Endothelin-1 Analogues Substituted at Both Position 18 and 19: Highly Potent Endothelin Antagonists with No Selectivity for either Receptor Subtype ET_A or ET_B

Takashi Kikuchi,* Kazuki Kubo, Tetsuya Ohtaki, Nobuhiro Suzuki, Taiji Asami, Norio Shimamoto, Mitsuhiro Wakimasu, and Masahiko Fujino

Discovery Research Division, Takeda Chemical Industries, Ltd., Wadai-10, Tsukuba, Ibaraki 300-42, Japan

Received July 26, 1993®

Novel endothelin-1 (ET-1) analogues which are highly potent endothelin antagonists at both receptor subtype ET_A and ET_B are reported. The replacement of Asp[18] with the Thr[18] and of Ile[19] with a hydrophobic amino acid whose side-chain branches on the γ-carbon such as Leu, cyclohexylalanine, and γ-methylleucine (γ-MeLeu) resulted in loss of or significantly decreased the biological activity of ET-1, while high affinity for the ET_A (IC_{50} = 0.42–0.70 nM) and ET_B (IC_{50} = 0.17–0.43 nM) receptor was retained. These compounds were shown to have high antagonist activities in ET-1-induced vasoconstriction of porcine coronary artery (pA_2 7.4–7.7) and in Sarafotoxin S6c-induced vasoconstriction of rabbit pulmonary artery ([Thr[18],γ-MeLeu[19]]ET-1: pA_2 8.4). Among these compounds, [Thr[18],γ-MeLeu[19]]ET-1 has the desirable characteristic of possessing no agonist activity at either receptor subtype.

Endothelin-1 (ET-1), a potent vasoconstrictor peptide consisting of 21 amino acids (Figure 1), was isolated from conditioned medium of cultured porcine aortic endothelial cells.[1] Genomic analysis has identified two additional isopeptides designated ET-2 and ET-3,[2] and the existence of two receptor subtypes has been suggested:[3,4] one selective for ET-1 and ET-2 and the other nonselective for all three isopeptides. These receptors have been cloned and termed ET_A and ET_B, respectively.[5,6] The ET_A receptor is expressed predominantly in the cardiovascular system[7] and mediates vasoconstriction, while the ET_B receptor is predominant in the central nervous system in vascular endothelial cells.[8] The ET_B receptor may be functionally linked to vasodilation via release of endothelium derived relaxing factor (EDRF) in endothelial cells.[9,10] However, various ET_B selective agonists have been discovered, and some of them also cause vasoconstriction and increase blood pressure.[11,12] These facts indicate that the ET_B receptor may be linked to not only vasodilation but also vasoconstriction. In addition, the existence of other receptor subtypes has been suggested.[13–15] Therefore, potent endothelin antagonists, not only selective but also nonselective, are necessary to determine the physiological and/or pathophysiological role of endothelin and its receptor subtypes.

Recently, several peptide endothelin antagonists have been reported. Two of them are ET-1 derivatives: [Dpr[1]-Asp[15]]ET-1(1–21) (the 1–15 cystine linkage is replaced with an amide linkage between the α-amino group of 2,4-diaminobutyric acid in position 1 and the β-carboxyl group of aspartic acid in position 15) reported by Spinella and co-workers[16] and [Cys[11]-Cys[15]]ET-1(11–21) (IRL 1038) reported by Urade and co-workers.[17] The affinity of [Dpr[1]-Asp[15]]ET-1(1–21) for rat pulmonary smooth muscle cells is about 2 orders of magnitude less than that of ET-1. Its receptor selectivity has not yet been clarified, but available results suggest that it may be an ET_A-selective antagonist.[16] IRL 1038 has been found to have much higher affinity for ET_B receptors than for ET_A receptors but 350–1800-fold less affinity than ET-1 for ET_B receptors.[17] On

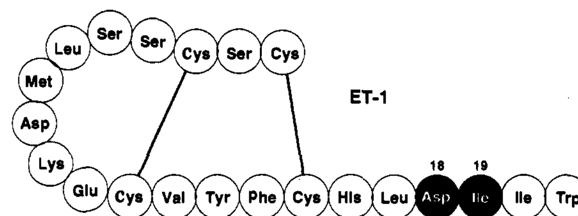

Figure 1. Structure of ET-1.

the other hand, BQ-123[18] and FR 139317[19] which were designed from a cyclic pentapeptide lead isolated from *Streptomyces misakiensis* fermentation products have also been reported as potent endothelin antagonists. These antagonists are highly selective for receptor subtype ET_A.[18,19] Very recently, a hexapeptide antagonist, PD 145065, has been reported by Cody and co-workers. This antagonist inhibited the binding of [^{125}I]ET-1 to receptor subtypes ET_A and ET_B with IC_{50} values of 3.5 and 15 nM, respectively.[20]

We have attempted to design endothelin antagonists on the basis of the SAR studies on ET-1. Nakajima et al.[21] and Hunt et al.[22] have shown that four carboxylates in ET-1 (Asp[8], Glu[10], Asp[18], and the C-terminal) are important for both binding to ET receptors and expression of biological activities. Our own SAR studies demonstrated that Asp[18] was essential for activation of the ET_A receptor rather than binding. We synthesized various analogues substituted at position 18 and found that the analogues substituted at position 18 with a hydrophobic amino acid (Val, Nva, Leu, Ile, γ-MeLeu, or Phe) were ET_A receptor antagonists.[23] One of them, [Leu[18]]ET-1 (**3**), inhibited binding of [^{125}I]ET-1 to the ET_A receptor with an IC_{50} value of 8.60 nM and inhibited ET-1-induced vasoconstriction of porcine coronary artery with a pA_2 value of 5.3 (Table I). On the other hand, the analogues substituted at position 18 with a hydrophilic amino acid (Thr, Ser, or Asn) retained agonistic activity. These results suggested that a certain interaction, probably hydrogen bond formation, between a functional group at position 18 and the corresponding site of the receptor is essential for activation of the ET_A receptor.[23] Among these analogues substituted

® Abstract published in *Advance ACS Abstracts*, November 1, 1993.

