Comparative Study of the Active Site Caging of Serine Proteases: Thrombin and Factor Xa†

Jan Willem Thuring,‡ Hui Li,§ and Ned A. Porter*,§

Department of Chemistry, Duke University, Durham, North Carolina 27708, and Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37235

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ABSTRACT: Bovine thrombin and human factor Xa were acylated at their active site selectively with inhibitors derived from the parent compound 4-guanidinophenyl (*E*)-4-diethylamino-2-hydroxy-α-methylcinnamate hydrochloride, **1b**. Peptidyl side chains were attached to the phenol ring via amide connection, which served as a recognition motif in inhibiting different serine proteases. Upon irradiation with 366 nm light, the *trans*-cinnamate attached to the active-site serine isomerizes to the cis isomer which then rapidly lactonizes to release the free enzyme. The peptidyl side chain sequences specific for each serine protease were revealed via constructing and screening a library of homologous compounds. This methodology may be applied to other proteases. One application based on enzyme-specific, photoactivatable inhibitors is to isolate a designated active protease from a mixture of several proteases. Thus, a cinnamate inhibitor with a biotin moiety, **1d**, was synthesized. A solution of enzyme-specific, biotinylated inhibitor was added into a mixture of proteases containing a target enzyme. The target enzyme was acylated at the active site and subsequently bore a biotin tail. An avidin column was used to separate the biotinylated enzyme from the unmodified ones, by a strong binding between biotin and avidin. After a brief irradiation on the avidin column, the retained enzymes were released from the biotin tag and eluted off the column. To demonstrate the idea, thrombin and factor Xa have been separated from each other by this strategy.

The photochemical control of the activity of a vast array of biologically important molecules has been the subject of numerous studies over the past 30 years. One approach to control biological activity with light involves the covalent modification of a functional group in the biologically active compound that is critical for its activity, by a photolabile mask rendering the "caged" molecule biologically inactive. Subsequent photolysis will "uncage" the molecule, restoring its activity in a well-defined spatial and temporal manner. Examples of biomolecules that have been modified by this photoprotection/deprotection strategy and that have been used in intact cell systems are as diverse as neurotransmitters (*1*), nucleotides, Ca^{2+} chelators, and inositol phosphates (*2, 3*). The activity of large biomolecules such as enzymes may also be controlled using this strategy as has been demonstrated for a photoactivatable lysozyme (*4*). It should be noted, however, that very few examples of caged proteins exist in the literature (*5, 6*), the major obstacle being that it is difficult to target a suitable caging group to the desired site in the protein. Our own efforts in this field have been focused on the acylation by a *trans*-cinnamoyl ester **1** of the catalytic serine residue in the active site of serine proteases such as thrombin and FXa[1] (clotting enzymes, **1a**−**b**), as well as the digestive enzymes trypsin (**1a**−**b**) and α-chymotrypsin (**1c**). The proteolytically inactive acyl-enzymes thus obtained can be isolated and show reasonably good dark stability. Upon illumination, however, a rapid trans to cis photoisomerization occurs, followed by a dark lactonization reaction (Scheme 1), thus restoring the activity of these caged acyl-enzymes (*7*−*11*). Following this acylation−photodeprotection scheme, we have been able to initiate plasma coagulation both in vivo and in vitro (*12*).

The positively charged *p*-amidino and *p*-guanidino phenol leaving groups in **1** mimic the preferred arginine cleavage

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‡ Duke University.

§ Vanderbilt University.

[1] Abbreviations: FXa, factor Xa; ^{t}BuOK, potassium *tert*-butoxide; DMF, *N*,*N*-dimethylformamide; HMTA, hexamethylenetetraamine; NMM, *N*-methylmorpholine; DMAP, 4-*N*,*N*-dimethylaminopyridine; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; TFA, trifluoroacetic acid; EtOAc, ethyl acetate; EtOH, ethyl alcohol; MeOH, methyl alcohol; THF, tetrahydrofuran; DI, deionized; RP-HPLC, reverse-phase high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; ^{t}Bu, *tert*-butyl; Boc, *tert*-butoxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; TES, triethylsilane; PyBOP, benzotriazol-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate; DIPEA, diisopropylethylamine; HOBt, *N*-hydroxybenzotriazole; MsCl, methanesulfonyl chloride; Ala, alanyl; Arg, arginyl; Asp, aspartyl; Asn, asparagyl; Cha, cyclohexylalanyl; Glu, glutaryl; Gln, glutamyl; Gly, glycyl; Ile, isoleucyl; Lys, lysyl; Orn, ornithyl; Phe, phenylalanyl; Phe(*p*-NO_2), *p*-nitrophenylalanyl; Phe(*p*-F), *p*-fluorophenylalanyl; Phg, phenylglycyl; Pro, prolyl; Trp, tryptophyl; Tyr, tyrosyl; Val, valyl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Suc, succinyl; equiv, equivalent(s); NHEt, ethylamino; PMB, *p*-methoxybenzyl; Cbz, benzyloxycarbonyl; *p*NA, *p*-nitroaniline; T/F, the ratio of the inhibition rate on thrombin over FXa; SPPS, solid-phase peptide synthesis; PBS, phosphate-buffered saline.

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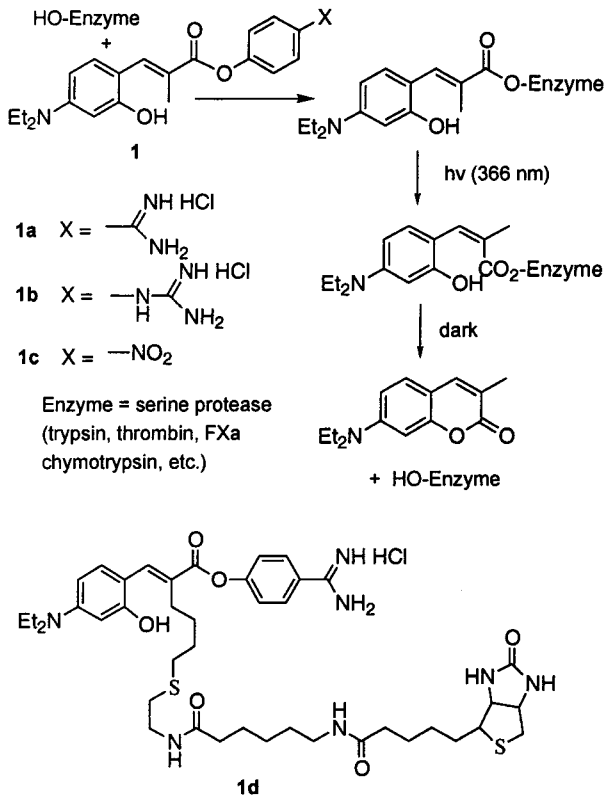
Scheme 1



sites of the coagulation proteases thrombin and FXa (trypsin-like proteases). As the positive charge in **1** is not a constituent of the acyl site of the scissile ester bond (P_1 recognition site), these inhibitors are referred to as "inverse substrates" (*13*−*15*). Inverse substrates are useful to probe the catalytic serine residue of these proteases as any functionality can at least in theory be covalently attached to this active-site serine (*16*). To demonstrate this, we have introduced an affinity label, viz., a biotin tag, onto thrombin by tethering biotin at the α-carbon of the cinnamate **1d**, which enables one to purify the acyl-enzyme by (strept)avidin affinity chromatography (*17*). In addition, inverse substrates have recently been shown as promising tools for protease-catalyzed peptide synthesis (*18*, *19*).

In contrast to other reports on the caging of proteins, which are rather nonspecific with respect to the site of action or that require site-directed mutagenesis (*4*), our approach shows a clear advantage. The caging cinnamate is targeted to the active site of serine proteases since it is a mechanism-based inhibitor; i.e., the mechanism of inhibition is the same as the one by which serine proteases cleave their natural substrates.

Still, a clear shortcoming of the inverse substrates **1** is their lack of selectivity toward a broad range of serine proteases, in particular trypsin-like enzymes. This hampers the targeting of one specific protease when it is present in a mixture, i.e., any biological sample. We report here our first efforts to address this issue, which involve the preparation of a library of peptide-substituted *p*-guanidinosalicyl cinnamates and their analysis as irreversible inhibitors of an artificial mixture of bovine thrombin and human FXa. In addition, several inverse peptidyl esters of a cinnamate were synthesized and assayed. The strategy has also been applied to the separation of proteases in a mixture by the use of a biotinylated cinnamate, **1d**.

MATERIALS AND METHODS

General. The chemicals used for solid-phase synthesis were purchased from Novabiochem (Pasadena, CA) and Bachem (Basel, Switzerland). Other chemicals were purchased from Aldrich-Sigma without further purification. Thrombin (bovine) was purchased from Johnson & Johnson Medical Inc. (Austin, TX). FXa (human) was purchased from Enzyme Research Inc. (IN). The chromogenic substrates were purchased from Diapharma Group Inc. (Cleveland, OH). The Hewlett-Packard 8452A Diode Array Spectrophotometer and SPECTRAmax Microplate Spectrophotometer (Molecular Devices, Oakland, CA) were used for the chromogenic assays. The HI-TOP System (PolyWhat Kit 004, Polyfiltronics, Cambridge, MA) was used for the parallel synthesis of the library. Sephadex G-25 (fine) was purchased from Sigma. Disposable gel filtration columns were purchased from Knotes Scientific Glassware/Instruments (Vineland, NJ). Avidin columns and eluting buffers were purchased from Pierce (Rockford, IL) and were used according to instructions. A hand-held 4W Mineralight UVSL-25 lamp (Ultra-Violet Products Inc., San-Garbiel, CA) was used for enzyme photoactivation.

