Structure−Activity Studies of the Phe[4] Residue of Nociceptin(1−13)-NH_2: Identification of Highly Potent Agonists of the Nociceptin/Orphanin FQ Receptor

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Received May 21, 2001

A total of 32 compounds was prepared to investigate the functional role of Phe[4] in NC(1−13)-NH_2, the minimal sequence maintaining the same activity as the natural peptide nociceptin. These compounds could be divided into three series in which Phe[4] was replaced with residues that would (i) alter aromaticity or side chain length, (ii) introduce steric constraint, and (iii) modify the phenyl ring. Compounds were tested for biological activity as (a) inhibitors of the electrically stimulated contraction of the mouse vas deferens; (b) competitors of the binding of [^{3}H]-NC-NH_2 to mouse brain membranes; and (c) inhibitors of forskolin-stimulated cAMP accumulation in CHO cells expressing the recombinant human OP_4 receptor. Results indicate that all compounds of the first and second series were inactive or very weak with the exception of [N(CH_3)Phe[4]]NC(1−13)-NH_2, which was only 3-fold less potent than NC(1−13)-NH_2. Compounds of the third series showed higher, equal, or lower potencies than NC(1−13)-NH_2. In particular, [(pF)Phe[4]]NC(1−13)-NH_2 (pF) and [(pNO_2)Phe[4]]NC(1−13)-NH_2 (pNO_2) were more active than NC(1−13)-NH_2 by a factor of 5. In the mVD, these compounds showed the following order of potency: (pF) = (pNO_2) ≥ (pCN) > (pCl) > (pBr) > (pI) = (pCF_3) = (pOCH_3) > (pCH_3) > (pNH_2) = (pOH). (oF) and especially (mF) maintained high potencies but were less active than (pF). Similar orders of potency were observed in binding competition and cAMP accumulation studies. There was a strong ($r^2 \geq 0.66$) correlation between data observed in these assays. Biological activity data of compounds of the third series were plotted against some Hansch parameters that are currently used to quantify physicochemical features of the substituents. In the three biological assays agonist potency/affinity positively correlates with the electron withdrawal properties of the groups in the p-position of Phe[4] and inversely with their size.

Introduction[1]

Nociceptin (NC)/orphanin FQ is a neuropeptide which modulates several biological functions via activation of the OP_4 receptor (see for a recent review ref 2). On structural and transductional grounds, the NC/OP_4 and opioid systems appear to be related. However, NC does not bind to opioid receptors and conversely opioid ligands do not bind to the OP_4 receptor.

To increase our knowledge of the physiopathological roles of the NC/OP_4 receptor system, selective and potent ligands of either a peptide or a non-peptide nature are required.

Over the last several years, we have performed systematic structure−activity studies on NC.[3,4] Initially we studied the activities of NC fragments to determine the minimal sequence (NC(1−13)-NH_2) that retains full agonist activity.[5] NC(1−13)-NH_2 has since served as a template for the design of further compounds. Second, in an attempt to protect the N-terminus from degradation by aminopeptidases,[6] we prepared [Phe[1]Ψ(CH_2−NH)Gly[2]]NC(1−13)-NH_2 and noted that it behaves as a OP_4 antagonist in a variety of in vitro assays, while acting as a potent agonist in most in vivo assays.[2] This compound, which has been widely used in NC studies, is in fact a partial agonist whose intrinsic activity strongly depends on the efficiency of the stimulus/response coupling (for a detailed discussion of this topic see ref 2). Third, using a variety of different approaches we modified the N-terminal Phe of NC(1−13)-NH_2 and identified [Nphe[1]]NC(1−13)-NH_2.[7] This compound has been shown to be a pure and selective antagonist in vitro at recombinant human OP_4 receptors[8] and at native peripheral[9−11] and central[12,13] OP_4 sites. Moreover, in vivo this compound has antagonist properties at OP_4 receptors controlling pain threshold,[10] locomotor activity,[14] food intake,[15] spatial memory,[16] and cardiovascular parameters.[17]

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10.1021/jm010221v CCC: $20.00 © 2001 American Chemical Society
Published on Web 10/13/2001

