Dioxapyrrolomycin Biosynthesis in *Streptomyces fumanus*

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Streptomyces fumanus, intramurally coded as culture LL-F42248, produces a series of pyrrolomycins including dioxapyrrolomycin (**1**) as the principal component. Our biosynthetic studies revealed that feeding labeled acetate to growing cultures of *S. fumanus* yielded pyrrolomycins labeled in the phenyl ring only. When L-[*methyl*-^{13}C]methionine was fed, the labeled carbon atom was found in the methoxy group of pyrrolomycins H−J and in the methylenedioxy bridge of dioxapyrrolomycin. A $Na^{15}NO_3$-enriched medium was employed to produce ^{15}N-labeled pyrrolomycins in which both nitrogen atoms were highly enriched, whereas feeding of ^{15}N-labeled L-proline furnished pyrrolomycins labeled in the pyrrole moiety. Thus, *S. fumanus* elaborates the pyrrolomycin skeleton from proline and a polyketide precursor. Since the organism readily converted ^{13}C- or ^{15}N-labeled pyrrolomycin C, G, or H into the correspondingly labeled dioxapyrrolomycin, these minor pyrrolomycins are actually precursors of the ultimate product, dioxapyrrolomycin.

Our original work with culture LL-F42248 resulted in the isolation of the pyrrole-containing antibiotics dioxapyrrolomycin (**1**, first reported as F42248α) and pyrrolomycin C (**2**).[1] The name dioxapyrrolomycin was proposed in an independent publication of the same compound.[2] The discovery of this intriguing natural product triggered a research effort in our company that resulted in the chemical synthesis of novel insecticidal compounds, culminating in chlorfenapyr,[3] which was subsequently marketed as Pirate.[4] The producing organism, *Streptomyces fumanus* (LL-F42248), was noted for its ability to generate highly halogenated metabolites and, most remarkably, to nitrate the pyrrole[5] moiety. In view of the organism's unique ability to specifically and directly substitute the pyrrole with a nitro group derived from nitrate, we carried out studies with a series of stable isotope-labeled precursors to probe the biosynthesis of dioxapyrrolomycin (**1**). In the course of examining fermentation extracts of culture LL-F42248, we discovered that pyrrolomycins G−J (**3**−**6**)[6] were produced as minor metabolites. To understand their biosynthetic relevance with respect to the synthesis of **1**, we included these compounds in our analysis of label incorporation and transformation.

As noted, the biosynthetic mechanism for the introduction of the nitro group in dioxapyrrolomycin is of considerable interest. Our experiments have shown that nitration is favored—in the sense that relatively more **1** is formed in comparison to pyrrolomycin C—when nitrate is present in the medium.[5] Omitting chloride from the medium reduces pyrrolomycin production, but does not significantly alter the product profile. This organism's facility to incorporate halogen atoms in secondary metabolites rivals that of marine algae.[7] On a weight percentage basis, these compounds are among the most heavily halogenated compounds produced by Actinomycetes. As indicated previously,[5] the organism will synthesize both chlorinated and/or brominated metabolites depending upon the halide content of the medium. It is particularly noteworthy that in the presence of high levels of bromide the predominant product is pyrrolomycin F_1[8] rather than the corresponding brominated analogue of **1**. In this paper we report on the origin of the pyrrolomycin skeleton as well as the sequence of the later steps in the biosynthetic sequence leading to dioxapyrrolomycin (**1**).

