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Synthesis and biological evaluation of new biphalin analogues with non-hydrazine linkers

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Abstract—Biphalin is a potent opioid peptide agonist, with a palandromic structure, composed of two enkephalin-like active fragments connected tail to tail by a hydrazine linker (Tyr-D-Ala-Gly-Phe-NH-NH<-Phe<-Gly<-D-Ala<-Tyr). This study presents the synthesis and in vitro bioassays of six new biphalin analogues with three different non-hydrazine linkers, some of which have higher binding affinity and bioactivity than biphalin.

1. Introduction

Biphalin (Fig. 1) is an opioid octapeptide with a dimeric structure based on two identical portions derived from enkephalins joined tail to tail by a hydrazide bridge. This particular structure enhances the antinociceptive activity of the enkephalins with an unknown mechanism, probably based on a cooperative binding.[1] Biphalin has excellent binding affinity for μ and δ receptors with a EC_{50} of about 1–5 nM at both μ and δ receptors. It is a highly potent analgesic (250 times that of morphine; i.c.v. administration; tail flick test), as potent or more potent than etorphine.[2] A definitive explanation of the extraordinary in vivo potency shown by this compound, which has pronounced efficacy in pain modulation, is still not available; it has been suggested, however, that its high agonist activity at both μ and δ receptor may be related.[3] Biphalin has significantly higher potency than other analgesics with novel biological profiles;[4] in particular, most recent data show that biphalin is unlikely to produce dependency in chronic use.[5]

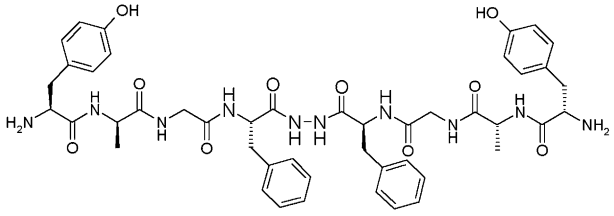

Figure 1. Biphalin.

In the past 10 years, there have been many attempts to modify its structure to obtain products unaffected by the action of enkephalinases, to enhance its antinociceptive activity, and to modify the BBB penetration.[3,4,6] Structure–activity relationship studies (SAR) also were made in order to understand the elements responsible for biphalin's high activity.

SAR studies have shown that the Tyr[1] moiety is required for interaction with the opioid receptors; the 4 and 4′ positions can be modified with rigid constrained

Abbreviations: 3H-DAMGO, [D-Ala(2),N-Me-Phe(4),Gly-ol(5)]enkephalin; ^{3}H-DPDPE, [^{3}H]-c[2-D-penicillamine,5-D-penicillamine]enkephalin; BBB, Blood Brain Barrier; DCM, Dichloromethane; DMF, *N*,*N*-Dimethyl Formamide; DMSO, Dimethylsulfoxide; EDC, 1-Ethyl-(3-dimethylaminopropyl)carbodiimide; Et_2O, Diethyl Ether; EtOAc, Ethyl acetate; GPI/LMMP, Guinea pig ileum/longitudinal muscle myenteric plexus (μ opioid receptors); HOBT, 1-hydroxybenzotriazole; I.C.V., intracerebroventricular; MVD, mouse vas deferens (δ opioid receptors); TEA, Triethylamine; TFA, Trifluoroacetic Acid; TOCSY, Total Correlation Spectroscopy

Keywords: Biphalin analogues; Opioid agonists; Linkers; Structure–activity relationship.

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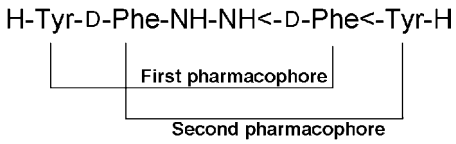


Figure 2. Opioid ligand with two overlapping pharmacophores. Relative affinities (K_i; nM) for μ and δ binding sites in guinea pig membranes: 31±2 (μ); 187±15 (δ). See Ref. 8.

Phe analogues or with electron rich Phe analogues increasing the activity.[7] Modification of the hydrazide bridge by using alkyl diamines led to reduced activity, probably because of the higher degree of freedom around the diamide bridge.[1a]

Figure 2 shows how the hydrazide bridge was used as an alternative scaffold to design opioid ligands with two overlapping opioid pharmacophores. It was found in Lipkowski's laboratory that this compound possesses potent analgesic properties after intraperitoneal injection.[8] This compound can be viewed as an analogue of a truncated/modified biphalin.

