Synthesis and Biological Activities of Angiotensin II, Sarilesin, and Sarmesin Analogues Containing Aze or Pip at Position 7[†]

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Analogues of [Sar[1]]angiotensin II, Sarilesin (type I antagonist), and Sarmesin (type II antagonist) with L-azetidine-2-carboxylic acid (Aze) and L-pipecolic acid (Pip) at position 7 have been prepared by the solid-phase method, purified by reverse-phase HPLC, and bioassayed in the rat uterus. Analogues of the superagonist [Sar[1]]ANGII with Aze or Pip at position 7 and sarcosine (Sar) or aminoisobutyric acid (Aib) at position 1 had high intrinsic activity in the rat isolated uterus assay (34–184%). Analogues of Sarilesin ([Sar[1],Ile[8]]ANGII) with Aze or Pip at position 7 and Sar or Aib at position 1 retained high antagonist activity (pA_2 = 7.1–8.3). Analogues of Sarmesin ([Sar[1],Tyr(OMe)[4]]ANGII) with Aze and Pip at position 7 had pA_2 values of 7.4 and 6.5, respectively. [Aze[7]]-ANGII and [Pip[7]]ANGII had low activities (12% and 1%, respectively), and deletion of Sar at position 1 of Sarmesin analogues abolished binding (or affinity) as judged from pA_2 values. Nuclear Overhauser effect (NOE) spectroscopy studies of [Sar[1],Aze[7]]ANGII in DMSO-d_6 have indicated a clustering of the three aromatic rings (Tyr, His, Phe) and proximity of Sar C_α and Arg C_δ protons to the Tyr/Phe ring protons. These data emphasize that replacement of Pro with the lower and higher homologs Aze and Pip does not greatly alter the structural requirements necessary for expression of agonist or antagonist activity, when sarcosine occupies position 1, but not when Asp occupies position 1, suggesting that there is an intimate relationship between the N-terminal and penultimate residues of the molecule in the biologically active conformation of the molecule.

Introduction

The octapeptide angiotensin II (ANGII, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) acts at receptors at central and peripheral sites in numerous target tissues to elicit contractible and secretory responses and has been implicated in blood pressure regulation and the pathogenesis of hypertension and congestive heart failure.[1,2] Substitution of Pro at position 7 of ANGII with D-Pro results in a dramatic loss of activity, whereas substitution with other secondary amino acids such as sarcosine, N-methylalanine, and nipecotic acid moderately decreases the biological activity.[3,4] Similarly, substitution of Pro with 5,5-dimethylthiazolidine-4-carboxylic acid and other constrained amino acids results in analogues with reduced agonist or antagonist activity.[5] It has been furthermore reported that replacement of Pro by Pip in ANGII resulted in an inactive analogue;[6,7] inactivity of [Pip[7]]ANGII was attributed to loss of rigidity and subsequently loss of specificity because of ring expansion. Previous conformational studies on angiotensin II have illustrated that the peptide molecule takes up a folded structure characterized by a trans His–Pro bond, a clustering of the three aromatic rings which lay above the proline residue and a charge relay system involving the triad Tyr hydroxyl–His imidazole–Phe carboxylate.[4,8] A γ-turn within the C-terminal tripeptide invoked by the presence of a secondary amino acid at position 7 stabilizes the folded structure.[9,10] A tight turn also involving residues Ile[5], His[6], Pro[7] and responsible for determining the mode of binding has been recently reported to be present in a bound conformation of ANGII.[11]

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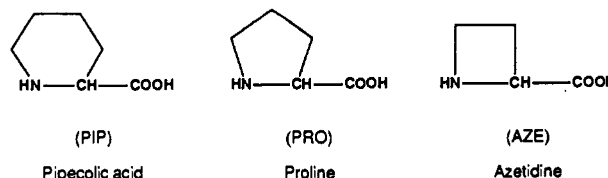
In the present study we have replaced the Pro residue in ANGII, Sarilesin, Sarmesin, and analogues thereof with the lower and higher homologs azetidine and pipecolic acid, respectively, to investigate further the role of Pro[7] in maintaining the conformational integrity of agonists and antagonists for binding with high affinity to angiotensin receptors.

Results

Tables I and II give the chemical data and biological activities, respectively, of the various angiotensin analogues synthesized in the present study. As is evident from the data for the first six peptides listed in Table II, substitution of Aze and Pip at position 7 in [SAR[1]]ANGII resulted in analogues which retained high activity. Thus [Sar[1],Aze[7]]-ANGII expressed the same degree of activity (98%) as angiotensin II while [Sar[1],Pip[7]]ANGII was found to be much potent (184%). Substitution of Sar by Aib in these peptides results in moderately decreased activity (34% and 65%). The same activity (35%) is retained upon deletion of the N-terminal amino acid of the Aze[7] analog.



