Aromatic polyketides are biologically synthesized via dedicated pathways, which often encompass as many as 20 to 30 individual steps (7, 16). Early stages of aromatic polyketide biosynthesis resemble fatty acid synthesis in bacteria, in which acetyl and malonyl coenzyme A (CoA) precursors are combined in repeated Claisen condensations to form a carbon backbone. However, unlike in fatty acid synthesis, polyketide synthases do not reduce the elongating carbon chain during synthesis, and subsequently a long polyketide is produced. This highly reactive polyketide chain is subsequently reduced, aromatized, and cyclized in a controlled manner, resulting in the formation of the various polyaromatic carbon skeletons. Differences in the stereo- and regiochemistries of cyclization and further tailoring reactions, such as hydroxylations, reductions, and methyllations, explain the vast number of compounds belonging to this class of natural products.

In order to understand how the structural diversity of BIQ metabolites is reflected in the composition of the biosynthetic enzymes, the alnumycin gene cluster has previously been cloned and expressed heterologously in Streptomyces albus (35). Molecular genetic studies have clarified that the biosynthesis is initiated from a butyryl starter unit (46), while cyclization of the third ring is catalyzed by enzymes belonging to entirely different enzyme families than those found from the actinorhodin and granaticin pathways (35). Recently, the novel pathway leading to the attachment and formation of the sugar-like 4'-hydroxy-5'-hydroxymethyl-2',7'-dioxane moiety was characterized in vitro and in vivo (34).

Many aromatic polyketides, such as the anthracyclines, the isochromanquinones, and the angucyclines, are p-quinones (16). Since this arrangement does not directly arise from the polyketide backbone upon cyclization, biosynthesis usually includes a tailoring step, which introduces a hydroxyl group in the para-arrangement in relation to a hydroxyl originating from the polyketide backbone. The polyphenolic dihydroquinones thus formed may readily oxidize nonenzymatically to the final p-quinone structures. Typically, enzymes catalyzing these steps belong to one of three enzyme families: the flavoprotein hydroxylases (22, 25), the so-called cofactorless monooxygenases (10,14, 38), and the two-component flavin-dependent monooxygenases (36, 44). In effect, the actinorhodin gene cluster harbors genes for both of the two latter classes, whose products are able to catalyze quinone formation (36). The alnumycin biosynthetic cluster (35), on the other hand, only contains genes for the putative two-component mono-
oxygenase system, alnT and alnH, which encode a flavin-dependent monooxygenase and a flavin reductase, respectively. The AlnT/AlnH pair is homologous to the ActVA-ORF5/ActVB from the actinorhodin pathway, respectively, which has been studied in recent years using molecular genetics (36) and biochemistry (42–44). The lack of a cofactorless oxygenase homologous to ActVA-ORF6 on the alnumycin pathway suggests a closer evolutionary relationship to the granaticin (20) and medermycin (21) (Fig. 1) gene clusters, which both encode genes only for the two-component system.

The aim of the work described in this paper was to uncover the molecular basis for formation of the p-quinone arrangement in the lateral instead of the central ring in alnumycin A. We demonstrate that alnT and alnH affect the same biosynthetic step, as both single-gene deletion mutants produced the same novel naphthodianthrone derivative, thalnumycin A (ThA), and medermycin (21) (Fig. 1) gene clusters, which both encode genes only for the two-component system.

The open reading frames (ORFs) alnH and alnT were deleted from the cosmid pAlnuori by using the λ Red recombinease system (8) through a two-step homologous recombination process. The primers used for inactivation of alnT and alnH were amplified from Streptomyces sp. CM020 genomic DNA. The primers used for amplification of alnH were alnHforB (EcoRI site in bold), 5’-CTAAAGATCATAACCGCCACGGCGGCGCC, and alnHrevH (HindIII site in bold), 5’-CTAAAGTCTACCGGGCGGCGCCGCC. The PCR product generated was cloned as a BglII-HindIII fragment into a modified pBAD/Hisp vector (23), resulting in pBAD/HisB Aluminum. The construction of pBAD/HisBAlnT was executed in a similar manner. The primers used for amplification of alnT were alnTforB (BglII site in bold), 5’-CACAGATCTCGCAAGCAGCGACGGCC, and alnTrev (EcoRI site in bold), 5’-CACAGAATTCGCTACCGGGCGCCCGTCG. The product was cloned in the same vector as a BglII-EcoRI fragment. Both constructs were verified by sequencing.

