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Elastase. I. A New Inhibitor, 1-Bromo-4-(2,4-dinitrophenyl)butan-2-one*

L. Visser,† D. S. Sigman,‡ and E. R. Blout§

ABSTRACT: The hydrolytic action of elastase on both its natural substrate, elastin, and a synthetic ester, *p*-nitrophenyl *tert*-Boc-L-alaninate is inhibited irreversibly and stoichiometrically by a new inhibitor, 1-bromo-4-(2,4-dinitrophenyl)-butan-2-one.

The site of reaction of the inhibitor with the enzyme is the γ-carboxyl group of a glutamic acid—tentatively identified as Glu-6. Evidence for the esterification of this glutamic acid residue is obtained from amino acid analysis of the modified enzyme after Lossen rearrangement and from leucine aminopeptidase digestion of its cyanogen bromide peptides.

Elastase possesses remarkable similarities in its chemical, physical, and biological properties to two other pancreatic proteolytic enzymes, trypsin and chymotrypsin. All three enzymes possess a highly reactive serine which can be phosphorylated by diisopropyl fluorophosphate, four homologous disulfide bridges, and two histidine residues in homologous sequences (Brown *et al.*, 1967). Elastase is unique, however, in its ability to degrade elastin, a connective tissue protein with a very high content of glycine, alanine, and proline (Partridge and Davis, 1955).

Considerable work with site-specific irreversible inhibitors has been carried out with trypsin and chymotrypsin and has demonstrated one of the two homologous histidines in both chymotrypsin and trypsin to be essential for catalytic activity (Schoellmann and Shaw, 1963; Shaw *et al.*, 1965). Few examples of specific chemical modifications of elastase have been reported, due perhaps in part to the lack of a convenient assay system for this enzyme. The recent development of a spectrophotometric assay with *p*-nitrophenyl *tert*-Boc-L-alaninate (NBA)[1] as substrate (Visser and Blout, 1969) has made such studies more practicable.

The apparent specificity of elastase for alanine derivatives suggested that either the chloromethyl ketone or diazo ketone derivatives of alanine would be useful chemical modification reagents to demonstrate that elastase, like trypsin and chymotrypsin, possesses a histidine residue essential for enzymic activity. As will be shown below, none of the several alanine derivatives synthesized inhibited elastase activity. However, 1-bromo-4-(2,4-dinitrophenyl)butan-2-one [DPBB (XIII)] was found to react irreversibly and stoichiometrically with elastase, abolishing its esterolytic (with NBA as sub-

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† Present address: Department of Biochemistry, University of Natal, Pietermaritzburg, Republic of South Africa.

‡ Department of Biological Chemistry, School of Medicine, The Center for the Health Sciences, University of California, Los Angeles, Los Angeles, Calif. 90024.

§ To whom correspondence should be addressed.

[1] The abbreviations used are: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; NBA, *p*-nitrophenyl *tert*-Boc-L-alaninate; DPBB, 1-bromo-4-(2,4-dinitrophenyl)butan-2-one; PMSF, phenylmethanesulfonyl fluoride; HFIP, hexafluoro-2-propanol; DPB-elastase, dinitrophenylbutanonyl-elastase; LAP, leucine aminopeptidase; DEPP, diethyl *p*-nitrophenyl phosphate; NMT, *N*-methyl-*N*-nitroso-*p*-tosylamide.

CHART I

Compound	No.
$C_6H_5SO_2NHCH(CH_3)COCH_2Cl$	I
$C_6H_5SO_2NHCH(CH_3)COCHN_2$	II
$4\text{-}H_3CC_6H_4SO_2NHCH_2CH_2COCH_2Cl$	III
$4\text{-}H_3CC_6H_4SO_2NHCH_2CH_2COCHN_2$	IV
$C_6H_5CH_2CH_2COCH_2Br$	V
$2,4\text{-}(O_2N)_2C_6H_3CH_2COCH_2Br$	VI
$4\text{-}O_2NC_6H_4COCH_2Br$	VII
$2,4\text{-}(O_2N)_2C_6H_3CH_2CH_2COOH$	VIII
$4\text{-}H_3CC_6H_4SO_2NHCH(CH_3)COCH_2Cl$	IX
$4\text{-}H_3CC_6H_4SO_2NHCH(CH_3)COCHN_2$	X
$BrCH_2CONHCH(CH_3)COOH$	XI
$BrCH_2CONHCH(CH_3)COOC_6H_4NO_2$	XII
$2,4\text{-}(O_2N)_2C_6H_3CH_2CH_2COCH_2Br$	XIII
$2,4\text{-}(O_2N)_2C_6H_3CH_2CH_2COCHN_2$	XIV
$2,4\text{-}(O_2N)_2C_6H_3CH_2CH_2CO^{14}CH_2Br$	XV
$2,4\text{-}(O_2N)_2C_6H_3NHCH_2CH_2COCH_2Br$	XVI
$2,4\text{-}(O_2N)_2C_6H_3NHCH_2CH_2COCHN_2$	XVII

strate) as well as its elastolytic (with orcein–elastin as substrate) activity.

A detailed account of the specific inhibition of elastase with 1-bromo-4-(2,4-dinitrophenyl)butan-2-one is the subject of the present communication. The site of the reaction of the inhibitor with the enzyme is shown to be a COOH of a glutamyl residue, as has been suggested previously (Visser and Blout, 1969).

