RES-701-2, a Novel and Selective Endothelin Type B Receptor Antagonist Produced by *Streptomyces* sp.

II. Determination of the Primary Structure

KEIICHI YANO, MOTOO YAMASAKI*, MAYUMI YOSHIDA, YUZURU MATSUDA and KAZUO YAMAGUCHI

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machidashi, Tokyo 194, Japan

(Received for publication July 20, 1995)

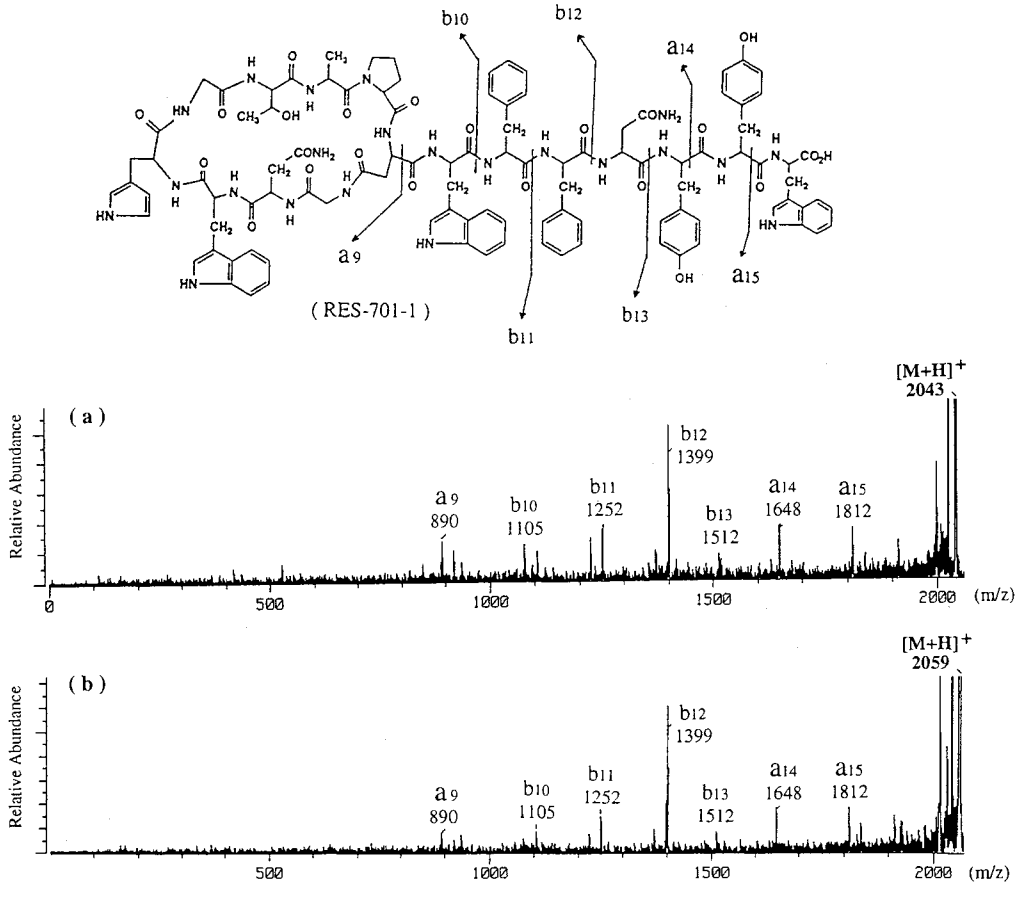
Endothelins (ETs) are a family of potent vasoactive peptides found in at least three distinct isoforms: ET-1, ET-2, and ET-3[1]. Two major subtypes of endothelin receptors, termed ET_A and ET_B, have been identified in various target cells[2]. It is well known that ETs act on these G protein-coupled receptors and exert multiple pharmacological effects[3]. As the development of potent and selective endothelin receptor antagonists facilitates the understanding of endothelin[4], the function of ET_B receptors is yet to be fully elucidated.

In a preceding paper, we elucidated the isolation, physico-chemical and biochemical properties, and structural determination of RES-701-1, a novel endothelin type B receptor antagonist[5,6]. In the course of further screening programs for ET receptor antagonists from culture broths of microorganisms, we discovered other structural related peptides, RES-701-2, -3 and -4 in the culture broth of *Streptomyces* sp.[7]. This paper describes the primary structure elucidation of RES-701-2 including DL-amino acid analysis.

FAB-mass spectrometry analysis revealed that the molecular weight of RES-701-2 is 2059, which is 16 mass higher than RES-701-1[6]. The molecular formula of RES-701-2 was confirmed as $C_{103}H_{115}N_{23}O_{24}$ by high resolution-FAB-MS analysis (calcd: 2058.8503 for M + H, found: 2058.8496) and amino acid analysis (found: Asx3, Gly2, His1, Thr1, Ala1, Pro1, Tyr2, Phe2, Trp2). The amino acid configuration was found to be the L-form by precolumn derivatization with chiral reagent, (+)-1-(9-fluorenyl)ethyl-chloroformate and reversed phase HPLC analysis.

