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Dipeptide Side Chain-Side Chain Hydrophobic Interactions as Conformational Core for Chymotrypsin Inhibition

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A series of dipeptides with the sequence of H-D-Leu-L-Phe-R, where R denotes 4-phenylpiperidide, 4-phenylpiperazide, and their derivatives, have been synthesized to examine their ability to inhibit chymotrypsin. Dipeptide phenylpiperidide and phenylpiperazide inhibited chymotrypsin fairly strongly (K_i=7.5×10^{-4} M and 1.4×10^{-3} M, respectively) (1 M=1 mol dm^{-3}), while their phenyl-substituted analogs were inactive. In high resolution ^{1}H NMR measurements, it was found that the proton signals of D-Leu γ-CH and β-CH$_2$ shifted considerably to the upfield when compared with those of H-D-Leu-L-Ala-OH, suggesting that these upfield shifts are due to the magnetic anisotropy effect by the benzene ring of the Phe residue. This was confirmed by measuring NOE between D-Leu-isobutyl and L-Phe-phenyl groups. These NMR data indicate that the side chains of both D-Leu and L-Phe are in close proximity, resulting in formation of a hydrophobic complex. It was suggested that, in the inhibitory conformation of dipeptides against chymotrypsin, the phenyl group of phenylpiperidide or phenylpiperazide moiety fits the S_1 site, while the side chain-side chain hydrophobic complexing core fits the S_2 site.

The discovery of various chymotrypsin-like proteases that play crucial roles in pathological lesions has required highly specific and selective inhibitors for depressing these proteases.[1] We have recently reported that dipeptide methyl esters containing 2,3-methanophenylalanine (▽Phe),[2] H-▽Phe-L-Phe(or Leu)-OMe, inhibits chymotrypsin fairly strongly (K_i=1.6—14×10^{-4} M).[3] It was assumed that these dipeptide methyl esters construct an inhibitory conformation with a hydrophobic core between ▽Phe-phenyl and ester-methyl groups.[4,5] Such inhibitory conformation was considered to be compelled by the extremely constrained three-membered ring of ▽Phe, which restricts the ϕ and ψ angles very severely.[5,6] Similar inhibitory conformation was also found for H-D-Phe-L-Phe-OMe, which inhibited chymotrypsin strongly (K_i=9.0×10^{-5} M).[7]

In these inhibitory conformations, the hydrophobic core was assumed to fit the chymotrypsin S_2 site, while the L-Phe or L-Leu residue in the position 2 fits the S_1 site.[3,4,7] The formation of a hydrophobic core appears to be the most important structural requirement for this particular inhibition. If this formation occurs without compulsion, the molecular conformation may stabilize much more strongly. Deber and Joshua[8] reported the synthesis of a series of linear dipeptides of the type H-L-Phe-L- or -D-Xxx-OH, where Xxx represented various amino acid residues, and found the presence of an intramolecular side chain-side chain complexing for H-L-Phe-D-Xxx-OH, but not for H-L-Phe-L-Xxx-OH. In the present study, we noted this side chain-side chain interaction in aromatic dipeptides as one of possible ways to construct a hydrophobic core for chymotrypsin inhibition.

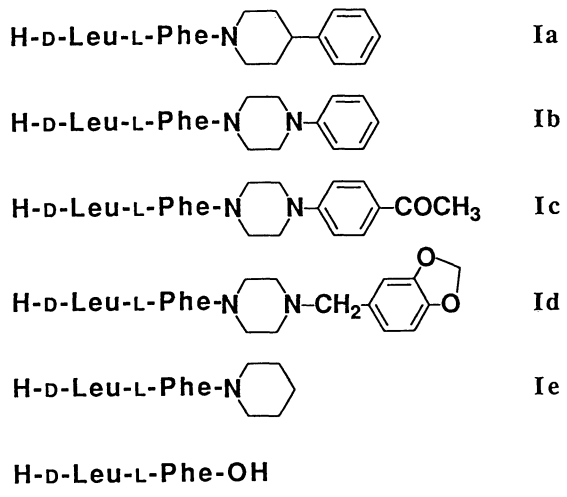

Fig. 1. Structure of dipeptide phenylpiperidide (**Ia**), phenylpiperazide (**Ib**), and their derivatives.