Table 1. Abbreviated Names and Analytical Properties of NC Analogues

no.	abbreviated names	purity, %	t_R[a] (min)		MH+[b]	
			I	II	calcd	found
1	[Cha4]NC(1−13)-NH2	>99	10.17	11.16	1388.5	1388.8
2	[(pPh)Phe4]NC(1−13)-NH2	>98	16.01	16.92	1458.5	1458.6
3	[1Nal4]NC(1−13)-NH2	>99	15.08	15.91	1432.5	1432.7
4	[2Nal4]NC(1−13)-NH2	>99	15.63	16.40	1432.5	1432.8
5	[Cβ(Ph)Phe4]NC(1−13)-NH2	>99	14.67	15.61	1458.5	1458.8
6	[Phg4]NC(1−13)-NH2	>99	8.00	8.74	1368.5	1368.6
7	[Hfe4]NC(1−13)-NH2	>99	10.19	10.76	1396.5	1396.3
8	[Nphe4]NC(1−13)-NH2	>98	11.63	12.23	1382.5	1382.2
9	[N(CH3)Phe4]NC(1−13)-NH2	>98	12.10	12.85	1396.5	1396.8
10	[Cα(CH3)Phe4]NC(1−13)-NH2	>98	12.19	12.69	1396.5	1396.5
11	[Cα(CH3)-D-Phe4]NC(1−13)-NH2	>98	12.76	13.38	1396.5	1396.1
12	[Cβ(CH3)-Treo-Phe4]NC(1−13)-NH2 (1)	>97	10.97	11.80	1396.5	1396.2
13	[Cβ(CH3)-Treo-Phe4]NC(1−13)-NH2 (2)	>97	12.69	13.31	1396.5	1396.6
14	[Cβ(CH3)-Eritro-Phe4]NC(1−13)-NH2 (1)	>98	11.20	12.32	1396.5	1396.7
15	[Cβ(CH3)-Eritro-Phe4]NC(1−13)-NH2 (2)	>98	12.30	13.17	1396.5	1396.4
16	[Atc4]NC(1−13)-NH2 (1)	>99	12.74	13.47	1408.5	1408.2
17	[Atc4]NC(1−13)-NH2 (2)	>98	13.66	14.78	1408.5	1408.4
18	[Aic4]NC(1−13)-NH2	>99	11.84	12.44	1394.5	1394.7
19	[Tic4]NC(1−13)-NH2	>99	11.03	11.95	1394.5	1394.1
20	[(pF)Phe4]NC(1−13)-NH2	>99	11.31	11.93	1400.5	1400.2
21	[(pCl)Phe4]NC(1−13)-NH2	>99	12.78	13.59	1417.0	1417.5
22	[(pBr)Phe4]NC(1−13)-NH2	>99	14.11	14.60	1461.4	1461.7
23	[(pI)Phe4]NC(1−13)-NH2	>99	15.95	16.69	1508.4	1508.1
24	[(mF)Phe4]NC(1−13)-NH2	>99	12.28	13.15	1400.5	1400.2
25	[(oF)Phe4]NC(1−13)-NH2	>99	11.12	12.25	1400.5	1400.6
26	[(pNO2)Phe4]NC(1−13)-NH2	>99	12.19	12.62	1427.5	1427.6
27	[(pCN)Phe4]NC(1−13)-NH2	>99	10.72	11.28	1407.5	1407.5
28	[(pCF3)Phe4]NC(1−13)-NH2	>99	14.18	15.35	1450.5	1450.1
29	[(pOCH3)Phe4]NC(1−13)-NH2	>99	10.97	11.89	1412.5	1412.6
30	[(pCH3)Phe4]NC(1−13)-NH2	>99	12.14	12.73	1396.5	1396.3
31	[(pNH2)Phe4]NC(1−13)-NH2	>99	12.23	12.82	1397.5	1397.7
32	[(pOH)Phe4]NC(1−13)-NH2	>99	8.97	9.53	1398.5	1398.5

[a] t_R is the retention time determined by analytical HPLC. [b] The mass ion (MH^+) was obtained by MALDI TOF mass spectrometry. (1) and (2) see Experimental Section.

In the present study, we have focused on the Phe4 residue, the pharmacophore of the message domain of NC(1−13)-NH2. This residue is crucial for receptor occupation and activation, as suggested by both Ala scan[18,19] and D-amino acid scan studies.[19] In addition, the chemical requirements of position 4 appear to be very rigorous, as substitution with Tyr or Leu are tolerated in position 1 but not in position 4.[5,19] Therefore, we have performed a systematic structure activity analysis on Phe4 of NC(1−13)-NH2 by examining the effects of (i) modifications of the aromaticity, length, and position of the amino acid side chain, (ii) conformational restrictions, and (iii) insertion of different chemical groups in the phenyl ring. These modifications will alter the electronic, steric, and lipo/hydrophilic features of this residue. We describe the structure of several novel ligands which behave as highly potent agonists of the OP_4 receptor.

Result and Discussion

Thirty-two analogues of NC(1−13)-NH2, modified in the Phe4 pharmacophore, were synthesized (Table 1). As shown in Figure 1, compounds of the first series were modified in their aromaticity (**1**−**5**), length (**6** and **7**), or position (**8**) of the Phe4 side chain. Compounds of the second series (Figure 2, compounds **9**−**19**) contain conformational constraints of the Phe4 residue, while those of the third series (Figure 3, compounds **20**−**32**) were substituted on the aromatic ring of Phe4 with different chemical functionalities.

Peptides **1**−**32** (Table 1) were prepared by solid-phase peptide synthesis. The Cβ(CH3)-Phe was prepared as described in the literature[20] as racemic erythro (SS, RR) and racemic threo (SR, RS). Chemical characteristics of these new compounds are presented in Table 1. Retention time values were determined in two (I and II) solvent systems to assess the purity of each compound and mass ion values (MH^+) are also reported.

Peptides of the three series were tested for their ability to inhibit electrically evoked contractions (twitch response) of the mouse vas deferens (mVD), a NC sensitive pharmacological preparation.[5,21] If these compounds were found to be inactive as agonists, they were assayed as antagonists against the reference agonist NC(1−13)-NH2. Results of this biological assay are presented in Table 2 as pEC_{50} to describe agonist potency and pK_B to describe antagonist potency. Compounds **20**−**32** were also tested in a receptor binding assay performed using mouse forebrain membranes and [3H]-NC-NH2 as a radioligand.[22] Binding data are presented as pK_I and are shown in Table 3. Compounds **20**−**32** were then tested in CHO cells stably expressing the human recombinant OP_4 receptor, for their ability to inhibit forskolin-stimulated cAMP accumulation.[23] The results of these experiments are presented in Table 4.

Results summarized in Table 2 indicate that removal of aromaticity (Cha/Phe4) leads to inactivity (**1**). Enhancement in aromatic character obtained using naphthyl rings (**3** and **4**) or with other modifications (**2** and **5**) markedly reduced or eliminated activity. However, these latter modifications not only increased aromaticity but also steric bulkiness of the residue, and this may prevent interaction with the receptor.

