Design, Synthesis, and Incorporation of a β-Turn Mimetic in Angiotensin II Forming Novel Pseudopeptides with Affinity for AT_1 and AT_2 Receptors

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A benzodiazepine-based β-turn mimetic has been designed, synthesized, and incorporated into angiotensin II. Comparison of the mimetic with β-turns in crystallized proteins showed that it most closely resembles a type II β-turn. The compounds exhibited high to moderate binding affinity for the AT_2 receptor, and one also displayed high affinity for the AT_1 receptor. Molecular modeling showed that the high-affinity compounds could be incorporated into a previously derived model of AT_2 receptor ligands.

Introduction

The potent vasoactive octapeptide hormone angiotensin II (Ang II) acts through the AT_1 and the AT_2 receptors, both of which belong to the G-protein-coupled receptor superfamily. The AT_1 receptor mediates most of the known actions of Ang II, such as vasoconstriction, aldosterone release, and sodium and water retention.[1−3] Recently it has been established that the AT_2 receptor has a physiologic role in the brain and in cardiovascular and renal functions in adults, as well as in the modulation of various processes associated with cell differentiation and tissue repair.[2] In some cells, AT_2 receptor activation inhibits proliferation and also mediates differentiation in neural cell lines.[4] Often the effects of AT_2 receptor activation oppose those mediated by the AT_1 receptor, and it is therefore not surprising that the AT_2 receptor, in recent years, has attracted interest as a new target for potential therapeutics.

Despite only 32−34% sequence homology,[5,6] AT_1 and AT_2 receptors bind Ang II with high affinity. As deduced from conformational analyses of conformationally constrained analogues, there is supporting evidence that Ang II adopts a turn centered at Tyr^4 when activating the AT_1 receptor.[7−16] While less research has been devoted to molecular recognition involving the interaction of Ang II with the AT_2 receptor, binding data and conformational analyses of linear and cyclic Ang II analogues suggest that the AT_1 and AT_2 receptors accommodate Ang II differently.[9,17−28] We have previously prepared a series of scaffolds,[10,26−28] for examples see Figure 1, designed to mimic γ-turn secondary structure motifs. Of the pseudopeptides obtained by incorporation of these motifs into Ang II to substitute the backbone centered around Tyr^4, only that with scaffold **A** exhibited high affinity for the AT_1 receptor and acted as an AT_1 agonist, inducing contraction of blood vessels in vitro.[10] In contrast, several of the scaffolds were tolerated with respect to AT_2 receptor recognition.[26,27] It was proposed that the turn region around Tyr^4, the guanidino group of the Arg^2 residue, the N-terminal end, and their relative orientations in space are critical for favorable interaction with the AT_2 receptor.[26,27]

β-Turn secondary structures are much more common than γ-turns in proteins, as shown by Rose et al.,[29] and several scaffolds mimicking β-turns have been reported in recent years.[30−33] We felt encouraged to elaborate on the benzodiazepine structure and to synthesize β-turn mimetics with the benzodiazepine core as template.

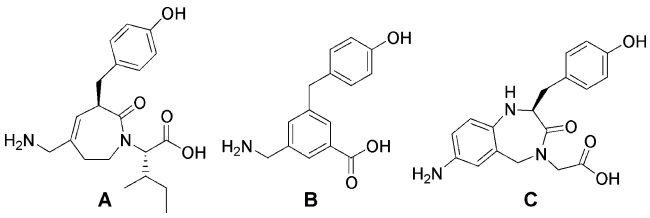

Figure 1. Some γ-turn mimetics (**A**−**C**) previously incorporated in Ang II.[10,26,28]

We herein report the synthesis and preliminary pharmacological evaluation of Ang II analogues **1**−**3** (Chart 1) comprising a benzodiazepine-based type II β-turn mimetic.

Results

Chemistry. The benzodiazepine-based Fmoc-protected β-turn mimetic **18** was synthesized in solution and incorporated into Ang II using solid-phase methodology to deliver the pseudopeptides **1**−**3**. The bicyclic core structure was utilized as a turn template to replace the peptide fragments Val^3-Tyr^4-Ile^5-His^6 (**1**), Val^3-Tyr^4-Ile^5 (**2**), and Tyr^4-Ile^5 (**3**) in Ang II.

