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Neuroprotectins A and B, Bicyclohexapeptides Protecting Chick Telencephalic Neurons from Excitotoxicity

II. Structure Determination

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In the course of our search for neuroprotective agents of microbial origin against kainate-induced neurotoxicity, we have succeeded in the isolation of two new bicyclohexapeptides, neuroprotectins A and B, together with a known compound, complestatin, from the fermentation broth of *Streptomyces* sp. Q27107. They are closely related in structure to complestatin and possess an oxindolylalanine moiety in place of the tryptophan residue present in complestatin.

In the preceding paper[1], we described the fermentation, isolation, physico-chemical properties and neuronal cell protecting activity of neuroprotectins A (**1**) and B (**2**, Fig. 1), which were isolated from the culture broth of *Streptomyces* sp. Q27107. Neuroprotectins A and B completely protected the primary cultured chick telencephalic cells from glutamate- and kainate-induced neurotoxicities. Compounds **1** and **2** possess an oxindolylalanine moiety in their structure, which is different from those of complestatin[2] and chloropeptin[3,4], an artefact formed by acid treatment of complestatin. In this paper, we report the structure elucidation of **1** and **2**.

Results and Discussion

Structure Determination

Structure of Neuroprotectin A (1)

The structure of **1** was determined to be an aromatic peptide by various one- and two-dimensional NMR experiments including ^{1}H NMR, ^{13}C NMR, DEPT, DQF-COSY, HMQC and HMBC. The ^{1}H and ^{13}C NMR data are summarized in Table 1. In the ^{1}H NMR spectra measured in DMSO-d_6 or CD_3OD, nine exchangeable protons were observed in the down-field region between 7.10 to 10.60 ppm due to hydroxyl or amide protons which were quenched by the addition of D_2O. Eighteen aromatic methine protons appeared in aromatic region between 6.73

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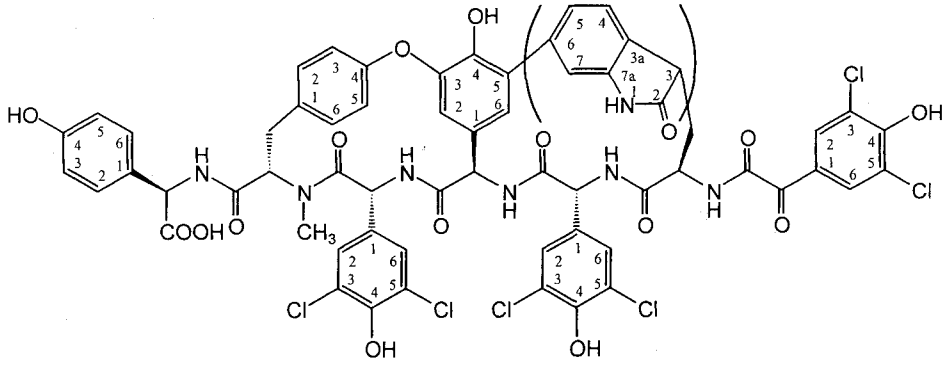
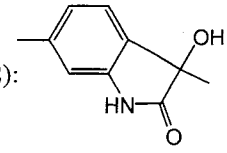
Fig. 1. Structures of neuroprotectins, complestatin and chloropeptin.

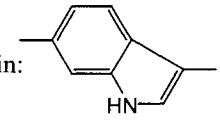


Neuroprotectin A (**1**)

Neuroprotectin B (**2**):



Complestatin:



Chloropeptin:

