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Iron is an essential element for the vital activity of all living organisms on Earth. However, most organisms suffer from iron-deficiency stress because most iron exists as poorly water-soluble trivalent iron salts in the earth’s current environment under conditions of high-oxygen partial pressure. To acquire insoluble iron efficiently, some kinds of microorganisms have developed an original strategy characterized by the synthesis and secretion of siderophores as an efficient iron chelator and by the specific uptake of the resulting iron(III) complex.\textsuperscript{1} Not surprisingly, siderophores have been a subject of intensive research due to not only their physiological importance but also their interesting chemical properties, such as a high affinity for metal ions and a noteworthy structural change after complexation.\textsuperscript{2} Enterobactin (1) is a well-recognized siderophore produced by various Gram-negative bacteria, and its affinity for ferric ion is one of the highest among known siderophores (Figure 1).\textsuperscript{3} Thus, the development of enterobactin analogs as functionalized molecules has been strenuously studied, and their application to the detection of bacteria\textsuperscript{4} and the enhancement of antibacterial agent activity\textsuperscript{5} was recently reported. We also focused on the high iron affinity of enterobactin and planned to apply it to an iron sensor. Herein, we report a new fluorescent probe based on enterobactin for the detection of iron ion.

There have been several examples of iron ion sensors by the assembly of siderophores and fluorescent organic molecules.\textsuperscript{6} For example, Su et al. developed a highly selective iron ion sensor named—FIDFO, which consists of fluorescein and desferioxamine.\textsuperscript{6a} Thus, we expected that the combination of enterobactin and fluorescence organic molecules might form an efficient iron sensor due to enterobactin’s highest affinity for iron ion relative to other siderophores.

![Figure 1. Molecular design of iron sensor.](image-url)
Tetrahedron

On the other hand, fluorescent organic molecules connecting to enterobactin adopted the 1,3a,6a-triazapentalene (TAP, 2) as a compact fluorophore that does not disturb iron complexation or uptake by bacteria. We recently discovered that the 1,3a,6a-triazapentalene skeleton without an additional fused ring system is a compact and highly fluorescent chromophore, and that it exhibits various useful fluorescence properties such as a large Stokes shift and a positive fluorescence solvatochromism. Since the initial disclosure of the fluorescence system of TAPs, we have strenuously studied the application development of TAPs and other groups also reported the alternative synthetic method of highly fluorescent TAPs. Interestingly, the fluorescence wavelength and intensity of 1,3a,6a-triazapentalenes vary widely with the electron density of 2-substituents. Based on this fluorescence property, we designed the TAP-enterobactin (3), which possesses a TAP system on the catechol rings of enterobactin. The coordination of catechol to the iron ion should induce a change in the electron state of the phenyl ring as the 2-substituent of TAP, and this would mean a simultaneous change in the fluorescence of TAP. In addition, the structural change in TAP-enterobactin (3) induced by complexation is also expected to change the fluorescence of TAP (Figure 1).

We first tried to synthesize a catechol moiety possessing a TAP system, and began this attempt with iodovanillin (4). The hydroxyl groups of 4 were protected by the SEM group after demethylation to give 5. The CO insertion reaction of 5 in methanol proceeded smoothly to afford methyl ester 6 in 79% yield, although the neighboring SEM group was removed. Thus, the free hydroxyl group of 6 was protected by the SEM group again to give bis-SEM-protected 7, which was treated with diazophosphonate (8) to afford alkyne 9 in 83% yield. Finally, the method of synthesizing 1,3a,6a-triazapentalenes developed by our group was applied to alkyne 9, and the reaction of 9 with azidotridiflate (10) in the presence of a catalytic amount of copper(I) salt proceeded smoothly to give the desired TAP-catechol derivative (11) in 80% yield (Scheme 1).

Scheme 1. Synthesis of TAP-catechol unit (11).

Having prepared TAP-labeled catechol derivative (11), condensation with the serine trimer (13) was the next examination. After the hydrolysis of the methyl ester of 11 by treatment with 1.0 equiv of lithium hydroxide at 85 °C followed by the evaporation of the solvents, the resulting lithium salt (12) was directly treated with the serine trimer (13) in the presence of DMT-MM (14) as a condensing reagent, which is available in both alcohol and water solvents, as reported by Kunishima. However, the desired product (15) was not obtained, and neither were various other conditions. Thus, we next tried to construct TAP rings after the condensation of catechol-alkyne with serine trimer (13). The methyl ester of alkyne (9) was hydrolyzed under similar conditions to give lithium salt (14). After the solvents were removed, the condensation of the resulting (14) with (13) by the combined action of DMT-MM and NMM·HCl in methanol proceeded to afford the desired condensing product in 56% yield. The subsequent TAP-forming reaction of the resulting alkyne proceeded, and we observed the formation of the desired (15) by a direct 1H NMR measurement of the reaction mixture after evaporation. However, the isolation of (15) was very difficult due to its poor stability under the conditions of workup and silica gel column chromatography. Thus, we should increase the stability of the TAP system on the catechol ring.

