Isolation of Salsolinol, a Tetrahydroisoquinoline Alkaloid, from the Marine Sponge *Xestospongia* cf. *vansoesti* as a Proteasome Inhibitor

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Salsolinol (1), a tetrahydroisoquinoline alkaloid, was isolated from the marine sponge *Xestospongia* cf. *vansoesti* collected in Indonesia as a proteasome inhibitor, along with three salsolinol derivatives, norsalsolinol (2), *cis*-4-hydroxysalsolinol (3), and *trans*-4-hydroxysalsolinol (4). Compounds 1 and 2 inhibited the chymotrypsin-like activity of the proteasome with IC_{50} values of 50 and 32 μg/ml, respectively, but 3 and 4 showed no inhibitory effect even at 100 μg/ml.

Key words tetrahydroisoquinoline alkaloid; proteasome; chymotrypsin-like activity; marine sponge

The proteasome functions as a proteolytic machine in the ubiquitin-dependent proteolytic pathway called the ubiquitin-proteasome system.[1,2] The 26S proteasome consists of two subcomplexes, the 20S core particle (the 20S proteasome) and the 19S regulatory particle, and a client protein is degraded by the proteolytic active sites of the 20S proteasome after polyubiquitination. The 20S proteasome is classified as a threonine protease that contains two pairs of three different sites, which catalyze chymotrypsin-like, trypsin-like, and caspase-like activities. Since the level of proteasome activity is increased especially in tumor cells, it is reasonable to develop specific compounds targeting the ubiquitin-proteasome system for cancer treatment. The recent approval of bortezomib (PS-341, Velcade®), a synthetic proteasome inhibitor, for the treatment of relapsed multiple myeloma has opened the way to the discovery of drugs targeting the proteasome.[3,4] Significantly, bortezomib is effective against various tumor cells that are resistant to conventional chemotherapeutic agents.[5] Currently, three proteasome inhibitors, salinosporamide A (NPI-0052),[6—8] carfilzomib (PR-171),[9,10] and CEP-18770,[11,12] are undergoing phase I and II clinical trials. So far, structurally-diverse proteasome inhibitors have been developed by chemical synthesis and also by searching natural sources and chemical libraries as drugs for the clinical treatment of cancer and also as molecular tools for the investigation of cellular events.[13]

During a search for natural products exhibiting biological activity, we screened extracts of natural sources for inhibitory activity against the proteasome. To date, based on the inhibition of the chymotrypsin-like activity of the proteasome, we isolated agosterols,[14] mycalolides,[15] *cis*-hinokiresiol,[16] and aaptamine derivatives[17] from natural sources as proteasome inhibitors. Here, we report the isolation of salsolinol (**1**) and its derivatives (**2**—**4**) from the marine sponge *Xestospongia* cf. *vansoesti*, and their two biological activities, inhibitory activity against the chymotrypsin-like activity of the proteasome and cytotoxic activity.

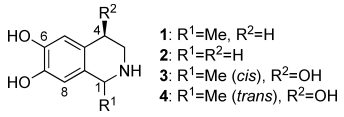

Chart 1

Specimens of *Xestospongia* cf. *vansoesti* were collected in Indonesia. The EtOH extract of the sponge was evaporated, and the aqueous residue was extracted with EtOAc and then *n*-BuOH. The *n*-BuOH (6.0 g) and H_2O fractions, which showed inhibitory activity against the chymotrypsin-like activity of the proteasome and cytotoxicity, were subjected to octadecyl silyl (ODS) column chromatography and ODS HPLC to afford salsolinol (**1**, 191.8 mg, 0.048% wet weight), norsalsolinol (**2**, 4.96 mg, 0.0012%), *cis*-4-hydroxysalsolinol (**3**, 1.35 mg, 0.00034%), and *trans*-4-hydroxysalsolinol (**4**, 3.68 mg, 0.00092%) (Chart 1).

The ^{1}H-NMR spectrum of salsolinol (**1**) showed a doublet methyl signal at δ 1.58 (d, J=6.4 Hz), two pairs of methylene signals at δ 2.86/2.95 and 3.30/3.46, and a methine signal at δ 4.41 (q, J=6.4 Hz), and two singlet aromatic signals at δ 6.56 and 6.61. The ^{13}C-NMR spectrum of **1** showed six hydrogen-bearing carbons at δ 19.7 (CH_3), 25.8 (CH_2), 40.9 (CH_2), 52.4 (CH), 113.5 (CH), and 116.1 (CH) and four quaternary carbons at δ 123.3, 125.3, 146.0, and 146.6; the latter two indicated to be oxygenated by chemical shifts. Analysis of 2D-NMR data strongly suggested that **1** was 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol).[18] The ^{1}H- and ^{13}C-NMR spectra of norsalsolinol (**2**) indicated that the methyl group at C-1 in **1** is replaced with a hydrogen atom in **2** to be a methylene group, δ_H 4.10 (2H, s) and δ_C

