Tailoring Enzymes Involved in the Biosynthesis of Angucyclines Contain Latent Context-Dependent Catalytic Activities

Pekka Prikainen,1 Pauli Kallio,1 Keqiang Fan,3 Karel D. Klika,2 Khaled A. Shaaban,4 Pekka Mäntsäälä,1 Jürgen Rohr,4 Keqian Yang,3 Jarmo Niemi,1 and Mikko Metsä-Ketela1,*
1Department of Biochemistry and Food Chemistry
2Department of Chemistry
University of Turku, 20014 Turku, Finland
3State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PRC
4Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0596, USA
*Correspondence: mikko.mk@gmail.com
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SUMMARY

Comparison of homologous angucycline modification pathways from five closely related Streptomyces species (pga, cab, jad, urd, lan) allowed us to deduce the biosynthetic steps responsible for the three alternative outcomes: gaudimycin C, dehydrodrabolomycin, and 11-deoxylandomycinone. The C-12b-hydroxylated urdamycin and gaudimycin metabolites appear to be the ancestral representatives from which landomycins and jadomysins have evolved as a result of functional divergence of the ketoreductase LanV and hydroxylase JadH, respectively. Specifically, LanV has acquired affinity for an earlier biosynthetic intermediate resulting in a switch in biosynthetic order and lack of hydroxyls at C-4a and C-12b, whereas in JadH, C-4a/C-12b dehydroxylation has evolved into an independent secondary function replacing C-12b hydroxylation. Importantly, the study reveals that many of the modification enzymes carry several alternative, hidden, or ancestral catalytic functions, which are strictly dependent on the biosynthetic context.

INTRODUCTION

Enzymes have been classically renowned for their precision and ability to act as highly stereo- and regiospecific catalysts. However, in recent years the promiscuity of enzymes has raised considerable attention (Copley, 2003; Khersonsky et al., 2006; Hult and Berglund, 2007). This holds especially true for proteins involved in the biosynthesis of natural products, which have been valued for their ability to utilize “unnatural” substrates (Niemi et al., 2008). In effect the phenomenon appears to be an inherent property of secondary metabolic pathways, where selective pressure for diversification of function may supersed desperate efficiency and accuracy. Substrate promiscuity may therefore be considered as a highly beneficial trait, which facilitates the rapid evolution of the biosynthetic pathways that ultimately has led to the exceptional numbers of metabolites isolated from natural sources. The diversification process requires adaptation of the enzymes to new biosynthetic contexts and may involve changes in substrate recognition, stereo- and regiochemistry of the reaction, or even appearance of a completely new function. Such refunctionalization would be more challenging to accomplish with more efficient and fine-tuned enzymes such as those found in primary metabolism. Importantly, the broad substrate specificity of enzymes in secondary metabolism has enabled the use of pathway engineering, e.g., combinatorial biosynthesis, for generation of novel natural products (Rix et al., 2002; Niemi et al., 2008). Therefore, detailed understanding of changes in enzyme function would also be important for elucidating the boundaries and possibilities of current pathway engineering technologies.

Angucyclines are a specific group of aromatic polyketides, which have been associated with various biological activities (Antal et al., 2005; Jakeman et al., 2009; Korynevská et al., 2007; Lombó et al., 2009). These compounds are found widely in nature and are mainly produced by soil-dwelling Streptomyces bacteria. The biosynthesis of angucyclines diverges from other type II aromatic polyketides by the action of the specific cyclase that closes the fourth ring of the polyketide into an angular orientation producing UWM6 (1) (Figure 1) (Kulowski et al., 1999; Metsä-Ketela et al., 2003). The biosynthesis continues through a cascade of oxidation/reduction reactions that result in the formation of the gaudimycin (cab/pga) (Palmu et al., 2007), landomycin (lan) (Weber et al., 1994), urdamycin (urd) (Rohr and Zeeck, 1987), and jadomycin (jad) (Ayer et al., 1991) metabolites (see Figure S1 available online). The enzymes responsible for the modification of the polyketide aglycone belong to two well-characterized families: NADPH-dependent flavoprotein mono-oxygenases, and short-chain alcohol dehydrogenases/reductases (SDRs). The biosynthetic genes are distributed in the clusters in a conserved order (Figure 2), and the gene products share substantial sequence identity, which has made functional predictions challenging based on primary amino acid sequence alone. Each gene cluster encodes for a NADPH-dependent flavoprotein hydroxylase (PgaE, UrdE, LanE, CabE, and JadH), and an additional
flavoprotein oxygenase that may be found in several different combinations with an SDR component (Figure 2).