The synthesis started with a benzylic oxidation of 2-fluoro-*m*-xylene to give the diacid **5** (Scheme 1). The diacid was transformed into the monoester **6** in one step or via the diester. Nitration of the monoester **6** gave **7**, which was transformed to the acid chloride and further reacted in a Friedel−Crafts acylation. The substituted benzophenone was obtained as a 2:5 mixture of the ortho- and para-substituted isomers.

The substituted benzophenone **8a** was first reduced to the diphenylmethane derivative **9** to decrease the potential for the fluorine ipso-carbon to undergo nucleophilic aromatic substitution (Scheme 2). In the next step, the methyl ester was reduced[34] to the alcohol, and Swern oxidation was employed to reoxidize the alcohol **10** to the aldehyde. Reductive amination of **11** with 2-aminoethanol produced a primary alcohol that was protected with a *tert*-butyldimethylsilyl group (TBDMS). Fmoc-L-Ile-OH was coupled with the secondary amine **12**, and the Fmoc group was removed. Subsequent internal nucleophilic aromatic substitution delivered the benzodiazepine core structure as a 1:3 mixture of the TBDMS-protected form and the free alcohol **14**.

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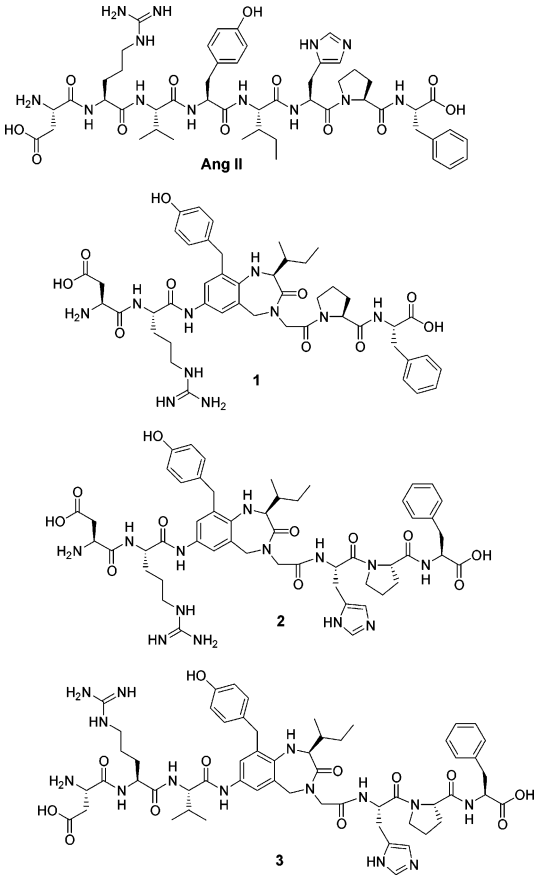
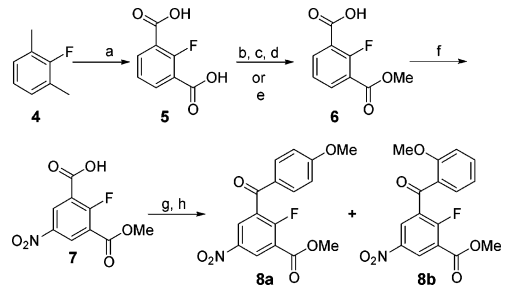
Chart 1



Scheme 1[a]



[a] Reagents: (a) $KMnO_4$, KOH, H_2O, 58%; (b) $SOCl_2$; (c) MeOH, 99%; (d) LiOH, THF/MeOH/H_2O, 47%; (e) MeOH, H_2SO_4, 40%; (f) HNO_3, H_2SO_4, 97%; (g) $SOCl_2$; (h) $AlCl_3$, anisole, CH_2Cl_2, 37% (**8a**), 15% (**8b**).

Treatment of the product mixture with tetrabutylammonium fluoride (TBAF) gave benzodiazepine **14**.

