A suitable stereoisomer of vibrioferrin probes for iron uptake of *Vibrio parahaemolyticus*

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Suitable Stereostructures of vibrioferrin probe for iron uptake of *Vibrio parahaemolyticus* was revealed. Stereoisomers of dansyl labeled vibrioferrin at the 2''-position were synthesized and their uptake activities were evaluated. *Vibrio parahaemolyticus* take in both isomers at the 2''-position. In addition to *Vibrio parahaemolyticus*, several bacteria have also taken up the (*R*)-isomer.

Keywords: vibrioferrin, siderophore, fluorescent probe

Iron is an essential element for all creatures on the earth. However, iron is mainly existing as water insoluble trivalent iron salts under the current global environment of high oxygen partial pressure, and many lives suffer from iron deficiency.[1] To acquire trivalent iron efficiently, poaceous plants and many bacteria have developed unique strategy characterized by the secretion of efficient iron chelators called siderophore and take in iron as its complex through the specific transporters.[2] Mugineic acid (MA) was the first identified phytosiderophore from the roots of barley in 1972 by Takagi[3] (Figure 1), and we succeeded in development of the fluorescent probes of MA that could be visualized the state being incorporated into the cells overexpressing MA•Fe(III) complex transporter in 2010.[4] On the other hand, vibrioferrin (VF) was initially isolated as a siderophore of *Vibrio parahaemolyticus*, a type of halophilic Gram-negative bacillus, by Yamamoto in 1992 (Figure 1).[5] The same group later revealed the structure of VF by chemical degradation analysis in 1994, demonstrating that the C2''' position is epimeric mixture and the configuration at the C2'' position is *S*.[6] In addition, the chemical synthesis of VF by Takeuchi et al. revealed that the configuration at the C2 position is *R* and its epimer is inactive.[7] However, the effect of the configuration at the C2'' position for iron uptake has been still unclear. In the case of phytosiderophore MA, the epimer at the C2' position also showed similar iron transport activity to the natural MA, and the fluorescent probe that connected the fluorophore via the hydroxy group of the C2' epimer visualized cellular uptake.[4] Recently, Wang's group reported an excellent fluorescent probe of VF for selective targeting of vibrios, in which the fluorophore was linked from the methyl group at the C2'' position.[8] Based on our experience of fluorescent siderophores,[4,9] we considered that the epimer at the C2'' position of VF would be also suitable stereoisomer for introducing various labelling groups.

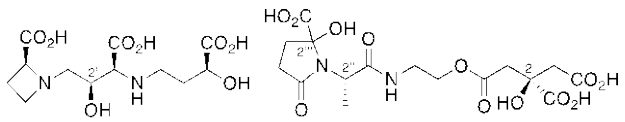


Figure 1. Structure of mugineic acid (MA) and vibrioferrin (VF)

First, we analyzed the conformation of iron complex of VF by DFT calculation (Figure 2). The iron complexes of possible epimers at C2''' and C2'' positions were optimized. The DFT analysis indicated that the epimer (*R* configuration) at the C2'' position, with *R* configuration at the C2''' position, was the most stable structure (Complex A) among four stereoisomers (Complexes A~D), suggesting that the introduction of functional molecules such as fluorophores to the methyl moiety of the epimer at the C2" position likely provide a more stable iron complex than that of natural form (Table 1). On the other hand, since bacteria recognize their siderophore-iron complexes, the uptake activity cannot be discussed solely by the stability of the complexes. Therefore, to elucidate the suitable stereoisomer of VF for probe development, we attempted to synthesize the fluorescent probes of both stereoisomers of VF and evaluate their incorporation into bacteria.

