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Chlorogentisylquinone, a New Neutral Sphingomyelinase Inhibitor, Produced by a Marine Fungus

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Chlorogentisylquinone, a new inhibitor of neutral sphingomyelinase activity, was purified from the culture broth of a fungal strain FOM-8108 isolated from a marine environment by solvent extraction, silica gel chromatography and Sephadex LH-20 chromatography. Its chemical structure was elucidated by spectroscopic studies including ^{1}H, ^{13}C, DEPT, HMQC and HMBC NMR experiments. Chlorogentisylquinone inhibited neutral sphingomyelinase activity of rat brain membranes with an IC_{50} value of 1.2 μM.

Sphingomyelin pathway is involved in the cell signaling system initiated by hydrolysis of plasma membrane phospholipid sphingomyelin to generate ceramide[1~3]. Ceramide released through activation of neutral sphingomyelinase (nSMase) and/or acid sphingomyelinase (aSMase) plays an important role in cell growth, differentiation and apoptosis as a second messenger[4~9]. Therefore, regulation of sphingomyelin pathway by a specific inhibitor may give a clue to elucidate the mechanism of cell signal transduction, further, leading to a new therapeutic drug for diseases such as cancer, inflammation, autoimmune disorders and so on, in which ceramide generation participates.

Previously, we showed that altenusin of fungal origin inhibits nSMase activity[10]. During our continuous screen for SMase inhibitors of microbial origin, two potent inhibitors were purified from the culture filtrate of a fungal strain FOM-8108 isolated from sea sand. One inhibitor was identified as gentisylquinone (**2**, Fig. 1), originally isolated as a quinhydrone complex[11], but the other was found to be a new compound designated chlorogentisylquinone (**1**, Fig. 1). In this paper, we describe the fermentation, isolation, structure elucidation and biological properties of these compounds.

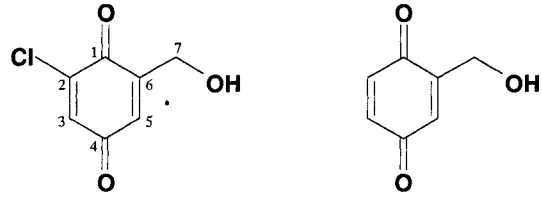
Materials and Methods

General

The fungal strain FOM-8108 was isolated from marine sand collected at Katase Enoshima Beach, Kanagawa, Japan, and was used for production of **1** and **2**. The taxonomic study on the producing fungus will be published elsewhere.

EI-MS spectrometry was conducted on a JEOL JMS-AX505 H spectrometer. UV and IR spectra were measured with a Beckman DU640 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer,

Fig. 1. Structures of chlorogentisylquinone (**1**) and gentisylquinone (**2**).



1 **2**

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respectively. NMR spectra were obtained with JEOL EX-270 and Varian XL-400 spectrometers.

Fermentation Media

For production of **1** and **2**, the seed medium was used containing 2.0% glucose, 0.2% yeast extract (Oriental Yeast Co.), 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% Polypepton (Daigo Nutritive Chemicals), 0.1% KH_2PO_4 and 0.1% agar, in 50% sea water (Tokaikisen). The pH was adjusted to 6.5 prior to sterilization. The production medium was composed of 2.4% Potato Dextrose Broth (Difco) and 1.0% $Mg_3(PO_4)_2 \cdot 8H_2O$ in 50% sea water.

Materials

[Choline methyl-^{3}H]sphingomyelin (500 mCi/6.2 nmol/ml) was purchased from American Radiolabeled Chemicals. aSMase (human placenta), sphingpmyelin (bovine brain), and synthetic quinone and related derivatives were purchased from Sigma. Rat brain membrane was prepared according to the established method[12] and was used as enzyme source of nSMase. Unless otherwise noted, the other reagents utilized were in a special grade.

Quantitative Analysis of **1** and **2** by HPLC

Analytical HPLC was carried out with the HP1100 system (Hewlett-Packard) for determination of the amounts of **1** and **2** in the culture broths. The samples (ethyl acetate extracts) dissolved in methanol were analyzed under the following conditions: column, Symmetry C18/3.5 μm (i.d. 2.1×150 mm, Waters); mobile phase, a 20-minute linear gradient from 0.05% H_3PO_4 to 40% CH_3CN/0. 05% H_3PO_4; flow rate, 0.2 ml/minute; detection, UV at 254 nm. Compounds **1** and **2** were eluted as peaks with retention times of 11.3 and 7.2 minutes, respectively.

