Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 15, pp. 1595-1600, 1995
Elsevier Science Ltd
Printed in Great Britain
0960-894X/95 $9.50+0.00

0960-894X(95)00264-2

RES-701-1, COMPARATIVE STUDY OF THE SYNTHETIC AND THE MICROBIAL-ORIGIN COMPOUNDS

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Abstract: RES-701-1 is an ET_B receptor selective antagonist obtained from *Streptmyces* sp. (IC_{50} = 10 nM). The chemically synthesized RES-701-1 which exhibits only micromolar affinity for ET_B receptor was examined by ^1H NMR and FAB-MS. The results indicated that the primary structures of the synthetic and the authentic RES-701-1 are identical but the 3D structure is completely different between them.

Introduction:

Endothelin (ET) exhibits the most potent and long lasting activity of any vasoconstrictor known[1]. Two isoforms (ET-2, ET-3) have been identified so far[2]. These three peptides mediate many biological responses in cardiovascular and non-cardiovascular tissue through binding to two different types of receptors, ET_A and ET_B[3-8]. Recently we have isolated a novel ET_B selective antagonist RES-701-1 (IC_{50}=10 nM)[9]. The primary structure of RES-701-1 was determined by the sequencing of peptide fragments obtained by limited chemical hydrolysis, and FAB-MS[10]. The amino acid sequence of RES-701-1 is shown in Figure 1 . It possesses a novel internal linkage between β-carboxyl group of Asp9 and α-amino group of Gly1. To aid understanding, we have named Gly1-Asp9 the "ring", and Trp10-Trp16 the "tail".

Recently, He *et al.* reported that RES-701-1 prepared by liquid/solid phase synthesis exhibits only micromolar-order affinity and does not possess the selectivity for ET_B[11].

In this report, we synthesized RES-701-1 as described below and examined it by ^1H NMR and FAB-MS. The results were compared with those for the authentic RES-701-1 and they revealed why the authentic RES-701-1 exhibits potent ET_B selective antagonistic activity and the synthetic RES-701-1 does not.

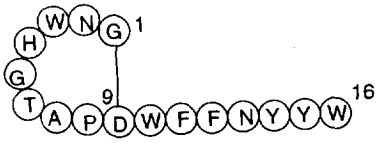


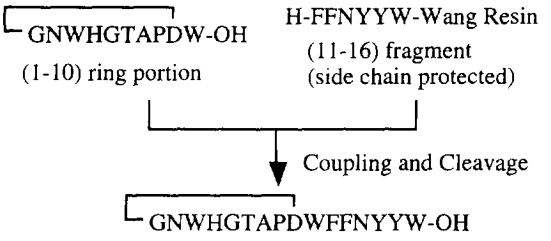
Figure 1. Amino acid sequence of RES-701-1.

Experimental Procedure:

Synthesis The synthetic RES-701-1 was obtained by solid phase condensation of the N-terminal (1-10) ring portion and the C-terminal (11-16) fragment on Wang resin illustrated in Scheme 1. Solid phase peptide syntheses were performed using a standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthetic strategy on PSSM-8 peptide synthesizer (Shimadzu Corp., Japan) according to the method recommended by Shimadzu[12] .

C-terminal protected (1-10) linear fragment was prepared by the condensation of N^{α} and side chain protected N-terminal (1-9) fragment, prepared using a 2-chlorotritylchloride resin[13], and Trp-benzylester followed by sidechain deprotection[14] and N^{α} deprotection. Cyclization of this linear fragment was carried out with PyBOP (2 equiv.) / HOBt (2 equiv.) / NMM (3 equiv.) system in DMF (20 mg/ml) at 4° C. Cyclized

product was purified by preparative reversed-phase HPLC and C-terminal benzylester was deprotected by catalytic hydrogenation with hydrogen / palladium-carbon. This N-terminal (1-10) ring portion dissolved in 1.2 ml DMF containing PyBOP (2 equiv.), HOBt (2 equiv.) and NMM (3 equiv.) at 0° C was added to C-terminal (11-16) fragment on Wang resin (2 equiv. to the ring portion) and stirred overnight at 4° C. The peptides were cleaved from the resin, sidechain deprotected[14] and purified by HPLC. The primary structure of the synthetic RES-701-1 was confirmed by FAB-MS and amino acid analysis.



