Structure–Activity Studies on the C-Terminal Amide of Substance P[1]

Emanuel Escher,*[†] Réjean Couture,[†] Constantinos Poulos,[‡] Nikos Pinas,[‡] Jacques Mizrahi,[†] Dimitrios Theodoropoulos,[‡] and Domenico Regoli[†]

Department of Organic Chemistry, University of Patras, Patras, Greece, and Department of Physiology and Pharmacology, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada. *Received January 22, 1982*

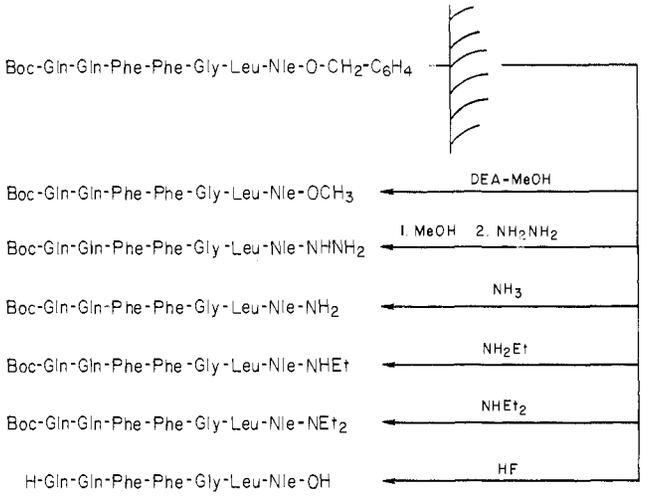
Twelve C-terminal heptapeptide analogues of substance P have been synthesized by solid phase and by the classical solution method. The modifications concerned all the C-terminal primary amide of SP and should therefore help to understand the biological significance of this carboxamide, as evaluated by in vivo and in vitro bioassays. From the results it can be seen that not the slightest change of the two amide protons is tolerated without an important loss of activity: replacement of one or two amide protons with alkyl groups, extension of the amide to the hydrazide and its alkyl analogues, and exchange of the amide with an ester or a carboxylic acid all reduce the relative activity/affinity at least by 2-fold. It is not clear for what reason all these modifications produce such a drastic activity reduction.

In a previous study[2] we have investigated the biological importance of the primary amides on the glutamine residues in positions 5 and 6 of substance P (SP, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2). No special function could be found for the side-chain amide in position 5, while in position 6 some increase of the in vivo biological activity was observed with more lipophilic amide substitutions. Moreover, all attempts to remove the carboxy terminal Met-NH_2 resulted in peptides devoid of SP-like activity.[3] The only modifications that were tolerated were replacements of Met by Nle, Ala, and Leu, which, however, produced variable loss of activity. Ala^{11}-SP[4] retains about 10–20% of affinity, compared to SP, and Leu^{11}-SP retains 26%[5] and Nle^{11}-SP retains 50% of affinity, according to the most recent findings.[6] The replacement of the carboxamide group by a carboxylate results in an analogue almost inactive in all bioassay,[4,7,8,9] but the methyl ester analogue of SP is reported with considerable but somewhat divergent biological activities on the same bioassays.[8,10]

We therefore decided to carry out a more detailed analysis of the C-terminal amide in order to learn more about the significance of this amide and its contribution to the hormone–receptor interaction. The C-terminal amide was modified in two independent series of peptides, one derived from the heptapeptide SP(5–11) and the other from Nle^{11}-SP(5–11). In both series the primary amide was mono- and dialkylated, replaced by the carboxylate and by an ester, and extended as a hydrazide. The biological activities were established with the two standard tests, in vivo, the hypotensive effect on the rat blood pressure, and in vitro on guinea pig ileum. Some of these compounds have been already preliminarily communicated.[11]

Syntheses. The C-terminal analogues of Met^{11}-SP(5–11) were synthesized by the classical solution method using a stepwise chain elongation from the C terminal combined with fragment couplings. The analogues of Nle^{11}-SP(5–11) have been prepared by the solid-phase method; the protected heptapeptide sequence was built up on chloromethylated polystyrene resin, and, according to the desired peptide, different methods were used for the peptide–resin cleavage (see Scheme I). All peptides were purified by gel filtration and partition chromatography and were identified with the standard analytical procedures. The purity and identity of the products from the classical solution method were shown by amino acid analysis, elementary analysis of products and fragments, and by TLC. The products from the solid-phase synthesis were tested by amino acid analysis, TLC, HPLC, and reversed-phase

Scheme I



TLC. The identity of the peptides was mainly shown by the reversed-phase TLC, which showed large differences between, for example, the methyl ester **9** and the amide

[†] University of Patras.
[‡] University of Sherbrooke.

