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Synthesis of an analogue of the substance P C-terminal hexapeptide with modification at the glutaminyl and methioninyl residues and increased activity in NK-2 receptor type: structure–activity relationships

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Summary — Analogues of [Orn^6]-SP$_{6-11}$ have been synthesized in which the Met^{11} residue is replaced by Hse(CH$_3$), Hse(Bzl), Nva(5-OCH$_3$), Nva(5-OBzl) and Abu. These analogues were tested in 3 *in vitro* preparations representative of NK-1, NK-2 and NK-3 receptor types. The Hse(Bzl) analogue is 16.6-fold more potent than the parent hexapeptide at the NK-2 receptor and 2.4-fold more potent at the NK-3 receptor. The Nva(5-OCH$_3$) analogues howed weak antagonist activity in NK-2 and NK-3 receptor types, being a full agonist at NK-1. It is concluded from structure–activity correlations that the role of Met^{11} side chain in substance P is associated with activity and/or efficacy, as appropriate modifications in the side chain may result either in agonists with increased activity compared to the parent hexapeptide or selective agonists or may induce antagonism.

homoserine derivatives / 5-hydroxynorvaline derivatives / NK-2 agonist / NK-3 antagonist / substance P analogues / tachykinins

Introduction

Mammalian tachykinins – substance P(SP), neurokinin A(NKA) and neurokinin B(NKB) – are a family of peptides which share the common C-terminal sequence Phe-X-Gly-Leu-Met-NH$_2$ (X = Phe, Val) and which have a wide spectrum of similar biological properties. Receptors for SP, NKA and NKB have been classified into 3 subgroups named NK-1, NK-2 and NK-3 respectively [1]. The above classification has been based upon the relative order of potency in various bioassays, of natural peptides, their fragments and synthetic selective agonists toward one of the receptor subtypes [2, 3]. Very recently several novel agonists and antagonists have been synthesized which are either peptides resulting after appropriate modifications in the natural neurokinins or their C-terminal fraaments [4–8] or non-peptides [9], characterized by high potency and selectivity. The C-terminal hexapeptide of substance P, H-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$ (SP$_{6-11}$), is the minimal peptide fragment of substance P (SP) that retains substantial SP-like activity in most biological preparations [10–13]. In view of the importance of the SP C-terminal hexapeptide as regards biological activity, this sequence should provide a basis for examining some aspects of the structure–activity relationship in tachykinin agonists.

In previous studies [14–18] it has been shown that the SP$_{6-11}$ sequence of SP 'is' crucial for activation of neurokinin receptors, while modifications at the methionyl residue have several effects *eg* reduce or increase the potency or induce selectivity; thus the Met^{11} residue, and especially the nature of its side chain, is one of the most important elements for the activity of SP and related peptides. The above results prompted us to further investigate the role of the SCH$_3$ group of Met^{11} by replacing it with OCH$_3$ OCH$_2$Ph, CH$_2$OCH$_3$, CH$_2$OCH$_2$Ph and H in the model hexapeptide H-Orn-Phe-Phe-Gly-Leu-Met-NH$_2$ **1** [19]. The synthesized analogues were tested in 3 different preparations representative of the proposed NK-1, NK-2 and NK-3 tachykinin receptors [1], and structure–activity correlations have been reported.

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Abbreviations: AcOEt: ethylacetate; Boc: *tert*-butyloxycarbonyl; Bzl: benzyl; Cpt-Cl: 1-oxo-1-chlorophospholane; DMF: *N,N*-dimethylformamide; NMM: *N*-methylmorpholine; THF: tetrahydrofuran; TFA: trifluoro acetic acid; TLC: thin layer chromatography; (CH$_3$O)Z: *p*-methoxybenzyloxycarbonyl.

