Bioorganic & Medicinal Chemistry 16 (2008) 6924–6935

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Ligands to the (IRAP)/AT4 receptor encompassing a 4-hydroxydiphenylmethane scaffold replacing Tyr$^2$

Hanna Andersson [a], Heidi Demaegdt [b], Georges Vauquelin [b], Gunnar Lindeberg [a], Anders Karlén [a], Mathias Hallberg [c,*]

[a] Department of Medicinal Chemistry, Uppsala University, Box 574, SE-751 23 Uppsala, Sweden
[b] Department of Molecular and Biochemical Pharmacology, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium
[c] Department of Pharmaceutical Biosciences, Division of Biological Research on Drug Dependence, Uppsala University, PO Box 591, SE-751 24 Uppsala, Sweden

ARTICLE INFO

*Article history:*
Received 22 January 2008
Revised 9 May 2008
Accepted 22 May 2008
Available online 27 May 2008

*Keywords:*
Angiotensin IV
Insulin-regulated aminopeptidase (IRAP)
Cystinyl aminopeptidase (CAP)
Aminopeptidase N (AP-N)
Structure–activity relationship
Peptide synthesis
Peptide mimetic
4-Hydroxydiphenylmethane
Tyrosine mimetic

ABSTRACT

Analogues of the hexapeptide angiotensin IV (Ang IV, Val$^1$-Tyr$^2$-Ile$^3$-His$^4$-Pro$^5$-Phe$^6$) encompassing a 4-hydroxydiphenylmethane scaffold replacing Tyr$^2$ and a phenylacetic or benzoic acid moiety replacing His$^4$-Pro$^5$-Phe$^6$ have been synthesized and evaluated in biological assays. The analogues inhibited the proteolytic activity of cystinyl aminopeptidase (CAP), frequently referred to as the insulin-regulated aminopeptidase (IRAP), and were found less efficient as inhibitors of aminopeptidase N (AP-N). The best Ang IV mimetics in the series were approximately 20 times less potent than Ang IV as IRAP inhibitors. Furthermore, it was found that the ligands at best exhibited a 140 times lower binding affinity to the membrane-bound IRAP/AT4 receptor than Ang IV. Although the best compounds still exert lower activities than Ang IV, it is notable that these compounds comprise only two amino acid residues and are considerably less peptidic in character than the majority of the Ang IV analogues previously reported as IRAP inhibitors in the literature.



## 1. Introduction

Proteolytic degradation of neuropeptides often provides products that are essentially inactive. However, there are also numerous examples disclosed where the fragments formed demonstrate pronounced biological effects and outcomes that frequently differ considerably from those exerted by the parent peptide.[1,2] Angiotensin IV (Ang IV, Val$^1$-Tyr$^2$-Ile$^3$-His$^4$-Pro$^5$-Phe$^6$), a component in the renin–angiotensin system, provides a good example of a degradation product that at least partly is exhibiting different effects than its precursors.[3–5] The angiotensin IV receptor, first described as the specific high-affinity binding site for Ang IV,[6–8] has been identified as a membrane bound enzyme of the M1 metallopeptidase family denoted cystinyl aminopeptidase (CAP),[9] a peptidase that is more often referred to as the insulin-regulated membrane aminopeptidase (IRAP; EC 3.4.11.3).[9,10]

The ability of Ang IV to facilitate learning and memory in behavioural models has attracted a particular interest in recent years.[11–18] As deduced from autoradiographic studies, the high-affinity Ang IV binding site but not the AT1 receptor is abundant in neocortex, cerebellum, dentate gyrus and CA1–CA3 subfields of the hippocampus.[19–22] These structures are all representing important brain areas believed to be involved with cognitive processes. A precise mechanism explaining the observation that Ang IV acts as a potent cognitive enhancer has not yet been presented, but some hypotheses have emerged. The fact that IRAP is abundantly occurring in vesicles containing the insulin-sensitive glucose transporter GLUT4,[10,23] led to the hypothesis that Ang IV is able to enhance memory and learning by modulating the translocation of GLUT4 to the cell surface and consequently increase glucose uptake in neurons.[3] Alternatively, since IRAP/AT4 receptor ligands have been shown to act by binding to IRAP and thereby inhibiting the catalytic activity of the enzyme,[24,25] it has been proposed that Ang IV at least partly exerts its action by prolonging the half-life of one or more of its neuropeptide substrates, such as somatostatin or vasopressin that are recognized for their ability to facilitate learning and memory.[4,10,26] It has also been suggested that alternative targets of Ang IV exist and that IRAP may act as a classical receptor.[4,25,27] Aminopeptidase N (AP-N) constitutes one potential target candidate because the catalytic activity was found to be sensitive to Ang IV.[28] Notably, it was recently shown that Ang IV was able to bind with high affinity to the CAP apo-enzyme, but neither to the native CAP,

* Corresponding author. Tel.: +46 18 471 4141; fax: +46 18 501920.
*E-mail address:* Mathias.Hallberg@farmbio.uu.se (M. Hallberg).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.05.046

the native AP-N nor to the AP-N apo-enzyme.[29] A receptor subtype that has been demonstrated to be involved at least partially in the mediation of the observed cognitive effects of Ang IV is the D2 dopamine receptor. It was clearly shown that D2 receptor blockade abolished all cognitive effects mediated by Ang IV.[30] Hence, it was proposed that Ang IV promotes release of dopamine and possibly also acetylcholine in vivo[31] and that the potential liberation of these transmitter substances might play a fundamental role downstream from IRAP/AT4 for the enhancement of the cognitive effects observed.[30] However, the link between Ang IV analogues and the IRAP/AT4 receptor in relation to dopamine and the D2 receptor is still not clear, but the close location of the IRAP/AT4 and D2 receptors in brain areas associated with learning and memory should be favourable for interactions.[32–34] Furthermore, a recent study showed that the effect of Ang IV on dopamine release was not a result of inhibition of the IRAP and AP-N enzyme activity, hence it was hypothesized that the effect is a result of activation of IRAP and/or AP-N acting as receptors.[35]

Regardless of the detailed underlying mechanism, the IRAP/AT4 receptor has recently evolved into a new target for pharmaceuticals aimed for treatment of various cognitive disorders. We are convinced that non-peptidic metabolically stable drug-like Ang IV analogues that efficiently can cross the blood–brain barrier would be attractive as research tools and enable extensive studies of the impact of Ang IV on the in vivo physiology in complex animal models. Our long-term goal is to design and synthesize such ligands. In addition, non-peptidic ligands could possibly serve as potential lead structures for further optimization in drug discovery programmes.

We herein report that incorporation of a 4-hydroxydiphenylmethane scaffold as a substitute for $Tyr^2$, in combination with a phenylacetic acid or benzoic acid scaffold as substitute for the $His^4$-$Pro^5$-$Phe^6$ amino acid residues in Ang IV deliver more drug-like IRAP/AT4 receptor ligands, although with lower potency than Ang IV both with regard to receptor binding and IRAP inhibition (**1**–**11**, Table 1). Further truncations and/or internal deletions render compounds that are essentially inactive (**12**–**15**, Table 1). A comparison of the $K_i$ values from the receptor binding and IRAP inhibition assays indicates some differences in the structure–activity relationships (SAR).

## 2. Results and discussion

### 2.1. Synthesis of Ang IV analogues

The scaffolds utilized in the synthesis of the target analogues (**1**–**15**, Table 1) were prepared via modifications of previously reported procedures.[36–38] The diphenylmethane scaffold **20**[36] was synthesized according to the route outlined in Scheme 1. The first step was conducted by a Negishi coupling[39,40] using the acid chloride of the commercially available benzoic acid and the organozinc reagent of *p*-bromoanisole. Conversion of *p*-bromoanisole to the corresponding Grignard reagent followed by transmetallation afforded the organozinc reagent which was used immediately. A solid supported scavenger, 3-(1-thioureido)propyl-functionalized silica gel,[41] was used prior to the reduction of ketone **16**[36] to avoid palladium-catalyzed side reactions. Triethylsilane and triflic acid in $TFA/CH_2Cl_2$ were employed for the quantitative reduction providing **17**.[36] The hydrolysis of the ester was performed with LiOH in $THF/H_2O$/MeOH affording compound **18**[36] in a quantitative yield. Compound **19**[36] was obtained by hydrogenation of the nitro group using Pd/C in absolute ethanol followed by Fmoc protection of the resulting aniline. Demethylation of the *p*-methoxy group to produce the corresponding phenol **20**[36] was accomplished with boron trifluoride-methyl sulfide complex in $CH_2Cl_2$.

Building blocks **21a**[42] and **21b**[37,38] (Scheme 2) were prepared essentially according to a reported three-step procedure.[37,38] Initially, a solvent-free protocol[43] was used to obtain the brominated intermediates ethyl 2-(bromomethyl)benzoate and ethyl 2-(bromomethyl)phenylacetate, but the reaction was found to be sluggish and gave complex reaction mixtures. Therefore, a protocol employing methyl acetate as a solvent and microwave-assisted heating was modified and applied.[44] The commercially available ethyl 2-methylbenzoate and ethyl 2-methylphenylacetate were reacted with *N*-bromosuccinimide (NBS) and 2,2′-azobis(2-methylpropionitrile) (AIBN) in MeCN at 90 °C for 15 min to obtain ethyl 2-(bromomethyl)benzoate and ethyl 2-(bromomethyl)phenylacetate. The transformations of the bromomethyl groups into the corresponding azidomethyl groups were achieved by treatment with $NaN_3$ in DMF. Finally, the esters were hydrolyzed with LiOH in $THF/MeOH/H_2O$ to obtain the corresponding carboxylic acids **21a** and **21b**.

The target angiotensin IV analogues **2**–**15** were prepared by manual solid-phase synthesis techniques using Fmoc protection as described previously (Scheme 2).[45] Initial coupling of **21a** and **21b** to 2-chlorotrityl chloride resin furnished **22a** and **22b**,[46] which were further elongated with L-proline (**23a** and **23b**), L-leucine (**24a** and **24b**), L-isoleucine (**25a** and **25b**) and **20** (**26b**). The elongation was performed by a slightly modified literature procedure[37] involving addition of tributylphosphine to a mixture of resin bound azide and activated amino acid in $CH_2Cl_2$ or **20** in $CH_2Cl_2$/MeCN. Prolonged reaction times were used for the subsequent couplings of **20**, L-valine and L-norleucine as these reactions were expected to proceed slowly. Analogues **1** and **15** were prepared in a similar fashion by consecutive coupling of **20** and L-valine to **22c** and **22d**, respectively.

The Fmoc-protected scaffold **20** was used in the pseudopeptides to replace the $Tyr^2$, $Val^1$-$Tyr^2$, $Tyr^2$-$Ile^3$ or $Val^1$-$Tyr^2$-$Ile^3$ residues in Ang IV. The building blocks **21a** and **21b** were utilized in compounds **2**–**14** as γ-turn mimicing scaffolds to replace $His^4$-$Pro^5$-$Phe^6$ in Ang IV.

### 2.2. Biochemical evaluation

The Ang IV analogues were tested and compared for their binding affinity to IRAP natively expressed in CHO-K1 cells as well as their ability to inhibit the catalytic activity of recombinant human IRAP and aminopeptidase N transiently transfected in HEK293 cells. The chemical structures, the binding affinities and protease inhibition data of the compounds are presented in Table 1.

