Differences in Backbone Structure between Angiotensin II Agonists and Type I Antagonists

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Type I angiotensin II antagonists with *O*-methyl-L-homoserine [HSer(γ-OMe)] and δ-methoxy-L-norvaline [Nva(δ-OMe)] at position 8 have been prepared by the solid-phase method, purified by reverse-phase HPLC, and bioassayed in the rat uterus, and their backbone conformational properties were investigated by nuclear Overhauser effect (NOE) spectroscopy. [Sar^1,HSer(γ-OMe)^8]ANGII, [HSer(γ-OMe)^8]ANGII, [Des^1,HSer(γ-OMe)^8]ANGII, [Sar^1,Nva(δ-OMe)^8]-ANGII, and [Des^1,Nva(δ-OMe)^8]ANGII had, respectively, the following antagonist activities, pA_2: 7.6, 7.5, <6.0, 7.1, and 6.9. Analogs of [Sar^1]ANGII with δ-hydroxy-L-norvaline [Nva(δ-OH)], δ-methoxy-L-norvaline [Nva(δ-OMe)], 4′-carboxyphenylalanine [Phe(4′-COOH)], and 4′-(trifluoromethyl)phenylalanine [Phe(4′-CF_3)] at position 4 were also prepared by solid phase and bioassayed in the rat uterus. [Sar^1,Nva(δ-OH)^4]ANGII, [Aib^1,Nva(δ-OMe)^4]ANGII, [Sar^1,DL-Phe(4′-COOH)^4]ANGII, and [Sar^1,DL-Phe(4′-CF_3)^4]ANGII had, respectively, agonist activities as follows: 4%, 1.5%, 3%, <0.1%, and <0.1%. These data emphasize that replacement of Ile^8 in Sarilesin with the higher homologs HSer(γ-OMe) and Nva(δ-OMe) does not greatly alter the structural requirements necessary for expression of type I antagonist activity, while replacement of the tyrosine hydroxyl in [Sar^1]ANGII by the carboxylate or the trifluoromethyl group abolishes activity, suggesting that the tyrosinate pharmacophore cannot be replaced by any negatively charged or electronegative group. Conformational investigation of the ANGII type I antagonists [HSer(γ-OMe)^8]ANGII and [Sar^1 Nva(δ-OMe)^8]ANGII in DMSO by 1D-NOE spectroscopy revealed that the Tyr-Ile-His bend, a conformational property found in ANGII and [Sar^1]ANGII (*J. Biol. Chem.* **1994**, *269*, 5303) is not present in type I antagonists, providing for the first time an important conformational difference between angiotensin II agonists and type I antagonists.

Introduction

The octapeptide angiotensin II (ANGII: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) acts at receptors in numerous target tissues to elicit contractile and secretory responses and has been implicated in the pathogenesis of hypertension and congestive heart failure.[1,2] Type I and II antagonist analogs have been identified from structure–activity studies. Type I antagonists are obtained by substituting the aromatic Phe^8 residue with an aliphatic amino acid (Ile, Ala, and Thr) and are characterized by slow receptor resensitization rates.[3–7,19] Type II antagonists are produced by methylating or omitting the Tyr hydroxyl group in [Sar^1]ANGII and are reversible and competitive antagonists.[8–11] Replacement of the tyrosine 4 hydroxyl by chlorine has produced an ANGII antagonist,[12] while deletion of the Phe^8 residue has been reported to produce also ANGII antagonists.[13] A triad of interacting groups (Tyr OH–His imidazole–Phe COO^-) in angiotensin II has been also postulated to create the tyrosinate anion pharmacophore responsible for receptor activation/triggering.[14–17] Recent conformational studies on angiotensin II and [Sar^1]ANGII have illustrated that the agonist peptide molecule takes up a folded structure characterized by a trans His–Pro bond, a clustering of the three aromatic rings, and a charge relay system involving the triad Tyr hydroxyl–His imidazole–Phe carboxylate analogous to the one found in serine proteases.[15–18] A tight Tyr–Ile–His bond revealed by nuclear Overhauser effect (NOE) studies stabilizes the folded structure and determines the mode of binding.[18]

In the present study we have replaced the Ile residue at position 8 in sarilesin with HSer(γ-OMe) and the higher homolog Nva(δ-OMe), respectively, to investigate further the role of the aliphatic side chain at position 8 in maintaining the conformational integrity of type I antagonists for binding with high affinity to angiotensin II receptors. Furthermore, we have investigated the effects on bioactivity of substituting the Tyr^4 residue in [Sar^1]ANGII with other anionic or electronegative amino acids in order to delineate aspects of this pharmacophore which provide for its receptor triggering and desensitizing properties. In particular, the amino acids Nva(δ-OH) and Nva(δ-OCH_3), as well as the aromatic amino acids Phe(4′-COOH) and Phe(4′-CF_3), were investigated. NOE studies on the type I ANGII antagonists [HSer(γ-OMe)^8]ANGII and [Sar^1,Nva(δ-OMe)^8]-ANGII carried out under the same conditions as for ANGII and [Sar^1]ANGII, showed that the agonists

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Table 1. Biological Activities of Angiotensin II Analogs[a]

	agonist activity (% of ANGII)	antagonist activity (pA_2)
[Sar^1,HSer(γ-OMe)^8]ANGII	<0.1	7.6 (3)
[HSer(γ-OMe)^8]ANGII	<0.1	7.5 (2)
[Des^1,HSer(γ-OMe)^8]ANGII	<0.1	<6.0 (4)
[Sar^1,Nva(δ-OMe)^8]ANGII	<0.1	7.1 (3)
[Des^1,Nva(δ-OMe)^8]ANGII	<0.1	6.9 (4)
[Sar^1,Nva(-OH)^4]ANGII	4 (4)	<6.0 (4)
[Aib^1,Nva(δ-OH)^4]ANGII	1.5 (4)	<6.0 (4)
[Sar^1,Nva(δ-OMe)^4]ANGII	3 (4)	<6.0 (4)
[Sar^1,Nva(δ-OH)^4,Ile^8]ANGII	<0.1	<5.0
[Aib^1,Nva(δ-OH)^4,Ile^8]ANGII	<0.1	6.2 (2)
[Sar^1,DL-Phe(4′-COOH)^4]ANGII	<0.1	<5.0 (4)
[Sar^1,DL-Phe(4′-CF_3)^4]ANTII	<0.1	<5.0 (4)

[a] Values are given as mean number of experiments with human ANGII as the standard; standard error of the mean were 10–20% for agonist activities and 0.1 for antagonist pA_2 values. Abbreviations are standard except HSer(γ-OMe), *O*-methyl-L-homoserine or γ-methoxy-L-homoserine; HSer, homoserine; Nva(δ-OH), δ-hydroxy-L-norvaline; Nva(δ-OMe), δ-methoxy-L-norvaline; Phe(4′-COOH), 4-carboxyphenylalanine; Phe(4′-CF_3), 4-(trifluoromethyl)phenylalanine.

possess a Tyr-Ile-His bend whereas the antagonists lacked this conformational property, and this may account for the expression of antagonist activity.

Results

Tables 1 and 2 give the biological activities and the chemical data, respectively, of the various angiotensin II analogs synthesized in the present study. As it is evident from the data for the first five peptides listed in Table 1, substitution at position 8 with HSer(γ-OMe) and Nva(δ-OMe) resulted in analogs of moderately decreased antagonist activity compared to [Sar^1,Ile^8]-ANGII (pA_2 = 8.1). Thus, [Sar^1,HSer(γ-OMe)^8]ANGII and [Sar^1,Nva(δ-OMe)^8]ANGII had antagonist activities of 7.6 and 7.1, respectively. Substitution of Sar by Asp in [Sar^1,HSer(γ-OMe)^8]ANGII retained activity (pA_2 = 7.5) while deletion of the N-terminal residue resulted in loss of activity (pA_2 < 6). The pharmacological profile and duration of action of these type I antagonists was similar to that observed for [Sar^1,Ile^8]ANGII.[3,19] In the case of [Sar^1,Nva(δ-OMe)^8]ANGII (pA_2 = 7.1), deletion of Sar^1 did not substantially decrease antagonist activity (pA_2 = 6.9). Replacement of Tyr 4 by Nva(δ-OH) and Nva(δ-OMe) in the superagonist [Sar^1]ANGII resulted in a marked reduction in agonist activity (4% and 1.5%, respectively) and no detectable antagonist activity (pA_2 < 6). Similarly, [Aib^1,Nva(δ-OH)^4]ANGII had low but measurable agonist activity (3%). Replacement of Tyr by Nva(δ-OH) in [Sar^1,Ile^8]ANGII resulted in loss of antagonist activity (pA_2 < 5.0), while replacement of Sar by Aib afforded an analog with measurable antagonist activity (pA_2 = 6.2). Both, 4′-carboxy- and 4′-(trifluoromethyl)phenylalanine derivatives have been synthesized by novel syntheses described in this work (see the Experimental Section) and incorporated in the position 4 of the [Sar^1]ANGII peptide molecule to test the role of these residues in possible receptor triggering and expression of activity. It was found that replacement of Tyr by 4′-carboxyphenylalanine and 4′-(trifluoromethyl)phenylalanine in [Sar^1]ANGII resulted in completely inactive analogs (<0.1% and pA_2 < 5).

