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Synthesis of Fluorescent Probes Based on the Pyochelin Siderophore Scaffold

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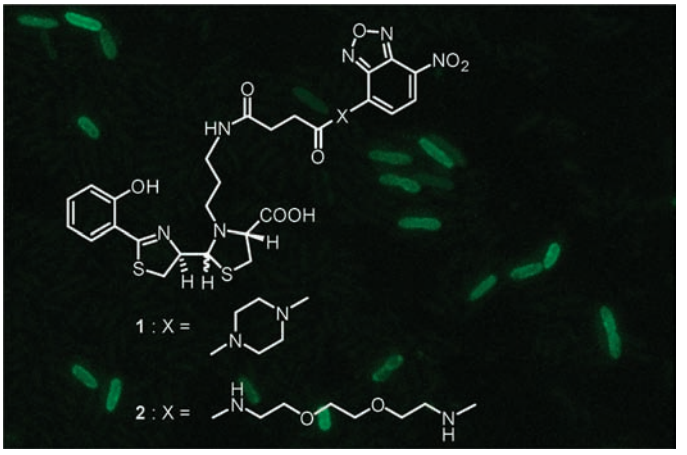
ABSTRACT



Pyochelin is a siderophore common to several pathogenic bacterial strains. Two conjugates, 1 and 2, between the NBD (4-nitrobenzo[1,2,5]oxadiazole) fluorophore and an N3′′-functionalized pyochelin were synthesized. These fluorescent probes unexpectedly increased their fluorescence in an aqueous medium in the presence of iron(III) and were transported into bacterial cells.

During infections, bacteria require iron concentrations in the micromolar range. Higher eukaryotes contain substantial amounts of this crucial element, which is tightly associated with transport and storage proteins and is not freely available to pathogens. To overcome this problem, pathogenic microorganisms synthesize and excrete small molecules, called siderophores, into the extracellular medium. These compounds are able to scavenge iron from the host through their very high affinity for iron(III).[1,2] In Gram-negative bacteria, specific transporters translocate ferric siderophores into the cytoplasm.[1b,2] Pyochelin is a siderophore produced by *Pseudomonas aeruginosa* and *Burkholderia cepacia*, which are two opportunistic Gram-negative bacteria (Scheme 1). These bacterial species cause severe infections, which are often lethal for cystic fibrosis patients.[3] Because iron is crucial for bacterial growth, the pyochelin-dependent iron uptake pathway is a potential therapeutic target. Fluorescent siderophores are invaluable tools to investigate the molecular mechanisms involved in bacterial iron acquisition.[4] To date, such an approach has not been possible for the pyochelin-dependent iron uptake pathway, because the instrinsic fluorescence of

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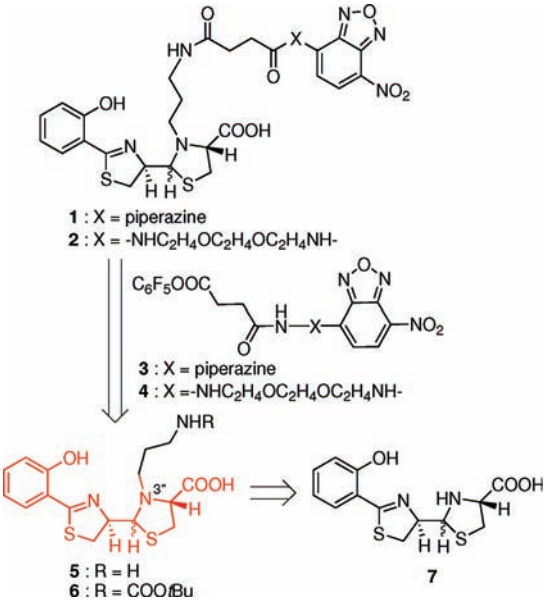
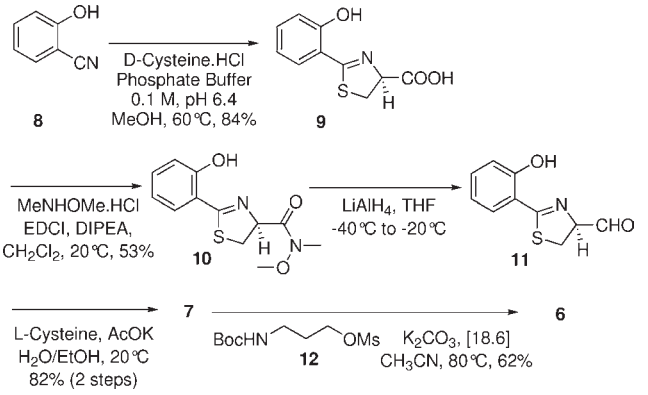
this siderophore is very weak.[4d] The present study describes the synthesis of two fluorescently labeled pyochelins **1** and **2**, which are suitable for further applications in biological media. The 4-nitro-benzo[1,2,5]oxadiazole (NBD) fluorophore was chosen according to its size and photophysical properties in aqueous media.[4a−c] The NBD and pyochelin were connected through two types of spacer arm, a short succinic spacer arm, and a longer spacer which minimized the steric hindrances with the proteins involved in ferric pyochelin uptake. The two NBD-spacer arm building blocks were prepared under the form of the two pentafluorophenyl esters **3** and **4**. These building blocks were connected to the free amine **5** of the functionalized pyochelin **6**. Pyochelin analog **6** was synthesized starting from *nor*-pyochelin **7** (Scheme 1).

