New Mechanism-Based Inactivators of Trypsin-like Proteinases. Selective Inactivation of Urokinase by Functionalized Cyclopeptides Incorporating a Sulfoniomethyl-Substituted *m*-Aminobenzoic Acid Residue

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In order to obtain selective suicide substrates of trypsin-like proteases including plasminogen activators, plasmin, and thrombin, a series of cyclopeptides cyclo[Arg or Lys-aB(CH$_2$X)-Gly$_4$], in which a substituted *o*- or *m*-aminobenzoyl group constitutes a latent electrophile, have been prepared. Treatment of the corresponding phenyl ethers cyclo[P$_1$-aB(CH$_2$OC$_6$H$_5$)-Gly$_4$] with HBr/HOAc or R^{1}R^{2}S/TFA gives the bromides (X = Br) or the sulfonium salts (X = $^+$SR^{1}R^2 with R^1 = R^2 = Me or R^1 = Me and R^2 = C$_6$H$_5$), respectively. These water-soluble cyclopeptides behave as time-dependent inhibitors of bovine trypsin and human urokinase (u-PA) but have no effect on tissue plasminogen activator (t-PA) and no or poor effect on plasmin and thrombin. The compounds containing a *m*-aminobenzoic acid residue are more efficient inactivators than their anthranilic analogues. The kinetic criteria expected for a suicide inhibition are met. A mechanism of inhibition involving the formation of a quinonimmonium methide intermediate is proposed. The activity of the inhibitors is very sensitive to the nature of the X benzylic substituent. An increased efficiency for the inactivation of human urokinase is observed with the sulfonium salts. The selectivity of the inactivation of u-PA compared to t-PA could be of therapeutical significance in controlling cell proliferation and invasion.

Serine proteinases play important roles in numerous physiological processes, and some of them are involved in pathological states. As such, there is a considerable interest in the development of efficient and selective low-molecular-weight synthetic serine proteinases inhibitors for fundamental studies of enzyme mechanisms, in vivo biochemical investigations, and development of potential therapeutic drugs.[1-5] Thrombin, plasminogen activators, and plasmin are particularly interesting targets since these trypsin-like proteinases are implicated in several pathological states such as coagulation or thrombolytic disorders and tumor invasion.[6,7]

Mechanism-based inactivators ("suicide" substrates), which generate reactive species exclusively at the active site of a target enzyme, are expected to display a maximum selectivity since their inhibitory activity requires discrimination in binding steps, catalytic activation by the enzyme, and irreversible modification of the active center.[8] Previously, a number of heterocyclic structures including enol lactones,[9] ynenol lactones,[10] halomethylated dihydrocoumarins,[11-15] isocoumarins,[16] and β-lactams[17,18] have been shown to be suicide substrates of serine proteases. Functionalized 3,4-dihydrocoumarins, such as A (Y = O) (Figure 1), were the first efficient general inhibitors of this type, but were not very selective.[13] Functionalized 3,4-dihydroquinolinones A (Y = NH) were inefficient, probably because of the s-cis configuration of the amide bond imposed by the six-membered ring.[19] Therefore we considered their macrocyclic analogs B, in which the scissile amide bond may have a s-trans configuration.[20,21] These designed potential "suicide" substrates of serine proteinases display a latent electrophilic quinonimmonium methide function included a cyclopeptide vector. Compared
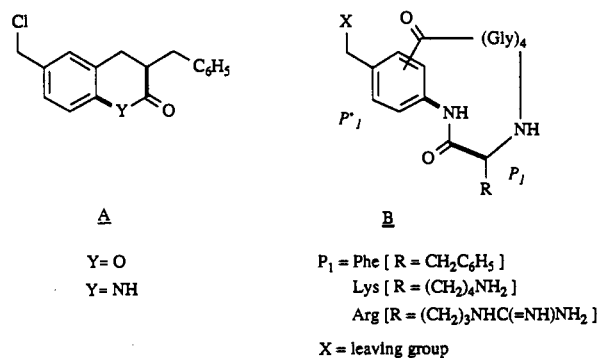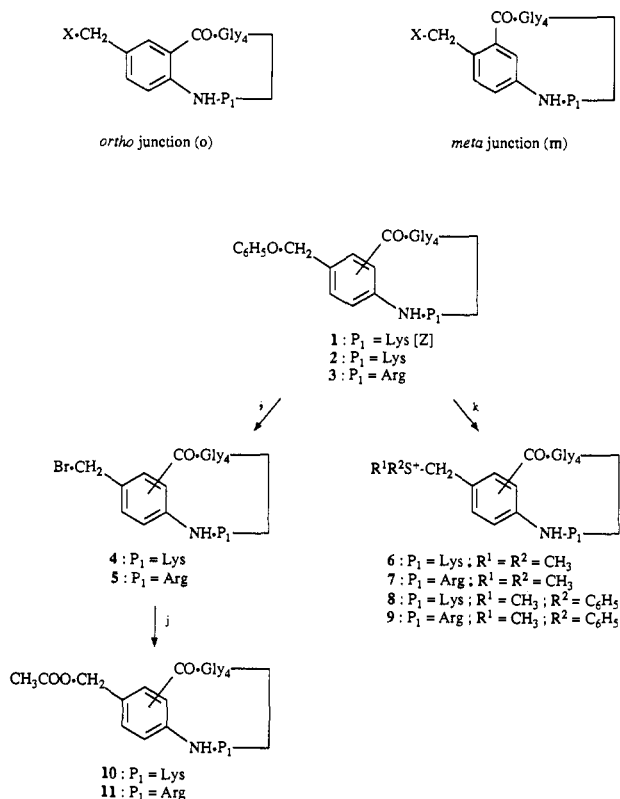

Figure 1. Structure of the functionalized cyclopeptides B compared to the related functionalized 3,4-dihydrocoumarins (A; Y = O) and 3,4-dihydroquinolinones (A; Y = NH).

with simple heterocyclic structures such as A, compounds B have structural similarity with normal peptide substrates. This should contribute to the discrimination in binding steps and thus to a greater selectivity toward a particular protease. The latent electrophile is constituted by a functionalized *o*- or *m*-aminobenzoic acid P'$_1$ residue (*o*-aB[CH$_2$X] or *m*-aB[CH$_2$X]), having a benzylic leaving group X in para position to the nitrogen atom (Figures 1 and 2). This latent electrophile is introduced next to a P$_1$ residue (Schechter and Berger notation)[22] having a good affinity for the S$_1$ binding site of the target proteinase, in order to induce the selective cleavage of the P$_1$–P'$_1$ amide bond which will unmask the electrophile. The cyclic nature of these designed inhibitors should prevent diffusion of the demasked quinonimmonium methide function out of the active site during the lifetime of the acyl enzyme.

Figure 2. Synthesis of the functionalized cyclopeptides: (i) HBr/AcOH, (j) CH$_3$COOK/DMF, (k) R^{1}R^{2}S/TFA.

On this basis, cyclopeptides of type B with P$_1$ = Phe and X = Cl or Br have been shown to be selective irreversible inhibitors of α-chymotrypsin.[21] A study has now been undertaken to extend this type of inhibition toward proteinases of tryptic specificity. Previous experiments using model cyclopeptides with P$_1$ = Lys or Arg and lacking the leaving group (X = H) showed that these compounds are selective substrates of bovine trypsin and human urokinase. The functionalized parent cyclopeptides 2–3 or 10–11 (o and/or m) (Figure 2) containing a benzylic phenoxy or acetoxy group (X = OC$_6$H$_5$ or OCOCH$_3$) were also good substrates of trypsin and urokinase, but failed to inactivate both enzymes.[23] We presumed that substitution of the phenoxy or acetoxy substituents by better leaving groups could transform these substrates into mechanism-based inactivators. In this paper, we report a study on the synthesis and the inhibitory activity toward trypsin, urokinase-type (u-PA) and tissue-type (t-PA) plasminogen activators, plasmin, and thrombin of the functionalized cyclopeptides 4–9 (o and/or m) (Figure 2) with P$_1$ = Arg or Lys and X = Br, $^+$S(CH$_3$)$_2$, $^+$S(CH$_3$)C$_6$H$_5$.

