Investigating the Biosynthetic Origin of the Nitro Group in Pyrrolomycins

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Feasible modes of introducing the nitro group into pyrrolomycin antibiotics were investigated based on incorporation of ^{15}N-labeled arginine and proline into dioxapyrrolomycin, produced by the actinomycete culture LL-F42248. Biosynthesis of nitrated pyrrolomycins was unaffected by the presence of nitric oxide synthase (NOS) inhibitors. The culture was able to grow in nitrogen-free (minimal) media and produce nitrated secondary metabolites. These results indicate that LL-F42248 is capable of fixing nitrogen.

Pyrrolomycins are a group of polyhalogenated metabolites produced by *Actinosporangium* and *Streptomyces* species.[1] Diagnostic structural features of these metabolites include, a dichloropyrrole moiety linked to a halogenated-hydroxyphenyl ring via a one-carbon bridge. However, the most remarkable feature of these compounds is the occurrence of a 3-nitropyrrole moiety in some analogues (pyrrolomycin A, B, E, G, H and dioxapyrrolomycin).[1a–c,e] Pyrrolomycins have long been recognized for their potent antimicrobial[1] properties. Among these antibiotics, dioxapyrrolomycin is described for its anthelmintic[2] and neuropeptide-activity modulatory[3] properties, while pyrrolomycin B is reported to exhibit immunomodulatory[4] activity.

During our preliminary work with a dioxapyrrolomycin-producing actinomycete strain from the Wyeth culture collection, LL-F42248 (*Streptomyces fumanus*), we discovered several new members of the pyrrolomycin family of antibiotics (pyrrolomycins G−J) along with formerly described analogues, pyrrolomycin C and dioxapyrrolomycin.[1b] In a subsequent study, we uncovered the biosynthetic origin of the carbon skeleton of dioxapyrrolomycin.[5] The biosynthetic precursors were proposed on the basis of collective results obtained from precursor-feeding experiments with ^{13}C- and ^{15}N-labeled L-proline, ^{13}C-labeled acetates, and ^{13}C- and ^{2}H-labeled L-methionine. Accordingly, in dioxapyrrolomycin, the pyrrole ring is derived from L-proline and the phenyl moiety is derived from condensation of three acetate units. Furthermore, the methylenedioxy bridge is derived from L-methionine. We also demonstrated that pyrrolomycin C is nitrated to produce pyrrolomycin G, which is successively transformed into other nitrated pyrrolomycins. Despite these efforts, the biosynthetic origin of the nitro group remained obscure.

Our continued interest in nitrated pyrrolomycins was prompted by the rarity of such aryl-nitro compounds in nature and the insufficient understanding of the biosynthetic origin of the nitro group. Some examples of naturally occurring aryl-nitro compounds include pyrrolnitrin,[6] aureothin,[7] chloramphenicol,[8] and the thaxtamins.[9]

Nitration in biological systems typically occurs via oxidation of an amino function[10] or via involvement of a nitric oxide synthase (NOS).[9] Nitration could also occur through a process analogous to direct aromatic nitration by nitrites. In fact, in a previous study using $K^{15}N^{18}O_3$ as the sole nitrogen source, we demonstrated that the nitro group of dioxapyrrolomycin is introduced into the antibiotic as an intact NO_2 species.[11] This observation supported the likelihood of a direct biochemical nitration in pyrrolomycin antibiotics. On the basis of sequence analysis of the pyrrolomycin biosynthetic gene clusters from *Actinosporangium vitaminophilum* (ATCC 31673) and *Streptomyces* sp. UC11065, Parry and coworkers suggested that nitrate reductase encoded within the pyrrolomycin gene cluster may play a role in generating a reactive nitrosylating species.[12] While recent advances have been made in understanding the biosynthesis of the pyrrolomycins, the biochemical details of pyrrolomycin nitration remain ambiguous.