This analogue still maintained opioid like activity and represents a good example of molecular simplification.

In this letter we describe the synthesis and biological evaluation of six new biphalin analogues in which the hydrazide bridge has been replaced with three different diamines containing an aromatic or aliphatic cyclic structure: 1,4-phenylenediamine, 1,2-phenylenediamine, and piperazine. Both the native biphalin pharmacophore Tyr-D-Ala-Gly-Phe and the shorter pharmacophore Tyr-D-Phe were used in the new derivatives.

2. Chemistry

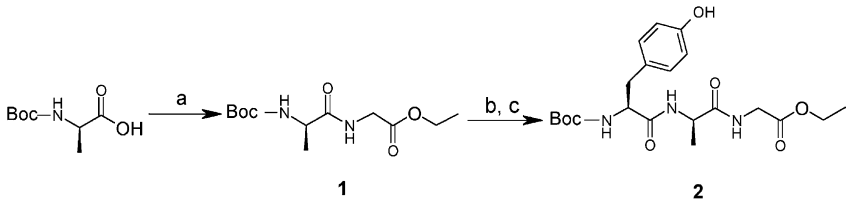
The syntheses of the new analogues were performed in the solution phase following an N^{α}-Boc strategy. The peptides N^{α}-Boc-D-Ala-Gly-OEt (**1**) and N^{α}-Boc-Tyr-D-Ala-Gly-OEt (**2**) were synthesized by the asymmetric anhydride method[9] obtained with isobutyl chloroformate and TEA at −15 °C (Scheme 1)[10]. Dipeptide **1** was used for the next step without further purification. Tripeptide ester **2** was purified by silica gel chromatography with EtOAc/DCM 1:4 as eluant, and then crystallized in EtOAc/Et_2O. The ethyl ester group was hydrolyzed with NaOH 1 N (6 equiv) in methanol at rt. The solvent was removed under vacuum; the basic aqueous solution was acidified with 1 N HCl to pH 3 and extracted with EtOAc. The solvent was removed under vacuum to give the crude product as an oil. All the other coupling reactions were performed with the standard method[9] of HOBt·H_2O/EDC/TEA in DMF (Schemes 2 and 3).[11]

The deprotection of the N^{α}-*tert*-butyloxycarbonyl group (Boc) was performed by dissolving each N^{α}-Boc protected product into a mixture of TFA 50% in DCM for 30 min at rt, under nitrogen atmosphere. The DCM and the TFA were removed under vacuum and the resultant intermediate products were used in the next step without further purification. With the exception of **1** and **2**, all the N^{α}-Boc protected products and the final products as TFA salts **6–8** and **12–14** were purified by reverse-phase HPLC using a Vydac (C_{18}-bonded, 300 Å; 10 mm × 25 cm) column and a gradient of 10–90% acetonitrile in 0.1% aqueous TFA over 40 min at a flow rate of 3 mL/min the extent of purity was monitored at 220, 254, 280, and 350 nm. Approximately 10 mg of crude peptide was injected each time, and the fractions containing the purified peptide were collected and lyophilized to dryness.

