Exploration of the Role of Phenylalanine in the Thrombin Receptor Tethered-Ligand Peptide by Substitution with a Series of Trifluorophenylalanines

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(Received April 13, 2000)

The thrombin receptor-tethered ligand SFLLRNP (abbreviation formed by one letter amino acid codes expressing Ser–Phe–Leu–Leu–Arg–Asn–Pro) consists of the Phe-2 residue essential for the receptor activation. In order to explore the molecular characteristics of this Phe-2-phenyl, a series of trifluorophenylalanines [(F_3)Phe] was incorporated into this S/Phe/LLRNP for evaluation of their ability to induce the human platelet aggregation. A complete set of (F_3)Phe in the L-configuration, namely, (2,3,4-F_3)Phe, (2,3,5-F_3)Phe, (2,3,6-F_3)Phe, (2,4,5-F_3)Phe, (2,4,6-F_3)Phe, and (3,4,5-F_3)Phe, was prepared from trifluorobenzyl bromides and diethyl acetamidomalonate. S/(2,3,4-F_3)Phe/LLRNP was equipotent to S/Phe/LLRNP, while (2,4,5-F_3)Phe-containing analog was almost twice as potent as those. (2,4,6-F_3)Phe-analog exhibited about a half of the activity of S/Phe/LLRNP. (3,4,5-F_3)Phe-, (2,3,5-F_3)Phe-, and (2,3,6-F_3)Phe-analogs were very weak. The analysis of these assay results suggested that Phe-2-phenyl of SFLLRNP is in the edge-to-face CH/π interaction with the receptor aromatic group, utilizing the Phe-2-phenyl edge along with benzene hydrogens at position 2–3 or 5–6. The computer-assisted semi-empirical molecular orbital calculations by MOPAC showed that the fluorine atom decreases the electron density of its *ortho*, *meta*, and *para* hydrogens, and thus increases their acidity more strongly in that order. All these suggested that H → F replacements reinforce the edge-to-face CH/π interaction to enhance biological activity. The H → F replacement on the Phe–phenyl group was found to render an effective structural examination; i.e., to identify the hydrogens in the CH/π interaction, and to intensify the CH/π interaction.

In the ligand/receptor interaction, amino acid residues of the peptide ligand are involved in the specific interactions with each receptor counterpart. The electrostatic bonding, hydrogen bonding, and hydrophobic bonding are major constituents of such specific interactions. Among these interactions, the nature of hydrophobic bondings is not so definitive. The hydrophobic interactions are usually constructed among hydrophobic amino acids, which possess alkyl or aromatic side chains. Phenylalanine (Phe),[1] a hydrophobic amino acid and also grouped into aromatic amino acids, has the phenyl group at the side chain, and its phenyl-benzene ring is counted upon to play an important role in the intermolecular interaction of peptides and proteins. The Phe residue in the biologically active peptides is often crucially important to elicit an intrinsic activity.[2,3] However, the molecular mechanism of Phe-phenyl in the peptide ligands has never been elucidated in detail.

The structure and activation mechanism of thrombin receptor is extraordinary.[4] Thrombin receptor is activated by a serine proteinase thrombin, which binds to the receptor to cleave the peptide bond between Arg-41 and Ser-42. A newly exposed N-terminal moiety of the thrombin receptor, Ser–Phe–Leu–Leu–Arg–Asn–Pro (SFLLRNP), functions as a ligand to activate the receptor by itself.[4] The receptor is also activated with exogenously administered synthetic peptide SFLLRNP with no thrombin. This has allowed the extensive structure-activity studies for exploration of the structural essentials of receptor-tethered SFLLRNP,[5—11] and the Phe-2-phenyl group has been recognized as one of the most important structural elements (SFLLRNP is denoted hereafter as S/Phe/LLRNP). We reported that *para*-fluorophenylalanine [(4-F)Phe] at position 2 of S/Phe/LLRNP enhances the activity several times in the assays of phosphoinositide-turnover in human epithelial-like SH-EP cells[12] and of human platelet aggregation. (4-F)Phe is now utilized for almost all the synthetic peptide analogs of thrombin receptor-tethered ligands to substantiate this high level of potency.[13—16] In our recent efforts to clarify the genuine functional role of Phe, we have suggested the presence of the edge-to-face π/π interaction between the Phe-phenyl group of thrombin receptor-tethered ligand peptide and the receptor aromatic group.[17]

Phenylalanine can be involved in two different types of the π/π interactions; i.e., the face-to-face π/π stacking interaction and the edge-to-face π/π interaction (Fig. 1).[18] In the edge-to-face π/π interaction, Phe-phenyl acts as a donor of benzene-CHs. Thus, this edge-to-face π/π interaction is also denoted as an edge-to-face CH/π interaction. We have postulated that these two types of the π/π interactions can