The structures and preparation of the protected alcohol precursors that were used in the syntheses of the cinnamate esters **6**−**12a** (Chart 1) are presented in Supporting Information. FAB−HRMS generally gave peaks at (M) and (M+H), and the reported and calculated value presented here is for the more intense peak of the two. The SPPS syntheses of the resin-bound peptidols on SASRIN resin (Bachem, Switzerland) and *o*-Chlorotrityl resin (Novabiochem, CA), as well as their cleavage from the solid support, were performed according to the manufacturer's instructions (*20*, *21*).

*Ethyl (E)-4-N,N-Diethylamino-2-(4-methoxybenzyloxy)-α-methylcinnamate (**15**; See Scheme 4).* The cinnamate **14** (2.73 g, 9.85 mmol) (*22*) was dissolved in DMF (20 mL) and placed under an argon atmosphere. To this solution were subsequently added *p*-methoxybenzyl chloride (1.85 g, 11.8 mmol) and ^{t}BuOK (1.22 g, 10.8 mmol) in portions. The mixture was heated (50−60 °C) and stirred for 2.5 h. DMF was removed in vacuo, and the residue was partitioned between saturated NH_4Cl and ethyl acetate. The aqueous phase was extracted twice with ethyl acetate, and the combined organic extracts were washed with water (2×) and brine. After drying ($MgSO_4$), the crude product was obtained as a reddish oil (4.4 g), which was found sufficiently pure for the subsequent reaction. The product could be purified by flash chromatography (SiO_2, hexanes/EtOAc 4:1 to 3:1) to give the product as a yellowish oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 (br s, 1H), 7.34 (d, 2H, J = 8.8 Hz), 7.28 (d, 1H, J = 8.8 Hz), 6.87 (d, 2H, J = 8.8 Hz), 6.25 (dd, 1H, ^4J = 2.8 Hz, 8.8 Hz), 6.18 (d, 1H, ^4J = 2.8 Hz), 5.02 (s, 2H), 4.20 (q, 2H, J = 7.2 Hz), 3.78 (s, 3H), 3.31 (q, 4H, J = 7.2 Hz), 2.08 (br s, 3H), 1.29 (t, 3H, J = 7.2 Hz), 1.12 (t, 6H, J = 7.2 Hz). FAB−HRMS calcd for $C_{24}H_{31}NO_4^{+\bullet}$, 397.2278; found, 397.2253.

*(E)-4-N,N-Diethylamino-2-(4-methoxybenzyloxy)-α-methylcinnamic Acid (**2**).* The above compound was dissolved in a mixture of ethanol (40 mL) and 10% aqueous NaOH (40

mL). The solution was stirred 1 h at 80−90 °C. Ethanol was removed in vacuo and to the residue was added water and the alkaline solution was acidified to pH 6 with 1 N HCl. The product was extracted into dichloromethane (3 × 25 mL), and the combined organic extracts were washed with brine, dried with $MgSO_4$, and concentrated in vacuo to afford the crude product, which was recrystallized from EtOAc to give a yellow solid. Yield: 2.14 g (56% over 2 steps). IR (KBr): ν 2973 (broad), 1670, 1603, 1514, 1267 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.10 (br s, 1H), 7.33 (d, 2H, J = 8.8 Hz), 7.32 (d, 1H, J = 8.8 Hz), 6.88 (d, 2H, J = 8.8 Hz), 6.25 (dd, 1H, ^4J = 2.8 Hz, 8.8 Hz), 6.16 (d, 1H, ^4J = 2.8 Hz), 5.03 (s, 2H), 3.78 (s, 3H), 3.31 (q, 4H, J = 7.2 Hz), 2.10 (br s, 3H), 1.11 (t, 6H, J = 7.2 Hz). FAB−HRMS calcd for $C_{22}H_{27}NO_4^+$, 369.1940; found, 369.1934.

*2-Hydroxy-5-N,N′-bis(tert-butoxycarbonyl)guanidinobenzoic Acid (**13**; See Scheme 3).* The 2-hydroxy-5-aminobenzoic acid (5.0 g, 32.7 mmol) was suspended in 100 mL of DMF, and *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine (10.0 g, 32.3 mmol) was added. The mixture was stirred for 10 h at room temperature to give a dark-brown solution. The DMF was evaporated under vacuum, and the oily crude product was redissolved in dichloromethane. After extensive washing with water (6−7 times), the organic phase was dried with Na_2SO_4. The product was triturated from dichloromethane with hexanes. Yield: 10.0 g (78%); mp 240 °C (dec). ^1H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, 1H, J = 2.8 Hz), 7.45 (dd, 1H, J = 2.8 Hz, 8.8 Hz), 6.95 (d, 1H, J = 8.8 Hz), 1.55 (s, 9H), 1.45 (s, 9H). Elemental analysis calcd. for $C_{18}H_{25}N_3O_7$: C 54.68%, H 6.37%, N 10.63%; found: C 54.44%, H 6.27%, N 10.52%. FAB−HRMS calcd for $C_{18}H_{26}N_3O_7^+$, 396.1770; found, 396.1778.

*4′-N,N′-Bis(tert-butoxycarbonyl)guanidino-2′-carboxy-1′-phenyl (E)-4-N,N-Diethylamino-2-(4″-methoxybenzyloxy)-α-methylcinnamate [**3**-(PMB,Boc)].* Compound **2** (1.845 g, 5 mmol) was dissolved in 50 mL of dry dichloromethane under argon and subdued light. After being cooled to −20 °C, NMM (0.604 mL, 5.5 mmol) was added followed by isobutyl chloroformate (0.713 mL, 5.5 mmol). The solution was stirred for 15 min at −20 °C and gradually returned to room temperature. After the solvent was evaporated, dry ethyl acetate was added, and insoluble NMM salt was removed by filtration. Then the ethyl acetate was removed to give an orange oil. The 5-*N*,*N*′-bis(*tert*-butoxycarbonyl)guanidino-2-hydroxybenzoic acid (2.37 g, 6 mmol) was dissolved in dry pyridine (50 mL) at 0 °C in 5 min with stirring. This solution was added to the activated cinnamic acid derivative and was stirred at 0 °C for 1 h. The stirring was continued for another 3.5 h at room temperature. The solvent was removed under vacuum, and ethyl acetate and 5% $KHSO_4$ (20 mL each) were added. The aqueous layer was extracted with ethyl acetate twice, and the organic fractions were combined, dried, and evaporated. The crude product was purified with chromatography (SiO_2, EtOAc/hexanes 1:3 to 2:1). Yield: 51%; mp 102−103 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 10.41 (s, 1H), 8.24 (m, 1H, J = 1.2 Hz), 8.12 (dd, 1H, J = 8.8 Hz, 2.8 Hz), 8.01 (d, 1H, J = 2.8 Hz), 7.39 (d, 1H, J = 8.8 Hz), 7.36 (d, 2H, J = 8.8 Hz), 7.19 (d, 2H, J = 8.8 Hz), 6.87 (d, 1H, J = 8.8 Hz), 6.30 (dd, 1H, J = 8.8 Hz, 2.4 Hz), 6.21 (d, 1H, J = 2.4 Hz), 5.04 (s, 2H), 3.78 (s, 3H), 3.35 (q, 4H, J = 7.2 Hz), 2.21 (d, 3H, J = 1.2 Hz), 1.54 (s, 9H), 1.50 (s, 9H), 1.16 (t, 6H, J = 7.2 Hz). FAB−HRMS calcd for $C_{40}H_{51}N_4O_{10}^+$, 747.3604; found, 747.3594.

*General Procedure for the DCC Coupling of **2** and β-Amino Alcohols.* To a solution of the acid **2** (0.208 mmol) and the amino alcohol (0.229 mmol, prepared as described in the Supporting Information) in dichloromethane (2 mL) at 0 °C were added DMAP (0.229 mmol) and DCC (0.104 mmol). The mixture was allowed to warm slowly to room temperature. After 3 h, the mixture was cooled to 0 °C, and more DCC (0.062 mmol) was added. After another 3 h, the addition of DCC (0.062 mmol) was repeated 1 more time. The mixture was allowed to stir overnight. The solvent was removed in vacuo, and ethyl acetate (4 mL) was added to the residue. DCU was removed by filtration, and the filtrate was evaporated under reduced pressure. The desired products were purified by flash chromatography using silica gel.

*General Procedure for the Deprotection of Peptidyl Esters To Make Compounds **6**−**9** and **11**−**12a** (See Chart 1).* The protected cinnamate ester (0.041 mmol) was dissolved in a mixture of TFA and anisole 95:5 (v/v, 1 mL). After stirring for 30 min at room temperature, the mixture was concentrated, residual TFA was stripped off by coevaporation with toluene, and the residue was purified by flash chromatography (SiO_2, dichloromethane/MeOH 9:1 to 4:1). In the case of compounds **7a**, **8a**−**b**, **9b**, and **12a**, the corresponding HCl salts were prepared by treating the product TFA salt with thionyl chloride (10 equiv) in MeOH (1 mL). After 1 h, the mixture was concentrated and purified by flash chromatography under the same conditions. The combined fractions were lyophilyzed from water/ethanol. Alternatively, compounds **6a**−**c**, **7b**−**c**, **9a**, and **11a**−**b** as the TFA salts were purified by cation-exchange chromatography using cellulose CM as the stationary phase (20 cm long × 1.5 cm diameter), and eluted with aqueous NH_4OAc (0.1 M × 100 mL, 0.3 M × 100 mL, and 0.5 M × 100 mL). The combined fractions were lyophilyzed.

