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# Conformational constraints in angiotensin IV to probe the role of Tyr$^2$, Pro$^5$ and Phe$^6$

**Aneta Lukaszuk,[a] Heidi Demaegdt,[b] Isabelle Van den Eynde,[a] Patrick Vanderheyden,[b] Georges Vauquelin[b] and Dirk Tourwé[a]***

**The aromatic amino acids Tyr and Phe in angiotensin IV (Ang IV) were conformationally constrained by the use of β-Me substituted analogs, or cyclic constrained analogs. None of these modifications was allowed for Tyr[1], while only *e*-β-MePhe[6] substitution resulted in an AngIV analog with high IRAP potency and selectivity *versus* AP-N or the $AT_1$ receptor. This indicates an important role of the orientation of the Phe[6] for inducing selectivity. Pro[5] replacement with 2-aminocyclopentanecarboxylic acid maintained IRAP potency and abolished $AT_1$ affinity. These results confirm the importance of conformational constrained amino acids to generate selectivity in bioactive peptides. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.**

**Keywords:** angiotensin IV; insulin-regulated aminopeptidase; $AT_4$ receptor; conformational constraints; aminopeptidase N

## Introduction

Angiotensin IV (Ang IV: H-Val-Tyr-Ile-His-Pro-Phe-OH) is one of the bioactive degradation products in the renin-angiotensin system [1]. This hexapeptide which has ability to improve learning and memory [2–7] is also known to stimulate DNA synthesis [8] and has vascular and renal actions [9]. The use of Ang IV analogs to treat dementia, seizure and ischemia has been suggested [10]. The $AT_4$ receptor, suggested to be cystinyl aminopeptidase (EC3.4.11.3, CAP, also denoted as IRAP) was described as the specific high-affinity binding site for Ang IV [11]. IRAP is a membrane-associated zinc-dependent metallopeptidase of the M1 family. It was proposed that $AT_4$ receptor ligands exert their biological effects by inhibition of the IRAP catalytic activity [12,13], resulting in a delayed catabolism of other bioactive peptides (e.g. vasopressin, oxytocin, Lys-bradykinin, angiotensin III, Met-enkephalin, dynorphin A 1–8, neurokinin A, neuromedin B, somatostatin and cholecystokinin 8) [14–16] or by triggering intracellular processes in the event that IRAP also acts as a cellular receptor [13,17]. There is also evidence that in addition to IRAP, aminopeptidase N (EC3.4.11.2, AP-N) may also be a mediator for the effects of Ang IV [18,19]. Hence, AP-N is susceptible to represent an alternative target for Ang IV, especially as it was reported to undergo homodimerization and to trigger intracellular responses after the binding of antibodies [20]. This peptidase preferentially cleaves peptides with *N*-terminal neutral or basic amino acids [21]. However, 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. Recent finding, however, challenge the assumption that IRAP is the $AT_4$ receptor [10,22]. The tritium labeled metabolically stable Ang IV analog [$^3$H]AL-11 allowed the labeling of native IRAP under physiologically relevant conditions, i.e. without addition of metal chelators which result in the generation of the apo-form of IRAP, and clearly showed that native Ang IV inhibits the catalytic activity of IRAP with low potency, and that the active and apo-forms of IRAP have a distinct pharmacological profile [23].

Using a β-homo-amino acid scan, we were able to identify AL-11: H-(*R*)$\beta^2$hVal-Tyr-Ile-His-Pro-$\beta^3$hPhe-OH as a potent and metabolically stable Ang IV analog with high selectivity for IRAP *versus* AP-N and *versus* the $AT_1$ receptor [24]. We showed subsequently, that the replacement of His$^4$-Pro$^5$ in Ang IV by the conformational constrained dipeptide analog 2-(4-amino-1,2,4,5-tetrahydroindolo[2,3-c]azepin-3-one)acetic acid (Aia-Gly) together with incorporation of (*R*)-$\beta^2$hVal at the *N*-terminus resulted in potent, selective and metabolically stable analog H-(*R*)-$\beta^2$hVal-Tyr-Ile-Aia-Gly-Phe-OH (AL-40) [25].

As part of our ongoing studies on the role of the individual amino acid residues an Ang IV, while studying the inhibition of IRAP activity rather than the binding to the apo-enzyme as was done in previous studies [26–30], we now report the results of the

* *Correspondence to: Dirk Tourwé, Department of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium. E-mail: datourwe@vub.ac.be*

a *Department of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium*

b *Department of Molecular and Biochemical Pharmacology, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium*

**Abbreviations used:** *TES, triethylsilane; TFMSA, trifluoromethanesulfonic acid; Dmt, 2′,6′-dimethyltyrosine; Hat, 2-amino-6-hydroxy-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid; 6-Htc, 1,2,3,4-tetrahydro-6-hydroxyisoquinoline-3-carboxylic acid; 7-Htc, 1,2,3,4-tetrahydro-7-hydroxyisoquinoline-3-carboxylic acid; eβMeTyr, erythro-β-methyl-Tyr; tβMeTyr, threo-β-methyl-Tyr; Atc, 2-amino-1,2,3,4-tetrahydro-naphthalene-2-carboxylic acid; Aic, 2-aminoindane-2-carboxylic acid; eβMePhe, erythro-β-methyl-Phe; tβMePhe, threo-β-methyl-Phe; DMEM, Dulbecco's modified essential medium; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; L-Leu-pNA, L-leucine-p-nitroanilide; HEK293 cells, human embryonic kidney cells; CHO-K1 cells, Chinese hamster Ovary cells; IRAP, insulin-regulated aminopeptidase; 1,10-Phe, 1,10 phenanthrolin.*

