34 were subjected to treatment with spleen phosphodiesterase I (10 units/mg of protein) (Merck, Darmstadt, West-Germany), snake venom phosphodiesterase II (0.019 unit/mg of protein) (Sigma, St. Louis, MO), and human serum. Incubation with the test compounds (1 mg/mL) were done with phosphodiesterase I (at 0.002 unit/mL) and phosphodiesterase II (at 0.2 units/mL) for 0, 1, 4, 24, and 48 h at 37 °C. Hydrolysis products were separated by thin-layer chromatography (TLC) and characterized by running appropriate standards in parallel. They were quantitated by estimation of the intensity of the spots on the TLC plates under UV. For comparison, compound **13**, araAMP, araA, and ara-hypoxanthine (araHX) were incubated with the enzymes under identical experimental conditions, and the reaction products were analyzed.

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Synthesis and Immunological Evaluation of N-Terminal, Noncrossreactive Tachykinin Antigens

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The N-terminal hexa- or pentapeptide sequences of the three mammalian tachykinins substance P, neurokinin A, and neurokinin B have been synthesized by the conventional solid-phase procedure with 6-aminocaproyl-*S*-(acetamidomethyl)cysteine as a C-terminal spacer and attachment function. A fourth sequence, with an additional N-terminal 6-aminocaproyl residue on the substance P–hapten sequence, was cyclized N- to C-terminally. For this purpose, a four-level protection scheme has been applied: BOC-TFA for N-terminal protection and cleavage; TFA-stable but HF-labile anchoring function and side-chain protection; *S*-acetamidomethyl for semipermanent thiol protection. The side chain amino function of Lys was protected with NO_2Z, stable against HF but readily cleaved with hydrogenation. The hapten sequences were coupled to maleimidated BSA, after the Acm group was removed by mercury/hydrogen sulfide treatment. Mice immunized with the three linear hapten sequences produced sera that were specific in enzyme-linked immunosorbant assay for the presented hapten and the respective tachykinin but displayed no crossreactivity at all toward the other haptens nor to one of the other tachykinins. It is concluded that this approach produced antisera, specific and selective for its respective mammalian tachykinins.

During the last few years, mammalian tachykinins have gained widespread attention. Substance P (SP) has been known for a very long time[1] and its physiological role is now considered to be that of a neurotransmitter[2] or a neuromodulator[3] peptide. Apart from SP, several other peptides from lower vertebrates belong to this family, all characterized by the same common C-terminal sequence—Phe-Xxx-Gly-Leu-Met-NH_2 (Xxx is either Val, Phe, or Tyr). For many years SP was the only tachykinin known to exist in mammals, and radioimmunoassays (RIA) were conducted assuming no other peptides reacted with the antisera. Recently, however, two new members of this family were discovered in mammals: neurokinin A (NKA)[4] and neurokinin B (NKB).[5] These new compounds have still mostly unknown, probably neurophysiological functions. Therefore, highly specific assays for each mammalian tachykinin are necessary in order to carry out conclusive studies on these agents.

Therefore, due to the particular chemistry of the tachykinins, most of the hitherto available immunoassays for SP were crossreactive with the other tachykinins. The chemically inert C-terminal heptapeptide of SP (see structural formulas below) did not permit the preparation of haptens with anything else exposed other than the C-terminus. All classical conjugation methods, like those using glutaraldehyde or dicyclohexylcarbodiimide conjugation, have always coupled the N-terminal part of SP and the other tachykinins to the carrier (e.g. BSA or thyroglobulin).

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substance P (SP):

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

neurokinin A (NKA):

H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2

neurokinin B (NKB):

H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2

For this reason, a strategy was designed to produce immunogens displaying the N-terminus of the three mammalian tachykinins.

Hapten Syntheses. In order to expose the N-terminal part of the three tachykinins, a C-terminal function was introduced for the new specific hapten–carrier coupling. The function chosen was the acetamidomethyl-protected cysteine, which, after liberation of the free sulfhydryl function, can couple specifically to a maleimidated protein carrier.[6] In order to suppress potential crossreactivity, the common C-terminal pentapeptide Phe-Val(orPhe)-Gly-Leu-Met-NH_2 of the tachykinins was eliminated and the nonspecific spacer amino acid 6-aminocaproic acid (Aca) was used to connect the C-terminus to the sulfhydryl-bearing moiety Cys(Acm). The hapten sequences were therefore built up into the following sequences:

SP–hapten	H-Arg-Pro-Lys-Pro-Gln-Gln-Aca-Cys(Acm)OH	(1)
NKA–hapten	H-His-Lys-Thr-Asp-Ser-Aca-Cys(Acm)OH	(2)
NKB–hapten	H-Asp-Nle-His-Asp-Phe-Aca-Cys(Acm)OH	(3)
SP–prec hapten	⌜Aca-Arg-Pro-Lys-Pro-Gln-Gln-Aca-Cys(Acm)⌝	(4)

In order to detect additionally the prohormone of SP and not only the matured peptide, a further antigen sequence, peptide 4, which mimicks a N-terminally prolonged SP, was prepared. Antibodies specific against the N-terminal part of the peptide, without "seeing" the α-amino function, could be helpful for the detection of such precursor peptides, even if those antibodies could display crossreactivity with free SP. Such an eventual crossreactivity could be subtracted by using antibodies specific to the N-terminus, produced with the hapten sequence 1 for free SP, "seeing" only SP. This midsequence hapten peptide 4 was therefore prepared by N-terminal extension with 6-aminocaproic acid, followed by cyclization to the free carboxylic function of the anchoring Cys(Acm) and finally by conjugation to a maleimidated carrier protein. This cyclic peptide 4 would expose the sequence Arg-Pro-Lys-Pro-Gln as a "loop" and not as a "tail" as in peptide 1.

Because of the cyclization of this peptide in the presence of a thiol function, a four-level orthogonal protection scheme had to be applied. For sulfhydryl protection, the acetamidomethyl group (Acm) was chosen, a group that is stable to hydrogenolysis and acidolysis.[7] For lysine, which had to be protected at its ϵ-amino function during cyclization in peptide 4, the acidolytically stable 4-nitrobenzyloxycarbonyl function (NO_2Z) was used. This protecting group is stable even against liquid HF but readily cleaved by hydrogenolysis or reductive acidolysis.[8,9] All α-amino functions were blocked with the *tert*-butoxycarbonyl function, cleavable with dilute trifluoroacetic acid (TFA). The guanidino function of Arg and the imidazole function of His were protected with the tosyl group, the Ser and Thr hydroxyls with the benzyl, and the β-carboxyl function of Asp with the cyclohexyl group. Peptide synthesis was carried out by classical solid-phase synthesis with chloromethylated, cross-linked polystyrene, esterified to Boc-Cys(Acm). Chain elongation was carried out in a stepwise manner with TFA as the cleaving reagent, and the completed sequence was cleaved from the solid support in liquid HF. After purification, the N-terminal peptide of SP, peptide 1, was subjected to hydrogenolysis in order to cleave the ϵ-Lys protection. The intersequence hapten 4, however, was first cyclized and then subjected to hydrogenation. All peptides were conjugated to maleimidobenzoyl-BSA (MBSA) after cleaving the thiol-protecting group Acm with the mercury and hydrogen sulfide treatment.[10]

Table I. ELISA Determination of Serum Specificity[a]

immunogen	ELISA antigen SP	NKA	NKB
1–MBSA	+++	–	–
2–MBSA	–	+++	–
3–MBSA	–	–	+++

[a] Antisera obtained after immunization with the respective immunogen 1–MBSA, 2–MBSA, or 3–MBSA were tested each individually in ELISA against the respective tachykinins. (+++) indicates strong recognition, (–) indicates nondetectable interaction; all antisera also had crossreactivity with native BSA. Experimental details are described in the text.

Results and Discussion

The peptide synthesis yielded the required peptide sequences without complications, as shown with FAB-MS. The cyclic hapten 4 was obtained with ease; after HF cleavage and workup in acetic acid, some material cyclized spontaneously to the desired sequence; the main fraction, however, had to be cyclized, producing a peptide identical with the spontaneously formed product 4. The proposed four-level protection scheme was carried out without problems, a scheme involving the already orthogonal protections α-amino-Boc combined with the side-chain protections, the HF-stable NO_2Z on peptide 1 and 4, and the Acm group.

