Haploporic acid B and C, New Antibacterial Sesquiterpenoids from the Basidiomycete *Haploporus odorus*

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Two new dimeric drimane sesquiterpenoid ethers, haploporic acid B (**2**) and C (**3**), were isolated from the fruit-bodies of fungus *Haploporus odorus*, together with two known sesquiterpenoid dimmers (**1** and **4**). These structures were determined by spectroscopic and chemical methods. Haploporic acid B (**2**) showed strong antibacterial activity against *Staphylococcus aureus*.

Keywords : Haploporic acid, Drimane sesquiterpenoid, Unsymmetrical dimer, Basidiomycete, *Haploporus odorus*, Antibacterial activity

1. Introduction

In the course of our investigation on chemical components from Basidiomycetes, we have reported the chemotaxonomy of Russulaceae and Boletaceae fungi[1-5], some biologically active compounds from Aphyllophorales fungi.[6-9] *Haploporus odorus* (*Ezoshiroamitake* in Japanese, Polyporaceae) is a white-rotting fungus growing mainly on a willow tree, rarely on a cherry tree, in a cold district. Its fruit-body is a white-pale yellow semicircle having a sweet smell like an anise. We have reported the isolation and structural determination of a novel symmetrical dimeric drimane sesquiterpenoid ether of isocitric acid, haploporic acid A (**1**) form the fruit-bodies of *H. odorus*.[8] Further investigation of this fungus, we have been found that the extract of *H. odorus* had the antibacterial activity against gram-positive bacteria. In this paper, we wish to report the isolation and structural determination of the antibacterial compounds from this fungus.

2. Materials and Methods

The dried fruit-bodies of *H. odorus* (110g) were extracted with dichloromethane, and its extract showed antibacterial activity against *Staphylococcus aureus*. The extract was purified with SiO_2, ODS, and recrystallized to afford compounds **1**-**4**. The yield of compounds **1**-**4** were 714.7, 34.6, 28.9, and 419.4 mg, respectively.

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（令和 2 年 1 月 23 日受付）

3. Results and Discussion

Compound **2** (haploporic acid B) was isolated as a colorless oil and the FAB (positive)-MS showed $[M + Na]^+$ at *m/z* 871, $[M-H+2Na]^+$ at *m/z* 893, indicating that the molecular weight of **2** is 848. The IR spectrum showed the absorptions of carboxylic acid (3500-2600 and 1715cm^{-1}), ester carbonyl (1740cm^{-1}), and olefine (1640cm^{-1}). In the ^{1}H-NMR spectrum for **2**, four olefinic proton signals due to two exomethylene [δ_H 4.93(1H, *d*, *J*=1.8Hz), δ_H 4.87(1H, *d*, *J*=1.2Hz), δ_H 4.79(1H, *d*, *J*=1.8Hz), δ_H 4.77(1H, *d*, *J*=1.2Hz)], three methoxyl (δ_H 3.78, 3.69, 3.68, each 3H, *s*) and four *tert*-methyl (δ_H 0.80, 0.76, 0.75, 0.73, each 3H, *s*) groups.

The proton signals due to isocitrate and drimane sesquiterpene moieties were also observed in the ^{1}H-NMR spectrum for **2**, which were supported by ^{1}H-^{1}H COSY and HMBC. The ^{13}C-NMR spectrum showed 45 carbon signals. The analysis of DEPT and HMQC spectrum suggested it to be as follows ; *tert*-CH_3 x 4, CH_2 x 12, CH x 6, C x 4, OCH_3 x 3, OCH_2 x 4, OCH x 2, >C=CH_2 x 2, COO x 4, COOH x 2, OH x 1. These results suggested that the molecular formula for **2** was $C_{45}H_{68}O_{15}$. The general details of the ^{1}H- and ^{13}C-NMR spectrum were very similar to those of **4** which has been reported from the fungus *Haploporus odorus*[8] and *Cryptoporus volvatus*[10], although small differences were observed. On the other hand, the molecular weight clarified by FAB-MS data and the molecular formula of **2** agreed with those of **4**. Thus, **2** was suggested to be the isomer of **4**. Finally, further interpretation of HMBC spectrum of **2** clarified that the location of dimerization was at C-4' and C-15'', in which the long range couplings were observed between H-15'' (δ_H 3.87, 3.64) and C-4' (δ_C 170.0), showing in Fig. 1. The absolute stereochemistry of **2** was confirmed by chemical transformation. Methylation with diazomethane, reduction with LAH and acetylation of **2** afforded to **6** showing in Fig. 2. The spectral data of **6** were identical to those of the tetraacetate prepd. from **1** in same methods. Therefore, the structure of **2** including the absolute configuration was determined as **2** showing in Fig. 1.

