Tritiated Peptides. 12. Synthesis and Biological Activity of [4-^{3}H-Phe^8]Substance P

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Substance P has been prepared ^{3}H labeled at Phe^8 by catalytic deiodination of a protected precursor. Synthesis of the precursor was by solid-phase methodology on polydimethylacrylamide resin and by condensation in solution of fragments covering sequences 1–4, 5–7, and 8–11. Free peptide made by each route analyzed satisfactorily and had the same chromatographic characteristics as unlabeled substance P. It was indistinguishable from the latter by radioimmunoassay when N and C terminally directed antisera were used and in the ability to cause contractions of isolated guinea pig ileum. Specific radioactivity was 23 Ci/mmol.

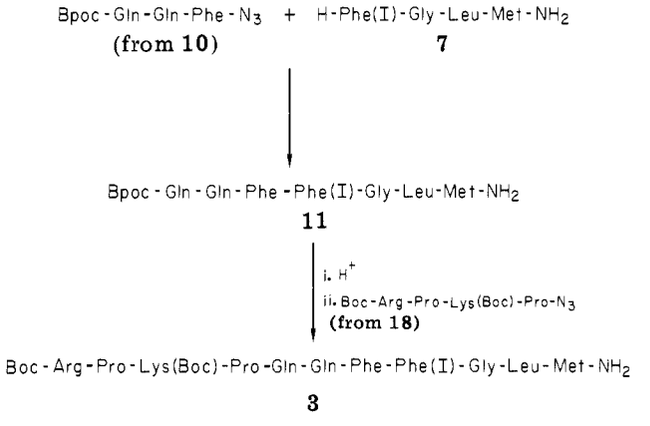
Substance P has been proposed as a neurotransmitter or neuromodulator in the central nervous system (CNS).[1] The key to determining its functional importance in nervous system function must involve a better understanding of the nature and location of the receptors with which substance P can interact in the CNS. The development of ligand binding techniques has proved very useful for studies of interaction of drugs with receptor sites for conventional amine and amino acid neurotransmitters[2] as well as for peptides.[3] These receptor binding studies require a labeled ligand with physical properties and biological activities, including receptor binding characteristics, preferably identical with the original ligand. The most common approach for labeling peptides has been incorporation of iodine-125 into tyrosine residues or addition of an extra residue already containing this isotope. Both of these modifications would alter the physical properties of substance P. Although the specific radioactivities of such derivatives are high, they do not necessarily have the binding properties of the parent compound.

Tritiated–halogen exchange of *p*-chlorophenylalanine, incorporated into a substance P derivative, has been reported[4] but without supporting analytical data. The conditions required for complete exchange could cause severe damage (desulfurization) to the methionine residue. To circumvent this problem, a *p*-chlorophenylalanine substance P derivative with methionine replaced by norleucine has been used.[5] A preparation of [^{3}H]substance P by catalytic gas-exposure exchange labeling has been reported, but the product was only 80% biologically active.[6] This is the first report of the preparation of fully characterized pure tritium-labeled substance P. We have previously reported the preparation of a number of peptides labeled with tritium to a high level at specific sites.[7] The basis of the method is synthesis of a protected precursor molecule containing an iodine-substituted aromatic amino acid, followed by replacement of iodine with tritium by catalytic exchange in an aprotic solvent. After acidolytic deprotection and purification, the desired labeled product is obtained in quantities that allow rigorous chemical analysis.

Results and Discussion

The protected precursor **3** was synthesized both by the solid-phase technique and by fragment condensation in solution. The solid-phase procedure used a polar resin based on polyacrylamide and incorporating acryloylsarcosine methyl ester.[8] When the fluorenylmethyloxycarbonyl (Fmoc) group was used to protect the growing peptide chain, this method has been reported[9] to furnish substance P of acceptable purity in good yield. The *tert*-butyloxycarbonyl group was used for arginine N$^\alpha$ protection and for the side chain of lysine. After ammonolysis of the protected peptide from the resin, the product **3** was isolated by ion-exchange chromatography and preparative high-pressure liquid chromatography (HPLC).

Scheme I. Synthesis of Compound 3 by Fragment Condensation



The same precursor **3** was also synthesized by fragment condensation with azide couplings as shown in the Scheme I. Fragments corresponding to the peptide sequences 1–4 (**18**), 5–7 (**10**), and 8–11 (**7**) were assembled by stepwise condensation. Compounds **10** and **7** were obtained without difficulty; however, compound **18** was noncrystalline, as were its precursors, compounds **12–15** and **17**. Nevertheless, satisfactory elemental analyses were obtained for compounds **17** and **18**. Literature methods for the preparation of Boc-Arg-OH[10] or its hydrochloride[11] either failed

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or worked poorly in our hands. We report an easy, reliable synthesis using $(t\text{-BuO})_2CO$ and conversion of the product to Boc-Arg-OH·HCl in which form it is suitable for use in DCC couplings. After coupling and purification by CCD in a solvent system containing HOAc, the chloride counterion was observed to have been replaced by acetate ion. Difficulty was experienced in the purification of compound **3** stemming not from any gross impurity of the coupling product, but from the similar physical properties of those few impurities present to the desired product. It is probable that the impurities were produced as a result of the practical difficulties encountered during coupling. These difficulties were almost entirely due to the low solubility of the amino component (**19**) under the conditions used and necessitated its portionwise addition, over 2 h, to the generated azide to allow stirring and effective cooling. It is possible that local heating effects and their less than optimal dissipation may have caused partial degradation of the azide to the corresponding isocyanate.

