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SYNTHESIS OF NEW 3-HYDROXY-4(1*H*)-PYRIDINONE DIRECTLY ATTACHED TO QUINOXALINE AND ITS FLUORESCENCE PROPERTY UPON COMPLEXATION TO METAL IONS

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Abstract – A new fluorophore (**3**), in which the bidentate ligand, 3-hydroxy-2-methyl-4(1*H*)-pyridinone is directly attached to the fluorescent 2,3-dimorpholino-quinoxaline at C-6 position. The bidentate ligand (**3**) formed 3:1 complexes with Fe(III), Al(III), Ga(III), and Cr(III). The fluorescence was efficiently quenched by the metal complex formation *via* the Perrin model of static quenching, the quenching efficiency being in order of Fe(III) >> Al(III) > Ga(III) > Cr(III). The fluorescence was recoverd by removal of Fe(III) with the *N*-benzoyl analogue of a naturally occurring siderophore, desferrioxamine B.

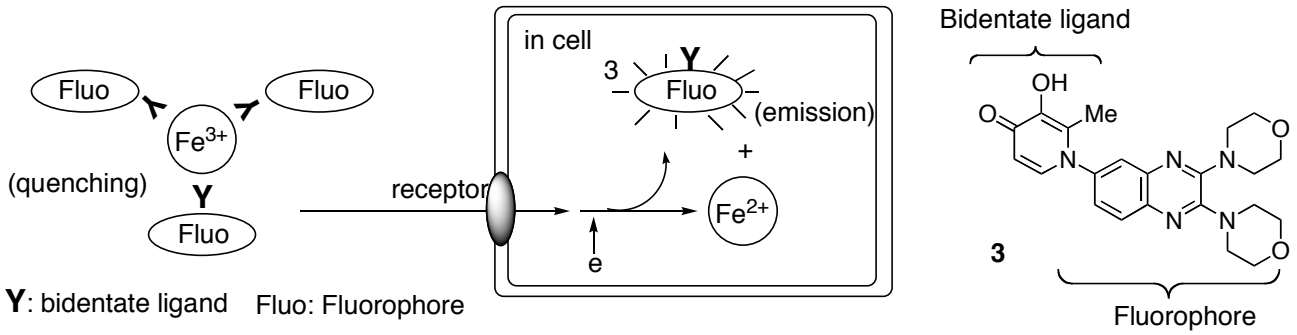
INTORODUCTION

Microorganisms excrete low-molecular-weight ligands specific for Fe(III) termed “siderophore”, which is necessary for the growth, for sequestering iron from the environment and transporting it into a cell through the membrane.[1,2] Two common functional groups found in siderophores are hydroxamic acid and catechol, which act as strong bidentate ligands to Fe(III). A mechanism of the iron-uptake by microorganisms has been examined by means of the isotope-labelling (^{14}C and ^{59}Fe) technique,[3,4] but there are many disadvantages in viewpoint of safety, toxcity, and environmental pollution. Recently fluorescent molecular sensors have become increasingly important for the detection of intracellular cations.[5] However, only a few papers concerning the detection of iron in cell and the environment have been reported.[6-9] In 1996, Shanzer and co-workers introduced fluorescent-labeled ferrichrome analogues, which composed of trihydroxamic acid, a spacer, and a fluorophore, as the diagnostic tool for the detection and identification of pathogenic bacteria and fungi.[10]

Previously we demonstrated that 3-hydroxy-2-methyl-1-phenyl-4(1*H*)-pyridinone and *N*-

hydroxydiazinones exhibit the growth-promotion activities for *Arthrobacter flavescens* JG-9,[11] and 6-aminoquinoxalines emit a strong fluorescence, being applicable to new fluorescence derivatization reagents for carboxylic acids.[12]

