pyroglutamic acid means that the compound SP_{6-11} is a mixture of the glutaminyl and pyroglutaminyl forms. Estimates based on HPLC measurements suggest that the ratio of the two forms is approximately 65:35]; atropine sulfate and indomethacin Sigma; methysergide bimaleate, Sandoz; mepyramine maleate, generous gift of May and Baker Ltd. All other compounds were of Analar quality.

Indomethacin was dissolved in 10% $NaHCO_3$, all other drugs were dissolved in physiological salt solution.

Bioassays were performed on the guinea pig ileum and rat colon muscularis mucosae preparations as previously described[2,21] but with the following modifications: (i) atropine (1 μM), mepyramine (1 μM), methysergide (1 μM), and indomethacin (1 μM) were included in the bathing media for all experiments; (ii) the time cycle for administration of agonist doses was arranged such that there was a 7-min interval between doses in the guinea pig ileum and a 10-min interval in the rat colon. Pilot experiments had demonstrated that between dose interactions were minimized for SP when these schedules were used.

Formal 3 + 3 assays (guinea pig ileum) or 2 + 2 assays (rat colon) were performed on all analogues by using a randomized block design.[21,22] Results were analyzed by analysis of variance, and only those assays which satisfied tests for parallelism, linearity, difference of curvature, and regression were accepted for estimates of relative activities. All results are expressed as relative activities, taking SP_{1-11} as 1.0.

Samples of peptides were prepared in 0.01 M acetic acid or dimethyl-sulfoxide (the latter solvent being used for the less soluble analogues), at a stock concentration of 1-10 mM. This solution was divided into aliquots which were stored at -30 °C until required for use. A sample of solution was dried under nitrogen and prepared for amino acid analysis to provide an accurate estimate of peptide concentration. A further sample of each compound was subjected to FAB spectrometry to confirm its structure.

During the experiments, dilutions were made in physiological salt solution. Control experiments with Me_2SO showed that, at the concentrations used in the present experiments, this compound did not effect the response of the tissue to any of the peptides tested.

Acknowledgment. Thanks are due to Prof. K. Barlos for providing us with a sample of Boc-Thr(OCH_3)-OH. This work was partially supported by the 1984 Research Fund of the Royal Society of Chemistry. We wish to acknowledge Mrs. T. A. Crees for excellent technical assistance and Miss A. Ward and Mrs. A. Brett for typing the manuscript.

(21) Gater, P. R.; Jordan, C. C.; Owen, D. G. *Br. J. Pharmacol.* **1982**, *75*, 341.

[pGlu^6,Pro^9]SP_{6-11}, a Selective Agonist for the Substance P P-Receptor Subtype

Ralph Laufer,[†] Chaim Gilon,[‡] Michael Chorev,[§] and Zvi Selinger*[†]

Department of Biological Chemistry, Institute of Life Sciences, Departments of Organic Chemistry and Pharmaceutical Chemistry, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. *Received July 8, 1985*

Substitution of a single amino acid residue, proline for glycine-9 in [pGlu^6]SP_{6-11}, a hexapeptide analogue of substance P, confers on the peptide selective agonist activity toward the SP-P receptor subtype. [pGlu^6,Pro^9]SP_{6-11} had 20% and 75% of the activity of [pGlu^6]SP_{6-11} in stimulating, respectively, K^+ release from rat parotid slices and contraction of the isolated guinea pig ileum, via the SP-P receptor subtype. In contrast, [pGlu^6,Pro^9]SP_{6-11} had substantially reduced activity on SP-E systems such as the hamster urinary bladder and rat duodenum, being about 20-fold less potent than [pGlu^6]SP_{6-11} and 200-670-fold less potent than neurokinin B. In the guinea pig ileum [pGlu^6,Pro^9]SP_{6-11} had very low activity on the neuronal tachykinin receptor, being 325 times less potent than [pGlu^6]SP_{6-11} and 1000 times less potent than neurokinin B. Because of its discrimination between the muscular and neuronal receptors in the guinea pig ileum (muscular/neuronal potency ratio = 600), [pGlu^6,Pro^9]SP_{6-11} can be used to specifically desensitize the muscular receptor of this tissue. This procedure enables a selective and sensitive bioassay of the neuronal receptor.

Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is an undecapeptide that acts as a neurotransmitter/modulator in the mammalian peripheral and central nervous systems.[1,2] Substance P is part of a family of peptides known as tachykinins that share the common C-terminal sequence Phe-X-Gly-Leu-Met-NH_2.[3] This family includes the peptides eledoisin, physalaemin, and kassinin, which are all of nonmammalian origin.[3] Recently, two additional mammalian tachykinins were isolated and termed neurokinin A and neurokinin B.[4,5]

Although the tachykinins exert similar biological actions, their rank order of potencies varies between different pharmacological preparations, indicating that there may be more than one type of tachykinin receptor.[6,7] three main types of tachykinin receptors have been suggested to exist in the periphery, the SP-P, SP-E, and SP-N receptors.[7-9] While on SP-P systems such as the guinea pig ileum and the rat parotid[8-10] all tachykinins have similar potencies, SP-E systems such as the urinary bladder of hamster and mouse and the rat duodenum are characterized by a much higher potency of eledoisin, kassinin, neurokinin A, and neurokinin B than substance P and physalaemin.[8,9,11] In addition to the classical SP-P re-

[†] Department of Biological Chemistry.
[‡] Department of Organic Chemistry.
[§] Department of Pharmaceutical Chemistry.

