Journal of Peptide Science
J. Pept. Sci. 2006; **12**: 481–490
Published online 20 March 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.753

JOURNAL OF PEPTIDE SCIENCE

Synthesis of enzymatically resistant nociceptin-related peptides containing a carbamic acid residue

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Received 11 January 2006; Revised November 2005; Accepted 12 January 2006

Abstract: Nociceptin, a 17-amino acid peptide (FGGFTGARKSARKLANQ, N/OFQ), is the endogenous ligand of the nociceptin/orphanin FQ (NOP) receptor. This receptor–ligand system is involved in various physiological as well as pathophysiological mechanisms, but owing to the peptidic structure, it is rapidly degraded by enzymes. The enzymatic digestion of nociceptin involves mainly aminopeptidases and yields Noc(2-17)-OH and other smaller fragments. We aimed at increasing the enzymatic stability against aminopeptidases in the case of peptide Noc(1-13)-NH_2, which possesses the minimum sequence capable of interacting with the NOP receptor. Therefore we developed a new procedure for the synthesis of peptides with the carbamic acid residue [···-NH-CH(R)-CO-NH-CO-NH-CH(Q)-CO-···]. A set of four carbamic acid-nociceptin derivatives were produced. The carbamic acid residue was incorporated into the inner part of the peptides, building on solid phase, by using a suitable dipeptide fragment with carbamic acid residue produced by a simple and efficient three-step solution phase procedure. Enzymatic stability of carbamic acid peptides was studied in the presence of aminopeptidase M (AP-M) and in rat brain membrane homogenate. The receptor-binding properties were also studied by radioligand binding assay on crude rat brain membranes and the activity of the ligands were analyzed on isolated mouse *vas deferens* (MVD) tissues. We found that incorporation of the carbamic acid residue into the *N*-terminal part of nociceptin significantly increases the resistance against AP-M. We observed the decrease of binding affinities to the NOP receptor in case of the peptides modified in the *N*-terminal portion. Consequently, the incorporation of the carbamic acid residue into peptides can be proposed as a promising and reasonable tool for increasing enzymatic stability, where the native molecule is less sensitive for carbamic acid residue–related structural change. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: carbamic acid residue; enzymatic stability; aminopeptidase M; rat brain membrane homogenate; nociceptin; NOP receptor; radioligand binding; GTPγS assay; mouse vas deferens bioassay

INTRODUCTION

Cloning of μ, κ and δ opioid receptors have been followed by the identification of a new, structurally similar, but functionally distinct, receptor called *nociceptin/orphanin FQ receptor* (NOP receptor) [1,2]. Searching for endogenous ligands for this receptor resulted in the discovery of a 17-amino acid peptide, nociceptin/orphanin FQ (FGGFTGARKSARKLANQ, N/OFQ) [3,4]. Nociceptin is derived from its larger molecular mass precursor polypeptide pronociceptin [5,6]. *In vitro* and *in vivo* studies demonstrated that this receptor–ligand system mediates a variety of biological processes, and is widely distributed in the central and peripheral nervous systems, immune system and also in peripheral organs. Nociceptin is involved in the induction of different physiological responses as well as in pathophysiological events [7–9].

Development of peptides as clinically useful drugs is frequently limited by their poor metabolic stability and low bioavailability. Because of the peptidic structure, N/OFQ is not resistant against enzymatic degradation and its metabolites are weakly active or inactive. N/OFQ is degraded primarily by aminopeptidases present in the plasma to yield Noc(2–17)-OH and other smaller fragments [10,11]. *N*-terminal truncation leads to complete loss of activity, while the removal of a few amino acids from the *C*-terminus does not terminate affinity to the receptor. Structure–activity studies suggest that Noc(1–13)-OH is the minimum sequence capable of interacting with the NOP receptor. The amidated analogues of N/OFQ and Noc(1–13)-OH were also prepared and it was observed that Noc(1–13)-NH_2 possesses approximately the same biological properties as native nociceptin [12].

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Our aim was to increase the enzymatic stability of N/OFQ against aminopeptidases by producing derivatives with the carbamic acid residue [···-NH-CH(R)-CO-NH-CO-NH-CH(Q)-CO-···] incorporated into the truncated and amidated analogue of N/OFQ, the Noc(1–13)-NH_2 peptide. To the best of our knowledge, the synthesis and enzymatic degradation of derivatives of nociceptin or other peptides containing carbamic acid residue incorporated into the inner part of the backbone sequence have not been reported so far. This is a first set of nociceptin-related peptides containing the carbamic acid residue in the *N*-terminal part with increased stability against aminopeptidases (Table 2). According to data published until now, carbamic acid residue has been applied as *N*-terminal protecting group of amino acids and dipeptides [13].

It should be noted that several *N*-terminally modified nociceptin derivatives have been described in the literature. For example, [$Phe^1\Psi(CH_2$-NH)Gly^2]Noc(1–13)-NH_2 is a partial agonist, and [$Nphe^1$]Noc(1–13)NH_2 behaves as an ORL1 receptor antagonist, but most of the *N*-terminally modified peptides exhibited no or reduced activity [14–17]. It has been reported that the full length of the *N*-terminal 'message' tetrapeptide unit Phe^1-Gly^2-Gly^3-Phe^4 and moreover the dibasic repeats Arg^8-Lys^9 and Arg^{12}-Lys^{13} are essential for NOP receptor binding and/or its activation [14,18]. Previous experiments also showed that the length of the N/OFQ peptide backbone, the distance between Phe^1 and Phe^4, seems to be crucial. In view of these data we substituted Gly^2 or Gly^3 by a carbamic acid residue and incorporated a β-amino acid (β-alanine or β-homophenylalanine) simultaneously to retain the length of the peptide backbone. The presence of the carbamic acid residue instead of glycine does not change the acidic–basic characteristic of the peptide and presumably does not produce new side chain–specific interactions.

In this paper, we describe a new procedure for the synthesis of a new set of nociceptin-related peptides with the carbamic acid residue in the *N*-terminal tetrapeptide part. Furthermore, we report on the enzymatic stability of the synthetic peptides in the presence of aminopeptidase M (AP-M) and in rat brain membrane homogenate. The influence of the structural modifications on the receptor binding was also studied by using radioligand binding assay on crude rat brain membranes, and the activity of new ligands were analyzed on isolated mouse *vas deferens* (MVD) tissues.