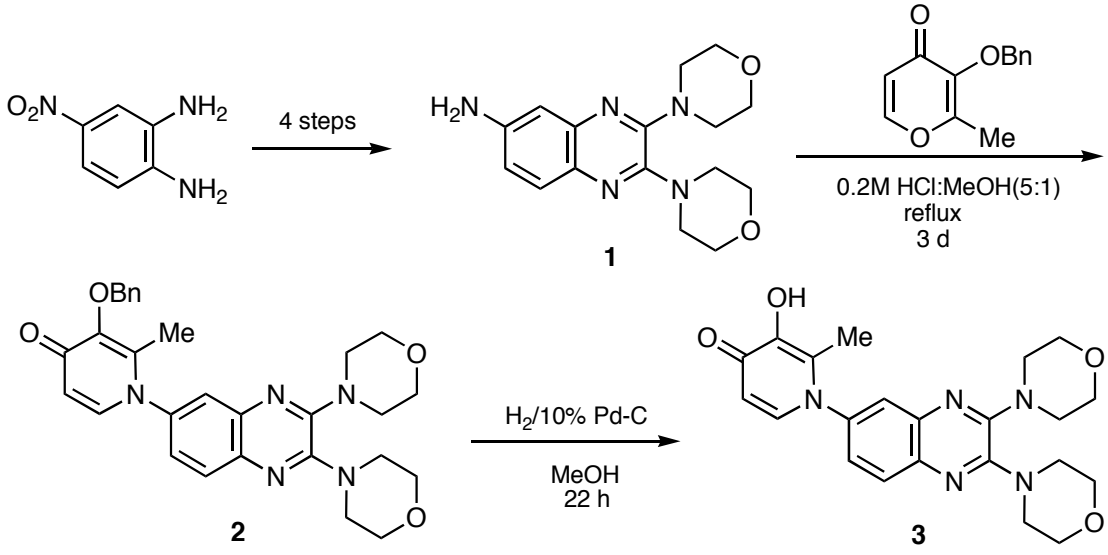
We describe here the synthesis of a new fluorescent heterocyclic bidentate ligand-quinoxaline conjugate (**3**) and the evaluation of fluorescence characteristics, especially quenching of fluorescence upon complexation with Fe(III) and other various transition metals and recovering of fluorescence upon metal ion release. If the quenching of fluorescence upon Fe(III) complexation (fluorescence *off*), the uptake of Fe(III) complex through the receptor into a cell, and subsequent release of iron upon dissociation of the bidentate ligand (fluorescence *on*) as shown in Scheme 1 occur smoothly, the iron-uptake would be observed with digital fluorescence microscopy.



Scheme 1 Schematic diagram of microbial iron-uptake by the bidentate ligand-fluorophore conjugate.

RESULTS AND DISCUSSION

The synthetic procedure for bidentate ligand (**3**) was depicted in Scheme 2. The condensation of 6-aminoquinoxaline (**1**)[12] with *O*-benzylmaltol under the acidic condition and subsequent removal of the protecting group of compound (**2**) with the catalytic hydrogenation afforded the product (**3**), which emitted a strong fluorescence at λmax 445 nm when the excitation wavelength of 365 nm was applied.



Scheme 2 Synthesis of 3-hydroxy-2-methyl-1-(2,3-dimorpholinoquinoxalin-6-yl)pyridin-4(1*H*)-one

The Fe(III)-complex-forming tendency of compound (**3**) was examined in 50% aqueous DMF solution at an apparent pH 7 by plotting the absorbance at λmax 455 nm as a function of the molar ratio [Fe(III)]/[(**3**)]. Compound (**3**) showed an intersection at a ratio of 0.32, indicating the formation of 3:1 Fe(III) complex (Figure 1). It was also supported from ^{1}H NMR spectral data of diamagnetic Ga(III) complex of compound (**3**) in $CDCl_3$ solution. A singlet and two doublets due to methyl and olefinic protons at C-5 and C-6 positions of the 4(1*H*)-pyridinone ring apparently shifted to the down field compared to those of free ligand (**3**); Δδ 0.16 for 2-Me, 0.68 for 5-H, and 0.38 ppm for 6-H.

