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Cysteine Proteases such as Papain are not Inhibited by Substrate Analogue Peptidyl Boronic Acids

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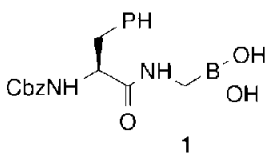
Abstract—Peptidyl boronic acids that are close structural analogues of good substrates have been prepared and evaluated as potential transition state analogue inhibitors of the representative cysteine protease, papain. However, no inhibition could be detected at concentrations up to 10 mM. The reasons for the lack of inhibition were sought from molecular modeling. Molecular mechanics and semi-empirical quantum mechanics calculations indicated that the absence of inhibition was due to boronic acid–cysteine protease tetrahedral complexes being 0.79 kcal mol^{-1} less stable than their preceding noncovalent EI-complexes. In contrast, an analogous boronic acid–serine protease tetrahedral complex was calculated to be 2.74 kcal mol^{-1} more stable than its precursor Michaelis EI-complex. It thus appears that boronic acids are ineffective inhibitors of cysteine proteases due to the thermodynamic favoring of a weak EI-complex preceding tetrahedral intermediate formation, and that any oxyanion hole stabilization of the subsequent tetrahedral intermediate cannot overcome this energy handicap. © 1997 Elsevier Science Ltd.

Introduction

Cysteine and serine proteases are involved in many disease processes and developing effective inhibitors for both groups of enzymes is of major pharmaceutical interest.[1] The mechanisms of cysteine and serine protease catalyses are similar in a number of respects.[2] Both types of enzymes employ a histidine–imidazole residue as a proton shuttle, and a nucleophile, cysteine thiol or serine hydroxyl respectively, is acylated during the process. Furthermore, inhibitors of serine proteases very often inhibit cysteine proteases,[3] although inhibitors that are selective for specific enzymes can be designed.[4]

Boronic acids are often very potent transition-state analogue[5] inhibitors of serine proteases,[6] by forming tetrahedral adducts with the active site serine that are highly stabilized by oxyanion hole interactions. In particular, peptidyl boronic acids that are close substrate analogues inhibit serine proteases very strongly.[7] In contrast, our literature search revealed no information on the inhibition of cysteine proteases by boronic acids, with the exception of one report,[8] which suggested that a mutant of β-lactamase, in which the active site serine has been replaced with cysteine, was noncompetitively inhibited by boric acid. Accordingly, in order to explore this lacuna of cysteine protease inhibition, and to provide additional information on the still incompletely understood nature of the oxyanion hole stabilizations of cysteine proteases,[9] compounds **1** and **2** were prepared as potential inhibitors of papain. The design of these peptidyl boronic acid structures was based on the substrate specificity, and X-ray structures,[2] of papain (E.C.3.4.22.2), which favors binding of Phe residues in its S_2 pocket and Gly or Ala in S_1.



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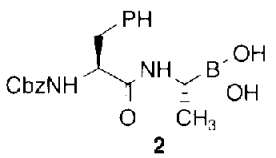

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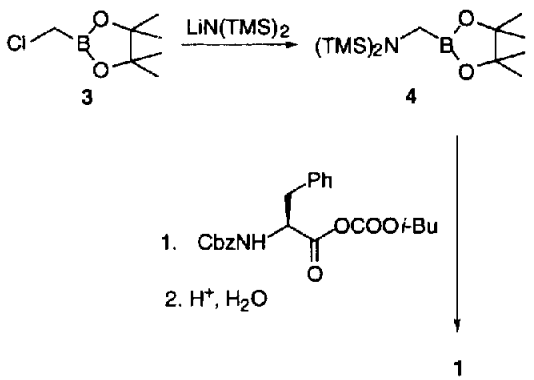
Results and Discussion

