

## (3*R*,4*aR*,5*S*,6*R*)-6-Hydroxy-5-methylramulosin: a New Ramulosin Derivative from a Marine-Derived Sterile Mycelium

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**(3*R*,4*aR*,5*S*,6*R*)-6-Hydroxy-5-methylramulosin (1) was isolated from a culture of a sterile mycelium, which was derived from the green alga, *Codium fragile*, along with (–)-5-methylmellein (2), (–)-5-hydroxymethylmellein (3), and (–)-(3*R*,4*R*)-*cis*-4-hydroxy-5-methylmellein (4). The absolute configuration of 1 was determined by the NMR data along with the lactone sector rule by circular dichroism (CD). Compound 1 exhibited moderate cytotoxic activity against HeLa cells.**

**Key words** 6-hydroxy-5-methylramulosin; sterile mycelium; marine-derived fungus

Marine microorganisms have attracted considerable attention as important sources of structurally diverse secondary metabolites and as potential leads for drug discovery.<sup>1–3</sup> In the course of our search for new biologically active compounds from marine-derived organisms,<sup>4–6</sup> we report here the isolation and structure elucidation of (3*R*,4*aR*,5*S*,6*R*)-6-hydroxy-5-methylramulosin (1), along with three known compounds, (–)-5-methylmellein (2), (–)-5-hydroxymethylmellein (3), and (–)-(3*R*,4*R*)-*cis*-4-hydroxy-5-methylmellein (4), which were isolated from *Valsa ceratosperma*, the pathogenic fungus of apple canker.<sup>7</sup>

The marine-derived fungus was isolated from the green alga, *Codium fragile* (SURINGAR) HARIOT, collected in Toyama Bay in the Japan Sea. The fungus was grown on agar plates composed of 50% seawater with nutrients. The culture plates were extracted with EtOH. The extract was partitioned between EtOAc and H<sub>2</sub>O. The EtOAc-soluble fraction (4.5 g) was subjected to ordinary and reverse-phase silica gel column chromatography and HPLC to furnish 1 (16.0 mg), 2 (12.0 mg), 3 (26.0 mg), and 4 (3.6 mg).

Compound 1 has a molecular formula of C<sub>11</sub>H<sub>16</sub>O<sub>4</sub> as established by HR-FAB-MS, and the molecular formula requires four degrees of unsaturation. The <sup>1</sup>H-NMR spectrum in CDCl<sub>3</sub> (Table 1) revealed two doublet methyl groups [ $\delta$  0.81 (3H, d, *J*=7.0 Hz, 5-Me) and 1.38 (3H, d, *J*=6.8 Hz, 3-Me)], two methines bearing an oxygen functional group [ $\delta$  4.10 (ddd, *J*=10.8, 6.5, 3.2 Hz, H-6) and 4.76 (ddq, *J*=1.5, 5.4, 6.8 Hz, H-3)], two hydrogen signals [ $\delta$  1.63 (br s, 6-OH) and 13.27 (s, 8-OH)], and six methylene and methine signals ( $\delta$  1.5–2.9). The <sup>1</sup>H–<sup>1</sup>H correlation spectroscopy (<sup>1</sup>H–<sup>1</sup>H COSY) experiment of 1 indicated the presence of a partial structure in bold lines (Fig. 1). The heteronuclear multiple-bond correlation (HMBC) experiment of 1 showed the correlations between H<sub>2</sub>-7 and two quaternary carbons C-8 ( $\delta$

173.1) and C-8a ( $\delta$  93.1) and between three hydrogens H-4 $\beta$ , H-4a, and H-5 and C-8a (Fig. 1). HMBCs were observed from H-3 to a carbonyl carbon C-1 ( $\delta$  171.7) and from the downfield hydroxy resonance 8-OH to C-8 and C-8a, which clarified the connectivity of the quaternary carbons C-1, C-8, and C-8a. Thus, the planar structure of 1 was shown (Fig. 1). The relative stereochemistry of 1 was elucidated by its NOE experiment. NOE correlations between H-4a and two signals H-3 and H-6 indicated that they were oriented on the same side (Fig. 1) at axial positions; hence the 3-Me and 6-OH groups were in equatorial orientation. While the correlations between 5-Me and two hydrogens H-4 $\alpha$  ( $\delta$  1.90) and H-7 $\alpha$  ( $\delta$  2.31) revealed that they were the opposite side of H-3, H-4a, and H-6 and that they were in axial orientation. The absolute stereochemistry of 1 was substantiated by applying the lactone sector rule by CD measurement, in which the signs, used in the ketone octant rule, are reversed for lactone sectors.<sup>8</sup> The sector projection, in which 1 is viewed in the plane of the lactone group along the line of the carboxyl group and its attached carbon atom, is shown in Fig. 2. The CD spectrum of 1 showed the positive Cotton effect observed at 260 nm ( $\Delta\epsilon$ =+126) due to the lactone group with the exocyclic  $\alpha,\beta$ -double bond and with the  $\beta$ -hydroxy group bearing hydrogen bonding to the oxygen at C-1. As shown in Fig. 2, the positive contribution was exhibited for 1. Therefore, the structure of 1 was established as (3*R*,4*aR*,5*S*,6*R*)-6-hy-

