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Nociceptin/Orphanin FQ(1–13)NH_2 Analogues Modified in the Phe[1]-Gly[2] Peptide Bond

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Abstract—The synthesis and pharmacological activity of novel nociceptin/orphanin FQ (N/OFQ) analogues modified in the Phe[1]-Gly[2] peptide bond are reported. The aim of the present work was to elucidate the importance of this peptide bond for the N/OFQ receptor (NOP) interaction. Our study indicates that the first peptide bond in N/OFQ is important but not crucial for interaction with the N/OFQ receptor; for instance, substitution with a methyleneoxy bond generates an agonist derivative just 3-fold less potent than the reference compound.

A few years after the identification of nociceptin/orphanin FQ [N/OFQ: H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH and its receptor (N/OFQ peptide receptor (NOP)][1] a vast amount of information regarding this novel peptide/receptor system are available.[2] It has been demonstrated that the N/OFQ-NOP receptor system regulates several biological functions both at peripheral and central levels.[3] Therefore, the NOP receptor is now considered a novel target for drug development.

To fully understand the biological role(s) of this system, selective ligands for NOP receptor are needed. To this aim, in the last few years, we performed a series of structure–activity studies on N/OFQ.[4,5] We divided the N/OFQ into a message [N/OFQ(1–4)] which includes the two pharmacophores Phe[1] and Phe[4] important for receptor activation and an address sequence [N/OFQ(5–17)] which contains the two couple of basic residues Arg[8,12]-Lys[9,13] crucial for receptor occupation.[6] We identified a series of original peptide ligands for the NOP receptor including: selective agonists such as N/OFQ(1–13)NH_2[7] or [(pF)Phe[4]]N/OFQ(1-13)NH_2,[8] non selective agonists such as [Tyr[1]]N/OFQ(1-13)NH_2,[9] selective partial agonists like [Phe[1]Ψ(CH_2–NH)Gly[2]]N/OFQ(1-13)NH_2 ([F/G]NC(1–13)NH_2),[10,11] and pure antagonists, as [Nphe[1]]N/OFQ(1–13)NH_2[12,13] and more recently UFP-101.[14] Worthy of mention is the fact that NOP antagonists were exclusively identified by modifications of the message domain of N/OFQ.

In the present study, we have further investigated the importance of the first peptide bond of N/OFQ(1–13)NH_2 for the interaction with the NOP receptor.

The synthesis of the reference compounds N/OFQ(1–13)NH_2 and [F/G] N/OFQ(1–13)NH_2 have been already reported,[6,10] while the other peptides were obtained by means of a mixed solid-phase/solution peptide synthesis approach. In particular, compound **4** was synthetised condensing Boc-Phe-CHO on the [Sar[2]]N/OFQ(2–13)-PAL-PEG-PS resin, obtained by Fmoc standard solid-phase peptide synthesis, following the procedure previously described by us.[10] For the synthesis of peptides **5**–**7** we condensed the pseudodipeptides Boc-PheΨ(CH_2–O)-Gly-OH,[15] Boc-PheΨ(CH_2–S)-Gly-OH,[16] and Boc-PheΨ(CO–CH_2)-Gly-OH,[17] with N/OFQ(3–13)-PAL-PEG-PS resin in the presence of 1-ethyl-3-[3′-(dimethylamino)propyl]-carbodiimide hydrochloride (EDCl) and 1-hydroxybenzotriazole (HOBt) as

