Full Paper

1-Pentanoyl-*N*-{[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl}-pyrrolidine-2-carboxamide: Investigation of Structural Variations

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We recently reported a series of 1-acyl-*N*-(biphenyl-4-ylmethyl)pyrrolidine-2-carboxamides as AT_1 receptor ligands. The most potent compound of the series, 1-pentanoyl-*N*-{[2'-(1*H*-tetrazol-5-yl)-biphenyl-4-yl]methyl}-pyrrolidine-2-carboxamide, showed an interesting affinity for the receptor. To investigate the influence of structure variations on affinity, the synthesis of additional compounds belonging to this series has been performed. Biological tests run on the newly synthesized compounds on CHO-hAT_1 cells stably expressing the human AT_1 receptor confirm our previous hypothesis, i.e. that, within this series, the length of the acyl chain, the substitution of the amidic group and the nature of the acidic one are crucial for the receptor interaction, being a valeric chain, a secondary amidic function and the tetrazole moiety, respectively, the optimal ones.

Keywords: AT_1 receptors / binding affinity / sartans / tetrazoles

Received: October 6, 2010; Revised: January 17, 2011; Accepted: January 20, 2011

DOI 10.1002/ardp.201000296

Introduction

The renin–angiotensin system (RAS) plays a central role in the regulation and the management of hypertension [1, 2]. Angiotensin II, the major player in this system elicits a wide array of biological actions, including vascular smooth muscle contraction and growth of smooth muscle cells and cardiac myocytes [3]. These effects are triggered by activation of angiotensin type 1 receptors [4]. As these receptors have a major role in the regulation of cardiovascular homeostasis, there has been much interest in developing nonpeptide antagonists for the clinical treatment of hypertension and congestive heart failure [5, 6]. Among the numerous antagonists, a few non peptide AT_1 antagonists well known as sartans, are approved by FDA and are available for the treatment of hypertension. These include losartan, the first non-peptide AT_1, antagonist which represents the prototype of the sartans (Fig. 1) and other drugs (valsartan or irbesartan). All these drugs have a common biphenyl fragment bearing an acidic moiety and differ in the nature of the pendent heterocyclic system connected to the *para*-position of the distal phenyl ring by means of a methylene group. Almost all of the chemical manipulations within the fundamental skeleton of sartans, concerned the substitution or modification of the imidazole ring of losartan [7]. According to these investigations, in a previous work we reported the synthesis and the evaluation of a series of 1-acyl-*N*-(biphenyl-4-ylmethyl)pyrrolidine-2-carboxamides as novel AT_1 receptor ligands, some of which showed a slight affinity for the receptor [8]. We found out that 1-pentanoyl-*N*-{[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl}-pyrrolidine-2-carboxamide **1** (Fig. 1) was the most potent of the series showing a K_i value of 0.6 μM. Thus, this work is focused on the study of structure-activity relationships of this compound, in order to establish which groups are crucial for receptor interaction. We turned our attention on four different portions of the molecule preparing some derivatives related to **1** (Table 1). First of all, the amidic nitrogen atom was alkylated by introducing a methyl group (compound **2c**) with the aim to verify

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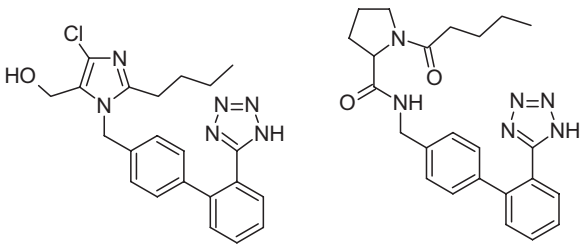


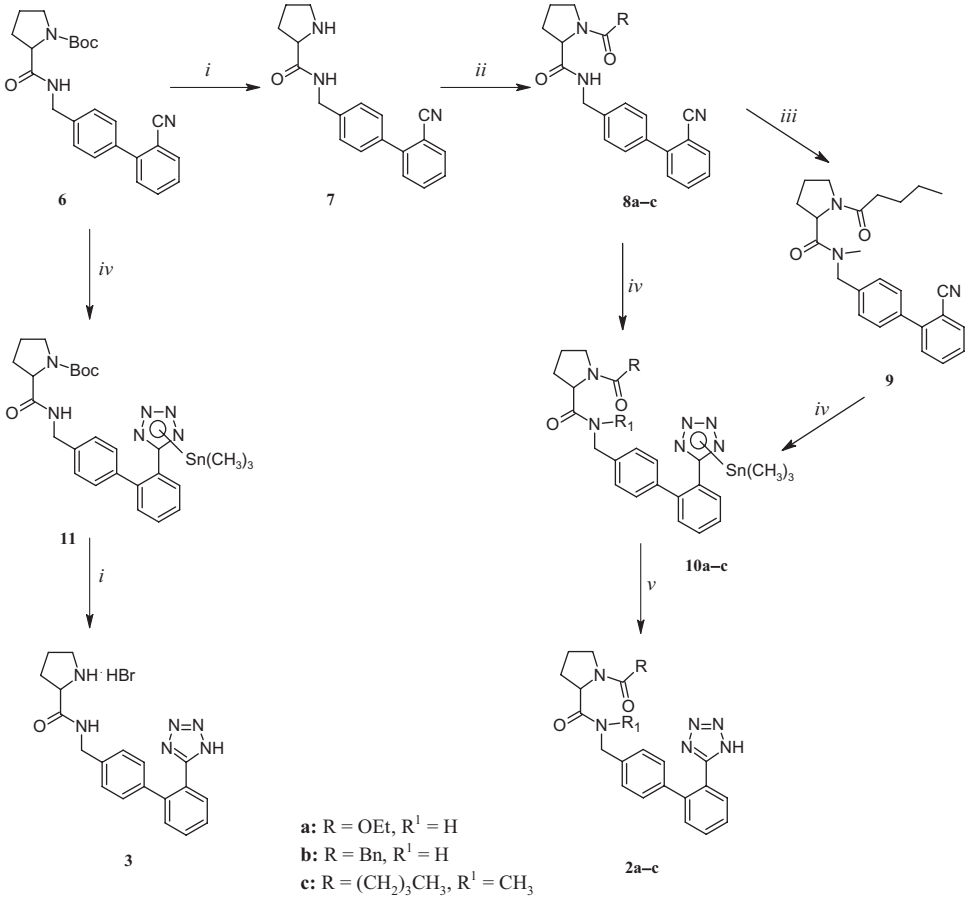
Figure 1. Structures of Losartan and compound **1**

if the secondary amide function was crucial for receptor interaction. Secondly, we modified the alkyl chain on the nitrogen atom of the pyrrolidine ring (compounds **2a**,**b** and **3**) to investigate the importance of the nature of this chain on the AT_1 receptor affinity; then, the tetrazole ring (RCN_4H) was replaced by a carboxylic moiety (RCO_2H, compound **4**) since it has been long held that RCN_4H may serve as non classical isostere of the RCO_2H [9]. Finally, basing on the dramatic results obtained with compound **4**, we envisaged the insertion of a retroamidic bridge side chain into the biphenyl structure (compound **5**) in order to position the carboxylic group in a better orientation for a ionic interaction with the receptor.

