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Bioorganic & Medicinal Chemistry 14 (2006) 5963–5972
Bioorganic & Medicinal Chemistry

Short pseudopeptides containing turn scaffolds with high AT_2 receptor affinity

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Received 19 December 2005; revised 4 May 2006; accepted 15 May 2006
Available online 5 June 2006

Abstract—Two pentapeptides, Ac-Tyr-Ile-His-Pro-Phe/Ile, were synthesized and shown to have angiotensin II AT_2 receptor affinity and agonistic activity. Based on these peptides, a new series of 13 pseudopeptides was synthesized via introduction of five different turn scaffolds replacing the Tyr-Ile amino acid residues. Pharmacological evaluation disclosed subnanomolar affinities for some of these compounds at the AT_2 receptor. Substitution of Phe by Ile in this series of ligands enhanced the AT_2 receptor affinity of all compounds. These results suggest that the C-terminal amino acid residues can be elaborated on to enhance the AT_2 receptor affinity in truncated Ang II analogues.

1. Introduction

The octapeptide angiotensin II (Ang II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is the endogenous ligand for the AT_1- and AT_2-receptors. Both of these receptors are G-protein coupled. They differ considerably by sharing only 32–34% sequence homology,[1,2] and by having different signaling pathways as well as exerting different physiological effects.[3,4] Vasoconstriction, aldosterone release, and stimulation of sympathetic transmission, and cellular growth are generally attributed to AT_1 receptor activation.[3–5] The role of the AT_2 receptor is less clear, largely because of its low level of expression in adults. The AT_2 receptor is the predominant angiotensin receptor in fetal tissues, but is rapidly down-regulated after birth, suggesting an involvement in fetal development.[6] Importantly, it is up-regulated in certain pathological conditions such as heart failure, myocardial infarction, brain lesions, vascular injury, and wound healing.[3,4,7,8] It has also been demonstrated that AT_2 receptor activation mediates antiproliferation, cellular differentiation, programmed cell death (apoptosis), and even vasodilation.[3,4,7] Furthermore, agonistic activation of the AT_2 receptor in neuronal cells induces neurite outgrowth and elongation, promotes cellular migration, and modulates neuronal excitability.[9] The mechanism of action involves activation of the nitric oxide/guanyl cyclase/cGMP pathway[10] and a sustained increase in $p42/p22^{mapk}$ activity.[11]

Losartan was the first selective non-peptide AT_1 receptor antagonist on the market, launched in 1994 as an anti-hypertensive drug and developed from a non-peptide lead.[12,13] Recently, our group reported the first selective non-peptide AT_2 receptor agonist.[14] It has been proposed that such AT_2 receptor agonists might find a role as a therapeutic agent in the cardiovascular area since it has been demonstrated that AT_2 receptor activation affects cardiac remodeling and leads to reduced blood pressure.[7,15] The selective AT_2 receptor agonists originate from an agonist (L-162,313) that is structurally related to the sartans and

Keywords: Angiotensin II; AT1 receptor; AT2 receptor; AT2 receptor agonist; Pseudopeptides; Turn scaffolds.
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doi:10.1016/j.bmc.2006.05.019

that activates both the AT_1 receptor[16,17] and the AT_2 receptor[18]. Hence, the receptor-selective AT_2 agonists[14] and the AT_1 antagonists[13] were developed by classical medicinal chemistry approaches from non-peptide leads. An alternative way to produce novel lead series would be to start from the bioactive peptide and stepwise remove structural elements less crucial for receptor activation and at the same time strengthen important ligand receptor interactions.[19] To make peptidomimetics by such an iterative procedure is a challenging task. Nevertheless, in recent years a number of elegant examples have been disclosed.[20–22] We have been intrigued by and are addressing the question whether Ang II can be converted to low-molecular weight drug-like AT_2 receptor agonists via a stepwise process.

The ligand binding interactions with the AT_1- and AT_2-receptors seem to be different. The AT_1 receptor is sensitive to fairly small changes in the amino acid sequence of Ang II. For example, substitution of Phe^8 by Ile^8 results in an AT_1 receptor antagonist.[23] The AT_2 receptor, on the contrary, seems to be more tolerant both toward structural alterations in Ang II[24,25] and also with regard to truncation of the peptide as de Gasparo et al. reported in a study from 1991.[26] In that study, it was found that the acetylated pentapeptides Ac-Tyr-Val-His-Pro-Phe and Ac-Tyr-Val-His-Pro-Ile both bound to the AT_2 receptor with high selectivity and with K_i values in the nanomolar range.[26] Unfortunately, no data were disclosed concerning the agonistic and/or antagonistic properties of these compounds.

We herein report that pentapeptide derivatives of Ang II, with either an N-terminal comprising an acetyl group (**1**) or a free amine (**3**), are high-affinity AT_2 receptor ligands (Chart 1 and Table 1). Substitution of the C-terminal Phe by Ile in these pentapeptides results in **2** and **4**, which both have higher AT_2 receptor affinities, as compared to **1** and **3**, respectively. We also report that compounds **1** and **2** exerted agonistic effects at the AT_2 receptor based on their ability to induce neurite outgrowth. Furthermore, we disclose that highly potent AT_2 receptor ligands can be obtained starting from these pentapeptides by substitution of the dipeptide fragment Tyr-Ile of **1**–**4** with properly substituted benzodiazepine or diphenylmethane scaffolds, that is, **A**, **B**, **C**, **D**, or **E** (Chart 2). Two of these pseudopeptides were also shown to be AT_2 receptor agonists.

Table 1. Binding affinities for the AT_1- and AT_2-receptors

Compound	K_i (nM) ± SEM	
	AT_1 (rat liver membranes)	AT_2 (pig uterus zmyometrium)
Ang II	0.95	0.2
[4-NH_2-Phe^6]Ang II	>10,000	0.7
1	>10,000	37.9 ± 2.5
2	>10,000	3.0 ± 0.2
3	31.0 ± 1.7	11.0 ± 1.3
4	>10,000	4.2 ± 0.2
5	>10,000	23.9 ± 1.3
6	>10,000	114.6 ± 2.7
7	>10,000	12.1 ± 0.8
8	9.4 ± 0.7	9.2 ± 0.2
9	>10,000	12.3 ± 1.5
10	195.9 ± 5.1	24 ± 2.2
11	>10,000	38.7 ± 1.2
12	566.6 ± 17.4	102.7 ± 1.1
13	27.7 ± 1.8	0.8 ± 0.02
14	>10,000	2.5 ± 0.1
15	>10,000	0.5 ± 0.03
16	>10,000	21.8 ± 0.7
17	>10,000	0.7 ± 0.04

