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Enzyme-Controlled Molecular Recognition: Selective Targeting of Trypsin with a Substrate-Inhibitor Supramolecular Complex

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Abstract: We have synthesized and studied the host compound (**1**) that forms a noncovalent complex (K = 480 ± 30 M^{-1}) with 1,4-diamidinobenzene (**2**) at pD 8.0 in D_2O. Guest **2** has been shown to inhibit the proteases specific for cationic side chains (K_i values for thrombin, plasmin, and trypsin being 1.0 ± 0.4, 0.6 ± 0.1, and 0.88 ± 0.07 mM, respectively). Host **1** and the **1·2** complex are shown to undergo the trypsin-catalyzed cleavage that results in the increased concentration of the free form of **2** and, therefore, induces the enzyme inhibition. Plasmin and thrombin are shown to cleave **1** at a significantly lower rate than trypsin. Thus, the complex of **1** with **2** represents the first example of a selective enzyme-sensitive supramolecular system capable of enzyme targeting through a "trojan horse"-type mechanism in which a bioactive compound acts on the same molecule that provides its release from a delivery system.

Among multiple branches of supramolecular chemistry,[1] one of the most interesting directions is focused on the creation of new synthetic host−guest systems engaged in molecular recognition of biological compounds, such as peptides and proteins, in their native environment, i.e., aqueous solutions.[2] When the recognition targets are enzymes, the synthetic molecular assemblies can be tailored not only to bind to the target, but also to serve as enzyme substrates. As a variation of such an approach, enzyme-induced decapsulation[3] has been previously used as a selective controlled release system.

Further development of synthetic enzyme-controlled molecular recognition systems may have important implications for medicinal chemistry. Indeed, various methods of drug latentiation have been used to transform known bioactive compounds to their enzyme-labile derivatives thereby improving their pharmacokinetic profiles.[4] A similar approach has been used to build enzyme-sensitive delivery systems in which a drug molecule bound to a carrier is released upon specific enzymatic attack.[5]

The design of the system described below was based on the idea that an enzyme-targeted delivery system would work best if the enzyme catalyzing the release would also be the target for the released drug (Scheme 1). The catalyzed cleavage of the host carrier (in its free or guest-complexed form) would

(1) Lehn, J.-M. *Supramolecular Chemistry*. *Concepts and Perspectives*; VCH: Weinheim, 1995.

(2) (a) Still, W. C. *Acc. Chem. Res*. **1996**, *29*, 155−163. (b) Schneider, H.-J. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 848. (c) Torneiro, M.; Still, W. C. *Tetrahedron* **1997**, *53*, 8739−8750. (d) Albert, J. S.; Goodman, M. S.; Hamilton, A. D. *J. Am. Chem. Soc*. **1995**, *117*, 1143−1144. (e) Peczuh, M. W.; Hamilton, A. D.; Sanchezquesada, J.; de Mendoza, J.; Haack, T.; Giralt, E. *J. Am. Chem. Soc*. **1997**, *119*, 9327−9328. (f) Hamuro, Y.; Calama, M. C.; Park, H. S.; Hamilton, A. D. *Angew. Chem., Int. Ed. Engl*. **1997**, *36*, 2680−2683. (g) Hinzen, B.; Seiler, P.; Diederich, F. *Helv. Chim. Acta* **1996**, *79*, 942−960.

(3) Menger, F. M.; Johnston, D. E. *J. Am. Chem. Soc*. **1991**, *113*, 5467−5468.

(4) (a) Korolkovas, A. *Essentials of Medicinal Chemistry*, 2nd ed.; Wiley-Interscience: New York, 1988. (b) Wermuth, C. G.; Gaignault, J.-C.; Marchandeau, C. Designing Prodrugs and Bioprecursors. 1. Carrier Prodrugs. In: *The Practice of Medicinal Chemistry*; Wermuth, C. G., Ed.; Academic Press: London, 1996.

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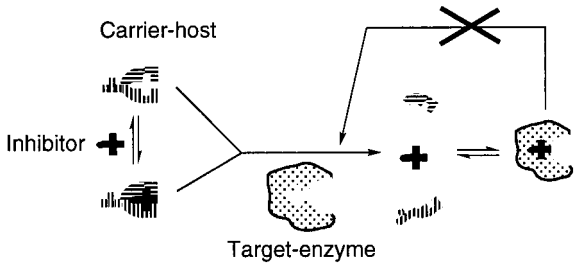
Scheme 1



thus increase concentration of the free form of the enzyme inhibitor and thereby close the negative feedback loop, as shown in Scheme 1. Such a Trojan horse-type complex may be potentially used as a delivery system that discharges the inhibitor at the immediate site of its action providing precision of targeting at the molecular level.

