J. Biochem. 128, 225–232 (2000)

Edge-to-Face CH/π Interaction between Ligand Phe-Phenyl and Receptor Aromatic Group in the Thrombin Receptor Activation

Ayami Matsushima, Tsugumi Fujita, Takeru Nose, and Yasuyuki Shimohigashi[1]

Laboratory of Structure-Function Biochemistry, Department of Chemistry, Faculty and Graduate School of Sciences, Kyushu University, Fukuoka 812-8581

Received April 24, 2000; accepted May 17, 2000

In the ligand/receptor interaction, the side chain phenyl group of phenylalanine (Phe) is involved in a so-called hydrophobic interaction, in which the Phe-phenyl group functions as a π element or merely as a hydrophobic element. The thrombin receptor-tethered ligand SFLLRNP consists of the Phe-2 residue essential for receptor activation. In order to explore the molecular characteristics of this Phe-2-phenyl group, a complete set of S/Phe/LLRNP peptides comprising six different difluorophenylalanine isomers [(F_2)Phe] was newly synthesized and assayed to evaluate their ability to induce the aggregation of human platelets. The assay results clarified several important structural elements to conclude that Phe-2-phenyl of S/Phe/LLRNP is in the edge-to-face CH/π interaction with the receptor aromatic group, utilizing the Phe-phenyl edge along with adjacent benzene hydrogens at positions (2–3) or (5–6). It was also found that the fluorine atom at position 4 increases the acidity of the hydrogen mainly at its *ortho* position, resulting in a reinforcement of the CH/π interaction and thus in an enhancement of biological activity. The H→F replacement in the benzene ring was found to provide an effective structural examination to the Phe residue; *i.e.*, to identify the hydrogens in the CH/π interaction, and to strengthen the CH/π interaction.

Key words: CH/π interaction, fluorophenylalanine, phenylalanine, thrombin receptor.

Phenylalanine (Phe), an aromatic amino acid, has a phenyl group at the side chain, and its phenyl-benzene ring is counted upon to play a structural role in the intermolecular interactions of peptides and proteins, or in intramolecular interactions for the structural stabilization of peptides and proteins. In the ligand–receptor interaction, amino acid residues in the ligand peptide are involved in each specific interaction with the receptor counterparts. Specific interactions usually comprise the interaction such as the electrostatic bonding, hydrogen bonding, and the hydrophobic bondings. Among these interactions, the nature of so-called hydrophobic bonding is not definitive. The Phe residue in the biologically active peptides is often crucially important for eliciting intrinsic activity (*1*, *2*). However, the molecular mechanism of the interaction of Phe has never been elucidated in detail, particularly for the side chain benzene ring, namely, the phenyl group. One of reasons for this is that the Phe residues are inevitably involved in the hydrophobic interaction.

Thrombin, a serine proteinase, binds to its receptor and cleaves the specific peptide bond between Arg^{41} and Ser^{42}. A newly exposed N-terminal fragment of the thrombin receptor, Ser-Phe-Leu-Leu-Arg-Asn-Pro (SFLLRNP), functions as a ligand to activate the receptor by itself (*3*). Although the structure and activation mechanism of the thrombin receptor are extraordinary, the receptor is activated by exogenously administered synthetic peptide SFLLRNP without thrombin. This has allowed the extensive structure-activity studies for exploration of the structural essentials of receptor-tethered SFLLRNP (*4–10*), and the Phe-2-phenyl group has been recognized as one of the most important structural elements. We reported that *para*-fluorophenylalanine [(4-F_1)Phe] at position 2 of SFLLRNP enhances the activity several fold in assays of phosphoinositide-turnover in human epithelial-like SH-EP cells (*11*) and of human platelet aggregation (SFLLRNP is denoted hereafter as S/Phe/LLRNP). *para*-Fluorophenylalanine is now utilized for almost all synthetic peptide analogs of thrombin receptor-tethered ligands to substantiate this high level of potency (*12–15*). We suggested that the ligand Phe-2 benzene ring is involved in the π–π interaction with thrombin receptor (*11*).

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 CH/π interaction (Fig. 1). We have postulated that these interaction can be differentiated by incorporating fluorophenylalanines into the peptide (*16*). 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). It

[1] To whom correspondence should be addressed. Tel/Fax: +81-92-642-2584, E-mail: shimoscc@mbox.nc.kyushu-u.ac.jp

Abbreviations: Boc, *tert*-butoxycarbonyl; Cha, cyclohexylalanine; (F_2)Phe, difluorophenylalanine; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPTLC, high-performance thin layer chromatography; MBHA, *p*-methylbenzhydrylamine; (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; SFLLRNP or S/Phe/LLRNP, Ser-Phe-Leu-Leu-Arg-Asn-Pro-NH_2; and TFA, trifluoroacetic acid.

is highly likely that multiple fluorine replacements of the benzene hydrogens will clarify whether essential hydrogens exist on Phe-phenyl or whether the π system is required for the interaction with the receptor. Our previous results for S/Phe/LLRNP analogs containing monofluorophenylalanines suggested that the Phe-2-phenyl group is in the edge-to-face CH/π interaction with the receptor aromatic group (*16*).

In the present study, a complete series of difluorinated phenylalanines, difluorophenylalanines [(F_2)Phe], were prepared and incorporated into the peptide S/Phe/LLRNP (Fig. 2). 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 biological activity.

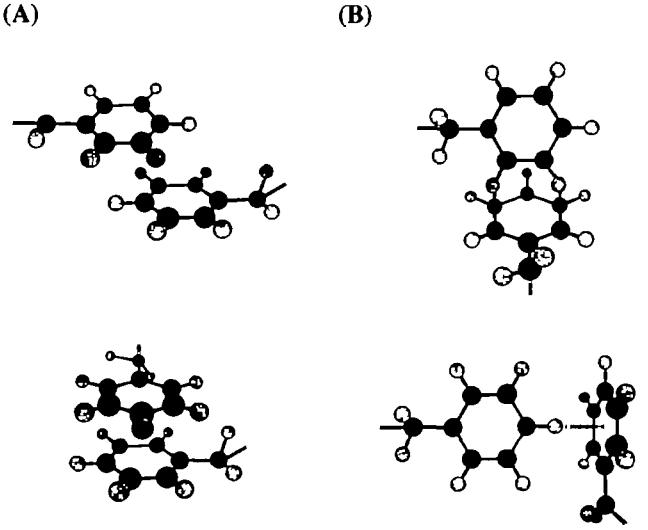


Fig. 1. **The mode of π–π interaction between Phe-phenyls.** (A) the face-to-face π–π stacking interaction, and (B) the edge-to-face CH/π interaction.