(1) Symbols and abbreviations are in accordance with the recommendation of the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 977 (1971)]. Other abbreviations used are: SP; DMF, dimethylformamide; THF, tetrahydrofuran; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; DCHA, dicyclohexylamine; ONP, *n*-nitrophenol; DEA, diisopropylethylamine; TEA, triethylamine; NMM, *N*-methylmorpholine.
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Table I. Biological Activities of C-Terminally Modified Substance P(5–11) Analogues[a]

no.	C terminal modification of SP(5–11)	rat blood pressure: in vivo		guinea pig ileum: in vitro			
		RBP	n	pD_2	RA	α_E	n
1	Met^{11}-NH_2	100	97	8.77	100	1	108
2	Met^{11}-OCH_3	1.0	7	7.30	3.37	1	10
3	Met^{11}-$NHCH_3$	5	7	6.77	1	(0.85)	9
4	Met^{11}-$N(CH_3)_2$	0.2	5		<0.01		10
5	Met^{11}-$NHNH_2$	0.4	9	6.80	1	1	10
6	Met^{11}-$NHN(CH_3)_2$	<0.05	5	6.60	0.53		10
7	Nle^{11}-NH_2	14	8	7.93	14.5	1	16
7	Nle^{11}-NH_2	100	8	7.93	100	1	16
8	Nle^{11}-OH	1.5	8	6.34	2.6	1	8
9	Nle^{11}-OCH_3	1.5	8	6.47	3.5	1	8
10	Nle^{11}-$NHCH_2CH_3$	0.3	7	5.37	0.3		7
11	Nle^{11}-$N(CH_2CH_3)_2$	0.2	6	6.03	1.3	1	8
12	Nle^{11}-$NHNH_2$	5.2	6	6.63	5.0	1	8

[a] RBP is the relative blood pressure potency in percent; n is the number of experiments; RA is the relative affinity in percent; pD_2 is the negative log of the dose which causes half-maximal contraction; α_E is the intrinsic activity. The relative potencies of 8–12 are expressed in percent compared to 7 (lower half), which itself is compared to 1 (upper half). Standard errors are maximally 20% of each RA or RBP value and are omitted for the sake of clarity.

7. Also, the increase of lipophilicity from peptide **7** to **10** to **11** is well demonstrated with this technique (see Table II).

All peptides were tested in the standard bioassays, the guinea pig ileum and the rat blood pressure. The biological data are expressed by the intrinsic activity (α_E), the affinity (pD_2), the relative affinity (RA) as defined earlier,[12] and the relative blood pressure potency (RBP) as shown in Table I.

It can be seen immediately that any modification of the primary amide lowers the relative affinity/potency by at least 20-fold. Full sequence modification should produce similar biological effects with the same modification on shorter but active fragments: The Nle^{11} modification of SP results in a 50% weaker analogue; the same modification on the C-terminal heptapeptide retains only 14% in both bioassays. This difference seems acceptable and has been reported to be even stronger on the pentapeptide fragment.[13] It is, however, difficult to understand why the methyl ester analogues of the heptapeptide sequences (**2** and **9**) have 30- to 100-fold reduced activity compared to the undecapeptide methyl ester.[8,10]

All modifications of the primary amide either increase the size or introduce a charge and/or prevent the formation of hydrogen bridges with the amide protons. Several rationalizations can be made in an attempt to explain these properties: (A) The binding locus of the amide on the SP receptor does not accept anything larger than the primary amide. In this case not even the carboxylate is accepted because of its ionic character at or near physiologic pH and the ensuing hydrate shell. (B) Both amide hydrogens are participating in some interactions with the receptor and/or intramolecularily with some carboxy of the peptide, which is our favorite model: In a recently proposed model[14] for the SP conformation, it was shown that in one form the C-terminal amide favorably hydrogen bonds simultaneously to the Phe^7 and Phe^8 carbonyls. Already monoalkylation would breakup such a conformation.

From the presented data it can be concluded that on SP heptapeptide no modification of the C-terminal amide is tolerated on the guinea pig ileum and rat blood pressure bioassays. The important differences between the SAR of SP heptapeptide and undecapeptide cannot be explained easily if the same mode of action is accepted for both. Due to several independent studies on SP undecapeptide methyl ester and due to the identical results of the two series of peptides obtained on totally different ways for this study, no simple experimental error can be credited for this unexplained discrepancy.

