2-Aroylbenzoyl Serine Proteases: Photoreversible Inhibition or Photoaffinity Labeling?

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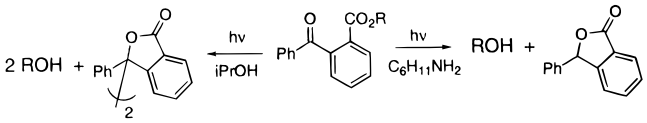
Abstract: Phenyl esters of 2-benzoylbenzoates were determined to be inhibitors of the serine protease enzymes chymotrypsin and thrombin. Thus, *p*-guanidinophenyl 2-benzoylbenzoate (**1b**) inhibited thrombin while the corresponding *p*-nitrophenyl ester (**1a**) inhibited chymotrypsin activity. Other *p*-nitrophenyl esters were prepared that display activity as chymotrypsin inhibitors, three having methoxy group substitution on the benzoyl ring: 2-methoxy (**2a**), 2,5-dimethoxy (**3a**), and 2,4,5-trimethoxybenzoyl (**4**). After incubation with **1b**, an acyl thrombin was isolated that showed no lytic activity but which slowly regained activity in pH 7.4 buffer. Irradiation of this acyl enzyme with 366 nm light led to an enzyme that showed no gain of lytic activity over time. Incubation of chymotrypsin with **1a**−**3a** and **4** led to acyl enzymes which showed no activity but which regained activity slowly. Irradiation of these inactive acyl enzymes with 366 nm light led to a rapid increase in enzyme activity. The formation of acylchymotrypsins that can be photochemically deacylated is suggested by these data. Experiments that relate to the mechanism of enzyme acylation and the subsequent photochemistry of the acylenzymes are reported.

In the last 30 years, knowledge of organic photochemistry has been combined with a rapidly increasing knowledge of biochemistry to mimic natural photobiological switches. Metal binding,[1,2] protein/ligand interactions,[3] and enzyme activity[4,5] have all been controlled photochemically. We have previously reported a photoreversible serine protease inhibitor based on the cinnamate chromophore.[6−8] This compound inhibits a variety of serine proteases, such as thrombin, Factor X_a, and chymotrypsin, by acylating the active site serine hydroxyl. A *Z*−*E* photoisomerization of the acyl cinnamate results in deacylation of the active site serine and formation of the native enzyme. This photochemistry has been exploited in an immunoassay strategy,[9] used clinically in the annealment of skin grafts,[10] and used in the in vivo photoactivation of thrombin.[11] This approach to control enzyme activity with light involves a photoremovable alcohol protecting group strategy with the enzyme acting as the alcohol.

Scheme 1



More recently, we reported a photolabile alcohol protecting group[12] which used the photoreduction of 2-benzoyl benzoates to create a nucleophile in proper position to lactonize and free the alcohol (Scheme 1). These benzophenones underwent 1-electron reduction when irradiated in the presence of hydrogen donors (e.g., 2-propanol) and 2-electron reduction when irradiated in the presence of electron donors (e.g., cyclohexylamine). Given the recent interest in enzyme photoactivation, we sought to learn whether this benzophenone strategy would be applicable to photochemical enzyme control. We report here the synthesis of *p*-nitrophenyl and *p*-guanidinophenyl esters of 2-aroyl benzoate esters and their study as chymotrypsin, thrombin, and Factor X_a photoreversible inhibitors.

Results and Discussion

Synthesis. A series of *p*-nitrophenyl esters, **1a**−**3a** and **4**−**7**, were prepared by dicyclohexylcarbodiimide (DCC) coupling of *p*-nitrophenol and the corresponding 2-aroylbenzoic acid. These esters, shown in Figure 1, were prepared as potential chymotrypsin inhibitors. The analogous *p*-guanidinophenyl esters,

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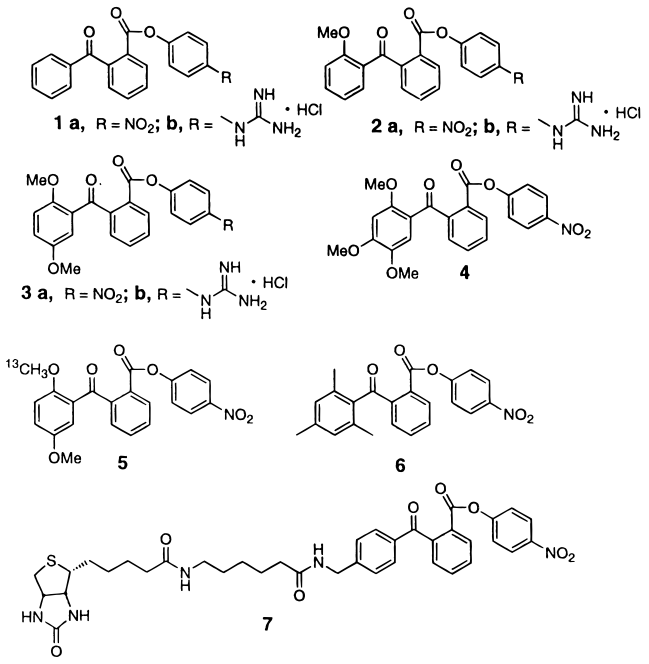
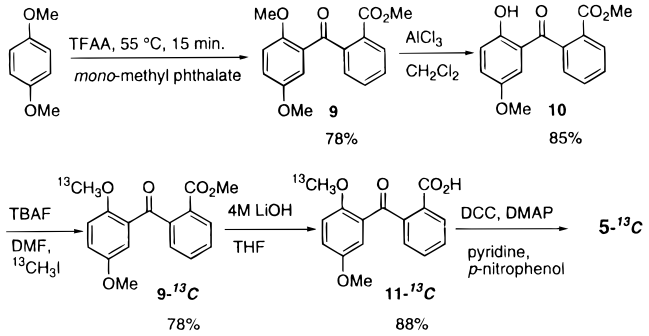


Figure 1. *p*-Nitrophenyl 2-aroylbenzoates used for chymotrypsin inhibition experiments and *p*-guanidinophenyl 2-aroylbenzoates used for thrombin and Factor X_a inhibition experiments.

Scheme 2

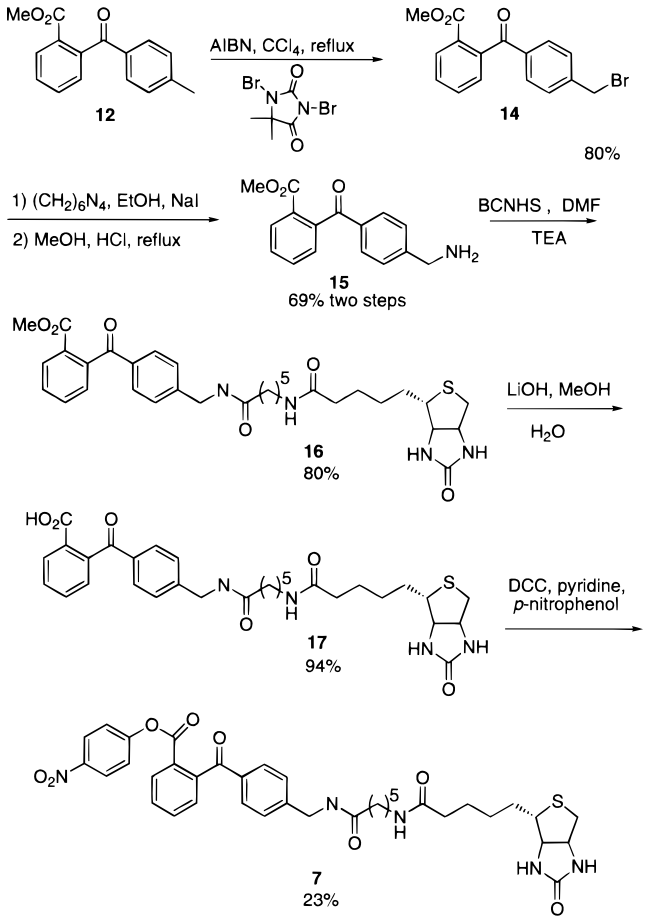


1b−**3b**, were prepared as potential thrombin or Factor X_a inhibitors. These compounds were prepared by DCC coupling of the carboxylic acid with *p*-*N*,*N*′-bis-BOC-guanidinophenol (**8**). Subsequent removal of the BOC groups gave the indicated esters.

Scheme 2 shows the synthesis of a ^{13}C-labeled *p*-nitrophenyl ester. Methyl 2-(2,5-dimethoxybenzoyl)benzoate (**9**) was prepared by Friedel−Crafts acylation of dimethoxybenzene.[12] The *o*-methoxy group was selectively cleaved by aluminum chloride to give phenol **10**. The phenol was alkylated by $^{13}CH_3I$ to give **9**-*13C*. Hydrolysis of the ester gave the carboxylic acid (**11**-*13C*), which was coupled to *p*-nitrophenol using DCC to obtain **5** (2*,5-$(OMe)_2$).[13]

The synthesis of a biotinylated benzophenone inhibitor is shown in Scheme 3. Methyl *p*-toluoylbenzoate[14] (**12**) was prepared by esterification of *p*-toluoylbenzoic acid (**13**). The ester was brominated with 3,3-dimethyl-*N*,*N*′-dibromohydantoin and AIBN in CCl_4 to give **14**. The bromide was displaced by hexamethylenetetramine and the salt hydrolyzed by refluxing

Scheme 3



methanolic HCl to give amine **15**. This amine was acylated by BCNHS **(**niotin caproyl *N*-hydroxysuccinimide**)** to give **16**. Hydrolysis of the methyl ester with methanolic LiOH gave carboxylic acid **17**. DCC coupling with *p*-nitrophenol gave **7** (biotin tether) in 23% yield.

