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Supporting Information

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BN/CC Isosteric Compounds as Enzyme Inhibitors: *N*- and *B*-ethyl-1,2-azaborine Inhibit Ethylbenzene Hydroxylation as Non-Convertible Substrate Analogues**

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Supporting Information

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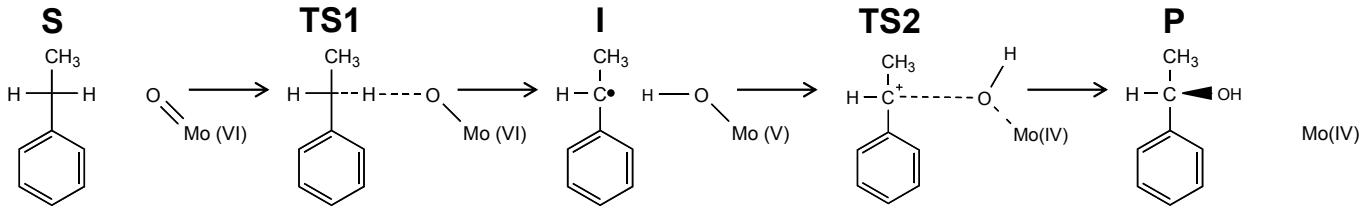
1. Catalytic Mechanism of EbDH

The proposed catalytic mechanism of oxygen-independent ethylbenzene hydroxylation by EbDH should proceed as it is shown in supporting figure 1



Supporting Figure 1. Proposed catalytic mechanism of oxygen-independent ethylbenzene hydroxylation by EbDH. S: Substrate state (ethylbenzene); TS1: Transition state 1: Approach of the hydrogen atom from the C1 atom of the ethyl substituent of ethylbenzene to the oxo-ligand of the molybdenum atom; I: (radical) intermediate state: Formation of a substrate radical by abstraction of a proton and an electron from the C1 atom of the substrate by the oxo-ligand of the molybdenum atom to form a hydroxyl group (molybdenum reduced from oxidation sate VI to V); TS2: Transition state 2: Carbocation formation and subsequent rebound of the hydroxyl group from the molybdenum atom including transfer of the second electron from the substrate to the molybdenum atom (molybdenum reduced from oxidation sate V to IV); Product state ((*S*) 1-phenylethanol).

2. Enzyme Preparation

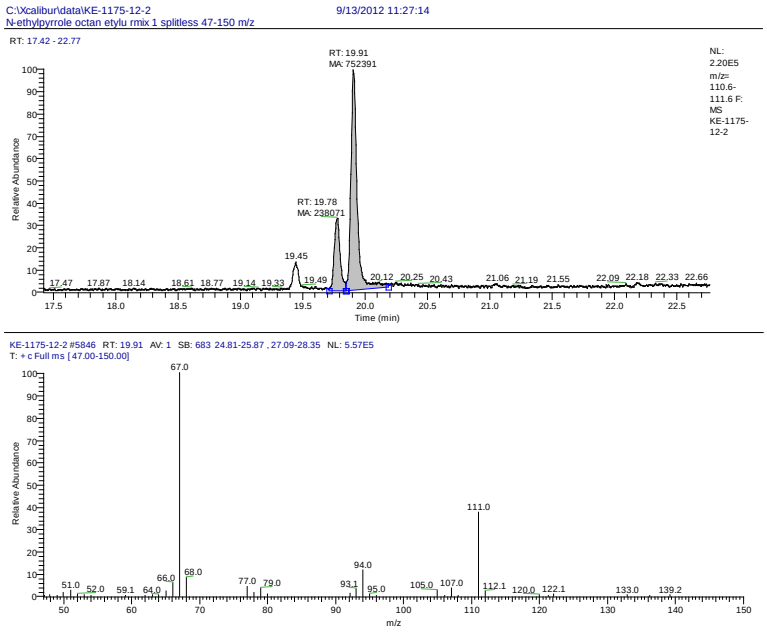
Ethylbenzene dehydrogenase (EbDH) was purified from *"Aromatoleum aromaticum"* strain EbN1 grown anaerobically on ethylbenzene.[1,2] *"A. aromaticum"* was grown with 3.5 mM of ethylbenzene as carbon and electron source and 10 mM nitrate as electron acceptor in mineral salt medium in a 100 l fermenter (Braun Melsungen). Cells were harvested at an OD_{578} of 3 and about 300 g of wet cell mass was usually obtained. Crude extracts were prepared from 40 g aliquots of wet cells suspendend in 40 ml of basal buffer (10 mM Tris/Cl, 10 % (v/v) glycerol, 100 µM ferricenium tetrafluoroborate, pH 7.5) with DNase I (0.05 mg/ml) and lysozyme (0.05 mg/ml) and passed 3 times through a French press cell (American Instruments Company) at 137 MPa, followed by a ultracentrifugation step at 100.000 x g to remove cell debris. The supernatant was then loaded to a DEAE sepharose fast flow column (50 ml column volume; BIO-RAD) and washed with 2 column volumes of basal buffer. The flow-through fractions with EbDH activity were collected and loaded on a ceramic hydroxyapatite (type II) column (50 ml column volume, BIO-RAD). The column was washed with 2 column volumes buffer 1 (10 mM Tris/acetate pH 7.5, 10 mM potassium phosphate, 10 % (v/v) glycerol, 100 µM ferricenium tetrafluoroborate, pH 7.5) and eluted with 10 column volumes using a linear salt gradient from 0 to 300 mM potassium phosphate in buffer 1. EbDH activity typically eluted between 160-220 mM potassium phosphate. These fractions were pooled and concentrated with a ViaSpin centrifuge concentrator (50 kDa MWCO; Millipore). Aliquots of 200 µl of the concentrated enzyme were shock-frozen in liquid nitrogen and further stored at –80°C. Protein concentrations were determined by the Coomassie dye binding assay.[3] All purification steps were performed aerobically with an Äkta FPLC system (GE Healthcare).

