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Direct Selection for Catalysis from Combinatorial Antibody Libraries Using a Boronic Acid Probe: Primary Amide Bond Hydrolysis

Changshou Gao, Brian J. Lavey, Chih-Hung L. Lo, Anita Datta, Paul Wentworth, Jr., and Kim D. Janda*

Contribution from the Department of Chemistry, The Scripps Research Institute and the Skaggs Institute for Chemical Biology, 10550 N. Torrey Pines Road, La Jolla, California 92037

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Abstract: This report describes a joint hybridoma and combinatorial antibody library approach to elicit catalysts for primary amide bond hydrolysis. By immunization with a trigonal boronic acid hapten **3a** and construction of a Fab (antigen-binding fragment) library, a diastereoselective catalyst for hydrolysis of the tripeptide primary amide substrate **1a** was selected. In contrast, no antibody catalyst was isolated by standard hybridoma methods of monoclonal antibody production following immunizations with hapten **3a**. The active Fab, BL25, obeys Michaelis−Menten kinetic behavior (k_{cat}/k_{uncat} *ca.* 4×10^4, $K_m = 150\ \mu$M) and is competitively inhibited by a boronic acid hapten analog **3b** ($K_i = 9\ \mu$M). Kinetic and binding studies both point to Fab selection of a hydrated tetrahedral anionic form of the boronic acid hapten **3a** which serves to mimic the putative transition state **4** for catalysis of water addition to the primary amide bond. Fab-BL25 exhibits exquisite substrate selectivity, as a methyl ester analog of **1a** is not accepted as a substrate. This work emphasises the power of the direct selection strategy when linked to screening of antibody combinatorial libraries and discloses the utility of boronic acids as haptens in acyl transfer processes.

Since the first reports of antibody catalysis,[1] a defining tenet for catalyst generation has been the mimicry and incorporation of a reaction's transition-state stereoelectronics into hapten design.[2] Success necessarily depends upon accurate assumptions of mechanistic details, the extent to which the hapten is a model of the transition state and how the immune system responds to the antigen. While this approach has proven to be largely successful for over 50 chemical reactions, there are some transformations which have proved resistant to this methodology. One example of this is the unassisted hydrolysis of an amide bond, a biochemical process of extreme importance.[3]

By utilizing our direct selection for catalysis approach[4] coupled with an α-amino boronic acid hapten **3a**, an antibody Fab that catalyzes efficient regio- and stereoselective primary amide bond hydrolysis of tripeptide **1a** to carboxylic acid **2a** has been isolated from a combinatorial antibody library (Scheme 1).

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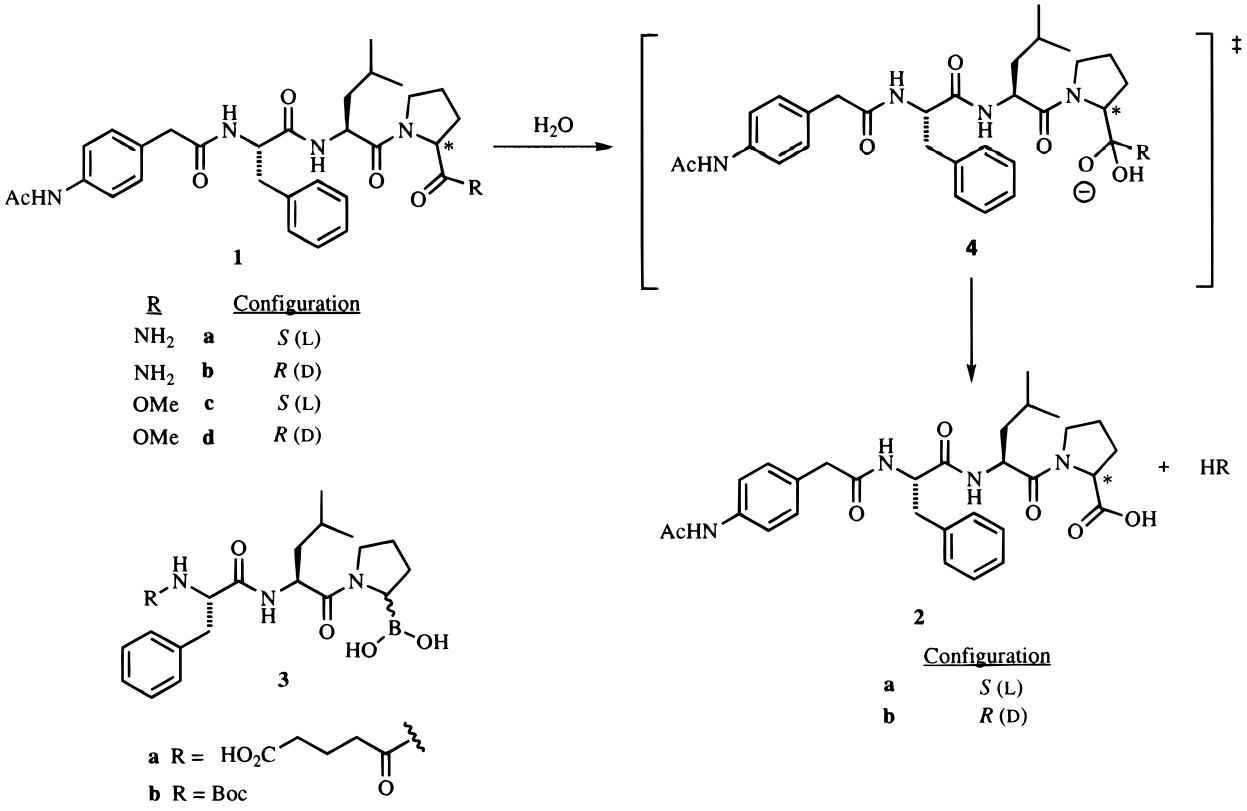
Scheme 1. α-Amino Boronic Acid Hapten **3a** Was Utilized in a Direct Selection Strategy from Antibody Combinatorial Libraries To Elicit Antibody-Fab Catalysts for Primary Amide Bond Hydrolysis of Either **1a** or **1b**