0022-2623/93/1836-4087$04.00/0 © 1993 American Chemical Society

Table I. Relative Activities of the ET-1 Analogues Substituted at Positions 18 and 19 for the ET_A Receptor

no.	analogue	binding assay[a]	vasoconstriction max.[b]	antagonism[c]
1	ET-1	0.15 ± 0.0064	120 (6)	
2	ET-3	5.90 ± 0.31	>80 (6)	
3	[Leu[18]]ET-1	8.60 ± 0.62	0 (4)	5.3 (8)
4	[Thr[18]]ET-1	0.19 ± 0.0075	76 (4)	NT[d]
5	[Leu[19]]ET-1	0.75 ± 0.034	73 (6)	NT
6	[Thr[18],Leu[19]]ET-1	0.42 ± 0.013	4 (4)	7.7 (4)
7	[Thr[18],Cha[19]]ET-1	0.61 ± 0.033	2 (4)	7.7 (4)
8	[Thr[18],γ-MeLeu[19]]ET-1	0.70 ± 0.015	0 (4)	7.4 (6)
9	[Thr[18],Val[19]]ET-1	0.18 ± 0.0082	128 (6)	NT
10	[Thr[18],Nva[19]]ET-1	0.20 ± 0.0065	112 (6)	NT

[a] All data are expressed as mean of three or more determinations ± SEM nM IC_{50} values. Competitive binding versus [^{125}I]ET-1 was determined in porcine cardiac ventricular membrane. [b] Test compounds are added to the preparation in a cumulative manner. Values of vasoconstriction are normalized by that induced by 60 mM KCl as 100%, and the number of determinations is indicated in parentheses. Max. means the maximal value of vasoconstriction induced by each test compound. The highest concentration used was 1 μM. [c] Antagonism of ET-1-induced vasoconstriction was expressed as pA_2 values calculated by van Rossum's method, and the number of determinations is indicated in parentheses. [d] NT = not tested.

with a hydrophilic amino acid, [Thr[18]]ET-1 (4) showed almost the same affinity for the ET_A (IC_{50} = 0.19 nM) receptor as ET-1 (IC_{50} = 0.15 nM). Nevertheless, the maximal vasoconstriction induced by compound 4 in porcine coronary artery was significantly decreased (Table I). This leads us to believe that Asp[18] of ET-1 directly interacts with the ET_A receptor and that this interaction induces a conformational change of the receptor and subsequent activation of the signal-transduction pathways. Therefore, modification in the vicinity of position 18, even if only subtle, would significantly affect the agonist activity. We therefore thought that subtle modification in the vicinity of position 18 of compound 4 would further reduce the agonistic activity without significantly decreasing the affinity for the ET_A receptor. In this paper, we present [Thr[18]]ET-1 (4) analogues substituted at position 19 which are highly potent antagonists at both receptor subtype ET_A and ET_B.[24]

Synthesis

All ET-1 analogues were prepared using standard Boc solid-phase synthetic methods[25] on an Applied Biosystems 431A peptide synthesizer. Synthesis of the analogues was initiated from Boc-Trp(CHO)-OCH_2-Pam resin containing 0.5 mmol of Trp. N^{α}-*tert*-butyloxycarbonyl (Boc) amino acid derivatives were activated by *N,N*-dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) and coupled to the growing peptide resin whose N^{α}-Boc groups were deprotected by 50% trifluoroacetic acid (TFA)/dichloromethane (DCM) and neutralized by diisopropylethylamine (DIEA). After coupling the last amino acid, the N^{α}-Boc-protected peptide resin was treated with thiophenol in DMF to remove the 2,4-dinitrophenyl (Dnp) group on His followed by treatment with TFA/DCM. The obtained resin was then subjected to anhydrous HF in the presence of *p*-cresol and 1,4-butanedithiol as scavengers with stirring at 0 °C for 1 h. All protecting groups and peptide-resin linkages were cleaved by this treatment. The obtained tetrahydro peptides were then cyclized by air-oxidation. The insolubility of the tetrahydro peptides under neutral pH and the liability to aggregate during oxidation significantly decreased the yields of the final products. To solve these problems, we took the following measures. The tetrahydro peptides were dissolved up to a concentration of 0.2 mM in a mixture of ethanol, 1-butanol, and water (1:1:2) containing 0.1 M ammonium hydroxide. The pH of the solution was then adjusted to 8.0–8.5 with acetic acid, and the solution was kept at room temperature for 20 h with stirring. Cyclized peptides were sized by Sephadex G-50 gel filtration in 60% acetic acid/water to separate out oligomers. Since ET-1 has four Cys residues, it can demonstrate three isomeric forms of disulfide bridging—types I, II, and III—as shown in Figure 2. Gel-filtration products gave two major peaks on reverse-phase HPLC analysis. Each peak component was purified by preparative HPLC and characterized by fast atom bombardment (FAB) mass spectrometry. Since the results of molecular mass measurement for both components were virtually the same, they were expected to be disulfide bond isomers. To confirm their disulfide structures, we performed FAB mapping method,[26] and these two components were finally identified as type I and type II isomers. All peptides were analyzed for homogeneity and structural integrity by analytical HPLC, fast atom bombardment mass spectrometry (FABMS), and amino acid analysis (AAA).

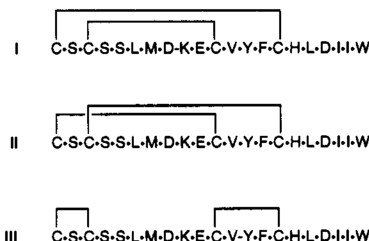

Figure 2. Structure of three possible isomers of ET-1.

Table II. Relative Activities of the ET-1 Analogues Substituted at Positions 18 and 19 for the ET_B Receptor

no.	analogue	binding assay[a]	$[Ca^{2+}]_i$[b]	antagonism[c]
1	ET-1	0.12 ± 0.0037	100 (3)	
2	ET-3	0.093 ± 0.0013	100 (3)	
3	[Leu[18]]ET-1	0.45 ± 0.026	NT[d]	NT
4	[Thr[18]]ET-1	0.33 ± 0.025	NT	NT
5	[Leu[19]]ET-1	0.17 ± 0.013	NT	NT
6	[Thr[18],Leu[19]]ET-1	0.17 ± 0.013	28 (3)	8.7[e] (3)
7	[Thr[18],Cha[19]]ET-1	0.42 ± 0.022	10 (3)	7.8[e] (3)
8	[Thr[18],γ-MeLeu[19]]ET-1	0.25 ± 0.0085	0 (3)	8.4[f] (4)

[a] All data are expressed as mean of three or more determination ± SEM nM IC_{50} values. Competitive binding versus [^{125}I]ET-1 was determined in bovine cerebrum membrane. [b] The $[Ca^{2+}]_i$ increase in murine peritoneal macrophages (MPMs) induced by each compound at a concentration of 100 nM. $[Ca^{2+}]_i$ increase induced by 100 nM ET-1 is defined as 100%, and the number of determinations is indicated in parentheses. The higher concentration of compounds 6–8 cannot induce a greater increase in $[Ca^{2+}]_i$. [c] Antagonism of sarafotoxin S6c-induced vasoconstriction in rabbit pulmonary artery. Both ET_A and ET_B receptors are expressed in this preparation. The number of determination is indicated in parentheses. [d] NT = not tested. [e] Antagonism is expressed as pD_2' value. [f] Antagonism is expressed as pA_2 value calculated by van Rossum's method.

