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Design and Synthesis of Side-Chain Conformationally Restricted Phenylalanines and Their Use for Structure–Activity Studies on Tachykinin NK-1 Receptor[†]

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Constrained analogues of phenylalanine have been conceptually designed for analyzing the binding pockets of Phe[7] (S_7) and Phe[8] (S_8), two aromatic residues important for the pharmacological properties of SP, i.e., L-tetrahydroisoquinoleic acid, L-diphenylalanine, L-9-fluorenylglycine (Flg), 2-indanylglycine, the diastereomers of L-1-indanylglycine (Ing) and L-1-benz[*f*]indanylglycine (Bfi), and the *Z* and *E* isomers of dehydrophenylalanine (Δ^ZPhe, Δ^EPhe). Binding studies were performed with appropriate ligands and tissue preparations allowing the discrimination of the three tachykinin binding sites, NK-1, NK-2, and NK-3. The potencies of these agonists were evaluated in the guinea pig ileum bioassay. According to the binding data, we can conclude that the S_7 subsite is small, only the gauche (−) probe [(2*S*,3*S*)-Ing[7]]SP presents a high affinity for specific NK-1 binding sites. Surprisingly, the [Δ^EPhe[7]]SP analogue, which projects the aromatic ring toward the trans orientation, is over 40-fold more potent than the *Z* isomer, [Δ^ZPhe[7]]SP. A plausible explanation of these conflictual results is that either the binding protein quenches the minor trans rotamer of [(2*S*,3*S*)-Ing[7]]SP in solution or this constrained amino acid side chain rotates when inserted in the protein. In position 8, the high binding affinities of [Flg[8]]SP and [(2*S*,3*S*)-Bfi[8]]SP suggest that the S_8 subsite is large enough to accept two aromatic rings in the gauche (−) and one aromatic ring in the trans direction. Peptides bearing two conformational probes in positions 7, 8, or 9 led to postulate that S_7, S_8, and S_9 subsites are independent from each other. The volumes available for side chains 7 and 8 can be estimated to be close to 110 and 240 Å^3, respectively. The large volume of the S_8 subsite raises question on the localization of the SP-binding site in the NK-1 receptor. If SP were to bind in the transmembrane domains, the cleft defined by the seven transmembrane segments must rearrange during the binding process in order to bind a peptide in an α-helical structure and at least one large binding subsite in position 8. Thus, indirect topographical analysis with constrained amino acids might contribute to the analysis of the receptor/ligand dynamics. Finally, this study demonstrates that a good knowledge of the peptidic backbone structure and a combination of constrained amino acids are prerequisites to confidently attribute the preferred orientation(s) of an amino acid side chain.

Introduction

Five tachykinins have been isolated from mammals: substance P (SP),[1] neurokinins A and B (NKA, NKB),[2–4] neuropeptide-γ (NP-γ),[5] and neuropeptide K (NPK).[6] Three types of tachykinin receptors have been characterized both pharmacologically[7,8] and by molecular cloning:[9] SP has the highest affinity for the NK-1-type, NKA for the NK-2-type, and NKB for the NK-3-type receptors, respectively. These three mammalian receptors belong to the family of G-protein-coupled seven transmembrane (7TMS) receptors. Comparison of binding and pharmacological data with agonists and antagonists has recently demonstrated the existence of species differences for the pharmacological properties and structure of NK-1 receptors[11,12] and has also led to the hypothesis that NK-1[13,14] and NK-2[15,16] receptor subtypes might also exist.

For the past years, our aim has been to determine the bioactive fingerprint of the NK-1 receptor as a negative image of topographical probes derived from the preferred endogenous ligand SP, i.e., SP-constrained analogues with reasonably stable conformations.[7] A traditional approach to determine stable conformations of a bioactive peptide involves conformational constraints. A disulfide or amide bridge between nonadjacent residues,[17] substitution by proline[18] or lactam[19] residues, and N-methylation[18–20] of the backbone carboxamide are now widely used as intramolecular or local conformational restrictions. Such modifications introduced in the sequence of SP have enabled us to deduce favored conformations for the SP backbone: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. The core of SP from Lys[3](CO) to Phe[8](NH) can adopt either an α- or 3_{10}-helix structure or two consecutive β-turns, type II′ and I, if the chiral center C_α of residue 5 has been inverted.[21–23] The C-terminal tripeptide Gly-Leu-Met-NH_2 should exist in a more or less extended conformation, i.e., a 2–7 ribbon type.[7,18] These conclusions have been drawn from biological data and structural analysis.[7,24,25]

† The abbreviations for the amino acids are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Chem.* 1984, *138*, 9–37). The symbols represent the L-isomer except when indicated otherwise. The other abbreviations used are as follows: 7TMS, seven transmembrane segments; AAA, amino acid analysis; Bfi, 2-(1-benz[*f*]indanyl)glycine; Boc, (*tert*-butyloxy)-carbonyl; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; Dip, 3,3-diphenylalanine; Flg, 2-(9-fluorenyl)glycine; HOBt, 1-hydroxybenzotriazole; HPh, homophenylalanine; Ing, 2-(1-indanyl)glycine; 2Ing, 2-(2-indanyl)glycine; NMP, *N*-methyl-2-pyrrolidone; Pen, penicillamine; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Tle, *tert*-leucine; GPI, guinea pig ileum; GPT, guinea pig trachea.

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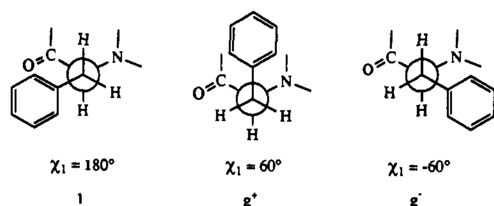


Figure 1. Side-chain orientations for α-amino acids (χ_1 torsional angle).

Except for the proline residue, the amino acid side chains are generally flexible, their orientations depending on the value of the torsional angle χ_1 which can take three different limit values (Figure 1). Thus, the second step of our approach has involved the design of conformationally constrained amino acids with a particular χ_1 value, allowing us to probe the length and width of the binding pockets for the important side chains of the bioactive peptide. New constrained analogues of phenylalanine have been synthesized for the first time; they have been conceptually designed for the analysis of the binding pockets of Phe^7 and Phe^8, two important aromatic residues for the pharmacological properties of SP. (In this paper, we have extended the nomenclature used for enzyme subsites, and subsites occupied by the side chains of residues 7 and 8 in the NK-1 receptor will be named S_7 and S_8.) Recently, attention has been focused on the incorporation of conformationally restricted Phe or Tyr to better assess the structural requirements of these aromatic side chains in the sequence of various peptides.[26-31] The structures of the various amino acids, **1–16**, introduced in position 7 or 8 in the sequence of SP are reported in Figure 2. In preliminary reports, the original procedure we have developed for the diastereoselective synthesis of some of these constrained analogues of phenylalanine has been briefly described for L-diphenylalanine (Dip) (**7**), L-9-fluorenylglycine (Flg) (**8**),[32] and the diastereoisomers of L-1-indanylglycine (Ing), **9** and **10**, and L-1-benz[f]indanylglycine (Bfi), **11** and **12**.[33] (Detailed experimental part for the diastereoselective synthesis of these amino acids, **7–12**, has been included in the Experimental Section of this full report.) In this paper, the consequences on the conformation of SP-substituted analogues, **17–39**, have been analyzed according to their binding and pharmacological potencies.

General Considerations on the Conformational Probes

The volumes of the various side chains calculated by the volume function of the SYBYL program vary from 73 (Tle) to 153 (Dip) Å^3 (Figure 2). Since the respective side chains are more or less flexible, the space which can be potentially occupied in the three rotameric orientations g^+, g^-, and t should correspond to the volume in the case of the symmetric Tle side-chain and, except for the *Z* and *E* rotamers of dehydrophenylalanine, should be at least 3 times the value reported in Figure 2 in the other cases. Schematic views of the different probes' cross sections have been drawn according to two different axes, Figure 3a corresponding to a Newman projection of the C_β substituents and Figure 3b to a projection centered on C_β (three atoms bound to C_β plus C_α). The limits of the volumes which can be inserted into the S_7 and S_8 binding subsites of the NK-1 receptor have been firstly estimated by increasing the volumes of the β- and γ-substituted side chains, i.e., Tle (73 Å^3), Pen(S-Me) (91.8 Å^3), and Pen-

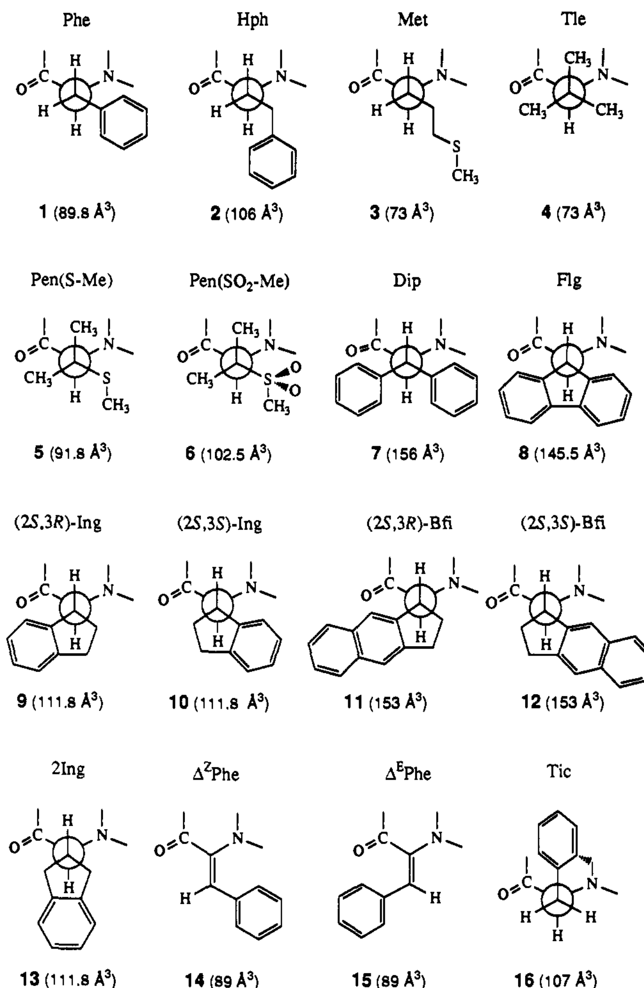


Figure 2. Newman projections of the conformational probes used in this study.

(SO_2-Me), (102.5 Å^3), and then by incorporating specific rotameric probes. Pen(SO_2-Me)-substituted peptides should also give information on an eventual role for electrostatic interactions.

Considering the conformationally restricted analogues of phenylalanines, **7–16**, the three rotameric directions g^+, g^-, and t are not sterically equivalent, as demonstrated by calculations (Table 1). When the energy of each conformer was calculated by minimizations of a model substituted amino acid, CH_3CO-X-$CONH_2$ (X = **4**, **8**, **9**, or **10**), for a given ϕ and ψ values, differences in the energies obtained for the various rotamers were indeed observed. For example, for (2*S*,3*S*)-Ing (**10**) in a B conformation, (ϕ, ψ = −150°, 150°), the t rotamer corresponded to the lowest energy for the side chain; however, in an A conformation (ϕ, ψ = −70°, 50°), the g^- conformer was the most stable. Thus, depending on the conformation adopted by the constrained amino acid, the side chain will explore different orientations of the space and could be used as either a t, g^+, or g^- probe. Within a backbone-restricted matrix, the specific orientation of these constrained side chains is also related to the conformation of the peptidic backbone. For example, the presence of two turns of helix will prevent a bulky side chain from adopting a g^+ orientation in the second turn of the helix; consequently, all the bulky side chains will adopt g^- and/or t orientations. For example, for a (2*S*,3*S*)-Ing incorporated in an α-helix, the g^+ rotamer being excluded for structural considerations, the difference between the energies of the g^- and t rotamers (3 kcal/mol) suggested that (2*S*,3*S*)-Ing could be regarded as a g^- probe.

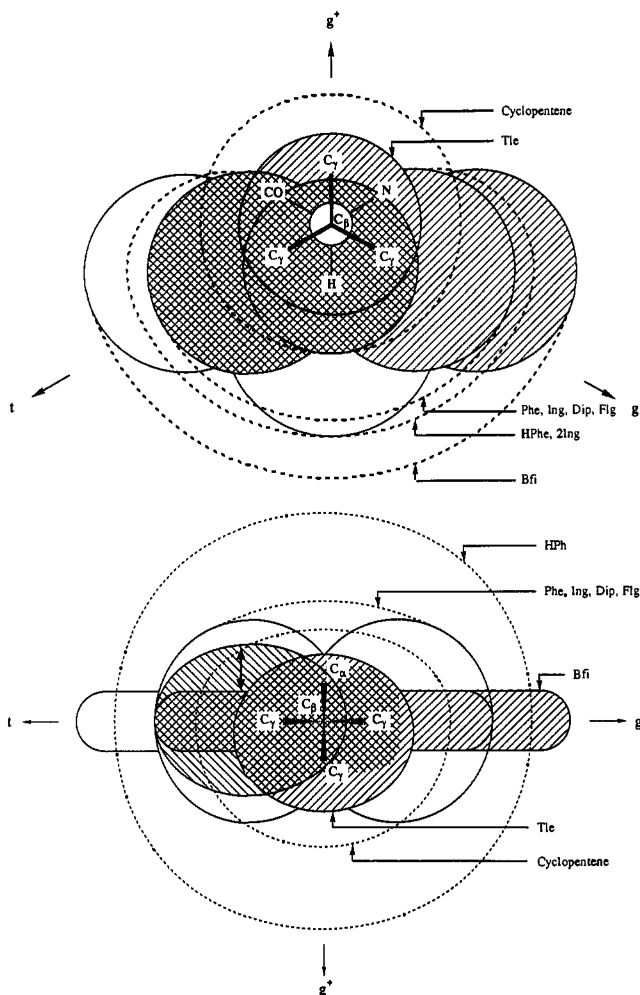


Figure 3. Cross sections of accessible volumes of the different side chains: (top) projection in a plane perpendicular to the C_α–C_β bond (Newman projection) and (bottom) projection in a plane perpendicular to the previous one centered on the C_β atom. The shift from top to bottom projections corresponds to a 120° rotation in the C_β,C_α,H_α plane. The orientations of the different side chains with the restrictions imposed by an α-helix backbone were schematically drawn; aromatic ring(s) and cyclopentene were approximately depicted as circles or ellipses: (broken circles) possible (accessible) conformers; (solid circles) stable conformers (energetically favored); (forward slashes) orientations accepted in the S_7 subsite of specific NK-1 binding site; and (backward slashes) orientations accepted in the S_8 subsite of specific NK-1 binding site.