Mild conditions were required for the conversion of the alcohol function in **14** to a carboxylic acid.[26] The protecting group on the phenolic hydroxy group in **15** was changed to a Boc group to facilitate the solid-phase peptide synthesis. Finally, the nitro group in **17** was reduced and the aniline nitrogen was protected with FmocCl. Standard Fmoc/*t*-Bu SPPS methodology was used to incorporate the protected benzodiazepine-based turn mimetic **18** into Ang II.

Molecular Modeling. (A) Comparison of the *β*-Turn Mimetic Moiety and Protein *β*-Turns. Conformational analysis was performed on model compound **19** (Figure 2). This resulted in 30 low-energy conformations (within 5 kcal/mol of the lowest energy minimum). The five conformations within 1 kcal/mol of the lowest energy conformation were evaluated by comparing them with an in-house database of 13 784 extracted *β*-turns from protein X-ray structures obtained from the Protein Data Bank (PDB).[35,36] A complete atom-to-atom-based comparison between the *β*-turn mimetic and the protein *β*-turns was not possible because there is no exact match between the first two amino acid residues in the turn and the mimetic. For the comparison, we choose to include the C_α and C_β atoms of the $i + 1$ and $i + 2$ residues in the protein *β*-turns to represent the direction of the side chains. To allow for a fit of the peptide backbone, the C_α atoms of $i - 1$, $i + 3$, and $i + 4$ and the carbonyl oxygen atoms of $i - 1$, $i + 2$, and $i + 3$ were also included. Atoms in the *i* residue were not considered because of the lack of correspondence in the turn mimetic. The corresponding atoms in model compound **19** used for comparison are shown in Figure 2.

The investigated conformations were superimposed with the lowest root-mean-square (rms) atomic pair distances (0.75 Å cutoff) on type II and type IV *β*-turns. One example of **19** superimposed on a type II *β*-turn is shown in Figure 3. By increase of the rms distance criterion to include superimpositions up to 1 Å, other turns, such as types I, II′, and VIII, could also fit the mimetic.

(B) Common Binding Mode for Ligands at the AT_2 Receptor. Conformational analysis of model structures of **2** and **3** resulted in 2320 and 1562 conformations, respectively. When these conformations were superimposed on a previously derived AT_2 receptor binding model,[28] conformations with a common binding mode could be identified (Figure 4).

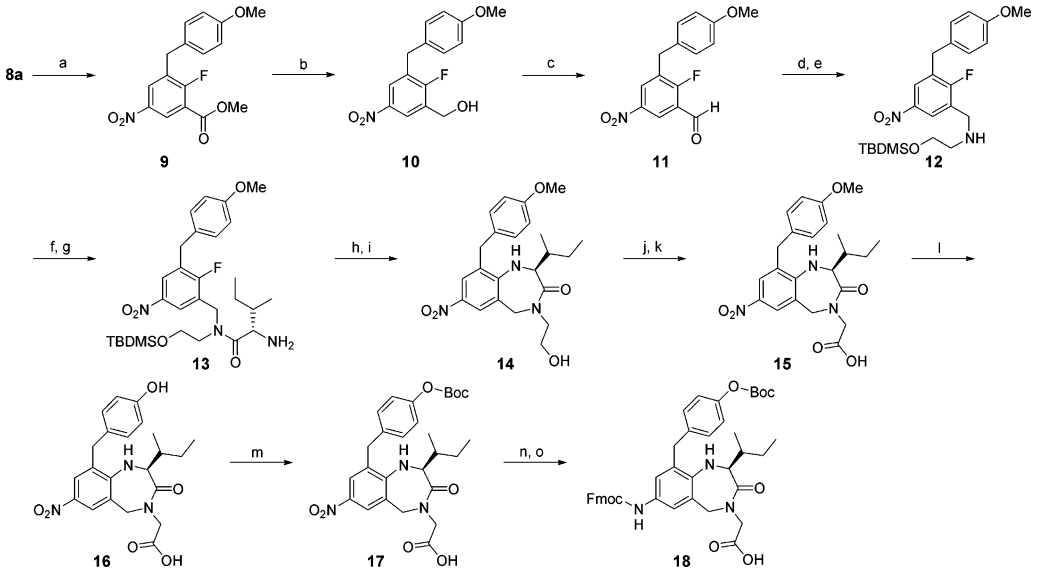
In Vitro Binding Affinity. Ang II analogues **1**−**3** were evaluated in radioligand binding assays using displacement of [^{125}I]Ang II from AT_1 receptors in rat liver membranes[37] and from AT_2 receptors in pig uterus membranes[38] (Table 1). The natural ligand Ang II and the highly AT_2 selective analogue [4-NH_2-Phe^6]Ang II[22] were used as reference substances. Compound **1**, in which the *β*-turn mimetic is substituted for the Val^3-Tyr^4-Ile^5-His^6 segment, has moderate binding affinity for the AT_2 receptor and only weak binding affinity for the AT_1 receptor. In **2**, which contains the His^6 residue, the AT_2 receptor binding affinity was increased, while the AT_1 receptor affinity remained weak. Compound **3**, which contains both Val^3 and His^6, exhibited considerable binding affinity for the AT_1 and AT_2 receptors.

