Potential Transition State Analogue Inhibitors for the Penicillin-Binding Proteins†

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ABSTRACT: Penicillin-binding proteins (PBPs) are ubiquitous bacterial enzymes involved in cell wall biosynthesis. The development of new PBP inhibitors is a potentially viable strategy for developing new antibacterial agents. Several potential transition state analogue inhibitors for the PBPs were synthesized, including peptide chloromethyl ketones, trifluoromethyl ketones, aldehydes, and boronic acids. These agents were characterized chemically, stereochemically, and as inhibitors of a set of low molecular mass PBPs: *Escherichia coli* (EC) PBP 5, *Neisseria gonorrhoeae* (NG) PBP 3, and NG PBP 4. A peptide boronic acid was the most effective PBP inhibitor in the series, with a preference observed for a D-boroAla-based over an L-boroAla-based inhibitor, as expected given that physiological PBP substrates are based on D-Ala at the cleavage site. The lowest K_I of 370 nM was obtained for NG PBP 3 inhibition by Boc-L-Lys(Cbz)-D-boroAla (**10b**). Competitive inhibition was observed for this enzyme−inhibitor pair, as expected for an active site-directed inhibitor. For the three PBPs included in this study, an inverse correlation was observed between the values for log K_I with **10b** and the values for log(k_{cat}/K_m) for activity against the analogous substrate, and K_m/K_I ratios were 90, 1900, and 9600 for NG PBP 4, EC PBP 5, and NG PBP 3, respectively. These results demonstrate that peptide boronic acids can be effective transition state analogue inhibitors for the PBPs and provide a basis for the use of these agents as probes of PBP structure, function, and mechanism, as well as a possible basis for the development of new PBP-targeted antibacterial agents.

Penicillin-binding proteins (PBPs)[1] are ubiquitous bacterial enzymes that catalyze the final steps of cell wall biosynthesis (reviewed in refs *1*−*5*). In *Escherichia coli* these enzymes catalyze the reactions shown in Figure 1. Variations of the pentapeptide substrate are known in other bacteria and include D-IsoGln ($GluNH_2$) in place of γ-D-Glu, L-Lys in place of diaminopimelic acid (DAP), and bridging amino acids on the ϵ-amino group of Lys or DAP (reviewed in ref *6*). Additional variation of the carboxy terminus is associated with vancomycin resistance and includes replacing the terminal D-Ala in position 5 by D-Lac (*7*−*9*). In view of the variation naturally present in pentapeptide substrates, it is noteworthy for the development of peptide-mimetic PBP inhibitors that D-Ala is highly conserved as the fourth (penultimate) residue, and a high degree of specificity for this residue has been observed in model enzyme−substrate specificity studies (reviewed in ref *10*).

Bacteria possess a number of different PBPs (*E. coli* has at least eight) which can be divided into two groups, the low molecular mass (LMM) PBPs and the high molecular mass (HMM) PBPs (*3*). The LMM PBPs are composed of a single transpeptidase/hydrolase domain whereas the HMM PBPs possess an additional domain N-terminal to the PBP domain which is in some cases a penicillin-insensitive transglycosylase. Both LMM and HMM PBPs can each be further subdivided into three classes (A, B, and C) on the basis of sequence homology (*3*). Active site motifs within the PBPs include the highly conserved active site serine containing the SXXK motif and the S(Y)XN and K(H)T(S)G motifs (were X is a variable amino acid and parentheses denote a less frequently occurring substitution) (reviewed in refs *4* and *11*). Both HMM and LMM PBPs are inhibited by β-lactam antibiotics, but it is the HMM PBPs which are the lethal targets for β-lactam antibiotics, whereas LMM PBPs are not lethal targets. An additional feature of the PBPs is that the LMM PBPs give readily detectable enzyme activity against simple ~D-Ala-D-Ala-based substrates, whereas the HMM PBPs do not, for reasons which are not yet understood. The precise role of individual PBPs in the cell wall biosynthesis process is also poorly understood, although the broad outlines of this process have been emerging (reviewed in refs *5* and *12*). Although it is the HMM PBPs which are essential for bacterial cell viability, the LMM *E. coli* PBP 5 (EC PBP 5) is essential for normal cell shape (*13*−*15*).

The active site serine involved in PBP-catalyzed acyl-transfer reactions is the target of the β-lactam antibiotics,

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[1] Abbreviations: EC, *Escherichia coli*; DCM, dichloromethane; HMDS, 1,1,1,3,3,3-hexamethyldisilazane; HMM, high molecular mass; IBCF, isobutyl chloroformate; LMM, low molecular mass; NG, *Neisseria gonorrhoeae*; NMM, *N*-methylmorpholine; PBP, penicillin-binding protein; THF, tetrahydrofuran.

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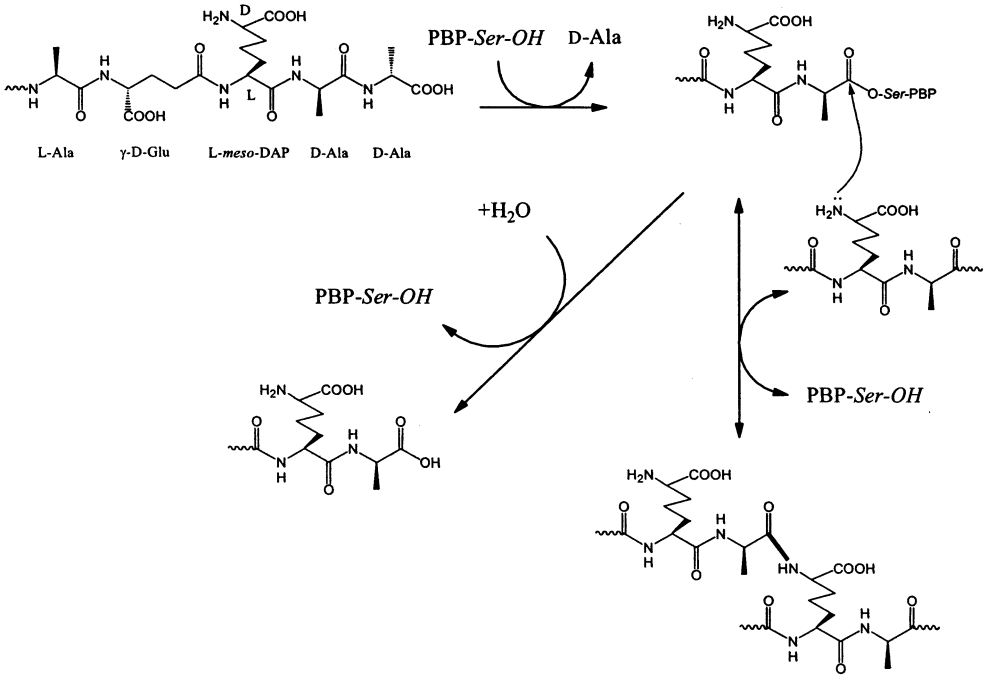


FIGURE 1: PBP-catalyzed bacterial cell wall biosynthesis reactions in *E. coli*. A PBP attacks the pentapeptide cell wall precursor to release the terminal D-Ala residue and form an acyl-enzyme intermediate. The acyl group can either be hydrolyzed ($+H_2O$) (hydrolase activity) or be transferred to an amino group on an adjacent pentapeptide (down arrow) to form a cross-link (transpeptidase activity). Cross-linked peptides are also cleaved by PBPs (up arrow) (endopeptidase activity).

which act as suicide substrates for the PBPs by forming stable acyl-enzyme intermediates (*16*−*18*). Bacterial resistance to β-lactam antibiotics has emerged primarily through two mechanisms: the production of β-lactam-degrading enzymes (β-lactamases) (*19*) and the accumulation of mutations in their HMM PBP targets which render them less susceptible to inhibition by β-lactam antibiotics (*4*, *20*). The emergence and spread of β-lactam-resistant bacteria is a serious public health issue, especially in view of the simultaneous emergence and spread of resistance to other classes of antibiotics. New antibacterial agents are urgently needed to combat drug-resistant bacteria. Alternative non-β-lactam inhibitors for the PBPs could provide such new antibacterial agents.

