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Syntheses and Biological Activities of Substance P Analogs Containing L- and D-Homoglutamine and L- and D-Pyrohomoglutamic Acid at Position 5 and 6

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Synopsis. Six substance P (SP) analogs substituted by L- and D-homoglutamine and L- and D-pyrohomoglutamic acid (6-oxo-2-piperidinecarboxylic acid) at position 5 and 6 were synthesized by standard solid phase method. The contractile activities of the analogs were compared with that of SP on isolated guinea-pig ileum and trachea.

Substance P[1] (SP), an undecapeptide with the sequence [H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$[2]], is a putative neurotransmitter or a neuromodulator. Up to date, numerous SP analogs have been synthesized and their structure-activity relationships have been studied. The potencies of SP related peptides, pGlu-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$ **1** and pGlu-Phe-Phe-Gly-Leu-Met-NH$_2$ **2**, have been found to be higher than the contracting activity of SP itself on guinea-pig ileum[3] (Table 1).

Our study[4,5] on the structure-activity relationships of SP and the SP related peptide **1** revealed that the elongation of the methylene group of the side chain in the Gln residue(s), that is, the substitution of L-homoglutamine (Hgn) for Gln moiety at position 5 and 6, enhanced the contractile activity on the smooth muscle of isolated guinea-pig ileum, and the replacement of the Gln residue(s) with D-Hgn moiety brought the decrease of the activity on the ileum assay. On pyrohomoglutamic acid (pHgu, 6-oxo-2-piperidinecarboxylic acid) peptide analogs[6] (Table 1) of the SP related peptides, the potency of [pHgu^6]-SP (6—11) **5** was higher than the activity of [D-pHgu^6]-SP (6—11) **6**, while [pHgu^5]-SP (5—11) **3** showed lower activity than that of [D-Hgu^5]-SP (5—11) **4**.

This paper describes syntheses and biological properties of six analogs (Fig. 1) of SP and SP related peptides: [L-Hgn^5, D-Hgn^6]- and [D-Hgn^5, L-Hgn^6]-SP and SP (4—11) and [L-pHgu^5, D-Hgn^6]- and [D-pHgu^5, L-Hgn^6]-SP (5—11). The contracting activities of the synthetic peptides were compared with that of our synthetic SP in order to examine the biological effects of the substitution and confirm the greater importance of the L-configuration of the amino acid residue at the position 6 than that of the residue at the position of 5 for the contractile activities of SP.

The SP analogs were synthesized in the same way with the preparation of L-Hgn analogs[4] by the solid phase technique on an automated peptide synthesizer by standard solid phase method[7] starting from *t*-butoxycarbonyl(Boc)-Met-benzhydrylamine-resin. α-Amino functions were protected by Boc group. The side-chain-protecting groups were benzyloxycarbonyl for Lys, tosyl for Arg and Boc for the amide nitrogen

	1 4 5 6 11	
[Hgn^5, D-Hgn^6]-SP	H-Arg-Pro-Lys-Pro—Hgn-D-Hgn-Phe-Phe-Gly-Leu-Met-NH$_2$	**7**
[D-Hgn^5, Hgn^6]-SP	H-Arg-Pro-Lys-Pro-D-Hgn—Hgn-Phe-Phe-Gly-Leu-Met-NH$_2$	**8**
[Hgn^5, D-Hgn^6]-SP (4-11)	H-Pro—Hgn-D-Hgn-Phe-Phe-Gly-Leu-Met-NH$_2$	**9**
[D-Hgn^5, Hgn^6]-SP (4-11)	H-Pro-D-Hgn—Hgn-Phe-Phe-Gly-Leu-Met-NH$_2$	**10**
[pHgu^5, D-Hgn^6]-SP (5-11)	pHgu-D-Hgn-Phe-Phe-Gly-Leu-Met-NH$_2$	**11**
[D-pHgu^5, Hgn^6]-SP (5-11)	D-pHgu—Hgn-Phe-Phe-Gly-Leu-Met-NH$_2$	**12**

Fig. 1. Synthetic SP analogs.

Table 1. Relative Contracting Activities of Synthetic Analogs on Guinea-Pig Ileum

	Peptide	Amino acid sequence	Relative activity
		1 5 6 11	
	SP	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$	1
1	[pGlu^5]-SP (5—11)	pGlu-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$	1.7
2	[pGlu^6]-SP (6—11)	pGlu-Phe-Phe-Gly-Leu-Met-NH$_2$	2.0
3	[pHgu^5]-SP (5—11)	pHgu-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$	0.65
4	[D-pHgu^5]-SP (5—11)	D-pHgu-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$	1.76
5	[pHgu^6]-SP (6—11)	pHgu-Phe-Phe-Gly-Leu-Met-NH$_2$	1.50
6	[D-pHgu^6]-SP (6—11)	D-pHgu-Phe-Phe-Gly-Leu-Met-NH$_2$	0.17

(N^{ca}) of Hgn. The Hgn residue can be incorporated into a peptide chain by the usual solid phase technique and solution method using N^{α},N^{ca}–(Boc)$_2$–Hgn–OH[4,8], which is derived from the ester of (Boc)$_2$–Lys–OH by ruthenium tetraoxide oxidation.[9,10] The protecting group Boc on the amide of N^{α},N^{ca}–(Boc)$_2$–Hgn–OH can be removed by the same deblocking reagents as those for the protecting group on the α-amino function to produce the Hgn moiety.[8,10] Our investigation[6,11] developed a simple method for synthesis of pHgu-peptide by cyclization of Hgn residue at N-terminus in peptide chain.