2. Results

2.1. Chemistry

The turn scaffolds **A**, **B**, **C**, and **E** either N-acetylated or with a free N-terminal were synthesized and introduced

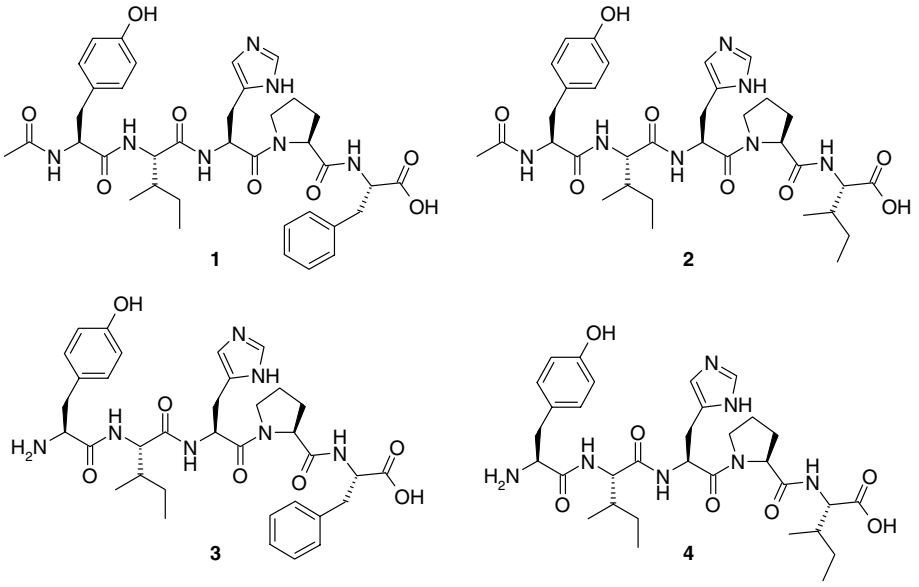


Chart 1.

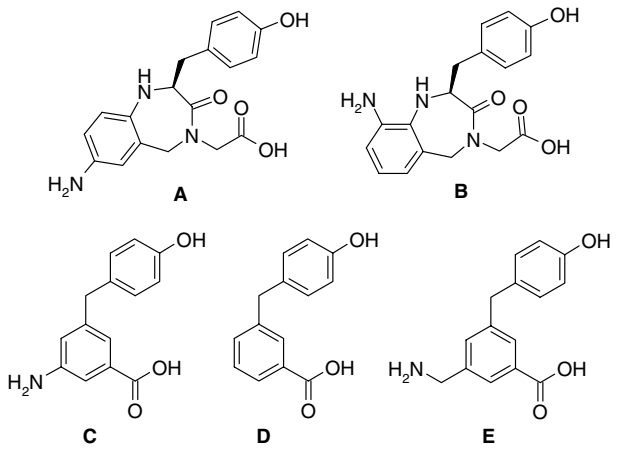
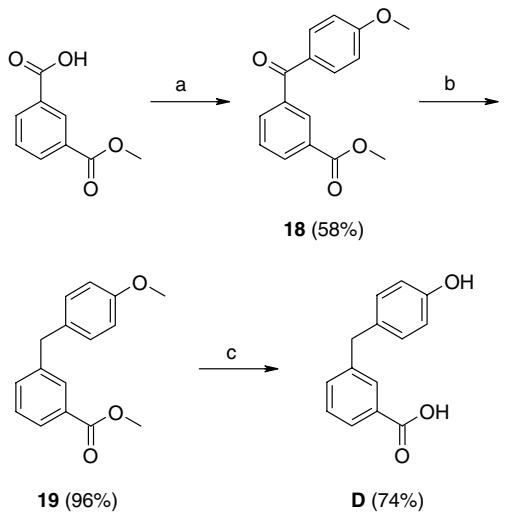


Chart 2.

instead of Tyr-Ile in **5**–**17** (Chart 3). The synthetic routes to the benzodiazepine γ-turn scaffolds **A**[27] and **B**,[28] and the aromatic γ-turn scaffolds **C**,[29] and **E**[29] were previously published. The synthesis of scaffold **D** was performed as outlined in Scheme 1. Compound **18** was prepared by a Friedel–Crafts acylation of anisole with the acid chloride of the commercially available monomethyl isophthalate. The acylation gave *ortho* and *para* regioisomers where the major product was found to be the *para* substituted **18**.[30] Separation of the two regioisomers was conducted with column chromatography and 58% of **18** was isolated. The subsequent reduction of the carbonyl group of **18**, to afford **19**, was performed under acidic conditions with triethylsilane as hydride source.[31] The deprotection of the methoxy group was accomplished with boron trifluoride dimethylsulfide complex in DCM.[32] Without any further purification the mild hydrolysis was conducted with LiOH in a mixture of THF/H_2O/MeOH to yield compound **D**.

The peptides **1**–**4** and the pseudopeptides **5**–**17** were prepared using standard Fmoc-strategy solid-phase peptide synthesis (SPPS) with Phe-Wang or Ile-Wang resins as



Scheme 1. Reagents and conditions: (a) i—$SOCl_2$, reflux; ii—anisole, $AlCl_3$, DCM; (b) $(H_3CH_2C)_3SiH$, F_3CSO_3H, TFA; (c) i—$BF_3{\cdot}S(CH_3)_2$, DCM; ii—LiOH, THF/MeOH/H_2O.

outlined in Scheme 2. The subsequent Fmoc-protected amino acids were coupled using either HBTU or PyBOP as coupling reagent. Piperidine in DMF was used for deprotection of the N-terminal. Scaffolds **A**, **B**, **C**, **D**, and **E** (**A**, **B**, **C**, and **E** were N-terminal Fmoc-protected) were introduced with the identical coupling conditions as above but with a prolonged reaction time. The pseudopeptides containing an acetylated N-terminal were finally coupled to acetic acid, again using standard conditions. The final cleavage of the products from the resin was performed using 95% aq TFA.

2.2. Receptor affinity studies

Compounds **1**–**17** were evaluated in radioligand binding assays relaying on displacement of [^{125}I]Ang II from AT_1 receptors in rat liver membranes[33] and from AT_2 receptors in pig uterus membranes[34] (Table 1). Ang II and [4-NH_2-Phe^6]Ang II were used as reference substances. The results are given as K_i values in Table 1.

