

Artificial Siderophore Modification and Metal Coordination Providing Selectivity for Imaging of Bacterial Infections

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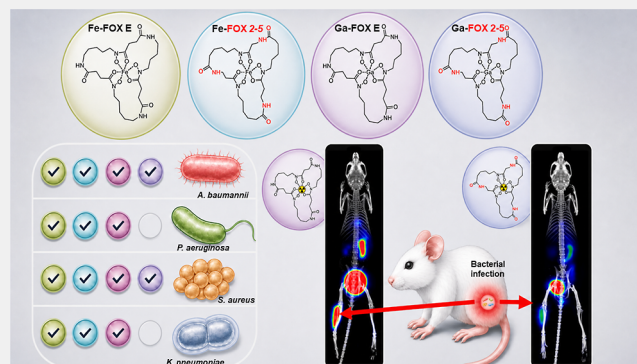
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ABSTRACT: Purpose: diagnosing bacterial infections can be challenging, as the source of infection is often difficult to identify. Positron emission tomography (PET) imaging with bacteria-specific agents offers a promising approach to localizing and potentially characterizing infection sites. In this study, we compared the selectivity of two iron chelators, the natural siderophore FOX E and its synthetic variant FOX 2–5, for targeting different pathogens when radiolabeled for molecular imaging. Methods: Both siderophores were evaluated across various bacteria, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, and *Escherichia coli*. The siderophores were labeled with radioactive gallium-68 and iron-59 for short-term uptake assays. In addition, ^{68}Ga -labeled siderophores were applied in a murine infection model of the respective pathogens for PET imaging. Results: Our *in vitro* results demonstrated that both siderophores were taken up by bacteria when labeled with iron-59. However, when labeled with gallium-68, FOX 2–5 exhibited strain-specific differences: while gallium-68 uptake was similar for both chelators in *S. aureus* and *A. baumannii*, FOX 2–5 failed to accumulate in *P. aeruginosa* and *K. pneumoniae* unlike FOX E. These findings were confirmed *in vivo* with corresponding differences in the PET images of mouse infection models. Conclusion: minor modifications in the structure of siderophores can alter bacterial specificity, particularly when coordinated with gallium. Additionally, this study revealed a distinction in recognition between gallium and iron complexes in the Gram-negative *P. aeruginosa* and *K. pneumoniae*. These findings highlight artificial siderophores as versatile, customizable agents for characterizing and potentially diagnosing bacterial infections.

KEYWORDS: siderophores, infection imaging, PET, bacterial infection, gallium-68, iron-59, molecular imaging



INTRODUCTION

Iron is an essential nutrient for almost all living cells and plays a crucial role in many physiological processes.¹ Despite its high abundance in the environment, iron is often biologically inaccessible due to its low solubility under physiological conditions.^{2,3} Microorganisms have evolved various strategies to adapt to habitats with limited iron availability. One such strategy is the high-affinity iron uptake system known as “siderophore iron acquisition”.⁴ Siderophores are low-molecular-weight molecules with a high affinity for iron and other metals, which are secreted to scavenge environmental iron.^{5–7}

Siderophores function as critical virulence factors during infection, facilitating the acquisition of iron from the host organism.⁸ Since the host tightly sequesters iron within high-affinity binding proteins such as hemoglobin and transferrin, pathogenic microorganisms have evolved specialized siderophore biosynthetic pathways and dedicated transport systems.⁹ These uptake systems recognize and internalize not only endogenous siderophores but can also effectively utilize

siderophores produced by other microorganisms, a phenomenon known as *axenosiderophore utilization*.¹⁰

The ionic radius of gallium closely matches that of iron, enabling isosteric substitution in many iron-binding ligands, including siderophores.¹¹ Siderophores form highly stable complexes with gallium that are in general recognized and internalized by iron uptake systems.¹² This property has been used in diagnostic imaging, especially positron emission tomography (PET), by using the positron-emitting isotope Ga-68.¹³

Localizing infection sites and identifying the causative pathogens simultaneously would be highly beneficial for

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routine clinical practice. When combined with PET imaging, pathogen-specific tracers offer a rapid, noninvasive alternative to traditional diagnostic methods, such as tissue sampling and microbiological cultures. Since these procedures can be time-consuming, optimizing them and investigating faster alternatives are vital for providing effective patient care in clinical settings.

From a tracer chemistry standpoint, developing pathogen-specific imaging agents requires an understanding of the structural features that influence bacterial recognition and uptake. Therefore, investigating structural determinants is essential for the rational optimization of the tracer performance and specificity.

Of the various approaches explored for pathogen-specific imaging, those using siderophore-based tracers have emerged as particularly promising candidates due to their selective uptake by microorganisms. Consequently, considerable effort has been devoted to evaluating different siderophores as imaging agents for pathogens.^{14,15} ⁶⁸Ga-labeled pyoverdine has been shown to enable PET imaging of *P. aeruginosa* infections.¹⁶ Similarly, Ornibactin has also been successfully labeled with Ga-68 for PET imaging of infection sites like pneumonia and myositis.¹⁷ ⁶⁸Ga-labeled triacetylfusarinine C exhibited strong infection-targeting properties in rats for fungal infections.¹³ The clinically approved Deferoxamine B (DFO B, Desferrioxamine B, Desferal) has proven useful in detecting both bacterial¹⁸ and fungal infections¹⁹ and has recently shown promising results in a clinical trial.²⁰

The chemical mimicry is the foundation for developing gallium-labeled siderophores as infection-specific imaging agents. Several siderophores have demonstrated efficacy in this matter in both *in vitro* and *in vivo* studies: One notable example is the hydroxamate-type siderophore deferoxamine B, which is produced by the bacterium *Streptomyces pilosus* and is utilized by many microorganisms as a xenosiderophore.^{21,22} Furthermore, it is registered as the drug Desferal to treat iron-overload disorders. DFO B has been evaluated for its imaging capabilities and has shown promise in detecting infections caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus*.¹⁸

Ferrioxamine E (FOX E, Nocardamine) is a ferrioxamine-type siderophore like DFO B. However, unlike DFO B, which is a linear trihydroxamate, FOX E is a cyclic trihydroxamate produced by various bacteria.^{23,24} FOX E has demonstrated strong potential for the uptake of specific siderophore when labeled with Ga-68. It has been used in multiple PET studies due to its ability to be utilized by certain fungal and bacterial species as a xenosiderophore.^{25–27}

