Tetrapeptide Tachykinin Antagonists: Synthesis and Modulation of the Physicochemical and Pharmacological Properties of a New Series of Partially Cyclic Analogs

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Received January 14, 1993

We report on the synthesis and the pharmacological properties of a new series of tachykinin antagonists based on the pseudopeptide pharmacophore cyclo[-Abo-Asp(D-Trp-Phe-N(Me)Bzl)-] which contains the 2-azabicyclo[2.2.2]octane-3(*S*)-carboxylic acid (Abo) residue. Variation of the substituents on the tryptophan indole nitrogen was shown to modulate water solubility and transport properties of the analogs as well as potency in classical in vitro response and binding assays. One water-soluble compound, **16**, in which the substituent was 3-carbonylpropionate, strongly prolonged the reaction time in the mouse hot-plate test both after iv or oral administration and was devoid of degranulating activity in rat peritoneal mast cells.

Peptide and non-peptide antagonists of substance P and related tachykinins have been the object of intense search over the years, both as tools for the elucidation of the mechanisms of pain transmission and as potential analgesic and antiinflammatory drugs (for a review, see ref 2). Progress in the discovery of neurokinin antagonists has followed the general strategy for the rational design of peptide drugs[3] in which a natural sequence is modified step by step in order to increase its potency, selectivity, and stability. Introduction of a D-aromatic amino acid in positions 7 and 9 was the crucial modification of the natural sequence of substance P which converted it into an antagonist and which culminated in spantide I[4] and II.[5] The discovery of a different hormonal message in the N- and C-terminal parts of substance P[6] focused the studies on the C-terminal fragment and led to the synthesis of shorter antagonists such as [D-Pro^4,D-Trp^{7,9}]SP$_{4-11}$,[7] Ac-Thr-D-Trp(For)-Phe-N(Me)-Bzl (FR113680),[8] or N^2-[(4*R*)-4-hydroxy-1-[(1-methyl-1*H*-indol-3-yl)carbonyl]-L-prolyl]-*N*-methyl-*N*-(phenylmethyl)-3-(2-naphthyl)-L-alaninamide (FK888).[9] Finally, screening on a radioreceptor assay using labeled substance P as the ligand led to identification of non-peptide antagonists,[10-13] which display high selectivity toward NK-1 and NK-2 receptors. Their therapeutical relevance still remains to be established.

The purpose of our work was to improve the pharmacological properties of a low molecular weight, potent, stable, and nontoxic NK-1-targeted pseudopeptide antagonist. Our design was based on the rationale that the pharmacophore should incorporate the dipeptide D-Trp-Phe which is present in most of the known neurokinin receptor antagonists and should include substituents which would increase affinity and stability. To this end, a dipeptide unit was attached to the N-terminus and a secondary amine to the C-terminus of D-Trp-Phe. Stability was aimed at by partial cyclization of the dipeptide into a diketopiperazine ring, by attachment of this ring via the side chain of Asp (instead of an usual peptide bond), and by N-methylation of the benzylamide bond. It was anticipated that the sole remaining unprotected peptide bond between D-Trp and Phe would be less susceptible to proteolytic degradation due to the D configuration of Trp. Screening of the possible residues in the diketopiperazine moiety, including nonproteinogenic amino acids, led to Abo-Asp as the best choice, a dipeptide containing L-azabicyclooctanecarboxylic acid (Abo), a proline analog synthesized first in our laboratory.[14]

In vitro degradation studies in human blood serum confirmed the relative stability of the analogs which were unsubstituted on Trp-*N*-indole, such as compound **8** (Table I). In contrast, a rapid hydrolysis of the *N*-indole-substituted analogue **9** was demonstrated, giving rise to compound **8**. These observations led to the design of the series of derivatives **9** to **18** (Figure 1) in which hydrophobicity, solubility, and stability could be modulated via the choice of the Trp substituent and of the substituent connecting bond.

We report here on the chemical synthesis and preliminary pharmacological evaluation of 11 compounds **8**-**18** which have emerged from our investigation and which are displayed in Figure 1.

Chemistry. Abo was prepared as described by Vincent et al.[15] and the L-enantiomer obtained from its diastereoisomeric tartric salt. Pseudopeptide **8**, the precursor of most other final compounds, was prepared by the classical methods of peptide synthesis in solution[16] using differential protection and selective deprotection for the synthesis of the dipeptide fragments followed by fragment condensation (Figure 2). Coupling of protected amino acids or peptide fragments was carried out with DCC[17] or with TBTU[18] in presence of HOBt. No final deprotection was required. The various substituents on the indole nitrogen were introduced by acylation of compound **8**. For example, reacting compound **8** with [[6-(Fmoc-amino)hexyl]oxy]-carbonyl chloride and treating the product with piperidine for deprotection yielded compound **18**. Compound **10** was an exception, which was directly prepared from N^{ind}-Me-D-Trp, instead of unsubstituted D-Trp. Due to the poor nucleophilicity of indole, the appropriate acylation method

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0022-2623/93/1836-1654$04.00/0

Table I. Analytical Data of the Final Compounds 8–18

compd	$[\alpha]^{23}_D$, deg (c = 1)	FAB-MS (MH^+) m/e	TLC[a] R_f(A)	TLC[a] R_f(B)	HPLC[b] K'	log P	water solubility, mg/mL
8[f]	−12.1 (AcOH)	689	0.74	0.80	1.18	3.18	<0.01
9	−1.5 (AcOH)	717	0.70	0.83	1.45	3.47	<0.01
10	−6.1 (AcOH)	703	0.70	0.85	1.99	3.85	<0.01
11	−8.7 (AcOH)	731	0.67	0.85	1.69	3.69	<0.01
12	−10.7 (AcOH)	747	0.71	0.88	2.19	4.05	<0.01
13	−9.6 (AcOH)	823	0.77	0.90	6.76	5.66	<0.01
14	−10.7 (AcOH)	789	0.76	0.89	6.18	5.33	<0.01
15	−7.8 (AcOH)	815	0.79	0.90	21.30	7.29	<0.01
16[f]	−12.7 (AcOH)	789[d]	0.70	0.63	1.14	3.15	45[e]
17[f]	−16.7 (H_2O)	816	0.36	0.50	0.72	2.48	43
18	−19.5 (H_2O)	832	0.38	0.45	1.00	2.95	40

[a] Solvent systems: A = BuOH/AcOH/H_2O (72:7:21), B = $CHCl_3$/MeOH/AcOH (70:25:4.5:0.5). [b] Capacity factor K' for isocratic elution in acetonitrile/water (1:1) containing 0.1% TFA. [c] P = partition coefficient in octanol/water. [d] MH^+ of sodium salt = 811. [e] Sodium salt. [f] Company codes: 8 = S15890; 16 = S16474; 17 = S16375.

