Supporting Information for

Structure and Biosynthesis of Amychelin, an Unusual Mixed-Ligand Siderophore from *Amycolatopsis sp.* AA4

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**General procedures.** UV-visible absorbance spectra were collected on an Amersham Biosciences Ultrospec 5300 Pro Spectrophotometer. HPLC purifications were carried out on an Agilent 1200 Series analytical HPLC system equipped with a photo diode array detector, or on an Agilent 1200 Series preparative HPLC system also equipped with a diode array detector. Low resolution HPLC-MS analysis was performed on an Agilent 1200 Series HPLC system equipped with a diode array detector and a 6130 Series ESI mass spectrometer using an analytical Phenomenex Luna C18 column (5 μm, 4.6 × 100 mm) operating at 0.7 mL/min with a gradient of 10 % MeCN in H2O (+0.1 % formic acid) to 100 % MeCN (+0.1 % formic acid) over 25 min. High resolution HPLC-ESI-MS (HR-MS) and tandem MS (MS/MS) were carried out in the lab of Prof. Suzanne Walker (Harvard Medical School) on an Agilent 1200 series HPLC equipped with a diode array detector and a 6520 Series LC/Q-TOF using the same column and gradient as above for HPLC-MS analysis. The HR-MS was calibrated to within 3 ppm, while the MS/MS was calibrated to within 12 ppm. 1H, 13C, and 2D NMR spectra were recorded in the inverse-detection probe of a Varian Inova spectrometer (600 MHz for 1H, 150 MHz for 13C). 13C NMR spectra were recorded on the same instrument with a broad-band probe. Chemical shifts were referenced to the residual solvent peaks in D2O and DMSO-d6.

**Cultivation of Amycolatopsis sp. AA4, bioassays, and generation targeted mutants.** Initial screening for interactions between actinomycetes was done by plating 1 μL droplets of dense spore solutions 0.5 cm apart on R2YE agar. After 5 days of growth, the resulting colonies were observed for alterations in developmental morphology, an example of which is shown in Fig 1 in the text. Preparative-scale cultivation of *Amycolatopsis sp.* AA4 was carried out in a minimal medium consisting of 25 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), pH 7.2, 2.34 mM MgSO4, 0.5 mM NaH2PO4, 0.5 mM K2HPO4, 55.4 mM glucose, 85.5 mM NaCl, and 66.5 mM glycine. One mL of R2YE trace element solution1 was also added to each liter of the minimal medium. Four 2.8 L baffled Fernbach flasks, each containing 0.5 L of the medium above, were inoculated with 20 μL of a concentrated spore solution and cultivated on a shaker/incubator for 8 days at 240 rpm and 30°C. Tests for biological activity were conducted by plating a lawn of *S. coelicolor* (10⁵ spores/plate) on solid R2YE medium followed by incubation for 16 hours (overnight) at 30°C. One cm wells were then punched into the agar. For assay-guided fractionation, 50 μL of the test solution were mixed with 250 μL of sterile, melted 0.7% agar and the resulting 300 μL were added to agar wells. *S. coelicolor* cell morphology was monitored over the next 24-48 hours.

Targeted mutations in the amc gene cluster of *Amycolatopsis sp.* AA4 were made by
using the ReDirect methodology described by Gust et. al. (2003). Briefly, individual genes on fosmids were replaced with apramycin resistance cassettes, and the modified fosmids were conjugated into *Amycolatopsis sp.* AA4 via a nonmethylating *E. coli* donor strain to allow for homologous recombination. Mutations were confirmed by PCR and/or direct sequencing. A knockout mutant lacking amcG did not produce any amychelin.

