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Chymotrypsin Inhibition Induced by Side Chain-Side Chain Intramolecular CH/π Interaction in D-Thr-L-Phe Benzylamide

Iori Maeda,* Yasuyuki Shimohigashi,*,[1] Koichi Ikesue,* Takeru Nose,* Yuzuru Ide,* Keiichi Kawano,† and Motonori Ohno*

*Laboratory of Biochemistry, Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812-81; and †Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060

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The dipeptide benzyl amide H-D-Thr-Phe-NH-CH_2-C_6H_5 was found to inhibit chymotrypsin strongly ($K_i = 4.5 \times 10^{-6}$ M) in a competitive manner. When a series of phenyl amides H-D-Thr-Phe-NH-$(CH_2)_n$-C_6H_5 ($n = 0$-4) were tested, inhibitory potency peaked at $n = 1$ (benzyl amide). Incorporation of a methyl group into the benzyl methylene resulted in formation of stereoisomers, H-D-Thr-Phe-NH-(*R* or *S*)-CH(CH_3)-C_6H_5, with considerably different inhibitory potencies. The *R*-isomer was as active as the benzyl amide, while the *S*-isomer was about 30-fold less active than the benzyl amide. Furthermore, when a fluorine atom was introduced into the *para*-position of the amide-benzyl group, the resulting H-D-Thr-Phe-NH-CH_2-C_6H_4(*p*-F) showed considerably enhanced inhibitory activity (about 5-fold, $K_i = 9.1 \times 10^{-7}$ M). In conformational analysis by 400 MHz ^{1}H-NMR, all dipeptides having D-Thr-Phe backbone structure showed large upfield shifts of D-Thr-βO*H* (shifts in ppm, 0.09-0.17), D-Thr-βC*H* (0.23-0.32), and D-Thr-γCH_3 (0.38-0.53), indicating the presence of shielding effects from the benzene ring. In addition, NOE enhancements between the D-Thr-γCH_3 and Phe-phenyl groups were evidenced by measurements of two-dimensional NOESY spectra and NOE difference spectra. These observations demonstrated the spatial proximity of these side chains, which is due to side chain-side chain CH/π interaction. All these results support the idea that the amide-benzyl group binds at the chymotrypsin S_1 site, while the hydrophobic core with CH/π interaction binds at the S_2 or S_1' site.

Key words: CH/π interaction, chymotrypsin, dipeptide inhibitor, enzyme inhibitor, inhibitory conformation.

Serine proteinases play crucial roles in various physiological processes. However, they also possess an intrinsic ability to destroy important proteins of cells and tissues if uncontrolled. In recent years, various chymotrypsin-like proteases have been found in cells from pathological lesions, such as those present in muscular dystrophy, carcinoma, rheumatoid arthritis, leukemia, and allergy (*1-5*). It is expected that inhibitors of these chymotrypsin-like enzymes would suppress the enzyme actions and might improve the symptoms. Only a limited number of compounds, however, are known to be specific inhibitors of chymotrypsin or chymotrypsin-like enzymes (*6*). Ac-Pro-Ala-Pro-phenylalaninal (*7*) and boro-Phe derivatives (*8*) are effective reversible chymotrypsin inhibitors, although they are rather hazardous because of the presence of aldehyde and boric acid, respectively, as key structural elements for inhibition. We have recently designed and synthesized inhibitors with a simple dipeptide benzyl ester structure (*9*). H-D-Leu-Phe-OBzl, one of the most effective of them, inhibited chymotrypsin strongly in a competitive manner.

^{1}H-NMR conformational analyses of H-D-Leu-Phe-OBzl have suggested that the side chains of D-Leu (isobutyl) and Phe (phenyl) are in close proximity (*9*). A key structural feature of the dipeptide for chymotrypsin inhibition appeared to be this hydrophobic side chain-side chain interaction. The resulting side chain hydrophobic core was assumed to bind at the chymotrypsin S_2 or S_1' site, while the C-terminal ester-benzyl group binds to the S_1 site. To examine this hypothesis, we have tested several structures in the present study, picking D-Thr-Phe benzyl amide as a lead compound. Since dipeptide benzyl esters were degraded gradually by chymotrypsin, we designed benzyl amide derivatives to prevent the degradation. Another feature of D-Leu-Phe dipeptides was their poor water-solubility. To get better solubility, we replaced hydrophobic leucine (D-Leu-1) with the hydrophilic β-hydroxyl amino acid threonine (D-Thr).

[1] To whom correspondence should be addressed. E-mail: shimoscc@mbox.nc.kyushu-u.ac.jp

Abbreviations: Ac, acetyl; ATEE, Ac-Tyr-OEt; Bzl, benzyl (-CH_2-C_6H_5); Boc, *tert*-butoxycarbonyl; DQF-COSY, two-dimensional double-quantum-filtered correlated spectroscopy; FID, free induction decay; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement and exchange spectroscopy.

MATERIALS AND METHODS

Peptide Synthesis—A series of dipeptide benzylamides were synthesized by a two-step coupling procedure: *i.e.*, (1) the coupling of Boc-Phe-OH with various amines by water-soluble carbodiimide, and (2) the coupling of Boc-D-Thr-OH with the resulting phenylalanine amides. The removal of the Boc group was carried out by treatment with trifluoroacetic acid for Boc-Phe amides and 4.6 M HCl in dioxane for Boc-D-Thr-Phe amides. Purification of the final amino-liberated dipeptide amides was carried out by recrystallization from methanol-diethyl ether. The purity was verified by high-performance thin-layer and liquid chromatography.