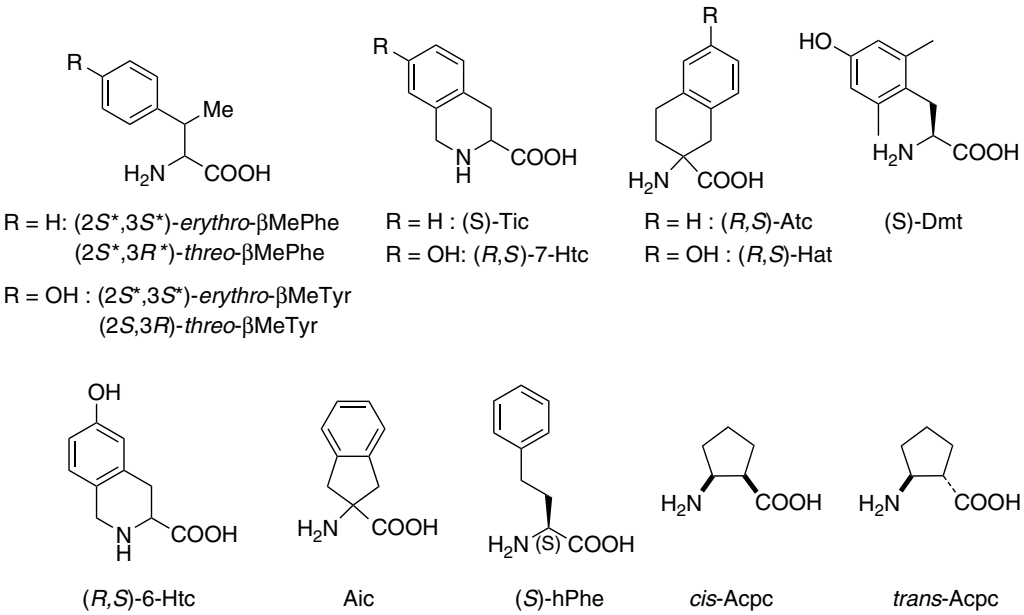


**Figure 1.** Structure of the amino acids used as Tyr, Pro and Phe replacements in Ang IV.

modification of Tyr[2], Pro[5] and Phe[6]. Introducing conformationally, constrained amino acids in a peptide has been shown to be a powerful method to confer selectivity and stability [31–40]. Constraints may eventually improve potency by reducing the entropy change during binding to the active site of a receptor or enzyme, and may also lead to increased metabolic stability and selectivity [41]. In this study we used Dmt, Hat, *eβ*MeTyr, *tβ*MeTyr, 6-Htc, 7-Htc as Tyr replacements (Figure 1). These conformationally constrained amino acids are known to induce receptor selectivity and metabolic stability [40]. As Pro analogs, we investigated the various stereoisomers of *cis*- and *trans*-aminocyclopentanecarboxylic acid (Acpc). As Phe substitutions, homo-Phe and conformationally constrained Phe analogs Atc, Aic, *eβ*MePhe, *tβ*MePhe, Tic have been used. Those residues are known to have profound effects on the affinity, selectivity and ability of the ligands to generate intracellular responses [39,40,42].

## Materials and Methods

### Materials

L-Leu-*p*NA was obtained from Sigma–Aldrich (Bornem, Belgium) and *p*-nitroaniline from VWR International (Leuven, Belgium). Monoiodinated Ang IV was obtained by iodination of Tyr[4] using Iodogen iodination reagent from Pierce (Erembodegem, Belgium) and $^{125}$I from MP Biomedicals (Asse, Belgium) and was isolated on a C18 monomeric 120 Å RP-HPLC column (25 cm × 4.6 mm, 5 µm) from Grace Vydac (Hesperia, CA, USA). It was stored at −20 °C in 10 mM $KH_2PO_4$, pH 6.5 containing 45% ethanol. CHO-K1 cells were kindly obtained from the Pasteur Institute (Brussels, Belgium).

Fmoc-Phe-Wang- and 2-chlorotrityl chloride resins, $CH_2Cl_2$, TES, Fmoc-protected amino acids were obtained from Fluka (Bornem, Belgium), TBTU was from Senn Chemicals (Gentilly, France), DMF and DIPEA were from Sigma-Aldrich. Conformational constrained amino acids were synthesized in Department of Organic Chemistry, Vrije Universiteit Brussel (Brussels, Belgium).