Compound **3** (haploporic acid C) was isolated as a colorless oil. It showed very similar IR absorption to that of **2**. The ^{1}H- and ^{13}C-NMR data for **3** were also similar to those of **2** (see Table 1 and 2), except for one methoxyl proton signal (δ_H 3.72, 3H, *s*, δ_C 52.6). On the other hand, the HMBC analysis revealed that the location of dimerization were at C-4' (δ_C 170.2) and C-15'' (δ_H 3.89, 3.69, δ_C 70.5), C-4''' (δ_C 169.7) and C-15 (δ_H 4.47, 3.24, δ_C 71.1) like as **1**. Thus, **3** was suggested to be demethyl compound of **1**, which was supported by the FAB-MS data (*m/z* 825 $[M + Na]^+$). Methylation of **3** with diazomethane afforded to **3**-trimethyl, whose analytical data were identical to those of **5** prepd. from **1** in same method. Therefore, the structure of **3** including the absolute configuration was determined as **3** showing in Fig. 1.

Compound **1** (colorless powder, mp 203-204˚C, $[\alpha]_D$ +30.0˚) and **4** (colorless oil, $[\alpha]_D$ +47.0˚) were identified with haploporic acid A and cryptoporic acid E, respectively, by directly comparing their spectral data with those of authentic samples.[7, 10]

Table 1. ^{1}H-NMR Data for Compounds **1**-**3** and **5**[#,$]

position		**1**	**2**	**3**	**5**
1	a	1.00, *ddd* (12.4, 12.4, 3.2*)	1.13, *m*	1.35, *m*	1.26, *m*
	b	1.72, br *d* (12.4)	1.66, br *d* (13.2)	1.70, *m*	1.79, br *d* (12.6)
2	a	1.45, *m*	1.55, *m*	1.54, *m*	1.50, *m*
	b	1.55, *m*	1.55, *m*	1.60, *m*	1.60, *m*
3	a	1.15, *ddd* (13.2, 13.2, 3.6)	1.25, *m*	1.25, *m*	1.23, *m*
	b	1.45, *m*	1.45, *m*	1.63, *m*	1.60, *m*
5		1.45, *m*	1.47, *dd* (12.6, 2.4)	1.26, *m*	1.72, *dd* (12.6, 2.4)
6	a	1.32, *m*	1.34, *m*	1.30, *m*	1.31, *ddd* (13.2, 13.2 4.2)
	b	1.67, *m*	1.59, *m*	1.50, *m*	1.50, *m*
7	a	2.05, *m*	2.09, *ddd* (12.6, 12.6, 4.8)	1.86, *m*	1.88, *ddd* (13.2, 13.2 5.4)
	b	2.46, br *d* (13.6)	2.40, *m*	2.31, br *d* (15.1)	2.24, br *d* (13.2)
9		1.94, br *d* (10.8)	2.01, br *d* (7.8)	2.13, *m*	2.32, br *d* (6.0)
11	a	3.89, *dd* (12.8, 1.2)	3.60, *dd* (10.2, 3.0)	3.69, *m*	3.62, *dd* (9.6, 3.6)
	b	3.98, *dd* (12.8, 11.2)	3.99, *dd* (10.2, 9.0)	4.08, *dd* (9.5, 9.5)	3.82, *dd* (9.6, 9.6)
12	a	4.84, br *s*	4.77, *d* (1.2)	4.55, *s*	4.29, *s*
	b	5.01, br *s*	4.87, *d* (1.2)	4.84, *s*	4.75, *s*
13		0.74, *s*	0.76, *s*	0.76, *s*	0.80, *s*
14		0.79, *s*	0.75, *s*	0.79, *s*	0.82, *s*
15	a	3.55, *d* (11.2)	3.09, *d* (11.4)	3.24, *d* (11.2)	3.60, *d* (11.4)
	b	4.45, *d* (11.2)	3.43, *d* (11.4)	4.47, *d* (11.2)	3.88, *d* (11.4)
1'		4.05, *d* (2.0)	4.08, *d* (5.4)	4.47, *d* (7.1)	4.18, *d* (5.4)
2'		3.49, *ddd* (11.2, 3.2, 2.0)	3.38, *ddd* (7.2, 6.0, 5.4)	3.42, *ddd* (7.8, 7.1, 6.1)	3.45, *ddd* (9.6, 5.4, 4.2)
3'	a	2.77, *dd* (16.4, 3.2)	2.71, *dd* (16.2, 7.2)	2.67, *dd* (17.3, 6.1)	2.58, *dd* (16.8, 4.2)
	b	2.92, *dd* (16.4, 11.2)	2.91, *dd* (16.2, 6.0)	2.80, *dd* (17.3, 7.8)	2.80, *dd* (16.8, 9.6)
1"	a		1.13, *m*	1.22 *m*	
	b		1.71, br *d* (12.6)	1.70 *m*	
2"	a		1.55, *m*	1.54 *m*	
	b		1.55, *m*	1.60 *m*	
3"	a		1.25, *m*	1.25 *m*	
	b		1.45, *m*	1.50 *m*	
5"			1.44, *dd* (12.6, 2.4)	1.26 *m*	
6"	a		1.34, *m*	1.30 *m*	
	b		1.59, *m*	1.68 *m*	
7"	a		1.95, *ddd* (12.6, 12.6, 4.2)	1.86 *m*	
	b		2.36, *m*	2.26, br *d* (16.3)	
9"			1.86, br *d* (8.4)	2.10 *m*	
11"	a		3.67, *dd* (10.2, 3.0)	3.69 *m*	
	b		3.93, *t* (10.2)	3.73 *m*	
12"	a		4.79, *d* (1.8)	4.89, *s*	
	b		4.93, *d* (1.8)	4.70, *s*	
13"			0.73, *s*	0.72, *s*	
14"			0.80, *s*	0.76, *s*	
15"	a		3.64, *d* (11.2)	3.68, *d* (10.9)	
	b		3.97, *d* (11.2)	3.89, *d* (10.9)	
1'"			4.29, *d* (4.2)	4.30, br *s*	
2'"			3.51, *ddd* (10.2, 4.2, 3.6)	3.68, *m*	
3'"	a		2.80, *dd* (17.4, 10.2)	2.64, *dd* (16.6, 4.6)	
	b		2.88, *dd* (17.4, 3.6)	2.96, *dd* (16.6, 8.8)	
OMe		3.68, *s*	3.68, *s*	3.72, *s*	3.71, *s*
			3.69, *s*		3.71, *s*
			3.78, *s*		