SMase Assay

nSMase and aSMase activities were measured according to the method of QUINTERN *et al.*[13] with some modifications. The reaction mixture for nSMase activity contained 25 μM [^{3}H]sphingomyelin (0.01 μCi), 20 mM HEPES-NaOH buffer (pH 7.4), 6.5 mM $MgCl_2$, 0.1% Triton X-100, 0.75 unit a membrane fraction prepared from rat brains as enzyme source and a test sample (5 μl in 50% CH_3OH) in a total volume of 50 μl. As for aSMase activity, the reaction mixture contained 25 μM [^{3}H]sphingomyelin (0.01 μCi), 250 mM sodium acetate buffer (pH 5.0), 0.1% NP-40 (Sigma), aSMase (0.5 unit) and a test sample in 50 μl. The reaction was started by adding the enzyme solution. After incubation at 37°C for 30 minutes, the reaction was stopped by addition of $CHCl_3$ - MeOH (2 : 1, 200 μl) to the mixture. After vortexing, the mixture was centrifuged and the aqueous layer (50 μl) containing the released [^{3}H]phosphocholine was measured for radioactivity by a liquid scintillation counter (Beckman).

Antimicrobial Activity

Antimicrobial activity against 16 species of microorganisms was measured by a paper disk method. Media for each microorganism are as follows: GAM agar (Nissui Seiyaku Co.) for *Bacteroides fragilis*; Waksman agar for *Mycobacterium smegmatis*; Bacto PPLO agar (Difco) supplemented with 15% horse serum, 0.1% glucose, 0.2% phenol red (5 mg/ml) and 1.5% agar for *Acholeplasma laidlawii*; nutrient agar for the other bacteria; a medium composed of 1.0% glucose, 0.5% yeast extract, and 0.8% agar for fungi and yeasts. A paper disk (i.d. 6 mm, ADVANTEC) containing 10 μg of sample was placed on an agar plate. Bacteria except *Xanthomonas oryzae* were incubated at 37°C for 24 hours. Yeasts and *Xanthomonas oryzae* were incubated at 27°C for 24 hours. Fungi were incubated at 27°C for 48 hours. Antimicrobial activity was expressed as diameter (mm) of inhibitory zone.

Cytotoxic Activity

P-388, a tumor cell line was cultured in the RPMI1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin and 0.01 μM 2-mercaptoethanol at 37°C. After the cells were treated with various concentrations of quinones or related compounds for 18 hours, the growth of the cells was determined by MTT assay[14]. Cytotoxic activity was defined as an IC_{50} value of a drug for the P-388 growth.

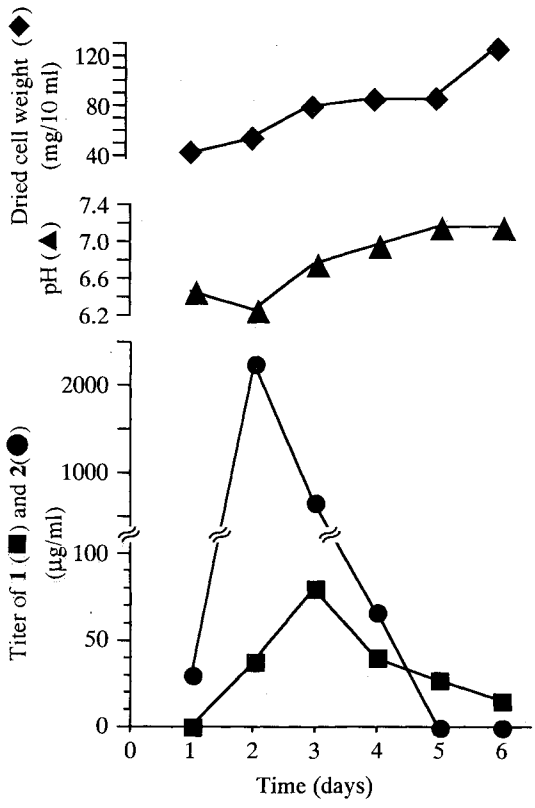
Results

Fermentation

A stock culture of strain FOM-8108 was inoculated into a 80-ml test tube containing 10 ml of the seed medium and incubated on a shaker at 27°C for 3 days. Then, 2 ml of this culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium and incubated on a rotary shaker at 27°C for 2 days. The main culture was started by transferring 1 ml of the seed culture into a 500-ml Erlenmeyer flask containing 100 ml of the production medium, and the fermentation was carried out at 27°C on a rotary shaker (210 rpm). A typical time course of the production of **1** and **2** is shown in Fig. 2. The pH and dried cell weight gradually increased from initiation of the

