# Protein Surface Recognition by Synthetic Receptors: A Route to Novel Submicromolar Inhibitors for α-Chymotrypsin

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**Abstract:** A family of synthetic receptors for protein surface recognition has been prepared. The receptors are based on a design in which four peptide loops are arrayed around a central calilx[4]arene core. By varying the sequence of the loop regions a range of differently functionalized receptor surfaces approximately 450 Å$^2$ in area can be prepared. From this family we have identified potent inhibitors of chymotrypsin that function by binding to the surface of the protein. The most potent of these (**1**) shows slow binding kinetics in an analogous manner to several of the natural protein proteinase inhibitors. Detailed kinetic analysis showed **1** to be a competitive inhibitor with $K_i$ and $K_i$* values of 0.81 and 0.11 $\mu$M, respectively.

## Introduction

Every protein contains a unique distribution of functional groups (charged, hydrogen bonding, and hydrophobic) on its exterior surface. In many cases, these regions are involved in important interactions to other proteins as part of multisubunit, reactivity modulating, or signaling complexes.[1] The design of synthetic agents that recognize and bind to specific regions of a protein surface offers a new approach to enzyme inhibitors[2] or to the disruption of key protein−protein interactions (as in Figure 1). We are interested in developing a modular and potentially general approach to synthetic receptors that bind to protein surfaces. Our design takes its origin from the antigen recognition sites of antibodies that use changes in the sequence and conformation of six hypervariable loops to complex a wide range of protein surfaces.[3] We have recently introduced a new class of protein surface receptors in which four peptide loops are attached to a central calixarene scaffold (Figure 1).[4] The result is a concave molecular surface approximately 450−500 Å$^2$ in area whose recognition characteristics can be readily changed by varying the sequence of the cyclic peptide. In the present paper we report the preparation of a family of tetra-loop receptors and from them identify potent surface binding inhibitors of α-chymotrypsin.[5]

The serine proteases represent interesting targets for protein surface recognition due to their central role in biological and medicinal chemistry.[6] Also, a large family of natural protein inhibitors is known to bind to surface regions and/or the active sites of different serine proteases.[7] The crystal structure of α-chymotrypsin (ChT) bound to Kunitz inhibitor shows extensive interactions from negatively charged regions on the inhibitor to several basic residues on the chymotrypsin surface close to the active site.[8] Figure 2 shows the disposition of lysines and arginines on the surface of chymotrypsin and suggests that a complementary synthetic receptor might bind to this region of the protein and block the approach of a substrate to the active site.

## Results and Discussion

**Inhibitor Design and Synthesis.** Our approach to surface binding inhibitors of chymotrypsin involved the synthesis by solid-phase methods of a series of cyclic peptides[9] containing a 5-nitro-3-aminomethylbenzoic acid dipeptide mimetic (Figure 3A).[10] Reduction of the nitro group followed by reaction with the tetra-acid chloride derivative of calilx[4]arene tetracarboxylic acid and removal of any side chain protecting groups gave a series of receptors containing four identical peptide loops (Figure 3B).

In targeting the predominantly cationic active site region of α-chymotrypsin, our initial approach was to construct receptors in which each peptide loop contained two anionic residues (GDGD, receptor **1**) or one anionic and one hydrophobic residue (GDGY, **2**), such that they might stretch across the surface of the protein and contact multiple basic residues. For comparison purposes we also prepared the corresponding receptor containing four bis-cationic (GKGK, **3**) peptide loop derivatives, a simplified octa-carboxylic acid derivative of calixarene **4** and the nitro-substituted single loop analogue **5**.

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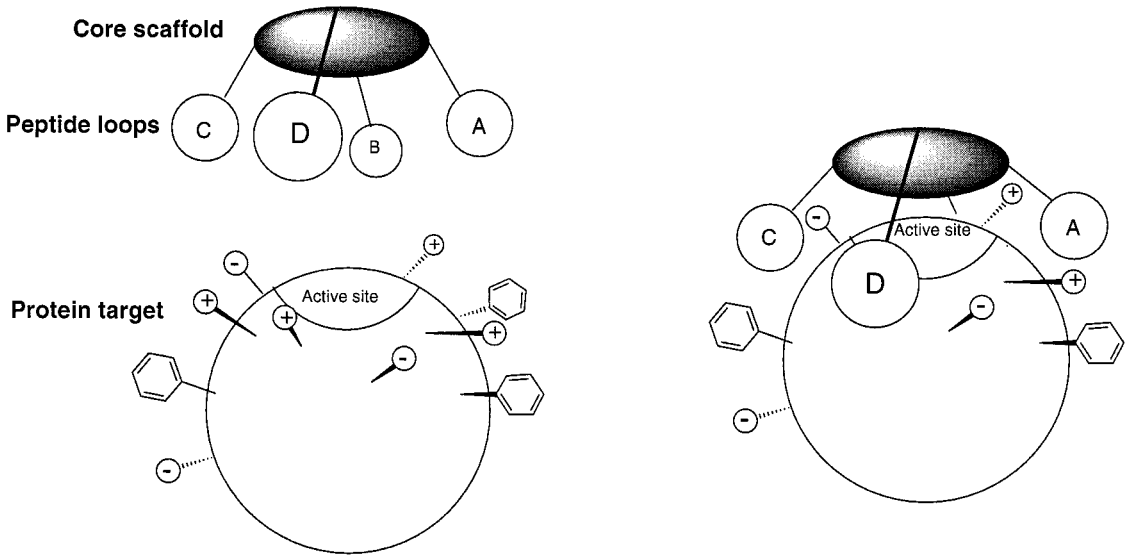



