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Utilizing an iron(III)-chelation masking strategy to prepare mono- and bis-functionalized aerobactin analogues for targeting pathogenic bacteria†

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A direct and selective method for the functionalization of aerobactin has been described. Selectivity was achieved by masking the functioning carboxylate *via* iron-chelation, leaving the two remaining carboxylates for direct modification. Both mono- and bis-functionalized aerobactin effectively targeted pathogenic bacteria, showing a facile method with prospective applications.

Iron, which is a key component of many biological processes, is essential to the survival of all beings, including bacteria. Iron availability is limited by hosts as a nutrient barrier to prevent the proliferation of malicious invaders. To overcome this nutritional barrier during infection, siderophores, a class of iron chelators with exceptionally high affinity, are adopted by microbial pathogens to scavenge iron from their hosts.[1] The necessity of siderophore production during infection and their ability to carry iron(III) across the membrane into the cytoplasm provide an ideal target for therapeutic applications.[2] In fact, natural siderophore-toxin conjugates, also known as sideromycins, such as microcin E492, albomycin and salmycin, have drawn as much attention as their intriguing conjugate design.[3] Artificial sideromycin mimics have also been synthesized, and many of them have shown promising antimicrobial activities.[4] Additionally, siderophore-based vaccine conjugates have recently been demonstrated as potential therapeutic alternatives to eliminate bacterial infections in animal models.[5] However, the challenge of the general synthesis of diverse siderophore conjugates has prevented their applications.

Aerobactin is a representative example of a hydroxamate–citrate hybrid siderophore that is widely used by both terrestrial and marine bacteria. This hexadentate chelator is composed of two N^6-acetyl-N^6-hydroxy-L-lysines (AHLs) that are symmetrically linked to one citric acid through two amide bonds. The two AHL hydroxamates and the central α-hydroxyl carboxylate of citrate are the iron(III) chelating moieties. Epidemiological studies have considered aerobactin to be a virulence factor with certain correlation with bacterial pathogenicity.[6] In pathogenic strains such as hypervirulent *K. pneumoniae*[7] and uropathogenic *E. coli*,[8] the occurrence of aerobactin is markedly higher than that in avirulent strains. On the WHO priority pathogens list in 2017,[9] carbapenem-resistant and third-generation cephalosporin-resistant *Enterobacteriaceae*, including *K. pneumoniae* and *E. coli*, have been flagged as "critical". Therefore, it is worthwhile to develop functional probes targeting these pathogens.

Although aerobactin and its cognate receptor, IutA, are frequently found in many pathogenic bacteria, it remained unknown whether aerobactin can be used as a bacterial targeting unit. More specifically, it was not clear how to modify aerobactin to prepare functional conjugates that can be recognized by bacteria. Aerobactin was first isolated in 1969,[10] and the first total synthesis of aerobactin was reported in 1982.[11] Despite previous synthetic efforts, aerobactin cannot be used to immediately prepare aerobactin conjugates or analogues. To date, no well-defined synthetic aerobactin conjugates or analogues have been reported.

In aerobactin, the two AHL carboxylates are potential modification sites because they are not involved in iron chelation. However, the free central α-hydroxyl carboxylate in citrate poses a synthetic challenge for selective modification without a sophisticated protection strategy. Alternatively, we turned our focus on the well-studied iron(III) coordination of aerobactin.[12] Copper(II) chelation has been widely used to mask α-amino acids, rendering robust and selective side chain modifications.[13] In our case, we envisioned that the central α-hydroxyl carboxylate in the citrate moiety can be masked by iron(III) chelation. In other words, the central carboxylate could be considered as being "protected" by iron(III). Using this chelation strategy, we might be able to directly and selectively modify native aerobactin.

