Immunosuppressive Boronic Acid Dipeptides: Correlation between Conformation and Activity

Terence A. Kelly,*,† Julian Adams,† William W. Bachovchin,‡ Randall W. Barton,§ Scot J. Campbell,∥ Simon J. Coutts,† Charles A. Kennedy,§ and Roger J. Snow†

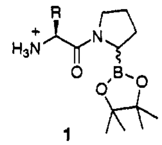
Departments of Medicinal Chemistry, Pharmacology, and Analytical Sciences
Boehringer Ingelheim Pharmaceuticals Inc.
175 Briar Ridge Road, Ridgefield, Connecticut 06877
Department of Biochemistry
Tufts University School of Medicine
Boston, Massachusetts 02111

Received September 27, 1993

Dipeptidyl peptidase IV (DPP4; CD26) is a serine protease present on the surface of $CD4^+$ cells.[1] Inhibition of this enzyme has been shown to suppress IL-2 production[2] and antigen-induced T-cell proliferation[3] *in vitro*. Recently, potentially therapeutic effects of inhibitors of DPP4 have been demonstrated using *in vivo* models of immunosuppression.[4] Furthermore, CD26 has been identified as the adenosine deaminase binding protein,[5] a molecule involved in the severe combined immunodeficiency disease (SCID), and has also been proposed to be a coreceptor on the surface of $CD4^+$ cells for the human immunodeficiency virus (HIV),[6] the causative agent of the acquired immune deficiency syndrome (AIDS).

The DPP4 enzyme cleaves a two amino acid unit from the amino terminus of polypeptides which possess proline at the P^1 position.[7] Although its native substrate remains unknown, mutagenesis studies[8] have demonstrated that the enzymatic activity of DPP4 is important in its role in the activation of T-cells.

In order to explore more fully the role of this enzyme in immunosuppression we decided to examine the properties of the prolineboronic acid-containing dipeptides **1** (X_{aa}-boroPro).[9] These compounds have been shown by Bachovchin to be potent inhibitors of DPP4.[10] The literature on boronate ester polypeptides indicates that they gain activity upon exposure to aqueous buffer, presumably through hydrolysis to the active boronic acid (*e.g.*, **2**).[11] In contrast, dipeptides **1** have been reported to *lose* activity in a time-dependent manner upon exposure to an aqueous medium,[10] suggesting a more complicated scenario.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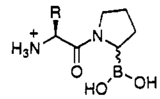
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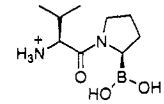
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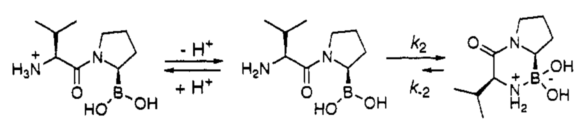
To simplify the anlaysis of this apparent contradiction, we elected to synthesize the dipeptide in the unprotected boronic acid form (Scheme I). A single stereoisomer of (1*S*,2*S*,3*R*,5*S*)-pinanediol pyrrolidine-2(*R*)-boronate (**4**)[12] was coupled to Boc-valine to produce **5**.[13] Next, the pinanediol group was cleaved by treatment of the dipeptide with basic sodium periodate.[14] Finally, removal of the Boc group from **6** with HCl–Et_2O afforded the HCl salt of compound **3** (val-boroPro).[15]

Val-boroPro (**3**) is a remarkably potent inhibitor of DPP4 (IC_{50} = 16 nM).[16,17] However, the inhibitory activity of the material decreases rapidly upon standing in pH = 7.8 buffer, thus pointing to an intrinsic instability in the dipeptide motif.

We hypothesized that the loss of inhibition was due to a cyclization taking place to generate a compound of structure **7** (eq 1). This compound is a boron analog of a diketo piperazine, often a side product in peptide chemistry.



(1)

3 Active   **7** Inactive

Compound **7** could be isolated by treatment of **3** with aqueous base followed by purification via ion exchange chromatography. The cyclic structure of **7** was confirmed by ^{11}B-NMR. Compound **3** shows a peak in the ^{11}B spectrum at δ 28.0 ppm (relative to $BF_3{\cdot}Et_2O$ δ = 0 ppm) while compound **7** shows a peak at δ 3.5, indicative of a tetracoordinated boron atom. This structure is supported further by X-ray data obtained for a protected analog.[18]

A ^{1}H-NMR experiment was designed to follow the course of the reaction.[19] Compound **3** was dissolved in an aqueous buffer adjusted to pH = 7.8. Over time, increasing amounts of **7** were observed. The relative proportion of each compound was assessed at different time points by integrating the peaks at δ 3.09 and 4.05 (**3**) and at δ 2.46 and 2.65 (**7**) (Figure 1, curve 1). Kinetic

† Department of Medicinal Chemistry, Boehringer Ingelheim.
‡ Tufts University School of Medicine.
§ Department of Pharmacology, Boehringer Ingelheim.
∥ Department of Analytical Sciences, Boehringer Ingelheim.

