5612 J. Med. Chem. 2009, 52, 5612–5618
DOI: 10.1021/jm900651p

Journal of Medicinal Chemistry Article

The Replacement of His(4) in Angiotensin IV by Conformationally Constrained Residues Provides Highly Potent and Selective Analogues

Aneta Lukaszuk,[†] Heidi Demaegdt,[‡] Debby Feytens,[†] Patrick Vanderheyden,[‡] Georges Vauquelin,[‡] and Dirk Tourwé*,[†]

[†]*Department of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium, and* [‡]*Department of Molecular and Biochemical Pharmacology, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium*

Received May 18, 2009

The histidine residue in angiotensin IV was replaced by various conformationally constrained amino acids. The substitution of the His[4]−Pro[5] dipeptide sequence by the constrained Trp analogue Aia−Gly, in combination with β^2hVal substitution at the *N*-terminus, provided a new stable analogue H-(*R*)-β^2hVal-Tyr-Ile-Aia-Gly-Phe-OH (**AL-40**) that is a potent ligand for the Ang IV receptor IRAP and selective versus AP-N and the AT1 receptor.

Introduction

The renin−angiotensin system (RAS) mediates important physiological functions including body water and electrolyte homeostasis, blood pressure, sexual behavior, and the regulation of gland hormones. These functions are mediated by angiotensin II (Ang II), which interacts with the AT1 receptor subtype. The hexapeptide angiotensin IV (Ang IV, H-Val-Tyr-Ile-His-Pro-Phe-OH), derived from Ang II by cleavage of the two *N*-terminal amino acids, binds to the AT1 and AT2 receptors with low affinity. A specific AT4 receptor[1−4] has been identified as the cystinyl aminopeptidase (EC3.4.11.3, CAP[a]), also denoted as insulin regulated aminopeptidase (IRAP).[5,6] Among a wide range of physiological actions, Ang IV is responsible for cognitive enhancement, improving memory acquisition,[7,8] and inhibition of pharmacologically induced seizures[9] as well as for vascular and renal actions.[10] Ang IV exerts its effects by binding to AT4 receptors, which are widely distributed across tissues. Three hypotheses for explaining the effects of the AT4 receptor ligands were proposed: (i) Ang IV acts as a potent IRAP inhibitor, which prolongs the action of endogenous promnestic peptides,[11,12] (ii) Ang IV might modulate glucose uptake by modulating trafficking of GLUT4,[5,13] or (iii) IRAP might also act as a receptor and trigger intracellular processes.[4,5,14,15] Moreover, it has been suggested that aminopeptidase N (AP-N) is also a potential target of Ang IV.[16]

To precisely study the mechanism by which Ang IV exerts its biological effects, there is great need to improve the metabolic stability of Ang IV and to prepare analogues that are highly selective for the AT4 receptor.

We previously reported a study in which each amino acid in Ang IV, except His, was replaced by its β^2-homo- or β^3-homoanalogue.[17] β-Homoamino acids diverge from α-amino acids by having an extra methylene group (homo) either between the carboxyl group and the α-carbon (β^3-homoamino acid) or between the amine group and the α-carbon (β^2-homoamino acid).[18] Incorporation of such β-homoamino acids has been used to create peptidomimetics that not only retain biological activity[19] but that are also resistant to proteolysis.[20−30] Our study resulted in the development of H-β^2hVal-Tyr-Ile-His-Pro-β^3hPhe-OH **1** (**AL-11**), a potent, selective, and stable AT4 ligand, in which β^2hVal provides selectivity for IRAP versus AP-N, and stability against its aminopeptidase activity and in which β^3hPhe is responsible for selectivity toward the AT1 receptor.[17]

Because histidine was the only residue in the Ang IV sequence for which the β^2- and β^3-homoamino acid analogues are difficult to obtain,[31] this amino acid was not modified in our previous study.

Another successful approach for enhancing the receptor selectivity and stability of biologically active peptides is the incorporation of conformationally constrained amino acids.[32,33] The conformations of the side chains in a peptide are highly important for biological activity and the introduction of constrained side chains is at the basis of the design in χ-space, which resulted in many potent peptidomimetics.[34,35] The conformations of the amino acid side chains are described by the torsional angles χ^1, χ^2,...[35] For aromatic residues such as Phe, Tyr, Trp, or His, three low energy conformations exist: *gauche* (−) ($\chi^1 = -60°$), *trans* ($\chi^1 = 180°$), and *gauche* (+) ($\chi^1 = +60°$). These low energy conformations can be constrained by structural modifications that limit the rotation around the $C^\alpha-C^\beta$ (and $C^\beta-C^\gamma$) bond.[35,36]

We now describe the results of the replacement of His[4] in Ang IV by conformationally constrained aromatic residues. In this study, we selected two types of constraints (Figure 1).

*To whom correspondence should be addressed. Phone: (+32) 2 629 32 95. Fax: (+32) 2 629 33 04. E-mail: datourwe@vub.ac.be.

[a] Abbreviations: Aba, 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one; Aia, 4-amino-1,2,4,5-tetrahydro-indolo[2,3-*c*]-azepin-3-one; AP-N, aminopeptidase N; BSA, bovine serum albumin; CAP, cystinyl aminopeptidase; CHO cells, Chinese hamster ovary cells; DIOZ, 4-isopropyl-5,5-diphenyloxazolidin-2-one; DIPEA, diisopropylethylamine; DMEM, Dulbecco's modified essential medium; EDTA, ethylenediaminetetraacetic acid; GLUT4, insulin-regulated glucose transporter; HEK293 cells, human embryonic kidney cells; IRAP, insulin regulated aminopeptidase; L-Leu-*p*NA, L-leucine-*p*-nitroanilide; PBS, phosphate-buffered saline; Spi, 4,5,6,7-tetrahydro-3*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TES, triethylsilane; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Tris, tris(hydroxymethyl)aminomethane.

