Structure−Activity Study of the Nociceptin(1−13)-NH_2 N-Terminal Tetrapeptide and Discovery of a Nociceptin Receptor Antagonist

Girolamo Calo', Remo Guerrini,† Raffaella Bigoni, Anna Rizzi, Clementina Bianchi, Domenico Regoli, and Severo Salvadori*,†

Department of Experimental and Clinical Medicine, Section of Pharmacology and Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, 44100 Ferrara, Italy

Received November 25, 1997

In the present study, the minimal fragment sequence required to fully activate the nociceptin (NC) receptor, namely NC(1−13)-NH_2, was used as template for the design of a series of new compounds. Changes were made in the N-terminal tetrapeptide Phe-Gly-Gly-Phe, which has been shown to be essential for receptor occupation and activation. The new compounds were tested for their ability to inhibit the electrically evoked contraction of the mouse vas deferens, a pharmacological preparation sensitive to NC. Results obtained indicate that (a) the replacement of Gly^2 or Gly^3 with an aromatic residue (Phe) of L or D chirality eliminates the ability of the peptide to occupy the NC receptor; (b) the distance between Phe^1 and Phe^4 of NC appears to be critical, since any alteration of it leads to a marked decrease or a total elimination of biological activity; and (c) the insertion of a pseudopeptide bond between Phe^1 and Gly^2 maintains affinity but eliminates the ability of the peptide to activate the NC receptor and leads to antagonism. The peptide [$Phe^1\psi(CH_2-NH)Gly^2$]-NC(1−13)-NH_2 acts as a selective NC receptor antagonist and is inactive on opioid receptors. The results summarized in this paper confirm and extend our previous findings by showing that the structural requirements for NC binding to its receptor are clearly different from those of opioids; in addition, this structure−activity study has led to the identification of the first NC receptor selective antagonist.

Introduction[1]

Nociceptin (NC) is a recently identified heptadecapeptide (H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH)[2,3] which acts as an endogenous agonist of the ORL_1 receptor. Binding[2−4] as well as biological studies[5−8] demonstrated that, despite the structural similarity of NC and its receptor with peptides and receptors of the opioid family, the two systems are pharmacologically distinct. In fact, naloxone antagonizes opioid-evoked effects but is inactive against NC.

The following biological actions have been described for NC in the periphery: inhibition of neurotransmitter release from sympathetic,[6,9] parasympathetic,[5,10] or peptidergic nerves,[7,8,11] hypotension and bradycardia,[12,13] and diuresis.[14] NC actions on the central nervous system include the following: induction of hyperalgesia[2,3] and antiopioid effects[15] in the brain, analgesia in the spinal cord,[16,17] inhibition of locomotor activity,[3] impairment of spatial learning,[18] stimulation of food intake,[19,20] and inhibition of glutamate,[21] acetylcholine,[22] and dopamine[23] release. These findings suggest an important role of the NC/NC receptor system in different physiological functions. However, the lack of a selective NC receptor antagonist has prevented, until now, a definite pharmacological characterization of the biological actions exerted by NC.

As for other neuropeptides, the effects of NC are related to the degradation of the peptide by specific peptidases. NC biotransformation in the brain mainly depends on the activity of two different enzymes, an aminopeptidase N which cleaves the peptide linkage Phe^1-Gly^2, and the endopeptidase 24.15 which cuts Ala^7-Arg^8, Ala^{11}-Arg^{12}, and Arg^{12}-Lys^{13} bonds.[24] Aminopeptidase N and endopeptidase 24.15 inhibitors potentiate behavioral effects mediated by NC, confirming the involvement of these enzymes in NC inactivation[25] in vivo.

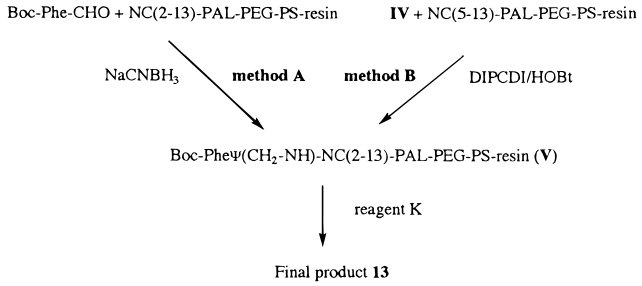
In previous reports,[5,9] using the mouse vas deferens (mVD) in vitro assay, we have shown that NC(1−13)-NH_2 has the same activity as that of the naturally occurring ligand NC and that of the synthetic analogue NC-NH_2. In the present study, the truncated ligand NC(1−13)-NH_2 (the minimal fragment sequence required to fully activate NC receptor[26]) was therefore used as the template for the design of a series of new compounds. Changes were made in the N-terminal tetrapeptide Phe-Gly-Gly-Phe, which has been shown to be essential for receptor occupation and activation.[26] First, one of the two glycine residues was replaced by L- or D-Phe to increase the aromaticity of the peptide. Second, the distance between Phe^1 and Phe^4 was altered by deletion or addition of glycine residues or by replacing glycines with β, γ, or δ amino acids or a proline residue. Third, to obtain protection from aminopeptidases, the peptide bond between Phe^1 and Gly^2 was replaced with CH_2-NH, or Gly^2 was substituted by D-Ala.

* Correspondence address: Severo Salvadori, Dipartimento di Scienze Farmaceutiche, Università di Ferrara, via Fossato di Mortara 17/19, 44100 Ferrara, Italy. Fax: +39-532-291283. E-mail: s.salvadori@ifeuniv.unife.it.

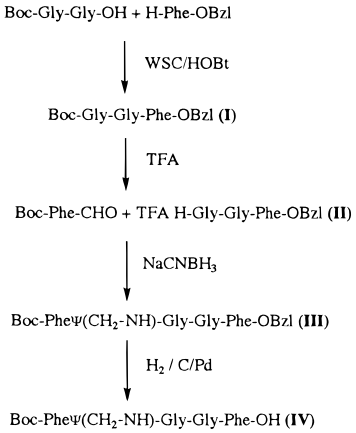
† Department of Pharmaceutical Sciences and Biotechnology Center.

