Inhibition of Human Leukocyte Elastase by Phosphate Esters of *N*-Hydroxysuccinimide and Its Derivatives: Direct Observation of a Phosphorylated Enzyme by ^{31}P Nuclear Magnetic Resonance Spectroscopy†

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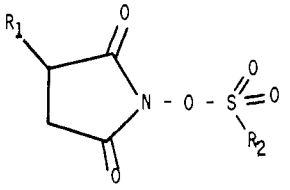
ABSTRACT: A series of phosphate esters derived from *N*-hydroxysuccinimide and 3-alkyl-*N*-hydroxysuccinimide have been synthesized and found to be potent time-dependent irreversible inhibitors of human leukocyte elastase (HLE). The observed inhibitory activity in this series of compounds correlated well with the known preference of HLE for substrates with small hydrophobic side chains. Maximum potency was reached when a favorable aromatic interaction involving a phenyl group present in the inhibitor and an aromatic residue located in the vicinity of the S_2' subsite was operative. ^{31}P NMR spectroscopy was used to probe the mechanism of action of these compounds. Direct evidence is presented in support of a mechanism involving phosphorylation of the active site serine. These compounds constitute a new class of hydrolytically stable phosphorylating agents.

The degranulation of polymorphonuclear leukocytes (PMNs) in inflammatory states results in the release of several enzymes, including the proteolytic enzyme elastase (Baggiolini et al., 1978). The chronic and unrestrained degradative action of elastase on elastin, the elastic component of lung connective tissue, and other matrix components is believed to lead to the onset of lung disease (Mittman & Taylor, 1988; Crystal, 1990). Elastase has been the target of extensive structural and mechanistic studies (Stein et al., 1985, 1987), primarily because of its likely involvement in the pathophysiology of pulmonary emphysema, cystic fibrosis, and, possibly, other connective tissue ailments (Wewers, 1989; Fujita et al., 1990). These efforts have included modulation of the activity of the renegade enzyme through the use of synthetic and naturally occurring inhibitors (Weinbaum & Groutas, 1990; Bode et al., 1989).



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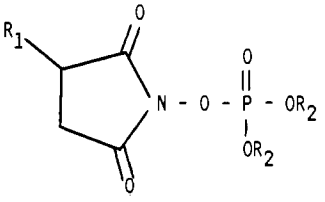

II

We have recently reported the mechanism-based inactivation of serine proteases by 3-alkyl-*N*-hydroxysuccinimide derivatives (structure I) (Groutas et al., 1989a,b, 1990a). Here, we describe the serendipitous discovery of a new class of phosphorylating agents (structure II); as well as pertinent mechanistic and inhibitory studies.

Materials and Methods

^{31}P (121.4-MHz) NMR spectra were obtained on a Varian XL-300 Fourier transform spectrometer using a 60° pulse. The spectra were broad-band decoupled and recorded at 30 °C. The number of transients acquired was in the 5000–10000 range. Chemical shifts are in parts per million for ^{31}P NMR spectra and referenced to 85% H_3PO_4.

Table I: Phosphorus Derivatives of *N*-Hydroxysuccinimides[a]

compound	R_1	R_2	$k_{obs}/[I]$ (M^{-1} s^{-1})
1	hydrogen	ethyl	970
2	hydrogen	benzyl	3170
3	hydrogen	*N*-propyl	7550
4	methyl	ethyl	1060
5	methyl	benzyl	1260
6	ethyl	ethyl	1610
7	ethyl	*N*-propyl	9430
8	*N*-propyl	ethyl	2310
9	*N*-propyl	*N*-propyl	14710
10	*N*-butyl	methyl	550
11	*N*-butyl	ethyl	2570
12	*N*-butyl	*N*-propyl	17240
13	*N*-butyl	*N*-butyl	11300
14	*N*-butyl	phenyl	3110
15	*N*-butyl	benzyl	3520
16	*N*-hexyl	ethyl	4020
17	*N*-hexyl	*N*-propyl	7450
18	isopropyl	ethyl	2050
19	isopropyl	benzyl	4650
20	isobutyl	ethyl	2740
21	isobutyl	*N*-propyl	10920
22	isobutyl	benzyl	6180
23	benzyl	hydrogen	inactive
24	benzyl	ethyl	5900
25	benzyl	*N*-propyl	*b*
26	benzyl	benzyl	3480
27	*m*-fluorobenzyl	ethyl	4730

[a] R_1 and R_2 refer to substituents on structure II. [b] Inactivation was too fast to measure by ordinary sampling techniques.