Transition state analogues are compounds that mimic the transition state of enzyme-catalyzed reactions and are often potent enzyme inhibitors (reviewed in refs *21* and *22*). Transition state analogues are also useful for mechanistic, structural, and functional studies of enzymes. Several classes of such inhibitors have been developed for the serine proteases, including peptide boronic acids (*23*−*25*), peptide aldehydes (*26*), peptide chloromethyl ketones (*27*, *28*), and peptide tri- and difluoromethyl ketones (*29*−*31*) (Figure 2). Given that both serine proteases and PBPs form transient serine-linked acyl-enzyme intermediates with their substrates during catalysis, it seems reasonable that one or more classes of serine protease inhibitors might be effective PBP inhibitors. In related studies, transition state analogues have been developed for the β-lactamases, including boronic acids (*32*−*36*) and phosphonates (*37*−*39*), and may provide new β-lactamase inhibitors to help combat β-lactam-resistant bacteria. A phosphonate monoester inhibitor for a PBP has been described briefly (*38*), but potent inhibitors have not emerged from these studies (*40*).

As part of our effort to develop new non-β-lactam inhibitors for the PBPs that could provide a basis for new antibacterial agents, we describe here the synthesis and characterization of a series of potential transition state analogue inhibitors for the PBPs. The classes of agents synthesized were peptide aldehydes, peptide trifluoromethyl ketones, peptide chloromethyl ketones, and peptide boronic acids. These compounds were characterized as inhibitors against a set of LMM PBPs with readily detectible enzyme activity against ~D-Ala-D-Ala-based substrates: EC PBP 5, *Neisseria gonorrhoeae* (NG) PBP 3, and NG PBP 4. This set includes one class A (EC PBP 5) and two class C (NG PBP 3 and NG PBP 4) LMM PBPs.

EXPERIMENTAL PROCEDURES

General. Tris, D-Ala, horseradish peroxidase (HRP, type X, 21 mg/mL as an ammonium sulfate suspension, 250 units/mg), ampicillin, and FAD were purchased from Sigma Chemical Co. (St. Louis, MO). Pig kidney D-amino acid oxidase (DAO, 6.0 mg/mL as an ammonium sulfate suspension, 12 units/mg) was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Boc-L-Lys(Cbz) and other protected amino acids were purchased from Sigma or Bachem (King of Prussia, PA). The two PBP substrates, diacetyl-L-Lys-D-Ala-D-Ala (Ac_2-KAA) and Boc-γ-D-Glu-L-Lys(Cbz)-D-Ala-D-Ala, and the synthetic intermediates, Boc-L-Lys(Cbz)-D-Ala and Boc-L-Lys(Cbz)-L-Ala, were synthesized using standard methods of solution-phase peptide synthesis (*41*, *42*). The fluorescent HRP substrate 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red, AR) was purchased from Molecular Probes (Eugene, OR). Reagents for syntheses of intermediates and inhibitors, general reagents, and solvents were purchased from Aldrich (St. Louis, MO). HPLC was performed on a Hewlett-Packard series 1050 system equipped with a diode array detector on C18 columns (Econosphere 3.2 × 250 mm, 5.0 μm particles) at 0.6 mL/min with an elution gradient of solvent A (0.1% TFA in water) for 1 min,

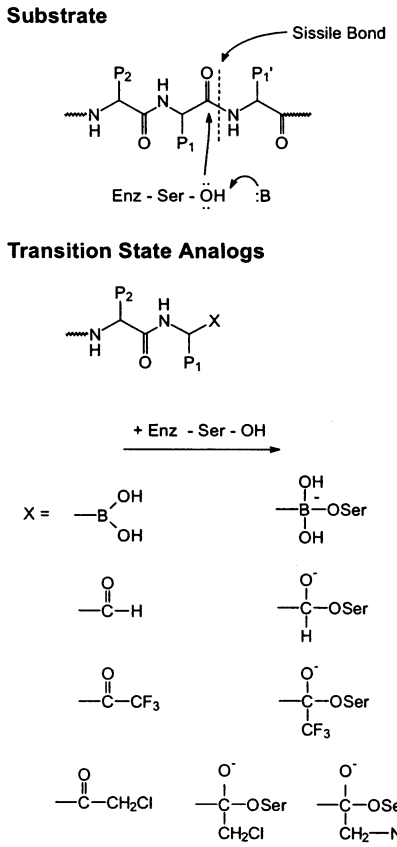
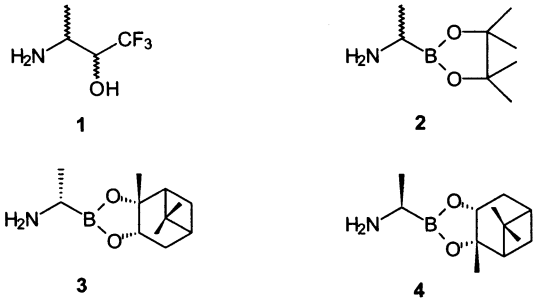


FIGURE 2: Substrate specificity and corresponding transition state analogues for serine proteases and related enzymes. The P2−P1−//−P1′ terminology is from Schechter and Berger (*59*), where // designates the proteolytic cleavage site. The four transition state analogue classes shown at the bottom can form tetrahedral adducts with the active site serine. In the case of chloromethyl ketones an additional alkylation reaction is possible with an active site nucleophile, which in the case of serine proteases is often the active site histidine.

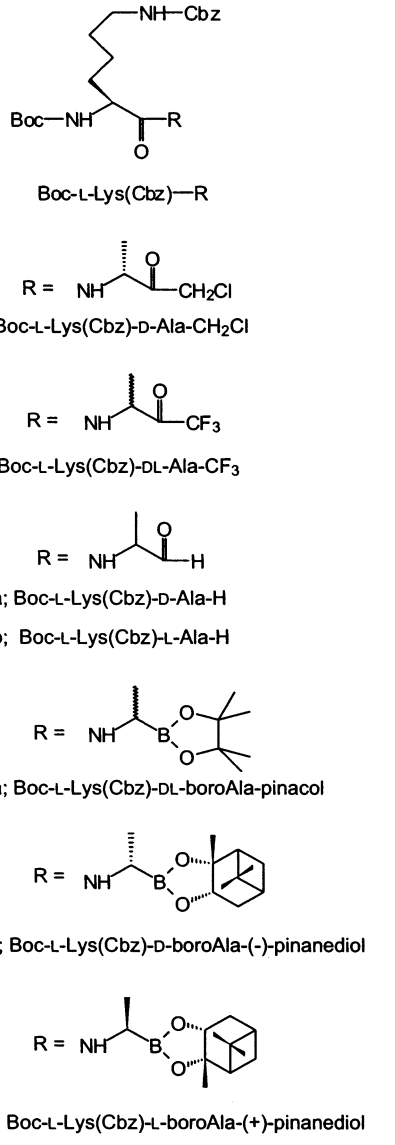
then 0−100% of solvent B (0.1% TFA in 30% water, 70% acetonitrile) in 10 min, and then 0−100% of solvent C (0.1% TFA in acetonitrile) in 5 min. Preparative HPLC was performed on a C18 column (Econosil, 22 × 250 mm, 10 μm particles) at 5 mL/min with an elution gradient of 10% of solvent B initially, then 10−100% of solvent B in 30 min, and then 100% of solvent B to 100% of solvent C in 30 min. TLC was performed on Riedel-deHaën Kieselgel 60 F 254 plates, layer thickness 0.2 mm, elution system 9:1 $CHCl_3$:MeOH, and detection with 0.5% ninhydrin in ethanol and heating. LCMS was carried out on an aQa ThemoQuest (Finnigan) system equipped with an atmospheric pressure ionization (API) electrospray source or by direct infusion. Proton spectra were obtained using a Bruker AC-250 spectrometer on samples in $CDCl_3$ as the solvent. Chemical shifts are reported in parts per million relative to tetramethylsilane as internal standard. Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN).