Thrombin and Factor X_a Inhibition. None of the *p*-guanidinophenyl esters shown in Figure 1 (**1b**−**3b**) were found to be Factor X_a inhibitors. Even when 100 equiv of compound was added to buffered solutions of Factor X_a, no change in Factor X_a activity, as measured by chromogenic assay,[15] was observed. Likewise, only one of these compounds was found to inhibit thrombin. Enzyme activity vs time plots for the inhibition of thrombin by 20 equiv of compounds **1b**−**3b** are shown in Figure 2a. Only compound **1b** (Phenyl) displays any tendency toward thrombin inhibition.

Photolysis of a solution of thrombin inhibited by 20 equiv of **1b** (phenyl) did not result in any photoactivation. In fact, photolysis of the inhibited enzyme resulted in a loss of activity. Thrombin inhibited by **1b** (phenyl) and kept in the dark slowly regains lytic activity as the enzyme hydrolyzes the ester. On the other hand, if this solution is photolyzed for 60 min early in the inhibition, no increase in enzyme activity is observed during photolysis. Nor does this photolyzed solution *ever* regain enzyme activity (Figure 2b). This suggests that irradiation of the inhibited thrombin leads only to photoaffinity labeling of the active site. The labeled thrombin is unable to hydrolyze in the normal serine protease fashion.

Chymotrypsin Inhibition. Unlike the case with thrombin and Factor X_a, the compounds shown in Figure 1 were found

(13) For both the inhibitors and corresponding acyl enzymes, the compound number will be followed by the substitution pattern on the aromatic ring opposite that containing the carboxyl group. For example, *p*-nitrophenyl ester **3a** would appear as **3a** (2,5-$(OMe)_2$) and acyl-chymotrypsin as Chymo-**1** (phenyl).

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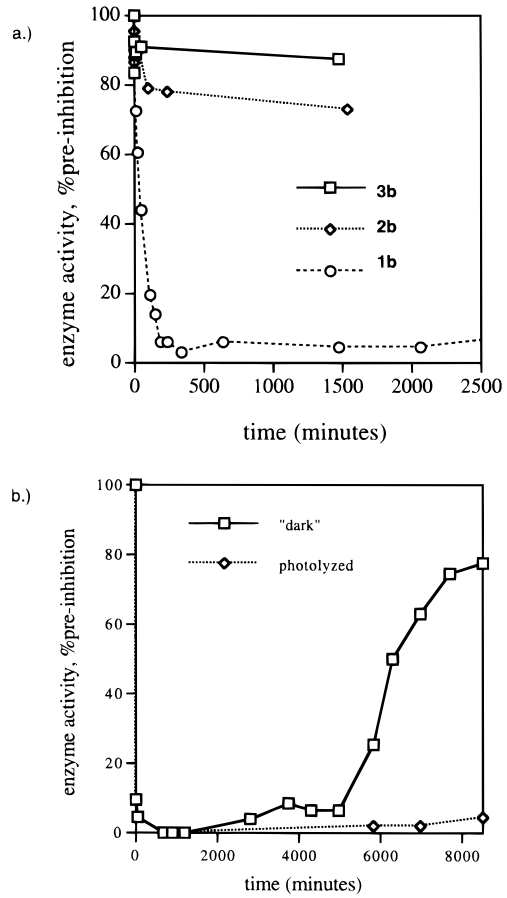


Figure 2. (a) Inhibition of thrombin by 20 equiv of **1b** (phenyl), **2b** (2-OMe), or **3b** (2,5-$(OMe)_2$) in the dark. Conditions: pH 7.4 Tris buffer (50 mM), 150 mM NaCl, [Thrombin] = 0.9 μM. Inhibitors were added to aqueous thrombin as solutions in methanol (v/v < 5%). (b) Inhibition and photolysis of thrombin by 20 equiv of **1b** (phenyl) in pH 7.4 Tris buffer (50 mM), 150 mM NaCl. The curve designated "dark" was inhibited and the enzyme activity followed over time while the sample was kept in the dark. The curve designated "photolyzed" was treated exactly as for the "dark" sample with the exception that the sample was irradiated (1000 W Hg/Xe arc lamp, grating monochromoter used to isolate λ = 366 nm) for 60 min while enzyme activity was zero (800−860 min). [Thrombin] = 0.9 μM.

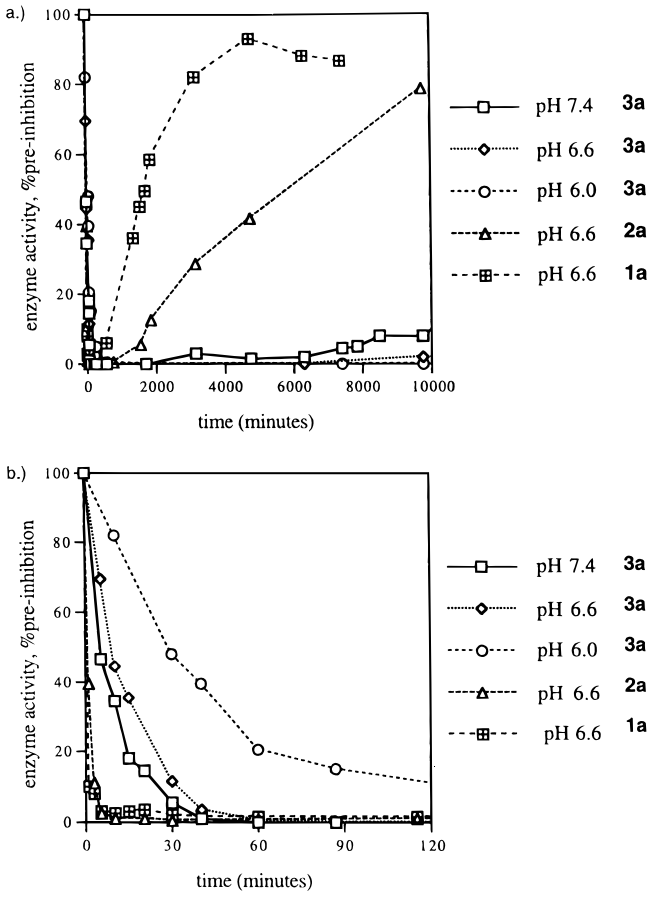


Figure 3. (a) Inhibition of chymotrypsin (3−10 μM) by 1 equiv of **1a** (phenyl), **2a** (2-OMe), or **3a** (2,5-$(OMe)_2$). Phosphate buffered saline was used as the solvent ([KH_2PO_4] = 0.05 M, [NaCl] = 0.15 M). This graph indicates a long time course for the inhibition. Compare to panel b, which shows the same inhibition over a shorter time course. (b) Inhibition of chymotrypsin (3−10 μM) by 1 equiv of **1a** (phenyl), **2a** (2-OMe), or **3a** (2,5-$(OMe)_2$). Phosphate buffered saline was used as the solvent ([KH_2PO_4] = 0.05 M, [NaCl] = 0.15 M). This graph indicates a short time course for the inhibition.

to be excellent chymotrypsin inhibitors. Figure 3 shows the inhibition of chymotrypsin by three of these compounds, **1b**−**3b**. Inhibition is rapid and, in the case of **3b** (2,5-$(OMe)_2$), indefinite. The acyl enzymes can be isolated by size-exclusion chromatography (SEC), shown in Figure 4. In addition to these three compounds, **4** (2,4,5-$(OMe)_3$) inhibits chymotrypsin. The inhibition is slower than that for **1b**−**3b** and the acyl enzyme hydrolyzes more rapidly than for any of these three inhibitors. Compound **6** (2,4,6-$(CH_3)_3$) inhibits chymotrypsin very slowly. Only after 4 days is enzyme activity reduced to less than 10% of the initial value when 25 equiv of **6** is added to chymotrypsin. The fact that **6** is so sluggish as an inhibitor suggests that, in forming the acyl enzyme, the benzophenone functionality becomes planar. With two ortho substituents, **6** would resist this. Scheme 4 shows the formation of acyl enzymes.

Observation of enzyme activity vs time for solutions of acyl enzyme kept in the dark allow the calculation of a pseudo-first-order rate constant for hydrolysis of the acyl enzyme. The rate constants and half-lives for the acyl enzymes are tabulated in Table 1. At pH 6.0 Chymo-**3** (2,5-$(OMe)_2$) is stable indefinitely. After 10 days less than 8% of the acyl enzyme hydrolyzes.

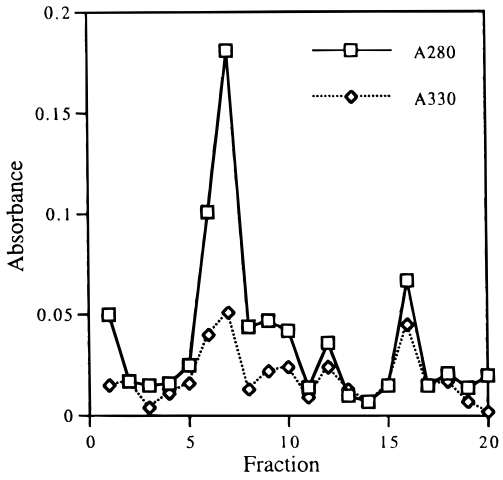
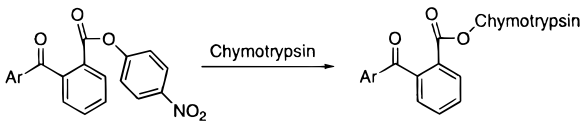


Figure 4. Isolation of acyl-chymotrypsin Chymo-**3** (2,5-$(OMe)_2$) by size-exclusion chromatography (Sephadex G-25), pH 6.0 PBS eluent. Chymotrypsin was inhibited by excess **3a** in pH 6.6 PBS ([chymotrypsin] = 8 μM, [KH_2PO_4] = 50 mM, [NaCl] = 150 mM).

