Natural Product Synthesis

DOI: 10.1002/anie.200805140

Formation of the Pyridazine Natural Product Azamerone by Biosynthetic Rearrangement of an Aryl Diazoketone**

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Compounds containing N–N bonds have been made synthetically for over a hundred years. However, it wasn't until 1951 that the first naturally occurring compound containing a dinitrogen group, the azoxy-containing toxin macrozamin, was reported.[1] Since that time, natural products containing hydrazine, nitrosamine, azoxy, diazo, and other N–N-bonded functional groups have been identified. The enzymes responsible for N–N bond formation could make valuable biocatalysts, yet the biosynthesis of this rare structural unit is poorly understood.

Biosynthetic investigations of the azoxy-containing antibiotic valanimycin (**1**)[2] and the antifungal antibiotic pyridazomycin (**2**)[3a] (Figure 1) have shown that both of these natural products are derived from the condensation of amino acids. Pyridazomycin[3b] was the only natural product known to contain a pyridazine ring until the phthalazinone meroterpenoid azamerone (**3**) was isolated from the marine sediment-derived bacterium *Streptomyces* sp. CNQ-766.[4] It was first speculated that the pyridazine ring moiety in **3** was also derived from the cyclization of amino acid residues, but identification of coproduced chlorinated meroterpenoids, such as A80915C (**4**), 3″-hydroxy-7-methylnapyradiomycin B2 (**5**), and analogues possessing a diazo functional group (**6**–**8**)[5] suggested that **3** may rather be derived from a naphthoquinone precursor (Scheme 1). Herein, we report the biosynthesis of **3** from a series of stable isotope tracer experiments, which validate its biosynthetic relationship to the napyradiomycin family of meroterpenoid antibiotics.

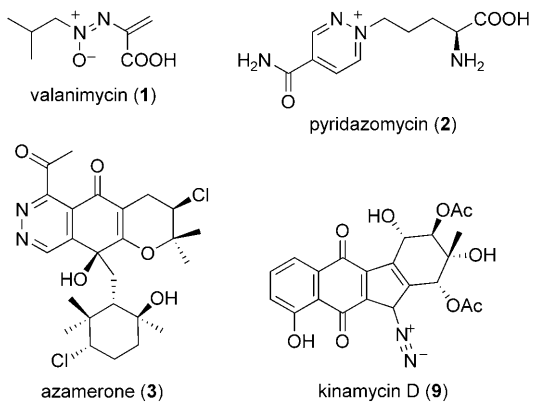

Figure 1. Selected natural products containing a N–N bond.

The original isolation of **3** from *S.* sp. CNQ-766 generated approximately 0.25 mg L^{-1},[4] which was insufficient for labeling experiments. Different fermentation conditions were thus evaluated to improve production yields that resulted in a >30-fold increase in **3** at 8.5 mg L^{-1}.[5a,6] In addition to exploring the biosynthesis of **3** with stable isotopes, we also examined **4** and the diazo meroterpenoids SF2415A3 (**6**) and A80915D (**7**) to probe the biosynthetic relationship of this extended structural family. Feeding experiments with [1,2-$^{13}C_2$]acetate clearly revealed that the naphthoquinone core of **4** was derived from the symmetrical pentaketide 1,3,6,8-tetrahydroxynaphthalene (THN) and that the two isoprenoid units originated from the mevalonate pathway (Scheme 1).[6] These findings were consistent with previous isotopic experiments in this compound series from *Chainia rubra* MG802-AF1[7] and from the analysis of the 43 kbp napyradiomycin biosynthetic gene cluster (*nap*) from *Streptomyces aculeolatus* NRRL 18422 and *Streptomyces* sp. CNQ-525.[8]

^{13}C NMR spectroscopic analysis of [1,2-$^{13}C_2$]acetate-enriched **3** also showed that all carbons except for the C8 methyl group originate from acetate (Figure 2).[6] The bicyclic phthalazinone unit clearly harbored two distinct ^{13}C-labeling patterns of equal proportions, thereby implying that it too derives from the symmetrical THN unit common to the napyradiomycins. Further analysis of L-[methyl-^{13}C]methionine-enriched **3** revealed the 26% selective enrichment of C8, confirming that the acetyl methyl group of **3** is methionine-derived.[6] Importantly, if labeled materials were administered to *S.* sp. CNQ-766 during the initial production of **3**, which is roughly five days after inoculation during stationary growth, no enrichment could be detected. Labeled materials had to be added at the time of inoculation in order for them to be efficiently incorporated, thereby suggesting that **3** originates from a methylated intermediate produced earlier in the fermentation process.