We have designed a series of H-D-Leu-L-Phe-R (**Ia—e**), where R denotes 4-phenylpiperidide, 4-phenylpiperazide, and their derivatives (Fig. 1). In this paper the synthesis of dipeptides and their inhibitory activity for chymotrypsin will be described together with their conformational analysis by ^{1}H NMR.

Results and Discussion

The synthetic scheme is depicted in Fig. 2. A series of Boc-L-Phe amides (**IIa—e**) were prepared by a mixed anhydride coupling method between Boc-L-Phe-OH and amines in yields of 66—95%. The resulting amides were treated with trifluoroacetic acid (TFA) to remove the Boc group, and then coupled with Boc-D-Leu-OH using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in the presence of 1-hydroxybenzotriazole (HOBt) (43—77% yields). The desired dipeptide amides (**Ia—e**) as hydrochlorides were obtained from Boc-D-Leu-L-Phe amides by treatment with hydrogen chloride in dioxane.

Inhibitory activity for chymotrypsin was analyzed

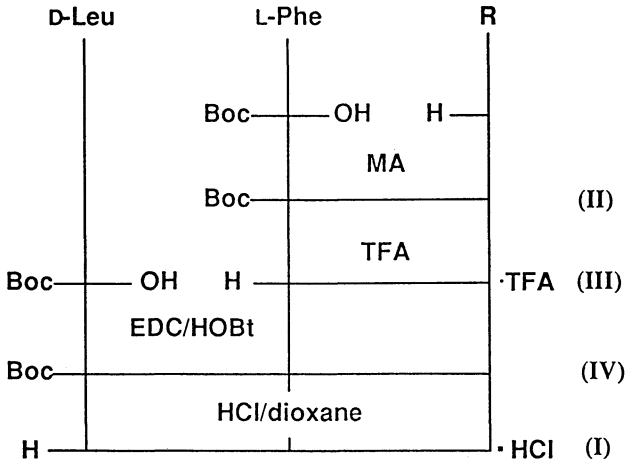

Fig. 2. Synthetic scheme of H-D-Leu-L-Phe-R.
R designates the amide derivatives of 4-phenylpiperidine, 1-phenylpiperazine, 4'-piperazinoacetophenone, 1-piperonylpiperazine, and piperidine.

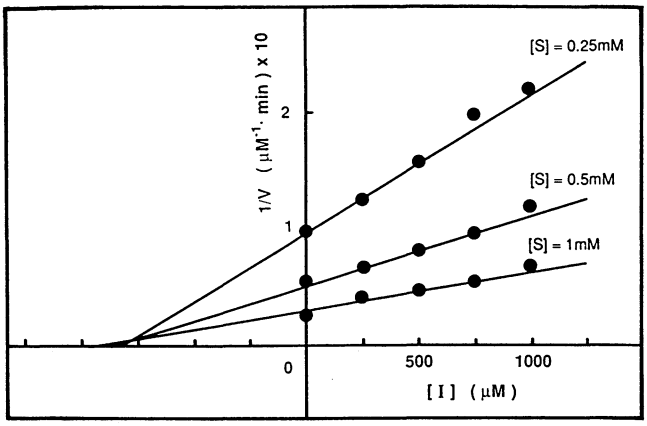

Fig. 3. Dixon plot for hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*NA by chymotrypsin in the presence of H-D-Leu-L-Phe-phenylpiperidide (**Ia**) at pH 7.8. Concentrations of the substrate are indicated in the figure.

