β-Homo-amino Acid Scan of Angiotensin IV

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Angiotensin IV, a metabolite of angiotensin II, inhibits the enzyme insulin regulated aminopeptidase or IRAP and also, although with lower potency, aminopeptidase-N (AP-N). When both β^2-homo amino acid- and β^3-homo amino acid substitutions were used, allowed the identification of H-(*R*)β^2hVal-Tyr-Ile-His-Pro-β^3hPhe-OH as a potent and stable Ang IV analog with high selectivity for IRAP versus AP-N and the AT1 receptor.

Introduction

Angiotensin IV (Ang IV: H-Val-Tyr-Ile-His-Pro-Phe-OH) is a physiologically active metabolite of Ang II in the renin-angiotensin system.[1] It improves memory acquisition and has vascular and renal actions.[2] It was proposed that Ang IV may exert its effects by binding to AT4 receptors. Those were recently identified as cystinyl aminopeptidase (EC3.4.11.3, CAP, also denoted as insulin regulated aminopeptidase or IRAP[a]), a membrane-associated zinc-dependent metallopeptidase of the M1 family. This class of enzymes possesses two characteristic motifs in the C-terminal domain: the zinc-binding motif HEXXH-X-E and the exopeptidase motif GXMEN.[3,4] The soluble form of IRAP has been referred to as placental leucine aminopeptidase (P-LAP) or oxytocinase[5,6] and was characterized as the main enzyme present in maternal serum responsible for the breakdown of oxytocin.[3] IRAP is also able to hydrolyze vasopressin, Lys-bradykinin, angiotensin III, Met-enkephalin, dynorphin A 1–8, neurokinin A, neuromedin B, somatostatin, and cholecystokinin 8.[7–9]

It is still not clear how Ang IV exerts its biological effects. This could be through inhibition of the IRAP catalytic activity, resulting in a delayed catabolism of other bioactive peptides (e.g., vasopressin, oxytocin), or by triggering intracellular processes in the event that IRAP also acts as a cellular receptor.[10,11] Presently, the existence of other cellular targets for Ang IV can still not be excluded. In this respect, it has been documented that Ang IV is also capable to inhibit the activity of aminopeptidase N (EC3.4.11.2, AP-N, M1), an IRAP-related enzyme.[12,13] Hence, AP-N is susceptible to represent an alternative target for Ang IV, especially since it was reported to undergo homodimerization and to trigger intracellular responses after the binding of antibodies.[14] Yet, both enzymes display distinct pharmacological profiles and it is noteworthy that Ang IV inhibits the IRAP activity with higher potency than the AP-N activity.

An important handicap for the studies dealing with the physiological role of Ang IV is its rapid degradation by different proteases.[15,16] Hence, there is a need for metabolically stabilized Ang IV analogues. Especially for the studies that are focused on IRAP, these analogues should also preferably display pronounced selectivity for this enzyme, not only versus AP-N but also versus the AT1-type receptors for Ang II. Indeed, although Ang IV has relatively low affinity for the AT1 receptors, several of its in vivo actions have been reported to be susceptible to inhibition by AT1 receptor-selective nonpeptide antagonists.

High affinity sites for radiolabeled Ang IV have been referred to as "AT4-receptor" and earlier structure–activity relationship studies established that the first three amino acids of Ang IV are critical for this high affinity binding.[17] An N-terminal primary α-amine, an L-configuration of the first amino acid, an activated aromatic ring in the side chain of residue 2, and a hydrophobic amino acid in position 3 are also required.[18,19] These studies also suggested that positions 4, 5, and 6 of Ang IV are more tolerant and could accommodate a variety of amino acids.[17] Based on the observation that radiolabeled Ang IV displays high affinity binding to IRAP, this enzyme was proposed to personify the "AT4-receptor". However, in nearly all of these structure–activity relationship studies, the high affinity binding of radiolabeled Ang IV was measured in the presence of metal chelators. Recent studies also revealed that high affinity binding of radiolabeled Ang IV to IRAP only takes place in the presence of such chelators and, in fact, that it only occurs to the catalytically inactive zinc-depleted form of this enzyme. In contrast, no high affinity binding of radiolabeled Ang IV could be detected for the catalytically active as well as apo-forms of AP-N.[7,20,21] These findings question the physiological relevance of structure–activity relationship studies based on radiolabeled Ang IV binding.

In this study the amino acids of Ang IV, except His, were systematically replaced by both β^2- and β^3-homo-amino acids.

