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N^{α}-Dimethylcoprogens

Three novel trihydroxamate siderophores from pathogenic fungi

Mahbubul A. F. Jalal, Steve K. Love, and Dick van der Helm

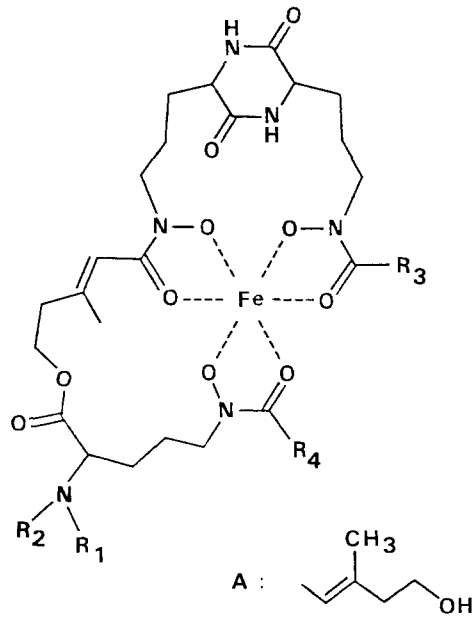
Department of Chemistry, The University of Oklahoma, Norman, OK 73019, USA

Summary. Three novel siderophores have been isolated from a highly pathogenic strain of *Alternaria longipes* (ATCC 26293). The compounds are N^{α}-dimethylated analogs of coprogen, neocoprogen I and isoneocoprogen I. Structures of the compounds have been determined by ^{1}H- and ^{13}C-NMR, fast-atom-bombardment (FAB) mass spectroscopy and partial hydrolysis. One of the new compounds, N^{α}-dimethylcoprogen, is also produced, as the major siderophore, in another fungus, *Fusarium dimerum*.

Key words: Siderophores — N^{α}-dimethylcoprogens — Pathogenic fungi — *Alternaria longipes* — *Fusarium dimerum*

Introduction

In nature, iron exists in an extremely insoluble polymeric ferric hydroxide form. Most aerobic microorganisms produce and excrete low-molecular-mass (500–1500 daltons) compounds called siderophores for the purpose of solubilizing and transporting extracellular iron into the cell (Neilands 1981; van der Helm et al. 1987). Fungi generally produce L-ornithine-based hydroxamate-type siderophores, among which the coprogen family forms a prominent and ubiquitous group. The trihydroxamate members of this family are characterized by a linear ligand structure with three hydroxamate groups made from three N^{δ}-hydroxy-N^{δ}-acylornithine units. Two of these units are joined to form a diketopiperazine ring and the third one is linked to this dimer by an ester bond (Structure 1). So far, five compounds of this group, coprogen (**1**), coprogen B (**2**), neocoprogen I (**3**), ferric triornicin (isoneocoprogen I) (**4**), and neocoprogen II (**5**) have been reported from various fungi (Diekmann 1970; Frederick et al. 1981; Frederick et al. 1982; Hossain et al. 1987; Zähner et al. 1963). In this communication, structures of three novel members of this family, isolated from a highly phytopathogenic strain of *Alternaria longipes* (ATCC 26293) and from *Fusarium dimerum*, are described.



1 R_1=H R_2=$COCH_3$ R_3=R_4=A
2 R_1=R_2=H R_3=R_4=A
3 R_1=H R_2=$COCH_3$ R_3=CH_3 R_4=A
4 R_1=H R_2=$COCH_3$ R_3=A R_4=CH_3
5 R_1=H R_2=$COCH_3$ R_3=R_4=CH_3
6 R_1=R_2=CH_3 R_3=R_4=A
7 R_1=R_2=CH_3 R_3=CH_3 R_4=A
8 R_1=R_2=CH_3 R_3=A R_4=CH_3

Offprint requests to: D. van der Helm

Materials and methods

Production and isolation. A. longipes (ATCC 26393) and *F. dimerum* were maintained on agar slants made of the Grimm-Allen's iron-deficient medium and grown in the liquid culture of the same composition to produce siderophores. The conditions for growth and production have been described earlier (Jalal et al. 1984a). The crude extract obtained after the addition of $FeCl_3$ to the culture medium was passed through a column of XAD-2. The column was washed thoroughly with H_2O and the ferric complexes of the siderophores were eluted with methanol. Repeated chromatography of the methanolic extract on silica gel columns (solvent: chloroform/methanol/water; 35:12:2) followed by a C_{18} reversed-phase column (solvent: 0–40% methanol/water gradient) (Jalal et al. 1984b) separated the N^{α}-dimethylcoprogens from each other and from the other siderophores of the fungi.