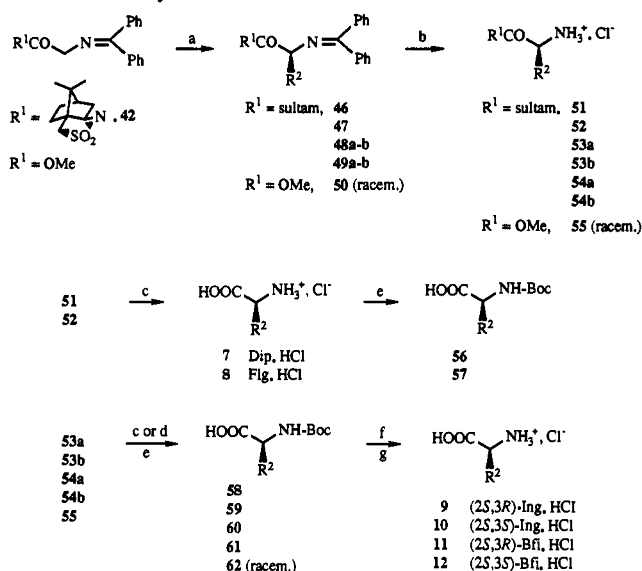
Table 1. Energies (kcal/mol) of the Different Populations of CH_3-CO-X-$CONH_2$ in Different Conformations, Calculated by Energy Minimization Using the SYBYL Program[a]

X	rotamers	B	A	A_β	E
(2*S*,3*S*)-Ing	g⁻	4.51	3.29	4.51	3.34
	g⁺	4.90	4.28	5.07	3.20
	t	3.06	6.24	6.73	3.48
(2*S*,3*R*)-Ing	g⁻	1.51	4.03	4.14	2.76
	g⁺	2.44	1.65	1.92	*b*
	t	2.24	2.92	2.92	3.8
(2*S*)-Flg	g⁻, t	1.61	3.68	4.87	1.70
	g⁺, g⁻	1.71	2.05	1.58	3.08
	t, g⁺	1.25	2.69	3.82	3.43
(2*S*)-Tle	g⁻, t, g⁺	−3.53	−1.43	0.42	−1.65

[a] B: $\phi, \psi = -150°, 150°$. A: $\phi, \psi = -70°, -50°$. E: $\phi, \psi = -80°, 80°$. In the A_β structure, the amino acid X has been inserted in a type I β-turn structure in position $i + 1$; $\phi, \psi = -60°, -30°$.[79] [b] Rotamer incompatible with an E structure for steric hindrance.

In the particular case of SP, the core of the peptide, Lys[3](CO)–Phe[8](NH), is folded in an α-helical structure,[21] Phe[7] and Phe[8] being at the beginning of the second turn of the helix. Therefore, according to the above-mentioned considerations, a Tic residue introduced in position 7 or 8 will disrupt the α-helical structure of the core of SP since a g^+ orientation of the side chain in the second turn of an α-helix is excluded.

Scheme 1. Syntheses of Constrained Amino Acids **7–13**[a]



[a] (a) *n*-BuLi, R^2Br with R^2 = bromodiphenylmethane, 9-bromoflurene, 1-bromoindane (**43**), 1-bromobenz[*f*]indane (**44**), and LDA, R^2Br with R^2 = 2-bromoindane (**45**), yielding compounds **46**, **47**, **48a,b**, **49a,b**, and **50**, respectively; (b) 0.5 N $HCl/CHCl_3$; (c) LiOH; (d) $Na_2S \cdot 9H_2O$, THF/H_2O or LiOH for compound **55**; (e) Boc_2O; (f) TFA/CH_2Cl_2, 1:1; (g) 1 N HCl.

If one assumes that the other constrained amino acids will not dramatically perturb the helical structure of SP, one can postulate that:

(i) the Flg and Dip probes will simultaneously occupy the g^- and t orientations;

(ii) the two diastereoisomers (2*S*,3*S*) and (2*S*,3*R*) of Ing and Bfi will correspond to a g^- and t probe, respectively, since the χ_1 torsional angle is controlled by the chirality of C_β whereas the cyclopentene ring imposes the orientation of the aromatic ring, $\chi_2 = +60°$ and $-60°$ for the 2*S*,3*S* and 2*S*,3*R* diastereoisomers, respectively;

(iii) the two isomers of dehydrophenylalanine, Δ^ZPhe and Δ^EPhe, will correspond to a g^- and t orientation, respectively.

This working hypothesis has been confirmed, in the case of SP, by the NMR analysis of the SP analogues incorporating these constrained side-chain analogues (H. Josien, Thèse Doctorat d'Université, Paris VI, France, October 25, 1993, manuscript in preparation).

Results and Discussion

Syntheses. The *S*-methyl derivative of penicillamine, Pen(S-Me) (**5**), has been obtained by alkylation of penicillamine by chloromethane in liquid ammonia. Oxidation of **5** by hydrogen peroxide in glacial acetic acid yielded the sulfone Pen(SO_2-Me) (**6**); these amino acids were isolated as their N^α-(*tert*-butyloxy)carbonyl derivatives **40** and **41**, respectively. The general strategy we have developed for the synthesis of new, optically pure, constrained analogues of phenylalanine, i.e., amino acids **7–12**, is outlined in Scheme 1. DL-Diphenylalanine has been previously incorporated in the sequence of angiotensin II,[34] and more recently, another diastereoselective synthesis of Dip has

Table 2. Physicochemical Properties of the Constrained Amino Acids **7–12** and/or Their N^α-Boc Derivatives **56–62**

compd	structure[a]	mp (°C)	$[\alpha]^{20}_D$ (deg)[c]	TLC[d]	HPLC[e]
7	Dip, HCl	205–208	+43	0.67	10.40
8	Flg, HCl	>270	−26	0.64	9.50
9	(2*S*,3*R*)-Ing, HCl	227–230[b]	−9.7	0.60	3.60
10	(2*S*,3*S*)-Ing, HCl	185–188[b]	+53.9	0.59	3.65
11	(2*S*,3*R*)-Bfi, HCl	221–225[b]	−69.9	0.66	11.90
12	(2*S*,3*S*)-Bfi, HCl	192–194[b]	+90.9	0.66	11.60
56	Boc-Dip	129–131	+33.6	0.41	24.90
57	Boc-Flg	206–209	+49.7	0.40	24.40
58	Boc-(2*S*,3*R*)-Ing	109–111	+10.8	0.39	21.70
59	Boc-(2*S*,3*S*)-Ing	foam	+28.6	0.39	22.30
60	Boc-(2*S*,3*R*)-Bfi	213–216[b]	−96.2	0.38	26.40
61	Boc-(2*S*,3*S*)-Bfi	102–106	+112.7	0.40	27.30
62	Boc-DL-2Ing	175–177		0.39	22.50

[a] Unnatural amino acid abbreviations are given as a corresponding footnote to the title and are drawn in Figure 2. [b] Decomposition on Kofler melting point apparatus. [c] Value for *c* 0.5; 1 N HCl for **7–8**, AcOH for **9–12**, MeOH for **56–59** and **61**, and DMF for **60**. [d] TLC conditions: *n*-BuOH/AcOH/H_2O (6:2:2) for **7–12** and $CHCl_3$/MeOH/AcOH (95:5:0.5) for **56–62**. [e] Lichrospher 100 RP-8e column in gradient mode (0.25 M triethylammonium phosphate buffer, pH 3.0, and acetonitrile) at a flow rate of 1.5 mL/min monitored at λ = 210 nm. Gradient mode: 12–48% CH_3CN, linear, 30 min for **7–12**; and 16–64% CH_3CN, linear, 30 min for **56–62**.

been described.[35] The key step for our synthesis of amino acids **7–12** involved an asymmetric alkylation of a Schiff base from a sultam-derived glycinate, **42**, by the corresponding electrophiles, i.e., bromodiphenylmethane for Dip (**7**), 9-bromofluorene for Flg (**8**), 1-bromoindan (**43**) for (2*S*,3*R*)-Ing (**9**) and (2*S*,3*S*)-Ing (**10**), and 1-bromobenz[*f*]indan (**44**) for (2*S*,3*R*)-Bfi (**11**) and (2*S*,3*S*)-Bfi (**12**), respectively. The yields of the alkylation step varied from 68% to 88%, the diastereoisomeric excess at the C_α chiral center was over 95% as estimated from the NMR analysis, and only a slight induction on the C_β center for the 1-indanyl and 1-benz[*f*]indanyl probe was observed. Purification and recrystallization afforded the alkylated products with a de over 99%. The racemic amino acid DL-2Ing (**13**) has been obtained by alkylation of an achiral Schiff base of methyl glycinate, $(C_6H_5)_2C{=}N{-}CH_2{-}COOCH_3$, with 2-bromoindan (**45**). Attempts to alkylate the sultam-derived glycinate **42** with 2-bromoindan or 2-[(methylsulfonyl)oxy]indan were unsuccessful. Hydrolysis of the alkylated Schiff bases yielded the corresponding *N*-aminoacylsultam derivatives **51**, **52**, **53a–b**, **54a–b**, and **55**. At this stage, the 1-indanyl, **53a–b**, and 1-benz[*f*]indanyl, **54a–b**, diastereoisomers were separated by silica gel chromatography. The sultam or methyl ester group was then cleaved by either LiOH or $Na_2S{\cdot}9H_2O$ for the sterically hindered amino acids. Amino acids **5–13** were isolated and characterized as their hydrochloride salts and/or the N^α-Boc-protected derivative (Table 2). The synthesis of L-Tic (**16**) was accomplished according to Julian *et al.*[36]

These amino acids were introduced by solid-phase peptide synthesis in the sequence of SP, yielding peptides **17–25** and **28–39** (Table 3). To introduce the ΔPhe probes in position 7 of SP, the dipeptide analogues Boc-Gln-Δ^ZPhe azlactone (**64**) and Boc-Gln-Δ^ZPhe-OH (**65**) were synthesized following classical procedure[37] and coupled with DMAP activation or DCC activation, respectively, to provide [Δ^ZPhe[7]]SP (**26**). The [Δ^EPhe[7]]SP analogue (**27**) was obtained by photoisomerization of its Δ^Z isomer, in reasonable yield (15%), according to Nitz's procedure.[38]

Binding Studies. The binding potencies of the various SP-modified analogues (Table 3) have been determined

Table 3. Physicochemical Properties of Substance P Analogues

compd	structure[a]	$[\alpha]^{20}_D$ (deg) (c 0.5; 10% AcOH)	TLC[c] I	TLC[c] II	HPLC[d] t_R	HPLC[d] % CH_3CN
17	[HPh[7]]SP	−69.0	0.17	0.65	17.40	23
18	[Tic[7]]SP	−98.8	0.16	0.64	10.70	22
19	[Dip[7]]SP	−70.9	0.17	0.65	15.30	24
20	[Flg[7]]SP	−63.0	0.17	0.65	10.75	26
21	[(2*S*,3*R*)-Ing[7]]SP	−67.8	0.16	0.65	16.85	23
22	[(2*S*,3*S*)-Ing[7]]SP	−73.4	0.17	0.67	11.15	26
23	[(2*S*,3*S*)-Bfi[7]]SP	−26.4	0.16	0.68	14.15	26
24	[2Ing[7]]SP	−70.0	0.18	0.65	7.70	26
25	[D-2Ing[7]]SP	*e*	0.21	0.66	13.50	26
26	[Δ^ZPhe[7]]SP	−60.1	0.21	0.63	8.80	23
27	[Δ^EPhe[7]]SP	*e*	0.21	0.61	8.40	25
28	[HPh[8]]SP	−77.2	0.17	0.64	15.10	23
29	[Tle[8]]SP	−113.8	0.17	0.66	10.75	18
30	[Pen(S-Me)[8]]SP	−54.8[b]	0.17	0.64	14.00	18
31	[Pen(SO_2-Me)[8]]SP	−43.7[b]	0.15	0.61	8.00	17
32	[Tic[8]]SP	−115.9	0.16	0.64	9.10	22
33	[Dip[8]]SP	−77.2	0.17	0.65	11.00	26
34	[Flg[8]]SP	−49.6	0.16	0.66	11.60	26
35	[(2*S*,3*R*)-Bfi[8]]SP	−76.1	0.16	0.68	12.25	26
36	[(2*S*,3*S*)-Bfi[8]]SP	−43.4	0.16	0.67	12.15	26
37	[Dip[8], Pro[9]]SP	−101.5	0.17	0.66	14.80	27
38	[Flg[8], Pro[9]]SP	−97.1	0.16	0.66	11.15	26
39	[(2*S*,3*S*)-Ing[7], Flg[8]]SP	−56.0	0.18	0.69	11.90	27
SP	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2					

[a] Unnatural amino acid abbreviations are given as a corresponding footnote to the title and are drawn in Figure 2. [b] Value for *c* 0.5; AcOH. [c] TLC conditions for I and II: see the Experimental Section. [d] Analytical HPLC: t_R = retention time in min. HPLC conditions are described in the Experimental Section. [e] Not enough material prepared.

for the three tachykinin binding sites presently discovered in mammals with appropriate ligands and tissue preparations, i.e., [^{125}I]BHSP-specific bindings on rat brain synaptosomes and guinea pig ileum membranes for NK-1 binding sites, [^{3}H]NKA-specific binding on membranes from rat duodenum for specific NK-2 binding sites, and [^{125}I]BHELE-specific binding on rat brain synaptosomes for NK-3 binding sites.[7]

The influence of the incorporation of constrained phenylalanine analogues on the binding potency of SP for rat specific NK-1 binding sites will only be considered for the topographical analysis of this binding protein.

Conformational Probes Introduced in Position 7 (Table 4). Replacement of the aromatic residue Phe[7] by a methionine drastically reduced (2370-fold decrease) the affinity for specific NK-1 binding sites. The propensity of a methionine to be found in an α-helical structure is similar to that of a phenylalanine residue, according to calculations and statistical analysis of the presence of both amino acids in proteins.[39] Therefore, the methionine residue should not disrupt the α-helical structure of the core of SP, and the weak binding potency of [Met[7]]SP should be attributed to the loss of an important interaction of the aromatic side chain of residue 7 in the S_7 binding subsite of the NK-1 receptor. Considering the respective IC_{50} values of SP and [Met[7]]SP, the contribution of this aromatic interaction might be estimated to be close to 5 kcal/mol.

Structural considerations indicated that a constrained analogue of phenylalanine which will lock the aromatic side chain of residue 7 in a g^+ orientation, such as a Tic residue, will destabilize the α-helical structure of the core of SP, a prerequisite for high binding affinity to specific NK-1 binding sites.[21–23] Indeed, the low binding potency of [Tic[7]]SP demonstrated that a g^+ orientation of the aromatic side chain in position 7 was excluded.

Table 4. Binding Affinities of SP Analogues Modified at Position 7 on Specific NK-1, NK-2, and NK-3 Binding Sites and Biological Activities in the GPI Bioassay[a]

	NK-1[b]				GPI		
peptides	$\Delta\Delta G$[f]	IC_{50} (nM)	NK-2[c] IC_{50} (nM)	NK-3[d] IC_{50} (nM)	IC_{50} (nM)[e]	EC_{50} (nM); (α^E)[g]	pK_B[h]
SP		0.64 ± 0.07	200 ± 80	130 ± 10	1.6 ± 0.3	2.5 ± 0.7 (1.0)	n.d.
[HPh[7]]SP	2.8	77 ± 2	1900 ± 500	>10000	19 ± 2	5.8 ± 0.4 (1.01)	7.25 ± 0.03
[Met[7]]SP	4.9	2700 ± 1100	n.d.	7400 ± 5500	n.d.	225 ± 30 (0.97)	n.d.
[Tic[7]]SP	4.9	2400 ± 900	520 ± 90	1900 ± 400	n.d.	417 ± 112 (0.98)	n.d.
[Dip[7]]SP	4.5	1300 ± 300	3600 ± 300	17000 ± 7000	n.d.	226 ± 156 (1.01)	n.d.
[Flg[7]]SP	4.5	1400 ± 600	690 ± 360	3100 ± 100	110 ± 40	9.5 ± 2.1 (0.98)	7.1 ± 0.1[j]
[(2*S*,3*R*)-Ing[7]]SP	2.6	54 ± 17	530 ± 100	2100 ± 700	23 ± 11	6.9 ± 2.8 (0.97)	7.06 ± 0.07
[(2*S*,3*S*)-Ing[7]]SP	0.9	3.1 ± 0.6	380 ± 10	2200 ± 1300	n.d.	1.2 ± 0.6 (1.02)	n.d.
[(2*S*,3*S*)-Bfi[7]]SP	2.5	48 ± 15	120 ± 60	1600 ± 900	49 ± 10	6.9 ± 2.9 (1.0)	7.3 ± 0.1
[2Ing[7]]SP	4.8	2300 ± 1000	1700 ± 100	>10000	n.d.	1300 ± 65 (1.04)[i]	n.d.
[D-2Ing[7]]SP	5.0	2800 ± 900	3900 ± 800	>10000	n.d.	4168 ± 708 (1.03)	n.d.
[Δ^Z-Phe[7]]SP	4.9	2700 ± 1200	4900 ± 1500	>10000	n.d.	3100 ± 640 (0.98)[i]	n.d.
[Δ^EPhe[7]]SP	2.7	63 ± 6	950 ± 160	11700 ± 7000	n.d.	8.4 ± 2.5 (1.05)	7.15 ± 0.1

[a] Binding data represent results obtained from two to eight independent experiments run in triplicate. Peptide concentrations required to inhibit the specific binding of the radioligand to either synaptosomes or membranes to 50% (IC_{50}) were estimated from the Hill plots of the titration curves. Nonspecific binding, determined in the presence of 10^{-6} M of the corresponding unlabeled ligand, represented 25%, 11%, 25%, and 29% of the total binding in the four different binding assays, respectively. In general, the EC_{50} values of each peptide in various experiments (n = 3–10) did not show variations >25%. Preparations that showed large variations of sensitivity were discarded. [b] Rat brain synaptosomes, [^{125}I]BHSP. [c] Rat duodenum membranes, [^{3}H]NKA. [d] Rat brain synaptosomes, [^{125}I]BHELE. [e] Guinea pig membranes, [^{125}I]BHSP. [f] $\Delta\Delta G = 1.36 \log IC_{50}/IC_{50}(SP)$ (kcal/mol). n.d.: not determined. [g] α^E: average values obtained from 3 to 10 independent experiments, standard errors (data not shown) ranged from 0.02 to 0.08. [h] $pK_B = \log(\text{dose ratio} - 1) - \log([\text{RP-67,580}])$ one concentration of antagonist RP-67,580: 10^{-7} M; and three independent pK_B determinations. [i] In atropine-treated GPI, the EC_{50} values were not significantly different, being 688 ± 136 (α^E 0.97) and 4168 ± 708 (α^E 0.92) nM for [2Ing[7]]SP and [Δ^ZPhe[7]]SP, respectively. [j] pA_2 7.5 ± 0.2, slope of the Schild plots 1.00 ± 0.07, two independent determinations with [RP-67,580] 10^{-7}, 3×10^{-7}, and 10^{-6} M.