Synthesis. The compounds listed in Table I were conveniently synthesized by reacting equivalent amounts of the appropriate *N*-hydroxysuccinimide, dialkyl phosphite, and triethylamine in carbon tetrachloride. A general synthetic procedure and the relevant spectral data for the compounds listed in Table I are reported in the supplementary material (see pragraph at end of paper regarding supplementary material). The 3-alkyl-N-hydroxysuccinimides were prepared as previously described (Groutas et al., 1989a).

Chemicals. The chemicals used were generally of the highest purity. Baker-analyzed 60–200 mesh silica gel was used for flash chromatography. Methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide and *N*-acetyl-L-tyrosine methyl ester

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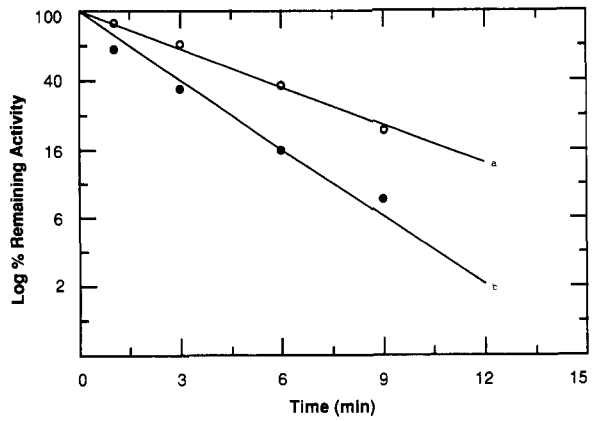

FIGURE 1: (a) Kinetics of inactivation of human leukocyte elastase by compound 11. The enzyme (182 nM) was incubated with compound 11 (1.82 μM) in 0.1 M HEPES buffer, pH 7.25, and 1% DMSO. Aliquots were removed periodically and assayed for enzymatic activity by using methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide. (b) Protection of the enzyme by substrate. The conditions were the same as in part a, except that the enzyme was incubated with the inhibitor in the presence of the substrate (212 μM) and aliquots were removed at different time intervals and assayed for enzymatic activity.

were purchased from Sigma Chemical Co., St. Louis, MO.

α-Chymotrypsin was purchased from Sigma as a three times crystallized and lyophilized salt-free type II powder. Active-site titration of the enzyme (Schonbaum et al., 1961) yielded 80–90% active sites.

Human leukocyte elastase was purchased from Elastin Products Co., Owensville, MO.

Enzyme Assays and Inhibition Studies. Human leukocyte elastase was assayed by mixing 10 μL of an 18.2 μM enzyme solution (in 0.05 M sodium acetate buffer, pH 5.5), 10 μL of dimethyl sulfoxide, and 980 μL of HEPES buffer, pH 7.2, in a thermostated test tube. A 100-μL aliquot was transferred to a thermostated cuvette containing 880 μL of HEPES buffer and 20 μL of a 4.25 mM solution of methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide, and the change in absorbance was monitored at 410 nm for 2 min. In a typical inhibition run, 10 μL of inhibitor (182 μM) in dimethyl sulfoxide was mixed with 10 μL of 18.2 μM enzyme solution and 980 μL of HEPES buffer, pH 7.2, and placed in a constant-temperature bath. Aliquots of 100 μL were withdrawn at different time intervals and transferred to a cuvette containing 20 μL of methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (4.25 mM) and 880 μL of HEPES buffer. After a 30-s incubation, the absorbance was monitored at 410 nm for 2 min. The pseudo-first-order rate constants (k_{obs}) were obtained from plots of ln (v_t/v_o) vs t and expressed in terms of the apparent second-order inactivation rate constants $k_{obs}/[I]$ M^{-1} s^{-1}. These are the average of two or three determinations.

Modeling. The commercially available computational chemistry software SYBYL 5.1 along with AMPAC 1.0 as distributed with SYBYL by Tripos Associates, 1699 S. Hanley Road, Suite 303, St. Louis, MO 63144, was used in conjunction with a Silicon Graphics personal IRIS workstation.