Hydrolysis of Dipeptides by Chymotrypsin—The stability of dipeptide benzyl ester and a series of amide derivatives to chymotrypsin hydrolysis was monitored by HPLC at intervals (t=5-120 min). The peptide (1 mM) was incubated in phosphate buffer (pH 7.0; total volume, 3.0 ml) with chymotrypsin (1.3 μg/ml) at 25°C. An aliquot (200 μl) of the incubation mixture was added to acetic acid (10 μl), and the solution was injected into the HPLC. The % hydrolysis was calculated from the peak integration ratio between each compound at t min and that (=100) at 0 min. Analytical HPLC was performed on a reversed-phase column, Wakosil-II 5C18 HG (4.6×150 mm), in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile (10-60%) for 60 min.

Solubility Test—The extent to which dipeptide amides can dissolve in aqueous alcohol was assessed by inspecting the presence or absence of precipitation after centrifugation of the mixed and stirred solution. Dipeptides (5-6 mg, the amount sufficient to make 50 mM final concentration) was added to 0, 10, 30, or 50% MeOH (250 μl) and the solutions were vortexed for 10 min at room temperature and centrifuged (10,000 rpm for 2 min). When precipitation was observed, peptides were first dissolved in neat MeOH (25 μl for 10%, 75 μl for 30%, and 125 μl for 50%) and then diluted with water to 250 μl total volume.

Enzyme Assay—Dipeptides synthesized were tested for their inhibitory activity against bovine chymotrypsin (Worthington Biochemical Co., Freehold, NJ, USA). Substrate Ac-Tyr-OEt (ATEE) was purchased from the Peptide Institute (Osaka). For the kinetic analyses of interactions between dipeptides and chymotrypsin, a standard method was employed. Briefly, the substrate ATEE was dissolved in 50 mM phosphate buffer (pH 7.0) with or without inhibitors. The enzyme reaction was initiated by adding an aliquot (20 μl) of the chymotrypsin solution (final concentration, 1.3 μg/ml) to the solution of substrate and inhibitor (980 μl) at 25°C. The final concentrations of the substrate and inhibitors varied, depending upon the potencies of the compounds. Dipeptides were directly dissolved in water. When H-D-Thr-Phe-NH-$(CH_2)_n$-C_6H_5 (n=0-4) were assayed, the dipeptides were first dissolved in MeOH (1 ml) and then diluted with water. For further dilution, 10% MeOH was used to adjust the concentration (2%) of MeOH in the final assay solutions. The rate of hydrolysis of the substrates was determined in duplicate or triplicate by the spectroscopic measurement of change in absorbance at 237 nm using a Hitachi 100-60 spectrometer. Inhibition constants, K_I, were determined by Dixon plot analysis (*10*).

^{1}H-NMR Measurements—Samples were prepared by dissolving 5 mg of the peptide in 0.5 ml of DMSO-d_6 (99.96% d, Aldrich, Milwaukee, WI, USA) or D_2O (99.9% d, Isotec, Miamisburg, OH, USA). All ^{1}H NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 400 MHz, at 25°C. The chemical shifts were determined using tetramethylsilane as an internal standard and the signals were assigned by 2D phase-sensitive DQF-COSY. COSY spectra were obtained by using the standard method (*11-13*), and 512 FIDs were acquired with successive t_1 values. The digital resolution was 9.76 Hz/pt. 2D NOESY spectra were recorded according to the ordinary procedure (*14*) with 512 FIDs per t_1 and five different mixing times in a range of 50-1,000 ms. The H-{H} nuclear Overhauser effect (NOE) data were recorded by using 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 the selected proton signal with a low decoupling power for 3 s before acquisition.

RESULTS

Stability of Dipeptides to Chymotrypsin Hydrolysis—The stability of synthetic dipeptides to chymotrypsin was assessed by reversed-phase HPLC monitoring (Fig. 1). Peaks corresponding to the starting dipeptide ester or amide and its free acid derivative were integrated at intervals. Ac-Tyr-OEt (ATEE) was completely degraded by chymotrypsin in 20 min. Although H-D-Leu-Phe-O-CH_2-C_6H_5 was fairly stable, it was also eventually hydrolyzed to H-D-Leu-Phe-OH in 90 min. This indicated that the benzyl ester H-D-Leu-Phe-O-CH_2-C_6H_5 is a partial inhibitor, being also a substrate of chymotrypsin.

When the benzyl amide H-D-Thr-Phe-NH-CH_2-C_6H_5 was tested, no hydrolysis was observed even after 2 h (Fig. 1). Even after prolonged incubation (24 and 48 h), the hydrolyzate H-D-Thr-Phe-NH_2 did not emerge at all (data not shown). None of the dipeptide amides was degraded by chymotrypsin.

Solubility of Dipeptide Amides—It was found that D-Leu-containing dipeptide amides are difficult to dissolve in

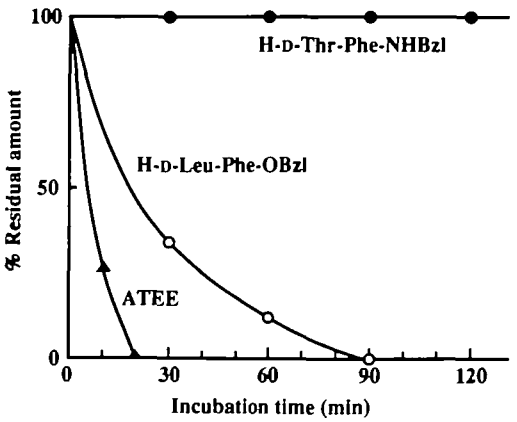

Fig. 1. **Chymotrypsin hydrolysis of dipeptide inhibitors and substrate Ac-Tyr-OEt (ATEE).** H-D-Thr-Phe-NHBzl (●) and H-D-Leu-Phe-OBzl (○) along with ATEE (▲). The % residual amount of compounds was estimated from the HPLC (ODS column) peak integration relative to the amount at 0 min.

water (unpublished observations). Even in aqueous MeOH (30%) they did not dissolve directly. They did dissolve in 30% MeOH as long as they were first dissolved into a small amount of neat MeOH and then diluted to 30% solution. However, D-Thr-containing dipeptide amides dissolved easily in water, although alkyl benzyl amides such as H-D-Thr-Phe-NH-$(CH_2)_3$ or $_4$-C_6H_5 required a small amount (2%) of MeOH.

