A Selective AT_2 Receptor Ligand with a γ-Turn-Like Mimetic Replacing the Amino Acid Residues 4−5 of Angiotensin II

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Three angiotensin II (Ang II) analogues encompassing a benzodiazepine-based γ-turn-like scaffold have been synthesized. Evaluation of the compounds in a radioligand binding assay showed that they had no affinity to the rat liver AT_1 receptor. However, one of the compounds displayed considerable affinity to the pig uterus AT_2 receptor (K_i = 3.0 nM) while the other two lacked affinity to this receptor. It was hypothesized that the reason for the inactivity of one of these analogues to the AT_2 receptor was that the guanidino group of the Arg^2 residue and/or the N-terminal end of the pseudopeptide could not interact optimally with the receptor. To investigate this hypothesis, a conformational analysis was performed and a comparison was carried out with the monocyclic methylenedithioether analogue cyclo(S−CH_2−S)[$Cys^{3,5}$]-Ang II which is known to bind with high affinity to the AT_2 receptor (K_i = 0.62 nM). This comparison showed that, in the compounds with high AT_2 receptor affinity, the guanidino group of the Arg^2 residue and the N-terminal end could access common regions of space that were not accessible to the inactive compound. To examine the importance of the guanidino group for binding, the Arg side chain was removed by substituting Arg^2 for Ala^2 in the analogue having the high affinity. This analogue lacked affinity to AT_2 receptors, which supports the role of the guanidino group in receptor binding.

Introduction

The octapeptide angiotensin II (Ang II) exerts its actions by binding to two pharmacologically distinct receptor subtypes designated AT_1 and AT_2. The AT_1 receptor mediates the well-known physiological effects of Ang II, such as vasoconstriction, aldosterone release, stimulation of sympathetic transmission, and cellular growth.[1−3] AT_1 receptor antagonists (the sartans) are currently used as effective clinical antihypertensives. The function of the AT_2 receptor subtype, which was cloned more recently and found to share only 32−34% sequence identity with the AT_1 receptor,[4,5] remains elusive and somewhat controversial. It has been suggested that it plays a role in mediating antiproliferation, cellular differentiation, apoptosis, and vasodilation.[6−9] Importantly, the receptor is expressed at low densities in adult tissues but is up-regulated in pathological conditions such as heart failure, renal failure, myocardial infarction, brain lesions, vascular injury, and wound healing.[10−15] The AT_2 receptor has recently attracted considerable interest as a new potential therapeutic target.

Knowledge of the conformation of Ang II when bound to the two receptor subtypes is invaluable for the understanding of the topological requirements within the AT_1 and AT_2 receptor−peptide complexes. Since the preferred solution conformations of the flexible linear octapeptide do not necessarily correspond to those in receptor−peptide complexes, strategies relying on constrained analogues should provide valuable information.[16] Cyclization can be a powerful tool for imposing conformational constraints. In addition, properly selected cyclization methodologies could serve to facilitate the stepwise transformation of peptide leads into peptidomimetic molecules.[17,18] Models of Ang II bound to the AT_1 receptor have been proposed and there is growing evidence that the peptide adopts a turn centered at the Tyr^4 residue.[19−27] Considerably less is known about the structural requirements for AT_2 receptor affinity, but there was strong early support, based on studies of linear and cyclic [Sar^1]Ang II analogues, for significant differences from those for AT_1 receptor affinity.[21,28−30] However, recently, as a result of photoaffinity labeling experiments combined with site-directed mutagenesis, Deraët et al. proposed that Ang II binds in an almost identical fashion and adopts an extended β-strand-like conformation at both the AT_1 and the AT_2 receptors,[31] contrary to previous predictions.

Our long-term objective is to enable transformation of peptides to nonpeptides by iterative incorporation of well-defined secondary structure mimetics in the target peptides with a special focus on AT_1 and AT_2 receptor ligands. This paper reports the design, synthesis, and binding data of the four Ang II analogues **1**−**4**, encompassing γ-turn-like mimicking scaffolds (Chart 1). One of these pseudopeptides, compound **2**, exhibits very high AT_2 receptor selectivity. We have also evaluated the previously synthesized Ang II analogues **5a** and **5b**, which contains a similar γ-turn scaffold, for AT_2 receptor affinity. Finally, we present molecular modeling studies

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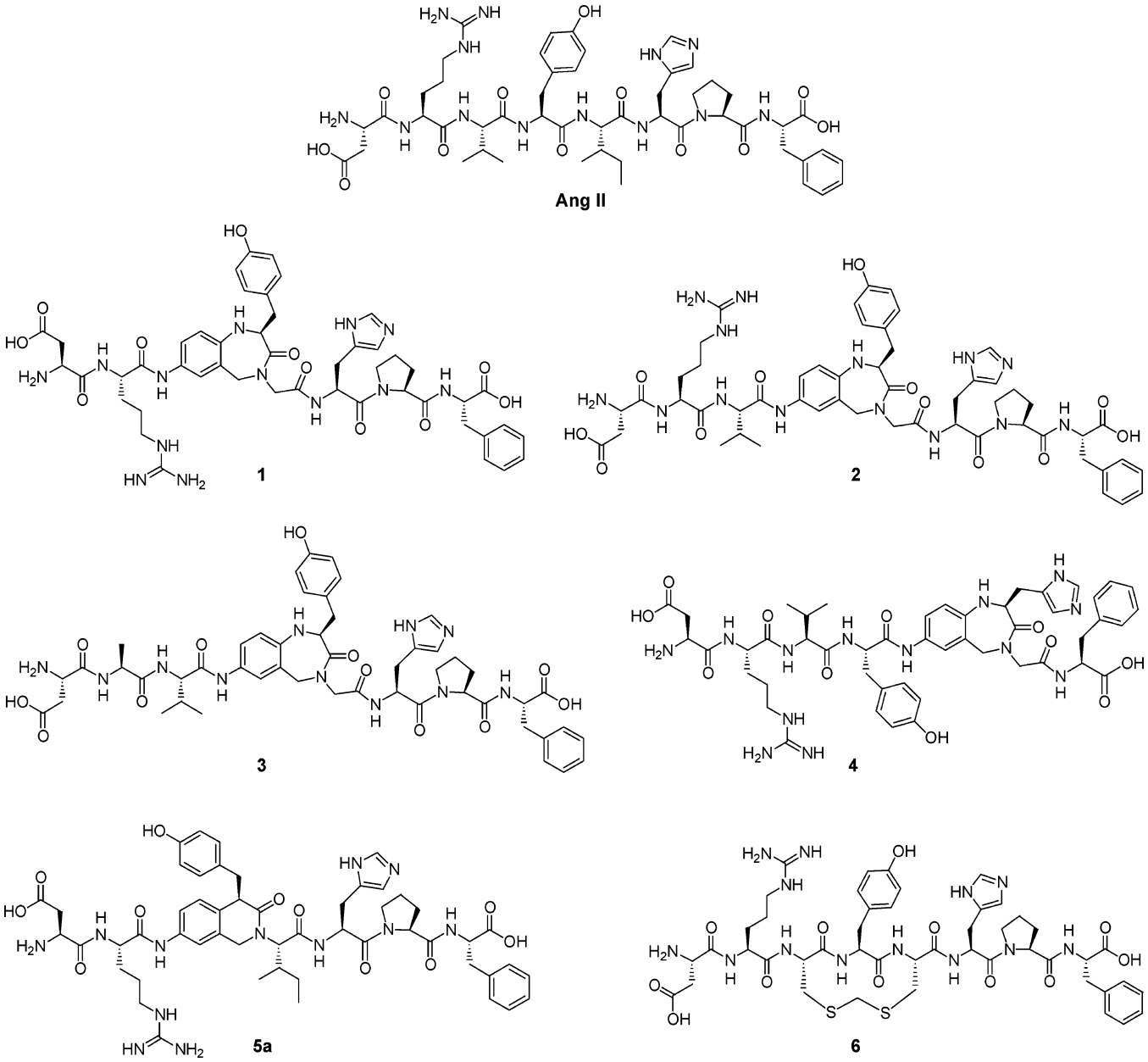
Chart 1



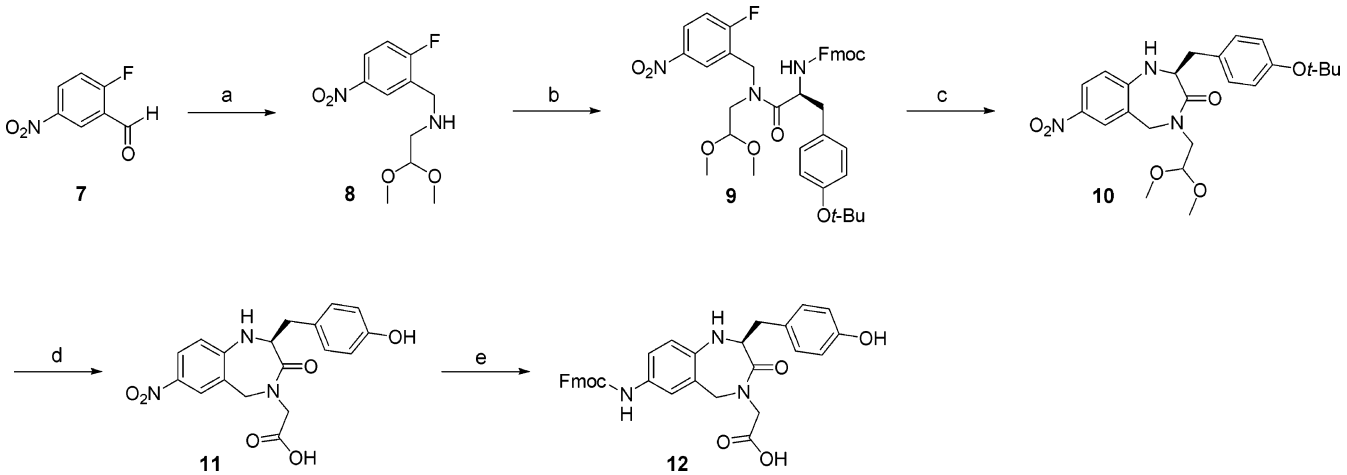
of **1**, **2**, and **5a** and a comparison with compound **6**, which has been shown to have high affinity to the AT_2 receptor.[32]

Results

Chemistry. The bicyclic core structure of **1**−**4**, prepared by solution chemistry, was incorporated into Ang II using solid-phase methodology. The synthesis of pseudopeptide **5**, encompassing a γ-turn mimetic region centered at Tyr^4 has been previously reported.[20] Insertion of NH into this γ-turn mimic gives the benzodiazepine core structure that has previously been recognized and successfully applied as a turn mimic.[33] The benzodiazepine system was used as a turn template for substituting Val^3-Tyr^4-Ile^5 (**1**), Tyr^4-Ile^5 (**2** and **3**), or Ile^5-His^6-Pro^7 (**4**) segments of Ang II.