RESULTS

Inactivation Kinetics. A series of compounds represented by structure II were synthesized and subsequently evaluated for their inhibitory activity toward human leukocyte elastase (HLE). The inactivation of HLE by these compounds was found to be time-dependent, rapid, and irreversible. A typical example is the time-dependent inactivation of HLE by compound 11 (Figure 1a). The synthesized compounds and the second-order inactivation rate constants ($k_{obs}/[I]$) are listed in Table I. With a few exceptions (vide infra), first-order inactivation plots were linear for four half-lives. Centricon-10 filtration (centrifugation twice at 5 °C for 1 h after the addition of fresh 0.08 M Tris buffer, pH 7.3) did not regenerate any enzyme activity. For example, incubation of inhibitor 9 ($R_1 = R_2 = n$-propyl) with HLE lead to a rapid and total loss of enzymatic activity. No regain in activity was observed after a 48-h period.

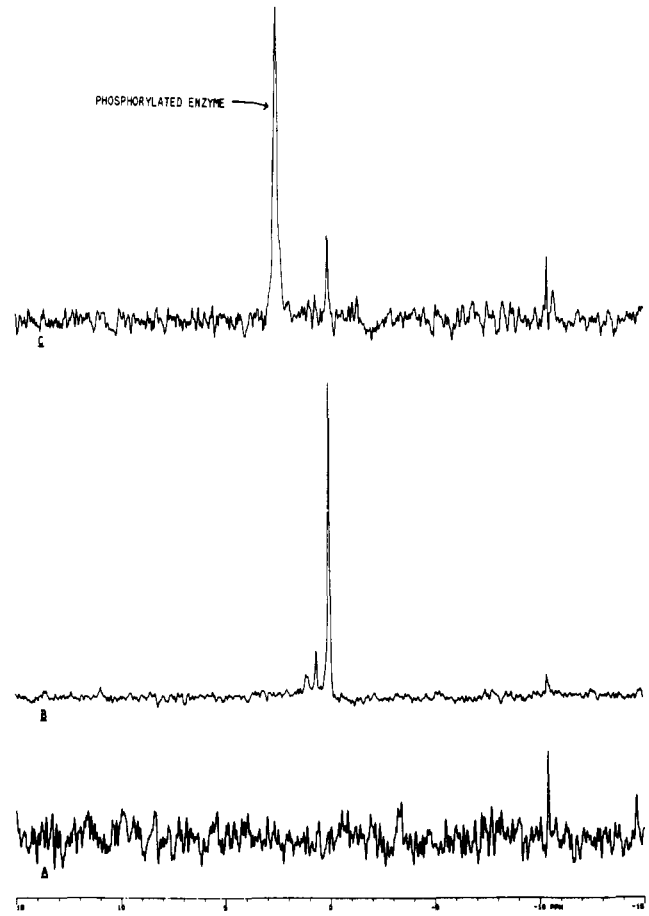

FIGURE 2: (A) α-Chymotrypsin (0.87 mM) in 0.08 M Tris buffer, pH 7.3, 0.1 M $CaCl_2$, 30% D_2O, and 10% DMSO. The resulting solution was used as a control for spectrum C, thus the same conditions apply. (B) Inhibitor 2 (2.3 mM) in 0.08 M Tris buffer, pH 7.3, 0.1 M $CaCl_2$, 30% D_2O, and 10% DMSO. (C) α-Chymotrypsin (0.87 mM) in 0.08 M Tris buffer, pH 7.3, 0.1 M $CaCl_2$, 30% D_2O, and inhibitor 2 (2.3 mM) was incubated (30 °C, 10% DMSO) for 3.6 h and checked for remaining activity (0%) against the control. The solution was filtered and concentrated through a Centricon-10 filter. The concentrate was taken up in 0.08 M Tris buffer, pH 7.3, 0.1 M $CaCl_2$, and 30% D_2O to a final volume of 900 μL, adjusted to pH 7.3, and checked for remaining activity against the control (0%). ^{31}P NMR spectra were recorded with a Varian XL-300 FT-NMR spectrometer. Spectra A and C were acquired by using a 60° pulse, broad-band decoupling, and 30, 6593, and 9217 transients, respectively, as well as a 1.6-s acquisition time, NOE enhancement, an 8-Hz line broadening, a pH of 7.3, and a 6.6-s delay between pulses. Spectrum B was acquired as above, except the acquisition time was 2 s, there were 4922 transients, and there was a 5-s delay between pulses. All spectra were externally referenced to 85% H_3PO_4. The two peaks at 0.57 and 1.06 ppm have been assigned as inhibitor degradation products. The peak at 2.53 ppm has been assigned to phosphorylated α-chymotrypsin.