Because of the very slow nature of this reaction it proved impossible to accurately measure the rate constant, but it could be estimated and an upper limit projected. Even at pH 7.4 this acyl enzyme has a half-life of over 3 weeks. The least stable

Scheme 4



Ar = C_6H_5	1a	Chymo-1
Ar = 2-(OMe)-C_6H_4	2a	Chymo-2
Ar = 2,5-$(OMe)_2C_6H_3$	3a	Chymo-3
Ar = 2,4,5-$(OMe)_3C_6H_2$	4	Chymo-4
Ar = 2-(O^{13}Me), 5-(OMe) C_6H_3	5	Chymo-5
Ar = 2,4,6-$(CH_3)C_6H_2$	6	Chymo-6
Ar = biotinylated tether	7	Chymo-7

Table 1. Half-lives and Rate Constants for Hydrolysis of Acyl-Chymotryspins

acyl enzyme	pH	$k_{hydrolysis}$	half-life
Chymo-**3** (2,5-$(OMe)_2$)	7.4	3.4×10^{-7} s^{-1}	23.8 days
Chymo-**3** (2,5-$(OMe)_2$)	6.0	0.006×10^{-7} s^{-1} [a]	4 years[a]
Chymo-**2** (2-OMe)	6.0	4.81×10^{-6} s^{-1}	40 h
Chymo-**4** (2,4,5-$(OMe)_3$)	6.0	7.83×10^{-5} s^{-1}	2.5 h
Chymo-**1** (phenyl)	6.0	1.11×10^{-5} s^{-1}	17 h
Chymo-**7** (biotinylated)	6.0	4.06×10^{-5} s^{-1}	4.7 h

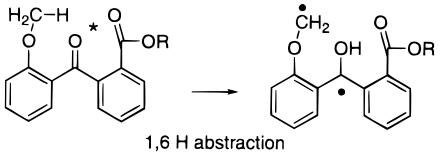
[a] Estimated.

acyl-chymotrypsin [Chymo-**4** (2,4,5-$(OMe)_3$)] has a half-life of only 2.5 h at pH 6.0.

Acyl-Chymotrypsin Photoactivation. Irradiation of most of the acyl-chymotrypsins leads to a sharp increase in lytic activity. Figure 5 shows enzyme activity vs time of photolysis for acyl-chymotrypsins Chymo-**1**−**3**. In the case of Chymo-**2** (2-OMe) and Chymo-**3** (2,5-$(OMe)_2$) enzyme activity increases very quickly when the acyl enzyme is irradiated with 366 nm light. Acyl-chymotrypsin Chymo-**1** (phenyl) photoactivates much slower and to a lesser extent than do Chymo-**2** (2-OMe) or Chymo-**3** (2,5-$(OMe)_2$). Acyl-chymotrypsin Chymo-**4** (2,4,5-$(OMe)_3$) photoactivates similarly to Chymo-**2** (2-OMe) and Chymo-**3** (2,5-$(OMe)_2$) with approximately 70−80% return of lytic activity, although this activation required longer photolysis times than did Chymo-**2** and Chymo-**3**.

Acyl-chymotrypsin Chymo-**6** (2,4,6-$(CH_3)_3$) did not photoactivate to a significant extent. Two hours of photolysis led to less than a 10% return of lytic activity. The lack of photoactivation of Chymo-**6** (2,4,6-$(CH_3)_3$) is probably due to photoenolization of this substrate.[16,17] The presence of ortho alkyl groups in aryl ketones is known to significantly diminish reactivity toward hydrogen or electron donors. The pattern of photoactivation observed for the other acyl-chymotrypsins is less explicable. It is interesting that two of the acyl enzymes, Chymo-**2** and Chymo-**3**, photoactivate so much more efficiently than the other acyl enzymes. The two that photoactivate the best have the possibility of an intramolecular 1,6-hydrogen abstraction from the *o*-methoxy group to the phenone carbonyl.[18] Examination of the photochemistry of the corresponding methyl esters of **2** and **5** revealed no tendency toward 1,6-hydrogen abstraction upon photolysis. One compound, designed to be more susceptible to 1,6-hydrogen abstraction, methyl 2-(2-benzyloxy-5-methoxybenzoyl)benzoate also did not undergo 1,6-hydrogen abstraction upon photolysis in degassed benzene. Presumably, the *o*-carboxyl group alters the reactivity of the phenone so that intramolecular hydrogen abstraction, observed by Wagner in the analogous non-ester-containing benzophenones, does not occur.[19]

Furthermore, 1,6-hydrogen abstraction does not explain the photoactivation of Chymo-**1** (phenyl). If Chymo-**2**−**4** (methoxy



containing) were to photoactivate by 1,6-hydrogen abstraction followed by lactonization, a second mechanism would still be required to explain the photoactivation of Chymo-**1** (phenyl) since this pathway is not available for this substrate. Any mechanism that explains photoactivation of Chymo-**1** (phenyl) is most likely to depend on hydrogen or electron transfer from the enzyme to which it is bound—essentially an intramolecular reaction. The hydrogen abstraction from *o*-alkoxy groups in aryl ketones is known not to be competitive with hydrogen or electron transfer from solvent. [19]

There are reports of a benzophenone-containing chymotrypsin inhibitor that photolabels the active site and leaves the labeled enzyme with attenuated lytic activity.[20,21] On the basis of this precedent, the apparent photoactivation may be no more than a photolabeling of the active site. To answer this question, two labeling experiments were performed.

The first experiment used ^{13}C-labeled inhibitor, **3** (2*,5-$(OMe)_2$), in order that the reaction could be monitored by NMR. Chymotrypsin was inhibited by 1 equiv of the labeled inhibitor. Once lytic activity had reached zero, the solution was irradiated using a 1000 W Hg/Xe arc lamp and grating monochromator to isolate 366 nm light. The solution regained about 70% of the initial enzyme activity. Spectra were obtained (^{13}C) for both the acyl enzyme and the photolyzed solution. The acyl enzyme displays a signal at δ 61.80. After photoactivation, two signals appear—a strong one at δ 59.45 and a weaker signal at δ 62.28. The signal at δ 59.45 appears as a quartet in a non-decoupled spectrum with a C−H coupling constant of 145.3 Hz. The chemical shift and coupling constant of this signal is very nearly identical with that of 2-(2,5-dimethoxybenzoyl)benzoic acid obtained under similar conditions. For this compound in pH 6.6 deuterated phosphate buffered saline (PBS) and a concentration of chymotrypsin of approximately 600 μM, the chemical shift is δ 59.34 and the coupling constant is 145.2 Hz. Furthermore, when a solution of acyl-chymotrypsin Chymo-**3** (2,5-$(OMe)_2$) is photoactivated and then extracted with ethyl acetate, the organic layer contains a product with *m*/*z* 286. This corresponds to the molecular ion of 2-(2,5-dimethoxybenzoyl) benzoic acid. Taken together these data indicate that the product of acyl-chymotrypsin photoactivation is the corresponding carboxylic acid.

The labeling experiment provides no data regarding the yield of the acid or active enzyme. That is, the chromogenic assay used to assess enzyme activity does not indicate if fully active enzyme is generated. The photoactivation may give, for example, fully active enzyme in 60% yield or a quantitative yield of enzyme that has only 60% activity. Affinity-labeled enzyme resulting from photolysis may be proteolytically active,

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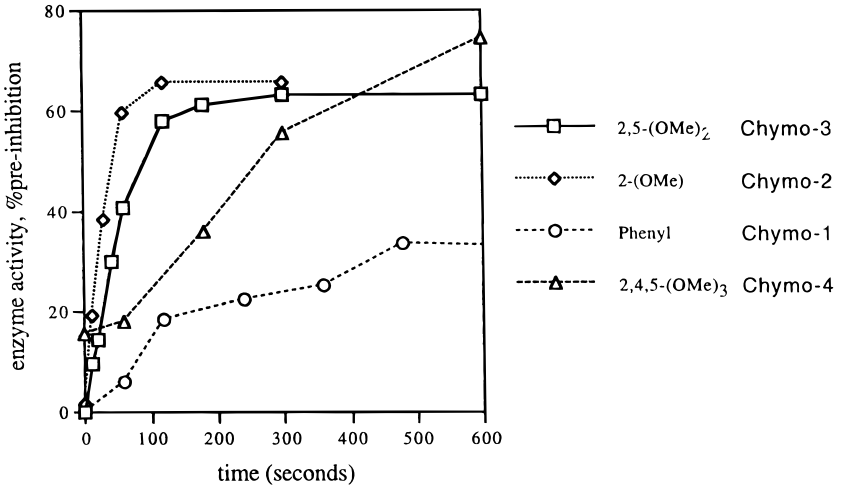

Figure 5. Photoactivation of acyl-chymotrypsins Chymo-**1** (phenyl), Chymo-**2** (2-OMe), Chymo-**3** (2,5-$(OMe)_2$), and Chymo-**4** (2,4,5-$(OMe)_3$). Conditions: pH 6.0 PBS, 1000 W Hg/Xe arc lamp, $\lambda = 366$ nm, [chymotrypsin] = 5−10 μM.

or it may have partial activity. A second labeling experiment was therefore performed to address this question.