Results and discussion

The analogues of the C-terminal hexapeptide of substance P were synthesized in solution by coupling the protected N-terminal tetrapeptide acid Boc-Orn (Boc)-Phe-Phe-Gly-OH [19] to the C-terminal dipeptides H-Leu-X-NH_2 [X = Hse(CH_3), Hse(Bzl), Nva(5-OCH_3), Nva(OBzl), Abu] with the mixed carboxylic–phosphinic anhydride method [20–22]. This method involves formation of the corresponding mixed phosphinic anhydride by reacting the peptide acid with 1-oxo-1-chlorophospholane (Cpt-Cl) [23] in the presence of NMM followed by aminolysis. Peptide fragments were synthesized by the stepwise procedure using the REMA method [23] for coupling reactions, while Nα-amino protection in all cases was performed with the *t*-butyloxycarbonyl group which was then removed with HCl in acetic acid.

Structural modifications in the model hexapeptide H-Orn-Phe-Phe-Gly-Leu-Met-NH_2 **1** involved replacement of the methionine SCH_3 group by OCH_3, OBzl, CH_2OCH_3, CH_2OBzl, and H. The resulting analogues were tested in guinea-pig ileum longitudinal smooth muscle preparation (GPI), rat colon muscularis mucosae (RC) and rat portal vein (RPV). Equipotent molar ratios (EPMR) were expressed as the ratio EC_{50} test compound EC_{50} standard. The standards used were substance P methyl ester (SP–OCH_3) in GPI, neurokinin A (NKA) in RC and neurokinin B (NKB) in RPV; the results are summarized in table I.

In GPI (NK-1 receptor type) all the analogues showed decreased activity compared to the parent hexapeptide; but all were full agonists with the exception of analogue **21**, which is a partial agonist. The lower potency of our analogues compared to that of SP-OCH_3 or SP is also supported by the behavior of the isosteric analogues [Lys^6, Nle^{11}]-SP_{6-11} [25], [Nle^{11}]-SP_{5-11} [14] and [Nle^{11}]-SP [26] and is in agreement with a previous observation that the sulfur of Met^{11} is more important for the C-terminal hexapeptide than for SP [27]. Comparing either lipophilicity or size of the groups that replaced the Met^{11} SCH_3 group and activity in the resulting analogues **17–21**, no obvious correlation could be found between them. On the other hand, the analogue **21**, resulting from the replacement of the SCH_3 group in **1** by hydrogen, had very low activity and a maximum effect which was 52% of that of SP-OCH_3 at 30 μM, thus showing its importance for the SCH_3 group. The above results indicate that the Met^{11} SCH_3 group contributes to the affinity and/or the efficacy of SP and that its role might be associated with the stereochemistry of this group. This is further supported by the activity of the analogue [Orn^6, $Glu(OBu^t)^{11}$]-SP_{6-11} which is 2.8-fold more potent than the parent hexapeptide and equipotent to SP–OCH_3 [17]. We have also observed that the analogues **18** and **20** which have an aromatic ring at the side chain of the amino acid at position 11 have higher activity than the corresponding analogues **17** and **19** which do not have an aromatic ring. This is in agreement with previous results [16, 17] and may be attributed to the high lipophilicity and nature of the benzene ring which is better diffused than aliphatic alkyl groups through biological barriers and/or the cell membrane.

The analogue [Orn^6, $Nva(5\text{-}OCH_3)^{11}$]-SP_{6-11} **19** shows selectivity for the NK-1 receptor, but at this reduced level of potency a high degree of selectivity cannot be quantified. Analogue **19** and analogues [$Met(O_2)^{11}$]-SP and [$Met(O)^{11}$]-SP [18], which result from modifications in the Met^{11} side chain and which are NK-1 selective agonists, have a common structural feature, *ie* the presence of an oxygen atom at the ε-position in the side chain of the amino acid at position 11. In addition, the groups SO_2CH_3, $SOCH_3$ and CH_2OCH_3 which replaced the SCH_3 group in the above analogues have a much lower lipophilic character than the SCH_3 group, a similar size [28, 29] and are strong

Table I. Agonist and antagonist activity of peptides H-Orn-Phe-Phe-Gly-Leu-X-NH_2.