**Purification of apo-, Fe- and Ga-amychelin.** Cultures of *Amycolatopsis sp.* AA4 (2 L total) were centrifuged at 7000 rpm for 15 min and the remaining supernatant was further clarified using 0.2 micron Nalgene filter units. The filtrate was then extracted using 1:1 volume of ethyl acetate. The remaining 2 L of the aqueous phase was concentrated in vacuo to 0.35 L and subsequently passed over a methanol-activated 0.5 g Sep-Pak C18 cartridge (Waters). The aqueous flow through was passed over a 0.5 g Sep-Pak C8 cartridge (Waters) and the flow through was concentrated in vacuo to volume of 40 mL. This 40 mL fraction had robust siderophore and biological activity.

To obtain apo-amychelin, 10 mL of this fraction was purified on a preparative Phenomenex Luna C18 column (5 µm, 21.2 × 250 mm) operating at 13 mL/min using 5 × 2 mL injections. Amychelin was eluted with a gradient of 10 % MeCN in H₂O (+0.1 % formic acid) to 39 % MeCN in H₂O (+0.1 % formic acid) over 25 min. The fractions containing amychelin, identified by the UV spectrum of the hydroxybenzoyl moiety (~22 % MeCN), were pooled, concentrated in vacuo and applied onto a Phenomenex Luna C8 column (5 µm, 21.2 × 250 mm) operating at 13 mL/min using 2 injections. Amychelin was eluted with a gradient of 8 % MeCN in H₂O (+0.1 % formic acid) to 32 % MeCN in H₂O (+0.1 % formic acid) over 23 min. The fractions containing amychelin (~15 % MeCN) were pooled and dried in vacuo. The biological and iron chelating activities were lost after application of the first C18 column. This procedure provided 4 mg of 2.

To obtain Fe- or Ga-amychelin, 8 mL of a ~1 mg/mL solution of amychelin (from the 40 mL fraction above) were transferred to a 40 mL vial equipped with a magnetic stir bar and stirred gently at room temperature. Then 10-fold excess of solid GaBr₃ (or FeCl₃) was added slowly over ~5 min. Gentle stirring was continued for another 10 min. The reaction was then incubated overnight at 4°C. Fe- or Ga-amychelin were purified on a preparative Phenomenex Luna C18 column (5 µm, 21.2 × 250 mm) operating at 13 mL/min. Fe-amychelin was eluted with a gradient of 10 % MeCN in H₂O to 26 % MeCN in H₂O over 14 min. Elution of Fe-amychelin was monitored using its MLCT band at 435 nm (see Fig. S1) and occurred at 14 % MeCN. This procedure was repeated to give 5 mg of Fe-amychelin with >95 % purity. Ga-amychelin was
eluted with a gradient of 10 % MeCN in H₂O to 39 % MeCN in H₂O over 25 min. Elution of Ga-amychelin was monitored using its MLCT band at 335 nm (see Fig. S1) and occurred at 13 % MeCN. This procedure provided 6 mg Ga-amychelin with >90% purity.

**X-ray diffraction analysis of Fe-amychelin.** Crystals of Fe-amychelin were grown by dissolving ~1-3 mg of Fe-amychelin in ~250 µL of CH₂Cl₂ and gently heating the mixture to aid dissolution. The solution was then transferred to a small crystallization tube and layered with ~250 µL of hexanes. The mixture was allowed to mix slowly at 4°C, and bright red crystals appeared after 4-5 days. A crystal of Fe-amychelin was mounted on a diffractometer and data were collected at 100 K. The intensities of the reflections were collected by means of a Bruker APEX II DUO CCD diffractometer (Cu Kα radiation, λ=1.54178 Å), and equipped with an Oxford Cryosystems nitrogen flow apparatus. The collection method involved 1.0° scans in ω at 30°, 55°, 80° and 105° in 2θ. Data integration down to 0.84 Å resolution was carried out using SAINT V7.46 A (Bruker diffractometer, 2009) with reflection spot size optimization. Absorption corrections were made with the program SADABS (Bruker diffractometer, 2009). The structure was solved by the direct methods procedure and refined by least-squares methods using SHELXS-97 and SHELXL-97 (Sheldrick, 2008). Non-hydrogen atoms were refined anisotropically, and hydrogen atoms were allowed to ride on the respective atoms. Crystal data as well as details of data collection and refinement are summarized in Table S4.