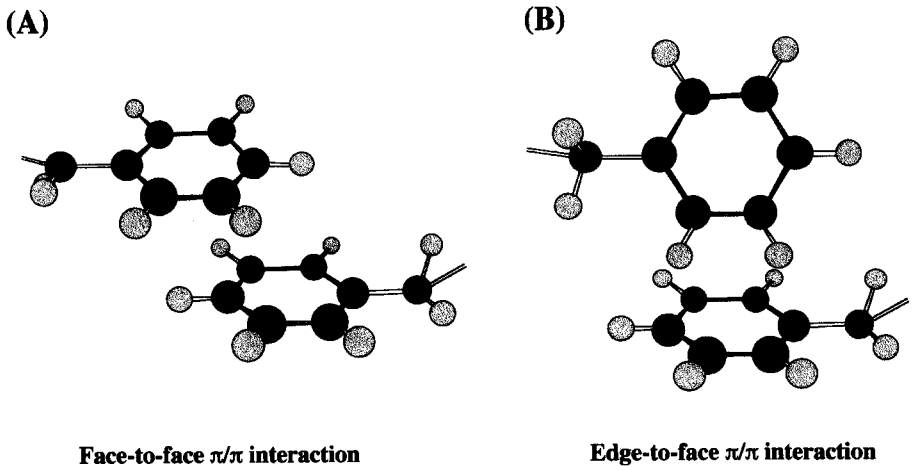

Fig. 1. The putative modes of π/π interaction between Phe-phenyls. (A) the face-to-face π/π stacking interaction, and (B) the edge-to-face CH/π (or π/π) interaction.

be differentiated by replacing Phe with fluorophenylalanines (F_n)Phe.[17] Fluorine can replace the benzene hydrogens (CHs) without changing the atomic size, because the van der Waals radii of fluorine and hydrogen atoms are similar to each other (1.35 Å for fluorine and 1.20 Å for hydrogen). Multiple fluorine replacements would clarify whether essential hydrogens exist on Phe-phenyl or whether the π system is required for the interaction with the receptor. In the present study, a complete set of trifluorinated phenylalanines, trifluorophenylalanines [(F_3)Phe] (Fig. 2), were prepared and incorporated into the peptide S/Phe/LLRNP. Peptides were evaluated in the assay for human platelet aggregation, and the Phe-phenyl CHs in the edge-to-face CH/π interaction were identified as structural essentials for eliciting a biological activity.

Experimental

Materials. Boc-L-(2,3,4,5,6-F_5)Phe–OH and Boc-L-cyclohexylalanine (Boc–Cha–OH) were purchased from Watanabe (Hiroshima). Pentafluorophenylalanine (2,3,4,5,6-F_5)Phe is denoted merely as (F_5)Phe hereafter, because only one isomer is present. Boc-derivatives of other ordinary amino acids and *p*-methylbenzhydrylamine (MBHA) resin were obtained also from Watanabe. Pre-coated silica gel plates for high-performance thin-layer chromatography (HPTLC) were purchased from Merck (Darmstadt, Germany), and the solvent system for HPTLC was 1-butanol : acetic acid : pyridine : water = 4 : 1 : 1 : 2 (v/v). All the chemicals were of the best grade available.

Synthesis of Trifluorophenylalanine [(F_3)Phe]. Diethyl 2-Acetamido-2-(trifluorobenzyl)malonates. The synthesis and optical resolution of trifluorophenylalanines [(F_3)Phe] were carried out by the method essentially as described.[19] Six different trifluorobenzyl bromides **1** (120 mmol) (JRD Fluoro-chemicals Ltd., Surrey, UK) were coupled with diethyl acetamidomalonate (100 mmol), respectively, in abs. EtOH suspended with Na (120 mmol) or NaOEt (120 mmol) by refluxing for 2 h. After filtration and evaporation, the residue was treated with cold water to collect the precipitate, which was recrystallized from EtOH–water to afford diethyl 2-acetamido-2-(trifluorobenzyl)malonate **2** (av yield, 87%).

Acetyl-DL-trifluorophenylalanines. Diethyl ester **2** (70 mmol) was then subjected to a simultaneous saponification in MeOH (200 ml)/2 M NaOH (280 mmol) at 40 °C for 3 h (M = mol dm^{-3}). The resulting malonic acid derivatives **3** were liberated by 6 M HCl and extracted with ethyl acetate. Since **3** appeared to be unstable, the residue of evaporation was directly refluxed in *para*-xylene for 2 h to afford decarboxylated acetyl-DL-trifluorophenylalanines (Ac-DL-(F_3)Phe, **4**; av yield, 95%).

Optical Resolution and Boc-Protection. Ac-DL-(F_3)Phe was digested with *Aspergillus genus* acylase (Tokyo Chemical Industry) to resolve into Ac-D-(F_3)Phe and L-(F_3)Phe. To a solution (pH 8.0) of Ac-DL-(F_3)Phe (50 mmol) in 0.2 M NaOH (275 ml) was added acylase (400 mg) in 0.2 M AcONa, and the solution was incubated at 38 °C for 48 h. After filtration of activated charcoal for removal of the enzyme, Ac-D-(F_3)Phe was extracted with EtOAc from the aqueous solution (pH 3.0) and purified by recrystallization (av yield, 92%). The liberated free amino acid L-(F_3)Phe was collected and purified by applying to a column (3.8×22 cm) of Dowex 50×8 (H^+ form) eluted with 2 M NH_4OH (av yield after recrystalization, 80%). Optical purity of L-(F_3)Phe was confirmed on a high-performance liquid chromatography (HPLC) using a chiral column SUMICHIRAL OA 5000 (0.46×15 cm, 5 μm; Sumika Chemical Analysis Service, Osaka). Boc-derivatives were prepared by using

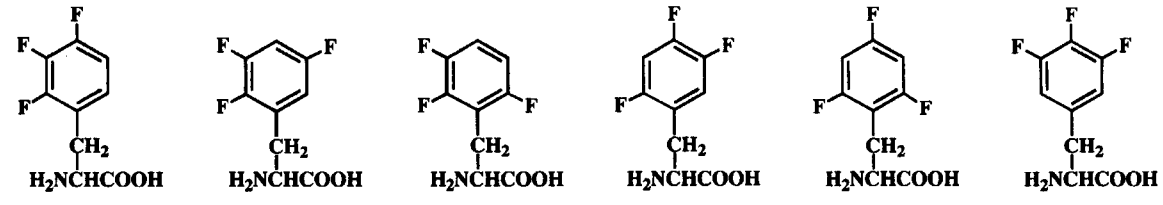

Fig. 2. Chemical structure of all six structural isomers of trifluorophenylalanines [(F_3)Phe].

di-*t*-butyl dicarbonate according to the method reported (av yield, 94%).[20] The analytical data for each series of compounds are shown in Tables 1 and 2.