Experimental Section

All amino acids are of the L configuration. Capillary melting points are determined on a Büchi SMP-20 apparatus and are reported uncorrected. Optical rotations were measured with a Carl Zeiss precision polarimeter (0.005°). For TLC, loads of 10–15 g were applied to precoated plates of silica gel, F 254 (E. Merck or Riedel de Haen), and were developed until the solvent ascended 10–15 cm. The following systems (all V/V) were used for TLC: (A) 1-butanol–acetic acid–water (4:1:1), (B) chloroform–methanol–acetic acid (95:5:3), (C) 1-butanol–acetic acid–water–pyridine (30:6:24:20), (D) 1-butanol–pyridine–water (20:10:11), (E) 1-butanol–acetic acid–water (4:1:5) upper phase, (F) 1-butanol–acetic acid–water (5:2:3), (RP) reversed-phase TLC on KC 18 precoated plates (Whatman) eluted with 30% acetonitrile–7% 2-propanol in 0.5 M $AcONH_4$ at pH 7. The products were detected by UV, chlorination, followed by *o*-tolidine and ninhydrin. Compounds **7–12** were also tested analytically on isocratic HPLC (C_{18} reversed-phase, Bio-Rad, eluted with 20% acetonitrile–2.5% 2-propanol in 0.25 M $AcONH_4$ at pH 5.0); all products produced single peaks with the same order of retention as observed on RP TLC KC18.

Boc-Met-N(CH_3)$_2$. To a solution of Boc-Met-OH·DCHA (4.30 g, 10 mmol) in THF (30 mL) cooled to −10 °C was added ethyl chloroformate (1.09 g, 10 mmol). After 2 min, a solution of dimethylamine hydrochloride (2.43 g, 30 mmol) in 10 mL of THF–H_2O (7:3, v/v) was neutralized with TEA (3.03 g, 30 mmol) and mixed immediately with the anhydride as previously described.[15] The product was an oil (2.34 g, 85%): TLC R_f (A) 0.71, R_f (B) 0.92; IR ν_{max} (Nujol) 3300 (NH), 1710 (CO), 1655 (CO) cm^{-1}; NMR ($CDCl_3$) δ 6.0 (1 H, d, NH, J = 9 Hz), 3.0 [6 H, d, $N(CH_3)_2$, J = 10 Hz], 2.0 (3 H, 5, SCH_3), 1.40 [9 H, s, $C(CH_3)_2$].

Boc-Leu-Met-N(CH_3)$_2$. A portion of Boc-Met-N(CH_3)$_2$ (1.8 g, 6.5 mmol) was deprotected with 1.75 N HCl in CH_3COOH (15 mL). The resulting HCl salt was dissolved in CH_2Cl_2 (15 mL) and neutralized with NMM. This was added to a solution of Boc-Leu-OH·H_2O (1.61, 6.5 mmol) and DCC (1.34 g, 6.5 mmol) in CH_2Cl_2 (15 mL) precooled at 0 °C. The reaction mixture was left at 0 °C for 2 h and for an additional 24 h at room temperature. The precipitated DCU was filtered, and the filtrate was washed

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Table II. Analytical Data for Gln-Gln-Phe-Phe-Gly-Leu-X[a]

