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Discovery of Dipeptides with High Affinity to the Specific Binding Site for Substance P_{1-7}

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Substance P 1−7 (SP_{1-7}, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH) is the major bioactive metabolite of substance P. The interest in this heptapeptide originates from the observation that it modulates, and in certain cases opposes the effects of the parent peptide, e.g., the nociceptive effect. The μ-opioid receptor agonist endomorphin-2 (EM-2, H-Tyr-Pro-Phe-Phe-NH_2) has been found to also interact with the specific binding site of SP_{1-7} with only a 10-fold lower affinity compared to the native peptide. Considering the smaller size of EM-2 compared to the target heptapeptide, it was selected as a lead compound in the development of low-molecular-weight ligands to the SP_{1-7} binding site. An alanine scan and truncation study led to the unexpected discovery of the dipeptide H-Phe-Phe-NH_2 (K_i = 1.5 nM), having equal affinity as the endogenous heptapeptide SP_{1-7}. Moreover, the studies show that the C-terminal phenylalanine amide is crucial for the affinity of the dipeptide.

Introduction

The undecapeptide substance P (SP[a]), discovered in 1931, was the first mammalian neuropeptide to be identified.[1] SP is a neurotransmitter and neuromodulator at G-protein coupled neurokinin-1 (NK-1) receptors and is involved in a variety of processes in different tissues. In the past, its role in pain transmission in the central nervous system (CNS) has attracted the most attention.[2] However, lately, interest has focused on the involvement of SP in mental disorders like depression[3] and anxiety.[4] Despite worldwide efforts toward development of SP related drugs, aprepitant (MK-869, approved as an antiemetic agent) remains the only NK-1 receptor antagonist on the market.[5]

Neuropeptides are in many cases degraded into bioactive fragments with similar or modified biological activity. Additionally, it seems that the degradation products often exert a modulatory effect on the response of the parent peptide.[6] Substance P is cleaved enzymatically into at least five metabolites, of which the N-terminal heptapeptide SP_{1-7} (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH) is the major fragment.[7,8] SP_{1-7} has been shown not only to modulate several actions of SP but also oppose several effects of SP, e.g., the nociceptive effect,[9] inflammatory effect,[10] and the potentiating effect on opioid withdrawal symptoms.[11,12] In addition, SP_{1-7} has also been shown to promote memory function.[13]

Although the actions of SP_{1-7} are well-known, no explicit receptor for this peptide fragment has yet been identified. However, specific binding sites for SP_{1-7} in mouse and rat spinal cord and in the ventral tegmental area of the rat brain have been found.[14−16] SP_{1-7} displayed greater affinity to these binding sites than SP and other related degradation fragments, e.g., SP_{1-6}, SP_{1-8}, and SP_{1-9} (100−2000 times lower). Moreover, established ligands [Sar^9, $Met(O_2)^{11}$]-SP, R396, and senktide of the NK-1, NK-2, and NK-3 receptors, respectively, showed weak or negligible affinity toward the SP_{1-7} binding sites. Negligible binding of SP_{1-7} to any of the known tachykinin and μ-opioid receptors indicates the occurrence of a specific binding site for SP_{1-7}, which most probably is a specific receptor according to data reported for this bioactive fragment.[14,15]

Recent published data by Zhou et al. indicates that the attenuating effect on morphine withdrawal by SP_{1-7} might be related to the σ receptor system because a σ receptor agonist could reverse the SP_{1-7} related effect observed in the study.[17] However, the same σ receptor agonist showed weak binding affinity to the SP_{1-7} binding site, which indicates that SP_{1-7} is modulating the effect seen for the σ receptor system rather than working directly as a ligand for the σ receptor.

Historically, the identification of receptors (mostly G-protein coupled receptors, GPCRs) combined with the development of nonpeptide antagonists and agonists have been crucial for the elucidation of the functional role of neuropeptides.[18,19] Because they are encoded in the genome, the function of a neuropeptide and its role in the mechanism behind diseases can be studied by knockout techniques in transgene animals.[20] By nature, the specific biological effects of metabolites of genome-encoded peptides cannot be studied by such a technique. Thus, metabolically stable and selective nonpeptide ligands (or peptidomimetics) of neuropeptide metabolites are essential pharmacological tools for such studies.

