Novel Neuronal Nitric Oxide Synthase (nNOS) Selective Inhibitor, Aplysinopsin-Type Indole Alkaloid, from Marine Sponge *Hyrtios erecta*

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Two novel aplysinopsin-type indole alkaloids, 1 and 2, and three known indole alkaloids were isolated from the marine sponge *Hyrtios erecta*. These compounds exhibited selective inhibitory activity against the neuronal isozyme of nitric oxide synthase (nNOS). Furthermore, new quinolone 7 was also isolated from the same marine sponge. The chemical structures of these new compounds were elucidated on the basis of spectroscopic analysis.

Key words nitric oxide synthase; indole alkaloid; marine sponge

Nitric oxide (NO) is known to be an important second messenger having numerous functions which regulate many physiological processes, *e.g.*, inflammation, regulation of blood pressure, platelet adhesion, neurotransmission, and defense mechanisms.[1—3] Since NO is a reactive molecule having one unoccupied electron, its excessive production causes a number of disease states such as post-ischemic stroke damage,[4] schizophrenia,[5] development of colitis,[6] tissue damage and pathological inflammation.[7] Therefore, the rational control of NO production is deemed to be an efficient approach to treat these afflictions.

The biosynthesis of NO is catalyzed by nitric oxide synthase (NOS), which is classfied into three isoforms: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Each isozyme uses L-arginine as a substrate and requires reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, and tetrahydrobiopterin (THB) as cofactors. nNOS and eNOS are constitutive Ca^{2+}/calmodulin-regulated enzymes, whereas iNOS is a Ca^{2+}/calmodulin-independent emzyme induced in macrophage.[8] Therefore, a selective inhibitor of each NOS isozyme is expected to have significant therapeutic potential.[9]

In the course of our study of bioactive substances from marine organisms, we started a search for new substances which exhibit selective inhibitory effect against each isozyme of NOS. We found novel aplysinopsin-type indole alkaloids, **1** and **2**, as selective inhibitors against nNOS from the Okinawan marine sponge of *Hyrtios erecta*. In this paper, we describe the details of the structure elucidation of **1** and **2** together with novel quinolone **7**.

Results and Discussion

In this screening, two types of NOS isozymes, iNOS prepared from mouse macrophage and nNOS prepared from rat cerebellum, were used. The acetone extract of the titled fresh sponge was partitioned into an AcOEt–H_2O mixture to provide an AcOEt soluble portion, which showed nNOS selective inhibitory activity (94% and 21% inhibition against nNOS and iNOS, respectively at 125 μg/ml). The AcOEt soluble portion was subjected to bioassay-guided separation (inhibition of nNOS and iNOS). The active fractions, which were obtained by repeated SiO_2 column chromatography, were further purified by HPLC to provide compounds **1**—**7** (Chart 1). Compounds **3**, **4**, and **5** were respectively identified as 6-bromo-2′-demethylaplysinopsin (*Z*),[10] 5,6-dibromo-3-(2-methylaminoethyl)indole,[11] and 5,6-dibromo-3-(2-aminoethyl)indole[11] by comparison of MS and NMR spectral data with those of reported compounds.

Compound **1** was obtained as a yellow powder. The electron impact mass spectrum (EI-MS) of **1** showed the molecular ion at *m*/*z* 396/398/400 with relative intensities as 1 : 2 : 1, suggesting the presence of two bromine atoms. The molecular formula of **1** was established as $C_{13}H_{10}Br_2N_4O$ by high-resolution electron impact EI-MS (HR-EI-MS). The IR spectrum of **1** showed an absorbance of amide carbonyl group (1697 cm^{-1}). The UV spectrum [λ_{max} (MeOH) 383 nm (ε 20700), 294 nm (ε 4400), 247 nm (ε 17300)] of **1** supported the presence of an aromatic system. The ^{1}H- and ^{13}C-NMR data for **1** showed the signals of three aromatic protons [δ, 8.43 (s), 8.21 (s), 7.85 (s)] and eight olefinic carbons [δ_C, 135.7, 130.6, 127.8, 123.6, 116.9, 116.8, 115.2, 108.9]. The correlation between all proton and carbon signals was clarified as shown in Table 1 by heteronuclear multiple quantum coherence (HMQC) spectrum. Heteronuclear multiple bond connectivity (HMBC) analysis suggested the presence of a 5,6-disubstituted indole skeleton and clarified that compound **1** was a 5-brominated analogue of 6-bromo-2′-demethylaplysinopsin (**3**),[10] which has been isolated as a diastereomeric mixture from a marine sponge of *Dendrophyllia* sp. The geometry of the C-8(1′) olefin was determined to be *Z* by comparison of the chemical shift of the H-2 proton and C-8 carbon with those of 6-bromo-2′-demethylaplysinopsin (**3**).

