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A new route to trihydroxamate-containing artificial siderophores and synthesis of a new fluorescent probe

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Abstract—A fluorescent labelled artificial siderophore **1** was synthesized by coupling a 7-nitrobenz-2-oxa-1,3-diazole (NBD) derivative to the terminal amino group of a new trihydroxamate-containing amine **2**, a ferrichrome-type siderophore that was obtained from tris(hydroxymethyl)aminomethane. Compound **1** was shown to be a suitable tool for experiments on siderophore transport and uptake processes in various organisms cells and particularly in *Candida albicans* cells.

1. Introduction

Although iron is one of the most abundant elements in the earth's crust, in aerobic environments, it is found as Fe(III) ion forming very insoluble oxides and hydroxides. In order to overcome this low solubility and bioavailability of iron, microorganisms synthesize strong iron(III) chelators, called siderophores, and release them into the environment where they selectively trap iron and mediate its transport and deposition into the cell.[1,2]

Recent results have shown that iron uptake from hydroxamate siderophores, and particularly ferrichrome-type siderophores, occurs in *Candida albicans* and is mediated by one or more high-affinity transport systems.[3] For a better understanding of the mechanisms of iron transport in *C. albicans*, we undertook the synthesis of ferrichrome analogues. It is important from a medical point of view to understand how *C. albicans* acquires iron. Indeed, proteins that mediate iron transport in *C. albicans* represent potential targets for the development of anti-*Candida* therapies.

Many ferrichrome analogues were synthesized by Shanzer and co-workers[4,5] with the aim of studying the influence of the various zones of the molecules on the iron(III)-binding, recognition by the cells of *Pseudomonas putida* and penetration into these cells. It has been shown that the *R′* zone, can be modified without affecting the iron binding, or the interaction with membrane proteins for which the influence of the *R* substituents is very sensitive[5] (Scheme 1).

In this paper, we report the synthesis of a fluorescent-ferrichrome analogue **1** from a new trihydroxamate-containing amine **2** (Scheme 1). The iron-binding part of these molecules incorporates the glycyl chain (R = H), which allows them to fully mimic the natural ferrichrome.[4]

2. Results and discussion

2.1. Chemistry

Shanzer co-workers used *R′*-substituted pentaerythritols as starting materials for the synthesis of ferrichrome analogues. This strategy compelled them to reproduce in each case the harsh building of the trihydroxamate parts of the molecules (Scheme 1).[4,5] We synthesized amine **2** as a source of various amino-substituted derivatives expected to have the ferrichrome siderophore properties. This organic specificity is similar to those

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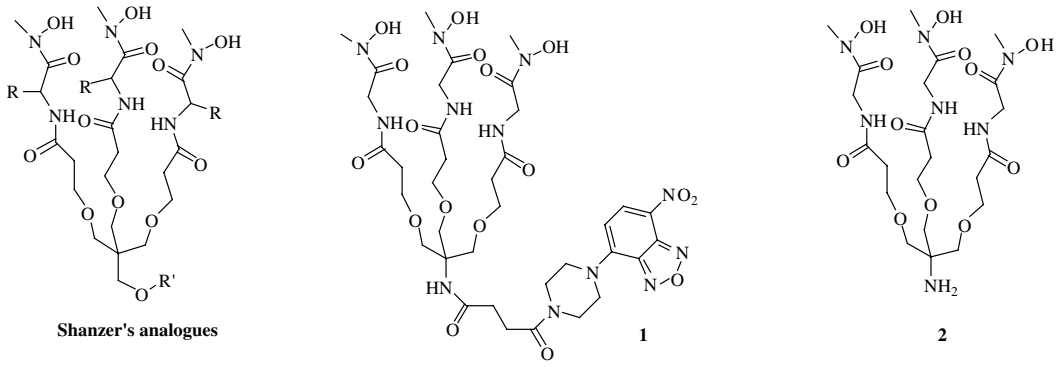
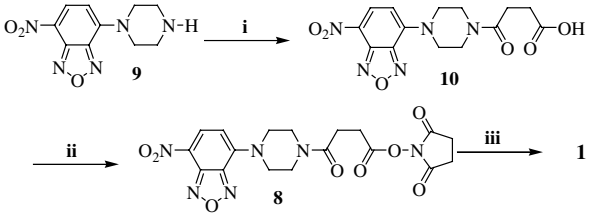


Scheme 1.

