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## Articles

# Structure–Activity Relationships of C-Terminal Endothelin Hexapeptide Antagonists

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The discovery of selective endothelin (ET) receptor antagonists will facilitate identification of the physiological and pathological roles for ET and its isopeptides. Structure–activity studies of the C-terminal hexapeptide of ET have been carried out to elucidate those amino acids important for receptor binding and agonist or antagonist activity. Binding studies were performed in rat heart ventricle, rabbit renal artery vascular smooth muscle cells, and rat cerebellum. In addition, many of the compounds have been evaluated functionally for their effects on endothelin-1-induced arachidonic acid release and inositol phosphate accumulation in specific cell lines. Selected compounds have been evaluated in a functional bioassay in tissue preparations specifically expressing either $ET_A$ or $ET_B$ receptors. We have previously described the structure–activity relationships in the hydrophobic C-terminal hexapeptide of ET, a region known to be highly important for receptor recognition. A mono-D-amino acid scan of the ET[16–21] revealed that substitution at $His^{16}$ gave rise to analogs with significantly enhanced binding affinity. We have further evaluated the C-terminal region and will describe the design, synthesis, and pharmacological evaluation of several novel and potent ET peptide receptor antagonists.

## Introduction

There is considerable interest in vascular control mechanisms and especially in the vasomotor role of endogenous peptides. Endothelin (ET) is a potent vasoconstrictor peptide that was first isolated and characterized from porcine aortic endothelial cells.[1,2] Immediately after the first report on ET it was demonstrated that there were in fact three distinct genes encoding a family of isopeptides, ET-1, ET-2, and ET-3. One of these genes encoded a peptide identical to ET, and thus ET and ET-1 are now often used interchangeably in the literature. These peptides have 21 amino acids including two disulfide bonds between positions 1–15 and 3–11.[3–7] In addition to being a potent and long acting vasoconstrictor, ET-1 elicits many other important biological properties reviewed recently.[3–7] It is currently not known whether ET-1 is secreted as a paracrine or autocrine regulator or whether its actions are physiological and/or pathophysiological in nature. The discovery of selective endothelin (ET) receptor antagonists will facilitate identification of the physiological and/or pathological roles for ET-1 and its isopeptides.

Many of the structure–activity studies of endothelin analogs and fragments have evaluated compounds in a variety of animal tissues with unknown receptor subtype populations.[8–10] An exciting advance in the ET field occurred with the cloning and expression of two ET receptor subtypes termed $ET_A$ and $ET_B$ from bovine and rat lung, respectively.[11,12] More recently, the human receptors have also been cloned.[13,14] In addition, a report of the cloning and characterization of an ET-3 specific

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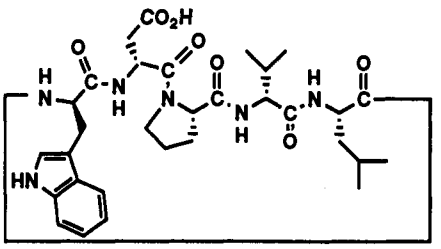
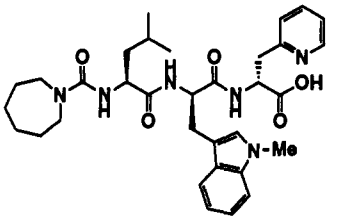
receptor from Xenopus melanophores has appeared.[15] The $ET_A$ receptor clearly mediates the vasoconstrictor and mitogenic responses[16] of ET and is widely localized in vascular smooth muscle of cardiovascular tissue origin and in certain regions of the brain.[11,17] Several groups have recently reported the discovery of $ET_A$ specific antagonists that should prove useful in elucidating the role of the $ET_A$ receptor in disease models. An ET-1 analog with a lactam bridge replacing one of the disulfide linkages was the first reported ET receptor antagonist.[18] Shortly thereafter, several groups reported $ET_A$ selective antagonists, discovered by random screening techniques. Thus, compound 1 (BQ-123), a cyclic pentapeptide, and 2 (FR139317),



D-TRP-D-ASP-PRO-D-VAL-LEU

Compound 1 (BQ-123)

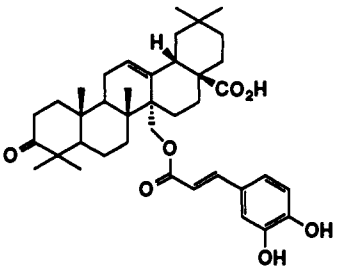


(Hexahydro-1H-azepinyl)carbonyl-Leu-(N-Me)-D-Trp-D-2-Pya

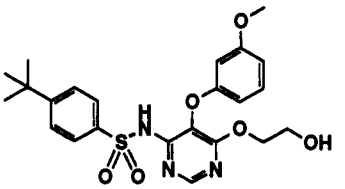
Compound 2 (FR 139317)

a linear tripeptide, are representative classes of molecules binding selectively to the $ET_A$ receptor.[19-23] Interestingly, 1 has been reported to antagonize both ET-1- and angiotensin II-induced contraction in rabbit aorta.[24] Very recently, the first nonpeptide $ET_A$ receptor antagonist 3 was reported, isolated from bayberry, *Myrica cerifera*.[25] This compound selectively antagonized specific binding of [$^{125}$I]ET-1 but not of [$^{125}$I]ET-3 to rat cardiac membranes. Compound 3 also antagonized ET-1-induced increase in intracellular free calcium concentration in Swiss 3T3 fibroblasts and ET-1 induced contraction of rat aortic strips. Several other nonpeptide antagonists[26,27] have recently been reported including the sulfonylamide Ro 46-2005 (compound 4).[27]

The $ET_B$ or nonselective receptor recognizing the ET isopeptides with equal affinity was originally known as the nonvascular smooth muscle receptor, but this latter description has been found to be inaccurate.[12] This receptor is now known to be localized on endothelial cells and on vascular smooth muscle of certain tissues. The nonselective receptor has been associated with vasodilator activity perhaps through the release of endothelium-derived relaxing factor (EDRF) or various arachidonic acid metabolites.[28,29] However, several tissues including rabbit pulmonary artery recognize all three isopeptides with equal



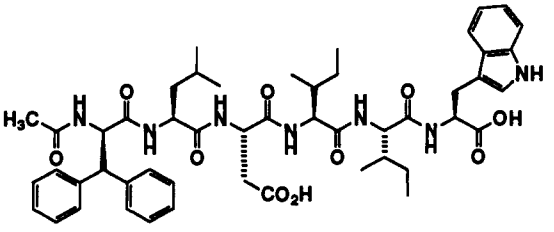
Compound 3



Compound 4 (Ro 46-2005)

affinity (thus it has $ET_B$ like characteristics) and mediate vasoconstrictor responses.[30,31] Several other groups have also reported that selective $ET_B$ agonists cause vasoconstrictor as well as vasodilator activities.[32-34] The related snake venom peptide sarafotoxin 6c (SRTX-6c), a selective $ET_B$ agonist, induced a transient decrease in blood pressure, followed by a long-lasting pressor response accompanied by marked renal and mesenteric vasoconstriction when administered *in vivo*.[31] A linear analog, *N*-acetyl-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp (BQ-3020), elicited endothelium-dependent vasodilatation in norepinephrine-precontracted porcine pulmonary artery but caused vasoconstriction in rabbit pulmonary artery.[34] Thus, it is clear that the $ET_B$ receptor mediates both vasoconstrictor and vasodilator responses in different tissue beds, but it is not clear whether the $ET_B$ receptor localized to endothelial cells is the same as that present in the brain and/or vascular smooth muscle. Pharmacological evidence for the presence of $ET_B$ subtypes has begun to appear in the literature.[35] In addition, it is now becoming evident that species differences will prove to be important in endothelin pharmacology.[36]

We have recently reported the discovery of linear hexapeptide analogues of the C-terminal region of endothelin that bind to both $ET_A$ (rabbit renal VSMC) and $ET_B$ (rat cerebellum) receptors.[37-39] From this work we discovered the hexapeptide PD 142893, compound 5, to be a functional antagonist inhibiting ET-1 induced contraction at both $ET_A$ and $ET_B$ type tissues.[37-43]