No pyroglutamyl heptapeptide (**20**) formation by cyclization of N-terminal glutamine was observed during coupling, although a small quantity (<5%) of compound **20** was generated during the N-deprotection of the protected heptapeptide (**11**). The usual method for Bpoc removal with aqueous acetic acid[12] gave a virtually quantitative yield of compound **20**, and this finding led to the use of the "strong acid" conditions for Bpoc removal described in the text. The small quantity of compound **20** that was present was removed by filtration, since it was almost completely insoluble in organic solvents.

Notwithstanding these problems, purification of compound **3** by extensive CCD treatment (in four different solvent systems) gave the product in 44% yield. Compound **3** analyzed for the diacetate salt instead of the expected monoacetate. On conversion to the chloride salt, the dichloride was obtained.

Tritiated substance P was prepared by catalytic dehalogenation from the two samples of the protected precursor **3**. Use of the protected, rather than free, peptides affords two advantages. Protection of ionizable amino functions, together with the use of an aprotic solvent, minimizes potential dilution by exchange of tritium with protium before labeling. This would lower the specific activity achievable. Additionally, the use of deprotected free peptides results in the loss of most of the substrate by irreversible adsorption to the catalyst. Protected peptides do not adsorb even to charcoal-based catalysts. Examples of adsorption are provided by studies on the synthesis of [^{3}H]angiotensin[13] and [^{3}H]oxytocin.[14] Although both syntheses afforded products of high specific activity, yields were, respectively, 10 and 2.5%. In contrast, we have reported a yield as high as 87% using a protected peptide[15] and generally observe yields in the range of 40–50%.[7]

The reported conditions for tritiation are based on our previous experiences[7] and are designed to be reasonably practical. In order for one to obtain an acceptably high degree of tritium–iodine (as opposed to protium–iodine) replacement, reaction must be reasonably rapid (<30 min), so that exchange both with protons in the substrate and with extraneous protons unavoidably present as moisture does not greatly dilute the tritium before reduction can occur. Our reported conditions meet this requirement, as evidenced by the achieved specific activities (ca. 79% of theoretical). Extended reaction times in this instance led to desulfurization of methionine[16] and generation of the undecapeptide derivative containing a [^{3}H]aminobutyric acid residue at position 11. This side-reaction is not significant (≤2%) under the experimental conditions reported and is, in any case, not a serious drawback in the present synthesis, since the derived impurity is separable from the product when HPLC is used at the final purification stage.

Table I. Amino Acid Analysis After Acidic or Enzymic Hydrolysis of [^{3}H]Substance P Samples and Their Chromatographic Purities

	source of compd 3			
	solid phase		fragment condensation	
amino acid	acid	enzyme	acid	enzyme
Arg	0.99	0.95	0.99	0.97
Gln		1.85		1.94
Glu	1.99		1.97	
Gly	1.08	1.01	1.01	0.98
Leu	1.02	1.00	1.00	0.98
Lys	0.99	0.97	1.00	1.00
Met	0.89	0.99	0.96	0.99
Phe	2.00	2.00	2.00	2.00
Pro	1.97	1.92	2.00	1.96
Specific activity (Ci mmol^{-1})	22.95		22.9	
TLC purity				
solvent E	96.0 ± 1.1		100 ± 0.1	
solvent F[a]	91.0		93.2	
HPLC purity				
system A	86.7 ± 7.5		98.6 ± 0.2	
system B	94.5 ± 5.8		99.3 ± 1.0	
system C	92.1 ± 4.0		99.3 ± 0.6	

[a] Unreliable: solvent caused sulfoxidation (possibly due to traces of pyridine *N*-oxide).

The final products were available in sufficient quantity to allow amino acid analysis after both acidic and enzymic digestion. The analytical results (Table I) suggest a slight superiority of the "classical" solution synthesis method over the solid-phase method, which is not unexpected. Our solid-phase results, however, demonstrated the quality of product that can be obtained relatively rapidly (compared to the more laborious solution-phase synthesis) with the polyacrylamide-based resin.[8] Enzymic digestion also demonstrated that ≥99% of the tritium was associated with phenylalanine.[17] It also was a test for the efficiency of removal of the α-aminobutyryl[11] analogue. In both preparations, the amount of tritium associated with α-aminobutyric acid was <0.1%. Small amounts (≤1%) of tritium were associated with methionine.[16] Radiochemical purity of the products was assessed in two TLC and three HPLC systems. The two batches of tritiated substance P behaved exactly as standard substance P in two complementary radioimmuno assays (Figure 1a,b). The spasmogenic activity of both samples was equal to that of standard substance P on isolated guinea pig ileum, and identical dose–response curves were obtained (Figure 2).