Slowly reversing type I antagonists,[12] the pharmacological properties of which have been described in detailed previously[13,14] were produced by substitution of Pro at position 7 with Aze or Pip in [Sar[1],Ile[8]]ANGII. Both analogues, [Sar[1],Aze[7],Ile[8]]ANGII and [Sar[1],Pip[7],Ile[8]]-ANGII, retained high antagonist activity (pA_2 = 7.9 and

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Table I. Chemical Data on [Aze^7]- and [Pip^7]angiotensin II Analogues[a]

analogue	TLC R_f		amino acid analysis							
	BPAW	CMAW	Asp	Arg	Val	Tyr	Ile	His	Aze (Pip)	Phe
[Sar^1,Aze^7]ANGII	0.61	0.46		1.00	0.81	0.87	0.76	0.79		1.14
[Aib^1,Aze^7]ANGII	0.58	0.46		1.00	0.86	0.92	0.81	0.78		1.04
[Aze^7]ANGII	0.59	0.46	0.92	1.06	0.83	0.93	0.80	0.81		1.12
[Des^1,Aze^7]ANGII	0.59	0.54		1.02	0.83	0.88	0.78	0.77		1.08
[Sar^1,Pip^7[ANGII	0.55	0.46		1.28	1.21	1.15	1.17	1.00		1.10
[Aib^1,Pip^7]ANGII	0.53	0.50		1.24	1.18	1.12	1.15	0.98		1.06
[Pip^7]ANGII	0.66	0.56	0.99	1.00	1.06	0.95	1.27	0.97		1.32
[Sar^1,Aze^7,Ile^8[ANGII	0.48	0.44		1.00	0.88	0.93	0.95	0.99		0.95
[Aib^1,Aze^7,Ile^8]ANGII	0.52	0.47		0.99	0.86	0.95	0.93	0.97		0.98
[Sar^1,Pip^7,Ile^8]ANGII	0.55	0.50		1.35	1.09	1.24	1.11	1.00		1.11
[Aib^1,Pip^7,Ile^8]ANGII	0.50	0.51		1.31	1.00	1.18	1.09	0.98		1.08
[Sar^1,Tyr(Me)^4,Aze^7]ANGII	0.46	0.49		1.00	0.89	0.74	0.90	0.73		1.00
[Sar^1,Tyr(Me)^4,Pip^7]ANGII	0.57	0.56		1.14	1.00	1.05	0.90	0.90		0.95
[Des^1,Tyr(Me)^4,Aze^7]ANGII	0.57	0.51		1.02	0.91	0.78	0.91	0.75		1.01
[Des^1,Tyr(Me)^4,Pip^7]ANGII	0.62	0.55		1.12	1.00	1.02	0.91	0.89		1.01

[a] Conversion of the Tyr(Me) to Tyr during acid hydrolysis is not always quantitative. N-Methylated amino acids give very low color yields by amino acid analysis and could not be reliably estimated.

Table II. Rat Uterus Biological Activities of Angiotensin II, Sarilesin, and Sarmesin Analogues Containing Azetidine and Pipecolic Acid[a] at Position 7

analogue	agonist activity (% of ANGII)	antagonist activity (pA_2)
[Sar^1]ANGII	160	
[Sar^1,Aze^7]ANGII	98 (9)	
[Aib^1,Aze^7]ANGII	34 (9)	
[Aze^7]ANGII	12 (9)	
[Des^1,Aze^7]ANGII	35 (5)	
[Sar^1,Pip^7]ANGII	184 (6)	
[Aib^1,Pip^7]ANGII	65 (4)	
[Pip^7]ANGII	1.0 (6)	<5.0
[Sar^1,Ile^8]ANGII	0.1	8.1
[Sar^1,Aze^7,Ile^8]ANGII	<0.1 (6)	7.9
[Aib^1,Aze^7,Ile^8]ANGII	<0.1 (4)	7.1
[Sar^1,Pip^7,Ile^8]ANGII	<0.1 (2)	8.3
[Aib^1,Pip^7,Ile^8]ANGII	<0.1 (6)	7.6
[Sar^1,Tyr(Me)^4]ANGII	<0.1	7.7
[Sar^1,Tyr(Me)^4,Aze^7]ANGII	0.5 (4)	7.4
[Sar^1,Tyr(Me)^4,Pip^7]ANGII	<0.1 (4)	6.5
[Des^1,Tyr(Me)^4,Aze^7]ANGII	<0.1 (4)	<5.0
[Des^1,Tyr(Me)^4,Pip^7]ANGII	<0.1 (4)	<5.0

[a] Values are given as mean (number of experiments) with human ANGII as the standard; standard errors of the mean were 10–20% for agonist activities and 0.1 for antagonist pA_2 values; abbreviations are standard except: Aib, 2-aminoisobutyric acid; Aze, azetidine-2-carboxylic acid; Pip, pipecolic acid; Des analogues of ANGII are ANGIII analogues. Data for [Pip^7]ANGII consistent with previous results from refs 6 and 7.

8.3, respectively) compared to [Sar^1,Ile^8]ANGII (pA_2 = 8.1). Substitution of Sar by Aib in these analogues results in moderately decreased antagonist activity (pA_2 = 7.1 and 7.6, respectively). The pharmacological profile and duration of action of these type I antagonists was similar to that observed for [Sar^1,Ile^8]ANGII.[13]

Competitive type II antagonists[15,16] were produced by substitution of Pro at position 7 with Aze and Pip in [Sar^1,Tyr(OMe)^4]ANGII (Sarmesin pA_2 = 7.7). Whereas, [Sar^1,Tyr(OMe)^4,Aze^7]ANGII retained a strong antagonist potency (pA_2 = 7.4), [Sar^1,Tyr(OMe)^4,Pip^7]ANGII was found to be a weak antagonist (pA_2 = 6.5). Deletion of the Sar^1 residue resulted in dramatic loss of activity in both analogues. Thus [Des^1,Tyr(OMe)^4,Aze^7]ANGII and [Des^1,Tyr(OMe)^4,Pip^7]ANGII were in each case biologically inactive at the dose tested, as in the case for [Des^1,Tyr(Me)^4]ANGII.

