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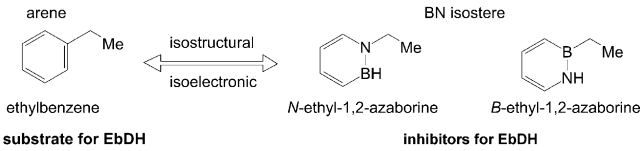
Boron Mimetics

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

Daniel H. Knack, Jonathan L. Marshall, Gregory P. Harlow, Agnieszka Dudzik, Maciej Szaleniec, Shih-Yuan Liu, and Johann Heider**

BN/CC isosterism has recently emerged as a viable strategy to increase structural diversity.[1,2] In particular, the chemistry of 1,2-dihydro-1,2-azaborines (hereafter abbreviated as 1,2-azaborines), which are BN isosteres of the family of arenes, has attracted attention as novel aromatic compounds relevant to biomedical research and materials science. In the past decade, significant advances have been made in the synthesis and characterization of 1,2-azaborines.[3,4] However, virtually no information is available on the behavior of 1,2-azaborines in a biological context. We previously demonstrated that *N*-ethyl-1,2-azaborine and the parent 1,2-azaborine bind inside the hydrophobic pocket of the L99A mutant of T4 lysozyme.[5] The demonstration of a biochemically active role of 1,2-azaborines, for example, as substrate or inhibitor of an enzyme, has to date remained elusive. In view of the dominance of arenes in pharmaceuticals, the study of the biochemical reactivity of 1,2-azaborines is significant: the new chemical space made available by 1,2-azaborines could open up opportunities in drug discovery and biomedical research. Herein, we reveal that the BN isosteres of ethylbenzene, *N*- and *B*-ethyl-1,2-azaborine, are actually strong inhibitors for the hydroxylation of ethylbenzene by ethylbenzene dehydrogenase (EbDH), providing the proof of concept that BN/CC isosterism can lead to novel biochemical behavior (Scheme 1). Ethylbenzene dehydrogenase is a molybdenum enzyme catalyzing the oxygen-independent hydroxylation of ethylbenzene to (*S*)-1-phenylethanol, initiating anaerobic ethylbenzene mineralization in the denitrifying betaproteobacterium "*Aromatoleum aromaticum*".[6–10] Oxygen-independent hydroxylation of a non-activated hydrocarbon is an unusual and unique feature of this enzyme.[8] A potential reaction mechanism involving consecutive radical and carbocation derivatives of the substrates as reaction intermediates was derived from quantum chemical calculations and experimental data.[11–16] The enzyme hydroxylates an extraordinarily broad spectrum of alkylated aromatic and heteroaromatic compounds in an enantioselective manner,[12] making it a very suitable system to investigate 1,2-azaborines as potential substrates.



Scheme 1. Effect of BN/CC isosterism on enzyme reactivity.

Herein, we explore *N*-ethyl-1,2-azaborine and *B*-ethyl-1,2-azaborine as substrates for EbDH (Scheme 1). Somewhat surprisingly, we did not detect enzymatic turnover for either of the 1,2-azaborines by photometric assay or by GC-MS analysis of liquid-phase extractions of reactions incubated for up to 12 h. However, we were able to identify both *N*-ethyl-1,2-azaborine and *B*-ethyl-1,2-azaborine as very strong inhibitors of EbDH (see Figure 1a). In particular, *N*-ethyl-1,2-azaborine turned out to be a very efficient inhibitor with an IC_{50} value of 2.8 μM whereas *B*-ethyl-1,2-azaborine had an IC_{50} value of 100 μM. The nature of inhibition with *N*-ethyl-1,2-azaborine was investigated by a more detailed inhibitory kinetic analysis (Figure 1b). We determined *N*-ethyl-1,2-azaborine to be a mixed-type inhibitor with a very low competitive inhibition constant ($K_{ic} = 0.55$ μM), which is basically identical to the K_m of ethylbenzene (0.45 μM).[11] The uncompetitive inhibition constant ($K_{iu} = 9$ μM) is 16-fold higher than that value, suggesting the presence of a second binding site. The experimental observations are consistent with the very similar molecular/geometric structures between *N*-ethyl-1,2-azaborine and the native substrate ethylbenzene,