S0022-2623(97)00805-4 CCC: $15.00
Published on Web 08/05/1998

Scheme 1. Synthesis of [Phe$^1\psi$(CH_2−NH)Gly^2]-HC(1−13)-NH_2 by Mixed Solution-Solid-Phase Method



Scheme 2. Synthesis of [Phe$^1\psi$(CH_2−NH)Gly^2]-NC-Tetrapeptide Protected Fragments



Results and Discussion

Peptides **1**−**14** were prepared by solid-phase peptide synthesis performed on a Milligen 9050 synthesizer using a Fmoc-PAL-PEG-PS-resin (0.16 mmol/g, 0.2 g in all syntheses). Peptides were assembled using Fmoc-protected amino acids (4 equiv) and DIPCDI (4 equiv) and HOBt (4 equiv) as coupling agents for 1 h for each coupling. Side chain protecting groups used were Pmc for arginine, Trt for asparagine, Boc for lysine, and tBu for serine and threonine. Peptide **13** was synthesized by mixed solution-solid-phase methods by means of two distinct synthetic approaches, as depicted in Scheme 1. In a first approach we synthesized the dipeptide Boc-Pheψ(CH_2−NH)Gly-OH and the tripeptide Boc-Pheψ-(CH_2−NH)Gly-Gly-OH; however, their low solubility both in MeOH and in DMF prevented their condensation with NC(3−13)-PAL-PEG-PS-resin or NC(4−13)-PAL-PEG-PS-resin, respectively. Thus, the tetrapeptide Boc-Pheψ(CH_2−NH)Gly-Gly-Phe-OH was synthesized in solution following Scheme 2 and then condensed as a final acylation step on nociceptin fragment NC(5−13)-PAL-PEG-PS-resin via manual injection mode. The same derivative was also obtained by condensing off-line Boc-Phe-CHO [27] with the nociceptin fragment NC-(2−13)-PAL-PEG-PS-resin and reducing the intermediate imine derivative in situ with $NaBH_3CN$, to minimize racemization.[28] The same synthetic approach was adopted for compound **14**. Boc-Pheψ(CH_2−NH)-Gly-Gly-Phe-OH was obtained by condensation via WSC/HOBt of Boc-Gly-Gly-OH and H-Phe-OBzl to obtain a protected tripeptide. Boc was removed by acidic treatment with TFA, followed by reductive alkylation with Boc-Phe-CHO in the presence of $NaBH_3$-CN.[28] Catalytic removal of the C-terminal ester function by H_2/Pd gave the N-protected tetrapeptide. All protected peptides were cleaved from the resin by treatment with TFA/H_2O/phenol/ethanedithiol/thioanisole (reagent K) (82.5:5:5:2.5:5, v/v) at room temperature for 1 h.[29]

The chemical features of the new compounds are presented in Table 1 using a *K′* which was determined in two (I and II) solvent systems to assess the purity of each compound. The diastereomer compounds **13** and **14** showed the same *K′* in solvent systems I and II; we also performed HPLC analysis on peptides **13** and **14**, using different solvent systems (data not shown) but were unable to get a satisfactory separation of the two diastereomers. Therefore, even if the pseudopeptide bond ψ(CH_2−NH) between L or D Phe^1 and Gly^2 was synthesized according to the method described by Ho et al.[28] which minimizes racemization, we cannot exclude the presence in the final product of a small amount of diastereomer impurity. Mass ion data obtained experimentally were found to be very similar to expected values obtained by calculation.

The compounds were tested for their ability to inhibit the electrically evoked contraction (twitch response) of the mouse vas deferens (mVD), a pharmacological preparation recently shown to be sensitive to NC.[6,9] They were also assayed as antagonists of the reference agonist, NC(1−13)-NH_2 (compound **1**). Results of biological assays are presented in Table 2 in terms of pEC_{50} to describe their agonistic potency and in terms of pA_2 to indicate affinity as antagonists. The naturally occurring compound NC, the synthetic analogue NC-NH_2, and the fragment NC(1−13)-NH_2 show the same affinity and evoke similar maximal responses in this preparation. The activities of the new compounds can be analyzed and discussed as follows. The replacement of Gly^2 or Gly^3 by L- or D-Phe (compounds **2**−**5**) leads to compounds inactive as agonists or as antagonists on the NC receptor. [D-Phe^3]-NC(1−13)-NH_2 (compound **5**) is a weak agonist whose effect is completely prevented by naloxone, indicating that it activates not the NC receptor but an opioid receptor. Thus, the addition of an aromatic residue of L or D chirality between Phe^1 and Phe^4 eliminates the ability of the peptide to occupy the NC receptor. This contrasts with what happens for dermorphin tetrapeptide analogues[30] and the recently identified opioid tetrapeptide endomorphins,[31] where a C-terminal Trp-Phe or Phe-Phe sequence improves selectivity and potency for the μ opioid receptor type. The deletion of the spacer Gly^2-Gly^3 (compound **6**), as well as different lengths of the spacer (compounds **7**, **8**, **9**, and **10**) are not tolerated. In fact, compounds **7**, **8**, and **9** are completely inactive; compound **10** maintains slight agonist activity, but is more than 100-fold less potent than the reference compound. Therefore, the distance between Phe^1 and Phe^4 of NC appears to be critical for preserving the affinity and the full biological activity. On the contrary, in some opioid peptides (i.e., endomorphin, morphiceptin, deltorphin, or dermorphin) the presence of an L or D chirality second residue between the two aromatic pharmacofores (Tyr^1 and Phe^3) leads to potent μ or δ opioid receptor ligands.[31−34]

Table 1. Abbreviated Names and Analytical Properties of NC(1−13)-NH_2 and Its Analogues