[Sar^1,Aze^7]ANGII was subjected to COSY and 1D-NOE NMR studies suitable for resonance assignment and distance information. The one-dimensional NMR spectrum in DMSO-d_6 showed a complex downfield region with broad overlapping NH and $C_\alpha H$ resonances. To simplify the aromatic region so that it was possible to study intramolecular proton–proton interactions between aromatic rings, the 1D-NOE NMR spectra were run after the NH's were exchanged with D_2O.

Figure 1 shows the COSY spectrum of [Sar^1,Aze^7]ANGII in DMSO-d_6 + D_2O, while Figure 2 shows the NOE difference spectrum resulting after saturation of the His C_2 proton at δ = 7.48 ppm. As seen, weak enhancements of the Tyr meta and ortho proton resonances at δ = 6.99 ppm (0.39%) and δ = 6.61 ppm (0.42%) as well as of the Phe protons at δ = 7.15 ppm (0.66%) were observed, indicating proximity of the three aromatic rings (Tyr, His, Phe). However, upon saturation of the Tyr meta proton resonance at δ = 6.99 ppm no enhancement was observed for the His C_2 and C_4 proton resonances at δ = 7,48 ppm and δ = 6.88 ppm. Only the Tyr ortho proton at δ = 6.61 ppm in the aromatic region under scrutiny was enhanced (30%) as expected. The latter saturation serves as a control experiment to show the minimal contribution of partial saturation to the enhancements of the Tyr meta and ortho and the Phe proton signals in Figure 2.

Saturation of Sar C_α proton at δ = 3.15 ppm and Arg C_δ proton at δ = 3.05 ppm, resulted in enhancements of Tyr and Phe ring protons (Figure 3, Table III), revealing (1) a bend in the N-terminal domain and (2) close proximity of the Sar/Arg residues with the ring cluster.

It has been shown that biological activity is related to ring clustering and the formation of a charge relay mechanism involving the triad Tyr hydroxyl–His imidazole and Phe carboxylate.[4] To investigate proximity of the Tyr, His, Phe rings, NOE experiments were carried out by saturating the His C_2 and Tyr meta and ortho protons, in [Sar^1,Aze^7]ANGII. Upon saturation of the His C_2 proton, NOE enhancement of the Tyr meta and ortho protons as well as of the Phe ring protons is observed (Figure 2). The selected lines were irradiated 20 times for 100 ms (total irradiation time 2.0 s). Although the observed effects were weak they could be confirmed through monitoring the NOE build up by employing other irradiation times (0.2, 0.5, 1.0, 2.0, and 5.0 s) and also by control experiments. This procedure used a very low decoupler power setting (typically 10B lower than for a standard NOE experiment) so that it was possible to avoid partial saturation of resonances in close proximity. Each line required a total of 1000 scans and the relaxation time was