*General Procedure for the Deprotection of Peptidyl Esters **10a**,**b**.* For the neutral compounds **10a**,**b**, the PMB group was removed by adding anisole (20 equiv) and TFA (20 equiv) to a solution of the protected ester (0.082 mmol) in dichloromethane (2 mL). After stirring for 2 h at room temperature, the mixture was concentrated, residual TFA was stripped off by coevaporation with methanol, and the residue was purified by column chromatography (SiO_2, hexanes/EtOAc 3:1, 2:1, 1:1, 2:3). The combined fractions were lyophilyzed from water/ethanol.

Preparation of the Library. The library of compounds was prepared via the standard solid-phase peptide synthesis method, in a parallel format (12 × 8) from three batches. Compounds **3**-Xaa-NH_2 were purified with RP-HPLC and analyzed with ESI-MS. The quantity of each compound was estimated with UV/Vis (absorption maximum around 360 nm in Tris buffer, pH 7.4). The extinction coefficient of **3**-Ala-OMe is 2.98×10^4 M^{-1} cm^{-1} at 360 nm in Tris buffer, pH 7.4, with 5% EtOH. A portion of the **3**-Xaa_1-Xaa_2-NH_2 was analyzed with ESI-MS.

General Procedure for Enzyme Inhibition and Assays. All enzyme inhibition experiments were carried out under subdued light at room temperature. The enzyme solutions were made by diluting frozen or lyophilized enzymes from commercial sources with Tris buffer (Tris, 50 mM; NaCl, 150 mM, pH 7.4 or 6.6). The active concentration was kept

in the range of 1−2 μM and was determined by chromogenic assays (see below). At time zero, a known amount of inhibitor solution (5−100 equiv from 1 to 2 mM ethanolic solution) was added to the enzyme stock solution while the ethanol content was kept below 5% (v/v).

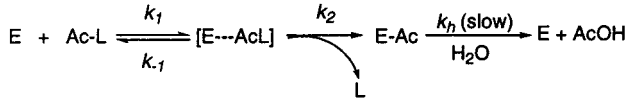
A typical enzyme activity assay was performed as follows (thrombin). To a disposable polystyrene cuvette were added 100 μL of 1 mM chromogenic substrate solution (S2238 in DI water) and 1 mL of Tris buffer (50 mM Tris, 250 mM NaCl, pH 8.3, with 1 mg/mL BSA), and the solution was vortexed. Then an aliquot of 20 μL of enzyme from the stock solution (with or without inhibitor) was added and mixed quickly. The release of *p*-nitroaniline was monitored at 402 nm for 72 s. The experimental initial rate of hydrolysis was used to calculate active enzyme concentration based on known K_M and k_{cat} values (*7*, *10*). For FXa and α-chymotrypsin, 200 and 150 μL of 1 mM chromogenic substrate (S2222 and S2586) solution were used, respectively, and for α-chymotrypsin, the Tris buffer (pH 8.3) consists of 30 mM Tris, 3 mM $CaCl_2$, and 400 mM NaCl.

Parallel enzymatic assays were carried out on a microplate reader which can read an array of assaying solutions at a time. A stock solution (about 1.0 μM) of enzyme was prepared and distributed into a 96-well incubation plate of 100 μL/well (Plate-E). An aliquot of 5.0 μL was taken from each well into a 96-well assay plate (Plate-A). Then 195 μL of substrate solution (for S2238, 0.1 mM; for S2222, 0.2 mM) was added to each well using a multiple pipet as quickly as possible, and reading was started by the software SOFTmax PRO. To get reasonable and reproducible kinetic points, 3 rows of enzyme (24 wells) were assayed per reading rather than 12 rows altogether. Duplicate assays were run when necessary. A typical reading time lasts 90 s at 6 s intervals, and a 3 s shaking was employed before scanning. After the initial enzyme concentration was determined, 96 aliquots of 5.0 μL of inhibitor solution were taken from the inhibitor stock solution plate (Plate-I, 0.8 mM of each inhibitor in 10% ethanol/water when 40 equiv of inhibitor is applied; otherwise adjust inhibitor concentration accordingly) and added to the corresponding wells of a freshly prepared Plate-E. The starting time of inhibition was recorded.

In our experiments, since the enzymes are acylated by the cinnamate-type inhibitors much slower than the turnover of the chromogenic substrates, a discontinuous assay method was utilized to obtain residual enzyme activity at various time points. An aliquot of (inhibited) enzyme sample (5 or 20 μL) was withdrawn from the enzyme stock solution at defined time and assayed directly. In our study, the binding and acylation of the enzymes by the cinnamate-type inhibitors (<1 μM in the assaying solution) can be neglected while the active enzymes are hydrolyzing the chromogenic substrates (in the range of 90−180 μM). The observed inhibition rate constant, k_i, was obtained by solving the equation in Scheme 2. In most cases, reasonable fits were found with the correlation coefficients ranging from 0.95 to 1.00. The hydrolysis (turnover) of the acylated enzyme during the inhibition can be neglected, because the hydrolysis is very insignificant as long as the inhibition time is limited to within 12 h.

Acyl-Enzyme Purification. After completion (<5% of initial activity) of the enzyme inhibition, the mixture of acyl-enzyme and excess of inhibitors is loaded onto a gel filtration column. Tris buffer (pH 6.6 or 7.4) was used for elution. The fractions were collected (typically 1 mL each) in polymethylacrylate disposable cuvettes, and the 280 nm absorption was measured with UV/Vis to identify the fractions containing acyl-enzyme.

Scheme 2



E = enzyme; AcL = acylating inhibitor;
Ac = acyl group; L = leaving group
$d[E]/dt = k_i [E] [I]$
At time zero, $[E] = [E]_0$, $[I] = [I]_0$

Separation of Biotinylated Enzymes via an Avidin Column. The prepacked Immunopure immobilized columns consist of 2 mL of 4% cross-linked agarose beads with monomeric avidin covalently attached. These columns have approximately 1 cm diameters, and the filled column length is approximately 2.5 cm. The column was first washed with PBS buffer (2 × 4 mL), the Biotin Blocking & Elution buffer (3 × 2 mL), the Regeneration buffer (3 × 4 mL), and PBS (or Tris) buffer (2 × 4 mL). Then the biotinylated acyl-enzyme solution (with other components) was loaded on the column under subdued light. PBS buffer (or Tris buffer) washed out the unbound components, which were collected in 1 mL fractions, until no more UV absorption was observable at 280 and 360 nm. The avidin column was then irradiated with a 4 W hand-held UV lamp about 2 cm from the column for 4 min, rotating 90° after every minute. The deacylated enzyme was eluted with PBS (or Tris buffer) in 1 mL fractions until the 280 nm absorption returned to baseline. Photoactivation of each fraction was also performed to measure the acyl-enzyme content of each fraction. Acyl-enzyme elution never exceeded a few percent of enzyme applied to the column. The regeneration of the column was done by washing with the Biotin Blocking & Elution buffer (3 × 2 mL), Regeneration buffer (2 × 4 mL), and 5 mL of PBS buffer with 0.05% sodium azide as preservative.

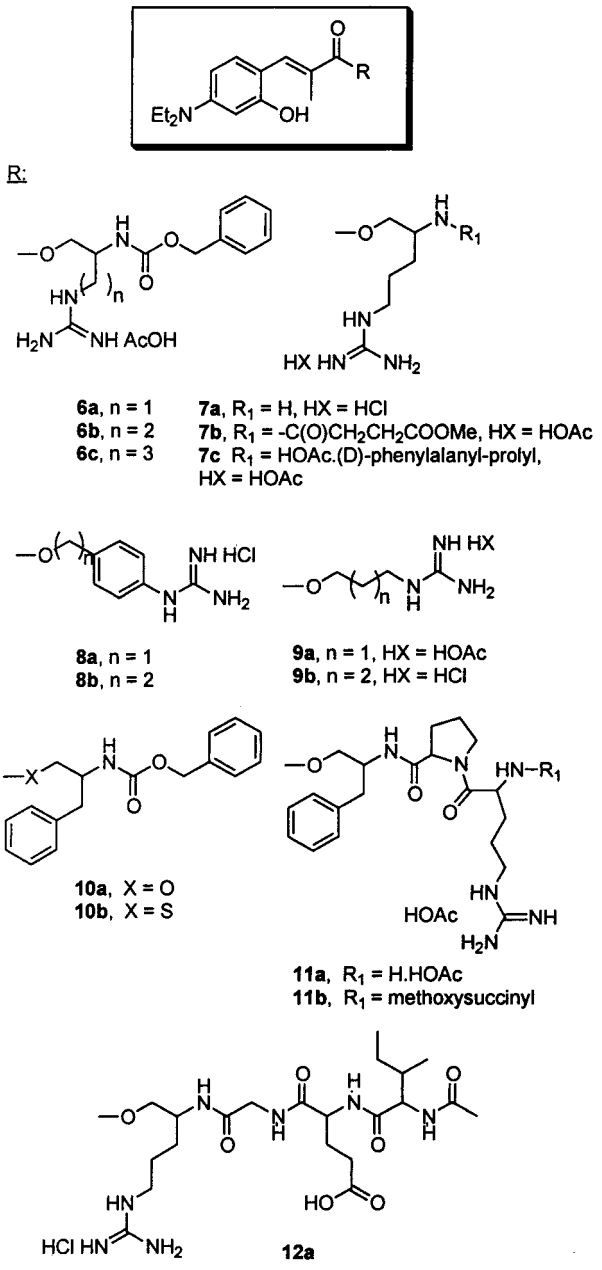
Photoactivation of Acyl-Enzymes. (1) For photoactivation of individual samples of acylated enzymes, an aliquot of 20 μL of (acyl-)enzyme solution was drawn in the polypropylene pipet tip and subjected to the irradiation of a 4 W UVSL-25 Mineralight hand-held UV lamp (366 nm). The distance is kept at approximately 1.5 cm from the center of the illumination window. (2) For photoactivation of an array of acyl-enzymes, the plate containing the samples was placed beneath the lamp by 1 cm. The size of the illumination window allows 3 columns of wells (3 × 8) to be irradiated at a time.