Pharmacology

Inhibition of the binding of [^{125}I]ET-1 to receptor subtypes ET_A and ET_B was determined in porcine cardiac ventricular membrane and bovine cerebrum membrane, respectively. These tissues selectively express one endothelin receptor subtype,[27] and the IC_{50} values of ET-1 and ET-3 in Table I and II also support this. Agonist activity at the ET_A receptor was measured by vasocon-

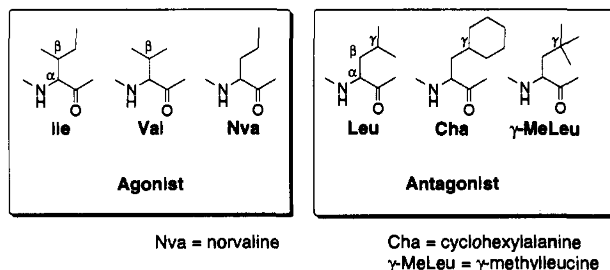

Figure 3. Side-chain structures of position 19 of the analogues with threonine in position 18.

striction in porcine coronary artery. Antagonist activity at the ET_A receptor was measured by inhibition of ET-1-induced vasoconstriction in isolated porcine coronary artery where the ET_A receptor is predominant,[18] and antagonist activity at the ET_B receptor was measured by inhibition of vasoconstriction induced by sarafotoxin S6c (S6c) in rabbit pulmonary artery.[14] Agonist-induced increases in cytosolic free calcium concentration ($[Ca^{2+}]_i$) were measured in murine peritoneal macrophages (MPMs) which express only the ET_B receptor.[28]

Results and Discussion

Table I shows the binding affinities for the ET_A receptor and the agonist and antagonist activities in porcine coronary artery.

Compounds **4** ([Thr[18]]ET-1) and **5** ([Leu[19]]ET-1) inhibited [^{125}I]ET-1 binding to the ET_A receptor with IC_{50} values of 0.19 and 0.75 nM, respectively. The maximal vasoconstriction induced by these compounds in porcine coronary artery was 76% and 73% of vasoconstriction induced by 60 mM KCl. These values are proportionate to 63% and 61% of the vasoconstriction induced by ET-1, respectively, showing that these compounds are partial agonists. Surprisingly, compound **6** ([Thr[18],Leu[19]]ET-1) which was substituted at both positions 18 and 19 caused almost no vasoconstriction (Table I) in spite of having high affinity for the ET_A receptor (IC_{50} = 0.42 nM) and was a potent antagonist inhibiting ET-1-induced vasoconstriction with a pA_2 value of 7.7. Compounds **7** and **8** were substituted at position 18 with Thr and at position 19 with cyclohexylalanine (Cha) and γ-methylleucine (γ-MeLeu) (Figure 3), unusual amino acids analogous to Leu, respectively. These substitutions caused loss of vasoconstriction activity without significant loss of affinity for the ET_A receptor, and both compounds also showed potent antagonist activities (pA_2 = 7.7 and 7.4, respectively). On the other hand, compounds **9** and **10** which were substituted at position 18 with Thr and at position 19 with Val and norvaline (Nva) (Figure 3), respectively, were agonists equipotent to ET-1.

The fact that both compounds **4** and **5** were partial agonists with high affinity for the ET_A receptor suggested that position 19 as well as position 18 was candidate site for the design of endothelin antagonists. Therefore, we synthesized compound **6** since it was expected that simultaneous substitution at positions 18 and 19 would produce an additive or synergistic effect. The results, as shown above, were even more desirable than expected. There is a very subtle structural difference between compound **4** and **6**: the substitution of Ile[19] with Leu[19]. Ile and Leu are isomers that are distinguished by whether the side-chain branches on the β-carbon or the γ-carbon. Compounds **7** and **8** were substituted at position 19 with the unusual amino acids Cha and γ-MeLeu, respectively, whose side chains branched on the γ-carbon like Leu (Figure 3). These compounds were also antagonists. On the contrary, compounds **9** and **10** were agonists equipotent to ET-1. The amino acids introduced at position 19 of these compounds are Val whose side-chain branches on the β-carbon and Nva which has a linear side chain (Figure 3). These results strongly suggest that branching on the γ-carbon of the position 19 side chain is essential for the antagonistic action of compounds **6–8**.

Table II shows the binding affinities for the ET_B receptor, activities to increase cytosolic free calcium concentration ($[Ca^{2+}]_i$) mediated through the ET_B receptor, and antagonistic activities against sarafotoxin S6c (S6c)-induced vasoconstriction in rabbit pulmonary artery. Compounds **6–8** also have high affinity for the ET_B receptor (IC_{50} = 0.17, 0.42, and 0.25 nM, respectively). ET-1 and ET-3 cause the same increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) in murine peritoneal macrophages (MPMs) where the ET_B receptor is predominantly expressed[27] and produce a maximal response at 1 nM (data not shown). Compound **8** did not induce an increase in $[Ca^{2+}]_i$ in MPMs, while compounds **6** and **7** had partial agonist activities (maximal $[Ca^{2+}]_i$ increase was 28% and 10% of that induced by ET-1, respectively, Table II). Compounds **6–7** also induced vasoconstriction in rabbit pulmonary artery, but the maximal vasoconstriction induced by these compounds was strikingly decreased (data not shown) and they had antagonist activities. Compounds **6** and **7** not only shifted the S6c dose–contraction curve to the right but also decreased the maximal level of dose-dependent contraction induced by S6c. These facts suggest that compounds **6** and **7** act as noncompetitive antagonists with PD_2' values of 8.7 and 7.8, respectively. On the other hand, compound **8** induced no vasoconstriction in rabbit pulmonary artery (data not shown) and caused competitive antagonism with a pA_2 value of 8.4.

As shown above, compound **8** is a potent antagonist which shows no agonist activity at either receptor subtype ET_A or ET_B. Compounds **6** and **7**, although having partial agonist activity at the ET_B receptor, also have antagonist activity at both subtypes. These results lead us to believe that the vicinity of position 18 of ET-1 may play a key role in activating not only the ET_A but also the ET_B receptor. The noncompetitive antagonism of compounds **6** and **7**, in contrast to the competitive antagonism of compound **8**, may result from desensitization or down-regulation induced by the partial agonist activity of these compounds.