The pentapeptides **1**–**4** all exhibit nanomolar affinity for the AT_2 receptor. In addition, peptide **3** exerts affinity for the AT_1 receptor. All of the pseudopeptide ligands **5**–**17** displayed nanomolar affinity for the AT_2 receptor and exhibited K_i values ranging from 0.5 nM to 114.6 nM. Four of the compounds (**8**, **10**, **12**, and **13**) are non-selective and their AT_1 receptor affinities range from K_i = 9.4 nM to 567 nM.

2.3. In vitro morphological effects induced by compounds 1, 2, 8, and 15 in NG 108-15 cells

To study the effects of compounds **1**, **2**, **8**, and **15** on cell differentiation, NG108-15 cells were used. In their undifferentiated state, neuroblastoma × glioma hybrid NG108-15 cells have a rounded shape and divide actively. It has previously been shown by us that these cells express only the AT_2 receptor[35,36] and that a three-day treatment with Ang II or the selective peptidic AT_2 receptor agonist CGP 42112A induces neurite outgrowth.[36]

Compounds **1**, **2**, **8**, and **15** were first tested at various concentrations ranging from 1 pM to 1 μM and none of the doses induced cell death. When the cells were stimulated for three days with each of the compounds, they induced neurite outgrowth. The 3-day treatment increased the number of cells with neurites longer than one cell body from 8.32 ± 0.29% in control cells to 17.29 ± 0.13%, 17.85 ± 0.55%, 18.21 ± 0.12%, and 16.28 ± 0.69% in the cells treated with compound **1** (0.01 μM), **2** (0.01 μM), **8** (0.1 μM), and **15** (0.01 μM), respectively (Fig. 1). This effect was mediated through the AT_2 receptor, since co-incubation of **1**, **2**, **8**, and **15** with the AT_2 receptor antagonist, PD123,319 (10 μM), virtually halted neurite elongation. We have previously shown that PD123,319 administered alone does not alter the morphology of the untreated cells.[11,36]

2.4. Rabbit thoracic aorta strips, AT_1 functional assay

The effect of compound **8** was tested at six different concentrations ranging from 10 nM to 3 μM at the AT_1

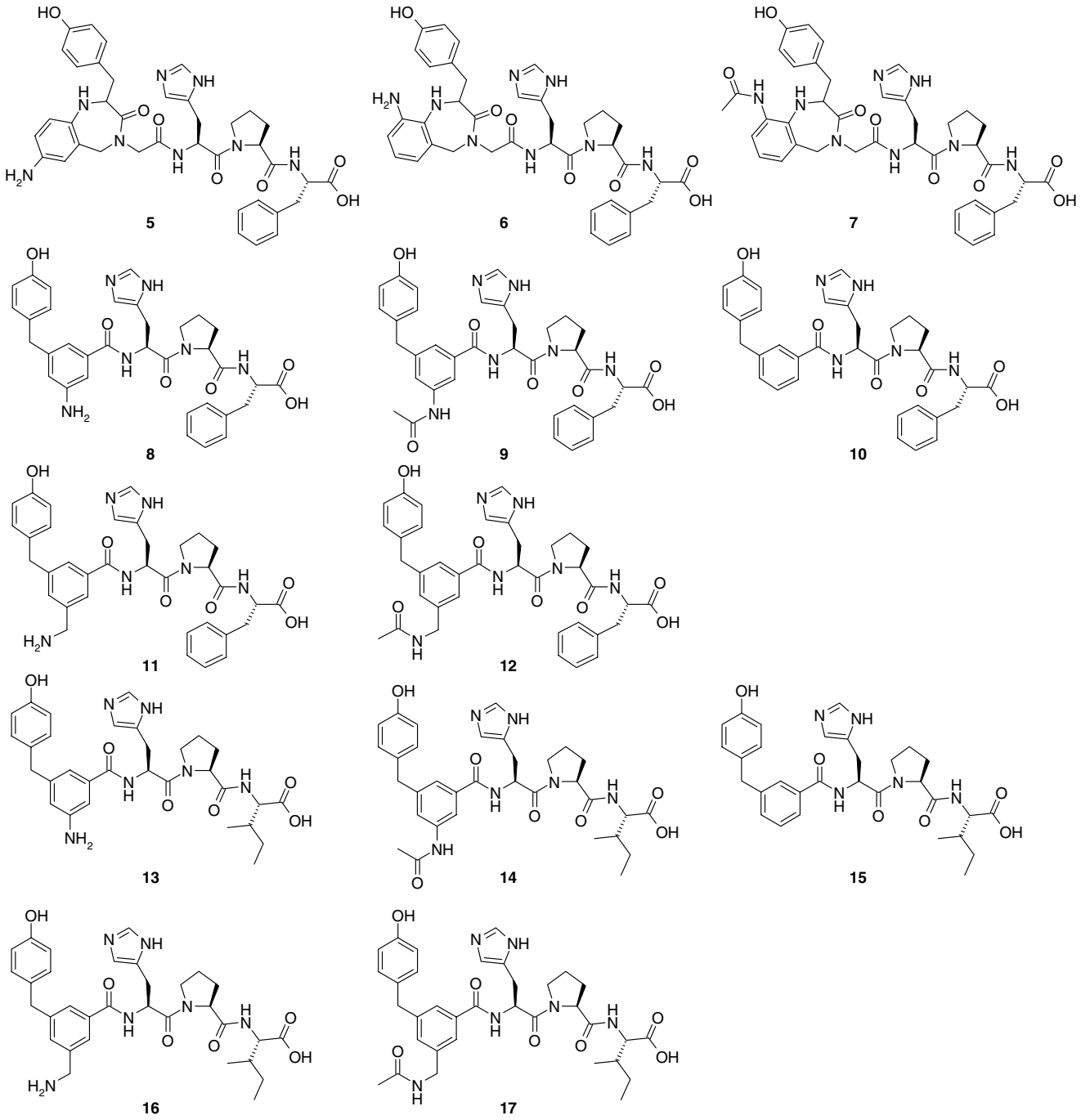
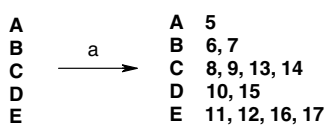


Chart 3.

receptor using the rabbit aorta strips according to the literature procedure.[37] At these experimental settings compound **8** did neither induce contraction nor inhibit Ang II induced contraction at concentrations as high as 3 μM.



Scheme 2. Reagents: (a) i—His-Pro-Phe-Wang or His-Pro-Ile-Wang resins, HBTU or PyBOP, DIEA, DMF; ii—20% piperidine in DMF; iii—95% aq TFA.

