Table I. Steady-State Concentration of *n*-Hexyl-*tert*-butyl Nitroxide Generated from 6.6×10^{-2} M *n*-Heptanoyl Peroxide and NtB in Benzene at 40 °C

$[NtB] \times 10^3$, M	$[RT\cdot]_{ss} \times 10^6$, M	$(k_1[T]/[RT\cdot]_{ss}) \times 10^{-9}$ [a]	$2k_3$ [a,b]
28.4	11.6	21.8	140
14.2	13.0	9.72	103
5.67	10.8	4.67	128
2.84	10.8	2.34	94
0.57	5.0	1.01	158
0.28	3.2	0.78	132

[a] In M^{-1} sec^{-1} units. [b] Calculated from eq 4 using $v_i = 2 \times 10^{-8}$ M s^{-1} and $k_2 = 6.8 \times 10^8$ M^{-1} s^{-1} which gives the minimum variation in $2k_3$.

[PBN]/[NtB] ratios of 24 and 32 the k^T_{NtB}/k^T_{PBN} ratios extrapolated to $t = 0$ were 64.8 and 60.8, respectively.

Additional rate constants can be readily obtained from experiments of this type. For example, it has previously been shown[3] that the rate of decomposition of a peroxide, v_i, can be obtained by monitoring the initial rate of formation of the spin adduct. However, it has not previously been pointed out that with most traps it should be possible to use the steady-state concentration of the adduct to determine the rate constants for the two processes that normally will lead to its consumption, viz.,

$$R\cdot + RT\cdot \xrightarrow{k_2} \text{products} \quad (2)$$

$$RT\cdot + RT\cdot \xrightarrow{2k_3} \text{products} \quad (3)$$

For initiation and reactions 1-3, the appropriate equation is

$$\left(\frac{k_1[T]}{[RT\cdot]_{ss}} - k_2\right) \Big/ \left(\frac{k_1[T]}{[RT\cdot]_{ss}} + k_2\right) = \frac{2k_3}{v_i}[RT\cdot]_{ss}^2 \quad (4)$$

The data in Table I illustrate this application of spin trapping for R = *n*-hexyl and T = NtB in benzene at 40 °C. Measurement of the rate of RT· formation gave $v_i = 2 \times 10^{-8}$ M s^{-1}.[19] With this value the tabulated data could best be fitted to equation 4 by taking $k_2 = 6.8 \times 10^8$ M^{-1} s^{-1} which gave $2k_3$ 125 ± 20 M^{-1} s^{-1}. The value found for k_2 is in the range that has been found for addition of other alkyls to sterically hindered dialkyl nitroxides such as 2,2,6,6-tetramethyl-4-oxopiperidine-*N*-oxyl,[20-23] e.g., 4×10^8 M^{-1} s^{-1} for the cyclopentyl addition.[23,24] The value found for $2k_3$ was checked by direct measurement of the rate of decay of *n*-hexyl-*tert*-butyl nitroxide. This radical was generated from its hydroxylamine by photolysis in benzene in the presence of di-*tert*-butyl peroxide.[25] The exact value of $2k_3$ could not be determined because we were unable to prepare the *N*-*n*-hexyl-*N*-*tert*-butylhydroxylamine completely free from *N,N*-di-*tert*-butylhydroxylamine and di-*tert*-butyl nitroxide.[26] The effect of these impurities is to accelerate the decay of *n*-hexyl-*tert*-butyl nitroxide. However, under conditions of minimum impurity we were able to establish that *n*-hexyl-*tert*-butyl nitroxide decayed with second-order kinetics and that $2k_3 \leq 246 \pm 47$ M^{-1} s^{-1} at 40 °C.

This work is currently being extended to other spin traps and other temperatures.