Compound No	*X*	*Equipotent molar ratio (EPMR)*			*pK_B*		
		GPI NK-1 SP-OCH_3 = 1	*RC NK-2 NKA = 1*	*RPV NK-3 NKB = 1*	*GPI NK-1*	*RC NK-2*	*RPV NK-3*
1	Met	2.6	148.1	2 000	–	–	–
17	Hse(OCH_3)	75.2	834	692	–	–	–
18	Hse(OBzl)	4.9	8.9	828	–	–	–
19	Nva(5-OCH_3)	181	> 588[b]	> 2 500[c]	–	< 5.0	5.2
20	Nva(5-OBzl)	62.4	86.7	1 000	–	–	–
21	Abu	7 925[a]	19 444	> 1 754[d]	–	–	< 4.5

[a]Maximum effect 52% of that of SP-OCH_3 at 30 μM; [b]maximum effect 6% of that of NKA at 10 μM; [c]no significant effect at 10 μM; [d]no significant effect at 30 μM.

H-bonding acceptors through their oxygen. These factors may be a coincidence, but on the other hand they may be factors that induce selectivity in the NK-1 receptor by promoting and stabilizing a certain conformation. In NK-2 and NK-3 receptors the analogue **19** is almost devoid of agonist activity, but shows weak antagonist activity (table I). These results are rather surprising, but are indicative of the multiple role of the amino acid side chain at position 11 of SP; further work is required to establish that appropriate modification in the Met^{11} side chain may induce antagonism.

In RC (NK-2 receptor type) all the analogues show a different behavior compared to that in GPI. Increasing the lipophilic character of the group attached at the γ-position in the Met^{11} side chain resulted in an increase in activity of the analogues compared to the parent hexapeptide. Thus, the analogues **18** and **20** are 16.6- and 1.7-fold more potent than **1**. The analogue **17**, in which OCH_3 is less lipophilic than SCH_3, is 5.6-fold less potent than the parent hexapeptide; while the analogue **19**, in which the CH_2OCH_3 is the least lipophilic of all the groups used has only very low agonist activity and is a weak antagonist, as discussed above. Structural requirements of NK-1 and NK-2 receptors for the Met^{11} side chain thus appear to be different. The side chain of the Hse(Bzl) residue (analogue **18**) seems to meet these requirements, while the lipophilicity of the side chain at position 11 and especially the presence of an aromatic ring at a certain position in the side chain are additional factors for successful interaction with the NK-2 receptor. These results are further supported by the behavior of [Orn^6, $Glu(OBzl)^{11}$]-SP_{6-11}, which is more potent than the parent hexapeptide [16] but less potent than the corresponding Hse(Bzl) analogue **18**. It is also interesting to note that the Hse(OBzl) analogue **18** maintains good affinity for the NK-2 receptor, although structurally it is more an SP-like peptide than a NKA-like peptide; and it is ≈ 10-fold more potent than SP (the EPMR value at NK-2 is 98.2) [30] while analogue **20** is equipotent to SP.

In RPV (NK-3 receptor type) analogues **17**, **18** and **20** are full agonists and are more potent than the parent hexapeptide, although they show very low activity compared to NKB and can be characterised as SP-like peptides. Analogue **19** showed very low activity, no significant effect even at 10 μM being observed; however, it showed weak antagonist activity as discussed above. The presence of the SCH_3 group seems to be important because its replacement by hydrogen in the parent hexapeptide results in an inactive analogue, which exerts weak antagonist activity although the same is observed in analogue **19** where the SCH_3 group has been replaced by CH_2OCH_3. When in the latter group one hydrogen of the methyl group is replaced by a benzene ring, the resultant analogue not only becomes a full agonist but is even more potent than the parent hexapeptide. Comparing lipophilicity of the groups used to replace the SCH_3 group and the activity of the resulting analogues, no correlation is found, while the presence of an aromatic ring in the side chain does not seem to contribute greatly to the activity of the corresponding analogues as it does in the NK-1 receptor type.

In conclusion, it appears that there is a specific requirement which is different for each receptor subtype for the sulfur-containing Met^{11} side-chain regarding activation of the neurokinin receptors, and that this is determined by the SCH_3 group. This requirement seems to be fulfilled for the NK-2 receptor subtype by the OBzl group and to a lesser extent by the CH_2OBzl group, while the presence of the aromatic ring seems to be very important. In the NK-1 and NK-3 receptor preparations, lipophilicity of the side chain of the amino acid at position 11 does not seem to be an important factor for activation of the receptor. All these factors point to the hypothesis that the SP Met^{11} side chain is associated with affinity and/or efficacy; and that the stereochemistry of the group attached at the γ-position in the amino acid side chain at position 11 appears to be a determining factor for activity. Simple but appropriate modifications in the side chain may result in agonists with increased activity compared to the parent hexapeptide or selective agonists; or may induce antagonism.