Because of its broad applicability, FOX E was selected as a lead structure for the synthesis of analogues designed to potentially achieve better species specificity. Mular et al. described a series of cyclic FOX E analogues, labeled with Ga-68 and demonstrated that the pathogenic fungus *Aspergillus fumigatus* and the bacterial Gram-positive pathogen *Staphylococcus aureus* are able to utilize FOX E and its synthetic analogues.^{28,29} These studies also revealed that FOX 2–5, a candidate with the same molecular weight as FOX E that differs only in the position of the amides, was most promising, see Figure 1.²⁸

However, further internal studies of FOX 2–5 yielded inconsistent results: while Fe-FOX 2–5 efficiently promoted bacterial growth, uptake of [⁶⁸Ga]Ga-FOX 2–5 by *P. aeruginosa* was negligible to variable compared with [⁶⁸Ga]Ga-FOX E. These findings suggested that siderophore

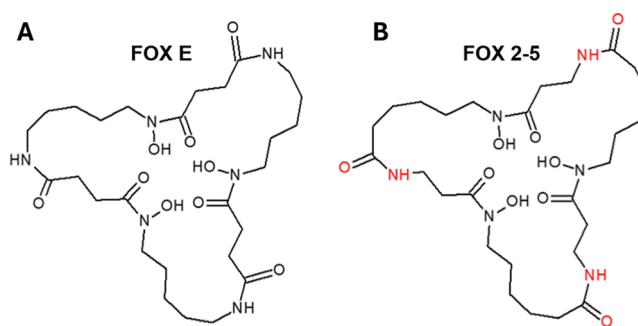


Figure 1. Structure of FOX E (A) and FOX 2–5 (B). The two siderophores differ in the arrangement of their amide groups, which are positioned in a retro orientation, highlighted in red, for FOX 2–5, compared to FOX E.

complexes loaded with iron or gallium may be processed differently by the bacteria. Therefore, this study investigated the uptake selectivity of [⁶⁸Ga]Ga-FOX 2–5 and [⁶⁸Ga]Ga-FOX E across several clinically relevant bacterial species, including *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. In addition, differences in bacterial uptake between ⁶⁸Ga- and ⁵⁹Fe-labeled FOX 2–5 and FOX E were evaluated through complementary *in vitro* and *in vivo* experiments, including murine myositis models, to further assess their potential for pathogen-specific infection imaging (see Figure 1).

RESULTS AND DISCUSSION

In Vitro Comparison of Iron and Gallium Siderophore Complexes

To evaluate the utilization of the siderophores FOX E and FOX 2–5 by the tested species, we conducted a growth-promotion assay to determine if the siderophores support bacterial growth, thereby confirming their uptake and use by the respective strains. The assay included *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and a *P. aeruginosa* mutant deficient in endogenous siderophore biosynthesis (Δ pvdF Δ pchA) and another additionally lacking uptake via the ferrioxamine transporter FoxA (Δ pvdF Δ pchA Δ foxA).³⁰ These mutants were selected to minimize potential interference from pyoverdine- or pyochelin-mediated cross-chelation of the radiometal under iron-limiting conditions and to facilitate the interpretation of siderophore-dependent uptake mechanisms. The results of the growth promotion assay are shown in Figure 2.

The results indicate that there was no difference in growth promotion observed between Fe-FOX E and Fe-FOX 2–5. Growth of *P. aeruginosa* and *A. baumannii* increased with higher Fe-siderophore concentrations, while no increase was observed with Ga-complexed siderophores. *K. pneumoniae* showed only a moderate increase in growth at higher Fe-siderophore concentrations, while *E. coli* exhibited no increase. Previous studies have shown that *E. coli* cannot efficiently utilize ferrioxamine-type siderophores.^{31,32} *S. aureus* was not included since Mular et al.²⁹ had already shown comparable growth promotion of Fe-FOX 2–5 and Fe-FOX E. As expected, no growth-promoting effect was observed for Ga-siderophores in any of the tested strains. In *P. aeruginosa*, both Fe-FOX E and Fe-FOX 2–5 promoted similar growth in the wild-type (WT) strain and in the strain deficient in siderophore production (the Δ pvdF Δ pchA mutant). However,