Inactivation of alnT and alnH. The open reading frames (ORFs) alnH and alnT were deleted from the cosmid pAlnuori by using the λ Red recombinease system (8) through a two-step homologous recombination process. The primers used for inactivation consisted of a 50-nucleotide (nt) region homologous to the target sequence followed by a 20-nt priming sequence (in bold) for amplification of the Cm’ gene with flanking FRT (FLP recognition target) sites. Elimination of the resistance gene was subsequently executed using the helper plasmid pFPL2 (17) expressing the FLP recombinease enzyme. The primers used for inactivation of alnH were alnHforCm, 5’-GACAGGACACGCCCGCGCAGTGCGACCGCAGCTGCGAAGCTGGACACCCCTCTGCAGGCTGATGCTAGGCTGGACTCCT, and alnHrevCm, 5’-TACGGGAGCGCCGCGCGGCTGCTGGTCGAGGAATGGGAGATGGGAGTCGAGCTGTTGACCCCTCTGCAGGCTGATGCTAGGCTGGACTCCT, and alnHrevCm. For alnT, the primers were alnTforCm, 5’-GTACCGGGAACAGCGCCGGCGCGGCGGCTGCTGGTCGAGGAATGGGAGATGGGAGTCGAGCTGTTGACCCCTCTGCAGGCTGATGCTAGGCTGGACTCCT, and alnTrevCm, 5’-TACGGGAGCGCCGCGCGGCTGCTGGTCGAGGAATGGGAGATGGGAGTCGAGCTGTTGACCCCTCTGCAGGCTGATGCTAGGCTGGACTCCT.

It was observed that the gene of interest was replaced by a 50-bp fragment from the 5’ end, a 103-bp scar sequence, and 50 bp from the 3’ end of the gene, resulting in pAlnuoriΔalnH. Likewise, the inactivation of alnT was performed using the primers alnTforCm and alnTrevCm. The PCR product generated was cloned as a BglII-HindIII fragment into a modified pBAD/Hisp vector (23), resulting in pBAD/HisBΔalnT. The construction of pBAD/HisBΔalnT was executed in a similar manner. The primers used for amplification of alnT were alnTforB (BglII site in bold), 5’-CACAGATCTCGCAAGCAGCGACGGCC, and alnTrev (EcoRI site in bold), 5’-CACAGAATTCGCTACCGGGCGCCCGTCG. The product was cloned in the same vector as a BglII-EcoRI fragment. Both constructs were verified by sequencing.
the 3′ end, resulting in pAoriΔalnT. After conjugation into S. albus, the mutated cosmids were reisolated, transformed back into E. coli TOP10, and verified by restriction enzyme analysis. Expression and purification of AlnH and AlnT. Recombinant AlnH protein was produced in 500 ml 2×YT medium (in a 2-liter Erlenmeyer flask) supplemented with 100 μg/ml ampicillin and inoculated with a 5-ml preculture of E. coli TOP10/pBADHisBdΔalnH. Induction was performed at an optical density at 600 nm (OD600) of 0.6 with L-arabinose to flask) supplemented with 100 mg/ml ampicillin and inoculated with a 5-ml preculture of E. coli TOP10/pBADHisBdΔalnH. Induction was performed at an optical density at 600 nm (OD600) of 0.6 with L-arabinose to

Enzyme reaction conditions. The enzyme assay mixture included 100 mM phosphate buffer (pH 7.0), 5 μM FMN, 1 mM NADH, approximately 200 μM ThA, and 0.25% (vol/vol) dimethyldisulfide in a final volume of 200 μl. AlnT and AlnH were added to the reaction mixture at 8 mM and 0.4 μM (final concentrations), respectively, and the reaction mixture was incubated at room temperature for 10 min. Two successive 50-μl chloroform extractions were performed. The extracted compounds were then vacuum concentrated and resuspended in acetone for high-performance liquid chromatography (HPLC) analysis. For the negative-control reaction, AlnT was incubated in boiling water for 15 min before addition to the reaction mixture.