The relative orientation of the phenylalanine side chain in position 7, within the α-helical structure of the backbone, has been estimated with constrained analogues of phenylalanine which preferentially adopt t and/or g^- orientations(s) when inserted in the second turn of an α-helical structure. The diphenylalanine- and fluorenylglycine-constrained analogues of phenylalanine [Dip[7]]SP and [Flg[7]]SP should explore both the t and g^- conformational directions which could be adopted by the flexible aromatic ring of phenylalanine. From the low binding potencies of [Dip[7]]SP and [Flg][7]SP, we deduced that the S_7 subsite of the NK-1 receptor cannot simultaneously accept the t and g^- orientations. Therefore, the shape and size of the S_7 subsite has been estimated with the probes which should adopt, as predicted by energy calculations, the t or g^- orientation. From energy calculations of CH_3CO-(2*S*,3*S*)-Ing-NH_2 and CH_3CO-(2*S*,3*R*)-Ing-NH_2 (Table 1), the diastereoisomers of 1-indanylglycine inserted into an α-helix prefer the g^- rotamer for (2*S*,3*S*)-Ing and the t rotamer for (2*S*,3*R*)-Ing. The [(2*S*,3*S*)-Ing[7]]SP analogue was only 5 times less potent than SP and almost 20 times more potent than [(2*S*,3*R*)-Ing[7]]SP in inhibiting [^{125}I]-BHSP-specific binding. From these results, we tentatively concluded that the g^- side-chain orientation fitted into the S_7 subsite in a more appropriate way than the t rotamer.

The width of the S_7 subsite in this g^- direction was further estimated by the addition of another aromatic ring to (2*S*,3*S*)-Ing with the (2*S*,3*S*)-Bfi-constrained amino acid. [(2*S*,3*S*)-Bfi[7]]SP was about 15 times less potent than [(2*S*,3*S*)-Ing[7]]SP, indicating that the S_7 subsite is not very large and accepts only one aromatic ring. The small size of S_7 was further supported by the low potency of [HPh[7]]-SP, an analogue of phenylalanine with an extra methylene group. Interestingly enough, the affinity of [2Ing[7]]SP, which can be regarded as a constrained homophenylalanine analogue, was 30 times higher than that of [HPh[7]]SP (IC_{50} = 2700 nM). When folded in an A structure, the side chain of [2Ing[7]]SP should be exclusively orientated at an intermediate position between the t and g^- orientation as depicted in Figure 2.

From these results, it was tempting to speculate that the S_7 subsite accepted only one aromatic residue in the gauche (−) orientation. However, the small size of this binding subsite and the small difference between the energies of the CH_3CO-Ing-NH_2 g^- and t rotamers in an α-helical structure, i.e., 2.95 and 1.11 kcal/mol for the 2*S*,3*S* and 2*S*,3*R* diastereoisomers, respectively (Table 1), suggested to be very cautious about the conclusion. For this reason, the *Z* and *E* isomers of dehydrophenylalanine were introduced in position 7, the energy barrier between Δ^ZPhe and Δ^EPhe being too high to allow an interconversion of an isomer during the binding process. In contrast to the results obtained with the (2*S*,3*S*)-Ing probe, the g^- probe Δ^ZPhe yielded the weakest binding competitor; [Δ^ZPhe[7]]-SP was 67 times less potent than [Δ^EPhe[7]]SP incorporating the t probe.

A plausible interpretation for these apparently conflictual results is that the S_7 subsite accepts only one aromatic ring in the t direction since the Δ^EPhe isomer corresponds to a t probe and that either the g^- rotamer of [(2*S*,3*S*)-Ing[7]]SP rotates around the C_α–C_β bond yielding the t rotamer when inserted in the receptor or the receptor selects the minor t rotamer in solution, as recently evidenced for the binding of cyclosporin to cyclophilin.[40] The low binding affinity of [(2*S*,3*R*)-Ing[7]]SP compared to that of [(2*S*,3*S*)-Ing[7]]SP suggests that the minor t rotamer of [(2*S*,3*S*)-Ing[7]]SP fits in the S_7 subsite in a more appropriate way than the major t rotamer of [(2*S*,3*R*)-Ing[7]]SP. This difference comes either from the steric hindrance of the pentene ring which does not overlap in both t rotamers or from the difference in the χ_2 angle of the two probes. The high binding potency of the [(2*S*,3*S*)-Ing[7]]SP analogues indicates that the NK-1 receptor recognizes a $\chi_2 = +60°$ orientation of the aromatic side chain.

Altogether, these results obtained with constrained analogues of phenylalanine suggested that, in position 7 of SP, a trans conformation, $\chi_1 = +60°$ with $\chi_2 = +60°$, for the aromatic side chain should be preferred and that

Table 5. Binding Affinities of SP Analogues Modified at Position 8 on Specific NK-1, NK-2, and NK-3 Binding Sites and Biological Activities in the GPI Bioassay[a]

peptides	NK-1[b] ΔΔG[f]	NK-1[b] IC_{50} (nM)	NK-2[c] IC_{50} (nM)	NK-3[d] IC_{50} (nM)	GPI IC_{50} (nM)[e]	GPI EC_{50} (nM); (α^E)[g]	GPI pK_B[h]
SP		0.64 ± 0.07	200 ± 80	130 ± 10	1.6 ± 0.3	2.5 ± 0.7 (1.0)	n.d.
[HPh⁸]SP	2.7	62 ± 12	450 ± 130	1900 ± 900	74 ± 27	2.8 ± 0.2 (0.97)	7.2 ± 0.1
[Met⁸]SP	2.0	18 ± 6	n.d.	1200 ± 800	20 ± 6	2.5 ± 0.7 (0.95)	7.1 ± 0.1
[Tle⁸]SP	0.8	2.6 ± 0.2	9 ± 2	65 ± 30	n.d.	2.5 ± 1.2 (1.0)	n.d.
[Pen(S-Me)⁸]SP	0.8	2.4 ± 0.9	68 ± 46	170 ± 70	n.d.	1.7 ± 0.9 (1.05)	n.d.
[Pen(SO_2-Me)⁸]SP	1.5	7.8 ± 1.4	160 ± 80	190 ± 80	n.d.	1.1 ± 0.5 (1.06)	n.d.
[Tic⁸]SP	4.4	1200 ± 400	18000 ± 13000	20000 ± 9900	2500 ± 300	83 ± 17 (0.94)	7.2 ± 0.1
[Dip⁸]SP	3.9	500 ± 170	670 ± 40	2200 ± 600	260 ± 140	15 ± 1 (1.01)	6.9 ± 0.2
[Flg⁸]SP	0.8	2.5 ± 0.8	41 ± 18	91 ± 50	n.d.	5.7 ± 2.3 (0.96)	n.d.
[(2S,3R)-Bfi⁸]SP	2.2	28 ± 4	57 ± 6	180 ± 40	n.d.	2.8 ± 1.7 (1.01)	n.d.
[(2S,3S)-Bfi⁸]SP		0.6 ± 0.3	29 ± 16	110 ± 30	n.d.	1.7 ± 0.7 (1.05)	n.d.

[a] Same legend, footnotes b–h, as Table 4.

Table 6. Binding Affinities of SP Analogues Modified at Positions 7 and 8 or 8 and 9 on Specific NK-1, NK-2, and NK-3 Binding Sites and Biological Activities in the GPI Bioassay[a]

peptides	NK-1[b] ΔΔG[f]	NK-1[b] IC_{50} (nM)	NK-2[c] IC_{50} (nM)	NK-3 IC_{50} (nM)	GPI IC_{50} (nM)[e]	GPI EC_{50} (nM); (α^E)[g]	GPI pK_B[h]
SP		0.64 ± 0.07	200 ± 80	130 ± 10	1.6 ± 0.3	2.5 ± 0.7 (1.0)	n.d.
[Pro⁹]SP	0.9	2.9 ± 0.4	>10000	>10000	n.d.	2.0 ± 0.5 (1.02)	6.97 ± 0.07
[Dip⁸, Pro⁹]SP	4.9	2500 ± 1100	6000 ± 300	>10000	260 ± 60	20 ± 5 (1.0)	7.25 ± 0.08[i]
[Flg⁸, Pro⁹]SP	0.9	3.3 ± 0.4	1300 ± 400	15000 ± 9000	n.d.	1.7 ± 0.8 (1.10)	n.d.
[(2S,3S)-Ing⁷, Flg⁸]SP	2.2	29 ± 4.5	110 ± 59	450 ± 100	7.9 ± 2.1	3.4 ± 1.6 (1.03)	7.1 ± 0.1

[a] Same legend, footnotes b–h, as Table 4. [i] pA_2 7.4 ± 0.2, slope of the Schild plots 0.80 ± 0.15, two independent determinations with [RP-67,580] 10^{-7}, 3×10^{-7}, and 10^{-6} M.

the S_7 subsite is not wide, accommodating in the binding pocket only one aromatic ring in this trans direction.

Conformational Probes Introduced in Position 8 (Table 5). The same systematic modifications have been introduced in position 8 of SP. As for position 7, the g^+ probe, i.e., the Tic residue, introduced in position 8, should disrupt the required α-helical structure; indeed, [Tic⁸]SP was a weak competitor of specific NK-1 binding sites.

In contrast to position 7, the aromaticity of the side chain in position 8 was not as important, as shown by the binding potency of [Met⁸]SP. Therefore, the structural requirements for the side chain in position 8 were further investigated with various alkyl side chains. A *tert*-leucine residue in position 8 should destabilize the α-helical structure since the three methyl groups of this symmetric amino acid occupied simultaneously the three rotameric orientations g^+, g^-, and t. However, the destabilizing interaction introduced by a methyl group in the g^+ orientation in the second turn of the α-helix has been estimated by energy calculations to be small, close to 2 kcal/mol, in contrast to the one induced by a Tic residue. Indeed, [Tle⁸]SP was a potent NK-1 agonist. Since [Pen(S-Me)⁸]SP and [Pen(SO_2-Me)⁸]SP were also potent NK-1 agonists, the width of the S_8 subsite corresponding to the β-position of the side chain should be wide enough to adopt larger aromatic probes, i.e., diphenylalanine and fluorenylglycine.

The S_8 subsite of the receptor was indeed large enough to accommodate simultaneously the t and g^- rotamers but only when the aromatic rings of the modified side chains were coplanar, such as in [Flg⁸]SP. [Dip⁸]SP was weakly recognized by specific NK-1 binding sites. This difference should be related to the respective orientations of the two aromatic rings in Dip and Flg residues. For the diphenylalanine analogue, the aromatic rings are not coplanar but in two different planes almost perpendicular to those occupied by the aromatic rings of the fluorenylglycine analogue, as observed by X-ray analysis of a *N*-protected diphenylalanine amide analogue.[32]

Since [Flg⁸]SP was a good competitor of [¹²⁵I]BHSP-specific binding, indicating that both rotamers g^- and t were accepted, the 1-indanyl probes were not used but the substituted 1-indanyl analogues (2*S*,3*S*)-Bfi and (2*S*,3*R*)-Bfi were directly introduced in position 8. The g^- probe [(2*S*,3*S*)-Bfi⁸]SP was more efficient than [Flg⁸]SP and as efficient as SP in displacing the [¹²⁵I]BHSP radioligand from NK-1 binding sites, indicating that the S_8 subsite can accommodate a second aromatic ring in the g^- direction, adding a stabilizing interaction of 0.8 kcal/mol. In contrast, when the second aromatic ring was oriented in the t direction, as in [(2*S*,3*R*)-Bfi⁸]SP, a destabilizing interaction of 2.2 kcal/mol was introduced.

Considering the rearrangement observed with the 1-indanyl probes in position 7, the question remains whether or not these 1-benz[*f*]indanylglycine diastereoisomers rotate in the S_8 subsite. To answer this question, a synthesis of the *Z* and *E* isomers of dehydronaphthylalanine has to be developed. Since these molecules are not readily available, we will tentatively conclude that in position 8 of SP, the gauche (−) and trans orientations for the aromatic side chain are accepted and the S_8 subsite of the NK-1 receptor is large enough to accept two aromatic rings in the g^- direction and one aromatic ring in the t direction.

Conformational Probes Introduced Simultaneously in Position 7, 8, and/or 9 (Table 6). The insertion of two adjacent probes in the same molecule should allow to determine if there is any spatial cooperativity between the S_7, S_8, and S_9 subsites of the NK-1 receptor. The (2*S*,3*S*)-Ing probe introduced in position 7 and the Flg probe accepted by the S_8 subsite were simultaneously introduced in the sequence of SP, i.e., [(2*S*,3*S*)-Ing⁷,Flg⁸]-SP. Since, as previously established, the introduction of a proline residue in position 9, which locks the ϕ angle (ϕ_9

= −70 ± 20°), led to a selective NK-1 agonist,[41] positions 8 and 9 were concomitantly modified, i.e., [Flg[8],Pro[9]]SP.

The destabilizing interaction in the peptides bearing two conformational probes were more or less additive (within experimental error) when compared to the peptides substituted by only one probe. [Flg[8], Pro[9]]SP was as potent and as selective as [Pro[9]]SP in binding to selective NK-1 binding sites. Thus, the S_7, S_8, and S_9 subsites of the NK-1 receptor can be considered as independent from each other.

Selectivity for Specific NK-1 Binding Sites Versus Specific NK-2 and NK-3 Binding Sites. For position 7, no clear-cut evidence was observed in the selectivity of SP analogues for specific NK-1 binding sites versus specific NK-2 and/or NK-3 binding sites. In the case of SP analogues modified in position 8, one modification led to a very interesting result. [Tle[8]]SP was found to be 200 times more potent than SP in inhibiting [^{3}H]NKA-specific binding on NK-2 binding sites, being even 5 times more potent than NKA. This high potency for NK-2 receptors was confirmed by its high activity in the rabbit pulmonary artery bioassay (data not shown). This result indicates that [Tle[8]]SP should adopt both the helical structure for interacting with NK-1 receptors and the already postulated extended conformation for this residue as in NKA and NKA agonists.