Boc-L-Lys(Cbz)-D-Ala-CH_2Cl (5). This compound was synthesized by the method of Kettner and Shaw (*43*). To a solution of 0.451 g of Boc-L-Lys(Cbz)-D-Ala-OH (1 mmol) and 0.12 mL of NMM (1.1 mmol) in 15 mL of THF at −15 °C was added 0.136 mL of IBCF (1.05 mmol). After 20 min at this temperature, the solid was filtered off and washed with 10 mL of THF. The combined THF solutions were treated with etheral diazomethane obtained from 2.15 g (10 mmol) of Diazald (Aldrich). After overnight at 0 °C, the reaction mixture was concentrated on a water bath, and the residue was diluted with 10 mL of diethyl ether and treated with 3 mL of 2 M hydrogen chloride in diethyl ether (6 mmol). After 30 min, the solvent was evaporated, and the residue was treated with ether (50 mL) and water (20 mL). The ether layer was separated, washed with 20 mL of water, dried over anhydrous magnesium sulfate, and evaporated to dryness to give 0.32 g of a brown crystalline mass. This material was dissolved in 10 mL of chloroform and treated with 20 mL of hexane to give a white precipitate which was

Scheme 1



Scheme 2



filtered and dried to give 0.23 g (0.48 mmol, 48%) of product (R_f 0.65). This product was 98% pure by HPLC. ^{1}H NMR: δ 1.3−1.9 [m, 6H, C$H_2\beta$, C$H_2\gamma$, C$H_2\delta$ (lysine)], 1.38 (d, J = 7.5 Hz, 3H, CH_3CH), 1.43 (s, 9H, *t*Bu), 3.19 [m, 2H, C$H_2\epsilon$ (lysine)], 4.06 [m, 1H, C$H\alpha$ (lysine)], 4.28 (s, 2H, COCH_2Cl), 4.72 (m, 1, CH$_3$CH), 4.94 (m, 1H, CbzNH), 5.10 (s, 2H, CH_2Ph), 5.24 (d, J = 7.25 Hz, 1H, BocNH), 6.97 (d, J = 7 Hz, 1H, LysNH), 7.35 (s, 5H, Ph). LCMS: *m*/*e* 483.8 ([M]$^+$). Anal. Calcd for $C_{23}H_{34}N_3O_6Cl$: C, 57.08; H, 7.08; N, 8.68; O, 19.83; Cl, 7.33. Found: C, 56.03; H, 7.15; N, 8.45; O, 19.20; Cl, 8.36.

Boc-L-*Lys(Cbz)-NHCH(CH$_3$)CH(OH)CF$_3$ (**6**).* To a solution of 1.14 g of Boc-L-Lys(Cbz) (3 mmol) and 0.88 mL of NMM (8 mmol) in 20 mL of THF maintained at −15 °C was added 0.39 mL of IBCF (3 mmol). After 20 min at this temperature, a solution of 0.539 g of 2-amino-1-(trifluoromethyl)-1-propanol hydrochloride (**1**) [prepared as described previously (*44*)] (3 mmol) in 3 mL of DMF was added to the reaction mixture. After 12 h at room temperature, the reaction mixture was concentrated under reduced pressure. The residue was diluted with 20 mL of diethyl ether and washed with 10 mL of 1 M hydrochloric acid, 10 mL of water, 10 mL of saturated aqueous sodium bicarbonate, and 10 mL of water. The ether layer was dried over anhydrous magnesium sulfate and evaporated to dryness to give 1.33 g (2.64 mmol, 88%) of product as a white foam (R_f 0.65). This product was used without further purification for the preparation of **7**. LCMS: *m*/*e* 506.3 ([M + H]$^+$).

Boc-L-*Lys(Cbz)*-DL-*Ala-CF$_3$ (**7**).* This compound was synthesized from **6** by the method of Imperiali and Abeles (*44*). To a solution of 0.224 g of Boc-L-Lys(Cbz)-NHCH(CH$_3$)-CH(OH)CF$_3$ (**6**) (0.44 mmol) in 5 mL of *tert*-butyl alcohol maintained at 0 °C was added 2.2 mL of a 0.6 M solution of sodium hydroxide (1.33 mmol) and 3.5 mL of 6% aqueous potassium permanganate (1.33 mmol). After 3 h, the reaction was quenched by the addition of 10 mL of methanol. After 10 min, the reaction was poured into a mixture of 25 mL of ether and 25 mL of water. The ether layer was separated and washed with 20 mL of 10% citric acid and 20 mL of water, dried over anhydrous magnesium sulfate, and evaporated to dryness to give 0.297 g of product as an oil. After being dried over P_2O_5 under vacuum, 0.218 g (0.43 mmol, 98%) of a glass product was obtained (R_f 0.66, broad). LCMS: *m*/*e* 504.3 ([M + H]$^+$) and 522.4 ([M + H + H$_2$O]$^+$). Anal. Calcd for $C_{23}H_{32}N_3O_6F_3$: C, 54.86; H, 6.41; N, 8.35; F, 11.32. Anal. Calcd for $C_{23}H_{34}N_3O_7F_3$ (hydrate): C, 52.97; H, 6.57; N, 8.06; F, 10.93. Found: C, 53.19; H, 6.72; N, 8.00; F, 10.71.

Boc-L-*Lys(Cbz)*-D-*Ala-N(CH$_3$)OCH$_3$ (**8a**).* A solution of 1.03 g of Boc-L-Lys(Cbz)-D-Ala (2.28 mmol) and 0.3 mL of NMM (2.7 mmol) in 25 mL of DCM maintained at −15 °C was treated with 0.33 mL of IBCF (2.6 mmol). After 30 min at this temperature, a mixture of 0.25 g of *N*,*O*-dimethylhydroxylamine hydrochloride (2.6 mmol) and 0.43 mL of triethylamine (3 mmol) in 15 mL of acetonitrile was added, which was prepared 10 min before addition with intensive shaking. After being stirred for 12 h the reaction mixture was concentrated under reduced pressure. The residue was diluted with 75 mL of ethyl acetate and washed with 15 mL of 1 M hydrochloric acid, 15 mL of water, 15 mL of saturated aqueous sodium bicarbonate, and 15 mL of water. The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness to give 1.15 g (2.33 mmol nominally, 102%, containing residual solvent as an impurity) of a syrup, which slowly crystallized upon storage (R_f 0.63). ^{1}H NMR: δ 1.3−1.9 [m, 6H, C$H_2\beta$, C$H_2\gamma$, C$H_2\delta$ (lysine)], 1.33 (d, J = 6.0 Hz, 3H, CH_3CH), 1.44 (s, 9H, *t*Bu), 3.19 [m, 5H, C$H_2\epsilon$ (lysine), CH_3N], 3.77 (s, 3H, CH_3ON), 4.13 [m, 1H, C$H\alpha$ (lysine)], 4.92 (m, 1H, CH$_3$CH), 5.00 (m, 1H, CbzNH), 5.09 (s, 2H, PhCH_2), 5.22 (d, J = 7.5 Hz, 1H, BocNH), 6.91 (d, J = 6.0 Hz, 1H, LysNH), 7.35 (s, 5H, Ph). LCMS: *m*/*e* 495.2 ([M + H]$^+$).