[HSer(γ-OMe)^8]ANGII and [Sar^1,Nva(δ-OMe)^8]ANGII were subjected to COSY and 1D-NOE NMR studies in DMSO-d_6 suitable for resonance assignment and distance information. The one-dimensional NMR spectra showed fairly well resolved aromatic and $C_\alpha H$ regions, allowing assignment and distance information. Figure 1 shows the COSY spectrum of [HSer(γ-OMe)^8]ANGII in DMSO-d_6 while Figure 2 shows the NOE difference spectra resulting after saturation of the His $C_\alpha H$ (4.65 ppm), Tyr $C_\alpha H$ (4.45 ppm), His $C_4 H$ (6.88 ppm), and Tyr ortho H (6.62 ppm) proton resonances. As seen, no proton enhancements of the Tyr C_α or His C_α resonances were observed upon saturation of the His C_α or Tyr C_α proton resonances, indicating the absence of a Tyr-Ile-His bend. Saturation of the His C_4 proton resonance (6.88 ppm) resulted in enhancement (7.1%) of the Tyr meta proton at δ = 7.02 ppm. Saturation of the His C_2 proton resonance (7.52 ppm) resulted in weak enhancements of the Tyr meta (2.25%) and Tyr ortho (1.66%) proton resonances. These enhancements indicate proximity of the Tyr/His rings. Saturation of the Tyr meta proton at δ = 7.02 ppm resulted as expected in strong enhancements of the Tyr ortho proton at δ = 6.62 ppm (25.30%). Similarly saturation of the Tyr ortho proton resonance at δ = 6.62 ppm resulted in enhancement of the Tyr meta proton at δ = 7.02 ppm (19.20%). No other enhancements were observed. Figure 3 shows the NOE difference spectra of [Sar^1,Nva(δ-OMe)^8]ANGII resulting after saturation of His $C_\alpha H$ (4.63 ppm) and Tyr $C_\alpha H$ (4.48 ppm) proton resonances. As seen, no proton enhancements of the Tyr C_α or His C_α resonances were observed upon saturation of the His C_α or Tyr C_α proton resonances indicating also lack of a Tyr-Ile-His bend in this type I ANGII antagonist. We used NOE experimental conditions (saturation of control areas, low power, different *T* preirradiation times) so that partial saturation and spin diffusion would be visibly mini-

Table 2. Chemical data of Angiotensin II Analogs[a]

	TLC								
analog	BPAW[b]	CMAW[c]	Arg	Val	Tyr	Ile	His	Pro	Phe
[Sar^1,Nva(δ-OH)^4]ANGII	0.55	0.80	0.86	0.91	–	0.97	0.89	1.00	1.05
[Aib^1,Nva(δ-OH)^4]ANGII	0.57	0.80	0.72	0.76	–	0.79	0.76	1.00	1.10
[Sar^1,Nva(δ-OH)^4,Ile^8]ANGII	0.59	0.79	0.86	0.94	–	1.95	0.86	1.00	–
[Aib^1,Nva(δ-OH)^4,Ile^8]ANGII	0.47	0.79	0.87	0.95	–	1.90	0.86	1.00	–
[Des^1,Nva(δ-OMe)^8]ANGII	0.54	0.72	0.85	0.97	0.94	0.96	0.90	1.00	–
[Sar^1,Nva(δ-OMe)^8]ANGII	0.61	0.67	0.85	0.96	1.00	0.94	0.85	1.00	–
[Des^1,HSer(γ-OMe)^8]ANGII	0.55	0.58	1.03	0.95	1.00	0.97	1.28	1.01	–
[HSer(γ-OMe)^8]ANGII	0.54	0.52	1.03	0.95	1.00	0.97	1.28	1.10	–
[Sar^1,HSer(γ-OMe)^8]ANGII	0.65	0.52	1.00	1.04	0.82	0.76	0.72	0.94	–
[Sar^1,DL-Phe(4′-COOH)^4]ANGII	0.70	0.41	1.00	0.77	–	0.97	0.78	0.97	1.22
[Sar^1,DL-Phe(4′-CF_3)^4]ANGII	0.50	0.54	1.00	0.83	–	0.79	0.83	0.88	1.04

[a] δ-Hydroxy-L-norvaline, homoserine, and phenylalanine derivatives give low color yields by amino acid analysis and could not be reliably estimated. Identity of analogs was confirmed by FAB MS.[29] [b] 1-Butanol–pyridine–acetic acid–water (15:10:3:6). [c] Chloroform–methanol–acetic acid–water (15:10:2:3).

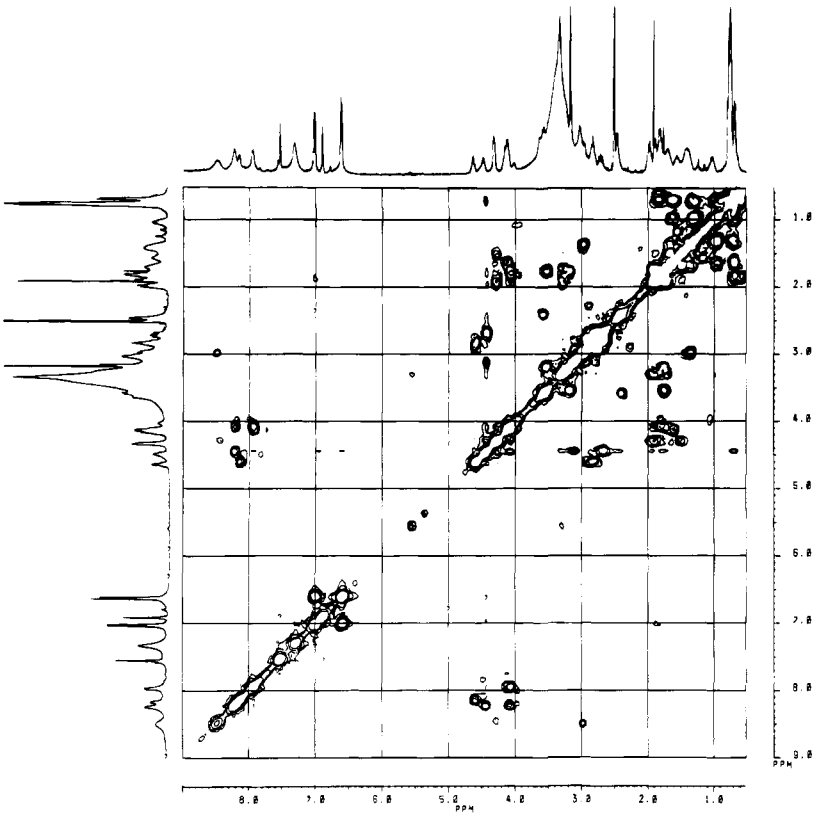


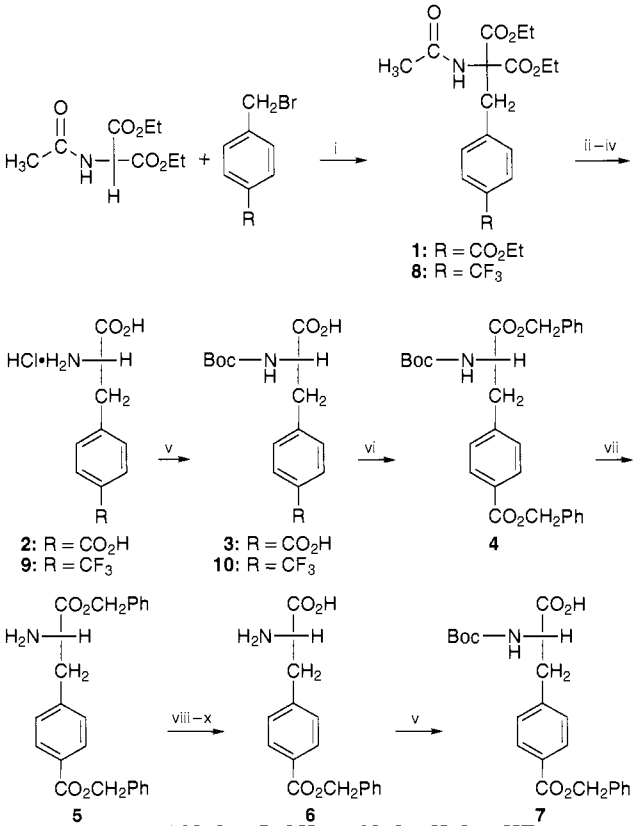
Figure 1. Two-dimensional contour plot of a 400 MHz COSY spectrum for [HSer(γ-OMe)^8]ANGII in DMSO-d_6.