We aim at developing metabolically stable SP_{1-7} mimetics to be used as research tools for the identification of the SP_{1-7}

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[a] Abbreviations: SP, substance P; NK-1, neurokinin-1; CNS, central nervous system; NK-2, neurokinin-2; NK-3, neurokinin-3; GPCR, G-protein coupled receptor; SAR, structure−activity relationship; EM-2, endomorphin-2; EM-1, endomorphin-1; FHDoE, focused hierarchical design of experiments; Phg, phenylglycine; Tyr(OMe), *O*-methyltyrosine; Phe(2-Me), 2-methylphenylalanine; Thi, 2-thienylalanine; Cha, cyclohexylalanine; Phe(3-F), 3-fluorophenylalanine; tciz, theoretical chemically intuitive *z*-scale; ACE, angiotensin-converting enzyme; Boc, *tert*-butoxycarbonyl; NKB, neurokinin B. Abbreviations used for amino acids are according to the rules of the IUPAC-IUB in Jones, J. H. *J. Pept. Sci.* **2003**, *9*, 1−8.

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target (for example a receptor) and for functional studies in complex animal models. In the long term, such compounds could be of pharmaceutical interest, i.e., as analgesics, anxiolytics, or anti-inflammatory drugs. Previously, we reported a thorough structure−activity relationship (SAR) study of SP_{1-7} involving an alanine-scan, truncations, and C- and N-terminal modifications of the heptapeptide.[21] It was demonstrated that the C-terminal part of the heptapeptide and especially the phenylalanine in position 7 is most essential for binding. Moreover, C-terminal amidation potentiated the ligands 5−10-fold; an effect also observed in an in vivo study where the amidated analogue of SP_{1-7} was shown to reduce opioid withdrawal signs in rats more effectively than the native heptapeptide.[17] The SAR study further led to the identification of the tripeptide H-Gln-Gln-Phe-NH_2 (K_i = 1.9 nM) showing equal affinity as the parent heptapeptide (K_i = 1.6 nM). The tripeptide constitutes a good starting point toward low-molecular-weight drug-like compounds.

These results indirectly explained a previous observation by Botros et al. that the tetrapeptide endomorphin-2 (EM-2, H-Tyr-Pro-Phe-Phe-NH_2), having a C-terminal phenylalanine amide, is a relative efficient binder to the SP_{1-7} binding site with only a 10-fold reduction in affinity compared to SP_{1-7}.[15] EM-2 together with endomorphin-1 (EM-1, H-Tyr-Pro-Trp-Phe-NH_2) were identified as late as 1997 and constitute the most potent endogenous ligands for the μ-opioid receptors.[22] Even though EM-1 contains a C-terminal phenylalanine, it does not possess any significant affinity to the SP_{1-7} binding site, suggesting the presence of a discriminating binding pocket not able to fit a tryptophan adjacent to the phenylalanine. The SAR of EM-1 and EM-2 for the affinity to the μ-opioid receptors is rather well-explored, and in this case the N-terminal amine and the phenolic tyrosine part are crucial.[23−25] Thus, an alanine scan of EM-2, examining the importance of each amino acid residue for the μ-opioid receptor interaction, showed that the substitutions of tyrosine and proline for an alanine reduced affinity 20000- and 5000-fold, respectively. However, when substituting the two C-terminal phenylalanines, the affinity dropped only 400- and 100-fold, respectively.[23]

We felt prompted to perform a SAR study of EM-2 toward its binding to the SP_{1-7} binding site. Thus, we herein present the results from an alanine scan and truncation study of EM-2 and report on the discovery of the dipeptide H-Phe-Phe-NH_2, found to have higher affinity to the SP_{1-7} binding site than the parent tetrapeptide EM-2 and equal affinity as the heptapeptidic endogenous ligand SP_{1-7}. The structural and stereochemical requirements for the binding of H-Phe-Phe-NH_2 have been further studied via small dipeptide libraries based on this lead.

Results

Affinity Studies toward the SP_{1-7} Binding Site. Peptides **1**−**20** were prepared using solid-phase peptide synthesis. The purities determined by RP-HPLC (at 220 nm) were ≥96%. In Table 1, the analytical data and the purities of the synthesized peptides are shown. The affinity of these to the SP_{1-7} binding site was determined in a binding assay using spinal cord membrane from Sprague−Dawley rats and radioactive [^{3}H]-SP_{1-7} as a tracer. Competition experiments were performed at six different concentrations, as illustrated in Figure 1. The K_i values of **1**−**20** are reported in Table 2.