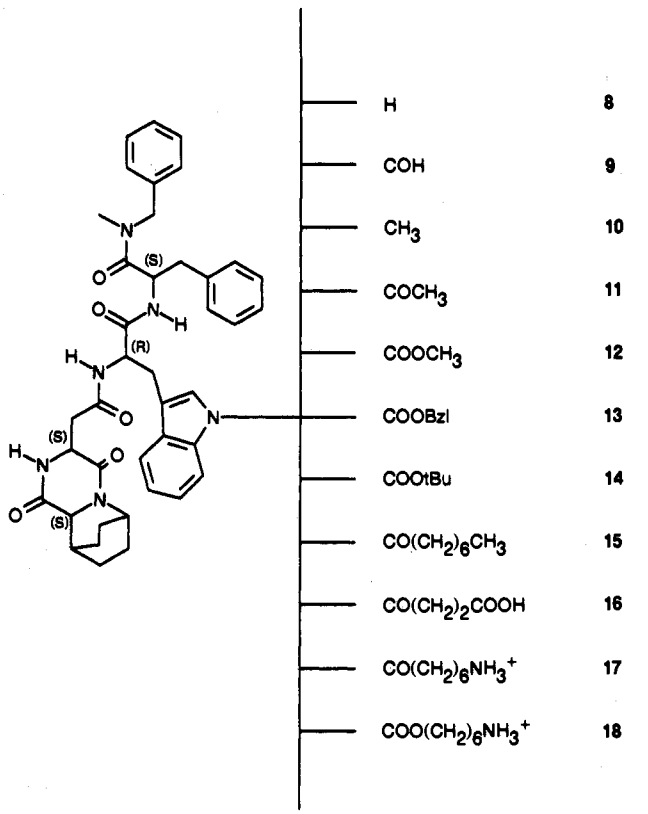
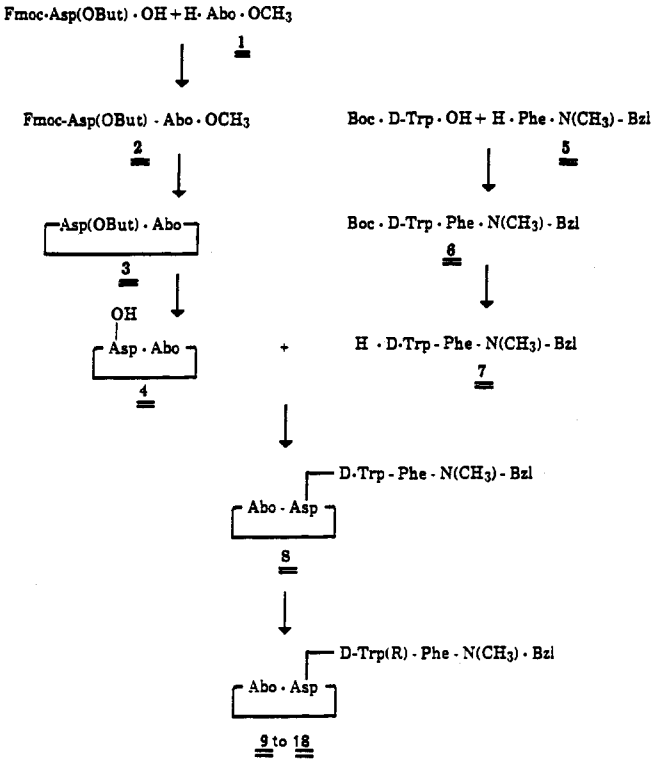


Figure 1. Structural formula of the pseudopeptide analogs. Abo = 2-azabicyclo[2.2.2]octane-3(*S*)-carboxylic acid.



R = HCO, CH_3, CH_3CO, CH_3OCO, BzlOCO, tBuOCO, $CH_3(CH_2)_6CO$, $HOOC(CH_2)_2CO$, $^-H_3N(CH_2)_6CO$, $^+H_3N(CH_2)_6OCO$.

Figure 2. Synthesis scheme of 8 and of its indole N-substituted derivatives 9–18. Abo = 2-azabicyclo[2.2.2]octane-3(*S*)-carboxylic acid.

had to be selected for each individual case. A general procedure is nevertheless given for the preparation of most of the derivatives of 8, using a phase-transfer-catalyzed acylation,[19] while the synthesis of compounds **16** and **17** succeeded best in the presence of a crown ether and KF.

Since pharmacokinetic properties of the analogs are likely to be influenced by hydrophobicity and solubility, the partition coefficients in octanol/water and the solubility in water were determined by classical methods.

Biochemical and Pharmacological Assays

Proteolysis. Since one of our goals was to increase the resistance of the analogs toward enzymatic degradation, the stability of the new pseudopeptides was assessed in proteolytic media in vitro[20] and compared to reference peptides.

Binding. The potency of the analogs to displace tritiated SP from its binding sites was assessed in a microsomal rat brain membrane preparation according to a described procedure.[21] In order to measure the affinity on pure NK-1 sites and to evaluate species differences, binding was also performed on cloned rat and human NK-1 and NK-2 receptors stably expressed in Chinese hamster ovary cells according to a described methodology.[22] Finally, in order to test the specificity toward a number of non-neurokinin receptor sites, in particular toward opiate receptors, binding experiments were carried out according to classical protocols (see the Experimental Section) and using specific radiolabeled ligands in appropriate membrane preparations.

Bioassays. The pharmacological tests in vitro were performed on three isolated organs, the rabbit vena cava (RJV), the rabbit pulmonary artery without endothelium (RPA), and the rat portal vein (RPV), the contractile responses of which are mediated exclusively by NK-1, NK-2, and NK-3 receptors, respectively.[23]

Table II. pA_2 (±sem) Values of the New Analogs and of Reference Peptides Measured against the Standard Agonists SP, Neurokinin A, or Neurokinin B in the Rabbit Vena Cava (NK-1), Rabbit Pulmonar Artery (NK-2), and Rat Portal Vein (NK-3) Bioassays

analog	NK-1	NK-2	NK-3	n^a
8	7.0 ± 0.6	5.7 ± 0.5	5.8 ± 0.5	6
9	6.5 ± 0.4	4.9 ± 0.1	4.9 ± 0.1	12
10	6.7 ± 0.4	5.0 ± 0.2	5.1 ± 0.2	6
11	7.3 ± 0.7	6.9 ± 0.7	7.0 ± 0.7	6
12	7.0 ± 0.8	4.8 ± 0.1	6.8 ± 0.6	6
13	6.8 ± 0.5	5.9 ± 0.3	5.8 ± 0.3	6
14	6.9 ± 0.5	4.9 ± 0.1	5.9 ± 0.2	6
15	4.9 ± 0.1	4.8 ± 0.1	4.9 ± 0.1	6
16	6.9 ± 0.7	4.8 ± 0.2	4.9 ± 0.2	6
17	6.7 ± 0.6	4.7 ± 0.2	5.7 ± 0.3	12
18	7.4 ± 0.6	5.0 ± 0.2	5.0 ± 0.2	12
FR 113680	7.1 ± 1.1	5.4 ± 0.4	5.8 ± 0.5	12
spantide I	6.8 ± 0.7	5.8 ± 0.9	6.2 ± 0.7	12

[a] n = number of independent experiments. FR 113680 = Ac-Thr-D-Trp(For)-Phe-N(Me)Bzl.