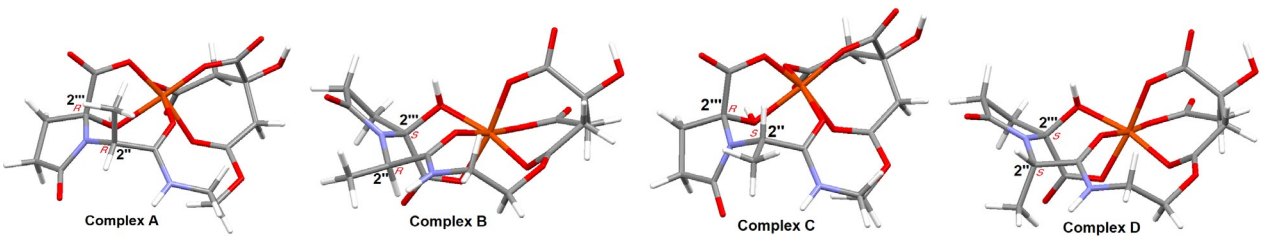


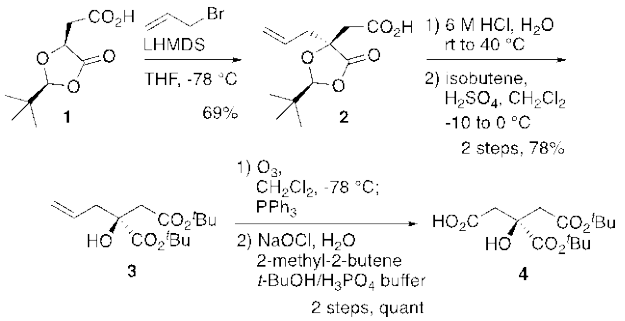
Figure 2. Optimized conformations of the iron complexes of VF and its epimer at the C2" position.

Vib.Fe(III)complex	Complex A (*R*,*R*)	Complex B (*S*,*R*)	Complex C (*R*,*S*)	Complex D (*S*,*S*)

E	-1756.971694	-1756.956869	-1756.967251	-1756.962554
ΔE (kcal/mol)	0	9.3	2.8	5.7

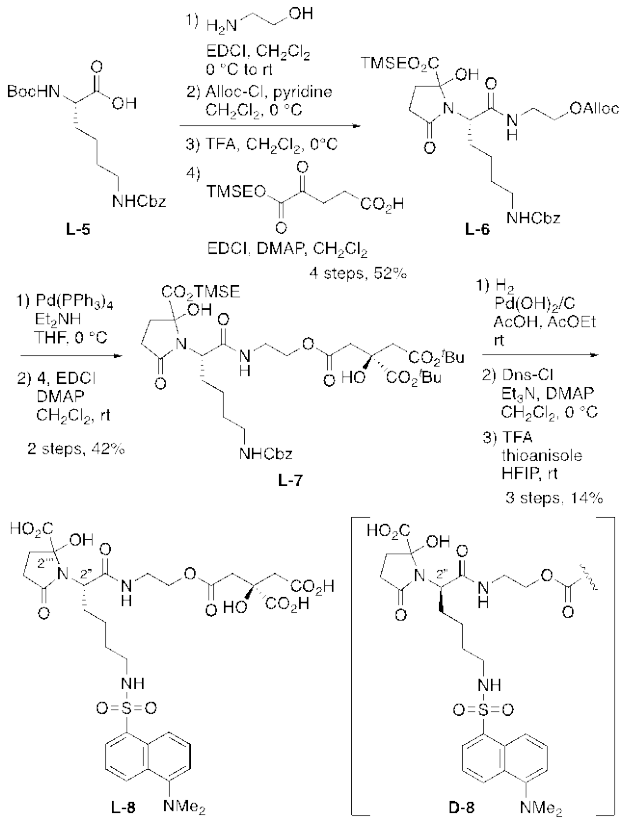
Table 1. Optimized conformations of the iron complexes of VF and their relative energies

Although optically pure citric acid fragments of VF have been obtained by optical resolution in the previous synthesis of VF and its analogs, we adopted the asymmetric synthesis this time because the epimer at the C2 position was known to be inactive. The chiral allyl dioxolanone **2** was obtained by the asymmetric allylation reaction of **1** derived from L-malic acid.[10,11] Removal of acetal followed by *tert*-butyl esters formation afforded the masked citric acid **3**. Finally, oxidative cleavage of the terminal olefin and subsequent Pinnick oxidation[12] of the resulting aldehyde afforded the desired desymmetrized citric acid **4** (Scheme 1).



Scheme 1. Asymmetric synthesis of the citric acid fragment **4**.

With reference to the Wang's synthesis,[8] we synthesized a protected VF analog **7**, in which the L-alanine fragment of VF was replaced with L-lysine. Starting with protected L-lysine **L-5**, sequential operation of a condensation with aminoethanol, a protection with alloc group, removal of Boc group, and a condensation with ketoglutaric acid analog afforded **L-6** in 52% yield from **L-5**. Next, removal of alloc group followed by a condensation with desymmetrized citric acid **4** gave the desired **L-7**. While Wang's group introduced a fluorescein by the formation of propargyl carbamate with the primary amine in the lysine moiety and subsequent click reaction[13], we tried to directly introduce organic fluorophores because each fragment possessed different protecting groups. As the organic fluorophore, a dansyl group was adopted due to its good stability and fluorescence intensity. After hydrogenation of **L-7**, the resulting free amine was condensed with dansyl chloride followed by treatment with TFA for removal of TMSE and *tert*-butyl groups to give dansyl labeled VF **L-8** as a 1:1 diastereomeric mixture at the C2''' position (Scheme 2). To elucidate the effect of the C2'' position, we similarly synthesized the **D-8** as the epimer at the C2'' position from D-lysine (Scheme 2). Although diastereomers at the C2''' position could be separated by PTLC before the deprotection leading to **D-8**, the separated diastereomers were readily epimerized back to diastereomeric mixtures.