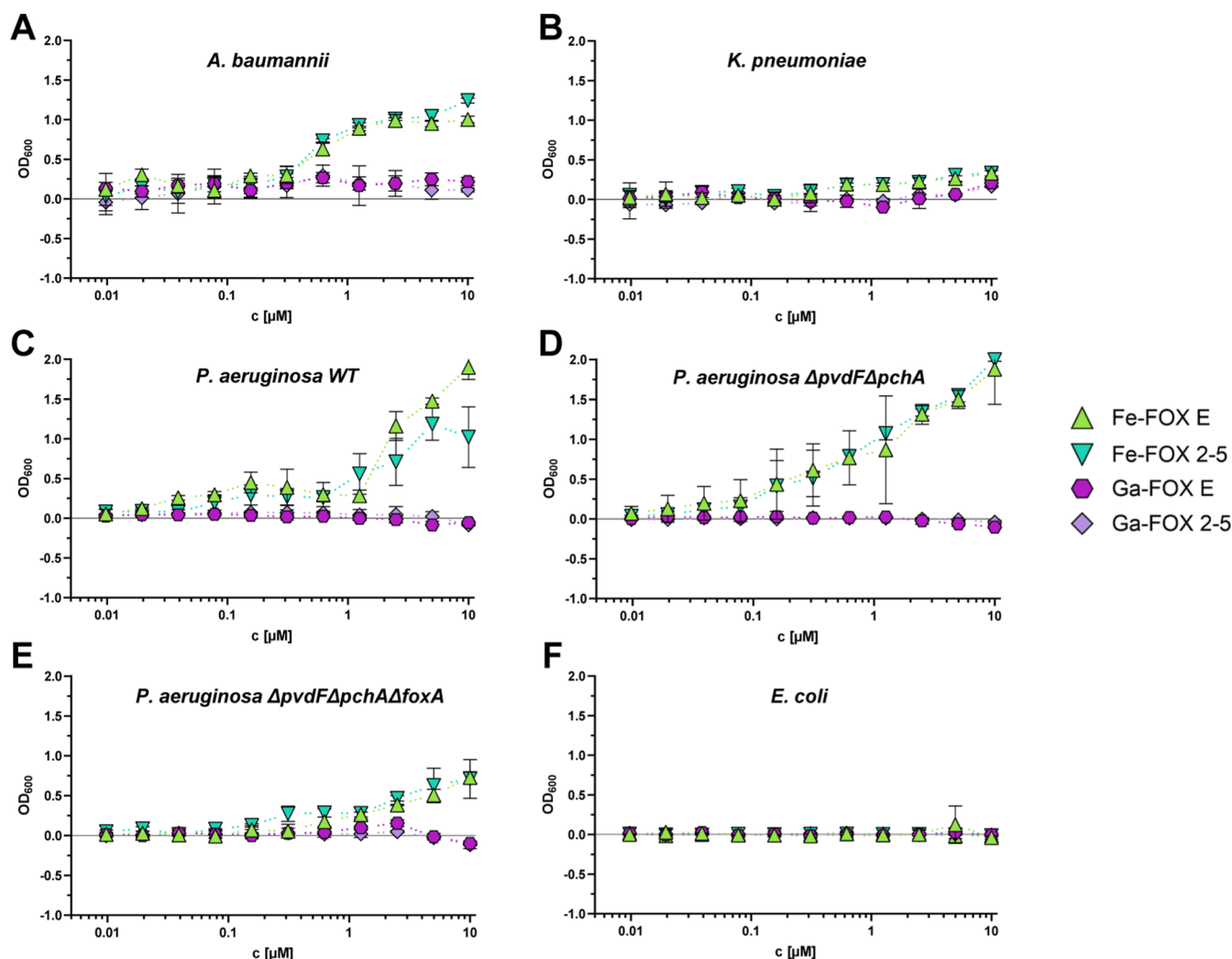


Figure 2. Both FOX E and FOX 2–5 promoted growth in the tested species. Growth promotion analysis of (A) *A. baumannii*, (B) *K. pneumoniae*, and three different *P. aeruginosa* strains (C) WT, (D) $\Delta pvdF\Delta pchA$, and (E) $\Delta pvdF\Delta pchA\Delta foxA$, as well as (F) *E. coli* with siderophores FOX E and FOX 2–5. Complexes were coordinated with either nonradioactive iron or gallium. FOX E is shown in upward triangle, green (iron) and hexagon, purple (gallium), while FOX 2–5 is shown in downward triangle, turquoise (iron) and diamond, lilac (gallium). The y-axis represents optical density (bacterial growth), and the x-axis shows the concentration of the respective metal - siderophore complex. No difference in growth promotion between Fe-FOX E and Fe-FOX 2–5 for *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, and *E. coli* were observed. Ga-complexed siderophores did not promote any growth.

this growth promotion was reduced upon additional inactivation of FoxA (the $\Delta pvdF\Delta pchA\Delta foxA$ mutant), the tonB-dependent transporter (TBDT) responsible for ferrioxamine-Fe uptake.³⁰ Growth promotion by Fe-FOX E and Fe-FOX 2–5 in *P. aeruginosa* was not affected by inactivation of siderophore biosynthesis ($\Delta pvdF\Delta pchA$ mutant) compared to the wild-type (WT), but it was significantly decreased by the additional inactivation of FoxA ($\Delta pvdF\Delta pchA\Delta foxA$ mutant), a ferrioxamine-transporters.³⁰

Since FOX E and FOX 2–5 were able to promote bacterial growth when complexed with iron, indicating successful iron acquisition and utilization, we next investigated whether differences existed in the uptake of iron- and gallium-loaded complexes. To address this question, a competitive uptake assay was performed by using the radioactive isotopes Fe-59 and Ga-68. The simultaneous application of both radiometals enabled a comparison of iron and gallium uptake and provided further insight into the biological relevance of the differences observed between FOX E and FOX 2–5.

Radiolabeling of both FOX E and FOX 2–5 was straightforward both with Ga-68 and Fe-59. Thin-layer chromatography (TLC) analysis demonstrated a labeling efficiency of over 99% for both [⁶⁸Ga]Ga-FOX 2–5 and [⁶⁸Ga]Ga-FOX E using 0.1 M citrate buffer as the mobile phase (Figure S1). Reversed-phase radio-HPLC analysis revealed a single radioactive peak, at approximately 10 min, indicating successful labeling, high radiochemical purity, and efficient yield of the ⁶⁸Ga-labeled siderophores [⁶⁸Ga]Ga-FOX E and [⁶⁸Ga]Ga-FOX 2–5 (Figure S2). For [⁵⁹Fe]Fe-FOX E and [⁵⁹Fe]Fe-FOX 2–5, labeling efficiencies analyzed with TLC were over 95%, respectively.

Results of short-term uptake assays are shown in Figure 3 comparing uptake of FOX E vs FOX 2–5 complexing Ga-68 vs Fe-59 under iron limitation (–Fe) and sufficiency (+Fe) in various bacterial strains. Overall, the uptake of the two tested siderophores, whether loaded with Ga-68 or Fe-59, was significantly higher under iron-limitation conditions than under iron-sufficient conditions in *P. aeruginosa*, *K. pneumo-*