Experimental Section

Hapten and Substrate Synthesis. General Procedures. Unless otherwise stated, all reactions were performed under an inert atmosphere with dry reagents and solvents and flame-dried glassware. Analytical thin-layer chromatography (TLC) was performed using 0.25 mm coated silica gel Kieselgel 60 F_{254} plates. Visualization of the chromatogram was by UV absorbance, methanolic sulfuric acid, aqueous potassium permanganate, iodine, and *p*-anisaldehyde. Liquid chromatography was performed using compressed air (flash chromatography) with the indicated solvent system and silica gel 60 (230−400 mesh). Preparative TLC was performed using Merck 1 mm coated silica gel Kieselgel 60 F_{254} plates. ^{1}H NMR spectra were recorded on either a Bruker AM-250 or a Bruker AMX-400 spectrometer. Chemical shifts are reported in parts per million (ppm) on the δ scale from an internal standard. ^{13}C NMR (proton decoupled) spectra were recorded on a Bruker AMX-500 spectrometer at 125 MHz. ^{31}P NMR (proton decoupled) spectra were recorded on a Bruker AMX-400 spectrometer at 101 MHz. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-VSE mass spectrometer.

1-((1,1-Dimethylethoxy)carbonyl)pyrrolidine-2-boronic Acid 6.[5] The title compound was prepared in two steps from Boc-pyrrole. Step one involves the synthesis of 1-(1,1-dimethylethoxy)carbonyl)pyrrole-2-boronic acid[6] by reaction of Boc-pyrrole **5**, lithium tetramethylpiperidine (52 mmol), and triethyl borate (30 mL, 176 mmol) in THF (100 mL) at −78 °C. The product was isolated as a white crystalline solid (8.9 g, 85%): mp 100−101 °C (lit. mp 101.0−101.5 °C); ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.55 (s, 9H), 6.37 (t, J = 3 Hz, 1H), 7.05 (dd, J = 1.6, 3.2 Hz, 1H), 7.15 (s, 2H), 7.44 (dd, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 27.9, 85.5, 112.0, 127.0, 128.7, 152.0; FABMS *m*/*z* 212 (MH^+). In step two, a stirred suspension of the pyrrole from above (6.15 g, 24 mmol) and Adam's catalyst (500 mg) in ethyl acetate (EtOAc) (100 mL) was hydrogenated at 50 psi for 8 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated *in vacuo* to give **6** as a white solid (11 g, 99%): mp 101−102 °C (lit. mp 101 °C); ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.42 (s, 9H), 1.6−2.15 (m, 5H), 3.1−3.6 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) 25.1, 25.7, 28.4, 45.6, 46.2, 78.6, 154.5; HRFABMS calcd for $C_9H_{18}BNO_4$ 215.1329, obsd 215.1328.

(1*S*,2*R*,3*R*,5*S*)-Pinanediol 1-((1,1-Dimethylethoxy)carbonyl)pyrrolidine-2-boronate 7. A solution of **6** (1.52 g, 7.1 mmol) and (1*S*,2*R*,3*R*,5*S*)-(+)-pinanediol (1.36 g, 8.0 mmol) in ether (25 mL) was stirred at room temperature for 2 h. Removal of the solvent *in vacuo* and flash chromatography gave **7** as a 1:1 mixture of diastereomers, which were not separated (1.2 g, 85%): ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.52 (s, 3H), 1.10 (s, 9 H), 1.61 (s, 3H), 1.2−2.2 (m, 8H), 3.1−3.6 (m, 3H), 6.37 (t, J = 3 Hz, 1H), 7.05 (dd, 1.6, 3.2 Hz, 1H), 7.15 (s, 2H), 7.44 (dd, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.9, 26.6, 27.1, 27.3, 28.4, 28.6, 28.8, 36.0, 38.2, 39.9, 85.5, 112.0, 127.0, 128.7, 152.0; HRFABMS calcd for $C_{19}H_{32}BNO_4$ 349.2424, obsd 349.2427.

(1*S*,2*R*,3*R*,5*S*)-Pinanediol Pyrrolidine-2-boronic Acid 8. A solution of **7** (224 mg, 0.64 mmol) in ether (90 mL) was treated with dry HCl at 0 °C for 35 min. The mixture was then stirred at room temperature. After the mixture was cooled on ice, a precipitate formed, which was collected and washed with cold ether. This yielded **8** as a white crystalline solid (113 mg, 62%): mp 248 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.82 (s, 3H), 1.14 (d, 1H), 1.30 (2, 3H), 1.48 (s, 3H), 1.85−2.15 (m, 6H), 2.15−2.50 (m, 3H), 3.20 (bs, 1H), 3.45 (bs, 2H), 4.40 (d, 1H), 8.80 (bs, 1H), 10.56 (bs, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.9, 24.5, 26.5, 27.0, 27.2, 28.5, 34.9, 28.1, 39.4, 45.8, 51.2, 79.0, 87.8; HRFABMS calcd for $C_{14}H_{24}BNO_2$ 249.1900, obsd 249.1903.

Boc-Phe-Leu-boro-Pro-OPinane 9. To a stirred solution of Boc-Phe-Leu-OH (200 mg, 0.25 mmol) in DCM (5 mL) was added EDC (100 mg, 0.37 mmol), hydroxybenzotriazole (85 mg, 0.37 mmol), and **8** (125 mg, 0.37 mmol). The mixture was stirred overnight at room temperature and then evaporated to dryness *in vacuo*. The residue was purified by preparative TLC to give the title compound as a viscous oil (100 mg, 85%): ^{1}H NMR (250 MHz, CD_3OD) δ 0.84 (m, 4H), 0.90 (d, 3H), 0.94 (d, 3H), 1.14, (s, 9H), 1.17 (d, 1H), 1.30 (2, 3H), 1.48 (s, 3H), 1.80−2.20 (m, 6H), 2.20−2.50 (m, 4H), 2.84 (m, 1H),

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3.15 (bs, 1H), 3.20 (m, 1H), 3.45 (bs, 2H), 3.68 (m, 1H), 4.35 (d, 1H), 4.67 (dd, 2H), 8.80 (bs, 1H), 10.56 (bs, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 22.0, 22.2, 23.7, 23.9, 24.5, 25.1, 26.3, 27.0, 27.1, 27.2, 28.5, 34.0, 34.9, 35.8, 38.8, 39.4, 41.7, 45.8, 47.3, 50.8, 51.2, 55.7, 79.7, 87.9, 98.9, 127.7, 129.4, 129.5, 130.3, 138.2, 172.2, 173.5, 176.7; HRFABMS calcd for $C_{34}H_{52}BN_3O_6$ 609.3949, obsd 609.6043.