Our long-term objective is to develop drug-like Ang IV mimetics that exhibit a high metabolic stability and oral bioavailability. We foresee that such compounds, characterized by a considerably longer duration of action than the parent peptide Ang IV, would serve as attractive research tools, in particular when experiments in complex animal models are performed. One approach to peptide mimetics is to induce constrains by cyclizations and in those cases where potent cyclized peptides are identified, proceed by conducting a conformational analysis to determine the bioactive conformation of the target peptide. Subsequent displacement of the peptide backbone with proper organic scaffolds, which mimic the secondary structure of the bioactive conformation, followed by structural optimization should eventually provide drug-like peptide mimetics.[47,48]

Recently, we demonstrated that 1 to 3 cyclization of the N-terminal of Ang IV, for example, oxidative cyclization of $Cys^1$ with $Cys^3$ where $Val^1$ and $Ile^3$ in Ang IV had been displaced, delivered a peptide with an approximately 1000-fold lower binding affinity than Ang IV itself.[49] Since $Cys^1/Cys^3$ cyclized peptides preferentially adopt γ-turns, it was concluded that it is not likely that a γ-turn is present in the N-terminal of Ang IV when the peptide is

**Table 1**
Structures, binding affinities and inhibition activities of compounds **1**–**15** and Ang IV

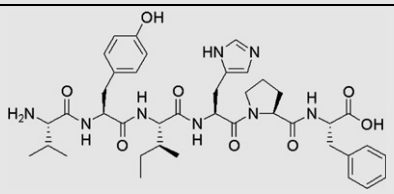
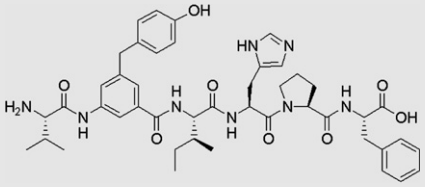
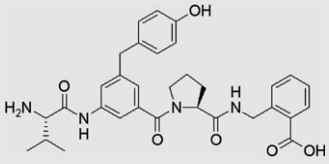
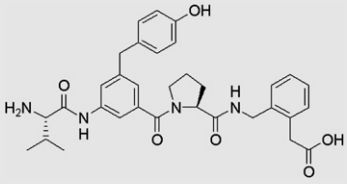
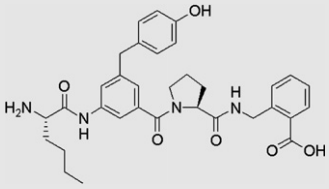
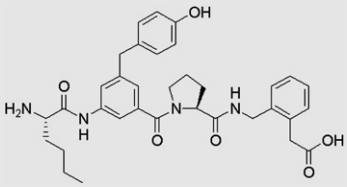
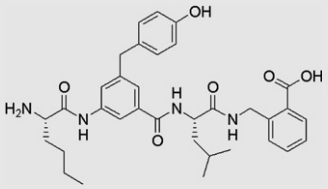
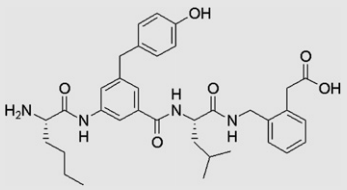
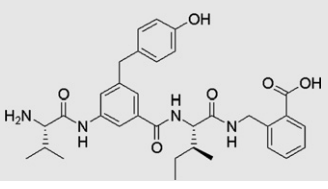
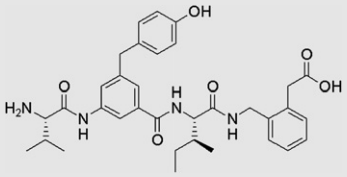
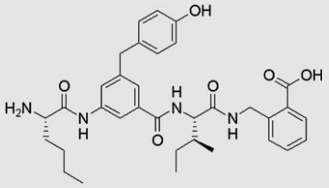
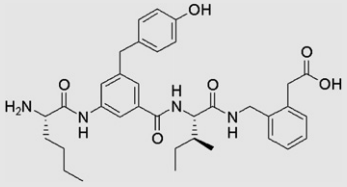
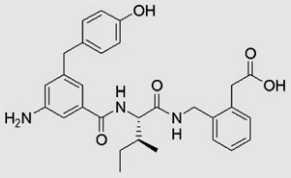
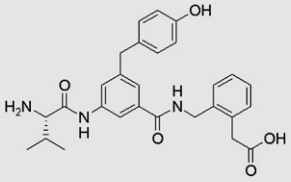
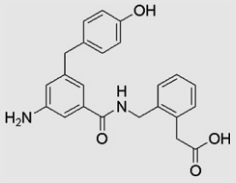
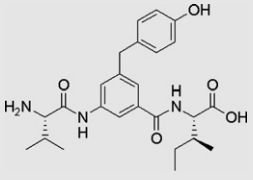
| Compound | Structure | Binding affinity $K_i \pm SD$[a] (μM) | IRAP enzyme inhibition $K_i \pm SD$[b] (μM) | AP-N enzyme inhibition $K_i \pm SD$[c] (μM) |
|---|---|---|---|---|
| Ang IV[d] |  | 0.007 ± 0.003 | 0.06 ± 0.04 | 0.83 ± 0.10 |
| **1** |  | 6.8 ± 1.0 | 1.2 ± 0.6 | 5.0 ± 0.3 |
| **2** |  | 13 ± 0.3 | 12 ± 3.9 | 55 ± 8.4 |
| **3** |  | 14 ± 0.6 | 7.4 ± 0.0 | 49 ± 1.9 |
| **4** |  | 2.5 ± 0.1 | 6.3 ± 0.0 | 17 ± 2.5 |
| **5** |  | 1.4 ± 0.1 | 3.3 ± 0.9 | 11 ± 0.5 |
| **6** |  | 3.9 ± 0.1 | 3.7 ± 1.1 | 10 ± 0.6 |
| **7** |  | 3.1 ± 0.2 | 3.9 ± 2.5 | 16 ± 3.1 |
| **8** |  | 12 ± 1.1 | 1.3 ± 0.5 | 20 ± 4.0 |

**Table 1** (*continued*)

| Compound | Structure | Binding affinity $K_i \pm SD$[a] (μM) | IRAP enzyme inhibition $K_i \pm SD$[b] (μM) | AP-N enzyme inhibition $K_i \pm SD$[c] (μM) |
|---|---|---|---|---|
| **9** |  | 8.9 ± 1.7 | 1.3 ± 0.5 | 21 ± 1.6 |
| **10** |  | 2.3 ± 0.2 | 1.8 ± 1.8 | 17 ± 2.5 |
| **11** |  | 1.0 ± 0.1 | 1.4 ± 0.6 | 13 ± 1.1 |
| **12** |  | >100 | 97 ± 38 | >100 |
| **13** |  | 86 ± 7.9 | 16 ± 0.3 | 33 ± 5.2 |
| **14** |  | >100 | 35 ± 4.9 | >100 |
| **15** |  | >100 | 76 ± 7.0 | 69 ± 7.7 |

SD, standard deviation.

[a] [$^{125}$I]Angiotensin IV competition binding in CHO-K1 cell membranes.

[b] Evaluated in an enzyme assay comprising recombinant human IRAP, transiently transfected in HEK293 cells.

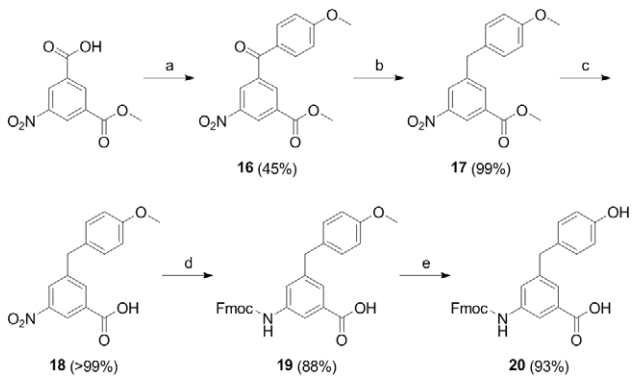
[c] Evaluated in an enzyme assay comprising recombinant human AP-N, transiently transfected in HEK293 cells.

[d] Data for Ang IV are taken from Ref. 29.

binding to its target protein. However, by applying larger ring systems (i.e., Cyc$^1$/Hcy$^3$, Hcy$^1$/Cys$^3$ and Hcy$^1$/Hcy$^3$ cyclizations) thereby providing higher conformational flexibility more potent ligands could be obtained.[49]

Prior to the incorporation of more complex backbone mimetics to replace residues 1–3, we wanted to assess if a simpler one-residue backbone mimetic could be successfully employed. A series of derivatives comprising a 4-hydroxydiphenylmethane scaffold to replace the Tyr$^2$ residue in Ang IV were designed and synthesized. All except analogue **1** contain only two or less amino acid residues.

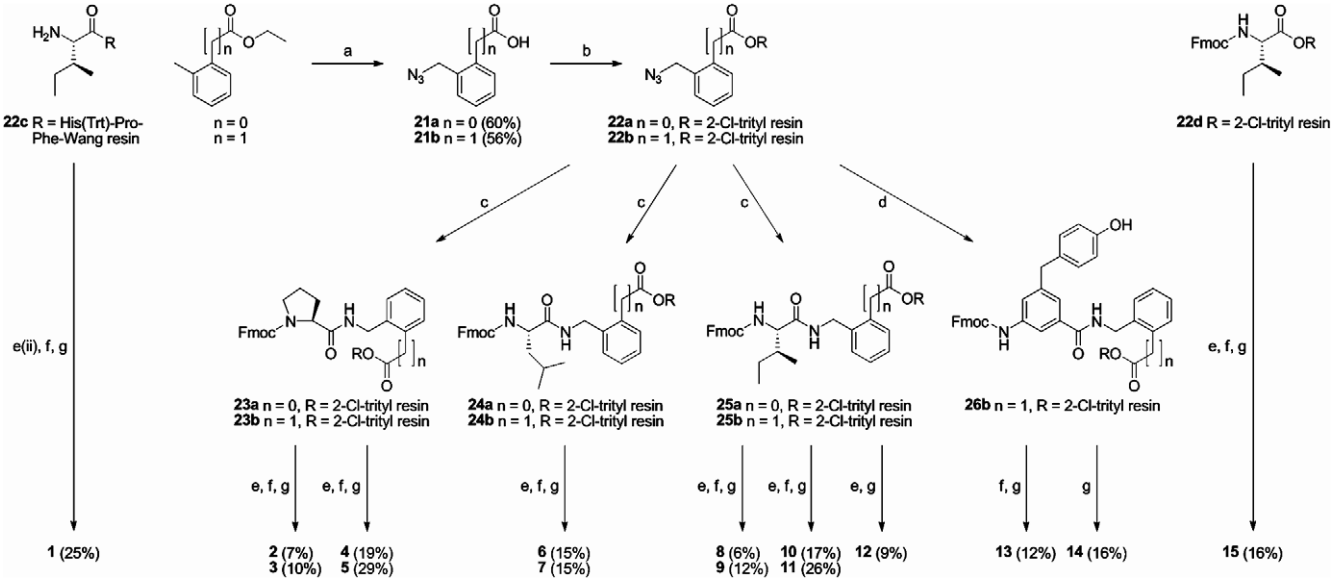
Diphenylmethane is one of the most common scaffolds in drugs. The 4-hydroxydiphenylmethane fragment contains this privileged structure[50] and has previously been incorporated to replace the amino acid residues centred at tyrosine in the octapeptide




**Scheme 1.** Reagents: (a) i—$SOCl_2$; ii—4-$MeOC_6H_4ZnCl$, $PdCl_2(PPh_3)_2$, THF; iii—3-(1-thioureido)propyl-functionalized silica gel, THF; (b) $Et_3SiH$, $CF_3SO_2OH$, TFA, DCM; (c) LiOH, THF/MeOH/$H_2O$; (d) i—$H_2$, Pd/C, EtOH; ii—Fmoc-Cl, $Na_2CO_3$ (aq), dioxane; (e) $BF_3{\cdot}S(CH_3)_2$, DCM.

angiotensin II. Potent pseudopeptides with high affinity for the AT2 receptor were identified.[36] However, we found that incorporation of a 4-hydroxydiphenylmethane scaffold encompassing simply an N-terminal aminomethyl group to substitute $Val^1$-$Tyr^2$ or $Val^1$-$Tyr^2$-$Ile^3$ in Ang IV rendered, in experiments where porcine frontal cortex membranes were used, ligands that were 1000-fold less potent than Ang IV or inactive, respectively.[45] Similarly, with the anilide-based 4-hydroxydiphenylmethane scaffold employed in the present study we likewise obtained a 1000-fold drop in binding affinity as compared to Ang IV, despite the presence of both $Val^1$ and $Ile^3$ (cf. Ang IV and **1**). Notably though, a comparison of the IRAP activity inhibition data revealed that **1** is only 20 times less potent than Ang IV. Similarly, **1** is only six times less active as an AP-N inhibitor compared to Ang IV. The incorporation of the 4-hydroxydiphenylmethane scaffold in Ang IV to afford **1** rendered a considerably less bioactive ligand suggesting that the aromatic ring system applied herein induces constrain leading to unfavourable spatial arrangement of the side chains of the N-terminal. The extended back bone and non-optimal locations of the peptide bonds of **1** can provide alternative rationales for the lower activity. As we recently demonstrated some large macrocylic rings in the N-terminal are in fact accepted[49] but as apparent from the present study small alterations of the backbone can exert a dramatic impact on the bioactivity. Efforts, including modelling as were previously successfully applied for the C-terminal to determine the active conformation will now be devoted to the identification of optimal structural elements in the N-terminal of Ang IV.