Boronic acid **1** was prepared as outlined in Scheme 1. The chloromethylpinacol ester **3** was smoothly converted to the *bis*(trimethylsilyl)amino boronate **4**. This was then desililated in situ with water, and reacted immediately with isobutyl-*Cbz*-phenylalanylformate according to the mixed anhydride procedure developed for similar compounds by Kinder and Katzenellenbogen.[10] The pinacol ester moiety was cleaved during the acidic work up of this sequence, to give (*Cbz*-phenylalanyl)aminomethylboronic acid (**1**) in 21% overall yield from **3**. For the other boronic acid, the

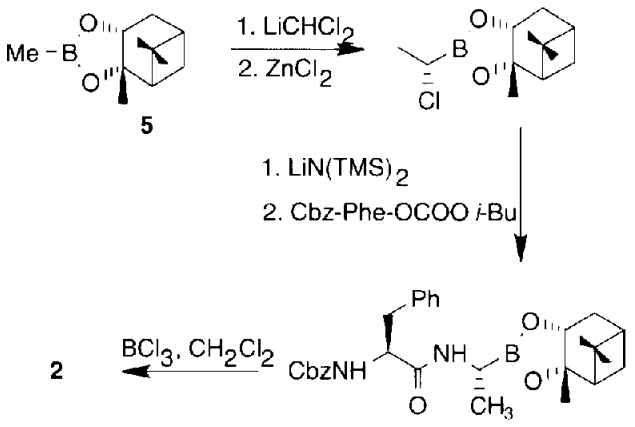


Scheme 1.

route shown in Scheme 2, based on the chemistry of Matteson et al.[11] with *(S)*-pinanediol as the chiral auxiliary, was followed. The target compound, (1*R*)-(*Cbz*-phenylalanylamino)ethyl boronic acid (**2**), was obtained from the pinanediol ester **5** in 11% overall yield.

The peptidyl boronic acids **1** and **2** were only slightly soluble in water, and 10% acetonitrile was therefore added as a cosolvent in the inhibition studies. No detectable inhibition of papain was observed at the maximum obtainable concentrations, 10 mM, for each of the inhibitors under these conditions, thus reflecting actual K_I values well in excess of this concentration.

In contrast to this absence of inhibition for a representative cysteine protease, boronic acids with equivalent substrate analogue characteristics for serine proteases are outstanding competitive inhibitors, often with subpicomolar K_I values.[6] Furthermore, ketone analogues of good substrates are known to be good transition-state analogue inhibitors of cysteine proteases.[12] Insights into the reasons why papain does not interact with such close substrate analogue boronic acid structures in the serine protease manner were sought using molecular modelling. The molecular mechanics and dynamics simulations carried out were based on the crystallographic data for serine and cysteine proteases that are available from the Protein Data Bank.[13] While for serine proteases, boronic acids have been shown to be capable of forming tetrahedral complexes with either the active site serine or histidine residues,[14] the trend appears to be that good substrate analogues bind to serine and poor substrate analogues to histidine, although binding of a single inhibitor to give both a serine- and a histidine-adduct has been reported.[15] Accordingly, by analogy for papain, only tetrahedral complexes involving cysteine addition to the good substrate analogues **1** and **2** were considered. According to the X-ray structures of tetrahedral complexes of boronic acids with serine proteases,[16] one of the oxygens on the boron is located in the oxyanion hole, while the second is oriented towards the active site histidine. This is consistent with the fact that the oxyanion hole



Scheme 2.

participates in the stabilization of the tetrahedral intermediate during serine protease catalysis. At the same time, there is a controversy on the role of the oxyanion hole in the catalysis by cysteine proteases.[9]