Antinociceptive activity was evaluated in the classical mouse hot-plate[24] and rat tail flick[25] tests, after iv and oral administration or after intrathecal injection, respectively.

Degranulation was assessed in vitro in isolated rat peritoneal mast cells according to Taylor et al.[26] at concentrations of the analogs varying between 10^{-7} and 10^{-4} M, after incubation for 10 min at 37 °C, and histamine was quantitated using a fluorescence assay.[27]

Results

Hydrophobicity, Solubility, and Stability. The measured values of the partition coefficient P in the solvent system octanol/water[28] were as follows: compound **9**, log P 3.47; compound **13**, log P 5.66. The estimated values based on the capacity factor K' (Table I) and using **9** and **13** for calibration varied from log P 2.48 (compound **17**) to log P 7.29 (compound **15**). Water solubility was conferred on the series by introduction of an aminoalkyl substituent or a carboxylate anion on the indole moiety, thus improving the suitability for the pharmacological tests. Three members of the series, compounds **16**, **17**, and **18**, were highly soluble in water at room temperature (solubility ≥40 mg/mL), due to the formation of their sodium carboxylate or ammonium chloride salt, respectively (Table I).

In human blood serum no detectable degradation of unsubstituted **8** at concentration of 10^{-3} M was found after 24 h at 37 °C, while the derivative **9** was converted into **8** (deformylation) with a half-life of 3.5 h under the same conditions. Deformylation was also observed with the reference FR 113680 with a comparable half-life. All other substituted analogs of **8** were also stable for 24 h in this assay, despite the variable attachment to *N*-indole via an alkyl, acyl, or carbamate bond. In a rat kidney homogenate (10 mg of tissue/mL), which readily degrades bradykinin, the compounds **8**–**18** were found to be stable at least for 22 h at 37 °C, except for compounds **9** and FR 113680 which were deformylated with a half-life of 8 h.

Bioassays. The pA_2 values displayed in Table II for the NK-1 receptor using the rabbit vena cava preparation varied between 6.5 and 7.4, with compound **15** (substituent R = octanoyl, pA_2 = 4.9) being the sole notable exception. Selectivity for NK-1 with respect to the NK-2 and NK-3 receptors was observed, the pA_2 being by 1–2 units higher on NK-1 than on the other sites. Compound **11** (R = acetyl) was not selective. The whole series of analogs was at least as active and selective as the reference peptides FR 113680 and spantide I. The best NK-1 antagonist in these bioassays was compound **18** (R = [(6-aminohexyl)-oxy]carbonyl), with a pA_2 of 7.4 and a NK-1 selectivity toward NK-2 and NK-3 by 2.4 log units.

Binding Affinity and Selectivity. Displacement studies of tritiated substance *P* from its binding sites on membrane preparations from both rat and mouse brain revealed only low affinities of the analogs **8**–**18**. None of the K_I values was lower than 10^{-6} M, compared to the corresponding values for substance P_{4-11} (7.5×10^{-7} M), spantide I (2.6×10^{-6} M) and FR 113680 (3.2×10^{-7} M, results not shown). While K_I values in binding studies with cloned rat NK-1 receptor were also in the order of 10^{-6} M, those with the cloned rat NK-2 and human NK-1 and NK-2 receptors were 40–150 times lower, corresponding to affinities in the 10–100 nM range (Table III).

Specificity for neurokinin receptors relative to opiate receptors was assessed in competitive binding experiments which showed no significant affinity of the tested compounds for the classical opiate μ-, δ-, and κ-sites in rat brain membranes:[29] IC50 toward [^{3}H]DAGO (μ), [^{3}H]DPDPE (δ), and [^{3}H]U69593 (κ) were higher than 3×10^{-6} M in all cases. Compound **9**, as an example, showed also a specificity for neurokinin receptors by at least 3 orders of magnitude, compared to the reference ligand (in parentheses) in the following binding assays: in guinea pig ileum membranes, bradykinin B2 ([^{3}H]bradykinin);[30] in rat brain membranes, 5HT1A (2-(*N*,*N*-di[2,3-^{3}H]propylamino)-8-hydroxy-1,2,3,4-tetrahydronaphthalene);[31] 5HT2 ([^{3}H]ketanserin);[32] α-1 (-)-([^{3}H]prazosin);[33] α-2 (+)-([^{3}H]clonidine);[34] α-2 (-)-([^{3}H]rauwolscine);[35] in adenosine deaminase-treated rat brain membranes: adenosine A1 ((-)-*N*^{6}-R-[8-^{3}H]phenylisopropyladenosine);[36] adenosine A2 (5′-*N*-ethylcarboxamido[8-^{3}H]adenosine).[37]

Antinociceptive Activity. When administered intravenously (mouse hot-plate test) at 0.5 mg/kg, the analogs induced antinociceptive effects (Table III), with compounds **8**, **9**, **16**, **17**, and **18** being the most active. They were approximately as potent as morphine. However, naloxone did not antagonize the antinociceptive effect (results not shown), thus confirming its nonopiate character. The kinetics of the antinociceptive effect of compound **8** after iv and oral administration is shown as an example in Figure 3. When the compounds were injected intravenously, the activity reached a peak of 50–65% increase of reaction time (RT) after 5–10 min (e.g. **8** in Figure 3A). These values compared well with those obtained after morphine treatment. Comparable increases of RT were obtained after 0.5–1 h and were still present after 2 h when the analogs were given orally at 40 mg/kg (e.g. **8** in Figure 3B). The effect of codeine was in the same range, also lasting for over 2 h. The onset and the maximum intensity of the antinociceptive effect after iv or oral administration were the same as for morphine or codeine, respectively. No effect on behavior or locomotion was observed under these experimental conditions with any of the compounds.

