Substance P Analogs Containing Homoglutamine at Position 5 and 6

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Synopsis. Six substance P (SP) analogs, in which the glutaminyl residue(s) at the position(s) 5 or/and 6 were replaced by homoglutamine (Hgn), were prepared using a novel N^{α},N^{ca}-$(Boc)_2$-Hgn-OH by the solid phase method. The activity of the analogs was assayed on isolated guinea pig organs. Results indicate that the elongation of the methylene on glutaminyl residue(s) in SP enhances the activity on isolated guinea pig ileum.

Chang and Leeman[1] isolated in 1970 substance P (SP) from bovine hypothalami and the amino acid sequence was elucidated by Chang, et al.,[2] to be H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2.[3] Studies on the relationship between peptide chain length and activity[4] of SP revealed that the contractile activity of certain C-terminal fragments was as high as or even higher than that of SP itself on the smooth muscle of isolated guinea pig ileum. Although the C-terminal pentapeptide had much lower activity, addition of Gln residue(s) at the N-terminal of the pentapeptide leads to an enhancement of biological activity. The conformation analyses suggest a U shape for the C-terminal part of the molecule, providing an explanation for the observation that full biological activity is retained in SP hexapeptide, and that bioactive structures are stabilized by the formation of intramolecular hydrogen bond between the amide proton (or amino proton) of the glutamine residue at position 6 and the carbonyl group of the C-terminal amide.[5] These imply that the Gln residue(s) of SP is very important for inducing the activity.

This paper describes the first synthetic example of bioactive peptide analogs substituted with homoglutamine (Hgn; α-aminoadipamic acid). In order to examine the biological effect of the elongation of methylene group in the Gln residue(s) at positions of 5 or/and 6 by the displacement with Hgn, we synthesized six analogs and submitted them for biological assay to compare with our synthetic SP.

The SP analogs were synthesized by the solid phase technique[6] using an automated peptide synthesizer. Deprotection and dicyclohexylcarbodiimide (DCC) coupling were programed as given in Table 1. *t*-Butoxycarbonyl (Boc) protection was used for α-amino function and the amide nitrogen (N^{ca}) of Hgn. The Hgn residue was incorporated into peptide chains as usual manner using N^{α},N^{ca}-$(Boc)_2$-Hgn-OH,[7,8] which was derived from an ester of $(Boc)_2$-Lys-OH by ruthenium tetraoxide oxidation. This oxidative transformation[9,10] made it possible to produce quite conveniently the novel Hgn derivative which is applicable to synthetic studies (on the structure-activity relationships of peptides). The protecting group on the amide can be deblocked by the same reagents as those for the groups on α-amino function to yield Hgn residue.[8] Other side-chain protecting groups were Z for Lys and tosyl (Tos) for Arg. After the completion of the chain elongations, the peptide resin was treated with liquid hydrogen fluoride (HF)[11] in the presence of anisole to cleave the peptide from the resin with simultaneous removal of the protecting groups and formation of the carboxyl terminal amide. The crude product was purified by preparative reverse-phase high-performance liquid chromatography (HPLC) using 0.1% TFA in acetonitrile (CH_3CN) as eluent. Highly purified peptides were obtained after gel filtration on Sephadex G-10 column. The yield was sacrificed for the purity. Homogeneity of the peptides was demonstrated by analytical HPLC and thin-layer chromatography (TLC). When single peaks and single spots were observed for a peptide in all chromatographic systems, the sample

Table 1. Program for Solid Phase Peptide Synthesis

	Reagents	Operation	Mix time / min
1	CH_2Cl_2	3×Wash	1.5
2	TFA (25% in CH_2Cl_2)	Prewash	1.5
3	TFA (25% in CH_2Cl_2)	Deprotection	30
4	CH_2Cl_2	3×Wash	1.5
5	CH_2Cl_2	3×Wash	1.5
6	TEA (10% in CH_2Cl_2)	3×Neutralization	1.5
7	CH_2Cl_2	6×Wash	1.5
8	Boc-Amino Acid (in CH_2Cl_2 or DMF)	Mix	5
9	DCC	Mix	120
10	CH_2Cl_2	6×Wash	1.5

