31 (6H, s), 0.78 (3H, s), δ_c 145.7, 140.5, 119.0, 117.5, 83.4, 79.2] ascribable to the abeo-9(10–19)-androstane-type steroidal skeleton with the oxabicyclo[3.2.1]-octene unit in **5**, except for lacking the signals of the isoquinoline unit. Detailed HMBC analysis of the signals assignable to the diene moiety [δ 5.23 (dd-like, J=2.5, 5.0 Hz), δ_c 117.5, 140.5, and 5.68 (d-like, J=2.5 Hz), δ_c 119.0, 145.7] and the allylic methylenes [δ 2.14 (br dd, J=10.4, 17.8 Hz), 2.28 (m), δ_c 28.6 and δ 2.46 (br dd, J=15.4, 15.4 Hz), 2.23 (ddd, J=2.5, 2.5, 15.4 Hz), δ_c 28.8] suggested the presence of the 1(10),9(19)-diene system in the AB rings of **1**. To replace the isoquinoline unit, the ^1H and ^{13}C NMR spectra of **1** showed the signals ascribable to the side chain part [two doublet methyls (δ 0.91 (3H, d, J=6.6 Hz), 0.86 (3H, d, J=6.6 Hz), δ_c 18.7, 17.4), *N*-methyl (δ 2.24 (3H, s), δ_c 46.6), five methylenes (δ_c 64.4, 56.6, 32.3, 31.4, 29.6), and three methines (δ_c 42.0, 36.2, 36.0)]. Detailed analysis of the 2D-NMR (COSY, HMQC, and HMBC) spectra of **1** clarified the presence of the terminal *N*-methyl piperidine unit, and consequently the planar structure of cortistatin E (**1**) was determined as shown in Figure 2.

Next, the relative stereostructure of cortistatin E (**1**) was elucidated on the basis of NOESY correlations and $^3J_{HH}$ coupling constants (Fig. 3). Thus, the correlation between H-3 and H-6a and H-6b revealed the β-axial orientation for the H-3 proton and the geometry of the 5,8-oxide bridge in the seven-membered ring B. The correlations between the H-14 and H-17; H-7a and 18-CH_3 revealed the trans-axial orientation for the H-14 and 18-CH_3. The β orientation of the side chain at the C-17 position was deduced from the correlation between 18-CH_3 and 21-CH_3, and the relative stereostructure of the abeo-9(10–19)-androstane-type steroidal skeleton in **1** was determined as shown in Figure 3.

Table 1. ^1H NMR data for cortistatins E (**1**), F (**2**), G (**3**), and H (**4**) (at 600 MHz in $CDCl_3$)