Ac-D-Dip-Leu-Asp-Ile-Ile-Trp

Compound 5 (PD 142893)

Structure–activity studies of ET-1-related peptides have indicated that the C-terminal Trp residue is important

for the vasoconstrictor activity of endothelin in porcine coronary artery strips ($ET_A$).[44] ET[1–15] has been shown to be inactive,[45] while the hydrophobic C-terminal hexapeptide ET[16–21] has been reported to be a partial agonist in guinea pig isolated bronchus, rat vas deferens, and rabbit pulmonary artery.[46,47] Structure–activity studies of the C-terminal hexapeptide of ET-1 have indicated the importance of individual amino acids to receptor recognition in a variety of tissue beds.[48,49] However, we have found that this fragment is devoid of functional activity in several tissue preparations including rat aorta and rabbit pulmonary artery.[49] Other studies attempting to demonstrate the agonist activity originally reported for the ET[16–21] fragment have likewise met with little success.[50,51] Maggi et al. have explained this disparity by invoking that the agonist activity of ET[16–21] in certain tissues may be due to its selective action at a single receptor subtype not expressed in cardiovascular tissues.[28,29] However, it is clear that the C-terminal hexapeptide, possessing very weak binding affinity in a number of animal tissues at both known $ET_A$ and $ET_B$ receptor subtypes (50–70 $\mu$M), is important for receptor recognition.[49] We have carried out structural modifications of the C-terminal region of ET-1 in an attempt to enhance its activity and to discover $ET_A$ and $ET_B$ antagonists. Using rational design techniques, we have discovered potent analogs of the C-terminal hexapeptide that are functional ET receptor antagonists.[39–43] These compounds should prove useful to study the role of ET in normal physiology and in the pathophysiology of human diseases.

## Chemistry

**Peptide Synthesis and Characterization.** All of the peptides were synthesized by solid-phase synthesis techniques.[52–54] The analogs were prepared using either an *N*-$\alpha$-Boc protection scheme with either a PAM (phenylacetamidomethyl) resin or an FMOC protection scheme on an HMP resin [[4-(hydroxymethyl)phenoxy]methyl]. The peptides prepared by Boc chemistry were deprotected and cleaved from the resin using anhydrous liquid HF/anisole or HF/*p*-cresol (9:1 v/v) followed by N-terminal acetylation with 90% acetic acid and an excess of acetic anhydride. The peptides prepared by an *N*-$\alpha$-FMOC protection scheme were deprotected with 20% piperidine in *N*-methylpyrrolidinone. Final deprotection and cleavage from the resin was carried out with a mixture of TFA–thioanisole–ethanedithiol–$H_2O$ (89:5:2:3:1). In both cases, the protected peptide could be acetylated while the peptide was still on the resin in methylene chloride with an excess of 1-acetylimidazole.

Crude peptides were then purified by preparative reversed-phase HPLC eluting with a linear gradient of 0.1% aqueous TFA with increasing concentrations of acetonitrile. Peptides were isolated by lyophilization and assessed for homogeneity by analytical HPLC and TLC and characterized by amino acid analysis, FAB mass spectroscopy, proton NMR spectroscopy and elemental analysis (Table I). The solid-phase synthesis of compound **5** is shown in Scheme I as an example. The unnatural amino acid 3,3-D-diphenylalanine (D-Dip) was synthesized according to a previously reported method.[55] The disodium salt of the peptides could be synthesized by treatment of the free diacid in methanol with aqueous sodium hydroxide solution (2 equiv) followed by filtration and lyophilization.

## Results and Discussion

A mono-D-amino acid scan of the 16–21 region of ET-1 (compounds **6–11**) gave rise to several analogs with enhanced binding affinity over the natural C-terminal hexapeptide Ac-His$^{16}$-Leu-Asp-Ile-Ile-Trp$^{21}$, compound **6** (Table I). Ac-D-His$^{16}$-Leu-Asp-Ile-Ile-Trp$^{21}$, **12**, exhibited a >10-fold enhancement in binding affinity compared with **6** (see Table II) in rat heart ventricle, rabbit pulmonary artery, and rat aorta.[49] When the existence of receptor subtypes was first reported we began to evaluate compounds at tissues known to express the individual subtypes. We discovered that **12** was approximately equipotent at both $ET_A$ and $ET_B$ receptors.[38] Compound **12** is also a functional antagonist of ET-1-induced inositol phosphate (IP) accumulation in rat skin fibroblasts and inhibits ET-1-induced arachidonic acid release (AAR) in rabbit renal vascular smooth muscle cells, both $ET_A$ functional assays.[7,37,40,56] SRTX-6c, a selective $ET_B$ agonist, does not induce arachidonic acid release in these cells. Unfortunately, compound **12** did not inhibit ET-1-induced vasoconstrictor activity in a bioassay with either known $ET_A$ (rabbit femoral or renal artery) or $ET_B$ (rabbit pulmonary artery) tissues (Figure 1). Thus, we attempted to increase the potency of **12**.

Earlier in our research program we reported that the full length analog, ET-1[16-*L*-Phe], was a 5-fold more potent agonist than ET-1 itself in the rat aorta ($ET_A$) and in rabbit pulmonary artery ($ET_B$).[31] Substitution of the D-His$^{16}$ residue with D-Phe$^{16}$ in the C-terminal hexapeptide series also led to an slight enhancement in binding affinity at both receptor subtypes. Functional antagonism as measured by inhibition of ET-1-induced arachidonic acid release was essentially identical for both compounds **12** and **13**. Comparison of the binding affinities in vascular smooth muscle cells ($ET_A$) and in rat cerebellum ($ET_A$) indicates that compound **13** is a reasonably nonselective $ET_A/ET_B$ antagonist.

In an attempt to understand the importance of each of the residues in this hexapeptide series to binding at each receptor subtype, several substitutions were incorporated at each of the positions in turn in **13** (Table III). Comparison of compounds **6** with **12** and **13** with **15** indicates that D-stereochemistry at position 16 affords a large increase in binding affinity and antagonist activity at both $ET_A$ and $ET_B$ receptors. This is a fairly general phenomenon throughout the series. In addition, N-terminal acetylation in the D-Phe series causes an increase in receptor binding affinity (compounds **13** and **14**). Interestingly, residue Leu$^{17}$ can be replaced with acidic, basic, neutral, or aromatic groups or removed, without loss of antagonist activity, as indicated by comparison of compound **13** with compounds **17–21**. Aromatic residues at Asp$^{18}$ and Leu$^{17}$ maintain reasonable $ET_A$ receptor binding affinity while increasing $ET_B$ receptor binding affinity substantially (compounds **20–22**). Indeed some selective $ET_B$ ligands (compounds **21** and **22**) can be obtained by these substitutions. However, few substitutions are tolerated in the Ile$^{19}$-Ile$^{20}$-Trp$^{21}$ tripeptide without marginal losses in antagonist activity at the $ET_A$ receptor, illustrated by compounds **24–32**. Clearly, the structural requirements for the $ET_A$ receptor are more stringent, since some modifications in the C-terminal tripeptide have maintained $ET_B$ binding (compounds **24** and **30**).