Compound **2** was also acquired as a yellow powder. The HR-EI-MS of **2** implied the same molecular formula with **1**. The UV, IR and NMR spectra of **2** were similar to those of **1**, except for the signals assignable to H-2 (δ 8.90), H-4 (δ 8.14) and C-8 (δ_C 115.0). These data indicated that **2** was the C-8(1′) *E* isomer of **1** since the same differences were observed between 6-bromo-2′-demethylaplysinopsin (*Z*) (**3**) and 6-bromo-2′-demethylaplysinopsin (*E*).[10] The two dimentional (2D)-NMR data for **2** also supported the chemical structure of **2**.

Compound **6** was obtained as a yellow powder. The IR

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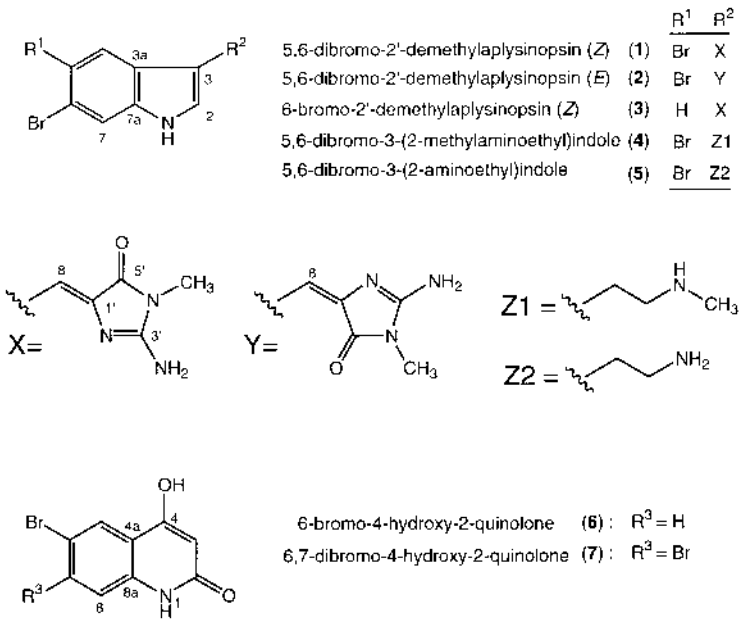

Chart 1

Table 1. ^{1}H- and ^{13}C-NMR Data for **1**, **2** and **7** in DMSO-d_6

No.	**1**		**2**		**7**	
	δ_C	δ (mult., int.)	δ_C	δ (mult., int.)	δ_C	δ (mult., int.)
1		12.31 (s, 1H)		12.19 (s, 1H)		12.07 (s, 1H)
2	130.6	8.21 (s, 1H)	132.6	8.88 (s, 1H)	165.0	
3	108.3		108.2		134.3	8.08 (s, 1H)
3a	127.8		128.4			
4	123.6	8.43 (s, 1H)	122.5	8.14 (s, 1H)	106.9	3.36 (s, 1H)
4a					126.8	
5	116.9		116.9		124.4	8.28 (s, 1H)
6	115.2		115.2		115.5	
7	116.8	7.85 (s, 1H)	117.2	7.91 (s, 1H)	116.1	
7a	135.8		135.8			
8	108.9	7.21 (s, 1H)	115.1	7.32 (s, 1H)	116.9	7.86 (s, 1H)
8a					136.0	
1′	135.7		135.7			
3′	155.9		152.7			
5′	163.2		160.8			
6′	26.0	3.15 (s, 3H)	26.0	3.15 (s, 3H)		

spectrum of **6** showed the presence of an OH group (3283 cm^{-1}) and amide carbonyl group (1697 cm^{-1}). The MS and NMR spectra of **6** suggested the molecular formula of **6** as $C_9H_6BrNO_2$. The ^{1}H- and ^{13}C-NMR analysis assisted by the HMQC experiment of **6** displayed the presence of nine carbon signals attributable to four aromatic methines, one amide carbonyl carbon and four aromatic quaternary carbons. Furthermore, the HMBC correlations suggested that compound **6** was 6-bromo-4-hydroxy-2-quinolone. The isolation of compound **6** from a natural source is reported for the first time, but **6** has been already reported as a synthetic compound without NMR assignment.[12)]

Compound **7** was also isolated as a yellow powder. The EI-MS of **7** showed the molecular ion at *m/z* 317/319/321, implying the existence of two bromine atoms. The molecular formula of **7** as $C_9H_5Br_2NO_2$ was defined by HR-EI-MS. Similarity in the NMR spectra of **6** and **7** revealed that both **6** and **7** have the same carbon skeleton. Furthermore, the chemical shift of C-8 carbon in **7** was shifted to higher field (8.5 ppm) than that of **6**, and the proton signal assignable to H-7 in **6** was not observed in **7** (Table 1). These findings suggested that compound **7** was 6,7-dibromo-4-hydroxy-2-quinolone.

