Pergamon

Tetrahedron Letters 39 (1998) 6303–6306

TETRAHEDRON LETTERS

Agosterol A, a Novel Polyhydroxylated Sterol Acetate Reversing Multidrug Resistance from a Marine Sponge of *Spongia* sp.

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Received 20 May 1998; revised 22 June 1998; accepted 26 June 1998

Abstract: Agosterol A (**1**) has been isolated from a marine sponge of *Spongia* sp. and the absolute stereostructure elucidated. Agosterol A (**1**) is a novel polyhydroxylated sterol acetate, which completely reverses multidrug resistance in human carcinoma cells caused by overexpression of two kinds of membrane glycoprotein.

Keywords: steroids and sterols; sponges; antitumour compounds; stereochemistry

Multidrug resistance (MDR) in tumor cells has been recognized as a major obstacle to successful cancer chemotherapy. Overexpression of membrane glycoprotein [1,2] (*e.g.*, P-glycoprotein: P-gp) has been observed in MDR tumor cell lines and P-gp is believed to function as an energy-dependent efflux pump. So, a substance which inhibits the action of those membrane glycoproteins would have high possibility for solving the MDR problems in cancer chemotherapy. In the course of our study of bioactive substances from marine organisms, [3,4] we focused on a search for reversing substances of MDR in tumor cells and isolated a novel polyhydroxylated sterol acetate named agosterol A (**1**) from a marine sponge of *Spongia* sp. This paper describes the elucidation of the absolute stereostructure of agosterol A (**1**).

An acetone extract of the titled frozen sponge (20 kg collected in July at Ago Bay, Mie Prefecture) was partitioned into a water-AcOEt mixture to provide the AcOEt soluble portion (172 g). The AcOEt soluble portion showed strong growth inhibition at 10 μg/ml concentration against P-gp overexpressing MDR tumor cells (KB-C2) [5] in the presence of 0.1 μg/ml of colchicine, while it exhibited little cytotoxicity against parental KB-3-1 cells at 10 μg/ml. This fraction was subjected to bioassay-guided separation (growth inhibition assay against KB-C2 in the presence of colchicine). Repeated SiO_2 column chromatography (*n*-hexane-AcOEt) of the AcOEt soluble portion (7.0 g) afforded the active fraction (560 mg) [67 % inhibition at 3 μg/ml (0.1 μg/ml colchicine) against KB-C2], which was further

0040-4039/98/$ - see front matter
PII: S0040-4039(98)01336-7

separated by HPLC (Cosmosil 5SL, Et_2O; mightysil RP-18 GP, MeOH:H_2O=5:1) to provide agosterol A (**1**) (91 mg) (1.3 % yield from the AcOEt soluble portion).

Agosterol A (**1**) was obtained as a colorless amorphous solid: $[\alpha]_D$ + 27.3 (c= 0.1, MeOH). The IR spectrum of **1** showed the strong absorption bands due to ester (1746 cm^{-1}) and hydroxyl (3461 cm^{-1}) groups. The FAB MS of **1** 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 in conjunction with NMR analysis. The ^1H- and ^{13}C-NMR data of **1** indicated the presence of three secondary methyls, two tertiary methyls, an olefinic proton, and five oxymethine protons together with three acetyl groups. Acetylation of **1** (Ac_2O/pyridine, r.t.) furnished the pentaacetate [6]. The COSY spectrum of **1** revealed the presence of three partial structures (fragment A: C-1 to C-7, fragment B: C-9, C-11 and C-12, fragment C: C-14 to C-27) as shown in Fig. 1. The presence of these partial structures has also been substantiated by TOCSY experiment of **1**. The connectivities between these three partial structures, three quaternary carbons, and two tertiary methyls (C-18 and C-19) have been figured out on the basis of the following HMBC correlations: 1) adjacency of fragments A and B through C-8 and C-10: cross peaks between H-1, H-5, H_3-19 and C-10; H-5, H-7 and C-9; H-9 and C-8; 2) adjacency of fragments B and C through C-8 and C-13: cross peaks between H-12 and C-13, C-14, C-17; Ha-15, Ha-16, H_3-18 and C-13. These adjacencies were also supported by allylic couplings between H-7 and H-9, H-14. The connectivities of three acetoxyl moieties were also clarified by the HMBC correlations between each acetylmethyl proton and C-3, C-4, and C-6, respectively. Based on the accumulated evidence, the planar structure of agosterol A has been elucidated as **1** (Fig. 1).