One water-insoluble (compound **9**) and one water-soluble analog (**17**), were tested for their potency to increase the RT in the rat tail flick test after intrathecal injection, against the NK-1 selective agonist [Sar^9,Met(O$_2$)^{11}]-substance P (results not shown). The two analogs blocked

Table III. Binding Affinity (IC50 (nM) ± sem) at Cloned Human and Rat NK-1 and NK-2 Receptors and Antinociceptive Activity (% Increase of Reaction Time) in the Mouse Hot-Plate Test

compd	human NK-1[a]	rat NK-1[a]	human NK-2[a]	rat NK-2[a]	hot plate[b] iv	hot plate[c] po
8	165 ± 26	6400 ± 450	65 ± 14	65 ± 9	66**	74*
9	54 ± 14	10400 ± 900	17 ± 6	142 ± 26	51*	33*
10	127 ± 23	16100 ± 850	79 ± 11	489 ± 48	17	10
16	85 ± 15	14450 ± 1450	129 ± 13	388 ± 30	42*	53*
17	21 ± 5	2900 ± 290	45 ± 9	188 ± 23	54*	62*
18	34 ± 8	3140 ± 100	46 ± 6	244 ± 17	40*	62*
FR 113680	6 ± 1	3400 ± 200	99 ± 9	824 ± 106	nd[d,e]	nd[d,f]

[a] $n = 3$ displacement curves for each analog and assay. [b] Measurement of reaction time 10 min after iv injection of 0.5 mg/kg; percent increase compared to reaction time with vehicle alone; $n = 12$–24 mice, $*p < 0.05$, $**p < 0.01$. [c] Measurement 60 min after oral administration of 40 mg/kg. [d] Insoluble under the same test conditions. [e] Morphine 58%, $p < 0.01$. [f] Codeine 48%, $p < 0.01$.

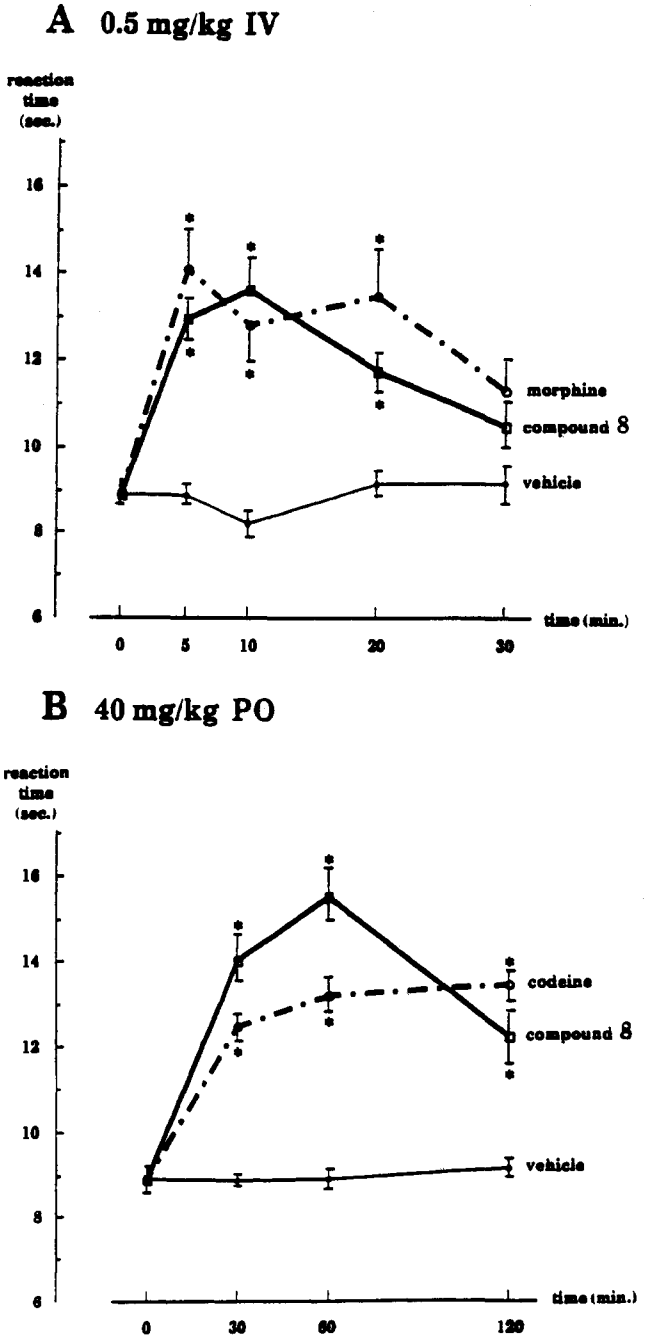


Figure 3. Percent increase of the mean reaction time (11 s) in the hot-plate test (mouse) after iv (A) or po (B) administration of compound 8, compared to morphine (iv) or codeine (po). Doses: 0.5 mg/kg (iv) and 40 mg/kg (po). $P < 0.001$.

in a dose-dependent and reversible way the decrease of RT induced by the agonist. The hyperalgesic effect of 6.5 nmol of agonist (40 μg/kg, 35% reduction of RT) injected 15 min after injection of 0.65 nmol (2.4 μg/kg) of **17** was completely abolished, while it was fully restored in a subsequent test after 24 h in the absence of the antagonist. Compound **9** was about 10 times less active. The antagonists **9** and **17** had no agonistic effect on RT of their own and no adverse effects on locomotion at antagonist doses lower than 65 nmol.

Degranulating Effects. Five among the most active compounds were tested for their ability to promote degranulation of rat peritoneal mast cells. While a significant degranulation (IC50 13 μM) was observed with the free amine analogues **17** and **18**, no degranulation could be detected with compounds **8**, **9**, and **16** when applied in a concentration of 0.1 mM or lower.

Discussion

This study describes chemical and pharmacological properties of a series of compounds derived from the pharmacophore **8**, a pseudotetrapeptide including two residues in a diketopiperazine ring attached via the side chain of the aspartyl residue to a dipeptide amide. Variations of the substituent on the tryptophan indole nitrogen produced a series of chemically well characterized analogs and modulated the physicochemical and pharmacological properties of the pharmacophore.

Potencies in the pharmacological bioassays were modest on NK-1 sites ($pA_2 = 7.0 \pm 0.5$) and about 1 or 2 log units lower on NK-2 and NK-3 sites, thus showing moderate NK-1 selectivity in this test. No clear structure–activity relationship could be derived since the pA_2 values were not significantly different, except for the *N*-octanoyl derivative **15** (pA_2 4.9) on NK-1 sites, the most hydrophobic compound of the series.