Table 1. Analytical Data and Purities of Peptides **1**−**20**

compd	molecular mass	M + H$^+$	purity (%)[a]	purity (%)
1	571.3	572.3	99.8	>99.9[b]
2	479.3	480.3	98.5	>99.9[b]
3	545.3	546.3	99.8	>99.9[b]
4	495.3	496.3	99.6	>99.9[b]
5	495.3	496.3	99.7	>99.9[b]
6	572.3	573.3	>99.9	>99.9[b]
7	408.2	409.1	>99.9	>99.9[c]
8	311.2	312.1	>99.9	>99.9[b]
9[d]	164.1	165.0		
10	312.2	313.1	>99.9	96.2[c]
11	353.2	354.1	>99.9	>99.9[c]
12	311.2	312.1	98.4	>99.9[c]
13	311.2	312.1	>99.9	>99.9[c]
14	311.2	312.1	99.3	99.4[c]
15	297.2	298.1	>99.9	99.8[c]
16	277.2	278.1	>99.9	99.7[c]
17	355.2	356.2	>99.9	>99.9[c]
18	317.1	318.1	>99.9	>99.9[c]
19(I)	297.2	298.1	>99.9	>99.9[c]
19(II)	297.2	298.1	>99.9	>99.9[c]
20	335.2	336.2	>99.9	>99.9[c]

[a] The purity was determined by RP-HPLC with a H_2O/MeCN gradient with 0.1% TFA and UV detection at 220 nm using the column: ACE 5 C18 (50mm × 4.6 mm). [b] The purity was determined by RP-HPLC using the column: ACE 5 Phenyl (50 mm × 4.6 mm). [c] The purity was determined by RP-HPLC using the column: Thermo Hypersil Fluophase RP (50 mm × 4.6 mm). [d] The peptide was of analytical grade from commercial source and tested without any further purification.

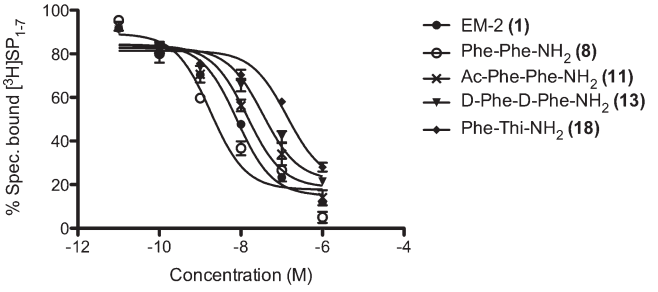

Figure 1. Inhibition of [^{3}H]-SP_{1-7} binding by selected representative peptides. Data represent three independent experiments in triplicate as mean ± SEM.

To determine the importance of the EM-2 amino acid side chains for the binding to the SP_{1-7} binding site, each amino acid residue was sequentially substituted for an alanine. The three N-terminal amino acid residues (tyrosine, proline, and the internal phenylalanine) could be substituted for an alanine without significantly affecting the affinity (cf. **1** with **2**, **3**, and **4**). However, the same substitution of the C-terminal phenylalanine resulted in a dramatic loss of affinity (see compound **5**). These results prompted the synthesis and development of N-terminally truncated analogues **7**−**9**. While the removal of the N-terminal tyrosine was well tolerated, the removal of the two N-terminal amino acids resulted in a 6-fold higher binding affinity (**8**) compared to the parent peptide **1**. However, further truncation to the single phenylalanine amide **9** was deleterious for the affinity. To assess the influence of the C-terminal primary amide, carboxylic acid derivatives **6** and **10** were prepared. Introduction of a carboxylic acid in the tetrapeptide **6** resulted in a 3-fold drop in binding affinity, while this modification in the corresponding dipeptide gave an inactive compound.

N-terminal acetylation of the dipeptide was accompanied with a 10-fold decrease in affinity. The preferred configuration of the two phenylalanines of H-Phe-Phe-NH_2 was determined via the evaluation of the four L and D-variants **8** and **12**−**14**. The natural L,L configuration gave the highest binding affinity.

To investigate the effect on affinity from small variations in side chain size and polarity, six new dipeptide analogues of **8** were designed using the focused hierarchical design of experiments method (FHDoE).[26] FHDoE is a statistical molecular design method for selecting a diverse set of analogues based on an active peptide. Each peptide in the set is designed to retain a high resemblance to the active peptide, thus increasing the probability of obtaining new active compounds. Using the FHDoE method, the six dipeptides **15**−**20** shown in Table 2 (for an illustration of the used amino acids see Figure 2) were selected and prepared. Three of the new dipeptides were inactive (**15**, **17**, and **20**, K_i > 10000 nM) and the other three (**16**, **18**, and **19**) had significantly lower affinity compared to **8** as shown in Table 2.