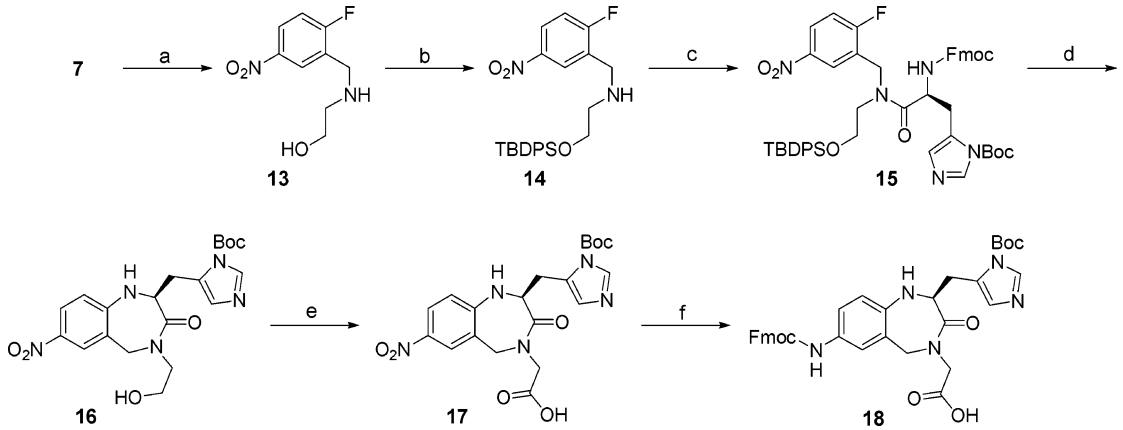
The synthesis of **12**, outlined in Scheme 1, was based on the strategy described by Keenan at al.,[33] starting with a reductive amination of the substituted benzaldehyde **7** with the glycine equivalent 2,2-dimethoxyethylamine. Glycine or its methyl ester could not be used as building blocks because of the facile formation of a diketopiperazine in a later step. The subsequent coupling of the secondary amine **8** with Fmoc-L-Tyr(*t*-Bu)-OH was performed using *O*-(7-azabenzothiazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) as activating reagent. The Fmoc group was removed with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) rather than with the more commonly used piperidine, since the latter was found to undergo nucleophilic aromatic substitution at the fluorine *ipso*-carbon. The deprotected amine **9** underwent an internal nucleophilic aromatic substitution under the basic conditions used. A significantly improved yield of the benzodiazepine scaffold was achieved in the cyclization if the deprotected amine was first purified through column chromatography to remove the deprotection side products and thereafter immediately treated with an aqueous triethylamine/DMSO solution. Treatment of **10** with TFA/CH_2Cl_2 removed both the acetal protection and the

Scheme 1[a]



[a] Reagents: (a) 2,2-dimethoxyethylamine, HOAc, $NaCNBH_3$, MeOH, 72%; (b) Fmoc-L-Tyr(*t*-Bu)-OH, HATU, DIEA, CH_2Cl_2, 90%; (c) (i) DBU, THF, (ii) Et_3N, DMSO, H_2O, 64%; (d) (i) TFA, CH_2Cl_2, (ii) $NaClO_2$, NaH_2PO_4, cyclohexene, *t*-BuOH, THF, H_2O, 77%; (e) (i) $HCOONH_4$, Pd/C, MeOH, (ii) FmocCl, Na_2CO_3 (aq), dioxane, 47%.

Scheme 2[a]



[a] Reagents: (a) 2-aminoethanol, HOAc, $NaCNBH_3$, MeOH, 85%; (b) TBDPSCl, DBU, CH_3CN, 98%; (c) Fmoc-L-His(Boc)-OH, HATU, DIEA, CH_2Cl_2, 86%; (d) (i) DBU, THF, (ii) Et_3N, DMSO, H_2O, 81%; (e) (i) ClCOCOCl, DMSO, Et_3N, CH_2Cl_2, (ii) $NaClO_2$, NaH_2PO_4, cyclohexene, *t*-BuOH, H_2O, 55%; (f) (i) $HCOONH_4$, Pd/C, MeOH, (ii) FmocCl, Na_2CO_3 (aq), dioxane, 33%.

tert-butyl group. The unstable aldehyde was immediately converted to the carboxylic acid **11** using mild oxidation by sodium chlorite in a mixture of *tert*-butyl alcohol, THF, and sodium dihydrogenphosphate solution at 0 °C.[34] At higher temperatures or with other oxidizing agents (e.g. Jones reagent), the carboxylic acid **11** was not detected. Instead, Jones reagent produced a mixture of products where benzylic oxidation followed by cleavage of the tyrosine side chain was the predominant reaction. Reduction of the nitro group was performed with ammonium formiate and Pd/C. The zwitterion obtained was not isolated but directly reacted with FmocCl.[35] Compound **12** decomposed during column chromatography and was therefore purified by repeated extractions.

The peptides **1**, **2**, and **3** were obtained by incorporation of the Fmoc derivative **12** into Ang II using standard Fmoc/*t*-Bu SPPS methodology. Some of the couplings, particularly the acylation of the anilinic nitrogen, were expected to proceed slowly and require long reaction times. Therefore, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) rather than 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as activating agent in order to eliminate the risk of formation of tetramethylguanidino derivatives. No protection was used for the phenolic hydroxyl group. According to MS analysis, 2 equiv of the Fmoc-amino acid were incorporated in each of the following coupling steps. However, the presumed phenyl ester was cleaved smoothly in the ensuing deprotection reaction. The target peptides **1**, **2**, and **3** were liberated from the resin by treatment with TFA in the presence of water and triethylsilane, isolated by precipitation with ether, and purified by RP-HPLC on a C18 column.

The histidine analogue **18** was synthesized employing a similar route, outlined in Scheme 2. Aminoethanol was now used instead of amino acetal as glycine ester equivalent, to avoid the need for acidic deprotection. The benzaldehyde **7** underwent a reductive amination with unprotected 2-aminoethanol using 5 equiv of amine and with $NaCNBH_3$ as the reducing agent. A longer reaction time or higher temperature lowered the yield. The alcohol **13** was protected with *tert*-butyldiphenylchlorosilane (TBDPSCl) to circumvent side reactions, such as Boc migration, and to simplify the purification. Coupling of **14** with Fmoc-L-His(Boc)-OH, deprotection of the Fmoc-group, and the subsequent cyclization proceeded smoothly. The TBDPS-protecting group was spontaneously cleaved during the cyclization, probably

Table 1. Binding Affinities for the AT_1 and AT_2 Receptors

compound	AT_1 (rat liver membranes) K_i (nM) ± SEM	AT_2 (pig uterus myometrium) K_i (nM) ± SEM
Ang II	0.24 ± 0.07	0.23 ± 0.01
[4-NH_2-Phe^6]Ang II	>10000	2.1 ± 0.5
1	>10000	>10000
2	>10000	3.0 ± 1.1
3	>10000	>10000
4	>10000	>10000
5a	>10000	61 ± 2
5b	>10000	>10000
6[32]	44 ± 1	0.62 ± 0.04

assisted by the fluoride ions liberated. Since the direct oxidation of the alcohol to carboxylic acid with TEMPO and sodium hypochlorite[36] failed, the oxidation was performed in two steps. Swern oxidation gave the aldehyde in high yield. However, the aldehyde was found to decompose on silica and during storage and was therefore directly oxidized to the carboxylic acid **17** using sodium chlorite at 0 °C. In contrast to the observations for the tyrosine derivative, THF had to be excluded from the reaction mixture to produce the acid **17** in a satisfactory yield. Reduction of the nitro group with ammonium formiate and Pd/C gave the zwitterion, which was subsequently protected using FmocCl. It was necessary to keep the reaction time short and to add FmocCl before the base in order to prevent migration of the Boc group from the imidazole to the aniline nitrogen. The building block **18** was incorporated into Ang II, using the SPPS procedure described for **12** above, to yield the analog **4**. However, in this case a combination of ion-exchange chromatography and RP-HPLC was required for purification of the final product.

Biological Results. Compounds **1**−**5** were evaluated in radioligand binding assays relying on 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). Ang II and the highly AT_2 receptor-selective [4-NH_2-Phe^6]Ang II were used as reference substances. Compound **2** and compound **5a** exhibited high AT_2/AT_1 selectivities, and the former compound (**2**) displayed a considerable binding affinity to the AT_2 receptor (K_i = 3.0 nM). Compounds **1**, **3**, **4**, and **5b** were devoid of binding affinity to either receptor subtype.

Conformational Preferences. Conformational analysis was performed on the model compounds **1m**, **2m**, and **6m** (see Figure 1) of the pseudopeptides **1**, **2**, and **6** using the MMFFs force field and the General Born Solvent Accessible (GB/SA)[39] water solvation in MacroModel 7.1.[40] We previously used the AMBER* force field and the GB/SA water solvation model in MacroModel to calculate the conformational preferences of turn mimetics and peptide analogues (see, for example, 20, 41, 42). However, when using the AMBER* force field and the GB/SA water solvation model for the compounds in this study, there seemed to be a strong bias toward conformations in which the guanidino group formed intramolecular hydrogen bonds to the backbone carbonyl groups. Because of these hydrogen bonds and/or the lack of high quality force field parameters for the benzamide moiety, the amide bond near the aromatic ring became distorted in **1m** and **2m**. Since this distortion was not observed with MMFFs, this force field was used in the further analysis. The number of conformations within 5 kcal/mol of the global energy minimum found for **1m**, **2m**, and **6m** were 4342, 2901, and 1273, respectively.[43] Three different ring conformations of the diazepine system in **1m** and **2m** were identified; two of these were very similar to the inverse γ-turn and one was similar to the classic γ-turn.[44]

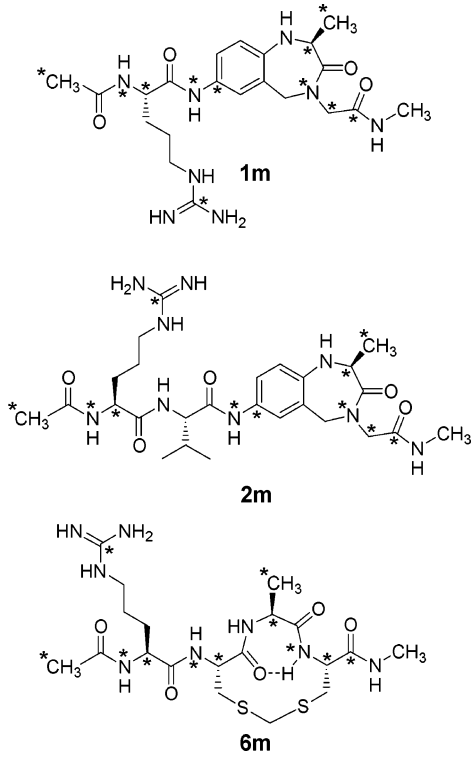


Figure 1. Model compounds used in conformational analysis and molecular modeling. A star indicates those atoms that were superimposed in order to reduce the number of identified conformations.