Rabbit Thoracic Aorta String. AT_1 Functional Assay. Compound **3**, which has AT_1 and AT_2 receptor affinity, was also evaluated for agonistic activity at the AT_1 receptor in thoracic aorta, according to a procedure published by Pendleton et al.[39] The agonist Ang II that was used as a reference compound induced a concentration-dependent contraction of the rabbit thoracic aorta, which was inhibited by the AT_1 receptor antagonist saralasin. Compound **3** was tested at seven concentrations from 3.0 nM to 3.0 *μ*M but did not produce any contraction of this tissue.

Discussion

It has previously been shown that several cyclic analogues of Ang II have good AT_1 and AT_2 receptor affinity.[9,23,40] Furthermore, a number of Ang II analogues containing a benzodiazepine-based *γ*-turn mimetic or a thiazabicycloalkane dipeptide mimetic have shown considerable binding affinity for the AT_2 receptor and high AT_2/AT_1 selectivity.[24,26,27] Also, an Ang II pseudopeptide containing a small aromatic ring as a *γ*-turn mimicking moiety was recently found to have high affinity for the AT_1 and AT_2 receptors. Although several *γ*-turn mimetics have been introduced in Ang II,[10,14,26−28] *β*-turn mimetics have rarely been utilized, even though it has been suggested that Ang II adopts a *β*-turn around Tyr^4 during

Scheme 2[a]



[a] Reagents: (a) Et_3SiH, CF_3SO_2H, TFA, CH_2Cl_2, 98%; (b) $NaBH_4$, H_2O, dioxane, 65%; (c) ClCOCOCl, DMSO, Et_3N, CH_2Cl_2, 100%; (d) 2-aminoethanol, $NaCNBH_3$, HOAc, MeOH; (e) TBDMSCl, DBU, THF, 71%; (f) Fmoc-L-Ile-OH, HATU, DIEA, CH_2Cl_2; (g) DBU, THF, 92%; (h) Et_3N, DMSO; (i) TBAF, THF, 90%; (j) ClCOCOCl, DMSO, Et_3N, CH_2Cl_2; (k) $NaClO_2$, NaH_2PO_4, cyclohexene, *t*-BuOH, H_2O, 70%; (l) BF_3·Me_2S, CH_2Cl_2, 78%; (m) Boc_2O, Et_3N, DMAP, THF/H_2O, 74%; (n) H_2, Pd/C, MeOH; (o) FmocCl, Na_2CO_3 (aq), dioxane, 32%.

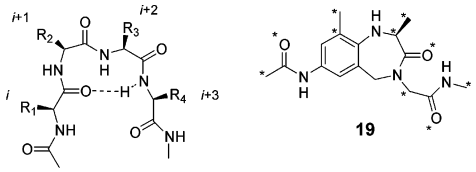


Figure 2. A β-turn (left) and a model compound of the investigated β-turn mimetic (**19**) (right). Atoms used in the β-turn mimetic for superimposing onto crystallized β-turns are marked by an asterisk.