Recorded at 500MHz in $CDCl_3$.

$ Signals were assigned by COSY, HMQC and HMBC.

* *J*(Hz)

Table 2. ^{13}C-NMR Data for Compounds **1**-**3** and **5**[#,$]

position	**1**	**2**	**3**	**5**
1	39.3	38.3	38.5	39.4
2	18.8	18.6	18.5	18.5
3	36.6	35.2	35.4	35.6
4	38.8	37.4	38.5	37.3
5	47.8	47.8	46.4	46.7
6	23.0	23.4	23.2	23.3
7	36.4	37.1	36.9	36.9
8	145.4	146.4	146.8	147.7
9	51.9	54.9	56.7	55.4
10	37.9	38.4	37.3	39.7
11	64.0	68.0	69.6	70.7
12	109.3	108.2	107.9	107.4
13	15.9	15.8	15.6	14.8
14	17.4	17.7	17.6	17.9
15	69.7	71.8	71.1	71.3
1'	73.0	77.8	77.2	79.5
2'	44.4	44.6	43.5	44.1
3'	32.5	33.1	31.5	31.8
4'	169.3	170.0	170.2	170.6
5'	170.1	171.1	171.9	171.4
6'	179.7	178.0	176.7	172.0
1"		38.8	38.6	
2"		18.4	18.5	
3"		35.8	35.5	
4"		37.9	38.8	
5"		47.2	46.4	
6"		23.2	23.4	
7"		37.0	37.1	
8"		145.7	147.8	
9"		54.3	54.1	
10"		38.4	37.7	
11"		67.4	68.5	
12"		108.8	107.3	
13"		15.7	15.7	
14"		17.8	17.8	
15"		70.9	70.5	
1"'		76.8	79.4	
2"'		43.6	44.6	
3"'		31.4	32.2	
4"'		171.5	169.7	
5"'		170.9	173.0	
6"'		178.4	176.2	
OMe	52.3	52.3	52.6	52.0
	-	52.3	-	52.3
	-	52.4	-	-

[#] Recorded at 125Hz in $CDCl_3$

[$] Signals were assigned by DEPT, HMQC and HMBC.

