Isolations of *N*-Methyl-D-aspartic Acid-Type Glutamate Receptor Ligands from Micronesian Sponges

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The bioassay-guided fractionation of the water-soluble extract of the marine sponge *Cribrochalina olemda* collected in Palau resulted in the isolation of a new amino acid cribronic acid (**1**): (2*S*,4*R*,5*R*)-5-hydroxy-4-sulfooxypiperidine-2-carboxylic acid. However, aqueous extracts of *Stylotella aurantium* and *Axinella carteri* collected in Yap State, Micronesia, afforded a known *N*-methyl-D-aspartic acid (NMDA)-type glutamate receptor agonist, (2*S*,4*S*)-4-sulfooxypiperidine-2-carboxylic acid (**2**), as a common active principle. Both **1** and **2** induced convulsive behaviors in mice upon intracerebroventricular (icv) injection with ED_{50} values of 29 ± 3.0 and 20 ± 2.8 pmol/mouse, respectively. Radioligand binding assay using rat cerebrocortical membrane demonstrated that **1** and **2** inhibit the binding of the labeled NMDA receptor ligand [^{3}H]CGP39653 at IC_{50} values of 83 ± 15 and 214 ± 20 nM, respectively. However, **1** and **2** did not displace [^{3}H]kainic acid or [^{3}H]AMPA. These data indicated that **1** is a selective NMDA-type glutamate receptor ligand with potent convulsant activity in mice.

Glutamate receptors (GluRs) play central roles in mammalian CNS, not only in excitatory neurotransmissions but also in complex brain functions such as memory formation and learning. GluRs are a highly diverse receptor group and are divided largely into ionotropic and metabotropic receptors. Ionotropic GluRs (iGluRs) are further subdivided into three subtypes on the basis of their pharmacological preference toward selective agonists, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainic acid, and *N*-methyl-D-aspartic acid (NMDA)-type receptors.[1] Several naturally occurring amino acids are known as selective ligands of iGluRs. Among them, marine red algae-derived iGluR ligands such as kainic acid and domoic acid are used as standard tools in neurobiology.[2] We previously found the potent epileptogenic amino acid dysiherbaine (Figure 1) from an aqueous extract of the sponge *Dysidea herbacea*[3] and reported its unusual pharmacological properties toward neuronal and recombinant iGluRs.[4,5] This result suggested that novel iGluR ligands with unexpected structural and functional diversity might be widely distributed in marine benthic organisms. We thus further screened Micronesian marine benthic organisms for novel GluR ligands using a mouse assay, since GluR ligands can induce various convulsive behaviors in mice.[4] Because aqueous extracts of the sponges *Cribrochalina olemda*, *Stylotella aurantium*, and *Axinella carteri* showed potent convulsive activity in mice, the active principles of each of these organisms were isolated. Here, we report the isolation, structural determination, and in vitro and in vivo biological activities of a new amino acid, cribronic acid, (2*S*,4*R*,5*R*)-5-hydroxy-4-sulfooxypiperidine-2-carboxylic acid (**1**),[6] from the Palauan sponge *C. olemda*, and (2*S*,4*S*)-4-sulfooxypiperidine-2-carboxylic acid (**2**) from the Micronesian sponges *A. carteri* and *S. aurantium* as a common active principle.

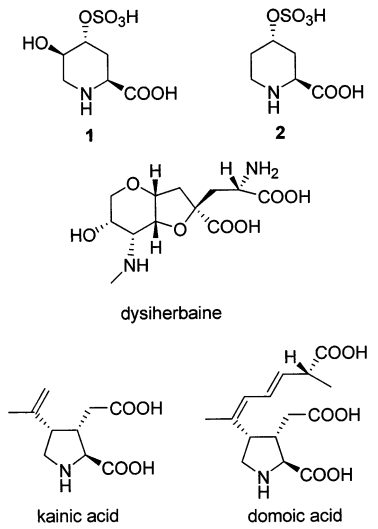

Figure 1. Structures of **1**, **2**, and other marine-derived excitatory amino acids.

Results and Discussion

An aqueous extract of *C. olemda* was separated by a series of gel filtrations, and combined bioactive fractions were finally recrystallized to give pure **1** (1.6×10^{-3}% of wet sample). The molecular formula for **1** was assigned to be $C_6H_{11}NO_7S$ on the basis of its high-resolution FABMS (HRFABMS) and NMR data (Table 1).