The low binding affinity found in the radioligand assay on rat and mouse brain membranes using SP as the ligand as well as in the cloned rat substance P receptors, compared to the higher affinity on human NK-1 sites, is in line with species differences reported by other investigators.[38,39] These differences, although less pronounced, also exist between cloned rat and human NK-2 receptors. Furthermore, the apparent NK-1 selectivity observed in the bioassays (rabbit) is in contrast to the NK-2 selectivity observed in the binding assays on cloned rat receptors. These results therefore suggest that the relatively high antinociceptive activity found in the mouse hot-plate and rat tail flick tests is unlikely to be due to a NK-1 antagonistic activity. Indeed, an apparent correlation is observed between the binding affinities to the cloned rat NK-2 receptors and the percent increase of the reaction time in the iv mouse hot-plate test (columns 5 and 6 in Table III; correlation coefficient $R = 0.9$, $n = 6$). Although this correlation should be taken with great care, due to the difference in species, it might suggest that NK-2 receptors

are involved in pain transmission as evaluated in the mouse hot-plate test.

The results for the most active compounds (Figure 3) are comparable with those obtained with morphine under the same conditions. Indeed, the two agents have a rapid onset of action (maximum in 5–10 min), and their effects persist for at least 20 min. Similar effects are observed when the analogs are given orally, at 80 times higher doses, a reasonable factor considering the generally poor absorption of peptide drugs. It is also noteworthy that the most active and soluble compounds have a log P close to 2, a value found for a large variety of structurally unrelated CNS drugs which cross the blood brain barrier.[40]

Most relevant for the pharmacokinetics, introduction of ionizable substituents on tryptophan results in water solubilization of the analogs **16** (sodium salt) and **17** and **18** (hydrochlorides), which greatly facilitates their administration. Use of an organic solvent (DMSO) in the rat tail flick test (intrathecally) and incorporation of the analogs into liposomes in the mouse hot-plate test can be avoided for these compounds.

All analogs of the series are highly stabilized against proteolysis as assessed both in human blood serum and in rat kidney homogenate preparations, thus confirming the expected effects of chemical modifications in the N- and C-terminal ends of the molecule. The only exception is the observed deformylation of compound **9** to give stable compound **8**. This unexpected and unreported degradation also occurred for FR 113680 under the same conditions. The relatively long duration of action of compounds **8** and **16–18** as the most representative examples is probably the result of this chemical stabilization and fading primarily caused by nonproteolytic elimination processes. Oral activity also reflects a favorable balance between hydrophobicity and solubility and good stability toward proteolysis. No prodrug properties can be expected from the analogs, since despite the variable link between tryptophan–indole nitrogen and the substituent, no rapid conversion of the analogs **10–18** to give the potent metabolite **8** is observed.

Another important goal, which was to prevent the histamine-releasing activity reported for several neurokinin antagonists such as spantide,[41] was achieved for most members of the series. Only analogs **17** and **18** showed significant mast cell degranulation, an effect which could be related to the presence of a free amino function in the indole substituent.

In conclusion, a series of partially cyclic and chemically modified tetrapeptides have been produced, some of them displaying high analgesic potency in in vivo tests in the mouse and the rat, significant oral activity in the mouse, high affinity for cloned rat NK-2 and human NK-1 and NK-2 receptors, despite their modest in vitro potency and affinity in bioassays and binding assays in rodent preparations. No apparent toxicity at therapeutically relevant doses was observed. Good water solubility and prolonged stability against proteolytic degradation was also achieved. In particular, water-soluble **16**, which is antinociceptive in the mouse and which shows no degranulating effects of rat peritoneal mast cells, is a good candidate for further pharmacological evaluation and may represent a valuable alternative to non-peptide tachykinin antagonists.[42]

Experimental Section

General Methods. Precoated plates (Merck F254 silica gel) were used for ascending TLC in the following solvent systems (v/v): (A) BuOH/AcOH/H_2O (72/7/21); (B) $CHCl_3$/MeOH/H_2O/AcOH (70/25/4.5/0.5). Reversed-phase HPLC was performed on a Waters 625 LC system equipped with a Waters 991 photodiode array UV detector, utilizing a Deltapack C18 (spherical 5 μm) column (3.9 × 150 mm). Retention times (t_R) are given for isocratic elution at 1 mL/min in the binary solvent system 0.1% TFA in water/0.1% TFA in acetonitrile (1:1). Preparative HPLC was routinely performed on a Waters Prep LC 3000 system equipped with a Waters 490E multiwavelength detector on a PrePak cartridge (47 × 300 mm) filled with a C18-silica (300 Å, 15 μm) phase. The operational flow rate was 60 mL/min.

For amino acid analyses, peptides were hydrolyzed in 6 N HCl (0.3 mL) for 20 h at 110 °C in sealed tubes. Hydrolysates were analyzed with a Varian LC 90 Star system. The whole procedure including liquid transfer, mixing, Fmoc-derivatization, pentane extraction, and separation on a Aminotag C18 (5-μm) column (4.6 × 150 mm) is completed within 40 min. Molecular weights of peptides were determined by FAB mass spectrometry on a Nermag R10-10C apparatus. The samples were dissolved in a glycerol–thioglycerol matrix (1/1), and ionization was effected by a beam of krypton atoms accelerated through 6–8 keV. Specific optical rotations were measured (g/100 mL) on a Perkin-Elmer 241 polarimeter. ^{1}H NMR spectra were recorded on Bruker spectrometers at 200 or 400 MHz as indicated, with Me_4Si as external standard.

The partition coefficients in the solvent system octanol/water were determined by the shake/flask method[28] for the two analogs **8** and **13**, while for the other analogs they were estimated from the capacity factor on reversed-phase HPLC, using the log P values of **8** and **13** for calibration.

H-Abo-OMe, HCl (1). A solution of 5 g (32 mmol) of L-Abo in 250 mL of dry methanol was treated with 7 mL (96 mmol) of thionyl chloride at 0 °C. After 1 h reaction at 0 °C, the temperature was raised to 60 °C for 3 h. The solvent was evaporated and the residue triturated in benzene and reprecipitated from methanol/benzene. Yield: 6.4 g (96%). Mp: 115–120 °C. TLC: R_f(A) 0.28, R_f(B) 0.49. $[\alpha]^{23}_D$: 6.6° (c 1.0, H_2O). Anal. $C_9H_{16}NO_2Cl$ (205.7): C, H, N, Cl, H_2O. ^{1}H NMR (200 MHz, [$^2H_6$]DMSO): δ 1.30–2.15 (m, 8H), 2.2 (m, 1H), 3.40 (m, 1H), 3.80 (s, 3H), 4.18 (m, 1H), 8.30 (d, NH), 10.65 (t, NH^+).