 Published on Web 08/27/2009

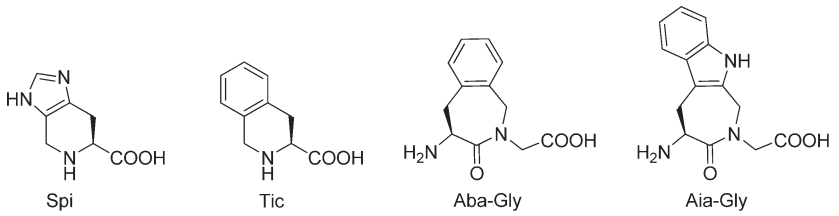

Figure 1. Conformationally constrained aromatic residues used as His replacements in Ang IV.

Table 1. Inhibition of Enzyme Activity by Ang IV Analogues in Membranes of Transfected HEK 293 Cells

code	Angiotensin IV: H-Val-Tyr-Ile-His-Pro-Phe−OH	HEK293+IRAP pK_i ± SD	HEK293+AP-N pK_i ± SD	K_i (AP-N)/K_i (IRAP)
2	H-Val-Tyr-Ile-**Spi**-Pro-Phe-OH	6.29 ± 0.09	5.52 ± 0.26	5
3	H-Val-Tyr-Ile-**Tic**-Pro-Phe-OH	6.66 ± 0.01	5.61 ± 0.09	11
4	H-Val-Tyr-Ile-**Aia-Gly**-Phe-OH	6.74 ± 0.03	5.07 ± 0.12	50
5	H-Val-Tyr-Ile-**Aba-Gly**-Phe-OH	7.30 ± 0.04	5.69 ± 0.04	50
6	H-(*R*)-**β^2hVal**-Tyr-Ile-**Aba-Gly**-Phe-OH	7.90 ± 0.06	5.53 ± 0.19	250
7 (AL-40)	H-(*R*)-**β^2hVal**-Tyr-Ile-**Aia-Gly**-Phe-OH	8.07 ± 0.05	6.10 ± 0.61	100
8	H-(*R*)-**β^2hNle**-Tyr-Ile-**Aba-Gly**-Phe-OH	6.89 ± 0.03	5.32 ± 0.49	33
9	H-(*R*)-**β^2hNle**-Tyr-Ile-**Aia-Gly**-Phe-OH	7.26 ± 0.06	5.40 ± 0.37	100
Ang IV	H-Val-Tyr-Ile-His-Pro-Phe-OH[49]	7.25 ± 0.14	6.08 ± 0.02	14
[Nle^1]Ang IV	H-**Nle**-Tyr-Ile-His-Pro-Phe-OH	7.28 ± 0.03	4.97 ± 0.23	200
1 (AL-11)	H-(*R*)-**β^2hVal**-Tyr-Ile-His-Pro-**β^3hPhe**-OH[17]	7.56 ± 0.21	5.23 ± 0.04	200
10	H-(*R*)-**β^2hVal**-Tyr-Ile-His-Pro-**Phe**-OH[17]	7.00 ± 0.15	5.35 ± 0.15	50

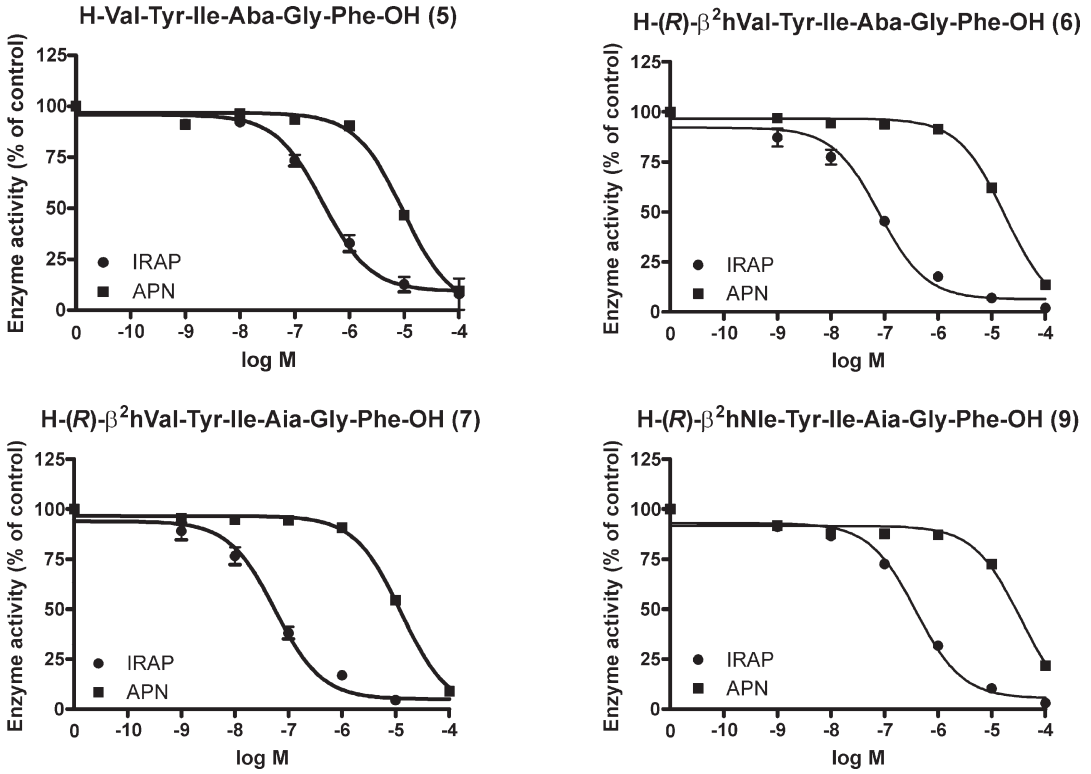

Figure 2. Inhibition of the enzymatic activity in membrane homogenates of HEK293 cells transfected with human IRAP (■) or AP-N (▼) (corresponding to 1.5 × 105 cells/incubation) with compounds **5**, **6**, **7**, and **9**. Cells were incubated at 37 °C with 1.5 mM L-Leu-*p*NA in the absence (control activity) or presence of increasing concentrations of compound. The rate of L-Leu-*p*NA cleavage (corresponding to enzyme activity and expressed as a percentage of control) was 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 peptide−enzyme combinations are given in Table 1.

