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Biomimetic enterobactin analogue mediates iron-uptake and cargo transport into *E. coli* and *P. aeruginosa*

The design, synthesis and biological evaluation of the artificial enterobactin analogue EntKL and several fluorophore-conjugates thereof are described. EntKL provides an attachment point for cargos such as fluorophores or antimicrobial payloads. Showing similar iron binding behaviour compared to the natural product, EntKL exhibited potent concentration-dependent growth promoting effects in siderophore biosynthesis deficient mutants of *E. coli* and *P. aeruginosa* and was demonstrated to be able to deliver fluorescent molecular cargos via the bacterial iron transport machinery into the bacteria.

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Biomimetic enterobactin analogue mediates iron-uptake and cargo transport into \textit{E. coli} and \textit{P. aeruginosa}\textsuperscript{†}

Robert Zscherp,\textsuperscript{†a} Janetta Coetzee,\textsuperscript{†b,c} Johannes Vornweg,\textsuperscript{d} Jörg Grunenberg,\textsuperscript{a} Jennifer Herrmann,\textsuperscript{b,c} Rolf Müller\textsuperscript{b,c} and Philipp Klahn\textsuperscript{a,†}

The design, synthesis and biological evaluation of the artificial enterobactin analogue Ent \textsubscript{aL} and several fluorophore-conjugates thereof are described. Ent \textsubscript{aL} provides an attachment point for cargos such as fluorophores or antimicrobial payloads. Corresponding conjugates are recognized by outer membrane siderophore receptors of Gram-negative pathogens and retain the natural hydrolyzability of the tris-lactone backbone. Initial density-functional theory (DFT) calculations of the free energies of solvation (ΔG(solv)) and relaxed Fe–O force constants of the corresponding [Fe-Ent\textsubscript{aL}]\textsuperscript{3+} complexes indicated a similar iron binding constant compared to natural enterobactin (Ent). The synthesis of Ent \textsubscript{aL} was achieved via an iterative assembly based on a 3-hydroxylysine building block over 14 steps with an overall yield of 3%. A series of growth recovery assays under iron-limiting conditions with \textit{Escherichia coli} and \textit{Pseudomonas aeruginosa} mutant strains that are defective in natural siderophore synthesis revealed a potent concentration-dependent growth promoting effect of Ent \textsubscript{aL} similar to natural Ent. Additionally, four cargo-conjugates differing in molecular size were able to restore growth of \textit{E. coli} indicating an uptake into the cytosol. \textit{P. aeruginosa} displayed a stronger uptake promiscuity as six different cargo-conjugates were found to restore growth under iron-limiting conditions. Imaging studies utilizing BODIPY\textsubscript{FL}-conjugates, demonstrated the ability of Ent \textsubscript{aL} to overcome the Gram-negative outer membrane permeability barrier and thus deliver molecular cargos via the bacterial iron transport machinery of \textit{E. coli} and \textit{P. aeruginosa}.

Introduction

The development of bacterial resistance towards antimicrobial drugs is an intrinsic part of bacterial evolution and this, in turn, necessitates the continuous development of novel drugs able to kill these life-threatening multi-drug resistant human pathogens.\textsuperscript{1–3} Facing the current spread of bacterial resistance against clinically used antibiotics, the development of novel antimicrobial drugs and innovative concepts is of vast importance to counteract this serious threat for public health.\textsuperscript{4,5,3}

Four of the six ESKAPE pathogens,\textsuperscript{6,7} represent bacterial species considered as significant threat for public health due to a very high occurrence of multi-drug resistance, are Gram-negative bacteria. In addition, the pathogens recently prioritized by the WHO with a critical, high or medium need for the development of novel antimicrobial drugs are primarily Gram-negative bacteria, including β-lactam-resistant \textit{Escherichia coli} and \textit{Pseudomonas aeruginosa} or fluoroquinolone-resistant \textit{Salmonella} and \textit{Shigella} species.\textsuperscript{8} However, the development of novel antimicrobial drugs against Gram-negative bacteria is challenging, not only due to the presence of drug exporters as also found in Gram-positive bacteria, but because many antibiotic compounds fail to overcome the Gram-negative outer membrane. As a consequence, many potential drugs displaying antibacterial activity against Gram-positive bacteria remain inactive against Gram-negative bacteria, although their biological targets are generally present, as they fail to translocate over the cell envelope barrier.\textsuperscript{9}

An innovative approach to enable translocation of antimicrobial drugs or reporter molecules over the Gram-negative cell envelope barrier and to develop novel antimicrobial drugs against these pathogens is the conjugation and hybridization with siderophores.\textsuperscript{4,6,8–12} Siderophores are small molecule iron chelators,\textsuperscript{13,14} produced and secreted by bacteria, fungi and plants to ensure their supply with iron, an essential growth factor for all living organisms.\textsuperscript{15} Beyond iron uptake,
Siderophores have multiple other biological functions in bacteria, e.g., they are important virulence factors contributing to pathogenesis. After coordination of Fe(III), the resulting ferri-siderophores are recognized by specific siderophore receptors, transmembrane proteins embedded in the outer membrane, and are actively transported into the cytosol of bacteria. Siderophore drug conjugates can enter bacterial cells via the same pathway through siderophore receptor mediated uptake.