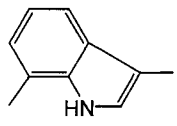
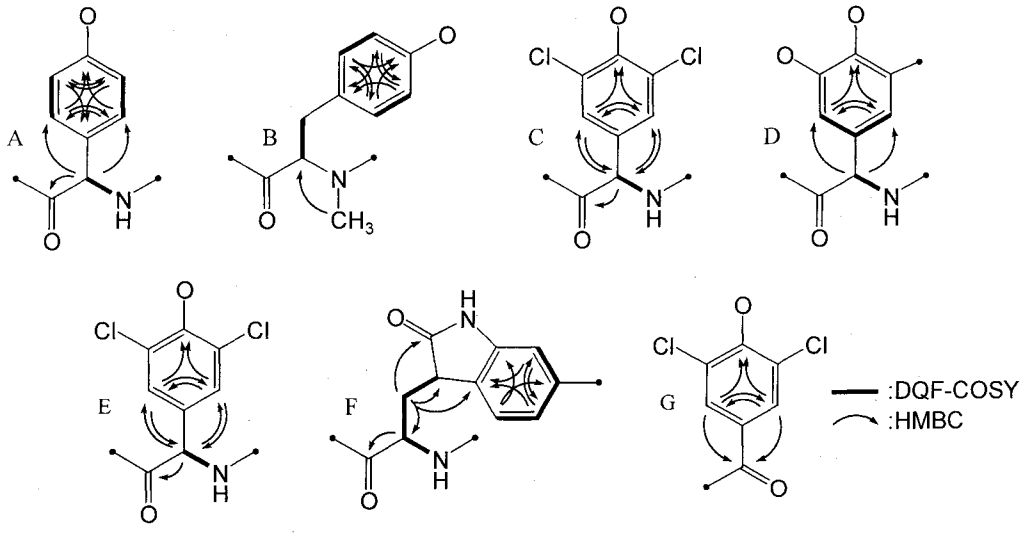


Fig. 2. Partial structures of neuropeptin A elucidated by DQF-COSY and HMBC experiments.



to 7.92 ppm in addition to *meta*-coupled aromatic protons at 5.76 and 5.68 ppm. Furthermore, six methine protons assignable to α-methine protons of peptides were observed together with two methylene protons at 3.02, 3.06 and 1.98 ppm and *N*-methyl protons at 2.99 ppm. The ^{13}C NMR and DEPT spectra revealed the presence of a methyl, two methylenes, 7 sp^3 methines and 19 sp^2 methines, 16 sp^2 quaternary carbons, 7 oxygenated sp^2 quaternary carbons

Table 1. ^{1}H and ^{13}C NMR spectral data of neuroprotectin A in DMSO-d_6.

Positions	δ_H	δ_C	Positions	δ_H	δ_C
A (4-Hydroxyphenylglycine)			E (3,5-Dichloro-4-hydroxyphenylglycine)		
αCH	5.05 (1H, d, *J*=6.0)[a]	55.8	αCH	5.54 (1H, d, *J*=8.0)	54.2
1		127.7	1		130.9
2,6	7.09 (2H, d, *J*=8.4)	128.2	2,6	7.02 (2H, s)	126.2
3,5	6.74 (2H, d, *J*=8.4)	115.3	3,5		121.9
4		157.2	4		148.5
CO		171.4	4-OH	10.02 (1H, br. s)	
NH	8.46 (1H, d, *J*=6.0)		CO		170.7
			NH	7.10 (1H, d, *J*=8.0)	
B (*N*-Methyltyrosine)					
αCH	5.08 (1H, m)	61.2	F (β-3-Oxindolylalanine)		
βCH_2	3.02 (2H, m)	35.0	αCH	3.48 (1H, m)	48.1
1		134.1	βCH_2	3.06 (1H, m)	30.0
2	7.17 (1H, m)	130.3		1.98 (1H, d, *J*=12.3)	
3	7.15 (1H, m)	121.4	1	10.60 (1H, br. s)	
4		156.0	2		178.6
5	6.76 (1H, m)	122.9	3	3.67 (1H, t, *J*=3.0)	43.8
6	7.78 (1H, dd, *J*=9.0, 2.0)	131.6	3a		125.9
CO		168.5	4	7.09 (1H, m)	125.3
$N\text{-}CH_3$	2.99 (3H, s)	31.2	5	6.73 (1H, m)	123.1
			6		141.9
C (3,5-Dichloro-4-hydroxyphenylglycine)			7	6.75 (1H, m)	110.7
αCH	5.18 (1H, d, *J*=6.0)	51.6	7a		142.6
1		131.2	CO		167.1
2,6	7.36 (2H, s)	127.0	NH	9.62 (1H, d, *J*=7.8)	
3,5		122.0			
4		148.7	G (2-(3,5-Dichloro-4-hydroxyphenyl)-2-oxoacetic acid)		
4-OH	10.11 (1H, br. s)				
CO		169.3	αCO		184.1
NH	8.92 (1H, d, *J*=6.0)		1		128.5
			2,6	7.92 (2H, s)	130.6
D (3,4-Dihydroxyphenylglycine)			3,5		122.4
αCH	5.69 (1H, d, *J*=8.9)	55.1	4		156.0
1		127.1	4-OH	9.49 (1H, br. s)	
2	5.76 (1H, d, *J*=2.0)	112.9	CO		163.4
3		149.5			
4		141.0			
4-OH	9.48 (1H, br. s)				
5		130.2			
6	5.68 (1H, d, *J*=2.0)	125.9			
CO		167.6			
NH	8.55 (1H, d, *J*=8.9)				

