Inhibition of Trypsin with Active-Site-Directed Enzyme-Activated *N*-Nitrosoamide Substrates†

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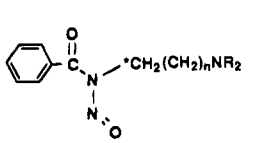
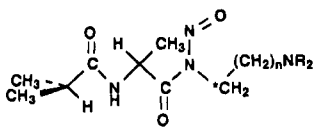
ABSTRACT: A series of active-site-directed enzyme-activated nitrosoamide inhibitors of trypsin has been designed, synthesized, and tested. The inhibitors contain an *N*-nitrosoamide group that can generate an alkylating agent and a positively charged ammonium ion group at the end of an aliphatic carbon chain that provides specificity. The half-lives of inhibition under normal conditions were 0.6 to 2 min for compounds in series **1**. One of the compounds, *N*-(4-amino-1-butyl)-*N*-nitrosobenzamide (**1b**), is a very efficient inhibitor; its partition ratio, k_2/k_{inact}, is zero suggesting that it may be a useful titrant for trypsin and related enzymes. The extent of inhibition is substantially decreased by the competitive inhibitor benzamidine, indicating that the inhibitors were operating in the active site. Two modes of inhibition were noted: reversible and irreversible. The *N*-nitrosoamide inhibitors bind to the trypsin binding pocket guided by the primary specificity. They then acylate the enzyme (at Ser-195), producing a leaving group that generates diazonium ions (or) carbocations in the active site; these react with a proximal carboxylic acid side chain of the enzyme to form a carboxylic acid ester, presumably that of Asp-194. If primary amino groups are present on the alkyl group, the ester (^{13}C NMR δ 67.2 ppm for $R_1COO^{13}\underline{CH_2}$-$(CH_2)_nNH_2$) rearranges into the amide form (^{13}C NMR δ 62.9 ppm for $R_1CONH(CH_2)_n{}^{13}\underline{CH_2}OH$) through an O → N acyl migration; an irreversibly inhibited enzyme results. A model based on the orientation of the site-specific group of *N*-(4-amino-1-butyl)-*N*-nitroso-*N'*-isobutyryl-D-alaninamide (**D2a**) and its L antipode in the active site of trypsin is proposed to explain the preferential inhibition of trypsin by the D-isomer. Analysis of the structure−inhibition relationship revealed four factors that determine the inhibition modes of the inhibitors: 1, length of the alkyl group; 2, stability of the acyl-enzyme; 3, the option O−N acyl migration; and 4, chirality.

Trypsin and trypsin-like enzymes are involved in many important biological activities such as regulation of transmembranes, signal transduction by release of signal peptides, blood coagulation and fibrinolysis, hormones and growth factors, reproduction, and neutral development (Neurath, 1986; Hörl & Heidland, 1988; Festoff & Hantai, 1990). Recently, these enzymes were also found to be involved in the virulency of viruses (Shimamoto *et al.*, 1991), in cancer cell-mediated degradation (Koivunen *et al.*, 1991), and in activation of G-protein-coupled receptors (Chen *et al.*, 1994; Nystedt *et al.*, 1994). High resolution X-ray crystal structures have not been determined for most trypsin-like enzymes, and they are not well characterized. Studies on the mechanisms of action of trypsin-like enzymes such as thrombin and the design of therapeutic drugs for the diseases in which those enzymes play a role generally rely on knowledge obtained from the study of trypsin (Hijikata-Okunomiya *et al.*, 1988; Matsuzaki *et al.*, 1988; Chow *et al.*, 1990; Turk *et al.*, 1991). Although many active-site−directed enzyme-activated inhibitors (mechanism-based inhibitors) have been examined with respect to serine enzymes such as α-chymotrypsin (White *et al.*, 1981; Westkaemper & Abeles, 1983; Daniels & Katzenellenbogen, 1986) and β-lactamase (Brenner & Knowles, 1981; Faraci & Pratt, 1985), only a few have been reported for trypsin and trypsin-like enzymes: examples are isocoumarin-based inhibitors that inactivate trypsin and blood coagulation serine proteases (Gelb & Abeles, 1986) and isatoic anhydride-based inhibitors of trypsin and thrombin (Kam *et al.*, 1988).

We have designed and synthesized new enzyme-activated inhibitors of trypsin, **1**−**3**, based on knowledge of the primary specificity of trypsin, on structural information concerning the interaction between trypsin and its naturally occurring inhibitors (Huber *et al.*, 1974 a,b; Stroud *et al.*, 1974; Huber & Bode, 1978; Laskowski *et al.*, 1981; Mangel *et al.*, 1990; and on experience with nitrosoamide inhibitors of α-chymotrypsin (White *et al.*, 1975, 1977a,b, 1981, 1990, Donadio *et al.*, 1985). The compounds consist of modified amino acids capable of forming an ammonium ion at one end

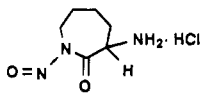


1a R=CH_3, n=2
1b R=H, n=3 (hydrochloride)
1c R=CH_3, n=3



DL **2a** R=H, n=3 (hydrochloride)
D **2a**
D **2b** R=CH_3, n=3

• = $^{13}CH_2$ or $^{12}CH_2$



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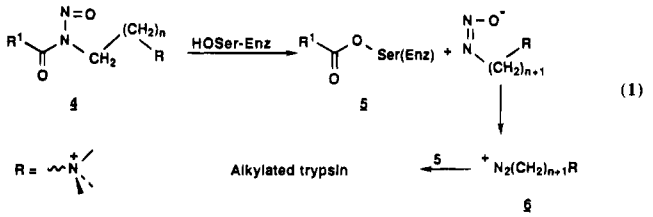
to allow a lysine-type binding to trypsin, and a nitrosoamide group at the other that utilizes trypsin activation. Reaction of the nitrosoamide moiety with the serine residue of the catalytic triad produces an acyl enzyme and, in a series of fast steps, a diazonium ion (or a carbocation) that alkylates the enzyme and renders it inactive (eq. 1).



(1)

Of the inhibitors prepared, compounds **1a**, **1b**, and **1c** are benzamide derivatives and compounds DL**2a**, D**2a**, and D**2b** are derivatives of alanine. The compounds are "inverse" substrates of trypsin in that the arrangement of the carbonyl and amide nitrogen functional groups is "inverse" of that present in the normal substrates of the enzyme (Tanizawa *et al.*, 1977). The inhibitors proved to be rapid-acting, efficient inhibitors of trypsin; in some cases, incubation of the inhibited enzymes in buffers led to a regeneration of enzyme activity (either fully or partially), whereas in others the inhibition was irreversible. The mechanism of action of the inhibitors was elucidated through application of NMR[1] spectrometric studies (^{1}H and ^{13}C), kinetic measurements, and model studies.

EXPERIMENTAL PROCEDURES

Methods and Materials. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian XL-400 and Bruker AMX-300 spectrometers; chemical shifts were reported in parts per million downfield from internal tetramethylsilane except where noted. Polarimetric measurements were made on a Perkin-Elmer 141 polarimeter at the sodium D line (589 nm). Mass spectroscopic measurements were made on a VG 70-S Gas Chromatograph−Mass spectrometer.

Materials. Trypsin (EC 3.4.21.4, Type XIII, TPCK treated from bovine pancrease, dialyzed and lyophilized, essentially salt free; Sigma Chemical Company) was used without further purification. The approximate concentration of trypsin was calculated from its molecular mass = 23 281 daltons (Barman, 1969), and the weights were used. Trypsin enzyme activity was assayed by measuring the change in absorbance at 247 nm during the hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) at pH 8.1 in a Tris hydrochloride buffer (Hummel, 1959; Worthington, 1988). α-Chymotrypsin (EC 3.4.4.5, Type I-S, Sigma Chemical Company) activity was assayed by measurement of the change in the absorbance at 256 nm during the hydrolysis of benzoyltyrosine ethyl ester (BTEE) in a pH 7.8 phosphate buffer (Hummel, 1959). TAME, BTEE, and Tris were Sigma Chemical Co. products. Constant-boiling HCl (6N) was purchased from Pierce Chemical Company. Sephadex gel (G-25, superfine grade) was obtained from Pharmacia. All other chemicals were of the highest purity available. All aqueous solutions for enzyme work were prepared with deionized water. The syntheses of 4-dimethylaminobutyronitrile [1-^{13}C] (**7**), *N*-(4-dimethylamino-1-butyl)benzamide [1-^{13}C] (**8**), and *N*-(4-dimethylamino-1-butyl)-*N*-nitrosobenzamide [1-^{13}C] (**1c**) are reported elsewhere (White & Chen, 1993). The synthesis of ***N-nitroso-N-(4-amino-1-butyl)-N′-isobutyryl-D-alaninamide hydrochloride (D2a)*** is reported in this paper for exemplary purposes; the syntheses of the remaining inhibitors (**1**−**3**) will be reported elsewhere.[2] All inhibitors used were NMR-pure except for inhibitors **1a** and **3**, which were used without separation of the nitrosoamide product from their amide precusors; the latter did not inhibit trypsin. Inhibitor D**2a** was purified by reverse phase HPLC.