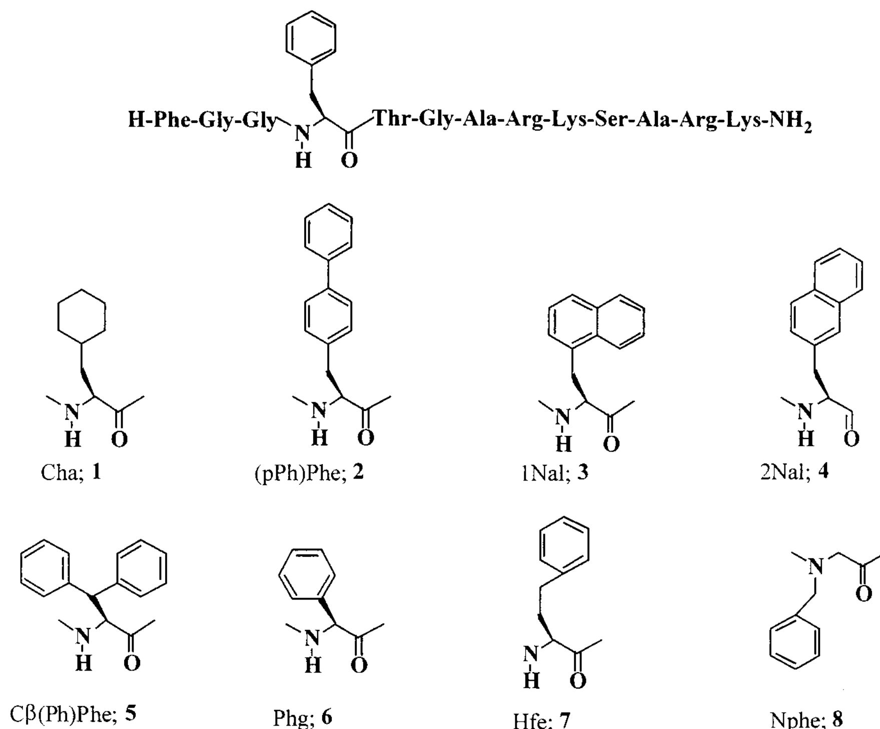

Figure 1. Analogues of NC(1−13)-NH_2 modified in the aromaticity, length, or position of Phe[4] side chain.

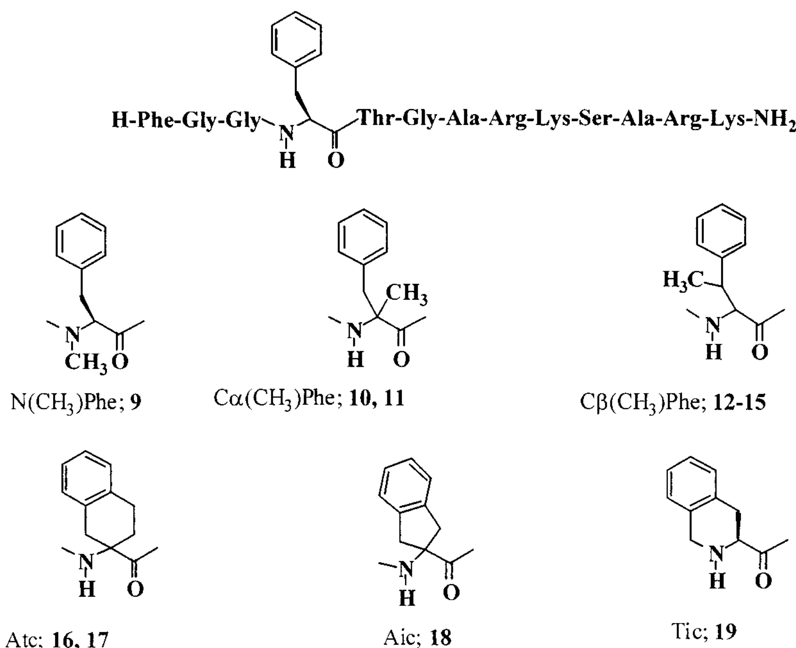

Figure 2. Analogues of NC(1−13)-NH_2 with conformational restrictions at Phe[4] residue.

Changing the distance between the aromatic ring of Phe[4] and the peptide backbone as in analogues **6** and **7** results in inactivity. Shift of the Phe[4] side chain from Cα to N (**8**) leads to inactivity both as agonists and antagonists. Thus, SAR obtained with some compounds of this series differ from those obtained with identical substitutions in Phe.[1] Indeed, Cha was well tolerated in position 1,[4] and [Nphe[1]]NC(1−13)-NH_2[4] acted as a pure and competitive antagonist in a variety of assays.[2] It thus appears that the chemical requirements of Phe[4] are much more rigorous than those of Phe[1]. Based on prior studies (see Introduction) and on the profile displayed by compounds **1**−**9**, it is suggested that the receptor sites interacting with Phe[1] and Phe[4] residues have different chemical requirements.

Compounds of the second series (**9**−**19**, Figure 2) with conformational restrictions obtained by methylating the N (**9**), Cα (**10** and **11**), or Cβ (**12**−**15**) atoms or by intramolecular cyclization of the side chain with the Cα (**16**−**18**) or N (**19**) atoms display no activity. The only exception is compound **12**, which however is 30-fold less potent than NC(1−13)-NH_2.

Compounds of the third series (**20**−**32**) primarily substituted in the p-position of Phe[4] show variable potencies where [(pF)Phe[4]]NC(1−13)-NH_2 is 5-fold more potent than NC(1−13)-NH_2 and the other p-halogenated

