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Hydrogen Bonding and Attenuation of the Rate of Enzymic Catalysis

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Abstract: Hydrogen bonds between a small molecule and an enzyme can potentially contribute significantly to the stability of the complex. Such electrostatic interactions can also lower energy barriers for reactions by solvation of high-energy species. A novel type of inhibitor is described in this report, which was designed to take advantage of a hydrogen bond that it makes to the active-site histidine of chymotrypsin to attenuate its basicity. Substrates of chymotrypsin acylate the active-site serine (of the catalytic triad), and the acyl-enzyme intermediate undergoes deacylation in a second step of the catalytic turnover. The active-site histidine (of the catalytic triad) serves as the general base in both steps of the turnover process. Such attenuation of basicity by hydrogen bonding was expected to impair catalysis by the enzyme. Two molecules of this type were synthesized that are based on the structure of the chymotrypsin substrate Ac-L-Ala-L-Ala-Gly-L-Phe methyl ester. These were methyl (2*R*,3*R*)-5-(*N*-acetyl-L-alanyl-L-alanyl)amino-2-benzyl-3-hydroxylpentanoate (**1**) and methyl (2*R*,3*S*)-5-(*N*-acetyl-L-alanyl-L-alanyl)amino-2-benzyl-3-hydroxylpentanoate (**2**). Compound **1** acylated chymotrypsin, but the acyl-enzyme species resisted deacylation. On the other hand, compound **2** did not even have the ability to acylate the active-site serine. Molecular modeling supported the assertion that compound **1** makes a critical hydrogen bond to the active-site histidine at the acyl-enzyme stage, whereas compound **2** does so at the preacylation complex. The concepts described herein are of general interest and should find applications for inhibition of enzymes that employ general acid−base chemistry for their catalytic processes.

Hydrogen bonding is an electrostatic interaction that is ubiquitous in biological molecules.[1] It is one of the factors that contributes to protein folding, to substrate recognition by enzymes, and to the energetics of turnover in enzymic chemistry. An enzyme may make hydrogen bonds to the ground-state or transition-state species. If the strength of a given hydrogen bond to the transition-state species is stronger than that to the ground state, the hydrogen bond lowers the energy barrier for the enzymic reaction by solvating the high-energy transition-state species. The effects of these interactions could range from modest to significant. A typical hydrogen bond may contribute as much as 2−3 kcal/mol toward binding or lowering of energy barriers, although effect as much as 11 kcal/mol have been documented for enzymic systems.[2] Furthermore, the possibility for existence of low-barrier or short, strong hydrogen bonds, which may have the potential to contribute to enzymic catalysis even more significantly, has been the subject of much discussion recently.[3] Indeed, the manifestation of the effects of such an interaction between an enzyme and substrate can be realized even at sites relatively remote from the seat of the reaction.[4]

The contribution of hydrogen bonds has often been a factor in design of molecules that mimic either the transition-state or ground-state species in design of inhibitors.[5] We disclose herein a novel type of enzyme inhibitor that exploits the nature of a

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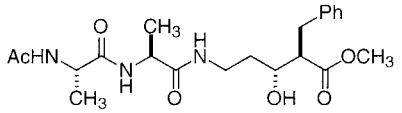
specific hydrogen bond between the inhibitor and the enzyme to shut down the catalytic machinery.

We decided to apply this method of inhibition to chymotrypsin, a prototypic serine protease for which high-resolution crystal structures are known. The catalytic machinery of chymotrypsin, as in other serine proteases, is dominated by the catalytic triad of Asp-His-Ser, of which serine undergoes acylation and deacylation in the course of substrate turnover. The histidine of the triad activates the serine hydroxyl for the acylation process and a water molecule for the deacylation step. Hence, it serves as a general base in both steps. We reasoned that the donation of a hydrogen bond from a substrate analogue to the histidine imidazole group would attenuate its basicity. One could envision this in two ways: the hydrogen bond would tie up the lone pair of electrons on the imidazole ring, so it would not be available for activation of the active-site serine, and the additional hydrogen bond solvates the general base effectively. Each principle would lower the basicity of the general base and would impair the catalytic machinery of chymotrypsin in either the preacylation complex or the acyl-enzyme intermediate stage.