No.	1	2	3	4
1	5.23 (dd-like, 2.5, 5.0)	6.08 (dd, 2.5, 9.9)	5.23 (dd-like, 2.0, 5.2)	5.23 (dd-like, 2.2, 4.8)
2_{eq}	2.28 (m)	5.78 (d-like, 9.9)	2.32 (m)	2.28 (m)
2_{ax}	2.14 (br dd, 10.4, 17.8)		2.16 (dd-like, 11.5, 18.6)	2.14 (dd-like, 10.5, 17.9)
3	2.64 (m)	3.42 (br d, 10.7)	2.78 (br s)	2.68 (m)
4_{eq}	1.97 (m)	1.86 (dd, 4.4, 11.2)	1.98 (m)	1.95 (m)
4_{ax}	1.85 (m)	1.95 (m)	1.86 (m)	1.83 (m)
6a	1.99 (m)	2.02 (dd-like, 11.1, 11.1)	2.01 (m)	1.98 (m)
6b	1.83 (m)	1.63 (ddd, 7.4, 11.1, 11.1)	1.85 (m)	1.82 (m)
7b	2.02 (m)	2.21 (m)	2.02 (m)	1.99 (m)
7a	1.82 (m)	1.71 (m)	1.83 (m)	1.81 (m)
11_{ax}	2.46 (br dd, 15.4, 15.4)	5.37 (dd-like, 2.7, 5.2)	2.48 (br dd, 12.6, 16.7)	2.45 (m)
11_{eq}	2.23 (ddd, 2.5, 2.5, 15.4)		2.27 (dd-like, 3.0, 16.7)	2.23 (m)
12_{eq}	1.99 (m)	2.32 (dd, 5.2, 18.1)	1.97 (m)	1.98 (m)
12_{ax}	1.30 (m)	2.18 (br d, 18.1)	1.36 (m)	1.30 (m)
14	1.95 (m)	2.25 (m)	1.99 (m)	1.94 (m)
15b	1.72 (m)	1.77 (m)	1.70 (ddd-like, 2.0, 9.7, 19.4)	1.73 (m)
15a	1.45 (m)	1.53 (m)	1.46 (m)	1.46 (m)
16	1.93 (m)	1.99 (m)	1.78 (m)	1.92 (m)
	1.33 (m)	1.35 (m)	1.40 (m)	1.32 (m)
17	1.20 (dd-like, 9.6, 9.6)	1.34 (m)	1.39 (m)	1.25 (m)
18	0.78 (3H, s)	0.73 (3H, s)	0.84 (3H, s)	0.78 (3H, s)
19	5.68 (d-like, 2.5)	5.82 (s-like)	5.69 (d-like, 2.0)	5.68 (d-like, 2.2)
20	1.35 (m)	1.34 (m)	2.32 (m)	1.48 (m)
21	0.91 (3H, d, 6.6)	0.90 (3H, d, 5.8)	1.14 (3H, d, 6.6)	1.05 (3H, d, 6.6)
22	1.32 (m)	1.32 (m)	6.16 (dd, 8.8, 15.7)	1.66 (m)
	1.09 (m)	1.09 (m)		1.31 (m)
23	1.47 (m)	1.48 (m)	6.43 (d, 15.7)	2.65 (ddd, 4.6, 13.4, 13.4)
	1.11 (m)	1.12 (m)		2.43 (m)
24	0.80 (m)	0.82 (m)		
25	1.39 (m)	1.43 (m)		
26_{eq}	2.75 (br d, 11.0)	2.78 (br d, 9.9)	8.33 (s)	8.32 (s)
26_{ax}	1.55 (dd, 11.0, 11.0)	1.58 (m)		
27	0.86 (3H, d, 6.6)	0.87 (3H, d, 6.6)	2.29 (3H, s)	2.27 (3H, s)
28_{eq}	1.70 (m)	1.71 (m)	7.24 (d, 4.9)	7.02 (d, 4.9)
28_{ax}	1.24 (m)	1.26 (m)		
29_{eq}	2.86 (br d, 12.6)	2.88 (br s)	8.33 (s-like)	8.33 (s-like)
29_{ax}	1.83 (m)	1.85 (m)		
N–$(CH_3)_2$	2.31 (6H, s)	2.30 (6H, s)	2.37 (6H, s)	2.31 (6H, s)
N–CH_3	2.24 (3H, s)	2.26 (3H, s)		

Table 2. ^{13}C NMR data for cortistatins E (**1**), F (**2**), G (**3**), and H (**4**) (at 150 MHz in $CDCl_3$)