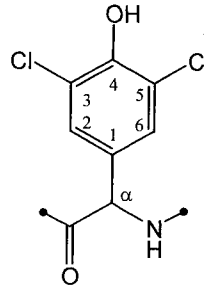
NMR spectra were measured on a JEOL JNM-A500 spectrometer, with ^{1}H NMR at 500 MHz and with ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as an internal standard.
[a]Proton resonance integration, multiplicity and coupling constants (*J*=Hz) in parenthesis.

and 9 carbonyl carbons. The HMQC spectrum[5] revealed the one-bond ^{1}H-^{13}C connectivities and the presence of five symmetrical aryl methines at 7.92 (s), 7.36 (s), 7.09 (d), 7.02 (s) and 6.74 (d) ppm. The above ^{1}H and ^{13}C NMR spectra were closely related to those of complestatin, suggesting that **1** was a peptidic compound composed of aromatic amino acids.

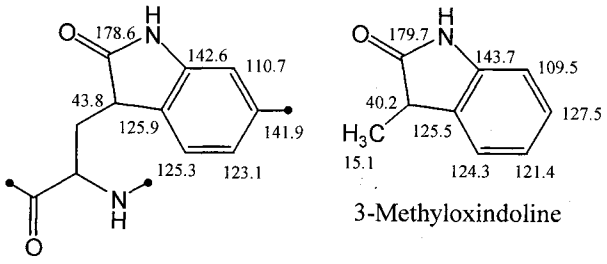
The partial structures A to G consisting of amino acid

residues were established by the DQF-COSY[6] and HMBC[7] experiments, as shown in Fig. 2. The 3,5-dichloro-4-hydroxyphenyl moieties assignable to partial structures C, E and G, and an oxindoline residue F were confirmed by comparison of the ^{13}C chemical shifts with those of 3,5-dichloro-4-hydroxyphenyl groups in complestatin and 3-methyloxindoline. These chemical shift values for neuroprotectin A were in good agreement with those of the corresponding carbons of the 3,5-dichloro-4-hydroxyphenyl moiety and 3-methyloxindoline as shown in Fig. 3. These partial structures were connected by detailed interpretation of the HMBC spectrum, which showed the correlations between the carbonyl carbon and amide proton of the neighboring amino acid as shown in Fig. 4. In addition to the hexapeptide sequence, the long-range correlations from H-6 (δ_H 5.68) in partial unit D to the quaternary carbon C-6 (δ_C 141.9) of the partial unit F and from the methine protons H-5 (δ_H 6.73) and H-7 (δ_H 6.75) in partial unit F to the quaternary aromatic carbon C-5 (δ_C 130.2) in partial unit D established a 16-membered peptidic macrocycle. Further structural information for **1** was obtained from the comparison of ^1H and ^{13}C NMR spectral data with those of complestatin. Non-equivalent proton and carbon chemical shifts of 1,4-disubstituted benzene for the partial unit B suggested that the partial units B and D should be bridged by an ethereal linkage as shown in the cases of complestatin and kistamicins[8]. Finally, the remaining subunit G was deduced to be connected to the amide carbonyl carbon at 163.4 ppm. The structure of **1** was quite similar to that of complestatin, the difference being that tryptophan in complestatin was replaced with β-3-oxindolylalanine in **1**.

Fig. 3. ^{13}C chemical shifts of the 3,5-dichloro-4-hydroxyphenyl moiety and oxindole in neuroprotectin A and known compounds.



