Chemistry & Biology
Article

# Discovery of a Two-Component Monooxygenase SnoaW/SnoaL2 Involved in Nogalamycin Biosynthesis

Vilja Siitonen,[1] Bastian Blauenburg,[1] Pauli Kallio,[1] Pekka Mäntsälä,[1] and Mikko Metsä-Ketelä[1,*]
[1]Department of Biochemistry and Food Chemistry, University of Turku, Turku FIN-20014, Finland
*Correspondence: mikko.mk@gmail.com
DOI 10.1016/j.chembiol.2012.04.009

## SUMMARY

**Nogalamycin is an anthracycline polyketide antibiotic that contains two deoxysugars, at positions C-1 and C-7. Previous biosynthetic studies conducted in vivo affiliated *snoaL2* with an unusual C-1 hydroxylation reaction, but in vitro activity was not established. Here, we demonstrate that inactivation of either *snoaL2* or *snoaW* resulted in accumulation of two nonhydroxylated metabolites, nogalamycinone and a novel anthracycline 3′,4′-demethoxy-nogalose-nogalamycinone. The C-1 hydroxylation activity was successfully reconstructed in vitro in the presence of the two enzymes, NAD(P)H and the substrates. Based on relative reaction efficiencies, 3′,4′-demethoxy-nogalose-nogalamycinone was identified as the likely natural substrate. A biosynthetic model was established where the atypical short-chain alcohol dehydrogenase SnoaW reduces the anthraquinone to a dihydroquinone using NADPH, which enables activation of oxygen and formation of a hydroperoxy intermediate. Finally, protonation of the intermediate by SnoaL2 yields the 1-hydroxylated product.**

## INTRODUCTION

Polyketide antibiotics comprise a structurally diverse class of secondary metabolites, which have been well established for their biological activities and applications in medicine (Newman and Cragg, 2007). Aromatic polyketides, which are a major subclass of polyketides produced mainly by soil-dwelling *Streptomyces* bacteria, commonly consist of a polyaromatic carbon skeleton that is further modified in tailoring reactions such as glycosylations, hydroxylations, decarboxylations, and methylations (Hertweck et al., 2007). Differences in the shape and size of the aglycone moiety and the degree of tailoring explain the vast number of compounds belonging to this family.

One large class of aromatic polyketides is the anthracyclines, which have been chemically defined to consist of a 7,8,9,10-tetrahydro-5,12-naphtacenoquinone aglycone chromophore onto which one or more deoxysugars are attached, most commonly at the C-7 position (Hutchinson, 1997; Metsä-Ketelä et al., 2008). Nogalamycin (**1**, Figure 1) is an intriguing member of anthracyclines as it contains an aminosugar, nogalamine, attached to the polyketide aglycone in an unique manner through two bonds, O-glycosylation at C-1 and via a carbon-carbon bond between C-2 and C-5″ (Arora, 1983). The compound was further developed into a semisynthetic derivative, menogaril, which progressed to clinical trials, where it showed promising antitumor activity (Neil et al., 1979) with reduced acute toxicity (McGovren et al., 1979), but the development of the compound nevertheless ceased at phase II. In spite of this, the promising features of menogaril and the structural peculiarity of **1** in general was the driving incentive for the isolation of the biosynthetic gene cluster, which was used in a wide range of molecular genetic studies (Kantola et al., 2000; Siitonen et al., 2012; Torkkell et al., 1997, 2000, 2001; Ylihonko et al., 1996a, 1996b). These experiments led to the generation of several novel anthracycline metabolites and identified the functions of dozens of gene products residing on the pathway.

In recent years, several families of cofactor-independent oxygenases have been identified and characterized (Fetzner, 2002; Fetzner and Steiner, 2010). The oxygenases have generated considerable interest in the scientific community as direct addition of $O_2$ to organic molecules is a spin forbidden reaction, which is usually overcome by utilization of transition metals or organic cofactors to break the spin-barrier (Fetzner and Steiner, 2010; Mattevi, 2006). Intriguingly, two pairs of unrelated putative cofactor-independent enzymes, SnoaB/AknX and SnoaL2/AclR, have been demonstrated to be involved in oxygen transfer reactions in the biosynthesis of the anthracyclines nogalamycin and aclacinomycin. Mechanistic aspects of the quinone forming C-12 monooxygenases SnoaB (Grocholski et al., 2010) and AknX (Chung et al., 2002) have suggested that catalysis proceeds via a carbanion intermediate, which is in agreement with the findings from their actinorhodin counterpart, ActVA-ORF6 (Sciara et al., 2003). In addition, early molecular genetic studies identified the product of another small open reading frame *snoaL2* to be involved in anthracycline C-1 hydroxylation through expression of the gene in *Streptomyces galilaeus* strain H063, which resulted in the production of 1-hydroxylated aklavinone (Torkkell et al., 2001). This prompted further interest in SnoaL2 and AclR, which based on sequence similarity appeared to be evolutionarily related to polyketide cyclases, such as SnoaL (Sultana et al., 2004; Torkkell et al., 2000), AknH (Kallio et al., 2006), and DnrD (Kendrew et al., 1999) that catalyze cyclization of the fourth ring through aldol condensation, rather than to known cofactor-independent oxygenases.

Determination of the 3D structures of SnoaL2 and AclR by protein crystallography from the nogalamycin and aclacinomycin/cinerubin pathways, respectively, verified that the fold of the two enzymes was indeed similar to the polyketide cyclases

 

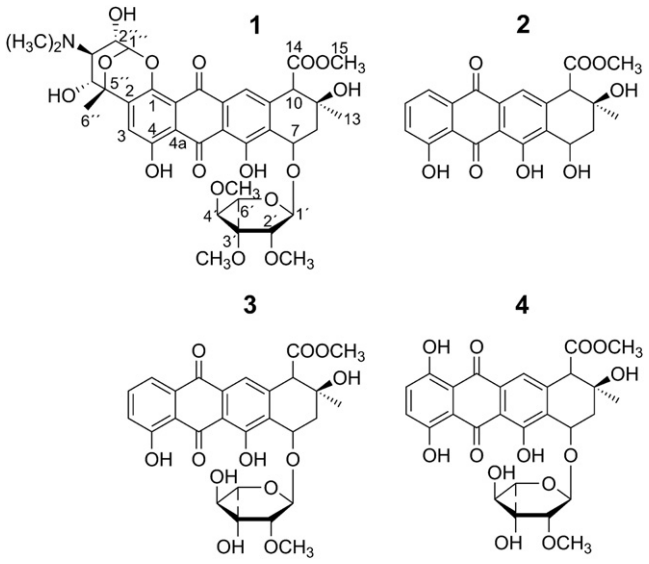


**Figure 1. Structures of the Anthracyclines Investigated in This Study**
(1) Nogalamycin.
(2) Nogalamycinone.
(3) 3′,4′-Demethoxy-nogalose-nogalamycinone.
(4) 3′,4′-Demethoxy-nogalose-1-hydroxy-nogalamycinone.

(Beinker et al., 2006). Despite extensive effort, however, in vitro activity for the C-1 hydroxylation reaction could not be established at the time which hindered structure-function studies that would explain how these two enzyme pairs (SnoaL/SnoaL2 and AknH/AclR, approximately 25% sequence identity) have evolved to catalyze such different chemical reactions.

In this paper, we demonstrate that inactivation of either *snoaL2* or *snoaW*, which encodes an "atypical" short-chain alcohol dehydrogenase (SDR) (Persson et al., 2009), resulted in similar production profiles and in the accumulation of nonhydroxylated metabolites. The C-1 hydroxylation activity could be subsequently reconstituted in vitro using substrates isolated from the knockout strains, NAD(P)H, and the two enzymes SnoaL2 and SnoaW. Finally, extensive biochemical characterization of the reaction led us to present a mechanistic model inspired by flavin chemistry for the C-1 hydroxylation reaction catalyzed by the novel two-component monooxygenase system SnoaW/SnoaL2.