Study of a biotinylated inhibitor, **7**, allows for the separation of photoaffinity-labeled enzyme from unlabeled enzyme using affinity chromatography. The avidin/biotin interaction is one of the strongest known non-covalent interactions.[22] The K_D for dissociation of biotin from avidin is 10^{-15} M, and it is almost completely unaffected by changes in pH, organic solvent, or denaturing agents. This is the case for avidin in its tetrameric state. In monomeric form the interaction with biotin is still strong but reversible enough for use as an affinity chromatographic reagent.[23−25] Following photoactivation of the biotinylated acyl-chymotrypsin Chymo-**7**, any enzyme that has been photoaffinity labeled will be retained by an avidin column, while enzyme that is not labeled will not be retained.

When 20 equiv of inhibitor **7** (biotinylated) is added to chymotrypsin at pH 6.6, enzyme activity is reduced to <5% in a few minutes. The acyl enzyme Chymo-**7** (biotinylated) was isolated by SEC using pH 6.0 PBS as an eluent. Hydrolysis of Chymo-**7** (biotinylated) at pH 6.0 occurred with a rate constant of 4.1×10^{-5} s^{-1}, giving this acyl enzyme a half-life of 4.7 h. Photolysis of Chymo-**7** (biotinylated) led to a 72% increase in enzyme activity after 90 min. Both the acyl enzyme and the crude photolysis mixture were chromatographed over immobilized monomeric avidin. The avidin chromatograms for these two solutions are shown, respectively, in parts a and b of Figure 6.

Figure 6a shows that Chymo-**7** (biotinylated) is bound to the avidin column. Thus, uninhibited chymotrypsin elutes from the avidin column with pH 6.0 PBS while the acyl enzyme elutes only when 2 mM biotin is included in the eluent. This shows that the biotin on the inhibitor is free to associate with avidin outside of the enzyme-binding pocket. All of the enzyme activity of the solution placed on the column elutes in fraction 2. Fractions 8 and 9 display no enzyme activity, and these fractions undergo photoactivation and dark hydrolysis exactly like the acyl enzyme isolated by SEC.

Figure 6b shows that most of the enzyme is not bound to biotin following photoactivation. Fractions 2−4, eluting with only pH 6.0 PBS and containing only unbiotinylated material,

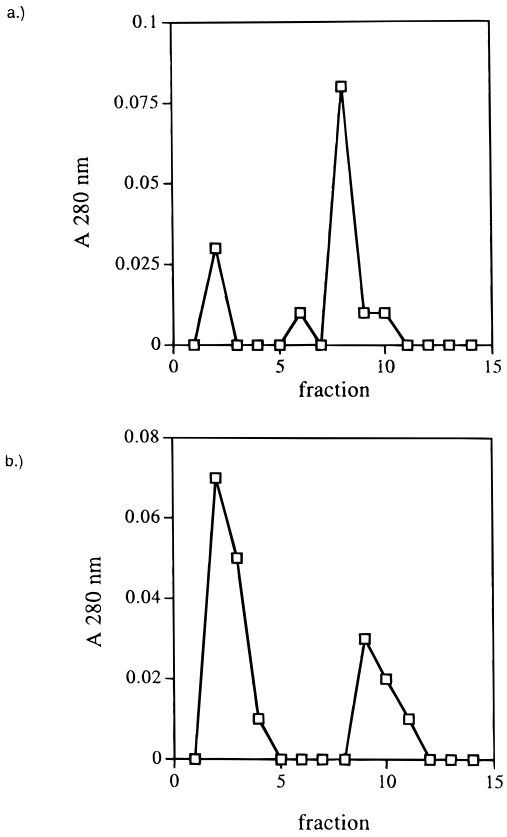

Figure 6. (a) Avidin affinity chromatography of Chymo-**7** (biotinylated acyl-chymotrypsin). Eluent: fractions 1−6, pH 6.0 PBS; fractions 7−14, pH 6.0 PBS containing 2 mM biotin. Fraction volume was 2 mL. Nonbiotinylated material elutes in fractions 1−6, while biotinylated material elutes in fractions 7−14. (b) Avidin affinity chromatography of photoactivated Chymo-**7** (biotinylated acyl-chymotrypsin). Eluent: fractions 1−7, pH 6.0 PBS; fractions 8−14, pH 6.0 PBS containing 2 mM biotin. Fraction volume was 2 mL. Nonbiotinylated material elutes in fractions 1−7, while biotinylated material elutes in fractions 8−14.

accounts for >95% of the active enzyme placed on the avidin column. Fractions 9−11, eluting with pH 6.0 PBS containing 2-mM biotin and all biotinylated material, contain both labeled enzyme and the biotinylated byproduct of photoactivation. These fractions contain only very slight lytic activity (0.02−0.03 μM). This demonstrates that most of the 2-aroyl benzoate inhibitor acts as a true photoreversible inhibitor and not as a photoaffinity label. Also, because the biotin-containing material contains such

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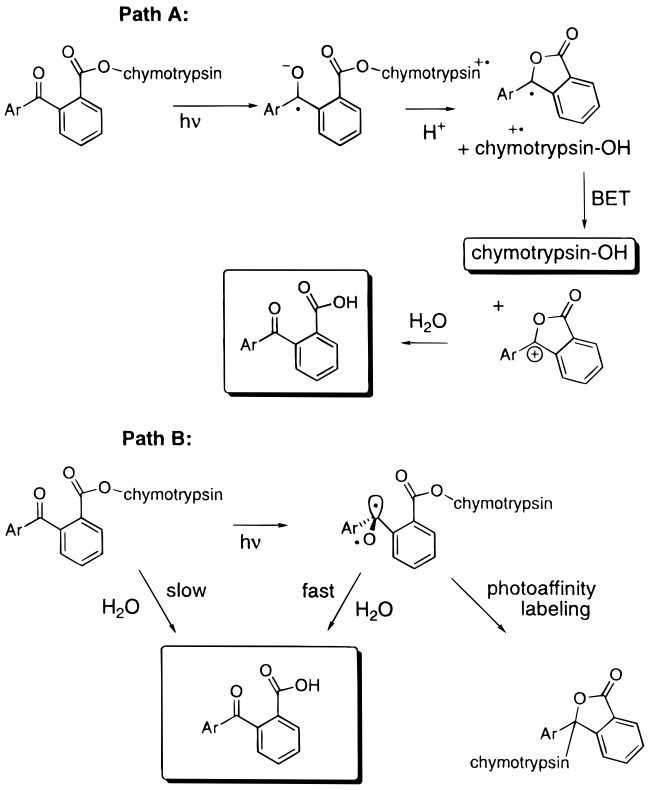
Scheme 5



a small amount of enzyme activity, *all* of the recovered enzyme activity upon photolysis must be due to enzyme that is not photoaffinity labeled. These data suggest that the recovery of the enzyme activity described in the experiments with inhibitors **2** and **3** results from fully active enzyme and that the remainder of the enzyme is lost to photoaffinity labeling.

These experiments do not account for the mechanism of carboxylic acid production in the photoactivation step. Scheme 5 shows two possibilities. The first possible mechanism shown involves an electron transfer. The excited inhibitor abstracts an electron from the protein to give a radical ion pair. The ketyl radical anion lactonizes to give the active site serine hydroxyl and a phthalide radical. Back electron transfer returns the enzyme to its "normal" state and leaves a phthalide cation. Trapping of the cation by water produces the carboxylic acid. The back electron transfer would become more favorable with the addition of electron-donating groups to the benzophenone. This may explain the increase in enzyme yield versus photoaffinity labeling for these acyl-chymotrypsins.

Path B in Scheme 5 shows a simple increase in hydrolysis rate results from a change in geometry of the benzophenone. The fact that **6** (2,4,6-$(CH_3)_3$) inhibits so slowly suggests that in the acylation of the serine hydroxyl the benzophenone becomes planar. If this were true, the change in conformation (planar to tetrahedral)[26] of the benzophenone upon excitation could explain the photoactivation.

Furthermore, the observed efficiencies of photoactivation versus photoaffinity labeling fit this model. Wagner has studied the effect of substitution on excited-state aryl ketone hydrogen abstraction in detail.[27] This work indicates that electron-donating substituents on benzophenones slows the rate of intermolecular hydrogen abstraction upon photolysis. Electron-withdrawing groups increase the rate of this reaction. According to these reports, the rates of intermolecular hydrogen abstraction for the acyl-chymotrypsins discussed above should follow as Chymo-**1** (phenyl) > Chymo-**2** (2-OMe) > Chymo-**3** (2,5-$(OMe)_2$) > Chymo-**4** (2,4,5-$(OMe)_3$). As hydrogen atom abstraction becomes faster, photoaffinity labeling should increase at the expense of photoactivation. Therefore, the better hydrogen atom abstractor should photoactivate in the lowest yield. This is what is observed, although more data, with a variety of substituents is needed to say with any certainty that the ability of the benzophenone to abstract hydrogen is related to efficiency of photoactivation.

