Studies on Proline Boronic Acid Dipeptide Inhibitors of Dipeptidyl Peptidase IV: Identification of a Cyclic Species Containing a B-N Bond[1]

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Abstract: The proline boronic acid dipeptides AlaboroPro (**14**), ProboroPro (**15**), and ValboroPro (**16**) are very potent inhibitors of the enzyme dipeptidyl peptidase IV (DPP IV or CD26), found on the surface of T-cells, and are a new class of immunosuppressants. The efficient synthesis of the free boronic acids as single enantiomers is described, and the absolute configuration determined. These compounds are known to lose DPP IV inhibitory activity in solution: this is shown to be due to the reversible formation of a cyclic species analogous to a diketopiperazine, containing a B-N bond. The cyclic compounds, both as the free boronic acids (**17-19**) and as the pinanediol esters (**11-13**), have been isolated and characterized by ^1H and ^{11}B NMR, and in one case by X-ray crystallography. The cyclization is pH dependent, with the open form favored at low pH, while the cyclic form predominates at neutral pH. Both the rate and extent of cyclization depend on the N-terminal amino acid. The rates of cyclization have been measured by ^1H NMR and shown to correlate with the decrease in DPP IV inhibitory activity. ValboroPro cyclizes more slowly, and to a lesser extent than AlaboroPro or ProboroPro, which is predicted to lead to greater immunosuppressive potency *in vivo*.

Introduction

Dipeptidyl peptidase IV (DPP IV)[2] is an extracellular membrane-bound enzyme present on several cell types, in particular $CD4^+$ T-cells,[3] as well as kidney, liver, and intestine.[4] In T-cells DPP IV has been shown to be identical to the antigen CD26.[5,23] This molecule appears to be involved in modulating the immune response when the cells are stimulated via the T-cell receptor,[6] and has been reported to associate with both CD45[7] and adenosine deaminase.[8] CD26 has also recently been reported to be involved in the infection of T-cells by the human immunodeficiency virus,[9] although this has been questioned.[9b] DPP IV is a serine protease which cleaves a dipeptide from the N-terminus of a polypeptide, where the second residue is proline. Substrates with hydroxyproline or alanine at this position are also cleaved, though less efficiently.[2b,10] Details of the involvement of DPP IV in the immune response are still unclear, and the endogenous substrate has yet to be identified. There are conflicting reports as to whether the catalytic activity is,[11] or is not,[12] essential for its function as a costimulator in T-cell

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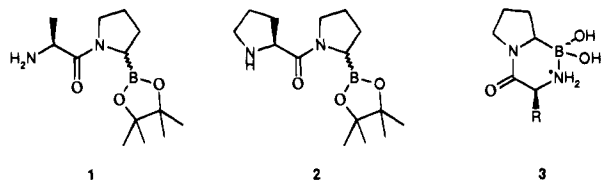
activation. Recently it has been shown that inhibitors of DPP IV block the proliferative response of T-cells to antigenic stimulation,[13] and suppress IL-2 production.[14] In particular, the dipeptides of proline boronic acid[15] AlaboroPro and ProboroPro, prepared as the pinacol esters **1** and **2**, have been reported by Flentke and Bachovchin[16] to be extremely potent inhibitors of DPP IV, and show immunosuppressive activity both in cell culture[16] and *in vivo*.[17] However, these compounds were also reported to lose their inhibitory activity in aqueous solution at neutral pH.[16] As part of a study to evaluate the therapeutic potential of these boronic acids as immunosuppressants, we wished to determine the cause of this change in activity. We reasoned that a plausible explanation might be the formation of a cyclic species such as **3**,[1,18] analogous to a diketopiperazine,



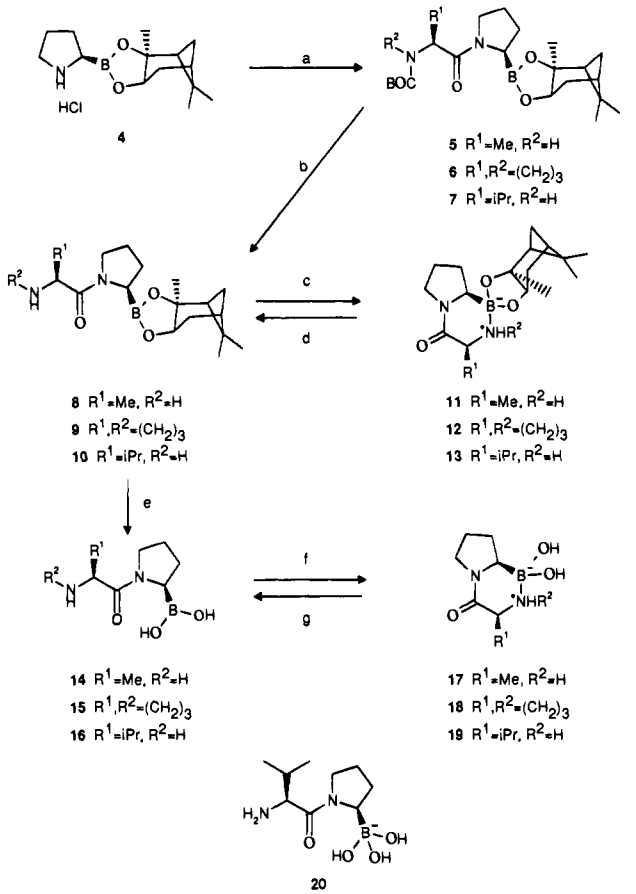
1 **2** **3**

in which the amine nitrogen coordinates to the boron atom. Here we report that cyclic dipeptides of this type are indeed formed, and have been isolated and characterized. The cyclization has been shown to be reversible, and pH dependent, with the open form favored at low pH. Furthermore, we show that the rate of cyclization correlates with the decay in biological activity.

Results

Synthetic Chemistry. In order to carry out these studies we required the proline boronic acid dipeptides as single isomers. Recently we reported an improved synthesis and resolution of proline boronic acid pinanediol ester hydrochloride **4**,[19] which could be routinely obtained in >98% diastereomeric excess. This was coupled with protected amino acids using a water-soluble carbodiimide to prepare the protected dipeptides of alanine **5**, proline **6**, and valine **7** in almost quantitative yield (Scheme 1). Originally **1** and **2** were obtained from racemic proline boronic acid,[16,20] and although the diastereoisomers of **2** have since been separated by HPLC,[18] this is the first time the dipeptides have been obtained in quantity as single isomers. Deprotection with HCl gave the dipeptides **8**–**10** with the

Scheme 1[a]



[a] Reagents: (a) BOC-amino acid, EDC, HOBT, *N*-methylmorpholine, CH_2Cl_2, 0 °C to rt; (b) HCl, Et_2O, 0 °C to rt; (c) Na_2CO_3(aq), CH_2Cl_2; (d) maleic acid or $MeSO_3H$, CH_2Cl_2/MeOH; (e) $PhB(OH)_2$, H_2O, hexane, rt; (f) Dowex 50X2-200, elute with aqueous NH_3; (g) $MeSO_3H$, MeCN.

boronic acid still protected. Pinanediol boronates are reported[21] to be very stable to hydrolysis, and the conditions described[21] for removing the pinanediol are incompatible with the other functional groups. However, Coutts *et al.*[22] have recently reported two mild methods for the deprotection of pinanediol boronates. For the present studies, efficient deprotection was achieved by transesterification of the free amine dipeptides **8**–**10** with phenylboronic acid in a two-phase system of water at low pH and hexane. Pinanediol phenylboronate was recovered in high yield from the organic layer. The boronic acids were isolated by passing the aqueous layer through an ion exchange column, and eluting the product with aqueous ammonia. This yielded the amine free bases as the cyclic forms **17**–**19** (see below). The open compound **16** could be conveniently prepared in solid form by adding methanesulfonic acid to an acetonitrile solution of **19**, which precipitated the salt of ValboroPro (ValBP) (**16**), analytically pure. This proved to be the best method of purifying the material. The free boronic acids of AlaboroPro (AlaBP) (**14**) and ProboroPro (ProBP) (**15**) were prepared in the same way. All the open chain compounds with a free terminal amine, both with and without pinanediol, showed potent inhibitory activity in an *in vitro* enzyme assay[23] (Table 1), and

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Table 1. Inhibition of Human DPP IV by Proline Boronic Acid Dipeptides

compound	IC_{50}[a] (nM)	IC_{50} (nM) at equilibrium[b]	k_{cyc}[c] (s^{-1})	k_{inact}[d] (s^{-1})
8	24.0 ± 2.8			
9	23.5 ± 1.8			
10	16.9 ± 1.3			
14	32.7 ± 2.9	7800 ± 2600	$(3.2 \pm 0.25) \times 10^{-4}$	$(1.4 \pm 0.04) \times 10^{-3}$
15	18.5 ± 1.1	35300 ± 2300	$(7.5 \pm 0.14) \times 10^{-5}$	$(1.7 \pm 0.04) \times 10^{-3}$
16	16.9 ± 1.3	1500 ± 200	$(6.4 \pm 0.15) \times 10^{-5}$	$(6.4 \pm 0.7) \times 10^{-4}$

[a] IC_{50} for inhibition of DPP IV in end-point assay. Each figure is the mean of at least three separate determinations, ± SEM. [b] Solutions of inhibitor were allowed to equilibrate for 24 h in Tris buffer at pH 7.8 at room temperature, before the IC_{50} was determined. [c] Observed rate constant for cyclization in D_2O, pD 7.8, from a nonlinear regression fit of the data in Figure 6 to eq 2, ± standard error. [d] Observed rate constant for loss of activity in H_2O, pH 7.8, from data in Figure 8, over the first 60 min, fit by nonlinear regression to an exponential decay, ± standard error.