## RESULTS

### Inactivation of Genes Encoding SnoaW and SnoaL2, and Complementation

Direct manipulation of *Streptomyces nogalater* genomic DNA has been notoriously challenging due to an efficient restriction modification system present in the strain. However, we have recently been able to express a large part of the biosynthetic gene cluster in the heterologous host *Streptomyces albus* using the cosmid pSnogaori (Siitonen et al., 2012). For this study, *snoaL2* and *snoaW* were knocked out from pSnogaori in *Escherichia coli* using λ Red recombination as described in the Experimental Procedures. Analysis of the metabolites produced by *S. albus*/pSnoΔaL2 and *S. albus*/pSnoΔaW revealed similar

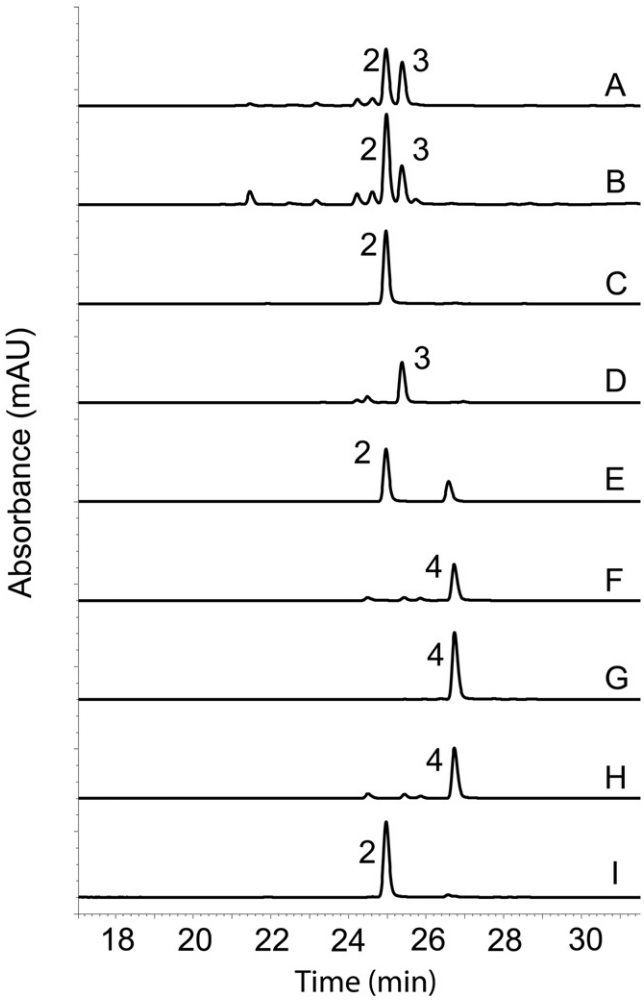


**Figure 2. HPLC Traces Recorded at 460 nm of the Various Metabolites Investigated in This Study**
(A) Production profile of the strain *S. albus*/pSnoΔaL2. The main metabolites produced are nogalamycinone (**2**) and 3′,4′-demethoxy-nogalose-nogalamycinone (**3**).
(B) Production profile of the strain *S. albus*/pSnoΔaW, which produces the same metabolites as the *snoaL2* knockout strain.
(C) Authentic **2** obtained from previous studies (Siitonen et al., 2012).
(D) Purified **3** obtained from cultures of *S. albus*/pSnoΔaW.
(E) Reaction of **2** with SnoaW and SnoaL2 in the presence of NADPH.
(F) Conversion of **3** into 3′,4′-demethoxy-nogalose-1-hydroxy-nogalamycinone (**4**) by SnoaW and SnoaL2 in the presence of NADPH.
(G) Authentic **4** obtained from previous studies (Siitonen et al., 2012).
(H) Conversion of **3** into **4** by SnoaW and SnoaL2 in the presence of NADH.
(I) Reaction of **2** with SnoaW and SnoaL2 in the presence of NADH.

production profiles and indicated the presence of two new major peaks with characteristic spectra of anthracyclines by high-performance liquid chromatography (HPLC)-UV/Vis (Figures 2A and 2B).

In order to rule out any possible polar effects caused by the deletion of *snoaW* to the transcription of downstream genes, we next introduced the plasmid pIJTaW, which contained PCR amplified *snoaW* under the control of TipA-promoter, into the corresponding deletion mutant strain. The introduction of pIJTaW

**Table 1. $^{1}H$ NMR and $^{13}C$ Assignments of 3 in MeOD/*d6*-DMSO at 25°C**

| Position | $^{1}H$ | $^{13}C$ |
|---|---|---|
| 1 | 7.53 dd, 1.19, 7.58 | 119.4 |
| 2 | 7.67 dd, 7.57, 8.32 | 137.4 |
| 3 | 7.25 dd, 1.12, 8.42 | 124.4 |
| 4 | | 161.2 |
| 4-OH | Exchangeable | |
| 4a | | 115.7 |
| 5 | | 191.8 |
| 5a | | 114.5 |
| 6 | | 161.5 |
| 6-OH | Exchangeable | |
| 6a | | 131.1 |
| 7 | 4.96 dd, 1.09, 5.54 | 72.6 |
| 8 | 2.005 d, 14.2 | 38.7 |
| | 2.65 dd, 14.19, 5.62 | |
| 9 | | 67.9 |
| 9-OH | Exchangeable | |
| 10 | 4.04 s | 57.38 |
| 10a | | 143.1 |
| 11 | 7.35 s | 119.5 |
| 11a | | 132.15 |
| 12 | | 180.7 |
| 12a | | 133.1 |
| 13 | 1.37 s, 3H | 29.2 |
| 14 | | 170.9 |
| 15 | 3.61 s, 3H | 52.0 |
| 1′ | 5.26 d, 1.00 | 101.8 |
| 2′ | 2.99 d, 1.10 | 85.1 |
| 2′-O-CH3 | 3.44 s, 3H | 58.9 |
| 3′ | | 71.5 |
| 3′-OH | 4.06 s | |
| 3′-CH3 | 1.01 s, 3H | 18.7 |
| 4′ | 3.21 d, 9.56 | 74.7 |
| 4′-OH | Exchangeable | |
| 5′ | 3.59 | 68.2 |
| 6′ | 1.21 d, 6.5, 3H | 18.3 |

The chemical shifts of $^{1}H$ and $^{13}C$ nuclei were internally referenced to MeOD. The spectra were recorded with a 500 MHz instrument. s, singlet; d, doublet; dd, doublet of doublets.
See also Figure S1.

into *S. albus*/pSnoΔaW complemented the mutation and the production profile of the strain was converted essentially back to that of the original nonmutated strain *S. albus*/pSnogaori (data not shown). The gene *snoaL2* resides putatively in its own operon (Siitonen et al., 2012), and therefore it was unlikely to cause any downstream effects upon gene inactivation.

## Structure Elucidation of the Metabolites and Identification of an Anthracycline Intermediate

One of the metabolites detected in the *S. albus*/pSnoΔaW cultivations was readily inferred as nogalamycinone (**2**, Figure 1) based on the HPLC elution time and UV/Vis spectral properties, which were identical to an authentic sample obtained from previous studies (Figure 2C) (Siitonen et al., 2012). This assignment was also supported by further LC-ESI(−)-HR-MS analysis ($[M\text{-}H]^-$ observed 397.0929, calculated 397.0929).