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(9) In brief: $C_6H_5C(O)O\cdot$ + PBN, $k_1 = (1-10) \times 10^5$ M^{-1} s^{-1}, at 40 °C in benzene;[3,5] $(CH_3)_3CO\cdot$ + PBN, $k_1 = 55 \times 10^5$, $(CH_3)_3CO\cdot$ + NtB, $k_1 = 15 \times 10^5$, both at 25 °C in benzene;[4] $C_6H_5\cdot$ + PBN, $k_1 = 120 \times 10^5$, at 25 °C in methanol;[5] $(CH_3)_3COCO$ + NtB, $k_1 = (11-55) \times 10^5$, $[(CH_3)_3CO]_2\dot{C}H$, $k_1 = (9-45) \times 10^5$, both at 40 °C in di-*tert*-butyl peroxide;[6,7] $CH_3O\cdot$ + NtB, $k_1 = 1.3 \times 10^6$, at −45 °C in methanol.[8]
(10) Some "known" rate constants have been estimated from data obtained under very different conditions, sometimes on rather flimsy grounds. Even the most reliable of these "known" rate constants (i.e., that for $2(CH_3)_3C\cdot \rightarrow$ products which was used in ref 6 and 7) may be uncertain under the trapping conditions by a factor of 5 (see footnote, p 81, ref 7).
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(13) The acronym most often used. It stands for nitroso-*tert*-butane.
(14) Cf. 3.15 G for the $^{13}CH_3\cdot$ adduct: K. Sommermeyer, private communication.
(15) Cf. 4.38 G for $(Me_3{}^{13}C)_2NO\cdot$: R. Briere, H. Lemaire, and A. Rassat, *J. Chem. Phys.*, **48**, 1429 (1968).
(16) Unimolecular decompositions that may be more facile[17] such as,

$$RN(\dot{O})CMe_3 \rightarrow RN{=}O + Me_3C\cdot$$

and

$$C_6H_5CHRN(\dot{O})CMe_3 \rightarrow C_6H_5\dot{C}HR + Me_3CN{=}O$$

are not expected to affect the $[HT\cdot]/[CT\cdot]$ ratio.
(17) See, e.g., O. W. Maender and E. G. Janzen, *J. Org. Chem.*, **34**, 4072 (1969).
(18) The range of concentrations available is limited by the need to obtain approximately equal concentration of the two radicals.
(19) This yields a rate constant for the decomposition of *n*-heptanoyl peroxide to free radicals of 3×10^{-7} s^{-1} in benzene at 40 °C, which is in good agreement with other data. See, e.g., J. E. Guillet, T. R. Walker, M. F. Meyer, J. P. Hawk, and E. B. Towne, *Ind. Eng. Chem., Prod. Res. Devel.*, **3**, 257 (1964).
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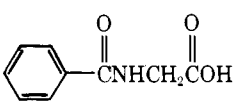
Aminomethaneboronic Acids. Synthesis and Inhibition of a Boron Analogue of Esterase Substrates

Sir:

We have synthesized a boronic acid analogue of hippuric acid (*n*-benzoylglycine) (**1**), an N-blocked amino acid, the esters and amides of which are used as substrates by proteolytic enzymes. The analogue synthesized (**2**) represents an example



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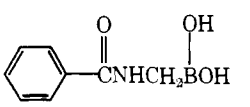

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of an amino acid substrate where the carboxyl carbon has been replaced by boron. Kinetic inhibition studies with α-chymotrypsin show that **2** is a potent, competitive inhibitor, and that it binds three orders of magnitude more tightly than does **1** to this enzyme. Our results suggest that this inhibitor acts as a possible analogue (**3**) of the metastable tetrahedral reaction

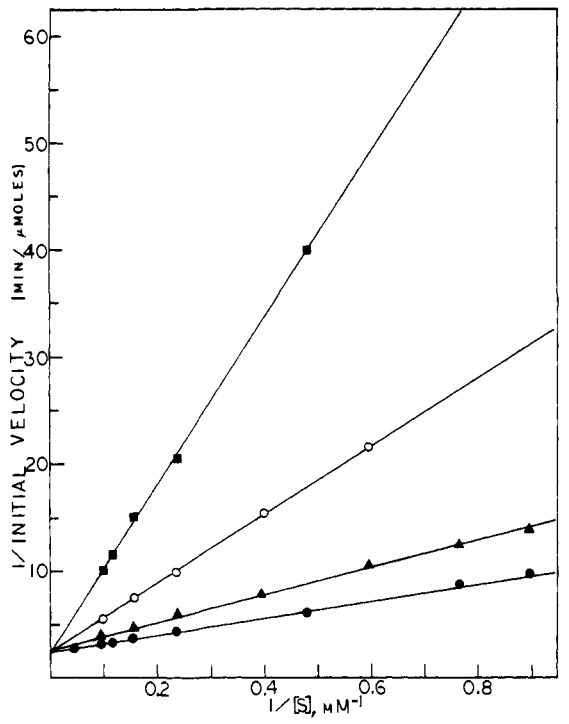
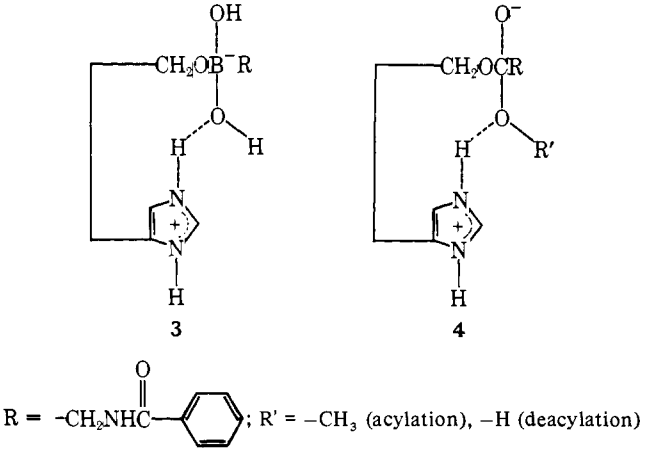