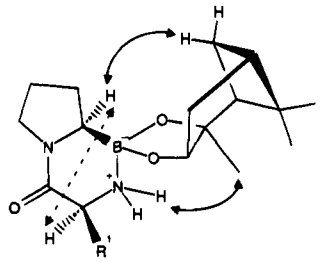

Figure 1. Selected NOEs observed in the 2D NOESY spectra of **11** (R^1 = Me), dashed arrow, and **13** (R^1 = iPr), solid arrows.

as reported by Bachovchin,[16] all lost activity rapidly on standing in solution at pH 7.8.

Structural Studies of Pinanediol Boronates. Initial studies to identify the proposed cyclic species were carried out on the pinanediol boronates. If the proposed cyclization were occurring, the change from trigonal to tetrahedral boron should be readily discernible by ^{11}B NMR.[24] The first evidence for this came from studies on **10**, which showed the expected ^{11}B signal for a trigonal boronate as a broad peak centered at 30 ppm downfield from boron trifluoride etherate. On extracting the free base of **10** into an organic solvent, the initially formed product was converted within 15–30 min to a less polar species as monitored by TLC. This was isolated by flash chromatography, and shown to still contain pinanediol by ^1H NMR and MS. The ^{11}B NMR spectrum now consisted of a single, much sharper peak at δ 7.8, consistent with a tetrahedral boron, and provided strong evidence that this material has the cyclic structure **13**. ^1H NMR of **13** showed it to be predominantly one diastereoisomer (90%) at the newly-formed chiral center at boron, but a minor component was always present. This component may be the other diastereoisomer, but it could not be separated by chromatography, possibly because of interconversion of the species. Further proof that **13** was indeed cyclic came from the 2D NOESY spectrum, which revealed NOEs between an NH and a methyl group of pinanediol (see Figure 1), as well as between the boroPro α proton and one of the pinanediol methylene bridge protons. This also revealed the absolute configuration at boron to be as shown in Figure 1. This stereochemistry results from attack by the nitrogen on the face of the boronate ring which bears the pinanediol methyl group. From examination of models, this was the expected diastereoisomer, since the methylene bridge of the pinanediol shields the face of the boronate ring adjacent to it, and prevents the approach of the nitrogen from this side. The cyclic analogs of alanine **11** and proline **12** were obtained similarly, and showed ^{11}B NMR signals at δ 9.2 and 8.9, respectively. In the case of **11**, an NOE was observed between the two α protons (Figure 1), again consistent with the cyclic structure. In the NOESY

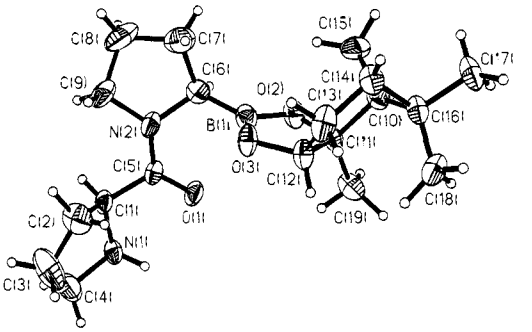

Figure 2. ORTEP drawing of the X-ray structure of the methanesulfonate salt of **9**, with the methanesulfonic acid omitted for clarity. ψ_1(N1–C1–C5–N2) = 173°.

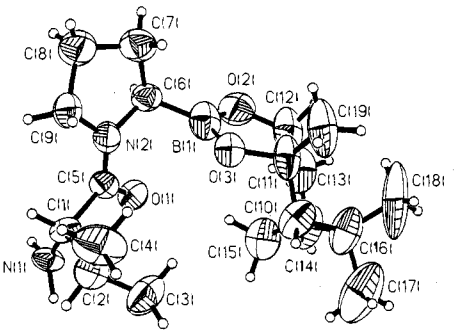

Figure 3. ORTEP drawing of the X-ray structure of the hydrogen maleate salt of **10**, with the maleic acid omitted for clarity. ψ_1(N1–C1–C5–N2) = 149°.

spectrum of **12**, however, overlapping peaks prevented assignment of NOEs which would confirm the structure.

To investigate whether the cyclization is reversible, **13** was treated with acid in an organic solvent, whereupon it was reconverted to the salt of **10**. This also provided a means of preparing a variety of salt forms for purification and crystallization experiments.

Rigorous proof of the cyclic structure and stereochemistry was obtained by X-ray crystallography. The open forms of **9** and **10** were crystallized as the methanesulfonate and maleate salts, respectively (Figures 2 and 3). This confirmed the absolute configuration of the proline boronic acid as *R*, corresponding to an L-amino acid, by correlation with the terminal L-Pro or L-Val residue, as expected from the biological activity. The conformations of **9** and **10** in the crystal are similar to the minimum energy conformations predicted by calculation;[25] in particular for **9** the observed ψ_1 = 173° is close to the value calculated for ProProNH$_2$ (ψ_1 = 170°)[25a] while for **10** ψ_1 = 149°, which is similar to the calculated value for

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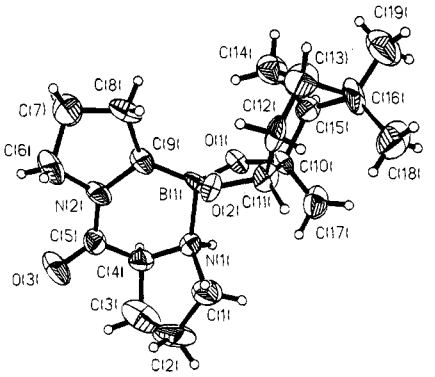

Figure 4. ORTEP drawing of the X-ray structure of **12**. The unit cell contains two independent molecules of chloroform, which were disordered, and are omitted for clarity.

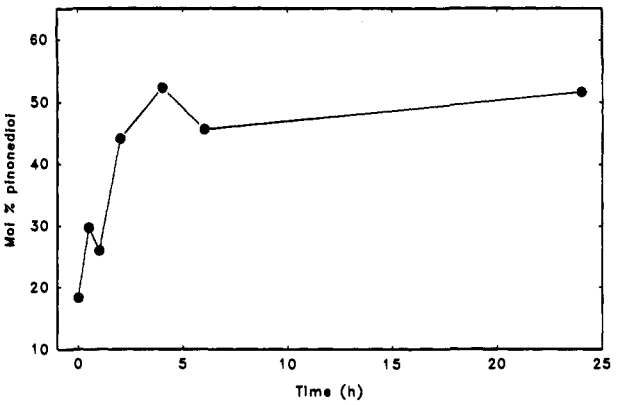

Figure 5. GC measurement of the formation of pinanediol over time from **10** in Tris buffer at pH 7.0.

AlaProNH$_2$ (ψ_1 = 147°).[25c] The cyclic form of the proline analog **12** crystallized as a chloroform solvate, shown in Figure 4. This clearly shows a nitrogen–boron bond of 1.74 Å, and also the tetrahedral geometry of both boron and the proline nitrogen, with the *S* configuration in each case. The configuration at boron is also in agreement with that determined for **13** by NMR. A search of the Cambridge Crystallographic Database retrieved 16 examples of nitrogen coordinated to tetrahedral boron, with B–N distances ranging from 1.55 to 1.73 Å. Only three structures[26,27] contained boron bonded to one carbon, two oxygens, and one nitrogen atom, the most closely related being two triphenylboroxines with B–N bond lengths of 1.66 and 1.73 Å.[24] This appears to be the first example of a tetrahedral boronate coordinated to a secondary amine, and the first with a B–N bond as part of a ring.