In an attempt to discover smaller antagonists, we have evaluated deletions in compound **13** (Table IV). Clearly,

Table I

| compd | HPLC $t_R$[a,b] | HPLC purity (%) | FAB/MS $(m/z)^+$ calcd mass | FAB/MS $(m/z)^+$ obsd mass | AAA[c] (cal) obtained |
|---|---|---|---|---|---|
| 5 | 17.6[b] | 99 | 924.12 (M) | 946.6 (M + Na) | Leu (1) 1.13, Asp(1) 1.00, Ile(2) 1.68[d] |
| 6 | 17.2[b] | 98 | 837.98 (M) | 838.1 (M + H)<br>860.1 (M + Na) | His(1) 0.96, Leu(1) 1.00, Asp(1) 1.17, Ile(2) 1.86 |
| 7 | 17.8[b] | 97 | 837.98 (M) | 838.7 (M + H)<br>860.8 (M + Na) | His(1) 0.86, Leu(1) 1.00, Asp(1) 0.89, Ile(2) 1.48[d] |
| 8 | 18.7[b] | 98 | 837.98 (M) | 838.4 (M + H) | His(1) 1.45, Leu(1) 1.00, Asp(1) 1.28, Ile(2) 0.85,[d] Trp(1) 0.68 |
| 9 | 18.2[b] | 99 | 837.98 (M) | 838.4 (M + H) | His(1) 1.21, Leu(1) 1.00, Asp(1) 0.77, Ile(2) 0.85[d] |
| 10 | 17.0[b] | 98 | 837.98 (M) | 838.8 (M + H) | His(1) 0.99, Leu(1) 1.00, Asp(1) 1.00, Ile(2) 1.14[d] |
| 11 | 16.8[b] | 98 | 837.98 (M) | 838.4 (M + H) | His(1) 1.13, Leu(1) 1.00, Asp(1) 0.90, Ile(2) 1.00,[d] Trp(1) 0.50 |
| 12 | 17.6[b] | 97 | 837.98 (M) | 838.6 (M + H)<br>860.9 (M + Na) | His(1) 0.99, Leu(1) 1.00, Asp(1) 1.28, Ile(2) 1.15[d] |
| 13 | 21.9[b] | 99 | 848.02 (M) | 848.4 (M + H)<br>870.9 (M + Na) | Phe(1) 0.94, Leu(1) 1.00, Asp(1) 1.25, Ile(2) 1.27[d] |
| 14 | 17.2[a] | 98 | 805.98 (M) | 806.4 (M + H)<br>828.3 (M + Na) | Phe(1) 1.01, Leu(1) 1.00, Asp(1) 1.25, Ile(2) 1.46[d] |
| 15 | 16.6[a] | 99 | 848.02 (M) | 847.3 (M + H)<br>870.2 (M + Na) | Phe(1) 1.07, Leu(1) 1.00, Asp(1) 1.26, Ile(2) 1.42[d] |
| 16 | 15.3[a] | 95 | 805.98 (M) | 806.7 (M + H)<br>828.7 (M + Na) | Phe(1) 0.87, Leu(1) 1.41, Asp(1) 1.39, Ile(2) 2.00 |
| 17 | 14.3[a] | 99 | 863.97 (M) | 864.1 (M + H)<br>886.0 (M + Na) | Phe(1) 1.00, Glu(1) 1.04, Asp(1) 1.06, Ile(2) 1.19[d] |
| 18 | 15.2[a] | 97 | 849.01 (M) | 849.1 (M + H)<br>871.0 (M + Na) | Phe(1) 1.00, Orn(1) 1.00, Asp(1) 1.38, Ile(2) 1.62[d] |
| 19 | 15.8[a] | 99 | 805.94 (M) | 806.4 (M + H)<br>828.4 (M + Na) | Phe(1) 1.26, Ala(1) 1.09, Asp(1) 1.00, Ile(2) 1.37[d] |
| 20 | 18.8[b] | 99 | 882.04 (M) | 904.3 (M + Na) | Phe(2) 2.00, Asp(1) 0.95, Ile(2) 0.93[d] |
| 21 | 17.4[b] | 99 | 882.04 (M) | 904.1 (M + Na) | Phe(2) 2.00, Asp(1) 1.14, Leu(1) 0.97, Ile(2) 1.35[d] |
| 22 | 21.7[b] | 99 | 860.02 (M) | 880.7 (M + H)<br>902.4 (M + Na) | Phe(2) 2.00, Leu(1) 0.79, Ile(2) 1.63[d] |
| 23 | 20.0[b] | 99 | 861.10 (M) | 861.1 (M + Na) | Phe(1) 1.41, Leu(1) 1.00, Lys(1) 0.54, Ile(2) 1.44[d] |
| 24 | 16.8[a] | 99 | 805.94 (M) | 806.4 (M + H)<br>828.3 (M + Na) | Phe(1) 1.11, Leu(1) 1.00, Asp(1) 1.37, Ala(1) 1.26, Ile(2) 1.21[d] |
| 25 | 17.4[a] | 99 | 863.97 (M) | 864.1 (M + H)<br>886.2 (M + Na) | Phe(1) 1.02, Leu(1) 1.00, Asp(1) 1.01, Glu(1) 1.16, Ile(1) 0.96[d] |
| 26 | 16.7[a] | 98 | 863.03 (M) | 863.1 (M + H)<br>885.1 (M + Na) | Phe(1) 1.00, Leu(1) 1.00, Asp(1) 1.01, Lys(1) 0.96, Ile(1) 0.98[d] |
| 27 | 16.5[a] | 97 | 805.94 (M) | 806.5 (M + H)<br>828.5 (M + Na) | Phe(1) 0.84, Leu(1) 0.88, Asp(1) 1.03, Ile(1) 0.94,[d] Ala(1) 1.00 |
| 28 | 15.7[b] | 99 | 863.04 (M) | 886.3 (M + Na) | Phe(1) 1.00, Leu(1) 0.74, Asp(1) 1.37, Ile(2) 0.87,[d] Glu(1) 1.02 |
| 29 | 16.4[b] | 99 | 863.03 (M) | 864.2 (M + H) | Phe(1) 1.00, Leu(1) 1.00, Asp(1) 1.06, Ile(1) 0.95, Lys(1) 1.19 |
| 30 | 17.4[a] | 99 | 824.98 (M) | 825.5 (M + H)<br>847.5 (M + Na) | Phe(1) 1.05, Leu(1) 1.00, Asp(1) 1.13, Ile(2) 1.31,[d] Tyr(1) 0.98 |
| 31 | 17.9[b] | 99 | 865.07 (M) | 864.9 (M + H)<br>887.1 (M + Na) | Phe(1) 1.00, Leu(1) 1.22, Asp(1) 1.13, Ile(2) 1.53[d] |
| 32 | 15.82[a]<br>15.95 | 99 | 798.94 (M) | 799.3 (M + H)<br>821.3 (M + Na) | Phe(1) 1.10, Leu(1) 1.00, Asp(1) 0.90, Ile(2) 0.98,[d] His(1) 1.20 |
| 33 | 16.6 | 98 | 734.86 (M) | 735.1 (M + H)<br>757.2 (M + Na) | Phe(1) 1.00, Asp(1) 1.25, Ile(2) 0.97[d] |
| 34 | 17.5[a] | 98 | 734.86 (M) | 735.5 (M + H)<br>757.8 (M + Na) | Phe(1) 0.98, Leu(1) 1.00, Asp(1) 1.25, Ile(1) 1.02 |
| 35 | 17.7[a] | 98 | 782.9 (M) | 783.1 (M)<br>805.7 (M + Na) | Phe(1) 1.03, Ile(2) 1.39,[d] Tyr(1) 1.00 |
| 36 | 18.0[a] | 98 | 619.9 (M) | 619.7 (M)<br>641.6 (M + Na) | Phe(1) 1.00, Ile(2) 1.21[d] |
| 37 | 16.3[a] | 99 | 506.61 (M) | 507.2 (M + H)<br>529.1 (M + Na) | Phe(1) 1.00, Ile(1) 0.92 |
| 38 | 17.3[a] | 99 | 768.87 (M) | 769.7 (M + H)<br>791.6 (M + Na) | Phe(2) 1.64, Asp(1) 1.00, Ile(1) 1.07 |
| 39 | 16.3[a] | 99 | 864.02 (M) | 864.0 (M + H)<br>886.3 (M + Na) | Tyr(1) 1.04, Leu(1) 1.00, Asp(1) 0.93, Ile(2) 1.40[d] |
| 40 | 18.4[a] | 97 | 887.05 (M) | 887.0 (M + H)<br>908.5 (M + Na) | Leu(1) 1.00, Asp(1) 1.19, Ile(2) 1.36[d] |
| 41 | 18.7[b] | 99 | 898.08 (M) | 899.0 (M + H)<br>921.0 (M + Na) | Leu(1) 1.00, Asp(1) 1.25, Ile(2) 1.17[d] |
| 42 | 16.6[b] | 97 | 924.12 (M) | 925.3 (M + H)<br>946.8 (M + Na) | Leu(1) 1.11, Asp(1) 1.00, Ile(2) 1.63[d] |
| 43 | 18.4[a] | 97 | 882.08 (M) | 882.1 (M)<br>904.1 (M + Na) | Leu(1) 1.00, Asp(1) 1.45, Ile(2) 1.61[d] |
| 44 | 15.9[a] | 99 | 925.10 (M) | 925.1 (M)<br>947.1 (M + Na) | Orn(1) 1.29, Asp(1) 1.00, Ile(2) 1.69[d] |
| 45 | 17.2[a] | 98 | 940.07 (M) | 940.5 (M + H))<br>962.4 (M + Na) | Glu(1) 0.73, Asp(1) 1.00, Ile(2) 1.74[d] |
| 46 | 17.4[b] | 98 | 956.20 (M) | 978.3 (M + Na) | Leu(1) 1.00, Phe(1) 1.10, Ile(2) 1.38[d] |