Table 2. K_i Values of EM-2 Derivatives and Phe−Phe Analogues for Inhibition of [^{3}H]-SP_{1-7} Binding to Rat Spinal Cord Membrane

compd	sequence	K_i ± SEM (nM)
SP_{1-7}	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH	1.6 ± 0.1
1 (EM-2)	H-Tyr-Pro-Phe-Phe-NH_2	8.7 ± 0.1
	Alanine Substituted and Terminally Modified Peptides	
2	H-Ala-Pro-Phe-Phe-NH_2	11.5 ± 0.1
3	H-Tyr-Ala-Phe-Phe-NH_2	10.2 ± 0.3
4	H-Tyr-Pro-Ala-Phe-NH_2	9.4 ± 0.1
5	H-Tyr-Pro-Phe-Ala-NH_2	1460 ± 15
6	H-Tyr-Pro-Phe-Phe-OH	30.2 ± 1.7
	Truncated Peptides	
7	H-Pro-Phe-Phe-NH_2	10.9 ± 0.7
8	H-Phe-Phe-NH_2	1.5 ± 0.1
9	H-Phe-NH_2	5028 ± 31
	Terminally Modified Phe−Phe Peptides	
10	H-Phe-Phe-OH	> 10000
11	Ac-Phe-Phe-NH_2	18.5 ± 1.7
	Phe−Phe Analogues	
12	(L)-Phe-(D)-Phe-NH_2	540 ± 20
13	(D)-Phe-(D)-Phe-NH_2	64 ± 3
14	(D)-Phe-(L)-Phe-NH_2	175 ± 13
	Phe−Phe Analogues Having Noncoded Amino Acids (FHDoE Generated)	
15	H-Phe-Phg-NH_2	> 10000
16	H-Leu-Phe-NH_2	10.2 ± 1.0
17	H-Tyr(OMe)-Phe(2-Me)-NH_2	> 10000
18	H-Phe-Thi-NH_2	251 ± 4
19[a]	H-Phg-Phe-NH_2	(I) 2247 ± 115 (II) 182 ± 7
20	H-Cha-Phe(3-F)-NH_2	> 10000

[a] I and II: either of the diastereomers (L-Phg-L-Phe-NH_2, D-Phg-L-Phe-NH_2).

Human NK-1 and NK-3 Receptor Binding of H-Phe-Phe-NH_2. The possible binding of H-Phe-Phe-NH_2 (**8**) to the human neurokinin receptors NK-1 and NK-3 was studied. The affinity of the compound was tested at a single concentration of 10 μM in agonist radioligand binding assays relying on displacement of [Sar^9, Met(O_2)^{11}]-SP from NK-1 receptors and [MePhe^7]-NKB from NK-3 receptors, respectively. Compound **8** showed neither affinity for the NK-1 receptor nor for the NK-3 receptor.

Discussion

The design of low-molecular-weight drug-like molecules as mimetics of bioactive peptides aims at eliminating problems inherent to the peptide structure such as low bioavailability and rapid degradation by proteolytic enzymes. Nonpeptide ligands have in the past worked as valuable tools in the investigation of neuropeptide receptors and their endogenous ligands.[19] Specifically, nonpeptide receptor antagonists have played an important role in understanding the physiological function of neuropeptides.[18]

In previous studies, it was found that the tetrapeptide EM-2 interacted with the SP_{1-7} site with only a 10-fold reduction in affinity. Furthermore, a few other Tyr-Pro-containing opioid related neuropeptides such as Tyr-MiF-1, β-casomorphin, and morphiceptin showed moderate affinity to the SP_{1-7} binding site.[15] Considering the smaller size of EM-2 compared with the target heptapeptide, it was chosen as a starting point for further development of low-molecular-weight SP_{1-7}-mimetics. A systematic survey of the influence of each amino acid of EM-2 for affinity to the SP_{1-7} binding site was initiated via an alanine scan and truncation study.

The results from the alanine scan and the N-terminal truncation study of EM-2 were conclusive and comparable with the previous SAR study of SP_{1-7}. Thus, the nature of the three N-terminal amino acids is not important for binding because these could be replaced by alanine without significant change in affinity (cf. **1** with **2**−**4**). In fact, the presence of the N-terminal Tyr-Pro sequence reduces the binding strength because its removal affords a dipeptide with 6-fold higher affinity (**8**, K_i = 1.5 nM) as compared with EM-2 (**1**, K_i = 8.7 nM). This terminated early speculation about the involvement of the Tyr-Pro sequence in binding to the SP_{1-7} binding site.[15] The C-terminal phenylalanine is required for strong binding to this site, as seen by the 170-fold drop in affinity (compared with EM-2) for the tetrapeptide **5**, having an alanine instead of phenylalanine.