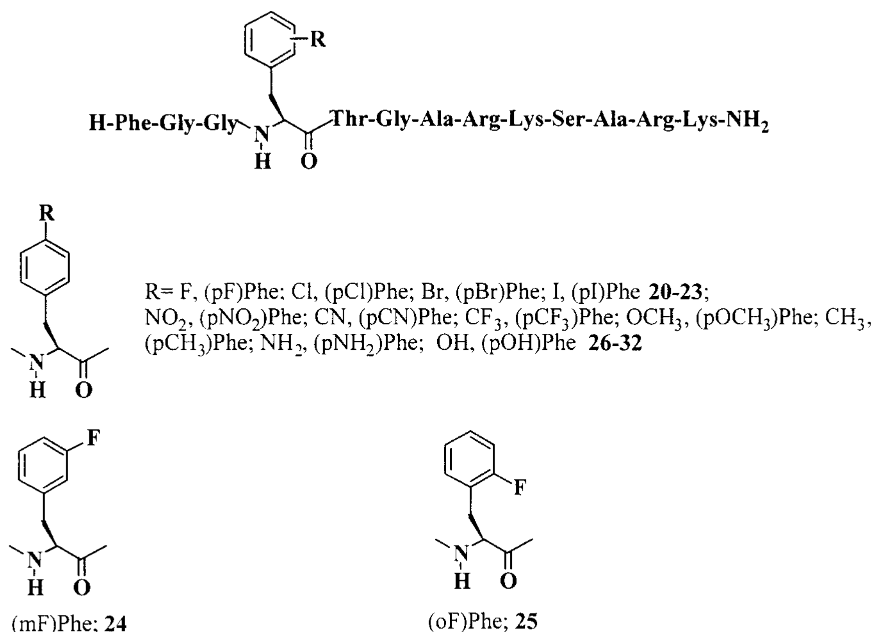

Figure 3. Analogues of NC(1−13)-NH_2 substituted in the different position of Phe^4 aromatic ring.

Table 2. Effects of NC(1−13)-NH_2 Analogs in the Electrically Stimulated Mouse Vas Deferens[a]

no.	abbreviated names	agonist pEC$_{50}$ (CL$_{95\%}$)	agonist E_{max}	antagonist pK_b
	NC	7.43 (0.17)	−84 ± 4%	ND
	NC(1−13)-NH_2	7.49 (0.11)	−82 ± 3%	ND
1	[Cha^4]NC(1−13)-NH_2	at 10 μM causes −34 ± 7%		inactive
2	[(pPh)Phe^4]NC(1−13)-NH_2	inactive		inactive
3	[1Nal^4]NC(1−13)-NH_2	5.30 (0.40)	−80 ± 6%	ND
4	[2Nal^4]NC(1−13)-NH_2	5.60 (0.40)	−75 ± 8%	ND
5	[Cβ(Ph)Phe^4]NC(1−13)-NH_2	inactive		inactive
6	[Phg^4]NC(1−13)-NH_2	inactive		inactive
7	[Hfe^4]NC(1−13)-NH_2	inactive		inactive
8	[Nphe^4]NC(1−13)-NH_2	at 10 μM causes −16 ± 8%		inactive
9	[N(CH_3)Phe^4]NC(1−13)-NH_2	7.10 (0.50)	−84 ± 2%	ND
10	[Cα(CH_3)Phe^4]NC(1−13)-NH_2	inactive		inactive
11	[Cα(CH_3)-D-Phe^4]NC(1−13)-NH_2	inactive		inactive
12	[Cβ(CH_3)-Treo-Phe^4]NC(1−13)-NH_2 (I)	6.05 (0.50)	−86 ± 5%	ND
13	[Cβ(CH_3)-Treo-Phe^4]NC(1−13)-NH_2 (II)	inactive		inactive
14	[Cβ(CH_3)-Eritro-Phe^4]NC(1−13)-NH_2 (I)	inactive		inactive
15	[Cβ(CH_3)-Eritro-Phe^4]NC(1−13)-NH_2 (II)	inactive		inactive
16	[Atc^4]NC(1−13)-NH_2 (I)	inactive		inactive
17	[Atc^4]NC(1−13)-NH_2 (II)	inactive		inactive
18	[Aic^4]NC(1−13)-NH_2	inactive		inactive
19	[Tic^4]NC(1−13)-NH_2	inactive		inactive
20	[(pF)Phe^4]NC(1−13)-NH_2	8.19 (0.25)	−85 ± 2%	ND
21	[(pCl)Phe^4]NC(1−13)-NH_2	7.09 (0.32)	−82 ± 5%	ND
22	[(pBr)Phe^4]NC(1−13)-NH_2	6.68 (0.27)	−85 ± 3%	ND
23	[(pI)Phe^4]NC(1−13)-NH_2	6.13 (0.34)	−76 ± 5%	ND
24	[(mF)Phe^4]NC(1−13)-NH_2	8.03 (0.30)	−90 ± 6%	ND
25	[(oF)Phe^4]NC(1−13)-NH_2	7.48 (0.54)	−94 ± 2%	ND
26	[(pNO_2)Phe^4]NC(1−13)-NH_2	8.15 (0.13)	−86 ± 2%	ND
27	[(pCN)Phe^4]NC(1−13)-NH_2	8.13 (0.15)	−84 ± 4%	ND
28	[(pCF_3)Phe^4]NC(1−13)-NH_2	6.27 (0.50)	−75 ± 12%	ND
29	[(pOCH_3)Phe^4]NC(1−13)-NH_2	6.42 (0.52)	−80 ± 7%	ND
30	[(pCH_3)Phe^4]NC(1−13)-NH_2	5.96 (0.37)	−73 ± 10%	ND
31	[(pNH_2)Phe^4]NC(1−13)-NH_2	at 10 μM causes −23 ± 9%		inactive
32	[(pOH)Phe^4]NC(1−13)-NH_2	inactive		5.47 (0.40)

[a] The antagonist properties of these compounds were evaluated using NC(1−13)-NH_2 as an agonist. ND: not determined because these compounds are full agonists. Inactive: inactive up to 10 μM. None of the effects of these compounds were affected by 1 μM naloxone. These data are the mean of at least five experiments.

analogues (**21**−**23**) show lower potencies (from 2 to 30-fold) varying with the increasing size of the halogen atom. Similar potency increases (5-fold) are observed with compounds **26** and **27**, containing NO_2 and CN in the p-position of Phe^4, respectively. Addition of CF_3 (**28**), OCH_3 (**29**) or CH_3 (**30**) at the p-position of the phenyl ring results in more than a 10-fold decrease in potency, and that of NH_2 (**31**) or OH (**32**) leads to inactivity. Compound **32** displays weak antagonistic activity (pK_B 5.47).

