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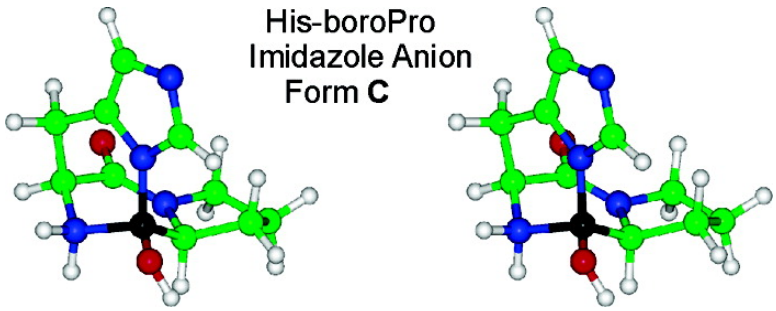
J. Am. Chem. Soc., **2005**, 127 (22), 8112-8119• DOI: 10.1021/ja050215e • Publication Date (Web): 13 May 2005



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Autochelation in Dipeptide Boronic Acids: pH-Dependent Structures and Equilibria of Asp-boroPro and His-boroPro by NMR Spectroscopy

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Abstract: Many dipeptide boronic acids of the type H_2N-**X**−**Y**-$B(OH)_2$ are potent protease inhibitors. Interest in these compounds as drugs for cancer, diabetes, and other diseases is growing. Because of the great mutual B−N affinity, cyclization through the N- and B-termini, forming six-membered rings, is a common occurrence at neutral pH and higher where the terminal amino group is unprotonated. Here we report the discovery that when **X**, the N-terminal amino acid, contains a side chain having a functional group with boron affinity and suitable geometry, additional cyclization in the form of bidentate intramolecular chelation or "autochelation" may occur, predominantly at mid pH. NMR studies of two compounds, L-Aspartyl-L-boroProline (Asp-boroPro) and L-Histidyl-L-boroProline (His-boroPro), are reported here from pH 0.5 to pH 12 by ^{1}H, ^{15}N, ^{13}C, and ^{11}B NMR. Both of these previously unreported autochelates contain two fused six-membered rings, *cis*-proline, chiral boron, and $-NH_2^+$ protons in slow exchange with water, even at 25 °C and pH as high as 4. Using microscopic acid−base equilibrium constants, we show that at high pH (>8 for Asp-boroPro and >10 for His-boroPro) hydroxide competes with the side chains for boron, reducing the chelates from bidentate to monodentate. At low pH (<0.5), proton competition for N-terminal nitrogens causes both compounds to become noncyclic. High chelate stability causes a reduction of the apparent acidic dissociation constant of the protonated N-terminal amino group greater than eight units. In the His-boroPro autochelate, imidazolate anion is produced at the extraordinarily low pH value of ∼9.

Introduction

FDA approval of the dipeptide boronic acid bortezomib[1] (Velcade) for treatment of multiple myeloma, and a number of other boronic acid-based drugs being in various stages of research and development,[2] have made these organoboron compounds increasingly significant.

At pH values above a certain threshold (generally ∼8), many dipeptide boronic acids have been shown to undergo reversible cyclization, forming six-membered rings analogous to diketopiperazines through B−N bond formation.[3] Rate and equilibrium constants of these pH-dependent cyclization reactions have been determined,[3] and structures of several cyclic products have been elucidated in solids by X-ray crystallography[3] and in solution by NMR.[4] ^{1}H NMR spectra of L-Histidyl-L-boroProline (His-boroPro) revealed the presence of multiple forms at high pH values.[5] The possibility that a B−N bond could be formed through a His ring nitrogen, rather than the terminal amino nitrogen atom, was suggested,[5] and a species with an eight-membered ring was proposed.

Here we prove by more extensive multinuclear NMR studies that His-boroPro actually cyclizes through multiple B−N (and in the case of L-Aspartyl-L-boroProline (Asp-boroPro), B−N and B−O) bonds, forming bicyclic six-membered ring structures. We refer to the phenomenon as "autochelation". The fact that tridentate chelates of suitably designed boronic acids can be predicted justifies this broader term, although "cyclization" is clearly adequate to describe the formation of their monodentate chelates.

Results and Discussion

L-Asp-L-boroPro. Representative 1D 600 MHz ^{1}H NMR spectra of 0.03 M Asp-boroPro in 90% H_2O/10% D_2O at 25 °C and three different pH values are shown in Figure 1. The pH 1.04 spectrum (bottom) is a mixture of two species in slow exchange with a ratio of about 5:2. (Slow exchange on the NMR time scale typically means lifetimes greater than about 0.1 s.) The minor component, with resonances labeled **A**, partially overlaps the major component, labeled **B**, in the spectrum where it is observed in pure form at pH 4.00. Through the use of 2D ^{1}H/^{1}H COSY, ^{1}H/^{1}H NOESY, and natural abundance ^{1}H/^{13}C HSQC spectra, all resonances for all Asp-boroPro species were assigned, as shown in Table 1. The important α proton resonance of Asp appears at ∼4.6 ppm, very close to H_2O (4.766 at 25 °C), so the use of low temperature (e.g., 5 °C) for shifting the

(1) Palombella, V. J. et al. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15671−15676.
(2) Yang, W. *Med. Res. Rev.* **2003**, *23*, 346−368.
(3) Snow, R. J. et al. *J. Am. Chem. Soc.* **1994**, *116*, 10860−10869.
(4) Sudmeier, J. L.; Gunther, U. L.; Gutheil, W. G.; Coutts, S. J.; Snow, R. J.; Barton, R. W.; Bachovchin, W. W. *Biochemistry* **1994**, *33*, 12427−12438.
(5) Coutts, S. J. et al. *J. Med. Chem.* **1996**, *39*, 2087−2094.