Results and Discussion

Origin of the Pyrrolomycin Skeleton. Growing cultures of *S. fumanus* with [^{13}C]-enriched acetates labeled only the phenyl moiety of the pyrrolomycins, as shown by the incorporation data for **1** in Table 1. When [1-^{13}C]-acetate was included in the fermentation medium, C-8, -10, and -12 of **1** were significantly enriched. Similarly, the label derived from [2-^{13}C]-acetate was found in C-7, -9, and -11. Individual acetate units were defined by feeding [1,2-$^{13}C_2$]-acetate. The antibiotic obtained from the doubly labeled acetate experiment showed three pairs of coupled resonances, C-7, -8 ($J = 66.4 \pm 0.3$ Hz), C-9, -l0 ($J = 68.3 \pm 0.3$ Hz), and C-11, -12 (AB pattern, J not determined). The remaining portion of the carbon skeleton of **1** is derived from L-proline, as determined by incorporation experiments using [1-^{13}C]-, [2-^{13}C, ^{15}N]-, and [U-^{13}C]-labeled L-prolines. Enrichments obtained in **1** by including [1-^{13}C]-L-proline in the fermentation medium are summarized in Table 1. The exceptionally high enrichment observed for C-6 (>60 times natural abundance) strongly suggested intact incorporation of L-proline. This was confirmed by feeding uniformly ^{13}C-labeled L-proline, which yielded pyrrolomycins in which all pyrrole carbons and C-6 were highly enriched. In the ^{13}C NMR spectrum of **1** derived from the [U-^{13}C]-L-proline, resonances for C-2, C-3, C-4, C-5, and C-6 appeared as greatly enhanced multiplets in comparison to the remaining singlet carbon resonances of **1**, indicating that proline was incorporated as a complete unit. That the nitrogen atom of proline was not metabolized during the feeding experiments was corroborated by feeding of doubly labeled [2-^{13}C, ^{15}N]-L-proline. With the proline nitrogen atom becoming the pyrrole nitrogen of **1**, the ^{13}C NMR spectrum of double-labeled **1** showed a greatly enhanced doublet (enrichment factor = 28) for C-2 (d, $J_{NC} = 14.3$ Hz). As this spectrum displayed only a ^{13}C-doublet for C-2, but no ^{13}C-singlet NMR resonance, it was clear that L-proline was incorporated into **1** as an intact unit. Hence, L-proline plus three acetate residues are condensed to form the basic skeleton of dioxapyrrolmycin.

Final Steps in the Biosynthesis of Dioxapyrrolomycin. To probe the sequence of biosynthetic modifications leading to **1**, pyrrolomycin components labeled with ^{15}N or ^{13}C were prepared for reintroduction to growing cultures to track their incorporation. All of the incorporation experiments were followed by LC/MS analysis, under low-resolution conditions, which was adequate to determine the extent of enrichment in most instances. Fourier transform ion cyclotron resonance tandem mass spectrometry (FT-MS^n) was employed for the confirmation of both the extent and position of enrichment for selected compounds. The ultrahigh resolution routinely achieved in FTMS measurements can reveal the isotopic fine structure in great detail. One ppm accuracy in mass measurement makes the assignment of isotope peaks unambiguous. For the location of labeled atoms, specific ions can be isolated and then activated by collision with inert gas or by irradiation with a

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Table 1. Incorporation of ^{13}C into **1** as Determined by ^{13}C NMR[a] Spectroscopy

		1-^{13}C acetate	2-^{13}C	1,2-$^{13}C_2$ acetate	1-^{13}C proline	$^{13}CH_3$ -methionine
pos.	δ_C	EF[b]	EF[b]	*J* (Hz)	EF[b]	EF[b]
2	130.0	1.0	0.8		1.00[c]	1.0
3	131.9	1.2	0.7		0.9	1.2
4	106.0	1.0	0.6		0.9	1.0
5	116.4	1.3	0.5		1.2	1.0
6	69.8	0.7	0.9		60.9	1.0
7	124.5	0.9	4.1	66.3	1.1	1.9
8	148.7	6.8	0.6	66.6	1.0	1.3
9	123.0	1.9	6.6	68.1	1.2	1.7
10	129.9	6.7	0.9	68.5	0.8	1.0
11	126.5	1.7	4.9	AB	1.1	1.0
12	126.2	8.3	1.2		0.8	1.0
13	91.4	1.0	1.0		1.0	88.4

[a] Spectra of **1** recorded on acetone-d_6 solutions at 75 MHz. [b] Enrichment factor (EF) is the ratio of normalized signals between labeled and natural abundance spectra. [c] Signal is largely split into a doublet by coupling to C-6; intensity calculated by summation of peak height.

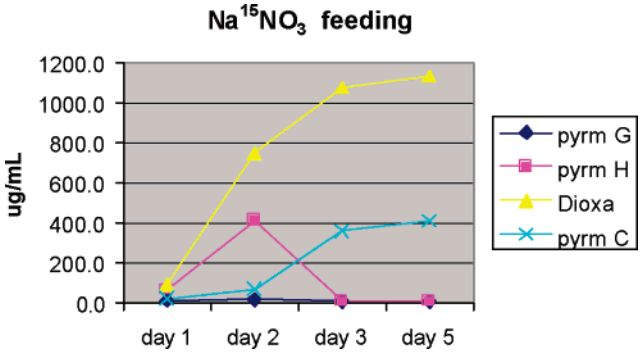
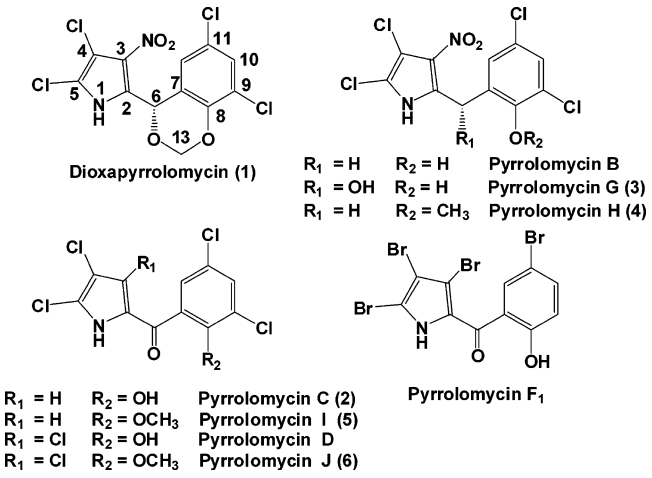