MATERIALS AND METHODS

Materials—A series of difluorophenylalanines (F_2)Phe was prepared from the starting materials of difluorobenzyl bromides and diethyl acetamidomalonate. All difluorophenylalanines were obtained in the L-configuration after enzymatic optical resolution of acetyl-DL-difluoropheninylalanines. Boc-derivatives were prepared using di-*t*-butyl dicarbonate according to the method reported (*17*). The details were reported elsewhere (*18*). Boc-L-(2,3,4,5,6-F_5)Phe-OH, Boc-L-(3,4,5-F_3)Phe-OH, and Boc-L-cyclohexylalanine (Cha)-OH were obtained from Watanabe (Hiroshima). Boc-L-(3,4,5-F_3)Phe-OH is the only isomer commercially available among trifluorophenylalanines. Boc-derivatives of other ordinary amino acids and *p*-methylbenzhydrylamine (MBHA) resin were obtained from Watanabe. The configuration of L-amino acids will not be shown hereafter.

Pre-coated silica gel plates for high-performance thin layer chromatography (HPTLC) were purchased from Merck (Darmstadt, Germany). All other chemicals were of the best grade available.

Peptide Synthesis—Analogs of SFLLRNP were synthesized by the manual method for solid phase peptide synthesis (*10*). The side chain protecting groups of the Boc-amino acids were benzyl for Ser and *p*-tolylsulfonyl (Tos) for Arg. All six isomers of Boc-L-(F_2)Phe-OH prepared were utilized without any trouble in peptide syntheses just as ordinary amino-protected amino acids. To obtain C-terminal peptide amides, Boc-Pro-MBHA resin was utilized. Coupling reac-

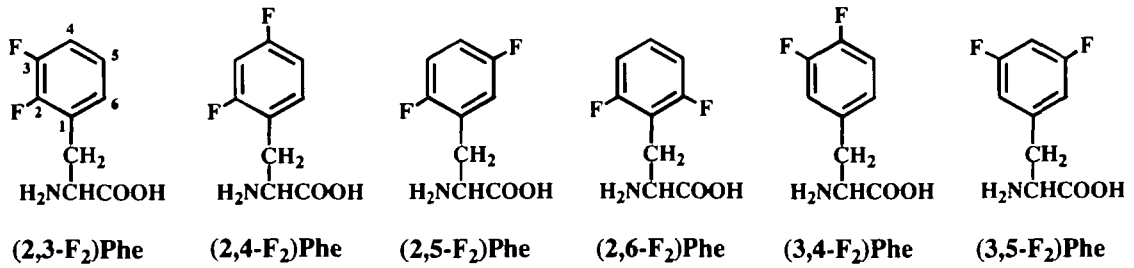


Fig. 2. **Chemical structure of the six structural isomers of difluorophenylalanines (F_2)Phe.** Only the side chain benzyl moieties are shown for comparison.

TABLE I. **The analytical data of synthetic peptides S/(F_2)Phe/LLRNP from 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	Leu	Arg	Asn	Pro
(2,3-F_2)Phe	44	881.90	27.8	0.82	2.00	0.95	0.97	0.96
(2,4-F_2)Phe	39	881.93	26.8	0.81	2.00	0.94	0.96	0.97
(2,5-F_2)Phe	41	882.01	25.7	0.86	2.00	0.91	0.93	0.94
(2,6-F_2)Phe	32	882.05	25.1	0.88	2.00	0.90	0.94	0.92
(3,4-F_2)Phe	44	881.95	31.5	0.81	2.00	0.94	0.96	0.97
(3,5-F_2)Phe	58	881.93	25.6	0.81	2.00	0.95	0.97	0.97

[a]Peptides S/(F_2)Phe/LLRNP are shown by the amino acid residue of (F_2)Phe. [b]Total yield from the Boc-Pro-MBHA resin. [c]Values express the mass number (*m*/*z*) of $(M+H)^+$. Calculated value is 881.96. [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. Difluorophenylalanines [(F_2)Phe] were not determined.

tions (0.1 mol scale) were carried out with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxy-benzotriazole (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 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 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 the peptides were measured on a mass spectrometer Voyager™ DE-PRO (PerSeptive Biosystems, Framingham, MA) by the method of matrix assisted laser desorption ionization time-of-flight (MALDI-TOF). The analytical data are shown in Table I.

Platelet Aggregation Assay—The assay was carried out essentially as previously described (*16*). Briefly, blood was obtained from healthy donors who denied taking any medication for the previous one 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.

GAUSSIAN Calculation—The molecular orbital calculation was carried out using the GAUSSIAN-94 series program with the basis function named 6-31G* on the workstation of the Silicon Graphics Octane (*19*). The total atomic charge values were obtained for each atom of the 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.

TABLE II. **The biological activity of synthetic peptides S/(F_n)Phe/LLRNP in 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
(3,4,5-F_3)Phe	15 ± 2.1	18
(2-F_1)Phe	9.4 ± 4.6	29
(3-F_1)Phe	4.6 ± 2.7	59
(4-F_1)Phe	1.1 ± 0.47	250
(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

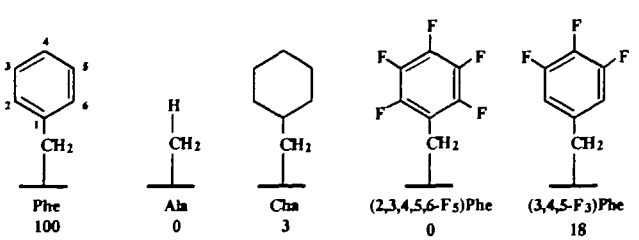
Biological activities of S/(F_1)Phe or (F_5)Phe/LLRNP peptides have been previously reported (Ref. *16*). [a]Peptides S/Xaa/LLRNP are shown by the amino acid residue of Xaa.