To obtain native aerobactin, we revised the preparation of aerobactin (Scheme 1). For the citric acid portion, the reported *t*-butyl citrate protected at the central carboxylate[14] was chosen

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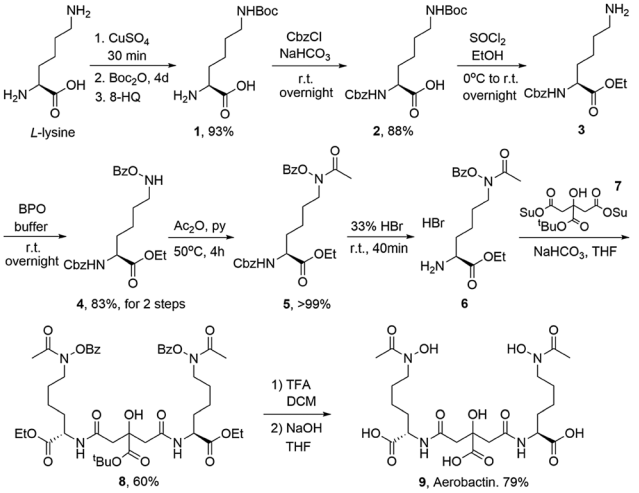
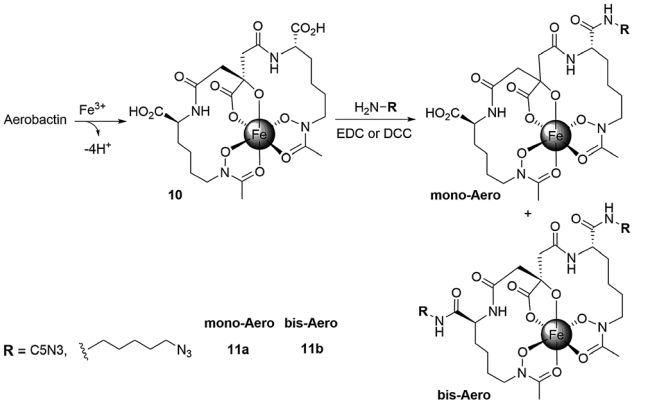


Scheme 1 Alternative chemical synthesis of aerobactin.



Scheme 2 The iron(III)-chelation masking strategy for the synthesis of mono- and bis-substituted aerobactin analogues. See the ESI† for the extended substrate scope.

to prevent undesired imide formation and subsequent amide shift.[15] For the AHL portion, we started with L-lysine using modified methods from previous analogous schemes.[16] In short, two amino groups were sequentially protected by Boc and then by Cbz groups. The esterification was achieved by treatment with $SOCl_2$ and EtOH at low temperatures; Boc can be removed at the same time. Then, without purification, the resulting product **3** was subjected to benzoyl peroxide (BPO) oxidation[17] under optimized buffer conditions to give the desired hydroxylamine derivatives **4**. After acetylation, the fully protected AHL **5** was obtained in good yield and after only four separation steps.

Using dicyclohexylcarbodiimide (DCC), the *t*-butyl citrate was pre-activated with *N*-hydroxysuccinimide (HOSu) to give **7**. Cbz-deprotection of **5** was achieved with HBr in AcOH to afford **6** for immediate use.[16] The typical Cbz-deprotection by catalytic hydrogenation led to complex mixtures, where the intra-molecular transacetylation was the major side product. The construction of aerobactin was accomplished by amide bond formation between **6** and **7**. To prevent amide shift, a slight excess of **6** was introduced. Finally, after global deprotection, aerobactin was obtained in 32% overall yield from L-lysine.

We then investigated the feasibility of functionalization by iron(III)-chelation masking (Scheme 2). To test this idea, a set of amino linkers with different functional groups was examined. In the typical coupling reactions, aerobactin was mixed with different amounts of linkers and coupling regents (Table S1, ESI†). The reactions were monitored directly by LC/MS. Notably, in the presence of 2 equivalents of iron(III), only the mono- and bis-substituted amide products were detected, even when excess linkers (10–40 equiv.) and coupling reagents (100 equiv.) were used; no tri-substituted products were observed. In contrast, in the absence of iron(III), tri-substituted products were easily observed, and similar results were observed when iron(III) was insufficient (0.5 equiv.). These results clearly indicated that iron(III) masked one carboxylate from amide coupling. When excess coupling reagents were used, DCC was a better option due to the easy removal of its by-product dicyclohexylurea (DCU). The mono-substituted compound could not be obtained as the major end-product by simply controlling the amounts of coupling reagents or linkers. Instead, it could only be obtained by monitoring the reaction progress (Fig. S4, ESI†). Using optimal iron(III)-masking conditions, we were able to prepare the mono- and bis-substituted aerobactin analogues **11a** and **11b** in separate reactions. These analogues can be purified by HPLC on the milligram scale with >90% purity (Fig. S2 and S3, ESI†). Interestingly, the purified mono-substituted analogue **11a** exhibited two close HPLC peaks with identical masses, which is consistent with mono-substitution at two AHL carboxylates generating a pair of diastereomers (Fig. S1, ESI†). This result strengthened the iron(III)-chelation masking strategy.