The SAR revealed in this study is very interesting from the point that in peptide ligands like endothelin a minor positional change of one methyl group can convert an agonist to an antagonist. If we can determine why compounds **6–8** cannot function as agonists, it will help clarify the signal-transduction mechanism of endothelin receptors, and highly potent antagonists such as compounds **6–8** with no endothelin receptor-subtype selectivity will be useful as novel tools to clarify the physiological and/or pathophysiological significance of endothelin and its receptor subtypes and as therapeutic drugs for diseases in which endothelins are involved in the pathogenesis.

Experimental Section

Abbreviations used follow IUPAC–IUB nomenclature.[29] Additional abbreviations are Cha, cyclohexylalanine; γ-MeLeu, γ-methyl-L-leucine; Nva, norvaline; TFA, trifluoroacetic acid;

Table III. Peptide Analytical Data

HPLC[a] t_R[b]	FABMS $(M + H)^+$	AAA: molar ratios[c]
		[Leu[18]]ET-1 (3)
41.4	2488.9	Asp 1.00 (1); Ser 2.62 (3); Glu 1.05 (1); Cys +; Val 0.95 (1); Met 0.96 (1); Ile 0.77 (2); Leu 2.60 (3); Tyr 0.89 (1); Phe 1.01 (1); Lys 1.05 (1); His 0.89 (1)
		[Thr[18]]ET-1 (4)
41.8	2477.0	Asp 1.00 (1); Thr 0.82 (1); Ser 2.51 (3); Glu 0.96 (1); Cys +; Val 1.02 (1); Met 0.98 (1); Ile 1.06 (2); Leu 2.16 (2); Tyr 0.87 (1); Phe 0.99 (1); Lys 1.03 (1); His 0.98 (1)
		[Leu[19]]ET-1 (5)
38.6	2490.8	Asp 2.00 (2); Ser 2.59 (3); Glu 1.09 (1); Cys +; Val 0.98 (1); Met 0.93 (1); Ile 0.93 (1); Leu 3.01 (3); Tyr 0.91 (1); Phe 1.05 (1); Lys 1.00 (1); His 0.98 (1)
		[Thr[18],Leu[19]]ET-1 (6)
40.4	2477.1	Asp 1.00 (1); Thr 0.93 (1); Ser 2.56 (3); Glu 1.06 (1); Cys +; Val 0.99 (1); Met 0.99 (1); Ile 0.94 (1); Leu 3.08 (3); Tyr 0.97 (1); Phe 1.02 (1); Lys 1.00 (1); His 1.19 (1)
		[Thr[18],Cha[19]]ET-1 (7)
42.4	2517.2	Asp 1.00 (1); Thr 0.94 (1); Ser 2.55 (3); Glu 1.05 (1); Cys +; Val 0.97 (1); Met 1.01 (1); Ile 0.94 (1); Leu 2.04 (2); Tyr 0.92 (1); Phe 1.01 (1); Lys 1.00 (1); His 1.19 (1)
		[Thr[18],γ-MeLeu[19]]ET-1 (8)
41.0	2491.3	Asp 1.00 (1); Thr 0.94 (1); Ser 2.51 (3); Glu 1.05 (1); Cys +; Val 0.98 (1); Met 1.00 (1); Ile 0.92 (1); Leu 2.07 (2); Tyr 1.04 (1); Phe 0.99 (1); Lys 1.01 (1); His 1.00 (1)
		[Thr[18],Val[19]]ET-1 (9)
39.0	2463.0	Asp 1.00 (1); Thr 0.91 (1); Ser 2.48 (3); Glu 1.06 (1); Cys +; Val 1.66 (2); Met 0.92 (1); Ile 0.75 (1); Leu 1.97 (2); Tyr 0.86 (1); Phe 0.98 (1); Lys 0.98 (1); His 0.95 (1)
		[Thr[18],Nva[19]]ET-1 (10)
39.7	2462.7	Asp 1.00 (1); Thr 0.89 (1); Ser 2.48 (3); Glu 1.04 (1); Cys +; Val 0.88 (1); Met 0.98 (1); Ile 0.86 (1); Leu 1.96 (2); Tyr 0.85 (1); Phe 0.92 (1); Lys 1.00 (1); His 0.87 (1)

[a] Analytical HPLC runs in a linear gradient of CH_3CN from 0% to 80% over 80 min in 0.1% aqueous TFA. [b] t_R means retention time (min). [c] Peptides are hydrolyzed with 6 N HCl at 110 °C for 24 h. Qualitative determination of the presence of Cys indicated by "+" is due to difficult quantitation. Ile values are low due to incomplete hydrolysis, and Trp is not reported since it is destroyed during hydrolysis. Values in parentheses are theoretical ones.

HPLC, high-performance liquid chromatography; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; NMP, *N*-methylpyrrolidone; DCM, dichloromethane; DIEA, diisopropylethylamine; Bzl, benzyl; OcHex, cyclohexyl ester; BrZ, 2-bromobenzyloxycarbonyl; MeBzl, 4-methylbenzyl; ClZ, 2-chlorobenzyloxycarbonyl; Dnp, 2,4-dinitrophenyl; CHO, formyl; and Pam, phenylacetamidomethyl.

Peptide Synthesis and Purification. Peptide syntheses were performed by the solid-phase method[25] utilizing an automated synthesizer (Applied Biosystems Inc. Model 431 A) with a standard-scale Boc chemistry option. N^α-Boc-Trp(CHO)-OCH_2-Pam resin and N^α-Boc-amino acid derivatives were purchased from Applied Biosystems Japan. Fast atom bombardment mass spectrometry (FABMS) was recorded on a JEOL JMS-HX110HF double-focusing mass spectrometer. Amino acid analyses were performed on hydrolyzates (6 N HCl/24 h/110 °C) using a Hitachi amino acid analyzer L-8500. HPLC analyses were performed with a Shimadzu gradient system equipped with a variable-wavelength detector and integrator. A Shimadzu ODS-H (4.0-mm-i.d. × 150-mm) column was used and eluted with a linear gradient of 0–80% acetonitrile in water containing 0.1% TFA. The analytical data for all peptides are summarized in Table III.

Preparation of [Leu[18]]ET-1 (3). (a) Protected [Leu[18]]ET-1-resin. Synthesis was initiated from Boc-Trp(CHO)-OCH_2-Pam resin (0.5 mmol). All N^α-*tert*-butyloxycarbonyl amino acids were employed as the following side-chain-protected derivatives: Asp(OcHex), Cys(MeBzl), Glu(OcHex), His(Dnp), Lys-(ClZ), Ser(Bzl), Thr(Bzl), and Tyr(BrZ). Each amino acid derivative (2.0 mmol) activated by *N,N*-dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) was coupled to the growing peptide resin whose N^α-Boc-groups were deprotected. NMP was used as the solvent during the activation and coupling, and DIEA was used as the base. NMP and DCM were used as the solvent for washing. After coupling the last amino acid, the Boc-protected peptide resin was treated with 10 mL of DMF containing 1 mL of thiophenol for 2 h at room temperature to remove the Dnp group on His followed by treatment with 10 mL of 50% trifluoroacetic acid (TFA)/dichloromethane (DCM) containing 0.1% indole. After this treatment, the peptide-resin was dried in vacuo to yield the protected [Leu[18]]ET-1-resin (2.26 g).