3. NMR spectroscopy

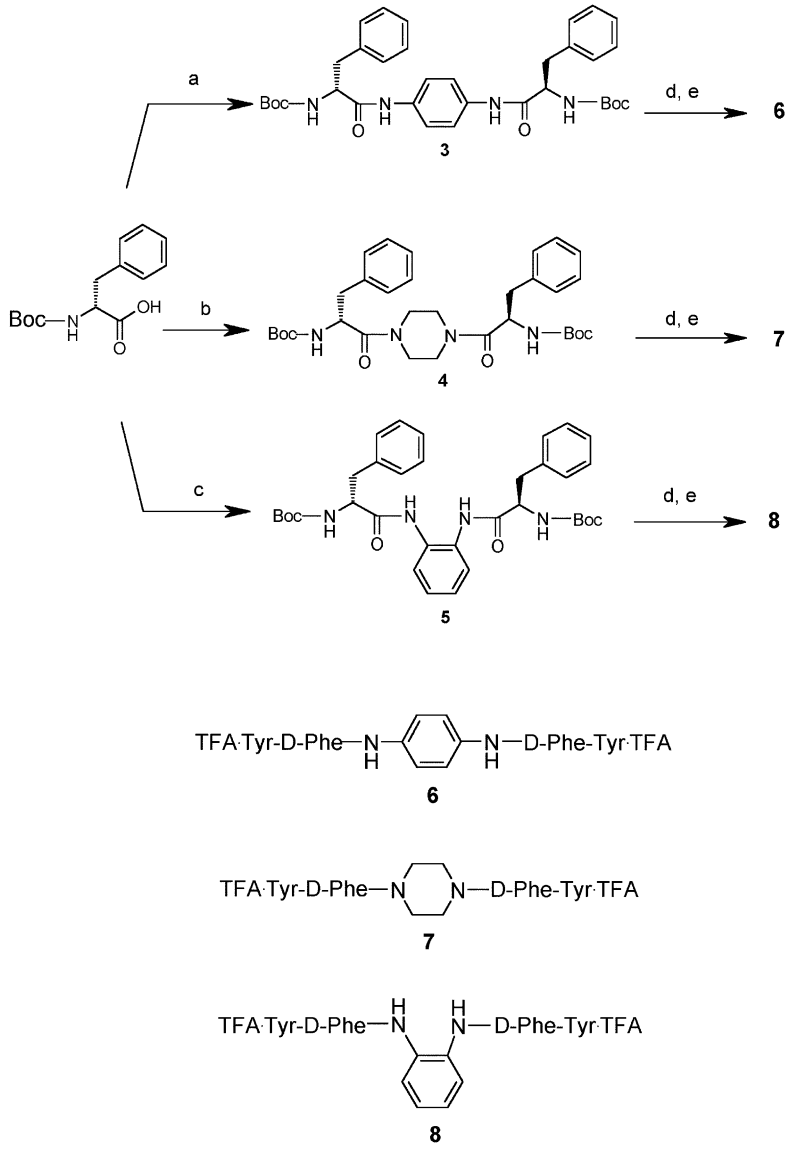
All NMR spectra were acquired on a Bruker DRX-600 spectrometer at 25 °C using a Nalorac triple-resonance single-axis gradient 5 mm probe, and the data processed with the Bruker software XWINNMR.

NMR analysis of the intermediate N^{α}-Boc protected products were performed in DMSO-d_6, although in the case of product **2** a mixture of $CDCl_3$ 90%/DMSO-d_6 10% was used. In the case of the final products **6–8** and **12–14**, the samples were dissolved as TFA salts in 90% H_2O/10% D_2O solution buffered at pH 4.5 containing 50 mM sodium acetate-d_3/HCl buffer and 1.0 mM NaN_3 as preservative, at a peptide concentration of ca. 10 mM. 1D ^1H spectra were acquired using the Watergate sequence[12a] using *z*-axis gradients, with a τ delay of 240 μs and 3–9–19 pulses at full power. 2D TOCSY spectra[12b] were acquired in TPPI mode[12c] with 512 t_1 increments and a mixing time of 73 ms, including 2.5 ms trim pulses before and after the MLEV-17 sequence. Water suppression was achieved with Watergate using *z*-axis gradients, with a τ delay of 330 μs. The power level for spin-lock, trim pulses, and 3–9–19 pulses was 8.3 kHz.



1 2

Scheme 1. Reagents and conditions: (a) H-Gly-OEt·HCl (1 equiv), *i*-BuOCOCl (1.1 equiv), TEA (1.1 equiv), DCM (20 mL), −15 °C for 15 min then rt for 12 h; (b) TFA 50%, DCM, rt for 30 min under N_2 atmosphere; (c) N^{α}-Boc-Tyr-OH (1.1 equiv), *i*-BuOCOCl (1.1), TEA (1.1 equiv), DCM (20 mL), −15 °C for 15 min then rt for 12 h . (Overall yield 85%).