Our design of the inhibitor(s) commenced with the crystal structure of chymotrypsin modified by Pro-Gly-Ala-Tyr.[6] Molecular modeling indicated that a hydroxyl moiety β to the ester carbonyl would be poised for interaction with the His-57 imidazole. Furthermore, both *R* and *S* stereoisomers at that site could allow for such an interaction, although the hydrogen bond by the *R* stereoisomer to the imidazole ring appeared to be stronger. Therefore, we envisioned two potential inhibitors, compounds **1** and **2**. These compounds are peptidomimetic analogues of the chymotrypsin substrate **3**. Since evaluation of the effect of the hydroxyl group in **1** and **2** was central to our concept for inhibition, we also decided to study compound **4**, which lacks that functional group.



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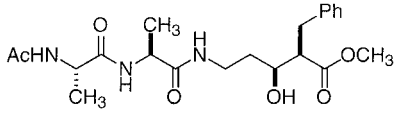

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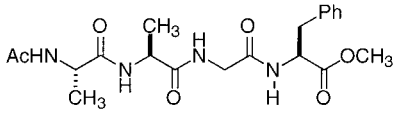

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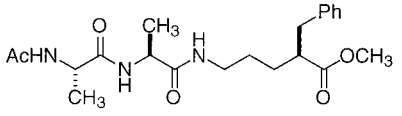

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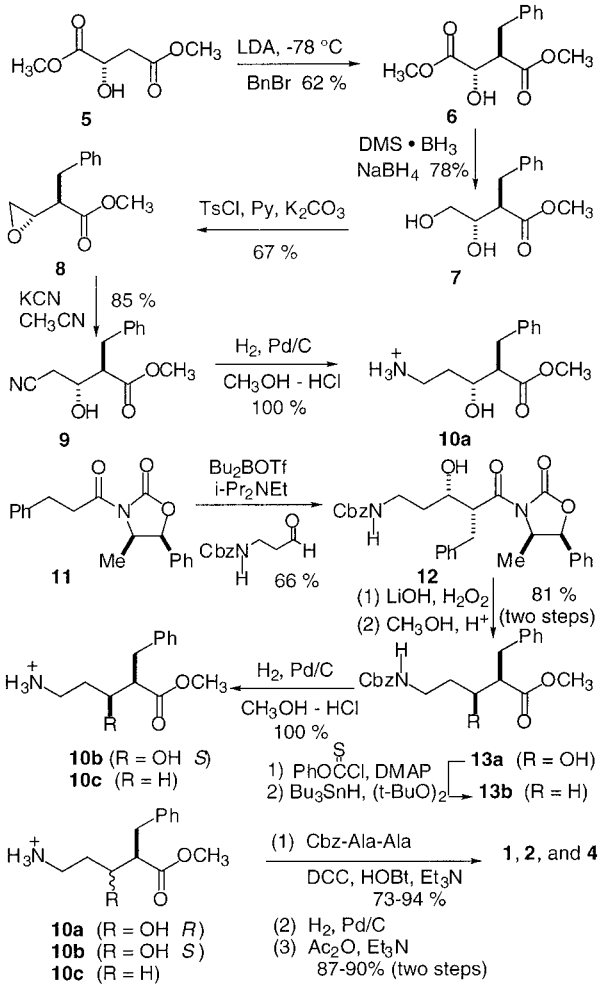
Compounds **1**, **2** and **4** were synthesized according to Scheme 1. Dimethyl L-malate (**5**) was stereoselectively benzylated by following the procedure reported by Seebach and Wasmuch[7] to give **6**. No diastereomeric impurity was detected when the optical purity of the product was analyzed by NMR. The conversion of **6** to **7** was effected in 78% yield by the general procedure for regioselective reduction with the borane−dimethyl sulfide complex in the presence of a catalytic amount of sodium borohydride in THF.[8] Treatment of **7** with *p*-toluenesulfonyl chloride gave selective tosylation of the primary hydroxyl group. Subsequent treatment of the tosylate with potassium carbonate in methanol afforded **8**. Nucleophic opening of the epoxide ring with potassium cyanide gave **9** in 85% yield. Medium-pressure catalytic hydrogenation of **9** in the presence of palladium-on-charcoal yielded **10a**, which was subsequently coupled with *N*-Cbz-L-Ala-L-Ala. The Cbz protecting group was replaced by the acetyl group to afford methyl (2*R*,3*R*)-5-(*N*-acetyl-L-alanyl-L-alanyl)amino-2-benzyl-3-hydroxypentanoate (**1**). The (2*R*,3*S*) analogue was synthesized starting from the *N*-acylated oxazo-