Scheme 1.

Receptor binding Assay The binding activity of the synthetic RES-701-1 was examined as described[15].

MS experiment FAB-MS measurement was carried out on the first of two mass spectrometers using four sectors of the JEOL JMS-HX/HX110, operating at 10kV accelerating voltages. The DMSO solution of the synthetic RES-701-1 was dissolved in NBA/glycerol matrix and used for the measurement. High resolution FAB-MS spectrum was measured using polyethylene glycol as a standard.

NMR experiment The synthetic RES-701-1 was dissolved in deuterated dimethyl sulphoxide (DMSO-d_6) at a concentration of 3 mM. NMR spectra were recorded at 30 ^{o}C on BRUKER AM-500 spectrometer. Chemical shifts were referenced to the methyl proton resonance of DMSO at 2.5 ppm.

Two dimensional nuclear Overhauser effect spectroscopy (NOESY)[16], double quantum filtered shift correlated spectroscopy (DQF-COSY)[17] and homonuclear Hartmann Harn spectroscopy (HOHAHA)[18] spectra were recorded in a phase sensitive mode using time proportional phase incrementation. Spectra were collected with 512 complex points in t_1 and 2048 complex points in the t_2 dimension. The t_2 FIDs were multiplied by a shifted sine-bell square window function and then Fourier transformed. The t_1 FIDs were zero-filled up to 2048 points, multiplied by the same window function and Fourier transformed.

Results:

HPLC analysis The HPLC elution time of the synthetic RES-701-1 obtained by our strategy was clearly distinct from that of the authentic RES-701-1 (Figure 2). This synthetic RES-701-1 showed very low affinity for ET_B receptor (about 20% inhibition of ET-1 binding at 0.5μM). This result is consistent with the recent report[11].

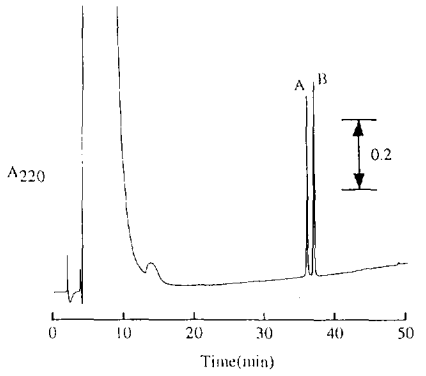


Figure 2. HPLC analysis of the synthetic and the authentic RES-701-1. Samples were co-injected (1μg each dissolved in DMF) on C18 reverse phase column (YMC, Japan, 150 X 6mm, 1ml/min) and eluted by linear gradient from 0 to 90% acetonitrile in 0.1% TFA solution. Peaks A and B represent the synthetic and the authentic sample, respectively.

MS spectra Since the HPLC elution time of the synthetic RES-701-1 was different from that of the authentic RES-701-1, MS spectrum of the synthetic RES-701-1 was examined. The FAB-MS spectrum showed the protonated molecular ion at $(M+H)^+$ = m/z 2043. The molecular formula of the synthetic RES-701-1 was determined to be $C_{103}H_{115}N_{23}O_{23}$ on the basis of the positive ion high resolution FAB-MS ($(M+H)^+$=m/z 2042.8503, calculated for (M+H, $C_{103}H_{116}N_{23}O_{23})^+$ 2042.8614). The molecular weight and molecular formula of the synthetic RES-701-1 were the same as that of the authentic RES-701-1 reported before[10].