[*] D. H. Knack, Prof. Dr. J. Heider
Laboratory for Microbial Biochemistry, Philipps University of Marburg
35043 Marburg (Germany)
E-mail: heider@biologie.uni-marburg.de
J. L. Marshall, G. P. Harlow, Prof. Dr. S.-Y. Liu
Department of Chemistry, University of Oregon
Eugene, OR, 97403-1253 (USA)
E-mail: lsy@uoregon.edu
A. Dudzik, Dr. M. Szaleniec
Jerzy Haber Institute for Catalysis and Surface Chemistry, Polish Academy of Sciences
30-239 Kraków (Poland)

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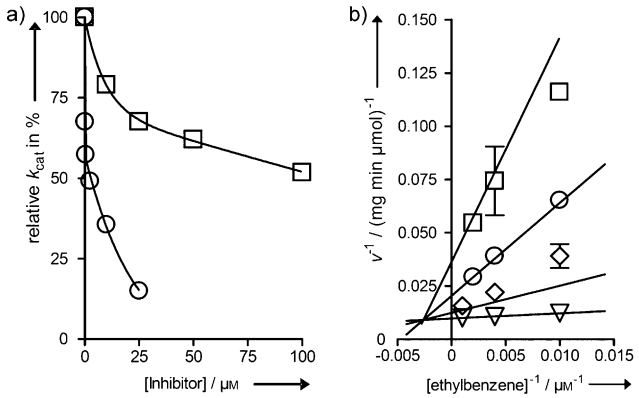

Figure 1. A: Nonlinear plots of the remaining ethylbenzene-dependent activity of EbDH when preincubated with different concentrations of *N*-ethyl-1,2-azaborine (circles) and *B*-ethyl-1,2-azaborine (squares). B: Double reciprocal plot of ethylbenzene-dependent enzymatic activity obtained when preincubated with different concentrations of *N*-ethyl-1,2-azaborine: 0 μM (triangles), 2.5 μM (diamonds), 10 μM (circles) 25 μM (squares). The kinetic data were fitted using the model for mixed inhibition[17] (see also Supporting Information).

which should make it hard for the enzyme to distinguish the two compounds.

We further performed a MM (molecular mechanics) modeling (see Supporting Information) of the 1,2-azaborines inside the EbDH substrate binding pocket and of bound ethylbenzene to probe this hypothesis (Figure 2 and Supporting Information). Indeed, both 1,2-azaborines fit nicely in the pocket. In contrast to *N*-ethyl-1,2-azaborine, which is almost congruent with ethylbenzene, *B*-ethyl-1,2-azaborine seems to bind in a slightly different orientation to the active site. As can be seen from Figure 1 a, *B*-ethyl-1,2-azaborine is a less-efficient inhibitor than *N*-ethyl-1,2-azaborine. Our modeling suggests that the lower inhibitory effect of *B*-ethyl-1,2-azaborine in comparison to *N*-ethyl-1,2-azaborine is a result of weaker binding through less-favorable van der Waals interactions with the hydrophobic residues in the active site (see Supporting Information for details).

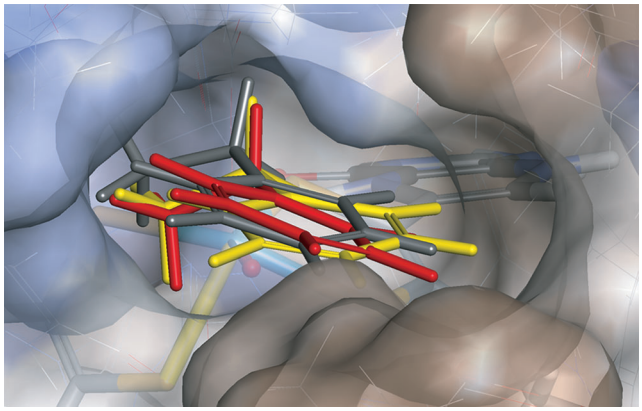

Figure 2. Overlay of *N*-ethyl-1,2-azaborine (red), *B*-ethyl-1,2-azaborine (yellow), and ethylbenzene (gray) modeled into the EbDH active site. The protein surface is given in grades of hydrophobicity (brown = hydrophobic, blue = hydrophilic). The view leads from the substrate channel towards the molybdenum cofactor. Note that force-field parameters had to be estimated for modeling the 1,2-azaborines, which may cause some deviations from the actual binding mode (see Supporting Information)