Scheme 1



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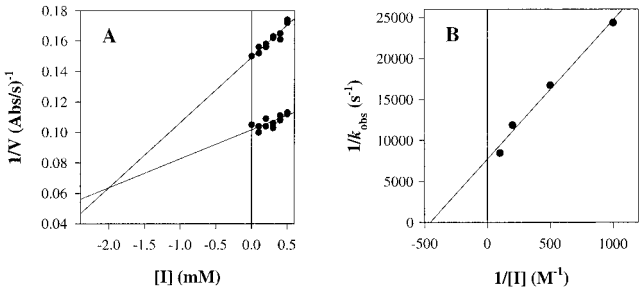

Figure 1. (A) Dixon plot for reversible competitive inhibition of chymotrypsin by compound **2**. (B) The double-reciprocal plot of the first-order rate constant for inactivation of chymotrypsin vs concentrations of compound **1**.

lidinone **11**[9] in the presence of 2 equiv of diisopropylethylamine with *N*-Cbz-3-aminopropanal to afford the *erythro*-isomer **12**.[10] Removal of the Cbz group from compound **13a** facilitated a spontaneous cyclization of the product to give the corresponding six-membered lactam. The absolute stereochemistry at positions 2 and 3 were established by determination of the X-ray structure of this lactam. The *erythro*-adduct **12** was readily hydrolyzed without racemization of either center to the corresponding acid upon treatment with 1.5 equiv of lithium hydroxide and 4 equiv of hydrogen peroxide in THF−water (0 °C). Following the esterification, **13a** was transformed into the corresponding deoxy methyl ester **13b** by treatment with phenyl chlorothionoformate and DMAP, followed by tributyltin hydride and di-*tert*-butylperoxide in good yield. As described above, when **13a** was subjected to deprotection in methanol, almost a quantitative yield of the corresponding lactam was obtained, which proved helpful in the establishment of the absolute chemistry. However, when the deprotection was performed in methanol containing 2 equiv of HCl, hydrochloric acid salts of **10b** and **10c** were obtained. Compounds **10b** and **10c** were transformed into the corresponding tetrapeptide analogues **2** and **4** according to the procedure described above.

Compound **4** is simply a substrate for chymotrypsin ($k_{cat} = 0.054 \pm 0.003$ min^{-1} and $K_m = 420 \pm 50$ μM, $k_{cat}/K_m = 2.1 \pm 0.3$ M^{-1} s^{-1}), which means that it acylated the enzyme and the complex underwent deacylation. The following two substrates have similar structures to our compounds and they have been studied with chymotrypsin: Ac-L-Ala-L-Ala-L-Phe-OMe ($K_m = 30$ μM, $k_{cat} = 60$ s^{-1}, $k_{cat}/K_m = 2 \times 10^6$ M^{-1} s^{-1})[11a] and Suc-L-Ala-L-Ala-L-Pro-L-Phe-pNA ($K_m = 43$ μM, $k_{cat} = 45$ s^{-1}, $k_{cat}/K_m = 1 \times 10^6$ M^{-1} s^{-1}).[11b] Compound **4** is a poorer substrate for chymotrypsin by a few orders of magnitude. The reason for this difference in tunover property may have to do with the fact that the amine moiety of the C-terminal amino acid of the substrate makes a critical hydrogen bond to the main-chain carbonyl of Ser-214 of chymotrypsin.[6,12] This interaction is obviously absent for the binding of **4** to the active site.