3. Kinetic Measurements

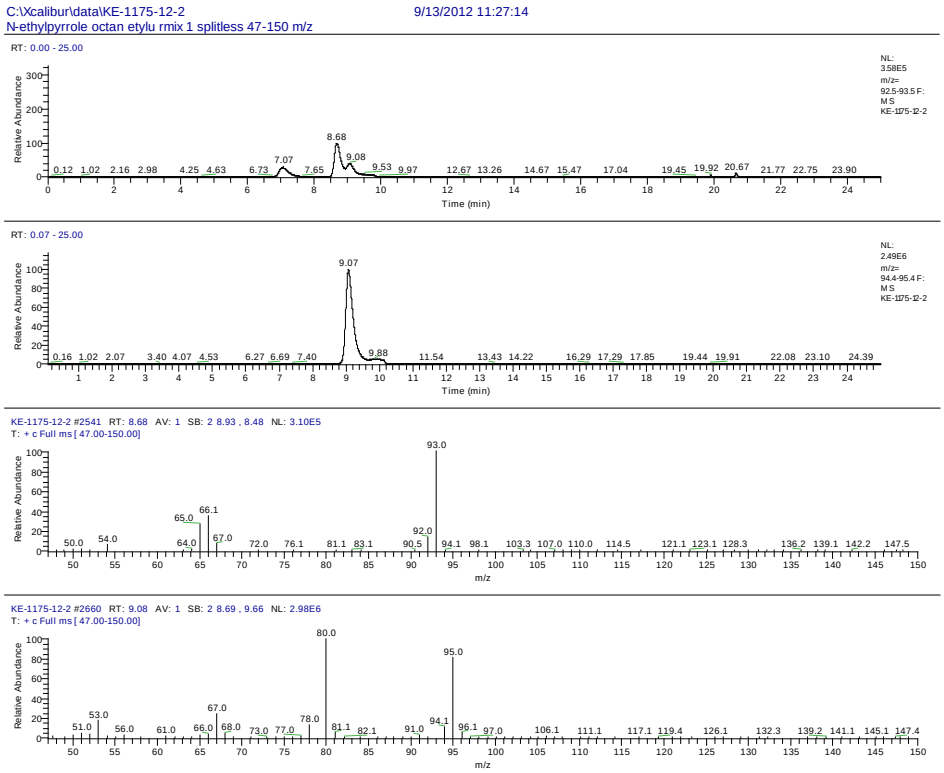
Enzymatic activity was determined in quartz cuvettes under an N_2 atmosphere in the absence of oxygen by a photometric test as described before.[4,5] Purified EbDH (10 -100 μg) was added to the reaction mixture (1.5 ml of 100 mM Tris-HCl [pH 7.5] with 200 μM ferricenium-tetrafluoroborate as electron acceptor), and the photometric test was started by the addition of the substrate (stock solutions in *tert*-butanol as nonreactive solvent). The reduction of ferricenium was followed at 290 nm (ε = 6.2 mM^{-1} cm^{1}), 300 nm (ε = 3.6 mM^{-1} cm^{-1}) or 310 nm (ε = 1.7 mM^{-1} cm^{-1}), depending on the substrate used. Especially for the *N*- and *B*-ethyl-1,2-azaborines, the assays needed to be monitored at 310 nm because of the still significant UV absorption of these compounds at lower wavelengths. Kinetic parameters were determined by nonlinear fitting of the enzyme activities obtained at varied substrate concentrations to the standard Michaelis-Menten equation using the GraphPad Prism software package. Inhibition parameters (IC_{50} values) were calculated from kinetic data obtained with the native substrate ethylbenzene at up to five different inhibitor concentrations and in controls without inhibitor. The inhibition kinetic parameters of *N*-ethyl-1,2-azaborine were determined by nonlinear fitting of the kinetic data obtained for ethylbenzene hydroxylation at three different *N*-ethyl-1,2-azaborine concentrations and in controls without inhibitor. The nonlinear fitting was performed using the equations for different modes of inhibition (competitive, uncompetitive, mixed) using the program LEONORA.[6] For choosing a plausible inhibition model for the inhibitor, the overall quality of fitting and the obtained inhibition constants K_{ic} (competitive inhibition) and/or K_{iu} (uncompetitive inhibition) were evaluated. If a model was far off the experimental data or produced very high standard deviations (>50%), very high absolute values, or negative values for either K_{ic} or K_{iu}, it was regarded as implausible.