1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (Tic) and 4,5,6,7-tetrahydro-3*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid (Spi), analogues of Phe and His, respectively,[37,38] restrict the side chain conformation to a χ^1 of −60° or +60°.[39] The 2-azepinone-type residues 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one (Aba) and 4-amino-1,2,4,5-tetrahydroindolo[2,3-*c*]-azepin-3-one (Aia), which are analogues of Phe and Trp, respectively, restrict the side chain conformation to a χ^1 of +60° or 180°.[36,40,41] Because the latter constraint is obtained by linking the aromatic ring in the side chain to the

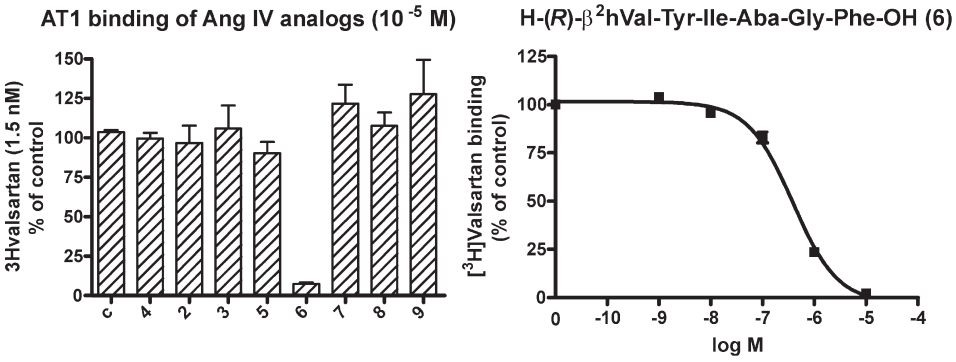

Figure 3. Interaction of the compounds with the AT1 receptor (left) and dose−response curve for **6** (right). CHO cells stably transfected with the AT1 receptor (CHO−AT1) were incubated for 40 min at 37 °C with 10^{-5} M of compound and 1.5 nM [^{3}H]Valsartan. The full dose competition to determine the pK_i was performed for **6**. Data refer to specific binding of [^{3}H]Valsartan (expressed as percent of control binding, i.e., binding in medium only), calculated by subtracting nonspecific binding in the presence of 1 μM Candesartan from total binding.

nitrogen of the succeeding amino acid through a methylene bridge,[36] the Pro^5 residue of Ang IV was replaced by Gly. Those constraints have been successfully applied in peptides and peptide mimetics.[35,36,38,42−45] Following our previous finding that metabolic stability is linked to modification of the Val^1 residue, the incorporation of these conformational constraints at position four of Ang IV is combined with the use of (*R*)-β^2hVal and (*R*)-β^2hNle residues at position one.

Results

Synthesis. The conformationally constrained amino acids Tic and Spi were prepared from Phe and His, respectively, by a Pictet−Spengler condensation with formaldehyde according to literature procedures.[37,38] The Aba−Gly and Aia−Gly dipeptide analogues were prepared as described in our previous publications.[36,41] The β^2-homoamino acids (*R*)-β^2hVal and (*R*)-β^2hNle were synthesized by an asymmetric aminomethylation reaction using the DIOZ chiral auxiliary.[46,47] All amino acid analogues were Boc-protected and incorporated into the Ang IV sequences by solid phase peptide synthesis using a Merrifield resin. The peptides were purified by semipreparative HPLC to a purity >98%.

Enzyme Activity. Determination of the aminopeptidase catalytic activity was performed in membrane homogenates of HEK293 cells transiently transfected with human IRAP or AP-N and was based on the cleavage of the substrate L-leucine-*p*-nitroanilide (L-Leu-*p*NA) into L-leucine and *p*-nitroaniline in the presence of increasing concentrations of the Ang IV analogues.[48] The Ang IV analogues produced a concentration-dependent inhibition of the catalytic activity. The results of the inhibition of enzyme activity by the Ang IV analogues are presented in Table 1, and selected curves are shown in Figure 2.

For the analogues containing native Val at position 1, a comparison with the data for native Ang IV reveals that the substitution of His^4 by Tic in **3** or Spi in **2** results in a drop in inhibitory potency both for IRAP and AP-N. The 2-benzazepinone constraint on the contrary leads to a compound with comparable potency as Ang IV in case of the Aba−Gly substitution (**5**) and to a slightly less potent compound in the case of the Aia−Gly substitution (**4**). The best results were obtained for the analogues in which β^2-homoamino acids were combined with the 2-benzazepinone constraint. The (*R*)-β^2hVal-containing analogues **6** and **7** displayed very similar pK_i values for inhibition of IRAP, the latter one being more potent than our best analogue so far, peptide **1**. These two compounds exhibited at least a hundred-fold IRAP selectivity versus AP-N. In contrast to native Ang IV in which the replacement of Val^1 by Nle results in an increase in IRAP versus AP-N selectivity (Table 1), this is not the case for the β^2hNle-containing analogues **8** and **9**.

Binding to the AT1 Receptor. The affinity of the Ang IV analogues for the AT1 receptor was evaluated on intact CHO-AT1 cells by measuring the ability of 10^{-5} M of compound to inhibit the specific binding of the selective AT1 receptor antagonist Valsartan (1.5 nM). Except for **6**, none of the analogues inhibited Valsartan binding and hence showed no affinity for the AT1 receptor (Figure 3).

A full dose-inhibition study was conducted for **6** and a p$K_i = 6.85 \pm 0.11$ was determined.

Stability Experiments. Stability experiments were performed in membrane homogenates of CHO-K1 cells containing 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 [^{3}H]Ang IV (3 nM) for 30 min at 37 °C. Nonspecific binding was measured with Ang IV (10 μM). In the presence of the chelators, ethylenediaminetetraacetic acid (EDTA) and 1,10-phenantroline, which have the ability to block IRAP, AP-N, and other metalloprotease activity, no metabolic breakdown was taking place.[50] In the absence of these chelators, peptides susceptible to metalloprotease degradation will be rapidly metabolized.

Omission of metal chelators during the preincubation step resulted in a rightward shift of the **4** and **5** inhibition curves (Figure 4), suggesting that these compounds were quickly metabolized by IRAP and/or other metalloproteases present in the cell membranes.