These results support the suggestion[3,4] that Phe^4 is the active site of NC and indicate that the peptide/

Table 3. Receptor Binding Affinities of NC(1−13)-NH_2 Analogs in Mouse Forebrain Membranes[a]

no.	abbreviated names	pK_I ($CL_{95\%}$)	K_I (nM)
	NC	8.72 (0.24)	1.91
	NC(1−13)-NH_2	9.04 (0.20)	0.91
20	[(pF)Phe[4]]NC(1−13)-NH_2	9.40 (0.20)	0.40
21	[(pCl)Phe[4]]NC(1−13)-NH_2	9.10 (0.23)	0.79
22	[(pBr)Phe[4]]NC(1−13)-NH_2	8.74 (0.32)	1.82
23	[(pI)Phe[4]]NC(1−13)-NH_2	7.76 (0.40)	17
24	[(mF)Phe[4]]NC(1−13)-NH_2	9.05 (0.25)	0.89
25	[(oF)Phe[4]]NC(1−13)-NH_2	8.44 (0.28)	3.63
26	[(pNO_2)Phe[4]]NC(1−13)-NH_2	9.52 (0.31)	0.30
27	[(pCN)Phe[4]]NC(1−13)-NH_2	9.22 (0.26)	0.60
28	[(pCF_3)Phe[4]]NC(1−13)-NH_2	9.00 (0.28)	1.00
29	[($pOCH_3$)Phe[4]]NC(1−13)-NH_2	7.05 (0.36)	89
30	[(pCH_3)Phe[4]]NC(1−13)-NH_2	7.84 (0.35)	14
31	[(pNH_2)Phe[4]]NC(1−13)-NH_2	5.75 (0.38)	1778
32	[(pOH)Phe[4]]NC(1−13)-NH_2	6.30 (0.27)	501

[a] These data are the mean of at least four experiments.

Table 4. Effects of NC(1−13)-NH_2 Analogs on Forskolin Stimulated cAMP Accumulation in CHO_{hOP4}[a]

		agonist		
no.	abbreviated names	pEC_{50} ($CL_{95\%}$)	E_{max}	antagonist pK_B
	NC	9.66 (0.25)	−103 ± 1%	ND
	NC(1−13)-NH_2	9.49 (0.17)	−102 ± 2%	ND
20	[(pF)Phe[4]]NC(1−13)-NH_2	9.80 (0.40)	−103 ± 1%	ND
21	[(pCl)Phe[4]]NC(1−13)-NH_2	9.57 (0.41)	−101 ± 1%	ND
22	[(pBr)Phe[4]]NC(1−13)-NH_2	8.85 (0.20)	−103 ± 1%	ND
23	[(pI)Phe[4]]NC(1−13)-NH_2	8.14 (0.49)	−106 ± 1%	ND
24	[(mF)Phe[4]]NC(1−13)-NH_2	9.60 (0.32)	−104 ± 5%	ND
25	[(oF)Phe[4]]NC(1−13)-NH_2	9.00 (0.29)	−104 ± 5%	ND
26	[(pNO_2)Phe[4]]NC(1−13)-NH_2	9.87 (0.47)	−103 ± 2%	ND
27	[(pCN)Phe[4]]NC(1−13)-NH_2	9.52 (0.16)	−103 ± 2%	ND
28	[(pCF_3)Phe[4]]NC(1−13)-NH_2	7.50 (0.35)	−105 ± 5%	ND
29	[($pOCH_3$)Phe[4]]NC(1−13)-NH_2	7.20 (0.30)	−100 ± 2%	ND
30	[(pCH_3)Phe[4]]NC(1−13)-NH_2	7.96 (0.13)	−106 ± 3%	ND
31	[(pNH_2)Phe[4]]NC(1−13)-NH_2	6.59 (0.54)	−105 ± 1%	ND
32	[(pOH)Phe[4]]NC(1−13)-NH_2	6.87 (0.25)	−103 ± 3%	ND

[a] ND: not determined because these compounds are full agonists. Inactive: inactive up to 10 μM. These data are the mean of at least four experiments.

receptor interaction can be improved by the presence of adequate chemical groups in the p-position of Phe[4]. Placement of F in m- (**24**) or o- (**25**) positions led to compounds with slightly higher (**24**) or similar (**25**) potency to NC(1−13)-NH_2.

Compounds **20**−**32** were also tested for competition with [^{3}H]NC-NH_2 binding to mouse brain membranes and the inhibition of forskolin stimulated cAMP accumulation in CHO cells stably expressing the human OP_4 receptor. Results summarized in Tables 3 and 4 confirm those of the biological assay by showing that compounds **20** (pF) and **26** (pNO_2) display pK_I values of 9.40 and 9.52, respectively, which are about 3-fold higher than that of NC(1−13)-NH_2 (pK_I 9.04). Compound **24** (mF) and **25** (oF) are 3- and 10-fold weaker than [(pF)Phe[4]]NC(1−13)-NH_2. Potencies of the other p-substituted derivatives are similar (**21, 22, 27, 28**) or inferior (**23, 29**−**32**) to that of NC(1−13)-NH_2. Collectively, these binding data (Table 3) are in good agreement with those of the tissue bioassay as indicated by the correlation coefficient (r^2) of 0.66.

The order of potency for inhibition of forskolin stimulated cAMP formation in CHO_{hOP4} cells of p-halogenated analogues (Table 4) is F (**20**) > Cl (**21**) > Br (**22**) > I (**23**), with the F derivative being 2-fold more potent than NC(1−13)-NH_2. The presence of F in the p-position gives the most potent compound, while F in the m- and o-positions reduces potency by 2 and 10-fold, respectively. These differences could be attributed to the magnitude and direction of the dipole moment of the Phe[4] side chain, which may influence the interaction of NC (1−13)-NH_2 with the OP_4 receptor.