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Table 1. ^{1}H- and ^{13}C-NMR Data for **3** and **4** in D_2O

	3			**4**		
	δ_H, J in Hz	δ_C, mult.	HMBC	δ_H, J in Hz	δ_C, mult.	HMBC
1	4.37 q 6.4	51.3 CH		4.50 q 6.4	50.0 CH	3, 8a
3	3.32 br d 12.0	47.1 CH_2		3.24 br d 12.2	43.6 CH_2	
	3.41 br d 12.0			3.51 br d 12.0		
4	4.77 m	62.1 CH		4.78 m	61.9 CH	
4a		124.7 qC			124.3 qC	
5	6.82 s	116.2 CH	4, 6, 7, 8a	6.81 s	115.8 CH	4, 6, 7, 8a
6		143.9 qC			144.0 qC	
7		145.0 qC			145.0 qC	
8	6.71 s	112.5 CH	1, 4a, 6, 7	6.64 s	113.1 CH	1, 4a, 6, 7
8a		125.2 qC			125.4 qC	
1-Me	1.55 d 6.4	18.1 CH_3	1, 8a	1.44 d 6.4	18.4 CH_3	1, 8a

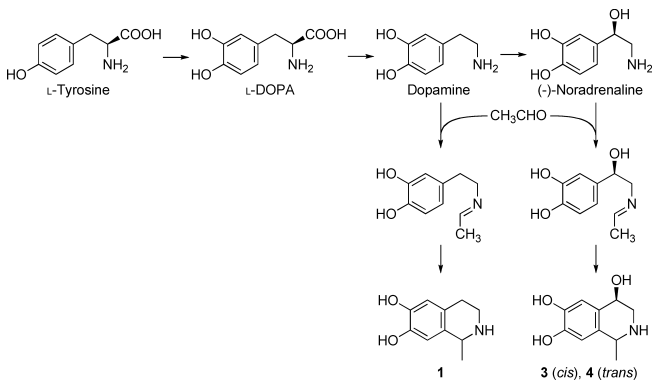

Chart 2. Possible Biosynthetic Pathway of **1**, **3**, and **4**

43.9 (CH_2), which was supported by the FAB-MS spectrum. Thus, **2** was identified as 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (norsalsolinol).[19] The molecular formulas of the *cis*- (**3**) and *trans*-4-hydroxysalsolinols (**4**), $C_{10}H_{13}NO_3$, were established by high resolution (HR)-FAB-MS and both isomers were found to contain an additional oxygen atom when compared to **1**. Their NMR spectra were similar to those of **1**, except for the absence of the methylene group in **1** and the presence of additional methine signals in the lower field in **3** and **4**, δ 4.77 (m) and 4.78 (m), respectively (Table 1). Analysis of 2D-NMR data indicated that **3** and **4** were 4-hydroxy derivatives of **1**. Although **3** and **4** have yet to be isolated from natural sources, they have been chemically synthesized.[20] From the NMR data, **3** and **4**, isolated from the sponge, were identified as *cis* and *trans* isomers, respectively. The specific rotation of **1**, $[\alpha]_D$ 0, indicated a racemic mixture, as confirmed by flat circular dichroism (CD) curves from 200 to 400 nm. The racemic character of **1** could be rationalized by considering its biosynthetic pathway (Chart 2), in which it is generated by the condensation of dopamine and acetaldehyde. On the other hand, **3** and **4** would be biosynthesized by the condensation of (−)-noradrenaline and acetaldehyde, and so have the 4*R* configuration (Chart 2). We prepared **3** and **4** from (−)-noradrenaline and measured the specific rotations. However, the values of the synthetic **3** and **4** were almost zero and we could not determine the absolute configurations on the basis of the values.

Thus, we isolated salsolinol (**1**), norsalsolinol (**2**), *cis*-4-hydroxysalsolinol (**3**), and *trans*-4-hydroxysalsolinol (**4**) from the marine sponge *Xestospongia* cf. *vansoesti* collected in Indonesia. Proteasome inhibitory and cytotoxic activities of **1** and **2** are listed in Table 2. Compounds **1** and **2** inhibited the chymotrypsin-like activity of the proteasome with IC_{50} values of 50 and 32 μg/ml, respectively, and also showed cytotoxicity against human cervix epithelioid carcinoma (HeLa) cells with IC_{50} values of 17 and 7 μg/ml, respectively. Compounds **3** and **4** showed no proteasome inhibitory activity even at 100 μg/ml and no cytotoxicity against HeLa cells at 50 μg/ml. In addition, the cytotoxicity of **1** was tested against murine leukemia (L1210), human amnion (FL), human oral epidermoid carcinoma (KB), and human lung adenocarcinoma (A549) cell lines. The respective IC_{50} values were 8, 13, 20, and 27 μg/ml.