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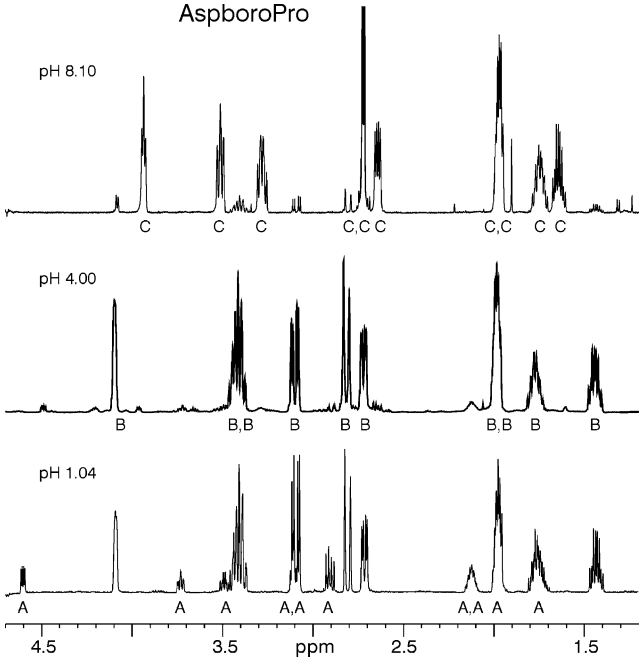


Figure 1. Stack plot of presaturated 1D ^{1}H 600 MHz NMR spectra of 0.03 M Asp-boroPro in 90% H_2O/10% D_2O at 25 °C and various pH values.

Table 1. Chemical Shifts and Spin-Coupling Constants of L-Asp-L-boroPro at 25 °C

	form **A**; pH < 0	form **B**; e.g., pH 4	form **C**; pH > 8
	L-Asp		
	^{1}H Chemical Shifts (ppm vs DSS)		
A	∼4.6[a]	4.08	3.94
1B	∼2.9[a,b]	2.81	2.72
2B	∼3.1[a,b]	3.09	2.72
1F (N−H)	−	6.59	−
2F (N−H)	−	7.14	−
	Spin-Coupling Constants (Hz)		
A−1B	8.8	∼0	∼6.2
A−2B	4.2	6.3	∼6.2
A−1F	−	∼0	−
A−2F	−	∼2	−
1B−2B	−18.0[c]	−18.5[c]	∼−18[c]
1F−2F	−	−13.0[c]	−
	L-boroPro		
	^{1}H Chemical Shifts (ppm vs DSS)		
A	3.10	2.71	2.64
1B	∼1.74	1.43	1.64
2B	2.11	1.97	1.97
1G	2.11	1.97	1.97
2G	∼1.97	1.76	1.75
1D	3.49	3.42	3.28
2D	3.74	3.39	3.51
	^{11}B Chemical Shifts (ppm vs H_3BO_3)		
boron atom	(broad)	−17.4	−17.1
	Spin-Coupling Constants (Hz)		
A−1B	?	12.8	11.9
A−2B	?	5.0	6.0

[a] Slightly pH-dependent, i.e., a few hundredths of a ppm. [b] Assignments of 2B and 1B uncertain, may be interchanged. [c] Negative sign assumed by analogy with known H−C−H geminal couplings.

H_2O peak (to 5.004 ppm) was sometimes employed in making these assignments. At low temperature and very low pH with WATERGATE excitation to avoid saturation transfer, a broad peak (∼60 Hz wide) was observed at 8.3 ppm, which we attribute to the exchangeable protons on the $-NH_3^+$ group (see a in Figure 1S).

The ratio of the species **A** to **B** increases slightly at decreasing pH, but the effect seems to level off at pH < 0.5, perhaps in part due to the effect of increasing ionic strength on the equilibrium. We were unable at any pH to make **A** more than about 20% more abundant than **B**. Form **A** is believed to be a fully extended, fully protonated species in which proline is in the *trans* conformation. In the pH range 0.5−1.5, we also noted a slight upfield shift in the Asp α (A) and β (1B and 2B) resonances of species **A**, as footnoted in Table 1. This must be due to deprotonation of the carboxyl group of the dangling Asp side chain, as will be discussed below. The resonances assigned to species **B**, however, are pH-independent in the range 0.5 to about 8, where **B** finally disappears, as shown in the top spectrum in Figure 1. At high pH, a third species appears, labeled **C**, clearly in slow exchange with **B**.

It is remarkable to observe a species such as **B**, whose pH independence extends to such a wide pH range. Most simple boronic acid dipeptides cyclize with an apparent pK_a of ∼6 or 7,[6] in contrast to this apparent pK_a of ∼0.5 for Asp-boroPro. What is responsible for the extraordinary stability of the cyclization product in this case?

The answer lies in a detailed analysis of form **B**. The 1D ^{1}H NMR spectra in Figures 1S and 2 show two well-resolved, slowly exchanging −NH proton resonances at 6.59 and 7.14 ppm. The line widths of these peaks (<3 Hz) is small enough even to reveal fine structure caused by the rarely observed geminal H−N−H spin-coupling (−13.0 Hz). It is unusual to observe −N−H protons from terminal amino groups at temperatures as high as 25 °C and pH values as high as 4, because their exchange is base-catalyzed. Such −N−H protons normally undergo saturation transfer in water-presaturated spectra such as that shown in Figure 2. But no chemical exchange peaks are detected in the NOESY spectrum in Figure 2. Furthermore, WATERGATE excitation such as that employed in Figure 1S gives virtually the same intensity for the two peaks at ∼6.7 and 7.2 as comparable presaturation spectra, although the peak at 8.3 disappears with presaturation. Raising the temperature to 25 °C (c and d in Figure 1S) causes a slight shift and broadening of the two more upfield −N−H protons, but less than 25% loss of area compared to the spectrum second from the bottom. The fact that these two exchange-resistant protons actually belong to species **B** is confirmed by the NOESY spectrum in Figure 2.

The −N−H protons are labeled "D1F" and "D2F", where "D" is the one-letter code for Asp, and "1F" and "2F" give absolute stereochemistry, as used in standard X-ray crystallography nomenclature. The NOESY spectrum of Asp-boroPro at pH 4.00 (Figure 2) indicates five cross-peaks involving the −N−H protons: (1) Protons D1F and D2F correlate with each other (not shown in the truncated display of Figure 2), as they also do in the COSY spectrum, confirming that they are a geminal H−N−H pair, (2) D1F and D2F both correlate with the vicinal DA proton, showing nearly equal intensities (lower left), (3) D1F correlates with only D2B, one of the Asp β H−C−H pair, and (4) in the red square (left side), D2F interacts across the amino acids, correlating with PA, the proline α hydrogen.

(6) Lai, J. H.; Zhou, Y.; Sudmeier, J. L.; Wu, W.; Sanford, D. G.; Hliang, M.; Poplawski, S.; Bachovchin, W. W. *Adv. Exp. Med. Biol.* **2003**, *524*, 333−338.