Peptide Synthesis. Analogs of S/Phe/LLRNP were synthesized by the manual method for solid-phase peptide synthesis.[10] The side chain protecting groups of Boc-amino acids were benzyl for Ser and *p*-tolylsulfonyl (Tos) for Arg. To obtain C-terminal peptide amides, Boc–Pro–MBHA resin was utilized. Coupling reactions (0.1 mmol scale) were carried out with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxybenzotriazole (HOBt) in a mixed solvent of *N*-methylpyrrolidone and *N*,*N*-dimethylformamide (1 : 2, v/v) for 30 min. Peptides were liberated from the resin by treatment with anhydrous liquid hydrogen fluoride containing 10% *p*-cresol at 0 °C for 1 h, and purified by gel filtration with Sephadex G-15 followed by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) (Cica–Merck, LiChrospher 100 RP-18 (e) (5 μm): 25×250 mm). The elution conditions employed were as follows: solvent system, 0.1% aqueous trifluoroacetic acid (TFA)-(A solution) and acetonitrile containing 20% A solution-(B solution); flow rate, 5 ml min^{-1}; temperature, 25 °C; UV detection, 225 nm. Elution was performed with a linear concentration gradient of the B solution (20—60%) over 40 min.

The purity of peptides was verified by analytical RP-HPLC (LiChrospher 100 RP-18 (e) (5 μm): 4.0×250 mm, under the same conditions except for a flow rate of 0.75 ml min^{-1}. For amino acid analyses, the hydrolysis of peptide samples was carried out in constant-boiling hydrochloric acid (110 °C, 24 h). The amino acids analyses were carried out on a Hitachi L-8800 amino acid analyzer. Mass spectra of peptides were measured on a mass spectrometer Voyager™ DE-PRO (PerSeptive Biosystems Inc., Framingham, MA) with the method of matrix assisted laser desorption ionization time-of-flight (MALDI-TOF).

Platelet Aggregation Assay. The assay was carried out essentially as previously described.[21] Briefly, blood was obtained from healthy donors who denied taking any medication during the previous week. The collected blood was anticoagulated with citrate (1 part 3.8% sodium citrate to 9 parts blood), and platelet-rich plasma (PRP) was obtained by centrifugation at 80 × *g* for 10 min. Platelet-poor plasma (PPP) was also prepared by centrifugation at 2,000 × *g* for 10 min. The prepared PRP was used within 4 h, and the aggregation test was carried out at 37 °C by the standard turbidimetric procedure using NKK hema tracer PAT-4M (MC Medical, Tokyo), PPP being used as a reference. The peptide concentration required for half-maximal platelet aggregation was obtained by three determinations.

MOPAC Calculation. The semi-empirical molecular orbital calculation was carried out using MOPAC version 6.00 with PM3 parameters running on the workstation of the Silicon Graphics O_2.[22] The values of net atomic charges and dipole contributions were obtained for each atom of phenylalanines, and the effects of H → F replacements on the atom electron density were calculated for the fluorophenyl groups by subtracting each value of Phe-phenyl.

Results and Discussion

Synthesis of Trifluorophenylalanines and S/(F_3)-Phe/LLRNP Peptides. In the present study, we achieved the synthesis of all six isomers of optically pure L-trifluorophenyl-alanines [(F_3)Phe]. Optical purity of obtained L-(F_3)Phe was confirmed on a chiral column HPLC showing no

Table 1. The Melting Points of Compounds Related to Trifluorophenylalanines

	Malonate **2**[a]	Ac–DL-(F_3)Phe	Ac–D-(F_3)Phe	L-(F_3)Phe	Boc–L-(F_3)Phe
2,3,4-F_3	142[b]	153	133	226[c]	63
2,3,5-F_3	138	156	168	216[c]	116
2,3,6-F_3	116	173	163	230[c]	85
2,4,5-F_3	141	143	169	224[c]	106
2,4,6-F_3	91	181	173	217[c]	110
3,4,5-F_3	153	164	158	211[c]	110

The data of elemental analyses indicated that the values analyzed were consistent with those calculated. R_f values in HPTLC were measured in the solvent system of 1-butanol : acetic acid : pyridine : water = 4 : 1 : 1 : 2 (v/v), and those were 0.88—0.95 for malonate **2**, 0.57—0.64 for Ac–DL-(F_3)Phe, 0.58—0.65 for Ac–D-(F_3)Phe, 0.44—0.48 for L-(F_3)Phe, and 0.76—0.79 for Boc–L-(F_3)Phe. Mass spectra of L-(F_3)Phe were measured on a MALDI-TOF, and the values observed were consistent with that calculated (*m*/*z* 220.19 as $(M+H)^+$. a) Diethyl 2-acetamido-2-(trifluorobenzyl)malonate. b) The values indicate the mean with ± 1 °C. c) Decomp.