Scheme 2. Synthesis of dansyl-VF analogs.

Having prepared fluorescent labeled **VF** analogs, the uptake of these compounds into cells was evaluated (Figure 3). To 100 μL of a solution of *Vibrio parahemolyticus* strain in culture medium was added 2 μL of a solution of iron complex of **L-8**, which was prepared by treatment of **L-8** with 1.0 equiv of $FeCl_3$•$6H_2O$ in H_2O (30 mM). The mixture was incubated at room temperature for 5 min. Then, 10 μL of the mixture was dropped on a glass slide and fluorescently observed by confocal laser microscope. *Vibrio parahaemolyticus* treated with dansyl labeled **L-8** showed fluorescence along the bacterial shape (Figures 3a-b), and it means that **L-8** was recognized as a siderophore and incorporated into *Vibrio parahaemolyticus*. As reported by Wang, the introduction of a fluorophore from the methyl group at the C2'' position was confirmed to be effective. Next, we investigated the effect of the stereocenter at the C2" position by using **D-8**. The bacteria treated with the iron complex of **D-8** also showed fluorescence similar to that of **L-8** (Figures 3c-d). Therefore, both stereoisomers at the C2'' position were found to be incorporated into bacteria as the DFT analysis suggested. Furthermore, treatment of only **D-8** without iron source did not show fluorescence from bacteria (Figures 3e-f), suggesting that the bacteria recognized the iron complex of **D-8**, i.e., stereoisomer at the

C2” position was also recognized as their siderophore. Moreover, treatment of only methanol and saline did not show fluorescence, indicating that the fluorescence in bacteria was not from cell death or autofluorescence (Figures 3g and 3h).

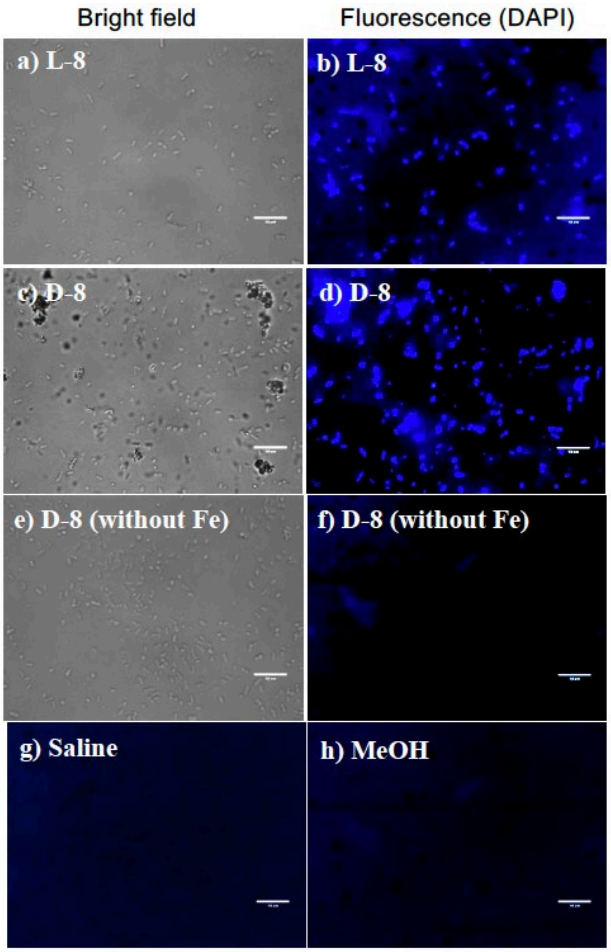


Figure 3. Evaluation of labelled VF analogs for uptake of *Vibrio parahaemolyticus*. Living bacteria solution (100 μL) in culture medium was treated with 2 μL of various solutions such as 0.03 M solution of Fe(III)•**L-8** complex in H_2O (**a**: bright field, **b**: fluorescence), 0.028 M solution of Fe(III)•**D-8** complex in MeOH (**c**: bright field, **d**: fluorescence), 0.028 M solution of only **D-8** in MeOH (**e**: bright field, **f**: fluorescence), Saline (**g**: fluorescence) and MeOH (**h**: fluorescence). The uptake of labelled VF analogs was monitored by confocal microscopy (EVOS FL; Thermo) in a fluorescence image with excitation and emission wavelengths of 360 and 447 nm, respectively (**b**, **d**, **f, g**, **h**). Scale bar = 10 μm.