Table 1. FTMS/MS Analysis of the Proportion of ^{15}N-Isotope at the Nitro Group of **1**

growth condition[a]	% of $^{15}N_1$-**1**	$^{15}NO_2$ % of $^{15}N_1$-**1**	$^{14}NO_2$ % of $^{15}N_1$-**1**
A	54%	7%	93%
B	50%	77%	23%
C	53%	85%	15%
D	22%	12%	88%

[a] Availabe nitrogen sources under each of the growth conditions: (A) L-[^{15}N]-proline, L-arginine; (B) L-[$^{15}N_2$]-guanidino-arginine, L-proline; (C) L-[$U^{15}N_4$]-arginine, L-proline; (D) L-[^{15}N]-proline.

Considering how efficiently LL-F42248 produces nitrated pyrrolomycins, we speculated that the organism might employ multiple nitrosylation strategies. Consequently, we decided to examine the role of NOS activity in the pyrrolomycin-producing culture LL-F42248.

In mammalian cells, nitric oxide (NO) is produced during the enzymatic oxidation of L-arginine to L-citrulline.[13] Initially one of the terminal guanidino nitrogens in L-arginine is oxidized by NO synthases to form the *N*-hydroxy-L-arginine [N-OH-L-Arg] intermediate. N-OH-L-Arg is further oxidized, and the internal guanidino-C−N bond cleaved to yield nitric oxide. NOS-mediated biosynthetic nitration likely involves oxidized forms of NO such as nitrate/nitrite. One such example of an aromatic nitration by bacterial-NOS (*Streptomyces* spp.) has been described during the biosynthesis of thaxtomin A.[14]

In order to explore the involvement of NOS activity in pyrrolomycin nitration, culture LL-F42248 was incubated with four different ^{14}N- and ^{15}N-labeled nitrogen sources (Table 1).[15] The nitrogen sources, L-[$^{15}N_2$]-guanidino arginine, L-[^{15}N]-proline, L-[$U^{15}N_4$]-arginine, L-[^{14}N]-proline, and L-[^{14}N]-arginine were added at concentrations of 1.0 g/L. The fate of the ^{15}N-labeled precursors was followed by negative-mode SORI-CID[16] ESI-FTMS/MS analysis of the ^{15}N-enriched dioxapyrrolomycin (Figure 1, **1** and **2**) isolated from 3-day fermentations.

The negative-mode FTMS analysis detected $^{15}N_1$-labeled **1** (*m*/*z* 382) with ^{15}N-label at either the nitro group or the pyrrole moiety, and the $^{15}N_2$-labeled ion (*m*/*z* 383) corresponding to ^{15}N-enrichment at both nitrogen atoms in **2**. Under each of the growth conditions A−D (Table 1), the proportion of ^{15}N-isotope at the nitro group of **1** was calculated on the basis of the relative abundance of $^{14}NO_2^-$ and $^{15}NO_2^-$ fragment ions in the FTMS/MS spectrum of the deprotonated molecular ion, $[M - H]^-$ *m*/*z* 382.[17]

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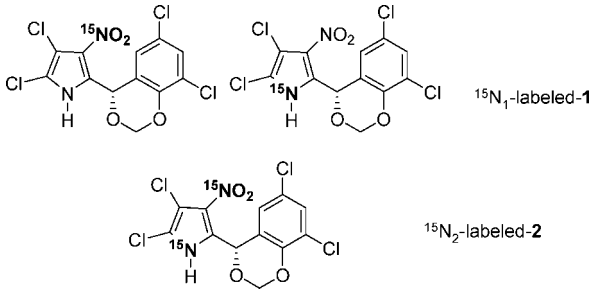

Figure 1. $^{15}N_1$- and $^{15}N_2$-Dioxapyrrolomycins **1** and **2**.

When L-[^{15}N]-proline and L-[^{14}N]-arginine were the only sources of nitrogen in the medium (condition A, Table 1), the $^{14}NO_2^-$ fragment ion was detected with 93% abundance, indicating that the nitro group in **1** was minimally labeled. Under conditions B and C, when L-[$^{15}N_2$]-guanidino-Arg and L-[U$^{15}N_4$]-Arg were the only available ^{15}N-substrates, the $^{15}NO_2^-$ fragment ion was detected with high abundance (77% and 85%, respectively), indicating that the majority of **1** carried an ^{15}N-enriched nitro group. Consequently, the ^{15}N/^{14}N-labeling pattern extracted from the NO_2 fragment ion spectra of **1** suggested that, under conditions A−C (Table 1), the NO_2 group of **1** originated from the guanidino group of L-arginine. It follows that these results could be indicative of NOS activity in the bacterial culture during pyrrolomycin nitration.