Figure 1. Time course of pyrrolomycin production in NO_3^- medium.

CO_2 laser to induce decomposition. The structures of the resulting fragment ions were deduced to establish the location of the enriched atoms.



Cultures of *S. fumanus* grown on a defined medium, with $Na^{15}NO_3$ as the sole nitrogen source, produced antibiotics **1**−**4**, in which each nitrogen atom was highly enriched by ^{15}N. The time course of production of **1**, **2**, **3**, and **4** in this medium is illustrated in Figure 1. To take advantage of the higher titers of **3** and **4** present early in the course of the fermentation, this material was harvested after 2 days of cultivation. Figure 2 illustrates the ^{15}N enrichment of **4** obtained in this experiment. The isolated ^{15}N-labeled pyrrolomycins **2**, **3**, and **4** (Table 2) were reintroduced to cultures of *S. fumanus* to track their metabolic fates.

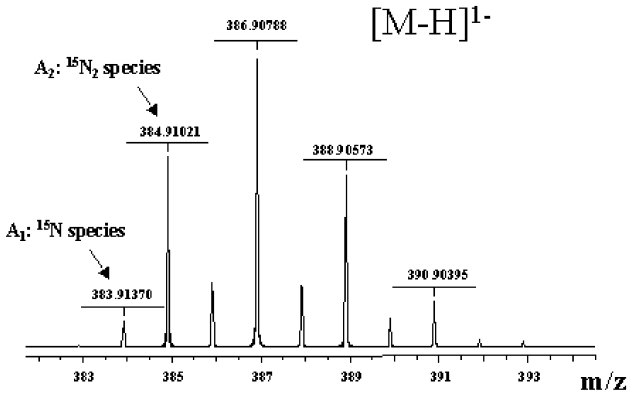


Figure 2. Mass scale-expanded segment of the negative mode ESI FTMS mass spectrum for ^{15}N-enriched pyrrolomycin H (**4**), showing the deprotonated molecular ion $[M - H]^-$. A_1 corresponds to $C_{12}H_7{}^{35}Cl_4{}^{15}N{}^{14}NO_4^-$ and A_2 to $C_{12}H_7{}^{35}Cl_4{}^{15}N_2O_4^-$. The proportion of the two ^{15}N isotopes was calculated based on the relative peak heights as follows: $(A_2 - 0.1359A_1)/(A_1 + A_2 - 0.1359A_1) = 88\%$.

Table 2. Percentage Enrichment Determined by MS

compound	precursor	^{15}N (position)	^{13}C at C-13
1	$^{15}NO_3$	87% (N-1 & $-NO_2$)	
2	$^{15}NO_3$	82% (N-1)	
3	$^{15}NO_3$	86% (N-1 & $-NO_2$)	
4	$^{15}NO_3$	88% (N-1 & $-NO_2$)	
1	^{15}N-**2**	8.3% (N-1)	
1	^{15}N-**4**	79% (N-1 & $-NO_2$)	
4	^{15}N-**3**	86% (N-1 & $-NO_2$)	
1	^{13}C-met		85%
4	^{13}C-met		85%
1	^{13}C-**4**		57%

When ^{15}N-labeled pyrrolomycin C (**2**) was fed, ^{15}N-labeled **1** was recovered with 8−18% retention of the ^{15}N label at N-1 depending on the time of harvest. A time course of this experiment monitored by LC/MS indicated initial heavy enrichment of the $^{15}N_1$ species at day 1, which was rapidly diluted through de novo biosynthesis between days 2 and 5. The specificity of the labeling at N-1 was firmly established by FTMS/MS experiments that showed none of the ^{15}N enrichment in the $-NO_2$ species. As shown in Figure 3, in the IRMPD FTMS spectrum of the dioxapyrrolomycin isolated in this experiment, the NO_2^- fragment ion was entirely at *m*/*z* 46, corresponding to the ^{14}N isotope (exptl, 45.99347, calcd, 45.99345, $\Delta = 0.02$ mmu). Although the extent of labeling in **1** was reduced during the course of production, it was also apparent that the pyrrolomycin C (**2**) detected after day 2 was devoid of ^{15}N enrichment. These results indicate essentially quantitative conversion of ^{15}N-labeled **2** to **1**.