**Figure 1.** Schematic representation of enzyme inhibition by protein surface recognition.

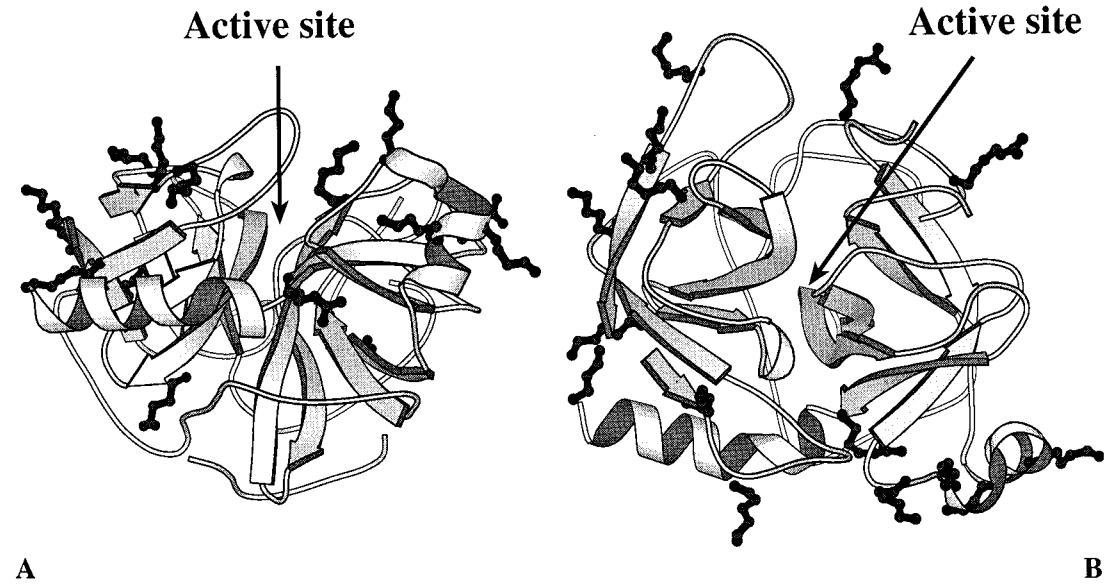
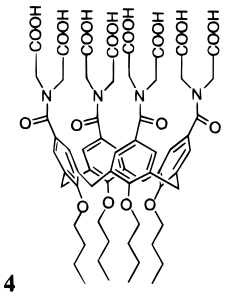



**Figure 2.** Distribution of basic residues on the surface of chymotrypsin (A, side view; B, top view).



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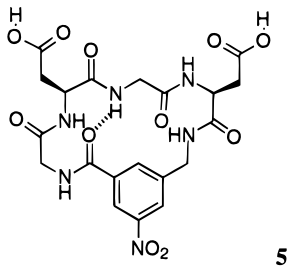

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**Initial Screening for Inhibition Activity.** This family of receptors was screened for its ability to inhibit bovine pancreatic α-chymotrypsin by treating the enzyme ($3.03 \times 10^{-7}$ M) with solutions of **1**, **2**, or **3** ($9.2 \times 10^{-6}$ M) in phosphate buffer for a 24 h induction period followed by addition of the chromogenic substrate, *N*-benzoyltyrosine-*p*-nitroanilide (BTNA). Receptor **1** (GDGD) was found to decrease hydrolysis to 10% of that of the untreated enzyme compared to little or no inhibition by **2** and **3**. In contrast, another serine protease, elastase, showed no inhibition with **1**, **2**, or **3**, underlining the selectivity of **1** for the surface of α-chymotrypsin. The inhibition depended on the length of time that **1** was incubated with the enzyme before addition of the substrate. A plot of initial velocity vs incubation time at [**1**] = $3.09 \times 10^{-5}$ M and [ChT] = $4.29 \times 10^{-6}$ M (Figure 4) shows that slow binding is occurring with a half-life of 135 ± 18 min. This property of slow binding inhibition is similar to that seen with certain protein inhibitors of the serine proteases (e.g., $\alpha_1$-trypsin inhibitor with kallikrein[7] or modified pancreatic trypsin inhibitor with trypsin[11]) whose half-lives are also measured in hours. In this initial screening no detectable inhibition was seen with the monomeric cyclic peptide **5** (with GDGD sequence) clearly pointing to the importance of multiple peptide loops in enzyme binding.

That the inhibition of α-chymotrypsin by **1** was due to specific complex formation was confirmed by nondenaturing gel electrophoresis on agarose gel (Figure 5). Comparison of lanes 1 and 2 shows that **1** and α-chymotrypsin (1 equiv) form a new species with opposite polarity to the uncomplexed enzyme. No binding is seen to soybean trypsin inhibitor (TI; lanes 3 and 4) and the ChT-TI complex migrates toward the anode in a similar but distinct manner (lane 5).

(11) Vincent, J. P.; Lazdunski, M. *Biochemistry* **1972**, *11*, 2967−2977.