RESULTS

Structural Requirement of Phe-2 in Thrombin Receptor Activation—The assay results of a series of S/Xaa/LLRNP analogs are shown in Table II. For easy understanding of the structure–activity relationships, the structures of peptides are depicted as the amino acid residue Xaa at position 2 together with their relative potencies (Set 1–Set 5). The values in parentheses shown for Set 3–Set 5 are the potencies relative to the parent SFLLRNP containing monofluorophenylalanines (F_1)Phe. The first set (Set 1) exhibits the activity comparison of five different S/Xaa/LLRNP peptides to demonstrate the importance of Phe-2-phenyl benzene hydrogens.

For thrombin receptor–tethered ligand peptide S/Phe/LLRNP, the importance of Phe-2 immediately became apparent by its replacement with alanine. When S/Phe/LLRNP was fully active with an EC_{50} value of 2.7 μM, the peptide concentration to elicit half maximal platelet aggregation, S/Ala/LLRNP was completely inactive (Table II, Set 1). Also, the importance of the π character of β-phenyl, namely, the aromacity, became apparent from the fact that the cyclohexylalanine(Cha)-containing analog, S/Cha/LLRNP, exhibited an essential loss of activity (Set 1). 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, and is thus devoid of aromaticity. Another important structural element is ring conformation, since the cyclohexyl ring adopts a chair conformation in energy minimization (*20*). This structure may not be fully complementary with the receptor binding site. It is thus clear that S/Phe/LLRNP requires the benzene ring in planarity, namely, the Phe-phenyl group, at the position 2.

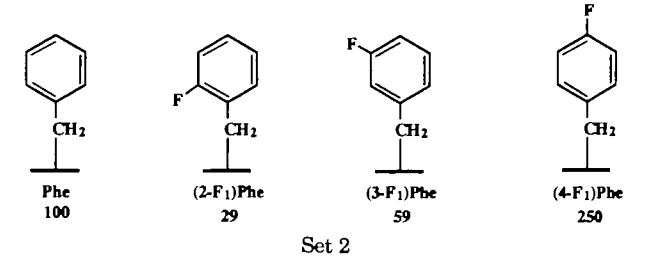
In this study, it was confirmed that S/(2,3,4,5,6-F_5)Phe/LLRNP does not elicit platelet aggregation at all. S/(2,3,4,5,6-F_5)Phe/LLRNP was completely inactive as previously reported (*16*). Clearly, the replacement of all five hydrogen atoms of the Phe-2-phenyl benzene ring by fluorine totally eliminates the ability of S/Phe/LLRNP to bind to the receptor. In contrast, when the hydrogens were put back on the (2,3,4,5,6-F_5)Phe residue at positions 2 and 6, the resulting peptide S/(3,4,5-F_3)Phe/LLRNP was found to be fully active and to have about 15% of the activity of



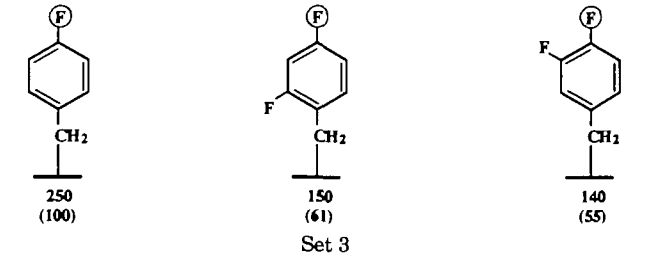
Set 1

SFLLRNP (Table II, Set 1). It is obvious that none of the fluorine atoms of (2,3,4,5,6-F_5)Phe are involved in receptor interaction. The activity recovery by S/(3,4,5-F_3)Phe/LLRNP implies that the hydrogen atom at position 2 or/and 6 is essential for receptor interaction.

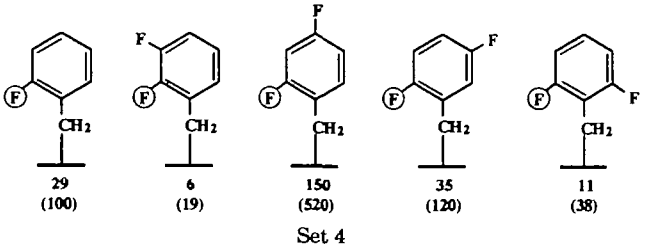
Similarly, it was found that S/(2,6-F_2)Phe/LLRNP fully activates the thrombin receptor (Table II), suggesting that hydrogen atom 3, 4, or/and 5 is also essential. In this concern, however, the hydrogen at position 4 (*para* position) is judged not to be required in the interaction with the receptor, since S/(4-F_1)Phe/LLRNP lacking *para*-hydrogen was rather more potent than parent S/Phe/LLRNP. Although the benzene ring of Phe-phenyl possesses six edges, the edges in which both of *ortho*- and *meta*-hydrogens exist are the edge along with the position (2–3) and the edge along with the position (5–6) (see Phe in Set 1). Collectively, all these results strongly suggest that two adjacent hydrogens at the (2–3)-edge or (5–6)-edge are necessary for full receptor recognition.