Table 2. Optical Properties of Trifluorophenylalanines and Their Derivatives

	Retention time in HPLC[a]		Optical rotation $[\alpha]_D^{20}$/°		
	L-(F_3)Phe	D-(F_3)Phe[b]	Ac–D-(F_3)Phe[c]	L-(F_3)Phe[d]	Boc–L-(F_3)Phe[c]
2,3,4-F_3	33.7	41.1	−25.3	−32.7	−1.2
2,3,5-F_3	21.7	28.5	−28.8	−4.3	−4.0
2,3,6-F_3	11.8	16.5	−16.8	+15.0	−13.3
2,4,5-F_3	20.8	28.0	−26.9	−14.7	−3.5
2,4,6-F_3	10.9	15.6	−17.4	+11.8	−6.9
3,4,5-F_3	47.6	72.7	−26.6	−21.2	+2.0

a) The data of retention time (min) were obtained on a chiral column SUMICHIRAL OA 5000 (0.46×15 cm, 5 μm). b) D-(F_3)Phe for this HPLC analysis was obtained from Ac-D-(F_3)Phe by the treatment with 4 M HCl-dioxane (1:1, v/v) under reflux for 5 h (av yield, 94%). c) *c* 1.0, MeOH. d) *c* 1.0, H_2O.

contamination of the D-isomer and any other materials. The synthetic route is shown in Fig. 3 and the physical constants of the products and intermediates are shown in Tables 1 and 2. This procedure starting from the coupling between fluorobenzyl bromides and diethyl acetamidomalonate were also applied successfully for the syntheses of L-difluorophenylalanines [(F_2)Phe][8] and appeared to be excellent to prepare the aromatic amino acids.

In this study, we further prepared Boc derivatives of all of L-(F_3)Phe isomers, and these Boc–L-(F_3)Phe isomers were used for the syntheses of thrombin receptor-tethered ligand peptide S/Phe/LLRNP. Although Boc–L-(3,4,5-F_3)-Phe are available from several commercial sources, the data of physicochemical constants of free amino acid L-(3,4,5-F_3)Phe and even of Boc–L-(3,4,5-F_3)Phe are not available to date. Thus, we included those in the tables (Tables 1 and 2). Eventually, all six L-(F_3)Phe isomers were incorporated into the position 2 of S/Phe/LLRNP to replace Phe. Peptides were synthesized by the manual solid phase synthesis method without any troubles (Table 3).

Full Activation by Substitution of (F_5)Phe with (F_3)-Phe. Table 4 shows the platelet aggregation activity of a series of analogs of S/Phe/LLRNP, in which Phe-2 was replaced by various amino acids. With the inactivity of S/Ala/LLRNP and drastically reduced activity of S/Cha/LLRNP, the fundamental structural importance of Phe-phenyl and its aromaticity in the thrombin receptor activation have been acknowledged.[17] Cha possesses the cyclohexyl group, a saturated form of the phenyl group, and is nearly isosteric with Phe. Since Cha lacks the quadrupole moment associated with an aromatic ring, it is devoid of the aromaticity. The importance of benzene hydrogens (CH) of Phe-2 in S/Phe/LLRNP was demonstrated by a series of analogs containing mono-, di-, and pentafluorophenylalanines.[17]

In the present study, inactivity of S/(F_5)Phe/LLRNP was confirmed again (Table 4). S/(F_5)Phe/LLRNP exhibited absolutely no activation in the human platelet aggregation assay

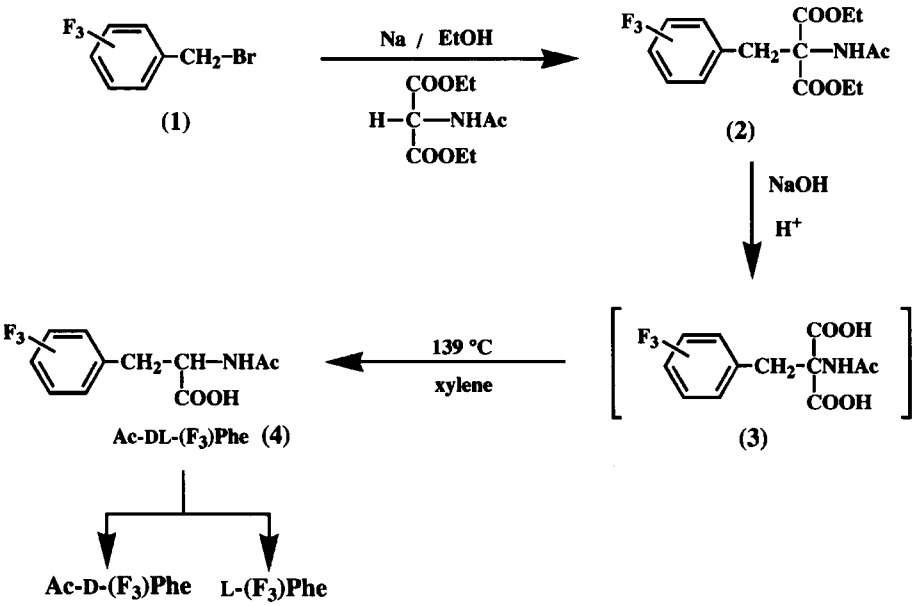

Fig. 3. Synthetic route of trifluorophenylalanines [(F_3)Phe].