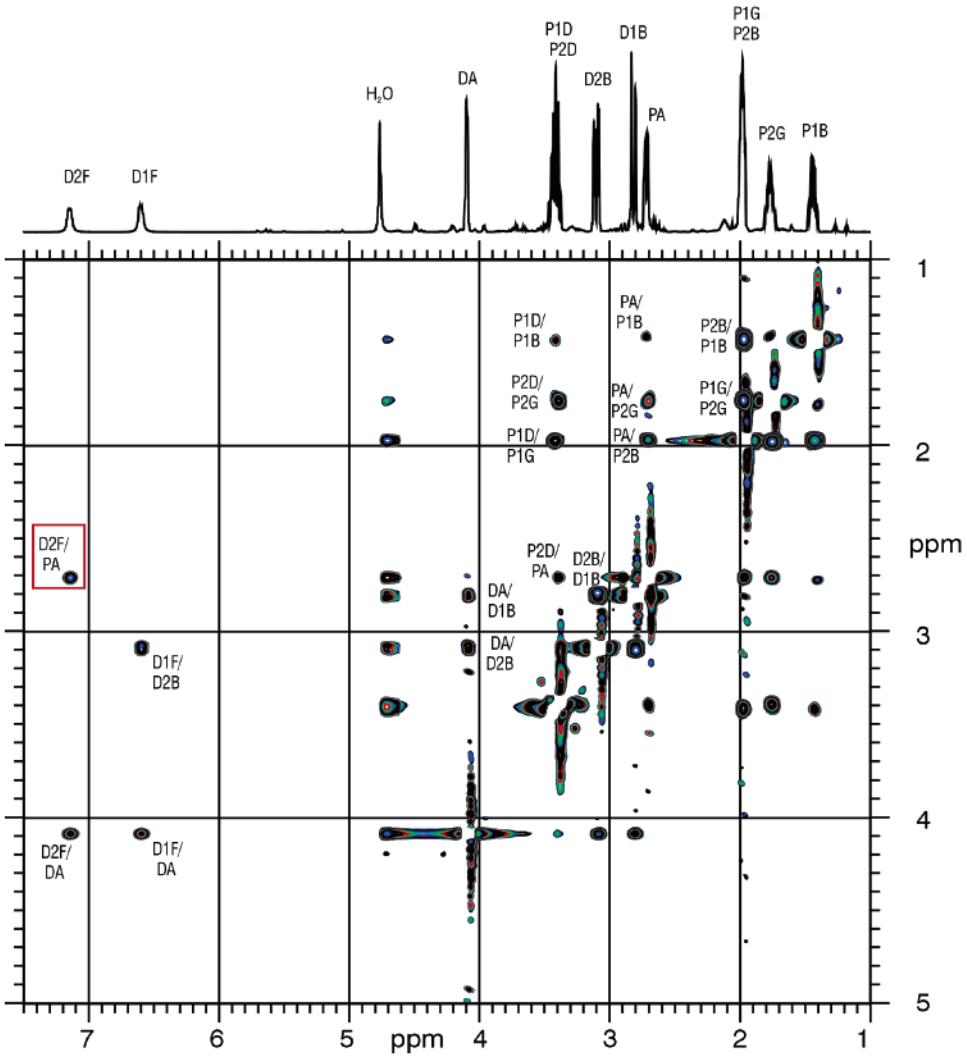


Figure 2. Presaturated $^1H/^1H$ 600 MHz NOESY spectrum of 0.03 M Asp-boroPro in 90% H_2O/10% D_2O at 25 °C and after pH = 4.00 (Form **B**), using 700-ms mixing time.

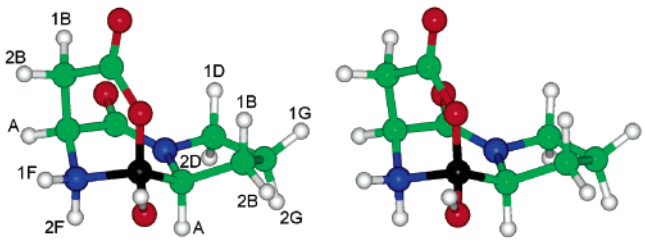


Figure 3. Stereo drawing of Asp-boroPro autochelate (form **B**).

There is only one structure that can satisfy all the above observations, and that is the multicylic structure shown in Figure 3 in which the Asp side chain forms an additional six-membered ring cyclizing to boron in addition to the expected six-membered B−N containing ring. This structure was constructed and energy-minimized using the B3LYP density-functional treatment in the standard 6-31G** basis set (B3LYP/6-31G**).[7] Because in Figure 3 we now have boron not only *cyclized*, but bound to a self-contained *bidentate* ligand, we term species **B** an "autochelate" (i.e., a chelate formed by additional bonds to a covalently bound metal). This structure is also consistent with the ^{11}B spectra, which show a single, rather sharp resonance at ∼−17 ppm versus H_3BO_3, indicative of tetrahedral sp^3 boron, at all pH values. Any ^{11}B signal from planar sp^2 boron due to the $-B-(OH)_2$ group present at pH < 1 would, based on our observations of scores of boronic acids, resonate at 8−11 ppm and would be so broad as to be barely distinguishable from baseline. The specific NOESY interactions mentioned above give us the

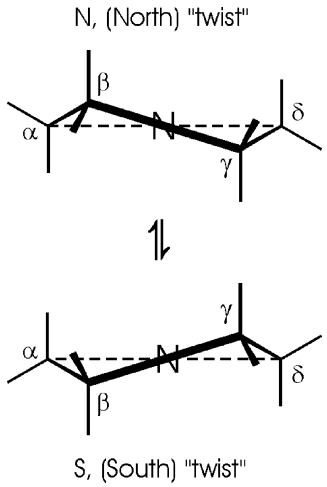


Figure 4. Schematic drawing of N- and S-conformers of proline ring.

(7) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648−5652.