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Conclusion

The above experiments demonstrate that *p*-guanidinophenyl 2-aroylbenzoyl benzoates do not inhibit either thrombin or Factor X_a or the inhibited enzyme does not photoactivate. The analogous *p*-nitrophenyl 2-aroylbenzoates serve as effective photoreversible inhibitors of chymotrypsin. Acyl-chymotrypsins are produced that are isolable by size-exclusion chromatography. These inactive enzymes are stable to hydrolysis for times varying from several hours to months. Irradiation of the acyl-chymotrypsin with 366 nm light produces a sharp increase in enzymatic activity. Up to 80% of preinhibition activity is returned within minutes of photolysis. Labeling experiments with ^{13}C indicate that some of the benzophenone inhibitor acts as a photoaffinity labeling reagent. The same experiments show that the product of the photoactivation is the carboxylic acid corresponding to the *p*-nitrophenyl ester used as inhibitor. Biotin-labeling experiments confirm that most of the enzyme is photoactivated and any photoaffinity-labeled enzyme is proteolytically inactive. The mechanism of the photoactivation remains unknown.

Experimental Section

General Synthetic Procedures. Melting points are uncorrected. Chemicals for which a synthesis is not reported were purchased commercially. Reactions were carried out in flame-dried glassware under argon. Gas chromatography was performed on a Hewlett-Packard 5890A gas chromatograph, with flame ionization detection, coupled to a Hewlett-Packard 3393A integrator (15 m SPB-5, 0.20 mm i.d.). Photolysis was carried out with a 450 W medium-pressure mercury lamp (Hanovia), 1000 W mercury/xenon arc lamp (Oriel), or a handheld 4 W mercury lamp (Spectronics) as indicated. All ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using a Varian INOVA spectrometer at 300 MHz for ^1H and 75.4 MHz for ^{13}C unless otherwise noted. Low-resolution mass spectra (MS) were recorded using a Hewlett-Packard GC/MS system 5988A mass spectrometer by chemical ionization (CI) at 70 eV with CH_4/NH_3 as reagent gas. Fast atom bombardment (FAB) mass spectra were recorded using a JEOL JMS-SX102A mass spectrometer with xenon as the fast atom. Infrared spectra were recorded using a Bomem MB-100 infrared spectrometer and a NaCl chamber cell, NaCl window, or KBr disk. Elemental analysis was performed by Atlantic Microlabs (Atlanta, GA). Sephadex G-25 was purchased from Sigma Chemical Co. Immunopure Immobilized Monomeric Avidin was purchased in kit form from Pierce (catalog no. 20227).

***p*-Nitrophenyl 2-Benzoylbenzoate (1a).** To a solution of 2-benzoylbenzoic acid (1.2 g, 5 mmol), *p*-nitrophenol (0.834 g, 6 mmol), and DMAP (0.122 g, 1 mmol) in pyridine (50 mL) was added DCC (1.23 g, 6 mmol), and the resulting solution was stirred at 25 °C under argon for 60 h. The reaction mixture was chilled and filtered. The filtrate was poured into 1 M HCl, and the solution was extracted 2 × 100 mL of EtOAc. The combined organic layers were washed with 2 × 100 mL of 1 M HCl, 2 × 100 mL of saturated sodium bicarbonate, and 1 × 50 mL of brine, dried ($MgSO_4$), and concentrated in vacuo. The crude oil was purified via flash column chromatography (SiO_2, CH_2Cl_2) to give a white solid (0.539 g, 1.55 mmol, 31%), mp 86−87 °C:

^{1}H ($CDCl_3$, ppm) 8.17 (d, 1H, J = 16.0 Hz), 8.14 (d, 2H, J = 9.3 Hz), 7.77 (d, 2H, J = 7.1 Hz), 7.74 (td, 1H, J = 1.4, 7.5 Hz), 7.67 (td, 1H, J = 1.4, 7.6 Hz), 7.55 (dt, 2H, J = 7.5, 16.0 Hz), 7.44 (t, 2H, J = 7.9 Hz), 6.99 (d, 2H, J = 9.2 Hz); ^{13}C ($CDCl_3$, ppm) 196.17, 163.42, 154.91, 141.87, 136.85, 133.51, 133.39, 130.83, 130.10, 129.52, 128.71, 128.39, 127.96, 125.07, 122.09. Anal. Calcd for $C_{20}H_{13}NO_5$: C, 69.16; H, 3.77; N, 4.03; found: C, 69.18; H, 3.81; N, 3.99.

***p*-*N*,*N*′-Bis-BOC-guanidinophenyl 2-Benzoylbenzoate.** To a solution of 2-benzoylbenzoic acid (0.678 g, 3.0 mmol), *p*-*N*,*N*′-bis-BOC-guanidinophenol (1.05 g, 3.0 mmol), and DMAP (0.037 g, 0.3 mmol) in pyridine (5 mL) was added DCC (1 M in pyridine, 6 mL, 6.0 mmol), and the resulting solution was stirred at 25 °C under argon for 48 h. The reaction mixture was chilled and filtered. The filtrate was poured into 1 M HCl, and the solution was extracted 3 × 100 mL of EtOAc. The combined organic layers were washed with 2 × 100 mL of 1 M of HCl, 2 × 100 mL of 10% aqueous KOH, 2 × 50 mL of water, and 1 × 50 mL of brine. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The crude oil was purified via flash column chromatography (SiO_2, 3% acetone in chloroform) to give a white solid (0.850 g, 1.52 mmol, 51%), mp 150−160 °C (gas evolution): IR ($CHCl_3$, cm^{-1}) 3555, 3161, 2996, 2930, 1787, 1737, 1710, 1691, 1677, 1658, 1640, 1564; ^{1}H ($CDCl_3$, ppm) 11.59 (bs, 1H), 10.28 (bs, 1H), 8.17 (d, 1H, J = 8.0 Hz), 7.73 (d, 2H, J = 8.4 Hz), 7.70 (td, 1H, J = 1.2, 7.6 Hz), 7.62 (td, 1H, J = 1.2, 7.6 Hz), 7.53 (t, 1H, J = 7.6 Hz), 7.44 (m, 4H), 6.72 (d, 1H, J = 8.8 Hz), 1.49 (s, 9H), 1.45 (s, 9H); ^{13}C ($CDCl_3$, ppm) 209.70, 173.70, 164.49, 153.42, 144.91, 141.91, 141.84, 137.12, 133.22, 132.90, 130.90, 130.69, 129.81, 129.47, 128.59, 128.09, 122.99, 121.46, 78.15, 28.14, 28.04; HRMS calcd for $[C_{31}H_{33}N_3O_7 + H]^+$ 560.2398, found 560.2390.

***p*-Guanidinophenyl 2-Benzoylbenzoate Hydrochloride (1b).** To a solution of *p*-*N*,*N*′-bis-BOC-guanidinophenyl 2-benzoylbenzoate (0.158 g, 0.28 mmol) in $CHCl_3$ (10 mL) was added trifluoroacetic acid (5 mL). The resulting yellow solution was stirred at 25 °C for 24 h. The solvent was removed in vacuo and the residue dissolved in 1 M HCl in ether and stirred for 2 h. Solvent was removed in vacuo to give a chalky, white powder (0.095 g, 0.24 mmol, 86%): ^{1}H (CD_3OD, ppm) 8.21 (d, 1H, J = 8.0 Hz), 7.83 (td, 1H, J = 1.6, 7.6 Hz), 7.76 (m, 3H), 7.62 (tt, 1H, J = 1.6 Hz, 7.2 Hz), 7.50 (m, 3H), 7.25 (d, 2H, J = 9.2 Hz), 6.97 (d, 2H, J = 9.2 Hz); ^{13}C (CD_3OD, ppm) 198.36, 166.26, 158.20, 150.91, 143.08, 138.32, 134.73, 134.46, 134.00, 131.70, 131.46, 130.63, 129.85, 129.42, 128.09, 124.06; HRMS calcd for $[C_{21}H_{17}N_3O_3 + H]^+$ 360.1349, found 360.1342.

***p*-Nitrophenyl 2-(2-Methoxybenzoyl)benzoate (2a).** To a solution of 2-(2-methoxybenzoyl)benzoic acid[28] (1.27 g, 4.7 mmol), *p*-nitrophenol (0.784 g, 5.64 mmol), and DMAP (0.122 g, 1 mmol) in pyridine (40 mL) was added DCC (1.16 g, 5.64 mmol). The solution was stirred at 25 °C under argon for 60 h. The mixture was poured into 1 M HCl, and the resulting solution was extracted 2 × 100 mL of EtOAc. The combined organic layers were washed 2 × 100 mL of 1 M HCl, 2 × 100 mL of saturated sodium bicarbonate, 1 × 50 mL of brine, dried, and concentrated in vacuo. The resulting oil was dissolved in approximately 5 mL of ether. After 2 days the desired product was obtained as white needles (0.539 g, 1.43 mmol, 30%), mp 80−82 °C: ^{1}H ($CDCl_3$, ppm) 8.19 (d, 2H, J = 9.2 Hz), 8.03 (m, 1H), 7.71 (dd, 1H, J = 1.8 Hz, 7.7 Hz), 7.62 (m, 2H), 7.49 (m, 2H), 7.13 (d, 2H, J = 9.2 Hz), 7.01 (m, 1H), 6.93 (d, 1H, J = 8.4 Hz), 3.57 (s, 3H); ^{13}C ($CDCl_3$, ppm) 195.04, 159.02, 155.29, 143.80, 134.47, 132.60, 131.58, 130.01, 129.95, 129.84, 128.27, 128.17, 126.72, 125.05, 122.03, 120.69, 112.17, 55.60. Anal. Calcd for $C_{21}H_{15}NO_6$: C, 66.84; H, 4.01; N, 3.71;. Found: C, 66.96; H, 4.06; N, 3.73.

