Annonamine, a New Aporphine Alkaloid from the Leaves of *Annona muricata*

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Atypical Parkinsonism in the Caribbean Island Guadeloupe is thought to be associated with the consumption of plants of the Annonaceae family, especially *Annona muricata* (soursop). In this study, a new aporphine alkaloid named annonamine (1) was isolated from the leaves of *A. muricata* L. together with four known benzyloquinoline alkaloids (2–5). The structures of the isolated compounds were elucidated by the spectroscopic method.

Key words *Annona muricata*; Annonaceae; aporphine; alkaloid; annonamine

Parkinson's disease (PD) is a common neurodegenerative disorder. Over ten genes have been identified as causes of familial PD so far.¹⁾ However, in the case of idiopathic PD (accounting for over 90% of PD cases), endogenous and/or exogenous environmental factors are believed to be crucial for the onset of symptoms.^{2,3)} In 1999, Caparros-Lefebvre *et al.* suggested the high prevalence of atypical Parkinsonism in the Caribbean Island Guadeloupe was linked with consumption of Annonaceous fruits and herbal tea prepared from *Annona muricata* and *A. squamosa*.⁴⁾ In 2005, Höglinger *et al.* described the neurotoxic effects of annonacin, the most abundant acetogenin in *A. muricata*, which inhibited the complex I of the mitochondrial respiratory chain.⁵⁾ On the other hand, we have previously identified 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1BnTIQ) alkaloids as one of the endogenous neurotoxins that could induce PD in an animal model.⁶⁾ In this study, we have phytochemically investigated the leaves of the title plant in detail to characterize the chemical constituents especially on alkaloids, because the leaves of *A. muricata* are used as tea for cardiotonics and it has been known that *A. muricata* produced benzyloquinoline type alkaloids.^{7,8)} The present study describes the isolation and structural elucidation of a new aporphine alkaloid, annonamine (1) together with four known alkaloids (2–5). The neurotoxicities of these compounds (1–5) were also investigated against human neuroblastoma cell line, SH-SY5Y.

Air-dried leaves of *A. muricata* were extracted with MeOH three times by maceration. The combined MeOH extract was evaporated and partitioned with *n*-hexane, CHCl₃, EtOAc and 1-BuOH successively to give *n*-hexane, CHCl₃, EtOAc and 1-BuOH-soluble fractions, respectively. CHCl₃ and EtOAc-soluble fractions were combined because of the similarity of TLC pattern. The residue of 1-BuOH-soluble fraction and the combined mixture of CHCl₃ and EtOAc-soluble fractions were subjected to various kinds of column chromatography (CC) to yield five alkaloids (1–5) (Fig. 1).

Annonamine (1) was obtained as a colorless amorphous powder and the high-resolution electrospray-ionization time-of-flight mass spectroscopy (HR-ESI-TOF-MS) provided the positive-ion peak at *m/z*=296.1644, which was corresponding

to C₁₉H₂₂NO₂. The IR absorption at 3360 cm⁻¹ indicated the presence of hydroxy group. In the ¹³C-NMR spectrum, 12 *sp*² carbon signals were ascribable to two aromatic rings. The substitution manners of each ring were determined by a coupling pattern analysis of five aromatic protons, of which a singlet aromatic proton (δ_H 6.78), and four *ortho*- and *meta*-coupled protons (δ_H 8.32, 7.40, 7.37, 7.31) suggested the presence of penta-, and *ortho*-disubstituted aromatic rings, respectively (Table 1). Three downfield-shifted singlet methyl groups (δ_H 3.62 on δ_C 60.8, δ_H 3.42 on δ_C 54.2, and δ_H 3.08 on δ_C 43.8) were attributable to the *O*-methyl, *O*- or *N*-methyl, and *N*-methyl groups, respectively. In the heteronuclear multiple bond connectivity (HMBC) spectrum, δ_H 3.42 and δ_H 3.08 were correlated with each other, which indicated the both methyl groups (δ_H 3.42 on δ_C 54.2 and δ_H 3.08 on δ_C 43.8) were connected to the same hetero atom, *i.e.* nitrogen (Fig. 2). In addition, these two methyl groups were also correlated with both δ_C 70.8 and δ_C 62.8, which unveiled the presence of a quaternary amine in its structure (Fig. 2). The other methoxy group (δ_C 60.8) must be located on C-1, because of the relatively downfield-shifted chemical shift value caused by a steric effect of *ortho*-disubstitution. The contiguities of the remaining two methylenes (δ_C 62.8, 24.7), and a methylene and a methine (δ_C 70.8, 30.9) groups were also elucidated