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[a] Abbreviations: TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; DIPEA, diisopropylethylamine; TES, triethylsilane; TFMSA, trifluoromethanesulfonic acid; TFA, trifluoroacetic acid; DMEM, Dulbecco's modified essential medium; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; L-Leu-pNA, L-leucine-*p*-nitroanilide; HEK293 cells, human embryonic kidney cells; CHO-K1 cells, Chinese hamster ovary cells; IRAP, insulin regulated aminopeptidase.

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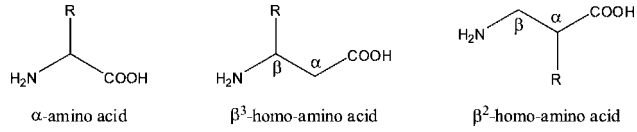


Figure 1. General structures of α- and *β*-amino acids.

The effect of these substitutions was then examined on the interaction of Ang IV with the catalytic forms of IRAP and AP-N as well as with the AT1 receptor. Binding studies are also performed but only with focus on the metabolic stability of the newly generated molecules. In general terms, the incorporation of *β*-homo-amino acids has been used to create peptidomimetics that not only retain biological activity[22] but that are also resistant to proteolysis.[23–25] Compared to α-amino acids, *β*-homo-amino acids contain an extra methylene group (homo). Depending on the position of the side chain on the 3-aminoalkanoic acid skeleton, β^2-homo-amino acids (side chain on the α-carbon) or β^3-homo-amino acids (side chain on the *β*-carbon) can be obtained (Figure 1).[26] *β*-Homo-amino acids are also known to be turn-inducing residues with the potential to enhance the structural stability of short peptides.[27] The results of the present study support the role of some *β*-homo-amino acids in increasing the enzymatic stability of Ang IV and in generating receptor selectivity.

Results

Synthesis. The peptides were prepared by solid phase peptide synthesis, using three different resins, depending on the protecting groups of the *β*-homo-amino acids. Merrifield resin was used for the synthesis of peptides with Boc-protected *β*-homo-amino acids, **2**, **3**, **4**, and **12**, and Wang resin was used for peptides with Fmoc-protected *β*-homo-amino acids, **1**, **6**, **7**, **8**, **9**, **10**, **11**, **14**, **15**, and **16**. 2-Chlorotrityl chloride resin was used when the first *β*-homo-amino acid had to be loaded to the resin (peptides: **5**, **13**, **17**). The β^3-homo-amino acids were of (*S*)-configuration. The β^2-homo-amino acids were used as racemates[28] and, after peptide synthesis, two diastereoisomeric peptides were obtained. Separation of two diastereoisomeric peptides was achieved for some of the analogues, but this was not always possible. The separated isomers of unkown absolute configuration were labeled as "a" or "b", depending on the elution order in the HPLC. Unseparable mixtures were labeled as "ab". Because Ile[2] can be replaced in Ang IV by Leu without loss of affinity,[20] β^2-homo-Leu was used because of its easier synthesis.[28] Homochiral (*S*)- or (*R*)-β^2hVal was prepared according to the reported procedure.[29] This allowed the identification of **6** as the 2-(*R*)-isomer and **7** as the 2-(*S*)-isomer.

Enzyme Activity. Catalytic activity was measured in membrane homogenates of HEK293 cells transiently transfected with human IRAP or AP-N in the presence of different concentrations of compound. Determination of the aminopeptidase catalytic activity was based on cleavage of the substrate L-leucine-*p*-nitroanilide (L-Leu-pNA) into L-leucine and *p*-nitroaniline. Formation of *p*-nitroaniline was followed by measuring at 37 °C the absorption at 405 nm every 5 min between 10 and 50 min. The enzymatic activities were calculated by linear regression analysis of the time-wise increase of the absorption.

The new Ang IV analogues were assayed in this manner, (Figure 2), and the results are collected in Table 1. For the analogues containing β^3-homo amino acids, the pK_i values for inhibition of IRAP activity increased from compound with β^3-hVal to β^3-hPhe (**1** to **5**). A substitution with a β^3-homo-amino acid more distant from the *N*-terminus seems to be more

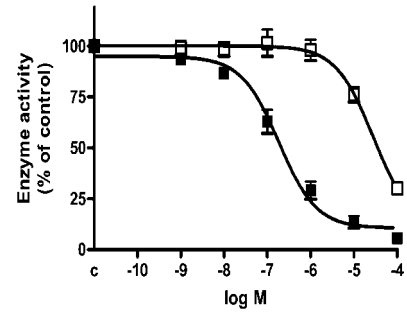
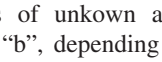