NMR spectra ^{1}H NMR spectra of the authentic and the synthetic RES-701-1 were examined. The assignments of the authentic and the synthetic RES-701-1 were performed by the method of Wüthrich[19]. Tables 1 and 2 show the ^{1}H NMR chemical shifts of the authentic and the synthetic RES-701-1, respectively. The results of the sequential assignments of the authentic and the synthetic RES-701-1 were consistent with the amino acid sequence of RES-701-1 reported before[10]. Moreover, the NOE between CβH of Asp9 and NH of Gly1, which is the linkage position, was also observed for both peptides. Therefore, it was confirmed that the primary structure of the authentic RES-701-1 is consistent with that reported before and the primary structure of the synthetic RES-701-1 was the same as that of the authentic RES-701-1. However, ^{1}H NMR chemical shifts of the synthetic RES-701-1 (Table 2) are completely different from those of the authentic RES-701-1 (Table 1). Thus, in order to obtain the information of a secondary structure, NOE connectivities were examined. The NOE connectivities of the authentic and the synthetic RES-701-1 are shown in Figures 3a and 3b, respectively. For Pro8, the presence of a strong NOE between the CαH of Ala7 and the CδH of Pro8 of the authentic and the synthetic RES-701-1 indicates the exclusive adoption of a trans peptide bond for both peptides. In the authentic RES-701-1 (Figure 3a), many NOEs were observed between the "tail" and the "ring". In addition, β-sheet structure was observed between the segments of 7-9 and 12-14. It is considered that the authentic RES-701-1 adopts very rigid structure. Contrary to this, no long-range NOEs were observed for the synthetic RES-701-1, nor was there any NOE between the "ring" and the "tail" (Figure 3b). Since the primary structure of the synthetic RES-701-1 was the same as that of the authentic RES-701-1, it is suggested that the 3D structure of the synthetic

RES-701-1 is different from that of the authentic RES-701-1. The synthetic RES-701-1 adopts a much more flexible conformation than that of the authentic RES-701-1.

Table 1. ^{1}H Chemical Shift Table of RES-701-1 in DMSO at 30°C[a].

residue	NH	CαH	CβH	others
G1	8.54	4.25,3.50		
N2	9.10	4.92	Hβ3 2.46,Hβ2 3.20	NH_2 7.51,7.20
W3	8.06	4.36	2.91	H2 6.94,H4 7.42,H5 6.83,H6 7.02,H7 7.30, NH 10.78
H4	7.64	4.82	Hβ3 2.76,Hβ2 3.00	H2 9.07,H4 6.89
G5	7.97	4.40,3.60		
T6	7.92	4.33	4.41	γMe 1.10
A7	7.86	4.76	1.22	
P8		4.96	2.00	γH 1.88,1.96, δH 3.68
D9	7.64	4.53	2.66, 3.08	
W10	7.80	4.00	Hβ3 3.06, Hβ2 2.84	H2 6.92,H4 7.42,H5 6.96,H6 7.02, H7 7.30, NH 10.73
F11	7.22	4.38	2.53,2.62	H2,6 6.95,H3,5 7.16,H4 7.24
F12	8.54	4.25	Hβ3 3.22,Hβ2 2.80	H2,6 6.95,H3,5 7.16 H4 7.28
N13	8.02	5.48	Hβ3 1.98,Hβ2 2.25	NH_2 6.76,6.53
Y14	8.49	4.83	2.66	H2,6 6.90, H3,5 6.60
Y15	8.43	4.51	2.84	H2,6 6.88, H3,5 6.53
W16	7.57	4.43	Hβ3 2.84,Hβ2 3.06	H2 7.23,H4 7.47,H5 6.96,H6 7.02,H7 7.33,NH 10.48

a.Chemical shifts are referenced internally to the methyl resonance of DMSO-d6 at 2.5 ppm.

Table 2. ^{1}H Chemical Shift Table of the synthetic RES-701-1 in DMSO at 30°C[a].

residue	NH	CαH	CβH	others
G1	8.07	3.93,3.53		
N2	8.06	4.47	2.55,2.44	NH_2 6.98,7.48
W3	8.22	4.36	2.91	H2 7.09, H4 7.53, H5 6.98, H6 7.06, H7 7.33, NH 10.71
H4	8.10	4.47	3.00,3.16	H2 9.07,H4 6.
G5	8.07	3.93,3.64		
T6	8.00	4.11	4.23	γMe 1.06
A7	7.57	4.55	1.22	
P8		4.20	1:85,1.43	γH 1.80,1.74, δH 3.60,3.47
D9	8.08	4.53	2.58,2.23	
W10	7.57	4.44	3.00,2.83	H2 6.99, H4 7.48, H5 6.92, H6 7.02, H7 7.30, NH 10.68
F11	7.98	4.47	2.96,2.77	H2,6 7.18, H3,5 7.14, H4 7.24
F12	7.94	4.53	2.96,2.77	H2,6 7.18, H3,5 7.14 H4 7.28
N13	8.22	4.62	2.58, 2.42	NH_2 7.43,6.93
Y14	7.94	4.32	2.83,2.63	H2,6 6.93, H3,5 6.58
Y15	8.22	4.44	2.93,2.73	H2,6 7.03, H3,5 6.64
W16	8.02	4.49	3.13,3.05	H2 7.17, H4 7.49, H5 6.92, H6 7.06, H7 7.33, NH 10.80