In the present study, we modelled the putative boronic acid–papain tetrahedral complex assuming that one of the oxygens of the boron binds to the oxyanion hole. The high resolution X-ray structure of papain[2a] was energy-minimized using the Biosym 'Discover' program and the boronic acid *Cbz*-Phe-(boro)Ala(OH)$_2$ (**2**) was docked into the active site by superimposing it on the inhibitor structure of the X-ray structure of the *Cbz*-Phe-Ala-CMK-papain[2b] covalent complex. One of the oxygen atoms of the two hydroxyl groups attached to the boron atom was directed to Nε of His159, while the other was placed in the oxyanion hole formed by the side-chain NH_2 of Gln19 and backbone NH of Cys25. This system was solvated with a 5 Å layer of water molecules. Energy minimization by molecular mechanics was followed by molecular dynamics simulation for 20 ps. Analysis of the trajectory showed that the tetrahedral **2**-complex oriented itself at the active site such that one oxygen remained in the oxyanion hole while the other was hydrogen-bonded to Nε of His159. This result is similar to those of our simulations of the tightly bound boronic acid–serine protease EI-complexes,[7g,17] and thus indicates that for cysteine proteases, the presence of an active site environment capable of eliciting good interactions with the oxyanion hole is insufficient on its own to ensure a favourable (low K_I) boronic acid–cysteine protease EI-complex.

We then directed our attention towards considerations of the stabilities of the tetrahedral complexes themselves. Kollman and co-workers have recently investigated the catalytic pathway of cysteine[18] and serine[19] proteases using the combination of molecular mechanics and semi-empirical quantum mechanics. We followed a similar approach in evaluating the stabilities of the tetrahedral complexes of boronic acids with cysteine and serine proteases in comparison with their corresponding precursor Michaelis EI-complexes. For cysteine proteases, the above papain–**2** complex was used, while our previous (boro)Phe(OH)$_2$–subtilisin Carlsberg complex,[16] was employed as the model for serine proteases. The coordinates of the catalytic triad, the boronic acid, the residues forming the oxyanion hole, and one of the solvent water molecules closest to boron, were extracted from each complex. In order to make the calculations more manageable, each system was simplified, with cysteine and serine being represented by methyl mercaptan and MeOH, histidine by methyl imidazole, and asparagine and aspartate by acetamide and acetate, respectively. Also, the residues making up the oxyanion holes were replaced by positioning two water molecules in the locations of the hydrogen bonding atoms. The overall charge of the cysteine protease model was zero, while for the serine protease model a charge of −1 was assigned.

AM1[20] calculations were performed on both the model tetrahedral intermediates and the Michaelis EI-complexes, in which the SH or OH nucleophiles were initially positioned 5 Å from the boron atom. For the tetrahedral complexes, there was little change in the positions of the atoms after minimizations. For the Michaelis EI-complexes, as expected, the most stable arrangement for the cysteine protease involved the imidazolium–thiolate ion pair, while the imidazole–alcohol combination was more stable for the serine protease model. However, the calculations showed that in the cysteine protease case, the tetrahedral complex was 0.79 kcal mol^{-1} less stable than its precursor Michaelis EI-complex. In contrast, the oxyanion hole-stabilized serine protease tetrahedral intermediate was favored by 2.74 kcal mol^{-1} over its preceding non-covalent EI-complex. Thus, it appears that boronic acids are ineffective inhibitors of cysteine proteases due to the thermodynamic favoring of the weak EI-complex preceding tetrahedral intermediate formation, and that the magnitude of any oxyanion hole stabilization of the subsequent tetrahedral intermediate is insufficient to overcome this energy handicap.

Experimental

General methods

Unless otherwise stated, all reactions were performed under nitrogen using oven-dried glassware. Anhydrous reagents and solvents were prepared according to literature procedures.[21] Analytical thin-layer chromatography was performed on Merck plates (silica gel F_{254}, 0.25 mm). Compounds that were not visible under UV light were detected by spraying with a mixture of ninhydrin (0.3 g) and acetic acid (3 mL) in EtOH (100 mL) followed by heating. Preparative flash column chromatography was performed using silica gel 60 (40–63 microns), supplied by Toronto Research Chemicals Inc. Melting points were obtained on a Fisher–Johns melting-point apparatus, and are uncorrected. Boiling points are of Kugelrohr distillations and are uncorrected. Optical rotations were measured in a Perkin–Elmer 243 B polarimeter in a thermostatted cell. IR spectra were determined on KBr pellets (for solids) or films (for liquids) on a Nicolet 5DX FTIR spectrophotometer. NMR (^{1}H, ^{13}C) spectra were recorded on a Gemini 200 spectrometer (at 200 or 50 MHz, respectively) unless otherwise indicated. Mass spectra were measured on a Bell and Howell 21-490 (low resolution) or an AEI MS3074 (high resolution) instrument. Elemental analyses were by Galbraith Laboratories, Knoxville, Tenessee.