In contrast all analogues containing a β^2-homoamino acid at the *N*-terminus (**6**−**9**) were stable under these conditions.

Discussion

In this study, we examined the potential of the replacement of His^4 for obtaining more stable and more IRAP-selective Ang IV analogues. The introduction of conformationally constrained amino acid analogues is a powerful method to confer receptor selectivity and metabolic stability to the modified peptides.[31,32] Therefore we have replaced His^4 in Ang IV by several constrained analogues of His, Phe, and Trp.

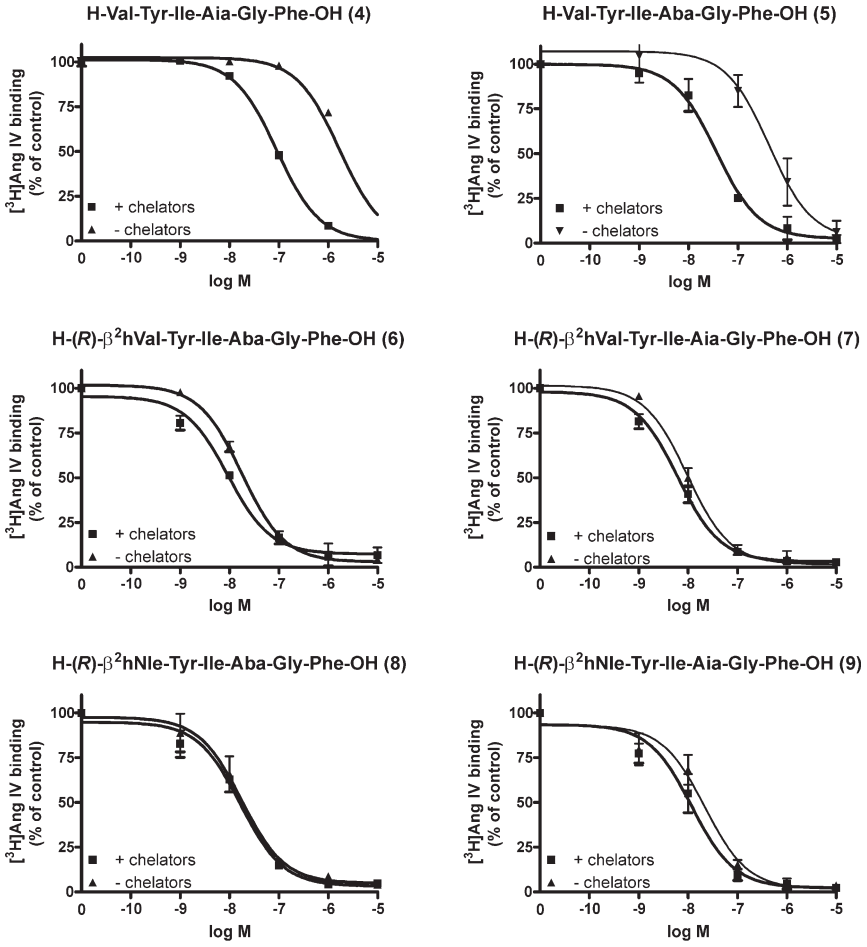

Figure 4. Stability experiments performed in membrane homogenates of CHO-K1 cells. Membranes were preincubated for 40 min at 37 °C with increasing concentrations of compounds in the presence (■) or absence (▲) of chelators. Then cells were incubated for 30 min with 3 nM [^{3}H]AngIV. Data refer to specific binding of [^{3}H]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 substitution of His by its analogue Spi (**2**) or by the Phe analogue Tic (**3**), both of which adopt a *gauche* (+) conformation within a peptide sequence,[39] led to a substantially decreased ability to inhibit the hydrolysis of Leu-*p*-NA by IRAP.[39] This indicates that a *gauche* (+) ($\chi^1 = +60°$) orientation of the His side chain results in a less efficient interaction with IRAP. The replacement of the His−Pro dipeptide fragment by Aba−Gly on the contrary, which restricts the side chain conformation to a χ^1 of +60° or 180°,[36,40,41] is leading to a compound (**5**) with a potency comparable to that of Ang IV. Because the *gauche* (+) orientation was excluded by the results of **2** and **3**, it can be concluded that the *trans* orientation of the aromatic side chain at position 4 of Ang IV is preferred and that the imidazole ring is not a requirement. In fact, an indole ring is also accepted at this position, the Aia−Gly substitution (**4**) leading to a compound that is only slightly less potent. Importantly, these substitutions maintain the selectivity for IRAP versus AP-N. As can be expected, however, the analogues that were not modified at the *N*-terminus were not stable against the aminopeptidase activity of IRAP. Our strategy to introduce (*R*)-β^2hVal was also successful in this study. Analogues **6** and **7** do not only have an increased affinity for IRAP, but also a good selectivity versus AP-N, and are metabolically stabilized. Binding studies reported by Sardinia[51] indicated that [Nle^1]Ang IV had about a 1000-fold higher affinity than native AngIV. This was, however, not confirmed in the studies by Lew,[11] where a factor of only 10 was observed. Our results do not indicate an increased potency in the enzyme assay but an increased IRAP versus AP-N selectivity for [Nle^1]Ang IV (Table 1). The change of β^2hVal in analogues **6** and **7** to β^2hNle in **8** and **9** did not result in analogues having a better profile.

These results indicate that neither the Pro^5 ring nor the His^4 imidazole ring are required for binding to IRAP. Previous studies have demonstrated that the entire His-Pro-Phe sequence in Ang IV can be replaced by *o*-aminomethylphenyl acetic acid.[52] However, potency, selectivity, and stability were less than for the analogues reported here.