a.Chemical shifts are referenced internally to the methyl resonance of DMSO-*d6* at 2.5 ppm.

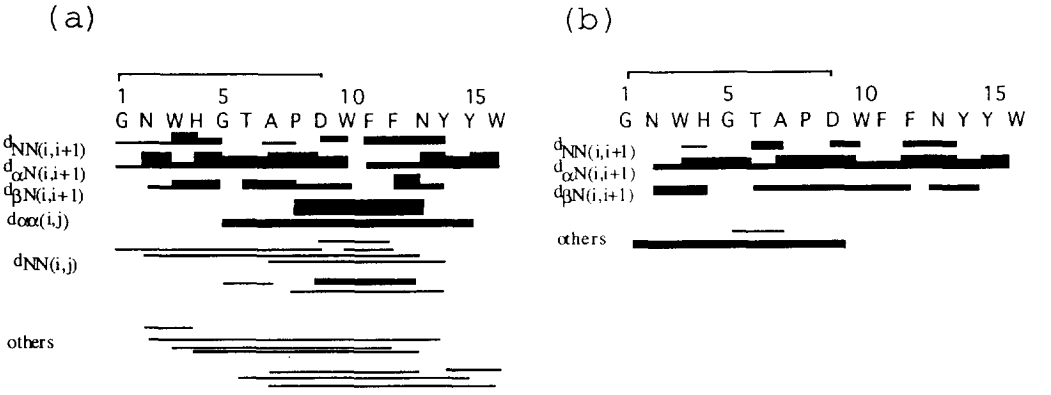


Figure 3. NOE connectivities of (a) the authentic RES-701-1 and (b) the synthetic RES-701-1. The thickness of bars indicates the intensity of NOE cross peaks. The CδH proton was substituted for the NH proton for Pro8.

Discussion:

Recently He *et al.* reported that RES-701-1 prepared by liquid/solid phase synthesis exhibits only micromolar-order affinity for ET_A and ET_B receptors[11]. They suggested that the reported amino acid sequence of RES-701-1 might be incorrect. Our evaluation of the synthetic RES-701-1 also revealed that it exhibits much lower receptor binding activity compared to the authentic RES-701-1, as reported by them. However, we confirmed the amino acid sequence of RES-701-1 reported by Yamasaki *et. al.*[10] on the basis of the ^1H NMR analysis of the authentic and the synthetic RES-701-1. As indicated above, the authentic and the synthetic RES-701-1 peptides possess the same molecular formula, the same molecular weight, and the same primary structure. However, as shown in Figure 2, the elution time of HPLC of the synthetic RES-701-1 is different from that of the authentic RES-701-1. In addition, the secondary structure of the authentic RES-701-1 is remarkably different from that of the synthetic one. Thus, in conclusion, the 3D structure of the authentic RES-701-1 is different from that of the synthetic RES-701-1. We propose that the difference of the receptor binding activity between the authentic and the synthetic RES-701-1 is a result of the difference of the 3D structure. As reported elsewhere, structure calculation of the authentic RES-701-1 revealed that it adopts an extraordinary folding[20]. Therefore it is considered that a peptide possessing such an extraordinary folding is difficult to be synthesized by the ordinary method[21].

Acknowledgment: R.K. greatly thanks Dr. N.Kagami for his carefully reading this manuscript.

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21. We obtained the biologically active RES-701-1 as a crude sample (estimated about 5% content by ion-pair chromatography) by cyclization of non-protected full-length RES-701-1 linear peptide as described in our Patent Application (WO93/13218).

(*Received in Japan* 20 *April* 1995; *accepted* 6 *June* 1995)