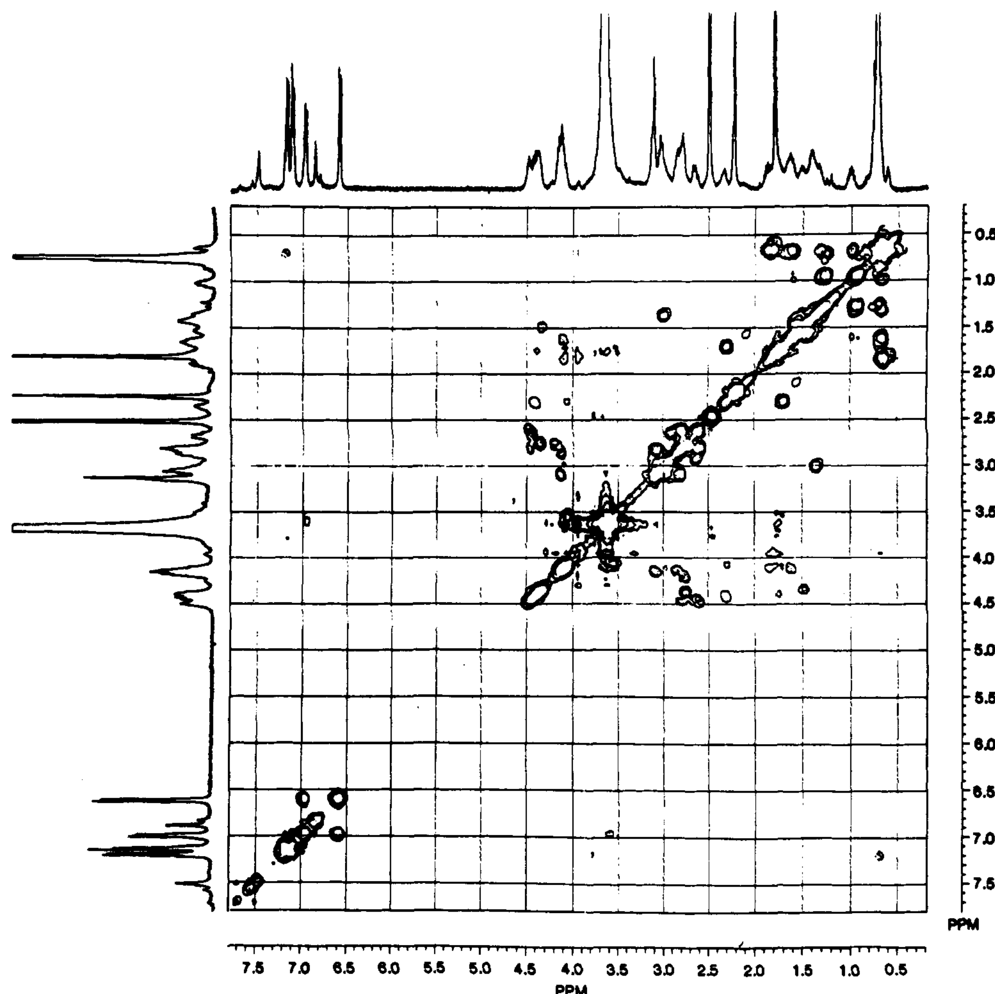

Figure 1. Two-dimensional contour plot of a 400-MHz COSY spectrum for [Sar[1],Aze[7]]ANGII in DMSO-d_6 after D_2O exchange.

2 s. Time of acquisition for each transient was $\simeq$3 s. Saturation of the Tyr meta protons resonance at δ = 6.99 ppm resulted in enhancement of the Tyr ortho protons at δ = 6.61 ppm but not of the His C_2 and C_4 proton resonances or of the Phe ring protons. This control experiment favors the absence of partial saturation effects under the experimental conditions used, validating the data in Figure 2 showing proximity of the three aromatic rings in DMSO-d_6. We used NOE experimental conditions (low power, different T preirradiation times, saturation of control areas) so that spin diffusion and partial saturation would be visibly minimized for the discussed interactions. These experiments show that the interaction between Tyr, His, and Phe 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 protons and Phe ring protons, the reverse effect is not observed upon saturation of the Tyr meta, ortho, and Phe ring protons. The probable reason for this is that Tyr ortho, meta, and Phe ring 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 para protons, while the Tyr meta protons can relax through the Tyr ortho and C_β protons. Similarly a Phe ring proton can relax through the rest of the ring protons.

Discussion

We have recently reported the synthesis and conformational properties of *N-tert*-butyloxycarbonyl, *O*-phenacyl (PE) derivatives of Pro, Aze, Pip, and other proline-containing dipeptides.[17,18] While both cis and trans isomers about the urethane amide bond of *N-t*-Boc-Pro derivatives were observed by ^1H and ^{13}C NMR spectroscopy, only one isomer was apparently indicated in the case of *N-t*-Boc-Aze-PE. This prompted us to synthesize ANGII analogues with Aze at position 7, in which expectantly only one isomer (cis or trans) might be favored. However, 1D- and 2D-NMR results in [Sar[1],Aze[7]]ANGII indicated the presence of both isomers about the His[6]–Aze[7] amide bond. The equilibrium situation was not strikingly different from that observed in [Sar[1]]ANGII in which a ratio 1/5 in favor of the trans conformer was observed, from the relative intensity of the His C_2 and C_4 proton resonances in both isomers of the two analogues. Examination of the ^1H NMR spectrum of ANGII taken under exactly the same conditions used for the spectra of [Sar[1]]ANGII and [Sar[1],Aze[7]]ANGII (0.5 mM in DMSO-d_6) shows instead a 10/1 ratio for the two isomers in favor of the trans as indicated by NOE experiments (unpublished results). This difference in the cis/trans ratio between ANGII and [Sar[1]]ANGII or Sar[1],Aze[7]]ANGII indicates a role for Sarcosine at the N-terminus in determining the His[6]–Pro[7] cis/trans equilibrium. This long-range effect of Sar can be explained on the basis of recent NOE experiments showing a folding of the N-terminal sector toward the ring cluster (Tyr, His, Phe), with the Sar residue in close proximity to the His side chain.[19]

Previous structure–activity studies on angiotensin and analogues have shown that the secondary amino acid Pro