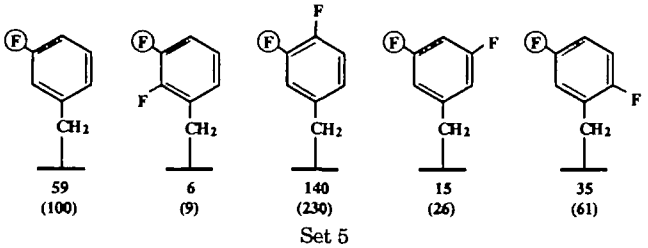
Set 2



Set 3



Set 4



Set 5

Effect of Placement of the Second Fluorine in the Monofluorinated Benzene Ring—As mentioned above, S/(4-F_1)-Phe/LLRNP enhanced the activity in human platelet aggregation several fold. However, when the fluorine atom was placed at the *ortho* or *meta* position, the resulting analogs S/(2-F_1)Phe/LLRNP and S/(3-F_1)Phe/LLRNP exhibited a diminished activity (about 30 and 60% of SFLLRNP, respectively) (Set 2), indicating that H→F replacements at the *ortho* or *meta* positions are unfavorable (*16*).

In the present study, we further examined the effect of placement of the second fluorine atom on these monofluorinated Phe-2-benzene rings. We prepared a series of SFLLRNP analogs containing all of structural isomers of difluorophenylalanines (F_2)Phe; *i.e.*, (2,3-F_2)Phe, (2,4-F_2)Phe, (2,5-F_2)Phe, (2,6-F_2)Phe, (3,4-F_2)Phe, and (3,5-F_2)Phe (Fig. 2). The biological activities of these six different S/(F_2)Phe/LLRNP analogs were compared to those of three derivatives of S/(F_1)Phe/LLRNP to examine the effect of substitution.

When another fluorine was placed on the benzene ring of (4-F_1)Phe to replace the *ortho* or *meta* hydrogen, the resulting difluorinated analogs reduced the activity. S/(2,4-F_2)-Phe/LLRNP and S/(3,4-F_2)Phe/LLRNP exhibited 61 and 55% activity of S/(4-F_1)Phe/LLRNP (Set 3), and again it is clear that H→F replacements at the *meta* and *ortho* positions are unfavorable. Apparently, the presence of fluorine at these positions is disadvantageous for the interaction of S/Phe/LLRNP with the thrombin receptor, suggesting that the particular hydrogens at the edge (2–3) or (5–6) are involved in receptor interaction. It should be noted, however, that both analogs are still more potent (140–150%) than the parent peptide S/Phe/LLRNP (Table II, Set 3). This activity enhancement is obviously due to the H→F replacement at the *para* position. When the activity of S/(2,4-F_2)Phe/LLRNP is compared with that of S/(2-F_1)Phe/LLRNP, the activity increment is 5.2-fold. The increment between S/(3,4-F_2)Phe/LLRNP and S/(3-F_1)Phe/LLRNP is 2.3-fold. Clearly, the fluorine atom at the *para*-position of Phe-2-phenyl is a direct cause of reinforcement in the receptor interaction.

When a second fluorine atom was placed on the benzene ring of *ortho*-fluorinated (2-F_1)Phe at four different positions, the resulting derivatives, S/(2,X-F_2)Phe/LLRNP, showed mixed activity profiles involving enhancement and suppression (Set 4). As mentioned above, when H→F replacement occurs at the *para* position, the resulting S/(2,4-F_2)Phe/LLRNP increases agonist activity 5.2-fold, eliciting a platelet aggregation at a lower concentration range. In contrast, when H→F replacement occurred at the *ortho* or *meta* position, the resulting S/(2,3-F_2)Phe/LLRNP and S/(2,6-F_2)Phe/LLRNP reduced the activity considerably [about 20 and 40% activity of S/(2-F_1)Phe/LLRNP, respectively]. S/(2,5-F_2)Phe/LLRNP sustained the activity, although it is still much less potent (35% activity) than the parent S/Phe/LLRNP. These results indicate that Phe-phenyl benzene-hydrogens at the *ortho* and *meta* positions are intrinsically necessary for eliciting a full receptor activation.

When the assay results of a series of analogs S/(3,X-F_2)Phe/LLRNP were compared with those of S/(3-F_1)Phe/LLRNP, an activity profile similar to that of S/(2,X-F_2)Phe/LLRNP was obtained (Set 5). The benzene ring of *meta*-fluorinated (3-F_1)Phe possesses four benzene-hydrogens at

positions 2, 4, 5, and 6. When one of these was replaced with fluorine, only S/(3,4-F_2)Phe/LLRNP showed enhanced activity in platelet aggregation. All other derivatives, S/(2,3-F_2)Phe/LLRNP, S/(3,5-F_2)Phe/LLRNP, and S/(3,6-F_2)-Phe/LLRNP, showed reduced activity (about 10–60%). Collectively, it is clear that H→F replacement at the *ortho* or *meta* position is unfavorable, again indicating that benzene-hydrogens at the *ortho* and *meta* positions are themselves indispensable in eliciting receptor activation.

DISCUSSION

A characteristic feature of the thrombin receptor is that the receptor protein molecule itself contains a ligand, S/Phe/LLRNP, in the N-terminal extracellular portion (*3*). Since the synthetic heptapeptide having this sequence activates the receptor without thrombin reaction, studies on the structure-activity relationships of tethered ligands have evolved with a number of peptide analogs to find the structural essentials for receptor recognition and activation. In the present study, it became evident that benzene-hydrogens of Phe-phenyl are essential at the *ortho* and *meta* positions for receptor recognition and activation.

The fact that Phe-2 in S/Phe/LLRNP requires the benzene hydrogen(s) for its receptor interaction indicates that the benzene hydrogen *per se* is involved in the interaction. This interaction should involve hydrogen-bonding with the aromatic group. For instance, as shown in Fig. 1B, two phenylalanines can form an edge-to-face CH/π interaction, in which CH of Phe-phenyl binds to the π-system of another Phe-phenyl (*21–24*). The CH/π interaction is a kind of hydrogen-bonding, and Phe is also able to make this with the other π systems of aromatic amino acids such as histidine, tryptophan, and tyrosine (*25*). Thus, it is likely that the benzene hydrogens at the edge (2–3) or (5–6) participate into hydrogen-bondings with the receptor aromatic group. The H→F replacement would disconnect a CH/π hydrogen-bonding, resulting in a drop in biological activity.

