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# Ferroverdins, Inhibitors of Cholesteryl Ester Transfer Protein Produced by *Streptomyces* sp. WK-5344

## II. Structure Elucidation

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The structures of ferroverdins B and C, novel inhibitors of cholesteryl ester transfer protein, were elucidated by spectroscopic studies including various NMR measurements. They are the complex between one $Fe^{2+}$ and three ligands, that is, two common *p*-vinylphenyl-3-nitroso-4-hydroxybenzoates and one hydroxy *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate for ferroverdin B and one carboxylic acid *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate for ferroverdin C.

Ferroverdins B and C along with known ferroverdin[1,2] (ferroverdin A in this paper) were isolated from the culture broth of *Streptomyces* sp. WK-5344 as inhibitors of cholesteryl ester transfer protein (CETP). As described in the preceding paper[3], ferroverdin B was found to show potent inhibition against CETP activity with an $IC_{50}$ value of 0.62 $\mu$M[3]. Ferroverdins are structurally related, and ferroverdin A, originally reported as a green pigment[1,2], is a complex of one iron and three nitrosophenyl ligands[2]. In this paper, we report the structure elucidation of ferroverdins B and C.

## Materials and Methods

### Materials

Ferroverdins were isolated from the culture broth of *Streptomyces* sp. WK-5344 as described in the preceding paper[3].

### General Experimental Procedures

UV spectra were recorded on a Shimadzu UV-200S spectrophotometer. IR spectra were recorded on a Horiba FT-210 infrared spectrometer. Optical rotations were obtained with a JASCO DIP-370 digital polarimeter. EI-MS spectra were recorded on a JEOL JMS-D 100 mass spectrometer at 20 eV. FAB-MS spectra were recorded on a JMS-DX300 mass spectrometer. The various NMR spectra were obtained on a Varian XL-400 spectrometer.

## Results

### Physico-chemical Properties of Ferroverdins

Physico-chemical properties of ferroverdins A, B and C are summarized in Table 1. They were all obtained as green powders, and the UV spectra showed the maxima at 285 ($\varepsilon$ 44,000), 305 sh ($\varepsilon$ 24,000), 440 ($\varepsilon$ 6,000) and 690 nm ($\varepsilon$ 5,300). These physico-chemical properties indicated that ferroverdins B and C are iron complexes of *p*-substituted *o*-nitrosobenzoates as well as ferroverdin A[1,2].

### Structure of Ferroverdin B

The molecular formula of ferroverdin B was determined to be $C_{45}H_{30}N_3O_{13}Fe$ on the basis of HRFAB-MS measurement (*m/z*, found 877.1203, calcd 877.1206 for $C_{45}H_{31}N_3O_{13}Fe$ $[M+H]^+$). The $^{13}C$ NMR spectrum ($CD_3OD$) (Table 2) obtained 45 carbons which were classified into two $sp^2$ methylene, twenty five $sp^2$ methine

Table 1. Physico-chemical properties of ferroverdins A, B and C.