3. Discussion

To develop a bioactive and less peptide-like molecule affecting a certain peptide receptor utilizing the endogenous ligand as lead structure is an intricate task. One approach is to determine the pharmacophore elements of the peptide and then their 3D-relationship. Some of the tools used to determine the pharmacophore elements are truncation of the peptide and Ala-scans. The 3D-relationship can be studied using, for example,

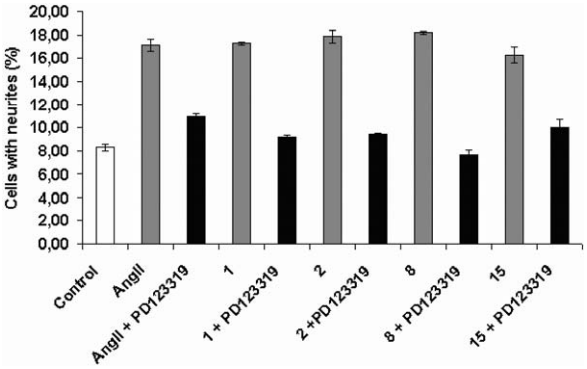


Figure 1. The effect on neurite outgrowth in NG108-15 cells. White, untreated cells; grey, cells treated with Ang II, **1**, **2**, **8** or **15**; black, cells treated with the same compounds in combination with the AT_2 receptor antagonist, PD 123,319. Cells with at least one neurite longer than a cell body were counted as positive for neurite outgrowth. The number of cells with neurites represents the percentage of the total number of cells in the micrographs (at least 290 cells according to the experiment). Concentrations used in the assay: 0.1 μM of Ang II, 0.01 μM of **1**, 0.01 μM of **2**, 0.1 μM of **8**, 0.1 μM of **15**, and 10.0 μM of PD 123,319.

cyclization of the peptide followed by replacement of these cyclic fragments for well-defined secondary structure mimics.[19]

De Gasparo et al. synthesized the truncated pentapeptide analogues Ac-Tyr-Val-His-Pro-Phe and Ac-Tyr-Val-His-Pro-Ile of Ang II and showed that they had binding affinity to AT_2 receptors.[26] We were intrigued by this finding and decided to resynthesize these compounds, although using Ile (compounds **1** and **2** as in Ang II) instead of Val. We also synthesized the compounds with a free N-terminal group (compounds **3** and **4**) to investigate the importance of the acetyl group and the charge in this position. Compounds **1**–**4** were evaluated in the AT_1 and AT_2 receptor binding assays and compounds **1** and **2** in the agonist assay using NG 108-15 cells. All four compounds showed affinity to the AT_2 receptor with K_i values ranging between 3.0 nM and 37.9 nM (Table 1). Peptides **2** and **4** containing the C-terminal Ile residue had somewhat higher affinity as compared to the Phe analogues, which was also observed by de Gasparo et al. Furthermore, the charged and acetylated N-terminal groups had about the same affinity. Finally, both compounds **1** and **2** were shown to be AT_2 receptor agonists. It was therefore foreseen that these truncated analogues could be an excellent starting point in the search for less peptidic AT_2 receptor agonists.

Previous studies including 3–5 disulfide monocyclizations and other types of cyclizations have provided support for the hypothesis that Ang II adopts a γ-turn-like conformation in the Val^3-Tyr^4-Ile^5 region when binding to AT_2 receptors.[27,39] A series of γ-turn mimicking scaffolds (**A**, **B**, **C**, and **E**) have therefore been incorporated into Ang II to replace the Val-Tyr-Ile and Tyr-Ile residues.[27,29,38,40] The benzodiazepine based scaffolds **A** and **B** mimic a γ-turn well but they are synthetically very demanding to prepare.[27,38] The aromatic scaffolds **C** and **E** on the other hand are easily accessible and position the N- and C-terminal in a correct position relative to each other as compared to a γ-turn.[29,40] Since previous studies have shown that all of these scaffolds can substitute Tyr-Ile in Ang II with retained AT_2 receptor affinities, we planned to further reduce the peptide character of the pentapeptides by introducing these scaffolds in this position.

Scaffolds **A**, **B**, **C**, and **E** were introduced in the pentapeptides to give compounds **5**, **6**, **8**, and **11** which all showed nanomolar affinity for the AT_2 receptor (Table 1). Interestingly, the basicity of the N-terminal does not appear to be critical for good affinity since, for example, the free benzyl amine **11** (calcd $pK_a \sim 9.2$) has a K_i of 38.7 nM and the aniline **8** (calcd $pK_a \sim 3.5$) a K_i of 9.2 nM. The corresponding acetylated compounds (**7**, **9**, and **12**) were also synthesized for comparison (except for scaffold **A**). Compound **7** gained 10-fold in affinity (K_i = 12.1 nM) compared to its non-acetylated equivalent **6**. On the other hand, **9** exhibits a K_i of 12.3 nM, which is comparable to the free aniline **8**. The affinity of **12** (K_i = 102.7) dropped by a factor of 3 as compared to that of **11** (K_i = 38.7). Apparently, the N-terminal end can be both neutral and charged, and still good affinities are obtained. We therefore incorporated scaffold **D**, where the nitrogen is absent, in the Tyr-Ile position. Interestingly, compound **10** displayed a K_i value of 24 nM showing that the N-terminal nitrogen is not necessary for binding. The eight pseudopeptides (**5**–**12**) discussed so far all showed good affinity to the AT_2 receptor with K_i values ranging from 9.2 nM to 114.6 nM.

The results of peptides **1**–**4** revealed that substitution of Phe in the C-terminal for Ile afforded ligands with up to 10-fold higher affinities. Therefore, a series of pseudopeptides encompassing Ile in the C-terminal were synthesized. Because of their simplicity and synthetic accessibility the aromatic scaffolds **C**, **D**, and **E** were chosen for incorporation in the Tyr-Ile part. As expected, compounds **13**–**17** exhibited better affinity for the AT_2 receptor than their Phe equivalents. It is interesting to note that regardless of which scaffolds were used and whether the N-terminal is free, acetylated or not present, the AT_2 receptor affinities were enhanced by 2–145 times for the series of Ile analogues as compared to the corresponding Phe analogues. Thus, the present data suggest that the C-terminal isoleucine side chain is a key residue that promotes enhanced AT_2 receptor affinity and selectivity toward the AT_1 receptor. However, the importance of the N-terminal appears to be of less significance. In fact, the Ile analogues **13**, **15**, and **17** are all equipotent with Ang II.