N-(4-Amino-1-butyl)-N′-isobutyryl-D-alaninamide Hydrochloride (9). To a solution of 2-nitrophenyl *N*-isobutyryl-D-alaninate (2.00 g, 7.14 mmol) in dry ethyl acetate (25 mL) was added dropwise, over 15 min, a freshly prepared solution of 1,4-diaminobutane (0.861 mL, 8.56 mmol) in dry ethyl acetate (20 mL) with vigorous stirring under nitrogen at room temperature. An orange precipitate formed 5 min after the addition was complete. TLC on silica gel showed that the starting ester had completely reacted. The orange solid obtained by filtration was washed with hexane and with ethyl acetate. The yellow solid was dried *in vacuo* to give relative pure amide (2.49 g, 95%), mp 175−180 °C. TLC on silica gel (CH_3OH: $NH_3 \cdot H_2O$:H_2O, 8:1:1, by volume) showed one major spot, $R_f = 0.30$ (ninhydrin detection). The crude *N*-(4-amino-1-butyl)-*N′*-isobutyryl-D-alaninamide 2-nitrophenate salt was desalted and purified by flash column chromatography (Still *et al.*, 1978) (silica gel, 70−230 mesh, 60 Å) with the eluent CH_3OH:$NH_3 \cdot H_2O$:H_2O (18:1:1, v/v/v). The fractions containing the product were evaporated to dryness *in vacuo*. The residue was dissolved in 1 N HCl (7.1 mL), and the aqueous solution was lyophilized to give **9** as a pale solid (1.14 g, 63%), mp 202−205 °C: $[\alpha]^{25}D + 8.7°$ (c 1.00, glacial acetic acid); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.06 (broad s, 3H), 7.98 (m, 2H), 4.21 (quintet, $J = 7.2$ Hz, 1H), 3.05 (m, 2H), 2.75 (m, 2H), 2.46 (septet, $J = 6.8$ Hz, 1H), 1.55 (m, 2H), 1.45 (m, 2H), 1.18 (d, $J = 7.2$ Hz, 3H), 0.977 (d, $J = 6.8$ Hz, 6H); ^{13}C NMR (400 MHz, DMSO-d6) δ 175.8, 172.4, 48.0, 38.4, 37.7, 33.5, 25.9, 24.3, 19.5, 19.3, 18.4; IR (KBr) 3293, 2968, 1956 (broad), 1633, 1548, 1512, 1446, 1374, 1234, 1169, 1094, 694 cm^{-1}; TLC on silica gel (CH_3OH:$NH_3 \cdot H_2O$:H_2O, 18:1:1, by volume) $R_f = 0.46$. Anal. Calcd: $C_{11}H_{24}ClN_3O_2$: C, 49.71; H, 9.10. Found: C, 49.29; H, 9.27. Caution: recrystallization from an acetonitrile−ethanol mixture at 70 °C gave a largely racemic product, $[\alpha]^{25}D = 0°$ (c 0.40, ethanol); $[\alpha]^{25}D = +1°$ (c 0.40, methanol).

N-Nitroso-N-(4-amino-1-butyl)-N′-isobutyryl-D-alaninamide Hydrochloride (D2a). (Note: *This reaction should be carried out in a good fume hood, and protective gloves should be used.*) The nitrosoamide **D2a** was prepared by an adaptation of the nitrosation method of White and Aufdermarsh (1961). *N*-(4-Amino-1-butyl)-*N′*-isobutyryl-D-alaninamide hydrochloride (**9**) (20 mg, 0.075 mmol) was ground into a fine powder that was dried *in vacuo* for 1 h. The flask containing the powder was filled with dry nitrogen

[1] Abbreviations: BTEE, benzoyltyrosine ethyl ester; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; PTI, pancreatic trypsin inhibitor; r, R value of regression analysis; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; TLC, thin layer chromatography; TMS, tetramethylsilane; Tris, tris(hydroxymethyl)-aminomethane; TSP, 3-(trimethylsilyl)propionic acid-2,2,3,3-d_4, sodium salt; UV, ultraviolet; Vis, visible.

[2] The syntheses and physical characterization of the inhibitors will be reported in *Org. Prep. Prop. Int.* (1995).

and quickly fitted with a rubber septum; a balloon filled with nitrogen was attached, and acetic anhydride (10 mL) was introduced via a syringe. The suspension was stirred and cooled to 0 °C for 15 min. Gaseous N_2O_4 (8.5 mL, 0.30 mmol) was introduced via a long syringe needle during a period of 5 min; the reaction mixture became yellow in color. After 20 min the solution was evaporated to dryness *in vacuo*. To the yellow residue was added 0.90 mL of acetonitrile, and 0.15 mL of the solution was taken and diluted to 0.90 mL with acetonitrile for spectrophotometric measurements. The yield of nitrosation was estimated to be 56% (ϵ_{425} = 84; see below). After the crude *N*-nitrosoamide was washed with methylene chloride or ethyl ether, the ^{1}H NMR spectrum showed the presence of the *N*-nitrosoamide derivative (5.31 ppm), the starting amide (4.18 ppm), and a small amount of probably 4-amino-1-butyl nitrate (4.55 ppm). Reverse-phase HPLC (μ Bondpak C18 column, CH_3CN) was used to purify the material; pure **D2a** gave R_f (HPLC, CH_3CN) 7.0 min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.49 (d, J = 5.9 H2, 1H), 7.59 (broad s, 4H), 5.33 (t, J = 6.7 Hz, 1H), 3.72 (t, J = 6.4 Hz, 2H), 2.74 (m, 2H), 2.47 (m, 1H), 1.40 (d, J = 7.3 Hz, 3H), 1.36 (m, 4H), 1.003 (d, J = 6.9 Hz, 3H), 1.000 (d, J = 6.9 Hz, 3H). UV(CH_3CN) λ_{max} 244 nm (ϵ 7505); Vis (CH_3CN) λ_{max} 425 nm (ϵ 84), 408 (88).

Benzoyltrypsin. Benzoyltrypsin was prepared on the basis of the published method for preparation of benzoyl-α-chymotrypsin (Vishnu & Caplow, 1969; Amshey *et al.*, 1975). Although benzoyltrypsin had been prepared earlier without isolation using 4-amidinophenyl benzoate (Markwardt *et al.*, 1972), the use of benzoylimidazole was found to be more convenient. Trypsin (45 mg, 1.93 μmol) was dissolved in 50 mM Tris-HCl buffer at pH 7.2 (2.95 mL). *N*-Benzoylimidazole, which was prepared from imidazole and benzoyl chloride (Vishnu & Caplow, 1969) (20 mg, 116 μmol) in CH_3CN (65 μL), was added to the enzyme solution; a white precipitate formed. After incubation for 20 min, the mixture was adjusted to pH ~3 with 2N HCl. Assay of the enzyme activity at this point showed only 1% of trypsin activity relative to a control. The mixture was centrifuged, and the supernatant was desalted on a Sephadex G-25 column. The pool of the fractions containing benzoytrypsin was lyophilized to give a white puffy solid (2.13 mg).

Hydrolysis of Benzoyltrypsin. Benzoyltrypsin (2.13 mg) was dissolved in 1 mM HCl (1.0 mL). To an aliquot (20 μL) of this stock solution 50 mM Tris-HCl at pH 7.2 (0.98 mL) was added; the solution was shaken and incubated at 25 °C (enzyme concentration 1.8×10^{-6} M). Aliquots (100 μL) were taken at intervals for assay of the enzyme activity (enzyme concentration 6.0×10^{-8} M) (Hummel, 1959). The deacylation rate followed first-order kinetics [$k = (4.9 \pm 0.13) \times 10^{-4}$ s^{-1}, r = 0.990]; half-life of deacylation = 24 ± 0.6 min. An aliquot (20 μL) of the stock solution was treated as described above except that 20 μL of 1 M NH_2-OH pH 7.2 was used to substitute for 20 μL of 0.98 mL Tris-HCl buffer. The enzyme activity remaining after 2 h of incubation with 20 mM NH_2OH was used as the maximum value of the regeneration of the acyl-enzyme activity in a pH 7.2 Tris-HCl buffer. A value of 6.4 min ($k = 1.8 \times 10^{-3}$ s^{-1}) was reported by Markwardt *et al.* (1972) for the half-life of hydrolysis of benzoyltrypsin in 0.154 M NaCl at pH 7.2 (25 °C); the benzoyltrypsin was prepared from the reaction of trypsin with 4-aminophenyl benzoate, but not isolated. A more recent publication from one of the authors (Stürzebecher, 1986) cited the same value for the half-life; in neither case was the buffer identified.

*4-Amino-1-butyl benzoate (**10**).* A solution of 4-amino-1-butanol (50 mg, 0.56 mmol) in 0.5 mL of $CDCl_3$ in a hydrolysis tube was degassed via three freeze-thaw cycles *in vacuo* (~10^{-2} torr). The inlet stopcock was closed and the inlet was fitted with a rubber septum. Hydrogen chloride gas (19 mL, 0.85 mmol) was introduced through the septum, and the tube was cooled in liquid nitrogen. A solution of benzoyl chloride (79 mg, 0.56 mmol) in 0.5 mL of $CDCl_3$ was introduced through the septum. The reaction mixture was degassed again by the freeze−thaw method. The hydrolysis tube was then heated in an oven at 61 °C for 66 h. During the first 12 h, the reaction mixture was cloudy; then it turned into a golden yellow, clear solution. The solution was cooled to room temperature, and chloroform (5 mL) was added. The mixture was extracted with water (5.0 mL, 0 °C), and the aqueous layer was separated and made basic with 1 mL of triethylamine. The aqueous layer was then extracted with $CHCl_3$ (3 × 5.0 mL). The organic layers were pooled, dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo* to give **10** in the form of a golden, oil (41 mg, 38%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, t, J_1 = 7.4 Hz, J_2 = 1.2 Hz, 2H), 7.55 (t, t, J_1 = 7.4 Hz, J_2 = 1.3 Hz, 1H), 7.43 (t, t, J_1 = 7.7 Hz, J_2 = 1.6 Hz, 2H), 4.33 (t, J = 6.6 Hz, 2H), 3.08 (b s, 2H); 2.83 (t, J = 6.8 Hz, 2H), 1.83 (quintet, J = 7.2 Hz, 2H), 1.71 (quintet, J = 7.6 Hz, 2H); ^{13}C NMR (300 MHz, $CDCl_3$ at 77 ppm) δ 166.6 (CO), 132.9 (C-4′) 129.5 (C-1′, C-2′), 128.3 (C-3′) 64.6 (C-1), 41.2 (C-4), 28.8 (C-2), 26.1 (C-3).

*Half-Lives of Hydrolysis of Inhibitors **1**−**3**.* The inhibitor in acetonitrile was mixed with a buffer solution (Tris-HCl, 50 mM, pH 7.2, 8.1, etc.) in a quartz cell (final acetonitrile concentration ~3%). The change of absorbance at 408 nm was monitored with time at 25 °C.

