Pergamon

Tetrahedron 55 (1999) 13965–13972

TETRAHEDRON

Reversal of Multidrug Resistance in Human Carcinoma Cell Line by Agosterols, Marine Spongean Sterols.

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Received 2 September 1999; accepted 4 October 1999

Abstract: We have isolated agosterol A (**1**) from a marine sponge of *Spongia* sp. as a reversing substance to multidrug resistance (MDR) in human carcinoma cell lines, KB-C2 and KB-CV60, overexpressing P-glycoprotein and MRP, respectively. Further investigation led us to isolate analogous sterols, agosterols B (**2**), C (**5**), A_4 (**7**), D_2 (**10**), A_5 (**13**) and C_6 (**14**) from the same sponge and determine their structures. From the structure-activity relationship study, each of the 3,4,6-acetoxyl groups and 11,22-hydroxyl groups was elucidated to be crucial for reversing MDR in tumor cells.

Keywords: steroids and sterols; multidrug resistance; marine metabolites; sponge

Introduction

The development of resistance to multiple anti-cancer agents in tumor cells has been recognized as a major problem which impedes to successful cancer chemotherapy. A major mechanism underlying this multidrug resistance is overexpression of membrane glycoprotein, which behaves as an energy-dependent efflux pump of anticancer agents. One of these membrane glycoproteins is well known as P-glycoprotein (P-gp)[1]. Accordingly, a substance which inhibits the action of these membrane glycoproteins would have high potential to realize more successful cancer chemotherapy. In the course of our study of bioactive substances from marine organisms[2], we focused on a search for reversing substances of MDR in tumor cells and isolated agosterol A (**1**)[3] from a marine sponge of *Spongia* sp. collected in Mie Prefecture, Japan. Compound **1** was a novel polyhydroxylated sterol acetate and completely reversed multidrug resistance caused by overexpression of membrane glycoproteins, P-gp or multidrug-resistance-associated protein (MRP)[4]. Recently, we further isolated agosterols B (**2**), C (**5**), A_4 (**7**), D_2 (**10**), A_5 (**13**) and C_6 (**14**) from the same sponge. In this paper, we describe the structure elucidation and the structure-activity relationships of these related compounds.

Results and Discussion

An acetone extract of the titled frozen sponge was partitioned into a water-AcOEt mixture to provide the AcOEt soluble portion. The AcOEt soluble portion was subjected to repeated SiO_2 column chromatography and HPLC (ODS, MeOH-H_2O) to furnish agosterol A (**1**) and its analogous sterols named agosterols B (**2**), C (**5**), A_4 (**7**),

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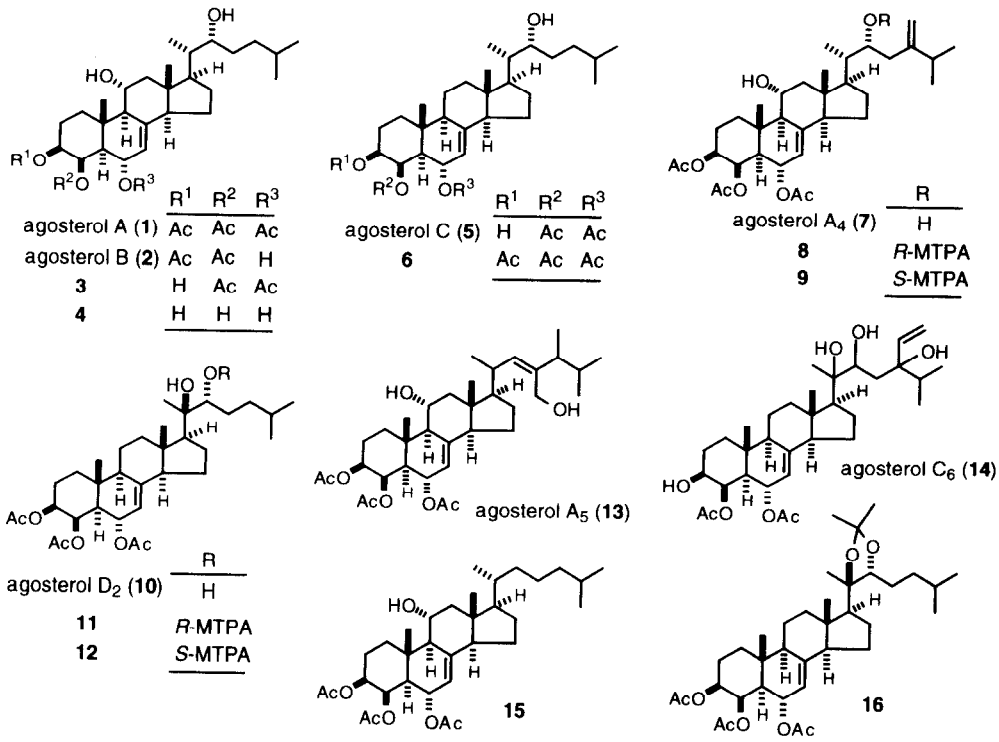
PII: S0040-4020(99)00870-4



D_2 (**10**), A_5 (**13**), and C_6 (**14**) as minor constituents.

These compounds were analyzed by 2D-NMR (COSY, HMQC, HOHAHA, HMBC, and NOESY) and all proton and carbon signals were assigned as shown in Table 1a and 1b. Compound **1** was treated with 0.1 % NaOMe at 0 °C to give 3-deacetyl derivative **3** and 3,4,6-trideacetyl derivative **4**. **5** was treated with Ac_2O in pyridine at 0 °C to furnish 3-acetyl derivative **6**. Compound **1** was treated with CBr_4 and Ph_3P to afford a 22-brominated compound, which was further treated with Bu_3SnH and AIBN to give 22-dehydroxy derivative **15**.