Table 3. The Analytical Data of Synthetic Peptides S/(F_3)Phe/LLRNP from the Mass Spectrometry, Reversed-Phase High-Performance Liquid Chromatography, and Amino Acid Analysis together with Synthetic Yield

Peptides[a]	Yield[b]	MALDI-TOF-MS[c]	RP-HPLC[d]	Amino acid analysis[e]					
	%	Found	RT/min	Ser	(F_3)Phe	Leu	Arg	Asn	Pro
(2,3,4-F_3)Phe	26	900.16	27.3	0.89	0.92	2.00	1.00	0.95	1.03
(2,3,5-F_3)Phe	20	900.00	26.0	1.05	0.92	2.00	1.11	1.07	1.03
(2,3,6-F_3)Phe	25	899.73	24.9	0.96	1.00	2.00	1.02	0.99	1.02
(2,4,5-F_3)Phe	29	899.88	26.2	0.95	1.10	2.00	1.03	10.7	1.06
(2,4,6-F_3)Phe	28	899.96	25.1	0.94	0.94	2.00	1.01	0.97	1.03
(3,4,5-F_3)Phe	29	900.05	29.4	0.93	0.98	2.00	0.97	0.97	0.94

a) Peptides S/(F_3)Phe/LLRNP are shown by the amino acid residue of (F_3)Phe. b) Total yield from the Boc–Pro–MBHA resin. c) Values express the mass number (*m/z*) of $(M+H)^+$. Calculated value is 900.01. d) Retention time (RT) was measured on an analytical column (Cica–Merck, LiChrospher 100 RP-18 (e) (5 μm): 4.0×250 mm) with a linear gradient of 0.1% TFA and 80% acetonitrile. e) Amino acid analysis was carried out by the ion-exchange method with ninhydrin colorimetric determination, and the values were normalized for leucine as an internal standard.

Table 4. The Biological Activity of Synthetic Peptides S/(F_3)Phe/LLRNP in the Human Platelet Aggregation

Peptides[a)]	EC_{50} μM	Relative potency %
Phe	2.7 ± 1.1	100
Ala	inactive	0
Cha	86 ± 7.1	3
(F_5)Phe	inactive	0
(2,3,4-F_3)Phe	2.9 ± 0.1	98
(2,3,5-F_3)Phe	130 ± 3.5	2
(2,3,6-F_3)Phe	35 ± 8.0	8
(2,4,5-F_3)Phe	1.5 ± 0.35	180
(2,4,6-F_3)Phe	5.7 ± 0.35	49
(3,4,5-F_3)Phe	18 ± 2.1	16
(2,3-F_2)Phe	49 ± 16	6
(2,4-F_2)Phe	1.8 ± 0.12	150
(2,5-F_2)Phe	7.6 ± 1.3	35
(2,6-F_2)Phe	25 ± 0.35	11
(3,4-F_2)Phe	2.0 ± 0.35	140
(3,5-F_2)Phe	18 ± 2.1	15
(2-F)Phe	9.4 ± 4.6	29
(3-F)Phe	4.6 ± 2.7	59
(4-F)Phe	1.1 ± 0.47	250

Biological activities of the six different isomers of S/(F_2)-Phe/LLRNP and three isomers of S/(F)Phe/LLRNP were previously reported in the literatures (Refs. 17 and 24). a) Peptides S/Xaa/LLRNP are shown by the amino acid residue of Xaa.

(Fig. 4). The incorporation of (F_5)Phe would affect the interaction with the receptor in at least two different ways. Since the quadrupole moment of Phe should be reversed by substituting with (F_5)Phe,[23] the change in quadrupole moment of the ring puts the (F_5)Phe ring at a disadvantage. When the (F_5)Phe ring mimics sterically the orientation of Phe in S/Phe/LLRNP, the ring quadrupole moment would be inverted as compared to S/Phe/LLRNP. In another case, the (F_5)Phe ring would turn through 90° and thereby align itself

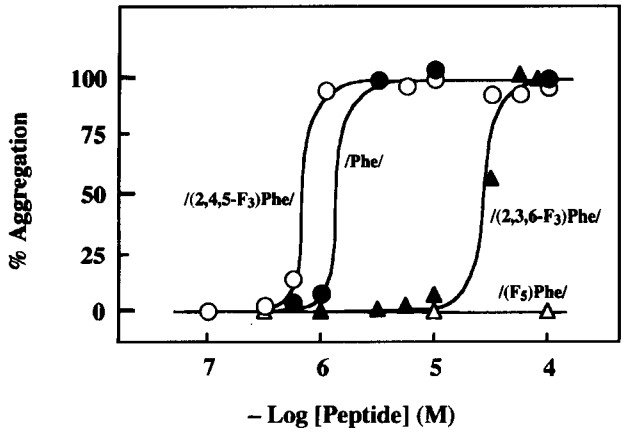

Fig. 4. Dose-response curves of S/Phe/LLRNP and its analogs in human platelet aggregation assay. ○—○: S/(2,4,5-F_3)Phe/LLRNP, ●—●: S/Phe/LLRNP, ▲—▲: S/(2,3,6-F_3)Phe/LLRNP, and △—△: S/(F_5)Phe/LLRNP.

according to the electrostatic influences of the quadrupole moment. These conditions would lead the peptide to the conformation unfavorable to interact with the receptor. As to (F_3)Phe ring, however, the differences in the ring quadrupole moments of trifluorophenyl rings and in the dipole moments of (F_3)Phe residues are very subtle among six (F_3)Phe isomers, and it is unlikely that such subtle differences provide the conformations with varied activities in a huge range, 2—180% activity of parent S/Phe/LLRNP as seen in Table 4. Indeed, in spite of almost the same dipole moments of (2,4,5-F_3)Phe and (2,4,6-F_3)Phe, their peptides exhibited drastically different potencies, namely, 180% and 49% activity of parent S/Phe/LLRNP.