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(9) Prolineboronic acid is the analog of proline where the carboxylic acid is replaced by a boronic acid. It is abbreviated as boroPro.

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(15) All compounds were characterized by ^{1}H-, ^{11}B-, and ^{13}C-NMR, CI-MS, and melting point. Compound **3** was further characterized by microanalysis for C, H, N, and B.

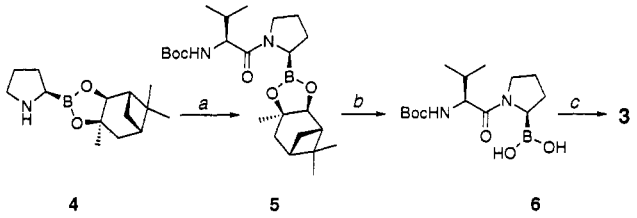
(16) The IC_{50} number refers to the amount of compound required to inhibit 50% of enzymatic activity for the duration of the assay.[17] This number greatly underestimates the true activity of these compounds due to the cyclization process that is occurring over the 1-h course of the assay. K_i values for this series of compound have been estimated at <100 pM.[22]

(17) DPP4 enzyme assay: This assay (based on the following: Smith, R. E.; Van Frank, R. M. *Frontiers of Biology Volume 43: Lysosomes in Biology and Pathology*; Neuberger, A., Tatum, E. L., Eds.; Amsterdam: North Holland, 1975; pp 193–249) relies on the ability of the enzyme to cleave the substrate L-alanyl-L-prolinyl-2-(4-methoxy)naphthylamide (Ala-Pro-MNA).

(18) Snow, R. J.; Coutts, S. J.; Kelly, T. A.; Krolikowski, D. A.; Campbell, S.; Leonard, S.; Barton, R. W.; Kennedy, C. A.; Adams, J. A. Manuscript in preparation.

(19) The ^{1}H-NMR experiment was performed on a Bruker Instruments AF-270 spectrometer. A homonuclear presaturation routine using a spatially selective composite observe pulse (based on the following: Bax, A. *J. Magn. Reson.* **1985**, *65*, 142) was employed for solvent suppression. The sample was

Scheme 1



4 5 6

[a] 2 equiv of Boc-valine, DCC, THF, 2 h, then filter and add to 4 and *N*-methylmorpholine in $CHCl_3$ (96%).[b] $NaIO_4$, acetone, H_2O (61%).[c] HCl, EtOAc (90%).

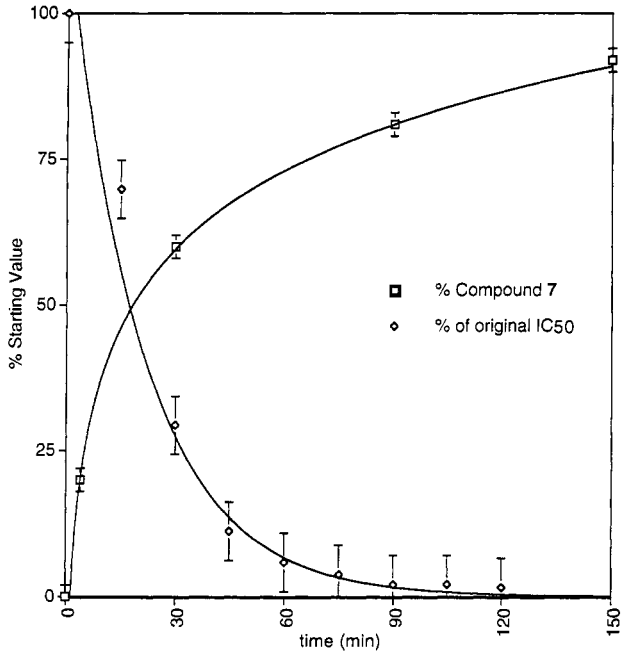


Figure 1. Curves showing that the cyclization of **3** to **7** (curve 1, squares, determined by ^{1}H-NMR) corresponds to the loss of ability to inhibit the DPP4-catalyzed hydrolysis of substrate (curve 2, diamonds). Both experiments were performed at pH = 7.8. Kinetic data for curve 2 are fitted up to t = 75 min since at high [**7**] the cumulative inhibition demonstrated in Figure 2 becomes significant over the time required to run the assay (ref 16).

analysis of the data gave an observed first-order rate constant[20] of $3.9 \times 10^{-4} \pm 0.7 \times 10^{-4}$ s^{-1} for the cyclization of **3** to **7**. A corresponding biochemical experiment was run measuring the time-dependent ability of the compound to inhibit DPP4 after also standing in an aqueous buffer at pH = 7.8. As expected, the compound became less active over time and the inactivation proceeded with a similar observed rate constant[20] ($6.6 \times 10^{-4} \pm 0.6 \times 10^{-4}$ s^{-1}, Figure 1, curve 2). The correlation demonstrated between the structural and enzymatic experiments establishes that the cyclization is indeed responsible for the loss in activity.