No.	**1**	HMBC (^1H)	**2**	HMBC (^1H)	**3**	HMBC (^1H)	**4**	HMBC (^1H)
1	117.5	2_{eq}, 2_{ax}, 19	127.7	19	117.0	2_{ax}, 19	117.4	2_{eq}, 2_{ax}, 19
2	28.6	1, 4_{eq}, 4_{ax}	132.1	4_{eq}	27.9	4_{eq}, 4_{ax}	28.4	4_{eq}, 4_{ax}
3	59.1	1, 4_{eq}, 4_{ax}, N–$(CH_3)_2$	60.7	1, 4_{eq}, 4_{ax}, N–$(CH_3)_2$	59.1	1, 2_{ax}, 4_{eq}, 4_{ax}, N–$(CH_3)_2$	59.0	1, 2_{ax}, 4_{eq}, 4_{ax}, N–$(CH_3)_2$
4	36.5	2_{eq}	31.2	2	36.5	6b	36.7	2_{eq}, 6b
5	79.2	1, 4_{eq}, 4_{ax}, 7a, 19	78.9	1, 4_{eq}, 4_{ax}, 6b, 7b, 19	79.1	1, 4_{eq}, 4_{ax}, 6b, 19	79.2	1, 4_{eq}, 4_{ax}, 19
6	39.2	4_{eq}, 4_{ax}, 7b	38.4	4_{ax}	39.0	4_{ax}	39.1	4_{eq}, 4_{ax}, 7b
7	33.3	6, 14	30.8	6b, 14	33.2	14	33.2	6a, 14
8	83.4	7b, 11_{eq}, 14, 15a, 19	82.2	6a, 7b, 11, 14, 19	83.4	6a, 19	83.3	7b, 11_{eq}, 15b, 19
9	145.7	7b, 11_{eq}, 11_{aq}, 19	140.9	7b, 12_{eq}, 12_{ax}, 19	145.6	7b, 12_{eq}	145.5	7b, 12_{eq}
10	140.5	1, 2_{eq}, 4_{eq}, 6a, 6b, 19	139.8	2, 4_{eq}, 6b	140.5	2_{ax}, 4_{eq}	140.5	2_{ax}, 2_{eq}, 4_{eq}, 6b
11	28.8	12_{eq}, 12_{ax}, 19	123.0	12_{eq}, 12_{ax}, 19	28.8	19	28.8	12_{eq}, 12_{ax}, 19
12	39.4	17, 18	43.1	14	39.3	14, 18	39.4	17, 18
13	43.6	11_{eq}, 12_{eq}, 12_{ax}, 14, 15b, 17	42.0	18	43.7	12_{ax}, 14, 15b, 18	43.7	12_{ax}, 14, 17, 18
14	53.9	7b, 12_{eq}, 15a, 18	51.9	12_{eq}, 18	53.9	7b, 18	53.9	7b, 12_{eq}, 15b, 18
15	20.5	14, 16	20.4	14	20.6	14, 17	20.5	14
16	28.2	14, 15a, 17	28.3		28.4	15a, 15b, 17	28.1	17
17	56.8	12_{ax}, 14, 18, 21, 22	56.9	18, 21	56.0	16, 18, 20, 21, 22	56.3	14, 16, 18, 21
18	12.0	12_{eq}, 12_{ax}, 14, 17	14.5	12_{ax}, 14	12.3	12_{ax}, 14	12.0	12_{ax}, 14, 17
19	119.0	1, 11_{eq}	121.3	1, 11	119.1		119.0	1
20	36.2	17, 21	35.9	21	40.9	21, 22, 23	36.1	17, 21
21	18.7	17	18.4		20.5	17, 22	18.9	
22	32.3	21	32.4	17, 21	142.5	20, 21	35.7	
23	29.6	20, 25	29.5		123.5	20, 28	29.6	28
24	42.0	26_{eq}, 27	42.0	27	144.5	22, 23, 26, 27, 29	150.5	27
25	36.0	27	35.9	27	130.1	23, 26, 27, 28	131.6	26, 27, 28
26	64.4	27, 29_{ax}, N–CH_3	64.6	27, N–CH_3	151.2	27	150.8	27, 29
27	17.4		17.4	—	16.8		16.2	26
28	31.4		31.2	—	119.5	23	123.7	23, 29
29	56.6	26_{eq}, 26_{ax}, 28_{eq}, N–CH_3	56.2	N–CH_3	147.6	26, 28	147.7	26, 28
N–$(CH_3)_2$	41.4	N–$(CH_3)_2$	40.8	N–$(CH_3)_2$	41.0	N–$(CH_3)_2$	41.2	N–$(CH_3)_2$
N–CH_3	46.6		46.0					

Table 3. ^1H and ^{13}C NMR data for cortistatin A (**5**) (at 600 or 150 MHz in $CDCl_3$)