We report here a demonstration of the above general concept, implemented in a model synthetic host carrier that is capable of complexing with an inhibitor of several serine proteases and at the same time serves as a selective substrate for one of the proteases.

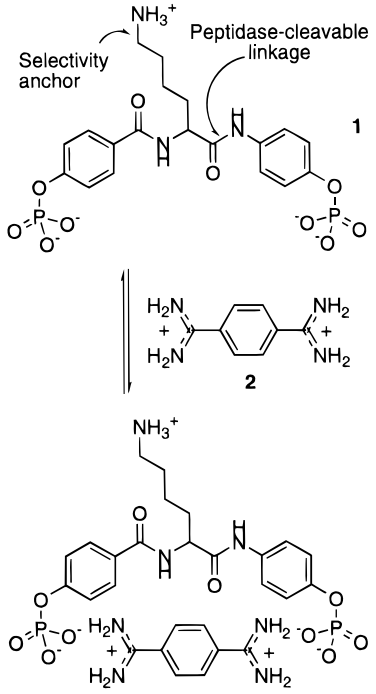
Design and Synthesis of the Host−Inhibitor System. The enzymes chosen as potential targets were trypsin, thrombin, and plasmin, proteases that cleave peptide bonds specifically at the sites containing positively charged residues, Arg and Lys.[6] The host−inhibitor combination was designed to meet the requirements of Scheme 1, as described below.

1,4-Diamidinobenzene **2** has been previously shown to inhibit trypsin via binding to its cation-recognition site.[7] Our preliminary studies have shown that **2** exhibits similar inhibitory properties toward thrombin and plasmin. The K_i values of **2** for thrombin (from bovine plasma), plasmin (from human plasma), and trypsin (type-IX from porcine plasma) were found to be 1.0 ± 0.4, 0.6 ± 0.1, and 0.88 ± 0.07 mM, respectively. Our measured K_i for trypsin differed from the previously reported value (5 mM,[7] perhaps, because of somewhat different experimental conditions).

Host compound **1** has been designed to contain two "arms" that would be capable of binding to **2** via the formation of up to four salt bridges between the phosphate and amidinium groups (Scheme 2).[8] Formation of the guanidinium−phosphate salt bridges has been shown to be an effective recognition mechanism in natural and synthetic complexes.[9] Molecular modeling of the **1·2** complex (Sybyl/Tripos software) has shown that the phosphate groups (d(P−P) = 14.0 Å in the energy-minimized conformation) are located favorably for the formation of two sets of the ion pairs with the dication of **2** (d(C(amidine)−C(amidine)) = 7.0 Å), as shown in Figure 1. The two phosphate-containing halves of **1** have been linked together by peptide bonds through a lysine residue that can serve as an anchor rendering the host compound a potential substrate for proteases specific to cationic side chains. The enzymatic cleavage of **1** should thus result in the increased concentration of the free form of **2** that can subsequently inhibit the protease.

Host **1** was synthesized via amide formation and phosphorylation procedures (Scheme 3) and isolated as its tetraammonium salt. Its binding with **2** was studied by the ^{31}P NMR titration in aqueous solution at pD 8.0, close to the optimum for the protease action.[6] Considerable complexation-induced ^{31}P chemical shifts were indicative of the expected ionic binding mechanism. The titration curves (Figure 2) were satisfactory fitted by a 1:1 binding equation[10] and yielded the binding constant value of 480 ± 30 M^{-1} (ΔG = 15.3 kJ/mol). The observed host−inhibitor affinity is slightly lower than one should expect from the formation of four salt bridges in the aqueous solution.[11] Such a difference may be attributed to the formation of an internal ion pair between the lysine ammonium and one of the phosphate groups in **1** which effectively decreases the host negative charge. The stability of the host−inhibitor

