Synthesis and Biological Activities of Substance P Antagonists[†]

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Several substance P analogues containing various D-amino acid modifications have been synthesized by the solid-phase procedure, detached from the solid support by ammonolysis, and purified by gel filtration combined with reversed-phase chromatography. Three compounds were fair to very potent competitive antagonists of substance P on three bioassays, i.e., guinea pig ileum, rabbit mesenteric vein, and guinea pig trachea. [Arg⁶,D-Trp¹⁰]SP(6–11) is a reasonable antagonist in all three bioassays and [D-Pro⁴,D-Trp⁷,⁹]SP(4–11) is a very potent competitive antagonist with pA_2 values ranging around 6.0.

Substance P (SP) has been found to be present in the central nervous system[1,2] and is suspected of being a neuromodulator[1,3,4] involved in the transmission of pain.[4,5] Inhibition of this peptide in vivo by specific antagonists is expected to produce analgesia. Numerous analogues of substance P have therefore been synthesized and tested for antagonism,[6-11] but the success, until now, has been moderate. Two groups of compounds have been shown to exert some antagonism: (1) superagonists, such as [Ile⁸]SP, [*arg*⁹]SP and [des-α-amino-Arg¹]SP and (2) weak agonists, such as [*phe*⁷]SP,[6] or inactive compounds, containing two substitutions, such as [*leu*⁸,*phe*⁹]SP.[8] While the antagonistic effects of the superagonists is probably due to desensitisation of the guinea pig ileum[12,13] the effect of [*leu*⁸,*phe*⁹]SP could be a true antagonistic one, but it is very weak.[8] Affinity of antagonists appears to be increased by triple substitutions, such as in [*pro*²,*phe*⁷,*trp*⁹]SP and [*pro*²,*trp*⁷,⁹]SP,[9] two compounds that have shown some activity in vitro and in vivo.[9,11] Inhibitory concentrations of these compounds are, however, very high, still in the range of 10^{-5} M.[9,11]

An interesting feature of SP, as well as of other tachykinins,[14,15] and of some neuropeptides (e.g., neurotensin[16]) is that some C-terminal fragments, such as 4–11, 5–11, or 6–11 of SP[17] maintain full activity and are even more active than the entire undecapeptide in some pharmacological preparations.[15,17] We therefore thought that the affinity of antagonists could be further improved by using some of the above-mentioned substitutions in the fragment sequences. Thus, Phe⁷ and Gly⁹ were replaced by *trp* in the partial sequences 4–11, 5–11, and 6–11. The choice of *trp* as replacement residue was dictated by the knowledge that *trp* confers antagonistic properties to LH-RH,[18] to neurotensin,[19] and to SP[9,10] when placed in critical positions.

Because multiple replacements in peptide sequences are known to produce drastic reductions of affinity (see discussion by Rudinger[20]), in a second series of experiments, we used *trp* to replace one by one the four intermediate residues of the C-terminal hexapeptide sequence H-Arg-Phe-Phe-Gly-Leu-Met-NH₂, in which Arg was used instead of Gln, to increase the solubility of the compounds in water.

Peptide Synthesis and Purification. The peptides were built up by the stepwise solid-phase method on chloromethylated polystyrene as a solid support, and the coupling was achieved with preformed symmetrical anhydrides. Boc was used for α-amino protection. The

guanido group of Arg was left as an HCl salt. After completion of the synthesis, the protected peptides were cleaved by ammonolysis from their solid support[21] to give fully protected peptide amines containing Boc-Arg·HCl. It was important to obtain protected peptides, because free SP sequences exhibit very often serious solubility problems which easily jeopardize any purification attempt.

The fully protected peptides were purified by gel filtration and silica gel adsorption chromatography, while gel filtration and reversed-phase preparative liquid chromatography were used for the Arg-containing peptides. The purified peptides, still Boc protected, were deprotected with TFA and subjected to a final gel filtration. The critical step was the application of the preparative reversed-phase chromatography, which enabled a purification not obtainable with other more classical methods like gel filtration and ion exchange and partition chromatog-

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[†] Symbols and abbreviations are in accordance with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, (1971)]. In 'addition, the following abbreviations are used: SP, substance P; TFA, trifluoroacetic acid; DMF, dimethylformamide; Et₂S, ethyl sulfide. L-Amino acids are abbreviated with the usual three letter code, Aaa; D-amino acids are abbreviated as *aaa*.

raphy. The final peptides were pure on several TLC systems, electrophoresis, and analytical HPLC, and amino acid compositions were confirmed by amino acid analysis.