Biological Activity in the Guinea Pig Ileum Bioassay. The incorporation of aromatic constrained analogues of phenylalanine has led to the discovery of antagonists for the angiotensin II receptor[34] and, more recently, for the enkephalin receptor of the δ-type.[42] Thus, in parallel to their binding affinities, the potencies of these SP-modified analogues were evaluated in the guinea pig ileum bioassay (GPI). The ileal longitudinal muscle mainly contains NK-1 and NK-3 receptors and a lower concentration of NK-2 receptors.[43,44] The guinea pig ileum tissue is, however, the most commonly used preparation since other more specific bioassays are inadequate for large screening analysis, i.e., dog carotid artery,[45] SP-induced salivation in conscious rats,[46] or metabolism of phosphatidylinositol on cortical astrocytes from the newborn mouse in primary culture.[47] In guinea pig trachea (GPT), only NK-1 and NK-2 tachykinin receptors coexist;[48] however, NK-1 receptors appear to have some unusual characteristics when adequate protease inhibitors and selective NK-1 antagonists are used.[44] CP-96,345 and RP-67,580 are only weak inhibitors against NK-1 agonists, being over 10-fold to 100-fold more potent in GPI compared to in GPT. Similar peculiar results have also been observed in rabbit iris sphincter[51] and guinea pig airways,[52] suggesting that different NK-1 receptors might exist in these tissues. Thus, in spite of the pharmacological disadvantage of the GPI bioassay, screening with this tissue has allowed the discovery of peptidic antagonists such as spantide II,[53] GR-71251 and GR 82334,[54] FR 113680,[55] and FK 224[56] and also non-peptidic antagonists.[49,50] Recently, we have also described new NK-1 antagonists[14] (manuscript submitted), based on the GPI pharmacological test. The specificity of thus unveiled NK-1 antagonists is then ascertained with more or less specific NK-2 and NK-3 bioassays, for example, rabbit pulmonary artery[45] and rat portal vein.[43,45]

In the present study, none of the SP analogues modified in position 7 or 8 and/or 9 presents antagonist properties; all are full agonists, α^E ranging from 0.95 to 1.10. When

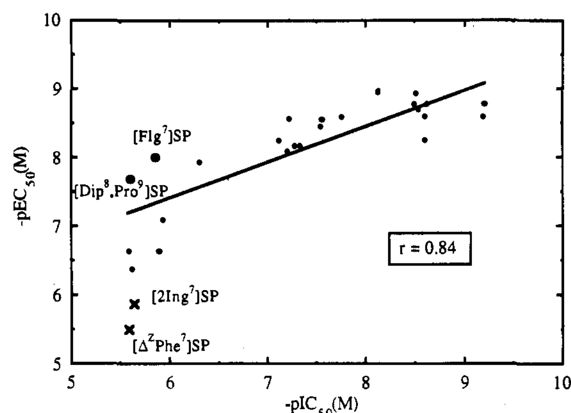


Figure 4. Correlation between the binding on rat brain synaptosomes (IC_{50}) and the activity on guinea pig ileum (EC_{50}). [2Ing[7]]SP and [Δ^ZPhe[7]]SP are excluded from the correlation line.

the activity of these analogues, $-\log EC_{50}$ (M), was plotted versus their respective affinity, a correlation with a correlation factor of r = 0.84 was found (Figure 4). Considering in more details the different compounds, half of them are 10-fold more potent (EC_{50}) in the GPI bioassay than in inhibiting [^{125}I]BHSP-specific binding (IC_{50}); two of them, i.e., [Flg[7]]SP and [Dip[8], Pro[9]]SP, are even over 100-fold more potent. When the binding potencies of these peculiar compounds were measured on membranes from guinea pig ileum using the same radioligand, [^{125}I]BHSP, no clear-cut difference in their IC_{50}'s was found, except for [Flg[7]]SP and [Dip[8], Pro[9]]SP which presented a 10-fold increase in their affinities for membranes from guinea pig ileum (Tables 4–6). A similar 10-fold increase has already been observed for septide and septide-like molecules.[13] All these peptides interact with NK-1-type receptors in GPI since a non-peptide NK-1 antagonist, RP-67,580, inhibits with similar pK_B values all these peculiar SP analogues, with pK_B's ranging from 6.9 to 7.3. For the most divergent analogues, [Flg[7]]SP and [Dip[8], Pro[9]]SP, pA_2 values were also measured to show that this antagonism was competitive. Indeed, RP-67,580 competitively inhibits the contractile activity of both compounds with similar pA_2 values of 7.5 ± 0.2 and 7.4 ± 0.2 for [Flg[7]]SP and [Dip[8], Pro[9]]SP, respectively (Tables 4 and 6). Schild plots were linear and showed slopes not significantly different from unity, i.e., 1.00 ± 0.07 and 0.80 ± 0.15, respectively.

In conclusion, with constrained amino acids, an indirect topographical analysis of specific binding sites of rodent tachykinin NK-1 has been carried out, based on the IC_{50} of SP analogues modified in position 7 or/and 8. The side-chain constrained amino acids, i.e., diphenylalanine, 9-fluorenylglycine, 2-indanylglycine, and the 2*S*,3*R* and 2*S*,3*S* diastereoisomers of 1-indanylglycine and 1-benz[*f*]indanylglycine have been designed to determine the orientation of Phe[7] and Phe[8] side chains.

From this study, we can tentatively conclude that the S_7 subsite of the NK-1 binding sites is relatively small and can adopt only one aromatic probe in the t direction, the S_8 subsite being wider since it can accommodate one aromatic probe in the t direction and two in the g^- direction. The conformation of [(2*S*,3*S*)-Ing[7],Flg[8]]SP, depicted in Figure 5, has been built up taking into account, firstly, the data previously obtained from structure–activity relationships, secondly, the ϕ and ψ angles for the Gly[9] residue, deduced from the potency of the selective NK-1 agonist

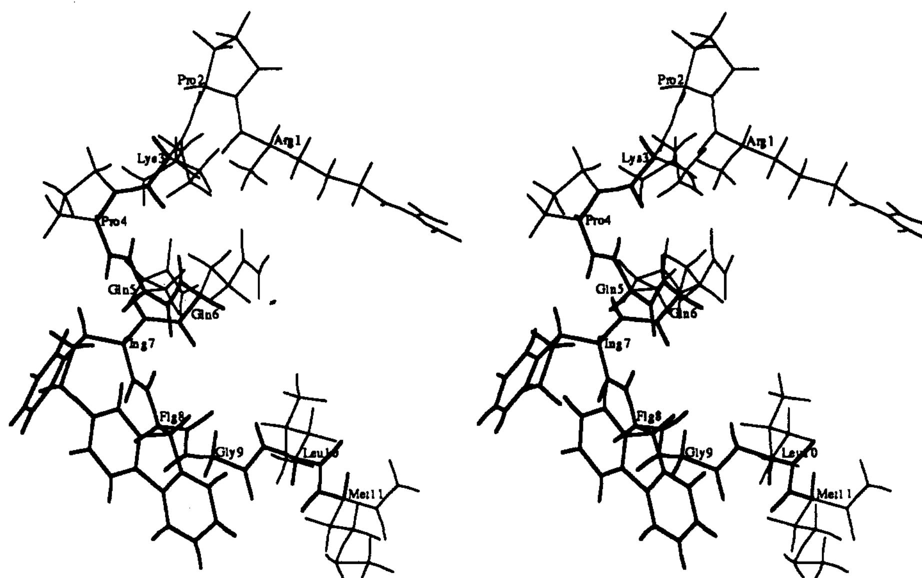


Figure 5. Stereoview of [(2*S*,3*S*)-Ing[7], Flg[8]]SP. Thin lines correspond to orientations based on energy calculations; thick lines correspond to orientations deduced from structure–activity relationships with constrained analogues of SP.

[Pro[9]]SP[41] and confirmed by the absence of conformational cooperativity between the S_8 and S_9 binding subsites (this study), and, thirdly, the orientations of the aromatic rings in positions 7 and 8 (this study). The orientations of the other side chains were obtained by energy minimization, but their rotameric preferences remain to be determined with new constrained amino acids to be designed. Thus, this structure must be regarded as a partial fingerprint of the NK-1 binding site. Since the two phenylalanines are contiguous and because of the t, (t, g$^-$) preferences for residues 7 and 8, respectively, the peptidic backbone should be preferentially folded in a 3_{10}-helical structure and the volumes available for the side chains being localized in two more or less orthogonal planes. The volume available for the side chain 7 is around 110 Å^3 [(2*S*,3*S*)-Ing] and for the side chain 8 240 Å^3 (2*S*,3*S*)-Bfi and Flg. The smaller size of the S_7 subsite is in agreement with the specific requirement for the phenylalanine residue which is highly conserved in the tachykinin family. In position 8, various aromatic or alkyl amino acids are found among tachykinins; thus, a wider S_8 subsite will allow a better adaptation for these various residues. Indeed, slight modifications of residue 7, without alteration of the peptidic backbone structure, usually induce a large decrease in the binding potency, whereas a residue introduced in position 8 can fit into the S_8 subsite and establish more or less stabilizing interaction depending on the nature of the residue and those of the amino acids present in the S_8 subsite.

The surprisingly large volume of the S_8 subsite ($\approx$240 Å^3), however, raises major comments. Where is the SP-binding site localized in the NK-1 receptor? Mutated NK-1 receptors have suggested that residues belonging to the extracellular loops and the transmembrane domains are involved in the binding of SP.[59–62] If SP binds to the extracellular loops, such a large S_8 subsite may be conceivable. If the S_8 subsite resides in the helical transmembrane segments, as it has been proposed for monoaminergic neuromediators[63] and more recently for SP,[64] the cleft inside the 7TMS must accept a peptide in an α- or 3_{10}-helical structure, a large side chain in position 8, and probably others, which have to be defined. Binding of the ligand will disturb the original environment of the cleft, according to the "wobbling" hypothesis proposed by Hibert et al.[63] And, whenever the ligand is docked in the receptor, a large binding subsite, S_8, must be created in the protein even though the network of intramolecular interactions between the side chains of the 7TMS receptor has to be perturbed. Thus, such an indirect topographical study with constrained amino acids might contribute to the analysis of the receptor/ligand dynamics, although, paradoxically, these conformational probes were initially designed to lock a particular side chain in a well-defined position.

Secondly, this study demonstrated that constrained amino acids, mimicking preferred rotamer(s), have to be used with caution. It has been previously shown with various neuropeptide/receptor complexes that the binding proteins recognized more or less selectively one diastereoisomer of β-modified amino acids.[29,30] In fact, the crucial issue is whether or not, from this diastereoselectivity, the side-chain orientation in the receptor can be deduced. Indeed, a new chiral center in the β-position creates two diastereoisomers, *SS** and *SR**, which both can adopt three different conformations, i.e., $SS(g^-) \Leftrightarrow SS(t) \Leftrightarrow SS(g^+)$ and $SR(g^-) \Leftrightarrow SR(t) \Leftrightarrow SR(g^+)$. The design of the diastereoisomers of 1-indanylglycine and 1-benz[*f*]indanylglycine as constrained analogues of phenylalanine and 2-naphthylalanine, respectively, increased the energy differences between the different rotamers compared to β-methylphenylalanine and β-methylnaphthylalanine. However, this difference may not be high enough since the concomitant use of the *Z* and *E* isomers of dehydrophenylalanine with the (2*S*,3*S*)- and (2*S*,3*R*)-indanylglycine analogues suggests that the receptor may bind the less stable isomer. Thus, a definitive answer concerning the orientation of a particular phenylalanine side chain can only be ascertained by using a combination of constrained amino acids, i.e., χ_1-blocked residues such as Δ^E- and Δ^Zdehydrophenylalanine and χ_2-blocked amino acids such as the 1-indanylglycines. Whenever possible, analogous χ_1- and χ_2-blocked residues should be designated for the different lateral side chains if one wants to confidently attribute the privileged orientation(s).

Finally, the three-dimensional structure of the peptidic backbone and the preferred rotamer of a constrained amino

acid side chain are intimately related; therefore, the knowledge of the three-dimensional structure of such a modified peptide is a prerequisite for a topographical analysis.

Experimental Section

Chemistry. Melting points were determined on a Kofler melting point apparatus and are uncorrected. NMR spectra were recorded on Bruker AC-200 (200 MHz) and Jeol GSX400 (400 MHz) spectrometers with chemical schifts expressed in ppm (δ) relative to tetramethylsilane. Elementary analyses, performed by the Service Regional de Microanalyses, Paris VI, were within 0.4% of the theoretical values calculated for C, H, and N, as noted. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Values were obtained from the means of 10 successive 5-s integrations. Analytical TLC was carried out with Merck silica gel 60 F_{254} TLC plates, and compound visualization was effected with a UV lamp, phosphomolybdic acid oxidation, or ninhydrin (after TFA exposure for the *N*-Boc derivatives). The following eluting systems were used (all v/v), A, cyclohexane–ethyl acetate (8:2), B, chloroform–ethyl acetate (7:3), C, chloroform–methanol–acetic acid (95:5:0.5), and D, chloroform–methanol–acetic acid (80:20:0.5). Column chromatography was performed with Merck silica gel 60, 0.063–0.200 mm (atmospheric pressure), and Merck basic alumina activity II–III, 70–230 mesh or Merck silica gel 60, 0.040–0.063 mm (flash chromatography).

Synthesis of the N^{α}-Boc Penicillamine Derivatives 40 and 41. N^{α}-[(*tert*-Butyloxy)carbonyl]-*S*-methyl-L-penicillamine (40) [Boc-Pen(S-Me)]. A suspension of L-penicillamine (1.00 g, 6.70 mmol) in dry liquid ammonia was saturated with stirring, at −55 °C, with chloromethane over a period of 1 h. After evaporation of the solvents, the resulting powder was submitted to the classical N^{α}-Boc-protection procedure. The Boc-*S*-methylpenicillamine was purified by recrystallization of its Boc-*S*-methylpenicillamine DCHA salt to provide 1.75 g (58%) of white crystals: mp 152–154 °C (lit.[65] mp 152.5–154 °C); $[\alpha]^{20}_{D}$ −10° (*c* 1; MeOH); ^{1}H NMR (200 MHz, CD_3OD) δ 4.05 (s, 1 H), 1.43 (s, 9 H), 1.38 (s, 3 H), 1.30 (s, 3 H).

N^{α}-[(*tert*-Butyloxy)carbonyl]-*SO_2*-methyl-L-penicillamine (41) [Boc-Pen(SO_2-Me)]. To a mixture of *S*-methyl-L-penicillamine (1.00 g, 6.13 mmol) in glacial acetic acid was added 30% hydrogen peroxide (2 mL), and the reaction was stirred at room temperature for 24 h. The crude product obtained after evaporation of the solvent was submitted to the classical N^{α}-Boc-protection procedure. The Boc-*SO_2*-methylpenicillamine was purified by recrystallization of its Boc-*SO_2*-methylpenicillamine DCHA salt to give 2.25 g (70%) of white crystals: mp 161–162 °C; $[\alpha]^{20}_{D}$ +1.4° (*c* 1; 10% AcOH); ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.0 (d, 1 H), 4.6 (d, 1 H), 2.95 (s, 3 H), 1.51 (s, 3 H), 1.59 (s, 3 H), 1.43 (s, 9 H).

Preparation of the Chiral Synthon. The chiral inductor sultam (10,10-dimethyl-5-thia-4-azatricyclo[5.2.1.0]decane 5,5-dioxide) was prepared according to the original literature.[66,67] The (−)-antipode was used for the synthesis of L-amino acids.