Analytical RP-HPLC was performed using an Agilent 1100 Series system (Waldbronn, Germany) with a Discovery® BIO Wide Pore (Supelco, Bellefonte, PA, USA) RP C18 column (25 cm × 4.6 mm, 5 µm, 300 Å) using UV detection at 215 nm. The mobile phase was water/acetonitrile, containing 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 RP C18 column (Supelco, Discovery® BIO Wide Pore 25 cm × 21.2 mm, 10 µm, 300 Å) was used under the same conditions as the analytical RP-HPLC, but with a flow rate of 20 ml/min. MS was recorded on a VG Quattro II spectrometer using ESI, data collection was done with Masslynx software.

### Amino Acid Synthesis

All conformationally constrained amino acids (Figure 1) were previously prepared in our laboratory: *rac-β* MePhe [43] and *rac-β* MeTyr [44], *rac*-6-Htc [45], *rac*-7-Htc [46], L-Tic [47], *rac*-Atc [47], *rac*-Hat [48] and Aic [47]. L-Dmt and L-hPhe are commercially available. *Rac-cis-* and *rac-trans-Acpc* were a generous gift from F. Fülöp (Szeged, Hungary) [49].

### Peptide Synthesis

Synthesis of all peptides was carried out by solid phase peptide synthesis using *N*-9-fluorenylmethoxycarbonyl (Fmoc) *N*-terminal protected amino acids. The peptides were synthesized on Fmoc-Phe-Wang resin (0.76 mmol/g) (peptides: **1 to 4, 5, 6**) and 2-chlorotrityl chloride resin (1.5 mmol/g) (peptides: **9 to 14**) and side chain protection groups were: Tyr(*t*-Bu), His(Trt) for Fmoc strategy and Tyr(2,6-di-Cl-Bzl).

*Loading of the 2-chlorotrityl chloride resin*

The amino acid (3 equiv. relative to the resin) and 6 equiv. of DIPEA were dissolved in $CH_2Cl_2$ (10 ml per 1 g of the resin), containing, if necessary, a small amount of DMF to facilitate dissolution of the amino acid. The 3-chlorotrityl chloride resin was pre-swollen in $CH_2Cl_2$ for 1 h, and after that the solution containing the amino acid



  

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was added and the resin was shaken for 30–120 min. Afterwards the resin was washed 3× with $CH_2Cl_2$/MeOH/DIPEA (17 : 2 : 1), then 2× DMF and 3×$CH_2Cl_2$. Deprotection of the first amino acid was prolonged (2 × 30 min).

*Coupling procedure*

Amino acid (3 equiv.), TBTU (3 equiv.), and DIPEA (6 equiv.) were mixed together and left for 3 min for activation. The solution was added to the resin and shaken for 2 h, except for coupling to the amino groups of the $C^{\alpha}$-tetrasubstituted residues and to the secondary amines of Tic and 6- or 7-Htc which were reacted overnight [50,51].

*Deprotection*

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.

*Cleavage from the resin*

Peptides were cleaved from the Wang and 2-chlorotrityl chloride resin by treating with TFA/$H_2O$/TES (95 : 2.5 : 2.5) for 2 h.

*Purification*

Peptides were purified by RP-HPLC on a SUPELCO Discovery® BIO Wide Pore preparative C18 column. Each peptide was at least 98% pure as assessed by analytical RP-HPLC. The molecular weights were confirmed by ESI-MS (Table 1).

## Biological Evaluation Procedures

### Cell Culture, Transient Transfection and Membrane Preparation

CHO-K1, CHO-$AT_1$ and HEK293 cell lines were cultured in 75 and 500 $cm^2$ culture flasks in 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 non-essential amino acids, 1 mM sodium pyruvate and 10% (v/v) fetal bovine serum (Invitrogen). 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 [52] carrying the complete human aminopeptidase N (AP-N) cDNA [53]. The transient transfection was performed as described previously with 8 µl/ml LipofectAMINE (Invitrogen) and 1 µg/ml plasmid DNA [53]. After transfection, the cells were cultured for 2 more days. IRAP and AP-N transfected HEK293 cells displayed a 8 and 10 times higher enzyme activity than non-transfected cells.

CHO-K1 cell and transfected HEK293 cell membranes were prepared as described previously. 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 (30 000 *g* at 4 °C). The pellet was resuspended in 50 mM Tris–HCl, centrifuged (30 min 30 000 *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-Leu-*p*NA [54] 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 Me sulfonyl fluoride. The incubation mixture comprised 50 µl membrane homogenate, 200 µl L-Leu-*p*NA (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 Tecan M200 96-well-reader. The enzymatic activities were calculated by linear regression analysis of the time-wise increase in the absorption.

### Stability Experiments

The stability of compounds 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). Pre-incubations 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 non-specific binding). Then the binding assay was initiated by adding 50 µl 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-Phe, the final [$^3$H]Ang IV concentration was 3 nM and the final unlabeled ligand concentrations are indicated in Figure 4. After incubation, the mixture was vacuum filtered using an Inotech 24 well cell-harvester through GF/B glass fiber filters (Whatman) pre-soaked in 1% (w/v) BSA. After drying, the radioactivity retained in the filters was measured [after adding 3 ml scintillation liquid (Optiphase Hisafe)] using a beta-counter (Perkin-Elmer). [$^3$H]Ang IV was characterized previously [22].