| | Ferroverdin A | Ferroverdin B | Ferroverdin C |
|---|---|---|---|
| Appearance | Green powder | Green powder | Green powder |
| Molecular formula | $C_{45}H_{30}N_3O_{12}Fe$ | $C_{45}H_{30}N_3O_{13}Fe$ | $C_{46}H_{30}N_3O_{14}Fe$ |
| Molecular weight | 860 | 876 | 904 |
| FAB-MS (*m/z*) | | | |
| Negative | 860 $[M]^-$ | 876 $[M]^-$ | 904 $[M]^-$ |
| | 592 | 608 | 636 |
| | 268 | 592 | 592 |
| | | 284 | 312 |
| | | 268 | 268 |
| Positive | 861 $[M+H]^+$ | 877 $[M+H]^+$ | 905 $[M+H]^+$ |
| | 883 $[M+Na]^+$ | 899 $[M+Na]^+$ | 927 $[M+Na]^+$ |
| HRFAB-MS (*m/z*) (positive) | | | |
| | $C_{45}H_{31}N_3O_{12}Fe$ | $C_{45}H_{31}N_3O_{13}Fe$ | $C_{46}H_{31}N_3O_{14}Fe$ |
| Calcd: | 861.1257 | 877.1206 | 905.1155 |
| Found: | 861.1258 | 877.1203 | 905.1151 |
| UV $\lambda_{max}^{CH_3OH}$ nm (ε) | 285 (44,000) | 285 (44,000) | 285 (44,000) |
| | 305 sh (24,000) | 305 sh (24,000) | 305 sh (24,000) |
| | 440 (6,000) | 440 (6,000) | 440 (6,000) |
| | 690 (5,300) | 690 (5,300) | 690 (5,300) |
| IR $\nu_{max}^{KBr}$ ($cm^{-1}$) | 3400, 1728, 1597, | 3400, 1728, 1701, | 3400, 1718, 1701, |
| | 1506, 1431, 1385, | 1597, 1493, 1385, | 1597, 1508, 1385, |
| | 1279, 1207, 1101 | 1284, 1207, 1105 | 1281, 1196, 1105 |
| $[\alpha]_D^{25}$ (*c* 0.01, $CH_3OH$) | −414000° | −3000° | −1000° |
| Solubility | | | |
| Soluble: | $CH_3OH$, $CHCl_3$, $CH_3CN$, Acetone, $C_2H_5OH$, Ethyl acetate | $CH_3OH$, $CHCl_3$, $CH_3CN$, Acetone, $C_2H_5OH$, Ethyl acetate | $CH_3OH$, $CHCl_3$, $CH_3CN$, Acetone, $C_2H_5OH$, Ethyl acetate |
| Insoluble: | $H_2O$, *n*-Hexane | $H_2O$, *n*-Hexane | $H_2O$, *n*-Hexane |
| Color reaction | | | |
| Positive: | 50% $H_2SO_4$ | 50% $H_2SO_4$ | 50% $H_2SO_4$ |

and eighteen $sp^2$ quaternary carbons by analysis of the DEPT spectra. Some carbon signals overlapped, and they were assigned by the gated decoupling experiment and HMBC experiment. The $^1H$ NMR spectrum displayed 29 proton signals (Table 2). To fulfill the molecular formula of ferroverdin B, the presence of a hydroxyl group was suggested. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum.

Analyses of $^1H$-$^1H$ coupling observed in COSY spectrum and $^{13}C$-$^1H$ long-range couplings of $^2J$ and $^3J$ in the HMBC spectrum revealed the four partial structures as shown in Fig. 2. 1) $^1H$-$^1H$ coupling between H-5 ($\delta$ 7.20) and H-6 ($\delta$ 8.19) and the long-range couplings from H-2 ($\delta$ 7.906) to C-1 ($\delta$ 116.9), C-3 ($\delta$ 160.6), C-4 ($\delta$ 181.9), C-6 ($\delta$ 138.4) and C-7 ($\delta$ 166.5), from H-5 to C-1 and C-3, and from H-6 to C-2 ($\delta$ 114.7), C-4 and C-7 showed 1,3,4-trisubstituted benzene as the partial structure I. 2) $^1H$-$^1H$ coupling between H-9 ($\delta$ 7.17) and H-10 ($\delta$ 7.49) and between H-12 ($\delta$ 6.76) and $H_2$-13 ($\delta$ 5.24, 5.77) and the long-range couplings from H-9 to C-8 ($\delta$ 152.1), C-9 ($\delta$ 123.0) and C-11 ($\delta$ 136.8), from H-10 to C-8, C-9, C-10 ($\delta$ 128.2) and C-12 ($\delta$ 137.3), from H-12 to C-10 and C-11, and from $H_2$-13 to C-11 and C-12 showed the partial structure II of the *p*-vinylphenyl moiety.

Furthermore, the long-range coupling of $^4J$ were observed from H-9 to C-7, and chemical shifts of C-7 and C-8 supported the presence of an ester bond. Thus, the bigger partial structure III of the *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate moiety was suggested.

