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Production of Stealthin C Involves an S−N-Type Smiles Rearrangement

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Supporting Information

ABSTRACT: The kinamycin family of aromatic polyketide natural products contains an atypical angucycline ring system substituted with a diazo group. The enzymatic chemistry involved in constructing both of these structural features has been largely unexplored. Here we report the *in vivo* and *in vitro* production of seongomycin, a shunt product from this pathway, and stealthin C, a proposed biosynthetic precursor to the kinamycins. We show that a single enzyme, the flavin-dependent monooxygenase AlpJ, can generate these metabolites from *N*-acetyl-L-cysteine and L-cysteine, respectively, and that the synthesis of stealthin C likely proceeds via a nonenzymatic S−N-type Smiles rearrangement. This unexpected route to stealthin C reveals a distinct approach to install aromatic amino groups in metabolites and raises questions about the intermediacy of this species in kinamycin production.

The diazofluorenes are a group of DNA-damaging natural products produced by both soil and marine Actinomycetes (Figure 1A).[1,2] Family members, including the kinamycins and the lomaiviticins, share a common diazotetrahydrobenzo[*b*]-fluorene core scaffold that has been a rich source of inspiration for synthetic chemists and chemical biologists who have pursued syntheses of the natural products as well as simplified analogs for biological evaluation.[1]

Despite intense interest in these molecules, our understanding of diazofluorene biosynthesis is largely limited to *in vivo* experiments involving the kinamycins, monomeric metabolites produced by *Streptomyces murayamaensis* and *Streptomyces ambofaciens*.[3] Gould and co-workers identified additional metabolites from *S. murayamaensis*, including the aromatic polyketide dehydrorabelomycin (**1**). **1** is a key intermediate in the production of all angucycline polyketides, including the atypical angucyclines gilvocarcin and jadomycin.[4] Feeding deuterated **1** to *S. murayamaensis* confirmed its connection to kinamycin D, suggesting a biosynthetic route involving C-ring contraction and nitrogen insertion events (Figure 1B).[5]

Seongomycin (**2**), stealthin C (**3**), and kinobscurinone (**4**) are kinamycin-related metabolites isolated from *S. murayamaensis* mutants generated via chemical mutagenesis.[6] Feeding studies implicated both **3** and **4** as biosynthetic intermediates (Figure 1B),[6a,b] whereas **2** and its counterpart from lomaiviticin biosynthesis (homoseongomycin) were proposed to be shunt products derived from the reaction of the diazo-containing metabolites prekinamycin and homoprekinamycin with cellular thiols.[7] Notably, **2**−**4** were produced upon heterologous expression of a partial kinamycin biosynthetic gene cluster.[8]

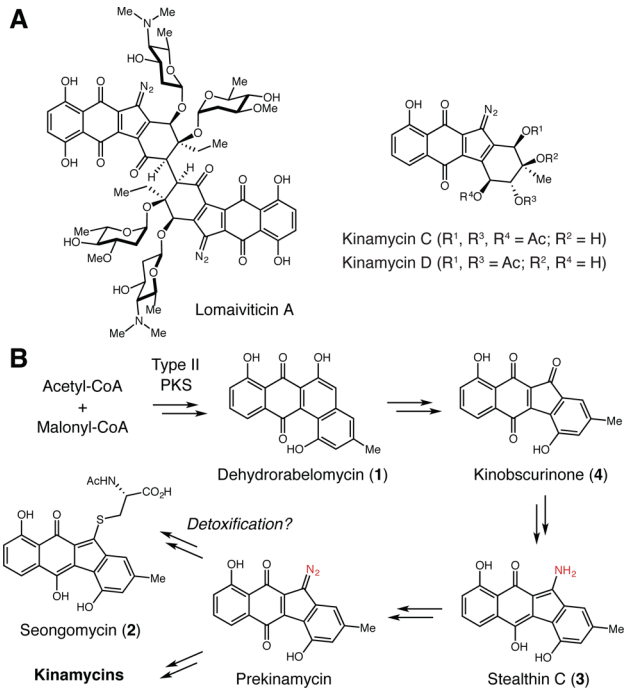

Figure 1. Biosynthesis of the diazofluorene natural products. (A) Representative diazofluorene natural products. (B) Proposed biosynthetic pathway to the kinamycins and seongomycin.