The ^1H- and ^{13}C-NMR spectra (Table 1a) of agosterol B (**2**) were very similar to those[3] of **1**, except for lacking the signals assignable to the 6-acetoxyl moiety in **1**. The H-6 proton and C-6 carbon signals in **2** were observed in higher field. On the basis of 2D-NMR analysis of **2**, agosterol B was confirmed to be 6-deacetyl analogue (**2**) of **1**.

The FAB-MS of agosterol C (**5**) showed a quasimolecular $(M+Na)^+$ ion peak at *m/z* 541 and the molecular formula was determined as $C_{31}H_{50}O_6$ by HR-FAB MS. The ^1H- and ^{13}C-NMR spectra of **5** were also similar to those[3] of **1**. The NMR signals assignable to the 3-acetoxyl moiety in **1** were not observed in **5** and the proton and carbon signals at 3 and 11 positions were observed in higher field compared with those of **1**. From this evidence and the 2D-NMR analysis, the chemical structure of agosterol C (**5**) was determined to be 3-deacetyl-11-dehydroxy analogue of **1**.

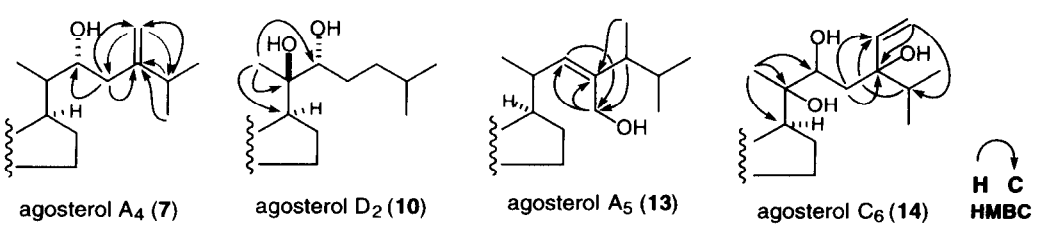
The FAB-MS of agosterol A_4 (**7**) showed a quasimolecular $(M+Na)^+$ ion peak at *m/z* 611 and the molecular formula was determined as $C_{34}H_{52}O_8$ by HR-FAB MS. The ^1H- and ^{13}C-NMR spectra of **7** were closely similar to those of **1** except for the signals assignable to exomethylene [δ 4.83, 4.95 (both s); δc 109.9 (t), 153.2 (s)]. As shown in Fig. 1, the position of the exomethylene group in **7** was defined to be C-24 on the basis of the

Table 1a ^{1}H- and ^{13}C-NMR Data for Agosterols B (**2**), C (**5**) and A_4 (**7**). (600 MHz and 150 MHz in $CDCl_3$)

	2		**5**		**7**	
No.	^{13}C δc	^{1}H δ (mult., *J* (Hz))	^{13}C δc	^{1}H δ (mult., *J* (Hz))	^{13}C δc	^{1}H δ (mult., *J* (Hz))
1	38.8 (t)	2.54 (dt, 14.0, 3.6)	36.8 (t)	1.85 (dt, 13.5, 3.6)	38.6 (t)	2.57 (dt, 14.0, 3.8)
		1.41 (m)		1.23 (m)		1.40 (m)
2	22.4 (t)	1.93 (m), 1.70 (m)	25.0 (t)	1.73 (m), 1.69 (m)	22.4 (t)	1.90 (m), 1.62 (m)
3	72.4 (d)	4.79 (dt, 11.3, 4.4)	71.5 (d)	3.71 (m)	72.0 (d)	4.80 (dt, 12.2, 3.8)
4	69.6 (d)	5.50 (br.s)	69.8 (d)	5.34 (br.s)	66.8 (d)	5.48 (br.s)
5	51.6 (d)	1.47 (dd, 9.9, 2.7)	47.7 (d)	1.68 (m)	47.8 (d)	1.75 (dd, 9.1, 3.0)
6	63.9 (d)	3.90 (m)	68.2 (d)	5.38 (d-like, 10.7)	67.3 (d)	5.33 (d-like, 10.4)
7	123.8 (d)	5.33 (br.s)	118.3 (d)	5.09 (d, 1.7)	120.9 (d)	5.17 (br.s)
8	137.7 (s)	-	142.5 (s)	-	139.1 (s)	-
9	57.7 (d)	1.76 (m)	50.1 (d)	1.76 (m)	57.6 (d)	1.78 (m)
10	36.3 (s)	-	35.6 (s)	-	36.5 (s)	-
11	69.0 (d)	3.92 (m)	21.1 (d)	1.58 (m), 1.50 (m)	69.0 (d)	3.98 (m)
12	50.8 (t)	2.32 (dd, 11.8, 4.9)	38.9 (t)	2.04 (m)	50.8 (t)	2.34 (dd, 11.8, 4.9)
		1.33 (m)		1.26 (m)		1.33 (m)
13	42.5 (s)	-	43.9 (s)	-	43.6 (s)	-
14	54.4 (d)	1.90 (m)	54.3 (d)	1.84 (m)	54.5 (d)	1.92 (m)
15	22.7 (t)	1.60 (m), 1.41 (m)	22.9 (t)	1.55 (m), 1.42 (m)	22.8 (t)	1.68 (m), 1.41 (m)
16	27.1 (t)	1.79 (m), 1.40 (m)	27.1 (t)	1.82 (m), 1.38 (m)	27.0 (t)	1.80 (m), 1.43 (m)
17	52.8 (d)	1.29 (m)	53.0 (d)	1.26 (m)	52.7 (d)	1.35 (m)
18	12.6 (q)	0.56 (s)	11.9 (q)	0.56 (s)	12.8 (q)	0.59 (s)
19	15.4 (q)	1.12 (s)	15.4 (q)	1.09 (s)	15.5 (q)	1.24 (s)
20	43.4 (d)	1.66 (m)	42.5 (d)	1.65 (m)	40.7 (d)	1.85 (m)
21	12.7 (q)	0.94 (d, 6.6)	12.6 (q)	0.92 (d, 6.8)	12.6 (q)	0.98 (d, 6.7)
22	73.7 (d)	3.60 (d-like, 9.4)	73.9 (d)	3.61 (d-like, 11.6)	69.6 (d)	3.74 (d-like, 9.8)
23	27.7 (t)	1.33 (m), 1.22 (m)	27.7 (t)	1.32 (m), 1.22 (m)	35.9 (t)	2.20 (dd, 15.5, 6.8), 1.96 (m)
24	36.0 (t)	1.41 (m), 1.16 (m)	36.0 (t)	1.40 (m), 1.16 (m)	153.2 (s)	-
25	28.1 (d)	1.55 (m)	28.1 (d)	1.53 (m)	33.2 (d)	2.21 (m)
26	22.9 (q)	0.90 (d, 6.8)	22.8 (q)	0.90 (d, 6.6)	21.6 (q)	1.08 (d, 6.7)
27	22.8 (q)	0.89 (d, 6.8)	22.4 (q)	0.89 (d, 6.6)	22.4 (q)	1.06 (d, 6.7)
28	-	-	-	-	109.9 (t)	4.83 (s), 4.95 (s)
3-Ac	21.0 (q)	2.02 (s)	-	-	21.0 (q)	1.95 (s)
	170.3 (s)	-	-	-	170.3 (s)	-
4-Ac	20.9 (q)	2.18 (s)	21.1 (q)	2.10 (s)	21.0 (q)	2.06 (s)
	172.1 (s)	-	172.2 (s)	-	170.3 (s)	-
6-Ac	-	-	21.2 (q)	2.06 (s)	21.2 (q)	2.02 (s)
			171.1 (s)	-	171.2 (s)	-