Scheme 1. Structure and Retrosynthesis of Fluorescent Conjuguates **1** and **2**. Structure of Pyochelin (colored in red)



Although the thiazolidine nitrogen N3″ of pyochelin is involved in iron chelation,[5] this atom can host a three-carbon long aliphatic extension bearing a terminal amine function. Other positions were shown to be crucial for either metal chelation or biological recognition and were not suitable for substitution.[5] The synthesis of *nor*-pyochelin **7** started with the condensation of D-cysteine with the 2-hydroxybenzonitrile **8** under buffered conditions in an hydromethanolic medium.[6] The resulting thiazoline **9** was used without further purification to synthesize the corresponding Weinreb amide **10** using *N*,*O*-dimethylhydroxylamine in the presence of EDCI. The hydroxamic ester **10** was then reduced to the corresponding aldehyde **11** using $LiAlH_4$.[7] This aldehyde is very sensitive and was used straight away without further purification. Condensation with L-cysteine, in the presence of potassium acetate in a hydroethanolic medium, led to the expected *nor*-pyochelin **7**. The latter was converted into the functionalized pyochelin **6** by coupling with the methanesulfonic acid 3-*tert*-butoxycarbonyl-aminopropyl ester **12**.[8] The best results for this reaction were obtained when the *nor*-pyochelin **7** and the linker precursor **12** were reacted in the presence of K_2CO_3 and crown-ether[18.6] in acetonitrile at 80 °C (Scheme 2). In these conditions, the expected compound **7** was cleanly isolated with a 62% yield. Other methods using different bases (Cs_2CO_3, tertiary amines), solvents (acetone, dioxane), or temperature conditions led to poor yields of the desired product. The *O*-alkylation product on phenol has never been isolated. This observation could be due to the presence of a hydrogen bond between the phenol and thiazoline nitrogen.[5a]

Scheme 2. Synthesis of the Functionalized Pyochelin **6**



The two NBD-spacer arm building blocks **3** and **4** were prepared in parallel. The corresponding *N*-hydroxysuccinimide ester for pentafluorophenyl ester **3** has been previously described in the literature.[4b] In our hands, this compound was produced at a moderate yield and presented technical difficulties when used in our approach (low stability, lack of solubility, final product contamination with *N*-hydroxysuccinimide). We then developed the synthesis of the alternative building block **3**. The *tert*-butyl-hemisuccinate **13** was converted into the corresponding pentafluorophenyl ester **14**, a white crystalline solid, stable and easy to handle. This succinic diester **14** was reacted with piperazinyl-NBD **15**,[4a] and the *tert*-butyl protecting group of the resulting adduct **16** was further cleaved in the presence

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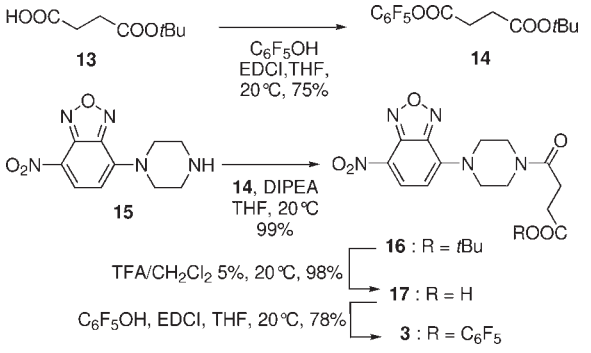
of TFA. The carboxylic acid **17** was then esterified with pentafluorophenol in the presence of EDCI leading to the synthesis of the expected building block **3** (Scheme 3).[9]