		K′[a]		MH^{+}[b]	
no	abbreviated names	I	II	calculated	found
1	NC(1−13)-NH_2	2.17	3.81	1382.5	1382.7
2	[Phe^2]-NC(1−13)-NH_2	3.07	3.91	1472.7	1472.8
3	[D-Phe^2]-NC(1−13)-NH_2	3.29	4.16	1472.7	1472.4
4	[Phe^3]-NC(1−13)-NH_2	3.52	4.37	1472.7	1472.4
5	[D-Phe^3]-NC(1−13)-NH_2	3.41	4.26	1472.7	1472.5
6	[desGly^{2,3}]-NC(1−13)-NH_2	2.83	4.08	1268.5	1268.4
7	[β-Ala^2,desGly^3]-NC(1−13)-NH_2	3.39	4.51	1339.5	1339.4
8	[Gaba^2,desGly^3]-NC(1−13)-NH_2	3.45	4.32	1353.0	1353.4
9	[Ava^2,desGly^3]-NC(1−13)-NH_2	3.67	4.57	1367.0	1367.2
10	[Phe-(Gly)$_3$−Phe]-NC(5−13)-NH_2	3.18	4.39	1439.6	1439.7
11	[Pro^2,desGly^3]-NC(1−13)-NH_2	3.21	4.08	1365.6	1366.0
12	[D-Ala^2]-NC(1−13)-NH_2	3.08	3.91	1396.5	1396.8
13	[Phe$^1\psi$(CH$_2$−NH)Gly^2]-NC(1−13)-NH_2	3.73	4.25	1368.5	1368.8
14	[D-Phe$^1\psi$(CH$_2$−NH)Gly^2]-NC(1−13)-NH_2	3.73	4.25	1368.5	1369.2

[a] K′ is the capacity factor determined by analytical HPLC. I and II refer to the HPLC gradients as described in the paragraph Peptide Purification and Analytical Determinations of the Experimental Section. [b] The mass ion (MH^{+}) was obtained by MALDI-TOF mass spectrometry.

Table 2. Effects of NC, NC(1−13)-NH_2, and NC(1−13)-NH_2 Analogues in the Electrically Stimulated Mouse Vas Deferens

		agonist		antagonist
no	abbreviated names	pEC$_{50}$[a] (CL$_{95\%}$)	E_{max}[b]	pA$_2$[c]
	NC	7.84 (0.07)	−83 ± 3%	ND[d]
	NC−NH_2	7.74 (0.11)	−79 ± 4%	ND
1	NC(1−13)-NH_2	7.75 (0.14)	−84 ± 4%	ND
2	[Phe^2]-NC(1−13)-NH_2	I[e]		I
3	[D-Phe^2]-NC(1−13)-NH_2	I		I
4	[Phe^3]-NC(1−13)-NH_2	I		I
5	[D-Phe^3]-NC(1−13)-NH_2	5.68 (0.34)	−75 ± 12%	I
6	[desGly^{2,3}]-NC(1−13)-NH_2	I		I
7	[β-Ala^2,desGly^3]-NC(1−13)-NH_2	I		I
8	[Gaba^2,desGly^3]-NC(1−13)-NH_2	I		I
9	[Ava^2,desGly^3]-NC(1−13)-NH_2	I		I
10	[Phe-(Gly)$_3$−Phe]-NC(1−13)-NH_2	5.49 (0.43)	−73 ± 11%	I
11	[Pro^2,desGly^3]-NC(1−13)-NH_2	I		I
12	[D-Ala^2]-NC(1−13)-NH_2	6.0 (0.45)	−81 ± 4%	ND
13	[Phe$^1\psi$(CH$_2$−NH)Gly^2]-NC(1−13)-NH_2	I		6.75
14	[D-Phe$^1\psi$(CH$_2$−NH)Gly^2]-NC(1−13)-NH_2	I		I

[a] pEC$_{50}$: the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect; CL$_{95\%}$: 95% confidence limits. [b] E_{max}: the maximal effect induced by an agonist expressed as percent inhibition of electrically induced twitches. [c] pA$_2$: the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit the original submaximal response; the antagonistic properties of these compounds were tested using NC(1−13)-NH_2 as agonist. [d] ND: not determined because these compounds are full agonists. [e] I: inactive at 10 μM. The effects of these compounds, except for compound 5 whose action was completely blocked by the opioid receptor antagonist, were not affected by naloxone, 1 μM.

Deletion of Gly^3 and replacement of Gly^2 with Pro ([Pro^2,desGly^3]-NC(1−13)-NH_2, compound **11**) or D-Ala^2 ([D-Ala^2,desGly^3]-NC(1−13)-NH_2[26]) are incompatible with NC receptor interaction, therefore differing from opioids (see above). Replacement of Gly^2 with D-Ala^2 in the NC sequence (which has been found to improve potency and duration of action of enkephalin)[35,36] reduces the biological activity on NC receptors while maintaining binding affinity.[37] Compound **12** is a full agonist with reduced (by about 100-fold) potency, which is in agreement with the above-mentioned data.

The insertion of a pseudopeptide bond between Phe^1 and Gly^2 (compound **13**) maintains affinity but eliminates the ability of the peptide to activate the NC receptor and gives a NC receptor antagonist. The same chemical modification in dynorphin A (1−11)-NH_2 gave an analogue which acts as a κ opioid receptor agonist with reduced affinity.[38] [Phe$^1\psi$(CH$_2$−NH)Gly^2]-NC(1−13)-NH_2 requires the L chirality of Phe^1, since the D-Phe diastereomer (compound **14**) is inactive. Compound **13** is selective for the NC receptor; in fact, as shown in Figure 1, the inhibitory effect of NC in the mVD is not modified by naloxone but is reduced by compound **13**. On the contrary, the effect of the δ opioid receptor selective agonist, deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), is antagonized by naloxone but is not affected by compound **13**. The displacement to the right of the NC concentration response curve by compound **13** (Figure 1, panel A) as well as that of deltorphin I by naloxone (Figure 1, panel B) is parallel to the control curve, suggesting that the antagonism is of the competitive type. Moreover, [Phe$^1\psi$(CH$_2$−NH)Gly^2]-NC(1−13)-NH_2 when applied at concentrations up to 10 μM does not affect either the inhibitory action of dermorphin (a μ opioid receptor selective agonist) in the guinea pig ileum[39] or that of U69593 (a κ opioid receptor selective agonist) in the rabbit vas deferens (control: pEC$_{50}$ 7.58 ± 0.28, E_{max} − 100%; in the presence of 10 μM [Phe$^1\psi$(CH$_2$−NH)Gly^2]-NC(1−13)-NH_2: pEC$_{50}$ 7.54 ± 0.33, E_{max} −100%).