To reduce the number of identified conformations and facilitate the analysis, the minimization convergence criterion was increased, and only key atoms that describe important structural features were superimposed (Figure 1). This reduced the number of conformations to 1726, 1217, and 803 for **1m**, **2m**, and **6m**, respectively. Inspection of the results showed that in many of the conformations, the guanidino group still formed a hydrogen bond with one of the carbonyl oxygens of the peptide backbone. The hydrogen bond was especially pronounced in **2m**, since this molecule is more flexible and has an extra carbonyl group as compared to **1m**. This probably does not apply to the case when **2m** binds to the receptor since the Arg^2 side chain in this case is more likely to participate in receptor interactions[45] rather than in intramolecular hydrogen bonds. Even though the conformational distribution was biased toward these conformations, we believe that the general conformational properties of the pseudopeptides are relevant for our purposes.

Discussion

In comparison to the current situation for the AT_1 receptor, few investigations of the structure−activity relationships of the AT_2 receptor have been reported.[2,21,30,32,45−47] Also, little information about the functional properties of AT_2 receptor ligands, except for a few compounds (e.g. PD123,319, CGP-42112 and

[4-NH_2−Phe^6]Ang II), is available. Most of the binding affinity studies performed to date show that modification of the linear peptides Ang II or [Sar^1]Ang II is well tolerated by the AT_2 receptor. The amino acid residue Asp^1 is not important for affinity to the receptor since Ang III (Ang II(2−8)) and Ang II have similar affinity and since many modifications in position 1 are allowed.[47] Ang IV (Ang II(3−8)) has been reported to have a very low affinity to the AT_2 receptor, indicating a role for Arg^2 in receptor binding.[47] The importance of the charge and the length of the amino acid side chain in position two in Ang II has also been shown from mutagenesis studies of the AT_2 receptor.[45] Furthermore, Miura et al. recently introduced Ala into several positions in Ang II and only observed a minor decrease in affinity to the AT_2 receptor for most of the synthesized Ala analogues.[30] The side chains of Arg^2, Tyr^4 and His^6 seemed to be sensitive to this modification, implying their possible role as pharmacophore groups.

Several cyclic analogues of Ang II have also been synthesized and evaluated for AT_2 receptor affinity. Marshall's group performed disulfide monocyclizations in the 3−5 region of [Sar^1]Ang II that resulted in a more than 10-fold reduction of the affinity to the AT_2 receptor, while affinities to the AT_1 receptor subtype were essentially unaffected.[21] Notably, disulfide-based bicyclizations in the same region delivered [Sar^1]Ang II analogues with high affinities for the AT_2 receptor but with lower AT_1 receptor affinities in general.[21] We recently observed that monocyclic methylenedithioether analogues with Cys and Hcy in the 3 and 5 positions of Ang II had binding preference for the AT_2 receptor. The most potent analogue (**6**) in the series, encompassing a 12-membered Cys^{3,5} cyclized ring system, exhibited a K_i of 0.62 nM. It preferentially adopted low energy inverse γ-turn conformations centered at Tyr^4, as deduced from conformational analysis of model tripeptides.[32] These results suggested that incorporation of rigid bicyclic γ-turn mimicking scaffolds in this region could be a relevant first approach toward less peptide-like AT_2 receptor ligands derived from the native octapeptide.

In this work, two different γ-turn mimicing bicyclic scaffolds, a benzodiazepine and a isoquinoline, differing only in the presence (**1**−**4**) or absence (**5a** and **5b**) of an NH group have been studied (Chart 1). The isoquinoline scaffold, which was previously introduced in the 3−5 position of Ang II and evaluated for AT_1 receptor affinity, delivered two diastereoisomers **5a** and **5b** (**a** and **b** denote single diastereoisomers of unassigned absolute stereochemistry).[20] It was confirmed in this study that both of the diastereoisomers lacked affinity to the AT_1 receptor. However, when evaluated for AT_2 receptor affinity, it was shown that one of the diastereoisomers bound fairly well to this receptor (K_i = 61 nM). We hypothesized that the stereochemistry of this diastereoisomer corresponds to the natural L-configuration (**5a**).

The other γ-turn-like mimetic incorporated in Ang II in this study, to give **1**−**4**, contains the benzodiazepine structure (Chart 1). The benzodiazepine scaffold is more bulky than the peptide γ-turn, and the angle at which the peptide chain enters the turn is different from that

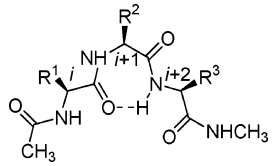


Figure 2. A γ-turn with the *i*, *i*+1, and *i*+2 residues indicated.

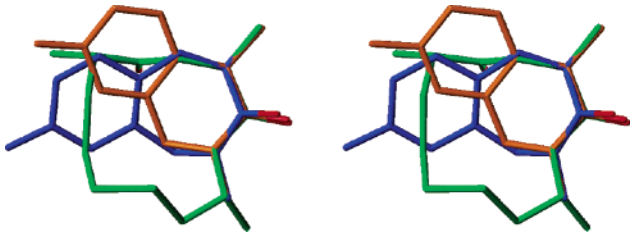
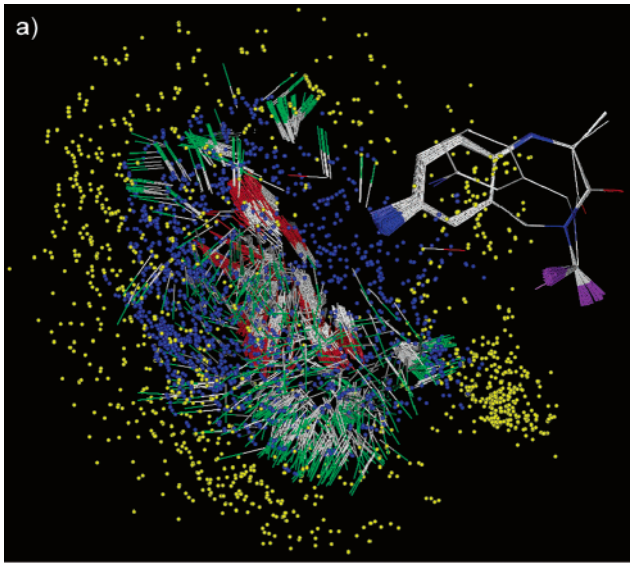


Figure 3. Stereopresentation (relaxed) of the best fit of the γ-turn mimetic moiety of **1m**/**2m** (blue) and a model compound of **5a** (orange) to **6m** (green). The atoms in the *i*+1 residue (except the carbonyl oxygen) and the N atom and C_α atom of the *i*+2 residue were superimposed in **1m**/**2m** and **6m**. The corresponding atoms in **5a** (with Csp^2 in the *i*+1 residue instead of N) were fitted to **6m**. Only the γ-turn/γ-turn mimetic region is shown, with hydrogens omitted for clarity. The global minimum was used for **1m**, the lowest energy conformation with an equatorial Tyr substituent for **5a** and the lowest energy conformation with a γ-turn conformation for **6m**.

at which it leaves. An atom-to-atom comparison of the backbone atoms of compound **1** (or **2**) and **6** shows that an exact match can be obtained from the *i*+1 residue and onward to the C-terminus (Figure 1). However, the *i* residue of the γ-turn-like mimetic region in **1** cannot be mapped onto **6**. This difference in geometry will affect the relative position of the Asp-Arg residues as compared to the rest of the peptide and will thus affect the ability to adopt similar conformations. Preliminary conformational analysis of the γ-turn/γ-turn mimetic region in **1**, **5a**, and **6**, followed by superimposition of the *i*+1 and *i*+2 residue, indicated that the residues entering the γ-turn region are directed quite differently (Figure 3). Because of the geometric differences, we synthesized not only compound **1**, which is more similar to **5a**, but also compound **2** with the Val^3 residue. The lipophilic Ile^5 side chain was omitted in **1** and **2** for synthetic reasons and also because it is considered only to have a conformational stabilizing role and to induce a turn motif.[19,48]

Both compound **1** and compound **2** lacked affinity (K_i > 10 000 nM) to the AT_1 receptor. Compound **1** also lacked affinity (K_i > 10 000 nM) to the AT_2, receptor while, interestingly, compound **2** bound with high affinity to the same receptor (K_i = 3.0 nM). We hypothesized that the reason for the lack of affinity of **1** was not that Val^3 was missing per se but rather that the guanidino group of the Arg^2 residue and/or the N-terminal end of the pseudopeptide could not interact optimally with the receptor due to the geometric differences between **1** and **6** (Figure 3). In comparison, Val^3 could function as a spacer in compound **2** and allow the Arg residues and the N-terminal end in the two active compounds **2** and **6** to interact in similar positions despite the geometric difference. To test this hypothesis, we performed conformational analysis of the model compounds of **1**, **2**, and **6** as shown in Figure 1. As a basis for the modeling we omitted the Asp residue, since it is known not to be important for binding to the AT_2