Regarding modifications of the C-terminal part of Ang IV, we previously reported that replacement of $His^4$-$Pro^5$-$Phe^6$ in Ang IV with a 2-aminomethylphenylacetic acid moiety delivered a ligand with an equipotent binding affinity as compared to Ang IV when the cortex membrane binding assay was employed.[45] In analogy, we have now found that **1** can be subjected to the same transformation with essentially no loss of activity, concerning neither receptor affinity nor enzyme inhibition capacity, cf. **1** and **9**. A small loss of activity was encountered with a C-terminal benzoic acid instead of the phenylacetic acid fragment, cf. **8** and **9**. In general, it was observed that that the benzoic acid derivatives were less active than the corresponding phenylacetic acid derivatives. The importance of the C-terminal part was demonstrated by the truncated analogue **15**, the binding affinity was lost and the enzyme inhibition dropped 60 times when the benzoic and phenylacetic acid scaffolds were deleted, cf. **8**, **9** and **15**. The attempts to impose more steric constrain by replacing $Ile^3$ of **8** and **9** by proline provided less active compounds (**2** and **3**) in particular with regard to the enzyme inhibition. Both of the proline derivatives **2** and **3** are less active than **1** in particular with regard to AP-N inhibition. Replacement of the isopropyl side chain of $Val^1$ by a non-branched butyl group, that is, norleucine, delivered more efficient ligands to the IRAP/AT4 receptor, cf. **8** with **10** and **9** with **11**, respectively. The beneficial effect of a norleucine residue in position 1 in Ang IV analogues is well established from recent binding studies[51,52] and we assume therefore that the novel less peptidic ligands bind to the receptor protein essentially in the same fashion as the native hexapeptide does. In addition, with proline in position 3, a norleucine residue in position 1 was favourable also for IRAP inhibition, cf. **2** with **4** and **3** with **5**, respectively. However, with isoleucine in position 3, replacing valine by norleucine seemed to exert only minor influences on the enzyme inhibition, cf. **8** with **10** and **9** with **11**. Concerning position 3, a one carbon migration of a methyl group, that is, a leucine instead of an isoleucine, had an unfavour-




**Scheme 2.** Reagents: (a) i—NBS, AIBN, MeCN; (ii) $NaN_3$, DMF; (iii) LiOH, THF/MeOH/$H_2O$; (b) 2-chlorotrityl chloride resin, DIEA, $CH_2Cl_2$; (c) i—Fmoc-L-Xaa-OH, DIPCDI, HOBt, $CH_2Cl_2$; ii—$PBu_3$; (d) i—**20**, DIPCDI, HOBt, $CH_2Cl_2$/MeCN; ii—$PBu_3$; (e) i—20% piperidine/DMF; ii—**20**, PyBOP, DIEA, DMF; (f) i—20% piperidine/DMF; ii—Fmoc-L-Xaa-OH, PyBOP, DIEA, DMF; (g) i—20% piperidine/DMF; ii—$Et_3SiH$, TFA/$H_2O$.

able impact on both affinity and IRAP inhibition, cf. **6** with **10** and **7** with **11**, respectively. Thus, it appears that both positions 1 and 3 in this series of ligands are important for the molecular recognition and that even minor structural modifications of the lipophilic side chains alter the bioactivity.

The weak potency of analogues **1**–**11** confirms the importance of the N-terminal part both regarding binding and inhibition.[49,51–54] These analogues incorporate a large 4-dihydroxidiphenylmethane scaffold in place of tyrosine at position 2. To elucidate whether a tyrosine containing di- or tripeptide (i.e., $Val^1$-$Tyr^2$, $Tyr^2$-$Ile^3$ or $Val^1$-$Tyr^2$-$Ile^3$) could be replaced by the diphenylmethane scaffold whilst still retaining the activity, an additional set of analogues (**12**–**14**) were synthesized. Although the analogues containing four units (**2**–**11**) were found to be more potent, the three and two unit analogues contributed some additional information. The importance of the amino acid residue in position 1 was reflected by the N-terminally truncated analogue **12**, which was inactive. Deletion of the residue in position 3 produced the least negative outcome. Thus, analogue **13** showed a 10-fold drop in enzyme inhibition and a 100-fold lower binding affinity compared to the best compounds in the series of four unit analogues (**2**–**11**). C-Terminal truncation of analogue **13** produced a small non-peptidic compound (**14**) consisting of the 4-hydroxydiphenylmethane scaffold connected to the 2-(aminomethyl)phenylacetic acid moiety. Whereas no binding affinity was encountered it acted as a weak IRAP inhibitor.

Ang IV displays moderate selectivity for IRAP compared to AP-N.[29] More selective inhibitors and/or ligands could serve as valuable tools for investigation of the involvement of IRAP and AP-N in mediating the effects of Ang IV. The present analogues exhibited the similar selectivity as Ang IV.

## 3. Conclusions

Ang IV peptide mimetics that retain binding affinity to the IRAP/AT4 receptor and with a capacity to inhibit the proteolytic activity of IRAP have been designed and synthesized. The most potent ligand in the series, compound **11**, was found to be 140 times less active than Ang IV in the binding assay but only about 25 times less efficient as an IRAP inhibitor. Concerning structure–activity relationships, some differences were observed for receptor binding and enzyme inhibition. However, it is difficult to explain that the compounds in general exhibit an equal or higher potency in the IRAP inhibition assay than in the binding assay whereas Ang IV has the reverse profile. Characteristic features of the new pseudopeptides are the benzene ring replacing the methine carbon of $Tyr^2$ and a phenylacetic acid or a benzoic acid replacing $His^4$-$Pro^5$-$Phe^6$. Further truncations and/or deletions were deleterious for activity. The best Ang IV analogues in this study comprise only two amino acid residues and are consequently much less peptidic in character than the majority of the Ang IV pseudopeptides previously reported in the literature. Ang IV peptide mimetics are foreseen to be of considerable interest as research tools and the ligands presented herein will be subjected to further optimization with the primary objectives to improve potency as well as stability to protease degradation.

## 4. Experimental

### 4.1. Chemistry

Microwave-heated reactions were performed in a Smith Synthesizer™ (Biotage AB, Uppsala, Sweden) producing controlled irradiation at 2450 MHz. Thin-layer chromatography (TLC) was conducted on aluminium sheets precoated with silica gel 60 $F_{254}$ (0.2 mm, Merck) and the analytes were visualized by UV-light and iodine. Column chromatography was performed using silica gel 60 (40–63 μm, Merck). Low resolution mass spectra were recorded on a GC–MS instrument equipped with a CP-Sil 8 CB capillary column (30 m × 0.25 mm, 0.25 μm) utilizing electron impact (EI) at an ionization energy of 70 eV. The oven temperature (GC) was 40–300 °C. Analytical RP-HPLC-MS was performed on a Gilson HPLC system with a Finnigan AQA quadrupol mass spectrometer using an Onyx monolithic C18 column (50 × 4.6 mm) with gradients of MeCN/$H_2O$ (0.05% HCOOH) at a flow rate of 4 mL/min. Both UV (DAD, 214 and 254 nm) and MS (ESI) detection were utilized. Preparative RP-HPLC was performed using a Zorbax SB-C8 column (5 μm, 21.2 × 150 mm) with gradients of MeCN/$H_2O$ (0.1% TFA) at a flow rate of 5 mL/min and UV detection at 230 nm. The pseudopeptide purities were determined on an ACE 5 C18 column (5 μm, 4.6 × 50 mm) and an ACE 5 Phenyl column (5 μm, 4.6 × 50 mm) using the same buffer system at a flow rate of 2 mL/min and with UV detection at 220 nm. NMR spectra were recorded on a Varian Mercury plus spectrometer ($^1H$ at 399.8 MHz and $^{13}C$ at 100.5 MHz or $^1H$ at 399.9 MHz and $^{13}C$ at 100.6 MHz) at ambient temperature. Chemical shifts ($\delta$) are reported in ppm referenced indirectly to TMS via the solvent residual signal. Exact molecular masses were determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source at the Department of Pharmaceutical Biosciences, Uppsala University, Sweden. Amino acid analyses were performed at the Department of Biochemistry and Organic Chemistry, Uppsala University, Sweden. THF was freshly distilled over Na/benzophenone, $CH_2Cl_2$ was distilled over calcium hydride and *N*-bromosuccinimide (NBS) was recrystallized from acetic acid. Other chemicals were used without further purification. Compounds **16**–**20**,[36] **21a**[42] and **21b**[37,38] are known. Characterization data were consistent with those previously reported.

#### 4.1.1. Methyl 3-(4-methoxy-benzoyl)-5-nitrobenzoate[36] (16)

5-Nitroisophthalic acid monomethyl ester (7.88 g, 35.0 mmol) was refluxed in 70 mL thionyl chloride for 2 h. After evaporation of the thionyl chloride, 100 mL dry $CH_2Cl_2$ was added and evaporated. The resulting acid chloride was kept under vacuum overnight. A dry three-necked round-bottomed flask was charged with magnesium turnings (919 mg, 37.8 mmol), sealed with a septum, evacuated and filled with nitrogen. THF (28 mL), *p*-bromoanisole (4.38 mL, 35 mmol) and a catalytic amount of methyl iodide (10 droplets) were added sequentially under stirring. The temperature of the spontaneously warmed reaction mixture was slowly decreasing. After 40 min it was heated to 60 °C and kept at that temperature for approximately 3 h. The freshly prepared Grignard reagent was allowed to attain ambient temperature and subsequently added over a 15 min period to a stirred solution of zinc chloride (5.01 g, 36.8 mmol) in 28 mL dry THF at 0 °C. The reaction mixture was stirred at 0 °C for 15 min and then at room temperature for approximately 4 h. The resulting slurry of the precipitated white organozinc reagent was cooled to 0 °C and dichlorobis(triphenylphosphine)palladium (737 mg, 1.05 mmol) was added. The reaction vessel was flushed with nitrogen and a solution of the previously synthesized acid chloride in 40 mL dry THF was added dropwise. The reaction mixture was stirred at room temperature over night (16 h), poured into 300 mL 0.5 M HCl and extracted with EtOAc (3× 200 mL). The combined organic phases were washed with $H_2O$ (150 mL), dried over $MgSO_4$, filtered and evaporated. Purification by column chromatography (*i*-hexane/EtOAc 17:3–13:4, and subsequently, toluene/EtOAc 96:4) gave **16** as a yellow solid (4.99 g). To eliminate traces of palladium, **16** (3.58 g) was dissolved in 55 mL THF and heated to 60 °C for 80 min in the presence of 3-(1-thioureido)propyl-functionalized silica gel (3.82 mg, loading: 1.1 mmol/g, 200–400 mesh). The functionalized silica was subsequently filtered off and washed with 50 mL THF. The

combined filtrates were evaporated to give **16**[36] as a pale yellow solid (3.55 g, 45% overall).

#### 4.1.2. Methyl 3-(4-methoxybenzyl)-5-nitrobenzoate[36] (17)

Trifluoroacetic acid (TFA) (8.45 mL, 110 mmol) and triflic acid (0.10 mL, 1.1 mmol) were added to a stirred solution of **16** (3.48 g, 11 mmol) in 25 mL $CH_2Cl_2$ at 0 °C. After dropwise addition of triethylsilane (5.59 mL, 35 mmol), the reaction mixture was allowed to attain ambient temperature. It was stirred for 3 h and then slowly poured into a cold saturated solution of $NaHCO_3$ (100 mL). The aqueous phase was extracted with $CH_2Cl_2$ (2× 50 mL) and the combined organic phases were washed with $H_2O$ (2× 75 mL), dried over $MgSO_4$, filtered and evaporated. Excessive triethylsilane was removed by repeated addition and evaporation of toluene. Compound **17**[36] was collected as a pale yellow solid (3.29 g, 99%).

