# Additional Pyrrolomycins from Cultures of *Streptomyces fumanus*

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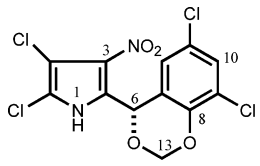
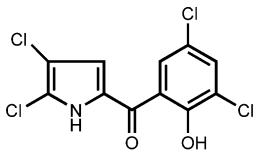
Along with dioxapyrrolomycin (**1**), four new pyrrolomycin antibiotics, namely, pyrrolomycin G (**3**), pyrrolomycin H (**4**), pyrrolomycin I (**5**), and pyrrolomycin J (**6**), were produced in cultures of *Streptomyces fumanus*. Apart from dioxapyrrolomycin, pyrrolomycin G and pyrrolomycin H are the only other chiral members of the pyrrolomycin family of antibiotics, and their absolute stereochemistry was deduced to be 13*S*. Here, we report the isolation, structure elucidation, and antimicrobial activity of these new pyrrolomycins.

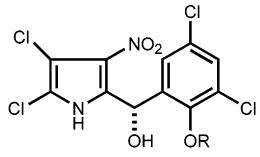
Owing to our interest in understanding how biological systems perform nitrations of organic compounds, we have carried out fermentation experiments with *Streptomyces fumanus* (culture LL-F42248) to study the production of dioxapyrrolomycin (**1**, originally reported as F42248α[1]). In addition to culture LL-F42248, pyrrolomycin-type antibiotics have reportedly been produced by other *Streptomyces* sp.,[1−3] *Actinosporangium* sp.,[4−6] and an unidentified actinomycete.[7,8] All pyrrolomycins can be considered derivatives of pyrrolomycin A,[7] a nitrated dichloropyrrole that is typically linked to a substituted phenyl group via a methine or methylene bridge, except for pyrrolomycin E,[4] which lacks the bridging carbon. A characteristic feature of the pyrrolomycins is their high halogen content, which is usually represented by four chlorine substituents, with the exception of pyrrolomycin D, which has five chlorine, and pyrrolomycin E, which carries three chlorine atoms. Replacement of up to three chlorine atoms by bromine has been described for pyrrolomycins $F_1$−$F_3$.[5] Reportedly, all pyrrolomycins exhibit antimicrobial activity, while anthelmitic activity[9] has been mentioned less frequently. While previous work with culture LL-F42248 resulted in the isolation of **1** and **2** (pyrrolomycin C),[1] our present investigation has yielded four additional pyrrolomycins (**3**−**6**) that we wish to report here.



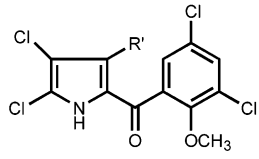
Dioxapyrrolomycin (**1**)



Pyrrolomycin C (**2**)



Pyrrolomycin G (**3**) R = H
Pyrrolomycin H (**4**) R = $CH_3$



Pyrrolomycin I (**5**) R' = H
Pyrrolomycin J (**6**) R' = Cl

The harvested pellet from a 5-day fermentation of culture LL-F42248 (500 mL) was treated with acetone, concentrated, and subsequently extracted with ethyl acetate. The crude extract was then purified by reversed-phase HPLC. This procedure yielded pyrrolomycin G (**3**, 20 mg) as a yellow solid together with F42248α (**1**, 80 mg). Pyrrolomycin H (**4**, 14 mg) was obtained as a yellow solid, but this required a second reversed-phase HPLC step. Although small quantities of pyrrolomycins I (**5**) and J (**6**) were also obtained during the chromatography, these were not purified further. Later, pure samples of **5** (0.7 mg) and **6** (0.5 mg) were isolated from a second fermentation, where $^{13}$C-methyl-L-methionine was fed to the culture in an otherwise identical procedure. Pyrrolomycin C (**2**, 7.5 mg) was also purified from this second fermentation.