Figure 2. Inhibition of the enzymatic activity in membrane homogenates of HEK293 cells transfected with human IRAP (■) or AP-N (□; corresponded to 1.5 × 105 cells/incubation) with compound **13**. Cells were incubated at 37 °C with 1.5 mM L-Leu-pNA in the absence (control activity) or presence of increasing concentrations of compound. The rate constants of L-Leu-pNA cleavage (corresponding to enzyme activity and expressed as a percentage of control) were calculated by linear regression analysis of the absorption (at 405 nm) vs time curves with measurements made every 5 min (between 10 and 50 min). The pK_i values of all the peptides are given in Table 1.

Table 1. Inhibition of Enzyme Activity by Ang IV Analogues with *β*-Homo-amino Acids in Membranes of Transfected HEK 293 Cells[a]

Nr	Compounds	HEK293 + IRAP pKi ± SD	HEK293 + AP-N pKi ± SD
1	H-Ile-β^3hVal-Tyr-Ile-His-Pro-Phe-OH	5.27 ± 0.20	<4
2	H-Val-β^3hTyr-Ile-His-Pro-Phe-OH	6.76 ± 0.17	5.34 ± 0.23
3	H-Val-Tyr-β^3hIle-His-Pro-Phe-OH	6.83 ± 0.17	5.12 ± 0.42
4	H-Val-Tyr-Ile-His-β^3hPro-Phe-OH	6.84 ± 0.31	5.27 ± 0.02
5	H-Val-Tyr-Ile-His-Pro-β^3hPhe-OH	7.69 ± 0.12	5.61 ± 0.40
6	H-(*R*)-β^2hVal-Tyr-Ile-His-Pro-Phe-OH (a)	7.00 ± 0.15	5.35 ± 0.15
7	H-(*S*)-β^2hVal-Tyr-Ile-His-Pro-Phe-OH (b)	5.24 ± 0.06	3.06 ± 0.34
8	H-Val-β^2hTyr-Ile-His-Pro-Phe-OH (ab)	5.97 ± 0.10	4.99 ± 0.07
9	H-Val-Tyr-β^2hLeu-His-Pro-Phe-OH (a)	6.70 ± 0.08	5.26 ± 0.22
10	H-Val-Tyr-β^2hLeu-His-Pro-Phe-OH (ab)	6.75 ± 0.01	4.96 ± 0.48
11	H-Val-Tyr-Ile-His-(R)-β^2hPro-Phe-OH	7.09 ± 0.15	5.53 ± 0.15
12	H-Val-Tyr-Ile-His-Pro-β^2hPhe-OH (ab)	6.96 ± 0.26	5.61 ± 0.44
13	H-(*R*)-β^2hVal-Tyr-Ile-His-Pro-β^3hPhe-OH	7.56 ± 0.21	5.23 ± 0.04
14		5.67 ± 0.82	4.46 ± 0.54
15	H-β^2hNle-Tyr-Ile-His-Pro-Phe-OH (ab)	6.20 ± 0.37	5.03 ± 0.64
16	H-β^2hLeu-Tyr-Ile-His-Pro-Phe-OH (ab)	5.88 ± 0.10	3.85 ± 0.07
17	H-Ile-β^2hLeu-Tyr-Ile-His-Pro-β^3hPhe-OH (ab)	6.78 ± 0.10	4.16 ± 0.05
18	H-Val-Tyr-Ile-His-Pro-Phe-OH[21]	7.25 ± 0.14	6.08 ± 0.02

[a] The separated isomers of unknown absolute configuration were labelled as "a" or "b", depending on the elution order in the HPLC. Unseparable mixtures were labelled as "ab".

favorable for the potency of the analogue. In the case of the β^2-homo amino acid containing peptides, the analogues with β^2-hVal and β^2-hPro (**6**, **11**) are the most active for IRAP. All compounds inhibited the activity of recombinant IRAP more potently than the activity of recombinant AP-N. The analogues that were at least a 100 times selective (**5**, **6**, **11** and **13**, **17**) were further used in this study.