mized. The selected lines were irradiated 20 times for 100 ms (total irridation time 2.0 s). Each line required a total of 1000 scans, and the relaxation time was 2 s. For each transient the acquisition time was ≈3 s.

Chemistry

The synthesis of *N*-(*tert*-butyloxycarbonyl)-4-(benzoxycarbonyl)phenylalanine (**7**) and *N*-(*tert*-butyloxycarbonyl)-4-(trifluoromethyl)phenylalanine (**10**) used as position 4 residues in the synthesis of angiotensin II analogs is depicted in Scheme 1. Compound **7** was synthesized using diethyl acetamidomalonate as starting material which was condensed in a Gabriel synthesis with bromo-*p*-toluic acid ethyl ester to afford the acetamido triester 1 in crystalline form and high yield. Hydrolysis of **1** with sodium hydroxide afforded 4-carboxyphenylalanine (**2**) in hydrochloride form. Protection of the amino group of **2** with the *N*-*tert*-butyloxycarbonyl group was carried out using *tert*-butyl 4,6-dimethylpyrimidine-2-thiolocarbonate as described by Nagasawa[26] to afford *N*-(*tert*-butoxycarbonyl)-4-carboxyphenylalanine (**3**) in 64% yield. Compound **3** was esterified using benzyl alcohol, dicyclohexylcarbodiimide and (dimethylamino)pyridine (DMA) in dichloromethane, to afford *N*-*t*-Boc dibenzyl ester 4 which was purified by flash chromatography. Deprotection of the amino group of 4 with 50% trifluoroacetic acid in CH_2Cl_2 afforded 4-carboxyphenylalanine dibenzyl ester **5**. Treatment of the dibenzyl ester **5** with $CuSO_4 \cdot 5H_2O$ in 1 N NaOH solution following the method of Prestige et al.[21] allowed selective hydrolysis of the α-benzyl ester group, affording 4-(benzoxycarbonyl)phenylalanine (**6**) in which the Phe ring carboxylate remained protected as benzyl ester. Compound **6** was finally converted to *N*-(*tert*-butoxycarbonyl)-4-(benzyloxycarbonyl)phenylalanine (**7**) by the method of Nagasawa[26] or using similar method described in Experimental Section. *N*-*t*-Boc-4-(benzyloxycarbonyl)phenylalanine was incorporated at position 4 of the [Sar^1]angiotensin peptide sequence using solid-

Scheme 1[a]



[a] Reagents: (i) NaOEt/EtOH; (ii) NaOH/H_2O–THF; (iii) acetic acid, Δ; (iv) concentrated HCl, Δ; (v) *tert*-butyl 5-(4,6-dimethylpyrimidine-2-thiolocarbonate, Et_3N, THF–H_2O, room temperature, 24 h or 2-[[(tert-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile, Et_3N, dioxane–H_2O, 21 h; (vi) benzyl alcohol, DCC, DMAP, CH_2Cl_2; (vii) TFA, CH_2Cl_2; (viii) $CuSO_4$, pH = 8; (ix) HCl, pH = 3; (x) EDTA sodium salt.

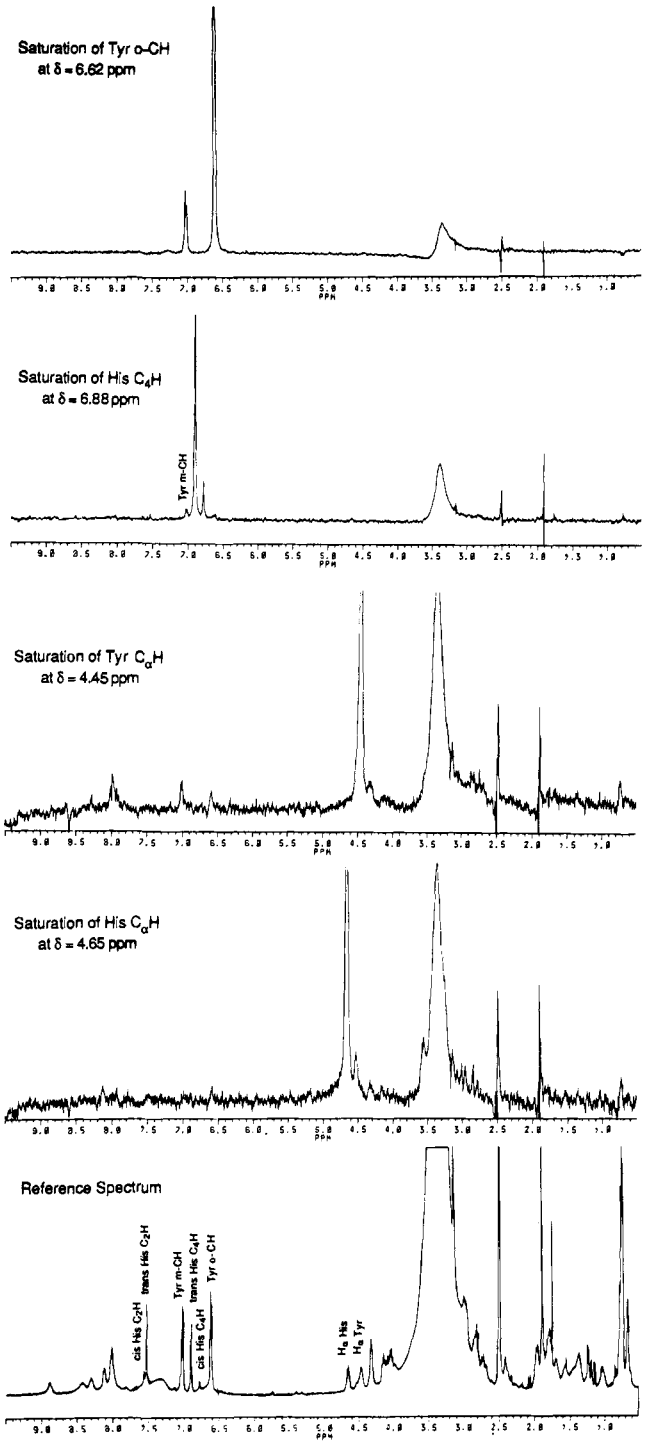


Figure 2. ^{1}H NMR NOE difference spectra for [HSer(γ-OMe)^8]-ANGII in DMSO-d_6.

phase methods. Deprotection of the Phe ring carboxyl from its benzyl group took place simultaneously with deprotection of other residues (Arg, His) during the cleavage of the peptide from resin upon treatment with HF. The obtained analog, [Sar^1,DL-Phe(4′-COOH)^4]-ANGII was purified by HPLC, identified by FABMS and amino acid analysis, and bioassayed in the rat uterus.