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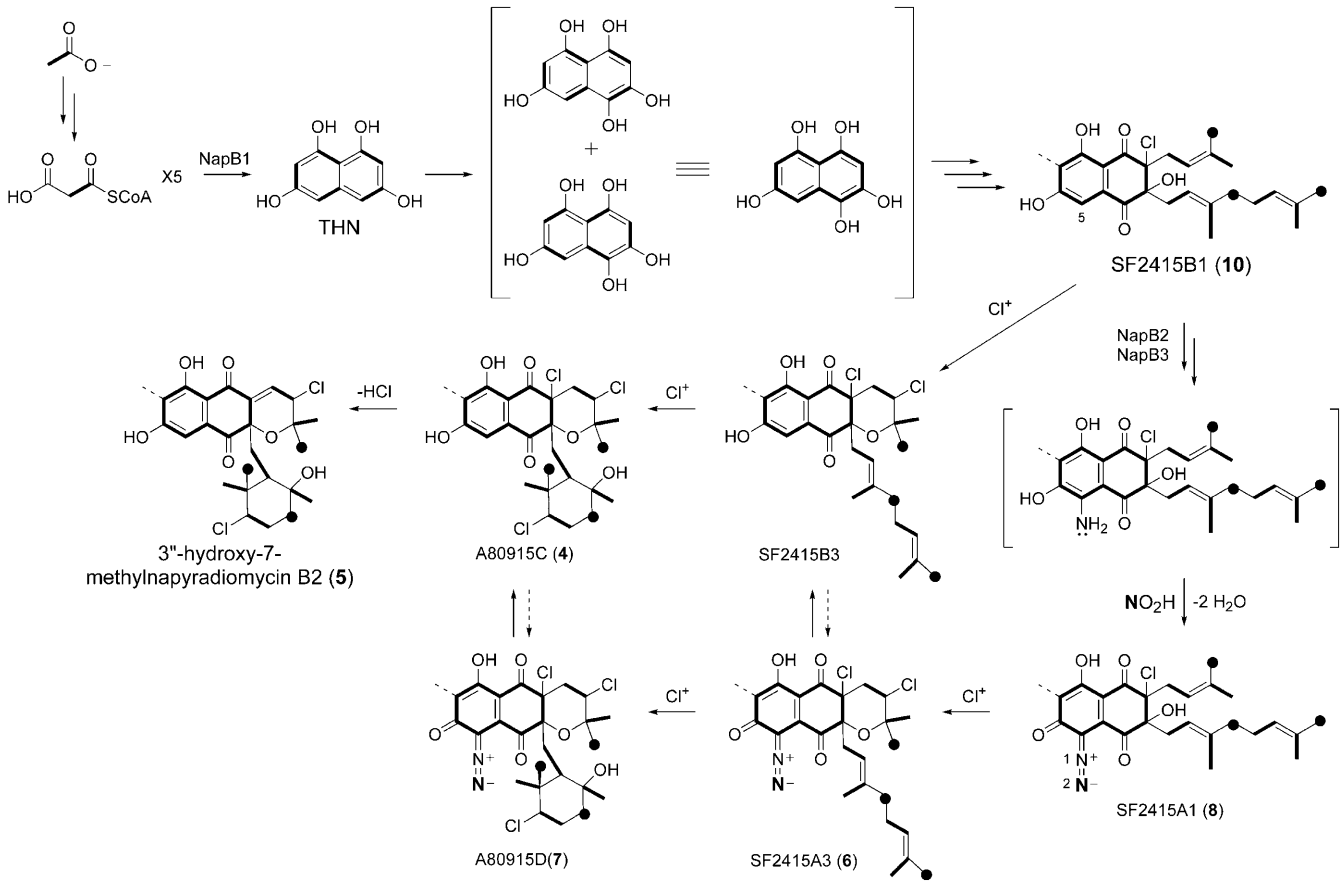
[**] This work was supported by the California Sea Grant Program (R/NMP-98 to B.S.M.) and by a Ruth L. Kirschstein National Research Service Award to J.M.W. from the NIH Training Program in Marine Biotechnology (GM067550). We thank Dr. W. Fenical, Dr. P. R. Jensen, Dr. H. C. Kwon, and Dr. J. Y. Cho (University of California at San Diego (USA)) for *Streptomyces* sp. CNQ-766 and standard azamerone, and Dr. R. J. Parry (Rice University (USA)), Dr. T. F. Molinski, Dr. T. A. M. Gulder, and Dr. A. A. Mrse for helpful advice.