**Determination of pFe³⁺ for amychelin.** Determination of pFe³⁺ for amychelin was carried out as reported by Abergel et al. essentially without modifications.³ Briefly, purified Fe-amychelin, prepared as described above, was dissolved in Hepes buffer (10 mM Hepes, 0.1 M KCl, pH 7.4) and three different concentration ranges of ETIDA were added from EDTA stock solutions also prepared in Hepes buffer. Each reaction consisted of a total volume of 0.25 mL, a final concentration of 0.1 mM Fe-amychelin and a range of 5-fold–8000-fold EDTA (relative to Fe-amychelin) in Hepes buffer. The reaction was allowed to equilibrate at room temperature for at least 24 h, and UV-visible spectra were subsequently recorded. The composite spectra contain contributions from both Fe-EDTA and Fe-amychelin. The ε of both species as a function of λ were determined in Hepes buffer (see Fig. S1 for Fe-amychelin) and used to deconvolute the spectra. The contribution of Fe-amychelin was subtracted from the composite spectra using the 435 nm absorption band and the ε of both Fe-amychelin and Fe-EDTA were used to quantify the proportion of each species in solution. Concentrations of apo-EDTA and apo-amychelin were calculated by subtracting [Fe-EDTA] (or [Fe-amychelin]) from total initial EDTA (or Fe-
The log [EDTA]/[amychelin] was plotted against log [Fe-EDTA]/[Fe-amychelin] and the data were fit to Eq. S1, which has been derived by Abergel et al.\(^3\) One data set is shown in Fig. 2C; the two additional data sets are shown in Fig. S14 yielding a \(\text{pFe}^{\text{III}}\) of 30.0 ± 1.6 for Fe-amychelin.

\[
\log \left( \frac{[\text{Fe-EDTA}]}{[\text{Fe-amychelin}]} \right) = \log \left( \frac{[\text{EDTA}]}{[\text{amychelin}]} \right) + \Delta \text{pFe}^{\text{III}} \quad (S1)
\]
Figure S1. UV-visible spectra of apo-amychelin (black trace), Ga-amychelin (blue trace), and Fe-amychelin (red trace). Ga- and Fe-amychelin were prepared as described above. The inset shows a magnified view of the MLCT bands.
Figure S2. $^1$H NMR spectrum of Ga-amyhelin in DMSO-$d_6$.

Figure S3. gCOSY NMR spectrum of Ga-amyhelin in DMSO-$d_6$. 
Figure S4. TOCSY NMR spectrum of Ga-amycel in DMSO-$d_6$.

Figure S5. ROESY NMR spectrum of Ga-amycel in DMSO-$d_6$. 
Figure S6. Multiplicity-edited gHSQC NMR spectrum of Ga-amychealin in DMSO-d$_6$. Correlations of CH and CH$_2$ groups are shown in blue and red, respectively.

Figure S7. gHMBC NMR spectrum of Ga-amychealin in DMSO-d$_6$. 
Figure S8. $^1$H NMR spectrum of 2 in D$_2$O.

Figure S9. gCOSY NMR spectrum of 2 in D$_2$O.
Figure S10. TOCSY NMR spectrum of 2 in D$_2$O.

Figure S11. ROESY NMR spectrum of 2 in D$_2$O.
Figure S12. Multiplicity-edited gHSQC NMR spectrum of 2 in D$_2$O. Correlation of CH and CH$_2$ groups are shown in blue and red, respectively.