Compound **10** was synthesized using diethyl acetamidomalonate and *p*-(trifluoromethyl)benzyl bromide as starting materials in dry ethanol containing sodium ethoxide. The Gabriel condensation afforded compound **8** in high yield (91%). Hydrolysis of **8** with sodium hydroxide followed by acidification afforded 4-(trifluoromethyl)phenylalanine (**9**). Protection of the amino group of **9** with the *N*-*tert*-butyloxycarbonyl group was

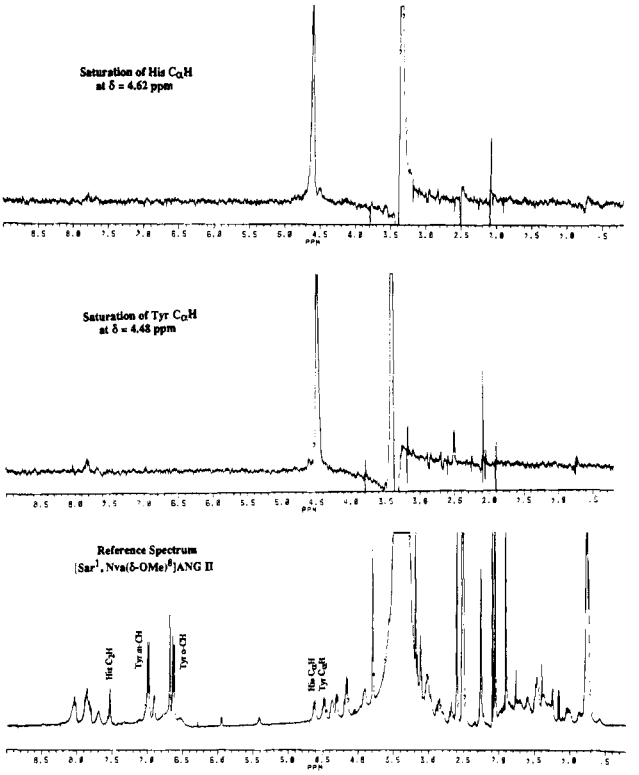


Figure 3. ^{1}H NMR NOE difference spectra for [Sar^1,Nva(δ-OMe)^8]ANGII in DMSO-d_6.

carried out in a similar way as for compound **3** using the method of Nagasawa.[26] *N*-*t*-Boc-4-(trifluoromethyl)-phenylalanine (**10**) was used for the synthesis of analog [Sar^1,DL-Phe(4′-CF_3)^4]ANGII, which was purified by HPLC identified by FAMBS and amino acid analysis and bioassayed in the rat uterus.

Discussion

Structure–Activity Studies. [Sar^1,Ile^8]ANGII (sarilesin) is a potent type I inhibitor ($pA_2 = 8.1$) characterized by protracted antagonist effects on isolated smooth muscle tissues. Therefore, it was of interest to investigate structure–activity relationships in sarilesin analogs with modification at positions 1, 4, and 8 as well as conformational properties which could account for its antagonist activity. In the present study we have found that substitution of HSer(γ-OMe) and Nva(δ-OMe) at position 8 in [Sar^1]ANGII resulted in analogs with antagonist activities of $pA_2 = 7.6$ and 7.1, respectively. These findings indicate that replacement of Ile^8 in Sarilesin with HSer(γ-OMe) and its higher homolog Nva(δ-OMe) does not substantially change the bioactive conformation important for receptor recognition and expression of type I antagonist activity. However, the lower antagonist activity of [Sar^1,Nva(δ-OMe)^8]ANGII bearing a longer aliphatic side chain at position 8 suggests that the proper length of chain at the C-terminal residue of type I antagonist is important for a favorable binding interaction. With the exception of [Des^1,HSer(γ-OMe)^8]ANGII which was found to have very low antagonist activity ($pA_2 < 6$), variation in activity of the synthesized analogs illustrated a preference for the lower homolog HSer(γ-OMe) over Nva(δ-OMe) for the expression of maximum type I antagonist activity. Replacement of Sar by Asp at position 1 in [Sar^1,HSer(γ-OMe)^8]ANGII retained high antagonist activity ($pA_2 = 7.5$) while subsequent deletion of the

N-terminal residue of position 1 resulted in loss of antagonist activity ($pA_2 < 6$), indicating that the effect of the positive charge at the N-terminus derives from its precise location.[9] Substitution of Nva(δ-OMe) in position 4 of Sarilesin resulted in a drastic loss of activity ($pA_2 < 5$) whereas for the analog [Aib[1],Nva(δ-OH)[4],Ile[8]]ANGII, a low binding affinity ($pA_2 = 6.2$) was observed, illustrating the importance of a free hydroxyl group at position 4 for the expression of antagonist activity.

Previous studies on ANGII have implicated that a triad of interacting groups (Tyr OH−His imidazole−Phe COO^-) creates a tyrosinate anion pharmacophore (tyranophore) responsible for receptor activation/triggering. This hypothesis is based on SAR, NMR, and fluorescence studies which further illustrated the importance of the Tyr hydroxyl in receptor activation.[15−18] In the present study, we have investigated the effects on bioactivity by substituting the Tyr residue in [Sar[1]]-ANGII with other anionic or electronegative amino acids as Nva(δ-OH) and Nva(δ-OMe) and with two aromatic amino acids in which the Tyr hydroxyl has been replaced by the COOH and CF_3 groups, respectively. The phenylalanine derivatives, 4-(benzyloxycarbonyl)-phenylalanine and 4-(trifluoromethyl)phenylalanine were synthesized by novel methods. A variation of the Gabriel synthesis utilizing diethyl acetamidomalonate[20−22] was used to prepare the (D,L) amino acids **2** and **9** as depicted in Scheme 1. These amino acids were incorporated in the [Sar[1]]ANGII molecule at position 4 in order to delineate aspects of this pharmacophore which provides for its receptor triggering properties. Deprotection of the 4-(benzyloxycarbonyl)-phenylalanine residue during treatment of the peptide-resin with HF resulted in [Sar[1],D,L-Phe(4′-COOH)]ANGII which was found to be biologically inactive. Furthermore, the synthesis of δ-hydroxyl-L-norvaline [Nva(δ-OH)] was undertaken[23] in order to determine if an aliphatic hydroxyl group appropriately positioned within the ANGII molecule would be able to mimic the tyrosine hydroxyl group and participate in a charge relay system.[17] The present study shows that [Sar[1],Nva(δ-OH)[4]]-ANGII displays low agonist activity (4%) and presumably has little tendency to participate in a stable charge relay system. Methylation of the hydroxyl group produces an analog, [Sar[1],Nva(δ-OMe)[4]]ANGII, with similar activity (3%), suggesting that the electronegative oxygen atom in both cases may act as a weak proton acceptor and thereby produces a weak receptor response (3−4%).

We thought it of interest to investigate if a naked anion such as a carboxylate could act as a proton acceptor in place of the phenolate group of tyrosine in ANGII. Interestingly, [Sar[1],DL-Phe(4′-COOH)[4]]ANGII was virtually biologically inactive, illustrating that the naked COO^- anion does not contribute in the receptor activation process. Since both the carboxylate of Phe-(4′-COOH) and the phenolate group of tyrosine at position 4 can act as proton acceptors from the receptor, the vast difference in activity may indicate the importance of having a nucleophilic group (rather than a negative charge) provided by the tyrosinate species. The nature of the aromatic ring of position 4 influences the binding affinity of ANGII analogs and shows that sufficient perturbation of the receptor by an aromatic ring can produce a partial response or eliminate the response. It has also been shown that replacement of the Tyr hydroxyl by fluorine as in [Sar[1],Phe(4′-F)[4]]-ANGII produces an antagonist ($pA_2 = 7.5$)[12] while polyfluorination of the Phe ring in [Sar[1],Phe[4]]ANGII as in [Sar[1],Phe(F_5)[4]]ANGII abolishes both agonist and antagonist activity, indicating that the affinity of ANGII analogs for receptors depends on the electronegative and quadrupole properties of the aromatic ring.[24] A recent report[25] has provided evidence that the apparent binding affinity of ANG II analogs is associated with a perpendicular interaction of the position 4 aromatic ring with a receptor-based group. In this study, replacement of the hydroxyl group by the trifluoromethyl group CF_3 produced an analog without agonist or antagonist activity. The loss of activity illustrates that both electrostatic and steric properties of the aromatic ring in position 4 are subtle and highly relevant as suggested previously.[24,25]