All the known substrates of EbDH are alkylaromatic compounds substituted at the C atoms of the aromatic ring.[11,12] To demonstrate that the relatively strong inhibitory behaviors of 1,2-azaborines originate from the combination of geometrical similarity and electronic structure differences between ethylbenzene and the corresponding 1,2-azaborines (BN/CC isosterism) rather than simple electronic structure changes through alkyl substitution at a heteroatom, we investigated the reactivity of EbDH with *N*-ethylimidazole and *N*-ethylpyrrole as other heteroatom-substituted heterocyclic substrate analogues. In doing so, we found that *N*-ethylimidazole is another inhibitory compound rather than a substrate, showing albeit a very weak inhibitory effect (IC_{50} value of 2200 μM) compared to *N*- and *B*-ethyl-1,2-azaborine. On the other hand, we detected clear enzymatic activity with *N*-ethylpyrrole, although it had a threefold lower rate than ethylbenzene (k_{cat}: 0.16 s^{-1} and 0.4 s^{-1}, respectively) and showed a relatively low affinity to EbDH (K_m(app) value: 130 μM). The hydroxylation of *N*-ethylpyrrole proceeded with an observed enantiomeric excess (*ee*) of 60% for the *S*-enantiomer of the product 1-(1*H*-pyrrol-1-yl)ethanol, determined by chiral GC coupled with mass spectrometry (see Supporting Information). We also detected a dehydrogenated side product (1-vinyl-1*H*-pyrrole), derived from a carbocation intermediate postulated to be generated by EbDH.[12] 1-Vinyl-1*H*-pyrrole may react non-enzymatically with water, resulting in a racemic mixture of the 1-(1*H*-pyrrol-1-yl)ethanol enantiomers, which would explain the relatively low stereoselectivity observed for *N*-ethylpyrrole hydroxylation.

To correlate the enzyme kinetic parameters of substrate conversion and enzyme inhibition to the molecular and electronic properties of the heteroatom-substituted compounds, calculations of the stabilities of corresponding radical and carbocation intermediates relative to those of ethylbenzene were performed (Table 1).[14] The data for *N*-ethylpyrrole show that radical formation is slightly less favored than for ethylbenzene. However, carbocation formation is energetically more favorable than for ethylbenzene, consistent with the involvement of a carbocation intermediate in the oxidation of *N*-ethylpyrrole by EbDH. Because substrate activation by radical formation is believed to be the rate-limiting step,[16] these values may also explain the lower enzymatic activity with *N*-ethylpyrrole than with ethylben-

Table 1: Relative calculated energy levels for radical and carbocation formation from different ethylbenzene analogues.

Compound[a]	ΔΔ*G* radical [kJ mol^{-1}]	ΔΔ*G* carbocation [kJ mol^{-1}]
N-ethyl-1,2-azaborine	23.69	12.28
B-ethyl-1,2-azaborine	15.41	24.43
N-ethylimidazole	23.0	37.9
ethylbenzene	0	0
***N*-ethylpyrrole**	18.56	−14.46

[a] Compounds in bold are substrates, the other compounds are inhibitors of EbDH.

zene. For the inhibitory compounds including the 1,2-azaborines, both calculated energies for radical and carbocation formation are substantially higher than those for ethylbenzene (Table 1). Therefore, it appears plausible that the energy barriers for forming the radical and carbocation intermediates cannot be overcome with these compounds, leading to inhibition by formation of non-productive enzyme–ligand complexes. These new data are consistent with the model for the catalytic mechanism of EbDH and will help to refine and expand the quantitative structure–activity relationship (QSAR) analysis for EbDH.[11]

In summary, this study provides the first evidence that non-natural 1,2-azaborine compounds can influence enzyme activity as structural mimics of aromatic compounds. The differences in electronic structures between ethylbenzene and its BN isosteres result in a role change from substrate to inhibitor in EbDH-mediated hydroxylation reactions. Control experiments with other heteroatom-substituted alkylarenes suggest that the combined effects of geometric similarity and electronic structure differences provided by BN/CC isosterism are responsible for the potent inhibition. The data reported herein demonstrate the proof-of-concept that BN/CC isosterism can lead to novel behavior in a biological context. The extended chemical space made available by azaborines should provide chemists with new tools to explore structure–activity relationships in biomedical research and medicinal chemistry.