For structure elucidation of the other previously undetected metabolite, the strain was grown in large scale and the polyketide produced was purified by open column chromatography and preparative HPLC. The UV/Vis spectrum of the compound was identical to that of **2** with maxima at 258, 289, and 430 nm, suggesting that the polyketide aglycone chromophore was the same. The molecular formula $C_{29}H_{32}O_{12}$ was verified by LC-ESI-HR-MS ($[M\text{-}H]^-$ observed 571.1807, calculated 571.1821) indicating that the product might be a monoglycosylated nogalamycin intermediate. This was finally confirmed by $^{1}H$ and $^{13}C$ NMR spectra (Table 1), and COSY, HSQC, HMBC measurements (Figure S1 available online), which unequivocally proved that the compound was, to our knowledge, a novel anthracycline 3′,4′-demethoxy-nogalose-nogalamycinone (**3**, Figures 1 and 2D).

## Production and Purification of SnoaW and SnoaL2

For production of histidine tagged recombinant proteins, *snoaW* and *snoaL2* were cloned into a modified pBAD/HisB plasmid (Kallio et al., 2006) in *E. coli* TOP10 as described in Experimental Procedures. The cloning procedure introduced an additional AHHHHHHHRS N-terminal sequence into the polypeptides. Both proteins could be purified to homogeneity in a single step using affinity chromatography. The yield of SnoaL2 (17.0 kDa) was 17 mg/liter culture medium, while SnoaW (30.4 kDa) was produced in a lower yield of 12 mg/liter culture medium. Addition of 1% reduced Triton X-100 (v/v) was required for stabilization of SnoaW.

## In Vitro C-1 Hydroxylation Activity of SnoaW/SnoaL2

When 1 μM SnoaW and 20 μM SnoaL2 were incubated with 100 μM **2** in the presence of 1 mM NADPH, approximately 20% conversion was observed in an overnight incubation at room temperature as estimated by HPLC (Figure 2E). However, when **3** was used as a substrate, the color of the reaction mixture shifted from yellow to red within minutes, and near to complete conversion was achieved (Figure 2F). The reaction was accompanied with several changes in the spectral properties, which could be attributed to (1) consumption of the cosubstrate NADPH at 340 nm, (2) consumption of the substrate at 435 nm, and (3) formation of the product at 490 nm (Figure 3). The end product of the reaction was identified as 3′,4′-demethoxy-nogalose-1-hydroxy-nogalamycinone (**4**, Figure 1) by comparison of the spectral properties to an authentic standard (Siitonen et al., 2012) and by HPLC (Figure 2G).

## Activity of SnoaW Alone Leads to a Nonproductive Cycle

To determine the order of the reactions catalyzed by SnoaL2 and SnoaW, the proteins were incubated individually with **3** and NADPH. While catalytic activity could not be observed for SnoaL2, the SnoaW reaction resulted in a decrease of the absorbance signal at 340 nm, specific for NADPH. The reaction took place only in the presence of the substrate, yet no conversion product could be initially detected in HPLC analysis; the mixture

 

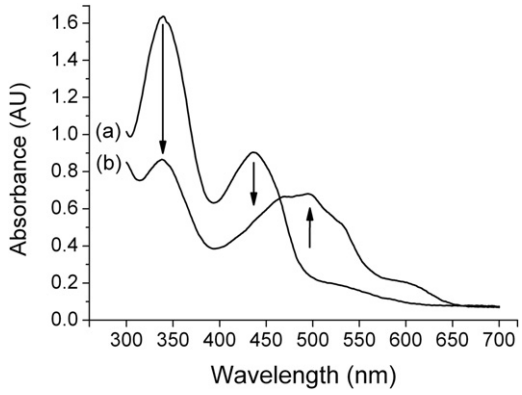


**Figure 3. Monitoring of the In Vitro Reaction by UV/Vis Spectrophotometry**

(A) Directly after initiation of the reaction.

(B) After 20 min of the reaction. The decrease of absorbance at 340 nm correlates with the oxidation of NADPH, while the changes at 435 and 490 nm correspond to the conversion of the substrate to the hydroxylated product.

See also Figure S2 for reaction kinetics calculated from the spectral data.

solely contained **3** in quantities comparable to those initially supplied. When the substrate consumption was monitored at 435 nm, the signal first decreased in proportion to the amount of NADPH, but eventually returned toward the starting levels (Figure 4A) suggesting that the product of the reaction had converted back into the substrate. However, when the reaction was conducted under near single turnover conditions with a high concentration of SnoaW followed by removal of oxygen after 30 s and quenching of the reaction using organic solvents, a reaction product could finally be detected by HPLC (Figure 4B). The metabolite displayed a clear hypsochromic shift in comparison to **3**, and the UV/Vis maximum had changed from 435 to 370 nm (Figure 4B). Further characterization of the intermediate was hindered by oxidation of the reaction product, since after 45 min only **3** could be detected by reinjection of the same sample into HPLC (Figure 4B). Consistent with our other observations, if SnoaL2 was added to the SnoaW reaction mixture after depletion of NADPH, no conversion into the 1-hydroxylated product could be detected. However, if the NADPH depleted SnoaW reaction was enriched with both SnoaL2 and additional NADPH, the end product **4** was obtained normally.

### Formation of $H_2O_2$ during the Reaction Cascade

In flavin chemistry, a (hydro)peroxy intermediate is formed when reduced flavin reacts with $O_2$. Such a peroxyflavin is unstable and decays to form hydrogen peroxide and oxidized flavin (van Berkel et al., 2006). In order to obtain experimental evidence for the existence of a peroxy anthracycline intermediate, we used the horseradish peroxidase (HRP)/2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay system to measure formation of $H_2O_2$. When the nonproductive SnoaW reaction was titrated with NADPH, cosubstrate concentration-dependent formation of $H_2O_2$ was observed (Figure 4C). In contrast, hydrogen peroxide was not detected in an efficiently coupled SnoaW/SnoaL2 reaction.

### Oxygen Dependence and Metal Independence of SnoaW/SnoaL2

If the coupled SnoaW/SnoaL2 reaction was performed in a Thunberg cuvette under anaerobic conditions, the reaction did not proceed and NADPH consumption could not be detected (Figure 4D). When oxygen was introduced into the reaction mixture, the concentration of NADPH started to decrease (Figure 4D) and product was formed as in the default conditions. In order to verify that the incorporated oxygen derived from molecular oxygen, the reaction was next conducted under $^{18}O_2$ atmosphere. Mass spectrometry analysis confirmed that the mass of the reaction product was increased by 2.0033 Da, which was consistent with incorporation of 1/2 $^{18}O_2$. Finally, the reaction was conducted in the presence of 10 mM EDTA without any interference, which excluded the involvement of metal ions in catalysis.

### Cosubstrate Specificity and SnoaW Reaction Kinetics

Sequence analysis revealed that SnoaW was homologous to atypical SDR enzymes such as triphenylmethane reductase TMR (Kim et al., 2008b) and quinone oxidoreductase QOR2 (Kim et al., 2008a) with sequence features showing preference for NADPH over NADH. However, in the hydroxylation of **3**, SnoaW was in effect able to utilize both NADPH (Figure 2F) and NADH (Figure 2H) as cosubstrates. This was in contrast to the conversion of **2**, where only NADPH could be used (Figure 2E), while utilization of NADH did not lead to product formation (Figure 2I). Kinetic comparison showed that SnoaW did not conform to typical Michaelis-Menten saturation kinetics as the *v*/[S] profiles were nonhyperbolic, which suggested modulation by both substrate activation and inhibition (Figure S2). The observed maximum reaction velocity was nearly threefold higher with NADH than with NADPH (7.26 ± 1.22 $min^{-1}$ versus 2.68 ± 0.46 $min^{-1}$), but was reached only at much higher concentrations of **3** (50 versus 10 μM).