4. GC-MS Analysis

Chiral GC-MS analysis of reaction mixtures was performed on a Trace DSQ 1000 Thermo Finnigan gas chromatograph with a mass spectrometer, using an electron ionization detector. Reaction mixtures were extracted with ethyl acetate and dried over anhydrous $MgSO_4$, and 0.5 μl of the extracts were injected in the GC-MS instrument. The separations were conducted on a Cyclodex-B column (60 m, 0.25mm [inner diameter], 0.25 μm). The analyses were carried out in a splitless mode with constant flow of helium (1.2 ml/min) as a carrier gas with injector at 200°C. The temperature program comprised of a 4-min temperature hold at 80°C, followed by a slow temperature gradient (5°C/min) up to 175°C with a final 7-min temperature hold at 175°C. Supporting figure 2 shows the GC-MS results for the two enantiomeric hydroxylated products of *N*-ethylpyrrole (shaded in grey), supporting figure 3 those for the additional minor dehydrogenated product, along with a substrate control.



Supporting Figure 2. The GC-MS analysis of *N*-ethylpyrrole yielded two alcohol product peaks at 19.78 min and 19.91 min (shaded) with M^+ of 111 m/z and characteristic fragmentation peaks corresponding to the M-OH $[M-17]^+$ and $M–H_2O$ $[M-18]^+$ ions (results shown for the larger peak). The elution order of enantiomers was assigned by analogy to other secondary alcohols with aromatic or heterocyclic substituents (i.e. the (*R*)- before the (*S*)-enantiomer).



Supporting Figure 3. A second product was detected close to substrate peak (9.08 min) at 8.68 min (upper spectrum). It had an M^+ of 93 m/z (consistent with 1-ethenyl-1H-pyrrole) and showed a $[M-27]^+$ fragmentation ion signal indicating loss of a C_2H_3 group. The lower spectrum and m/z map represents a substrate control with unreacted *N*-ethylpyrrole.

5. Molecular Modeling

Modeling of the 1,2-azaborine ligands in the active site of EbDH was conducted using the Discovery Studio 3.5 software (Accelrys). The initial model was obtained from QM:MM modeling based on the crystallographic structure of the alpha subunit of EbDH, which was modified to represent the oxidized form (15895 atoms with 466 water molecules present in crystal structure) and optimized in Gaussian 09 on B3LYP/6-31g(d,p)/AMBER level of theory, using an electronic embedding approach. The initial position of ligands was taken from an overlay with the position of ethylbenzene in the transition state associated with C-H activation (TS1).[7] Two different conformations of the 1,2-azaborine rings were considered with the BH/NH groups pointing in both possible directions toward MGD-Q (conformer 1) or MGD-P (conformer 2).