The NMR of the partial structure III′ was similar to that of the partial structure III, but the two carbon signals of C-12′ ($\delta$ 126.1) and C-13′ ($\delta$ 140.4) were different. The long-range couplings from H-9′ ($\delta$ 7.22) to C-8′ ($\delta$ 153.1), C-9′ ($\delta$ 122.7) and C-11′ ($\delta$ 134.9), from H-10′ ($\delta$ 7.60) to C-8′,

Table 2. $^{1}H$ and $^{13}C$ NMR chemical shifts of ferroverdins A, B and C.

| Carbon No. | Ferroverdin A | | | Ferroverdin B | | | Ferroverdin C | | |
|---|---|---|---|---|---|---|---|---|---|
| | $^{13}C$ chemical shifts ppm[a] | $^{1}H$ chemical shifts ppm[b] | | $^{13}C$ chemical shifts ppm[a] | $^{1}H$ chemical shifts ppm[b] | | $^{13}C$ chemical shifts ppm[a] | $^{1}H$ chemical shifts ppm[b] | |
| C-1 | 116.9 | | | 116.9 | | | 116.9 | | |
| C-2 | 114.7 | 7.90 | (3H, d, *J*=2.0 Hz) | 114.7 | 7.906 | (2H, d, *J*=2.0 Hz) | 114.7 | 7.90 | (2H, d, *J*=2.0 Hz) |
| C-3 | 160.6 | | | 160.6 | | | 160.6 | | |
| C-4 | 181.9 | | | 181.9 | | | 181.9 | | |
| C-5 | 122.7 | 7.20 | (3H, d, *J*=9.0 Hz) | 123.3 | 7.20 | (2H, d, *J*=9.0 Hz) | 122.7 | 7.19 | (2H, d, *J*=9.0 Hz) |
| C-6 | 138.3 | 8.20 | (3H, dd, *J*=9.0, 2.0 Hz) | 138.4 | 8.19 | (2H, dd, *J*=9.0, 2.0 Hz) | 138.3 | 8.18 | (2H, dd, *J*=9.0, 2.0 Hz) |
| C-7 | 166.5 | | | 166.5 | | | 166.5 | | |
| C-8 | 152.1 | | | 152.1 | | | 152.1 | | |
| C-9 | 123.0 | 7.10 | (6H, d, *J*=8.5 Hz) | 123.0 | 7.17 | (4H, d, *J*=8.5 Hz) | 123.0 | 7.16 | (4H, d, *J*=8.5 Hz) |
| C-10 | 128.2 | 7.50 | (6H, d, *J*=8.5 Hz) | 128.2 | 7.49 | (4H, d, *J*=8.5 Hz) | 128.2 | 7.48 | (4H, d, *J*=8.5 Hz) |
| C-11 | 136.8 | | | 136.8 | | | 137.2 | | |
| C-12 | 137.3 | 6.70 | (3H, dd, *J*=17.5, 11.0 Hz) | 137.3 | 6.76 | (2H, dd, *J*=17.5, 11.0 Hz) | 137.3 | 6.75 | (2H, dd, *J*=17.5, 11.0 Hz) |
| C-13 | 114.2 | 5.30 | (3H, dd, *J*=11.0, 1.0 Hz) | 114.2 | 5.24 | (2H, dd, *J*=11.0, 1.0 Hz) | 114.2 | 5.23 | (2H, dd, *J*=11.0, 1.0 Hz) |
| | | 5.75 | (3H, dd, *J*=17.5, 1.0 Hz) | | 5.77 | (2H, dd, *J*=17.5, 1.0 Hz) | | 5.76 | (2H, dd, *J*=17.5, 1.0 Hz) |
| C-1′ | | | | 116.8 | | | 116.9 | | |
| C-2′ | | | | 114.7 | 7.91 | (1H, d, *J*=2.0 Hz) | 114.7 | 7.72 | (1H, d, *J*=2.0 Hz) |
| C-3′ | | | | 163.1 | | | 160.6 | | |
| C-4′ | | | | 181.9 | | | 181.9 | | |
| C-5′ | | | | 122.7 | 7.20 | (1H, d, *J*=9.0 Hz) | 122.7 | 7.11 | (1H, d, *J*=9.0 Hz) |
| C-6′ | | | | 138.4 | 8.20 | (1H, dd, *J*=9.0, 2.0 Hz) | 138.3 | 8.06 | (1H, dd, *J*=9.0, 2.0 Hz) |
| C-7′ | | | | 166.5 | | | 166.5 | | |
| C-8′ | | | | 153.1 | | | 152.1 | | |
| C-9′ | | | | 122.7 | 7.22 | (2H, d, *J*=8.5 Hz) | 123.0 | 7.15 | (2H, d, *J*=8.5 Hz) |
| C-10′ | | | | 129.6 | 7.60 | (2H, d, *J*=8.5 Hz) | 128.2 | 7.42 | (2H, d, *J*=8.5 Hz) |
| C-11′ | | | | 134.9 | | | 138.3 | | |
| C-12′ | | | | 126.1 | 6.50 | (1H, d, *J*=16.0 Hz) | 127.6 | 6.71 | (1H, d, *J*=9.0 Hz) |
| C-13′ | | | | 140.4 | 7.46 | (1H, d, *J*=16.0 Hz) | 110.3 | 7.48 | (1H, d, *J*=9.0 Hz) |
| C-14′ | | | | | | | 171.6 | | |
| 14′-OH | | | | | | | | 8.54 | (1H, s) |