To verify that the central α-hydroxyl carboxylate remains intact after amide coupling, we observed the photoreactivity of the α-hydroxyl carboxylate in many siderophores, such as aerobactin, vibrioferrin, and petrobactin.[18] Structural analysis by NMR was not feasible due to the strong paramagnetic iron(III) bound in the analogues. Iron removal was time-consuming and not necessary because iron-bound aerobactin analogues can be used directly in biological tests. Photolysis was facilitated by the chelated iron(III), which was reduced to iron(II), dissociated and oxidized back to iron(III) by air before being re-chelated with the product (Fig. 1a). Only the iron-bound α-hydroxyl carboxylate can undergo photolysis, unlike the AHL carboxylates. **10**, **11a**, and **11b** were irradiated with 419 nm light and then subjected to HPLC/MS analysis. All three compounds gave the corresponding photolysis products (Fig. 1 and Fig. S5, S6, ESI†). LC/MS/MS analysis for the mono- and bis-substituted **11a** and **11b** was also conducted to verify the regioselectivity of the amide coupling. The results were consistent with photolysis (Fig. S7 and S8, ESI†).

We next examined whether the mono- and bis-substitution affected the iron-uptake ability of aerobactin. Neither substitution affected the iron binding, as shown in the characteristic red iron-bound complex due to the charge-transfer absorption. To determine whether the mono- or bis-substitution affected bacterial binding, we prepared aerobactin–fluorophore conjugates by click chemistry. Although direct functionalization required fewer steps,

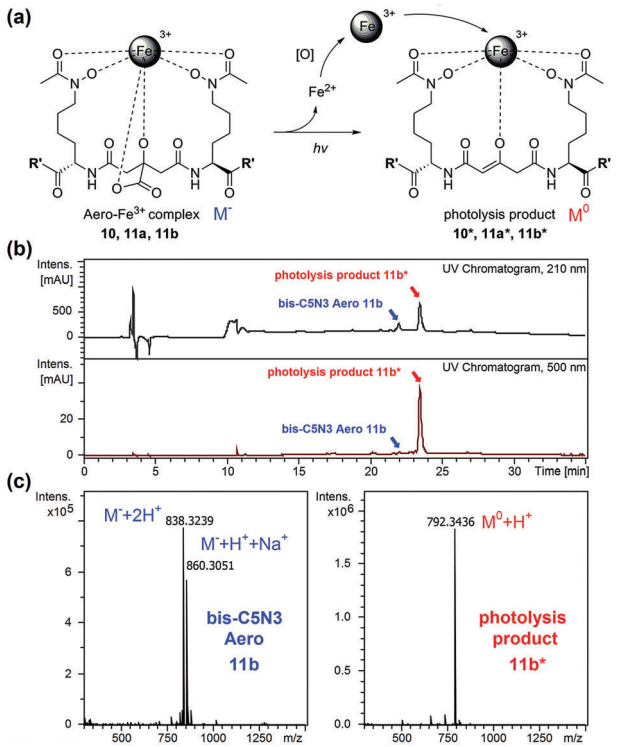


Fig. 1 Photolysis reaction of bis-C5N3 Aero **11b**. (a) The general photolysis scheme on the α-hydroxyl carboxylate moiety in the presence of iron(III). (b) HPLC chromatograms for the photolysis of bis-C5N3 Aero at 210 nm and 500 nm. (c) MS spectra of bis-C5N3 Aero **11b** and its corresponding photolysis product **11b***.