Table I (Continued)

| compd | HPLC $t_R$[a,b] | HPLC purity (%) | FAB/MS $(m/z)^+$ calcd mass | FAB/MS $(m/z)^+$ obsd mass | AAA[c] (cal) obtained |
|---|---|---|---|---|---|
| 47 | 19.8[a] | 98 | 910.09 (M) | 910.6 (M + H)<br>932.3 (M + Na) | Leu(1) 1.00, Asp(1) 1.13, Val(1) 0.83, Ile(1) 0.75 |
| 48 | 20.2 | >99 | 910.09 (M) | 910.3 (M + H)<br>933.2 (M + Na) | Leu(1) 1.00, Asp(1) 1.68, Ile(1) 0.96, Val(1) 0.89 |
| 49 | 19.0[a] | >95 | 901.08 (M) | 901.7 (M + H)<br>923.4 (M + Na) | Leu(1) 1.00, Asp(1) 1.17, Ile(2) 1.52,[d] Tyr(1) 0.93 |

[a] 10:90–76:24 $CH_3CN$ with 0.1% TFA–0.1% aqueous TFA; linear gradient over 20 min. Flow rate 1.5 mL/min (column conditions in Experimental Section). [b] 20:80–14:86 $CH_3CN$ with 0.1% TFA–0.1% aqueous TFA; linear gradient over 22 min. Flow rate 1.5 mL/min (column conditions in Experimental Section). [c] Trp was not stable to the hydrolysis conditions and was not measured. Leucine (Leu) used as the standard amino acid. [d] Incomplete hydrolysis of the Ile–Ile bond was routinely observed.

**Scheme I.** Synthesis of Compound 5

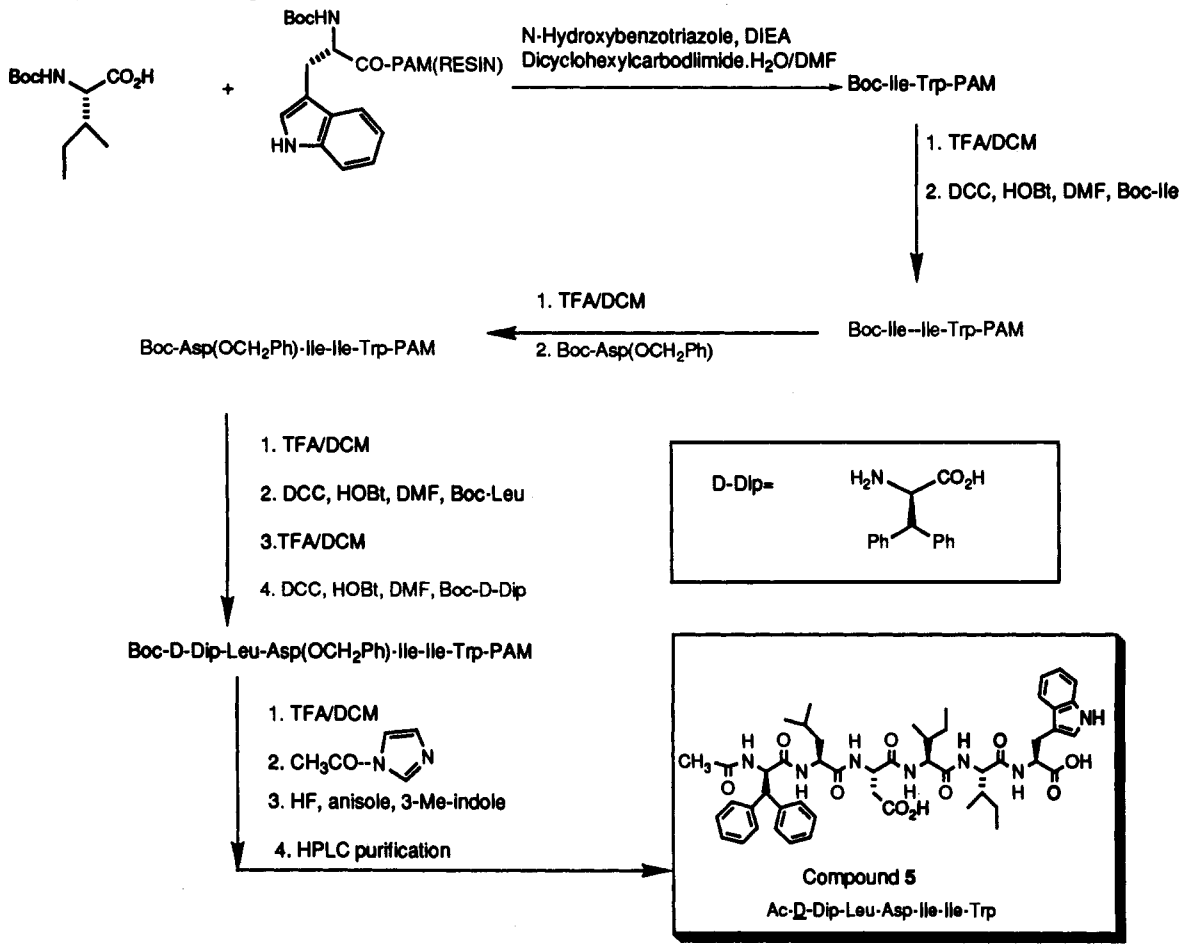



**Table II.** Analogs of ET[16-21]

| compd | | $IC_{50}/\mu M$ binding[a] | $IC_{50}/\mu M$ IP[b] |
|---|---|---|---|
| 6 | Ac-His-Leu-Asp-Ile-Ile-Trp | 58 | 50 |
| 7 | Ac-His-Leu-Asp-Ile-Ile-D-Trp | >50 | c |
| 8 | Ac-His-Leu-Asp-Ile-D-Ile-Trp | 16.9[d] | >100 |
| 9 | Ac-His-Leu-Asp-D-Ile-Ile-Trp | 169[d] | c |
| 10 | Ac-His-Leu-D-Asp-Ile-Ile-Trp | 37 | >50 |
| 11 | Ac-His-D-Leu-Asp-Ile-Ile-Trp | 39 | c |
| 12 | Ac-D-His-Leu-Asp-Ile-Ile-Trp | 4.1[d] | 1.4 |

[a] Rat heart ventricle. [b] Inhibition of ET-1 stimulated inositol phosphate accumulation in rat fibroblasts. [c] Not tested. [d] $n = 2$ $IC_{50}$ determinations. All other values represent one $IC_{50}$ determination. $IC_{50}$ values were derived from single competition experiments in which are data points were measured in triplicate. Binding data was computer analyzed by nonlinear least-squares analysis giving the best fit for a one-site model.

shortening the peptide leads to some loss in binding affinity at both receptor subtypes (compounds 33-38).