Experimental protocols

Capillary melting points were determined on a Büchi SMP-20 apparatus and are reported uncorrected. Optical rotations were measured with a Carl Zeiss precision polarimeter (±0.005°). Analysis by TLC was on precoated plates of silica gel F254 (Merck) with the following solvent systems: R_{f1} chloroform–methanol (6:1), R_{f2} 1-butanol–acetic acid–water (4:1:1) and R_{f3} 1-butanol–acetic acid–water–pyridine (30:6:24:20). The products on TLC plates were detected by UV light and either chlorination followed by a solution of 1% starch–1% KI (1:1 v/v) or ninhydrin. Retention times (t_R) of peptides were measured by RP–HPLC with Lichrosorb RP-18 column 250 x 4 mm 5 μ with the following solvent systems: A 0.1% TFA in water, B 0.1% TFA in CH_3CN, 90%–10% (A:B) isocratic elution for 5 min followed by linear gradient 90%–10% (A:B) to 40%–60% (A:B) for 20 min (for compound **17**), 70%–30% (A:B) isocratic elution for 5 min followed by linear gradient 70%–30% (A:B) to 20%–80% (A:B) for 20 min (for compounds **18**–**21**), UV detection at 257 nm, flow rate 2 ml/min. The elemental analysis of amino acid derivatives and dipeptides were within ± 0.40% of the calculated values. Methodology for amino-acid analyses of the final products and for FAB mass spectral analysis have been previously reported [16]. DMF was distilled immediately before use over CaH_2. Abbreviations used are in accordance with the rules of the IUPA–IUB Commission on Biochemical Nomenclature [31]. Other abbreviations have been given on p 949.

Preparation of α-amides of amino acid derivatives
To a solution of the N^{α}-*t*-butyloxycarbonyl amino acid derivative (3 mmol) in THF (10 ml) cooled to 15°C NMM (3 mmol) was added followed by isobutylchloroformate (3 mmol). After 2 min a solution of 58% NH_4OH (0.9 ml, 4.5 mmol) precooled to –15°C was added and the reaction mixture left to stand at the above temperature for 2 h, then warmed to room temperature. Finally saturated $NaHCO_3$ was added and the mixture was extracted with AcOEt then washed in 5% $NaHCO_3$, water and dried (Na_2SO_4). The solvent was evaporated *in vacuo* and the residue was crystallized with the addition of petroleum ether 60–80°C to yield the corresponding amides (table II).

Deprotection of the t-butyloxy and p-methoxybenzyloxy carbonyl groups
A sample (2 mmol) of the N^{α}-Boc or N^{α}-(CH_3O)Z protected amino acid derivative or peptide was dissolved in HCl 1 N acetic acid (10 ml). After 1 h at room temperature the solvent was removed *in vacuo* at 25°C, and the residue solidified by the addition of dry ether. The resulting hydrochloride salt was filtered, washed in dry ether, dried *in vacuo* over KOH pellets and then used in the coupling without further purification.

Preparation of dipeptides 5–11
To a solution of the N^{α}-protected leucine (4.8 mmol) in THF (8 ml) cooled to –15°C NMM (4.8 mmol) was added followed by isobutylchloroformate (4.8 mmol). After 2 min a solution of the hydrochloride salt of the amino component (3 mmol) in DMF (5 ml) precooled to –15°C was added and the reaction mixture left to stand at the above temperature for 3 h and then allowed to warm to room temperature. Finally the solvent was evaporated *in vacuo* and the residue dissolved in AcOEt then washed in 5% $NaHCO_3$, water, 10% citric acid, water and dried (Na_2SO_4). The solvent was removed *in vacuo* and the residue solidified with the addition of petroleum ether 60–80°C to yield the desired product (table II). The above procedure was applied to the synthesis of dipeptides **5–9**. Peptides **10** and **11** were prepared from **8** and **9** respectively by ammoniolysis with gaseous ammonia in methanol.