In order to verify the preceding observations, we sought to evaluate the effect of NOS inhibitors on pyrrolomycin nitration. We reasoned that if NOS-mediated nitration is prevalent in LL-F42248, then addition of NOS inhibitors to the growth medium should suppress the production of nitrated pyrrolomycin analogues.

Accordingly, NOS inhibitors NMMA (N^G-methyl-L-arginine) and AG (aminoguanidine) were added into the growing culture at a range of concentrations between 0.5 and 20.0 mM. Control experiments were set up under identical nutritional conditions but without the addition of NOS inhibitors.[15] The effect of the NOS inhibitors was monitored by LC/UV/MS analysis. However, LC/UV/MS profiles failed to reflect any adverse effects of NOS inhibitors on the biosynthesis of nitrated pyrrolomycin analogues. The titers of the metabolites were not significantly different between the control and the NOS-inhibitor-treated fermentations. When the concentration of AG was higher than 6 mM, toxic effects were observed. On the basis of these results,[18] the catabolism of L-arginine during the biosynthesis of pyrrolomycin metabolites must occur independent of NO synthases, probably via alternative routes of degradation,[19] thereby contributing to the nitrogen pool.

Having ruled out the involvement of a bacterial NO synthase-mediated pathway in the biosynthesis of nitrated pyrrolomycins, we subsequently focused on the anomalous $^{15}NO_2/^{14}NO_2$-enrichment pattern obtained under growth condition D (Table 1). In this case when LL-F42248 was grown on a defined medium, with L-[^{15}N]-proline as the sole nitrogen source, the FTMS SORICID mass spectrum of **1** (Figure 2) showed a predominant $^{14}NO_2^-$ fragment ion (exptl, 45.99333, calcd, 45.99345, $\Delta = -0.12$ mmu) with 88% abundance, indicating that the nitro group was primarily unlabeled. The isotope ratio of $^{15}NO_2/^{14}NO_2$ (about 1:7) implied that, in addition to the presupplied ^{15}N in the growth medium (in the form of L-[^{15}N]-proline), the organism must have an alternative means of accumulating ^{14}N. This observation suggested that LL-F42248 (family *Streptomycetaceae*) may have the ability to acquire atmospheric $^{14}N_2$ by fixation. Furthermore, it can be reasoned that N_2 fixation may have been triggered under condition D as a viable alternative to the catabolism of L-[^{15}N]-proline as a supply of nitrogen. Evidence of N_2-fixation by other actinomycete bacterial strains has been reported in the literature.[20]

However, it should be highlighted that, under conditions A−D (Table 1), a nylon-based resin (polyamide SC 6.6; 15 g/L) was

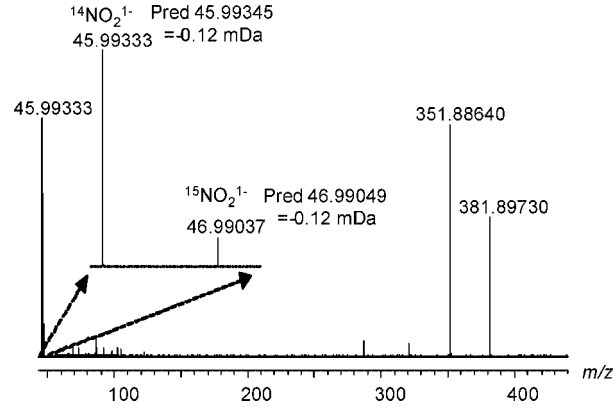

Figure 2. Negative-mode ESI FTMS SORI-CID mass spectrum of the deprotonated molecular ion $[M - H]^{1-}$ (m/z 382) of **1** under condition D. The expanded view on the top left shows the isotopic distribution of the NO_2^- fragment ions.