A large range of aromatic substitutions are tolerated at position 16 (Table V). SAR studies around the D-Phe[16] position with different aromatic substitutions (compounds 39–42, 5) have led to the potent functional $ET_A/ET_B$ antagonist, compound 5 (PD 142893).[40] Compound 5 displays moderate binding selectivity (10-fold) for the $ET_A$ receptor over the $ET_B$ subtype. Moreover, 5 is a functional antagonist of endothelin-induced arachidonic acid release in vascular smooth muscle cells ($ET_A$) and antagonizes ET-1-stimulated contraction in the rabbit femoral, renal ($ET_A$), and pulmonary arteries ($ET_B$) (Figure 2).

Some amino acid substitutions in 5 are shown in Table VI. Removal of the N-terminal acetyl group (compound 43) resulted in a substantial loss in binding affinity at both $ET_A$ and $ET_B$ receptors, as expected from the D-Phe series structure–activity relationships (SAR). As for the D-Phe[16]-containing hexapeptide, substitution at Leu[17] with Orn or Glu was well tolerated (compounds 44 and 45). In the D-Phe series, substitution with aromatic groups at the Asp[18] position gave rise to increased $ET_B$ selectivity, and

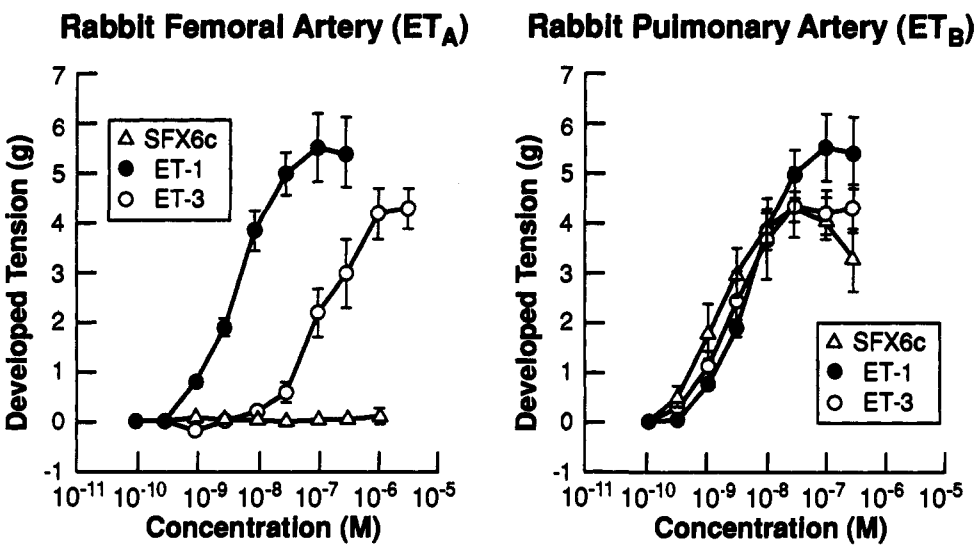



**Figure 1.** Effects of increasing concentrations of ET-1, ET-3, and SRTX-6c on contractile force in denuded femoral (left panel) and pulmonary (right panel) arteries isolated from rabbits. Each point represents the mean ± SEM of 6–12 rings isolated from 3–6 animals.

**Table III.** Analogs of the 16-D-Phe Series

| compd | | binding $IC_{50}/\mu M$ RHV | binding $IC_{50}/\mu M$ $ET_A$[a] | functional $IC_{50}/\mu M$ $ET_B$[b] | functional $IC_{50}/\mu M$ AAR[d] |
|---|---|---|---|---|---|
| 12 | Ac-D-His-Leu-Asp-Ile-Ile-Trp | 4.5 | 8.9[e] | 9.1[e] | 3.2 |
| 13 | Ac-D-Phe-Leu-Asp-Ile-Ile-Trp | 0.74 | 1.8[e] | 2.0[e] | 3.1 |
| 14 | D-Phe-Leu-Asp-Ile-Ile-Trp | 12.4[e] | >10 | 2.6 | c |
| 15 | Ac-Phe-Leu-Asp-Ile-Ile-Trp | c | 18.4 | 18.5 | c |
| 16 | Phe-Leu-Asp-Ile-Ile-Trp | c | >100 | 2.6 | c |
| 17 | Ac-D-Phe-Glu-Asp-Ile-Trp | 0.74 | 0.93[e] | 0.84[e] | 0.6 |
| 18 | Ac-D-Phe-Orn-Asp-Ile-Ile-Trp | 0.68 | 0.58[e] | 2.45[e] | 2.0 |
| 19 | Ac-D-Phe-Ala-Asp-Ile-Ile-Trp | 0.42 | 0.42[e] | 0.29[e] | 0.33[f] |
| 20 | Ac-D-Phe-Phe-Asp-Ile-Ile-Trp | 0.33 | 0.20 | 0.06[e] | 2.6 |
| 21 | Ac-D-Phe-D-Phe-Asp-Ile-Ile-Trp | c | 0.91[f] | 0.02[f] | 2.2[e] |
| 22 | Ac-D-Phe-Leu-Phe-Ile-Ile-Trp | 0.38 | 1.4 | 0.036[f] | 4.5 |
| 23 | Ac-D-Phe-Leu-Lys-Ile-Ile-Trp | 12.0 | >10 | >10 | c |
| 24 | Ac-D-Phe-Leu-Asp-Ala-Ile-Trp | 1.61 | 3.6[f] | 0.45[f] | 1.5 |
| 25 | Ac-D-Phe-Leu-Asp-Glu-Ile-Trp | 9.0 | 6.7 | >10 | >10 |
| 26 | Ac-D-Phe-Leu-Asp-Lys-Ile-Trp | >50 | >10 | >10 | c |
| 27 | Ac-D-Phe-Leu-Asp-Ile-Ala-Trp | 6.83 | 8.0 | >10 | >10 |
| 28 | Ac-D-Phe-Leu-Asp-Ile-Glu-Trp | 5.2 | c | c | c |
| 29 | Ac-D-Phe-Leu-Asp-Ile-Lys-Trp | >50 | c | c | c |
| 30 | Ac-D-Phe-Leu-Asp-Ile-Ile-Tyr | c | >10 | 0.15[e] | c |
| 31 | Ac-D-Phe-Leu-Asp-Ile-Ile-Bta | 3.8 | 14 | 9.8 | >10 |
| 32 | Ac-D-Phe-Leu-Asp-Ile-Ile-DL-His | c | >10 | >10 | c |

[a] Rabbit renal artery vascular smooth muscle cells. [b] Rat cerebellum. [c] Not tested. [d] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. RHV = rat heart ventricle. Bta = 3-benzothienylalanine. [e] $n = 2$ $IC_{50}$ determinations. [f] $n = 3$ $IC_{50}$ determinations. All other values represent one $IC_{50}$ determination. $IC_{50}$ values were derived from single competition experiments in which are data points were measured in triplicate. Binding data was computer-analyzed by nonlinear least-squares analysis giving the best fit for a one-site model.

the same trend is observed in the PD 142893 series (compound **46**). Since we knew that the C-terminal tripeptide region did not tolerate many changes from the earlier work, we carried out only a limited number of conservative substitutions. Thus, compounds **47** and **48** illustrate the effects of Val for Ile substitutions in **5**. Indeed, there is a fall in binding affinity when Val is substituted in the 20 position (compound **49**) while it appears to be well tolerated at the 19 position (compound **47**). The $Tyr^{21}$ analog compound **49** exhibited a loss in activity at both receptor subtypes, unlike the D-Phe-containing compound **30** which is an $ET_B$ selective ligand.