***N*-(Diphenylmethylene)glycinesultam (42).** To a stirred solution of sultam (12.92 g, 60 mmol) in anhydrous toluene (150 mL), under argon, was added dropwise in 5 min by syringe, 36 mL of a 2 N trimethylaluminum solution in hexane: an evolution of methane ensued, which stopped when 1 equiv of reagent had been added. After 15 min, methyl *N*-(diphenylmethylene)-glycinate[68] (18.24 g, 72 mmol), in anhydrous toluene (100 mL), was added and the mixture was heated at 50 °C for 2 days. The cooled solution was slowly poured into 600 mL of ice-cooled H_2O/CH_2Cl_2 (1:1) and filtered through a pad of Celite (rinsed with 2 × 100 mL of CH_2Cl_2). The filtrate was decanted and extracted with CH_2Cl_2 (2 × 100 mL). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure, and the residue was purified by flash chromatography (SiO_2; cyclohexane/AcOEt, 7:3) to provide 25.3 g (97%) of a slightly yellow foamy compound: R_f (A) 0.15; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (dd, 2 H, arom), 7.50–7.29 (m, 6 H, arom), 7.18 (m, 2 H, arom), 4.62 (AB system, 2 H), 3.89 (dd, 1 H), 3.40 (AB system, 2 H), 2.20 (m, 1 H), 2.07 (m, 1 H), 1.98–1.81 (m, 3 H), 1.47–1.26 (m, 2 H), 1.11 (s, 3 H), 0.94 (s, 3 H).

Preparation of the Electrophiles. 1-Bromoindan (43).[69] Pure indene was saturated with stirring at 0 °C with HBr gas for 30 min and then degassed from excess HBr by two freeze–thaw cycles, with liquid nitrogen *in vacuo*. This electrophile should be stored at 0 °C and used the same day: ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.50–7.15 (m, 4 H, arom), 5.60 (dd, 1 H), 3.18 (dd, 1 H), 2.88 (dt, 1 H), 2.56 (m, 2 H).

1-Bromobenz[*f*]indan (44). A mixture of *endo*- + *exo*-4,9-epoxy-3a,4,9,9a-tetrahydro-1*H*-benz[*f*]indene[70] (5.64 g, 31 mmol) and 10% aqueous H_2SO_4 (57 mL) was refluxed for 5 h. The cold solution was slowly poured into 200 mL of $CHCl_3$ and 400 mL of ice-cooled saturated $NaHCO_3$. After decantation and extraction with $CHCl_3$ (2 × 50 mL), the combined organic layers were washed with water (100 mL), dried ($MgSO_4$), and evaporated to give 4.61 g (90%) of a white solid: mp 162–163 °C (lit.[71] mp 163–164 °C); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.95–7.70 (m, 3 H, arom), 7.78 (s, 1 H, arom), 7.53–7.32 (m, 2 H, arom), 7.01 (m, 1 H), 6.64 (m, 1 H), 3.54 (m, 2 H).

A solution of this solid (4.55 g, 27.4 mmol) in $CHCl_3$ (50 mL) was saturated at 0 °C with HBr gas for 30 min. The solvent was removed under reduced pressure without heating, and the residue was recrystallized in pentane/Et_2O (9:1) to provide 6.41 g (95%) of a yellow-brown product: mp 67–69 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.90 (s, 1 H, arom), 7.88–7.74 (m, 2 H, arom), 7.70 (s, 1 H, arom), 7.55–7.35 (m, 2 H, arom), 5.72 (dd, 1 H), 3.36 (m, 1 H), 3.04 (m, 1 H), 2.68–2.53 (m, 2 H).

2-Bromoindan (45). To a stirred solution of 2-indanol (6.71 g, 50.0 mmol) and triphenylphosphine (13.77 g, 52.5 mmol) in dry CH_2Cl_2 under argon was added bromine (2.70 mL, 52.5 mmol) at 0 °C. The cooling bath was removed, and the reaction was stirred at room temperature for an additional 30 min. The mixture was concentrated under reduced pressure with heating (40 °C), and the hot residue was poured into Et_2O (100 mL) with vigorous stirring. The solution was cooled at 0 °C for 15 min and then filtered. The filtrate was washed with 5% Na_2SO_3 (75 mL) and H_2O (75 mL), dried ($MgSO_4$), and evaporated to give 9.85 g (100%) of an oil:[72] ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35–7.15 (m, 4 H, arom), 4.76 (tt, 1 H), 3.52 (dd, 2 H), 4.34 (dd, 2 H).

General Procedure for the Alkylation of *N*-(Diphenylmethylene)glycinesultam (42). *N*-(Diphenylmethylene)-L-3,3-diphenylalaninesultam (46). *Bromodiphenylmethane was washed with pentane before use, to ensure proper quality.* Under argon, to a solution of the Schiff base 42 (3.52 g, 8.06 mmol) in anhydrous THF (40 mL) and anhydrous HMPA (11 mL) was added, over 2 min, at −78 °C, 1.6 N *n*-BuLi in hexane (5.05 mL, 8.06 mmol). The mixture was warmed to room temperature, and bromodiphenylmethane (4.00 g, 16.2 mmol) in anhydrous THF was injected. After 1 h, the system was closed (septum) and stirred at room temperature for 5 days. The reaction was quenched with a few drops of AcOH and the mixture diluted with 150 mL of Et_2O and washed six times with saturated NH_4Cl (6 × 100 mL). After drying ($MgSO_4$) and concentration under reduced pressure, the residue was adsorbed on basic alumina and purified by flash chromatography (basic alumina; cyclohexane/AcOEt, 85:15). Recrystallization from CH_2Cl_2/Et_2O (9:1) afforded 3.27 g (68%) of white crystals: mp 236–237 °C; R_f (A) 0.28; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (dd, 2 H, arom), 7.59 (dd, 2 H, arom), 7.38–7.05 (m, 14 H, arom), 6.41 (br d, 2 H, arom), 5.25 (d, *J* = 5.0 Hz, 1 H), 5.10 (d, *J* = 5.0 Hz, 1 H), 3.80 (dd, 1 H), 3.34 (AB system, 2 H), 1.88–1.55 (m, 4 H), 1.34 (m, 1 H), 1.21 (m, 1 H), 1.03 (m, 1 H), 0.88 (s, 3 H), 0.86 (s, 3 H). Anal. ($C_{38}H_{38}N_2O_3S$) C, H, N.

***N*-(Diphenylmethylene)-L-2-(9-fluorenyl)glycinesultam (47).** The standard procedure described above for the synthesis of compound 46 was followed, starting from 42 (3.52 g, 8.06 mmol) and 1.5 equiv of 9-bromofluorene (3.12 g, 12.73 mmol) and stirring for 3 days at room temperature. Workup, flash chromatography (basic alumina; cyclohexane/AcOEt, 8:2, and then $CHCl_3$), and recrystallization (Et_2O/CH_2Cl_2, 9:1) provided 3.47 g (72%) of white crystals: mp >270 °C; R_f (A) 0.21; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (dd, 2 H, arom), 7.65 (dd, 2 H, arom), 7.52–7.00 (m, 12 H, arom), 6.83 (br d, 2 H, arom), 5.56 (d, *J* = 2.2 Hz, 1 H), 4.57 (d, *J* = 2.2 Hz, 1 H), 3.98 (dd, 1 H), 3.42 (AB system, 2 H), 2.25–1.75 (m, 5 H), 1.53–1.26 (m, 2 H), 1.04 (s, 3 H), 0.92 (s, 3 H). Anal. ($C_{38}H_{36}N_2O_3S$) C, H, N.

***N*-(Diphenylmethylene)-L-2-(1-indanyl)glycinesultam (48a,b).** The general procedure described above for the synthesis of compound **46** was followed, starting from **42** (4.38 g, 10.0 mmol) and 1-bromoindan (**43**) (3.94 g, 20.0 mmol). The latter was degassed from excess HBr before introduction by four freeze–thaw cycles with liquid nitrogen under high vacuum. This electrophile, dissolved in anhydrous THF (20 mL) under argon, was injected onto the carbanion at −20 °C, and the solution was stirred for 2 h at this temperature and 2 h at room temperature. The diastereoisomeric ratio (mixture of 2*S*,3*S* and 2*S*,3*R* isomers), 2% in favor of the 2*S*,3*S* isomer after workup, did not change after flash chromatography (basic alumina; cyclohexane/AcOEt, 8:2, and then $CHCl_3$) and two consecutive crystallizations (Et_2O/CH_2Cl_2 and then pentane/Et_2O). These treatments afforded 3.79 g (69%) of a white powder: R_f (A) 0.32. Anal. ($C_{34}H_{36}N_2O_3S$) C, H, N.

For structural analysis, each isomer of this mixture was obtained by a transimination reaction[62] of benzophenone imine with the pure diastereoisomer (2*S*,3*S*)- or (2*S*,3*R*)-*N*-aminoacylsultam hydrochloride and separated after hydrolysis of the imine function. (i) *N*-(Diphenylmethylene)-(2*S*,3*R*)-2-(1-indanyl)glycinesultam (**48a**):[73] ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (dd, 2 H), 7.53–6.88 (m, 10 H, arom), 6.68 (br d, 2 H), 5.10 (d, *J* = 1.4 Hz, 1 H), 4.05 (br d, 1 H), 3.92 (dd, 1 H), 3.38 (AB system, 2 H), 3.23 (m, 1 H), 2.89 (m, 1 H), 2.76 (m, 1 H), 2.18–1.97 (m, 3 H), 1.95–1.80 (m, 3 H), 1.50–1.18 (m, 2 H), 1.14 (s, 3 H), 0.95 (s, 3 H). Anal. ($C_{34}H_{36}N_2O_3S$) C, H, N. (ii) *N*-[*N*-(Diphenylmethylene)-(2*S*,3*S*)-2-(1-indanyl)glycinyl]sultam (**48b**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (dd, 2 H, arom), 7.58–7.08 (m, 9 H, arom), 7.02 (m, 1 H), 6.63 (br d, 2 H, arom), 4.96 (d, *J* = 6.6 Hz, 1 H), 3.90 (dd, 1 H), 3.84 (m, 1 H), 3.32 (AB system, 2 H), 2.87 (m, 1 H), 2.74 (m, 1 H), 2.30 (m, 1 H), 2.22–2.03 (m, 2 H), 1.95–1.75 (m, 4 H), 1.45–1.27 (m, 2 H), 0.97 (s, 3 H), 0.90 (s, 3 H). Anal. ($C_{34}H_{36}N_2O_3S$) C, H, N.

***N*-(Diphenylmethylene)-L-2-(1-benz[*f*]indanyl)glycinesultam (49a,b).** The standard procedure described above for the synthesis of compound **46** was followed, starting from **42** (7.85 g, 17.98 mmol) and 1.2 equiv of 1-bromobenz[*f*]indan (**44**) (5.35 g, 21.6 mmol). The latter, dissolved in anydrous THF (20 mL) under argon, was added to the carbanion at −20 °C; the solution was stirred for 2 h at this temperature and 2 h at room temperature. Workup and flash chromatography (basic alumina; cyclohexane/AcOEt, 7:3, and then $CHCl_3$) provided 8.63 g (80%) of a white powder. The diastereoisomeric ratio was 33% in favor of the 2*S*,3*S* isomer: R_f (A) 0.33. Anal. ($C_{38}H_{38}N_2O_3S$) C, H, N.

By analogy with the *N*-(diphenylmethylene)-L-2-(1-indanyl)glycinesultam derivatives, the main spectral characteristics of the two isomers of the mixture have been noted. (i) *N*-(Diphenylmethylene)-(2*S*,3*R*)-2-(1-benz[*f*]indanyl)glycinesultam (**49a**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.52 (br d, 2 H), 5.28 (d, *J* = 2.3 Hz, 1 H), 3.41 (s, 2 H), 1.16 (s, 3 H), 0.94 (s, 3 H). (ii) *N*-(Diphenylmethylene)-(2*S*,3*S*)-2-(1-benz[*f*]indanyl)glycinesultam (**49b**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.70 (br d, 2 H), 5.11 (d, *J* = 6.5 Hz, 1 H), 3.30 (s, 2 H), 0.83 (s, 3 H), 0.75 (s, 3 H).

Alkylation of Methyl *N*-(Diphenylmethylene)glycinate. Methyl *N*-(Diphenylmethylene)-2-(2-indanyl)glycinate (50). The general procedure described above for the synthesis of compound **46** was followed, starting from methyl *N*-(diphenylmethylene)glycinate[68] (6.33 g, 25 mmol) as Schiff base, using 2 N LDA in hexane (12.5 mL, 25 mmol) for the deprotonation and 2-bromoindane (**45**) (9.85 g, 50 mmol) as electrophile. After 3 h at room temperature, the reaction was quenched with AcOH (1 mL) and the mixture treated. Purification by flash chromatography (SiO_2; cyclohexane/AcOEt, 7:3) gave 3.08 g (33%) of a yellow oil. An analytical sample was slowly recrystallized from pentane/Et_2O: mp 116–118 °C; R_f (A) 0.45; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.62–7.05 (m, 14 H, arom), 4.22 (d, *J* = 6.9 Hz, 1 H), 3.76 (s, 3 H), 3.27 (dd, 1 H), 3.20–2.85 (m, 3 H), 2.67 (dd, 1 H). Anal. ($C_{25}H_{23}NO_2$) C, H, N.

Hydrolysis of the Imine Function. L-3,3-Diphenylalaninesultam Hydrochloride (51). Silica gel (5 g) and 0.5 N HCl (7.2 mL, 3.60 mmol) were added to a solution of **46** (1.81 g, 3 mmol) in $CHCl_3$ (20 mL), and the mixture was sonicated in a cleaning bath at 40 °C for 2 h. After concentration to dryness on a Rotovap apparatus, with a fritted disc adaptator to keep SiO_2 in the round-bottom flask, the residue was subjected to flash chromatography (SiO_2; $CHCl_3$, for benzophenone and then $CHCl_3$/MeOH, 9:1, for the product) and recrystallized from CH_2Cl_2/Et_2O (9:1) to afford 1.27 g (89%) of a white solid: mp 118–119 °C; R_f (B) 0.39; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.48–7.00 (m, 10 H, arom), 4.84 (d, *J* = 11 Hz, 1 H), 4.25 (d, *J* = 11 Hz, 1 H), 3.79 (dd, 1 H), 3.44 (AB system, 2 H), 1.95–1.55 (m, 5 H), 1.40–1.15 (m, 2 H), 0.87 (s, 3 H), 0.65 (s, 3 H).

L-2-(9-Fluorenyl)glycinesultam Hydrochloride (52). The Schiff base **47** (1.80 g, 3.0 mmol) was hydrolyzed as described above for the synthesis of compound **51** to yield 1.30 g (92%) of a white solid: mp 149–152 °C; R_f (B) 0.36; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.83–7.65 (m, 3 H, arom), 7.45–7.18 (m, 5 H, arom), 4.83 (d, *J* = 2.1 Hz, 1 H), 4.44 (br d, *J* = 2.1 Hz, 1 H), 4.05 (dd, 1 H), 3.56 (s, 2 H), 2.35–2.10 (m, 2 H), 2.07–1.81 (m, 3 H), 1.58–1.33 (m, 2 H), 1.15 (s, 3 H), 0.98 (s, 3 H).