Boc-L-*Lys(Cbz)*-D-*Ala-H (**9a**).* Following the method of Fehrentz and Castro (*45*) and Nahm and Weinreb (*46*), 2 mL of 1 M lithium aluminum hydride in THF (2 mmol) was added to a solution of 0.495 g of Boc-L-Lys(Cbz)-D-Ala-N(OCH$_3$)CH$_3$ (**8a**) (1 mmol) in 10 mL of THF at 0 °C. By the end of the addition a thick gel was formed. After 30 min at 0 °C, 50 mL of ethyl acetate and 15 mL of 1 M hydrochloric acid were added to the reaction mixture. The ethyl acetate layer was separated and washed with 10 mL of water, 10 mL of saturated aqueous sodium bicarbonate, and 10 mL of water, dried over anhydrous magnesium sulfate, and evaporated to dryness to give 0.419 g (0.96 mmol, 96%) of product as an oil (R_f 0.56). ^{1}H NMR: δ 1.3−1.9 [m, 6H, C$H_2\beta$, C$H_2\gamma$, C$H_2\delta$ (lysine)], 1.33 (d, J = 7.25 Hz, 3H, CH_3CH), 1.44 (s, 9H, *t*Bu), 3.17 [m, 2, C$H_2\epsilon$ (lysine)], 4.13 [m, 1H, C$H\alpha$ (lysine)], 4.44 (m, 1H, CH$_3$CH), 5.00−5.15 (m, 3H, CbzNH, CH$_2$Ph), 5.31 (m, 1H, BocNH), 7.00 (m, 1H, LysNH), 7.35 (s, 5H, Ph), 9.50 (s, 1H, CHO). LCMS: *m*/*e* 436.3 ([M + H]$^+$).

Boc-L-*Lys(Cbz)*-L-*Ala-N(CH$_3$)OCH$_3$ (**8b**).* This compound was prepared as described for **8a** starting from 0.677 g of Boc-L-Lys(Cbz)-L-Ala (1.5 mmol). The yield was 0.713 g (1.44 mmol, 96%) of product as a syrup (R_f 0.63). ^{1}H NMR: δ 1.3−1.9 [m, 6H, C$H_2\beta$, C$H_2\gamma$, C$H_2\delta$ (lysine)], 1.33 (d, J = 6.0 Hz, 3H, CH_3CH), 1.44 (s, 9H, *t*Bu), 3.13 (s, 3H, CH_3N), 3.20 [m, 2H, C$H_2\epsilon$ (lysine)], 3.75 (s, 3H, CH_3ON), 4.12 [m, 1H, C$H\alpha$ (lysine)], 4.89 (m, 1H, CH$_3$CH), 5.08−5.30 (m, 4H, CbzNH, CH_2Ph, BocNH), 6.70 (d, J = 9 Hz, 1H, LysNH), 7.35 (s, 5H, Ph). LCMS: *m*/*e* 495.1 ([M + H]$^+$).

Boc-L-*Lys(Cbz)*-L-*Ala-H (**9b**).* This compound was prepared as described for **9a** by reduction of 0.495 g of Boc-L-Lys(Cbz)-L-Ala-N(OCH$_3$)CH$_3$ (**8b**) (1 mmol) to give 0.419 g (0.96 mmol, 96%) of product as an oil (R_f 0.56). ^{1}H NMR: δ 1.3−1.9 [m, 6H, C$H_2\beta$, C$H_2\gamma$, C$H_2\delta$ (lysine)], 1.33 (d, J = 7.25 Hz, 3H, CH_3CH), 1.44 (s, 9H, *t*Bu), 3.18 [m, 2H, C$H_2\epsilon$ (lysine)], 4.12 [m, 1H, C$H\alpha$ (lysine)], 4.45 (m, 1H, CH$_3$CH), 5.00−5.15 (m, 3H, CbzNH, CH_2Ph), 5.32 (d, J = 6 Hz, 1H, BocNH), 6.94 (m, 1H, LysNH), 7.35 (s, 5H, Ph), 9.52 (s, 1H, CHO). LCMS: *m*/*e* 436.2 ([M + H]$^+$).

*HPLC Purification and Stereochemical Analysis of Peptide Aldehydes **9a** and **9b**.* Peptide aldehydes prepared by reduction of Weinreb amides are reported to retain the optical integrity of the amino acid aldehyde residue (*45*). However, the crude aldehydes **9a** and **9b** demonstrated high backgrounds in the redox reaction-based PBP activity assays, and additional purification by preparative HPLC was required. Peptide aldehydes are highly susceptible to racemization, and the HPLC-purified peptide aldehydes were analyzed for racemization by conversion to the corresponding carboxylic acids by reduction with sodium borohydride and oxidation with potassium permanganate (*47*). This was followed by

hydrolysis with 6 N hydrochloric acid at 120 °C for 6 h, derivatization with Marfey's reagent, and separation of the D- and L-Ala diastereomeric Marfey's derivatives by analytical HPLC with detection at 340 nm (*48*, *49*). This analysis demonstrated 20% racemization of **9a** (to **9b**), and 20% racemization of **9b** (to **9a**).

*Pinacol 1-Amino-1-ethylboronate Hydrochloride (**2**) (*DL-*BoroAla-pinacol Hydrochloride).* This compound was prepared similarly to the trifluoroacetate salt as described by Kettner and Shenvi (*24*). To a solution of 2.25 g of 1-[bis-(trimethylsilyl)amino]ethylboronate (7.14 mmol) in 10 mL of ether at −80 °C was added 11 mL (22 mmol) of a 2 M solution of hydrogen chloride in ether. The reaction mixture was allowed to warm to room temperature and diluted with 25 mL of hexane. The precipitate formed upon hexane addition was filtered, washed with hexane, and dried to give 1.46 g (7.07 mmol, 99%) of a white crystalline solid. ^{1}H NMR: δ 1.279 (s, 12H, pinacolyl), 1.506 (d, J = 7.5 Hz, 3H, CH_3CHB), 2.92−3.10 (br s, 1H, CH_3CHB), 8.10−8.35 (br s, 3H, NH_3^+).

*Boc-*L*-Lys(Cbz)-*DL*-boroAla-pinacol (**10a**).* To a solution of 0.761 g of Boc-L-Lys(Cbz) (2.0 mmol) and 0.286 mL (2.6 mmol) of NMM in 10 mL of DCM at −20 °C was added 0.272 mL of IBCF (2.1 mmol). After 30 min at this temperature, 0.425 g of DL-boroAla-pinacol hydrochloride (**2**) (2.05 mmol) and 0.286 mL of NMM (2.6 mmol) were added to the reaction mixture. After 2 h at room temperature, the reaction mixture was diluted with 70 mL of ethyl acetate, washed with 20 mL of 1 M hydrochloric acid, 10 mL of water, 10 mL of saturated sodium bicarbonate solution, and 10 mL of water, dried over anhydrous magnesium sulfate, and evaporated to give 1.03 g (1.92 mmol, 96%) of product as a white solid foam. The product obtained showed a single spot on TLC (R_f 0.52). ^{1}H NMR: δ 1.15 [d, J = 7.25 Hz, 1.5H, CH_3CH (D- or L-boroAla)] and 1.16 [d, J = 7.25 Hz, 1.5H, CH_3CH (L- or D-boroAla)], 1.22 (s, 12H, pinacolyl), 1.3−1.9 [m, 6H, C$H_2\beta$, C$H_2\gamma$, C$H_2\delta$ (lysine)], 1.43 (s, 9H, *t*Bu), 2.91 (s, 1H, CH_3CHB), 3.17 [m, 2H, C$H_2\epsilon$ (lysine)], 4.13 [m, 1H, C$H\alpha$ (lysine)], 5.05−5.13 (m, 4H, CbzN*H*, CH_2Ph, BocN*H*), 6.85 [m, 0.5H, LysN*H* (D- or L-boroAla)], 6.95 [m, 0.5H, LysN*H* (L- or D-boroAla)], 7.35 (s, 5H, Ph). Mass (infusion): *m*/*e* 534.4 ([M + H]$^+$). Anal. Calcd for $C_{27}H_{44}N_3O_7B$: C, 60.79; H, 8.31; N, 7.88; B, 2.03. Found: C, 60.04; H, 8.69; N, 7.60; B, 1.83.