Compounds **3**−**6** were characterized by standard spectroscopic methods. The UV spectra of **1**, **3**, and **4** with absorption maxima at 210, 226 (sh), 282, and 342 nm suggested that their chromophores were identical. Further, LCMS analysis of **3**, **4**, **5**, or **6** revealed a typical cluster of ions due to isotopic distribution of four or five chlorine atoms and was therefore indicative of a pyrrolomycin derivative.

Structure **4** revealed such an ion cluster between 383 and 389 due to four chlorine atoms, indicating that **4** was related to **1**. A difference between **4** and **1**, however, was observed in their $^1$H and $^{13}$C NMR spectra. The resonances for the C-13 methylene group ($\delta_H$ 5.54 and $\delta_C$ 91.4) in the $^1$H and $^{13}$C NMR of **1** were absent, and instead, a methyl group resonance at $\delta_H$ 3.86 and $\delta_C$ 61.9 was found in its place, suggesting that **4** was the ring-open version of **1**. This contention was supported by high-resolution Fourier transform ion cyclotron resonance (HRFT-ICR) mass spectrometry, which established the molecular formula of **4** as $C_{12}H_8Cl_4N_2O_4$ (*m/z* 382.91661 $[M - H]^-$ calcd 382.91654) for the monoisotopic species. The complete assignment of the $^1$H and $^{13}$C NMR data of **4**, acquired in acetone-$d_6$ (Table 1), was as follows. The two aromatic resonances $\delta_H$ 7.43 (H-10, $J$ = 2.6 Hz) and 7.28 (H-12, $J$ = 2.6 Hz) were placed meta to each other due to the observed coupling constants, flanking C-11 ($\delta_C$ 122.9), to which both protons showed two-bond HMBC correlations. Further, mutual HMBC correlations were noted between H-10 and C-12 ($\delta_C$ 127.8) and H-12 and C-10 ($\delta_C$ 130.5). Both protons also correlated to the oxygen-bearing carbon C-8 ($\delta_C$ 154.0), with further observable correlations from H-12 to C-7 ($\delta_C$ 138.7) and from H-10 to the two chlorinated quaternary carbons C-9 ($\delta_C$ 129.0) and C-11, thus fixing the assignment for the phenyl moiety. The four remaining quarternary $^{13}$C-resonances of **4** at $\delta_C$ 105.9, 115.1, 129.1, and 136.6 were attributed to the pyrrole moiety. Especially useful in assigning these resonances were HMBC correlations from the oxymethine H-6 ($\delta_H$ 6.69) to C-2 ($\delta_C$ 136.6) and C-3 ($\delta_C$-

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**Table 1.** NMR Spectral Data for **3**−**5** Recorded in Acetone-$d_6$ at 400 MHz

| | **3** | | **4** | | | **5** | |
|---|---|---|---|---|---|---|---|
| pos. | $\delta_C$ | $\delta_H$ (mult., $J$ = Hz) | $\delta_C$ | $\delta_H$ (mult., $J$ = Hz) | HMBC | $\delta_C$ | $\delta_H$ (mult., $J$ = Hz) |
| 1 | | 12.20 (br s) | | 12.20 (br s) | | | 12.2 0 (br s) |
| 2 | 136.5 | | 136.6 | | | 130.6 | |
| 3 | 129.7 | | 129.1 | | | 121.0 | 6.79 (s) |
| 4 | 105.8 | | 105.9 | | | 91.7 | |
| 5 | 114.9 | | 115.1 | | | 123.3 | |
| 6 | 64.9 | 6.74 | 65.1 | 6.69 (s) | C2, C3, C7, C8, C12 | 181.2 | |
| 7 | 131.8 | | 138.7 | | | 133.1 | |
| 8 | 150.4 | | 154.0 | | | 154.3 | |
| 9 | 125.1 | | 129.0[a] | | | 130.8 | |
| 10 | 129.8 | 7.36 (d, 2.4) | 130.5 | 7.43 (d, 2.6) | C8, C9, C11, C12 | 134.0 | 7.69 (d, 2.6) |
| 11 | 122.4 | | 129.9[a] | | | 130.1 | |
| 12 | 127.5 | 7.25 (d, 2.4) | 127.8 | 7.28 (d, 2.6) | C6, C7, C8, C10, C11 | 129.9 | 7.46 (d, 2.6) |
| 13 | | | 61.9 | 3.86 (s) | | 64.5 | 3.81 (s) |
| 6-OH | | 11.97 (br s) | | 11.9 (br s) | | | |
| 8-OH | | 11.70 (br s) | | | | | |