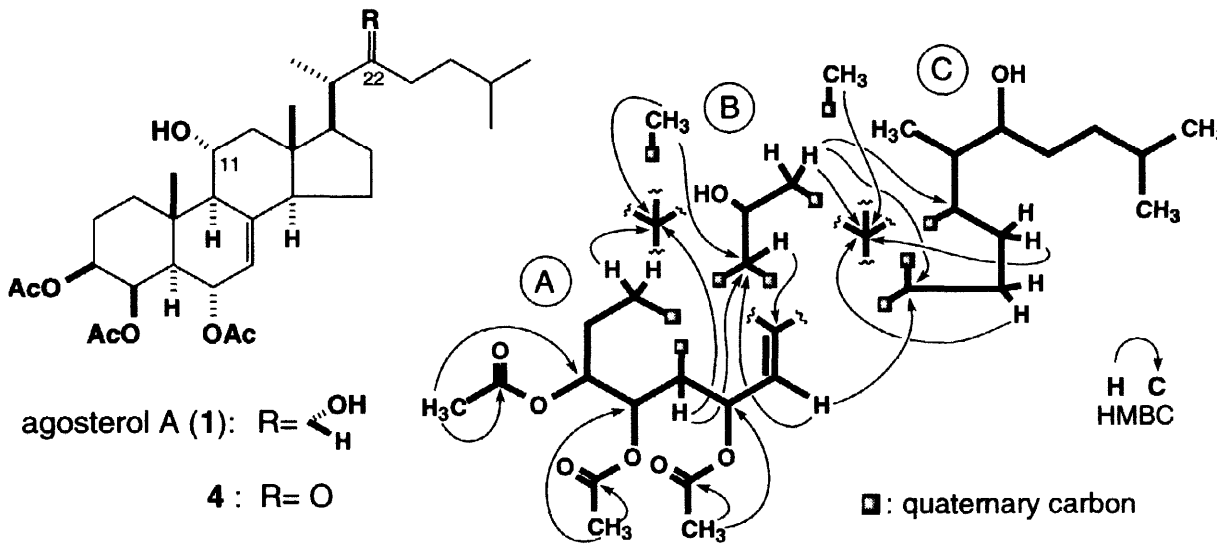
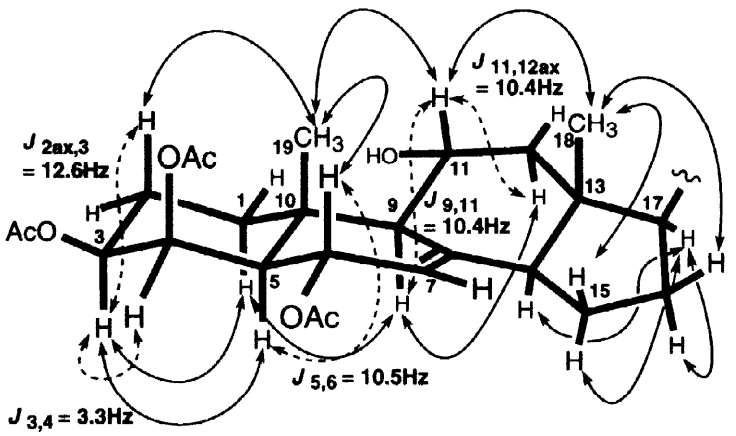


Fig. 1 HMBC correlations among three partial structures

Next, we tried to elucidate the absolute stereostructure of agosterol A (**1**). As shown in Fig. 2, the relative stereostructure of the ring part in **1** was elaborated on the basis of the ROESY correlations and the $^3J_{HH}$ couplings. Furthermore, in order to elucidate the

absolute configurations at C-11 and C-22 in **1**, we applied modified Mosher's method [7]. Thus, **1** was treated with *R*-(+)- or *S*-(-)-2-methoxy-2-phenyl-2-trifluoromethylacetic acid (MTPA), dicyclohexylcarbodiimide, and *N*,*N*-dimethylaminopyridine in CH_2Cl_2 to furnish the 22-*O*-*R*-(+)-MTPA ester **2a**, 11,22-*O*-*R*-(+)-MTPA ester **3a**, 22-*O*-*S*-(-)-MTPA ester **2b**, and 11,22-*O*-*S*-(-)-MTPA ester **3b**, respectively. All proton signals of **2a**, **2b**, **3a**, and **3b** were assigned and the absolute configurations at both C-11 and C-22 were determined as *R* by the analysis of Δδ values (Fig. 3).