Experimental Section

Materials. Phenylmethanesulfonyl fluoride was obtained from California Biochemical Corporation; *N*-1-naphthylethylenediamine, *p*-toluenesulfonyl chloride, bromoacetyl bromide, and cyanogen bromide were obtained from Eastman Organic Chemicals. Hexafluoro-2-propanol was supplied by E. I. du Pont de Nemours Company. *N*-Nitroso-*N*-methylurea was purchased from Columbia Organic Chemicals, *N*-methyl-[^{14}C]*N*-nitroso-*p*-tosylamide from New England Nuclear Corporation, *N*-methyl-*N*-nitroso-*p*-tosylamide from J. T. Baker & Company, *N*-2,4-dinitrophenylglycine from Sigma Chemical Company, and leucine aminopeptidase from Nutritional Biochemicals Corporation. 1-Chloro-L-3-benzenesulfonamidobutan-2-one (I) and its diazo precursor (II) were gifts from Dr. G. Hein (Boston University). Dr. S. Hartman (Chemistry Department, Boston University) donated 1-chloro-4-tosylamidobutan-2-one (III) and its 1-diazo precursor (IV).

The following compounds were synthesized: 1-bromo-4-phenylbutan-2-one (V), 1-bromo-3-(2,4-dinitrophenyl)propan-2-one (VI), α-bromo-4-nitroacetophenone (VII), and 3-(2,4-dinitrophenyl)propionic acid (VIII); *N*-tosyl-L-alanine, *N*-tosyl-L-alanylchloride [1-chloro-L-3-tosylamidobutan-2-one (IX) and its 1-diazo precursor (X); *N*-bromoacetyl-L-alanine (XI) and its *p*-nitrophenyl ester (XII)]; 1-bromo-4-(2,4-dinitrophenyl)butan-2-one (XIII), its 1-diazo precursor (XIV), and radioactively labeled 1-^{14}C isotope (XV); 1-bromo-4-(2,4-dinitroanilino)butan-2-one (XVI) and its 1-diazo precursor (XVII).

Methods

Synthesis of IX and X. L-Alanine was the starting material for the stepwise synthesis of X and IX.

N-Tosyl-L-alanine was synthesized from L-alanine according to the method of Greenstein and Winitz (1961). The colorless crystals had mp 129–131°, lit. mp 132–134°; $\epsilon_{263\,nm}^{CH_3CN}$ 4.7×10^2 M^{-1} cm^{-1}; $[\alpha]_D^{23}$ 14° (*c* 0.243, CH_3CN).

N-Tosyl-L-alanyl chloride was synthesized from *N*-tosyl-L-alanine by the procedure of Popenoe and du Vigneaud (1954) using PCl_5 (mp 91–93°, lit. 93–94°).

The diazo derivative X was synthesized from *N*-tosyl-L-alanyl chloride with the aid of ethereal diazomethane (Arndt, 1943). The procedure is generally the same as that of Schoellmann and Shaw (1963). A solution of tosyl-L-alanyl chloride (3.9 g, 15 mmoles) in 100 ml of anhydrous ether was added dropwise to a gently stirred (Teflon bar) anhydrous ether solution at 0° containing 30 mmoles of CH_2N_2 (generated from *N*-nitroso-*N*-methylurea with KOH and dried according to Arndt, 1943). It was stirred for about 45 min, then left at room temperature overnight. The ether was removed under a stream of dry nitrogen; the residue redissolved in ether and evaporated. The infrared spectrum of the yellow syrup has a strong band at 2100 cm^{-1} which is diagnostic of the diazo (C=N=N) stretching frequency (Bellamy, 1956). Attempts to crystallize X were unsuccessful, but elution from a silica gel column with chloroform followed by ethyl acetate gave two 360-nm absorbing fractions. The first fraction eluted with $CHCl_3$ had infrared characteristics compatible with an *N*-methyl derivative of X, while the second fraction had infrared characteristics consonant with X and was used in inhibition studies.

Conversion of X into IX was accomplished by carefully "titrating" a solution of diazo derivative with concentrated HCl until no further N_2 was evolved. The solvent was evaporated after HCl treatment, the residue taken up in ethyl acetate and extracted with water (4 × 25 ml) until the extract was neutral, the ethyl acetate layer dried over Na_2SO_4 and evaporated, the residue taken up in a small volume of chloroform, *n*-heptane added to cloud point, and the solution kept in a cold room. Within a week, shiny needle-type crystals formed which were dried *in vacuo* over $CaCl_2$ after filtration: mp 62–64°; $[\alpha]_D^{23}$ −52.6° (*c* 1.38, CH_3CN); infrared band of diazo precursor X at 2108 cm^{-1} is lost and C=O absorption frequency appears at 1737 cm^{-1} in IX. *Anal.* Calcd for $C_{11}H_{14}ClNO_3S$: C, 47.9; H, 5.12; Cl, 12.86; N, 5.08; S, 11.63. Found: C, 47.6; H, 5.12; Cl, 11.5; N, 5.14; S, 12.2. The eventual yield of IX after numerous intermediate unsuccessful crystallization attempts was only about 30%.

Synthesis of 1-Bromo-4-(2,4-dinitrophenyl)butan-2-one (XIII). A syrup of 2,4-dinitrophenylpropionyl chloride (15.3 g) was obtained by refluxing 15 g of 2,4-dinitrophenylpropionic acid (VIII) with 10 ml of thionyl chloride (Riley, 1952), evaporating the solvent, and drying on a vacuum pump. The infrared spectrum of the syrup contained a strong carbonyl absorption band at 1790 cm^{-1}, typical of an acid chloride.

The diazo compound XIV was synthesized from 2,4-dinitrophenylpropionyl chloride and ethereal diazomethane (1:2 molar ratio) in a manner analogous to that described above for X. After ether had been removed, the yellow residue was taken up in warm chloroform and treated with charcoal. Hexane was added to the filtrate to the cloud point. Yellow-orange crystals formed in the cold room: yield, 50%; mp 65–66°; infrared band (very strong) at 2100 cm^{-1}; $\epsilon_{248\,nm}^{CH_3CN}$ 2.46×10^4 M^{-1} cm^{-1}. *Anal.* Calcd for $C_{10}H_8N_4O_5$: C, 45.44; H, 3.05; N, 21.22. Found: C, 45.38; H, 3.11; N, 21.36.