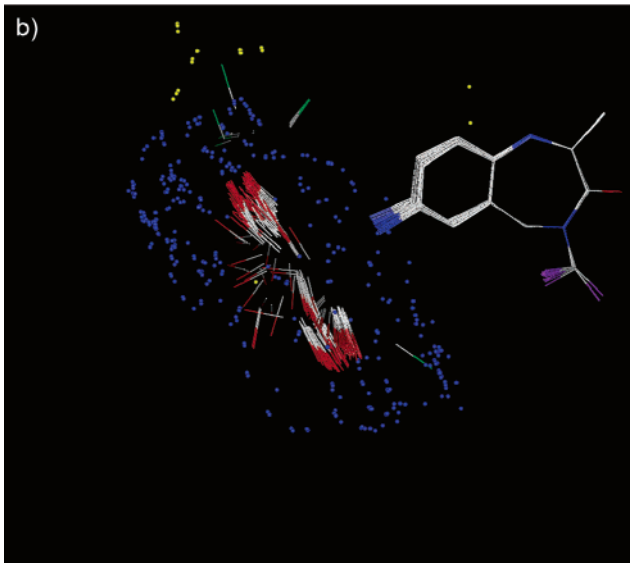


Figure 4. (a) RMS best fit of **1m/2m** to **6m** using the atoms defined in Figure 3. Only the bicyclic moiety, the carbon in the guanidino group in **1m** (blue) and in **2m** (yellow), and the N-terminal CH_3−CO bond vector in **1m** (methyl carbon red, carbonyl carbon white) and in **2m** (methyl carbon green, carbonyl carbon white) are shown. The benzodiazepine scaffold that does not seem to overlap corresponds to the classic γ-turn conformation. (b) Only those conformations where **1m** and/or **2m** could position the carbon of the guanidino group and the N-terminal methyl carbon near those of the corresponding groups in **6m** are shown.

receptor. The His-Pro-Phe residues were also omitted, since we assumed that they occupy similar conformational space in the three compounds.

On the basis of the results from the conformational analysis, the cyclic moieties were superimposed and the position of the carbon in the guanidino group in **1m** (blue) and in **2m** (yellow) were plotted (Figure 4a) for all identified conformations. We also plotted the N-terminal CH_3−CO bond vector in **1m** (methyl carbon red, carbonyl carbon white) and in **2m** (methyl carbon green, carbonyl carbon white). It can be seen that the carbon in the guanidino group in **2m** reaches further out from the bicyclic scaffold and occupies different

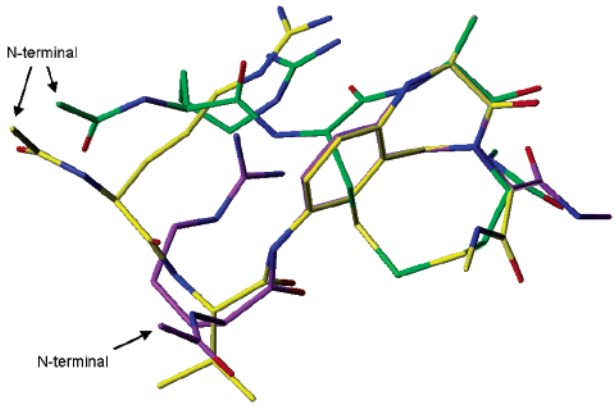


Figure 5. Superimposition of **2m** (yellow carbons) and **6m** (green carbons) showing the similar positioning of the Arg side-chain, the N-terminal backbone direction and γ-turn/γ-turn mimetic moiety. When the γ-turn mimetic moiety is used as the basis for superimposition, the guanidino group in compound **1m** (violet carbons) and the N-terminal acetyl group cannot reach the same binding site.

regions of space as compared to the guanidino carbon in **1m**, and that the N-terminal end of the peptide backbone (CH_3−CO bond vector) also seems to be oriented quite differently.

To examine whether **1m** and/or **2m** could position the carbon of the guanidino group and the N-terminal methyl carbon in the same region of space as the corresponding groups in **6m** when the γ-turn/γ-turn mimetic regions are superimposed, we searched for conformations that had an root-mean-square (RMS) distance between these atoms below 3 Å (Figure 4b). Inspection of Figure 4b shows that the common spatial positions of these atoms in **1m**/**6m** and **2m**/**6m** is different and that there are more conformations of compounds **1m**/**6m** (blue dots) having these atoms in a similar position than for compounds **2m**/**6m** (yellow dots). This is most likely because compounds **1m** and **6m** more closely resemble each other. However, according to the above reasoning, none of the sets of conformations of **1m/6m** should correspond to the "bioactive" conformation. In contrast, there are two areas in Figure 4b that are unique regarding the positioning of the Arg side chain in **2m** and **6m** and which cannot be adopted by **1m**/**6m**. In Figure 5 one of these sets of conformations from the area behind the azepine ring is shown along with the conformation of **1m** with the guanidino carbon closest to this set. It can be seen that the direction of the N-terminal group in **2m** and **6m** is similar but slightly separated in distance. The direction of the outgoing C-terminal group (C(O)-$NHCH_3$) is also different, but the energy penalty for **2m** to adopt the same direction as in **6m** is only about 1 kcal/mol (MMFFs force field). **2m** and **6m** also have more of an extended backbone conformation in accordance with the hypothesis of Deraët et al.[31] To rationalize the above results, we therefore suggest that the extra Val residue in **2** enables the guanidino group and the N-terminal chain to adopt optimal conformation(s) for binding (as defined by the strong binder **6**) when the γ-turn/γ-turn mimetic regions are superimposed. This conformation(s) cannot be adopted by the inactive analogue **1**, assuming the overlay shown in Figure 5. To challenge the hypotheses regarding the importance of the Arg side chain, we

synthesized compound **3** in which Arg has been substituted for Ala. This compound lacked affinity for AT_2 receptors thus supporting a role of the guanidino group of the Arg^2 residue in receptor binding.

The γ-turn-like scaffold in **1** and **2** was also introduced in the 5−7 region of Ang II since previous studies have shown that the AT_1 receptor can accommodate peptides cyclized in this position.[49] In that study, the bicyclic [Sar^1,Hcy^3,Mpt^5]Ang II analogue had an affinity of 1.3 nM to the AT_1 receptor and the [Sar^1,Hcy^5,Mpc^7]Ang II analogue exhibited an affinity of 20 nM. These data suggest that Ang II might adopt a turn also centered at His^6 when binding to the AT_2 receptor. Notably, compound **4** was inactive at both the AT_1 and AT_2 receptors. With regard to AT_2 receptor binding, support for the presence of a turn motif located in the 5−7 region of Ang II has to the best of our knowledge so far not been reported.

Conclusion

In summary, four analogues of angiotensin II encompassing a benzodiazepine-based γ-turn-like peptidomimetic have been synthesized. One of these analogues (**2**) binds with high affinity to the Ang II AT_2 receptor. Our conformational analysis suggested that the guanidino group of the Arg^2 residue and the N-terminal end of the active analogues **2** and **6** could access common regions of space inaccessible to the compound that lacked affinity (**1**). To challenge this hypothesis, compound **3**, which lacks the Arg side chain, was synthesized. The latter compound had no affinity to the AT_2 receptor, which supports the hypothesis and emphasizes the importance of the Arg side chain for binding to the receptor.

Experimental Section

General Methods. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX400 at 400 and 100.5 MHz, respectively. Spectra were recorded at ambient temperature unless otherwise noted. Chemical shifts are reported as δ values (ppm) referenced to δ 7.26 ppm $CHCl_3$, δ 77.0 ppm $CDCl_3$, δ 2.50 ppm, δ 39.5 (DMSO-d_6), δ 1.98 CH_3CN, δ 117.7 CD_3CN, CH_3-OH δ 3.30 ppm and CD_3OD δ 49.15 ppm. Optical rotations were measured on a Perkin-Elmer model 241 polarimeter. Elemental analyses were performed by Mikro Kemi AB, Uppsala Sweden. Mass spectra were recorded on LC-MS utilizing electron-spray ionization (ESI) or on a GC-MS, equipped with a nonpolar capillary column, utilizing electron impact (EI) at an ionizing energy of 70 eV.

Thin-layer chromatography (TLC) was performed using aluminum sheets precoated with silica gel 60 F_{254} (0.2 mm, E. Merck). The spots were identified using UV-detection and/or by spraying with 3% methanolic solution of ninhydrin followed by heating. Flash column chromatography was performed using Merck silica gel 60 (40−63 μm). The starting material **7** was synthesized according to a previously published procedure.[50]

Solid-Phase Peptide Synthesis (SPPS). The starting Fmoc-His(Trt)-Pro-Phe-Wang resin (0.25 mmol) was synthesized manually, in a 5 mL disposable syringe equipped with a porous polyethylene filter, using standard Fmoc/*t*-Bu conditions. Removal of the Fmoc group was achieved by treatment with 20% piperidine/DMF for 2 + 10 min. The Fmoc amino acids (0.75 mmol) were coupled by reaction with HBTU (0.75 mmol) and diisopropylethylamine (DIEA) (1.50 mmol) in DMF (1.2 mL) for 45 min.

The angiotensin II analogues were synthesized in 2 mL syringe vessels using a similar procedure. However, PyBOP/DIEA were used for activation, and prolonged reaction times (ca. 20 h) were allowed for incorporation of compounds **12** and **18** as well as for the following amino acid. The Fmoc amino acids were protected as follows: Asp(O*t*-Bu), Arg(Pbf), Tyr(*t*-Bu), His(Trt).

Completeness of the coupling reactions was ascertained by PDMS analysis of cleaved resin samples (ca 1 mg) before and/or after deprotection.

(2,2-Dimethoxyethyl)(2-fluoro-5-nitrobenzyl)amine (8). HOAc (2.00 mL, 35.0 mmol) and 2,2-dimethoxyethylamine (24.97 g, 47.3 mmol) were added to the aldehyde **7** (4.01 g, 23.7 mmol) dissolved in 50 mL of MeOH. The reaction mixture was stirred overnight before $NaCNBH_3$ (3.01 g, 47.9 mmol) was added in portions. After another night, water was added and the MeOH was evaporated. The remaining aqueous solution was extracted with EtOAc, dried over Na_2SO_4, and evaporated. Purification by column chromatography (gradient system CH_2Cl_2 to 2% MeOH in CH_2Cl_2) gave **8** as a yellow oil (4.39 g, 72%). ^1H NMR ($CDCl_3$) δ: 8.34 (m, 1H, CH), 8.12 (m, 1H, CH), 7.15 (m, 1H, CH), 4.47 (t, J = 5.3 Hz, 1H, CH), 3.91 (s, 2H, CH_2), 3.36 (s, 6H, CH_3), 2.74 (d, J = 5.3 Hz, 2H, CH_2), 2.02 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 164.3 (d, J = 257.5 Hz, CF), 144.3 (C), 129.9 (d, J = 16.9 Hz, C), 125.9 (d, J = 6.9 Hz, CH), 124.5 (d, J = 10.7 Hz, CH), 116.1 (d, J = 24.6 Hz, CH), 103.6 (CH), 54.1 (CH_3), 50.4 (CH_2), 46.2 (CH_2). GC-MS (M^+): 258. Anal. ($C_{11}H_{15}FN_2O_4$) C, H, N.