Further, feeding of $^{15}N_2$ -labeled **3** produced $^{15}N_2$-labeled **4** with 86% ^{15}N enrichment at day 2 of the fermentation. The LCMS spectrum of the ^{15}N-labeled **4** (Figure 4b) reflects nearly complete incorporation of ^{15}N, as the envelope of the molecular ion region has shifted two mass units higher (compare Figure 4a), confirming that **3** is the biosynthetic precursor of **4**. The intermediacy of **4** was established in an analogous manner where ^{15}N-labeled **4** was converted to **1** in the course of a 5-day incorporation experiment.

The formation of the methylenedioxy bridge in dioxapyrrolomycin (**1**) was subsequently studied in more detail. Cultures of *S. fumanus* grown with L-[*methyl*-^{13}C]-methionine yielded **1**, **4**, **5**, and **6**, in which C-13 was >85% enriched in ^{13}C, confirming that the C-13 methyl group (or methylenedioxy group of **1**) is derived from methionine. The mechanism by which the methyl group of **4** is transformed into the methylenedioxy bridge of **1** was further examined by feeding L-[*methyl*-$^{13}CD_3$]-methionine. The dioxapyrrolomycin (**1**) thus obtained had molecular ions three nominal mass units higher (Figure 5b) than unlabeled material (Figure 5a),

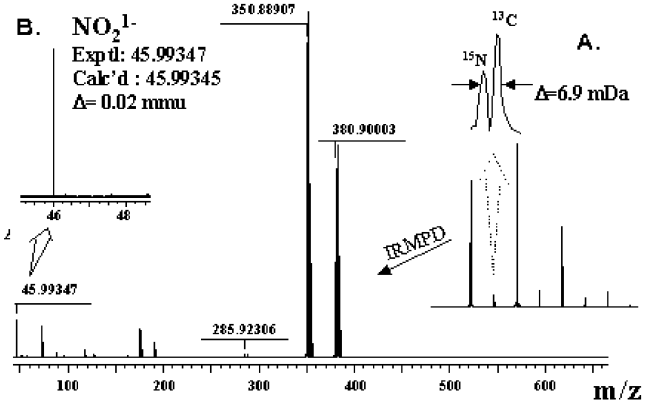


Figure 3. Negative mode ESI FTMS IRMPD mass spectrum for ^{15}N-enriched dioxapyrrolomycin (**1**) derived in the feeding experiment with ^{15}N-enriched pyrrolomycin C (**2**). ^{15}N-labeling was revealed by resolution of the ^{15}N species ($C_{12}H_5Cl_4N^{15}NO_4^-$) from the proximal ^{13}C species of the deprotonated molecular ion ($C_{11}{}^{13}CH_5Cl_4N_2O_4^-$), as shown in the inset A. The abundance of the ^{15}N peak (A_1) was 0.0981, relative to the monoisotopic ion peak (A_0) at *m*/*z* 381 as 1. The extent of ^{15}N-labeling was calculated as $(A_1 - 0.0074A_0)/(A_0 + A_1 - 0.0074A_0) = 8.31\%$. The monoisotopic nature of the NO_2^- fragment ion, generated by IRMPD, is illustrated in inset B, where only the ^{14}N species is significant.

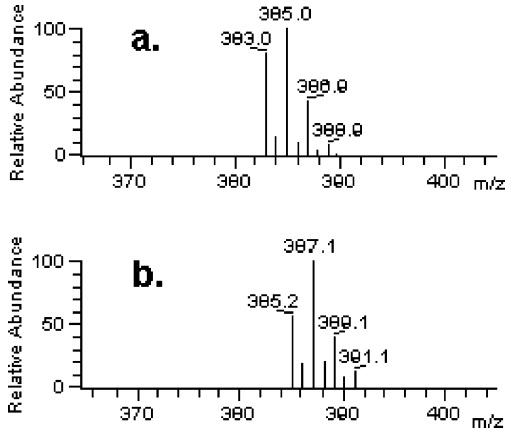


Figure 4. LCMS spectra of pyrrolomycin H (**4**), $[M - H]^-$: (a) unlabeled, (b) derived from ^{15}N-labeled **3**.