H_2N-Phe-Leu-boro-Pro-OPinane 10. To a stirred solution of **9** (125 mg, 0.37 mmol) in DCM (2 mL) was added TFA (1 mL), and the reaction mixture was stirred at room temperature for 30 min. The mixture was then evaporated to dryness *in vacuo*. The residue was used without further purification.

Glutaroyl-Phe-Leu-boro-Pro-OPinane 11. To a stirred solution of **10** (100 mg, 0.33 mmol) and DMAP (87 mg, 0.80 mmol) in DCM (5 mL) was added glutaric anhydride (27 mg, 0.35 mmol). The reaction mixture was stirred overnight and then concentrated *in vacuo* to give a crude oil, which was purified by preparative TLC to yield the title compound as a white solid (157 mg, 68%): ^{1}H NMR (250 MHz, CD_3OD) δ 0.84 (m, 4H), 0.90 (d, 3H), 0.94 (d, 3H), 1.17 (d, 1H), 1.30 (2, 3H), 1.48 (s, 3H), 1.78−2.15 (m, 12H), 2.20−2.50 (m, 4H), 2.84 (m, 1H), 3.15 (bs, 1H), 3.20 (m, 1H), 3.45 (bs, 2H), 3.68 (m, 1H), 4.35 (d, 1H), 4.67 (dd, 2H), 8.80 (bs, 1H), 10.56 (bs, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 22.0, 22.2, 23.7, 23.9, 24.5, 25.1, 26.5, 27.0, 27.1, 27.2, 28.5, 34.0, 34.9, 35.8, 38.8, 39.4, 41.7, 45.8, 47.3, 50.8, 51.2, 55.7, 79.7, 87.9, 98.9, 127.7, 129.4, 129.5, 130.3, 138.5, 172.4, 173.3, 175.2, 178.2; HRFABMS calcd for $C_{34}H_{50}BN_3O_7Cs$ 756.2802, obsd 756.2826.

Antibody Library Production and Panning Procedures. Hybridoma Methodology. Monoclonal antibodies were generated as described previously.[7]

Combinatorial Library Generation. 1. Preparation of cDNA Template. Total RNA was purified from a mouse spleen that had been hyperimmunized with the KLH-**3a** bioconjugate (RNA isolation kit, Stratagene). First-strand cDNA was synthesized from the above-total RNA using a "first-strand cDNA synthesis" kit (Pharmacia) with Not I-$(dT)_{18}$ primer.

2. PCR Amplification. PCR amplification of the immunoglobulin Fd of heavy-chain and light-chain fragments were done separately with distinct primers.[8] Upstream primers were designed to hybridize to partially conserved sequences in the framework 1 (FR1) region of V_H (variable heavy-chain) or V_L (κ) (variable Iight-chain) sequences, and downstream primers were designed to hybridize to constant domain sequences. The primers preserved a full-length light (*L*) chain. The heavy-chain primers were designed such that the gene is terminated just 3′ of the first cysteine codon in the hinge exon. The resulting gene product is intended to correspond to an Fd of IgG isotype and conserves the heavy/light-chain disulfide bond. PCR experiments were carried out with *Thermus aquaticus* DNA polymerase (Promega). The reactions were subjected to 35 rounds of amplification (Perkin-Elmer 9600 thermal cycler) at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1.5 min followed by a final incubation at 72 °C for 30 min.

3. Combinatorial Fab Library Construction. The PCR-amplified light-chain DNA fragments were gel-purified, digested with *Sac* I and *Xba* I, and further gel-purified. The product was ligated with *Sac* I/*Xba*-I linearized pComb3H vector[9] at room temperature overnight. Following ligation, DNA was washed with equal volumes of phenol and chloroform. The DNA was then precipitated with ethanol, washed with 70% ethanol, and dried under vacuum. The pellet was resuspended in water (15 mL) and transformed by electroporation into 300 μL of *Eschericia coli* XL-1 Blue.[10] After transformation, SOC medium[11] (3 mL) was added and the culture was shaken at 250 rpm for 1 h at 37 °C, after which super broth (SB)[12] containing carbenicillin (20 μg/mL) and tetracycline (10 μg/mL) was added. At this stage, samples (1, 0.1, and 0.01 μL) were removed for library size determination, which was found to be 5×10^7 (light chain) and the culture was grown for an additional hour at 37 °C while shaking at 300 rpm. The culture was added to 100 mL of SB containing carbenicillin (50 μg/mL) and tetracycline (20 μg/mL) and was grown overnight at 37 °C. Phagemid DNA containing light-chain library was prepared from this overnight culture.