XIV (10 mmoles, 2.64 g) in 10 ml dry acetone was "titrated" by dropwise addition (with stirring) of 48% HBr (specific gravity 1.51, ~9 M) until N_2 evolution stopped (1.2 ml, 10.8 mequiv of HBr required). After evaporation of the solvent, the residue was dissolved in 100 ml of warm 95% ethanol, treated with charcoal, and filtered, and hexane was added to the cloud point. Sheen-like yellow crystals formed overnight

in the cold room; yield, 2.5 g (90%); mp 71–72°; infrared no longer shows 2100 cm^{-1} band of diazo compound, but a doublet 1715 and 1725 cm^{-1}; $\epsilon_{251\,nm}^{1\%\ CH_3CN}$ 1.67 × 10^4 M^{-1} cm^{-1}. *Anal.* Calcd for $C_{10}H_9BrN_2O_5$; C, 37.85; H, 2.86; N, 8.84; Br, 25.21. Found: C, 38.15; H, 2.96; N, 8.85; Br, 24.85.

Synthesis of [1-^{14}C]DPBB (XV). Radioactive [^{14}C]CH_2N_2 was generated on a microscale from *N*-methyl-[^{14}C]*N*-nitroso-*p*-tosylamide (NMT) according to the procedure of Hartman (1963); 3.2 mg of [^{14}C]NMT (7 mCi/mole, total activity 0.1 mCi) and 40.8 mg of carrier NMT (total NMT, 0.2 mmole) in 1.25 ml of anhydrous ether were treated in a closed distillation system with 1 ml of sodium octanol (10 mg of Na/ml) and the liberated [^{14}C]CH_2N_2 collected in a flask on a Dry Ice–*tert*-butyl Cellosolve freezing mixture. To this was added 13 mg (0.05 mmole) of 2,4-dinitrophenylpropionyl chloride in 1 ml of ether, and the synthesis was then continued as for XIV and XIII above. The infrared of the diazo intermediate showed the expected peak at 2100 cm^{-1} which disappeared upon conversion into the bromo derivative. Crystallization of XV from ethanol gave a first crop of 3.8 mg, with the correct carbonyl infrared frequency (1725 cm^{-1}) and melting point (71–72°). Its specific activity of 0.483 μCi/μmole (or 1.075 × 10^6 cpm/mole) was equal to 97% of the expected value calculated from the dilution of the starting material, [^{14}C]NMT. From the mother liquor a second crop of [1-^{14}C]DPBB of lower specific activity (0.112 μCi/μmole) was recovered after seeding with 16 mg of unlabeled DPBB.

Synthesis of 4-(2,4-Dinitroanilino)-1-bromobutan-2-one (XVII). This compound and its precursor XVIII were synthesized in an analogous fashion to XIII and XIV above. The material used in the inhibition trials was not crystalline, but had the expected infrared characteristics.

Synthesis of XII, the p-Nitrophenyl Ester of XI. Various attempts to synthesis XII by dicyclohexylcarbodiimide coupling of XI with *p*-nitrophenol according to a procedure used for the preparation of *N*-bromoacetyl-α-aminoisobutyric *p*-nitrophenyl ester (Lawson and Schramm, 1965) were unsuccessful. The difficulty is probably due to azlactone formation from the active ester. The desired compound was eventually synthesized from millimole quantities of L-alanine *p*-nitrophenyl ester and bromacetyl bromide in ethyl acetate–pyridine at 4° (Greenstein and Winitz, 1961). Light yellow crystals from ethanol–ether melted at 111–112°: yield, 95%; infrared shows strong active ester C=O absorption frequency at 1760 cm^{-1}, amide I (1635 cm^{-1}) and amide II (1540 cm^{-1}); $\epsilon_{268\,nm}^{CH_3CN}$ 9.4 × 10^3 M^{-1} cm^{-1}. *Anal.* Calcd for $C_{11}H_{11}BrN_2O_5$: C, 39.88; H, 3.32; N, 8.46; Br, 24.17. Found: C, 39.32; H, 3.48; N, 8.18; Br, 23.88.

Conditions for Elastase Inhibition by DPBB. A 50- to 100-fold molar excess of inhibitor over enzyme with a final DPBB concentration of 5 × 10^{-4} M in a 5% CH_3CN solution was regularly employed. Buffer or buffered enzyme solution was always added to a DPBB aliquot in order to prevent precipitation of the sparingly soluble inhibitor. Inhibition was carried out at 37° in 0.05 M sodium phosphate buffer, pH 6.5 or 7.0. It was sometimes advantageous, because of decomposition of DPBB, to add the inhibitor in two portions, one at the start and the second at a later stage. Progress of inhibition was followed by assaying periodically against either orcein–elastin (Sachar *et al.*, 1955) or the *p*-nitrophenyl ester of *tert*-Boc-L-alanine as substrate (Visser and Blout, 1969). Ordinarily, inhibition mixtures contained 10^{-4} M $CaCl_2$ to stabilize elastase against thermal inactivation during incubations at 37°. Excess inhibitor was removed at the end of the incubation by dialysis (>24 hr) against frequent changes of distilled water in the cold room or by gel filtration on a Sephadex G-25 column. In some experiments with radioactive [^{14}C]DPBB, excess inhibitor was extracted from the aqueous inhibition mixture with ether (four times, equal volume) followed by hexane (once) until the organic extracts had less than 5% of the original radioactivity.

Stock solutions (10^{-1} M or 10^{-2} M) of DPBB in acetonitrile or methanol were stored, protected from light, in the cold room.

