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Legonoxamines A-B, two new hydroxamate siderophores from the soil bacterium, *Streptomyces sp.* MA37

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1. Introduction

Iron is an essential element for the growth of almost all forms of life, serving as a cofactor in enzymatic processes, oxygen metabolisms, electron transfer, and DNA and RNA syntheses.¹ It is also required for biofilm formation because Iron regulates surface motibility and stabilizes the polysaccharide matrix.² Because of the low bioavailability of iron in the environment, microorganisms have developed specific uptake strategies such as production of siderophores, metal-chelating molecules with low molecular weight (200-2000Da) that are produced by microorganisms, especially under iron-limiting conditions.^{3,4} The role of siderophores is to scavenge the extracellular iron from different terrestrial and aquatic habitats and transport them to microbial and plant cells via specific high-affinity uptake receptors.^{3,5}

Siderophores are a highly diverse group of compounds and they are classified into four main categories based on the chemical nature of the moieties donating the oxygen ligands for Fe³⁺ coordination.¹ These four classes are: hydroxamates (N-OH

ABSTRACT

Two new siderophores belonging to the hydroxamate class, Legonoxamine A (1) and B (2) have been isolated from the soil bacterium, *Streptomyces sp.* MA37, together with one known compound, desferrioxamine B (3). Their structures were elucidated based on spectroscopic methods including 1D, 2D NMR, MS, as well as by comparison with the relevant literatures. To our knowledge, this is the first report describing a siderophore containing the *N*-hydroxyl phenylacetyl cadaverine (HPAC) moiety in the structure. Based on bioinformatics analysis and previous knowledge of the biosynthesis of the hydroxamate-type siderophore, the biosynthetic gene cluster (*lgo*) responsible for the production of **1-3** was identified in the annotated genome of the producing strain. The supplementation of phenylacetate and benzoate analogues with meta substitution into the cultures of *Streptomyces sp.* MA37 resulted in the production of new legonoxamine A derivatives as observed in LC-HR-ESIMS, suggesting that the legonoxamine biosynthetic pathway has a good degree of natural flexibility of accepting unnatural precursors with different functional groups.

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amides) (i.e. desferrioxamines)^{6,7}, catecholates (i.e. enterobactins)⁸, carboxylates (i.e. achromobactin)^{9,10}, and siderophores with mixed ligands.¹¹ There are more than 500 different siderophores, of which only about 270 have been structurally characterized.¹

In our screening program to discover novel natural products from Ghanaian isolates^{12–15}, we have identified a talent soil bacterium, *Streptomyces sp.* MA37, a Ghanaian isolate that has the capacity of producing two pyrrolizidine alkaloid legonmycins¹⁶, one carbazole alkaloid neocarazostatin A^{17–19} and a range of fluorinated metabolites ^{20,21}.

In this study, we report the isolation and characterization of two new desferrioxamine siderophore derivatives from *Streptomyces sp.* MA37, which were named legonoxamine A **1** and B **2**, after their association with Legon, Ghana, the location of the University of Ghana. Both legonoxamines contain a unique moiety, *N*-hydroxyl phenylacetyl cadaverine (HPAC), which, to our best knowledge, has not been found in other known siderophores. Interestingly, we also isolated the known

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siderophore, desferrioxamine B (3) that have been known to be produced by various bacteria.¹

In silico analysis of the annotated genome of *Streptomyces sp.* MA37 allowed the identification of the biosynthetic gene clusters (*lgo*). A precursor-directed biosynthesis approach was employed in order to access new legonoxamine derivatives. The biosynthetic machinery exhibited relaxed substrate specificity with meta-substituted phenylacetate and benzoate analogues that were administered.

2. Results and Discussion

Streptomyces sp. MA37 was cultivated in the presence of HP20 resin (35g/L medium). After 7-day fermentation (12L), the aqueous solution was filtered and HP20 resin was extracted with methanol (3x500 ml). The crude extract was concentrated under vacuum, followed by fractionation using the reversed phase solid phase extraction (SPE). Subsequently the 100% methanol fraction was then subjected to semi-preparative reversed-phase HPLC resulting in the isolation of compounds **1** (5.3 mg), **2** (3.2 mg), and **3** (4.5 mg).