Structural Studies of Boronic Acids. With secure evidence for the formation of a cyclic product in the case of the pinanediol esters, we turned our attention to the free boronic acids, which are assumed to be the active inhibitors. Both forms of the free boronic acid of ValBP (**16** and **19**) were isolated and characterized as described above. The material isolated from the ion exchange column after lyophilization showed a single peak at δ 2.7 in the ^{11}B NMR spectrum in D_2O, consistent with the cyclic structure **19**, and ^{1}H NMR showed the valine α proton at δ 3.55. On acidification of the solution, the ^{11}B signal moved to δ 28, and the valine α proton moved downfield to δ 4.14. Both these observations closely parallel the changes seen in the conversion of **13** to **10**. The IR spectra were also consistent with the structures shown: **16** displayed a strong band at 1350 cm^{-1}, typical of a trigonal boronic acid,[28] which was absent in **19**. One of the main pieces of evidence for the cyclic structure **19** is the ^{11}B NMR;[29] a possible structure which might also fit the NMR data is the borate **20**. To address this question, **16** was dissolved in D_2O buffered to pD 10.5, and the ^{1}H NMR spectrum run immediately. This showed a single species, different from **16** or **19**, which was presumed to be **20**. Over several hours, this converted to **19** as monitored by ^{1}H NMR. A small, but detectable, change from δ 6.1 to δ 2.7 was also observed in the ^{11}B NMR over the same period. At high pH, **20** should be formed rapidly from **16**, in a prototropic equilibrium, whereas conversion to **19** requires isomerization of the amide bond, and is relatively slow. Thus, **19** is the preferred form at neutral or alkaline pH. It should be noted that the activity of boronic acid inhibitors of other enzymes does not change at alkaline pH.[20] Thus, simply forming a tetrahedral boron does not lead to loss of activity. To date we have not succeeded in obtaining X-ray quality crystals of a free boronic acid in either open or cyclic form, but taken together, we believe that the above data establish the cyclic structure for **19**.

Studies on Pinanediol Hydrolysis. Peptide boronic acid enzyme inhibitors have generally been prepared as boronate esters, for example, with pinacol, which are rapidly hydrolyzed to the free boronic acid in aqueous solution.[30] As noted above, pinanediol boronates are reported to be much more stable to hydrolysis. Since the pinanediol esters **8**–**10** were extremely potent in the enzyme assay (Table 1), it was necessary to determine whether the activity was due to the free boronic acid generated by hydrolysis, or whether the esters themselves were active. This was studied initially by ^{1}H NMR using a solution of **10** in phosphate buffer in H_2O at pH 7.8. Even at early time points, the formation of free pinanediol was readily discernible, along with peaks corresponding to the free boronic acid **16**. The amount of pinanediol increased over several hours, and two new sets of peaks appeared, one of which was the cyclic pinanediol boronate **13**, and the other was the cyclic form of the free boronic acid **19**. After 24 h, the only species present by NMR were **13**, **19**, and pinanediol, and no further change was observed after this time. The extent of pinanediol formation was estimated to be 40–50%. Qualitatively similar results were obtained using a GC assay to measure pinanediol: approximately 20% hydrolysis was observed after 5 min, which leveled off at 50–60% after 5 h (Figure 5). From these observations we concluded that the process illustrated in Scheme 2 was occurring. Hydrolysis of pinanediol does occur, but is incomplete. Both the boronate ester and the free boronic acid undergo cyclization at neutral pH, with the equilibrium strongly favoring the cyclic form. The cyclic pinanediol ester **13** does not interconvert directly with **19**, since the mechanism for hydrolysis of a boronate presumably involves attack by water on the vacant orbital of boron as the first step. In **13** this coordination site is occupied by the amine. At lower pH (ca. 3), cyclization does not occur, but the rate of hydrolysis as determined by ^{1}H NMR is much slower than at pH 7.8. The extent of hydrolysis at pH 7.8 was surprising, in view of the reported stability of pinanediol boronates,[21] and the observed stability of **7**, under hydrolytic conditions.

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Scheme 2. Equilibrium Established on Dissolving ValboroPro Pinanediol Ester **10** in Water at pH 7.8

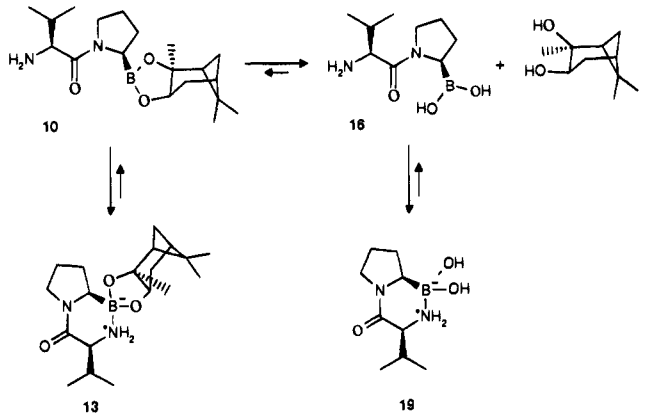


Table 2. Effect of Pinanediol on Inhibition of DPP IV by Continuous Enzyme Assay

addition to reaction[a]	relative velocity[b]	addition to reaction[a]	relative velocity[b]
DMSO control	1.00	**10**, pinanediol	0.59
(+)-pinanediol	1.01	**16**	0.23
10	0.34	**16**, pinanediol	0.57

[a] Inhibitors were added at 1.1×10^{-7} M. Pinanediol was added at 3×10^{-3} M. [b] Velocities were determined by a linear regression fit to the equilibrium portion of the raw data, and expressed relative to the DMSO control. In all cases the linear correlation coefficient was >0.98.

Since hydrolysis to the free boronic acid clearly occurs in water, it seemed likely that this could account for the inhibition seen with the pinanediol ester **10**, but in view of its potency we wanted to determine whether all the activity is due to **16**, or whether **10** itself has activity. To do this, the rate of inhibition of DPP IV by **10** and **16** was measured using a continuous enzyme assay, in the presence of excess pinanediol. The data (Table 2) indicated very similar levels of inhibition by either 1.1×10^{-7} M boronic acid **16** or the pinanediol ester **10**. It is not possible to determine which compound is more inhibitory from this result, since the pinanediol ester **10** contains significant concentrations of the free boronic acid **16** (ca. 20%) at the start of the enzyme reaction. The addition of 3×10^{-3} M (+)-pinanediol to either **16** or **10** would be expected to significantly decrease the equilibrium concentration of free boronic acid **16** (Scheme 2). The data indicate that the level of inhibition is reduced to the same extent in each case, suggesting that **16** is considerably more inhibitory than the pinanediol ester **10**.

NMR Studies of Cyclization Rates. With methods available to prepare the free boronic acids in both the open and cyclic forms, their interconversion was studied by ^{1}H NMR. Solutions (25 mM) of the dipeptides **14**–**16** were prepared in phosphate buffer in D_2O, pD 7.8.[31] In each case a smooth conversion from the protonated open form to the cyclic form was observed (Figure 6). After 24 h equilibrium had been reached, with none of the open form detectable by NMR. Cyclization of AlaBP (**14**) was rapid, $t_{1/2}$ = 44 min, whereas cyclization of ProBP (**15**) and ValBP (**16**) was significantly slower, $t_{1/2}$ = 160 and 190 min, respectively. These rates, measured in D_2O, are somewhat slower than previously reported rates measured in H_2O.[1,16,18] This is due to a solvent deuterium isotope effect, which leads to differences in pK_a in D_2O compared to H_2O, and is discussed in more detail below. The behavior of **16** was studied in more detail. At pD 4 or below no change was seen, but above pD 4, the cyclic form **19** started to appear. At pD 5.0–5.5 an equilibrium mixture was obtained, in which both species were present in approximately equal amounts. Similarly, **14** was unchanged at pD 3 over time, but at higher pD, **17** appears, reaching 50% at pD 4.0–4.5. **15** cyclizes to the extent of 15% even at pD 3, despite the higher pK_a of proline, and is 50% cyclized at pD 3.5–4.0.[32]

Analogous experiments were carried out starting from the cyclic compounds. As expected, no change was seen at pD 7.8, except for **19**, which appears to contain a small amount of open form in the solid state; this converts to the cyclic form in solution. On dissolving **17** or **19** in D_2O at pD 3.0, disappearance of the cyclic form was almost immediate (Figure 7). In addition to the open trans form, a new species was observed, which is thought to be the protonated, open cis form (cAH in Scheme 3). This is assumed to be formed rapidly as the B–N bond is broken by protonation, and then isomerizes to trans over time. In the case of **18**, ring opening was markedly slower, with the cyclic form still present after 2 h.

From these observations we propose the model outlined in Scheme 3. The protonated dipeptides exist predominantly in the trans form (AH). The steps required to form the cyclic species (B) are (1) amide bond isomerization, (2) deprotonation (which could occur in either order), and (3) B–N bond formation. The overall process is thus complex, involving three reversible steps. The data do not allow us to determine rate constants for each step. Isomerization of AH to give the cis form, cAH, is expected to be relatively slow, whereas the prototropic interconversion between cAH and cA should be very fast, and these species must be in equilibrium. A detailed mechanistic analysis is outside the scope of the present work, and we are not able to determine, from the kinetics, whether the isomerization or the B–N bond forming step is rate limiting. For our purposes in measuring the disappearance of the active inhibitor AH, we were interested in the macroscopic rate of cyclization, since the only species observed in this experiment are AH and the cyclic form B. The overall reaction can be written as AH $\rightleftharpoons$ B + H$^+$. Analysis of this process leads to an integrated rate equation[33] for the formation of B that includes both a first-order term and a zero-order term (eq 1). [A_T] is

$$[B]/[A_T] = k_1/(k_1 + k_{-1}[H^+]) - \{k_1/(k_1 + k_{-1}[H^+])\} \exp[-(k_1 + k_{-1}[H^+])t] \quad (1)$$

the total concentration of dipeptide, and [H$^+$] is constant since the solution is buffered. Under the conditions used for the cyclization studies, at pD 7.8, the open form is undetectable by NMR at equilibrium, implying that the back-reaction is much slower than the forward reaction, i.e., $k_1 \gg k_{-1}[H^+]$. With this approximation, the equation simplifies to a first-order process (eq 2). Since [A_T] = [AH] + [B], the fraction in the open form

$$1 - [B]/[A_T] = \exp[-(k_1 + k_{-1}[H^+])t] \quad (2)$$

([AH]/[A_T]) was fit to eq 2 by nonlinear regression, from which observed rate constants $k_{cyc} = (k_1 + k_{-1}[H^+])$ were derived (Table 1), which are measurements of the overall rate of the cyclization reaction.