(2*S*,3*R*)-2-(1-Indanyl)glycinesultam and (2*S*,3*S*)-2-(1-Indanyl)glycinesultam (53a,b). A mixture of diastereoisomers **48a,b** (de 2%, 6.63 g, 12 mmol), $CHCl_3$ (15 mL), Et_2O (15 mL), and 0.5 N HCl (36 mL, 18 mmol) was sonicated in a cleaning bath for 4 h. The solvents were evaporated to give a gum which was triturated in Et_2O (100 mL) and filtered. To the resulting solid were added propylene oxide (20 mL) and Et_2O (100 mL), and the solution was stirred at room temperature overnight. After concentration to dryness, the *N*-aminoacylsultams were separated by silica gel chromatography (column measurements *h* = 1.5 m, o.d. = 3 cm; Merck silica gel 60, 0.063–0.200 mm/0.04–0.063 mm (95:5); 1.5 g of crude for each run of purification; eluent CH_2Cl_2/AcOEt, 85:15) and recrystallized from CH_2Cl_2/Et_2O (9:1) to provide first (i) (2*S*,3*S*)-2-(1-indanyl)glycinesultam (**53b**) (1.98 g, 83%) as white crystals [mp 178–180 °C; R_f (B) 0.33; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32–7.10 (m, 4 H, arom), 4.12 (d, *J* = 6.5 Hz, 1 H), 3.94 (dd, 1 H), 3.54 (m, 1 H), 3.49 (AB system, 2 H), 3.03 (m, 1 H), 2.81 (m, 1 H), 2.25 (m, 1 H), 2.16–1.82 (m, 6 H), 1.64 (NH_2), 1.48–1.30 (m, 2 H), 1.11 (s, 3 H), 0.97 (s, 3 H). Anal. ($C_{21}H_{28}N_2O_3S$) C, H, N] and then (ii) (2*S*,3*R*)-2-(1-indanyl)glycinesultam (**53a**) (1.89 g, 83%) as white crystals [mp 150–151 °C; R_f (B) 0.27; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35–7.10 (m, 4 H, arom), 4.44 (d, *J* = 4.3 Hz, 1 H), 3.92 (dd, 1 H), 3.68 (m, 1 H), 3.45 (AB system, 2 H), 3.00 (m, 1 H), 2.84 (m, 1 H), 2.15–1.80 (m, 7 H), 1.50 (NH_2), 1.60–1.25 (m, 2 H), 1.00 (s, 3 H), 0.93 (s, 3 H). Anal. ($C_{21}H_{28}N_2O_3S$) C, H, N].

(2*S*,3*R*)-2-(1-Benz[*f*]indanyl)glycinesultam and (2*S*,3*S*)-2-(1-Benz[*f*]indanyl)glycinesultam (54a,b). The hydrolysis of the mixture of diastereoisomers **49a,b** (de 33%, 8.60 g, 14.3 mmol) proceeded as described above for the synthesis of **53a,b** to give, after chromatography on the same column (eluent CH_2Cl_2/AcOEt, 88:12) and in the same order of elution, the 2*S*,3*S* diastereoisomer followed by the 2*S*,3*R* diastereoisomer, which were recrystallized from CH_2Cl_2/Et_2O (9:1). (i) (2*S*,3*S*)-2-(1-Benz[*f*]indanyl)glycinesultam (**54b**): 3.06 g, 74%, white crystals; mp 209–210 °C; R_f (B) 0.36; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (s, 1 H, arom), 7.81–7.70 (m, 2 H, arom), 7.63 (s, 1 H, arom), 7.42–7.34 (m, 2 H, arom), 4.21 (d, *J* = 6.7 Hz, 1 H), 3.95 (dd, 1 H), 3.65 (dd, 1 H), 3.48 (AB system, 2 H), 3.17 (m, 1 H), 2.97 (m, 1 H), 2.29 (m, 1 H), 2.17–2.06 (m, 2 H), 2.03–1.81 (m, 4 H), 1.64 (NH_2), 1.47–1.32 (m, 2 H), 1.04 (s, 3 H), 0.95 (s, 3 H). Anal. ($C_{25}H_{30}N_2O_3S$) C, H, N. (ii) (2*S*,3*R*)-2-(1-Benz[*f*]indanyl)glycinesultam (**54a**): 1.50 g, 72%, white crystals; mp 207–208 °C; R_f (B) 0.29; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78–7.70 (m, 2 H, arom), 7.66 (s, 1 H, arom), 7.65 (s, 1 H, arom), 7.43–7.33 (m, 2 H, arom), 4.43 (br d, *J* = 6.0 Hz, 1 H), 3.88 (m, 1 H), 3.72 (m, 1 H), 3.38 (s, 2 H), 3.19 (m, 1 H), 2.98 (m, 1 H), 2.27 (m, 1 H), 2.15–1.95 (m, 3 H), 1.93–1.73 (m, 3 H), 1.57 (NH_2), 1.43–1.27 (m, 2 H), 0.84 (s, 3 H), 0.56 (s, 3 H). Anal. ($C_{25}H_{30}N_2O_3S$) C, H, N.

Methyl 2-(2-Indanyl)glycinate Hydrochloride (55). To a mixture of **50** (1.11 g, 3.00 mmol) in Et_2O (10 mL) was added 1 N HCl (3.6 mL, 3.60 mmol), and the reaction vessel was sonicated for 2 h in a cleaning bath. The solution was diluted with Et_2O (100 mL) and H_2O (100 mL), and the aqueous layer was washed with Et_2O and lyophilized to afford 525 mg (72%) of a white solid: R_f (D) 0.76; ^{1}H NMR (200 MHz, CD_3OD) δ 7.27–7.05 (m, 4 H, arom), 4.19 (d, *J* = 6.5 Hz, 1 H), 3.70 (s, 3 H), 3.26–2.85 (m, 5 H).

Removal of the Sultam and N^α-Boc Protection. L-3,3-Diphenylalanine Hydrochloride (7) [Dip, HCl].[32] To a

solution of **51** (3.56 g, 7.49 mmol) in acetonitrile (75 mL) were added LiBr (3.26 g, 37.5 mmol), tetrabutylammonium bromide (0.97 g, 3.00 mmol), and $LiOH \cdot H_2O$ (1.26 g, 30.0 mmol), and the mixture was stirred at room temperature for 8 h. The solvent was evaporated, and the residue was taken up in $CHCl_3$ (50 mL) and H_2O (50 mL). After separation of the layers, two additional extractions with $CHCl_3$ (2 × 30 mL) provided recovered sultam (which could be separated from salts after filtration through a pad of silica gel with CH_2Cl_2/AcOEt, 9:1, as eluent; 91%). The aqueous layer was acidified with 1 N HCl (50 mL), concentrated to about 10 mL, and purified through a BIO REX 70 column (H^+ form, rinsing with 1 N HCl). The appropriated fractions were concentrated under reduced pressure, and the residue was recrystallized from EtOH to give 1.57 g (76%) of thin white crystals: mp 198–201 °C; $[\alpha]^{25}_D$ −41° (*c* 0.5; 1 N HCl); R_f (D) 0.27; ^{1}H NMR (200 MHz, CD_3OD) δ 7.57–7.10 (m, 10 H, arom), 4.83 (d, *J* = 11 Hz, 1 H), 4.45 (d, *J* = 11 Hz, 1 H).

***N*$^\alpha$-[(*tert*-Butyloxy)carbonyl]-L-3,3-diphenylalanine (56) [Boc-Dip].** The N^α-Boc protection was accomplished through a slight modification of the original method.[74] A mixture of **7** (1.42 g, 5.11 mmol), $NaHCO_3$ (1.50 g), and di-*tert*-butyl dicarbonate (1.40 g, 6.1 mmol) in absolute EtOH (50 mL) was sonicated in a cleaning bath for 4 h. The solution was filtered through a pad of Celite and concentrated to dryness. The residue was taken up in CH_2Cl_2 (100 mL), washed with ice-cooled aqueous 10% citric acid (2 × 50 mL), and dried ($MgSO_4$). After evaporation of the solvent, the crude product was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH/AcOH, 95:5:0.5) and recrystallized from cyclohexane/pentane to give 1.46 g (86%) of a white solid: mp 129–131 °C; $[\alpha]^{20}_D$ +33.6° (*c* 0.5; MeOH); R_f (C) 0.41; ^{1}H NMR (400 MHz, $CDCl_3$) mixture of trans (85%) and cis (15%) isomers (Boc function), trans δ 7.45–7.10 (m, 10 H, arom), 5.08 (dd, *J* = 8.7 and 7.5 Hz, 1 H), 4.83 (dd, *J* = 8.7 Hz, NH), 4.50 (d, *J* = 7.5 Hz, 1 H), 1.37 (s, 9 H); cis (detected peaks) δ 5.35 (m, 1 H), 4.36 (m, 1 H). Anal. ($C_{20}H_{23}NO_4$) C, H, N.

L-2-(9-Fluorenyl)glycine Hydrochloride (8) [Flg, HCl].[32] To a stirred solution of **52** (3.55 g, 7.50 mmol) in THF (35 mL) was added a mixture of $LiOH \cdot H_2O$ (1.26 g, 30.0 mmol) in H_2O (7 mL) at 0 °C. The cooling bath was removed, and the reaction was stirred at room temperature for 3 h. The mixture was diluted with H_2O (100 mL) and extracted with $CHCl_3$ (3 × 75 mL) to allow the sultam recovery (97%). The aqueous phase was treated as described above for the synthesis of **7** to afford, after recrystallization from H_2O/EtOH (9:1), 1.27 g (84%) of thin white crystals: mp >270 °C; $[\alpha]^{25}_D$ +27° (*c* 0.5; 1 N HCl); R_f (D) 0.09; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (dd, 2 H, arom), 7.87 (dd, 1 H, arom), 7.58 (dd, 1 H, arom), 7.43 (dt, 2 H, arom), 7.36 (dt, 2 H, arom), 4.78 (d, *J* = 2.6 Hz, 1 H), 4.66 (br s, 1 H).

***N*$^\alpha$-[(*tert*-Butyloxy)carbonyl]-L-2-(9-fluorenyl)glycine (57) [Boc-Flg].** The procedure described for the synthesis of **56** was followed, starting from **8** (1.52 g, 5.51 mmol), to provide the title compound (1.46 g, 78%) as a white solid: mp 206–209 °C; $[\alpha]^{20}_D$ +49.7° (*c* 0.5; MeOH); R_f (C) 0.40; ^{1}H NMR (200 MHz, CD_3OD) δ 7.78 (dt, 2 H, arom), 7.62 (dd, 1 H, arom), 7.41 (dd, 1 H, arom), 7.46–7.17 (m, 4 H, arom), 4.94 (d, *J* = 3.9 Hz, 1 H), 4.57 (d, *J* = 3.9 Hz, 1 H), 1.22 (s, 9 H). Anal. ($C_{20}H_{21}NO_4$) C, H, N.

***N*$^\alpha$-[(*tert*-Butyloxy)carbonyl]-(2*S*,3*R*)-2-(1-indanyl)glycine (58) [Boc-(2*S*,3*R*)-Ing].** To a stirred solution of **53a** (1.61 g, 4.15 mmol) in THF (12 mL) was added a mixture of $Na_2S \cdot 9H_2O$ (1.50 g, 6.24 mmol) in H_2O (11 mL) at 0 °C. The cooling bath was removed, and the reaction was stirred at room temperature for 5 h. The mixture was diluted with H_2O (100 mL) and extracted with $CHCl_3$ (3 × 75 mL) to allow the sultam recovery (95%). The aqueous layer was acidified under a hood with 1 N HCl (30 mL) and concentrated under reduced pressure to provide 2.3 g of the crude amino acid hydrochloride together with the salt. To this raw material were added $NaHCO_3$ (1.50 g), absolute EtOH (50 mL), and di-*tert*-butyl dicarbonate (0.93 g, 4.15 mmol), and the mixture was sonicated in a cleaning bath for 5 h. By analogy with **56**, workup, purification by flash chromatography (SiO_2; CH_2Cl_2/MeOH/AcOH, 95:5:0.5), and recrystallization (CH_2Cl_2/pentane) afforded 0.76 g (63%) of a white solid: mp 109–111 °C; $[\alpha]^{20}_D$ +10.8° (*c* 0.5; MeOH); R_f (C) 0.39; ^{1}H NMR (200 MHz, $CDCl_3$) mixture of trans (90%) and cis (10%) isomers (Boc function), trans δ 7.37–7.10 (m, 4 H arom), 4.99 (d, *J* = 9.0 Hz, NH), 4.87 (dd, *J* = 9.0 and 3.9 Hz, 1 H), 3.78 (m, 1 H), 3.10–2.85 (m, 2 H), 2.15 (m, 1 H), 1.89 (m, 1 H), 1.36 (s, 9 H). Anal. ($C_{16}H_{21}NO_4$) C, H, N.

(2*S*,3*R*)-2-(1-Indanyl)glycine Hydrochloride (9) [(2*S*,3*R*)-Ing, HCl].[33] A solution of the N^α-Boc derivative **58** (291 mg, 1.00 mmol) in 10 mL of CH_2Cl_2/TFA (5:1) was stirred at room temperature for 30 min. The mixture was concentrated under reduced pressure to afford the pure amino acid (305 mg, 100%) as its TFA salt. A simple exchange in 1 N HCl gave the corresponding HCl salt: mp 227–230 °C dec; $[\alpha]^{20}_D$ −9.7° (TFA salt; *c* 0.5; AcOH); R_f (D) 0.24; ^{1}H NMR (200 MHz, CD_3OD) δ 7.40–6.95 (m, 4 H, arom), 4.39 (d, *J* = 2.8 Hz, 1 H), 3.89 (m, 1 H), 2.89 (ddd, 2 H), 2.16 (m, 1 H), 1.89 (m, 1 H).

***N*$^\alpha$-[(*tert*-Butyloxy)carbonyl]-(2*S*,3*S*)-2-(1-indanyl)glycine (59) [Boc-(2*S*,3*S*)-Ing].** Following the procedure described above for the synthesis of **58**, **53b** (1.74 g, 4.48 mmol) was hydrolyzed (24 h) and then N^α-Boc protected (5 h) to provide, after purification by flash chromatography (SiO_2; CH_2Cl_2/MeOH/AcOH, 95:5:0.5), 0.74 g (57%) of a slightly yellow foam: $[\alpha]^{20}_D$ +28.6° (*c* 0.5; MeOH); R_f (C) 0.39; ^{1}H NMR (400 MHz, $CDCl_3$) mixture of trans (85%) and cis (15%) isomers (Boc function), trans δ 7.33–7.10 (m, 4 H, arom), 4.74 (d, *J* = 9.6 Hz, NH), 4.66 (dd, *J* = 9.6 and 3.2 Hz, 1 H), 3.88 (m, 1 H), 2.98 (m, 1 H), 2.87 (m, 1 H), 2.36 (m, 1 H), 2.08 (m, 1 H), 1.42 (s, 9 H); cis (detected peaks) δ 5.28 (m, NH), 4.41 (m, 1 H), 3.74 (m, 1 H). Anal. ($C_{16}H_{21}NO_4$) C, H, N.

(2*S*,3*S*)-2-(1-Indanyl)glycine Hydrochloride (10) [(2*S*,3*S*)-Ing, HCl].[33] The title compound was prepared from its N^α-(*tert*-butyloxy)carbonyl derivative **59** following the procedure described for the preparation of **9**. **10**: mp 185–188 °C dec; $[\alpha]^{20}_D$ +53.9° (TFA salt; *c* 0.5; AcOH); R_f (D) 0.23; ^{1}H NMR (200 MHz, CD_3OD) δ 7.40–6.92 (m, 4 H, arom), 4.23 (d, *J* = 3.6 Hz, 1 H), 3.78 (m, 1 H), 2.87 (ddd, 2 H), 2.32 (m, 1 H), 2.00 (m, 1 H).