(1) Nicoll, R. A.; Schenker, C.; Leeman, S. E. *Annu. Rev. Neurosci.* **1980**, *3*, 227.
(2) Pernow, B. *Pharmacol. Rev.* **1983**, *35*, 85.
(3) Erspamer, V. *Trends Neurosci.* **1981**, *4*, 267.
(4) Kimura, S.; Okada, M.; Sugita, Y.; Kanazawa, I.; Munekata, E. *Proc. Jpn. Acad. 59 Ser. B* **1983**, 101.
(5) Kangawa, K.; Minamino, N.; Fukuda, A.; Matsuo, H. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 533.
(6) Falconieri-Erspamer, G.; Erspamer, V.; Piccinelli, D. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1980**, *311*, 61.
(7) Lee, C. M.; Iversen, L. L.; Hanley, M. R.; Sandberg, B. E. B. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1982**, *318*, 281.
(8) Watson, S. P.; Sandberg, B. E. B.; Hanley, M. R.; Iversen, L. L. *Eur. J. Pharmacol.* **1983**, *87*, 77.
(9) Laufer, R.; Wormser, U.; Friedman, Z. Y.; Gilon, C.; Chorev, M.; Selinger, Z. *Proc. Natl. Acad. Sci. U.S.A.*, in press.
(10) Hanley, M. R.; Lee, C. M.; Jones, L. M.; Michell, R. M. *Mol. Pharmacol.* **1980**, *18*, 78.
(11) Nawa, H.; Doteuchi, M.; Igano, K.; Inouye, K.; Nakanishi, S. *Life Sci.* **1984**, *34*, 1153.

0022-2623/86/1829-1284$01.50/0

ceptor, which is located on the muscle cells, the guinea pig ileum contains also a neuronal tachykinin receptor that mediates the release of acetylcholine from cholinergic neurons of the myenteric plexus.[9,12,13] In contrast to the SP-E receptor subtype, this neuronal receptor is stimulated by neurokinin B more potently than by neurokinin A, kassinin, or eledoisin.[9] We therefore proposed to designate this receptor, whose endogenous ligand might be neurokinin B, "SP-N".[9]

The characterization of receptor subtypes, as well as studies of their functional role, would be facilitated by the availability of selective agonists for each type of receptor. Indeed, the finding of Watson et al.[8] that substance P methyl ester selectively acts on SP-P systems has provided strong support for the concept of multiple tachykinin receptors.[8,9] Since it has been shown that [pGlu^6]SP$_{6-11}$, a C-terminal hexapeptide analogue of substance P, is equipotent with the undecapeptide in SP-P systems,[14,15] this peptide has been widely used as parent compound in structure–activity studies.[15-17] We now report that [pGlu^6,Pro^9]SP$_{6-11}$, an analogue of [pGlu^6]SP$_{6-11}$ in which a proline residue replaces Gly^9, discriminates between two receptor subtypes in the same tissue preparation, the guinea pig ileum. [pGlu^6,Pro^9]SP$_{6-11}$ is a very potent and selective agonist for SP-P receptors, with very low potencies for the neuronal receptor in the guinea pig ileum, as well as for classical SP-E receptor subtypes.

Results

Peptide synthesis was based on the "excessive mixed carbonic-carboxylic acid anhydrides" method introduced by Tilak[18] and first employed for the synthesis of substance P by Beyerman et al.[19] The *tert*-butyloxycarbonyl group, used for N protection of the α-amino function, was removed by 1 N HCl in glacial acetic acid.[20] Analogue **10** was characterized by TLC, HPLC, amino acid analysis, potassium-cationized field-desorption mass spectrometry, and fast atom bombardment (FAB)/tandem mass spectrometry. These mass spectrometric procedures were recently found to be very helpful for the elucidation of structure and evaluation of purity of oligopeptides.[21,22]