Next, we investigated whether the epimeric analog **D-8** could be taken up by other bacteria as well as *Vibrio parahaemolyticus* (Figure 4). Various bacteria were treated with Fe(III)•**D-8** complex under the similar condition and observed by the confocal laser microscope. Interestingly, while fluorescence was observed in *Staphylococcus aureus* and *Porphyromonas gingivalis* (Figures 4a-d), *E. coli* and *Pseudomonas aeruginosa* did not show fluorescence (Figures 4e-h), demonstrating that there are bacteria that take in Fe(III)•**D-8** and bacteria that do not. In particular, despite *P. gingivalis* is known not to produce siderophores,[14] it strongly incorporated Fe(III)•**D-8**. The mechanistic elucidation of these bacterial selectivity is the next subject.

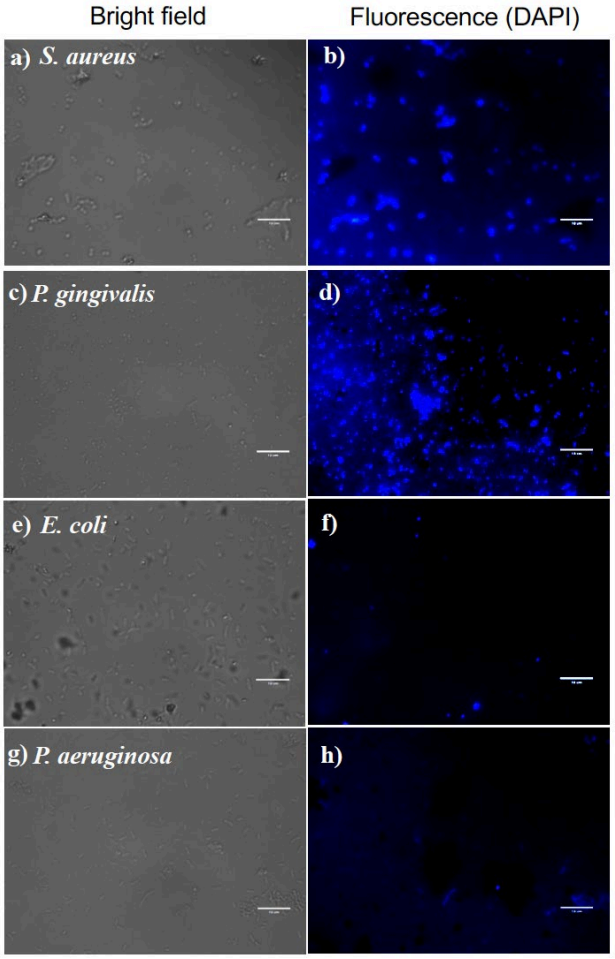


Figure 4. Evaluation of labelled VF analogs for uptake of bacteria other than *Vibrio parahaemolyticus*. 2 μL of 0.03 M solution of Fe(III)•**D-8** complex in H_2O were applied to various living bacteria solutions (100 μL) including *Staphylococcus aureus* (**a**: bright field, **b**: fluorescence), *Porphyromonas gingivalis* (**c**: bright field, **d**: fluorescence), *E. coli* (**e**: bright field, **f**: fluorescence), and *Pseudomonas aeruginosa* (**g**: bright field, **h**: fluorescence). The uptake of Fe(III)•**D-8** was monitored by confocal microscopy (EVOS FL; Thermo) in a fluorescence image with excitation and emission wavelengths of 360 and 447 nm, respectively (**b**, **d**, **f, h**). Scale bar = 10 μm.

In conclusion, we demonstrated that the epimer at the C2’’ position is also useful VF-based probe. We established the asymmetric synthesis of citric acid fragment **4** and applied it to the synthesis of VF analogs, in which L-alanine fragment was replaced by L-lysine and D-lysine. The dansyl group as the fluorophore was directly introduced to the primary amine moiety in lysine fragment, and subsequent global deprotection afforded the fluorescent probe of VF as the diastereomeric mixture at the C2’’’ position. Both analogs **L-8** and **D-8** derived from L-lysine and D-lysine

respectively were clearly showed fluorescence in bacteria. Indeed, application of **D-8** was able to fluorescently visualize the movement of bacteria *vibrio parahaemolyticus*. This study revealed that the D-lysine analog of VF that corresponds to the epimer at the C2'' position is also suitable probe for the mechanistic investigation of VF in the future.

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Supporting Information is available on http://dx.doi.org/10.1246/cl.******.

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