MATERIALS AND METHODS

Chemicals

N^{α}-*tert*-butyloxycarbonyl and N^{α}-fluorenylmethyloxycarbonyl protected amino acids (Boc-Ala-OH, Boc-Gly-OH, Boc-Phe-OH, Boc-Thr(Bzl)-OH, Boc-Ser(Bzl)-OH, Boc-Arg(Tos)-OH.ethyl acetate, Boc-Lys(Z(2Cl))-OH, Fmoc-β-Ala-OH, Fmoc-Gly-OH and Fmoc-Phe-OH) were purchased from Reanal (Budapest, Hungary); amino acid benzyl ester tosylates (H-Phe-OBzl. Tos, H-β-Ala-OBzl. Tos and H-Gly-OBzl. Tos) were from Bachem (Bubendorf, Switzerland); 4-methylbenzhydryl-amine (MBHA) resin was the product of Calbiochem-Novabiochem (Läufelfingen, Switzerland); 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate was obtained from Quantum Biotechnologies (Montreal, Canada); *N*-hydroxybenzotriazole, *N*, *N*′-diisopropylcarbodiimide, anisole, *N*-methylmorpholine, HF, Pd/C catalyst, tris-(hidroxymethyl)-amino-methane (Tris HCl) and potassium cyanate were from Fluka (Buchs, Switzerland); *N*-ethyldiisopropylamine and *N*-methylpyrrolidone (NMP) were purchased from Merck (Darmstadt, Germany); trifluoroacetic acid, benzene, dichloromethane, *N*,*N*-dimethylformamide and methanol (MeOH) were obtained from Reanal (Budapest, Hungary); piperidine, isobutyl chloroformate, thionyl chloride and acetonitrile (MeCN) were from Sigma-Aldrich (St Louis, MO, USA) and H_2 was from Linde (Wiesbaden, Germany). Fmoc-amino acid chlorides (Fmoc-β-Ala-Cl, Fmoc-Phe-Cl, Fmoc-Gly-Cl) were prepared according to Carpino *et al.* with minor modifications [19]. Boc-β-homophenylalanine was synthesized according to Müller *et al.* [20].

Synthesis

Preparation of carbamoyl-amino acid benzyl esters. Carbamoyl amino acid benzyl esters were prepared according to Corwin *et al.* with some modifications [21]. Briefly, amino acid benzyl ester tosylate salt (10 mmol) [H-Phe-OBzl. Tos (4.27 g); H-β-Ala-OBzl. Tos (3.49 g); H-Gly-OBzl. Tos (3.37 g)] was dissolved in 15 ml MeOH (if necessary, a few drops of 1.0 M sodium hydroxide solution was added to facilitate solubilization) and potassium cyanate (811 mg, 10 mmol) dissolved in 1 ml H_2O was added. The mixture was stirred for 24 h at room temperature. The reaction was monitored by TLC (system B). After completion of the reaction, the solvent was removed at reduced pressure, the product was crystallized and the purity was checked by TLC and RP-HPLC, and melting points were determined (Table 1). NH_2-CO-Phe-OBzl and NH_2-CO-Gly-OBzl have already been described by Chung and Corwin [21,22]. The novel derivative, NH_2-CO-β-Ala-OBzl, was analyzed by elemental analysis and mass spectrometry (MS) (Table 1) and its structure was improved by IR and NMR.

IR main peaks for NH_2-CO-β-Ala-OBzl: 3430, 3369, 1731, 1659, 1601, 1549, 1183, 751 and 704 cm^{-1}; ^1H NMR (500 MHz; DMSO-d_6) data: δ 2.50 (t, $J = 4.7$ Hz, 2H, $C_{\beta}H_2$, β–Ala), 3.29 (qa, $J = 4.7$ Hz, 2H, $C_{\alpha}H_2$, β-Ala), 6.21 (br, t, $J = 4.7$ Hz, 1H, HNC_{α}, β-Ala), 5.66 (br, s 2H, $CONH_2$), 5.09 (s, 2H, CH_2, Bzl), 7.40–7.27 (m, 5H, Ph, Bzl). ^{13}C NMR (125 MHz; DMSO-d_6): δ 172.6, 159.9, 137.0, 129.3, 128.8, 128.7, 66.4, 36.2, 35.7.

Synthesis of Fmoc-dipeptide benzyl esters containing carbamic acid residue. A solution of 5 mmol Fmoc-amino acid chloride [Fmoc-Phe-Cl (2.03 g); Fmoc-β-Ala-Cl (1.65 g); Fmoc-Phe-Cl (2.03 g); Fmoc-Gly-Cl (1.57 g)] and 5 mmol of the respective carbamoyl amino acid benzyl ester [NH_2-CO-β-Ala-OBzl (1.11 g); NH_2-CO-Phe-OBzl (1.49 g); NH_2-CO-Gly-OBzl (1.04 g); NH_2-CO-Phe-OBzl (1.49 g)] in absolute benzene was refluxed under nitrogen. The reaction was monitored by TLC (system A). After the reaction was completed, the solvent was

Table 1 Characteristics of novel intermediate products

Compound	Crystallization	Yield (%)	R_f[a]	R_t[b] (min)	C, H, N (%)[c] calc./meas.	Mol. Mass.[d] calc./meas.	m.p.[e] (°C)	$[\alpha]_{576}^{25}$[f]
NH_2-CO-βAla-OBzl	H_2O	58	0.46[D] 0.69[A]	10.9	59.4/58.9; 6.4/6.6; 12.6/12.3	222.1/222.0	117–118	—
Fmoc-Phe-NH-CO-βAla-OBzl	Ethyl acetate	63	0.86[D] 0.88[A]	n.d.	n.d.	591.2/591.2	142–144	n.d.
Fmoc-βAla-NH-CO-Phe-OBzl	Ethanol/ *n*-hexane	59	0.78[D] 0.84[A]	n.d.	n.d.	591.2/591.2	115–117	n.d.
Fmoc-Phe-NH-CO-Gly-OBzl	Methanol	51	0.51[E] 0.85[G]	n.d.	n.d.	577.2/577.2	149–151	n.d.
Fmoc-Gly-NH-CO-Phe-OBzl	Ethyl acetate/ *n*-hexane	67	0.48[F] 0.81[G]	n.d.	n.d.	577.2/577.2	141–143	n.d.
Fmoc-Phe-NH-CO-βAla-OH	Ethyl acetate/ *n*-hexane	69	0.70[B] 0.60[C]	19.3	n.d.	501.2/501.2	118–120	−4.7
Fmoc-βAla-NH-CO-Phe-OH	Ethyl acetate/ *n*-hexane	56	0.67[B] 0.58[C]	19.1	n.d.	501.2/501.2	86–89	−2.5
Fmoc-Phe-NH-CO-Gly-OH	Ethyl acetate/ *n*-hexane	72	0.60[B] 0.56[C]	19.0	n.d.	487.1/487.1	165–167	+119.7
Fmoc-Gly-NH-CO-Phe-OH	Ethyl acetate/ *n*-hexane	87	0.63[B] 0.60[C]	18.6	n.d.	487.1/487.1	88–90	−3.5