## DISCUSSION

Enzymatic addition of molecular oxygen into organic molecules has been the focus of intensive research ever since the initial discovery in the 1950s, that pyrocatechase (Hayaishi et al., 1955) and phenolase (Mason et al., 1955) utilize atmospheric oxygen as a substrate. A direct reaction between molecules in singlet and triplet states is spin forbidden, but due to the diradical nature of molecular oxygen, it can react with other molecules with unpaired electrons. Oxygenases can typically achieve such activation of oxygen through utilization of cofactors such as flavin, heme, or transition metals (Fetzner, 2002; Fetzner and Steiner, 2010; Torres Pazmiño et al., 2010).

In spite of these complications in oxygen activation, an increasing number of monooxygenases, which are devoid of any coenzymes, have been described in recent years. Examples of these include (1) a peculiar member of the crotonase family of enoyl-CoA isomerase/dehydratases, DpgC, involved in vancomycin biosynthesis (Widboom et al., 2007), (2) the quinone forming ActVA-ORF6 from the actinorhodin biosynthetic pathway (Sciara et al., 2003), and (3) ring-cleaving 2,4-dioxygenases Qdo and Hod involved in catabolism of N-heteroaromatic compounds (Fischer et al., 1999). In addition to monooxygenases, urate oxidase has been shown to be capable of activating

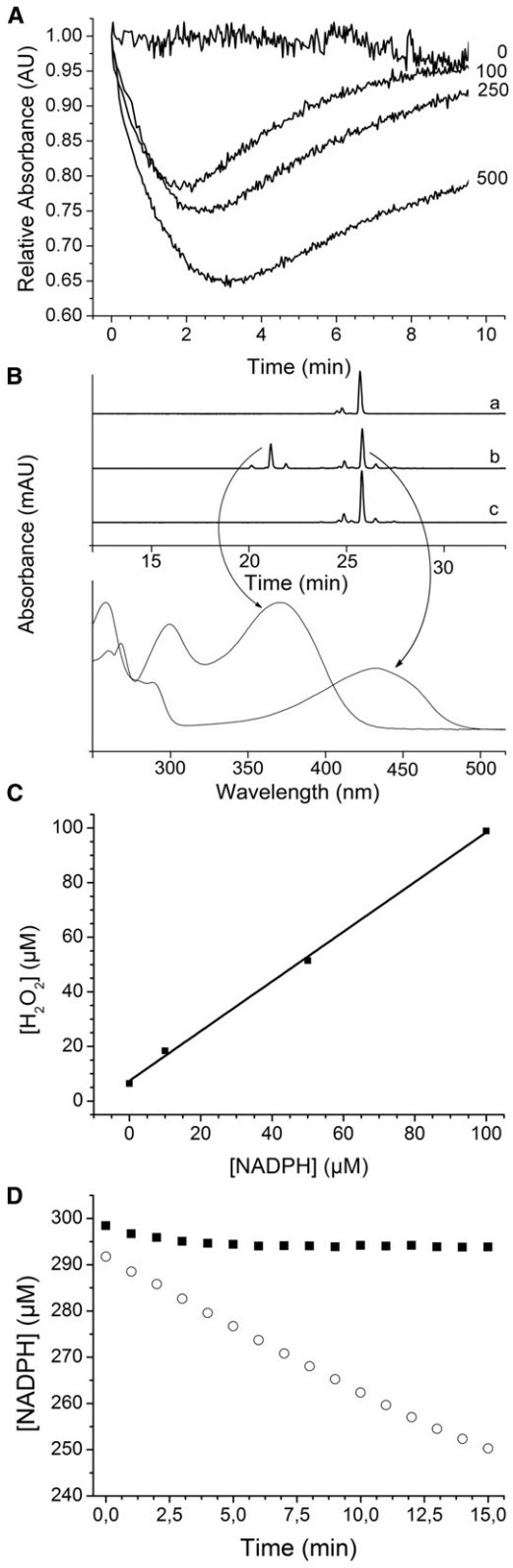


**Figure 4. In Vitro Characterization of the SnoaW/SnoaL2 Reaction**

(A) NADPH-dependent (0–500 μM) reduction of the substrate by SnoaW and its nonenzymatic reoxidation demonstrated at 435 nm with varying concentrations of NADPH.

(B) Detection of the product of the SnoaW reaction. HPLC traces recorded at 370 nm of (a) the substrate **3**, (b) directly after quenching and extraction of the reaction metabolites, and (c) reinjection of the reaction mixture after 45 min. The spectra of the compounds are marked with arrows.

(C) Correlation between NADPH consumption and formation of $H_2O_2$.

(D) Inhibition of the coupled SnoaW/SnoaL2 reaction under anaerobic conditions (black squares) and subsequent consumption of NADPH after oxygen was reintroduced into the reaction mixture (white circles).

See also Figure S3.

oxygen without cofactors (Colloc'h et al., 2008; Kahn and Tipton, 1998).

Two possible reaction mechanisms have been discussed in context with cofactor free monooxygenases. Early studies proposed a radical mechanism for TcmH from the tetracenomycin pathway (Shen and Hutchinson, 1993). However, with the exception of TcmH, a common catalytic concept inspired by flavin chemistry has started to emerge for other cofactor-independent oxygenases (Fetzner, 2002; Fetzner and Steiner, 2010). It seems that the common initial step is the formation of a carbanion intermediate, which could react with molecular oxygen and donate a single electron to dioxygen to yield a [substrate radical − superoxide radical] pair.

In this paper, we have unequivocally confirmed that SnoaW and SnoaL2 form a two-component monooxygenase system, which is responsible for the hydroxylation of the C-1 position of **3** in nogalamycin biosynthesis. The reaction was dependent on the presence of oxygen and incorporation experiments with $^{18}O_2$ verified that the hydroxyl group was derived from molecular oxygen. We further demonstrate that while the reaction could be stated to be cofactor independent, it was found to be cosubstrate dependent due to the requirement of NAD(P)H by SnoaW, much like other members of the SDR family (Kavanagh et al., 2008). However, despite this terminological complication, the challenge for oxygen activation remained, since no cofactors typically associated with monooxygenation reactions (flavin, heme, etc.) were detected during protein purification. In addition the coupled reaction could be conducted under high concentrations of EDTA, which indicated that neither component required metal ions for catalysis.

It is tempting to propose that the SnoaW/SnoaL2 pair would function in a similar manner to how the two-component flavin-dependent monooxygenases (Ballou et al., 2005; van Berkel et al., 2006), such as the much studied *p*-hydroxyphenyl acetate hydroxylase pHPAH $C_1/C_2$ (Chaiyen et al., 2001; Sucharitakul et al., 2005, 2006 ) and ActVA-ORF5/ActVB from the actinorhodin pathway (Valton et al., 2004, 2006; Okamoto et al., 2009), utilize and activate flavin mononucleotide (FMN). In these systems, a separate flavin reductase utilizes NADH for reduction of the FMN quinoid via hydride ($H^-$) transfer (Figure 5A). The reduced $FMN_{red}$ cofactor is subsequently transferred to the monooxygenase component, where it reacts with dioxygen according to the rules governed by classical flavin chemistry (Massey, 1994, 2000; Mattevi, 2006); i.e., first bound flavin ($FIH^-$) reacts with molecular oxygen forming a caged radical pair, followed by conversion into a flavin hydroperoxide (FlOOH), which is able to hydroxylate the substrate (Alfieri et al., 2007; Valton et al., 2008). The difference between the actinorhodin and nogalamycin systems would be that in the