No.	^1H	^{13}C	HMBC (^1H)	No.	^1H	^{13}C	HMBC (^1H)
1	4.09 (d-like, 9.6)	73.7	2, 19	15b	2.05 (m)	20.5	14
2	3.33 (dd, 9.6, 9.6)	74.1	1, 4_{eq}	15a	1.84 (m)		
3	2.43 (ddd, 3.1, 9.6, 12.7)	62.2	2, 4_{ax}, 4_{eq}, N–$(CH_3)_2$	16b	2.35 (m)	26.4	17
4_{eq}	1.93 (dd, 3.1, 12.7)	29.1	6b	16a	2.21 (m)		
4_{ax}	1.89 (dd, 12.7, 12.7)			17	3.15 (dd, 9.0, 11.0)	56.9	12_{eq}, 16b, 18
5		79.5	4_{eq}, 4_{ax}, 6b, 19	18	0.54 (3H, s)	15.2	12_{ax}, 14, 17
6a	2.19 (m)	39.7		19	6.25 (d-like, 2.2)	119.5	1, 11
6b	1.66 (ddd, 8.5, 10.5, 10.5)			1′	9.22 (br s)	152.3	3′, 8′
7b	2.28 (m)	30.5	6b, 14	3′	8.49 (br d, 5.2)	142.5	1′, 4′
7a	1.78 (ddd, 8.5, 8.5, 12.2)			4′	7.63 (d-like, 5.2)	120.1	3′, 5′
8		81.9	7b, 11, 14	4a′		134.7	1′, 3′, 6′, 8′
9		139.5	7a, 7b, 12_{eq}, 12_{ax}	5′	7.76 (d-like, 8.5)	125.8	4′
10		139.8	1, 6b, 19	6′	7.59 (dd, 1.6, 8.5)	132.0	17, 8′
11	5.44 (dd-like, 2.2, 5.2)	121.5	12_{eq}, 12_{ax}, 19	7′		139.9	17, 5′
12_{ax}	2.38 (d-like, 17.6)	40.0	14, 18	8′	7.78 (d-like, 1.6)	126.3	17, 6′
12_{eq}	1.97 (dd, 5.2, 17.6)			8a′		128.5	4′, 5′
13		44.8	11, 12_{ax}, 14, 17, 18	N–$(CH_3)_2$	2.30 (3H, s)	40.1	N–$(CH_3)_2$
14	2.51 (m)	51.6	12_{eq}, 18				

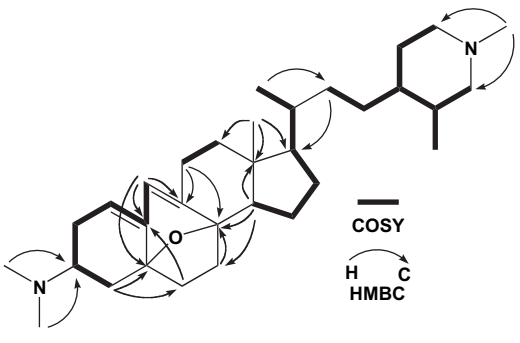

Figure 2. Key HMBC correlations in cortistatin E (**1**).

Furthermore, the correlations between H-26_{ax} (δ 1.55) and H-24, H-29_{ax} (δ 1.83); H-25_{ax} and H-28_{ax} (δ 1.24) and the large coupling constants (dd, J=11.0, 11.0 Hz) of the H-26_{ax} revealed the relative configuration of the C-24 and C-25 positions in the *N*-methyl piperidine ring.

Cortistatin F (**2**) was obtained as a colorless powder. The ESI-TOF MS of **2** gave a molecular ion [$(M+H)^+$] peak at m/z 479 and the molecular formula was determined as $C_{32}H_{50}N_2O$ by HR ESI-TOF MS. The ^1H and ^{13}C NMR spectra of **2** showed closely similar signals, which were assignable to the side chain consisting of the terminal *N*-methyl piperidine ring, to those of **1** (Tables 1 and 2). The

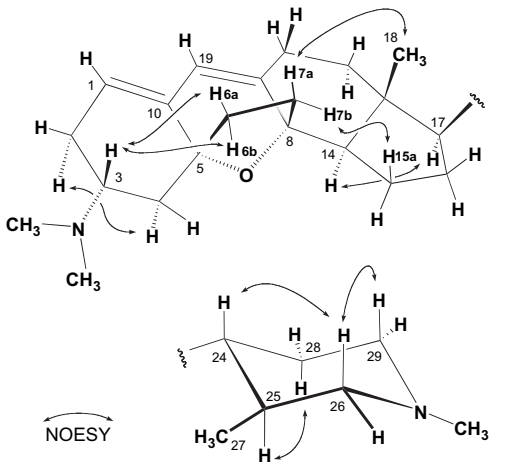

Figure 3. Key NOESY correlations in cortistatin E (**1**).

six olefinic carbons' signals (δ_c 121.3, 123.0, 127.7, 132.1, 139.8, and 140.9) and the UV absorptions at 269, 280, and 292 nm in **2** suggested the presence of the conjugated triene system in **2**. The detailed analysis of the HMBC and NOESY spectra of **2** revealed the relative stereostructure having a 1,9(11),10(19)-triene system (Fig. 4). Consequently, cortistatin F (**2**) was determined to be the 1,9(11),10(19)-triene analogue of cortistatin E (**1**).