n.d. – no data.
[a] TLC eluent systems: pyridine/acetic acid/H_2O/ethyl acetate (20/6/11/480, v/v) (system A); pyridine/acetic acid/H_2O/ethyl acetate (20/6/11/120, v/v) (system B); *n*-dichloromethane/methanol/acetic acid (90/8/2, v/v) (system C); ethyl acetate (system D); ethyl acetate/*n*-hexane (1/1, v/v) (system E); ethyl acetate/*n*-hexane (2/1, v/v) (system F); dichloromethane/methanol/acetic acid (40/20/1, v/v) (system G).
[b] HPLC conditions: Shimadzu HPLC apparatus, Vydac 218TP C18 RP-HPLC column (250 × 4.6 mm, 5 μm, 300 Å), eluent A: 0.045% TFA in H_2O and eluent B: 0.036% TFA in MeCN; linear gradient elution: 5–95% in 30 min, $\upsilon = 1$ ml/min, $\lambda = 220$ nm.
[c] C, H, N elemental analysis by Vario EL III, Elementar Analysensysteme.
[d] Bruker Esquire 3000plus ion trap mass spectrometer equipped with electrospray ionization source; positive ionization mode in the range of 15–1800 m/z; the source housing was kept at room temperature; samples were dissolved in acetonitrile : water = 1 : 1, 0.1% acetic acid (v/v) solvent mixture; samples were injected into the mass spectrometer using a syringe pump, at a flow rate of 10 μl/min.
[e] Melting points were measured by a Buchi 530 apparatus.
[f] Carl Zeiss Polamat A polarimeter, 25 °C, $c = 0.4$ g/100 ml in DMF.

evaporated in vacuum. The residue was dissolved in ethyl acetate and was extracted subsequently with water, saturated $NaHCO_3$ solution and 10% NaCl solution. The organic phase was separated, dried over $MgSO_4$, filtered and the solvent was evaporated. After crystallization the pure product was analyzed by TLC and MS and melting points were determined (Table 1).

Preparation of Fmoc-dipeptides containing carbamic acid residue. Fmoc-dipeptide benzyl esters containing carbamic acid residue were transformed into *C*-terminal free derivatives, which are appropriate for coupling to peptide resin. For cleavage of *C*-terminal benzyl protecting group two methods were tested: HF treatment (A) and hydrogenation (B).

Method A: 2.5 mmol Fmoc-dipeptide benzyl ester containing carbamic acid residue [Fmoc-Phe-NH-CO-β-Ala-OBzl (1.48 g); Fmoc-β-Ala-NH-CO-Phe-OBzl (1.48 g)] was reacted with anhydrous HF (15 ml) in the presence of anisole (1.5 ml) at −5 °C for 1 h. HF was removed under reduced pressure and diethyl ether was added to the remaining material. The precipitated product was filtered off and washed with diethyl ether and recrystallized.

Method B: 10% Pd-on-charcoal catalyst (144 mg) was added to 2.5 mmol Fmoc-dipeptide benzyl ester containing the carbamic acid residue [Fmoc-Phe-NH-CO-Gly-OBzl (1.44 g); Fmoc-Gly-NH-CO-Phe-OBzl (1.44 g)] dissolved in DMF. The mixture was hydrogenated for 1 h. The reaction was monitored by TLC (system E). After the reaction was complete, the catalyst was removed by filtration and the solvent by evaporation in vacuum and the products were crystallized.

The purity and yield of the products obtained by hydrogenation were slightly higher (Table 1).

The crystalline products were checked for purity by TLC and RP-HPLC, and melting points and optical rotations were determined (Table 1). The structures of new derivatives, prior to coupling to peptide-resin by the solid phase peptide synthesis (SPPS) method, were improved by MS (Table 1), IR and NMR.

IR main peaks for *Fmoc–Phe–NH–CO–Gly–OH*: 3414 (sh), 3314, 1708 (br, unresolved), 1682, 1533, 1261, 1218, 758, 742 and 702 cm^{-1}; for *Fmoc–Phe–NH–CO–β–Ala–OH*: 3414 (sh), 3311, 1708 (br, unresolved), 1683 (sh), 1537, 1250, 1219, 759, 741 and 700 cm^{-1}; *Fmoc–Gly–NH–CO–Phe–OH*: 3411, 3307, 1704 (br, unresolved), 1537, 1248, 1214, 1182, 760, 741 and 701 cm^{-1}; *Fmoc–β–Ala–NH–CO–Phe–OH*: 3413, 3300, 1700 (br, unresolved), 1540, 1248, 1217, 1183, 760, 742 and 702 cm^{-1}; ^1H NMR (500 MHz; DMSO-d_6) data:

Fmoc–Phe–NH–CO–Gly–OH: δ 3.01 and 2.80 (two d,d, J = 13.5 Hz, J = 3.4 Hz, and J = 13.5 Hz, J = 11.3 Hz, 2H, $C_\beta HH'$, Phe), 3.91 (d, J = 6.0 Hz, 2H, $C_\alpha H_2$, Gly), 4.10–4.22 (m, 3H, H-9 and CH_2, Fmoc), 4.38 (m, 1H, $C_\alpha H$, Phe), 7.20–7.39 (m, 9H, H-2,3,6,7, Fmoc and Ph,Phe), 7.60 and 7.62 (two d, J = 7.7 Hz, 2H, H-1,8, Fmoc), 7.85 (d, J = 7.6 Hz, 2H, H-4,5, Fmoc), 7.80 (d, J = 8.3 Hz, 1H, HNC_α, Phe), 8.55 (br ~s, 1H, HNC_α, Gly), 10.73 (s, 1H, CO–NH–CO), ~12.7 (very br, 1H, CO_2H). ^{13}C NMR (125 MHz; DMSO-d_6): δ 175.0, 172.0, 156.9, 154.8, 144.6, 141.5, 138.4, 129.5, 129.1, 128.9, 128.5, 127.9, 126.3, 121.0, 66.6, 57.6, 47.4, 42.2, 37.5; *Fmoc–Phe–NH–CO–β–Ala–OH*: δ2.46 (t, J = 4.9 Hz, 2H, $C_\beta H_2$, β-Ala), 2.99 and 2.77 (two d,d, J = 13.7 Hz, J = 3.4 Hz, and J = 13.7 Hz, J = 11.5 Hz, 2H, C_β HH′, Phe), 3.37 (qa, J = 4.7 Hz, 2H, $C_\alpha H_2$, β-Ala), 4.13–4.19 (m, 3H, H-9 and CH_2, Fmoc), 4.32 (m, 1H, C_α H, Phe), 7.19–7.40 (m, 9H, H-2,3,6,7, Fmoc and Ph, Phe), 7.59 and 7.62 (two d, J = 7.7 Hz, 2H, H-1,8, Fmoc), 7.85 (d, J = 7.6 Hz, 2H, H-4,5, Fmoc), 7.77 (d, J = 8.3 Hz, 1H, HNC_α, Phe), 8.43 (br ~s, 1H, HNC_α, β-Ala), 10.60 (s, 1H, CO-NH-CO), ~12.3 (very br, 1H, CO_2H). ^{13}C NMR (125 MHz; DMSO-d_6): δ 175.0, 174.0, 157.1, 154.0, 144.7, 141.6, 138.5, 129.4, 129.1, 128.8, 128.5, 128.0, 126.2, 121.0, 66.6, 57.5, 47.4, 37.5, 35.9, 34.8; *Fmoc–Gly–NH–CO–Phe–OH*: δ 3.11 and 3.00 (two d,d, J = 13.7 Hz, J = 5.1 Hz, and J = 13.7 Hz, J = 7.3 Hz, 2H, C_β HH′, Phe), 3.74 (d, J = 6.1 Hz, 2H, $C_\alpha H_2$, Gly), 4.22 (t, J = 7.0 Hz, 1H, H-9, Fmoc), 4.30 (d, J = 7.0 Hz, 2H, CH_2, Fmoc), 4.52 (m, 1H, C_α H, Phe), 7.15 (d, J = 7.6 Hz, 2H, H-2,6, Ph, Phe), 7.19 (t, J = 7.6 Hz, 1H, H-4, Ph, Phe), 7.26 (t, J = 7.6 Hz, 2H, H-3,5, Ph, Phe), 7.32 (t, J = 7.7 Hz, 2H, H-2,7, Fmoc), 7.41 (t, J = 7.7 Hz, 2H, H-3,6, Fmoc), 7.70 (d, J = 7.7 Hz, 2H, H-1,8 Fmoc), 7.88 (d, J = 7.7 Hz, 2H, H-4,5, Fmoc), 7.58 (t, J = 6.1 Hz, 1H, HNC_α, Gly), 8.45 (br ~s, 1H, HNC_α, Phe), 10.41 (s, 1H, CO-NH-CO), ~13.0 (very br, 1H, CO_2H). ^{13}C NMR (125 MHz; DMSO-d_6): δ 173.2, 172.6, 157.4, 153.4, 144.7, 141.7, 137.6, 130.0, 129.2, 128.5, 128.0, 127.6, 126.1, 121.0, 66.7, 54.5, 47.5, 44.5, 37.8; *Fmoc–β–Ala–NH–CO–Phe–OH*: δ 2.47 (t, J = 5.9 Hz, 2H, $C_\beta H_2$, β-Ala), 3.10 and 2.99 (two d,d, J = 13.7 Hz, J = 5.1 Hz, and J = 13.7 Hz, J = 6.9 Hz, 2H, C_β HH′, Phe), 3.20 (qa, J = 5.9 Hz, 2H, $C_\alpha H_2$, β-Ala), 4.19 (t, J = 7.0 Hz, 1H, H-9, Fmoc), 4.26 (d, J = 7.0 Hz, 2H, CH_2, Fmoc), 4.52 (m, 1H, C_α H, Phe), 7.16 (d, J = 7.6 Hz, 2H, H-2,6, Ph, Phe), 7.20 (t, J = 7.6 Hz, 1H, H-4, Ph, Phe), 7.26 (t, J = 7.6 Hz, 2H, H-3,5, Ph, Phe), 7.32 (t, J = 7.7 Hz, 2H, H-2,7, Fmoc), 7.40 (t, J = 7.7 Hz, 2H, H-3,6, Fmoc), 7.67 (d, J = 7.7 Hz, 2H, H-1,8, Fmoc), 7.87 (d, J = 7.7 Hz, 2H, H-4,5, Fmoc), 7.35 (t, J = 6.1 Hz, 1H, HNC_α, β-Ala), 8.64 (br ~s, 1H, HNC_α, Phe), 10.39 (s, 1H, CO-NH-CO), ~13.0 (very br, 1H, CO_2H). ^{13}C NMR (125 MHz; DMSO-d_6): δ 174.1, 173.2, 156.9, 153.6, 144.8, 141.6, 137.6, 130.0, 129.2, 128.5, 128.0, 127.6, 126.1, 121.0, 66.3, 54.4, 47.6, 37.9, 36.9, 36.8.

Peptide synthesis. Peptide synthesis was carried out by Boc/Bzl SPPS strategy [23], using MBHA resin [24]. The polypeptide chain was assembled by coupling with a mixture of 2 equiv. of adequately side-chain protected Boc-amino acid derivative and 1.9 equiv. HBTU coupling reagent in the presence of 2.1 equiv. DIEA in DMF [25]. The following side-chain protecting groups were used: Tos for Arg, Z(2Cl) for Lys and Bzl for Ser and Thr. Coupling reactions was monitored by the ninhydrin test [26]. Two equiv. of Fmoc-dipeptides containing the carbamic acid residue was coupled with two equiv. of DIPCI and HOBt coupling reagents [27] in two steps: first for 1.5 h and the second overnight. The day after, the ninhydrin test was negative. Boc deprotection was performed by treatment with 30% TFA in DCM for 5 + 25 min, and 10% DIEA in DCM was used for neutralization after cleavage. For removal of *N*-terminal Fmoc protecting group of the incorporated building block, 30% piperidine in DMF was applied for 3 + 17 min. Cleavage from the resin and side-chain deprotection were carried out simultaneously with liquid HF (10 ml/1 g peptide-resin) in the presence of anisole (1 ml/1 g peptide-resin), at −5 °C for 1.5 h [28,29]. After evaporation of HF, diethyl ether was added to the residue. The resin and the precipitated product were filtered off and washed with diethyl ether twice. The peptide was dissolved in water and the resin was filtered off. Aqueous solution of the peptide was extracted three times with diethyl ether and then the inorganic phase was separated and evaporated. The residue was dissolved in water and freeze-dried.

Crude products were purified by preparative reversed-phase fast protein liquid chromatography (FPLC) and purified peptides were freeze-dried. Purification was performed on a Pharmacia FPLC apparatus (two P-500 pump, LCC-500 Plus system controller, FRAC-100 fraction collector, REC-2 recorder (Pharmacia, Uppsala, Sweden); Büchi UV-vis filter-photometer (BÜCHI Labortechnik, Flawil, Switzerland)), using Vydac 218TP 480 × 25 mm C18 RP-HPLC column, particle size 20–30 μm, pore size 300 Å (Vydac, Hesperia, CA, USA), with eluent A: 0.045% TFA in H_2O and eluent B: 0.036% TFA in MeCN. Linear gradient elution: 5–40% B was developed with 14 ml/min flow rate, during 60 min. Detection of peaks was done at λ = 220 nm.

Purified compounds were characterized by analytical HPLC and electrospray ionization MS (Table 2).

Analytical Methods

TLC analyses were performed on precoated plates, silica gel 60 F_{254} from Merck (Darmstadt, Germany). For the development of the chromatograms the following eluent systems were used: pyridine/acetic acid/H_2O/ethyl acetate (20/6/11/480, v/v) (system A); pyridine/acetic acid/H_2O/ethyl acetate (20/6/11/120, v/v) (system B); dichloromethane/methanol/acetic acid (90/8/2, v/v/v) (system C); ethyl acetate (system D); ethyl acetate/*n*-hexane (1/1, v/v) (system E); ethyl acetate/*n*-hexane (2/1, v/v) (system F); dichloromethane/methanol/acetic acid (40/20/1, v/v/v) (system G). Spots were detected by UV light and/or with chlorine/toluidine.