Stability Experiments. Stability experiments were performed in membrane homogenates of CHO-K1 cells which contain endogenous IRAP. Preincubations of the membranes with different concentrations of compound were carried out for 40 min at 37 °C in the presence or absence of metal chelators. Then, a competition binding assay was performed by adding

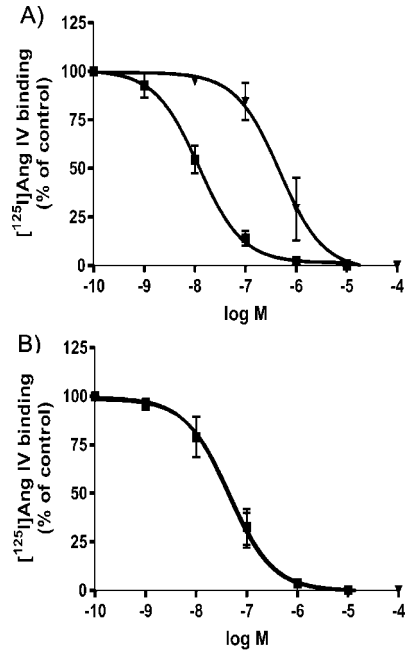


Figure 3. Stability experiments performed in membrane homogenates of CHO-K1. Membranes were preincubations for 40 min at 37 °C with increasing concentrations of compounds **5** (A) and **13** (B) in the presence (■) or absence (▼) of chelators. Then, cells were incubated for 30 min with 1 nM [^{125}I]AngIV. Data refer to specific binding of [^{125}I]AngIV (expressed as percent of control binding), calculated by subtracting nonspecific binding in the presence of 10 μM unlabeled Ang IV from total binding. The corresponding pK_i values are given in Table 2.

[^{125}I]Ang IV (1 nM) for 30 min at 37 °C. Nonspecific binding was measured with Ang IV (10 μM).

For the stability experiments, the four most active compounds were chosen: **5**, **6**, **11** and **13**, **17**. Preincubation without metal chelators gave rise to an inhibition curve shifted to the right (Figure 3), suggesting that, in the absence of the chelators, compounds **5** and **11** were quickly metabolized by IRAP or other metalloproteases present in the cell membranes. On the other hand, no shift was observed for compounds **6**, **13**, and **17**, demonstrating that these peptides are stable in the presence of CHO-K1 cell membranes. (Table 2).

Binding to the AT1 Receptor. Affinity of the compounds to the AT1 receptor was tested on intact CHO-AT1 cells. Cells were incubated with different concentrations of compound and the radiolabeled AT1 receptor-selective nonpeptide antagonist [^{3}H] Valsartan (1.5 nM) for 40 min at 37 °C. Nonspecific binding was measured with Candesartan (1 μM). After the incubation, cells were washed on ice and the remaining radioactivity was measured.

Figure 4 illustrates that peptides **5**, **11**, **13**, and **17** do not interact with AT1 receptor, meaning that those compounds are selective for IRAP or the AT4 receptor. Compound **6** binds to the AT1 receptor with similar potency as Ang IV (pK_i = 6.26 ± 0.23 vs 6.39 ± 0.01), this can be explained by the unchanged C-terminal part of the molecule, which is important for AT1 receptor binding.

Discussion

To firmly establish the identity of the cellular recognition sites that are responsible for the various physiological effects of Ang IV and, especially to discriminate the involvement of IRAP from other potential targets like AP-N and AT1 receptors, there is a need for more stable and more IRAP-selective Ang IV analogues. Such compounds may eventually be used as leads for therapeutic drugs. In this study, we used β-homo-amino acid replacements for stabilization and to induce selectivity. In this respect, previous studies revealed that Ang II (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH) analogues in which Asp^1 was replaced by β^3-D-hAsp and β^3-L-hAsp stimulated AT1 receptors more potently than the parent compound and that their effect lasted 2–3 times longer.[30] In contrast, examination of β^3-hTyr^4- and β^3-hIle^5-containing Ang II analogues revealed that they were less hypertensive (an AT1 receptor-mediated effect) than the parent peptide but still highly resistant to degradation by chymotrypsin. Taken together, these studies establish the role of some β-homo-amino acid replacements in increasing the metabolic stability of Ang II. Yet, such substitutions do not warrant the potency of the parent peptide to be retained. The present results with the β-homo-amino acid-substituted Ang IV analogues proffer essentially the same conclusion.