culture to day 6. The production of **2** reached over 2 mg/ml at day 2, then decreased rapidly. The maximum production of **1** was observed at day 3, suggesting that **1** was derived from **2**.

Fig. 2. A typical time course of **1** and **2** production by the fungal strain FOM-8108.



Isolation

The isolation procedure for **1** and **2** is summarized in Fig. 3. The four-day old culture broth (5 liters) was centrifuged to obtain the supernatant. After adjusted to pH 9 with 2 N NaOH, the supernatant was extracted with an equal volume of ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a brown oil (1.48 g). It was applied on a silica gel column (75 g, Silica gel 60, 63~200 μm, Merck) previously equilibrated with hexane, and materials were eluted stepwise with hexane-ethyl acetate solutions. Compound **1** was eluted with hexane - ethyl acetate (4 : 1), and **2** with hexane - ethyl acetate (10 : 3). The fractions containing **1** and **2** were collected separately and concentrated under reduced pressure to give crude **1** (103 mg) and **2** (59 mg) as brown powders. They were finally purified by Sephadex LH-20 column chromatography (i.d. 1.0×110 cm, Amersham Pharmacia; solvent, CH_3OH). The active fractions were concentrated to dryness to give pure **1**

Fig. 4. HMBC experiments for **1**.

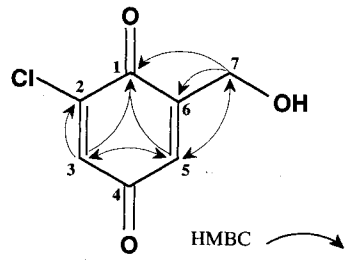


Fig. 3. Purification procedure of **1** and **2**.

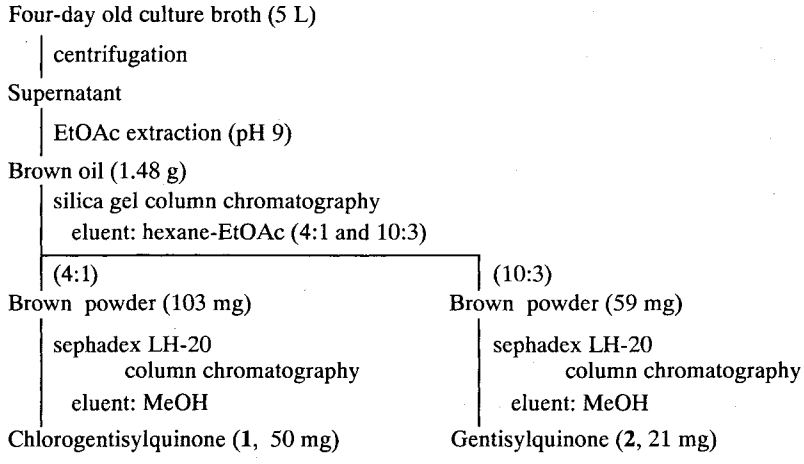


Table 1. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	Brown needle	Brown powder
Molecular formula	$C_7H_5ClO_3$	$C_7H_6O_3$
EI-MS (*m/z*)	171, 173	138
HR-MS calcd	171.9927 $(M)^+$	138.0317 $(M)^+$
Found	171.9927 $(M)^+$	138.0328 $(M)^+$
UV λ_{max}^{MeOH}nm (ε)	258 (37,100) 325 (1,500)	244 (36,900)
IR ν_{max}^{KBr} cm^{-1}	3473, 1654, 1631, 1594, 1108	3482,1655, 1638, 1591
Solubility		
Soluble	$CHCl_3$, EtOAc, MeOH	$CHCl_3$, EtOAc, MeOH
Insoluble	Hexane, H_2O	Hexane, H_2O

Table. 2. ^1H and ^{13}C NMR chemical shifts of **1** and **2**.