Purity of intermediates and peptides were analyzed by RP-HPLC, carried out on Vydac 218TP C18 column, 250 × 4.6 mm, particle size 5 μm, pore size 300 Å (Vydac, Hesperia, CA, USA) using a Shimadzu HPLC apparatus (two LC-6A pump, SPD-6AV UV-detector, SIL-6B auto injector, SCL-6B system controller (Shimadzu Corporation, Kyoto, Japan)); eluent A: 0.045% TFA in H_2O and eluent B: 0.036% TFA in MeCN. Linear gradient was developed with 1 ml/min flow rate, during 30 min. Peaks were detected at λ = 220 nm.

Identification of the products was achieved by mass spectrometry. Mass spectrometric experiments were performed either on a Bruker Esquire 3000plus (Bremen, Germany) ion trap mass spectrometer, or on a Perkin-Elmer SCIEX API-2000 triple-quadrupole instrument (Perkin-Elmer, Wellesley, MA, USA), both equipped with electrospray ionization source.

Table 2 Structure and characteristics of synthetic peptides

Code	Sequence	R_t[a] (min)	Mol. Mass.[b] calc./meas.	Half-life, $t_{1/2}$ (h)[c]	
				AP-M	Rat brain membrane
1	H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	13.3[aa]	1381.6/1381.5	0.7	2.7
2	H-Phe-NH-CO-βAla-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	20.9[ab]	1381.6/1381.2	3.1	2.8
3	H-Phe-βAla-NH-CO-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	21.7[ab]	1381.6/1381.2	3.1	n.d.
4	H-βHph-Gly-NH-CO-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	15.8[aa]	1381.6/1380.8	3.0	n.d.
5	H-Phe-NH-CO-Gly-βHph-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	14.6[aa]	1381.6/1381.2	3.5	2.6
6	H-Phe-βAla-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	12.6[aa]	1338.6/1338.3	0.7	2.1

[a] RP-HPLC: Vydac 218TP C18 RP-HPLC column (250 × 4.6 mm, 5 μm, 300 Å), Shimadzu HPLC apparatus, eluent A: 0.045% TFA in H_2O, eluent B: 0.036% TFA in MeCN:
[aa] Gradient elution: 5–40% in 30 min, $v = 1$ ml/min, $\lambda = 220$ nm.
[ab] Gradient elution: 5–25% in 30 min, $v = 1$ ml/min, $\lambda = 220$ nm.
[b] Mass spectrometric experiments were performed on a Perkin-Elmer SCIEX API-2000 triple-quadrupole instrument equipped with electrospray ionization source; spectra were acquired in positive ionization mode, in the range of 15–1800 m/z; the source housing was kept at room temperature; samples were dissolved in acetonitrile : water = 1 : 1, 0.1% acetic acid (v/v) solvent mixture; samples were injected into the mass spectrometer using a syringe pump, in a flow rate of 10 μl/min.
[c] Half-lives of synthesized peptides in presence of aminopeptidase-M enzyme or in rat brain membrane homogenate.

Spectra were acquired in positive ionization mode, in the 15–1800 m/z range. Samples were dissolved in acetonitrile: water = 1 : 1, 0.1% acetic acid (v/v) solvent mixture. Samples were injected into the mass spectrometer using a syringe pump, in a flow rate of 10 μl/min.

Carbamoyl amino acid derivatives and Boc-β-Hph-OH were also characterized by C, H, N elemental analysis (Vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany).

NMR data were obtained on a BRUKER DRX 500 spectrometer (Bremen, Germany) at 300 K. Assignments are based on 2D-COSY-, 2D-HMQC- and 2D-HMBC measurements.

Optical rotations, $[\alpha]_{578}$, were determined using a Polamat A polarimeter (Carl Zeiss AG, Jena, Germany). Experiments were performed at room temperature, with the compounds dissolved in DMF (0.4 g/100 ml).

Melting points were measured by a Buchi 530 apparatus (Buchi Labortechnik AG, Flavil, Switzerland).

IR spectra were recorded on a Bruker Equinox 55 FT-IR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) at a resolution of 2 cm^{-1} in KBr pellets.

Enzymatic Degradation

Digestion with aminopeptidase M. Enzymatic stability of synthetic peptides was investigated in the presence of aminopeptidase M (AP-M; EC 3.4.11.2. Merck, from pancreas, lyophilized) according to Pfleiderer [30–34]. Peptides were dissolved in 0.1 M Tris HCl buffer (pH 7.4) to give a final concentration 1 mg/ml ($\sim 10^{-3}$ M). One hundred micrograms of AP-M enzyme was added to the peptide solutions to achieve 100 μg/ml final enzyme concentration ($\sim 10^{-6}$ M). Solutions were incubated at 37 °C (M91 Micro incubator, F.LLI GALLI, Milan, Italy) for up to 24 h. Fifty microliter aliquots from the solution were taken after 0, 15, 30, 45, 60, 180, 360 min and 24 h. The samples were stored at −20 °C. The amount of the intact peptide was determined by RP-HPLC as a function of time. The curves obtained by the data were hyperbolic. The fitting was made by Microcal Origin 5.0 program. Half-life values ($t_{1/2}$) were determined from the curves at 50% of the Y-axis. Two independent sets of experiments were performed in every case. As control experiments, we investigated the stability of peptides as well as AP-M (at the same concentration) alone in 0.1 M Tris HCl buffer (pH 7.4) at 37 °C for 24 h.

RP-HPLC measurements were carried out on an Eurosphere-100 equipment with a C-18, 250 × 4 mm column, particle size 5 μm, pore size 100 Å (Knauer, Berlin, Germany) using an Aquasil C18 precolumn (Keystone Scientific., Bellefonte, PA, USA) on a Knauer laboratory assembled system (HPLC Pump Type 364, Variable Wavelength monitor A0293, A0258 injector, Dynamic Mixing Chamber A0285 (Knauer, Berlin, Germany)). Gradient elution was applied using eluent A: 0.1% TFA in H_2O and eluent B: 0.1% TFA in 80% MeCN–20% H_2O; gradient developed: 5–30% B, 25 min, 30–60% 15 min, at flow rate: 1 ml/min; detection was performed at $\lambda = 214$ nm. Twenty microliters of the sample was injected in every case.