A number of investigators have shown that tachykinins elicit contraction of the guinea pig ileum by a direct atropine-insensitive action on the smooth muscle and also indirectly by stimulating myenteric cholinergic neurons.[9,12,13,23] The potencies of tachykinins in stimulating the muscular receptors of the guinea pig ileum, which have been classified as SP-P receptors,[7] are usually determined in the presence of the muscarinic blocker atropine.[7-9,13,23] The activities on the neuronal receptors were determined after desensitization of the muscular receptors by pretreatment with the selective SP-P agonist substance P methyl ester. We have previously shown[9] that pretreatment of the ileum with a desensitizing dose of substance P methyl ester abolishes the response mediated via the muscular SP-P receptor, as demonstrated by the loss of contractile action elicited by substance P methyl ester, as well as by a 30-fold shift of the substance P dose–response curve. The tachykinin-induced contraction that remains intact after inactivation of the SP-P receptor was shown to be due to stimulation of neuronal receptors, since it is sensitive to atropine and tetrodotoxin. The neuronal tachykinin receptors ("SP-N") that mediate acetylcholine release from enteric neurons can thus be assayed following desensitization of the muscular ("SP-P") receptors with substance P methyl ester. This treatment does not affect the activity of SP-N receptors, as demonstrated by the unchanged dose–response curve to neurokinin B, the most potent SP-N agonist.[9]

Table I. Discrimination by [pGlu^6,Pro^9]SP$_{6-11}$ between Muscular and Neuronal Receptors in the Guinea Pig Ileum[a]

peptide	EC_{50}, nM: muscular receptor	EC_{50}, nM: neuronal receptor	potency ratio muscular/neuronal receptor receptor
[pGlu^6]SP$_{6-11}$	1.6	4	2.5
[pGlu^6,Pro^9]SP$_{6-11}$	2.2	1300	600

[a] EC_{50} values were determined in the presence of atropine, 10^{-6} M (muscular receptor), or substance P methyl ester, 10^{-7} M, 2-min preincubation (neuronal receptor). Results are mean values from two to five experiments; standard errors less than 25%.

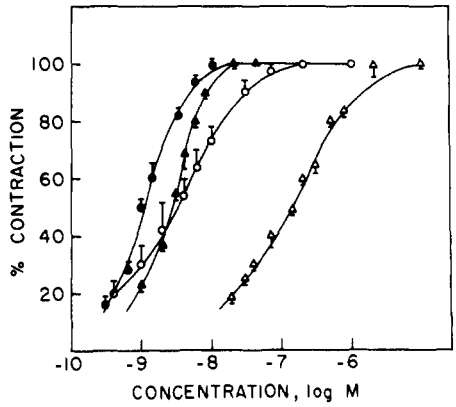


Figure 1. Contractile effects of substance P and neurokinin B in the guinea pig ileum, before and after pretreatment with [pGlu^6,Pro^9]SP$_{6-11}$. The guinea pig ileum preparation was pretreated with [pGlu^6,Pro^9]SP$_{6-11}$ (**10**) (2×10^{-7} M, 2 min) and not washed before application of a test dose of peptide. Dose–response curves were determined before (closed symbols) and after (open symbols) pretreatment. ▲, △) Substance P, (●, ○) neurokinin B.

As shown in Table I, analogue **10** is almost as potent as the parent compound, [pGlu^6]SP$_{6-11}$, in stimulating the SP-P receptor but 325 times less active than [pGlu^6]SP$_{6-11}$ in its action on the neuronal receptor. As expected for a selective agonist of the muscular receptor, the contractile action of analogue **10** (EC_{50} = 3 nm) is not affected by 10^{-6} M of atropine. Analogue **10** acts on the SP-P receptor 600 times more potently than on the neuronal receptor and thus clearly discriminates between different receptor subtypes in the guinea pig ileum.

Consequently, it was examined whether analogue **10** could be used instead of substance P methyl ester to specifically desensitize the muscular receptor in the guinea

(12) Yau, W. M.; Youther, M. L. *Eur. J. Pharmacol.* **1982**, *81*, 665.
(13) Fosbraey, P.; Featherstone, R. L.; Morton, I. K. M. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1984**, *326*, 111.
(14) Yanaihara, N.; Yanaihara, C.; Hirohashi, M.; Sato, H.; Iizuka, Y.; Hashimoto, T.; Sakagami, M. In *Substance P*; von Euler, U. S., Pernow, B., Eds.; Raven: New York, 1977; pp 22–44.
(15) Laufer, R.; Chorev, M.; Gilon, C.; Friedman, Z. Y.; Wormser, U.; Selinger, Z. *FEBS Lett.* **1981**, *123*, 291.
(16) Sandberg, B. E. B.; Lee, C. M.; Hanley, M. R.; Iversen, L. L. *Eur. J. Biochem.* **1981**, *114*, 329.
(17) Chorev, M.; Rubini, E.; Gilon, C.; Wormser, U.; Selinger, Z. *J. Med. Chem.* **1983**, *28*, 129.
(18) Tilak, M. A. *Tetrahedron Lett.* **1970**, 849.
(19) Beyerman, H. C.; Rammeloo, T.; Renirie, J. F. M.; Syrier, J. L. M.; Zon, A., *Recl. Trav. Chim. Pays-Bas* **1976**, *95*, 143.
(20) Merrifield, R. B. *J. Am. Chem. Soc.* **1964**, *86*, 304.
(21) Deutsch, J.; Gilon, C.; Chorev, M. *Int. J. Pept. Protein Res.* **1981**, *18*, 203.
(22) Eckart, K.; Schwarz, H.; Chorev, M.; Gilon, C. *Biochemistry*, submitted for publication.
(23) Costa, M.; Furness, J. B.; Pullin, C. O.; Borstein, J. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1985**, *328*, 446.