*Rate of Trypsin-Catalyzed Hydrolysis of ^{13}C-Labeled Inhibitor **1c**.* The title inhibitor (3.81 μmol) in CH_3CN (50 μL) was mixed with trypsin (45 mg, 1.93 μmol) in a pH 7.2 42 mM phosphate buffer (2.95 mL) containing 15% D_2O. The absorbance at 425 nm was recorded with time versus the buffer alone as the reference. After 20 min, the solution was adjusted to pH 3 with 2 N HCl. The absorbance at 425 nm was stable at this pH for several hours, and this value was taken as the final absorbance value. The second-order rate constant was obtained using the rate law equation of Atkin (1982), with the assumption that at the initial stage (0−1 min) one inhibitor molecule reacts with one enzyme molecule (over that time span, the inhibitor and benzoyltrypsin are stable) ([Trypsin] = 6.43×10^{-4} M; [1c] = 1.27×10^{-3} M). Also, from the curve of absorbance versus time, and using the KaleidaGraph program, the following fitting equation was found (regression factor r = 0.998):

$$A(t) = -0.02926/[\mathrm{EXP}\,(-0.7929t - 0.682) - 1]$$

where A = absorbance at 425 nm and t = reaction time. The calculated half-life of tryptic hydrolysis of inhibitor **1c** was essentially the same as obtained by the Atkin approach.

*Inhibition of Trypsin by N-Nitroso Inhibitors **1**−**3** (General Procedure).* Each *N*-nitroso compound in 0.05 mL of acetonitrile was slowly mixed with 0.95 mL of 1 mg/mL trypsin solution in Tris-HCl buffer (the final buffer concentration was 50 mM with 10 mM $CaCl_2$ at pH 7.2 for

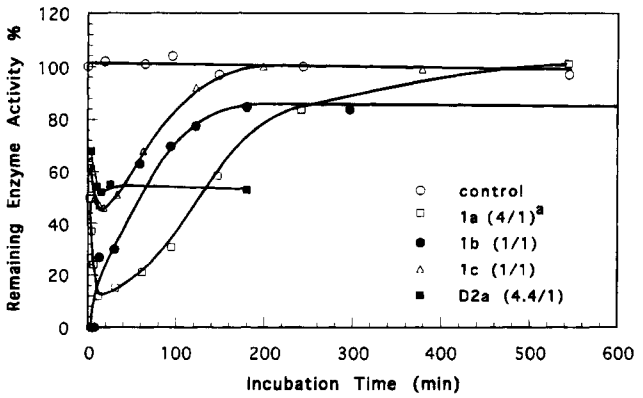


FIGURE 1: Time-dependent inhibition of trypsin by *N*-nitrosoamide inhibitors (conditions listed in Experimental Procedures). [a]*Ratio* of inhibitor to enzyme.

inhibitors **1a**, **1b**, and **1c**; or 40 mM with 10 mM $CaCl_2$ at pH 8.1 for inhibitors DL**2a**, D**2a**, D**2b**, and **3**) during a period of 2 min (enzyme concentration, 4.29×10^{-6} M). Both the control (without inhibitor) and the sample were shaken and incubated at 25 °C unless noted elsewhere. Aliquots of 20 μL of the incubated solution were taken from the control and the sample, respectively, and diluted to 1.00 mL in each case with 1 mM HCl (enzyme concentration, 8.6×10^{-7} M). The diluted samples and controls were kept in an ice-bath for the assay of the enzyme activity (enzyme concentration in the assay solution = 2.86×10^{-8} M) using TAME by the method of Hummel (1959). The percentage of inhibition was calculated by comparing the activity of the inhibited enzyme with that of a control (Table 1).

Inhibitions in the Presence of Benzamidine Hydrochloride. Solutions of benzamidine, inhibitor **1c**, and trypsin (molar ratio = 236:4:1) and benzamidine, inhibitor **2a**, and trypsin (molar ratios = 236:5.2:1 or 236:32:1) were incubated for 30 min in a pH 6.5, 0.036 M NaH_2PO_4–0.021 M Na_2HPO_4 buffer (ionic strength μ = 0.1) (Boyd, 1965). A parallel control run without the competitive inhibitor was also carried out for comparison. The procedures of inhibition and the assay of the enzyme activity were the same as described in the entry on the inhibition of trypsin (the final enzyme concentration in the assay solution was 2.86×10^{-8} M).

*Inhibition of α-Chymotrypsin with **DL2a**.* A solution containing the inhibitor and enzyme (43:1 molar ratio; enzyme = 4.3×10^{-5} M) in a pH 7.8, 50 mM phosphate buffer was incubated at 25 °C. Aliquots were taken and diluted using the procedures described for the inhibition of trypsin and they were assayed using BTEE by the method of Hummel (1959).

Reactivation of Inhibited Trypsin (General Procedure). Reactivation of trypsin inhibited by **1**–**3** was studied by monitoring the recovery of enzyme activity with time at 25 °C. The media were the same as listed for the inhibition step (*vide supra*). Inhibition was determined on the basis of the control activity; with respect to assays of **1a**, **1b**, and **cc**, trypsin, at pH 7.2 in 50 mM Tris-HCl buffer, lost about 5–10% activity after 24 h of incubation. For DL**2a** and D**2a**, trypsin lost 10–15% activity after 24 h of incubation at pH 8.1. Buffer solutions were 50 mM (pH 7.2) or 40 mM (pH 8.1) Tris-HCl with 10 mM $CaCl_2$. For example, a solution of [1-^{13}C] **1c**-inhibited enzyme (8.6×10^{-7} M, after G-25 chromatography) was prepared in 1.00 mL of 50 mM (pH 7.2) Tris-HCl buffer containing 10 mM $CaCl_2$. A control solution with active trypsin was prepared in the same way. Aliquots (100 μL) were removed from the inhibited enzyme solution at intervals, and the enzyme activity (enzyme concentration 2.86×10^{-8} M) was assayed (Figure 1).

Hydroxylamine Runs. Solutions of [1-^{13}C] **1c**-inhibited enzyme containing different concentrations of the buffered hydroxylamine were prepared in an identical manner as described above, and the enzyme activity was also assayed periodically. Pseudo-first-order kinetic constants of regeneration of the inhibited enzyme were obtained from these experiments.

*O → N Acyl Migration of 4-Amino-1-butyl Benzoate (**10**).* One milliliter of D_2O was added to 2.0 mg of the title compound. The initially turbid solution became clear within a few min (pH ~ 9). The rearrangement was followed by ^{1}H NMR spectroscopy (and subsequently at pH 12).

^{13}C NMR Spectra of Labeled Inhibited Trypsin and Its Hydrolysates (General Procedure). ^{13}C NMR spectra were measured on inhibited trypsin in 0.006 N HCl made up to contain 15% D_2O. Acquisition parameters were: pulse width = 17.5 μs (a 90° pulse = 21 ms), and an acquisition time = 0.54 s with no delay (10 000–40 000 transients). The decoupling parameters were set based on the "Gamma-H2 Test" (a program included in the Varian XL-400 software programs), and tested each time with the use of the same solvent and tube containing dioxane before the protein samples were run. A line broadening of 10–20 Hz was usually taken in Fourier transformation (acetonitrile was used as a reference; chemical shift = 2.92 ppm at pH ~ 3 relative to the chemical shift of 0.0 ppm for TSP in D_2O). In case of hydrolysates (12–45 mg per run), the samples were dissolved in 0.50 mL of 0.1 N DCl in D_2O, and ^{13}C NMR spectra were recorded at 100 MHz. Acquisition parameters were an acquision time of 0.74 s with 1 s delay and a pulse width of 19.0 μs (a 90° pulse width = 21 μs); 20 000–40 000 transients were usually acquired. A line broadening of 5 Hz was applied (except as otherwise noted).

*Preparation and Reactions of [1-^{13}C] **1c**- and **D2a**-Inhibited Trypsin and ^{13}C NMR Studies. (1) Inhibition of Trypsin by [1-^{13}C] **1c** and **D2a**.* Trypsin (45.0 mg, 1.93 μmol) was dissolved in 2.95 mL of a 15% D_2O (pH 7.2) phosphate buffer (42 mM, ionic strength μ = 0.1) [two aliquots (6.68 μL) of the enzyme solution were taken and diluted to 8.6×10^{-7} M with 1 mM HCl as controls for assay of the enzyme activity]. ^{13}C-Labeled *N*-nitrosobenzamide **1c** (0.965 mg, 3.59 μmol) (15.5 μmol for [1-^{13}C] D**2a**) in CH_3CN (50 mL) was added with gentle stirring during a period of 2 min. After 18 min (30 min for [1-^{13}C] D**2a**), two aliquots (6.68 mL) were taken and diluted to 8.6×10^{-7} M with 1 mM HCl for assay of enzyme activity. The remaining solution was adjusted to pH 3.0 with 2 N DCl (pH 2.2 for [1-^{13}C] D**2a**), and ^{13}C NMR spectra were measured relative to a control with native trypsin (45 mg).

(2) ^{13}C NMR Spectra after G-25 Chromatography. The inhibited trypsin was chromatographed on Sephadex G-25 with elution *via* 1 mM HCl at a flow rate of 1.07 mL/min. Fractions containing the inhibited trypsin were pooled and lyophilized to give a white, puffy solid (30 mg for [1-^{13}C] **1c** and 34 mg for [1-^{13}C] D**2a**). The lyophilized enzyme from **1c** was dissolved in 2 mL of 1 mM HCl [15% D_2O, with 1.7% CH_3CN as a reference (2.92 ppm relative to TSP

in D_2O)] (6 mM HCl for [1-^{13}C] D**2a**); ^{13}C NMR spectra were then measured.

(3) Enzyme Activity. The enzyme activity was monitored in each step using the general procedure given earlier for the inhibition of trypsin. The inhibition values were corrected for the loss of activity of native enzyme (control).