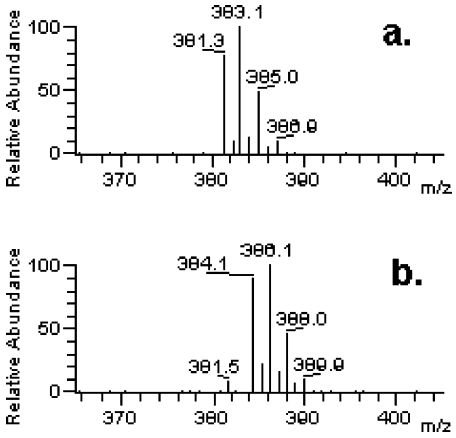
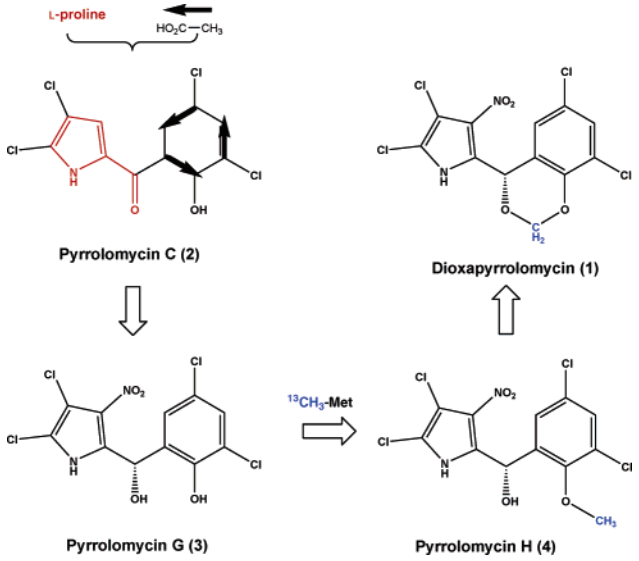


Figure 5. LCMS spectra of dioxapyrrolomycin (**1**), $[M - H]^-$: (a) unlabeled **1**, (b) $^{13}CD_2$-labeled **1** (derived from $^{13}CD_3$-labeled methionine).

demonstrating that the $^{13}CD_2$ unit is incorporated in the methylenedioxy bridge without significant hydrogen−deuterium exchange.

From these results a late stage biosynthetic sequence can be developed as outlined in Scheme 1. Conversion of pyrrolomycin C (**2**) to pyrrolomycin G (**3**) initiates the production of dioxapyrrolomycin through nitration and ketone reduction. Pyrrolomycin G (**3**) is then O-methylated by *S*-adenosylmethionine to give pyrrolomycin H (**4**). Oxidative cyclization of the methyl group resulting in acetal formation finally yields the methylenedioxy bridge of dioxapyrrolomycin (**1**).

Scheme 1. Final Steps in the Biosynthetic Pathway of Diaoxapyrrolomycin (**1**)



Conclusion

Feeding [1-^{13}C]-, [2-^{13}C]-, or [1,2-$^{13}C_2$]-acetate yielded **1** where ^{13}C enrichment occurred only in the phenyl ring, indicating its derivation via a triketide precursor, while feeding variously labeled L-prolines revealed that the pyrrole ring and the bridging carbon C-6 of **1** are derived from L-proline. These results are consistent with the presence of a PKS/NRPS biosynthetic pathway in *S. fumanus* analogous to the reported gene cluster responsible for the biosynthesis of pyoluteorin,[9,10] which incorporates the same skeleton as dioxapyrrolomycin.

Although the biosynthesis of the dioxapyrrolomycin skeleton is essentially complete with the generation of pyrrolomycin C (**2**), its nitration and further elaboration are unique to the biosynthesis of **1**. The complete conversion of labeled **2** to labeled **1** demonstrates unambiguously that **2** is a real intermediate and not simply a shunt product of the biosynthesis. Therefore, **2** is the most likely candidate to undergo further modification, but whether it is the exact substrate used for the direct biochemical nitration is still debatable. Since no nitrated intermediates have been isolated that contain a ketone at C-6, its reduction either precedes the nitration reaction or is concomitant with it. This reasoning predicts that pyrrolomycins D,[11] F,[8] I,[6] and J,[6] all C-6-keto derivatives, are shunt products derived from competing halogenations at C-3. Similarly, pyrrolomycin B formation can be viewed as an over-reduction at C-6 as a result of the coupled nitration−reduction process.