1 4 5 6 11		
H-Arg-Pro-Lys-Pro-Hgn-Gln- Phe-Phe-Gly-Leu-Met-NH_2	[Hgn^5]-SP	**1**
H-Arg-Pro-Lys-Pro-Gln- Hgn-Phe-Phe-Gly-Leu-Met-NH_2	[Hgn^6]-SP	**2**
H-Arg-Pro-Lys-Pro-Hgn-Hgn-Phe-Phe-Gly-Leu-Met-NH_2	[$Hgn^{5,6}$]-SP	**3**
H-Pro-Hgn-Gln- Phe-Phe-Gly-Leu-Met-NH_2	[Hgn^5]-SP (4−11)	**4**
H-Pro-Gln- Hgn-Phe-Phe-Gly-Leu-Met-NH_2	[Hgn^6]-SP (4−11)	**5**
H-Pro-Hgn-Hgn-Phe-Phe-Gly-Leu-Met-NH_2	[$Hgn^{5,6}$]-SP (4−11)	**6**

Fig. 1. Synthetic SP analogs.

Table 2. Physical Properties and Yield of the Peptides

Analog	$[\alpha]_D^{25}$ (c 0.5, 3M AcOH)/°	Retention time[a] min	R_f^I	R_f^{II}	Yield %
1	−80.4	9.8	0.00	0.56	30.8
2	−86.2	10.2	0.00	0.57	18.2
3	−82.2	11.0	0.00	0.56	28.2
4	−54.0	13.2	0.13	0.68	24.7
5	−58.0	13.6	0.14	0.74	20.2
6	−52.8	15.0	0.13	0.69	25.5

a) Condition of analytical HPLC: column, NOVA-PAK C_{18}; flow rate, 1 ml min^{-1}; Detection, 210 nm; eluent system, linear gradient from 21% to 35% CH_3CN (15 min) in 20 mM phosphate buffer (pH 3.0).

Table 3. Amino Acid Analyses of the Peptides

Analog	Found (Calcd)									
	Lys	Arg	Glu	Pro	Gly	Hgu[a]	Met	Leu	Phe	NH_3
1	1.10 (1)	1.13 (1)	0.98 (1)	1.77 (2)	0.97 (1)	1.10 (1)	0.94 (1)	0.98 (1)	2.03 (2)	3.84 (3)
2	1.12 (1)	1.10 (1)	0.98 (1)	1.85 (2)	0.95 (1)	1.08 (1)	0.97 (1)	0.98 (1)	1.98 (2)	3.82 (3)
3	0.94 (1)	1.04 (1)	—	1.77 (2)	1.05 (1)	2.18 (2)	0.99 (1)	1.00 (1)	2.04 (2)	3.29 (3)
4	—	—	1.00 (1)	0.85 (1)	0.98 (1)	1.15 (1)	0.98 (1)	0.98 (1)	2.05 (2)	3.85 (3)
5	—	—	1.01 (1)	0.89 (1)	1.00 (1)	1.03 (1)	0.98 (1)	0.98 (1)	2.10 (2)	3.86 (3)
6	—	—	—	0.93 (1)	1.02 (1)	2.20 (2)	0.96 (1)	0.98 (1)	1.92 (2)	3.67 (3)

a) Homoglutamic acid; α-aminoadipic acid.

Table 4. Relative Potencies of SP Analogs on Guinea Pig Organs

Analog	Relative contractile activity to SP=1	
	Ileum	Trachea
1	1.81	2.09
2	1.25	1.20
3	3.41	2.09
4	3.41	4.36
5	1.97	1.00
6	1.50	2.75

was considered appropriately pure for bioassay. These chromatographic data were reinforced by the amino acid analytical data.

Biological properties of the synthetic SP analogs in this study were examined on isolated guinea pig organs and the contractile activities of the analogs were compared with that of synthetic SP. The results are shown in Table 4.

The elongation of the methylene group of Gln residue(s) at position 5 or/and 6 of SP enhanced the biological activity on isolated guinea pig ileum, and [$Hgn^{5,6}$]-SP **3** possessed the highest potency among the three analogs (**1**—**3**). Octapeptide analogs **4** and **5** showed about 340 and 200% of the activity of SP respectively and their potencies were higher than those of **1** and **2** replaced at the same position, while [$Hgn^{5,6}$]-SP (4—11) **6** had significant activity, but its potency was less than those of analogs **4** and **5**, and about a half of the undecapeptide **3**.

The analogs **1**, **3**, **4**, and **6** were also found to be highly active on the trachea assay, while **2** and **5** were as high as that of SP. Octapeptide analog **4** had the highest activity on the both bioassays. Thus the potencies of Hgn^5 analogs **1** and **4** were higher than the activities of Hgn^6 analogs **2** and **5** respectively. The potencies of all analogs prepared in the present investigation were as high as or higher than the activity of SP itself. This indicates probably that resistibility of the peptides to metabolic breakdown is increased by the introduction of Hgn residue(s) and the Hgn analogs may possess a U-shape conformation and hydrogen bond very similar to natural SP.