The general concept of this Trojan Horse approach is derived from natural anetotypes. The sideromycins, such as albomycins or the microcins E492 (ref. 37–40) and H47 (ref. 41) are utilized by different bacteria to defend their ecological niche against competing bacteria. These natural siderophore drug conjugates reveal a remarkable increase in antibacterial activity compared to the parental free antibiotics. Inspired by this natural concept researchers have developed artificial siderophore drug conjugates based on synthetically modified siderophore analogues, leading to active drug accumulation and tremendously increased antibacterial activity compared to the parental drugs. Furthermore, siderophore drug conjugates can expand the activity of Gram-positive-only drugs towards Gram-negative pathogens. Additionally, this strategy allows for the design of narrow-spectrum antibiotics, selectively targeting specific virulent pathogens, thereby reducing the selection pressure on the bacterial resistome. Importantly, the siderophore-β-lactam hybrid cefiderocol developed by Shionogi Pharmaceuticals was recently approved by the FDA for the treatment of pneumonia caused by Gram-negative bacteria.

In this context, the tris-catechol siderophore enterobactin ([Ent, Scheme 1A]) displaying an extraordinarily high Fe(III) binding constant ($K_d = 10^{49}$ M$^{-1}$) as well as its glycosylated derivatives, the salmochelins evading lipocandin-2 inactivation, are of high interest. Ent and salmochelins are biosynthesized by Gram-negative bacteria of the family Enterobacteriaceae, such as E. coli, Klebsiella pneumonia, Salmonella typhimurium, Versinia enterococitica and Shigella flexneri, to ensure their iron supply. However, Ent has also been found to be produced by Gram-positive Streptomyces species and it serves as a xenosiderophore for the opportunistic human pathogen P. aeruginosa, where internalization is mediated by the outer membrane siderophore receptor PfeA. Ent and salmochelins also play a crucial role during infection and have been demonstrated to sequester iron from human transferrin. Previously reported artificial Ent analogues used for the development of siderophore drug conjugates or siderophore-based imaging tools were based on modifications at one of the catechol units and the natural tris-serine lactone backbone was substituted by artificial moieties to generate an attachment point for payloads in the backbone of the siderophore. Although a co-crystal structure of [Fe-Ent]$^{3+}$ with its E. coli siderophore receptors of FepA and Iron$^{42}$ is not available, recognition of [Fe-Ent]$^{3+}$ at other proteins such as FeuA, VctP, PfeA or lipocalin-2 (ref. 43) suggests that the attachment of larger payloads at the catechol units might interfere with [Fe-Ent]$^{3+}$ receptor recognition.

Therefore, we envisaged the synthesis of the novel, biomimetic enterobactin analogue EntKL (Scheme 1B). Our synthesis is based on the incorporation of the 3-hydroxy lysine derivative into Ent backbone, aiming to generate an attachment point for antimicrobial payloads in the backbone out of the recognition site of the siderophore and simultaneously retain the natural hydrolyzability of the tris-lactone backbone.

### Result and discussion

Retrosynthetically, the final siderophore could be derived from a modified asymmetric tris-lactone backbone by decoration with catechol units via amide coupling (Scheme 1B). As the template mediated trans-esterification approach utilized earlier, by Shanzer, later by Gutierrez and Raymond for the synthesis of the enterobactin triserine lactone backbone, leads exclusively to the formation of the thermodynamic, symmetric product, it is not applicable for the synthesis of the envisaged asymmetric tris-lactone backbone. Therefore, we decided to follow an approach of iterative assembly of a linear trimer containing the 3-hydroxy lysine derivative with subsequent macro lactonization as used in the early synthesis of Ent by Corey, Rastetter and Rogers in order to generate the desired asymmetric backbone of EntKL. In order to estimate if the envisaged substituent at the asymmetric tris-lactone backbone would negatively impact the
solution thermodynamics and/or the kinetic stability of the Fe(III) complex $[\text{Fe-Ent}_{\text{KL}}]^3^-$, we computed the free energies of solvation $\Delta G_{\text{sol}}$ as well as the relevant (relaxed) Fe–O force constants for $[\text{Fe-Ent}]^3^-$ and two stereoisomers of $[\text{Fe-Ent}_{\text{KL}}]^3^-$ bearing an axial or equatorial substituent (Table 1) following the protocol of Baramov et al.55 (see ESI†).