The whole model was typed with a CHARMm force field (c36b2)[8], and MM point charges of the protein were calculated according to the Momany-Rone scheme.[9] The CHARMm parameters for boron were estimated automatically during the typing procedure and verified for correctness in a separate geometry minimization of the azaborine ligands in vacuum. The MM point charges for the ligands (ethylbenzene and 1,2-azaborines), the Mo-cofactor and the iron-sulfur cluster present in the alpha subunit of EbDH were fitted to the electrostatic potential according to the Merz-Singh-Kollman scheme.[10]

The MM energy minimization was conducted *in vacuo* with 1000 steps of steepest descent with a RMS gradient tolerance of 3, followed by conjugate gradient minimization with gradient tolerance of 0.004 kJ/mol. All residues penetrating a 3 Å radius around the ligand were allowed to move during minimization, while the rest of the protein was kept frozen. The single point energies were calculated with implicit solvent approach with a distance-dependent dielectrics method with ε=4. The binding energies were approximated from the energy difference of the enzyme-ligand complex (E^{ES}) and the sums of energies of the non-minimized enzyme without ligand ($E\ enz^{rigid}$) and the ligands in either rigid (i.e. conformation as in active site) or relaxed conformations ($E\ ligand^{rigid}$, resp. $E\ ligand^{relaxed}$). The results are shown in supporting table 1.

$$\Delta E^{\ binding} = E^{ES} - (E\ enz^{rigid}) - (E\ ligand^{rigid/relaxed}).$$

Supporting Table 1. Calculated potential energies (in kJ/mol) for enzyme-ligand complexes (E^{ES}), enzyme without ligand ($E\ enz^{rigid}$), and ligands (E ligand) in either rigid or relaxed forms. The resulting binding energies (ΔE binding) were calculated from these values for rigid or relaxed ligands in both possible conformers.

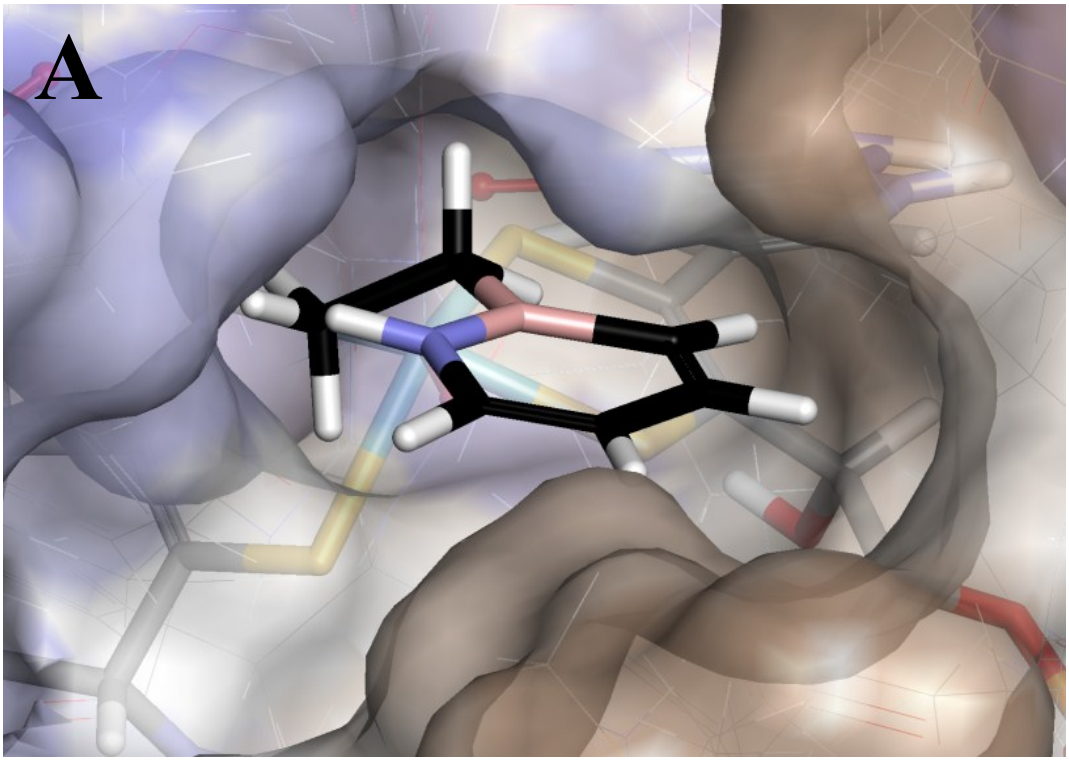
Type and conformer of ligand	E^{ES}	$E\ enz^{rigid}$	$E\ ligand^{rigid}$	$\Delta E\ binding^{rigid}$	$E\ ligand^{relaxed}$	$\Delta E\ binding^{relaxed}$
B-ethyl-1,2-azaborine conformer 1	**-25478.64**	-25451.02	14.56	**-42.18**	-5.19	**-22.43**
B-ethyl-1,2-azaborine conformer 2	-25471.82	-25458.47	25.31	-38.66	-6.44	-6.90
N-ethyl-1,2-azaborine conformer 1	-25479.85	-25448.92	23.85	-54.77	-0.042	-30.88
N-ethyl-1,2-azaborine conformer 2	**-25483.48**	-25419.14	13.31	**-77.70**	-0.042	**-64.35**
ethylbenzene	-25453.28	-25448.13	33.05	-38.20	3.56	-8.74