The coupling of the haptenic sequences to MBSA was achieved without a very high substitution on the carrier, however. In amino acid analysis no clear change of the composition was visible and SDS-PAGE indicated only a relatively small molecular change of a few kilodaltons. This indicates that approximatively two to three sequences were coupled to the MBSA. At higher substitutions, a significant change in the amino acid composition would have been visible, as well as in SDS-PAGE. The immunogens 1–MBSA, 2–MBSA, 3–MBSA, and 4–MBSA were injected into mice, and after the usual booster injections, a clear, specific antigenic response was visible for the first three antigens. The fourth antigen, 4–MBSA, has not been tested yet because of lack of the two other tachykinin intersequence antigens of NKA and NKB and of N-terminally extended SP analogues as tracers. In the enzyme-linked immunosorbent assay (ELISA), the sera of the immunized mice only interacted with the presented immunogen and the corresponding tachykinin but not at all with the other antigens or the other two tachykinins

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(see Table I). We can therefore conclude that the approach to build up N-terminal hapten sequences of the mammalian tachykinin peptides and to attach them specifically through a sulfhydryl–maleimide coupling to a carrier protein has produced highly selective antisera, specific for each tachykinin without any noticeable crossreactivity.

Current efforts are under way, both to the obtention of monoclonal antibodies from these immunized animals and to the production of the two remaining NKA and NKB intersequence haptens. The latter is in the hope to induce antisera with specific recognition of the precursor peptides of the individual tachykinins for immunocytochemical studies.

Experimental Section

General Procedures. Amino acid derivatives used as starting materials, peptide reagents, and chloromethylated resin (copolysterene–1% divinylbenzene) were purchased from Bachem Fine Chemicals or Chemalog Chemical Dynamics Corp. or Aldrich Chemical Co. The following amino acid derivatives were prepared in our laboratory: L-Boc-Lys(NO_2Z),[9] L-Boc-Nle, Boc-Aca (*N*-(butyloxycarbonyl)-6-aminocaproic acid). Dicyclohexylcarbodiimide (DCC) was purified by dissolving the commerical product in dry diethyl ether; the insoluble material, dicyclohexylurea, was removed by filtration, and the ether was evaporated in vacuo. All solvents and reagents used for solid-phase synthesis were of analytical quality and the solvents were redistilled before use. All mixtures of liquids are given in volume/volume. Preparative reversed-phase chromatography was performed on 30 μm C-18 material (Nucleosil, Macherey-Nagel, Duren, Germany) in Michel-Miller glass columns (diameter 2.5 cm or 0.8 cm) at a mean pressure of 5–10 atm. Analytical high-pressure liquid chromatography (HPLC) was performed on a Waters-M-45 system equipped with a VYDAC 218 TP 104 column and a 214-nm UV detector; peptides were considered pure if the peak integral was at least 95% % of the combined peptide-peak integrals. TLC was performed on Merck DC-Alufoils Kieselgel 60 F_{254} in the solvent systems BAW (BuOH/AcOH/H_2O 5:2:3), and TRUTH ($CHCl_3$/MeOH/AcOH 95:5:3). Visualization was carried out with UV fluorescence and either with Pauly's reagent (4-sulfobenzenediazonium hydroxide in 2 N Na_2CO_3) or with a modified Reindel-Hoppe procedure[11] or with ninhydrin. The technique of fast atom bombardment mass spectrometry (FAB-MS) has been used to confirm the peptide structures with a Hewelett-Packard instrument, type 5988 A. FAB-MS analyses were made directly from a thioglycolic solution of the sample.

Peptide Synthesis. The syntheses were carried out with a Peptomat automatic peptide synthesizer, by procedures previously described.[12] The first amino acid, L-Boc-Cys(Acm), was attached to the resin by the cesium salt procedure.[13] N-Boc protection was used for all amino acids. This temporary protection was removed, prior to the next coupling, by reaction with TFA in CH_2Cl_2 (2:3) for 20 min at room temperature. A 5% solution of diisopropylethylamine (DEA) in CH_2Cl_2 was used for neutralization of the free amino function of the growing peptide. The ensuing coupling was performed with the symmetrical anhydride of the next Boc-amino acid in a 4–6-fold excess.[14] Boc-Gln was coupled with 1 equiv of DCC and *N*-hydroxybenzotriazole (HOBT), all in 4-fold excess. The completion of every coupling was checked with the ninhydrin test[15] and repeated if necessary. The peptides were cleaved from the resin by exposure for 1 h at 0 °C to approximately 20 mL of liquid anhydrous HF, containing 10% anisole and 1% diethyl sulfide.