Structural Elements for Chymotrypsin Inhibition—Using ATEE as a substrate, the chymotrypsin-inhibitory activity of dipeptide amides was examined. It was found that H-D-Thr-Phe-NH-CH_2-C_6H_5 very strongly inhibits chymotrypsin, and from Dixon plots (*10*) and Lineweaver-Burk plots (*15*) the inhibition was judged to be competitive (Fig. 2). The K_i value was calculated to be 4.5×10^{-6} M (Table I). The L-L isomer H-L-Thr-Phe-NH-CH_2-C_6H_5 also inhibited chymotrypsin in a competitive manner, although it was much weaker (only 9% in terms of potency) than the D-L isomer (Table I).

Dipeptide amides lacking the C-terminal phenyl group, namely H-D-Thr-Phe-$NHCH_3$ and H-D-Thr-Phe-NH_2, were completely inactive. Thus, the C-terminal phenyl group is crucially important for dipeptides to interact with chymotrypsin. Although the removal of phenyl from Phe-2, resulting in formation of H-D-Thr-Ala-NHBzl, preserved competitive inhibition, this peptide showed a sharp drop (about 170-fold) in potency (Table I). These results indicated that the C-terminal phenyl and Phe-2-phenyl groups are essential structural elements for chymotrypsin inhibition.

Optimal Spacer Length between the Two Phenyl Groups—If the two essential phenyl groups in H-D-Thr-Phe-NH-CH_2-C_6H_5 interact with chymotrypsin specifically, they should be placed spatially at a proper distance. Thus, an optimal spacer length may exist to locate them at each binding site. A series of dipeptide benzyl amides H-D-Thr-Phe-NH-$(CH_2)_n$-C_6H_5 ($n=0$-4) were synthesized and assayed for inhibition. The inhibitory potencies were compared in terms of the reciprocal of the inhibitory constants, namely, $1/K_i$. As shown in Fig. 3, the inhibition peaked at $n=1$. The phenylbutyl amide ($n=4$) and phenyl amide ($n=0$) were about 30- and 50-fold less active than the benzyl amide ($n=1$), respectively. Also, the phenethyl ($n=2$) and phenylpropyl ($n=3$) amides were considerably weaker than the benzyl amide.

Increase in the number of methylene chains would increase the molecular hydrophobicity of dipeptides. This may cause non-specific hydrophobic interactions between dipeptides and the enzyme, resulting in reinforcement of inhibitory activity. In this study, the molecular hydrophobicity was evaluated in terms of the retention time in analytical HPLC with a reversed-phase ODS column. As expected, dipeptides with increasing number of methylene chains emerged later with prolonged retention time (Fig. 3), indicating that the hydrophobicity of dipeptides indeed increased with increasing number of methylenes. It should be noted, however, that this apparent increase in hydrophobicity is completely independent of the inhibitory potencies of the dipeptides (Fig. 3). Thus, the maximal inhibitory activity by benzyl amide ($n=1$) was judged to be due to the structural optimization of the distance between the two phenyls, not to the molecular hydrophobicity.

Stereospecific Inhibition—The recognition of the chymo-

TABLE I. **Inhibitory potency of Thr-Phe dipeptides against chymotrypsin.**

Dipeptide	Inhibitory potency	
	K_i (μM)	Relative potency
L-Thr-Phe-NH-CH_2-C_6H_5	50±2.5	100
D-Thr-Phe-NH-CH_2-C_6H_5	4.5±0.41	1,100
D-Thr-Ala-NH-CH_2-C_6H_5	770±25	6
D-Thr-Phe-NH-CH_3	Inactive	0
D-Thr-Phe-NH_2	Inactive	0
D-Thr-Phe-NH-(*R*)-CH(CH_3)-C_6H_5	7.8±1.5	640
D-Thr-Phe-NH-(*S*)-CH(CH_3)-C_6H_5	240±23	21
D-Thr-Phe-NH-CH_2-C_6H_4(*p*-F)	0.91±0.07	5,500

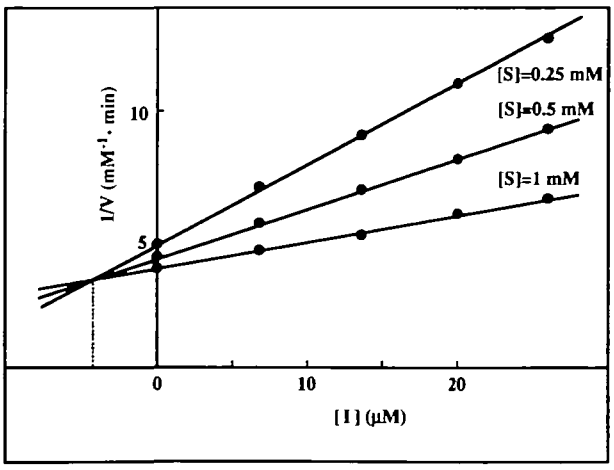

Fig. 2. **Dixon plot analysis for chymotrypsin hydrolysis of ATEE in the presence of H-D-Thr-Phe-NHBzl as an inhibitor.** The assay was performed at pH 7.0 (50 mM phosphate buffer) and 25°C. The substrate concentrations used are indicated in the figure, and the K_i value was estimated from the crossing point of three lines as 3.6×10^{-6} M.