Substrate Protection. The inactivation of HLE by structure II involves the active site of the enzyme. Evidence for this is provided by the observed decrease in inactivation rates in the presence of substrate. The inactivation of HLE by compound 11 in the presence of substrate (212 μM) lead to a large decrease in the inactivation rate constant [$k_{obs}/[I]$ decreased from 2570 to 1590 M^{-1} s^{-1}, (Figure 1b)].

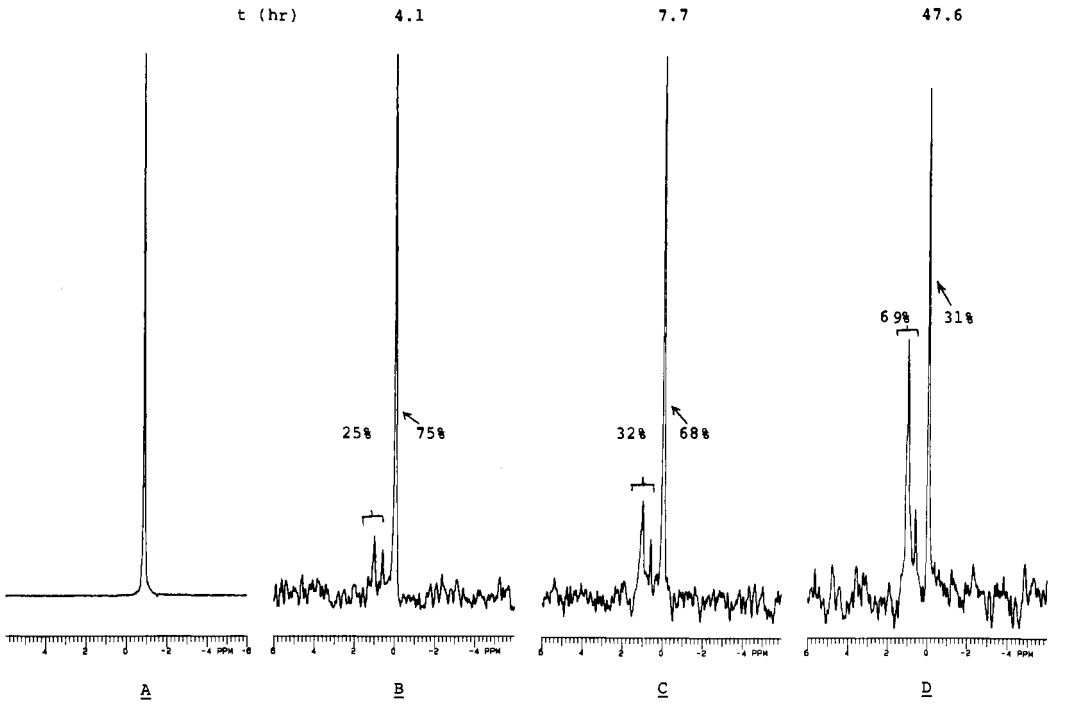

FIGURE 3: Stability of compound 2 in buffer as a function of time. (A) Inhibitor 2 in $CDCl_3$; (B–D) inhibitor 2 (92.0 mM) in 0.08 M Tris buffer, pH 7.3, 0.1 M $CaCl_2$, 30% D_2O, and 10% DMSO. Spectra A, B, and C were acquired at 4.1 h, 7.7 h, and 47.6 h, respectively, after initial mixing. ^{31}P (121.4-MHz) NMR spectra were obtained on a Varian XL-300 FT-spectrometer. Spectrum A was acquired with a 45° pulse, broad-band decoupling, at 30 °C, with 128 transients, a 1.6-s acquisition time, and a 1.6-s delay between pulses. Spectra B–D were acquired with a 45° pulse, broad-band decoupling, at 30 °C, with 1280 transients, a 2.0-s acquisition time, NOE enhancement, 8-Hz broadening, at pH 7.3, with a 5-s delay between pulses. All spectra were externally referenced to 85% H_3PO_4.