Partial Str.	C	E	reported values
α	51.6	54.2	51.5 ~ 55.2
1	131.2	130.8	131.0 ~ 132.2
2	127.0	126.1	126.7 ~ 127.2
3	122.0	121.9	121.7 ~ 122.1
4	148.7	148.5	148.1 ~ 148.8
5	122.0	121.9	121.7 ~ 122.1
6	127.0	126.1	126.7 ~ 127.2



Structure of Neuroprotectin B (2)

The physico-chemical properties and ^1H NMR spectrum of **2** were very similar to those of neuroprotectin A and complestatin. **2**, however, had an additional oxygen atom than **1**. The structure of **2** was also assigned on the basis of the NMR spectral analysis. In the ^1H NMR spectra measured in DMSO-d_6 (Table 2), **2** showed one additional exchangeable proton at 6.21 ppm in addition to 9 hydroxyl or amide protons found in **1**. Other ^1H signals were well matched to those of **1**. The ^{13}C NMR and DEPT spectra of **2** were very similar to those of **1** except that an oxygenated quaternary carbon at 75.4 ppm in **2** replaced a methine carbon (C-3 of F) at 43.8 ppm in **1**. Further structural

Fig. 4. HMBC correlations between the partial structures A~G for neuroprotectin A.

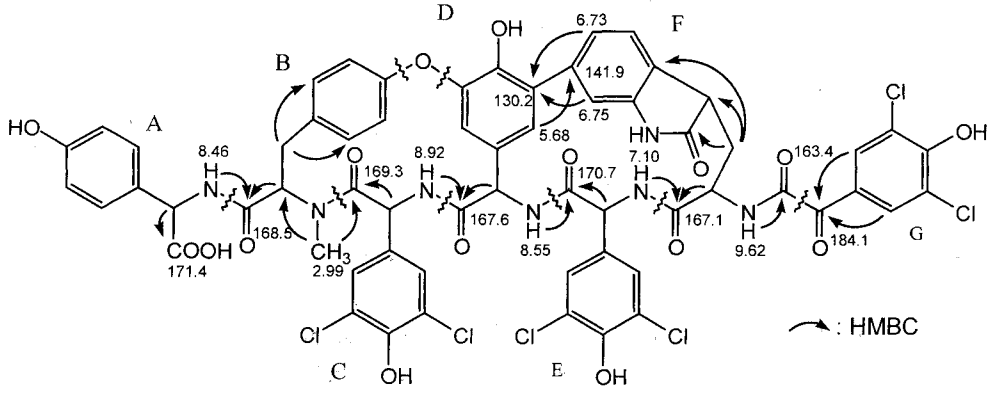


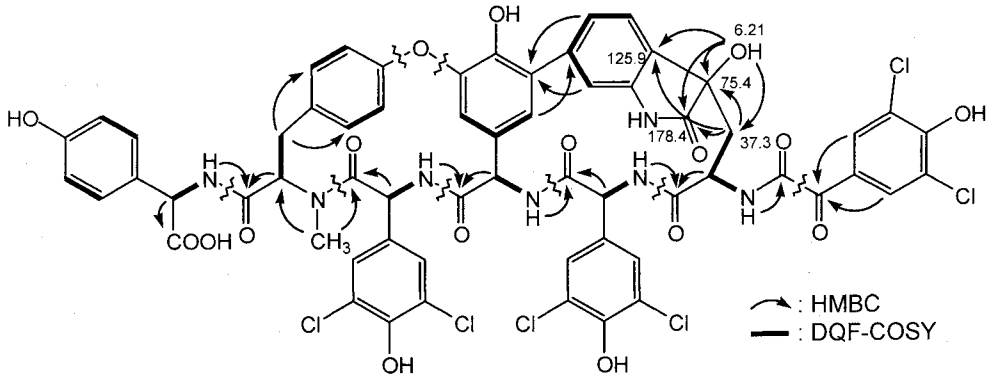
Table 2. ^{1}H and ^{13}C NMR spectral data of neuroprotectin B in DMSO-d_6.