These initial studies left many biosynthetic questions unanswered, including the identities of the enzymes involved in benzo[*b*]fluorene ring formation and diazo generation. The discovery of the complete kinamycin and lomaiviticin biosynthetic gene clusters has provided new opportunities to investigate these intriguing transformations.[3c,9] Here we report the *in vivo* and *in vitro* characterization of enzymatic machinery capable of synthesizing **2** and **3** from **1** and either *N*-acetyl-L-cysteine or L-cysteine. Assays with labeled substrates and substrate analogs suggest that formation of **3** involves a nonenzymatic S−N-type Smiles rearrangement. This discovery calls into question the proposed role of stealthin C as a biosynthetic intermediate.

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We began by identifying the enzymes involved in generating the benzo[*b*]fluorene ring system. Yang and co-workers reported two oxidases from *S. ambofaciens* (AlpJ/K) that produce **2** from **1** *in vivo* and *in vitro*.[10] Simultaneously, we pursued a similar line of inquiry that revealed these enzymes are also capable of producing proposed biosynthetic intermediate **3** *in vivo* and *in vitro*. We uncovered candidate benzo[*b*]fluorene ring-forming enzymes by comparing the *kin* and *lom* biosynthetic gene clusters to gene clusters that produce other angucycline polyketides.[11] In the jadomycin and gilvocarcin pathways, flavin dependent monooxygenases JadG and GilOII perform a Baeyer–Villiger-type oxidative C–C bond cleavage reaction.[4] We located homologous flavin-dependent monooxygenases encoded in the *kin* and *lom* clusters (AlpJ/KinG and Lom28). These JadG homologues were always colocalized with a second flavin-dependent oxidase (AlpK/KinO1 and Lom27) that was absent from the *gil* and *jad* gene clusters (Figure S1), suggesting that this enzyme might also play a role in benzo[*b*]fluorene formation.

To initiate our studies, we cloned *alpJ* and *alpK* from *S. ambofaciens* into the *Streptomyces* expression vector pUWL201PW in an operon under the control of the *ermE** promoter. The resulting plasmid pAlpJK was transformed into *Streptomyces lividans* TK64 to afford recombinant strain pAlpJK/TK64. Feeding **1** to pAlpJK/TK64 led to production of **2** (m/z $[M + H]^+$ = 454.0960) (Figures 2 and S2), confirming that the AlpJK enzyme pair catalyzed the C–C bond cleavage and ring contraction reactions.

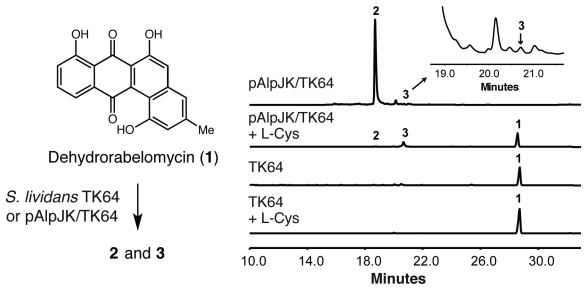

Figure 2. AlpJ converts dehydrorabelomycin to seongomycin and stealthin C *in vivo*. HPLC analysis of culture extracts.

Interestingly, we also detected a minor metabolite that had a similar UV–visible absorption spectrum to stealthin C (**3**) (m/z $[M + H]^+$ = 308.0924) (Figures 2 and S3).[6b] This observation was unexpected as aminobenzo[*b*]fluorene formation is proposed to require at least one additional enzyme for nitrogen transfer.[6b] Though this result suggested that AlpJK could generate **3** *in vivo*, the yield of this metabolite was extremely low compared to that of **2**. This difference could arise from the availability of biosynthetic precursors to these metabolites. The *N*-acetyl-cysteine group of **2** is likely derived from mycothiol, which is present at high concentrations in *S. lividans* cells (~2.2 mM).[12] We wondered if the amino group of **3** might derive from L-cysteine (present at ~0.2 mM).[12] We fed **1** and L-cysteine (~11 mM) to pAlpJK/TK64 and observed both a loss of **2** and an 11-fold increase in **3** (Figure 2). Feeding **1** and ^{15}N-labeled L-cysteine confirmed that the nitrogen atom of this amino acid is incorporated into stealthin C (Figure S4).