#### 4.1.3. 3-(4-Methoxybenzyl)-5-nitrobenzoic acid[36] (18)

LiOH (1.30 g, 54.2 mmol) was added to a solution of **17** (3.25 g, 10.8 mmol) in THF (130 mL), MeOH (43 mL) and $H_2O$ (43 mL). After stirring over night the reaction mixture was concentrated, diluted with $H_2O$ and acidified with 1 M HCl. EtOAc (250 mL) was added and the two phases were separated. The aqueous phase was extracted with EtOAc (50 mL) and the combined organic phases were washed with brine (2× 100 mL) and $H_2O$ (100 mL), dried over $MgSO_4$, filtered and evaporated to give **18**[36] as a solid (3.09 g, quant.).

#### 4.1.4. 3-(9H-Fluoren-9-ylmethoxycarbonylamino)-5-(4-methoxybenzyl)benzoic acid[36] (19)

A mixture of compound **18** (3.07 g, 10.7 mmol) and 10% Pd/C (1.14 g, 1.07 mmol) in 150 mL absolute ethanol was stirred under hydrogen at atmospheric pressure and room temperature for approximately 5 h. The crude product obtained after filtration and evaporation, when analyzed by LC–MS, was considered to be of sufficient purity to be used in the next step without further purification. An aqueous solution of $Na_2CO_3$ (0.3 M, 125 mL) was slowly added to a solution of the aniline in 100 mL dioxane at 0 °C. 9-Fluorenylmethyl chloroformate (Fmoc-Cl) (3.36 g, 11.8 mmol) in 100 mL dioxane was added dropwise, whereafter the reaction mixture was allowed to reach ambient temperature and left under continuous stirring for approximately 44 h. The reaction mixture was slowly acidified with 1 M HCl and EtOAc (200 mL) was added. The aqueous phase was extracted with EtOAc (2× 100 mL) and the combined organic phases were washed with brine (2× 100 mL) and $H_2O$ (2× 100 mL), dried over $MgSO_4$, filtered and evaporated. Purification by column chromatography (*i*-hexane/EtOAc/HCOOH 74:25:1) and an additional extraction using EtOAc and $H_2O$ gave **19**[36] as a solid (4.52 g, 88%).

#### 4.1.5. 3-(9H-Fluoren-9-ylmethoxycarbonylamino)-5-(4-hydroxybenzyl)benzoic acid[36] (20)

Boron trifluoride-methyl sulfide complex (10.2 mL, 97 mmol) was added dropwise to **19** (4.65 g, 9.70 mmol) in 145 mL dry $CH_2Cl_2$ at 0 °C under nitrogen. The reaction mixture was allowed to reach ambient temperature and left under continuous stirring for 26 h. The reaction was quenched by very slow addition of the mixture to cold 0.5 M HCl under stirring. Subsequent extraction with EtOAc was followed by washing with $NaHCO_3$, brine and $H_2O$, drying over $MgSO_4$, filtration and evaporation. Purification by column chromatography (*i*-hexane/EtOAc/HCOOH 65:34:1 followed by EtOAc to elute the product) gave **20**[36] as a solid (4.18 g, 93%).

#### 4.1.6. 2-(Azidomethyl)benzoic acid[42] (21a)

Ethyl 2-methylbenzoate (1.91 mL, 12.0 mmol), NBS (2.35 g, 13.2 mmol), AIBN (98.5 mg, 0.60 mmol) and MeCN (18 mL) were added to a 20 mL Smith Process Vial™ containing a stirring bar. After capping and evacuation of the vial, the reaction mixture was heated to 90 °C by microwave irradiation for 15 min, cooled to room temperature, transferred to a round-bottomed flask and concentrated under reduced pressure. Subsequent precipitation of succinimide by *i*-hexane/$CH_2Cl_2$, filtration, evaporation and purification by column chromatography (*i*-hexane/ether 98:2) gave ethyl 2-(bromomethyl)benzoate as a pale yellow oil (1.97 g, 67%). $^1$H NMR ($CDCl_3$) $\delta$ 7.96 (dd, 7.8, 1.4 Hz, 1H), 7.50–7.43 (m, 2H), 7.36 (ddd, $J$ = 7.8, 6.8, 2.0 Hz, 1H), 4.95 (s, 2H), 4.40 (q, $J$ = 7.1 Hz, 2H), 1.42 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR ($CDCl_3$) $\delta$ 166.7, 139.1, 132.5, 131.7, 131.3, 129.6, 128.6, 61.4, 31.7, 14.3. EI$^+$-MS $m/z$ 243 ($M^+$). The residual compound obtained above (1.94 g, 7.99 mmol) was reacted with $NaN_3$ (0.62 g, 9.58 mmol) in 10 mL DMF at room temperature for 22 h. The reaction was quenched with $H_2O$ and extracted with EtOAc. The combined organic layers were washed with brine and $H_2O$, dried over $Na_2SO_4$, filtered and evaporated. Purification by column chromatography (*i*-hexane/EtOAc 95:5) gave ethyl 2-(azidomethyl)benzoate as a colourless oil (1.56 g, 95%). $^1$H NMR ($CDCl_3$) $\delta$ 8.02 (dd, $J$ = 7.8, 1.5 Hz, 1H), 7.55 (ddd, $J$ = 7.5, 7.5, 1.5 Hz, 1H), 7.49 (ddd, $J$ = 7.8, 1.5, 0.5 Hz, 1H), 7.40 (ddd, $J$ = 7.5, 7.5, 1.5 Hz, 1H), 4.82 (s, 2H), 4.39 (q, $J$ = 7.1 Hz, 2H), 1.41 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR ($CDCl_3$) $\delta$ 166.9, 137.3, 132.7, 131.2, 129.9, 129.3, 128.2, 61.4, 53.2, 14.4. LiOH (0.89 g, 37.0 mmol) was added to a solution of the residual compound obtained above (1.52 g, 7.41 mmol) in THF (14 mL), MeOH (2 mL) and $H_2O$ (4 mL). After stirring for 17 h, the reaction mixture was diluted with $CH_2Cl_2$ and $H_2O$, and extracted with $CH_2Cl_2$. The aqueous phase was acidified with 3 M HCl and extracted with $CH_2Cl_2$. The combined organic phases were washed with brine and $H_2O$, dried over $Na_2SO_4$, filtered and evaporated to give **21a**[42] as a white solid (1.24 g, 95%). $^1$H NMR (acetone-$d_6$) $\delta$ 8.08 (dd, $J$ = 7.8, 1.5 Hz, 1H), 7.64 (ddd, $J$ = 7.5, 7.5, 1.5 Hz, 2H), 7.56 (dd, $J$ = 7.8, 1.5 Hz, 1H), 7.50 (ddd, $J$ = 7.5, 7.5, 1.5 Hz, 2H), 4.87 (s, 2H). $^{13}$C NMR (acetone-$d_6$) $\delta$ 168.2, 138.3, 133.5, 132.1, 131.2, 130.4, 129.1, 53.4. ESI$^-$-MS $m/z$ 176.0 $(M-H)^-$, 222.0 $(M+HCOOH-H)^-$, 353.1 $(2M-H)^-$, 572.3 $(3M-H)^-$.

#### 4.1.7. 2-(Azidomethyl)phenylacetic acid[37,38] (21b)

2-(Azidomethyl)phenylacetic acid was prepared according to the procedure described above. Ethyl 2-methylphenylacetate (2.14 mL, 12.0 mmol), NBS (2.35 g, 13.2 mmol), AIBN (98.5 mg, 0.60 mmol) and MeCN (18 mL) were used in the first step. Ethyl 2-(bromomethyl)phenylacetate was obtained as a pale yellow oil (2.39 g, 77%). $^1$H NMR ($CDCl_3$) $\delta$ 7.37 (m, 1H), 7.30–7.24 (m, 3H), 4.60 (s, 2H), 4.16 (q, $J$ = 7.2 Hz, 2H), 3.79 (s, 2H), 1.26 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CDCl_3$) $\delta$ 171.2, 136.5, 133.6, 131.4, 130.8, 129.3, 128.1, 61.3, 38.5, 31.9, 14.3. EI$^+$-MS $m/z$ 257 ($M^+$). The residual compound obtained above (2.19 g, 8.52 mmol), $NaN_3$ (0.66 g, 10.2 mmol) and DMF (10 mL) were used in the second step. Ethyl 2-(azidomethyl)phenylacetate was obtained as a pale yellow oil (1.85 g). $^1$H NMR ($CDCl_3$) $\delta$ 7.34–7.29 (m, 4H), 4.42 (s, 2H), 4.15 (q, $J$ = 7.2 Hz, 2H), 3.71 (s, 2H), 1.25 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CDCl_3$) $\delta$ 171.2, 134.1, 133.3, 131.3, 130.0, 129.0, 127.9, 61.2, 52.9, 38.7, 14.3. The ethyl 2-(azidomethyl)phenylacetate obtained above (1.85 g), LiOH (1.01 g, 42.2 mmol), THF (14 mL), MeOH (2 mL) and $H_2O$ (4 mL) were used in the last step. The crude product was purified by filtration trough a pad of silica followed by recrystallization (toluene/ether 1:3) and column chromatography (*i*-hexane/EtOAc/HCOOH 60:40:1) to afford **21b**[37,38] as a white solid (1.20 g, 73% over two steps). $^1$H NMR (acetone-$d_6$) $\delta$ 7.43–7.29 (m, 4H), 4.54 (s, 2H), 3.77 (s, 2H). $^{13}$C NMR (acetone-$d_6$) $\delta$ 172.4, 135.4, 135.0, 132.1, 130.5, 129.4, 128.2, 53.1, 38.4. ESI$^-$-MS $m/z$ 190.0 $(M-H)^-$, 236.1 $(M+HCOOH-H)^-$, 381.2 $(2M-H)^-$, 572.3 $(3M-H)^-$.

#### 4.1.8. 2-(Azidomethyl)benzoyl-2-chlorotrityl resin (22a)

2-(Azidomethyl)benzoic acid **21a** (101 mg, 0.57 mmol) was reacted with 2-chlorotrityl chloride resin (584 mg, 0.86 mmol) in $CH_2Cl_2$ (6 mL) in the presence of *N*,*N*-diisopropylethylamine (DIEA)

(0.39 mL, 2.24 mmol). After 4 h, MeOH (0.5 mL) was added and the mixture was stirred for another 25 min. The resin was washed with several portions of, in turn, $CH_2Cl_2$, DMF and $CH_2Cl_2$, and dried in vacuo overnight. Two batches were made and the resulting substitution degree was determined to 0.86 and 0.89 mmol/g based on the weight increase.

#### 4.1.9. 2-(Azidomethyl)phenylacetyl-2-chlorotrityl resin (22b)

2-(Azidomethyl)phenylacetic acid **21b** (101 mg, 0.53 mmol) was reacted with 2-chlorotrityl chloride resin (556 mg, 0.82 mmol) in $CH_2Cl_2$ (6 mL) in the presence of DIEA (0.37 mL, 2.12 mmol) following the procedure described above. Two batches were made and the resulting substitution degree was determined to 0.84 and 0.85 mmol/g from the weight increase. Another batch was made using 2-(azidomethyl)phenylacetic acid **21b** (104 mg, 0.54 mmol), 2-chlorotrityl chloride resin (534 mg, 0.79 mmol) and DIEA (0.37 mL, 2.1 mmol). Substitution degree: 0.88 mmol/g.

#### 4.1.10. Ile-His(Trt)-Pro-Phe-Wang resin (22c)

The Ile-His(Trt)-Pro-Phe-Wang resin was synthesized with a Symphony instrument using Fmoc protection. The starting polymer was Fmoc-Phe-Wang resin (297 mg, 0.25 mmol). Removal of the Fmoc group was achieved by reaction with 20% piperidine in DMF (2× 2.5 mL, 5 + 10 min). The amino acids (125 μmol) were coupled in DMF using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (125 μmol) in the presence of *N*-methylmorpholine (NMM) (500 μmol). Double couplings (2 × 30 min) were used for all amino acids. At the end of each coupling cycle, the remaining amino groups were capped by addition of 20% acetic anhydride in DMF (1.25 mL) to the reaction mixture and allowing the reaction to proceed for 5 min. After completion of the synthesis the Fmoc group was removed and the partially protected peptide resin was washed with several portions of DMF and $CH_2Cl_2$ and then dried in a stream of nitrogen and in vacuo. The resulting substitution degree was determined to 0.55 mmol/g based on the weight increase.