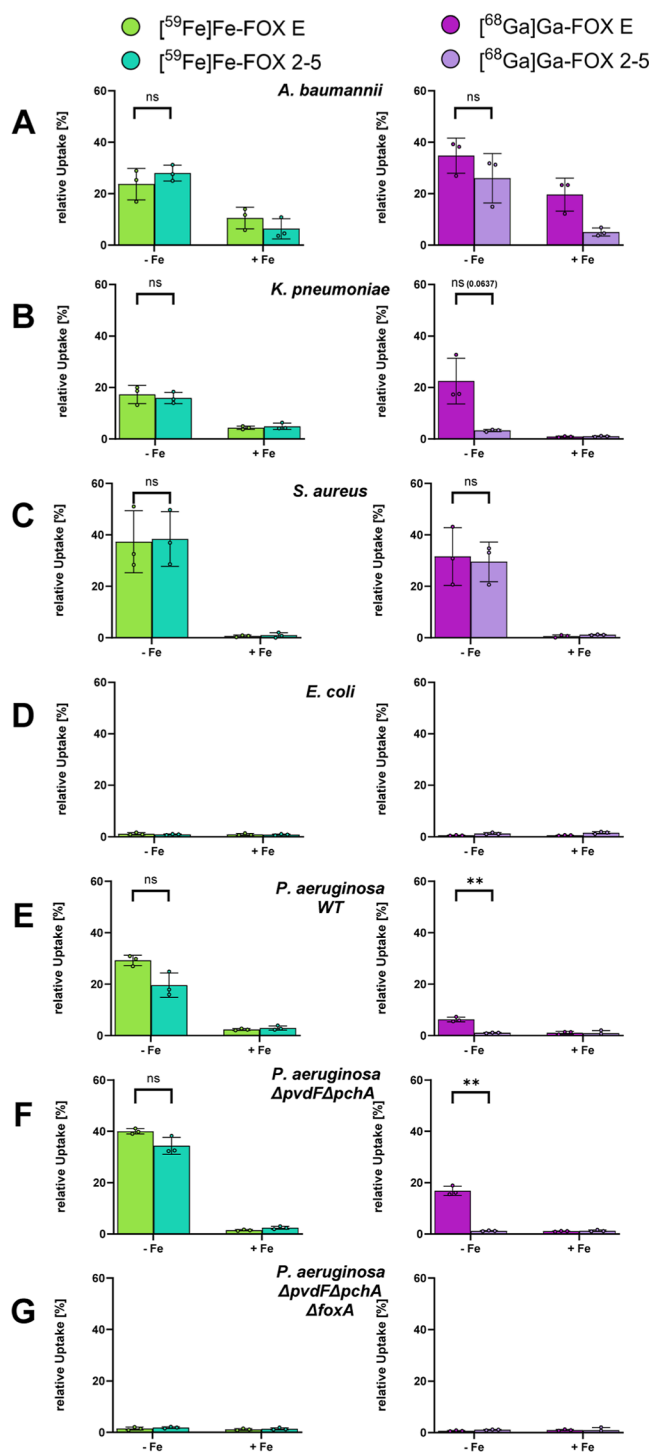


Figure 3. Short-term uptake analysis of FOX E and FOX 2–5 labeled with either Fe-59, bar charts on the right, ^{59}Fe -labeled FOX E, green, and ^{59}Fe -labeled FOX 2–5, turquoise, or Ga-68, bar charts on the left, ^{68}Ga -labeled FOX E, purple, and FOX 2–5, lilac. Uptake was assessed in iron-limitation (–Fe) and iron-saturated (+Fe) media conditions. (A) *A. baumannii*, (B) *K. pneumoniae*, (C) *S. aureus*, (D) *E. coli*, (E) *P. aeruginosa* WT, (F) *P. aeruginosa* $\Delta pvdF\Delta pchA$, and (G) *P. aeruginosa* $\Delta pvdF\Delta pchA\Delta foxA$.

niae, *A. baumannii*, and *S. aureus*. These data are consistent with specific uptake by siderophore transporters, as their expression is generally induced under iron limitation conditions^{33,34,35}. This specific uptake is further supported by

the lack of uptake in *E. coli*, for which it is stated that it cannot utilize ferrioxamines efficiently^{31,32}. This also corresponds to the growth promotion assays shown in Figure 2.

No significant differences in uptake of Fe-59 were observed when complexed with FOX E and FOX 2–5 (^{59}Fe)-FOX E and ^{59}Fe)-FOX 2–5 across all four ferrioxamine-utilizing species (Figure 3). Analyzing the ^{68}Ga -uptake, no statistically significant differences were observed for *E. coli*, *S. aureus*, and *A. baumannii*. However, we could, for the first time, observe a striking difference in the handling of the gallium versus the iron siderophore complex in *P. aeruginosa* and *K. pneumoniae*, but only for the artificial siderophore FOX 2–5. Uptake of ^{68}Ga -siderophore was significantly lower when chelated by FOX 2–5 compared to FOX E in *P. aeruginosa*, and a similar trend was observed in *K. pneumoniae*, although it did not reach statistical significance ($p = 0.0637$). In a *P. aeruginosa* mutant strain lacking siderophore biosynthesis ($\Delta pvdF\Delta pchA$ mutant), the uptake of Ga-68 was significantly reduced when chelated by FOX 2–5 compared to FOX E, mirroring the pattern observed in the wild-type (WT) strain. However, additional inactivation of *FoxA* ($\Delta pvdF\Delta pchA\Delta foxA$ mutant) abolished the uptake of Fe-59 and Ga-68 regardless of whether they were chelated by FOX E or FOX 2–5.

These data indicate that *FoxA* can internalize both iron and gallium when chelated by FOX E, whereas uptake via FOX 2–5 is significantly more efficient for iron than for gallium.

Although FOX E and FOX 2–5 differ only in the reversed orientation of their amide groups, this structural modification was apparently sufficient to lead to a lack of bacterial recognition of the gallium complex in *P. aeruginosa* and *K. pneumoniae*. Interestingly, this is not the case for *A. baumannii*, also a Gram-negative bacterium, with no detectable difference between the two metals.

Gram-negative species use TonB-dependent outer-membrane receptors/transporters for siderophore import, such as *FoxA* for uptake of ferrioxamine-type siderophores in *P. aeruginosa*, whereas Gram-positive bacteria rely on highly specific ABC-type transport systems.^{36–38}

On the basis of these differences, we initially anticipated distinct uptake profiles between Gram-positive and Gram-negative bacteria. Contrary to our expectations, Gram-negative *K. pneumoniae* and *P. aeruginosa* exhibited similar uptake behavior, and the Gram-positive *S. aureus* and Gram-negative *A. baumannii* demonstrated comparable trends.

