NOTES

Isolation and Structure Elucidation of Novel Neuronal Cell Protecting Substances, Carbazomadurins A and B Produced by *Actinomadura madurae*

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It was recently reported that the extracellular concentration of L-glutamate, an excitatory amino acid, acting as a major neurotransmitter in the brain, increases after brain ischemic attack[1,2]. In addition, neuronal cell death is suggested to be related to excessive excitement of glutamate receptors and inhibition of the uptake of cystine, a precursor of glutathione which is the major reducing substance in the cells[3,4]. Therefore, antagonists for glutamate receptors and radical scavengers may be useful candidates to cure brain ischemia injury. In the course of our screening for substances protecting central nervous system from glutamate toxicity using neuronal hybridoma N18-RE-105 cells as an *in vitro* ischemia model, we isolated several compounds[5~11]. Further investigation has resulted in the isolation of novel compounds designated carbazomadurins A and B (**1** and **2**, respectively, Fig. 1).

The producing organism of carbazomadurins, identified as *Actinomadura madurae* 2808-SV1, was cultivated in 500-ml Erlenmeyer flasks containing 100 ml (totally 3 liters) of the medium consisting of glycerol 1.5%, Pharmamedia 1.5%, L-glutamate 0.5%, NaCl 0.3%, pH 7.4 before sterilization at 27°C for 5 days on a rotary shaker. The mycelial acetone extract was concentrated to a small volume and the aqueous residue was extracted with EtOAc. The solvent layer was dried over Na_2SO_4 and concentrated to dryness in the dark to give an oily brownish residue. This material was subjected to a silica gel column packed with *n*-hexane-EtOAc-MeOH (20 : 5 : 1), and eluted with the same solvent system in the dark. Further purification of the active eluate by HPLC using PEGASIL ODS (Senshu-Pak 20 i.d. × 250 mm) developed with 60% CH_3CN gave pure **1** and **2** (1.6 mg and 2.0 mg, respectively).

The physico-chemical properties of **1** and **2** are summarized in Table 1. Both **1** and **2** showed identical UV and visible spectra, which resembled those of carbazole compounds such as epocarbazolins[12] and carbazomycin B[13]. The molecular formulae of **1** and **2** were established by high-resolution FAB-MS as

Fig. 1. Structure of carbazomadurins A (**1**) and B (**2**).

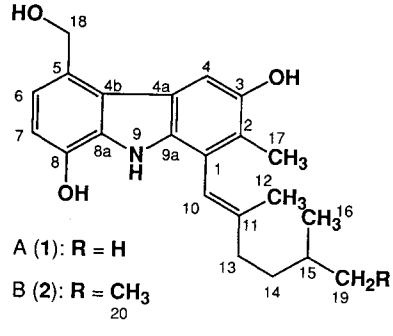


Table 1. Physico-chemical properties of carbazomadurins A (**1**) and B (**2**).

	A	B
Appearance	Pale yellow powder	Pale yellow powder
MP (°C)	108~110 (dec.)	108~110 (dec.)
Molecular formula	$C_{22}H_{27}NO_3$	$C_{23}H_{29}NO_3$
HRFAB-MS found:	353.2015 $(M)^+$	367.2158 $(M)^+$
calcd:	353.1991	367.2148
$[\alpha]_D^{24}$		+4° (*c* 0.05, MeOH)
UV λ_{max}^{MeOH} nm (ε)	237 (31,700), 250 (25,800, sh), 289 (7,200, sh), 299 (9,400), 345 (6,100), 357 (6,600)	237 (35,700), 249 (29,100, sh), 289 (7,400, sh), 299 (10,400), 347 (6,500), 357 (7,300)
UV $\lambda_{max}^{MeOH+NaOH}$ nm (ε)	236 (30,200), 255 (24,000), 303 (8,000), 362 (6,100)	236 (33,600), 255 (27,300), 304 (8,100), 363 (6,600)
IR ν_{max} (KBr) cm^{-1}	3440 (br), 1630, 1585, 1440, 1380	3440 (br), 1620, 1580, 1440, 1380

Table 2. ^{13}C and ^{1}H chemical shifts of carbazomadurins A (**1**) and B (**2**).

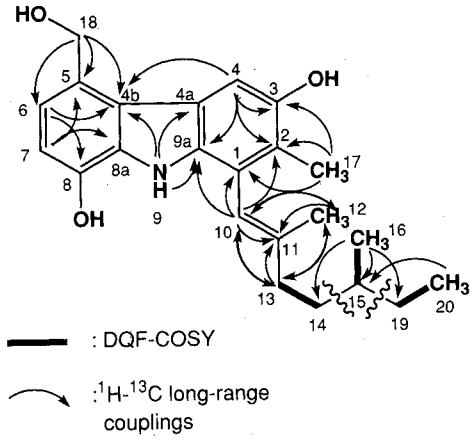
No.	A		B		No.	A		B	
	δ_C	δ_H	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
1	122.4		122.1		11	142.8		142.5	
2	122.4		122.1		12	18.0	1.55	17.7	1.56
3	150.0		149.7		13	37.8	2.34	37.1	2.35
4	107.0	7.59	106.6	7.59	14	38.0	1.55	35.3	1.70, 1.46
4a	121.5		121.1		15	28.6	1.69	34.7	1.50
4b	123.6		123.2		16	22.9	0.99	11.4	0.99
5	128.4		128.1		17	13.6	2.26	13.2	2.26
6	118.9	6.91	118.5	6.92	18	63.8	5.01	63.4	5.01
7	109.7	6.72	109.3	6.72	19	22.9	0.99	29.7	1.48, 1.26
8	143.0		142.7		20			19.1	0.94
8a	130.6		130.2		9-NH		8.92		8.89
9a	133.7		133.3		*OH		7.80		7.80
10	120.4	6.43	120.1	6.43	*OH		3.90		3.90

$C_{22}H_{27}NO_3$ and $C_{23}H_{29}NO_3$, respectively. The ^{1}H and ^{13}C NMR spectral data are shown in the Table 2. Their structures were elucidated as follows.