Cortistatin G (**3**) was obtained as a colorless powder. The ESI-TOF MS of **3** gave a molecular ion [(M+H)$^+$] peak at *m/z* 459 and the molecular formula was determined as $C_{31}H_{42}N_2O$ by HR ESI-TOF MS. The ^{1}H and ^{13}C NMR signals ascribable to the abeo-9(10–19)-androstane-type steroidal skeleton in **3** (Tables 1 and 2) were closely similar to those of **1**, and cortistatin G (**3**) was deduced to have the same steroidal skeleton. Whereas, the presence of a 3-methylpyridine unit and the 22*E*-ene moiety was disclosed from the HMBC correlations of the signals observed at δ 8.33 (2H, s), 7.24 (d, *J*=4.9 Hz), 6.43 (d, *J*=15.7 Hz), 6.16 (dd, *J*=8.8, 15.7 Hz), δ_c 151.2, 147.6, 144.5, 142.5, 130.1, 123.5, and

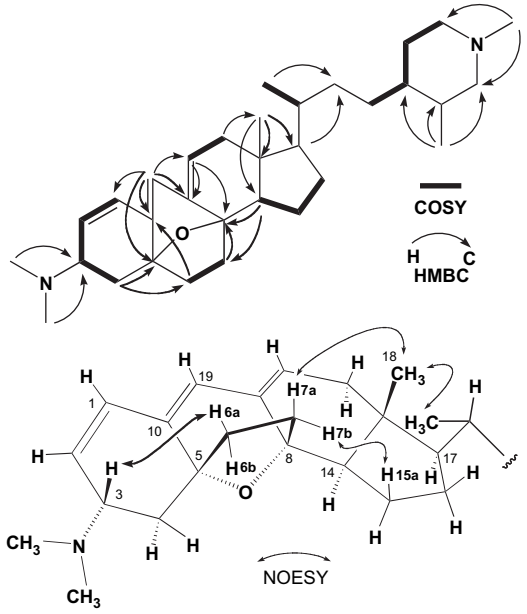

Figure 4. Key HMBC and NOESY correlations in cortistatin F (**2**).

119.5 (Table 2). Thus, the chemical structure of cortistatin G (**3**) was determined as shown in Figure 1.

Cortistatin H (**4**) was obtained as a colorless powder. The ESI-TOF MS of **4** gave a molecular ion [(M+H)$^+$] peak at *m/z* 461 and the molecular formula was determined as $C_{31}H_{44}N_2O$ by HR ESI-TOF MS. The ^{1}H and ^{13}C NMR spectra of **4** (Tables 1 and 2) were closely similar to those of **3**, except for the signals assignable to C-22 and C-23 methylenes [δ 1.66 (m), 1.31 (m) and 2.65 (ddd, *J*=4.6, 13.4, 13.4 Hz), 2.43 (m), δ_c 35.7 and 29.6]. On the basis of 2D-NMR analysis of **4**, the structure of cortistatin H (**4**) was determined to be the 22,23-dihydro analogue of **3** (Fig. 1).

Cortistatins were clarified to be unique abeo-9(10–19)-stigmastane-type steroidal alkaloids having isoquinoline, *N*-methyl piperidine, or a 3-methylpyridine unit in the side chain. Previously, several abeo-9(10–19)-type steroidal alkaloids have been isolated from terrestrial plants of *Buxus* sp. and *Cimicifuga* sp.[6–9] and a marine sponge of *Corticium* sp.[10] Stigmastane-type steroidal alkaloids having the *N*-methyl piperidine unit together with the *N*-methyl pyrrolidine unit in the side chain have been also isolated from marine sponges of *Corticium* sp.[11,12] In 1970, F. Khuong-Huu suggested that the reactive 9β,19-cyclo system encountered in some Buxus alkaloids might be the biogenetic precursor of the conjugated abeo-9(10–19)-diene system.[13–15] Furthermore, many of the isolated stigmastane alkaloids from a marine sponge of *Corticium* sp. have a 7-ene moiety. On the basis of this information, cortistatins are presumed to be biosynthesized from a 3,29-diaminosterol **6**. Thus, the abeo-9(10–19)-diene system is formed from compound **6** through a 9β,19-cyclo system. Then, the 6-ene unit is oxidized to afford a 5,8-oxide ring system. On the other hand, the piperidine-type side chain in **7** is formed from cyclization of the 29-amino group in **6**. Then, the piperidine unit is dehydrated to afford a 3-methylpyridine unit, which is further converted to an isoquinoline unit by cyclization and demethylation of the C-21 or C-26 methyl group (Fig. 5).