In a first step, we tried to reproduce the experimentally well-known free energy of complexation ($\Delta G_{\text{sol}}$) as well as the relevant (relaxed) Fe–O force constants for $[\text{Fe-Ent}]^3^-$ and two stereoisomers of $[\text{Fe-Ent}_{\text{KL}}]^3^-$ as bearing an axial or equatorial substituent (see Table 1) following the protocol of Baramov et al.55 (see ESI†).

Our calculated value of $-64.5$ kcal mol$^{-1}$ for $\Delta G_{\text{sol}}$ for $[\text{Fe-Ent}]^3^-$ is very close to the literature ref. 59 and 60 positively validating our applied in silico procedure. Interestingly, the artificial ferri-enterobactin analogues seem to be even better iron binders than the unsubstituted $[\text{Fe-Ent}]^3^-$ by several kcal mol$^{-1}$. Furthermore, the value for $[\text{Fe-Ent}_{\text{KL}}(\text{eq})]^3^-$ was calculated to be energetically more favored as compared to $[\text{Fe-Ent}_{\text{KL}}(\text{ax})]^3^-$ by a value of $3.5$ kcal mol$^{-1}$.

Nevertheless, the absolute values need to be considered with caution and we hypothesize that both artificial enterobactin analogues, Ent$_{\text{KL}}(\text{eq})$ and Ent$_{\text{KL}}(\text{ax})$, lead to complexes which are at least of similar thermodynamic stability compared to $[\text{Fe-Ent}]^3^-$ and that there is no significant energetic advantage of one analogue over the other. While siderophore Fe(III) complexes are thermodynamically very stable, they are kinetically labile. This is in contrast to, for example, their chromic counterparts, which are both thermodynamically and kinetically stable. In order to find out if this kinetic lability holds true also for our artificial ferri-enterobactin analogues, we calculated the relaxed Fe–O force constants, a method which was recently successfully applied in several other weakly bound systems. According to our computational results, all three complexes seem to be kinetically labile with relaxed Fe–O force constants well below 1 N cm$^{-1}$ (see Table 1), indicating very weak bonds with less or no covalency.96–100 (Note, that the averaged Cr–O value for $[\text{CrEnt}]^3^-$ is 1.25 N cm$^{-1}$; unpublished results J. G.) Furthermore, our calculated Fe–O values are very similar. In summary, all three complexes seem to be kinetically labile, while being thermodynamically stable, as found earlier for $[\text{Fe-Ent}]^3^-$ by Raymond and co-workers.101,102

Thus, expecting no negative impact of an axial backbone substituent on the Fe(III) complex stability, we developed a robust, scalable synthetic route to the required 3-hydroxy lysine derivative 1 starting from commercially available 4-aminobutanol (2) as outlined in Scheme 2. A sequence of Boc protection, Swern oxidation of the resulting N-Boc-4-aminobutanol (3) to aminal 4 and final Wittig olefination in presence of methyl (triphenyl-phosphoranylidene) acetate (5) at $100^\circ$ C led to formation of the E-configured $\alpha,\beta$-unsaturated methyl ester 6 in excellent diastereoselectivity of $E:Z = 100:1$ and 89% yield over 3 steps with only a single chromatographic purification step being required. An asymmetric sharpless dihydroxylation using AD-mix-$\alpha$ finally gave diol 7 in 83% yield. The formation of the corresponding cyclic sulfite in the presence of thionyl chloride and triethyl amine at 0 °C and subsequent $\alpha$-inversion in the presence of sodium azide afforded $\alpha$-azido methyl ester 8 in 64% yield. At this step the absolute

![Table 1](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>$[\text{Fe-Ent}]^3^-$</th>
<th>$[\text{Fe-Ent}_{\text{KL}}(\text{ax})]^3^-$</th>
<th>$[\text{Fe-Ent}_{\text{KL}}(\text{eq})]^3^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G_{\text{sol}}$ [kcal mol$^{-1}$]</td>
<td>$-64.5$</td>
<td>$-67.2$</td>
</tr>
<tr>
<td>$\Delta G_{\text{sol}}$ [kJ mol$^{-1}$]</td>
<td>$-269.9$</td>
<td>$-281.6$</td>
</tr>
<tr>
<td>Fe–O bond force constant (av) [N cm$^{-1}$]</td>
<td>0.95</td>
<td>0.97</td>
</tr>
</tbody>
</table>

$^a$ Computed for DMSO as solvent. $^b$ Separate consideration of all hydronium ions.
configuration at the C3 position introduced by the sharpless dihydroxylation was determined to be S-configured with an enantiomeric excess of 94%ee using Mosher’s method.103

Finally, a Staudinger reduction and subsequent Cbz protection generated the methyl ester 9 in 87% yield, which was saponified in the presence of trimethyl hydroxide in 1,2-dichloro ethane at 65 °C using Nicolaou’s protocol184 yielding the desired 3-hydroxy lysine derivative 1 in 94%.