no.	X	formula	M_r	yield, %	TLC R_f (system)	amino acid analysis					
						Glx	Phe	Gly	Leu	Met	Nle
1	Met-NH_2	$C_{41}H_{60}O_9N_{10}S\cdot C_2H_4O_2$	929.11		0.71 (C), 0.45 (F), 0.46 (RP)						
2	Met-OCH_3	$C_{42}H_{61}O_{10}N_9S\cdot HCl$	920.54	60	0.66 (C), 0.47 (A), 0.40 (E)	2.01	1.95	1.00	1.04	0.88	
3	Met-$NHCH_3$	$C_{42}H_{62}O_9N_{10}S$	883.09	40–50	0.80 (B), 0.17 (A), 0.14 (E)	1.94	2.00	1.00	0.96	0.87	
4	Met-$N(CH_3)_2$	$C_{43}H_{64}O_9N_{10}S$	897.12	40–50	0.64 (C), 0.28 (A), 0.20 (E)	2.02	2.06	1.00	0.98	0.92	
5	Met-$NHNH_2$	$C_{41}H_{61}O_9N_{11}S\cdot 2HCl$	957.00	62	0.67 (C), 0.42 (A), 0.33 (E)						
6	Met-$NHN(CH_3)_2$	$C_{43}H_{65}O_9N_{11}S\cdot 2HCl$	985.05	40–50	0.65 (C), 0.13 (A), 0.10 (E)	2.01	1.97	1.00	1.02	0.96	
7	Nle-NH_2	$C_{42}H_{62}O_9N_{10}\cdot C_2H_4O_2$	911.07	15	0.67 (C), 0.47 (F), 0.38 (RP)	1.98	2.01	0.98	0.93		1.00
8	Nle-OH	$C_{42}H_{61}O_{10}N_9$	852.01	40	0.67 (C), 0.49 (F), 0.51 (RP)	1.91	1.97	1.01	1.02		1.00
9	Nle-OCH_3	$C_{43}H_{63}O_{10}N_9\cdot C_2HO_2F_3$	980.06	28	0.68 (C), 0.49 (F), 0.22 (RP)	2.01	1.95	0.96	1.00		0.97
10	Nle-$NHCH_2CH_3$	$C_{44}H_{66}O_9N_{10}\cdot C_2H_4O_2$	939.12	26	0.68 (C), 0.48 (F), 0.29 (RP)						
11	Nle-$N(CH_2CH_3)_2$	$C_{46}H_{70}O_9N_{10}\cdot C_2H_4O_2$	967.18	7	0.68 (C), 0.44 (F), 0.00 (RP)						
12	Nle-$NHNH_2$	$C_{42}H_{63}O_9N_{11}\cdot C_4H_2O_4F_6$	1094.09	18	0.68 (C), 0.45 (F), 0.48 (RP)						

[a] Amino acid analyses have not been carried out on peptides 10–12 because they stem from the already known sequence. Yields are based on the yield of the last deprotection step and the final purification. For definitions of systems A, B, C, E, F, and RP, see Experimental Section.

with 5% $NaHCO_3$, 10% citric acid, and water and dried (Na_2SO_4). The solvent was removed in vacuo and the residue was solidified upon trituration with *n*-hexane. The solid product was recrystalized from ethyl acetate–*n*-hexane (3:7, v/v): yield 2.15 g (78%); mp 126–127 °C; $[\alpha]^{22}_D$ −42.3° (0.5% DMF); TLC R_f (A) 0.78, R_f (B) 0.60. Anal. ($C_{17}H_{33}N_3O_4S$) C, H, N.

Boc-Met-$NHCH_3$. This was prepared from Boc-Met-OH·DCHA (4.30 g, 10 mmol) and methylamine hydrochloride (2.03 g, 30 mmol) according to the procedure described for Boc-Met-$N(CH_3)_2$: yield 2.17 g (83%); mp 90–91 °C [AcOEt–petcoleum ether (60–80 °C), 2:5, v/v]; $[\alpha]^{22}_D$ −19.2° (1% DMF); TLC R_f (A) 0.72, R_f (C) 0.77. Anal. ($C_{11}H_{22}H_2O_3S$) C, H, H.

Boc-Leu-Met-$NHCH_3$. This was prepared from Boc-Leu-OH·H_2O (1.61 g, 6.5 mmol) and the deprotected Boc-Met-$NHCH_3$ (1.7 g, 6.5 mmol) according to the procedure described for Boc-Leu-Met-$N(CH_3)_2$: yield 1.99 g (82%); mp 130–132 °C [AcOEt–petroleum ether (60–70 °C), 1:3, v/v]; $[\alpha]^{22}_D$ −27° (0.5% DMF); TLC R_f (A) 0.69, R_f (C) 0.74. Anal. ($C_{18}H_{35}N_3O_4S$) C, H, N.

Boc-Met-$NHN(CH_3)_2$. To a colution of Boc-Met-OH·DCHA (4.5 g, 10.4 mmol) in THF (30 mL) cooled to −10 °C was added isobutyl chloroformate (0.42 g, 10.4 mmol). After 2 min, a solution of $(CH_3)_2NNH_2$ (1.87 g, 31.3 mmol) in THF (5 mL) was mixed immediately with the anhydride. The reaction mixture was left to stand at room temperature for 1 h and then was filtered off. The filtrate was evaporated in vacuo, and the residue was taken up in ethyl acetate, washed with 5% $NaHCO_3$ and water, and dried (Na_2SO_4). The solvent was removed under reduced pressure, and the residue was solidified upon trituration with cold petroleum ether (high boiling). The solid was recrystallized from petroleum ether and yielded 2.12 g (70%): mp 93–94 °C; $[\alpha]^{22}_D$ −19.5 (1% EtOH); TLC R_f (A) 0.63, R_f (C) 0.82. Anal. ($C_{12}H_{25}N_3O_3S$) C, H, N.