RESULTS

Syntheses of Peptidol Inverse Inhibitors. Based on previous reports by Walker (*23*, *24*), we synthesized a number of inverse peptidyl esters, **6**−**12a** shown in Chart 1.

The reaction conditions to mediate the coupling of the protected β-amino alcohols (**I-VIIa**, see Supporting Information) with the cinnamic acid **2** were studied in detail (Scheme 3). Many standard conditions furnished the desired product in very low yields or not at all. It appears that the problematic

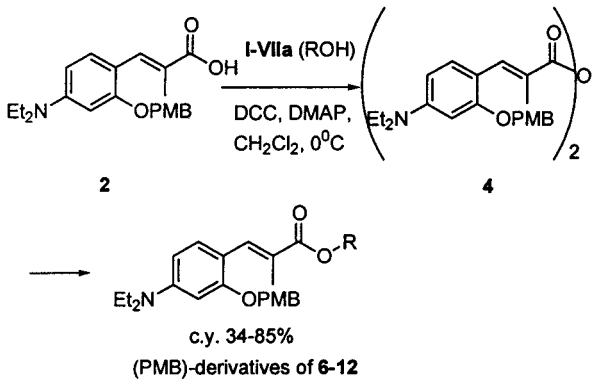
Chart 1



coupling is a result of the high electron density at the cinnamate carbonyl, and the relatively poor nucleophilicity of the aliphatic alcohols as compared to phenolates. Under optimal conditions, the peptidyl esters **6**−**12a** were obtained in 34−85% yield.

It was found that the coupling reaction occurred via the intermediacy of the deeply yellow colored symmetric anhydride **4**, as evidenced by TLC and isolation of the compound (see Supporting Information). Its formation is a result of the strong nucleophilicity of the cinnamic acid carboxylate in **2**. We found that the gradual addition of DCC to the mixture of the acid **2** and the alcohol in the presence of 1.1 equiv of DMAP in dichloromethane at 0 °C gave satisfactory results. The symmetric anhydride is thus slowly regenerated after reaction with the alcohol. The yields of the DCC coupling reactions and the final deprotections (TFA with 5% anisole v/v) are reported in Supporting Information. Some of the lower yields after deprotection were obtained

Scheme 3



after cation exchange chromatography, where only the very pure fractions were collected.

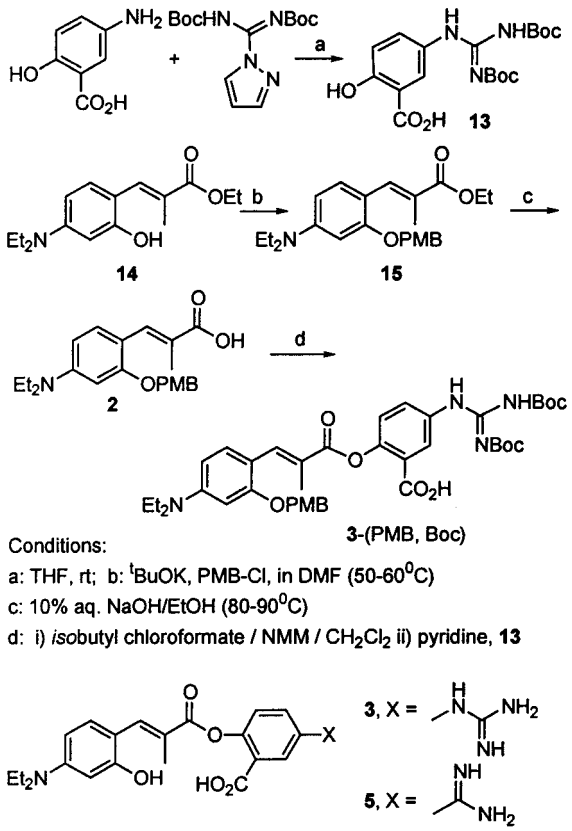
Activity of Peptidol Inverse Inhibitors. The peptidyl ester **6c** was examined against thrombin and FXa. This inhibitor bears the positively charged guanidino function at the P_1 position, and it can be extended with various amino acid residues beyond this position. Walker et al. have shown (*23*, *24*) that the analogous *p*-methoxybenzoate esters with an inverse peptidol were comparable to known highly potent "normal-type" peptide inhibitors, such as the chloromethyl ketones, that are irreversible inhibitors of some selected serine proteases. Unfortunately, when **6c** (40 equiv) was assayed for inhibitory activity toward thrombin and FXa, only a weak competitive inhibition was observed, with K_i's in the range of 0.2−0.3 mM. Enzyme activity could not be restored by irradiation at 366 nm. Similarly, compounds **6a**−**b**, **8a**−**b**, and **9a**−**b** were found to be weak inhibitors against thrombin. This suggests that the distance between the positively charged guanidino function (P_1 recognition site) and the cinnamoyl carbonyl was not the determinant factor for their inhibitory capability.

Modifications beyond the P_1 argininol residue (**7a**−**c**) were also examined. Compound **7c**, which has the same inverted sequence as the thrombin-selective chromogenic substrate S2238, did not exhibit appreciable inhibitory activity either, nor did the compounds **7a**−**b**. Compound **12a**, bearing the inverted peptidol sequence of the FXa-selective chromogenic substrate S2222, did not inhibit FXa.

The introduction of aromatic amino acid residues at the P_1 position, which had also been used in Walker's study on α-chymotrypsin, did not render an irreversible inhibitor of this enzyme (**10a**, **11a**−**b**). For example, the addition of 40 equiv of inhibitor **10a** to a 2.1 μM α-chymotrypsin solution in PBS buffer (pH 7.4) reduced the enzyme activity immediately to 75% of the original activity, but it did not decrease further during prolonged incubation. An additional 120 equiv of inhibitor further reduced the activity of the enzyme to 62% but did not change further. This observation is characteristic of reversible inhibition, with an estimated k_i value in the range of 0.25−0.50 mM. Substitution of the alkoxy leaving group in **10a** by a sulfanyl function as in **10b** showed similar K_i values for α-chymotrypsin.

In analogy to the study of Walker on anisic acid functionalized peptidyl esters (*23*, *24*), the extended inhibitors **11a**−**b** were prepared. A very slow inhibition of α-chymotrypsin was now observed, but inhibition by acylation cannot be explicitly differentiated from reversible inhibition. Ir-

Scheme 4

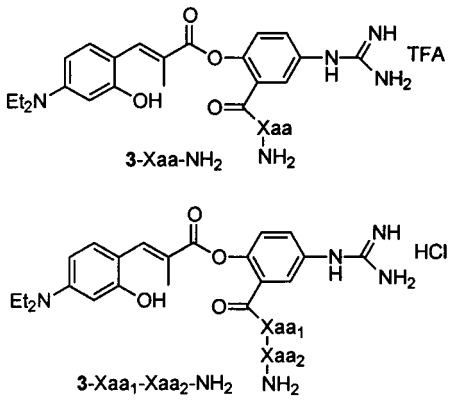


radiation using a 366 nm hand-held 4 W lamp restored a fraction of the activity. Irrespective of the exact inhibition mechanism that is operable, the inhibitors presented above are of no practical value in the inhibition and controlled photoactivation of serine proteases.

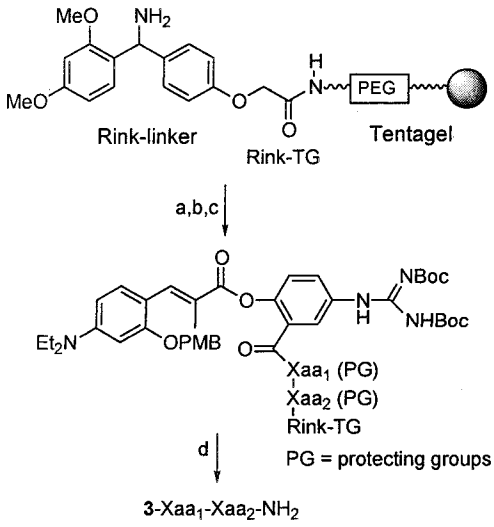
4-Guanidinosalicylate-Derived Inhibitors. The selection of cinnamates with leaving groups of the guanidino and amidino salicylate type (**3**, **5**) allows for the introduction of various oligopeptide sequences through the carboxylate handle. The report that 2′-substituted amidinophenyl benzoates showed inhibitory properties toward thrombin (*25*) encouraged us to embark on the synthesis of the guanidinophenyl scaffold **3**-(PMB, Boc) (Scheme 4). The readily available PMB-protected cinnamic acid **2** and the Boc-protected 5-guanidinosalicylic acid **13** were coupled via the mixed anhydride method, obtained by the reaction of **2** and isobutyl chloroformate. Thus, **3**-(PMB, Boc) was obtained in multigram quantities. Experiments to prepare the corresponding amidino salicyl cinnamate via the same strategy are described in Supporting Information. All attempts to generate the free acid **5** failed and led to instantaneous decomposition. Therefore, we concentrated our efforts to modify the carboxylic acid function on the guanidino derivative **3**.