	1		**2**	
Carbon No.	^{13}C chemical shifts (ppm)[a]	^1H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^1H chemical shifts (ppm)[b]
1	180.4		188.5	
2	145.0		137.5	6.75 m (1H)
3	134.6	7.05 d (1H, *J*=2.5)	137.8	6.75 m (1H)
4	186.5		189.1	
5	131.5	6.78 dd (1H, *J*=2.3, 2.5)	131.3	6.75 m (1H)
6	150.0		150.0	
7	59.4	4.45 d (2H, *J*=2.3)	58.8	4.43 d (2H, *J*=2.0)

[a] Chemical shifts are shown with reference to CD_3OD at 49.0 ppm
[b] Chemical shifts are shown with reference to CD_3OD at 3.31 ppm

(50 mg) as brown needles and pure **2** (21 mg) as a brown powder.

Physico-chemical Properties and Structure Elucidation

From the physico-chemical properties (Table 1) and NMR spectral data (Table 2), **1** and **2** appeared structurally related. The structure of **2** was identified with gentisylquinone by comparison with the reported spectral data[15]. The structure of **1** was elucidated as described below. The molecular formula of **1** ($C_7H_5O_3Cl$) and a strong absorption at 1108 cm^{-1} in IR spectrum (Table 1) suggested that a proton in **2** was replaced with a chloride. The ^{13}C NMR spectrum (Table 2) of **1** resembled that of **2** expect carbon signals of C-1 and C-2. The connectivity of proton and carbon atoms was established by ^{13}C-^1H HMQC spectrum, indicating the presence of a chloride atom at C-2 in **1**. The ^{13}C-^1H long-range couplings of ^2J and ^3J in the HMBC experiments are shown in Fig. 4. The ^{13}C-^1H long-range coupling of ^4J from 7-H (δ 4.45) to C-4 (δ 186.5) was observed. Thus, the structure of **1** was finally elucidated as 2-chloro-6-hydroxymethyl-1, 4-benzoquinone.

Biological Properties

Inhibition of nSMase by **1** and **2**

Effects of **1** and **2** on SMases are shown in Fig. 5. Both **1** and **2** inhibited nSMase activity in a dose-dependent manner with IC_{50} values of 1.2 and 5.9 μM, respectively, while they showed no inhibitory effect on aSMase activity even at 100 μM. Furthermore, the inhibitory activity of quinones and related compounds (Fig. 6) against SMases was tested and the results are summarized in Table 3. 1,4-Benzoquinone (**3**) and 2-chloro-1,4-benzoquinone (**4**) also showed specific nSMase inhibition with IC_{50} of 20 and 12 μM, respectively. However, 2,5-dimethoxybenzyl alcohol (**5**) and 3-chlorobenzyl alcohol (**6**) with a benzene ring instead of a quinone ring lost the inhibitory activity. Thus, **1** is the most potent nSMase inhibitor among the compounds tested.

In order to elucidate the mechanism of nSMase inhibition by **1**, steady-state kinetics were obtained (Fig. 7). The Lineweaver-Burk plots (Fig. 7A) showed that **1** inhibited nSMase in a mixed manner with respect to the substrate sphingomyelin. The Dixon plots (Fig. 7B) were nonlinear, that is, the inhibition appeared to synergistically increase as **1** increased.

Antimicrobial and Cytotoxic Activity

Antimicrobial activity of **1**~**6** is summarized in Table 4. Compounds **1**~**4** showed broad antimicrobial activity against Gram-positive bacteria and Gram-negative bacteria. But **5** and **6** showed no antimicrobial activity against these microorganisms.

The cytotoxic activity of **1**~**6** against P-388 cells is shown in Table 5. The IC_{50} values of **1**~**4** were 7.6, 14.7, 64.8, and 84.5 μM, respectively, while **5** and **6** showed no cytotoxic activity at 100 μM.

Discussion

Although activation of the sphingomyelin pathway by nSMase and aSMase has been shown to increase the production of ceramide in several cell lines, the biological properties of ceramide generation is still not completely

Table 3. Effects of quinones and related compounds on nSMase and aSMase activities.

Compound	IC_{50} (μM)	
	nSMase	aSMase
1	1.2	>100
2	5.9	>100
3	20	>100
4	12	>100
5	>100	>100
6	>100	>100

Table 4. Antimicrobial activity of **1**~**6**.