(b) [Leu[18]]ET-1. The protected peptide-resin (0.5 g of 2.26 g, 110 μmol) was subjected to 5.0 mL of liquid anhydrous HF in the presence of 0.5 g of *p*-cresol and 0.5 mL of 1,4-butanedithiol as scavengers with stirring at 0 °C for 1 h. After removal of HF in vacuo, the residue was precipitated and washed with anhydrous ether followed by extraction with cold glacial TFA (30 mL, 4 °C). The extract was evaporated to a viscous syrup followed by trituration with ether. The resultant yellow powder was then dissolved in 800 mL of a mixture of ethanol, 1-butanol, and water (1:1:2) containing 0.1 M ammonium hydroxide. The pH of the solution was then adjusted to 8.0–8.5 with acetic acid. After being stirred for 20 h at room temperature, the solution was evaporated and lyophilized to a dry yellow powder. The completion of disulfide bond formation was determined by analytical HPLC. The lyophilized powder was sized by Sephadex G-50F gel filtration using 60% aqueous acetic acid as the solvent. The fraction corresponding to the monomeric peptides was collected and evaporated at 30 °C to a volume of about 10 mL. Lyophilization of this solution yielded a pale-yellow powder (77 mg, 31 μmol, 28%). The obtained powder was dissolved in 60% acetic acid (about 30 mg/0.8 mL, three times, total 97 mg) and subjected to preparative HPLC (Beckman) on a C18 column (YMC Co. Ltd., 20 × 250 mm) with a mobile phase of 34.5% acetonitrile in a 0.1% aqueous TFA solution (flow rate of a mobile phase: 8.0 mL/min). The two major peaks which were eluted successively were collected separately and evaporated to a volume of about 1 mL. Each fraction was diluted to 10 mL with water and lyophilized to yield a white powder (peak 1 = 2.8 mg, 3%; peak 2 = 21 mg, 21%; each peak is numbered according to the order of the retention time). The results of molecular mass measurements for peaks 1 and 2 were virtually the same, and they were disulfide bond isomers. Their disulfide structure was confirmed by FAB mapping method as described previously.[26,30] The component of peak 2 was finally identified as [Leu[18]]ET-1 having correct disulfide linkages (Cys[1]-Cys[15] and Cys[3]-Cys[11]). The purity of the final product was verified by analytical HPLC, and

the structure assignment was corroborated by the amino acid analysis and FABMS results (Table III).

[Thr[18]]ET-1 (4). Compound 4 was synthesized and purified in a manner similar to that employed for compound 3. The protected peptide-resin (0.50 g of 2.30 g, 109 μmol) was subjected to deprotection followed by oxidation. After oxidation, peptides were sized by Sephadex G-50F gel filtration, and the monomer fraction was collected (109 mg, 42%). Gel-filtered peptides (28 mg) were purified by preparative HPLC on a C18 column with a mobile phase of 32.0% acetonitrile in 0.1% aqueous TFA. The two major peaks which were eluted successively were collected separately (peak 1, 0.8 mg, 3%; peak 2, 5.8 mg, 21%). The results of molecular mass measurement for peaks 1 and 2 were virtually the same, and they were disulfide bond isomers. Their disulfide structures were confirmed by FAB mapping method as described previously.[26,30] The component of peak 2 was finally identified as [Thr[18]]ET-1 having correct disulfide linkages (Cys[1]-Cys[15] and Cys[3]-Cys[11]). The purity of the final product was verified by analytical HPLC, and the structure assignment was corroborated by the amino acid analysis and FABMS results (Table III).

[Leu[19]]ET-1 (5). Compound 5 was synthesized and purified in a manner similar to that employed for compound 3. The protected peptide-resin (0.60 g of 2.31 g, 130 μmol) was subjected to deprotection followed by oxidation. After oxidation, peptides were sized by Sephadex G-50F gel filtration, and the monomer fraction was collected (133 mg, 44%). Gel-filtered peptides (46 mg) were purified by preparative HPLC on a C18 column with a mobile phase of 30.0% acetonitrile in 0.1% aqueous TFA. The two major peaks which were eluted successively were collected separately (peak 1, 1.3 mg, 3%; peak 2, 11 mg, 25%). The results of molecular mass measurement for peaks 1 and 2 were virtually the same, and they were disulfide bond isomers. Their disulfide structures were confirmed by FAB mapping method as described previously.[26,30] The component of peak 2 was finally identified as [Leu[19]]ET-1 having correct disulfide linkages (Cys[1]-Cys[15] and Cys[3]-Cys[11]). The purity of the final product was verified by analytical HPLC, and the structure assignment was corroborated by the amino acid analysis and FABMS results (Table III).

[Thr[18],Leu[19]]ET-1 (6). Compound 6 was synthesized and purified in a manner similar to that employed for compound 3. The protected peptide-resin (0.50 g of 2.28 g, 110 μmol) was subjected to deprotection followed by oxidation. After oxidation, peptides were sized by Sephadex G-50F gel filtration, and the monomer fraction was collected (128 mg, 51%). Gel-filtered peptides (31 mg) were purified by preparative HPLC on a C18 column with a mobile phase of 30.0% acetonitrile in 0.1% aqueous TFA. The two major peaks which were eluted successively were collected separately (peak 1, 1.3 mg, 4%; peak 2, 5.5 mg, 18%). The results of molecular mass measurement for peaks 1 and 2 were virtually the same, and they were disulfide bond isomers. Their disulfide structures were confirmed by FAB mapping method as described previously.[26,30] The component of peak 2 was finally identified as [Thr[18],Leu[19]]ET-1 having correct dislufide linkages (Cys[1]-Cys[15] and Cys[3]-Cys[11]). The purity of the final product was verified by analytical HPLC, and the structure assignment was corroborated by the amino acid analysis and FABMS results (Table III).