On the other hand, both **1** and **2** inhibited the enzyme, and each did so in a different manner. Compound **2** served as a competitive inhibitor of the enzyme ($K_i = 2.2 \pm 0.8$ mM; Figure 1A). Dilution of the inhibited chymotrypsin mixture into a solution of substrate for the enzyme allowed for the immediate recovery of full activity. Furthermore, analysis of the inhibition mixture for over 20 h did not reveal the formation of any products of turnover of this compound in the presence of the enzyme. These results collectively indicate that **2** formed the preacylation complex for its competitive inhibition of the enzyme, but resisted serine acylation. However, **1** behaved as a mechanism-based inactivator for chymotrypsin, as evidenced by a time-dependence for loss of activity associated with enzyme acylation. Once the acyl-enzyme intermediate formed, it resisted deacylation. The enzyme inactivation proceeded with the following kinetic parameters: $k_{inact} = (1.4 \pm 0.2) \times 10^{-3}$ s^{-1}, $K_I = 2.8 \pm 0.7$ mM, and $k_{inact}/K_I = 0.6 \pm 0.1$ M^{-1} s^{-1} (Figure 1B). The inhibited complex underwent deacylation with the attendant recovery of activity in a very slow process with $k_{rec} = (4.2 \pm 0.5) \times 10^{-6}$ s^{-1} (i.e., $t_{1/2}$ for recovery of activity was 1.9 days). The dissociation constant for the formation of the preacylation complex with this enzyme inactivator (K_i) was evaluated at 620 ± 150 μM.[13] Therefore, as per design principles, we believe that this compound interacted with the histidine imidazole in the second step, such that once the acyl-enzyme species formed, it could not undergo ready deacylation. However, comparison of the values for k_{inact}/K_I for compound **1** with that for k_{cat}/K_m for **4** indicates that acylation of the active-site serine is not a problem for compound **1** (the two values are comparable). One discerns this from the following argument. Serine proteases operate by the following kinetic scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E\text{–}S \xrightarrow{k_3} E + P$$

For this scheme, the following kinetic parameters are derived: $k_{cat} = (k_2k_3)/(k_2 + k_3)$, $K_m = (k_3K_s)/(k_2 + k_3)$, and $k_{cat}/K_m = k_2/K_s$, assuming that $k_{-1} > k_2$. The ratios of k_{cat}/K_m and, by analogy, of k_{inact}/K_I, are directly proportional to the rate of enzyme acylation.

Modeling was attempted to rationalize these findings. Whereas the confirmation of these observations should await crystallographic findings, preliminary modeling indicated that the hydroxyl group of compound **1** interactes with the active-site histidine at the acyl-enzyme intermediate and that of **2** does so at the preacylation complex. Hence, **1** acylates the active-site serine and resists deacylation, whereas **2** is incapable of enzyme acylation.

Compounds **1** and **2** serve as prototypes for this novel type of enzyme inhibitor, and they demonstrate clearly the proof of the concept disclosed herein. The strategy described in this report should in principle find applications for many enzymes that employ general acid−base chemistry for their catalytic processes.[14]

Material and Methods

^{1}H and ^{13}C NMR spectra were recorded on either a Varian Gemini-300 or a Varian Unity-500 spectrometer. Chemical shifts are reported in ppm from tetramethylsilane on the δ scale. Optical rotations were determined with a Jasco DIP-370 digital polarimeter. Infrared and mass spectra were recorded on Nicolet 680 DSP and Kratos MS 80RFT

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(10) Interestingly, the aldol condensation in the presence of 1.0 equiv of the base led to the selective formation of the *anti*-aldol; the experimental details for this observation will be described elswhere.

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(13) K_i is a dissociation constant, whereas K_I is a more complex parameter for inhibition, such as K_m is for turnover.

