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Short communication

Structural Elucidation of *Diploptera punctata* Allatostatin-7

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Juvenile hormones belong to sesquiterpenoid compounds, which control the development and reproduction of insects. They are synthesized and secreted in the corpora allata. It was found that allatostatin secreted from the insect brain inhibits biosynthesis of juvenile hormones. The first allatostatin was separated from the cockroach *Diploptera punctata*.[1,2] Up to now, 13 allatostatins were found based on the preproallatostatin precursor as listed in Table 1.[3]

They all regulate biosynthesis of juvenile hormones, especially juvenile hormone-III, as well as have common structures. Their C-terminal sequence is Y(F)-Xaa-F-G-L-NH_2. Besides *D. punctata*, other allatostatins were discovered in the cockroaches *Periplaneta americana, Blaberus craniifer, Blatta orientalis, Supella longipalpa, Blattella germanica,* and *Schistocerca gregaria*.[3] In addition, peptides with similar C-terminal sequences were found in other insects such as moth, locust and fly.[4-7] They have similar C-terminal sequences as well as inhibit biosynthesis of juvenile hormone-III. Twenty allatostatins were separated from crab *Carcinus maenas*, which showed an allatostatic effect regulating juvenile hormone-III.[8-11] Another Crustacea, crayfish *Orconectes limosus* has three peptides showing the allatostatic effect.[12] Since the discovery of *D. punctata* allatostatins, more than 50 peptides were found,[11] showing the allatostatic effect which was considered to be caused by their common C-terminal sequences.

Interestingly, even though the peptide separated from tobacco hornworm *Manduca sexta* does not have the C-terminal sequence mentioned above and its C-terminal is not amidated, it shows the allatostatic effect. Its C-terminal sequence is P-I-S-C-F-OH.[13,14] On the contrary, the peptide separated from the locust *S. gregaria* consists of 18 amino acids, and though 11-18 residues of its C-terminal are exactly the same as those of *D. punctata* allatostatin-2 (L-P-V-Y-N-F-G-L-NH_2), it does not show the allatostatic effect.[7]

Therefore, it can be said that the allatostatic effect is not caused by the amidated C-terminal sequence such as Y(F)-Xaa-F-G-L-NH_2. In other cases, structure-activity relationships of peptides depend upon their three dimensional structures instead of the primary sequences. As a result, the structural elucidation must precede the study of relationships between the structures of allatostatins and the allatostatic effects, which can give information about the development of inhibitors of biosynthesis of juvenile hormone-III, assumed to be one of the biological pesticides.

In this study, the structure of one of *D. punctata* allatostatins, Dp-AST7, was elucidated using NMR spectroscopy. Dp-AST7 consists of 13 amino acids, A-P-S-G-A-Q-R-L-Y-G-F-G-L-NH_2, showing the strongest allatostatic effect among the 13 *D. punctata* allatostatins. It was synthesized using ABI peptide synthesizer and separated by Sephadex G25 and preparative HPLC (reverse phased C18). The NMR sample of Dp-AST7 was prepared in 100% D_2O. In order to observe all amide protons, a separate sample was prepared in 50% D_2O/50% H_2O (vol/vol). Sample concentrations were approximately 1.67 mM in the above solvent conditions. All NMR experiments were carried out on a Bruker AVANCE 400 (9.4T) spectrometer equipped with a 5 mm dual probe. The probe temperature was maintained at 298 K. In all experiments, solvent suppression was achieved using selective saturation and a proton pulse length of 9.7μsec. Presaturated one-dimensional NMR spectra were measured using a spectral width of 4006.41 Hz and 32 K of data points. Two dimensional experiments were performed by the time proportional phase incrementation (TPPI) technique in all cases except for the COSY experiments. Two-dimensional COSY, TOCSY, ROESY, and NOESY spectra were acquired with 2 K data points in t2 and 256 t1 increments. 256 t1 FIDs were collected in each experiment with 256 scans per t1 value. Spectral width of 4006.41 Hz was used in all experiments. The pulse sequence for COSY was magnitude N-type with presaturation during the relaxation delay.[15] The pulse sequence used for TOCSY was an MLEV17 spin-lock where a 2.5 msec trim pulse was employed.[16] A mixing time of 125 msec was used in phase sensitive TOCSY experiments. Phase sensitive NOESY[17] and ROESY[18] spectra were measured using 100, 500, and 1000 msec mixing times. Phase-shifted sine-bell squared functions were applied prior to Fourier transformation. All data were zero-filled to 4 K data points in the t1 dimension.