[Phe$^1\psi$(CH$_2$−NH)Gly^2]-NC(1−13)-NH_2 represents the first example of a selective NC receptor antagonist. In fact, only antagonists which act nonselectively and with low affinity (carbetapentane and rimcazole)[40] or molecules which act as partial agonists (hexapeptides derived from a combinatorial library)[41] were reported

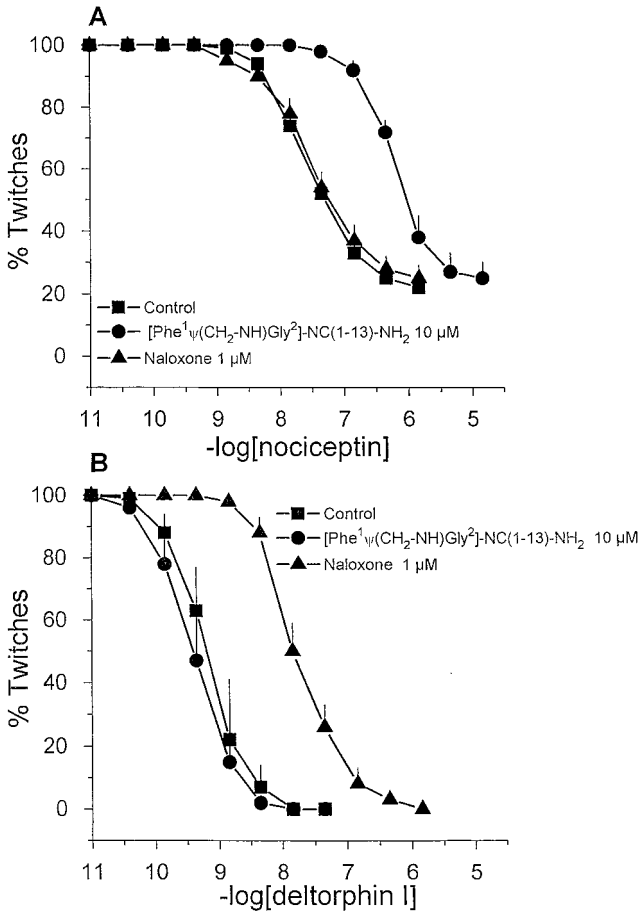


Figure 1. Effects of naloxone (1 μM) and [Phe$^1\psi$(CH_2NH)-Gly^2]-NC(1−13)-NH_2 (10 μM) on concentration−response curves to nociceptin (panel A) or deltorphin I (panel B) in the electrically stimulated mouse vas deferens. Data are mean ± SEM of at least five experiments.

in the literature. [Phe$^1\psi$(CH_2−NH)Gly^2]-NC(1−13)-NH_2 which is still a long peptide with relatively low affinity on the NC receptor should be considered as a prototype for the development of new, smaller, and more potent antagonists.

In chemical terms, on one hand, the replacement of CO by CH_2 in compound **13** eliminates the possibility of forming hydrogen bonds; on the other hand, it increases the flexibility of the N-terminal portion of the molecule by transforming the amide into an amine function. Furthermore, the new N-terminal tetrapeptide, which is considered to be the active site of NC,[2] becomes more basic, and this may prevent receptor activation.

The use of a pseudopeptide bond between Phe^1 and Gly^2, in addition to conferring the ability to act as a NC receptor antagonist, may protect the new peptide from degradation by aminopeptidases. Worthy of mention is also the fact that NC(1−13)-NH_2 is already (at least in part) protected from degradation by carboxypeptidases, as suggested by the significantly higher potency of NC-(1−13)-NH_2 compared to that of NC(1−13)-OH.[26] Thus, [Phe$^1\psi$(CH_2−NH)Gly^2]-NC(1−13)-NH_2 appears to be stable against exopeptidases acting at the N and C termini and may therefore have a prolonged duration of action in vivo. Studies are under way to clarify this point.

Experimental Section

Materials. Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, or Fluka (Switzerland). The resin [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxy-phenoxy)-valeric acid] on the poly(ethylene glycol)/polystyrene support (Fmoc-PAL-PEG-PS) was from Millipore (Waltham, MA). Naloxone was from Tocris Cookson (Bristol, U.K.) and U69593 from Amersham International plc (Buckinghamshire, U.K.). Stock solutions (1 mM) of peptides were made in distilled water and kept at −20 °C until use. Krebs solution (gassed with 95% O_2 and 5% CO_2, pH 7.4) had the following composition (in mmol): NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, and $CaCl_2$ 2.5, glucose 10. All other reagents were from Sigma Chemical Co. (St. Louis, MO) or E. Merck (Darmstadt, Germany) and were of the highest purity grade available.

Peptide Purification and Analytical Determinations. Crude peptides were purified by preparative reversed-phase HPLC using a Waters Delta Prep 4000 system with a Waters PrepLC 40-mm assembly C_{18} column (30 × 4 cm, 300 A, 15-μm spherical particle size column). The column was perfused at a flow rate of 50 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0 to 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) in 25 min was adopted for the elution of peptides. Analytical HPLC analyses were performed on a Bruker liquid chromatography LC 21-C instrument fitted with an Alltech C_{18} (4.6 × 150 mm 5-μm particle size) column and equipped with a Bruker LC 313 UV variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (BX-10). Analytical determination and capacity factor (K′) of the peptides were determinated using HPLC conditions in the above solvent system (solvents A and B) programmed at flow rates of 1 mL/min using the following linear gradients: (I) from 0% to 50% solvent B in 25 min, (II) from 0% to 20% solvent B in 25 min, and (III) from 0% to 100% B in 25 min. All analogues showed less than 1% impurities when monitored at 220 nm.

Amino acid analyses were carried out using Pico-Tag methodology and PITC as an amino acid derivatization reagent. Non-natural amino acids and amino acids involved in peptide bond modification [L or D Phe$^1\psi$(CH_2−NH)Gly^2] were not quantitatively determined during the amino acid analysis. Lyophilized samples of peptides (50−1000 pmol) were placed in heat-treated borosilicated tubes (50 × 4 mm), sealed, and hydrolyzed using 200 μL of 6 N HCl containing 1% phenol in the Pico-Tag workstation for 1 h at 150 °C. A Pico-Tag column (15 × 3.9 mm) was employed to separate the PITC-amino acid derivatives. The peptide compositions were as expected.

Molecular weights of compounds were determined by a MALDI-TOF analysis using a Hewlett-Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as the matrix. The values are expressed as MH^+.

TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: (a) CH_2Cl_2/methanol/toluene (17:2:1, v/v/v), (b) 1-butanol/acetic acid/H_2O (3:1:1, v/v/v). Ninhydrin (1%) or chlorine/iodine spray reagents were employed to detect the peptides. Melting points were determined on a Reicher-Kofler apparatus and are uncorrected. Optical rotation used a Perkin-Elmer 241 polarimeter with a 10-cm cell using methanol or DMF at a peptide concentration of 1%. ^{1}H NMR spectroscopy was obtained with a 200 MHz Bruker instrument and are recorded in δ units.

Solid-Phase Peptide Synthesis. NC(1−13)-NH_2 (1). Fmoc-PAL-PEG-PS-resin (0.16 mmol/g, 0.2 g) was treated with piperidine (20%) in DMF and linked with N^α-Fmoc-N^ϵ- Lys-(Boc), via its *N*-hydroxybenzotriazole active ester. The following N^α-Fmoc amino acids were sequentially coupled to the growing peptide chain: N^α-Fmoc-N^ω-Arg-(Pmc), N^α-Fmoc-Ala, N^α-Fmoc-O-(tBu)-Ser, N^α-Fmoc-N^ϵ-(Boc)-Lys, N^α-Fmoc-N^ω-(Pmc)-Arg, N^α-Fmoc-Ala, N^α-Fmoc-Gly, N^α-Fmoc-O-(tBu)-Thr,

N^{α}-Fmoc-Phe, N^{α}-Fmoc-Gly, N^{α}-Fmoc-Gly, N^{α}-Fmoc-Phe. All the N^{α}-Fmoc amino acids (4 equiv) were coupled to the growing peptide chain by using 1,3-diisopropylcarbodiimide (4 equiv) and 1-hydroxybenzotriazole (4 equiv) in DMF, and the coupling reaction time was 1 h. Piperidine (20%) in DMF was used to remove the Fmoc group at all steps. After deprotection of the last N^{α}-Fmoc group, the peptide−resin was washed with methanol and dried in vacuo to yield the protected NC(1−13)-PAL-PEG-PS-resin. The other peptide amides (compounds **2**−**12**) were synthesized in the same manner. The protected peptide−resin was treated with reagent K (10 mL/0.2 g of resin) for 1 h at room temperature. After filtration of the exhausted resin the solvent was concentrated in vacuo and the residue triturated with ether. The crude peptide was purified by preparative reverse phase HPLC to yield a white powder after lyophilization.

Boc-Pheψ(CH_2−NH)-NC(2−13)-PAL-PEG-PS-resin (V), Method A. NC(2−13)-PAL-PEG-PS-resin (0.2 g, 0.16 mmol/g, 0.032 mmol) was swelled in methanol containing 1% (v/v) acetic acid (2 mL). After 20 min, a solution of Boc-Phe-CHO (0.012 g, 0.048 mmol) and $NaBH_3CN$ (0.006 g, 0.096 mmol) dissolved in methanol (0.3 mL) was added and the reaction mixture stirred for 1 h. After this time, the resin was washed with methanol and treated with reagent K as for the synthesis of compound **1**. This final product (compound **13**), after purification, is identical to the compound obtained by method B, and both have the same pharmacological activity (data not shown).

Boc-D-Pheψ(CH_2−NH)-NC(2−13)-PAL-PEG-PS-resin. NC(2−13)-PAL-PEG-PS-resin (0.2 g, 0.16 mmol/g, 0.032 mmol) was swelled in methanol containing 1% (v/v) acetic acid (2 mL). After 20 min, a solution of Boc-D-Phe-CHO (0.012 g, 0.048 mmol) and $NaBH_3CN$ (0.006 g, 0.096 mmol) dissolved in methanol (0.3 mL) was added and the reaction mixture stirred for 1 h. After this time, the resin was washed with methanol and treated with reagent K, as for the synthesis of compound 1, to obtain compound **14**.

Solution Peptides Synthesis. Fmoc-Ava-OH.[42] To a stirred solution of H-Ava-OH (1.17 g, 10 mmol) in 10% Na_2CO_3 (23 mL) at 0 °C was added Fmoc-Cl (2.46 g, 9.5 mmol) dissolved in dioxane (15 mL). The mixture was stirred at room temperature for 2 h, diluted with H_2O (150 mL), and acidified with concentrated HCl. The gelatinous precipitate was extracted with EtOAc and the organic phase washed with brine, dried on Na_2SO_4, and evaporated to dryness. Trituration of the residue with Et_2O yielded a white powder 2.24 g (67%); R_f (b) 0.88; mp 149−150 °C; ^{1}H NMR (DMSO) δ= 1.41−1.43 (4H, $2CH_2$, m), 2.13−2.33 (2H, CH_2, m), 2.95−2.98 (2H, CH_2, m), 4.15−4.38 (3H, CH_2 + CH, m), 7.28−7.90 (9H, Ar + NH, m), 12.04 (1H, COOH, bs).

Boc-Gly-Gly-Phe-OBzl (I). To a stirred solution of Boc-Gly-Gly-OH (0.58 g, 2.5 mmol) and HCl·H-Phe-OBzl (0.73 g, 2.5 mmol) in DMF (10 mL) at 0 °C, were added HOBt (0.42 g, 2.75 mmol), WSC (0.52 g, 2.75 mmol), and TEA (0.35 mL, 2.5 mmol). The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After evaporation of the DMF, the residue was solubilized in EtOAc and washed with citric acid (10%), $NaHCO_3$ (5%), and brine. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was an oil, yield 0.97 g (83%): R_f (a) 0.78; K′ (III) 9.25; $[\alpha]^{20}_D$ −1.4 (MeOH); ^{1}H NMR ($CDCl_3$) δ= 1.41 (9H, $3CH_3$, s), 3.12 (2H, CH_2, dd, J = 15.2, 7.9 Hz), 3.69 (2H, CH_2, bs), 3.87 (2H, CH_2, bs), 4.52−4.68 (1H, CH, m), 5.13 (2H, CH_2, s), 6.4−6.6 (1H, NH, bs) 6.87−7.15 (10H, $2C_6H_5$, m), 8.15 (1H, NH, d, J = 8.3 Hz), 8.57 (1H, NH, t, J = 8.7 Hz).