[a] Assignments may be interchanged.

129.1), which corroborated the assignment of C-2 and C-3. The resonances at $\delta_C$ 105.9 and 115.1 were assigned to C-4 and C-5, respectively, based on the following C−N coupling between N-1 and C-4 and N-1 and C-5 observed on a sample of $^{15}$N-labeled pyrrolomycin H (**4**) (a detailed study on labeled pyrrolomycins and a biosynthetic scheme will be published separately). The $^{13}$C NMR spectrum of $^{15}$N-labeled **4** showed three resonances that appeared as doublets due to direct coupling of $^{13}$C with $^{15}$N. Two of these, C-2 ($\delta_C$ 136.6, $J_{CN}$ = 14.0 Hz) and C-3 ($\delta_C$129.1, $J_{CN}$ = 23.2 Hz), were already assigned with observations discussed above. The third doublet at $\delta_C$ 115.1 belonged to C-5 ($^1J_{CN}$ = 16.3 Hz) since this was the only remaining carbon bound to nitrogen. C-4 ($\delta_C$ 105.9) showed a small coupling of 4.5 Hz appropriate for two-bond C−N interactions. The connection between the pyrrole moiety and the phenyl ring was evident by HMBC correlations from H-6 to both C-2 and C-3 (pyrrole moiety) as well as to C-7, C-8, and C-12 (phenyl moiety).

The NMR spectra of pyrrolomycin G (**3**) lacked a resonance for either a methyl or methylene group, suggesting that this group was absent. HRFT-ICR mass spectrometry of **3** established the molecular formula of $C_{11}H_6Cl_4N_2O_4$ (*m/z* 368.90082 $[M - H]^-$ calcd 368.90089) for the monoisotopic species, confirming that the difference between **3** and **1** was a methylene group, as inferred from the NMR data. This was also supported by the differences in chemical shifts of C-7, C-8, C-9, and C-11 (Table 1).

LCMS analysis of pyrrolomycin I (**5**) revealed the familiar molecular ion cluster between 336 and 342, due to the isotopic distribution of four chlorine atoms. HRFT-ICR mass spectrometry readily established the molecular formula of **5** as $C_{11}{}^{13}CH_7Cl_4NO_2$ (*m/z* 336.91926 $[M - H]^-$ calcd for 336.91917) for the $^{13}$C-monoisotopic species, indicating that this molecule lacked the usual nitro group. Thus, the $[C_4H_2Cl_2N]^{1-}$ fragment ion at *m/z* 133.95695 (calcd 133.95698) became diagnostic for the substitution pattern at the pyrrole unit, still revealing the presence of two chlorine atoms but the absence of the nitro group here. Another fragment ion observed at *m/z* 175.97534 (calcd 175.97565), representing a composition of $[C_6{}^{13}CH_5Cl_2O]^-$, was identical to that observed for **4** and implied that the substitutions at the phenyl moiety were identical for both compounds. However, the UV and NMR spectra of **4** and **5** were clearly distinguished. The UV spectrum of **5** showed absorption maxima at $\lambda_{max}$ 206 and 314 nm, the same chromophore as seen with pyrrolomycin C (**2**). The $^1$H NMR spectrum revealed the presence of a proton at C-3 (singlet at $\delta_H$ 6.79) that was attached (HSQC) to a carbon at $\delta_C$ 121.0, replacing the nitro group present in **4**. Furthermore, the presence of a carbonyl group, instead of the oxymethine group present in **4**, was indicated by a carbon resonance at $\delta_C$ 181.0 (C-6) in the $^{13}$C NMR spectrum, leading to the structure assignment of pyrrolomycin I (**5**) as shown. Therefore, pyrrolomycin I (**5**) is the naturally occurring methylated form of pyrrolomycin C (**2**).