Although *P. aeruginosa* and *A. baumannii* are often compared and their transporters are commonly regarded as similar, detailed analysis reveals that even minor structural differences can result in significant changes in how they interact with molecules, even when the molecules themselves differ only slightly.³⁹ Therefore, while differences between Gram-positive and Gram-negative bacteria are the obvious conclusion, one cannot simply assume that all members within these bacterial groups will behave similarly. What is needed for a complete understanding of each transporter is a detailed characterization of the mechanistic and structural aspects of the siderophore uptake systems in different bacterial species, which is known for *FoxA* of *P. aeruginosa* but still lacking for other strains.^{40,41}

There are some limitations of this *in vitro* study: first, Fe-59 and Ga-68 come from different production sources. Whereas Ga-68 is generator-produced, resulting in a carrier-free radionuclide with very high specific activity (1.5 PBq/mg Ga), Fe-59 is carrier-added (specific activity of 128 MBq/mg Fe). This results in higher molar concentrations used in *in vitro*

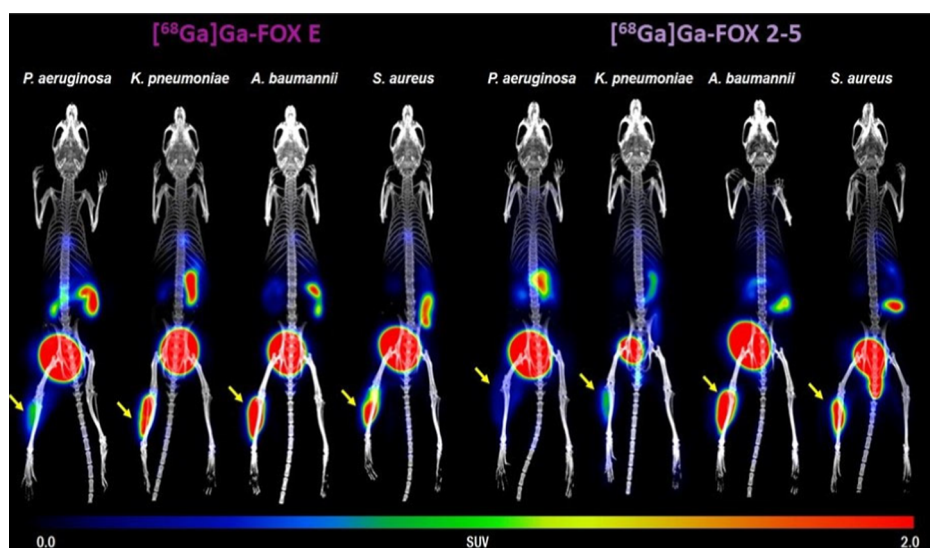


Figure 4. Static PET/CT images of $[^{68}\text{Ga}]\text{Ga-FOX E}$ and $[^{68}\text{Ga}]\text{Ga-FOX 2-5}$ in infected BALB/c mice, acquired 5 h postinfection. Mice were infected with *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, or *A. baumannii* in the right hind leg (indicated by a yellow arrow) and received *E. coli* (5×10^7 CFUs) in the left hind leg.

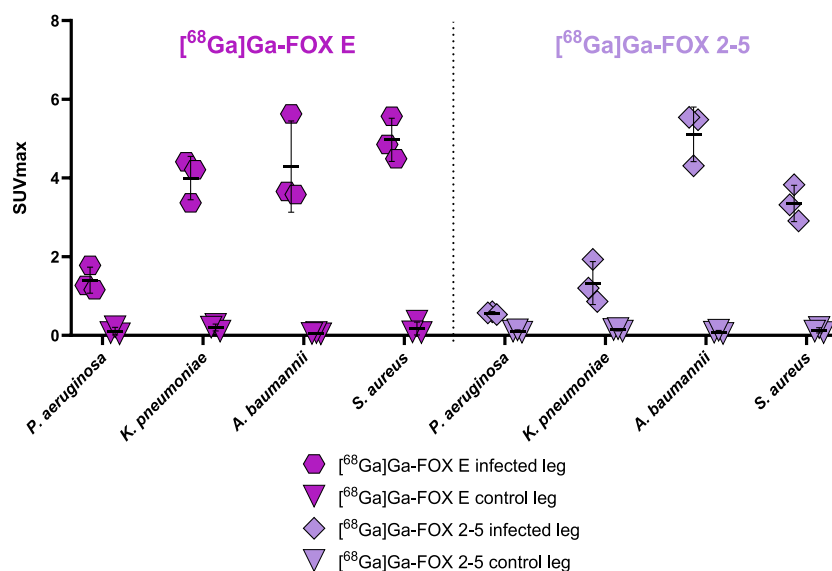


Figure 5. SUV_{max} values from Static PET/CT images of $[^{68}\text{Ga}]\text{Ga-FOX E}$ (left side) and $[^{68}\text{Ga}]\text{Ga-FOX 2-5}$ (right side) in infected BALB/c mice, acquired 5 h postinfection. Mice ($n = 3$) were infected with *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, or *A. baumannii* in the right hind leg and received *E. coli* (all 5×10^7 CFUs) in the left hind leg as a control (represented as a downward triangle). Data is represented as individual values, including SD and mean. Detailed information is provided in Table S1.

assays for the ^{59}Fe -labeled siderophores and makes a direct comparison difficult. In a control experiment (see Figure S3), we addressed this issue by aiming to simulate equal concentrations by the addition of nonradioactive gallium. This was expressed in pmol/mL/OD_{600} instead of relative uptake and showed that the uptake trends remained the same when normalized to an equimolar result. This method of normalization is also used by Will et al. 2024 and is comparable to our data, although they used the radioactive iron isotope Fe-55 , which introduces additional methodological differences.³⁰

Another limitation is that siderophore transporter expression is induced by iron starvation, whereas it is downregulated under iron-sufficient conditions. Not all culture conditions could be optimized in a way that these conditions were reached; in particular for *A. baumannii* and *K. pneumoniae*,

uptake under iron supplementation was observed, indicating an insufficient iron supply to suppress siderophore transporter expression. However, this only had an effect on the control conditions without impacting the final conclusions. Additionally, comparing the relative uptake per CFU across different microorganisms is also difficult, as each strain exhibits distinct behavior in growth and expression levels of siderophore transporters due to iron starvation. Therefore, relative uptake showed a certain inter- and intra-assay variability, and interpretation was done with caution.