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[1] Z. Liu, T. B. Marder, *Angew. Chem.* **2008**, *120*, 248 – 250; *Angew. Chem. Int. Ed.* **2008**, *47*, 242 – 244.
[2] H. B. Zhou, K. W. Nettles, J. B. Bruning, Y. Kim, A. Joachimiak, S. Sharma, K. E. Carlson, F. Stossi, B. S. Katzenellenbogen, G. L. Greene, J. A. Katzenellenbogen, *Chem. Biol.* **2007**, *14*, 659 – 669.
[3] For a Review of azaborine chemistry, see: a) P. G. Campbell, A. J. V. Marwitz, S.-Y. Liu, *Angew. Chem.* **2012**, *124*, 6178 – 6197; *Angew. Chem. Int. Ed.* **2012**, *51*, 6074 – 6092; b) M. J. D. Bosdet, W. E. Piers, *Can. J. Chem.* **2009**, *87*, 8 – 29; c) A. J. Ashe III, *Organometallics* **2009**, *28*, 4236 – 4248.
[4] a) E. R. Abbey, L. N. Zakharov, S.-Y. Liu, *J. Am. Chem. Soc.* **2008**, *130*, 7250 – 7252; b) A. J. V. Marwitz, M. H. Matus, L. N. Zakharov, D. A. Dixon, S.-Y. Liu, *Angew. Chem.* **2009**, *121*, 991 – 995; *Angew. Chem. Int. Ed.* **2009**, *48*, 973 – 977; c) P. G. Campbell, E. R. Abbey, D. Neiner, D. J. Grant, D. A. Dixon, S.-Y. Liu, *J. Am. Chem. Soc.* **2010**, *132*, 18048 – 18050; d) E. R. Abbey, L. N. Zakharov, S.-Y. Liu, *J. Am. Chem. Soc.* **2011**, *133*, 11508 – 11511; e) A. N. Lamm, E. B. Garner, D. A. Dixon, S.-Y. Liu, *Angew. Chem.* **2011**, *123*, 8307 – 8310; *Angew. Chem. Int. Ed.* **2011**, *50*, 8157 – 8160; f) A. Chrostowska, S. Xu, A. N. Lamm, A. Mazière, C. D. Weber, A. Dargelos, P. Baylère, A. Graciaa, S.-Y. Liu, *J. Am. Chem. Soc.* **2012**, *134*, 10279 – 10285; g) A. J. V. Marwitz, E. R. Abbey, J. T. Jenkins, L. N. Zakharov, S.-Y. Liu, *Org. Lett.* **2007**, *9*, 4905 – 4908.
[5] L. Liu, A. J. Marwitz, B. W. Matthews, S. Y. Liu, *Angew. Chem.* **2009**, *121*, 6949 – 6951; *Angew. Chem. Int. Ed.* **2009**, *48*, 6817 – 6819.
[6] H. A. Johnson, D. A. Pelletier, A. M. Spormann, *J. Bacteriol.* **2001**, *183*, 4536 – 4542.
[7] H. A. Johnson, A. M. Spormann, *J. Bacteriol.* **1999**, *181*, 5662 – 5668.
[8] O. Kniemeyer, J. Heider, *J. Biol. Chem.* **2001**, *276*, 21381 – 21386.
[9] R. Rabus, M. Kube, A. Beck, F. Widdel, R. Reinhardt, *Arch. Microbiol.* **2002**, *178*, 506 – 516.
[10] D. P. Kloer, C. Hagel, J. Heider, G. E. Schulz, *Structure* **2006**, *14*, 1377 – 1388.
[11] M. Szaleniec, C. Hagel, M. Menke, P. Nowak, M. Witko, J. Heider, *Biochemistry* **2007**, *46*, 7637 – 7646.
[12] D. H. Knack, C. Hagel, M. Szaleniec, A. Dudzik, A. Salwinski, J. Heider, *Appl. Environ. Microbiol.* **2012**, *78*, 6475 – 6482.
[13] M. Szaleniec, T. Borowski, K. Schuhle, M. Witko, J. Heider, *J. Am. Chem. Soc.* **2010**, *132*, 6014 – 6024.
[14] M. Szaleniec, M. Witko, J. Heider, *J. Mol. Catal. A* **2008**, *286*, 128 – 136.
[15] M. Szaleniec, M. Witko, R. Tadeusiewicz, J. Goclon, *J. Comput.-Aided Mol. Des.* **2006**, *20*, 145 – 157.
[16] M. Szaleniec, A. Salwinski, T. Borowski, J. Heider, M. Witko, *Int. J. Quantum Chem.* **2012**, *112*, 1990 – 1999.
[17] A. Cornish-Bowden, *Analysis of Enzyme Kinetic Data*, Oxford University Press, Oxford, **1995**.