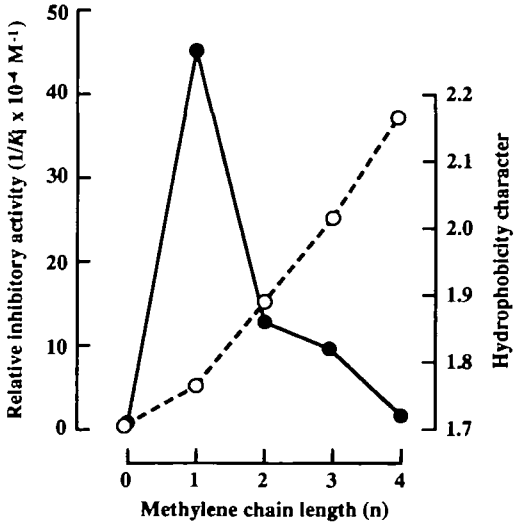

Fig. 3. **Relationship of chymotrypsin inhibitory activity and molecular hydrophobicity of H-D-Thr-Phe-NH-$(CH_2)_n$-C_6H_5 as a function of methylene chain length *n*.** Inhibitory activity is shown by a solid line (●) and molecular hydrophobicity by a broken line (○). The inhibitory potency was evaluated as the reciprocal of the inhibitory constants. The molecular hydrophobicity was estimated as the ratio of the retention time relative to that of H-D-Thr-Phe-NH_2, which has no phenyl group.

trypsin S_1 site by substrates is stereospecific (*16*). If the C-terminal phenyl group fits the chymotrypsin S_1 site, the interaction should also be stereospecific. When a methyl group is introduced into the methylene group present at the C-terminal benzyl moiety, two stereoisomers, H-D-Thr-Phe-NH-(*R* or *S*)-$CH(CH_3)$-C_6H_5, can be formed. We synthesized both isomers and tested them for chymotrypsin inhibition. It was found that both isomers inhibit chymotrypsin in a competitive manner. However, only the *R*-isomer was very potent ($K_i = 7.8 \times 10^{-6}$ M). The *S*-isomer was an extremely weak inhibitor (approximately 30-fold less active than the *R*-isomer and benzyl amide).

Activity Enhancement by para-Fluorophenyl Derivative—When the C-terminal phenyl group binds to the S_1 site, the head of this group would face toward Ser-189 in the S_1 pocket (*17*). Expecting hydrogen bonding to Ser-