[a] Each sample was dissolved in $CD_3OD$. Chemical shifts are shown with reference to $CD_3OD$ as 49.8 ppm.
[b] Chemical shifts are shown with reference to $CD_3OD$ as 3.30 ppm.

C-9′, C-10′ ($\delta$ 129.6) and C-12′ ($\delta$ 126.1), from H-12′ ($\delta$ 6.50) to C-10′ and C-11′, and from H-13′ ($\delta$ 7.46) to C-11′ and C-12′ showed the presence of hydroxy-*p*-vinylphenyl moiety as shown in the partial structure II′ (Fig. 2). To confirm the coupling constants of certain proton signals, the crowded or overlapped signals were analyzed by differential selective proton decoupling experiments. The irradiation at H-12′ ($\delta$ 6.50) simplified the signal of H-13′ ($\delta$ 7.46). The proton coupling constant was 16.0 Hz between H-12′ and H-13′, indicating that the olefin has the *trans* configuration.

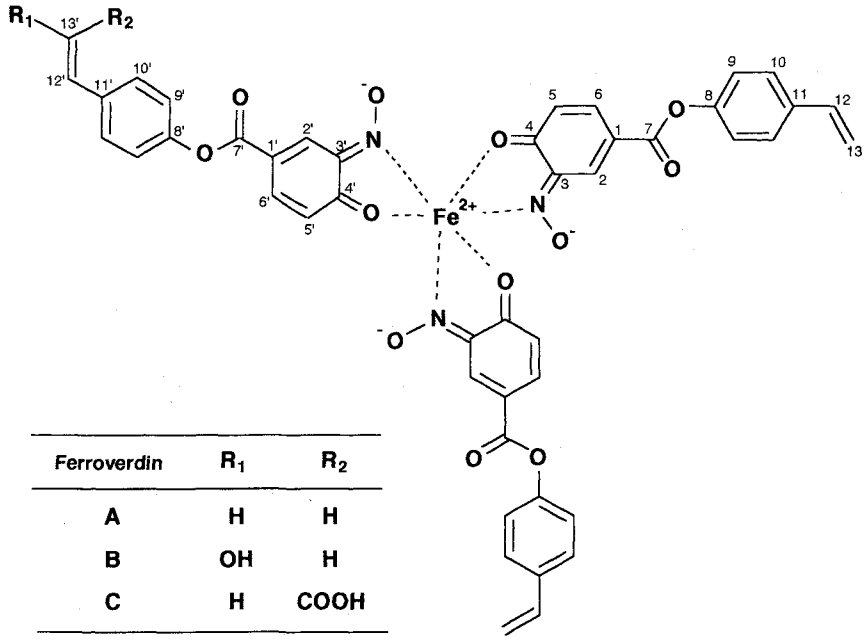
The integration of $^{13}C$ and $^{1}H$ signals revealed that the ratio of the partial structure III : III′ was 2 : 1. This structure was also explainable by the fragment ion peak analysis of FAB-MS (*p*-nitrooctylether as matrix was used.) (Fig. 3).

Taken together, the structure of ferroverdin B was elucidated as shown in Fig. 1.