Table 2. Biological Activities of **1** and **2**

Compound	Proteasome inhibition IC_{50} (μg/ml)	Cytotoxicity IC_{50} (μg/ml)				
		HeLa	L1210	FL	KB	A549
1	50	17	8	13	20	27
2	32	7	—*a)*	—*a)*	—*a)*	—*a)*

Proteasome inhibition was tested for the chymotrypsin-like activity with a 20S proteasome preparation from human erythrocytes (BostonBiochem, E-360). *a*) Not tested.

Salsolinol (**1**) is detected in human and animal brains, and is thought to be synthesized from dopamine.[21] Kicha *et al.* isolated **1** as salts with steroidal sulfates from the starfish *Lethasterias nanimensis chelifera*,[18] and reported that the *R*-isomer was predominantly incorporated into the salts. Liu *et al.* found the ratio of (*R*)- to (*S*)-**1** to be 1.6 in the cerebral ganglion of *Aplysia californica* and 1.07 in a dried banana, by gas chromatography-mass spectrometry with cyclodextrin chiral columns.[22] In addition, **1** has been isolated from Asclepiadaceae,[23] Papaveraceae,[23] and Aristolochiaceae[24] plants. However, this is the first time that **1** has been isolated from a marine sponge and it should be noted that its yield, 0.048% (wet weight), is relatively high. As **2** was detected in dopamine-rich areas of the human brain[25,26] and both **3** and **4** have been only chemically synthesized,[20] this is the first isolation of **2**—**4** as secondary metabolites from a natural source.

In mammalian brains, salsolinol (**1**) has neurotoxic effects in dopaminergic cells, and its metabolites, including its *N*-

methylated derivative *N*-methylsalsolinol, are implicated in the etiopathogenesis of Parkinson's disease.[21] In dopaminergic neuroblastoma SH-SY5Y cells, **1** causes both apoptosis and necrosis.[27] Despite its neurotoxic activity, **1** induces the release of prolactin from the anterior lobe of the pituitary gland by binding an unknown receptor,[21,28] implying that it functions as a neuromodulator.

In this study, we first found that salsolinol (**1**) and its derivative (**2**) function as proteasome inhibitors. In connection with our findings, it should be noted that dopamine inhibits a chymotrypsin-like activity of purified human 20S proteasome.[29] Furthermore, it has been reported that lactacystin, a proteasome inhibitor, causes loss of nigral dopaminergic neurons.[30] Thus, it can be inferred that **1** induces dopaminergic neuron death possibly through the inhibition of the proteasome.

Experimental

Optical rotations were determined with a JASCO P-1020 polarimeter in H_2O. CD spectra were measured on a JASCO J-720WI spectropolarimeter in MeOH. NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer in CD_3OD or D_2O. Chemical shifts in CD_3OD were referenced to the residual solvent peaks, δ_H 3.3 and δ_C 49.0. In D_2O, chemical shifts of ^{1}H-NMR were referenced to the peak of residual solvent at δ_H 4.65, and those of ^{13}C-NMR to the peak of dioxane at δ_C 66.5. Mass spectra were measured on a JMS AX-500 or JMS HX-110 mass spectrometer.

Extraction and Isolation The marine sponge was collected at a depth of 10 m in North Sulawesi, Indonesia, in September 2006 and soaked in EtOH immediately. The sponge was identified as *Xestospongia* cf. *vansoesti*. A voucher specimen (RMNH POR. 4807) has been deposited in the National Museum of Natural History, The Netherlands. The sponge (400 g, wet weight) was extracted with EtOH. The extract was evaporated, and the aqueous residue was extracted with EtOAc and then *n*-BuOH. The *n*-BuOH fraction (6.0 g) and H_2O fraction showed inhibitory activity against the chymotrypsin-like activity of the proteasome and cytotoxicity. The *n*-BuOH fraction was partitioned between hexane and 90% MeOH–H_2O, and the methanolic fraction (5.0 g) was subjected to ODS column chromatography with a step-wise gradient using H_2O and MeOH. The fraction eluted with H_2O was purified by ODS HPLC with H_2O to afford salsolinol (**1**, 175.8 mg), *cis*-4-hydroxysalsolinol (**3**, 0.33 mg), and *trans*-4-hydroxysalsolinol (**4**, 1.2 mg). The H_2O fraction obtained by the partition between *n*-BuOH and H_2O was purified by the same procedure to afford **1** (16.0 mg), norsalsolinol (**2**, 4.96 mg), **3** (1.02 mg), and **4** (2.48 mg).