Scheme 2. Reagents and conditions: (a) Boc-D-Phe-OH (2.1 equiv), EDC (2.1), HOBt·H_2O (2.1 equiv), TEA (3.2 equiv), 1,4-phenylenediamine·2HCl (1 equiv), DMF (20 mL), 30 min in 0 °C, then rt for 6 h; (b) Boc-D-Phe-OH (2.1 equiv), EDC (2.1 equiv), HOBt·H_2O (2.1 equiv), TEA (2.2 equiv), piperazine·HCl (1 equiv), DMF (20 mL), 30 min in 0 °C, then rt for 6 h; (c) Boc-D-Phe-OH (2.1 equiv), EDC (2.1 equiv), HOBt·H_2O (2.1 equiv), TEA (3.2 equiv), 1,2-phenylenediamine·2HCl (1 equiv), DMF (20 mL), 30 min in 0 °C, then rt for 6 h; (d) TFA 50% in DCM 30 min in rt under N_2 atmosphere, then Boc-Tyr-OH (2.1 equiv), EDC (2.1 equiv), HOBt·H_2O (2.1 equiv), TEA (3.2 equiv), DMF (20 mL), 30 min in 0 °C, then rt for 6 h; (e) TFA 50% in DCM, 30 min rt under N_2 atmosphere. (Overall yield 30–50%).

3.1. Radioligand labeled binding assays

Receptor binding affinities to the δ and μ opioid receptors were evaluated as previously described.[13a,b] The ligands used were [^{3}H]DPDPE and [^{3}H]DAMGO for δ and μ receptors, respectively.

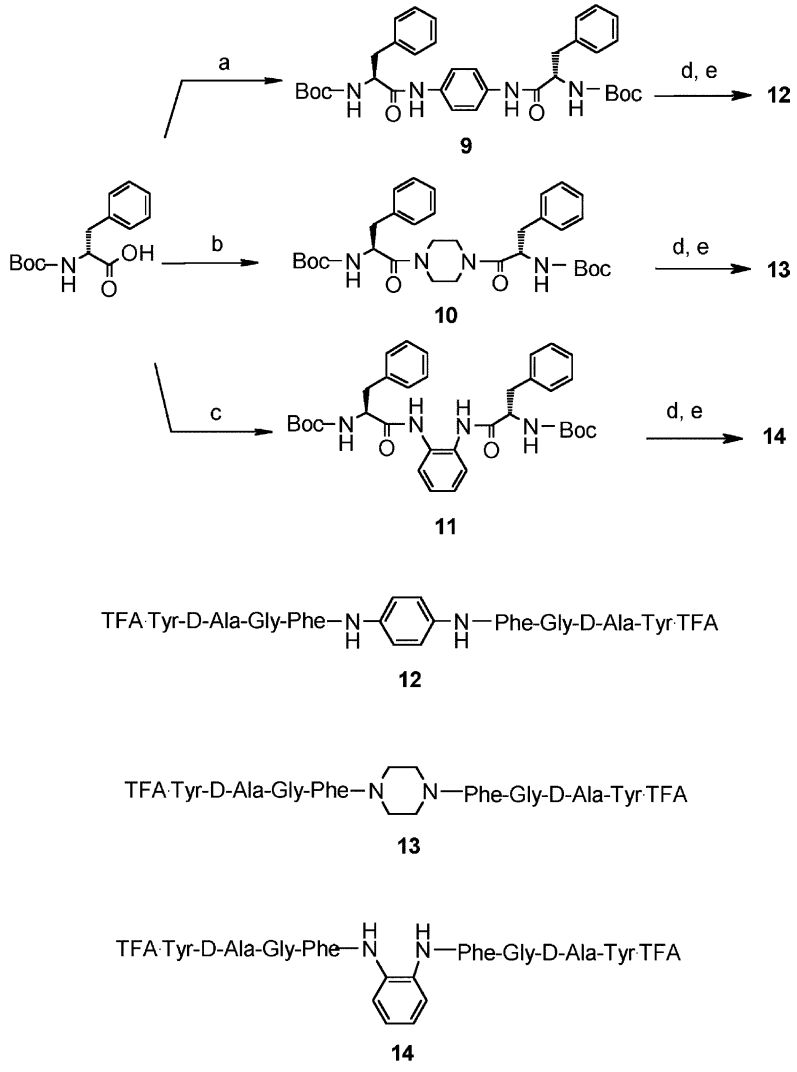
3.2. GPI and MVD in vitro bioassays

The in vitro tissue bioassays were performed as described previously[14] (see Table 1).

IC_{50} values represent the mean of no less than four experiments. IC_{50} values, relative potency estimates, and their associated standard errors were determined by fitting the data to the Hill equation by a computerized non-linear least-square method.