TFA·H-Gly-Gly-Phe-OBzl (II). Boc-Gly-Gly-Phe-OBzl (O.8 g, 1.7 mmol) was treated with TFA (3 mL) for 0.5 h at room temperature. A solution of Et_2O/petroleum ether (1:1) was added to the solution until the product precipitated, yield 0.76 g (93%): R_f (a) 0.38; K' (III) 6.81; mp 96−98 °C; $[\alpha]^{20}_D$ −0.5 (MeOH); ^{1}H NMR ($CDCl_3$) δ= 3.06 (2H, CH_2, dd, J = 15.7, 7.5 Hz), 3.72 (2H, CH_2, bs), 3.91 (2H, CH_2, bs), 4.72−4.76 (1H, CH, m), 5.06 (2H, CH_2, s), 7.09−7.33 (10H, $2C_6H_5$, m), 8.06 (1H, NH, d, J = 7.8 Hz), 8.3−8.6 (3H, NH_3+ bs), 8.67 (1H, NH, t, J = 7.7 Hz).

Boc-Pheψ(CH_2−NH)Gly-Gly-Phe-OBzl (III). To a stirred solution of TFA·H-Gly-Gly-Phe-OBzl (0.58 g, 1.2 mmol) and TEA (O.17 mL, 1.2 mmol) in methanol (20 mL) containing 1% (v/v) of acetic acid were added Boc-Phe-CHO (0.33 g, 1.32 mmol) and $NaBH_3CN$ (0.23 g, 3.6 mmol) dissolved in methanol (3 mL). After 1 h, the reaction mixture was evaporated and the residue dissolved in EtOAc. The organic layer was washed with $NaHCO_3$ (5%) and brine, dried, and evaporated to dryness. The residue was purified by silica gel column chromatography with solvent system (a) (see under Peptide Purification and Analytical Determinations above), and trituration of pure fractions with Et_2O/petroleum ether (1:1) yielded a white powder, 0.53 g (73%): R_f (a) 0.66; K' (III) 13.21; mp 104−106 °C; $[\alpha]^{20}_D$ −5.7 (MeOH); ^{1}H NMR (DMSO) δ 1.3 (9H, $3CH_3$, s), 2.6−2.8 (2H, CH_2 m), 2.88−3.08 (3H, CH_2 + CH, m), 3.32−3.39 (4H, $2CH_2$, bs), 3.68−3.74 (3H, CH_2 + NH, m), 4.53 (1H, CH, dd, J = 6.9, 8.2 Hz), 5.07 (2H, CH_2 s), 6.73 (1H, NH, d, J = 8.2 Hz), 7.16−7.35 (15H, $3C_6H_5$, m), 8.05 (1H, NH, t, J = 7.7 Hz), 8.47 (1H, NH, d, J = 8.3 Hz).

Boc-Pheψ(CH_2−NH)Gly-Gly-Phe-OH (IV). To a stirred solution of Boc-Pheψ(CH_2−NH)Gly-Gly-Phe-OBzl (0.3 g, 0.5 mmol) in DMF (30 mL) was added C/Pd (10%, 0.2 g), and H_2 was bubbled through for 2 h at room temperature. After filtration, the solution was evaporated to dryness and the residue triturated with Et_2O, yield 0.21 g (83%): R_f (b) 0.86; K' (III) 11.19; mp 202−203 °C; $[\alpha]^{20}_D$ −0.8 (DMF); ^{1}H NMR (DMSO) δ 1.41 (9H, $3CH_3$, s), 2.62−2.76 (2H, CH_2, m), 2.91−3.12 (3H, CH_2 + CH, m), 3.30−3.36 (4H, $2CH_2$, bs), 3.70−3.74 (2H, CH_2, bs), 4.53 (1H, CH, dd, J = 7.1, 8.4 Hz), 6.73 (1H, NH, d, J = 7.8 Hz), 7.16−7.35 (10H, $2C_6H_5$, m), 8.05 (1H, NH, t, J = 8.1 Hz), 8.47 (1H, NH, d, J = 8.4 Hz) 8.88−8.93 (2H, NH_2^+, bs).

Boc-Pheψ(CH_2−NH)-NC(2−13)-PAL-PEG-PS-resin (V), method B. Boc-Pheψ(CH_2−NH)Gly-Gly-Phe-OH (0.065 g, 0.128 mmol) dissolved in DMF (2 mL) was added to HOBt (0.02 g, 0.128 mmol) and DIPCDI (20 μL, 0.128 mmol). After 5 min, this solution was injected by manual inject port into the peptide synthesizer containing NC(5−13)-PAL-PEG-PS-resin (0.2 g, 0.16 mmol/g, 0.032 mmol) and recycled for 16 h. The protected peptide−resin was treated with reagent K, as in compound **1**.

Bioassay. Swiss male mice weighing 25−30 g and New Zeland male rabbits weighing 1−1.5 Kg were used. The vas deferens (mVD) was prepared according to Hughes et al.[43] and suspended in 10 mL organ baths containing Mg^{2+}-free Krebs solution at 33 °C. The tissues were stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1-ms duration and 0.1-Hz frequency. The resting tension was maintained at 0.3 g for the mouse tissue and at 1 g for the rabbit tissue. The electrically evoked contractions were measured isotonically by means of a Basile strain gauge transducer and recorded on a Linseis multichannel chart recorder (model 2005). After an equilibration period of about 2 h, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration−response curves (crc) for NC, NC(1−13)-NH_2, and NC(1−13)-NH_2 analogues U69593 or deltorphin I were performed (0.5 log unit steps). When required, antagonists were added to the Krebs solution 15 min before performing crc for the agonists.

Data Analysis and Terminology. The data are expressed as mean ± standard error of the mean of at least four experiments. For pEC_{50} values the confidence limits at 95% were given. Data have been statistically analyzed using the Student's two-tailed t test or one-way analysis of variance followed by the Dunnet test for multiple comparison via a software package.[44] P values lower than 0.05 were considered to be significant.

The pharmacological terminology adopted in this study is in line with the following recent IUPHAR recommendations:[45,46] the agonist apparent affinities are given as pEC_{50} = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the possible maximal re-

sponse; apparent affinities of antagonists are given in terms of pA_2 = the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit the original submaximal response. Antagonist apparent affinities have been evaluated using the Gaddum Schild equation, $pA_2 = -\log(CR - 1/[\text{antagonist}])$ assuming a slope value equal to unity.

Acknowledgment. We thank Dr. M. Remelli for chiral HPLC analysis (not mentioned in the text). This work was supported by the Italian Ministry of the University (40% and 60% grants).