Results

Synthesis. Both series of cyclopeptides incorporating an o-(o) or m-(m) aminobenzoic acid residue were prepared according to the reaction sequence shown in Figure 2. The starting cyclopeptides 1 and 3, functionalized by a phenoxymethyl group (X = OC$_6$H$_5$), were obtained by peptide synthesis in solution.[23]

Treatment of 1o and 1m with HBr/AcOH, conditions used by Trudelle et al.[24] for the cleavage of O-benzyltyrosines, cleaved both the benzyl ether and the Z protecting group of the lysine side chain to give the bromomethyl compounds 4o and 4m. Similarly, the bromides 5 were

obtained from 3. Substitution of the benzylic bromides 4m and 5m by potassium acetate in DMF gave the corresponding acetates 10m and 11m.

The sulfonium groups were introduced upon treatment of the benzylic ethers 1 and 3 by dimethyl sulfide or thioanisole in TFA, according to deprotection conditions of O-benzyltyrosines established by Kiso et al.,[25] which gave the cyclopeptides 6–9, functionalized by either X = Me$_2$S$^+$ or X = C$_6$H$_5$(Me)S$^+$.

Enzymology. Inactivation Kinetics. The functionalized cyclopeptides 4–9, all soluble in water, behaved (except 4o) as time-dependent inhibitors of various trypsin-like proteases, with no significant inactivating effect on porcine pancreatic elastase and bovine chymotrypsin. They displayed large differences in the inactivation efficiency, depending on the nature of the peptidic ring and of the target trypsin-like protease (Table I). The reversible or irreversible character of the inhibition was explored after removing the excess of inhibitor by filtration–centrifugation (Centricon 10). After 16–24-h incubation at 4 °C, the filtered enzymes regained less than 3% of their initial activity. In the same conditions, the spontaneous loss of activity of control samples was less than 5%. Addition of buffered hydroxylamine (0.75 M, pH 8.5, 25 °C) to the inhibited enzymes generally resulted in 0.1% to ≤2% reactivation.

Using the preincubation method under pseudo-first-order conditions (excess inhibitor over enzyme), first-order inactivation processes were observed and characterized by rate constants k_{obs}. In the case of urokinase, inactivation kinetics were usually linear for ~1 and 2–4 half-lives (for X = Br and $^+$SR^{1}R^2, respectively). k_{obs} was obtained from the slope of the initial linear portion of the semilogarithmic plot of enzyme activity (E/E_0) versus time (not shown). Deviations from linear may be attributed to a depletion of inhibitor concentration due to a large partition ratio, a product inhibition, or a too fast inactivation.[8] The preincubation method was used when the first-order rate constants were small enough to be determined with a sufficient degree of accuracy. Saturation kinetics depending on inhibitor concentration were observed (5m, 8m, urokinase), and the double reciprocal plot of k_{obs} against the inhibitor concentration yielded the apparent dissociation constant of an enzyme–inhibitor complex (K_I) and the first-order inactivation rate constant k_{inact} at infinite inhibitor concentration (Figure 3). This is consistent with the minimal kinetic scheme described in eq 1. The ratio k_{inact}/K_I is an apparent second-order rate constant characterizing the inhibitor potency. For poor inactivations, this ratio was obtained as $k_{obs}/[I]$ at low inhibitor concentrations (eq 3). The kinetic parameters k_{inact} and K_I were determined using the progress curve method for the most efficient inhibitors (4m, 5m, 8m, 9m: trypsin; 7m, 9m; urokinase). The experimental data (Figure 4) fit the scheme shown in eq 4, as evidenced by the kinetic analysis of changing slopes with time of the progress curves for the hydrolysis of the chromogenic substrate (BAPNA for trypsin and S-2444 for urokinase), run in the presence of various inhibitor concentrations. In some cases, we verified that a good agreement between the two methods was obtained for the determination of the inactivator potency. For example, for 9m/urokinase, k_{inact}/K_I was found equal to 2170 M^{-1} s^{-1} by the preincubation method and 2330 M^{-1} s^{-1} by the progress curve method.[26]

Table I. Kinetic Parameters for the Inactivation of Bovine Trypsin and Human Urokinase, Plasmin, t-PA, and Thrombin by the Functionalized Cyclopeptides 4–9 at pH 7.5 and 25 °C

| compd | P_1 | X | bovine trypsin | | | urokinase | | | plasmin | t-PA | thrombin |
			k_{inact} (s^{-1})	$10^3 K_I$ (M)	k_{inact}/K_I (M^{-1} s^{-1})	k_{inact} (s^{-1})	$10^3 K_I$ (M)	k_{inact}/K_I (M^{-1} s^{-1})	k_{inact}/K_I (M^{-1} s^{-1})	k_{inact}/K_I (M^{-1} s^{-1})	k_{inact}/K_I (M^{-1} s^{-1})
4o	Lys	Br			NI[a]			NI	NI	NI	NI
4m	Lys	Br	0.02	0.08	250			5[b]	NI	NI	NI
5o	Arg	Br			~5[b]			14	NI	NI	NI
5m	Arg	Br	0.031 (0.041)[c]	0.055 (0.02)[c]	560 (2050)[c]	0.017	0.092	185	~2[b]	NI	NI
6m	Lys	$^+$S(CH$_3$)$_2$			NI			~1[b]	NI	NI	NI
7m	Arg	$^+$S(CH$_3$)$_2$			~1[b]	0.014	0.041	341	NI	NI	NI
8o	Lys	$^+$S(CH$_3$)C$_6$H$_5$			NI			~25[b]	NI	NI	NI
8m	Lys	$^+$S(CH$_3$)C$_6$H$_5$	0.0075	0.0087	862	0.077	0.5	154	75	NI	NI
9o	Arg	$^+$S(CH$_3$)C$_6$H$_5$			50[b]			14[b]	NI	NI	NI
9m	Arg	$^+$S(CH$_3$)C$_6$H$_5$	0.011	0.012	916	0.021[d]	0.009[d]	2330[d]	40[d]	~1[b,d]	~12[b]

K_I is an apparent binding constant determined either by Kitz and Wilson[38] or by Hart and O'Brien[39] analysis and Wilkinson statistical treatment.[40] The error limits are ±15%. [a] NI: no inhibition. [b] Obtained as $k_{obs}/$[I] at low inhibitor concentration. [c] Porcine trypsin. [d] Reference 26.
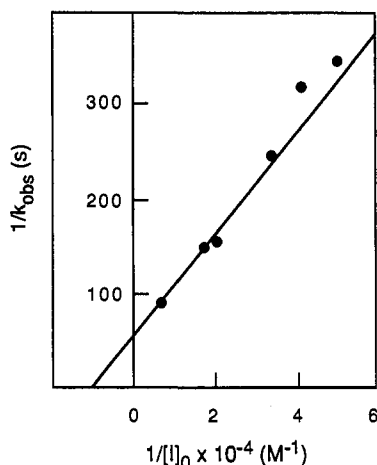

Figure 3. Saturation kinetics for the inactivation of urokinase by the cyclopeptide 5m at 25 °C. Urokinase (55 μM) was incubated with various concentrations of 5m (20–150 μM) in 0.05 M phosphate (pH 7.5), 0.1 M NaCl, 0.05% Tween 80. Aliquots were assayed periodically for catalytic activity using S-2444. The inhibition rate constant k_{obs} was obtained from the slope of the initial linear portion of a semilogarithmic plot of enzyme activity ln (E/E$_0$) versus time (not shown). The double reciprocal plot of k_{obs} versus inhibitor concentrations gives a straight line with an x-intercept of $-K_I$ and an y-intercept of k_{inact}.

For inactivating agents,[27] the rate constants k_{obs} for the inactivation of the enzyme is proportional to the concentration of inhibitor, according to eq 6. The order in the inactivation reaction for the inhibitor can be determined from a plot of k_{obs} versus [I] by fitting the experimental data to the theoritical curve for the inactivation ($k = k_{inact}/K_I$). Tentatively, this kinetic analysis of the reaction of urokinase with 5m, 8m (Figure 5), and 9m was performed, giving respectively n = 1.04, 1.02, and 1.08 (standard error = 0.01). This suggests that urokinase interacts with a single molecule in the course of inactivation and is in agreement with eq 3 at low inhibitor concentration ([I]$_0$ < K_I).