Alternatively, if Phe-phenyl requires its benzene hydrogens for CH/π interaction with the receptor, (F_5)Phe would be unable to interact with the receptor owing to the replacement of hydrogens with fluorine. Thus, (F_5)Phe/Phe-substitution would disconnect such essential CH/π interaction, resulting in inactivity. The fact that all (F_3)Phe-containing peptides could elicit a complete aggregation of the platelets strongly suggests that benzene CHs of Phe-2-phenyl are involved in the interaction with thrombin receptor. As six different ways exist to place three fluorine atoms on the Phe-phenyl group, six different ways exist to place back two hydrogens to the (F_5)Phe-phenyl group. It should be noted that, in spite of a total lack of basal activation by S/(F_5)-Phe/LLRNP, all these six (H_2,F_3)Phe-containing peptides activated fully the human platelets, showing the activation from the basal to 100% aggregation of platelets. Figure 4 shows the dose-response curves of four typical S/Phe/LLRNP related peptides, namely, S/Phe/LLRNP itself, highly potent S/(2,4,5-F_3)Phe/LLRNP, considerably less potent S/(2,3,6-F_3)Phe/LLRNP, and inactive S/(F_5)Phe/LLRNP.

Structure–Activity Relationships of S/(F_3)Phe/LLRNP. S/(2,3-F_2)Phe/LLRNP was very weak (about 6% of the parent S/Phe/LLRNP) in the human platelet aggregation assay (Table 4).[24] When the third fluorine is placed on the benzene ring of (2,3-F_2)Phe-phenyl, three different places are available, namely, the positions 4, 5, and 6. It was found that S/(2,3,5-F_3)Phe/LLRNP and S/(2,3,6-F_3)Phe/LLRNP are very weak, showing only 2% and 8% activities of S/Phe/LLRNP, respectively (Table 4). Placement of fluorine at positions 5 and 6 indeed induced no reinforcement in activity. In contrast, S/(2,3,4-F_3)Phe/LLRNP was found to be 16 times more potent than S/(2,3-F_2)Phe/LLRNP, and became almost equipotent to parent S/Phe/LLRNP. Apparently, unlike the positions 5 and 6, H → F replacement at position 4 enhances greatly the activity in platelet aggregation. Similar activity enhancement by H → F replacement at position 4 was also previously observed for S/(4-F)Phe/LLRNP, S/(2,4-F_2)Phe/LLRNP, and S/(3,4-F_2)-Phe/LLRNP (Table 4).[24] S/(4-F)Phe/LLRNP was 2.5-fold more potent than S/Phe/LLRNP, and this activity enhancement was observed only for (4-F)Phe-containing analog, but not for (2-F)Phe- and (3-F)Phe-containing analogs. S/(2,4-F_2)Phe/LLRNP and S/(3,4-F_2)Phe/LLRNP were about 1.5-fold more potent than S/Phe/LLRNP, but they were 5.2- and

2.3-fold more potent than S/(2-F)Phe/LLRNP and S/(3-F)-Phe/LLRNP, respectively.[24]

S/(2,3,4-F_3)Phe/LLRNP possesses the benzene CHs at positions 5 and 6. Again, it should be noted that S/(F_5)-Phe/LLRNP was completely inactive. When two hydrogens were placed back at positions 5 and 6 on the pentafluorophenyl group of S/(F_5)Phe/LLRNP, the resulting S/(2,3,4-F_3)Phe/LLRNP became fully active. This clearly indicates that these hydrogens are crucially important to interact with the thrombin receptor. When benzene CHs are in the intermolecular interaction, they are in a so-called CH/π interaction, in which benzene CHs participate in a kind of hydrogen bonding with the complementary aromatic ring. Since CHs are present at the edge of the benzene aromatic ring, this interaction is also called an edge-to-face CH/π interaction as mentioned earlier. In Fig. 5A, the edge-to-face CH/π interaction in which both hydrogens 5-H and 6-H of (2,3,4-F_3)Phe in S/(2,3,4-F_3)Phe/LLRNP participate are depicted to show a fully accessible form in interacting with the receptor aromatic group.

Enhanced Activity of S/(2,4,5-F_3)Phe/LLRNP by Edge-to-Face CH/π Interaction. The S/(F_3)Phe/LLRNP isomers in which the (F_3)Phe residue possesses the fluorine atom at position 4 of phenyl benzene ring are S/(2,4,5-F_3)Phe/LLRNP, S/(2,4,6-F_3)Phe/LLRNP, and S/(3,4,5-F_3)-Phe/LLRNP, besides S/(2,3,4-F_3)Phe/LLRNP (Fig. 6). The benzene ring of (2,4,6-F_3)Phe has hydrogens at the *meta* positions 3 and 5, and S/(2,4,6-F_3)Phe/LLRNP exhibited the activity about a half (49%) of S/Phe/LLRNP (but about 4.5 times more potent than S/(2,6-F_2)Phe/LLRNP). (3,4,5-F_3)-Phe consists of hydrogens at the *ortho* positions 2 and 6, and the activity of S/(3,4,5-F_3)Phe/LLRNP was much reduced (16% of S/Phe/LLRNP and almost equipotent to S/(3,5-F_2)-Phe/LLRNP). If the fluorine atom acts just to disconnect the CH/π interaction, these results suggest that the contribution of hydrogens to the CH/π interaction is greater at the *meta* positions than at the *ortho* positions.