The biosynthesis of dioxapyrrolomycin is completed following the reductive nitration of **2** yielding **3**, which contains the required 6-hydroxyl group. Methylation of the phenolic hydroxyl group generates **4**. This O-methyl transferase can methylate variously substituted pyrrolomycins, as evidenced by compounds **4**, **5**, and **6**, independent of the substitution status at C-6. Feeding of ^{15}N-labeled **3** confirmed that **4** is indeed derived from **3**, while feeding experiments using ^{13}C- and ^{15}N-labeled **4** established unequivocally that **4** is the ultimate precursor of **1**.

Our experiments with doubly labeled L-[*methyl*-$^{13}CD_3$]-methionine established that the C-13 methyl group in **4** is converted to the methylendioxy bridge of **1** without significant exchange of the

deuterium label. This result is consistent with the reported oxidative cyclization mechanism leading to methylenedioxy bridge formation in berberine studied in plant cell culture.[12] Among the antibiotics produced by Actinomycetes there are only three classes of compounds that feature a 4*H*-1,3-dioxine ring, i.e., dioxapyrrolomycin, hexacyclic xanthones such as simaomicin[13] and lysolipin,[14] and the streptovaricins.[15,16] Although reports on their biosyntheses did include the observations that methionine is the precursor for the bridging methylendioxy group, they did not elaborate on a cyclization mechanism. Natural products possessing methylendioxy bridges are more common among plant metabolites, especially those that bear 1,3-dioxole rings resulting from the initial methylation of dopamine moieties in alkaloids such as reticuline or scoulerine and subsequent action of a cytochrome P-450 enzyme complex.[17] It is important to note that there are several regiospecific methylendioxy bridge-forming P-450 enzymes involved in berberine biosynthesis.[17] While a specific oxidative enzyme is suspected to be responsible for constructing the methylendioxy bridges of the antibiotics mentioned above, none have yet been reported in the literature.

Experimental Section

General Experimental Procedures. An HPLC system with diode array detection employing a YMC ODS-A (4.6 × 250 mm, 5 μm) C_{18} HPLC column using a linear gradient from 30 to 90% mobile phase B (0.01% TFA in CH_3CN) mixed with mobile phase A (0.01% TFA in water) over 30 min was used to analyze the pyrrolomycins and other F42248 culture extracts. Preparative purification of the pyrrolomycins was achieved by using a semipreparative YMC ODS-A column (1 × 25 cm, 5 μm) and a flow rate of 4 mL/min instead of 1 mL/min, under otherwise identical conditions.[6] UV spectra were recorded concomitantly during the analyses. LC/MS data were obtained on a Thermoquest-Finnigan LCQ Deca instrument equipped with a diode array detector and a YMC ODS-A column (0.2 × 10 cm, 3 μm; flow 0.3 mL/min (solvent A: 0.025% formic acid in water; solvent B: 0.025% formic acid in CH_3CN; gradient: 5% B to 95% B into A over 15 min, then holding for 10 min); the MS detection system uses alternating positive and negative ESI modes. All 1D and 2D NMR spectra were recorded on a Bruker DPX-400 spectrometer at 400 and 100 MHz for ^1H and ^{13}C, respectively, using a 3 mm broadband probe, unless noted otherwise. Proton-detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{C-H} = 140$ Hz) and HMBC (optimized for $^nJ_{C-H} = 8.3$ Hz) pulse sequences. High-resolution mass spectra (HRMS) were obtained using a Bruker (Billerica, MA) APEXII FT-ICR mass spectrometer equipped with an actively shielded 9.4 T superconducting magnet (Magnex Scientific Ltd., UK), an external Bruker APOLLO ESI source, and a Synrad 50W CO_2 CW laser. The molecular ions were isolated using correlated sweep and then dissociated using infrared multi-photon dissociation (IRMPD). All HPLC solvents were EM Omnisolv quality and used without further purification.

Fermentation. General fermentation conditions were as described in our previous publication.[6] Changes pertaining to the biosynthetic studies are noted in the respective sections below. As described in previous reports,[5,6] polyamide resin was added to the fermentation broth for the facile isolation of the pyrrolomycins. The resin plus cell mass was extracted with acetone, the extract was concentrated, and the individual pyrrolomycin components were subsequently purified from this crude complex by reversed-phase HPLC.