*(−)-Pinanediol (1S)-(1-Aminoethyl)boronate Hydrochloride (**3**) (*D*-BoroAla-(−)-pinanediol Hydrochloride).* To a solution of 3.1 mL of HMDS (14.5 mmol) in 15 mL of THF at −100 °C was added 9.1 mL of a 1.6 M solution of *n*-buthyllithium in hexane (14.5 mmol) down the cold side of the reaction flask. The reaction mixture was allowed to warm to room temperature and then cooled to −100 °C. This was followed by the addition of 3.07 g of (−)-pinanediol (1*R*)-(1-chloroethyl)boronate (prepared as described in ref *50*) (12.7 mmol). The reaction mixture was allowed to warm to room temperature and stand for 24 h. After this period, the reaction mixture was cooled to −80 °C followed by addition of 21.7 mL (43.5 mmol) of a 2 M solution of hydrogen chloride in ether. The reaction mixture was allowed to warm to room temperature and concentrated under reduced pressure to give a brown solid. This material was washed with 40 mL of ethyl acetate, then dissolved in chloroform (250 mL), and filtered. The resulting solution was dried under reduced pressure to give 2.2 g (8.5 mmol, 67%) of product. ^{1}H NMR: δ 0.83−2.4 (m, 16H, pinanyl), 1.52 (d, J = 7.25 Hz, 3H, CH_3CHB), 3.07 (br s, 1H, CH_3CHB), 4.39 (d, J = 8.5 Hz, 1H, C*H*OB), 8.23 (br s, 3H, NH_3^+).

Stereochemical Characterization of D*-BoroAla-(−)-pinanediol (**3**).* This was performed by NMR analysis of the acetyl derivative according to the method of Matteson et al. (*51*). TEA (63 μL, 0.45 mmol) was added to a solution of 40 mg of D-boroAla-(−)-pinanediol hydrochloride (**3**) (0.15 mmol) in 3 mL of DCM at −10 °C, followed by addition of 17 μL of acetyl chloride (0.23 mmol). After 1 h at room temperature, the reaction mixture was diluted with 7 mL of DCM and washed with 3 mL of 1 M hydrochloric acid, 3 mL of water, 3 mL of saturated sodium bicarbonate solution, and 3 mL of water. The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness to give 38 mg (0.14 mmol, 96%) of the product (−)-pinanediol (1*S*)-(1-acetamidoethyl)boronate as a glass. Recrystallization from 0.7 mL of acetonitrile gave 28 mg (0.10 mmol, 70%) of crystals. ^{1}H NMR: δ 9.058 to 8.409 (N*H*) ratio 25:1 (the same ratio as for the product before crystallization). This result demonstrates 4% of L-boroAla present in D-boroAla-(−)-pinanediol (**3**).

*Boc-*L*-Lys(Cbz)-*D*-boroAla-(−)-pinanediol (**10b**).* To a solution of 0.266 g of Boc-L-Lys(Cbz) (0.7 mmol) and 88 μL of NMM (0.8 mmol) in 5 mL of DCM at −20 °C was added 97 μL of IBCF (0.75 mmol). After 30 min at this temperature, 0.182 g of D-boroAla-(−)-pinanediol hydrochloride (**3**) (0.7 mmol) and 88 μL of NMM (0.8 mmol) were added, and the reaction mixture was allowed to warm to room temperature. After 2 h, the reaction mixture was diluted with 25 mL of ethyl acetate and washed with 10 mL of 1 M hydrochloric acid, 10 mL of water, 10 mL of saturated sodium bicarbonate solution, and 10 mL of water. The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness to give 0.388 g (0.67 mmol, 95%) of product as a white foam (R_f 0.67). ^{1}H NMR: δ 0.83−2.4 [m, 22H, pinanyl, C$H_2\beta$, C$H_2\gamma$, C$H_2\delta$ (lysine)], 1.20 (d, J = 7.25 Hz, 3H, CH_3CHB), 1.43 (s, 9H, *t*Bu), 3.08 (br s, 1H, CH_3CHB), 3.17 [m, 2H, C$H_2\epsilon$ (lysine)], 4.07 [m, 1H, C$H\alpha$ (lysine)], 4.29 (d, J = 7.25, 1H, C*H*OB), 4.95−5.20 (m, 4H, CbzN*H*, CH_2Ph, BocN*H*), 6.38 [br s, 0.04H, LysN*H* (L-boroAla, assigned as described below)] and 6.54 [br s, 1H, LysN*H* (D-boroAla, assigned as described below)], 7.35 (s, 5H, Ph). LCMS: *m*/*e* 586.4 ([M + H]$^+$). Anal. Calcd for $C_{31}H_{48}N_3O_7B$: C, 62.77; H, 8.26; N, 7.17; B, 1.85. Found: C, 62.77; H, 8.52; N, 6.72; B, 1.64.

*Chemical Shift Assignment for BoroAla NH Diastereomers in Boc-*L*-Lys(Cbz)-*DL*-boroAla-(−)-pinanediol.* (−)-Pinanediol (0.102 g, 0.6 mmol) was added to a solution of 0.32 g of Boc-L-Lys(Cbz)-DL-boroAla-pinacol (**10a**) (0.6 mmol) in 3 mL of wet (1% H_2O) acetonitrile. After 3 days at room temperature, the transesterification reaction was completed (TLC, starting R_f 0.52, product R_f 0.67). Acetonitrile was evaporated under reduced pressure, and the residue was dissolved in 20 mL of ethyl acetate and washed with saturated sodium chloride solution (3 × 10 mL). The ethyl acetate layer was dried with anhydrous magnesium sulfate and concentrated to dryness to give 0.37 g of the product Boc-L-Lys(Cbz)-DL-boroAla-(−)-pinanediol. This product contained residual pinacol and was purified by preparative HPLC to give an analytical sample. ^{1}H NMR: δ 6.39 to

6.55 (boroAla N*H*) ratio 1:1.28. After addition of an approximately equal amount of Boc-L-Lys(Cbz)-D-boroAla-(−)-pinanediol (**10b**) to the sample, NMR showed a δ 6.42 to 6.56 (boroAla N*H*) ratio of 0.26:1. Hence, the former signal was assigned to the boroAla N*H* of Boc-L-Lys(Cbz)-L-boroAla-(−)-pinanediol and the latter to the boroAla N*H* of Boc-L-Lys(Cbz)-D-boroAla-(−)-pinanediol.

*(+)-Pinanediol (1R)-(1-Aminoethyl)boronate Hydrochloride (**4**) (L-BoroAla-(+)-pinanediol Hydrochloride).* This compound was prepared as described for **3** using (+)-pinanediol as the chiral directing agent. To a solution of 2.56 mL of HMDS (12.9 mmol) in 5 mL of THF at −100 °C was added 8.1 mL of a 1.6 M solution of *n*-butyllithium in hexane (12.9 mmol) down the cold side of the reaction vessel. The reaction mixture was allowed to warm to room temperature and then cooled to −100 °C followed by the addition of 2.73 g of (+)-pinanediol (1*S*)-(1-chloroethyl)boronate [prepared as described previously (*50*)] (11.3 mmol). The reaction mixture was allowed to warm to room temperature and stirred for 48 h. After this period, the reaction mixture was cooled to −80 °C followed by the addition of 17.7 mL of 2 M hydrogen chloride in diethyl ether (35.5 mmol). The reaction mixture was allowed to warm to room temperature and concentrated under reduced pressure to give a brown solid. This residue was washed with 20 mL of ethyl acetate, then dissolved in chloroform, and filtered. The resulting solution was concentrated to dryness under reduced pressure to give 2.32 g (8.9 mmol, 79%) of product as a light brown foam.