Table 1. Inhibitory Activities of Dipeptides for Chymotrypin

Peptides	K_i/M
Ia	7.5×10^{-4}
Ib	1.4×10^{-3}
Ic	No inhibition
Id	No inhibition
Ie	No inhibition
H-D-Leu-L-Phe-OH	No inhibition
H-D-Leu-L-Ala-OH	No inhibition

using succinyl-Ala-Ala-Pro-Phe-*p*NA as a substrate,[9] because significant UV absorption due to the inhibitors at wavelengths below 250 nm made the spectroscopic monitor at 237 nm of hydrolysis of another popular substrate Ac-Tyr-OEt difficult. It was found that the dipeptide phenylpiperidide (**Ia**) and phenylpiperazide (**Ib**) inhibit chymotrypsin moderately. Analyses by Dixon plots[10] and Lineweaver-Burk plots[11] indicated the inhibition manner to be competitive. In Fig. 3 the Dixon plot of compound **Ia** is shown. The K_i values of **Ia** and **Ib** were 7.5×10^{-4} and 1.4×10^{-3} M, respectively (Table 1). It should be noted that the inhibitory potencies of these dipeptides are almost equal to those of ▽Phe-containing dipeptides.[3] This suggests that incorporation of a sterically constrained amino acid such as ▽Phe is not necessarily crucial for constructing the inhibitory conformation.

Dipeptides with phenyl-substituted piperazide (**Ic—d**) showed no inhibition. Also, H-D-Leu-L-Phe piperidide (**Ie**) did not inhibit chymotrypsin at all. These results imply that the phenyl group in the R moiety of H-D-Leu-L-Phe-R should not be substituted to elicit an inhibitory activity against chymotrypsin.

In 400-MHz ^{1}H NMR measurements, the signal assignments were carried out by the two-dimensional COSY experiment. The chemical shift values were carefully read off for comparison. When the chemical shifts of D-Leu side chain protons were compared with those of H-D-Leu-L-Ala-OH, considerable upfield shifts became prominent for all dipeptides having the D-Leu-L-Phe sequence: δ=0.13—0.38 for D-Leu β-CH_2; and δ=0.28—0.49 for D-Leu γ-CH. Even protons of δ-methyl groups showed a small upfield shift (δ=0.12—0.15). These results indicate that the side chain of D-Leu is in very close proximity to the phenyl group and undergoes the anisotropy effect of the benzene ring. Since H-D-Leu-L-Phe-OH and H-D-Leu-L-Phe piperidide (**Ie**), which has no phenyl in the *C*-terminal R

Table 2. Chemical Shifts of Leucine Side Chain Protons of Dipeptides

Dipeptide	Chemical shifts (ppm) of leucine residue					
	βCH_2		γCH		δCH_3	
H-D-Leu-L-Ala-OH	1.526	(0.000)	1.678	(0.000)	0.890	(0.000)
H-D-Leu-L-Phe-OH	1.147	(0.379)	1.299	(0.379)	0.763	(0.127)
Ia	1.275	(0.251)	1.275	(0.403)	0.764	(0.126)
	1.384	(0.142)	1.384	(0.294)	0.823	(0.067)
Ib	1.268	(0.258)	1.268	(0.410)	0.764	(0.126)
Ic	1.293	(0.233)	1.293	(0.385)	0.774	(0.166)
Id	1.184	(0.342)	1.184	(0.494)	0.741	(0.149)
Ie	1.399	(0.127)	1.399	(0.279)	0.772	(0.118)

portion, also showed such upfield shifts of D-Leu side chain (Table 2), the shielding phenyl group apparently belongs to L-Phe in the position 2.

The presence of side chain interaction between D-Leu–isobutyl and L-Phe–phenyl was confirmed by measurements of the nuclear Overhauser effect (NOE). H–{H} NOE difference spectra of H–D-Leu–L-Phe-piperidide (**Ie**) were obtained by irradiation of each selected proton signal (Fig. 4). When the proton signals of the D-Leu–β-methylene, γ-methine, and δ-methyl groups were irradiated separately, the enhancement as positive NOE was observed for the proton signals of L-Phe-phenyl in the aromatic envelope (Figs. 4B and 4C). Similar enhancement was also observed for the D-Leu β-methylene, γ-methine, and δ-methyl protons by irradiation of L-Phe–phenyl proton signals (Fig. 4D). These NOE observed indicate that the side chains of D-Leu and L-Phe in D-Leu–L-Phe dipeptides are spatially in close proximity to each other.