### $AT_1$ Receptor Binding

CHO-K1 cells stably expressing the human angiotensin II $AT_1$ receptor (CHO-$AT_1$) [55] were used to test the affinity of the compounds to the $AT_1$ 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. Non-specific 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 (Optiphase Hisafe, Perkin-Elmer).

### Data Analysis

All experiments were performed at least 3 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].

## Results

### Synthesis

All conformationally constrained amino acids (Figure 1) were previously prepared in our laboratory according to the published procedures [43–48], except the Acpc isomers which were a gift from F. Fülöp [49]. L-Dmt and L-hPhe are commercially available. Peptide synthesis was performed on solid phase using the Fmoc strategy. As Phe is the first amino acid in the peptide sequence, the Fmoc-protected Phe analogs were loaded onto 2-chlorotrityl chloride resin. In all other case, a preloaded Fmoc-Phe-Wang resin was used. After cleavage from the resin, the crude peptides were purified by HPLC and characterized by MS (Table 1). As many constrained amino acids were used as racemates, after peptide synthesis two diastereoisomeric peptides were obtained. The separated isomers of unknown absolute configuration were labeled as 'a' or 'b', depending on the elution order in the HPLC. No attempts to assign absolute configuration were made because no analog turned out to be substantially better than previous ones.

### Enzyme Activity

Measurement 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-Leu-*p*NA into L-leucine and *p*-nitroaniline in the presence of increasing concentrations of the Ang IV analogs [54]. Formation of *p*-nitroaniline was followed at 37 °C by measuring the absorption at 405 nm. The enzymatic activities were calculated by linear regression analysis of the time-wise increase in the absorption. The Ang IV analogs produced a concentration-dependent inhibition of the catalytic activity. The results of the inhibition of enzyme activity by the Ang IV analogs are presented in Table 2 and selected curves are shown in Figure 2.

All replacements of Tyr$^2$ by constrained analogs resulted in a large drop in IRAP inhibitory potency, indicating the essential role

**Table 1.** Analytical data of the peptide analogs

| Number | Compound | $t_R$ (min) | (M+H$^+$) calculated | (M+H$^+$) found | Yield (%) |
|---|---|---|---|---|---|
| **1** | H-Val-**Dmt**-Ile-His-Pro-Phe-OH | 9.65 | 803.43 | 803.53 | 33 |
| **2a** | H-Val-**Hat**-Ile-His-Pro-Phe-OH | 9.68 | 801.42 | 801.33 | 11 |
| **2b** | H-Val-**Hat**-Ile-His-Pro-Phe-OH | 10.07 | 801.42 | 801.33 | 24 |
| **3a** | H-Val-***e*β*MeTyr**-Ile-His-Pro-Phe-OH | 9.64 | 789.42 | 789.55 | 7 |
| **3b** | H-Val-***e*β*MeTyr**-Ile-His-Pro-Phe-OH | 10.57 | 789.42 | 789.34 | 5 |
| **4** | H-Val-**(*S*)-*t*β*MeTyr**-Ile-His-Pro-Phe-OH | 10.15 | 789.42 | 789.33 | 2 |
| **5a** | H-Val-**6-Htc**-Ile-His-Pro-Phe-OH | 9.53 | 787.40 | 787.32 | 9 |
| **5b** | H-Val-**6-Htc**-Ile-His-Pro-Phe-OH | 9.96 | 787.40 | 787.32 | 22 |
| **6a** | H-Val-**7-Htc**-Ile-His-Pro-Phe-OH | 9.52 | 787.40 | 787.80 | 3 |
| **6b** | H-Val-**7-Htc**-Ile-His-Pro-Phe-OH | 9.72 | 787.40 | 787.80 | 20 |
| **7a** | H-Val-Tyr-Ile-His-***trans*-Acpc**-Phe-OH | 9.40 | 789.42 | 789.35 | 10 |
| **7b** | H-Val-Tyr-Ile-His-***trans*-Acpc**-Phe-OH | 10.18 | 789.42 | 789.35 | 18 |
| **8a** | H-Val-Tyr-Ile-His-***cis*-Acpc**-Phe-OH | 9.27 | 789.42 | 789.30 | 10 |
| **8b** | H-Val-Tyr-Ile-His-***cis*-Acpc**-Phe-OH | 10.21 | 789.42 | 789.30 | 1 |
| **9a,b** | H-Val-Tyr-Ile-His-Pro-**Atc-OH** | 10.40 | 801.42 | 801.30 | 7 |
| **10** | H-Val-Tyr-Ile-His-Pro-**Aic-OH** | 9.00 | 787.40 | 787.30 | 13 |
| **11a** | H-Val-Tyr-Ile-His-Pro-***e*β*MePhe-OH** | 9.91 | 789.42 | 789.30 | 10 |
| **11b** | H-Val-Tyr-Ile-His-Pro-***e*β*MePhe-OH** | 10.40 | 789.42 | 789.30 | 5 |
| **12** | H-Val-Tyr-Ile-His-Pro-(***S*)-*t*β*MePhe-OH** | 9.69 | 789.42 | 789.36 | 28 |
| **13** | H-Val-Tyr-Ile-His-Pro-**Tic-OH** | 9.94 | 787.40 | 787.73 | 3 |
| **14** | H-Val-Tyr-Ile-His-Pro-**hPhe-OH** | 10.00 | 789.42 | 789.30 | 9 |
| **15** | H-Val-Tyr-Ile-His-Pro-β$^3$**hPhe**-OH | 9.58 | 789.42 | 789.30 | 6 |