(14) The crystal structure of chymotrypsin modified by the mechanism-based inactivator 3-benzyl-6-chloro-2-pyrone indicates that a carboxylate from the inhibitor, which is generated on active-site modification of the enzyme, interacts weakly (3.4 Å) with the active-site histidine (Ringe, D.; Mottonen, J. M.; Gelb, M. H.; Abeles, R. H. *Biochemistry* **1986**, *25*, 5633). The complex undergoes slow deacylation, which may be attributable to this weak interaction and the fact that the acyl-enzyme species is an $\alpha-\beta$-unsaturated ester.

spectrometers, respectively. Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Analytical high-performance liquid chromatography was carried out on a Perkin-Elmer Series 410 LC, using a Vydac C-18 column. Thin-layer chromatography was performed with Whatman reagents 0.25 mm silica gel 60-F plates. All other reagents were purchased from the Aldrich Chemical Co. α-Chymotrypsin and *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide were purchased from Sigma Co. Spectrophotometric studies were performed on Hewlett-Packard 8453 diode array instrument. Nonlinear regression analysis was performed by the use of the SigmaPlot program (Jandel Scientific). Other calculations were performed by the MS Excel program. The PDB codes for the chymotrypsin that were used for the design of the compounds are 1gct.pdb and 2gct.pdb (both at 1.8 Å resolution).[6] The numbering of the residues is according to the PDB nomenclature, as described earlier.[6] The optical rotation, melting point, IR, NMR, and MS for each compound are provided in the Supporting Information.

Dimethyl (2*R*,3*S*)-2-Benzyl-3-hydroxysuccinate (6). Butyllithium (2.5 M solution in hexane; 28 mL, 70 mmol) was added dropwise to a solution of diisopropylamine (9.9 mL, 70 mmol) in anhydrous THF (50 mL) at 0 °C under a nitrogen atmosphere, and the mixture was stirred for 30 min. Dimethyl (*S*)-2-hydroxysuccinate (5.3 g, 33 mmol) in anhydrous THF (10 mL) was added dropwise to the solution of lithium diisopropylamine, described above, at −78 °C, and the mixture was stirred for 1 h. Benzyl bromide (6.0 g, 35 mmol) was added and stirring was continued at −78 °C for 1 h and subsequently at −50 °C for 5 h. Saturated aqueous ammonium chloride (20 mL) was added to the mixture, and the solution was then washed with ethyl acetate. The organic layer was washed with 5% $NaHCO_3$ and saturated NaCl, was dried over $MgSO_4$, and was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc:hexane, 1:4) to afford the title compound as a colorless oil (5.16 g, 62%).

Methyl (2*R*,3*S*)-2-Benzyl-3,4-dihydroxybutanoate (7). A borane−methyl sulfide solution (2.0 M solution in THF; 2.32 mL, 4.64 mmol) was added dropwise to a solution of **6** (1.17 g, 4.64 mmol) in anhydrous THF (10 mL) at 0 °C under a nitrogen atmosphere, and the mixture was stirred for 1 h. Sodium borohydride (8.8 mg, 0.23 mmol) was added to the mixture, and stirring was continued for 30 min at room temperature. The mixture was then quenched by the addition of dry methanol (3 mL), followed by an additional 30 min of stirring. The solvent was removed by evaporation in vacuo to give a colorless oil that was purified by column chromatography (silica gel, EtOAc:hexane, 1:1) to give the title compound as a colorless oil (810 mg, 78%).

Methyl (2*R*,3*S*)-2-Benzyl-3,4-epoxybutanoate (8). Freshly distilled pyridine (4 mL) and toluenesulfonyl chloride (7.63 g, 40 mmol) were added to a solution of **7** (4.48 g, 20 mmol) in dry dichloromethane (48 mL) at 0 °C under a nitrogen atmosphere. The mixture was stirred for 12 h at 0 °C and was then acidified (pH 3−4) with 1N HCl. The organic layer was washed with water and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo to give the monotosylate as a semisolid (5.67 g). This residue was dissolved in methanol (50 mL) and was treated with potassium carbonate (2.07 g, 15 mmol) while being chilled in ice−water temperature. After 1 h, the mixture was quenched by the addition of water and was washed with ethyl acetate. The ethyl acetate layer was washed with saturated NaCl and dried over Na_2SO_4, and the solvent was evaporated. The residue was purified by column chromatography (silica gel, EtOAc:hexane, 1:4) to give the title compound as a colorless oil (2.75 g, 67%).

