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Sequential Action of Two Flavoenzymes, PgaE and PgaM, in Angucycline Biosynthesis: Chemoenzymatic Synthesis of Gaudimycin C

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SUMMARY

Tailoring steps in aromatic polyketide antibiotic biosynthesis are an important source of structural diversity and, consequently, an intriguing focal point for enzymological studies. PgaE and PgaM from *Streptomyces* sp. PGA64 are representatives of flavoenzymes catalyzing early post-PKS reactions in angucycline biosynthesis. This in vitro study illustrates that the chemoenzymatic conversion of UWM6 into the metabolite, gaudimycin C, requires multiple closely coupled reactions to prevent intermediate degradation. The NMR structure of gaudimycin C confirms that the reaction cascade involves C12- and C12b-hydroxylation, C2,3-dehydration, and stereospecific ketoreduction at C6. Enzymatic ^{18}O incorporation studies verify that the oxygens at C12 and C12b derive from O_2 and H_2O, respectively. The results indicate that PgaM deviates mechanistically from flavoprotein monooxygenases, and suggest an alternative catalytic mechanism involving a quinone methide intermediate.

INTRODUCTION

Aromatic polyketides are complex natural compounds well established for their biological activities and applications in medicine (Newman et al., 2000). They are produced by a number of different organisms, but especially *Streptomyces* bacteria have been a rich source of natural product diversity (Bentley et al., 2002; Omura et al., 2001). Angucyclines comprise a distinct group of aromatic polyketides (Rohr and Thiericke, 1992), which are characterized by the angular shape of the polyaromatic tetracyclic ring that differentiates these compounds from the closely related anthracyclines (Strohl et al., 1997; Niemi et al., 2002) and tetracyclines (Hunter, 2002). Typical examples of angucyclines are urdamycin (Drautz et al., 1986) and landomycin (Henkel et al., 1990), products of the two most extensively studied biosynthetic pathways. Angucyclines have been shown to exhibit a variety of activities of medical interest (Rohr and Thiericke, 1992) including antitumor (Korynevska et al., 2007; Antal et al., 2005) and antibacterial activities (Antal et al., 2005; Abdelfattah et al., 2003), as well as platelet aggregation inhibition (Omura et al., 1988; Kawashima et al., 1989). Angucyclines were first discovered in 1965 (Dann et al., 1965), but still only a few hundred structures have been described. Recent genetic screening studies have indicated that angucycline gene clusters are much more abundant than expected based on traditional cultivation methods (Metsä-Ketelä et al., 2002), which suggests that many potentially important angucyclines are yet to be discovered. The *pga* and *cab* systems discussed in this article are examples of such cryptic gene clusters, and they were initially cloned to increase the biosynthetic potential for combinatorial biosynthesis applications (Palmu et al., 2007).

The carbon skeleton of angucyclines is based on the four-ringed benz[*a*]anthracene system (Rohr and Thiericke, 1992). Biosynthetically, they belong to the family of aromatic type II polyketides (Hopwood, 1997; Hertweck et al., 2007), which means that the initial steps in the biosynthesis resemble those of bacterial type II fatty acid synthesis. The biosynthesis begins by the iterative action of the minimal PKS complex, which consists of a ketosynthase/chain length factor (KS_α/CLF or KS_α/KS_β) heterodimer and an acyl carrier protein. The complex polymerizes 10 acetate building blocks to form a highly reactive 20 carbon polyketide chain, which is subsequently folded in a controlled manner into the first stable intermediate, UWM6 (**1**, Figure 1) by ketoreductases, aromatases, and cyclases (Kulowski et al., 1999; Metsä-Ketelä et al., 2003). After this point, different angucycline pathways diverge through the action of numerous tailoring or post-PKS enzymes. These biosynthetic tailoring steps are important for generation of the vast chemical diversity of the compounds (Rix et al., 2002) and, consequently, are of special interest for genetic and enzymological studies.

Several in vivo studies have suggested that early angucycline post-PKS tailoring steps are commonly accompanied by further bioconversion events in *Streptomyces*. The reactions have been shown to deviate from the pathways to shunt products (Palmu et al., 2007; Rix et al., 2003, 2005; Mayer et al., 2005) and to result in unexpected alterations in the compounds produced (Palmu et al., 2007). In parallel, possible involvement of nonenzymatic reactions (Palmu et al., 2007; Metsä-Ketelä et al., 2003), unusual bifunctionality, and atypical enzymology (Mayer et al., 2005; Chen et al., 2005) have been associated with these biosynthetic steps. As a result, separation of the individual catalytic events from nonspecific spontaneous or endogenous reactions has been challenging, and has complicated the functional

