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SUBSTANCE P ANALOGUES CONTAINING p-FLUORO-L-PHENYLALANINE

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Three substance P analogues containing p-fluoro-L-phenylalanine were synthesized by Merrifield's solid phase method. The spectroscopic behaviors in CD- and ^{19}F NMR-spectra as well as pharmacological properties of the fluorine containing substance P analogues are described.

Fluorine-19 is a nucleus possessing spin number 1/2 and is only one isotope of this element occurs in nature. ^{19}F chemical shift in NMR is comparatively large and sensitive to the chemical environments. Furthermore, C-F bond is chemically inert and the F atom is small enough to cause little steric complication in the molecule. The range of chemical shift of ^{19}F is extremely wide compared with the chemical shift of ^{1}H. The ^{19}F-^{19}F and ^{19}F-^{1}H spin coupling constants are also distinctly larger than those between proton. These characteristics of ^{19}F NMR seem to be a great advantage over ^{1}H NMR in the application to the studies of conformation of biomolecules, especially of biologically active peptides or proteins.[1] Recent studies have disclosed that the interaction of hormone or neuropeptide with phospholipid bilayer on the receptor membrane is a critical step in the expression of biological activities.[2] ^{19}F NMR will be particularly of use in such an investigation because the ^{19}F resonance is not masked by ^{1}H.

In this study, three analogues of substance P(SP) which contain p-fluoro-L-phenylalanine as shown below, have been synthesized and spectroscopic properties were investigated. All the three peptide derivatives exhibited quantitatively the same contracting activity as the native substance P on the smooth muscle of guinea pig ileum.

Substance P H-Arg-Pro-Lys-Pro-Gln-Gln-Phe(X)-Phe(Y)-Gly-Leu-Met-NH_2

F7-SP: X=F, Y=H F8-SP: X=H, Y=F F7,8-SP: X=F, Y=F

The peptide derivatives were synthesized by Merrifield's solid phase method[3] starting from butyloxycarbonyl(Boc)-Met-benzhydrylamine-resin (Met contents of the resin: 0.26 mmol/g of resin). Boc-group of α-amino group was removed by the treatment of 50% trifluoroacetic acid (TFA) in CH_2Cl_2 containing 1% $HSCH_2CH_2SH$ for 30 min at room temperature. Protective groups for secondary functions of amino acids used are benzyloxycarbonyl for ε-NH_2 group of lysine and p-toluensulfonyl for guanido group of arginine, respectively. Boc-Phe(F) was prepared by the reaction of $(Boc)_2CO$[4] and p-fluoro-L-phenylalanine. All coupling reactions were carried out

by Peptide Synthesizer (990B, Beckman) with the aid of dicyclohexylcarbodiimide/ 1-hydroxybenztriazole method as usual manner. After the completion of the chain elongations, the peptide resin was treated with anhydrous HF in the presence of anisole and thioanisole at 0 °C for 1 h. The excess HF was removed in vacuo, the residue was taken up by 10% aqueous AcOH and the resin was filtered off. The AcOH solution was passed through the column of Dowex 1x2 (AcO^- form) and the eluate was lyophilized. The crude product thus obtained was gel-filtrated by the column of Sephadex LH-20 eluting with 50% CH_3CN in 0.1 M AcOH. Further purification was carried out by reverse phase high performance liquid chromatography (HPLC) using the column of Nucleosil 5C18 (10.7 x 250 mm) under the gradient elution of aqueous CH_3CN containing 0.1% of TFA. The elution pattern of F7-SP, F8-SP, F7,8-SP, and native SP are shown in Fig. 1. Amino acid composition of the acid hydrolysates (6 M HCl, 24 h at 110 °C) of the synthetic peptides were consistent with the calculated values.

The pharmacological activities of F7-SP, F8-SP, and F7,8-SP were determined by the contracting activities on guinea pig ileum. Longitudinal strip taken from guinea pig ileum was placed in a 5 ml organ bath containing Tyrode's solution under bubbling through the gas mixture of O_2 (95%) and CO_2 (5%) at 37 °C. The muscle preparation was equilibrated for 60 min. The contractions were recorded isotonically under a resting load of 0.5 - 1.0 g using FD-pick up (TB-612T, Nihon Koden) connected with carrier amplifier (AP801G, Nihon Koden) and recorder. Peptides were applied at 20 - 30 min intervals. Relative potency was determined as the ratio of EC_{50} of SP / EC_{50} of the synthetic peptides, where EC_{50} is the concentration of the peptide required to give a half maximum response. The contracting activities of the synthetic fluorine containing SP related peptides were approximately same with