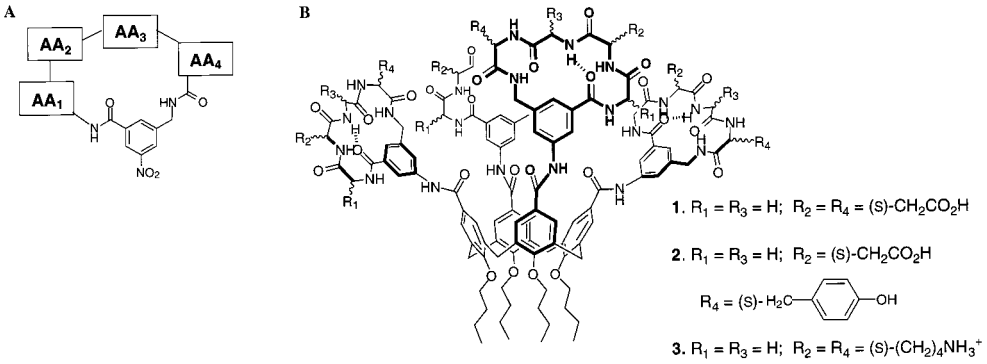



**Figure 3.** Designs of artificial receptors for chymotrypsin surface recognition.

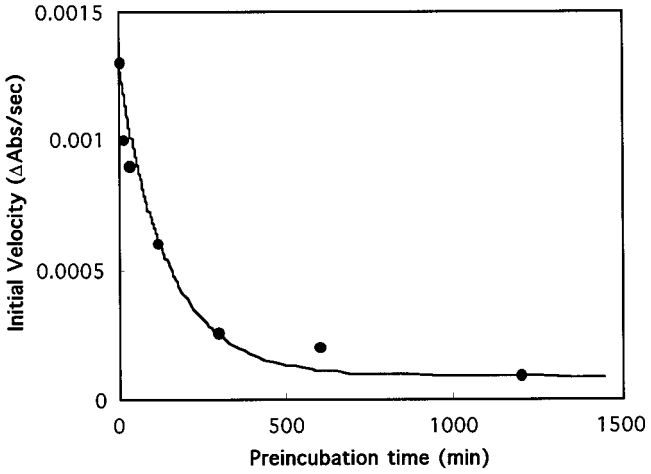



**Figure 4.** Time course for the inhibition of chymotrypsin by **1**.

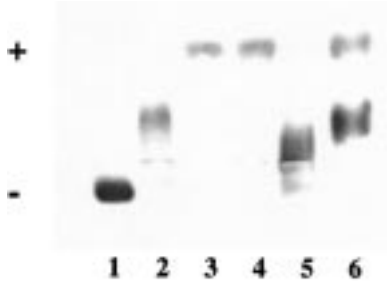


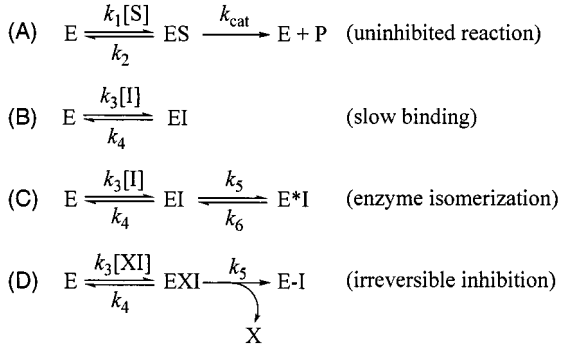

**Figure 5.** Nondenaturing gel electrophoresis on 1% agarose at pH 7.4, sample loaded in the center of the gel (lane 1, ChT; lane 2, ChT+**1**; lane 3, TI; lane 4, TI+**1**; lane 5, ChT+TI; lane 6, ChT+TI+**1**).

**Slow Binding Inhibition.** Slow binding inhibition is encountered more rarely than classical or tight binding inhibiton and can result from three essential mechanisms (Scheme 1):[12] simple slow binding (mechanism B), in which the inhibitor binds to the enzyme like a classical inhibitor but with a slow binding rate; enzyme isomerization (mechanism C), in which a rapid inhibitor binding step is followed by a slower conformational change or isomerization; and irreversible inhibition (mechanism D).[13] In the case of mechanism B, the inhibition constant ($K_i$) is defined simply as the equilibrium dissociation constant.

$$K_i = \frac{k_4}{k_3} = \frac{[E][I]}{[EI]} \quad (1)$$

However, in mechanism C two inhibition constants must be considered. The first ($K_i$) is the dissociation constant for the initial EI complex (as in eq 1), and the second ($K_i$*) corresponds to the dissociation constant of the second enzyme inhibitor complex E*I, as defined by eq 2.

**Scheme 1.** Mechanisms of Time-Dependent Enzyme Inhibition




$$K_i^* = \frac{K_i k_6}{k_5 + k_6} = \frac{[E][I]}{[EI] + [E^*I]} \quad (2)$$

These different slow binding inhibition mechanisms can be investigated by preincubation of enzyme with inhibitor followed by the measurement of initial velocities for substrate hydrolysis as a function of preincubation time. In these cases, the inhibition can be described by eq 3,

$$v_i/v_0 = \exp(-k_{obs}t) \quad (3)$$

where $v_0$ and $v_i$ are the initial velocities of uninhibited and inhibited reaction, respectively, and $k_{obs}$ is the first-order rate constant for the interconversion between the initial and the steady state. Figure 6 shows the fractional velocities for *N*-benzoyltyrosine-*p*-nitroanilide hydrolysis by α-chymotrypsin for different concentrations of inhibitor **1**. Correlation of $k_{obs}$ (obtained for each curve in Figure 6) against [**1**] gave a nonlinear plot (Figure 7) that is consistent with slow binding inhibition through a two-step, enzyme-isomerization mechanism (Scheme 1C).[12] Nonlinear curve fitting of these data by using eq 4 gave values for $K_i^{app}$, $k_5$, and $k_6$ (Table 1).

$$k_{obs} = k_6 + \frac{k_5[I]}{K_i^{app} + [I]} \quad (4)$$

A similar analysis was applied to the much weaker inhibitor **2**. A plot of [**2**] vs $k_{obs}$ gave a linear relationship (Figure 8) that is characteristic of a single step slow binding mechanism (Scheme 1B), as described by eq 5.[12] However, in this case we cannot exclude the possibility of mechanism C, since if $K_i^{app}$

(12) Copeland R. A. In *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*; Wiley-VCH: New York, 1996; p 237.
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**Table 1.** Summary of Rate and Equilibrium Constants for the Inhibition of Chymotrypsin[a] by Receptors

| receptors[b] | $k_3$ ($M^{-1}$ $min^{-1}$)[c] or $k_5$ ($min^{-1}$)[d] | $k_4$ ($min^{-1}$)[c] or $k_6$ ($min^{-1}$)[d] | $K_i$ ($10^{-6}$ M) | $K_i$* ($10^{-6}$ M) |
|---|---|---|---|---|
| **1** (GDGD) | 0.00652 ± 0.00005 | 0.000986 ± 0.000048 | 0.813 ± 0.033 | 0.107 ± 0.007 |
| **2** (GDGY) | 79.8 ± 9.6 | 0.00121 ± 0.00003 | 15.2 ± 1.5 | |
| **3** (GKGK) | | | 35.7 ± 3.2 | |
| **4** | | | 26.4 ± 0.2 | |
| **5** | | | >50 | |