However, this single phenylalanine is not sufficient for binding as can be observed for **9** with almost 600-times weaker binding affinity than EM-2. N-Terminal acetylation (dipeptide **11**) provides a 10-fold affinity reduction compared to the dipeptide amide **8**, suggesting that an interaction, possibly a hydrogen bond or an electrostatic interaction with the free amine at the second (N-terminal) phenylalanine, is lost. The C-terminal primary amide is also essential for the affinity; the corresponding tetrapeptide carboxylic acid (**6**)

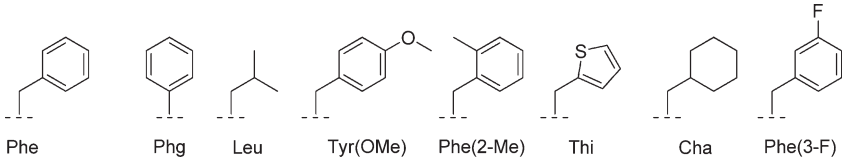

Figure 2. Formulas of the side chains of coded and noncoded amino acids used for the synthesis of FHDoE generated analogues.

has more than three times lower affinity. The same substitution in the dipeptide series results in a completely inactive carboxylic acid compound (**10**). The total loss of binding is more drastic than expected because the corresponding carboxylic acid tetrapeptide **6** still exhibits an affinity of 30 nM. Possibly, the C-terminal carboxylic acid and the C-terminal primary amide induce different conformations of the dipeptide. Altogether, is seems that a dipeptide with a free α-amino group and with a C-terminal phenylalanine amide fits well into a small discrete binding pocket for the C-termini of SP_{1-7} and EM-2. The penultimate phenylalanine is of less importance because the affinity remains unchanged in the tetrapeptide having alanine in this position.

The newly identified dipeptide lead **8** was further studied. The stereochemical requirements for binding to the SP_{1-7} binding site were evaluated by synthesis of the three remaining L and D variants **12**, **13**, and **14**. Obviously, the preferred configuration of the two phenylalanines in **8** is the natural L,L configuration. Of the other variants, the D,D compound **13** showed the highest affinity although 40-times lower than the L,L compound. For the D,L and the L,D dipeptides (**12** and **14**), the affinity was significantly reduced (120- and 360-fold, respectively). Thus, it seems that the mutual arrangement of the two phenylalanine side chains is important. It would be of interest to study these compounds for functional activity as well because there are several examples where an L to D switch of an amino acid within a neuropeptide results in antagonistic properties. However, such functional assays are still awaited.

To investigate the effects of structural modifications of **8** on binding affinity, the FHDoE method was used to design a small series of dipeptides with similar physicochemical properties as **8**. Because the FHDoE method yields relatively conservative modifications, the probability of obtaining high-affinity peptides should be rather high. However, all dipeptides selected using the FHDoE method were either inactive (K_i > 10 000 nM) or had considerably less affinity than **8**. This indicates that modifications of the dipeptide side chains are not well tolerated. It appears that the C-terminal phenylalanine is the one most sensitive toward modification because shortening of the side chain to give phenylglycine (**15**) resulted in inactivity. Furthermore, replacement of the phenyl ring for the bioisosteric thiophene ring as in compound **18** reduced the affinity 170-fold. *ortho*-Methyl and *meta*-fluoro substituents on the phenyl moiety gave inactive compounds (**17** and **20**). However, in these cases, the N-terminal amino acid was also changed which complicates the interpretation. The pocket for the side chain of the N-terminal amino acid seems to be less discriminating because the replacement of phenylalanine by leucine was rather well accepted, albeit with a 7-fold reduction in affinity (cf. **16** with **8**).

In summary, we have been able to identify a dipeptide H-Phe-Phe-NH_2 with affinity in the nanomolar range to the binding site of the endogenous heptapeptide SP_{1-7}. We are optimistic about the future conversion of this lead into stable nonpeptide ligands. In fact, dipeptides have previously served as successful starting points for the elegant design and development of orally bioavailable nonpeptides, e.g., antagonists to the NK-1, NK-2, and NK-3 receptors.[27] Several angiotensin-converting enzyme (ACE) inhibitors on the drug market can be seen as modified dipeptides,[28] and the dipeptide Boc-Phe-Phe-NH_2 was the lead for the development of PD161182, a nonpeptide NK-3 receptor selective antagonist.[29,30] Apart from the N-protecting Boc-group, it is identical to our dipeptide lead **8**, a fact that of course raises the question whether SP_{1-7} and the ligands tested here indeed target the NK-3 receptor. The possible binding of **8** to NK-1 and NK-3 receptors was therefore evaluated. However, no binding was achieved at a concentration of 10 μM. In addition, two independent research groups have found that established NK-3 agonists, i.e., the [Pro^7]NKB and senktide, are inactive at the SP_{1-7} binding site,[14,15] which further strengthens our hypothesis of an explicit, not yet isolated receptor with specific features for the SP_{1-7} fragment.