(31) pD values were determined as the pH meter reading + 0.4: Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188–191.

(32) Further NMR work has led to a measurement of the equilibrium constants. Values for k_{off} for **14**–**16** have also been determined: Pargellis, C. A.; Pitner, T. P.; Pav, S.; Snow, R. J.; Graham, E. Manuscript in preparation.

(33) Equation 1 was obtained by adapting the equation for a reversible mixed first- and second-order process: Schmid, R.; Sapunov, V. N. *Non-Formal Kinetics*; Verlag Chemie: Weinheim, 1982; p 20.

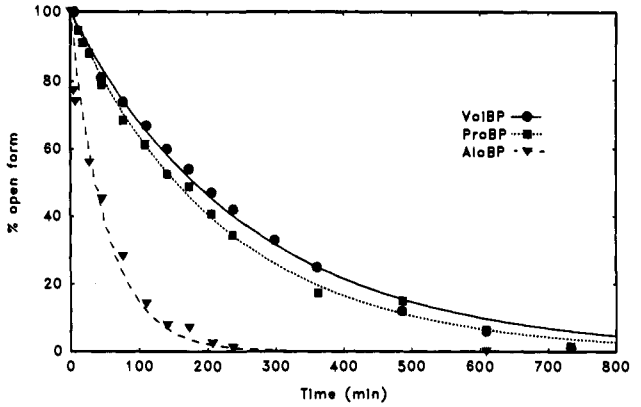


Figure 6. ^{1}H NMR measurement of the cyclization of **14**−**16**, in 0.5 M phosphate in D_2O at pD 7.8, 25 °C. Curves indicate the fit of data to eq 2 by nonlinear regression.

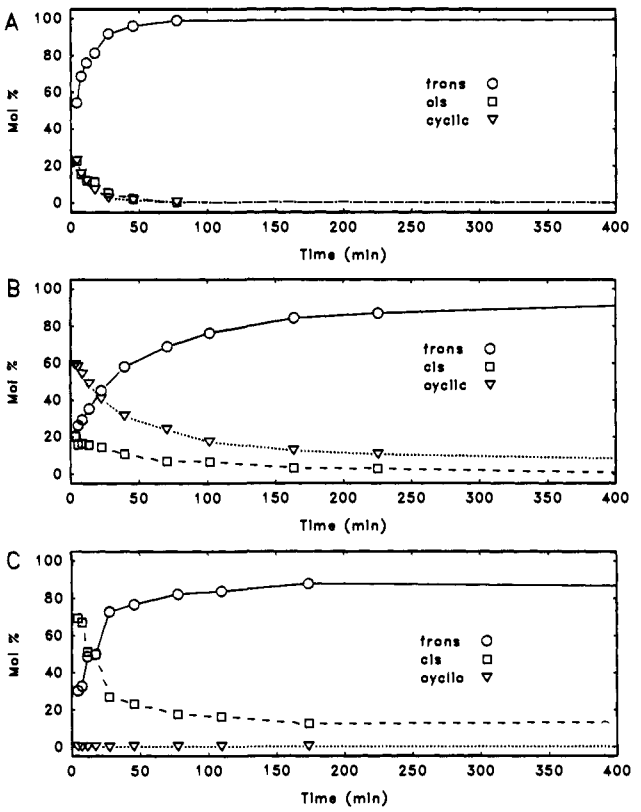
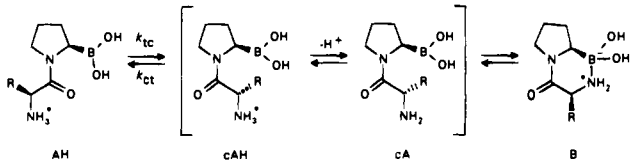


Figure 7. NMR studies of the ring opening of cyclic boronic acids in 0.5 M phosphate in D_2O at pD 3.5, showing the cyclic form, the open trans form, and a third species believed to be the open cis form: (A) AlaboroPro **17**, (B) ProboroPro **18**, (C) ValboroPro **19**.

Scheme 3. Model of the Steps Involved in the Cyclization Process



Enzyme Inhibition Studies. Since our goal is to determine the usefulness of these compounds as immunosuppressants, we chose to use an end-point enzyme assay, which measures the amount of substrate processed in a fixed time.[23] The proline boronic acids are slow tight-binding inhibitors,[34] so the inhibition is time dependent, and because of the cyclization, the concentration of inhibitor changes over time. The IC_{50} values determined

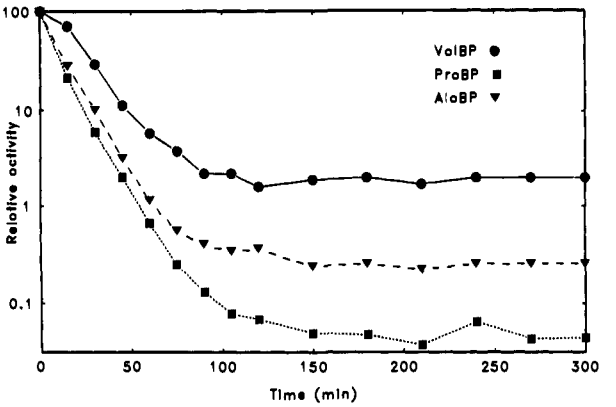


Figure 8. Change in DPP IV inhibitory activity over time for boronic acids in Tris buffer at pH 7.8, normalized to inhibition at $t = 0$.

thus represent a combination of the binding affinity in the presence of substrate, the enzyme concentration, and the rate of cyclization for each compound, and hence underestimate the K_i. In the absence of a complex rate equation describing all of these concurrent processes, the IC_{50} value gives the simplest indication of the ability of the compound to inhibit DPP IV *in vivo*. Potent inhibition was observed in this assay, starting with either the free boronic acids or the pinanediol esters, with only small differences between the analogs (Table 1). Experiments were carried out to correlate the interconversion of open and cyclic forms with the inhibition of DPP IV. Solutions of the inhibitors were prepared in Tris buffer, pH 7.8, at room temperature. Aliquots were removed at intervals, and the IC_{50} was determined and plotted as a fraction of the value at $t = 0$ (Figure 8). As expected, all compounds rapidly lost activity. At early time points the decrease in activity was approximately first order, from which the observed rate of inactivation, k_{inact}, was derived by fitting the data to an exponential decay by nonlinear regression. The value obtained for k_{inact} is a combination of both the rate of cyclization and the enzyme/inhibitor association rate constant, and represents the effective lifetime of the active inhibitor in solution. In order to determine whether the decrease in activity is due to cyclization, the values of k_{inact} were compared with the values of k_{cyc} determined by NMR. The rates k_{inact}, measured in H_2O, are all faster than the corresponding figures for k_{cyc}, measured in D_2O, by a factor of 5−20. Most of this difference is presumably due to the solvent deuterium isotope effect, since direct measurements of cyclization in H_2O by NMR[1a] or HPLC[18,35] give rates which are 5−10 times faster than those in D_2O. One factor is the difference in pK_a in D_2O compared to H_2O. The experiments were carried out at pD 7.8, i.e., pH meter reading 7.4; however, it is known for a wide range of compounds that the pK_a increases by about the same amount as the correction to the meter reading.[36] This would lead to about a 3-fold decrease in the fraction of the unprotonated species. Furthermore, it is apparent from Scheme 3 that deprotonation of cAH is necessary for cyclization, so a deuterium isotope effect would be expected.[36] The two effects are probably sufficient to account for the observed difference in k_{cyc} and k_{inact}, particularly since k_{inact} is measured indirectly. In both the NMR and the enzyme experiment, the slowest rate is observed for ValBP (**16**); however, there is a difference in the rank order of AlaBP (**14**) and ProBP (**15**). ProBP cyclizes at approximately the same rate as ValBP (**16**) and more slowly

(34) The values of k_{on}, k_{off}, and K_i for **15** have been determined as 5.02 × 10^6 M^{-1} s^{-1}, 77.8 × 10^{-6} s^{-1}, and 15.5 pM, respectively.[18]

(35) Betageri, R. Unpublished observation on **16**.