Worthy of mention is the observation that compound **32** behaves as a receptor agonist, while acting as an antagonist in the mouse vas deferens. Thus, **32** resembles [Phe$^1\psi$(CH_2−NH)Gly[2]]NC(1−13)-NH_2 [6] which also acts as an antagonist in the mouse vas deferens[24] and as a full agonist in CHO_{hOP4} cells.[23] For a detailed discussion of the possible reasons that may explain the spectrum of pharmacological actions of [Phe$^1\psi$(CH_2−NH)Gly[2]]NC(1−13)-NH_2, the reader is refereed to the review article by Calo' et al.[2]

There is good agreement between data obtained in the mVD, mB membranes, and CHO_{hOP4} assays as indicated by the high correlation coefficients between cAMP−mouse vas deferens, r^2 = 0.81, and cAMP−receptor binding, r^2 = 0.79.

To determine if changes in potency/affinity correlate with physicochemical properties of the substituents (namely hydrophobicity, electronic, and steric features), we have analyzed our data according to Hansch,[27] using the following as parameters: (i) the Hammett constant σ_p which is the difference between the log of the ionization constant for benzoic acid in water at 25 °C and the log of the ionization constant for the p-derivative under the same experimental conditions. Positive values of σ_p represent electron withdrawal by the substituent from the aromatic ring, and negative σ_p values indicate electron release to the aromatic ring. (ii) The Hansch hydrophobic constant π which is the difference between the log of the partition coefficient of benzoic acid in water/octanol at 25 °C and the log of the partition coefficient of the p-substituted derivative under the same experimental conditions. Positive values of π indicate that the substituent favors the octanol phase (i.e. hydrophobic character), and negative values indicate a hydrophilic character relative to H. (iii) The Taft steric constant, Es, which can be defined as the ratio between the rate constant for acid hydrolysis of the esters X-CH_2COOR and CH_3COOR. This parameter depends essentially on the steric hindrance of the X substituent. Values of parameters σ_p, π, and Es, taken from Hansch and Leo,[27] are available as Supporting Information.

Simple regression analysis (i.e. $y = f(x)$) was performed between the independent variables σ_p, π, and Es and the values of potency estimated for the p-position substituted analogues in the mouse vas deferens assay (Table 2) and in the cAMP accumulation assay on CHO_{hOP4} cells (Table 4) or the values of affinity estimated for the same compound in the receptor binding assay (Table 3). As an example, Figure 4 displays the regression analysis obtained between receptor binding affinity and the σ_p. For each regression analysis the determination coefficient (r^2) was calculated, and these values are summarized in Table 5. Determination coefficient values obtained using π or Es as independent variables were very low for all sets of data. However, with σ_p as the independent variable, statistically significant values of the determination coefficients for all

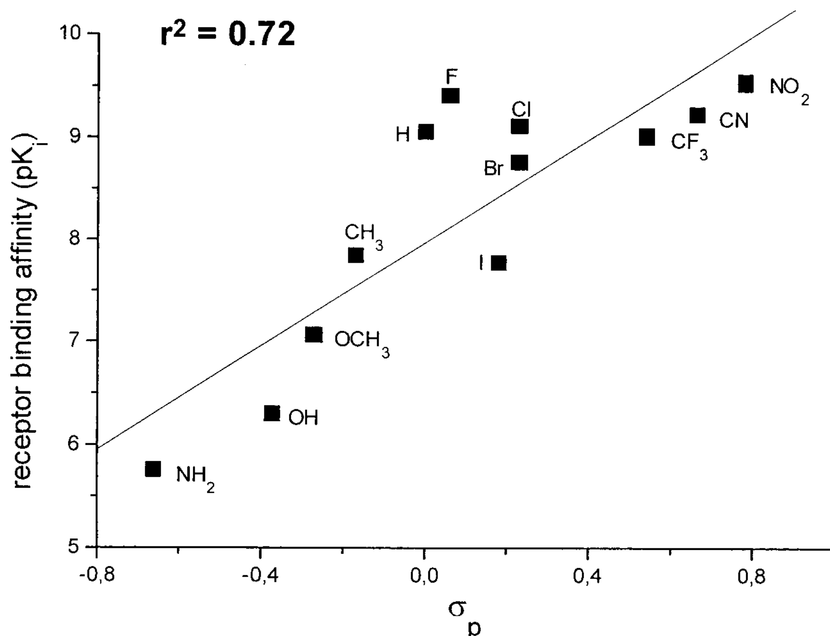

Figure 4. Correlation between receptor binding data and the Hansch parameter σ_p for the compounds of the third series.

Table 5. Determination Coefficients (r^2) Obtained by Simple Regression between the Independent Variables σ_p, π, and Es and the Values of Potency/Affinity Estimated in the Mouse Vas Deferens, Receptor Binding, and cAMP Assays for Analogues of the Third Series Substituted in the p-Position of Phe[4]

	σ_p	π	Es
cAMP-CHO$_{hOP4}$	0.47[a]	0.05	0.01
mVD	0.51[a]	0.01	0.01
receptor binding	0.72[a]	0.20	0.09

[a] $p < 0.05$.

three sets of data (Table 5) were observed. These results indicate that lipophilicity and steric features of the chemical groups used for substituting the H atom in the p-position of Phe[4] may not be relevant for biological activity, while the electron withdrawing/releasing effect of the p-substituted chemical group from/to the aromatic ring of Phe[4] appears to be important. Indeed, the higher the electron withdrawal effect of the substituent group from the aromatic ring, the higher the potency/affinity value.

To determine if the lipophilic and steric characteristics together with the electronic effects of the substituents may better correlate with the biological data, a multiple regression analysis (i.e. $y = f(x,z)$) was performed using, as independent variables, (σ_p, π) and (σ_p, Es). Determination coefficients r^2 of the regression analysis performed with (σ_p, π) as independent variables were not significantly different from those obtained using σ_p alone (data not shown). In contrast, when (σ_p, Es) were used as independent variables, the determination coefficients were higher than those obtained using only σ_p i.e., from 0.51 (σ_p) to 0.76 (σ_p, Es) for the mouse vas deferens data, from 0.47 (σ_p) to 0.67 (σ_p, Es) for the cAMP-CHO$_{hOP4}$ data, and from 0.72 (σ_p) to 0.78 (σ_p, Es) for the receptor binding data. These increases in r^2 values are statistically significant ($p < 0.05$) for the mouse vas deferens and cAMP-CHO$_{hOP4}$ data and approached ($p = 0.14$) statistical significance for the receptor binding data. Details of the calculated equations and their parameters are available as Supporting Information.