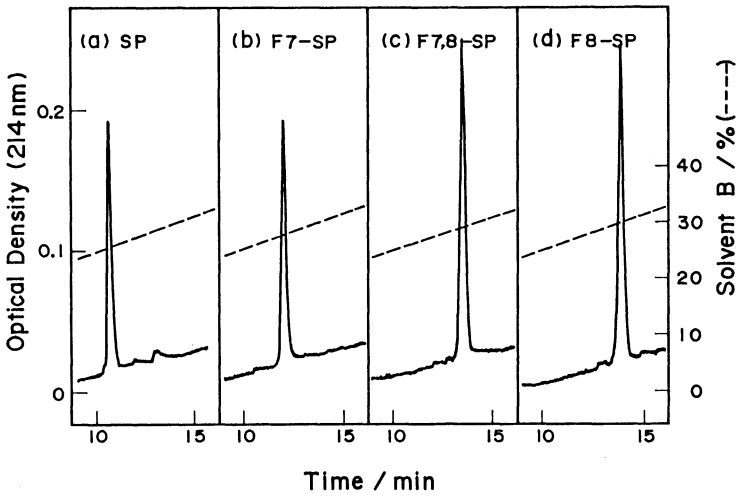

Fig. 1. Analytical HPLC of SP(a), F7-SP(b), F8-SP(c), and F7,8-SP(d). Column: TSK gel ODS--80TM (4.6 x 150 mm), Eluant: (A) 0.1% TFA, (B) 80% CH_3CN containing 0.1% TFA, Flow rate: 1.0 ml/min.

Table 1. Contractile activities[a] of SP, F7-SP, F8-SP, and F7,8-SP on guinea pig ileum

	EC_{50}	Relative-potencies
SP	2.1 x 10^{-9}M	1.00
F7-SP	1.9 x 10^{-9}M	1.11
F8-SP	2.3 x 10^{-9}M	0.95
F7,8-SP	2.0 x 10^{-9}M	1.05

a) n= 4-6.