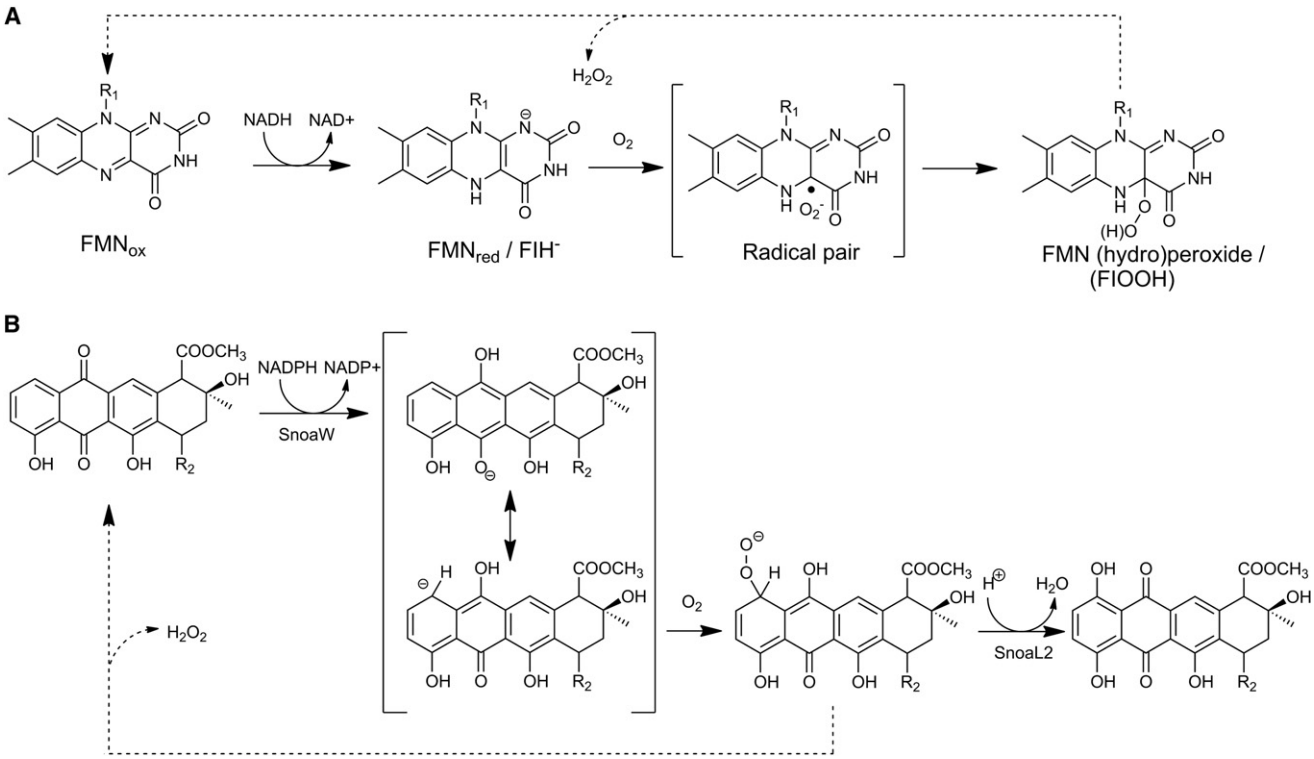


**Figure 5. Comparison of Activation of Flavin by Two-Component FMN Monooxygenases and the Proposed C-1 Hydroxylation of 3 by SnoaW/SnoaL2**

(A) Classical mechanism of activation of flavin for reaction with molecular oxygen. Adapted from references (Massey, 1994; Mattevi, 2006; Valton et al., 2008).
(B) Proposed model for C-1 hydroxylation of **3**.

ActVA-ORF5/ActVB system FMN is a cofactor, while in the SnoaW/SnoaL2 system the anthracycline is the actual substrate. The similarity to cofactor-independent monooxygenases (Fetzner and Steiner, 2010) would stem from the observation that dioxygen would in effect be activated by the organic substrate itself rather than by either of the proteins.

In nogalamycin biosynthesis, we propose that SnoaW uses NAD(P)H to reduce the quinone moiety of the anthracycline into a dihydroquinone (Figure 5B). Our experiments demonstrate that the product of the SnoaW reaction was converted back to the original substrate in the absence of SnoaL2; much like $FMN_{red}$ is readily reoxidized to $FMN_{ox}$ in aerobic conditions (Massey, 1994). Furthermore, the extensive hypsochromic shift observed in the chromophore of the transient SnoaW reaction product (Figure 4B) is similar to the one that takes place during flavin reduction (Massey, 2000). Finally, the recent characterization of a related SDR enzyme QOR2 as a quinone oxidoreductase supports our functional assignment for SnoaW (Kim et al., 2008a). The striking similarity between the highly conjugated reduced FMN and the proposed anthracycline intermediate (Figure 5) implies that the reduced substrate might react with dioxygen in a similar manner to flavin in the next step. The proposal is strengthened by the detection of $H_2O_2$ during the nonproductive SnoaW reaction, which yields strong evidence toward the existence of an anthracycline peroxy intermediate. Finally, protonation of this intermediate by SnoaL2 would result in formation of the final 1-hydroxylated product, **4** (Figure 5B).

It is curious to note that the SnoaW reaction did not proceed in the absence of molecular oxygen. This could surprisingly indicate that the reduced dihydroanthracycline would react with oxygen already in the active site of SnoaW, and that the intermediate transferred to SnoaL2 would be the hydroperoxy form. The benefit of such a system might be to ensure correct regiochemistry of the reaction, and to prevent hydroxylation at the chemically equivalent C-11 position. In any case, detection of $H_2O_2$ during the nonproductive SnoaW cycle demonstrates that SnoaL2 is not required for formation of the anthracycline peroxy intermediate. This is in contrast to the two-component FMN monooxygenases, where kinetic studies have suggested that it is the reduced flavin, which is transferred to the oxygenase component prior to the reaction with dioxygen (Sucharitakul et al., 2007). The concerted action of SnoaW and SnoaL2 appears to be efficiently coupled, since no hydrogen peroxide was detected in the presence of SnoaL2. Crystallization trials currently in progress with SnoaW, SnoaL2, and the monoglycosylated substrate/product will hopefully provide further verification for the proposed biosynthetic model and enable structure-function studies of this new family of monooxygenases.

In closing, the experiments also verified the timing of the C-1 hydroxylation event in nogalamycin biosynthesis, which demonstrated that in contrast to earlier suggestions (Beinker et al., 2006) SnoaW/SnoaL2 function at rather late stage, since the monoglycosylated anthracycline **3** was preferred over **2** as a substrate.

## SIGNIFICANCE

**Nogalamycin is an aromatic polyketide antibiotic with peculiar structural features, which have raised interest toward the individual biosynthetic steps. This compound produced by *Streptomyces nogalater* comprises a four-ring aglycone, a neutral sugar at position C-7, and an aminosugar attached via O-glycosylation at position C-1 and a carbon-carbon bond between C-2 and C-5″. Previous studies conducted in vivo affiliated the open reading frame *snoaL2* with the C-1 hydroxylation step prior to glycosylation. Subsequent structure determination of SnoaL2 revealed a curious similarity to polyketide cyclases like SnoaL and implied that the enzyme belonged to a new family of cofactor-independent oxygenases. However, the in vitro activity of SnoaL2 could not be established at that time. Recently the nogalamycin cluster was expressed in the heterologous host *S. albus*, which enabled genetic engineering of the cluster and more detailed studies. In this work, we show that not only *snoaL2* but also another open reading frame, *snoaW*, residing on the cluster is associated with the C-1 hydroxylation reaction prior to the second glycosylation event. The finding describes a two-component monooxygenase system relying on NAD(P)H and molecular oxygen, which consists of an atypical SDR enzyme, SnoaW, and a hydroxylase, SnoaL2. Extensive biochemical characterization of the reaction led to a mechanistic proposal where in the initial stage (1) SnoaW reduces the monoglycosylated anthracycline substrate to a dihydroquinone, which (2) activates the substrate for reaction with oxygen and formation of a hydroperoxy intermediate that is (3) finally protonated by SnoaL2 to yield the end product of the reaction.**