Supporting Information Available: Table of amino acid hydrolysis (1 page). Ordering information is given on any current masthead page.

References

(1) Abbreviations follow IUPAC-IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides: *J. Biol. Chem.* **1985**, *260*, 1442. Additional abbreviations used herein are as follows: Ava (5-aminovaleric acid), β-Ala (3-aminopropionic acid), Boc (*tert*-butoxycarbonyl), DIPCDI (1,3-diisopropylcarbodiimide), Fmoc [(9-fluorenylmethyl)oxycarbonyl], Fmoc-PAL-PEG-PS [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)-valeric acid] on the poly(ethylene glycol)/polystyrene support), Gaba (4-aminobutyric acid), HOBt (1-hydroxybenzotriazole), MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight), OBzl (benzyl ester), PITC (phenylisothiocyanate), Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl), tBu (*tert*-butyl), TEA (triethylamine), TFA (trifluoroacetic acid), Trt (trityl), WSC (1-ethyl-3-(3′ dimethylaminopropyl)carbodiimide·HCl).

(2) Meunier, J. C.; Mollereau, C.; Toll, L.; Suaudeau, C.; Moisand, C.; Alvinerie, P.; Butour, J. L.; Guillemot, J. C.; Ferrara, P.; Monserrat, B.; Mazarguil, H.; Vassart, G.; Parmentier, M.; Costentin, J. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* **1995**, *377*, 532−535.

(3) Reinscheid, R. K.; Nothacker, H. P.; Bourson, A.; Ardati, A.; Henningsen, R. A.; Bunzow, J. R.; Grandy, D. K.; Langen, H.; Monsma, F. J., Jr.; Civelli, O. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* **1995**, *270*, 792−794.

(4) Dooley, C. T.; Houghten, R. A. Orphanin FQ: receptor binding and analog structure activity relationships in rat brain. *Life Sci.* **1996**, *59*, 23−29.

(5) Calo', G.; Rizzi, A.; Bodin, M.; Neugebauer, W.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Regoli, D. Pharmacological characterization of nociceptin receptor: an in vitro study. *Can. J. Physiol. Pharmacol.* **1997**, *75*, 713−718.

(6) Berzetei-Gurske, I. P.; Schwartz, R. W.; Toll, L. Determination of activity for nociceptin in the mouse vas deferens. *Eur. J. Pharmacol.* **1996**, *302*, R1−R2.

(7) Giuliani, S.; Maggi, C. A. Inhibition of tachykinin release from peripheral endings of sensory nerves by nociceptin, a novel opioid peptide. *Br. J. Pharmacol.* **1996**, *118*, 1567−1569.

(8) Giuliani, S.; Maggi, C. A. Prejunctional modulation by nociceptin of nerve-mediated inotropic responses in guinea-pig left atrium. *Eur. J. Pharmacol.* **1997**, *332*, 231−236.

(9) Calo', G.; Rizzi, A.; Bogoni, G.; Neugebauer, V.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Regoli, D. The mouse vas deferens: a pharmacological preparation sensitive to nociceptin. *Eur. J. Pharmacol.* **1996**, *311*, R3−R5.

(10) Patel, H. J.; Giembycz, M. A.; Spicuzza, L.; Barnes, P. J.; Belvisi, M. G. Naloxone-insensitive inhibition of acetylcholine release from parasympatethic nerves innervating guinea-pig trachea by the novel opioid, nociceptin. *Br. J. Pharmacol.* **1997**, *120*, 735−736.

(11) Helyes, Z.; Nemeth, J.; Pinter, E.; Szolcsanyi, J. Inhibition by nociceptin of neurogenic inflammation and the release of SP and CGRP from sensory nerve terminals. *Br. J. Pharmacol.* **1997**, *121*, 613−615.

(12) Giuliani, S.; Tramontana, M.; Lecci, A.; Maggi, C. A. Effect of nociceptin on heart rate and blood pressure in anaesthetized rats. *Eur. J. Pharmacol.* **1997**, *333*, 177−179.

(13) Champion, H. C.; Kadowitz, P. J. Nociceptin, an endogenous ligand for the ORL(1) receptor, has novel hypotensive activity in the rat. *Life Sci.* **1997**, *60*, PL241−PL245.

(14) Kapusta, D. R.; Sezen, S. F.; Chang, J. K.; Lippton, H.; Kenigs, V. A. Diuretic and antinatriuretic responses produced by the endogenous opioid-like peptide, nociceptin (orphanin FQ). *Life Sci.* **1996**, *60*, PL15−PL21.

(15) Mogil, J. S.; Grisel, J. E.; Reinsheid, R. K.; Civelli, O.; Belknap, J. K.; Grandy, D. K. Orphainin FQ is a functional anti-opioid peptide. *Neuroscience* **1996**, *75*, 333−337.

(16) Stanfa, L. C.; Chapman, V.; Kerr, N.; Dickenson, A. H. Inhibitory action of nociceptin on spinal dorsal horn neurones of the rat, in vivo. *Br. J. Pharmacol* **1996**, *118*, 1875−1877.

(17) Xu, X. J.; Hao, J. X.; Wiesenfeld-Hllin, Z. Nociceptin or antinociceptin: potent spinal antinociceptive effect of orphanin FQ/nociceptin in the rat. *NeuroReport* **1996**, *7*, 2092−2094.

(18) Sandin, J.; Georgieva, J.; Schott, P. A.; Ogren, S. O.; Terenius, L. Nociceptin/Orphanin FQ microinjected into hippocampus impairs spatial learnig in rats. *Eur. J. Neurosci.* **1997**, *9*, 194−197.

(19) Pomonis, J. D.; Billington, C. J.; Levine, A. S. Orphanin FQ, agonist of orphan opioid receptor RORL1, stimulates feeding in rats. *NeuroReport* **1996**, *8*, 369−371.

(20) Stratford, T. R.; Holahan, M. R.; Kelley A. E. Injections of nociceptin into nucleus accumbens shell or ventromedial hypothalamic nucleus increase food intake. *NeuroReport* **1997**, *8*, 423−426.

(21) Nicol, B.; Lambert, D. G.; Rowbotham, D. J.; Smart, D.; McKnight, A. T. Nociceptin induced inhibition of K^+ evoked glutamate release from rat cerebrocortical slices. *Br. J. Pharmacol.* **1996**, *119*, 1081−1083.