For cloning of the heavy chain, the heavy chain PCR fragment and phagemid DNA containing light chain were digested as described above except that the restriction enzymes *Xho* I and *Spe* I were used. The resulting linearized vector and Fd fragment were purified by agarose gel electrophoresis. Ligation of this vector with a Fd DNA fragment proceeded as described above for the light chain. After transformation into *E. coli*, SOC media (5 mL) was added and the culture was shaken at 250 rpm for 1 h at 37 °C. Then SB containing carbenicillin (20 μg/mL) and tetracycline (10 μg/mL) was added (at this stage samples were removed for titering as described above, this showed that the Fab library contained 2×10^8 members) and the culture was shaken at 300 rpm for an additional 1 h. This culture was added to 100 mL of SB containing carbenicillin (50 μg/mL) and tetracycline (20 μg/mL) and was then shaken for 1 h at 37 °C. Helper phage VCSM13 (10^{12} plaque forming units) was added, and the culture was shaken at 300 rpm for 2 h at 37 °C. After this time, kanamycin (70 mg/mL) was added and the culture was incubated at 30 °C overnight. The supernatant was cleared by centrifugation (9000 rpm for 5 min in a JLA-10500 rotor) at 4 °C. Phage were precipitated by addition of $^1/_5$ of the volume of 20% PEG-8000 and 2.5 M NaCl followed by incubation on ice for 30 min and centrifugation (9000 rpm for 20 min in a JLA-10500 rotor). The phage pellet was resuspended in 1% BSA in phosphate-buffered saline (PBS; 50 mM sodium phosphate/150 mM sodium chloride, pH 7.4) (2 mL per 100 mL of supernatant) and microcentrifuged at full speed for 5 min to pellet any debris. The supernatant was transferred to a fresh tube and contained the phage library ready for panning.

4. Direct Selection Panning Procedure. The panning procedure utilized is a modification of that originally described.[13] In the first round, six wells of a microtiter plate (Costar 3690) were coated overnight at room temperature with 25 μL of the BSA-**3a** conjugate (10 μg/mL) in PBS. The wells were washed 3−5 times with water and blocked with 3% BSA in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) (100 μL/well), and the plate was incubated at 37 °C for 1 h. Block solution was shaken off, and 50 μL/well of the phage Fab solution (typically 10^{11}−10^{12} colony-forming units) was added, and the plate was incubated for 2 h at 37 °C. The phage were removed, and the plate was washed 3 times with water, 1 time with wash buffer (TBS/0.5% tween 20), 1 time with 8 M urea (37 °C for 30 min), and 3 times with water. The adherent phage were then eluted with elution buffer (0.1 M HCl, adjusted to pH 2.2 with solid glycine and containing 1% BSA) (50 μL). The elution buffer was pipetted up and down several times, removed, and neutralized with 3 μL of Tris-base (2 M) per 50 μL of elution buffer used. Eluted phage were then used to infect 10 mL of fresh *E. coli* XL-1 blue cells ($OD_{600} = 1$) for 15 min at room temperature, after which 10 mL of SB containing carbenicillin (20 μg/mL) and tetracycline (10 μg/mL) was added. At this stage, titering was performed as described *vide supra* to determine the number of phage eluted from the plate. The culture was shaken for 1 h at 37 °C and then added to 100 mL of prewarmed SB containing carbenicillin (50 μg/mL) and tetracycline (20 μg/mL) and was then shaken for 1 h at 37 °C. Helper phage VCSM13 were added, and the culture was incubated overnight. Phage solution was prepared as described above. The phage solution was then ready for further panning. For the next three rounds of panning, the washing procedures were modified as follows; second round, 3 times with washing buffer, 2 times with 8 M urea (37 °C for 30 and 10 min, respectively); third round, 5 times with washing buffer, 3 times with 8 M urea (37 °C for 30, 10, and 10 min,

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respectively); fourth round, 10 times with washing buffer, 4 times with 8 M urea (37 °C for 30, 10, 10, 10 min, respectively).

5. ELISA Screening of the Soluble Fab Fragment. After the fourth round of panning, the phagemid DNA was purified as described above, digested with *EcoR* and *Spe* I. The 1.5 kb fragment containing the Fab gene was subcloned into pWPY501 (pET-**3a** derivative vector) vector where protein expression is under the control of the T7 polymerase and transformed into *E. coli* BL21(DE3) (Novagen) cells. The transformed cells were plated onto LB (Luria-Bertani) agar containing carbenicillin (100 μg/mL) and incubated overnight at 37 °C. Thirty single colonies were picked from the plate and inoculated into SB (5 mL) containing carbenicillin (100 μg/mL). The cells were incubated at 37 °C with shaking. When the OD_{600} of the culture reached 0.6−1.0, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.05 mM to induce the T7-polymerase gene placed under the lac UV5 promoter. After induction, the cells were incubated at 25−30 °C with shaking overnight. The screening for soluble Fab recognition of the BSA-**3a** conjugate was performed with this culture supernatant. The ELISA plate was coated with 25 μL/well of BSA-**3a** (5 μg/mL in PBS) at room temperature overnight. The plate was washed 5 times with water, blocked with 50 mL of Blotto (2% w/v skimmed milk powder in PBS), and incubated at 37 °C for 1 h. Blotto was shaken off, and 25 μL/well of the culture supernatants were added and incubated at 37 °C for 1 h. The plate was washed 5 times with water followed by addition of 25 μL/well of HRP-conjugated goat antimouse $F(ab')_2$ (Pierce). The plate was then incubated at room temperature for 2 h and washed 5 times with water before addition of the TMB substrate kit; 26 of the 30 colonies picked showed binding to the BSA-**3a** conjugate.

6. Antibody Expression and Purification. Fermentation of the selected Fab (BL25) was carried out in a 20 L fermentor with LB media supplemented with M9 salts, 0.4% glycerol, and carbenicillin (100 μg/mL). After equilibration of the medium at 37 °C, a seed culture of freshly transformed *E. coli* BL21(DE3) cells was added into 10 L of media and incubation at 37 °C was continued until OD_{600} = 5.0−10.0. BL25 Fab expression was induced by addition of IPTG (2.5 mM).

The cells were removed by centrifugation (9000 rpm for 20 min at 4 °C). The 12 L of growth media was concentrated to 200 mL using a hollow fiber concentrator (Amicon) equipped with three 100 000 NMWL Hollow fiber cartridges. The resulting solution was centrifuged at 30 000 g for 30 min at 4 °C to remove precipitated material. A FPLC affinity column was prepared from γ-bind Plus Sepharose (Pharmacia) and goat antimouse $F(ab')_2$ IgG (Eappel) as previously reported.[14] Two buffers were used in the affinity chromatography procedure: 0.1 M HCl−glycine pH 3.0 (buffer A) and PBS pH 7.4 (buffer B). The column was equilibrated with buffer B, and the concentrated supernatant was added to the top of the column followed by extensive washing with PBS until the base line reading at 280 nm had returned to base line. The Fab was elated by dropping the pH with buffer A. Eluted fractions were collected, neutralized, concentrated, and then loaded onto a HIPrep TM 26/60 Sephacryl S-100HR ion-exchange column (Pharmacia) preequilibrated with PBS (pH 7.4) buffer. The Fab fractions were collected, concentrated, and dialyzed exhaustively into CHES buffer (100 mM, pH 9.0) for kinetic studies.