Synthesis of a Library Based on Structure 3. Pilot experiments showed that **3**-Ala-OCH_3 has a greater inhibitory selectivity between thrombin and FXa than the parent inhibitor **1b**. Irradiation of the inhibited enzyme using light of 366 nm fully recovered enzyme activity. On the other hand, compound **3**-(D)-Phe-OH showed no appreciable inhibitory activity toward thrombin, and only a very weak inhibitory effect toward FXa. These data suggest the potential to elicit inhibitor specificity by a proper choice of residues

Chart 2



Scheme 5



coupled to the carboxylic acid group in **3** and set the stage for a systematic evaluation of various amino acids and dipeptides attached to the salicylate carboxyl group (Chart 2and Scheme 5).

Thus, a library consisting of 23 single amino acid analogues (**3**-Xaa-Z, see Table 1) and 112 dipeptides (**3**-Xaa_1-Xaa_2-NH_2, Chart 2, Xaa_1 = Ala, Arg, Asp, Cha, Glu, Gly, Ile, Lys, Orn, Phe, Phg, Pro, Trp, Tyr; Xaa_2 = Asp, Gly, Ile, Lys, Phe, Phg, Tyr, Val) was prepared by conventional solid-phase parallel synthesis (Scheme 5) to assess the acylation rate difference of thrombin and FXa.

The clean and high-yield coupling of the scaffold **3**-(PMB, Boc) with the free amino group on the solid support was demonstrated by the individual solid-phase synthesis and characterization of **3**-Ala-NH_2. ^{1}H NMR analysis of the crude solid from workup showed the desired product (>90%) and two minor components both containing an alanine residue, but no indication of the cinnamate moiety. The isolated yield was greater than 85%. TLC gave only one spot for the crude product, which turned fluorescent quickly if irradiated with

Table 1: Kinetic Study of the Inhibition of Thrombin and FXa by **3**-Xaa-Z (k_i, M^{-1} s^{-1})[a]

compound	Tris buffer, pH 6.6			Tris buffer, pH 7.4			ESI MS found (MH_2^{2+}, MH^+) calcd (M)
	thrombin	FXa	T/F	thrombin	FXa	T/F	
3-Gly-NH_2	3.66	1.08	3.4	6.88	1.31	5.3	242.2, 483.3 482.2
3-Ala-NH_2	6.35	1.03	6.2	13.1	1.31	10.0	249.4, 497.3 496.2
3-Ala-OCH_3	4.61	1.01	4.6	8.66	0.89	9.7	256.8, 512.2 511.2
3-D-Ala-OCH_3	S-shaped[c]	<0.5		S-shaped	<0.5		256.9, 512.5 511.2
3-Val-NH_2	12.7	2.32	5.5	20.1	3.75	5.4	263.2, 525.3 524.3
3-D-Val-NH_2	3.06	1.05	2.9	3.34	0.80	4.2	263.3, 525.2 524.3
3-Ile-NH_2	26.4	8.47	3.1	35.5	13.2	2.7	270.3, 539.4 538.3
3-Cha-NH_2	9.43	1.21	7.8	14.7	2.39	6.1	290.3, 579.3 578.3
3-Asn-NH_2	1.85	0.76	2.4	5.04	1.43	3.5	270.8, 540.3 539.2
3-Gln-NH_2	4.85	0.65	7.4	9.13	0.99	9.2	277.7, 554.2 553.3
3-Phe-NH_2	17.4	7.62	2.3	22.1	3.56	6.2	287.3, 573.3 572.3
3-Phe(*p*-NO_2)-NH_2	18.2	6.33	2.9	28.7	4.10	7.0	309.8, 618.3 617.3
3-Phe(*p*-F)-NH_2	7.67	2.46	3.1	22.4	4.24	5.3	96.3, 591.5 590.3
3-Phg-NH_2	11.0	2.33	4.7	24.2	4.30	5.6	80.2, 559.1 558.3
3-Tyr-NH_2	4.66	1.79	2.6	10.0	3.80	2.6	95.3, 589.3 588.3
3-D-Phe-OH				nd[b]	0.17		574.28[e] 573.26
3-Trp-NH_2	2.96	1.11	2.7	2.16	1.21	1.8	306.8, 612.4 611.3
3-Pro-NH_2	3.08	1.12	2.7	5.38	1.76	3.1	62.2, 523.3 522.3
3-Arg-NH_2	4.69	1.25	3.8	11.3	2.36	4.8	91.7, 582.2 581.3
3-Lys-NH_2	10.3	0.99	10.5	22.6	2.31	9.8	77.8, 554.3 553.3
3-Orn-NH_2	11.5	1.17	9.8	23.9	2.48	9.6	70.8, 540.4 539.3
3-Asp-NH_2	1.45	1.45	1.0	1.71	1.55	1.1	71.2, 541.1 540.2
3-Glu-NH_2	1.24	3.14	0.4	1.89	1.04	1.8	78.2, 555.3 554.2
3-OCH_3	S-shaped	S-shaped		S-shaped	S-shaped		441.2122[d] 440.2060
3-NH_2	S-shaped	S-shaped		S-shaped	S-shaped		426.2133 425.206
1b	1.47	0.51	2.9	4.38	1.36	3.2	184.7, 368.3 367.2
1a	67	4	17	12	0.7	17	see ref *8*
1d	0.5	7.0	0.07	4.0	8.5	0.47	see ref *17*

[a] The inhibition was carried out in Tris buffer at room temperature under subdued light. Error is estimated as ±20%. The initial active enzyme concentration was 1.0 μM, except for **1b**, **3**-Ala-OCH_3, and **3**-D-Phe-OH (between 1.4 and 1.6 μM). Typically 20 equiv of inhibitors was used for thrombin inhibition, and 100 equiv for FXa inhibition, except for **1b**, **3**-Ala-OCH_3, and **3**-D-Phe-OH (40 equiv for both enzymes), **3**-OCH_3 and **3**-D-Ala-OCH_3 (10 equiv for both enzymes), and **3**-NH_2 (5 equiv for both enzymes). [b] nd = no detectable inhibitory effect. [c] The enzyme activity first decreased and then recovered and then decreased again, like a transposed "S". [d] FAB-HRMS (matrix 3-nba) MH^+ peak found. [e] FAB-MS (matrix 3-nba) MH^+ peak found.

an UV lamp (366 nm), indicating coumarin formation. It was found that samples of **3**-Ala-NH_2 without chromatographic purification gave consistent assay results (k_i). Three samples of **3**-Ala-NH_2 (one purified, two from different batches without purification) assayed under the same condition gave close k_i values (1.2:1.0:1.0). Since the free amino acid or the dipeptides are not inhibitors of the proteases, the yield and purity are satisfactory for a library study. Using the same solid-phase protocol (*21*), the compound **3**-Phg-Tyr-OH was prepared from Rink Acid Resin, **3**-Phg-Tyr-OCH_3 was obtained from **3**-Phg-Tyr-OH using CH_3I/Cs_2CO_3 in THF, and **3**-Phg-Tyr-NHEt was synthesized from the Sieber Resin. The inhibitors **3**-OCH_3 and **3**-NH_2 were also prepared for comparison purposes. All **3**-Xaa-Z compounds were purified by RP-HPLC (except for **3**-Ala-OCH_3 by normal-phase chromatography), quantified by UV/Vis spectroscopy, and

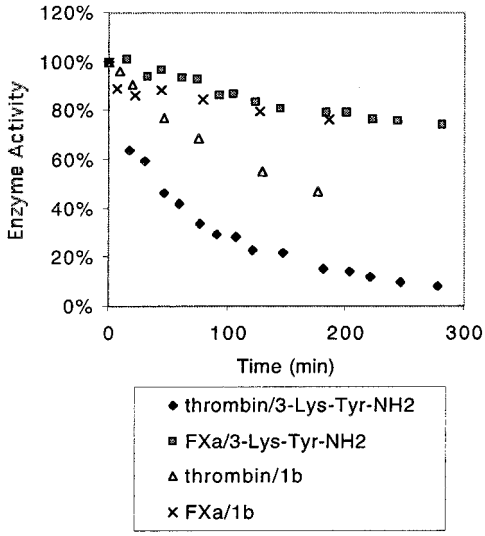


FIGURE 1: Inhibition time course of **3**-Lys-Tyr-NH_2 and **1b** on thrombin and FXa, respectively. All of the inhibitions were performed in Tris buffer (pH 6.6, 50 mM Tris, with 150 mM NaCl). The remaining enzyme activity as a percentage of the initial activity was plotted against time.

assayed against thrombin and FXa separately in a parallel manner.