Digestion with rat brain membrane homogenate. Stability of synthetic peptides was also investigated in the rat brain membrane homogenate. Crude membrane fractions of the brain cells (obtained from the Biological Research Center, Szeged, Hungary) were prepared from Wistar rats according to the method of Pasternak with a minor modification described by Simon *et al.* [35]. Briefly, the animals were decapitated, and the brains without the cerebella rapidly removed and washed with ice-cold 0.05 M Tris HCl buffer (pH 7.4) at 4 °C. The brains were homogenized in 20 volumes (v/w) of the same buffer with a Braun Teflon glass homogenizer and the homogenate was filtered through four layers of gauze and centrifuged with a Sorvall centrifuge (40 000*g*, 4 °C, 20 min). The pellet was resuspended in the above-mentioned buffer in 20 volumes (v/w) and incubated at 37 °C for 30 min to remove any endogenous opioids that might be present. Centrifugation

was repeated and the final pellet was resuspended in five volumes (v/w) of 0.05 M Tris.HCl buffer (pH 7.4) at 4 °C containing 0.32 M sucrose to give a final protein concentration of 3–4 mg/ml. Aliquots of 5 ml were stored at −70 °C until use, then diluted, centrifuged (40 000g, 4 °C, 20 min) and suspended in an appropriate buffer. Protein content of the homogenate was determined by the Bradford method [36].

Peptides were dissolved in 0.1 M Tris HCl buffer (pH 7.4) to give a final concentration 1 mg/ml ($\sim 10^{-3}$ M). One hundred microliters of rat brain homogenate was added to the peptide solutions (1 : 10 v/v dilution, 10 v/v% of rat brain homogenate). The mixture was carefully homogenized (syringe, needle 27G) and incubated at 37 °C. Experiments were performed under sterile conditions. One hundred microliter samples from the reaction mixture were taken after 0, 60, 120, 180, 360 min and 24 h. The enzymatic reaction was stopped and the proteins of the samples were precipitated by addition of perchloric acid (5% v/v). The supernatant was analyzed by RP-HPLC measurements and curve fitting was done as described above. Samples were centrifuged (SeisystemBio, Servintern, Budapest, Hungary) at 2000 rpm for 5 min before RP-HPLC measurements. As control experiments we determined the stability of peptides and rat brain membrane homogenate alone at the same concentration (1 mg/ml and 10 v/v%, respectively) in 0.1 M Tris HCl buffer (pH 7.4) at 37 °C for 24 h.

Pharmacological Assays

Competition binding studies. Receptor-binding experiments were carried out on crude membrane fractions prepared from Wistar rat brains in 50 mM Tris HCl buffer (pH 7.4) in a final volume of 1 ml, containing 0.2–0.4 mg/ml membrane protein, as described previously [37]. Rat brain membrane preparation was incubated with [^{3}H]nociceptin(1–17)-NH_2 in the presence of unlabeled compounds (10^{-5} to 10^{-11} M). For this, up to 0.045 nM of radioligand was prepared in 1 mg/ml protease-free bovine serum albumin solution (Protease-free BSA, Fraction V, Sigma). The reaction was terminated by rapid filtration under vacuum, and washed three times with 5 ml ice-cold 50 mM Tris HCl (pH 7.4) buffer through Whatman GF/C glass fibers by using a Brandel M24R Cell Harvester. The filter fiber was presoaked for 30 min in 0.3% polyethyleneimine solution (pH 10). After filtration, the filter fiber was dried and the bound radioactivity was measured in UltimaGold scintillation cocktail using Packard Tricarb 2300TR Liquid Scintillation Analyzer (Packard Instruments, Meriden, CT, USA). Protein concentration was determined by the Bradford method using bovine serum albumin as standard [36]. Receptor-binding experiments were performed in duplicates and repeated at least three times. Data were analyzed by GraphPad Prism (version 3.0, San Diego, CA, USA). Displacement curves were fitted by nonlinear regression using the one-site competition fitting option. IC_{50} values were used for the calculation of the K_i values (the equilibrium inhibition constant) according to the Cheng–Prusoff equation [38].

Mouse vas deferens (MVD) assay. *Vasa deferentia* taken from NMRI mice weighing 35–40 g were prepared, mounted and field-stimulated as described previously [16,39]. Nociceptin derivatives were first tested at 10^{-5} M concentration and if a remarkable inhibition was observed, the compounds were further studied in a noncumulative manner at 4–6 concentration levels. Agonist potencies were characterized by the 50% inhibitory concentrations (IC_{50}) calculated from the logarithmic regressions of individual concentration–response curves. To the pooled data points, nonlinear curve fitting was used according to the Hill equation (Sigmaplot 8.0). A synthetic hexapeptide NOP receptor antagonist, Ac-RYYRIK-NH_2 [40], was used to inhibit effects exerted by an agonist peptide, and a 30-min preequilibration period was applied for the antagonist. Four parallel experiments were performed with each peptide studied.

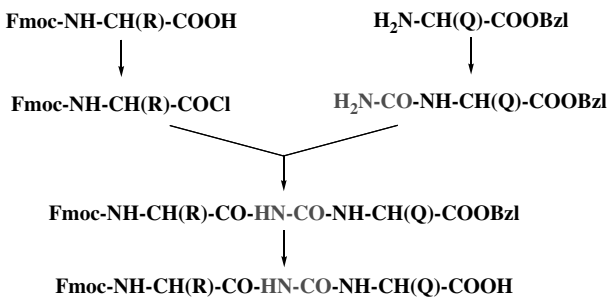
RESULTS

Synthesis

A new procedure was developed for the synthesis of peptides containing carbamic acid residue in their sequence for increasing enzymatic stability. The synthetic strategy is outlined in Scheme 1. First we prepared the dipeptide building block containing the carbamic acid residue, Fmoc-NH-CH(R)-CO-NH-CO-NH-CH(Q)-COOH, by solution phase method. Afterwards, it was incorporated into the target peptide by SPPS. The *C*-terminally protected building block was prepared by the reaction of appropriate Fmoc amino acid chloride with temporary *C*-terminally protected carbamoyl amino acid benzyl ester. After the removal of the benzyl ester protecting group, the free acid derivative was used for SPPS. The new carbamic acid peptides (**2–5**) produced are listed in Table 2. We also synthesized Noc(1–13)-NH_2 (**1**) and [Phe-β-Ala-Phe]-Noc-(5–13)-NH_2 (**6**) peptides as control compounds for enzymatic degradation experiments (Table 2). The latter was prepared to confirm that the increase in stability could be primarily due to the incorporation of carbamic acid residue, but not to the presence of the beta amino acid. Analytical data of peptides are also listed in Table 2.

Enzymatic Stability

The enzymatic stability of the carbamic acid–nociceptin derivatives was compared to that of peptides Noc(1–13)-NH_2 and [Phe-β-Ala-Phe]-Noc-(5–13)-NH_2 in the presence of AP-M as well as in rat brain membrane



Scheme 1 Synthesis strategy of the dipeptide fragment with carbamic acid residue.

homogenate. In these experiments the amount of intact peptide was measured as a function of time by RP-HPLC and the half-life ($t_{1/2}$) of the peptide was calculated (Table 2). According to control experiments no loss of the enzyme activity was observed during the experiment (data not shown).