If the Phe-phenyl group rotates freely about the axis passing through β-CH_2 and *para*-H or F, both edges of (2–3) and (5–6) would be accessible to the receptor aromatic group for CH/π interaction. The edge availability seems to be an important structural factor in determining the stability of the ligand-receptor interaction. For instance, this makes S/(2,4-F_2)Phe/LLRNP and S/(3,4-F_2)Phe/LLRNP interact with the receptor in the two different ways as shown in Fig. 3. It should be noted that *ortho* and *meta* hydrogens are adjacent in the edge along with the position (5–6). In the edge along with the position (2–3), one hydrogen exists at the *ortho* or *meta* position, which would provide a single CH/π interaction in the receptor interaction (Fig. 3). The availability of two kinds of edges would provide an interaction strong enough to sustain full receptor activity. However, enhanced activities of S/(2,4-F_2)Phe/LLRNP (150%) and S/(3,4-F_2)Phe/LLRNP (140%) appear not to be substantiated merely by such edge availability. For a rational explanation, the effect of fluorine on the hydrogen acidity should be taken into consideration and this is discussed later by calculation of the electron density.

In the case of (2,5-F_2)Phe, each edge contains one hydrogen at the *ortho* or *meta* position as shown in Fig. 4, and both of these edges might participate in the CH/π interaction equally well. In one of these interactions, (2,5-F_2)Phe should be designated as (3,6-F_2)Phe, the structure of which comes out by 180° rotation of (2,5-F_2)Phe. Since these provide a single CH/π interaction, the interaction of S/(2,5-F_2)Phe/LLRNP must be much weaker than parent S/Phe/LLRNP [indeed, S/(2,5-F_2)Phe/LLRNP did show 35% activ-

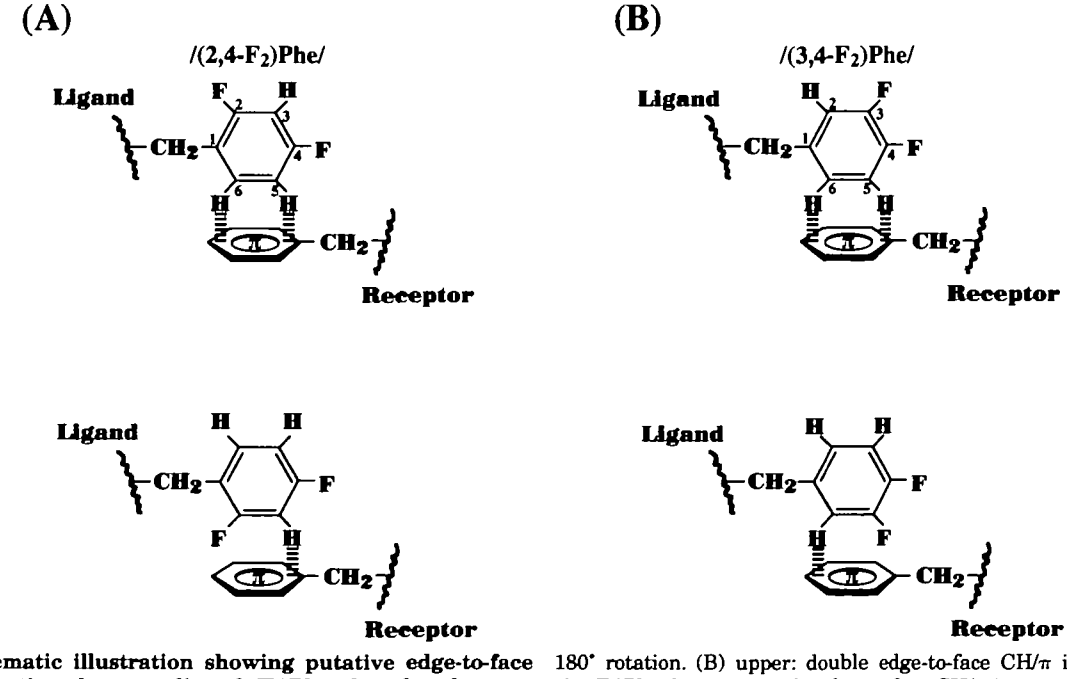


Fig. 3. **Schematic illustration showing putative edge-to-face CH/π interactions between ligand (F_2)Phe-phenyl and receptor aromatic group.** (A) upper: double edge-to-face CH/π interactions by (2,4-F_2)Phe; lower: single edge-to-face CH/π interaction by 180° rotation. (B) upper: double edge-to-face CH/π interactions by (3,4-F_2)Phe; lower: a single edge-to-face CH/π interaction by 180° rotation. The receptor aromatic group is shown in a symbolic form with the letter π in the benzene ring.

ity of S/Phe/LLRNP].

S/(2,6-F_2)Phe/LLRNP, S/(3,5-F_2)Phe/LLRNP, and S/(3,4,5-F_3)Phe/LLRNP exhibit nearly the same activity (11–18%; Table I). It should be noted that, in contrast to (2,4-F_2)Phe, (3,4-F_2)Phe, and (2,5-F_2)Phe, the phenyl groups of (2,6-F_2)-Phe, (3,5-F_2)Phe, and (3,4,5-F_3)Phe are absolutely symmetric about the axis (Fig. 4), and thus only one kind of the edge with one hydrogen is available at the *ortho* or *meta* position for the CH/π interaction. These structural similarities may afford a similar ability to activate the receptor.