NMR spectroscopy. Proton-NMR spectra were determined at 300 MHz and ^{13}C-NMR spectra at 75.4 MHz with a Varian XL300 instrument. ^{1}H and ^{13}C resonances were assigned on the basis of selective homo- and hetero-nuclear decoupling experiments, deuterium-exchange studies and chemical shift correlation with known siderophores of analogous structures (Hossain et al. 1987; Jalal et al. 1986). In addition, $-CH_3$, $-CH_2-$, $-\overset{|}{C}H-$, $=\overset{|}{C}-$ and $-\overset{|}{C}=O$ carbon atoms were distinguished by an APT pulse sequence program (LeCocq and Lallemand 1981; Patt and Shoolery 1982).

Positive-ion FAB mass spectrometry. Nominal and accurate masses of the ions of the N^{α}-dimethylcoprogens were determined with a VG ZAB-E FAB mass spectrometer. To determine accurate mass by peak matching, coprogen (MH^+, *m/z* 822.30982) was used as standard for N^{α}-dimethylcoprogen and neocoprogen I (MH^+, *m/z* 752.2680) for N^{α}-dimethylneocoprogen I. External calibration was done with CsI.

Thin-layer chromatography. All purification and isolation steps were followed by thin-layer chromatography on silica gel 60 H with solvent systems (i) chloroform/methanol/water (35:12:2) and (ii) butanol/acetic acid/water (4:1:5; upper phase). In some cases, other solvent systems (chloroform/methanol/hexane, 1:1:1; chloroform/methanol, 1:3 and chloroform/methanol, 3:1) were also used.

Paper electrophoresis. Paper electrophoresis of N^{α}-dimethylcoprogens was carried out along with authentic coprogen and coprogen B, on Whatman 3M paper at a field strength of 1000 V for 1 h.

Deferriation. N^{α}-Dimethylcoprogens were deferriated by 8-hydroxyquinoline method. A large excess of recrystallized 8-hydroxyquinoline was added to a solution of the ferric siderophore (10 mg in 5 ml H_2O) and the mixture was left overnight at room temperature. The greenish black suspension was then extracted with chloroform repeatedly until the unused 8-hydroxyquinoline and its ferric complex were removed from the clear aqueous siderophore solution, which was freeze-dried to a white powder.

Purification of degradation products. Degradation products of the N^{α}-dimethylcoprogens were purified by chromatographic procedures using Bio-Gel P2/water and silica-gel/chloroform/methanol/water systems.

Results and discussion

N^{α}-Dimethylcoprogens (compounds **6**–**8**) have been extracted and purified from the culture broth by a combination of chromatographic procedures used earlier for similar siderophores (Jalal et al. 1984b). N^{α}-Dimethylcoprogen (**6**) showed two prominent peaks in the FAB mass spectrum, at *m/z* (relative intensity) 808 (100) and 830 (43), which correspond to the MH^+ and MNa^+ ions, respectively. On the basis of the accurate mass of the MH^+ ion (808.3321), the molecular formula of **6** has been determined as $C_{35}H_{55}N_6O_{12}Fe$ (calculated for MH^+, 808.33056). The visible absorption maximum of **6** in aqueous solution (436 nm) is typical of a ferric hydroxamate complex. Stability of the absorption maximum in the pH range 7.0–2.0 suggests that it is a 1:1 ferric trihydroxamate complex (Sayer and Emery 1968; Schwarzenbach and Schwarzenbach 1963).

The ^{1}H- and ^{13}C-NMR spectroscopy on its deferri derivative (**9**) showed that the compound is a close relative of coprogen (**1**). Most of the resonances of deferricoprogen (Hossain et al. 1987) are present in the spectra of deferri **6** (**9**). The differ-