The structure of pyrrolomycin J (**6**) was elucidated in a similar manner. HRFT-ICR mass spectrometry established the molecular formula of **6** as $C_{11}{}^{13}CH_6Cl_5NO_2$ (*m/z* 370.88038 $[M - H]^-$ calcd 370.88020) for the $^{13}$C-monoisotopic species, suggesting that in this compound the nitro group was replaced by chlorine. This was also reflected in the composition of a fragment ion representing the pyrrole unit at *m/z* 167.91808 (calcd 167.91800) that consisted of $[C_4HNCl_3]^-$ demanding a pyrrole that was substituted with three chlorine atoms. By comparison to **5**, the $^1$H NMR spectrum indicated that the singlet at $\delta_H$ 6.79 (H-3) was absent in **6** and must have been replaced by a chlorine atom. Otherwise, both the $^1$H and $^{13}$C NMR data of **5** and **6** were virtually identical, leading to the conclusion that **6** is the chlorinated analogue of **5**. Therefore pyrrolomycin J (**6**) is the naturally occurring methylated form of pyrrolomycin D.[4]

Among the members of the pyrrolomycin family, only dioxapyrrolomycin (**1**), pyrrolomycin G (**3**), and pyrrolomycin H (**4**) possess a chiral center. The absolute stereochemistry of **1** at its C-13 chiral center was previously determined to be ***S*** from the crystal structure of its *N*-methyl derivative.[2] For **3** and **4**, we applied CD spectroscopy to deduce the absolute stereochemistry at the chiral carbon C-13 and compared them with **1**. The CD spectrum of **1** has a negative elipticity at 202 ($\Delta\epsilon$ −13) and a positive elipticity at 215 ($\Delta\epsilon$ +12) nm. Likewise, **3** and **4** display CD spectra with minima/maxima at 197 ($\Delta\epsilon$ −6.6), 209 ($\Delta\epsilon$ +3.4) nm and 197 ($\Delta\epsilon$ −44), 211 ($\Delta\epsilon$ +39) nm, respectively, suggesting that **1**, **3**, and **4** have the same absolute stereochemistry at C-13.

The four new pyrrolomycins (**3**−**6**) were tested against selected Gram-positive and Gram-negative bacteria and showed modest in vitro activity. The minimum inhibitory concentrations (MICs), obtained by the standard broth dilution method ranged between 1 and 32 $\mu$g/mL (see Table 2). From CD studies, the absolute stereochemistry of **3** and **4** was deduced to be 13***S***, the same configuration as that reported for **1**.[2] Except for **6**, the new pyrrolomycins reported here can be considered biosynthetic precursors of **1**. Conclusions on the biosynthetic pathway of the pyrrolomycins will be reported separately.

**Table 2.** Antimicrobial Data for **3**, **4**, and **6** (MIC in $\mu$g/mL, Agar Dilution Method)[a]

| | 3 | 4 | 6 |
|---|---|---|---|
| *S. aureus* WT | 8 | 1 | 1 |
| *S. aureus* MRSA | 8 | 2 | 2 |
| *E. faecium* VR | 8 | NT | NT |
| *E. coli* WT | >128 | 128 | >128 |
| *E. coli* imp | 32 | 4 | 8 |

[a] WT: wild type, MRSA: menthicillin resistant, VR: vancomycin resistant, imp: permeability mutant, NT: not tested.