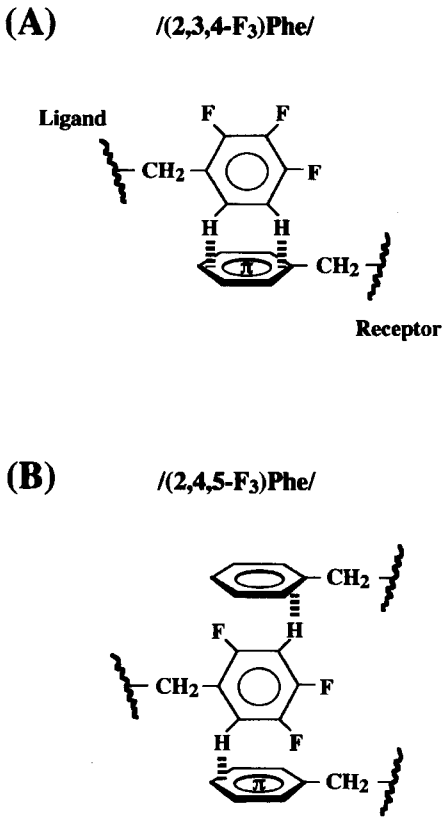

Fig. 5. Schematic illustration showing putative edge-to-face CH/π interactions between ligand (F_3)Phe-phenyl and receptor aromatic group. (A) double edge-to-face CH/π interactions by (2,3,4-F_3)Phe, and (B) two single edge-to-face CH/π interactions by (2,4,5-F_3)Phe at both edges. The receptor aromatic group is shown in a symbolic form with the letter π in the benzene ring.

In contrast to these inferior activities, S/(2,4,5-F_3)-Phe/LLRNP was found to be very potent (180%). This high activity of S/(2,4,5-F_3)Phe/LLRNP clearly indicates that the effect of H → F replacement is not only a disconnection of the CH/π interaction, but also an activity enhancement. An enhanced activity of S/(2,4,5-F_3)Phe/LLRNP seemed to be explained by at least two structural conditions, namely, the edge availability and CH acidity. The thought of the "edge availability" is based on the idea of accessibility of benzene-hydrogens for the CH/π interaction. As mentioned above, among five benzene-hydrogens, 4-H at the *para* position is not involved in the CH/π interaction. The other four hydrogens are able to be in the CH/π interaction and are present at the two different edges; i.e., the edge along with the position (2–3) and the one along with the position (5–6). (2,4,5-F_3)-Phe has two benzene-hydrogens in both edges, 3-H present at the *meta* position in the (2–3)-edge and 6-H at the *ortho* position in the (5–6)-edge. In the case of (2,4,6-F_3)Phe and (3,4,5-F_3)Phe, however, two benzene-hydrogens are present simultaneously at the *meta* positions and at the *ortho* positions, respectively, as described above. Since these structural differences eventually resulted in different extents of biological activity, it appears that the relative location of hydrogens in the benzene ring is a key structure to substantiate the CH/π interaction essential for receptor activation.

It is evident that (2,4,5-F_3)Phe stabilizes the peptide/receptor interaction more strongly than (2,4,6-F_3)Phe and (3,4,5-F_3)Phe. If the (2,4,5-F_3)Phe-phenyl group utilizes either 3-H or 6-H for the CH/π interaction, no such difference should emerge between S/(2,4,5-F_3)Phe/LLRNP and S/(2,4,6-F_3)Phe or (3,4,5-F_3)Phe/LLRNP. This is because these three trifluorophenylalanines share at least one hydrogen at either the (2–3)- or the (5–6)-edge. These results strongly suggest that both hydrogens 3-H and 6-H of (2,4,5-F_3)Phe-phenyl are involved simultaneously in the receptor interaction as shown in Fig. 5B, while the combinations of hydrogens (3-H and 5-H) for (2,4,6-F_3)Phe and (2-H and 6-H) for (3,4,5-F_3)Phe are disadvantageous for the interaction.

The phenyl group of (2,3,4-F_3)Phe has only one edge, but consists of two hydrogens (Fig. 6). The phenyl groups of (2,3,5-F_3)Phe and (2,3,6-F_3)Phe also have one edge consisting of one hydrogen. These structural features might be disadvantageous to gain an effective interaction to bind to the

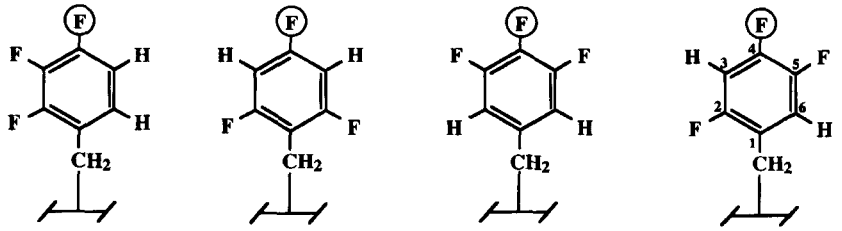

Fig. 6. Structural comparison of (F_3)Phe in S/(F_3)Phe/LLRNP: Availability of edges loading hydrogen and fluorine atoms. The structures of side chain of (F_3)Phe in S/(F_3)Phe/LLRNP are shown, and the fluorine atom at the *para* position was marked by the circle. % activity exhibits the potency of each peptide ligand relative to S/Phe/LLRNP.

receptor.