However, more important was the confirmation of these *in vitro* data by the specific uptake in the animal infection model, supporting the potential of translational approaches.

In Vivo Assessment of Gallium- and Iron-Labeled Siderophores

PET/CT images of a murine model of acute myositis are summarized in Figures 4 and 5. All studies demonstrated rapid renal excretion, with minimal intestinal activity that did not compromise image quality. [^{68}Ga]Ga-FOX E was efficiently taken up in infection sites of *S. aureus*, *K. pneumoniae*, and *A. baumannii*. While *P. aeruginosa* also showed uptake, it was markedly lower compared to the other strains. The artificial analogue [^{68}Ga]Ga-FOX 2–5 exhibited comparably high uptake in sites of *S. aureus* and *A. baumannii* infection but reduced uptake in *K. pneumoniae* infection and practically no accumulation in *P. aeruginosa* infection sites. All contralateral-control sites with *E. coli* infection did not reveal any accumulation of ^{68}Ga -labeled siderophores, indicating the specificity of the imaging signal. SUV_{max} (standardized uptake value maximum) values are displayed in Figure 5. SUV_{max} values in infected legs varied from 0.5–6.0; all values in infected legs were higher than in noninfected, indicating specific accumulation of the tracer at sites of infection.

Our *in vitro* studies of ^{68}Ga -labeled FOX E and FOX 2–5 were confirmed by PET/CT imaging in an infection model (Figures 4 and 5): [^{68}Ga]Ga-FOX E demonstrated efficient uptake at infection sites of *S. aureus*, *K. pneumoniae*, and *A. baumannii*, whereas uptake in *P. aeruginosa* was present, but lower than in the other strains. On the other hand, [^{68}Ga]Ga-FOX 2–5 showed strong accumulation at infection sites only of *S. aureus* and *A. baumannii*, moderate uptake in *K. pneumoniae*, but no accumulation at *P. aeruginosa* infection sites.

Overall, in this study, we demonstrated that the metal coordinated to siderophores (iron or gallium) influences uptake. This effect was particularly evident with the biomimetic, artificial siderophore FOX 2–5, where the gallium complex was no longer recognized by certain pathogens. This may be related to the inability of gallium to undergo redox cycling under physiological conditions, whereas iron can be reduced during uptake and intracellular release processes. In addition, differences in affinity of the metal-siderophore complexes for their transport systems may contribute to the observed effects; however, as indicated in our previous paper,²⁹ Ga(III) complexes of FOX E and FOX 2–5 ($\log\beta_{\text{GaFOX E}} = 29.79$ and $\log\beta_{\text{GaFOX 2–5}} = 29.50$) are slightly less stable than corresponding Fe(III) complexes ($\log\beta_{\text{FeFOX E}} = 32.21$, $\log\beta_{\text{FeFOX 2–5}} = 31.32$), and this behavior is typical for siderophores.¹² Of importance, it is generally accepted that the Fe(III) and Ga(III) complexes of siderophores are isostructural, and both are present in solution as a mixture of rapidly interconverting stereoisomers;^{42–46} both siderophores are achiral, and thus Δ and Λ stereoisomers are true mirror images of each other and are chemically indistinguishable.

It is also possible that gallium-loaded siderophores interfere with uptake mechanisms in certain organisms by acting as transport inhibitors, although this proposed mechanism remains hypothetical and requires further investigation. Taken together, these findings support the rationale that structural modifications of siderophores have the potential to not only improve production and pharmacokinetic properties but also enable species-specific delivery of radioactivity for molecular infection imaging, which is particularly important in the context of rising antibiotic resistance and the growing need for rapid, accurate infection diagnostics. PET imaging with siderophore-based probes may serve as an effective tool, as it

could provide earlier interventions and more precise treatment strategies. Understanding which siderophore structures are selectively recognized and internalized by pathogens is therefore key to developing effective targeted strategies for diagnosing and treating bacterial infections.

CONCLUSION

Our research indicates that tailoring siderophore structures to enhance their selectivity for specific bacterial strains has the potential for developing bacteria-selective infection imaging molecules. First, it was established that there is a difference depending on whether gallium or iron is bound to the siderophore. Second, it was determined that a small change, such as a slight rearrangement in amide orientation while retaining the same molecular weight, can prevent some bacteria from recognizing Ga complexes of FOX E and FOX 2–5.

This approach may offer a useful foundation for the development of more selective imaging probes and, potentially, targeted antimicrobial strategies, which should ultimately be beneficial for detecting infections in the body fast, strain-specific, and noninvasively.

MATERIALS AND METHODS

All reagents and solvents were acquired from commercial suppliers (Sigma-Aldrich, Merck, and Fluka Analytical) unless otherwise stated and employed without any further purification. FOX E was obtained from Pol-Aura (Zabrze, Poland), and FOX 2–5 from TriMen Chemicals (Łódź, Poland).

Ga-68 Radiolabeling

For *in vitro* assays, [^{68}Ga]GaCl₃ was eluted from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator (Eckert & Ziegler Eurotope GmbH, Berlin, Germany) by fractionated elution using 0.1 N HCl. FOX E and FOX 2–5 were complexed with [^{68}Ga]GaCl₃ by using 1.75 μg of siderophore (1 mg/mL solution) with 25 μL of [^{68}Ga]Ga-Eluate (~ 5 MBq) and 5 μL of 1.1 M sodium acetate, resulting in a pH of 4.5.

For *in vivo* assays, [^{68}Ga]GaCl₃ was eluted from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator (Eckert & Ziegler Eurotope GmbH, Berlin, Germany) by fractionated elution using 0.1 N HCl. FOX E and FOX 2–5 were complexed with [^{68}Ga]GaCl₃ by using 10 μg of siderophore (1 mg/mL solution) with 300 μL of [^{68}Ga]Ga-Eluate (~ 60 MBq) and 30 μL of 1.1 M sodium acetate resulting in a pH of 4.5.

Successful labeling was checked via reversed-phase high-performance liquid chromatography (RP-HPLC) and thin-layer chromatography (TLC).