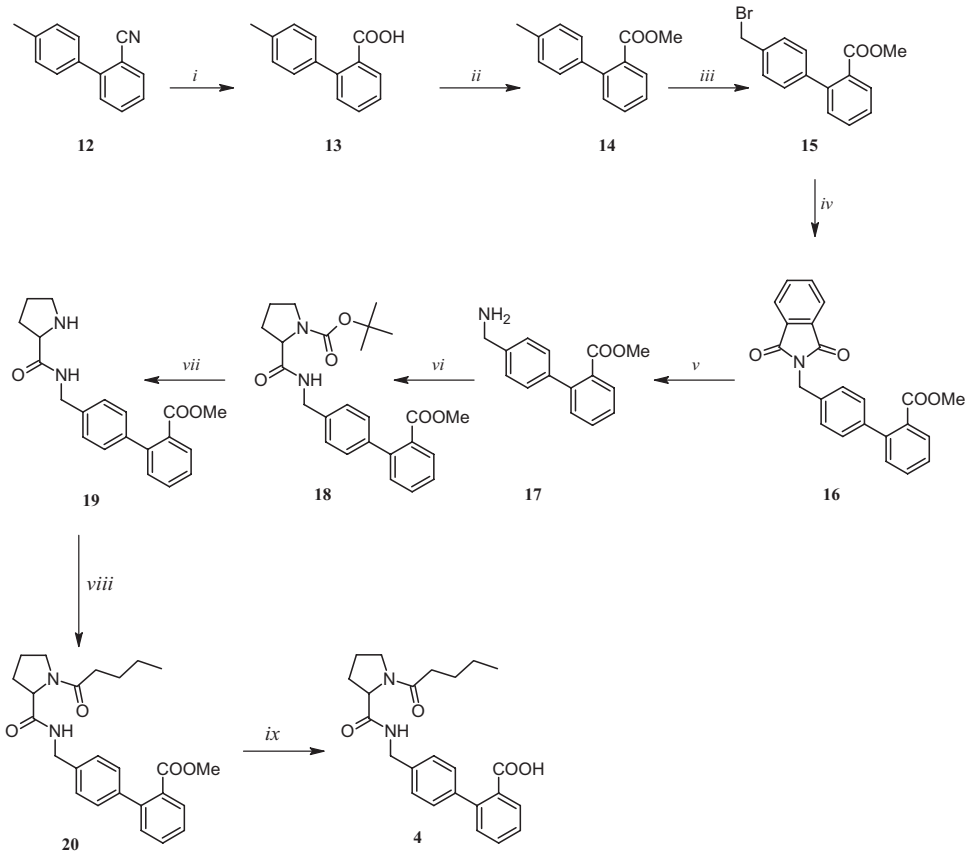
Results and discussion

Chemistry

Compounds **2a–c** and **3** were prepared as depicted in Scheme 1. Compound **7**, prepared as previously described [8], was converted into the acyl derivatives **8a**,**b**,**c** by reacting with diethylpyrocarbonate or phenylacetyl chloride or valeric acid, respectively. Compound **8c** was alkylated with iodomethane to give compound **9**. Compounds **8a**,**b** and **9** were converted into their corresponding trimethyltintetrazole



Scheme 1. *Reagents and conditions*: i) 48% HBr, EtOAc, rt; ii) diethylpyrocarbonate, 1 N NaOH, THF, rt (for **8a**); $Ph(CH_2)COOH$, $SOCl_2$, Et_3N, THF, reflux (for **8b**); $CH_3(CH_2)_3COOH$, EEDQ, Et_3N, $CHCl_3$, reflux (for **8c**); iii) 95% NaH, CH_3I, CH_3CN, 0°C; iv) $(CH_3)_3SnN_3$, toluene, reflux; v) silica gel (for **2a**); HCl (g), toluene/THF (for **2b**,**c**).

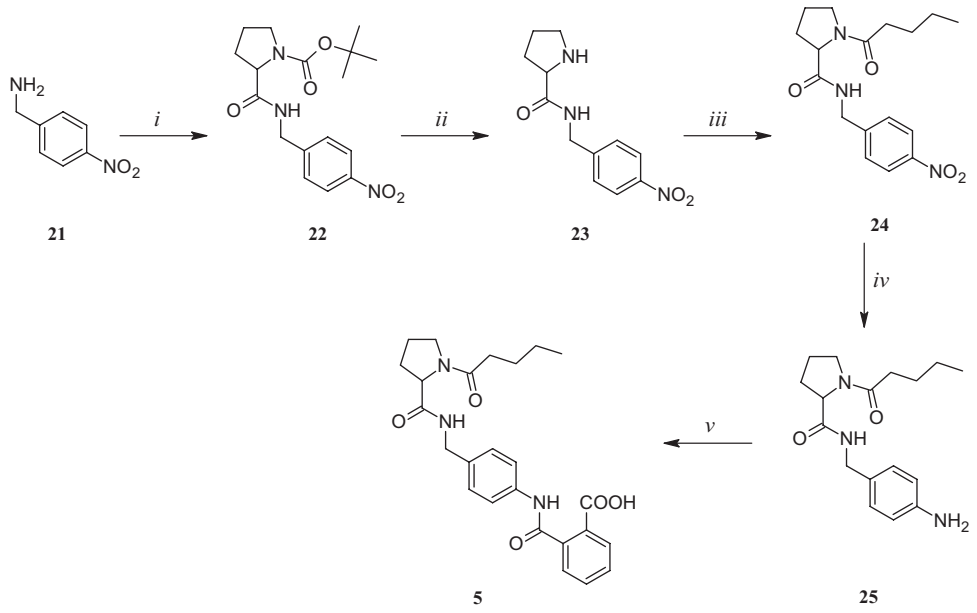


Scheme 2. *Reagents and conditions:* i) KOH, ethylene glycol, reflux; ii) CH_3I, K_2CO_3, acetone, reflux; iii) *N*-bromosuccinimide, $(PhCO)_2O_2$, CCl_4, reflux; iv) phthalimide, K_2CO_3, CH_3CN, reflux; v) $N_2H_4 \cdot H_2O$ (55%), MeOH/abs. EtOH, reflux; vi) 1-*N*-Boc-pyrrolidine-2-carboxylic acid, EEDQ, Et_3N, $CHCl_3$, reflux; vii) 48% HBr, EtOAc, rt; viii) valeric acid, $SOCl_2$, THF, reflux; ix) 1 N NaOH, abs. EtOH, reflux.

derivatives **10a–c** by reacting with azidotrimethyltin. Compound **2a** was directly obtained by column chromatography of **10a** [8, 10], while compounds **2b,c** by treatment of **10b,c** with gaseous HCl [8]. For the preparation of compound **3**, compound **6** was converted into its corresponding trimethyltintetrazole derivative **11** as above described for **10a–c**; then, treatment of **11** with gaseous HBr brought to both the removal of Boc and deprotection of tetrazole. The synthesis of compound **4** was obtained as shown in Scheme 2. Hydrolysis of commercial 4'-methyl-1,1'-biphenyl-2-carbonitrile **12** with KOH [11] gave the carboxylic acid **13**, which was converted into the corresponding ester **14** with iodomethane. Bromination of **14** provided the bromo derivative **15** which was submitted to a Gabriel reaction to give the phthalimido derivative **16**. Amine **17** was obtained starting from **16** as reported in the literature [12]. Successively, it was reacted with (*RS*)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid in the presence of EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline) to afford compound **18**. The following deprotection of **18** with HBr (48%) gave compound **19**, which was acylated by reaction with valeric acid and $SOCl_2$ to give compound **20**. Saponification [13] of this latter intermediate gave the titled compound **4**. The synthesis of compound **5** was achieved by the procedure shown in Scheme 3. Compound **24** was obtained starting from **21** following the same procedures used in Scheme 2. Reduction of the nitro group of **24** using tin chloride gave amine **25**, which was reacted with phthalic anhydride to give the desired compound **5**.