Scheme 3. Synthesis of the NBD-Spacer Arm Block **3**

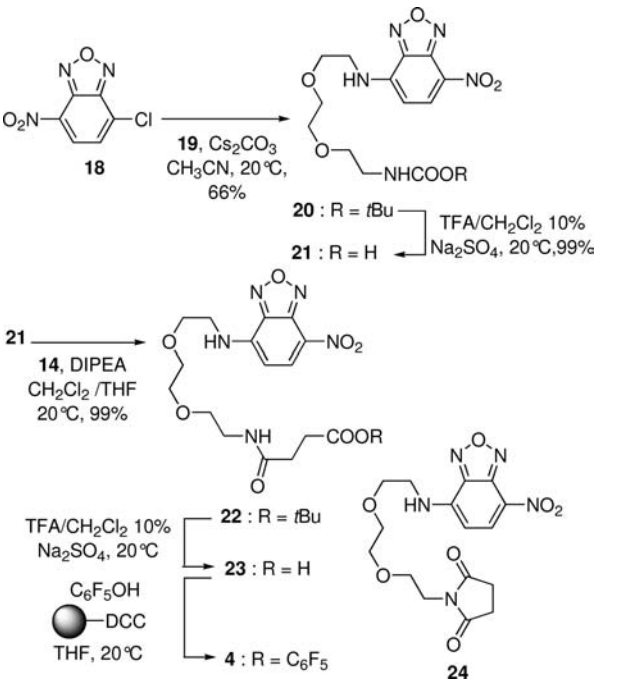


Synthesis of the second NBD-spacer building block **4** started with the reaction of {2-[2-(2-amino-ethoxy)-ethoxy]-ethyl}-carbamic acid *tert*-butyl ester **19**[10] with commercially available NBD chloride **18** in the presence of cesium carbonate. The Boc group of the resulting compound **20** was then cleaved using 10% TFA/CH_2Cl_2. However this reaction had to be performed under anhydrous conditions to avoid the formation of several unexpected side products. Addition of oven-dried sodium sulfate to the reaction mixture led to higher yields in expected free amine **21**. Compound **21** was then reacted with the succinate derivative **14** leading to the *tert*-butyl ester **22**. Free carboxylic acid **23** was generated from **22** under the previous anhydrous deprotection conditions. The last step was the activation of acid **23** under the form of pentafluorophenyl-ester **4**. Purification of compound **4** on a silica gel quantitatively led to the succinamide derivative **24**, resulting from an intramolecular reaction between the amide function and the activated ester. However, activation of carboxylic acid **23** in the presence of polymer supported DCC led to the activated ester **4**,[11] which was isolated and then used without any further purification (Scheme 4).

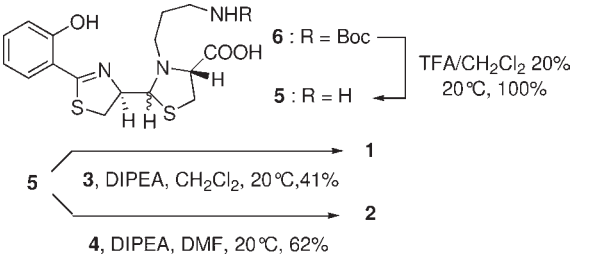
The amine function of pyochelin analog **6** was then deprotected using 20% TFA/CH_2Cl_2. The resulting amine **5** was conjugated with the two NBD-spacer arm building blocks **3** and **4** in the presence of DIPEA. The expected conjugates **1** and **2** were isolated in two steps from functionalized pyochelin **6** in 41% and 62% yields, respectively (Scheme 5).

Iron(III) usually induces a quenching of chelator fluorescence, especially in aqueous media. However, the intrinsic fluorescence of probes **1** and **2** dissolved in Tris buffer (pH 8.0) was strikingly enhanced in the presence of iron(III). For probe **1**, the highest level of fluorescence was reached after addition of 1 equiv of iron(III) whereas, for probe **2**, 5 equiv of iron(III) were necessary to observe the maximal fluorescence. The fluorescence enhancements were 290% for probe **1** and 320% for probe **2** (Figure 1). Job's plot analysis gave the ligand/iron(III) complex stoichiometry and the association constants of probes **1** (2: 1, $K = 6.3 \times 10^{10}\ M^{-2}$) and **2** (3: 1, $K = 2.6 \times 10^{19}\ M^{-3}$) with iron(III).[12] These constants are higher than that described for natural pyochelin ($K = 2.4 \times 10^{5}\ M^{-2}$) in methanol.[13]

Scheme 4. Synthesis of the NBD-Spacer Arm Block **4**



Scheme 5. Conjugation of Functionalized Pyochelin **6** with the Fluorophore-Spacer Arm Building Blocks **3** and **4**



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(13) Cox, C. D.; Graham, R. *J. Bacteriol*. **1979**, *137*, 357–364. This constant, estimated in methanol, might be higher in aqueous media.