Fe-59 Radiolabeling

FOX E was labeled with Fe-59 by mixing 100 μg FOX with 147.4 kBq [^{59}Fe]FeCl₃ (specific activity was 128.54 MBq/mg Fe on 18.10.2024) in 0.1 M HCl solution (POLATOM, Otwock, Poland) and 14 μL 1.1 M sodium acetate, resulting in a pH of 4.5. For FOX 2–5, 40 μg were used and labeled with 58.9 kBq [^{59}Fe]FeCl₃ in 0.1 M HCl and 6 μL of 1.1 M sodium acetate, resulting in a pH 4.5. Successful labeling was verified by TLC.

Thin-Layer Chromatography

For TLC analysis, 2.5 μL of the labeling solution was applied to an ITLC-SG strip (Varian, Lake Forest, CA, USA), which was then placed in a 0.1 M sodium citrate buffer (pH 5) for chromatographic development. Afterward, the strip was exposed under a detection foil (1 min for Ga-68, 5 min for Fe-59) and analyzed using a Phosphor Imager (Cyclone Plus, PerkinElmer, Waltham, MA, USA). TLC results were evaluated with OptiQuant software (Version 5.0; PerkinElmer, Waltham, MA, USA) by comparing the region of interest at the origin with the signal at the solvent front of the strip; background signal was subtracted accordingly. Results were expressed

Table 1. Strains Used in This Study

strain	collection ID	genotype	reference
<i>Pseudomonas aeruginosa</i> PAO1	PAO1	wild-type strain	PMID: 10984043
$\Delta pvdF\Delta pchA$. <i>aeruginosa</i>	PAS283	PAO1; <i>pvdF</i> and <i>pchA</i> chromosomally deleted	PMID: 26718479
$\Delta pvdF\Delta pchA\Delta foxA$ <i>P. aeruginosa</i>	PAS535	PAO1; <i>pvdF</i> , <i>pchA</i> and <i>foxA</i> chromosomally deleted	PMID: 32902248
<i>Escherichia coli</i>	NCTC 13351	wild-type strain	National Collection of Type Cultures, UK Health Security Agency, UK
<i>Klebsiella pneumoniae</i>	NCTC 13465	wild-type strain	National Collection of Type Cultures, UK Health Security Agency, UK
<i>Acinetobacter baumannii</i>	NCTC 13301	wild-type strain	National Collection of Type Cultures, UK Health Security Agency, UK
<i>Staphylococcus aureus</i>	CCM 5971	wild-type strain	Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic

as the relative percentage of the radiolabeled siderophore over the total applied activity.

Reversed Phase-HPLC

RP-HPLC was performed using an UltiMate 3000 system (Thermo Fisher Scientific, Vienna, Austria), equipped with an RS UHPLC pump, autosampler, column oven maintained at 25 °C, and a variable wavelength detector set to 220 nm for UV detection. Radiodetection was conducted using a Gabi Star radiodetector (Raytest, Straubenhardt, Germany) connected in series with the UV detector. Chromatographic separation was achieved on a Jupiter Proteo column (250 × 4.6 mm, 4 μm particle size, 90 Å pore size; Phenomenex Inc., Torrance, CA, USA) using a mobile phase consisting of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B), with a gradient from 10% to 60% B over 18 min at a flow rate of 1 mL/min. For the analysis, the radiolabeling solution was diluted 1:10 with d_4H_2O , and 5 μL was injected.

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in Table 1. Bacteria were initially cultivated in liquid LB medium at 37 °C and 150 rpm for 18 h. The overnight cultures were then used either for the growth promotion assay (see below) or to inoculate the liquid shaking cultures. For the latter purpose, 100 mL of liquid minimal medium (MM)⁴⁷ containing 1% (w/v) glucose and 20 mM glutamine as the respective carbon and nitrogen sources were prepared in 500 mL Erlenmeyer flasks. Casein enzymatic hydrolysate (MilliporeSigma, Burlington, MA, USA) was added to the medium for *E. coli*, *K. pneumoniae*, *A. baumannii*, and *S. aureus* at a final concentration of 2 g/L. For iron-deplete (-Fe) conditions, iron was omitted, and for iron-replete (+Fe) conditions, $FeSO_4$ was added to a final concentration of 0.03 mM. Each flask was inoculated with 4.5 mL of the corresponding overnight culture and incubated at 37 °C with shaking at 150 rpm for 18 h. After incubation, the cultures were washed, resuspended in fresh corresponding medium, and adjusted to an optical density at 600 nm (OD_{600}) of 1.5.

CFUs were determined by the viable colony counting. The correlation between CFUs and optical density at 600 nm (OD_{600}) is shown in Table S2.

Growth Promotion Assay

To determine the microbial utilization of FOX E and FOX 2–5, a growth promotion assay was performed. Stable (nonradioactive) gallium or iron was therefore complexed with FOX E or FOX 2–5, respectively, using equimolar amounts of $GaCl_3$ or $FeCl_3$ in a 1 M sodium acetate buffer solution (pH 4.5) at room temperature for 10 min, after which the sample was characterized by RP-HPLC. Overnight cultures were washed, resuspended in fresh MM with 1 μM $FeSO_4$, and supplemented with casein enzymatic hydrolysate where appropriate (see above). The OD_{600} was measured, and the cultures were adjusted. A total of 80 μL of the bacterial suspensions were transferred into each well of a 96-well microtiter plate (Nunc MicroWell 96-well; Thermo Fisher Scientific Inc., Waltham, MA,

USA) and incubated at 37 °C for 2 h. 20 mL of serially diluted Ga/Fe-FOX E and Ga/Fe-FOX 2–5 (starting at 10 μM) were added to the wells, resulting in a final cell amount of 1×10^4 cells per well. $Ga(NO_3)_3$ was used as a growth inhibition control. The plates were incubated at 37 °C. To evaluate growth, the OD_{600} was measured at the beginning and after 18 h of incubation using a microplate reader (CLARIOstar Plus, BMG Labtech, Ortenberg, Germany). All samples were prepared in triplicate.