## Functional Evaluation

Several of the compounds were evaluated for their ability to inhibit ET-1-induced contractile activity in rabbit femoral and renal ($ET_A$) arteries and SRTX-6c-induced contractile activity in pulmonary ($ET_B$) arteries (Figure 1). Compounds **12** and **13** and many of the analogs were completely inactive at both tissues at concentrations of up to 10 μM. Figure 2 illustrates the dose–response curves for the effects of compound **5** as the disodium salt at each of the tissues. Clearly, the compound shifts the dose–

**Table IV.** Truncated Analogs of Compound 13

| compd | | binding $IC_{50}/\mu M$ RHV | binding $IC_{50}/\mu M$ $ET_A$[a] | functional $IC_{50}/\mu M$ $ET_B$[b] | functional $IC_{50}/\mu M$ AAR[d] |
|---|---|---|---|---|---|
| 13 | Ac-D-Phe-Leu-Asp-Ile-Ile-Trp | 0.72[e] | 1.8[e] | 2.0[e] | 3.1 |
| 33 | Ac-D-Phe-Asp-Ile-Ile-Trp | 2.15 | 9.1 | 9.3 | c |
| 34 | Ac-D-Phe-Leu-Asp-Ile- - -Trp | 24.5 | >10 | >10 | c |
| 35 | Ac-D-Phe-Tyr- - -Ile-Ile-Trp | c | >10 | >10 | c |
| 36 | Ac-D-Phe- - -Ile-Ile-Trp | c | >10 | >10 | c |
| 37 | Ac-D-Phe- - -Ile- - -Trp | c | 8.0 | >10 | c |
| 38 | Ac-D-Phe-Asp-Phe-Ile-Trp | c | >10 | 4.6 | c |

[a] Rabbit renal artery vascular smooth muscle cells. [b] Rat cerebellum. [c] Not tested. [d] Inhibition of ET-1 stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. RHV = rat heart ventricle. [e] $n = 2$ $IC_{50}$ determinations. All other values represent one $IC_{50}$ determination. $IC_{50}$ values were derived from single competition experiments in which are data points were measured in triplicate. Binding data was computer-analyzed by nonlinear least-squares analysis giving the best fit for a one-site model.

**Table V.** Aromatic Substitutions

| compd | | $IC_{50}/\mu M$ $ET_A$[a] | $IC_{50}/\mu M$ $ET_B$[b] | $IC_{50}/\mu M$ AAR[d] | $IC_{50}/\mu M$ IP[e] |
|---|---|---|---|---|---|
| 13 | Ac-D-Phe-Leu-Asp-Ile-Ile-Trp | 1.8[g] | 2.0[g] | 3.1 | 0.86 |
| 39 | Ac-D-Tyr-Leu-Asp-Ile-Ile-Trp | 0.40 | 7.0 | 0.25 | 0.43 |
| 40 | Ac-D-Trp-Leu-Asp-Ile-Ile-Trp | 0.13 | 1.8 | 0.45 | c |
| 5[40] | Ac-D-Dip-Leu-Asp-Ile-Ile-Trp | 0.025[g] | 0.14[g] | 0.07 | c |
| 5 | Ac-D-Dip-Leu-Asp-Ile-Ile-Trp-2Na | 0.04[h] | 0.06[h] | 0.07[g] | c |
| 41 | Ac-D-2-Nal-Leu-Asp-Ile-Ile-Trp | 1.0 | 1.0 | 1.9 | 0.63 |
| 42 | Ac-D-Bip-Leu-Asp-Ile-Ile-Trp | 4.4 | 3.5 | c | c |

[a] Rabbit renal artery vascular smooth muscle cells. [b] Rat cerebellum. [c] Not tested. [d] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [e] Inhibition of ET-1 stimulated inositol phosphate accumulation in rat skin fibroblasts. RHV = rat heart ventricle. Bip = 4,4′-biphenylylalanine. [f] All compounds are TFA salts except where noted. [g] $n = 2$ $IC_{50}$ determinations. [h] $n = 5$ $IC_{50}$ determinations. All other values represent one $IC_{50}$ determination. $IC_{50}$ values were derived from single competition experiments in which are data points were measured in triplicate. Binding data was computer-analyzed by nonlinear least-squares analysis giving the best fit for a one-site model.

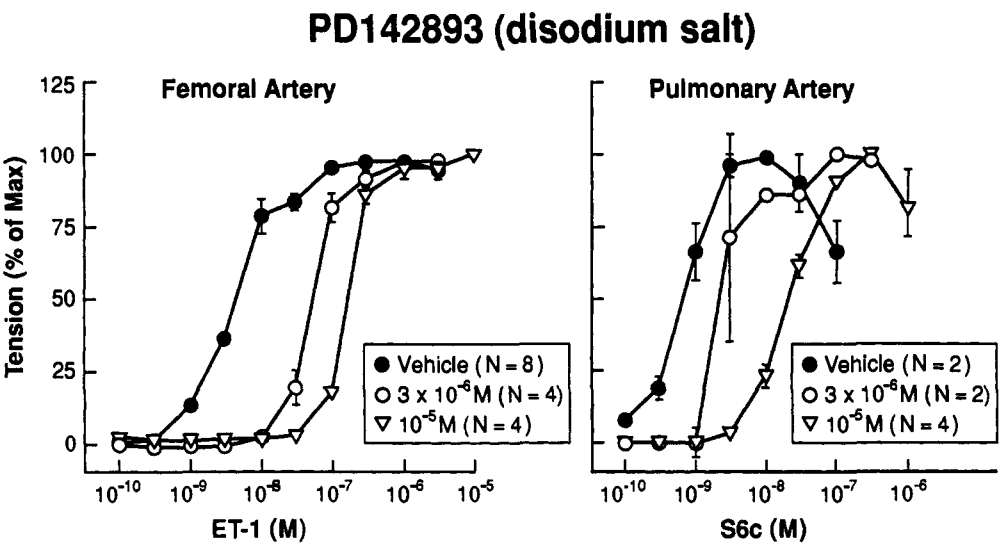



**Figure 2.** Effects of increasing concentrations of compound 5 on ET-1-induced contractile force in denuded femoral arteries (left panel) and SRTX-6c-induced contractile force in denuded pulmonary arteries (right panel) isolated from rabbits. Each point represents the mean ± SEM of 6–12 rings isolated from 3–6 animals.

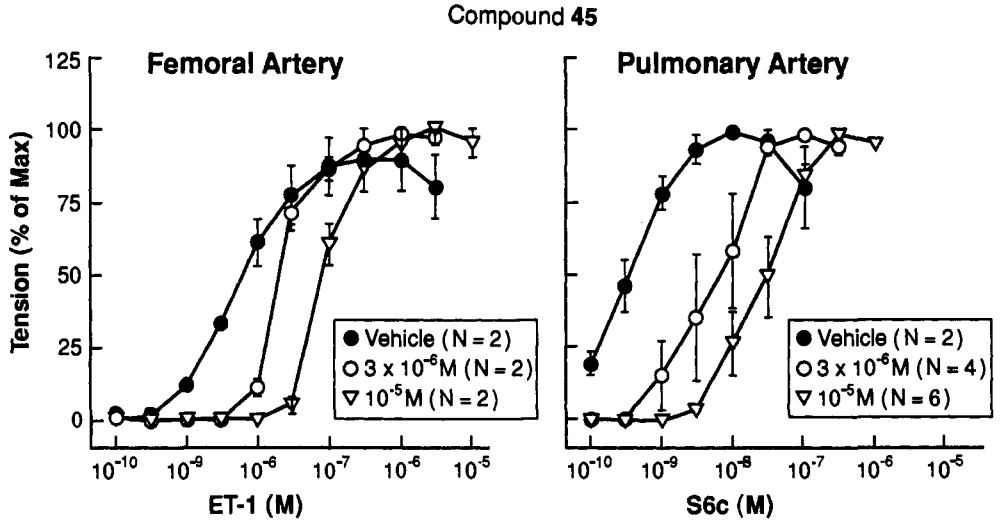



**Figure 3.** Effects of increasing concentrations of compound 45 on ET-1-induced contractile force in denuded femoral arteries (left panel) and SRTX-6c (or S6c) induced contractile force in denuded pulmonary arteries (right panel) isolated from rabbits. Each point represents the mean ± SEM of 6–12 rings isolated from 3–6 animals.