[a] Concentration of chymotrypsin was fixed as $3.5 \times 10^{-7}$ M for **1**, **2**, and **4** and $7.0 \times 10^{-7}$ M for **3** and **5** in 5% DMSO, pH 7.4, 5 mM phosphate buffer at 25 °C. Substrate was BTNA and its concentration was fixed as $1.4 \times 10^{-4}$ M. [b] [**1**] = $(0.555-7.40) \times 10^{-6}$ M, [**2**] = $(5.83-11.7) \times 10^{-6}$ M, [**3**] = $(1.00-5.00) \times 10^{-5}$ M, [**4**] = $(1.05-4.20) \times 10^{-5}$ M, [**5**] = $(1.00-5.00) \times 10^{-5}$ M. [c] For **2**. [d] For **1**.

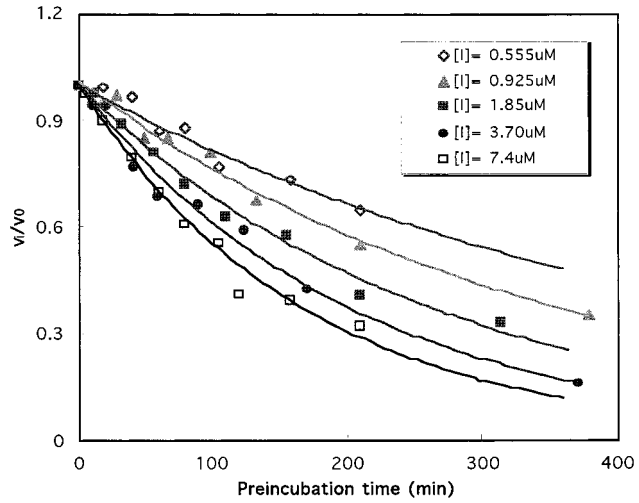


**Figure 6.** Plot of fractional velocity vs preincubation time for several concentrations of **1**.

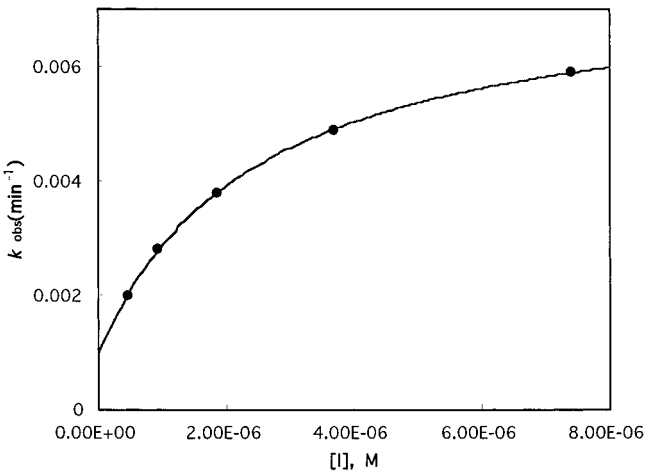


**Figure 7.** Plot of $k_{obs}$ vs [**1**].

$\gg K_i$*[app], the concentration of the EI complex would be negligible and a linear relationship between [I] and $k_{obs}$ would be seen.[12]

$$k_{obs} = k_4\left(1 + \frac{[I]}{K_i^{app}}\right) \quad (5)$$

To determine the type of slow binding inhibition (e.g., competitive, noncompetitive, etc.) and hence to obtain appropriate values for $K_i$ or $K_i$*, we measured the dependence of $k_{obs}$ on the concentration of substrate. With receptor **1**, $k_{obs}$ was seen to decrease as the substrate concentration increased, which is behavior unique to the competitive mechanism of slow binding inhibition (Figure 9).[14,15] Full data for inhibition of α-chymotrypsin by each receptor are summarized in Table 1. For **3**, **4**, and **5**, time dependent effects could not be observed due to the very weak inhibition (less than 20% inhibition after 5 h of incubation). In these cases, Lineweaver−Burk analysis was used and the inhibition constants measured by this method are collected in Table 1. Receptor **1** binds tightly to chymotrypsin in both its initial ($K_i$ = 0.81 μM) and final ($K_i$* = 0.11 μM) enzyme−inhibitor complexes. In contrast, receptors **2** and **3** with less complementary recognition surfaces show significantly lower inhibition potency ($K_i$ = 15 and 36 μM, respectively). However, electrostatic effects are not the sole contributor to strong binding since derivative **4**, with a similar number of carboxylic acid groups, is a much weaker inhibitor of α-chymotrypsin than **1**.

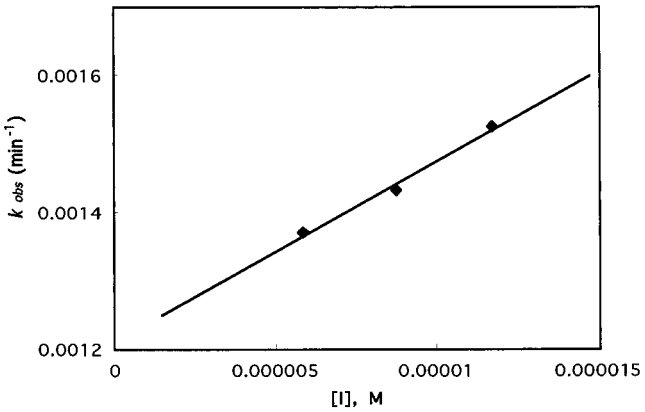


**Figure 8.** Plot of $k_{obs}$ vs [**2**].

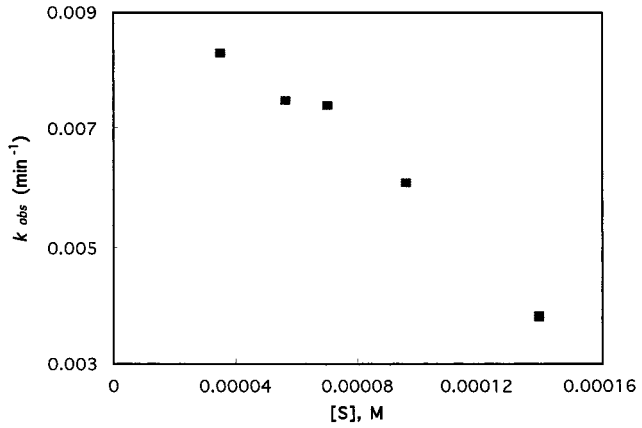


**Figure 9.** Plot of $k_{obs}$ vs [BTNA] at fixed concentrations of chymotrypsin and **1**.

(14) In the case of slow binding competitive inhibition the appropriate relationships are:

$$k_{obs} = \frac{k}{1 + [S]/K_m} \quad \text{and} \quad K_i^{app} = K_i\left(1 + \frac{[S]}{K_m}\right)$$

See: Tian, W. X.; Tsou, C. L. *Biochemistry* **1981**, *34*, 808.

(15) It should be noted that sufficiently high concentrations of substrate could not be reached to observe saturation effects.

The observation of competitive inhibiton of α-chymotrypsin by **1** lends support to the proposal that the receptor is binding close to the active site of the enzyme and blocking the approach of the substrate.[16] In addition, a competitive binding experiment showed, by nondenaturing gel electrophoresis (Figure 2, lane 6), that **1** (3.5 equiv) is able to displace trypsin inhibitor from its 1:1 complex with α-chymotrypsin. This suggests that **1** is binding to a comparable region of α-chymotrypsin, possibly interacting with several cationic residues (including lysines 36, 90, 175, 177 and arginine 145) near the active site cleft. This interaction may either lead to a disruptive conformational change in the protein or interfere with the approach of the substrate to the active site. However, receptor **1** is also not modified by the enzyme, suggesting no direct contact with the active site. Incubation of α-chymotrypsin and **1** for 5 h followed by denaturation of the complex and reverse phase HPLC analysis (elution solvent, 7−90% acetonitrile:water containing 0.1% TFA) showed that unreacted receptor was recovered.

In summary, we have developed a novel series of inhibitors of α-chymotrypsin based on the specific interaction of a synthetic receptor with the exterior of the protein. We are currently extending this strategy to less symmetrical receptors and to different classes of proteins.

## Experimental Section

**Materials and Instruments.** Buffer reagents were purchased from Bio-Rad, and α-chymotrypsin (ChT), porcine pancreatic elastase and *N*-benzoyltyrosine-*p*-nitroanilide(BTNA) were purchased from Sigma and used without purification. Spectra Max 250 (Molecular Devices) was used for simultaneous 96-well microplate assays. HPLC data were obtained from DYNAMAX with Microsorb-MB (C18-reverse phase column) column (Rainin). All equipment and reagents for electrophoresis were purchased from Bio-Rad. $^1$H and $^{13}$C NMR data were collected on a Bruker AM 500 NMR.

**General Conditions for Enzyme Assay.** For kinetic studies, a 5 mM sodium phosphate buffer of pH 7.4 was used. All solutions were kept at 25 °C for the kinetic analyses and also for the incubation periods. α-Chymotrypsin and different concentrations of the receptors were mixed and kept at 25 °C for a fixed incubation period. An aliquot (190 μL) of the incubated solution was taken and to it was added 10 μL of a stock solution of BTNA. Hydrolysis was followed by monitoring product formation at 410 nm.

**Electrophoresis.** 1% of melted agarose in 5 mM of sodium phosphate was poured on the glass plate to a thickness of 1 mm. Small wells (5 μL volume) were made in the middle of the gel with a small comb. Individual samples (4 μL) in buffer (plus 0.1% of tracking dye and glycerol) were loaded on to the gel and a constant voltage (100 V) was applied for aproximately 1 h to run the electrophoresis. At the end of this time the gel was fixed in 25% acetic acid solution for 20 min and acetone for 10 min. The gel was dried by hot air and then immersed in a 0.5% Coomasie Blue staining solution (10% acetic acid, 40% methanol aqueous solution). The gel background was destained with 10% acetic acid, 40% methanol, and 50% water solution until the protein bands could be seen.

**General Procedure for Solid-Phase Synthesis of Cyclic Peptide Sequences. (a) cyclo(Gly-Asp(O$^t$Bu)-Gly-Asp(O$^t$Bu)-Amab).** To a 25 mL vessel was added MBHA resin (0.50 g, substitution level = 1.01 mmol/g), HMPB (0.360 g, 1.5 mmol), BOP (0.664 g, 1.5 mmol), HOBt (0.203 g, 1.5 mmol), DIEA (0.26 mL, 1.5 mmol), and 5 mL of peptide synthesis grade DMF. The mixture was shaken at room temperature overnight. After all the liquids were removed, the resin was washed thoroughly (20 mL volumes) with DMF (2×), MeOH (2×), $CH_2Cl_2$ (2×), MeOH (2×), and DMF (2×). Kaiser test showed there was no free amine left on the resin. To the resin was added Fmoc-Gly-OH (0.744 g, 2.5 mmol), DCC (0.516 g, 2.5 mmol), and DMAP (0.306 g, 2.5 mmol) in DMF/$CH_2Cl_2$ (2 mL/8 mL) and the vessel was put in the refrigerator for 6 h. After filtration the resin was washed extensively by using the same procedure as above. The resin was coupled with 2.5 mmol of a preformed symmetric anhydride of Fmoc-Gly-OH and DMAP (0.306 g, 2.5 mmol) and the vessel was shaken at room temperature for 4 h. The following steps were then performed: (a) The resin was washed with DMF (2×), MeOH (2×), $CH_2Cl_2$ (2×), MeOH (2×), and DMF (2×); (b) the Fmoc group was removed by using 20% piperidine in DMF for 45 min; (c) the resin was washed using the same procedure as in step a; (d) Fmoc-Amnb-OH (0.420 g, 1.0 mmol, 2 equiv), BOP (0.443 g, 1.0 mmol, 2 equiv), HOBt (0.135 g, 1.0 mmol, 2 equiv), and DIEA (0.18 mL, 1.0 mmol, 2 equiv) in 10 mL of DMF were added and the reaction vessel was shaken for 2 h; and (e) the ninhydrin test was performed to check the completion of the coupling, and recoupling was performed if necessary. Steps a→e were repeated for the other amino acid residues using the same equivalents until the desired linear peptide was assembled.