Conformational Studies. Our recent investigations by NOE spectroscopy in the rotating frame (ROESY) and 1D-NOE studies of ANGII and [Sar[1]]ANGII have shown proximity of the three rings (Tyr, His, Phe), a *trans*-His−Pro bond and a Tyr-Ile-His bend, which seems to be a critical factor for the manifestation of agonist activity.[16−18] By saturating distinct resonances at the well-resolved C_α proton region in the NMR spectra of the type I ANGII antagonists [HSer(γ-OMe)[8]]-ANGII and [Sar[1],Nva(δ-OMe)[8]]ANGII, we were able to shed light on the backbone conformation of these molecules. Thus, saturation of the His C_α proton resonance in both antagonists did not produce any enhancement of the Tyr C_α proton resonance, in contrast to what was the case for ANGII and [Sar[1]]ANGII.[18] Similarly, saturation of the Tyr C_α proton did not again produce any enhancement for the His C_α proton. Lack of such enhancements in [HSer(γ-OMe)[8]]ANGII and [Sar[1],Nva(δ-OMe)[8]]ANGII indicate a loose backbone conformation at this position of the molecules deprived of a Tyr-Ile-His bend, which is a characteristic feature of ANGII, [Sar[1]]ANGII, and agonist analogs.[18] The His C_α and Tyr C_α proton resonances constitute an excellent probe for investigating backbone conformation and their chemical shifts are well-established at $\delta = 4.65$ ppm and $\delta = 4.45$ ppm for [HSer(γ-OMe)[8]]ANGII and at $\delta = 4.63$ ppm and $\delta = 4.48$ ppm for [Sar[1],Nva(δ-OMe)[8]]ANGII through COSY cross-peaks with their $C_{\beta\beta'}$ protons at $\delta = 2.90$ ppm/2.85 ppm and $\delta = 2.88$ ppm/2.82 ppm, respectively. $C_{\beta\beta'}$ protons of aromatic residues (Tyr, His, Phe) appear as distinct and easily recognizable AB quarters upon saturation of their adjacent C_α protons when NMR experiments are carried out using DMSO-d_6/D_2O (9/1) as solvent. Also, our previous NMR studies have constantly shown that the His C_α and Tyr C_α protons resonate at lower field than other C_α protons, and their saturation in our NOE studies provided useful information concerning backbone conformation.[15−18] Extension of these studies in sarilesin (unpublished results) supported our findings and indicated that a Tyr-Ile-His bend as revealed by 1D-NOE studies is a conformational feature of agonists but not of type I antagonists. These findings may open new avenues for designing non-peptide agonists as we have done with non-peptide ANGII receptor antagonists.[32−34] Our investigations of [HSer(γ-OMe)[8]]ANGII by 1D-NOE experiments have indicated also proximity of the Tyr and His rings. Thus, saturation of the His C_4 proton at $\delta =$

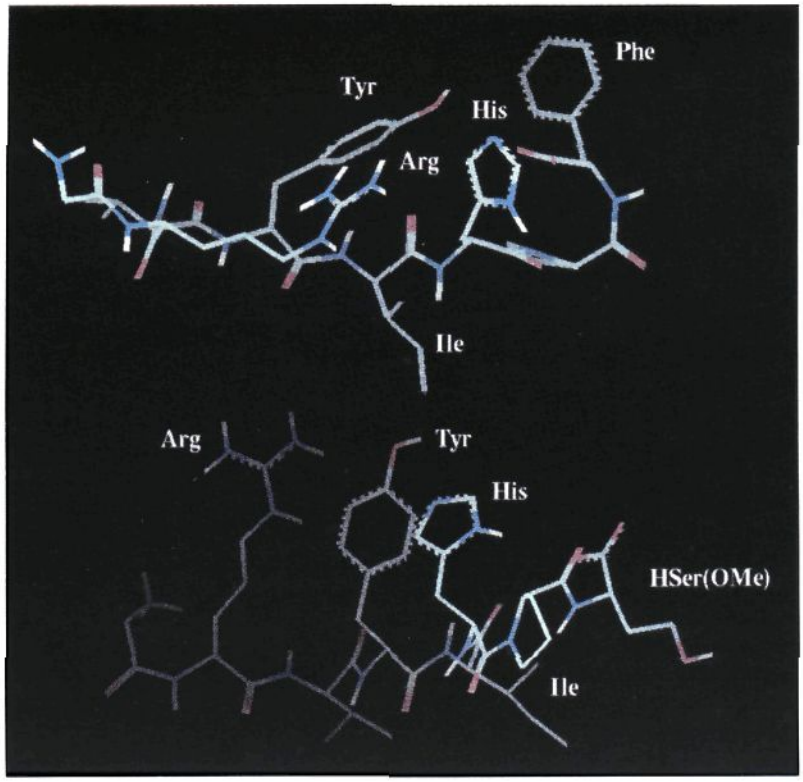


Figure 4. Low-energy conformers of [Sar^1]ANGII (top) and [HSer(γ-OMe)^8]ANGII (bottom).

6.88 ppm resulted in enhancement (7.1%) of the Tyr meta proton at $\delta = 7.02$ ppm (Figure 2). Saturation of the Tyr ortho proton resonance at $\delta = 6.60$ ppm resulted in enhancement of the Tyr meta proton at $\delta = 7.02$ ppm but not of the His C_2 and C_4 proton resonances. These experiments show that the interaction between His and Tyr rings is not a reverse relaxation phenomenon. Thus, while the His C_2 and C_4 protons can relax through the closely spaced Tyr meta and ortho ring protons, the reverse effect is not observed upon saturation of the Tyr meta and ortho protons. The probable reason for this is that the Tyr ortho and meta protons have relaxation pathways which are not available to the His C_2 and C_4 protons. The Tyr ortho protons can relax through the Tyr meta and hydroxyl protons, while the meta protons can relax through the Tyr ortho and $C_{\beta\beta'}$ protons. In our studies, meta and ortho protons refer to the hydroxyl group of tyrosine. The NOE data suggest a loose backbone conformation for the type I ANGII antagonists [HSer(γ-OMe)^8]ANGII and [Sar^1,Nva(δ-OMe)^8]ANGII in DMSO-d_6, in contrast to ANGII and [Sar^1]ANGII which are characterized by a compact folded conformation due to the Tyr-Ile-His bend present in agonists.[18] A comparison of the conformations of [Sar^1]ANGII with [HSer(γ-OMe)^8]ANGII is shown in Figure 4. These models were derived using NOE data and represent low-energy structures. Details of constructing such models are given in a previous publication.[18]

Conclusion

This research aims at refining present knowledge of the angiotensin II backbone conformation as well as of the roles of residues 4 and 8 in activity and conformation of angiotensin II agonist and antagonist analogs. A Tyr-Ile-His bend exists in ANGII and [Sar^1]ANGII but not in type I antagonist analogs with an aliphatic amino acid at position 8. This conformational difference between agonists and type I antagonists, as revealed by 1D-NOE studies, may constitute a major conformational feature distingushing the activity of agonists and antagonists and may provide new avenues in designing non-peptide ANGII agonists as has been possible for non-peptide ANGII antagonists.[32-36] A phenolate anion at position 4, but not a phenylalanine carboxylate anion, is required for the expression of agonist activity, suggesting a unique involvement of the tyrosine hydroxyl in receptor activation. Proper charge positioning or nucleophilicity is better provided by the tyrosinate species and accounts for its triggering ability. The SAR data also indicate that for type I antagonists, substitution of Ile^8 in Sarilesin with HSer(γ-OMe) and Nva(δ-OMe) does not significantly change the active conformation important for receptor recognition and expression of antagonist activity. Variations in activity of the type I antagonist analogues illustrated a preference for Ile or Ala over HSer(γ-OMe) and Nva(δ-OMe). These data indicate that increasing the length of the position 8 aliphatic side chain results in reduction of antagonist activity and influences the conformation necessary for maximum interaction of a type I antagonist with the receptor.