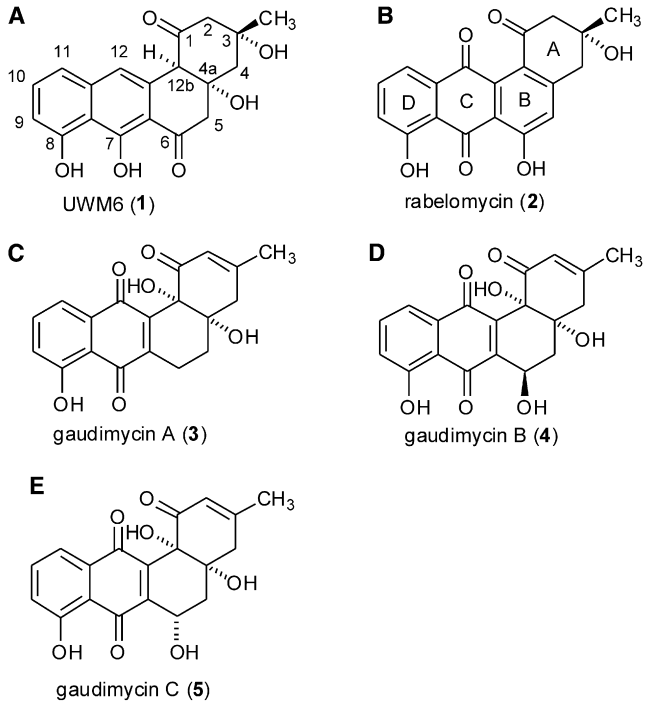

Figure 1. Structures and Numbering of Angucycline Metabolites

(A) The substrate of PgaE, UWM6 (**1**).

(B) The observed in vivo product of PgaE, rabelomycin (**2**).

(C) The in vivo product of PgaE/PgaM, gaudimycin A (**3**).

(D) The in vivo product of the related *cab* pathway, gaudimycin B (**4**).

(E) The in vitro product of PgaE/PgaM, gaudimycin C (**5**).

assignment of the biosynthetic genes. The catalytic steps and enzymology of angucycline early post-PKS tailoring reactions are yet to be comprehensively described in vitro.

The most common enzymes associated with these reactions are homologous to flavoprotein monooxygenases and short chain alcohol dehydrogenase/reductases (SDRs). PgaE (Koskiniemi et al., 2007) and PgaM (Kallio et al., 2007) covered in this article are representatives of such enzymes. They originate from the silent angucycline gene cluster, *pga*, isolated from *Streptomyces* sp. PGA64, which is not active under laboratory conditions (Metsä-Ketelä et al., 2004). PgaE is homologous to the well-characterized flavoenzyme, *p*-hydroxybenzoate hydroxylase (Schreuder et al., 1989), and orthologous genes are found in most angucycline gene clusters (Palmu et al., 2007). According to heterologous expression studies, it uses **1**, the first stable intermediate of the pathway, as a substrate, and has been associated with the C12-hydroxylation reaction. However, the in vivo reaction of PgaE was shown to be accompanied by additional chemical changes resulting in the formation of a putative shunt product, rabelomycin (**2**, Figure 1) (Palmu et al., 2007). PgaM is a two-domain flavoprotein that contains an N-terminal domain homologous to FAD-dependent monooxygenases, which is fused to a domain homologous to SDRs via a short linker region (Kallio et al., 2007; Metsä-Ketelä et al., 2004). The oxygenase domain of PgaM shares sequence similarity with PgaE (50% amino acid sequence identity) and *p*-hydroxybenzoate hydroxylase, which designates it as a member of subclass A of flavoprotein monooxygenases based on sequence information (van Berkel et al., 2006). The SDR domain of PgaM has been shown to catalyze 2,3-dehydration, whereas the N-terminal oxygenase domain has been associated with an enzymatically unusual C12b-hydroxylation (Palmu et al., 2007). Curiously, the in vivo product of the PgaM reaction, gaudimycin A (**3**, Figure 1), had lost the C6-hydroxyl group, which was inconsistent with the expected functions of the tailoring genes used in the study (Palmu et al., 2007). Genes homologous to *pgaM* are infrequently encountered in *Streptomyces* genomes and not found outside angucycline biosynthesis, but the functions of homologous genes from urdamycin (Rix et al., 2003) and landomycin (Zhu et al., 2005) pathways have been elucidated.