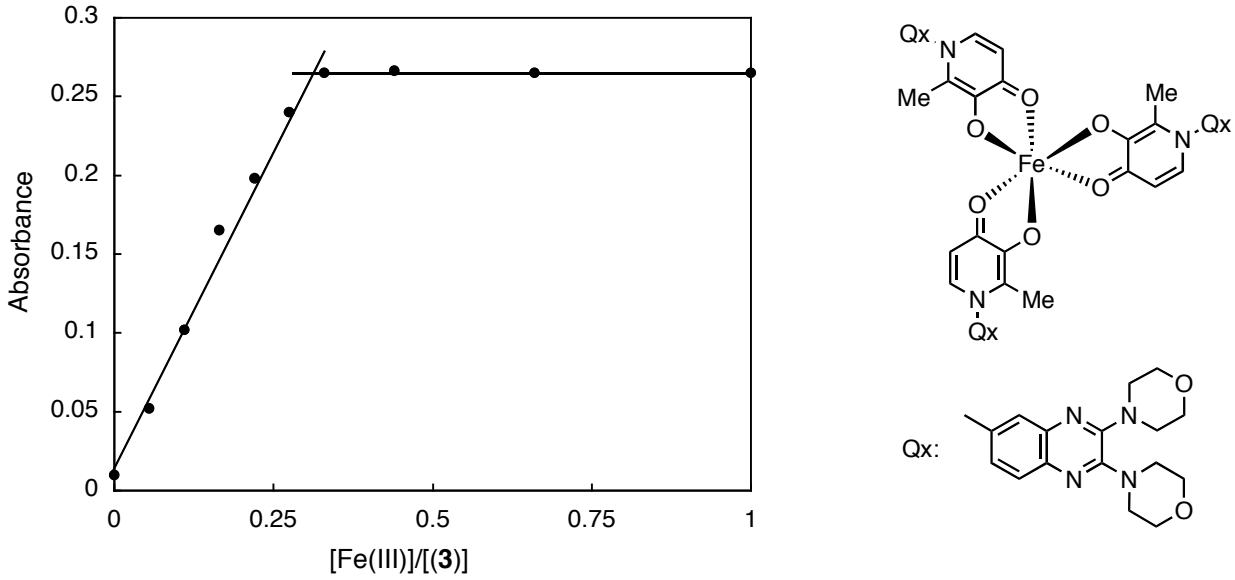


Figure 1 Plot of absorbance at 455 nm vs ratio of Fe(III) to ligand (**3**) in 50% aqueous DMF solution at an apparent pH 7; [(**3**)]=1x10^{-4} M.

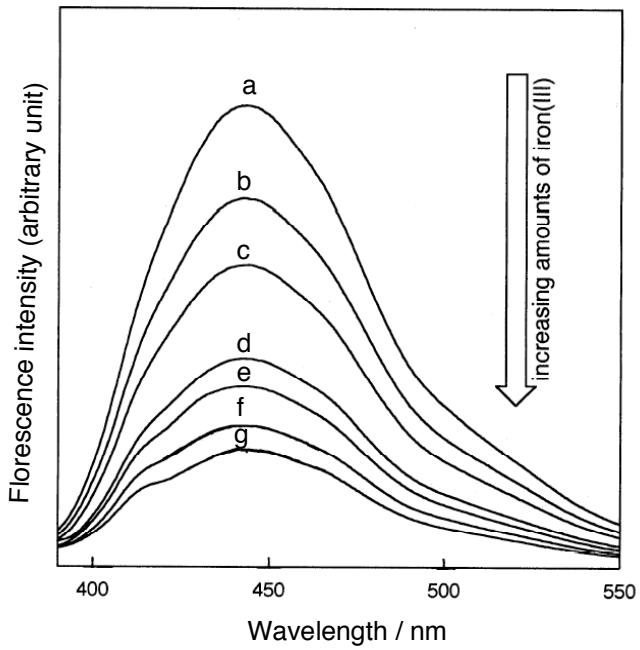


Figure 2 Fluorescence Fe(III) titration curves with ligand (**3**) in MeCN; [(**3**)]=1x10^{-5} M; [$Fe(ClO_4)_3 \cdot 6H_2O$]=0 for **a**, 0.06 for **b**, 0.11 for **c**, 0.17 for **d**, 0.22 for **e**, 0.28 for **f**, and 0.33 equiv for **g**.