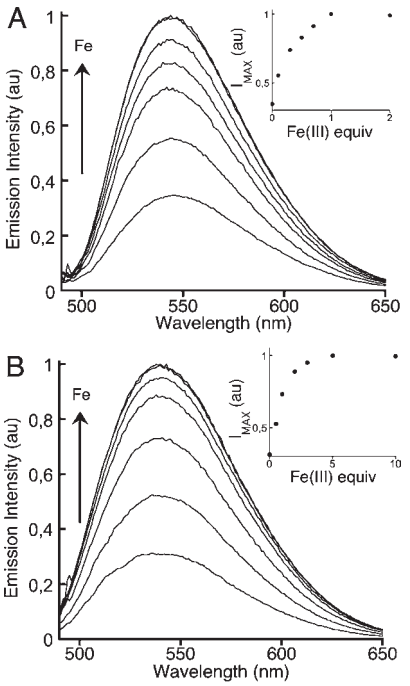

Figure 1. Emission spectra of probes **1** (A, 15 μM) and **2** (B, 2 μM) in Tris buffer (pH 8.0, 150 mM) in the presence of increasing $FeCl_3$ equivalents. Insets: Intensity plots at maximal emission wavelength $\lambda_{em}^{max}(A,B) = 545$ nm.

In the presence of iron(III), the intrinsic fluorescence of pyochelin is completely quenched[4d] when the fluorescence of precursors **16** and **22** appeared to be unaffected (see Supporting Information). The photophysical behavior of probes **1** and **2** is therefore related to the conjugation between pyochelin and NBD and to the interaction of iron(III) with the resulting siderophore−fluorophore conjugate. Determination of the fluorescence quantum yields indicated that the fluorescence of NBD is quenched by pyochelin (see Supporting Information). This effect is lowered upon iron addition, leading to an increase of NBD fluorescence. Although examples of chelation-enhanced fluorescence (CHEF) induced by iron(III) have been previously reported, very few molecules conserved this property in aqueous media.[14] Among these examples, our probes are the first ones based on a siderophore from a pathogenic bacteria. The ability of compounds **1** and **2** to interact with the pyochelin pathway in *P. aeruginosa* strains was investigated by epifluorescence microscopy. When probe **1** was incubated with the PAD07 strain of *P. aeruginosa*, the bacteria were labeled (Figure 2). In contrast, the DH51 strain, which did not express the pyochelin outer membrane receptor, was not labeled. Similar results were obtained using probe **2** (see Supporting Information). These observations showed that probes **1** and **2** are specifically recognized by the pyochelin specific outer membrane receptor.

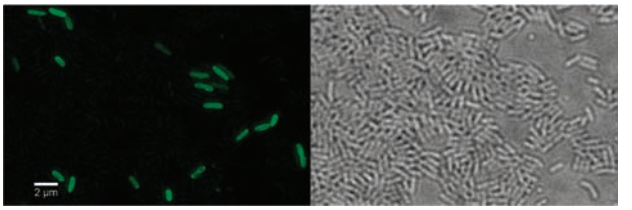

Figure 2. Epifluorescence microscopy of *P. aeruginosa* PAD07 strain complemented with probe **1**. The left picture was taken using the filter set: exc. 472 ± 30 nm/em, 520 ± 30 nm/dichroic 502−730 nm. The right picture was taken by light microscopy. Scale bar: 2 μm.

Probes **1** and **2** will be useful molecular tools to investigate the pyochelin-dependent iron uptake pathway. This could potentially lead to therapeutic developments against pathogenic bacteria expressing this biological system. In this context, our results suggest that functionalized pyochelin **6** is a promising candidate to vectorize antibiotics using a Trojan-horse prodrug strategy.[15] This approach is currently under investigation in our group.

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Supporting Information Available. Experimental procedures and analytical/spectral data. Fluorescence measurements, microscopy images, and protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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