Peptides 7 and 8, which were initially synthesized as the [Boc-Gln[6]] and [Boc-Gln[5]] analogue, respectively, have been cyclized after deprotection to the final pyroglutamic N terminus.[22] This modification, however, produced two products devoid of enough solubility in water and various other solvents to permit the utilization of the compounds for biological studies.

Results of Biological Assays. The new peptides were tested in three pharmacological preparations (guinea pig ileum, rabbit mesenteric vein, and guinea pig trachea), in order to determine the activities of the new compounds in intestinal, vascular, and respiratory smooth muscles, and to compare these activities with those of the standards SP, SP(4–11), and SP(6–11).

We were able to measure the biological activities of six of the eight newly synthesized compounds. These activities were compared to those of SP and of the two partial sequences (octapeptide 4–11 and hexapeptide 6–11) in the three pharmacological preparations. Moreover, the antagonistic activity of 6, [Arg[6],*trp*[10]]SP(6–11), and that of 9, [*pro*[4],*trp*[7,9]]SP(4–11), were compared with that of the only commercially available (from Peninsula Laboratory, Marina Del Rey, CA) inhibitor of substance P, namely, [*pro*[2],*phe*[7],*trp*[9]]SP, described by Folkers et al.[23a] The results of pharmacological assays, summarized in Table I, show the following: (1) The replacement of glutamine by arginine at the N terminus of the hexapeptide 6–11 of SP induced a decrease of affinity. The compound still maintains almost 50% of the activity of SP, it is perfectly soluble in water, and it is suitable for pharmacological assays. (2) The double substitutions with Arg in position 6 of SP(6–11) and *trp* either in position 7, 8, or 9 give compounds that appear to be full agonists but show drastic reductions of affinity to values lower than 1% of that of SP. (3) Compound 6, [Arg[6],*trp*[10]]SP(6–11) is totally inactive as agonist in two of the three preparations and is an antagonist of SP. The small quantity of compound 6 available and the high concentrations required for the test of antagonism allowed us to evaluate the antagonistic property of this compound only in the guinea pig ileum and the rabbit mesenteric vein. Still, 6 appears to be more active than the inhibitor described by Folkers et al.,[23] as shown at the bottom of Table I. (4) Compound 9, [*pro*[4],*trp*[7,9]]SP(4–11), was found to be inactive as agonist in two of the three preparations, but exerted a fairly potent antagonism against SP and SP(4–11) octapeptide in the guinea pig ileum, the rabbit mesentric vein, and particularly in the guinea pig trachea, where this antagonist showed a p*A*$_2$ value of almost 6.0. This is definitely the most active antagonist of SP tested in the present study. The other two promising compounds, 7 and 8, which had also Phe[7] and Gly[9] substituted by *trp* but a pGln N-terminus, were too insoluble in water and, therefore, could not be evaluated adequately in a pharmacological assay.

Experimental Section

Syntheses. *N*-Butyloxycarbonyl-protected amino acids, peptide reagents, and chloromethylated resin (copolystyrene–1% divinylbenzene; 0.75 mmol of Cl/g of resin) were obtained either from Bachem Fine Chemicals Inc. or from Chemalog, Chemical Dynamics Corp. The purity of amino acids was tested in TLC before use. We purified dicyclohexylcarbodiimide (DCC) by