Substrate Protection. The addition of substrates to enzyme inhibition mixtures resulted in a significant decrease in inactivation constants. For example, the rate constant $k_{obs}/$[I] for the inactivation of urokinase (0.66 μM) by 9m (4 μM) was decreased by a factor of 1.6 in the presence of 260 μM S-2444, compared to that obtained in the absence of chromogenic substrate. A protection by leupeptin of the inactivation of trypsin by 5m was also

observed. This was confirmed with competitive substrate assays: it was verified that increasing amounts of substrate S-2444 (or BAPNA) at fixed inhibitor concentrations protected urokinase (trypsin) against inactivation by 7m, 9m (4m, 5m, 8m, 9o,m).

Ultimate Activity Assays. The inactivation efficiency of some cyclopeptides was characterized by determination of the partition ratio, which represents the average number of "enzyme turnovers per inactivation".[8] The intercept (–1, assuming a 1:1 stoichiometry) with the x axis of the linear plot[28] of the fraction of enzyme activity [E]/[E]$_0$ at infinite time versus the molar excess of inhibitor over enzyme [I]$_0$/[E]$_0$ gives the partition ratio (Figure 6). The partition ratio for the inactivation of bovine trypsin by 8m and 9m is 17 and 19, respectively. Deviations from linearity are attributed to product inhibition or product protection of the enzyme toward further inactivation.[8]

Discussion

Inactivation Mechanism. The present cyclopeptides were designed on the basis of the "suicide" inactivation process occurring through the postulated mechanism depicted in Figure 7. It first implies that the active serine of the proteinase selectively reacts with the carbonyl group of the P_1 residue of the cyclopeptide to form an acyl enzyme. A second crucial step is the unmasking of a latent quinonimmonium methide electrophile in the active site. Activation of the benzylic function results from the transformation of a *p-amido*benzylic P_1' fragment in the starting molecule, into a *p-amino*benzylic one in the acyl enzyme.[29] Owing to the strong releasing effect of a *p*-amino substituent (σ^+ = –1.30),[30] the nucleophilic substitution of the benzylic group by an active site nucleophile Nu may occur through an elimination–addition process.[20] Therefore, the nature of the leaving group X, on which depends the rate of elimination of X$^-$, is expected to play a crucial role in the efficiency of the inhibition.

The inactivation of the tested trypsin-like enzymes fulfills the criteria expected for a suicide-type inactivation: first-order loss of enzyme activity and saturation kinetics. The inactivation process is irreversible since no reactivation of the inhibited enzymes, either spontaneously or after treatment by hydroxylamine, is observed. The latter observation rules out an inhibition through the formation of a stable acyl enzyme. A synthetic substrate (or a reversible inhibitor: leupeptin for trypsin) protects urokinase and trypsin against inactivation by the func-
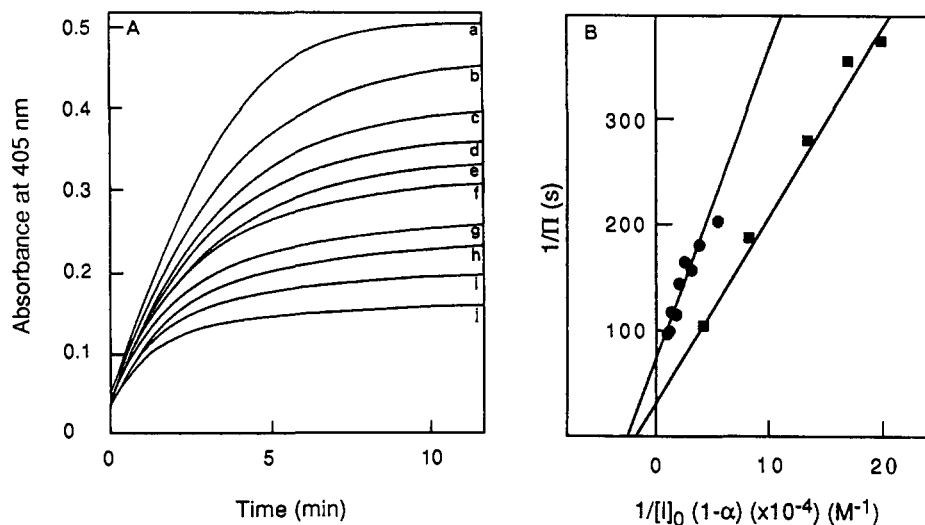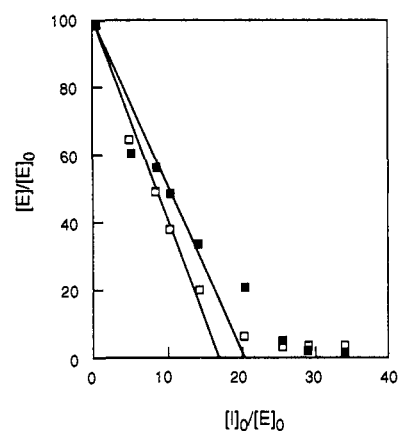
Figure 4. (A) Progress curves for the inhibition of human urokinase by 7m in the presence of the chromogenic substrate S-2444 at 25 °C. Absorbance at 405 nm was recorded in phosphate buffer solution (0.05 M, pH 7.5, 0.1 M NaCl, 0.05% Tween 80) containing 31 nM urokinase, 58.5 μM S-2444, and the following concentrations of 7m: 0 (a), 43.75 (b), 62.5 (c), 75 (d), 93.75 (e), 125 (f), 156 (g), 187.5 (h), 250 (i), and 312.5 μM (j). (B) Determination of the inactivation kinetic parameters k_{inact} and K_I using the competitive substrate assay of human urokinase (a) and bovine trypsin (b) in the presence of the inhibitors 7m and 5m, respectively. The experimental conditions for 7m are given above, and for 5m were 0.1 M Tris, pH 7.2, 0.01 M CaCl$_2$, 0.5 μM trypsin, 0.3 mM BAPNA, 6–30 μM 5m. The first derivative of the progress curves (velocity) as shown in A were obtained directly from a computer-assisted spectrophotometer. A semilogarithmic plot of these velocities versus time gives a straight line with a slope $-\pi$ depending on the inhibitor concentration. A plot of the inverse of π versus the inverse of $[I](1 - [S]/K_M + [S])$ gives a straight line with an x-intercept of $-K_1$ and an y-intercept of k_{inact}.
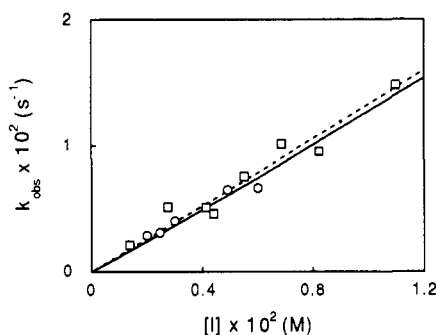
Figure 5. Plots of the first-order inactivation rate constants of human urokinase versus inhibitor concentrations at pH 7.5 and 25 °C. Experimental points: (O) 5m, (□) 8m; theoritical curves generated by eq 6 and the k_{inact}/K_1 values from Table I: (–) 5m, (- -) 8m.

Figure 6. Ultimate activity assay. Bovine trypsin (7 μM) was incubated at 25 °C and pH 7.5, with various concentrations of 8m (□) and 9m (■). After incubation to completion, residual enzyme amidolytic activity was determined on the chromogenic substrate BAPNA.

tionalized cyclopeptides. This demonstrates that the chemical modification occurs at the active site. Treatment of the observed rate constants of inactivation according to the method of Levy et al.[27] gives an order of 0.8–0.9 for the tested cyclopeptides when they react with urokinase, suggesting a stoichiometry approaching 1:1. The partition ratio can be used to characterize the efficiency or selectivity of a suicide substrate.[28] For the sulfonium 9m, a better result is obtained toward urokinase than toward trypsin (partition ratios of 4 and 19, respectively). No significant change of the partition ratio is observed when an arginine residue is substituted by a lysine one in the peptidic ring (values of 17 and 19 for 8m and 9m toward trypsin, respectively).