HMBC correlations in **7**. To confirm the absolute configuration of the 22-hydroxyl group in **7**, the modified Mosher method[5] was applied. A comparative analysis of all the proton signals of 22-*R*-MTPA (**8**) and 22-*S*-MTPA ester (**9**) clarified 22-*R* configuration in **7**. Furthermore, 20*S* configuration in **7** was tentatively presumed on the basis of the similarity of the 21-carbon signal. Consequently, the chemical structure of agosterol A_4 was determined as **7**.

The FAB-MS of agosterol D_2 (**10**) showed a quasimolecular $(M+Na)^+$ ion peak at *m/z* 599 and the molecular



agosterol A_4 (**7**) agosterol D_2 (**10**) agosterol A_5 (**13**) agosterol C_6 (**14**)

Fig. 1 HMBC correlations in the side chain part of agosterols

Table 1b ^{1}H- and ^{13}C-NMR Data for Agosterols D_2 (**10**), A_5 (**13**) and C_6 (**14**). (600 MHz and 150 MHz in $CDCl_3$)

	10		**13**		**14**	
No.	^{13}C δc	^{1}H δ (mult., *J* (Hz))	^{13}C δc	^{1}H δ (mult., *J* (Hz))	^{13}C δc	^{1}H δ (mult., *J* (Hz))
1	38.9 (t)	1.87 (m)	38.7 (t)	2.58 (dt, 14.3, 3.3)	37.0 (t)	1.86 (m)
		1.28 (m)		1.41 (m)		1.22 (m)
2	22.4 (t)	1.84 (m), 1.64 (m)	22.5 (t)	1.90 (m), 1.63 (m)	25.0 (t)	1.70 (2H, m)
3	71.9 (d)	4.80 (dt, 12.4, 3.8)	72.0 (d)	4.81 (dt, 11.6, 4.4)	71.4 (d)	3.72 (m)
4	66.8 (d)	5.45 (br.s)	66.8 (d)	5.48 (br.s)	69.8 (d)	5.35 (br.s)
5	47.6 (d)	1.72 (dd, 10.7, 3.3)	47.9 (d)	1.73 (dd, 10.7, 3.0)	47.8 (d)	1.66 (dd, 10.9, 3.3)
6	67.6 (d)	5.35 (d-like, 10.2)	67.4 (d)	5.32 (d-like, 10.3)	68.1 (d)	5.38 (d-like, 10.7)
7	118.9 (d)	5.10 (br.s)	120.9 (d)	5.16 (d, 1.8)	119.2 (d)	5.10 (d, 1.9)
8	141.9 (s)	-	139.2 (s)	-	141.7 (s)	-
9	49.9 (d)	1.75 (m)	57.6 (d)	1.80 (d-like, 9.6)	50.0 (d)	1.76 (m)
10	36.3 (s)	-	36.5 (s)	-	35.6 (s)	-
11	20.9 (d)	1.56 (m), 1.50 (m)	69.1 (d)	3.99 (td, 10.7, 5.0)	20.8 (d)	1.60 (m), 1.52 (m)
12	39.4 (t)	2.15 (d-like, 12.0)	50.8 (t)	2.32 (dd, 12.0, 5.2)	39.4 (t)	2.16 (m)
		1.30 (m)		1.35 (m)		1.32 (m)
13	44.3 (s)	-	43.2 (s)	-	44.2 (s)	-
14	54.7 (d)	1.82 (m)	54.9 (d)	1.92 (m)	54.8 (d)	1.84 (m)
15	22.5 (t)	1.63 (m), 1.57 (m)	22.7 (t)	1.50 (m), 1.35 (m)	21.5 (t)	1.53 (m), 1.40 (m)
16	22.9 (t)	1.83 (m), 1.45 (m)	28.1 (t)	1.75 (m), 1.18 (m)	22.5 (t)	1.62 (m), 1.33 (m)
17	54.0 (d)	1.60 (m)	56.0 (d)	1.33 (m)	54.1 (d)	1.55 (m)
18	13.6 (q)	0.73 (s)	13.2 (q)	0.59 (s)	13.5 (q)	0.72 (s)
19	15.5 (q)	1.12 (s)	15.5 (q)	1.23 (s)	15.4 (q)	1.10 (s)
20	76.6 (s)	-	35.0 (d)	2.48 (m)	77.2 (s)	-
21	20.7 (q)	1.21 (s)	20.9 (q)	1.02 (d, 6.6)	21.1 (q)	1.26 (s)
22	76.2 (d)	3.39 (d-like, 9.0)	135.4 (d)	5.07 (d, 9.9)	72.0 (d)	3.65 (d-like, 8.6)
23	29.2 (t)	1.40 (m), 1.20 (m)	139.9 (s)	-	36.2 (t)	1.78 (m), 1.70 (m)
24	36.0 (t)	1.40 (m), 1.20 (m)	45.6 (d)	1.95 (m)	87.9 (s)	-
25	28.1 (d)	1.52 (m)	31.5 (d)	1.65 (m)	33.9 (d)	2.30 (m)
26	22.5 (q)	0.91 (d, 6.8)	19.4 (q)	0.86 (d, 6.9)	20.8 (q)	0.88 (d, 7.2)
27	22.3 (q)	0.90 (d, 6.8)	21.8 (q)	0.84 (d, 6.6)	21.0 (q)	0.90 (d, 7.2)
28	-	-	17.0 (q)	0.98 (d, 6.9)	135.6 (d)	5.55 (dd, 18.1, 11.8)
29	-	-	60.4 (t)	4.07 (d, 11.5), 4.16 (d, 11.5)	116.9 (t)	5.26 (dd, 11.8, 1.4)
						5.15 (dd, 18.1, 1.4)
3-Ac	21.0 (q)	1.98 (s)	21.3 (q)	1.98 (s)	-	-
	170.3 (s)	-	170.3 (s)	-	-	-
4-Ac	21.2 (q)	2.08 (s)	20.9 (q)	2.08 (s)	21.1 (q)	2.06 (s)
	170.3 (s)	-	170.3 (s)	-	171.1 (s)	-
6-Ac	21.6 (q)	2.04 (s)	21.9 (q)	2.04 (s)	21.0 (q)	2.11 (s)
	171.3 (s)	-	171.2 (s)	-	172.1 (s)	-