As shown in Table 2, compounds **1**—**5** showed selective inhibitory activity against nNOS. Notably, compounds **1** and **2** showed no inhibitory activity against iNOS. On the other hand, N^{ω}-monomethyl-L-arginine (L-NMMA),[13)] a known NOS inhibitor, showed no selectivity against both isozymes.

Experimental

General Experimental Procedures IR spectra were obtained with a JASCO FT-IR 5300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured with JEOL Lambda-500 (500 MHz) or AL-300 (300 MHz) or Varian inova-600 (600 MHz) spectrometer with Me_4Si (0 ppm) as the internal standard. 2D-NMR spectra were recorded on JEOL Lambda-500 (500 MHz) or Varian

Table 2. Inhibitory Effects of Compounds **1**—**7** against nNOS and iNOS

	1	**2**	**3**	**4**	**5**	**6**	**7**	L-NMMA
nNOS (125 μg/ml)	100	100	100	104	103	22	22	—
(25 μg/ml)	32	13	22	25	13	1	4	60
iNOS (125 μg/ml)	0.0	0.0	7.5	20	29	6.7	9.9	—
(25 μg/ml)	0.0	0.0	0.0	9.7	14	0.0	8.3	67

Data represents inhibition (%) of enzyme activity.

inova-600 (600 MHz) spectrometer. UV spectra were obtained with Hitachi 330 spectrometer. EI-MS spectra were recorded on a JEOL JMS 600 mass spectrometer.

Materials Tris was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NADPH was purchased from Oriental Yeast Co. (Tokyo, Japan). $CaCl_2$ was purchased from Fluka (Buchs, Switzerland). L-NMMA, THB and iNOS were purchased from Cayman Chemical Co. (Michigan, U.S.A.). L-[^{3}H]-arginine (1424.5 GBq/mmol) was purchased from Dupont NEN (Boston, MA, U.S.A.). Ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), L-arginine, FMN, and FAD were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Extraction and Isolation The frozen sponge (6 kg), which has been collected at Iriomote-Island, Okinawa Prefecture, was extracted with acetone (10 l) at room temperature 3 times. The residue obtained by evaporation of the solvent under reduced pressure was partitioned into an AcOEt/H_2O mixture. Then, the AcOEt phase was evaporated to give an AcOEt–soluble portion (40 g). The AcOEt–soluble portion, which showed selective inhibition against nNOS (94% and 21% inhibition against nNOS and iNOS, respectively at 125 μg/ml) was subjected to bioassay-guided separation. The AcOEt–soluble portion (2 g) was separated by SiO_2 column (eluted with AcOEt–acetone–MeOH/$CHCl_3$) to give three fractions [fr. A (0.79 g), fr. B (0.14 g), fr. C (0.71 g)]. Fr. B which showed selective inhibition against nNOS (71% inhibition against nNOS and 4.8% against iNOS at 125 μg/ml) was further separated by SiO_2 column ($CHCl_3$: MeOH : H_2O=40 : 3 : 1) and reversed phase HPLC [Cosmosil 5C18-AR, 65% aq. MeOH containing 0.1% trifluoroacetic acid (TFA)] to obtain 5,6-dibromo-2′- demethylaplysinopsin (*Z*) (**1**, 12 mg, 0.6% from AcOEt extract), 5,6-dibromo-2′-demethylaplysinopsin (*E*) (**2**, 1.5 mg, 0.07%), and 6-bromo-2′-demethylaplysinopsin (*Z*) (**3**, 4.4 mg, 0.22%). Fraction C which also showed nNOS selective inhibition (96% inhibition against nNOS and 0% against iNOS at 125 μg/ml) was subjected to SiO_2 column ($CHCl_3$: MeOH : H_2O=40 : 3 : 1–30 : 3 : 1–MeOH) to give fr. C1 (0.16 g) and fr. C2 (0.44 g). Fraction C2 was further purified by HPLC (Cosmosil 5C18-AR, 33% aq. CH_3CN–0.1% TFA) to afford 5,6-dibromo-3-(2-methylaminoethyl)indole (**4**, 1.6 mg, 0.08%) and 5,6-dibromo-3-(2-aminoethyl)indole (**5**, 6.7 mg, 0.33%). Fraction C1 was also purified by HPLC (Cosmosil 5C_{18}-AR, 55% aq. MeOH–0.1% TFA) to afford 6-bromo-4-hydroxy-2-quinolone (**6**, 1.5 mg, 0.08%) and 6,7-dibromo-4-hydroxy-2-quinolone (**7**, 4 mg, 0.20%).