Radioactivity Measurements. Protein samples were precipitated from inhibition mixtures with trichloroacetic acid, washed with ethanol, dissolved with Hyamine hydroxide, and counted in a toluene liquid scintillation fluid (0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis[2-(5-phenyloxazolyl)]benzene) according to the procedure of Hartman (1963), except that a trichloroacetic acid precipitation period of 30 min at 0° was used instead of 5 min at room temperature. The efficiency of counting on a Packard liquid scintillation spectrometer was determined with the aid of a ^{14}C standard.

Lossen Rearrangement of DPB-Elastase. The technique of Gallop *et al.* (1960) as applied by Gross and Morell (1966) on pepsin was used. Treatment of a protein containing a side-chain aspartate or glutamate ester with hydroxylamine, dinitrofluorobenzene, and alkali leads to the conversion of such a glutamate or aspartate residue into diaminobutyric or diaminopropionic acid, respectively, which can easily be identified on amino acid chromatograms after acid hydrolysis. Amounts of DPB-elastase and elastase ranging from 2.5 mg to 10 mg were used in this procedure.

Amino Acid Analysis. The standard automatic procedure of Spackman *et al.* (1958) was carried out with a Beckman Model 120B amino acid analyzer on 24 hr, 6 N HCl hydrolysates of proteins or peptides, or on the supernatants after LAP digestions.

Cyanogen Bromide Cleavage of Elastase and DPB-Elastase. Elastase has 2 methionine residues at which it can be cleaved with CNBr. The original cleavage method of Gross and Witkop (1962) with some recent modifications (Gross, 1967) gave satisfactory results.

[^{14}C]DPB-elastase (50 mg, 2 μmoles of enzyme or 4 μmoles of methionine) was dissolved in 6 ml of 70% formic acid and the yellow solution added to 12.7 mg of CNBr in 1 ml of 70% HCOOH (*i.e.*, a 30-fold excess of CNBr relative to methonine content). The flask was stoppered, covered with aluminum foil for protection against light, and stirred for 24 hr at room temperature. After two lyophilizations, the residue was dissolved in 1.5 ml of HCOOH, and the deep-orange solution oxidized with performic acid according to the procedure of Hirs (1967). Finally, the oxidation mixture was diluted with water and lyophilized twice to remove the performic acid. The same procedure was applied to 12.5 mg (0.5 μmole) of an elastase control.

Digestion of Cyanogen Bromide Fragments of DPB-Elastase with Leucine Aminopeptidase. Digestion of 5–10 mg of radioactively labeled peak D (Table IV, below) with manganese-activated leucine aminopeptidase was performed at 37° for 48 hr, pH 8.0 (Light and Greenberg, 1965), in centrifuge tubes. After digestion and centrifugation, the supernatants were treated with 10% trichloroacetic to acid precipitate any undigested protein. The supernatants were assayed for radioactivity and analyzed for free amino acid content after removal of trichloroacetic acid by ether extraction.

Column Chromatography of Peptides after Cyanogen Bromide Cleavage of Elastase. The peptides were placed on a column of SE-Sephadex C-25, fine grade, and eluted by 0.001 N

TABLE I: Alanine Analogs as Elastase Inhibitors.

Compound	% Inhibition[a]
N-Tosyl-L-alanyl chloride	4
L-3-Tosylamido-1-diazo-2-butanone (X)	17[b]
L-3-Tosylamido-1-chloro-2-butanone (IX)	21
L-3-Benzenesulfonamido-1-chloro-2-butanone (I)	30
D-3-Benzenesulfonamido-1-chloro-2-butanone	0
4-Tosylamido-1-diazo-2-butanone (IV)	13
4-Tosylamido-1-chloro-2-butanone (III)	11
N-Bromoacetyl-L-alanine (XI)	0
N-Bromoacetyl-L-alanine *p*-nitrophenyl ester (XII)	0

[a] Inhibition measurement was carried out after incubation of elastase at pH 7 (0.05 M phosphate), 25° for 24 hr with 50–100-fold molar excess of test compound. [b] Compound not crystalline, but a chromatographically purified fraction.

TABLE II: Inhibition of Elastase by DPBB.

Inhibitor Concentration (M)	Concentration Dependence[a] k_{obsd} (sec^{-1} × 10^4)	$k_{calcd} = \frac{k_{obsd}}{DPBB}$ (M^{-1} sec^{-1} × 10)
10^{-3}	2.3	2.3
5×10^{-4}	1.21	2.4
10^{-4}	0.31	3.1
5×10^{-5}	0.15	3.0

[a] Incubated at 37°, pH 7 with 5×10^{-6} M elastase in 0.05 M phosphate–5% CH_3CN.

acetic acid and 1 N ammonia, in that order, starting at a pH of ~4.5. The effluent was monitored at 230 nm for absorption by peptide bonds and aliquots of each tube were assayed for radioactivity. A radioactive peptide which was eluted at a pH of about 7 was rechromatographed on SE-Sephadex C-25 equilibrated with 33% hexafluoro-2-propanol. The column was developed with 33% aqueous HFIP followed by 33% HFIP in 1 N NH_4OH. This second chromatographic step was carried out to ensure the complete dispersion of CNBr peptides which often remain aggregated in weaker solvents. The method is a modification of one in which 50% hexafluoroacetone and Sephadex G-25 were used to separate CNBr peptides of chymotrypsinogen (Burkhardt and Wilcox, 1967). Chromatography was conducted inside a well-ventilated hood to prevent eye damage by the noxious solvent vapors.

Results

Inhibition by Phenyl Methanesulfonyl Fluoride and Alanine Analogs. Elastase is rapidly inactivated by phenyl methanesulfonyl fluoride at pH 7.0 with a second-order rate constant (calculated from a first-order plot) of 3.8 M^{-1} sec^{-1}. This rate constant is about an order of magnitude faster than the value of 0.36 M^{-1} sec^{-1} found for diethyl *p*-nitrophenyl phosphate inhibition of elastase (Visser and Blout, 1971), and is comparable to that reported for PMSF and trypsin (Fahrney and Gold, 1963).