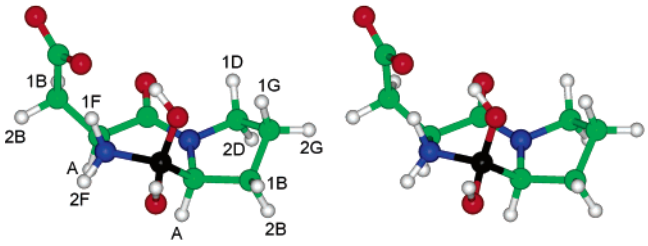
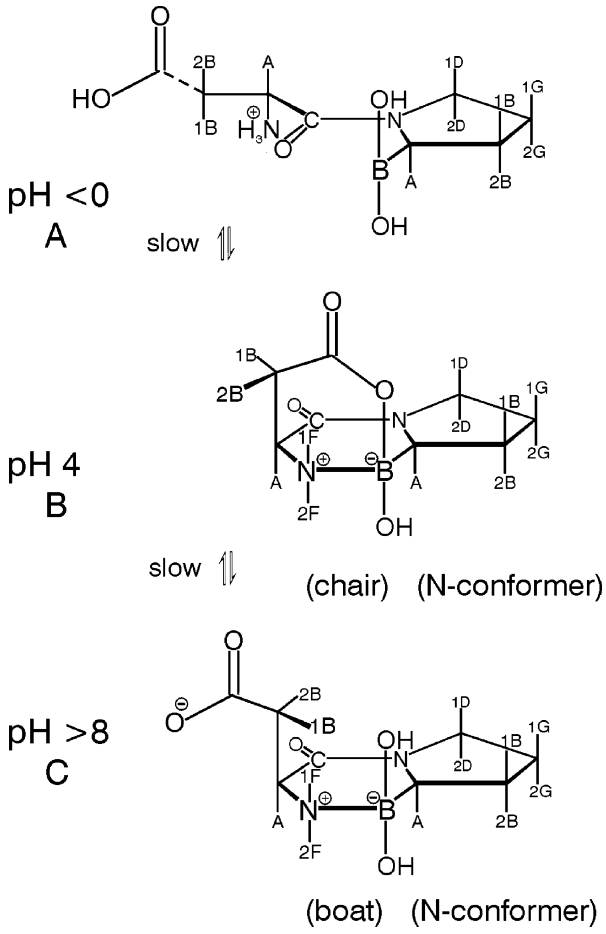


Figure 5. Stereo drawing of Asp-boroPro (form **C**).

Scheme 1. Summary of Asp-boroPro Structures and Proton Labeling at Various pH Values



absolute stereochemical assignments of all Asp resonances D1B, D2B, D1F, and D2B for form **B**, as listed in Table 1.

The proline in species **B** must be *cis* with respect to the peptide bond in order to cyclize. What can be deduced about its proline ring conformation? Virtually complete overlap of proline resonances P1G and P2B complicates the task of conformational analysis based on vicinal coupling constants as we have done previously.[4] However, with a clear view of only P1B, the most upfield resonance (total width ~44 Hz based on sum of all 1B spin-coupling constants), we can shortcut to the conclusion that this proline is >99% in the N conformation.[4] The S conformation would produce a total sum of 1B spin-coupling constants of ~25 Hz, with any mixtures of N and S being a linear average of 44 and 25 Hz. Figure 4 shows a schematic drawing of N- and S-conformers of the proline ring.

The unusual stability of species **B** (Figure 3) can be explained by standard arguments for the "chelate effect", the increase in translational entropy brought about by the release of solvent

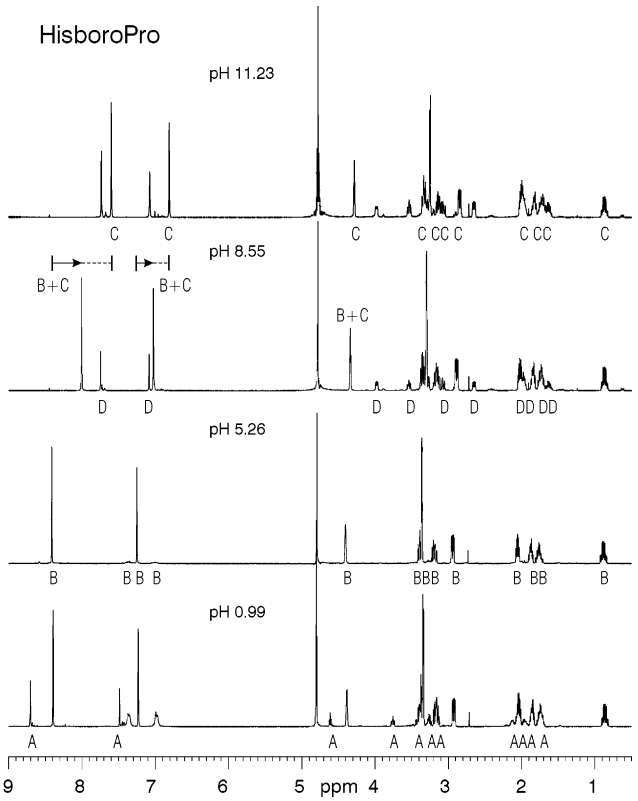


Figure 6. Stack plot of presaturated 1D ^{1}H 500 MHz NMR spectra of 0.03 M His-boroPro in 90% H_2O/10% D_2O at 25 °C and various pH values.

molecules when rings are closed compared to the binding of analogous monodentate ligands.[8] Note that in this structure, boron has become an additional chiral center. Note also that boron still has one open coordination site and could accept an internal *tridentate* ligand of appropriate geometry.

At high pH, something happens to species **B** as shown in Figure 1 (top spectrum, pH 8.10). A new species **C**, in slow exchange with **B**, is produced. The NOESY spectrum shown in Figure 2S gives an important clue in the form of a new interresidue cross-peak between DA and PA, the two α hydrogens. This can happen only when the primary six-membered B−N ring is intact. But now this six-membered B−N ring has flipped from chair to boat, enabling the 1−4 "flagpole" interaction of the two α hydrogens.

This must mean that the carboxylate group of the Asp side chain has released the boron atom, no doubt due to increased competition for boron by hydroxide ion. NMR resonance P1B is still ~44 Hz wide, indicating that the N configuration lives on in species **C**. Figure 5 shows the structure of species C in stereo obtained through energy minimization using a DFT method (B3LYP/6-31G**),[7] which also produced the boat for the B−N ring and the N conformer for proline.

Scheme 1 summarizes what we have learned about the structures of Asp-boroPro as a function of pH. The sugarlike representation of most rings helps to simplify the structures, especially for labeling the protons as they appear in Table 1.

L-His-L-boroPro. Representative 1D 500 MHz ^{1}H NMR spectra of 0.03 M His-boroPro in 90% H_2O/10% D_2O at 25 °C and four different pH values are shown in Figure 6. The pH

(8) Bowmaker, G. A.; Hanna, J. V.; Hart, R. D.; Healy, P. C.; White, A. H. *Aust. J. Chem.* **1994**, *47*, 25−45.