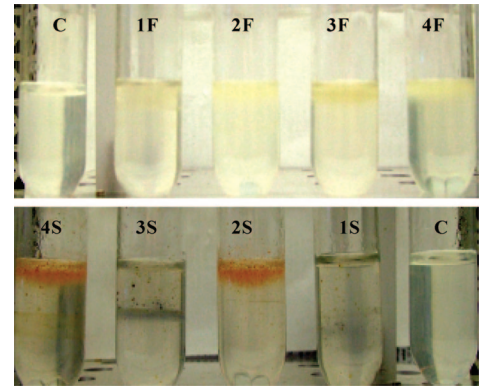

Figure 3. Growth of LL-F42248 (1F−4F) and *S. coelicolor* (1S−4S) in semisolid agar in the presence and absence of $(NH_4)_2SO_4$. Tubes 1F, 1S: 6.5 mg innoculum, − $(NH_4)_2SO_4$. Tubes 2F, 2S: 6.5 mg innoculum, + $(NH_4)_2SO_4$. Tubes 3F, 3S: 13.0 mg innoculum, − $(NH_4)_2SO_4$. Tubes 4F, 4S: 13.0 mg innoculum, + $(NH_4)_2SO_4$. Tubes C = control (media only).

present in the growth media to facilitate extraction of the pyrrolomycin metabolites produced during the fermentation. At the end of the fermentation, the antibiotic-bound resin was separated from the growth medium by centrifugation. The antibiotics were recovered from the resin by extracting with acetone or methanol. Therefore, the unexpected ^{14}N/^{15}N ratio (^{15}N-isotope dilution) observed under condition D (Table 1) could have resulted from consumption of nylon as a source of nitrogen by the bacterial culture. In order to test this hypothesis, the polyamide resin was replaced with HP-20, a nitrogen-free resin, and LL-F42248 was cultured under condition D. After 3 days, singly labeled dioxapyrrolomycin (**1**) was isolated and analyzed by FTMS/MS. The analysis showed that ~40% of **1** carried a $^{15}NO_2$ group, whereas ~60% carried a $^{14}NO_2$ group. Even though the isotope ratio of $^{15}NO_2/^{14}NO_2$ was greater than the previous result (Table 1, condition D), the percentage of **1** carrying an unlabeled NO_2 group was noticeably higher than the $^{15}NO_2$-labeled species.

To further investigate the N_2-fixation capacity of LL-F42248, we decided to test the ability of this organism to grow in defined semisolid media in the presence and absence of $(NH_4)_2SO_4$ (Figure 3, 1F−4F).[15] Semisolid conditions (microanaerobic; unshaken) were used to enhance nitrogenase activity, since nitrogenase could be inhibited by oxygen (vigorous shaking). As a negative control, *S. coelicolor* M145, a non-nitrogen-fixing bacterium, was grown under identical conditions, stationary at 28 °C for 9 days (Figure 3, 1S−4S). Tube C (Figure 3) contained uninoculated media.

From these experiments, it was apparent that LL-F42248 was able to grow in media rich in $(NH_4)_2SO_4$ (tubes 2F, 4F), as well as media lacking a nitrogen source (tubes 1F, 3F). As anticipated, growth occurred in a thin band (pellicle growth) just below the surface of the medium (Figure 3). In contrast, *S. coelicolor* M145 grew only in media containing a nitrogen source (Figure 3, 2S, 4S). These visual observations of pellicle growth provided indirect evidence of N_2 fixation by culture LL-F42248, thereby unambiguously demonstrating that the bacterial culture LL-F42248 is able to utilize atmospheric N_2 for growth and primary metabolism.

Subsequently, further studies were initiated to investigate whether the culture was able to produce nitrated secondary metabolites in nitrogen-free minimal media using atmospheric N_2 as the sole nitrogen source. Accordingly, culture LL-F42248 was grown on defined nitrogen-free minimal medium for up to 27 days and analyzed by low-resolution LC/MS at ~5−7 day intervals. Cultures analyzed after 7 days of fermentation showed insignificant production of pyrrolomycin analogues. However, fermentation samples collected between 14 and 27 days showed clear indications of the production of nitrated pyrrolomycin analogues. HRFTMS analysis identified pyrrolomycins C, G, H and dioxapyrrolomycin in these fermentations.

To the best of our knowledge, this is the first report that describes the production of nitrated secondary metabolites by actinomycete bacteria using atmospheric N_2 as the sole nitrogen source.