Furthermore, the affinity of the reported compounds for the AT1 receptor was studied by measuring the ability of 10^{-5} M of compound to compete with the binding of the nonpeptide AT1 receptor antagonist [^{3}H]Valsartan (1.5 nM) to recombinant CHO cells stably expressing the AT1 receptor. None of the peptides bound to the AT1 receptor at this concentration, except **6**. This compound shows a binding affinity ($pK_i = 6.85 \pm 0.11$) comparable to that of Ang IV ($pK_i = 6.39 \pm 0.01$) (Figure 3). Our previous studies[17] showed that the *C*-terminal part of Ang IV is crucial for AT1 receptor binding, the introduction of a β^3hPhe residue being responsible for the loss of AT1 affinity. In the present study, the *C*-terminus was not modified. Therefore the conformational constraints introduced at position 4 are also responsible for

IRAP vs AT1-selectivity for all analogues except **6**. Remarkably, **6** with a sequence H-(*R*)-β^2hVal-Tyr-Ile-Aba-Gly-Phe-OH, was the only analogue showing weak AT1 affinity, in contrast to the related Val[1]-containing analogue **5**, and to **7**, which contains an indole ring instead of the benzene ring in **6**. This observation is difficult to explain but indicates that subtle changes in the peptide main chain, combined with side chain modifications, can influence the interaction with the AT1 receptor while not having a major effect on IRAP affinity.

Conclusion

In this study we used a new approach to develop Ang IV analogues that are selective for IRAP versus AP-N and versus the AT1 receptor and that are resistant to aminopeptidase degradation. The replacement of the His[4]−Pro[5] dipeptide sequence by residues that have a side chain constrained in the *trans* conformation was very effective to provide this selectivity. In combination with a (*R*)-β^2hVal substitution to provide metabolic stability, the new ligand H-(*R*)-β^2hVal-Tyr-Ile-Aia-Gly-Phe-OH, **7**, was developed with improved potency compared to the previously reported **1**. This ligand contains a constrained Trp residue as a replacement for His[4]. It will be an improved tool to study the physiological roles of Ang IV and IRAP. These results further demonstrate the power of conformational constraints as a tool to overcome the well-known limitations of natural peptides.

Experimental Section

General. L-Leu-pNA was obtained from Sigma-Aldrich (Bornem, Belgium) and *p*-nitroaniline from VWR International (Leuven, Belgium). All other reagents were of the highest grade commercially available. CHO-K1 cells were kindly obtained from the Pasteur Institute (Brussels, Belgium). [^{3}H]Valsartan was kindly supplied by Novartis (Basel, Switzerland), and [^{3}H]Angiotensin IV was obtained from G. Tóth, Biological Research Center (Szeged, Hungary).[53]

Fmoc-Phe-Wang resin, CH_2Cl_2, TES, and Fmoc-protected amino acids were obtained from Fluka (Bornem, Belgium), TBTU was from SennChemicals (Gentilly, France), and DMF and DIPEA were from Sigma-Aldrich (Bornem, Belgium). 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) contained 0.1% TFA. The standard gradient consisted of a 20 min run from 3 to 97% acetonitrile 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, and data collection was done with Masslynx software.

Each peptide was at least 98% pure as assessed by analytical RP-HPLC. The molecular weights were confirmed by ESI-MS (Supporting Information).

Peptide Synthesis. The synthesis of all peptides was carried out by solid-phase peptide synthesis using *tert*-butoxycarbonyl (Boc) *N*-terminal protected amino acids. The peptides were synthesized on Boc-Phe Merrifield resin (0.57 mmol/g) and side chain protecting groups were: Tyr(2,6-di-Cl-Bzl), His(Tos). The removal of the Boc protection was carried out in 50% TFA in CH_2Cl_2 (2 × 10 min) and 10% TEA in CH_2Cl_2 (2 × 5 min) was used for neutralization. Coupling of amino acids (3 equiv) was performed in DMF/CH_2Cl_2 (1v:1v) using TBTU (3 equiv) and DIPEA (6 equiv), and the reaction was monitored using the Kaiser test. The peptides were cleaved from the resin by treating it with TFA/TFMSA/TES (20:2:3). The peptides were purified by RP-HPLC on a SUPELCO DiscoveryBIO wide pore preparative C18 column, and the pure peptides were then lyophilized. Purities and structures were confirmed using analytical RP-HPLC and ESI-MS.

Peptide 7. The peptide was obtained with a yield of 3.8%. HPLC: t_R = 13.02 min (purity > 99%); MS (ESI): (M + H$^+$) calculated: 810.40; (M + H$^+$) found: 810.40.

The remaining peptides were synthesized and characterized according to the method described above. Purities, yields, HPLC, and MS data can be found in the Supporting Information.

Cell Culture, Transient Transfection, and Membrane Preparation.[17] 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[38] carrying the complete human AP-N cDNA.[54] The transient transfection was performed as described previously with 8 μL/mL Lipofectamine (Invitrogen, Merelbeke, Belgium) and 1 μg/mL plasmid DNA.[54] 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.[48] In short, the cells were harvested with 0.2% EDTA (w/v) (in phosphate-buffered saline (PBS), pH 7.4) and centrifuged for 5 min at 500*g* at room temperature. After resuspending in PBS, the number of cells was counted and they were 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, 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.[17] Determination of the aminopeptidase catalytic activity was based on the cleavage of the substrate L-leucine-*p*-nitroanilide (L-Leu-*p*NA)[48] 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) bovine serum albumin (BSA), and 100 μM phenyl methyl sulfonyl fluoride. The incubation mixture comprised 50 μL of membrane homogenate, 200 μL of L-Leu-*p*NA (1.5 mM), and 50 μL of 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 Tecan M200 96-well reader. The enzymatic activities were calculated by linear regression analysis of the time-wise increase of the absorption.

Stability Experiments.[17] The stabilities of the compounds were 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 of membrane homogenate (corresponding with 4 × 105 CHO-K1 cells), 50 μL enzyme assay buffer without or with 30 mM EDTA/600 μM 1,10-phenantrolin, 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 [^{3}H]Ang IV (18 nM, without or with 30 mM EDTA/600 μM 1,10-phenanthrolin), 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-phenantrolin, the final [^{3}H]Ang IV concentration was 3 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 (after adding 3 mL of scintillation liquid (Optiphase Hisafe)) using a β-counter (Perkin-Elmer). [^{3}H]Ang IV was characterized previously.[53]

AT1 Receptor Binding.[17] Chinese hamster ovary cells stably expressing the human angiotensin II AT_1 receptor (CHO-AT_1)[55] were used to test the affinity of the compounds for 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 concentrations of compound 10^{-5} (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 3 times with cold PBS (4 °C). The cell bound radioactivity in each well was subsequently solubilized with 500 μL of sodium hydroxide (0.2 M) and counted for 3 min in a liquid scintillation counter after addition of 3 mL of scintillation liquid (Optiphase Hisafe, Perkin-Elmer).