Fmoc-Asp(OBut)-Abo-OMe (2). A solution of **1** (7.12 g, 34.6 mmol) and triethylamine (4.8 mL, 34.6 mmol) in 70 mL of DMF was mixed with a solution of Fmoc-Asp(OBut)-OH (Bachem, 14.2 g, 34.6 mmol) and HOBt (Fluka, 5.85 g, 34.6 mmol) in 50 mL of DMF. After addition of DCC (8.57 g, 41.6 mmol) previously dissolved in 20 mL of DMF, the reaction mixture was kept with stirring for 14 h at room temperature. Insoluble material was then removed by filtration and the solvent evaporated in vacuo. The crude residue was taken up in ethyl acetate (400 mL) and washed successively three times with citric acid (5%), sodium bicarbonate (5%), and a saturated solution of sodium chloride. The residue obtained after drying over solid sodium sulfate and evaporation was purified by chromatography on silica gel in ethyl acetate/pentane (1/1, v/v). Yield: 18.7 g (96%). TLC: R_f(A) 0.79, R_f(B) 0.91. $[\alpha]^{23}_D$: −66.8° (c 1.0, MeOH). MS: m/e MH^+ 563. Anal. $C_{32}H_{38}N_2O_7$ (562.7): C, H, N, H_2O.

cyclo[-Asp(OBut)-Abo-] (3). The dipeptide **2** was first dissolved in 20% piperidine/DMF (60 mL) and kept for 2 h at room temperature. After evaporation of the solvent under reduced pressure, pure **3** was then obtained by recrystallization from methanol/ether. Yield: 2.6 g (69%). Mp: 132 °C. TLC: R_f(A) 0.68, R_f(B) 0.85. $[\alpha]^{23}_D$: −19.8° (c 1.0, MeOH). MS: m/e MH^+ 309. Anal. $C_{16}H_{24}N_2O_4$ (308.4): C, H, N.

cyclo[-Asp-Abo-] (4). Compound **3** (2.7 g) was dissolved in a mixture of 80 mL of TFA and dichloromethane (1/1, v/v) and stirred for 1 h at room temperature. Trituration of the residue in ether and filtration afforded pure **4**. Yield: 1.74 g (80%). TLC: R_f(A) 0.46, R_f(B) 0.51. $[\alpha]^{23}_D$: −38.9° (c 1.0, MeOH). MS: m/e MH^+ 253. Anal. $C_{12}H_{16}N_2O_4$ (252.3): C, H, N. ^{1}H NMR (200 MHz, [$^2H_6$]DMSO): δ 1.30–1.80 (m, 8H), 2.30 (m, 1H), 2.55 (dd, 1H), 2.70 (dd, 1H), 4.20 (m, 2H), 8.20 (s, 1H), 12.50 (s, 1H).

H-Phe-N(Me)-Bzl, HCl (5). To the solution in 200 mL of DMF of 50 g (188 mmol) Boc-Phe-OH, 66.4 g (207 mmol) TBTU, 34.9 g (207 mmol) HOBt, and 26.3 g (207 mmol) methylbenzylamine was added 36 mL (207 mmol) of diisopropylethylamine under cooling at 0 °C. After a 1-h reaction, the solution was

stirred for 16 h at room temperature. The solvent was then evaporated and the crude residue taken up in ethyl acetate and washed and dried as was compound **2**. The residue obtained after evaporation of the solvent was triturated in pentane from which it crystallized. Yield: 58 g (83%). TLC: R_f(A) 0.86, R_f(B) 0.74. The solid was then treated with 250 mL of a 3.2 N solution of gaseous HCl in ethyl acetate for 90 min at room temperature. Evaporation and reprecipitation from ethyl acetate/pentane afforded 35.3 g of **5** (overall yield 60%). TLC: R_f(A) 0.48, R_f(B) 0.53. $[\alpha]^{23}_D$: 30.5° (*c* 1.0, H_2O). Anal. $C_{17}H_{21}N_2OCl$ (304.8): C, H, N, Cl, H_2O.

H-D-Trp-Phe-N(Me)-Bzl, HCl (7). To the solution in 80 mL of DMF of 15 g (49 mmol) of Boc-D-Trp-OH, 7.3 g (54 mmol) of HOBt, 17.4 g (54 mmol) of TBTU, and 15 g (49 mmol) of compound **5** was added 14 g (108 mmol) of diisopropylethylamine under cooling to 0 °C. After 16 h at room temperature the reaction mixture was separated from the solvent and the crude residue washed as for compound **2** in ethyl acetate. The oily residue was then triturated in ether and pentane until a solid compound was obtained which was separated by filtration. Yield 22.3 g (81%) of a chromatographically pure product (**6**). TLC: R_f(A) 0.72, R_f(B) 0.75. Deprotection occurred by treatment of portions of this material (10 g, 18 mmol) with a 3.2 N solution of HCl in ethyl acetate for 45 min at room temperature. After evaporation of the solvent and stirring in dry ether for 2 h, a powdery product could be filtered off, rinsed, and dried. Yield: 9.8 g (overall yield 80%). TLC: R_f(A) 0.53, R_f(B) 0.48. $[\alpha]^{23}_D$: −29.6° (*c* 1.0, MeOH). Anal. $C_{28}H_{31}N_4O_2Cl$ (491.0): C, H, N, Cl.

cyclo[-Abo-Asp(D-Trp-Phe-N(Me)-Bzl)-] (8). The following reaction components were dissolved together in 50 mL of dichloromethane: 4.6 g (18 mmol) of cyclo[-Abo-Asp-], 3.3 g (20 mmol) of HOBt, 6.4 g (20 mmol) of TBTU, and 9 g (18 mmol) of H-D-Trp-Phe-N(Me)-Bzl, HCl. Diisopropylethylamine (5.17 g, 40 mmol) was then added under cooling at 0 °C and the mixture slowly brought to room temperature. Stirring for 24 h, evaporation of the solvent, washing in ethyl acetate with the same aqueous solutions as for compound **2**, drying, and evaporating led to a crude product which could be obtained as a filterable powdery compound when triturated in dry ether: 12.2 g (96%). The final product could be recrystallized from acetonitrile, rinsed with ether, and dried. Yield: 9.5 g (75%). Mp: 130 °C. Anal. $C_{40}H_{44}N_6O_5$ (688.8): C, H, N. AAA Abo 1.04 (1), Asp 1.01 (1), Phe 0.94 (1), Trp nd. ^{1}H NMR (400 MHz, [^{6}H]DMSO): δ 1.75–1.91 (m, 2H$_\beta$, 2H$_\gamma$, Abo), 2.42 (s, 1H$_\delta$, Abo), 2.50 (t, 1H$_\beta$, Asp), 2.8 (t, 1H$_\beta$, Asp), 2.9 (s, 3H, NMe), 3.08 (m, 2H$_\beta$, Phe), 3.1 (t, 1H$_\beta$, Trp), 3.35 (t, 1H$_\beta$, Trp), 4.0 (s, 1H, Abo), 4.15 (t, 1H$_\alpha$, Asp), 4.25 (d, 1H$_\beta$, Phe), 4.7 (d, 1H$_\beta$, Phe), 4.82 (m, 1H$_\alpha$, Trp), 5.15 (m, 1H$_\alpha$, Phe), 7.10–7.15 (m, 7H, Phe and Trp), 7.4 (m, 7H, Phe), 7.65–7.70 (m, 3H, Trp and Phe), 9.35 (d, 1H, NH Trp).