of desferrioxamine B, a linear trihydroxamate-type siderophore with an amino-terminal group, from which various derivatives have been synthesized.[6–8] For the preparation of **2**, we opted for the synthesis of intermediate **3**, a (poly-ether)amine, colloquially known as Lin's amine[9] (Scheme 2). It was obtained in two steps from tris(hydroxymethyl)aminomethane ('Tris'), a convenient and inexpensive starting material, previously used for the synthesis of dendritic macromolecules. Amine **3** was protected with the benzyl chloroformate (Cbz) group, yielding **4**. Subsequently, the removal of the ethyl esters without conflict with the protecting group was obtained, by using 1 M aqueous sodium hydroxide in ethanol at low temperature. Another way to obtain triacid **5** from 'Tris' has been described by Cardona and Gawley,[10] a three-step synthesis using an amino-polyether tri-*tert*-butyl ester as an intermediate. The triacid **5** obtained was treated with 3 equiv of hydroxamate-bearing glycyl residue[11] after activation of the carboxylic groups by pentachlorophenolate groups yielding **7**. The benzyl chloroformate group was easily removed from **7** by hydrogenation with Pd/C in ethanol and amine **2** was obtained quantitatively.

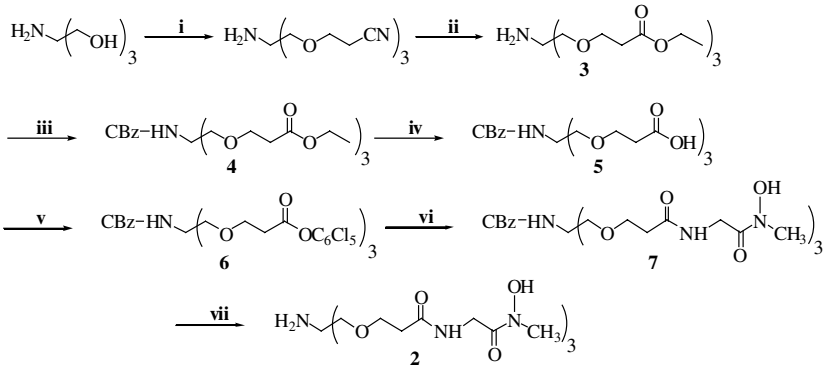
The fluorescent-ferrichrome analogue **1** was prepared by coupling amine **2** with the hydroxysuccinimidyl ester-fluorescent probe **8** (Scheme 3). Compound **8** was obtained in two steps from monosubstituted piperazinyl-NBD[5] **9** by condensation with succinic anhydride followed by esterification of the obtained acid **10** with NHS.



Scheme 3. Synthesis of NBD-ferrichrome analogue conjugate **1**. Reagents and conditions: (i) succinic anhydride, toluene/DMF (3:1, v/v), Δ ; (ii) NHS, DCC, THF/DMF (4:3, v/v), 25 °C; (iii) **2**, THF/DMF (3:6, v/v), 25 °C.

2.2. Chelation of iron(III) by 1

Adding the NBD label to the amine **2** imparts fluorescent properties to the free iron carrier. Excitation of **1** in methanol (80%)/aqueous sodium acetate (20%) at 474 nm and monitoring of the emission spectra over the 480–650 nm range reveal a single peak at 538 nm, that is quenched upon loading of the carrier with ferric iron (Fig. 1). In agreement with Shanzer and co-workers,[5] the observed fluorescence quenching of this ferrichrome analogue follows Perrin's model of static quenching (Fig. 1 inset) and, as expected, the fluore-



Scheme 2. Synthesis of trihydroxamate-containing amine **2**. Reagents and conditions: (i) CH_2=CHCN, aqueous KOH, dioxane; (ii) H_2SO_4, ethanol, reflux; (iii) benzylchloroformate, K_2CO_3, dioxane; (iv) 1 N aqueous NaOH, ethanol/water (7:3, v/v), 0 °C; (v) C_6Cl_5OH, DMAP, DCC, THF, 0 °C; (vi) $H_2NCHCONCH_3OH$,[11] Et_3N, THF, 25 °C; (vii) H_2/Pd, ethanol.