The molecular formula of **1** was established as $C_{31}H_{53}N_6O_8$ by high resolution electron spray ionization mass spectrometry (HR ESIMS) (*m*/*z* 637.3904 [M+H]⁺ Δ -1.957 ppm) (Fig. S1-S3). The molecular formula suggested 9 degrees of unsaturation. Interpretation of ¹H, HMBC and edited HSQC NMR data (Table 1, Fig. S4-S8)) of **1** revealed the presence of 20 methylenes, 5 methines and 6 quaternary carbons leading to the sub-formula $C_{31}H_{45}$. The presence of 5 methines (127.0-129.2 ppm) and 1 quaternary carbon (135.0 ppm) suggested the presence of one aromatic ring, which together with the presence of five additional quaternary carbonyl carbons (171.0-174.7 ppm) accounted for a total of 9 double bond equivalents.

Analysis of the ¹H-¹H COSY spectrum revealed 6 spin systems with repeating motifs, one consisting of H-1 through H-5, H-1' through H-5', and H-1" through H-5", and the other comprising H-7 through H-8 and H-7' through H-8', and the presence of one aromatic spin system H-8" through H-13".

The long-range couplings observed in the HMBC experiments established the connectivity of the 6 substructures including two succinyl units (substructures B and D) and three N-hydroxy-cadaverine units (substructures A, C and E) (Fig. S9). The cross peaks from H-7 (2.49 ppm) and H-8 (2.79 ppm) to C-6 (172.8 ppm) and C-9 (174.7 ppm), and H-7' (2.49 ppm) and H-8' (2.79 ppm) to C-6' (172.9 ppm) and C-9' (173.8 ppm), indicated the presence of two succinyl groups. The HMBC correlation from nitrogen-bearing methylene at H-5 (3.65 ppm) to the carbonyl carbon at C-6 (172.8 ppm) confirmed the connectivity of substructures A with B by an amido bond between C-5 and C-7. Similarly, the connectivities of substructures B with C, C with D, and D with E were elucidated by HMBC correlations from H-1' (3.17 ppm) to C-9 (174.7 ppm), H-5' (3.65 ppm) to C-6' (172.9 ppm), and H-1" (3.17 ppm) to C-9' (173.8 ppm), respectively. The remaining atoms were estimated to be hydroxyl groups attached to nitrogen, from the molecular formula of 1 to form three *N*-hydroxy groups.

Analysis of the 2D NMR data in **1** revealed the presence of two *N*-hydroxy-*N*-succinyl cadaverine (HSC) units, which were in agreement with bisucaberin B, a linear hydroxamate class siderophore from the marine bacterium *Tenacibaculum mesophilum*, which contains similar HSC functional groups in the structure.²²

The singlet proton signal at 3.79 ppm (H-7") showed HMBC correlations to the quaternary aromatic carbon C-8" (135.0ppm)



Figure 1. Legonoxamine A 1 with COSY (—) and key HMBC (\rightarrow) correlations, Legonoxamine B 2, and the known compound desferrioxamine B 3

and the carbonyl carbon at C-6" (173.6 ppm), signifying the presence of a phenyl acetyl moiety in the structure (substructure F). The connectivity of this moiety to the HSC residue (substructure E) was established on the basis of HMBC correlations from H-5" (3.65 ppm) and H-7" (3.79 ppm) to C-6" (173.6 ppm) (Fig. S9) to form one *N*-hydroxy-*N*-phenylacetyl cadaverine (HPAC) unit, respectively. The identity of the phenylacetyl moiety was further supported by comparison of the HPLC analysis of the hydrolysate of legonoxamine A 1 against the standard phenyl acetic acid. A prominent peak (RT = 14.4 min) observed in the hydrolysate shared the same RT as the one of phenyl acetic acid (Fig. S23).

The molecular ion peak of **2** was established as $C_{17}H_{25}N_2O_5^+$ by HR ESIMS (*m*/z 337.1760 [M+H]⁺ Δ -0.287 ppm), which required 7 degrees of unsaturation (Fig. S10-S12).

Based on the 1D and 2D NMR data, **2** showed a similar HPAC pattern as in **1** with a succinyl moiety in the structure (Fig. S13-S16). The cross peaks from H-3' (2.57 ppm) and H-2' (2.43 ppm) to C-4' (176.0 ppm) and C-1' (174.0 ppm) supported the presence of the succinyl residue.

Inspection of the ¹H, ¹³C (Table 1), and HR ESIMS data (m/z 561.3583 amu [M+H]⁺ Δ -2.117 ppm) (Fig. S17-S22) of **3** indicated that it was the known compound, desferrioxamine B which can be isolated from various *Streptomyces* species.^{5,23–25}

Final structural analysis of **1** and **2** were completed by comparison of the 1D and 2D NMR to known spectra for desferrioxamine compounds.^{22–27} It was evident that the proton and carbon chemical shifts at C-7" (in **1**) and C-7 (in **2**) had remarkably changed from 2.1 ppm (¹H) and 19.0 ppm (¹3C) in desferrioxamine B to 3.79 ppm (¹H) and 39.8 ppm (¹³C) in **1** and **2**, respectively. The presence of the aromatic ring (¹³C 127-135 ppm) makes **1** and **2** unique from other siderophores (Table 1, Fig. 1).