## Structures of Ferroverdin C

The molecular formula of ferroverdin C was determined to be $C_{46}H_{30}N_3O_{14}Fe$ on the basis of HRFAB-MS measurement (*m/z*, found 905.1151, calcd 905.1155 for $C_{46}H_{31}N_3O_{14}Fe$ $[M+H]^+$), which was $CO_2$ bigger than ferroverdin A. The $^{13}C$ NMR spectrum of ferroverdin C (Table 2) was similar to that of ferroverdins A and B, except for the presence of additional carbonyl carbon ($\delta$ 171.6) at the C-14′ position in ferroverdin C. From the $^{1}H$-$^{1}H$ COSY and HMBC experiments, the long-range couplings as shown in Fig. 4 suggested that a carboxylic acid was attached to the vinyl moiety. The proton coupling constant was 9.0 Hz between H-12′ ($\delta$ 6.71) and H-13′ ($\delta$ 7.48), indicating that the olefin has the *cis* configuration. The fragment ion peak analysis of FAB-MS (Fig. 3) also supported the structure.

Fig. 1. Structures of ferroverdins A, B and C.

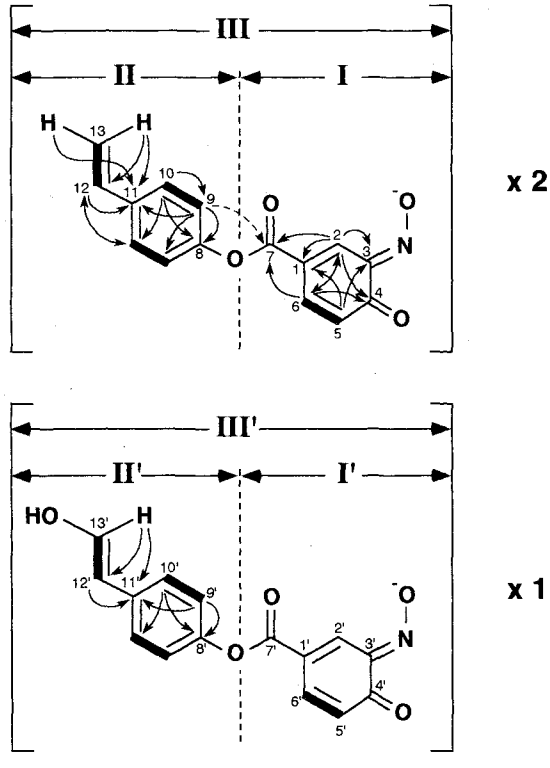


| Ferroverdin | $R_1$ | $R_2$ |
|---|---|---|
| A | H | H |
| B | OH | H |
| C | H | COOH |

Taken together, the structure of ferroverdin C was elucidated as shown in Fig. 1.

Fig. 2. $^{1}H$-$^{1}H$ COSY, $^{13}C$-$^{1}H$ HMQC and $^{13}C$-$^{1}H$ HMBC experiments of ferroverdin B.

$^{1}H$-$^{1}H$ COSY: —, $^{13}C$-$^{1}H$ HMBC: H→C.




## Discussion

Ferroverdin A[1,2], viridomycins A[4~7], E and F[8], actinoviridin[9] and 4-hydroxy-3-nitrosobenzamide ferrous chelate[10], previously isolated as green pigments produced by actinomycetes, were complexes between iron and *p*-substituted *o*-nitrosophenol ligands. Regarding the aromatic residues, viridomycin A and actinoviridin were reported to have *o*-nitrosophenols, while 4-hydroxy-3-nitrosobenzamide[10] and viridomycin F[8] possessed quinone-oxime forms by the chemical shifts of C-3 ($\delta$ 158~160) and C-4 ($\delta$ 179~182)[8,10]. The chemical shifts (Table 1) also accounted for the quinone-oxime forms for ferroverdins. Although viridomycins A and E were first reported as complexes of one iron and *o*-nitrosophenol dimers, analytical studies of their iron complexes revealed that most are complexes of one iron and three *o*-nitrosophenols[8]. Furthermore, most contain homotrimers, whereas viridomycin F and ferroverdins B and C contain hetero-trimers in their complexes.

Fig. 3. FAB-MASS experiments of ferroverdins A, B and C.