One of striking findings is that the fluorine atom at the *para*-position of Phe-2-phenyl enhances the activity of S/Phe/LLRNP in platelet aggregation. There is a possibility that the activity enhancement is based on the ability of the fluorine atom *per se* to make hydrogen-bond as a hydrogen acceptor. For instance, this has recently been demonstrated for a potent chymotrypsin inhibitor, H-D-Leu-Phe-NH-CH_2-C_6H_4(p-F) (*26*). When the dipeptide/γ-chymotrypsin complex was analyzed crystallographically, the C-terminal *para*-fluorobenzyl amide group of this dipeptide was found in the S_1 hydrophobic pocket of the enzyme, and at the bottom of this pocket, the *para*-fluorine atom was hydrogen-bonded to chymotrypsin Ser189 *via* a water molecule (*27*). This hydrogen-bonding resulted in a several-fold enhancement of inhibitory activity. In contrast, no such activity enhancement was observed for derivatives having a fluorine at the *ortho* and *meta* positions (*28*). The pentafluorinated derivative H-D-Leu-Phe-NH-CH_2-C_6F_5 possessing fluorine atoms at all *ortho*, *meta*, and *para* positions retains full inhibitory activity against the enzyme. It should be noted, however, this is not the case of the thrombin receptor–tethered ligand S/Phe/LLRNP in the present study, because the pentafluorinated derivative of S/Phe/LLRNP, namely, S/(2,3,4,5,6-F_5)Phe/LLRNP, was completely inactive in the assay for human platelet aggregation. These results clearly indicate that the fluorine atoms at the *para* positions of the highly potent S/(4-F_1)Phe/LLRNP, S/(2,4-F_2)Phe/LLRNP, and S/(3,4-F_2)Phe/LLRNP do not function as hydrogen acceptor.

Another effect of a fluorine atom introduced in the benzene ring is to activate hydrogen atoms as described above. When the atom charges of phenyl groups of fluorophenylalanines and ordinary Phe were calculated by the computer-assisted molecular orbital calculation GAUSSIAN method, several characteristics were noted. First, when the electron densities of the benzene carbons were computed, carbons at the *ortho* and *para* positions of a particular fluorine atom increased to nearly the same extent as the net charge or electron density, +0.0870 ± 0.0085 for *ortho* carbons and +0.137 ± 0.0018 for *para* carbons (Table III). The electron density of the carbon at the root of the fluorine atom is much reduced (−0.6775 ± 0.0025), and that of *meta* carbons also reduced (−0.0137 ± 0.0020). All these results reflect the fact that fluorine is an *ortho-para* director.

For benzene hydrogens, however, it was found that all hydrogens in the monofluorophenyl groups reduce the net electron density, although the extents in reduction vary depending upon where the hydrogen exist in relation to the specific fluorine: −0.0219 ± 0.0005 for the *ortho* hydrogen, −0.0106 ± 0.0003 for the *meta* hydrogen, and −0.0053 ± 0.0002 for the *para* hydrogen. 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 hydrogen exist relative to each fluorine (data not shown). This calculation estimates the change in the net electron density of benzene hydrogens of (2,4-F_2)Phe: *i.e.*, −0.0438 for the hydrogen at position 3, −0.0272 for the hydrogen at position 5, and −0.0212 for the hydrogen at

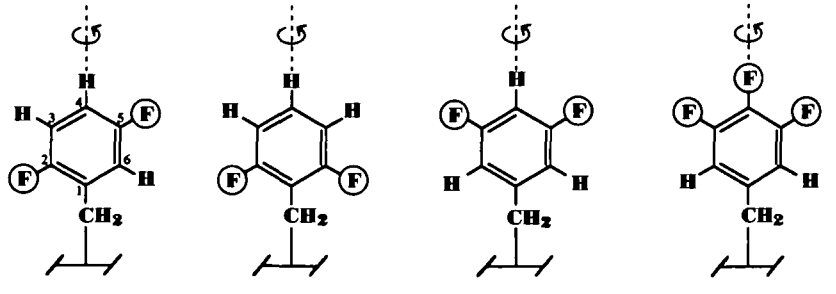


Fig. 4. **Structural comparison of (F_2)Phe in S/(F_2)Phe/LLRNP: Availability of edges loading hydrogen and fluorine atoms.** The structures of the side chain of (F_2)Phe in S/(F_2)Phe/LLRNP are shown. Fluorine atoms are marked by circles. % activity reflects the potency of each peptide ligand relative to S/Phe/LLRNP. The arrow indicates the direction of rotation about the axis along with the β-carbon and *para*-hydrogen.

TABLE III. **Changes in the electron density of monofluorinated phenylalanine benzene carbons and hydrogens.**

Position on benzene ring	Benzene-C			Benzene-H		
	(2-F_1)Phe	(3-F_1)Phe	(4-F_1)Phe	(2-F_1)Phe	(3-F_1)Phe	(4-F_1)Phe
ortho	+0.0796	+0.0820	+0.0828	−0.0220	−0.0210	−0.0222
	+0.1052	+0.0873	+0.0848	—[a]	−0.0220	−0.0224
meta	−0.0125	−0.0118	−0.0144	−0.0105	−0.0109	−0.0107
	−0.0173	−0.0116	−0.0144	−0.0100	—[a]	−0.0109
para	+0.0129	+0.0119	+0.0162	−0.0055	−0.0051	—[a]
root[b]	−0.6805	−0.6778	−0.6743	—[c]	—[c]	—[c]

[a]No benzene-H because of the presence of βCH_2. [b]The position to which the fluorine atom attaches. [c]No benzene-H because of the fluorine atom substitution.

position 6. Those of (3,4-F_2)Phe were–0.0325 for the hydrogen at position 2, −0.0325 for the hydrogen at position 5, and −0.0159 for the hydrogen at position 6. These reductions in electron density can be interpreted as an increase in the acidity of the benzene CHs. Apparently, the difference in such electron density or acidity may affect the strength of the CH/π interactions and further the activity. The CH/π interactions formed by hydrogens on the edge (5–6) of S/(2,4-F_2)Phe/LLRNP and S/(3,4-F_2)Phe/LLRNP would be almost the same due to the similar electron density, but stronger than those by S/Phe/LLRNP. If the slight activity difference between S/(2,4-F_2)-Phe/LLRNP and S/(3,4-F_2)Phe/LLRNP is significant, the difference in electron density on the other edge might be a cause: *i.e.*, −0.0438 for the hydrogen at position 3 of (2,4-F_2)Phe, and −0.0325 for the hydrogen at position 2 of (3,4-F_2)Phe.