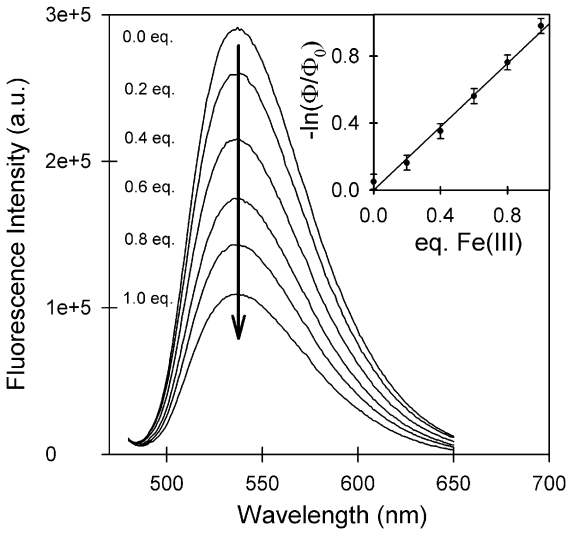


Figure 1. Fluorescence iron (III) titration curves with labelled ferrichrome analogue **1**. Aliquots of stock solutions of **1** in MeOH were treated with aliquots of methanolic solutions of $FeCl_3$ (0, 0.2, 0.4, 0.6, 0.8 and 1 equiv) and diluted with MeOH (80%)–0.1 N aqueous NaOAc (20%) to a final concentration of 5 μM.

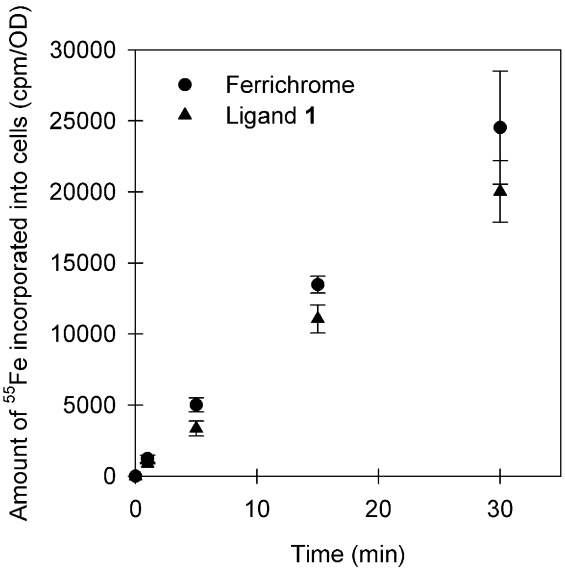


Figure 2. Iron uptake by *S. cerevisiae* from either ^{55}Fe-ferrichrome or ^{55}Fe bound to ligand **1** (fluorescent analogue of ferrichrome). Cells were grown overnight in YPD medium. Cells were then diluted 10-fold in the same medium and cultured for 6 h before iron uptake was measured from 1 μM ferrichrome (squares) or from 1 μM fluorescent analogue of ferrichrome (**1**) (circles). Means ± SE from three experiments.

scence of the NBD derivative **10** was not affected by addition of similar concentrations of iron salts.

The selectivity of our probe has been tested in the presence of other biologically relevant metal ions such as Ca(II), Mg(II) and Cu(II) metal ions. Ca^{2+} and Mg^{2+} had no effect on fluorescence signal of **1** and Cu^{2+} produced significant quenching but clearly less important than with Fe^{3+}.

2.3. Biological activity

We first tested that the iron bound to the fluorescent analogue of ferrichrome **1** was taken up by *Saccharomyces cerevisiae* cells as efficiently as the iron bound to commercially-available ferrichrome. Figure 2 shows that the kinetics of iron uptake from both ligands are similar. Then we checked that the fluorescent derivative of ferrichrome itself actually entered cells that are known to have specific ferrichrome transport mechanisms: *S. cerevisiae*[12] and *C. albicans*[13] Figure 3A and B shows that it was indeed the case. In both *S. cerevisiae* (Fig. 3A) and in *C. albicans* (Fig. 3B) cells, fluorescence accumulated preferentially in *peri*-vacuolar vesicles, which is consistent with the endocytosis-mediated mechanism of siderophore uptake proposed by Kim et al.[14] Cells that appeared full of fluorescence (Fig. 3A) were probably dead cells, freely permeable to the siderophore. *Tritrichomonas foetus* is a pathogenic flagellate of the uro-genital tract of cattle that has an unusually high nutritional requirement for iron.[15] Siderophore used by this organism has never been shown. Figure 3C shows that *T. foetus* accumulated **1** into small vesicles that could be pinocytosis vesicles. An extensive study of siderophore iron uptake/used by *T. foetus* will be published elsewhere. Figure 3 shows that ligand **1** is a useful tool in order to study ferrichrome uptake and used by various organisms.