*(4) ^{13}C NMR Spectra of the [1-^{13}C] D**2a**-Inhibited Trypsin Incubated with Hydroxylamine.* One-half of the solution of inhibited trypsin from the ^{13}C NMR study (Part 2 above) containing 17 mg of the enzyme was brought to pH ~ 7 with 2 N NaOD, and 0.1 mL of 1 N hydroxylamine at pH 7.2 was added (final pH = 7.2 ± 0.1). The solution became turbid. Incubation was carried out for 6 h and then the pH was adjusted to 2.2 with 2 N DCl; the solution became almost clear. The solution was centrifuged, and the ^{13}C NMR spectrum of the supernatant (10-mm tube) was obtained (Figure 3). The remaining half of the inhibited enzyme (from Part 2 above) was treated with hydroxylamine for 1 h as described above, and then assayed for activity. After a further incubation at 25 °C for 12 h, enzyme activity was again assayed. In both cases, 73−76% inhibition was observed.

*(5) ^{13}C NMR of 6 N HCl Hydrolysates of [1-^{13}C] **1c**- and D**2a**-Inhibited Trypsin.* [1-^{13}C] **1c**-Inhibited trypsin (12 mg) after G-25 chromatography was hydrolyzed (White *et al.*, 1990) [native trypsin (12 mg) as a control was treated in the same way]. The residue from the hydrolysis was dissolved in 0.60 mL of 0.1 N DCl in D_2O containing 1.7% CH_3CN. The D**2a**-inhibited enzyme solution (17 mg) (from part 4 above), after incubation with 0.09 M hydroxylamine at pH 7.2 for 6 h, was lyophilized. The residue was hydrolyzed (White *et al.*, 1990), and the hydrolysates were dissolved in 0.50 mL of 0.1 N DCl in D_2O; ^{13}C NMR spectra were obtained (Figure 2).

RESULTS

Syntheses. The preparation of the *N*-nitroso modified amide and peptide inhibitors (**1**−**3**) posed two major problems: (1) the necessity of nitrosating an amide group in the presence of the more basic primary amino function; this problem was largely solved by keeping the amino group in its protonated form, but carefully defined conditions were necessary since the acids required for protonation also lead to denitrosation of the desired nitrosoamide grouping (White, 1955); (2) the presence in some of the inhibitors of two amide groups, only one of which was to be nitrosated; this problem was solved by limiting the amount of nitrosating agent used, by controlling the reaction temperature and time, and by design, in which steric congestion was built into one of the amide linkages to inhibit access sterically by the nitrosating agent.[2]

Hydrolytic Stability of Inhibitors. Inhibitors DL**2a** and D**2a** exhibit half-lives of ~3 min at pH 8.1 and ~14 min at pH 6.5 in 40 mM Tris-HCl buffers. Inhibitors **1c** and D**2b** exhibit half-lives of ~2 h in a 50 mM (pH 7.2) Tris-HCl buffer, while inhibitor **1b** has an intermediate half-life of ~ 30 min in the same buffer. Thus, inhibitors with dimethyl substitution on the terminus of the putrescine moiety are more stable than those bearing the unsubstituted amino group. In the former case, direct hydrolysis of the labile nitrosoamide grouping is responsible for the instability observed, whereas in the latter, a neighboring group nucleophilic interaction of

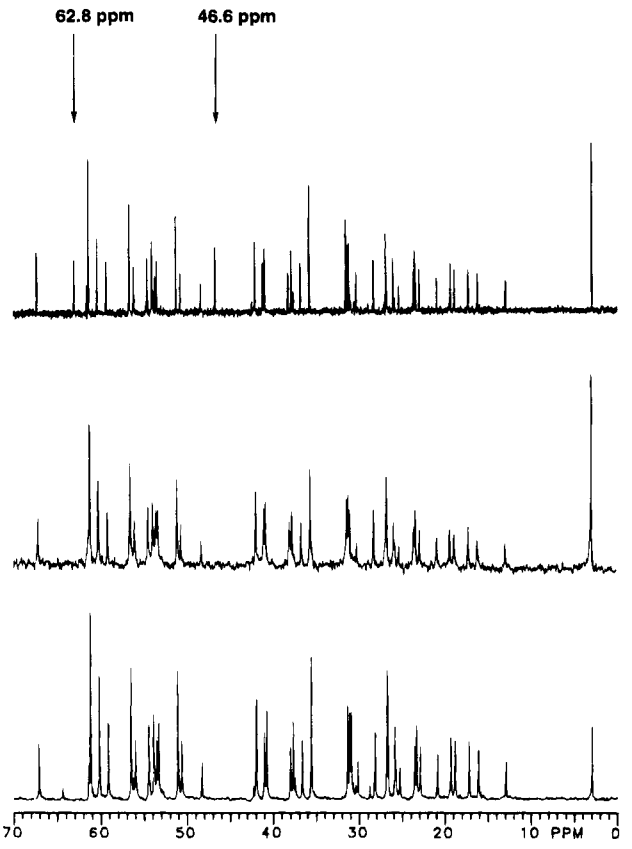
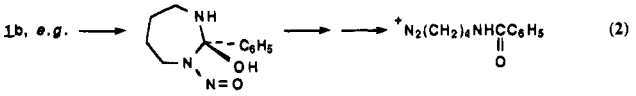


FIGURE 2: ^{13}C NMR spectra of 6 N HCl hydrolysates of [^{13}C] D**2a**-inhibited trypsin (top curve), [^{13}C] **1c**-inhibited trypsin (Middle Curve), and native trypsin (bottom curve).

the amino function with the carbonyl group is probably involved (eq. 2).



(2)

Inhibition Kinetics. All of the inhibitors except **3** react with trypsin very rapidly. The reactions are complex since both irreversible and reversible alkylations are occurring (Figure 1). The inhibition of trypsin by these inhibitors was determined at 25 °C in 50 mM Tris-HCl with 10 mM $CaCl_2$ at pH 7.2 for inhibitors **1a**, **1b**, and **1c** or 40 mM Tris-HCl with 10 mM $CaCl_2$ at pH 8.1 for inhibitors DL**2a**, D**2a**, **2b**, and **3** (enzyme concentration = 4.3 × 10^{-5} M). The initial rate of inhibition by **1a** at an inhibitor−enzyme ratio of 10/1 followed a pseudo-first-order rate law with $t_{1/2}$ = ~1.5 min. Inhibitor **1b** at an inhibitor/enzyme ratio of 1/1 inhibited trypsin 100% in 3.8 min. When trypsin was incubated with **1c** at an inhibitor/trypsin ratio of 2/1, a second-order rate was measured for the initial reaction with a rate constant k_{inact} of 18.4 ± 0.3 $M^{-1}\cdot s^{-1}$ (enzyme concentration = 6.43 × 10^{-4} M) (half-life = 37 sec). For inhibitors DL**2a** (inhibitor/enzyme = 64/1) and D**2a** (inhibitor/enzyme = 30/1), the half-lives of inhibition were less than 9 and 5 min, respectively. The time to reach maximum inhibition for inhibitor D**2b** (inhibitor/enzyme = 20/1) was 3 min.

Reaction of the Inhibitors with Trypsin. All of the inhibitors produced some inhibition of trypsin at least over a short term (Table 1). The inhibition by **1c** was fully reversible (upon incubation in buffer), those of **1a** and **1b** were mostly reversible, that of D**2b** was predominantly

Table 1: Inhibition of Trypsin by *N*-Nitrosoamides, $RCON(NO)^{a}CH_2(CH_2)_nNR'_2$

inhibitor			molar ratio (inhibitor/	maximum inhibition	reversible portion[b]	irreversible portion[c]
R	R′	*n*′	enzyme)	(%)	(%)	(%)
$R = C_6H_5$						
1a	CH_3	2	4	88	88	0
			10	92	80	12
				(91)[d]	75[e]	16
1b	H	3	1	100	85	15
1c	CH_3	3	1	58 ± 4	58 ± 4	0
			4/1	87		
			(59/4/1)[f]	0		
			17	96 ± 1	96	0
$R = (CH_3)_2CHCONHCH(CH_3)$						
DL**2a**	H	3	12	29 ± 2	0	29
			40	75 ± 6	0	75
D**2a**	H	3	1	14 ± 5	0	14
			4.4	41	0	41
			5.2	50	0	50
			(44/5.2/1)[f]	5		
			30−32	82 ± 3	0	82
			(237/31/1)[f]	21		
			60	91 ± 2	0	91
D**2b**	CH_3	3	20	32 ± 2	12	20
			60	45	20	25
3			~100	19		

[a] ^{13}C at this position for **1c** and D**2a**. [b] Trypsin activity regenerated after long incubation in the buffers (*e.g.*, 24 h) [corrected for trypsin denaturation (5−10%)]. [c] Maximum inhibition-reversible portion. [d] After Sephadex G-25 separation. [e] Inhibited trypsin that was incubated with 0.02M NH_2OH. [f] In the presence of benzamidine (benzamidine/inhibitor/enzyme).

irreversible, whereas those of DL**2a** and D**2a** were totally irreversible.

Benzamide-Based Inhibitors. ***1c***. Inhibitor **1c** led to 58% inhibition of trypsin at a ratio of inhibitor enzyme of 1:1 (Figure 1), and 96% inhibition at a ratio of 17:1. In both cases, the activity of trypsin could be fully regenerated on incubation in buffers (Figure 1 and Table 1). The **1c**-inhibited trypsin from Sephadex G-25 column purification was incubated in a 50 mM Tris-HCl buffer (pH 7.2) with 10 mM $CaCl_2$. Trypsin activity was regenerated following first-order kinetics, $k = 5.0 \times 10^{-4}$ s^{-1}, $r = 0.997$ (half-life = 23 min). The inhibited trypsin produced by inhibitor **1c** was reactivated completely regardless of the inhibitor/enzyme ratio used. The first-order aspect suggests that the enzyme was modified at only one site. The half-life of reactivation observed is essentially the same as that measured for benzoyltrypsin (24 min). In confirmation of the view that the inhibited enzyme was benzoyltrypsin, the reactivation of **1c**-inhibited trypsin was accelerated by 20 mM hydroxylamine, $t_{1/2} = 13.2$ min ($k = 1.1 \times 10^{-3}$ s^{-1} with $r = 1.000$). Further confirmation was obtained from ^{13}C NMR studies (*vide infra*).