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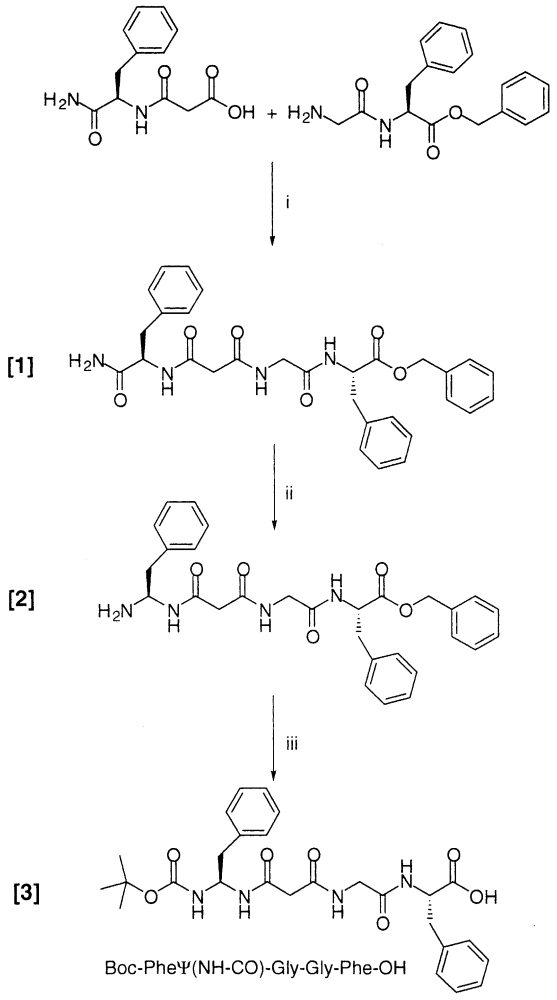
activating agents. In an attempt to adopt a similar scheme for the synthesis of compound **8**, we approach the synthesis of the pseudodipeptide Boc-PheΨ(NH–CO)-Gly-OH following the method for the solid phase synthesis of retro-inverso peptides proposed by Pessi et al.[18] that considers the conversion, with bis(trifluoroacetoxy)-iodobenzene (TIB), of the amide function of the pseudodipeptide malonil-D-Phe-amide into an amine function following protection with Boc. However, this reaction did not produce the desired compound but a series of other derivatives which were not further investigated. Therefore, an alternative way was considered for the synthesis of compound **8**: it was obtained condensing the pseudotetrapeptide Boc-PheΨ(NH–CO)-Gly-Gly-Phe-OH with the N/OFQ(5–13)-PAL-PEG-PS resin in the presence of EDCl and HOBt as activating agent. The pseudotrateptide Boc-PheΨ(NH–CO)-Gly-Gly-Phe-OH was obtained following Scheme 1 condensing the malonyl-D-Phe-amide[18] with H-Gly-Phe-OBzl.[19] The rearrangement of the amide function to amine function with TIB, the protection with Boc and the removal of the benzyl ester afforded the desired pseudotetrapeptide.[20]



Scheme 1. (i) EDCl, HOBt, Et_3N, DMF, 76%; (ii) TIB, CH_3CN/H_2O 6:4, 62%; (iii) $(Boc)_2O$, DMF; 10% Pd/C, H_2, MeOH, 78%.

Compounds **4–8** were purified by RP-HPLC (≥98% purity) and analysed on a MALDI-TOF mass spectrometer, yielding mass values in line with the expected chemical composition.

All the peptides were tested for their ability to inhibit the electrically evoked contraction of the mouse vas deferens (mVD), a pharmacological preparation sensitive to N/OFQ.[7] Cumulative concentration response curves were performed with N/OFQ(1–13)NH_2 and compounds **4–8.** When the peptides were found to be inactive as agonist, they were assayed as antagonists against the reference agonist N/OFQ(1–13)NH_2. The pharmacological results of the assays are presented in Table 1. The agonist and antagonist potencies were measured as pEC_{50} and pA_2, respectively. pA_2 values were determined by applying the Gaddum Schild equation [$pA_2 = -\log((CR-1)/[Antagonist])$], assuming a slope equal to unity. The adopted pharmacological terminology is in line with IUPHAR recommendations.[21]

N/OFQ(1–13)NH_2 produced a concentration dependent inhibition of the electrically induced twitch with pEC_{50} and E_{max} values of 7.85 and 90%, respectively (Table 1).

Reduction of the Phe[1]-Gly[2] peptide bond produced [F/G]N/OFQ(1–13)NH_2 which, in the mVD, is inactive as agonist and behaves as a competitive antagonist with a pA_2 value of 6.75.[11] The antagonist properties of this peptide were confirmed in some in vitro preparations while in others the peptide behaves as a partial agonist or even as a full antagonist [for a detailed description and discussion of the pharmacological profile of [F/G]N/OFQ(1–13)NH_2, see ref 22].

The introduction of the methyl substituent onto the Ψ(CH_2–NH) nitrogen, as in compound **4**, produced an inactive derivative. This result is in line with that obtained by methylation of the first Phe[1]-Gly[2] peptide bond in N/OFQ(1–13)NH_2; in fact, [Sar[2]]N/OFQ(1–13)NH_2 was demonstrated to behave as a NOP receptor agonist 100-fold less potent than N/OFQ(1–13)NH_2.[5] The nitrogen methylation of the first peptide bond ([Sar[2]]N/OFQ(1–13)NH_2) or of the reduced peptide bond Ψ(CH_2–NH) (**4**) probably limit the possible conformations of the N-terminal part of N/OFQ(1–13)NH_2, in particular of the side chain of Phe[1], thus causing a reduced biological activity. Moreover, nitrogen methylation improves the basic character of the reduced peptide bond and this may also prevent NOP receptor occupation.