Data Analysis.[17] 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 the equilibrium dissociation constant (K_D) of [^{3}H]Ang IV (from saturation binding experiments) or the Michaelis−Menten constant (K_m) for substrate cleavage.[56]

Acknowledgment. We thank the "The Fund for Scientific Research—Flanders" (FWO Vlaanderen) for financial support. Heidi Demaegdt is a postdoctoral researcher, Debby Feytens is a research assitant of the "Fund for Scientific Research—Flanders" (FWO Vlaanderen), and Patrick Vanderheyden is holder of a VUB research fellowship. We are grateful to G. Tóth and E. Szemenyei (Biological Research Center, Szeged, Hungary) for the tritiation of AngIV.

Supporting Information Available: Characterization of compounds **2**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) de Gasparo, M.; Catt, K. J.; Inagami, T.; Wright, J. W.; Unger, T. International Union of Pharmacology. XXIII. The angiotensin II receptors. *Pharmacol. Rev.* **2000**, *52*, 415–472.
(2) Gasparo de, M.; Husain, A.; Alexander, W.; Catt, K. J.; Chiu, A. T.; Drew, M.; Goodfriend, T.; Harding, J. W.; Inagami, T.; Timmermans, P. B. Proposed update of angiotensin receptor nomenclature. *Hypertension* **1995**, *25*, 924–927.
(3) Swanson, G. N.; Hanesworth, J. M.; Sardinia, M. F.; Coleman, J. K.; Wright, J. W.; Hall, K. L.; Miller-Wing, A. V.; Stobb, J. W.; Cook, V. I.; Harding, E. C.; et al. Discovery of a distinct binding site for angiotensin II (3−8), a putative angiotensin IV receptor. *Regul. Pept.* **1992**, *40*, 409–419.
(4) Chai, S. Y.; Fernando, R.; Peck, G.; Ye, S. Y.; Mendelsohn, F. A.; Jenkins, T. A.; Albiston, A. L. The angiotensin IV/AT4 receptor. *Cell. Mol. Life. Sci.* **2004**, *61*, 2728–2737.
(5) Albiston, A. L.; McDowall, S. G.; Matsacos, D.; Sim, P.; Clune, E.; Mustafa, T.; Lee, J.; Mendelsohn, F. A.; Simpson, R. J.; Connolly, L. M.; Chai, S. Y. Evidence that the angiotensin IV (AT4) receptor is the enzyme insulin-regulated aminopeptidase. *J. Biol. Chem.* **2001**, *276*, 48623–48626.
(6) Rogi, T.; Tsujimoto, M.; Nakazato, H.; Mizutani, S.; Tomoda, Y. Human placental leucine aminopeptidase/oxytocinase. A new member of type II membrane-spanning zinc metallopeptidase family. *J. Biol. Chem.* **1996**, *271*, 56–61.
(7) Delorenzi, A.; Locatelli, F.; Romano, A.; Nahmod, V.; Maldonado, H. Angiotensin II (3−8) induces long-term memory improvement in the crab *Chasmagnathus*. *Neurosci. Lett.* **1997**, *226*, 143–146.
(8) Wallis, M. G.; Lankford, M. F.; Keller, S. R. Vasopressin is a physiological substrate for the insulin-regulated aminopeptidase IRAP. *Am. J. Physiol. Endocrinol. Metab.* **2007**, *293*, E1092–E1102.
(9) Stragier, B.; De Bundel, D.; Sarre, S.; Smolders, I.; Vauquelin, G.; Dupont, A.; Michotte, Y.; Vanderheyden, P. Involvement of insulin-regulated aminopeptidase in the effects of the renin−angiotensin fragment angiotensin IV: a review. *Heart Failure Rev.* **2008**, *13*, 321–337.
(10) Wright, J. W.; Krebs, L. T.; Stobb, J. W.; Harding, J. W. The angiotensin IV system: functional implications. *Front. Neuroendocrinol.* **1995**, *16*, 23–52.
(11) Lew, R. A.; Mustafa, T.; Ye, S.; McDowall, S. G.; Chai, S. Y.; Albiston, A. L. Angiotensin AT4 ligands are potent, competitive inhibitors of insulin regulated aminopeptidase (IRAP). *J. Neurochem.* **2003**, *86*, 344–350.
(12) Albiston, A. L.; Mustafa, T.; McDowall, S. G.; Mendelsohn, F. A.; Lee, J.; Chai, S. Y. AT4 receptor is insulin-regulated membrane aminopeptidase: potential mechanisms of memory enhancement. *Trends Endocrinol. Metab.* **2003**, *14*, 72–77.
(13) Keller, S. R.; Scott, H. M.; Mastick, C. C.; Aebersold, R.; Lienhard, G. E. Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from Glut4 vesicles. *J. Biol. Chem.* **1995**, *270*, 23612–23618.
(14) Vauquelin, G.; Michotte, Y.; Smolders, I.; Sarre, S.; Ebinger, G.; Dupont, A.; Vanderheyden, P. Cellular targets for angiotensin II fragments: pharmacological and molecular evidence. *J. Renin Angiotensin Aldosterone Syst.* **2002**, *3*, 195–204.
(15) Wright, J. W.; Harding, J. W. The brain angiotensin system and extracellular matrix molecules in neural plasticity, learning, and memory. *Prog. Neurobiol.* **2004**, *72*, 263–293.
(16) Garreau, I.; Chansel, D.; Vandermeersch, S.; Fruitier, I.; Piot, J. M.; Ardaillou, R. Hemorphins inhibit angiotensin IV binding and interact with aminopeptidase N. *Peptides* **1998**, *19*, 1339–1348.
(17) Lukaszuk, A.; Demaegdt, H.; Szemenyei, E.; Toth, G.; Tymecka, D.; Misicka, A.; Karoyan, P.; Vanderheyden, P.; Vauquelin, G.; Tourwe, D. Beta-homo-amino acid scan of angiotensin IV. *J. Med. Chem.* **2008**, *51*, 2291–2296.
(18) Lelais, G.; Seebach, D. Beta^2-amino acids syntheses, occurrence in natural products, and components of beta-peptides. *Biopolymers* **2004**, *76*, 206–243.
(19) Ondetti, M. A.; Engel, S. L. Bradykinin analogs containing beta-homoamino acids. *J. Med. Chem.* **1975**, *18*, 761–763.
(20) Steer, D. L.; Lew, R. A.; Perlmutter, P.; Smith, A. I.; Aguilar, M. I. Beta-amino acids: versatile peptidomimetics. *Curr. Med. Chem.* **2002**, *9*, 811–822.
(21) Frackenpohl, J.; Arvidsson, P. I.; Schreiber, J. V.; Seebach, D. The outstanding biological stability of beta- and gamma-peptides toward proteolytic enzymes: an in vitro investigation with fifteen peptidases. *ChemBioChem.* **2001**, *2*, 445–455.
(22) Zubrzak, P.; Williams, H.; Coast, G. M.; Isaac, R. E.; Reyes-Rangel, G.; Juaristi, E.; Zabrocki, J.; Nachman, R. J. Beta-amino acid analogs of an insect neuropeptide feature potent bioactivity and resistance to peptidase hydrolysis. *Biopolymers* **2007**, *88*, 76–82.
(23) Hook, D. F.; Bindschadler, P.; Mahajan, Y. R.; Sebesta, R.; Kast, P.; Seebach, D. The proteolytic stability of "designed" beta-peptides containing alpha-peptide-bond mimics and of mixed alpha, beta-peptides: Application to the construction of MHC-binding peptides. *Chem. Biodiversity* **2005**, *2*, 591–632.