On the basis of the evidence in this study, 1 and 2 were confirmed as new siderophores belonging to the hydroxamate



Figure 2. A. The biosynthetic gene cluster of legonoxamines 1 and 2, and desferrioxamine B 3 in *Streptomyces sp.* MA37. B. A proposed biosynthetic model of 1-3.

class, for which the names of legonoxamines A and B are proposed, respectively, after their association with Legon, Ghana.

The MS/MS fragmentation data of 1 (m/z 690.3016 amu [M+Fe–2H]) suggested the presence of legonoxamine A (1) chelated with iron ($C_{31}H_{50}N_6O_8Fe$). The LC MS isotopic pattern confirmed the presence of the four stable isotopes (⁵⁴Fe, ⁵⁶Fe, ⁵⁷Fe, ⁵⁸Fe) of the iron-siderophore complex (Fig. S2-S3). Two molecules of 2 was found bound to Fe³⁺ at m/z 726.2557

 $[2M+Fe-3H]^{2+},\ C_{34}H_{46}N_4O_{10}Fe$ and three molecules at m/z 1062.4258 [3M+Fe-4H] ⁺, C_{51}H_{70}N_6O_{15}Fe (Fig. S11-S12). Each hydroxamate group in the structure provides two oxygens (-CONOH-) which form a bidentate ligand with iron, resulting in a highly stable, hexadentate octahedral iron (III) complex.

In silico analysis of the annotated genome of Streptomyces sp. MA37 allowed the identification of the putative biosynthetic gene cluster (*lgo*) (Fig. 2A) that directs the biosynthesis of **1-3**. The *lgo* gene cluster spans ~40 kbp of genomic DNA and contains 6 open reading frames (ORFs) with predicted functions that were assigned based on homology analysis.²⁸

The *lgo* cluster contains a cassette of four genes (*lgo*A-D) that typically direct the biosynthesis of **1-3** and two auxiliary genes responsible for transcriptional and transport functions (*lgo*E and F, respectively), and shows high homology with the reported biosynthetic gene clusters for desferrioxamines.^{28–33} Based on bioinformatics analysis and our current understanding of the desferrioxamine-type natural products,^{30–32,34} a possible legonoxamine biosynthetic pathway is proposed (Fig. 2B).

The *lgoA* and *lgoB* genes encode enzymes with similarity to PLP-dependent amino acid decarboxylases and FAD-dependent amine monooxygenases, respectively, and are proposed to catalyze the first two steps of the biosynthesis of the legonoxamines **1-2** and desferrioxamine **B 3**. The protein encoded by *lgoC* is similar to acyl-CoA transferase DesC in the desferrioxamine pathway from *Streptomyces coelicolor*.²⁹ It is believed that LgoC catalyses the acylation of *N*-hydroxycadaverine **5** using both acetyl CoA and phenylacetyl CoA as substrates to give the hydroxamic acids **6**, which undergo ATP-dependent oligomerization to give the **1** and **3**, both catalyzed by LgoD. Legonoxamine **B 2** is likely to be either a breakdown product of **1** during the chemical workup or a degraded shunt metabolite during the biosynthesis of **1**.

Precursor-directed biosynthesis is a powerful approach to enable the incorporation of unnatural precursors with diverse functional groups, expanding the pool of 'unnatural' natural products.³⁵ Additionally, region- and chemo- selectively modified and modifiable functional groups can be introduced using this approach. Utilizing this approach, new analogues of bioactive natural products can be prepared in a matter of days compared to a more lengthy and costly synthetic approach.³⁶ Thus, our next focus on interrogating the natural degree of flexibility of the legonoxamine biosynthetic pathway.