A mechanism-based inhibition process is also supported by the behaviour as *substrates* of the corresponding unfunctionalized compounds: we have shown previously that the ortho or meta cyclopeptides with P_1 = Lys or Arg and containing no leaving group (X = H) are efficiently cleaved by trypsin and urokinase. A specific cleavage at the P_1–P'_1 (aB) bond has also been demonstrated.[23] Thus,

one can assume that the same initial cleavage occurs in the course of inactivation by the structurally related functionalized cyclopeptides. However, the occurrence or the absence of inhibition is also directly related to the nature of the leaving group X. It has been observed that the cyclopeptides containing poorer leaving groups, phenolate and acetate, only behave as substrates.[23] The fact that only introduction of better leaving groups, bromide or sulfide, results in inactivation, is in agreement with a faster unmasking of the quinonimmonium methide electrophile in the active site. The opportunity, in the present system, to synthesize and compare structurally related substrates and inhibitors may be emphasized. So far, inactivation of a given protease by a functionalized cyclopeptide with X = Br or $^+SR^1R^2$ is only observed when the parent compounds with X = H and/or OC$_6$H$_5$, OCOCH$_3$ act as substrates of the same enzyme.
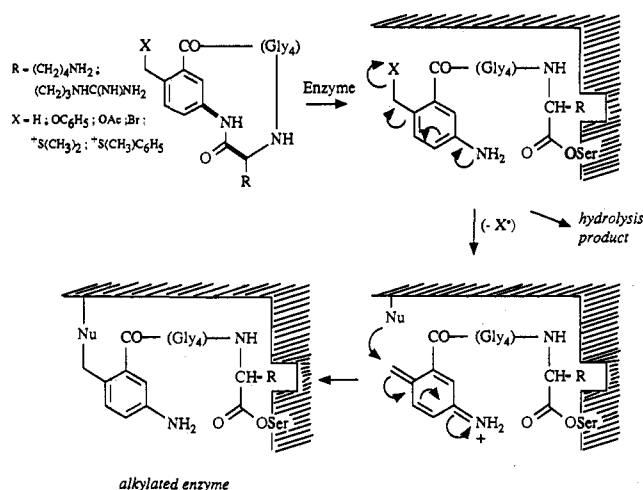
Figure 7. Postulated mechanism for mechanism-based inactivation of trypsin-like serine proteinases by functionalized cyclopeptides 4m–9m containing a good leaving group X = Br or $^+SR^1R^2$.

Influence of the Ortho vs Meta Junction of the P'_1 Aminobenzoic Residue and of the Nature (Arg vs Lys) of the P_1 Residue. Table I shows that for a same X group, the meta cyclopeptides are generally more reactive inhibitors than the corresponding ortho compounds. The k_{inact}/K_I constants (meta vs ortho) differ by factors of 112 (Arg; Br; trypsin), 13 (Arg; Br; urokinase), 18 (Arg; ^+S-$(CH_3)C_6H_5$; trypsin), and 166 (Arg; $^+S(CH_3)C_6H_5$; urokinase). In a similar manner, substitution of the P_1 lysine residue by an arginine leads to a much more efficient inactivation of both trypsin and urokinase. Thus, the expected preference of urokinase for an arginine P_1 residue[31] is observed. On the other hand, plasmin is slightly better inactivated by 8m (P_1 = Lys) than by 9m (P_1 = Arg).

Influence of the X Group. Reactivity–Selectivity Properties of the Sulfonium-Containing Cyclopeptides. As far as reactivity for inactivation of trypsin and urokinase is concerned, all meta cyclopeptides except 6m have k_{inact}/K_I constants in the range 10^2–10^3 M^{-1} s^{-1} (Table I). This is still at a lower level than the known best mechanism-based inactivators of trypsin-like enzymes, in which the enzyme-catalyzed bond cleavage involves an ester rather than an amide bond: 3-benzyl-6-(chloromethyl)-3,4-dihydrocoumarin[12,14,32] or more recently the excellent 3-alkoxy-4-chloro-7-guanidinoisocoumarins[33] have $k_{obs}/[I]$ constants in the range 10^3–10^4 and 10^3–10^5 M^{-1} s^{-1}, respectively. However, the present results show that what is lost so far in reactivity is regained in selectivity. From that point of view, the sulfonium-containing meta cyclopeptides are especially interesting, since the presence of the $^+SR^1R^2$ group enhances the selectivity for urokinase. For P_1 = Lys, the ratio r = $[k_{inact}/K_I]_{urokinase}/[k_{inact}/K_I]_{trypsin}$ relative to the inactivation of urokinase versus trypsin varies from 0.02 for 4m (X = Br) to ~0.2 for 8m (X = ^+S $(CH_3)C_6H_5$). For P_1 = Arg, this ratio is even reversed, varying from r = 0.3 for X = Br (5m) to r = 2.5 (9m) and ~340 (7m) for X = $^+SR^1R^2$. Altogether, the sulfonium-containing cyclopeptides 7m and 9m not only distinguish the family of trypsin-like enzymes from serine proteinases of different specificity, chymotrypsin and elastase, as expected from the choice of their P_1 arginine residue, but also combine a reasonable reactivity with a good selectivity in the inactivation of urokinase. Comparison between 7m, 9m, and 3-benzyl-6-(chloromethyl)-3,4-dihydrocoumarin[12]

(compound A; X = O in Figure 1) as inactivators of urokinase, t-PA, plasmin, and thrombin is instructive: respective k_{inact}/K_I values (M^{-1} s^{-1}) of 341/0/0/0 (7m), 2330/~1/40/12 (9m),[26] and 2100/187/136/30000 (dihydrocoumarin)[14,32] are observed. The sulfonium 7m is also highly selective for urokinase versus trypsin.

The two forms of plasminogen activators, t-PA and u-PA (urokinase), convert plasminogen into the broad spectrum protease plasmin. However, they appear to have different biological functions. The primary role of t-PA is dissolution of blood clots in vessels whereas urokinase is believed to be primarily involved in extracellular proteolysis including normal and pathological tissue destruction.[34] Evidences for the involvement of urokinase on cell proliferation and invasion have been reported.[35] Thus, a selective synthetic inactivator of urokinase with no effect on t-PA could behave as an antimetastatic agent.

The easy access to the sulfonium-containing from the phenoxy-containing cyclopeptides by treatment with aryl alkyl or dialkyl sulfides in TFA allows structural modifications of the sulfur substituents, which might further improve the reactivity/selectivity properties of such inhibitors. Sulfonium group has been seldom considered in protein modification studies: Horton and Tucker[36] have used 2-hydroxy-5-nitrobenzyl dimethyl sulfonium salts as tryptophan-modifying agent. More recently, Shaw has demonstrated the interest of peptidyl sulfonium salts as a new class of cysteine proteases inhibitors, clearly pointing out the opportunity to take advantage of the departing group region of the active center for possible increases in affinity or selectivity.[37]

Conclusion

J. C. Powers has recently stated the following concluding remarks about the current mechanism-based inactivators: "It is clear that small molecular-weight heterocyclic molecules can be very effective inhibitors for serine proteases. However, it is currently difficult to introduce absolute specificity into small-molecule non peptide inhibitors. This is especially true for the family of trypsin-like enzymes, which contains many members with similar active site structures and primary substrate specificity".[33] We believe that the present new class of mechanism-based inhibitors in which the latent electrophile is included in a cyclopeptidic vector might be a convenient solution to the selectivity problem. The initial model compounds, designed in view of a selective inactivation of trypsin-like enzymes over other serine proteinases of different specificity, behave as expected. These functionalized cyclopeptides possess interesting features for future developments: (i) They are soluble in water. (ii) Their cyclic nature avoids possible degradation by amino- or carboxypeptidases. (iii) They are subject to easy structural modifications and thus can be designed to inactivate a large variety of proteases. Possible structural modifications concern change of the ring size by introduction of more or less amino acid residues, and change of the leaving group including possibilities of modulation of the alkyl/aryl substituents of sulfoniums. (iv) They are subject to variation of the peptidic sequence, which is of main interest since replacement of the Gly_4 chain of the present cyclopeptides by selected amino acid residues P_n–P_2 according to the secondary binding subsites S_n–S_2 of a target protease should likely allow discrimination between members of the same family of trypsin-like enzymes. The

fibrinolytic and coagulation enzymes are interesting targets for our inhibitors, and the above mentioned structural parameters are currently explored in search of an increased potency.