Recent studies have shown that on the gaudimycin pathway, the flavoenzyme PgaE utilized 2,3-dehydro-UWM6 (2) as a substrate and catalyzed two consecutive steps, C-12 (3) and C-12b (4) hydroxylations, which were followed by the action of the SDR enzyme CabV to produce gaudimycin C (5) (Kallio et al., 2011). The oxidation of the hydroquinone to the C-7,12-quinone structure is generally thought to occur nonenzymatically. The two reactions catalyzed by PgaE were dependent on the presence of NADPH and molecular oxygen and were shown to occur at different phases due to competitive inhibition between 2 and 3. The second reaction catalyzed by PgaE initiated only after most of the original substrate was used, which allowed the isolation and use of the intermediate 3 as an alternative substrate. Efficient coupling of the second PgaE reaction to that of CabV was a strict requirement for the formation of 5 because in the absence of the SDR, the biosynthesis diverted onto a degradation pathway (Kallio et al., 2011). The ketoreduction step could be performed in an identical manner by either CabV or the two-domain PgaM, which implicated indirectly that the N-terminal oxygenase domain of PgaM was not required during conversion of 2 to 5 (Kallio et al., 2008b, 2011). The flavoenzyme JadH from the jadomycin pathway has also been investigated in detail by Chen et al. (2010), which has revealed that somewhat surprisingly, JadH behaves differently than PgaE despite the considerable amino acid sequence identity (58%). Although both enzymes utilize 2 as a substrate and catalyze C-12 hydroxylation, JadH additionally is able to perform a C-4a/C-12b dehydration reaction as seen in the structure of dehydrorabelomycin (7) (Figure 1) instead of the secondary hydroxylation at C-12b. Furthermore, conversion of 2 into jadomycin A has been established using cell-free extracts containing the flavoprotein mono-oxygenases JadH and JadF together with JadG, which is homologous to anthrone oxygenases (Chen et al., 2005). The functional assignments of the gene products from the landomycin and urdamycin pathways are mainly based on molecular genetic evidence. On the landomycin pathway, UrdE was originally postulated to be responsible for the C-12b hydroxylation (Decker and Haag, 1995), but later results suggested that the hydroxylation would be catalyzed by the oxygenase domain of UrdM (Faust et al., 2000), whereas the SDR domain was associated with the ketoreduction at C-6 (Mayer et al., 2005). On the landomycin pathway, LanV has been proposed to be responsible for the aromatization of ring A and 6-ketoreduction in landomycin A biosynthesis (Mayer et al., 2005).

In the present study we have characterized enzymes from five different angucycline pathways (pga, cab, urd, lan, and jad) in
order to determine the functional differences that explain the natural end product diversity. We have used different combinations of enzymes and reaction conditions to understand the order of the biosynthetic steps, and the cosubstrate and oxygen dependence of the individual enzymes. The first part of the study concentrates on the functions of the enzymes from the lan, urd, and jad pathways in their native contexts. In the latter part we examine the flexibility and specificity of the target enzymes in different combinations (combinatorial enzymatic synthesis) and conditions in order to deduce their catalytic behavior under nonnative environments.

