RES-701-1, A NOVEL AND SELECTIVE ENDOTHELIN TYPE B RECEPTOR ANTAGONIST PRODUCED BY *Streptomyces* sp. RE-701

II. DETERMINATION OF THE PRIMARY SEQUENCE

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The structure of RES-701-1, a novel microbial endothelin type B receptor antagonist, was determined. Protein chemical data and FAB-MS have identified RES-701-1 to be a cyclic polypeptide consisting of 16 common L-amino acids. This compound is so stable against proteolysis that its enzymatic digestion under standard conditions proved to be very difficult. Therefore, the primary sequence of RES-701-1 was determined by the application of advanced protein chemical methods to be Gly^1-Asn^2-Trp^3-His^4-Gly^5-Thr^6-Ala^7-Pro^8-Asp^9-Trp^{10}-Phe^{11}-Phe^{12}-Asn^{13}-Tyr^{14}-Tyr^{15}-Trp^{16}. The compound is cyclized between the β-carboxyl group of Asp^9 and the α-amino group of Gly^1.

Endothelins (ET)-1, which consists 21 amino acid residues, has been isolated from the culture media of porcine endothelial cells and shown to be one of the most potent vasocontrictors known[1]. Two isopeptides of ET-1, designated ET-2 and ET-3, have been discovered in mammalian tissues[2]. It is well known that the ET family of isopeptides exert diverse biological effects through specific receptors in a wide variety of tissues and cell types[2,3]. Furthermore, ET-1 may have a possible role in development of cerebral vasospasm after arachnoid hemorrhage[4], myocardial infraction[5] and acute renal failure[5]. Thus, substances which specifically inhibit the binding of endothelin to its receptors may have therapeutic potential in putative endothelin-related disease.

In the course of a screening program for ET receptor antagonists from culture broths of microorganisms, we discovered a novel cyclic peptide in a culture of *Streptomyces* sp. RE-701. In a preceding paper[6], the fermentation of the producing organism, isolation, physico-chemical and biological properties of the RES-701-1 have been described. In this paper, we report the primary structure of RES-701-1 including DL-amino acid analyses.

Results and Discussion

Structural Elucidation

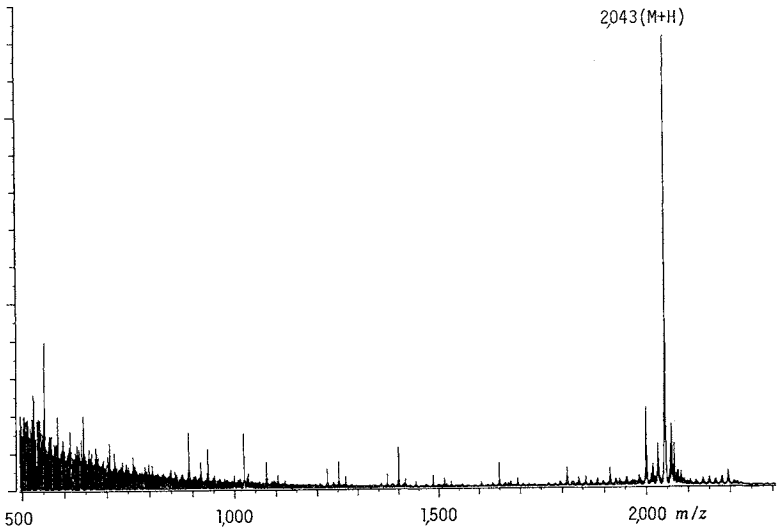
Isolation

RES-701-1 was obtained as a white powder from the fermentation broth of strain RE-701 by isolation procedures described in a preceding paper[6].

FAB-MS

The $M+H^+$ ion for RES-701-1 was measured at 2,043 by FAB-MS as shown in Fig. 1.

Fig. 1. FAB-mass spectrum of RES-701-1.



m-Nitrobenzyl alcohol-glycerol was used as matrix. Acceration voltage was 10 kV.

Table 1. Amino acid composition of RES-701-1.

Amino acids (theoretical)		Observed
Aspartic acid	(3)	3.1
Glycine	(2)	2.2
Histidine	(1)	1.0
Threonine	(1)	0.9
Alanine	(1)	1.0
Proline	(1)	1.1
Tyrosine	(2)	1.9
Phenylalanine	(2)	1.9
Tryptophan	(3)	3.4[a]

[a] The value of tryptophan is relative to the value of Ala as 1.0.

Table 2. HPLC data for DL-amino acid analysis of RES-701-1.