**Table VI.** Selected Analogs of Compound 5

| | compd | $EC_{50}/\mu M$ $ET_A$[a] | $ET_B$[b] | AAR[d] |
|---|---|---|---|---|
| 5 | Ac-D-Dip-Leu-Asp-Ile-Ile-Trp[e] | 0.04[g] | 0.06[g] | 0.07 |
| 43 | D-Dip-Leu-Asp-Ile-Ile-Trp | 2.1 | 1.9 | 1.92 |
| 44 | Ac-D-Dip-Orn-Asp-Ile-Ile-Trp | 0.013[f] | 0.15[f] | 0.03[f] |
| 45 | Ac-D-Dip-Glu-Asp-Ile-Ile-Trp | 0.025 | 0.052 | 0.13[f] |
| 46 | Ac-D-Dip-Leu-Phe-Ile-Ile-Trp | 0.58[h] | 0.06[h] | 1.3[f] |
| 47 | Ac-D-Dip-Leu-Asp-Val-Ile-Trp | 0.017[f] | 0.05[f] | 0.029[f] |
| 48 | Ac-D-Dip-Leu-Asp-Ile-Val-Trp | 6.0 | 6.0 | 2.60 |
| 49 | Ac-D-Dip-Leu-Asp-Ile-Ile-Tyr | 2.7[f] | 6.1[f] | 4.3 |

[a] Rabbit renal artery vascular smooth muscle cells. [b] Rat cerebellum. [c] Not tested. [d] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [e] All compounds are TFA salts except where noted where the disodium salt was synthesized. [f] $n = 2$ $IC_{50}$ determinations. [g] $n = 5$ $IC_{50}$ determinations. [h] $n = 3$ $IC_{50}$ determinations. All other values represent one $IC_{50}$ determination.

response curve to ET-1-induced contraction at the $ET_A$ (rabbit femoral) receptors ($pA_2$ = 6.6) and SRTX-6c-induced contraction at the $ET_B$ (rabbit pulmonary artery) ($pA_2$ = 6.3) receptors. Figure 3 illustrates the antagonist activity of compound 45 in the same assays. Thus, compound 45 inhibited ET-1-induced contraction at the $ET_A$ (rabbit femoral) receptors ($pA_2$ = 6.1) and SRTX-6c-induced contraction at the $ET_B$ (rabbit pulmonary artery) ($pA_2$ = 6.9) receptors. Compounds 5 and 45 showed no intrinsic agonist activity at concentrations of up to 10 μM.

## Conclusions

Ac-D-Phe[16]-Leu-Asp-Ile-Ile-Trp[21] (13) is a non-selective $ET_A/ET_B$ antagonist causing inhibition of endothelin-induced arachidonic acid release in vascular smooth muscle cells. SAR studies of the D-Phe[16] series have shown that a large range of aromatic substitutions are tolerated at position 16. D-stereochemistry at position 16 affords a large increase in binding affinity and antagonist activity at both $ET_A$ and $ET_B$ receptors. N-terminal acetylation causes an increase in receptor binding affinity and antagonist activity. Leu[17] can be replaced with acidic, basic, neutral or aromatic groups or removed without loss of binding affinity. Aromatic residues at Asp[18] and Leu[17] maintain $ET_A$ receptor binding affinity while increasing $ET_B$ receptor binding. However, substitution of Lys at

position 18 leads to a drop in affinity at the $ET_A$ receptor. Few changes are tolerated in the $Ile^{19}$-$Ile^{20}$-$Trp^{21}$ tripeptide without marginal losses in antagonist activity at the $ET_A$ receptor. Interestingly, $Tyr^{21}$ appears to be well tolerated at the $ET_B$ receptor in the D-$Phe^{16}$ (compound 13) series while it is not tolerated at either receptor subtype in the PD 142893 (compound 5) series.

SAR studies around the D-$Phe^{16}$ position have led to the potent functional $ET_A/ET_B$ antagonist 5. The disodium salt of compound 5 exhibits equipotent binding affinity for the $ET_A$ and $ET_B$ receptor subtypes in the animal tissues examined.

Compounds 5 and 45 are functional antagonists of ET-1-induced arachidonic acid release in vascular smooth muscle cells and of ET-1-stimulated contraction in the rabbit femoral- ($ET_A$) and SRTX-6c-stimulated contraction in rabbit pulmonary arteries ($ET_B$) (Figures 2 and 3). The ET antagonists described in this paper should prove useful as pharmacological tools to elucidate the physiological responsibilities of the two receptor subtypes and the potential role of endothelin in the pathophysiology of human diseases.

## Experimental Section

All reagents and solvents for peptide synthesis were reagent grade and used without further purification.

**Peptide Synthesis.** All of the peptides were synthesized by solid-phase synthesis techniques[52-54] on an Applied Biosystems Model 430A peptide synthesizer. The analogs were prepared using either (a) an *N*-α-Boc protection scheme with a PAM resin (Applied Biosystems); the amino acid side chain protection used was OBzl (Asp, Glu), 2-Cl-Z (Lys), Bom (His), or formyl (Trp); acetylation was carried out on the resin in methylene chloride with an excess of 1-acetylimidazole (20 equiv), and the peptides were then deprotected and cleaved from the resin using anhydrous liquid HF/anisole or HF/*p*-cresol (9:1 v/v), or (b) an FMOC protection scheme on an HMP resin [[(hydroxymethyl)phenoxy]-methyl] (Applied Biosystems); the amino acid side-chain protection used was O$^t$Bu (Asp, Glu, Tyr), trityl (His), or Boc (Lys, Orn); these peptides were FMOC-deprotected with 20% piperidine in *N*-methylpyrrolidinone, and acetylation was carried out on the resin in methylene chloride with an excess of 1-acetylimidazole (20 equivalents); deprotection and cleavage from the resin was carried out with TFA–thioanisole–ethanedithiol–$H_2O$ (89:5:2:3:1). α-Boc amino acids (Bachem, Torrance, CA) were coupled via *N*,*N*-dicyclohexylcarbodiimide (Applied Biosystems) in DMF (EM Science). α-FMOC amino acids (Bachem Bioscience) were coupled via dicyclohexylcarbodiimide in *N*-methylpyrrolidinone (Applied Biosystems). The crude peptides were precipitated with anhydrous diethyl ether and separated from ether-soluble nonpeptide material by filtration.

**Peptide Purification.** Crude peptides were dissolved in a mixture of aqueous TFA and acetonitrile (exact ratio depended on the solubility of the peptide) and then purified by preparative reversed-phase HPLC using a C18 preparative-scale Vydac column (218TP1022) (2.2 × 25 cm, 10–20 μM particle size) eluting with a linear gradient of 0.1% aqueous TFA with increasing concentrations of $CH_3CN$ (Mallinkroft or Burdick and Jackson) at 13–15 mL/min. The preparative HPLC system used included a Waters prep LC, Waters 490E variable-wavelength detector (214 and 280 nM), and Waters 600E system controller. Peptides were isolated by lyophilization.

**Peptide Homogeneity and Characterization.** Peptides were assessed for homogeneity by analytical RP HPLC and TLC. Analytical HPLC analysis was carried out on a Vydac RP-HPLC column (218TP54) (0.46 × 25 cm, 5-μM particle size). The analytical system used was the same as that described in detail above (peptide purification). Two different conditions for analytical HPLC analysis were used: (a) 10:90–76:24 $CH_3CN$ with 0.1% TFA–0.1% aqueous TFA, linear gradient over 20 min at a flow rate of 1.5 mL/min, and (b) 20:80–14:86 $CH_3CN$ with 0.1% TFA–0.1% aqueous TFA, linear gradient over 22 min at a flow rate of 1.5 mL/min. The peptides were characterized by amino acid analysis, FAB mass spectroscopy, and proton NMR spectroscopy (Table I).