H-Arg-Pro-Lys-Pro-Gln-Gln-Aca-Cys(Acm)-OH (1). Ten grams of chloromethylated resin was esterified with L-Boc-Cys-(Acm)OCs to yield a substitution of 0.88 mequiv/g of resin, according to the picric acid test.[16] Boc-Aca was coupled to the Cys(Acm)-resin which was split into several portions afterward. Two grams of this resin was completed to the above sequence and split in two equal portions. One of these (1.2 g) was treated with liquid HF. The cleavage reagent was removed by flushing with dry N_2, followed by evacuation through KOH pellets, first at 11 mmHg and then under high vacuum, both for 15 min. The peptide was extracted with 2 N AcOH and lyophilized. The crude peptide was purified by LH-20 gel filtration in DMF and lyophilized from 0.2 M HOAc. A second purification with reversed-phase C-18 column chromatography and a gradient of 0–50% CH_3CN in 0.05% TFA yielded 186.1 mg of 1: TLC (BAW) R_f 0.13; FAB/MS M + 1 at 1220; HPLC shows homogeneity of the product. Fourteen milligrams of this product was hydrogenated in 1 mL of 50% HOAc in the presence of 1.1 mg of 10% palladium on charcoal (Alfa Inorganics) at a pressure of 8 atm of H_2 in a previously described setup.[17] After 90 min the catalyst was filtered off through Celite, the vessel and the filter were rinsed with 4 mL of 2 N HOAc, and the filtrate was lyophilized. The crude peptide 1 was purified successively by LH 20 gel filtration and C-18 reversed-phase chromatography. The yield of pure 1 after lyophilization was 11 mg: TLC (BAW) R_f 0.03; HPLC shows homogeneity of the product; FAB/MS M + 1 at 1041.

H-His-Lys-Thr-Asp-Ser-Aca-Cys(Acm)OH (2). Successive couplings to the Boc-Aca-Cys(Acm)-resin (1.5 g) gave the protected peptide-resin (1.65 g). The HF treatment, as described for 1, yielded crude 2, which was purified by LH-20 gel filtration and reversed-phase C-18 column chromatography. The yield of 2 was 87.5 mg: TLC (BAW) R_f 0.35; FAB/MS M + 1 at 875; HPLC shows homogeneity of the product.

H-Asp-Nle-His-Asp-Phe-Aca-Cys(Acm)OH (3). Successive coupling to a further portion of the Boc-Aca-Cys(Acm)-resin (0.75 g) gave the protected peptide-resin (0.85 g). HF treatment, as described for 1, yielded crude 3, which was purified by LH-20 gel filtration and reversed-phase C-18 column chromatography as described above. The yield of 3 was 30.9 mg: TLC (BAW) R_f 0.38; FAB/MS M + 1 at 934; HPLC shows homogeneity of the product.

H-Aca-Arg-Pro-Lys(NO_2Z)-Pro-Gln-Gln-Aca-Cys(Acm)-OH. The second portion of the protected octapeptide sequence prepared as described for 1 was completed by coupling with Boc-Aca. This resin (1.15 g) was cleaved by treatment with HF and the crude peptide was purified as described for 1, producing 111.8 mg of linear, NO_2Z-protected 4: TLC (BAW) R_f 0.15; FAB/MS M + 1 at 1334; homogeneity shown in HPLC. From the reversed-phase purification was recovered a second fraction (24.3 mg): TLC (BAW) R_f 0.42; FAB/MS M + 1 at 1316; homogeneous by HPLC. This byproduct was identical with cyclic, NO_2Z-protected 4.

***cyclo*-(Aca-Arg-Pro-Lys-Pro-Gln-Gln-Aca-Cys(Acm)) (4).** The linear peptide was cyclized by very slow injection of a DMF solution of 5.2 mg of this peptide (5 mL) to a solution of 4.8 mg of DCC, 2.1 mg of HOBT, and 1.35 μL of DEA in 7 mL of DMF for 6 h at room temperature and with stirring overnight. The product was filtered, the solution was partially evaporated, and the cyclic peptide was purified by LH-20 gel filtration in DMF, yielding 2.8 mg of product: TLC (BAW) R_f 0.42; FAB/MS M + 1 at 1316; HPLC showed homogeneity of the product. This product was identical with the byproduct recovered above. Of this still NO_2Z protected, cyclic peptide, 8.3 mg was hydrogenated as described for peptide 1 (TLC (BAW) R_f 0.15), separated from the formed toluidine by LH-20 chromatography, and directly processed to the antigen coupling (see below) without further characterization.