Positions	δ_H	δ_C	Positions	δ_H	δ_C
A (4-Hydroxyphenylglycine)			E (3,5-Dichloro-4-hydroxyphenylglycine)		
αCH	5.05 (1H, d, *J*=6.0)[a]	55.8	αCH	5.55 (1H, d, *J*=9.0)	54.2
1		127.7	1		130.8
2,6	7.09 (2H, d, *J*=8.4)	128.2	2,6	7.00 (2H, s)	126.1
3,5	6.74 (2H, d, *J*=8.4)	115.3	3,5		121.9
4		157.2	4		148.5
CO		171.4	4-OH	10.01 (1H, br. s)	
NH	8.45 (1H, d, *J*=6.0)		CO		170.6
			NH	7.16 (1H, d, *J*=9.0)	
B (*N*-Methyltyrosine)					
αCH	5.08 (1H, m)	61.2	F (β-3-(3-Hydroxyoxindolyl)alanine)		
βCH_2	3.02 (2H, m)	34.7	αCH	3.49 (1H, m)	47.2
1		134.1	βCH_2	3.00 (1H, m)	37.3
2	7.17 (1H, m)	130.4		1.91 (1H, d, *J*=13.0)	
3	7.14 (1H, m)	121.5	1	10.54 (1H, br. s)	
4		155.8	2		178.4
5	6.78 (1H, dd, *J*=8.0, 2.0)	122.9	3		75.4
6	7.79 (1H, dd, *J*=8.0, 2.0)	131.7	3-OH	6.21 (1H, br. s)	
CO		168.5	3a		125.9
N-CH_3	2.98 (3H, s)	31.2	4	7.12 (1H, d, *J*=8.0)	124.6
			5	6.75 (1H, d, *J*=8.0)	123.3
C (3,5-Dichloro-4-hydroxyphenylglycine)			6		143.0
αCH	5.18 (1H, d, *J*=6.0)	51.6	7	6.71 (1H, m)	110.7
1		131.2	7a		140.6
2,6	7.36 (2H, s)	127.0	CO		166.8
3,5		122.0	NH	9.52 (1H, d)	
4		148.7			
4-OH	10.11 (1H, br. s)		G (2-(3,5-Dichloro-4-hydroxyphenyl)-2-oxoacetic acid)		
CO		169.3	αCO		184.1
NH	8.92 (1H, d, *J*=6.0)		1		128.5
			2,6	7.89 (2H, s)	130.5
D (3,4-Dihydroxyphenylglycine)			3,5		122.8
αCH	5.72 (1H, d, *J*=9.0)	54.9	4		156 0
1		127.4	4-OH	9.49 (1H, br. s)	
2	5.75 (1H, d, *J*=2.0)	112.9	CO		163.4
3		149.5			
4		141.0			
4-OH	9.49 (1H, br. s)				
5		130.2			
6	5.68 (1H, d, *J*=2.0)	125.4			
CO		167.6			
NH	8.56 (1H, d, *J*=9.0)				

NMR spectra were measured on a JEOL JNM-A500 spectrometer, with ^{1}H NMR at 500 MHz and with ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as an internal standard.
[a]Proton resonance integration, multiplicity and coupling constants (*J*=Hz) in parenthesis.

assignments of **2** were carried out by DQF-COSY and HMBC experiments, which established the seven partial structures (Fig. 5). Comparison of the partial structures in **1** and **2** revealed that the oxindole moiety for partial unit F in **1** was replaced with a 3-hydroxyoxindole in **2**. Therefore, the structure of **2** was determined as shown in Fig. 5 and confirmed by interpretation of the HMBC spectrum, which exhibited critical correlations from a hydroxyl proton at

Fig. 5. DQF-COSY and HMBC data of neuroprotectin B.



6.21 ppm to oxindole carbons at 178.4, 125.9 and 75.4 ppm.

The stereochemistries of complestatin and chloropeptin, a derivative formed by acid treatment of complestatin had been determined by NMR spectral analysis of an acid hydrolysate of complestatin and conformational analysis through molecular dynamics calculations and Monte Carlo techniques[9]. The ^{1}H and ^{13}C NMR chemical shifts of **1** and **2** were almost identical to those of complestatin and chloropeptin, except for the indolyl moiety characteristic to **1** and **2**. Isolation of complestatin from the cultural broth of neuroprotectins-producing strain Q27107 strongly suggested stereochemical and conformational identity of these compounds. The stereochemistry of C-3 of the oxindole moiety in neuroprotectins remains to be determined.

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