The first property to be studied for all the generated Ang IV analogues was their potency to inhibit IRAP activity compared to APN activity. As outlined in the introduction, studying the inhibition of enzyme activity is physiologically more relevant than the high affinity competition binding with radiolabeled Ang IV.[7,21,31] Inhibition of enzyme activity by these analogues was tested in membranes of HEK293 cells transfected with recombinant IRAP or AP-N. It was shown that, after the replacement of the various amino acids of Ang IV by the corresponding β-homo-amino acids, the analogues were still able to inhibit the IRAP activity more potently than the AP-N activity (Table 1). β^3-Homo-amino acid substitutions at the C-terminal part resulted in more potent analogues than corresponding substitutions at the N-terminal part. In the case of the β^2-homo-amino acid containing peptides, the analogues with β^2-hVal and β^2-hPro **6** and **11** were the most potent inhibitors of IRAP activity. Compounds **5**, **6**, **11**, and **13** were the most IRAP-selective; they inhibited the IRAP activity about 100-fold more potently than the AP-N activity. Compared to Ang IV (pK_i IRAP: 7.25, pK_i AP-N: 6.08),[21] they were not only more IRAP versus AP-N selective, but also more potent (Figure 2). Because [Nle^1]Ang IV is reported to be more potent than Ang IV,[19] we also prepared the β^2-hNle analogue **15**. This analogue, however, turned out to have a low potency and selectivity. Similarly, β^2-hLeu incorporation **16** resulted in a large drop in potency, but β^3-hPhe incorporation improved potency in compound **17**. The use of the commercially available achiral 1-aminomethyl-cyclopentane carboxylic acid in position 1 also resulted in an analogue **14** that was less potent than **6**. This demonstrated the importance of the isopropyl side chain in the N-terminal β^2-homo-amino acid.

The susceptibility for metabolic cleavage of the four most IRAP selective compounds **5**, **6**, **11**, and **13**, and Ang IV was subsequently compared. To this end, CHO-K1 cell membranes were preincubated with increasing concentrations of each compound either in the presence or absence of the chelators EDTA and 1,10-phenanthroline before the binding assay with [^{125}I]Ang IV (which was performed in the presence of both chelators). EDTA and 1,10-phenanthroline are well-known for their ability to block IRAP, AP-N, as well as the activity of other other metalloproteinases. Hence, when both chelators are present during the preincubation phase, no degradation of Ang IV and its analogues is supposed to take place. Compared to the competition curves obtained under such conditions, the curves will be shifted to the right for analogues that undergo degradation when the preincubation is carried out without these chelators, while the curves for the metabolically stable analogues

Table 2. [^{125}I]Ang IV Competition Binding in CHO-K1 Membranes after Preincubation in the Presence or Absence of EDTA and 1,10-Phenanthroline (Chelators)

Nr	compounds	CHOK1 + chelators pK_i ± SD	CHOK1 − chelators pK_i ± SD
5	H-Val-Tyr-Ile-His-Pro-β^3hPhe-OH	8.24 ± 0.22	6.67 ± 0.45
6	H-(R)-β^2hVal-Tyr-Ile-His-Pro-Phe-OH (a)	7.58 ± 0.08	7.64 ± 0.01
13	H-(R)-β^2hVal-Tyr-Ile-His-Pro-β^3hPhe-OH	7.70 ± 0.32	7.73 ± 0.31
17	H-β^2hLeu-Tyr-Ile-His-Pro-β^3hPhe-OH (ab)	7.70 ± 0.03	7.63 ± 0.05
18	H-Val-Tyr-Ile-His-Pro-Phe-OH[41]	8.10 ± 0.09	6.42 ± 0.23

will not undergo such shift.[32] According to this principle, Ang IV is rapidly degraded by metalloproteases present in the CHO-K1 cell membranes (Figure 3, Table 2). A shift was also observed for peptides **5** and **11**, but not for **6** and **13**. This indicates that only compounds with N-terminal modifications were protected against metabolic breakdown. This is consistent with the high metabolic stability reported for the reduced amide bond analogue Divalinal (ValΨ(CH_2−NH)Tyr-ValΨ(CH_2−NH)His-Pro-Phe-OH).[17]

Finally, the affinity of compounds **5**, **6**, **11**, **13**, and **17** for the AT1 receptor was studied. In contrast to IRAP, which contains a single membrane-spanning α-helical domain and a large exodomain containing a zinc-associated catalytic site, the AT1 receptor makes part of the G protein coupled receptor subfamily and its seven-membrane-spanning α-helical domains carry the binding site.[33] The outspoken difference in the structure of the ligand binding site of IRAP and the AT1 receptor provides a rationale for their distinct pharmacological profile.[34] Here, this distinction is also clearly illustrated by the very low affinity of **5**, **11**, **13**, and **17** for the AT1 receptor. These findings indicate that changes in the Phe and Pro backbone result in loss of AT1 receptor affinity. In contrast, only Ang IV and **6** showed measurable affinity (and equipotency) for the AT1 receptor. This is in accordance with the demonstration in earlier studies that the C-terminal part of Ang IV is crucial for binding to the AT1 receptor.