Deber and Joshua[8] found a similar type of side chain interactions for L-Phe–D-Xxx dipeptides. They observed distinct upfield shifts (0.2—0.7 ppm) for the signals of D-Xxx β- and γ-methylene protons in 100-MHz ^{1}H NMR. Although they suggested that these upfield shifts are due to the shielding effect of L-Phe–phenyl, no direct evidence has been presented. Since the side chain interactions were not observed for L-Phe–L-Xxx dipeptides, it seems that this side chain–side chain interaction in dipeptides is stabilized in the configurational sequences of L–D and D–L. In fact, the building study using the CPK model indicated that a tight side chain complexing is feasible for and L–D and D–L.

The hydrophobic complex formed between the side chains of the adjacent residues in D-Leu–L-Phe dipeptides appears to be too bulky to fit the S_1 hydrophobic pocket of chymotrypsin active center. Since the S_2 site of chymotrypsin is Trp-215,[12] the moiety in hydrophobic complexing seems to interact with this Trp hydrophobic site. In the case, the phenyl group of phenylpiperidide and phenylpiperazide should occupy the S_1 pocket. Inactivity of dipeptides having phenyl-substituted piperidine residues (Table 1) is due probably to their bulkiness that makes the dipeptide incapable to fit the S_1 site, while inactivity of H–D-Leu–L-Phe–OH would be explained by lack of the substituent at the *C*-terminal carboxyl group.

We have previously reported the inhibitory conformation of H–D-Phe–L-Phe–OMe,[7] in which D-Phe–phenyl and ester-methyl groups construct a hydrophobic core responsible for interaction with the chymotrypsin S_2 site. This was demonstrated by NOE experiments. As proven in the present study, it is also likely that a conformer with the side chain–side chain complex exists in the enzyme assay solution, although we could not confirm it by NOE experiments because of overlapping of the proton signals of the two phenyl groups. How-

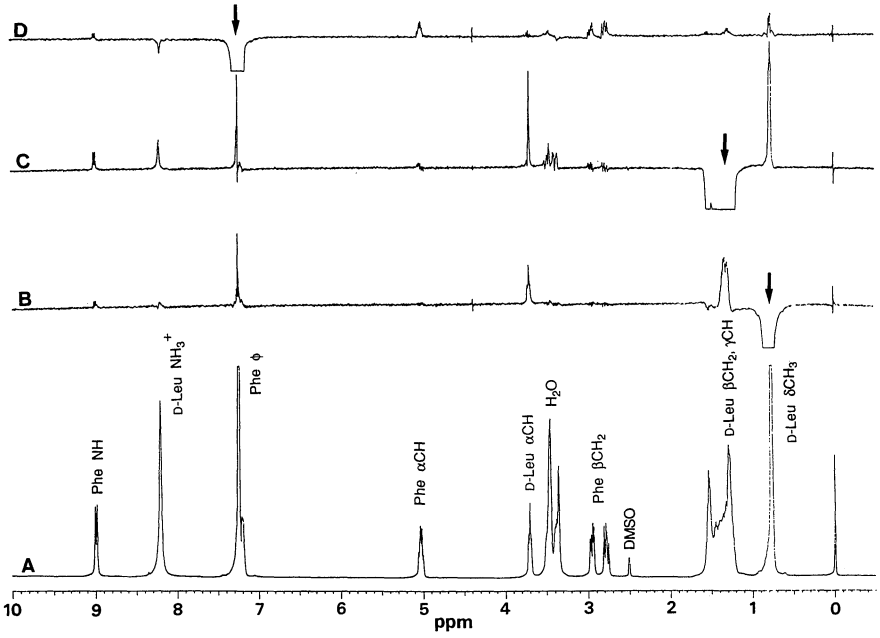

Fig. 4. 400-MHz ^{1}H NMR spectra of H–D-Leu–L-Phe–piperidide (**Ie**) hydrochloride in DMSO-d_6 at 25 °C.
A: The control spectrum acquired with irradiation in a blank region of the spectrum.
B—D: The NOE difference spectra obtained by subtraction of the control spectrum from the spectra acquired with irradiation of particular signals indicated by arrows; D-Leu δCH_3(B), D-Leu βCH_2 and γCH (C), and Phe–phenyl (D).