[Thr[18],Cha[19]]ET-1 (7). Compound 7 was synthesized and purified in a manner similar to that employed for compound 3. The protected peptide-resin (0.50 g of 2.31 g, 108 μmol) was subjected to deprotection followed by oxidation. After oxidation, peptides were sized by Sephadex G-50F gel filtration, and the monomer fraction was collected (116 mg, 46%). Gel-filtered peptides (21 mg) were purified by preparative HPLC on a C18 column with a mobile phase of 32.0% acetonitrile in 0.1% aqueous TFA. The two major peaks which were eluted successively were collected separately (peak 1, 0.9 mg, 4%; peak 2, 4.5 mg, 22%). The results of molecular mass measurement for peaks 1 and 2 were virtually the same, and they were disulfide bond isomers. Their disulfide structures were confirmed by FAB mapping method as described previously.[26,30] The component of peak 2 was finally identified as [Thr[18],Cha[19]]ET-1 having correct disulfide linkages (Cys[1]-Cys[15] and Cys[3]-Cys[11]). The purity of the final product was verified by analytical HPLC, and the structure assignment was corroborated by the amino acid anaysis and FABMS results (Table III).

[Thr[18],γ-MeLeu[19]]ET-1 (8). Compound 8 was synthesized and purified in a manner similar to that employed for compound 3. The protected peptide-resin (0.50 g of 2.28 g, 110 μmol) was subjected to deprotection followed by oxidation. After oxidation, peptides were sized by Sephadex G-50F gel filtration, and the monomer fraction was collected (115 mg, 46%). Gel-filtered peptides (44 mg) were purified by preparative HPLC on a C18 column with a mobile phase of 31.0% acetonitrile in 0.1% aqueous TFA. The two major peaks which were eluted successively were collected separately (peak 1, 0.8 mg, 2%; peak 2, 8.0 mg, 18%). The results of molecular mass measurement for peaks 1 and 2 were virtually the same, and they were disulfide bond isomers. Their disulfide structures were confirmed by FAB mapping method as described previously.[26,30] The component of peak 2 was finally identified as [Thr[18],γ-MeLeu[19]]ET-1 having correct disulfide linkages (Cys[1]-Cys[15] and Cys[3]-Cys[11]). The purity of the final product was verified by analytical HPLC, and the structure assignment was corroborated by the amino acid analysis and FABMS results (Table III).

[Thr[18],Val[19]]ET-1 (9). Compound 9 was synthesized and purified in a manner similar to that employed for compound 3. The protected peptide-resin (0.50 g of 2.37 g, 105 μmol) was subjected to deprotection followed by oxidation. After oxidation, peptides were sized by Sephadex G-50F gel filtration, and the monomer fraction was collected (80 mg, 32%). Gel-filtered peptides (46 mg) were purified by preparative HPLC on a C18 column with a mobile phase of 30.0% acetonitrile in 0.1% aqueous TFA. The two major peaks which were eluted successively were collected separately (peak 1, 2.0 mg, 4%; peak 2, 14 mg, 31%). The results of molecular mass measurement for peaks 1 and 2 were virtually the same, and they were disulfide bond isomers. Their disulfide structures were confirmed by FAB mapping method as described previously.[26,30] The component of peak 2 was finally identified as [Thr[18],Val[19]]ET-1 having correct disulfide linkages (Cys[1]-Cys[15] and Cys[3]-Cys[11]). The purity of the final product was verified by analytical HPLC, and the structure assignment was corroborated by the amino acid analysis and FABMS results (Table III).

[Thr[18],Nva[19]]ET-1 (10). Compound 10 was synthesized and purified in a manner similar to that employed for compound 3. The protected peptide-resin (0.50 g of 2.30 g, 109 μmol) was subjected to deprotection followed by oxidation. After oxidation, peptides were sized by Sephadex G-50F gel filtration, and the monomer fraction was collected (122 mg, 49%). Gel-filtered peptides (39 mg) were purified by preparative HPLC on a C18 column with a mobile phase of 30.0% acetonitrile in 0.1% aqueous TFA. The two major peaks which were eluted successively were collected separately (peak 1, 2.9 mg, 7%; peak 2, 10 mg, 26%). The results of molecular mass measurements for peaks 1 and 2 were virtually the same, and they were disulfide bond isomers. Their disulfide structures were confirmed by FAB mapping method as described previously.[26,30] The component of peak 2 was finally identified as [Thr[18],Nva[19]]ET-1 having correct disulfide linkages (Cys[1]-Cys[15] and Cys[3]-Cys[11]). The purity of the final product was verified by analytical HPLC, and the structure was corroborated by the amino acid analysis and FABMS results (Table III).

Biological Assays. Binding Assay. (a) Preparation of Porcine Cardiac Ventricular Membrane. Porcine cardiac membrane was prepared following the method described by Jones et al.[31] Briefly, cardiac ventricular muscle was minced and homogenized with a Polytron homogenizer in 10 mM $NaHCO_3$ buffer containing 0.5 mM EDTA, 0.5 mM PMSF, 1.0 μg/mL pepstatin, and 0.02% NaN_3. The homogenate was centrifuged at 14000*g* for 15 min. The resultant supernatant was ultracentrifuged at 100000*g* for 1 h to obtain a membrane pellet. The membrane was washed with 0.6 M KCl–20 mM phosphate buffer (pH 7.0) containing the protease inhibitors and stored at −70 °C as a suspension in 20 mM phosphate buffer (pH 7.0) containing 0.25 M sucrose, 0.3 M KCl, and the protease inhibitors.

(b) Preparation of Bovine Cerebrum Membrane. Bovine brain was homogenized with a Polytron homogenizer in 0.25 M sucrose buffer (pH 7.4) containing 20 mM Tris, 10 mM EDTA, 0.5 mM PMSF, 20 μg/mL leupeptin, 4 μg/mL E-64, and 1 μg/mL

pepstatin. The homogenate was centrifuged at 680g for 15 min, and the supernatant was ultracentrifuged at 100000g for 1 h. The pellet was washed and suspended in 20 mM Tris buffer containing 5 mM EDTA and the protease inhibitors (pH 7.4).

(c) **Assay.** The membrane fractions of porcine cardiac ventricular muscle and bovine cerebrum were diluted with an assay buffer consisting of 20 mM Tris, 2 mM EGTA, 5 mM magnesium acetate, 0.1% BSA, 0.5 mM PMSF, 20 μg/mL leupeptin, 4 μg/mL E-64, 1 μg/mL pepstatin, and 0.03% NaN_3 (pH 7.2) to 12 and 150 μg/mL, respectively. An aliquot of the diluted membrane was incubated with 100 pM [^{125}I]ET-1 (Amersham) and various concentrations of a test compound at 25 °C for 1 h. The mixture was diluted with chilled assay buffer supplemented with 0.05% CHAPS and filtered through a glass fiber filter GF/F (Whatmann). Radioactivity on the filter was counted in a γ-counter to determine bound [^{125}I]ET-1. The concentration of test compound causing 50% inhibition of the specific binding (IC_{50} value) was derived by fitting the data into a pseudo Hill equation:

$$\log[\%SPB/(100 - \%SPB)] = n[\log C - \log(IC_{50})]$$

where %SPB is specific binding as a percentage of maximum specific binding, n is a pseudo Hill constant, and C is the concentration of test compound.