RESULTS AND DISCUSSION

The Flavoenzyme UrdE and the Reductase Domain of UrdM Are Sufficient for Production of Gaudimycin C

Purified UrdE was shown to convert 2 in two separate NADPH/O₂-dependent steps (Figure S2) as previously described for PgaE by Kallio et al. (2011), and when coupled to the independently expressed SDR domain UrdMred, the corresponding reaction efficiently produced 5 (Figures 3A and 3B). This was equivalent to the biosynthetic reaction order UrdE-UrdE-UrdMred (Figure 4A) (Kallio et al., 2011), and attributed UrdE responsible for the consecutive hydroxylations at positions C-12 and C-12b (Figure 1). The significance of the finding was evident because UrdE had been previously associated with only one of the two hydroxylation steps in urdamycin biosynthesis (Decker and Haag, 1995; Faust et al., 2000). Instead, one possibility may be that the oxygenase domain has a similar role to JadF (Pahari et al., 2012) and LanM2 (Kharel et al., 2012), which have been very recently shown to be responsible for the release of the thioester-tethered polyketide from the acyl carrier protein.

Enzymatic Synthesis of 11-Deoxylandomycinone by LanE and LanV

LanE was shown to utilize 2 and to catalyze two successive oxygen-dependent steps (Figure S2) in the same manner as UrdE, PgaE (Kallio et al., 2011), and CabE (Koskiniemi et al., 2007). At first this was a surprising finding because natural landomycins do not contain a hydroxyl group at C-12b. However, incubation of LanE and LanV together with 2 resulted in the half could not be recovered by itself. In contrast both the full-length UrdM and the oxygenase domain UrdMox alone were highly unstable, whereas the SDR UrdMred domain could be isolated in soluble form at quantities of over 200 mg/l culture. The results show that the N-terminal flavoprotein oxygenase domain of UrdM is not required for C-12b hydroxylation, even though the enzyme has been linked with the reaction in the past by Faust et al. (2000). Instead, one possibility may be that the oxygenase domain has a similar role to JadF (Pahari et al., 2012) and LanM2 (Kharel et al., 2012), which have been very recently shown to be responsible for the release of the thioester-tethered polyketide from the acyl carrier protein.

Figure 3. Representative HPLC Traces at 428 nm of the Native In Vitro Reactions with 2 as a Substrate

A) The substrate 2 without added enzymes. (B) UrdE/UrdMred results in the formation of 5. (C) LanE/LanV yields 8. (D) JadH produces 7. (E) JadH/CabV also yields 7. Production of 7 by JadH was not affected by the presence of any of the SDR enzymes. Spontaneous formation of 7 (Kallio et al., 2011) was avoided by ensuring quantitative conversion of 3 before metabolite extraction. See also Figure S2.
The appearance of a previously undetected metabolite in HPLC (Figure 3C), whereas 5 was not observed. LC-MS and UV-Vis properties of this new compound were in agreement with the landomycin pathway intermediate 11-deoxylandomycinone (8) (Figure 1) (Shaaban et al., 2011), and further supported by the molecular formula C_{19}H_{14}O_{5} ([M-H]- observed 321.0780, calculated 321.0763) inferred by ESI-HR-MS. The structure was subsequently confirmed as 8 by comparison to an authentic standard by coelution in HPLC, UV/Vis-spectra, and CD-spectra (Figure S2). The structure of the product supported the functional assignment of LanE as the C-12 hydroxylase (Zhu et al., 2005), LanV as the C-6 ketoreductase (Mayer et al., 2005), and further clarified that one of the enzymes was responsible for an additional dehydration between C-4a/C-12b.