The incorporation of phenylacetyl moiety into 1 and 2 is unprecedented, suggesting that the acyltransferase LgoC, responsible for the biotransformation of acyl CoA and Nhydroxycadaverine into hydroxamic acids, may have a high degree of natural flexibility, which renders this position ideal for modification through precursor-derived biosynthesis. This provided encouragement that the incorporation of a variety of phenyl acetate and benzoate derivatives into legonoxamines might be feasible. To validate this hypothesis, six phenylacetate (PAc) derivatives and benzoate (Table S1) were added to individual cultures of Streptomyces sp. MA37 at the onset of siderophore production. The cultures were harvested after 7-day fermentation and the extracts were analyzed by LC-HR-ESIMS. Notably, the supplemented PAc analogues substituted at meta position and benzoate resulted in the production of new legonoxamine A 1 derivatives with the expected molecular ion being detected (Fig. S24-26, Table S1). Correct incorporation of the PAc/benzoate analogues were confirmed through inspection of the MS-MS spectra. Furthermore, the incorporation of 3-(trifluoromethyl) phenylacetate was further confirmed in the ¹⁹F-NMR of the sample at 600 MHz. A new organofluorine signal at 77ppm was observed in the fermentation culture when supplemented with the trifluoro precursor (Fig. S27). Interestingly, no incorporation were observed for PAAs substituted at para position or trans-cinnamic acid, for example, 4-chlorophenylacetic acid, and 4-(bromomethyl)phenylacetic acid.

Legonoxamine A (1) were initially screened for antibacterial and antifungal activities but does not show any significant activity.

4

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	Desferrioxamine B (3)		Legonoxamine A (1)		Legonoxamine B (2)	
	¹³ C	¹ H mult (J, Hz)	¹³ C	¹ H mult. (J, Hz)	¹³ C	¹ H mult. (J, Hz)
1	40.1	2.92, t (7)	40.0	2.91, t (7)	39.9	3.62, t (7)
2	29.7	1.53, m	29.6	1.51, m	29.7	1.50, m
3	24.6	1.33, m	24.9	1.35, m	26.0	1.30, m
4	27.1	1.63, m	27.1	1.65, m	27.0	1.64, m
5	48.8	3.63, t (7)	48.4	3.65, m	48.6	3.63, t (7)
6	172.2	-	172.8	-	170.6	- ·
7	25.8	2.58, t (7)	25.9	2.49, t (7)	39.8	3.79, s
8	28.5	2.76, t (7)	28.5	2.79, t (7)		
9	174.8	-	174.7	-		
1'	39.7	3.17, t (7)	39.2	3.17, t (7)	174.0	-
2'	29.7	1.53, m	29.6	1.51, m	31.3	2.43, t (7)
3'	24.6	1.33, m	24.9	1.35, m	30.1	2.57, t (7)
4'	27.1	1.63, m	27.1	1.65, m	176.0	-
5'	48.8	3.63, t (7)	48.4	3.65, m		
6'	172.2	-	172.9			
7'	25.8	2.45, t (7)	25.9	2.49, t (7)		
8'	28.5	2.76, t (7)	28.5	2.79, t (7)		
9'	173.4	-	173.8	-		
1"	39.7	3.17, t (7)	39.2	3.17, t (7)		
2"	29.7	1.53, m	29.6	1.51, m		
3"	24.6	1.33, m	24.9	1.35, m		
4"	27.1	1.63, m	27.1	1.65, m		
5"	48.8	3.63, t (7)	48.4	3.65, m		
6"	172.2	-	173.6	-		
7"	19.9	2.09, s	39.8	3.79, s		
8"(1) / 8 (2)			135.0	-	135.0	
9" (1) / 9 (2)			129.2	7.28, m	129.4	7.28, m
10" (1) / 10 (2)			127.4	7.21, m	127.4	7.21, m
11" (1) / 11 (2)			128.0	7.30, m	128.2	7.30, m
12" (1) / 12 (2)			127.4	7.21, m	127.4	7.21, m
13" (1) / 13 (2)			129.2	7.28, m	129.4	7.28, m

Table 1. NMR Spectroscopic Data for Legonoxamine A 1, B 2 and 3 (600 MHz, CD₃OD, 298K)

3. Conclusion

In summary, we have discovered two new structures containing phenylacetyl moiety, legonoxamine A 1 and B 2 together with the known siderophore metabolite, desferrioxamine B 3 from a soil isolate, Streptomyces sp. MA37. Genome mining of the annotated genome of the producing MA37 strain allowed the identification of the biosynthetic gene cluster (lgo) of legonoxamines (1-2) and desferrioxamine B 3. The supplemented PAc analogues with meta substitution and benzoate resulted in the production of new analogues of legonoxamines as observed in LC-HR-ESIMS and MS/MS fragmentation analysis, suggesting that the putative acyltransferase, LgoC, may display a good degree of substrate tolerance of LgoC is under evaluation and will be reported accordingly.