Spectroscopic analysis of NADH consumption by AlnH. Kinetic measurements of NADH and NAPDH consumption were performed in triplicate 200-μl reaction mixtures on a 96-well plate (Multiskan GO; Thermo Scientific) by measuring absorbance at 340 nm. Concentrations of NADH, NAPDH, FMN, and FAD were determined using the extinction coefficients 6,290 M−1 cm−1 (340 nm), 6,620 M−1 cm−1 (340 nm), 12,500 M−1 cm−1 (450 nm) (27), and 11,300 M−1 cm−1 (450 nm) (27), respectively. The reaction assay was performed in 20 mM Tris-HCl (pH 7.5) with varied concentrations of FMN, NADH, FAD, or NAPDH. All other components were at non-rate-limiting concentrations (3 times the determined Km values). Kinetic values were obtained by a nonlinear regression analysis of the initial reaction rates using Origin Pro (version 8.0). All reagents were purchased from Sigma except FMM (TCI Europe).

Analysis of metabolites and reaction mixtures. The XAD-7 resin from the cultivations of both S. albus/pAoriΔalnH and S. albus/pAoriΔalnT was separated from the culture by repeated washing with tap water and decanting. The compounds bound to the XAD-7 were extracted using acetonitrile for subsequent HPLC analysis using an SCL-10Avp HPLC system equipped with an SPD-M10Avp (Shimadzu) diode array detector. A 5-μm LiChroCART 250-4 RP-18 column (Merck) was used with a 25-min gradient from 40% acetonitrile in 0.1% formic acid to 100% acetonitrile at 0.5 ml/min for metabolite analysis. Extracts from reaction mixtures were analyzed using the same HPLC system but with a 3.5-μm Sunfire C18 (Waters) column, which was used with a 25-min gradient from 15% acetonitrile in 0.1% formic acid to 100% acetonitrile at 0.25 ml/min.

Purification of metabolites. For large-scale purification of ThA and DHPA, S. albus/pAoriΔalnH was cultivated for 5 days. One day prior to harvesting, XAD-7 was added to the culture medium. The cell and XAD pellet were collected via centrifugation and stored at −20°C until extraction. Once thawed, the pellet was resuspended in tap water and the XAD-7 was separated as described above. Metabolites were extracted using acetonitrile. The compounds were fractionated with a 0-to-100% methanol gradient in CHCl3 using normal-phase column chromatography (silica gel 60, 0.040 to 0.063 mm; Merck). For the final polishing step for ThA and DHPA, fractions obtained from the silica column were applied to a preparative LiChroCART 250-10 RP-18 column (10 μm; Merck) with an L-6200A HPLC system (Merck Hitachi) and eluted with aqueous 0.1% formic acid and a 50-to-100% acetonitrile gradient at 2.5 ml/min. For large-scale purification of ThB, the reaction volume was scaled up to 1.5 l and a total of 17 reactions were carried out using in total, approximately 2.2 mg ThA. Two successive chloroform extractions (500 μl for every reaction mixture) were performed. The extracted compounds were then vacuum concentrated and resuspended in acetone for purification by a preparative HPLC step using the Merck L-6200A apparatus and a 5-μm Sunfire prep C18 250– by 10-mm column (Waters) with a 25-min gradient from 15% acetonitrile with 0.1% formic acid to 100% acetonitrile at 2.5 ml/min. The fractions containing ThB were extracted by addition of water and chloroform and subsequently vacuum dried. The approximate yield of ThB was 1.6 mg. The HPLC buffers, water, and chloroform were bubbled with nitrogen gas for at least 10 min prior to use. Nitrogen gas was used to fill the vacuum after drying ThB to prevent unwanted oxidation. Nuclear magnetic resonance (NMR) measurements were conducted immediately on the day of enzymatic synthesis at 283 K, since several degradation products of ThB could be observed already on the next day by proton NMR and HPLC.