The effects on elastase activity of a series of alanine derivatives are given in Table I. None of these compounds caused any marked inhibition, but it may be noted parenthetically that *N*-bromoacetyl-L-alanine *p*-nitrophenyl ester is a good substrate.

Effect of 1-Bromo-4-(2,4-dinitrophenyl)butan-2-one on Elastase. Incubation of elastase with DPBB results in the progressive disappearance of enzyme activity. The absence of a regain of activity upon gel filtration or dialysis to remove excess inhibitor indicates that the enzyme–inhibitor complex is stable. Direct evidence for a covalent reaction between DPBB and elastase came from spectral studies and the incorporation of radioactivity from [^{14}C]DPBB (*vide infra*).

Initial results with DPBB showed that elastase activity is inhibited to similar extents upon incubation whether orcein–elastin or NBA is the test substrate. For example, when 5×10^{-5} M elastase was incubated with 5×10^{-4} M DPBB for 72 hr at room temperature in a pH 7.0 buffer, esterase activity was inhibited 82% and elastolytic activity, using orcein–elastin as substrate, was inhibited 73%. Subsequently, all effects of DPBB were investigated with the more convenient ester substrate.

When an elastase solution that is 80% inhibited, as determined by NBA hydrolysis, is titrated in an "all-or-none" assay (Koshland *et al.*, 1962) with diethyl *p*-nitrophenyl phosphate, 20% of kinetically active enzyme is found. Thus, the remaining 20% activity after DPBB inhibition in this case is not due to an enzyme that has fully reacted with DPBB and then possessed an altered activity. Instead, it is due to the presence of 20% unreacted enzyme. The fact that activity can be reduced to 5% by increasing the DPBB concentration to 10^{-3} M (*cf.* Table II) is also in agreement with such a mode of inhibition.

pH Dependence of Inhibition by DPBB. The bell-shaped pH–rate profile for the inhibition of elastase by DPBB in Figure 1 has a maximum between pH 6 and 7. The inhibitor–enzyme mixtures above pH 7 are all colored after incubation, from a pale yellow at pH 7 to a deep wine red above pH 10. The inhibitor alone displays the same color changes with pH. Since the inhibitor is apparently decomposed at alkaline pH values, the falloff in inhibition rate above pH 7.5 may be largely due to the destruction of the inhibitor.

Kinetics of DPBB Inhibition. The rate of inactivation of elastase by DPBB was studied as a function of inhibitor concentration at pH 7 and 37°. Table II indicates how the first-order rate constant for the inactivation of the enzyme varies as a function of inhibitor concentration. Also included in Table II is the apparent second-order rate constant obtained by dividing the observed first-order rate constant by the inhibitor concentration. Since the calculated second-order rate constants decrease with increasing inhibitor concentration, the following reaction scheme may be valid for the inhibition of elastase with DPBB

$$E + DPBB \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}DPBB \quad (1)$$

$$E\text{-}DPBB \xrightarrow{k_2} E\text{-}DPB \quad (2)$$

where E is free enzyme, E–DPBB is a noncovalent complex

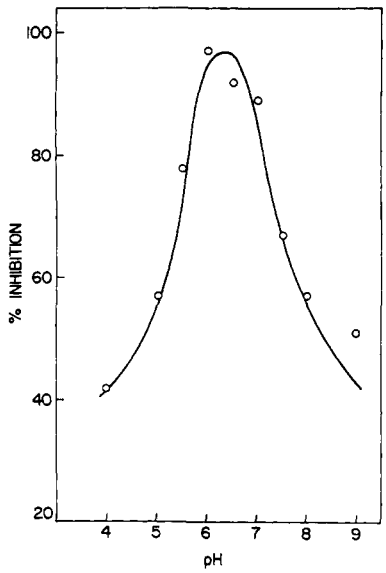

FIGURE 1: DPBB inhibition of elastase as a function of pH. Inhibition mixtures contained 5×10^{-6} M elastase and 5×10^{-4} M DPBB in 5% CH_3CN–0.045 M buffer, 10^{-4} M $CaCl_2$. Aliquots (100 μl) with 12.5 μg of enzyme were assayed after 10 hr at 37° against NBA at pH 6.5: controls, no DPBB added; buffers, pH 4–6, acetate; pH 6–8, phosphate; pH 8–9, Tris.

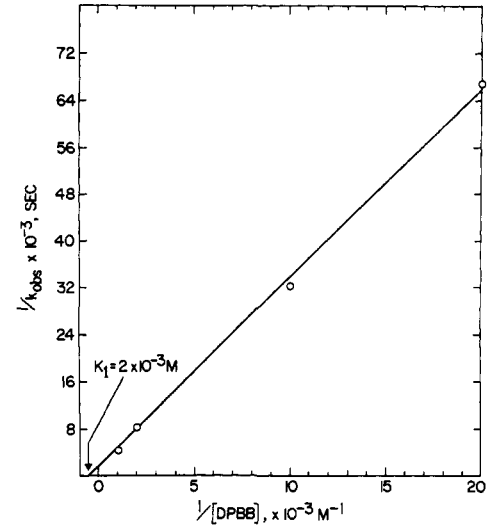

FIGURE 2: Kinetics of elastase inhibition by DPBB. The rate constant of inhibition (k_{obsd}) by DPBB was determined from first-order plots of the remaining activity after 5×10^{-6} M elastase had been incubated for various time intervals with different concentrations of DPBB (37°, 5% CH_3CN–0.045 M sodium phosphate, pH 7.0); aliquots with 12.5 μg of enzyme were assayed against NBA for activity. K_I is determined according to eq 4.