***p*-*N*,*N*′-Bis-BOC-guanidinophenyl 2-(2-Methoxybenzoyl)benzoate.** To a solution of 2-(2-methoxybenzoyl)benzoic acid[27] (0.853 g, 2.98 mmol), *p*-*N*,*N*′-bis-BOC-guanidinophenol (1.29 g, 3.80 mmol), and DMAP (0.037 g, 0.3 mmol) in pyridine (5 mL) was added DCC (1 M in pyridine, 12 mL, 12.0 mmol), and the resulting solution was stirred at 25 °C under argon for 48 h. The reaction mixture was chilled and filtered. The filtrate was poured into 1 M HCl, and the solution was extracted 3 × 100 mL of EtOAc. The combined organic layers were washed with 2 × 100 mL of 1 M HCl, 2 × 100 mL of 10% aqueous KOH, 2 × 50 mL of water, and 1 × 50 mL of brine. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The crude oil was purified via flash column chromatography (SiO_2, 2% acetone in chloroform) to give a white foam (1.346 g, 2.17 mmol, 73%), mp 82−85 °C: IR ($CHCl_3$, cm^{-1}) 3451, 3277, 3155, 2988, 2936, 1725, 1710, 1691, 1654, 1640, 1630, 1547; ^{1}H ($CDCl_3$, ppm) 11.61 (bs, 1H), 10.34 (bs, 1H), 8.02 (dd, 1H, J = 0.8, 7.8 Hz), 7.68 (dd, 1H, J = 1.6, 7.2 Hz), 7.59 (td, 1H, J = 1.6, 7.6 Hz), 7.53 (td, 1H, J = 1.6,7.6 Hz), 7.44 (m, 4H), 6.96 (td, 1H, J = 0.8, 7.4 Hz), 6.88 (d, 1H, J = 8.4 Hz), 6.82 (d, 2H, J = 9.2 Hz), 3.82 (s, 3H), 1.50 (s, 9H), 1.45 (s, 9H); ^{13}C ($CDCl_3$, ppm) 195.28, 172.39, 165.27, 159.07, 153.45, 147.54, 144.09, 134.26, 132.27, 131.63, 129.93, 129.49, 128.83, 127.84, 126.97, 123.17, 121.76, 120.55, 112.21, 55.62, 28.07; HRMS calcd for $[C_{32}H_{35}N_3O_8 + H]^+$ 590.2504, found 590.2484.

***p*-Guanidinophenyl 2-(2-Methoxybenzoyl)benzoate (2b).** Dry HCl gas was bubbled through a solution of *p*-*N*,*N*′-bis-BOC-guanidinophenyl 2-(2-methoxybenzoyl)benzoate (0.103 g, 0.17 mmol) in 5 mL of EtOAc for 5 h. The solution was chilled to 0 °C and the precipitate collected, washed with ether (100 mL), and dried in vacuo to give the desired product as a hygroscopic white powder (0.0364 g, 0.086 mmol, 50%): ^{1}H (CD_3OD, ppm) 8.05 (dd, 1H, J = 1.2, 7.2 Hz), 7.69 (m, 3H), 7.57 (td, 1H, J = 1.6, 8.8 Hz), 7.42 (dd, 1H, J = 1.6,7.2 Hz), 7.28 (d, 2H, J = 8.8 Hz), 7.06 (m, 4H), 3.57 (s, 3H); ^{13}C (CD_3OD, ppm) 197.44, 187.49, 177.52, 174.26, 167.14, 160.71, 158.19, 151.12, 145.29, 136.06, 133.70, 132.44, 131.07, 130.88, 128.91, 128.08, 124.13, 121.68, 113.67, 56.13; HRMS calcd for $[C_{22}H_{19}N_3O_4 + H]^+$ 390.1454, found 390.1445.

2-(2,5-Dimethoxybenzoyl) *p*-(Nitrophenyl)benzoate (3a). A mixture of 2-(2,5-dimethoxybenzoyl)benzoic acid[29] (200 mg, 0.7 mmol), dicyclohexylcarbodiimide (173 mg, 0.84 mmol, 1.2 equiv), *p*-nitrophenol (117 mg, 0.84 mmol, 1.2 equiv), and 10 mol % DMAP in 7 mL of pyridine was stirred at room temperature for 48 h. The reaction mixture was then poured into a separatory funnel containing 50 mL of 1 M HCl and 50 mL of EtOAc. The organic layer was then washed 2 × 50 mL of 1 M HCl, 4 × 50 mL of saturated sodium carbonate solution (until the aqueous wash was no longer yellow), 1 × 50 mL of distilled water, and 1 × 50 mL of brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was dissolved in 1 mL of dichloromethane and cooled to −10 °C. The mixture was then passed through a filter syringe and concentrated to give a yellow paste. The paste was dissolved in 3 mL of diethyl ether and allowed to sit for 2 h. At this time yellow crystals were observed in the flask. These were collected and shown to be the desired product (63.8 mg, 0.16 mmol, 23%), mp 109−110 °C: IR ($CHCl_3$) 1780, 1740, 1630 cm^{-1}; ^{1}H NMR ($CDCl_3$, ppm) 8.22 (d, 2H, J = 9 Hz), 8.06 (d, 1H, J = 8.1 Hz), 7.63 (m, 2H), 7.44 (d, 1H, J = 6.6 Hz), 7.32 (d, 1H, J = 3.3 Hz), 7.17 (d, 2H, J = 9.0 Hz), 7.07 (d, 1H, J = 3.0 Hz), 6.88 (d, 1H, J = 9.0 Hz), 3.77 (s, 3H), 3.49 (s, 3H); ^{13}C NMR ($CDCl_3$, ppm) 194.8, 164.7, 155.3, 153.5, 153.4, 145.3, 144.1, 135.9, 132.7, 130.0, 128.0, 127.8, 126.9, 125.6, 125.0, 122.2, 121.4, 114.7, 114.0, 56.2, 55.8; GC/CIMS (CH_4/NH_3(g)) m/z 408 (MH^+). Anal. Calcd for $C_{22}H_{17}NO_7$: C, 64.86; H, 4.21; N, 3.44. Found: C, 64.79; H, 4.26; N, 3.48.

Methyl 2-(2-Hydroxy-5-methoxybenzoyl)benzoate (10). Methyl 2-(2,5-dimethoxybenzoyl)benzoate[12] (1.595 g, 5.32 mmol) was dissolved in CH_2Cl_2 (20 mL). Aluminum chloride (5 equiv, 4.4 g, 26.6 mmol) was added in one portion. The color quickly went from colorless to dark red to yellow-green upon addition of the aluminum chloride. After being stirred for 8 h at ambient temperature, the reaction mixture was partitioned between ice water and EtOAc (200 mL each). The aqueous layer was brought to pH 2 with addition of 1 M HCl, and the layers were separated. The aqueous layer was extracted twice more with 150 mL EtOAc. The combined organic layers were washed with 150 mL of brine, dried over magnesium sulfate, and concentrated in vacuo. The yellow residue was chromatographed (Silica gel, 2% acetone in chloroform) to give the desired compound as a yellow solid (1.296 g, 4.53 mmol, 85%), mp 71−72 °C: IR (Nujol, cm^{-1}) 3400−2800 (bs), 2920 (ss), 1740, 1640, 1620, 1615, 1480, 1460, 1400; ^{1}H NMR ($CDCl_3$, ppm) 11.52 (s, 1H), 8.07 (dd, 1H, J = 1.2, 8.0 Hz), 7.65 (td, 1H, J = 1.2, 7.4 Hz), 7.58 (td, 1H, J = 1.2, 7.8 Hz), 7.37 (dd, 1H, J = 1.6, 7.6 Hz), 7.08 (dd, 1H, J = 3.0, 8.8 Hz), 6.98 (d, 1H, J = 9.2

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Hz), 6.51 (d, 1H, $J = 1.6$ Hz), 3.69 (s, 3H), 3.58 (s, 3H); ^{13}C NMR ($CDCl_3$, ppm) 202.34, 166.00, 156.87, 151.58, 140.03, 132.52, 130.34, 129.89, 128.72, 127.35, 123.86, 119.70, 119.09, 115.26, 55.78, 52.41; HRMS calcd for $[C_{16}H_{14}O_5]^+$ 286.0841, found 286.0832.

Methyl 2-(2-*^{13}C*-,5-Dimethoxybenzoyl)benzoate (9-*^{13}C*). Tetrabutylammonium flouride (1 M in THF, 25 mL, 25 mmol) was concentrated in vacuo. To the flask containing the TBAF was added methyl 2-(2-hydroxy-5-methoxybenzoyl)benzoate (1.77 g, 6.2 mmol) in dissolved in DMF (8 mL). Once this solution was homogeneous iodomethane (1.00 g, 7 mmol) was added via syringe. The initial red color began dissipating instantly. After 5 h the color had become pale yellow. The reaction mixture was poured into 50 mL of 2 M NaOH and extracted 3×50 mL of EtOAc. The combined organic layers were washed 2×100 mL of 2 M NaOH, 2×100 mL of water, and 1×100 mL of brine, dried over magnesium sulfate, and concentrated in vacuo to give a tan solid. This solid was chromatographed (Silica gel, 20−35% EtOAc in hexanes) to give the desired product as a white solid (1.442 g, 4.81 mmol, 78%): ^{1}H ($CDCl_3$, ppm) 7.89 (d, 1H, $J = 7.2$ Hz), 7.58 (t, 1H, $J = 7.2$ Hz), 7.48 (t, 1H, $J = 7.2$ Hz), 7.43 (d, 1H, $J = 7.6$ Hz), 6.88 (d, 1H, $J = 8.8$ Hz), 6.82 (d, 1H, $J = 2.8$ Hz), 6.80 (s, 1H), 6.62 (d, 1H, $J = 2.8$ Hz), 3.85 (s, 3H), 3.65 (s, 3H). 3.50 (d, 3H, ($^{13}C-^1H$) $J = 148$ Hz). All other spectra matched those of **9**.