One interesting aspect of our results is that **8** is a naturally occurring cleavage product from the first step in the catabolism of EM-2.[31] In vivo, EM-2 is cleaved by dipeptidylpeptidase IV at the Pro^2-Phe^3 peptide bond and the dipeptides generated are then hydrolyzed into amino acids by an aminopeptidase. It is enticing to propose that some of the bioactivity exerted by SP_{1-7} might be attributed to the dipeptide acting at the SP_{1-7} binding site because co-occurrence of SP and EM-2 in the dorsal spinal cord has been established.[32]

Conclusion

A systematic evaluation of the affinity of peptide ligands originating from the tetrapeptide lead EM-2 toward the SP_{1-7} binding site has been performed. Thus, we were able to identify the dipeptide H-Phe-Phe-NH_2 with equal affinity as the endogenous heptapeptide ligand SP_{1-7} and higher affinity than the tetrapeptide lead EM-2. Further SAR studies suggest that the C-terminal phenylalanine amide is crucial for binding and that the L-configuration gives the preferred side chain arrangement. The results also indicate that the binding pocket interacting with the side chain of the N-terminal amino acid is more tolerant for changes. Conversion of the dipeptide lead H-Phe-Phe-NH_2 into stable nonpeptide ligands is in progress. H-Phe-Phe-NH_2 and nonpeptide ligands thereof will be explored further in functional animal studies to achieve a more thorough understanding of the physiological function of SP_{1-7}.

Experimental Section

General Methods. Preparative RP-HPLC was performed on a system equipped with a Zorbax SB-C8 column (150 mm × 21.2 mm) or a 10 μm Vydac C18 column (250 mm × 22 mm), in both cases with UV detection at 230 nm. Analytical RP-HPLC-MS was preformed on a Gilson-Finnigan ThermoQuest AQA system (Onyx monolithic C18 column, 50 mm × 4.6 mm; MeCN/H_2O gradient with 0.05% HCOOH) in ESI mode, using UV (214 and 254 nm) and MS detection. The purity of each of the peptides was determined by RP-HPLC using the columns ACE 5 C18 (50 mm × 4.6 mm) and ACE 5 Phenyl (50 mm × 4.6 mm) or Thermo Hypersil Fluophase RP (50 mm × 4.6 mm) with a H_2O/MeCN gradient with 0.1% TFA and UV detection at 220 nm. All peptides showed purity above 95%.

NMR spectra were recorded on a Varian Mercury plus spectrometer (^1H at 399.8 MHz and ^{13}C at 100.5 MHz or ^1H at 399.9 MHz and ^{13}C at 100.6 MHz) at ambient temperature. Chemical shifts (δ) are reported in ppm referenced indirectly to TMS via the solvent residual signal. Exact molecular masses were determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source at the Department of Pharmaceutical Biosciences, Uppsala University, Sweden. Amino acid analyses were performed at the Department of Biochemistry and Organic Chemistry, Uppsala University, Sweden. All chemicals and solvents were of analytical grade from commercial sources.

General Synthesis of Peptides 1−6. The peptides **1**−**6** were synthesized with a Symphony instrument (Protein Technologies, Inc., Tucson, AZ) using standard Fmoc chemistry as earlier described.[21] Cleavage and purification were carried out as

described below. The reported yields are based on the loading of the starting resin and with correction for peptide content according to amino acid analysis.

Peptide 8 (H-Phe-Phe-NH_2). To an ice-cold solution of Z-Phe-OSu (200 mg, 0.505 mmol) and the hydrochloride salt of H-Phe-NH_2 (111 mg, 0.555 mmol) in EtOAc (15 mL) *N*,*N*-diisopropylethylamine (DIEA, 97 μL, 0.555 mmol) was added and the mixture stirred at room temperature. After 1.5 h, an additional portion of DIEA (97 μL, 0.555 mmol) was added and the mixture was stirred overnight. The precipitate was filtered off and dried under vacuum to yield Z-Phe-Phe-NH_2 (131 mg) as a white solid. The crude product was analyzed by LC-MS and found to be adequate for further reaction.