Compound **8** was selected for functional studies using both the AT_2 receptor agonist assay and the rabbit aorta strip AT_1 receptor assay because it had affinity to both these receptors. A compound with a pharmacological profile corresponding to both an AT_1 receptor antagonist, and AT_2 receptor agonist should, for example, be interesting as a therapeutic agent in the cardiovascular area (see Section 1).[7,15] The functional assays revealed

that compound **8** had AT_2 receptor agonistic properties but neither agonistic nor antagonistic activities were detected at the AT_1 receptor. Compound **15** was also evaluated as an AT_2 receptor agonist because of its high affinity and peptidomimetic character and was also shown to be an agonist. Taken together, the identification of compounds **8** and **15** has led to the discovery of less peptidic AT_2 receptor agonists and opens up for further developments of drug-like compounds.

Concerning the AT_1 receptor very few truncated peptide analogues of Ang II have shown affinity to this receptor.[41] It was therefore very surprising that the pentapeptide **3** had a K_i of 31 nM. It has also been very difficult to introduce secondary structure mimetics in Ang II and preserve the affinity. The AT_1 receptor binding affinities obtained with **8**, **10**, **12**, and **13** were therefore much unexpected. These compounds are anticipated to further elucidate the recognition requirements for AT_1 receptor ligands.

4. Conclusion

In summary, a new class of ligands for the Ang II AT_2 receptor is presented. Initially, four pentapeptides were synthesized of which two were confirmed to be agonists. Subsequently, five tyrosine-based scaffolds encompassing a benzodiazepine or a benzene ring were introduced N-terminally to generate a range of pseudopeptides with high affinity to the AT_2 receptor. C-terminal substitution of Phe by Ile in this series of ligands enhanced the AT_2 receptor affinity of all compounds. Three of these pseudopeptides (**13**, **15**, and **17)** were equipotent to the endogenous ligand Ang II. Furthermore, it was shown that the N-terminal nitrogen was not needed obtaining high AT_2 receptor affinity. Four of the compounds **1**, **2**, **8** and **15** were selected for examination in a functional assay and all were found to act as AT_2 receptor agonists.

5. Experimental

5.1. General

^{1}H NMR and ^{13}C NMR were obtained on a JEOL JNM-400 spectrometer or on a Varian Mercury plus spectrometer (^{1}H at 400 MHz, ^{13}C at 100.6 MHz). Spectra were recorded at ambient temperature unless otherwise stated. Chemical shifts are reported as δ values (ppm) referenced to δ 7.26 ppm for $CHCl_3$, δ 77.0 ppm for $CDCl_3$, δ 2.05 ppm and δ 29.84 ppm for acetone-d_6 and δ 1.94 and 1.32 ppm for MeCN-d_3. GC–MS was performed with an electron impact (70 eV) mass–selective detector, and an HP-1 capillary column using a 70–305 °C temperature gradient. The preparative RP-HPLC column used was a 10 μm Vydac C18 column (22 mm × 250 mm) with a mobile phase of MeCN in 0.1% aqueous TFA at a flow rate of 5 ml/min. Analytical RP-LC/MS was performed on a Gilson HPLC system with a Finnigan AQA quadropole mass spectrometer using a Chromolith Performance RP–18 4.6 mm × 100 mm column (Merck) at a flow rate of 4 mL/min. The pseudopeptide purities were determined on a Thermo Hypersil HyPurity C4, 5 μm, 4.6 mm × 50 mm column and a ACE 5 C18, 4.6 mm × 50 mm column. MikroKemi AB, Uppsala, Sweden or Analytische Laboratorien, Industriepark Kaiserau, Lindlar, Germany performed elemental analyses. Molecular masses (HRMS) were determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source. Column chromatography was performed using commercially available silica gel 60 (particle size: 0.040–0.063 mm Merck). Thin–layer chromatography was performed with aluminum sheets coated with silica gel 60 F_{254} (0.2 mm, E. Merck) and the analytes were visualized with UV-light. Dichloromethane used in the reactions was distilled over calcium hydride. Other chemicals were used without further purification. The AT_1 functional assay was performed at Cerep, France.

5.2. 3-(4-Methoxybenzoyl)-methyl benzoate (18)

Monomethyl isophthalate (4.78 g, 26.5 mmol) was refluxed in 35 mL thionyl chloride for 1 h. The thionyl chloride was evaporated under reduced pressure. An aliquot of the crude product (1.35 g, 6.80 mmol) was dissolved in 15 ml of dry DCM. $AlCl_3$ (2.72 g, 20.4 mmol) was suspended in 10 ml of dry DCM and cooled to 0 °C. The acid chloride solution was added slowly to the cooled reaction mixture and was stirred at 0 °C for 45 min. Anisole (2.2 mL, 20.4 mmol) was added dropwise and the reaction mixture was stirred at room temperature over night. The reaction mixture was poured into a mixture of 100 g ice and 100 mL of 1 M HCl and extracted with DCM. The organic phase was washed with $NaHCO_3$, H_2O and brine, dried over Na_2SO_4 and evaporated. After subsequent purification by column chromatography (DCM/isohexane 3:2), **18** (1.06 g, 58%) was isolated as a clear oil. ^{1}H NMR ($CDCl_3$) δ: 8.39 (ddd, J = 1.8, 1.7, 0.7 Hz, 1H, CH), 8.24 (ddd, J = 7.9, 1.7, 1.3 Hz, 1H, CH), 7.96 (ddd, J = 7.7, 1.8, 1.3 Hz, 1H, CH), 7.82 (m, 2H, 2× CH), 7.58 (ddd, J = 7.9, 7.7, 0.7 Hz, 1H, CH), 6.98 (m, 2H, 2× CH), 3.94 (s, 3H, CH_3), 3.90 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 194.6 (CO), 166.4 (CO), 163.5 (C), 138.6 (C), 133.8 (CH), 132.7 (CH), 132.6 (2× CH), 130.7 (CH), 130.2 (C), 129.6 (C), 128.5 (CH), 113.8 (2× CH), 55.5 (CH_3), 52.4 (CH_3). MS m/z (70 eV) 270 (M^+). Anal. ($C_{16}H_{14}O_4$) C, H, N.