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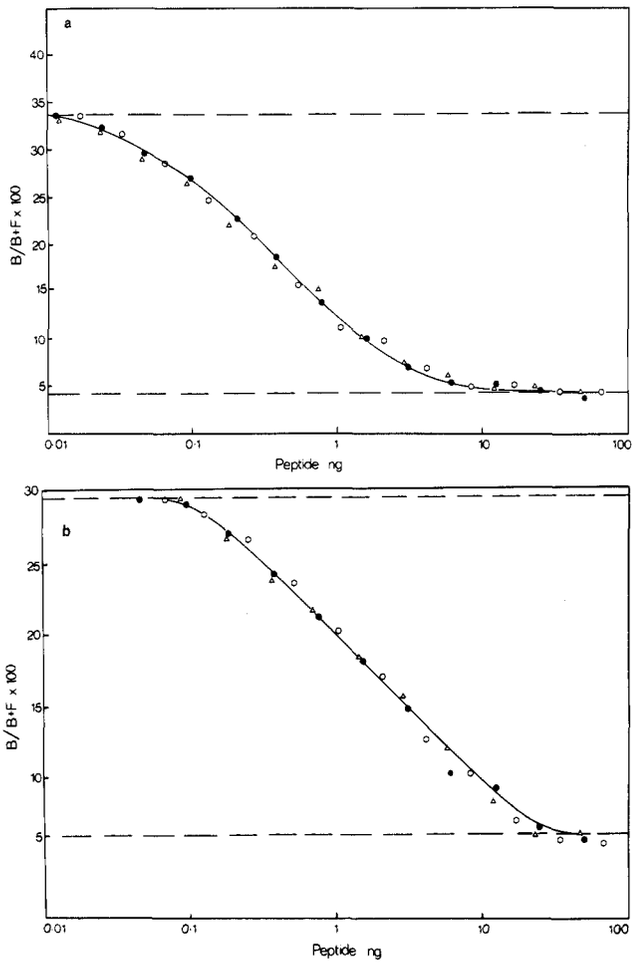

Figure 1. Cross-reactivity of [^{3}H]substance P (solid phase) (○), [^{3}H]substance P (solution) (△), and standard substance P (●) with C-terminal-directed (a) and N-terminal-directed (b) antisera. B = antiserum bound tracer, F = free (unbound) tracer.

Tritiated substance P prepared according to this procedure has been an important tool for assessing the role of substance P in the CNS and has been used for in vitro,[18,19] as well as in vivo[20] studies.

Experimental Section

Abbreviations for amino acids and their use in the formulation of derivatives follow the IUPAC–IUB recommendations.[21] The following abbreviations are used: Bpoc, biphenylylisopropyloxycarbonyl; CCD, countercurrent distribution; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DIEA, diisopropylethylamine; DMA, dimethylacetamide; DMF, dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole hydrate; HPLC, high-pressure liquid chromatography; Phe(4-I), 4-iodophenylalanine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TsOH, toluene-4-sulfonic acid.

Melting points are uncorrected. Microanalyses were performed by CHN Analysis Ltd., South Wigston, Leicester. Optical rotations were measured in a 1-dm tube with a Bellingham-Stanley P.70-2 polarimeter. CCD was performed in a Steady State Distribution Machine, Model 20 (123 tubes, 10 mL each phase), from Quickfit and Quartz Ltd., Stone, Staffs., with the following solvent systems: A, *n*-BuOH/HOAc/H_2O (4:1:5); B, $CHCl_3$/$C_6H_5CH_3$/MeOH/H_2O (5:5:8:2); C, *t*-BuOH/1.25% TsOH/$C_6H_5CH_3$ (10:10:3); D, MeOH/H_2O/$CHCl_3$/CCl_4 (12:3:4:7).

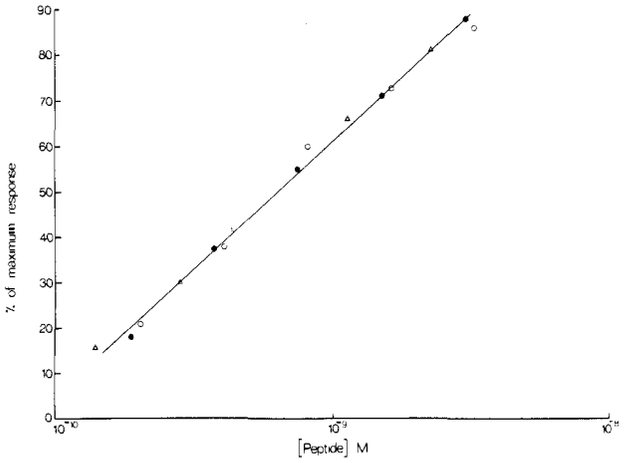

Figure 2. Contractile potencies of [^{3}H]substance P (solid phase) (○), [^{3}H]substance P (solution) (△), and standard substance P (●) on the guinea pig ileum.