Table 2. Chemical Shifts and Spin-Coupling Constants of L-His-L-boroPro at 25 °C

	form **A**; pH < 0	form **B**; e.g., pH 4	form **C**; pH > 10	form **D**; pH > 10
		1-His		
				2:1
		^{1}H Chemical Shifts (ppm vs DSS)		
A	4.61	4.39	4.28	3.98
1B	3.40	3.35	3.25	3.27
2B	3.40	3.35	3.25	3.07
2D (ring)	7.50	7.24	6.81	7.07
1E (ring)	8.70	8.40	7.60	7.73
1F (N−H)	−	6.98	−	−
2F (N−H)	−	7.36	−	−
		^{13}C Chemical Shifts (ppm vs DSS)		
D (ring)	121.7	119.0	126.6	120.3
E (ring)	137.3	136.8	141.3	139.4
		^{15}N Chemical Shifts (ppm vs NH_3 (and HNO_3))		
D (ring)	175.7, (−201.8)	193.6, (−183.9)	187.9, (−189.6)	178.3, (−199.2)
E (ring)	174.2, (−203.3)	172.5, (−205.0)	244.5, (−133.0)	232.2, (−145.2)
		Spin-Coupling Constants (Hz)		
A−1B	6.7	3.8	3.4	4.5
A−2B	6.7	3.8	3.4	10.2
A−1F	−	∼0	−	−
A−2F	−	3.8	−	−
1B−2B	−	−	−	−15.2[a]
1F−2F	−	−13.0[a]	−	−
		L-**boroPro**		
		^{1}H Chemical Shifts (ppm vs DSS)		
A	3.15	2.93	2.84	2.65
1B	1.73	0.87	0.87	1.63
2B	2.14	2.05	2.01	1.97
1G	2.03	1.87	1.83	1.98
2G	1.96	1.75	1.72	1.78
1D	3.26	3.18	3.13	3.32
2D	3.76	3.38	3.33	3.54
		^{11}B Chemical Shifts (ppm vs H_3BO_3)		
boron atom	(broad)	−19.8	−20.1	−17.5
		Spin-Coupling Constants (Hz)		
A−1B	?	12.9	12.8	11.4
A−2B	?	5.0	5.2	5.7

[a] Negative sign assumed by analogy with known H−C−H geminal couplings.

0.99 spectrum (bottom) is a mixture of two species in slow exchange with a ratio of about 3:1. Again, the minor component, with resonances labeled **A**, partially overlaps the major component, labeled **B**, in the spectrum above (second from bottom) where it is observed in pure form at pH 5.26. Through the use of 2D ^{1}H/^{1}H COSY, ^{1}H/^{1}H NOESY, and natural abundance ^{1}H/^{13}C HSQC spectra, all resonances for all His-boroPro species were assigned, as shown in Table 2. Species **A** and **B** are strictly in slow-exchange with each other, with the relative abundance shifting in favor or **A** as the pH is lowered. As in the case of Asp-boroPro, it was impossible to reduce the pH enough to obtain a spectrum of pure **A**.

Species **B** of this compound likewise exhibits two spin-coupled, slowly exchanging geminal H−N−H protons attributed to 1F and 2F of His, this time at 7.36 and 6.98 ppm, respectively.

At pH 5.26, these protons have almost disappeared from the effects of water presaturation and/or base-catalyzed exchange. At pH 3.19, however, the −N−H resonances of His-boroPro are far more prominent, as shown in the 1D spectrum on top of the NOESY spectrum. The NMR evidence that form **B** of His-boroPro is autochelated into a multicyclic structure is even stronger in this case. On the left side of Figure 7 are three red boxes indicating interresidue through-space NOESY interactions. They are H2F/PA, showing, as in the case of Asp-boroPro, the interaction of only one of the −N−H protons with the proline α hydrogen. But in addition, the aromatic C−H ϵ proton on the imidazole side chain correlates with both proline β hydrogens, producing the H1E/P1B and H1E/P2B cross-peaks. As additional proof, notice the chemical shift of the proline β hydrogen labeled P1B. At 0.87 ppm, it is the most upfield proline resonance we have observed (e.g., 1.43 ppm in Table 1 for Asp-boroPro is the next closest value), undoubtedly the result of ring current shielding from an imidazole ring that has moved very close to proline in the autochelate of His-boroPro, form **B**, depicted in Figure 8.

The total width of the proline 1B resonance is 44 Hz, indicating the N conformation of the proline ring. A complete seven-spin simulation of the proline ring in His-boroPro, form **B**, using WINDNMR71 software (Prof. Hans Reich, University of Wisconsin) is shown in Figure 3S. The spin-coupling constants are as follows: A−1B = 12.9; A−2B = 5.0; 1B−2B = −12.3; 2B−1G = 1.3; 2B−2G = 6.0; 1B−1G = 6.1; 1B−2G = 12.3; 1G−2G = −12.4; 1D−1G = 7.1; 1D−2D = −10.4; 1D−2G = 10.1; 2D−1G = 1.8; 2D−2G = 10.4.

As the pH of His-boroPro is raised above 6, two new species appear, labeled **C** and **D**. Above pH 11, they occur in a ratio of 2:1 with respect to each other. **C** is in rapid exchange with **B** and in slow exchange with **D**. The rapid exchange process