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Table I. Pharmacological Parameters of Agonist and Antagonist Peptides Related to Substance P[a]

no.	peptide	guinea pig ileum				rabbit mesenteric vein				guinea pig trachea			
		αE	pD_2	ED$_{50}$	RA	αE	pD_2	ED$_{50}$	RA	αE	pD_2	ED$_{50}$	RA
0	substance P (SP)	1.0	8.74	1.8×10^{-9}	100	1.0	7.58	2.6×10^{-8}	100	1.0	6.75	1.7×10^{-7}	100
1	octapeptide SP(4–11)	1.0	9.28	0.5×10^{-9}	346	1.0	7.79	1.6×10^{-8}	157	1.0	6.74	1.8×10^{-7}	97
2	hexapeptide SP(6–11)	1.0	8.91	1.2×10^{-9}	146	1.0	7.10	8.0×10^{-8}	33	1.25	7.46	3.4×10^{-8}	507
3	[Arg[6]]SP(6–11)	1.0	8.43	3.7×10^{-9}	49	1.0	7.10	7.9×10^{-8}	30	1.0	6.30	5.0×10^{-7}	35
4	[Arg[6],*trp*[7]]SP(6–11)	0.9	6.60	2.5×10^{-7}	0.7		5.56	2.7×10^{-6}	0.9		5.18	6.6×10^{-6}	2.8
5	[Arg[6],*trp*[8]]SP(6–11)	0.9	6.44	3.6×10^{-7}	0.5						5.38	4.2×10^{-6}	4.2
6	[Arg[6],*trp*[9]]SP(6–11)		5.88	1.3×10^{-6}	0.1						6.06	8.6×10^{-7}	20.3
	[Arg[6],*trp*[10]]SP(6–11)			inactive			6.0	9.7×10^{-7}	2.7	0.5		inactive	
	pA_2			5.01				4.96	<0.1				
	pA_2 – pA_{10}			0.82				0.69					
9	[*pro*[4],*trp*[7,9]]SP(4–11)[b]			inactive					<0.1			inactive	
	pA_2			5.65				5.65				5.96	
	pA_2 – pA_{10}			1.00				1.00				1.01	
	[*pro*[2],*phe*[7],*trp*[9]]SP[23]												
	pA_2			4.67	0.1				0.1				
	pA_2 – pA_{10}			0.45									

[a] αE = intrinsic activity; pD_2 = –log of the concentration producing 50% of the maximum effect; RA = relative (to SP) affinity; pA_2, pA_{10} = –log of the concentration producing 50% of the maximum effect; ED$_{50}$ = molar (M) concentration of peptide producing 50% of the maximum effect; RA = relative (to SP) affinity; pA_2, pA_{10} = –log of antagonist concentrations that reduce the effect of a double (pA_2) or a 10 times (pA_{10}) higher dose of agonist to that of a single dose. Peptides 7 and 8 were too insoluble for biological determinations. pD_2, pA_2, and pA_{10} values and ED$_{50}$ concentrations were calculated from 10–12 determinations. Variations of ED$_{50}$ were inferior to 15%. [b] Compound 9 is available from BACHEM, Switzerland.

Table II. Physicochemical Properties of Substance P Analogues

no.		yield,[a] %
0	$\overset{1}{\text{H-Arg}}\text{-}\overset{2}{\text{Pro}}\text{-}\overset{3}{\text{Lys}}\text{-}\overset{4}{\text{Pro}}\text{-}\overset{5}{\text{Gln}}\text{-}\overset{6}{\text{Gln}}\text{-}\overset{7}{\text{Phe}}\text{-}\overset{8}{\text{Phe}}\text{-}\overset{9}{\text{Gly}}\text{-}\overset{10}{\text{Leu}}\text{-}\overset{11}{\text{Met}}\text{-NH}_2$ SP	
1	H-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$ SP(6-11)	
2	H-Arg————————NH$_2$ [Arg^6]SP(6-11)	27
3	H-Arg-*trp*——————NH$_2$ [Arg^6,*trp*^{7}]SP(6-11)	41
4	H-Arg——*trp*————NH$_2$ [Arg^6,*trp*^{8}]SP(6-11)	25
5	H-Arg————*trp*——NH$_2$ [Arg^6,*trp*^{9}]SP(6-11)	8
6	H-Arg————————*trp*——NH$_2$ [Arg^6,*trp*^{10}]SP(6-11)	20
7	pGlu-*trp*-Phe-*trp*-Leu-Met-NH$_2$ [pGlu^6,*trp*^{7,9}]SP(6-11)	20
8	pGlu-Gln-*trp*-Phe-*trp*-Leu-Met-NH$_2$ [pGlu^5,*trp*^{7,9}]SP(5-11)	20
9	H-*pro*-Gln-Gln-*trp*-Phe-*trp*-Leu-Met-NH$_2$ [*pro*^{4},*trp*^{7,9}]SP(4-11)	16