The correlation NMR (COSY, HMQC, and HMBC) for **1** readily established a framework of 4,5-disubstituted piperidine-2-carboxylic acid (pipecolic acid) (Figure 2a). The molecular formula of **1** requires one hydroxyl group and one sulfooxy group for the substituent at C-4 and -5. In the ^{1}H NMR spectrum, a signal for H-4 appeared rather downfield at δ 4.5 relative to that for H-5, which appeared at δ 4.0. These data indicated that the sulfated oxygen atom is attached to C-4, while the hydroxyl group is attached to C-5. The relative stereochemistry of three substituents on the piperidine ring was assigned by *J* analysis in the ^{1}H NMR. H-2, an α proton of the pipecolic

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Table 1. NMR Data for **1** and **2** in D_2O

	1[a]		**2**[b]	
position	^{1}H (δ, mult. J Hz)	^{13}C	^{1}H (δ, mult. J Hz)	^{13}C
1		174.3		174.5
2	3.65, dd, 12.9, 3.4	54.6	3.60, dd, 3.4, 13.0	54.3
3	2.26, dt, 15.4, 3.2	27.5	1.95, brd, 15.4	31.5
	2.07, ddd, 15.4, 12.9, 2.4		1.69, brm	
4	4.41, brq, 3.4	73.3	4.58, brs	71.8
5	4.01, brd, 2.7	63.4	2.28, brdd, 15.1, 2.4	26.6
			1.69, brm	
6	3.20, dd, 13.7, 2.2	45.3	3.10, ddd, 2.2, 4.7, 12.7	38.8
	3.17, dd, 13.9, 3.0		3.00, dt, 3.2, 13.2	

[a] 294 K. [b] 285 K.

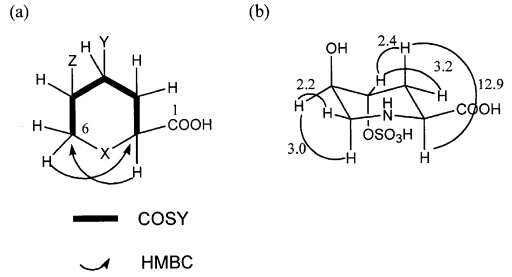

Figure 2. Key NMR correlations (a) and ^3J analysis (b) for **1**.

acid resonating at δ 3.7, appeared as a double doublet with J values of 12.9 and 3.4 Hz. A large coupling of 12.9 Hz between H-2 and one of the adjacent methylene protons clearly indicated that H-2 is axially oriented and that the piperidine ring is in a chair conformation. However, H-4 and H-5 were both assigned to be equatorial, because both H-4 and H-5 showed small J values to the adjacent methines ($^3J_{H3ax-H4}$ and $^3J_{H3eq-H4}$ = 3.2 and 2.4 Hz, respectively; and $^3J_{H6ax-H5}$ and $^3J_{H6eq-H5}$ = 3.0 and 2.2 Hz, respectively) (Figure 2b). Since the CD spectrum of **1** was nearly identical to that of (2*S*,4*S*)-4-sulfooxypiperidine-2-carboxylic acid (**2**), the absolute stereochemistry at C-2 was defined to be *S*. These data considered together secured the structure for **1** to be (2*S*,4*R*,5*R*)-5-hydroxy-4-sulfooxypiperidine-2-carboxylic acid.

An aqueous extract of *S. aurantium* was separated on a DE 52 anion exchange column. The active fraction was further separated on a BioGel P2 column. A fraction containing **2** was finally purified by a C30 reversed-phase HPLC to afford pure **2** (3.8×10^{-4}% of wet sample). The same separation scheme was applied to the extract of *A. carteri* to afford **2** also as the active principle. The molecular formula of **2**, $C_6H_{11}NO_6S$, was assigned on the basis of the HRFABMS and NMR data. Conventional NMR analysis (^{1}H, ^{13}C, COSY, HMQC, HMBC) revealed a 4-substituted piperidine-2-carboxylic acid structure for **2**, which left the remaining formula of HSO_4, and thus the sulfooxy group was assigned on C-4. The coupling pattern of H-2 (dd, 3.4 and 13.0 Hz) and H-4 (br s) suggested the respective axial and equatorial orientation of these protons. These data were assignable to the structure 4-sulfooxypiperidine-2-carboxylic acid, which was finally confirmed by direct comparisons (^{1}H NMR, TLC) with the synthetic sample provided by Professor Roberto Pellicciari at the University of Perugia. Both the sign and value of the optical rotation for **2** matched well with that for (2*S*,4*S*)-4-sulfooxypiperidine-2-carboxylic acid (*trans*-4-hydoroxypipecolic acid sulfate, *t*-HPIS) reported previously,[7] indicating that **2** has the same absolute stereochemistry as the reported compound.