1a. This inhibitor differs from **1c** only in possessing one fewer carbon unit in the aliphatic chain. At a low **1a**/enzyme ratio (4/1) only reversible inhibition (88%) was observed (Figure 1). At a ratio of 10/1, 92% inhibition was observed distributed as 80% reversible, 12% irreversible (Table 1). After purification on Sephadex G-25, the amount of inhibition remained high (91%, Table 1). When the desalted inhibited trypsin was treated with 20 mM NH_2OH at pH 7.2 (50 mM Tris-HCl with 10 mM $CaCl_2$), trypsin activity was regained (of the original 91%, 75% was reversible and 16% irreversible: Table 1). The half-life of regeneration of trypsin activity was 30 min in the same buffer used for the **1c**-inhibited enzyme; this value is close to the half-life of 24 min as observed for benzoyltrypsin.

1b. Inhibitor **1b**, bearing a free amino group at the end of the 4-carbon aliphatic side chain, led to inactivation of trypsin in both the reversible and irreversible modes. At an inhibitor/enzyme ratio of 1:1, the enzyme was inhibited to the extent of 100% within 4 min: 85% of the inhibited trypsin was in the reversible form and 15% was in the irreversible form (Table 1). Under the same conditions used for the 1c-inhibited enzyme the time course of regeneration of the enzyme activity, which followed first-order kinetics, gave a half-life of 29 min for the regeneration of trypsin activity. The inhibitor is so potent that the partition ratio (k_2/k_{inact}) is zero, the theoretical limit for inhibitors (Walsh *et al.*, 1978). This inhibitor is one of the most potent known inhibitors of trypsin; it may well be a useful titrant for trypsin and related enzymes (Bender *et al.*, 1965; Chase & Shaw, 1967).

Alanine-Based Inhibitors. ***D2a***. Inhibitions of trypsin by DL**2a** and D**2a** are totally irreversible under our incubation conditions (Table 1). At an inhibitor D2a/enzyme ratio of 4.4:1, 41% inhibition was observed (see Table 1 for other ratios). After an incubation of 4 h at pH 8.1 using the same buffer used with inhibitors in the 1 series, no enzyme activity was regained. (Note: Since inhibitor D**2a** has a half-life of ~3 min in the same buffer, no effective amounts of inhibitor remained after ~20 min; that is, the inhibition could not be attributable to the large initial excess of inhibitor used.)

D2b. More complicated results were obtained with this inhibitor; it contains an alanine moiety as in inhibitor D**2a**, but the 4-position of the putrescine moiety is modified by two methyl groups. At an inhibitor/enzyme ratio of 20/1, 32% inhibition occurred at 3 min (Table 1), of which 20% was irreversible and 12% was reversible under the experimental conditions.

Lysine-Based Inhibitor. Inhibitor **3** is a cyclic DL-lysine derivative. At an inhibitor/enzyme ratio of ~100, trypsin became 19% inhibited (Table 1). The inhibitor is not very selective since chymotrypsin was inhibited to the extent of 27% using an inhibitor **3**/enzyme ratio of 46/1.

Competitive Inhibitor Studies. In the presence of the competitive inhibitor benzamidine (Mares-Gula & Shaw, 1965), the inhibition of trypsin was affected dramatically in the case of both inhibitors **1c** and D**2a**. With a benzamidine/**1c** ratio of 59/4, and an inhibitor/enzyme ratio of 4/1, no inhibition of trypsin by inhibitor **1c** was observed, whereas 87% inhibition occurred when the competitive inhibitor was absent (Table 1). Similarly, inhibitor D**2a** inactivated trypsin activity to the extent of 50% (inhibitor/enzyme = 5.2/1) in the absence of benzamidine, whereas in its presence at a ratio to D**2a** of 44/5.2, only 5% inhibition was obtained (Table 1). The results indicate that these *N*-nitrosoamide inhibitors are active-site-directed enzyme-activated substrates and that the modification of trypsin occurs in the active site.

Selectivity in the Inhibition of Trypsin and Chymotrypsin. With a 40/1 ratio of inhibitor to enzyme, inhibitor DL**2a** inactivated trypsin activity to the extent of 75% while only 4% inhibition was observed in the inhibition of α-chymotrypsin at a 43/1 inhibitor to enzyme ratio. Thus, as expected from the presence of the charged side-chain, DL**2a** is more efficient in the inhibition of trypsin than of chymotrypsin.

*^{13}C NMR Studies of ^{13}C-Labeled **1c**- and D2a-Inhibited Trypsin. **1c**-Inhibited Enzyme.* Bovine trypsin was inhibited with ^{13}C-labeled **1c** in a pH 7.2 phosphate buffer for 18 min (inhibitor/enzyme = 1.9; 86~88% inhibition). ^{13}C NMR spectra of the reaction mixture adjusted to pH 3 with 2 N DCl was compared with that of native trypsin under the same conditions. The difference spectra showed new peaks for the inhibited enzyme mixture in the region between 0 and 130 ppm (relative to TSP at 0). The chemical shifts (relative peak heights) were 121.0 (8%); 67.8 (4%); 66.8 (2%); 62.9 (31%); 41.1 (49%); 24.1 (6%). The major peaks can be assigned as follows: 62.9 ppm ([1-^{13}C] 4-dimethylamino-1-butanol), 41.1 ppm ([1-^{13}C] *N*-(4-dimethylamino-1-butyl)-benzamide (amide precursor of **1c**), and 24.1 ppm ([1-^{13}C] 4-dimethylamino-2-butanol). The 121.0 ppm peak might stem from the minor by-product, [1-^{13}C] 4-dimethylamino-1-butene; the minor peaks at 67.8 and 66.8 ppm are unidentified. The assignments were based on the ^{13}C and ^{1}H NMR spectra of the decomposition products of inhibitor **1c** in a pH 7.2 buffer. The large amount of amide results from acid-catalyzed denitrosation of inhibitor **1c** in the pH 3 medium (White, 1955). The formation of the secondary alcohol is expected from the carbocation nature of the reaction (White, 1955).

The inhibited trypsin was purified on Sephadex G-25 to remove small molecular weight compounds. The ^{13}C NMR spectrum of the recovered enzyme mixture was essentially the same as that of trypsin in the same medium; no additional peaks were observed. In order to search further for the ^{13}C-label, the desalted, inhibited trypsin (12 mg) was hydrolyzed with 6 N HCl at 115 °C for 22 h. The ^{13}C NMR of the lyophilized hydrolysate spectrum in 0.1 N DCl (45 mg of enzyme) showed no new peaks relative to the hydrolysate of native trypsin (45 mg) (Figure 2). This result implies that either the inhibited enzyme contained no ^{13}C or that the ^{13}C-labeled moiety was freed during the hydrolysis and then volatilized during the lyophilization. The latter possibility seems to be ruled out because no product containing the dimethylamino group should be volatilized during lyophilization since the amino group will be in the form of the relatively nonvolatile hydrochloride salt. Thus, inhibition by **1c** does not involve attachment of the ^{13}C-labeled alkyl group. It should be noted that the half-life for the regeneration of enzyme activity from **1c**-inhibited trypsin (23 min) is essentially the same as that measured for "benzoyltrypsin" (see Experimental Procedures section). Both the ^{13}C NMR spectra and the kinetics offer evidence that the reversibly inhibited enzyme formed from inhibitor **1c** is "the acyl enzyme," benzoyltrypsin.

D2a-Inhibited Trypsin. Trypsin was treated with 8 molar equivalents of the ^{13}C-labeled inhibitor **D2a** in a (pH 7.2) phosphate buffer (15% D_2O) for 30 min (70% inhibition); then the reaction mixture was adjusted to pH 2.2 with 2*N* DCl. The inhibited trypsin was desalted on a Sephadex G-25 column, and the ^{13}C NMR spectrum was measured (Figure 3, top). A ^{13}C NMR spectrum of the native enzyme was obtained in the same way (Figure 3, bottom). A strong new peak was observed at 67.2 ppm in a region expected for $-COO^{13}\underline{CH_2}-$ groups; for example, ester **10** shows a signal at 67.2 ppm in D_2O. On standing a gradual shift occurs to 62.9 ppm attributed to the amide form (O→N acyl migration). To verify this general assignment, one-half of the sample (17 mg) was treated with 0.09 M NH_2OH at pH 7.2 for 6 h,

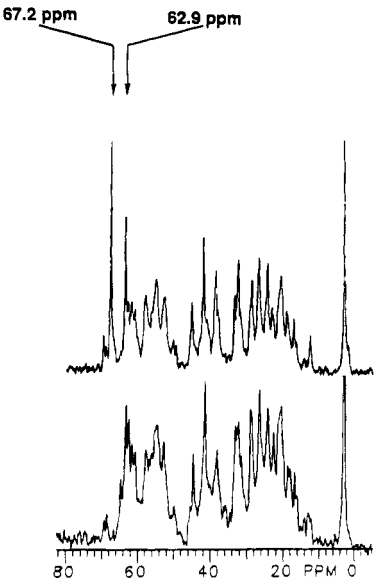
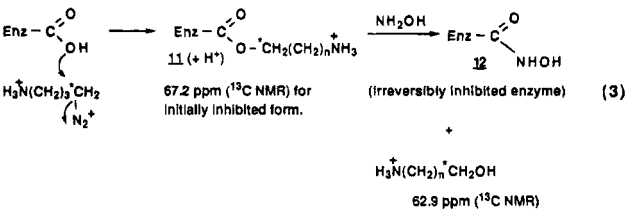


FIGURE 3: ^{13}C NMR spectra of **D2a**-inhibited trypsin (top spectrum) and native trypsin (bottom spectrum). The peaks at 2.9 and 67.2 ppm were truncated to fit the format.