5.3. 3-(4-Methoxy-benzyl)-methyl benzoate (19)

A solution of **18** (1.8 g, 6.66 mmol) in 40 mL of dry DCM was cooled to 0 °C and TFA (5.1 mL, 66.6 mmol) and triflic acid (15 μL, 0.17 mmol) was added. Triethylsilane (3.2 mL, 20.0 mmol) was added dropwise from an addition funnel to the solution. The ice bath was removed and the reaction mixture was stirred at ambient temperature over night. The reaction mixture was washed with 1 M NaOH and brine, dried over $MgSO_4$ and evaporated. Compound **19** (1.64 g, 96%) was isolated as a clear oil. ^{1}H NMR ($CDCl_3$) δ: 7.89–7.86 (m, 2H, 2× CH), 7.36 (m, 1H, CH), 7.35 (m, 1H, CH), 7.10 (m, 2H, 2× CH), 6.84 (m, 2H, 2× CH), 3.97 (m, 2H, CH_2), 3.90 (s, 3H, CH_3), 3.78 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 167.2 (CO), 158.1 (C), 141.9 (C), 133.4 (CH), 132.6

(C), 130.3 (C), 129.9 (CH), 129.8 (2× CH), 128.5 (CH), 127.3 (CH), 114.0 (2× CH), 55.2 (CH_3), 52.1 (CH_3), 40.8 (CH_2). MS *m/z* (70 eV) 256 (M^+). Anal. ($C_{16}H_{16}O_3$) C, H, N.

5.4. 3-(4-Hydroxybenzyl)-benzoic acid (D)

Compound **19** (1.3 g, 5.1 mmol) was dissolved in dry DMC (100 mL). The reaction vessel was fitted with a septum, cooled in an ice bath, and $BF_3{\cdot}S(CH_3)_2$ (1.4 mL, 12.7 mmol) was added slowly. The reaction mixture was allowed to reach ambient temperature and was left stirring over night. It was poured into 1 M HCl aqueous (100 mL) and extracted with DCM. The organic phase was washed with H_2O and brine, dried with $MgSO_4$ and evaporated. The crude product (5.1 mmol) was dissolved in THF (100 mL) and MeOH (12.5 mL) and H_2O (25 mL) was added. An 1 M aqueous solution of LiOH (25 mL) was added dropwise and the reaction was stirred over night. The reaction mixture was concentrated and then portioned between 5 N NaOH and ethyl acetate. The aqueous phase was acidified with concd HCl and extracted with ethyl acetate. The organic phase was washed with H_2O and brine, dried with $MgSO_4$ and evaporated to give **D** (864 mg, 74%) as an off-white solid. ^{1}H NMR (acetone-d_6) δ: 11.22 (br s, 1H), 8.20 (br s, 1H), 7.89–7.86 (m, 2H, 2× CH), 7.36 (m, 1H, CH), 7.35 (m, 1H, CH), 7.10 (m, 2H, 2× CH), 6.84 (m, 2H, 2× CH), 3.97 (m, 2H, CH_2), 3.90 (s, 3H, CH_3), 3.78 (s, 3H, CH_3). ^{13}C NMR (acetone-d_6) δ: 167.2 (CO), 158.1 (C), 141.9 (C), 133.4 (CH), 132.6 (C), 130.3 (C), 129.9 (CH), 129.8 (2× CH), 128.5 (CH), 127.3 (CH), 114.0 (2× CH), 55.2 (CH_3), 52.1 (CH_3), 40.8 (CH_2). LC/MS (M: 228): 457 (2M+H). Anal. ($C_{14}H_{12}O_3$) C, H, N.

5.5. General synthesis of peptides

The peptides **1**–**4** were synthesized with a Protein Technologies *Symphony* instrument using standard Fmoc/*t*-Bu chemistry as earlier described.[24] Acetylation was accomplished by allowing the resin to react with 20% acetic anhydride in DMF for 30 min directly after the last Fmoc deprotection, that is, without intermediate washing. Cleavage and purification was carried out as described below.

5.5.1. Peptide 1. Purification of 10.3 mg of the crude cleavage product gave 6.4 mg (57% based on amount of starting resin) of the target peptide. LC/MS (M: 717.3): 718.3 ($M+H^+$). Amino acid analysis: Tyr, 1.00; Ile, 0.86; His, 0.89; Pro, 1.00; Phe, 1.01. HRMS (M+1) calcd: 718.3564. Found: 718.3555.

5.5.2. Peptide 2. An aliquot (13.5 mg) of the crude product was purified to yield 10.0 mg (80%). LC/MS (M: 683.4): 684.3 ($M+H^+$). Amino acid analysis: Tyr, 1.00; Ile, 1.86; His, 0.89; Pro, 1.00. HRMS (M+1): Calcd: 684.3721. Found: 684.3713.

5.5.3. Peptide 3. Preparative RP-HPLC of 22.4 mg of the crude material afforded 11.1 mg (57%) of the pure peptide. LC/MS (M: 675.3): 676.4 ($M+H^+$), 338.9 ($[M+2H^+]/2$), 359.4 ($[M+MeCN+2H^+]/2$). Amino acid analysis: Tyr, 1.00; Ile, 0.91; His, 0.92; Pro, 1.01; Phe, 0.99. HRMS (M+1) calcd: 676.3459. Found: 676.3467.

5.5.4. Peptide 4. Part of the crude material (33.0 mg) was purified to give 15.8 mg (49%) of the desired peptide. LC/MS (M: 641.4): 642.2 ($M+H^+$), 321.7 ($[M+2H^+]/2$), 342.3 ($[M+MeCN+2H^+]/2$). Amino acid analysis: Tyr, 1.00; Ile, 1.87; His, 0.90; Pro, 1.00. HRMS (M+1) calcd: 642.3615. Found: 642.3602.

5.6. General synthesis of pseudopeptides

5.6.1. Coupling. Fmoc-His(Trt)-Pro-Phe-Wang resin[27] or Fmoc-His(Trt)-Pro-Ile-Wang resin[27] was weighed into a 2 mL disposable syringe equipped with a porous polyethylene filter and allowed to swell in DMF (1.5 mL) for 1 h. 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 washed with DMF (6× 1.5 mL, 6× 1 min). The turn scaffold, Fmoc-protected **A**, Fmoc-protected **B**, Fmoc-protected **C**, **D** or Fmoc-protected **E** (1.25 equiv), and PyBOP (1.25 equiv) (or HBTU (1.25 equiv) for couplings to the Fmoc-His(Trt)-Pro-Ile-Wang resin) were dissolved in DMF (1.5 mL) in the presence of DIEA (2.5 equiv) and allowed to react with the resin over night (18 h). LC/MS analysis after cleavage of an analytical sample showed complete reaction. The rest of the resin was washed with DMF (6× 2 mL, 6× 1 min). The Fmoc-group was cleaved as described above and the resin was washed with DMF (6× 2 mL, 6× 1 min), DCM (5× 2 mL, 5× 1 min) and MeOH (5× 2 mL, 5× 1 min) before being dried in vacuum over night. For the acetylated analogues, an aliquot of the resin was swelled in DMF (2 mL). The acetic acid (5 equiv) was dissolved together with PyBOP (5 equiv) or HBTU (5 equiv) and DIEA (10 equiv) in DMF (0.5 mL). The solution was added to the resin and agitated by rotation over night. The resin was deprotected, washed and dried as above.