Biological results

The compounds described herein were tested as racemic mixtures on CHO-hAT_1 cells (Chinese hamster ovary cells expressing the human AT_1 receptor) [14]. The receptor binding affinity was measured by the ability to displace [^{3}H]valsartan from its specific binding sites. None of the



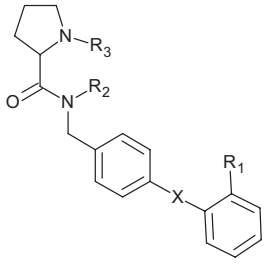
Scheme 3. *Reagents and conditions:* i) 1-(*RS*)-*N*-Boc-pyrrolidine-2-carboxylic acid, EEDQ, Et_3N, $CHCl_3$, reflux; ii) HBr (48%), EtOAc, rt; iii) valeric acid, $SOCl_2$, THF, reflux; iv) $SnCl_2 \cdot H_2O$, abs EtOH, reflux; v) phthalic anhydride, THF, rt.

described compounds was active towards AT_1 receptor showing a $K_i \geq 10$ μM (Table 1). The alkylation of the amidic nitrogen atom of **1** by introducing a methyl group (compound **2c**) resulted in a dramatic loss of affinity towards the AT_1 receptor, underlying the importance of a secondary amidic function. A possible explanation for this result may be envisaged: the hydrogen atom may create an intramolecular hydrogen bonding interaction with the carbonyl oxygen, in this modulating the correct orientation of the side chain and favouring the transoid conformation at the amido group; furthermore, a direct interaction of the hydrogen atom with the receptor cannot be ruled out. Results for compounds **2a**,**b** and **3** showed that isosteric substitution with an oxygen atom in the same chain, as in **2a**, introduction of a bulky phenylacetic moiety, as in **2b**, and removal of the amidic chain from the pyrrolidine nitrogen atom, as in **3**, led to the loss of affinity. These results suggest that, not only the presence, but also the nature of the acyl chain plays a key role in the affinity. Replacement of the tetrazole moiety with a carboxylic acid group, as in **4**, also led to a complete loss of affinity towards the AT_1 receptor. Since all the AT_1 antagonists have an acidic moiety at 2'-biphenyl position, ionized at physiological pH, which has been demonstrated to interact with the Lys199 [15], pK_a values of compounds **1** and **4** for comparison purpose were experimentally measured, following the procedure previously reported [16]. Results obtained suggest that a different acidity may be responsible for the negative result obtained with **4**, being its pK_a value lower than that of its parent compound **1** (i.e., **4**, 3.66 ± 0.02 versus **1**, 4.34 ± 0.02). Finally, introduction of a retroamido bridge [17] between the two aromatic rings of compound **1** did not give any positive result, being compound **5** not active.

Conclusion

The synthesis and biological evaluation of new compounds belonging to the series of 1-acyl-*N*-(biphenyl-4-ylmethyl)pyrrolidine-2-carboxamides are reported. Biological data confirm our previous hypothesis that, within this series, there are some features that are essential for receptor interaction. In fact, starting from the most potent compound of the series previously described [8], 1-pentanoyl-*N*-{[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl}-pyrrolidine-2-carboxamide **1** (Table 1), we found that the introduction of a methyl group onto the amidic function, or the modification of the valeryl chain on the pyrrolidine ring, or the substitution of the tetrazole group with a carboxylic one, not even by introducing a retroamidic bond between the two aromatic groups, brought to the loss of AT_1 receptor affinity. These findings confirm that the length of the acyl chain, the substitution of the amidic group and the nature of the acidic one are crucial for the receptor interaction, being a valeric chain, a secondary amidic function and the tetrazole moiety the optimal ones.

Table 1. Structure and AT_1 binding properties of newly synthesized compounds.



Compd.	R_1	X	R_2	R_3	K_i [μM][a]
1	Tetrazole	–	H	$CO(CH_2)_3CH_3$	0.60
2a	Tetrazole	–	H	$COOCH_2CH_3$	>10
2b	Tetrazole	–	H	$COCH_2Ph$	> 10
2c	Tetrazole	–	CH_3	$CO(CH_2)_3CH_3$	> 10
3	Tetrazole	–	H	H	> 10
4	COOH	–	H	$CO(CH_2)_3CH_3$	> 10
5	COOH	CONH	H	$CO(CH_2)_3CH_3$	> 10
Losartan					0.0158[b]
Irbesartan					0.00148[b]
Valsartan					0.00128[b]

[a] Affinities were calculated from competition curves by using nonlinear regression analysis and GraphPad Prism. [b] See [20]

Experimental section

Chemistry

Materials and Methods

Chemicals were purchased from Sigma-Aldrich or Lancaster. Yields refer to purified products and were not optimized. The structures of the compounds were confirmed by routine spectrometric and spectroscopic analyses. Compounds **6**, **7**, and **8c** were prepared as previously described [8]. Only spectra for compounds not previously described are given. Melting points were determined on a Gallenkamp apparatus in open glass capillary tubes and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer (Norwalk, CT) Spectrum One FT spectrophotometer and band positions are given in reciprocal centimeters (cm^{-1}). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian VX Mercury spectrometer operating at 300 and 75 MHz for ^{1}H and ^{13}C, respectively, using $CDCl_3$ as solvent unless otherwise indicated. Chemical shifts are reported in parts per million (ppm) relative to the residual non-deuterated solvent resonance: $CDCl_3$, δ 7.26 (^{1}H-NMR) and δ 77.3 (^{13}C-NMR), DMSO-d_6, δ 2.50 (^{1}H-NMR) and δ 39.5 (^{13}C-NMR). *J* values are given in Hz. Gas chromatography (GC)/mass spectroscopy (MS) was performed on a Hewlett-Packard 6890–5973 MSD at low resolution. Liquid chromatography (LC)/mass spectroscopy (MS) was performed on a spectrometer Agilent 1100 series LC-MSD Trap System VL. Elemental analyses were performed on a Eurovector Euro EA 3000 analyzer. Chromatographic separations were performed on silica gel columns by flash chromatography (Kieselgel 60, 0.040–0.063 mm, Merck, Darmstadt, Germany) as described by Still et al. [18]. TLC analyses were performed on precoated silica gel on aluminum sheets (Kieselgel 60 F_{254}, Merck).