formed between the enzyme and DPBB and E–DPB is the alkylated enzyme. The total enzyme concentration (E_t) at any time is then represented by eq 3. If the equilibrium described by eq 1 is rapidly achieved relative to the alkylation reaction then

$$E_t = E + E\text{-}DPBB + E\text{-}DPB \quad (3)$$

$$\frac{d(E\text{-}DPB)}{dt} = k_2(E\text{-}DPBB) = \frac{k_2(DPBB)(E_t - (E\text{-}DPB))}{(DPBB) + K_I} \quad (4)$$

where $K_I = k_{-1}/k_1$. As a result the pseudo-first-order rate constant for elastase inhibition, k_{obsd}, indicated in Table II, is defined by this scheme to be

$$k_{obsd} = \frac{k_2(DPBB)}{(DPBB) + K_I} \quad (5)$$

or

$$\frac{1}{k_{obsd}} = \frac{1}{k_2} + \frac{K_I}{k_2(DPBB)} \quad (6)$$

The concentration of E–DPBB is small relative to the total concentration of DPBB added.

In Figure 2, $1/k_{obsd}$ is plotted *vs.* 1/(DPBB). Since $1/K_I = -1/(DPBB)$ when $1/k_{obsd} = 0$, Figure 2 shows K_I equal to 2×10^{-3} M.

Specificity and Stoichiometry of DPBB Inhibition. The ultraviolet absorption spectra of elastase, DPBB, and DPBB-inhibited elastase in aqueous solutions are presented in Figure 3. The inhibited enzyme spectrum is approximated by a linear combination of those of DPBB and elastase. The most marked change in the latter spectrum upon inhibition is an increase in absorbance in the trough region near 250 nm, which correlates with the fact that the absorption maximum of DPBB is at 251 nm. The ratio of absorbance at 282 nm:250 nm decreases from >2.5 in the native elastase to about 1.5 in the completely inhibited enzyme and provides a useful diagnostic tool with which to follow the reaction. A 1:1 stoichiometry for the enzyme–inhibitor reaction follows from the spectral properties of DPB-elastase.

The 1:1 stoichiometry was confirmed by the extent of incorporation of radioactivity from [1-^{14}C]DPBB into elastase (Figure 4). The number of moles of [^{14}C]DPBB incorporated

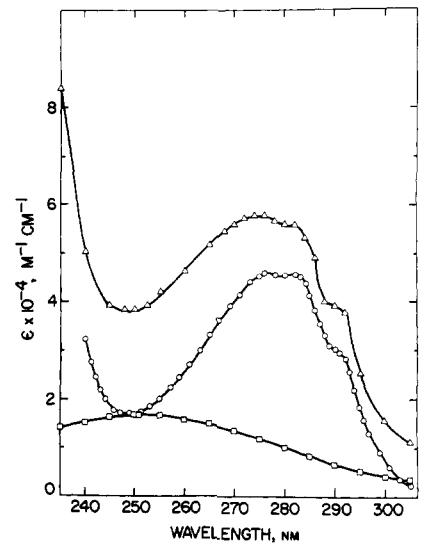

FIGURE 3: Absorption spectra of elastase, DPB-elastase, and DPBB. The spectrum of DPBB (□—□) was run in 1% CH_3CN–0.05 M sodium phosphate, pH 7.0, while the spectra of elastase (○—○) and DPB-elastase (△—△) were obtained after exhaustive dialysis against distilled water.

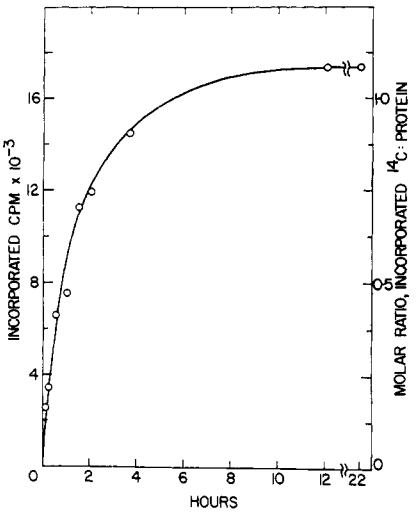

FIGURE 4: ^{14}C incorporation into elastase from [^{14}C]DPBB. Duplicate aliquots containing 0.5 mg of protein from an inhibition mixture (4×10^{-4} M [^{14}C]DPBB, 4×10^{-6} M elastase in 3.75% CH_3CN–0.046 M sodium phosphate, pH 7.0, 37°) were assayed for radioactivity.

per mole of elastase in several experiments averaged to 1.0 ± 0.2. Radioactivity was corrected for efficiency of ^{14}C counting in a toluene scintillation fluid (Hartman, 1963), and the kinetically active elastase concentration was established by DEPP titration of a control before inhibitor was added. The kinetics of ^{14}C incorporation are pseudo-first-order over at least 80% of the reaction (Figure 5) and elastase inhibition, as measured by the concurrent falloff in enzymic activity toward NBA, appears to essentially parallel ^{14}C incorporation.

The specificity of DPBB inhibition was further investigated in experiments where known reversible inhibitors and the native substrate elastin were added to inhibition mixtures. The results in Table III illustrate that in the presence of 0.001

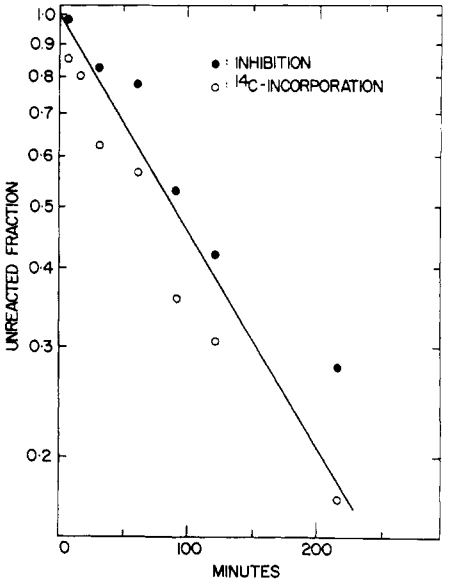

FIGURE 5: Rates of ^{14}C incorporation, inhibition of elastase. Inhibition mixtures of DPBB and elastase (see Figure 4 for details) were assayed for radioactivity, as well as remaining enzyme activity toward NBA as a function of time.