Short-Term In Vitro Uptake Assay

FOX 2–5 and FOX E were labeled with Fe-59 and Ga-68, respectively, and diluted to final concentrations of 30 nM [^{68}Ga]Ga-siderophore and 350 nM [^{59}Fe]Fe-siderophore per well. Multiscreen 96-well plates with hydrophilic PVDF membranes (0.22 μm pore size; Merck, Darmstadt, Germany) were prewashed with 150 μL of sterile PBS. Subsequently, 180 μL of bacterial cultures in iron-depleted (-Fe) or iron-replete (+Fe) media were added to the wells. Thereafter, 50 μL of FOX E or FOX 2–5 labeled with Ga-68 or Fe-59 were added under the following conditions: [^{59}Fe]Fe-FOX E and [^{68}Ga]Ga-FOX E; [^{68}Ga]Ga-FOX E alone, [^{59}Fe]Fe-FOX 2–5 and [^{68}Ga]Ga-FOX 2–5; [^{68}Ga]Ga-FOX 2–5 alone. After a 90 min incubation period at 37 °C with 150 rpm, the content was removed by filtration, and filters were washed three times with 150 μL ice-cold TRIS-buffered saline (10 mM, pH 7.3). Filters were dissected into tubes, and radioactivity was measured with a 2480 Automatic Gamma Counter Wizard2 3" (Perkin Elmer, Waltham, MA, USA). All conditions were tested in triplicate, with standards (50 μL of each radiolabeled siderophore) as references. Standards were measured in quadruplicate. Relative uptake was calculated by normalizing measurements to the mean of the standard reference (100%). Measurements were performed in the 511 keV channel for 30 s Ga-68 and, after decay of Ga-68, in the 1099 keV channel of Fe-59 for 90 s.

Values were determined for [^{68}Ga]Ga-siderophore, with [^{68}Ga]Ga-siderophore as the reference for gallium measurements and the iron standard for Fe-59/Ga-68 mixtures, which was measured after decay of Ga-68. Results were presented as means ± standard deviation (SD) of triplicates.

Animal Experiments

Animal experiments were performed on female Balb/c mice (between 8 and 10 weeks old) (Envigo, Horst, The Netherlands). Before the experiments, animals were allowed to acclimate to the laboratory environment for 1 week. They were housed under standard conditions in individually ventilated cages with sawdust bedding and had free access to food and water. All procedures were carried out in compliance with the Czech Animal Protection Act (No. 246/1992), approved by the Czech Ministry of Education, Youth, and Sports (MSMT-9847/2019-5 and MSMT-24421/2021-4), and by the institutional Animal Welfare Committee of the Faculty of Medicine and Dentistry, Palacký University in Olomouc. Throughout the course of the experiments, the animals' general health and body weight were monitored. To reduce the number of animals used in the

study, a sample size of $n = 3$ per group was utilized. Small animal imaging was carried out under 2% isoflurane anesthesia (FORANE, Abbott Laboratories, Abbott Park, IL) to reduce motion artifacts and minimize distress to the animals.

Animal Infection Model

The murine model of acute myositis was performed in mice immunosuppressed by intraperitoneal injection of cyclophosphamide (Endoxan, Baxter, Prague, Czech Republic) 5 days and 1 day before infection (receiving 150 mg/kg and 100 mg/kg doses, respectively). The induction of acute myositis was facilitated by the intramuscular injection of bacteria. The immunodeficient mice were inoculated with 5×10^7 CFUs of either *K. pneumoniae*, *A. baumannii*, *S. aureus*, or *P. aeruginosa* in the left hind leg and with 5×10^7 CFUs of *E. coli* as a negative control in the right hind leg. The infections were then left to develop for 5 h prior to the imaging.

Animal Imaging

The infected mice were placed under inhalation anesthesia and retro-orbitally injected with either [^{68}Ga]Ga-FOX 2–5 or [^{68}Ga]Ga-FOX E ($\sim 6\text{--}8$ MBq/mice, approximately ~ 2 μg of FOX 2–5 or FOX E). At 45 min after injection, the mice were placed in the prone position in the μPET /computed tomography (CT) machine (Mediso NanoScan PET/CT imaging system for small animals, Mediso Medical Imaging Systems, Budapest, Hungary). First, a single FOV (98.55 mm) 10 min PET scan was acquired, followed immediately by whole-body helical CT (50 kVp/908 μA , 720 projections). Mediso Tera-Tomo iterative 3D PET reconstruction (Mediso Medical Imaging Systems, Budapest, Hungary) was used for image reconstruction. Image visualization, processing, and quantification were performed using Mediso Inter-View FUSION software (Mediso Medical Imaging Systems, Budapest, Hungary). All images were normalized to the injected activity and animal weight. Quantitative analysis was performed by measuring the activity within a region of interest (ROI). The ROIs were created based on the anatomical structures visualized in the CT scans. Both infected and control infected mice's hind legs were measured and compared separately. The results were expressed as SUV_{max} .

Statistics

Statistical analyses were performed using GraphPad Prism version 10.5.0 (774) (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Welch's *t*-test was applied to triplicate measurements of the indicated values. Differences were considered statistically significant at $p < 0.05$. Results were presented as means $\pm$ standard deviation (SD) of triplicates.

Visualizations

Graphs were generated using GraphPad Prism version 10.5.0 (774) (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

■ ASSOCIATED CONTENT

Data Availability Statement

Statements and Declarations. The raw data supporting the findings of this study are available from the corresponding author upon reasonable request. All animal studies were carried out in compliance with the Czech Animal Protection Act (No. 246/1992), approved by the Czech Ministry of Education, Youth, and Sports (MSMT-9847/2019-5 and MSMT-24421/2021-4), and by the institutional Animal Welfare Committee of the Faculty of Medicine and Dentistry, Palacky University in Olomouc.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/cbmi.6c00132>.

Quantified PET/CT imaging data; correlation between CFUs and OD₆₀₀ measurements; TLC analysis of radiolabeled compounds; reversed-phase HPLC radio-

chromatograms; and *in vitro* uptake studies of ^{68}Ga - and ^{59}Fe -labeled FOX compounds (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CFUs	colony-forming units
CT	computed tomography
DFO B	deferoxamine B
FOX E	ferrioxamine E
MM	minimal medium
PET	positron emission tomography
RP-HPLC	Reversed-phase high-performance liquid chromatography
SD	standard deviation
SUVmax	Standardized Uptake Value maximum
TLC	thin-layer chromatography
TBDT	tonB-dependent transporter.

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