Exploiting the observations that (*R*)-β^2-hVal^1 **6** substitution in Ang IV confers metabolic stability, that β^3-hPhe^6 **5** substitution abolishes AT1 receptor affinity and that both substitutions confer IRAP- versus AP-N-selectivity, the double substituted analogue **13** was prepared. As expected, this analogue showed high metabolic stability as well as a high affinity and selectivity for IRAP. The equivalent [β^2-hLeu^1, β^3-hPhe^6] analogue **17** also showed excellent IRAP- versus APN-selectivity, but was less potent than its [β^2-hVal^1] analogue.

Conclusion

Replacing the amino acids of Ang IV by both β^2- and β^3-homo-amino acids resulted in interesting effects on metabolic stability and on IRAP versus APN and AT1 receptor selectivity. A combination of (*R*)-β^2-hVal and β^3-homo-Phe substitution allowed the development of **13**, a metabolically stable Ang IV analogue with high affinity and selectivity for IRAP. It is, therefore, an ideal candidate for further studies to unravel the molecular action mechanism of Ang IV.

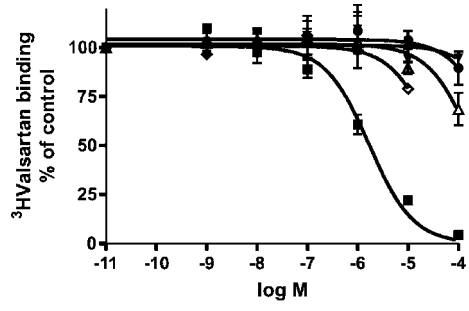


Figure 4. Affinity of the compounds **6** (■), **5** (Δ), **13** (●), **11** (▼), **17** (◊) to the AT1 receptor. CHO cells stably transfected with the AT1 receptor (CHO-AT1) were incubated for 40 min at 37 °C with different concentrations of compound and 1.5 nM ^{3}HValsartan. Data refer to specific binding of ^{3}HValsartan (expressed as percent of control binding), calculated by subtracting nonspecific binding in the presence of 1 μM Candesartan from total binding.

Experimental Section

Materials. L-Leucine-*p*-nitroanilide (L-Leu-pNA) and L-alanine-p-nitroanilide were obtained from Sigma-Aldrich (Bornem, Belgium) and *p*-nitroaniline from VWR International (Leuven, Belgium). Tyr^4 of Ang IV was iodinated using the Iodogen iodination reagent from Pierce (Erembodegem, Belgium). ^{125}I was obtained from MP Biomedicals (Asse, Belgium). Monoiodinated Ang IV was isolated on a GraceVydac C18 monomeric 120Å reverse-phase HPLC column (Hesperia, CA) and stored at −20 °C in 10 mM KH_2PO_4, pH 6.5, containing 45% ethanol. All other reagents were of the highest grade commercially available. CHO-K1 cells were kindly obtained from the Pasteur Institute (Brussels, Belgium).

Boc-Phe Merrifield-, Fmoc-Phe Wang-, and 2-chlorotrityl chloride resin, CH_2Cl_2, TES, Boc- or Fmoc-protected amino acids were obtained form Fluka (Bornem, Belgium), protected β^3-homo-amino acids were from Peptisyntha (Brussels,Belgium), TBTU was from SennChemicals (Gentilly, France), DMF, DIPEA, and (*R*)-Boc-β^2hPro, were from Sigma-Aldrich (Bornem, Belgium). Racemic Boc-β^2-homo-amino acids were synthesized by us, as reported elsewhere.[28] The Boc-(*S*)-β^2hVal and Boc-(*R*)-β^2hVal were prepared according to the described method.[29] Boc-1-aminomethylcyclopentane carboxylic acid was obtained from NeoMPS (Strasbourg, France).