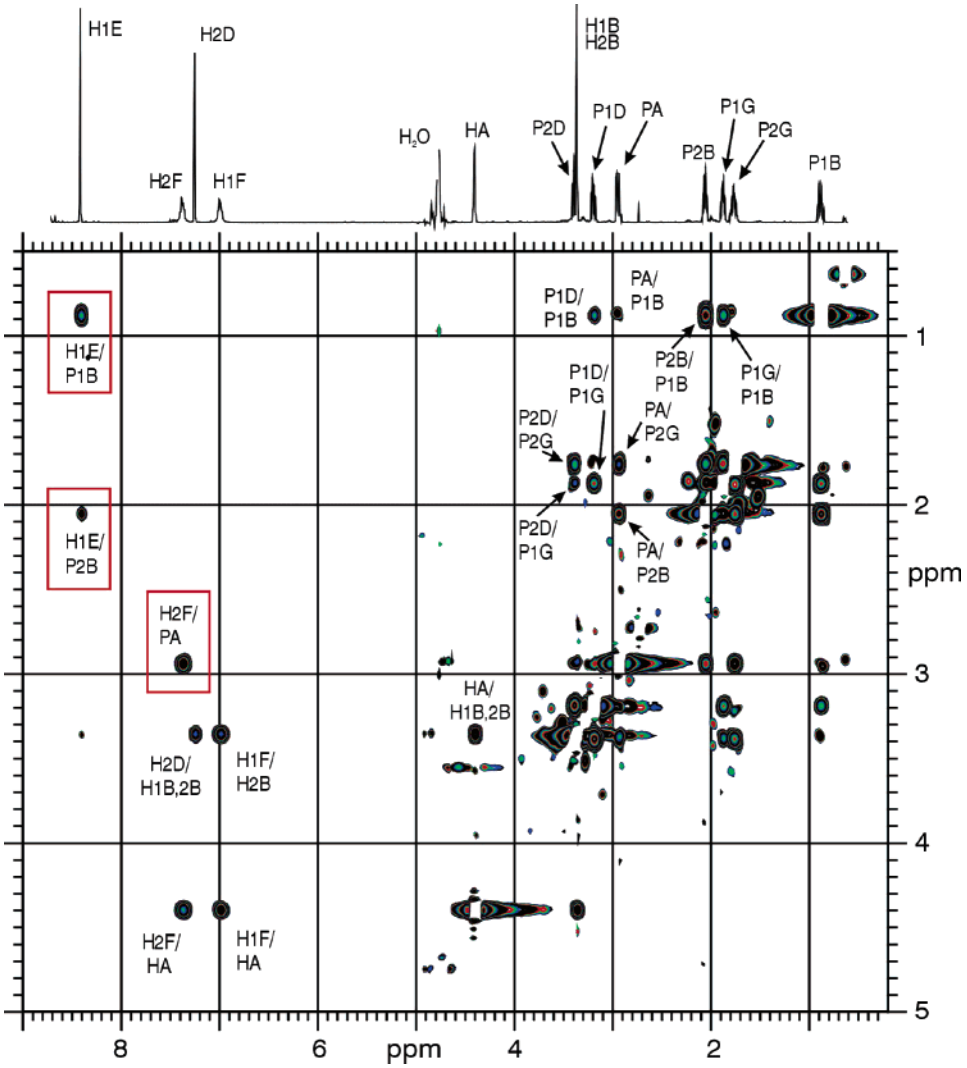


Figure 7. Presaturated $^{1}H/^{1}H$ 600 MHz NOESY spectrum of 0.03 M His-boroPro in 90% H_2O/10% D_2O at 25 °C and after pH = 3.19 (Form **B**), using 700-ms mixing time.

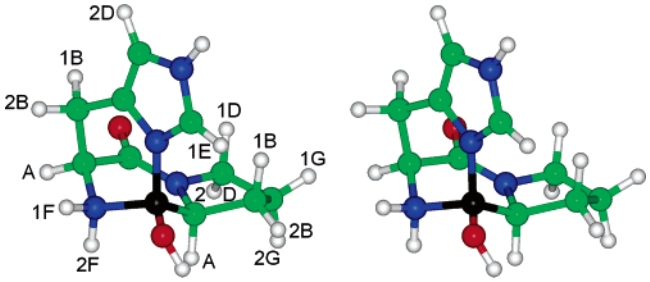


Figure 8. Stereo drawing of His-boroPro autochelate (form **B**).

causes a gradual upfield shift of practically all resonances of the **B** and **C** mixture, the largest amplitude being on the imidazole ring (see Figure 6, pH 8.55, second from top), and gradually decreasing as one moves toward the proline ring (see Table 2), with no effect on proline 1B. By pH 11.23, this shifting has reached its limit, as shown in the top spectrum of Figure 6. Several spectra at pH values between 6 and 11 show the gradual upfield shift of the combined **B** and **C** peak. A pK_a value of 8.6 was calculated from the chemical shift of this peak versus pH.

What is the identity of species **C** in rapid exchange with **B**? Replacement of imidazole by hydroxide should be kinetically slow, judging by the precedent of Asp-boroPro. We considered a number of alternatives, including dissociation of one of the His −N−H protons 1F or 2F.

The answer was provided by ^{15}N and ^{13}C chemical shifts of the imidazole ring obtained from $^{1}H/^{15}N$ and $^{1}H/^{13}C$ HSQC spectra in natural abundance at various pH values (Table 2). As shown by Bachovchin,[9] protonated imidazole nitrogen atom always has a chemical shift of 175 ppm or less. Unprotonated imidazole nitrogen atoms have chemical shifts of 235 ppm or greater. We discovered that the $N^{\epsilon 1}$−H proton dissociates in species **C**, yielding a chemical shift of 244.5 ppm. This was most unexpected, since unprotonated imidazolate anion is typically not observable in water, having a pK_a of 14.5,[10] rather than the value of 8.6 observed in this case.

Further evidence comes from the $^{13}C^{\delta 2}$ chemical shift of imidazole in species **C**. We have shown previously that only in the rare $N^{\delta 1}$ tautomer (where $N^{\delta 1}$ is protonated and $N^{\epsilon 1}$ is not) is the chemical shift of $^{13}C^{\delta 2}$ greater than about 122.5 ppm.[11] For His-boroPro we find a chemical shift of 126.6 only for form

(9) (a) Bachovchin, W. W.; Kanamori, K.; Vallee, B. L.; Roberts, J. D. *Biochemistry* **1982**, *21*, 2885−2892. (b) Bachovchin, W. W.; Kaiser, R.; Richards, J. H.; Roberts, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 7323−7326. (c) Bachovchin, W. W. *Magn. Reson. Chem.* **2001**, *39*, S199−S213.
(10) Eicher, T.; Hauptmann, S. *The Chemistry of Heterocycles: Structure, Reactions, Syntheses, and Applications*; Wiley-VCH: Weinheim, Germany, 2003.

C and less than 122 for all others. This is really an expansion of the original case, since B−N$^{\delta 1}$ has been subtituted for H−N$^{\delta 1}$. If *boronation* has the same, or nearly the same, electronic effect on imidazole as *protonation*, we should not have been surprised at the lowered pK_a (8.6) of imidazole in form **C**. Indeed, a pK_a value of 6.5 would be normal for dissociation of one −N−H proton with the opposite N protonated. A pK_a value of 8.6 caused by boronation is about a 75% reduction from 14.5 compared to the eight units reduction caused by protonation (pK_a 6.5 versus 14.5).