MS and NMR measurements. The analysis of samples for mass determination was performed with a high-resolution HPLC-electron spray ionization-mass spectrometry (MS) system using a MicrOTOF-Q mass spectrometer (Bruker) with 4-kV capillary voltage, 573 K dry heater temperature, and a nebulizer pressure of 1.6 × 105 Pa. An Agilent Technologies 1200 series HPLC system equipped with a diode array detector and Sunfire C18 column (Waters) with a 25-min gradient from 15% to 100% acetonitrile in 0.1% formic acid at 0.25 ml/min was used. NMR spectra were collected with a Bruker Avance 500-MHz spectrometer for both ThA and ThB.

RESULTS
Sequence analysis of AlnH and AlnT. A BLAST search of all nonredundant proteins as well as a CD search (28) with the AlnH sequence in GenBank uncovered a large number of proteins homologous to the FlaRed enzyme superfamily (NCBI accession number cl00801). These enzymes contain a flavin reductase domain or a domain typically associated with flavoprotein oxygenases. The sequence identified with highest similarity was a puta-
tive flavin reductase from *Streptomyces* sp. SA3_actF (GenBank accession number ZP_07976974.1; 57% sequence identity). The well-characterized NADH-dependent FMN reductase ActVB (accession number NP_629242.1 [36, 43, 44]), involved in the biosynthesis of actinorhodin, showed 49% sequence identity. A multiple sequence alignment with four closely related sequences is shown in Fig. S3 of the supplemental material.

Proteins homologous to AlnT belonged to the acyl-CoA dehydrogenase (ACAD) superfamily (NCBI accession number c09933). The sequence identified with highest similarity was a putative oxygenase (GenBank accession number AEM44243.1) from an uncultured bacterium (54% identity). More relevant in terms of putative function were the angucyclinone hydroxylase LndZ5 (37) from *Streptomyces globisporus* (53% identity) and ActVA-ORF5 (36, 42–44) from *Streptomyces coelicolor* A3(2) (48% identity), involved in biosynthesis of the aromatic polyketides landomycin and actinorhodin, respectively. Sequence comparison with the monoxygenase component of *p*-hydroxyphenylacetate hydroxylase (see Fig. S4 in the supplemental material) from *Acinetobacter baumannii* (1) identified conserved residues in AlnT that may be involved in catalysis (H396) and formation of hydrophobic interactions (W210) or hydrogen bonds (S171) with the flavin. According to the classification reported by van Berkel et al. (45), AlnT and AlnH form a class D flavoprotein monoxygenase.

**Inactivation of alnT and alnH.** The genes *alnT* and *alnH* reside in different operons approximately 15.3 kb apart within the alnuacin gene cluster (35). In order to experimentally verify that the gene products formed a two-component monoxygenase system and were involved in the same biosynthetic step, both genes were individually inactivated from cosmid pAlnuori as described in Materials and Methods by using RED/ET recombineering (8) in *E. coli* K-12. The resulting cosmids pAoriΔalnT and pAoriΔalnH, containing the *alnT* and *alnH* deletions, respectively, were introduced into the heterologous host *S. albus* through intergeneric conjugation from *E. coli* ET12567/pUZ8002 (24). Since both *alnT* and *alnH* were situated as last genes in their respective operon structures, the gene inactivation experiments were unlikely to stimulate any downstream effects.

**Analysis of metabolites produced by the mutant strains.** The strains *S. albus*/pAoriΔalnT and *S. albus*/pAoriΔalnH were cultivated for 5 days, and the metabolites produced were analyzed by HPLC. Encouragingly, the production profiles of the strains were identical, and three novel metabolites, not detected from cultivation of the parental strain *S. albus*/pAlnuori, were observed (Fig. 2). One of the compounds was readily identified as K1115 A (Fig. 1), which has been previously obtained through inactivation of the aldo-ketoreductase *aln4* (35). For structure elucidation of the second remaining metabolite, the strain *S. albus*/pAoriΔalnT was grown on a large scale, and the compounds produced were purified by open column chromatography and preparative HPLC.