Analytical RP-HPLC was performed using an Agilent 1100 Series system (Waldbronn, Germany) with a Supelco Discovery BIO Wide Pore (Bellefonte, PA) RP C-18 column (25 cm × 4.6 mm, 5 μm) using UV detection at 215 nm. The mobile phase (system 1, water/acetonitrile; system 2, water/methanol) contained 0.1% TFA. The standard gradient consisted of a 20 min run from 3 to 97% acetonitrile (system 1) or methanol (system 2) at a flow rate of 1 mL/min. Preparative HPLC was performed on a Gilson apparatus and controlled with the software package Unipoint. The reversed phase C18-column (Discovery BIO Wide Pore 25 cm × 21.2 mm, 10 μm) was used under the same conditions as the analytical RP-HPLC but with a flow rate of 20 mL min^{-1}. Mass Spectrometry (MS) was recorded on a VG Quattro II spectrometer using electrospray (ESP) ionization, data collection was done with Masslynx software.

Peptide Synthesis. Synthesis of all peptides was carried out by solid phase peptide synthesis using *tert*-butoxycarbonyl (Boc) or *N*-9-fluorenylmethoxycarbonyl (Fmoc) N-terminal protected amino acids. The peptides were synthesized on Boc-Phe Merrifield resin (0.57 mmol/g; peptides: **2**, **3**, **4**, **12**), Fmoc-Phe Wang resin (0.76 mmol/g; peptides: **1**, **8**, **9**, **10**, **11**, **14**, **15**, **16**), and 2-chlorotrityl chloride resin (1.5 mmol/g; peptides: **5**, **6**, **7**, **13**, **17**), and side chain protection groups were Tyr(*t*-Bu), β^3-hTyr(t-Bu), and His(Trt) for Fmoc strategy and Tyr(2,6-di-Cl-Bzl) and His(Tos) for Boc strategy. The Fmoc protecting group was removed by a solution of 20% piperidine in DMF (2 × 5 min.), cleavage of Boc-protection was carried out in 50% TFA in DCM (2 × 10 min.) and 10% TEA in DCM (2 × 5 min.) was used for neutralization. Coupling of amino acids was performed in DMF/DCM (1v/1v) using TBTU (3 equiv) and DIPEA (6 equiv). Peptides were cleaved from the resin by

treating with TFA/H_2O/TES (95:2.5:2.5) for 2 h, either HF or TFA/TFMSA/TES (20:2:3) in the case of Merrifield resin. Peptides were purified by RP-HPLC on a Supelco DiscoveryBIO Wide Pore preparative C18 column. Each peptide was at least 98% pure as assessed by TLC and analytical RP-HPLC. The molecular weights were confirmed by ESI-MS (Supporting Information).

Cell Culture, Transient Transfection, and Membrane Preparation. CHO-K1, CHO-AT1, and HEK293 cell lines were cultured in 75 and 500 cm^2 culture flasks in Dulbecco's modified essential medium (DMEM) supplemented with L-glutamine (2 mM), 2% (v/v) of a stock solution containing 5000 IU/mL penicillin and 5000 μg/mL streptomycin (Invitrogen, Merelbeke, Belgium), 1% (v/v) of a stock solution containing nonessential amino-acids, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (Invitrogen, Merelbeke, Belgium). The cells were grown in 5% CO_2 at 37 °C until confluent.

HEK293 cells were transiently transfected with plasmid DNA, pCIneo containing the gene of human IRAP (kindly obtained from Prof. M. Tsujimoto, Laboratory of Cellular Biochemistry, Saitama, Japan) or pTEJ4[35] carrying the complete human aminopeptidase N (AP-N) cDNA.[36] The transient transfection was performed as described previously with 8 μL/mL LipofectAMINE (Invitrogen, Merelbeke, Belgium) and 1 μg/mL plasmid DNA.[36] After transfection, the cells were cultured for 2 more days. IRAP and AP-N transfected HEK293 cells displayed a 10 and 8 times higher enzyme activity than nontransfected cells.

CHO-K1 cell and transfected HEK293 cell membranes were prepared as described previously.[37] In short, the cells were harvested with 0.2% EDTA (w/v; in PBS, pH 7.4) and centrifuged for 5 min at 500 g at room temperature. After resuspending in PBS, the number of cells were counted and washed. The cells were then homogenized in 50 mM Tris-HCl (at pH 7.4) using a Polytron (10 s at maximum speed) and Potter homogenizer (30 strokes at 1000 rpm) and then centrifuged for 30 min (30000 g at 4 °C). The pellet was resuspended in 50 mM Tris-HCl and centrifuged (30 min 30000 g at 4 °C), and the supernatant was removed. The resulting pellets were stored at −20 °C until use.