Analysis by HPLC of the radioactive products was effected on columns (25 × 0.46 cm) of the stated bonded phases with constant volume (20 mL) gradients as detailed with the following solvent systems: A, 40–80% MeOH in H_2O (+0.1% H_3PO_4) on Nucleosil 10C_{18}; B, 15–35% MeCN in H_2O (+0.1% H_3PO_4) on Nucleosil 10CN; C, 20–40% MeCN in H_2O (+0.2 N triethylammonium phosphate, pH 3.0) on Phenyl-bondapak.

Analysis by TLC was on precoated plates of silica gel (Merck F254) with the following solvent systems: A, $CHCl_3$/MeOH/HOAc (85:10:5); B, $CHCl_3$/MeOH/HOAc (80:10:10); C, $CHCl_3$/MeOH (95:5); D, *n*-BuOH/HOAc/H_2O (4:1:5); E, $CHCl_3$/MeOH/NH_4OH (sp gr 0.88) (60:45:20); F, EtOAc/C_5H_5N/HOAc/H_2O (5:5:1:3).

Evaporations of solvent were carried out in vacuo at a temperature below 40 °C. All solvents for CCD and HPLC were distilled before use. Conditions for amino acid analysis are detailed elsewhere.[22] Phe(I) was estimated as described earlier.[23] Fmoc-substituted amino acids, except Fmoc-Phe(4-I), were purchased from Chemical Dynamics Corp., South Plainfields, NJ, Fmoc chloride (FLUKA) was purchased from Fluorochem Ltd., Glossop, Derbys, England, and pure L-amino acids were purchased from Cambrian Chemicals Ltd., Croydon, Surrey, England. DIEA for solid phase use was distilled from ninhydrin and then from solid KOH and then stored in the dark. Fmoc-Cl was recrystallized from Et_2O before use. Polydimethylacrylamide resin (0.30 mmol of sarcosine/g of resin), prepared by copolymerization of a mixture of dimethylacrylamide, ethylenebisacrylamide, and acryloylsarcosine methyl ester,[8] was a gift from Dr. R. C. Sheppard, Laboratory of Molecular Biology, Cambridge. Tritium gas (98%) was purchased from Amersham International Ltd., Amersham, Bucks, England.

Fmoc-Phe(4-I) (1). A solution of 9-fluorenylmethoxycarbonyl chloride (0.71 g, 2.75 mmol) in dioxane (5 mL) was added dropwise to a vigorously stirred solution of Phe(I) (0.73 g, 2.5 mmol) in 10% Na_2CO_3 (7.5 mL) and then stirred for 3 h at room temperature. The mixture was diluted with H_2O (100 mL) and extracted with Et_2O (2 × 30 mL), and the aqueous layer was acidified to pH 3 with saturated citric acid solution. The aqueous phase was extracted with EtOAc (3 × 50 mL), and the combined extracts were washed with H_2O, dried (Na_2SO_4), and evaporated. Crystallization of the residue from $CHCl_3$–*n*-hexane gave the product (1.15 g, 89.8%) contaminated with a small quantity of a single impurity (TLC in $CHCl_3$–MeOH, 80:20, v/v). Passage of a portion of this material as a solution in $CHCl_3$ through a pad of silica gel gave the clean product on elution with $CHCl_3$–MeOH (90:10). Crystallization of this material from $CHCl_3$–*n*-hexane gave the protected amino acid: mp 175–177 °C; $[\alpha]_D^{27}$ +37.6 ± 0.5° (*c* 0.75,

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$CHCl_3$). Anal. ($C_{24}H_{20}INO_4 \cdot 0.5H_2O$) C, H, I, N.

Boc-Arg-Pro-Lys(Boc)-Pro-Gln-Gln-Phe-Phe(4-I)-Gly-Leu-Met-resin (2). Boc-Met-resin (515 mg) (0.21 mmol of methionine/g of resin) was treated with TFA/CH_2Cl_2 (1:1) (15 mL) for 30 min and then washed for 2 min each with CH_2Cl_2 (3 × 10 mL), DMA (3 × 10 mL), DIEA/DMA (1:9) (5 × 10 mL), and DMA (5 × 10 mL). Sixfold excesses of the preformed symmetrical anhydrides of Fmoc-Leu, Fmoc-Gly, Fmoc-Phe, Fmoc-Phe(4-I), Fmoc-Pro, and Fmoc-Lys(Boc), all dissolved in DMA (10 mL), were coupled as appropriate with the resin-bound peptide. At each stage, after complete reaction as judged by the ninhydrin test, the resin was washed with DMA (10 × 10 mL) for 1 min and deprotected with piperidine/DMA (1:4) (2 × 10 mL) for 3 and 7 min. The resin was then washed with DMA (10 × 10 mL for 1 min) before the next Fmoc-substituted amino acid derivative was introduced. Fmoc-Gln (0.9 mmol) was coupled as the *p*-nitrophenyl ester in the presence of HOBt (0.9 mmol). Boc-Arg-OH·HCl (1.8 mmol) was incubated with DCC (0.9 mmol) in DMA (10 mL) for 5 min before being added to the resin.