Figure S13. gHMBC NMR spectrum of 2 in D$_2$O.
Figure S14. EDTA competition assays with Fe-amychelin. Two additional data sets for titration of Fe-amychelin with EDTA are shown. The red lines describe fits to Eq. S1. These data sets together with the data in Fig. 2C yield a ΔpFeIII of +6.6 ± 1.6 for amychelin. See the methods section above for details.
**Table S1.** NMR spectral data for Ga-amyhelin in DMSO-$d_6$. The numbering scheme for amyhelin is shown above below.

![Amyhelin structure](image)

| C/H | δ$_H$ | Multiplicity (Hz) | δ$_C$ | HMBC     |
|-----|-------|-------------------|-------|----------|
| 1   |       |                   | 169.7 |          |
| 2a  | 3.30  | m                 | 49.6  |          |
| 2b  | 3.36  | m                 | 49.6  |          |
| 3   | 1.87  | m                 | 20.5  |          |
| 4a  | 1.65  | m                 | 25.3  |          |
| 4b  | 1.89  | m                 | 25.3  | C1       |
| 5   | 4.73  | m                 | 47.1  |          |
| 6 (N-H) | 7.87 | d, 9.6           | -     | C7       |
| 7   |       |                   | 173.0 |          |
| 8   | 4.62  | m                 | 52.1  | C7, C9, C10 |
| 9a  | 1.43  | m                 | 30.0  | C10      |
| 9b  | 1.66  | m                 | 30.0  | C10      |
| 10a | 1.06  | m                 | 18.5  |          |
| 10b | 1.64  | m                 | 18.5  |          |
| 11  | 3.29  | m                 | 51.9  | C9, C10  |
| 12  | 7.96  | s                 | 153.9 |          |
| 13 (N-H) | 7.56 | d, 8.5           | -     | C14      |
| 14  |       |                   | 169.8 |          |
| 15  | 3.96  | dt, 3.4, 6.1      | 58.2  | C14      |
| 16a | 3.63  | m                 | 61.7  |          |
| 16b | 3.74  | m                 | 61.7  |          |
| 17 (O-H) | 4.88 | t, 5.4           | -     | C15, C16 |
| 18 (N-H) | 8.65 | d, 6.5           | -     | C15, C19 |
| 19  |       |                   | 172.5 |          |
| 20  | 4.18  | m                 | 57.4  | C19, C21 |
| 21  | 3.53  | m                 | 60.9  | C19      |
| 22 (O-H) | 4.97 | t, 5.4           | -     | C20, C21 |
|       |       |       |       |
|-------|-------|-------|-------|
| 23 (N-H) | 8.47  | d, 4.6 | C20, C21, C24 |
| 24     |       |       | 170.6 |
| 25     | 4.18  | m     | 55.6  |
| 26a    | 3.63  | m     | 61.5  |
| 26b    | 3.82  | m     | 61.5  |
| 27 (O-H) | 4.84  | t, 5.9 | C26   |
| 28 (N-H) | 7.33  | d, 4.6 | C29   |
| 29     |       |       | 169.7 |
| 30     | 4.49  | dd, 3.6, 9.4 | 64.9 | C29 |
| 31a    | 4.53  | dd, 3.5, 8.9 | 72.2 | C32 |
| 31b    | 4.57  | t, 9.1  | 72.2  | C32 |
| 32     |       |       | 169.2 |
| 33     |       |       | 109.2 |
| 34     | 7.52  | dd, 1.8, 8.0 | 129.6 | C36, C38 |
| 35     | 6.57  | m     | 115.2 | C33, C37 |
| 36     | 7.31  | m     | 135.8 | C34, C38 |
| 37     | 6.67  | d, 8.4 | 122.6 | C32, C33, C35 |
| 38     |       |       | 168.7 |
**Table S2.** Single crystal X-ray diffraction data for Fe-amychelin<sup>a</sup>.