***N*$^\alpha$-[(*tert*-Butyloxy)carbonyl]-(2*S*,3*R*)-2-(1-benz[*f*]indanyl)glycine (60) [Boc-(2*S*,3*R*)-Bfi].** By analogy with the synthesis of **58**, **54a** (1.01 g, 2.30 mmol) was hydrolyzed (4 h) and then N^α-Boc protected (8 h), with several modifications in the workup and purification procedures due to the poor solubility of the final product: the final reaction mixture was filtered through a pad of Celite and concentrated under reduced pressure. The residue was taken up in CH_2Cl_2 (75 mL) and washed with ice-cooled 10% citric acid (2 × 50 mL), while keeping the solid at the interface in the organic layer. This layer was homogenized by addition of MeOH (50 mL) and dried ($MgSO_4$). The residue obtained after evaporation of the solvents was rinsed with cyclohexane and purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH/AcOH, 90:10:0.5) to afford 486 mg (62%) of a white solid: mp 213–216 °C dec; $[\alpha]^{20}_D$ −96.2° (*c* 0.5; DMF); R_f (C) 0.38; ^{1}H NMR (200 MHz, CD_3OD) mixture of trans (80%) and cis (20%) isomers (Boc function), trans δ 7.62 (s, 1 H, arom), 7.82–7.56 (m, 3 H, arom), 7.42–7.25 (m, 2 H, arom), 4.77 (d, *J* = 5.4 Hz, 1 H), 3.84 (m, 1 H), 3.24–2.85 (m, 2 H), 2.32–1.95 (m, 2 H), 1.28 (s, 9 H); cis (detected peaks) δ 4.63 (m, 1 H). Anal. ($C_{20}H_{23}NO_4$) C, H, N.

(2*S*,3*R*)-2-(1-Benz[*f*]indanyl)glycine Hydrochloride (11) [(2*S*,3*R*)-Bfi, HCl].[33] The title compound was prepared from its N^α-(*tert*-butyloxy)carbonyl derivative **60** following the procedure described for the preparation of **9**. **11**: mp 221–225 °C dec; $[\alpha]^{20}_D$ −69.9° (TFA salt; *c* 0.5; AcOH); R_f (D) 0.20; ^{1}H NMR (200 MHz, CD_3OD) δ 7.86 (s, 1 H, arom), 7.72 (s, 1 H, arom), 7.95–7.56 (m, 2 H), 7.52–7.25 (m, 2 H, arom), 4.72 (d, *J* = 3.9 Hz, 1 H), 4.12 (m, 1 H), 3.13 (ddd, 2 H), 2.32 (m, 1 H), 2.10 (m, 1 H).

***N*$^\alpha$-[(*tert*-Butyloxy)carbonyl]-(2*S*,3*S*)-2-(1-benz[*f*]indanyl)glycine (61) [Boc-(2*S*,3*S*)-Bfi].** By analogy with the synthesis of **58**, **54b** (2.37 g, 5.40 mmol) was hydrolyzed (8 h) and then N^α-Boc protected (8 h) to give, after purification and recrystallization (Et_2O/pentane) 0.96 g (52%) of a white solid: mp 102–106 °C; $[\alpha]^{20}_D$ +112.7° (*c* 0.5; MeOH); R_f (C) 0.40; ^{1}H NMR (400 MHz, $CDCl_3$) mixture of trans (80%) and cis (20%) isomers (Boc function), trans δ 7.76 (dd, 2 H, arom), 7.68 (s, 1 H, arom), 7.64 (s, 1 H, arom), 7.47–7.35 (m, 2 H, arom), 4.84 (d, *J* = 9.4 Hz, NH), 4.76 (dd, *J* = 9.4 and 3.3 Hz, 1 H), 3.99 (m, 1 H), 3.11 (m, 1 H), 2.99 (m, 1 H), 2.41 (m, 1 H), 2.14 (m, 1 H), 1.40 (s, 9 H); cis (detected peaks) δ 5.88 (m, NH), 4.47 (m, 1 H), 3.77 (m, 1 H). Anal. ($C_{20}H_{23}NO_4$) C, H, N.

(2*S*,3*S*)-2-(1-Benz[*f*]indanyl)glycine Hydrochloride (12) [(2*S*,3*S*)-Bfi, HCl].[33] The title compound was prepared from

its N^{α}-(*tert*-butyloxy)carbonyl derivative **61** following the procedure described for the preparation of **9**. **12**: mp 192–194 °C dec; $[\alpha]^{20}_{D}$ +90.9° (TFA salt; *c* 0.5; AcOH); R_f (D) 0.18; ^{1}H NMR (200 MHz, CD_3OD) δ 7.79 (s, 1 H, arom), 7.71 (s, 1 H, arom), 7.93–7.62 (m, 2 H, arom), 7.50–7.33 (m, 2 H), 4.49 (d, *J* = 4.4 Hz, 1 H), 4.00 (m, 1 H), 3.13 (ddd, 2 H), 2.49 (m, 1 H), 2.20 (m, 1 H).

N^{α}-[(*tert*-Butyloxy)carbonyl]-2-(2-indanyl)glycine (62) [Boc-DL-2Ing]. To a mixture of **55** (0.46 g, 1.90 mmol) in CH_3CN (10 mL) was added a solution of LiOH·H_2O (0.32 g, 7.5 mmol) in H_2O (10 mL), and the reaction was stirred at room temperature for 2 h. The solution was concentrated under reduced pressure to 5 mL, 10 mL of 1 N HCl was added, and the solvent was evaporated. The crude product was recrystallized from EtOH/H_2O to provide 381 mg of pure amino acid hydrochloride. This compound was subjected to N^{α}-Boc protection following the procedure described for the synthesis of **56**. During workup, while the mixture was washed with ice-cooled 10% citric acid (2 × 50 mL), a solid appeared at the interface which was kept in the organic layer and then dissolved by addition of MeOH before drying over $MgSO_4$. Purification yielded 346 mg (64%) of a white solid: mp 175–177 °C; R_f (C) 0.39; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.25–7.05 (m, 4 H, arom), 5.07 (d, *J* = 8.7 Hz, NH), 4.46 (m, 1 H), 3.18–2.68 (m, 5 H), 0.94 (s, 9 H). Anal. ($C_{16}H_{21}NO_4$) C, H, N.

Boc-Gln-DL-Phe(β-OH)-OH (63). To a solution, under argon, of Boc-Gln-OH (7.38 g, 30 mmol) and *N*-ethylmorpholine (3.9 mL, 31 mmol) in THF (50 mL) was added, at −18 °C, isobutylchloroformate (3.9 mL, 30 mmol), and the mixture was stirred for 45 min. A solution of DL-*threo*-3-phenylserine hydrate (6.00 g, 33 mmol) in 1 N NaOH (43 mL) was introduced at −10 °C, and the reaction mixture was stirred for 30 min at this temperature, 2 h at 0 °C, and 4 h at room temperature. It was concentrated to remove the THF, diluted with H_2O (50 mL), and washed with AcOEt (2 × 50 mL). The aqueous layer was then acidified with solid citric acid and extracted with AcOEt (3 × 100 mL) to provide, after drying ($MgSO_4$) and concentration under reduced pressure, 7.74 g (63%) of a colorless oil: R_f (C) 0.22.

Boc-Gln-Δ^{Z}Phe Azlactone (64). A solution, under argon, of **63** (7.55 g, 18.4 mmol) and freshly fused AcONa (2.00 g, 22.3 mmol) in acetic anhydride (150 mL) was vigorously stirred for 14 h at room temperature. The reaction mixture was poured into crushed ice, diluted with 5% $NaHCO_3$ (300 mL) and CH_2Cl_2 (500 mL), triturated, and filtered. After decantation, the organic layer was washed with 5% $NaHCO_3$ (3 × 200 mL) and dried ($MgSO_4$). The solvent was removed, and the residue was recrystallized from CH_2Cl_2/cyclohexane to give 4.33 g (63%) of a yellow solid: mp 155–156 °C; R_f (C) 0.35; ^{1}H RMN (200 MHz, $CDCl_3$) δ 8.15–8.00 (m, 2 H, arom), 7.53–7.37 (m, 3 H, arom), 7.22 (s, 1 H), 5.90 (br s, 1 H), 5.68 (br s, 1 H), 5.41 (d, *J* = 7.8 Hz, 1 H), 4.76 (m, 1 H), 2.43 (dd, 2 H), 2.31 (m, 1 H), 2.16 (m, 1 H), 1.47 (s, 9 H).

Boc-Gln-Δ^{Z}Phe-OH (65). To a mixture of **64** (1.12 g, 3 mmol) in CH_3CN (40 mL) was added a solution of LiOH·H_2O (165 mg, 3.9 mmol) in H_2O (20 mL), and the reaction was stirred for 30 min. After evaporation of CH_3CN *in vacuo*, the remaining solution was diluted with H_2O (100 mL) and washed with Et_2O (2 × 75 mL). This aqueous layer was then acidified with aqueous 10% citric acid (200 mL) and quickly extracted with AcOEt (4 × 75 mL). The combined organic layers were washed (H_2O) and dried ($MgSO_4$) to provide, after evaporation, 1.12 g (95%) of a slightly yellow solid: R_f (D) 0.32; ^{1}H RMN (200 MHz, CD_3OH) δ 9.53 (s, 1 H, NH), 7.72–7.15 (m, 8 H), 6.94 (d, *J* = 7.4 Hz, 1 H, NH), 4.22 (m, 1 H), 2.40 (t, 2 H), 2.13 (m, 1 H), 1.98 (m, 1 H), 1.47 (s, 9 H).

General Method for the Synthesis and Purification of Substance P Analogues. All reagents and solvents were reagent grade and used without further purification, except TEA and TFA which were distilled from ninhydrin for TEA before use in synthesis and chromatographic buffers. Ascending TLC was carried out on precoated silica gel 60 plates (Merck) using the following solvent systems (all v/v): (I) *n*-BuOH/AcOH/H_2O (4:1:5, upper phase) and (II) *n*-BuOH/pyridine/AcOH/H_2O (5:5:1:4). Amino acid analyses (AAA) were performed on a Pico-Tag Work Station (Waters Associates), using phenyl isothiocyanate derivatization. The peptides were hydrolyzed with 6 N HCl containing a small amount of phenol at 110 °C for 24 h in deaerated Eppendorf tubes. Pierce standards H were used for calibration. The presence of any unnatural amino acid was confirmed after injection of the pure compound. Molecular weights of peptides were characterized by electrospray ionization mass spectrometry, performed by the group of Prof. J. C. Tabet, Université P. et M. Curie, Paris.

Derivatized amino acids were purchased from Propeptide, Neosystem, Novabiochem, or Bachem. The N^{α}-amino function was protected with the Boc group, and side-chain protection was tosyl (Arg), 2-Cl-Z (Lys). Tic was synthesized as described elsewhere in literature.[36]

1. Peptide Synthesis. Peptide synthesis was carried out on a 0.1-mmol scale, either on an ABI Model 431A peptide synthesizer or manually for part or for the whole sequence, starting from a *p*-methylbenzhydrylamine resin (MBHA resin, typical substitution 0.77). All N^{α}-Boc-amino acids, in 5- or 10-fold excess, were assembled using DCC and HOBt as coupling agents. In a typical manual procedure, the following steps were performed in each cycle, with coupling efficiency monitored with the Kaiser test: (1) activation of the N^{α}-Boc-amino acid in CH_2Cl_2/DMF (5:1) with HOBt (1.5 equiv) and DCC (1.0 equiv) for 10–20 min, (2) removal of DCU, introduction in the reactor vessel, and mixing for 3–4 h, (3) washing with CH_2Cl_2, MeOH (2 × 1 min), and CH_2Cl_2 (2 × 1 min), (4) monitoring the completion of the reaction with the Kaiser test,[47] (5) Boc removal for 30 min with 50% TFA in CH_2Cl_2 and 1 mg of indole/100 mL of the solution, (6) washing with CH_2Cl_2 (2 × 1 min), (7) neutralization with 10% DIEA in CH_2Cl_2 (2 × 1 min), and (8) washing with CH_2Cl_2, MeOH (2 × 1 min), and CH_2Cl_2 (2 × 1 min). After removal of the last N^{α}-Boc-protecting group, the resin was dried *in vacuo*. The peptide–resin was transferred into the Teflon vessel of an HF apparatus, and the peptide was cleaved from the resin by treatment with 1.5 mL of anisole, 0.25 mL of dimethyl sulfide, and 10 mL of anhydrous HF per gram of peptide–resin for 30 min at −20 °C and 30 min at 0 °C. After evaporation *in vacuo* of the HF and the solvents over 2 h, the resin was first washed three times with Et_2O and then subsequently extracted three times with 10% AcOH. Lyophilization of the extract gave crude product containing mainly, as estimated by HPLC, the expected peptide and its sulfoxide side product(s).

2. Peptide Purification and Characterization. Peptides were purified by using one of the following methods. **Method A**: purification was performed by preparative reverse-phase HPLC with an Applied Biosystems apparatus, using a 10- × 250-mm Brownlee column packed with Aquapore Octyl, 300-Å pore size. The separation was accomplished with various acetonitrile gradients in aqueous 0.1% TFA (see below) at a flow rate of 6 mL/min with UV detection fixed at 220 nm. **Method B**: the crude peptide was purified on CM-32 carboxymethyl cellulose (Whatman) cation-exchange chromatography (1.5 cm × 15 cm) using a linear gradient (400 mL, 0.01 M NH_4OAc, pH 4.5/CH_3CN, 1:1, 400 mL, 0.*x* M NH_4OAc, pH 6.5; *x* = positive charges of the peptide + 1). Final purification was achieved by partition chromatography on a Sephadex G-25F column with the solvent system I.

Before pooling, the collected fractions were checked for purity by analytical HPLC, performed on a Waters Associates apparatus coupled to a D-2000 chromatointegrator (Merck). The separation was accomplished on a Lichrospher 100 RP-8e column (Merck) in isocratic mode (0.25 M triethylammonium phosphate buffer, pH 3.0, and acetonitrile) at a flow rate of 1.5 mL/min with UV detection fixed at 210 nm.

The configurations of the 2Ing-substituted peptides could not be determined unambiguously; therefore, they have been assigned on the basis of the HPLC retention times of the SP analogues (the D-substituted peptides in position 7 or 8 always being eluted after the L-isomer) and their biological activities.

[HPh^7]SP (17). The title compound was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid. Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 74 mg (48%). Purity: 98.0%. Electrospray: *m*/*z* $(M + H)^+$ calcd, 1361.75; found 1361.33 AAA: $Arg_{1.00}Glu_{1.88}Gly_{1.01}Leu_{1.02}Lys_{1.03}Met_{1.02}Phe_{1.05}Pro_{2.03}$.

[Tic^7]SP (18) was manually synthesized with 5-fold excess of each N^{α}-Boc-amino acid. Purification: method B. Yield: 31 mg (20%). Purity: 97.3%. Electrospray: *m*/*z* $(M + H)^+$ calcd,

1359.74; found, 1359.70. AAA: $Arg_{1.04}Glu_{2.18}Gly_{1.04}Leu_{0.92}Lys_{0.90}Met_{0.90}Phe_{0.95}Pro_{1.93}$.

[Dip⁷]SP (19) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-Dip (5 equiv). Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 71 mg (44%). Purity: 99.2%. Electrospray: m/z $(M + H)^+$ calcd, 1423.77; found 1423.32. AAA (48-h hydrolysis required): $Arg_{1.08}Glu_{2.13}Gly_{1.12}Leu_{1.05}Lys_{0.91}Met_{0.91}Phe_{0.93}Pro_{1.89}$.

[Flg⁷]SP (20) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-Flg (5 equiv). Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 27 mg (17%). Purity: 98.7%. Electrospray: m/z $(M + H)^+$ calcd, 1421.75; found, 1421.75. AAA (48-h hydrolysis required): $Arg_{0.94}Glu_{2.32}Gly_{1.12}Leu_{0.89}Lys_{0.93}Met_{0.95}Phe_{0.91}Pro_{1.98}$.

[(2*S*,3*R*)-Ing⁷]SP (21) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-(2*S*,3*R*)-Ing (5 equiv). Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 58 mg (37%). Purity: 98.6%. Electrospray: m/z $(M + H)^+$ calcd, 1373.75; found, 1373.65. AAA: $Arg_{0.95}Glu_{1.94}Gly_{1.11}Leu_{1.11}Lys_{0.89}Met_{1.08}Phe_{1.00}Pro_{2.15}$.