The resin was thoroughly washed with DMF (2×), MeOH (2×), $CH_2Cl_2$ (3×), and MeOH (2×) and dried in vacuo overnight. The resin was treated with 1% TFA/$CH_2Cl_2$ (5×, 2 min each), and the filtrates were drained into 1−2 mL of ice cold DMF. The $CH_2Cl_2$ was removed by evaporation in vacuo and the peptide was precipitated by dropping the DMF solution into 100 mL of $H_2O$. The precipitate was collected on a glass filter, washed with $H_2O$, and dried in vacuo (0.35 g). The Fmoc group was removed subsequently by treating the peptide with 10% $Et_2NH$/ACN (20 mL) for 2 h. The solvents were then removed in vacuo and the dibenzofulvene was extracted from the solid residue with 100 mL of $Et_2O$. The peptide was cyclized by treatment with BOP (0.443 g, 1.0 mmol), HOBt (0.135 g, 1.0 mmol), and DIEA (0.18 mL, 1.0 mmol) in 200 mL of DMF at room temperature overnight. The DMF was evaporated in vacuo and the crude product was taken up in 100 mL of EtOAc and washed with 1 N NaOH (50 mL × 2), 1 N HCl (50 mL × 2), and brine (50 mL) and dried over $Na_2SO_4$. The solvent was then evaporated and the residue was applied to a silica gel column with 5% MeOH/$CH_2Cl_2$ as the eluent to produce the desired cyclic peptide (0.111 g, 32% overall yield from resin): mp >350 °C; $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 9.58 (t, $J$ = 5.0 Hz, 1H), 8.81 (t, $J$ = 6.0 Hz, 1H), 8.53 (s, 1H), 8.37 (s, 1H), 8.32 (d, $J$ = 9.4 Hz, 1H), 8.05 (t, $J$ = 4.0 Hz, 1H), 7.85 (t, $J$ = 5.6 Hz, 1H), 4.82 (m, 1H), 4.78 (m, 1H), 4.73 (m, 1H), 4.14 (dd, $J$ = 16.8, 4.6 Hz, 1H), 3.96 (dd, $J$ = 13.0, 6.1 Hz, 1H), 3.90 (dd, $J$ = 13.3, 5.5 Hz, 1H), 3.78 (dd, $J$ = 11.0, 6.0 Hz, 1H), 3.73 (dd, $J$ = 8.4, 4.3 Hz, 1H), 2.83 (dd, $J$ = 15.5, 5.3 Hz, 1H), 2.62 (dd, $J$ = 15.9, 8.4 Hz, 1H), 2.40 (dd, $J$ = 15.5, 9.2 Hz, 1H), 2.34 (dd, $J$ = 15.9, 6.6 Hz, 1H), 1.40 (s, 9H), 1.38 (s, 9H); LR FAB-MS *m/e* calcd for $C_{28}H_{39}N_6O_{11}$ $[M + H]^+$ 635.3, found 635.5.

To a solution of cyclo(Gly-Asp(O$^t$Bu)-Gly-Asp(O$^t$Bu)Amnb) (0.217 g, 0.32 mmol) in 15 mL of MeOH was added 10% Pd/C (100 mg, 0.094 mmol) carefully. The suspension was stirred under 1 atm of $H_2$ at room temperature for 7 h. The catalyst was removed by filtration through Celite. The filtrate was concentrated and the residue was applied to a chromatography column ($SiO_2$, 5% MeOH/$CH_2Cl_2$, 10% MeOH/$CH_2Cl_2$) to give the title compound (0.060 g, 31%): mp 184−185 °C dec; $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 8.78 (dd, $J$ = 6.4, 3.9 Hz, 1H), 8.37 (dd, $J$ = 7.0, 5.2 Hz, 1H), 8.23 (d, $J$ = 9.5 Hz, 1H), 8.08 (t, $J$ = 5.9 Hz, 1H), 7.96 (d, $J$ = 8.9 Hz, 1H), 6.81 (s, 2H), 6.54 (s, 1H), 5.24 (s, 2H), 4.83 (m, 2H), 4.46 (dd, $J$ = 16.3, 7.4 Hz, 1H), 4.01 (dd, $J$ = 16.5, 7.0 Hz, 1H), 3.95 (dd, $J$ = 17.3, 6.7 Hz, 1H), 3.86 (dd, $J$ = 16.2, 4.5 Hz, 1H), 3.66 (dd, $J$ = 13.8, 5.6 Hz, 1H), 3.60 (dd, $J$ = 11.8, 3.6 Hz, 1H), 2.82 (dd, $J$ = 15.3, 5.4 Hz, 1H), 2.64 (dd, $J$ = 15.8, 8.5 Hz, 1H), 2.40 (dd, $J$ = 15.3, 9.0 Hz, 1H), 2.27 (dd, $J$ = 15.8, 6.4 Hz, 1H), 1.37 (s, 9H), 1.34 (s, 9H); LR FABMS *m/e* calcd for $C_{28}H_{41}N_6O_9$ $[M + H]^+$ 605.3, found 605.4.