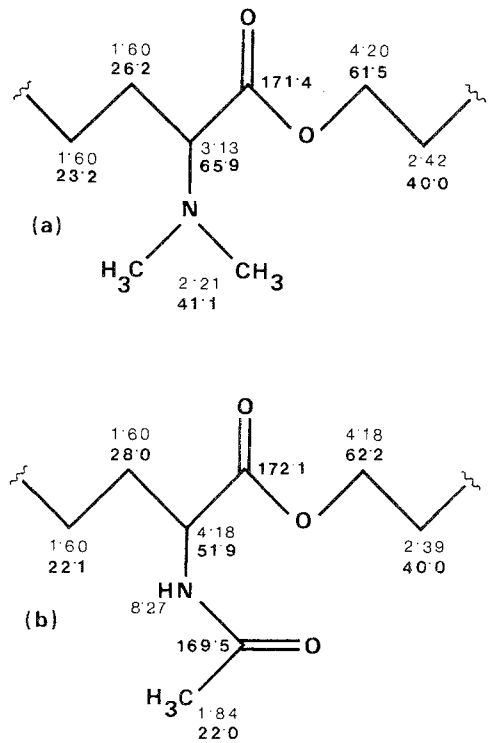


Fig. 1a, b. ^{1}H- and ^{13}C-NMR chemical shifts in the N^{α} regions of the ester-linked ornithine residues in compound **9** (deferri form of **6**) **(a)** deferricoprogen **(b)**

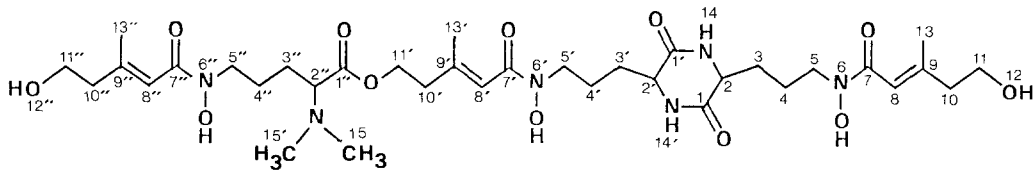
ences in the proton and ^{13}C signals of the two compounds are observed in the C^{α} region of the ester-liked ornithyl residue (Fig. 1). The singlet for the N^{α}-acetyl CH_3 group and the doublet for the $N^{\alpha}H$ proton of deferricoprogen (at $\delta = 1.84$ and 8.27 ppm respectively) are absent in the proton spectra of **9**. Similarly, the ^{13}C signals of the N^{α}-acetyl group ($\delta = 22.0$ ppm, CH_3 and 169.5 ppm, C=O) of coprogen are also absent in the new compound. Spectra of **9** show a new singlet corresponding to two methyl groups (six protons) at $\delta = 2.21$ ppm, while their ^{13}C signal appears at $\delta = 41.1$ ppm. Chemical shift values of these CH_3 signals and the absence on the N^{α}-acetyl signals suggest that **9** is an analogue of deferricoprogen, in which the N^{α}-acetyl group has been substituted by an N^{α}-dimethyl group. Further evidence for this substitution is supplied by the change in the NMR chemical shifts of the adjacent nuclei. The signal for the $C^{\alpha}H$ proton shifts upfield from $\delta = 4.18$ ppm to produce a triplet at $\delta = 3.13$ ppm, while its ^{13}C signal shifts downfield from $\delta = 51.9$ ppm to $\delta = 65.9$ ppm. Less pronounced shifts occur in the ^{13}C signals of $C^{\beta}H_2$ from $\delta = 28.0$ ppm to $\delta = 26.2$ ppm and of C=O from $\delta = 172.1$ ppm to $\delta = 171.4$ ppm. NMR signals of the rest of the molecule are virtually identical to those of deferricoprogen (Hossain et al. 1987).

The structure of **6** derived from the NMR results is consistent with the molecular formula obtained from the high-resolution mass spectroscopy and with its chromatographic and electrophoretic properties. Compound **6** (R_f 0.32) moves slightly faster than coprogen (R_f 0.28) in chloroform/methanol/water (35:12:2, freshly prepared) owing to its slightly less polar character. At pH 5.0, coprogen is neutral on paper electrophoresis, while compound **6** travels as a cation (3.2 cm/h at 1000 V field strength). The N^{α}-dimethyl group is basic and protonates at the experimental pH value.

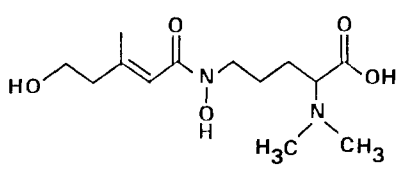
Additional evidence for the structure comes from the partial degradation of compound **6**. It degrades slowly during normal laboratory handling at room temperature, into a more polar compound (R_f in chloroform/methanol/water 0.03) which can be separated from **6** by gel filtration on Bio-Gel P2 (moves ahead of **6**). On deferriation, this compound yields two ligands, one of which is more polar (R_f 0.2 in chloroform/methanol/water) than the other (R_f 0.38). The faster-moving compound is spectroscopically and chromatographically identical to dimerum acid (**10**) (Jalal et al. 1986). It is likely that the degradation of **6** occurs due to the facile hydrolysis of the ester bond, thereby producing a mixed ferric complex of dimerum acid and N^{α}-dimethylated *trans*-fusarinine (**11**) (which forms the ester-linked part of **6**).