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



  

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**Table 2.** Inhibition of enzyme activity by Ang IV analogs in membranes of transfected HEK 293 cells

| Number | Compound | HEK293 + IRAP $pK_i \pm D$ | HEK293 + AP-N $pK_i \pm SD$ | Selectivity $K_i$(AP-N)/$K_i$(IRAP) |
|---|---|---|---|---|
| **1** | H-Val-**Dmt**-Ile-His-Pro-Phe-OH | 5.51 ± 0.02 | 4.34 ± 0.11 | 14.8 |
| **2a** | H-Val-**Hat**-Ile-His-Pro-Phe-OH | 4.73 ± 0.12 | 3.70 ± 0.35 | 10.7 |
| **2b** | H-Val-**Hat**-Ile-His-Pro-Phe-OH | 5.24 ± 0.02 | 4.35 ± 0.04 | 7.8 |
| **3a** | H-Val-***e*β*MeTyr**-Ile-His-Pro-Phe-OH | 5.69 ± 0.06 | 3.84 ± 0.07 | 70.8 |
| **3b** | H-Val-***e*β*MeTyr**-Ile-His-Pro-Phe-OH | 5.20 ± 0.02 | 3.66 ± 0.17 | 34.7 |
| **4** | H-Val-**(*S*)-*t*β*MeTyr**-Ile-His-Pro-Phe-OH | 5.21 ± 0.05 | 4.35 ± 0.09 | 7.2 |
| **5a** | H-Val-**6-Htc**-Ile-His-Pro-Phe-OH | 4.90 ± 0.01 | 3.48 ± 0.01 | 26.3 |
| **5b** | H-Val-**6-Htc**-Ile-His-Pro-Phe-OH | 4.40 ± 0.08 | 3.42 ± 0.14 | 9.5 |
| **6a** | H-Val-**7-Htc**-Ile-His-Pro-Phe-OH | 5.06 ± 0.02 | 4.21 ± 0.41 | 7.1 |
| **6b** | H-Val-**7-Htc**-Ile-His-Pro-Phe-OH | 4.81 ± 0.13 | 3.60 ± 0.47 | 16.2 |
| **7a** | H-Val-Tyr-Ile-His-***trans*-Acpc**-Phe-OH | 7.08 ± 0.14 | 5.14 ± 0.07 | 87.1 |
| **7b** | H-Val-Tyr-Ile-His-***trans*-Acpc**-Phe-OH | 6.78 ± 0.02 | 5.12 ± 0.15 | 45.7 |
| **8a** | H-Val-Tyr-Ile-His-***cis*-Acpc**-Phe-OH | 7.30 ± 0.02 | 5.76 ± 0.03 | 34.7 |
| **8b** | H-Val-Tyr-Ile-His-***cis*-Acpc**-Phe-OH | 6.89 ± 0.02 | 5.43 ± 0.30 | 28.8 |
| **9a,b** | H-Val-Tyr-Ile-His-Pro-**Atc-OH** | 6.73 ± 0.09 | 5.41 ± 0.04 | 20.9 |
| **10** | H-Val-Tyr-Ile-His-Pro-**Aic-OH** | 6.67 ± 0.18 | 5.55 ± 0.03 | 13.2 |
| **11a** | H-Val-Tyr-Ile-His-Pro-***e*β*MePhe-OH** | 6.76 ± 0.01 | 5.67 ± 0.08 | 12.3 |
| **11b** | H-Val-Tyr-Ile-His-Pro-***e*β*MePhe-OH** | 7.12 ± 0.08 | 5.18 ± 0.03 | 87.1 |
| **12** | H-Val-Tyr-Ile-His-Pro-(***S*)-*t*β*MePhe-OH** | 6.92 ± 0.07 | 5.53 ± 0.09 | 24.5 |
| **13** | H-Val-Tyr-Ile-His-Pro-**Tic-OH** | 6.76 ± 0.03 | 6.20 ± 0.80 | 3.6 |
| **14** | H-Val-Tyr-Ile-His-Pro-**hPhe-OH** | 7.00 ± 0.07 | 5.27 ± 0.06 | 53.7 |
| **15** | H-Val-Tyr-Ile-His-Pro-$\beta^3$**hPhe-OH** | 7.69 ± 0.12 | 5.31 ± 0.40 | 239.9 |
| **AL-11** | **H-(*R*)$\beta^2$hVal**-Tyr-Ile-His-Pro-$\beta^3$**hPhe-OH** | 7.56 ± 0.21 | 5.23 ± 0.04 | 213.8 |
| **Ang IV** | H-Val-Tyr-Ile-His-Pro-Phe-OH [57] | 7.25 ± 0.14 | 6.08 ± 0.02 | 14.8 |

The separated isomers of unknown absolute configuration were labeled as 'a' or 'b', depending on the elution order in the HPLC.