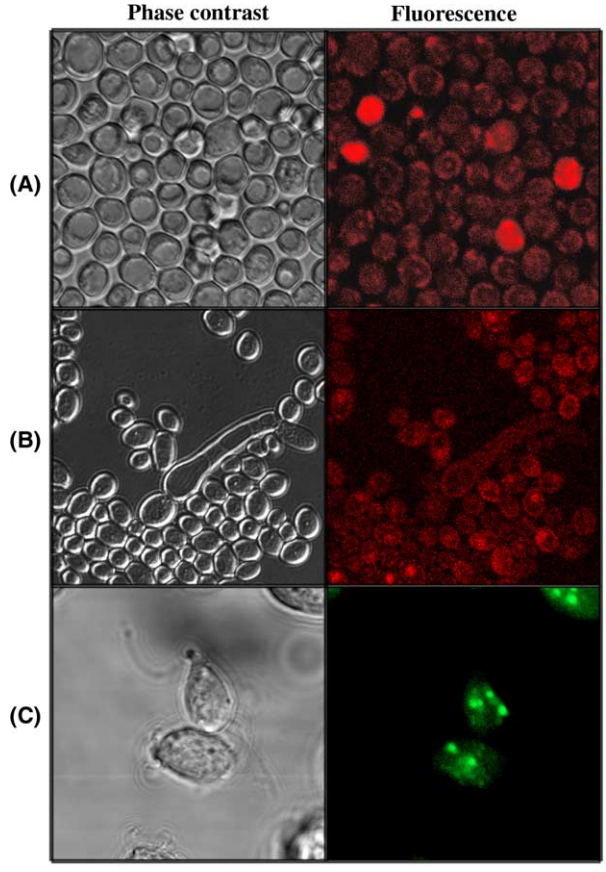


Figure 3. Confocal micrographs of *S. cerevisiae* (A), *C. albicans* (B) and *T. foetus* (C) cells grown overnight in the presence of 10 μM of the fluorescent analogue of ferrichrome (**1**).

3. Conclusion and prospects

A simple method to produce a ferrichrome-type siderophore bearing a free amino group is reported. A fluorescent labelled artificial siderophore (**1**) was prepared by linking a fluorescent nucleus to the amino group. This compound was actually uptaken by the cell and was used as a source of iron, like the ferrichrome itself, in *C. albicans* and in *S. cerevisiae* but also in *T. foetus* for which such a result has never been shown. This synthetic fluorescent probe **1** is a promising tool for studying siderophore uptake mechanisms as the intracellular localization of the ligand but its behaviour in the cell could also be monitored.

Compound **2**, as easily derivatized as desferrioxamine B, will be very useful in order to have access to new biological tools. It could also be investigated as the siderophore component of conjugates, particularly siderophore-antifongic agent conjugates.

4. Experimental

4.1. Chemistry

All reagents were analytical grade, dried and purified when necessary.

TLC analyses were carried out on pre-coated plates of silica or alumina gel 60 F254 (Merck). The visualization was realized by using a two-wavelength (365 and 254 nm) detection system or iodine and, in the case of the hydroxamate ligands, a $FeCl_3$ solution. Silica gel used for column chromatography was 60A granulometry 6–35 or 40–63 μm (SDS) or Florisil® 100–200 mesh (ACROS).

Spectra were obtained as follows: ^{1}H NMR spectra were recorded at 270 MHz on a Jeol GSX 270 WB spectrometer or at 500 MHz on a Brucker AVANCE DRX 500 and ^{13}C NMR spectra were recorded at 125.75 MHz on the Brucker AVANCE DRX; FTIR spectra were recorded on a BIO-RAD spectrometer; ESI-MS spectra were recorded on a JMS-700 (JEOL LTD, Akishima, Tokyo, Japan) double focusing mass spectrometer with reversed geometry, equipped with a pneumatically assisted electrospray ionization (ESI) source and MALDI-TOF-MS spectra on a Brucker BIFLEX III.

Fluorescence experiments were performed by using a QM-4/QuantaMaster™ fluorometer from PTI®.