Boc-Arg-Pro-Lys(Boc)-Pro-Gln-Gln-Phe-Phe(4-I)-Gly-Leu-Met-NH_2 (3). Compound **2** (250 mg) was suspended in MeOH saturated with NH_3 at 0 °C (10 mL), and the mixture was kept sealed at room temperature for 4 h. The resin was filtered off and washed with MeOH (3 × 5 mL) and DMA (5 mL). The combined filtrates were evaporated, and the residue was dissolved in 5 mM NH_4OAc, pH 5.0/MeOH (1:1) (6 mL) and applied to a SP-Sephadex column (14 × 1.5 cm) equilibrated with the above buffer. The column was eluted with the same buffer, the eluate was evaporated, and the residue was dissolved in 10% HOAc (10 mL) and lyophilized to give 40 mg of peptide material. HPLC revealed two minor impurities, one eluting before the main peak and one after. Partition chromatography on a column (60 × 2.7 cm) of Sephadex G-25 equilibrated with *n*-BuOH/HOAc/water (4:1:5) did not give a homogeneous product. Peptide material (11 mg) recovered from the SP-Sephadex column was purified by HPLC in portions (1–1.5 mg) on a column (30 × 0.39 cm) of μBondapak C_{18} eluted isocratically with CH_3CN/10 mM NH_4OAc, pH 4.0 (550:450). The eluate corresponding to the main peak (254 nm) was evaporated, and the residue was dissolved in 10% HOAc (5 mL). Lyophilization yielded 7 mg of product (41% based on the methionine content of the resin).

Boc-Gly-Leu-Met-NH_2 (4). We prepared this compound by stepwise synthesis starting from Met-NH_2·HCl and using DCC couplings of Boc-Leu and Boc-Gly in turn with HCl in HOAc for amino deprotection: mp 128–130 °C; $[\alpha]^{28}_D$ −39.4° (*c* 0.8, MeOH) [lit.[24] mp 115–117 °C, $[\alpha]_D$ −31° (*c* 1, HOAc); lit.[25] mp 107–110 °C, $[\alpha]_D$ −29.5° (*c* 1, HOAc); lit.[26] mp 159–160 °C, $[\alpha]_D$ −30.8° (*c* 1, HOAc)]. Anal. ($C_{18}H_{34}N_4O_6S$) C, H, N, S.

Gly-Leu-Met-NH_2·HCl (5). Compound **4** (3.0 g, 6.9 mmol) was dissolved in saturated HCl in HOAc (100 mL) and kept for 1 h. Evaporation gave product **5** as a foam in 100% yield. It was pure as judged by TLC in solvent B.

Boc-Phe(4-I)-Gly-Leu-Met-NH_2 (6). Boc-Phe(4-I)-OH[23] (1.35 g, 3.46 mmol) and HOBt (0.53 g, 3.46 mmol) were dissolved in DMF (5 mL), and DCC (0.79 g, 3.81 mmol) in DMF (1 mL) was added. The solution was stirred for 1 h at 0 °C and then for 1 h at 10 °C, and a solution of **5** (1.28 g, 3.46 mmol) and DIEA (0.6 mL, 3.46 mmol) in DCC (5 mL) was added. The mixture was stirred at 8 °C overnight. After DCU was removed by filtration and the filtrate was evaporated, trituration of the residue with *n*-BuOH/EtOAc (1:1) afforded material that was crystallized from MeOH to give product **6** (1.55 g, 64%): mp 214–216 °C; $[\alpha]^{29}_D$ −34.4° (*c* 1.2, DMF). Anal. ($C_{27}H_{42}IN_5O_6S$) C, H, I, N, S.

Phe(4-I)-Gly-Leu-Met-NH_2·HCl (7). This was prepared from compound **6** as described for compound **4** in a yield of 100%. It was pure as judged by TLC in solvent B.

Gln-Phe-OMe·HCl (8). Z-Gln-Phe-OMe[27] (8.8 g, 20 mmol) was dissolved in TFE (373 mL) containing H_2O (22 mL) and 1 N HCl (20 mL, 20 mmol) and 10% Pd/C (4.0 g) and stirred under H_2 for 4 h. The mixture was filtered and evaporated to a foam, which was pure as judged by TLC in solvent B.