(2*S*)-3-(4-*tert*-Butoxyphenyl)-*N*-(2,2-dimethoxyethyl)-2-[(9*H*-fluoren-9-ylmethoxylcarbonyl)amino]-*N*-(2-fluoro-5-nitrobenzyl)propionamide (9). Fmoc-Tyr(*t*-Bu)-OH (5.12 g, 11.1 mmol), HATU (4.25 g, 11.2 mmol) and DIEA (5.00 mL, 28.7 mmol) were added to a solution of the amine **8** (2.71 g, 10.5 mmol) in 50 mL of CH_2Cl_2. The reaction mixture was stirred overnight. After addition of water, the mixture was extracted with EtOAc. The organic phase was washed with 1 M $KHSO_4$, water, saturated $NaHCO_3$ solution, and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (pentane:EtOAc 3:1) to give **9** as a yellow foam (6.65 g, 90%). $[\alpha]^{22}_D$ = −13° (c = 1.02, MeOH). The product was observed as rotamers at 20 °C in NMR, increasing the temperature to 120 °C results in one set of peaks, but they are still very broad in the aliphatic region. ^1H NMR (DMSO-d_6) δ: 8.20 (m, 1H, CH), 8.08 (m, 1H, CH), 8.02 and 7.95 (minor) (m, 1H, NH), 7.87 (m, 2H, 2 × CH), 7.67 and 7.62 (minor) (m, 2H, 2 × CH), 7.49 (m, 1H, CH), 7.40 (m, 2H, 2 × CH), 7.30 (m, 2H, 2 × CH), 7.18 and 7.05 (minor) (m, 2H, 2 × CH), 6.81 and 6.74 (minor) (m, 2H, 2 × CH), 4.73 and 4.50 (minor) (m, 1H, CH), 4.71 (m, 1H, CH_2), 4.58 (m, 1H, CH_2), 4.38 (m, 1H, CH), 4.20 (m, 1H, CH_2), 4,14 (m, 1H, CH_2), 3.83 and 3.57 (minor) (m, 1H, CH_2), 3.30−3.18 (m, 7H, 2 × CH_3 + CH_2), 2.95−2.81 (m, 2H, CH_2), 1.19 and 1.15 (s, 9H, 3 × CH_3). ^{13}C NMR (DMSO-d_6) δ (major peaks): 173.0 (C), 163.6 (d, J = 253 Hz, CF), 156.0 (C), 155.6 (C), 153.6 (C), 144.0 (C), 143.7 (C), 140.7 (C), 132.3 (C), 129.8 (CH), 127.6 (CH), 127.2 (CH), 125.3 (CH), 124.84 (CH), 124.81 (CH), 123.5 (CH), 120.1 (CH), 116.8 (d, 24.5, CH), 103.3 (CH), 77.6 (C), 65.9 (CH), 55.3 (CH_3), 54.3 (CH_3), 52.3 (CH), 50.0 (CH_2), 46.6 (CH_2), 44.3 (CH_2), 36.5 (CH_2), 28.5 (CH_3). LC-MS ($M + H^+$): 700.0. Anal. ($C_{39}H_{42}FN_3O_8$) C, H, N.

(2*S*)-2-(4-*tert*-Butoxybenzyl)-4-(2,2-dimethoxyethyl)-7-nitro-1,2,4,5-tetrahydrobenzo[1,4]diazepin-3-one (10). DBU (1.41 mL, 9.43 mmol) was added to **9** (6.06 g, 8.66 mmol) in dry THF (15 mL). After 2 h, the reaction was complete according to TLC (2% MeOH in CH_2Cl_2). The reaction mixture was evaporated, and the residue was purified by column chromatography (CH_2Cl_2 to elute Fmoc-related side products, then 2% MeOH in CH_2Cl_2). The free amine was obtained in 3.98 g. The amine (3.98 g, 8.34 mmol) was dissolved in 170 mL of DMSO and 1.7 mL of water and 3.4 mL of Et_3N were added. After 3 days at room temperature, EtOAc and saturated $NaHCO_3$ (aq) were added. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with water and brine, dried (Na_2SO_4) and evaporated. The crude residue was purified by column chromatography (gradient CH_2Cl_2 to 1% MeOH in CH_2Cl_2) to give **10** as a yellow foam (2.55 g, 64% over two steps).

$[\alpha]^{22}_D = -327°$ ($c = 1.03$, MeOH). ^{1}H NMR ($CDCl_3$) δ: 7.89−7.40 (m, 2H, CH and CH), 7.19 (m, 2H, 2 × CH), 6.97 (m, 2H, 2 × CH), 6.35 (m, 1H, CH), 5.37 (d, $J = 16.6$ Hz, 1H, CH_2), 5.00 (dd, $J = 5.3$, 9.0 Hz, 1H, CH), 4.42 (bs, 1H, NH), 4.34 (dd, $J = 4.8$, 5.4 Hz, 1H, CH), 4.06 (d, $J = 16.6$ Hz, 1H, CH_2), 3.71 (dd, $J = 4.8$, 14.0 Hz, 1H, CH_2), 3.54 (dd, $J = 5.4$, 14.0 Hz, 1H, CH_2), 3.37 (s, 3H, CH_3), 3.36 (dd, $J = 5.3$, 14.7 Hz, 1H, CH_2), 3.21 (s, 3H, CH_3), 2.89 (dd, $J = 9.0$, 14.7 Hz, 1H, CH_2), 1.34 (s, 9H, 3 × CH_3). ^{13}C NMR ($CDCl_3$) δ: 169.1 (C), 154.5 (C), 151.0 (C), 137.8 (C), 130.9 (C), 129.6 (CH), 126.6 (CH), 125.3 (CH), 124.6 (CH), 118.4 (C), 115.3 (CH), 103.3 (CH), 78.6 (C), 54.9 (CH and CH_3), 54.8 (CH_3), 52.1 (CH_2), 49.5 (CH_2), 36.1 (CH_2), 28.8(CH_3). LC-MS ($M + H^+$): 457.9. Anal. ($C_{24}H_{31}N_3O_6$) C, H, N.

[(2*S*)-2-(4-Hydroxybenzyl)-7-nitro-3-oxo-1,2,3,5-tetrahydrobenzo[1,4]diazepin-4-yl]acetic Acid (11). Compound **10** (400 mg, 0.87 mmol) was dissolved in 16 mL of TFA/CH_2Cl_2 (1:1). After 1 h, the aldehyde was deprotected according to TLC (10% MeOH in CH_2Cl_2), and the reaction mixture was evaporated. The residue was dissolved in EtOAc and water, and the layers were separated. The organic layer was washed with water, dried over Na_2SO_4, and evaporated. The obtained aldehyde was dissolved in 3 mL of THF and 4.5 mL of *t*-BuOH. The solution was cooled on an ice-bath, and cyclohexene (1.43 mL, 14.1 mmol) was added. A freshly prepared ice-cold solution of $NaClO_2$ (752 mg, 80%, 6.65 mmol) and $NaH_2PO_4 \cdot H_2O$ (760 mg, 5.53 mmol) in 3.5 mL of water was added dropwise. After 45 min at 0 °C, the reaction was complete according to TLC (20% MeOH in CH_2Cl_2). EtOAc and water were added, and the layers were separated. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated. After purification by column chromatography (gradient 2% MeOH in CH_2Cl_2 to 30% MeOH in CH_2Cl_2), the product was redissolved in EtOAc. The EtOAc solution was washed with 1 M $KHSO_4$, dried over Na_2SO_4, and evaporated to give **11** as a yellow foam (249 mg, 77%). $[\alpha]^{22}_D = -399°$ ($c = 0.54$, MeOH). ^{1}H NMR (CD_3CN) δ: 7.92 (m, 1H, CH), 7.88 (m, 1H, CH), 7.24 (m, 2H, 2 × CH), 6.79 (m, 2H, 2 × CH), 6.59 (m, 1H, CH), 6.37 (br s, 2H, 2 × OH), 5.48 (d, $J = 17.2$ Hz, 1H, CH_2), 5.46 (bs, 1H, NH), 5.12 (dd, $J = 6.2$, 7.7 Hz, 1H, CH), 4.29 (d, $J = 17.6$ Hz, 1H, CH_2), 4.14 (d, $J = 17.6$ Hz, 1H, CH_2), 4.03 (d, $J = 17.2$ Hz, 1H, CH_2), 3.24 (dd, $J = 6.2$, 14.3 Hz, 1H, CH_2), 2.81 (dd, $J = 7.7$, 14.3 Hz, 1H, CH_2). ^{13}C NMR (CD_3CN) δ: 170.2 (C), 170.1 (C), 156.0 (C), 152.6 (C), 137.4 (C), 130.9 (CH), 128.8 (C), 126.5 (CH), 125.3 (CH), 118.9 (C), 115.5 (CH), 115.4 (CH), 55.4 (CH), 51.6 (CH_2), 48.5 (CH_2), 35.5 (CH_2). LC-MS ($M + H^+$): 371.8. Anal. ($C_{18}H_{17}N_3O_6 \cdot 1/2\ H_2O$) C, H, N.