TABLE III: Protectors of Elastase against DPBB Inhibition.

Experiment	Addition	% Residual Activity as Esterase[a]
1	None (enzyme alone)	100
	DPBB (40-fold molar excess 12 hr, 25°)	35
	DPBB (40-fold molar excess 12 hr, 25°) + 10^{-3} M Boc-L-alanine	50
2	None (enzyme alone)	100
	DPBB (100-fold molar excess 10 hr, 37°)	13
	DPBB (100-fold molar excess 10 hr, 37°) + 0.1 M NaCl	54
	DPBB (100-fold molar excess 10 hr, 37°) + 0.1 M NaCl + elastin (400-fold excess by weight)	71

[a] Aliquots were assayed against NBA in 5% CH_3CN–0.05 M phosphate, pH 6.5, 24° after incubation at 37°.

M Boc-L-alanine, 0.1 M NaCl, or 0.1 M NaCl plus elastin, the rate of inhibition of elastase by DPBB is retarded. When the protective effect of the substrate elastin is being investigated, NaCl should be present to slow down the breakdown of elastin by elastase.

Other compounds closely related to DPBB in structure were found to have little or no inhibitory action; these include V, VI, XVI, and XVII.

Site of Inhibition by DPBB. Amino acid analysis of acid hydrolysates of DPBB-inhibited elastase (with or without performic oxidation) did not reveal any obvious changes in composition. The covalent linkage was therefore presumed to be labile under the conditions of acid hydrolysis. Inhibitors of pepsin such as 1-diazo-4-phenylbutan-2-one (Hamilton *et al.*, 1967) and *p*-bromophenacyl bromide (Gross and Morell, 1966) are known to react with β-carboxyls of aspartate residues. Also, aryl α-halomethyl ketones form esters in a facile fashion with carboxylic acid salts (Moreland, 1956). The structural and functional similarity of DPBB with these compounds led to the consideration of a possible ester bond between DPBB and elastase. Incubation of [^{14}C]DPB-elastase (38,250 cpm/mg) with 1 M NH_2OH at pH 9 (16 hr, 37°) caused the release of 80% of the radioactivity into a trichloroacetic acid soluble supernatant (7840 cpm/mg was recovered in the protein precipitate). After hydroxylamine treatment, the inhibited enzyme gave a positive (pink) color with *N*-1-naphthylethylenediamine in a modified Segal test (Bergmann and Segal, 1956) for hydroxamates. These observations are consistent with the presence of an ester bond from which the inhibitor moiety can be displaced by the nucleophile hydroxylamine. The enzyme is still inactive after hydroxamate formation.

To distinguish between an aspartyl or glutamyl ester, a Lossen rearrangement followed by acid hydrolysis of DPB-elastase was performed (for reaction scheme, see Gross and Morell (1966)). Amino acid analysis of the hydrolysate indicated that an average of 1 residue of diaminobutyric acid per 9

TABLE IV: Amino Acid Analyses of a Radioactive Fragment (D) Isolated from [^{14}C]DPB-Elastase Treated with CNBr.[a]

Amino Acid	Fragment[b]				S		S + Alkali	
	D	I	II	III	Found	Calcd	Found	Calcd
Lys	0.4	0	2	1	Nd	0	Nd	0
His	0.8	1	3	2	Nd	0	Nd	0
HSL[c]	0.7	1	1	0	Nd	0	Nd	0
Arg	1.2	3	5	4	Nd	1	Nd	1
$CySO_3H$	0.6	1	3	4	0.2	0	0.0	0
Asp	2.7	2	15	7	0.6	0	0.4	1
Thr	2.2	4	12	4	0.8	1	0.5	1
Ser	2.6	6	9	7	1.5	1	1.0	1
Glu	2.4	5	12	2	0.2	1	1.9	2
Pro	0.8	1	4	2	0.0	0	0.0	0
Gly	3.3	5	10	10	1.8	2	2.2	2
Ala	2.0	2	11	4	1.0	1	1.0	1
Val	2.5	3	14	10	3.7[d]	2	3.7	2
Ile	1.0	2	5	3	0.3	0	0.1	0
Leu	2.1	2	13	3	0.9	0	0.3	0
Tyr	0	0	8	2	0.0	0	0.0	0
Phe	0.3	0	1	2	0.3	0	0.0	0

[a] Calculated on the basis of the expected alanine content of CNBr-treated, oxidized elastase. [b] I, II, and III: expected composition of CNBr fragments from elastase; S: supernatant after digestion of D with leucine aminopeptidase. [c] HSL = homoserine lactone obtained from methionine after CNBr cleavage. [d] The high valine values are probably due to a better yield from the two N-terminal valines upon LAP digestion.

residues of histidine plus lysine (*i.e.*, per mole of enzyme) was obtained. Apart from implicating a γ-glutamyl ester link between elastase and the inhibitor, the result also confirmed the equimolar stoichiometry deduced from the spectroscopic and radioactivity measurements (*cf.* Methods).

Further evidence for the participation of a glutamic acid residue in the covalent bond between the enzyme and inhibitor was provided by a preliminary investigation of cyanogen bromide fragments from [^{14}C]DPB-elastase. The amino acid sequence of elastase contains two methionines, at positions 41 and 172, and four glutamic acids at positions 6, 50, 59, and 69. Three peptide fragments are therefore expected after BrCN cleavage at the methionines and performic acid oxidation to break the four intramolecular disulfide bonds, fragment I consisting of residues 1–41, fragment II consisting of residues 42–172, and fragment III consisting of residues 173–240.