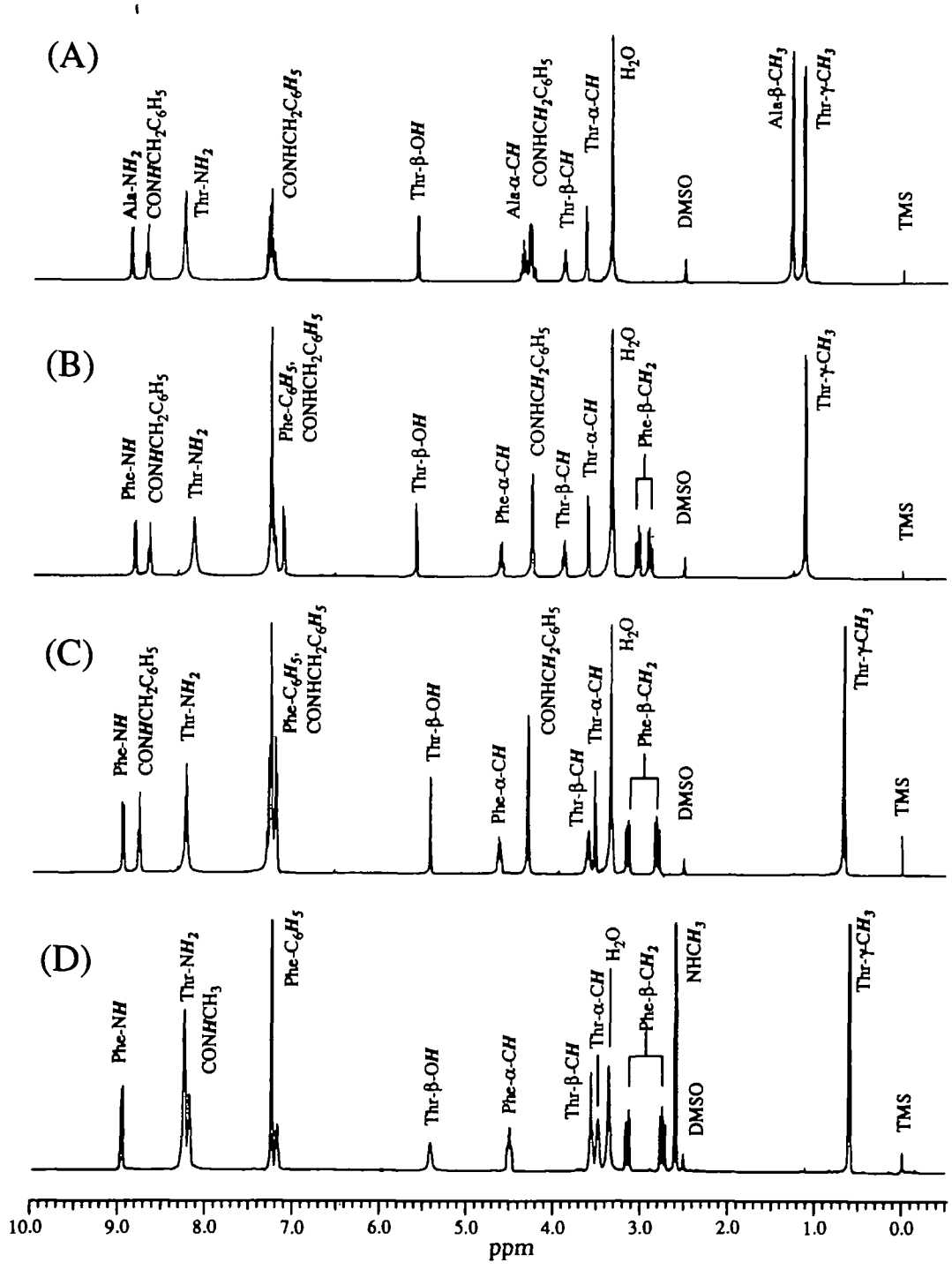

Fig. 4. **Comparison of chemical shifts of 400-MHz ^{1}H-NMR 1D spectra of Thr-containing dipeptides.** (A) H-D-Thr-Ala-NHBzl, (B) H-L-Thr-Phe-NHBzl, (C) H-D-Thr-Phe-NHBzl, and (D) H-D-Thr-Phe-$NHCH_3$. The spectra were recorded in DMSO-d_6 at 25°C, and each signal was assigned on the basis of 2D-COSY measurements.

189-βOH, we prepared the analog having a C-terminal *para*-fluorophenyl group instead of phenyl. The resulting H-D-Thr-Phe-NH-CH_2-C_6H_4(p-F) inhibited chymotrypsin very strongly ($K_i = 9.1 \times 10^{-7}$ M) in a competitive manner, indicating that the substitution of hydrogen to fluorine increased the inhibitory activity about five times (Table I).

Side Chain-Side Chain Interaction Analyzed by ^{1}H-NMR—For a series of benzyl amide analogs, ^{1}H-NMR measurements were carried out in DMSO-d_6 at room temperature. Signal assignments were accomplished basically with 2D DQF-COSY spectra. When the chemical shifts in DMSO-d_6 of Thr side chain protons of H-D-Thr-Phe-NHBzl were compared with those of H-D-Thr-Ala-NHBzl, large upfield shifts were observed for Thr-βOH (0.159 ppm), Thr-βCH (0.288 ppm), and γCH_3 (0.472 ppm) (Fig. 4) (Table II). No shifts were observed for L-Thr-side chain in H-L-Thr-Phe-NHBzl (Fig. 4) (Table II). All other dipeptides amides having D-Thr-Phe backbone structure exhibited large upfield shifts of proton signals of the D-Thr side chain; βOH (0.09-0.17 ppm), βCH (0.22-0.32 ppm), and γCH_3 (0.38-0.53 ppm) (Table II). It should be noted that the NMR spectra of H-D-Thr-Phe-NH_2 and H-D-Thr-Phe-$NHCH_3$ also showed large upfield shifts of D-Thr-side chain proton signals. Since these dipeptides lack the C-terminal amide-phenyl group, there is only the benzene ring of Phe-2. It was thus concluded that the upfield shifts observed for the D-Thr side chain are attributed to the shielding effect from the Phe-phenyl group, but not to that from the amide-phenyl group at the C-terminus. This is also apparent from the absence of upfield shifts of D-Thr-side chain protons in H-D-Thr-Ala-NHBzl. These results imply that the Phe-phenyl group is in close proximity to the D-Thr-side chain, and this was confirmed by measurements of NOESY spectra as described below.

NOE Enhancements between Side Chains—The presence of a side chain-side chain interaction between the Thr-methyl and Phe-phenyl groups was further confirmed by measurements of 2D NOESY spectra and 1D nuclear Overhauser effect (NOE) difference spectra for H-D-Thr-Phe-NHBzl and H-D-Thr-Phe-$NHCH_3$. The NOESY spectrum of H-D-Thr-Phe-NHBzl showed a cross-peak between Thr-γ-CH_3 and the aromatic envelope, but no such peak was observed for H-L-Thr-Phe-NHBzl (data not shown). Although the signals of the two benzene rings of Phe-2 and C-terminal phenyl emerged at the same region of the aromatic envelope, it appeared that the signal (*ca.* 7.2 ppm) of *ortho*-Hs of Phe-phenyl is slightly apart from those (*ca.* 7.3 ppm) of *meta* and *para*-Hs (Fig. 4). When the NOESY spectrum of H-D-Thr-Phe-$NHCH_3$ was measured, the cross-peak between Thr-γ-CH_3 and *meta* and *para*-Hs was also distinctly observed (Fig. 5). This peak was confirmed as a genuine NOE by measurements with a series of mixing times (50-1,000 ms). Since H-D-Thr-Phe-$NHCH_3$ lacks the C-terminal phenyl group, this cross-peak is certainly due to

TABLE II. **Upfield shifts of proton chemical signals of D-Thr-side chain in 400 MHz ^{1}H-NMR 1D measurements.**

Dipeptide	Upfield shifts (ppm)		
	Thr-βOH	Thr-βCH	Thr-γCH_3
D-Thr-Ala-NH-CH_2-C_6H_5	0.0 (5.581)[a]	0.0 (3.885)	0.0 (1.136)
D-Thr-Phe-NH-C_6H_5	0.124	0.222	0.387
D-Thr-Phe-NH-CH_2-C_6H_5	0.159	0.288	0.472
D-Thr-Phe-NH-$(CH_2)_2$-C_6H_5	0.153	0.301	0.507
D-Thr-Phe-NH-$(CH_2)_3$-C_6H_5	0.136	0.288	0.479
D-Thr-Phe-NH-$(CH_2)_4$-C_6H_5	0.156	0.297	0.487
D-Thr-Phe-NH-CH_3	0.174	0.316	0.529
D-Thr-Phe-NH_2	0.091	0.276	0.460
D-Thr-Phe-NH-(R)-CH(CH_3)-C_6H_5	0.143	0.237	0.376
D-Thr-Phe-NH-(S)-CH(CH_3)-C_6H_5	0.140	0.231	0.404
D-Thr-Phe-NH-CH_2-C_6H_4(p-F)	0.164	0.266	0.445
L-Thr-Phe-NH-CH_2-C_6H_5	0.012	0.002	0.023

[a]Values in the parenthesis show the chemical shifts (ppm) of protons of the D-Thr-side chain in D-Thr-Ala-NH-CH_2-C_6H_5.