Methyl (2*R*,3*S*)-2-Benzyl-4-cyano-3-hydroxybutanoate (9). Anhydrous potassium cyanide (146 mg, 2.32 mmol) was added to a solution of **8** (240 mg, 1.16 mmol) in acetonitrile (5 mL), and the mixture was stirred overnight at room temperature. The solvent was evaporated in vacuo, and the residue was purified by column chromatography (silica gel, EtOAc:hexane, 1:3) to give the title compound as a colorless oil (230 mg, 85%).

Methyl (2*R*,3*R*)-5-Amino-2-benzyl-3-hydroxypentanoate Hydrochloride (10a). A solution of **9** (200 mg, 0.858 mmol) in anhydrous methanol (10 mL) containing HCl (2.58 mmol) was hydrogenated under 40 psi at room temperature in the presence of 10% palladium on carbon (200 mg) for 2 h. After removal of the catalyst by filtration, the filtrate was concentrated in vacuo to give the title compound as a white solid (234 mg, 100%).

Methyl (2*R*,3*R*)-5-(*N*-Cbz-L-alanyl-L-alanyl)amino-2-benzyl-3-hydroxypentanoate (14a). To a solution of *N*-Cbz-L-alanyl-L-alanine (294 mg, 1 mmol) in CH_2Cl_2 (10 mL) was added 1-hydroxybenzotriazole (150 mg, 0.9 mmol) and dicyclohexylcarbodiimide (185 mg, 0.9 mmol), and the mixture was stirred for 1 h. Subsequently, **10a** (220 mg, 0.8 mmol) and triethylamine (81 mg, 0.8 mmol) were added to the reaction mixture, which was stirred for an additional 24 h at room temperature. After filtration, the filtrate was concentrated in vacuo, and the residue was taken up in ethyl acetate, washed with 1 N HCl, 5% $NaHCO_3$, and saturated NaCl, and dried over $MgSO_4$. The solvent was evaporated, and the residue was purified by column chromatography (silica gel, $CHCl_3$:MeOH, 20:1) to give the title compound as a white solid (375 mg, 73%).

Methyl (2*R*,3*R*)-5-(*N*-Acetyl-L-alanyl-L-alanyl)amino-2-benzyl-3-hydroxypentanoate (1). A solution of **14a** (126 mg, 0.2 mmol) in methanol (10 mL) containing HCl (0.4 mmol) was hydrogenolyzed at room temperature in the presence of 10% palladium on carbon for 30 min. Removal of the catalyst and concentration of the filtrate under reduced pressure gave the corresponding deprotected compound (83 mg, 100%). This product was dissolved in 10% triethylamine methanol, to which was added acetic anhydride (24.5 mg, 0.24 mmol, 22 μL) while stirring vigorously. After acetylation was completed, the solvent was evaporated under reduced pressure, and the residue was purified by column chromatography (silica gel, $CHCl_3$:MeOH, 20:1) to give the title compound as a white solid (76 mg, 90%).

(4*R*,5*S*)-3-[(2*R*,3*S*)-5-Cbz-amino-2-benzyl-3-hydroxypentanoyl]-4-methyl-5-phenyloxazolidin-2-one (12). Di-*n*-butylboryltriflate (1 M in CH_2Cl_2, 12 mL, 12 mmol) was added dropwise to a stirred solution of the *N*-acyloxazolidinone **11** (3.09 g, 10 mmol) in CH_2Cl_2 (50 mL) at 0 °C. After the mixture was stirred at 0 °C for 10 min, diisopropylethylamine (DPEA; 1.55 g, 2.1 mL, 12 mmol) in CH_2Cl_2 (10 mL) was added dropwise to the reaction mixture. The mixture was stirred at 0 °C for 30 min and was cooled to −78 °C. To the above enolate solutionwas added a solution of *N*-Cbz-aminopropylaldehyde (2.47 g, 12 mmol) and DPEA (2.1 mL) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at −78 °C for 4 h, allowed to warm to 0 °C, and the reaction was then quenched with a mixture of methanol (60 mL), aqueous phosphate buffer (pH 7.0, 40 mL), and 30% H_2O_2 (40 mL). The aqueous layer was extracted with CH_2Cl_2. The combined organic portion was washed with saturated aqueous NH_4Cl and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc:hexane, 1:2) to give the title compound as colorless oil (3.40 g, 66%). The unused starting oxazolidinone was also recovered (1.05 g, 34%).