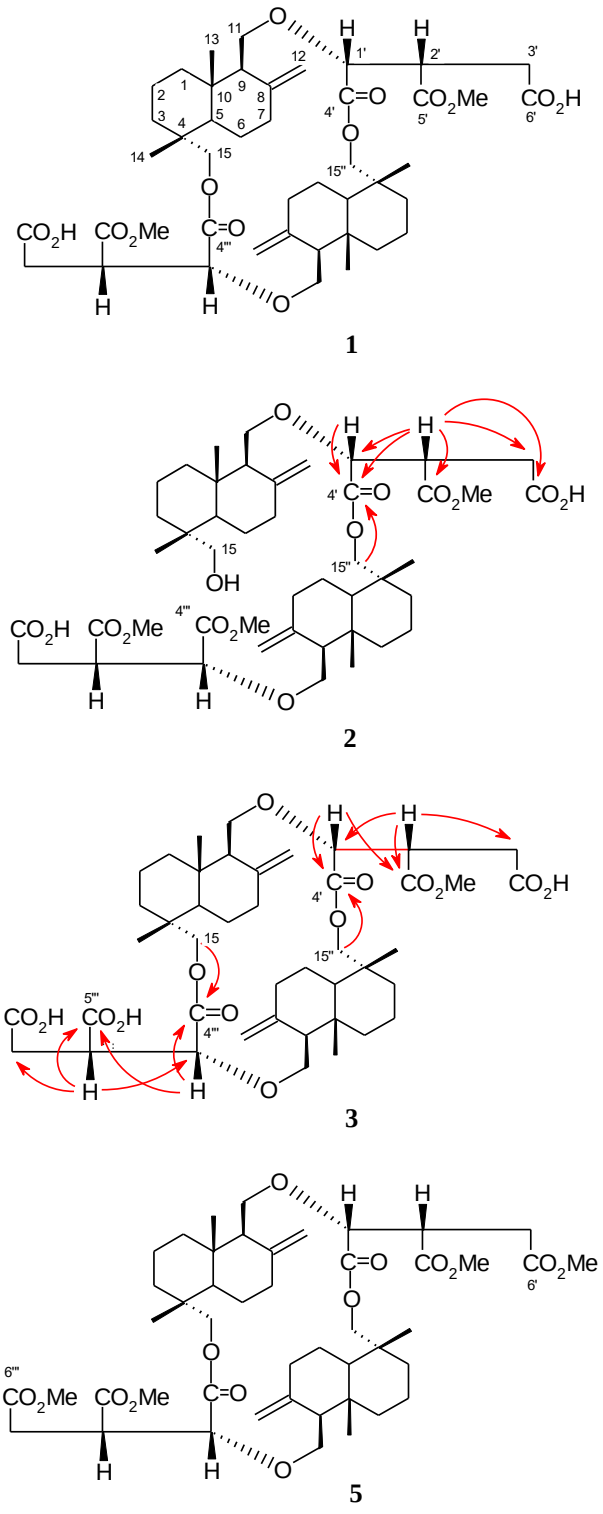

Fig. 1. Structures of Compounds **1**-**3**, and **5**

Arrows show the diagnostically significant C-H correlation found by HMBC.

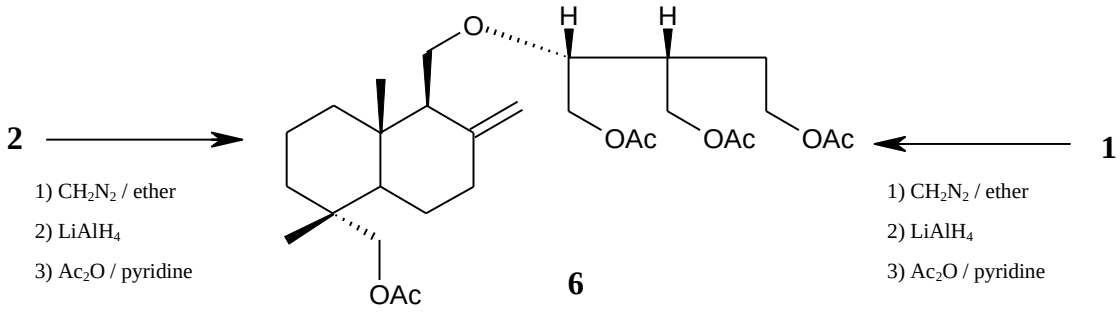

Fig. 2. Chemical transformation to a related compound **6**.