Amino acid analyses were performed using an Applied Biosystems (ABI) automated derivatizer/analyzer Model 420A with a two-component aqueous trifluoroacetic acid/acetonitrile buffer system. Automated hydrolysis was carried out with 6 N HCl at 155–160 °C for 75 min. PTC derivatization (phenyl isothiocyanate) in the presence of diisopropylethylamine was carried out followed by separation of the derivatized amino acids using an ABI Model 130A separation system. Data analysis was performed on an ABI Model 610A system (data analysis program version 1.2).

FAB mass spectra were measured with a VG analytical 7070E/HF mass spectrometer in either a thioglycerol or 3-nitrobenzyl alcohol matrix using xenon as the target gas.

Proton NMR spectra were measured with a Varian XL 200 or Bruker AM 250 instrument using tetramethylsilane as an external standard in DMSO-$d_6$.

**Synthesis of Compound 5.** The linear hexapeptide was prepared by standard solid-phase synthetic peptide methodology utilizing a Boc/Benzyl strategy.[52-54] The protected peptide resin was prepared on an Applied Biosystems 430A peptide synthesizer, utilizing protocols supplied for a *N*,*N*′-dicyclohexylcarbodiimide mediated coupling scheme (standard 1.0, version 1.40). Starting with 0.81 g of *N*-α-Boc-Trp-PAM resin 0.43 mequiv of Boc-Trp total) the protected peptide was prepared by the stepwise coupling of the following amino acids: *N*-α-Boc-D-Dip, *N*-α-Boc-Leu·$H_2O$, *N*-α-Boc-Asp(Bzl), and *N*-α-Boc-Ile·0.5$H_2O$. A typical cycle for the coupling of each individual amino acid residue is illustrated below. All the single couple reaction vessel cycles conformed to the following pattern: (a) 33% TFA in dichloromethane (DCM) for 80 s, (b) 50% TFA in DCM for 18.5 min (c) three DCM washes, (d) 10% DIEA in DMF for 1 min, (e) 10% DIEA in DMF for 1 min, (f) five DMF washes, (g) coupling period, and (h) five DCM washes.

After the coupling of *N*-α-Boc-D-Dip, the Boc group was removed with the end-$NH_2$ cycle and the free amine was acetylated with *N*-acetylimidazole (1.0 g, 120 min) in 20 mL of dichloromethane (DCM). The resin was washed with DCM (3 × 20 mL) and dried under reduced pressure. The peptide was liberated from the solid support and the carboxylate of aspartic acid deprotected by treatment with anhydrous hydrogen fluoride (ca. 9.0 mL) and anisole (0.5 mL) (60 min, 0 °C). After the hydrogen fluoride was removed under a stream of nitrogen, the resin was washed with diethyl ether (3 × 30 mL) and extracted with 20% HOAc in water (3 × 30 mL) and glacial HOAc (2 × 30 mL). The aqueous extractions were combined, concentrated under reduced pressure, and lyophilized (350 mg). The crude peptide was dissolved in 4.0 mL of 50% TFA/$H_2O$, filtered through a 0.4-μm syringe filter, and chromatographed on a Vydac 218TP1022 column (2.2 × 25.0 cm, 15.0 mL/min; A, 0.1% TFA/$H_2O$; B, 0.1% TFA/$CH_3CN$; gradient, 0% B for 10 min, 30%–60% B over 120 min). Individual fractions were collected and combined based upon analysis by analytical HPLC. The combined fractions were concentrated under reduced pressure (10 mL), diluted with $H_2O$ (50 mL), and lyophilized (150 mg). The homogeneity and structure of the resulting peptide was confirmed by analytical HPLC, proton nuclear magnetic resonance spectroscopy ($^1$H-NMR), and fast atom bombardment mass spectroscopy (FAB-MS), M + Na *m*/*z* 946.6.

**Endothelin Receptor Binding Assay Protocol.** The binding assay protocol using rat heart ventricle has been reported previously.[49] The $ET_A$ and $ET_B$ binding assays were carried out as follows:[37] Incubations were performed in 12 × 75 mm polypropylene tubes containing 20 mM tris(hydroxymethyl)-aminomethane hydrochloride (Trizma) buffer, 2 mM ethylenediamine tetraacetate (EDTA), 100 μM phenylmethylsulfonyl fluoride (PMSF), 100 μM bacitracin, 30 pM [$^{125}$I]-Et-1 (2,000 Ci/mmol), and 5 μg of rabbit renal artery vascular smooth muscle membranes ($ET_A$) or 5 μg of rat cerebellar membranes from adult blue laurie rats ($ET_B$) (total volume of 250 μL) (pH 7.4 at 37 °C). The order of the additions (tubes on ice) were (i) test compound, (ii) [$^{125}$I]-ET-1, and (iii) membranes. Test compounds were diluted in a buffer (20 mM Trizma, 2 mM EDTA, 1 mg/mL BSA,

and 1% DMSO) to five times the final incubation concentration. [$^{125}$I]-ET-1 was diluted in the same buffer without DMSO. Membranes were diluted in buffer containing 100 μM PMSF and 100 μM bacitracin, without bovine serum albumin (BSA) or DMSO. Immediately following the last addition, the incubation was initiated by hand agitation. Tubes were then incubated at 37 °C for 2 h. Incubations were terminated by filtration through Whatman GF/B filters which were presoaked with 50 mM Trizma containing 0.2% BSA and 100 μM bacitracin (pH 7.3 at 5 °C). Nonspecific binding is defined as binding in the presence of 100 nM ET-1, and specific binding was defined as total binding minus nonspecific binding. $IC_{50}$ values were calculated by weighted nonlinear regression curve fitting to the mass action equation (giving the best fit for a one-site model).[57]

**Functional Assay Protocols. Arachidonic Acid Release (AAR) Assay.**[37,58] The loading media (LM) used for the [$^3$H] arachidonic acid assay consisted of Dulbeccos modified Eagles/Ham's nutrient mixture F12 (DME/F12)(1:1) which contained 0.5% fetal calf serum (0.5% FCS) and 0.25 μCi/mL [$^3$H] arachidonic acid (218 Ci/mmol) (Amersham). Confluent monolayers of cultured rabbit renal artery vascular smooth muscle cells were incubated in 0.5 mL of the LM over 18 h, at 37 °C, in 5% $CO_2$. The LM was aspirated, and the cells were washed once with the assay buffer (Hank's salts BSS + 10 mM HEPES buffer + fatty acid-free BSA (1 mg/mL)) and incubated for 5 min with 1 mL of the prewarmed assay buffer. This solution was aspirated, followed by an additional 1 mL of prewarmed assay buffer, and further incubated for another 5 min. A final 5-min incubation was carried out in a similar manner. The same procedure was repeated with the inclusion of 10 mL of the test compound (1 nM to 1 μM) and 10 mL of ET-1 (0.3 nM), and the incubation was extended for 30 min. This solutuion was then collected, 10 mL of scintillation cocktail was added, and the amount of [$^3$H] arachidonic acid was determined in a liquid scintillation counter.

**Inositol Phosphate Accumulation (IP) Assay.** Rat skin fibroblast cells were prelabeled with [$^3$H]inositol, and endothelin-1-stimulated accumulation of total [$^3$H]inositol, and endothelin-1-stimulated accumulation of total [$^3$H]inositol phosphates in the presence of $Li^+$ was monitored using anion-exchange chromatography.[37] Antagonist activity of compounds was assessed by the reduction of endothelin-stimulated inositol phosphate accumulation.

**In Vitro Contractility Studies.** Vascular rings of rat femoral artery ($ET_A$) or rabbit pulmonary artery ($ET_B$) were placed in 37 °C-maintained organ baths containing oxygenated Krebs bicarbonate solution and gassed continuously with 5% $CO_2$ in oxygen.[30] Peptides were added to the endothelium-intact preparations. Vascular contractile responses were measured isometrically. The ET-1 ($ET_A$) or SRTX-6c ($ET_B$) response in the presence of novel compounds was expressed as a percent of the ET-1 or SRTX-6c response in the absence of compounds.

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