No Change of the fluorescence intensity was observed when $Fe(ClO_4)_3 \cdot 6H_2O$ was added to a solution of compound (**2**) in MeCN. On the other hand, the fluorescence of compound (**3**) was efficiently quenched by gradual addition of $Fe(ClO_4)_3 \cdot 6H_2O$ in MeCN, the fluorescence intensity being remarkably decreased. (Figure 2) The observed fluorescence quenching, ϕ_q/ϕ_0, of compound (**3**) was not linear with the quencher Fe(III) concentration, thus failed to obey the Stern-Volmer model of dynamic quenching. (Figure 3-**A**) On the contrary, plots of $\ln(\phi_q/\phi_0)$ vs [Fe(III)] gave a straight line, suggesting that the quenching proceeds *via* the Perrin model of static quenching. (Figure 3-**B**)

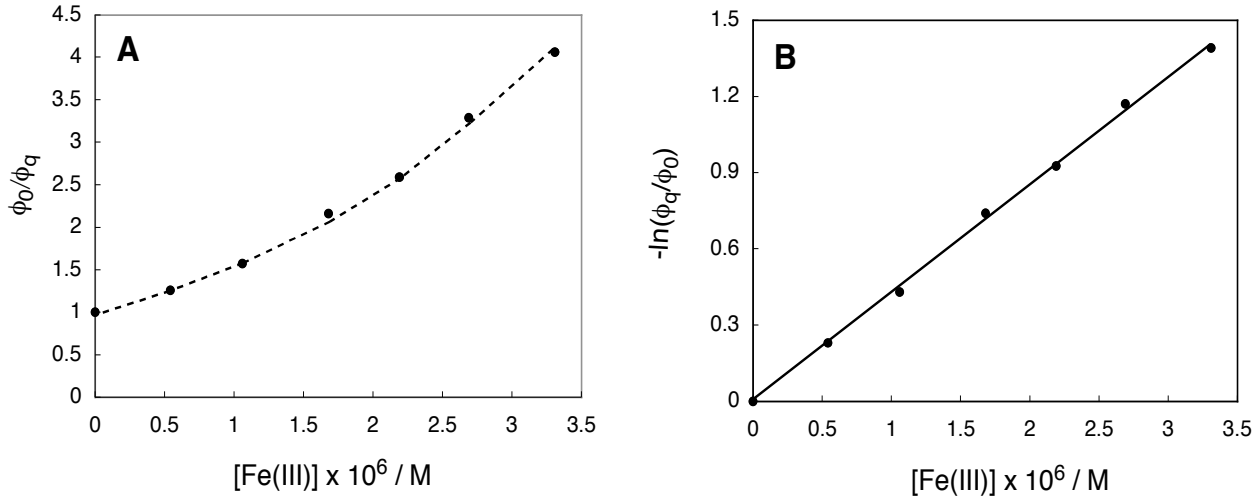
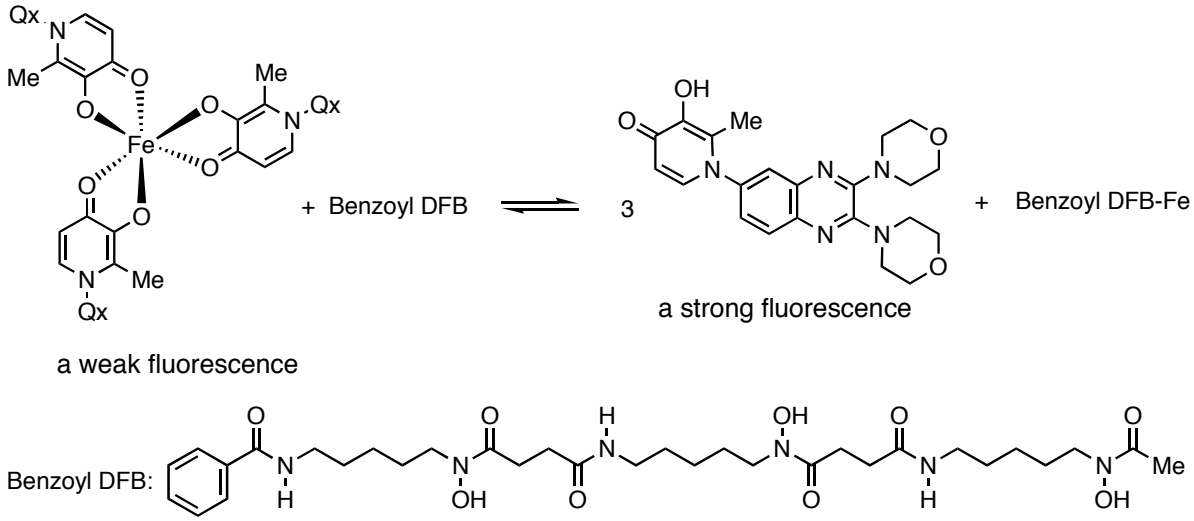