With 1 in hands, we achieved the assembly of the linear trimer 17 as outlined in Scheme 3. First, the 2-methylthraquinyl ester 11 was formed with 2-bromomethylthraquinone 10 (MaqBr) in the presence of DBU in 55% yield.13 A two times consecutive sequence of Steglich esterification with Cbz-Ser(TBS)-OH 12 and subsequent desilylation in the presence of a mixture of aqueous, concentrated HF solution and TBAF led to the Maq-protected linear trimer 16 obtained in 64% yield over 4 steps. Ester 16 was then photolytically cleaved at 360 nm in the presence of N-methyl morpholine (NMM) in a mixture of iso-propanol and chloroform giving the unprotected linear trimer 17 as precursor for the backbone cyclization in 65% yield.

As expected for the formation of a 12-membered ring with several axial substituents, the cyclization of 17 to the tris-lactone 18 was challenging.

The activation of the carboxylic acid via several reagents predominantly led to elimination under formation of the corresponding dihydro amino acid (Table 2).

After extensive screening of different methods for the macro lactonization, a first success with a low yield of 13% was achieved using Shiina’s reagent185 (Entry 1, Table 2). Any other screened coupling reagent such as different carbodiimides, PyBOP, T3P (propylphosphonic anhydride) or Yamaguchi’s thraquinone trimer10 yielding the desired 3-hydroxy lysine derivative 1 in 94%.

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However, as the yield of this reaction was hardly reproducible, we finally applied 2,2′-dipyridyl disulfide to form the intermediate thioester and furnished macro lactonization in the presence of silver acetate giving 32% yield of the desired tris-lactone 18 reliably (Entry 4, Table 2).

The final quantitative, hydrogenolytic Cbz deprotection required 20 atm of hydrogen gas in a mixture of methanol, ethyl acetate and TFA. Subsequent acylation under Schotten–Bau mann conditions with 2,3-diacetoxybenzoic acid chloride 19 or 2,3-dimethoxybenzoic acid chloride 20, respectively, led to the formation of (AcO)EntKL and (MeO)EntKL. EntKL could be obtained in low yield from (AcO)EntKL by mild saponification in diluted methanolic ammonia.

However, as it has been reported that acetylated siderophore-prodrugs can be activated in situ and are favorable to prevent the inactivation of catecholates by enzymatic methylolation,48,76,109,110 we employed (AcO)EntKL as siderophore-prodrug and (MeO)EntKL as starting point for the synthesis of corresponding negative control probes, unable to bind iron or mediate uptake in our approach. In order to determine if the synthesized enterobactin derivatives are internalized by E. coli and P. aeruginosa during iron limitation, growth recovery assays were done with mutant strains that lack the ability to bio-synthesize siderophores, and therefore require external siderophores to be able to grow under iron-limiting conditions. Importantly, iron-limiting conditions are typically found at the site of infections being part of the host immune response to prevent bacterial growth.38,111,112 E. coli K-12 ΔentA grew to OD600 ≈ 0.35 in 50% MHB II medium (37 °C, t = 24 h), and this value decreased to <0.05 when 200 µM 2,2′-bipyridyl (DP) was added to the media in order to simulate iron-limiting conditions.

Scheme 3  Assembly of a linear trimer, cyclization and final catechol decoration.
conditions. Low-micromolar concentrations of Ent restored growth, and the E. coli cultures reached OD₆₀₀ = 0.1, 0.15 and 0.3 in the presence of 1.0 μM, 10 μM and 15 μM Ent, respectively (Fig. 1A).

Intriguingly, the addition of (AcO)EntKL led to restore the growth of E. coli K-12 ΔentA in the presence of DP in a concentration-dependent manner and with a similar efficiency as the natural siderophore Ent (Fig. 1A and B). A significant increase in growth was observed starting at 1 μM concentration, while at 10 μM and 15 μM the cultures grew to OD₆₀₀ ≈ 0.15 and 0.3, respectively, indicating the ability of (AcO)EntKL to be internalized into the cytosol of E. coli. Similarly, P. aeruginosa K648 Δpvd/pch grew to OD₆₀₀ ≈ 0.4 (37°C, t = 24 h) and this value decreased to <0.25 in the presence of 600 μM DP. Supplementation of the iron-reduced growth medium with 1.0 μM of Ent resulted in the restoration of P. aeruginosa growth to OD₆₀₀ = 0.35 (Fig. 1D).