4. Results and discussion

As reported in Table 1, compounds **6**–**8** showed weak binding affinity and in vitro activity at the opioid receptors, probably because the Tyr and Phe moieties are not in a favorable position to accomplish the overlapping of the previously postulated pharmacophore. Compounds **12**–**14** showed exceptionally good binding affinity and bioactivity. Analogue **12** was comparable with biphalin, and compound **13** binds the receptors with three to five times higher affinity than biphalin, with the in vitro



Scheme 3. Reagents and conditions: (a) Boc-L-Phe-OH (2.1 equiv), EDC (2.1 equiv), HOBt·H_2O (2.1 equiv), TEA (3.2 equiv), 1,4-phenylenediamine·2HCl (1 equiv), DMF (20 mL), 30 min in 0 °C, then rt for 6 h; (b) Boc-L-Phe-OH (2.1 equiv), EDC (2.1 equiv), HOBt·H_2O (2.1 equiv), TEA (2.2 equiv), piperazine·HCl (1 equiv), DMF (20 mL), 30 min 0 °C, then 6 h in rt; (c) Boc-L-Phe-OH (2.1 equiv), EDC (2.1 equiv), HOBt·H_2O (2.1 equiv), TEA (3.2 equiv), 1,2-phenylenediamine·2HCl (1 equiv), DMF (20 mL), 30 min in 0 °C, then rt for 6 h; (d) TFA 50% in DCM 30 min rt under N_2 atmosphere, then Boc-Tyr-D-Ala-Gly-OH (2.1 equiv), EDC (2.1 equiv), HOBt·H_2O (2.1 equiv), TEA (3.2 equiv), DMF (20 mL), 30 min, 0 °C, then rt for 6 h; (e) TFA 50% in DCM, 30 min rt under N_2 atmosphere. (Overall yield 30–50%).

Table 1. Binding affinities and in vitro activity

Drugs	Binding IC_{50}[a] (nM)		Bioassay IC_{50}[a] (nM)	
	δ	μ	MVD	GPI/LMMP
Biphalin[b]	**2.6 ± 0.4**	**1.4 ± 0.2**	**27 ± 1.5**	**8.8 ± 0.3**
6	2400 ± 1000	8200 ± 1800	17% at 1 μM	43% at 20 μM
7	400 ± 98	2700 ± 370	8.1% at 1 μM	25.5% at 20 μM
8	640 ± 44	3010 ± 1300	47% at 10 μM	61% at 20 μM
12	3.17 ± 0.6	1.27 ± 0.08	35.6 ± 6.4	40 ± 16.0
13	0.65 ± 0.30	0.48 ± 0.06	9.3 ± 0.30	2.5 ± 0.6
14	0.19 ± 0.04	1.93 ± 0.20	0.72 ± 0.20	40 ± 13.0

[a] ± SEM.
[b] Data according to Ref. 7c.

bioassay potency reflecting the same pattern. Analogue **14** shows a 1:10 selectivity for the δ versus μ opioid receptors in binding. This preference is more evident in the bioassays where the bioactivity for the δ receptors is 50 times higher than at the μ receptor. As compared with the activity of biphalin, all the above reported results show how a reduced degree of freedom between the two pharmacophore moieties and their consequent relative position can influence the binding affinity and selectivity toward different receptors. In the case of the

shorter pharmacophore (compounds **6**–**8**) the affinity for the receptors is significantly reduced. The presence of the classic pharmacophore (**12**–**14**) maintains or improves the binding affinity. Whereas the CO–NH of the linker fragment in compounds **6**, **8**, **12**, and **14** should adopt the usually more favorable *trans* conformation, this limitation is not present in compounds **7** and **13** which contain tertiary amide bonds at the two piperazine nitrogen atoms and thus are free to choose between equivalent conformers. The remarkable activity of compound **13** leads to the hypothesis that the *NH* moiety of hydrazine in biphalin is not related to the binding at the opioids receptors. We can conclude that the hydrazine linker is not fundamental for activity or binding, and it can be conveniently substituted by different conformationally constrained cycloaliphatic diamine linkers. Future in vivo studies will give us more information about the biological effects of this type of modification.