The replacement of CO–NH bond with CH_2–O (**5**) produced an agonist 3-fold less potent than N/OFQ(1–13)NH_2 (Table 1), while substitution with CH_2–S (**6**) produced a reduction of both potency and efficacy. In fact compound **6** behaves as a partial agonist with an intrinsic activity (α) of 0.6. These peptide bond substitutions maintain the hydrogen bond acceptor but lose the donor properties. Quantitatively the CH_2–S moiety, due to reduced electronegativity and increased size of the sulfur atom, is inferior as hydrogen bond acceptor compared to CH_2–O modification. However, the

Table 1. Effects of [Phe[1]Ψ(**X**)Gly[2]]N/OFQ(1–13)NH_2 peptides in the electrically stimulated mVD assay

Compd	X	Agonist		Antagonist[a]
		pEC_{50} ($CL_{95\%}$)	E_{max} (%)	pA_2 ($CL_{95\%}$)
N/OFQ(1–13)NH_2	CO–NH	7.85 (0.15)	90±2	ND[b]
[F/G]N/OFQ(1–13)NH_2	CH_2–NH	Inactive[c]		6.75 (0.21)
4	CH_2–N(CH_3)	Crc incomplete[d]		Inactive
5	CH_2–O	7.38 (0.43)	73±7	ND
6	CH_2–S	6.79 (0.22)	54±5*	6.25 (0.14)
7	CO–CH_2	6.21 (0.18)	86±3	ND
8	NH–CO	Inactive		Inactive

[a]The antagonistic properties of these compounds were tested using N/OFQ(1–13)NH_2 as agonist.
[b]ND, not determined because these compounds are full agonists.
[c]Inactive: inactive up to 10 μM.
[d]Crc incomplete: only a slight effect (<50% inhibition) was detected at the highest concentration tested (10 μM). These data are mean of at least five biossay experiments.
*$p < 0.05$ versus N/OFQ(1–13)NH_2 effect, according to ANOVA followed by the Dunnet test.

hydrogen bond donor/acceptor properties of the Phe[1]-Gly[2] bond do not seem to be particularly relevant for the biological activity. On the other hand, the geometries of the CH_2–O and CH_2–S moieties are comparable to that of the trans amide bond, but the distances between the Cαi–Cαi + 1 appear to be slightly increased in the case of the CH_2–S bond[23] (i.e., CO–NH: 3.8 A; CH_2–O: 3.7 A; CH_2–S: 4.2 A). Being Phe[1] and Phe[4] the most important residues responsible for NOP receptor activation [5], the increased distance between Cαi-Cαi + 1 (and therefore between Phe[1] and Phe[4]) could be relevant for the decrease of efficacy of compound **6**. This is suggested by the fact that the shift of the side chain of Phe[1] from the Cα to the nitrogen (which produced a bigger increase of distance between Phe[1] and Phe[4]) fully eliminated intrinsic activity generating the first pure antagonist for the NOP receptor: [Nphe[1]]N/OFQ(1–13)NH_2.[12,13]

The introduction of a CO–CH_2 bond (**7**) lead to a weakly potent agonist derivative. Moreover, the effects of compound **7** were slightly sensitive to the classical opioid receptor antagonist naloxone which at 1 μM produced a 3-fold rightward shift of the concentration response curves to [Phe[1]Ψ(CO–CH_2)Gly[2]]N/OFQ(1–13)NH_2, demonstrating that this modification produced a decrease in the selectivity of action of the ligand. This peptide bond modification eliminates the NH group (and its hydrogen bond donor/acceptor properties) but maintains the carbonyl function as a possible hydrogen bond acceptor moiety. This may indicate that the nitrogen of the first peptide/preudopeptide bond is important for biological activity. However, the relative high potency of compounds **5** and **6** (in which the N atom was replaced with O and S, respectively) indicated that a heteroatom is required rather than specifically nitrogen. Finally, despite the close geometrical mimicry[24] between the standard peptide bond and the retro-inverso peptide bond (**8**), this latter modification generated an inactive analogue, indicating that the position of the nitrogen atom is also crucial for receptor occupation.