Boc-Leu-Met-$NHN(CH_3)_2$. To a solution of Boc-Leu-OH and H_2O (1.68 g, 6.76 mmol) in THF (30 mL) cooled to −10 °C was added TEA (0.7 g, 6.76 mmol) and isobutyl chloroformate (0.92 g, 6.76 mmol). After 2 min, a solution of H-Met-$NHN(CH_3)_2$, prepared by deprotection of Boc-Met-$NHN(CH_3)_2$ (1.97 g, 6.76 mmol) with 1.75 N HCl in CH_3COOH and neutralization of the resulting salt with TEA (1.4 g, 13.5 mmol), in THF (20 mL) was added to the anhydride. The reaction mixture was left to stand at room temperature for 1 h and then was filtered off. The filtrate was evaporated in vacuo, and the residue was taken up in ethyl acetate, washed with 5% $NaHCO_3$ and water, and dried (Na_2SO_4). The solvent was evaporated under reduced pressure, and the oily residue was solidified upon trituration with ether–*n*-hexane: yield 1.71 g (63%); mp 125–127 °C (ether–*n*-hexane, 1:5, v/v); $[\alpha]^{22}_D$ −26° (1%, DMF); TLC R_f (A) 0.56, R_f (C) 0.67. Anal. ($C_{19}H_{38}N_4O_4S$) C, H, N.

Preparation of Boc-Gln-Gln-Phe-Phe-Gly-OBzl. A portion of Boc-Gln-Phe-Phe-Gly-OBzl,[16] (3 mmol) was deprotected with 11 mL of TFA–anisol (10:1, v/v). The resulting trifluoroacetate was dissolved in DMF (10 mL), neutralized with NMN, and allowed to react with a sample of Boc-Gln-OH (3 mmol) dissolved in DMF (5 mL) and preactivated at 0 °C for 0.5 h with HOBt (4.8 mmol) and DCC (3.0 mmol). The reaction mixture was left to stand for 2 h at 0 °C and then for 24 h at room temperature. The precipitated DCU was filtered, and the solvent was removed in vacuo. The remaining residue was solidified upon trituration with water. The resulting solid was washed several times with 5% $NaHCO_3$, 10% citric acid, and water and dried (P_2O_5). The product was further purified by recrystallization from DMF/AcOEt (1:2): $[\alpha]^{22}_D$ −22.5° (1% DMF); TLC R_f (A) 0.84, R_f (C) 8.88; yield 62%.

Preparation of Boc-Gln-Gln-Phe-Phe-Gly-OH. A sample of Boc-Gln-Gln-Phe-Phe-Gly-OBzl (1.5 mmol) was dissolved in 50 mL of DMF–H_2O (9:1, v/v) and hydrogenated over 10% Pd/C. The progress of the reaction was monitored by TLC. At the end, the solvent was removed in vacuo, and the residue was solidified upon trituration with ether. The solid was filtered, washed several times with ether, and dried (P_2O_5): mp 209–213 °C dec; $[\alpha]^{22}_D$ −27.4° (1% DMF); TLC R_f (A) 0.73, R_f (C) 0.70; yield 94%; mp

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Table III

compd	recrystn solvent	mp, °C dec	$[\alpha]^{22}_D$,[b] deg	yield, %	TLC R_f A	TLC R_f C
Boc-3	EtOH	246–250	−29.4	50	0.74	0.87
Boc-4	EtOH	222–226	−17.2	65	0.81	0.73
Boc-6	DMF/Et_2O[a]	219–223	−17.8	68	0.63	0.78

[a] 1:3. [b] 0.5% DMF.

Table IV

no.	purifications	mp, °C dec	$[\alpha]^{22}_D$,[a]	TLC R_f A	TLC R_f C	TLC R_f E
3	partition Sephadex G-25, 1.6 × 80 cm, BAW, 4:1:5, upper	252–257	−24.0	0.28	0.64	0.20
4	gel filtration, Sephadex G-15, 2.5 × 76 cm, 2 M AcOH	226–231	−27.7	0.17	0.80	0.14
6	gel filtration, Sephadex G-15, 1.6 × 80 cm, 2 M AcOH	240–244	−20.2	0.13	0.65	0.10

[a] 0.1% DMF.