AP-M, a zinc-containing metalloprotease (M.W. 280 kDa) from swine kidney microsomes [30–34, 41–43], removes amino acids sequentially from the *N*-terminal part of peptides and proteins having a free α-amino or α-imino group. However, in peptides containing an X-Pro sequence, where X is a bulky hydrophobic residue (Leu, Tyr, Trp, Met sulfone), cleavage does not occur.

Analysis of stability of peptides and proteins in rat brain membrane homogenate is frequently used to evaluate the catabolism of neuropeptides in the central nervous system. The brain membrane homogenate, however, contains different peptidases to which nociceptin may not be exposed during its action. In addition, the distribution of proteolytic enzymes in the brain is not uniform. Since the NOP receptor is a membrane-bound receptor, its ligands compete for binding in the extracellular space and could be exposed to membrane-bound proteases [44].

In the AP-M study, we observed significant differences in the degradation profiles and also in the half-lives between the carbamic acid peptides and control peptides (Table 2, Figure 1). We found that the simultaneous incorporation of the carbamic acid residue and β-amino acid residue into peptides significantly increases the enzymatic stability. The pattern of degradation curves for the control peptides (**1**) and (**6**) are very similar. In contrast, compound (**2–5**) exhibited different profiles of decomposition from the control peptides, while the patterns of their degradation curves were also similar. The half-lives of the modified nociceptin derivatives (**2–5**) were significantly higher than those of the control peptides (**1**) and (**6**). The $t_{1/2}$ value of the all α-amino acid containing peptide (**1**) was 0.7 h. Interestingly, essentially no increase was detected after the replacement of Gly-Gly by β-alanine ($t_{1/2} = 0.7$ h) for peptide (**6**). However, the presence of the carbamic acid residue, regardless of its position, resulted in a four- to fivefold increase in $t_{1/2}$ values (e.g. $t_{1/2} = 3.5$ h for peptide (**5**)). The pronounced increase in stability of peptides (**2–5**) was reflected in their kinetics profile as compared to control peptides. It is important to emphasize that no elevated stability was observed in case of peptide (**6**), which contains only β-alanine but no carbamic acid residue. This shows that the incorporation of β-alanine alone does not influence the enzymatic stability significantly. We can conclude that the effect observed is primarily due to the incorporation of carbamic acid residue and not to the presence of the β-amino acid. Considering that the $t_{1/2}$ values of modified peptides (**2–5**) are similar, the increased stability appears to be independent of the position of the carbamic acid residue and the β-amino acid. These data suggest that the incorporation of the carbamic acid residue into the *N*-terminal part of peptides is capable of increasing the enzymatic stability against AP-M.

On the basis of these data, two carbamic acid peptides (**2**) and (**5**) and control peptides (**1**) and (**6**) were selected for stability studies with rat brain membrane homogenate. In these experiments no increase in enzymatic stability of the carbamic acid–nociceptin derivatives was observed as compared to the control peptides. This finding could be due to the simultaneous presence of endo- and carboxypeptidase activities in the brain membrane homogenate (Table 2, Figure 2), which catalyzed the hydrolysis of the test- and the control peptides similarly.

Pharmacological Assays

The influence of the modifications on the receptor–ligand interaction as well as on the biological activity of the ligands was studied by using pharmacological assays, such as displacement binding experiments and functional tissue assay.

Binding to rat brain membranes. In order to characterize the binding affinity of the peptides, competition binding assays were performed on crude rat brain membranes. In this experiment the binding of a traceable radioligand is displaced with the binding of an

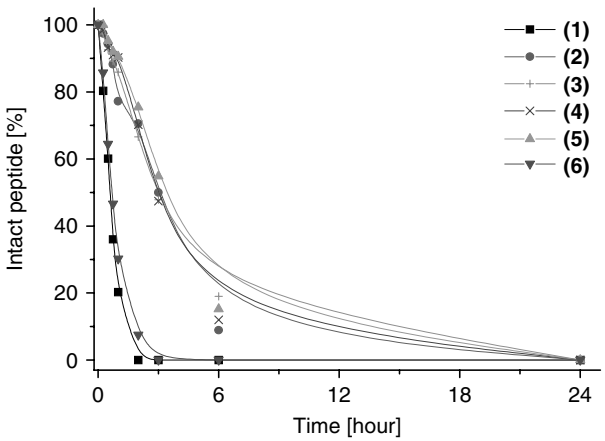


Figure 1 Degradation of peptides in presence of aminopeptidase M. Peptides were dissolved in 0.1 M Tris HCl buffer (pH 7.5) to give a final concentration of 1 mg/ml, and 100 µg AP-M enzyme was added. Solutions were incubated at 37 °C. Fifty-microliter aliquots from the solution were taken after 0, 15, 30, 45, 60, 180, 360 min and 24 h. As control experiments, stability of peptides as well as AP-M was studied at the same concentration alone in 0.1 M Tris HCl buffer (pH 7.4) at 37 °C for 24 h. The amount of the intact peptide was analyzed by RP-HPLC as a function of time. The curves obtained by the data are hyperbolic. The fitting was made by Microcal Origin 5.0 program. Half-life values ($t_{1/2}$) were determined from the curves at 50% of the *Y*-axis.

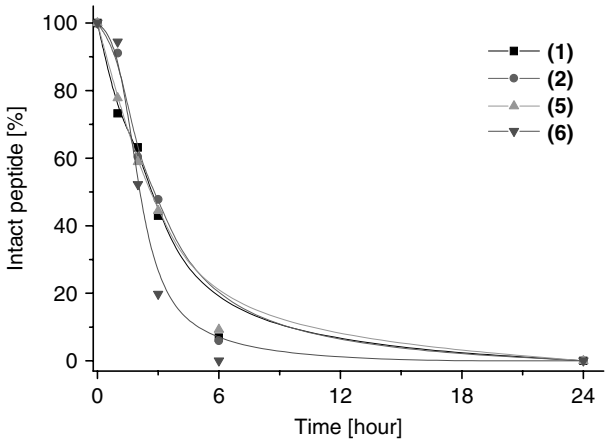


Figure 2 Degradation of peptides in presence of rat brain membrane homogenate. Peptides were dissolved in 0.1 M Tris.HCl buffer (pH 7.5) to give a final concentration of 1 mg/ml, and 100 µl of the rat brain homogenate was added (1/10 dilution) in every case. Mixtures were incubated at 37 °C. Samples of 100 µl were taken after 0, 60, 120, 180, 360 min and 24 h. Peptide solution in the same concentration, but without added rat brain homogenate was used as control. The enzymatic reaction was stopped and the proteins of the samples were precipitated by addition of perchloric acid (5% v/v) and centrifuged at 2000 rpm for 5 min before RP-HPLC measurements. The concentration of the intact peptide in the supernatant was analyzed by RP-HPLC as a function of time. The curves obtained by the data are hyperbolic. The fitting was made by Microcal Origin 5.0 program. Half-life values ($t_{1/2}$) were determined from the curves at 50% of the *Y*-axis.

unlabeled ligand in a competitive manner. The concentration of the displacing ligand required to decrease the signal to 50% of the original value (IC_{50}) was used to characterize the binding. Displacement curves were fitted by nonlinear regression using the one-site competition fitting option. From this assay, an estimate of the equilibrium dissociation constant (K_i) of the ligand–receptor complex can also be obtained [38]. The K_i values were calculated according to the Cheng–Prusoff equation [38]. We found that two peptides (compound (**2**) and (**3**)) had no measurable binding in this experimental system (pK_i values were less than 5), while peptides (**4**) and (**5**) exhibited only low binding affinities ($pK_i = 5.71 \pm 0.11$ and $pK_i = 5.75 \pm 0.05$, respectively). In contrast, the pK_i value of Noc(1–13)-NH_2 was considerably higher: $pK_i = 9.40$.