Table II. Biological Activities of Mammalian Tachykinins and Synthetic Analogues[a]

	relative potency				
	SP-P systems			SP-E systems	
test peptide	K^+ release from rat parotoid slices	guinea pig ileum	SP-N system: Guinea pig ileum	hamster bladder	rat duodenum
neurokinin B	ND	1	1	1	1
neurokinin A	ND	0.12	0.04	1	0.30
substance P	1	1.0	0.03	0.010	0.054
$[pGlu^6]SP_{6-11}$	1.0	1.5	0.38	0.035	0.083
$[pGlu^6,Pro^9]SP_{6-11}$	0.2	1.1	0.001	0.0015	0.005

[a] Relative potency (neurokinin B = 1, except for rat parotid assay: $[pGlu^6]SP_{6-11}$ = 1) is defined as the ratio of EC_{50} of neurokinin B (or $[pGLu^6]SP_{6-11}$): EC_{50} of peptides tested. Potencies on the guinea pig ileum SP-P and SP-N receptors were determined in the presence of atropine (10^{-6} M) or substance P methyl ester (10^{-7} M, 2-min preincubation), respectively.[9] Results are mean values of relative potencies calculated in two to ten experiments; standard errors less than 30%. EC_{50} values for neurokinin B (nM): guinea pig ileum (SP-P), 2.4; (SP-N), 1.5; hmaster bladder: 50; rat duodenum: 6.5. EC_{50} value for $[pGlu^6]SP_{6-11}$, rat parotid slices: 40 nM. ND, not determined.

pig ileum and thus constitute a new tool for the selective assay of the neuronal receptor. After pretreatment of the guinea pig ileum with a high concentration of analogue **10** (2×10^{-7} M, 2 min), the preparation became insensitive to further application of this peptide. The EC_{50} value for analogue **10** was increased from 3 nM to greater than 1 μM, indicating that the SP-P receptor had been inactivated by desensitization. Under these conditions, no contraction could be obtained even with a high concentration (10^{-6} M) of substance P methyl ester. The inactivation of the SP-P receptor is also reflected in the right-shift of the substance P dose–response curve (Figure 1). The contractile activity of the ileum that remains after pretreatment with analogue **10** is due to release of acetylcholine from enteric neurons: Atropine (10^{-6} M) totally inhibited the contractile response to substance P (10^{-7} M) and neurokinin B (10^{-7} M) measured in the presence of analogue **10** (10^{-7} M). This further demonstrates inactivation of the SP-P receptor, which is atropine insensitive. Pretreatment with analogue **10** does not, however, affect the activity of the neuronal SP-N receptor, as shown by the almost unchanged effect of neurokinin B in the presence of analogue **10** (Figure 1).

Following desensitization of the muscular receptor with analogue 10, the following relative potencies were obtained for the neuronal receptor: neurokinin B, defined as 1; substance P, 0.02; analogue **10**, <0.004. (For absolute potencies, see Figure 1.) These results are similar to the relative potencies for the SP-N receptor that were obtained after inactivation of the muscular receptor with substance P methyl ester (see Table II). It seems therefore that analogue **10** can be used instead of substance P methyl ester as a tool for the selective assay of the neuronal SP-N receptor in the guinea pig ileum.

Table II summarizes the biological activites of analogue **10** and of its parent compound $[pGlu^6]SP_{6-11}$, as well as those of mammalian tachykinins, in SP-P, SP-N, and SP-E systems. Potencies are expressed relative to that of neurokinin B, which potently stimulates all three receptor subtypes. The high potency of analogue **10** on the SP-P receptor is further demonstrated in the rat parotid slice system, where it is only slightly less active than $[pGlu^6]SP_{6-11}$ in causing K^+ release. However, in SP-E systems, analogue **10** is a weak agonist, about 20-fold less active than $[pGlu^6]SP_{6-11}$ and 200 and 670 times less potent than neurokinin B in contracting the isolated rat duodenum and hamster urinary bladder, respectively. Moreover, at concentrations up to 40 μM, analogue **10** was without effect in another SP-E system,[7] the electrically stimulated rat vas deferens.

Discussion

Substitution of a proline residue for the Gly^9 residue of $[pGlu^6]SP_{6-11}$ gives rise to a potent and selective hexapeptide agonist of the SP-P receptor subtype. Analogue **10** possesses activities comparable to those of $[pGlu^6]SP_{6-11}$ and substance P in causing contraction of the guinea pig ileum, as well as K^+ release from rat parotid slices. It is noteworthy that the free hexapeptide analogue $[Pro^9]SP_{6-11}$ was recently reported to possess very low activities (233 and 40 times less than substance P) in the guinea pig ileum and the in vivo rat salivation assay, respectively.[24] The striking difference in the potencies of these two compounds seems to be due to the enhancing effect of the (pyroglutamyl)[6] residue on activity, as has been previously reported.[25]

The finding of Watson et al.[8] that substance P methyl ester is a selective agonist for the SP-P receptor is among the strongest experimental supports for the classification of substance P receptors into SP-P and SP-E subtypes. The present finding of a second selective agonist with a considerably different structure lends further support to this classification.