Bpoc-Gln-Gln-Phe-OMe (9). Compound **7** (3.7 g, 8.85 mmol) and Bpoc-Gln-ONp (5.0 g, 10 mmol) were dissolved in DMF (100 mL), and DIEA (1.53 mL, 8.85 mmol) was added. The mixture was stirred overnight and then evaporated. The residue was triturated successively with ice-cold 0.2 N HCl, H_2O, Et_2O, and EtOAc, and the resulting solid was crystallized from MeOH/H_2O to give the product **9** (4.15 g, 70%): mp 183–185 °C; $[\alpha]^{29}_D$ −26.4° (*c* 1, MeOH). Anal. ($C_{36}H_{43}N_5O_8$) C, H, N.

Bpoc-Gln-Gln-Phe-N_2H_3 (10). A solution of **9** (2.0 g, 2.97 mmol) and $N_2H_4 \cdot H_2O$ (2.88 mL, 59.4 mmol) in DMF (25 mL) was stirred for 16 h under N_2. After the solvent was evaporated, the residue was triturated with ice–water, and the solid obtained was crystallized from DMF/MeOH/H_2O to give the product **10** (0.75 g, 37%): mp 193–195 °C; $[\alpha]^{28}_D$ −13.9 ± 0.6° (*c* 1, DMF). Anal. ($C_{35}H_{43}N_7O_7 \cdot 0.5H_2O$) C, H, N.

Bpoc-Gln-Gln-Phe-Phe(4-I)-Gly-Leu-Met-NH_2 (11). Compound **10** (1.37 g, 2.03 mmol) was dissolved in DMF (8 mL), the solution was cooled to −10 °C, and 3.2 N HCl in EtOAc (1.58 mL, 5.07 mmol) and then *t*-BuONO (0.24 mL, 2.03 mmol) were added at that temperature. The mixture was stirred for 10 min at −10 °C, and DIEA (0.87 mL, 5.23 mmol) was added, followed by a solution at −10 °C of **7** (1.22 g, 2.03 mmol) and DIEA (0.35 mL, 2.03 mmol) in DMF (8 mL). The mixture was stirred for 1 h at −10 °C and then for 40 h at 8 °C. The solvent was evaporated, and trituration of the residue with ice–water gave a solid, which was crystallized from DMF/MeOH/H_2O to yield 1.58 g (63%) of **11**: mp 258 °C dec; $[\alpha]^{29}_D$ −19.7° (*c* 0.65, DMF). Anal. ($C_{57}H_{73}IN_{10}O_{11}S \cdot H_2O$) C, H, I, N, S.

Z-Lys(Boc)-Pro-OMe (12). A solution of Z-Lys(Boc)-OH (38 g, 0.1 mmol) and HOBt (15.6 g, 0.1 mmol) in DMF (200 mL) was cooled to 0 °C. A solution of DCC (22.8 g, 0.11 mol) in DMF (20 mL) was added, and the mixture was stirred for 1 h at 0 °C and then for 1 h at 10 °C. A solution of Pro-OMe·HCl (17 g, 0.1 mol) and Et_3N (14.4 mL, 0.103 mol) in DMF (100 mL) at 0 °C was added slowly to the active ester solution, while the temperature was kept below 10 °C. The mixture was stirred at 8 °C overnight, DCU was filtered off, and the filtrate was evaporated. The residue was dissolved in EtOAc (100 mL) and washed with portions (2 × 250 mL) of H_2O, 10% citric acid, H_2O, saturated $NaHCO_3$, and H_2O. The solution was dried (Na_2SO_4) and evaporated to an oil, which was dissolved in Et_2O (200 mL) and chromatographed on a pad (4 × 11 cm) of silica gel, which was eluted with Et_2O, collecting 250 mL fractions. Fractions 3–12 afforded 11.6 g of crude product, which when chromatographed a second time yielded 9.7 g (20%) of product **12** as an oil. It was pure as judged by TLC in solvent C.

Lys(Boc)-Pro-OMe·HCl (13). Compound **12** (10 g, 20 mmol) was dissolved in a mixture of MeOH (100 mL) and HOAc (10 mL), 5% Pd/C (0.7 g) was added, and the mixture was stirred under H_2 for 3 h. The catalyst was removed by filtration (Celite), and the filtrate was evaporated. The residue was dissolved in EtOAc (40 mL), and 3.2 N HCl in EtOAc (6.4 mL, 20.5 mmol) was added dropwise while the solution temperature was kept below 0 °C. Light petroleum (250 mL) was added, and the solvents were evaporated to give a gum (5.8 g, 72%), which was stored in vacuo over solid NaOH.

Z-Pro-Lys(Boc)-Pro-OMe (14). This was prepared from Z-Pro-OH (1.4 g, 5.6 mmol) and **13** (2.2 g, 5.6 mmol) by the HOBt–DCC coupling procedure as detailed for compound **12**. After extraction, the crude material was purified by chromatography on a silica gel column (27 × 3.5 cm), which was developed with mixtures of MeOH in $CHCl_3$ (up to 5%). Fraction selection was by analytical TLC (solvent C), and pure product **14** (1.2 g, 36%) was obtained as an oil.