Thus, the activity of S/(F_2)Phe/LLRNP seems to be explained by at least two structural conditions, namely, the edge availability and CH acidity. The activity difference between S/(2,5-F_2)Phe/LLRNP (35%) and S/(3,5-F_2)Phe/LLRNP (15%) is a good example, for instance, to explain these structural factors. As to the edge availability, S/(2,5-F_2)Phe/LLRNP provides two kinds of edges, each of which contains one hydrogen at the *ortho* or *meta* position. Both of these edges might be involved in the CH/π interaction equally well. In contrast, S/(3,5-F_2)Phe/LLRNP provides only one kind of edge with one hydrogen at the *ortho* position. This difference in edge availability between (2,5-F_2)-Phe and (3,5-F_2)Phe might be one of reasons why S/(2,5-F_2)Phe/LLRNP is more active than S/(3,5-F_2)Phe/LLRNP. If the (2,5-F_2)Phe-phenyl group utilizes both edges for simultaneous CH/π interaction with the receptor aromatic groups, benzene-hydrogens at positions (3, 6) or (2, 4) may be involved. Our recent results with a series of substitutents revealed the importance of such an interaction at position 3 (unpublished data). As for the electron density, the change (−0.0325) calculated for *meta*- or *ortho*-hydrogens of (2,5-F_2)Phe is larger, indicating greater acidity, than that (−0.0257) for *ortho*-hydrogens of (3,5-F_2)Phe. This might also affect their intrinsic ability to activate the receptor. On the other hand, the low activity of S/(2,3-F_2)Phe/LLRNP appears to be due to the availability of only one edge (5–6) with a slight increase in acidity (−0.0159, −0.0159).

It is now highly likely that the double CH/π interaction exists between the ligand and receptor, in which CHs at the edge (2–3) or (5–6) participate in hydrogen-bondings with the receptor aromatic group. Although the enthalpy for a single unit CH/π interaction is small (about 1 kcal/mol) (*25*), the total enthalpy of such CH/π interaction would become sizable. It is stressed that this kind of interaction is advantageous, especially entropically, in that the chance for interaction is increased by organizing the CHs and/or π groups into a discrete chemical structure (*25*). It thus appears that the CH/π interaction would bring about a very high specificity or selectivity in molecular interactions. The Phe-2-phenyl of SFLLRNP might be in such a specific CH/π interaction. In our attempt to carry out molecular modeling of the thrombin receptor, a seven transmembrane domain receptor exhibited a dense disposition of aromatic amino acid residues in the portion proximal to the extracellular side of the fifth transmembrane domain, the amino acid sequence of which is **YYAYYF**SA**F**SAV**FFF**VPLIIS-TVCYVSII. The first 15 amino acid residues include 9 aromatic amino acids (four tyrosines and five phenylalanines in bold letters in the sequence). The π part of CH/π interaction might be constructed by these aromatic residues.

Collectively, the present study shows the importance of the edge-to-face CH/π interaction between the ligand peptide SFLLRNP and the thrombin receptor. Also, the usefulness of fluorophenylalanines incorporated into the ligand peptides has become apparent, especially for evaluating such CH/π interactions. For the CH/π interaction involving the Phe-phenyl benzene ring, the replacement of benzene-hydrogens with fluorine elicits two different structural effects. One is the reinforcement of the CH/π interaction due to the increase in acidity of the benzene CH, which is adjacent to the fluorine atom. The other is the interference in the CH/π interaction when a fluorine atom is placed at the position where the specific CH participates in the CH/π interaction. These novel structural examinations are useful for evaluating the structural elements in biologically active peptides essential for their receptor recognition and activation, and contribute greatly to the understanding of the construction principal of biomolecules.

REFERENCES

1. Shimohigashi, Y. (1986) Design principles: Enkephalins with predictable mu/delta receptor specificity in *Opioid Peptides: Medicinal Chemistry* (Rapaka, R.S., Barnett, G., and Hawks, R.L., eds.) NIDA Research Monograph 69, pp. 65–100, NIDA-DHHS, US Government Printing Office, Rockville
2. Hruby, V.J., Li, G., Haskell-Luevano, C., and Shenderovich, M. (1997) Design of peptides, proteins, and peptidomimetics in chi space. *Biopolymers* **43**, 219–266
3. Vu, T.-K.H., Hung, D.T., Wheaton, V.I., and Coughlin, S.R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057–1068
4. Hui, K.Y., Jakubowski, J.A., Wyss, V.L., and Angleton, E.L. (1992) Minimal sequence requirement of thrombin receptor agonist peptide. *Biochem. Biophys. Res. Commun.* **184**, 790–796
5. Chao, B.H., Kalkunte, S., Maraganore, J.M., and Stone, S.R. (1992) Essential groups in synthetic agonist peptides for activation of the platelet thrombin receptor. *Biochemistry* **31**, 6175–6178
6. Vassallo, R.R., Jr., Kieber-Emmons, T., Cichowski, K., and Brass, L.F. (1992) Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.* **267**, 6081–6085
7. Scarborough, R.M., Naughton, M.A., Teng, W., Hung, D.T., Rose, J., Vu, T.-K.H., Wheaton, V.I., Turck, C.W., and Coughlin, S.R. (1992) Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J. Biol. Chem.* **267**, 13146–13149
8. Sabo, T., Gurwitz, D., Motola, L., Brodt, P., Barak, R., and Elhanaty, E. (1992) Structure-activity studies of the thrombin receptor activating peptide. *Biochem. Biophys. Res. Commun.* **188**, 604–610
9. Sakaguchi, K., Kodama, H., Ogino, Y., Costa, T., Nose, T., and Shimohigashi, Y. (1994) Structural essentials of Ser-1 in tethered peptide ligand of human thrombin receptor for phosphoinositide hydrolysis. *Bull. Chem. Soc. Jpn.* **67**, 1659–1663
10. Nose, T., Shimohigashi, Y., Okazaki, M., Satoh, Y., Costa, T., Shimizu, N., Ogino, Y., and Ohno, M. (1995) Different roles of two consecutive leucine residues in a receptor-tethered ligand peptide (SFLLRNP) in thrombin receptor activation. *Bull. Chem. Soc. Jpn.* **68**, 2695–2698
11. Nose, T., Shimohigashi, Y., Ohno, M., Costa, T., Shimizu, N.,