formula was determined as $C_{33}H_{52}O_8$ by HR-FAB MS. The chemical structure of the ring part of **10** was clarified by detailed ^{1}H- and ^{13}C-NMR comparison with agosterol A (**1**) and C (**5**). Thus, it was elucidated that **10** has the 11-dehydroxyl ring structure of **1**. As for the side chain part of **10**, the characteristic NMR signals were observed at δ 1.21 (3H, s) and δc 76.5 (s), which were assignable to the 21-methyl proton and the C-20 carbon by HMBC analysis, respectively. Furthermore, **10** was treated with 2,2-dimethoxypropane and PPTS to furnish the acetonide derivative **16**. The chemical shifts of the 21-methyl proton signals (δ 1.53 for **10** in d_5-pyridine and δ 1.14 for **16** in $CDCl_3$) clarifieded 20*R*,22*R* configuration[6]. The absolute configuration of the side chain part of **10** was further confirmed by application of the modified Mosher method to the 22-hydroxyl group. A comparative analysis of the proton signals of 22-*R*-MTPA ester (**11**) and 22-*S*-MTPA ester (**12**) clarified 22-*R* configuration in **10**. Consequently, the chemical structure of agosterol D_2 was elucidated to be **10**.

The FAB-MS of agosterol A_5 (**13**) showed a quasimolecular $(M+Na)^+$ ion peak at *m/z* 625 and the molecular formula was determined as $C_{35}H_{54}O_8$ by HR-FAB MS. From the ^{1}H- and ^{13}C-NMR analysis, it was clarified

that **13** had the same ring structure as that of **1**. As for the side chain part of **13**, the characteristic NMR signals of tri-substituted olefin [δ 5.07 (d, *J*=9.9 Hz), δc 135.4 (d), 139.9 (s)], secondary methyl [δ 0.98 (d, *J*=6.9 Hz)], and oxymethylene [δ 4.07, 4.16 (both d, *J*=11.5 Hz), δc 60.4 (t)] were observed. Furthermore, the plane structure of the side chain part in **13** was constructed by HMBC correlations as shown in Fig. 1. The *E* geometry of Δ^{22} double bond was assigned by NOESY correlations between H-22 and H-24 and H-28. Consequently, the chemical structure of agosterol A_5 was elucidated as **13.** The stereostructures at C-20 and C-24 in **13** were not determined.

The FAB-MS of agosterol C_6 (**14**) showed a quasimolecular $(M+Na)^+$ ion peak at *m/z* 599 and the molecular formula was determined as $C_{33}H_{52}O_8$ by HR-FAB MS. From the similarity of the ^1H- and ^{13}C-NMR spectra, **14** was found to have the same ring structure as that of agosterol C (**5**). As for the side chain part of **14**, several characteristic NMR signals assignable to a vinyl group [δ 5.55 (dd, *J*=18.1, 11.8 Hz), 5.26 (dd, *J*=11.8, 1.4 Hz), 5.15 (dd, *J*=18.1, 1.4 Hz)], two oxygenated quaternary carbons [δc 77.2 (s), 87.9 (s)], and a carbinol methine [δ 3.65 (d-like), δc 72.0 (d)] were observed. These functional groups were connected by the HMBC correlations as shown in Fig. 1. Consequently, the chemical structure of agosterol C_6 was elucidated as **14.** The stereostructures at C-20, C-22, and C-24 in **14** were not determined.