Preparation of protected hexapeptide analogues
A sample of Boc-Orn(Boc)-Phe-Phe-Gly-OH (3 mmol) was dissolved in DMF (10 ml) followed by the addition of NMM (3 mmol) and Cpt-Cl (3 mmol) at 0°C. After a time of 30-min activation period, the hydrochloride salt of the amino component (3 mmol) was added, followed by an equivalent amount of NMM (3 mmol). The reaction mixture was left for 24 h at 0°C and the pH was kept at 7.5–8 by adding NMM. Finally the solvent was removed *in vacuo* and the residue solidified by the addition of saturated $NaHCO_3$. The solid was filtered, on the filter with water, followed by 10% citric acid then water and dried *in vacuo* over P_2O_5. Recrystallization from DMF–ether gave the desired product (table III).

Table II. Physical constants of amino acid and dipeptide derivatives.

Compound No	*Peptide*	*mp*[a]	*Yield*	$[\alpha]_D^{25}$	*TLC*	
		(°C)	*(%)*	*(deg)*	R_{f1}	R_{f2}
2	Boc-Hse(Bzl)-NH_2	128–130	60	– 9.30[b]	0.66	0.72
3	Boc-Nva(5-OBzl)-NH_2	100–101	61	+ 4.45[b]	0.60	0.67
4	Boc-Abu-NH_2	129–131	49	+ 4.54[c]	0.54	0.66
5	Boc-Leu-Hse(Bzl)-NH_2	127–128	73	– 40.90[b]	0.62	0.68
6	Z(OCH_3)-Leu-Nva(5-OBzl)-NH_2	172–174	88	– 5.64[b]	0.58	0.71
7	Z(OCH_3)-Leu-Abu-NH_2	177–180	77	– 24.48[d]	0.63	0.70
8	Boc-Leu-Hse(CH_3)-OCH_3	84–85	76	– 43.68[b]	0.66	0.85
9	Z(OCH_3)-Leu-Nva(5-OCH_3)-OCH_3	80–81	83	– 6.50[c]	0.65	0.72
10	Boc-Leu-Hse(CH_3)-NH_2	152–154	91	– 41.21[b]	0.58	0.60
11	Z(OCH_3)-Leu-Nva(5-OCH_3)-NH_2	196–197	90	– 8.90[c]	0.56	0.70

[a]Recrystallization solvents: AcOEt–petroleum ether 60–80°C for **6** and **8**, *n*-hexane for **11**, ether–petroleum ether 60–80°C for **12**; [b]c = 1 DMF; [c]c = 2 DMF; [d]c = 2 CH_3OH.

Table III. Physical constants of peptides Boc-Orn(Boc)-Phe-Phe-Gly-Leu-X-NH_2.

Compound No	*X*	*mp*	*Yield*	$[\alpha]_D^{25}$*(deg)*	*TLC*		
		(°C)	*(%)*	*c = 1 DMF*	R_{f1}	R_{f2}	R_{f3}
12	Hse(CH_3)	213–216	70	– 35.17	0.57	0.72	0.78
13	Hse(Bzl)	198–200	69	– 45.53	0.66	0.78	0.81
14	Nva(5-OCH_3)	228–230	65	– 24.56	0.46	0.78	0.81
15	Nva(5-OBzl)	212–214	64	– 22.30	0.50	0.76	0.79
16	Abu	236–238	74	– 38.76	0.53	0.78	0.82

Table IV. Physical constants of peptides H-Orn-Phe-Phe-Gly-Leu-X-NH_2.