#### 4.1.11. Fmoc-Ile-2-chlorotrityl resin (22d)

Fmoc-Ile-OH (142 mg, 0.40 mmol) was reacted with 2-chlorotrityl chloride resin (341 mg, 0.50 mmol) in $CH_2Cl_2$ (6 mL) in the presence of DIEA (0.28 mL, 1.61 mmol) following the procedure described for **22a**. Substitution degree: 0.93 mmol/g.

### 4.2. General procedure for the preparation of resins 23a, 23b, 24a, 24b, 25a, 25b and 26b

A round-bottomed flask was charged with the appropriate amino acid or **20**, 1-hydroxybenzotriazole (HOBt) and *N*,*N*′-diisopropylcarbodiimide (DIPCDI) in dry $CH_2Cl_2$ or $CH_2Cl_2$/MeCN. After preactivation under nitrogen for 10 min, **22a** or **22b** was added, followed by tributylphosphine after another 10 min. The reaction mixture was stirred under nitrogen for 4–6 h. The resin was filtered off and washed with several portions of, in turn, $CH_2Cl_2$, DMF, MeOH and $CH_2Cl_2$, and finally dried in in vacuo overnight.

#### 4.2.1. Fmoc-Pro-2-(aminomethyl)benzoyl-2-chlorotrityl resin (23a)

Resin **23a** was prepared according to the general procedure using Fmoc-Pro-OH (263 mg, 779 μmol), HOBt (105 mg, 779 μmol), DIPCDI (122 μL, 779 μmol), $CH_2Cl_2$ (15 mL), **22a** (303 mg, 262 μmol) and tributylphosphine (150 μL, 520 μmol). Reaction time: 6 h. Yield: 373 mg.

#### 4.2.2. Fmoc-Pro-2-(aminomethyl)phenylacetyl-2-chlorotrityl resin (23b)

Resin **23b** was prepared according to the general procedure using Fmoc-Pro-OH (260 mg, 771 μmol), HOBt (104 mg, 771 μmol), DIPCDI (121 μL, 771 μmol), $CH_2Cl_2$ (15 mL), **22b** (283 mg, 242 μmol) and tributylphosphine (149 μL, 514 μmol). Reaction time: 6 h. Yield: 354 mg.

#### 4.2.3. Fmoc-Leu-2-(aminomethyl)benzoyl-2-chlorotrityl resin (24a)

Resin **24a** was prepared according to the general procedure using Fmoc-Leu-OH (379 mg, 1.07 mmol), HOBt (145 mg, 1.07 μmol), DIPCDI (168 μL, 1.07 mmol), $CH_2Cl_2$ (20 mL), **22a** (315 mg, 281 μmol) and tributylphosphine (206 μL, 710 μmol). Reaction time: 4 h. Yield: 379 mg.

#### 4.2.4. Fmoc-Leu-2-(aminomethyl)phenylacetyl-2-chlorotrityl resin (24b)

Resin **24b** was prepared according to the general procedure using Fmoc-Leu-OH (269 mg, 760 μmol), HOBt (105 mg, 774 μmol), DIPCDI (119 μL, 761 μmol), $CH_2Cl_2$ (20 mL), **22b** (305 mg, 258 μmol) and tributylphosphine (147 μL, 510 μmol). Reaction time: 4 h. Yield: 373 mg.

#### 4.2.5. Fmoc-Ile-2-(aminomethyl)benzoyl-2-chlorotrityl resin (25a)

Resin **25a** was prepared according to the general procedure using Fmoc-Ile-OH (275 mg, 779 μmol), HOBt (105 mg, 779 μmol), DIPCDI (122 μL, 779 μmol), $CH_2Cl_2$ (15 mL), **22a** (301 mg, 260 μmol) and tributylphosphine (150 μL, 520 μmol). Reaction time: 6 h. Yield: 378 mg.

#### 4.2.6. Fmoc-Ile-2-(aminomethyl)phenylacetyl-2-chlorotrityl resin (25b)

Resin **25b** was prepared according to the general procedure using Fmoc-Ile-OH (273 mg, 771 μmol), HOBt (104 mg, 771 μmol), DIPCDI (121 μL, 771 μmol), $CH_2Cl_2$ (15 mL), **22b** (301 mg, 257 μmol) and tributylphosphine (149 μL, 514 μmol). Reaction time: 6 h. Yield 381 mg.

#### 4.2.7. 3-(9H-Fluoren-9-ylmethoxycarbonylamino)-5-(4-hydroxybenzyl)benzoyl-2-(aminomethyl)phenylacetyl-2-chlorotrityl resin (26b)

Resin **26b** was prepared according to the general procedure using **20** (246 mg, 528 μmol), HOBt (77.7 mg, 575 μmol), DIPCDI (82.2 μL, 525 μmol), $CH_2Cl_2$ (30 mL), MeCN (5 mL), **22b** (399 mg, 351 μmol) and tributylphosphine (203 μL, 700 μmol). Reaction time: 5 h. Yield: 517 mg.

### 4.3. General synthesis of angiotensin IV analogues 1–15

Resin **22c**, **22d**, **23a**, **23b**, **24a**, **24b**, **25a**, **25b** or **26b** was weighed into a 2 mL disposable syringe fitted with porous polyethylene filter. The Fmoc group was removed by treatment with 20% piperidine in DMF (2× 1.5 mL, 10 + 15 min) and the polymer was washed with DMF (5× 1.5 mL, 5 × 1 min). Coupling of **20** and/or the appropriate amino acid was performed by agitation over night in DMF (1.5 mL) using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) in the presence of DIEA. The resin was washed with DMF (5× 1.5 mL, 5 × 1 min) and subsequently deprotected and washed as described above. After completion of the coupling cycle, the resin was also washed with several portions of, in turn, $CH_2Cl_2$, MeOH and $CH_2Cl_2$ before it was dried in in vacuo. The pseudopeptide was cleaved by treatment with TFA/$H_2O$/triethylsilane (90:5:5 ca. 1 mL/100 mg resin) for 2 h. The resin was filtered off and washed with TFA (4× 0.3–0.5 mL). The filtrate was collected in a centrifuge tube and concentrated in a stream of nitrogen. Cold diethyl ether (ca. 13 mL) was used to precipitate the product, which was collected by centrifugation, washed with cold diethyl ether (⩽3× 7 mL) and dried. The

crude pseudopeptide was dissolved in MeCN/0.1% aqueous TFA, filtered through a 0.45 μm nylon membrane and purified in 1–2 runs by RP-HPLC. Selected fractions were analyzed by RP-HPLC and RP-HPLC-MS, and those containing pure product were pooled and lyophilized.

#### 4.3.1. Analogue 1

Except for the initial Fmoc deprotection, the analogue was prepared according to the general procedure using **22c** (251 mg, 138 μmol), **20** (80.2 mg, 172 μmol), PyBOP (89.6 mg, 172 μmol) and DIEA (59.9 μL, 344 μmol) in the first coupling followed by Fmoc-Val-OH (142 mg, 418 μmol), PyBOP (217 mg, 417 μmol) and DIEA (144 μL, 829 μmol) in the second. Reactiontime: 18 h for both couplings. Purification by preparative RP-HPLC gave **1** as a white solid (11.3 mg, 25%). HPLC purity: C18 column 99.0%, Phenyl column 99.1%. HRMS ($M+H^+$): 837.4277, $C_{45}H_{57}N_8O_8$ requires 837.4299. Amino acid analysis: Val 1.05; Ile 0.99; His 0.98; Pro 0.98; Phe 1.00.

#### 4.3.2. Analogue 2

The analogue was prepared according to the general procedure using **23a** (170 mg, 120 μmol), **20** (85.7 mg, 184 μmol), PyBOP (95.8 mg, 184 μmol) and DIEA (64.1 μL, 368 μmol) in the first coupling followed by Fmoc-Val-OH (100 mg, 294 μmol), PyBOP (153 mg, 294 μmol) and DIEA (103 μL, 589 μmol) in the second. Reaction time: 17 h and 18 h, respectively. Purification by preparative RP-HPLC gave **2** as a white solid (5.1 mg, 7%). $^1$H NMR ($CD_3OD$) $\delta$ (3:1 mixture of rotamers, major rotamer reported) 7.98 (dd, $J$ = 7.8, 1.4 Hz, 1H), 7.73 (m, 1H), 7.58 (m, 1H), 7.52 (m, 1H), 7.46 (m, 1H), 7.35 (m, 1H), 7.25 (m, 1H), 7.03 (m, 2H), 6.70 (m, 2H), 4.78 (app. s, 2H), 4.60 (dd, $J$ = 8.2, 5.8 Hz, 1H), 3.92 (app. s, 2H), 3.73 (d, $J$ = 5.9 Hz, 1H), 3.68 (m, 1H), 3.56 (m, 1H), 2.35–2.17 (m, 2H), 2.06–1.97 (m, 2H), 1.87 (m, 1H), 1.11 (d, $J$ = 6.9 Hz, 3H), 1.08 (d, $J$ = 6.9 Hz, 3H). HPLC purity: C18 column >99.9%, Phenyl column 98.3%. HRMS ($M+H^+$): 573.2711, $C_{32}H_{37}N_4O_6$ requires 573.2713. Amino acid analysis: Val, 1.13; Pro, 0.87 (72% peptide).

#### 4.3.3. Analogue 3

The analogue was prepared according to the general procedure using **23b** (148 mg, 101 μmol), **20** (84.8 mg, 182 μmol), PyBOP (92.5 mg, 178 μmol) and DIEA (63.5 μL, 364 μmol) in the first coupling followed by Fmoc-Val-OH (99.4 mg, 293 μmol), PyBOP (153 mg, 293 μmol) and DIEA (102 μL, 586 μmol) in the second. Reaction time: 16 h for both couplings. Purification by preparative RP-HPLC gave **3** as a white solid (5.9 mg, 10%). $^1$H NMR ($CD_3OD$) $\delta$ (3:1 mixture of rotamers, major rotamer reported) 7.73 (m, 1H), 7.46 (m, 1H), 7.39 (m, 1H), 7.29–7.20 (m, 4H), 7.02 (m, 2H), 6.70 (m, 2H), 4.54 (dd, $J$ = 8.1, 5.8 Hz, 1H), 4.50 (d, $J$ = 15.0 Hz, 1H), 4.46 (d, $J$ = 15.0 Hz, 1H), 3.91 (app. s, 2H), 3.75 (app. s, 2H), 3.73 (d, $J$ = 5.9 Hz, 1H), 3.63 (m, 1H), 3.52 (m, 1H), 2.35–2.17 (m, 2H), 2.05–1.95 (m, 2H), 1.86 (m, 1H), 1.11 (d, $J$ = 6.9 Hz, 3H), 1.08 (d, $J$ = 6.9 Hz, 3H). $^{13}$C NMR ($CD_3OD$) $\delta$ (major rotamer reported) 175.4, 174.4, 171.6, 168.0, 157.0, 145.2, 139.1, 138.2, 138.0, 134.4, 132.5, 131.8, 131.0, 129.8, 128.6, 124.9, 123.1, 117.7, 116.4, 62.2, 60.5, 51.8, 42.1, 41.7, 39.1, 31.7, 31.2, 26.4, 19.0, 17.8. One carbon signal in the aromatic area is missing due to overlapping signals. HPLC purity: C18 column 99.4%, Phenyl column 99.4%. HRMS ($M+H^+$): 587.2854, $C_{33}H_{39}N_4O_6$ requires 587.2870. Amino acid analysis: Val, 1.04; Pro, 0.96 (77% peptide).