Pro-Lys(Boc)-Pro-OMe (15). Compound **14** (4.4 g, 7.5 mmol) was dissolved in MeOH (110 mL), 10% Pd/C (1.8 g) was added, and the mixture was stirred under H_2 for 3 h. The catalyst was

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removed by filtration, and the filtrate was evaporated to give **15** (3.4 g, 100%), which was pure as judged by TLC in solvent C. The oil solidified on standing and was recrystallized from EtOAc/petroleum ether: mp 102–104 °C; $[\alpha]^{25}_D$ −81.2° (*c* 1, MeOH). Anal. ($C_{22}H_{38}N_4O_6 \cdot 0.5H_2O$) C, H, N.

Boc-Arg-OH·HCl (16). Arginine hydrochloride (21.05 g, 0.1 mol) was dissolved in a mixture of dioxane (200 mL) and 0.5 N NaOH (200 mL), the solution was cooled to 0 °C (t-BuO)$_2$CO (24 g, 0.14 mol), and 4 N NaOH (12.5 mL, 0.05 mol) was added with vigorous stirring, which was continued for 2 h. Some insoluble material was removed by filtration, and the filtrate was extracted with Et_2O (2 × 100 mL). The aqueous phase was cooled to 0 °C, acidified (pH 5) with 1 N HCl, extracted with $CHCl_3$ (3 × 100 mL), neutralized (pH 7.3) with 1 N NaOH, and evaporated. The residue was dissolved in n-BuOH (200 mL), insoluble material was filtered off, and the filtrate was evaporated. The residue was dissolved in H_2O (100 mL) and lyophilized. The foam (27.4 g, 100 mmol) obtained was dissolved in MeOH (65 mL), and 2.5 N HCl in MeOH (40 mL, 100 mmol) was added to the solution at −20 °C. After evaporation, the residue was washed with a mixture of H_2O (20 mL) and CH_3CN (100 mL) and then crystallized from H_2O to give 23.2 g (75%) of **16**, mp 114–116 °C. A sample recrystallized from H_2O (60% yield) had mp 118–121 °C; $[\alpha]^{25}_D$ −8.9° (*c* 2.1, H_2O) [lit.[11] mp 117–119 °C; $[\alpha]_D$ −8.8° (*c* 2, H_2O)].

Boc-Arg-Pro-Lys(Boc)-Pro-OMe (17). Compounds **15** (3.4 g, 6.8 mmol) and **16** (3.5 g, 10.2 mmol) were dissolved in DMF (10 mL), and the solution was cooled to 0 °C. A solution of DCC (2.53 g, 11.2 mmol) in DMF (1 mL) was added, and the mixture was stirred for 2 h at 0 °C. Additional compound **16** (1.06 g, 3.4 mmol) and DCC (0.85 g, 3.74 mmol) were added, and the mixture was stirred for 1 h at 0 °C, and then for 16 h at 8 °C. DCU was removed by filtration, and the filtrate was evaporated. The crude material was purified by CCD with solvent system A to yield 1.95 g (35%) of amorphous product **17**, which was obtained as the monohydrate of the acetate salt after lyophilization. Anal. ($C_{33}H_{58}N_8O_9 \cdot CH_3COOH \cdot H_2O$) C, H, N.

Boc-Arg-Pro-Lys(Boc)-Pro-N_2H_3 (18). A solution of **17** (1.75 g, 2.27 mmol) and $N_2H_4 \cdot H_2O$ (1.3 mL, 26.8 mmol) in MeOH (9 mL) was stirred under N_2 for 18 h. More $N_2H_4 \cdot H_2O$ (1.3 mL, 26.8 mmol) was added, and the solution was stirred for an additional 18 h. The solution was evaporated, and the crude residue was purified by CCD with solvent system A to yield 1.28 g (73%) of amorphous diacetate hydrate **18** after lyophilization. Anal. ($C_{32}H_{58}N_{10}O_8 \cdot 2CH_3COOH \cdot H_2O$) C, H, N.

Gln-Gln-Phe-Phe(4-I)-Gly-Leu-Met-NH_2·HCl (19). Compound **11** (0.75 g, 0.61 mmol) was dissolved in DMF (30 mL), and saturated HCl in HOAc (30 mL) was added. After 15 min, the solvent was evaporated, the residue was dissolved in MeOH (30 mL), and the solution was stored at 5 °C for 16 h. The product **19** (565 mg, 90%) was collected by filtration. Analysis by TLC (solvent D) showed that some pyroglutamyl heptapeptide was present.

Glp-Gln-Phe-Phe(4-I)-Gly-Leu-Met-NH_2 (20). Compound **11** (1.48 g, 1.20 mmol) was dissolved in 80% aqueous HOAc (24 mL) and stirred for 1 h at 50 °C. The solid that formed was collected by filtration, dried, and then crystallized from DMF/MeOH/H_2O to yield 0.48 g (49%) of **20**: mp 314 °C; $[\alpha]^{28}_D$ −35.8° (*c* 0.8, DMF). Anal. ($C_{41}H_{56}IN_9O_9S \cdot H_2O$) C, H, I, N, S.