Compound No	X	mp (°C)	Yield (%)	$[\alpha]_D^{25}$ (deg)	TLC		HPLC t_R	FAB–MS m/z	Amino acid analysis[d]		
					R_{f2}	R_{f3}	(min)	$(M + H)^+$	Leu	Gly	Phe
17	Hse(CH_3)	156–158	83	– 32.32[a]	0.23	0.55	18.5	712	1.02	1.00	1.98
18	Hse(Bzl)	132–135	63	– 35.60[a]	0.27	0.59	17.0	788	1.00	1.01	1.96
19	Nva(5-OCH_3)	168–171	72	– 33.13[a]	0.27	0.56	11.0	726	1.00	0.98	1.99
20	Nva(5-OBzl)	154–157	67	– 32.00[b]	0.35	0.61	14.5	802	1.02	1.00	1.94
21	Abu	180–183	66	– 15.15[c]	0.22	0.55	10.8	682	0.99	1.00	1.98

[a]c = 1 DMF; [b]c = 0.7 DMF; [c]c = 0.3 CH_3COOH; [d]Orn, Hse, Nva(5-OH) present but not measured.

Preparation of H-Orn-Phe-Phe-Gly-Leu-X-NH_2

A sample of Boc-Orn-Phe-Phe-Gly-Leu-X-NH_2 (200–300 mg) was deprotected according to the general procedure described above. The deprotected hexapeptides were dissolved in water, filtered through a Millipore filter and lyophilized. The peptides **17–19** were purified by partition chromatography on Sephadex G25F (2 x 85 cm) with 1-butanol-acetic acid–water (4:1:5 v/v, upper phase), while peptides **20** and **21** were first submitted to gel filtration on Sephadex G-15 (2 x 85 cm) using as eluent 0.5 M and 1 M acetic acid respectively and then submitted to partition chromatography as described above. For physical constants see table IV.

Bioassays

Agonist activity at NK-1 receptors as determined from contractile responses of guinea pig ileum longitudinal smooth muscle (GPI) recorded under isotonic conditions at 37°C in the presence of atropine (1 μM), mepyramine (1 μM), methylsergide (1 μM) and indomethacin (1 μM). Agonist activity at NK-2 receptors was determined from contractile responses of the rat colon muscularis mucosae preparation (RC) in the presence of antagonists as described for GPI. Tissues were mounted in 2-ml organ baths and doses of agonist were added in volumes of < 220 μl (exposure time 20 s for GPI 45 s for RC). Preparations were washed thoroughly between doses, with an inter-dose interval of either 7 min (GPI) or 15 min (RC).

Experiments were conducted as 3 + 3 (GPI) or 2 + 2 (RC) assays against SP–OCH_3 or NKA respectively as standards using a randomized block design. Each agonist concentration was tested 4 times. Data were analyzed by analysis of variance. Only those assays demonstrating signifiant deviation from parallelism and no signifiant deviation from linearity were used to calculate equipotent molar ratios (EPMR).

Agonist activity at NK-3 receptors was determined from contractile activity in the everted rat portal vein preparation [32] (RPV) at 25°C. Isometric contractions (resting tension 0.5 g) were recorded in response to serially applied doses of agonist administered at 15-min intervals. Concentration–response curves were established for standard (neurokinin B) and test compound. Results were standardized by determining EPMR values for each compound; EC_{50} values were determined.

Guinea pig ileum and rat colon preparations were immersed in Tyrode solution of the following composition: (mM) Na^+ 149.1, K^+ 2.8, Ca^{2+} 1.8, Mg^{2+} 2.1, Cl^- 147.5, $H_2PO_4^-$ 0.3, HCO_3^- 11.9, glucose 5.6, and bubbled with 95% oxygen 5% carbon dioxide. Rat portal vein preparations were immersed in Krebs–Henseleit solution of the following composition: (mM) Na^+ 143, K^+ 5.9, Ca^{2+} 1.25, Mg^{2+} 0.6, Cl^- 125.2, $H_2PO_4^-$ 1.2, HCO_3^- 25, SO_4^{2-} 0.6, glucose 11.1, and bubbled with 95% oxygen 5% carbon dioxide.

For determination of antagonist activity, isolated tissue preparations were prepared as for the agonist studies. Antagonists were pre-equilibrated with the tissue for 15 min. Antagonist-induced parallel displacements of agonist concentration–response curves were quantified as the ratio of equiactive molar concentrations and estimated graphically at the level of half-maximal response. The apparent affinity (pK_B) of the antagonist was estimated as the mean (± SEM) of the individual values using the relationship:

pK_B = log(concentration ratio-1) – log(molar antagonist concentration).

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