Kinetic Studies. Steady-State Antibody Kinetics. The reactions were performed in 100 mM CHES, pH 9.0 with 2% DMF in the presence of substrates **1a**−**d** with or without antibodies. DMF was included to increase the substrate's solubility. At appropriate times, a 50 μL aliquot was removed and quenched into 50 μL of 70% water (0.1% TFA) and 30% acetonitrile which contained 3-chloroacetanilide as an external standard. The formation of the respective acids **2a**−**b** was monitored by reversed-phase HPLC using a C_{18}-VYDAC201TP54 column with an isocratic mobile phase (70% water (0.1% TFA) and 30% acetonitrile) at 254 nm and 1 mL min^{-1} flow rate. The product concentrations were determined by comparisons of peak height and area ratios (relative to standard) against calibrated ratios of product standard. Initial velocities were determined from five data points on the linear part of the progression curve. The kinetic parameters were determined by treatment of the initial rate data (between 2−5% of reaction conversion) with the computer program Enzfitter. Antibody concentrations were determined by UV assay (OD_{280}/1.35 = concentration in mg mL^{-1}).

Competitive ELISA Fab-BL25 Binding Studies. Ninety six-well microtiter plates were coated with the BSA-**3b** conjugate as described *vide supra*. Varying concentrations of either the haptenic inhibitor **3b**, the amide substrates **1a**,**b**, or ester substrates **1c**,**d** were then incubated with Fab-BL25 at 37 °C for 5 min at a pH range of 6.0−11.0. This mixture was then applied to the microtiter plates and incubated at 37 °C for 90 min and developed as described *vide supra*. The pH versus OD_{450} data was then fit to an equation for p*K*a determination using the Grafit computer program.

Results and Discussion

The key selection step is linked to antibody recognition of the trigonal α-amino boronic acid hapten **3a**, while this is the first example of the utility of boron as a hapten for catalytic antibody generation, there seemed to be two possible ways by which it may serve to elicit catalysts for acyl transfer reactions.

First, while the precise nature of how the immune system responds to boronic acid haptens is unknown, it was assumed that the Lewis-acid hapten **3a** could select for a complementary Lewis base in the antibody-combining site during antibody production (Scheme 2). This is supported by evidence from boronic acid inhibitors of serine proteases which are known to bind by coordination of the active-site serine or histidine to form a high-affinity tetrahedral anionic mimic of the transition state for peptide bond cleavage.[15] Antibody selection of such a Lewis base may then lead to recruitment of nucleophilic catalysis and/or transition-state stablilization (by recognition of the tetrahedral adduct formed after coordination) for the hydrolysis of **1a** or **1b**. Second, there is a well-characterized p*K*a associated with the equilibrium hydration of trigonal boronic acids, and this tetrahedral hydrate may be an excellent mimic of the transition state for the catalysis of water addition to a carbonyl center. For α-amino boronic acids, this p*K*a is around 8.0 (Scheme 3),[15b] therefore, by immunizing mice which have a plasma pH of *ca.* 7.4, there will be a high proportion of this hydrated form available to the immune system. Likewise, by modifying the pH at which panning occurs, the proportion of trigonal to tetrahedral forms of **3a** can be changed during antibody combinatorial library screening.

Hapten **3a** and its inhibitor analog **3b**, both trigonal α-amino boronic acids, were synthesized in eight steps, *via* proline boronic acid pinanediol ester **8** as a mixture of two diastereomers which were not separated (Scheme 4). Synthesis of 1-(1,-1dimethylethoxy)carbonyl)pyrrolidine-2-boronic acid **6** was performed as described previously.[5] The boronic acid **6** was then protected as its (+)-pinanediol ester **7** to increase its stability and facilitate purification of the intermediates along the synthetic route. On the basis of previous precedence, it was rationalized that by immunization with a diastereomeric hapten mixture antibodies could be produced which catalyze the hydrolysis of either the all L-isomer substrate **1a** or its D-proline