Experimental Section

***N-t*-Boc Methodology.** Solid-phase synthesis of [Nva(δ-OH)^4]- and [Nva(δ-OMe)^4]angiotensin II and Sarilesin analogs was accomplished using *tert*-butyloxycarbonyl-substituted amino acids and 1% cross-linked divinylbenzene resins (0.4–0.6 mequiv/g) as solid support and procedures previously described.[10] *N-t*-Boc methodology was used also for the synthesis of [Phe(4′-COOH)^4]- and [Phe(4′-CF_3)^4]angiotensin II analogs. *tert*-Butyloxycarbonyl-blocked amino acids were synthesized in our laboratories[26] or were purchased from Peninsula Laboratories, Bachem Inc., or Chemical and Biopharmaceutical Laboratories of Patras (CBL). Protected amino acids, resins, ANGII, and sarilesin were purchased from Peninsula

Laboratories. Nva(δ-OH) and Nva(δ-OMe) were synthesised by methods previously described[23] or purchased from CBL and converted to *N*-*t*-Boc derivatives by the method of Nagasawa.[26] Phe(4′-COOH) and Phe(4′-CF_3) were synthesized by the Gabriel method described herein.[20-22] Solid-phase peptide synthesis was carried out with a Beckman 990 or 990B peptide synthesizer or with a reaction flask (2 × 12 cm) equipped with a bottom G-2 filter and tap connected to a water vacuum aspirator. Coupling steps with 2.5 equiv of Boc-protected amino acid and coupling reagent were employed for each amino acid; the coupling was mediated by *N*,*N*′-dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBt) for 3 h. Deprotection steps (2 and 25 min) utilized 35% CF_3-COOH in CH_2Cl_2 containing indole (0.1%). Neutralization steps (2 and 25 min) utilized 10% triethylamine in CH_2Cl_2. Peptides were synthesized on a 0.5-mmol scale. The completed peptide was removed from the resin and simultaneously deprotected by treatment with anhydrous HF (10 mL) in the presence of *p*-cresol (1 g) for 30 min at 0 °C. The peptide was dissolved in CF_3COOH (100 mL), and the resin was removed by filtration. The solvent was removed by rotary evaporation, and the crude product was isolated by trituration with ether and filtration. The crude product was dissolved in 7% acetic acid (10 mL), clarified by centrifugation, and purified by HPLC.

Synthesis of 4-Carboxy- and 4-(Trifluoromethyl)-phenylalanine. A variation of the Gabriel synthesis utilizing diethyl acetamidomalonate[21] was used to prepare the (D,L) amino acids **2** and **9** as depicted in Scheme 1. Selective deprotection of the benzyl ester **5** was achieved by the copper complex according to the method of Prestidge et al.[22] Amino acid **7** was obtained in 90% purity by ^{1}H-NMR with the impurity tentatively identified as the structural isomer Boc-NH-CH-(CH_2Ph-4-CO_2H)-CO_2CH_2Ph. The Boc-N-protected amino acids **7** and **10** were used in position 4 to prepare ANGII analogs.

Diethyl 2-Acetamido-2-[[4-(ethoxycarbonyl)phenyl]-methyl]malonate (1). Dry ethanol (68 mL) was added dropwise to pentane-washed sodium pieces (2.3 g, 0.1 mol) under an argon atmosphere. After completion of addition, heating was required to dissolve the last pieces of sodium. Solid diethyl acetamidomalonate (21.722 g, 0.1 mol) was added portionwise over 30 min, after which the reaction mixture became very thick and hard to stir and heating was required to solubilize it. α-Bromo-*p*-toluic acid ethyl ester (24.31 g, 0.1 mol, 70% pure, the balance being *p*-toluic acid ethyl ester 16.7% and α,α-dibromo-*p*-toluic acid ethyl ester) in dry ethanol (100 mL) was added dropwise over 90 min.[20] The mixture turned yellow, and a fine yellowish precipitate deposited on the sides of the flask. After the mixture was refluxed for 5 h, it was cooled, ether and H_2O were added, and the organic layer was separated. The aqueous layer was saturated with solid NaCl and extracted with ethyl acetate. The organic extracts were combined, washed with brine, dried over $MgSO_4$, and filtered. The solvents were removed *in vacuo*. Hexane–benzene, 4:1, was added to the residue, and the insoluble material was filtered out and washed exhaustively with 10:1 hexane–benzene. Then, it was allowed to air-dry giving 1 (23.4 g). A second crop (9.0 g) could be obtained after concentration of the mother liquor (85% total yield): ^{1}H-NMR ($CDCl_3$) δ 7.97–7.91 (m, 2H), 7.1–7.06 (m, 2H), 6.53 (bs, NH, 1H), 4.41–4.42 (overlapped quartets, OCH_2, 6H), 3.71 (s, CH_2-Ph, 2H), 2.03 (s, $COCH_3$, 3H), 1.41–1.26 (overlapped triplets, 9H); ^{13}C-NMR ($CDCl_3$) δ 169.1, 167.3, 166.3, 140.6, 129.8, 129.7, 129.5, 67.0, 62.8, 60.9, 37.8, 23.0, 14.3, 14.0; exact mass calcd for $C_{19}H_{25}NO_7$ 379.1631, found 379.1637.

4-Carboxyphenylalanine Hydrochloride (2). Sodium hydroxide (11.6 g, 0.29 mol) in H_2O (226 mL) was added to acetamido triester **1** (32.4 g, 85.4 mmol) in tetrahydrofuran (80 mL), and the mixture was stirred at room temperature overnight. After the bulk of tetrahydrofuran has been removed *in vacuo*, acetic acid (24.5 mL) was added to acidify the reaction and the mixture was refluxed for 1 h. Then the mixture was taken to dryness, 12 M hydrochloric acid (80 mL) was added, and the mixture was refluxed for 1 h. The mixture was taken to dryness, H_2O was added, and it was taken to dryness again. Water was added to the residue, and the insoluble material was filtered out and washed with H_2O, ethanol, and ethyl acetate, giving **2** (16.66 g). A second crop (2.06 g) was obtained (89% total yield): ^{1}H-NMR (D_2O) δ 7.85 (d, *J* = 8.2 Hz, 2H), 7.28 (d, *J* = 8.2 Hz, 2H), 4.23 (dd, *J* = 7.6, 5.9 Hz, 1H), 3.2 (m, 2H).

***N*-(*tert*-Butoxycarbonyl)-4-carboxyphenylalanine (3).** *tert*-Butyl 4,6-dimethylpyrimidine-2-thiolocarbonate (8.0 g, 32.3 mmol) was added to a solution of amino acid **2** (7.434 g, 30.3 mmol) and Et_3N (12.7 mL, 90.9 mmol) in tetrahydrofuran (68 mL) and H_2O (68 mL). The mixture was stirred at room temperature overnight according to the procedure of Nagasawa *et al.*[26] After workup **3** was obtained in 64% yield (5.945 g): ^{1}H-NMR (CD_3OD) δ 7.67 (d, *J* = 8.2 Hz, 2H), 7.07 (d, *J* = 8.2 Hz, 2H), 4.17 (dd, *J* = 9.1, 5.4 Hz, 1H), 2.82 (two sets of dd overlapping, 2H), 1.47 (s, 9H); ^{13}C-NMR (DMSO-d_6) δ 173.2 (CO_2H), 167.2 (CO_2H), 155.3 (CON), 143.3, 129.2, 129.1, 128.9, 78.0 (CO), 54.7 (CHN), 36.4 (CH_2-Ph), 28.1 (*t*-Bu); exact mass calcd for $C_{15}H_{20}NO_5$ (M^+ − OH) 292.1185, found 292.1191.

***N*-(*tert*-Butoxycarbonyl)-4-carboxyphenylalanine Dibenzyl Ester (4).** Dicyclohexylcarbodiimide (9.374 g, 45.4 mmol) was added to a mixture of **3** (6.692 g, 21.6 mmol), benzyl alcohol (4.913 g, 45.4 mmol), and DMAP (0.55 g) in CH_2-Cl_2 (100 mL). After the reaction mixture was stirred overnight, the precipitated dicyclohexylurea was filtered out and rinsed with the minimum amount of CH_2Cl_2. Workup according to the procedure of Hassner and Alexanian[22] gave crude **4**. Chromatography over flash silica eluting with 0–2% MeOH–$CHCl_3$ gave **4** in 63% yield (6.7 g): ^{1}H-NMR ($CDCl_3$) δ 7.94 (d, *J* = 8.2 Hz, 2H), 7.50–7.25 (m, 10H), 7.11 (d, *J* = 8.2 Hz, 2H), 5.37 (s, CH_2O, 2H), 5.13 (d, *J* = 1.2 Hz, CH_2O, 2H), 4.66 (m, 1H), 3.15 (m, CH_2Ph, 2H), 1.43 (s, *t*-Bu, 9H); ^{13}C-NMR ($CDCl_3$) δ 171.3 (CO_2), 166.1 (CO_2), 163.2 (CON), 141.5, 136.0, 134.9, 129.8, 129.4, 129.3, 128.8, 128.6, 128.2, 128.1, 80.0 (*t*-BuCO), 67.2 (CH_2O), 66.6 (CH_2O), 54.2 (CHN), 38.3 (CH_2Ph), 28.2 (*t*-Bu); exact mass calcd for $C_{25}H_{21}NO_5$ (M^+ − $C_4H_{10}O$) 415.1420, found 415.1434.