Fig. 1. Structures of the Isolated Alkaloids

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Table 1. ^1H - and ^{13}C -NMR Data for **1**

	C	H
1	146.9	—
1a	128.6	—
1b	120.2	—
2	152.9	—
3	116.5	6.78 s
3a	126.2	—
4	24.7	α^* 3.02 m β^* 3.34 m
5	62.8	α^* 3.79 ddd (13, 7, 1) β^* 3.73 td (13, 5)
6a	70.8	4.56 dd (14, 4)
7	30.9	α^* 3.05 m β^* 3.37 m
7a	133.5	—
8	129.4	7.40 d (7)
9	129.5	7.31 td (7, 1)
10	129.2	7.37 brt (8)
11	129.1	8.32 dd (8, 1)
11a	132.5	—
O-Me	60.8	3.62 (3H, s)
N ⁺ -Me α^*	43.8	3.08 (3H, s)
N ⁺ -Me β^*	54.2	3.42 (3H, s)

Measured in CD_3OD . δ in ppm. *The orientations were determined by phase-sensitive nuclear Overhauser exchange spectroscopic analysis.

Fig. 2. Important Correlations on COSY (Bold Lines) and HMBC (Arrows)

by double-quantum-filtered ^1H - ^1H correlation spectroscopy (COSY) and hetero-nuclear multiple quantum coherence spectra (Fig. 2). The planer structure was elucidated as shown in Fig. 2 by the detailed analysis of 2D-NMR spectra. Finally, the absolute stereochemistry of C-6a was determined to be *R* by the circular dichroism (CD) spectrum.⁹⁾ Thus, the structure of **1** was elucidated as (*R*)-2-hydroxy-1-methoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de,g*]quinolin-6-ium.

PD is a common neurodegenerative disorder marked by progressive loss of melanized, catecholamine producing neurons in the midbrain (substantia nigra) and brain stem (locus ceruleus). Therefore, we next evaluated the neurotoxicity against the human neuroblastoma SH-SY5Y which is a well-characterized catecholaminergic cell line often used as a neuronal model in PD research. Among the tested compounds (**1**–**5**), compound **3** was the most active (IC_{50} for **1**: 195.8 ± 17.0 , **2**: 186.6 ± 29.4 , **3**: 34.6 ± 3.5 , **4**: 97.1 ± 11.2 , **5**: $202.9 \pm 9.9 \mu\text{M}$).

In this study, we have successfully isolated a new aporphine alkaloid together with four known benzyloquinoline alkaloids from the leaves of *A. muricata*. It is noteworthy that anonaine (**3**) has been reported to inhibit dopamine biosynthesis at $0.05 \mu\text{M}$ by mainly reducing tyrosine hydroxylase activity in rat stable pheochromocytoma cells, PC12.¹⁰⁾ In this study, we presented the moderate neurotoxicity of **3** against SH-SY5Y. Thus, anonaine (**3**) might affect the development of the idiopathic atypical Parkinson's disease in the Caribbean Island Guadeloupe by inhibiting the dopamine biosynthesis at lower dose and by the neurotoxicity at higher dose. We are now planning to investigate the detailed biological function of these alkaloids on the several protein molecules concerning for the development of Parkinson's disease.

Experimental

General Experimental Procedures Silica gel CC was performed on silica gel 60 (Merck, Darmstadt, Germany), and reversed-phase [octadecyl silica gel (ODS)] open CC (RPCC) on Cosmosil 75C₁₈-OPN (Nacalai Tesque, Kyoto, Japan) ($\Phi=4\text{ cm}$, $L=20\text{ cm}$). HPLC was performed on ODS (Cosmosil; Nacalai Tesque, Japan; $\Phi=10\text{ mm}$, $L=250\text{ mm}$), and the eluate was monitored with a refractive index monitor.