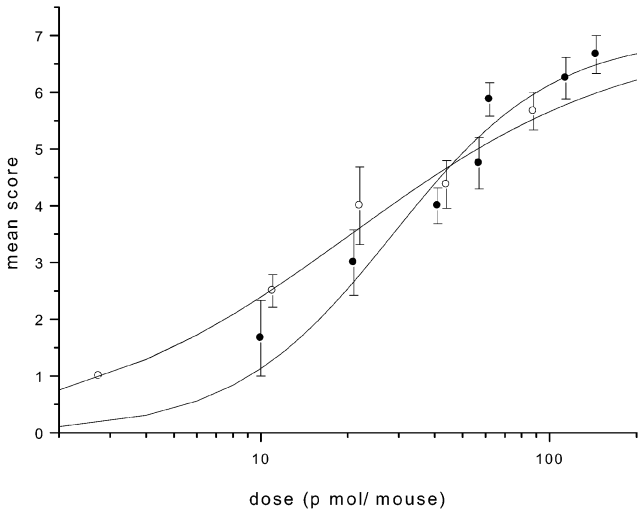

Figure 3. Dose-dependent behavioral changes in mice induced by icv administration of compound **1** (black circles) and natural compound **2** (open circles). ED_{50} values for **1** and **2** are 29 ± 3.0 and 20 ± 2.8 pmol/mouse, respectively.

In the present study, we observed that both **1** and **2** were potent convulsants in mice. Compound **2** was first reported from the seed of the legume *Pletophorum africanum*.[7] Later, its pharmacological properties were determined in vitro using three models: mice cortical wedge depolarization, the contraction of mice longitudinal muscle myenteric plexus preparation, and a radioligand binding assay to be a selective NMDA agonist.[8] The structural similarity of **1** and **2** suggested that **1** would also act toward the NMDA-type glutamate receptors, and the convulsant action of these compounds can be mediated by the NMDA receptors. We thus compared the biological actions of **1** and **2** both in vivo and in vitro. Intracerebroventricular (icv) injections of both **1** and **2** in mice induced convulsions in a dose-dependent manner. Upon icv injection of **1** (145 pmol/mouse), the mice immediately exhibited violent behaviors, including running, jumping, and tonic extension. Five animals out of six died within 60 min. At a lower dose (33 pmol/mouse), walking or hyperactive and stereotypical behaviors were the most noticeable symptoms, but these behaviors were transient in most animals and faded within 20−30 min. A dose−response relationship for **1** of between 10 and 145 pmol/mouse (n = 41) was established, in that the ED_{50} value was estimated to be 29 ± 3.0 pmol/mouse (Figure 3).

The injection of **2** also induced convulsant behaviors similar to **1**. The ED_{50} values for natural **2** (20 ± 3 pmol/mouse, n = 29, Figure 3) corresponded well with the synthetic sample (15 ± 5 pmol/mouse, n = 29, data not shown). It should be noted that synthetic (2*S*,4*R*)-derivative (*cis*-HPIS) did not show any noticeable convulsant activity at the highest dose tested (9 nmol/mouse). Since the behavioral profiles observed upon the injection of **1** or **2** were very similar to that induced by NMDA or TG (tetrazol-5-yl) glycine,[9] a highly selective NMDA-type agonist, the convulsions induced by **1** and **2** could be mediated by the NMDA receptors.[10]

The radioligand binding assay using rat cerebrocortical membrane preparations further confirmed that **1** acts on the NMDA receptors. Both compounds **1** and **2** displaced [^{3}H]CGP39653,[11] a selective NMDA-type ligand, in a dose-dependent manner with IC_{50} values of 83 ± 15 and 214 ± 20 nM, respectively (Figure 4). The potency of **1** was almost comparable to that of glutamate (IC_{50} = 69 ± 14 nM). However, neither of these compounds affected the bindings