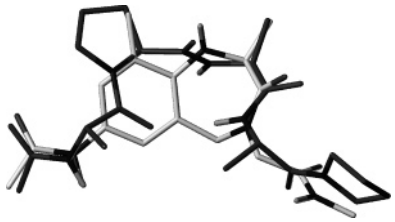


Figure 3. Minimized conformation of the synthesized benzodiazepine-based β-turn mimetic superimposed onto a type II β-turn (rms distance = 0.67 Å). The PDB code for the protein is 1H2C (chain A, turn region Gly^{141}-Pro^{146}). All torsion angles are within ±20° from the ideal torsion angles of a type II β-turn. For clarity, only the backbone and C_β atoms are shown.

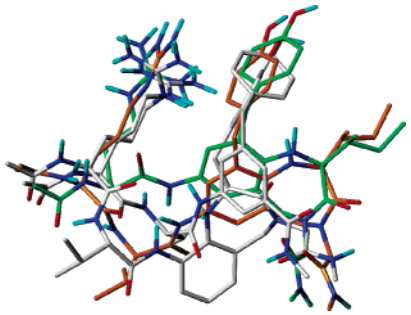


Figure 4. Conformations of the model structures of **2** (green carbons) and **3** (orange carbons) together with two of the compounds from the previously derived AT_2 receptor binding model (gray carbons).

Table 1. Binding Affinities for the AT_1 and AT_2 Receptors

	$K_i \pm$ SEM (nM)	
compd	AT_1 (rat liver membranes)	AT_2 (pig uterus myometrium)
Ang II	0.1	0.6
[4-$NH_2$$Phe^6$]Ang II	>10000	0.7
1	2108 ± 33	53.1 ± 1.7
2	1668 ± 20	4.7 ± 0.3
3	14.9 ± 0.4	1.8 ± 0.04

receptor interaction.[13,16] Two locations for a β-turn centered at Tyr^4 are possible in Ang II: the 2−5 and the 3−6 regions. We decided to synthesize a β-turn mimetic with a tyrosine side chain in the $i + 1$ position and an isoleucine side chain in the $i + 2$ position and introduce this turn mimetic into the 3−6 region of Ang II.

A β-turn is most often defined as any tetrapeptide unit occurring in a nonhelical region that causes a reversal of the direction of the peptide chain (Figure 2).[29] Further, the distance between C_α of the first (i) residue and the fourth ($i + 3$) residue in the peptide sequence should be less than 7 Å.[41] β-Turns comprise a rather diverse group of structures, and they have therefore been divided into a number of turn types (I, I′, II, II′, IV, VIa, VIb, VIII) according to the backbone dihedral angles ϕ and ψ of the $i + 1$ and $i + 2$ residues of the turn.[42] In addition, a number of slightly different turn types have been presented in the literature.[29,41,43] To classify the turn type mimicked by **19**, we focused on comparing the C_α−C_β bond vectors of amino acids $i + 1$ and $i + 2$ and the C_α atoms that have corresponding atoms in the turn mimetic (shown in Figure 2). The five conformations of the β-turn mimetic with the lowest energy (<1 kcal/mol) were compared with 13 784 β-turns extracted from X-ray structures of proteins from the PDB.[35,36] On the basis of this comparison, the investigated β-turns with the best fit to the mimetic were found to belong to the type II and the type IV classes. Since β-turn mimetics are primarily designed to mimic the classical turn types and since type IV turns comprise a diverse collection of nondefined turns, these were not considered further. Thus, of the classical β-turn types, scaffold **19** seems to best mimic a type II β-turn.

The synthesized β-turn mimetic was introduced into Ang II, and the three obtained pseudopeptides **1**−**3** showed binding

affinity for the AT_2 receptor. Compound **1**, which lacks the histidine side chain, displayed a moderate binding affinity for the AT_2 receptor ($K_i = 53.1$ nM). It has previously been shown that the binding affinity of Ang II for the AT_2 receptor drops by a factor of 10 when the histidine is replaced by an alanine[17] or a glycine.[25] In **2**, the histidine residue was present and the binding affinity for the AT_2 receptor was increased 10-fold ($K_i = 4.7$ nM). A slight gain in affinity was observed when an extra valine residue was also present, **3** ($K_i = 1.8$ nM).