The DQF-COSY and HMBC spectra of **2** revealed the 1,2,3,4-tetrasubstituted benzene ring with an alkyl side chain as shown in Fig. 2. In the DQF-COSY spectrum of **2**, an allylic methylene proton 13-H (2.35 ppm) was coupled to adjacent methylene protons 14-H (1.70 ppm, 1.46 ppm), while a methyl proton 16-H (0.99 ppm) was coupled to a methine proton 15-H (1.50 ppm). Another methyl proton 20-H (0.94 ppm) was coupled to methylene protons 19-H (1.48 ppm and 1.26 ppm). In the HMBC spectrum of **2**, the methylene proton 13-H was long-rage coupled to two sp^2 carbons C-10 (120.1 ppm) and C-11 (142.5 ppm) together with a methyl carbon C-12 (17.7 ppm). In addition, an allyl methyl proton 12-H (1.56 ppm) was long-range coupled to C-10, C-11 and C-13 (37.1 ppm). Thus, the sequence from C-11 to C-13 was revealed as shown in Fig. 2. The connectivities between C-14, C-15 and C-19 were established by observing ^{1}H-^{13}C long-range couplings from 16-H to C-14 (35.3 ppm), C-15 (34.7 ppm) and C-19 (29.7 ppm). These results together with other long-range couplings established the existence of the 2,5-dimethylheptene side chain moiety as shown in Fig. 2.

Since the UV spectrum of **2** suggested the presence of a carbazole nucleus, the remaining six sp^2 carbons except for C-17 (13.2 ppm) and C-18 (63.4 ppm) are considered to consist the carbazole skeleton. In the HMBC spectrum of **2**, an aromatic proton 4-H (7.59 ppm) was long-range coupled to aromatic carbons C-2 (122.1 ppm), C-3 (149.7 ppm), C-4b (123.3 ppm) and C-9a (133.3 ppm). In addition, long-range couplings from a methyl proton

Fig. 2. NMR analyses of carbazomadurin B (**2**).



17-H (2.26 ppm) to aromatic carbons C-1 (122.1 ppm), C-2 and C-3, and from an olefinic proton 10-H (6.43 ppm) to C-1, C-2 and C-9a established the positions of the methylated heptenyl side chain and the aromatic methyl residue on the benzene ring as shown in Fig. 2. A hydroxymethyl proton 18-H (5.01 ppm) was long-range coupled to C-4b, C-6 (118.5 ppm) and C-5 (128.1 ppm) which in turn long-range coupled to an aromatic proton 7-H (6.72 ppm). Thus, the hydroxymethyl moiety is deduced to be substituted at the C-5 position. By comparing the ^{13}C chemical shifts of aromatic carbons with those of epocarbazolins and by analyzing the long-range couplings from an exchangeable proton 9-NH (8.89 ppm) to C-4a (121.1 ppm), C-4b and C-9a, it was concluded that the nitrogen atom was connected to both C-8a (130.2 ppm) and C-9a. The remaining two hydroxyl residues were substituted at the C-3 and C-8 (142.7 ppm)

positions. These data revealed the structure of **2** as shown in Fig. 2.

The stereochemistry of the C_{10}=C_{11} double bond in the side chain of **2** was determined to be *E* by the NOE between 10-H and 13-H as well as by ^{13}C chemical shift of the methyl carbon C-12 (17.7 ppm) as shown in Fig. 2.

The structure of **1** was elucidated by comparing NMR spectral data with those of **2**. The NMR data of **1** was identical with those of **2** except for the signals due to the side chain. Detailed analysis of the DQF-COSY and HMBC spectrum of **1** proved the presence of an isoamyl residue in **1**. The stereochemistry of the C_{10}=C_{11} double bond in **1** was also determined to be *E* according to the ^{13}C chemical shift of methyl carbon C-12 (18.0 ppm). Thus, the structure of **1** was determined to be that shown in Fig. 1.

In the evaluation system we employed, **1** and **2** suppressed effectively the toxicity of L-glutamate in N18-RE-105 cells with EC_{50} values 97 nM and 84 nM, respectively. Since the carbazole nucleus, which constitutes the chromophore of **1** and **2**, was reported to exhibit potent antioxidative activity, we also examined the antioxidative activity of **1** and **2** by observing the inhibitory effect against buthionine sulfoximine (BSO^{14}), an inhibitor of glutathione synthesis) toxicity. It was reported that L-glutamate toxicity in N18-RE-105 cells was mediated by inhibiting the uptake of cystine, which led to the loss of intracellular reducing agent glutathione and the accumulation of intracellular peroxides. As expected, **1** and **2** also showed a protective effect from the BSO toxicity with identical EC_{50} values to those in the glutamate toxicity. These data suggested that the mode of action of carbazomadurins was based on the antioxidative activity. Further studies on the detailed biological activities are now under way.

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