Optical rotations were measured on a JASCO P-1030 polarimeter. IR spectrum was measured on a Horiba FT-710 Fourier transform infrared spectrophotometer. UV spectrum was obtained on a JASCO V-520 UV/Vis spectrometer. NMR spectra were taken on a JEOL ECA 600 spectrometer at 600 MHz for ^1H and 150 MHz for ^{13}C , respectively, with tetramethylsilane as an internal standard. Positive-ion HR-ESI-TOF-MS was recorded on an Applied Biosystem QSTAR XL spectrometer. CD spectra were obtained on a JASCO J-720 spectropolarimeter. VersaMax (Molecular Devices, Sunnydale, CA, U.S.A.) was used as a microplate reader.

Plant Material Leaves of *A. muricata* were collected in Okinawa, Japan, in November, 2004, and a voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Graduate School of Biomedical Sciences, Hiroshima University (No. 04-a.m.-Okinawa-1105).

Extraction and Isolation The air-dried leaves of *A. muricata* (520 g) were extracted with MeOH (2 L) three times by maceration. The MeOH extracts were combined and evaporated to dryness to afford a viscous gummy material (78.2 g). This residue was suspended in 1.5 L of H_2O and extracted with equal volume of *n*-hexane, CHCl_3 , EtOAc and 1-BuOH successively to afford 13.4, 34.9, 1.1 and 5.0 g of the residues, respectively. The remaining H_2O layer was concentrated to furnish an H_2O -soluble fraction (23.9 g). The 1-BuOH soluble fraction (5.0 g) was applied to a silica gel CC ($\Phi=2.5\text{ cm}$, $L=50\text{ cm}$) using a stepwise gradient elution with increasing amounts of MeOH in CHCl_3 [CHCl_3 (1 L), CHCl_3 -MeOH (20:1, 750 mL), (10:1, 750 mL), (5:1, 750 mL), and (3:1, 750 mL)], (CHCl_3 :MeOH: H_2O =15:6:1, 750 mL), and (MeOH, 750 mL). The residue (1.13 g) of the CHCl_3 :MeOH: H_2O =15:6:1 eluate obtained on silica gel CC was subsequently purified by HPLC (ODS) with 20% $\text{CH}_3\text{CN}/0.1\%$ trifluoroacetic acid (TFA) to afford **1** (25.8 mg) from the peak at 20.2 min (flow rate: 3.0 mL/min).

The combined residue of CHCl_3 and EtOAc soluble fractions (36.0 g) was applied to a silica gel CC ($\Phi=5.8\text{ cm}$, $L=38\text{ cm}$) using a stepwise gradient elution with increasing amounts of

MeOH in CHCl_3 [CHCl_3 (3 L), CHCl_3 -MeOH (100:1, 3 L), (50:1, 3 L), (30:1, 3 L), (20:1, 3 L) (10:1, 3 L), (5:1, 3 L), and (2:1, 3 L)], and (MeOH, 5 L). The residue (7.36 g) of the CHCl_3 :MeOH=10:1 eluate obtained on silica gel CC was subsequently subjected to RPCC with a stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90, 100% MeOH, 300 mL containing with 0.1% TFA). The residues (190 mg) obtained from 50% MeOH eluate was purified by HPLC (ODS) with 30% acetone/0.1% TFA to afford **5** (10.3 mg) from the peak at 10.4 min (flow rate: 2.8 mL/min). The residue (5.95 g) of the CHCl_3 :MeOH=5:1 eluate obtained on silica gel CC was subsequently subjected to RPCC with a stepwise gradient elution with increasing amounts of MeOH in H_2O (30, 50, 70, 90, 100% MeOH, 300 mL containing with 0.1% TFA). The residues (170 mg) obtained from 30% MeOH eluate was purified by HPLC (ODS) with 20% CH_3CN /0.1% TFA to afford **2** (4.9 mg) from the peak at 17.9 min (flow rate: 2.5 mL/min). The residues (220 mg) obtained from 50% MeOH eluate was purified by HPLC (ODS) with 30% acetone/0.1% TFA to afford **4** (7.0 mg) and **3** (5.3 mg) from the peaks at 5.2 and 14.6 min, respectively (flow rate: 2.8 mL/min).