Methyl (2*R*,3*S*)-5-*N*-Cbz-amino-2-benzyl-3-hydroxypentanoate (13a). A solution of **12** (1.03 g, 2.0 mmol) in a mixture of THF and water (4:1, 20 mL) was cooled to 0 °C. The sequential addition of 30% H_2O_2 (0.9 mL, 8.0 mmol) and a solution of LiOH (126 mg, 3.0 mmol) in water (3 mL) to the mixture was followed by 1 h of stirring at 0 °C. A solution of Na_2SO_3 (1.01 g, 8.0 mmol) in water (6 mL) was added, and the resulting solution was stirred for 15 min. The organic solvent was removed under reduced pressure, and the resulting aqueous phase (pH 12) was washed with CH_2Cl_2 (3 × 20 mL). The aqueous phase was cooled to 0 °C, acidified to pH ≈ 1 with 1 N HCl, and extracted with EtOAc (5 × 20 mL). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure to give the corresponding acid as a colorless oil (578 mg). The CH_2Cl_2 extract was dried over $MgSO_4$ and evaporated under reduced pressure. This procedure allowed the recovery of the chiral oxazolidinone for reuse (335 mg, 95%). The above acid was dissolved in methanol (20 mL) containing catalytic amount of HCl and was stirred at room temperature overnight. The solvent was removed in vacuo, and the residue was taken up in ethyl acetate (20 mL) and then washed with 5% $NaHCO_3$, water, and saturated NaCl, followed by drying over $MgSO_4$. The solvent was removed in vacuo, and the residue was purified by column chromatography (silica gel, EtOAc:hexane, 1:2) to give the title compound as a colorless oil (601 mg, 81%).

Methyl (2*R*,3*S*)-5-(*N*-Cbz-L-alanyl-L-alanyl)amino-2-benzyl-3-hydroxypentanoate (14b). A solution of **13a** (410 mg, 1.10 mmol) in

5 mL of anhydrous methanol containing HCl (2.2 mmol) was subjected to hydrogenolysis at room temperature in the presence of 10% palladium on carbon (400 mg). After filtration, the solvent was evaporated in vacuo to give a white solid (300 mg). To a solution of *N*-Cbz-L-Ala-L-Ala (485 mg, 1.65 mmol) in CH_2Cl_2 (5 mL) were added HOBt (148 mg, 1.1 mmol) and DCC (227 mg, 1.1 mmol). The mixture was stirred at room temperature for 1 h. To this mixture was added the above amine salt (300 mg, 1.1 mmol), followed by stirring at room-temperature overnight. After filtration, the filtrate was washed with 5% $NaHCO_3$, 1 N HCl, water, and saturated NaCl and dried over $MgSO_4$. The solvent was removed in vacuo, and the residue was purified by column chromatography (silica gel, $CHCl_3$:MeOH, 40:1) to give the title compound (485 mg, 86%).

Methyl (2*R*,3*S*)-5-(*N*-Acetyl-L-alanyl-L-alanyl)amino-2-benzyl-3-hydroxypentanoate (2). The title compound was prepared according to the procedure described for **1** from 126 mg (0.2 mmol) of **14b** to afford 75 mg (90%) of **2** as a white solid.