To explore these processes *in vitro*, AlpJ and the AlpK homologue KinO1 were expressed in *Escherichia coli* (Figure S5). Although purified AlpJ was colorless, purified KinO1 was bright yellow. LC-MS analysis of the supernatants of both enzymes revealed FAD (Figures S6 and S7). When **1** was incubated with AlpJ, the *E. coli* FAD reductase Fre,[13] FAD, and NADPH, we observed a new peak by HPLC that did not correspond to the hydroxyoxepinone intermediate noted previously in *in vitro* assays with **1** and GilOII or JadG.[4d] Subsequent LC-MS analysis indicated that this new compound had m/z $[M - H]^-$ = 581.1218 (Figure S8, trace ii and Figure S9), which corresponds to a dimeric benzofluorene (**5**). This product was also identified by Yang and co-workers.[10] When we replaced Fre with KinO1, the consumption of **1** was slower and **5** was not detected (Figure S8, trace iii). Furthermore, AlpJ alone generated **5** with reduced efficiency (Figure S8, trace i). These results show that AlpJ can catalyze both the oxidative C–C bond cleavage reaction and the subsequent ring-closing reaction. The altered product distribution in the presence of KinO1 implies this enzyme both reduces FAD and works as an ancillary oxygenase to enhance the production of hydroquinone-kinobscurinone and avoid **5**, a function also proposed by Yang and co-workers.[10]

We next added *N*-acetyl-L-cysteine to the HPLC assays described above and observed efficient formation of a new product with a mass corresponding to **2** (Figures S10 and S11). AlpJ produced **2** both in the presence and absence of KinO1/Fre, although the qualitative rate of production was lower without a partner oxidoreductase. The dispensability of KinO1/Fre suggests a supportive but nonessential role in the formation of **2** *in vitro*. This contrasts with the results of Yang and co-workers.

The discovery that **2** can be accessed directly from **1** revises our thinking about its origins. Our work, and that of Yang and co-workers, demonstrates an alternate route to **2** that does not require a diazo-containing metabolite. We propose that AlpJ first catalyzes the ring opening reaction via a Baeyer–Villiger oxidation mechanism, giving rise to a previously posited aldehyde/acid intermediate (Scheme S1).[4a,b] This species may then undergo ring closure, decarboxylation, and dehydration to give the benzofluorene ring. The C-5 position of benzofluorene could be further oxidized to give hydroquinone-kinobscurinone, which could be attacked by the thiol of *N*-acetyl-cysteine, affording **2**.

We then explored the reactivity of AlpJ and Fre toward **1** and L-cysteine *in vitro*. We observed the formation of two new peaks that displayed UV–vis absorbances similar to that of **3** (Figures S12 trace ii, S13, and S14). An assay time course suggested that one of these products (**3a**) was a reaction intermediate, as its depletion accompanied the formation of the second product (**3**) (Figure 3A). The two products were also generated by AlpJ and KinO1 (Figure S12, trace iii). Isolation and high-resolution mass spectrometry (HRMS) analysis of the final product revealed m/z $[M + H]^+$ = 308.0929, which matches the molecular formula of **3**. The characterization of this product was complicated by the fact that **3** is NMR-silent.[14] We therefore derivatized **3** obtained from a large scale assay with methyl iodide.[3b] This provided a new product (**6**) that coeluted with an authentic standard of dimethylstealthin C (Figures 3B and S15). Further HRMS analysis of intermediate **3a** (Figure S14) showed a molecular weight m/z $[M + H]^+$ = 531.0898 and fragmentation pattern consistent with appendage of a cysteine disulfide to the benzofluorene scaffold. Inclusion of other proteinogenic amino acids in AlpJ/Fre *in vitro* assays did not provide new products (data not shown).

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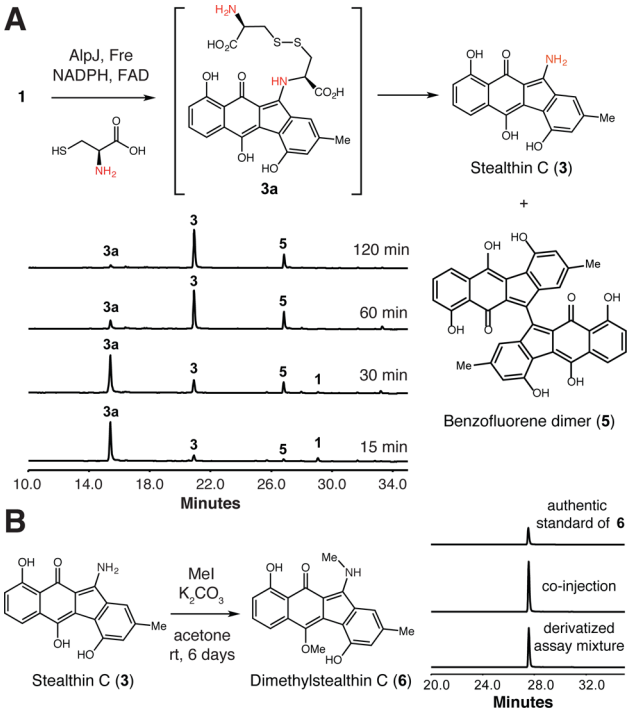