(24) Seebach, D.; Overhand, M.; Kuhnle, F. N. M.; Martinoni, B.; Oberer, L.; Hommel, U.; Widmer, H. β-peptides: Synthesis by Arndt−Eistert homologation with concomitant peptide coupling. Structure determination by NMR and CD spectroscopy and by X-ray crystallography. Helical secondary structure of a beta-hexapeptide in solution and its stability towards pepsin. *Helv. Chim. Acta* **1996**, *79*, 913–941.
(25) Seebach, D.; Gardiner, J. β-Peptidic peptidomimetics. *Acc. Chem. Res.* **2008**, *41*, 1366–1375.
(26) Seebach, D.; Rueping, M.; Arvidsson, P. I.; Kimmerlin, T.; Micuch, P.; Noti, C.; Langenegger, D.; Hoyer, D. Linear, Peptidase-Resistant β^2/β^3-Di-and α/β^3-Tetrapeptide Derivatives with Nanomolar Affinities to a Human Somatostatin Receptor. *Helv. Chim. Acta* **2001**, *84*, 3503–3510.
(27) Hook, D. F.; Gessier, F.; Noti, C.; Kast, P.; Seebach, D. Probing the Proteolytic Stability of β-Peptides Containing α-Fluoro- and α-Hydroxy-β-Amino Acids. *ChemBioChem.* **2004**, *5*, 691–706.
(28) Seebach, D.; Abele, S.; Schreiber, J. V.; Martinoni, B.; Nussbaum, A. K.; Schild, H.; Schulz, H.; Hennecke, H.; Woessner, R.; Bitsch, F. Biological and Pharmacokinetic Studies with β-Peptides. *Chimia* **1998**, *52*, 734–739.
(29) Heck, T.; Limbach, M.; Geueke, B.; Zacharias, M.; Gardiner, J.; Kohler, H.-P. E.; Seebach, D. Enzymatic Degradation of β- and Mixed α,β-Oligopeptides. *Chem. Biodiversity* **2006**, *3*, 1325–1348.
(30) Geueke, B.; Kohler, H. P. Bacterial beta-peptidyl aminopeptidases: on the hydrolytic degradation of beta-peptides. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 1197–1204.
(31) Lelais, G.; Micuch, P.; Josien-Lefebvre, D.; Rossi, F.; Seebach, D. Preparation of Protected β^2- and β^3-Homocysteine, β^2- and β^3-Homohistidine and β^2-Homoserine for Solid-Phase Syntheses. *Helv. Chim. Acta* **2004**, *87*, 3131–3159.
(32) Cowell, S. M.; Lee, Y. S.; Cain, J. P.; Hruby, V. J. Exploring Ramachandran and chi space: conformationally constrained amino acids and peptides in the design of bioactive polypeptide ligands. *Curr. Med. Chem.* **2004**, *11*, 2785–2798.
(33) Hruby, V. J.; Balse, P. M. Conformational and topographical considerations in designing agonist peptidomimetics from peptide leads. *Curr. Med. Chem.* **2000**, *7*, 945–970.
(34) Hruby, V. J. Design in topographical space of peptide and peptidomimetic ligands that affect behavior. A chemist's glimpse at the mind-body problem. *Acc. Chem. Res.* **2001**, *34*, 389–397.
(35) Gibson, S. E.; Guillo, N.; Tozer, M. J. Towards control of c-space: Conformationally constrained analogues of Phe, Tyr, Trp and His. *Tetrahedron* **1999**, *55*, 585–615.
(36) Tourwe, D.; Verschueren, K.; Frycia, A.; Davis, P.; Porreca, F.; Hruby, V. J.; Toth, G.; Jaspers, H.; Verheyden, P.; Van, B. G. Conformational restriction of Tyr and Phe side chains in opioid peptides: information about preferred and bioactive side-chain topology. *Biopolymers* **1996**, *38*, 1–12.
(37) Kazmierski, W.; Hruby, V. J. A new approach to receptor ligand design: synthesis and conformation of a new class of potent and highly selective [mu] opioid antagonists utilizing tetrahydroisoquinoline carboxylic acid. *Tetrahedron* **1988**, *44*, 697–710.
(38) Sánchez-Sánchez, M. M.; Tel-Alberdi, L. M.; Rioseras, M. J.; Rico-Ferreira, M. R.; Bermejo-González, F. The Pictet−Spengler Reaction on L-Histidine. Preparation of Conformationally Restricted (+)-Pilocarpine Analogs. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 191–195.
(39) Kazmierski, W. M.; Yamamura, H. I.; Hruby, V. J. Topographic Design of Peptide Neurotransmitters and Hormones on Stable Backbone Templates—Relation of Conformation and Dynamics to Bioactivity. *J. Am. Chem. Soc.* **1991**, *113*, 2275–2283.
(40) Ballet, S.; Mayorov, A. V.; Cai, M.; Tymecka, D.; Chandler, K. B.; Palmer, E. S.; Rompaey, K. V.; Misicka, A.; Tourwe, D.; Hruby, V. J. Novel selective human melanocortin-3 receptor ligands: use of the 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one (Aba) scaffold. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2492–2498.