Scheme 2



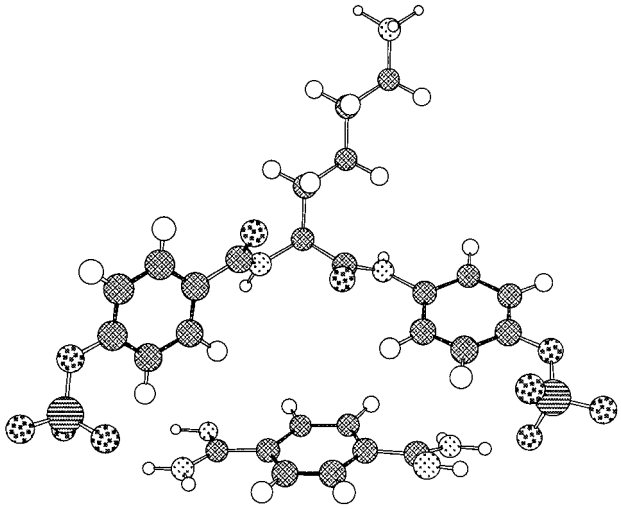


Figure 1. Energy-minimized molecular model of the **1·2** complex.

(6) Barman, T. E. *The Enzyme Handbook*; Springer-Verlag: New York, 1969; Vol. 2.

(7) Geratz, J. D. *Arch. Biochem. Biophys.* **1967**, *118*, 90.

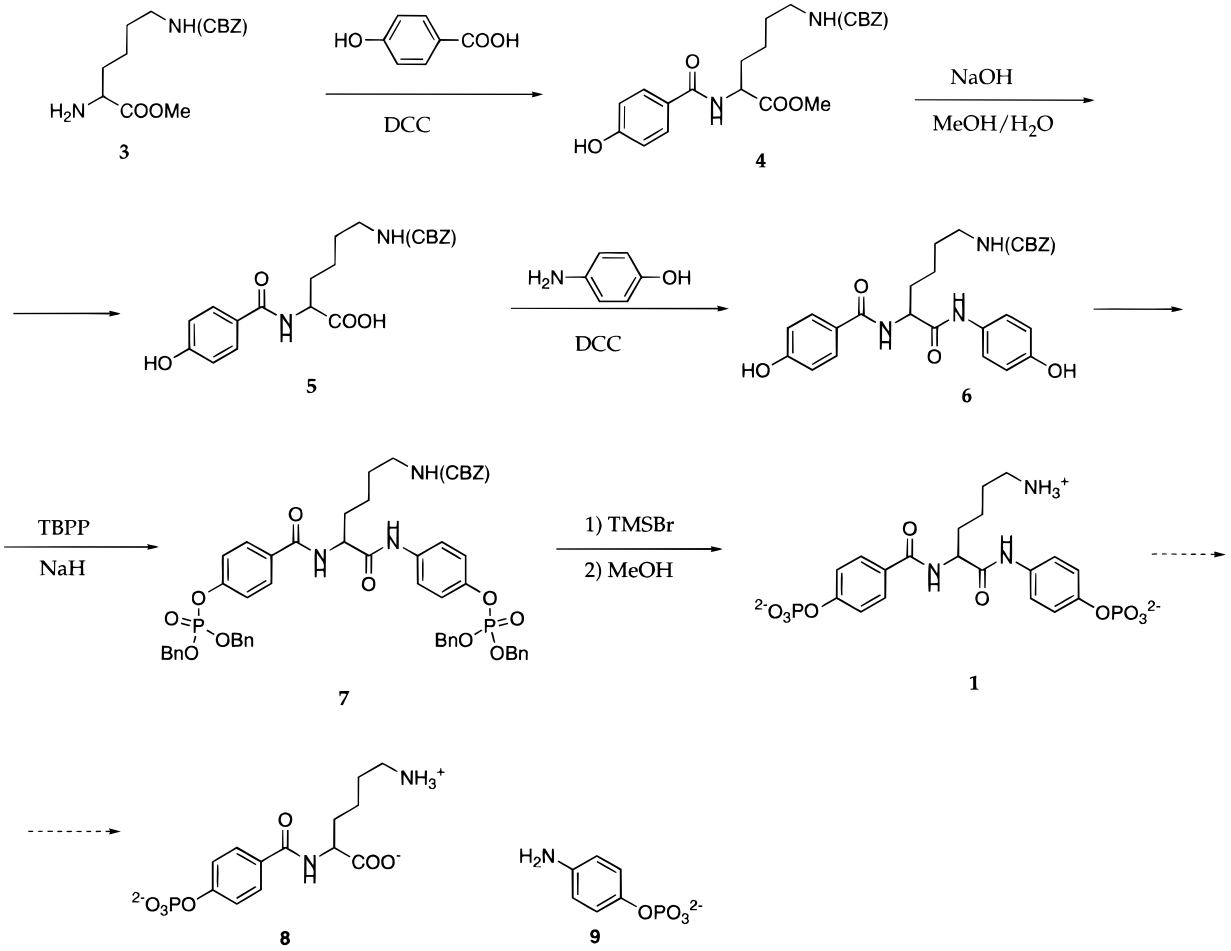
(8) As suggested by the reviewer, this binding mode may be alternatively described as a combination of two (2+1) salt bridges with possible formation of up to four ionic hydrogen bonds.