(36) Schowen, K. B. J. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; pp 225−283.

than AlaBP (**14**) by NMR, but in the enzyme assay ProBP loses activity slightly more rapidly than AlaBP, and both lose activity significantly faster than ValBP. The reason for this apparent discrepancy is thought to lie in differences in the association and dissociation rate constants for the three compounds. It is also possible that the cyclic compounds may revert to the open form, to varying extents, during the 1 h time period used for the enzyme assay. We believe that the rates k_{inact} and k_{cyc} are sufficiently similar, taking into account the isotope effect, to conclude that cyclization is the reason for the loss of inhibitory activity.

Another feature of the inactivation of the boronic acid inhibitors is apparent from Figure 8, namely, that although activity decreased, equilibrium was reached for each compound, with significant residual activity in the micromolar range (Table 1). This is believed to be due to small amounts of the open form present at equilibrium, which are not observable by NMR. There is a significant difference in the position of equilibrium for the three compounds, as judged by comparing the inhibitory potency of the equilibrium mixture with the open form, which gives an estimate of the equilibrium constant. The fact that equilibrium is reached indicates that the inactivation is reversible. The extent of cyclization is greatest for ProBP, and least for ValBP, which is consistent with the NMR observation that ProBP cyclizes at lower pD. This suggests that ring opening of the cyclic form is more facile for ValBP than ProBP.

The reversal of cyclization at low pH was studied by measuring the return of inhibitory activity. Cyclic compounds equilibrated at pH 7.8 were diluted into pH 3.5 acetate buffer, and aliquots were removed at intervals for assay as before. AlaBP and ValBP regained the full activity of the open form within 30–45 min, but consistent with the NMR data, ProBP was slower, reaching a maximum of 80% of the full activity after 1 h, with no further change.[37] From NMR, ProBP is partly cyclized at pH 3.5. As well as the effect of lowering the pH, we have previously demonstrated[1a] that DPP IV itself is able to trap the open form of ValBP, even at neutral pH, and drive the equilibrium in this direction.

Discussion

It is clear that the interconversion between active and inactive forms will play a significant role in the *in vivo* activity of these DPP IV inhibitors. The key step involves cis–trans isomerization of a proline-containing peptide. There has been considerable recent interest in this process, following the discovery of the peptidyl proline isomerase (PPIase) activity of cyclophilin[38] and FK506 binding protein,[39] known as immunophilins,[40] although these are probably not linked to the immunosuppressive properties of DPP IV inhibitors. The proline boronic acid dipeptides are an interesting example of the cis–trans isomerization, since both the trans (open) peptide and the cis (cyclic) form can be obtained, by varying the pH, and in principle the interconversion can be studied in both directions.

In the model proposed in Scheme 3, the rate of cyclization depends on the rate of trans-to-cis isomerization of the amide (k_{tc}), the equilibrium between the protonated and unprotonated forms, and the rate of formation of the B–N bond from the cis unprotonated amine. We suspected that the amide bond rotation is the rate-determining step, with relatively fast B–N bond formation. To see if this is likely, the observed rates of cyclization, k_{cyc} (Table 1), were compared to literature figures for k_{tc}. It should be noted that with a few exceptions,[41] most studies of proline isomerization have measured the cis-to-trans rate, k_{ct}, and have derived k_{tc} from the equilibrium constant. Published values for k_{tc} are in the range 1.2×10^{-3} to 7.4×10^{-3} s^{-1} [41–43] for AlaProOH and 7.7×10^{-4} s^{-1} for ValProOH.[42] Experiments by Harrison and Stein gave figures of 9.7×10^{-4} and 2.7×10^{-4} s^{-1} for an AlaPro and a ValPro peptide, respectively.[44] The 4-fold difference in rate is similar to the difference we observe between AlaBP and ValBP, and we attribute this to steric differences in the side chain. The values of k_{cyc} are somewhat lower than published figures for k_{tc}, but this is expected because k_{cyc} also includes the equilibrium between protonated and unprotonated forms. The data are consistent with amide bond isomerization being the rate-limiting step. There have been several studies of ProPro-containing peptides,[45] which have shown that the cis–trans equilibrium varies considerably with solvent and protonation state, but we are not aware of any direct measurements of isomerization rates for this peptide bond. Given the well-known propensity of ProPro peptides to form diketopiperazines,[46] the rate of cyclization of **15** seems slow: this may be due to the higher pK_a of proline, and thus a smaller fraction of the free amine.

As well as the difference in rates of cyclization, there is clearly a difference in the equilibrium position, with ProBP favoring the cyclic form more than the others. One explanation for this may be the steric interactions between the side chains favor the cyclic form more in the case of ProBP. The most obvious difference between the compounds is the secondary amine of proline: at first sight this might be expected to disfavor cyclization, since the higher pK_a of Pro (10.6) compared to Ala or Val (9.7)[47] would favor protonation and hence the open form, but the secondary amine of Pro is also a stronger Lewis base. It appears that the increase in Lewis basicity on going from a primary to secondary amine is greater than the increase in Bronsted basicity, leading to a stronger B–N bond. This is supported by measurements of ionization potential (IP),[48] which indicate that secondary amines have lower IP values than primary amines relative to their proton affinities. Increased B–N bond strength for secondary over primary amines complexed to trimethylborane has been determined experimentally[49] and by calculation.[50]

(37) This behavior of ProBP has also been noted by Gutheil and Bachovchin.[18]

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(47) pK_a values for the free amino acids, taken from Perrin, D. D. *Dissociation Constants of Organic Bases in Aqueous Solution*; Butterworths: London, 1965.

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(49) Brown, H. C.; Taylor, M. D. *J. Am. Chem. Soc.* **1947**, *69*, 1332–1336.

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Conclusions

We have shown that boroPro dipeptides interconvert with a cyclic form analogous to a diketopiperazine, and that this is a reversible, pH-dependent conformational change. Both species have been isolated in a stable, analytically pure form. Cyclization is the reason for the decrease in DPP IV inhibitory activity over time. The rate and extent of cyclization depend on the terminal amino acid, and both factors will influence the *in vivo* immunosuppressive activity. ValBP has been shown to cyclize more slowly and to a lesser extent than the previously reported inhibitors AlaBP and ProBP, which is expected to lead to increased potency. ValBP (**16**) has been shown to have more potent immunosuppressive activity *in vivo* than AlaBP (**14**) or ProBP (**15**), details of which will be published in due course.[51]

Experimental Section

General Directions. Melting points were determined in open capillaries on a Büchi 512 apparatus, and are uncorrected. ^{1}H NMR spectra were obtained on a Bruker AC250, AF270, or AM500 spectrometer. 2D NOESY spectra were obtained on the AM500 spectrometer using a mixing time of 500 ms. ^{13}C NMR spectra were run on the same instruments at 62.9, 67.9, and 125.8 MHz, respectively; those in D_2O are referenced to an external dioxane standard at δ 67.4. ^{11}B NMR spectra were obtained on the AF270 instrument at 86.7 MHz, using difference spectra to subtract out background signals from the probe; δ values are quoted in parts per million downfield from an extemal $BF_3{\cdot}Et_2O$ standard. All NMR spectra are of solutions in $CDCl_3$ unless otherwise stated. Mass spectra were recorded on a Finnegan 4000, operating in DCI mode, with methane as the reagent gas. Free boronic acids were derivatized by adding ethylene glycol immediately before running the spectrum. High-resolution mass spectra were recorded in FAB mode on a Kratos MS80RFAQ equipped with a cesium ion gun for LSIMS analysis. Glycerol was employed as both the matrix and intemal mass calibrant. A resolution of 9000 was obtained, and exact mass determinations were made with the peak matching system; a 1000 ppm sweep of 3 s duration was used. Free boronic acids were analyzed as the glycerol ester, which forms spontaneously in the matrix. IR spectra were obtained on a Perkin-Elmer PE983 instument, and are of KBr pellets unless stated otherwise. Optical rotations were measured using a Perkin-Elmer 241 polarimeter and a 1 dm path length cell. Capillary gas chromatograms were run on a Hewlett-Packard 5890 with a flame ionization detector. Elemental analyses were performed by Midwest Microlab, Indianapolis, IN, except for boron analyses, which were performed by Galbraith Laboratories, Knoxville, TN.

Materials and Methods. Protected amino acids were purchased from Advanced Chemtech. All other reagents were purchased from Aldrich Chemical Co., and used as received. Organic solutions which had been in contact with water were dried over $MgSO_4$ prior to evaporation under vacuum in a rotary evaporator.

Human DPP IV was purified in the full length form from the YT lymphoid cell line by detergent solubilization and three chromatographic steps as previously described.[23,52]

Kinetic data were analyzed using Sigmaplot for Windows, version 1.02a (Jandel Corp.), which uses a Marquardt-Levenburg algorithm for curve fitting.

Peptide Coupling. General Procedure. To a stirred, ice-cooled solution of BOC-protected amino acid (17.5 mmol) in CH_2Cl_2 (100 mL) was added hydroxybenzotriazole (2.37 g, 17.5 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (4.32 g, 22.8 mmol). After 30 min, solid (1*S*,2*S*,3*R*,5*S*)-pinanediol pyrrolidine-2(*R*)-boronate hydrochloride (5.00 g, 17.5 mmol) was added, followed by *N*-methylmorpholine (3.9 mL, 35.1 mmol), and the mixture allowed to warm slowly to room temperature over 20 h. The solution was washed in turn with water, 1 M $KHSO_4$, and Na_2CO_3 solution. The organic solution was filtered through a plug of silica gel, continuing elution with EtOAc. Evaporation of the filtrate yielded the protected dipeptides, pure by NMR, in 95−98% yield.