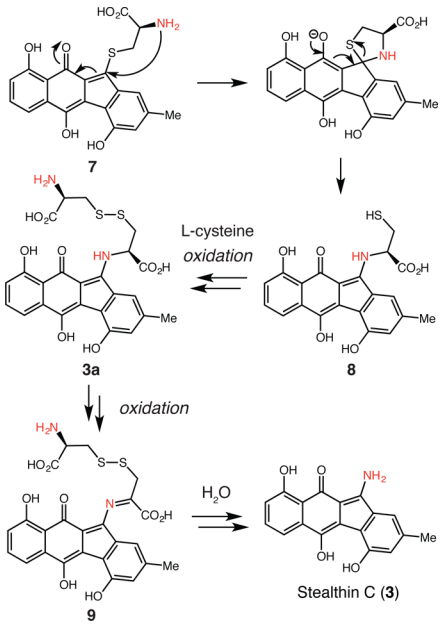
Figure 3. AlpJ converts **1** and L-cysteine to stealthin C (**3**). (A) HPLC analysis of *in vitro* assays containing AlpJ and Fre. **5** = diazofluorene dimer. Additional control assays are shown in Figures S16 and S17. (B) Derivatization of AlpJ assay product **3**.

The production of **3** from **1** and L-cysteine by AlpJ and KinO1/Fre is interesting from a mechanistic perspective. To verify the origin of the amino substituent of **3**, we performed the *in vitro* assay with ^{15}N-labeled L-cysteine (Figure S12, trace iv) and observed the masses of **3** and **3a** increase by +1 and +2 mass units, respectively (Figures S18 and S19). As AlpJ could convert **1** to either **3** in the presence of L-cysteine or **2** in the presence of *N*-acetyl-L-cysteine, we deduced that the presence of a free amine might be a critical determinant of reaction outcome. We hypothesize that in a manner analogous to the formation of **2**, generation of **3** involves attack by the L-cysteine thiol to form a thioether intermediate (*N*-deacetylseongomycin, **7**) followed by an intramolecular, S−N-type Smiles rearrangement (Scheme 1).[15] This reactivity resembles the S- to N-acyl transfer mechanisms of native chemical ligation and protein splicing.[16] The resulting N-linked intermediate (**8**) could form a disulfide bond with L-cysteine to afford **3a**, which could be further oxidized to imine **9**. Hydrolysis of this intermediate would provide **3**.

To test this mechanistic proposal, we ran AlpJ/Fre *in vitro* assays with **1** and a panel of alternative amino acid cosubstrates (Figure 4). L-Serine and *S*-methyl-L-cysteine failed to provide new products, confirming the essential role of a thiol nucleophile (Figure S20, traces i and ii). L-cysteine analogs containing non-nucleophilic amino groups, including the dipeptide γ-Glu-Cys and *N*-carbamoyl-L-cysteine, generated new *S*-linked products (**10** and **11**). This result shows that a nucleophilic amino group is necessary for C−N bond formation. (Figure S20, traces iii and iv, Figures S21 and S22). Assays with *N*-methyl-L-cysteine provided *N*-methyl-stealthin C (**12**) (Figure S20, trace v, Figure S23). We investigated the importance of the carboxylic acid of L-cysteine by examining cysteamine. HPLC and LC-HRMS analyses revealed a single new product (m/z [M − H]$^-$ = 441.0949) that likely corresponds to mixed disulfide **13** (Figure S20, trace vi, and Figure S24). This result suggests that the carboxylic acid is not needed for the Smiles rearrangement but is critical for the final oxidation. Assays with L-homocysteine gave a single product with a mass corresponding to homocysteine adduct **14** (m/z [M − H]$^-$ = 424.0862) (Figure S20, trace vii and Figure S25). The failure to form **3** could reflect a reduced rate of formation for a six membered ring-containing intermediate. Finally, assays with D-cysteine generated **3** with efficiencies similar to that observed for L-cysteine (Figure S20, trace viii).