Reagent grade chemicals were purchased from Aldrich. Papain (E.C.3.4.22.2) was purchased from Sigma as a suspension in sodium acetate and was further purified by affinity chromatography.[22] The thiol content of the enzyme was estimated with 5,5′-dithio-*bis*(2-nitrobenzoic acid).[23]

(*Cbz*-Phenylalanyl)aminomethylboronic acid (1, *Cbz*-Phe-(boro)Gly(OH)$_2$). A mixture of diisopropyl (chloromethyl)boronate[24] (3.57 g, 20 mmol) and pinacol (2.36 g, 20 mmol) in hexane (40 mL) was stirred overnight at 20 °C. Hexane was removed at atmospheric pressure and the remaining material distilled to give 2-(chloromethyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**3**, 3.10 g, 88%), bp 89–91 °C 28 mmHg^{-1} (bp 73 °C/12 mmHg).[25]

To a cooled (−78 °C) solution of hexamethyldisilazane (2.39 mL, 11.3 mmol) in THF (20 mL) was added *n*-BuLi (6.83 mL of 1.6 M solution in hexane, 10.9 mmol). The cooling bath was removed and the mixture was allowed to warm to 0 °C within 0.5 h and then stirred at 0 °C for an additional 0.5 h. The resulting solution of lithiohexamethyldisilazane was cooled again (−78 °C), and the chloromethyldioxaborolane **3** (1.84 g, 10.4 mmol) in THF (5 mL) was added within 10 min. The reaction mixture was then allowed to warm to 20 °C and stirred overnight, after which hexane (50 mL) was added and the precipitated LiCl filtered off. The filtrate was concentrated in vacuo and the residue Kugelrohr distilled to give 2-[*bis*(trimethylsilyl)aminomethyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**4**, 2.74 g, 83%) as a colorless liquid, bp 60–65 °C 3 mmHg^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.04 (s, 18 H), 1.20 (s, 12 H), 2.41 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 1.37, 24.65, 29.08, 83.22.

Cesium carbonate (605 mg, 1.86 mmol) was added at 20 °C to *Cbz*-phenylalanine (1.11 g, 3.71 mmol) in MeOH (10 mL). The mixture was stirred overnight, the MeOH then evaporated in vacuo, and the resulting cesium salt of *Cbz*-phenylalanine was dried over P_2O_5 at 0.2 mmHg for 24 h. It was then dissolved in dry DMF (5 mL) under N_2, the solution cooled to −15 °C, and isobutylchloroformate (0.48 mL, 3.71 mmol) added in one portion and the mixture stirred at −15 °C for 0.5 h. 2-[*Bis*(trimethylsilyl)aminomethyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (1.12 g, 3.71 mmol) was added with stirring, followed by water (0.25 mL, 13.8 mmol). After stirring for 3 h at −15 °C the reaction mixture was quenched with 1 M aqueous HCl (20 mL), extracted with EtOAc (30 mL), and washed with water (5 mL). The boronic acid was extracted from EtOAc into 1 M NaOH (3 × 3 mL), washed with EtOAc (2 × 10 mL), the aqueous solution acidified with 1 M aqueous HCl (11 mL), extracted with EtOAc (2 × 10 mL) and dried over $MgSO_4$. After filtering and concentrating in vacuo, the residue was chromatographed ($CHCl_3$:MeOH (3:2) elution) to give a white solid. This was dissolved at 20 °C in a minimum amount of acetone, then water was added to saturation, and the acetone then rapidly removed under vacuum. The resulting crystals were filtered and dried in vacuo to afford (*Cbz*-phenylalanyl)-aminomethylboronic acid (**1**, 330 mg, 25%), mp 52–55 °C, $[\alpha]_D^{23}$ = +20.40 (*c* 1.24; CH_3OH). IR (KBr) 3318, 3029, 2938, 1702, 1698, 1654, 1648, 1633, 1534, 1531, 1401, 1240, 698 cm^{-1}. ^{1}H NMR (CD_3OD) δ 2.32 (AB, *J* = 2.64 Hz, 2 H), 2.89–3.23 (m, 2 H), 4.58 (m, 1 H), 5.02 (s, 2 H), 7.24–7.29 (m, 10 H). ^{13}C NMR (CD_3OD) δ 31.63 (broad), 38.47, 55.23, 67.65, 127.95, 128.67,