As shown in Table 2, agosterol A (**1**) completely reversed the resistance against colchicine in KB-C2 cells[7] (P-gp overexpressing strain) and also the resistance against vincristine in KB-CV60 cells[8] (MRP overexpressing strain) at 1 μg/ml concentration, respectively. Compound **1** was not cytotoxic even at 10 μg/ml concentration. We further examined the reversing activity of the analogous compounds (**2**, **5**, **7**, **10**, **13**, and **14**) and their derivatives (**3**, **4**, **6**, and **15**). Among them, **7** and **13** having more complex side chain showed moderate reversing activity. And, **3** lacking a 3-acetyl group and **2** lacking a 6-acetyl group showed much weaker reversing activity compared with that of **1**. Furthermore, tri-deacetylated derivative **4** showed no activity. Thus, the three acetyl groups in ring AB are presumed to be crucial for reversing activity. Next, **6** lacking a 11α-hydroxyl group and **15** lacking a 22-hydroxyl group also showed much weaker activity. So, the 11- and 22-hydroxyl groups are also crucial for reversing activity. So far, agosterol A (**1**) showed the strongest activity

Table 2 Reversal of MDR in KB-C2 and KB-CV60 cells by agosterols and its derivatives

No.	Dose (μg/ml)	Growth Inhibition (%) KB 3-1	KB-C2	KB-CV60	No.	Dose (μg/ml)	Growth Inhibition (%) KB 3-1	KB-C2	KB-CV60
1	10	15 ± 3	88 ± 3	78 ± 1	**6**	10	16 ± 6	91 ± 1	76 ± 10
	3	5 ± 4	88 ± 3	80 ± 2		3	0 ± 0	30 ± 8	28 ± 12
	1	3 ± 5	77 ± 5	80 ± 2	**7**	10	5 ± 1	88 ± 3	82 ± 3
2	10	8 ± 3	85 ± 1	82 ± 2		3	1 ± 1	78 ± 4	75 ± 1
	3	8 ± 5	38 ± 10	77 ± 4		1	1 ± 1	33 ± 2	27 ± 5
	1	9 ± 8	22 ± 7	44 ± 16	**10**	10	29 ± 9	86 ± 3	73 ± 3
3	10	2 ± 3	53 ± 9	81 ± 1		3	1 ± 1	46 ± 2	23 ± 10
	3	0 ± 0	24 ± 5	62 ± 8	**13**	10	23 ± 7	90 ± 1	54 ± 7
	1	0 ± 0	2 ± 1	23 ± 8		3	17 ± 8	72 ± 4	24 ± 11
4	10	0 ± 0	1 ± 2	0 ± 0		1	13 ± 8	31 ± 6	20 ± 9
	3	0 ± 0	0 ± 0	0 ± 0	**14**	10	19 ± 7	15 ± 7	21 ± 4
5	10	11 ± 6	12 ± 11	8 ± 2		3	17 ± 8	13 ± 3	21 ± 3
	3	9 ± 8	9 ± 1	8 ± 2	**15**	10	15 ± 1	91 ± 1	52 ± 4
						3	0 ± 0	10 ± 3	0 ± 0

The value to KB 3-1 shows the cytotoxicity of each compound. The value to KB-C2 and KB-CV60 shows the growth inhibition in the presence of each compound and colchicine (0.1 μg/ml, for the assay using KB-C2) or vincristine (0.1 μg/ml, for the assay using KB-CV60) as an anti-tumor agent. Each value presents mean±S.D. Colchicine and vincristine were not cytotoxic against KB-C2 and KB-CV60 at 0.1 μg/ml concentration, respectively.

and many functional moieties (3,4,6-acetoxyl and 11,22-hydroxyl groups) are crucial for expressing reversing activity. The mechanistic study of the reversal of MDR of agosterol A (**1**) is under investigation.