The ^{15}N chemical shifts of imidazole reported in Table 2 also shed light on the tautomer fractions. The chemical shifts of N$^{\delta 1}$ bound to boron in forms **B** and **C** are 193.6 and 187.9 ppm, respectively. This is about 75% of the interval between ^{15}N shifts in unprotonated species in Table 2 (244 ppm) and protonated species (∼173 ppm). Therefore, as a rule of thumb, it appears that boron acts like ∼75% of a proton in its electronic effect upon imidazole in His-boroPro.

We have carried out extensive ^{15}N NMR studies of the enzyme α-lytic protease and its two types of boronic acid complexes.[9c] In Type 2 complexes, there is a direct B−N bond from the inhibitor to an imidazole nitrogen atom of active site His 57 that exhibits a chemical shift of 209.8 ppm in the tetrahedral adduct. This represents more like 50% of the protonation. But this bond is to the N$^{\epsilon 2}$ atom, not N$^{\delta 1}$ as in His-boroPro, and could have unfavorable geometry due to spatial constraints in the enzyme active site.

With the dissociation of the imidazole N−H proton causing the change of form **B** to form **C**, there is no change in the ring current shielding of the proline 1B hydrogen, which should be the case for ring systems containing the same number of π electrons.

Form **D** of His-boroPro has the imidazole ring displaced by hydroxide ion, exactly analogous to form **C** of Asp-boroPro. The NOESY spectrum (not shown) of form **D** gives exactly the same intraresidue cross-peak between the His and Pro α hydrogens, proving the six-membered B−N containing ring has flipped into a boat, just as in the case of Asp-boroPro, form **C**. The width of the P1B resonances remains ∼44 Hz wide in both forms **C** and **D**, indicating the N configuration.

If the **D** form has a dangling His side chain, freed from boron, the pH-independent imidazole ^{1}H chemical shifts (7.73 and 7.07 ppm) are somewhat greater than those of form **C** (7.60 and 6.81 ppm) or for that matter pure Histidine at pH 13 (7.64 and 6.90 ppm). What accounts for the 0.1 to 0.2 ppm increase is probably the effect of C═O anisotropic shielding upon the imidazole ring. This juxtaposition is the rationale behind the assignment of the His β protons 1B and 1B, based on the resulting vicinal angles and known A−1B and A−2B spin coupling constants.

The ^{11}B spectra of His-boroPro shown in Figure 9 reveal a divergence at high pH with about a 2:1 ratio. The low pH (0.55) ^{11}B sharp peak at −20 ppm is consistent with sp^3 hybridized boron. At pH 10.38, we observe two different types of sp^3 boron with about a 2:1 ratio, consistent with forms **C** and **D**.

Scheme 2 summarizes the structures of His-boroPro determined here as a function of pH.

Microscopic Acid−Base Equilibria. Scheme 3 summarizes the various species present at all pH values along with the relevant microscopic acid−base equilibrium constants. The estimation of macroscopic pK_1 for both compounds is done very simply from the 5:2 ratio of species **B** to **A** (conjugate base to acid) at pH 1.04 for Asp-boroPro and a ratio of 3:1 at pH 0.99 for His-boroPro. This leads to macroscopic pK_1 values of 0.6 and 0.5 for Asp-boroPro and His-boroPro, respectively. Considering that N-terminal amino groups and boronic acids typically dissociate with pK_a values in the range 9 to 10, the autochelation phenomenon leads to acid-strengthening of about nine units. The value of macroscopic pK_2 for Asp-boroPro is obtained similarly from the ratio of **B** to **C** at various pH values. For His-boroPro, it was easier to determine the microscopic equilibrium constant pk_{2N} by measuring the imidazole chemical

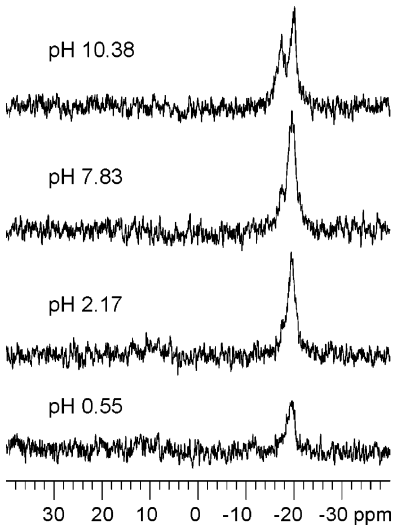
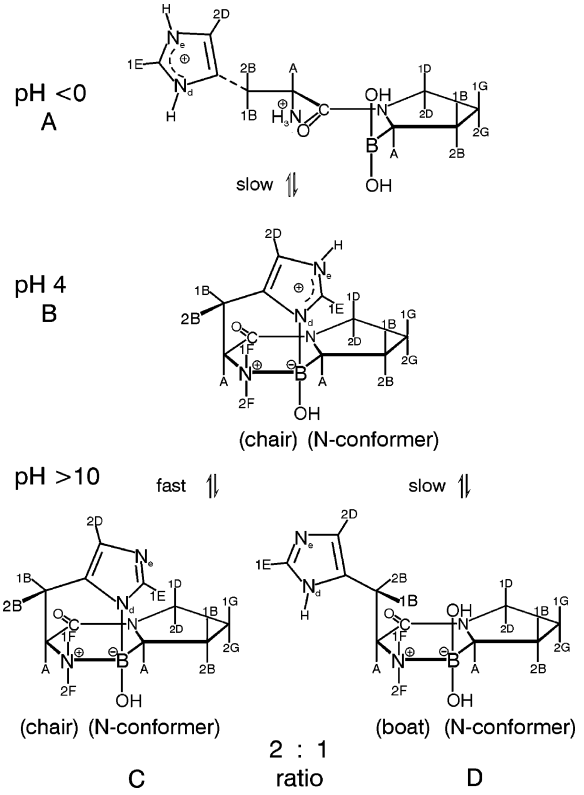