[(2*S*,3*S*)-Ing⁷]SP (22) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-(2*S*,3*S*)-Ing (5 equiv). Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 53 mg (34%). Purity: 99.6%. Electrospray: m/z $(M + H)^+$ calcd, 1373.75; found, 1373.93. AAA: $Arg_{0.96}Glu_{2.17}Gly_{1.03}Leu_{0.98}Lys_{0.95}Met_{0.97}Phe_{0.93}Pro_{1.96}$.

[2*S*,3*S*)-Bfi⁷]SP (23) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-(2*S*,3*R*)-Bfi (5 equiv). Purification: method A (gradient, 12–39% CH_3CN, linear, 30 min). Yield: 108 mg (67%). Purity: 99.8%. Electrospray: m/z $(M + H)^+$ calcd, 1423.77; found, 1423.61. AAA: $Arg_{0.96}Glu_{1.97}Gly_{1.07}Leu_{1.00}Lys_{1.04}Met_{1.01}Phe_{0.98}Pro_{2.01}$.

[2Ing⁷]SP and [D-2Ing⁷]SP (24–25) were assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-DL-2Ing (5 equiv) which was coupled manually following the classical method, e.g., DCC/HOBt for 4 h followed by Boc removal with TFA/CH_2Cl_2 and neutralization with DIEA. Purification: method A (gradient, 18–33% CH_3CN, linear, 30 min) provided first (i) [L-2Ing⁷]SP (24) (yield, 20 mg (13%); purity, 96.7%; electrospray, m/z $(M + H)^+$ calcd, 1373.75; found, 1373.74; AAA, $Arg_{0.99}Glu_{2.22}Gly_{1.09}Leu_{0.96}Lys_{0.92}Met_{0.94}Phe_{0.92}Pro_{1.91}$) and second (ii) [D-2Ing⁷]SP (25) (yield, 20 mg (13%); purity, 97.7%; AAA, $Arg_{0.94}Glu_{2.22}Gly_{1.06}Leu_{0.97}Lys_{1.03}Met_{1.00}Phe_{0.91}Pro_{1.89}$).

[Δ^ZPhe⁷]SP (26). (i) Synthesis *via* Boc-Gln-Δ^ZPhe Azlactone Coupling. The peptide was assembled on the resin (0.1-mmol scale) until the Gly⁹ residue and the Boc function were removed. Under argon, to a stirred mixture of Boc-Gln-Δ^ZPhe azlactone (64) (373 mg, 1.00 mmol) in anhydrous $CHCl_3$ (5 mL) was added DMAP (122 mg, 1.00 mmol). Two minutes later, the resin was introduced and the reaction mixture was stirred at room temperature for 24 h. The resin was washed with $CHCl_3$, AcOEt, NMP, and CH_2Cl_2 followed by Boc removal with TFA/CH_2Cl_2 and neutralization with DIEA. The last part of the sequence was assembled on the synthesizer. Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 15 mg (10%). Purity: 99.5%. Electrospray: m/z $(M + H)^+$ calcd, 1345.72; found, 1345.63.

(i) Synthesis *via* Boc-Gln-Δ^ZPhe-OH Coupling. The peptide was assembled on the synthesizer on a 0.5-mmol scale with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-Gln-Δ^ZPhe-OH (65) (1.18 g, 3 equiv) which was coupled manually with DCC activation in pure DMF for 48 h followed by Boc removal with TFA/CH_2Cl_2 and neutralization with DIEA. Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 149 mg (20%). Purity: 99.6%.

Photoisomerization of Δ^ZPhe⁷]SP. [Δ^EPhe⁷]SP (27). In a thermostated reactor fitted with an N_2 inlet tube, a solution of [Δ^ZPhe⁷]SP (30 mg) in deaerated MeOH (15 mL) was irradiated with a UV lamp at $\lambda = 310$ nm, for 2 h at 20 °C. This procedure was repeated on another 30 mg sample; the combined mixtures were diluted with H_2O, concentrated, and lyophilized. The peptides were separated using method A (gradient, 15–33% CH_3CN, linear, 30 min) to provide 9 mg (15%) of the desired compound (and 42 mg, 70%, of recovered [Δ^ZPhe⁷]SP). Purity: 98.6%. Electrospray: m/z $(M + H)^+$ calcd, 1345.72; found, 1345.61.

[HPh⁸]SP (28) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid. Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 40 mg (26%). Purity: 98.4%. Electrospray: m/z $(M + H)^+$ calcd, 1361.75; found, 1361.85. AAA: $Arg_{0.95}Glu_{1.87}Gly_{0.99}Leu_{1.02}Lys_{1.08}Met_{1.05}Phe_{1.06}Pro_{2.05}$.

[Tle⁸]SP (29) was manually synthesized with 5-fold excess of each N^{α}-Boc-amino acid. Purification: method B. Yield: 47 mg (31%). Purity: 99.1%. Electrospray: m/z $(M + H)^+$ calcd, 1313.75; found, 1313.45. AAA: $Arg_{0.91}Glu_{2.21}Gly_{0.99}Leu_{0.91}Lys_{0.89}Met_{0.99}Phe_{0.98}Pro_{1.94}$.

[Pen(S-Me)⁸]SP (30) was manually synthesized with 5-fold excess of each N^{α}-Boc-amino acid. Purification: method B. Yield: 34 mg (23%). Purity: 98.4%. Electrospray: m/z $(M + H)^+$ calcd, 1345.72; found, 1345.61. AAA: $Arg_{0.90}Glu_{2.21}Gly_{0.93}Leu_{1.00}Lys_{0.97}Met_{1.07}Phe_{0.97}Pro_{1.92}$.

[Pen(SO_2-Me)⁸]SP (31) was manually synthesized with 5-fold excess of each N^{α}-Boc-amino acid. Purification: method B. Yield: 39 mg (26%). Purity: 97.5%. Electrospray: m/z $(M + H)^+$ alcd, 1377.71; found 1377.71. AAA: $Arg_{0.91}Glu_{2.00}Gly_{0.99}Leu_{1.06}Lys_{1.02}Met_{1.06}Phe_{1.02}Pro_{2.05}$.

[Tic⁸]SP (32) was manually synthesized with 5-fold excess of each N^{α}-Boc-amino acid. Purification: method B. Yield: 34 mg (22%). Purity: 99.0%. Electrospray: m/z $(M + H)^+$ calcd, 1359.74; found, 1359.61. AAA: $Arg_{1.01}Glu_{1.98}Gly_{1.04}Leu_{0.98}Lys_{1.00}Met_{0.95}Phe_{1.00}Pro_{2.00}$.

[Dip⁸]SP (33) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-Dip (5 equiv). Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 79% mg (49%). Purity: 98.8%. Electrospray: m/z $(M + H)^+$ calcd, 1423.77; found, 1423.14. AAA (48-h hydrolysis required): $Arg_{0.95}Glu_{2.05}Gly_{1.11}Leu_{1.00}Lys_{0.98}Met_{0.88}Phe_{0.94}Pro_{1.90}$.

[Flg⁸]SP (34) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-Flg (5 equiv). Purification: method A (gradient, 12–36% CH_3CN, linear, 30 min). Yield: 34 mg (21%). Purity: 99.0%. Electrospray: m/z $(M + H)^+$ calcd, 1421.75; found, 1421.27. AAA (48-h hydrolysis required): $Arg_{0.99}Glu_{2.35}Gly_{1.11}Leu_{0.91}Lys_{0.91}Met_{0.90}Phe_{0.89}Pro_{1.95}$.

[(2*S*,3*R*)-Bfi⁸]SP (35) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-(2*S*,3*R*)-Bfi (5 equiv). Purification: method A (gradient, 12–42% CH_3CN, linear, 30 min). Yield: 75 mg (47%). Purity: 98.9%. Electrospray: m/z $(M + H)^+$ calcd, 1423.77; found, 1424.10. AAA: $Arg_{1.02}Glu_{1.91}Gly_{1.01}Leu_{1.04}Lys_{1.01}Met_{1.00}Phe_{1.07}Pro_{2.12}$.

[(2*S*,3*S*)-Bfi⁸]SP (36) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-(2*S*,3*S*)-Bfi (5 equiv). Purification: method A (gradient, 12–42% CH_3CN, linear, 30 min). Yield: 39 mg (24%). Purity: 99.9%. Electrospray: m/z $(M + H)^+$ calcd, 1423.77; found, 1423.78. AAA: $Arg_{1.02}Glu_{1.94}Gly_{0.94}Leu_{1.04}Lys_{1.03}Met_{1.00}Phe_{1.08}Pro_{2.15}$.

[Dip⁸, Pro⁹]SP (37) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-Dip (5 equiv). Purification: method A (gradient, 18–36% CH_3CN, linear, 30 min). Yield: 83 mg (50%). Purity: 99.7%. Electrospray: m/z $(M + H)^+$ calcd, 1463.80; found, 1463.31. AAA: $Arg_{1.09}Glu_{2.06}Leu_{0.98}Lys_{0.94}Met_{0.96}Phe_{0.96}Pro_{2.95}$.

[Flg⁸, Pro⁹]SP (38) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-Flg (5 equiv). Purification: method A (gradient, 15–39% CH_3CN, linear, 30 min). Yield: 73 mg (45%). Purity: 99.5%. Electrospray: m/z $(M + H)^+$ calcd, 1461.78; found, 1461.09. AAA: $Arg_{1.02}Glu_{1.98}Leu_{1.05}Lys_{0.98}Met_{0.96}Phe_{1.09}Pro_{3.15}$.

[(2*S*,3*S*)-Ing⁷, Flg⁸]SP (39) was assembled on the synthesizer with 10-fold excess of each N^{α}-Boc-amino acid, except for Boc-(2*S*,3*S*)-Ing and Boc-Flg (5 equiv). Purification: method A (gradient, 18–36% CH_3CN, linear, 30 min). Yield: 84 mg (52%). Purity: 99.2%. Electrospray: m/z $(M + H)^+$ calcd, 1447.77; found 1447.11. AAA: $Arg_{0.92}Glu_{1.92}Gly_{1.07}Leu_{1.10}Lys_{1.00}Met_{1.09}Pro_{2.15}$.

Binding Assays on Rat Brain Synaptosomes, NK-1 and NK-3 Assays. The radioligands [^{125}I]BHSP and [^{125}I]BHELE were obtained by acylation of SP and eledoisin, respectively, with ^{125}I-Bolton and Hunter reagent (2000 Ci/mmol, monoiodo derivative from Amersham). Crude synaptosomal preparations from rat brain minus cerebral cortex and from rat cerebral cortex were used for the [^{125}I]BHSP (NK-1) and [^{125}I]BHELE (NK-3)

assays, respectively. The specific activity of both radioligands was 2000 Ci/mmol. Synaptosomes were prepared as previously described.[75,76] Briefly, the final pellet was resuspended, at a concentration of 5 mL for 1 g of initial tissues, in Krebs–Ringer phosphate buffer containing bovine serum albumin (0.4 mg/mL), bacitracin (30 μg/mL), and glucose (1 mg/mL). Routinely, 20 μL of the synaptosomal fraction was incubated in Eppendorf tubes at 20 °C in 200 μL of Krebs–Ringer phosphate buffer containing 40 pM of [^{125}I]BHSP (5 min) or [^{125}I]BHELE (15 min) and increasing concentrations of peptides.[75,76] Finally, radioactivity bound to tissues was estimated using a Packard gamma counter. All assays were made in triplicate, and nonspecific binding was defined as the amount of labeled ligand bound in the presence of SP (10^{-6} M, NK-1 assay) or eledoisin (10^{-6} M, NK-3 assay).

[^{3}H]NKA Binding from Rat Duodenum, an NK-2 Assay. A membrane suspension from the smooth muscle of the rat duodenum was prepared according to a described procedure.[77] Twenty microliters of the membrane suspension (300 μg of protein) was incubated in Tris-HCl buffer, 50 mM, pH 7.4, containing bovine serum albumin (0.4 mg/mL), bacitracin (40 μg/mL), leupeptin (4 μg/mL), chymostatin (4 μg/mL), kelatorphan (10^{-6} M), and $MnCl_2$ (3 mM), for 25 min at 20 °C in a final volume of 200 μL with [^{3}H]NKA (0.9 nM, specific activity 75 Ci/mmol) with or without unlabeled ligand concentration. After centrifugation, the pellet-bound radioactivity was counted in a LKB-Wallace liquid spectrometer.[77] All assays were run in triplicate, and nonspecific binding was defined as the amount of labeled ligand bound in the presence of NKA (10^{-6} M).

[^{125}I]BHSP Binding from Guinea Pig Ileum, an NK-1 Assay. Membranes from male Hartley guinea pig ileum were prepared according to Morimoto et al.[56] with the following modification.[13] The mucus was scraped with a blunted scalpel, and guinea pig ileum smooth muscle tissues were finally cut with scissors. Tissues were dispersed in Tris-HCl buffer, 50 mM, pH 7.4, containing sucrose (0.25 M) and EDTA (0.1 mM), at 4 °C and homogenized with a Polytron (Kinematica) for 30 s. The homogenate was centrifuged (800 g, 10 min, 4 °C) to remove tissue clumps, and the supernatant was centrifuged (14000*g*, 20 min, 4 °C) to yield pellets which were washed in Tris-HCl (50 mM, pH 7.4). Final centrifugation (14000*g*, 20 min, 4 °C) yielded crude membrane fractions which were resuspended in Tris-HCl buffer (50 mM, pH 7.4) containing $MnCl_2$ (3 mM), bovine serum albumin (0.4 mg/mL), bacitracin (40 μg/mL), chymostatin (4 μg/mL), leupeptin (4 μg/mL), and thiorpan (10^{-6} M). For binding experiments, 20 μL of this membrane suspension was incubated in Eppendorf tubes at 20 °C for 5 min in a final volume of 200 μL with 40 pM of [^{125}I]BHSP (specific activity 2000 Ci/mmol) and increasing concentrations of peptides. After incubation, the pellet-bound radioactivity was estimated using a Packard gamma counter. All assays were made in triplicate, and nonspecific binding was defined as the amount of labeled ligand bound in the presence of SP (10^{-6} M).

Bioassay on Guinea Pig Ileum. Peptides were tested on tissues taken from male guinea pigs (Charles River, 300–400 g). The terminal portion of GPI was used to obtain a 1-cm long strip. Strips of GPI were suspended in a 12.5-mL organ bath containing oxygenated (95% O_2, 5% CO_2) Tyrode solution at 37 °C under a tension of 1*g*. The agonist was added cumulatively, i.e., the concentration in the bath was increased by a factor of 2 whenever a steady response to the previous concentration was reached. pK_B values (pK_B = log(dose ratio − 1) − log(molar concentration of antagonist)) were estimated for one concentration of the antagonist RP-67,580 preincubated for 5 min before the first addition of agonist. As the dose ratio was close to 2, pK_B values were close to pA_2 values. The pA_2 values (±SEM) were obtained by regression analysis of Schild plots as described by Arunlakshana and Schild.[78]

Computational Methods. The side-chain volumes were calculated by the volume function of the SYBYL program. Energy calculations were carried out using the SYBYL (version 5.5) program (TRIPOS). Calculations were performed on a Silicon graphics computer with an Evans and Sutherland PS 390 graphics system. The different conformers (A, B, A_β, E) and rotamers (g^-, g^+, t) for CH_3CO-X-$CONH_2$-protected amino acids (X = Ing, Flg, or Tle) were built up and subjected to full-energy minimization calculations using TRIPOS force fields. A distance-dependent dielectric function ($\epsilon = 2r$) was employed for these calculations. The partial atomic charges were calculated by a combination of Gasteiger and Hückel methods.

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