cyclo[-Abo-Asp(D-Trp(For)-Phe-N(Me)-Bzl)-] (9). Gaseous HCl was passed through a solution of the precursor **8** (5 g, 7.3 mmol) in formic acid (30 mL) until saturation, and the resulting mixture was stirred for 16 h at room temperature. After evaporation of the solvent under reduced pressure, the pink oil was triturated in diethyl ether and the crude product chromatographed on silica gel (column 2.5 × 40 cm) in $CHCl_3$/MeOH to give **9** (3.1 g, 59%).

cyclo[-Abo-Asp(D-Trp(Me)-Phe-N(Me)-Bzl)-] (10). This compound was obtained by using the same synthesis steps as compound **8** and in comparable yields, starting with N^{in}-Me-D-Trp,[43] instead of D-Trp. Yield of the last coupling of **4** (2 mmol) to H-D-Trp(Me)-Phe-N(Me)-Bzl (2 mmol) to give **10**: 860 mg (61%). AAA Abo 1.02 (1), Asp 0.99 (1), Phe 0.99 (1), Trp nd.

General Procedure for the Preparation of Compounds 11–15 and 18). The precursor **8** (2.06 g, 3 mmol) was dissolved upon warming in 80 mL of methylene chloride. The cooled solution was then treated with a small amount of the phase-transfer catalyst tetrabutylammonium hydrogen sulfate (10 mg, 0.03 mmol) and with solid sodium hydroxide (480 mg, 12 mmol), and after 10 min with a 3–4-fold excess of the corresponding acylating or carbamate-forming agent. The mixture was stirred for 16 h at room temperature and the reaction mixture washed with water and saturated NaCl solution. The crude product obtained after evaporation of the solvent was purified by preparative HPLC on reverse phase.

cyclo[-Abo-Asp(D-Trp(Ac)-Phe-N(Me)-Bzl)-] (11). This compound was obtained by the general procedure using acetyl chloride (3 equiv) as the acylating agent. Yield: 1.25 g (57%).

cyclo[-Abo-Asp(D-Trp(OCOMe)-Phe-N(Me)-Bzl)-] (12). This compound was prepared by the general procedure starting from **8** (500 mg, 0.73 mmol) and using methyl chloroformate (168 μL, 2.18 mmol) for carbamate formation. Yield: 313 mg (58%).

cyclo[-Abo-Asp(D-Trp(OCOBzl)-Phe-N(Me)-Bzl)-] (13). This compound was obtained by the general procedure using benzyl chloroformate (1.24 mL, 8.7 mmol) for acylation. Yield: 2.1 g (86%).

cyclo[-Abo-Asp(D-Trp(OCOtBu)-Phe-N(Me)-Bzl)-] (14). This compound was prepared by a slight modification of the general procedure, starting with 400 mg (0.58 mmol) of **8** and treating the solution in methylene chloride with a catalytic amount of (dimethylamino)pyridine (7 mg, 58 μmol) and di-*tert*-butyl dicarbonate (133 mg, 0.6 mmol). Yield: 180 mg (39%).

cyclo[-Abo-Asp(D-Trp(OC(CH$_2$)$_6$CH$_3$)-Phe-N(Me)-Bzl)-] (15). This compound was obtained by the general procedure using octanoyl chloride (1.21 g, 9 mmol) as the acylating agent. Yield: 1.15 g (47%).

cyclo[-Abo-Asp(D-Trp(OC(CH$_2$)$_2$COOH)-Phe-N(Me)-Bzl)-] (16). To a solution of the crown ether 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6) (960 mg, 3.6 mmol) in 40 mL of THF were added successively the precursor **8** (2.5 g, 3.6 mmol), succinic anhydride (450 mg, 4.5 mmol), solid KF (420 mg, 7.2 mmol), and diisopropylethylamine (790 μL, 4.5 mmol). After a 70-h reaction in the dark at room temperature, the solvent was evaporated and the residue washed successively in ethyl acetate three times with 5% $NaHCO_3$, 5% citric acid, and a saturated NaCl solution. Purification of the crude product was achieved by ion-exchange chromatography on Amberlite IRA-93 (free base) in water/acetonitrile (1:1) and elution with 0.1 M HCl in the same solvent. The solid crude product (544 mg, 24%) was reprecipitated from methanol/ether and converted to its sodium salt by dissolution in acetonitrile/water (1:1), addition of 1 equiv of 1 M NaOH, and lyophilization.

cyclo[-Abo-Asp(D-Trp(OC(CH$_2$)$_6$NH$_3^+$Cl$^-$)-Phe-N(Me)-Bzl)-] (17). This compound was prepared by the same method as **16**, by reacting **8** (2.5 g, 3.6 mmol) with Boc-NH(CH$_2$)$_6$CO-ONp (1.6 g, 4.5 mmol) in the presence of KF (422 mg, 7.2 mmol), the crown ether 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6) (960 mg, 3.6 mmol), and diisopropylethylamine (790 μL, 4.5 mmol) in THF, for 70 h in the dark at room temperature. The product was washed following the general procedure and purified by chromatography on silica gel (column 0, 5 × 40 cm) in chloroform/methanol (97/3). Yield: 1.6 g (48%). For deprotection, the product (1.5 g) was then treated with 3 M HCl in ethyl acetate (100 mL) for 30 min at room temperature, the solvent was evaporated, and the oily hydrochloride was triturated in ether and dried. Yield: 1.26 g (91%). Further purification was obtained by preparative HPLC of two fractions of 600 mg. Since the solvent system contained TFA, the product was submitted to ion-exchange chromatography on Amberlite IRA-93 in water/acetonitrile (1:1), and the residue was dissolved in a minimum volume of dioxane and treated with 3 M HCl in ethyl acetate (100 mL) for 5 min at room temperature, which precipitated the pure hydrochloride **17**. Yield: 800 mg (overall 14%).