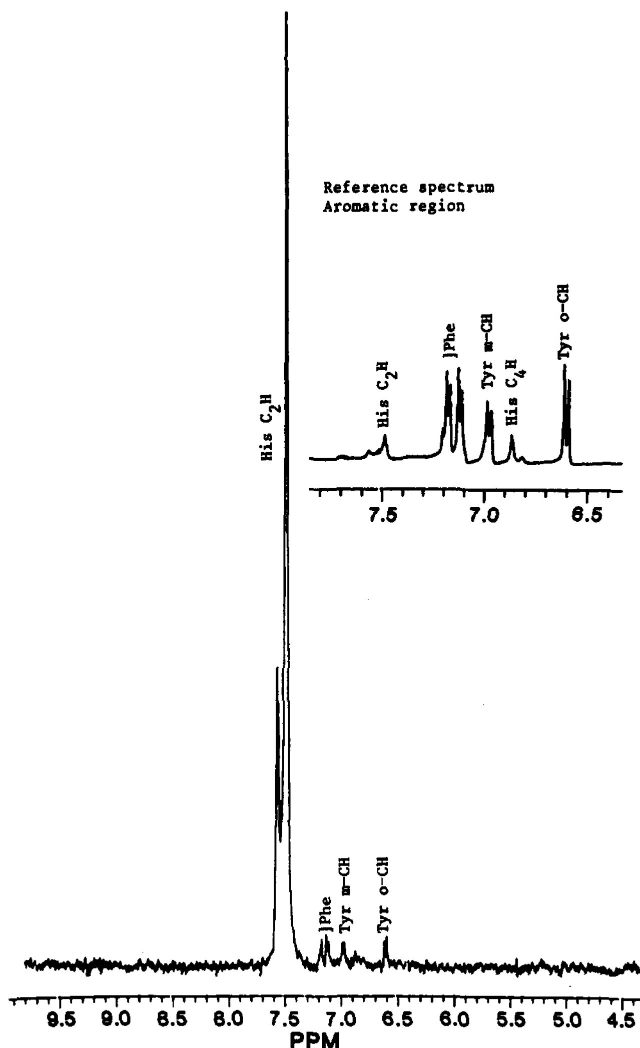

Figure 2. Reference spectrum and NOE difference spectrum for [Sar[1],Aze[7]]ANGII in DMSO-d_6 + D_2O obtained upon saturation of the His C_2 proton line at δ = 7.48 ppm.

occupying position 7 is of great importance in maintaining the conformational integrity of the hormone for binding with high affinity to angiotensin receptors. Conformational studies[9] on angiotensin II have shown the presence of a γ-turn at the C-terminal tripeptide His-Pro-Phe, in which the His[6]–Pro[7] amide bond exists predominantly in the trans form. In this form the rigid Pro ring serves to bring into close proximity the side chains of the functionally essential amino acids Tyr, His and Phe, thereby creating an appropriate stereo structure for hormone–receptor interaction. The His[6]–Pro[7] trans configuration and the clustering of the three aromatic rings in [Sar[1]]-ANGII have been demonstrated recently by nuclear Overhauser effect spectroscopy in the rotating frame.[8] The His[6]–Pro[7] trans configuration in Sarmesin and its analogues has also been well established in our NOE experiments from the observed enhancements of the Pro C_δ protons at δ = 3.2 ppm and δ = 3.6 ppm, upon irradiation of the well-resolved His C_α proton resonance at δ = 4.7 ppm.[19,20] However in [Sar[1],Aze[7]]ANGII such an experiment was not possible since the His C_α proton resonance was overlapped with the rest of the C_α proton resonances.

In the present study, we have found that substitution of Aze and Pip at position 7 in [Sar[1]]ANGII, Sarilesin, and Sarmesin resulted in analogues which retained high activity (Table II). These findings indicate that replacement of proline with its four- or six-membered-ring homologues Aze or Pip, does not significantly change the bioactive conformation important for receptor recognition and expression of agonist or antagonist activity. With the exception of the type II antagonist [Sar[1],Tyr(Me)[4],Pip[7]]-ANGII, which was found to have significantly lower antagonist activity (pA_2 = 6.5) when compared with [Sar[1],Tyr(Me)[4],Aze[7]]ANGII (pA_2 = 7.4), variations in activity of the synthesized analogues illustrated a preference for Sar over Aib at position 1, and Pip over Aze at position 7, for the expression of maximum activity of both agonist and type I antagonist analogues. It is known that substitution of Sar at position 1 results in an increase in potency of both agonist and antagonist analogues of ANG II—an effect contributed to increased binding affinity as well as to increased biological half-life of the peptide. As is evident from the data in Table II, modifying position 1 is a critical factor for affecting conformation and activity. Replacement of Pro by Aze and Pip at position 7 of [Sar[1]]-ANG II retained high activity (98% and 184%, respectively), indicating that [Sar[1],Aze[7]]ANG II and [Sar[1],Pip[7]]-ANG II retain all conformational requirements for maximum activity. Substitution of Sar by Aib (aminoisobutyric acid) at position 1 in these two analogues reduces activity (34% and 65%, respectively). Replacement of Sar by the native amino acid Asp at position 1 of [Sar[1],Aze[7]]ANG II and [Sar[1],Pip[7]]ANG II, resulted in analogues of considerably lower agonist activity (12% and 1%, respectively), indicating that the nature, size, and shape of the N-terminal amino acid appears to play an important role in conformation and activity. A similar situation is observed for the type II antagonist Sarmesin wherein replacement of Sar[1] with Asp causes a marked reduction in binding affinity to below detectable levels.[19] The interaction of the Sar[1] residue with the C-terminal domain of angiotensin II analogues is not well understood at the present time but may relate to its known proximity and possible interaction with the His[6] residue.[8]