Based on these calculations, the preferential conformers are predicted to be conformer 1 for *B*-ethyl-1,2-azaborine (NH group pointing towards MGD-Q) and conformer 2 for *N*-ethyl-1,2-azaborine (BH group pointing towards MGD-P). The obtained optimal docking structures are shown individually in supporting figures 4A-C, and an overlay figure of all three compounds in Figure 2 in the manuscript.

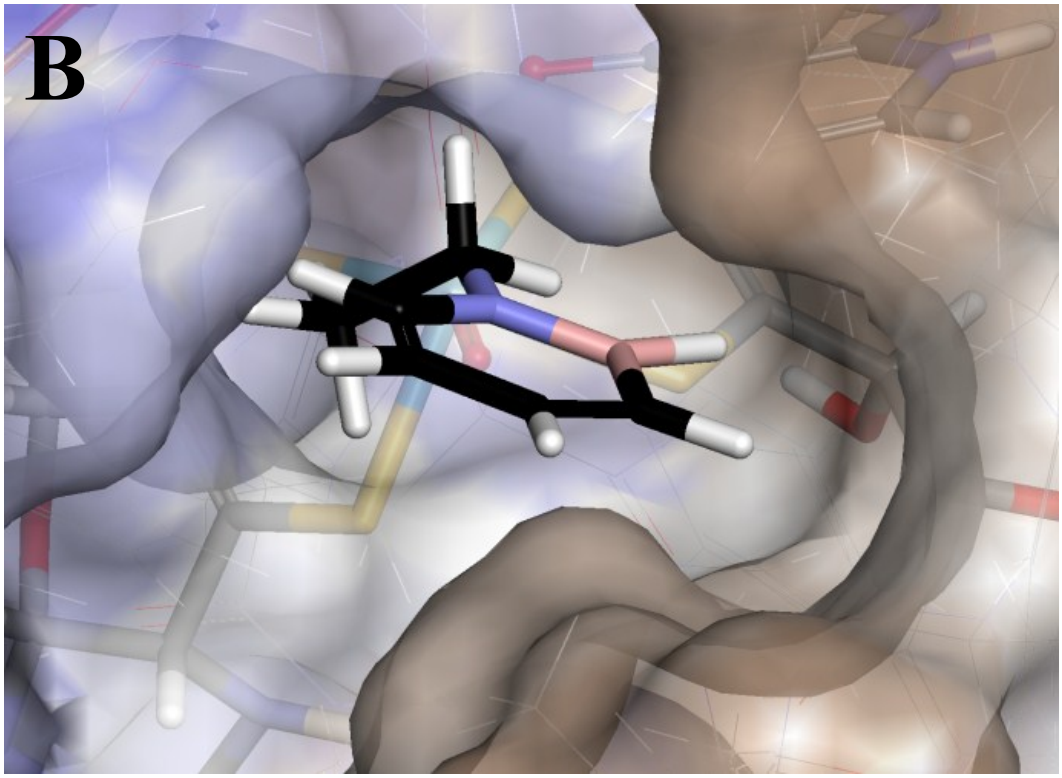
It should be emphasized here, that due to lack of proper CHARMm force field parameterization for the heteroaromatic boron atom, the interaction energies that include the B atom could only be estimated and may not be accurate (especially for van der Waals interactions). The actual errors of energy values will especially influence the *N*-ethyl-1,2-azaborine – enzyme complex, which exposes the B atom towards different sides of the active site in the two possible orientations of the ligand. Taking this into consideration, the modeled structures of ligand-enzyme complexes just indicate that it is plausible to assume an analogous binding of the 1,2-azaborines to that of the native substrate.
The interaction energies of ligands with EbDH were evaluated with the "Calculate Interaction Energy" protocol for these conformers as well as for the reference complex with ethylbenzene. The protocol quantified van der Waals and electrostatic interaction energies of the ligands with all amino acids penetrating a 5 Å radius around the ligand (supporting table 2).

Supporting Table 2. Total interaction energies calculated for ligands and amino acids within a 5 Å radius.