Methyl (2*R*)-5-*N*-Cbz-amino-2-benzylpentanoate (13b). DMAP (488 mg, 4 mmol) and phenyl chlorothionoformate (192 mg, 1.1 mmol) were added to the solution of **13a** (345 mg, 0.93 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred at room temperature for 4 h. Ethyl acetate (50 mL) was added to the reaction mixture, then washed with 1N HCl, 5% $NaHCO_3$, water, and saturated NaCl, and was dried over $MgSO_4$. The solvent was removed in vacuo, and the residue was purified by column chromatography (silica gel, EtOAc:hexane, 1:5) to give an unstable phenyl thiocarbonate as a yellowish oil (333 mg, 71%).

Tributyltin hydride (2 mL) and di-*tert*-butyl peroxide (12 mg) were added to the thiocarbonate (175 mg, 0.345 mmol), and the mixture was stirred at 50 °C for 3 h. The reaction mixture was directly subjected to column chromatography (silica gel, EtOAc:hexane, 1:3) to give the title compound as a colorless oil (98 mg, 80%).

Methyl (2*R*)-5-(*N*-Cbz-L-alanyl-L-alanyl)amino-2-benzylpentanoate (14c). The title compound was prepared according to the procedure for **14b** from 57 mg (0.16 mmol) of **13b** to afford 75 mg (94%) of **14c** as a white solid.

Methyl (2*R*)-5-(*N*-Acetyl-L-alanyl-L-alanyl)amino-2-benzylpentanoate (4). The title compound was prepared according to the procedure for **1** from 373 mg (0.75 mmol) of **14c** to afford 265 mg (87%) of **4** as a white solid.

Kinetics. A typical inactivation experiment was performed as described below. A solution of **1** in water was added to a solution of α-chymotrypsin (0.44 μM final concentration) in 50 mM Tris, 50 mM $CaCl_2$ buffer, pH 7.8, in a total volume of 200 μL, to give final concentrations of **1** in the range of 1−10 mM. Aliquots (10 μL) of the inactivation mixture were diluted into the 1-mL assay mixture containing 250 μM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide in the same buffer. The initial rate of hydrolysis of the substrate was monitored at 400 nm.

The recovery of activity of inactivated enzyme was studied in the following manner. After 20 h of incubation with 10 mM of compound **1** (residual enzyme activity was less than 5%), the solution was subjected to ultrafiltration to remove the excess of inhibitor. The inactive enzyme was reconstituted to the original volume with 100 mM phosphate buffer, pH 7.8. Enzyme activity was monitored over a number of hours.

A solution of α-chymotrypsin (final concentration 68 μM) in 50 mM Tris, 50 mM $CaCl_2$, pH 7.8, was mixed with a solution of **4** in the same buffer (final concentration 1.25 mM), and the mixture was incubated at room temperature. Aliquots (50 μL) were removed from the mixture at different time points. The reaction was stopped by separation of the low molecular weight species from the protein by ultrafiltration through a Microcon device, and 10-μL portions of the filtrate were analyzed for determination of concentration of **4** by HPLC: $t_R = 22.8$ min (Vydac C-18 4.6 mm × 25 cm, 25% acetonitrile isocratic for 10 min, linear gradient 25−95% acetonitrile for 10 min, 95% isocratic acetonitrile for 10 min, 0.5 mL/min). The eluent was monitored at 215 nm on a Perkin-Elmer UV detector, and the area under the signals was integrated by Dynamax MacIntegrator. Concentrations of **4** in the course of the kinetic runs were calculated from similar determinations for known concentrations of the compound used to generate a calibration curve. Kinetic parameters (k_{cat} and K_m) for enzymic turnover of the substrate were calculated from the complete hydrolysis curves, using the integrated form of the Michaelis−Menten equation.[15]

The dissociation constants (K_i) for compounds **1** and **2** for α-chymotrypsin were calculated by the method of Dixon.[16] A series of assay mixtures containing both *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide as the substrate (either 50 or 250 μM), and various concentrations of compounds as inhibitor (100−500 μM) were prepared in 50 mM Tris, 50 mM $CaCl_2$, pH 7.8. A portion of the enzyme was added to give a final concentration of 44 nM in a total volume of 1.0 mL. The enzyme activity was determined immediately.

Supporting Information Available: Detailed information for optical rotation, melting point, IR, NMR and MS for each compound is provided (5 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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