4.2. Benzyl *N*-Tris{[2-(2,3,4,5,6-pentachlorophenoxycarbonyl)ethoxy]methyl}methyl carbamate (6)

Triacid **5** (1.19 g, 2.52 mmol), pentachlorophenol (2.68 g, 10.09 mmol) and DMAP (24.6 mg, 0.20 mmol) were dissolved in THF (20 mL). The solution was cooled to 0 °C and a solution of DCC (2.08 g, 10.09 mmol) in THF (20 mL) was then added. The reaction mixture was stirred under N_2 at room temperature for 48 h. The precipitate of dicyclohexylurea was eliminated by filtration and the filtrate was concentrated to dryness. The resulting residue was purified, first by filtration on column (neutral alumina gel, AcOEt) then by column chromatography (silica gel, AcOEt/petroleum ether 35:65), yielding **6** (2.20 g, 72%), a colourless viscous oil, ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.91 (t, 6H, J = 5.9 Hz), 3.77 (s, 6H), 3.84 (t, 6H, J = 5.9 Hz), 5.03 (s, 2H), 5.22 (s, 1H), 7.27 (m, 5H). MS (ESI^+): m/z 1209 and good correlation with isotopic cluster.

4.3. Benzyl *N*-Tris{[2-[((*N*-hydroxy-*N*-methylcarbamoyl)methyl)aminocarbonyl]ethoxy]methyl}methylcarbamate (7)

To a solution of **6** (4.95 g, 4.07 mmol) in THF (60 mL), placed in a three necked round bottomed flask under N_2, was added a mixture of Et_3N (2.47 g, 24.42 mmol) and *N*-hydroxy-*N*-methylcarbamoylmethyl ammonium 2,2,2-trifluoroacetate[11] (5.33 g, 24.42 mmol) in suspension in THF (60 mL). The mixture was stirred at room temperature for 4 days and the solvent was then evaporated under reduced pressure. After addition of water (100 mL), the aqueous layer was extracted with CH_2Cl_2 (100 mL) and the organic phase was washed with water (3×50 mL). The aqueous phases were collected and concentrated to dryness. The resulting residue was purified by column chromatography (silica) by using AcOEt/MeOH (85:15) as eluent, giving **7** (1.45 g, 49%), an orange viscous oil, ^{1}H NMR (270 MHz, CD_3OD) δ 2.48 (t, 6H, J = 5.9 Hz), 3.18 (s, 9H), 3.7 (s and t, 12H, J = 5.9 Hz), 4.13 (s, 6H), 5.00 (s, 2H), 7.27–7.33 (m, 5H). ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.35 (t, 6H, J = 6.4 Hz), 3.07 (s, 9H), 3.50 (s, 6H), 3.54 (t, 6H, J = 6.4 Hz), 3.95 (d, 6H, J = 5.3 Hz), 4.96 (s, 2H), 6.56 (br s, 1H), 7.26–7.36 (m, 5H), 7.89 (t, 3H, J = 5 Hz), 9.91 (br s, 3H). MS (ESI^-): m/z 728 $(M-H)^-$, 842 $(M-H+CF_3CO_2H)^-$.

4.4. Tris{[2-[((*N*-hydroxy-*N*-methylcarbamoyl)methyl)-aminocarbonyl]ethoxy]methyl} methylamine (2)

Compound **7** (1.02 g, 1.40 mmol) was stirred with of 10% Pd/C (122 mg) in ethanol (100 mL) under H_2 at room temperature for 4 h 30 min. The palladium was filtered off and the solvent removed under reduced pressure, giving amine **2** (0.83 g, 100%), as a hygroscopic light yellow solid, ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.36 (t, 6H, J = 6.3 Hz), 3.07 (s, 9H), 3.17 (s, 6H), 3.55 (t, 6H, J = 6.3 Hz), 3.95 (d, 6H, J = 5.2 Hz), 7.91 (br s, 3H), 10.05 (br s, 3H). ^{1}H NMR (500 MHz, CD_3 OD) δ 2.37 (t, 6H, J = 5.8 Hz), 3.19 (s, 9H), 3.42 (s, 6H), 3.70 (t, 6H, J = 5.8 Hz), 4.14 (s, 6H). ^{13}C NMR (125.75 MHz, DMSO-d_6) δ 35.7, 39.7, 56.6, 67.2, 71.2, 168.9, 170.3. MS (MALDI TOF): m/z 596 $(M+H)^+$, 618 $(M+Na)^+$.