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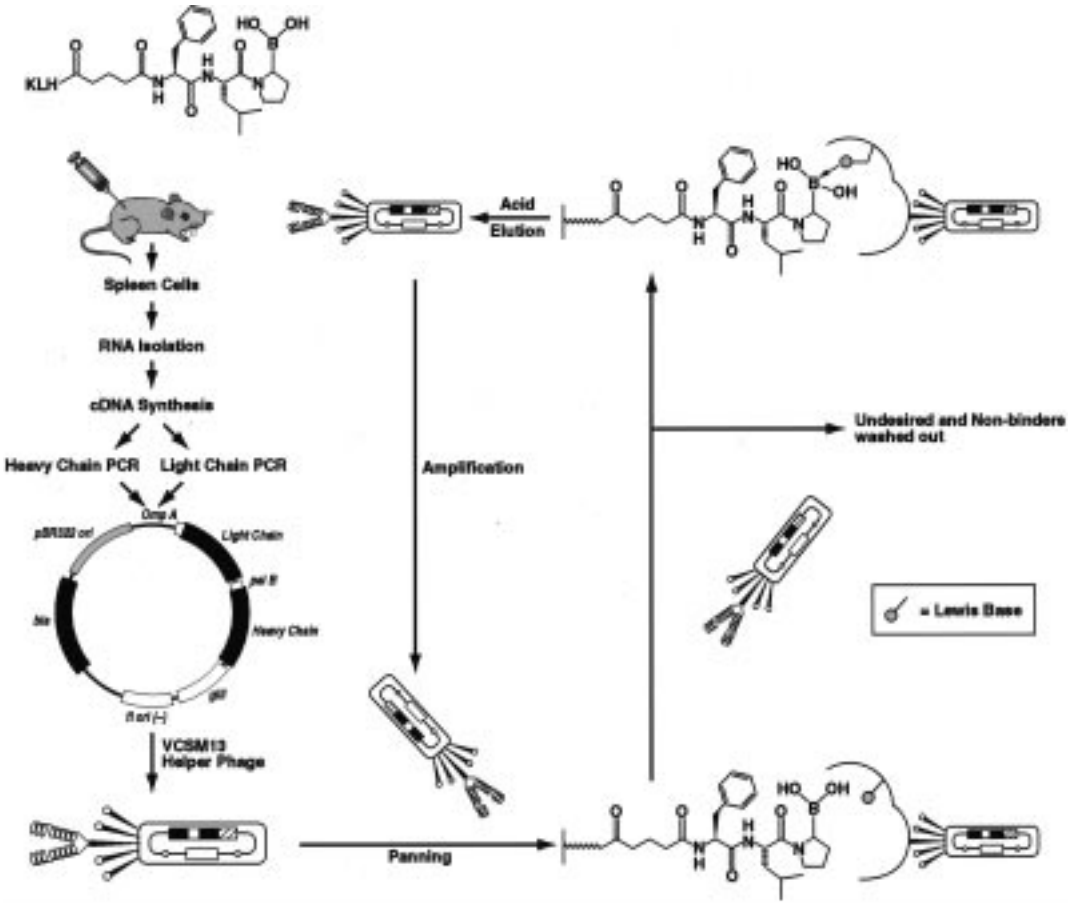
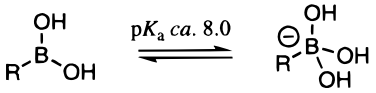
(15) (a) Powers, J. C.; Harper, J. W. In *Proteinase Inhibitors*; Barrett, A. J., Salvesen, G., Eds.; Elsevier: New York, 1986; pp 69−73. (b) Kettner, C. A.; Shenvi, A. B. J. *Biol. Chem.* **1984**, *259*, 15106−15114 (c) Kettner, C. A.; Bone, R.; Agard, D. A.; Bachovin, W. W *Biochemistry* **1998**, *27*, 7682−7688. (d) Tsilikounas, E.; Kettner, C. A.; Bachovin, W. *W. Biochemistry* **1993**, *32*, 12651−12655. (e) Bachovin, W. W.; Wong, S. F.; Shenvi, A. B.; Kettner, C. A. *Biochemistry* **1988**, *27*, 7689−7697. (f) Bone, R. A.; Frank, D.; Kettner, C. A.; Agard, D. A. *Biochemistry* **1989**, *28*, 7600−7609.

Scheme 2. Schematic Representation of Phage-Display of Fab Fragments To Select for Antibody-Combining Sites Containing Lewis Bases



Scheme 3. Hydration of Boronic Acids to Their Tetrahedral Form



diastereomer **1b**;[7,16] and therefore, no attempt was made to resolve the 1:1 mixture of diastereomers of **7**. The Boc group of **7** was removed with anhydrous HCl to give the amine **8** in 62% yield. Coupling of **8** with Boc-Phe-Leu-OH gave the fully protected boronate ester **9** in good yield (85%) following silica gel chromatography. The synthetic route to the haptenic inhibitor **3b** was completed by stirring **9** in phosphate-buffered saline (PBS, pH 7.4) for 1 h to remove the (+)-pinanediol protecting group.[15b] The glutaroyl linker of hapten **3a** was attached by an initial deprotection of the Boc protecting group in TFA and subsequent coupling of this amine with glutaric anhydride to give the protected hapten **11** in good yield (68%). The pinane diol ester was hydrolyzed to give **3a** as described for **3b**, *vide supra*.

The amide and ester substrates **1a**−**d** both incorporate the 4-acetamidophenyl group, as a UV chromophore to facilitate assaying methods during the measurement of both the background- and antibody-catalyzed hydrolysis processes. The chromophore is strategically located in the position occupied by the glutaroyl spacer in hapten **3a** and is expected to be a region of minimal antibody recognition. The primary amide substrates **1a** and **1b** were synthesized by treating the tripeptide ester substrates (4-acetamido)phenylacetic acid (PAP)Phe-Leu-(L)-Pro-OMe (**1c**) and PAP-Phe-Leu-(D)-Pro-OMe (**1d**), respectively, with a solution of ammonia in MeOH.[17]

Hapten **3a** was coupled to carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) following activation to its sulfo-*N*-hydroxysuccinimide ester. Gix^+ (129) mice were immunized with the KLH-**3a** conjugate, and antibody production occurred by two methods: standard hybridoma methodology[18] and combinatorial Fab (antigen binding fragment) library construction commencing from immunized mouse spleen RNA.