4-Carboxyphenylalanine Dibenzyl Ester (5). Trifluoroacetic acid (50 mL) was added to a solution of **4** (6.7 g) in CH_2Cl_2 (50 mL) at room temperature, and the progress of the reaction was followed by TLC. After 40 min, excess TFA and CH_2Cl_2 were removed *in vacuo*. Ethyl acetate (100 mL) was then added, and the organic layer was washed with H_2O (2 × 50 mL), 5% $NaHCO_3$ (3 × 50 mL), H_2O (50 mL), and brine (50 mL). Finally, it was dried over anhydrous $MgSO_4$, filtered, and evaporated, giving **5** in 98% yield (5.29 g): ^{1}H-NMR ($CDCl_3$) δ 8.06 (bs), 7.81 (d, *J* = 8.3 Hz, 2H), 7.48–7.0 (m, 12H), 5.22 (s, CH_2O), 5.0 (d, *J* = 2.8 Hz, CH_2O, 2H), 4.26 (m, 1H), 3.19 (d, *J* = 6.3 Hz, 2H); ^{13}C-NMR ($CDCl_3$) δ 172.3 (CO_2), 166.1 (CO_2), 141.5, 136.0, 134.9, 130.0, 129.4, 129.0, 128.5, 128.2, 128.1, 67.3 (CH_2O), 66.6 (CH_2O), 55.0 (CHN), 39.4 (CH_2Ph); exact mass calcd for $C_{24}H_{23}NO_4$ (M^+) 389.1627, found 389.1624.

4-(Benzyloxycarbonyl)phenylalanine (6). A solution of $CuSO_4$·$5H_2O$ (16.157 g) in H_2O (283 mL) was added to **5** (5.3 g, 13.6 mmol) in ethanol (113 mL), and the pH was adjusted to 8 with 1 N NaOH according to the procedure of Prestidge *et al.*[21] After the mixture was stirred at 33 °C for 1 h, the pH was lowered to 3 and the insoluble blue copper complex was filtered out and washed with H_2O, ethanol, ethyl acetate, and ether. Ethylenediaminotetraacetic acid disodium salt (6.32 g, 17 mmol) in water (81 mL) was added, and the solution was boiled. On cooling, a white solid precipitated. This was filtered and washed with water and was allowed to air-dry, giving **6** in 83% yield (3.4 g).

***N*-(*tert*-Butoxycarbonyl)-4-(benzyloxycarbonyl)-phenylalanine (7).** Amino acid **6** (1.4 g, 4.7 mmol) was stirred with 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile (1.264 g, 5.1 mmol) and Et_3N (1.8 mL) in dioxane (10 mL) and H_2O (10 m) at room temperature overnight. After workup, the crude product was triturated from 2:8 mixture of ethyl acetate/hexane, giving **7** in 85% yield (1.58 g). The ^{1}H-NMR spectrum showed the product to contain a 10% impurity, tentatively identified as an isomer of 7 in which the benzyl protecting group is on the α-carboxy group: ^{1}H-NMR ($CDCl_3$) δ 8.15 (bs), 8.02 (d, *J* = 8.2 Hz, 2H), 7.45–7.25 (m, 7H), 5.36 (s, CH_2O, 2H), 4.65 (m, 1H), 3.2 (m, 2H), 1.41 (s, *t*-Bu, 9H); ^{13}C-NMR ($CDCl_3$) δ 175.9 (CO_2), 166.2 (CO_2), 155.7 (CON), 142.0, 136.0, 130.0, 129.5, 129.0, 128.6, 128.2, 128.1, 80.1 (CO),

66.7 (CH_2Ph), 28.2 (*t*-Bu); exact mass calcd for $C_{22}H_{25}NO_6$ (M^+) 399.1682, found 399.1686.

Diethyl 2-Acetamido-2[[4-(trifluoromethyl)phenyl]-methyl]malonate (8). *p*-(Trifluoromethyl)benzyl bromide (2.0 g, 8.37 mmol) in dry ethanol (8.5 mL) was added dropwise over 30 min under argon to a solution of diethyl acetamidomalonate ester (1.817 g, 8.37 mmol) and sodium ethoxide in dry ethanol [prepared *in situ* by the reaction of sodium metal (0.192 g, 8.37 mmol) with ethanol (6.0 mL)]. After completion of addition, the mixture was refluxed for 5½ h, and then it was stirred at room temperature for 12 h. Water was added to dissolve the yellow precipitate, and the mixture was extracted with chloroform (3×). The combined organic extracts were washed with brine, dried over magnesium sulfate, and filtered, and the solvent was removed *in vacuo*. Chromatography of the residue over flash silica, eluting with 24–32% ethyl acetate/hexane, gave **8** in 91% yield (2.86 g): ^{1}H-NMR ($CDCl_3$) δ 7.53 (d, J = 8.1 Hz, 2H), 7.13 (d, J = 8.1 Hz, 2H), 6.54 (bs, NH, 1H), 4.28 (q, J = 7.2 Hz, CH_2O, 4H), 3.73 (s CH_2-Ph, 2H), 2.03 (s, CH_3CO, 3H), 1.30 (t, J = 7.1 Hz, 6H). Anal. Calcd for $C_{17}H_{20}F_3NO_5$: C, 54.40; H, 5.37; N, 3.73. Found: C, 54.4; H, 5.26; N, 3.74.

4-(Trifluoromethyl)phenylalanine Hydrochloride (9). The diester **8** (2.76 g, 7.35 mmol) was dissolved in tetrahydrofuran (10.0 mL) and water (13.0 mL). Solid sodium hydroxide (0.67 g, 16.5 mmol) was then added, and the mixture was stirred at room temperature for 18 h. Glacial acetic acid (2.0 mL) was added, and the mixture was boiled for 1 h. It was then concentrated under reduced pressure. Concentrated hydrochloric acid (10 mL) was added, and the resulting slurry was refluxed for 1 h. The mixture was taken to dryness, and the residue was taken up into ethanol. The ethanol was removed *in vacuo*. The remaining residue was refluxed once again with concentrated hydrochloric acid (10 mL) for 1 h, and then it was taken to dryness. Water was added to the residue, and the insoluble material was filtered out, washed with ether, and air-dried, giving **9** (1.0 g). The filtrate was taken to dryness, and dry ethanol was added. The insoluble material was filtered out. After evaporation of the ethanol filtrate, the residue was triturated with ether, giving a second crop of **9** (0.819 g). The hydrochloride **9** was obtained in 92% total yield: ^{1}H-NMR (CD_3OD) δ 7.67 (d, J = 8.1 Hz, 2H), 7.51 (d, J = 8.1 Hz, 2H), 4.33 (dd, J = 6.0, 7.3 Hz, $CHCO_2$, 1H), 3.43–3.20 (m, CH_2Ph, 2H), ^{13}C-NMR (CD_3OD) δ 170.9 (CO_2H), 140.4, 131.3, 130.9, 127.0, 126.93, 126.85, 54.8 (CHN), 37.0 (CH_2Ph). Anal. Calcd for $C_{10}H_{10}F_3NO_2$: C, 44.54; H, 4.11; N, 5.19. Found: C, 44.23; H, 3.95; N, 5.02.

***N*-*t*-BOC-4-(trifluoromethyl)phenylalanine (10).** To a solution of hydrochloride **9** (1.771 g, 6.57 mmol) and triethylamine (2.2 mL, 16.4 mmol) in dioxane (12.0 mL) and water (12.0 mL) was added 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile (BOC-ON, 1.643 g, 6.67 mmol), and the reaction mixture was stirred at room temperature for 21 h. Dioxane was removed *in vacuo*, and the mixture was diluted with water (30 mL). The basic aqueous layer was extracted with ether (3 × 50 mL). The aqueous layer was acidified with 5 N hydrochloric acid to pH 3, saturated with sodium chloride, and extracted with ethyl acetate (4 × 50 mL). The combined ethyl acetate extracts were dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Chromatography of the residue on a flash silica column eluting with 4–7% MeOH/$CHCl_3$ gave **10** in 67% yield (1.46 g): ^{1}H-NMR ($CDCl_3$ + 2 drops of CD_3OD) δ 173.8 (CO_2H), 155.3 (NCO), 140.5, 129.8, 128.9, 125.22, 80.0 (CO), 54.0 (NCH), 37.8 (CH_2Ph), 28.1 ($(CH_3)_3$). Anal. Calcd for $C_{15}H_{18}F_3NO_4$: C, 54.05; H, 5.44; N, 4.20. Found: C, 53.86; H, 5.34, N, 4.21.