[Sar[1],Ile[8]]ANG II (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 analogues with modifications at position 1 and 7. Replacement of Pro by Aze at position 7 in [Sar[1],Ile[8]]-ANG II retained high antagonist activity (pA_2 = 7.9) while subsequent replacement of Sar by Aib in [Sar[1],Aze[7],Ile[8]]-ANG II reduced activity (pA_2 = 7.1). The lower antagonist activity of the non-N-methylated analogue [Aib[1],-Aze[7],Ile[8]]ANGII bearing two C-methyl group at the carbon of the N-terminal acid suggests that general steric hindrance of a relatively nondiscriminating nature at position 1 is not sufficient to create a favorable binding interaction between the receptor and [Aze[7]]Sarilesin. Similarly, replacement of Pro by Pip at position 7 retained high antagonist activity (pA_2 = 8.3) while subsequent replacement of Sar by Aib in [Sar[1],Pip[7],Ile[8]]ANG II moderately reduced activity (pA_2 = 7.6), indicating again that the important steric influence of the methyl group at the N-terminus of Sarilesin or [Pip[7]]Sarilesin derives from its very precise location.

Methylation of the Tyr hydroxyl in [Sar[1],Aze[7]]ANG II and [Sar[1],Pip[7]]ANG II results in analogues of variable type II antagonist activity. Thus, [Sar[1],Tyr(Me)[4],Aze[7]]-ANG II retains high antagonist activity (pA_2 = 7.4)

Figure 3. ^{1}H NMR NOE difference spectra for [Sar^1,Aze^7]ANGII in DMSO-d_6 + D_2O.

compared to Sarmesin (pA_2 = 7.7), while [Sar^1,Tyr(Me)^4,Pip^7]ANG II was found to have lower antagonist activity (pA_2 = 6.5). For type II antagonists, it seems that the Aze ring, but not the Pip ring, in position 7 allows a favorable conformation for binding interaction with the receptor.[19] X-ray crystallography on the model compound *N*-*t*-Boc-Aze-OH has indicated a trans urethane bond, an sp^2 rather than sp^3 ring nitrogen hybridization and an almost planar ring geometry.[21] This may create a tighter γ-turn in the His-Aze-Phe terminal tripeptide which

Table III. NOE Enhancements for [Sar[1],Aze[7]]ANGII in DMSO-d_6

proton(s) saturated	enhancement	% proton	rationale
His C_2	Phe ring	0.66	interresidue NOE
His C_2	Tyr ortho	0.42	interresidue NOE
His C_2	Tyr meta	0.39	interresidue NOE
His C_2	Sar CH_3	1.11	interresidue NOE
His C_2	Val or Ile CH_3	4.00	interresidue NOE
His C_2	Sar C_α	1.91	interresidue NOE
His C_4	Tyr meta	4.38	interresidue NOE
His C_4	Tyr ortho	3.14	interresidue NOE
His C_4	Sar CH_3	1.44	interresidue NOE
His C_4	Val or Ile CH_3	7.25	interresidue NOE
His C_4	Phe ring	2.65	interresidue NOE
Tyr meta	Tyr ortho	30.61	intraresidue NOE
Tyr meta	Tyr C_α	1.63	intraresidue NOE
Tyr ortho	Tyr meta	22.08	intraresidue NOE
Tyr ortho	Tyr C_α	4.72	intraresidue NOE
Tyr ortho	Val or Ile CH_3	0.99	interresidue NOE
Sar C_α	Phe ring	0.90	interresidue NOE
Sar C_α	Tyr meta	0.40	interresidue NOE
Sar C_α	Tyr ortho	0.40	interresidue NOE
Phe ring	Tyr ortho	0.66	interresidue NOE
Arg C_δ	Tyr ortho	0.40	interresidue NOE
Arg C_δ	Arg $C_{\gamma\gamma'}$	2.90	intraresidue NOE

accounts for the strong agonist activity of [Sar[1],Aze[7]]ANG II and the strong type II antagonist activity of [Aze[7]]-Sarmesin. NOE experiments on [Sar[1],Aze[7]]ANG II showed an interaction between His/Phe rings, not previously seen in our ROESY investigation of [Sar[1]]ANG II, suggesting that a tighter bend in Aze[7]-ANG II analogues favors the ring clustering phenomenon. On the other hand, the six-membered ring of Pip in [Pip[7]]Sarmesin can take up chair and boat conformations which may not provide the best fit for receptor interaction in the case of type II antagonists.

The lack of antagonist activity, of [Des[1],Aze[7]]Sarmesin and [Des[1],Pip[7]]Sarmesin ($pA_2 < 5$), shows that the Sar residue occupying position 1 is of great importance for the manifestation of the antagonist activity of [Aze[7]]- and [Pip[7]]Sarmesin analogues. As mentioned above, similar considerations apply to the agonists wherein Sar[1] is replaced by Asp (Table II). The size and shape of the N-terminal residue of type II antagonists (and agonists) appears to be subject to unusually severe restrictions. As has been shown from structure–activity studies on Sarmesin,[22] inactive analogues result upon replacement of Sar with Asp, Ala, or Pro, illustrating that the nature of the secondary amino acid occupying position 1 is an important feature of the receptor binding process for Sarmesin. In other words, the important steric influence of the Sar methyl group at the N-terminus of Sarmesin, [Aze[7]]- and [Pip[7]]Sarmesin derives from its very precise location close to the ring cluster. Our recent investigations by nuclear Overhauser effect spectroscopy in the rotating frame (ROESY) and 1D-NOE studies have shown close proximity of the Sar residue to the His side chain, which seems to be a critical factor for manifestation of the maximum activity.[19] Sar[1] seems to have a stabilizing influence on type II antagonist conformation and also on the conformations of agonist analogues containing Aze[7] and particularly Pip[7] (Table II). Interestingly, type II antagonist activity does not depend on the presence of the C-terminal Phe residue of Sar[1] analogs including Sarmesin.[23] Bovy and co-workers[23] also found that for a series of Des-Phe[8] analogs, antagonist activity required Sar [but not Asp] at position 1, and required Pro [but not Sar] at position 7, again demonstrating codependency between residues 1 and 7. For agonist activity three rings are needed; for antagonist activity, only two.

