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# Bromoenterobactins as Potent Inhibitors of a Pathogen-Associated, Siderophore-Modifying *C*-Glycosyltransferase

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Many bacteria synthesize and secrete iron-binding small molecules, termed siderophores, under iron-limiting conditions to obtain iron that is required for survival.[1,2] Mammalian host serum is a surprisingly iron-poor environment for pathogenic bacteria, with an estimated free iron concentration of about $10^{-24}$ M.[3] As a result, many bacterial genes involved in iron acquisition are required for virulence.[1,4] However, siderophore production does not always correlate with virulence. For example, enterobactin (Ent) is biosynthesized by many nonpathogenic enteric bacteria, including *E. coli*. Mammalian neutrophils secrete a protein, lipocalin 2 (Lcn2), that binds both iron-free and iron-loaded Ent with high affinity ($K_d$ = 0.4 nM) (Fischbach et al., submitted) and thereby sabotages Ent-mediated iron acquisition (Figure 1A).[5,6] Certain Ent-producing bacteria (e.g., *E. coli* CFT073 and *Salmonella typhimurium* LT2) have evolved the five-gene *iroA* cluster to evade Lcn2. Proteins encoded by the *iroA* cluster catalyze the *C*-glucosylation of Ent to generate predominantly diglucosyl Ent (DGE, Figure 1B).[7−12] We recently demonstrated that Lcn2 does not bind DGE ($K_d$ > 1 $\mu$M), and the presence of the *iroA* cluster increases the virulence of *E. coli* strain H9409 in mice (Fischbach et al., submitted). Therefore, pathogenic bacterial strains use the *iroA* cluster to transform Ent to a siderophore that evades sequestration by the mammalian immune system (Figure 1B).[13] These findings suggest that inhibiting proteins encoded by the *iroA* cluster could be an effective strategy to combat *iroA*-harboring bacteria.

We have focused our initial inhibitor design efforts on the *C*-glucosyltransferase IroB because it is responsible for the first step in the *iroA*-mediated Ent modification process. We previously reported that IroB catalyzes the successive mono-, di-, and triglucosylation of Ent (Figure 1C).[9] In the absence of a crystal structure of IroB, we began by examining the substrate specificity of IroB to further understand the properties of its active site as an initial effort toward rationally designing inhibitors for IroB. Here we report the results of this study and the finding that brominated Ent analogues are potent inhibitors of IroB.

We designed and synthesized Ent analogues **1**−**5** (Figure 2) bearing a variety of substitution patterns on the benzoyl rings. Compounds **1**−**5** were not glucosylated when incubated with IroB and UDP-Glc under conditions that led to complete conversion of Ent to DGE. Furthermore, **1**−**5** are not effective inhibitors of IroB. When 128 $\mu$M of **1**−**5** was added to IroB-catalyzed Ent glucosylation reactions, the Ent glucosylation rate decreased less than 20%. The fact that **1**−**5** are neither substrates nor effective inhibitors of IroB suggests that IroB likely recognizes both hydroxyl groups on the 2,3-dihydroxybenzoyl (DHB) ring. This hypothesis is further supported by the observation that iron-bound Ent is not a substrate for IroB, possibly because the hydroxyl groups of the $Fe^{3+}$−Ent complex are not accessible to IroB (Supporting Information Figure S1).

We next prepared and characterized Ent analogues **6**−**8** (Figure 2) containing scaffolds other than the trilactone core. Analogues **6** and **7** are very poor substrates. Under conditions in which Ent is completely converted to DGE, less than 5% of **6** or **7** is converted to monoglucosylated product (Supporting Information Figure S2). The ring-opened Ent analogue **8** is not detectably accepted as a substrate. In addition, **6**−**8** are not effective inhibitors of IroB. Taken together, these data suggest that the DHB group and the trilactone core of Ent are key determinants of IroB recognition.

These findings led us to synthesize brominated Ent analogues **9**−**11** (Figure 2), in which both the trilactone core and the DHB moiety are conserved, while some or all of the positions to be glucosylated are blocked by substitution with bromine. We anticipated that the mono- (**9**) and dibromo-Ent (**10**) would remain substrates of IroB since each retains at least one DHB ring that could, in principle, be glucosylated. We were surprised to find that even monobromo-Ent **9** is not accepted as a substrate by IroB. Further studies reveal that **9**−**11** are potent inhibitors of IroB. The presence of compounds **9**−**11** changes the apparent $K_m$ value of IroB-catalyzed Ent glucosylation, but not the $k_{cat}$ value, suggesting that they are competitive inhibitors of IroB (see Supporting Information). In addition, preincubation of IroB with **9**−**11** does not change the inhibition profile, and IroB incubated with or without **9**−**11** gave the same mass, 42.5 kD, by MALDI-MS (Supporting Information), indicating that the inhibition does not involve covalent bond formation between IroB and inhibitors **9**−**11**. The $K_i$ values were determined to be 23 ± 3, 7.0 ± 1.5, and 3.3 ± 0.9 nM for compounds **9**, **10**, and **11**, respectively.