The Order of the Tailoring Steps Has Changed in Landomycinone Biosynthesis

Time course analysis of the coupled LanE/LanV reaction showed that 8 was accumulating already during the conversion of 2 (Figure 4B). This was direct proof that the LanV reaction took place immediately after the C-12 hydroxylation step but prior to the C-12b hydroxylation that was still inhibited at this phase (Kallio et al., 2011). The chronology indicated that LanV possessed catalytic affinity toward the C-12 hydroxylated intermediate 3, which was in clear contrast to the homologous enzymes involved in the biosynthesis of 5 (Figure 4A) (Kallio et al., 2011). To confirm that the steps after the initial C-12 hydroxylation were independent of oxygen as suggested by the structure of 8, the reaction was conducted under anaerobic conditions using the isolated 3 as the substrate. Consequently, 8 was produced (Figure 5A), but only in the simultaneous presence of both LanE and LanV. Despite the incomplete conversion, this had important implications in relation to the function of LanE in the native context: the experiment verified the catalytic order in the biosynthesis of 8 to be LanE-LanV-LanE, and attributed LanE indispensable for two separate catalytic steps. However, the second step could not be a hydroxylation because it was accomplished in the absence of oxygen. Instead, the chemical changes associated with the formation of 8 proposed that it would be the C-4a/C-12b-dehydration (Figure 1) equivalent to the secondary catalytic function of JadH (Chen et al., 2010). The NADPH titration profile (Figure 4B) confirmed that LanV used one equivalent of the cosubstrate in the production of 8, which was consistent with the expected reaction mechanism of SDR enzymes catalyzing similar ketoreduction reactions (Kavanagh et al., 2008).

Confirmation of the Opposite Stereoselectivities of LanV and CabV/UrdMred

The absolute stereochemistry of 8 has been previously confirmed as 6R via total synthesis by Roush and Neitz (2004) and Zhu et al. (2005), whereas to date, only the relative stereochemistry of 5 has been known. To ascertain the absolute configuration for 5, experimental and theoretically calculated
electronic circular dichroism (ECD) spectra were compared (Figures 6A, 6B, and S4), which was in good agreement with the 4aR,6S,12bS enantiomer shown in Figure 1. Similar experiments conducted for 2 (Figures 6C, 6D, and S4) also indicated an R configuration at C-4a, which thus supported the assignment for 5. The theoretically calculated ECD spectrum of 8 was also in good agreement with an R configuration at C-6 (Figures 6E, 6F, and S4) for this compound. The rigid polyaromatic aglycones limited the conformational freedom to rings A and B, and as a result only two (approximately 1:1 ratio), one and two (approximately 2:1 ratio), conformers of significant population were found for 2, 5, and 8, respectively, by molecular modeling. Importantly, the results verified that CabV and UrdMred were responsible for the 6S configuration in 5, whereas LanV catalyzed the formation of the opposite 6R configuration in 8 in the native pathways.

**JadH Catalyzes Dehydrorabelomycin Formation in a Single Step**

Despite high sequence identity with PgaE (58%), CabE (58%), UrdE (61%), and LanE (57%), JadH differed in function from the other hydroxylases and catalyzed the conversion of 2 into 7 (Figure 3D) via C-12 oxygenation and C-4a/C-12b dehydration (Figure 1) (Chen et al., 2010; Chen et al., 2005). One equivalent of NADPH was consumed in the process (Figure S3), and product degradation fragments were not observed. The C-12-hydroxylated product 3 could not be detected or isolated from the reaction, and in parallel, coinoculation with the purified SDRs from the
*cab*, *urd*, or *lan* pathways did not have any effect on the reaction profile, and only 7 was produced (Figure 3E). In addition JadH failed to convert isolated 3 into 7 under equivalent conditions (data not shown), further implying that (i) the native JadH reactions may proceed in a closely coupled sequence that cannot dissociate from one another, and possibly that (ii) the dehydration at C-4a/C-12b needs to take place first before the C-7/C-12 dihydroquinone formation can occur.