Effect on mouse vas deferens (MVD). In case of the MVD test, the NOP receptor agonist dose dependently inhibits the electrically stimulated twitches on the isolated MVD tissues and yields a concentration–response curve [45]. Agonist potencies can be characterized by the IC_{50} values calculated from the logarithmic regressions of the individual concentration–response curve. This value refers to the dose of the agonist that produces 50% of the maximal effect. Simultaneous presence of an antagonist can cause a shift of the dose–response curve of an agonist [46].

Nociceptin derivatives were first tested at 10^{-5} M concentration. Under the experimental condition used in this study, peptide (**5**) showed significant inhibition, while the other three derivatives (**2–4**) exhibited no effect. Peptide (**5**) displayed a weak agonistic character, which could be antagonized by the NOP receptor antagonist Ac-RYYRIK-NH_2 (data not shown). While nociceptin showed 50% inhibitory effect in a concentration of 10^{-9} M, peptide (**5**) displayed only $32.8 \pm 4.1\%$ inhibitory effect at 10^{-5} M concentration. Data obtained from the MVD assay show that only peptide (**5**) possesses activity, but this compound is an approximately 250 times less potent NOP receptor agonist than nociceptin in the field-stimulated MVD.

DISCUSSION

Nociceptin, a 17-amino acid peptide (FGGFTGARK-SARKLANQ, N/OFQ) is a major product from pronociceptin and the natural endogenous ligand of NOP receptor [1–6]. Nociceptin is degraded primarily by aminopeptidases in plasma to yield Noc-(2–17)-OH and other smaller fragments [10,11]. *N*-terminal truncation leads to complete loss of activity. Experiments with *C*-terminally truncated analogs of N/OFQ suggest that Noc(1–13)-OH is the minimum sequence capable for interaction with the NOP receptor, and its amidated analogue possesses the same biological properties as native nociceptin [12].

To increase the stability against biological degradation we synthesized Noc(1–13)-NH_2 analogues containing a carbamic acid residue at the *N*-terminal portion of the peptide. We developed a new procedure for the synthesis of carbamic acid peptides and produced four derivatives. New intermediates were also described. This procedure combines the solution phase and the solid phase approaches. We have demonstrated that carbamic acid residue can be incorporated into the inner part of peptides, building on solid phase, by using a dipeptide fragment with carbamic acid residue. This dipeptide unit with carbamic acid residue, suitable for coupling to the peptide resin, could be produced by a simple and efficient three-step solution phase procedure. The *N*-terminally protected Fmoc dipeptide containing carbamic acid residue was then coupled without difficulty to the growing peptide chain by the commonly used solid phase technology.

Enzymatic stability studies showed that the incorporation of carbamic acid residue into the *N*-terminal part of nociceptin increases the resistance against AP-M. Results with control peptides Noc(1–13)-NH_2 (**1**) and [Phe-β-Ala-Phe]-Noc-(5–13)-NH_2 (**6**) showed that the half-life values of the modified nociceptin derivatives

[Phe-β-Ala-NH-CO-Phe]-Noc(5–13)-NH_2 (**2**), [Phe-NH-CO-β-Ala-Phe]-Noc(5–13)-NH_2 (**3**), [β-Hph-Gly-NH-CO-Phe]-Noc(5–13)-NH_2 (**4**) and [Phe-NH-CO-Gly-β-Hph]-Noc(5–13)-NH_2 (**5**) are significantly higher than those of the control peptides (Table 2). The similar pattern in the degradation curves of peptide (**2–5**) suggest that the position of the carbamic acid residue has no effect on the improved enzymatic stability against AP-M, inasmuch as the presence of carbamic acid residue between either amino acid residues 1 and 2, or 2 and 3 resulted in elevated stability. These results also suggest that the increased stability could be due to the incorporation of carbamic acid residue and not to the presence of β-amino acid. In case of the rat brain membrane homogenate, these differences in enzymatic stability were not pronounced presumably because of the simultaneous presence of endo- and carboxypeptidase activities in the homogenate.

While these modifications in the *N*-terminal message segment resulted in enhanced enzymatic stability against AP-M, the binding properties of the peptides to NOP receptor decreased markedly. Despite the circumspect design of the new set of molecules (i.e. retaining of length of peptide backbone, the distance between Phe^1 and Phe^4 side chains), incorporation of the carbamic acid residue into the *N*-terminal part led to low or diminished binding. This result suggests that this modification at the *N*-terminal part of Noc(1–13)-NH_2 is not tolerated. It should be noted that several *N*-terminally modified nociceptin derivatives are described in the literature. A few of them are proved to be full or partial NOP receptor agonist or antagonist ligands; [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]Noc(1–13)-$NH_2$ is a partial agonist, [(*p*F)Phe^4]Noc(1–13)-NH_2 is a highly potent and selective NOP receptor agonist and [$Nphe^1$]Noc(1–13)NH_2 behave as an antagonist, but most of the *N*-terminally modified peptides, e.g. Ac-Noc(1–13)-NH_2, [Phe(NMe)^1]Noc(1–13)-NH_2, [Phe-$(Gly)_3$-Phe]Noc(1–13)-NH_2, [β-Ala^2, $desGly^3$]Noc(1–13)-NH_2 or [$Nphe^4$]Noc(1–13)-NH_2, exhibited no or reduced activity [14–17].

In conclusion, the incorporation of peptide segments with the carbamic acid residue can be a promising and reasonable tool for increasing enzymatic stability site-specifically. However, such modification could also result in decreased binding affinity if it occurs at positions that influence receptor–ligand interaction. Consequently, this type of modification can be applied for increasing stability only in those cases where the native molecule is less sensitive for the structural change caused by the incorporation of the carbamic acid residue.

Acknowledgements

The authors thank Gitta Schlosser for MS experiments, Elemér Vass for IR measurements and György Lakihegyi for the experimental work on enzymatic studies.

This work was supported by grants OTKA T-033078, F-034886 from the Hungarian Scientific Research Fund; by Medichem II. OM-00421/2004 from the Ministry of Culture and by RET-2004-DNT from the National Office for Research and Technology.

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