## EXPERIMENTAL PROCEDURES

### Strains and Culture Conditions

The *Escherichia coli* strains TOP10 (Invitrogen), K12, and ET12567/pUZ8002 (Chater and Wilde, 1980) were used for protein production, gene disruption, and intergeneric conjugation to *S. albus*, respectively. The *E. coli* strains were cultivated at 30°C–37°C in Luria broth, 2 × tryptone yeast extract (TY) or solid Luria agar plates with appropriate antibiotics for selection. The *Streptomyces* strains utilized in the study were *S. lividans* TK24 (Hopwood et al., 1985) and *S. albus* (Chater and Wilde, 1980). *Streptomyces* were grown at 30°C in liquid media TSB (Oxoid) for plasmid amplification, TSB supplemented with $MgCl_2$ (5 mM) and glycine (0.5% [w/v]), YEME (Kieser et al., 2000), supplemented with $MgCl_2$ (5 mM) and glycine (0.5% [w/v])) for protoplast generation, or NoS-soyE1 (Siitonen et al., 2012) for production of metabolites. Solid media for cultivation and storage were R2YE, MS (Kieser et al., 2000), and ISP4 agar (Difco).

### General DNA Methods and Cloning of Protein Overexpression Plasmids

Standard *Streptomyces* techniques for the manipulation of the strains were used (Kieser et al., 2000). Commercial kits were used for recovering DNA from agarose gels and for plasmid isolation (Fermentas and E.Z.N.A, Omega Bio-Tek, respectively). DNA modifying enzymes were purchased from Fermentas. The *snoaW* and *snoaL2* genes were amplified by PCR using Phusion-polymerase (Finnzymes), pSnogaori (Siitonen et al., 2012) as a template and primers *snoaW*FOR, *snoaW*REV, *snoaL2*FOR, and *snoaL2*REV (Supplemental Information). The genes were cloned as BglII-HindIII fragments (*snoaW* 0.83 kbp and *snoaL2* 0.42 kbp) to a modified pBAD/HisB (Invitrogen) vector (Kallio et al., 2006) resulting in pBAD/*snoaW* and pBAD/*snoaL2*. The constructs were verified by sequencing (Eurofins MWG Operon).

### Inactivation of SnoaW and SnoaL2 and Construction of the Complementation Plasmid

The *snoaW* and *snoaL2* gene disruption mutants were made using the λ Red recombinase system in *E. coli* (Datsenko and Wanner, 2000). The template for the modifications was the cosmid pSnogaori (Siitonen et al., 2012). The PCRs were performed with DyNAzyme II (Finnzymes) and the primers ΔaWFOR, ΔaWREV, ΔaL2FOR, and ΔaL2REV (Supplemental Information). The resistance gene was removed with help of pFLP2 (Hoang et al., 1998) resulting in the final expression constructs pSnoΔaW and pSnoΔaL2, which were transferred to *S. albus* by conjugation. The *snoaW* complementation plasmid was constructed from the protein production plasmid pBAD/*snoaW* by subcloning as NcoI-HindIII fragment (*snoaW* 0.86 kbp) into a BspHI-HindIII-digested pIJT486 vector (Siitonen et al., 2012) resulting in pIJTaW. The construct was cloned in *S. lividans* TK24 and transformed to the *S. albus*/ pSnoΔaW deletion mutant strain.

### Analysis of Metabolites

To analyze the secondary metabolites, the strains of interest were cultivated for 5 days in 30°C in 250 ml Erlenmeyer flasks with 30–50 ml of NoS-soyE1. The metabolites were recovered by overnight binding to Amberlite XAD-7 resin (Rohm and Haas, 20 g/l). After decanting the medium and washing the XAD-7 with water, the absorbed metabolites were extracted with MeOH. Alternatively, the metabolites were extracted from a 4-day-old culture broth with equal volume of ethyl acetate containing 1% acetic acid, dried and dissolved in MeOH. The extracted compounds were analyzed by HPLC (Shimadzu SCL-10Avp) with a SunFire C18, 3.5 μm, 2.1 × 150 mm column (Waters) using a gradient from 15% acetonitrile in 0.1% formic acid to 100% acetonitrile.

### Purification and Structure Determination of 3

For large-scale production of the unknown metabolite, a 4 liter cultivation of *S. albus*/pSnoΔaW was performed in 250 ml and 500 ml Erlenmeyer flasks. The cells were harvested after 5 days and the metabolites in the supernatant were collected using XAD-7 as described above. The compounds were isolated from XAD with a isopropanol:water gradient after which the fractions of interest were collected, combined, and extracted with chloroform and further purified with an open silica column (Silica gel 60, Merck) using a chloroform:MeOH gradient. Fractions of interest were further purified using a preparative scale HPLC (Merck Hitachi L-6200A) with a reverse-phase column (SunFire Prep C18, 5 μm, 10 × 250 mm, Waters) and a gradient from 15%–25% acetonitrile in 0.1% formic acid to 100% acetonitrile. The fractions containing the purified compound were combined, dried, and dissolved in MeOD/*d6*-DMSO for NMR analysis.

### LC-MS and NMR Measurements

HPLC-ESI-MS were acquired using a MicroTOF-Q mass spectrometer (Bruker Daltonics) linked to a HPLC-system (Agilent Technologies 1200 series) using the same column and similar conditions as in the analytical HPLC runs. NMR spectra were obtained in 25°C with a Bruker Avance 500 MHz NMR spectrometer using MeOD as internal reference for calibration of the $^{1}H$ and $^{13}C$ chemical shifts.

### Protein Production and Purification

The proteins SnoaL2 and SnoaW were expressed as His-tagged fusion proteins. The cells were grown in 2 × TY to $OD_{600}$ 0.6 and induced with L-arabinose (0.02% [w/v]). After 18–20 hr of shaking at 25°C, the cells were collected, washed, and lysed two times with French Pressure Cell Press (SLM Aminco) in buffer A (50 mM sodium phosphate, 50 mM NaCl, 5 mM imidazole, 10% glycerol [v/v], 1% TritonX-100 [v/v] [pH 7.5]), supplemented with 2 μg/ml DNase II. The cell debris were removed by centrifugation and the proteins were purified using a Talon Superflow Metal Affinity resin (Clontech) with 1 hr binding at 4°C and 15 min of washing with buffer A. The elution was done using buffer A containing 250 mM imidazole and the imidazole was subsequently removed from the collected fractions with PD-10 Desalting Columns (GE Healthcare). The purity of the proteins was verified by SDS-PAGE and the amount was estimated photometrically at 280 nm (NanoDrop2000, Thermo Scientific). Proteins were stored at −20°C in buffer C (50 mM sodium phosphate, 50 mM NaCl, 40% glycerol [v/v], 1% reduced TritonX-100 [v/v] (only for SnoaW) [pH 7.5]).

 

### Enzymatic Assays

The standard 200 μl end point assays were carried out at room temperature and contained 100 μM **2** or **3**, 1 μM SnoaW, 20 μM SnoaL2, and 0.5 mM NAD(P)H in reaction buffer (50 mM sodium phosphate, 50 mM NaCl [pH 7.5], 1.18–1.27% DMSO [v/v]). The reactions were quenched by chloroform extraction after which the organic phases were dried and dissolved in MeOH for HPLC analyses. Alternatively, the reactions were analyzed photometrically using the absorbance maxima of the substrate (435 nm), NADPH (340 nm), and the product (497 nm). The kinetic measurements were done as the end point assays with **3** as the substrate, but with 0.1 μM SnoaW. The concentration of **3** was varied from 5 to 250 μM with 1 mM NAD(P)H. The initial velocities were calculated by linear regression of the initial slopes (Origin 8, Origin Lab). The decoupled independent SnoaW reactions contained 100 μM **3**, 5 μM SnoaW, 0–500 μM NADPH. For detection of the SnoaW reaction product, the reaction containing 25 μM **3**, 10 μM SnoaW, 1 mM NADH was conducted in a Thunberg cuvette. After 30 s, the reaction was quenched with addition of 1 ml ethyl acetate with 1% acetic acid and removal of oxygen. The organic phase was evaporated and solubilized in 200 μl MeOH for analysis by HPLC. The metal dependency was tested in a standard assay supplemented and preincubated with 10 mM EDTA.