Amino acids	Retention time (minutes)	
	Authentic amino acids	Observed
D-Aspartic acid	35.6	—
L-Aspartic acid	36.0	36.2
D-Threonine	37.2	—
L-Threonine	38.0	38.2
D-Alanine	43.4	—
L-Alanine	44.2	44.2
D-Tyrosine	50.2	—
L-Tyrosine	52.4	52.2
D-Phenylalanine	56.8	—
L-Phenylalanine	59.4	59.0
D-Tryptophan	61.2	—
L-Tryptophan	62.6	62.4
D-Histidine	65.4	—
L-Histidine	69.4	68.6
L-Proline[a]	15.3	15.3
D-Proline[a]	16.8	—

— Not detected.

[a] DL-Proline was analysed separately under different chromatographic conditions referred in (6).

The molecular formula of RES-701-1 was determined to be $C_{103}H_{115}N_{23}O_{23}$ based on positive-ion high resolution FAB-MS (2,042.8722, Calcd for $(M+H, C_{103}H_{116}N_{23}O_{23})^+$ 2,042.8614) as shown in Fig. 1.

Amino Acid Analysis

The amino acid composition of RES-701-1 is shown in Table 1. This table shows RES-701-1 to be composed of 16 amino acids. Based on HRFAB-MS, the elemental composition $C_{103}H_{115}N_{23}O_{23}$ of RES-701-1 is proposed. This result shows that the MW of RES-701-1 corresponds to its amino acid composition minus the weight of one water molecule and indicates that the molecule must be cyclized.

Amino acid enantiomers were differentiated by RP-HPLC retention using the (+)-1-(9-

fluorenyl)ethylchloroformate (FLEC) method[7]. These analytical data of RES-701-1 indicated that all the amino acids were of the L-configuration (Table 2).

N-Terminal Sequence Analysis

N-Terminal sequence analysis was performed by automated Edman degradation but it did not yield any amino acid sequence information. This indicated that its terminal α-amino group was modified. This result also suggests that the molecule must be cyclized.

Limited HCl Hydrolysis

Early attempts to use enzymatic methods on the RES-701-1 failed. Therefore, to obtain fragments suitable for sequencing, limited acid hydrolysis techniques were used. Limited HCl hydrolysis (0.04 N HCl hydrolysis or 0.4 N HCl hydrolysis) yielded several fragments which could be isolated by RP-HPLC and sequenced as shown in Fig. 2. The sequenced data of 4 fragments are summarized in Fig. 3. Fig. 3 shows the total amino acid sequence of RES-701-1 as determined. This sequence indicates that two potential sites for cyclization with α-amino group in Gly^1 exist: The β-carboxyl group in Asp^9 or the α-carboxyl group in Trp^{16}.

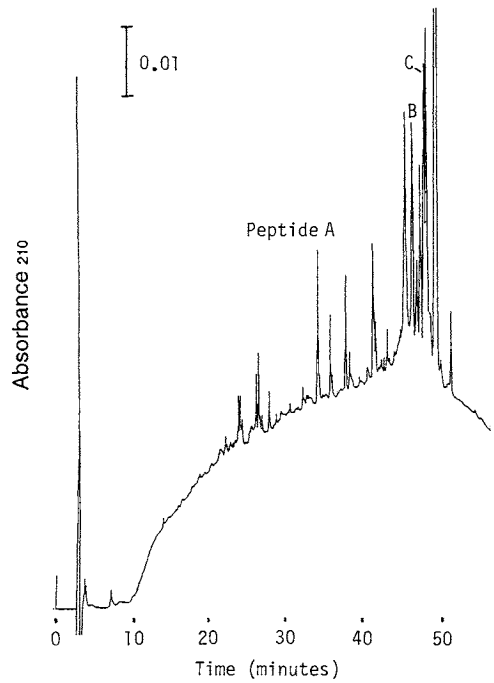
C-Terminal Amino Acid Analysis

C-Terminal amino acid analysis was performed by the hydrazinolysis[8] and yielded Trp. This result indicates that the α-carboxyl group in Trp^{16} is free and the potential site for cyclization with Gly^1 exists only in Asp^9.

Thus, the resulting complete structure of RES-701-1 was elucidated as shown in Fig. 4. This figure shows that RES-701-1 is a novel cyclic peptide, cyclized between the β-carboxyl group of Asp^9 and the α-amino group of Gly^1.