Experimental Section

Synthetic Procedures. Melting points were recorded on a Tottoli (Büchi) or a Mettler FP61 and are uncorrected. The ^{1}H NMR measurements were performed on a Brücker WM 300 instrument. The chemical shifts are reported in ppm, the deuterated solvents being used as internal standards: D_2O (4.80 ppm) and CD_3COOD (2.10 ppm). The coupling constants are given in hertz. The optical rotations were obtained on a Perkin-Elmer 241 polarimeter. Analyses were performed by the Services of CNRS at ICSN (Gif sur Yvette). The mass spectrometry measurements were performed by the Services of Pr. J. C. Tabet (Univ. Paris 6), Dr. A. Deroussent (Inst. G. Roussy, Villejuif), and for the FAB HRMS data, Dr. J. Favre-Bonvin (CNRS, Vernaison). Analytical thin-layer chromatography (tlc) and preparative column chromatography were performed on Kieselgel F 254 and on Kieselgel 60, 0.063–0.5 mm (Merck), respectively, with the following solvent systems (by vol.): I(x/y), MeOH $x\%$–CH_2Cl_2 $y\%$; II, AcOEt-n-BuOH-AcOH-H_2O (1/1/1/1); III, AcOEt-cyclohexane, 1/4; IV, CH_2Cl_2-MeOH-H_2O-AcOH (60/30/6/2).

Cyclo(-Lys-o-aB[CH$_2$Br]-Gly$_4$-) (4o). The cyclopeptide 1o[23] (0.100g; 0.14 mmol) was dissolved in warm AcOH (2.5 mL), the solution was cooled to room temperature, and a solution of 33% HBr/AcOH (2.5 mL) was added. The reaction mixture was stirred at room temperature for 1 h. Addition of ether (~75 mL) resulted in precipitation of a white solid. The precipitate was decanted and the supernatant discarded. This operation was repeated 8 to 10 times, until the supernatant ether was free of HBr and AcOH. The precipitate was then dried in vacuo at 25 °C to yield 0.100 g (100%) of bromide 4o as a white powder. Mp: 140 °C dec. R_f = 0.59 (II). ^{1}H NMR (D_2O): 7.66, d (1.9), 1 H (ArH); 7.60, dd (2.1; 8.3), 1 H (ArH); 7.43, d (8.3), 1 H (ArH); 4.60, m, 1 H (CH$^\alpha$ Lys); 4.57, s, 2 H (CH$_2$Br); 4.37, d (17.4), 1 H and 3.77, d (17.6), 1 H (CH$_2$ Gly); 4.34, d (17.5), 1 H and 3.89, d (17.9), 1 H (CH$_2$ Gly); 4.16, d (17.6) and 3.93, d (17.9), 2 H (CH$_2$ Gly); 3.90, s and 3.89, s, 2 H (CH$_2$ Gly); 2.98, m (t-like, 7.4), 2 H (CH$_2^\epsilon$ Lys); 1.9, m, 1.6, m, and 1.4, m, 6 H (CH$_2^{\beta,\gamma,\delta}$ Lys). [α]^{25} (c 0.13; H_2O) = +17.7° (589); +20.8° (578); +23.1° (546); +36.1° (436); +53.1° (365). MS (FAB$^+$): 570 (M, H$^+$ for ^{81}Br); 568 (M, H$^+$ for ^{79}Br). Anal. Calcd for C$_{22}$H$_{30}$N$_7$O$_6$Br·2HBr·H$_2$O: C, 35.31; H, 4.58; N, 13.10. Found: C, 35.58; H, 4.87; N, 13.12.

Cyclo(-Lys-m-aB[CH$_2$Br]-Gly$_4$-) (4m): obtained as above from 1m[23] (0.140g, 0.2 mmol) in AcOH (3.6 mL) and 33% HBr/AcOH (3.6 mL). Yield: 0.144 g (100%), white powder. Mp: 175–180 °C dec. R_f = 0.48 (II). ^{1}H NMR (D_2O): 7.67, dd (2.2; 8.4), 1 H (ArH); 7.50, d (8.4), 1 H (ArH); 7.40, d (2.2), 1 H (ArH); 4.72, d (10.4), 1 H and 4.63, d (10.4), 1 H (CH$_2$Br); 4.25, dd (5.7, 8.7), 1 H (CH$^\alpha$ Lys); 4.15–3.85, m, 8 H (CH$_2$ Gly); 2.96, m (t-like, 7.6), 2 H (CH$_2^\epsilon$ Lys); 1.87, m, 1.68, m, and 1.48, m, 6 H (CH$_2^{\beta,\gamma,\delta}$ Lys). [α]^{25} (c 0.11; H_2O) = +24.5° (589); +25.4° (578); +22.7° (546); –30.91° (436). MS (FAB$^+$): 570 (M, H$^+$ for ^{81}Br); 568 (M, H$^+$ for ^{79}Br); 488 (M, H$^+$ – HBr). HRMS (FAB$^+$): calcd for [C$_{22}$H$_{30}$N$_7$O$_6$Br,H$^+$] 568.1519 (^{79}Br), found 568.1508; calcd for [C$_{22}$H$_{30}$N$_7$O$_6$Br, H$^+$ – HBr] 488.2257, found 488.2275.

Cyclo(-Arg-o-aB[CH$_2$Br]-Gly$_4$-) (5o): obtained as above from 3o[23] (0.060 g, 0.1 mmol) in AcOH (1.8 mL) and 33% HBr/AcOH (1.8 mL). Yield: 0.045 g (67%), white powder. Mp: 190–210 °C dec. R_f = 0.61 (II). ^{1}H NMR (D_2O): 7.68, d (2), 1 H (ArH); 7.60 dd (2; 8.3), 1 H (ArH); 7.45, d (8.3), 1 H (ArH); 4.59, s, 2 H (CH$_2$Br); 4.6, m (masked), 1 H (CH$^\alpha$ Arg); 4.4–3.7, m, 8 H (4 CH$_2$ Gly); 3.20, m, 2 H (CH$_2^\delta$ Arg); 1.94, m and 1.60, m, 4 H (CH$_2^{\beta,\gamma}$ Arg). [α]^{25} (c 0.55; H_2O) = +27.3° (589); +27.3° (578); +30.9° (546); +47.3° (436); +80.1° (365). MS (FAB$^+$): 599 (M, H$^+$ for ^{81}Br); 597 (M, H$^+$ for ^{79}Br).

Cyclo(-Arg-m-aB[CH$_2$Br]-Gly$_4$-) (5m): obtained as above from 3m[23] (0.122 g, 0.2 mmol) in AcOH (3.6 mL) and 33% HBr/AcOH (3.6 mL). Yield: 0.128 g (100%), white powder. Mp: 130–140 °C dec. R_f = 0.53 (II). ^{1}H NMR (D_2O): 7.65, dd (2.2; 8.4), 1 H (ArH); 7.48, d (8.5), 1 H (ArH); 7.40, d (2.2), 1 H (ArH); 4.70, d (10.6), 1 H and 4.63, d (10.5), 1 H (CH$_2$Br); 4.25, dd (5.8;

8.5), 1 H (CH$^\alpha$ Arg); 4.1–3.9, m, 8 H (4 CH$_2$ Gly); 3.18, m (t-like, 6.7), 2 H (CH$_2^\delta$ Arg); 2.0–1.6, m, 4 H (CH$_2^{\beta,\gamma}$ Arg). [α]^{25} (c 0.16; H_2O) = –16.3° (589); –16.9° (578); –21.3° (546); –46.9° (436); –95.6° (365). MS (FAB$^+$): 570 (M, H$^+$ for ^{81}Br); 568 (M, H$^+$ for ^{79}Br); 516 (M, H$^+$ – HBr). HRMS (FAB$^+$): calcd for [C$_{22}$H$_{30}$N$_9$O$_6$-Br, H$^+$ – HBr] 516.2319, found 516.2361.