Salsolinol (**1**): ^{1}H-NMR (CD_3OD) δ: 1.58 (3H, d, J=6.4 Hz, 1-Me), 2.86 (1H, m, H-4), 2.95 (1H, m, H-4), 3.30 (1H, m, H-3), 3.46 (1H, m, H-3), 4.41 (1H, q, J=6.4 Hz, H-1), 6.56 (1H, s, H-5), 6.61 (1H, s, H-8). ^{13}C-NMR (CD_3OD) δ: 19.7 (CH_3, 1-Me), 25.8 (CH_2, C-4), 40.9 (CH_2, C-3), 52.4 (CH, C-1), 113.5 (CH, C-8), 116.1 (CH, C-5), 123.3 (qC, C-4a), 125.3 (qC, C-8a), 146.0 (qC, C-7), 146.6 (qC, C-6). ^{1}H-NMR (D_2O) δ: 1.42 (3H, d, J=6.4 Hz, 1-Me), 2.74 (1H, m, H-4), 2.79 (1H, m, H-4), 3.17 (1H, m, H-3), 3.35 (1H, m, H-3), 4.29 (1H, q, J=6.4 Hz, H-4), 6.52 (1H, s, H-5), 6.55 (1H, s, H-8). $[\alpha]_D^{25}$ 0 (c=2.9, H_2O). FAB-MS (positive) m/z: 180 $[M+H]^+$.

Norsalsolinol (**2**): ^{1}H-NMR (D_2O) δ: 2.85 (2H, t, J=6.4 Hz, H_2-4), 3.34 (2H, t, J=6.4 Hz, H_2-3), 4.10 (2H, s, H_2-1), 6.59 (1H, s), 6.64 (1H, s). ^{13}C-NMR (D_2O) δ: 23.7 (CH_2), 41.6 (CH_2), 43.9 (CH_2), 113.7 (CH), 115.8 (CH), 119.6 (qC), 123.7 (qC), 143.0 (qC), 143.8 (qC). FAB-MS (positive) m/z: 166 $[M+H]^+$.

cis-4-Hydroxysalsolinol (**3**): $[\alpha]_D^{29}$ +1.8 (c=3.6, H_2O). NMR data (D_2O): see Table 1. FAB-MS (positive) m/z: 196 $[M+H]^+$. HR-FAB-MS $[M+H]^+$ m/z: 196.0974 (Calcd for $C_{10}H_{14}NO_3$, 196.0974).

trans-4-Hydroxysalsolinol (**4**): $[\alpha]_D^{29}$ −0.68 (c=2.0, H_2O). NMR data (D_2O): see Table 1. FAB-MS (positive) m/z: 196 $[M+H]^+$. HR-FAB-MS $[M+H]^+$ m/z: 196.0973 (Calcd for $C_{10}H_{14}NO_3$: 196.0974).

Cytotoxicity Assay Cytotoxicity was evaluated in HeLa cells. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were seeded into 96-well microplates (3×10^3 cells/well) and pre-cultured for 1 d. The medium was replaced with that containing test compounds at various concentrations and the cells were further cultured at 37 °C for 3 d. The medium was then replaced with 50 μl 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 3 h. After the addition of 200 μl of dimethyl sulfoxide (DMSO), the optical density at 570 nm was measured with a microplate reader (MTP-810Lab, Corona Electric, Japan). Cytotoxicity against L1210, FL, KB, and A549 cells was tested according to the same procedure.

Proteasome Inhibition Assay The fluorogenic compound Suc-Leu-Leu-Val-Tyr-MCA (Peptide Institute, Inc., Osaka, Japan) was used as a substrate for the chymotrypsin-like activity of the 20S proteasome preparation from human erythrocytes (BostonBiochem, E-360). The proteasome (0.5 nM) in a mixture (100 μl) that contained 50 mM Tris–HCl, pH 7.8, 1 mM dithiothreitol, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% sodium dodecyl sulfate (SDS) was pre-incubated with test compounds at various concentrations at 30 °C for 10 min. Then, the substrate (10 μM) was added and the mixture was further incubated at 30 °C for 16 h. The reaction was stopped by adding 100 μl of 10% SDS and the fluorescence intensity owing to 7-amino-4-methylcoumarin (AMC) was measured (excitation, 360 nm; emission, 450 nm) with the microplate reader. The IC_{50}, the concentration required for 50% inhibition of the proteasome inhibitory activity, was calculated from duplicate measurements.

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