The broad reactivity observed with thiol nucleophiles and the lack of a preference for L- vs D-cysteine led us to question whether AlpJ and/or Fre catalyzed the thiol addition and Smiles rearrangement reactions involved in forming **2** and **3**. To examine this issue, we performed competition experiments in which AlpJ and Fre were incubated with **1** and equal concentrations of two thiols. Assays containing L-cysteine and *N*-acetyl cysteine, as well as L-cysteine and cysteamine, generated comparable amounts of both expected products (Figure S20, trace ix). A third product with a mass corresponding to a mixed disulfide was also produced in the assay containing L-cysteine

Scheme 1. Proposed Mechanism for Stealthin C Formation



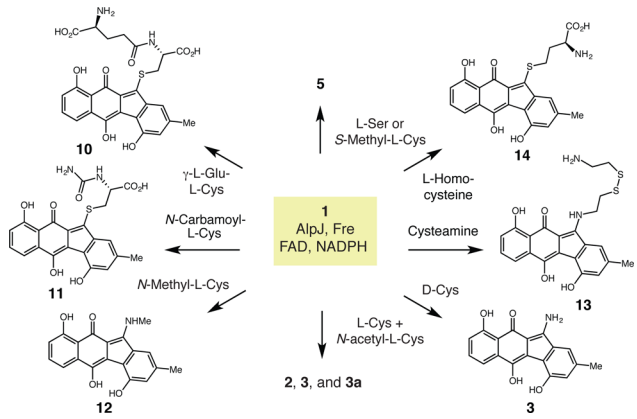


Figure 4. Reactivity of AlpJ-derived intermediates toward alternate nucleophiles.

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and cysteamine (Figure S20, trace x and Figure S26). Overall, the failure to observe a strong preference for L-cysteine over other thiols suggests this reactivity is nonenzymatic. Conversion of **3a** to **3** was observed when purified **3a** was incubated alone, as well as after assay mixtures containing **3a** were boiled to denature enzymes (Figure S27), indicating that the oxidation of **3a** and imine hydrolysis are likely also nonenzymatic.

The nonenzymatic generation of **3** from L-cysteine and an AlpJ-derived reaction partner *in vitro* has implications for the *in vivo* synthesis of this metabolite. **3** has only been isolated from mutant strains,[6b] consistent with nonenzymatic production from an accumulating, reactive species. This general phenomenon has been identified in other biosynthetic pathways.[17] The relevance of **3** to kinamycin biosynthesis was established using feeding experiments,[6b] and this result led to the hypothesis that diazo group construction involves N−N bond formation on the benzo[*b*]fluorene scaffold. However, the **3** used for these feeding experiments was carbon-labeled and only low levels of incorporation were observed. Though additional, unidentified enzymes could provide an alternate route to **3** *in vivo*, the observation that this metabolite is readily accessed via nonenzymatic reactions and the propensity of this ring system to undergo substitution chemistry led us to question the stability of **3** in the original feeding experiments and its role as a biosynthetic intermediate.

To investigate this possibility, we performed preliminary feeding experiments with the kinamycin producer *S. murayamaensis*. Feeding ^{15}N-labeled L-cysteine resulted in undetectable labeling of kinamycin D, whereas feeding $^{15}N_4$-labeled L-arginine as a control provided low but detectable incorporation (Table S3). The lack of kinamyin D enrichment upon feeding ^{15}N-labeled L-cysteine suggests that **3** arises from off-pathway reactions of a shunt product generated in mutants blocked in kinamycin synthesis. Significantly, this finding, along with recent investigations of hydrazide assembly in fosfazinomycin biosynthesis,[18] raises serious doubts about the long-assumed intermediacy of **3** in this pathway and will reshape future efforts to decipher the enzymatic chemistry of diazo formation.

Discovering new approaches for aromatic C−N bond formation is important because of this structural motif's predominance in pharmaceuticals and other bioactive compounds.[19] Interfacing the nonenzymatic S−N-type Smiles rearrangement with a natural product biosynthetic pathway represents a distinct strategy for accessing aromatic C−N bond containing compounds. Our findings should therefore inspire related strategies in synthetic biology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b10586.

Full experimental procedures and characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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