Scheme 2. Examination of synthesis of (15).

The TAP ring system possessing electron-donating substituents at the C2-position is basically stable, whereas the electron-donating derivatives readily decompose under acidic and oxidative conditions due to the high nucleophilicity at the C3-position of TAP. Although the catechol ring system of (15) probably decreased the stability of TAP, it would be difficult to further modify the catechol moiety to serve as an iron chelator. Thus, we next planned to introduce the substituents at the C3-position of TAP as a protecting group. Since Shi et al. also reported that the electron-donating groups at the C3-position increase the stability of 1,3a,6a-triazapentalenes, we attempted to introduce an electron-donating group. After comparing several substituents at the C3-position of TAP (See Supporting Information Table S1), we adopted an acetyl group as the best substituent. Acetylation at the C3-position of TAP was achieved by treating TAP (11) with acetic anhydride and triethylamine by reference to Hirobe’s procedure. The acetylated (16) was hydrolyzed to lithium salt, and subsequent condensation using DMT-MM with serine trimer (13) in methanol proceeded to give the desired condensing product in 62% isolated yield. The
acetylated TAP analog was stable enough for purification. Finally, the SEM groups were deprotected by treatment with anhydrous hydrogen chloride in 2-propanol to obtain the desired 17 in quantitative yield (Scheme 3).


With TAP-labeled enterobactin 17 in hand, we investigated the fluorescence change that occurs with the addition of iron(III). To a solution of 17 in DMSO were added various amounts of Fe(acac)$_3$ as an Fe$^{3+}$ ion source, and the mixtures were excited at 350 nm. The fluorescence maximum of 17 at 525 nm in DMSO did not shift with the addition of Fe(acac)$_3$, whereas the fluorescence intensity of 17 sensitively responded to the Fe$^{3+}$ ion. The fluorescence intensity decreased gradually according to the amount of Fe$^{3+}$ ion, and the fluorescence of 17 completely disappeared when 1.2 equiv of Fe(acac)$_3$ was added (Figure 2). The formation of a 1 : 1 complex of 17 with Fe$^{3+}$ ion was confirmed by HRMS spectroscopy. Similar behaviors of fluorescence intensity were also observed in DMF and tert-butanol. On the other hand, since 17 did not dissolve in water, its fluorescence behavior in water was not examined.

The metals other than iron did not show the complete quenching of the fluorescence of 17 even when 5.0 equiv of metal ions was added, whereas 1.2 equiv of Fe$^{3+}$ ion was enough to quench fluorescence completely. These differences were also observed by the naked eye. Figure 4 shows the fluorescent emissions in the presence of 5.0 equiv of metal ions in 1.0 x 10$^{-6}$ M DMSO solution. The fluorescence of 17 with Li$^+$ and Al$^{3+}$ ions were sufficiently observed with the results similar to the fluorescence spectral measurement. The other metals, such as Ca$^{2+}$, V$^{3+}$, Cu$^{2+}$, and Ni$^{2+}$, also showed visible fluorescence. Although the fluorescence with Ni$^{2+}$ ion was very weak, we were able to carefully observe the fluorescence. In clear contrast, the addition of only 1.2 equiv of Fe$^{3+}$ ion showed no fluorescence. Therefore, TAP-labeled enterobactin 17 was confirmed to be a highly selective and sensitive fluorescence-quenching sensor of Fe$^{3+}$ ion that was effective in 10$^{-6}$M solutions.
Tetrahedron

In conclusion, we developed a novel iron ion sensor 17 by combining enterobactin as a siderophore and TAP as a fluorescent organic molecule. Although the synthesis of TAP-labeled enterobactin was initially difficult due to the instability of TAP on the catechol ring, we later found that the 3-acetylated TAP derivative could be introduced to the catechol ring of enterobactin. The synthetic TAP-labeled enterobactin 17 was proven to be a highly sensitive and iron-specific quenching probe. Since the iron-specific fluorescence quenching mechanism could not be explained by simple photoinduced electron transfer (PET) mechanism, the mechanistic details remain to be seen. The computational studies are currently underway in our laboratory. To prompt the expansion of 17 to biological studies, such as an iron concentration measurement inside cells, the next subjects of study are how to increase fluorescence intensity and how to improve solubility in water.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://

References and notes

15. Since 17 was not stable under strongly acidic conditions, Fe(acac)2 was used for the complexation under neutral condition.
16. The solubility of 17 in ethyl acetate, diethyl ether, dichloromethane, and water is poor, whereas 17 readily dissolved in DMSO, DMF, t-BuOH and i-BuOH. The case of methanol needed diluted condition.