**(b) Butoxycalix[4]arene tetracyclo-(GDGD) (1).**[17] To a solution of butoxycalix[4]arene tetraacid[17] (86 mg, 0.10 mmol) and oxalyl chloride (254 mg, 2.0 mmol) in dry $CH_2Cl_2$ (10 mL) was added DMF (0.025 mL) through a silica gel filter and the mixture was stirred at room temperature for 8 h. The reaction mixture was evaporated in vacuo to obtain the acid chloride (102 mg). A solution of cyclo-(Gly-Asp(O$^t$Bu)-Gly-Asp(O$^t$Bu)-Amab) (266 mg, 0.44 mmol)[17] and DIEA

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(80 mg, 0.60 mmol) in dry $CH_2Cl_2$ (10 mL) was added to the evaporated residue. The mixture was stirred at room temperature for 14 h. The reaction mixture was purified by preparative TLC ($SiO_2$, first 10% MeOH/$CH_2Cl_2$, later 25% MeOH/$CH_2Cl_2$) to obtain the desired compound as a yellow powder (281 mg, 89%): mp >350 °C; $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 10.10 (broad s, 4H), 8.95 (broad, 4H), 8.49 (broad, 4H), 8.25 (d, $J$ = 8.7 Hz, 4H), 8.09 (broad, 4H), 7.99 (m, 8H), 7.67 (s, 4H), 7.60 (s, 8H), 7.37 (s, 4H), 4.81 (m, 8H), 4.51 (m, 8H), 3.99 (m, 20H), 3.63 (m, 8H), 3.46 (m, 4H), 2.79 (m, 4H), 2.62 (m, 4H), 2.31 (m, 8H), 1.97 (m, 8H), 1.47 (m, 8H), 1.35 (m, 72H), 1.02 (t, $J$ = 8.4 Hz, 12H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 120.1, 117.8, 80.0 (2C), 75.0, 49.1, 48.8, 44.2, 42.3, 41.4, 37.5, 36.9, 31.9, 30.5, 27.6, 18.9, 14.0. To the above compound (298 mg, 0.093 mmol) was added TFA (3 mL) and dry $CH_2Cl_2$ (3 mL) and the mixture was stirred at room temperature for 1 h and then evaporated under reduced pressure. The crude product was passed through anion-exchange resin (Amberlite IRA-400(OH), water) and cation-exchange resin (Amberlite IR 120 (plus), water) to remove ions. Water was lyophilized to give the title compound (229 mg, 90%): mp >350 °C; $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 10.02 (s, 4H), 8.81 (s, 4H), 8.48 (s, 4H), 8.31 (d, $J$ = 8.7 Hz, 4H), 7.97 (m, 12H), 7.64 (s, 4H), 7.55 (s, 8H), 7.30 (s, 4H), 4.76 (m, 8H), 4.52 (m, 8H), 4.0−3.4 (m, 32H), 2.8−2.6 (m, 8H), 2.5−2.3 (m, 8H), 1.97 (m, 8H), 1.50 (m, 8H), 1.01 (t, 8.1 Hz, 12 H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 172.2, 171.7, 170.9, 170.4, 169.3, 168.5, 165.1, 159.0, 140.2, 138.8, 134.4, 134.2, 128.7, 128.3, 121.4, 119.9, 117.7, 75.0, 49.1, 44.3, 42.2, 41.3, 36.1, 31.9, 30.5, 18.9, 14.0; HR FAB-MS *m/e* calcd for $C_{128}H_{145}N_{24}O_{44}$ (M + H$^+$) 2721.9767, found 2721.985.

**(c) Butoxycalix[4]arene tetracyclo-(GDGY) (2).** To a solution of butoxycalix[4]arene tetraacid[17] (22.5 mg, 0.027 mmol) in 5 mL of dry $CH_2Cl_2$ was added oxalyl chloride (146 mg, 1.1 mmol) and a catalytic amount of DMF (0.2 $\mu$L), and the mixture was stirred at room temperature overnight. The reaction mixture was evaporated in vacuo to give the acid chloride. A solution of cyclo-(Gly-Asp(O$^t$Bu)-Gly-Tyr(O$^t$Bu)-Amab) (78.0 mg, 0.12 mmol) and DIEA (15 mg, 0.12 mmol) in dry $CH_2Cl_2$ (5 mL) was added to the acid chloride and the mixture was stirred at room temperature for 2 days. The solvent and excess reagent were evaporated and the residue was applied to a Sephadex LH-20 gel filtration column eluted with $CH_2Cl_2$. The appropriate portions were collected and evaporated to give the fully protected product. Further treatment with 25% TFA/$CH_2Cl_2$ (5 mL) at room temperature for 2 h afforded the final product as a light-yellow powder (52.6 mg, 66%): mp >350 °C dec; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.99 (s, 4H), 9.15 (s, b, 4H), 8.74 (s, 4H), 8.42 (d, $J$ = 8.4 Hz, 4H), 8.41 (s, 4H), 8.19 (d, $J$ = 7.2 Hz, 4H), 7.96 (s, 4H), 7.84 (s, 8H), 7.63 (s, 4H), 7.55 (s, 4H), 7.31 (s, 4H), 6.99 (d, $J$ = 8.4 Hz, 8H), 6.64 (d, $J$ = 8.3 Hz, 8H), 4.75 (m, 4H), 4.58 (m, 8H), 4.43 (m, 4H), 4.01 (s, b, 8H), 3.94 (d, $J$ = 14.7 Hz, 4H), 3.74 (m, 12H), 3.57 (m, 8H), 2.90 (dd, $J$ = 13.8, 5.3 Hz, 4H), 2.80 (dd, $J$ = 16.4, 5.9 Hz, 4H), 2.63 (dd, $J$ = 13.6, 9.0 Hz, 4H), 2.48 (m, 4H), 1.96 (m, 8H), 1.49 (m, 8H), 1.03 (t, $J$ = 7.3 Hz, 12H); ES-MS calcd for $C_{148}H_{161}N_{24}O_{40}$ 2915.97 (M + H$^+$), found 2914.74 ± 0.38.