(9) (a) Perreault, D. M.; Cabell, L. A.; Anslyn, E. V. *Bioorg. Med. Chem.* **1997**, *5*, 1209−1220. (b) Schrader, T. *Chem. Eur. J.* **1997**, *3*, 1537−1541. (c) Schrader, T. H. *Tetrahedron Lett.* **1998**, *39*, 7, 517−520.

(10) K was determined from the nonlinear regression fitting of the titration data to the equation $\Delta\delta = \Delta\delta_\infty K[\mathrm{I}]/(1 + K[\mathrm{I}])$ that corresponds to the excess of the inhibitor over the complex maintained in the experiment.

(11) (a) Schneider, H.-J.; Theis, I. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 753. (b) Schneider, H.-J. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1417.

Scheme 3



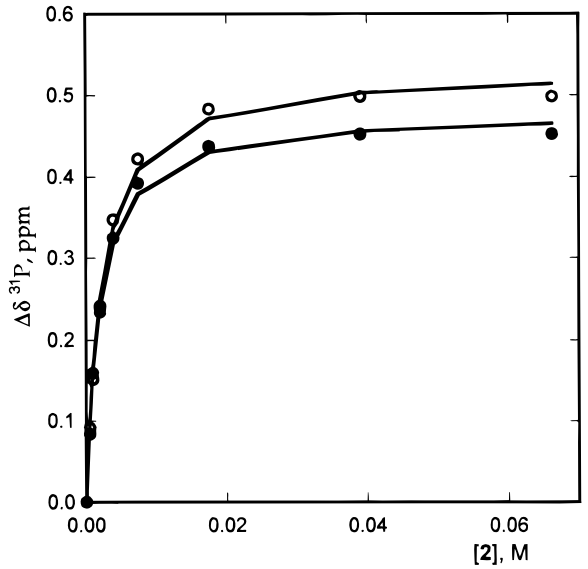


Figure 2. ^{31}P NMR titration of **1** (4.71 mM) with **2** at pD 8.1 and 20 °C. Changes in chemical shifts ($\Delta\delta$) are shown for two phosphate groups in **1**.

complex is similar to that commonly observed, for example, for drug−cyclodextrin complexes.[12]

Proteolytic Cleavage of the Host. Host **1** was then tested as a potential substrate for the proteases. The cleavage reactions performed at pH 8.5 and 37 °C in 0.1 M tris-HCl buffer were monitored by HPLC and by ^{1}H and ^{31}P NMR.

Incubation of **1** under the above conditions in the absence of enzymes showed no detectable cleavage within 54 h. Incubation of 10 mM **1** with thrombin (0.64 mM) for 24 h showed no changes in the substrate concentration and spectra, within experimental error. Similarly, an experiment with plasmin (0.06 mM)[13] showed <20% cleavage after 54 h. Such inactivity of the enzymes is not surprising given that the negatively charged side chains of **1** may strongly increase its K_m values. However, trypsin (0.81 mM) did catalyze the hydrolysis of **1**, as followed by HPLC (Figure 3). The progress of the hydrolysis reaction was also verified by the appearance of new signals in the ^{1}H NMR spectrum which were assigned to the cleavage products **8** and **9** by comparison with the independently synthesized standards (see the Experimental Section).

To test the trypsin effect on the inhibitor−host system, a similar cleavage experiment of **1** was performed in the presence of an equimolar concentration of **2**. The results shown in Figure 3 demonstrate that the cleavage was also observed in these conditions, although at a lower rate, as expected from the inhibitory action of **2** on the enzyme.