Methyl 2-(2-Benzyloxy-5-methoxybenzoyl)benzoate. To a solution of methyl 2-(2-hydroxy-5-methoxybenzoyl)benzoate (0.1442 g, 0.5 mmol) in DMF (6 mL) were added K_2CO_3 (0.345 g, 2.5 mmol) and 18-crown-6 (0.013 g, 0.05 mmol) in several portions. After the orange solution had stirred for 15 min, benzyl bromide (0.260 g, 0.2 mL, 1.5 mmol) was added. The solution stirred for 36 h at 25 ° C under argon. Over the course of the reaction the color faded to pale yellow. The reaction mixture was poured into EtOAc (100 mL), and the organic layer was washed with 3×50 mL of 10% aqueous KOH, 2×50 mL of water, and brine. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The crude oil was chromatographed (SiO_2, 5% acetone in chloroform. $R_f = 0.2$) and eluted as a yellow band to give the desired product as a yellow oil (0.200 g, 0.5 mmol, 100%): ^{1}H NMR ($CDCl_3$, ppm) 7.66 (m, 1H), 7.42 (dd, 1H, $J = 0.8$, 10.0 Hz), 7.39 (m, 1H), 7.28 (m, 2H), 7.20 (m, 3H), 7.02 (dd, 1H, $J = 3.2$, 8.8 Hz), 4.72 (s, 2H), 3.81 (s, 3H), 3.62 (s, 3H); ^{13}C NMR ($CDCl_3$, ppm) 195.51, 167.13, 153.62, 152.58, 144.19, 136.01, 131.59, 129.37, 128.94, 128.16, 127.64, 127.49, 127.28, 126.79, 121.01, 114.92, 114.53, 71.07, 55.83, 52.03; HRMS calcd for $[C_{23}H_{20}O_5]^+$ 376.1311, found 376.1295.

***p*-*N*,*N*′-Bis-BOC-guanidinophenyl 2-(2,5-Dimethoxybenzoyl)benzoate.** To a solution of 2-(2,5-dimethoxybenzoyl)benzoic acid[29] (0.853 g, 2.98 mmol), *p*-*N*,*N*′-bis-BOC-guanidinophenol (1.29 g, 3.80 mmol), and DMAP (0.037 g, 0.3 mmol) in pyridine (5 mL) was added DCC (1 M in pyridine, 12 mL, 12.0 mmol), and the resulting solution was stirred at 25 °C under argon for 48 h. The reaction mixture was chilled and filtered. The filtrate was poured into 1 M HCl, and the solution was extracted 3×100 mL of EtOAc. The combined organic layers were washed with 2×100 mL of 1 M HCl, 2×100 mL of 10% aqueous KOH, 2×50 mL of water, and 1×50 mL of brine. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The crude oil was purified via flash column chromatography (SiO_2, 2% acetone in chloroform) to give a white foam (1.346 g, 2.17 mmol, 73%), mp 147−49 °C (gas evolution): IR ($CHCl_3$, cm^{-1}) 3572, 3154, 2993, 2916, 1776, 1725, 1710, 1691, 1677, 1657, 1640, 1630, 1565. ^{1}H ($CDCl_3$, ppm) 11.60 (bs, 1H), 10.30 (bs, 1H), 8.04 (dd, 1H, $J = 1.2$, 7.6 Hz), 7.59 (td, 1H, $J = 1.2$, 7.6 Hz), 7.52 (td, 1H, $J = 1.6$, 7.6 Hz), 7.48 (d, 2H, $J = 8.8$ Hz), 7.38 (dd, 1H, $J = 1.2$, 7.2 Hz), 7.30 (d, 1H, $J = 7.2$ Hz), 7.02 (dd, 1H, $J = 3.2$, 9.2 Hz), 6.84 (m, 3H), 3.73 (s, 3H), 3.44 (s, 3H), 1.50 (s, 9H), 1.45 (s, 9H); ^{13}C ($CDCl_3$, ppm) 195.03, 165.18, 153.62, 153.51, 147.29, 144.42, 134.32, 132.29, 129.87, 129.29, 128.60, 127.45, 127.22, 123.10, 121.65, 121.16, 114.69, 114.11, 83.90, 79.90, 56.30, 55.84, 28.14, 28.07; HRMS calcd for $[C_{33}H_{37}N_3O_9 + H]^+$ 620.2605, found 620.2607.

***p*-Guanidinophenyl 2-(2,5-Dimethoxybenzoyl)benzoate Hydrochloride (3b).** Gaseous hydrogen chloride was bubbled through a solution of *p*-*N*,*N*′-bis-BOC-guanidinophenyl 2-(2,5-dimethoxybenzoyl)-benzoate (0.093 g, 0.15 mmol) in EtOAc (10 mL) for 3 h. The cloudy yellow solution was cooled to 0 °C and the precipitate collected. The white solid was washed with >25 mL of cold EtOAc and dried in vacuo to give the desired product (0.050 g, 73%): ^{1}H (CD_3OD, ppm) 8.06 (dd, 1H, $J = 0.8$, 7.2 Hz), 7.71 (td, 1H, $J = 1.2$, 7.6 Hz), 7.66 (td, 1H, $J = 1.6$, 7.6 Hz), 7.42 (dd, 1H, $J = 1.2$, 7.2 Hz), 7.28 (m, 3H), 7.30 (d, 1H, $J = 7.2$ Hz), 7.16 (dd, 1H, $J = 3.2$, 9.2 Hz), 7.05 (m, 3H), 3.75 (s, 3H), 3.49 (s, 3H); ^{13}C (CD_3OD, ppm) 197.06, 182.27, 167.06, 158.20, 156.00, 151.14, 145.45, 133.75, 130.96, 130.82, 129.81, 128.76, 128.12, 124.13, 122.09, 116.02, 115.45, 56.70, 56.23; HRMS calcd for $[C_{23}H_{21}N_3O_5 + H]^+$ 420.1559, found 420.1596.

***p*-Nitrophenyl 2-(2,4,5-Trimethoxybenzoyl)benzoate (4).** The following reagents were dissolved in dry CH_2Cl_2:2-(2,4,5-trimethoxybenzoyl)benzoic acid (0.612 g, 2 mmol), *p*-nitrophenol (0.417 g, 3 mmol), and DMAP (0.122 g, 1 mmol). To this solution was added DCC (0.618 g, 3 mmol) in one portion. The reaction mixture was then stirred, under argon, at 25 °C for 48 h. The solution was then washed 3×50 mL of 1 M NaOH, dried ($MgSO_4$), and concentrated in vacuo. The residue was flash chromatographed (SiO_2, 50% EtOAc in hexane) to afford a yellow solid, which was recrystallized from ether/hexane to afford the desired ester as white crystals (0.211 g, 0.48 mmol, 24%), mp 114−15 °C: ^{1}H ($CDCl_3$, ppm) 3.47 (s, 3H), 3.86 (s, 3H), 3.94 (s, 3H), 6.42 (s, 1H), 7.18 (d, 2H, $J = 9.2$ Hz), 7.37 (dd, 1H, $J = 1.1$, 7.5 Hz), 7.49 (s, 1H), 7.56 (td, 1H, $J = 1.3$, 7.6 Hz), 7.66 (td, 1H, $J = 1.3$, 7.5 Hz), 8.11 (dd, 1H, $J = 1.1$, 7.6 Hz), 8.22 (d, 2H, $J = 9.2$Hz); ^{13}C ($CDCl_3$, ppm) 193.66, 155.70, 155.31, 154.87, 146.10, 145.25, 143.53, 132.98, 130.12, 128.79, 126.91, 126.81, 125.11, 122.24, 118.06, 112.56, 112.52, 96.93, 56.38, 56.30, 56.18. Anal. Calcd. for $C_{23}H_{19}NO_8$: C, 63.16; H, 4.38; N, 3.20. Found: C, 63.08; H, 4.43; N, 3.21.

***p*-*N*,*N*′-Bis-BOC-guanidinophenol (8).** To a solution of *p*-aminophenol (6.54 g, 60 mmol), *N*,*N*′-bis-BOC-thiourea[30] (16.5 g, 60 mmol), and pyridine (15.8 g, 16.2 mL, 200 mmol) in DMF (150 mL) was added $HgCl_2$ (19.01 g, 70 mmol). The resulting thick, yellow solution was stirred for 48 h at 25 °C under argon. The solution was partitioned between chloroform (500 mL) and water (400 mL). The aqueous layer was extracted twice more with chloroform (500 mL total), and the combined organic layers were washed with 2×500 mL of saturated sodium bicarbonate, 2×500 mL of water, and brine (200 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo to give a thick red oil. The desired product was obtained as a pale tan powder (6.75 g, 19.9 mmol, 32%) from hot 2-propanol/water, mp gas evolution between 180 and 210 °C, decomposition at 210 °C: IR ($CHCl_3$, cm^{-1}) 3429, 3202, 3265, 2993, 2929, 1790, 1723, 1635, 1515, 1479, 1411, 1370; ^{1}H ($CDCl_3$, ppm) 11.58 (bs, 1H), 9.92 (bs, 1H), 6.99 (d, 2H, $J = 8.8$ Hz), 6.54 (d, 2H, $J = 8.8$ Hz), 6.82 (d, 2H, $J = 9.2$ Hz), 3.82 (s, 3H), 1.50 (s, 9H), 1.45 (s, 9H); ^{13}C ($CDCl_3$, ppm) 155.77, 155.34, 153.15, 126.93, 126.26, 125.87, 116.13, 83.77, 80.00, 28.10, 27.97; HRMS calcd for $[C_{17}H_{25}N_3O_5]^+$ 351.1794, found 351.1806.