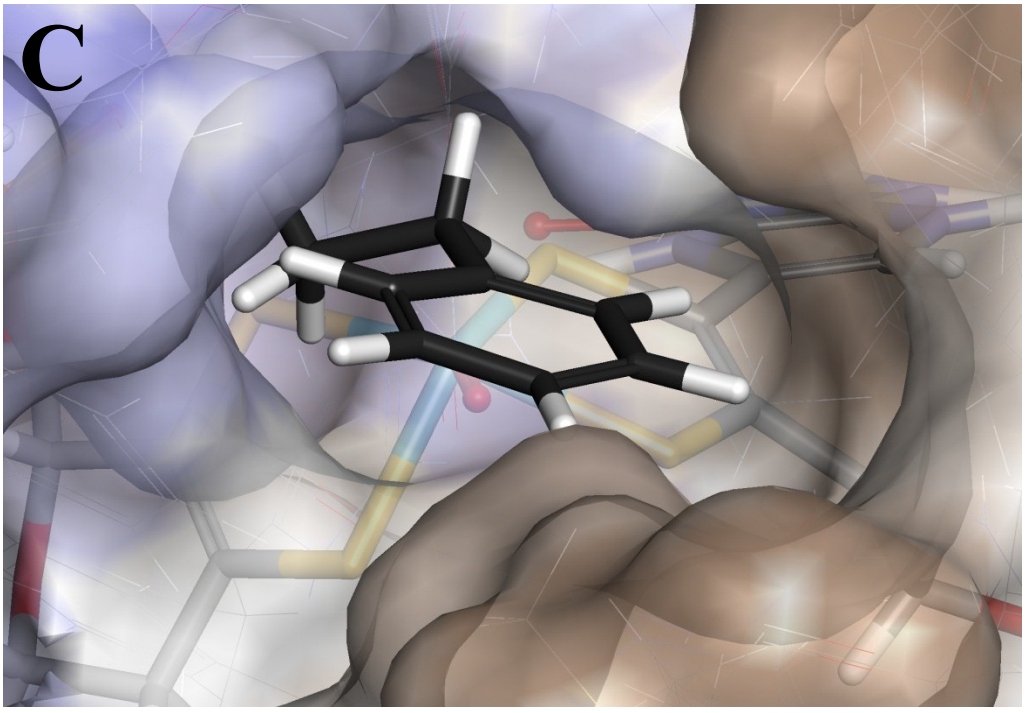
Ligand	Total Interaction Energy (kJ/mol)	Total VDW Interaction Energy (kJ/mol)	Total Electrostatic Interaction Energy (kJ/mol)
B-ethyl-1,2-azaborine conformer 1	-45.90	-43.77	-2.13
N-ethyl-1,2-azaborine conformer 2	-69.71	-67.53	-2.18
ethylbenzene	-55.77	-49.54	-6.19



Supporting Figure 4A. Modeling of *B*-ethyl-1,2-azaborine in the active site of EbDH. The protein surface is given in grade of hydrophobicity (brown=hydrophobic, blue=hydrophilic). The molybdenum atom in the background is shown in green.



Supporting Figure 4B. Modeling of *N*-ethyl-1,2-azaborine in the active site of EbDH. The protein surface is given in grade of hydrophobicity (brown=hydrophobic, blue=hydrophilic). The molybdenum atom in the background is shown in green.