128.94, 129.39, 129.52, 130.23, 137.67, 137.87, 158.00, 178.39. Anal. calcd for $C_{18}H_{21}BN_2O_5$: C, 60.70; H, 5.94; N, 7.86; B 3.03. Found: C, 60.52; H, 5.82; N, 7.88; B 2.83.

[1(*R*)-(*Cbz*-Phenylalanylamino)ethyl]boronic acid (2). The basic methodology used was as described previously.[26] MeMgBr (8.33 mL of 3 M solution in Et_2O, 25 mmol) was slowly added during 15 min to a solution of trimethyl borate (2.84 mL, 25 mmol) in Et_2O (80 mL) at −70 °C, and the resulting white suspension stirred for a further 1 h. The mixture was then allowed to warm to 20 °C and stirred for an additional 10 h. After work up, (*S*)-pinanediol (2.13 g, 12.5 mmol) was added with stirring. The reaction was monitored by the TLC, and more (*S*)-pinanediol added as needed. After apparent completion, the resulting solution was stirred for a further 1 h at 20 °C, filtered, concentrated in vacuo and Kugelrohr-distilled to furnish (*S*)-pinanediol methylboronate (**5**, 2.77 g, 41%), bp 60–65 °C (3 mmHg) (bp 37–41 °C (0.25 mmHg)),[27] $[\alpha]_D^{23}$ = +37.0 (*c* 3.58; $CHCl_3$). IR (film): 2931, 1458, 1414, 1390,1381, 1340, 1280, 1242, 1078, 1008 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.26 (s, 3 H), 0.81 (s, 3 H), 1.09 (d, *J* = 10.67 Hz, 1 H), 1.26 (s, 3 H), 1.36 (s, 3 H), 1.86–2.30 (m, 5 H), 4.23 (dd, *J* = 1.85 and 8.69 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 23.77, 26.22, 26.87, 28.45, 35.27, 37.93, 39.33, 51.14, 77.55, 85.36 (B–C not seen).

To (dichloromethyl)lithium (prepared at −100 °C from CH_2Cl_2 (1.02 mL, 16 mmol) in THF (30 mL) and *n*-BuLi (6.9 mL of 1.6 M in hexane, 11 mmol)) was added, in one portion, the above (*S*)-pinanediol methylboronate (**5**, 2.70 g, 10 mmol) in Et_2O (10 mL). The mixture was then stirred for 10 min, after which time a portion of rigorously dried anhydrous $ZnCl_2$[28] (0.56 g, 4.1 mmol) was added. Work up, followed by Kugelrohr-distillation, afforded (*S*)-pinanediol (1*S*)-(1-chloroethyl)boronate (**6**, 2.51 g, 79%), bp 58–60 °C (0.1 mmHg), (bp of 1*R*-enantiomer 80–82 °C (0.2 mmHg)),[29] $[\alpha]_D^{23}$ = +33.6 (*c* 2.35; toluene);. IR (film): 2928, 1456, 1412, 1394, 1380, 1339, 1284, 1240, 1076, 1006 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.82 (s, 3 H), 1.14 (d, *J* = 10.94 Hz, 1 H), 1.27 (s, 3 H), 1.40 (s, 3 H), 1.55 (d, *J* = 7.57 Hz, 3 H), 1.83–2.34 (m, 5 H), 3.55 (q, *J* = 7.51 Hz, 1 H), 4.34 (dd, *J* = 1.91 and 8.83 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 20.37, 23.75, 26.07, 26.23, 26.81, 28.21, 35.07, 38.06, 39.15, 51.05, 78.52, 86.73.