The Z-protected product was dissolved in MeOH:$CHCl_3$ (1:1, 10 mL) in a round-bottom flask and Pd/C (10%, 33 mg, 0.032 mmol) was added. The flask was sealed with a septum and purged, first with nitrogen and then with hydrogen gas. The reaction mixture was stirred under hydrogen atmosphere overnight and was then filtered and evaporated. The product was purified by RP-HPLC as described below to yield H-Phe-Phe-NH_2 in 19 mg (20%). HPLC purity: C18 column >99.9%, phenyl column >99.9%. LC/MS (M: 311.2): 312.1 ($M + H^+$). ^{1}H NMR (CD_3OD) δ 2.96 (dd, J = 8.5, 13.8 Hz, 1H), 3.01 (dd, J = 8.6, 14.3 Hz, 1H), 3.14 (dd, J = 6.2, 13.8 Hz, 1H), 3.26 (dd, J = 5.5, 14.3 Hz, 1H), 4.08 (J = 5.5, 8.6 Hz, 1H), 4.66 (dd, J = 6.2, 8.5 Hz, 1H), 7.17−7.39 (m, 10H). ^{13}C NMR (CD_3OD) δ 38.6, 39.1, 55.5, 56.0, 127.9, 128.9, 129.5, 130.2, 130.3, 130.5, 135.5, 138.2, 169.5, 175.3. Amino acid analysis: Phe, 2.00 (70% peptide).

General Synthesis of Peptides 7 and 10−20. Coupling. The peptides **7** and **10−20** were synthesized manually from Rink Amide MBHA resin (0.66 mmol/g) or Phe-2-chlorotrityl resin (0.85 mmol/g) in 2 mL disposable syringes fitted with porous polyethylene filters. Standard Fmoc conditions were used, and the Fmoc protecting group was removed by treatment with 20% piperidine in DMF (2 × 1.5 mL, 2 + 10 min) and the polymer was washed with DMF (6 × 1.5 mL, 6 × 1 min). Coupling of the appropriate amino acid, Fmoc-AA-OH (4 equiv) was performed in DMF (1.5 mL) using *N*-[(1*H*-benzotriazole-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU, 4 equiv) in the presence of DIEA (8 equiv). The resin was washed with DMF (5 × 1.5 mL, 5 × 1 min) and subsequently deprotected and washed as described above. After completion of the coupling cycle, the resin was also washed with several portions of DMF, CH_2Cl_2, and MeOH before it was dried in in vacuo.

Cleavage. The final peptide was cleaved from the resin by treatment with triethylsilane (100 μL) and 95% aqueous trifluoroacetic acid (TFA, 1.5 mL) followed by agitation for 2 h at room temperature. The resin was filtered off and washed with TFA (2 × 0.3−0.5 mL). The filtrate was collected in a centrifuge tube and concentrated in a stream of nitrogen. Cold diethyl ether (≤7 mL) was used to precipitate the product, which was collected by centrifugation, washed with cold diethyl ether (≤3 × 7 mL), and dried.

Purification. The crude peptide was dissolved in MeCN/0.1% aqueous TFA, filtered through a 0.45 μm nylon membrane and purified in 1−2 runs by RP-HPLC. Selected fractions were analyzed by RP-HPLC and RP-HPLC-MS, and those containing pure product were pooled and lyophilized. The reported yields are based on the loading of the starting resin and with correction for peptide content according to amino acid analysis.

[2,4-DehydroPro]SP_{1-7}. The precursor peptide for tritium-labeling [2,4-DehydroPro]SP_{1-7} was prepared by standard solid-phase peptide synthesis techniques using Fmoc/*t*-butyl protection and purified as described above. Tritium labeling of the precursor was performed by Amersham Biosciences (Cardiff, UK) and resulted in 370 MBq (10 mCi) of [^{3}H]-SP_{1-7} with a specific activity of 3.11 TBq/mmol (84 Ci/mmol).

Animal Experiment and Membrane Preparation. The preparations of receptor membranes were conducted using spinal cords from male Sprague−Dawley rats. The rats (Alab AB, Sollentuna, Sweden), weighing 200−250 g, were housed in groups of four in air-conditioned rooms (22−23 °C and a humidity of 50−60%) under an artificial light−dark cycle and had free access to food and water. Prior to tissue sampling, the rats were allowed to adapt to the laboratory environment for 1 week. The animals (n = 15) were killed by decapitation, and the spinal cords were rapidly removed and quickly frozen. Tissues were then kept at −80 °C until analyzed. The animal experiment was approved by the local ethical committee in Uppsala, Sweden.