Labeled Acetate and L-Proline Incorporation Experiments. In separate experiments [1-^{13}C]-sodium acetate, [2-^{13}C]-sodium acetate, and [1,2-$^{13}C_2$]-sodium acetate were added to the production medium 48 h post-inoculation, at a final concentration of 10 mM. In the proline experiments the labeled precursors were substituted for the unlabeled L-proline and incorporated with the same timing, at final concentrations of 2 mM. The fermentations were harvested after a total of 5 days. The yield of dioxapyrrolomycin in these experiments was typically 250 mg/L.

$Na^{15}NO_3$ Incorporation. The production medium was modified by substituting $Na^{15}NO_3$ for the unlabeled salt, and proline was omitted; the total volume was 2.0 L. The fermentation was harvested after 2 days. Following HPLC purification the ^{15}N-labeled components were obtained and then analyzed by FTMS. As an example, the FTMS data for ^{15}N-enriched pyrrolomycin H (**4**) are shown in Figure 2 with the description of how the incorporation rate is calculated. The ^{15}N-labeled incorporation rates for **1**, **2**, **3**, and **4** are summarized in Table 2.

$^{15}N_2$-Dioxapyrrolomycin (1): 482 mg. Two relevant ions were detected in the negative mode ESI FTMS mass spectrum with *m*/*z* 381.89776 and 382.89475, corresponding to the deprotonated molecular ions for ^{15}N-labeled **1** (calcd for $C_{12}H_5Cl_4N^{15}NO_4^-$ 381.89793) and $^{15}N_2$-labeled **1** (calcd for $C_{12}H_5Cl_4{}^{15}N_2O_4^-$ 382.89496), respectively. No unlabeled **1** was detected. The proportion of the $^{15}N_2$-labeled species was 87% based on the observed relative peak heights for the *m*/*z* 382 and 383 ions, and 13% as the mono-^{15}N-labeled **1**. In FTMS/MS experiments on the deprotonated molecular ion of the mono-^{15}N-labeled **1** at *m*/*z* 382, the only NO_2^- species detected corresponded to the $^{15}NO_2^-$ fragment ion, indicating that the nitro group was entirely ^{15}N enriched and that therefore the residual ^{14}N is at the N-1 position. Fragment: *m*/*z* 180.93595, calcd for $C_4HCl_2{}^{15}N_2O_2^-$ (180.93613). ^{15}N NMR-(acetone): 165.2 ppm (*N*-1), 363.4 ppm (NO_2).

^{15}N-Pyrrolomycin C (2): 40 mg. Two relevant ions were detected in the negative mode ESI FTMS mass spectrum with *m*/*z* 321.90005 and 322.89713, corresponding to the deprotonated molecular ions for unlabeled **2** (calcd for $C_{11}H_4Cl_4NO_2^-$ 321.90016) and ^{15}N-labeled **2** (calcd for $C_{11}H_4Cl_4{}^{15}NO_2^-$ 322.89713), respectively. The proportion of the ^{15}N-labeled species was 82% based on relative peak heights. Fragment: *m*/*z* 134.95376, calcd for $C_4H_2Cl_2{}^{15}N^-$ 134.95292. ^{15}N NMR-(acetone): 158.3 ppm (*N*-1).

$^{15}N_2$-Pyrrolomycin G (3): 10 mg. Three relevant ions were detected in the negative mode ESI FTMS mass spectrum with *m*/*z* 368.90164, 369.89816, and 370.89478, corresponding to the deprotonated molecular ions for unlabeled **3** (calcd for $C_{11}H_5Cl_4N_2O_4^-$ 368.90089), ^{15}N-labeled **3** (calcd for $C_{11}H_5Cl_4N^{15}NO_4^-$ 369.89793), and $^{15}N_2$-labeled **3** (calcd for $C_{11}H_5Cl_4{}^{15}N_2O_4^-$ 370.89496), respectively. The proportion of the $^{15}N_2$-labeled **3** species was 86% based on the observed relative peak heights for the *m*/*z* 369, 370, and 371 ions, and 12% as the mono-^{15}N-labeled **3**. Fragment: *m*/*z* 180.93564, calcd for $C_4HCl_2{}^{15}N_2O_2^-$ 180.93613.