*Stereochemical Characterization of L-BoroAla-(+)-pinanediol (**4**).* This compound was converted to the acetyl derivative for stereochemical characterization according to the method of Matteson et al. (*51*) as described above for **3**. TEA (84 μL, 0.6 mmol) was added to a solution of 52 mg of L-boroAla-(+)-pinanediol hydrochloride (**4**) (0.2 mmol) in 3 mL of DCM at −10 °C, followed by addition of 22 μL of acetyl chloride (0.3 mmol). After 1 h at room temperature, the reaction mixture was diluted with 7 mL of DCM and washed with 3 mL of 1 M hydrochloric acid, 3 mL of water, 3 mL of saturated sodium bicarbonate solution, and 3 mL of water. The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness to give 52 mg (0.196 mmol, 98%) of the product (+)-pinanediol (1*R*)-(1-acetamidoethyl)boronate as a glass. Recrystallization from 1 mL of acetonitrile gave 38 mg (0.143 mmol, 72%) of crystals. ^{1}H NMR: δ 8.59 to 9.26 (N*H*) ratio 1:55 (the same ratio for product before crystallization). This result demonstrates 2% of D-boroAla present in L-boroAla-(+)-pinanediol (**4**).

*Boc-L-Lys(Cbz)-L-boroAla-(+)-pinanediol (**10c**).* To a solution of 0.266 g of Boc-L-Lys(Cbz) (0.7 mmol) and 88 μL of NMM (0.8 mmol) in 5 mL of DCM at −20 °C was added 97 μL of IBCF (0.75 mmol). After 30 min at this temperature, 0.182 g of L-boroAla-(+)-pinanediol hydrochloride (**4**) (0.7 mmol) and 88 μL of NMM (0.8 mmol) were added, and the reaction mixture was allowed to warm to room temperature. After 2 h, the reaction mixture was diluted with 25 mL of ethyl acetate and washed with 10 mL of 1 M hydrochloric acid, 10 mL of water, 10 mL of saturated sodium bicarbonate solution, and 10 mL of water. The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness to give 0.408 g (99%) of product as a white foam (R_f 0.67). ^{1}H NMR: δ 0.83−2.4 [m, 22H, pinanyl, C$H_2\beta$, C$H_2\gamma$, C$H_2\delta$ (lysine)], 1.20 (d, J = 7.25 Hz, 3H, CH_3CHB), 1.43 (s, 9H, *t*Bu), 3.05−3.20 [m, 3H, CH$_3$C*H*B, C$H_2\epsilon$ (lysine)], 4.06 [m, 1H, C*H*α (lysine)], 4.28 (d, J = 7.5 Hz, 1H, C*H*OB), 4.95−5.20 (m, 4H, CbzN*H*, CH_2Ph, BocN*H*), 6.51 [br s, 1H, LysN*H* (L-boroAla, assigned as described below)], 7.35 (s, 5H, Ph). LCMS: *m*/*e* 586.4 ([M + H]$^+$). Anal. Calcd for $C_{31}H_{48}N_3O_7B$: C, 62.77; H, 8.26; N, 7.17; B, 1.85. Found: C, 62.71; H, 8.53; N, 6.74; B, 1.68.

Chemical Shift Assignment for BoroAla NH Diastereomers in Boc-L-Lys(Cbz)-DL-boroAla-(+)-pinanediol. Boc-L-Lys(Cbz)-DL-boroAla-pinacol was reesterified with (+)-pinanediol as described above for the chemical shift assignment for **10b**, and an analytical sample was prepared by HPLC. ^{1}H NMR: δ 6.53 to 6.64 (boroAla N*H*) ratio 1:2 (partially overlapping). (Note that HPLC purification enriched one of the stereoisomers, skewing the ratio away from 1:1.) After addition of an equal amount of Boc-L-Lys(Cbz)-L-boroAla-(+)-pinanediol to the sample, the δ 6.49 to 6.57 (boroAla N*H*) ratio was 1:5. Hence, the former signal was assigned to the boroAla N*H* of Boc-L-Lys(Cbz)-D-boroAla-(+)-pinanediol and latter to the boroAla N*H* of Boc-L-Lys(Cbz)-L-boroAla-(+)-pinanediol.

Penicillin-Binding Proteins. EC PBP 5 was expressed in *E. coli* and purified by Sepharose−ampicillin affinity chromatography as described previously (*52*). NG PBP 3 and NG PBP 4 were expressed in *E. coli* as fusion proteins with His$_6$-maltose-binding protein (H$_6$-MBP) in a modified version of pMAL-C2 expression vector (New England Biolabs, Beverly, MA) and purified from soluble fractions on a HiTrap chelating column (Amersham Biosciences, Piscataway, NJ) complexed with Ni^{2+}. Following cleavage of the PBP from H$_6$-MBP by tobacco etch virus protease C, the PBPs were purified by repassage over a Ni^{2+} HiTrap chelating column and elution with 15 mM imidazole. The proteins were concentrated to between 2 and 5 mg/mL, dialyzed against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10% glycerol, and frozen at −80 °C in small aliquots. Complete details of NG PBP 3 and NG PBP 4 expression and purification will be presented elsewhere.

PBP Inhibition Assays. PBP activity assays were performed as described previously (*53*, *54*). Stock solutions of inhibitors were prepared in 50% methanol/water. Enzyme assays were performed in a buffer comprised of 100 mM pyrophosphate, 100 mM NaCl, and 0.5 mg/mL alkylated BSA, at pH 8.5, with 5% methanol included as a cosolvent for solubilization of inhibitors. The 5% methanol had no effect on PBP activities. Assays to determine K_I values for PBP inhibitors were performed with 10 mM Ac$_2$-L-Lys-D-Ala-D-Ala as the substrate. This concentration is below the K_m of this substrate for all three PBPs included in this study (subsaturating conditions) (*54*; unpublished results). K_Is for inhibitors were determined by performing assays with serially diluted inhibitors (steps of two in inhibitor concentration) around the midpoint inhibitor concentration. For analysis of inhibition by peptide boronic acid esters, the boronic acid ester was preincubated in the assay buffer at least 15 min prior to addition of PBP to release the free boronic acid from the ester for binding to the enzyme (*24*). Assays were started by addition of PBP and incubated at 25 °C for 30−60 min. PBP activity was then inactivated and the D-Ala reaction

product quantitated using fluorescently detected DAO/HRP coupled enzyme assays (*53*). For inhibition at subsaturating substrate concentrations ([S] < K_m) the following equation will generally apply (except in the case of an uncompetitive inhibitor)

$$v = v_0/(1 + I/K_I) \quad (1)$$

where v is the observed enzyme-catalyzed rate, v_0 is the uninhibited rate, I is the inhibitor concentration, and K_I is the inhibitor dissociation constant. K_I values were obtained by fitting inhibition data with this equation by nonlinear regression using BMDP statistical software (SPSS Science, Chicago, IL).

*Kinetic Mechanism of Inhibition of NG PBP 3 by **10b**.* The most effective enzyme−inhibitor pair was NG PBP 3 inhibition by **10b**. To characterize the kinetic mechanism of inhibition, NG PBP 3 (210 pM) was incubated with a range of substrate concentrations [0−10 mM Boc-γ-D-Glu-L-Lys(Cbz)-D-Ala-D-Ala] in the absence and presence of compound **10b** at concentrations of 200, 400, and 600 nM.