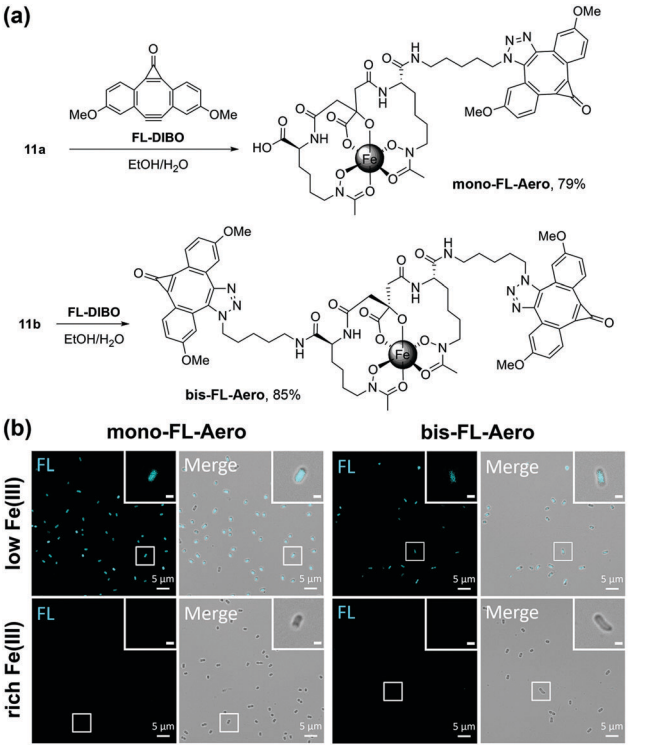


Fig. 2 (a) Functionalization of the azido aerobactin analogues **11a** and **11b** with **FL-DIBO** by SPAAC. (b) Microscopy images of *K. pneumoniae* treated with **mono-FL-Aero** and **bis-FL-Aero** under different iron supplementations (inset scale bar: 1 μm).

click chemistry was superior due to its synthetic flexibility. Both clickable azido analogues **11a** and **11b** (Scheme 2) were prepared to conduct copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) or strain-promoted azide–alkyne cycloaddition (SPAAC). The reactivity of the iron-bound aerobactin analogues in CuAAC varied, and it was difficult to find proper click pairs and conditions. Instead, SPAAC gave more reliable click products. Thus, a recently reported symmetric fluorogenic **FL-DIBO** was synthesized[19] and then clicked by SPAAC to prepare two fluorescent conjugates, **mono-FL-Aero** and **bis-FL-Aero**, both with purities >90% by HPLC (Fig. S2 and S3, ESI†).§

The bacterial targeting abilities of **mono-FL-Aero** and **bis-FL-Aero** were first tested in pathogenic *K. pneumoniae*. The bacteria were cultured in low iron (1 μM) minimal media (MM9) to induce the siderophore-mediated iron-uptake pathway before incubation with aerobactin–fluorophore conjugates. **mono-FL-Aero** was able to label *K. pneumoniae* with high efficiency, suggesting that the non-chelating AHL carboxylate was a proper conjugation site (Fig. 2b). Surprisingly, **bis-FL-Aero** also labeled *K. pneumoniae* with good efficiency, implying that neither AHL carboxylates interfered with receptor binding. Subsequently, high levels of iron (100 μM) were introduced during culture preparation to turn off the siderophore pathway. No significant fluorescence labeling was observed for either conjugate, indicating that the uptake process was due to the iron-dependent siderophore system. This also indicated that the clicked **FL-DIBO** did not bind to the bacteria non-specifically.