It should be noted that in the latter reaction, host **1** plays a dual role, that of the scavenger for the inhibitor and of the substrate for the enzyme. Thus the enzyme activity, affected by the released inhibitor, is probed by the rate of the host

(12) (a) Szejtli, J. *Cyclodextrins and Their Inclusion Complexes*; Akadémiai Kiadó: Budapest, 1982. (b) Duchêne, D. *New Trends in Cyclodextrins and Their Derivatives*; Editions de Santé: Paris, 1991.

(13) Plasmin was tested at a lower concentration than other proteases for solubility reasons and also because plasmin is known to be ca. 50 times more active than trypsin (per mM of enzyme) in the hydrolysis of their specific amide substrates (see Supporting Information).

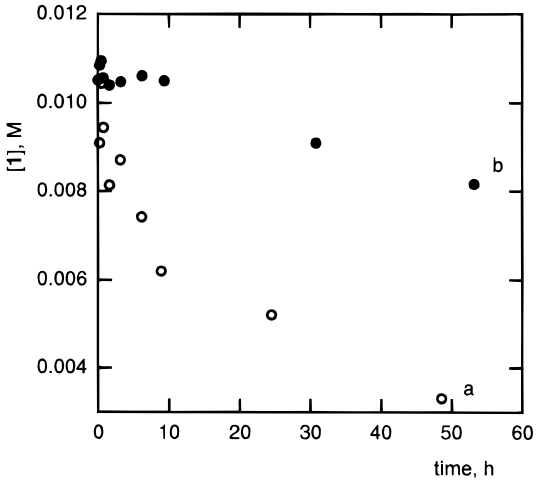


Figure 3. Time courses for the trypsin-catalyzed hydrolysis of **1** (10 mM) in the absence (a) and in the presence (b) of 10.6 mM **2** (each curve is an average of two independent runs, see text for details).

cleavage. The complex kinetics of this process makes it difficult to analyze the precise mechanism of the reaction, namely whether the host itself or its complex with the inhibitor acts as the enzyme substrate. However, regardless of the mechanism, the trypsin-catalyzed host cleavage leads to the increasing concentration of the inhibitor's *free* form in solution. Given the measured **1·2** stability constant, it can be estimated that under the experimental conditions (Figure 3), 64% of the inhibitor initially exists in the host-complexed form and therefore is effectively removed from the interaction with the enzyme. The subsequent host hydrolysis increases the fraction of the free inhibitor. The overall process of trypsin interaction with the **1·2** complex can thus be considered as a product-inhibited reaction. Apparently, the inhibitory effect of **2** results in deactivation of trypsin with respect to not only **1** but also any other potential substrate, thereby serving the general purpose of targeting.

Conclusion

We have described a host compound that scavenges the protease inhibitor via noncovalent complex formation and may release it upon proteolytic cleavage. An essential feature of this system is that the host increases the selectivity of the inhibitor action. Indeed, the K_i values of **2** are similar for all three tested enzymes, but only trypsin is capable of cleaving host **1** and the **2·1** complex. This is no surprise given that the substrate specificity is determined by the overall catalytic mechanism of the enzyme, while the inhibitor specificity reflects only the binding event.

Our current effort is focused on the development of systems that use reversible *covalent* bonds between the host and the inhibitor and, therefore, form stronger complexes that may lead to new therapeutic applications. The ultimate trojan horse-type system should act in a way similarly to the suicide enzyme inactivators[14] with two major differences: (1) while the product of the suicide reaction usually irreversibly links to the enzyme, the "trojan horse" product leaves the enzyme in its native state; and (2) the "trojan horse" approach requires no special inhibitor design, because there is a known inhibitor that can be used in the complex.

(14) (a) Sjoerdsma, A. *Clin. Pharm. Therap*. **1981**, *30*, 3−22. (b) Walsh C. T. *Annu. Rev. Biochem*. **1984**, *53*, 493−535.

Experimental Section

Materials and Methods. All reagents were purchased from Aldrich unless otherwise noted. *N*-ϵ-CBZ-L-lysine methyl ester, specific substrates for K_i determination, trypsin-type IX (15 900 units/mg of protein) from porcine pancreas, thrombin (98 units/mg of protein) from bovine plasma, and plasmin (3.9 units/mg of protein) from human plasma were purchased from Sigma. ^{1}H, ^{13}C, and ^{31}P NMR were recorded on Varian Unity Inova-400, Varian Unity Inova-300, and Varian Gemini (300 MHz) spectrometers. UV spectra were recorded with a Cary 3E UV−vis spectrophotometer. HPLC analyses were performed on a Beckman System Gold chromatograph equipped with a photodiode array UV detector.