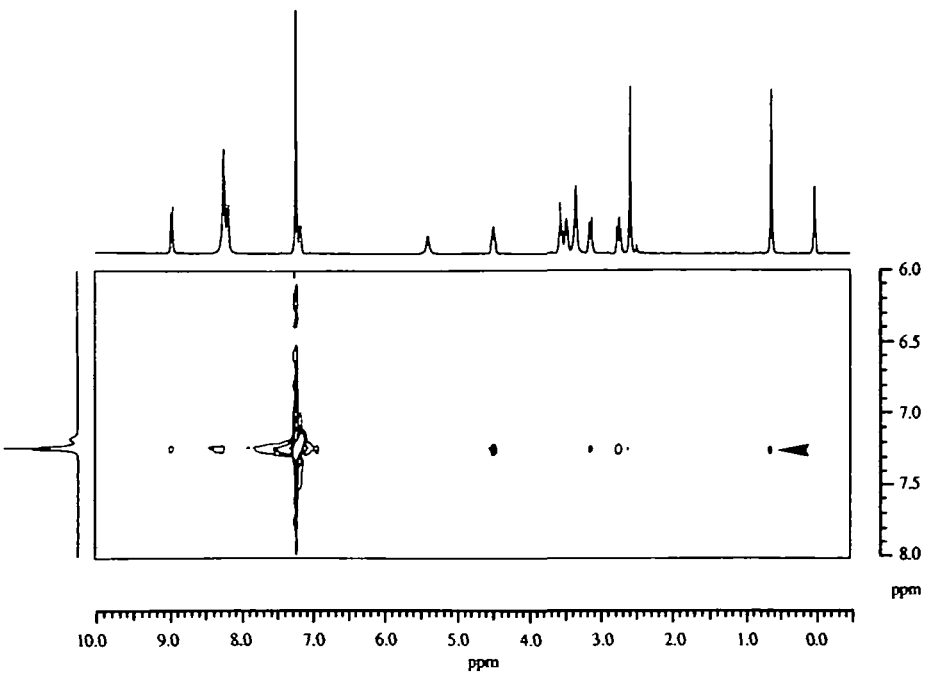

Fig. 5. **2D NOESY spectrum for pairs of the Phe-2 aromatic ring proton and aliphatic protons in D-Thr-Phe-$NHCH_3$, which possesses only one aromatic ring, that of Phe-2.** The cross-peak indicated with an arrowhead was assigned to the NOE between the Phe-phenyl and the D-Thr-γCH_3 groups.

NOE enhancements between side chains of D-Thr-1 and Phe-2.

H-{H} NOE difference spectra were obtained by irradiation of selected proton signals (data not shown). When the proton signals of D-Thr-β-CH and γ-CH_3 in H-D-Thr-Phe-$NHCH_3$ were irradiated separately, distinct NOE enhancements were found for signals at the aromatic envelope (7.2–7.3 ppm) in addition to those observed for signals of the D-Thr protons. No enhancement was observed for those of H-D-Thr-Ala-NHBzl, which lacks Phe-phenyl. These results further confirm that the side chains of D-Thr-1 and Phe-2 are in close proximity to each other.

Conformational Rigidity—The signals (double doublets) of protons of Phe-βCH_2 of H-D-Thr-Phe-NHBzl were nonequivalent, showing a splitting pattern ($\Delta\delta = 0.337$ ppm). The H_α-H_β's vicinal coupling constants of Phe were about 4.4 and 10.4 Hz. When the fractional populations P_I, P_{II}, P_{III} for three staggered rotamers about the C_α-C_β bond of Phe were calculated using these J couplings with Pachler's equation (*18*), the obtained average P_X values were (0.71, 0.16, 0.13) (the values of P_I and P_{II} are exchangeable) (Table III). It should be noted that P_I is much larger than P_{II}. In contrast, the P_I and P_{II} values of Phe in H-L-Thr-Phe-NHBzl are almost equal (Table III). These results indicate that H-D-Thr-Phe-NHBzl has a substantial rigidity of the Phe side chain around the χ_1 angle, while that of the L-Thr isomer is relatively flexible. This is clearly a reflection of the fact that the Phe-phenyl group is in close proximity to the D-Thr-side chain. Although the conformation of H-D-Thr-Phe-NHBzl appears to be rather rigid, it is unlikely that the side chains are completely fixed. It had been suggested that the Phe-phenyl is free from D-Leu-isobutyl during the hydrolysis of H-D-Leu-Phe-OBzl by chymotrypsin.

Judging from the P_X values for H-D-Leu-Phe-OBzl (Table III), it appears that H-D-Thr-Phe-NHBzl is in a conformation with almost the same rigidity as H-D-Leu-Phe-OBzl. Other amide analogs were also shown to have conformations similar to that of H-D-Thr-Phe-NHBzl (Table III). No particular difference in ^{1}H-NMR spectra was found between H-D-Thr-Phe-NH-(*R*)-$CH(CH_3)$-C_6H_5 and H-D-Thr-Phe-NH-(*S*)-$CH(CH_3)$-C_6H_5.

Conformation in Water—Similar conformational analyses by 400 MHz ^{1}H-NMR measurements were performed for H-D-Thr-Phe-NHBzl, H-D-Thr-Ala-NHBzl, H-D-Thr-Phe-$NHCH_3$, and H-L-Thr-L-Phe-NHBzl in D_2O. Large upfield shifts for D-Thr-βCH (0.21–0.28 ppm) and γCH_3 (0.20–0.28 ppm) were observed, but not in the L-Thr residue. Distinct NOESY cross-peaks between D-Thr-γCH_3 and Phe-phenyl were observed for H-D-Thr-Phe-$NHCH_3$ (data not shown), indicating that these groups are in close proximity. Calculated average P_X values were (0.61, 0.21, 0.18) for three staggered rotamers about the C_α-C_β bond of Phe in H-D-Thr-Phe-$NHCH_3$. All these results indicated that dipeptides with a D-Thr-Phe backbone structure adopt essentially similar conformations in water and DMSO-d_6.