At higher concentrations of Ent of 10 μM and 15 μM the growth promoting response was even exceeding the growth in absence of DP, with P. aeruginosa K648 Δpvd/pch growing to OD₆₀₀ ≈ 0.5 and 0.6, respectively. Again, (AcO)EntKL was also able to restore the growth of P. aeruginosa K648 Δpvd/pch under iron-limiting conditions in a concentration-dependent manner (Fig. 1D and E). While the growth promoting effect of (AcO)EntKL at a concentration of 1.0 μM was slightly smaller compared to Ent, the growth promoting was clearly surpassing the effect of Ent at 10 μM. In contrast to the uptake of Ent in E. coli, Ent never reaches the cytosol of P. aeruginosa as iron release takes place in the periplasm through cleavage of the siderophore backbone by the periplasmic esterase PfeE. This indicates that the compounds are accepted as substrates by PfeE. As expected, the permethylated probe (MeO)EntKL, was not able to restore growth of neither E. coli K-12 ΔentA nor P. aeruginosa K648 Δpvd/pch (Fig. 1C and F).

Table 2  Selected conditions for the cyclization of the linear trimer

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MNBA (5.0 eq.), DMAP (8.0 eq.), (CH₂Cl₂), 0–23 °C, 20 h</td>
<td>13%a</td>
</tr>
<tr>
<td>2</td>
<td>PPh₃ (1.5 eq.), DIAD (1.3 eq.), (THF), 0–23 °C, 24 h</td>
<td>14%a</td>
</tr>
<tr>
<td>3</td>
<td>(1) Corey’s disulfane² (1.4 eq.), PPh₃ (1.4 eq.), (CH₂Cl₂), 23 °C, 10 min; (2) AgBF₄ (5.0 eq.), (PhH), 25 °C, 16 h</td>
<td>5–50%ab</td>
</tr>
<tr>
<td>4</td>
<td>(1) 2,2’-Dipyridyldisulphide (1.4 eq.), PPh₃ (1.4 eq.) in (CH₂Cl₂), 30 °C, 90 min; (2) AgOAc (2.0 eq.), (PhH), 30 °C, 3 h</td>
<td>32%a</td>
</tr>
</tbody>
</table>

a Elimination. b Bad reproducibility.

Fig. 1  E. coli K-12 ΔentA and P. aeruginosa K648 Δpvd/pch growth recovery assays employing Ent in comparison to (AcO)EntKL and (MeO)EntKL (50% MHB II, ±200 or 600 μM DP (2,2'-bipyridine), t = 24 h, 37°C). Gray bars: OD₆₀₀ of bacteria cultured in the absence of DP. Black bars: OD₆₀₀ of bacteria cultured in the presence of 200 μM (E. coli) or 600 μM (P. aeruginosa) DP. (A) Ent promotes growth recovery of E. coli. (B) (AcO)EntKL affords growth recovery of E. coli. (C) (MeO)EntKL shows no growth recovery of E. coli. (D) Ent promotes growth recovery of P. aeruginosa. (E) (AcO)EntKL affords growth recovery of P. aeruginosa. (F) (MeO)EntKL shows no growth recovery of P. aeruginosa. Each bar indicates the average of three independent replicates (two wells per replicate) and the error bars are the standard deviation of the mean. As P. aeruginosa shows a growth of <0.25 OD in the absence of an external siderophore, Y-axis is shown starting at an OD₆₀₀ of 0.2. Statistical significance is indicated as a p-value < 0.05 and was determined by an unpaired t-test using GraphPad Prism 9 (version 9.1.1). ***(P-value: <0.001), **(P-value: <0.005) and *(P-value: <0.05).
Next, we synthesized a series of cargo-conjugates of (AcO)EntKL and (MeO)EntKL attaching fluorophores and dyes that differ in their size in order to investigate whether (AcO)EntKL is able to deliver different cargo molecules to E. coli and P. aeruginosa. Therefore, we cleaved the Boc group at the amino handle in the presence of TFA in dichloromethane and reacted the resulting free amine in the presence of N-hydroxy succinimide (NHS) esters of the corresponding fluorophores and dyes (SulfoCy5-NHS, BODIPYFL-NHS and MG-NHS) obtaining the conjugates (AcO)EntKL-BODIPYFL, (AcO)EntKL-MG and (AcO)EntKL-SulfoCy5 as well as their respective negative control probes (MeO)EntKL-BODIPYFL, (MeO)EntKL-MG and (MeO)EntKL-SulfoCy5 in moderate yields as outlined in Scheme 4. Similarly, initial Boc cleavage in the presence of TFA and subsequent installation of a PEG4-N3 chain via NHS ester coupling gave access to the corresponding (AcO)EntKL-PEG4-N3 and (MeO)EntKL-PEG4-N3. Final copper(I)-mediated azide–alkyne click reaction with alkyne functionalized BODIPYs (BODIPY-alkyne and BODIPYFL-alkyne) furnished the conjugates (AcO)EntKL-PEG4-BODIPY and (AcO)EntKL-PEG4-BODIPYFL as well as their respective negative control probes (MeO)EntKL-PEG4-BODIPY and (MeO)EntKL-PEG4-BODIPYFL in moderate to good yields (Scheme 4). Finally, we synthesized (AcO)EntKL-PFG-MG and (MeO)EntKL-PFG-MG via a sequence of initial Boc cleavage, installation of a PEG4-NH2Boc chain via NHS ester coupling, final Boc cleavage and reaction of the free amine with MG-NHS in moderate yields.