and Ogino, Y. (1993) Enhancement of thrombin receptor activation by thrombin receptor-derived heptapeptide with *para*-fluorophenylalanine in place of phenylalanine. *Biochem. Biophys. Res. Commun.* **193**, 694–699
12. Feng, D.-M., Veber, D.F., Connolly, T.M., Condra, C., Tang, M.-J., and Nutt, R.F. (1995) Development of a potent thrombin receptor ligand. *J. Med. Chem.* **38**, 4125–4130
13. Shimohigashi, Y., Nose, T., Okazaki, M., Satoh, Y., Ohno, M., Costa, T., Shimizu, N., and Ogino, Y. (1994) Differential roles of two consecutive phenylalanine residues in thrombin receptor-tethered ligand peptides (SFFLRNP) in thrombin receptor activation. *Biochem. Biophys. Res. Commun.* **203**, 366–372
14. Bernatowicz, M.S., Klimas, C.E., Hartl, K.S., Peluso, M., Allegretto, N.J., and Seiler, S.M. (1996) Development of potent thrombin receptor antagonist peptides. *J. Med. Chem.* **39**, 4879–4887
15. Ahn, H.-S., Foster, C., Boykow, G., Arik, L., Smith-Torhan, A., Hesk, D., and Chatterjee, M. (1997) Binding of a thrombin receptor tethered ligand analogue to human platelet thrombin receptor. *Mol. Pharmacol.* **51**, 350–356
16. Nose, T., Fujita, T., Nakajima, M., Inoue, Y., Costa, T., and Shimohigashi, Y. (1998) Interaction mode of the Phe-phenyl group of thrombin receptor-tethered ligand SFLLRNP in receptor activation. *J. Biochem.* **124**, 354–358
17. Moroder, L., Hallett, A., Wünsch, E., Keller, O., and Wersin, G. (1976) Di-*tert.*-butyldicarbonat-ein vorteihaftes reagenz zur einführung der *tert.*-butyloxycarbonyl-schutzgruppe. *Hoppe Seyler's Z. Physiol. Chem.* **357**, 1651–1653
18. Fujita, T., Nose, T., Matsushima, A., Okada, K., Asai, D., Yamauchi, Y., Shirasu, N., Honda, T., Shigehiro, D., and Shimohigashi, Y. (2000) Synthesis of a complete set of L-difluorophenylalanines, L-(F_2)Phe, as molecular explorers for the CH/π interaction between peptide ligand and receptor. *Tetrahedron Lett.* **41**, 923–927
19. Frisch, M.J., Trucks, G.W., Schlegel, H.B., Gill, P.M.W., Johnson, B.G., Robb, M.A., Cheeseman, J.R., Keith, T., Petersson, G.A., Montgomery, J.A., Raghavachari, K., Al-Laham, M. A., Zakrzewski, V.G., Ortiz, J.V., Foresman, J. B., Cioslowski, J., Stefanov, B.B., Nanayakkara, A., Challacombe, M., Peng, C.Y., Ayala, P.Y., Chen, W., Wong, M.W., Andres, J.L., Replogle, E.S., Gomperts, R., Martin, R.L., Fox, D.J., Brinkley, J.S., Defrees, D.J., Barker, J., Stewart, J.P., Head-Gordon, M., Gonzalez, C., and Pople, J.A., (1995) *GAUSSIAN 94, Reveision C.2*, Gaussian Inc., Pittsburgh
20. Gembitsky, D.S., Murnin, M., Ötvös, F.L., Allen, J., Murphy, R.F. and Lovas, S., (1999) Importance of the aromatic residue at position 6 of [Nle10]neurokinin A(4–10) for binding to the NK-2 receptor and receptor activation *J. Med. Chem.* **42**, 3004–3007
21. Singh, J. and Thornton, J.M. (1985) The interaction between phenylalanine rings in proteins. *FEBS Lett.* **191**, 1–6
22. Hunter, C.A., Singh, J., and Thornton, J.M. (1991) π–π Interactions: The geometry and energetics of phenylalanine-phenylalanine interactions in proteins. *J. Mol. Biol.* **218**, 837–846
23. Chipot, C., Jaffe, R., Maigret, B., Pearlman, D.A., and Kollman, P.A. (1996) Benzene dimer: A good model for π–π interactions in proteins? A comparison between the benzene and the toluene dimers in the gas phase and in an aqueous solution. *J. Am. Chem. Soc.* **118**, 11217–11224
24. Dougherty, D.A. (1996) Cation-π interactions in chemistry and biology: A new view of benzene, Phe, Tyr, and Trp. *Science* **271**, 163–168
25. Nishio, M., Umezawa, Y., Hirota, M., and Takeuchi, Y. (1995) The CH/π interaction: Significance in molecular recognition. *Tetrahedron* **51**, 8665–8701
26. Shimohigashi, Y., Maeda, I., Nose, T., Ikesue, K., Sakamoto, H., Ogawa, T., Ide, Y., Kawahara, M., Nezu, T., Terada, Y., Kawano, K., and Ohno, M. (1996) Chymotrypsin inhibitory conformation induced by amino acid side chain-side chain intramolecular CH/π interaction. *J. Chem. Soc., Perkin Trans.* **1**, 2479–2485
27. Kashima, A., Inoue, Y., Sugio, S., Maeda, I., Nose, T., and Shimohigashi, Y. (1998) X-ray crystal structure of a dipeptide-chymotrypsin complex in an inhibitory interaction. *Eur. J. Biochem.* **255**, 12–23
28. Maeda, I., Shimohigashi, Y., Ide, Y., Nose, T., Nezu, T., Terada, Y., Kawano, K., and Ohno, M. (1996) π hydrogen bond between dipeptide side chain as a structural essential for chymotrypsin inhibition, Peptide Chemistry 1995, Proceedings of the 33rd Symposium on Peptide Chemistry (Ed., Nishi, N.), pp. 309–312