To examine the primary structure of RES-701-2, N-terminal amino acid analysis was carried out with Edman degradation. However, no phenylthiohydantoin(PTH)-amino acid was released, suggesting that there is no free terminal amino group. Furthermore, attempts to obtain specific fragments by enzymatic digestion with thermolysin, chymotrypsin and proline specific endopeptidase were unsuccessful. Partial hydrolysis was therfore carried out in order to get the structural information. Amino acid sequence analyses of the

Fig. 1. FAB-MS/MS spectra of RES-701-1(a) and RES-701-2(b).



resulting peptides reveal that the sequence from residue 1 to 15 is identical to that of RES-701-1[6] and that the difference between RES-701-1 and RES-701-2 exists only in C-terminus.

In the FAB-MS/MS analysis of RES-701-1 or RES-701-2, each spectrum contains two prominent series of N-terminal fragment ions (a and b ions) which confirms the amino acid sequence in the C-terminus (Fig.1). Comparing the fragment ions between the two compounds, the spectrum patterns are almost the same but for the parent ions. This result also suggests that primary sequences except for the C-terminal amino acid are identical, including the cyclic structure described in Fig. 1.

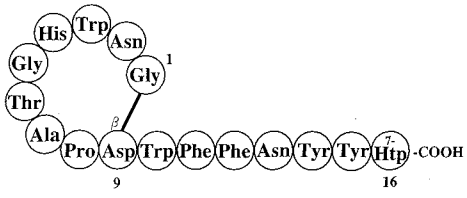
Surprisingly, the C-terminal amino acid of RES-701-2 is different from any the other 20 natural amino acids by an amino acid analysis after vapor phase hydrazinolysis[8]. Despite its stability to proteolytic digestion, carboxypeptidase A (CPase A), with relatively high amount (Enzyme/Substrate = 1/20), is found to release the C-terminal amino acid successfully. The released C-terminal amino acid was purified by preparative reversed phase HPLC and analyzed by ^{1}H and ^{13}C NMR spectroscopy. Chemical shifts for the C-terminal amino acid of RES-701-2 are shown in Table 1. Taking the molecular weight of the amino acid ($m/z = 221$) into consideration, hydroxylation might be occurred at position C7 of tryptophan. NOEs and C-H long-range coupling data also support the above (data not shown). On an optical rotatory power measurement of the C-terminal amino acid in water, a negative value ($[\alpha]_D^{20} = -22.6°$) was observed, indicating that the C-terminal amino acid has the L-configuration like the other amino acids found in RES-701-2.

Table 1. Chemical shifts for C-terminal amino acid of RES-701-2.

Position	$^{13}C^{a}$	$^{1}H^{b}$
α	52.8	4.02 (1H, dd, J=7.2, 5.3 Hz)
β	26.4	3.13 (1H, dd, J=7.2, 15.1 Hz), 3.22 (1H, dd, J=5.3, 15.1 Hz)
1		10.8 (1H, d, J=2.4 Hz)
2	124.1	7.11 (1H, d, J=2.4 Hz)
3	107.3	
4	109.2	7.02 (1H, d, J=7.9 Hz)
5	119.3	6.79 (1H, dd, J=7.9, 7.5 Hz)
6	105.5	6.50 (1H, d, J=7.5 Hz)
7	143.6	9.53
8	126.3	
9	128.9	
CO	170.7	

Chemical shifts expressed in ppm relative to DMSO.
[a] 125 MHz ^{13}C NMR in DMSO.
[b] 500 MHz ^{1}H NMR in DMSO.

Fig. 2. Primary structure of RES-701-2.



Cyclization was observed between α-amino group of Gly[1] and β-carboxyl group of Asp[9]. 7-Htp: 7-hydroxytryptophan

The primary structure of RES-701-2 is shown in Fig. 2. Cyclization is observed between the α-amino group of Gly1 and the β-carboxyl group of Asp9. It has been reported that anantin, an atrial natriuretic peptide receptor antagonist[9], and PR71955, an anti-HIV peptide[10], also have this novel cyclic form. The biosynthesis of such novel structures has never been reported so far. It may involve unique enzymes that form a lactam bridge between an amino group and a carboxyl group within the molecules. Recently the peptide synthesis of RES-701-1 has been reported by J. H. He *et al.*[11]. Although the synthetic compound had the precise primary structure, it showed less binding affinity for ET_B receptor than natural RES-701-1. We had also synthesized RES-701-1 using similar methods described in ref. 11), and found that the synthetic compound did not coelute with the natural RES-701-1 on analytical HPLC (data not shown), suggesting different tertiary structures for the two compounds. Precise structural determination by NMR analysis will be reported elsewhere.