When incubating E. coli K-12 ΔentA under iron-limiting conditions with (AcO)EntKL-PEG4-BODIPY, (AcO)EntKL-PEG4-BODIPYFL, (AcO)EntKL-BODIPYFL and (AcO)EntKL-SulfoCy5 led to a concentration-dependent growth recovery (Fig. 2A–D) clearly indicating the uptake of these cargo-conjugates to the cytosol of E. coli. The two conjugates (AcO)EntKL-MG and (AcO)EntKL-PEG4-MG bearing a malachite green derivative as cargo molecule did not lead to a growth recovery in E. coli (Fig. 2E and F). Furthermore, high concentrations of 10 μM and 15 μM seemed to reduce overall growth in E. coli K-12 ΔentA (Fig. 1E and F) as well as in the E. coli wild-type strain (see ESI: Fig. S7).

Scheme 4 Synthesis of different EntKL-cargo conjugates bearing different fluorophores and chromophores as payload.
and S8†). This might be due to the fact that these compounds are not taken up by the bacteria and therefore, they further reduce the available iron in the surrounding medium. Although, the malachite green derivative attached within (AcO)EntKL-MG and (AcO)EntKL-PEG3-MG has a large diameter of approximately 13.7 Å (extracted from an energy minimized structure by Chem3D® Ultra 15.1.0.144), size seems not to be the exclusive parameter for uptake. In comparison, (AcO)EntKL-SulfoCy5, bearing the rigid SulfoCy5 fluorophore with a length diameter of approximately 19.9 Å (extracted from an energy

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** E. coli K-12 ΔentA (A–F) and P. aeruginosa K648 Δpvd/pch (G–L) growth recovery assays employing (AcO)EntKL-PEG4-BODIPY, (AcO)EntKL-BODIPYFL, (AcO)EntKL-PEG4-BODIPYFL, (AcO)EntKL-SulfoCy5, (AcO)EntKL-MG and (AcO)EntKL-PEG3-MG (50% MHB II, ± 200 or 600 μM DP [2,2'-bipyridine], t = 24 h, 30 °C). Gray bars: OD600 of bacteria cultured in the absence of DP. Black bars: OD600 of bacteria cultured in the presence of 200 μM (E. coli) or 600 μM (P. aeruginosa) DP. (A) (AcO)EntKL-PEG4-BODIPY promotes growth recovery of E. coli. (B) (AcO)EntKL-SulfoCy5 promotes growth recovery of E. coli. (C) (AcO)EntKL-BODIPYFL promotes growth recovery of E. coli. (D) (AcO)EntKL-PEG4-BODIPYFL promotes growth recovery of E. coli. (E) (AcO)EntKL-MG is not able to restore growth of E. coli. (F) (AcO)EntKL-PEG3-MG is not able to restore growth of E. coli. As P. aeruginosa shows a growth of <0.25 OD in the absence of an external siderophore, Y-axis is shown starting at an OD600 of 0.2. (G) (AcO)EntKL-PEG4-BODIPY promotes growth recovery of P. aeruginosa. (H) (AcO)EntKL-SulfoCy5 promotes growth recovery of P. aeruginosa. (I) (AcO)EntKL-BODIPYFL promotes growth recovery of P. aeruginosa. (J) (AcO)EntKL-PEG4-BODIPYFL promotes growth recovery of P. aeruginosa. (K) (AcO)EntKL-MG promotes growth recovery of P. aeruginosa. (L) (AcO)EntKL-PEG3-MG promotes growth recovery of P. aeruginosa. Each bar indicates the average of three independent replicates (two wells per replicate) and the error bars are the standard deviation of the mean. Fig. S3–S8† contain the assay results for the corresponding negative control probes (MeO)EntKL-PEG4-BODIPY, (MeO)EntKL-BODIPYFL, (MeO)EntKL-PEG4-BODIPYFL, (MeO)EntKL-SulfoCy5, (MeO)EntKL-MG and (MeO)EntKL-PEG3-MG, none of which were able to restore growth in neither E. coli nor P. aeruginosa. Statistical significance is indicated as a p-value < 0.05 and was determined by an unpaired t-test using GraphPad Prism 9 (version 9.1.1). ***p-value: <0.001, **p-value: <0.005) and *p-value: <0.05).
minimized structure by Chem3D® Ultra 15.1.0.144) of its indocyanine-backbone, led to a growth recovery under same conditions. Looking at the crystal structure of the enterobactin specific siderophore receptor FepA, the transmembrane pore opening upon recognition of Ent seems to have an elliptical inner diameter between 20-30 Å, large enough to harbor any of the reported conjugated discussed here. Therefore, additional parameters beyond size seem to determine the uptake of a bound cargo at the outer membrane receptor.