*Ethyl 2-({[(2′-cyan-1,1′-biphenyl-4-yl)-methyl]amino}carbonyl)pyrrolidine-1-carboxylate **8a***

A stirred solution of amine **7** (1 g, 3.27 mmol) in 1 N NaOH (12 mL) was cooled at 0°C. Slowly a solution of 0.64 g (3.93 mmol) of diethylpyrocarbonate in THF (24 mL) was added. The mixture was allowed to stir at room temperature for 4 h. The solvent was removed under vacuum and the crude residue taken up with ethyl acetate and washed with 2 N NaOH. The alkaline phase was acidified with 2 N HCl and extracted twice with ethyl acetate. The combined organic phases were dried affording a white solid which was recrystallized from $CHCl_3$/hexane to give 0.95 g (79%) of white crystals: M.p.: 154–156°C; GC/MS *m*/*z* 377 (M^+, 10), 142 (100); ^{1}H-NMR: δ 1.0–1.38 (m, 3H), 1.90 (br s, 2H), 2.05–2.50

(m, 2H), 3.25–3.50 (m, 2H), 4.11 (br s, 2H), 4.25–4.70 (m, 3H), 6.85 (br s, 1H), 7.32–7.54 (m, 6H, Ar), 7.58–7.68 (m, 1H, Ar), 7.70–7.78 (m, 1H, Ar); ^{13}C-NMR: δ 14.8 (1C), 24.8 (1C), 28.7 (1C), 43.1 (1C), 47.3 (1C), 60.9 (1C), 62.0 (1C), 111.4 (1C), 118.9 (1C), 127.8 (2C), 127.9 (1C), 129.2 (1C), 130.2 (1C), 133.1 (1C), 134.0 (2C), 137.4 (1C), 139.2 (1C), 145.3 (1C), 155.0 (1C), 172.2 (1C); IR (KBr): 3313 (NH), 2228 (C≡N), 1668 (broad, C=O) cm^{-1}. Anal. calcd. for $C_{22}H_{23}N_3O_3 \cdot 0.50\ H_2O$ (386.44): C, 68.38; H, 6.26; N, 10.87. Found: C, 68.20; H, 6.03; N 10.82.

N-[(2′-Cyan-1,1′-biphenyl-4-yl)methyl]-1-phenylacethylpyrrolidine-2-carboxamide ***8b***

A solution of **7** (0.95 g, 3.11 mmol) and Et_3N (0.58 mL, 4.24 mmol) in anhydrous THF (150 mL) was stirred and brought to reflux under nitrogen atmosphere; then phenylacetyl chloride (0.38 g, 2.83 mmol), obtained by treatment of phenylacetic acid with $SOCl_2$, was added and the reaction was refluxed for 7 h. The solid was filtered off and the solvent was evaporated under reduced pressure. The residue, dissolved in ethyl acetate, was washed twice with 2 N HCl and once with 2 N NaOH. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Purification of the residue by column chromatography (EtOAc/petroleum ether 8:2) gave 0.81 g (68%) of a pale oil: GC/MS *m*/*z* 423 (M^+, 12), 70 (100); ^{1}H-NMR: δ 1.71–1.92 (m, 2H), 1.95–2.15 (m, 1H), 2.20–2.35 (m, 1H), 2.81 (br s, 1H, NH, exch. D_2O), 3.35–3.60 (m, 2H), 3.64 (s, 2H), 4.37 (d, J = 6.0 Hz, 2H), 4.52–4.62 (m, 1H), 7.10–7.50 (m, 10H, Ar), 7.52–7.64 (m, 2H, Ar), 7.66–7.72 (m, 1H, Ar); ^{13}C-NMR: δ 25.2 (1C), 28.1 (1C), 42.1 (1C), 43.1 (1C), 48.0 (1C), 60.3 (1C), 111.2 (1C), 119.0 (1C), 127.2 (2C), 127.9 (2C), 128.9 (2C), 129.1 (1C), 129.2 (2C), 130.2 (1C), 133.1 (1C), 134.0 (2C), 134.6 (1C), 137.1 (1C), 139.4 (1C), 145.3 (1C), 171.5 (1C), 171.8 (1C); IR (neat): 3301 (NH), 2223 (C≡N), 1643 (broad, C=O) cm^{-1}.

N-[(2′-Cyanobiphenyl-4-yl)methyl]-N-methyl-1-pentanoylpyrrolidine-2-carboxamide ***9***

Under nitrogen atmosphere a solution of 0.90 g (2.31 mmol) of compound **8c** and 0.33 g of 95% sodium hydride powder (13.9 mmol) in anhydrous CH_3CN (35 mL) was cooled in an ice bath for 1.5 h. Then, a solution of iodomethane (1.72 mL, 27.8 mmol) in anhydrous CH_3CN (25 mL) was slowly added dropwise. The mixture was stirred for 24 h at room temperature. The solvent was removed under vacuum and the residue taken up with ethyl acetate; then the organic layer was washed twice with water and dried affording 0.9 g (97%) of an oil: LC/MS *m*/*z* 426 [M^+ + 23]; ^{1}H-NMR: δ 0.89 (t, J = 7.4 Hz, 3H), 1.22–1.42 (m, 2H), 1.56–1.70 (m, 2H), 1.82–2.01 (m, 3H), 2.03 (m, 3H), 3.06 (s, 3H), 3.33–3.57 (m, 1H), 3.61–3.76 (m, 1H), 4.51–5.00 (m, 3H), 7.30–7.64 (m, 6H, Ar), 7.69–7.76 (m, 2H, Ar); ^{13}C-NMR: δ 14.1 (1C), 22.7 (1C), 25.0 (1C), 27.0 (1C), 29.1 (1C), 34.4 (1C), 35.2 (1C), 47.5 (1C), 51.3 (1C), 56.4 (1C), 111.3 (1C), 118.9 (1C), 127.7 (2C), 128.2 (1C), 129.2 (1C), 130.2 (1C), 133.1 (1C), 133.9 (2C), 137.2 (1C), 138.1 (1C), 145.3 (1C), 172.1 (1C), 172.8 (1C); IR (neat): 2223 (C≡N), 1733, 1635 (C=O) cm^{-1}.

Ethyl 2-[({[2′-(1-(trimethylstannyl)-1H-tetrazol-5-yl)-biphenyl-4-yl]methyl}amino)carbonyl] pyrrolidine–1-carboxylate ***10a***

A solution of compound **8a** (0.80 g, 2.12 mmol) in dry toluene (12 mL) and azidotrimethyltin (0.83 g, 4.03 mmol) was kept at 110°C under nitrogen atmosphere for 48 h. The precipitate was filtered off and washed with hot toluene to give 0.65 g (53%) of **10a** as a brown solid: M.p.: >250°C; LC/MS *m*/*z* 607 [M^+ + 23].

N-({2′-[1-(Trimethylstannyl)-1H-tetrazol-5-yl]biphenyl-4-yl}-methyl]-1-phenylacetyl pirrolidine-2-carboxamide ***10b***

Prepared in 89% yield as above described for compound **10a** starting from compound **8b**. Tan solid. M.p.: >250°C; IR (KBr): 1626 (broad, C=O) cm^{-1}.

N-({2'-[1-(Trimethylstannyl)-1H-tetrazol-5-yl]biphenyl-4-yl}methyl]-N-methyl–1-pentanoyl pyrrolidine-2-carboxamide ***10c***

Prepared in 88% yield as above described for **10a** starting from **9**. Brown solid: M.p.: >250°C; LC/MS *m*/*z* 631 [M^+ + 23]; IR (KBr): 1635 (broad, C=O) cm^{-1}.