#### 4.3.4. Analogue 4

The analogue was prepared according to the general procedure using **23a** (170 mg, 120 μmol), **20** (85.7 mg, 184 μmol), PyBOP (95.8 mg, 184 μmol) and DIEA (64.1 μL, 368 μmol) in the first coupling followed by Fmoc-Nle-OH (104 mg, 294 μmol), PyBOP (153 mg, 294 μmol) and DIEA (103 μL, 589 μmol) in the second. Reaction time: 17 h and 18 h, respectively. Purification by preparative RP-HPLC gave **4** as a white solid (13.6 mg, 19%). $^1$H NMR ($CD_3OD$) $\delta$ (3:1 mixture of rotamers, major rotamer reported) 7.96 (dd, $J$ = 7.8, 1.4 Hz, 1H), 7.73 (m, 1H), 7.57 (m, 1H), 7.51 (m, 1H), 7.47 (m, 1H), 7.34 (m, 1H), 7.24 (m, 1H), 7.02 (m, 2H), 6.70 (m, 2H), 4.77 (app. s, 2H), 4.60 (dd, $J$ = 8.1, 5.8 Hz, 1H), 3.96–3.91 (m, 3H), 3.62 (m, 1H), 3.51 (m, 1H), 2.32 (m, 1H), 2.05–1.81 (m, 5H), 1.44–1.35 (m, 4H), 0.95 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CD_3OD$) $\delta$ (major rotamer reported) 174.5, 171.7, 170.9, 168.7, 157.0, 145.2, 140.9, 139.2, 138.2, 133.3, 132.5, 132.0, 131.0, 129.8, 128.1, 124.8, 123.1, 117.7, 116.4, 62.2, 55.2, 51.7, 43.1, 41.7, 32.5, 31.1, 28.0, 26.3, 23.4, 14.1. One carbon signal in the aromatic area is missing. HPLC purity: C18 column 99.5%, Phenyl column 99.7%. HRMS ($M+H^+$): 587.2873, $C_{33}H_{39}N_4O_6$ requires 587.2870. Amino acid analysis: Nle, 1.01; Pro, 0.99 (77% peptide).

#### 4.3.5. Analogue 5

The analogue was prepared according to the general procedure using **23b** (148 mg, 101 μmol), **20** (84.8 mg, 182 μmol), PyBOP (92.5 mg, 178 μmol) and DIEA (63.5 μL, 364 μmol) in the first coupling followed by Fmoc-Nle-OH (91.4 mg, 253 μmol), PyBOP (132 mg, 253 μmol) and DIEA (88.2 μL, 506 μmol) in the second. Reaction time: 16 h for both couplings. Purification by preparative RP-HPLC gave **5** as a white solid (17.4 mg, 29%). $^1$H NMR ($CD_3OD$) $\delta$ (3:1 mixture of rotamers, major rotamer reported) 7.73 (dd, $J$ = 2.1, 1.6 Hz, 1H), 7.46 (dd, $J$ = 2.1, 1.6 Hz, 1H), 7.40 (m, 1H), 7.28–7.21 (m, 4H), 7.02 (m, 2H), 6.70 (m, 2H), 4.54 (dd, $J$ = 8.1, 5.8 Hz, 1H), 4.50 (d, $J$ = 15.0 Hz, 1H), 4.46 (d, $J$ = 15.0 Hz, 1H), 3.94–3.91 (m, 3H), 3.77 (d, $J$ = 16.0 Hz, 1H), 3.73 (d, $J$ = 16.0 Hz, 1H), 3.62 (m, 1H), 3.51 (m, 1H), 2.31 (m, 1H), 2.03–1.81 (m, 5H), 1.44–1.35 (m, 4H), 0.95 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CD_3OD$) $\delta$ (major rotamer reported) 175.4, 174.4, 171.6, 168.7, 157.0, 145.2, 139.2, 138.2, 138.1, 134.4, 132.5, 131.8, 131.0, 129.8, 128.6, 124.9, 123.1, 117.6, 116.4, 62.2, 55.2, 51.8, 42.0, 41.7, 39.1, 32.5, 31.2, 28.0, 26.4, 23.4, 14.1. One carbon signal in the aromatic area is missing due to overlapping signals. HPLC purity: C18 column 98.5%, Phenyl column 99.5%. HRMS ($M+H^+$): 601.3038, $C_{34}H_{41}N_4O_6$ requires 601.3026. Amino acid analysis: Nle, 1.01; Pro, 0.99 (59% peptide).

#### 4.3.6. Analogue 6

The analogue was prepared according to the general procedure using **24a** (100 mg, 74.2 μmol), **20** (115 mg, 247 μmol), PyBOP (130 mg, 249 μmol) and DIEA (86.8 μL, 498 μmol) in the first coupling followed by Fmoc-Nle-OH (96.5 mg, 272 μmol), PyBOP (130 mg, 249 μmol) and DIEA (86.8 μL, 498 μmol) in the second. Reaction time: 15 h and 17 h, respectively. Purification by preparative RP-HPLC gave **6** as a white solid (6.6 mg, 15%). $^1$H NMR ($CD_3OD$) $\delta$ (9:1 mixture of rotamers, major rotamer reported) 7.98 (m, 1H), 7.91 (dd, $J$ = 2.1, 1.6 Hz, 1H), 7.52–7.48 (m, 4H), 7.35 (m, 1H), 7.04 (m, 2H), 6.71 (m, 2H), 4.73 (m, 2H), 4.64 (m, 1H), 3.95–3.91 (m, 3H), 1.99–1.84 (m, 2H), 1.78–1.67 (m, 3H), 1.47–1.36 (m, 4H), 1.00–0.93 (m, 9H). $^{13}$C NMR ($CD_3OD$) $\delta$ (major rotamer reported) 174.8, 170.6, 170.2, 168.8, 157.0, 145.2, 141.0, 139.3, 136.5, 133.5, 132.5, 132.2, 131.0, 130.1, 128.3, 125.1, 124.6, 118.2, 116.4, 55.2, 54.1, 43.2, 41.7, 32.5, 28.0, 26.2, 23.4, 22.0, 14.1. One carbon signal in the aromatic area is missing and two carbon signals in the aliphatic area are missing due to overlapping signals. HPLC purity: C18 column >99.9%, Phenyl column 99.5%. HRMS ($M+H^+$): 603.3172, $C_{34}H_{43}N_4O_6$ requires 603.3183. Amino acid analysis: Nle, 1.01; Leu, 0.99 (89% peptide).

#### 4.3.7. Analogue 7

The analogue was prepared according to the general procedure using **24b** (151 mg, 101 μmol), **20** (174 mg, 374 μmol), PyBOP (96.7 mg, 187 μmol) and DIEA (130 μL, 74.8 μmol) in the first coupling followed by Fmoc-Nle-OH (133 mg, 374 μmol), PyBOP

(196 mg, 374 μmol) and DIEA (130 μL, 748 μmol) in the second. Reaction time: 16 h and 6 h, respectively. Purification by preparative RP-HPLC gave **7** as a white solid (9.8 mg, 15%). $^1$H NMR ($CD_3OD$) $\delta$ (9:1 mixture of rotamers, major rotamer reported) 7.91 (m, 1H), 7.52 (m, 1H), 7.50 (m, 1H), 7.32 (m, 1H), 7.26–7.20 (m, 3H), 7.03 (m, 2H), 6.71 (m, 2H), 4.63 (m, 1H), 4.47 (d, $J$ = 15.1 Hz, 1H), 4.43 (d, $J$ = 15.1 Hz, 1H), 3.95–3.91 (m, 3H), 3.73 (app. s, 2H), 2.00–1.84 (m, 2H), 1.80–1.63 (m, 3H), 1.47–1.36 (m, 4H), 0.98 (d, $J$ = 6.4 Hz, 3H), 0.96 (d, $J$ = 6.4 Hz, 3H), 0.95 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR ($CD_3OD$) $\delta$ (major rotamer reported) 175.4, 174.7, 170.1, 168.8, 157.0, 145.2, 139.3, 138.1, 136.5, 134.5, 132.5, 131.8, 131.0, 129.8, 128.64, 128.56, 125.1, 124.6, 118.2, 116.4, 55.2, 54.0, 42.0, 41.9, 41.7, 39.2, 32.5, 28.0, 26.2, 23.41, 23.40, 22.0, 14.1. HPLC purity: C18 column >99.9%, Phenyl column 99.2%. HRMS (M+H$^+$): 617.3350, $C_{35}H_{45}N_4O_6$ requires 617.3339. Amino acid analysis: Nle, 1.01; Leu, 0.99 (81% peptide).

#### 4.3.8. Analogue 8

The analogue was prepared according to the general procedure using **25a** (170 mg, 117 μmol), **20** (85.7 mg, 184 μmol), PyBOP (95.8 mg, 184 μmol) and DIEA (64.1 μL, 368 μmol) in the first coupling followed by Fmoc-Val-OH (99.9 mg, 294 μmol), PyBOP (153 mg, 294 μmol) and DIEA (103 μL, 589 μmol) in the second. Reaction time: 19 h and 18 h, respectively. Purification by preparative RP-HPLC gave **8** as a white solid (4.0 mg, 6%). $^1$H NMR ($CD_3OD$) $\delta$ (17:3 mixture of rotamers, major rotamer reported) 7.94 (m, 1H), 7.90 (m, 1H), 7.52 (m, 1H), 7.48–7.42 (m, 3H), 7.33 (m, 1H), 7.03 (m, 2H), 6.71 (m, 2H), 4.76 (d, $J$ = 15.4 Hz, 1H), 4.70 (d, $J$ = 15.3 Hz, 1H), 4.42 (d, $J$ = 8.2 Hz, 1H), 3.93 (app. s, 2H), 3.74 (d, $J$ = 5.9 Hz, 1H), 2.28 (m, 1H), 1.96 (m, 1H), 1.59 (m, 1H), 1.23 (m, 1H), 1.12 (d, $J$ = 6.9 Hz, 3H), 1.09 (d, $J$ = 6.9 Hz, 3H), 0.96–0.88 (m, 6H). HPLC purity: C18 column 99.1%, Phenyl column 99.3%. HRMS (M+H$^+$): 589.3029, $C_{33}H_{41}N_4O_6$ requires 589.3026. Amino acid analysis: Val, 1.02; Ile, 0.98 (70% peptide).

#### 4.3.9. Analogue 9

The analogue was prepared according to the general procedure using **25b** (170 mg, 115 μmol), **20** (84.8 mg, 182 μmol), PyBOP (94.8 mg, 183 μmol) and DIEA (63.5 μL, 364 μmol) in the first coupling cycle followed by Fmoc-Val-OH (100 mg, 292 μmol), PyBOP (152 mg, 292 μmol) and DIEA (102 μL, 584 μmol) in the second cycle. Reaction time: 18 h and 16 h, respectively. Purification by preparative RP-HPLC gave **9** as a white solid (8.3 mg, 12%). $^1$H NMR ($CD_3OD$) $\delta$ (9:1 mixture of rotamers, major rotamer reported) 7.91 (dd, $J$ = 2.2, 1.6 Hz, 1H), 7.53 (m, 1H), 7.47 (m, 1H), 7.34 (m, 1H), 7.25–7.20 (m, 3H), 7.03 (m, 2H), 6.71 (m, 2H), 4.48 (d, $J$ = 15.3 Hz, 1H), 4.44 (d, $J$ = 15.3 Hz, 1H), 4.40 (d, $J$ = 8.6 Hz, 1H), 3.93 (app. s, 2H), 3.78–3.70 (m, 3H), 2.28 (m, 1H), 1.97 (m, 1H), 1.60 (m, 1H), 1.23 (m, 1H), 1.11 (d, $J$ = 6.9 Hz, 3H), 1.08 (d, $J$ = 6.9 Hz, 3H), 0.95–0.90 (m, 6H). $^{13}$C NMR ($CD_3OD$) $\delta$ (major rotamer reported) 175.4, 173.7, 170.1, 168.2, 157.0, 145.3, 139.2, 138.0, 136.6, 134.5, 132.4, 131.8, 131.0, 130.0, 128.7, 128.6, 125.1, 124.5, 118.1, 116.4, 60.5, 60.0, 41.9, 41.7, 39.2, 37.9, 31.7, 26.3, 19.0, 17.8, 16.0, 11.2. HPLC purity: C18 column 99.0%, Phenyl column 99.5%. HRMS (M+H$^+$): 603.3181, $C_{34}H_{43}N_4O_6$ requires 603.3183. Amino acid analysis: Val, 1.05; Ile, 0.95 (79% peptide).