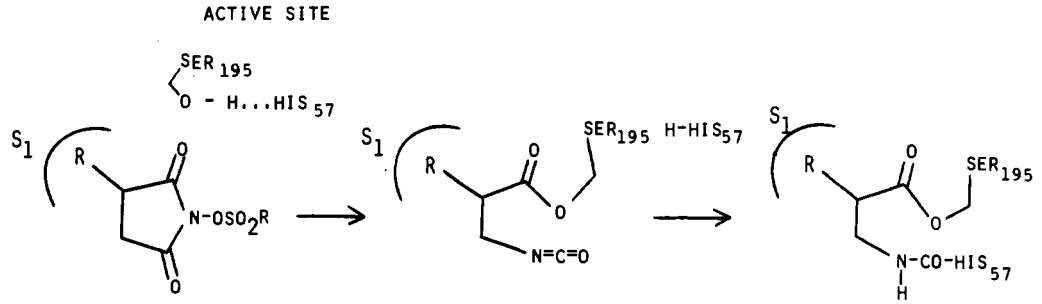

FIGURE 4: Proposed mechanism of inactivation of serine proteases by compounds represented by structure I.

NMR Studies

Mechanism of Inactivation. The mechanism of inactivation of HLE by structure II was investigated by using ^{31}P NMR. It was found to be expedient to use compound 2 and α-chymotrypsin in conducting the ^{31}P NMR experiments. Compound 2 inactivates α-chymotrypsin rapidly and irreversibly, and its ^{31}P NMR spectrum does not overlap the resonance of the phosphorylated enzyme. The use of α-chymotrypsin was based on the availability of a wealth of ^{31}P NMR data of phosphorylated derivatives of α-chymotrypsin (Gorenstein et al., 1989; Gorenstein, 1984; Adebodun & Jordan, 1989) and its low cost. The results of the ^{31}P NMR studies are summarized in Figure 2 and clearly demonstrate the formation of phosphorylated α-chymotrypsin.

Stability Studies. The stability of a representative member (compound 2) in Tris buffer, pH 7.3, was investigated by ^{31}P NMR, and the results are summarized in Figure 3. The half-life of compound 2 was found to be about 26 h.

Discussion

We have previously shown that compounds represented by structure I constitute a new class of mechanism-based inhibitors that inactivate serine proteases via an enzyme-induced Lossen rearrangement, as illustrated in Figure 4 (Groutas et al., 1989a,b). We initially intended to investigate the effect of the leaving group on the inactivation process, and it was anticipated that compounds represented by structure II would inactivate HLE by an analogous mechanism (Figure 5a).

Effect of Structure on Inhibitory Activity. All of the compounds listed in Table I were found to be highly effective irreversible inhibitors of HLE. Furthermore, the interaction of these compounds with the enzyme involves the active site. Appropriate structural variations in R_1 and R_2 lead to optimization of inhibitory activity and provided some early indications about an unanticipated mechanistic pathway for the inactivation process (vide infra). It is clear from Table I that maximum inhibitory activity is obtained when R_2 = *n*-propyl (compare, for example, compounds 1–3, 8–9, and 10–15). This is in accord with the known preference of neutrophil elastase for accommodating small hydrophobic side chains at its S_1 subsite (Bode et al., 1989). Varying the length of R_1 while keeping R_2 = *n*-propyl leads to compounds with enhanced inhibitory activity (compounds 3, 7, 9, and 12). Assuming that R_2 occupies the S_1 subsite (vide infra), this observation suggests the involvement of a favorable hydrophobic interaction of the R_1 group with the S_2' subsite of HLE. A branched R_1 group reduces inhibitory activity considerably (compare compounds 12 and 21), presumably because of steric constraints.