Boc-Arg-Pro-Lys(Boc)-Pro-Gln-Gln-Phe-Phe(4-I)-Gly-Leu-Met-NH_2 (3). Compound **18** (570 mg, 0.74 mmol) was dissolved in DMF (3 mL), and the solution was cooled to −10 °C. A solution of HCl (3 N) in EtOAc (0.62 mL, 1.86 mmol) was added below −10 °C, followed by t-BuONO (88.5 μL, 0.74 mmol), and the solution was stirred for 10 min at −10 °C. DIEA (32.2 μL, 1.86 mmol) was added, followed by four equal portions of compound **19** (141 mg, 0.124 mmol) and DIEA (21 μL, 0.124 mmol), which were added at 30 min intervals. The mixture was stirred at 8 °C for 65 h, and insoluble material (360 mg) was filtered off. The solid was purified by CCD with solvent system A to give 155 mg of product **3**. The filtrate was evaporated, and the residue yielded further pure **3** (215 mg) by CCD with systems A–D. After lyophilization, the yield was 370 mg (44%). Anal. ($C_{73}H_{113}IN_{18}O_{17}S \cdot 2CH_3COOH \cdot 4H_2O$) C, H, I, N, S.

A portion (50 mg, 27 μmol) of this material was dissolved in H_2O/MeOH (1:1) (4 mL) and passed through a column (6 mL) of Dowex 1 (chloride form) resin. After lyophilization, the product (35 mg, 72%) analyzed for the dihydrochloride. Anal. ($C_{73}H_{113}IN_{18}O_{17}S \cdot 2HCl \cdot 3H_2O$) C, H, Cl, I, N, S.

[4-^{3}H-Phe^8]Substance P (21). A solution of compound **3** (1.3–4.9 mg, 0.7–2.6 μmol in different experiments) in DMF (0.5 mL) was stirred with 98% $^3H_2$ gas (3.2 mL, 0.14 mmol, 8 Ci) in the presence of 10% Pd/C (ca. 5 mg) and 5% Rh/$CaCO_3$ (ca. 5 mg). After 40 min, the catalysts were removed by filtration through a pad of TLC-grade cellulose, and the filtrate was evaporated. The residue was dissolved in 90% TFA (5 mL) and kept for 30 min. The mixture was evaporated and the residue was dissolved in H_2O (1 mL) and passed through a column (0.5 mL) of Dowex 1 (acetate form) resin, which was eluted with H_2O (2 mL). The combined eluate and washings were evaporated, and the residue was dissolved in H_2O (0.5 mL). The solution was chromatographed on a column (3 × 0.7 cm) of carboxymethylcellulose ($HNMe_3^+$ form), which was developed with a linear gradient (40 mL total) of trimethylammonium acetate (0–0.5 M, pH 5.0). Fractions (1 mL) were collected automatically and analyzed for ^{3}H. The contents of the tubes containing radioactivity were combined, and buffer was removed by rotary evaporation with repeated addition of H_2O. The residue was dissolved in H_2O (0.52 mL) and the bulk (0.50 mL) of the solution was applied to a column (50 × 0.7 cm) of Nucleosil $10C_{18}$ using a Rheodyne six-port injection valve. The column was eluted at 6 mL min^{-1} with a constant volume (100 mL) gradient of MeCN/H_2O/H_3PO_4 (from 150:850:1 to 350:650:1). The eluate was monitored at 210 nm, and fractions (0.5 min) were collected automatically. Fractions, as indicated by the UV trace, were combined and evaporated to a volume of approximately 1 mL. The solution was passed through a column (0.5 mL) of Dowex 1 (acetate form) resin, and the eluate was evaporated to dryness. The residue was redissolved in H_2O and stored in liquid N_2 at a concentration of less than 5 mCi mL^{-1}. Analytical data are given in Table I. Yields of compound **20** from compound **3** were in the range 17–38% over a number of experiments.

Biological Tests: Radioimmunoassay. Immunoreactivity of [^{3}H]substance P prepared by either solid phase or solution methods was compared to standard synthetic substance P by serial dilution against either C-terminal specific or N-terminal specific antisera. Serially diluted aliquots of standard or [^{3}H]substance P were incubated with guinea pig C-terminal[23] (dilution 1:25 000) or rabbit N-terminal (1:4000) antisera for 48 h in 4 °C, with [^{125}I-Tyr^8]substance P as a tracer (added radioactivity approximately 10 000 cpm). Bound and free tracer were separated by addition of dextran-coated charcoal and centrifuged at 3000g for 10 min at 4 °C. The radioactivity in the supernatant ("bound") and the radioactivity in the charcoal pellet ("free") were counted in a Beckman Model 4000 gamma-counter.

Contraction of the Isolated Guinea Pig Ileum. Spasmogenic activity of the ileum in response to the applied peptides was recorded isotonically under a resting load of 0.5–0.8 g, with an Ealing isotonic transducer. The details of the assay have been described elsewhere.[14]

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