Table 3. Minimum Inhibitory Concentration of compounds **1**-**4** on Bacteria (MIC)

bacteria / compounds	concentration[#] (μg/disk)			
	B. subtilis	*S. aureus*	*P. fluorescens*	*E. coli*
1 (haploporic acid A)	50	6.25	NA[$]	NA
2 (haploporic acid B)	25	1.56	NA	NA
3 (haploporic acid C)	50	6.25	NA	NA
4 (cryptoporic acid E)	100	100	NA	NA

: Samples were tested at 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 μg/disk by the paper disk method. The minimum concentrations necessary to cause a clear inhibition zone over 8mm are listed.

$: NA; no activity at the highest does tested (200 μg/disk).

Antibacterial activity of dimeric drimane sesquiterpenes of isocitric acid (**1**-**4**) were measured by the paper disc method. The results are summarized in Table 3. Compound **2** (haploporic acid B) showed strong antibacterial activity, **1** (haploporic acid A) and **3** (haploporic acid C) showed moderate activity, and **4** (cryptoporic acid E) showed weak activity against two gram-positive bacteria. However, all compounds indicated no activity against two gram-negative bacteria. The activity-enhancing effect of these compounds on antibacterial activity was suggested to the location of dimerization, because the unsymmetrical dimer **2** esterizing at C-4' and C-15'' was higher activity than **4** esterizing at C-5' and C-15''.

4. Experimental

Instruments. NMR spectra (TMS as the internal standard) were obtained with a Bruker AC500 instrument, and IR spectra were recorded on a Jasco FT/IR-8000 spectrometer. MS spectra were measured with JEOL AX-500. The optical rotation was measured with a Jasco DIP-1000.

*Extraction and Isolation of compounds **1-4***. The dried fruiting bodies (110g) of *H. odorus* collected in Nagano prefecture, Japan, were extracted with methylene chloride (100ml) to afford a pale yellow paste (18g), which showed antibacterial activity (the minimum inhibitory concentration = 100μg per disc) against *Staphylococcus aureus*, The extract was chromatographed on silica gel, eluting with $CHCl_3$, $CHCl_3$-MeOH (20:1), (10:1), (5:1), (1:1), and MeOH, in that order. A $CHCl_3$-MeOH (10:1) eluent was rechromatographed on silica gel, and a $CHCl_3$-EtOAc (5:1) eluent was triturated with benzene to afford **1** (714.7mg). A $CHCl_3$-MeOH (1:1) eluent was chromatographed on silica gel, eluting with $CHCl_3$, $CHCl_3$-Me_2CO(5:1), (1:1), and Me_2CO, in that order, to afford eight fractions. Fraction 3 [$CHCl_3$-Me_2CO (5:1) eluent] gave **4** (419.4mg). A MeOH eluent was chromatographed on silica gel, eluting with $CHCl_3$-Me_2CO-AcOH (10:1:0.1), and MeOH, in that order, to afford fifteen fractions. The mixture of fractions 6-9 were rechromatographed on ODS gel, a MeOH-water (10:1) eluent gave **2** (34.6mg). The mixture of fractions 10-13 were also rechromatographed on ODS gel, a MeOH-water (3:1) eluent gave **3** (28.9mg).

*Compound **1** (haploporic acid A)*. Colorless powder, mp 203-204˚C, $[\alpha]_D$ +30.0˚ (*c* 0.1, $CHCl_3$).

*Compound **2** (haploporic acid B)*. Colorless oil, $[\alpha]_D$ +37.1˚ (*c* 0.1, $CHCl_3$) , FAB(positive)-MS: *m/z* 871 $[M + Na]^+$, *m/z* 893 $[M + 2Na]^+$. IR ν_{max} (KBr)cm^{-1}: 3500-2600, 2930, 1740, 1715, 1640, 1440, 1380, 1220, 1130, 1040, 890, 670. ^1H and ^{13}C-NMR spectral data are shown in Tables 1 and 2.

*Compound **3** (haploporic acid C)*. Colorless oil, $[\alpha]_D$ +42.1˚ (*c* 0.1, $CHCl_3$) , FAB(positive)-MS: *m/z* 825 $[M + Na]^+$, *m/z* 847 $[M + 2Na]^+$. IR ν_{max} (KBr)cm^{-1}: 3500-2600, 2930, 1730, 1710, 1640, 1440, 1380, 1220, 1130, 1040, 890. ^1H and ^{13}C-NMR spectral data are shown in Tables 1 and 2.