Figure 1. Inhibition of the chymotrypsin-catalyzed hydrolysis of methyl hippurate by *N*-benzoylaminomethaneboronic acid, 3×10^{-5} M (■), 7.7×10^{-6} M (○), 3×10^{-6} M (▲), no inhibitor (●), at pH 7.50 and 25 °C. The reaction mixtures contained 1-10 mM substrate, 0.1 M KCl, 10^{-5} M K_2HPO_4, and enzyme at a concentration of 6×10^{-6} M. No significant polymerization of enzyme is expected at this concentration in the pH range 5-10.[1] Initial velocities were calculated by following base uptake (0.02 N NaOH as titrant) for the first few percent of the reaction after the addition of 20 μl of the enzyme to the preincubated reaction solution. Stock solutions of α-chymotrypsin were prepared in 10^{-3} N HCl and did not change in activity over a period of several months when stored at 4 °C. The reciprocal of the initial velocity, which has been normalized to one concentration of enzyme, was plotted against the reciprocal of the substrate concentration 1/[S]. α-Chymotrypsin, the three-times-recrystallized activation product of the three-times-crystallized zymogen, dialyzed and salt-free, was purchased from Worthington Biochemical Corp. Methyl hippurate was prepared from hippuric acid and methanol by the method of Mill and Crimmin (P. J. Mill and W. R. C. Crimmin, *Biochim. Biophys. Acta*, **23**, 432 (1957)).



intermediates (**4**) for the acylation and deacylation which occur during chymotrypsin-catalyzed hydrolysis.

Aryl- and arylalkylboronic acids are potent competitive inhibitors of chymotrypsin[1-3] and subtilisin,[3,4] and it is likely that they act as transition state analogues. Crystallographic,[5] nuclear magnetic resonance,[6] and rapid temperature-jump[7,8] studies with subtilisin, and laser raman[9] and nuclear magnetic

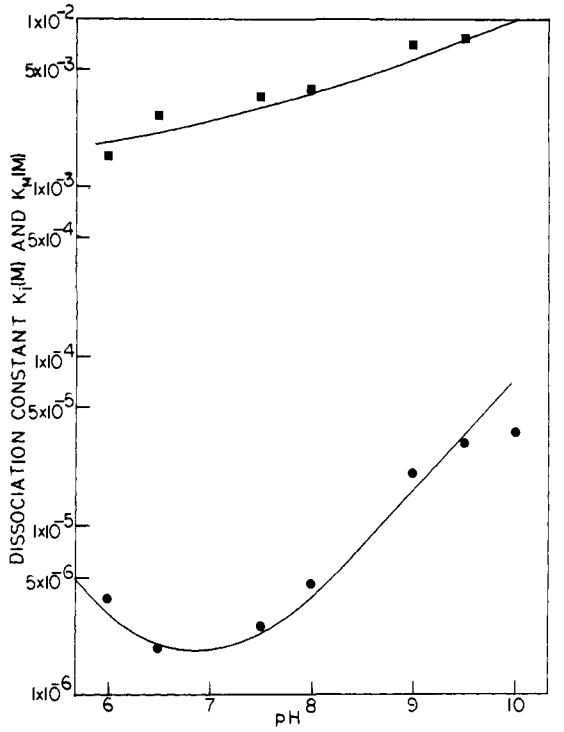


Figure 2. Semilogarithmic plots of Km (■) vs. pH and K_i (●) vs. pH for the inhibition of chymotrypsin-catalyzed hydrolysis of methyl hippurate by *N*-benzoylaminomethaneboronic acid. The conditions were as described in the legend in Figure 1. The kinetics can be described by the equation (M. Dixon and E. C. Webb, "Enzymes", Chapter 8, Academic Press, New York, N.Y., 1964)