no.	TLC			rotation		amino acid analysis					
	BAW	BAWP	RP	% in DMF	$[\alpha]^{22}_D$	Glx	Arg or Pro	Leu	Met	Phe	Gly
0	0.42	0.33	0.11								
1	0.65	0.78	0.62								
2	0.45	0.70	0.41	0.5	−15		0.97	1.01	0.49	2.04	1.00
3	0.45	0.70	0.35	0.55	−10		1.03	1.00	0.88	1.03	1.00
4	0.46	0.71	0.37	0.5	−5		1.00	1.07	0.82	1.10	1.08
5	0.49	0.73	0.33	0.5	−18		0.99	1.00	0.89	1.97	
6	0.45	0.68	0.37	0.2	+12.5		0.98		0.32	2.06	1.01
7	0.82	0.81	0.35	0.55	−30.9	1.00		1.00	0.43	1.00	
8	0.63	0.77	0.50	0.2	+20	1.98		1.00	0.20	1.07	
9	0.44	0.62	0.40	0.1	−15	2.00	0.93	1.01	0.48	1.00	

[a] All products are in the acetate form (except 7 and 8) and are calculated as such.

dissolving the commercial product (Aldrich Chemical Co., Inc.) in dry diethyl ether; the insoluble material (urea) was removed by filtration, and the ether was evaporated in vacuo. All solvents and reagents used for solid-phase synthesis were of "analytical" quality, and were redistilled in the appropriate manner before use. TLC was performed on Merck precoated silica gel plates (Type G60-F254) in the solvent systems BAW (1-butanol–acetic acid–water, 5:2:3), BAWP (1-butanol–acetic acid–water–pyridine, 30:6:20:12). Reversed-phase TLC (RP) was carried out on Whatman KC18 plates in the solvent system 50% acetonitrile in 0.5 M ammonium acetate, pH 7.0. Peptides were visualized by UV fluorescence and by a modified Reindel–Hoppe Procedure.[24] Elution of columns was monitored by TLC and by UV at 280 nm, all products were purified until they produced single spots. Peptide samples for amino acid analyses were hydrolyzed during 24 h at 110 °C in 6 N HCl + 1% butanethiol in vacuum-sealed tubes. The amino acid analyses were performed on a Technicon TMS analyzer equipped with an Autolab integrator by Dr. P. Schiller of the Clinical Research Institute in Montreal. Trp, however, was completely destroyed.

Peptide synthesis was carried out with a Burrel shaker and glass reaction vessels or an automated Beckman Model 990 synthesizer by procedures previously described.[21] The synthesis was started on Boc-Met substituted peptide resin ester (0.4 mequiv/g), prepared in the usual manner.[25] In order to avoid any destruction of tryptophan and methionine during the deprotection steps, 2.5% Et$_2$S and 5% ethanedithiol were added to the 40% TFA solution. Every deprotection step and every coupling step was monitored for completion by a ninhydrin test.[26]

[Arg^6]SP(6-11) (2). Boc-Met-resin ester (2.5 g) was elongated to the desired sequence with the following protected amino acids: Boc-Leu, Boc-Gly, Boc-Phe, and N$^\alpha$-Boc-Arg·HCl. All amino acids were used in sixfold excesses and were coupled as the symmetrical anhydrides[27] in DMF/CH$_2$Cl$_2$ (1:9); only Boc-Arg·HCl was coupled in DMF/CH$_2$Cl$_2$ (1:1). This peptide resin ester (2.5 g) was subjected in a pressure bottle to ammonolysis for 14 days at room temperature in DMF-2-propanol (1:1), saturated previously at 0 °C with gaseous ammonia.[21] After filtration and evaporation,