Compound **7** (N^{α}-dimethylneocoprogen I) and compound **8** (N^{α}-dimethylisoneocoprogen I) are structural isomers and both produce an intense MH^+ ion peak at *m/z* 738 (100) and a minor MNa^+ ion peak at *m/z* 760 in the FAB mass spectroscopy. The accurate mass of the MH^+ ion of compound **7** (738.2885) shows that the molecular formula of the compound is $C_{31}H_{49}N_6O_{11}Fe$ (calculated for MH^+, 738.2887).

Both **7** and **8** have the same mobility ($R_f = 0.32$) in silica gel thin layers using chloroform/methanol/water. The compounds separate from each other on a preparative C_{18} reversed-



9



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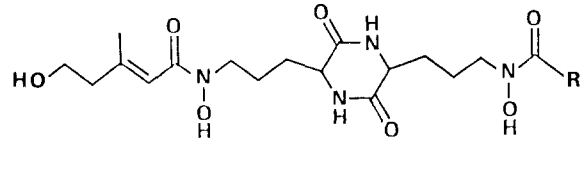

10 R = A (as in **1**)

12 R = CH_3

phase column chromatographed with a methanol–water gradient. Like **6**, both **7** and **8** are cationic at pH 5.0 and move 3.2 cm/h at 1000 V field strength towards the negative electrode during paper electrophoresis. Their absorption maxima in aqueous solution (430 nm and 432 nm for **7** and **8** respectively) are insensitive to pH change in the range of 2–7 suggesting that they are also trihydroxamate compounds.

On deferriation, **7** undergoes partial hydrolysis into two compounds. One of these products, which runs faster on silica gel thin layers in chloroform/methanol/water (R_f 0.42) shows an MH^+ ion peak at *m/z* 415 and an MNa^+ peak at *m/z* 437. Its ^{1}H-NMR spectrum is consistent with the dihydroxamate structure shown in **12.** The other hydrolysis product, which gives an MH^+ ion peak at *m/z* 289 and an MNa^+ peak at 311, is chromatographically identical with N^α-dimethylated *trans*-fusarinine (**11**) produced from **6**. This compound (**11**) is very unstable and it has not been characterized further. Compound **8**, after deferriation, also undergoes hydrolysis into two products, one of which is identified as dimerum acid on the basis of spectroscopic and chromatographic evidences.

The above spectroscopic and degradative results strongly suggest that **7** and **8** are similar to neocoprogen I (**3**) and isoneocoprogen I (ferric triornicin) (**4**) respectively, except that they possess N^α-dimethyl groups instead of the N^α-acetyl group. These compounds contain an N^δ-anhydromevalonoyl group at one end of the molecule and an N^δ-acetyl group at the other end. In **7** the N^δ-acetyl group is at the diketopiperazine end, while in **8**, this group is in the ester-linked ornithyl residue.

These novel siderophores (**6**–**8**) add both lipophilic as well as ionic character to the coprogen family of siderophores. The presence of two methyl groups on the N^α atom is likely to increase the lipophilicity of the molecular surface by partially covering the adjacent ester group. The N^α atom protrudes out on the surface of the ester loop, as shown by the X-ray structure of neocoprogen I (**3**) (Hossain et al. 1987). On the other hand, in the physiological pH range, N^α-dimethyl groups remain protonated and the siderophore molecule therefore acts as a positively charged species. In this respect, these novel siderophores resemble coprogen B, which contains a positively charged α-amino group. It will be interesting to see what kind of functional change is brought about in the iron-transport and receptor binding process by these siderophores with altered physical properties.

A. longipes (ATCC 26293), the producing organism of N^α-dimethyl coprogens, is a virulent strain which is highly pathogenic to plant hosts (Simmons 1981; Stavely and Slana 1973). *F. dimerum*, which also produces N^α-dimethylcoprogen (**6**) is known to have pathogenic strains that cause an eye infection (Zapater et al. 1972). It is possible that these new siderophores play a role in the pathogenicity of these fungi.