The synthetic products were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) using 0.1% trifluoroacetic acid in acetonitrile as eluent. Highly purified peptides (Tables 2 and 3) were obtained after gel filtration of Sephadex G-10 column. Homogeneity of the peptides was demonstrated by analytical HPLC and thin-layer chromatography (TLC). When single peak and single spot were observed for a peptide in all chromatographic systems used, the peptide was considered appropriately pure for bioassay. These chromatographic data were reinforced by the amino acid analytical data.

The agonistic effects of the synthetic peptides were compared with that of our synthetic SP on the isolated guinea-pig ileum and trachea. The results are presented in Table 4.

Table 2. Physical Properties and Yields of the Synthetic Peptides

Analog	$[\alpha]^{14}_{D}$ (*c* 0.5, 3M AcOH)/°	Retention time[a)] / min	R_f^{I}	R_f^{II}	Yield / %
7	−68.0	11.2	0.00	0.58	14.5
8	−53.8	11.3	0.00	0.58	20.6
9	−24.6	14.7	0.13	0.71	11.4
10	−39.4	14.5	0.13	0.71	20.1
11	−35.4[b)]	17.1	0.47	0.85	15.0
12	−46.2[b)]	17.2	0.46	0.85	13.3

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). b) *c* 0.5, DMF.

The potency of [D-Hgn^5,Hgn^6]-SP **8** was as high as the activity of SP itself on the guinea-pig ileum assay. The replacements of both Gln residues at the position 5 and 6 with Hgn-D-Hgn sequence brought the drastic decrease of the activities on the both assays. The C-terminal octapeptide analog **10** was found to have about 10% activity of **8** on the ileum assay. D-pHgu-peptide analog **12** possessed the highest potency among the six analogs **7**—**12** on the both assays, while pHgu-peptide analog **11** had little activities. The results indicate that the L-configuration of the amino acid residue at the position 6 may be more important than that of the residue at the position 5 for the contractile activities of SP and SP related peptides.

Experimental

Amino acid derivatives were purchased from Peptide Institute, Inc., Osaka, and benzhydrylamine (BHA) resin (available amine of the resin: 0.6 mmol g^{-1} of support) from Beckman Inc. Optical rotations were measured in a Nipponbunkoh DIP-4 Polarimeter. Amino acid analyses on samples previously hydrolyzed with 6 M HCl (1 M=1 mol dm^{-3}) (110 °C, 24 h) were performed on a Hitachi KLA-5 Amino Acid Analyzer. TLC were carried out on silica-gel plates (Merck). The following solvent systems were used: R_f^{I}, *n*-BuOH:AcOH:H$_2$O (4:1:5, upper phase); R_f^{II}, *n*-BuOH:pyridine:AcOH:H$_2$O (30:20:6:24). Analytical HPLC were 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 for 15 min from 21% to 35% CH_3CN in 20 mM phosphate buffer (pH 3.0).

Table 4. Biological Activities of the Synthetic SP Analogs on Guinea-Pig Organs

Analog	RA	
	GPI	GPT
7	0.04	0.04
8	1.00	0.17
9	0.01	0.13
10	0.11	0.13
11	0.02	<0.01
12	2.58	0.26

GPI, guinea-pig ileum; GPT, guinea-pig trachea; RA, relative contractile activity to SP = 1.

Table 3. Amino Acid Analyses of the Synthetic Peptides

Analog	Found (Calcd)								
	Lys	Arg	Pro	Gly	Hgu[a)]	Met	Leu	Phe	NH_3
7	1.04(1)	1.00(1)	1.93(2)	1.04(1)	2.13(2)	0.98(1)	0.95(1)	1.91(2)	4.04(3)
8	0.87(1)	0.90(1)	2.08(2)	1.20(1)	2.10(2)	1.00(1)	0.96(1)	1.89(2)	3.51(3)
9	—	—	0.89(1)	1.02(1)	2.10(2)	0.98(1)	0.96(1)	2.05(2)	3.13(3)
10	—	—	0.92(1)	1.13(1)	2.12(2)	1.00(1)	0.97(1)	1.86(2)	3.62(3)
11	—	—	—	0.92(1)	2.33(2)	0.93(1)	0.92(1)	1.90(2)	3.20(2)
12	—	—	—	1.02(1)	2.12(2)	0.99(1)	0.97(1)	1.89(2)	2.44(2)

a) Homoglutamic acid.

General Procedure for the Preparation of Analogs. The solid phase synthesis was carried out using a Beckman System 990C Peptide Synthesizer as described previously.[4] Half gram of BHA-resin hydrochloride for each analog served as the solid support. A 2.5 fold excess of amino acid derivative was used for all coupling. The coupling was effected with dicyclohexylcarbodiimide/1-hydroxybenzotriazole. The protected peptide resin was treated with anhydrous liquid HF[12] containing 10% anisole. After evaporation of HF in vacuo, the peptide was extracted with 50% AcOH. The extract of N-terminal Hgn-peptide was kept at 40 °C for 4 h to yield pHgu moiety.

Purification of the Peptides. The crude peptide was subjected to HPLC as reported previously.[4] 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 20—28% CH_3CN contents of the solvent system. The desired fraction was passed through a Sephadex G-10 column (16×930 mm) eluted with 2 M AcOH. Homogeneity of the peptides was analyzed by analytical HPLC and TLC.

Bioassay. The agonistic activity of the synthetic analog was measured on ileum and trachea taken from guinea-pig, as described before.[4] The contraction was recorded by means of an isotonic transducer (Nippon Kohden, TD-111T) with load of 1 or 2 g (for trachea and ileum respectively) on a Servocorder (Watanabe Instruments, SR6204). Concentration-response curves were obtained using a cumulative dose-assay, and the time between two consecutive dose-response curves was longer than 10 min.

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