Initially, 43 cross peaks out of a total of 53 proton resonances in the COSY spectrum were assigned. Based on the COSY assignments, 63 of the 69 cross peaks found in the

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Table 1. Thirteen allatostatins identified based on the preproallatostatin precursor of *Diploptera punctata* allatostatin.[3]

Dp-AST	Primary Sequences																		
1													L	Y	D	F	G	L	$-NH_2$
2	A	Y	S	Y	V	S	E	Y	K	R	L	P	V	Y	N	F	G	L	$-NH_2$
3											S	K	M	Y	G	F	G	L	$-NH_2$
4										D	G	R	M	Y	S	F	G	L	$-NH_2$
5											D	R	L	Y	S	F	G	L	$-NH_2$
6											A	R	P	Y	S	F	G	L	$-NH_2$
7						A	P	S	G	A	Q	R	L	Y	G	F	G	L	$-NH_2$
8										G	G	S	L	Y	S	F	G	L	$-NH_2$
9									G	D	G	R	L	Y	A	F	G	L	$-NH_2$
10			P	V	N	S	G	R	S	S	G	S	R	F	N	F	G	L	$-NH_2$
11								Y	P	Q	E	H	R	F	S	F	G	L	$-NH_2$
12													P	F	N	F	G	L	$-NH_2$
13											I	P	M	Y	D	F	G	I	$-NH_2$

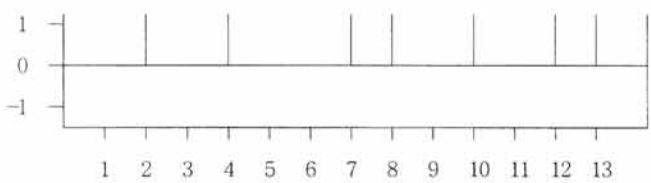

Fig. 1. The Wishart index of Dp-AST7 obtained from ^1H chemical shifts.

TOCSY spectrum were assigned as summarized in Table 2.

The methods using chemical shifts provide rapid and accurate technique for the type and location of the secondary structure of proteins from proton chemical shifts alone. This method introduced by Wishart is strongly based on the conformational chemical shift tendencies of α-protons.[19] The Wishart indices as a guide were used in the determination of the secondary structure (Fig. 1).

According to the Wishart indices, a minimum of three consecutive "1's" are needed to define the β-sheet and a minimum of four consecutive "-1's" to define the α-helix. As shown in Fig. 1, Dp-AST7 has neither α-helix nor β-sheet.

Even though the secondary structure does not exist, in cases of oligopeptides, the tertiary structure may be determined by NMR spectroscopy. Since nOe cross peaks and dihedral angles give information about the three dimensional structure, nOe peaks should first be collected. In Dp-AST7 no peaks were observed except for the scalar coupled cross peaks in NOESY and ROESY, so that instead of nOe cross peaks, dihedral angles were used for the structural determination based on the Karplus equations[20] as follows:

$$^3J_{HN\text{-}H\alpha} = 6.4\cos^2(\Phi - 60^\circ) - 1.4\cos(\Phi - 60^\circ) + 1.9$$
$$^3J_{H\alpha\text{-}H\beta 2} = 9.5\cos^2\chi_1 - 1.6\cos\chi_1 + 1.8$$

where Φ and χ_1 are dihedral angles of HN-H_α and H_α-$H_{\beta 2}$, respectively. The values of ^3J and dihedral angles are ranged within the narrow limits (Table 3) signifying that Dp-AST7 does not contain any constraints.

Table 2. Assignments of NMR data of Dp-AST7.

residue	NH	$C_\alpha H$	$C_\beta H$	others
ala		4.38	1.70	
pro		4.75	2.54	γ2.15/2.25 δ3.38/3.39
ser	8.56	4.69	4.23	
gly	8.27	4.18/3.98		
ala	8.24	4.41	1.65	
gln	8.34	4.33	2.62	γ2.30
arg	8.12	4.40	2.03	γ1.84 δ3.85
leu	7.85	4.56	1.82	δ1.10
try	7.98	4.68	3.29/3.15	
gly	8.19	4.11/3.99		
phe	8.03	4.74	3.28/3.39	
gly	8.69	4.12/4.00		
leu	8.00	4.47	1.81/1.66	δ1.03

Table 3. The values of ^3J of Dp-AST7 and dihedral angles calculated based on the Karplus equations.

residue	$^3J_{HN\text{-}H\alpha}$	Φ	$^3J_{H\alpha\text{-}H\beta 2}$	χ1
ala			6	139.43
pro			7	145.92
ser			7	145.92
gly	7	217.96		
ala	7	217.96	8	151.50
gln	7	217.96	7	145.92
arg	7	217.96	7	145.92
leu			9	158.02
try	7	217.96	7	145.92
gly				
phe	7	217.96		
gly	7	217.96		
leu	7	217.96	7	145.92

Therefore, at this time, it can be concluded that the three dimensional structure of Dp-AST7 does not exist.

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