However, size exclusion might still play a role at the ABC-type transporter mediating uptake of hydrolyzed fragments across the inner membrane.

When incubating the conjugates with P. aeruginosa K648 ΔpvdΔpch under iron-limiting media conditions all cargo conjugates led to clear concentration-dependent growth recovery, including (AcO)EntKL-MG and (AcO)EntKL-PEG3-MG, indicating the uptake of these cargo-conjugates to the periplasm of P. aeruginosa (Fig. S9). These results are in line with the findings of Nolan and co-workers reporting that P. aeruginosa exhibits greater promiscuity for the uptake compared to E. coli. Looking at the crystal structure of PfeA, the outer membrane siderophore receptor of P. aeruginosa, a similar elliptical inner diameter between 25–35 Å can be assumed. Consistently, none of the corresponding permethylated negative control probes led to a growth recovery neither in E. coli nor P. aeruginosa (see ESI, Fig. S3–S8†). The overall growth recovery response to the added compounds was significantly higher for P. aeruginosa K648 ΔpvdΔpch compared to E. coli K-12 ΔentA, under non-iron-limiting conditions (Fig. 3B). The comparison of the growth recovery over all tested compounds in E. coli revealed a similar efficiency of all conjugates compared to the natural siderophore Ent (Fig. 3A).

A similar picture was observed for the growth recovery in P. aeruginosa. However, for some of the compounds such as (AcO)EntKL and (AcO)EntKL-PEG4-BODIPY an even improved performance compared to Ent was observed (Fig. 3B). Furthermore, we were able to demonstrate that growth of P. aeruginosa mutants could be restored with (AcO)EntKL in the presence of comparably high concentrations (0.01 μM, 0.1 μM, 1.0 μM and 10 μM) of the human iron scavenger protein apo-transferrin (Kd = 10^-12 M)†† (see ESI, Fig. S9†).

Further, an additional growth recovery assay was conducted with (AcO)EntKL in the presence of high concentrations (1.0 μM, 10.0 μM, 50 μM and 100 μM) bovine serum albumin (see ESI, Fig. S9†). It is worth to mention, that growth recovery was achieved with (AcO)EntKL. Albumin is responsible for the transport of lipophilic compounds and it is therefore able to bind Ent. These results indicate the potential ability of our designed enterobactin derivative (AcO)EntKL to compete with human iron-binding and lipophilic transport serum proteins.

Next, we incubated (AcO)EntKL-BODIPYFL, (MeO)EntKL-BODIPYFL, (AcO)EntKL-PEG4-BODIPYFL and (MeO)EntKL-PEG4-BODIPYFL at different concentrations with E. coli K-12 ΔentA, P. aeruginosa K648 ΔpvdΔpch and their corresponding wild-type strains under iron-limiting conditions in order to proof whether uptake of the probes leads to fluorescence labelling of the bacteria, providing additional evidence for the uptake into the bacteria.

To our delight, all assessed bacteria were fluorescently labelled when treated with either (AcO)EntKL-PEG4-BODIPYFL or (AcO)EntKL-BODIPYFL at concentrations of 10 μM (Fig. 4A, B, D and E, see ESI, Fig. S11, S13, S16 and S18†), further confirming the uptake into the wild-type and mutant strains of E. coli and P. aeruginosa. Weaker fluorescence was observed at 1.0 μM concentration of the fluorophore conjugates on all tested strains (see ESI, Fig. S11, S13, S16, S18 and Table S3†). In general, the fluorescence uptake into both mutant strains was higher compared to the wild types, and E. coli showed a significantly higher uptake than P. aeruginosa (see ESI, Table S3†).

Furthermore, we observed slight differences in the labeling performance of (AcO)EntKL-BODIPYFL and (AcO)EntKL-PEG4-BODIPYFL for the different bacteria. While (AcO)EntKL-BODIPYFL led to more prominent labelling of E. coli BW25113
Corrected total cell fluorescence (CTCF) of each experiment see Table S3†. Consistently, no labelling was observed when cells were treated with either \((\text{MeO})\text{EntKL-BODIPY}FL\), \((\text{MeO})\text{EntKL-PEG4-BODIPY}FL\) or non-conjugated \(\text{BODIPY}FL\)-alkyne alone (see ESI, Fig. S12, S14, S15, S17, S19 and S20†). Fluorescence labeling of \(E.\ coli\ K-12\ \Delta\text{entA}\) and \(P.\ aeruginosa\ K468\ \Delta\text{pvd/pch}\) was also observed when treating the bacteria with mixtures of \((\text{AcO})\text{EntKL-BODIPY}FL\) or \(\text{Ent/1 : 10, 1 : 1, 10 : 1 or 10 : 10}\) (Fig. S21 and S22†).