Cortistatin A (**5**) showed cytostatic anti-proliferative activity against HUVECs at very wide-range concentrations (100 pM to 1 μM), in which the selective index was more than 3000-fold higher in comparison with those of normal human dermal fibroblast (NHDF) and several tumor cells [KB epidermoid carcinoma cells (KB3-1), human chronic myelogenous leukemia cells (K562), and murine neuroblastoma cells (Neuro2A)]. In contrast, cortistatins E (**1**), F (**2**), G (**3**), and H (**4**) showed only weak anti-proliferative activity (IC_{50} 0.35–1.9 μM) against HUVECs and no selectivity between HUVECs and other cell lines. Then, the diol moiety in the A ring and/or isoquinoline unit in the side chain might be important for the highly selective anti-proliferative property of cortistatin A (**5**).

Due to the scarcity of the isolated amount and the lack of remarkable anti-proliferative activity against HUVECs, the stereochemistry of the C-21 methyl in the side chain of **1**–**4** has not been examined. From the view point of the similarity of the chemical structures between cortistatins E (**1**)–H (**4**) and cortistatin A (**5**), the absolute stereostructure of the steroidal skeleton in **1**–**4** is presumed to be the same as

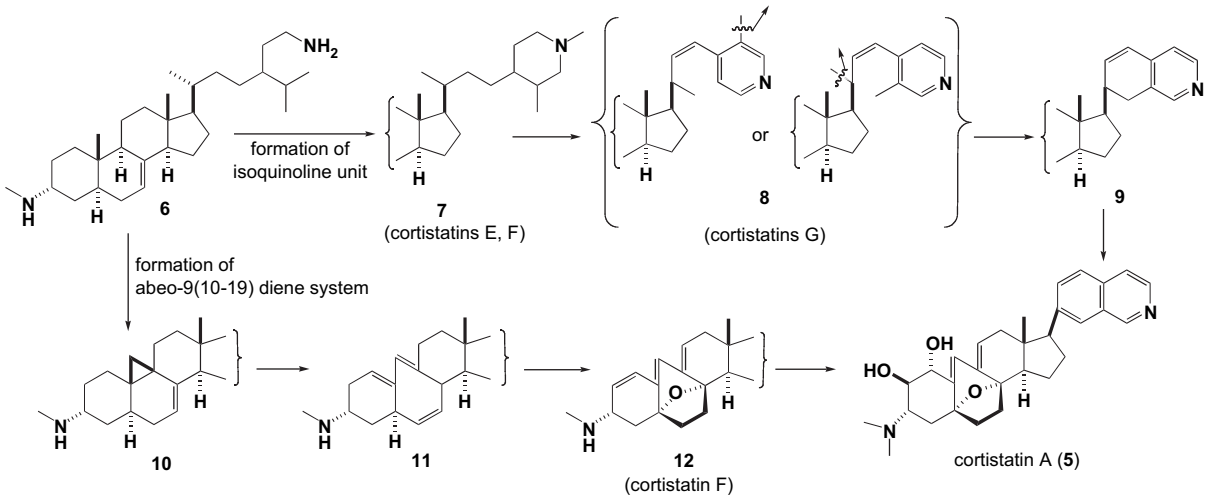

Figure 5. Plausible biogenesis of cortistatins.

that in **5**, which has been determined by the CD exciton chirality method.[5] Further detailed evaluation of the anti-angiogenic effect of cortistatins is under way.

3. Experimental

3.1. General experimental procedures

The following instruments were used to obtain physical data: a JASCO DIP-370 digital polarimeter for specific rotations; a JASCO FT/IR-5300 infrared spectrometer for IR spectra; Shimadzu UV-2450 for UV spectra; Micromass (Waters) Q-TOF MS Ultima LC–MS/MS spectrometer for ESI-TOF MS and HR ESI-TOF MS; a Varian Unity Inova 600 for ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectrum. In both the spectra, chemical shifts are recorded as parts per million relative to $CDCl_3$ (δ 7.26 or δ_c 77.0) as an internal standard.