To enable a comparison of the kinetically determined $K_i$ values with the actual binding constants, $K_d$ values for binding to IroB were obtained by measuring the change in the intrinsic fluorescence of IroB in the presence of various concentrations of **9**−**11**. It was noted in initial assays that the presence of UDP-Glc has no effect on the fluorescence. Therefore, subsequent binding assays were all carried out in the absence of UDP-Glc. These measurements revealed $K_d$ values of 34 ± 8, 12 ± 2, and 9 ± 4 nM for inhibitors **9**, **10**, and **11**, respectively. The $K_d$ values are essentially equivalent to the $K_i$ values obtained in kinetic assays, suggesting that the mechanism of IroB inhibition involves high affinity binding of **9**−**11** to IroB.

Using the same fluorescence method, the $K_d$ value for Ent binding to IroB was measured to be 2.3 ± 0.7 $\mu$M, similar to the $K_m$ value (3.5 ± 0.5 $\mu$M). Therefore, addition of a single Br atom at C5 of one DHB moiety increases the binding affinity about 60-fold. We speculate that the IroB active site contains a hydrophobic pocket

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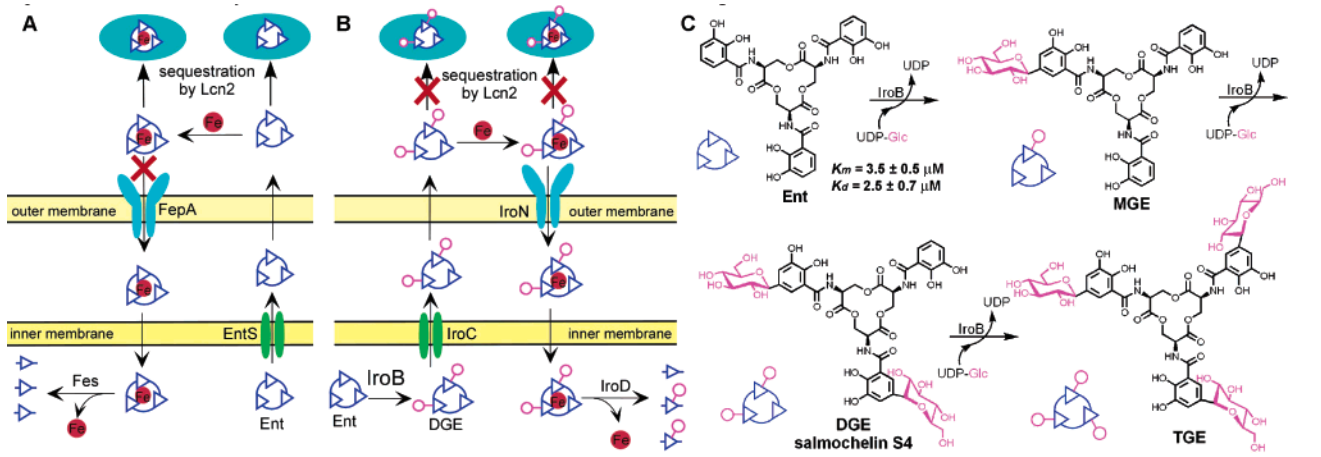


***Figure 1.*** The *C*-glycosyltransferase IroB converts Ent to glucosylated forms that escape sequestration by mammalian Lcn2 protein. (A) In strains that do not harbor the *iroA* cluster, Ent is secreted into the host, where it is tightly bound by host Lcn2, rendering Ent ineffective as a siderophore. (B) In strains harboring the *iroA* cluster, IroB coverts Ent to DGE, which cannot be sequestered by Lcn2 and therefore is an effective siderophore. (C) IroB catalyzes the successive mono-, di-, and tri-*C*-glucosylation of Ent to give MGE, DGE, and TGE *in vitro*. The symbols at the bottom left corner of each structure are simplified representations of the siderophores.