### Anaerobic Reactions and Oxygen Incorporation Studies

The anaerobic reactions and $^{18}O_2$ incorporation studies were carried out in a Thunberg cuvette, which was successively evacuated and treated with nitrogen. For the incorporation studies the cuvette was finally flushed with $^{18}O_2$. The anaerobic reactions were carried out with 300 μM NADPH, 120 μM **3**, 1 μM SnoaW, and 5 μM SnoaL2. After the anaerobic treatment NADPH was monitored at 340 nm for 45 min, after which the reaction was reoxygenated and NADPH was monitored for further 15 min. The oxygen incorporation reactions contained 5 μM SnoaW and 10 μM SnoaL2. The reactions were incubated for 20 min after which a chloroform extraction and HPLC-MS analysis were performed as described above.

### Detection of Hydrogen Peroxide

The $H_2O_2$ detection reactions were done using the HRP/ABTS assay system (Szutowicz et al., 1984) with 5 μM SnoaW, 30 μM SnoaL2, and 10–100 μM NADPH. After consumption of NADPH the formed $H_2O_2$ was quantified by adding 5 μM HRP and 500 μM ABTS to the reaction mixtures and measuring the absorbance at 405 nm.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2012.04.009.

## ACKNOWLEDGMENTS

The authors would like to acknowledge the help of M.Sc. Pekka Patrikainen in gene inactivation experiments and M.Sc. Maria Aromaa in purification of the metabolites. This work was supported by the Finnish Academy (grant 127844 to P.M. and 136060 to M.M.-K.).

Received: January 16, 2012
Revised: March 16, 2012
Accepted: April 4, 2012
Published: May 24, 2012

## REFERENCES

Alfieri, A., Fersini, F., Ruangchan, N., Prongjit, M., Chaiyen, P., and Mattevi, A. (2007). Structure of the monooxygenase component of a two-component flavoprotein monooxygenase. Proc. Natl. Acad. Sci. USA *104*, 1177–1182.

Arora, S.K. (1983). Molecular-structure, absolute stereochemistry, and interactions of nogalamycin, a DNA-binding anthracycline anti-tumor antibiotic. J. Am. Chem. Soc. *105*, 1328–1332.

Ballou, D.P., Entsch, B., and Cole, L.J. (2005). Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases. Biochem. Biophys. Res. Commun. *338*, 590–598.

Beinker, P., Lohkamp, B., Peltonen, T., Niemi, J., Mäntsälä, P., and Schneider, G. (2006). Crystal structures of SnoaL2 and AclR: two putative hydroxylases in the biosynthesis of aromatic polyketide antibiotics. J. Mol. Biol. *359*, 728–740.

Chaiyen, P., Suadee, C., and Wilairat, P. (2001). A novel two-protein component flavoprotein hydroxylase. Eur. J. Biochem. *268*, 5550–5561.

Chater, K.F., and Wilde, L.C. (1980). *Streptomyces albus* G mutants defective in the SalGI restriction-modification system. J. Gen. Microbiol. *116*, 323–334.

Chung, J.Y., Fujii, I., Harada, S., Sankawa, U., and Ebizuka, Y. (2002). Expression, purification, and characterization of AknX anthrone oxygenase, which is involved in aklavinone biosynthesis in *Streptomyces galilaeus*. J. Bacteriol. *184*, 6115–6122.

Colloc'h, N., Gabison, L., Monard, G., Altarsha, M., Chiadmi, M., Marassio, G., Sopkova-de Oliveira Santos, J., El Hajji, M., Castro, B., Abraini, J.H., and Prangé, T. (2008). Oxygen pressurized X-ray crystallography: probing the dioxygen binding site in cofactorless urate oxidase and implications for its catalytic mechanism. Biophys. J. *95*, 2415–2422.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA *97*, 6640–6645.

Fetzner, S. (2002). Oxygenases without requirement for cofactors or metal ions. Appl. Microbiol. Biotechnol. *60*, 243–257.

Fetzner, S., and Steiner, R.A. (2010). Cofactor-independent oxidases and oxygenases. Appl. Microbiol. Biotechnol. *86*, 791–804.

Fischer, F., Künne, S., and Fetzner, S. (1999). Bacterial 2,4-dioxygenases: new members of the alpha/beta hydrolase-fold superfamily of enzymes functionally related to serine hydrolases. J. Bacteriol. *181*, 5725–5733.

Grocholski, T., Koskiniemi, H., Lindqvist, Y., Mäntsälä, P., Niemi, J., and Schneider, G. (2010). Crystal structure of the cofactor-independent monooxygenase SnoaB from *Streptomyces nogalater*: implications for the reaction mechanism. Biochemistry *49*, 934–944.

Hayaishi, O., Katagiri, M., and Rothberg, S. (1955). Mechanism of the pyrocatechase reaction. J. Am. Chem. Soc. *77*, 5450–5451.

Hertweck, C., Luzhetskyy, A., Rebets, Y., and Bechthold, A. (2007). Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork. Nat. Prod. Rep. *24*, 162–190.

Hoang, T.T., Karkhoff-Schweizer, R.R., Kutchma, A.J., and Schweizer, H.P. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene *212*, 77–86.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M., and Schrempf, H. (1985). Genetic Manipulation of *Streptomyces*: A Laboratory Manual (Norwich, England: The John Innes Foundation).

Hutchinson, C.R. (1997). Biosynthetic studies of daunorubicin and tetracenomycin C. Chem. Rev. *97*, 2525–2536.

Kahn, K., and Tipton, P.A. (1998). Spectroscopic characterization of intermediates in the urate oxidase reaction. Biochemistry *37*, 11651–11659.

Kallio, P., Sultana, A., Niemi, J., Mäntsälä, P., and Schneider, G. (2006). Crystal structure of the polyketide cyclase AknH with bound substrate and product analogue: implications for catalytic mechanism and product stereoselectivity. J. Mol. Biol. *357*, 210–220.

Kantola, J., Kunnari, T., Hautala, A., Hakala, J., Ylihonko, K., and Mäntsälä, P. (2000). Elucidation of anthracyclinone biosynthesis by stepwise cloning of genes for anthracyclines from three different *Streptomyces* spp. Microbiology *146*, 155–163.

Kavanagh, K.L., Jörnvall, H., Persson, B., and Oppermann, U. (2008). Medium- and short-chain dehydrogenase/reductase gene and protein families : the SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. Cell. Mol. Life Sci. *65*, 3895–3906.

Kendrew, S.G., Katayama, K., Deutsch, E., Madduri, K., and Hutchinson, C.R. (1999). DnrD cyclase involved in the biosynthesis of doxorubicin: purification

  

and characterization of the recombinant enzyme. Biochemistry *38*, 4794–4799.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical *Streptomyces* Genetics (Norwich, England: The John Innes Foundation).

Kim, I.K., Yim, H.S., Kim, M.K., Kim, D.W., Kim, Y.M., Cha, S.S., and Kang, S.O. (2008a). Crystal structure of a new type of NADPH-dependent quinone oxidoreductase (QOR2) from *Escherichia coli*. J. Mol. Biol. *379*, 372–384.