TABLE III. **Pachler's coefficient (P_X) for three staggered rotamers about the Cα-Cβ bond of Phe-2 in Thr-Phe or Leu-Phe dipeptide benzyl amides and esters.**

Dipeptide	P_I	P_{II}	P_{III}
D-Leu-Phe-O-CH_2-C_6H_5	0.68	0.19	0.13
D-Thr-Phe-NH-C_6H_5	0.67	0.21	0.12
D-Thr-Phe-NH-CH_2-C_6H_5	0.71	0.16	0.13
D-Thr-Phe-NH-$(CH_2)_2$-C_6H_5	0.61	0.23	0.16
D-Thr-Phe-NH-$(CH_2)_3$-C_6H_5	0.70	0.19	0.11
D-Thr-Phe-NH-$(CH_2)_4$-C_6H_5	0.67	0.20	0.11
D-Thr-Phe-NH-CH_3	0.74	0.13	0.13
L-Leu-Phe-O-CH_2-C_6H_5	0.48	0.33	0.19
L-Thr-Phe-NH-CH_2-C_6H_5	0.51	0.33	0.16

DISCUSSION

It was found in the present study that the dipeptide benzyl amide H-D-Thr-Phe-NHBzl inhibits chymotrypsin much more strongly than reported chymotrypsin inhibitors such as aldehydes (*7*) and boric acid derivatives (*8*), which are characterized by poor water solubility and high toxicity (*6*). The potency ($K_I = 4.5 \times 10^{-6}$ M) of H-D-Thr-Phe-NHBzl for chymotrypsin inhibition was comparable to that of chymostatin (*19*). Competitive enzyme inhibitors usually have a structure similar to that of substrates. When H-D-Leu-Phe-OBzl was found to act as a competitive inhibitor of chymotrypsin, the Phe-phenyl group was thought to be directed toward the chymotrypsin S_1 site. Indeed, this residue occupied the S_1 site, since chymotrypsin eventually hydrolyzed the ester bond (*9*). However, it was suggested that H-D-Leu-Phe-OBzl adopts a conformation in which the D-Leu-isobutyl and Phe-phenyl are in proximity (*9*). In this unique conformation, the group binding to the S_1 site was assumed to be the ester-benzyl. In the present study, the first requisite was to confirm this.

For high inhibitory activity of H-D-Thr-Phe-NHBzl, several structural requirements were found. The first important requisite is the presence of Phe at position 2. When Phe-2 was replaced by Ala, the resulting H-D-Thr-Ala-NHBzl exhibited extremely low inhibitory activity (only 0.58% of that of H-D-Thr-Phe-NHBzl). The importance of Phe-phenyl is not just because of its interaction with the enzyme. The proximity of the Phe-phenyl group to the D-Thr-side chain was evidenced by the upfield chemical shifts and NOE enhancements in ^{1}H-NMR measurements (Table II and Figs. 4 and 5).

The interaction between the D-Thr-side chain and Phe-phenyl is well characterized in terms of CH/π interaction. Nishio *et al.* (*20*) have recently described in detail the concept of the CH/π interaction. The characteristics of CH/π interaction are: (i) it can play a role in polar media, such as biological environments, (ii) multiple CH groups as in methyl groups can participate simultaneously in interactions with the π system, and (iii) the π-electron density of the aromatic ring increases. Although the enthalpy for one unit of CH/π interaction is small (about 1 kcal/mol), the total enthalpy becomes sizeable when the CH/π interaction is multiple. Since upfield shifts of the proton signals were observed for all of the D-Thr-side chain including βOH, weak H-bondings may exist between protons of the D-Thr-side chain and Phe-2-phenyl. Thus, in the case of D-Thr-Phe dipeptides, the CH/π interaction between D-Thr-side chain and Phe-phenyl appears to be rather strong.

One of the characteristics of the CH/π interaction is a relatively large contribution from delocalization (charge transfer from π to σ*) and dispersive interaction as compared to the normal H-bonding (*20*). This is the basis of the crucial point that CH/π interaction can play a role in

polar media, the interaction being hardly disturbed by the presence of water. It should be noted that the CH/π interaction in H-D-Leu-Phe dipeptides is well retained in water.

However, it is not yet clear whether such a hydrophobic core induced by the CH/π interaction is responsible for chymotrypsin inhibition. An analog with non-π cyclohexyl at position 2 may answer this question. Another important structural requirement for chymotrypsin inhibition is the C-terminal phenyl group. Since elimination of the benzyl ($-CH_2C_6H_5$) or phenyl ($-C_6H_5$) group from H-D-Thr-Phe-NHBzl, creating H-D-Thr-Phe-NH_2 or H-D-Thr-Phe-$NHCH_3$, resulted in inactivity, the role of the phenyl group in inhibition is crucial. Furthermore, it was found that this C-terminal phenyl group should be placed at the C1 position from the amide NH group. Positioning of phenyl at a shorter or longer distance than C1 decreased the inhibitory activity drastically (Fig. 3). It is obvious that the presence of such an optimum distance between the dipeptide backbone and C-terminal phenyl group relates to the structure of enzyme, especially the sites to which the inhibitors bind. An important question about this C-terminal phenyl group is whether or not such complementary structure of chymotrypsin requires only the benzene ring. To address this point, derivatives with cyclohexyl or other alkyl groups should be tested.