Acknowledgements

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References and notes

1. (a) Lipkowski, A. W.; Koneka, A. M.; Sroczynska, I. *Peptides* **1982**, *3*, 697–700; (b) Lipkowski, A. W.; Konopka, M.; Osipiak, B.; Gumulka, W. S. In *Peptides 1982*; Bláha, K., Maloň, P., Eds.; de Gruyter: Berlin, 1983, pp 481–486.
2. Horan, P. J.; Mattia, A.; Bilsky, E. J.; Weber, S. J.; Davis, T. P.; Yamamura, H. I.; Malatynska, E.; Appleyard, S. M.; Slaninova, J.; Misicka, A.; Lipkowski, A. W.; Hruby, V. J.; Porreca, F. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 1446–1454.
3. Abbruscato, J.; Thomas, S. A.; Hruby, V. J.; Davis, T. P. *J. Neurochem.* **1997**, *69*, 1236–1245.
4. Abbruscato, T. J.; Williams, S. A.; Misicka, A.; Lipkowski, A. W.; Hruby, V. J.; Davis, T. P. *J. Pharmacol. Exp. Ther.* **1996**, *276*, 1049–1057.
5. Yamazaki, M.; Suzuki, T.; Nanta, M.; Lipkowski, A. W. *Life Sci.* **2001**, *69*, 1023–1028.
6. Hruby, V. J.; Davis, T. P.; Polt, R.; Bartosz-Bechowski, H.; Misicka, A.; Lipkowski, A.; Sharma, S. D.; Li, G.; Bonner, G., et al. In *Peptides: Chemistry, Structure and Biology*; Kaumaya, P. T. P., Hodges, R. S., Eds.; Mayflower Scientific: Kingswinford, 1996, pp 154–156.
7. (a) Misicka, A.; Lipkowski, A. W.; Horvath, R.; Davis, P.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. *Life Sci.* **1997**, *60*, 1263–1269; (b) Li, G.; Haq, W.; Xiang, L.; Lou, B.-S.; Hughes, R.; De Leon, I. A.; Davis, P.; Gillespie, T. J.; Romanowski, M.; Zhu, X.; Misicka, A.; Lipkowski, A. W.; Porreca, F.; Davis, T. P.; Yamamura, H. I.; O'Brien, D. F.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 555–560; (c) Lipkowski, A. W.; Misicka, A.; Davis, P.; Stropova, D.; Janders, J.; Lachwa, M.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2763–2766; (d) Misicka, A.; Lipkowski, A. W.; Stropova, D.; Yamamura, H. I.; Davis, P.; Porreca, F.; Hruby, V. J. *Peptide Sci.* **1999**, 726–727.
8. Lipkowski, A. W.; Konecka, A. M.; Sroczynska, I.; Przewlocki, R.; Stala, L.; Tam, S. W. *Life Sci.* **1987**, *40*, 2283–2388.
9. Han, So-Y.; Kim, Y.-A. *Tetrahedron* **2004**, *60*, 2447–2467.
10. Selected data: **1**: ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.18 (6H, m, D-Ala CH_3 and CH_3–CH_2–O), 1.38 (9H, s, *t*-butyl), 3.72–3.88 (2H, m, Gly CH_2), 4.0 (1H, m, D-Ala α-CH), 4.08 (2H, m, CH_3-CH_2–), 6.90 (1H, d, D-Ala NH), 8.15 (1H, t, Gly NH). FAB-MS 296.93 *m/e* (M^++Na). **2**: ^{1}H NMR (600 MHz, $CDCl_3$ 90% DMSO-d_6 10%) δ 1,26–1,29 (6H, m, CH_3CH_2O– and D-Ala CH_3), 1.49 (9H, s, *t*-butyl), 2.89–2.98 (2H, m, Tyr β-CH_2), 3.8–4.02 (2H, m, Gly α-CH_2), 4.16 (2H, m, CH_3CH_2O–), 4.23 (1H, m, Tyr α-CH), 4.46 (1H, m, α-CH D-Ala), 5.69 (1H, br, Tyr NH), 6.75 and 7.01 (4H, dd, aromatic), 7.34 (1H, br, D-Ala NH), 7.7 (1H, br, Gly NH), 8.71 (1H, s, Tyr OH). FAB-MS *m/e* 460.01 (M^++Na); MP 155–158 (EtOAc/Et_2O).