4. Experimental section

4.1 Extraction and Isolation

Streptomyces sp. MA37 was isolated from the rhizosphere of a Moracear Bark Cloth tree (*Antiaris toxicaria*), growing in the University of Ghana Botanical Gardens, Legon, Africa.²¹ The pure

bacterial isolate was cultured following the guidelines given by the International Streptomyces Project (ISP) at 28°C, supplemented with 2 mM potassium fluoride.

The crude sample was fractionated on a C18 solid phase extraction (SPE) column using 25% MeOH in water (SPE 25), followed by 50% MeOH (SPE 50), then by 100% MeOH (SPE 100). Finally, the column was flushed with 100% methanol containing 0.05% trifluoroacetic acid (SPE 100 TFA). The eluents were concentrated under reduced pressure and each fraction was subjected to LC MS analysis.

The compounds of interest were detected in the SPE 100 fraction by LC-MS and ¹H NMR analysis. Purification of this fraction was carried out by reversed phase C18 High Performance Liquid Chromatography, HPLC (ACE 10uM 10 x 250 mm column) using a mixture of methanol, water and TFA (0.05%) as an eluent with a linear gradient from 0 to 100% MeOH over 45 min and a solvent flow of 1.5 mL/min. All the employed chemicals were HPLC grade.

4.2 Structure Elucidation

The structure was deduced by High-Resolution Electrospray Ionisation Mass Spectrometry (HR-ESIMS), 1D and 2D NMR spectroscopy. HR-ESIMS was determined using LC MS Thermo Scientific MS system (LTQ Orbitrap) coupled to a Thermo Instrument HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump, C18 Sunfire 150×46 mm Waters®). The following parameters were used: capillary voltage 45 V, capillary temperature 320°C, auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100-2000 amu (maximum resolution 30000). High performance digital Bruker AVANCE III HD 400 MHz (AscendTM 9.4 Tesla) and Bruker AVANCE III HD 600 MHz (AscendTM 14.1 Tesla) with Prodigy TCITM cryoprobe were used to obtain the following information at 25°C: ¹H NMR, ¹³C NMR, ¹H-¹H COSY, ¹H-¹³C HSQC and ¹H-¹³C HMBC. Infrared spectrum was recorded using the PerkinElmer Spectrum Two Fourier Transform Infrared (FTIR) Spectrometer (2013) with ATR diamond cell. The optical rotation was measured using ADP 410 polarimeter (Bellingham + Stanley Ltd. 2007) equipped with a light emitting diode with interference filter.

4.3 Hydrolysis

The hydrolysis of legonoxamine A (1) was carried out using 5 N HCl (80° C, 1hr). The components of the hydrolysate was analysed by reversed phase C-18 HPLC (ACE 5 C-18 HL 150 x 4.6 mm) using the phenyl acetic acid as the standard (Sigma Aldrich). HPLC analysis for both hydrolysate and standard was performed under the following conditions: solvent A – 95% H₂O-5% methanol-0.05% trifluoroacetic acid, solvent B- methanol; linear gradient from 20% to 100% methanol, flow rate 1.0 mL/min, monitored at 254 nm.

4.4 Feeding Experiment

The MA37 culture was separately grown in 250-mL Erlenmeyer flasks containing 100-mL ISP2, each supplemented with commercially available and synthetic precursors of the benzoic acid and phenyl acetic acid moiety to make 1.0 mM of the solution. The benzoic acid was purchased from BDH Chemicals LTD, the 4-(bromomethyl)phenylacetic acid, 3-(methylthio) phenylacetic acid, 3-(trifluoromethyl)phenylacetic acid, and *trans*-cinnamic acid were purchased from Sigma Aldrich, and 4-chlorophenylacetic acid from Merck. The reagents used for the fermentation feeding experiment were 95-99.5% pure and were used as received.

The incubation temperature was maintained at 28°C in a rotary shaker. After 7 days of fermentation, it was added with Diaion® HP-20 (3g/50mL solution), and incubated overnight. The solution was filtered and the resin was extracted with methanol. The crude extract was concentrated in the rotary evaporator and each sample was subjected to LC MS analysis.

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Supplementary Material

LC MS, NMR Spectra of **1**, **2**, and **3**, including ¹H, ¹³C, COSY, HSQC in CD₃OD, and feeding experiment results are available in the Supplementary data.

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Highlights:

- Two new siderophores containing phenylacetate motif were characterized
- The putative biosynthetic gene cluster was identified
- Precursor-directed biosynthesis showed natural flexibility for unnatural precursors.

Graphical Abstract

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