the crude protected peptide was filtered over Sephadex LH20 (1.5 × 40 cm), eluted with DMF. The peptide fractions were pooled, evaporated, and further purified by ion-exchange chromatography on Sephadex SP C25 (1.5 × 30) [equilibrated with 0.01 M AcONH$_4$, pH 5.0-MeOH (1:1) and a linear gradient of 0.15 M AcONH$_4$-MeOH(1:1)]. The pure peptide fractions were pooled and lyophilized twice in order to remove AcONH$_4$, producing 210 mg of Boc-2. Deprotection was achieved by addition of 20 mL of TFA containing 5% Et$_2$S and 5% ethanedithiol and left standing at room temperature for 20 min. After evaporation in vacuo and repeated extraction with dry ethyl ether, the peptide was dissolved in 1.5 mL of glacial AcOH, diluted with 3 mL of 0.5 M AcOH$_4$ and 300 μL of 1-butanol, and applied onto Sephadex LH 20 (1.5 × 40 cm), eluted with 0.5 M AcOH$_4$ and 10% 1-butanol. The pure fractions were pooled, lyophilized several times, and produced 170 mg of 2.

[Arg^6,*trp*^{7}]SP(6-11) (3). To 1.5 g of Boc-Met-resin ester were coupled the protected amino acids mentioned before and Boc-*trp*. The completed peptide-resin ester (2.03 g) was subjected to ammonolysis for 10 days as described for 2. After filtration of the residual resin and evaporation of the solvent, the crude peptide was dissolved in MeOH and filtered over Sephadex LH20 (1.5 × 80 cm) with MeOH. The peptide-containing fractions were collected, evaporated in vacuo, redissolved in MeOH/H$_2$O (1:1), and applied to a column (1.5 × 30 cm) containing Nucleosil 30 μ C18 (Machery-Nagel, Germany). This reversed-phase column was eluted first with 400 mL of 20% CH$_3$CN-0.1 M AcONH$_4$, pH 7.0, followed by a 1600-mL linear gradient from this initial solution to 50% CH$_3$CN. The solvent was pumped with an FMI-lab pump at a speed of 9 mL/min and at a mean pressure of 8 atm. The peptide was eluted as a symmetrical peak around 40% CH$_3$CN. Afterwards, the column was flushed with 250 mL of 60% CH$_3$CN-0.1 M AcONH$_4$, followed by MeOH. The pure peptide fractions were pooled, evaporated, and twice lyophilized. The 273.5 mg of peptide Boc-3 was deprotected in a mixture of 8 mL of TFA, 1 mL of Et$_2$S, and 1 mL of ethanedithiol for 20 min at room temperature. After evaporation, the peptide was 3 times triturated with dry diethyl ether, dissolved in 200 μL of glacial AcOH, diluted to 20 mL with water, filtered, and lyophilized. Peptide 3 was dissolved in 0.5 M AcOH and filtered a last time over Sephadex LH 20, producing, after pooling and lyophilization, 226.9 mg of pure 3.

[Arg^6,*trp*^{8}]SP(6-11) (4). The same quantities, substances, and procedures were used to build up the peptide sequence and for the peptide–resin cleavage. For purification, the Boc-protected peptide was subjected to several attempts of ion-exchange

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chromatography on Sephadex SP C25; however, no satisfactory separation was obtained. The peptide-containing fractions were pooled and filtered over Sephadex LH20 in MeOH, and the filtrate was evaporated. This product was purified by partition chromatography on Sephadex G25 (1.5 × 40 cm) with the two-phase system dichloroethane–1-butanol–methanol–water (5:3:2:2), followed by a second partition chromatography with the solvent system dichloroethane–1-butanol–petrol-ether–methanol–water (3:2:1:3:3). The Boc-4 was deprotected and filtered as described for **3**, yielding 130 mg of pure **4**.

[Arg^6,trp^9]SP(6–11) (5). This peptide was prepared from 1.5 g of Boc-Met-resin in exactly the same manner as described for **3**. Pure Boc-**5** eluted from the reversed-phase column at about 45% CH$_3$CN; standard deprotection produced 48.7 mg of pure **5**.

[Arg^6,trp^{10}]SP(6–11) (6). This peptide was synthesized and purified with the quantities and techniques described for **3**; however, the crude, Boc-protected peptide was initially purified on Sephadex SP-C25 ion-exchange, followed by reversed-phase chromatography, and not with gel filtration as described for **3**. Deprotection and final gel filtration was carried out as described for **3** and produced 114.5 mg of pure **6**.