The second compound was confirmed as the decarboxylated form of K1115 A, DHPA (Fig. 1), which has previously been isolated from the marine *Streptomyces* sp. FX-58 (19). The structure elucidation was based on 1H- and 13C-NMR spectra (Table 1), and high-resolution MS measurements in the negative mode ([M − H]− observed 281.0848, calculated 281.0814). Consistent with this assessment, the UV-Vis spectrum of DHPA was identical to that of K1115 A (see Fig. S5 in the supplemental material).

For structure elucidation of the third metabolite, the molecular formula C17H17O4 was inferred from the high-resolution mass spectrometry data ([M − H]− observed 285.1123, calculated 285.1127). A literature search revealed that the 1H and 13C spectra (Table 1) for the compound were similar to those reported for HU235, a metabolite isolated from heterologous expression of the R1128 pathway (29). Significantly, only a single carbonyl carbon signal at 206.6 was present in the 13C NMR spectra, indicating that the compound isolated did not contain a quinone ring. The structure was finally verified by heteronuclear single quantum coherence and heteronuclear multiple bond correlation techniques (see Fig. S6 in the supplemental material), which demonstrated that the metabolite was a novel polyketide denoted as ThA (Fig. 1).

**In vitro activity of AlnT/AlnH and enzymatic synthesis of ThB.** Both AlnH and AlnT were produced in *E. coli* TOP10 as N-terminally histidine-tagged recombinant proteins that contained an additional AHHHHHHHRS sequence and purified to near homogeneity (see Fig. S1 and S2 in the supplemental material) as described in Materials and Methods. Based on sequence analysis, it was likely that AlnH was an NADH-dependent flavin reductase. In order to confirm this supposition, AlnH was incubated together with FMN and an excess of NADH. Monitoring of the reaction at 340 nm, specific for NADH, showed a clear decrease in absorbance over time, indicating oxidation of the cofactor to NAD+. Activity was also seen with FAD, although FMN appeared to be the preferred flavin species. Slow consumption of NADPH was also detected with FMN but not with FAD (Table 2). Similar to the closely related flavin reductase homologue ActVB (11, 12), NADH and FMN appeared to be the preferred cofactors (Table 2).

None of the metabolites isolated from the knockout strains contained correctly cyclized pyran rings, which indicates that quinone formation is likely to occur at an earlier stage during polyketide folding and that the natural substrate for AlnH is likely to be an unstable and highly reactive bicyclic intermediate of the pathway. Therefore, in order to demonstrate the regiochemistry of the putative monoxygenase component AlnT, various substrate analogues were screened in a reaction together with AlnH, AlnT, FMN, and NADH. While none of the synthetic compounds tested (e.g., dithranol and 1,8-dihydroxynaphthalene) proved promising, HPLC analysis identified a new peak when ThA was used as a substrate (Fig. 3A). The appearance of the product was dependent on the presence of all of the reaction mixture compo-
nents mentioned above and, importantly, a control reaction using heat-deactivated AlnT that did not lead to product formation (Fig. 3B). The UV-Vis spectrum of the reaction product was similar to that of ThA but displayed a bathochromic shift of 26 nm, which was consistent with our expectation of a hydroxylation product (see Fig. S5 in the supplemental material). This assumption was verified by high-resolution MS measurements in the negative mode ([M $\cdot$ H]$^{-}$ observed 301.1082, calculated 301.1076), which confirmed the molecular formula, C$_{17}$H$_{17}$O$_{5}$. Despite the instability of the compound, multiple enzymatic reactions provided sufficient material for structure elucidation by $^1$H NMR and detection of selected carbon signals indirectly via heteronuclear single-quantum correlation spectroscopy. The relatively large coupling constant (J, 8.4 Hz) between H-6 and H-7 indicated that the protons were vicinal, which thus excluded the m-position for the hydroxyl group at the lateral ring. The distinction between the o- and p-positions was accomplished by detection of the relatively large downfield change in the chemical shift for H-4 (Table 1), which confirmed that the product ThB was the p-hydroquinone (Fig. 1).