Collectively this QSAR analysis indicates that (i) the lipophilic character of the substituents is not relevant for biological activity, (ii) the electron withdrawal effect from the Phe[4] aromatic ring is crucial for biological activity, and (iii) the steric features of the substituents are not critical per se. However, these are important when considered together with electron withdrawal properties. In particular, for those substituents showing similar values of σ_p (e.g. Cl, Br, I) the biological activity is negatively related to their Es value, i.e., the smaller the group the higher the potency/affinity of the analogue. In other words, these findings suggest that the highest biological activity is obtained when a group with strong electron withdrawal properties (and less importantly with small dimensions) is present in the p-position of Phe[4].

QSAR parameters that we have considered (σ_p, π, and Es) do not take into account the hydrogen bonding properties of the substituents. In this regard, it is worthy of note that the substituents which are able to accept but not to donate a hydrogen bond (i.e. NO_2, CN, and under certain conditions F[28]) seem to favor biological activity. This also seems evident when comparing the activity of compounds **32** and **29**. In the former analogue, the hydroxy function acts as a hydrogen bond donor. This property associated with the electron releasing tendency is not tolerated. Methylation of this function, as in **29**, partially restored biological activity, possibly due to the hydrogen bond accepting ability of the OCH_3 group.

Currently little is known about the interaction of NC with its receptor. However, molecular modeling[29] and site directed mutagenesis[30] studies performed by Meunier's group suggest that Phe[4] of NC(1−13)-NH_2 sequence may interact with the Phe[220] (TM V) and Tyr[131] (TM III) residues of the OP_4 receptor (see Figure 6 of the paper by Topham et al.[29]). Electronic changes in the aromatic ring of Phe[4] induced by p-substitution with electron withdrawing groups might facilitate the interaction with the two aromatic residues of the receptor (Phe[220]/Tyr[131]). In addition to this, the pocket

of the receptor in which Phe[4] is located also contains Gln[286]. This residue plays a pivotal role in receptor transduction since its mutation with Ala generates a mutant receptor that normally binds NC but is totally unresponsive to agonists.[30] The ability of the substituents to accept (but not donate) a hydrogen bond might be important for the interaction of (pX)Phe[4] (peptide) with the amide function of the Gln[286] side chain (receptor). Of course, these initial working hypotheses require further experimentation.

Conclusions

In the present study we describe a systematic SAR analysis of the Phe[4] pharmacophore of the peptide NC(1−13)-NH_2. All attempts to reduce conformational freedom or to modify the aromaticity of the Phe[4] side chain were found to be detrimental for biological activity. Introduction of halogens into the phenyl ring of Phe[4] led to significant changes in activity with the most potent compound being the pF analogue. NO_2 and CN groups in the same position also increased the ability of the analogues to bind to and activate the OP_4 receptor. These compounds (e.g. [(pF)Phe[4]]NC(1−13)-NH_2) behaved as agonists of the OP_4 receptor displaying higher potency than the natural sequence.

Experimental Section

Materials. Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, Fluka (Switzerland), or Chem-Impex International (U.S.A).The resin [5-(4′-Fmoc-aminomethyl-3′, 5′-dimethoxyphenoxy)valeric acid]-poly(ethylene glycol)/polystyrene support (Fmoc-PAL-PEG-PS) was from Millipore (Waltham, MA). The $C\beta(CH_3)$-Treo-Phe and $C\beta(CH_3)$-Eritro-Phe were synthesized as reported.[20] Naloxone was from Tocris Cookson (Bristol, 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) was of the following composition (in mM): NaCl (118.5), KCl (4.7), KH_2PO_4 (1.2), $NaHCO_3$ (25), $CaCl_2$ (2.5), and glucose (10). All other reagents were from Sigma Chemical Co. (Poole, U.K.) or E. Merck (Darmstadt, Germany) and were of the highest purity available.

Peptide Purification and Analytical Determinations. Crude peptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 × 4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 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) over 25 min for the elution of peptides. Analytical HPLC analyses were performed on a Beckman 125 liquid chromatograph fitted with a Alltech C_{18} column (4.6 × 150 mm 5 μm particle size) and equipped with a Beckman 168 diode array detector. Analytical purity and retention time (t_R) of the peptides were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at a flow rate of 1 mL/min using the following linear gradients: (I) from 0% to 50% B over 25 min and (II) from 0% to 20% B over 25 min. The $C\beta(CH_3)$-Treo-Phe, $C\beta(CH_3)$-Eritro-Phe, and Atc amino acids were used, for the synthesis of the corresponding NC(1−13)-NH_2 derivatives, as enantiomeric mixtures; (1) and (2) in Table 1 indicate the corresponding diastereomeric NC(1−13)-NH_2 analogues with earlier and later elution times, respectively**.** All analogues showed > 97% purity when monitored at 220 nm. Molecular weights of compounds were determined by a MALDI-TOF (matrix assisted laser desorption ionization time-of-flight) analysis using a Hewlett-Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as the matrix. Values are expressed as MH^+.