$$\frac{1}{V} = \frac{Km(1 + [I]/K_i)}{k_{cat.}[E]_t[S]} + \frac{1}{k_{cat.}[E]_t}$$

where $[E]_t$, [S], and [I] are the concentrations of enzyme in all forms, substrate, and inhibitor, respectively, and where $V_m = k_{cat.}[E]_t$. The values of K_i, which is the dissociation constant of the enzyme inhibitor complex, were derived from the appropriate $1/v$ vs. 1/[S] plots and the equation $K_i = [I]/(V_m/(\text{slope})/Km - 1)$, where [I] is the inhibitor concentration, and the slope is from the plot with inhibitor present.

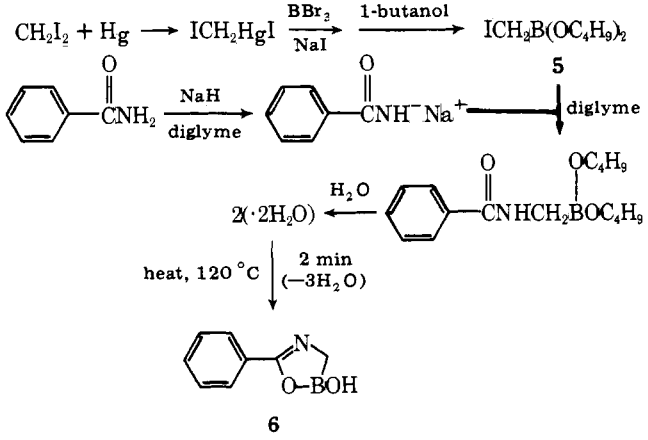
resonance[6] studies with chymotrypsin are consistent with the suggestion made by Koehler and Lienhard[1] that these boronic acids form stable tetrahedral adducts with active site serine residues.

Inhibitors of this type should be invaluable in elucidating enzyme-substrate interactions near the transition state. In order to better satisfy active site requirements, boronic acid analogues of amino acids or peptides should be used. It is probable that multisubsite interactions at the active site, such as those found with trypsin and soybean trypsin inhibitor,[10] occur generally in proteolytic enzymes and may affect interactions at the scissile bond. In an attempt to study the tetrahedral intermediate in chymotrypsin-catalyzed reactions with amino acid analogues which would form stable enzyme-inhibitor adducts, we have synthesized *N*-benzoylaminomethaneboronic acid (**2**) and have studied the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate in the presence of its boronic acid analogue over the pH range 6-10.

Synthesis of this boronic acid analogue was achieved by Scheme I.

Dibutyliodomethane boronate (**5**) was prepared by the general method of Matteson[11] and added dropwise to a cooled suspension of sodium benzamide in diglyme under argon. After stirring at room temperature for several days, water is added. After 1 h the mixture is extracted with ether. A precipitate of **2** then forms in the aqueous layer, which after filtration and air drying gives a white solid, mp 140-145 °C, yield 40%. After

Scheme I



being twice recrystallized from H_2O, the resulting white crystals have mp 156–158 °C; ^1H NMR (Me_2SO-d_6, D_2O (1:1)) δ 3.77 (s, NCH_2B), 7.67, 7.93 (m, aromatic) ppm. Anal. Calcd for $C_8H_{14}O_5NB$ dihydrate: C, 44.69; H, 6.51; N, 6.52. Found: C, 44.87; H, 6.34; N, 6.42. Thin layer chromatography of the ether and water solutions as well as precipitates which formed in both layers was performed by adding catechol before spotting. The boronic acids themselves do not move appreciably on silica gel plates, but, in the presence of 1,2-pyrocatechol, which presumably forms a diester complex [$-B(OR)_2$], R_f values of 7–8 are achieved in ethyl acetate/hexane (1:1). An analytical sample of **2**, which was dried for 2 min at 120 °C, appeared to lose 3 mol of H_2O and gave an elemental analysis consistent with structure **6**. No attempt was made to isolate or further characterize this compound. Anal. Calcd for $C_8H_8NO_2B$: C, 59.70; H, 4.96, N, 8,70. Found: C, 59.90; H, 5.04; N, 8.55.