Assay of the 3-X Library. Among all the **3**-Xaa-Z inhibitors examined, the values (Table 1) for thrombin inhibition vary from very small (D-Phe-OH, **3**-Asp-NH_2, **3**-Glu-NH_2) to relatively large (**3**-Ile-NH_2). In contrast, the various P_2 substituents have less influence on the inhibitory activity for FXa. Some of the most potent **3**-Xaa-Z thrombin inhibitors have bulky aliphatic residues, gaining a factor up to 16 in rate enhancement as compared to the reference inhibitor **1b**. However, no significant increase in T/F selectivity was observed for these inhibitors. In contrast, the basic residues Lys and Orn showed only a moderate increase of k_i toward thrombin, but increased the T/F selectivity factor up to 10.5, while the reference inhibitor **1b** has a T/F preferential factor of about 3. Briefly, those inhibitors with Xaa = Ala, Cha, Gln, Lys, and Orn are clearly thrombin-selective, while Xaa = Asp is essentially nonselective at pH 7.4 and 6.6 and Xaa = Glu is clearly FXa-selective at pH 6.6 and modestly thrombin-selective at pH 7.4. Inhibition rates are typically 2−3 times faster at pH 7.4 than at pH 6.6.

The 112 dipeptides (**3**-Xaa_1-Xaa_2-NH_2) were assayed against thrombin and FXa in a parallel manner, and a selection of the results on thrombin are presented in Figure 2 (see also Supporting Information Table 1). For this assay, the crude dipeptides were not purified after cleavage from the beads, and a 100% yield in the syntheses was assumed. The inhibition rate was clearly sequence-dependent. Statistically, the variations at P_3 have less effect on inhibitory activity than variations at P_2, based on the standard deviation of k_i among the different groups. It was observed that inhibitors with Xaa_1 = Ala, Arg, and Lys constitute most of the potent inhibitors toward thrombin. In contrast, many members with Xaa_1 = Gly, Asp, and Glu were relatively poor inhibitors. Also, many higher k_i values are associated with inhibitors with Xaa_2 = Ile, Val, Phg, and Lys. Inhibition curves are presented in Figure 1 for **3**-Lys-Tyr-NH_2, which prefers thrombin compared to the parent inhibitor **1b**. This inhibitor was purified and assayed individually. While

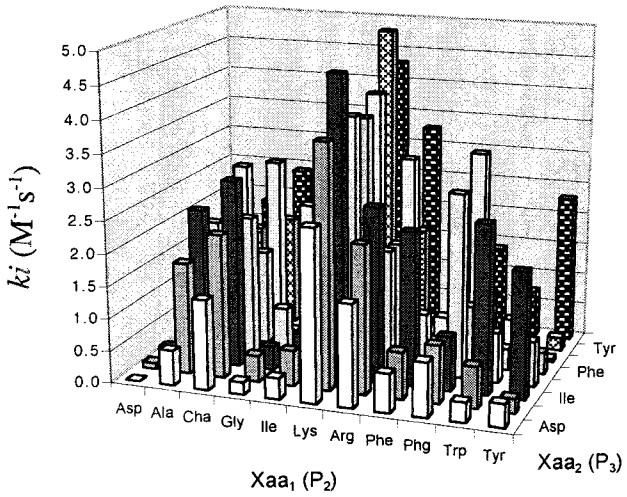


FIGURE 2: Diversity of inhibition rate by a library of inhibitors (**3**-Xaa_1-Xaa_2-NH_2) to thrombin at pH 6.6 (for data, see Supporting Information).

repurified **3**-Lys-Tyr-NH_2 displayed over twice the inhibitory activity of the crude reaction mixture used in the study of the library, the T/F selectivity determined for the purified compound was within 10% of the value determined from the crude reaction mixture.

All inhibitors except those with Xaa_1 = Pro behaved in a normal way; i.e., they reduced enzyme activity over time, and photolysis regenerated full enzyme activity. However, seven of eight inhibitors with Xaa_1 = Pro (Xaa_2 = Asp, Gly, Ile, Lys, Phe, Tyr, Val) showed a different inhibition pattern on thrombin. After a rapid reduction of active concentration, thrombin regained activity gradually in the absence of light. The spontaneous recovery is definitely much faster than the hydrolysis (turnover) of the acyl-enzyme, whose half-life is in the hundred hours range at pH 7.4 (*7*, *26*). This suggests that the expected acylation of the thrombin active site did not occur with the inhibitors having Xaa_1 = Pro, but rather that another mechanism was operable.

During the inhibition process, the average value of the remaining enzyme activity of the entire library and the standard deviation of the activity array (divided by the average k_i values) should reflect the potency and diversity of the whole ensemble of inhibitors, which indicates the effectiveness of library design. As shown in Figure 3, it is apparent that the remaining enzyme activity of the entire library decreased over time and could be fully recovered upon irradiation. On the other hand, the relative standard deviation, which reflects the diversity of inhibition rate constants among all of the inhibitors, increased over time but reverted to the background level after photorelease of the thrombin.

Since one goal of this study was to selectively acylate one serine protease over another, a series of competitive inhibition assays were carried out using a 1:1 molar ratio of thrombin and FXa for model study. Some of the more potent thrombin inhibitors were selected. The remaining enzyme activities after the addition of the inhibitor (40 equiv nominally, assuming quantitative yield from the SPPS) were assessed using the chromogenic substrates S2238 for thrombin and S2222 for FXa. The two substrates are quite specific to the two enzymes, with a minor cross-reactivity of 3.0% and 1.4%, respectively. The results of the competitive study are shown in Table 2.

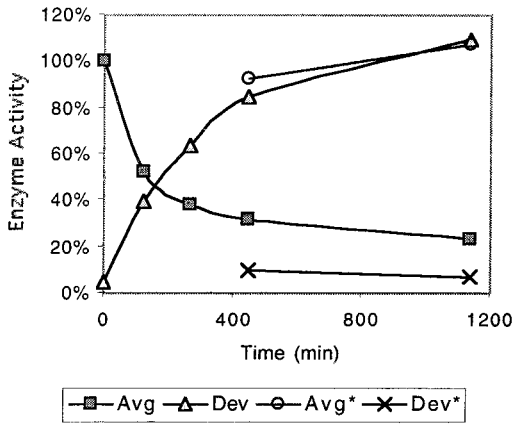


FIGURE 3: Overall activity index for inhibition and photoactivation of thrombin. The initial active enzyme concentration is 1.0 μM for every incubation well, in Tris buffer, pH 6.6, at room temperature. "Avg" = average thrombin activity among 96 wells. "Dev" = standard deviation of activities relative to average enzyme activity at a certain time point. "Avg*" = "Avg" after 5 min irradiation. "Dev*" = "Dev" after 5 min irradiation.

Table 2: Comparative Study of the Inhibition Rate Constants of **3**-Xaa$_1$-Xaa$_2$-NH$_2$ Compounds (Selected Members from the Library; k_i, M^{-1} s^{-1})[a]

compound[b]	Tris buffer, pH 6.6 thrombin	FXa	T/F	ESI MS (MH_2^{2+}, MH^+)	calcd MW
3-Ala-Ile-NH$_2$	3.00	0.65	4.6	305.8, 610.3	609.3
3-Ala-Phg-NH$_2$	3.18	0.41	7.7	315.8, 630.5	629.3
3-Cha-Phg-NH$_2$	2.87	0.55	5.2	356.9, 712.6	711.4
3-Lys-Gly-NH$_2$	3.30	1.28	2.6	306.3, 611.4	610.3
3-Lys-Ile-NH$_2$	3.76	1.17	3.2	334.4, 667.5	666.4
3-Lys-Lys-NH$_2$	3.40	0.31	10.9	341.9, 682.4	681.4
3-Lys-Phe-NH$_2$	3.09	0.54	5.7	351.4, 701.6	700.4
3-Lys-Phg-NH$_2$	3.59	0.69	5.2	344.4, 687.5	686.4
3-Lys-Tyr-NH$_2$	3.83	0.47	8.1	359.5, 717.5	716.4
3-Lys-Tyr-NH$_2$[c]	8.77	1.05	8.4	359.3, 717.4	716.4
3-Lys-Val-NH$_2$	3.61	0.66	5.5	327.4, 653.4	652.4
3-Arg-Phg-NH$_2$	3.47	0.71	4.9	358.4, 715.4	714.4
3-Arg-Val-NH$_2$	3.25	0.52	6.2	341.4, 681.4	680.4
3-Phg-Lys-NH$_2$	2.84	0.70	4.1	344.4, 687.4	686.4
3-Phg-Phg-NH$_2$	3.47	0.77	4.5	346.9, 692.4	691.3
3-Pro-Phg-NH$_2$	2.76	0.51	5.4	328.9, 656.4	655.3
3-Trp-Ile-NH$_2$	2.81	0.64	4.4	363.5, 725.5	724.4
3-Ile-Phg-NH$_2$	2.05	0.46	4.5	336.9, 672.4	671.3
3-Phe-Ile-NH$_2$	2.67	0.37	7.2	343.9, 686.4	685.4
3-Tyr-Val-NH$_2$	2.52	0.54	4.7	344.9, 688.4	687.3

[a] The inhibitors were assayed in a parallel manner with a microplate reader. Each inhibitor was added to a solution of 1:1 thrombin/FXa (0.8 μM each), and the residual enzymatic activity was monitored with chromogenic assays, using S2238 for thrombin and S2222 for FXa. The cross-talk between the enzymes and the substrates was corrected. Error is estimated as ±20%. [b] Taken from the library synthesis without purification. [c] Synthesized separately, purified with RP-HPLC, quantified with UV/Vis (absorbance maximum around 360 nm in Tris buffer, pH 7.4, with 5% EtOH), and assayed individually.