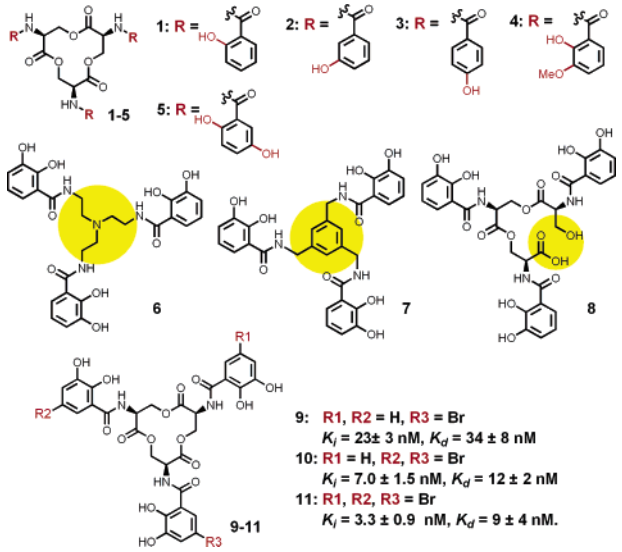


***Figure 2.*** Structures of Ent analogues used in this study.

that can accommodate the DHB ring, and that the presence of Br at C5 of DHB results in additional favorable enzyme−inhibitor van der Waals interactions or in more favorable entropy changes in water upon binding. Presumably, the presence of the C5 Br is inconsistent with the mechanism for glucosylation of C5, preventing compounds **9**−**11** from serving as substrates for IroB.

In summary, in the process of probing the substrate specificity of IroB with synthetic Ent analogues, we have discovered that brominated Ent derivatives **9**−**11** are potent inhibitors of IroB. Since IroB is responsible for the key modification of Ent in the bacterial strategy to evade mammalian Lcn2, we anticipate that IroB inhibitors could potentially be used as antibiotics against *iroA*-harboring pathogenic bacteria. Compounds **9**−**11** may therefore be promising lead compounds for the development of effective antibiotics that function by inhibiting IroB. The finding that the addition of a bromine atom can significantly increase the binding affinity of Ent to IroB may also have implications in designing inhibitors for other proteins in the iron acquisition pathway, such as the cell surface siderophore receptors IroN[7,14] and FepA.[15−17]

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**Supporting Information Available:** Synthesis, characterization, and enzymatic activity assay of Ent analogues, $K_i$ and $K_d$ determination for compounds **9**−**11**, and MALDI-MS of IroB. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Neilands, J. B. *J. Biol. Chem.* **1995**, *270*, 26723.
(2) Crosa, J. H.; Walsh, C. T. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 223.
(3) Raymond, K. N.; Dertz, E. A.; Kim, S. S. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3584.
(4) Snyder, J. A.; Haugen, B. J.; Buckles, E. L.; Lockatell, C. V.; Johnson, D. E.; Donnenberg, M. S.; Welch, R. A.; Mobley, H. L. T. *Infect. Immun.* **2004**, *72*, 6373.
(5) Goetz, D. H.; Holmes, M. A.; Borregaard, N.; Bluhm, M. E.; Raymond, K. N.; Strong, R. K. *Mol. Cell.* **2002**, *10*, 1033.
(6) Flo, T. H.; Smith, K. D.; Sato, S.; Rodriguez, D. J.; Holmes, M. A.; Strong, R. K.; Akira, S.; Aderem, A. *Nature* **2004**, *432*, 917.
(7) Hantke, K.; Nicholson, G.; Rabsch, W.; Winkelmann, G. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3677.
(8) Bister, B.; Bischoff, D.; Nicholson, G. J.; Valdebenito, M.; Schneider, K.; Winkelmann, G.; Hantke, K.; Sussmuth, R. D. *Biometals* **2004**, *17*, 471.
(9) Fischbach, M. A.; Lin, H.; Liu, D. R.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 571.
(10) Lin, H.; Fischbach, M. A.; Liu, D. R.; Walsh, C. T. *J. Am. Chem. Soc.* **2005**, *127*, 11075.
(11) Luo, M.; Lin, H.; Fischbach, M. A.; Liu, D.; Walsh, C. T.; Groves, J. T. *ACS Chem. Biol.* **2006**, *1*, 29.
(12) Zhu, M.; Valdebenito, M.; Winkelmann, G.; Hantke, K. *Microbiology* **2005**, *151*, 2363.
(13) Fischbach, M. A.; Lin, H.; Liu, D. R.; Walsh, C. T. *Nat. Chem. Biol.* **2006**, *2*, 132.
(14) Baumler, A. J.; Norris, T. L.; Lasco, T.; Voight, W.; Reissbrodt, R.; Rabsch, W.; Heffron, F. *J. Bacteriol.* **1998**, *180*, 1446.
(15) Ecker, D. J.; Matzanke, B. F.; Raymond, K. N. *J. Bacteriol.* **1986**, *167*, 666.
(16) Annamalai, R.; Jin, B.; Cao, Z.; Newton, S. M.; Klebba, P. E. *J. Bacteriol.* **2004**, *186*, 3578.
(17) Buchanan, S. K.; Smith, B. S.; Venkatramani, L.; Xia, D.; Esser, L.; Palnitkar, M.; Chakraborty, R.; van der Helm, D.; Deisenhofer, J. *Nat. Struct. Biol.* **1999**, *6*, 56.

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