229–231 °C dec. Anal. ($C_{35}H_{47}N_7O_{10}$) C, H, N.

Preparation of Peptides Boc-Gln-Gln-Phe-Phe-Gly-Leu-X. A portion of dipeptide Boc-Leu-X (1.0 mmol) was deprotected with 1.75 N HCl in CH_3COOH. The resulted hydrochloride salt was dissolved in DMF (6 mL), neutralized with NMM, and allowed to react with a sample of Boc-Gln-Gln-Phe-Phe-Gly-OH (1.0 mmol) dissolved in DMF (15 mL) and preactivated at 0 °C for 0.5 h with HOBt (1.8 mmol) and DCC (1.0 mmol). The reaction mixture was left to stand for 2 h at 0 °C and then for 24 h at room temperature. The precipitated DCU was filtered, and the solvent was removed in vacuo. The remaining residue was solidified upon trituration with water. The resulting solid was washed several times with 5% $NaHCO_3$, 10% citric acid, and water and dried (P_2O_5). The products were further purified by recrystallization from the appropriate solvent (see Table III).

Preparation of Peptides H-Gln-Gln-Phe-Phe-Gly-Leu-X (3, 4, and 6). A portion of peptide Boc-Gln-Gln-Phe-Phe-Gly-Leu-X (100–150 mg) was deprotected with 1.75 N HCl in CH_3COOH for 1 h. The solvent was removed in vacuo, and the residue, upon trituration with dry ether, gave the corresponding hydrochloride salt. This salt was neutralized with NMM in methanol. The precipitated NMM·hydrochloride was filtered, and the filtrate was evaporated under reduced pressure. The residue was dissolved in water, lyophilized, and purified as shown in Table IV. Yields were 40–50% based on the protected heptapeptide; the peptide hydrazide was obtained as the dihydrochloride salt.

Boc-Phe-Phe-Gly-Leu-Met-OCH_3. To a solution of Boc-Phe-Phe-Gly-OH (3.5 g, 7.48 mmol) in 25 mL of THF cooled to −10 °C was added TEA (0.755 g, 7.48 mmol) and isobutyl chloroformate (1.02 g, 7.48 mmol). After 5 min, a solution of HCl and H-Leu-Met-OCH_3 (3.37 g, 10.47 mmol) in 20 mL of THF was neutralized with TEA (1.06 g, 10.47 mmol) and mixed immediately with the anhydride. The reaction mixture was left to stand at room temperature for 2 h and then was filtered off. The filtrate was evaporated under reduced pressure, and the residue was taken up in ethyl acetate, washed with 5% $NaHCO_3$, H_2O, 10% citric acid, and water and dried (Na_2SO_4). The solvent was removed in vacuo, the residue was solidified upon trituration with petroleum ether, and the solid product was recrystallized from ethyl acetate/petroleum ether (1:4), v/v): yield 3.9 g (71%); mp 142–146 °C dec; $[\alpha]^{22}_D$ −46.4° (0.5% DMF); TLC R_f (A) 0.85, R_f (C) 0.80. Anal. ($C_{37}H_{53}N_5O_8S$) C, H, N.

Boc-Gln-Phe-Phe-Gly-Leu-Met-OCH_3. A sample of Boc-Phe-Phe-Gly-Leu-Met-OCH_3 (3.46 g, 4.7 mmol) was deprotected with 30 mL of 1.75 N HCl in CH_3COOH. The resulted hydrochloride salt was dissolved in DMF (10 mL), neutralized with NMM, and allowed to react at room temperature with a portion of Boc-Gln-ONP (1.72 g, 4.7 mmol), dissolved in DMF (6 mL), while the pH of the reaction mixture was adjusted to 7.5–8 with NMM. After 48 h the solvent was removed in vacuo, water was added to the solid residue, and the suspension was filtered; the residue was washed several times with 5% $NaHCO_3$, 10% citric acid, and water and dried (P_2O_5). The solid product was recrystallized from DMF/petroleum ether (1:3, v/v: yield 2.8 g (70%); mp 218–222 °C dec; $[\alpha]^{22}_D$ −31.4° (1% DMF); TLC R_f (A) 0.79, R_f (C) 0.88. Anal. ($C_{42}H_{61}N_7O_{10}S$) C, H, N.