cyclo[-Abo-Asp(D-Trp(OCO(CH$_2$)$_6$NH$_3^+$Cl$^-$)Phe-N(Me)-Bzl)-] (18). This compound was obtained by the general procedure, reacting **8** with the carbamate-formating agent Fmoc-NH(CH$_2$)$_6$OCOCl, prepared classically[44] from Fmoc-NH(CH$_2$)$_6$-OH with a 5-fold excess of phosgene in toluene. Product isolation was achieved by chromatography on silica gel (column 0, 3 × 40 cm) in chloroform/methanol (97/3). Yield: 853 mg (41%). For deprotection, the product (800 mg) was treated with 20% piperidine in DMF for 20 min at room temperature. Trituration in diethyl ether, filtration, and finally conversion to the hydrochloride by reprecipitation from 3 M HCl in ethyl acetate yielded 310 mg (47%).

Degradation Studies in Vitro. A known amount of the derivative (40 nM) was added to an aliquot (500 μL) of a heparinized plasma preparation and incubated for various durations at 37 °C in a water bath under gentle shaking. Reactions were stopped by addition of trifluoroacetic acid (10 μL) and acetonitrile. Samples were then centrifuged, and supernatants

were decanted off and injected (50 μL) for HPLC analysis. Identification of the metabolites was then performed by amino acid analysis and mass spectroscopy of the individual peaks of elution, as a function of time.

Binding. Each aliquot (1 mg of protein in 100 μL, protein concentration estimated according to Smith et al.[45]) of the membrane fraction of brains from male Wistar rats was incubated in 150 μL containing tritiated SP (Amersham, 40 Ci/mmol, final concentration 1 nM) and a variable concentration of the competing analog in 750 μL of buffer (Tris 50 mM, $CaCl_2$ 2 mM, $MgCl_2$ 2 mM, BSA 0.02%, pH 7.7), for 20 min at 25 °C. Incubation was followed by filtration, washing of the filters, and scintillation counting. Control experiments, subtraction of the nonspecific binding, and statistical evaluation were made using classical methods for each specific ligand (references given with the results). The K_I value of the antagonists was evaluated according to Cheng and Prusoff[46] on the basis of a direct binding experiment with the agonist alone.

Radioligand binding assays were performed with transfected CHO cells which expressed either recombinant rat NK-1 or NK-2 receptors[22,47] or human NK-1 or NK-2 receptors.[48] The cells were incubated for 2 h (to equilibrium) at 4 °C with [^{125}I-Tyr^1]SP or [^{125}I-His]NKA to label NK-1 and NK-2 sites, respectively. Radioligand concentrations used were approximately 0.1 nM, and the CHO cell expressed rat and human NK-1 receptors had K_d values of 1.5 and 0.7 nM, respectively, and the rat and human NK-2 receptors had K_d values of 2.1 and 1.7 nM, respectively. Binding in the presence of defined concentrations (3-fold dilutions) of the analogs was then estimated with a filtration assay and data processed as IC50 values.

Bioassays. The procedures for the isolation of the organs, their handling for pharmacological tests, and the recording system used for measuring the SP (in the rabbit vena cava), neurokinin A (in the rabbit pulmonary artery), and neurokinin B (in the rat portal vein) contractile effects have been described.[23] Antagonist apparent affinities were evaluated in terms of pA_2 by the Schild plot method.[49]

In Vivo Assays. The hot-plate test was performed on male CD1 mice (25–30 g) previously kept in a room with controlled temperature (20 ± 2 °C) and hygrometry (45–65%) and with a 12 h/12 h light–dark cycle and with free access to standard pellet diet and tap water. The animals were placed on a metal plate (DS-37, Apelex) maintained at 55 ± 0.2 °C, and latency time between heat stimulus application and licking of forepaws was measured 10 min after intravenous injection of the test compounds (0.5 mg/kg). In the kinetic assays, the compounds were administered by iv (0.5 mg/kg) or oral route (40 mg/kg), and the antinociceptive effect was measured as a function of time. In association with naloxone, compound 9 was administered by iv route 10 min after subcutaneous injection of 1 mg/kg naloxone. Water-soluble compounds were given in 0.9% NaCl (iv) or in water (orally). Insoluble compounds were given in 10% Tween 80 (iv) or in 0.2% hydroxypropylcellulose (orally). Randomly designed groups of 12 animals were used in each experiment. The means of treated groups were compared with the means of the vehicle-treated control group. Statistical evaluation was made by the Dunnett t-test.[50] The tail flick test followed the protocol of Couture et al.[51] for the implantation of the catheters, the pretreatment of the rats, and the measurement of the reaction time. The intensity of the heat stimulus was set to elicit a tail flick within 10–15 s, in order to optimize the hyperalgesic effect of the agonist [Sar^9,Met(O$_2$)^{11}]SP. The results were calculated as a percentage of the maximum possible effect and the statistical significance of the differences evaluated with the Student t-test. The antagonists were administered in a single dose of 0.65, 6.5, or 65 nmol (dose range of 2.0–240 μg/kg), 15 min before it injection of the agonist (6.5 nmol, 40 μg/kg). The antagonists were dissolved in DMSO, and artificial CSF (composition (mM): 128 NaCl, 2.6 KCl, 2.0 $MgCl_2$, 1.4 $CaCl_2$, pH 7.2) was added to obtain the desired solution (volume part of DMSO <30%).

Peritoneal Mast Cell Degranulation. For each experiment two or more male Sprague–Dawley rats weighing 350–400 g were killed by CO_2 asphyxiation and mast cells obtained by rinsing the peritoneal cavity (20 mL of buffer NaCl 50 mM, KCl 2.7 mM, $CaCl_2$ 0.9 mM, Na_2HPO_4 3 mM, KH_2PO_4 3.5 mM, glucose 5.6 mM, bovine serum albumin 1 mg/mL, pH 6.8). Washings pooled in conical polypropylene tubes were centrifuged at 400g for 10 min at 4 °C and resuspended in buffer to a density of 2 × 10^4 cells/mL). After a 10-min incubation at 37 °C, a solution (in water or DMSO) of the test compounds was added and incubation continued for 10 min in order to permit histamine release. The reaction was then quenched by placing the tubes in ice and centrifugation followed at 700g for 10 min at 4 °C. Supernatant and pellet were assayed fluorimetrically after condensation with o-phtalaldehyde, and histamine was released from the cell into the supernatant expressed as percentage of the total histamine content.

References

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