Supporting Information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805140.



Scheme 1. Proposed biosynthetic pathway for the diazo chlorinated meroterpenoids **6**–**8**[8] and their relation to the chlorinated dihydroquinones **4** and **5**. A summation of the two [1,2-$^{13}C_2$]acetate-labeling patterns are shown in bold (lines and dots signify double and single enrichments, respectively). Enrichment from L-[methyl-^{13}C]methionine is shown as a dashed line, and enrichment from [^{15}N]nitrite or [^{15}N]nitrate is shown in bold font.

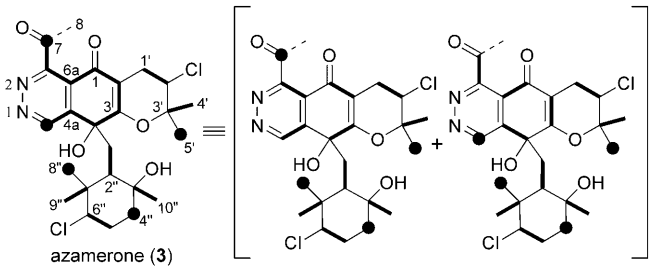


Figure 2. ^{13}C labeling of azamerone (**3**) derived from [1,2-$^{13}C_2$]acetate and L-[methyl-^{13}C]methionine. Two equally independent labeling patterns **3a** and **3b** were evident from the incorporation of [1,2-$^{13}C_2$]acetate (bold lines and dots signify double and single enrichments, respectively). The enrichment from L-[methyl-^{13}C]methionine is shown as a dashed line. The numbering scheme for azamerone has been adopted from Refs. [5c] and [8].

Altogether these data suggest that **3** is biosynthetically linked to the napyradiomycin family of natural products.

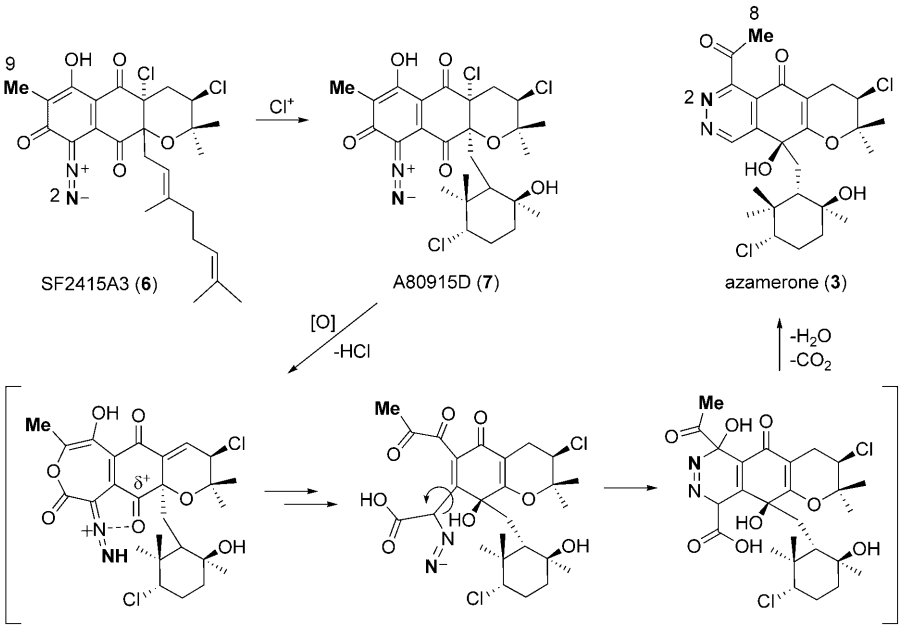
We turned our attention to the biosynthetic origin of the diazo functional group, as found in **6**–**8**, and its relation to **3**. Previous studies on the biosynthesis of the kinamycins with synthetic and natural intermediates revealed that its diazonium group is assembled from the stepwise addition of two nitrogen donors to a ketobenzo[b]fluorene precursor through an aminobenzo[b]fluorene intermediate into kinamycin D (**9**, Figure 1).[9] Considering a similar scenario, we administered different forms of ^{15}N-enriched substrates, including ammonium sulfate, nitrite, and nitrate, and measured their incorporation into **3**, **6**, and **7** by MS and NMR spectroscopy (Table 1).[6] MS analysis revealed that all ^{15}N-labeled precur-