Preparation of the BSA Conjugates. Bovine serum albumin (BSA; 680 mg, 10 μmol, Sigma) was dissolved in 10 mL of 0.1 M sodium phosphate buffer at pH 8.0, and 50 mg (120 μmol) of *N*-maleimidobenzoylsulfosuccinimide ester (Sulfo-MBS, Pierce) was added together with 5 μL of a 1 N NaN_3 solution. The

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mixture was stirred for 16 h at room temperature with maintenance of the pH at 8.0. The solution was dialyzed three times for a total of 24 h at 0 °C against water (10 L each, containing 100 μL of 1 N NaN_3) and the product, maleimidobenzoyl-BSA (MBSA), was lyophilized. The Acm group of the peptides 1–4 was cleaved by treatment of the Acm-protected peptides 1–4 dissolved in 2 mL of 3 M NaOAc at pH 4.0, with the addition of 4.2 mg of $Hg(OAc)_2$ for 20 min at room temperature. The reaction mixture was diluted with 4 mL of water, and gaseous H_2S was bubbled through for 30 min. The black suspension was filtered through Celite, the reaction vial and the filter were rinsed with 4 mL of water, and the filtrate fractions were combined and degassed in vacuo for 10 min. This solution was directly used for the coupling of the hapten peptides to the carrier by adding these solutions to MBSA in 15 mL of 50 mM Tris·HCl at pH 7.5 and nitrogen bubbling for 3 h. The reaction mixtures were dialyzed three times against water, as described above, and then lyophilized. Eleven milligrams of 1 coupled to 105 mg of MBSA produced 99.0 mg of conjugate, 9.5 mg of **2** to 102 mg of MBSA produced 90.0 mg, 10 mg of **3** to 100 mg of MBSA produced 97 mg, and 8.3 mg of 4 to 100 mg of MBSA finally produced 59.5 mg of conjugate.

Immunization Procedures. Female Balb/c mice (Charles River, St. Constant, Quebec) received weekly injections (500 or 50 ng/mL) of immunogen for 3 consecutive weeks. An additional immunization was given 3 weeks later. On each occasion the animals received a single intraperitoneal (0.1 mL) plus two intradermal (0.05 mL) injections of the immunogen in the form of an emulsion in complete Freund's adjuvant. Ten days after the last immunization, the mice were bled and the individual sera tested in an enzyme-linked immunosorbant assay (ELISA).[18] Crossreactivity with similar immunogens and titre of the sera were assessed. In the present study, 24 mice were used; six mice were immunized with each of the four immunogens 1–MBSA, 2–MBSA, 3–MBSA, and 4–MBSA.

ELISA Protocol. The enzyme-linked immunosorbent assay (ELISA) is a procedure that allows relatively rapid estimation of the specificity of antisera. In this test, the natural antigen (SP, NKA, NKB) is absorbed to the plastic surface of a well in a microtiter plate and serum potentially containing antibodies specific for these antigen is added. If the antigen is recognized and bound by antibodies, an indirect anti-antibody–enzyme test is used to visualize these immobilized antibodies.

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Drug-Induced Modifications of the Immune Response. 12. 4,5-Dihydro-4-oxo-2-(substituted amino)-3-furancarboxylic Acids and Derivatives as Novel Antiallergic Agents

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The synthesis of a series of novel 4,5-dihydro-4-oxo-2-(substituted amino)-3-furancarboxylic acids, salts, esters, and amides is described. The title compounds when tested in the mediator-induced dermal vascular permeability and active anaphylaxis assays in rats demonstrated moderate to potent antiallergic activity. The [2-*trans*-(4-methylphenyl)cyclopropyl]amino analogue **53** emerged as the most active derivative. Thus, when administered intraperitoneally to rats at a dose of 100 mg/kg, it inhibited the action of the mediators serotonin, histamine, and bradykinin by 100%. In the active anaphylaxis assay in rats, compound **30** suppressed the edema by 81% at a dose of 100 mg/kg, following intraperitoneal administration.

During the past few years our interest has been directed toward the synthesis of a new class of antiallergic agents, the 2(5*H*)-furanone derivatives **2** and **3**.[1,2] The γ-lactone amides **2** and **3** were obtained by a novel rearrangement of 2-(substituted amino)-4,5-dihydro-4-oxo-3-furancarboxylic acids (**1**) in the presence of 1 equiv of each

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(2) Georgiev, V. St.; Mack, R. A.; Walter, D. J.; Radov, L. A.; Baer, J. E. *Helv. Chim. Acta* **1987**, *70*, 1526.

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