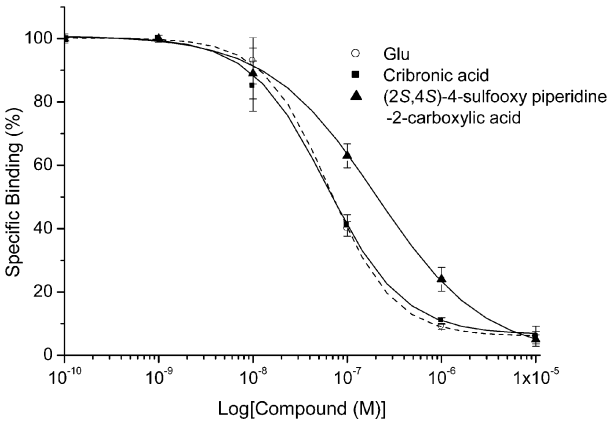

Figure 4. Inhibition of the [^{3}H]CGP39653 binding to the rat brain membrane by glutamate, cribronic acid (**1**), and (2*S*,4*S*)-4-sulfooxypiperidine-2-carboxylic acid (**2**). The IC_{50} values for glutamate, **1**, and **2** are 69 ± 14, 83 ± 15, and 214 ± 20 nM, respectively.

of [^{3}H] kainic acid and [^{3}H]AMPA (data not shown). These data indicated that cribronic acid (**1**), as in the case of **2**, is a selective NMDA-type glutamate receptor ligand and presumably is its agonist, with potent convulsant action in mice. An electrophysiological study to define the excitatory action of **1** against neuronal and recombinant receptors is underway and will be published elsewhere.

Pipecolic acid derivatives related to **1** and **2** are known as the constituents of many legume seeds, and their usefulness as a taxonomic marker has been described.[12] (2*S*,4*R*,5*R*)-4,5-Dihydoroxypipridine-2-carboxylic acid has previously been isolated from the seeds of *Brachystegia spiciformis*,[12] but its 4-*O*-sulfate derivative **1** has not been known.

Several marine-derived compounds that target the NMDA receptor have been reported. For example, the venomous peptides of fish-hunting snails, conotoxins T, G, and R, are NMDA antagonists,[13] and the cultured marine cyanobacterial products antillatoxin and kalikitoxin display cytotoxicity to cultured rat cerebellar granule neurons through NMDA receptor-mediated mechanisms.[14] Moreover, the NMDA receptor agonist activity was recorded in an extract of cultured bacteria associated with the marine sponge *Halichondria panicea*, although its active principles were not identified.[15] The NMDA itself was detected from the foot muscle of the blood shell *Scapharca broughtonii*.[16] Interestingly, nonfertilized eggs of the same organism contained a considerably high concentration of NMDA, but it was markedly decreased after fertilization up to 100-fold.[17] These findings, along with our results, suggest that marine organisms are an intriguing source of functionally and structurally interesting compounds that act toward mammalian glutamate receptors.

Experimental Section

General Experimental Procedures. The optical rotation was measured with a DIP 1000 digital polarimeter using a 10 × 0.35 mm cell. The NMR spectra were obtained on JEOL Lambda 400 and Varian Unity 600 spectrometers using D_2O as solvent. The ^{1}H chemical shift was reported in ppm relative to the HOD peak at δ 4.65 as the internal standard at the temperature indicated. The ^{13}C data were reported in ppm using a methanol-d_4 (add 20 μL) signal at δ 49.0 as the internal standard. FABMS was recorded on a JEOL JMS 700 instrument in the FAB mode using glycerol as matrix. The HR-FABMS data were measured with poly(ethylene glycol) 400 as the internal standard. The IR spectra were measured on a JASCO IR430 spectrophotometer. Authentic samples of synthetic *trans*- and *cis*-4-hydroxypipecolic acid sulfate were a gift from Professor Pellicciari at the University of Perugia. All commercially available agonists and antagonists were purchased form TOCRIS (UK).

Animal Materials. The sponges were identified by Dr. J. Hooper at the Queensland Museum, Australia, and voucher specimens of *C. olemda* (QMG306418), *S. aurantium* (QMG-314136), and *A. carteri* (QMG306987) were deposited at the museum.