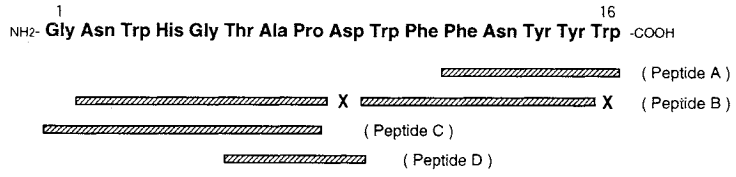
Recently, this type of cyclic peptide has been

Fig. 2. Separation of limited HCl hydrolysis products of RES-701-1.



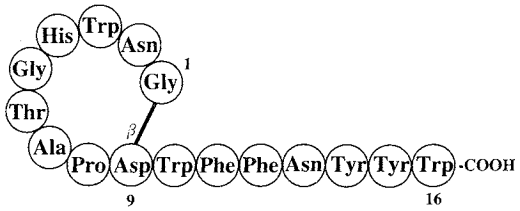
Analytical: 5 μg of RES-701-1 (0.04 N HCl hydrolysate). Column, C_{18} Silica, 150×6 mm i.d. (YMC, Japan). Linear 60 minutes gradient from 0~63% CH_3CN-0.1% TFA. Flow rate, 1 ml/minute.

Fig. 3. Sequence analysis of peptides after limited HCl hydrolysis.



▨ means an amino acid sequence of each peptide. X: Not determined. Peptide D was obtained from hydrolysate with 0.4 N HCl (data not shown).

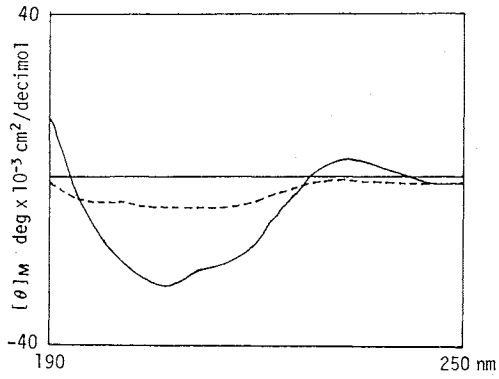
Fig. 4. Primary structure of RES-701-1.



The α-amino group of Gly^1 is connected to the β-carboxyl group of Asp^9.

Fig. 5. Circular dichroism spectra of RES-701-1 and the linear peptide of RES-701-1.

(——) RES-701-1, (------) linear peptide.



Measurement in methanol solution. Scan speed was 20 nm/minute with a step resolution 0.2 nm.

found in anantin[9], which is an atrial natriuretic peptide receptor antagonist and is cyclized between the β-carboxyl group of Asp^8 and the α-amino group of Gly^1. However, there is no sequence similarity between RES-701-1 and anantin. So, these peptides do not belong to the same family. There is also no significant sequence similarity between the ETs and RES-701-1, except that their *C*-terminal amino acid residue is Trp. This amino acid is reported to be especially important for the activity of ETs[10] as also is the *C*-terminal hydrophobic cluster (Compare ETs: position 19~21, and RES-701-1: position 14~16). Some of their structural similarities must be important for binding to the endothelin receptors. Since conservation of the charged amino acids, such Asp^8, Glu^{10}, His^{16} and Asp^{18} in ET-1 has been reported to be necessary for the activity[11], the absence of charged amino acids in comparable positions must be a reason for the loss of agonistic properties in RES-701-1.

To determine the secondary structure of RES-701-1, the far-UV CD spectra of the compound were measured (Fig. 5). This figure shows a negative CD (192~227 nm) observed in methanol solution in the case of RES-701-1 but absent in the case of the linear peptide of RES-701-1. This result suggests that the cyclization leads to the production of a certain secondary structure and that RES-701-1 in methanol could represent the 3D structure.

The 3D structure of RES-701-1 determined by NMR in solution will be reported elsewhere.

Experimental

FAB-mass spectrometry (FAB-MS) of RES-701-1 was measured using a JEOL JMS HX-110A employing an accelerating voltage of 10 kV. High resolution FAB mass spectrometry (HRFAB-MS) was also measured under the same conditions using polyethylene glycol as a reference.

Amino acid analysis was performed using vapor phase hydrolysis with 6 N HCl containing 1% phenol at 110°C for 20 hours. Only tryptophan was analyzed separately after hydrolysis with 4 M methane sulfonic acid (Pierce)[12]. The resulting amino acids were derivatized with phenylisothiocyanate and analyzed on a Waters automated amino acid analyzer. Precolumn derivatization with (+)-1-(9-fluorenyl)ethyl-chloroformate (Aldrich) followed by reverse phase HPLC analysis[7] was applied to distinguish between D- and L-amino acids.