ever, this conformation would not cause an inhibition of chymotrypsin, since no group to interact with the S_1 site is present in this conformation. For D-Leu-L-Phe dipeptides, the presence of a conformer with the hydrophobic complex formed between D-Leu-isobutyl and *C*-terminal phenyl groups, a type of complex observed for H-D-Phe-L-Phe-OMe, is also partly feasible. However, the competitive inhibition by this conformer is unlikely due to the undetectable inhibition by dipeptides **Ic** and **Id** (Table 1). Collectively, the conformation to induce the enzyme inhibition, namely the inhibitory conformation, appears to differ between D-Leu-L-Phe and D-Phe-L-Phe dipeptides, while both dipeptide inhibitors seems to resemble each other in the size and spatial arrangement of P_1 and P_2 sites.

In ^{1}H NMR spectrum of H-D-Leu-L-Phe-phenylpiperidide (**Ia**), which is the most potent inhibitor synthesized in this study (Table 1), two sets of proton signals were observed, suggesting the presence of conformers (Table 2). These conformers would be characterized by the difference in either axial or equatorial positioning of the phenyl group on the piperidine ring. Although we could not assign these conformations, it is clear that the conformer in the major conformation (65%) constructs the intramolecular hydrophobic side chain complex as shown by obvious upfield shifts of the D-Leu-isobutyl side chain (Table 2). It is thus suspected that the intrinsic inhibitory activity of this isomer would be much stronger than that observed for the mixture of isomers.

The present study indicates that the side chain-side chain hydrophobic interaction can be constructed in dipeptides with the D-L configurational sequence, and that a hydrophobic core formed has a conformation suitable to fit the active site of chymotrypsin. The hydrophobic interaction between the dipeptide and the enzyme should result in inhibition of the enzyme catalytic action. Analyses of such an inhibitory conformation of dipeptides might lead to elucidation of the subtle structure of the chymotrypsin active center.

Experimental

Synthesis. High performance (HP)-TLC was carried out on Silica Gel G (Merck, Frankfurt) with the following systems (v/v): R_f^1, $CHCl_3$-MeOH (9 : 1), R_f^2, *n*-BuOH-AcOH-pyridine-H_2O (4 : 1 : 1 : 2). Optical rotations were measured with a Union high sensitivity polarimeter PM-71. All melting points were uncorrected.

Boc-L-Phe-amides (IIa—e): To a solution of Boc-L-Phe-OH (1.327 g, 5.0 mmol) in tetrahydrofuran was added isobutyl chloroformate (0.65 ml, 5.0 mmol) at −15 °C. After 10 min, amine (4-phenylpiperidine, 1-phenylpiperazine, 4′-piperazinoacetophenone, 1-piperonylpiperazine, or piperidine) (10 mmol) was added. The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature, and evaporated. The residue was dissolved in EtOAc and the solution was washed successively with 4% $NaHCO_3$, 5% $KHSO_4$, and water, and then dried over Na_2SO_4. Physical properties of each compound are shown in Table 3.