Enzyme Assay. Determination of the aminopeptidase catalytic activity was based on the cleavage of the substrate L-leucine-*p*-nitroanilide (L-Leu-pNA)[38] into L-leucine and *p*-nitroaniline. This latter compound displays a characteristic light absorption maximum at 405 nm. Pellets, prepared as described above, were thawed and resuspended using a Polytron homogenizer in enzyme assay buffer containing 50 mM Tris-HCl (pH 7.4), 140 mM NaCl, 0.1% (w/v) BSA, and 100 μM phenyl methyl sulfonyl fluoride. The incubation mixture comprised 50 μL membrane homogenate, 200 μL L-Leu-pNA (1.5 mM), and 50 μL enzyme assay buffer alone or with test compound. The amount of membrane homogenate corresponded to 1.5×10^5 transfected HEK293 cells in each well. Assays were carried out at 37 °C in 96-well plates (Medisch Labo Service, Menen, Belgium) and the formation of *p*-nitroaniline was followed by measuring the absorption at 405 nm every 5 min between 10 and 50 min in a Bio-Whittaker ELISA reader. The enzymatic activities were calculated by linear regression analysis of the time-wise increase of the absorption.

Stability Experiments. The stability of angiotensin IV and compounds **5**, **6**, **11**, and **13** was compared in the presence of CHO-K1 cell membranes. Membrane pellets were thawed and resuspended using a Polytron homogenizer in 50 mM Tris-HCl (pH 7.4) enzyme assay buffer, and the assays were carried out in polyethylene 24-well plates (Elscolab, Kruibeke, Belgium). Preincubations were carried out for 40 min at 37 °C in 250 μL containing 150 μL membrane homogenate (corresponding with 4 × 105 CHO-K1 cells), 50 μL enzyme assay buffer without or with 30 mM EDTA/600 μM 1,10-Phe and 50 μL enzyme assay buffer without or with the different compounds or unlabeled Ang IV (60 μM for nonspecific binding). Then the binding assay was initiated by adding 50 μL of enzyme assay buffer containing [^{125}I] Ang IV (without or with 30 mM EDTA/600 μM 1,10-phenanthroline), and the mixture was further incubated for 30 min at 37 °C. Final chelator concentrations (when present) were 5 mM EDTA and 100 μM 1,10-Phe, the final [^{125}I] Ang IV concentration was 1 nM, and the final unlabeled ligand concentrations are indicated in Figure 3. After incubation, the mixture was vacuum filtered using an Inotech 24-well cell harvester through GF/B glass fiber filters (Whatman) presoaked in 1% (w/v) BSA. After drying, the radioactivity retained in the filters was measured using a Perkin-Elmer γ-counter.

AT1 Receptor Binding. Chinese hamster ovary cells, stably expressing the human angiotensin II AT_1 receptor (CHO-AT_1),[39] were used to test the affinity of the compounds to the AT1 receptor. Before the experiment, the plated cells were washed twice with PBS buffer at room temperature (0.5 mL per well) and then incubated with DMEM medium (400 μL) for 15 min at 37 °C. Next, a competition binding was performed by incubating the cells with different concentrations of compound (50 μL) and [^{3}H] Valsartan (final concentration of 1.5 nM, 50 μL) for 40 min at 37 °C. Nonspecific binding was measured with candesartan (final concentration of 1 μM, 50 μL). After incubation cells were washed three times with cold PBS (4 °C). The cell bound radioactivity in each well was subsequently solubilized with 500 μL sodium hydroxide (0.2 M) and counted for 3 min in a liquid scintillation counter after addition of 3 mL scintillation liquid (Optisafe, Perkin-Elmer).

Data Analysis. All experiments were performed at least two times with duplicate determinations each. The calculation of IC_{50} values from competition binding (or enzyme inhibition) experiments were performed by nonlinear regression analysis using GraphPad Prism 4.0. The equilibrium dissociation constants (K_i values) of the tested compounds in the binding and enzyme assays were calculated using the equation $K_i = [IC_{50}/(1 + [L]/K)]$ in which $[L]$ is the concentration of free radioligand (binding) or free substrate concentration (enzyme assay) and K is the equilibrium dissociation constant (K_D) of [^{125}I]Ang IV (from saturation binding experiments) or the Michaelis–Menten constant (K_m) for substrate cleavage.[40]

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Supporting Information Available: MS and HPLC data and HPLC traces of Ang IV analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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