From the screening of the **3**-Xaa$_1$-Xaa$_2$-NH$_2$ library, it was found that the most selective thrombin inhibitors provide selectivity up to a factor of 10, which is comparable to that obtained from the **3**-Xaa-Z series. The inhibitor **3**-Lys-Tyr-NH$_2$ was resynthesized, purified, and assayed individually, and the result showed that the T/F ratio is quite reproducible (Table 2). Although the inhibitors **3**-Asp-Xaa$_2$-NH$_2$ and **3**-Glu-Xaa$_2$-NH$_2$ were found to be relatively FXa-selective, we did not pursue these compounds because of their low reactivity to both enzymes. Instead, we chose the inhibitor **3**-Phg-Tyr-NH$_2$, with a T/F ratio close to unity, for further modifications.

Thus, a series of analogues with different end groups (**3**-Phg-Tyr-Z) was prepared to see how the end group affects selectivity (Table 3). For minor differences in structures, some variations in activity among this group are quite noteworthy. The **3**-Phg-Tyr-NHEt has a T/F ratio of 3.5 at pH 7.4, while **3**-Phg-Tyr-OMe has a selectivity factor of 2 in favor of Fxa at the same pH. These results not only indicate the importance of the end group, but also suggest the potential of extending the peptide chain further for more differentiation among the enzymes.

Separation of Thrombin and FXa. The biotinylated inhibitor **1d** offers the possibility of separating proteases based upon selective inhibition. An acyl-protease bearing the biotin side chain of the cinnamate **1d** would bind to an avidin or streptavidin column while acyl-enzymes not linked to biotin would not be retained, permitting separation of the acyl-enzymes. Photolysis of either of the modified enzymes (biotinylated or not) should recover the separated active enzymes.

Before using **1d** for separating proteases, a competitive inhibition assay was performed to examine the acylation process of **1d** in a mixture of thrombin and FXa. As shown in Figure 4, because of the difference in inhibition rate, after 20 min, about 10% of thrombin and about 40% of FXa were inhibited. Thus, if at this point the avidin column separation is performed, about one-fourth of the original enzymes will be retained on the column. The retained mixture of proteases can be recovered after photolysis of the column. The protease recovered in this way contains a 4:1 mixture of FXa/thrombin. The other three-fourths of the enzymes will contain a 3:2 ratio of thrombin/FXa. If the avidin column separation is performed after 4 h of incubation, then half of the thrombin can be recovered in high purity, with the rest of the FXa and thrombin collected in a 2:1 ratio. Figure 4 also confirmed that the biotinylated acyl-enzyme could be quantitatively photoactivated. Thus, irradiating the avidin column released all of the column-bound enzymes.

Furthermore, by combining one thrombin-selective inhibitor with another FXa-selective inhibitor, one of which has a biotin tag, higher separation efficiency of the two enzymes can be achieved. As shown in Figure 5, the enzyme mixture was first incubated with a thrombin-selective inhibitor, **3**-Lys-Tyr-NH$_2$. A large portion of thrombin was inhibited while only a small fraction of FXa was acylated. After removing the remaining excess inhibitor, **1d**, a FXa-selective inhibitor, as is evident from Figure 4, was used to acylate the remaining active enzymes, most of which was FXa. An avidin column retained the acyl-enzymes, and the **3**-Lys-Tyr-NH$_2$–inhibited portion plus the residual uninhibited enzyme were eluted as the first fraction. By this protocol, we have obtained results such that, by using either one biotinylated inhibitor (**1d**) or a combination of two selective inhibitors (**1d** with one of **1a** or **3**-Lys-Tyr-NH$_2$), one of the two enzymes could be isolated in reasonable yield with high purity (Figure 6, Table 4).

DISCUSSION

Alcohol Esters of the Substituted Cinnamate. The incubation of a series of alcohol-derived esters (rather than phenol-derived esters) of 4-*N*,*N*-diethylamino-2-hydroxy-α-methyl-

Table 3: End Group Effect on the Inhibition Rate Constants of **3**-Phg-Xaa$_2$-Z (k_i, M^{-1} s^{-1})

inhibitor[a]	Tris buffer, pH 6.6 thrombin	Tris buffer, pH 6.6 FXa	Tris buffer, pH 6.6 T/F	Tris buffer, pH 7.4 thrombin	Tris buffer, pH 7.4 FXa	Tris buffer, pH 7.4 T/F	ESI MS found (MH_2^{2+}, MH^+) calcd (M)
3-Phg-Tyr-OCH_3	2.48	5.01	0.50	6.28	12.3	0.51	369.3, 737.5 736.3
3-Phg-Tyr-OH	6.01	4.65	1.3	17.8	10.4	1.7	362.3, 723.4 722.3
3-Phg-Tyr-NH_2	2.50	2.10	1.2	6.76	10.6	0.64	361.8, 722.4 721.3
3-Phg-Tyr-NHEt	4.82	2.45	2.0	15.2	4.36	3.5	375.9, 750.3 749.4

[a] For each inhibition, a 1.0 μM solution of thrombin or FXa was incubated with 8.3−15.8 equiv of inhibitors (purified by RP-HPLC) in the indicated buffer at room temperature under subdued light. Error is estimated as ±20%.

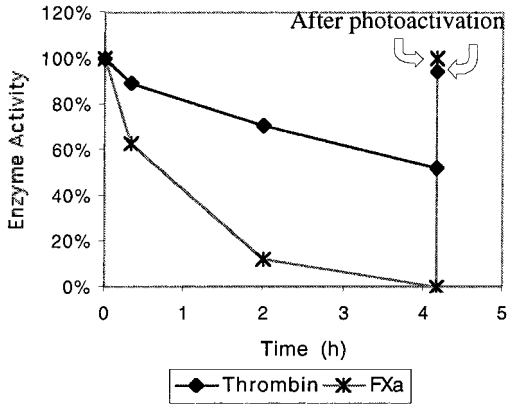


FIGURE 4: Competitive inhibition of **1d** on thrombin and FXa in Tris buffer, pH 6.6, at room temperature. Original activities of thrombin and FXa were 0.8 μM each, and the original concentration of **1d** was 6.4×10^{-5} M.

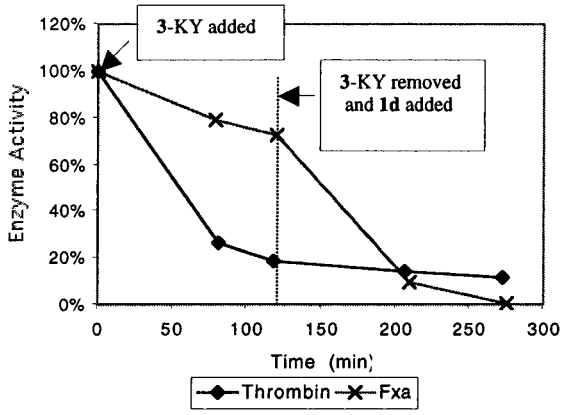


FIGURE 5: Schematic demonstration of the separation of thrombin and FXa by sequential inhibition with **3**-Lys-Tyr-NH_2 (**3**-KY) and **1d**.

cinnamate (**6**−**12**) with thrombin, FXa, or α-chymotrypsin gave no evidence for the formation of acyl-enzyme complex. The literature concerning the efficacy of inverse inhibitors with aliphatic alcohols as the leaving group is, indeed, conflicting. Jones et al. concluded that esters derived from phenylalaninol and tryptophan alcohol were not hydrolyzed by α-chymotrypsin (*27*). Similarly, aminobutyl acetate, which was designed as an irreversible inhibitor of trypsin, did not show the expected effect (*28, 29*). On the other hand, recent data published by Walker suggested that *p*-methoxybenzoate esters of some peptidols [Boc-Ile-Glu-Gly-Arg-Ψ-(CH_2O)-, Boc-(D)-Phe-Pro-Arg-Ψ-(CH_2O)-, Ac-Val-Pro-Phe-Ψ-(CH_2O)-, and Ac-Val-Pro-Val-Ψ-(CH_2O)-] were irreversible inhibitors for FXa, trypsin, chymotrypsin, and elastase, respectively, but not for thrombin, for which a reversible mode of inhibition was proposed (*23, 24*). The k_2/K_1 values for these inhibitors are quite high (4.5×10^5 M^{-1} min^{-1}, 2.5×10^5 M^{-1} min^{-1}, 6.6×10^6 M^{-1} s^{-1}, and 1.3×10^7 M^{-1} s^{-1} for the four enzymes above). It should be noted that Walker used a continuous assay method; i.e., the inhibitor, enzyme, and fluorogenic substrate were all mixed at time zero and monitored by fluorescence change continuously, in a time frame much shorter than in our study (*23, 24*).

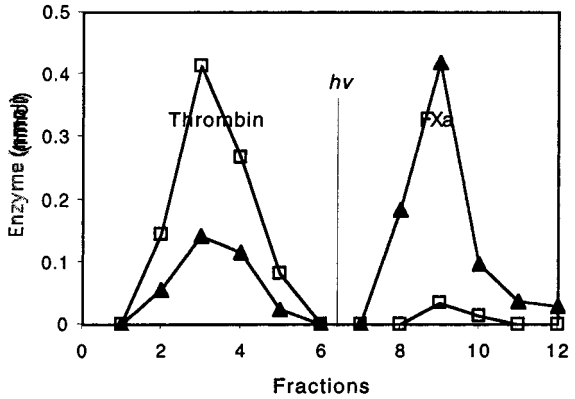


FIGURE 6: Separation of FXa from thrombin with **3**-Lys-Tyr-NH_2 and **1d**. Conditions: Tris buffer (50 mM, 150 mM NaCl, pH 6.6), room temperature. SEC using PD-10 Sephadex column (Supelco); final separation using avidin column (Pierce); photoactivation using a 4 W hand-held lamp (366 nm).