[(2*S*)-7-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-2-(4-hydroxybenzyl)-3-oxo-1,2,3,5-tetrahydrobenzo[1,4]-diazepin-4-yl]acetic Acid (12). HCO_2NH_4 (300 mg, 4.76 mmol) was added to **11** (80 mg, 0.22 mmol) and Pd/C (23 mg 10%, 0.02 mmol) in 2.2 mL of MeOH and stirred under nitrogen in a sealed tube. After 3 h, the reaction was completed according to TLC (20% MeOH in CH_2Cl_2). Pd/C was removed by filtration. The crude product obtained after evaporation was dissolved in 4 mL of 10% Na_2CO_3 (aq) and 2 mL of dioxane and cooled to 0 °C. FmocCl (112 mg, 0.43 mmol) dissolved in 2 mL of dioxane was added dropwise, and the reaction mixture was allowed to reach room temperature. After 24 h, 10% aqueous citric acid was added until pH 8−9. The mixture was washed several times with ether. New ether was added to the water phase, which was acidified to pH 3 by addition of 10% aqueous citric acid. The layers were separated, and the water layer was extracted with ether and EtOAc. The organic layers were combined and washed with water, dried over Na_2SO_4, and evaporated. The product **12** was obtained as white foam (57 mg, 47%). $[\alpha]^{22}_D = -107°$ ($c = 0.42$, MeOH). ^{1}H NMR (CD_3OD) δ: 7.76 (m, 2H, 2 × CH), 7.65 (m, 2H, 2 × CH), 7.36 (m, 2H, 2 × CH), 7.28 (m, 2H, 2 × CH), 7.14 (m, 2H, 2 × CH), 7.08−6.90 (m, 2H, 2 × CH), 6.71 (m, 2H, 2 × CH), 6.46 (m, 1H, CH), 5.35 (d, $J = 16.3$ Hz, 1H, CH_2), 4.79 (dd, $J = 5.6$, 8.1 Hz, 1H, CH), 4.48 (d, $J = 17.4$ Hz, 1H, CH_2), 4.39 (d, $J = 6.2$ Hz, 2H, CH_2), 4.20 (m, 1H, CH), 3.86 (m, 2H, CH_2 and CH_2), 3.15 (dd, $J = 5.6$, 14.3 Hz, 1H, CH_2), 2.72 (dd, $J = 8.1$, 14.3 Hz, 1H, CH_2). ^{13}C NMR (CD_3OD) δ: 173.9 (C), 173.5 (C), 157.2 (C), 156.4 (C), 145.4 (C), 143.7 (C), 142.8 (C), 131.6 (C), 131.5 (CH), 130.3 (C), 128.9 (CH), 128.3 (CH), 126.3 (CH), 122.3 (CH), 122.0 (CH), 121.3 (C), 121.1 (CH), 118.5 (CH), 116.7 (CH), 67.7 (CH_2), 57.7 (CH), 53.9 (CH_2), 50.3 (CH_2), 48.6 (CH), 37.4 (CH_2). LC-MS ($M + H^+$): 564.1. Anal. ($C_{33}H_{29}N_3O_6 \cdot H_2O$) C, H, N.

2-[(2-Fluoro-5-nitrobenzyl)amino]ethanol (13). The benzaldehyde **7** (1.00 g, 5.92 mmol) was dissolved in 60 mL of MeOH. HOAc (0.50 mL, 8.75 mmol), 2-aminoethanol (1.80 mL, 29.9 mmol), and $NaCNBH_3$ (0.370 g, 5.89 mmol) were added, and the reaction mixture was heated to reflux. The conversion was complete after 1.5 h according to TLC (10% MeOH in CH_2Cl_2). The mixture was cooled to room temperature, acidified to pH 1 by addition of 2 M HCl, and partly evaporated. The residue was washed with ether and 6 M NaOH was added to adjust the pH to 11. The water layer was extracted with diethyl ether, and the combined organic layers were dried over K_2CO_3, evaporated, and purified by column chromatography (3% MeOH in CH_2Cl_2) to give **13** as a yellow solid (1.07 g, 85%). ^{1}H NMR ($CDCl_3$) δ: 8.33 (m, 1H, CH), 8.17 (m, 1H, CH), 7.17 (m, 1H, CH), 3.91 (s, 2H, Ar−CH_2), 3.69 (m, 2H, CH_2), 2.80 (m, 2H, CH_2), 1.99 (br s, 2H, OH and NH). ^{13}C NMR ($CDCl_3$) δ: 164.4 (d, $J = 256$ Hz, CF), 144.0 (C), 129.0 (d, $J = 17.1$ Hz, C), 125.9 (d, $J = 6.1$ Hz, CH), 124.8 (d, $J = 9.8$ Hz, CH), 116.3 (d, $J = 24.4$ Hz, CH), 61.0 (CH_2), 50.5 (CH_2), 46.2 (CH_2). LC-MS ($M + H^+$): 214.8. Anal. ($C_9H_{11}FN_2O_3$) C, H, N.

[2-(*tert*-Butyldiphenylsilanyloxy)ethyl]-(2-fluoro-5-nitrobenzyl)amine (14). DBU (1.40 mL, 9.36 mmol) and TBDPSCl (2.40 mL, 9.38 mmol) were added to a solution of **13** (1.45 g, 6.78 mmol) in 35 mL of CH_3CN. The reaction mixture was stirred overnight. After evaporation, the residue was dissolved in CH_2Cl_2, washed with water and brine, dried over Na_2SO_4, and evaporated. Purification by column chromatography (2% MeOH in CH_2Cl_2) gave **14** as a yellow oil (3.02 g, 98%). ^{1}H NMR ($CDCl_3$) δ: 8.39 (m, 1H, CH), 8.17 (m, 1H, CH), 7.66 (m, 4H, CH), 7.48−7.36 (m, 6H, CH), 7.18 (m, 1H, CH), 3.96 (s, 2H, CH_2), 3.83 (t, $J = 5.1$ Hz, 2H, CH_2), 2.80 (t, $J = 5.1$ Hz, 2H, CH_2), 2.70 (br s, 1H, NH), 1.07 (s, 9H, 3 × CH_3). ^{13}C NMR ($CDCl_3$) δ: 164.7 (d, $J = 256.8$ Hz, CF), 144.3 (d, $J = 1.5$ Hz, C), 135.5 (C), 133.3 (C), 129.7 (CH), 127.9, 127.7 (CH), 126.0 (d, $J = 6.9$ Hz, CH), 124.6 (d, $J = 10.0$ Hz, CH), 116.2 (d, $J = 24.6$ Hz, CH), 62.8 (CH_2), 50.7 (CH_2), 46.1 (CH_2), 26.8 (CH_3), 19.1 (C). LC-MS ($M + H^+$): 454.2. Anal. ($C_{25}H_{29}FN_2O_3Si$) C, H, N.

1-(*tert*-Butoxycarbonyl)-5-[(2*S*)-2-[[2-(*tert*-butyldiphenylsilanyloxy)ethyl]-(2-fluoro-5-nitrobenzyl)carbamoyl]-2-(9*H*-fluoren-9-ylmethoxycarbonylamino)ethyl]imidazole (15). The amine **14** (4.60 g, 10.2 mmol) was dissolved in 80 mL of CH_2Cl_2. HATU (3.89 g, 10.2 mmol), Fmoc-L-His(Boc)-OH (4.89 g, 10.2 mmol), and DIEA (5.30 mL, 30.4 mmol) were added. The reaction mixture was stirred at room-temperature overnight. Water and EtOAc were added, and the layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layers were washed with 1 M $KHSO_4$, water, saturated $NaHCO_3$, and brine, dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography (CH_2Cl_2 to 2% MeOH in CH_2Cl_2) to give **15** as white foam (7.98 g, 86%). $[\alpha]^{23}_D = -19°$ ($c = 1.04$, MeOH). Rotamers in NMR. An attempt to run NMR at 120 °C in DMSO-d_6 failed since the substance was unstable at these conditions. ^{1}H NMR ($CDCl_3$) δ: 8.21 and 8.18 (minor) (m, 1H, CH), 8.13 and 8.10 (minor) (m, 1H, CH), 7.99 and 7.94 (minor) (m, 1H, CH), 7.76 (m, 2H, 2 × CH), 7.70−7.53 (m, 6H, 6 × CH), 7.47−7.28 (m, 10H, 10 × CH), 7.22−7.08 (m, 2H, 2 × CH), 6.01 and 5.87 (minor) (d, $J = 8.2$ Hz, 1H, NH), 5.06 (m, 1H, CH), 4.93 (d, $J = 15.9$ Hz, 1H, CH_2), 4.57 (d, $J = 15.9$ Hz, 1H, CH_2), 4.44−4.13 (m, 3H, CH_2 and CH), 3.89 (m, 2H, CH_2), 3.67 (m, 2H, CH_2), 3.05 (dd, $J = 6.2$, 14.8 Hz, 1H, CH_2), 2.93 (dd, $J = 6.6$, 14.8 Hz, 1H, CH_2), 1.56 (s, 9H, 3x CH_3), 1.02 (s, 9H, 3 × CH_3). ^{13}C NMR ($CDCl_3$) δ (major peaks): 172.3 (C), 164.0 (d, $J = 256.8$ Hz, CF), 155.6 (C), 146.6 (C), 144.4 (d, $J = 1.5$ Hz, C), 143.8 (C), 141.1 (C), 137.7 (C), 136.6 (CH), 135.4 (CH), 132.6 (C), 129.8 (CH), 127.7 (CH), 127.6 (CH), 127.0

(CH), 126.2 (C), 125.6 (d, J = 6.2 Hz, CH), 125.2 (CH), 124.7 (d, J = 10.0 Hz, CH), 119.8 (CH), 116.0 (d, J = 24.6 Hz, CH), 114.7 (CH), 85.6 (C), 67.0 (CH_2), 62.6 (CH_2), 50.2 (CH), 49.8 (CH_2), 47.0 (CH), 44.1 (CH_2), 31.3 (CH_2), 27.7 (CH_3), 26.7 (CH_3), 18.9 (C). LC-MS ($M + H^+$): 912.2. Anal. ($C_{51}H_{54}FN_5O_8Si \cdot H_2O$) C, H, N.