Among hydroxylated forms of tryptophan, 5-hydoxytryptophan has been well studied as a neurotransmitter in the central nervous system, and its biosynthesis including related enzymes has also been examined. 7-hydoxytryptophan, the C-terminal amino acid of RES-701-2, has never been reported as a natural compound so far, nor has its biosynthesis been elucidated. We could not detect any differences in biological activities between RES-701-1 and RES-701-2. It is noted that synthetic 7-hydroxytryptamine is about eight times more active as a vasocontrictor than tryptamine[12], although their antagonistic activities against the ET_B receptor are equivalent[7].

Experimental

RES-701-2 was isolated from the fermentation broth of *Streptomyces* sp. described in a preceding paper[7].

All mass spectra were acquired with a JEOL JMS HX/HX-110A tandem four sector mass spectrometer, which was operated at 10 kV accelating potential.

Amino acid analysis, DL-amino acid analysis with (+)-1-(9-fluorenyl)ethyl-chloroformate and vapor-phase hydrazinolysis were carried out with the same procedures described in ref. 6). N-terminal amino acid sequencing was performed on a Model 470A/120A protein sequencer (Applied Biosystems, U.S.A.).

Peptide fragments of RES-701-2 were obtained by partial hydrolysis with 0.04 N or 0.5 N HCl containing 20% methanol at 108°C for 2 hours. The resulting peptides were separated by reversed phase HPLC and

analyzed by a Shimadzu PPSQ-10 amino acid sequencer (Japan).

Carboxypeptidase A (Sigma C-9762) digestion was carried out in 0.1 M Tris-HCl (pH 8.0) containing 10% methanol at 37°C for 12 hours. Released C-terminal amino acid was purified on a reversed phase HPLC equipped with a Nucleosil 5C18 column (25 cm × 20 mm i.d.) at a flow rate of 10 ml/minute.

The optical rotation of 7-hydroxytryptophan was measured in water on a Jasco DIP-370 digital polarimeter (Japan) at ambient temperature.

All NMR spectra of 7-hydroxytryptophan dissolved in dimethyl sulfoxide were measured on a Bruker AM-500 spectrometer.

Acknowledgment

We thank Ms. YOSHIE OOISHI for her excellent technical assistance.

References

1) INOUE, A.; M. YANAGISAWA, S. KIMURA, Y. KASUYA, T. MIYAUCHI, K. GOTO & T. MASAKI: The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci. 86: 2863~2867, 1989
2) BAX, W. A. & P. R. SAXENA: The current endothelin receptor classification: time for reconsideration? Trends Pharmacol. Sci. 15: 379~386, 1994
3) MILLER, R. C.; J. T. PELTON & J. P. HUGGINS: Endothelins—from receptors to medicine. Trends Pharmacol. Sci. 14: 54~60, 1993
4) DOUGLAS, S. A.; T. D. MEEK & E. H. OHISTEIN: Novel receptor antagonists welcome a new era in endothelin biology. Trends Pharmacol. Sci. 15: 313~316, 1994
5) MORISHITA, Y.; S. CHIBA, E. TSUKUDA, T. TANAKA, T. OGATA, 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
6) YAMASAKI, M.; K. YANO, M. YOSHIDA, Y. MATSUDA & K. YAMAGUCHI: RES-701-1, a novel and selective endothelin type B receptor antagonist produced by *Streptomyces* sp. RE-701. II. Determination of the primary structure. J. Antibiotics 47: 276~280, 1994
7) OGAWA, T.; K. OCHIAI, T. TANAKA, E. TUKUDA, S. CHIBA, K. YANO, M. YAMASAKI, M. YOSHIDA & Y. MATSUDA: RES-701-2, -3 and -4, novel and selective endothelin type B receptor antagonists produced by *Streptomyces* sp. I. Taxonomy of producing strains, fermentation, isolation and biochemical properties. J. Antibiotics 48: 1213~1220, 1995
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). J. Antibiotics 44: 172~180, 1991
10) FRECHET, D.; J. D. GUITTON, F. HERMAN, D. FAUCHER, G. HELYNCK, B. M. DU SORBIER, J. P. RIDOUX, E. J. SURCOUR & M. VUILHORGNER: Solution structure of RP71955, a new 21 amino acid tricyclic peptide active against HIV-1 virus. Biochemistry 33: 42~50, 1994
11) HE, J. H.; W. L. CODY, M. A. FLYNN, K. M. WELCH, E. E. REYNOLDS & A. M. DOHERTY: RES-701-1, synthesis and a reevaluation of its effects on the endothelin receptors. Bioorg. Med. Chem. Lett. 5: 621~626, 1995
12) PAGE, I. H.: The vascular action of natural serotonin, 5- and 7-hydroxytryptamine and tryptamine. J. Pharmacol. Exptl. Therapy 105: 58~73, 1952