[*N*-[(1,1-Dimethylethoxy)carbonyl]-(*S*)-alanyl]pyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester (5): oil; IR (film) 3350, 1715, 1635, 1460, 1390, 1370 cm^{-1}; ^{1}H NMR δ 0.83 (s, 3 H), 1.27 (s, 3 H), 1.29 (d, J = 7 Hz, 3 H), 1.30 (s, 3 H), 1.40−1.43 (m, 1 H), 1.42 (s, 9 H), 1.60−2.15 (m, 9 H), 2.25−2.40 (m, 1 H), 3.18 (dd, J = 6.9 and 9.8 Hz, 1 H), 3.36−3.49 (m, 1 H), 3.62−3.75 (m, 1 H), 4.28 (dd, J = 2.2 and 9.1 Hz, 1 H), 4.44 (dq, J = 7 Hz, 1 H), 5.48 (d, J = 7 Hz, 1 H); ^{13}C NMR δ 18.2, 23.9, 26.1, 27.1, 28.3, 28.4, 35.4, 38.0, 39.5, 44.4, 46.3, 47.1, 51.1, 77.7, 79.2, 84.2, 85.7, 155.1, 170.8; MS (CI) *m/z* 421 (MH$^+$); HRMS *m/z* calcd for $C_{22}H_{38}BN_2O_5$ 421.2874, found 421.2853.

[*N*-[(1,1-Dimethylethoxy)carbonyl]-(*S*)-prolyl]pyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester (6): oil; IR (film) 1700, 1645, 1390, 1360 cm^{-1}; ^{1}H NMR δ 0.83 (s, 3 H), 1.27 (s, 3 H), 1.38 and 1.39 (2 s, 9 H, rotamers), 1.38−1.43 (m, 1 H), 1.43 (s, 3 H), 1.70−2.17 (m, 12 H), 2.26−2.38 (m, 1 H), 3.20 (ddd, J = 6.9, 9.8 and 18.0 Hz, 1 H), 3.34−3.47 (m, 2 H), 3.48−3.66 (m, 1.6 H), 3.79−3.84 (m, 0.4 H), 4.25 (dt, J = 2.6 and 9.0 Hz, 1 H), 4.34 (dd, J = 4.4 and 7.4 Hz, 0.6 H), 4.49 (dd, J = 3.0 and 7.9 Hz, 0.4 H); MS (CI) *m/z* 447 (MH$^+$, 100).

[*N*-[(1,1-Dimethylethoxy)carbonyl]-(*S*)-valyl]pyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester (7): mp 128−130 °C; IR (film) 3350, 1710, 1640, 1460, 1370 cm^{-1}; ^{1}H NMR δ 0.83 (s, 3 H), 0.91 (d, J = 6.7 Hz, 3 H), 0.97 (d, J = 6.7 Hz, 3 H), 1.27 (s, 3 H), 1.35−1.45 (m, 1 H), 1.39 (s, 3 H), 1.41 (s, 9 H), 1.72−2.14 (m, 9 H), 2.26−2.36 (m, 1 H), 3.15 (dd, J = 6.7 and 10.1 Hz, 1 H), 3.43−3.51 (m, 1 H), 3.70−3.81 (m, 1 H), 4.19−4.28 (m, 2 H), 5.29 (d, J = 9.2 Hz, 1 H); ^{13}C NMR δ 17.3, 19.2, 24.0, 26.3, 27.1, 27.2, 27.4, 28.4, 28.6, 31.4, 33.9, 35.5, 38.2, 39.6, 46.7, 51.2, 56.6, 77.8, 79.2, 85.8, 155.9, 170.2; ^{11}B NMR δ 31.0; MS (CI) *m/z* 449 (MH$^+$, 100), 393 (50). Anal. Calcd for $C_{24}H_{41}BN_2O_5$: C, 64.28; H, 9.22; N, 6.25. Found: C, 64.58; H, 9.33; N, 6.52.

Amine Deprotection. The protected dipeptide (3 mmol) was treated with a saturated solution of HCl in Et_2O (50 mL), with stirring at 0 °C. The solution was allowed to warm to room temperature over 3 h. The solvent was evaporated to yield the hydrochloride. Usually this was sufficiently pure for the next step, but could be further purified by recystallization (**9**), or by crystallization of another salt form obtained via the cyclic free base. On a larger scale it was preferable to pass dry HCl gas into a stirred Et_2O solution of the dipeptide, with ice cooling.

(*S*)-Alanylpyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester Methanesulfonate (8): mp 205−215 °C dec; $[\alpha]^{25}_D$ −46.4° (c 0.52, CH_2Cl_2); IR 3417, 3300−2800, 1638, 1391, 1375, 1250−1140, 1038 cm^{-1}; ^{1}H NMR δ 0.82 (s, 3 H), 1.20 (d, J = 11.0 Hz, 1 H), 1.27 (s, 3 H), 1.39 (s, 3 H), 1.51 (d, J = 6.9 Hz, 3 H), 1.75−1.91 (m, 3 H), 1.95−2.18 (m, 4 H), 2.21−2.38 (m, 2 H), 2.75 (s, 3 H), 3.24−3.38 (m, 2 H), 3.74−3.85 (m, 1 H), 4.26 (dd, J = 1.8 and 8.7 Hz, 1 H), 4.25−4.40 (m, 1 H), 7.81 (br, 3 H); ^{13}C NMR δ 16.1, 24.0, 26.1, 27.01, 27.07, 27.13, 28.5, 35.4, 38.1, 39.2, 39.4, 44.4, 46.4, 47.8, 51.1, 77.8, 86.0, 167.6; ^{11}B NMR δ 32.3; MS (CI) *m/z* 321 (MH$^+$, 100), 169 (40); HRMS *m/z* calcd for $C_{17}H_{30}BN_2O_3$ 321.2350, found 321.2337. Anal. Calcd for $C_{18}H_{33}BN_2O_6S$: C, 51.93; H, 7.99; N, 6.73; S, 7.70. Found: C, 51.63; H, 8.03; N, 6.65; S, 7.43.

(*S*)-Prolylpyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester Hydrochloride (9): mp 190 °C dec; $[\alpha]^{25}_D$ −114.2° (*c* 0.52, CH_2Cl_2); IR 2967, 2909, 2877, 2489, 1630, 1547, 1470, 1387, 1367 cm^{-1}; ^{1}H NMR δ 0.83 (s, 3 H), 1.19 (d, J = 10.7 Hz, 1 H), 1.28, (s, 3 H), 1.37 (s, 3 H), 1.78−2.16 (m, 11 H), 2.28−2.54 (m, 2 H), 3.32−3.42 (m, 3 H), 3.51−3.70 (m, 2 H), 4.27 (dd, J = 2.0 and 8.8 Hz, 1 H), 4.61 (br, 1 H), 7.20 (br, 2 H); ^{13}C NMR δ 23.8, 24.2, 25.9, 26.75, 26.79, 26.81, 28.4, 28.7, 35.1, 38.0, 39.2, 44.6, 46.4, 46.5, 50.9, 58.4, 77.8, 85.9, 165.7; ^{11}B NMR δ 33.3; MS (CI) *m/z* 347 (MH$^+$, 100). Anal. Calcd for $C_{19}H_{32}BClN_2O_3$: C, 59.63; H, 8.43; N, 7.32; B, 2.82; Cl, 9.26. Found: C, 59.56; H, 8.21; N, 7.25; B, 2.76; Cl, 9.40.

(*S*)-Valylpyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester Hydrogen Maleate (10): mp 145−146 °C; $[\alpha]^{25}_D$ −48.3° (*c* 0.57, CH_2Cl_2); IR 1629, 1583, 1483 (B−O stretch) cm^{-1}; ^{1}H NMR δ 0.84 (s, 3 H), 1.08 (d, J = 6.9 Hz, 3 H), 1.13 (d, J = 6.9 Hz, 3 H), 1.26−1.31 (m, 2 H), 1.29 (s, 3 H), 1.38 (s, 3 H), 1.72−2.15 (m, 7 H), 2.24−

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2.38 (m, 2 H), 3.28 (dd, J = 6.9 and 9.4 Hz, 1 H), 3.38−3.47 (m, 1 H), 3.73−3.78 (m, 1 H), 4.14 (d, J = 5.1 Hz, 1 H), 4.26 (d, J = 7.1 Hz, 1 H), 6.25 (s, 2 H), 7.5−9.0 (v. br, 4 H); ^{13}C NMR δ 17.0, 18.4, 24.0, 26.3, 27.0, 27.1, 28.7, 30.0, 35.4, 38.2, 39.5, 47.3, 51.2, 56.6, 78.1, 86.2, 135.6, 166.3, 169.5; ^{11}B NMR δ 29.6; MS (CI) m/z 349 (MH^+, 100), 197 (18). Anal. Calcd for $C_{23}H_{37}BN_2O_7$: C, 59.49; H, 8.03; N, 6.03. Found: C, 59.50; H, 8.13; N, 6.03.