General Procedures for the Solid-Phase Synthesis. As an illustrative example the synthesis of [(pF)Phe[4]]NC(1−13)-NH_2 (20) is described. Fmoc-PAL-PEG-PS resin (0.21 mmol/g, 0.5 g) was treated with 20% piperine/DMF and linked with N^{α}-Fmoc-N^{ϵ}-(Boc)-Lys by using [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] HATU[31] as coupling reagent. The following N^{α}-Fmoc amino acids were sequentially coupled to the growing peptide chain: N^{α}-Fmoc-N^{ω}-(Pmc)-Arg, 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-(pF)Phe, N^{α}-Fmoc-Gly, N^{α}Fmoc-Gly, and N^{α}-Fmoc-Phe. All the N^{α}-Fmoc amino acids (4 equiv) were coupled to the growing peptide chain by using HATU (4 equiv) in DMF, and the coupling reaction time was 1 h. Piperidine (20%)/DMF was used to remove the Fmoc group at every step. The peptide resin was washed with methanol and dried in vacuo to yield the protected [(pF)Phe[4]]NC(1−13)NH_2-Resin. The other peptides 2−32 were synthesized in a similar manner. The protected peptide-resin was treated with reagent K[32] (TFA/H_2O/phenol/ethanedithiol/thioanisole, 82.5:5:5: 2.5: 5; v/v; 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 was triturated with ether. The crude peptide was purified by preparative reverse phase HPLC to yield a white powder after lyophilization.

Mouse Vas Deferens Studies. Swiss male mice weighing 25−30 g were used, and bioassay experiments were performed as previously described.[7] Briefly, the vas deferens (mVD) was prepared according to Hughes et al.[33] 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 0.05 Hz frequency. Resting tension was maintained at 0.3 g. 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 to NC and NC related peptides were performed (0.5 log unit steps). When required, antagonists were added to the Krebs solution 15 min before performing crc for agonists.

Binding Studies with Mouse Brain Membranes. Male Swiss mice weighing 25−30 g were used, and binding experiments were performed as previously described.[7] Briefly, the mice were decapitated, and the forebrain was dissected on ice. The tissue was disrupted in a Polytron homogenizer (setting 5) in 20 volumes of 50 mM Tris HCl, 2 mM EDTA, 100 μM phenylmethylsulfonylfluoride (PMSF) at pH 7.4. The homogenate was centrifuged at 40 000g for 10 min, and the pellet was resuspended in the same buffer. After 30 min incubation at 37 °C, the membranes were centrifuged, and the resulting pellets were stored at −80 °C. Prior to freezing, an aliquot of the homogenate was removed for protein assay using the Bio Rad method,[34] with bovine albumin as a reference standard. The final pellet was resuspended in the same incubation buffer at a concentration of 200 μg protein/100 μL, and this homogenate was used in the binding assay. Displacement experiments were carried out in duplicate in a final volume of 250 μL in test tubes containing 0.5 nM [^{3}H]NC-NH_2, 50 mM Tris HCl buffer, 2 mM EDTA, 100 μM PMSF at pH 7.4, mouse forebrain membranes (200 μg protein/assay), and different concentrations of the ligand under study. Details of the synthesis of the radioligand [^{3}H]-NC-NH_2 have already been reported.[35] The inhibitory binding constant, K_I, was calculated from the IC_{50} value using the Cheng & Prusoff equation.[36] Nonspecific binding was defined as that in the presence of 10 μM NC-NH_2 and was about 30% of the total binding. Based on previous studies the incubation time was 120 min at 25 °C.[35] The incubation mixture was diluted with 3 mL of ice-cold incubation buffer then bound and free radioactivity separated by filtering the assay mixture through Whatman GF/C glass-fiber filters using a Brandel cell harvester. Filters were washed three times with 3 mL of incubation buffer, and

filter-bound radioactivity was counted in a Beckman LS-1800 Spectrometer (efficiency 55%). Under the experimental conditions described here we demonstrated that mouse forebrain membranes express a single class of binding sites for [^{3}H]-NC-NH_2 with a K_d value of 0.55 nM and a B_{max} value of about 100 fmol/mg protein. Moreover, the pharmacological profile of this site was identical to that of the NC receptor expressed in the mVD.[7,22]

Inhibition of cAMP accumulation in CHO cells. Accumulation of cAMP was measured in whole CHO_{hNCR} cells incubated in 0.3 mL volumes of Krebs-HEPES buffer containing BSA as described in detail by Okawa et al.[23] In addition, 1-isobutyl-4-methylxanthine (1 mM) and forskolin (1 μM) were also included. In concentration response curves to NC related peptides the maximum concentration tested was 10 μM. All incubations were for 15 min at 37 °C, and cAMP was extracted and assayed as previously described.[23]

Data Analysis and Terminology. Data are expressed as a mean of *n* experiments. For pEC_{50}, pK_B, and pK_I values the confidence limits at 95% ($CL_{95\%}$) are given. A weighted nonlinear least-squares curve fitting program LIGAND[37] was used for computer analysis of binding inhibition experiments. The pharmacological terminology adopted in this study is in line with IUPHAR recommendations.[38] 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 maximal possible effect. Apparent affinities of antagonists are given in terms of pK_B which were calculated using the Gaddum Schild equation: $pK_B = \log((CR - 1)/[\text{antagonist}])$ assuming a slope value equal to unity, where CR is the ratio between equieffective concentrations of agonist in the presence and absence of the antagonist. Ligand affinities obtained in binding competition experiments are given as pK_I = the negative logarithm to base 10 of the inhibition equilibrium constant.

Acknowledgment. This work was supported by the Italian Ministry of the University (Cofin 1999 grant to D.R.), by the University of Ferrara (60% grants to S.S. and G.C.), and by the British Council/Conferenza dei Rettori delle Universita' Italiane (travelling grant to G.C. and D.G.L.).

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(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: Boc (*tert*-butoxycarbonyl), DMF (*N,N*-dimethylformamide), Fmoc [(9-fluorenylmethyl)oxycarbonyl], Fmoc-PAL-PEG-PS [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)-valeric acid] on the poly(ethylene glycol)/polystyrene support), MALDI-TOF (matrix assisted laser desorption ionization time-of-flight), Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl), tBu (*tert*-butyl), TFA (trifluoroacetic acid).

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JM010221V