**DISCUSSION**

The great chemical diversity of aromatic polyketides is brought about via selection of starter units and the number of extension cycles used in the initial polyketide synthesis, variations in cyclization patterns, and through the action of numerous tailoring enzymes. As shown in Table 2, the apparent kinetic parameters for the reductase activities of AlnH and ActVB are quite similar, with the exception of FMN as a cosubstrate in AlnH.

**TABLE 1** NMR spectral data for DHPA, ThA, and ThB$^a$

<table>
<thead>
<tr>
<th>Position</th>
<th>DHPA</th>
<th>ThA</th>
<th>ThB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>162.5</td>
<td>1.38, s, 3H</td>
<td>26.2</td>
</tr>
<tr>
<td>1-OH</td>
<td>13.07, s</td>
<td>13.07, s</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.28, dd, 1.2, 8.4</td>
<td>6.99, d, 1.5</td>
<td>113.7</td>
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<tr>
<td>2-OH</td>
<td>135.6</td>
<td>138.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7.59, dd, 7.5, 8.5</td>
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<td>113.7</td>
</tr>
<tr>
<td>3a</td>
<td>132.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7.75, dd, 1.2, 7.5</td>
<td>6.99, d, 1.5</td>
<td>113.7</td>
</tr>
<tr>
<td>4a</td>
<td>132.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.65, d, 2.6</td>
<td>7.13, dd, 0.9, 8.2</td>
<td>113.7</td>
</tr>
<tr>
<td>5-OH</td>
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<td></td>
<td></td>
</tr>
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<td>6</td>
<td>150.9</td>
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</tr>
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<td>8</td>
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<td>8a</td>
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<td>1.11, t, 3H</td>
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</table>

$^a$ Chemical shifts are reported relative to tetramethyl silane, which was used as an internal standard. Measurements were conducted in CDCl$_3$ (DHPA) and acetone-d$_6$.

$^b$ Values were obtained indirectly via heteronuclear single quantum coherence spectroscopy measurements.

**TABLE 2** Apparent kinetic parameters for the reductase activities of AlnH and ActVB

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ ($\mu$M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>Cosubstrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlnH</td>
<td>FMN</td>
<td>1.15 ± 0.08</td>
<td>0.56 ± 0.01</td>
<td>NADH</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>FAD</td>
<td>12.3 ± 1.5</td>
<td>0.64 ± 0.02</td>
<td>NADH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>45.5 ± 2.8</td>
<td>1.05 ± 0.02</td>
<td>FMN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>2,687 ± 1,480</td>
<td>0.93 ± 0.38</td>
<td>FMN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>No activity</td>
<td></td>
<td>FAD</td>
<td></td>
</tr>
<tr>
<td>ActVB</td>
<td>FMN</td>
<td>1.0 ± 0.1</td>
<td>9.2 ± 0.4</td>
<td>NADH</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>FAD</td>
<td>8.7 ± 0.6</td>
<td>8.2 ± 0.7</td>
<td>NADH</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>6.6 ± 0.5</td>
<td>9.2 ± 0.4</td>
<td>FMN</td>
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</tr>
<tr>
<td></td>
<td>FMN</td>
<td>39.6</td>
<td>2.7</td>
<td>NADH</td>
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</table>
enzymes (7,16). Oxidative modifications are arguably one of the most common means of generating diversity in polyketide metabolites, together with methylations, glycosylations, and reductions. This holds especially true in the biosynthesis of polyaromatic metabolites, since many of the pathway intermediates can also react with molecular oxygen nonenzymatically, which could lead to the formation of undesirable shunt products (31). Therefore, in order to assert control over correct regiochemistry, particularly in p-quinone formation, a specific tailoring reaction is requisite.