Inhibition of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate in the presence of **2** was measured using a thermostatted Radiometer pH stat microtitration apparatus under argon flow. The kinetic data are presented in Figure 1. The linearity of the $1/v_0$ vs. $1/[S_0]$ plots and the common intercept on the $1/v_0$ axis indicate that the inhibition is apparently competitive over the range of inhibitor concentrations used. Similar plots indicate that competitive inhibition occurs at pH values between 6 and 10.

Each initial rate for the enzymatic reaction was obtained by subtracting the initial rate of base uptake in the absence of enzyme from that in its presence. In the most unfavorable case (pH 10) the background rate accounted for ~40% of the overall rate at the substrate concentrations used. The values of K_i for the inhibitor **2** are plotted as a function of pH in Figure 2. The values of *Km* for the substrate methyl hippurate are also plotted as a function of pH over the range 6–10. The values for *Km* (the apparent enzyme-substrate dissociation constant) are similar to those values obtained by Cuppett and Canady,[12] who observed the value of *Km* to increase from 2.5 $\times 10^{-3}$ M (pH 6) to ~1 $\times 10^{-2}$ (pH 10). Since acylation is the rate-determining step for the enzymatic hydrolysis of methyl hippurate over the pH range 6–10, *Km* values can be reasonably equated with enzyme-substrate dissociation constants.[12] A maximal velocity vs. pH profile similar to published data[12] was obtained with maximum rates occurring at pH 8.5–9.0. A rate constant of 9.45 min^{-1} was obtained at a pH of 7.5, which is close to previously published values.[1,12]

The pH-K_i profile for *N*-benzoylaminomethaneboronic acid is similar to those obtained for aralkylboronic acid inhibition of chymotrypsin[1,2] and subtilisin,[4] but it appears to be shifted slightly toward lower pH values with a maximum of about pH 6.5–7. As is the case with phenylethaneboronic acid, the pH-K_i profile suggests that the interaction of *N*-benzoylaminomethaneboronic acid and α-chymotrypsin may be dependent upon the state of ionization of two groups on the enzyme (such as his 57 and ile 16).[1] Titration of **2** in presence of mannitol[13] yields an apparent pK_a value >10.5. The pK_a for **2** in the absence of mannitol is higher and could not be determined owing to the interference from the titration of water. Any formation of the anionic form of **2** ($RB(OH)_3^-$) in the pH range studied will be insignificant. In addition, **2** binds to chymotrypsin ~25 times more tightly than does phenylethaneboronic acid at pH 7, and better than three orders of magnitude more tightly than does methyl hippurate, the substrate for which it is an analogue. *trans*-β-Styreneboronic acid[2] and benzeneboronic acid[3] bind, respectively, about 15 times and about two orders of magnitude less tightly than **2** does to α-chymotrypsin. Other competitive inhibitors such as hydrocinnamaldehyde, hydrocinnamamide, and 2-phenylethanesulfonic acid[2] bind two to four orders of magnitude less tightly to this enzyme than does **2**.

Boronic acid–enzyme adducts should be isosteric and isoelectronic with postulated tetrahedral enzyme–substrate intermediates. It appears that enzymes specifically stabilize such transition-state complexes[5] as opposed to the planar carbonyl ground-state substrate in the Michaelis (ES) complex. Interactions between the active site residues of subtilisin and phenylethane- and benzeneboronic acids complexed as covalent adducts in the crystalline form[5] clearly demonstrate the potential of such analogues. Further elucidation of detailed mechanisms of enzymatic catalysis should be possible with boronic acid peptide analogues which fit the multisubsite requirements as well as forming a tetrahedral transition-state adduct with the serine or cysteine residue at the enzyme active site.

The experimental results indicate that **2** is a potent, competitive inhibitor of α-chymotrypsin. The kinetic data for this amino acid analogue suggest that it may be forming a tetrahedral adduct with the hydroxyl group of serine 195 at the active site of α-chymotrypsin. Such an adduct would be considered an analogue of the tetrahedral reaction intermediate (i.e., a transition-state analogue) for the acylation and deacylation reactions which occur during chymotrypsin-catalyzed hydrolysis of amino acid ester and amide substrates.

The synthesis of more specific dipeptide boronic acids as substrate analogues for other proteolytic enzymes is in progress.

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