Cyclic Pinanediol Esters. The amine salt was partitioned between CH_2Cl_2 and aqueous Na_2CO_3, the organic layer dried, and the solvent evaporated. The product was purified by flash chromatography in CH_2Cl_2/MeOH (95:5); if recrystallized salt was used, further purification was not necessary.

cyclo-(*S*)-Alanylpyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester (11): mp 241−246 °C dec; $[\alpha]^{25}_D$ −70.0° (c 0.60, CH_2Cl_2); IR 3263, 3196, 1643, 1624 cm^{-1}; ^{1}H NMR δ 0.86 (s, 3 H), 1.27 (s, 3 H), 1.32 (s, 3 H), 1.33 (d, J = 9.8 Hz, 1 H), 1.50 (d, J = 6.7 Hz, 3 H), 1.66−1.76 (m, 2 H), 1.83−2.15 (m, 6 H), 2.27−2.36 (m, 1 H), 2.80 (dd, J = 6.0 and 10.4 Hz, 1 H), 3.27−3.38 (m, 1 H), 3.59 (dd, J = 8.0 and 12.4 Hz, 1 H), 3.76 (q, J = 6.7 Hz, 1 H), 3.92 (br, 2 H), 4.10 (dd, J = 2.6 and 8.7 Hz, 1 H); ^{13}C NMR (DMSO) δ 16.3, 24.2, 24.3, 25.9, 27.4, 29.5, 30.1, 37.1, 37.8, 39.8, 45.7, 49.6, 51.1, 52.8, 74.9, 80.9, 166.4; ^{11}B NMR δ 9.2; MS (CI) m/z 321 (MH^+, 100), 169 (61); HRMS m/z calcd for $C_{17}H_{30}BN_2O_3$ 321.2350, found 321.2374. Anal. Calcd for $C_{17}H_{29}BN_2O_3$·0.25H_2O: C, 62.88; H, 9.16; N, 8.63. Found: C, 62.91; H, 9.02; N, 8.55.

cyclo-(*S*)-Prolylpyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester (12): mp softens 108−120, melts 190 °C; $[\alpha]^{25}_D$ −28.0° (c 0.55, CH_2Cl_2); IR 3427, 3142, 1638, 1473, 1463 cm^{-1}; ^{1}H NMR δ 0.85 (s, 3 H), 1.27 (s, 3 H), 1.33 (s, 3 H), 1.43 (d, J = 10.1 Hz, 1 H), 1.66−2.10 (m, 11 H), 2.28−2.42 (m, 1 H), 2.84−2.91 (m, 2 H), 2.91−3.12 (m, 1 H), 3.13−3.35 (m, 2 H), 3.66 (dd, J = 9.8 and 10.3 Hz, 1 H), 3.39 (br, 1 H, N*H*), 4.00 (br.s, 1 H), 4.11 (dd, J = 2.0, 8.6 Hz, 1 H); ^{13}C NMR δ 22.9, 24.3, 25.2, 25.7, 25.9, 27.3, 30.3, 30.6, 37.1, 38.3, 40.3, 46.4, 53.1, 61.1, 76.9, 82.2, 165.5; ^{11}B NMR δ 8.9; MS (CI) m/z 347 (MH^+, 100), 167 (20); HRMS m/z calcd for $C_{19}H_{32}BN_2O_3$ 347.2506, found 347.2519.

cyclo-(*S*)-Valylpyrrolidine-2(*R*)-boronate (1*S*,2*S*,3*R*,5*S*)-Pinanediol Ester (13): mp 72−80 °C; $[\alpha]^{25}_D$ −62.6° (c 0.58, CH_2Cl_2); IR 3429, 3330, 3206, 3086, 1641, 1457 cm^{-1}; ^{1}H NMR δ 0.86 (s, 3 H), 1.00 (d, J = 6.8 Hz, 3 H), 1.05 (d, J = 6.8 Hz, 3 H), 1.27 (s, 3 H), 1.34 (s, 3 H), 1.39 (d, J = 7.7 Hz, 1 H), 1.65−1.80 (m, 2 H), 1.81−2.18 (m, 6 H), 2.29−2.36 (m, 1 H), 2.44−2.57 (m, 1 H), 2.80 (dd, J = 6.1 and 10.5 Hz, 1 H), 3.23−3.33 (m, 1 H), 3.2−3.7 (br, 2 H, NH_2), 3.42 (d, J = 4.3 Hz, 1 H), 3.60 (t, J = 11.9 Hz, 1 H), 4.15 (dd, J = 2.5 and 8.7 Hz, 1 H); ^{13}C NMR δ 17.1, 20.0, 24.3, 25.0, 26.7, 27.1, 27.3, 29.9, 30.6, 37.4, 38.2, 40.3, 46.0, 52.5, 58.5, 76.5, 82.0, 165.0; ^{11}B NMR δ 8.8; MS (CI) m/z 349 (MH^+, 100), 197 (54); HRMS m/z calcd for $C_{19}H_{34}BN_2O_3$ 349.2663, found 349.2668. Anal. Calcd for $C_{19}H_{33}BN_2O_3$·0.5H_2O: C, 63.87; H, 9.59; N, 7.84. Found: C, 63.99; H, 9.57; N, 7.82.

Boronate Deprotection. Cyclic Free Boronic Acid. A solution of the amine salt (10 mmol) in H_2O (100 mL) was adjusted to pH 2, if necessary, by addition of dilute HCl. Hexane (100 mL) and phenylboric acid (1.28 g, 10.5 mmol) were added and the mixture stirred vigorously. After 30 min the hexane was decanted and replaced with fresh hexane. This was repeated three more times, and the mixture stirred overnight, by which time a clear two-phase solution was obtained. The hexane layer was separated; evaporation of the combined organic layers yielded a 95−100% recovery of pinanediol phenylboronate. The aqueous layer was applied to a Dowex 50X2-100 ion exchange column in the H^+ form, and eluted with water until the eluate was neutral. Elution was continued with aqueous ammonium hydroxide (1:50 dilution), and appropriate fractions lyophillized to yield the cyclic free boronic acid in 90−95% yield.

cyclo-(*S*)-Alanylpyrrolidine-2(*R*)-boronic Acid (17): mp 80−92 °C dec; $[\alpha]^{25}_D$ −59.3° (c 0.58, H_2O); IR 3437, 3211, 3099, 1622 cm^{-1}; ^{1}H NMR (D_2O) δ 1.44 (d, J = 7.2 Hz, 3 H), 1.54−1.78 (m, 2 H), 1.91−2.00 (m, 2 H), 2.61 (dd, J = 6.0 and 11.9 Hz, 1 H), 3.30 (dt, J = 7.8 and 9.1 Hz, 1 H), 3.50 (t, J = 9.9 Hz, 1 H), 3.81 (q, J = 7.2 Hz, 1 H); ^{13}C NMR (D_2O) δ 16.6, 24.5, 27.9, 47.3, 49.9, 53.2, 168.9; ^{11}B NMR (D_2O) δ 2.8; MS (CI) m/z 169 (MH^+-H_2O, 100); HRMS (glycerol adduct) m/z calcd for $C_{10}H_{20}BN_2O_4$ 243.1516, found 243.1513. Anal. Calcd for $C_7H_{15}BN_2O_3$·0.3H_2O: C, 43.92; H, 8.21; N, 14.63. Found: C, 44.11; H, 8.00; N, 14.46.

cyclo-(*S*)-Prolylpyrrolidine-2(*R*)-boronic Acid (18): mp 215−219 °C; $[\alpha]^{25}_D$ −84.2° (c 0.5, H_2O); IR 3429, 3387, 3123, 1612, 1486, 1393, 1325 cm^{-1}; ^{1}H NMR (D_2O) δ 1.56−2.03 (m, 7 H), 2.24−2.33 (m, 1 H), 2.66 (dd, J = 5.9 and 12.0 Hz, 1 H), 3.10−3.33 (m, 3 H), 3.49 (dd, J = 8.8 and 11.9 Hz, 1 H), 3.99 (t, J = 8.8 Hz, 1 H); ^{13}C NMR (D_2O) δ 25.6, 26.8, 30.2, 31.2, 46.0, 47.1, 49.7, 64.1, 170.0; ^{11}B NMR (D_2O) δ 2.0; MS (CI) m/z 239 (MH^+, 100); HRMS (glycerol adduct) m/z calcd for $C_{12}H_{22}BN_2O_4$ 269.1673, found 269.1657.

cyclo-(*S*)-Valylpyrrolidine-2(*R*)-boronic Acid (19): mp 120−130 °C; $[\alpha]^{25}_D$ −81.0° (c 0.52, H_2O); IR 3400−3314, 3221−3108, 2961−2872, 1637, 1452−1369 cm^{-1}; ^{1}H NMR (D_2O) δ 0.97 (d, J = 7.0 Hz, 3 H), 1.06 (d, J = 7.0 Hz, 3 H), 1.59−1.80 (m, 2 H), 1.95−2.03 (m, 2 H), 2.41−2.51 (m, 1 H), 2.62−2.69 (m, 1 H), 3.23−3.32 (m, 1 H), 3.51−3.58 (m with overlapping doublet, J = 4.2 Hz, 2 H); ^{13}C NMR (D_2O) δ 19.0, 21.7, 27.3, 30.7, 29.9, 49.6, 61.0, 170.3; ^{11}B (D_2O) NMR δ 2.7; MS (CI) m/z 375 (M_2H^+ − 3H_2O, 90), 197 (MH^+ − H_2O, 100). Anal. Calcd for $C_9H_{19}BN_2O_3$: C, 50.50; H, 8.95; N, 13.09; B, 5.05. Found: C, 50.50; H, 8.67; N, 13.34; B, 5.06.