Experimental Section

General Experimental Procedures. High-resolution ESIMS analyses were performed on a Bruker (Billerica, MA) APEXII FTICR 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 CO2 CW laser.[21] Mass spectra were internally/externally calibrated using HP tuning mix. In the FTMS/MS experiments, the precursor ions [either the $^{15}N_1$-enriched ion of *m*/*z* 382 or the *m*/*z* 383 ion (corresponding to the $^{15}N_2$-enriched species or the ^{37}Cl isotope of the unlabeled species)] were isolated with only a single peak left using correlated sweep and then dissociated using a sustained off-resonance irradiation with collision-induced dissociation (SORI-CID). LC-MS data were obtained on a Thermo-Finnigan LCQ Deca instrument equipped with an Agilent 1100 LC system (diode array detector) and a YMC ODS-A 3 μm, 120 A, (100 × 2.0 mm) column. Semipreparative HPLC was performed on an Agilent 1100 series instrument (diode array detector) using a Phenomenex Luna 5 μm C18 (250 × 10 mm) column. Standard amino acids and NOS inhibitors were purchased from Sigma-Aldrich. Isotopic labeled amino acids were purchased from either Sigma-Aldrich or Cambridge Isotope Laboratories, Inc. (Andover, MA). All solvents were HPLC grade, purchased from either J. T. Baker, Inc. or EMD (OmniSolv).

General Fermentation Conditions. LL-F42248 was cultured as described previoulsy.[1b] Resin (15 g/L of either polyamide SC 6.6 or HP-20) was added to the growth media to facilitate extraction of the pyrrolomycin metabolites. Unmodified production medium unless otherwise specified contained the following components: 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% L-proline, 0.08% KF, and 1.5% resin.

General Extraction and Isolation Protocol. The harvested culture broth (50 mL) was poured into conical-bottom tubes and centrifuged at 4000 rpm for 15 min. The resulting aqueous supernatant was decanted, and the resin together with the cell mass was sonicated with acetone (150 mL × 2) in a room-temperature water bath and recentrifuged at 4000 rpm for 10 min. The acetone extracts were combined and concentrated to dryness in vacuo. The crude residue was dissolved in MeOH and chromatographed on a YMC-Pack-ODS-A column (250 × 20 mm, flow rate 10 mL/min, detection 254 nm) using a gradient of 65%−100% CH_3CN in H_2O (0.01% TFA) over 25 min to yield purified pyrrolomycin analogues.

Investigating the Involvement of NOS Activity in Pyrrolomycin Nitration. (a) Feeding Studies with ^{14}N- and ^{15}N-Labeled Nitrogen Sources (Table 1). The production medium was modified by omitting L-proline and $NaNO_3$ and adding 0.1% each of L-[^{15}N]-proline and L-[^{14}N]-arginine (condition A); L-[$^{15}N_2$]-guanidino arginine and L-[^{14}N]-proline (condition B); L-[U$^{15}N_4$]-arginine and L-[^{14}N]-proline (condition C); L-[^{15}N]-proline, (condition D). Nylon-based resin (polyamide SC 6.6; 1.5%) was present in all four conditions (A−D). Cultures were harvested and purified after 3 days according to the general extraction protocol described above.

(b) Effect of NOS Inhibitors on Pyrrolomycin Nitration. The production medium was modified by omitting L-proline and $NaNO_3$ and adding L-arginine (0.1%; condition 1), and omitting $NaNO_3$ and adding L-arginine (0.1%; condition 2). NOS inhibitors NMMA (N^G-methyl-L-arginine) and AG (aminoguanidine) were added into the above fermentation media (50 mL of medium, 250 mL Erlenmeyer flasks) at concentrations of 0.5, 1.0, 3.0, 6.0, 10.0, and 20.0 mM. Control experiments were set up under identical nutritional conditions but without the addition of NOS inhibitors. After 3 days at 28 °C and 200 rpm, the effects of NOS inhibitors on the production of nitrated pyrrolomycin cultures were analyzed by LC/UV/MS and compared to control fermentations.