| **Crystal data**          |                                                |
|---------------------------|------------------------------------------------|
| Chemical formula          | C<sub>30</sub>H<sub>39</sub>O<sub>14</sub>N<sub>8</sub>Fe |
| M<sub>r</sub>             | 791.52                                         |
| Crystal system, space group | Monoclinic, C2                              |
| Temperature (K)           | 100                                            |
| a, b, c (Å)               | 25.9580 (6), 9.0942 (2), 21.4570 (6)           |
| β (°)                     | 119.7623 (13)                                 |
| V (Å<sup>3</sup>)         | 4397.14 (19)                                  |
| Z                         | 4                                              |
| Radiation type            | Cu Kα                                          |
| μ (mm<sup>-1</sup>)       | 4.58                                           |
| Crystal size (mm)         | 0.24 × 0.18 × 0.10                            |

| **Data collection**       |                                                |
|---------------------------|------------------------------------------------|
| Diffractometer            | CCD area detector diffractometer               |
| Absorption correction     | Multi-scan                                     |
|                           | SADABS                                          |
| Tmin, Tmax                | 0.406, 0.658                                   |
| No. of measured, independent and observed [I > 2σ(I)] reflections | 26861, 6576, 5405|
| R<sub>i</sub>             | 0.068                                          |

| **Refinement**            |                                                |
|---------------------------|------------------------------------------------|
| R(I/F<sup>2</sup> > 2σ(I/F<sup>2</sup>), S | 0.063, 0.178, 1.04 |
| No. of reflections        | 6576                                           |
| No. of parameters         | 608                                            |
| No. of restraints         | 2                                              |
| H-atom treatment          | H atoms treated by a mixture of independent and constrained refinement |
| Δρ<sub>max</sub>, Δρ<sub>min</sub> (e Å<sup>-3</sup>) | 0.69, -0.52 |
| Absolute structure        | Flack H D (1983), Acta Cryst. A39, 876-881     |
| Flack parameter           | 0.012 (7)                                      |

<sup>a</sup> Computer programs used: APEX2 v2009.3.0 (Bruker-AXS, 2009), SAINT 7.46A (Bruker-AXS, 2009), SHELXS97 (Sheldrick, 2008), SHELXL97 (Sheldrick, 2008), Bruker SHELXTL.
Table S3. NMR spectral data for \textit{2} in D$_2$O. The numbering scheme for \textit{2} is shown above below.

\begin{table}[h]
\centering
\begin{tabular}{lllll}
\hline
C/H & $\delta_H$ & Multiplicity (Hz) & $\delta_C$ & HMBC \\
\hline
1 & & & 172.7 & \\
2 & 4.41 & dd, 5.1, 7.6 & 53.2 & C1, C3 \\
3a & 1.86 & m & 28.1 & C4, C5 \\
3b & 1.96 & m & 28.1 & C4, C5 \\
4 & 1.81 & m & 19.5 & C3 \\
5 & 3.31 & m & 50.3 & C3, C4 \\
6 & 166.9 & & & \\
7 & 4.43 & dd, 4.9, 10.3 & 50.6 & C6, C8, C9 \\
8a & 1.81 & m & 26.7 & C6, C7 \\
8b & 2.03 & m & 26.7 & C6, C7 \\
9a & 1.93 & m & 20.0 & C7 \\
9b & 2.01 & m & 20.0 & \\
10a & 3.60 & m & 51.8 & \\
10b & 3.67 & dd, 4.5, 10.8 & 51.8 & C9 \\
11 & 8.23 & s & ~156 & \\
12 & & & 172.0 & \\
13 & 4.52 & m & 55.7 & C12, C14 \\
14 & 3.91 & m & 61.1 & C12, C13 \\
15 & & & 171.5 & \\
16 & 4.47 & t, 5.1 & 55.9 & C15, C17 \\
17 & 3.90 & m & 61.1 & C15, C16 \\
18 & & & 171.6 & \\
19 & 4.57 & t, 5.3 & 55.9 & C18, C20, C21 \\
20 & 3.88 & m & 61.1 & C18, C19 \\
21 & & & 167.3 & \\
22 & 4.64 & dd, 3.7, 4.7 & 52.2 & C21, C23 \\
23a & 4.83 & dd, 3.7, 12.6 & 62.9 & C21, C22, C24 \\
23b & 4.91 & dd, 4.9, 12.6 & 26.9 & C21, C22, C24 \\
24 & & & 168.7 & \\
25 & & & 112.1 & \\
26 & 7.91 & dd, 1.4, 7.9 & 130.6 & C24, C28, C30 \\
\hline
\end{tabular}
\end{table}
Table S4. Annotation of the amychelin biosynthetic cluster.\(^a\)