Enzymes belonging to three different families have been identified to be responsible for p-quinone biosynthesis in various pathways, as indicated above. Gene clusters involved in the biosynthesis of BIQ metabolites harbor gene products homologous to the much-studied p-hydroxyphenyl acetate hydroxylase C1/C2 (1, 5, 40). Of these, ActVA-ORF5 and ActVB have been experimentally demonstrated to form a two-component monooxygenase system and to catalyze quinone formation in the biosynthesis of actinorhodin (36, 44). ActVA-ORF5 has been shown to be the actual FMN-dependent monooxygenase, while ActVB is a flavin: NADH oxidoreductase that provides reduced FMN for ActVA-ORF5. Analysis of the alunycin A biosynthetic gene cluster revealed that the most likely candidates for production of the quinone arrangement in the lateral ring were the putative monooxygenase AlnT and the flavin reductase AlnH.

Consistent with the assumption, the production profiles of alnT and alnH deletion mutants were highly similar, indicating that the gene products might be involved in the same biosynthetic step. Somewhat surprisingly, endogenous flavin reductases of S. albus were not able to complement the alnH mutation. Structural elucidation of the metabolites, however, revealed that all of the compounds lacked correct third ring cyclization, which suggested that the substrate for AlnH/AlnT would be a highly reactive bicy-
cyclic intermediate of the pathway. Retrosynthesis of K1115 A and DHPA (Fig. 4A) implies that the compounds isolated might have originated from an aldol condensation (41), in this case probably nonenzymatic, between carbons C-4 and C-17 (polyketide numbering) of the bicyclic intermediate. The timing of the decarboxylation of DHPA is more challenging to assess, but as β-keto acid decarboxylation is a well-established reaction in chemistry (26), it may be that the loss of the terminal carboxy group takes place prior to cyclization. In any case, subsequent dehydration and nonenzymatic oxidation of the intermediates would yield the final quinone products (Fig. 4A). In a similar manner, formation of ThA is likely to proceed via β-keto acid decarboxylation and C-3/C-16 cyclization of the same bicyclic intermediate (Fig. 4A).

We propose that in the alnumycin pathway, AlnH and AlnT form a two-component monoxygenase system that is responsible for hydroxylation of position C-11 (polyketide numbering) of the bicyclic intermediate of the pathway (Fig. 4B). Specifically, our data demonstrate that AlnH is a flavin:NADH oxidoreductase, which is required to provide FMN for AlnT for the actual monooxygenation reaction. The reduced FMN could next react with molecular oxygen, according to the rules established for classical flavin chemistry (30), and form an FMN peroxy intermediate that would be capable of hydroxylating the substrate. Although the probable natural substrate of the reaction is unstable and thus not available for in vitro studies, the enzymatic synthesis of ThB yielded strong evidence for the regiochemistry of the reaction and indicated that the hydroxylation would occur in the lateral ring in the para-position with regard to the hydroxyl group originating from the polyketide backbone.

Our results conclusively verify that both the timing and regiochemistry of alnumycin quinone formation differ from the equivalent reactions in the actinorhodin pathway (36) (Fig. 4C), which was somewhat unexpected due to the high sequence similarity of the monooxygenase components AlnT and ActVA-ORF5. It is interesting, however, that based on sequence similarity, AlnT is in effect more closely related to LndZ5, which is responsible for hydroxylation of the equivalent lateral ring in the biosynthesis of the larger four-ring angucycline antibiotic landmycin (37), than to ActVA-ORF5. It is plausible that an earlier hydroxylation step is required in the alnumycin pathway to prevent unwanted nonenzymatic quinone formation, as seen in the structures of K1115 A and DHPA. The results presented here also raise an interesting question regarding the timing of quinone formation in the biosynthesis of medermycin and granaticin and whether they follow the paradigms that have now been established for alnumycin (Fig. 4B) and actinorhodin (Fig. 4C) biosynthesis.

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