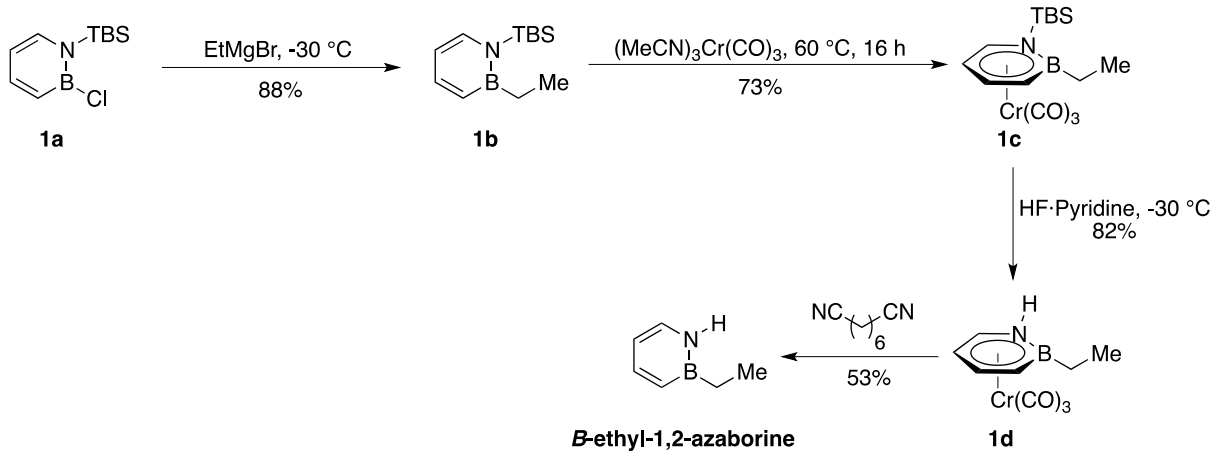


Supporting Figure 4C. Modeling of ethylbenzene in the active site of EbDH. The protein surface is given in grade of hydrophobicity (brown=hydrophobic, blue=hydrophilic). The molybdenum atom in the background is shown in green.

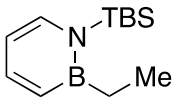
6. Synthesis of *B*-Ethyl-1,2-azaborine

Heterocycle *B*-ethyl-1,2-azaborine was prepared according to Scheme S1.

Scheme S1. Synthetic route toward *B*-ethyl-1,2-azaborine.

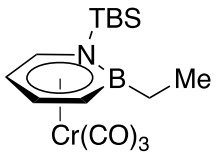


Compound 1b. In a glove box, a round bottom flask was charged with **1a**[11] (2.00 g, 8.79 mmol) in 40 mL THF. The flask was then cooled to –30 °C. Ethylmagnesium bromide (1.0 M solution in THF, 8.79 mL, 8.79 mmol) was added dropwise to the solution under stirring. The reaction was kept at –30 °C and stirred overnight (10 h) whereupon the solution was allowed to warm to room temperature. After stirring the mixture for an additional 2 hours, approximately three-fourths of the solvent was removed under reduced pressure. Then, 20 mL pentane was added to the reaction flask. The reaction mixture was passed through a medium-porosity frit, and the filtrate was concentrated under reduced pressure. The crude material was subjected to silica gel chromatography using 95:5 pentane:ether as an eluent to afford **1b** as a light yellow liquid (1.72 g, 88%).



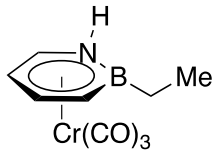
^{1}H NMR (500 MHz, CD_2Cl_2): δ 7.49 (dd, $^{3}J_{HH}$ = 11.2, 6.2 Hz, 1H), 7.27 (d, $^{3}J_{HH}$ = 6.8 Hz, 1H), 6.77 (d, $^{3}J_{HH}$ = 11.1 Hz, 1H), 6.21 (app. t, $^{3}J_{HH}$ = 6.3 Hz, 1H), 1.20 (q, $^{3}J_{HH}$ = 7.7 Hz, 2H), 1.10 (t, $^{3}J_{HH}$ = 7.6 Hz, 3H), 0.91 (s, 9H), 0.47 (s, 6H). ^{13}C NMR (125 MHz, CD_2Cl_2): δ 142.9, 138.5, 130.1 (br), 110.9, 26.7, 19.3, 13.7 (br), 11.6, –1.3. ^{11}B NMR (96 MHz, CD_2Cl_2): δ 41.6. FTIR (thin film) 3071, 2956, 2930, 2885, 2858, 1611, 1510, 1471, 1464, 1449, 1400, 1363, 1285, 1261, 1241, 1218, 1148, 1130, 1100, 1039, 1008, 988, 842, 823, 812, 786, 737, 695 cm^{-1}. HRMS (ESI) calcd for $C_{12}H_{25}BNSi$ (M^+) 222.1849, found 222.1849.

Compound 1c. In a glove box, a round bottom flask was charged with **1b** (1.72 g, 7.73 mmol) and trisacetonitrile(tricarbonyl)chromium(0) (3.61 g, 13.9 mmol) in 110 mL THF. The reaction mixture was heated to 60 °C and stirred for 16 h. At the conclusion of the reaction, the mixture was cooled to room temperature, and the solvent was removed under vacuum. The resulting crude material was filtered through a plug of silica gel. Concentration of the filtrate under reduced pressure and purification by silica gel chromatography using 10:1 pentane:ether as an eluent afforded the **1c** complex as a red/orange solid (2.02 g, 73%).