Experimental

Isolation The frozen sponge of *Spongia* sp. (20 kg), which was collected in July, 1988 at Ago Bay, Mie Prefecture, was initially steeped in acetone. 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 the AcOEt soluble portion (172 g). The AcOEt soluble portion (27 g) was separated by SiO_2 column ($CHCl_3$-MeOH and *n*-hexane-AcOEt) chromatography to give two fractions including agosterols [fractions I (81 mg) and II (2.1 g)]. Fraction I (81 mg) was subjected to HPLC (Mightysil RP-18 GP, MeOH:H_2O=6:1) to afford agosterol A_4 (**7**, 13 mg, 0.05 % yield from the AcOEt soluble portion). Fraction II (2.1 g) was separated by ODS open column (MeOH:H_2O=4:1) and further purified by HPLC (Mightysil RP-18 GP, MeOH:H_2O=5:1) to obtain agosterols A (**1**, 350 mg, 1.3 %), B (**2**, 9 mg, 0.03 %), C (**5**, 17 mg, 0.06 %), D_2 (**10**, 2 mg, 0.01 %), A_5 (**13**, 4 mg, 0.02 %), and C_6 (**14**, 9 mg, 0.03 %). **Agosterol B (2)**: $[\alpha]_D$ -5.5 (c=0.1, $CHCl_3$). HR-FAB MS: Obsd; *m/z* 557.3427. Calcd for $C_{31}H_{50}O_7Na$; *m/z* 557.3455 $(M+Na)^+$. IR (KBr); 3456, 1724 cm^{-1}. ^1H- and ^{13}C-NMR spectra; as shown in Table 1a. **Agosterol C (5)**: $[\alpha]_D$ +49.5 (c=0.7, $CHCl_3$). HR-FAB MS: Obsd; *m/z* 541.3517. Calcd for $C_{31}H_{50}O_6Na$; *m/z* 541.3505 $(M+Na)^+$. IR (KBr); 3460, 1739 cm^{-1}. ^1H- and ^{13}C-NMR spectra; as shown in Table 1a. **Agosterol A_4 (7)**: $[\alpha]_D$ +17.5 (c=0.9, $CHCl_3$). HR-FAB MS: Obsd; *m/z* 611.3564. Calcd for $C_{34}H_{52}O_8Na$; *m/z* 611.3560 $(M+Na)^+$. IR (KBr); 3489, 1745 cm^{-1}. ^1H- and ^{13}C-NMR spectra; as shown in Table 1a. **Agosterol D_2 (10)**: $[\alpha]_D$ +28.0 (c=0.2, $CHCl_3$). HR-FAB MS: Obsd; *m/z* 599.3564. Calcd for $C_{33}H_{52}O_8Na$; *m/z* 599.3559 $(M+Na)^+$. IR (KBr); 3489, 1743 cm^{-1}. ^1H- and ^{13}C-NMR spectra; as shown in Table 1b. **Agosterol A_5 (13)**: $[\alpha]_D$ +8.2 (c=0.3, $CHCl_3$). HR-FAB MS: Obsd; *m/z* 625.3741. Calcd for $C_{35}H_{54}O_8Na$; *m/z* 625.3716 $(M+Na)^+$. IR (KBr); 3466, 1745 cm^{-1}. ^1H- and ^{13}C-NMR spectra; as shown in Table 1b. **Agosterol C_6 (14)**: $[\alpha]_D$ +37.7 (c=0.2, $CHCl_3$). HR-FAB MS: Obsd; *m/z* 599.3564. Calcd for $C_{33}H_{52}O_8Na$; *m/z* 599.3559 $(M+Na)^+$. IR (KBr); 3468, 1734 cm^{-1}. ^1H- and ^{13}C-NMR spectra; as shown in Table 1b.

3-Deacetyl derivative 3 and 3,4,6-trideacetyl derivative 4 of agosterol A (1) Compound **1** (4.6 mg) was treated with 0.1 % NaOMe in MeOH (1.6 ml) and stirred at 0°C for 30 min. The reaction mixture was neutralized with Dowex HCR-W2 and poured into water, and the whole was extracted with AcOEt. Work-up of the AcOEt extract in the usual manner followed by SiO_2 column (n-hexane-AcOEt) afforded **3** (1.0 mg) and **4** (1.9 mg). **3**: HR-FAB MS: Obsd; *m/z* 557.3468. Calcd for $C_{31}H_{50}O_7Na$; *m/z* 557.3454 $(M+Na)^+$. IR (KBr); 3429, 1723 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 1.74 (m, H-2a), 1.68 (m, H-2b), 3.70 (dt-like, *J*=11.6, 4.3 Hz, H-3), 5.38 (br s, H-4), 5.36 (d-like, *J*=11.0 Hz, H-6), 2.06, 2.11 (each 3H, s, Ac-4, and Ac-6). **4**: HR-FAB MS: Obsd; *m/z* 473.3244. Calcd for $C_{27}H_{46}O_5Na$; *m/z* 473.3243 $(M+Na)^+$. IR (KBr); 3404 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 1.84 (m, H-2a), 1.60 (m, H-2b), 3.57 (ddd, *J*=11.6, 4.3, 3.4 Hz, H-3), 4.30 (br s, H-4), 1.19 (m, H-5), 4.45 (d-like, *J*=11.0 Hz, H-6), 5.37 (br s, H-7).

3-Acetyl derivative 6 of agosterol C (5) Compound **5** (2.7 mg) was treated with Ac_2O (6 μl) in pyridine (200 μl) at 0 °C for 5 h. The reaction mixture was neutralized with 0.1 N aq HCl and the whole was extracted with AcOEt. Work-up of the AcOEt extract in the usual manner followed by SiO_2 column (n-hexane-AcOEt) furnished 3-acetyl derivative **6** (1.0 mg). **6**: HR-FAB MS: Obsd; *m/z* 583.3653. Calcd for $C_{33}H_{52}O_7Na$; *m/z* 583.3611 $(M+Na)^+$. IR (KBr); 3456, 1746 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 1.90 (m, H-2a), 1.70 (m, H-2b), 4.80 (dt-like, *J*=11.6, 4.3 Hz, H-3), 5.44 (br s, H-4), 5.33 (d-like, *J*=11.0 Hz, H-6), 3.62 (d-like, *J*=9.5 Hz, H-22), 1.97 (3H, s, Ac-3), 2.06 (3H, s, Ac-4), 2.11 (3H, s, Ac-6).

22-*R*-MTPA Ester 8 and 22-*S*-MTPA ester 9 of agosterol A_4 (7) A solution of **7** (2.5 mg) in dichloroethane (1.0 ml) was treated with (*R*)-(+)-MTPA (5.1 mg), 1-[3-(dimethylamino)propyl]-3-