1-(*tert*-Butoxycarbonyl)-5-[(2*S*)-4-(2-hydroxyethyl)-7-nitro-3-oxo-2,3,4,5-tetrahydro-1*H*-benzo[1,4]diazepin-2-ylmethyl]imidazole (16). DBU (0.910 mL, 5.98 mmol) was added to a solution of **15** (5.318 g, 5.83 mmol) in 30 mL of THF. After 1 h at room temperature, the reaction was completed according to TLC (10% MeOH in CH_2Cl_2). The MeOH was evaporated and the product purified by column chromatography (gradient CH_2Cl_2 to 2% MeOH in CH_2Cl_2) to yield 3.70 g of the deprotected amine. The amine was dissolved in 90 mL of DMSO, and 0.90 mL of water and 2.60 mL of Et_3N were added. The reaction mixture was stirred for 72 h at room temperature. EtOAc and $NaHCO_3$ (aq) were added, and the layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layers were washed with water and brine, dried over Na_2SO_4, and evaporated. The residue was purified through column chromatography (gradient CH_2Cl_2 to 3% MeOH in CH_2Cl_2), and the product **16** was obtained as yellow foam (2.05 g, 81%). $[\alpha]^{23}_D = -300°$ (c = 1.05, MeOH). ^{1}H NMR ($CDCl_3$) δ: 8.02 (d, J = 1.4 Hz, 1H, CH), 7.89 (m, 1H, CH), 7.86 (m, 1H, CH), 7.28 (d, J = 1.4 Hz, 1H, CH), 6.50 (m, 1H, CH), 6.07 (d, J = 2.2 Hz, 1H, NH), 5.40 (d, J = 16.9 Hz, 1H, CH_2), 5.05 (ddd, J = 2.2, 4.5, 9.0 Hz, 1H, CH), 3.98 (d, J = 16.9 Hz, 1H, CH_2), 3.77 (m, 3H, CH_2 and CH_2), 3.60 (m, 1H, CH_2), 3.13 (dd, J = 4.5, 15.2 Hz, 1H, CH_2), 2.98 (dd, J = 9.0, 15.2 Hz, 1H, CH_2), 1.60 (s, 9H, 3 × CH_3). ^{13}C NMR ($CDCl_3$) δ: 169.9 (C), 151.7 (C), 146.7 (C), 138.9 (C), 137.4 (C), 136.9 (CH), 126.1 (CH), 125.6 (CH), 117.6 (C), 115.5 (CH), 115.1 (CH), 85.9 (C), 61.4 (CH_2), 54.4 (CH), 52.1 (CH_2), 50.5 (CH_2), 29.0 (CH_2), 27.9 (CH_3). LC-MS ($M + H^+$): 432.1. Anal. ($C_{20}H_{25}N_5O_6 \cdot H_2O$) C, H, N.

1-(*tert*-Butoxycarbonyl)-5-((2*S*)-4-carboxymethyl-7-nitro-3-oxo-2,3,4,5-tetrahydro-1*H*-benzo[1,4]diazepin-2-ylmethyl)imidazole (17). A solution of oxalyl chloride (58 μL, 0.68 mmol) in 3 mL of CH_2Cl_2 was cooled to −78 °C. DMSO (100 μL, 1.41 mmol) was added dropwise, and the mixture was stirred 15 min before a solution of **16** (250 mg, 0.58 mmol) in 2.5 mL of CH_2Cl_2 was added dropwise over 10 min. After another 30 min at −78 °C, Et_3N (275 μL, 2.01 mmol) was added and the reaction mixture was allowed to warm to −30 °C over 50 min. Water (2 mL) was added to quench the reaction. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were washed with 1 M $KHSO_4$, water, saturated $NaHCO_3$ solution, and brine, dried (Na_2SO_4), and evaporated. The aldehyde was obtained as yellow foam (233 mg, 94%). $[\alpha]^{22}_D = -266°$ (c = 1.03, CH_2Cl_2), ^{1}H NMR ($CDCl_3$) δ: 9.59 (s, 1H, CH), 8.13 (d, J = 1.3 Hz, 1H, CH), 7.90 (m, 1H, CH), 7.79 (m, 1H, CH), 7.34 (d, J = 1.3 Hz, 1H, CH), 6.60 (m, 1H, CH), 6.34 (br s, 1H, NH), 5.50 (d, J = 17.0 Hz, 1H, CH_2), 5.12 (m, 1H, CH), 4.67 (d, J = 18.5 Hz, 1H, CH_2), 4.15 (d, J = 18.5 Hz, 1H, CH_2), 3.73 (d, J = 17.0 Hz, 1H, CH_2), 3.17 (dd, J = 4.7, 15.1 Hz, 1H, CH_2), 3.06 (dd, J = 8.6, 15.1 Hz, 1H, CH_2), 1.61 (s, 9H, 3 × CH_3). ^{13}C NMR ($CDCl_3$) δ: 196.2 (CH), 169.6 (C), 151.7 (C), 146.5 (C), 138.4 (C), 137.4 (C), 136.6 (CH), 125.9 (CH), 125.6 (CH), 117.0 (C), 115.7 (CH), 115.3 (CH), 86.2 (C), 57.4 (CH_2), 54.0 (CH), 52.1 (CH_2), 27.9 (CH_2), 27.8 (CH_3).

A suspension of the aldehyde (220 mg, 0.47 mmol) in 13 mL of *t*-BuOH and 2.65 mL of cyclohexene was cooled to 0 °C, and a freshly prepared ice-cold solution of $NaClO_2$ (590 mg (80%), 5.22 mmol) and $NaH_2PO_4 \cdot H_2O$ (560 mg, 4.08 mmol) in 5.5 mL of water was added dropwise over 10 min. The reaction mixture was stirred for 1.5 h at 0 °C. Water and EtOAc were added, and the layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layers were washed with water and brine, dried (Na_2SO_4), and evaporated. After purification by column chromatography (gradient 2% MeOH in CH_2Cl_2 to 20% MeOH in CH_2Cl_2), the product was dissolved in EtOAc and 0.5 M $KHSO_4$, the layers were separated and the aqueous layer was extracted with EtOAc. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The product **17** (123 mg, 59%) was obtained as yellow foam. $[\alpha]^{22}_D = -316°$ (c = 0.53, MeOH). ^{1}H NMR (CD_3OD) δ: 8.17 (m, 1H, CH), 7.94 (m, 1H, CH), 7.85 (m, 1H, CH), 7.40 (m, 1H, CH), 6.60 (m, 1H, CH), 5.59 (d, J = 16.6 Hz, 1H, CH_2), 5.29 (m, 1H, CH), 4.39 (d, J = 17.5 Hz, 1H, CH_2), 4.15 (d, J = 17.5 Hz, 1H, CH_2), 4.08 (d, J = 16.6 Hz, 1H, CH_2), 3.20 (dd, J = 6.5, 15.0 Hz, 1H, CH_2), 2.89 (dd, J = 7.6, 15.0 Hz, CH_2), 1.61 (s, 9H, 3 × CH_3). ^{13}C NMR (CD_3OD) δ: 172.4 (C), 172.2 (C), 153.9 (C), 148.2 (C), 140.1 (C), 138.5 (CH), 138.4 (C), 127.5 (CH), 126.2 (CH), 119.5 (C), 116.8 (CH), 116.5 (CH), 87.3 (C), 54.5 (CH), 53.1 (CH_2), 49.8 (CH_2), 29.8 (CH_2), 28.1 (CH_3). LC-MS ($M + H^+$): 446.0. Anal. ($C_{20}H_{23}N_5O_7 \cdot H_2O$) C, H, N.

1-(*tert*-Butoxycarbonyl)-5-[(2*S*)-4-carboxymethyl-7-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-oxo-2,3,4,5-tetrahydro-1*H*-benzo[1,4]diazepin-2-ylmethyl]imidazole (18). HCO_2NH_4 (128 mg, 2.03 mmol) was added to a mixture of Pd/C (7 mg 10%, 7 μmol) and **17** (30 mg, 0.07 mmol) in 1 mL of MeOH. The reaction, was performed under nitrogen in a sealed tube, and was complete within 1.5 h according to TLC (20% MeOH in CH_2Cl_2). More MeOH was added, and the Pd/C was removed by filtration. After evaporation, the crude product was immediately suspended in 2 mL of dioxane and cooled to 0 °C. FmocCl (26 mg, 0.10 mmol) was added, followed by dropwise addition of 1 mL of 10% aqueous Na_2CO_3, pH 8−9. After 1.5 h, ether and water were added. The layers were separated, and the aqueous layer was washed with ether until TLC showed that all impurities with R_f values higher than that of the product had been removed. EtOAc was added to the aqueous layer, which was acidified to pH 3 using 10% citric acid. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with water, dried (Na_2SO_4), and evaporated to give **18** as white foam (14 mg, 33%). ^{1}H NMR (CD_3OD) δ: 8.12 (m, 1H, CH), 7.78 (m, 2H, 2 × CH), 7.67 (m, 2H, 2 × CH), 7.43−7.33 (m, 3H, 2 × CH and CH), 7.30 (m, 2 × CH), 7.11−6.92 (m, 2H, 2 × CH), 6.52 (m, 1H, CH), 5.49 (d, J = 16.4 Hz, 1H, CH_2), 4.97 (dd, J = 6.0, 7.7 Hz, 1H, CH), 4.51 (d, J = 17.4, 1H, CH_2), 4.41 (m, 2H, CH_2), 4.22 (m, 1H, CH), 3.90 (d, J = 17.4 Hz, 1H, CH_2), 3.86 (d, J = 16.4 Hz, 1H, CH_2), 3.13 (dd, J = 6.0, 14.8 Hz, 1H, CH_2), 2.83 (dd, J = 7.7, 14.8 Hz, 1H, CH_2), 1.61 (s, 9H, 3 × CH_3). ^{13}C NMR (CD_3OD) δ: 173.4 (C), 173.3 (C), 156.5 (C), 148.4 (C), 145.5 (C), 145.4 (C), 143.7 (C), 142.8 (C), 140.9 (C), 138.2 (CH), 130.3 (C), 129.0 (CH), 128.3 (CH), 126.3 (CH), 122.4 (CH), 122.1 (CH), 121.1 (CH), 118.5 (CH), 116.5 (CH), 87.2 (C), 67.8 (CH_2), 55.4 (CH), 53.9 (CH_2), 50.2 (CH_2), 48.6 (CH), 30.5 (CH_2), 28.9 (CH_3). Accurate mass ($C_{35}H_{35}N_5O_7$) calculated 637.2536, found 637.2519.