*Compound **4** (cryptoporic acid E)*. Colorless oil, $[\alpha]_D$ +47.0˚ (*c* 0.1, $CHCl_3$).

*Compound **5** prepd. by methanolysis of **1***. Compound **1** (180.0mg) was methylated with ethereal CH_2N_2 (12ml) in usual way to give a colorless solid, which was recrystallized from *n*-hexane-EtOAc (2:1) to afford compound **5** as colorless needles (133.0mg), mp 100-102˚C, $[\alpha]_D$ +18.0˚ (*c* 0.1, $CHCl_3$), FAB(positive)-MS: *m/z* 867 $[M + Na]^+$, *m/z* 889 $[M + 2Na]^+$. IR ν_{max} (KBr)cm^{-1}:2930, 1740, 1635, 1440, 1380, 1280, 1130, 1000, 890. ^1H and ^{13}C-NMR spectral data are shown in Tables 1 and 2.

*Compound **6** prepd. by reduction and acetylation of **5***. To a suspension of $LiAlH_4$ (100mg) in dry Et_2O (10ml) was added compound **5** (116.3mg) in dry Et_2O (7ml) and stirred for 4 hrs at room temp. The reaction mixture was extracted with EtOAc, and the extract (88.6mg) was chromatographed on silica gel. The $CHCl_3$-EtOAc (5:1) eluent was acetylated with Ac_2O-pyridine (each 1ml) to give a colorless oil (compound **6** : 22.9mg), $[\alpha]_D$ +14.7˚ (*c* 0.1, $CHCl_3$), EIMS: *m/z* 538 $[M]^+$, IR ν_{max} (KBr)cm^{-1}:2930, 1740, 1640, 1440, 1380, 1360, 1230, 1100, 1040, 890, 640. ^1H-NMR δ_H($CDCl_3$): 4.86(1H, *d*, *J*=1.1Hz), 4.64(1H, *d*, *J*=1.1Hz), 4.29(1H, *dd*, *J*=11.9, 3.9Hz), 4.12(5H, *m*), 3.85(1H, *d*, *J*=10.9Hz), 3.75(1H, *q*, *J*=8.2Hz), 3.63(1H, *d*, *J*=10.9Hz), 3.60(1H, *m*), 3.47(1H, *m*), 2.37(1H, br *d*, *J*=13.5Hz), 2.09, 2.08, 2.06, 2.05(each 3H, *s*), 1.97(1H, *m*), 1.82-1.55(6H, *m*), 1.43-1.17(7H, *m*), 0.82(3H, *s*), 0.75(3H, *s*). ^{13}C-NMR δ_C($CDCl_3$): 171.3, 170.9, 170.8(x 2), 146.6, 107.7, 77.6, 72.8, 67.4, 64.3, 63.6, 62.4, 56.1, 48.9, 38.7, 38.6, 37.3, 36.9, 36.8, 35.7, 27.1, 23.7, 21.0(x 4), 18.4, 17.6, 15.8.

*Methanolysis of compound **3***. Compound **3** (3.0mg) was methylated with ethereal CH_2N_2 (5ml) to give a colorless crystal (2.9mg), mp 100-103˚C, $[\alpha]_D$ +18.5˚ (*c* 0.1, $CHCl_3$), whose spectral data were identical to those of **5** prepd. from compound **1**.

*Preparation of tetraacetate from compound **2***. Compound **2** (22.1mg) was methylated with ethereal CH_2N_2 (5ml), following by reduction with $LiAlH_4$ (30mg) in Et_2O (3ml) to afford a residue, which was chromatographed on silica gel. The $CHCl_3$-MeOH (5:1) eluent was acetylated with the same reagents as described above to furnish a tetraacetate (9.3mg) as a colorless oil, $[\alpha]_D$ +14.7˚ (*c* 0.1, $CHCl_3$), of which spectral data were identical to those of **6** prepd. from compound **1**, completely.

Measurement of antibacterial activity. Compounds **1-4** were tested at 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 μg per disc by the paper disc method (ϕ=6mm, thin, TOYO) against *Bacillus subtilis* NBRC3007, *Staphylococcus aureus* NBRC3060, *Escherichia coli* NBRC3301 and *Pseudomonas fluorescens* NBRC3081. The minimum inhibitory concentrations of compounds **1-4** necessary to cause a clear inhibitory zone over 8 mm are shown in Table 3.

5. Acknowledgement

The authors thank Professor Y. Asakawa and Dr. T. Hashimoto, Tokushima Bunri University for their kind suggestions and discussions.

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