ethylcarbodiimide (EDCI, 4.1 mg), and DMAP (1.5 mg) at 60 °C for 48 h under an N_2 atmosphere. The reaction mixture was partitioned into an AcOEt-water mixture and the AcOEt extract was purified by SiO_2 column (n-hexane-AcOEt) to furnish 22-*R*-MTPA ester **8** (1.8 mg). A solution of **7** (2.3 mg) in dichloroethane (1.0 ml) was similarly treated with (*S*)-(-)-MTPA (4.6 mg), EDCI (3.8 mg), and DMAP (1.5 mg) to afford 22-*S*-MTPA ester **9** (1.8 mg). **8**: HR-FAB MS: Obsd; *m/z* 827.3983. Calcd for $C_{44}H_{59}O_{10}F_3Na$; *m/z* 827.3960 $(M+Na)^+$. IR (KBr); 1741 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 2.56 (dt, *J*=14.2, 3.8 Hz, H-1a), 1.41 (m, H-1b), 1.90 (m, H-2a), 1.65 (m, H-2b), 4.80 (dt-like, *J*=11.8, 4.4 Hz, H-3), 5.49 (br. s, H-4), 1.74 (d-like, *J*=11.0 Hz, H-5), 5.36 (m, H-6), 5.17 (d-like, *J*=1.7 Hz, H-7), 1.76 (d-like, *J*=11.0 Hz, H-9), 3.99 (ddd, *J*=11.0, 9.9, 5.2 Hz, H-11), 2.56 (dd, *J*=12.0, 5.2 Hz, H-12a), 1.36 (m, H-12b), 1.94 (m, H-14), 1.70 (m, H-15a), 1.49 (m, H-15b), 1.93 (m, H-16a), 1.62 (m, H-16b), 1.35 (m, H-17), 0.59 (3H, s, H-18), 1.28 (3H, s, H-19), 1.89 (m, H-20), 1.02 (3H, d, *J*=6.9 Hz, H-21), 5.35 (m, H-22), 2.17 (m, H-23a), 1.71 (m, H-23b), 2.12 (m, H-25), 0.96 (3H, d, *J*=7.1 Hz, H-26), 0.94 (3H, d, *J*=7.1 Hz, H-27), 4.62 (s, H-28a), 4.69 (s, H-28b), 1.98 (3H, s, Ac-3), 2.09 (3H, s, Ac-4), 2.05 (3H, s, Ac-6). **9**: HR-FAB MS: Obsd; *m/z* 827.4011. Calcd for $C_{44}H_{59}O_{10}F_3Na$; *m/z* 827.3958 $(M+Na)^+$. IR (KBr); 1741 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 2.54 (d-like *J*=14.5, H-1a), 1.40 (m, H-1b), 1.90 (m, H-2a), 1.65 (m, H-2b), 4.80 (dt-like, *J*=11.8, 3.6 Hz, H-3), 5.49 (br. s, H-4), 1.74 (dd, *J*=10.9, 3.0 Hz, H-5), 5.36 (m, H-6), 5.18 (br.s , H-7), 1.76 (d-like, *J*=10.3 Hz, H-9), 3.96 (m, H-11), 2.28 (m H-12a), 1.32 (m, H-12b), 1.91 (m, H-14), 1.68 (m, H-15a), 1.48 (m, H-15b), 1.92 (m, H-16a), 1.61 (m, H-16b), 1.31 (m, H-17), 0.58 (3H, s, H-18), 1.24 (3H, s, H-19), 1.82 (m, H-20), 0.80 (3H, d, *J*=6.7 Hz, H-21), 5.35 (m, H-22), 2.25 (m, H-23a), 1.71 (m, H-23b), 2.21 (m, H-25), 1.02 (3H, d, *J*=6.1 Hz, H-26), 1.02 (3H, d, *J*=6.1 Hz, H-27), 4.78 (s, H-28a), 4.87 (s, H-28b), 1.98 (3H, s, Ac-3), 2.09 (3H, s, Ac-4), 2.05 (3H, s, Ac-6).

22-*R*-MTPA Ester 11 and 22-*S*-MTPA ester 12 of agosterol D_2 (10) A solution of **10** (1.2 mg) in CH_2Cl_2 (1.0 ml) was treated with (*R*)-(+)-MTPA (2.5 mg), EDCI (2.0 mg), and DMAP (1.0 mg) at 25 °C for 24 h under an N_2 atmosphere. The reaction mixture was partitioned into an AcOEt-water mixture and the AcOEt extract was purified by SiO_2 column (n-hexane-AcOEt) to furnish 22-*R*-MTPA ester **11** (1.0 mg). A solution of **10** (1.0 mg) in CH_2Cl_2 (1.0 ml) was similarly treated with (*S*)-(-)-MTPA (2.1 mg), EDCI (2.0 mg), and DMAP (1.0 mg) to afford 22-*S*-MTPA ester **12** (0.9 mg). **11**: HR-FAB MS: Obsd; *m/z* 815.4031. Calcd for $C_{43}H_{59}O_{10}F_3Na$; *m/z* 815.3958 $(M+Na)^+$. IR (KBr); 1743 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 1.90 (m, H-1a), 1.30 (m, H-1b), 1.88 (m, H-2a), 1.67 (m, H-2b), 4.80 (dt-like, *J*=12.5, 3.6 Hz, H-3), 5.44 (br. s, H-4), 1.72 (dd, *J*=10.3, 3.0 Hz, H-5), 5.34 (d-like, *J*=9.7 Hz, H-6), 5.10 (d-like, *J*=1.2 Hz, H-7), 1.77 (m, H-9), 1.58 (m, H-11a), 1.52 (m, H-11b), 2.15 (d-like, *J*=12.2 Hz, H-12a), 1.32 (m, H-12b), 1.85 (m, H-14), 1.66 (m, H-15a), 1.50 (m, H-15b), 1.84 (m, H-16a), 1.45 (m, H-16b), 1.62 (m, H-17), 0.73 (3H, s, H-18), 1.12 (3H, s, H-19), 1.24 (3H, s, H-21), 5.01 (d-like, *J*=9.1 Hz, H-22), 1.47 (m, H-23a), 1.39 (m, H-23b), 1.01 (m, H-24a), 0.88 (m, H-24b), 1.40 (m, H-25), 0.73 (3H, d, *J*=6.7 Hz, H-26), 0.79 (3H, d, *J*=6.1 Hz, H-27), 1.98 (3H, s, Ac-3), 2.07 (3H, s, Ac-4), 2.03 (3H, s, Ac-6). **12**: HR-FAB MS: Obsd; *m/z* 815.3958. Calcd for $C_{43}H_{59}O_{10}F_3Na$; *m/z* 815.3959 $(M+Na)^+$. IR (KBr); 1747 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 1.90 (m, H-1a), 1.30 (m, H-1b), 1.88 (m, H-2a), 1.67 (m, H-2b), 4.80 (dt-like, *J*=12.2, 4.2 Hz, H-3), 5.44 (br. s, H-4), 1.72 (dd, *J*=10.3, 3.0 Hz, H-5), 5.34 (d-like, *J*=11.5 Hz, H-6), 5.10 (br. s, H-7), 1.77 (m, H-9), 1.55 (m, H-11a), 1.50 (m, H-11b), 2.13 (d-like, *J*=9.1 Hz, H-12a), 1.30 (m, H-12b), 1.84 (m, H-14), 1.65 (m, H-15a), 1.50 (m, H-15b), 1.82 (m, H-16a), 1.45 (m, H-16b), 1.59 (m, H-17), 0.71 (3H, s, H-18), 1.12 (3H, s, H-19), 1.20 (3H, s, H-21), 5.04 (d-like, *J*=8.5 Hz, H-22), 1.55 (m, H-23a), 1.47 (m, H-23b), 1.80 (m, H-24a), 1.20 (m, H-24b), 1.52 (m, H-25), 0.87 (3H, d, *J*=6.8 Hz, H-26), 0.85 (3H, d, *J*=6.1 Hz, H-27), 1.98 (3H, s, Ac-3), 2.07 (3H, s, Ac-4), 2.03 (3H, s, Ac-6).