Synthesis. (a) *N*-α-(4-Hydroxybenzoyl)-*N*-ϵ-CBZ-lysine Methyl Ester (4). N-ϵ-CBZ-L-lysine methyl ester hydrochloride was converted in its free base form **3** by ether extraction from aqueous solution at pH 10. DCC (2.20 g, 10.66 mmol) was added to a stirred solution of **3** (2.408 g, 8.19 mmol) and 4-hydroxybenzoic acid (1.13 g, 8.19 mmol) in anhydrous THF (20 mL) at 5 °C, and the temperature was raised to ambient. Upon completion of the reaction after 4 h, the solvent was evaporated in *vacuo* and the residue was dissolved in EtOAc (150 mL), extracted with 10% citric acid (2 × 75 mL), saturated $NaHCO_3$ solution (2 × 75 mL), brine (1 × 75 mL), and water (1 × 75 mL), and dried over anhydrous $MgSO_4$. Evaporation of the solvent yielded the oily product **4** (2.3291 g, 69%, R_f = 0.45, silica, 1:1 $CHCl_3$/EtOAc). Compound **4** was used in subsequent steps without further purification; ^{1}H NMR (300 Mz, DMSO) δ 8.38 (d, J = 7.3 Hz, 1H, −α-CH−N*H*−), 7.76 (d, J = 7.8 Hz, 2H, −CO−*Ph*-OH), 7.33 (m, 5H, −*Ph*), 7.22 (t, 1H, −ϵ-N*H*−COO−), 6.80 (d, J = 7.8 Hz, 2H, −CO−*Ph*−OH), 5.00 (s, 2H, −CH_2−Ph), 4.46−4.26 (m, 1H, −α-C*H*−), 3.63 (s, 3H, −COOCH_3), 2.98 (m, 2H, −CH_2−ϵ-NH−), 1.94−0.92 (m).

(b) *N*-α-(4-Hydroxybenzoyl)-*N*-ϵ-CBZ-lysine (5). To a stirred solution of **4** (2.568 g, 6.19 mmol) in 3:1 methanol−water (60 mL) were added NaOH pellets (1.24 g, 31.0 mmol) at 5 °C. After 2.5 h, when the TLC (silica, 1:1 $CHCl_3$/EtOAc) showed disappearance of all starting material, the solvent was evaporated and the residue was dissolved in water (50 mL) and acidified to pH 3.5 with a dilute solution of HCl. Extraction with EtOAc (2 × 125 mL), drying the organic layer over anhydrous $MgSO_4$, followed by the solvent evaporation and drying yielded the carboxylic acid derivative **5** (2.142 g, 86%) as an oily residue. The product **5** was used in subsequent steps without further purification; ^{1}H NMR (300 MHz, DMSO) δ 9.95 (br s, 1H, −Ph−O*H*), 8.23 (d, 1H, −α-NH−), 7.76 (d, 2H, −*Ph*−OH), 7.34 (m, 5H, −*Ph*), 7.22 (t, 1H, −N*H*−COO−), 6.81 (d, 2H, −*Ph*−OH), 4.99 (s, 2H, −CH_2−Ph), 4.31 (m, 1H, −α-C*H*−), 2.99 (m, 2H, −CH_2−ϵ-NH−), 1.88−1.68 (m, 2H, −α-CH−CH_2−), 1.55−1.22 (m, 4H, −(CH_2)$_2$−CH$_2$−ϵ-NH−).