***p*-Nitrophenyl 2-(2,4,6-Trimethylbenzoyl)benzoate (6).** To a solution of 2-(2,4,6-trimethylbenzoyl)benzoic acid[31] (1.07 g, 4.0 mmol), *p*-nitrophenol (0.695 g, 5.0 mmol), and DMAP (0.050 g, 0.4 mmol) in pyridine (4 mL) was added DCC (1 M in pyridine, 8 mL, 8 mmol). The reaction was stirred at 25 °C for 72 h, chilled, and filtered. The filtrate was poured into a separatory funnel containing 50 mL of 1 M HCl and 50 mL of EtOAc. The organic layer was then washed 2×50 mL of 1 M HCl, 4×50 mL of saturated sodium carbonate solution (until the aqueous wash was no longer yellow), 1×50 mL of distilled water, 1×50 mL of brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was dissolved in 1 mL of dichloromethane and cooled to −10 °C. The mixture was then passed through a filter syringe and concentrated to give a yellow paste. The paste was dissolved in 3 mL of diethyl ether and allowed to sit for 2 h. At this time yellow crystals were observed in the flask. These were collected and shown to be the desired product (0.388 g, 1.0 mmol, 25%), mp 99.5−100 °C: IR ($CHCl_3$, cm^{-1}) 3154, 2994, 2923, 2865, 1754, 1710, 1665, 1526; ^{1}H ($CDCl_3$, ppm) 8.27 (d, 2H, $J = 9.2$ Hz), 7.77 (dd, 1H, $J = 0.8$, 7.6 Hz), 7.66 (td, 1H, $J = 1.2$, 7.6 Hz), 7.51 (m, 3H), 7.42 (dd, 1H, $J = 1.2$, 7.6 Hz), 6.88 (s, 2H), 2.31 (s, 3H), 2.13 (s, 6H); ^{13}C ($CDCl_3$, ppm)

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199.65, 167.27, 155.79, 145.47, 139.71, 138.04, 135.37, 135.28, 132.73, 132.28, 131.29, 130.76, 129.33, 128.79, 125.24, 122.49, 21.18, 19.64; HRMS calcd for $[C_{23}H_{19}NO_5]^+$ 389.1263, found 389.1259.

Enzyme Assays and Inhibition Measurements. Enzyme solutions were assayed for protease activity by a chromogenic method. Substrates were purchased from Chromogenix and diluted to a concentration of 1.00 mM with deionized water. These substrates are non-natural peptides that contain an enzyme recognition sequence and a terminal *p*-nitroanilide. The protease acts to catalyze the hydrolysis of the *p*-nitroanilide, releasing *p*-nitroaniline, which can be measured by its UV−vis absorbance.

A polystyrene cuvette containing 1 mL of pH 8.3 Tris buffer (30 mM Tris, 3 mM $CaCl_2$, and 400 mM NaCl) and a set amount of the chromogenic substrate solution was prepared and the solution mixed thoroughly. The cuvette was angled and 20 mL of enzyme solution was added to the side of the cuvette. The enzyme and substrate were mixed by shaking the capped cuvette three times in as rapid manner as possible. The rate of change of the solution's absorbance at 402 nm (λ_{max} *p*-nitroaniline (pNA) $\epsilon = 10\,400$) was measured for periods ranging between 15 and 120 s. Michaelis−Menten kinetics describe the rate of pNA formation.

α-Chymotrypsin. α-Chymotrypsin (C-4129) was purchased from Sigma Chemical Co. The activity of α-chymotrypsin was measured by chromogenic assay using S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA − HCl; $K_m = 32.6$ mM, $k_{cat} = 26.77$ s^{-1})[7] in pH 8.3 buffer (30 mM Tris, 3 mM $CaCl_2$, and 400 mM NaCl) at room temperature. A polystyrene cuvette containing 1 mL of the pH 8.3 buffer and 150 mL of S-2586 (1.00 mM in H_2O) was prepared. The cuvette was angled, and 20 mL of chymotrypsin (1−5 mM) in pH 7.4 buffer (50 mM Tris and 150 mM NaCl) was added to the side of the cuvette. The enzyme and substrate were mixed by shaking the capped cuvette rapidly. The rate of change of the solution's absorbance at 402 nm (λ_{max} *p*-nitroaniline (pNA) $\epsilon = 10\,400$) was measured for 70 s. Michaelis−Menten kinetics describe the rate of pNA formation as described above.

Thrombin. Human thrombin (T-7009) was purchased from Sigma Chemical Co. The activity of thrombin was measured by chromogenic assay using S-2238 (H-D-Phe-Pip-Arg-pNA − 2HCl, K_m= 27.8 mM, $k_{cat} = 56.6$ s^{-1})[7] in pH 8.3 buffer (30 mM Tris, 3 mM $CaCl_2$, and 400 mM NaCl) at room temperature. A polystyrene cuvette containing 1 mL of the pH 8.3 buffer and 150 mL of the chromogenic substrate (1.00 mM in H_2O) was prepared and mixed thoroughly. The cuvette was angled, and 20 mL of enzyme solution was added to the side of the cuvette. The enzyme and substrate were mixed by shaking the capped cuvette rapidly three times before being placed in a UV−vis spectrophotometer. The rate of change of the solution's absorbance at 402 nm was measured for periods ranging between 15 and 120 s. Michaelis−Menten kinetics describe the rate of pNA formation as described above.

Factor X_a. Bovine Factor X_a (F-2027) was purchased from Sigma Chemical Co. The activity of Factor X_a was measured by chromogenic assay using S-2222 (Bz-Ile-Glu-Gly-Arg-pNA − HCl, K_m= 30.0 mM, $k_{cat} = 100$ s^{-1})[7] in pH 8.3 buffer (30 mM Tris, 3 mM $CaCl_2$, and 400 mM NaCl) at room temperature. A polystyrene cuvette containing 1 mL of the pH 8.3 buffer and 150 mL of the chromogenic substrate (1.00 mM in H_2O) was prepared and mixed thoroughly. The cuvette was angled and 20 mL of enzyme solution was added to the side of the cuvette. The enzyme and substrate were mixed by shaking the capped cuvette rapidly three times before being placed in a UV−vis spectrophotometer. The rate of change of the solution's absorbance at 402 nm was measured for periods ranging between 15 and 120 s. Michaelis−Menten kinetics describe the rate of pNA formation as described above.

Inhibitions. Inhibitions were performed by adding an excess of inhibitor dissolved in organic solvent (typically methanol, acetonitrile, or DMSO) to solutions of enzyme. The addition was performed in such a manner that the volume percentage of organic solvent never exceeded 3%. The activity of the resulting solutions was then assayed (as described above) as a function of time. The activity of the preinhibited enzyme solution was assigned a value of 100%. All inhibitions were performed in the dark at ambient temperature.

Acyl Enzyme Purification. In cases where a stable acyl enzyme was formed, as determined by plot of enzyme activity versus time following addition of an inhibitor, the acyl enzyme was isolated by gel filtration. The gel column was prepared by suspending Sephadex G-25 in the desired buffer and pouring into a plastic column. Ten milligrams of BSA was eluted through the column to block any nonspecific protein binding sites in the column. Eluent was collected until A_{280} was less than 0.005. The acyl enzyme solution was then loaded onto the top of the column and eluent passed through the column as fractions were collected. Each fraction was monitored by UV−vis spectroscopy for absorbance at 280 nm. Each fraction was approximately 1.0 mL and was collected into disposable polystyrene cuvettes. Fractions containing significant quantities of protein were collected and combined.

Photoactivation. Samples of acyl enzyme were irradiated by one of the two methods described below.

Method 1: Samples to be irradiated were placed either in plastic pipet tips or acrylic cuvettes and irradiated with a 4 W 366 nm lamp (Spectronics Corp., Westbury, NY). The gain of enzymatic activity was measured by chromogenic assay (as described above) on aliquots removed from the sample over time or, in the case of pipet tip photoactivations, the entire sample was assayed.

Method 2: Samples were placed in a polystyrene UV−vis cuvette and irradiated by a 1000 W Hg/Xe arc lamp, equipped with grating monochromoter, at a distance of 100 cm. The monochromoter was set to 366 nm with a 20 nm bandwidth. At specified times, 20 μL of the sample was removed and assayed.

Avidin Affinity Chromatography.[22−24] Samples containing potentially biotinylated protein were purified via affinity chromatography. The column was prepared by first blocking nonspecific or nonreversible binding sites by eluting 3 × 2 mL of pH 7.2 PBS containing 2 mM biotin through the column. Next, pH 2.8 glycine (0.1 M) buffer was eluted through the column (3 × 4 mL). The buffer in which the sample was dissolved was then passed through the column (4 × 4 mL). The sample was loaded onto the column in a 2 mL volume. Fractions were collected by stepwise addition of 2 mL eluent onto the column. UV absorbance was monitored. After at least five fractions were collected the eluent was switched to pH 6.0 PBS containing 2 mM biotin. Once all of the protein was washed off of the column it was regenerated by elution with glycine buffer.

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Supporting Information Available: Experimental procedures and characterization data for compounds **7** and **14**−**17** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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