The ability of analogue **10** to discriminate between the muscular and the neuronal receptors in the guinea pig ileum (with a potency ratio of 600) enables its use for selective desensitization of the SP-P receptor in this tissue, thus providing an easy and specific bioassay procedure for the neuronal receptor. The close similarity of results obtained in this bioassay, using either substance P methyl ester or analogue **10** for desensitization of the SP-P receptor, further strengthens the concept of two different receptor subtypes in the guinea pig ileum. It should be pointed out that the guinea pig ileum is a complex system[23,26] and that it might contain more than two tachykinin receptor subtypes. Thus, it has been proposed that the atropine-insensitive response to tachykinins, classically attributed to SP-P receptors,[7,8] might be mediated by both SP-P and SP-E receptor subtypes.[27] In the present work, analogue **10** was tested by functional bioassays of the three tachykinin receptors described so far: SP-P (rat parotid, guinea pig ileum), SP-E (rat vas deferens, hamster urinary bladder, rat duodenum), SP-N (enteric nerves of guinea pig ileum). Agonist **10** was found to be a potent agonist with high selectivity for SP-P systems only. However, it cannot be excluded that the atropine-insensitive contraction of the guinea pig ileum elicited by analogue **10** could be mediated by other, as yet undefined, tachykinin re-

(24) Piercey, M. F.; Dobry-Scheur, P. J. K.; Masiques, N.; Schroeder, L. A. *Life Sci.* **1985**, *36*, 777.
(25) Chorev, M.; Gilon, C.; Wormser, U.; Friedman, Z. Y.; Selinger, Z. In *Proceedings of the 16th European Peptide Symposium*; Brunfeld, K., Ed.; Scriptor: Copenhagen, 1981; pp 451–456.
(26) Regoli, D.; D'Orléans-Juste, P.; Escher, E.; Mizrahi, J. *Eur. J. Pharmacol.* **1984**, *97*, 161.
(27) Buck, S. H.; Burcher, E.; Shults, C. W.; Lovenberg, W.; O'Donohue, T. L. *Science (Washington, D.C.)* **1984**, *226*, 987.

ceptor subtypes, or by a subclass of SP-P receptors.

The selectivity of analogue **10** for classical SP-P systems[7,8] is probably the result of its preference for the SP-P receptor subtype rather than due to differences in metabolism or access to receptor sites between the different tissues. This is evident from the discrimination between muscular and neuronal receptors in the guinea pig ileum, which was determined in the same tissue preparation. Moreover, analogue **10** is likely to be metabolically more stable than substance P for the following reasons: We have previously shown that [pGlu^6]SP$_{6-11}$ is more resistant to degradation in vitro than substance P, perhaps due to the absence of a free amino terminus;[28] analogue **10** also contains the blocked pyroglutamyl residue at its amino terminal end. Analogue **10** might be even more stable than [pGlu^6]SP$_{6-11}$ because of the stabilizing effect of N-alkylation toward degradation by substance P degrading enzymes.[15,16] Thus, the lower activity of analogue **10** on SP-N and SP-E systems as compared to substance P and [pGlu^6]SP$_{6-11}$ is probably due to the detrimental effect of the Pro^9 substitution on receptor recognition.

As a pharmacological tool for studies of the SP-P receptor, analogue **10** presents some advantages over the undecapeptide substance P methyl ester. The absence of a hydrophilic amino-terminal sequence renders this peptide very hydrophobic and therefore more likely to pass the blood–brain barrier. Furthermore, analogue **10** is probably less prone to attack by proteases and esterases than substance P methyl ester. It is noteworthy that [pGlu^6]SP$_{6-11}$ and [pGlu^5]SP$_{5-11}$ have been used as parent compounds in a series of metabolically stable analogs of substance P.[15-17] Therefore, [pGlu^6,Pro^9]SP$_{6-11}$ might serve as the parent structure for the development of metabolically stable and selective agonists for the SP-P receptor.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer P141 photoelectric polarimeter or with a Cary 60 spectropolarimeter. HPLC analysis was performed on a Tracor 950 liquid chromatograph equipped with a Tracor 970A variable-wavelength detector. An Alltech C-18, 10-μm column (4.6 mm i.d. × 25 cm) was used. Compounds were detected at 210 nm. TLC was performed on precoated silica gel plastic plates: Polygram Sil NH-R/UV 254 purchased from Mackerey-Nagel Co. The following solvent systems were used: (A) $CHCl_3$/MeOH (9:1), (B) $CHCl_3$/MeOH (1:1), (C) $CHCl_3$/MeOH/AcOH (17:2:1), (D) *n*-BuOH/AcOH/H_2O (5:3:1), (E) $CHCl_3$/MeOH/AcOH/H_2O (17:2:1:0.3), (F) EtOAc/hexane (1:1). Peptides were visualized by ninhydrin spray (Merck), fluorescamine (Fluram, Hoffman La Roche & Co, AG), and iodine vapors. Amino acid analysis was performed on a LKB-4400 amino acid analyzer equipped with a Spectra-Physics SP-4100 printer–plotter computing integrator. Hydrolysis of peptide samples for amino acid analysis was carried out on 1-mg samples in constant-boiling HCl for 20 h at 110 °C in vacuum-sealed tubes. Elemental microchemical analysis was carried out by Dr. S. Blum and her staff at the Microanalytical Laboratory of the Chemistry Institute. Potassium-cationized field-desorption mass spectrometry and fast atom bombardment mass spectrometry were performed respectively by Dr. J. Deutsch, Department of Pharmaceutical Chemistry, The Hebrew University of Jerusalem, and Drs. K. Eckart and H. Schwarz, Technical University of Berlin, as described elsewhere.[21,22] DMF was distilled from benzoic anhydride under reduced pressure and then redistilled from ninhydrin. Chloroform was distilled from P_2O_5.