Test organism	Inhibition zone (mm)					
	1	**2**	**3**	**4**	**5**	**6**
Bacillus subtilis KB27	13	12	14	11	-	-
Staphylococcus aureus KB210	9	8	9	11	-	-
Micrococcus luteus KB212	9	8	7	10	-	-
Mycobacterium smegmatis KB42	-	-	-	-	-	-
Escherichia coli KB213	9	10	9	9	-	-
Escherichia coli KB176	9	7	7	7	-	-
Pseudomonas aeruginosa KB105	-	-	-	-	-	-
Xanthomonas oryzae KB88	9	10	8	10	-	-
Bacteroides fragilis KB169	-	-	-	-	-	-
Acholeplasma laidlawii KB174	10	9	10	12	-	-
Pyricularia oryzae KF180	-	-	-	-	-	-
Aspergillus niger KF103	-	-	-	8	-	-
Mucor racemosus KF223	-	-	-	-	-	-
Candida albicans KF1	-	-	-	-	-	-
Saccharomyces cerevisiae KF26	-	-	-	11	-	-

Papar disk assay. 10 μg sample on 6 mm disk.
-, No inhibition

Table 5. Cytotoxic activity of **1**~**6** against P-388 cells.

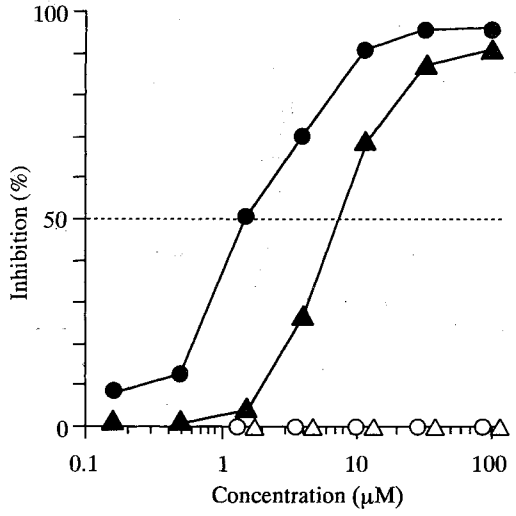
Compound	IC_{50} (μM)
1	7.6
2	14.7
3	64.8
4	84.5
5	>100
6	>100

elucidated. Therefore, specific inhibitors of SMases will be a useful tool to make their cellular function clear. Recently, several compounds from microbial origin, scyphostatin[16,17], F-11334s[18] and altenusin[10] were discovered as specific nSMase inhibitors. Among them, scyphostatin was utilized to elucidate the potential role of ceramide in the cellular inflammation process by showing dose-dependent inhibition of the lipopolysaccharide (LPS)-induced prostaglandin E_2 production and LPS-induced interleukin 1β production by the compound.

Gentisylquinone (**2**) and its chloro derivative (**1**) were purified as inhibitors of nSMase from the culture broth of a fungal strain isolated from a marine environment. Although **2** was previously reported as a constituent of the quinhydrone complex isolated from the culture of *Penicillium urticae* Bain[11,19], we could purify it as a free quinone. Free quinones seemed quite unstable, as also suggested from the dramatic decrease of the amount of **2** in the culture broth from day 2 to day 4 (Fig. 2). It was plausible that **1** is biosynthesized from **2** by incorporation of chloride ion from sea water at the C-2 position. More stable quinol type counterparts were not isolated as nSMase inhibitors from the culture, but gentisylalcohol was reported as an antibiotic from several microorganisms[19].