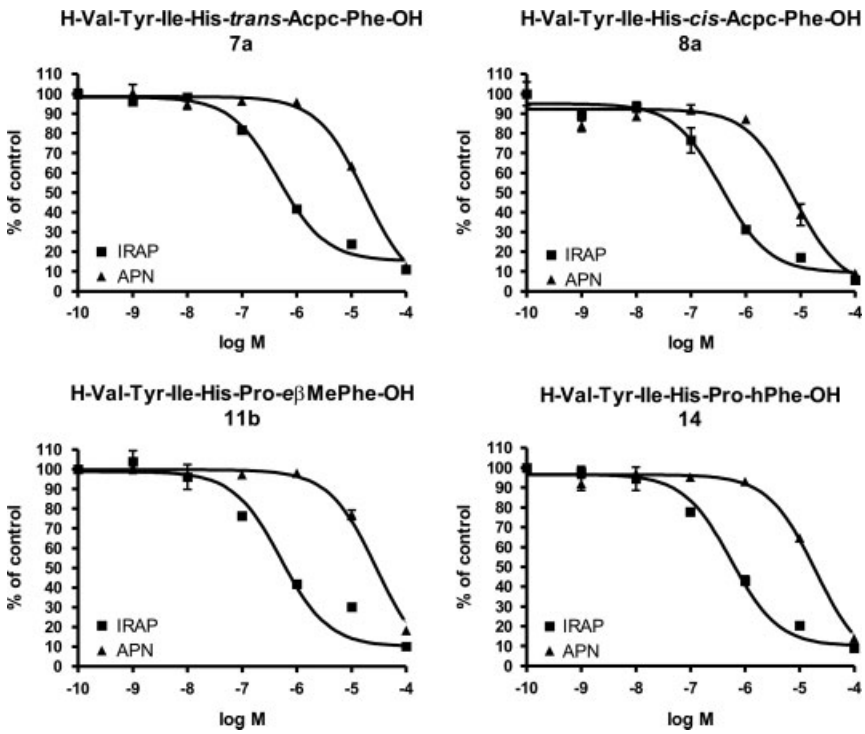


**Figure 2.** Inhibition of the enzymatic activity in membrane homogenates of HEK293 cells transfected with human IRAP (■) or AP-N (▲) (corresponded to 1.5 × $10^5$ cells/incubation) with compounds. Cells were incubated at 37 °C with 1.5 mM L-Leu-*p*NA in the absence (control activity) or presence of increasing concentrations of compound. The rate constants of L-Leu-*p*NA cleavage (corresponding to enzyme activity and expressed as a percentage of control) were calculated by linear regression analysis of the absorption (at 405 nm) *versus* 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 2.



  

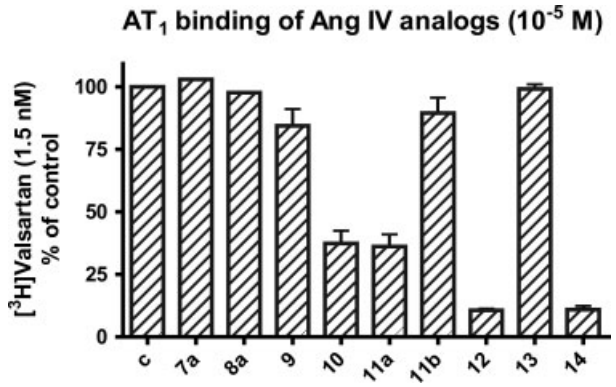


**Figure 3.** Affinity of the compounds to the $AT_1$ receptor. CHO cells stably transfected with the $AT_1$ receptor (CHO-$AT_1$) were incubated for 40 min at 37 °C with concentrations of compound $10^{-5}$ and 1.5 nM [$^3$H]Valsartan. Data refer to specific binding of [$^3$H]Valsartan (expressed as percent of control binding), calculated by subtracting non-specific binding in the presence of 1 μM Candesartan from total binding.

of this residue. As none of the analogs was potent, no further tests were performed.

One *trans*- and one *cis*-isomer of [Acpc$^5$]Ang IV **7a** and **8a** displayed high IRAP inhibitory potency and selectivity against AP-N. Analog **8a** was equipotent with Ang IV for inhibition of IRAP activity, and was slightly more selective. These two analogs were selected for further determination of their stability and $AT_1$ receptor affinity.

In general, most Phe replacements were rather well tolerated, although neither of the new analogs was more potent than Ang IV, nor more potent or selective than the [$\beta^3$hPhe]Ang IV analog **15** [24]. The incorporation of *e*-$\beta$ MePhe produced peptide **11b** which is very active and selective for IRAP *versus* AP-N. The two best ligands (**11b** and **14**), as well as **7a** were selected for additional evaluation.

## Binding to the At$_1$ Receptor

Affinity of the compounds to the $AT_1$ receptor was tested on intact CHO-$AT_1$ cells. Cells were incubated with different concentrations of compound and the radio-labeled AT1 receptor-selective non-peptide antagonist [$^3$H]Valsartan (1.5 nM) for 40 min at 37 °C. Non-specific binding was measured with Candesartan (1 μM). After the incubation, cells were washed on ice and the remaining radioactivity was measured. The two [Acpc$^5$]Ang IV analogs **7a** and **8a** did not displace the radioligand from the $AT_1$ receptor at a $10^{-5}$ M concentration, indication that they have a good selectivity for IRAP. The $AT_1$ affinity of the various Phe$^6$ replacements showed a strong dependence on the type of residue while the Tic residue in **13** and the *e*$\beta$MePhe residue in **11b** induced low affinity for $AT_1$, the *t*$\beta$MPhe residue in **12** and the hPhe residue in **14** were quite well tolerated by the $AT_1$ receptor (Figure 3).