A solution of lithiohexamethyldisilazane in THF (15 mL) was prepared from hexamethyldisilazane (1.29 mL, 6.10 mmol) and *n*-BuLi (3.86 mL of 1.6 M solution in hexane, 6.2 mmol) as described above for preparation of **4**, and reacted at −78 °C under N_2 with (*S*)-pinanediol (1*S*)-(1-chloroethyl)boronate (**6**, 1.94 g, 6.1 mmol) to give crude (*S*)-pinanediol (1*R*)-[1-*bis*(trimethylsilyl)aminoethyl]boronate, which was not characterized and was used directly in the next step without further purification.

To a solution of the cesium salt of *Cbz*-phenylalanine (2.15 g, 5.0 mmol) in dry DMF (7 mL) at −15 °C was added isobutylchloroformate (0.65 mL, 5.0 mmol) and the mixture stirred under N_2 at −15 °C for 0.5 h. To this mixture was added the (*S*)-pinanediol (1*R*)-[1-*bis*(trimethylsilyl)aminoethyl]boronate (obtained above) dissolved in DMF (2 mL), followed by water (0.30 mL, 16.7 mmol). After stirring for 3 h at −15 °C the reaction mixture was diluted with EtOAc (50 mL), the EtOAc phase was washed with water (3 × 10 mL), dried ($MgSO_4$), and filtered. The EtOAc was removed in vacuo and the resulting solid purified by column chromatography ($CHCl_3$:MeOH (20:1) elution) to give (*S*)-pinanediol [1(*R*)-(*Cbz*-phenylalanylamino)ethyl]-boronate (**7**, 490 mg, 19%), mp 80–83 °C, $[\alpha]_D^{23}$ = +2.93 (*c* 3.45; $CHCl_3$). IR (KBr) 3339, 3329, 2924, 2868, 1701, 1630, 1525, 1405, 1238, 701 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.84 (s, 3 H), 1.12 (d, *J* = 7.45 Hz, 3 H), 1.20 (d, *J* = 10.62, 1 H), 1.28 (s, 3 H), 1.40 (s, 3 H), 1.72–2.40 (m, 5 H), 2.96–3.16 (m, 3 H), 4.29 (dd, *J* = 2.00 Hz and 8.67 Hz, 1 H), 4.39 (q, *J* = 7.32, 1 H), 5.07 (s, 2 H), 5.38 (bd, 1 H), 5.96 (bd, 1 H), 7.20–7.35 (m, 10 H). ^{13}C NMR ($CDCl_3$) δ 16.14, 23.77, 26.06, 26.87, 28.37, 32.85 (broad), 35.40, 37.90, 38.50, 39.33, 51.27, 55.19, 66.79, 77.58, 85.54, 126.92, 127.98, 128.16, 128.53, 128.60, 129.51, 136.23, 136.46, 155.97, 171.75.