Figure 3 Plots for dynamic quenching (**A**) and static quenching (**B**) of ligand (**3**) by Fe(III). The conditions were as described in the legend of Figure 2.

The fluorescence-quenching efficiency of compound (**3**) by Fe(III) was calculated to be $k'=4.2 \times 10^5$ M^{-1}. This value was comparable or 4 times larger than the reported ones,[10] reflecting the effect of the proximity of the fluorophore moiety to the Fe(III)-coordination moiety by the direct linkage of ligand and fluorophore on the quenching process. Further, the quenching behaviors of Al(III), Ga(III), and Cr(III) complexes were also examined. Similarly, the quenching of these three complexes obeyed the Perrin model of static quenching. The magnitude of the quenching efficiency falled in order of Fe (4.2×10^5 M^{-1}) >> Al (2.1×10^5 M^{-1}) > Ga (1.3×10^5 M^{-1}) > Cr (0.6×10^5 M^{-1}).

The experiment on recovery of fluorescence by removal of Fe(III) with the *N*-benzoyl analogue (Benzoyl DFB) of a naturally occurring siderophore, desferrioxamine B (DFB), was carried out by monitoring the fluorescence intensity at 445 nm at appropriate intervals. The fluorescence apparently became strong with time, and the fluorescence intensity of the bidentate ligand (**3**) was recoverd to 67% after 20 h. (Scheme 3)



Scheme 3 Recovery of the fluorescence by removal of Fe(III) with benzoyl DFB

EXPERIMENTAL

Melting points were measured on a Mel-Temp apparatus in open capillaries and are uncorrected. IR, UV-VIS, and fluorescence spectra were recorded on JASCO FT/IR-230, Ubest V-550, and FP-6500 spectrophotometers, respectively. ^{1}H-NMR spectra were recorded on a JEOL JNM-LA400D NMR spectrometer in $CDCl_3$, and are reported in ppm (δ) downfield from internal Me_4Si. Thin layer chromatographic (TLC) analysis was performed on silica gel 60F-254 (Merck Co.) with a 0.2 mm layer thickness. Combustion analysis was performed on a Perkin Elmer series II CHNS/O analyzer 2400. 2,3-Dimorpholino-6-aminoquinoxaline (**1**)[12] and Benzoyl DFB[13] were prepared according to the literature methods.

3-Benzyloxy-2-methyl-1-(2,3-dimorpholinoquinoxalin-6-yl)pyridin-4(1*H*)-one (**2**)

A solution of compound (**1**) (380 mg, 1.21 mmol) and 3-benzyloxy-2-methyl-4-pyrone (171 mg, 0.8 mmol) in diluted HCl (6M HCl:H_2O=3:100; 3.3 mL) and MeOH (0.7 mL) was refluxed for 3 d. After evaporation of the solvents, to the residue was added 5% NaOH (10 mL) and the mixture was extracted with $CHCl_3$ (2x100 mL). The organic layer was washed with H_2O (2x30 mL), saturated NaCl (30 mL), and then dried over anhydrous Na_2SO_4. After evaporation of the solvent, the resudue was purified by column chromatogaraphy on silica gel with $CHCl_3$:acetone:EtOH (100:20:4) mixture, gel chromatography on Sephadex LH 20 with MeOH, and recrystallization from $CHCl_3$-hexane mixture to give the product (**2**) (143 mg, 35%) as white powders; mp: 140-145 °C; IR (KBr): 1622, 748, and 706 cm^{-1}; ^{1}H NMR (δ, $CDCl_3$, 400 MHz): 1.89 (3H, s, CH_3), 3.60-3.66 (8H, m, 2x-CH_2-N-CH_2-), 3.84-3.90 (8H, m, 2x-CH_2-O-CH_2-), 5.28 (2H, s, -CH_2Ph), 6.51 (1H, d, *J*=8 Hz, pyridone C5-H), 7.17 (1H, dd, *J*=2 and 9