An impure radioactive fragment has been isolated by chromatography (see Methods) from [^{14}C]DPB-elastase after CNBr cleavage. This fragment has an amino acid composition which corresponds closest to that expected for the N-terminal peptide ending at Met-41 (Table IV). Since the sequence Val^{1}-Met^{41} of elastase contains only one glutamic acid residue at position 6 (see Figure 6), it would appear that this is the site of interaction between the enzyme and inhibitor. This tentative conclusion is strengthened by results obtained from leucine aminopeptidase digests. Inspection of the amino acid sequence of elastase in Figure 6 reveals that LAP will only liberate amino acids 1–11 before its action is stopped

1 6 13
Val-Val-Gly-Gly-Thr-Glu-Ala-Gln-Arg-Asn-Ser-Trp-Pro-Ser-Gln-
Ile-Ser-Leu-Gln-Thr-Arg-Ser-Gly-Ser-Ser-Trp-Ala-His-Thr-Cys-Gly
41
Gly-Thr-Leu-Ile-Arg-Gln-Asn-Trp-Val-Met-(homoserine lactone)

FIGURE 6: Amino acid sequence of the N-terminal cyanogen bromide fragment of elastase (from elastase sequence by Shotton and Hartley, 1970). The numbering of the amino acids in this paper does not correspond to that used by Shotton and Hartley (1970), who began numbering the amino-terminal valine as 16. Thus, our Glu-6 corresponds to Shotton and Hartley's Glu-21.

by the presence of a proline residue at position 13. When the above radioactive peptide was digested with LAP at 37° for 48 hr at pH 8.0, only a marginal amount of glutamic acid (0.2 residues per residue of alanine) was detectable upon amino acid analysis of the supernatant of the digest. On the other hand, if the LAP digest of the radioactive fragment was treated for 5 hr at 70° with 0.1 N NaOH prior to amino acid analysis, the glutamic acid content increased to 1.9 residues per alanine (see Table IV). Esterification of Glu-6 by DPBB is consistent with these data. The net gain of 2 glutamic acid residues after alkaline hydrolysis can be attributed to the saponification of the esterified glutamic acid residue and 1 glutamine residue. There was no change in aspartic acid content upon basic hydrolysis. Attempts are now being made to isolate and sequence a smaller tryptic peptide carrying the ^{14}C label in order to provide conclusive evidence for the involvement of Glu-6 in the inhibition of elastase by DPBB.

Discussion

The results reported above demonstrate that 1-bromo-4-(2,4-dinitrophenyl)butan-2-one (DPBB) reacts specifically and stoichiometrically with elastase. Elastase modified by DPBB is inactive to elastin, the enzyme's true physiological substrate, to the synthetic substrate *tert*-Boc-alanine *p*-nitrophenyl ester and to the active-site titrant diethyl-*p*-nitrophenyl phosphate. The rate of ^{14}C incorporation from [^{14}C]-DPBB is paralleled by the rate of inhibition toward the synthetic substrate *tert*-Boc-L-alanine *p*-nitrophenyl ester. Known reversible inhibitors of elastase and the natural substrate protect the enzyme against inhibition by DPBB.

The site of chemical modification of elastase by DPBB is the γ-carboxyl group of a glutamic acid residue. This conclusion is supported by (1) a positive hydroxamate test after the modified enzyme had been incubated with hydroxylamine; (2) the identification of 1,4-diaminobutyric acid following a Lossen rearrangement of the modified enzyme; and (3) amino acid analysis of leucine aminopeptidase digests of peptides obtained after cyanogen bromide cleavage. Glu-6 seems to be the glutamic acid residue most likely esterified, but this conclusion must be regarded as tentative pending further confirmatory experiments.

Since no glutamic acid residues appear to be near the catalytic site (Ser-195, His-57) of elastase in the crystal structure (Shotton and Watson, 1970), a precise explanation for the lack of activity of DPB-elastase toward specific elastase substrates and the active-site titrant, diethyl-*p*-nitrophenyl phosphate, is difficult. The loss of activity to the titrant, even though the site of modification is distal to the active site, suggests either that the geometry of the catalytic site has been perturbed or access to the catalytic site has been impeded. The appreciable alteration in the circular dichroism

of DPB-elastase relative to the native enzyme favors the former interpretation and suggests the modified enzyme has been stabilized in a conformation distinct from that of the active enzyme (Visser and Blout, 1971).

The unusual feature of the inhibition of elastase by DPBB is its unique specificity. Although the inhibitor bears no obvious structural relationship with natural or synthetic substrates of elastase, the enzyme is modified by a mechanism which involves the formation of a reversible complex between the enzyme and the inhibitor. Furthermore, closely related homologs of DPBB suc has 1-bromo-4-(2,4-dinitroanilino)-butan-2-one (XVI), 1-bromo-4-phenylbutan-2-one (V), and 1-bromo-3-(2,4-dinitrophenyl)propan-2-one (VI) are not at all effective inhibitors of elastase.

The failure of closely related homologs to inhibit elastase suggests the distance between the binding site of the dinitrophenyl moiety and the carboxyl group of the modified glutamic acid residue is rigidly prescribed. If the site on the elastase molecule which binds the dinitrophenyl moiety is near the active site of the enzyme, as is suggested by the protection experiments (Table III), the modification of a distal glutamic acid residue must be a result of the orientation of the bromo ketone group away from the enzyme's catalytic center. In a sense then, DPBB may serve as a type of cross-linking reagent between a region near the active site and a glutamic acid residue on the periphery of the enzyme. It is possible that the *in vivo* function of the binding site of elastase which has affinity for the dinitrophenyl moiety is to bind the aromatic desmosine or isodesmosine residues of elastin.

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