### Stability Experiments

Stability experiments were performed in membrane homogenates of CHO-K1 cells which contain endogenous IRAP. Pre-incubations 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 EDTA and 1,10-phenantrolin. Then, a competition-binding assay was performed by adding [$^{125}$I]Ang IV (1 nM) for 30 min at 37 °C. Non-specific binding was measured with Ang IV (10 μM). EDTA and 1,10-phenantrolin are well-known for their ability to block IRAP, AP-N as well as the activity of other metalloproteinases [58]. Hence, when both chelators are present during the pre-incubation phase, no degradation of Ang IV and its analogs is supposed to take place. Compared to the competition curves obtained under such conditions, the curves will be shifted to the right for analogs that undergo degradation when the pre-incubation is carried out without these chelators, while the curves for the metabolically stable analogs will not undergo such shift [27].

These tests indicated a substantial metabolic breakdown for **7a, 11b** and **14** (Figure 4). These results were expected, in view of our previous results [24] that already showed that only *N*-terminal modifications provide stable peptides.

## Discussion

The introduction of conformational restrictions into biological active peptides may improve potency by reducing entropy change during binding to the active site of the receptor. Incorporation of such restricted amino acids may lead to increased metabolic stability and selectivity. In this study, we concentrated on Tyr$^2$, Pro$^5$ and Phe$^6$ structural modifications. The literature already reports analogs of Ang IV in which these amino acid in the sequence have been varied [9,26–28,30].

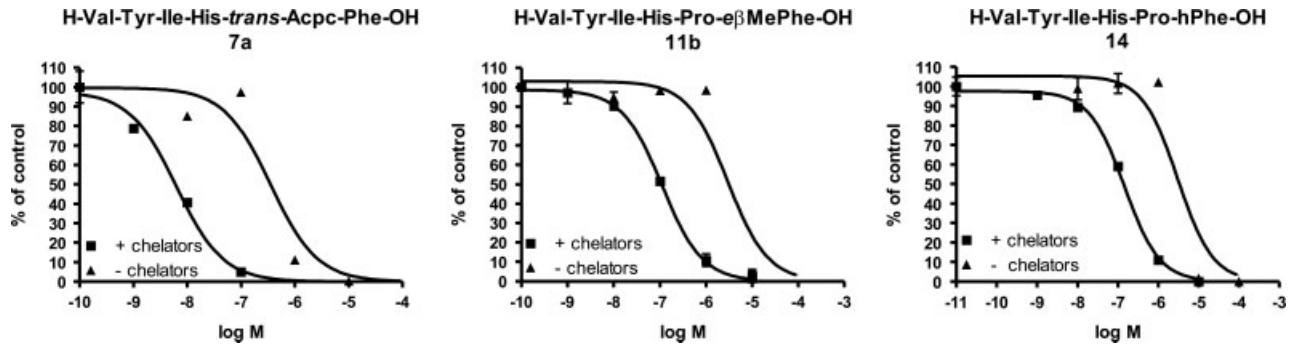


**Figure 4.** Stability experiments performed in membrane homogenates of CHO-K1. Membranes were pre-incubated for 40 min at 37 °C with increasing concentrations of compounds **7a, 11b** and **14** in the presence (■) or absence (▲) of chelators. Then, cells were incubated for 30 min with 1 nM [$^{125}$I]Ang IV. Data refer to specific binding of [$^{125}$I]Ang IV (expressed as percent of control binding), calculated by subtracting non-specific binding in the presence of 10 μM unlabeled Ang IV from total binding.



  

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Previous studies on D-amino acid and glycine scans of Ang IV have indicated that the side chain of Tyr and its L-configuration are essential [29]. Subsequently, it was demonstrated that the electron rich aromatic ring is important [28]. These studies, however, relied on a competition-binding assay with $^{125}$I-Ang IV to the apo-enzyme. More recently, the replacement of the Val-Tyr *N*-terminal dipeptide in Ang IV by a $\gamma$-turn mimetic was shown to result in a large decrease in potency for the inhibition of IRAP enzymatic activity [26]. Our results show that none of the cyclic Tyr analogs is tolerated by IRAP, and that even Me substitutions at the aromatic ring or at the $\beta$-carbon is detrimental for enzyme activity. Taken together, the Tyr analogs used allow the side chain to adopt each of the three staggered conformations at $\chi^1$. While the cyclic Tyr analogs Htc and Hat also constrain the $\chi^2$ angle and limit the orientation of the phenolic ring, this is not the case for the $\beta$MeTyr isomers. It must be concluded that the reason for the loss in IRAP activity should be ascribed to the Tyr-binding site in IRAP that is not able to accommodate any of the modifications used.