To a stirred solution of BCl_3 (4.8 mL of 1 M solution in CH_2Cl_2) in CH_2Cl_2 (15 mL) at −78 °C was added (S)-pinanediol [1(*R*)-(*Cbz*-phenylalanylamino)ethyl]boronate (**7**, 480 mg, 0.95 mmol) as a solid. The mixture was stirred at −78 °C for 1 h, worked up, and the residue chromatographed ($CHCl_3$:MeOH (3:2) elution), recrystallized from acetone:water (as described above for **1**), then dried in vacuo to give [1(*R*)-(*Cbz*-phenylalanylamino)ethyl]boronic acid (**2**, 240 mg, 71%), mp 101–104 °C, $[\alpha]_D^{23}$ = −43.10 (*c* 1.82; CH_3OH). IR (KBr) 3402, 3303, 3029, 2959, 1708, 1679, 1677, 1655, 1648, 1629, 1540, 1454, 1397, 1242, 699 cm^{-1}. ^{1}H NMR (CD_3OD) δ 0.96 (d, *J* = 7.24 Hz, 3 H), 2.63 (q, *J* = 7.05 Hz, 1 H), 2.93–3.20 (m), 4.55 (t, *J* = 7.32 Hz, 1 H), 5.03 (s, 2 H), 7.23–7.36 (m, 10 H). ^{13}C NMR (CD_3OD) δ 15.78, 38.36, 42.39 (broad), 54.42, 67.65, 128.34, 129.00, 129.24, 129.67, 129.86. 130.64, 137.57, 138.23, 158.33, 178.31. Anal. calcd for $2C_{19}H_{21}BN_2O_4 \cdot 1H_2O$: C, 63.18; H, 6.14; N, 7.76; B 2.99. Found: C, 63.39; H, 6.16; N, 7.80; B 2.33.

Inhibition studies

The basic kinetic procedures applied were as described previously.[8a] The inhibition constants survey for boronic acids **1** and **2** were performed with *N*-benzoyl-l-arginine-4-nitroanilide (BAPNA)[30] as the standard reference substrate, and observing the rate of *para*-nitroaniline formation by the absorption changes at 410 nm. Inhibitors were added as solutions in CH_3CN. Assay mixtures consisted of papain (4.2 mM), BAPNA (1 mM), sodium phosphate (50 mM), NaCl (0.2 M), EDTA (5 mM), CH_3CN (10%), and inhibitor (concentrations up to 10 mM) at pH 6.5 and 25 °C. No papain inhibition by **1** or **2** was observed under these conditions.

Computational methods

The simulations were performed with the Biosym Discover[31] program, on a Silicon Graphics 240 GTX computer, using the Consistent Valence Force Field (CVFF).[32] The X-ray structures of papain[2a] (Protein Data Bank[13] entry 9PAP, version of January 1992, 1.65-Å resolution) and of the *Cbz*-Phe-Ala-CMK-papain[2b] covalent complex (Protein Data Bank[13] entry 6PAD, version of January 1992, 2.80-Å resolution) were used as starting points. A nonbonded cutoff of 10 Å with a switching function between 7.5 and 9 Å was used. The nonbonded pair list was updated every 20 cycles and a dielectric constant of 1 was used in all calculations. The energy of the system was minimized with respect to all 3 N Cartesian coordinates until the maximum derivative of 0.1 kcal mol^{-1} Å^{-1} was reached. During the molecular dynamics simulations, the whole enzyme, with the exception of a 15 Å radius region around Cys25, was kept fixed, as were water atoms more than 20 Å away from Cys25. The MD simulations were performed for 20 ps with an initial equilibrium period of 10 ps and a time-step of 1 fs. Hydrogens were added at the pH (6.5) used for the kinetic measurements. This protonated all Lys, Arg, and His side chains, and the N-terminal residue, and deprotonated the side-chain COOH groups of Glu, Asp, and the C-terminal amino acid. In the calculations on the boronic acid–enzyme complexes, a tetrahedral carbon atom was used to mimic the boron atom since, as yet, no force field parameters have been reported for boron. To set up the initial structure for the energy minimization, the boron-equivalent carbon was covalently bound to the S-atom of the thiol group of the active site cysteine 25. Charges on Cys25 and the enzyme-bound boronic acid were generated by a single-point AM1/ESP[33] calculations (MOPAC 93)[34] and scaled to fit those of the CVFF library.

The AM1 calculations were carried out on HP 9000/755 computer, according to the protocols described in the text.

Acknowledgments

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