5.6.2. Cleavage. To cleave the peptide from the resin, triethylsilane (100 μL) and 95% aqueous TFA (1.5 mL) were added and the mixture was agitated for 1 h. The polymer was filtered off and washed with TFA (2× 0.3 mL). The filtrate was evaporated in a stream of nitrogen and the product was precipitated by addition of diethyl ether (13 mL). The precipitate was collected by centrifugation, washed with ether (3× 7 mL) and dried.

5.6.3. Purification. The crude peptide was purified by RP-HPLC. The separation was monitored at 230 nm and by analytical RP-HPLC and/or LC/MS of selected fractions.

5.6.4. Analogue 5. The synthesis was conducted as described above. An aliquot of the Fmoc-His(Trt)-Pro-Phe-Wang resin was deprotected, and coupled to Fmoc-protected **A** and after appropriate washing and deprotection, the resin was subjected to the standard cleavage and purification procedures to yield 6.0 mg, 8.2 μmol (30%), LC/MS (M: 722.3): 723.2 ($M+H^+$),

362.2 ([M+2H$^+$]/2), 382.8 ([M+MeCN+2H$^+$]/2), Amino acid analysis: His, 1.02; Pro, 0.99; Phe, 0.99. HRMS (M+1) calcd: 723.3255. Found: 723.3242.

5.6.5. Analogue 6. The synthesis was conducted as described above. An aliquot of the Fmoc-His(Trt)-Pro-Phe-Wang resin was deprotected, and coupled to Fmoc-protected **B** and after appropriate washing and deprotection, the resin was subjected to the standard cleavage and purification procedures to yield 11.2 mg, 15.5 μmol (54%), LC/MS (M: 722.3): 723.1 (M+H$^+$), 362.2 ([M+2H$^+$]/2), 382.8 ([M+MeCN+2H$^+$]/2), Amino acid analysis: His, 1.00; Pro, 1.00; Phe, 1.00. HRMS (M+1) calcd: 723.3255. Found: 723.3242.

5.6.6. Analogue 7. The synthesis was conducted as described above. An aliquot of the Fmoc-His(Trt)-Pro-Phe-Wang resin was deprotected, and coupled to Fmoc-protected **B** and after appropriate washing and deprotection, subsequent coupling of acetic acid. The resin was subjected to the standard cleavage and purification procedures to yield 9.9 mg, 12.9 μmol (59%), LC/MS (M: 764.3): 765.4 (M+H$^+$), 383.3 ([M+2H$^+$]/2), Amino acid analysis: His, 1.00; Pro, 1.00; Phe, 1.00. HRMS (M+1) calcd: 765.3360. Found: 765.3374.

5.6.7. Analogue 8. The synthesis was conducted as described above. An aliquot of the Fmoc-His(Trt)-Pro-Phe-Wang resin (70 mg, 42 μmol) was deprotected, and coupled to Fmoc-protected **C** and after appropriate washing and deprotection, the resin was subjected to the standard cleavage and purification procedures to yield 14.2 mg (54%). LC/MS (M: 624.3): 625.4 (M+H$^+$), 333.9 ([M+MeCN+2H$^+$]/2), 354.4 ([M+2MeCN+2H$^+$]/2). Amino acid analysis: His, 1.00; Pro, 0.99; Phe, 1.00. HRMS (M+1) calcd: 625.2775. Found: 625.2777.

5.6.8. Analogue 9. A portion of the Fmoc-His(Trt)-Pro-Phe-Wang resin (75 mg, 45 μmol) was deprotected and subjected to coupling of Fmoc-protected **C**, and subsequently with acetic acid (200 μmol) as outlined above. After cleavage, the crude product was purified according to the standard procedure. Yield 6.4 mg (23%). LC/MS (M: 666.3): 667.4 (M+H$^+$). Amino acid analysis: His, 1.00; Pro, 1.00; Phe, 1.00. HRMS (M+1) calcd: 667.2880. Found: 667.2885.

5.6.9. Analogue 10. The synthesis was conducted as outlined above, starting from Fmoc-His(Trt)-Pro-Phe-Wang resin (83 mg, 50 μmol). After the initial deprotection, the resin was coupled to **D** followed by deprotection, washing and drying. The standard procedure to cleave the product from the resin was used. The purification of the peptide differed slightly from the standard procedure. After purification on an ACE phenyl column instead of the standard 11.0 mg (36%) was recovered. LC/MS (M: 609.3): 610.2 (M+H$^+$). Amino acid analysis: His, 1.01; Pro, 0.99; Phe, 1.00. HRMS (M+1) calcd: 610.2666. Found: 610.2656.

5.6.10. Analogue 11. An aliquot of the Fmoc-His(Trt)-Pro-Phe-Wang resin (55 mg, 29 μmol) was deprotected, and coupled to Fmoc-protected **E**. The resin was washed, deprotected again, and then cleaved according to the general procedure. After purification on the ACE Phenyl column instead of the standard, 11.6 mg, 18.1 μmol (20%) were recovered. LC/MS (M: 638.29): 639.6 (M+H$^+$), 320.4 ([M+2H$^+$]/2), 340.9 ([M+MeCN+2H$^+$]/2), 361.4 ([M+2MeCN+2H$^+$]/2). Amino acid analysis: His, 1.00; Pro, 1.00; Phe, 1.01. HRMS (M+1) calcd: 639.2931. Found: 639.2938.

5.6.11. Analogue 12. The Fmoc-His(Trt)-Pro-Phe-Wang resin (58 mg, 30 μmol) was deprotected coupled to Fmoc-protected **E.** After the Fmoc-deprotection the resin was reacted with acetic anhydride (760 μmol) and DIEA (760 μmol) in DMF (0.5 mL) for 1 h. Standard cleavage gave 20.0 mg crude product which was purified on the ACE Phenyl column instead of the standard column. Yield: 3.4 mg, 5.0 μmol (16%); LC/MS (M: 680.30): 681.5 (M+H$^+$), 341.3 ([M+2H$^+$]/2), 361.9 ([M+MeCN+2H$^+$]/2). Amino acid analysis: His, 0.99; Pro, 1.00; Phe, 1.01. HRMS (M+1): Calcd: 681.3037. Found: 681.3032.