The cyclization is also reversible due to the dative nature of the B–N bond in **7**. We have observed that although activity against the enzyme diminishes by 3 orders of magnitude, the final IC_{50} is still submicromolar, suggesting that at pH = 7.8

(20) The rate of the cyclization is dependent on the position of the equilibrium between protonated and unprotonated **3** (see eq 1), so for the forward (*i.e.*, cyclization) reaction d[**7**]/dt is equal to k_2K_{eq}[**3**]/[H$^+$]. Since the solution is buffered, [H$^+$] is constant and the rate equation reduces to d[**7**]/dt = k_{obs}[**3**] where $k_{obs} = k_2K_{eq}/$[H$^+$].

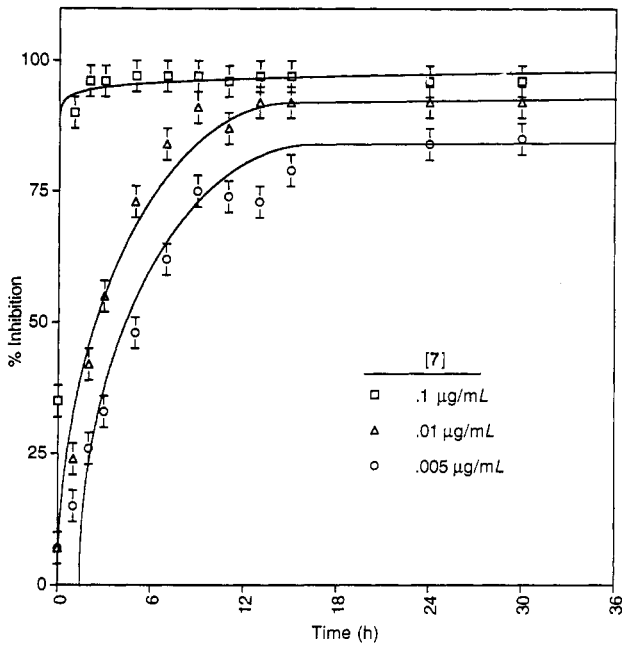


Figure 2. Recovery of inhibitory activity of **7** (via **3**) with time.

compound **7** is in equilibrium with a small amount of the open from **3**. We sought to exploit this process to see if the activity of compound **7** could be restored by driving the equilibrium back toward **3** using an acidic buffer. This proved possible. At pH = 3.0 the material reverted to its open form and completely regained its activity against the enzyme.[21]

Because boronic acid peptides are known to display tight-binding inhibition with a very slow off-rate,[22] the equilibrium has profound consequences for this series of immunosuppressants. When the enzyme is incubated in the presence of cyclized (*i.e.*, inactive) inhibitor under standard (pH = 7.8 buffer) conditions and then is assayed for the ability to hydrolyze substrate, a cumulative, time-dependent inhibition of the enzyme is observed (Figure 2). These data are consistent with the formation of the equilibrium between **3** and **7** being reestablished after the active open form **3** binds (and remains bound)[22] to the enzyme (eq 2). *Thus it is the presence of the enzyme itself that drives the equilibrium toward the active form.*

$$\mathbf{7} \rightleftharpoons \mathbf{3} + \text{Enz} \longrightarrow \text{Enz-}\mathbf{3} \qquad (2)$$

The results of these experiments show a system in which the ability to inhibit a serine protease relies on the position of an equilibrium between two isolable and interchangeable conformations. They also show that specific binding to an enzyme can drive an equilibrium if the off-rate is such that the enzyme can entrain one of the components from the system. The *in vivo* consequences of this phenomenon are currently under study.

Acknowledgment. We are grateful to the Analytical Science Department at Boehringer Ingelheim for their fine efforts in support of this work. In particular we thank Dr. Phil Pitner for many helpful discussions.

(21) The kinetic rationale for this phenomenon can be derived from the rate equation for the ring opening: d[**3**]/dt = $k_{-2}K_{eq}$[**7**][H$^+$]. As [H$^+$] is increased, the observed rate constant of the opening increases while the observed rate constant of the closing (which is inversely related to [H$^+$])[20] decreases.

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