Peptide Coupling Procedure. To a stirred solution of Boc-amino acid or Boc-peptide (1.2–1.4 equiv) in DMF, an equivalent of *N*-methylmorpholine (NMM) was added. The mixture was cooled to −20 °C and kept under nitrogen. Isobutyl chloroformate (IBCF) (1.2–1.3 equiv) was added and allowed to react for 2 min. A precooled solution of amino component hydrochloride (1.0 equiv) in DMF was added, followed by NMM (1.0 equiv). After completion of the reaction (1–2 h, monitored by Fluram), the temperature was allowed to reach 0 °C, and the reaction mixture was treated with 2 M $KHCO_3$ (3.0 equiv) for 30 min. (a) Addition of saturated aqueous NaCl solution (4 × volume of DMF) resulted in precipitate formation. The precipitate was collected and washed with water, until washings were free of Cl^-. The precipitate was dried over P_2O_5 in vacuo. (b) If a precipitate did not form after addition of NaCl solution to the reaction mixture, the latter was extracted with ethyl acetate. The organic phase was washed consecutively with a saturated aqueous NaCl solution, 0.5 N $KHSO_4$, NaCl solution, 5% $NaHCO_3$, and NaCl solution. It was then dried over $MgSO_4$, filtered, and taken to dryness under reduced pressure.

Deprotection. Boc-protected peptides (1 g/50 mL) were treated with 1 N HCl in glacial acetic acid[19] for 30 min at room temperature. The residue obtained after removal of solvent was dried overnight under vacuum, over KOH pellets. The product was then triturated with ether.

Boc-Leu-Met-OMe (1). Boc-Leu-OH (12 g, 48.1 mmol), dissolved in DMF (65 mL), was reacted with IBCF (5.7 mL, 40 mmol) in the presence of NMM (6.4 mL, 48.1 mmol). A solution of HCl·Met-OMe (8 g, 40 mmol) in DMF (80 mL) was added, followed by NMM (4.4 mL, 40 mmol). Treatment of the reaction mixture according to general procedure (a) yielded 13.2 g (88%) of **1**; mp 101–102 °C; $[\alpha]^{25}_D$ −31.7° (*c* 1.0, EtOH) [lit.[29] mp 97–99 °C, $[\alpha]^{22}_D$ −36.5° (*c* 1.0, EtOH)]; TLC R_f (F) 0.38. Anal. ($C_{17}H_{32}N_2O_5S$) C, H, N.

Boc-Leu-Met-NH$_2$ (2). A solution of **1** (12 g, 32 mmol) in 5 M NH_3 in methanol was allowed to stand at room temperature for 20 h. The reaction mixture was filtered, the solvent was evaporated under reduced pressure, and the product was washed with ether and dried over concentrated H_2SO_4 in vacuo. Yield: 10 g (87%) of **2**; mp 156–158 °C; $[\alpha]^{25}_D$ −33.2° (*c* 1.0, DMF) [lit.[29] mp 154–155 °C; $[\alpha]^{22}_D$ −35.1° (*c* 1.0, DMF)]; TLC R_f (A) 0.56; R_f (C) 0.65. Anal. ($C_{16}H_{31}N_3O_4S$) C, H, N.

HCl·Leu-Met-NH$_2$ (3). Compound **2** (8 g, 22.1 mmol) was deprotected according to the general procedure. The crude product was recrystallized from methanol/ether. Yeild: 6.0 g (90%); mp 189–190 °C; $[\alpha]^{25}_D$ +9.8° (*c* 1.0, H_2O) [lit.[29] mp 126–127 °C; $[\alpha]_D$ +10.2 (*c* 1.0, H_2O)]; TLC R_f (B) 0.48. Anal. ($C_{11}H_{23}N_3O_2S$) C, H, N.