(c) *N*-α-(4-Hydroxybenzoyl)-*N*-ϵ-CBZ-lysine 4-Hydroxyanilide (6). Carboxylic acid **5** (1.601 g, 3.99 mmol) and 4-aminophenol (0.436 g, 3.99 mmol) were dissolved in anhydrous THF (65 mL) in an argon atmosphere. The mixture was then cooled to 5 °C and to it was added DCC (1.07 g, 5.18 mmol). The reaction mixture was allowed to warm to room temperature and the progress of the reaction was monitored by TLC (silica, 7.5% MeOH in $CHCl_3$). On completion of the reaction after 4 h, the solvent was removed in *vacuo* and the residue was dissolved in EtOAc (150 mL) and extracted with saturated $NaHCO_3$ (2 × 75 mL), 10% citric acid (2 × 75 mL), brine water (75 mL), and water (75 mL). The EtOAc layer was then dried over anhydrous $MgSO_4$, evaporated, and dried under *vacuo* to yield product **5** (1.687 g, 86%). This crude product was used in the next step without further purification. A small portion of the product was purified by column chromatography (eluant: 7.5% MeOH in $CHCl_3$) for NMR spectroscopy; ^{1}H NMR (300 MHz, DMSO) δ 9.96 (s, 1H, −O*H*), 9.76 (s, 1H, −O*H*), 9.15 (s, 1H, −N*H*−Ph−OH), 8.18 (d, 1H, −N*H*−α-CH−), 7.80 (d, 2H, −CO−*Ph*−OH), 7.34 (m, 7H, −*Ph* + −NH−*Ph*−OH), 7.24 (t, 1H, −ϵ-N*H*−COO−), 6.81 (d, 2H, −CO−*Ph*−OH), 6.68 (d, 2H, −NH−*Ph*−OH), 5.00 (s, 2H, −CH_2−PH), 4.49 (m, 1H, −α-C*H*−), 3.01 (m, 2H, −CH_2−ϵ-NH−), 2.88−2.68 (m, 2H, −α-CH−CH_2−), 1.59−1.20 (m, 4H, −(CH_2)$_2$−CH$_2$−ϵ-NH−). ^{1}H−^{1}H COSY (300 MHz) indicated coupling between protons at δ 7.80 and 6.81 ppm and

between those at δ 7.34 and 6.68 ppm. FABMS *m*/*z* 492.3 $(M + H)^+$; calcd for $C_{27}H_{30}N_3O_6$ 492.5.

(d) Tetrabenzyl [*N*-α-(4-Benzoyl)-*N*-ϵ-CBZ-lys 4-anilide]diphosphate (7). To a stirred solution of **6** (0.2 g, 0.41 mmol) in anhydrous THF (14 mL) was added sodium hydride (dry 95%, 0.021 g, 0.83 mmol) under argon. After the suspension was stirred for 5 min a solution of tetrabenzylpyrophosphate (Fluka, 0.57 g, 1.06 mmol) in anhydrous THF (7 mL) was added dropwise. Within 15 min a white viscous substance precipitated out and TLC (eluant: 5% MeOH in $CHCl_3$) showed no presence of starting material **6**. After removing the solvent and drying in *vacuo*, the crude product was purified by silica chromatography (5% MeOH in $CHCl_3$) to yield compound **7** (0.257 g, 62%, $R_f = 0.54$); ^{1}H NMR (400 MHz, DMSO) δ 10.15 (s, 1H, −N*H*−Ph−), 8.59 (d, 1H, −NH−α-CH−), 7.91 (d, J = 8.8 Hz, 2H, −*Ph*−), 7.57 (d, J = 8.8 Hz, 2H, −*Ph*−), 7.33 (m, 27 H, $-CH_2-$*Ph* + −*Ph*−), 7.24 (t, 1H, −ϵ-N*H*−), 7.10 (d, J = 8.4 Hz, 2H, −*Ph*−), 5.12 (m, 6 H, −CH_2− Ph), 4.99 (m, 4H, −CH_2−Ph), 4.49 (m, 1H, −α-C*H*−), 2.98 (m, 2H, −CH_2−ϵ-NH−), 1.77 (m, 2H, −α-CH−CH_2−), 1.57−1.00 (m, 4H, −α-CH−CH_2−(CH_2)$_2$−); ^{31}P NMR (162 MHz, DMSO) δ −5.75, −6.35; FABMS *m*/*z* 1012.6 $(M + H)^+$; calcd for $C_{55}H_{55}N_3O_{12}P_2$ 1012.3.