Ethyl 2-[({[2′-(1H-tetrazol-5-yl)biphenyl-4-yl]-methyl}amino)carbonyl]pyrrolidine–1-carboxylate ***2a***

Compound **2a** was directly obtained (47%) by column chromatography (EtOAc/MeOH 9:1) of **10a** as a pink solid: M.p.: 109–111°C; LC/MS *m*/*z* 419 [M^+ − 1]; ^{1}H-NMR (DMSO-d_6): δ 1.0–1.25 (m, 3H), 1.79 (br s, 3H), 1.95–2.20 (m, 2H), 3.41 (br s partially overlapped to DMSO signal, 2H), 3.85–4.10 (m, 2H), 4.10–4.35 (m, 3H), 6.95–7.15 (m, 4H, Ar), 7.30–7.60 (m, 4H, Ar), 8.40–8.60 (m, 1H); ^{13}C-NMR (DMSO-d_6): δ 15.2 (1C), 23.8 (1C), 31.9 (1C), 42.3 (1C), 47.6 (1C), 60.4 (1C), 61.1 (1C), 127.0 (2C), 127.6 (2C), 129.5 (1C), 129.6 (1C), 130.8 (2C), 131.2 (1C), 138.5 (1C), 140.0 (1C), 141.4 (1C), 154.8 (1C), 155.0 (1C), 172.9 (1C); IR (KBr): 3419 (NH), 1674 (broad, C=O) cm^{-1}. Anal. calcd. for $C_{22}H_{24}N_6O_3 \cdot H_2O$ (438.46): C, 60.26; H, 5.98; N, 19.17. Found: C, 60.49; H, 5.58; N, 19.50.

N-{[2′-(1H-Tetrazol-5-yl)biphenyl-4-yl]methyl}-1-phenylacethylpyrrolidin-2-carboxamide ***2b***

A suspension of compound **10b** (0.60 g, 0.95 mmol) in a mixture of toluene (8 mL) and THF (2 mL) was stirred in an ice-bath. Then, gaseous hydrogen chloride was added to give a clear solution followed by precipitation of the desired prod-

uct. The precipitate was filtered and the filtrate was washed with toluene and purified by crystallization from MeOH/Et_2O to give 0.11 g (25%) of yellow crystals: M.p.: 109–110°C; LC/MS *m/z* 465 [M^+ – 1]; ^{1}H-NMR (DMSO-d_6): δ 1.02 (s, 1H), 1.60–2.40 (m, 6H), 3.40–3.80 (m, 3H), 4.0–4.60 (m, 2H), 6.80–7.40 (m, 9H, Ar), 7.40–7.80 (m, 4H, Ar), 16.2 (br s, 1H); ^{13}C-NMR (DMSO-d_6): δ 23.0 (1C), 25.0 (1C), 30.3 (1C), 42.1 (1C), 47.8 (1C), 60.4 (1C), 124.2 (2C), 126.9 (1C), 127.3 (2C), 128.3 (1C), 128.9 (1C), 129.3 (2C), 129.5 (1C), 131.3 (2C), 131.7 (2C), 136.2 (2C), 139.5 (1C), 142.0 (1C), 158 (1C), 169.8 (1C), 172.5 (1C); IR (KBr): 3308 (NH), 1634 (broad, C=O) cm^{-1}. Anal. calcd. for $C_{27}H_{26}N_6O_2 \cdot 2\ H_2O$ (502.53): C, 64.53; H, 6.02; N, 16.72. Found: C, 64.20; H, 5.77; N, 16.72.

N-{[2′-(1H-Tetrazol-5-yl)biphenyl-4-yl]methyl}-N-methyl-1-pentanoylpyrrolidine-2-carboxamide ***2c***

Prepared in 89% yield as above described for **2b** starting from **10c**. Tan crystals: M.p.: 218–220°C; LC/MS *m/z* 445 [M^+ – 1]; ^{1}H-NMR (DMSO-d_6): δ 0.85 (t, $J = 7.3$ Hz, 3H), 1.28 (sextet, $J = 7.4$ Hz, 2H), 1.45 (sextet, $J = 7.4$ Hz, 2H), 1.60–2.02 (m, 4H), 2.18–2.28 (m, 2H), 2.71 (s, 1H), 2.96 (s, 2H), 3.25–3.44 (m, 1H), 3.45–3.55 (m, 1H), 4.22–4.82 (m, 3H), 6.98–7.16 (m, 4H, Ar), 7.48–7.60 (m, 2H, Ar), 7.60–7.72 (m, 2H, Ar), 16.2 (br s, 1H); ^{13}C-NMR (DMSO-d_6): δ 14.5 (1C), 22.5 (1C), 25.0 (1C), 27.2 (1C), 29.7 (1C), 34.1 (1C), 35.4 (1C), 47.5 (1C), 52.5 (1C), 56.5 (1C), 124.1 (2C), 127.7 (2C), 128.4 (1C), 129.5 (1C), 131.3 (2C), 131.7 (1C), 137.4 (1C), 138.5 (1C), 141.9 (1C), 153.8 (1C), 170.9 (1C), 172.5 (1C); IR (KBr): 3410 (NH), 1660, 1599 (C=O). Anal. calcd. for $C_{25}H_{30}N_6O_2 \cdot 0.33\ H_2O$ (452.55): C, 66.35; H, 6.87; N, 18.57. Found: C, 66.53; H, 6.70; N, 18.43.

tert-Butyl 2-[({[2′-(1-(trimethylstannyl)-1H-tetrazol-5-yl)-biphenyl-4-yl]methyl}amino) carbonyl]pyrrolidine–1-carboxylate ***11***

Prepared in 32% yield as above described for compound **10a** starting from compound **6**. Used in the following step without any further characterization.

N-{[2′-(1H-Tetrazol-5-yl)biphenyl-4-yl]methyl}pyrrolidine-2-carboxamide · HBr ***3***

To a solution of 0.46 g (0.75 mmol) of compound **11** in ethyl acetate (12 mL), 5.5 mL of 48% HBr were added, keeping the mixture under stirring. The crude residue was taken up with ethyl acetate and washed twice with water, which was azeotropically removed (toluene/EtOH) affording 0.18 g (56%) of an orange solid, rinsed up with Et_2O: M.p.: 185–186°C; LC/MS *m/z* 349 [$M^+ + 1$]; ^{1}H-NMR (DMSO-d_6): δ 1.75–2.01 (m, 2H), 2.20–2.35 (m, 1H), 3.21 (br s partially overlapped to H_2O signal, 3H), 4.18 (br s, 1H), 4.25–4.45 (m, 2H), 7.06 (d, $J = 8.4$ Hz, 2H, Ar), 7.20 (d, $J = 8.0$ Hz, 2H, Ar), 7.48–7.70 (m, 4H, Ar), 8.61 (br s, 1H), 9.15 (br s, 1H), 16.2 (br s, 1H); IR (KBr): 1674 (C=O) cm^{-1}. Anal. calcd. for $C_{19}H_{20}N_6O \cdot 3\ HBr \cdot 3\ H_2O$ (627.17): C, 36.39; H, 4.34; N, 13.40. Found: C, 36.81; H, 4.03; N, 13.30.

4′-Methyl-1,1′-biphenyl-2-carboxylic acid ***13***

3.5 g of 4′-methyl-1,1′-biphenyl-2-carbonitrile **12** (18.1 mmol) was stirred for 48 h at 170°C in a mixture of ethylene glycol (21 mL) and 10 g (178 mmol) of KOH. The mixture was then acidified till pH 2 and extracted using ethyl acetate. The combined organic layers were extracted in 2 N NaOH, the aqueous layers were acidified with 2 N HCl and extracted three times with ethyl acetate. The organic layers were dried over anhydrous Na_2SO_4 affording 3.59 g (93%) of a brown solid: M.p.: 148–151°C [lit.: 140–142°C] [18]; GC/MS *m/z* 212 (M^+, 100); ^{1}H-NMR: δ 2.40 (s, 3H), 7.17–7.28 (m, 4H, Ar), 7.34–7.45 (m, 2H, Ar), 7.51–7.60 (m, 1H, Ar), 7.91–7.98 (m, 1H, Ar); ^{13}C-NMR: δ 21.4 (1C), 127.2 (3C), 128.6 (1C), 129.1 (1C), 130.8 (1C), 131.4 (1C), 132.2 (2C), 137.3 (1C), 138.3 (1C), 143.5 (1C), 173.1 (1C); IR (KBr): 3417 (OH), 1679 (C=O) cm^{-1}. Anal. calcd. for $C_{14}H_{12}O_2 \cdot 0.25\ H_2O$ (216.75): C, 77.58; H, 5.81. Found C, 77.69; H, 5.78.