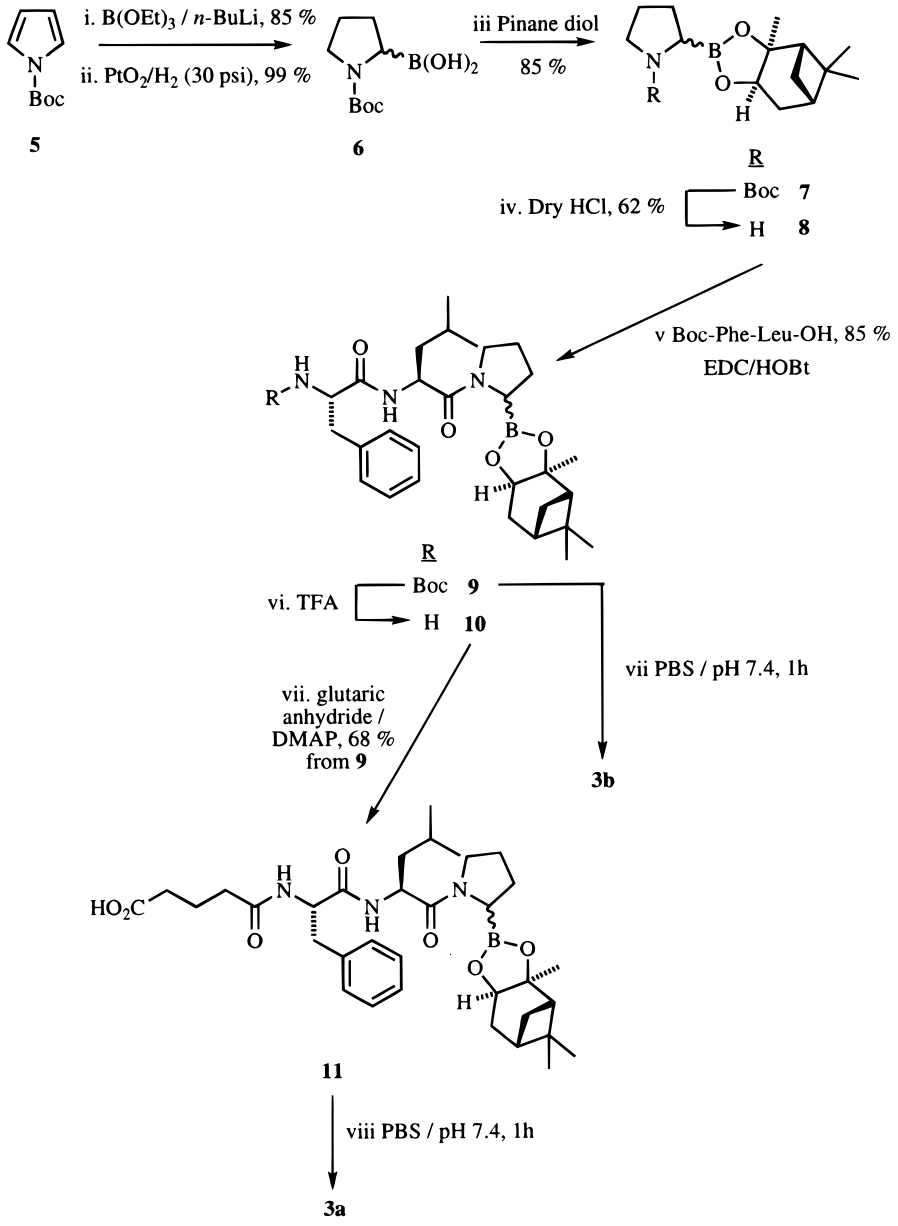
A combinatorial phage-Fab library was produced which contained *ca.* 2×10^8 members, and from this pool the key step for isolating a potential catalyst was found to be in the method of panning, *i.e.*, the method by which adherent phage-Fab members of the library are selectively isolated and enriched. The panning procedure was performed at pH 7.5, and under these conditions, the effective concentration of the trigonal and tetrahedral forms of hapten **3a** was expected to be approximately equivalent. Therefore, it was hoped that during panning antibodies would be isolated with high-affinity noncovalent recognition of the tetrahedral anionic boronate, a transition-state

(16) For leading papers where immunizations with chiral mixtures of haptens have elicited stereospecific or stereoselective catalysts, see: (a) Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Science* **1989**, *244*, 437−440. (b) Janda, K. D.; Shevlin, C.; Lerner, R. *A. Science* **1993**, *259*, 490−493. (c) Kitazume, T.; Lin, J. T.; Yamamoto T.; Yamazaki, T. *J. Am. Chem. Soc.* **1991**, *113*, 8573−8575.

(17) (a) Bodanszky, M.; Bodanszky, A. In *The Practice of Peptide Synthesis*, 2nd ed.; Springer-Verlag: New York, 1994; pp 165−166. (b) Hogberg, T.; Ström, P.; Ebner, M.; Rämsby, S. *J. Org. Chem.* **1987**, *52*, 2033−2036.

(18) (a) Köhler, G.; Howe, S. C.; Milstein, C. *Eur. J. Immunol.* **1976**, *6*, 292−295. (b) Köhler, G.; Milstein, C. *Eur. J. Immunol.* **1976**, *6*, 511−519.

Scheme 4. Synthetic Route to Boronic Acid Hapten **3a** and Inhibitor **3b**



analog for primary amide bond hydrolysis, in addition to antibodies containing Lewis bases, as discussed *vide supra*. After binding to BSA-**3a**, the phage-Fabs were sorted by a series of separate elutions. The nonbinders and weakly noncovalent binders were removed by repeated washings with 8 M urea. The adherent phage were then treated with an acidic wash (0.1 M HCl, pH 2.2),[5] and it was rationalized that this would serve to elute the phage-Fab by either perturbing the hydration equilibrium of **3a** back to the trigonal form and hence reducing antibody affinity or by directly hydrolyzing the boronate esters and amides formed by Lewis-acid coordination to **3a**.

Following infection of *E. coli* with the adherent phage library members, 30 colonies were randomly picked. From this sublibrary, based on its expression levels, Fab-BL25 was chosen for further investigation. The BL25-soluble Fab was over expressed in *E. coli* and purified by affinity chromatography and gel filtration chromatography, affording a protein fraction that was judged >98% pure Fab on inspection of native and denaturing gel analyses. ELISA studies showed that binding of Fab-BL25 to the BSA-**3a** bioconjugate is not perturbed under denaturing conditions (8 M urea), suggesting the contribution of either high-affinity noncovalent or covalent interactions in hapten-antibody recognition.