Table 3. Physical Properties of Synthetic Peptides

Peptides	Yield %	Mp °C	$[\alpha]_D^{20}$/° *c* 0.5, DMF	(HP)-TLC R_f
Boc-L-Phe-R (**II**)				
IIa	97	Oil	—	R_f^1=0.92
IIb	66	105—107	−19.2	0.84
IIc	93	152—155	−18.2	0.84
IId	75	Oil	—	0.76
IIe	>100	Oil	—	0.78
Boc-D-Leu-L-Phe-R (**IV**)				
IVa	52	123—125	−16.8	0.86
IVb	43	133—135	−17.2	0.84
IVc	66	139—140	−16.8	0.81
IVd	53	120—122	−17.4	0.81
IVe	77	134—136	−16.6	0.76
H-D-Leu-L-Phe-R(**I**)·HCl				
Ia	79	93—94	−16.2	R_f^2=0.81
Ib	92	213 (decomp)	−14.4	0.82
Ic	93	173 (decomp)	−15.1	0.79
Id	62	180 (decomp)	−15.6	0.67
Ie	72	88—89	−16.4	0.79

Boc-D-Leu-L-Phe-amides (IVa—e): Boc-L-Phe-amides (**IIa—e**) (2.0 mmol) were treated with TFA at 0 °C for 30 min. After evaporation and trituration with the aid of dry diethyl ether, the resulting powder (**IIIa—e**) was collected and dried. To a solution of this TFA salt and Et_3N (0.28 ml, 2.0 mmol) in DMF (10 ml) were added Boc-D-Leu-OH·H_2O (530 mg, 2.0 mmol), EDC·HCl (422 mg, 2.2 mmol), and HOBt (324 mg, 2.4 mmol) at 0 °C. The reaction mixture was treated as described above. Physical properties are shown in Table 3.

H-D-Leu-L-Phe-amide (Ia—e) Hydrochlorides: Compounds **IVa—e** (0.5 mmol) were dissolved in 4.6 M HCl in dioxane (2.2 ml, 10 mmol) at 0 °C for 2—5 h. After evaporation, the residue was crystallized from MeOH-diethyl ether. Physical properties are shown in Table 3.

Enzyme Assay. Dipeptides synthesized were tested for their inhibitory activity against bovine chymotrypsin (Worthington Biochemical Co., Freehold, New Jersey). Substrate succinyl-Ala-Ala-Pro-Phe-*p*NA was synthesized by the conventional solution method. For the kinetic analyses of interactions between dipeptides and chymotrypsin, the standard method was carried out. Briefly, substrate was dissolved in 0.1 M Tris-HCl buffer (pH 7.8) with or without inhibitors. The enzyme reaction was initiated by adding an aliquot (30 μl) of the chymotrypsin solution (final concentration, 1.3 μg ml^{-1}) to the solution of substrate and inhibitor (1470 μl). The final concentrations of the substrate and inhibitors varied as 6.25, 12.5, 25 μM and 0, 250, 500, 750, 1000 μM, respectively. The rate of hydrolysis of the substrate was determined in duplicate by the spectroscopic measurement of liberated *p*-nitroanilide at 410 nm using a Hitachi 124 spectrometer. Inhibition constants, K_i, were determined by the Dixon plot analysis[10] and summarized in Table 1.

^{1}H NMR Measurements. Samples were prepared by dissolving 10 mg of the peptide in 0.5 ml of DMSO-d_6 (99.96% *d*, Aldrich, Milwaukee, Wisconsin). All ^{1}H NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 400-MHz ^{1}H frequency at 25 °C. The chemical shifts were determined using tetramethylsilane as an internal standard and the signals were assigned by the two-dimensional COSY. The

COSY spectra were obtained by using the standard method.[13] Two hundreds fifty-six FIDs were acquired with successive t_1 values. The digital resolution was 9.76 Hz/pt. The H-{H} NOE data were recorded by a gated irradiation pulse sequence. The NOE difference spectra were obtained by subtracting the control spectrum from an original NOE spectrum produced by presaturation of selected proton signal with a low decoupling power for 3 s before pulse.

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2) Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; NOE, nuclear Overhauser effect; ∇Phe, 2,3-methanophenylalanine, or 1-amino-2-phenylcyclopropanecarboxylic acid; *p*NA, *p*-nitroanilide; and TFA, trifluoroacetic acid.

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