Methyl 4′-methyl-1,1′-biphenyl-2-carboxylate ***14***

To a solution of 3.0 g (14.1 mmol) 4′-methyl-1,1′-biphenyl-2-carboxylic acid **13** in acetone (300 mL), 2.4 g (16.2 mmol) K_2CO_3 were added. After 15 min, iodomethane (2.3 mL, 35.4 mmol) was added dropwise. The mixture was allowed to stir at reflux for 24 h. The solvent was removed under pressure and the residue taken up with ethyl acetate; the organic layer was washed twice with 2 N NaOH and dried over anhydrous Na_2SO_4 affording 3.1 g (96%) of a brown oil: GC/MS *m/z* 226 (M^+, 79), 195 (100); ^{1}H-NMR: δ 2.42 (s, 3H), 3.68 (s, 3H), 7.20–7.26 (m, 4H, Ar), 7.36–7.44 (m, 2H, Ar), 7.49–7.57 (m, 1H, Ar), 7.80–7.86 (m, 1H, Ar); ^{13}C-NMR: δ 21.5 (1C), 52.2 (1C), 127.2 (3C), 128.5 (1C), 129.1 (1C), 130.0 (1C), 131.0 (1C), 131.2 (1C), 131.5 (1C), 137.2 (1C), 138.7 (1C), 142.7 (1C), 169.5 (1C).

Methyl 4′-(bromomethyl)-1,1′-bipheny-2-carboxylate ***15***

Prepared from **14** in 88% yield as described in [19]. Purification by column chromatography (EtOAc/petroleum ether, 1.5:8.5); GC/MS *m/z* 305 ($M^+ < 1$), 225 (100); ^{13}C-NMR: δ 33.6 (1C), 52.2 (1C), 126.5 (2C), 127.6 (1C), 129.0 (1C), 129.3 (1C), 130.0 (1C), 130.2 (1C), 130.9 (2C), 131.6 (1C), 136.9 (1C), 141.8 (1C), 169.0 (1C); IR (neat): 1725 (C=O) cm^{-1}. ^{1}H-NMR data were found to match those reported in [19].

Methyl 4′-[(1,3-dioxo-1,3-diydro-2H-isoindol-2-yl)methyl]-1,1′-biphenyl-2-carboxylate ***16***

Prepared from **15** in 35% yield as described in [12] and purified by column chromatography (EtOAc/petroleum ether 1.5:8.5) and crystallization from $CHCl_3$/hexane. Spectroscopic data were in agreement with [12].

Methyl 4′-(aminomethyl)-1,1′-biphenyl-2-carboxylate ***17***

Prepared in 69% yield starting from **16** as described in [12]. Spectroscopic data were in agreement with [12].

Methyl 4′-{[(N-tert-butoxycarbonylpyrrolidine-2-ylcarbonyl)amino]methyl}-1,1′-biphenyl-2-carboxylate ***18***

To a solution of **17** (0.56 g, 2.31 mmol) in $CHCl_3$ (100 mL), 1-*N*-Boc-pyrrolidine-2-carboxylic acid (0.45 g, 2.10 mmol), EEDQ (0.62 mg, 2.52 mmol), and Et_3N (0.44 mL) were added. The reaction mixture was refluxed for 24 h. The solvent was evaporated and the residue was taken up with EtOAc and washed three times with 2 N HCl, three times with 2 N NaOH and once with water. The organic phase was dried (Na_2SO_4) and concentrated under vacuum to give a yellow oil which was purified by flash chromatography (EtOAc/petroleum ether, 8:2) giving 0.61 g (67%) of a white solid: M.p.: 134–136°C. LC/MS *m/z* 461 [$M^+ + 23$]; ^{1}H-NMR: δ 1.30 (br s, 9H), 1.85–1.95 (m, 4H), 2.05–2.20 (m, 1H), 3.15–3.23 (m, 2H), 3.69 (s, 3H), 4.32–4.35 (m, 3H), 7.18–7.56 (m, 7H, Ar), 7.89–7.95 (m, 1H, Ar); ^{13}C-NMR: δ 24.9 (1C), 28.6 (3C), 31.8 (1C), 43.3 (1C), 47.4 (1C), 52.2 (1C), 60.4 (1C), 80.8 (1C), 127.4 (3C), 128.8 (2C), 130.0 (2C), 131.0 (1C), 131.5 (1C), 137.4 (1C), 140.6 (1C), 142.3 (1C), 156.1 (1C), 169.2 (1C), 171.0 (1C); IR (KBr): 3440 (NH), 1727 (C=O), 1667 (broad, C=O) cm^{-1}.

Methyl 4′-{[(pyrrolidine-2-ylcarbonyl)amino]methyl}-biphenyl-2-carboxylate ***19***

To a stirred solution of 1.7 g (3.88 mmol) **18** in ethyl acetate (25 mL), 6.9 mL HBr (48%) were added. The crude residue was taken up with ethyl acetate and washed twice with water. The acidic aqueous phase was made alkaline with 2 N NaOH, then extracted twice with ethyl acetate: the combined organic layers dried over anhydrous Na_2SO_4 gave 1.06 g (81%) of a pale yellow oil: LC/MS *m/z* 361 [$M^+ + 23$]; ^{1}H-NMR: δ 1.65–1.82 (m, 2H), 1.90–2.05 (m, overlapping br s at 1.99 (m, 1H)), 1.99 (br s overlapping m at 1.90–2.05, exch. D_2O, 1H), 2.10–2.24 (m, 1H), 2.85–2.93 (m, 1H), 2.97–3.05 (m, 1H), 3.67 (s, 3H), 3.81 (q, $J = 4.5$ Hz, 1H), 4.48 (d, $J = 6.1$ Hz, 2H), 7.22–7.31 (m, 4H, Ar), 7.32–7.43 (m, 2H, Ar), 7.48–7.56 (m, 1H, Ar), 7.70–7.80 (m, 1H, Ar), 8.19 (br s, exch. D_2O, 1H); ^{13}C-NMR: δ 26.4 (1C), 31.0 (1C), 42.9 (1C), 47.5 (1C), 52.1 (1C), 60.9 (1C), 127.4 (3C), 128.8 (2C), 130.0 (2C), 131.0 (1C), 131.5 (1C), 137.9 (1C), 140.6 (1C), 142.4 (1C), 169.2 (1C), 175.3 (1C); IR (neat): 3224 (NH), 1724, 1658 (C=O) cm^{-1}.