The frozen spinal cords weighing approximately 250−300 mg/animal were thawed and placed on ice before being homogenized for 30 s in 30 volumes of ice-cold 50 mM Tris−HCl buffer (pH 7.4), containing 5 mM KCl and 120 mM NaCl, using a Polytron homogenizer. The homogenate was then centrifuged at 40000*g* for 20 min at 4 °C, and the supernatant was discarded. The resulting pellet was resuspended in 30 volumes of ice-cold 50 mM Tris−HCl buffer (pH 7.4), containing 300 mM KCl and 10 mM EDTA. Following incubation on ice for 30 min, the sample was centrifuged at 40000*g* for 20 min at 4 °C. The pellet obtained was diluted and homogenized in 30 volumes of ice-cold 50 mM Tris−HCl, containing 0.02% BSA, 5 mM, EDTA, 3 mM $MnCl_2$, and 40 μg bacitracin and recentrifuged twice as described above. The final pellet was resuspended and homogenized in 5 volumes of ice-cold 50 mM Tris−HCl buffer (pH 7.4) and immediately frozen at −80 °C until used in binding studies. The protein concentration of the membrane suspension was determined according to the method of Lowry[33] using bovine serum albumin as protein standard.

Radioligand Binding Assay. Assessment of the binding affinity for the various compounds analyzed in this study was carried out using the analogue [^{3}H]-SP_{1-7} as tracer. Assays were performed in tubes containing 50 μL of spinal cord membrane suspension (200 μg protein) and 0.9 nM of [^{3}H]-SP_{1-7} (specific activity: 3.11 TBq/mmol (84 Ci/mmol)) in a final volume of 0.5 mL 50 mM Tris binding buffer (pH 7.4), containing, 3 mM $MnCl_2$, 0.2% BSA, and peptidase inhibitors (40 μg/mL bacitracin, 4 μg/mL leupeptin, 2 μg/mL aprotinin, and 4 μg/mL phosphoramidon). The amounts of total and unspecific binding in percent of the total radioactivity added were approximately 7% and 1.7%, respectively. The competition experiments were conducted at six different concentrations varying between 0.01 nM and 1 μM of unlabeled compounds. Nonspecific binding was determined in the presence of 1 μM SP_{1-7}. Samples were incubated for 60 min at 4 °C before being terminated by rapid filtration under vacuum with a Brandel 24-sample cell harvester through Whatman GF/C glass fiber filters treated with a solution containing 50 mM Tris (pH 7.4), 0.3% polyethylenimine (PEI), and 0.5% Triton X-100 at 4 °C overnight. Filters were washed twice with 3 mL of cold 50 mM Tris−HCl (pH 7.4) complemented with 0.1 mg/mL BSA and 3 mM $MnCl_2$. The filters were air-dried for about 60 min before the bound radioactivity was determined using a liquid scintillation counter (Beckman LS 6000IC) at 63% efficiency in 5 mL of counting scintillant. The specific binding was determined as the difference between total and unspecific binding. All assays were run in triplicates, and each assay was repeated at least three times at different days. Data and statistics from the competition experiments were analyzed in the GraFit program (Erithacus Software, UK).

In Vitro Pharmacology: Human NK-1 and NK-3 Receptor Binding Assays. This study was performed at Cerep (France) according to literature.[34,35] One concentration (10 μM) of **8** was tested. [Sar^9, Met $(O_2)^{11}$]-SP was used as control agonist for the NK-1 receptor, and [$MePhe^7$]-NKB was used as control agonist for the NK-3 receptor.

Library Design using Focused Hierarchical Design of Experiments (FHDoE). A dipeptide library was designed using the FHDoE method, focused toward **8**. The method was directly adopted from the original article by Muthas et al.[26] in which

both the dipeptide design matrix and the principal properties for 113 α-amino acids are reported. The principal properties for the α-amino acids are obtained as $tciz_{\alpha}1$ and $tciz_{\alpha}2$ values and are mainly related to amino acid side chain size and polarity, respectively. A set of amino acids similar to phenylalanine was selected by considering only amino acids with $tciz_{\alpha}1$ and $tciz_{\alpha}2$ values similar to phenylalanine. The range was set to −1.23 to 1.93 for $tciz_{\alpha}1$ and −2.28 to 0.2 for $tciz_{\alpha}2$ (corresponding to ±25% of the total $tciz_{\alpha}1$ and $tciz_{\alpha}2$ span, respectively, centered on phenylalanine). The final selection within each list of possible amino acids used for substitution of Phe in each analogue was based on in-house availability. A more detailed description of the FHDoE method can be found in the Supporting Information.

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Supporting Information Available: Synthesis procedures and analytical data of peptides **1**−**20**, biological procedures including representative displacement curves, and description of the FHDoE method. This material is available free of charge via the Internet at http://pubs.acs.org.

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