**Timing of 6-Ketoreduction Determines Hydroxylase Activity**

Next, the enzymes from the parallel pathways were assayed in different combinations to deduce their functions in nonnative contexts. Incubation of the hydroxylases PgaE, CabE, UrdE, or LanE in any combination with either CabV or UrdMred and 2 resulted in the formation of 5. In contrast the corresponding coupled reactions with LanV produced exclusively 8. This was an unambiguous proof that outcome of the reaction was solely determined by the SDR and not the hydroxylase per se. Against the deduced native functions, this could now be explained by the substrate specificities of the different SDRs toward either 3 or 4, which ultimately defined the sequential reactions to proceed toward landomycins or the 12b-hydroxylated metabolites, respectively. Thus, it appears that a simple decrease in Km for 3 is the underlying cause for the functional diversification of LanV from the other SDRs and the key step responsible for the evolution of the landomycin antibiotics. This change in catalytic specificity, however, is not apparent at the level of primary structure because the sequence identity of LanV with its presumed ancestral counterparts CabV, UrdMred, and PgaMred still remains exceptionally high (over 65%) for members of the SDR family (Jörnvall et al., 1995, Persson et al., 2003).

The results also demonstrated that the hydroxylases, including LanE, all functioned in an identical context-dependent manner. In the presence of CabV or UrdMred, the catalysis proceeded via the hydroxylase-catalyzed C-12b oxygenation as in gaudimycins and urdamycins, whereas with LanV the reaction continued toward the dehydration at C-4a/C-12b as observed in landomycins. This showed that all the hydroxylases carried two alternative catalytic modes (hydroxylation and dehydration), the choice between which was determined by the timing of the SDR reaction. Thus, the lack of the C-12b hydroxyl group in landomycins is not due to a lack of an appropriate hydroxylase in the pathway, but intriguingly, the surrounding biosynthetic context in which the oxygenation reaction is overhauled by the previous step.

**The C-6 Stereoselectivity of LanV Is Context Dependent**

We then further corroborated our hypothesis of the reaction order by controlling the availability of oxygen with isolated intermediate 3 as a substrate. As shown above, incubation of LanV with LanE and 3 under strict anaerobic conditions resulted in the formation of 8 (Figure 5A). In striking contrast when the experiment was repeated in the presence of oxygen, conversion to 5 was observed (Figure 5B). Having now deduced the steps in the alternative pathways, this drastic change could be explained by the ability of both LanV and LanE to utilize and compete for the substrates 3. The hydroxylases apparently have higher affinity for 3 in comparison to LanV, and when O2 is available, this resulted in C-12b hydroxylation followed by C-6 ketoreduction as in the native *pga*, *urd*, and *cab* pathways (Figure 1). In the absence of oxygen, the C-12b hydroxylation is blocked, and consequently, the reaction order is reversed: LanV catalyzes C-6 ketoreduction, which then allows the hydroxylase to perform C-4a/C-12b dehydration as described for the formation of 8 (Figure 1). This scenario is equivalent to the native reaction sequence in landomycin biosynthesis, with the difference that here the C-12b hydroxylation was inhibited by the anaerobic conditions and not the presence of 2. In support of this proposed sequence, the same outcome could also be accomplished by coaddition of a small amount of 2 even when oxygen was present. The corresponding reactions with the SDRs CabV and UrdMred only produced trace quantities of the *lan* product 8 (Figure S5), indicating that the SDRs had a poor affinity toward 3 when the native pathway was restrained.

In addition to the switch in the reaction order itself, the experiment verified that LanV was in effect able to catalyze the formation of both 6R and 6S configurations in 8 and 5, respectively. The main determinant of the stereospecificity appears to be the C-12b substituent missing in 3 and present in 4, possibly by affecting the substrate orientation in the active site.