Boc-Gln-Gln-Phe-Phe-Gly-Leu-Met-OCH_3. A portion of Boc-Gln-Phe-Phe-Gly-Leu-Met-OCH_3 (2.5 g, 2.48 mmol) was deprotected with HCl in CH_3COOH, and the resulted hydrochloride salt was neutralized and allowed to react with Boc-Gln-ONP according to the procedure described for the preparation of Boc-Gln-Phe-Phe-Gly-Leu-Met-OCH_3: yield 1.75 g (72%); mp 226–230 °C dec (DMF/ether, 1:4, v/v); $[\alpha]^{22}_D$ −27.4° (0.5% DMF); TLC (R_f (A) 0.82, R_f (E) 0.89. Anal. ($C_{47}H_{69}N_9O_{12}S$) C, H, N.

H-Gln-Gln-Phe-Phe-Gly-Leu-Met-OCH_3·HCl (2). A portion of peptide Boc-Gln-Gln-Phe-Phe-Gly-Leu-Met-OCH_3 (100 mg, 0.1 mmol) was deprotected with 1.75 N HCl/CH_3COOH for 1 h. The solvent was removed in vacuo, and the residue was solidified upon trituration with dry ether. This salt was purified by gel filtration on Sephadex G-15 (80 × 1.6 cm) with 2 M AcOH as eluent (flow rate 8 mL/h) to give the desired analogue (V_e = 102 mL): yield, following lyophilization, 54 mg; mp 233–235 °C dec; $[\alpha]^{22}_D$ −48° (0.5% DMF). TLC showed one spot. Anal. ($C_{42}H_{61}N_9O_{10}S$·HCl) C, H, N. For amino acid analysis and TLC, see Table II.

Boc-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NHNH_2$. A portion of Boc-Gln-Gln-Phe-Phe-Gly-Leu-Met-OCH_3 (600 mg, 0.6 mmol) was dissolved in DMF (6 mL), and hydrazine monohydrate (75 mg, 1.5 mmol) was added. After 48 h at room temperature, the solvent was removed in vacuo, and the residue was solidified upon trituration with water. The solid was filtered, washed with water, dried (P_2O_5), and recrystallized from DMF/ether (1:2, v/v): yield 480 mg (80%); mp 233–238 °C dec; $[\alpha]^{22}_D$ −28° (0.5%, DMF); TLC R_f (A) 0.70, R_f (C) 0.81. Anal. ($C_{46}H_{69}N_{11}O_{11}S$) C, H, N.

H-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NHNH_2$·HCl (5). A sample of peptide Boc-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NHNH_2$ (100 mg, 0.1 mmol) was deprotected with 1.75 N HCl/AcOH for 1 h. The solvent was removed in vacuo, and the residue was solidifed upon trituration with dry ether. This salt was purified by gel filtration on Sephadex G-15 (80 × 1.6 cm) with 2 M AcOH as eluent (8 mL/h) to give the desired analogue: yield, following lyophilization, 60 mg; mp 238–252 °C; $[\alpha]^{22}_D$ −45° (0.5% DMF); TLC showed one spot. Anal. ($C_{41}H_{61}N_{11}O_9S$·2HCl) C, H, N. For amino acid analysis and TLC, see Table II.

Boc-Gln-Gln-Phe-Phe-Gly-Leu-Nle-resin. Peptide synthesis was carried out according to a previously described procedure:[17] 6 of chloromethylated polystyrene/1% divinylbenzene (Merrifield resin, Chemalog), 0.75 mequiv/g, was esterified to Boc-Nle. The resulting resin contained 0.45 mequniv/g of Boc-Nl, and the peptide chain was elongated by manual solid-phase peptide synthesis. Boc-Phe, Boc-Gly, and Boc-Leu were coupled by the symmetric anhydrides and Boc-Gln was coupled with a

(17) O. Leukart, E. Escher, D. Regoli, and R. Schwyzer, *Helv. Chim. Acta*, **62**, 546 (1979).

HOBT/DCC procedure, all in 4-fold excess. For the HOBT procedure, Boc-Gln was dissolved in DMF, 1.2 equiv of HOBT was added; this was then introduced into the reaction vessel. One equivalent of DCC, dissolved in CH_2Cl_2, was added and reacted during 60 min.