| Locus       | Gene | Predicted Function                        | Strand | Amino acids |
|-------------|------|-------------------------------------------|--------|-------------|
| SSMG_02531.1| amcR  | TetR-family transcriptional regulator     | –      | 597         |
| SSMG_02532.1| amcN  | Amino acid transporter                    | +      | 1491        |
| SSMG_02533.1| amcO  | Flavin-containing amine oxidase           | +      | 1362        |
| SSMG_02534.1| amcM  | Fe\(^{3+}\) periplasmic binding protein   | +      | 1233        |
| SSMG_02535.1| amcG  | Non-ribosomal peptide synthase            | –      | 3354        |
| SSMG_02536.1| amcF  | Non-ribosomal peptide synthase            | –      | 7089        |
| SSMG_02537.1| amcE  | Non-ribosomal peptide synthase            | –      | 4206        |
| SSMG_02538.1| amcD  | Non-ribosomal peptide synthase            | –      | 7002        |
| SSMG_02539.1| amcC  | mbtH-like protein                         | –      | 222         |
| SSMG_02540.1| amcB  | \(\alpha/\beta\) hydrolase                | –      | 807         |
| SSMG_02541.1| amcA  | Siderophore exporter                      | –      | 1176        |
| SSMG_02542.1| amcH  | Hydroxybenzoate-AMP ligase                | +      | 1548        |
| SSMG_02543.1| amcI  | Siderophore transport                      | +      | 984         |
| SSMG_02544.1| amcJ  | Siderophore transport                      | +      | 984         |
| SSMG_02545.1| amcL  | Salicylate synthase                       | –      | 1311        |
| SSMG_02546.1| amcK  | Lysine/ornithine-\(N\)-monooxygenase      | –      | 1281        |

\(^a\) Predicted functions are from FASTA, BLAST and InterProScan homology searches conducted between 7/15/2010-10/15/2010.
Table S5. Comparison of active site residues in A-domains in amychelin biosynthesis.\(^a\)

| A-domain     | Active site residues | Substrate                | Uniprot # |
|--------------|----------------------|--------------------------|-----------|
| amcD\(_A1\) | DVWHISLVVDK          | Ser                      | Q9RAH4\(^4\) |
| Nostopeptolide A | DVWHISLIDK         |                          |           |
| amcD\(_A2\) | DINYWGIGIK          | N-OH-\(N\)-formyl-Orn | Q9RK14\(^5\) |
| CDA antibiotic | DINYWGIGIK         |                          |           |
| amcE\(_A1\) | DAQEGGLVDK          | Gln                      | O66069\(^6\) |
| Lychenysin   | DAQLGVDK            |                          |           |
| amcF\(_A1\) | DVWHISLVDK          | Ser                      | Q9RAH4\(^4\) |
| Nostopeptolide A | DVWHISLIDK         |                          |           |
| amcF\(_A2\) | DVWHISLVDK          | Ser                      | Q9RAH4\(^4\) |
| Nostopeptolide A | DVWHISLIDK        |                          |           |
| amcG\(_A1\) | DLYNGLHIHK          | Cys                      | Q9RFM7\(^7\) |
| Pyochelin    | DLYNLSIWK           |                          |           |

\(^a\) Minor and major variations in the sequence are shown in italics and bold, respectively.
Supporting Information References

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