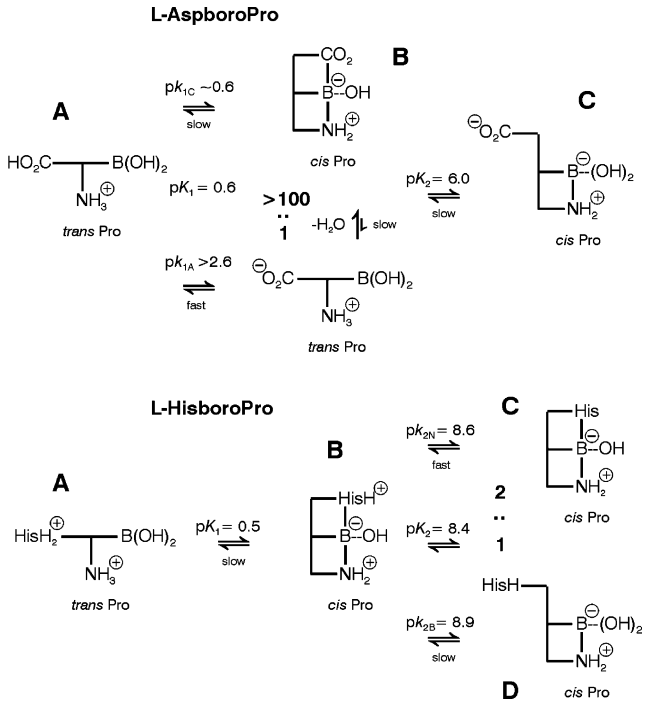
Figure 9. Stack plot of 1D ^{11}B 96.3 MHz NMR spectra of 0.03 M His-boroPro in 90% H_2O/10% D_2O at 25 °C and various pH values.

Scheme 2. Summary of His-boroPro Structures and Proton Labeling at Various pH Values



(11) Sudmeier, J. L.; Bradshaw, E. M.; Haddad, K. E.; Day, R. M.; Thalhauser, C. J.; Bullock, P. A.; Bachovchin, W. W. *J. Am. Chem. Soc.* **2003**, *125*, 8430−8431.

Scheme 3. Summary of Microscopic Acid−Base Equilibrium Constants and pH-Dependent Structural Changes of Asp-boroPro and His-boroPro



shifts in the interval between **B** and **C**. At pH 8.6 the imidazole shifts were at their titration midpoint, consistent with a value of 8.6 for pk_{2N}. Knowing that form **D** is half as abundant as form **C** in His-boroPro leads to a microscopic equilibrium constant pk_{2B} of 8.9, larger than pk_{2N} by log 2. The sum of microscopic equilibrium constants k_{2N} and k_{2B} leads to the macroscopic K_2, for which in this case pK_2 is 8.4.

At very low pH values (<1.5), where the fraction of form **A** of Asp-boroPro is small, the Asp side chain resonances A, 1B, and 2B were found to shift upfield by a few hundredths of a ppm with increasing pH. The higher the pH, the less **A** is present. Clearly we are seeing the proton rapidly come off the carboxylate group of the dangling Asp side chain with increasing pH. With the carboxylate deprotonated, this species must undergo *trans* to *cis* conversion and dehydration before going to form **B**. One or both processes are kinetically slow, accounting for the slow exchange between forms **A** and **B** (lifetimes > 0.1 s). At pH 4, there is no evidence of a second form of Asp-boroPro, and thus the unprotonated intermediate can be assumed to be 1% or less the amount of **B**. For 1% the value of pk_{1A} would be 2.6. If the intermediate were 0.1%, the pk_{1A} would be 3.6. Either value would be realistic, considering typical amino acid carboxylate group acidities, and will have negligible effect on the macroscopic value of pK_1 = 0.6. Studies of the rate and mechanism of these interconversions are underway.

Experimental Section

Syntheses. Asp-boroPro and His-boroPro were synthesized by standard methods.[5]

NMR Spectroscopy. All samples were about 0.03 M in concentration. ^{1}H and ^{13}C chemical shifts are reported relative to DSS, added as internal standard, ^{11}B spectra are referenced to external boric acid, and ^{15}N shifts are referenced to liquid NH_3, as well as the historical 1 M HNO_3 standard (−377.5 versus NH_3). NMR spectra were obtained using Bruker DRX600, AMX500, and DPX300 spectrometers. The DRX600 is equipped with a 5-mm triple resonance inverse probe with triple axis gradients and standard Avance electronics with digital filtering, digital ^{2}H field/frequency lock, and four pulse transmitter channels. The AMX500 has a standard-bore magnet, and 5-mm triple resonance inverse probe, multichannel interface, and Silicon Graphics O2 computer. The DPX300 uses a 5-mm inverse broadband probe, Avance electronics, and two pulse transmitter channels. All spectra were processed using Bruker software (XWINNMR 3.5 on the DRX600 and DPX300, and XWINNMR 2.6 on the AMX500) and imported into CorelDraw 9.0 for rendering.

The presaturated 1D spectra, such as those shown in the stack plots of Figures 1 and 6, were run on both the DRX600 and AMX500 using the composite pulse in Bruker's program *zgcppr*. Nonexcitation of water (WATERGATE) as shown in Figure 1S was achieved on the DRX600 using the program *p3919gp*. The presaturated ^{1}H/^{1}H NOESY spectra shown in Figures 2, 2S, and 7 were run using the standard Bruker program *noesyprtp*, with mixing time 700 ms and recycle time of 1.7 s, collecting 2 K of data in the observe dimension by typically 400−500 rows in the indirect dimension over a time of 8−15 h. The data were processed using the Gaussian-Lorentzian window function with LB2 = −10 Hz and GB2 = 0.02 and shifted sine bell squared using SSB = 2 in the indirect dimension.

1D ^{11}B spectra, such as the stack plot shown in Figure 9, were run at 96.3 MHz on the DPX300 spectrometer using a 5-mm inverse broadband probe. A read pulse of 10 us and recycle delay of 0.5 s were used. Quartz NMR tubes (Wilmad Glass, Buena Park, NJ) were used to reduce the borosilicate background. Because of Pyrex used in probe construction, some residual borosilicate background remains in the transformed spectra. This was reduced by subtracting a spectrum run on a water sample in a quartz tube.

Computations. The B3LYP/6-31G** density functional treatment was carried out with Spartan'04 software from Wavefunction, Inc., Irvine, CA.

Acknowledgment. The Bruker AMX500 NMR Spectrometer was funded by the NIH, and the Bruker DRX600 and DPX300 Spectrometers were funded by an NIH/NSF Shared Instrumentation Grant. The NMR simulation software WINDNMR71 was kindly provided by Prof. Hans Reich of the Chemistry Department, University of Wisconsin, Madison, WI.

Supporting Information Available: WATERGATE ^{1}H NMR spectra of Asp-boroPro (the 6−9 ppm portion) at various temperatures and pH values, NOESY spectrum of Asp-boroPro at 5 °C and pH = 8.34 (Form **C**), and an example of seven-spin ^{1}H NMR spectral simulation of proline in His-boroPro, form **B**. Complete citations for refs 1, 3, and 5. This material is available free of charge via the Internet at http://pubs.acs.org.

JA050215E