## Experimental Section

**General Experimental Procedures.** UV data were obtained on an HP1100 HPLC system equipped with a diode array detector (DAD). All 1D and 2D NMR spectra were recorded on a Bruker DPX-400 spectrometer at 400 and 100 MHz for $^1$H and $^{13}$C, respectively, using a 3 mm broadband probe. 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), 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.** Culture LL-F42248 was maintained on agar plates stored at 4 °C or as frozen aliquots of vegetative growth in a liquid medium containing 10% glycerol. For the first-stage seed, the culture was inoculated into a medium (7 mL) containing 1% glucose, 2% soluble starch, 0.5% yeast extract, 0.5% N-Z Amine type A, and 0.1% $CaCO_3$. After cultivation for 3 days at 28 °C at 200 rpm, this primary seed was used as the inoculum into 50 mL of the same medium and incubated for 24 h at 28 °C at 200 rpm. The second stage seed was used as a 5% inoculum for a 50 mL fermentation flask. The production medium consisted of 3% glucose, 0.1% $NaNO_3$, 0.01% $K_2HPO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.2% $CaCO_3$, 0.1% KCL, 0.1% proline, 0.08% KF, and 1.5% nylon particles (Machery Nagel MN-Polamid SC6). The fermentation was incubated at 28 °C at 200 rpm for 5 days.

**Isolation and Purification.** The harvested fermentation broth (500 mL) was centrifuged at 4000 rpm for 20 min, and the polyamide resin together with the cell mass was extracted twice with acetone (300 mL each). The combined acetone extracts were concentrated in vacuo, and the resulting aqueous suspension was extracted three times with ethyl acetate (100 mL each). The residue of the ethyl acetate extracts (230 mg) was then purified by reversed-phase HPLC (YMC ODS-A column, 20 × 250 mm, 5 $\mu$m) using a gradient of 40−90% acetonitrile/water containing 0.05% TFA over 40 min at a flow rate of 7 mL/min to yield pure pyrrolomycin G (**3**, 20 mg) eluting at 39 min. A fraction taken at 42 min was further purified by reversed-phase HPLC (YMC ODS-A column, 10 × 250 mm, 5 $\mu$m) using a gradient of 40−90% acetonitrile/water with 0.02% TFA over 23 min to yield pure pyrrolomycin H (**4**, 14 mg) at 14.8 min. Pyrrolomycins I (**5**) and J (**6**) were isolated from the centrifuged pellet obtained from 150 mL of culture, fed with $^{13}$C-methyl-L-methionine, after extraction with acetone (2 × 60 mL) and ethyl acetate (3 × 20 mL) as described above. Final purification of the crude extract (87 mg) was achieved by reversed-phase HPLC (YMC ODS-A column, 10 × 250 mm, 5 $\mu$m) using a gradient of 40−90% acetonitrile/water with 0.02% TFA over 23 min to yield pure pyrrolomycin C (**2**, 7.5 mg, 19.6 min), pyrrolomycin I (**5**, 0.7 mg, 19 min), and pyrrolomycin J (**6**, 0.5 mg, 20.2 min).

**Pyrrolomycin G (3):** yellow solid (20 mg), $[\alpha]_D$ −15.6° (*c* 0.28, MeOH); UV ($CH_3CN/H_2O$ gradient with 0.025% formic acid, HP1100 DAD) $\lambda_{max}$ (relative absorption) 210 (1), 226 (sh, 0.75), 282 (0.55), 342 (0.54), nm; CD $\lambda_{max}$ ($\Delta\epsilon$) ($3.4 \times 10^{-4}$ M, ACN) 197 (−6.6), 209 (+ 3.4) nm; $^1$H and $^{13}$C NMR data, see Table 1; HRFT-ICR *m/z* 368.90082 $[M - H]^-$, calcd for $C_{11}H_5Cl_4N_2O_4$ 368.90089.