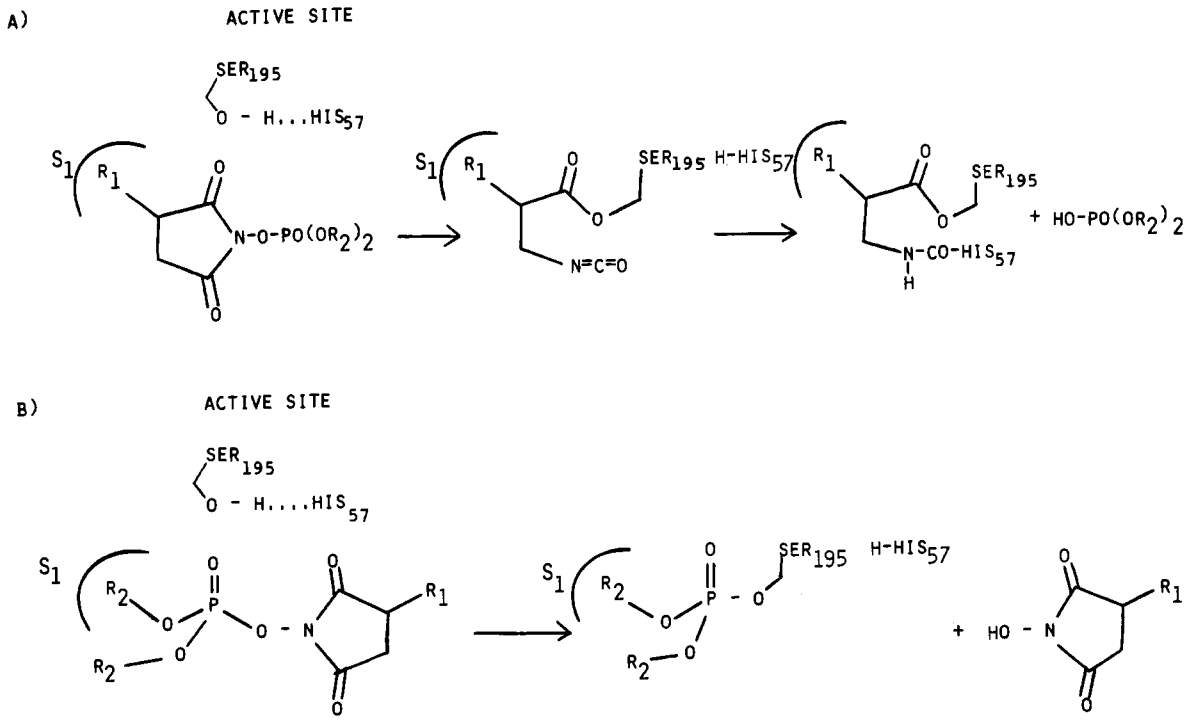

FIGURE 5: Possible binding modes of compounds represented by structure II to the active site of a serine protease. (A) The R_1 group of the inhibitor occupies the primary specificity site (S_1) of the enzyme. (B) The R_2 group occupies the S_1 subsite of the enzyme.

The drastic enhancement in inhibitory activity observed with compound 25 is likely due to a favorable aromatic interaction involving the phenyl group in the inhibitory and an aromatic residue that is located in the vicinity of the S_2' subsite, possibly Phe-41. An analogous favorable interaction was observed earlier with structure I, where R_1 = alkyl and R_2 = *trans*-styryl (Groutas et al., 1989a). Recently, there has been an increasing appreciation of the significance of aromatic interactions and their contribution to protein stability and substrate or inhibitor–enzyme interactions (Burley & Petsko, 1985; Jorgensen & Severance, 1990). Best fit superposition of compound 25 and the X-ray crystal structure of structure I (with R_1 = benzyl and R_2 = *trans*-styryl) is illustrated in Figure 6. The overlay of the two compounds reflects the similarity of their molecular shapes.

The mode of binding of structure II to the active site of the enzyme is illustrated in Figure 5b and is strongly supported by both the results of the structure–activity studies (compounds 1–3 and 23) and, more convincingly, by the NMR studies (vide infra).

Interestingly, the favorable aromatic interaction cited above affects the mode of binding of some of the members of this series to the active site of the enzyme. This is evident in the interaction of compounds 2, 15, and 22 with the enzyme, where incubation of excess inhibitor with HLE leads to time-dependent but incomplete inactivation of the enzyme. These compounds may bind to the active site as illustrated in Figure 5a. The observed kinetics (data not shown) suggest that these compounds are turned over with a partition ratio that is greater than zero (Silverman, 1988). The unpredictability of the mode of binding of structurally related molecular weight compounds has been noted earlier with porcine pancreatic elastase (Meyer et al., 1986) and, more recently, with papain (Varughese et al., 1989).