Reinforcement of CH/π Interaction. When Phe can form double CH/π interactions using two hydrogens, for example, at positions 5 and 6, 5-F of (2,4,5-F_3)Phe would disconnect a putative 5-H/π interaction, while its 6-H would still form a CH/π interaction. Meanwhile, S/(2,4,5-F_3)Phe/LLRNP was about twice as active as the parent S/Phe/LLRNP (Table 4). These strongly suggest that the CH/π interaction formed by (2,4,5-F_3)Phe is stronger than that by Phe with no fluorine. When the atom electron density of phenyl groups of fluorophenylalanines and ordinary Phe were calculated by the computer-assisted semi-empirical molecular orbital calculation MOPAC method, several characteristics were noted. First of all, when the electron density of benzene carbons were computed, carbons at the *ortho* and *para* positions of the particular fluorine atom increased the net density; av +0.0294 for *ortho* carbons and +0.0121 for *para* carbons. Here, the positive values indicate the increase in net formal charge obtained by subtraction of the charge value of particular atom from that of the corresponding atom in intact Phe-phenyl. Similarly, the minus values indicate the decrease in net formal charge, and the carbons at the root of the fluorine atoms indeed showed a much reduced value (−0.1691). Also, *meta* carbons showed a reduced value (−0.0232). All these results certainly reflect the fact that fluorine is an *ortho–para* director.

For benzene hydrogens, however, it was found that all of the hydrogens in the monofluorophenyl groups reduce the net electron density, although the extents in reduction vary depending upon the positions where the hydrogen exist against the particular fluorine: −0.0157 for the *ortho* hydrogen, −0.0040 for the *meta* hydrogen, and −0.0030 for the *para* hydrogen. These reductions in electron density can be interpreted as increases in acidity of benzene CHs. Apparently, the difference in such electron density or acidity affects the strength of CH/π interactions and thus the activity. Activity enhancement by (4-F)Phe is certainly due to such a reinforcement of CH/π interactions. It was also found that, in the case of multifluorinated phenyl groups, the net changes in electron density are additive depending upon the positions where the hydrogens exist against each fluorine. This calculation has estimated the change in the net electron density of benzene hydrogens of (2,4,5-F_3)Phe: i.e., −0.0357 for the hydrogen at position 3 and −0.0237 for the hydrogen at position 6. Those of (2,3,4-F_3)Phe were −0.0227 for the hydrogen at position 5 and −0.0110 for the hydrogen at position 6. Thus, if the CH/π interaction is formed by 6-H at the *ortho* position, the interaction of S/(2,4,5-F_3)Phe/LLRNP would be much stronger than that of S/(2,3,4-F_3)Phe/LLRNP.

The net electron densities of benzene hydrogens of (2,4,6-F_3)Phe are −0.0357 for 3-H and also −0.0357 for 5-H. The density of 5-H of (2,4,6-F_3)Phe is larger than that of 6-H of (2,4,5-F_3)Phe, although their 3-Hs have the same electron density (−0.0357). A much higher activity of S/(2,4,5-F_3)Phe/LLRNP than S/(2,4,6-F_3)Phe/LLRNP suggests an intrinsic involvement of 6-H in the CH/π interaction. In this concern, the H → F replacement at position 6 (*ortho* position) would disconnect this CH/π interaction and result in a reduction of the activity.

Collectively, S/(2,4,5-F_3)Phe/LLRNP appears to exhibits a highly enhanced activity due to the strengthened CH/π interactions formed in two edges of (2,4,5-F_3)Phe-phenyl ring. It was emphasized that the CH/π interaction is advantageous especially entropically in that the chance for interaction is increased by organizing CHs and/or π groups into a discrete chemical structure.[25] Thus, the intermolecular CH/π interaction would induce a very high specificity and selectivity. The present results strongly suggest that Phe-2-phenyl of SFLLRNP is in such specific CH/π interactions using both edges, (2–3) and (5–6), and presumably 3-H and 6-H.

In the present study, the usefulness of fluorophenylalanines incorporated into the ligand peptides has become evident, especially to evaluate the CH/π interactions. The H → F replacement on the Phe-phenyl benzene ring elicits two different types of structural effects. One is the reinforcement of the CH/π interaction due to the increase in acidity of benzene CHs, which are adjacent to the fluorine atom. The other is to hamper the CH/π interaction when fluorine was placed at the position where the particular CH participates in the CH/π interaction. When we made multiple H → F replacements for the same benzene ring as in the present study, these two structural effects would affect coincidentally the receptor interaction for recognition and activation. For instance, the activity difference between S/(2,4,6-F_3)Phe/LLRNP (49%)

and S/(3,4,5-F_3)Phe/LLRNP (16%) is well explained by these effects: i.e., for (2,4,6-F_3)Phe, strongly reinforced CH/π interaction by 3-H (−0.0357) and CH/π-disconnection by 6-F; and for (3,4,5-F_3)Phe, CH/π-disconnection by 3-F and moderately reinforced CH/π interaction by 6-H (−0.0227). These novel examinations are certainly useful to evaluate the structural elements essential for interaction of biologically active peptides.

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1 The abbreviations according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9—37 (1984), are used throughout. Unless otherwise specified, the amino acids are L-stereoisomers. Additional abbreviations are as follows: Boc, *t*-butoxycarbonyl; Cha, cyclohexylalanine; (F_n)Phe, fluorophenylalanines; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPTLC, high-performance thin-layer chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MBHA, *p*-methylbenzhydrylamine; (F_5)Phe or (2,3,4,5,6-F_5)Phe, pentafluorophenylalanine; PPP, platelet-poor plasma: PRP, platelet-rich plasma; RP-HPLC, reversed-phase high performance liquid chromatography; S/Phe/LLRNP or SFLLRNP, Ser–Phe–Leu–Leu–Arg–Asn–Pro; (F_3)Phe, trifluorophenylalanine; and TFA, trifluoroacetic acid.

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