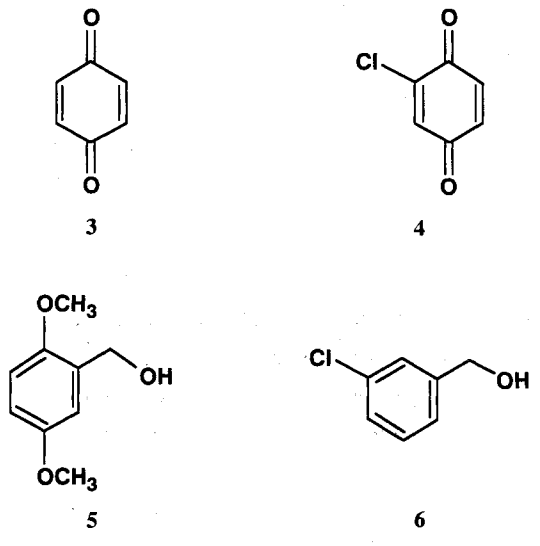
Compounds **1** and **2** inhibited nSMase activity of a membrane fraction prepared from rat brains (Fig. 5), whereas they did not exhibit inhibitory activity against aSMase from human placenta at all even at 100 μM, suggesting they are potent and specific inhibitors of mammalian nSMase activity. Since **1** and **2** have a simple quinone structure, other related compounds (Fig. 6) commercially available were also tested for their biological activities (Tables 3~5). Interestingly, compounds having a benzoquinone (**1**~**4**) exhibited nSMase inhibitory activity, antimicrobial activity, and cytotoxic activity. Although in our preliminary experiment compounds **1**~**4** showed no effect on nSMase from *Bacillus cereus* (Funakoshi), other related enzymes essential for growth in these

Fig. 5. Specific inhibition of nSMase activity by **1** and **2**.



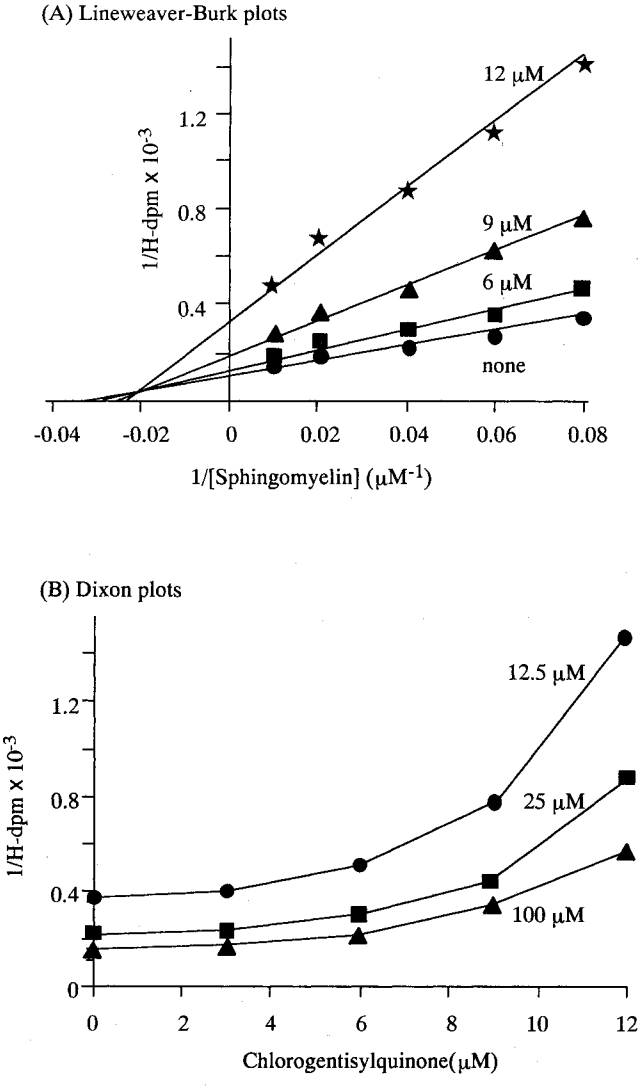
1; nSMase (●) and aSMase (○).
2; nSMase (▲) and aSMase (△).

Fig. 6. Structures of synthethic quinones and related compounds.



1,4-Benzoquinone (**3**), 2-chloro-1,4-benzoquinone (**4**), 2,5-dimethoxybenzyl alcohol (**5**) and 3-chloro-benzyl alcohol (**6**).

Fig. 7. Kinetics of nSMase inhibition by **1**.



microorganisms might be inhibited by the compounds.

We performed steady-state kinetic analyses of **1** to elucidate the mechanism of nSMase inhibition. The Lineweaver-Burk plots showed that **1** inhibited the enzyme activity in a mixed manner. The Dixon plots shown in Fig. 7B did not match a linear fit, suggesting its inhibition mechanism is complex. Similar data of kinetic analyses by scyphostatin, a potent nSMase inhibitor, were reported[16]. Detailed examination is required to elucidate the mechanism of nSMase inhibition.

Acknowledgments

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