The known compounds (**2**–**5**) were identified by comparison of spectroscopic data with those reported in the literature as follows. (*S*)-Norcorydine (**2**)¹¹ {ref. $[\alpha]_{\text{D}}^{23} +190$ ($c=0.2$, CHCl_3)}, $[\alpha]_{\text{D}}^{22} +151$ ($c=0.13$, CHCl_3), CD $\Delta\epsilon$ (nm): -7.81 (268), $+43.0$ (234) ($c=3.98 \times 10^{-5}$ M, MeOH), (*R*)-anonaine (**3**)¹² {ref. $[\alpha]_{\text{D}}^{20} -48$ ($c=0.1$, CHCl_3)}, $[\alpha]_{\text{D}}^{22} -34.9$ ($c=0.10$, CHCl_3), CD $\Delta\epsilon$ (nm): $+11.4$ (271), -52.5 (231) ($c=3.08 \times 10^{-5}$ M, MeOH), (*R*)-4'-*O*-methylcoclaurine (**4**)¹³ $[\alpha]_{\text{D}}^{22} +14.5$ ($c=0.19$, CHCl_3), CD $\Delta\epsilon$ (nm): -1.09 (288), -1.75 (230) ($c=3.18 \times 10^{-5}$ M, MeOH), (*R*)-*O,O*-dimethylcoclaurine (**5**)¹⁴ {ref. $[\alpha]_{\text{D}}^{25} +15.7$ ($c=0.4$, CHCl_3)}, $[\alpha]_{\text{D}}^{21} +10.9$ ($c=0.16$, CHCl_3), CD $\Delta\epsilon$ (nm): -1.15 (288), -2.00 (232) ($c=2.59 \times 10^{-5}$ M, MeOH). Other chemical constituents including megastigmane glucosides have been isolated previously and published elsewhere.¹⁵

Annonamine (**1**): Amorphous powder; $[\alpha]_{\text{D}}^{22} -12.5$ ($c=1.70$, MeOH); IR ν_{max} (film) cm^{-1} : 3360, 2964, 2937, 1680, 1431, 1354, 1259, 1200, 1183, 1136, 1007, 798; UV λ_{max} (MeOH) nm (log ϵ): 310sh (3.16), 283sh (3.76), 273 (3.90), 229 (3.99), 211 (4.15); ^1H - and ^{13}C -NMR (CD_3OD): Table 1; CD $\Delta\epsilon$ (nm): $+0.86$ (274), -6.15 (233) ($c=5.43 \times 10^{-5}$ M, MeOH); HR-ESI-TOF-MS (positive-ion mode) m/z : 296.1644 $[\text{M}]^+$ (Calcd for $\text{C}_{19}\text{H}_{22}\text{NO}_2$: 296.1645).

Cytotoxicity Assay The cytotoxicity assay was performed using human neuroblastoma cell line, SH-SY5Y by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. In brief, cells (5×10^3 cells/100 μL complete medium) were cultured in 96-well plates with different concentrations of the test compounds (200–6.25 μM ; 1% of dimethyl sulfoxide (DMSO) was present as a vehicle in all the experiments) for 72 h at 37°C. Then the culture supernatants were replaced with

100 μL of a MTT solution comprising 0.5 mg/mL of MTT in complete medium. After 3 h incubation at 37°C, the precipitate was dissolved in 100 μL of DMSO. The optical density values for each well were measured at 520 nm with a microplate reader.

The cytotoxicity was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (A_{\text{sample}} - A_{\text{background}}) / (A_{\text{DMSO}} - A_{\text{background}})] \times 100$$

where A_{DMSO} is the absorbance of the control reaction mixture (containing DMSO and all reagents except for the test compound).

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