Mechanism of Inactivation. Direct evidence in support of the mechanism shown in Figure 5b was obtained with ^{31}P NMR. According to this mechanism, inhibitor 2 binds to the active site of the enzyme with the R_2 of the inhibitor occupying the S_1 subsite (Schecter & Berger, 1967). This is then followed by phosphorylation of the active-site serine. The presence of the phosphorylated enzyme was established in three stages, as shown in Figure 2. Spectrum A shows the ^{31}P NMR spectrum of α-chymotrypsin in Tris buffer. Spectrum B shows the spectrum of inhibitor 2 under identical conditions. The resonance at 0.15 ppm is due to the inhibitor. The resonances at 0.57 and 1.06 ppm are inhibitor hydrolysis products. The latter resonance was conclusively shown to arise from dibenzyl phosphate through comparison of its ^{31}P NMR with that of an authentic sample. The resonance at 2.5 ppm in spectrum C has been ascribed to the phosphorylated enzyme (Gorenstein et al., 1989; Adebodun & Jordan, 1989).

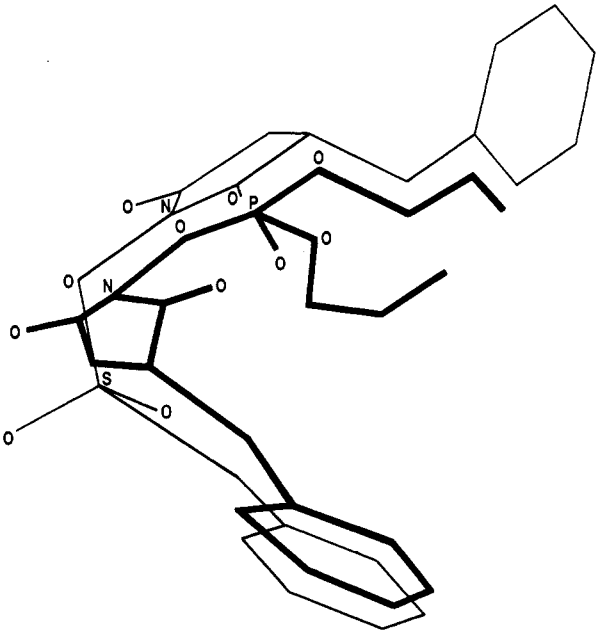

FIGURE 6: Overlap of compound 25 (solid line) with the X-ray crystal structure of I (R_1 = benzyl, R_2 = *trans*-styryl).

Stability Studies. The stability and chemical reactivity of phosphorylating agents are largely dependent on the electrophilicity of the phosphorus atom (Bartlett & Lamden, 1986;

Lamden & Bartlett, 1983). A shortcoming of many of the reported phosphorylating agents is their instability in aqueous media. Recently, a series of peptidyl aminoalkylphosphonate diphenyl esters that are highly effective phosphorylating agents of serine proteinases ($k_{obs}/[I]$ values with HLE were in the range of 1.5–7100 M^{-1} s^{-1}) and that also exhibit enhanced hydrolytic stability was reported by Oleksyszyn and Powers (1989).

The stability of a member (compound 2) of the class of phosphorylating agents described herein was examined by ^{31}P NMR as a function of time. Compound 2 was found to be fairly stable (half-life of 26 h) in Tris buffer, pH 7.3. The hydrolytic stability of compounds represented by structure II would be expected to be dependent on the pK_a of the leaving group. The pK_a's of 3-alkyl-*N*-hydroxyimides show substantial variation and are influenced by the nature of the alkyl group at the C-3 position (Ames & Grey, 1955). Thus, a finely tuned balance between hydrolytic instability and phosphorylating ability can, in principle, be achieved. We have observed that the introduction in structure II of a variety of *N*-hydroxyimides such as, for example, *N*-hydroxyhomophthalimide, 3-hydroxy-2,4-(1H,3H)quinazolinedione, etc., yields highly effective phosphorylating agents (unpublished observations). Lastly, tailoring specificity for a target proteinase by varying R_2 is feasible in this class of compounds.

Conclusion. This study has demonstrated that compounds represented by structure II constitute a new class of phosphorylating agents for serine proteinases. These compounds are of potential value as enzyme probes and therapeutic agents.

Supplementary Material Available

A table (3 pages) giving the synthetic methodology and physical properties of the phosphate esters of *N*-hydroxysuccinimide. Ordering information is given on any current masthead page.

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