Table 1: ^{15}N NMR spectroscopic data of **3** and **7** and incorporation percentages from nitrogen labeled precursors.

Labeled precursors	Enrichment of **3**[a] singly/doubly [%]	δ^{15}N [ppm][b]	Enrichment of **7**[a] singly/doubly [%]	δ^{15}N [ppm][c]
[$^{15}NO_2$]Na	77/6	−2	77/n.d.	−31
[$^{15}NO_3$]Na	74/3	−2	79/n.d.	−31
[$^{15}NH_4$]$_2$SO$_4$	11/n.d.	−2&13	7/n.d.	n.d.[d]
[$^{15}N_2H_4$]SO$_4$	5/0.3	−2&13	0/n.d.	n.d.[d]
[2-^{15}N, 9-^{13}C]**6**	11/6	−2	6/4	n.d.[d]

[a] Enrichment was calculated by *m/z*.[11] [b] ^{1}H–^{15}N HMBC spectra were measured in CD_3OD at 600 MHz and referenced internally to nitromethane ($\delta = 0$ ppm). [c] ^{15}N NMR spectra were measured in CD_3CN at 30.4 MHz and referenced externally to nitromethane ($\delta = 0$ ppm). [d] Chemical shift(s) were not measured, owing to inadequate sample size and low enrichment levels; n.d. = not determined.

sors were incorporated to different extents, with the oxidized forms having the highest assimilation rates. Whereas [^{15}N]ammonium sulfate singly enriched **3** and **7** by 7–11%, ^{1}H–^{15}N HMBC analysis of **3** showed that both nitrogen atoms were equally labeled. This result was in stark contrast to the enrichment by [^{15}N]nitrite and [^{15}N]nitrate, which were not only incorporated to a much greater extent at $>70\%$ but only labeled one of the two nitrogen atoms in the natural product. In the case of **7**, ^{15}N NMR spectroscopy showed selective enrichment of the distal diazo nitrogen ($\delta = -31$ ppm).[9c,10] ^{15}N-labeling of **3** gave rise to an enhanced signal at $\delta = -2$ ppm, which was assigned to the C6-bound nitrogen N2 (Figure 2) in the pyridazine ring based upon the J(H,N) coupling constants in the ^{1}H–^{15}N HMBC experiment.[6]



Scheme 2. Proposed oxidative rearrangement of the aryl diazoketone SF2415A3 (**6**) via A80915D (**7**) to azamerone (**3**) in *S.* sp. CNQ-766. Isotopically labeled C and N atoms from [2-^{15}N, 9-^{13}C]**6** are shown in bold font.