5.6.12. Analogue 13. An aliquot of the Fmoc-His(Trt)-Pro-Ile-Wang resin (80 mg, 50 μmol) was deprotected and coupled to Fmoc-protected **C.** The resin was subjected Fmoc-deprotection and cleavage. Purification was conducted according to the standard procedure. Yield 5.2 mg (18%). LC/MS (M: 590.3): 591.2 (M+H$^+$), 296.2 ([M+2H$^+$]/2), 316.8 ([M+MeCN+2H$^+$]/2). Amino acid analysis: His, 1.00; Pro, 1.00; Ile, 0.99. HRMS (M+1): Calcd: 591.2931. Found: 591.2935.

5.6.13. Analogue 14. A portion of the Fmoc-His(Trt)-Pro-Phe-Wang resin (80 mg, 50 μmol) deprotected and coupled to Fmoc-protected **C** followed by deprotection and subsequent coupling of acetic acid (200 μmol) as outlined above. After cleavage, the crude product was purified and 7.2 mg (23%) was isolated. LC/MS (M: 632.3): 633.3 (M+H$^+$). Amino acid analysis: His, 1.00; Pro, 1.01; Ile, 0.99. HRMS (M+1) calcd: 633.3037. Found: 633.3050.

5.6.14. Analogue 15. A part of the Fmoc-His(Trt)-Pro-Ile-Wang resin (80 mg, 50 μmol) was deprotected and further reacted with **D**, washed, deprotected, and cleaved according to the general method. After purification 4.6 mg (16%) were recovered. LC/MS (M: 575.3): 576.2 (M+H$^+$). Amino acid analysis: His, 1.00; Pro, 1.01; Ile, 1.00. HRMS (M+1): Calcd: 576.2822. Found: 576.2818.

5.6.15. Analogue 16. The Fmoc-His(Trt)-Pro-Ile-Wang resin (80 mg, 50 μmol) was subjected Fmoc-deprotection and coupling to Fmoc-protected **E**. The crude product was cleaved and purified according to the standard procedure. Yield 5.2 mg (17%). LC/MS (M: 604.3): 605.3 (M+H$^+$), 303.2 ([M+2H$^+$]/2), 323.7 ([M+MeCN+2H$^+$]/2). Amino acid analysis: His, 0.99; Pro, 1.01; Ile, 0.99. HRMS (M+1) calcd: 605.3088. Found: 605.3098.

5.6.16. Analogue 17. The Fmoc-His(Trt)-Pro-Ile-Wang resin (80 mg, 50 μmol) was subjected Fmoc-deprotection

and coupling to Fmoc-protected **E**. After deprotection it was subjected to coupling with acetic acid (200 μmol) as outlined above. After cleavage, the crude product was purified using the standard system. Yield 6.4 mg (20%). LC/MS (M: 646.3): 647.4 ($M+H^+$), 344.8 ([M+MeCN+2H$^+$]/2). Amino acid analysis: His, 1.00; Pro, 1.01; Ile, 0.99. HRMS (M+1) calcd: 647.3193. Found: 647.3191.

5.7. Rat liver membrane AT_1 receptor binding assay

Rat liver membranes were prepared according to the method of Dudley et al.[33] 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, 10 μM bacitracin, 10 μM pepstatin A, 10 μM bestatin, 10 μM captopril, 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 (0.01 nM–1.0 μM) 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. Non-specific binding was determined in the presence of 1 μM Ang II. The specific binding was determined by subtracting the non-specific binding from the total bound [^{125}I]Ang II. IC_{50} was determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK). The apparent dissociation constants K_i were calculated from IC_{50} values using the Cheng–Prusoff equation[42] ($K_d = 1.7 \pm 0.1$ nM, [L] = 0.057 nM). The binding data were best fitted with a one-site fit. All experiments were performed in triplicate.

5.7.1. Pig myometrial membrane AT_2 receptor binding assay. Myometrial membranes were prepared from porcine uteri according to the method of Nielsen et al.[34] 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 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 μM bacitracin, 10 μM pepstatin A, 10 μM bestatin, 10 μM captopril, 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 (0.01 nM–1.0 μM) 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. 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. IC_{50} was determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK). The apparent dissociation constants K_i were calculated using the Cheng–Prusoff equation[42] ($K_d = 0.7 \pm 0.1$ nM, [L] = 0.057 nM). The binding data were best fitted with a one-site fit. All experiments were performed in triplicate.

5.8. In vitro morphological effects

To study the in vitro morphological effects NG108-15 cells (provided by Drs M. Emerit and M. Hamon; INSERM, U. 238, Paris, France) were used as well as transfected NG108-15/pcDNA3 cells. The transfected cell line has previously been shown to have the same behavior as the native cell line.[43] Both cell lines were cultured (NG108-15 passage 18–28, NG108-15/pcDNA3 passage 12–18) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Burlington, ONT, Canada) with 10% fetal bovine serum (FBS, Gibco), HAT supplement (Hypoxanthine, Aminopterin, Thymidine from Gibco) and 50 mg/l gentamycin (Gibco) at 37 °C in 75 cm^2 Nunclon Delta flasks in a humidified atmosphere of 93% air and 7% CO_2, as previously described.[35] The transfected cell line was kept stable by addition of Geneticin (G-418, 200 μg/ml) to the media. Subcultures were performed at subconfluency. Under these conditions, cells express only the AT_2 receptor subtype.[35,36] Cells were stimulated during three days, once a day (first stimulation 24 h after plating). Cells were cultured for three subsequent days under these conditions. For all experiments, cells were plated at the same initial density of 3.6×10^4 cells/35 mm Petri dish. Cells were treated without (control cells), with [Val^5]-angiotensin II from Bachem (Marina Delphen, CA, USA) (100 nM) or **1**, **2** (10 nM), **8** or **15** (100 nM), in the absence or in the presence of PD 123,319 (RBI Natick, MA, USA) (10 μM), an AT_2 receptor antagonist introduced daily 30 min prior to Ang II, **1**, **2**, **8**, or **15**. During the three day treatment the transfected cell line was cultured without Geneticin.

5.9. Determination of cells with neurites

Cells were examined under a phase contrast microscope and micrographs were taken after three days under the various experimental conditions. Cells with at least one neurite longer than a cell body were counted as positive for neurite outgrowth. At least 290 cells were counted in three independent experiments.[10]

5.10. Vascular contractility studies

This study was performed at Cerep (France) according to literature.[37] Seven concentrations (3, 10, 30, 100, 300, 1000 nM) of **8** were tested. Ang II was used as reference agonist and saralasin (0.03 μM) as reference antagonist.

Acknowledgments

We gratefully acknowledge the financial support from the Swedish Research Council and the Swedish Foundation for Strategic Research. We also thank Kristofer Olofsson for linguistic revision.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.05.019.

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