Hz, quinoxaline C7-H), 7.34 (1H, d, *J*=8 Hz, pyridone C6-H), 7.27-7.47 (5H, m, Ph), 7.56 (1H, d, *J*=2 Hz, quinoxaline C5-H), and 7.78 ppm (1H, d, *J*=9 Hz, quinoxaline C8-H). *Anal*. Calcd for $C_{29}H_{31}N_5O_4 \cdot 0.8 H_2O$: C, 65.97; H, 6.22; N, 13.26. Found: C. 65.91; H, 6.43; N, 13.13.

3-Hydroxy-2-methyl-1-(2,3-dimorpholinoquinoxalin-6-yl)pyridin-4(1*H*)-one (3)

A suspension of 10% Pd-C (15 mg) in MeOH (20 mL) was prehydrogenated with H_2 for 30 min. To the suspension was added a solution of compound (**2**) (100 mg, 0.19 mmol) in MeOH (30 mL). After hydrogenation with H_2 under atmospheric pressure for 22 h, the catalyst was removed by filtration. The filtrate was concentrated to dryness, and the crude product was recrystallized from EtOH to give the pure product (**3**) (53 mg, 64%) as pale yellow powders; mp: 283-286 °C; IR (KBr): 3292 and 1622 cm^{-1}: ^1H NMR (δ, $CDCl_3$, 400 MHz): 2.17 (3H, s, CH_3), 3.62-3.67 (8H, m, -CH_2-N-CH_2-), 3.86-3.91 (8H, m, -CH_2-O-CH_2-), 6.52 (1H, d, *J*=8 Hz, pyridone C5-H), 7.27 (1H, dd, *J*=2 and 9 Hz, quinoxaline C7-H), 7.39 (1H, d, *J*= 8 Hz, pyridone C6-H), 7.63 (1H, d, *J*=2 Hz, quinoxaline C5-H), and 7.81 ppm (1H, d, *J*=9 Hz, quinoxaline C8-H). *Anal*. Calcd for $C_{22}H_{25}N_5O_4 \cdot 0.3H_2O$; C, 61.61; H, 6.02; N, 16.33. Found: C, 61.91; H, 6.21; N, 16.09.

Mole Ratio Determination of Fe(III) Complex

To each solution (1 mM, 0.5 mL) in DMF was added an appropriate amount of aqueous $Fe(NO_3)_3$ solution (1 mM). Deionized H_2O was added to the mixture to make a 50% aqueous DMF solution. The pH of the solution was adjusted to an apparent pH 7 by adding 0.01 or 0.1 M KOH. The resulting solution was diluted to a volume of 5.0 mL with 50% aqueous DMF. After 12 h the visible spectrum of the solution was measured at rt.

Gallium Complex Formation

Compound (**3**) (10 mg, 2.3×10^{-5} M) and $Ga(ClO_4)_3 \cdot 8H_2O$ (2.9 mg, 6.1×10^{-6} M) were dissolved in $CDCl_3$ (0.5 mL) , and the ^1H NMR spectrum was measured at rt; δ 2.33 (3H, s, CH_3), 3.61-3.68 (8H, m, 2x-CH_2-N-CH_2-), 3.83-3.90 (8H, m, 2x-CH_2-O-CH_2-), 7.20 (1H, br s, pyridone C5-H), 7.34 (1H, dd, *J*=2 and 9 Hz, quinoxaline C7-H), 7.77 (1H, br s, pyridone C6-H), 7.66 (1H, d, *J*=2 Hz, quinoxaline C5-H), and 7.84 ppm (1H, d, *J*=9 Hz, quinoxaline C8-H).

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