Experimental

Amino acid derivatives were purchased from Peptide Institute, Inc., Osaka, Japan and benzhydrylamine (BHA) resin (available amine of the resin: 0.6 mmol g^{-1} of support) from Beckman Inc., Palo Alto, Calif. Optical rotations were measured in a Nipponbunkoh DIP-4 Polarimeter. Amino acid analyses on samples previously hydrolyzed with 6M HCl (1 M=1 mol dm^{-3}) (110 °C, 24 h) were performed on a Hitachi KLA-5 Amino Acid Analyzer. TLC was carried out on silica-gel plates (Merck). The following solvent systems were used and allowed to ascend for 10 cm: R_f^I, *n*-BuOH : AcOH : H_2O (4 : 1 : 5, upper phase); R_f^{II}, *n*-BuOH: pyridine : AcOH : H_2O (30 : 20 : 6 : 24). Analytical HPLC was effected on the following systems: column, NOVA-PAK C_{18} (3.9×150 mm); flow rate, 1 ml min^{-1}, detection, 210 nm; eluent system, linear gradient of CH_3CN in 20 mM phosphate buffer (pH 3.0).

General Procedure for the Preparation of Analogs. The solid phase synthesis was carried out using a Beckman System 990C Peptide Synthesizer. Half gram of BHA-resin hydrochloride for each analog served as the solid support. For the incorporation of each N^{α}-Boc-protected amino acid, a program given in Table 1 was used. The amino acid derivatives were dissolved in dichloromethane, except Boc-Arg(Tos)-OH and Boc-Gln-OH which were dissolved in DMF, and employed for the coupling reaction. A 2.5 fold excess of the amino acid derivative was used for all couplings. One equivalent of HOBt was added as an additive to facilitate the repetitive coupling reaction, using the program

without step 1—4. In the case of Boc-Gln-OH, 2.5 equiv of HOBt was used for all couplings. The completion of the coupling reaction was assessed by a negative ninhydrin test. When the ninhydrin test was positive, even after repetition of the coupling two or three times, the unreacted amino groups were blocked completely by acetylation using an excess of Ac_2O (5 equiv of resin used) in the presence of pyridine (1 equiv) in dichloromethane for 20 min. The protected peptide resin was treated with anhydrous liquid HF containing 10% anisole. Approximately 5 ml of liquid HF was used for 0.25 g resin, and the reaction time was 1 h at 0 °C. After evaporation of HF in vacuo under ice-cooling, the peptide-resin mixture was washed with AcOEt. The peptide was extracted with three portions of 10% AcOH and the combined extracts were lyophilized.

Purification of the Peptides. The crude peptide was subjected to HPLC. The apparatus was composed of a model 590 pump and a U6K injector (Waters) connecting with a column (20×300 mm) of Chemcosorb ODS (Chemco). The eluates were monitored with a UV detector S-310A model-II (Soma) at 210 nm wavelength. CH_3CN-0.1% TFA solvent system was used as eluent at flow rate 10 ml min^{-1}. Each peptide was emerged at 40—60 min by isocratical elution with 21—23% CH_3CN contents of the solvent system. The desired fraction was passed through a Sephadex G-10 column (16×930 mm) eluted with 2M AcOH and the product was lyophilized. Homogeneity of the peptides was analyzed by analytical HPLC and TLC. The peptides on silica-gel plate were detected with ninhydrin and chlorine-*o*-tolidine reagents. When single peaks and single spots were observed for a peptide in all chromatographic systems and the amino acid compositions after hydrolysis of the peptides were consistent with the calculated values, the sample was submitted for bioassay.

Bioassay. Male guinea pig (weighing 200—250 g) was stunned by a blow to the back and exsanguinated. Ileum and trachea were rapidly isolated and kept for dissection in Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.6 mM glucose) at room temperature. Both organs were suspended in a 30 ml organ bath containing Tyrode's solution thermostated at 30 °C and bubbled with 95% O_2 and 5% CO_2 gas for 1 h. The contraction was recorded by means of isotonic transducer (Nippon Kohden, TD-111T) with load of 1 or 2 g (for trachea and ileum respectively) on a Servocorder (Watanabe Instruments, SR6204). Trachea was transversely cut into pieces and six pieces were connected to make a trachea preparation. Concentration-response curves were obtained using a cumulative dose-assay, the time between two consecutive dose-response curves being longer than 10 min.

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