#### 4.3.10. Analogue 10

The analogue was prepared according to the general procedure using **25a** (170 mg, 121 μmol), **20** (84.9 mg, 182 μmol), PyBOP (94.9 mg, 182 μmol) and DIEA (63.5 μL, 365 μmol) in the first coupling followed by Fmoc-Nle-OH (103 mg, 292 μmol), PyBOP (152 mg, 292 μmol) and DIEA (102 μL, 583 μmol) in the second. Reaction time: 18 h and 16 h, respectively. Purification by preparative RP-HPLC gave **10** as a white solid (12.5 mg, 17%). $^1$H NMR ($CD_3OD$) $\delta$ (19:1 mixture of rotamers, major rotamer reported) 7.99 (m, 1H), 7.90 (dd, $J$ = 2.1, 1.6 Hz, 1H), 7.52 (dd, $J$ = 2.1, 1.5 Hz, 1H), 7.49–7.45 (m, 3H), 7.35 (m, 1H), 7.03 (m, 2H), 6.71 (m, 2H), 4.78 (d, $J$ = 15.5 Hz, 1H), 4.73 (d, $J$ = 15.5 Hz, 1H), 4.42 (d, $J$ = 8.5 Hz, 2H), 3.95–3.92 (m, 3H), 2.00–1.84 (m, 3H), 1.59 (ddq, $J$ = 13.6, 3.5, 7.5 Hz, 1H), 1.47–1.36 (m, 4H), 1.24 (ddq, $J$ = 13.6, 9.0, 7.4 Hz, 1H), 0.97–0.90 (m, 9H). $^{13}$C NMR ($CD_3OD$) $\delta$ (major rotamer reported) 173.8, 170.4, 170.2, 168.8, 157.0, 145.3, 141.0, 139.3, 136.6, 133.5, 132.4, 132.2, 131.0, 130.3, 128.3, 125.0, 124.5, 118.0, 116.4, 60.1, 55.2, 43.1, 41.7, 37.8, 32.5, 28.0, 26.3, 23.4, 16.0, 14.1, 11.2. One carbon signal in the aromatic area is missing. HPLC purity: C18 column >99.9%, Phenyl column 99.5%. HRMS (M+H$^+$): 603.3199, $C_{34}H_{43}N_4O_6$ requires 603.3183. Amino acid analysis: Nle, 0.99; Ile, 1.01 (84% peptide).

#### 4.3.11. Analogue 11

The analogue was prepared according to the general procedure using **25b** (170 mg, 115 μmol), **20** (84.8 mg, 182 μmol), PyBOP (95.0 mg, 183 μmol) and DIEA (63.5 μL, 364 μmol) in the first coupling followed by Fmoc-Nle-OH (106 mg, 299 μmol), PyBOP (154 mg, 295 μmol) and DIEA (102 μL, 584 μmol) in the second. Reaction time: 18 h and 16 h, respectively. Purification by preparative RP-HPLC gave **11** as a white solid (18.5 mg, 26%). $^1$H NMR ($CD_3OD$) $\delta$ (19:1 mixture of rotamers, major rotamer reported) 7.91 (m, 1H), 7.52 (m, 1H),7.46 (m, 1H), 7.34 (m, 1H), 7.24–7.20 (m, 3H), 7.03 (m, 2H), 6.71 (m, 2H), 4.48 (d, $J$ = 15.2 Hz, 1H), 4.44 (d, $J$ = 15.2 Hz, 1H), 4.40 (d, $J$ = 8.6 Hz, 1H), 3.95–3.92 (m, 3H), 3.76 (d, $J$ = 16.3 Hz, 1H), 3.72 (d, $J$ = 16.3 Hz, 1H), 2.02–1.84 (m, 3H), 1.60 (ddq, $J$ = 13.6, 3.4, 7.6 Hz, 1H), 1.47–1.35 (m, 4H), 1.24 (ddq, $J$ = 13.6, 9.0, 7.4 Hz, 1H), 0.97–0.90 (m, 9H). $^{13}$C NMR ($CD_3OD$) $\delta$ (major rotamer reported) 175.4, 173.7, 170.1, 168.8, 157.0, 145.3, 139.3, 138.0, 136.6, 134.5, 132.5, 131.8, 131.0, 123.0, 128.7, 128.6, 125.0, 124.5, 118.0, 116.4, 60.1, 55.2, 41.9, 41.7, 39.2, 37.9, 32.5, 28.0, 26.3, 23.4, 16.0, 14.1, 11.2. HPLC purity: C18 column >99.9%, Phenyl column 99.2%. HRMS (M+H$^+$): 617.3327, $C_{35}H_{45}N_4O_6$ requires 617.3339. Amino acid analysis: Nle, 1.03; Ile, 0.97 (83% peptide).

#### 4.3.12. Analogue 12

The analogue was prepared according to the general procedure using **25b** (200 mg, 148 μmol), **20** (102 mg, 220 μmol), PyBOP (114 mg, 219 μmol) and DIEA (76.0 μL, 436 μmol). Reaction time: 18 h. Purification by preparative RP-HPLC gave **12** as a white solid (6.4 mg, 9%). $^1$H NMR ($CD_3OD$) $\delta$ (17:3 mixture of rotamers, major rotamer reported) 7.40 (m, 1H), 7.34 (m, 1H), 7.29 (m, 1H), 7.25–7.20 (m, 3H), 7.03 (m, 2H), 7.01 (m, 1H), 6.71 (m, 2H), 4.46 (m, 2H), 4.39 (d, $J$ = 8.5 Hz, 1H), 3.91 (app. s, 2H), 3.76 (d, $J$ = 16.3 Hz, 1H), 3.72 (d, $J$ = 16.3 Hz, 1H), 1.96 (m, 1H), 1.59 (ddq, $J$ = 13.6, 3.4, 7.6 Hz, 1H), 1.23 (m, 1H), 0.96–0.90 (m, 6H). HPLC purity: C18 column >99.9%, Phenyl column >99.9%. HRMS (M+H$^+$): 504.2509, $C_{29}H_{34}N_3O_5$ requires 504.2498.

#### 4.3.13. Analogue 13

The analogue was prepared according to the general procedure using **26b** (263 mg, 179 μmol), Fmoc-Val-OH (183 mg, 540 μmol), PyBOP (277 mg, 533 μmol) and DIEA (186 μL, 1.07 mmol). Reaction time: 19 h. Purification by preparative RP-HPLC gave **13** as a white solid (10.8 mg, 12%). $^1$H NMR ($CD_3OD$) $\delta$ 7.93 (dd, $J$ = 2.1, 1.6 Hz, 1H), 7.51 (dd, $J$ = 2.1, 1.5 Hz, 1H), 7.49 (m, 1H), 7.37 (m, 1H), 7.29–7.23 (m, 3H), 7.02 (m, 2H), 6.70 (m, 2H), 4.62 (app. s, 2H), 3.92 (app. s, 2H), 3.80 (app. s, 2H), 3.73 (d, $J$ = 5.9 Hz, 1H), 2.26 (dqq, $J$ = 5.9, 6.9, 6.9 Hz, 1H), 1.10 (d, $J$ = 6.9 Hz, 3H), 1.07 (d, $J$ = 6.9 Hz, 3H). $^{13}$C NMR ($CD_3OD$) $\delta$ 175.7, 169.6, 168.1, 157.0, 145.2, 139.2, 138.3, 136.7, 134.7, 132.5, 131.9, 131.0, 130.0, 128.7, 128.5, 124.9, 124.5, 118.2, 116.4, 60.5, 42.6, 41.8, 39.3, 31.7, 19.0, 17.8. HPLC purity: C18 column 98.2%, Phenyl column 98.1%. HRMS (M+H$^+$): 490.2331, $C_{28}H_{32}N_3O_5$ requires 490.2342.

#### 4.3.14. Analogue 14

Resin **26b** (222 mg, 150 μmol) was Fmoc deprotected and cleaved from the resin according to the general procedure. Purification by preparative RP-HPLC gave **14** as a white solid (9.4 mg, 16%). $^1$H NMR ($CD_3OD$) $\delta$ 7.56 (m, 1H), 7.44 (dd, $J$ = 2.2, 1.6 Hz, 1H), 7.36 (m, 1H), 7.29–7.23 (m, 3H), 7.09 (dd, $J$ = 2.2, 1.5 Hz, 1H), 7.03 (m, 2H), 6.71 (m, 2H), 4.62 (s, 2H), 3.93 (s, 2H), 3.80 (s, 2H). $^{13}$C NMR ($CD_3OD$) $\delta$ 175.6, 168.9, 157.1, 146.3, 138.2, 137.4, 134.6, 132.2, 132.0, 131.0, 130.1, 128.8, 128.6, 125.5, 124.8, 118.4, 116.4, 42.6, 41.5, 39.1. One carbon signal in the aromatic area is missing. HPLC purity: C18 column 99.5%, Phenyl column >99.9%. HRMS ($M+H^+$): 391.1652, $C_{23}H_{23}N_2O_4$ requires 391.1658.

#### 4.3.15. Analogue 15

The analogue was prepared according to the general procedure using **22d** (381 mg, 354 μmol), **20** (191 mg, 411 μmol), PyBOP (224 mg, 430 μmol) and DIEA (150 μL, 858 μmol) in the first coupling followed by Fmoc-Val-OH (334 mg, 982 μmol), PyBOP (512 mg, 981 μmol) and DIEA (342 μL, 1.96 mmol) in the second. Reaction time: 18 h and 19 h, respectively. Purification by preparative RP-HPLC gave **15** as a white solid (25.3 mg, 16%). $^1$H NMR ($CD_3OD$) $\delta$ 7.92 (dd, $J$ = 2.1, 1.6 Hz, 1H), 7.52 (dd, $J$ = 2.1, 1.5 Hz, 1H), 7.48 (m, 1H), 7.03 (m, 2H), 6.71 (m, 2H), 4.53 (d, $J$ = 6.2 Hz, 1H), 3.92 (app. s, 2H), 3.77 (d, $J$ = 6.0 Hz, 1H), 2.27 (dqq, $J$ = 6.0, 6.9, 6.9 Hz, 1H), 2.02 (m, 1H), 1.61 (ddq, $J$ = 13.6, 4.3, 7.5 Hz, 1H), 1.31 (ddq, $J$ = 13.6, 9.0, 7.4 Hz, 1H), 1.11 (d, $J$ = 6.9 Hz, 3H), 1.08 (d, $J$ = 6.9 Hz, 3H), 1.02 (d, $J$ = 6.8 Hz, 3H), 0.96 (m, 3H). $^{13}$C NMR ($CD_3OD$) $\delta$ 170.2, 168.2, 164.1, 157.0, 145.2, 139.2, 136.7, 132.5, 131.0, 125.1, 124.5, 118.1, 116.4, 60.5, 59.1, 41.7, 38.3, 31.7, 26.6, 19.0, 17.8, 16.1, 11.7. HPLC purity: C18 column 99.0%, Phenyl column 98.7%. HRMS ($M+H^+$): 456.2505, $C_{25}H_{34}N_3O_5$ requires 456.2498.

### 4.4. Biochemical evaluation

L-Leucine-*p*-nitroanilide (L-Leu-pNA) was obtained from Sigma–Aldrich (Bornem, Belgium). Tyr$^2$ of Ang IV was iodinated as described by Lahoutte et al.[55] using the Iodogen iodination reagent from Pierce (Erembodegem, Belgium).[55] $^{125}$I was obtained from MP Biomedicals (Asse, Belgium). Monoiodinated Ang IV was isolated on a GraceVydac C18 monomeric 120A reverse-phase HPLC column 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).

#### 4.4.1. Cell culture, transient transfection and membrane preparation

CHO-K1 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 non-essential amino acids, 1 mM sodium pyruvate and 10% (v/v) foetal 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, Laboratoty of Cellular Biochemistry, Saitama, Japan) or pTEJ4[56] carrying the complete human aminopeptidase N cDNA.[57] The transient transfection was performed as described previously with 8 μL/mL LipofectAMINE (Invitrogen, Merelbeke, Belgium) and 1 μg/mL plasmid DNA.[58] 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 non-transfected cells.

CHO-K1 cell and transfected HEK293 cell membranes were prepared as described previously.[59] 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.

#### 4.4.2. Enzyme assay

Determination of the aminopeptidase catalytic activity was based on the cleavage of the substrate L-Leu-pNA[59] 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 phenylmethylsulfonyl 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 $4 \times 10^5$ CHO-K1 cells and $1.5 \times 10^5$ 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.