11. Selected data: **6**: ^{1}H NMR (600 MHz, H_2O/D_2O) δ 2.83 and 2.95 (4H, m, Phe β-CH_2), 2.90 (4H, m, Tyr β-CH_2), 4.12 (2H, m, Tyr α-CH), 4.50 (2H, under water signal, Phe α-CH), 6.75–7.32 (22H, m, aromatic), 8.58 (2H, m, Phe NH), 9.73 (2H, s, *NH*-aromatic linker). FAB-MS *m/e* 729.3437 (M^+). **7**: ^{1}H NMR (600 MHz, H_2O/D_2O) δ 2.55–3.62 (8H, m, piperazine $4\times CH_2$), 2.68–78 (4H, m, Phe β-CH_2), 2.96–3.08 (4H, m, Tyr β-CH_2), 4.09 (2H, m, Tyr α-CH), 4.70 (2H, superimposed on H_2O signal, Phe α-CH), 6.78–7.34 (18H, m, aromatic), 8.48 (2H, m, Phe NH). FAB-MS *m/e* 706.3557 (M^+). **8**: ^{1}H NMR (600 MHz, H_2O/D_2O) δ 2.89–3.12 (8H, m, Phe β-CH_2 and Tyr β-CH_2), 4.09 (2H, m, Tyr α-CH), 4.75 (2H, superimposed on H_2O signal, Phe α-CH), 6.7–7.38 (22H, m, aromatics), 8.60 (2H, m, Phe NH), 9.40 (2H, s, aromatic liker *NH*). FAB-MS *m/e* 729.3407 (M^+). **12**: ^{1}H NMR (600 MHz, H_2O/D_2O) δ 1.05 (6H, d, D-Ala CH_3), 2.9–3.12 (8H, m, Phe β-CH_2 and Tyr β-CH_2), 3.85 (4H, m, Gly CH_2), 4.08 (2H, m, Tyr α-CH), 4.15 (2H, m, D-Ala α-CH), 4.65 (2H, superimposed on H_2O signal, Phe α-CH_2), 6.75–7.40 (22H, m, aromatic), 8.02 (2H, m, Phe NH), 8.32 (2H, m, Gly NH), 8.38 (2H, m, D-Ala NH), 9.58 (2H, s, aromatic linker *NH*). FAB-MS *m/e* 985.45 (M^+). **13**: ^{1}H NMR (600 MHz, H_2O/D_2O) δ 1.08 (6H, dd, D-Ala CH_3), 2.55–3.54 (8H, m, piperazine $4\times CH_2$), 2.85 and 2.99 (4H, m, Phe β-CH_2), 3.01 and 3.10 (4H, m, Tyr β-CH_2), 3.8 (4H, m, Gly CH_2), 4.09 (4H, m, Tyr α-CH and D-Ala α-CH), 4,75 (2H, superimposed on H_2O signal, Phe α-CH), 6.82–7.35 (18H, m, aromatic), 8.03 and 8.07 (2H, 2d, Phe NH, two conformers), 8.30 (2H, m, Gly NH), 8.4 (2H, m, D-Ala NH). FAB-MS *m/e* 963.48 (M^+). **14**: ^{1}H NMR (600 MHz, H_2O/D_2O) δ 1.05 (6H, d, D-Ala CH_3), 2.9–3.12 (8H, m, Phe β-CH_2 and Tyr β-CH_2), 3.75 (4H, m, α-CH_2 Gly), 4.04 (4H, m, D-Ala α-CH and Tyr α-CH), 4.75 (2H, superimposed on H_2O signal, Phe α-CH_2), 6.79–7.33 (18H, m, aromatic), 8.02 (2H, m, Phe *NH*), 8.18 (2H, m, Gly NH), 8.32 (2H, m, D-Ala NH), 9.28 (2H, s, aromatic linker *NH*). FAB-MS *m/e* 985.4546 (M^+).
12. (a) Sklenar, V.; Piotto, M.; Leppik, R.; Saudek, V. *J. Magn. Reson., Ser A* **1993**, *102*, 241–245; (b) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355–360; (c) Marion, D.; Ikura, M.; Bax, A. *J. Magn. Reson.* **1989**, *84*, 425–430.
13. (a) Polt, R.; Porreca, F.; Szabo, L.; Bilsky, E. J.; Davis, T.; Horvath, R.; Abbruscato, T. J.; Yamamura, H. J.; Hruby, V. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7114–7118; (b) Misicka, A.; Lipkowski, A. W.; Horvath, R.; Davis, P.; Kramer, T. H.; Yamamura, H. I.; Hruby, V. J. *Life Sci.* **1992**, *51*, 1025–1032.
14. Kramer, T. H.; Davis, P.; Hruby, V. J.; Burks, T. F.; Porreca, F. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 577–584.