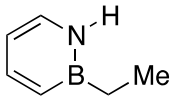
^{1}H NMR (500 MHz, CD_2Cl_2): δ 6.03 (d, $^{3}J_{HH}$ = 5.1 Hz, 1H), 5.99 (dd, $^{3}J_{HH}$ = 9.5 Hz, 6.0 Hz 1H), 5.21 (app. t, $^{3}J_{HH}$ = 5.0 Hz, 1H), 4.62 (d, $^{3}J_{HH}$ = 9.5 Hz, 1H), 1.27 (app. m, 1H), 1.15 (t, $^{3}J_{HH}$ = 7.5 Hz, 3H), 1.00 (app. m, 1H), 0.92 (s, 9H), 0.45 (s, 6H). ^{13}C NMR (125 MHz, CD_2Cl_2): δ 231.3, 109.3, 104.4, 84.9 (br), 83.4, 26.9, 20.0, 10.9, 10.3 (br), –0.8, –3.1. ^{11}B NMR (96 MHz, CD_2Cl_2): δ 27.4. FTIR (thin film) 3100, 3089, 3052, 3041, 2934, 2861, 1966, 1866, 1515, 1464, 1413, 1376, 1264, 1127, 1065, 1012, 984,

921, 840, 796, 731, 688, 673, 622 cm^{-1}. HRMS (ESI) calcd for $C_{15}H_{25}BNO_3SiCr$ (M^+) 358.1105, found 358.1102.



Compound 1d. To a stirred solution of complex **1c** (1.07 g, 3.00 mmol in 15 mL THF and 27 mL ether) was added dropwise a solution of HF-pyridine (2.0 M solution in THF of 70 wt% HF; 1.5 mL, 3.0 mmol) at –30 °C. The reaction was maintained at –30 °C with occasional stirring for 1.5 h. The mixture was warmed to room temperature and stirred an additional 0.5 h. The solution was concentrated under reduced pressure, and the resulting crude material was purified by silica gel chromatography using 60:40 pentane:ether as an eluent to yield **1d** as a red/orange solid. (0.598 g, 82%).

^{1}H NMR (500 MHz, CD_2Cl_2): δ 6.17 (t, $^3J_{HH}$ = 5.6 Hz, 1H), 5.88 (dd, $^3J_{HH}$ = 9.5, 6.1 Hz, 1H), 5.25 (app. t, $^3J_{HH}$ = 5.6 Hz, 1H), 5.06 (br s, 1H), 4.65 (d, $^3J_{HH}$ = 9.6 Hz, 1H), 1.07 (app. m, 5H). ^{13}C NMR (125 MHz, CD_2Cl_2): δ 230.6, 108.7, 98.8, 85.9 (br), 82.3, 8.9, 7.4 (br). ^{11}B NMR (96 MHz, CD_2Cl_2): δ 23.8. FTIR (thin film) 3339, 3115, 3101, 3053, 2964, 2937, 2916, 2877, 1967, 1946, 1883, 1863, 1846, 1537, 1479, 1445, 1407, 1395, 1348, 1261, 1142, 1135, 1116, 1068, 1017, 876, 847, 810 cm^{-1}. HRMS (EI) calcd for $C_9H_{10}BNO_3SiCr$ (M^+) 244.01603, found 244.01511.



***B*-Ethyl-1,2-Azaborine.** Under an N_2 atmosphere, complex **1d** (340 mg, 1.40 mmol) was dissolved in 1.5 mL 1,6-dicyanohexane (10.5 mmol) in a Schlenk tube and stirred at room temperature for 11 h. *B*-Ethyl-1,2-azaborine was then transferred at 60 °C under reduced pressure to a –78 °C cold trap to be isolated as a clear, colorless liquid. (78 mg, 53%).

^{1}H NMR (500 MHz, CD_2Cl_2): δ 7.87 (t, $^3J_{NH}$ = 49.4 Hz, 1H), 7.56 (dd, $^3J_{HH}$ = 11.4, 6.4 Hz, 1H), 7.27 (app. t, $^3J_{HH}$ = 7.3 Hz, 1H), 6.73 (d, $^3J_{HH}$ = 11.3 Hz, 1H), 6.21 (app. t, $^3J_{HH}$ = 6.6 Hz, 1H), 1.15 (app. m, 2H), 1.09 (app. m, 3H). ^{13}C NMR (125 MHz, CD_2Cl_2): δ 143.2, 133.5, 128.3 (br), 109.5, 10.2 (br), 9.6. ^{11}B NMR (96 MHz, CD_2Cl_2): δ 37.5. FTIR (thin film) 3381, 3072, 3019, 2953, 2910, 2872, 1615, 1537, 1461, 1423, 1354, 1229, 1124, 1089, 990, 756, 717 cm^{-1}. HRMS (EI) calcd for $C_6H_{10}BN$ (M^+) 107.09091, found 107.09063.

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