Kim, M.H., Kim, Y., Park, H.J., Lee, J.S., Kwak, S.N., Jung, W.H., Lee, S.G., Kim, D., Lee, Y.C., and Oh, T.K. (2008b). Structural insight into bioremediation of triphenylmethane dyes by Citrobacter sp. triphenylmethane reductase. J. Biol. Chem. *283*, 31981–31990.

Mason, H.S., Fowlks, W.L., and Peterson, E. (1955). Oxygen transfer and electron transport by the phenolase complex. J. Am. Chem. Soc. *77*, 2914–2915.

Massey, V. (1994). Activation of molecular oxygen by flavins and flavoproteins. J. Biol. Chem. *269*, 22459–22462.

Massey, V. (2000). The chemical and biological versatility of riboflavin. Biochem. Soc. Trans. *28*, 283–296.

Mattevi, A. (2006). To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes. Trends Biochem. Sci. *31*, 276–283.

McGovren, J.P., Neil, G.L., Denlinger, R.H., Hall, T.L., Crampton, S.L., and Swenberg, J.A. (1979). Chronic cardiotoxicity studies in rabbits with 7-con-O-methylnogarol, a new anthracycline antitumor agent. Cancer Res. *39*, 4849–4855.

Metsä-Ketelä, M., Niemi, J., Mäntsälä, P., and Schneider, G. (2008). Anthracycline biosynthesis: genes, enzymes and mechanisms. In Anthracycline Chemistry and Biology I: Biological Occurence and Biosynthesis, Synthesis and Chemistry, K. Krohn, ed. (Berlin, Heidelberg, Germany: Springer-Verlag), pp. 101–140.

Neil, G.L., Kuentzel, S.L., and McGovren, J.P. (1979). Treatment of mouse tumors with 7-con-O-methylnogarol and other analogs of the anthracycline antibiotic, nogalamycin. Cancer Treat. Rep. *63*, 1971–1978.

Newman, D.J., and Cragg, G.M. (2007). Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. *70*, 461–477.

Okamoto, S., Taguchi, T., Ochi, K., and Ichinose, K. (2009). Biosynthesis of actinorhodin and related antibiotics: discovery of alternative routes for quinone formation encoded in the act gene cluster. Chem. Biol. *16*, 226–236.

Persson, B., Kallberg, Y., Bray, J.E., Bruford, E., Dellaporta, S.L., Favia, A.D., Duarte, R.G., Jörnvall, H., Kavanagh, K.L., Kedishvili, N., et al. (2009). The SDR (short-chain dehydrogenase/reductase and related enzymes) nomenclature initiative. Chem. Biol. Interact. *178*, 94–98.

Sciara, G., Kendrew, S.G., Miele, A.E., Marsh, N.G., Federici, L., Malatesta, F., Schimperna, G., Savino, C., and Vallone, B. (2003). The structure of ActVA-Orf6, a novel type of monooxygenase involved in actinorhodin biosynthesis. EMBO J. *22*, 205–215.

Shen, B., and Hutchinson, C.R. (1993). Tetracenomycin F1 monooxygenase: oxidation of a naphthacenone to a naphthacenequinone in the biosynthesis of tetracenomycin C in *Streptomyces glaucescens*. Biochemistry *32*, 6656–6663.

Siitonen, V., Claesson, M., Patrikainen, P., Aromaa, M., Mäntsälä, P., Schneider, G., and Metsä-Ketelä, M. (2012). Identification of late-stage glycosylation steps in the biosynthetic pathway of the anthracycline nogalamycin. ChemBioChem *13*, 120–128.

Sucharitakul, J., Chaiyen, P., Entsch, B., and Ballou, D.P. (2005). The reductase of *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* requires *p*-hydroxyphenylacetate for effective catalysis. Biochemistry *44*, 10434–10442.

Sucharitakul, J., Chaiyen, P., Entsch, B., and Ballou, D.P. (2006). Kinetic mechanisms of the oxygenase from a two-component enzyme, *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii*. J. Biol. Chem. *281*, 17044–17053.

Sucharitakul, J., Phongsak, T., Entsch, B., Svasti, J., Chaiyen, P., and Ballou, D.P. (2007). Kinetics of a two-component *p*-hydroxyphenylacetate hydroxylase explain how reduced flavin is transferred from the reductase to the oxygenase. Biochemistry *46*, 8611–8623.

Sultana, A., Kallio, P., Jansson, A., Wang, J.S., Niemi, J., Mäntsälä, P., and Schneider, G. (2004). Structure of the polyketide cyclase SnoaL reveals a novel mechanism for enzymatic aldol condensation. EMBO J. *23*, 1911–1921.

Szutowicz, A., Kobes, R.D., and Orsulak, P.J. (1984). Colorimetric assay for monoamine oxidase in tissues using peroxidase and 2,2′-azinodi(3-ethylbenzthiazoline-6-sulfonic acid) as chromogen. Anal. Biochem. *138*, 86–94.

Torkkell, S., Ylihonko, K., Hakala, J., Skurnik, M., and Mäntsälä, P. (1997). Characterization of *Streptomyces nogalater* genes encoding enzymes involved in glycosylation steps in nogalamycin biosynthesis. Mol. Gen. Genet. *256*, 203–209.

Torkkell, S., Kunnari, T., Palmu, K., Hakala, J., Mäntsälä, P., and Ylihonko, K. (2000). Identification of a cyclase gene dictating the C-9 stereochemistry of anthracyclines from *Streptomyces nogalater*. Antimicrob. Agents Chemother. *44*, 396–399.

Torkkell, S., Kunnari, T., Palmu, K., Mäntsälä, P., Hakala, J., and Ylihonko, K. (2001). The entire nogalamycin biosynthetic gene cluster of *Streptomyces nogalater*: characterization of a 20-kb DNA region and generation of hybrid structures. Mol. Genet. Genomics *266*, 276–288.

Torres Pazmiño, D.E., Winkler, M., Glieder, A., and Fraaije, M.W. (2010). Monooxygenases as biocatalysts: Classification, mechanistic aspects and biotechnological applications. J. Biotechnol. *146*, 9–24.

Valton, J., Filisetti, L., Fontecave, M., and Nivière, V. (2004). A two-component flavin-dependent monooxygenase involved in actinorhodin biosynthesis in *Streptomyces coelicolor*. J. Biol. Chem. *279*, 44362–44369.

Valton, J., Fontecave, M., Douki, T., Kendrew, S.G., and Nivière, V. (2006). An aromatic hydroxylation reaction catalyzed by a two-component FMN-dependent Monooxygenase. The ActVA-ActVB system from *Streptomyces coelicolor*. J. Biol. Chem. *281*, 27–35.

Valton, J., Mathevon, C., Fontecave, M., Nivière, V., and Ballou, D.P. (2008). Mechanism and regulation of the Two-component FMN-dependent monooxygenase ActVA-ActVB from *Streptomyces coelicolor*. J. Biol. Chem. *283*, 10287–10296.

van Berkel, W.J.H., Kamerbeek, N.M., and Fraaije, M.W. (2006). Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J. Biotechnol. *124*, 670–689.

Widboom, P.F., Fielding, E.N., Liu, Y., and Bruner, S.D. (2007). Structural basis for cofactor-independent dioxygenation in vancomycin biosynthesis. Nature *447*, 342–345.

Ylihonko, K., Hakala, J., Kunnari, T., and Mäntsälä, P. (1996a). Production of hybrid anthracycline antibiotics by heterologous expression of *Streptomyces nogalater* nogalamycin biosynthesis genes. Microbiology *142*, 1965–1972.

Ylihonko, K., Tuikkanen, J., Jussila, S., Cong, L.N., and Mäntsälä, P. (1996b). A gene cluster involved in nogalamycin biosynthesis from *Streptomyces nogalater*: sequence analysis and complementation of early-block mutations in the anthracycline pathway. Mol. Gen. Genet. *251*, 113–120.

 