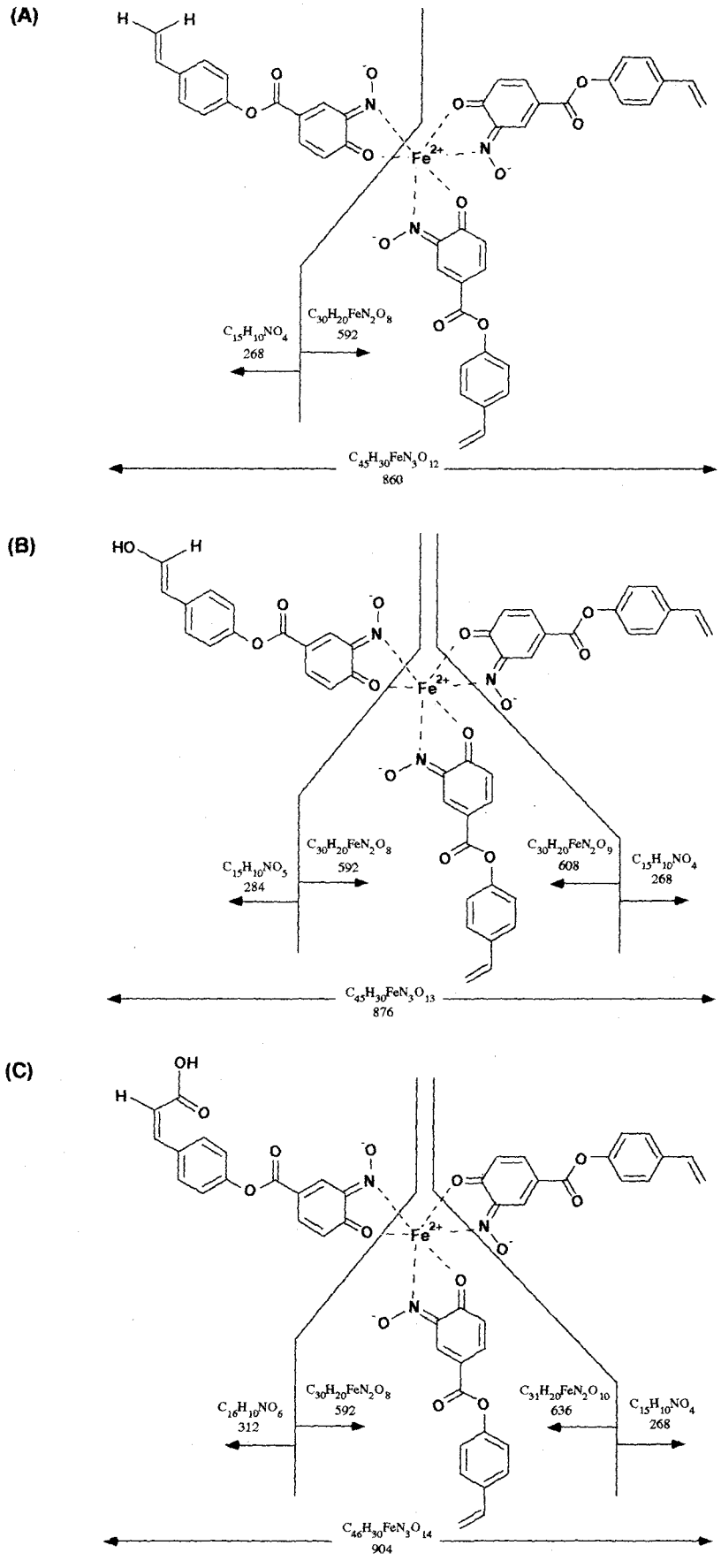
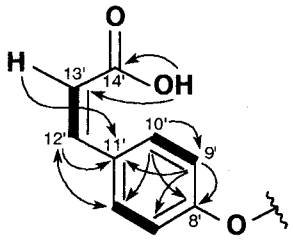


Fig. 4. $^{1}H$-$^{1}H$ COSY, $^{13}C$-$^{1}H$ HMQC and $^{13}C$-$^{1}H$ HMBC experiments of ferroverdin C.

$^{1}H$-$^{1}H$ COSY: —, $^{13}C$-$^{1}H$ HMBC: H→C.

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