Methyl 4′-({[(1-pentanoylpyrrolidine-2-yl)carbonyl]-amino}methyl)biphenyl-2-carboxylate ***20***

A solution of 1.0 g of compound **19** (2.96 mmol) and 0.86 mL (6.22 mmol) Et_3N in THF (110 mL) was stirred and brought to reflux. Then 0.9 mL (8.28 mmol) valeryl chloride were added and stirred for 16 h. The solid was filtered off and the solvent removed under vacuum. The crude residue was dissolved in ethyl acetate and washed three times with 2 N HCl and once with NaOH. Purification by flash chromatography (EtOAc/petroleum ether 8:2) gave 0.81 g (65%) of an slightly yellowish oil: GC/MS *m/z* 422 (M^+, 5), 70 (100); ^{1}H-NMR: δ 0.90 (t, $J = 7.3$ Hz, 3H), 1.25–1.41 (m, 1H), 1.55–1.68 (m, 2H), 1.78–1.80 (m, 2H), 1.90–2.02 (m, 1H), 2.05–2.25 (m, 1H), 2.32 (t, $J = 7.6$ Hz, 2H), 2.45–2.55 (m, 1H), 3.38–3.48 (m, 1H), 3.52–3.61 (m, 1H), 3.63 (s, 3H), 4.35–4.55 (m, 2H), 4.62–4.68 (m, 1H), 7.20–7.30 (m, 4H, Ar), 7.30–7.42 (m, 2H, Ar), 7.48–7.54 (m, 1H, Ar), 7.59 (br s, exch. D_2O, 1H), 7.80 (m, 1H, Ar); ^{13}C-NMR: δ 14.1 (1C), 22.7 (1C), 25.3 (1C), 27.1 (1C), 27.3 (1C), 34.6 (1C), 43.3 (1C), 47.8 (1C), 52.1 (1C), 59.8 (1C), 127.3 (3C), 128.7 (2C), 130.0 (2C), 130.9 (1C), 131.4 (1C), 137.6 (1C), 140.4 (1C), 142.4 (1C), 169.3 (1C), 171.5 (1C), 174.0 (1C); IR (neat): 3295 (NH), 1728 (C=O), 1648 (broad, C=O) cm^{-1}.

4′-({[(1-Pentanoylpyrrolidine-2yl)carbonyl]amino}-methyl)biphenyl-2-carboxylic acid ***4***

0.85 g (2.04 mmol) of compound **20** were dissolved in ethanol (38 mL) and 1 N NaOH (15 mL) was added keeping the mixture to stir and at reflux for 4 h. The solvent was removed under reduced pressure and the crude residue dissolved in ethyl acetate was washed three times with 2 N NaOH. The alkaline aqueous phases were acidified with 2 N HCl and extracted twice with ethyl acetate. The organic layers dried over anhydrous Na_2SO_4 gave 0.83 g (98%) of a transparent oil, which was crystallized from THF/hexane affording 0.54 g of white needles: M.p.: 147–149°C; LC/MS *m/z* 407 [$M^+ - 1$]; ^{1}H-NMR: δ 0.90 (t, $J = 7.4$ Hz, 3H), 1.25–1.41 (m, 2H), 1.50–1.69 (m, 2H), 1.74–2.00 (m, 2H), 2.10–2.25 (m, 2H), 2.32 (t, $J = 7.6$ Hz, 3H), 3.38–3.48 (m, 1H), 3.50–3.60 (m, 1H), 4.10 (dd, $J = 14.8$, 5.5 Hz, 1H), 4.28 (dd, $J = 14.8$, 6.3 Hz, 1H), 4.52 (d, $J = 6.0$ Hz, 1H), 6.96 (br s, exch. D_2O, 1H), 7.11 (d, $J = 8.0$ Hz, 1H, Ar), 7.20–7.35 (m, 3H, Ar), 7.35–7.45 (m, 1H, Ar), 7.45–7.55 (m, 2H, Ar), 7.88–8.02 (m, 1H, Ar); ^{13}C-NMR: δ 14.0 (1C), 22.6 (1C), 25.2 (1C), 27.0 (1C), 27.9 (1C), 34.5 (1C), 43.3 (1C), 47.8 (1C), 60.0 (1C), 127.3 (1C), 127.6 (2C), 128.9 (2C), 130.5 (2C), 131.2 (1C), 131.6 (1C), 137.7 (1C), 140.5 (1C), 142.9 (1C), 170.9 (1C), 171.5 (1C), 174.1 (1C); IR (KBr): 3261 (OH), 1715, 1657, 1618 (C=O) cm^{-1}. Anal. calcd. for $C_{24}H_{28}N_2O_4 \cdot 0.25\ H_2O$ (412.99): C, 69.80; H, 6.96; N, 6.78. Found: C, 70.07; H, 6.90; N, 6.78.

tert-Butyl 2-{[(4-nitrobenzyl)amino]carbonyl}pyrrolidine-1-carboxylate ***22***

Prepared as described for compound **18** starting from commercially available compound **21**. Purification by column chromatography (EtOAc) gave an oil, which was crystallized from EtOAc/hexane. Yellow crystals (90%): M.p.: 128–129°C; LC/MS *m/z* 372 [$M^+ + 23$]; ^{1}H-NMR: δ 1.42 (br s, 9H), 1.89 (br s, 3H), 2.00–2.41 (m, 1H), 3.40 (br s, 2H), 4.31 (br s, 1H), 4.51 (br s, 2H), 7.40 (m, 2H, Ar), 7.54 (br s, 1H), 8.14 (m, 2H, Ar); ^{13}C-

NMR: δ 24.9 (1C), 28.1 (3C), 28.6 (1C), 42.8 (1C), 47.5 (1C), 60.2 (1C), 80.9 (1C), 124.0 (2C), 128.1 (2C), 146.4 (1C), 147.4 (1C), 156.2 (1C), 172.6 (1C); IR (KBr): 3251 (NH), 1689 (C=O) cm^{-1}. Anal. calcd. for for $C_{17}H_{23}N_3O_5$ (349.38): C, 58.44; H, 6.64; N, 12.03. Found: C, 58.41; H, 6.64; N, 11.95.

N-(4-Nitrobenzyl)pyrrolidine-2-carboxamide ***23***

Prepared as described for compound **19** starting from compound **22**. Pale yellow oil (81%): LC/MS *m/z* 272 [$M^+ + 23$]; ^{1}H-NMR: δ 1.67–1.81 (m, 2H), 1.88–2.02 (m, 1H), 2.05–2.30 (m, 2H), 2.82–2.93 (m, 1H), 2.98–3.09 (m, 1H), 3.82 (q, $J = 4.7$ Hz, 1H), 4.51 (d, $J = 6.6$ Hz, 2H), 7.40 (d, $J = 4.7$ Hz, 2H, Ar), 8.10–8.30 (m, 3H, Ar + N*H*); ^{13}C-NMR: δ 26.5 (1C), 31.0 (1C), 42.4 (1C), 47.6 (1C), 60.8 (1C), 124.1 (2C), 128.3 (2C), 146.6 (1C), 147.4 (1C), 175.8 (1C); IR (neat): 3323 (NH), 1666 (C=O), 1525 (NO_2) cm^{-1}.