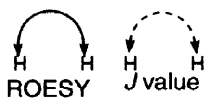


Fig. 2 The ROESY correlations in **1**

	R^1	R^2
2a	H	(*R*)-MTPA
2b	H	(*S*)-MTPA
3a	(*R*)-MTPA	(*R*)-MTPA
3b	(*S*)-MTPA	(*S*)-MTPA

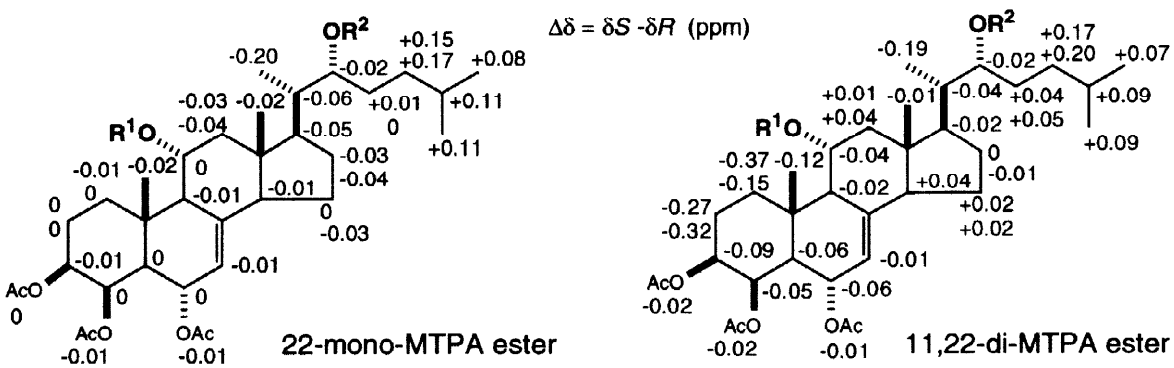


Fig. 3 Application of modified Mosher's method to **1**

Finally, the treatment of **1** with pyridinium dichromate in CH_2Cl_2 furnished the 22-keto derivative **4**. The CD spectrum of **4** showed a negative maximum (Δε –0.86) at 288 nm which was caused by asymmetric 22-ketocarbonyl, the same as an authentic (20*S*)-22-ketocholesterol (Δε –0.73 at 287.5 nm), and the absolute configuration at C-20 was determined as *S*. This result was also supported by ^{1}H- and ^{13}C-NMR comparison of the side chain part between **1** and (20*S*,22*R*)-22-hydroxy-cholesterol [8]. Consequently, the absolute stereostructure of agosterol A has been confirmed to be **1**.

Table 1 ^{1}H- and ^{13}C-NMR Data for Agosterol A (**1**). (500 MHz in $CDCl_3$)

No.	^{13}C δc	^{1}H δ (mult., *J* (Hz))	HMBC (^{13}C)[a]	No.	^{13}C δc	^{1}H δ (mult., *J* (Hz))	HMBC (^{13}C)[a]
1	38.6 (t)	2.57 (d-like, 14.0), 1.42 (m)	2, 10, 19	18	12.8 (q)	0.57 (s)	12, 13, 17
2	22.4 (t)	1.92 (m), 1.64 (m)	1, 3	19	15.5 (q)	1.24 (s)	1, 5, 9, 10
3	71.9 (d)	4.80 (dt, 12.6, 3.3)	2, 4, C=O (3-Ac)	20	42.4 (d)	1.67 (m)	
4	66.8 (d)	5.48 (br.s)	2, 3, 5, C=O (4-Ac)	21	12.6 (q)	0.95 (d, 6.7)	17, 20, 22
5	47.8 (d)	1.74 (d-like, 10.5)	9, 10, 19	22	73.7 (d)	3.60 (d-like, 10.2)	
6	67.3 (d)	5.33 (d, 10.5)	7	23	27.7 (t)	1.37 (m), 1.27 (m)	
7	120.9 (d)	5.17 (br.s)	9, 14	24	36.0 (t)	1.42 (m), 1.17 (m)	22
8	139.1 (s)	-		25	28.1 (d)	1.54 (m)	24, 26, 27
9	57.6 (d)	1.80 (m)	8, 11	26	22.9 (q)	0.90 (d, 6.7)	27
10	36.5 (s)	-		27	22.5 (q)	0.91 (d, 6.7)	24, 25, 26
11	69.0 (d)	3.98 (ddd, 10.4, 10.4, 4.5)	9, 10, 12	3-Ac	21.0 (q)	1.98 (s)	3, C=O (3-Ac)
12	50.8 (t)	2.33 (dd, 12.2, 4.5), 1.37 (m)	11, 13, 18		170.3 (s)	-	
13	43.5 (s)	-		4-Ac	20.9 (q)	2.08 (s)	4, C=O (4-Ac)
14	54.4 (d)	1.92 (m)	13		170.3 (s)	-	
15	22.7 (t)	1.57 (m), 1.44 (m)	13	6-Ac	21.2 (q)	2.04 (s)	6, C=O (6-Ac)
16	27.0 (t)	1.82 (m), 1.40 (m)	13		171.2 (s)	-	
17	52.8 (d)	1.28 (m)	18				

a) C coupled with H.

Agosterol A (**1**) completely reversed the resistance to colchicine in KB-C2 cells at 3 μg/ml and also the resistance to vincristine in KB-CV60 cells [9], which overexpress multidrug resistance-associated protein (MRP), at 1 μg/ml (The details will be reported elsewhere). So far, there are few agents [10,11] which reverse MDR caused by overexpression of MRP. Agosterol A (**1**) may be a pharmaceutical candidate for reversing MDR and also may be useful for detailed explication of the molecular mechanism of MRP.

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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[8] ^{1}H-NMR ($CDCl_3$, δ): 1.68 (m, H-20), 0.92 (d, *J*=6.7 Hz, H-21), 3.60 (d-like, *J*=10.1 Hz, H-22), 1.22, 1.33 (m, H-23), 1.42, 1.18 (m, H-24), 1.55 (m, H-25), 0.90 (6H, d, *J*=6.8 Hz, H-26, 27). ^{13}C-NMR ($CDCl_3$, δc): 42.6 (C-20), 12.4 (C-21), 74.1 (C-22), 27.7 (C-23), 36.0 (C-24), 28.1 (C-25), 23.0 (C-26), 22.5 (C-27).
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