By saturating distinct resonances, our NOE studies on [Sar[1], Aze[7]]ANG II were also able to detect a bend in the N-terminal domain. Thus saturation of the Sar C_α proton at $\delta = 3.15$ ppm and Arg C_δ proton at $\delta = 3.05$ ppm resulted in enhancements of the Phe and Tyr ring protons Table III), revealing proximity of the N-terminal Sar-Arg sector and the ring cluster. Assignment of the Arg C_δ proton at $\delta = 3.05$ ppm is well verified from cross peaks with the Arg C_γ proton at $\delta = 1.35$ ppm in the COSY spectrum. Proximity of Arg C_δ and Tyr ortho protons suggests the possibility of an interaction between the Arg and Tyr side chains not previously reported in our NMR studies of ANG analogues.[8,19] The Sar C_α proton resonance is also a characteristic broad singlet which appears at $\delta = 3.15$ ppm in all Sar[1] containing ANG II analogues (well verified from ROESY cross peaks with Sar NCH_3 proton at $\delta = 2.25$ ppm). Saturation of the Sar N CH_3 proton at $\delta = 2.25$ ppm did not enhance the Tyr/Phe ring protons and served as a control experiment for validation purposes (Figure 3). The NOE data suggest an extremely compact conformation for [Sar[1],Aze[7]]ANG II in DMSO in which all the aromatic and charged groups gather together and interact with one another.

Conclusion

This research aims at refining present knowledge of the roles of residues 1 and 7 in the ring clustering and the charge relay conformational model we have recently suggested. A γ-turn in the C-terminal tripeptide His-Pro-Phe, in which the His[6]–Pro[7] amide bond exists predominantly in the trans form, is important for stabilizing the folded receptor conformation. Replacement of Pro with its four- or six-membered-ring homologs Aze or Pip in [Sar[1]]ANGII (agonist), Sarilesin (antagonist), and Sarmesin (antagonist) resulted in analogues which retained high activity, whereas the activity of ANGII was substantially reduced. The SAR data indicate that, for peptides containing Sar[1], substitution of Aze[7] or Pip[7] does not significantly change the bioactive conformation important for receptor recognition and expression of agonist or antagonist activity. Variations in activity of the antagonist analogues illustrated a preference of Pip over Aze for maximum type I antagonist activity and vice versa for maximum type II antagonist activity. These data suggest that subtle changes in the ring properties of the secondary amino acid at position 7 (Aze = planar, Pro = envelope, Pip = boat/chair) influence the conformations necessary for maximum interaction of type I and type II antagonist. For all analogues studied, Sar was the preferred substitution over Aib, Asp, or Des at position 1 for the expression of maximum activity. Furthermore, the N-terminus plays an important role in determining the His[6]–Pro[7] cis/trans equilibrium and the stabilization of the tyrosinate species which triggers activity. 1D-NOE studies of [Sar[1],Aze[7]]ANGII in DMSO-d_6 have shown ring clustering (Tyr, His, Phe) and a bend in the N-terminal domain, resulting in close proximity of the Sar-Arg N-terminal dipeptide to the ring cluster, which seems to be a critical factor for manifestation of maximum activity.

Experimental Section

***N-t*-Boc Methodology.** Solid-phase synthesis of [Aze[7]]- and [Pip[7]]Angiotensin, Sarilesin, and Sarmesin analogues 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.[22]

tert-Butyloxycarbonyl-blocked amino acids were synthesized in our laboratories or were purchased from Peninsula Laboratories, Bachem Inc., or Biohellas. Protected amino acid-resins, ANGII and Sarilesin, were purchased from Peninsula Laboratories. Aze, Pip, and Tyr(Me) were purchased from Fluka and converted to *N-t*-Boc derivatives by the method of Nagasawa.[24] Solid-phase peptide synthesis was carried out with 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. When proved necessary by the ninhydrin test the coupling was repeated. Deprotection steps (2 and 25 min) utilized 35% CF_3COOH 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 up to the heptapeptide stage. Then the protected resin was divided in half, and the next Boc-amino acid was added to half. 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.

Fmoc Methodology. For the synthesis of [Aze[7]]- and [Pip[7]]-Sarmesin analogues we resorted to the Fmoc methodology, utilizing the 2-chlorotrityl-resin as solid support, as reported by Barlos et al.[25-27] The *N-t*-Boc methodology applied to these analogues resulted in crude material of very poor purity, and yields for the desired products were estimated by HPLC.