22-Dehydroxy derivative 15 of agosterol A (1) The CH_3CN solution (200 μl) of **1** (3.4 mg) was treated with Ph_3P (4.7 mg), 2,6-lutidine (0.3 mg, as a 350 μl of CH_3CN solution), and CBr_4 (7.8 mg) at 0 °C and stirred at 0 °C for 2 h. The solvent was removed by evaporation and the resulting residue was purified by

SiO_2 column (n-hexane-AcOEt) to obtain 22-brominated product (2.6 mg). 22-Brominated product (2.6 mg) was further treated with 2,2-azobisisobutyronitrile (AIBN, 5.0 mg) and Bu_3SnH (4.8 mg) in toluene (2 ml) and the reaction mixture was refluxed at 80 °C for 6 h. After evaporation, the resulting residue was purified by SiO_2 column (n-hexane-AcOEt) to furnish **15** (0.7 mg). **15:** HR-FAB MS: Obsd; *m/z* 583.3613. Calcd for $C_{33}H_{52}O_7Na$; *m/z* 583.3610 $(M+Na)^+$. IR (KBr); 3462, 1745 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 1.89 (m, H-2a), 1.63 (m, H-2b), 4.82 (dt-like, *J*=12.2, 4.3 Hz, H-3), 5.47 (br s, H-4), 5.32 (d-like, *J*=11.0 Hz, H-6), 1.35 (m, H-20), 1.33 (m, H-22a), 1.02 (m, H-22b), 1.97 (3H, s, Ac-3), 2.03 (3H, s, Ac-4), 2.08 (3H, s, Ac-6).

Acetonide derivative 16 of agosterol D_2 (10) Compound **10** (2.0 mg) was dissolved in 500 μl of 2,2-dimethoxypropane and treated with PPTS (12.5 mg). The reaction mixture was strried at 25 °C for 4 h and directly purified by SiO_2 column to furnish the acetonide derivative **16**. **16**: HR-FAB MS: Obsd; *m/z* 639.3885. Calcd for $C_{36}H_{56}O_8Na$; *m/z* 639.3872 $(M+Na)^+$. IR (KBr); 1743 cm^{-1}. ^1H-NMR ($CDCl_3$, δ); 1.14 (3H, s, H-21), 3.56 (d-like, *J*=9.0 Hz, H-22), 1.30, 1.40 (both 3H, s, H-acetonide).

Bioassay Human epidermoid carcinoma KB cells (KB-3-1) were used as the parental cell line for the present study. KB-3-1 cells were cultured in RPMI 1640 medium with 0.44 mg/ml of glutamine, 50 μg/ml of kanamycin sulfate, supplemented with 10 % newborn calf serum. Multidrug resistant (MDR) KB-C2 cells were selected and maintained from KB-3-1 in the medium containing 2 μg/ml of colchicine. MDR KB-CV60 cells were also selected and maintained in the medium containing 1 μg/ml of cepharanthine and 60 ng/ml of vincristine. Reversing activity and cytotoxicity of agosterols were measured by means of MTT colorimetric assay performed in 96-well plates. Equal numbers of cells (10,000) were inoculated into each well with 100 μl of the culture medium. After 24 h preincubation (37 °C, 5 % CO_2), a 50 μl solution of an anticancer agent (colchicine to KB-C2 or vincristine to KB-CV60) and testing sample were added to each of the wells, which were further incubated for 48 h. The cytotoxic activity of the testing sample was also examined by MTT assay using parental KB 3-1 cells. Thereafter, 25 μl of MTT solution (2 mg/ml in PBS) was added to each well and incubated for further 3 h. After removing the medium by aspiration, the resulting formazan was extracted with 200 μl of dimethylsulfoxide. The percentage of cell growth inhibition was calculated from the absorbance at 540 nm.

Acknowledgement The authors are grateful to the Naito Foundation, the Houansha Foundation, and the Ministry of Education, Science, Sports and Culture of Japan for financial support.

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