Bioassays. Male ddY mice (15−20 g, 4−6 weeks old, Owada experimental animals, Iwate Japan) were used. Intracerebroventricular (icv) administrations were performed according to the previously described method.[4,18] The mouse behavior was observed for 1 h. The convulsant behaviors were graded in order of severity as follows: normal = 0; hypoactive = 1; rigid posture, vigilant staring, or occasional head scratching = 2; occasional circling, walking = 3; running, frequent circling = 4; wild running, jumping = 5; tonic extension = 6; death = 7. The most severe symptom observed in the session was assigned as the behavioral score for each mouse. More than three animals were used at each dose. The total number of mice used for each experiment is indicated in the result. The dose−response curves were generated using the computer software Origin (Microcal Inc.).

Receptor binding studies were performed according to the method previously reported.[4,11,19,20] Briefly, a synaptic membrane was prepared from Sprague−Dawley rats (200−220 g). Labeled [^{3}H]kainic acid, [^{3}H]AMPA, and [^{3}H]CGP39653[11] were used as the ligands for the kainic acid, AMPA, or NMDA binding site. The conditions for each binding assay were as follows (ligand, ligand concentration, incubation temperature, incubation time, buffer): [^{3}H]KA, 1 nM, 4 °C, 1 h, 100 mM Tris-HCl (pH 7.1); [^{3}H]AMPA, 5 nM, 4 °C, 1 h, 50 mM Tris-HCl (pH 7.4); [^{3}H]CGP39653, 2 nM, 4 °C, 1 h, 5 mM Tris-HCl (pH 7.7).

Isolation of Compound 1. The *C. olemda* specimen was collected by scuba at a lagoon in Palau in February 2000. A frozen sample (188 g, wet wt) was thawed and homogenized with water (300 mL). The aqueous extract was then lyophilized to give a crude extract (10 g). Gel filtration using Sephadex LH20 (5 × 60 cm) of the crude extract afforded bioactive fractions. The TLC (silica gel) of the active fractions indicated the presence of a common spot at the R_f value of 0.13 (butanol−acetic acid−water, 4:1:1), which show a characteristic blue color by ninhydrin. The combined active fractions were then separated by Biogel-P2 (2.5 × 110 cm) followed by HW 40 (Toyo, 1.5 × 160 cm) to give an amorphous solid. The recrystallization of the solid from 1:1 methanol−water afforded pure cribronic acid (**1**) as very fine needles (3.0 mg). HPLC (Develosil C30, Nomura Chemical Co., 0.2% acetic acid) t_R =18.5 min; $[\alpha]^{18}_D$ −16.3° (*c* 0.24, H_2O): IR (KBr) ν 3540, 3376, 3241, 2609, 2421, 1626, 1405, 1330, 1277, 1226, 1042, 1011, 947 cm^{-1}; NMR data (Table 1); HRFABMS *m/z* 242.0323 [calcd for $C_6H_{12}NO_7S$ $(M + H)^+$ 242.0335].

Isolation of Compound 2. The *S. aurantium* and *A. carteri* specimens were collected in Yap State, Micronesia, in November 1999. A frozen sample of *S. aurantium* (800 g) was homogenized and then centrifuged (10 000 rpm, 30 min). The lyophilized aqueous supernatant was separated on an anion exchange (Whatman DE52, 5.5 × 20 cm) column using a linear gradient from 0.01 N acetic acid to 0.05 N ammonium formate. Convulsant activity was obtained between the fractions eluted with 30−40% of the buffer solution. The bioactive fraction of the above ion exchange column was further separated by BioGel P2 followed by HPLC (Develosil C30, Nomura Chemical Co., 0.2% acetic acid) to give (2*S*,4*S*)-4-sulfooxypiperidine-2-carboxylic acid (**2**, 2.8 mg, t_R = 17.2−21.0 min) as a colorless amorphous solid: $[\alpha]^{18}_D$ +6.8° (*c* 0.22, H_2O) (ref 7 +6.5°); IR (KBr) ν 3465, 1629, 1403, 1245, 1054, 959 cm^{-1}; NMR data (Table 1); HRFABMS *m/z* 224.0216 [calcd for $C_6H_{12}NO_7S$ $(M + H)^+$, 224.0229].

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