Cyclo(-Lys-m-aB[CH$_2$OCOCH$_3$]-Gly$_4$-) (10m). A mixture of bromide 4m (0.100 g, 0.154 mmol) and potassium acetate (0.038 g, 0.385 mmol) in DMF (4 mL) was stirred at room temperature for 2 h. After filtration, the solution was evaporated to dryness in vacuo. Column chromatography of the residue (SiO$_2$; eluent II), followed by evaporation of the product containing pooled fractions, dissolution of the residue in methanol, filtration, concentration to ca. 3 mL, precipitation with ether, and treatment of the precipitate as before (centrifugation, repeated washings with ether, and drying), yielded 0.032 g (34%) of acetate 10m as a white powder. Mp: 200 °C dec. R_f = 0.4 (II). ^{1}H NMR (D_2O): 7.71, dd (2.2; 8.3), 1 H (ArH); 7.52, d (8.4), 1 H (ArH); 7.43, d (2.2), 1 H (ArH); 5.27, d (12.7), 1 H and 5.18, d (12.7), 1 H (CH$_2$-OAc); 4.27, dd (5.7; 8.7), 1 H (CH$^\alpha$ Lys); 4.16, d (16.3) and 4.07, d (16.4), 2 H (CH$_2$ Gly); 4.15, d (17.2) and 3.97, d (16.6), 2 H (CH$_2$ Gly); 4.09, d (17.1) and 3.95, d (17.2), 2 H (CH$_2$ Gly); 3.99, d (16.3) and 3.91, d (16.1), 2 H (CH$_2$ Gly); 2.96, m (t-like, 7.5), 2 H (CH$_2^\epsilon$ Lys); 2.05, s, 3 H (OAc); 2.05–1.75, m, 2 H (CH$_2^\beta$ Lys); 1.87, s, 3 H (AcO–); 1.69, m, 2 H (CH$_2^\delta$ Lys); 1.51, m, 2 H (CH$_2^\gamma$ Lys). [α]^{25} (c 0.26; D_2O) = –37.0° (589); –37.0° (578); –41.5° (546); –80.0° (436); –150.9° (365). MS (FAB$^+$): 548 (M, H$^+$); 570 (M, Na$^+$); 586 (M, K$^+$); 488 (M, H$^+$ – AcOH). HRMS (FAB$^+$): calcd for [C$_{24}$H$_{33}$N$_7$O$_8$, H$^+$ – AcOH] 488.2257, found 488.2272.

Cyclo(-Arg-m-aB[CH$_2$OCOCH$_3$]-Gly$_4$-) (11m): obtained as above from the bromide 5m (0.100 g, 0.148 mmol). Yield: 0.069 g (73%), white powder. Mp: 224 °C dec. R_f = 0.55 (II). ^{1}H NMR (D_2O): 7.71, dd (2.2; 8.3), 1 H (ArH); 7.52, d (8.4), 1 H (ArH); 7.43, d (2.2), 1 H (ArH); 5.27, d (12.7), 1 H and 5.19, d (12.7), 1 H (CH$_2$OAc); 4.28, dd (5.8; 8.4), 1 H (CH$^\alpha$ Arg); 4.16, d (16.4) and 4.07, d (16.4), 2 H (CH$_2$ Gly); 4.15, d (17.2) and 3.97, d (17.2), 2 H (CH$_2$ Gly); 4.09, d (17.2) and 3.96, d (17.2), 2 H (CH$_2$ Gly); 3.99, d (16.3) and 3.92, d (16.3), 2 H (CH$_2$ Gly); 3.22, m (t-like, 6.7), 2 H (CH$_2^\delta$ Arg); 2.05, s, 3 H (OAc); 2.05–1.75, m, 2 H (CH$_2^\beta$ Arg); 1.88, s, 3 H (AcO–); 1.72, m, 2 H (CH$_2^\gamma$ Arg). [α]^{25} (c 0.13; D_2O) = –25.2° (589); –27.3° (578); –31.6° (546); –60.3° (436); –115.3° (365). MS (FAB$^+$): 576 (M, H$^+$); 614 (M,K$^+$). HRMS (FAB$^+$): calcd for [C$_{24}$H$_{33}$N$_9$O$_8$,H$^+$] 576.2530, found 576.2525.

Cyclo(-Lys-m-aB[CH$_2$S$^+$(CH$_3$)$_2$]-Gly$_4$-) (6m): To a mixture of peptide 1m[23] (0.100 g, 0.14 mmol) and dimethyl sulfide (1 mL, 14 mmol) was added TFA (4.5 mL). The solution was stirred at room temperature for 48 h. Addition of a large excess of ether led to precipitation of a white solid. The precipitate was collected by centrifugation, repeatedly washed with ether, and then centrifuged, dried, dissolved in acetic acid (ca. 2 mL), and chromatographed on preparative TLC plates (SiO$_2$; eluent II). Extraction with solvent mixture II, followed by filtration, complete evaporation of the solvents in vacuo at 25–30 °C, dissolution of the residue in ca. 2 mL of TFA, precipitation with ether, repeated washings of the precipitate with ether followed by centrifugation, and drying in vacuo at 25 °C, yielded 0.054 g (49%) of sulfonium 6m. Mp ~ 120 °C. R_f = 0.10 (II). ^{1}H NMR (CD$_3$COOD): 8.34, dd (1.9; 8.4), 1 H (ArH); 7.96 , d (2.1), 1 H (ArH); 7.64, d (8.6), 1 H (ArH); 4.89, d (13.5), 1 H and 4.84, d (13.5), 1 H (CH$_2$S$^+$); 4.61, dd (4.6; 9.5), 1 H (CH$^\alpha$ Lys); 4.38–4.05, m, 8 H (4 CH$_2$ Gly); 3.16, m (t-like, 7.4), 2 H (CH$_2^\epsilon$ Lys); 3.01, s and 3.00, s, 6 H (CH$_3$S$^+$); 2.1–1.6, m, 6 H (CH$_2^{\beta,\gamma,\delta}$ Lys). [α]^{25} (c 0.07; AcOH) = –35.0° (589); –35.4° (578); –46.2° (546); –81.6° (436); –163.3° (365). MS (FAB$^+$): 550 (M$^+$); 488 (M$^+$ – Me$_2$S). HRMS (FAB$^+$): calcd for [C$_{24}$H$_{36}$N$_7$O$_6$S$^+$] 550.2448, found 550.2499; calcd for [C$_{24}$H$_{36}$N$_7$O$_6$S$^+$ – Me$_2$S] 488.2257, found 488.2280.

Cyclo(-Arg-m-aB[CH$_2$S$^+$(CH$_3$)$_2$]-Gly$_4$-) (7m): obtained as above from the peptide 3m[23] (0.100 g, 0.15 mmol) and dimethyl sulfide (1.2 mL, 16.4 mmol) in TFA (4.6 mL). Yield: 0.067 g (56%), white powder. Mp: 140–150 °C dec. R_f = 0.12 (II). ^{1}H NMR (CD$_3$COOD): 8.29, d (broad; ~ 8.4), 1 H (ArH); 8.08, s (broad), 1 H (ArH); 7.64, d (8.3), 1 H (ArH); 4.87, s (broad), 2 H (CH$_2$S$^+$); 4.62, m, 1 H (CH$^\alpha$ Arg); 4.38–4.14, m, 8 H (4 CH$_2$ Gly); 3.34, m, 2 H (CH$_2^\delta$ Arg); 3.00, s, 6 H (CH$_3$S$^+$); 2.1–1.8, m, 4 H

(CH$_2^{\beta,\gamma}$ Arg). [α]^{25} (c 0.08; AcOH) = $-31.1°$ (589); $-31.7°$ (578); $-36.5°$ (546); $-71.8°$ (436); $-137.6°$ (365). MS (FAB$^+$): 578 (M$^+$); 539 (M, Na$^+$ – Me$_2$S); 516 (M$^+$ – Me$_2$S). HRMS (FAB$^+$): calcd for [C$_{24}$H$_{36}$N$_9$O$_6$S$^+$] 578.2509, found 578.2675; calcd for [C$_{24}$H$_{36}$-N$_9$O$_6$S$^+$ – Me$_2$S] 516.2319, found 516.2319.