Table 1. NMR Data (CDCl<sub>3</sub>) of 1

	$\delta_{\text{H}}$	<i>J</i> (Hz)	$\delta_{\text{C}}$	HMBC
1			171.7 C	
3	4.76 ddq	5.4, 1.5, 6.8	74.2 CH	C-1, C-4a
4	$\alpha$ 1.90 dt	5.4, 13.8	30.1 CH <sub>2</sub>	C-3, C-4a, 3-Me
	$\beta$ 1.59 ddd	1.5, 4.6, 13.8		C-4a, C-8a
4a	2.86 br d	13.8	29.6 CH	C-4, C-5, C-8a, 5-Me
5	2.06 m		37.2 CH	C-4, C-6, C-7, C-8a
6	4.10 ddd	10.8, 6.5, 3.2	68.5 CH	5-Me
7	$\alpha$ 2.31 dd	18.6, 10.8	34.2 CH <sub>2</sub>	C-6, C-8, C-8a
	$\beta$ 2.63 dd	18.6, 6.5		C-5, C-6, C-8, C-8a
8			173.1 C	
8a			93.1 C	
3-Me	1.38 d	6.8	20.2 CH <sub>3</sub>	C-3, C-4
5-Me	0.81 d	7.0	5.3 CH <sub>3</sub>	C-5, C-6, C-4a
6-OH	1.63 br s			
8-OH	13.27 s			C-7, C-8, C-8a

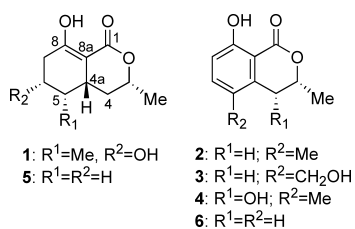


Chart 1

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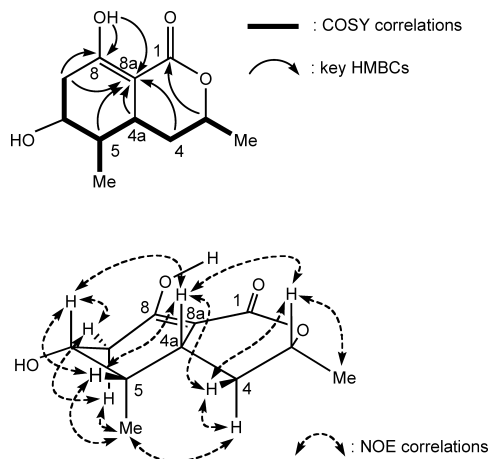


Fig. 1. COSY, Key HMBC, and NOE Correlations Observed for **1**

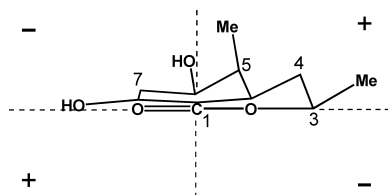


Fig. 2. Lactone Sector Projection of **1**

droxy-5-methylramulosin. Metabolites belonging to a family of ramulosin (**5**)<sup>9–13</sup> isolated so far contain commonly 3*R*,4*aR* configuration, and also mellein (**6**)<sup>7,14</sup> derivatives bear 3*R* configuration. Among them, 6-hydroxyramulosin, which was isolated from several fungi,<sup>9–11</sup> possesses a  $\beta$ -oriented hydroxy group at C-6, while the hydroxy group at C-6 in **1** was found to be  $\alpha$ -oriented.

Since the metabolites in the group of ramulosin (**5**) or mellein (**6**) exhibited a variety of biological activities, a number of the synthetic studies have been carried out to date. Recently, (+)-**6**, (–)-**5**, and their related natural products were synthesized as optically active form.<sup>15,16</sup> Compound **1** showed 65% growth inhibition against HeLa cells at a concentration of 50  $\mu\text{g/ml}$ , while compounds **2–4** were inactive.

#### Experimental

**General Experimental Procedures** Optical rotation was determined with a Horiba SEPA-300 high sensitive polarimeter. UV spectrum was measured on a Shimadzu UV-1600 UV–visible spectrometer. CD spectrum was measured on a JASCO J-820 spectropolarimeter. IR spectrum was recorded on a Shimadzu IR-460 infrared spectrophotometer. NMR spectra were recorded on a JEOL GSX500 in  $\text{CDCl}_3$ . Mass spectra were measured on a JEOL SX-102 mass spectrometer.

**Fungal Strain** The marine fungus was isolated from the green alga, *Codium fragile* (SURINGAR) HARIOT, collected in Toyama Bay in the Japan Sea. The identification of the fungus was evaluated by TechonoSuruga Co., Ltd. (Shizuoka, Japan), and it was just clarified to be a sterile mycelium. A voucher specimen is deposited at Kanazawa University with the code MF593.