Fmoc-blocked amino acids were synthesized in our laboratories as described previously[28] or were purchased from Biohellas Laboratories. Fmoc-His(Trt)-OH was purchased from Biohellas Laboratories. Fmoc-Arg(Pmc)-OH[29] was purchased from Bachem. The N-terminus amino acid was Boc-Sar. Attachment of the first protected amino acid (Fmoc-Phe) 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.[25]

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. Completion of coupling was verified by the ninhydrin test. A second coupling was employed in cases of incompleted coupling. 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. Removal of the protecting groups (Boc-Sar, Arg-Pmc, His-Trt) was achieved by treatment of the protected peptide with TFA–CH_2Cl_2 (1:1, 20 mL/g peptide) in presence of scavengers (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.

General Procedure for the Synthesis of Angiotensin II and III Analogues Using 2-Chlorotrityl Chloride Resin. I. Preparation of N^α-Fmoc-AA-2-chlorotrityl Chloride Resin. 2-Chlorotrityl chloride resin (1 g, 1.4–1.6 mequiv of Cl^-/g of resin) in dry dichloromethane (10 mL) was stirred in a round-bottom flask. Diisopropylethylamine (DIPEA) (0.545 mL, 3.2 mmol) and N^α-Fmoc-amino acid (N^α-Fmoc-AA-OH) (1 mmol) were added, and the solution was stirred for 20 min at room temperature. A mixture of MeOH (3 mL) and DIPEA (0.5 mL) was then added, and the mixture was stirred for another 10 min at room temperature. The Fmoc-AA-resin was filtered and subsequently washed with DMF (3 × 10 mL), DCM (2 × 10 mL), i-PrOH (2 × 10 mL), and Et_2O (2 × 10 mL) and dried in vacuo for 24 h at room temperature. The loading of the amino acid per gram of substituted resin (mmol AA/g of resin) was calculated by quantitative Kaiser test.

II. Preparation of Angiotensin II and III Analogues. The above Fmoc-AA-resin was divided in four. Each quarter was used for the synthesis of an individual analogue following the protocol described in ref 25. The finished peptide-resin was dried in vacuo, and was treated with the splitting mixture DCM–AcOH–TFE (7:1:2, 15 mL/g of resin) for 1 h at room temperature to remove the peptide from the resin. 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. Purification of the final product was achieved by HPLC, and identity was confirmed by amino acid analysis and FAB MS.

Preparative Reversed-Phase HPLC. Peptides were purified on a Varian HPLC system equipped with a Vista 401 microprocessor controller. A Bio-Rad Hi-Pore 318 reversed-phase preparative column (25.0 × 2.15 cm) was used. Separations were achieved with a stepped linear gradient of acetonitrile in 0.1% CF_3COOH at a flow rate of 7.5 mL/min. Peptides (5 × 5 mg) were injected through a nitrogen-pressurized Rheodyne injector with a 2.0-mL sample loop. One-fifth of the total sample was injected during each run for a 0.1-min inject period and by lowering the flow rate to 4.0 mL/min. One cycle consistent 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 201 fraction collector, and the elution time of the major product was 26–29 min. Elution of the peptide was determined simultaneously from the absorbances at 254 nm (Varian UV-1) and 230 nm (Kratos SF 769Z). Fractions containing the major peptide were pooled, and CH_3CN was removed on a rotary evaporator. After lyophilization the product was stored at −20 °C.

Amino acid analysis was performed on Beckman G300 high-performance analyzer. Compositional analysis data were collected from 6 M HCl hydrosylates (150 °C, 1 h). The purity of products was established by analytical HPLC reruns and by thin-layer chromatography (TLC). Two solvent systems were used: 1-butanol–pyridine–acetic acid–water (15:10:3:6) (BPAW) and chloroform–methanol–acetic acid–water (15:10:2:3) (CMAW). The identity of desired products was established by FABMS. FAB spectra run on a AEI M29 mass spectrometer. FAB gun run at 1 mA dischange current at 8 KV. The FAB matrix used was a mixture of dithiothreitol/dithioerythritol (6:1) (Cleland Matrix).[30]

Rat Uterus Bioassay. Defatted uterine horns from diethylstilbestrol-primed virgin Spague–Dawley rats (150–250 g) were used for the bioassay.[15,31] Each tissue was suspended under 1-g tension in a 3-mL tissue bath containing 150 mM NaCl, 5.6 mM KCl, 0.18 mM $NaHCO_3$, and 1.4 mM glucose at pH 7.0 gassed with oxygen. Contractions were monitored with Gould Metripak 763341-4202 isotonic transducers coupled to Gould 13-4615-50 transducer amplifiers. The agonist activity was determined by matching the response with an equivalent one to ANGII (human). The antagonist activity (pA_2) was determined as the negative logarithm of the concentration of antagonist required to reduce the response to an EC_{50} dose of ANGII to the response to half the EC_{50} dose. All peptide agonists used in this study were able to produce a maximum response if high enough doses were applied to the tissues.

NMR Experiment. NMR experiments were carried out using a Brucker 400-MHz NMR spectrometer. Three millgrams of

[Sar[1],Aze[7]]ANGII was dissolved in 0.5 mL of DMSO-d_6, and two drops of D_2O were added. 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.[32] The COSY (two-dimensional correlated spectroscopy) experiment provided contour plots which were symmetrized with respect to 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.[19,33,34]

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