(41) Pulka, K.; Feytens, D.; Van den Eynde, I.; De Wachter, R.; Kosson, P.; Misicka, A.; Lipkowski, A.; Chung, N. N.; Schiller, P. W.; Tourwe, D. Synthesis of 4-amino-3-oxo-tetrahydroazepino-[3,4-*b*]indoles: new conformationally constrained Trp analogs. *Tetrahedron* **2007**, *63*, 1459–1466.
(42) Kyle, D. J.; Martin, J. A.; Farmer, S. G.; Burch, R. M. Design and conformational analysis of several highly potent bradykinin receptor antagonists. *J. Med. Chem.* **1991**, *34*, 1230–1233.
(43) Schiller, P. W.; Weltrowska, G.; Berezowska, I.; Nguyen, T. M.; Wilkes, B. C.; Lemieux, C.; Chung, N. N. The TIPP opioid peptide family: development of delta antagonists, delta agonists, and mixed mu agonist/delta antagonists. *Biopolymers* **1999**, *51*, 411–425.
(44) Casimir, J. R.; Iterbeke, K.; Van Den, N. W.; Trescol-Biemont, M. C.; Dumortier, H.; Muller, S.; Gerlier, D.; Rabourdin-Combe, C.; Tourwe, D.; Paris, J. Conformational restriction of the Tyr53 side-chain in the decapeptide HE. *J. Pept. Res.* **2000**, *56*, 398–408.
(45) Ballet, S.; Frycia, A.; Piron, J.; Chung, N. N.; Schiller, P. W.; Kosson, P.; Lipkowski, A. W.; Tourwe, D. Synthesis and biological evaluation of constrained analogues of the opioid peptide H-Tyr-D-Ala-Phe-Gly-NH_2 using the 4-amino-2-benzazepin-3-one scaffold. *J. Pept. Res.* **2005**, *66*, 222–230.
(46) Hintermann, T.; Seebach, D. A useful modification of the Evans auxiliary: 4-isopropyl-5,5-diphenyloxazolidin-2-one. *Helv. Chim. Acta* **1998**, *81*, 2093–2126.
(47) Seebach, D.; Beck, A. K.; Capone, S.; Deniau, G.; Groselj, U.; Zass, E. Enantioselective Preparation of β-Amino Acid Derivatives for β-Peptide Synthesis. *Synthesis* **2009**, *2009*, 1–32.
(48) Demaegdt, H.; Vanderheyden, P.; De Backer, J. P.; Mosselmans, S.; Laeremans, H.; Le, M. T.; Kersemans, V.; Michotte, Y.; Vauquelin, G. Endogenous cystinyl aminopeptidase in Chinese hamster ovary cells: characterization by [^{125}I]Ang IV binding and catalytic activity. *Biochem. Pharmacol.* **2004**, *68*, 885–892.
(49) Demaegdt, H.; Lenaerts, P. J.; Swales, J.; De Backer, J. P.; Laeremans, H.; Le, M. T.; Kersemans, K.; Vogel, L. K.; Michotte, Y.; Vanderheyden, P.; Vauquelin, G. Angiotensin AT4 receptor ligand interaction with cystinyl aminopeptidase and aminopeptidase N: [^{125}I]angiotensin IV only binds to the cystinyl aminopeptidase apo-enzyme. *Eur. J. Pharmacol.* **2006**, *546*, 19–27.
(50) Irons, D. W.; Davison, J. M.; Baylis, P. H. Evaluation of enzyme inhibitors of cystinyl aminopeptidase and application to the measurement of immunoreactive atrial natriuretic peptide in human pregnancy. *Clin. Chim. Acta* **1994**, *231*, 185–191.
(51) Sardinia, M. F.; Hanesworth, J. M.; Krishnan, F.; Harding, J. W. AT4 receptor structure-binding relationship: N-terminal-modified angiotensin IV analogues. *Peptides* **1994**, *15*, 1399–1406.
(52) Axen, A.; Andersson, H.; Lindeberg, G.; Ronnholm, H.; Kortesmaa, J.; Demaegdt, H.; Vauquelin, G.; Karlen, A.; Hallberg, M. Small potent ligands to the insulin-regulated aminopeptidase (IRAP)/AT(4) receptor. *J. Pept. Sci.* **2007**, *13*, 434–444.
(53) Demaegdt, H.; Lukaszuk, A.; Buyser, E. D.; Backer, J.-P. D.; Szemenyei, E.; Tóth, G.; Chakravarthy, S.; Panicker, M.; Michotte, Y.; Tourwé, D.; Vauquelin, G. Selective labelling of IRAP by the tritiated AT4 receptor ligands [3H]Angiotensin IV and its stable analog [3H]AL-11. **2009**, submitted for publication .
(54) Olsen, J.; Cowell, G. M.; Konigshofer, E.; Danielsen, E. M.; Moller, J.; Laustsen, L.; Hansen, O. C.; Welinder, K. G.; Engberg, J.; Hunziker, W.; et al. Complete amino acid sequence of human intestinal aminopeptidase N as deduced from cloned cDNA. *FEBS Lett.* **1988**, *238*, 307–314.
(55) Vanderheyden, P. M. L.; Fierens, F. L. P.; De Backer, J. P.; Fraeyman, N.; Vauquelin, G. Distinction between surmountable and insurmountable selective AT1 receptor antagonists by use of CHO-K1 cells expressing human angiotensin II AT1 receptors. *Br. J. Pharmacol.* **1999**, *126*, 1057–1065.
(56) Yung-Chi, C.; Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.