Cyclo(-Lys-*o*-aB[CH$_2$S$^+$(C$_6$H$_5$)CH$_3$]-Gly$_4$-) (8o): obtained as above from the peptide 1o^{23} (0.150 g, 0.21 mmol) and thioanisole (2.1 mL, 21 mmol) in TFA (8.4 mL). Yield: 0.015 g (10%), white powder. R_f = 0.11 (II). ^{1}H NMR (D$_2$O): 7.7–7.1, m, 8 H (ArH and C$_6$H$_5$S$^+$); 4.9, m (masked), (CH$_2$S$^+$); 4.59, m, 1 H (CH$^\alpha$ Lys); 4.3–3.7, m, 8 H (4 CH$_2$ Gly); 3.28, s and 3.18, s, 3 H (CH$_3$S$^+$); 2.98, m (t-like, 7.5), 2 H (CH$_2^\epsilon$ Lys); 1.9, m, 1.7, m, and 1.4, m, 6 H (CH$_2^{\beta,\gamma,\delta}$ Lys). [α]^{25} (c 0.06; H$_2$O) = +21.7° (578); +23.3° (546); +40.0° (436); +55.0° (365). MS (FAB$^+$): 612 (M$^+$); 488 (M$^+$ – C$_6$H$_5$SMe).

Cyclo(-Lys-*m*-aB[CH$_2$S$^+$(C$_6$H$_5$)CH$_3$]-Gly$_4$-) (8m): obtained as above from 1m^{23} (0.100 g, 0.14 mmol) and thioanisole (1.64 mL, 14 mmol) in TFA (6.5 mL). Yield: 0.040 g (34%), white powder. Mp ~ 130 °C dec. R_f = 0.13 (II). ^{1}H NMR (D$_2$O): 7.8–7.3, m, 8 H (ArH and C$_6$H$_5$S$^+$); 5.20, d (12.6), 5.15, d (13.0) and 5.00, d (14.4), 4.95, d (13.9), 2 H (CH$_2$S$^+$); 4.27, m, 1 H (CH$^\alpha$ Lys); 4.2–3.8, m, 8 H (4 CH$_2$ Gly); 3.32, s and 3.30, s, 3 H (CH$_3$S$^+$); 2.97, m (t-like, 7.1), 2 H (CH$_2^\epsilon$ Lys); 1.9, m, 1.7, m, and 1.4, m, 6 H (CH$_2^{\beta,\gamma,\delta}$ Lys). ^{1}H NMR (CD$_3$COOD): 8.2–7.3, m, 8 H (ArH and C$_6$H$_5$S$^+$); 5.31, d (12.6), 5.26, d (13.0) and 5.14, d (12.4), 5.09, d (12.5), 2 H (CH$_2$S$^+$); 4.59, m, 1 H (CH$^\alpha$ Lys); 4.4–4.2, m, 8 H (4 CH$_2$ Gly); 3.46, s, 3 H (CH$_3$S$^+$); 3.15, m (t-like, 6.7), 2 H (CH$_2^\epsilon$ Lys); 2.1–1.6, m, 6 H (CH$_2^{\beta,\gamma,\delta}$ Lys). [α]^{25} c 0.08; AcOH) = $-33.0°$ (589); $-33.7°$ (578); $-41.2°$ (546); $-70.0°$ (436); $-142.5°$ (365). MS (FAB$^+$): 612 (M$^+$); 488 (M$^+$ – C$_6$H$_5$SMe). HRMS (FAB$^+$): calcd for [C$_{29}$H$_{38}$N$_7$O$_6$S$^+$] 612.2604, found 612.2623; calcd for [C$_{29}$H$_{38}$-N$_7$O$_6$S$^+$ – C$_6$H$_5$SMe] 488.2257, found 488.2262.

Cyclo(-Arg-*o*-aB[CH$_2$S$^+$(C$_6$H$_5$)CH$_3$]-Gly$_4$-) (9o): obtained as before from 3o^{23} (0.118 g, 0.176 mmol) and thioanisole (1.96 mL, 16.7 mmol) in TFA (7.5 mL). Yield: 0.040 g (27%), white powder. Mp: 140–150 °C dec. R_f = 0.12 (II). ^{1}H NMR (D$_2$O): 7.7–7.1, m, 8 H (ArH and C$_6$H$_5$S$^+$); 4.8, m (masked) (CH$_2$S$^+$); 4.6, m, 1 H (CH$^\alpha$ Arg); 4.3–3.7, m, 8 H (4 CH$_2$ Gly); 3.17, s, 3 H (CH$_3$S$^+$); 3.17, m, 2 H (CH$_2^\delta$ Arg); 1.9, m and 1.6, m, 4 H (CH$_2^{\beta,\gamma}$ Arg). [α]^{25} (c 0.09; H$_2$O) = +6.3° (578); +7.4° (546); +26.3° (436); +42.1° (365). MS (FAB$^+$): 516 (M$^+$ – C$_6$H$_5$SMe).

Cyclo(-Arg-*m*-aB[CH$_2$S$^+$(C$_6$H$_5$)CH$_3$]-Gly$_4$-) (9m): obtained from 3m^{23} (0.100 g, 0.15 mmol) and thioanisole (1.66 mL, 14 mmol) in TFA (6.4 mL). Yield: 0.028 g (22%), white powder. Mp ~ 190 °C dec. R_f = 0.14 (II). ^{1}H NMR (D$_2$O): 7.8–7.3, m, 8 H (ArH and C$_6$H$_5$S$^+$); 5.23, d (12.9), 5.15, d (~ 13) and 4.99, d (12.5), 4.95, d (~ 12.5), 2 H (CH$_2$S$^+$); 4.28, m, 1 H (CH$^\alpha$ Arg); 4.15–3.85, m, 8 H (4 CH$_2$ Gly); 3.32, s and 3.30, s, 3 H (CH$_3$S$^+$); 3.20, m, 2 H (CH$_2^\delta$ Arg); 1.9, m and 1.7, m, 4 H (CH$_2^{\beta,\gamma}$ Arg). ^{1}H NMR (CD$_3$COOD): 8.2–7.3, m, 8 H (ArH and C$_6$H$_5$S$^+$); 5.33, d (~ 12), 5.28, d (~ 12) and 5.16, d (~ 12), 5.11, d (~ 12), 2 H (CH$_2$S$^+$); 4.62, m, 1 H (CH$^\alpha$ Arg); 4.4–4.1, m, 8 H (4 CH$_2$ Gly); 3.46, s, 3 H (CH$_3$S$^+$); 3.34, m, 2 H (CH$_2^\delta$ Arg); 2.2–1.8, m, 4 H (CH$_2^{\beta,\gamma}$ Arg). [α]^{25} (c 0.03; AcOH) = $-21.1°$ (589); $-21.9°$ (578); $-28.1°$ (546); $-65.6°$ (436); $-100.0°$ (365). MS (FAB$^+$): 641 (M, H$^+$); 555 (M, K$^+$ – C$_6$H$_5$SMe); 516 (M$^+$ – C$_6$H$_5$SMe). HRMS (FAB$^+$): calcd for [C$_{29}$H$_{38}$N$_9$O$_6$S$^+$ – C$_6$H$_5$SMe] 516.2319, found 516.2361.

Enzymatic Studies. Bovine and porcine trypsin, human urinary urokinase (RFU 203, 95% pure in high-molecular-weight form), human two-chain t-pA, human plasmin, porcine pancreatic elastase (PPE), bovine α-chymotrypsin, and human thrombin were purchased from Sigma, Choay, Biopool, Kabi Vitrum, Serva, Cooper Biochemical and Boehringer–Mannheim, respectively. Active enzyme concentrations were determined by active-site titrations as described in ref 26.

The enzymes were assayed spectrophotometrically with the appropriate *p*-nitroanilide substrate: L-pyroglutamyl-L-glycyl-L-arginine *p*-nitroanilide (S-2444) for urokinase, D-valyl-L-leucyl-L-lysine *p*-nitroanilide (S-2251) for plasmin, and D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide (S-2238) for thrombin (purchased from Kabi Vitrum), benzoylarginine *p*-nitroanilide for trypsin, *N*-acetyl-L-tyrosine *p*-nitrophenyl ester for chymotrypsin, and succinyl-alanyl-alanyl-alanine *p*-nitroanilide for elastase (purchased from Sigma, Boehringer, and Serva, respectively). The experimental conditions were 0.025 M sodium
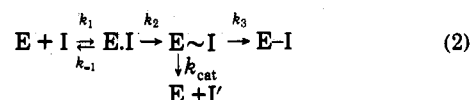
phosphate, 0.1 M NaCl (trypsin) and 0.05% Tween 80 (urokinase), 0.1 M phosphate, 25% glycerol (plasmin), 0.1 M Tris (elastase) and 0.025 M sodium phosphate, 0.1 M KCl (chymotrypsin). Assays were run at pH 7.5 and 25 °C in a Perkin-Elmer Lambda 5 spectrophotometer.