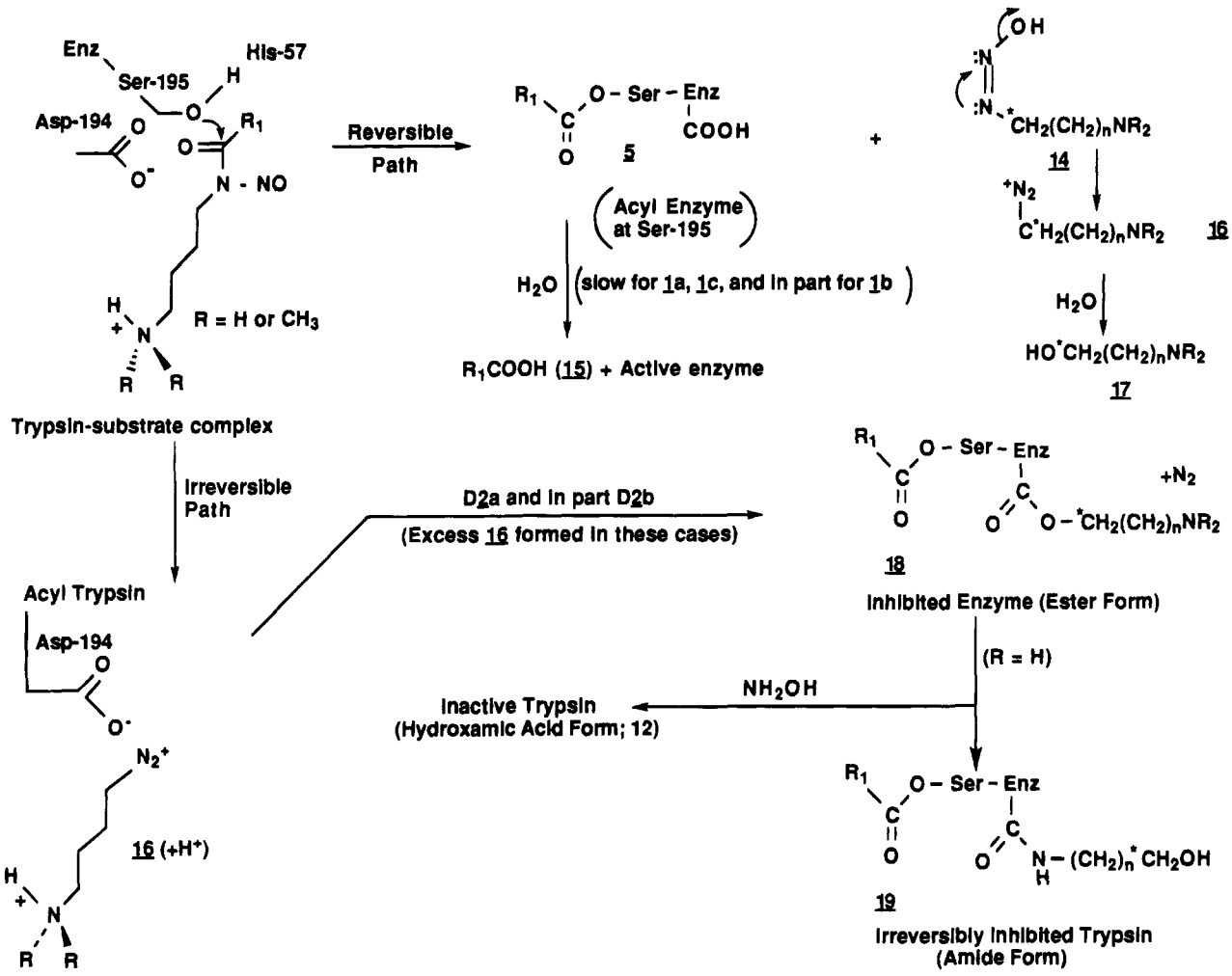
followed by acidification to pH 2.2. The ^{13}C NMR spectrum showed that two-thirds of the 67.2 ppm peak disappeared; the remaining third is also present in the spectrum of native trypsin and is attributed to the beta-carbons of the 10 threonine residues of the enzyme (White *et al.*, 1990). A peak at 62.9 ppm grew in intensity; this peak has the same chemical shift (62.9 ppm) as that of [1-^{13}C] 4-amino-1-butanol measured under the same conditions. The spectra support the hypothesis that a carboxyl group of the enzyme had been alkylated with the 4-amino-1-butyl diazonium ion (or the corresponding carbocation) and that treatment with hydroxylamine had released 4-amino-1-butanol from the ester with the formation of, presumably, a hydroxamic acid group (eq. 3). The remaining half of the inhibited enzyme (17 mg)



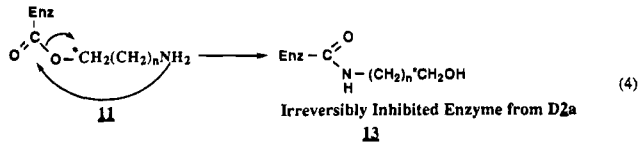
was treated with hydroxylamine for 1 h. The solution was adjusted to pH 7.2 and incubated at 25 °C for 12 h; measurement of the enzyme activity showed 73% inhibition. Thus, no regeneration of enzyme activity had occurred during the transformations.

A sample of the same 6 h hydroxylamine-treated enzyme (17 mg) was lyophilized and fully hydrolyzed in 6 N constant boiling HCl. The ^{13}C NMR spectrum of the hydrolysates (in 0.1 N DCl in D_2O) compared to that of hydrolysates of native trypsin showed two new peaks (Figure 2). One was the 62.8−62.9 ppm peak referred to above, stemming from 4-aminobutanol, and the other was a peak at 46.6 ppm that arose from 4-chloro-1-butylamine formed by action of hydrochloric acid on the alcohol. Pure 4-amino-1-butanol was treated with hydrochloric acid under the same conditions, and ^{13}C and ^{1}H NMR spectra showed that 4-chloro-1-butylamine was formed in 55% yield.

Scheme 1: Mechanism of Inactivation of Trypsin by Nitrosoamide Inhibitors **1** and **2**



O → N Acyl Migration. The *irreversibly inhibited* enzyme (from **D2a**, for example) was stable to buffer (pH 6.5−8.1) for long periods of time (>22 h). Based on the high rate of ester hydrolysis under basic conditions, enzyme activity should have been regenerated within the time of incubation, but it was not. In order to explain this observation, it was hypothesized that the 4-amino-1-butyl carboxylate ester formed in the inhibition of the enzyme underwent O → N acyl migration (Lemieux, 1964; Banthope, 1968; Deslongchamps *et al*, 1975, Chinsky *et al.*, 1989) to form the corresponding *N*-(4-hydroxyl-1-butyl)carboxamide, resulting in the formation of an *irreversibly inhibited* trypsin (**13**) (eq. 4). 4-Amino-1-butyl benzoate (**10**) was used to test the



(4)

possibility. The ^{1}H NMR spectrum of compound **10** was recorded at intervals after it was dissolved in 99% D_2O. Within 20 min, ~10% of *N*-(4-hydroxy-1-butyl)benzamide (δ $NHCH_2$ = 3.41 ppm) and 3% of 4-amino-1-butanol (δ OCH_2 = 3.65 ppm) were produced (migration/hydrolysis = ~3). The reaction medium was basified to pH ~12 (0.01*N* NaOD); no ester was detected after 30 min of incubation, but in its place were formed *N*-(4-hydroxy-1-butyl)benzamide (37%) and 4-amino-1-butanol (63%) (the ratio of the O → N acyl migration to hydrolysis = ~0.6). This 0.6 value is considerably lower than the value of ~3 found for the hydrolysis in water alone in the initial stage, presumably because of the enhanced rate of the bimolecular hydrolysis reaction with hydroxide ion.

DISCUSSION

The proposed mechanism of inhibition of trypsin by inhibitors **1**−**2** is given in Scheme 1. That the active-site competitive inhibitor benzamidine decreases the extent of inhibition of trypsin indicates that the inhibitors are active-site−directed. It is proposed that all the inhibitors first react with the enzyme to form the acyl trypsin (**5**). The diazonium ions (**16**) (or carbocations) formed in this process modify the carboxylic acid in closest proximity, Asp-194, to form the corresponding ester (**18**), or they are trapped by water to produce the alcohol (**17**), or to a lesser extent they alkylate other sites on the enzyme (noted especially when large inhibitor/enzyme ratios were used).

Four major variables governing the inhibitors have been identified.

(1) Inhibition vs Length of the Alkyl Chain. Compound **1b** is the most potent benzoyl based inhibitor, and 15% of the inhibition is irreversible when a 1/1 ratio of inhibitor to enzyme is used; the side chain in the binding pocket contains 5 heavy atoms in this case (Figure 4). Compound **1a** is a

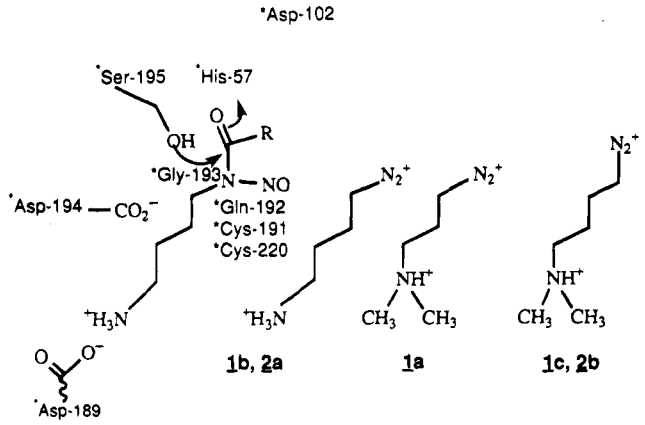


FIGURE 4: Schematic drawing of the proximity of the diazonium ions to nucleophiles in the vicinity of the active site of trypsin (* = enzyme residues).

little less reactive, but a similar pattern of inhibition is observed at a 10/1 ratio (Table 1); the total length of its side chain also consists of 5 heavy atoms (Figure 4). Compound **1c** possesses a side chain with a total length of 6 heavy atoms, and it leads solely to reversible inhibition. The diazonium ions are totally converted into hydrolysis products (1° and 2° alcohol and alkene) by water in this case. It is thus reasonable to assume that a total length of 5 heavy atoms (Figure 4) in the binding pocket brings the reactive $-CH_2^+$ terminus within bonding distance of the carboxyl group of aspartate 194 during the enzymatic reaction, whereas a 6-atom length forces the $-CH_2^+$ ($-CH_2N_2^+$) terminus past the aspartate carboxyl group and into the water medium, where it is rendered innocuous by conversion into the corresponding alcohol; in this case, the only inhibition stems from formation of the acyl enzyme, benzoyltrypsin. A muted, but similar effect of chain length is apparent in the comparison of inhibitors D**2a** and D**2b**. A study of Hartmann and Holler (1970) on the binding sites of trypsin has led to a similar conclusion that atoms 6–8 in a chain bound in the specificity pocket are exposed to the medium.