Vasoconstriction Assay. Right coronary arteries and rabbit pulmonary arteries were isolated from fresh porcine tissues and cut into 2 × 5-mm helical strips and 5-mm ring preparations, respectively. The endothelium of the preparations was denuded by rubbing the lumen. The helical strips and ring preparations were placed in organ baths containing Krebs-Henseleit solution bubbled with 95% O_2–5% CO_2 at 37 °C, and the tension was measured isometrically. Concentration–response curves for the peptides were obtained by cumulative application. The tension was normalized using 60 mM KCl-induced tension in each preparation. In antagonism experiments, test compounds were added 15 min before the application of ET-1 or S6c. pA_2 values were calculated by van Rossum's method.[32]

$[Ca^{2+}]_i$ Assay. Cytosolic free calcium ion concentration ($[Ca^{2+}]_i$) of murine peritoneal macrophages was measured by the method described previously with modification.[28] Briefly, peritoneal macrophages were collected from male ICR mice, treated intraperitoneally with 2 mL of 10% protease peptone 3 days earlier. The cells were plated on glass coverslips (1.5 × 10^6 cells/coverslip) in RPMI-1640 supplemented with 5% fetal calf serum and incubated at 37 °C for 1.5 h. The adherent cells on the coverslips were then loaded with 4 μM fura-2 acetoxymethyl ester at 24 °C for 1.5 h in a Hank's Balanced Salt Solution with 0.2 g/L $NaHCO_3$ and 0.05% bovine serum albumin (HBSS). The coverslips were mounted in quartz cuvettes in HBSS and maintained at 37 °C with constant stirring. Fluorescence was measured with a spectrofluorometer (Shimadzu RF-5000) set at 340 and 380 nm for excitation and 505 nm for emission. Maximum and minimum fluorescence was estimated after addition of ionomycin (2 μM) and glycol ether diamine *N,N,N',N'*-tetraacetic acid (8 mM), respectively. The transient increase in $[Ca^{2+}]_i$ induced by 20 nM of ET-3 was calculated to be 282 ± 15 nm (mean ± SE) according to the methods of Grynkiewicz et al.[33]

Acknowledgment. We wish to thank Drs. E. Mizuta, Y. Ishibashi, and S. Endo for their helpful advice. We also express our thanks to Mr. T. Watanabe, Mr. Y. Masuda, Mr. T. Sugoh, Mr. M. Harada, Miss M. Sato, and Miss M. Abe for biological assays, to Miss H. Yoshida for physicochemical analyses, and to Mr. J. A. Hogan for his critical reading of the manuscript.

Supplementary Material Available: Data for the disulfide-structure determination and charts of analytical HPLC for compounds 3–10 are provided (11 pages). Ordering information is given on any current masthead page.

References

(1) Yanagisawa, M.; Kurihara, H.; Kimura, S.; Tomobe, Y.; Kobayashi, M.; Mitsui, Y.; Yazaki, Y.; Goto, K.; Masaki, T. A Novel Potent Vasoconstrictor Peptide Produced by Vascular Endothelial Cells. *Nature* **1988**, *332*, 411–415.

(2) Inoue, A.; Yanagisawa, M.; Kimura, S.; Kasuya, Y.; Miyauchi, T.; Goto, K.; Masaki, T. The Human Endothelin Family: Three Structurally and Pharmacologically Distinct Isopeptides Predicted by Three Separate Genes. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2863–2967.

(3) Watanabe, H.; Miyazaki, H.; Kondoh, M.; Masuda, Y.; Kimura, S.; Yanagisawa, M.; Masaki, T.; Murakami, K. Two Distinct Types of Endothelin Receptors are Present on Chick Cardiac Membranes. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 1252–1259.

(4) Kloog, Y.; Bousso-Mittler, D.; Bdolah, A.; Sokolovsky, M. Three Apparent Receptor Subtypes for the Endothelin/Sarafotoxin Family. *FEBS Lett.* **1989**, *253*, 199–202.

(5) Arai, H.; Hori, S.; Aramori, I.; Ohkubo, H.; Nakanishi, S. Cloning and Expression of a cDNA Encoding an Endothelin Receptor. *Nature* **1990**, *348*, 730–732.

(6) Sakurai, T.; Yanagisawa, M.; Takuwa, Y.; Miyazaki, H.; Kimura, S.; Goto, K.; Masaki, T. Cloning of a cDNA Encoding a Non-Isopeptide-Selective Subtype of the Endothelin Receptor. *Nature* **1990**, *348*, 732–735.

(7) Hosoda, K.; Nakao, K.; Arai, H.; Suga, S.; Ogawa, Y.; Mukoyama, M.; Shirakami, G.; Saito, Y.; Nakanishi, S.; Imura, H. Cloning and Expression of Human Endothelin-1 Receptor cDNA. *FEBS Lett.* **1991**, *287*, 23–26.

(8) Ogawa, Y.; Nakao, K.; Arai, H.; Nakagawa, O.; Hosoda, K.; Suga, S.; Nakanishi, S.; Imura, H. Molecular Cloning of a Non-Isopeptide-Selective Human Endothelin Receptor. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 248–255.

(9) Takayanagi, R.; Kitazumi, K.; Takasaki, C.; Ohnaka, K.; Aimoto, S.; Tasaka, K.; Ohashi, M.; Nawata, H. Presence of Non-Selective Type of Endothelin Receptor on Vascular Endothelium and Its Linkage to Vasodilation. *FEBS Lett.* **1991**, *282*, 103–106.

(10) Saeki, T.; Ihara, M.; Fukuroda, T.; Yamagiwa, M.; Yano, M. [Ala^{1,3,11,15}]Endothelin-1 Analogs with ET_B Agonistic Activity. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 286–292.

(11) Hiley, C. R.; Jones, C. R.; Pelton, J. T.; Miller, R. C. Binding of [^{125}I]-Endothelin-1 to Rat Cerebellar Homogenates and Its Interactions with Some Analogues. *Br. J. Pharmacol.* **1990**, *101*, 319–324.

(12) Williams, D. L., Jr.; Jones, K. L.; Pettibone, D. J.; Lis, E. V.; Clineschmidt, B. V. Sarafotoxin S6c: An Agonist Which Distinguishes between Endothelin Receptor Subtypes. *Biochem. Biophys. Res. Commun.* **1991**, *175*, 556–561.

(13) Emori, T.; Hirata, Y.; Marumo, F. Specific Receptors for Endothelin-3 in Cultured Bovine Endothelial Cells and Its Cellular Mechanism of Action. *FEBS Lett.* **1990**, *263*, 261–264.