Preparation of Nitrogen-Free Growth Media. (a) Minimal Medium with Nitrogen [MMN$^+$]. Minimal media were prepared as described by Kieser et al.[22] Briefly, K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.2 g), and $(NH_4)_2SO_4$ (1.0 g) were dissolved in 1 L of distilled H_2O, and the pH was adjusted to 7.0. After autoclaving, 5 mL of a sterile 40% glucose solution and 1 mL of trace element solution A were aseptically added to the medium.

(b) Trace Element Solution A (L^{-1}). $ZnCl_2$ (40 mg), $FeCl_3 \cdot 6H_2O$ (200 mg), $CuCl_2 \cdot 2H_2O$ (10 mg), $MnCl_2 \cdot 2H_2O$ (10 mg), $Na_2B_4O_7 \cdot 10H_2O$ (10 mg), and $(NH_4)6Mo_7O_{24} \cdot 6H_2O$ (10 mg) were dissolved in 1 L of distilled H_2O and filter sterilized (MF75 sterilization filter units, cellulose nitrate membrane, Nalgene, 0.2 um).

(c) Minimal Medium without Nitrogen [MMN$^-$]. MMN$^-$ medium was identical to MMN$^+$ medium except $(NH_4)_2SO_4$ was omitted and trace element solution A was replaced with trace element solution B.

(d) Trace Element Solution B (L^{-1}). $ZnCl_2$ (40 mg), $FeCl_3 \cdot 6H_2O$ (200 mg), $CuCl_2 \cdot 2H_2O$ (10 mg), $MnCl_2 \cdot 2H_2O$ (10 mg), $Na_2B_4O_7 \cdot 10H_2O$ (10 mg), and Na_2MoO_4 (10 mg) were dissolved in 1 L of distilled H_2O and filter sterilized (MF75 sterilization filter units, cellulose nitrate membrane, Nalgene, 0.2 um).

Demonstrating the Ability to Grow in Semisolid, Nitrogen-Free Media. *S. coelicolor* M145 and LL-F42248 were inoculated into 7 mL of MYM medium (0.4% malt extract, 0.4% yeast extract, 1% maltose, pH 7.0; culture tube [25 o.d. × 150 L mm] with 2 glass beads/tube) and incubated at 28 °C and 200 rpm for 48 h. A 1.5 mL aliquot of cells was removed, the cells were pelleted by centrifugation and washed twice with 2 mL of phosphate-buffered saline (PBS), and the wet weight was determined. The cells were resuspended in PBS to give a final mass/vol ratio of 130 mg cells/mL. Aliquots of 50 and 100 μL were used to inoculate 7 mL of minimal media ± $(NH_4)_2SO_4$ (MMN$^+$ and MMN$^-$, respectively) containing 1.8 g/L of noble agar that had been dispensed into seed tubes. Following inoculation the medium was swirled to disperse the inoculum, and the tubes were incubated under stationary conditions at 28 °C for 9 days.

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Supporting Information Available: Analytical HPLC profile of major pyrrolomycin metabolites, HRFTMS spectra under growth conditions A−D, and calculations of the ^{15}N-isotope labeling in **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) 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. (b) Charan, R. D.; Schlingmann, G.; Bernan, V. S.; Feng, X.; Carter, G. T. *J. Nat. Prod.* **2005**, *68*, 277–279. (c) Ezaki, N.; Shomura, T.; Koyama, M.; Niwa, T.; Kojima, M.; Inouye, S.; Ito, T.; Nida, T. *J. Antibiot.* **1981**, *34*, 1363–1365. (d) Ezaki, N.; Koyama, M.; Kodama, Y.; Shomura, T.; Tashiro, K.; Tsuruoka, T.; Inouye, S. *J. Antibiot.* **1983**, *36*, 1431–1438. (e) Koyama, M.; Ezaki, N.; Tsuruoka, T.; Shigeharu, I. *J. Antibiot.* **1983**, *36*, 1483–1489.

(2) Conder, G. A.; Zielinski, R. J.; Johnson, S. S.; Kuo, Ming-Shang, 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. *J. Antibiot.* **1992**, *45*, 977–983.

(3) Masuda, K.; Suzuki, K.; Ishida-Okawara, A.; Mizuno, S.; Hotta, K.; Miyadoh, S.; Hara, O.; Koyama, M. *J. Antibiot.* **1991**, *44*, 533–540.