3.2. Isolation of cortistatins E (1), F (2), G (3), and H (4)

The dried marine sponge *C. simplex* (560 g), which was collected in July, 2001 at west end of Flores Island, Indonesia, was initially steeped in MeOH. The residue obtained by evaporation of the solvent under reduced pressure was partitioned into an AcOEt–water mixture (1:1), and the AcOEt layer was taken and evaporated to give an AcOEt soluble portion (12 g). The water phase was further partitioned with *n*-BuOH to give an *n*-BuOH soluble portion. To obtain alkaloid constituents, the *n*-BuOH and AcOEt soluble portions were partitioned into an AcOEt–5% aq HCl mixture, respectively. Each of the 5% aq HCl-soluble portion was neutralized with aq $NaHCO_3$ and further partitioned with AcOEt. The resulting alkaloid fraction from the *n*-BuOH soluble portion was subjected to LH-20 column chromatography (eluted with MeOH), SiO_2 column chromatography (eluted with $CHCl_3$–MeOH–H_2O containing 0.1% Et_2NH), and HPLC (COSMOSIL 5-NH_2 MS, eluted with CH_3CN–$CHCl_3$–H_2O=86:10:4) to furnish cortistatins E (**1**, 18 mg) and F (**2**, 2 mg). The alkaloid fraction from the AcOEt soluble portion was also subjected to LH-20 column chromatography (eluted with MeOH), SiO_2 column chromatography (eluted with $CHCl_3$–MeOH–H_2O containing 0.1% Et_2NH), and HPLC (COSMOSIL 5-NH_2 MS, eluted with CH_3CN–$CHCl_3$–H_2O=86:10:4; CAPCELL PAK C_{18}MG II, eluted with MeOH–H_2O=90:10 containing 0.1% Et_3N) to furnish cortistatins G (**3**, 9 mg) and H (**4**, 4 mg).

3.2.1. Cortistatin E (1). $[\alpha]_D^{20}$ −45.0 (*c* 0.50, $CHCl_3$). UV λ_{max} (MeOH) nm: 242 (ε 22,500). HR ESI-MS: obsd *m/z* 481.4159, calcd for $C_{32}H_{53}N_2O$ *m/z* 481.4158 $(M+H)^+$. IR (KBr) cm^{-1}: 1381, 1462. ^{1}H and ^{13}C NMR spectra: as shown in Tables 1 and 2.

3.2.2. Cortistatin F (2). $[\alpha]_D^{20}$ +142.7 (*c* 0.20, $CHCl_3$). UV λ_{max} (MeOH) nm: 269 (ε 20,900), 280 (ε 27,800), 292 (ε 21,700). HR ESI-MS: obsd *m/z* 479.3982, calcd for $C_{32}H_{51}N_2O$ *m/z* 479.4001 $(M+H)^+$. IR (KBr) cm^{-1}: 1381, 1460. ^{1}H and ^{13}C NMR spectra: as shown in Tables 1 and 2.

3.2.3. Cortistatin G (3). $[\alpha]_D^{20}$ −52.7 (*c* 0.88, $CHCl_3$). UV λ_{max} (MeOH) nm: 245 (ε 22,600). HR ESI-MS: obsd *m/z* 459.3374, calcd for $C_{31}H_{43}N_2O$ *m/z* 459.3375 $(M+H)^+$. IR (KBr) cm^{-1}: 1377, 1458, 1593. ^{1}H and ^{13}C NMR spectra: as shown in Tables 1 and 2.

3.2.4. Cortistatin H (4). $[\alpha]_D^{20}$ −57.8 (*c* 0.34, $CHCl_3$). UV λ_{max} (MeOH) nm: 242 (ε 20,200). HR ESI-MS: obsd *m/z* 461.3531, calcd for $C_{31}H_{45}N_2O$ *m/z* 461.3532 $(M+H)^+$. IR (KBr) cm^{-1}: 1381, 1454. ^{1}H and ^{13}C NMR spectra: as shown in Tables 1 and 2.

3.3. Assay for anti-proliferative activity against HUVECs and several cell lines

HUVECs and NHDF were obtained from Kurabo Inc. and were grown in HuMedia-EG2 medium and Medium 106s, respectively, with growth supplements (Kurabo Inc.) in humidified atmosphere of 5% CO_2 at 37 °C. KB3-1 were cultured in RPMI 1640 medium, and Neuro2A and K562 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS) and kanamycin (50 μg/ml) in humidified atmosphere of 5% CO_2 at 37 °C. A suspension of each cells were plated into each well of 96-well plates (1×10^3 cells/well/100 μl).

After 24 h, various concentrations of cortistatins were added, plates were incubated for an additional 96 h in a humidified atmosphere of 5% CO_2 at 37 °C, and cell proliferation was detected by WST-8 colorimetric reagent.

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