**JadH Carries the Ancestral C-12b Hydroxylase Activity**

The JadH reaction with 3 failed to produce 7 but, instead, resulted in gradual disappearance of the substrate as previously associated with enzyme-catalyzed degradation by PgaE (Figure S2) (Kalio et al., 2008b, 2011). When the reaction was repeated in the presence of either CabV, UrdMred or LanV, notably, 5 was obtained (Figures 5C–5E). Although the conversion was not quantitative, the finding demonstrated that JadH had retained the ability of the presumed ancestral enzymes to catalyze hydroxylation at position C-12b, a function masked by the C-4a/C-12b dehydratase activity in the native pathway. The experiment also encapsulated the utmost context dependence of the enzymes because in the coupled JadH/LanV reaction, neither of the enzymes functioned according to their native functions but catalyzed reactions characteristic for the corresponding enzymes in the gaudimycin and urdamycin pathways (Figure 1).

**Conclusions**

The study clarifies the redox modification steps of the *urd*, *lan*, and *jad* angucyclinone pathways and identifies the native enzyme-catalyzed steps responsible for the structural diversity of the alternative end products 5, 8, and 7, respectively (Figure 1). By comparing the catalytic functions of homologous proteins in vitro, we demonstrated how in the course of enzyme evolution, the SDR LanV and the flavoprotein hydroxylase JadH have differentiated into novel catalytic species. Specifically, LanV has acquired catalytic affinity toward the earlier biosynthetic intermediate 3 resulting in the switch of biosynthetic reaction order, and promoting LanE to catalyze C-4a/C-12b dehydration in place of the C-12b hydroxylation. In JadH the corresponding C-4a/C-12b dehydratase activity appears to have evolved into an SDR-independent secondary function, which effectively outcompetes the parallel C-12b oxygenase activity. Importantly, LanV and JadH represent the first branching points of the landomycin and jadomycin pathways, respectively, and thus appear...
to be ultimately responsible for the evolution of the corresponding angucycline families.

As one of the most important findings in this work, many of the enzymes were characterized by innate catalytic context dependence and could be coerced to perform several alternative functions by altering the reaction setups. For example the hydroxylases PgaE, CabE, UrdE, and LanE could all be harnessed to produce either 5 or 8 depending on the SDR involved, regardless of their original functions. In a similar manner under specified conditions JadH also could participate in the biosynthesis of 5 in addition to the native reaction producing 7. As for the SDRs, LanV could be applied to produce both 5 and 8, depending on the reaction conditions. From an evolutionary viewpoint this was fascinating because it demonstrated that both JadH and LanV have retained the ability to perform their ancestral catalytic activities and at the same time underlined the immense flexibility of the enzymes for acquisition of novel functions, which has enabled the biosynthesis of the vast number of secondary metabolites isolated from natural sources.

SIGNIFICANCE

In this study, we demonstrate the critical role of functional diversification of biosynthetic enzymes in the evolution of angucycline secondary metabolites in Streptomyces bacteria. We show that homologous flavoenzyme oxygenases and SDRs from related pathways (pga, cab, urd, lan, jad) are responsible for three alternative redox modification patterns, yielding either gaudimycin C, dehydrorabelogenes and SDRs from related pathways (Chionshte) in a disposable chromatography column (Bio-Rad) and eluted with 250 mM imidazole. Purity of the proteins was confirmed with SDS-PAGE, and the concentration of the proteins was estimated using Bradford dye binding method. LanE and LanV were stored at −20°C in 10 mM Na2PO4, 50 mM NaCl, 125 mM imidazole, and 50% glycerol (pH 7.6).

Cloning, Expression, and Purification of UrdE, UrdM, UrdMox, and UrdMed

The urdE, urdM, urdMox, and urdMed genes were amplified with Phusion DNA polymerase (Finnzymes) using Streptomyces flavidus AO (Trefzer et al., 2001) chromosomal DNA as a template. The following primers were used: 5′-TTA GAT CTG CTG TCA TGG TCG-3′ and 5′-TTT AGT CTT CCT TCG GAG CGG CCT CCA GGC GTG GCA-3′ for urdE; 5′-TTA GAT CTG TGG CCG CCT CTC TGG AG-3′ and 5′-TTT AGT CTT CCT TCG GAG CGG CCT CCA GGC GTG GCA-3′ for urdM; 5′-TTA GAT CTG TCG GCC CTC TCG AG-3′ and 5′-TTT AGT CTT CCT TCG GAG CGG CCT CCA GGC GTG GCA-3′ for urdMed.