**Extraction and Isolation** The fungus was cultivated on agar medium (20 ml  $\times$  500 plates) composed of 2.0% malt extract and 5.0% peptone in

50% natural seawater for 10 d at 25  $^{\circ}\text{C}$ . The culture was extracted with EtOH three times. The extract was evaporated under reduced pressure and partitioned between EtOAc and  $\text{H}_2\text{O}$ . The EtOAc fraction (4.5 g) was subjected to silica gel column chromatography using a step-wise gradient from hexane to EtOAc. The hexane/EtOAc (4 : 1, 2 : 1, and 1 : 1) fractions were combined and further purified by silica gel HPLC (hexane/EtOAc) and ODS HPLC ( $\text{MeOH}/\text{H}_2\text{O}$ ) to afford **1** (16.0 mg), **2** (12.0 mg), **3** (26.0 mg), and **4** (3.6 mg).

**1**: Colorless oil,  $[\alpha]_{\text{D}}^{25} +30^{\circ}$  ( $c=0.24$ , EtOH); UV  $\lambda_{\text{max}}$  (MeOH) nm (log  $\epsilon$ ): 263 (3.8); CD (MeOH)  $\Delta\epsilon_{260} +126$ ,  $\Delta\epsilon_{300} -35$ ; IR (film)  $\text{cm}^{-1}$ : 3370, 1647;  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR, see Table 1; FAB-MS (positive)  $m/z$ : 213  $[\text{M}+\text{H}]^+$ ; HR-FAB-MS (positive)  $m/z$ : 213.1124 (Calcd for  $\text{C}_{11}\text{H}_{17}\text{O}_4$  213.1127).

**Cytotoxicity Test** Cytotoxicity test was carried out with HeLa cells. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50  $\mu\text{g/ml}$ ) under a humidified atmosphere of 5%  $\text{CO}_2$  at 37  $^{\circ}\text{C}$ . The cells were seeded into 96-well microplates ( $3 \times 10^3$  cells/well) and pre-cultured for a day. The medium was replaced with that containing test compounds at various concentrations and the cells were further cultured at 37  $^{\circ}\text{C}$  for 3 d. The medium was then replaced with 50  $\mu\text{l}$  of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.2 mg/ml in medium) and the cells were incubated under the same conditions for 4 h. After the addition of 200  $\mu\text{l}$  of DMSO, the optical density at 570 nm was measured with a microplate reader.

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#### References

- 1) Feling R. H., Buchanan G. O., Mincer T. J., Kauffman C. A., Jensen P. R., Fenical W., *Angew. Chem., Int. Ed.*, **42**, 355–357 (2003).
- 2) Oh D.-C., Williams P. G., Kauffman C. A., Jensen P. R., Fenical W., *Org. Lett.*, **8**, 1021–1024 (2006).
- 3) Kwon H. C., Kauffman C. A., Jensen P. R., Fenical W., *J. Am. Chem. Soc.*, **128**, 1622–1632 (2006).
- 4) Kato H., Yoshida T., Tokue T., Nojiri Y., Hirota H., Ohta T., Williams R. M., Tsukamoto S., *Angew. Chem. Int. Ed.*, **46**, 2254–2256 (2007).
- 5) Tsukamoto S., Yoshida T., Hosono H., Ohta T., Yokosawa H., *Bioorg. Med. Chem. Lett.*, **16**, 69–71 (2006).
- 6) Tsukamoto S., Hirota H., Imachi M., Fujimuro M., Onuki H., Ohta T., Yokosawa H., *Bioorg. Med. Chem. Lett.*, **15**, 191–194 (2005).
- 7) Okuno T., Oikawa S., Goto T., Sawai K., Shirahama H., Matsumoto T., *Agric. Biol. Chem.*, **50**, 997–1001 (1986).
- 8) Jennings J. P., Klyne W., Scopes P. M., *J. Chem. Soc.*, **1965**, 7211–7229 (1965).
- 9) Tanenbaum S. W., Agarwal S. C., *Tetrahedron Lett.*, **1970**, 2377–2380 (1970).
- 10) Findlay J. A., Buthelezi S., Lavoie R., Pena-Rodriguez L., Miller J. D., *J. Nat. Prod.*, **58**, 1759–1766 (1995).
- 11) Stodola F. H., Cabot C., Benjamin C. R., *Biochem. J.*, **93**, 92–97 (1964).
- 12) Stierle D. B., Stierle A. A., Kunz A., *J. Nat. Prod.*, **61**, 1277–1278 (1998).
- 13) Osterhage C., König G. M., Jones P. G., Wright A. D., *Planta Med.*, **68**, 1052–1054 (2002).
- 14) Sasaki M., Kaneko Y., Oshita K., Takamatsu H., Asao Y., Yokotsuka T., *Agric. Biol. Chem.*, **34**, 1296–1300 (1970).
- 15) Islam M. S., Ishigami K., Watanabe H., *Tetrahedron*, **63**, 1074–1079 (2007).
- 16) Uchida K., Ishigami K., Watanabe H., Kitahara T., *Tetrahedron*, **63**, 1281–1287 (2007).