(14) Panek, R. L.; Major, T. C.; Hingorani, G. P.; Doherty, A. M.; Taylor, D. G.; Rapundalo, S. T. Endothelin and Structurally Related Analogs Distinguish between Endothelin Receptor Subtypes. *Biochem. Biophys. Res. Commun.* **1992**, *183*, 566–571.

(15) Moreland, S.; McMullen, D. M.; Delaney, C. L.; Lee, V. G.; Hunt, J. T. Venous Smooth Muscle Contains Vasoconstrictor ET_B-like Receptors. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 100–106.

(16) Spinella, M. J.; Malik, A. B.; Everitt, J.; Andersen, T. T. Design and Synthesis of a Specific Endothelin-1 Antagonist: Effects on Pulmonary Vasoconstriction. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *86*, 7443–7446.

(17) Urade, Y.; Fujitani, Y.; Oda, K.; Watakabe, T.; Umemura, I.; Takai, M.; Okada, T.; Sakata, K.; Karaki, H. An Endothelin B Receptor-Selective Antagonist: IRL 1038, [Cys^{11}-Cys^{15}]Endothelin-1(11–21). *FEBS Lett.* **1992**, *311*, 12–16.

(18) Ihara, M.; Noguchi, K.; Saeki, T.; Fukuroda, T.; Tsuchida, S.; Kimura, S.; Fukami, T.; Ishikawa, K.; Nishikibe, M.; Yano, M. Biological Profiles of Highly Potent Novel Endothelin Antagonists Selective for the ET_A Receptor. *Life Sci.* **1992**, *50*, 247–255.

(19) Aramori, I.; Nirei, H.; Shoubo, M.; Sogabe, K.; Nakamura, K.; Kojo, H.; Notsu, Y.; Ono, T.; Nakanishi, S. Subtype Selectivity of a Novel Endothelin Antagonist FR 139317, for the Two Endothelin Receptors in Transfected Chinese Hamster Ovary Cells. *Mol. Pharmacol.* **1993**, *43*, 127.

(20) Cody, W. L.; Doherty, A. M.; He, J. X.; DePue, P. L.; Topliss, J. G.; Haleen, S. J.; LaDouceur, D. M.; Flynn, M. A.; Hill, K. E.; Reynolds, E. E. Structure-Activity Relationships in the C-Terminus of Endothelin-1 (ET-1): The Discovery of Potent Antagonists. In *Peptides 1992, Proceedings of the 22nd European Peptide Symposium*; Schneider, C. H., Eberle, A. N., Eds.; ESCOM: The Netherlands, 1993; pp 687–688.

(21) Nakajima, K.; Kubo, S.; Kumagaye, S.; Nishio, H.; Tsunemi, M.; Inui, T.; Kuroda, H.; Chino, N.; Watanabe, T. X.; Kimura, T.; Sakakibara, S. Structure-Activity Relationship of Endothelin: Importance of Charged Groups. *Biochem. Biophys. Res. Commun.* **1989**, *163*, 424–429.

(22) Hunt, J. T.; Lee, V. G.; Stein, P. D.; Hedberg, A.; Liu, E. C.-K.; McMullen, D.; Moreland, S. Structure-Activity Relationships of Monocyclic Endothelin Analogs. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 33–38.

(23) Kikuchi, T.; Kubo, K.; Ohtaki, T.; Watanabe, T.; Wakimasu, M.; Fujino, M. Structure-Activity Relationships of Endothelin. In *Peptide Chemistry 1991*; Suzuki, A., Ed.; Protein Research Foundation: Osaka, 1992; pp 209–214.

(24) A part of this report was preliminarily disclosed as follows: Wakimasu, M.; Kikuchi, T.; Kubo, K.; Asami, T.; Ohtaki, T.; Fujino, M. Studies on Endothelin Antagonists. In *Perspectives in Medicinal Chemistry*; Testa, B., Kyburz, E., Fuhrer, W., Giger, R., Eds.; Verlag Helvetica Chimica Acta: Basel, 1993; pp 165–177.

(25) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154. (b) Stewart, J. M.; Young, J. D. In *Solid Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984.

(26) (a) Ishibashi, Y.; Kikuchi, T.; Wakimasu, M.; Mizuta, E.; Fujino, M. Assignment of Disulfide Bonds in Synthetic Endothelin Analog Isomers by FAB Mass Spectrometry. In *Peptide Chemistry 1991*; Suzuki, A., Ed.; Protein Research Foundation: Osaka, 1992; pp 333–338. (b) Ishibashi, Y.; Kikuchi, T.; Wakimasu, M.; Mizuta, E.; Fujino, M. Assignment of Disulfide Bonds in Synthetic Endothelin-1 Isomers by Fast Atom Bombardment Mass Spectrometry. *Biol. Mass Spectrom.* **1991**, *20*, 703–708.

(27) (a) Takayanagi, R.; Ohnaka, K.; Takasaki, C.; Ohashi, M.; Nawata, H. Multiple Subtypes of Endothelin Receptors in Porcine Tissues: Characterization by Ligand Binding, Affinity Labeling and Regional Distribution. *Regul. Pep.* **1991**, *32*, 23–37. (b) Hori, S.; Komatsu, Y.; Shigemoto, R.; Mizuno, N.; Nakanishi, S. Distinct Tissue Distribution and Cellular Localization of Two Messenger Ribonucleic Acids and Encoding Different Subtypes of Rat Endothelin Receptors. *Endocrinology* **1992**, *130*, 1885–1895.

(28) Kishino, J.; Hanasaki, K.; Kato, T.; Arita, H. Endothelin-Induced Intracellular Ca^{2+} Mobilization through Its Specific Receptors in Murine Peritoneal Macrophages. *FEBS Lett.* **1991**, *280*, 103–106.

(29) Nomenclature and symbolism for amino acids and peptides. *Eur. J. Biochem.* **1984**, *138*, 9–37.

(30) Methods and data for the disulfide-structure determination of compounds **3–10** can be obtained as supplementary material.

(31) Jones, L. R.; Besch, H. R., Jr.; Fleming, J. W.; McConnaughey, M. M.; Watanabe, A. M. Separation of Vesicles of Cardiac Sarcolemma from Vesicles of Cardiac Sarcoplasmic Reticulum. Comparative Biochemical Analysis of Component Activities. *J. Biol. Chem.* **1979**, *254*, 530–539.

(32) van Rossum, J. M. Techniques for the Making of Dose-Response Curves in Isolated Organs and Evaluation of Drug Parameters. *Arch. Int. Pharmacodyn.* **1963**, *143*, 299–331.

(33) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A New Generation of Ca^{2+} Indicators with Greatly Improved Fluorescence Properties. *J. Biol. Chem.* **1985**, 260, 3440–3450.