(2) The Acyl Moiety. With benzoyl-based inhibitors (**1a**–**c**), the benzoyl trypsin formed is relatively stable. Thus, initially, only a single molar equivalent of the reactive species (diazonium ions or carbocations) is formed, most of which react with water; slow hydrolysis of the acyl trypsin regenerates active enzyme. When the acyl moiety is an alanine derivative (DL**2a**, D**2a**, and D**2b**), the more "natural" acyl trypsin undergoes deacylation very rapidly so that new inhibitor molecules are continually converted into diazonium ions (and/or carbocations). These species alkylate Asp-194 and other sites on the enzyme.

(3) The Amino Terminus. The primary amino group in inhibitors **1d** and D**2a** supports the irreversible inhibition mode, and the dimethylamino group (in **1a**, **1c**, and D**2b**) supports the reversible mode (Table 1). The free amino moiety is capable of participating in an O → N acyl migration leading to an alkyl amide linkage (eq. 2) that is considerably more resistant to hydrolysis than the alkyl ester linkage, whereas the dimethylamino moiety cannot form a stable amide unit; migration does not occur, and the ester labels can be hydrolyzed to regenerate active enzyme. The chain-lengthening effect of the dimethylamino groups was addressed in section (1).

(4) Chirality. The D-alanyl isomer D**2a** is about four times as potent as the racemate, DL**2a** (41% inhibition with a ratio of D**2a**/enzyme = 4.4 and 29% inhibition with a ratio of DL**2a**/enzyme = 12, resp.) (Table 1). This result implies that the L-isomer might not be an irreversible inhibitor of trypsin. The saturation of inhibition (75% and 74% inhibition at inhibitor DL**2a**/enzyme ratios of 40/1 and 64/1, respectively) suggests that the L-isomer in DL**2a** could be a competitive inhibitor. This result is also rational based on the X-ray structure of the pancreatic trypsin inhibitor–trypsin complex (Huber *et al.*, 1974b). In Figure 5, the L- and D-isomers are sketched embedded in the active site of trypsin. The D-isomer has the same orientation of the α-methyl group at the chiral center as the natural inhibitor when it binds to trypsin, but for the L-isomer, the methyl orientation is opposite, and binding to the enzyme is presumably not efficient enough for the catalytic step, but adequate for general binding. A similar finding of enantiomeric differences with respect to the inhibition of α-chymotrypsin by *N*-benzyl-*N*-nitroso-*N'*-isobutyrylphenylalaninamide was reported by White *et al.* (1981). In that study, the L-isomer was found to be solely an innocuous substrate for the enzyme

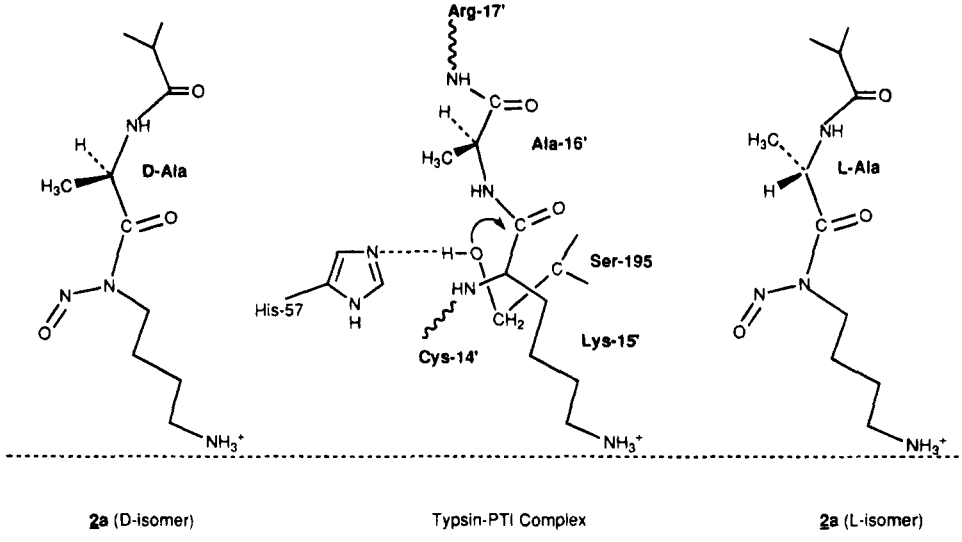


FIGURE 5: Orientation of alanine-based inhibitors in the active site of trypsin. The schematic drawing of the trypsin–PTI complex was adapted from figure 8 in Huber *et al.*, (1974), and from computer graphic displays.

(even though its reaction rate with the enzyme was greater) while the D-isomer inhibited the enzyme irreversibly.

Evaluation of the Inhibitors. Inhibitor **1c** inhibits trypsin only *reversibly* (Figure 1) even at a 20/1 ratio of inhibitor to enzyme. The use of ^{13}C-labeled 1c and NMR spectroscopy showed that the dimethylaminobutyl group is not attached to the enzyme, but is converted into dimethylaminobutanol during the inhibition. The diazonium ions (or carbocations) formed *in situ* do not modify any critical amino acid residue of trypsin, presumably because the bulky dimethylamino group has placed the reactive diazonium ion past the carboxyl group of aspartate-194 and into the medium (Figure 4). The inhibition stems from the formation of benzoyltrypsin; slow hydrolysis leads to the regeneration of active trypsin.

For inhibitor **1b**, the *reversible* inhibition should follow the same mechanism outlined above for inhibitor **1c**. The half-life of regeneration of the reversibly inhibited trypsin species (major portion) has a value of 29 min, similar to the 23 min found for **1c**-inhibited enzyme and 24 min for authentic benzoyltrypsin (the regeneration follows a first-order rate law). The small amount of *irreversible* inhibition observed at a low inhibitor/enzyme ratio (Table 1) probably follows the mechanism for the inhibition of trypsin by D**2a** (*vide infra*) because both inhibitors yield the same reactive species, 4-amino-1-butyl diazonium ion (**16**).

Inhibitor **1a**, as a dimethylamino-substituted and benzamide-based inhibitor, is related to **1c**, and the mechanism of inhibition at a low inhibitor-to-enzyme ratio is believed to be the same; that is, benzoyltrypsin formation and completely reversible inhibition. The total length of the binding moiety is the same as for **1b**, but **1a** is less efficient, presumably because the nitrogen atom in the $N(CH_3)_2$ group is one atom displaced from its normal position as the terminal nitrogen, and hydrogen-bonding options to the nitrogen atom are thus altered. At high inhibitor/enzyme ratios some irreversible alkylation of other sites occurs (Table 1).

Inhibitor D**2a** inhibits trypsin solely *irreversibly*. Approximately 10% of the 4-amino-1-butyl diazonium ions (**16**) are oriented favorably to react with the carboxylic acid group of Asp-194 in a displacement reaction to form "4-amino-1-butyl (aspartate) trypsin" (**18**) (Scheme 1). Evidence for this "ester" form of D**2a**-inhibited trypsin was obtained via ^{13}C NMR spectroscopy (δ at 67.2 ppm) using [(13C)] D**2a**-inhibited enzyme (Figure 3). Treatment of the "ester" form (**18**) with hydroxylamine released 4-aminobutanol while the enzyme remained inactive. This lack of hydroxylamine-mediated reversibility is consistent with the hypotheses outlined in eq. 3; that is, the aspartate ester (**18**) on reaction with hydroxylamine is converted into the corresponding hydroxamic acid. Since a negatively charged carboxylate group is required for enzyme activity (Huber & Bode, 1978), the "hydroxamate" enzyme is inactive.

The "ester" form of D**2a**-inhibited trypsin does not regenerate active trypsin during incubation at pH 6.5−8.1. Instead, an O → N acyl migration to form the stable "amide" form of the inhibited enzyme (eq. 4) occurs; that is, reaction of the "ester" form with a good nucleophile, hydroxylamine, is fast enough to compete with O → N acyl migration, but reaction with water and low concentrations of hydroxide ion is not.

The assumption that the "aspartic acid ester under discussion" relates to Asp-194 is supported by other studies (Aboderin & Fruton, 1966; Carraway *et al.*, 1969). Aboderin and Fruton's (1966) work suggested the participation of two carboxyl groups in the enzymatic action, while later it was shown (Carraway *et al.*, 1969) that one of those residues was Asp-194. From the X-ray structure of pancreatic trypsin inhibitor−trypsin complex (Huber *et al.*, 1974a), it can be seen that the reactive diazonium ion moiety is far from Asp-102, but fairly close to Asp-194 (Figure 4). Other nucleophiles that are within atom contact distance are Gln-192, Cys-191, Cys-220, and His-57. From the ^{13}C NMR studies, the possibility that alkylation occurs at the nitrogen atoms of Gln-192 or His-57 can be ruled out because no ^{13}C peaks in the region of 50−60 ppm (the $-\underline{C}H_2-N <$ region) were observed (White *et al.*, 1990) (*N*-alkyl derivative are stable to hydrolysis). S-Alkylation of Cys-191, 220 followed by cleavage to give S-alkyl cysteines is also ruled out based on the same rationale; that is, $-\underline{C}H_2-S-$ is expected to give a peak near 36 ppm (White *et al.*, 1990). Thus, alkylation occurs on oxygen and the carboxyl group of Asp-194 is the most likely alkylated site.