#### 4.4.3. Radioligand binding assays

The binding affinities of the compounds were assessed using 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, including 30 mM EDTA/600 μM 1,10-phenantroline (1,10-Phe). The assays were carried out in polyethylene 24-well plates (Elscolab, Kruibeke, Belgium). Preincubations were carried out for 40 min at 37 °C in a finale volume of 250 μL containing 150 μL membrane homogenate, 50 μL enzyme assay buffer (for total binding), 50 μL enzyme assay buffer with the compounds (for competition binding assays) or 60 μM unlabelled Ang IV (for non-specific binding). The binding assay was initiated by adding 50 μL enzyme assay buffer containing [$^{125}$I]Ang IV and the mixture was further incubated for 30 min at 37 °C. Final membrane concentrations were the same as for the enzyme assays, final chelator concentrations were 5 mM EDTA and 100 μM 1,10-Phe, the final [$^{125}$I]Ang IV concentration was 1 nM. After incubation, the mixture was vacuum filtered using an Inotech 24-well cell-harvester through GF/B glass fibre filters (Whatman) pre-soaked in 1% (w/v) BSA. After drying, the radioactivity retained in the filters was measured using a Perkin-Elmer γ-counter.

#### 4.4.4. Data analysis

All experiments were performed two times with duplicate determinations each. The calculation of $IC_{50}$ values from competition binding (or enzyme inhibition) experiments was 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 [$^{125}$I]Ang IV (from saturation binding experiments) or the Michaelis–Menten constant ($K_m$) for substrate cleavage.[60]

## Acknowledgments

We thank The Swedish Research Council, the Research Council of the 'Vrije Universiteit Brussel' (GOA-2002), the 'Geneeskundige Stichting Koningin Elisabeth' and the Institute for the Promotion of Innovation through Science and Technology in Flanders (I.W.T.-Vlaanderen) for economic support. We are also obliged to Matthias Bauwens and Ken Kersemans (Medical Imaging and Physical Sciences, Vrije Universiteit Brussel) for the preparation of radiolabelled Ang IV and Anders Johnsson for preparative work.

## Supplementary data

HPLC purity for analogues **1**–**15**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.05.046.

## References and notes

1. Hallberg, M.; Le Greves, P.; Nyberg, F. Neuropeptide processing. In *Proteases in biology Biology and disease. Disease. Proteases in the brainBrain*; Springer Science: New York, 2005; pp 203–234.
2. Hallberg, M.; Nyberg, F. *Curr. Protein Pept. Sci.* **2003**, *4*, 31–44.
3. Chai, S. Y.; Fernando, R.; Peck, G.; Ye, S. Y.; Mendelsohn, F. A.; Jenkins, T. A.; Albiston, A. L. *Cell. Mol. Life Sci.* **2004**, *61*, 2728–2737.
4. Vauquelin, G.; Michotte, Y.; Smolders, I.; Sarre, S.; Ebinger, G.; Dupont, A.; Vanderheyden, P. *J. Renin Angiotensin Aldosterone Syst.* **2002**, *3*, 195–204.
5. von Bohlen und Halbach, O. *Cell Tissue Res.* **2003**, *311*, 1–9.
6. de Gasparo, M.; Husain, A.; Alexander, W.; Catt, K. J.; Chiu, A. T.; Drew, M.; Goodfriend, T.; Harding, J. W.; Inagami, T.; Timmermans, P. B. M. W. M. *Hypertension* **1995**, *25*, 924–927.
7. de Gasparo, M.; Catt, K. J.; Inagami, T.; Wright, J. W.; Unger, T. *Pharmacol. Rev.* **2000**, *52*, 415–472.
8. 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 *Regul. Pept.* **1992**, *40*, 409–419.
9. Rogi, T.; Tsujimoto, M.; Nakazato, H.; Mizutani, S.; Tomoda, Y. *J. Biol. Chem.* **1996**, *271*, 56–61.
10. 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. *J. Biol. Chem.* **2001**, *276*, 48623–48626.
11. Braszko, J. J.; Kupryszewski, G.; Witczuk, B.; Wisniewski, K. *Neuroscience* **1988**, *27*, 777–783.
12. Delorenzi, A.; Locatelli, F.; Romano, A.; Nahmod, V.; Maldonado, H. *Neurosci. Lett.* **1997**, *226*, 143–146.
13. Delorenzi, A.; Maldonado, H. *Neurosci. Lett.* **1999**, *266*, 1–4.
14. Lee, J.; Albiston, A. L.; Allen, A. M.; Mendelsohn, F. A.; Ping, S. E.; Barrett, G. L.; Murphy, M.; Morris, M. J.; McDowall, S. G.; Chai, S. Y. *Neuroscience* **2004**, *124*, 341–349.
15. Olson, M. L.; Olson, E. A.; Qualls, J. H.; Stratton, J. J.; Harding, J. W.; Wright, J. W. *Peptides* **2004**, *25*, 233–241.
16. Pederson, E. S.; Harding, J. W.; Wright, J. W. *Regul. Pept.* **1998**, *74*, 97–103.
17. Wright, J. W.; Miller-Wing, A. V.; Shaffer, M. J.; Higginson, C.; Wright, D. E.; Hanesworth, J. M.; Harding, J. W. *Brain Res. Bull.* **1993**, *32*, 497–502.
18. Wright, J. W.; Stubley, L.; Pederson, E. S.; Kramar, E. A.; Hanesworth, J. M.; Harding, J. W. *J. Neurosci.* **1999**, *19*, 3952–3961.
19. Miller-Wing, A. V.; Hanesworth, J. M.; Sardinia, M. F.; Hall, K. L.; Wright, J. W.; Speth, R. C.; Grove, K. L.; Harding, J. W. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 1718–1726.
20. Roberts, K. A.; Krebs, L. T.; Kramar, E. A.; Shaffer, M. J.; Harding, J. W.; Wright, J. W. *Brain Res.* **1995**, *682*, 13–21.
21. Wright, J. W.; Krebs, L. T.; Stobb, J. W.; Harding, J. W. *Front. Neuroendocrinol.* **1995**, *16*, 23–52.
22. Fernando, R. N.; Larm, J.; Albiston, A. L.; Chai, S. Y. *J. Comp. Neurol.* **2005**, *487*, 372–390.
23. Keller, S. R.; Scott, H. M.; Mastick, C. C.; Aebersold, R.; Lienhard, G. E. *J. Biol. Chem.* **1995**, *270*, 23612–23618.
24. Lew, R. A.; Mustafa, T.; Ye, S.; McDowall, S. G.; Chai, S. Y.; Albiston, A. L. *J. Neurochem.* **2003**, *86*, 344–350.
25. Albiston, A. L.; Mustafa, T.; McDowall, S. G.; Mendelsohn, F. A.; Lee, J.; Chai, S. Y. *Trends Endocrinol. Metab.* **2003**, *14*, 72–77.
26. Wallis, M. G.; Lankford, M. F.; Keller, S. R. *Am. J. Physiol. Endocrinol. Metab.* 2007.
27. Wright, J. W.; Harding, J. W. *Prog. Neurobiol.* **2004**, *72*, 263–293.
28. Garreau, I.; Chansel, D.; Vandermeersch, S.; Fruitier, I.; Piot, J. M.; Ardaillou, R. *Peptides* **1998**, *19*, 1339–1348.
29. 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. *Eur. J. Pharmacol.* **2006**, *546*, 19–27.
30. Braszko, J. J. *Physiol. Behav.* **2006**, *88*, 152–159.
31. Lee, J.; Chai, S. Y.; Mendelsohn, F. A.; Morris, M. J.; Allen, A. M. *Neuropharmacology* **2001**, *40*, 618–623.
32. Ariano, M. A.; Fisher, R. S.; Smyk-Randall, E.; Sibley, D. R.; Levine, M. S. *Brain Res.* **1993**, *609*, 71–80.
33. von Bohlen und Halbach, O.; Albrecht, D. *Neuroendocrinology* **2000**, *72*, 218–223.
34. Wright, J. W.; Harding, J. W. *Brain Res. Brain Res. Rev.* **1997**, *25*, 96–124.
35. Stragier, B.; Demaegdt, H.; De Bundel, D.; Smolders, I.; Sarre, S.; Vauquelin, G.; Ebinger, G.; Michotte, Y.; Vanderheyden, P. *Brain Res.* **2007**, *1131*, 97–105.
36. Georgsson, J.; Skold, C.; Plouffe, B.; Lindeberg, G.; Botros, M.; Larhed, M.; Nyberg, F.; Gallo-Payet, N.; Gogoll, A.; Karlen, A.; Hallberg, A. *J. Med. Chem.* **2005**, *48*, 6620–6631.
37. Tang, Z. L.; Pelletier, J. C. *Tetrahedron Lett.* **1998**, *39*, 4773–4776.
38. Xu, J.; Guo, Z. *Carbohydr. Res.* **2002**, *337*, 87–91.
39. Negishi, E.-i.; Bagheri, V.; Chatterjee, S.; Luo, F.-T.; Miller, J. A.; Stoll, A. T. *Tetrahedron Lett.* **1983**, *24*, 5181–5184.
40. *Handbook of Organopalladium Chemistry for Organic Synthesis*; Negishi, E.-i., Ed.; Wiley-Interscience: New York, 2002; Vol. 1,.
41. Chaumel, F.; Beland, F. *Abstr. Papers Am. Chem. Soc.* **2002**, *224*, U184.
42. Wada, T.; Ohkubo, A.; Mochizuki, A.; Sekine, M. *Tetrahedron Lett.* **2001**, *42*, 1069–1072.
43. Togo, H.; Hirai, T. *Synlett* **2003**, 702–704.
44. Amijs, C. H. M.; van Klink, G. P. M.; van Koten, G. *Green Chem.* **2003**, *5*, 470–474.
45. Axén, A.; Andersson, H.; Lindeberg, G.; Ronnholm, H.; Kortesmaa, J.; Demaegdt, H.; Vauquelin, G.; Karlen, A.; Hallberg, M. *J. Pept. Sci.* **2007**, *13*, 434–444.
46. Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotiriu, P.; Yao, W. Q.; Schafer, W. *Tetrahedron Lett.* **1989**, *30*, 3943–3946.
47. Hruby, V. J. *Nat. Rev. Drug Discov.* **2002**, *1*, 847–858.
48. Hruby, V. J.; Balse, P. M. *Curr. Med. Chem.* **2000**, *7*, 945–970.
49. Axén, A.; Lindeberg, G.; Demaegdt, H.; Vauquelin, G.; Karlén, A.; Hallberg, M. *J. Pept. Sci.* **2006**, *12*, 705–713.
50. Patchett, A. A.; Nargund, R. P. *Annu. Rep. Med. Chem.* **2000**, *35*, 289–298.
51. Sardinia, M. F.; Hanesworth, J. M.; Krishnan, F.; Harding, J. W. *Peptides* **1994**, *15*, 1399–1406.
52. Kobori, T.; Goda, K.; Sugimoto, K.; Ota, T.; Tomisawa, K. Preparation of amino acid derivatives as angiotensin IV receptor agonists. WO 98/05624 A1, 1998.
53. Sardinia, M. F.; Hanesworth, J. M.; Krebs, L. T.; Harding, J. W. *Peptides* **1993**, *14*, 949–954.
54. Krishnan, R.; Hanesworth, J. M.; Wright, J. W.; Harding, J. W. *Peptides* **1999**, *20*, 915–920.
55. Lahoutte, T.; Mertens, J.; Caveliers, V.; Franken, P. R.; Everaert, H. *J. Nucl. Med.* **2003**, *44*, 1489–1494.
56. Johansen, T. E.; Scholler, M. S.; Tolstoy, S.; Schwartz, T. W. *FEBS Lett.* **1990**, *267*, 289–294.
57. Olsen, J.; Cowell, G. M.; Konigshofer, E.; Danielsen, E. M.; Moller, J.; Laustsen, L.; Hansen, O. C.; Welinder, K. G.; Engberg, J.; Hunziker, W.; Spiess, M.; Sjostrom, H.; Noren, O. *FEBS Lett.* **1988**, *238*, 307–314.
58. Le, M. T.; De Backer, J. P.; Hunyady, L.; Vanderheyden, P. M.; Vauquelin, G. *Eur. J. Pharmacol.* **2005**, *513*, 35–45.
59. Demaegdt, H.; Vanderheyden, P.; De Backer, J. P.; Mosselmans, S.; Laeremans, H.; Le, M. T.; Kersemans, V.; Michotte, Y.; Vauquelin, G. *Biochem. Pharmacol.* **2004**, *68*, 885–892.
60. Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.