**Pyrrolomycin H (4):** yellow solid (14 mg); $[\alpha]_D$ −91.0° (*c* 0.30, MeOH); UV ($CH_3CN/H_2O$ gradient with 0.025% formic acid, HP1100 DAD) $\lambda_{max}$ (relative absorption) 210 (1), 226 (sh, 0.75), 282 (0.25), 342 (0.15) nm; CD $\lambda_{max}$ ($\Delta\epsilon$) ($1.4 \times 10^{-4}$ M, ACN) 197 (−44), 211 (+39) nm; $^1$H and $^{13}$C NMR data, see Table 1; HRFT-ICR *m/z* 382.91661 $[M - H]^-$, calcd for $C_{12}H_7Cl_4N_2O_4$ 382.91654.

**Pyrrolomycin I (5):** yellow solid (0.7 mg); UV ($CH_3CN/H_2O$ gradient with 0.025% formic acid, HP1100 DAD) $\lambda_{max}$ (relative absorption) 206 (0.85), 314 (0.75) nm; $^1$H and $^{13}$C NMR data, see Table 1; HRFT-ICR *m/z* 336.91926 $[M - H]^-$, calcd for $C_{11}{}^{13}CH_6Cl_4NO_2$ 336.91917.

**Pyrrolomycin J (6):** yellow solid (0.5 mg); UV ($CH_3CN/H_2O$ gradient with 0.025% formic acid, HP1100 DAD) $\lambda_{max}$ (relative absorption) 206 (0.85), 314 (0.75) nm; $^1$H NMR (acetone-$d_6$ at 400 MHz) 7.68 (d, 2.6 Hz, H-10), 7.46 (d, 2.6 Hz, H-12); HRFT-ICR *m/z* 370.88038 $[M - H]^-$, calcd for $C_{11}{}^{13}CH_5Cl_5NO_2$ 370.88020.

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## References and Notes

(1) Carter, G. T.; Nietsche, J. A.; Goodman, J. J.; Torrey, M. J.; Dunne, T. S.; Borders, D. B.; Testa, R. T. *J. Antibiot.* **1987**, *40*, 233−236.
(2) Nakamura, H.; Shiomi, K.; Iinuma, H.; Naganawa, H.; Obata, T.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1987**, *6*, 899−903.
(3) Kaneda, M.; Nakamura, S.; Ezaki.; Iitaka, Y. *J. Antibiot.* **1981**, *10*, 1366−1368.
(4) Ezaki, N.; Koyama, M.; Shomura, T.; Tsuruoka, T.; Inouye, S. *J. Antibiot.* **1983**, *10*, 1263−1267.
(5) Ezaki, N.; Koyama, M.; Kodama, Y.; Shomura, T.; Tashiro, K.; Tsuruoka, T.; Inouye, S. *J. Antibiot.* **1983**, *11*, 1431−1438.
(6) Koyama, M.; Ezaki, N.; Tsuruoka, T.; Inouye, S. *J. Antibiot.* **1983**, *11*, 1483−1489.
(7) Ezaki, N.; Shomura, T.; Koyama, M.; Niwa, T.; Kojima, M.; Inouye, S.; Ito, T.; Niida, T. *J. Antibiot.* **1981**, *10*, 1363−1365.
(8) Koyama, M.; Kodama, Y.; Tsuruoka, T.; Ezaki, N.; Niwa, T.; Inouye, S. *J. Antibiot.* **1981**, *12*, 1569−1676.
(9) Conder, G. A.; Zielinski, R. J.; Johnson, S. S.; Kuo, M.-S. T.; Cox, D. L.; Marshall, V. P.; Haber, C. L.; DiRoma, P. J.; Nelson, S. J.; Conklin, R. D.; Lee, B. L.; Geary, T. G.; Rothwell, J. T.; Snagster, N. C. *J. Antibiot.* **1991**, *45*, 977−983.

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