In conclusion, the present results suggest that the Phe[1]-Gly[2] peptide bond is not crucial per se for NOP receptor interaction. Substitutions with different peptide bond isoster generate NOP ligands with different potencies and efficacies, independently from the hydrogen bond acceptor/donor properties of the peudopeptide bond. The reduction or elimination of the activity obtained substituting Phe[1]-Gly[2] peptide bond might be due to impaired ability of the N-terminal part of the peptide to adopt energetically favorable bioactive conformations. Worthy of mention is the finding that compound **5**, although slightly less potent than N/OFQ(1–13)NH_2, is a full agonist at NOP receptor; this analogue is probably more resistant than the natural sequence towards amino-peptidase-N action. In vivo evaluation of compound **5** is required for defining the utility of this latter peptide modification.

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20. Synthetic procedure: **H_2NCO-DPhe-Malonil-Gly-Phe-OBzl** (**1**): To a solution of H_2NCO-Dphe-malonic acid (5 g, 20 mmol) and the trifluoacetate salt of H-Gly-Phe-OBzl (8.52 g, 20 mmol) in DMF (30 mL) at 0 °C, HOBt (3.4 g, 22 mmol), EDCl (4.22 g, 22 mmol) and triethylamine (2.8 mL, 20 mmol) were added. The reaction was stirred for 1 h at 0 °C and overnight at room temperature. After evaporation of DMF, the residue was solubilized in EtOAc and washed with citric acid (10%) (3×30 mL), $NaHCO_3$ (5%) (3×30 mL) and brine. The organic phase was dried and concentrated under reduced pressure. The residue was cristallized from diethyl ether/light petroleum 1:1, yield 8.3 g (76%), melting point 161–166 °C, $[\alpha]_D^{20}$ −3.6 (*c* 1 methanol), MH^+ 545, NMR ^{1}H (DMSO-d_6, 200 MHz, δ): 3.12–3.31 (m 6H), 4.23 (s 2H), 4.78–4.92 (m 2H), 5.37 (s 2H), 5.81 (bs 1H), 6.73 (bs 1H), 7.17–7.38 (m 12H), 7.53 (bs 1H).

H-Phe-ψ(NH–CO)-Gly-Gly-Phe-OBzl (**2**): The precursor amide (1) (5.4 g, 10 mmol) was dissolved in acetonitrile/water 6:4 (50 mL) and flushed with N_2. To this stirred solution, TIB (4.7 g, 11 mmol) dissolved in 10 mL acetonitrile was added by siringe. The reaction was allowed to proceed for 4 h under N_2 then concentrated under reduced pressure at low temperature. The residue was suspended in $NaHCO_3$ (5%) (100 mL) and the product extracted with EtOAc (4×50 mL). The organic phase was washed with brine, dried and evaporated to dryness. The oil crude product was loaded onto a silica gel column and eluted with dichloromethane/methanol 9:1. The compound obtained from chromatography was cristallized from diethyl ether/light petroleum 1:1, yield 3.2 g (62%), melting point 122–127 °C, $[\alpha]_D^{20}$ + 5.7 (*c* 1 methanol), MH^+ 517, NMR ^{1}H (DMSO-d_6, 200 MHz, δ): 3.07–3.18 (m 6H), 3.31 (m 2H), 3.82 (s 2H), 4.81 (m 1H), 5.07 (m 1H), 5.28 (s 2H), 6.42 (bs 1H), 7.09–7.32 (m 17H).

Boc-Phe-Y(NH-CO)-Gly-Gly-Phe-OH (**3**): To a solution of (**2**) (2.6 g, 5 mmol) in DMF (20 mL), $(Boc)_2O$ (1 g, 5 mmol) was added and the reaction stirred for 6 h. After this time the solvent was evaporated in vacuo and the oil residue dissolved in methanol (100 mL) and hydrogenated for 1 h in the presence of 10% Pd/C. The catalyst was removed by filtration through Celite pad and the solvent evaporated under reduced pressure. The residue was cristallized from diethyl ether, yield 2 g (78%), melting point 175–180 °C, $[\alpha]_D^{20}$ + 15.5 (*c* 1 methanol), MH^+ 527, NMR ^{1}H (DMSO-d_6, 200 MHz, δ): 1.38 (s 9H), 2.92–3.02 (m 4H), 3.21 (s 2H), 3.93 (s 2H), 5.76 (bs 1H), 7.12–7.27 (m 12H), 7.86 (bs 1H).
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