$^{15}N_2$-Pyrrolomycin H (4): 181 mg. Two relevant ions were detected in the negative mode ESI FTMS mass spectrum with *m*/*z* 383.91370 and 384.91021, corresponding to the deprotonated molecular ions for the ^{15}N-labeled **4** (calcd for $C_{12}H_7Cl_4N^{15}NO_4^-$ 383.91358) and $^{15}N_2$-labeled **4** (calcd for $C_{12}H_7Cl_4{}^{15}N_2O_4^-$ 384.91061), respectively. No unlabeled **4** was detected. The proportion of the $^{15}N_2$-labeled **4** species was 88% based on the observed relative peak heights for the *m*/*z* 384 and 385 ions, and 12% as the mono-^{15}N-labeled **4**. Fragment: *m*/*z* 180.93643, calcd for $C_4HCl_2{}^{15}N_2O_2^-$ 180.93613. ^{15}N NMR(acetone): 156.6 ppm (*N*-1), 361.2 ppm (NO_2).

^{15}N-Pyrrolomycin C (2) Feeding. Purified ^{15}N-enriched pyrrolomycin C (82% ^{15}N) was added to the production medium at a concentration of 0.04 mg/mL at the time of inoculation, and the fermentation was harvested after 3 days. The dioxapyrrolomycin isolated contained 8.3% ^{15}N label specifically at N-1.

^{15}N-Pyrrolomycin G (3) Feeding. Purified ^{15}N-enriched pyrrolomycin G (86% $^{15}N_2$) was added to the production medium at the time of inoculation at a concentration of 0.18 mg/mL, and the fermentation was harvested after 2 days

^{15}N-Pyrrolomycin H (4) Feeding. Purified ^{15}N-enriched pyrrolomycin H was added to the production medium at the time of inoculation at a concentration of 0.15 mg/mL, and the fermentation was harvested after 1 day.

L-[*methyl*-^{13}C]-Methionine Feeding. A solution of L-[*methyl*-^{13}C]-methionine at a final concentration of 10 mM was added at the time of inoculation to 1 L of the production medium described above. The fermentation was harvested after 2 days. Pyrrolomycin H (23 mg) and dioxapyrrolomycin (157 mg) were isolated.

^{13}C-Dioxapyrrolomycin. Deprotonated molecular ions for both unlabeled and mono-^{13}C-labeled **1** were detected in the FTMS mass spectrum with *m*/*z* 380.90086 (calcd for $C_{12}H_5Cl_4N_2O_4^-$ 380.90089) and *m*/*z* 381.90414 (calcd for $C_{11}{}^{13}CH_5Cl_4N_2O_4^-$ 381.90426), respectively. Both ions were isolated in the ICR cell using correlated sweep and then fragmented using an infrared multi-photon dissociation (IRMPD). The same abundant fragment ion *m*/*z* 351 was detected in the IRMPD mass spectrum for both isolated precursor ions. On the basis of the assigned elemental composition from the accurate mass, this fragment ion *m*/*z* 351 was formed through the facile loss of formaldehyde from the methylenedioxy group, indicating that this functional group carried the entire ^{13}C-enrichment. The extent of ^{13}C-labeling for **1** therefore could be calculated as follows: $(A_2 - 0.1358A_1)$/

$(A_1 + A_2 - 0.1358A_1) = 85\%$ where A_1 (=0.1722) and A_2 (=1) are the observed relative peak heights for the *m*/*z* 381 and 382 ions, respectively, and the coefficient 0.1358 is the calculated ratios of the peak heights of a second (+1 Da) isotopic peak to the monoisotopic peak of the unlabeled **1** calculated using Bruker software Xmass (version 5.02).

^{13}C-Pyrrolomycin H (4). The extent of ^{13}C-labeling for H could be calculated as follows:

$$(A_2 - 0.1358A_1)/(A_1 + A_2 - 0.1358A_1) = 85\%$$

where A_1 (=0.169) and A_2 (=0.953) are the observed relative peak heights for the *m*/*z* 383 (no labeling) and 384 (C-13 labeling) peaks, respectively.

L-[*methyl*-^{13}CD$_3$]-Methionine Feeding. A solution of L-[*methyl*-^{13}CD$_3$]-methionine at a final concentration of 8.1 mM was added at the time of inoculation to 0.15 L of the production medium described above. The cultures were harvested after 46 h incubation, then extracted and concentrated for LC/MS analyses.

[^{13}C-13]Pyrrolomycin H (4) Feeding. Purified [^{13}C-13]-pyrrolomycin H (85%) was added to the production medium (50 mL) at a concentration of 0.2 mg/mL at the time of inoculation, and the fermentation was harvested after 2 days. The product was analyzed as a crude extract by FTMS without HPLC purification. The dioxapyrrolomycin analyzed in this sample contained 57% ^{13}C-enrichment exclusively in the methylenedioxy group.

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