With the gene cluster located at the pathogenicity islands, enteric bacteria secrete aerobactin as their virulence factor. To examine the selective targeting of our aerobactin conjugates, we surveyed several species of bacteria (Fig. 3). Four pathogenic Gram-negative bacteria, including *E. coli* O157:H7, *E. coli* CFT073, *S. enterica* and *V. cholerae*, were tested. *E. coli* and *S. enterica* exhibited high labeling efficiencies by both **mono-FL-Aero** and **bis-FL-Aero**. Consistent with a previous report, *V. cholerae* was not labeled by either of our probes.[20] Moreover, two Gram-positive bacteria, *B. subtilis* and *S. epidermidis*, were tested as negative controls because aerobactin is thought to be utilized only in Gram-negative bacteria. As expected, these two bacteria showed no labeling by either probe. When it comes to the epidemiologically important pathogens, *P. aeruginosa* and *S. aureus*, which were also tested, the results were inconclusive because their autofluorescence overlapped with the **FL-DIBO** emission. Nevertheless, our results demonstrated the good bacterial targeting feature of aerobactin in many pathogens.

In this study, we have developed an iron(III)-chelation masking strategy to prepare mono- and bis-functionalized analogues of native aerobactin without using complicated protecting protocols. This strategy could be generalized to other native siderophores carrying non-chelating functional groups, such as staphyloferrin A from *S. aureus*.[21] Currently, the reactions were limited to the few milligram scale by HPLC, but their expansion to a larger scale is possible after optimizing the separation schemes. Both mono- and bis-functionalized aerobactin conjugates were able to label a wide

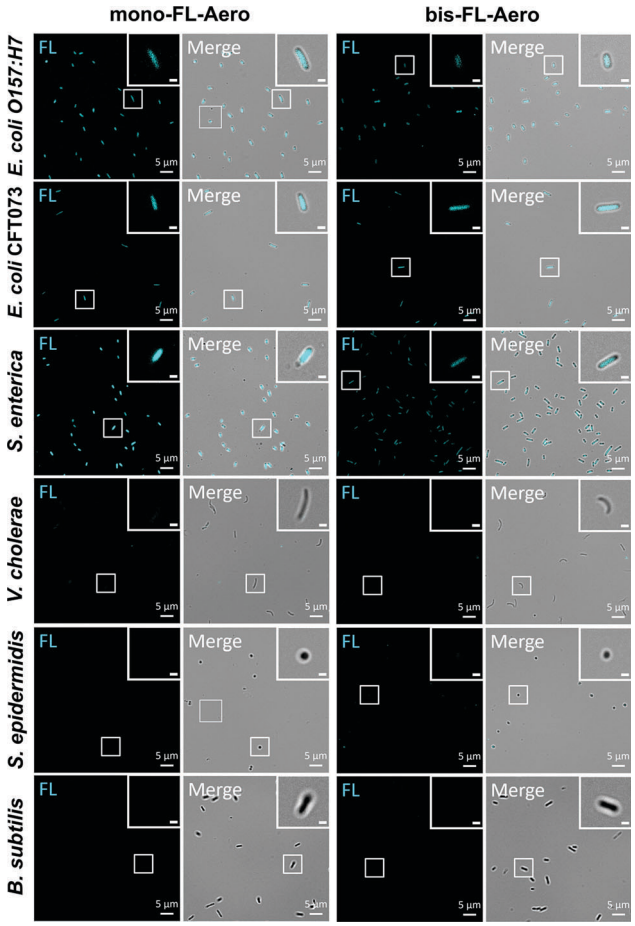


Fig. 3 Bacteria labeling experiments with the **mono-FL-Aero** and **bis-FL-Aero** conjugates under iron-limiting conditions (left: fluorescence channel and right: merge) (20 μM of either probe, cultured in MM9 minimal medium, more details are provided in the ESI†) (inset scale bar: 1 μm).

range of pathogenic bacteria. Our results also suggested the possibility of delivering more than one cargo by aerobactin at the same time. Aerobactin conjugates carrying two synergistic toxins might promote enhanced bacterial killing effects. For future therapeutic applications, the cellular locations of mono- and bis-functionalized conjugates after uptake should be confirmed as aerobactin analogues might be acquired into bacteria or stuck on surface receptors.

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Notes and references

§ Commercially available dibenzocyclooctyl (DBCO) reagents gave two regioisomers by SPAAC, which complicated the mono- and bis-functionalized probe preparations.

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