For inhibitor D**2b**, inhibition is inefficient because of the side-chain length, and partial reversibility was noted (Table 1); the latter could arise by hydrolysis of ester groups at Ser-195 and Asp-194 (O → N acyl migration is not possible).

A Comparison of N-Nitrosoamides 1−2 with Other Trypsin Inhibitors. Many conventional active-site-directed inhibitors of trypsin have been reported recently. S-ω-Aminoalkyl thioesters are "inverse" type of inhibitors (Kunieda *et al.*, 1983), the most efficient of which is *S*-4-aminobutylthiobenzoate; at an inhibitor/enzyme ratio of 35 it reversibly inhibited trypsin to the extent of 50% (1 h incubation at 30 °C). 1-(*N*-6-Amino-*n*-hexyl)carbamoylimidazole is a related example (Walker & Elemore, 1984); at an inhibitor/enzyme ratio of 30−76 it "irreversibly" inhibited trypsin and trypsin-like enzymes during 6−14 min of incubation, presumably via acylation of the Ser-195 hydroxyl group of trypsin to form carbamoylated serine stable to hydrolysis (Walker & Elemore, 1984). In comparison, the molar ratio of inhibitor used/enzyme inhibited at the initial stage was found to be 1 for inhibitor **1b**, ~2 for **1c**, and ~7 for D**2a** (Table 1). The half-lives of inhibition were found to be 0.6−2.0 min for the 1 series).

Compared with other "suicide" types of inhibitors, *N*-(4-amino-1-butyl)-*N*-nitrosoamide derivatives (**1b**, D**2a**) are also competitive. 7-(Aminomethyl)-1-benzylisatoic anhydride inhibits trypsin with a half-life of 10 min (inhibitor/enzyme = 25, enzyme concentration = 5×10^{-6} M) (Gelb & Abeles, 1986). The inhibition of trypsin by 4-chloro-7-guanidino-3-methyloxyisocoumarin proceeded with a half-life of 2.7 min ([enzyme] = 0.085 μM, [inhibitor] = 0.1−0.4 μM) (Kam *et al.*, 1988). Both of these inhibitors showed considerable recovery of trypsin (or thrombin) activity on incubation in buffer [thrombin inhibited by the isatoic inhibitor was 30% regenerated after 13 h (Gelb & Abeles, 1986), while trypsin inhibited by the coumarin inhibitor was 45% regenerated in the presence of 0.22 M NH_2OH (Kam *et al.*, 1988)]. In contrast, no reversible inhibition of trypsin was observed for inhibitor D**2a** within a range of inhibitor/enzyme ratios of 1−60.

REFERENCES

Aboderin, A. A., & Fruton, J. S. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 1252.

Amshey, J. W., Jr., Jinal, S. P., & Bender, M. L. (1975) *Arch. Biochem. Biophys. 169*, 1−6.
Atkins, P. W. (1982) *Physical Chemistry* 2nd ed, pp 929−931, W. H. Freeman and Co., San Francisco, CA.
Banthope, D. V. (1968) In *The Chemistry of the Amino Group* (Patai, S. Ed) pp. 585−667, Interscience Publishers, New York.
Barman, Th. E. (1969) *Enzyme Handbook* Vol. 2, 618 pp., Springer-Verlag, New York.
Bender, M. L., Killheffer, J. V., Jr., & Roeske, R. W. (1965) *Biochem. Biophys. Res. Commun. 19*, 161.
Boyd, W. C. (1965) *J. Biol. Chem. 240*, 4097−4098.
Brenner, D. G., & Knowles, J. R. (1981) *Biochemistry 20*, 3680.
Carraway, K. L., Spoerl, P., & Koshland, D. E., Jr. (1969) *J. Mol. Biol. 42*, 133.
Chase, T., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun. 29*, 508−514.
Chen, J., Ishii, M., Wang, L., Ishii, K., & Coughlin, S. R. (1994) *J. Biol. Chem. 269*, 16041−16045.
Chinsky, N., Margolin, L., & Klibanov, A. M. (1989) *J. Am. Chem. Soc. 111*, 386−388.
Chow, M. M., Meyer, E. F., Jr., Bode, W., Kam, C.-M., Radhskrishnan, R., Viyayalakshmi, J., & Powers, J. C. (1990) *J. Am. Chem. Soc. 112*, 7783−7789.
Daniels, S. B., & Katzenellenbogen, J. A. (1986) *Biochemistry 25*, 1436.
Deslongchamps, P., Dubé, S., Lebreux, C., Patterson, D. R., & Taillefer, R. J. (1975) *Can. J. Chem. 53*, 2791−2808.
Donadio, S., Perks, H. M., Tsuchiya, K., & White, E. H. (1985) *Biochemistry 24*, 2447−2458.
Faraci, W. S., & Pratt, R. F. (1985) *Biochemistry 24*, 903.
Festoff, B. W., & Hantai, D. (1990) *Serine Proteases and Their Serine Inhibitors in the Nervous System*, Plenum Press, New York.
Gelb, M. H., & Abeles, R. H. (1986) *J. Med. Chem. 29*, 585−589.
Hartmann, H., & Holler, E. (1970) *Eur. J. Biochem. 16*, 80−91.
Hijikata-Okunomiya, A., Okamoto, S., Tamao, Y., & Kikumoto, R. (1988) *J. Biol. Chem. 263*, 11269−11273.
Hörl, W. H., & Heidland, A. (1988) *Proteases II*, Plenum Press, New York.
Huber, R., & Bode, W. (1978) *Acc. Chem. 11*, 114−122.
Huber, R., Kukla, D., Steigemann, W., Deisenhofer, J., & Jones, A. (1974a) in *Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L. J., & Truscheit, E., Eds) pp 497−512, Springer-Verlag, Berlin.
Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., & Steigeman, W. (1974b) *J. Mol. Biol. 89*, 73−101.
Hummel, B. C. W. (1959) *Can. J. Biochem. Phys. 37*, 1393−1399.
Kam, C.-M., Fujikawa, K., & Powers, J. C. (1988) *Biochemistry 27*, 2547−2557.
Koivunen, E., Ristimaki, A., Itkonen, O., Osman, S., Vuento, M., & Stenman, U.-H. (1991) *Cancer Res. 51*, 2107−2112.
Kunieda, T., Ishizuka, T., & Hirobe, M. (1983) *Chem. Pharm. Bull. 31*, 3360−3362.
Laskowski, M., Jr., Empie, M. W., Kato, I., Kohr, W. J., Ardelt, W., Bogard, W. C., Jr., Weber, E., Papamokos, E., Bode, W., & Huber, R. (1981) in *Structural and Functional Aspects of Enzyme Catalysis* (Eggerer, H. & Huber, R., Eds.) pp 136−152, Springer-Verlag, New York.
Lemieux, R. U. (1964) In *Molecular Rearrangements* (de Mayo, P., Ed) Part 2, pp 709−769, Interscience: New York.
Mangel, W. F., Singer, P. T., Cyr, D. M., Umland, T. C., Toledo, D. L., Stroud, R. M., Pflugrath, J. W., & Sweet, R. M. (1990) *Biochemistry 29*, 8351−8357.
Mares-Gula, M., & Shaw, E. (1965) *J. Biol. Chem. 240*, 1579−1585.
Markwardt, F., Wagner, G., Walsmann, P., Horn, H., & Stürzebecher, J. (1972) *Acta Biol. Med. Ger. 28*, k19−25.
Matsuzaki, T., Sasaki, C., & Umeyama, H. (1988) *J. Biochem. 103*, 537−543.
Nystedt, S., Emilsson, K., Wahlestedt, C., & Sundelin, J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9208−9212.
Neurath, H. (1986) *J. Cell Biochem. 32*, 35−49.
Shimamoto, T., Shimamoto, T., Okada, Y., Yamada, N., Miyata, K., & Kiho, Y. (1991) *Cell Struct. Funct. 16*, 31−38.
Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923−2925.
Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974) *J. Mol. Biol. 83*, 185−208.
Stürzebecher, J. (1986) *Biomed. Biochim. Acta 45*, 1405.
Tanizawa, K., Kasaba, Y., & Kanaoka, Y. (1977) *J. Am. Chem. Soc. 99*, 4485−4488.
Turk, D., Stürzebecher, J., & Bode, W. (1991) *FEBS Lett. 287*, 133−138.
Vishnu, & Caplow, M. (1969) *J. Am. Chem. Soc. 91*, 6754−6758.
Walker, B., & Elemore, D. T. (1984) *Biochem. J. 221*, 277−280.
Walsh, C., Cromartie, T., Marcotte, P., & Spencer, R. (1978) *Method Enzymol 53D*, 437.
Westkaemper, R. B., & Abeles, R. H. (1983) *Biochemistry 22*, 3256−3264.
White, E. H. (1955) *J. Am. Chem. Soc. 77*, 6008.
White, E. H., & Aufdermarsh, Jr., C. A. (1961) *J. Am. Chem. Soc. 83*, 1179.
White, E. H., & Chen, Y. (1993) *J. Labelled Compd. Radiopharm. XXXIII*, 97−104.
White, E. H., Roswell, D. F., Politzer, I. R., & Branchini, B. R. (1975) *J. Am. Chem. Soc. 97*, 2290−2291.
White, E. H., Roswell, D. F., Politzer, I. R., & Branchini, B. R. (1977a) *Methods Enzymol. 46*, 216.
White, E. H., Jelinski, L. W., Perks, H. M., Burrows, E. P., & Roswell, D. F. (1977b) *J. Am. Chem. Soc. 99*, 3171.
White, E. H., Jelinski, L. W., Politzer, I. R., Branchini, B. R., & Roswell, D. F. (1981) *J. Am. Chem. Soc. 103*, 4231−4239.
White, E. H., Li, M., Cousins, J. P., & Roswell, D. F. (1990) *J. Am. Chem. Soc. 112*, 1956−1961.
Worthington, C. C. (1988) *Worthington Enzyme Manual* p. 320, Worthington Biochemical Corporation, Freehold, NJ.

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