5,6-Dibromo-2′-demethylaplysinopsin (*Z*) (**1**): A yellow powder. HR-EI-MS: *m*/*z*: 395.9224/397.9220/399.9182; Calcd for $C_{13}H_{10}Br_2N_4O$: 395.9220/397.9200/399.9180. UV λ_{max} (MeOH) nm (ε): 383 (20700), 294 (4400), 247 (17300). IR (KBr): 1697 cm^{-1}. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (150 MHz) (dimethyl sulfoxide (DMSO)-d_6): as shown in Table 1.

5,6-Dibromo-2′-demethylaplysinopsin (*E*) (**2**): A yellow powder. HR-EI-MS: *m*/*z*: 395.9211/397.9207/399.9176; Calcd for $C_{13}H_{10}Br_2N_4O$: 395.9220/397.9200/399.9180. UV λ_{max} (MeOH) nm (ε): 385 (8600), 293 (2700), 246 (8500). IR (KBr): 1694 cm^{-1}. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (150 MHz) (in DMSO-d_6): as shown in Table 1.

6-Bromo-4-hydroxy-2-quinolone (**6**): A yellow powder ^{1}H-NMR (300 MHz, DMSO-d_6): δ 11.98 (s, H-1), 8.01 (d, *J*=2.7 Hz, H-3), 3.37 (s, H-4), 8.07 (s, H-5), 7.39 (d, *J*=8.5 Hz, H-7), 7.26 (d, *J*=8.8 Hz, H-8). ^{13}C-NMR (75 MHz, DMSO-d_6): δ_C 165.3 (C-2), 133.4 (C-3), 106.9 (C-4), 127.6 (C-4a), 122.5 (C-5), 113.7 (C-6), 114.2 (C-7), 124.6 (C-8), 135.0 (C-8a).

6,7-Dibromo-4-hydroxy-2-quinolone (**7**): A yellow powder. HR-EI-MS: *m*/*z*: 316.8698/318.8672/320.8645; Calcd for $C_9H_5Br_2NO_2$: 316.8686/318.8666/320.8646. UV λ_{max} (MeOH) nm (ε): 226 (37400). IR (KBr): 3283, 1647 cm^{-1}. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) (DMSO-d_6): as shown in Table 1.

Preparation of Crude nNOS[14] Cerebellum (2.5 g) of male Wister rats (150 g) was homogenized with 12 ml of buffer solution [0.32 M sucrose, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 0.5 mM EDTA, 1 mM DTT] using a Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 2000 ***g*** for 15 min at 4 °C. The resulting supernatant was treated with AG50W-X8 column (Na^+ form, equilibrated with 2 ml of homogenate buffer, Bio-Rad Laboratories, Hercules, U.S.A.) to remove free arginine and the Ca^{2+} ion and used for nNOS assay.

NOS Assay The oxidation reaction of L-arginine was monitored by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline as described in a previous report.[15] In the case of nNOS assay, a solution containing 50 mM Tris (pH 7.4), 1 mM DTT, 100 μM NADPH, 1 mM $CaCl_2$, 0.5 mM EDTA, 50 μM THB, 16.2 nM L-arginin and 33.8 nM L-[^{3}H]-arginine was prepared. 10 μl of crude nNOS and a sample (5.5 μl of 10% aq DMSO solution) were added to the solution (184.5 μl) and then incubated at 20 °C for 30 min. The reaction mixture (170 μl) was added to 800 μl of a stop-solution (50 mM sodium citrate, 25 mM sodium tetraborate and 0.6 M sodium chloride). The resultant solution (100 μl) was analyzed by reversed-phase HPLC [TSK-gel Aminopak column (Tosoh, Tokyo, Japan), eluted by 50 mM sodium citrate, 25 mM sodium tetraborate and 0.6 M sodium chloride at 55 °C], and radioactivity of L-citrulline was measured by 515-TR Flow Scintillation analyzer (Packard, Groningen, Netherlands). In the case of iNOS assay, a solution containing 50 mM Tris (pH 7.4), 1 mM DTT, 100 μM NADPH, 5 μM FMN, 5 μM FAD, 50 μM THB, 16.2 nM L-arginine and 33.8 nM L-[^{3}H]-arginine was prepared. 100 μU of iNOS solution (10 μl) and a sample (5.5 μl of 10% aq DMSO solution) were added to the solution and then incubated at 37 °C for 30 min. The reaction mixture was treated by the same method as in the case of nNOS assay to measure production of L-citrulline.

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