Enzyme inhibitions were analyzed either by the preincubation method^{38} or by the progress curve method.^{39} In the first method, enzyme inactivation was initiated by addition of an aqueous stock solution of the inhibitor to the appropriate buffered enzyme solution. Aliquots (10 μL) were withdrawn at various time intervals, and the reaction was stopped either by simple dilution or dilution followed by filtration–centrifugation at 4 °C on Centricon 10 microconcentrator (Amicon) and washing with buffer. The samples were assayed for residual enzyme activity by addition of 1 mL of the appropriate standard assay mixture (see above). Enzyme and cyclopeptide concentrations in the incubation medium were for bovine trypsin: [E]$_0$ = 4.5 μM, [4o] = [6m] = [8m] = 1 mM, [5o] = 0.6–3.6 mM, [8o] = 0.6 mM; for urokinase: [E]$_0$ = 55 μM, [4o] = [4m] = [5o] = [6m] = [8o] = 100–400 μM, [5m] = [8m] = [9o] = 13–160 μM, [9m] = 2.75–41 μM; for plasmin: [E]$_0$ = 1 μM and [inhibitor] = 34–560 μM except for 8m: [E]$_0$ = 0.7 μM, [8m] = 46–210 μM; for t-pA: [E]$_0$ = 0.75 μM, [inhibitor] = 0.37–1 mM; for chymotrypsin: [E]$_0$ = 9 μM, [inhibitor] = 0.4–1 mM; for PPE: [E]$_0$ = 1.36 μM, [inhibitor] = 260–875 μM; for thrombin: [E]$_0$ = 0.1 μM, [inhibitor] = 300 μM.

The inactivation pseudo-first-order rate constants k_{obs} were obtained from least-squares analysis of the semilogarithmic plots of the percentage of remaining activity against time. The results were analyzed according to the simplified model described by Kitz and Wilson,^{38} implicating the formation of one enzyme–inhibitor complex (E*I) between the enzyme E and the inhibitor I, prior to formation of the inactivated enzyme (E–I) with the inhibitor covalently attached (eq 1).

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E^*I \overset{k_{inact}}{\rightarrow} E–I \qquad (1)$$
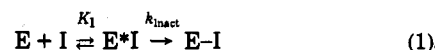
This expression is obtained from the general reaction of a suicide inactivator with a serine proteinase (eq 2), implicating the formation of an acyl enzyme, by combining k_2 and k_3 into a single constant and neglecting k_{cat}. In eq 2, E~I is the acyl enzyme and I′ the hydrolysis products I.
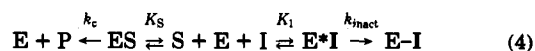
$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.I \overset{k_2}{\rightarrow} E{\sim}I \overset{k_3}{\rightarrow} E–I \qquad (2)$$
$$\quad\quad\quad\quad\quad \downarrow k_{cat}$$
$$\quad\quad\quad\quad\quad E + I'$$

The values of K_I and k_{inact} were determined by Wilkinson analysis of eq 3. In some cases, the ratio k_{inact}/K_I was obtained as $k_{obs}/[I]$ at low concentration of inhibitor.

$$1/k_{obs} = K_I/k_{inact}[I] + 1/k_{inact} \qquad (3)$$

In order to demonstrate that the inactivation was active-site directed, competition experiments were carried out with the substrate S-2444 (4 μM) for the inactivation of urokinase with 9m, and with leupeptin (5.8 μM), a trypsin-like enzyme inhibitor, for the inactivation of trypsin (3 μM) with 5m (150 μM).

The progress curves for the enzyme inactivation with the inhibitor I, run in the presence of a chromogenic substrate S, were analyzed as described previously^{26} according to the scheme of eq 4.^{39} ES is the enzyme–substrate complex and P the hydrolysis product of S. For example, the inhibition of bovine

$$E + P \overset{k_c}{\leftarrow} ES \overset{K_S}{\rightleftharpoons} S + E + I \overset{K_I}{\rightleftharpoons} E^*I \overset{k_{inact}}{\rightarrow} E–I \qquad (4)$$

trypsin (0.5 μM) was studied by adding 10 μL of stock enzyme solution to a buffered solution of the chromogenic substrate (0.3 mM) containing the inhibitor (3.6–15.3 μM) and 1.5% (v/v) DMSO. The inactivation of bovine trypsin was performed in analogous conditions of enzyme (0.5 μM) and substrate concentrations using 4m (12–50 μM), 5m (3–30 μM), 7m (284–426 μM), 8m (1.21–12.1 μM), 9o (19.7–330 μM), and 9m (1.8–8.5 μM with [E] = 0.7 μM). For porcine trypsin, the concentrations were [E]$_0$ = 0.3 μM; [5m] = 1.46–8.8 μM, and for urokinase [E]$_0$ = 31

nM; [S-2444] = 75 μM; [7m] = 44–312 μM. The rate v of change in absorbance at 405 nm due to hydrolysis of the appropriate substrate was obtained from the computer-assisted spectrophotometer with continuous comparison to a blank containing the same amount of inhibitor and substrate in buffer (25 °C). The kinetic constants K_I and k_{inact} were obtained from the graphs of $1/\pi$ versus $1/[I](1-\alpha)$ with $\alpha = [S]/K_M + [S]$ by use of eq 5 where $-\pi$ is the slope of the linear plot of $\ln(v)$ versus time at a given inhibitor concentration. In eq 5, [I] and [S] represent the initial

$$1/\pi = K_I/k_{inact}[I](1 - \alpha) + 1/k_{inact} \qquad (5)$$

concentration of inhibitor and chromogenic substrate, respectively, and K_M the Michaelis constant for the substrate. In both methods, error limits for k_{inact} and K_I were calculated by applicating the statistical treatment of Wilkinson[40] to the plots described by eqs 3 and 5.

For ultimate activity assays, solutions containing enzyme and inhibitor with various molar excess $[I]_0/[E]_0$ that would not totally inactivate the enzyme were incubated for 30 min at 25 °C, then for 6 h at 4 °C, [bovine trypsin] = 7 μM, [8m] = [9m] = 36–240 μM. At that time, the enzyme remaining activity was determined as described above for the incubation method. The order n of inactivator when it reacts with the enzyme is expressed by eq 6 where k is a constant.[27] The fitting of the data to eq 6 by nonlinear regression analysis may be used for low inhibitor concentrations ($[I]_0 < K_I$) to give an approximative evaluation of the stoichiometry of the reaction.

$$k_{obs} = k[I]_0^n \qquad (6)$$

Hydroxylamine reactivation assays were performed by treatment of inactivated enzyme solutions with the nucleophile hydroxylamine (0.75 M) at pH 8.5 and 25 °C during 30 min. Enzyme activity of filtered and washed aliquots versus a control solution containing no inhibitor was monitored.

Abbreviations. u-PA, urokinase-type plasminogen activator or urokinase; t-PA, tissue-type plasminogen activator; PPE, porcine pancreatic elastase; S-2238, D-phenylalanyl-L-pipecolyl-L-arginine p-nitroanilide; S-2244, L-pyroglutamyl-L-glycyl-L-arginine p-nitroanilide; S-2251, D-valyl-L-leucyl-L-lysine p-nitroanilide; BAPNA, benzoylarginine p-nitroanilide; Ac-Tyr-NE, N-acetyl-L-tyrosine p-nitrophenyl ester; Suc-Ala$_3$-NA, succinyl-alanyl-alanyl-alanine p-nitroanilide; o-aB[CH$_2$X], 2-amino-5-CH$_2$X-benzoic acid residue; m-aB[CH$_2$X], 5-amino-2-CH$_2$X-benzoic acid residue; for example, cyclo(-Lys-o-aB[CH$_2$Br]-Gly$_4$-) = cyclo(-lysyl-2-amino-5-bromomethyl-benzoyl-tetraglycyl-).

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