Interestingly, the fluorescence labelling for \(E.\ coli\) and \(P.\ aeruginosa\) mutant strains seems to be increased in the presence of \(\text{Ent}\), as lower concentrations of 1.0 \(\mu\text{M}\) of the fluorophore conjugates led to strong fluorescence signals of similar intensity (Fig. 4C and F, see also ESI, Table S3†) compared to the 10-fold higher concentration in absence of \(\text{Ent}\) (Fig. 4B and E, see also ESI, Table S3†). We assume that the overall fitness of the bacteria is increased in the presence of additional siderophore, leading to a more efficient uptake of the fluorophore conjugates. These results underline the potential of \(\text{EntKL}\)-based reporter conjugates to serve as molecular tools for infection detection.

Finally, \((\text{AcO})\text{EntKL}, (\text{MeO})\text{EntKL}\) and all derived cargo-conjugates were investigated for their antibacterial activity against the \(E.\ coli\) and \(P.\ aeruginosa\) strains and the compounds’ cytotoxic activity against human HepG2 cells was assessed (see ESI, Table S4†). All tested compounds lack antibacterial and cytotoxic activity, which is important looking at \((\text{AcO})\text{EntKL}\) in the context of being potentially used as safe carrier molecule for the future development of antimicrobial siderophore drug conjugates that help to prevent fast resistance development. In addition, all compounds lack any cytotoxic activity against human HepG2 cells, enabling their possible application in mammalian cells. Notably, although the close derivative of Miller’s artificial enterobactin analogue\(^{47}\) \((\text{AcO})\text{EntKL}\) displayed similar growth recovery efficacy in siderophore biosynthesis deficient mutants of \(E.\ coli\) and \(P.\ aeruginosa\) compared to \((\text{AcO})\text{EntKL}\) (see ESI, Fig. S10†), we found significant cytotoxic activity against human HepG2 cells with an \(\text{IC}_{50}\) value of 12.17 \(\mu\text{M}\) in our assay (see ESI, Table S4†).

**Conclusions**

In summary, we designed and synthesized the novel enterobactin derivative \((\text{AcO})\text{EntKL}\), which retains the natural hydrolysability of the tris-lactone scaffold, while providing an amino handle for easy attachment of cargos in the backbone. *In silico* studies at the DFT level of theory predicted a high thermodynamic stability combined with kinetic lability for the iron(\(\text{III}\)) complexes similarly as demonstrated earlier for the natural ferri-siderophore [Fe-Ent]\(^{3+}\).

Growth recovery experiments with siderophore biosynthesis deficient mutants of \(E.\ coli\) and \(P.\ aeruginosa\) under iron-limiting conditions revealed an uptake of \((\text{AcO})\text{EntKL}\) and other \(\text{EntKL}\)-based derivatives into the tested bacteria, while, in contrast, permethylated probes based on \((\text{MeO})\text{EntKL}\) unable to bind iron, did not lead to any growth recovery.

Furthermore, imaging experiments under iron-limiting conditions utilizing \(E.\ coli\) and \(P.\ aeruginosa\) deficient mutants and wild-type strains showed labelling of the bacteria, further underlining the uptake of cargo conjugates.
As fluorophore uptake into both, *E. coli* K-12 Δent*A* and *P. aeruginosa* K648 Δpvd/pch, increased in the presence of supplemented Ent, we assume that overexpression of Ent would not contribute to any resistance against Ent*KL*-based drug conjugates.

Since growth recovery was also observed in the presence of high concentrations of human apo-transferrin or albumin and no antimicrobial activity or cytotoxicity was observed, (AcO) Ent*KL* holds potential to serve as good starting point for the assembly of antimicrobial siderophore drug conjugates to tackle infections caused by Gram-negative bacterial pathogens in humans.

Further investigations towards the determination of the size exclusion limit of siderophore receptors and the synthesis of first drug conjugates are ongoing.

Data availability
All data are available in the ESI.

Author contributions
The research was conceived by P. K. The manuscript was written by P. K. with contributions of R. Z., J. C., J. G. and R. M. The ESI† was written by R. Z., P. K., J. C., J. H., J. G. and R. M. All compounds were synthesized by R. Z. Computations were planned by J. G. and conducted by J. V. Biological evaluation of the compounds was planned by J. H., R. M., J. C. and P. K. and conducted by J. C. All authors have given approval to the final version of the manuscript.

Conflicts of interest
There are no conflicts to declare.

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Notes and references