Fmoc Methodology. For the synthesis of [HSer(γ-OMe)^8] and [Nva(δ-OMe)^8]angiotensin II analogs we resorted to the Fmoc methodology utilizing the 2-chlorotrityl-resin as solid support as reported by Barlos *et al.*[27] Fmoc-blocked amino acids were synthesized in our laboratories as described previously or were purchased from CBL. Fmoc-His(Trt)-OH and Fmoc-Arg (Pmc)-OH were purchased from CBL and NOVA BIOCHEM. Attachment of the first Fmoc-protected amino acid, HSer(γ-OMe) or NVa(δ-OMe), to the resin (2-chlorotrityl) was achieved by a simple, fast, and racemization-free reaction using diisopropylethylamine (DIPEA) in dichloromethane (DCM) solution at room temperature.[27] Solid-phase peptide syntheses were carried out on 2-chlorotriphenylmethyl (trityl) resin (1.4–1.6 mmol Cl^-/g) using a manually handled reaction vessel (2 × 12 cm) equipped with a porous G filter (size 2) and tap at the bottom connected with a water vacuum aspirator. A vibrator was used for shaking the reaction vessel throughout the several steps. The protocol used for the peptide synthesis is previously described.[27] After preactivation with 1-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC), a 2.5-fold molar excess of Fmoc amino acids were used. Deprotection steps (5 and 20 min) utilized 20% piperidine in DMF. Splitting of the peptide from the 2-chlorotrityl-resin was carried out by treating the peptide-resin with the splitting solution, dichloromethane–acetic acid–2,2,2-trifluoroethanol (7:1:2, 15 mL/g resin) for 1 h. The mixture was filtered off, and the resin was washed with the splitting mixture and DCM several times. The solvent was removed on a rotary evaporator, and the obtained oily product was treated for 45 min at room temperature with 45–50% trifluoroacetic acid in DCM, containing 5% anisole as scavenger. The mixture was evaporated to ~0.5 mL, and the peptide precipitated from dry diethyl ether as a white amorphous solid. Removal of the protecting groups (Sar-Boc, Arg-Pmc, His-Trt) was achieved by treatment of the protected peptide with TFA–CH_2Cl_2 (1:1, 20 mL/g peptide) in the presence of scavenger (5% anisole). The solvent was removed by rotary evaporator, and the crude product was isolated by trituration with ether and filtration. The crude product was dissolved in 7% acetic acid (10 mL), clarified by centrifugation, and purified by HPLC.

Preparative Reversed-Phase HPLC. Purification of peptides was accomplished with use of a Varian HPLC system equipped with a Vista 401 microprocessor controller. Separations were achieved on a BioRad Hi-Pore 318 reversed-phase preparative column (25.0 × 2.15 cm) at 25 °C with a stepped linear gradient of acetonitrile in 0.1% CF_3CO_2H at a flow rate of 7.5 mL/min. Automated repetitive injections of peptides (5 × 5 mg) were made from a nitrogen-pressurized Rheodyne injector with a 2.0 mL sample loop. One-fifth of the total sample was injected during each run by lowering the flow rate to 4.0 mL/min for 0.1-min "inject" period. One cycle consisted of the following events: 0–10 min, 7.5 mL/min, 90% H_2O–10% of 1% aqueous CF_3CO_2H; 10–11 min, 4.0 mL/min; 11–11.1 min, "inject"; 11.1–13 min, 7.5 mL/min, 70% H_2O–20% CH_3CN–10% of 1% CF_3CO_2H; 13–30 min, 45% H_2O–45% CH_3CN–10% of 1% CF_3CO_2H; 30–42 min, 90% CH_3CN–10% of 1% CF_3CO_2H; 42–50 min, 100% H_2O. Fractions were collected at 0.1-min intervals with a Gilson Model 210 fraction collector programmed to collect for a 5-min period centered around the elution time (20–25 min) of the major product. The fraction collector was restarted by the Vista 401 at the beginning of each HPLC run so that the material eluted with the same retention time was repeatedly deposited in the same tubes. Elution of the peptide was detected simultaneously from the absorbances at 254 nm (Varian UV-1) and 230 nm (Kratos SF769Z). The elution times for these peptides were similar (22–24 min). Fractions containing the major product were pooled; after removal of CH_3CN on a rotary evaporator at 40 °C, the fractions were lyophilized and stored at −20 °C, yield 5–20%; purity >98%. Amino acid analyses (Beckman 121M) were carried out after acid hydrolysis in 6 N HCl (containing 1% cresol to prevent loss of tyrosine) at 100 °C for 18 h *in vacuo*.

Thin-Layer Chromatography (TLC). TLC was carried out with precoated silica gel on glass (Merck Kiesel gel 60 F254) TLC plates. The two-solvent systems used were 1-butanol–pyridine–acetic acid–water (15:10:3:6, BPAW) and chloroform–methanol–acetic acid–water (15:10:2:3, CMAW). Peptides were detected by fluorescence with ninhydrin (0.3%) in 1-butanol–acetic acid (100:3, v/v).

Rat Uterus Bioassay. The rat isolated uterus bioassay[8,28] was performed as described below. Female virgin Sprague–Dawley rats (150–200 g), injected 24 h previously with 25 μg of diethylstilbestrol in 0.05 mL of 20% polyvinylpyrrolidone (im), were killed by stunning and exsanguination. The uterine horns were exposed, cleaned of adherent fat *in situ*, and then removed to a Petri dish containing DeJalons solution (150 mM NaCl, 5.6 mM KCl, 0.18 mM $CaCl_2$, 1.8 mL $NaHCO_3$ and 1.4

mM glucose, PH 7.0) at room temperature where each was divided into two half lengths and arranged for mounting in a 3 mL tissue bath. Tissues thus prepared were tied to Grass force-displacement transducers under 1 g of tension and bathed at 29 °C in a constantly oxygenated DeJalons solution as prepared above. Contractions were monitored with Gould Metripak 763341-4202 isotonic transducers coupled to Gould 13-4615-50 transducer amplifiers housed in a Gould 2600S recorder. Samples were prepared initially as a 10^{-3} M solution in water. Agonist activities of peptides were determined by matching the response with an equivalent response to ANGII (human). For the measurement of antagonist activity of peptide ANGII receptor antagonists the analogs were given 2 min before ANGII. Antagonist activities (pA_2) were determined as the negative logarithm of the concentration of antagonist required to reduce the response to an ED_{50} dose of ANGII to that of an $ED_{50/2}$ dose. Each analog was tested in at least three different tissues, and the mean result was taken. A noncumulative dose–response curve to ANGII was first established in each tissue, and the EC_{50} dose and $EC_{50/2}$ response was determined. To avoid tachyphylaxis, the tissue was washed out at the peak response to the agonist and was allowed to rest for 10 min between agonist challenges.[8,28] Antagonist was applied to the tissue 2 min before the agonist, and the reproducibility of the EC_{50} agonist response was established before and after each antagonist challenge.

NMR Experiment. NMR experiments were carried out using a Brüker 400-MHz NMR spectrometer. [HSer(γ-OMe)^8]-ANGII and [Sar^1,Nva(δ-OMe)^8]ANGII were studied by dissolving three milligrams of each in 0.5 mL of DMSO-d_6. The chemical shifts were reported relative to the undeuterated fraction of the methyl group of DMSO-d_6 at 2.50 ppm with respect to TMS. One-dimensional spectra were recorded with a sweep width of 4.500 Hz and 32K (zero filled to 64K) data points and by methods previously described.[16] The COSY (two-dimensional correlated spectroscopy) experiment provided contour plots which were symmetrized with respect to the diagonal. One-dimensional NOE experiments were carried out in the difference mode using multiple irradiation. The methods used for the COSY and NOE experiments were similar to those previously described.[15,16,18,30,31]

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