Amino acid sequencing was performed on an Applied Biosystems Model 470A/120A automated gas phase sequencer.

Fragments of RES-701-1 were obtained by partial hydrolysis with 0.04 N or 0.4 N HCl containing 20% methanol at 108°C for 2 hours. The resulting peptides were separated by reverse phase HPLC and

analyzed by an amino acid sequencer.

Carboxyl terminal amino acid was identified by vapor-phase hydrazinolysis[8] at 95°C for 2 hours followed by precolumn derivatization with *o*-phthalaldehyde (Nacalai tesque, Japan)[13].

Circular dichroism spectrometry was measured in methanol solution from 190 nm to 250 nm using a Jasco J-500 spectropolarimeter (Japan).

The linear peptide of RES-701-1 was synthesized by a solid phase procedure on an Applied Biosystems Model 431A automated peptide synthesizer applying the Fmoc strategy.

Acknowledgment

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References

1) YANAGISAWA, M.; H. KURIHARA, S. KIMURA, Y. TOMOBE, M. KOBAYASHI, Y. YAZAKI, K. GOTO & T. MASAKI: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332: 411~415, 1988
2) LEMONNIER DE GOUVILLE, A. C.; H. L. LIPPTON, I. CAVERO, W. R. SUMMER & A. L. HYMAN: Endothelin—A new family of endothelium-derived peptides with widespread biological properties. Life Sci. 45: 1499~1513, 1989
3) MILLER, R. C.; J. T. PELTON & J. P. HUGGINS: Endothelins—from receptors to medicine. Trends Pharm. Sci. 14: 54~60, 1993
4) SUZUKI, H.; S. SATO, Y. SUZUKI, K. TAKEKOSHI, N. ISHIHARA & S. SHIMODA: Increased endothelin concentration in CSF from patients with subarachnoid hemorrhage. Acta Neuro. Scand. 81: 553~554, 1990
5) HAYNES, W. G.; A. P. DAVENPORT & D. J. WEBB: Endothelin: progress in pharmacology and physiology. Trends Pharm. Sci. 14: 225~228, 1993
6) MORISHITA, Y.; S. CHIBA, E. TSUKUDA, T. TANAKA, T. OGAWA, M. YAMASAKI, M. YOSHIDA, I. KAWAMOTO & Y. MATSUDA: RES-701-1, a novel and selective endothelin type B receptor antagonist produced by *Streptomyces* sp. RE-701. I. Characterization of producing strain, fermentation, isolation, physico-chemical and biological properties. J. Antibiotics 47: 269~275, 1994
7) EINARSSON, S.; P. JOSEFSSON, P. MOLLER & D. SANCHEZ: Separation of amino acid enantiomers and chiral amines using precolumn derivatization with (+)-1-(9-fluorenyl)ethylchloroformate and reversed-phase liquid chromatography. Anal. Chem. 59: 1191~1195, 1987
8) YAMAMOTO, A.; H. TODA & F. SAKIYAMA: Vapor-phase hydrazinolysis for microdetermination of carboxyl-terminal amino acids of proteins. J. Biochem. 106: 552~554, 1989
9) WYSS, D. F.; H.-W. LAHM, M. MANNEBERG & A. M. LABHARDT: Anantin—A peptide antagonist of the atrial natriuretic factor (ANF). II. Determination of the primary sequence by NMR on the basis of proton assignments. J. Antibiotics 44: 172~180, 1991
10) KIMURA, S.; Y. KASUYA, T. SAWAMURA, O. SHINMI, Y. SUGITA, M. YANAGISAWA, K. GOTO & T. MASAKI: Structure-activity relationships of endothelin: importance of the C-terminal moiety. Biochem. Biophys. Res. Commun. 156: 1182~1186, 1988
11) KLOOG, Y. & M. SOKOLOVSKY: Similarities in mode and sites of action of sarafotoxins and endothelins. Trends Pharmacol. Sci. 10: 212~214, 1989
12) SIMPSON, R. J.; M. R. NEUBERGER & T.-Y. LIU: Complete amino acid analysis of proteins from a single hydrolysate. J.B.C. 251: 1936~1940, 1976
13) ALTMANN, F.: Determination of amino sugars and amino acids in glycoconjugates using precolumn derivatization with *o*-phthalaldehyde. Anal. Biochem. 204: 215~219, 1992