Kinetic studies show that Fab-BL25 catalyzes the hydrolysis of the all L-isomer **1a** to the free acid **2a** with an initial velocity *versus* substrate concentration dependence at pH 9.0 (100 mM CHES) and 25 °C supporting a Michaelis−Menten model (k_{cat} = 0.003 min^{-1}, K_m = 150 μM, and k_{cat}/k_{uncat} *ca.* 4 × 10^4). Fab-BL25 exhibits remarkable diastereoselectivity as the D-proline primary amide diastereomer **1b** is not accepted as a substrate. Unfortunately, even with the UV chromophore to aid assay sensitivity, the background rate of primary amide bond hydrolysis of **1a** at pH 9.0 (100 mM CHES) and 25 °C is too low to accurately measure by HPLC methods. Consequently, a value of k_{uncat} = 7.0 × 10^{-8} min^{-1} was used based on literature precedence.[3,19] The observed rate enhancement supplied by BL25 reduces the half-life of the primary amide in **1a** from *ca.* 17.5 years to 3.9 h in the presence of the catalytic Fab and is >2 orders of magnitude higher than that observed for an antibody elicited by a phosphinate transition-state analog approach,[3] highlighting the power of this direct selection strategy

(19) Kahne, D.; Still, W. C. *J. Am. Chem. Soc.* **1988**, *110*, 7529−7533.

with the boronic hapten probe. Furthermore, the benefit of utilizing a combinatorial library approach for antibody generation was emphasized during this study because while 25 BSA-**3a** binding monoclonal antibodies were produced by hybridoma methodology, none of them catalyzed primary amide bond hydrolysis of either **1a** or **1b**. The explanation for why no catalytic clones were obtained by the hybridoma technique is undoubtedly complicated by the fact that, on average, only approximately 10% of the immune repertoire is sampled during the practical process involved. This insufficient screening of the immune library is a major reason for moving to alternative methods of monoclonal antibody generation, and phage display is a major step-forward in this respect.

Of major concern in generating antibodies with catalytic activity similar to naturally occurring enzymes is the risk of contamination. However, a number of experiments performed suggest that the observed catalytic activity is in fact antibody-mediated. First, there was no HPLC evidence of either cleavage of alternate amide bonds in the substrate **1a** or depletion of the desired dipeptide product **2a** by second- or higher-order amide hydrolyses, highlighting the exquisite regioselectivity of Fab-BL25. Furthermore, no catalysis above the background rate of the amide substrate **1b** or the alternate ester substrates **1c** and **1d** could be detected. The diastereomeric hapten analog **3b** exhibits competitive inhibition of catalytic activity in the kinetic HPLC assay (K_i = 9 μM), supporting the assumption that catalysis is taking place in the binding site of BL25. Finally, equally active protein was purified from three different fermentation batches. The remarkable substrate specificity of Fab-BL25 emphasized by the fact that the ester analog **1c** was not a substrate was studied in detail by competitive ELISA binding studies. No binding of **1c** to Fab-BL25 was observed up to the maximum concentration assayed, 2 mM, suggesting that the recognition of BL25 is highly focussed around the boronate center and that perhaps the methyl group of **1c** is either too bulky or does not have the requisite hydrogen bond donor−acceptor properties that are available for substrate **1a** and its putative transition state **4**.

A number of experiments have been performed to help dissect the catalytic mechanism of BL25 and, thus, elucidate how the boronic acid hapten elicited a biocatalyst. Although catalysis was, in general, followed for less than 1 turnover, assay times extended beyond 1 turnover demonstrated that Fab-BL25 is indeed catalytic. The reaction progress curve is linear for the entire period, arguing against burst-kinetics and a rate-determining deacylation step but not precluding nucleophilic catalysis as a component of the process.

The most illuminating piece of evidence of how the boronic acid **3a** may have elicited Fab-BL25 was gleaned from pH-dependant competitive ELISA binding studies. The BSA-**3a** conjugate was used to coat the ELISA plate, and free inhibitor **3b** was the competing ligand. The competition assay was measured over a pH range of 6−11, and a marked increase in Fab-BL25 affinity for the inhibitor **3b** with elevation in pH was observed (Figure 1).

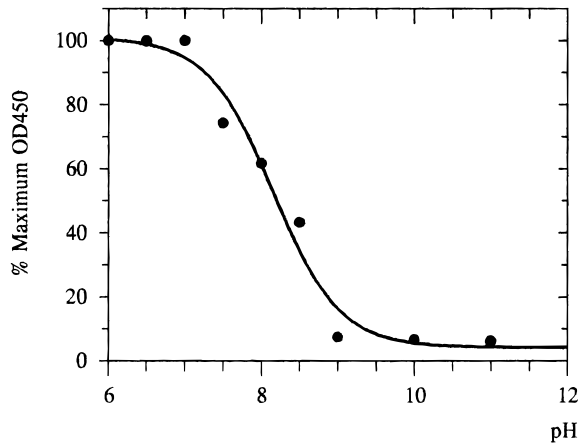


Figure 1. Competitive ELISA study. For this binding study, the ELISA plate was coated with BSA-**3a** conjugate (25 μL of a 5 μg mL^{-1} stock solution in PBS) and the competing ligand was boronic acid **3b**.

The 50% displacement point occurred at pH 8.0, which is the approximated p*K*a for the hydration of a trigonal α-amino boronic acid to its tetrahedral form (Scheme 3). This data is strong evidence that Fab-BL25 binds the tetrahedral form of hapten **3a** much more strongly than the trigonal form, suggestive that the catalytic power of BL25 is linked to transition-state stabilization for a process involving the catalytic addition of water to the primary amide substrate **1a** rather than by elicitation of a complementary Lewis base in the antibody binding site. As discussed *vide supra*, panning was performed at pH 7.5, resulting in an equal chance of highlighting antibodies capable of catalyzing this process as well as via a covalent mechanism. In future strategies to elicit Lewis bases to boronic acid haptens, lower pHs will be adopted during the critical panning phase of antibody selection.

Conclusions

By utilizing a trigonal boron hapten in our direct selection for catalysis strategy, we have isolated an antibody Fab which is the most efficient regio- and diastereoselective catalyst for unassisted primary amide bond hydrolysis yet reported. Steady-state kinetic assays and competitive ELISA binding assays both suggest that this catalyst was elicited to a hydrated tetrahedral boronate species and, thus, is acting by transition-state stabilization. This work reports the first example of boronic acids as haptens in acyl transfer processes, expands further our direct selection methodology, and serves to broaden the general utility of antibody catalysts into areas previously thought to be unattainable.

Acknowledgment. This work was supported in part by NIH Grant No. GM-43858 and The Skaggs Institute for Chemical Biology. The authors thank Dr. Jane Strouse and the UCLA NMR facility for invaluable help and advice.

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