It is uncertain whether the acyl-enzyme in Walker's study had indeed formed, or that a reversible inhibition was operable. In their study, attempts were made to isolate the anisoyl-α-chymotrypsin complex via size-exclusion chromatography (SEC) to remove excess inhibitor. A slow increase of enzyme activity was observed over time, seemingly consistent with a turnover process of an acyl-enzyme complex. It should be noted, however, that a large excess of inhibitor (20 000 equiv) was used in this experiment. It is thus possible that the excessive inhibitor was only partially removed, and a slow dissociation of the reversible inhibitor from α-chymotrypsin occurred to generate the observed phenomenon.

To shed more light on the conflicting results, we synthesized two of Walker's anisoyl esters, Ac-Ile-Glu-Gly-Arg-Ψ-(CH_2O)-CO-C_6H_4-OCH_3 and Ac-Val-Pro-Phe-Ψ-(CH_2O)-CO-C_6H_4-OCH_3, and assayed them against FXa and α-chymotrypsin, respectively (*23, 24*). We did not observe acyl-enzyme formation (100 equiv of inhibitor, pH 7.4) under

Table 4: Isolation and Recovery (Of Initial Amount) of Thrombin and FXa

	fraction 1 (before photolysis)			fraction 2 (after photolysis)			total recovery	
inhibitor(s)	thrombin[b] (%)	FXa[b] (%)	T/F	thrombin[b] (%)	FXa[b] (%)	F/T	thrombin (%)	FXa (%)
3-Lys-Tyr-NH_2/**1d**	55	20	2.8	2.4	45	19	58	64[a]
1a/**1d**	71	31	2.3	3.4	56	17	75	87
1d	53	3.7	14.4	22	83	3.9	75	87
1d	65	6.4	10	21	76	3.7	85	83

[a] For each enzyme, 11% of the original activity was lost due to extensive assays during the isolation procedures. [b] The effect of cross-reactivity of the enzymes was corrected.

conditions where the inhibitors **1a** and **1c** acylated FXa (k_i = 72 M^{-1} s^{-1}) and α-chymotrypsin (k_i = 45 M^{-1} s^{-1}). We strongly suspect that they are weak reversible inhibitors of FXa and chymotrypsin.

Salicylate-Based Inverse Inhibitors. The phenol/salicylic-type cinnamate esters represented by **1** and **3** do serve as irreversible inhibitors of FXa and thrombin. Good leaving groups such as phenolates compensate for the inherent disadvantage of inverse inhibitors "chemically". Indeed, the *p*-amidino moiety yields inhibitors that are roughly 50 or 270 times more potent than the guanidino counterparts, on FXa and thrombin, respectively (**1a** vs **1b**). The positive charge is important as a mimic of the naturally preferred arginine residue in the S_1 pocket of thrombin and FXa. In contrast, the corresponding *p*-nitrophenyl ester is completely inactive toward acylation of FXa and thrombin, even though the Hammett σ values of the nitro and amidino groups are very similar. Hence, the observed activity of **1a** and **1b** is not simply based on the chemical reactivity. The difference in activity of **1a** and **1b** is likely due to the difference in the distance of the positive charge to the carbonyl (*19*), and the higher chemical lability of the *p*-amidinophenol group. However, the *p*-amidinosalicylate was found to be incompatible with our parallel solid-phase protocol.

Although it is natural to assume that the inhibitors based on the chemotype **3** interact with similar regions of the enzyme as the natural substrates, directly grafting known sequences from natural substrates to the inverse inhibitor core did not lead to selective inhibitors. For example, **3**-Gly-Glu-Ile-NHEt was designed as being FXa-selective based on the structure of S2222 [chromogenic substrate for FXa, Bn-Ile-Glu(γ-OR)-Gly-Arg-*p*NA HCl, R = 50% H + 50% CH_3], whose turnover rate is 70−100 times faster with FXa than with thrombin. But **3**-Gly-Glu-Ile-NHEt actually favored thrombin over FXa, with k_i values of 5.02 and 0.93 M^{-1} s^{-1}, respectively (pH 6.6). A simple translation of the S2222 structure to inhibitors of type **3** is clearly inappropriate.

The introduction of a single amino acid as the P_2 substituent (**3**-Xaa-Z) produced distinct profiles for different enzymes, and a significant degree of diversity and selectivity among the two proteases was found, considering the relatively small size of the library. The presence of the key Asp189 in the S_1 binding pockets of both proteases determines the requirement for a positively charged substituent in the phenolic leaving group (Arg or analogues), preferably in the *para* position. There is a key difference of the presence of a Glu192 near the S_1 binding pocket in thrombin vs a Gln192 in FXa. The Glu192 is known to repel negatively charged residues on P_3 or P_3' of substrates (*30*). In fact, this difference has been advantageously utilized in the design of FXa-selective inhibitors where the carboxylate plays the decisive role in the FXa selectivity (*31*). The FXa-selective chromogenic substrate S2222 notably has an acidic residue at the P_3 position.

An examination of our results reveals that the inhibitors with acidic side chains (**3**-Asp-NH_2, **3**-Glu-NH_2, **3**-D-Phe-OH) were the least active inverse inhibitors to thrombin, which is in accord with the considerations mentioned above. On the other hand, the activity of these inhibitors did not increase significantly with FXa, leading to a modest overall increase of the F/T ratio as compared to the reference inhibitor **1b**. The observed high activity of bulky aliphatic residues at P_2 is in agreement with the extremely high propensity of Bz-Phe-Val-Arg-OCH_3 (*32*) to undergo thrombin-catalyzed hydrolysis. Yet among all inhibitors we investigated, those with basic residues at P_2 (**3**-Lys/Orn-NH_2) were the most selective for thrombin. This observation is also reflective of the interaction with the thrombin Glu192.

The screening of the **3**-Xaa_1-Xaa_2-NH_2 series of inhibitors did not lead to higher selectivity than was observed for the **3**-Xaa-NH_2 library. In fact, many inhibitors in the library were rather poor, and notable exceptions were again compounds with basic residues at P_2. Some of the more potent thrombin inhibitors were assayed in a competition experiment against 1:1 thrombin and FXa, and reasonably good T/F ratios were obtained. Overall, the P_3 (Xaa_2) position does not seem to be as critical as the P_2 (Xaa_2) position to differentiate the **3**-series inhibitors against thrombin and FXa.

The inhibitor structure changes reported here focus on the leaving group side of the inhibitor. The general trends observed make reasonable sense based upon known enzyme residues near the enzyme active site. The design principles that emerge from the study are, however, limited. An examination of substituent effects on the acyl side of the inhibitor is in progress and will be reported in due course. We note that introduction of the biotin side chain in **1d** has a profound effect on the T/F selectivity ratio (see Table 1). Thus, at pH 6.6, T/F for **1b** is 2.0 while that for **1d** is 0.07. Substitution on the acyl side of the inhibitor may well lead to compounds with a large range of enzyme selectivities.

Isolation of Thrombin or FXa from a 1:1 Mixture. The information obtained from the library screening provides the possibility to preferentially acylate one serine protease in the presence of others. If the acyl-enzyme has a "handle" (such as biotin) to be recognized by certain medium (such as immobilized avidin), the acyl-enzyme will stay with the medium and become separable from other enzymes. This idea in general is applicable to other families of enzymes or macromolecules (*33*).

By using the FXa-selective biotinylated inhibitor **1d** alone or in combination with a thrombin-selective inhibitor (**1a** or **3**-Lys-Tyr-NH_2), one enzyme could be isolated from a 1:1

mixture of two in good purity. The results demonstrate the feasibility of selectively "fishing out" one target enzyme from a multiple-component system. The most critical factor for the success of isolation is the substrate−enzyme specificity, indicated by the T/F ratio in this study. In addition, the isolation can be improved by the strategy using more than one acylating agent of orthogonal selectivity (one bearing a biotin tag, the others not). Furthermore, buffer pH and incubation time are also factors to determine the separation results, which can be optimized.

The total recovery of the two enzymes was generally less than quantitative because there was some loss of activity when the sample was passed through Sephadex and avidin columns (for entry 1 in Table 4, a significant amount of enzyme was consumed for assays). This disadvantage may be overcome by using avidin-bound agarose beads instead of the avidin column (*11*). The selectively biotinylated enzyme binds tightly to the solid phase and separates from the other enzymes. Compared to traditional column-based separation methods (affinity, ion exchange, or SEC), this photorelease approach has several advantages. The binding and detaching process is very simple, and the target enzyme can be released with light at any time.

SUPPORTING INFORMATION AVAILABLE

The synthesis and characterization of compounds **4**, **6**−**12**, **3**-Phg-Tyr-Z (Z = OH, OCH_3, NH_2, NHEt), **3**-Lys-Tyr-NH_2, **3**-Ala-OMe, **3**-Ala-NH_2, **3**-D-Phe-OH, and the library preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

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