Interestingly, **2** and **3** were capable of adopting binding modes similar to those found in a previously identified model of common binding modes of high-affinity AT_2 receptor ligands (Figure 4).[28] In this model, all the compounds contained a γ-turn-like mimetic scaffold in the same region as the investigated β-turn mimetic in **2** and **3**. Thus, both of these types of turn mimetics seem to be able to direct the recognition motifs in such a way that ligands with high affinity for the AT_2 receptor are obtained. However, although there are geometric similarities between the mimetics, there are also visible differences. For example, the isoleucine side chain in the β-turn mimetic does not have any equivalent in the compounds containing a γ-turn mimetic when superimposed as shown in Figure 4. Nonetheless, despite this extra volume, the compounds seem to be tolerated by the AT_2 receptor.

The benzodiazepine structure used in this study as a β-turn mimetic has also previously been used as a γ-turn mimetic (**C** in Figure 1). There is, however, a noticeable difference in the side chains, since the tyrosine side chain was moved from the benzodiazepine ring in the γ-turn mimetic to the aromatic ring in the β-turn mimetic. Both of these turn mimetic scaffolds have been incorporated in Ang II, replacing Val-Tyr-Ile. The pseudopeptide incorporating scaffold **C** lacked affinity for the AT_2 receptor ($K_i > 10{,}000$ nM), while **2** had excellent affinity ($K_i = 4.7$ nM). This indicates that the relative arrangement of the Arg and Tyr side chains is important for AT_2 receptor binding in the pseudopeptides, which has also been shown previously.[26]

Both pseudopeptides **1** and **2** exhibited very weak binding affinity for the AT_1 receptor, $K_i = 2108$ nM and $K_i = 1668$ nM, respectively. Interestingly, **3**, in which the Tyr^4-Ile^5 segment has been replaced by the β-turn mimetic, showed good binding affinity for the AT_1 receptor ($K_i = 14.9$ nM). The side chains of residues 2, 4, 6, and 8 in Ang II are considered key elements for binding to the AT_1 receptor.[18] In both **2** and **3**, all these side chains are present, but only **3** shows high binding affinity. This may be because the distance between the arginine side chain and the tyrosine side chain is more favorable for a correct AT_1 receptor interaction in **3**, which is similar to the results found for the AT_2 receptor ligands (see above).

Conclusion

A β-turn mimetic based on the benzodiazepine core structure has been synthesized and incorporated into Ang II. The β-turn mimetic was shown to best mimic a type II β-turn when compared with peptide β-turns in crystallized protein structures. Two of the three resulting pseudopeptides exhibited high AT_2 receptor affinity. One also showed good AT_1 receptor affinity but displayed no agonistic effect in an AT_1 receptor functional assay. Inclusion of the two compounds with the highest affinity in a previously published model of Ang II pseudopeptides showed that, despite the incorporation of different turn mimetics, the pseudopeptides were capable of similar binding modes.

Experimental Section

General Synthesis. The pseudopeptides were synthesized manually from Phe-2-chlorotrityl resin or His(Trt)-Pro-Phe-2-chlorotrityl resin[44] in 2 mL disposable syringes equipped with a porous polyethylene filter. Standard Fmoc/*t*-Bu conditions were used, and the Fmoc protecting group was removed by treatment with 20% piperidine/DMF for 5 + 10 min. The resin was washed with DMF after every coupling or deprotection step. The natural amino acids were coupled using the appropriate amino acid (5 equiv) in the presence of PyBOP (5 equiv) and DIEA (10 equiv) in DMF (0.5 mL). The turn templates (1.2 equiv) were reacted with the polymer in the presence of PyBOP (1.2 equiv) and DIEA (3.2 equiv) in DMF (0.5 mL). The coupling of the amino acid attached to the turn templates was repeated using first the conditions described above, and thereafter the resin was washed and recoupled with the same amino acid (10 equiv) using HATU (5 equiv) and DIEA (10 equiv) in 0.5 mL of DMF. After the final coupling step the Fmoc group was removed and the resin was washed with with DMF, CH_2Cl_2, and MeOH and dried in vacuo.