Properties of N^α-dimethylcoprogens 6–8

N^α-Dimethylcoprogen (**6**). Red amorphous substance soluble in water, methanol and ethanol; insoluble in ethyl acetate, acetonitrile and other nonpolar solvents. Absorption maximum at 436 nm (H_2O, pH 2.0–7.0). Paper electrophoresis, 3.2 cm/h towards cathode at pH 5.0 at 1000 V field strength. TLC, silica gel, (i) chloroform/methanol/water (35:12:2) (CMW), R_f 0.32; (ii) chloroform/methanol/hexane (1:1:1), R_f 0.11; (iii) chloroform/methanol (1:3), R_f 0.40; (iv) chloroform/methanol (3:1), R_f 0.18: (v) 1-butanol/acetic acid/water (4:1:5, upper phase), R_f 0.01. FAB mass spectroscopy, MH^+ *m/z* 808.3321 (100) (M, $C_{35}H_{55}N_6O_{12}Fe$), MNa^+ *m/z* 830 (43).

Deferri compound **9**: ^{1}H-NMR in $(CD_3)_2SO$, δ/ppm = 1.60 (m, 12H, 3,3′,3″,4,4′,4″), 2.02 (s, 9H, 13, 13′,13″), 2.21 (s, 6H, 15,15′), 2.24 (t, 4H, 10, 10″), 2.42 (t, 2H, 10′), 3.13 (t, 1H, 2″), 3.50 (m, 6H, 5, 5′, 5″), 3.53 (t, 4H, 11, 11″), 3.82 (m, 2H, 2, 2′), 4.20 (m, 2H, 11′), 4.58 (broad s, OH, 12, 12″), 6.22 (s, 3H, 8, 8′, 8″), 8.15 (s, 2H, 14, 14′), 9.73 (broad s, N—OH, 6, 6′, 6″). ^{13}C-NMR in $(CD_3)_2SO$, δ/ppm = 18.0 (13′), 18.2 (13, 13″), 22.1 (4,4′), 23.2 (4″), 26.2 (3″), 30.3 (3, 3′), 41.1 (15, 15′), 43.8 (10,10″), 46.7 (5,5′,5″), 53.7 (2,2′), 59.1 (11,11″), 61.5 (11′), 65.9 (2″), 116.2 (8, 8″), 117.1 (8′), 148.6 (9′), 150.9 (9,9″), 166.4 (7,7′,7″), 167.8 (1,1′), 171.4 (1″). Hydrolysis product: **10**, FAB mass spectroscopy, MNa^+ *m/z* 507 (17). ^{1}H-NMR in CD_3OD, δ/ppm = 1.80 (m, 8H), 2.07 (s, 6H), 2.37 (t, 4H), 3.68 (m, 4H), 3.72 (t, 4H), 4.00 (t, 2H), 6.31 (s, 2H). TLC in CMW R_f 0.38: chromatographically and spectroscopically identical with authentic dimerum acid.

N^α-Dimethylneocoprogen I (**7**). Red amorphous substance, solubility, mobility in paper electrophoresis and R_f in CMW on silica gel thin layers are similar to **6**. FAB mass spectroscopy, MH^+ *m/z* 738.2885 (100) (molecular formula, $C_{31}H_{49}N_6O_{11}Fe$), MNa^+ *m/z* 760 (4). Hydrolysis

products of the deferri compound: (a) **11**, FAB mass spectroscopy, MH^+ *m/z* 289 (9), MNa^+ *m/z* 311 (13); TLC, silica gel, CMW, R_f 0.10. (b) **12**, FAB mass spectroscopy, MH^+ *m/z* 415 (5), MNa^+ 437 (12). ^{1}H-NMR in CD_3OD, δ/ppm = 1.80 (m, 8 H, β and γ CH_2), 2.07 (s, 3 H, anhydromevalonoyl CH_3), 2.10 (s, 3 H, N^{δ}-CH_3), 2.37 (t, 2 H, $=\overset{|}{C}-CH_2-$), 3.66 (m, 4 H, δCH_2), 3.71 (t, 2 H, $-CH_2O-$), 4.00 (t, 2 H, α-CH), 6.30 (s, 1 H, $-CH=$); TLC, silica gel, CMW, R_f 0.42.

N^{α}-Dimethylisoneocoprogen I (**8**). Solubility, chromatographic and electrophoretic mobility similar to **7**. FAB mass spectroscopy, MH^+ *m/z* 738 (100), MNa^+ *m/z* 760 (7). Hydrolysis product of the deferri compound: dimerum acid (**10**).

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