In contrast to position 2, deletion of the Pro ring, or inversion of its configuration resulted in maintained affinity for the apo-enzyme [29]. Moreover, the His-Pro-Phe *C*-terminus could be replaced by an *o*-aminomethylphenylacetic acid moiety with high IRAP inhibitory potency. We previously demonstrated that Pro could be replaced by its $\beta^2$ or $\beta^3$ homologs with limited loss in IRAP activity [24]. We now selected the 2-aminocyclopentanecarboxylic acid stereoisomers Acpc, which are $\beta$-amino acid analogs of Pro, but having a primary amine and thus do not induce *cis-trans* isomerization of the amide bond. The Acpc isomers have been successfully used as Pro substitutes in a variety of applications [49]. Our results, using the Acpc stereoisomers confirm that the *trans* as well as the *cis*-Acpc isomers, **7a** and **8a**, can substitute Pro without loss of potency. Moreover, this substitution resulted in compounds with no affinity for the $AT_1$ receptor at $10^{-5}$ M. As expected, the analogs **7a** and **8a** are not metabolically stable due to a lack of modification at the *N*-terminus [24,25]. As none of these analogs was substantially better than previously published ones, no attempts to assign absolute configuration was made, nor was a $\beta^2$hVal introduced at *N*-terminus to induce metabolic stability.

It was demonstrated that the *C*-terminal part of Ang IV plays a less critical role in $AT_4$ receptor affinity [26,29]. However, we demonstrated [24] that changes in the Phe residue might have a great influence on $AT_4$ *versus* $AT_1$ receptor selectivity. It was shown that, after the replacement of the various amino acids of Ang IV by the corresponding constraints, all analogs were still able to inhibit the IRAP activity more potently than the AP-N activity (Table 2). Phe replacements gave very potent IRAP inhibitors. The incorporation of Atc and Aic residues at position 6, which have preferences for a gauche conformations [gauche(−) for the (*S*) and gauche(+) for the (*R*) enantiomer] and orient the aromatic ring perpendicular to the plane of the adjacent backbone atoms [51] lead to slight losses in IRAP activity, as is the incorporation of the Tic residue which is shown to adopt a gauche(+) conformation [51]. The incorporation of the $\beta$ MePhe isomers resulted in peptides **11a,b** and **12** which are active and selective for IRAP *versus* AP-N. The *threo* isomer is known to strongly reduce the occurrence of the gauche(+) rotamer and to have a slight preference for the *trans* conformation. The most potent isomer has the *e* configuration. This isomer is known to eliminate the *trans* rotamer and to prefer gauche conformations. Therefore, one might speculate that the bioactive conformation of Phe$^6$ in Ang IV adopts a gauche(−) conformation. *e*-$\beta$ MePhe resulted in peptide **11b** which is active and selective for IRAP *versus* AP-N. Also, homo-Phe incorporation gave a potent compound **14** (Figure 2). The tendency for metabolic cleavage of the selected compounds was compared. None of the tested compounds is protected against metabolic breakdown (Figure 4). This is consistent with our earlier reports [24,25] that only *N*-terminal modifications result in metabolic stability. As a final point, the affinity of compounds with Phe replacement for the $AT_1$ receptor was studied. It has been known that Phe$^8$ of Ang II plays a crucial role in binding to the $AT_1$ receptor and even minor changes in its structure have marked effects on biological activity [59]. The *C*-terminus of Ang II requires an appropriate orientation of the position 8 carboxyl group relative to the aromatic ring of the Phe residue [59]. This knowledge about structure of the ligand-binding site of the $AT_1$ receptor provides a rationale for their distinct pharmacological profile [59]. Here, the importance of the orientation is also clearly illustrated by the very low affinity of **9**, **11b** and **13** for the $AT_1$ receptor (Figure 3). However, not all *C*-terminal changes decreased $AT_1$ receptor binding. Atc incorporation (**9**) resulted in less affinity than replacing with Aic (**10**), which differs only by a $CH_2$ group. Furthermore, the two enantiomers of *e*-$\beta$ MePhe **11a,b** display diverse affinity, indicating that one enantiomer is preferred over the other.

Recently, highly potent disulfide constrained analogs of Ang IV were reported in which the His-Pro-Phe sequence was replaced by *o*-(aminomethyl)phenyl acetic acid [60]. A pronounced impact of the spatial arrangement in the *C*-terminal part of these constrained analogs was observed, confirming the observations made here on the importance of the orientation of the Phe$^6$ residue.

## Conclusion

All replacements of Tyr$^2$ by constrained analogs resulted in a large drop in IRAP inhibitory potency, indicating the essential role of this residue. Phe$^6$ replacement by *e*-$\beta$-MePhe resulted in an analog **11b** that was very selective for IRAP *versus* AP-N and $AT_1$. This indicates an important role of the orientation of the Phe$^6$ for inducing selectivity. Pro$^5$ replacement with 2-aminocyclopentanecarboxylic acid maintained IRAP potency and abolished $AT_1$ affinity. As expected, these analogs were not protected against metabolic degradation, but this would be easily achieved by the introduction of modification of the *N*-terminal Val residue. These results confirm the importance of conformational constrained amino acids generate selectivity in bioactive peptides.

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