Cleavage. The partially protected peptide resin was treated with triethylsilane (50 μL) and 95% aqueous TFA (1.5 mL) for 1.5 h. The polymer was removed by filtration and washed with TFA (2 × 0.3 mL). The combined filtrates were evaporated in a stream of nitrogen to 1.5 mL, and the product was precipitated with diethyl ether (12 mL). It was collected by centrifugation, washed with diethyl ether, and dried in vacuo.

Purification. The crude material was dissolved in a mixture of H_2O (2 mL) and CH_3CN (0.5 mL) and purified by RP-HPLC on a 5 μm ACE phenyl column (21.2 mm × 150 mm) using a 60 min gradient of 15−45% MeCN in 0.1% aqueous TFA at a flow rate of 5 mL/min. The separation was monitored by UV absorption at 220 nm and by LC/MS and/or analytical RP-HPLC of selected fractions.

Ang II Analogue 1. Fmoc-Pro-OH was coupled to Phe-2-chlorotrityl resin (27.9 mg, 23.7 μmol) according to the general procedure for 3 h. The Fmoc group was removed, and the turn template **18** was reacted with the polymer for 20 h. Fmoc-Arg-(Pbf)-OH was coupled using PyBOP for 20 h and HATU for 4 h. After deprotection the polymer was coupled with Fmoc-Asp(O*t*-Bu)-OH for 3 h. The yield of the deprotected, cleaved, purified, and lyophilized peptide was 6.6 mg (26%). LC/MS (M_{abs} 912.45): 913.6 ($M + H^+$), 457.5 ([$M + 2H^+$]/2). Amino acid analysis: Asp, 1.00; Arg, 1.01; Pro, 0.88; Phe, 0.99.

Ang II Analogue 2. Compound **18** was coupled to His(Trt)-Pro-Phe-2-chlorotrityl resin (60.0 mg, 36.0 μmol) (0.75 mL DMF) as outlined above for 20 h. The Fmoc group was removed, and the resin was washed with with DMF, CH_2Cl_2, and MeOH and dried in vacuo. Half of the material (38.7 mg, 18.0 μmol) was transferred to a second syringe and reacted with Fmoc-Arg(Pbf)-OH for 20 h. The coupling was repeated using HATU for 4 h. Coupling of Fmoc-Asp(O*t*-Bu)-OH for 10 h followed by deprotection, washing, cleavage, and purification produced the desired pseudopeptide in 8.5 mg (45%) yield. LC/MS (M_{abs} 1049.51): 1050.8 ($M + H^+$), 526.0 ([$M + 2H^+$]/2), 351.0 ([$M + 3H^+$]/3). Amino acid analysis: Asp, 1.00; Arg, 1.01; His, 0.97; Pro, 1.00; Phe, 1.02.

Ang II Analogue 3. Fmoc-Val-OH was coupled for 20 h with the second half (38.7 mg, 18.0 μmol) polymer collected after incorporation and deprotection of **18**. The coupling was repeated using HATU for 4 h. Successive couplings of Fmoc-Arg(Pbf)-OH and Fmoc-Asp(O*t*-Bu)-OH for 3 h produced the desired pseudopeptide. Deprotection, washing, cleavage, and purification gave the product in 9.1 mg (44%) yield. LC/MS (M_{abs} 1148.58): 1149.9 ($M + H^+$), 575.5 ([$M + 2H^+$]/2). Amino acid analysis: Asp, 1.00; Arg, 1.00; Val, 1.00; His, 0.96; Pro, 1.01; Phe, 1.02.

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Supporting Information Available: Experimental procedures including the synthesis of **5**−**18**, biological assays, molecular modeling, and ^{1}H, ^{13}C, and elemental analysis data of **6**−**18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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