RESULTS

Inhibitor Synthesis and Characterization. Inhibitors based on D-, L-, or DL-Ala (Scheme 2) were synthesized using established methods for these inhibitor classes. Elemental analysis of the chloromethyl ketone **5** gave a slightly higher than expected value for chlorine (8.36 found vs 7.33 expected), which is consistent with 1.25 wt % of residual $CHCl_3$ in the final product. Elemental analysis and LCMS data for the trifluoromethyl ketone **7**, as summarized in the Experimental Procedures, indicate that this compound was obtained as the trifluoromethyl ketone hydrate [−NHCH-$(CH_3)C(OH)_2CF_3$]. Elemental, LCMS, and NMR data for the other inhibitors were consistent with expected values. Stereochemical analysis was performed for both the aldehyde diastereomers (**9a** and **9b**) and the boronic acid diastereomers (**10b** and **10c**). The Weinreb reduction method for synthesizing aldehydes has been reported to result in products with high stereochemical purity (*45*). However, the initial aldehyde products contained impurities that interfered with the enzyme-catalyzed redox reactions used in the PBP assays, and HPLC purification of the two peptide aldehydes was required to remove the offending impurities. Peptide aldehydes are particularly sensitive to racemization, and stereochemical analysis of the peptide aldehydes after HPLC purification revealed about 20% racemization in both D- and L-Ala aldehyde products **9a** and **9b** used in the PBP inhibition study.

Three peptide boronic acid samples were synthesized for these studies; Boc-L-Lys(Cbz)-DL-boroAla-pinacol (**10a**), Boc-L-Lys(Cbz)-D-boroAla-(−)-pinanediol (**10b**), and Boc-L-Lys(Cbz)-L-boroAla-(+)-pinanediol (**10c**). Stereochemical analysis of D-boroAla (**3**) and L-boroAla (**4**) revealed 4% and 2% of the other boroAla stereoisomer, respectively, consistent with the use of pinanediol stereoisomers as chiral-directing agents in boronic acid chemistry (*50*, *55*). After incorporation of **3** and **4** into **10b** and **10c,** respectively, NMR analysis revealed that **10b** contained 4% of L-boroAla, and **10c** contained 2% of D-boroAla, demonstrating that racemization of boroAla did not occur during coupling. These results also demonstrate the final stereochemical purity of **10b** and **10c**.

Table 1: K_I Values for Transition State Analogue Inhibitors against NG PBP 3, NG PBP 4, and EC PBP 5

	inhibitor Boc-L-Lys(Cbz)-R where R =	K_I[a] NG PBP 3	NG PBP 4	EC PBP 5
5	D-Ala-CH_2Cl	~1 mM	>1 mM	>1 mM
7	DL-Ala-CF_3	60 (10) μM	>1 mM	>1 mM
9a	D-Ala-H (20% L)	60 (3) μM	>1 mM	>1 mM
9b	L-Ala-H (20% D)	79 (4) μM	ND	ND
10a	DL-boroAla-pinacol	0.43 (0.04) μM	30 (4) μM	14 (3) μM
10b	D-boroAla-(−)-pinanediol (4% L)	0.37 (0.03) μM	34 (8) μM	16 (5) μM
10c	L-boroAla-(+)-pinanediol (2% D)	5 (1) μM	>1 mM	290 (90) μM

[a] Standard errors are given in parentheses. ND = not determined.

Enzyme Inhibition. The synthesized compounds were tested as inhibitors for three PBPs: NG PBP 3, NG PBP 4, and EC PBP 5. Once the approximate range of inhibition was established for each inhibitor against each enzyme, a set of more focused assays with inhibitor concentrations centered around the midpoint of inhibition were performed. Inhibition data were then analyzed to give K_I values for each enzyme−inhibitor pair (Table 1). The peptide chloromethyl ketone (**5**), expected to be predominantly the D-Ala isomer, showed little or no inhibition after 30 min incubation with the PBPs, and thus no additional effort was made to determine the chiral purity of this compound or to synthesize the L-Ala diastereomer. The peptide trifluoromethyl ketone (**7**), which was expected to be a diastereomeric mixture of D- and L-Ala-trifluoromethyl ketones, showed modest inhibition of NG PBP 3. Stereospecific methods for the synthesis of trifluoromethyl ketones have not been developed to our knowledge, and no further analysis of D and L specificity was made with this agent. The D- and L-Ala-based peptide aldehydes **9a** and **9b** initially gave high background fluorescence in our redox-based assay for PBP activity, which precluded activity-based PBP inhibition studies. HPLC purification of **9a** and **9b** removed the high assay background but resulted in about 20% racemization of the alanine aldehyde (Ala-H) residue for both compounds. The two aldehydes, **9a** and **9b**, gave similar levels of inhibition against NG PBP 3, most likely because of the 20% diastereomeric impurity between the two samples. Given that the aldehydes were not outstanding inhibitors, no additional effort was made to improve the diastereomeric purity of the peptide aldehyde preparations.

The peptide boronic acid was initially synthesized as the diastereomer **10a** and found to be a good inhibitor for all three PBPs. Additional effort to stereospecifically prepare the individual D-boroAla and L-boroAla enantiomers appeared warranted and was performed using (+)- and (−)-pinanediol as a stereospecific-directing group as developed by Matteson and co-workers (*50*, *55*). NMR analysis of intermediates **3** and **4** and products **10b** and **10c** revealed a high level (96 and 98%, respectively) of diastereomeric purity. Characterization of **10b** and **10c** as PBP inhibitors demonstrated that the D-boroAla-based inhibitor **10b** was a >10-fold more effective inhibitor than the L-isomer-based **10c** (Table 1), with a K_I for NG PBP 3 of 370 nM.

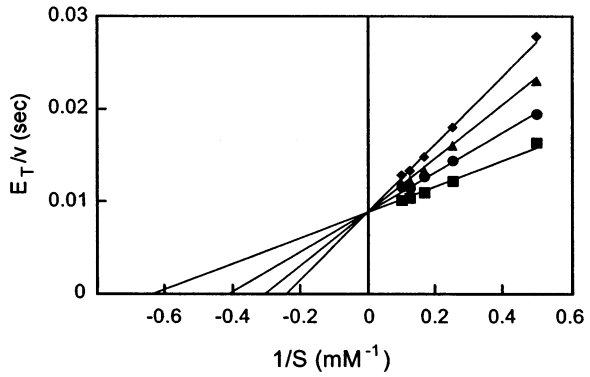


FIGURE 3: $1/v$ vs $1/S$ plots for NG PBP 3 inhibition by **10b**. The substrate was Boc-γ-D-Glu-L-Lys(Cbz)-D-Ala-D-Ala (0−10 mM). Data were collected with 210 pM NG PBP 3 and 0 (■), 200 nM (●), 400 nM (▲), and 600 nM (◆) **10b**. Data were analyzed by fitting with competitive, noncompetitive, and uncompetitive models. The competitive model was found to be statistically best, and the best fit lines for the competitive model are included.

Kinetic Mechanism of Inhibition. The kinetic mechanism for inhibition of NG PBP 3 by **10b** was assessed by performing assays over a range of substrate and inhibitor concentrations. Data from this experiment are presented in double-reciprocal form in Figure 3. These data were analyzed by nonlinear regression with models for competitive, noncompetitive, and uncompetitive inhibition. The model for competitive inhibition gave the best fit to a high degree of statistical significance ($P < 0.05$) (Figure 3).