(e) Tetraammonium [*N*-α-(4-Benzoyl)-lysine-4-anilide]diphosphate (1). Trimethylsilyl bromide (0.053 mL, 0.401 mmol) was added to a solution of **5** (0.074 g, 0.073 mmol) in anhydrous THF (2 mL) under argon at −40 °C. The reaction mixture was allowed to stir for 1 h while adjusting to ambient temperature. The progress of the reaction was followed by ^{31}P NMR (DMSO). Further trimethylsilyl bromide (0.053 mL, 0.401 mmol) was added four times at intervals of 0.5 h. The solvent from the reaction mixture was then removed and the residue was dissolved in MeOH (2 mL) and stirred for 2 days. The solvent was then removed, and the residue was dissolved in water (2 mL), pH adjusted to 7−8, and purified by ion-exchange chromatography (Sephadex DEAE 25, NH_4HCO_3 gradient of 0 to 1 M). Lyophilization of the eluant from the fractions containing 0.7−0.8 M salt yielded compound **1** (0.011 g, 29%, as determined by ^{1}H NMR) as a white fluffy solid; ^{1}H NMR (400 MHz, D_2O, pD =8.1) δ 7.60 (d, J = 6.4 Hz, 2H, −CO−Ph−), 7.11 (m, 4H, −CO−*Ph*− + −NH−*Ph*−), 7.00 (d, J = 8.0 Hz, 2H, −NH−*Ph*−), 4.40 (m, 1H, −α-C*H*−), 2.82 (t, J = 7.6 Hz, 2H, −CH_2−ϵ-NH_3^+), 1.90−1.65 (m, 2H, −α-CH−CH_2−), 1.63−1.50 (m, 2H, −CH_2−CH_2−ϵ-NH_3^+), 1.50−1.30 (m, 2H, −α-CH−CH_2−CH_2−); ^{1}H−^{1}H COSY (300 MHz) indicated coupling within the following pairs of signals: δ 7.6 and 7.11 ppm, δ 7.11 and 7.00 ppm, δ 2.82 and 1.63−1.50 ppm, and δ 1.90−1.65 and 1.50−1.30 ppm. ^{31}P NMR (162 MHz, D_2O, pD = 8.1) δ 0.343, 0.019. HPLC analysis of **1** (see Supporting Information) showed >95% purity. The structure of **1** was confirmed by the electrospray ionization (ESI) MS and MS/MS (see Supporting Information). The negative mode ESI MS (MeOH/water 1:1 v/v) showed a major singly charged molecular peak at *m*/*z* 516.1 corresponding to one negative charge on each phosphate group of **1** and one positive charge on the lysine ammonium. The positive mode ESI MS showed a singly charged peak at *m*/*z* 518.0 corresponding to fully protonated phosphate groups and positively charged ammonium.

Two byproducts of the reaction recovered from the fractions containing 0.5−0.6 and 0.6−0.7 M salt were identified as cleavage products **8** and **9**, respectively. **8**: ^{1}H NMR (400 MHz, D_2O, pD = 8) δ 7.61 (d, J = 8.0 Hz, 2H, *Ph*), 7.10 (d, J = 8.0 Hz, 2H, *Ph*), 4.20 (m, 1H, α-CH), 2.81 (t, J = 7.2 Hz, 2H, −CH_2−ϵ-NH_3^+), 1.8−1.6 (m, 2H, −α-CH−CH_2−), 1.6−1.5 (m, 2H, −CH_2−CH_2−ϵ-NH_3^+), 1.30 (t, J = 7.6 Hz, 2H, −α-CH−CH_2−CH_2−); ^{31}P NMR (162 MHz, D_2O, pD = 8) δ −3.05. **9**: ^{1}H NMR (400 MHz, D_2O, pD = 8) δ 7.25, 7.02 (dd); ^{31}P NMR (162 MHz, D_2O, pD = 8) δ −3.71. NMR data for **8** and **9** were later used to confirm assignments of the enzymatic cleavage products.

(f) 1,4-Diamidinobenzene Dihydrochloride (2). **2** was synthesized by a modified procedure of Garigipati.[15] 1,4-Dicyanobenzene (0.99 g, 7.72 mmol) was added as a solid to a 0.97 M stock solution of methylchloroaluminum amide in benzene,[16] and the resulting mixture was refluxed for 48 h under argon. The reaction mixture was then cooled and poured into a slurry of silica gel (20 g) in chloroform. The slurry was stirred for 5 min, filtered, resuspended in water, filtered, and washed with water (200 mL). The water and the chloroform filtrates were evaporated and the residues combined. The crude product was crystallized from water/EtOH to yield an off-white crystalline solid of **2** (1.14 g, 63%); ^{1}H NMR (300 MHz, D_2O + DMSO) δ 8.08 (s, −*Ph*−). Anal. Calcd for $C_8H_{10}N_4$·2HCl: C, 40.87; H, 5.14; N, 23.83; Cl, 30.16. Found: C, 40.97; H, 5.11; N, 23.71; Cl, 30.08.

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Supporting Information Available: K_i determination protocols, procedures for HPLC analysis of the cleavage reactions, and ESI MS and MS/MS spectra of **1** (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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