(4) Umezawa, K.; Ishizuka, M.; Sawa, T.; Takeuchi, T. *J. Antibiot.* **1984**, *37*, 1253–1256.
(5) Charan, R. D.; Schlingmann, G.; Bernan, V. S.; Feng, X.; Carter, G. T. *J. Nat. Prod.* **2006**, *69*, 29–31.
(6) Van Pee, K.-H.; Ligon, J. M. *Nat. Prod. Rep.* **2000**, *17*, 157–164.
(7) Hirata, Y.; Nakata, H.; Yamada, K.; Okuhara, K.; Naito, T. *Tetrahedron* **1961**, *14*, 252–274.
(8) Rebstock, M. C.; Crooks, H. M.; Controulis, J.; Bartz, Q. R. *J. Am. Chem. Soc.* **1949**, *71*, 2458–2462.
(9) Wach, M. J.; Kers, J. A.; Krasno, V, S. B.; Loria, R.; Gibson, D. M. *Nitric Oxide* **2005**, *12*, 46–53.
(10) Cardillo, R.; Fuganti, C.; Ghiringhell, D.; Diangrasso, D.; Grasselli, P.; Santopietro-Amisano, A. *Tetrahedron* **1974**, *30*, 459–461.
(11) Carter, G. T.; Nietsche, J. A.; Goodman, J. J.; Torrey, M. J.; Dunne, T. S.; Siegel, M. M.; Borders, D. B. *J. Chem. Soc., Chem. Commun.* **1989**, *40*, 1271–1273.
(12) Zhang, X.; Parry, R. J. *Antimicrob. Agents Chemother.* **2007**, *51*, 946–957.
(13) Stuehr, D. J. *Biochim. Biophys. Acta* **1999**, *1411*, 217–230.
(14) Kers, J. A.; Wach, M. J.; Krasnoff, S. B.; Widom, J.; Cameron, K. D.; Bukhalid, R. A.; Gibson, D. M.; Crane, B. R.; Loria, R. *Nature* **2004**, *429*, 79–82.
(15) See experimental section for a detailed description of the growth conditions and experimental protocols.
(16) Off-resonance irradiation with collision-induced dissociation: (a) Gauthier, J. W.; Trautman, T. R.; Jacobson, D. B. *Anal. Chim. Acta* **1991**, *246* (1), 211–225. (b) Feng, X.; Siegel, M. M. *Anal. Bioanal. Chem.* **2007**, *389*, 1341–1363.
(17) Details of the calculations of the ^{15}N-isotope labeling and FTMS spectra are given under the Supporting Information section.
(18) The results were based on the analysis of a single set of NOS-inhibitor-treated cultures and thus should be considered semiquantitative
(19) (a) Abdelal, A. T. *Annu. Rev. Microbiol.* **1979**, *33*, 139–168. (b) WU, G.; Morris, S. M., Jr. *Biochem. J.* **1998**, *336*, 1–17.
(20) (a) Gauthier, D.; Diem, H. G.; Dommergues, Y. *Appl. Environ. Microbiol.* **1981**, *41*, 306–308. (b) Valdés, M.; Pérez, N.-O.; Santos, P. E.; Caballero-Mellado, J.; Péna-Cabriales, J. J.; Normand, P.; Hirsch, A. M. *Appl. Environ. Microbiol.* **2005**, *71*, 460–466. (c) Ribbe, M.; Gadkari, D.; Meyer, O. *J. Biol. Chem.* **1997**, *272*, 26627–26633.
(21) (a) McDonald, L. A.; Barbieri, L. R.; Carter, G. T.; Kruppa, G.; Feng, X.; Lotvin, J. A.; Siegel, M. M. *Anal. Chem.* **2003**, *75*, 2730–2739. (b) Palmblad, M.; Hakansson, K.; Hakansson, P.; Feng, X.; Cooper, H. J.; Giannakopulos, A. E.; Green, P. S.; Derrick, P. J. *Eur. J. Mass Spectrom* **2000**, *6*, 267–275.
(22) Keiser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*; The John Innes Foundation: Crowes, Norwich, England, 2000.

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