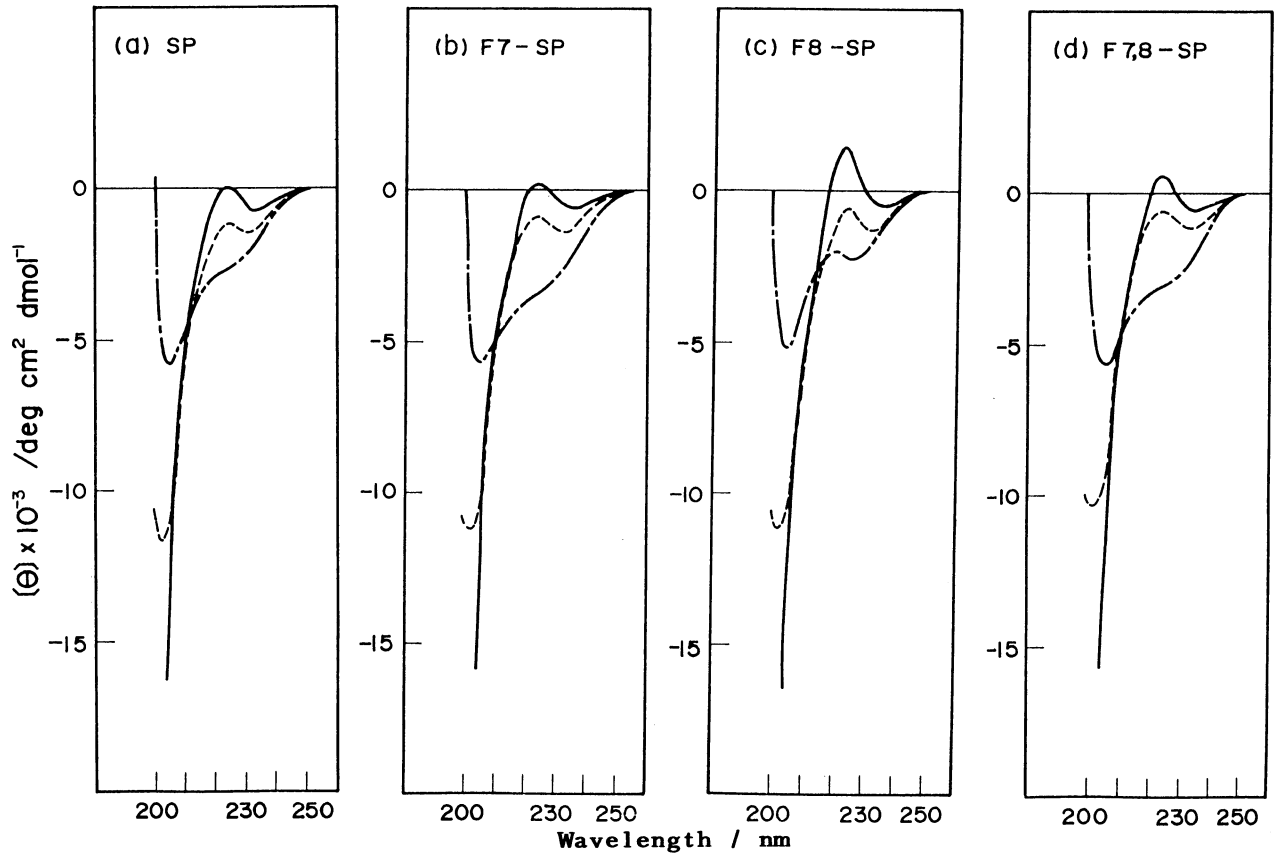

Fig. 2. CD spectra of SP(a), F7-SP(b), F8-SP(c), and F7,8-SP(d) in 50 mM Tris-HCl buffer (pH 7.2 containing 50 mM NaCl, 50 μM: ——), in 90% MeOH (50 μM:----) and in 50% TFE (50 μM:—·—·). The molar ellipticity [Θ] is divided by the number of amino acid residues, 11.

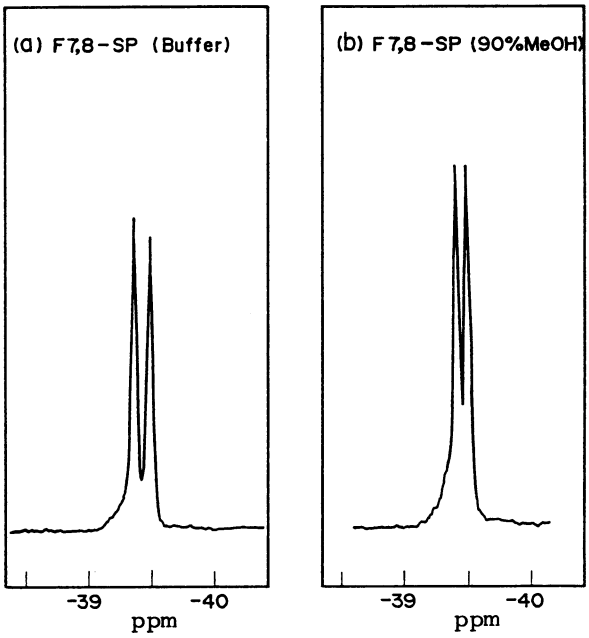

Fig. 3. ^{19}F-NMR spectra of F7,8-SP in Tris-HCl buffer (a) and in 90% MeOH (b) measured at 376 MHz under proton noise decoupling at 25 °C.

Table 2. ^{19}F chemical shifts (ppm)[a] of fluorine containin SP analogues in Tris-buffer and in 90% MeOH (376 MHz)

	Tris-buffer[b]	90% MeOH	Δδ[c]
F7-SP	-39.405	-39.331	0.074
F8-SP	-39.532	-39.402	0.130
F7,8-SP (Phe^7)	-39.357	-39.305	0.052
F7,8-SP (Phe^8)	-39.516	-39.415	0.101

a) ^{19}F chemical shifts (ppm) measured from CF_3CH_2OH. b) 50 mM Tris-HCl containing 50 mM NaCl, pH 7.2. c) Differences of ^{19}F chemical shift between Tris-buffer and 90% MeOH.

native SP as shown in Table 1.

The CD spectra of F7-SP, F8-SP, F7,8-SP and native SP in aqueous buffer (Tris-HCl, pH 7.2), in 90% MeOH and in 50% TFE were measured. As shown in Figs. 2(a) - 2(d), all four spectra resembled closely. In the Tris-buffer, the peptides are considered to be random coil by evaluating the curve pattern while in 90% MeOH, the peak at ca 220 nm lowered, the depth of the trough at ca 200 nm diminished and the inflexion point of the curve exhibited blue-shift. The tendency was enhanced preferentially in 50% TFE. These facts suggest the increase of ordered conformation especially of α-helical properties[5] in 90% MeOH as well as in 50% TFE. Since all four peptides showed nearly the same behaviors in CD-spectra, the attachment of fluorine atom at Phe^7 and Phe^8 or at both positions in SP molecule was proven to cause no significant influence on the shape of SP in solution.

On the other hand, unequivocal differences in ^{19}F chemical shifts among the three SP analogues were observed in ^{19}F NMR measured at 376 MHz on JEOL GX-400 both in aqueous solution and in 90% MeOH. The ^{19}F NMR spectra of F7,8-SP are shown in Fig. 3 and the ^{19}F chemical shifts of the SP analogues from CF_3CH_2OH as internal standard are compiled in Table 2. The ^{19}F signals of F7-SP and F8-SP are evidently distinguishable and assignable. Thus the application of ^{19}F labelled SP analogues will be especially useful in the study of interaction between SP and its receptor molecule, since ^{19}F signals are fully assignable without any complexity due to overlapping signals.

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