Boc-Pro-Leu-Met-NH$_2$ (4). Boc-Pro-OH (1.2 g, 5.6 mmol), dissolved in DMF (3 mL), was reacted with IBCF (0.63 mL, 5.6 mmol) in the presence of NMM (0.7 mL, 5.3 mmol). A solution of the amino component **3** (1.2 g, 4.0 mmol) in DMF (7 mL) was added, followed by NMM (0.5 mL, 4.0 mmol). Workup according to general procedure (b) yielded a crude product, which was recrystallized from ethyl acetate/methanol. Yield: 1.1 g (62%); mp 191 °C; $[\alpha]^{25}_D$ −86.8° (*c* 1.0, AcOH); TLC R_f (B) 0.75. Anal. ($C_{21}H_{38}N_4O_5S$) C, H, N.

HCl·Pro-Leu-Met-NH$_2$ (5). Compound **4** (1 g, 2.2 mmol) was deprotected according to the general procedure. Yield: 0.82 g (95%); mp 216–217 °C; $[\alpha]^{25}_D$ −72° (*c* 1.0, AcOH); TLC R_f (D) 0.30.

Boc-Phe-Pro-Leu-Met-NH$_2$ (6). Boc-Phe-OH (0.5 g, 1.82 mmol), dissolved in DMF (3 mL), was reacted with IBCF (0.23 mL, 1.74 mmol) in the presence of NMM (0.2 mL, 1.82 mmol). A solution of the amino component **5** (0.6 g, 1.52 mmol) in DMF (3 mL) and hexamethylphosphoric acid triamide (5 mL) was added, followed by NMM (0.16 mL, 1.52 mmol). The reaction mixture was treated according to method (b). Yield: 0.76 g (82%); mp 99–102 °C; $[\alpha]^{25}_D$ −67.3 (*c* 1.0, AcOH); TLC R_f (B) 0.77. Anal. ($C_{30}H_{47}N_5O_6S$) C, H, N.

HCl·Phe-Pro-Leu-Met-NH$_2$ (7). Compound **6** was deprotected according to the general procedure. Yield: 0.58 g (89%); mp 130–133 °C; TLC R_f (D) 0.54. Anal. ($C_{25}H_{39}N_5O_4S$) C, H, N.

Boc-Phe-Phe-Pro-Leu-Met-NH$_2$ (8). Boc-Phe-OH (0.15 g, 0.55 mmol), dissolved in DMF (1 mL), was reacted with IBCF

(28) Wormser, U.; Chorev, M.; Gilon, C.; Laufer, R.; Friedman, Z. Y.; Selinger, Z. *Biochim. Biophys. Acta* **1984**, *798*, 28.

(29) Lübke, K.; Schröder, E.; Schmiechen, R.; Gibian, H. *Liebigs Ann. Chem.* **1964**, *679*, 195.

(0.07 mL, 0.53 mmol) in the presence of NMM (0.06 mL, 0.55 mmol). A solution of the amino component **7** (0.25 g, 0.46 mmol) in DMF (2 mL) was added, followed by NMM (0.05 mL, 0.46 mmol). Workup according to general procedure (b) afforded a crude product, which was purified by chromatography on a column (2.5 × 50 cm) loaded with silica gel (Merck, 70–230 mesh) and eluted with $CHCl_3$/MeOH (95:5). The product obtained from chromatography was recrystallized from EtOAc–petroleum ether. Yield: 0.17 g (48%); mp 125–126 °C; $[\alpha]^{25}_D$ –61.8° (*c* 0.5, AcOH); TLC R_f (A) 0.59. Anal. ($C_{39}H_{56}N_6O_7S$) C, H, N.

HCl·Phe-Phe-Pro-Leu-Met-NH_2 (9). Compound **8** was deprotected according to the general procedure and the resulting product **9** (0.11 g, yield: 95%) was used without further characterization.

pGlu-Phe-Phe-Pro-Leu-Met-NH_2 (10). pGlu-OH (0.027 g, 0.21 mmol), dissolved in DMF (0.5 mL), was reacted with IBCF (0.023 mL, 0.17 mmol) in the presence of NMM (0.024 mL, 0.21 mmol). A solution of the amino component **9** (0.090 g, 0.13 mmol) in DMF (0.5 mL) was added, followed by NMM (0.015 mL, 0.13 mmol). Workup according to general procedure (a) yielded 0.02 g (20%) of (10); mp 118–121 °C. The product was pure in HPLC (H_2O/MeOH, 30:70, elution rate 1.5 mL/min, detection at 210 nm). TLC R_f (A) 0.32, R_f (B) 0.63; MS, *m/e* 764 $(M)^+$; FAB-MS, *m/e* 764 $(M)^+$, 765 $(M + H)^+$. Amino acid analysis: Glx 0.95, Phe 1.90, Pro 0.85, Leu 1.00, Met 0.95 (calcd: Glx 1.0, Phe, 2.0, Pro 1.0, Leu 1.0, Met 1.0).