4.5. 4-[4-(7-Nitro-2,1,3-benzoxadiazol-4-yl)piperazino]-4-oxobutanoic acid (10)

4-Nitro-7-piperazino-2,1,3-benzoxadiazole[5] (0.50 g, 2.0 mmol) and succinic anhydride (0.22 g, 2.2 mmol) were dissolved in toluene (30 mL) and DMF (10 mL). The

solution was refluxed overnight. After removal of the solvents under reduced pressure, the residue was purified by column chromatography (silica gel, 97:3 CH_2Cl_2/MeOH) yielded a red solid (0.65 g, 92%) identified to **10**, ^{1}H NMR (270 MHz, DMSO-d_6) δ 2.44 (t, 2H, J = 6.4 Hz), 2.59 (t, 2H, J = 6.4 Hz), 3.73–4.24 (4t, 8H, J = 5.1 Hz), 6.59 (d, 1H, J = 9 Hz), 8.50 (d, 1H, J = 9 Hz). MS (MALDI TOF): m/z 372 $(M+Na)^+$, 394 $(M+2Na-H)^+$.

4.6. *N*-Succinimidyl-4-[4-(7-nitro-2,1,3-benzoxadiazol-4-yl)piperazino]-4-oxobutanoate (8)

Fluorescent acid **10** (200 mg, 0.572 mmol), NHS (65.8 mg, 0.572 mmol) and DCC (118 mg, 0.573 mmol) were dissolved in THF (8 mL) and DMF (6 mL). The reaction mixture was stirred under N_2 at room temperature for 2 days. After removal of the precipitate by filtration, the filtrate was concentrated to dryness under reduced pressure. The resulting residue was purified by column chromatography (silica gel, AcOEt) giving ester **8** (0.17 g, 67%), a red solid, ^{1}H NMR (270 MHz, DMSO-d_6) δ 2.74–2.79 (m, 6H), 2.90 (t, 2H, J = 6.6 Hz), 3.73–4.24 (4 m, 8H, J = 5.1 Hz), 6.60 (d, 1H, J = 9.1 Hz), 8.51 (d, 1H, J = 9.1 Hz). MS (MALDI TOF): m/z 469 $(M+Na)^+$.

4.7. 4-[4-(7-Nitro-2,1,3-benzoxadiazol-4-yl)piperazino]-oxopropyl*N*-tris{[2-[((*N*-hydroxy-*N*-methylcarbamoyl)methyl)aminocarbonyl]ethoxy]methyl}methylamide (1)

Amine **2** (120 mg, 0.20 mmol) and ester **8** (108 mg, 0.24 mmol) were dissolved in THF (6 mL) and DMF (2.5 mL). The solution was stirred under N_2 at room temperature for 4 days then the solvents were evaporated under reduced pressure. The resulting residue was purified by column chromatography (silica gel, 85:15 CH_2Cl_2/MeOH), yielding **1**, a red viscous oil (38 mg, 20%), ^{1}H NMR (500 MHz, CD_3 OD) δ 2.50 (t, 6H, J = 5.8 Hz), 2.53 (t, 2H, J = 6.7 Hz), 2.68 (t, 2H, J = 6.7 Hz), 3.19 (s, 9H), 3.68 (t, 6H, J = 5.9 Hz), 3.72 (s, 6H), 4.15 (s, 6H), 4.28–3.88 (m, 8H, J = 5 Hz), 6.48 (d, 1H, J = 8,9 Hz), 8.42 (d, 1H, J = 8.9 Hz). ^{13}C NMR (125.75 MHz, CD_3OD) δ 29.3, 32.4, 36.4, 37.4, 41.4, 42.0, 45.3, 61.5, 68.5, 70.2, 104.0, 124.0, 136.7, 146.0, 146.3, 146.5, 170.9, 173.3, 174.4, 174.9.IR (KBr): ν 3421, 1648, 1553 cm^{-1}. MS (MALDI-TOF): m/z 949 $(M-H+Na)^+$, 950 $(M+Na)^+$.

4.8. Biological studies

Fluorescence confocal microscopy was performed as described by Ardon et al.[16] *C. albicans* (strain BWP17) and *S. cerevisiae* (strain BY4741) were grown on complete YPD medium (1% yeast extract, 1% peptone, 2% glucose). *T. foetus* was grown on Trypticase, yeast extract, maltose medium (TYM).[17] For microscopy experiments, cells were grown overnight in the presence of 10 μM of the ferri-siderophore and then washed with water (*S. cerevisiae*, *C. albicans*) or with isotonic buffer (*T. foetus*).

Iron uptake assays were performed as previously described[12] by using commercial ferrichrome (Sigma) or the newly synthesized fluorescent derivative of ferrichrome labelled with ^{55}Fe.

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