The mode of chymotrypsin inhibition by H-D-Thr-Phe-NHBzl was competitive as analyzed by Dixon plots, indicating that the substrate and inhibitor compete for the same S_1 site of chymotrypsin. The interactions between chymotrypsin and substrates or inhibitors at the S_1, S_1', and S_2 are all hydrophobic (*21*). The S_1 site is a hydrophobic pocket. The size of this S_1 pocket of chymotrypsin is reported to be 10-12 Å in depth and (3.5-4.0 Å) × (5.5-6.5 Å) in section (*22*), so that a benzene ring (3.5 Å in thickness and 6 Å in width) can fit into it. The recognition of the S_1 site is usually stereospecific, the L-aromatic amino acid being preferred. When two stereoisomers of H-D-Leu-Phe-NH-(*R* or *S*)-$CH(CH_3)$-C_6H_5 were tested for chymotrypsin inhibition, only the *R*-isomer was potent. The *S*-isomer was an extremely weak inhibitor. It should be noted that the *R*-configuration of C-terminal amide structure corresponds to the L configuration of amino acids, when the phenyl is thought of as a side chain. Judging from all these results, the C-terminal phenyl group is likely to be directed toward the S_1 site, and the side chain hydrophobic core to the S_2 or S_1' site (Fig. 6A).

When a fluorine atom was introduced into the *para*-position of C-terminal phenyl, the inhibitory potency of the resulting H-D-Thr-Phe-NH-CH_2-C_6H_4(*p*-F) considerably increased. If the C-terminal phenyl group binds to the S_1 site, this fluorine would face toward chymotrypsin Ser-189 in the S_1 pocket. It is then likely that hydrogen bonding between fluorine and Ser-189-OH would reinforce the enzyme-inhibitor interaction, resulting in increased inhibitory activity.

Nishio *et al.* (*20*) showed that several protein-ligand complexes exhibit CH/π interactions. In addition, they indicated the presence of a CH/π network in protein, which plays a role in stabilizing the structure of the ligand binding site. However, the present study shows another effect of CH/π interaction, which stabilizes a specific conformation of a small ligand molecule. This emphasizes the usefulness

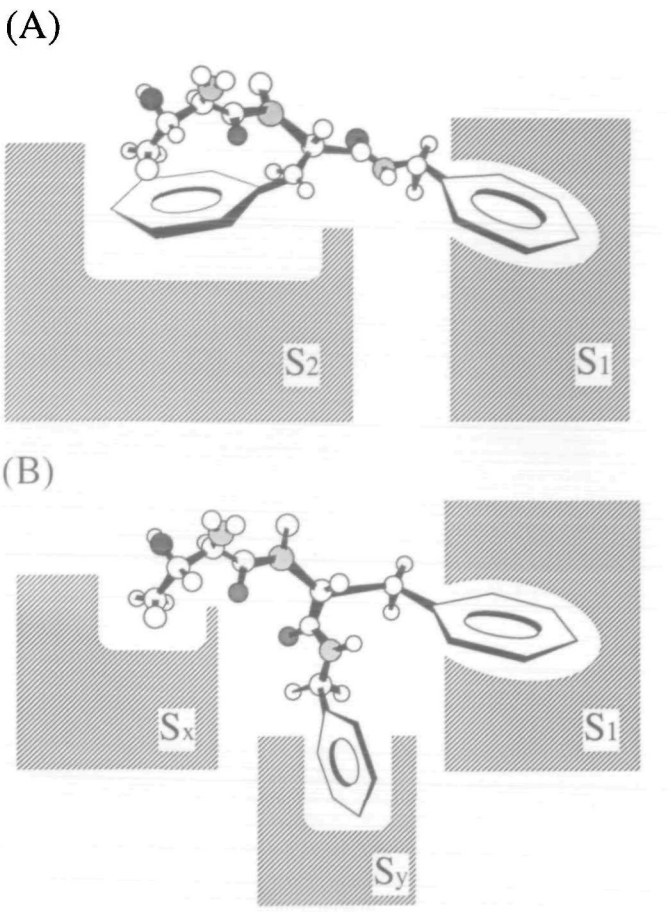

Fig. 6. **Possible binding models of dipeptide inhibitor H-D-Thr-Phe-NHBzl in the chymotrypsin active center.** (A) The C-terminal phenyl group binds to the S_1 site, while the hydrophobic core constructed by side chain-side chain CH/π interaction binds to the S_2 site. (B) The Phe-phenyl group occupies the S_1 site, and the C-terminal phenyl and D-Thr-side chain are directed toward each specific binding site.

of the CH/π interaction concept in designing molecules which interact specifically with acceptors (*23-25*).

In spite of the assumption described above, totally different inhibition modes may also be feasible. As mentioned, H-D-Leu-Phe-OBzl was eventually hydrolyzed by chymotrypsin. This was due to the occupation of the chymotrypsin S_1 site by Phe-2. Since D-Thr-Phe dipeptides are assumed to be in a conformational equilibrium between the structure having the side chain-side chain CH/π interaction and the extended structure without CH/π interaction, Phe-2 may occupy the chymotrypsin S_1 site (Fig. 6B). In this case, the C-terminal phenyl group, which is essential for inhibition, should interact with a site other than S_1 (S_y in Fig. 6B). These interactions might be sufficient to prevent the substrate from binding to these sites, and the insusceptibility of D-Thr-Phe dipeptide amides to chymotrypsin hydrolysis would sustain such an inhibiting action. Since the L-L isomer inhibited chymotrypsin only weakly (Table I), D-Leu should also possess a specific interaction in this inhibitory conformation (S_x in Fig. 6B). It is also possible that both mechanisms are involved in the effective inhibition of chymotrypsin. An X-ray analysis of dipeptide-chymotrypsin complex would resolve this question.

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