Biological Assays. Materials. Substance P and substance P methyl ester were purchased from Sigma, St. Louis, MO. Neurokinin A and neurokinin B were obtained from Cambridge Research Biochemicals, Harston, U.K. Neurokinin A was also purchased from Peninsula San Diego, CA. $[pGlu^6]SP_{6-11}$ was synthesized as described elsewhere.[17,25]

Isolated Smooth Muscle Preparations. Isolated smooth muscle preparations were suspended in a 10-mL organ bath containing Tyrode's solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 1.0, $NaHCO_3$ 25, and glucose 10), gassed with a 95% O_2–5% CO_2 mixture and maintained at 34 °C. Contractions were recorded isotonically. Peptides were applied at 2–3-min intervals with less than 30-s contact time (guinea pig ileum/rat duodenum) or at 15–30-min intervals with a contact time of 1–3 min (hamster urinary bladder). Potentiation of electrically stimulated rat vas deferens contraction was determined as described by Lee et al.[7] At the beginning of a desensitization experiment, the guinea pig ileum was first incubated with a high dose (10^{-7} M) of agonist until the response had faded to the base-line level (2–4 min). The tissue was then washed and immediately reincubated with the agonist (10^{-7} M) for 2 min. The contraction caused by a test peptide was then recorded. All test peptides produced similar maximal contractions in a given test preparation. Relative potencies were calculated from EC_{50} values (concentration of agonist producing 50% of the maximal contraction).

K^+ Release from Rat Parotid Slices. This assay was performed as described before.[15]

Registry No. 1, 2280-69-5; **2**, 2280-68-4; **3**, 2131-00-2; **4**, 101760-39-8; **5**, 101760-44-5; **6**, 101760-40-1; **7**, 101760-41-2; **8**, 101760-42-3; **9**, 101760-43-4; **10**, 79775-19-2; Boc-Leu-OH, 13139-15-6; HCl·Met-OMe, 2491-18-1; Boc-Pro-OH, 15761-39-4; Boc-Phe-OH, 13734-34-4; pGlu-OH, 98-79-3.

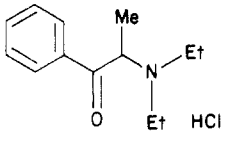
(*S*)-3-[(Benzyloxy)methyl]morpholine Hydrochloride: A Nonstimulant Appetite Suppressant without Conventional Neurotransmitter Releasing Properties

George R. Brown,*† Alan J. Foubister,† Gillian Forster,‡ and Donald Stribling*‡

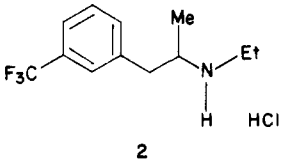
Chemistry and Bioscience Departments, ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire SK10 4TG, England. *Received August 6, 1985*

The synthesis and appetite-suppressant activity of (*S*)-3-[(benzyloxy)methyl]morpholine hydrochloride (**3**) in dogs are reported. The oral ED_{50} for appetite suppression in dogs of **3** was 12 mg/kg, and it was tolerated up to 200 mg/kg. **3** had no inhibitory effect on the release or uptake of noradrenaline, dopamine, or serotonin at 10^{-5} M. The (*R*) enantiomer (**4**) of **3** was not anorexiant.

Obesity, which affects some 30% of the adult population of the developed nations of the world, is a major risk factor for cardiovascular disease and non-insulin-dependant diabetes mellitus,[1] as well as creating social problems for the patient. Therapies for obesity range from slimming clubs, low-calorie diets, and behavioral modification to appetite suppressants and surgery but are in general ineffective. Obesity develops when food intake exceeds energy expenditure. For this reason the first choice for treatment is to prescribe a calorie-restricted diet. Generally two phases of weight loss occur: a short period of rapid loss related to fluid depletion followed by a much slower but significant rate of weight reduction. During this second period of treatment the patient may benefit from an appetite suppressant agent to assist with diet compliance. Currently used appetite suppressants such as amphetamines have serious side effects, including stimulant and cardiovascular actions.[2] This type of appetite suppressant, of which diethylpropion (**1**) is an example, is thought to exert its effect on food intake by acting through adrenergic and dopaminergic mechanisms at the feeding center in the hypothalamus.[3] Fenfluramine (**2**) does not depend on catecholamines for its appetite suppressant effect but is serotoninergic, giving the different side-effect profile of sedation and gastrointestinal actions. This involvement of neurotransmitters in the mechanisms of appetite sup-



1



2

† Chemistry Department.
‡ Bioscience Department.

(1) Salams, L. B. *Metabolic Control and Disease*, 8th ed.; Bondy, P. K., Rosenberg, L. E., Eds.; W. Saunders: Philadelphia, 1980; p 608.
(2) Bowman, W. C.; Rand, M. J. *Textbook* of Pharmacology, 2nd ed.; Blackwell Scientific Publications: Oxford, 1980; p 43.36.

0022-2623/86/1829-1288$01.50/0