The amplified genes were subcloned into pHBHΔ as BglII/HindIII fragments, and the constructs pHBHΔ/urdE, pHBHΔ/urdM, pHBHΔ/urdMox, and pHBHΔ/urdMed were verified by sequencing (Eurofins MWG Operon). In a similar manner to laiE, the sequence of the gene urdΔ was revised and deposited to GenBank under the accession number JQ782418. The urdE (54.7 kDa), urdM (71.1 kDa), urdMox (41.5 kDa), and urdMed (27.2 kDa) were overexpressed as N-terminal His-tagged fusion proteins in E. coli strain TOP10 (500 ml 2×YT medium in 2 liter Erlenmeyer flasks, 0.02% L-arabinose induction, incubation at 25°C with 250 rpm shaking for 21 hr). The proteins were purified to homogeneity in a single step using a 5 ml HisTrap Ni2+-affinity column (Amersham Biosciences) and eluted with 0.5 M imidazole. Purity of the proteins was confirmed with SDS-PAGE, and the concentration of the proteins was estimated using Bradford dye binding method. The proteins were stored at −20°C in 10 mM Na2PO4, 75 mM NaCl, 125–250 mM imidazole, and 50% glycerol (pH 7.4).

Expression and Purification of PgaE, CabE, CabV, and JadH

The proteins PgaE (Koskinen et al., 2007), CabE (Koskinen et al., 2007), CabV (Kallo et al., 2011), and JadH (Chen et al., 2010) were heterologously produced and purified as described earlier.

Enzyme Reactions In Vitro

In vitro enzyme reactions were carried out in conditions described earlier by Kallo et al. (2011). The enzyme concentrations used in the reactions were 160 nM JadH, 150 nM PgaE, 600 nM CabE, 150 nM LanE, 350 nM UrdE, and 150–6,000 nM CabV or LanV. Isolation of substrate and products from in vitro reactions was performed with repeated chloroform extractions as described earlier by Kallo et al. (2008b). Solid-phase extraction (SPE) columns (Discovery DSC-18/SUPELCO) were used to isolate 3 (Kallo et al., 2011). Reactions where 3 was used as a substrate were extracted with SPE columns, eluted with MeOH, and incubated with PgaE and NADPH in order to degrade all unused substrate that would be seen as 7 in HPLC (Kallo et al., 2011). This analysis confirms that 7 seen in HPLC is 7, and not 3. Anaerobic reaction conditions were achieved using a Thunberg cuvette or glucose oxidase/catalase treatment as described earlier by Kallo et al. (2011).

HPLC Analysis of the Substrate and Products

The compounds extracted from reactions were analyzed by RP-HPLC (Shimadzu VP series chromatography system with a diode array detector, SPD-M10Avp, Merck LiChropher 100 RP-18/5 μm column, gradient from 15% acetonitrile with 0.1% HCOOH to 100% acetonitrile, flow rate 0.5 ml/min, Tret 60 min). The substrate 2 and the products 5, 7, and 8 were identified...
based on retention times and UV-Vis spectra and compared on the basis of the peak areas at 406, 428, 459, or 449 nm, respectively.

**LC-MS Analysis**

The exact mass of the B form in vitro reaction was determined with a high-reso-

**Molecular Modeling, Experimental and Calculated Optical Rotation, and ECD Spectra**

DFT quantum chemical calculations were performed using Gaussian09 (Frisch et al., 2009) and GaussSum (O’Boyle et al., 2008) (version 2.2). Structures were first geom-

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**SUPPLEMENTAL INFORMATION**

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**Angucycline Tailoring Reactions**

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