[pGlu^6,trp^{7,9}]SP(6–11) (7). Boc-Met-resin ester (2.5 g) was elongated to the sequence Boc-Gln-trp-Phe-trp-Leu-Met-R in the usual manner, with the exception of Boc-Gln, which was coupled as the Boc-Gln-O-p-nitrophenyl active ester[28] in the presence of an equivalent of N-hydroxybenzotriazole. This resin (0.92 g) was ammonolyzed as usual during 14 days. After an initial gel filtration on Sephadex LH20 in the usual manner, the peptide was dissolved in CHCl$_3$ and applied to a lobar silica gel column, size B (Merck), eluted with a linear gradient up to 10% MeOH in CHCl$_3$. Pooling the pure fractions yielded 124 mg of pure Boc-protected peptide. Deprotection was carried out as usual (see **3**), and the residue was dissolved in 10% AcOH with 0.5% p-mercaptoacetic acid for cyclization during 16 h.[22] A white precipitate was filtered off, dissolved in MeOH, and chromatographied again on lobar silica gel, with 4% MeOH in CHCl$_3$. This produced 70.6 mg of pure **7**.

[pGlu^5,trp^{7,9}]SP(5–11) (8). The balance of nonammonolyzed Boc-protected peptide ester-resin (see **7**) was further elongated with Boc-Gln-O-p-nitrophenyl active ester in the presence of N-hydroxybenzotriazole. This resin (1.32 g; about half total quantity) was ammonolyzed, purified, and cyclized as described for **7**. The crude **8** was sparingly soluble in MeOH; it was dissolved in DMF and subjected to gel filtration on Sephadex LH20, eluted with MeOH: 80.2 mg of pure **8** was collected.

[pro^4,trp^{7,9}]SP(4–11) (9). The residual heptapeptide resin ester from **8** was coupled with Boc-pro, ammonolyzed, and filtered over LH20 in the manner described for **7**. The crude Boc-9 was

purified on Lobar silica gel, size B, eluted with a linear gradient from 5% up to 15% MeOH in CHCl$_3$, and, after collection and evaporation, reapplied to LH20 in MeOH. The pure peptide fractions were collected to give 85 mg of pure Boc-**9**. Deprotection and gel filtration on LH20 eluted with MeOH produced 70.0 mg of pure **9**. This product is actually available from BACHEM, Switzerland.

Biological Assays. Longitudinal strips of the guinea pig ileum were prepared according to the procedure of Rang,[29] helical strips of the rabbit mesenteric vein were made according to Bérubé et al.,[30] and helical strips of the guinea pig trachea were prepared as described recently by Mizrahi et al.[31] The tissues were suspended in 10-mL organ baths containing Krebs' solution, heated at 37 °C, and oxygenated with 95% O$_2$ and 5% CO$_2$. The composition of the Krebs' solution and other technical details have been recently described.[17,23] Changes of tension produced by the peptides were recorded isometrically with force transducers FD03C on a 7D Grass polygraph.

All compounds were tested (a) for agonistic activity by application of increasing (from 10^{-10} to 10^{-5} M) concentrations of each peptide into the bath and (b) for antagonism by injection of the compound in the bath at least 5 min before testing SP, octa-SP(4–11), or hexa-SP(6–11). In the test for antagonism, the concentration of peptides used was either an inactive one or the minimum active one. For each agonist, we tried to record a complete concentration–response curve, in order to evaluate the intrinsic activity (which is expressed as a fraction of that of SP) and the affinity [which is expressed in terms of pD_2, the –log of the concentration of peptide producing 50% of the maximum (for each compound) effect]. Affinity of antagonists is expressed in terms of pA_2, the –log of the concentration of antagonist that is able to reduce the effect of a double dose of agonist to that of a single dose. All peptides were soluble in water, except **7** and **8**. Concentrated solutions (1 or 5 mg/mL) were prepared in distilled water and kept at –20 °C in aliquots that were thawed and used for one experiment only. Concentrations of peptides are expressed in moles.

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