(22) Neal, M. J.; Cunningham, J. R.; Paterson, S. J.; McKnight, A. T. Inhibition by nociceptin of the light-evoked release of ACh from retinal cholinergic neurones. *Br. J. Pharmacol.* **1997**, *120*, 1399−1400.

(23) Murphy, N. P.; Ly, H. T.; Maidment, N. T. Intracerebroventricular orphanin FQ nociceptin suppresses dopamine release in the nucleus accumbens of anaesthetized rats. *Neuroscience* **1996**, *75*, 1−4.

(24) Montiel, J. L.; Cornille, F.; Roques, B. P.; Noble, F. Nociceptin/orphanin FQ metabolism: role of aminopeptidase and endopeptidase 24.15. *J. Neurochem.* **1997**, *68*, 354−361.

(25) Noble, F.; Roques, B. P. Association of aminopeptidase N and endopeptidase 24.15 inhibitors potentiate behavioral effects mediated by nociceptin/orphanin FQ in mice. *FEBS Lett.* **1997**, *401*, 227−229.

(26) Guerrini, R.; Calo', G.; Rizzi, A.; Bianchi, C.; Lazarus, L. H.; Salvadori, S.; Temussi, P. A.; Regoli, D. Address and message sequences for the nociceptin receptor: A structure−activity study of nociceptin(1−13) amide. *J. Med. Chem.* **1997**, *40*, 1789−1793.

(27) Fehrentz, J. A.; Castro, B. An efficient synthesis of optically active α-(*tert*-butoxycarbonylamino)-aldehydes from α-amino acids. *Synthesis* **1983**, 676−678.

(28) Ho, P. T.; Chang, D.; Zhong, J. W.; Musso, G. F. An improved low racemization solid-phase method for the synthesis of reduced dipeptides (ψCH_2NH) bond isosteres. *Pep. Res.* **1993**, *6*, 10−12.

(29) King, D. S.; Fields, C. G.; Fields, G. B. A cleavage method which minimizes side reactions following Fmoc solid-phase peptide synthesis. *Int. J. Pept. Protein Res.* **1990**, *36*, 255−266.

(30) Schiller, P. W.; Nguyen, T. M. D.; Lemieux, C. New types of opioid peptide analogs showing high μ-receptor selectivity and preference for either central or peripheral sites. In *Peptides 1988*; Jung, G., Bayer, E. Eds.; Berlin: Walter de Gruyter, **1988**, pp 613−615.

(31) Zadina, J. E.; Hackler, L.; Ge, L. J.; Kastin, A. J. A potent and selective endogenous agonist for the μ opiate receptor. *Nature* **1997**, *386*, 499−502.

(32) Broccardo, M.; Erspamer, V.; Falconieri Erspamer, G.; Improta, G.; Linari, G.; Melchiorri, P.; Montecucchi, P. C. Pharmacological data on dermorphins, a new class of potent opioid peptides from amphibian skin. *Br. J. Pharmacol.* **1981**, *73*, 625−631.

(33) Erspamer, V.; Melchiorri, P.; Falconieri Erspamer G.; Negri, L.; Corsi, R.; Severini, G.; Barra, D.; Simmaco, M.; Kreil, G. Deltorphins: a family of naturally occurring peptides with high affinity and selectivity for δ opioid binding sites. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5188−5192.

(34) Chang, K. J.; Killian, A.; Hazum, E.; Cuatrecasas, P. Morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2): a potent and specific agonist for morphine (μ) receptors. *Science* **1981**, *212*, 75−77.

(35) Morley, J. S. Structure−activity relationship of enkephalin-like peptides. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 81−110.

(36) Hruby, V. J.; Gehrig, C. A. Recent developments in the design of receptor specific opioid peptides. *Med. Res. Rev.* **1989**, *9*, 343−401.

(37) Reinscheid, R. K.; Ardati, A.; Monsma, F. J. Civelli, O. Structure−activity relationship studies on the novel neuropeptide orphanin FQ. *J. Biol. Chem.* **1996**, *271*, 14163−14168.

(38) Meyer, J. P.; Davis, P.; Lee, K. B.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. Synthesis using a Fmoc-based strategy and biological activities of some reduced peptide bond pseudopeptide analogues of dynorphin A. *J. Med. Chem.* **1995**, *38*, 3462−3468.

(39) Guerrini, R.; Calo', G.; Rizzi, A.; Bigoni, R.; Bianchi, C.; Salvadori, S.; Regoli, D. A new selective antagonist of the nociceptin receptor. *Br. J. Pharmacol.* **1998**, *123*, 163−165.

(40) Kobayashi, T.; Ikeda, K.; Togashi, S.; Itoh, N.; Kumanishi, T. Effects of sigma ligands on the nociceptin/orphanin FQ receptor

coexpressed with the G-protein-activated K+ channel in Xenopus oocytes. *Br. J. Pharmacol.* **1997**, *120*, 986−987.
(41) Dooley, C. T.; Spaeth, C. G.; Berzetei-Gurske, I. P.; Craymer, K.; Adapa, I. D.; Brandt, S. R.; Houghten, R. A.; Toll, L. Binding and in vitro activities of peptides with high affinity for the Nociceptin/Orphanin FQ receptor, ORL1. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 735−41.
(42) Carpino, A. L.; Han, G. Y. The 9-fluorenylmethoxycarbonyl amino-protecting group. *J. Org. Chem.* **1972**, *37*, 3404−3409.
(43) Hughes, J.; Kosterlitz, H. W.; Leslie, F. M. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. *Br. J. Pharmacol.* **1975**, *53*, 371−381.
(44) Tallarida, R. J.; Murray, R. B. Manual of pharmacologic calculations with computer programs. New York: Springer-Verlag, 1987.
(45) Vanhoutte, P. M.; Humphrey, P. P. A.; Spedding, M. X. International Union of Pharmacology recommendations for nomenclature of new receptor subtypes. *Pharmacol. Rev.* **1996**, *48*, 1−2.
(46) Jenkinson, D. H.; Barnard, E. A.; Hoyer, D.; Humphrey, P. P. A.; Leff, P.; Shankley, N. P. International Union of Pharmacology Commitee on receptor nomenclature and drug classification. XI. Recommendations on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* **1995**, 47, 255−266.

JM970805Q