Free Boronic Acid Dipeptides. Method A. To a stirred suspension of the cyclic boronic acid obtained above (25 mmol) in acetonitrile (190 mL) under nitrogen was added a solution of methanesulfonic acid (25 mmol) in acetonitrile (10 mL), dropwise over 5 min, and the mixture was stirred at room temperature for 2 h. The solid was collected by filtration, washed well with acetonitrile and Et_2O, and dried to afford the dipeptide salt in 80−90% yield.

Method B. To a solution of the cyclic boronic acid (2 mmol) in MeOH (10 mL) was added methanesulfonic acid (2 mmol) in MeOH (0.5 mL). After 30 min the solution was evaporated and the residue triturated with Et_2O. The supernatant was decanted and the solid dried to yield the boronic acid dipeptide.

(*S*)-Alanylpyrrolidine-2(*R*)-boronic Acid Methanesulfonate (14): (method B) mp 114−120 °C dec; $[\alpha]^{25}_D$ −47.5° (c 0.55, H_2O); IR 3400−2900 (br), 1642, 1513 1405, 1213, 1175, 1042 cm^{-1}; ^{1}H NMR (D_2O) δ 1.48 (d, J = 7.0 Hz, 3 H), 1.63−1.75 (m, 1 H), 1.84−2.01 (m, 1 H), 2.03−2.16 (m, 2 H), 2.78 (s, 3 H), 3.05 (ddd, J = 6.3, 8.0 and 9.7 Hz, 1 H), 3.69 (t, J = 8.0 Hz, 1 H), 4.31 (q, J = 7.0 Hz, 1 H); ^{13}C NMR (D_2O) δ 15.1, 26.97, 27.04, 38.7, 47.3, 48.1, 48.6, 168.2; ^{11}B NMR (D_2O) δ 31.4; MS (CI) (ethylene glycol adduct) m/z 213 (MH^+, 100), 142 (47); HRMS (glycerol adduct) m/z calcd for $C_{10}H_{20}BN_2O_4$ 243.1516, found 243.1527. Anal. Calcd for $C_8H_{19}BN_2O_6S$: C, 34.06; H, 6.79; N, 9.93; S, 11.37. Found: C, 33.97; H, 6.52; N, 9.77; S, 11.31.

(*S*)-Prolylpyrrolidine-2(*R*)-boronic Acid Methanesulfonate (15): (method A) mp 138−147 °C dec; $[\alpha]^{25}_D$ −103.1° (c 2.0, H_2O); IR 3407, 3159−3149, 2878, 1645, 1407, 1283, 1202, 1176, 1045 cm^{-1}; ^{1}H NMR (D_2O) δ 1.76−1.89 (m, 1 H), 2.02−2.25 (m, 6 H), 2.56−2.66 (m, 1 H), 2.89 (s, 3 H), 3.17 (dd, J = 6.8 and 10.8 Hz, 1 H), 3.47−3.58 (m, 3 H), 3.79 (t, J = 8.6 Hz, 1 H), 4.68 (t, J = 7.2 Hz, 1 H); ^{13}C NMR (D_2O) δ 24.1, 26.9, 27.0, 28.4, 38.7, 46.7, 47.4, 48.7, 59.2, 167.0; ^{11}B NMR (D_2O) δ 30.7; MS (CI) (ethylene glycol adduct) m/z 239 (MH^+, 50), 97 (100). Anal. Calcd for $C_{10}H_{21}BN_2O_6S$: C, 38.96; H, 6.81; N, 9.09; S, 10.40. Found: C, 38.88; H, 6.92; N, 9.13; S, 10.14.

(*S*)-Valylpyrrolidine-2(*R*)-boronic Acid Methanesulfonate (16): (method A) mp 181−182 °C; $[\alpha]^{25}_D$ −42.4° (c 1.0, H_2O, pH 2); IR 3387, 3000 (br), 2972, 2655, 1646, 1370 (B−O stretch), 1197 cm^{-1}; ^{1}H NMR (D_2O) δ 0.99 (d, J = 6.8 Hz, 3 H), 1.09 (d, J = 6.9Hz, 3 H), 1.69−1.75 (m, 1 H), 1.90−1.99 (m, 1 H), 2.10−2.14 (m, 2 H), 2.28−2.35 (m, 1 H), 2.80 (s, 3 H), 3.07 (dd, J = 7.0 and 11.2 Hz, 1 H), 3.46−3.51 (m, 1 H), 3.75 (t, J = 9.0 Hz, 1 H), 4.14 (d, J = 5.1 Hz, 1 H), the cis amide rotamer (ca. 3%) is also observed at 3.53−3.55 (m) and 3.83 (d, J = 6.2 Hz); ^{13}C NMR (D_2O) δ 16.2, 18.4, 26.9, 27.1, 29.0, 38.8, 47.9, 49.0, 57.2, 167.2, peaks due to the cis amide rotamer are observed at 16.8, 24.3, 29.9, 57.8, 167.5; ^{11}B NMR (D_2O) δ 31.5; MS (CI) (ethylene glycol adduct) m/z 241 (MH^+, 100). Anal. Calcd for $C_{10}H_{23}BN_2O_6S$: C, 38.72; H, 7.47; N, 9.03; B, 3.49; S, 10.34. Found: C, 38.49; H, 7.22; N, 8.90; B, 3.31; S, 10.34.

GC Assay for Pinanediol. A 2.6 mM solution of the hydrochloride of **10** in 0.1 M Tris·HCl buffer (25 mL), adjusted to pH 7.0 was prepared. At intervals 2 mL aliquots were removed and applied to a

Sep-Pak cartridge. The cartridge was washed with water (2 mL) and then with MeOH (9 mL). The eluate volume was adjusted to 10 mL with MeOH, and 1 μL samples quantitated by capillary GC, on a 25 m Ultra 1 column, calibrated to standard pinanediol solutions.

End-Point Enzyme Assay. The compounds were assayed using the procedure described previously,[23] based on the method of Smith and Van Frank.[53] Human DPP IV was purified[52] from YT cells.[54] Inhibitors were prepared as stock solutions in DMSO, and diluted with buffer immediately before assay. The substrate H-Ala-Pro-4-methoxy-2-naphthamide (AlaProMNA) and inhibitor were added simultaneously to assay buffer in a 96-well microtiter plate, and reaction was started by addition of enzyme, to give a total reaction volume of 0.11 mL containing 0.1 M Tris·HCl at pH 7.8, 455 μM substrate, 1% Triton X-100, 0.01% NaN_3, and 2.3% DMF, and incubated at 37 °C for 60 min. A solution of 4-(dimethylamino)cinnamaldehyde (3.3 mg/mL, 50 μL) was then added, and the optical density at 570 nm was measured.

Continuous Enzyme Assay. Enzyme activity was determined by monitoring the fluorescence change associated with the cleavage of AlaProNMA. Reactions were run at 23 °C in a final volume of 2.0 mL containing 0.1 M Tris at pH 8.0, 1.0% Triton X-100, 0.01% NaN_3, 0.5% DMF, and 1.0% DMSO. Purified human DPP IV was added to a final concentration of 7.0×10^{-10} M. Inhibitors were added to 1.10×10^{-7} M and (+)-pinanediol to 3.0 mM. The reaction was initiated by the addition of 1.00 mM AlaProNMA. Data acquisition was begun within 5 s after the addition of substrate. The time course of the reaction was monitored using an SLM Aminco Bowman series 2 Model SQ-340 fluorescence detector equipped with a high-speed two-place magnetic stirrer. The excitation maximum of the product, (4-methoxy-2-naphthyl)amine, was determined to be 342 nm with an emission maximum at 415 nm. The high voltage setting of the photomultiplier tube was set at 800 V. Data were collected for 4000 s at an acquisition interval of 10.0 s. Steady state velocities were determined from a linear regression to the final 300 data points. In all cases the linear correlation coefficient was greater than 0.98.

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Supplementary Material Available: Tables of atomic coordinates and thermal parameters for **9**·$MeSO_3H$, **10**·$C_4H_4O_4$, and **12**·$2CHCl_3$ (12 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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