Ang II Analogue 1. Fmoc-His(Trt)-Pro-Phe-Wang resin (143.7 mg, 58.9 μmol) was weighed into a 2 mL reaction vessel and allowed to swell in DMF (1.5 mL) for 20 min. The Fmoc group was removed by a three-step treatment with 20% piperidine in DMF (3 × 1.5 mL, 1 + 3 + 10 min), and the polymer was then washed with DMF (6 × 1.5 mL, 6 × 1 min). Compound **12** (41.0 mg, 72.8 μmol; contaminated with the NO_2 derivative **11**) and PyBOP (37.9 mg, 72.8 μmol) were dissolved in DMF (1.0 mL) in the presence of DIEA (30.4 μL, 175 μmol) and allowed to react with the resin for 20 h. LC/MS analysis after cleavage of an analytical sample showed that the coupling was incomplete. The resin was washed with DMF (6 × 1.5 mL, 6 × 1 min) and recoupled with **12** (25.0 mg, 44.4 μmol), PyBOP (23.1 mg, 44.4 μmol) and DIEA (15.5 μL, 88.8 μmol) in DMF (1 mL) for 17.5 h. After washing with DMF (6 × 1.5 mL, 6 × 1 min), the resin was deprotected and washed as described above. Fmoc-Arg(Pbf)-OH (130.7 mg, 174 μmol) was then coupled for 17 h using PyBOP (90.5 mg, 174 μmol) and DIEA (60.6 μL, 348 μmol) in DMF (0.7 mL). Fmoc-Asp(O*t*-Bu)-OH was introduced in the same way, using a coupling time of 4 h. The peptide resin was then deprotected, washed with DMF (6 × 1.5 mL, 6 × 1 min) and CH_2Cl_2 (6 × 2 mL, open column), and dried to yield 156.2 mg.

At all stages, unless otherwise indicated, PDMS analysis of deprotected samples showed the expected *m*/*z* value for the major peak. Incomplete couplings were not observed. However, after the introduction of **12**, the C-terminal tripeptide acylated by the nitro derivative **11** was detected as an impurity in all samples. Analysis of the Fmoc protected resin after coupling of Arg and Asp showed that 2 equiv of the amino acid were initially incorporated.

Triethylsilane (100 μL) and 95% aq TFA (4 mL) were added to the partially protected peptide resin (147.2 mg), and the mixture was agitated by rotation for 2 h. The polymer was filtered off and washed with TFA (3 × 0.7 mL). The yellow filtrate was evaporated in a stream of nitrogen to ca. 4 mL and the product precipitated by the addition of ether (25 mL). After cooling in an ice-bath, the precipitate was collected by centrifugation, washed with ether (4 × 15 mL), and dried to give 53.5 mg.

The crude peptide was dissolved in a mixture of H_2O (7.6 mL) and MeCN (0.4 mL) and purified in two runs by RP-HPLC on a 10 μm Vydac C18 column (2.2 × 25 cm) with an 80 min gradient of 5−45% MeCN in 0.1% aq TFA at a flow rate of 3 mL/min. The separation was monitored at 230 nm and by PDMS and/or analytical RP-HPLC of selected fractions. The yield of the purified and lyophilized peptide was 10.7 mg (19%). PDMS (Mw 994.1): 995.4 ($M + H^+$); amino acid analysis: Asp, 0.99; Arg, 1.00; His, 1.03; Pro, 0.98; Phe, 0.99.

Ang II Analogue 2. Reaction of the starting Fmoc-His(Trt)-Pro-Phe-Wang resin (95.5 mg, 39.2 μmol) essentially as described above for **1**, although including coupling of Val, produced 109.8 mg of the partially protected peptide resin. Cleavage (103.2 mg) with TFA−H_2O−triethylsilane gave 38.7 mg of the crude peptide, which was purified according to the above procedure. Yield: 9.1 mg (23%); PDMS (Mw 1093.2): 1095.2 ($M + H^+$); amino acid analysis: Asp, 0.99; Arg, 0.99; Val, 0.98; His, 1.01; Pro, 1.01; Phe, 1.00.

Ang II Analogue 3. This compound was similarly prepared from the same starting resin (31.9 mg, 25.6 μmol) by consecutive coupling of Val, Ala, and Asp. Cleavage of the partially protected peptide resin (42.1 mg) and purification by RP-HPLC produced 10.1 mg (39.1%) of the desired product. PDMS (Mw 1008.1): 1008.8 ($M + H^+$); amino acid analysis: Asp, 1.00; Ala, 0.99; Val, 1.00; His, 1.02; Pro, 1.00; Phe, 0.99.

Ang II Analogue 4. Fmoc-Phe-Wang resin (35.5 mg, 35.5 μmol) was deprotected and allowed to react with **18** (27.0 mg, 42.3 μmol), PyBOP (22.0 mg, 42.3 μmol), and DIEA (18.4 μL, 106 μmol) in DMF (0.9 mL) for 26 h. Fmoc-Tyr(*t*-Bu)-OH (64.3 mg, 140 μmol) was coupled with the aid of PyBOP (72.8 mg, 140 μmol) and DIEA (48.8 μL, 280 μmol) in DMF (0.7 mL) for 22 h. The same conditions were used for the remaining amino acids, but with reaction times of 2−2.5 h. The deprotected, washed, and dried peptide resin weighed 53.3 mg. TFA cleavage (48.1 mg) in the presence of H_2O and triethylsilane for 2 h gave 23.3 mg of the crude product.

PDMS analysis showed the expected *m*/*z* value for the major peak and, in addition, the presence of an impurity corresponding to the deletion peptide Asp-Arg-Val-Tyr-Phe. Since the two peptides seemed to coelute on the C18 column, a two-stage purification procedure was applied. The crude product (12.0 mg) was dissolved in 0.1% aq TFA containing 25% MeCN (2 mL) and applied onto a cation exchange column (Dyno NeoBar CS4, 0.65 × 6 cm, sodium form). The product was eluted using a gradient of NaCl (0−0.4 M in 30 min) in the same buffer at a flow rate of 3 mL/min and with UV detection (254 nm). The fractions corresponding to the major peak were pooled and evaporated to dryness in a vacuum. The residue was redissolved in 0.1% aq TFA (2.5 mL) and further purified by RP-HPLC on a Vydac C18 column (1 × 25 cm) using a flow rate of 3 mL/min and an 80-min gradient of 10−50% MeCN in 0.1% TFA. The separation was monitored at 230 nm and by PDMS and RP-HPLC of selected fractions. Yield of pure **4**: 1.9 mg (12%); PDMS (Mw 996.1): 997.7 ($M + H^+$); amino acid analysis: Asp, 1.02; Arg, 0.99; Val, 0.99; Tyr, 1.01; Phe, 0.99.

Rat Liver Membrane AT_1 Receptor Binding Assay. Rat liver membranes were prepared according to the method of Dudley et al.[37] Binding of [^{125}I]Ang II to membranes was conducted in a final volume of 0.5 mL containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA (bovine serum albumin), liver homogenate corresponding to 5 mg of the original tissue weight, [^{125}I]Ang II (80 000 cpm, 0.03 nM) and variable concentrations of test substance. Samples were incubated at 25 °C for 2 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets using a Brandel cell harvester. The filters were washed with 3 × 3 mL of Tris-HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a γ-counter. The characteristics of the Ang II binding AT_1 receptor were determined using six different concentrations (0.03−5 nmol/L) of labeled [^{125}I]Ang II. Nonspecific binding was determined in the presence of 1 μM Ang II. The specific binding was determined by subtracting the nonspecific binding from the total bound [^{125}I]Ang II. The dissociation constant ($K_d = 1.7 \pm 0.1$ nM, [L] = 0.057 nM) was determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK). The binding data were best fitted with a one-site fit. All experiments were performed in triplicate. K_i values were calculated using the Cheng and Prusoff equation ($K_d = 1.7432$).

Porcine (Pig) Myometrial Membrane AT_2 Receptor Binding Assay. Myometrial membranes were prepared from porcine uteri according to the method of Nielsen et al.[38] Potential interference by binding to AT_1 receptors was blocked by addition of 1 μM losartan. Binding of [^{125}I]Ang II to membranes was conducted in a final volume of 0.5 mL containing 50mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA, homogenate corresponding to 10 mg of the original tissue weight, [^{125}I]-Ang II (80 000 cpm, 0.03 mM), and variable concentrations of test substance. Samples were incubated at 25 °C for 1.5 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets using a Brandel cell harvester. The filters were washed with 3 × 3 mL of Tris-HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a γ-counter. The characteristics of the Ang II binding AT_2 receptor were determined using six different concentrations (0.03−5 nmol/L) of labeled [^{125}I]Ang II. Nonspecific binding was determined in the presence of 1 μM Ang II. The specific binding was determined by subtracting the nonspecific binding from the total bound [^{125}I]Ang II. The dissociation constant ($K_d = 0.7 \pm 0.1$ nM, [L] = 0.057 nM) was determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK). The binding data were best fitted with a one-site fit. All experiments were performed in triplicate. K_i values were calculated using the Cheng−Prusoff equation ($K_d = 0.7292$).

Conformational Analysis and Molecular Modeling. The conformational search was performed on structures **1m**, **2m**, and **6m** using the MMFFs force field as implemented in the program MacroModel 7.1.[40] The General Born Solvent Accessible (GB/SA) surface area method for water developed by Still et al.[39] was used in all calculations. Amide bonds were fixed in the trans configuration and the number of torsion angles allowed to vary simultaneously during each Monte Carlo step ranged from 1 to n - 1 where n equals the total number of rotatable bonds ($n = 17$ for **6m**, $n = 13$ for **1m**, and $n = 16$ for **2m**). Conformational searches were conducted using the Systematic Unbound Multiple Minimum (SUMM) search method[51] in the batchmin program. 5000 steps per rotatable bond were used. Conformations within 5 kcal/mol of the lowest found minimum were kept. The ring closure bond was defined as the bond between C_β and the sulfur atom of the *i* residue of the γ-turn in **6m** and the bond between N and C_α of the Ala residue. Torsional memory and geometric preoptimization were used. Truncated Newton Conjugate Gradient (TNCG) minimization with a maximum of 100 iterations was used in the conformational search, with derivative convergence set to 0.05 kJ/mol/Å. A maximum of 500 iterations of TNCG was used in the subsequent refinement minimization, with the convergence criteria set to 0.001 kJ/mol/Å and only the key atoms

defined by a star in Figure 1 were used as comparison atoms (COMP). Conformations within 5 kcal/mol from the lowest found minimum were saved. Molecular modeling and comparisons were performed in Sybyl 6.9.[52]

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(44) A γ-turn is defined by a three-residue turn forming a pseudo seven-membered ring. Often a hydrogen bond is formed between the carbonyl of the i residue and the amide NH of the $i+2$ residue. Two types of γ-turns exist, defined as inverse or classic. The inverse γ-turn with Φ_i+1 and Ψ_i+1 values corresponding to $-70°-(-85°)$ and $60°-70°$ and the classic with Φ_i+1 and Ψ_i+1 values corresponding to $70°-85°$ and $-60°-(-70°)$. The more common inverse γ-turn is identified by an equatorial $i+1$ side-chain orientation, while the classic γ-turn contains an axial $i+1$ side-chain.
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