**(d) Butoxycalix[4]arene tetracyclo-(GKGK) (3).** To a solution of butoxycalix[4]arene tetraacid (9.2 mg, 0.011 mmol) in 2 mL of dry $CH_2Cl_2$ was added oxalyl chloride (29 mg, 0.23 mmol) and a catalytic amount of DMF (0.1 $\mu$L), and the mixture was stirred at room temperature overnight. The reaction mixture was evaporated in vacuo to give the acid chloride. A solution of cyclo-(Gly-Lys(BOC)-Gly-Lys(BOC)-Amab) (32.0 mg, 0.044 mmol) and DIEA (6 mg, 0.048 mmol) in dry $CH_2Cl_2$ (2 mL) was added to the acid chloride and the mixture was stirred at room temperature for 2 days. The solvent and excess reagent were evaporated and the residue was applied to a Sephadex LH-20 gel filtration column eluted with $CH_2Cl_2$. The appropriate portions were collected and evaporated to give the fully protected product. Further treatment with 25% TFA/$CH_2Cl_2$ (3 mL) at room temperature for 2 h afforded the final product as its TFA salt (18 mg, 57%): mp >290 °C dec; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.97 (s, 4H), 8.71 (s, 4H), 8.42 (s, 4H), 8.33 (d, $J$ = 9.2 Hz, 4H), 8.23 (d, $J$ = 6.5 Hz, 4H), 7.95 (s, 4H), 7.85 (s, 8H), 7.67 (s, 4H), 7.49 (d, $J$ = 13.8 Hz, 4H), 7.29 (s, 4H), 4.50 (m, 8H), 4.22 (m, 8H), 3.94 (m, 24H), 3.75 (m, 4H), 3.59 (m, 4H), 2.76 (s, 16H), 1.93 (m, 12H), 1.68 (m, 4H), 1.51 (m, 28H), 1.30 (m, 20H), 1.02 (t, $J$ = 7.3 Hz, 12H); FAB-MS calcd for $C_{144}H_{201}N_{32}O_{28}$ 2827.28 (M + H$^+$), found 2827.4.

**(e) Iminodiacetic Acid Diethyl Ester.** A solution of iminodiacetic acid (2.66 g, 0.02 mol) and concentrated $H_2SO_4$ (2.0 mL) in 80 mL of EtOH was refluxed under $N_2$ for 4 h. The solvent was then evaporated and 100 mL of $H_2O$ was added. The resulting solution was neutralized with 1.0 N NaOH, and the solution was extracted with $Et_2O$ (150 mL × 2) and the organic layer was separated and dried over $MgSO_4$. The ether was evaporated to give the title compound as an oil (2.54 g, 67%): $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 4.18 (q, $J$ = 7.2 Hz, 4H), 3.44 (s, 4H), 1.83 (s, 1H), 1.26 (t, $J$ = 7.2 Hz, 6H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 172.0, 61.1, 50.4, 14.4; HR EI-MS *m/e* calcd for $C_8H_{15}NO_4$ [M]$^+$ 189.1001, found 189.1008.

**(f) Butoxycalix[4]arene Octaacid Ethyl Ester.** To a suspension of butoxycalix[4]arene tetraacid (84.2 mg, 0.102 mmol)[17] in 5 mL of $CH_2Cl_2$ was added $(COCl)_2$ (0.15 mL, 1.72 mmol) and a catalytic amount of DMF, and the mixture was stirred at room temperature overnight. The solvent and excess $(COCl)_2$ were evaporated and the residue was redissolved in 2.0 mL of $CH_2Cl_2$/THF (4/1). Iminodiacetic acid diethyl ester (1.3 M $CH_2Cl_2$/THF solution, 2.0 mL, 2.6 mmol) and 40 $\mu$L of DIEA were added to above solution, and the mixture was stirred at room temperature overnight. After evaporation, the residue was taken up into 20 mL of $CH_2Cl_2$ and the solution was washed with 0.1 N HCl and saturated brine and then dried over $Na_2SO_4$. The solvent was removed to give the product as an oil (0.148 g, 96%): $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 6.95 (s, 8H), 4.45 (d, $J$ = 13.1 Hz, 4H), 4.20 (m, 16H), 4.15 (s, 8H), 3.95 (m, 16H), 3.17 (d, $J$ = 13.1 Hz, 4H), 1.89 (p, $J$ = 7.7 Hz, 8H), 1.42 (m, 8H), 1.27 (t, $J$ = 7.0 Hz, 24H), 1.00 (t, $J$ = 7.4 Hz, 12H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 171.4, 169.4, 169.0, 134.5, 128.6, 128.1, 75.3, 61.6, 60.9, 52.1, 47.9, 32.0, 31.3, 19.3, 14.2, 14.0; HR FAB-MS *m/e* calcd for $C_{80}H_{109}N_4O_{24}$ [M + H]$^+$ 1509.7432, found 1509.7437.

**(g) Butoxycalix[4]arene Octaacid (5).** A solution of butoxycalix-[4]arene octaacid ethyl ester (0.135 g, 0.089 mmol) and LiOH (0.112 g, 2.67 mmol) in 10 mL of THF/$H_2O$ (1/1) was stirred at room temperature overnight. After THF was evaporated, $H_2O$ was added and the aqueous layer was washed with $Et_2O$ (10 mL × 2). The aqueous solution was acidified to pH 2.0 with 1 N HCl. The precipitate was filtered off and dried in vacuo to give a light yellow solid (90.7 mg, 79%): mp 186−188 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.21 (s, b, 8H), 6.84 (s, b, 8H), 4.35 (d, $J$ = 13.0 Hz, 4H), 3.94 (s, b, 24H), 3.28 (d, $J$ = 13.1 Hz, 4H), 1.88 (m, 8H), 1.44 (s, b, 8H), 0.99 (t, $J$ = 7.3 Hz, 12H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 170.3, 169.7, 157.7, 134.5, 127.9, 127.2, 74.7, 51.3, 48.2, 31.6, 30.3, 18.8, 13.8; LR FAB-MS *m/e* calcd for $C_{64}H_{77}N_4O_{24}$ [M]$^+$ 1285.3, found 1285.5.

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**Supporting Information Available:** Experimental details and results (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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