DISCUSSION

Transition state analogues are often potent enzyme inhibitors and can also be valuable tools for studies on the catalytic mechanism, substrate specificity, and structure−function correlations of target enzymes (*21*, *22*). The development of transition state analogue inhibitors for the PBPs would be of great utility for such studies and could also provide a basis for the development of new antibacterial agents. The goal of the present study was to prepare a series of potential peptide-mimetic transition state analogue inhibitors for the PBPs and to examine their inhibition of several available LMM PBPs. Boc-L-Lys(Cbz)-D-Ala was chosen as the parent structure, since it is a simple dipeptide homologue of the natural PBP substrate up to the cleavage site (Figure 1), and the orthogonal Boc and Cbz protecting groups on L-Lys provide for further elaboration of inhibitors. Lysine was used in place of diaminopimelic acid for several reasons. Foremost is that suitably protected DAP derivatives are not readily available. Also, Lys is often found in bacterial cell walls, and the difference of a remote carboxy group between Lys and DAP did not appear likely to have a major effect on the observation of inhibition. Established methods were used to prepare peptide chloromethyl ketones, peptide trifluoromethyl ketones, peptide aldehydes, and peptide boronic acids for testing as potential PBP inhibitors. Inhibitors were characterized by NMR, elemental analysis, LCMS, and/or chemical derivatization to establish the identity of each agent and, in some cases, stereochemical purity. The present study has benefitted greatly from the substantial research devoted to the development of transition state analogue inhibitors for the serine proteases, especially the pioneering studies of Matteson (*23*, *50*, *51*) and Kettner (*24*) on peptide boronic acids.

Peptide boronic acids were found to be the most effective inhibitors and were the only compounds to inhibit all three PBPs included in this study (Table 1). NG PBP 3 was sensitive to all of the inhibitors, although inhibition with the chloromethyl ketone (**5**) was very weak at 1 mM after 30 min incubation with the enzyme. After observing good inhibition by **10a**, a mixture of diastereomers, D- and L-boroAla-based enantiomers (**10b** and **10c**) were prepared stereospecifically as described by Matteson and co-workers (*50*, *55*) in 96% and 98% diastereomeric purity, respectively, and these compounds were tested as PBP inhibitors (Table 1). The D-boroAla-based **10b** gave significantly lower K_Is for all three PBPs, as expected given D-Ala at the cleavage site in the natural PBP pentapeptide substrates (Figures 1 and 2). If the apparent inhibition by the L-boroAla isomer (**10c**) was due to the presence of 2% of the D-boroAla isomer (**10b**), then the ratio of K_Is for **10c**/**10b** should be equal to about 50. However, ratios of 14, >30, and 18 are observed for NG PBP 3, NG PBP 4, and EC PBP 5, respectively. This observation suggests that, at least for NG PBP 3 and EC PBP 5, complete stereospecificity for D- vs L-based inhibitors may not be observed, and some inhibition by the L-boroAla isomer may be evident.

PBPs and the mechanistically related β-lactamases often exhibit complicated substrate kinetics due to multiple substrate binding sites (*56*−*58* and references cited therein). Such complicated substrate kinetics make kinetic characterization of an inhibitor difficult. In this study the tetrapeptide substrate Boc-γ-D-Glu-L-Lys(Cbz)-D-Ala-D-Ala, which has relatively good kinetic properties for NG PBP 3 ($K_m = 1.6$ mM, $k_{cat} = 115\ s^{-1}$) and, more importantly, lacks apparent substrate inhibition for NG PBP 3 (unpublished results), allowed the kinetic mechanism of **10b** to be assessed. Data from the kinetic characterization experiment are presented in Figure 3 in double-reciprocal form. These data demonstrate that **10b** is a competitive inhibitor of NG PBP 3 and that **10b** is therefore an active site-directed inhibitor.

The theory of transition state analogues (reviewed in ref *21*) predicts an inverse correlation between the K_I for a transition analogue and the k_{cat}/K_m for the corresponding enzyme-catalyzed reaction:

$$K_I \propto 1/(k_{cat}/K_m) \qquad (2)$$

Such a relationship has been observed in a number of examples, both in cases when inhibitors and substrates were varied, as well as cases where the enzyme was altered by site-directed mutagenesis (reviewed in ref *21*). In the present case the most effective PBP inhibitor, boronic acid **10b**, was assessed against three different enzymes (PBPs). Among the three enzymes included in this study, NG PBP 3 was the most sensitive, EC PBP 5 was of intermediate sensitivity, and NG PBP 4 was the least sensitive to inhibition by **10b**. This generally parallels the enzymatic activity of these enzymes against the analogous substrate, Boc-L-Lys(Cbz)-D-Ala-D-Ala (*54*; unpublished results), and a comparison of kinetic parameters for Boc-L-Lys(Cbz)-D-Ala-D-Ala hydrolysis by these enzymes with the K_I for inhibition by **10b** reveals an inverse relationship between log K_I values and log(k_{cat}/K_m) values (Figure 4). However, the slope of this relationship

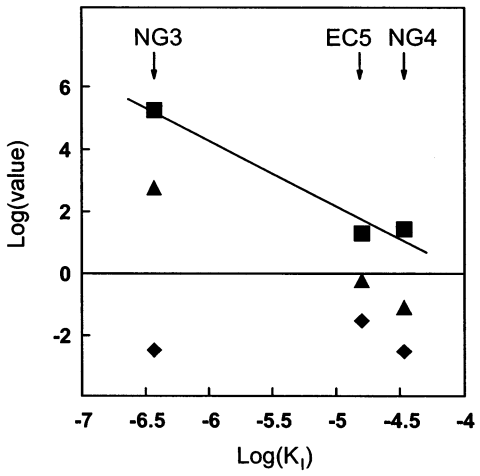


FIGURE 4: Correlation between log K_I values for **10b** and log(k_{cat}/K_m), log K_m, and log k_{cat} values for the analogous substrate [Boc-L-Lys(Cbz)-D-Ala-D-Ala] against the three PBPs: NG PBP 3 (NG3), NG PBP 4 (NG4), and EC PBP 5 (EC5). Log K_I values (Table 1) are plotted on the *x*-axis vs log values for k_{cat}/K_m (■), k_{cat} (▲), and K_m (◆) (*54*; unpublished results) on the *y*-axis. Arrows are used to indicate the PBP associated with a given set of points, and the line illustrates the correlation between log K_I and log(k_{cat}/K_m) values between the three PBPs.

in the present case is −2, rather than −1 predicted by theory (eq 2). The underlying reasons for this deviation from theory are presently unclear but might be related to a possible change in rate-determining step from acylation for EC PBP 5 and NG PBP 4 to deacylation for NG PBP 3 (unpublished observations). Another less quantitative criterion of transition state analogue behavior is the ratio of the K_I for a transition state analogue with the K_m for the analogous substrate. Transition state analogues are expected to have significantly lower K_I values than the substrate K_m values. In the present case with **10b** as the inhibitor and Boc-L-Lys(Cbz)-D-Ala-D-Ala as the substrate, the K_m/K_I ratios were 90 for EC PBP 5, 1900 for NG PBP 4, and 9600 for NG PBP 3. Therefore, even in the case of the weakest enzyme−inhibitor pair in this series (EC PBP 5−**10b**), nearly 100-fold tighter binding was observed for the transition state analogue over the corresponding substrate. The inverse correlation between K_I and k_{cat}/K_m values, as well as the low K_I values compared to K_m values, is evidence in favor of the peptide boronic acid **10b** acting as a transition state analogue for the PBPs studied here.

In summary, a series of potential transition state analogues for the PBPs were synthesized and characterized chemically and as PBP inhibitors. The most effective inhibitor, the peptide boronic acid Boc-L-Lys(Cbz)-D-boroAla (**10b**), was found to competitively inhibit NG PBP 3. A correlation between enzyme activity and inhibition was observed, and K_Is were substantially lower than K_ms, all observations consistent with **10b** acting as a transition state analogue inhibitor for the three PBPs used in this study. These results demonstrate that peptide boronic acids based on D-boroAla can be effective active site-directed inhibitors for the PBPs. In addition to their potential utility as PBP inhibitors, transition state analogues are also expected to be useful tools for investigating the catalytic mechanism and substrate recognition in the PBPs. Extension of these efforts to the development of effective new inhibitors for the HMM lethal target PBPs could provide a basis for the development of non-β-lactam inhibitors of the PBPs as novel antibacterial agents.

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