Results from the ^{15}N-enrichment studies suggested that the napyradiomycin diazo group is also formed in a stepwise manner. Oxidation of the naphthyl ring at C5 facilitates the introduction of the first nitrogen atom by a transamination reaction. The formation of the N–N bond arises from a nucleophilic attack of the aminodihydroquinone intermediate on nitrous acid (Scheme 1). The direct addition of a dinitrogen precursor was also probed with [$^{15}N_2$]hydrazine. MS analysis revealed that [$^{15}N_2$]hydrazine was only incorporated singly and at a low level into **3** (Table 1). Furthermore, ^{1}H–^{15}N HMBC analysis showed that both nitrogen atoms in **3** were labeled. Yet, unlike the [^{15}N]ammonium-labeled product, N2 was enriched to a slightly higher extent than N1, suggesting that metabolism of hydrazine in *S.* sp. CNQ-766 results in nonequivalent forms of single nitrogen species.[6]

The ^{15}N labeling studies indirectly supported that the diazo group, such as those in **6** and **7**, is a biosynthetic precursor to the pyridazine unit in **3** based on their similar enrichment characteristics. To unequivocally link the aryl diazoketones to **3**, we biosynthetically prepared [2-^{15}N, 9-^{13}C]**6** from [^{15}N]nitrite and L-[methyl-^{13}C]methionine and fed the labeled natural product back to *S.* sp. CNQ-766. HPLC–MS analysis of the other napyradiomycin derivatives indicated that **6** was converted into **7** and **3** with retention of both isotopes in the same molar ratio as in the substrate. Isolation and characterization by NMR spectroscopy of [2-^{15}N, 8-^{13}C]**6**-enriched **3** showed ^{13}C enrichment at C8 and ^{15}N enrichment at N2,[6] thereby confirming that not only is **6** a precursor of **3**, but the diazo N2 atom is biosynthetically equivalent with N2 of the pyridazine group in **3**.

These stable isotope experiments link **3** to the napyradiomycin family of chlorinated meroterpenoids, in which a Baeyer–Villiger-type oxidation of a diazonaphthoquinone, such as **7**, may initiate the biosynthetic interconversion (Scheme 2). Hydrolysis of the 7-membered heterocyclic intermediate would open the ring to facilitate both the 1,2-alkyl shift of the monoterpene subunit and the assembly of the pyridazine ring, in which the diazo group forms a new linkage with the C8 diketide. Aromaticity of the ring would be restored by subsequent decarboxylation and dehydration reactions.

The molecular basis for the formation of the diazo group and its rearrangement, however, is presently unresolved, even though the napyradiomycin biosynthetic gene cluster from the related *S* sp. CNQ-525 has been cloned and sequenced.[8] Further examination of this strain revealed that, like CNQ-766, it too synthesizes **3**. However inspection of the *nap* gene cluster did not reveal an obvious mechanism for diazo synthesis and rearrangement nor did its heterologous expression yield nitrogenated napyradiomycin analogues. The only gene that may encode a diazo biosynthetic enzyme is *nap*B3, which codes for a putative aminotransferase and may facilitate the transamination to the aminodihydroquinone intermediate. The *nap* cluster furthermore harbors a number of oxygenases whose functions have not yet been clarified that may be involved in the nitrogen biochemistry of **3**. It is, however, quite likely that some of the encoding genes are extraneous to the *nap* locus and may reside elsewhere in the *S.* sp. CNQ-525 genome, given that the expression of the cluster only yielded non-nitrogenated napyradiomycins.[8]

In summary, we established, using ^{13}C- and ^{15}N-labeled precursors, that azamerone (**3**) is biosynthesized via SF2415A3 (**6**), in which the aryl diazoketone undergoes a novel rearrangement wherein the aromatic ring is oxidatively cleaved to allow for its rearomatization with a dinitrogen group to give the unique phthalazinone core of **3**. This unprecedented biochemistry extends our limited knowledge

of the biosynthesis of natural products containing N–N bonds and opens the door to exploring and exploiting its molecular basis at the biochemical and genetic levels.

Received: October 20, 2008
Published online: December 15, 2008

Keywords: biosynthesis · biotransformations · isotopic labeling · natural products · nitrogen heterocycles

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