H-Gln-Gln-Phe-Phe-Gly-Leu-Nle-OH (8). The above resin, 1.2 g, was treated with 10 mL of anhydrous HF during 30 min at 0 °C in the presence of 1 mL of anisol. After evaporation of the HF and the anisol, the peptide was extracted with 50% acetic acid, diluted with water, and lyophilized.

The crude product was filtered over Sephadex LH 20 (1.5 × 60 cm), and the peptide-containing fractions were pooled and evaporated in vacuo. The product was further purified by a partition chromatography on Sephadex LH 20 (1.5 × 60 cm), with a gradient of 0.5 M AcOH to 0.5 M AcOH + 30% 2-propanol. The fractions were spotted on TLC, and the pure peptide fractions were pooled, evaporated, and lyophilized to produce 15 mg of **8**. For amino acid analysis, see Table II.

H-Gln-Gln-Phe-Phe-Gly-Leu-Nle-OCH_3 (9). Boc-protected heptapeptide resin ester, 1.4 g, was treated during 2 weeks at room temperature and occasional shaking with 100 mL of 1 M DEA in methanol. After filtration and evaporation, 205 mg of crude product was obtained. This material was dissolved in DMF and filtered over Sephadex LH 20 (1 × 40 cm), and the peptide fractions were combined and evaporated. This product, 50 mg, was deprotected by treating with 1 mL of TFA for 30 min at room temperature. The reaction was stopped by the addition of 15 mL of dry ethyl acetate and then evaporated and treated, twice again, with ethyl acetate. The final product was dissolved in water and lyophilized to produce 26.0 mg of peptide **9**. For amino acid analysis, see Table II.

H-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 (7). Protected heptapeptide resin ester, 1.4 g, was treated in a pressure bottle with 100 mL of a solution of DMF/2-propanol (1:1), saturated at 0 °C with gaseous ammonia. After 7 days, the solvent was changed, pooled after 14 days, and evaporated in vacuo, producing 172.9 mg of crude Boc-protected **7**. This product, 112 mg, was deprotected with 1 mL of TFA during 30 min at room temperature. After repeated addition of 10 mL of ethyl acetate and evaporation, the product was dissolved in DMF and filtered over Sephadex LH 20 (1.5 × 60 cm). The peptide-containing fractions were pooled, evaporated, and subjected to a second purification by partitioning on Sephadex LH 20 (1 × 40 cm) with a gradient of 0.5 M AcOH to 0.5 M AcOH + 30% 2-propanol. The pure fractions were pooled and yielded 18.2 mg of pure **7**. For amino acid analysis, see Table II.

H-Gln-Gln-Phe-Phe-Gly-Leu-Nle-$NHCH_2CH_3$ (10). Heptapeptide resin ester, 1.4 g, was treated exactly in the same manner as for **9** but with 2 M ethylamine in DMF/2-propanol (1:1). After 14 days, 359.6 mg was obtained. This crude product, 60 mg, was deprotected, filtered over Sephadex LH 20, and purified by partition exactly as described for **9**: 15.6 mg of pure **10** was obtained by this procedure.

H-Gln-Gln-Phe-Phe-Gly-Leu-Nle-$N(CH_2CH_3)_2$ (11). Heptapeptide resin ester, 1.4 g, was treated exactly as described for **10**, but diethylamine was used instead of ethylamine, producing 183.3 mg of crude Boc-protected **11**, which was deprotected and purified as described for **10**, producing 12.0 mg of pure **11**.

H-Gln-Gln-Phe-Phe-Gly-Leu-Nle-$NHNH_2$ (12). Protected, crude **9** 100 mg, was treated with 1 mL of hydrazine and 100 μL of water in 5 mL of DMF during 40 h at room temperature. The reaction mixture was evaporated, redissolved in DMF, and filtered over Sephadex LH 20 (1 × 40 cm). The peptide fractions were pooled, evaporated, and treated with 2 mL of TFA as described above. A final filtration over Sephadex LH 20 with DMF yielded 18 mg of pure **12**.

Biological Activities. Substance P and its analogues were dissolved with a minimum of 80% acetic acid, diluted with distilled water, neutralized with aqueous ammonia, and further diluted to the desired concentration with isotonic saline. These solutions were used the same day and discarded afterwards. The guinea pig ileum assay and the test for the hypotensive effects on the blood pressure of anesthetized rats were carried out as described earlier.[4] The results are expessed by the classical pharmacological parameters pD_2, α_E, relative affinity (guinea pig ileum), and relative potency (rat blood pressure); they are presented on Table I.

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