1-Pentanoyl-N-(4-nitrobenzyl)pyrrolidine-2-carboxamide ***24***

Prepared as described for compound **20** starting from compound **23**. Purification by column chromatography (EtOAc/petroleum ether 8:2) gave an oil, which was crystallized from $CHCl_3$/hexane giving 1.7 g (87%) of white crystals: M.p.: 115–116°C; GC/MS *m/z* 333 (M^+, <1), 70 (100); ^{1}H-NMR: δ 0.90 (t, $J = 7.3$ Hz, 3H), 1.26–1.40 (m, 2H), 1.54–1.66 (m, 2H), 1.74–1.90 (m, 2H), 1.92–2.20 (m, 2H), 2.31 (t, $J = 7.5$ Hz, 2H), 3.35–3.50 (m, 1H), 3.52–3.60 (m, 1H), 4.47 (d, $J = 6.3$ Hz, 2H), 4.63 (dd, $J = 7.8$, 1.6 Hz, 1H), 7.38 (d, $J = 8.8$ Hz, 2H, Ar), 7.91 (br s, 1H), 8.14 (d, $J = 8.8$ Hz, 2H, Ar); ^{13}C-NMR: δ 14.1 (1C), 22.7 (1C), 25.3 (1C), 28.1 (1C), 34.6 (2C), 42.9 (1C), 47.9 (1C), 59.8 (1C), 124.0 (2C), 128.2 (2C), 146.4 (1C), 147.3 (1C), 171.8 (1C), 174.3 (1C); IR (KBr): 3313 (NH), 1656, 1638 (C=O) cm^{-1}. Anal. calcd. for $C_{17}H_{23}N_3O_4$ (333.38): C, 61.25; H, 6.95; N, 12.60. Found: C, 61.09; H, 6.89; N, 12.49.

N-(4-Aminophenyl)-1-pentanoylpyrrolidine-2-carboxamide ***25***

To a stirred solution of 0.50 g (1.50 mmol) of compound **24** in absolute ethanol (20 mL), 1.70 g (7.50 mmol) of tin chloride(II)dihydrate were added in one portion. The mixture was brought to reflux and kept at this temperature for 2 h. The solvent was removed and the crude residue was made alkaline by addition of 2 N NaOH following by the extraction three times with ethyl acetate. The combined organic layers were dried affording 0.45 g (99%) of a pale brown oil: LC/MS *m/z* 326 [$M^+ + 23$]; ^{1}H-NMR: δ 0.87 (t, $J = 7.3$ Hz, 3H), 1.20–1.40 (m, 2H), 1.42–1.62 (m, 2H), 1.64–2.20 (m, 4H), 2.21 (t, $J = 7.5$ Hz, 2H), 3.00–3.80 (m, 4H), 4.20–4.40 (m, 2H), 4.57 (d, $J = 6.6$ Hz, 1H), 6.60 (d, $J = 8.2$ Hz, 2H, Ar), 7.02 (d, $J = 8.0$ Hz, 2H, Ar), 7.32 (br s, 1H); ^{13}C-NMR: δ 14.1 (1C), 22.7 (1C), 25.3 (1C), 27.1 (1C), 27.4 (1C), 34.6 (1C), 43.2 (1C), 47.7 (1C), 59.9 (1C), 115.4 (2C), 129.0 (2C), 129.3 (1C), 145.8 (1C), 171.3 (1C), 173.8 (1C); IR (neat): 3343 (NH), 1729 (broad, C=O) cm^{-1}.

2-{[(4-{[(N-Pentanoylpyrrolidine-2-ylcarbonyl)-amino]methyl}phenyl)amino]carbomoyl}benzoic acid ***5***

0.40 g (1.32 mmol) of amine **25** were dissolved in THF (10 mL), then 0.19 g (1.32 mmol) of phthalic anhydride were added in one portion and the mixture was allowed to stir at room temperature for 7 h. Then solvent was removed; the crude residue was taken up with ethyl acetate and washed three times with 2 N NaOH. The alkaline aqueous phases were acidified with 2 N HCl and extracted twice with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4 giving 0.50 g (84%) of an oil, which was crystallized from EtOH/Et_2O/iPr_2O to give 0.03 g of a grey powder: M.p.: 190–191°C; LC/MS *m/z* 450 [$M^+ - 1$]; ^{1}H-NMR (DMSO-d_6): δ 0.86 (t, $J = 7.2$ Hz, 3H), 1.10–1.55 (m, 4H), 1.70–2.35 (m, 6H), 2.45–2.55 (m, 2H), 3.50–3.65 (m, 1H), 4.25–4.40 (m, 2H), 7.32–7.40 (m, 4H, Ar), 7.84–7.98 (m, 4H, Ar), 8.35 (br t, $J = 6.1$ Hz, 1H), 8.67 (br t, $J = 5.8$ Hz, 1H); ^{13}C-NMR (DMSO-d_6): δ 14.6 (1C), 22.6 (1C), 25.0 (1C), 27.1 (1C), 30.2 (1C), 32.5 (1C), 34.1 (1C), 47.5 (1C), 60.3 (1C), 124.1 (2C), 127.9 (3C), 128.0 (1C), 128.3 (1C), 131.0 (1C), 132.2 (1C), 135.4 (1C), 140.1 (1C), 140.4 (1C), 167.8 (1C), 171.7 (1C), 171.8 (1C), 172.8 (1C); IR (KBr): 3420 (OH), 3287 (NH), 1724, 1650, 1627, 1603 (C=O) cm^{-1}. Anal. calcd. for $C_{25}H_{29}N_3O_5 \cdot 0.50\ H_2O$ (460.51): C, 65.20; H, 6.57; N, 9.12. Found: C, 65.59; H, 6.48; N, 9.15.

Cell culture and [^{3}H]valsartan binding

Chinese hamster ovary cells (CHO-K1) stably transfected with the human angiotensin II AT_1 receptor (denoted as CHO-AT_1 cells) [14] were cultured in 24-well plates in Dulbecco's modified essential medium (DMEM) supplemented with l-glutamine (2 mM), 2% of a stock solution containing penicillin (5000 IU mL^{-1}) and streptomycin (5000 μg mL^{-1}) (Life Technologies, Merelbeke, Belgium), and 10% fetal bovine serum (Life Technologies, Merelbeke, Belgium). The cells were grown in 5% CO_2 at 37°C until confluent, and before binding experiments, were washed three times with 0.5 mL $well^{-1}$ of DMEM at room temperature. The cells were then incubated at 37°C for 30 min in a final volume of 0.5 mL in each well containing 400 μL DMEM, 50 μL DMEM containing the investigated compound at increasing final concentrations between 10^{-9} and 10^{-5} M, and 50 μL [^{3}H]valsartan at a final concentration of 1.5 nM. At the end of the incubation, the cells were placed on ice and washed three times with ice-cold PBS containing $CaCl_2 \cdot 2\ H_2O$ (0.132 g L^{-1}), KCl (0.2 g L^{-1}), KH_2PO_4 (0.2 g L^{-1}), $MgCl_2 \cdot 6\ H_2O$ (0.1 g L^{-1}), NaCl (8 g L^{-1}), and $Na_2HPO_4 \cdot 2\ H_2O$ (1.44 g L^{-1}). To measure radioligand binding, 0.5 mL 1 M NaOH were added to the cells and then transferred into scintillation vials containing 3.5 mL scintillation liquid (Optiphase "Hisafe2", PerkinElmer). The bind-

ing values were normalized with total binding (that is, in the absence of competing ligand) and nonspecific binding (in the presence of the potent AT_1 receptor antagonist candesartan at 1 μM). IC_{50} values were determined by nonlinear regression analysis of the competition curves using GraphPad Prism and were converted into the corresponding K_i values as previously described [20].

The authors have declared no conflict of interest.

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