The aim of this study was to gain insight into the early, common tailoring steps in angucycline biosynthesis with PgaE and PgaM as model enzymes. Specifically, one of the initiatives was to investigate the enzymatically enigmatic C12b-hydroxylation reaction, which is a common step in the biosynthetic pathways of many angucyclines. Here we provide direct evidence that the angucycline intermediates involved are highly reactive, which causes consecutive catalytic steps to be dependent on efficient enzyme coupling, and explains many unexpected events observed in vivo. We also present the structure of a novel polyketide, gaudimycin C (**5**, Figure 1) formed in chemoenzymatic synthesis by PgaE and PgaM. The structure allowed us to deduce each step in the conversion, and confirmed the loss of the C6-hydroxyl group of **3** to result from endogenous reactions catalyzed by the host strain, *Streptomyces lividans* TK24. Furthermore, ^{18}O isotope incorporation studies showed that the oxygen incorporated by PgaM derives from H_2O. This implies that the oxygenase domain of PgaM is not a member of flavoprotein monooxygenases, despite sequence similarity, but catalyzes a novel hydroxylation reaction, possibly through a quinone methide (QM) intermediate.

RESULTS

Production of Proteins and Substrates

All proteins were produced and purified as N-terminal His-tag fusions, PgaE and PgaMox, which is a deletion mutant containing the N-terminal oxygenase domain of PgaM alone in *Escherichia coli* TOP10, and PgaM in *S. lividans* TK24. The soluble yield of PgaE expression was over 40 mg/l culture medium. Color of the final preparate (5 mg/ml) was intense yellow, consistent with the presence of FAD. The gene, *pgaM*, contains an unusual genetic organization of two overlapping reading frames, where the SDR reductase domain is produced as the C-terminal part of PgaM, but also as an independently translated reductase subunit, PgaMred, due to an internal start codon (Kallio et al., 2007). The two different isoforms were produced and copurified together in one nickel affinity chromatography step. These were seen in SDS-PAGE chromatography as separate bands migrating at 85 kDa and 27 kDa, which account for full-length, two-domain PgaM and independently encoded reductase PgaMred, respectively. The total purification yield was up to 2 mg/l culture, and the final preparate was light yellow in color, consistent with the binding motif for FAD found in sequence of the oxygenase subunit (Metsä-Ketelä et al., 2004). The purification yield of PgaMox was over 8 mg/l culture media, and again the protein

appeared to have retained bound FAD. The substrate, **1**, for enzymatic studies was produced and purified, yielding ~2 mg/l culture media. Color of **1** methanol stock used for activity assays (~0.1 mg/ml) was clear, bright yellow. Rabelomycin (**2**) was extracted and purified as a by-product of production of **1**.

PgaE Reaction with UWM6 Yields an Unstable Product

PgaE was shown to consume **1** as a substrate in the presence of molecular oxygen and NADPH, as detected spectrophotometrically as a linear decrease of absorption at the substrate maximum of 406 nm (Figure 2A). The loss of yellow color of **1** by PgaE was accompanied by the disappearance of the corresponding signal in HPLC (Figure 2B), but instead of yielding a single product, the reaction resulted in the appearance of several minor peaks (Figure 3A). The compounds detected exhibited increased hydrophilicity and absorption maxima below the visible wavelength range, and no plausible PgaE product candidates could be observed. According to LC-MS analysis, molecular ion mass of the main fragment with absorption maximum at 367 nm (Figure 3A) was only 325 amu, 18 amu less than that of **1**. The compounds reacted further in extended incubation of the reaction mixture after depletion of **1**. This was observed as gradual reduction of HPLC signal intensities together with a shift in the overall elution profile toward the void volume (Figures 3B–3C). In the absence of PgaE, **1** remained stable and did not show any signs of spontaneous conversion or degradation (Figure 2C).

PgaM Converts the PgaE Product into a Novel Compound

PgaM did not have any activity toward **1** under equivalent reaction conditions. Instead, a coupled reaction with both PgaE and PgaM resulted in the appearance of a new peak observed in HPLC UV-Vis (Figure 4A). The product had an absorption maximum at 428 nm, and, based on LC-MS analysis, a mass (356 *m/z*) 14 amu higher than that of **1**. The mass was 16 amu higher in comparison with the equivalent in vivo product, **3**, and the same as that of the in vivo product of the related *cab* pathway, gaudimycin B (**4**, Figure 1), produced in *S. lividans* TK24 (Palmu et al., 2007). RP-HPLC retention times of the compounds were clearly distinct and, accordingly, the product was named gaudimycin C (**5**).

The products detected in the PgaE reaction were shown not to be intermediates in the pathway, as they failed to produce **5** when isolated and incubated with PgaM (Figure 4B). In essence, pathway intermediates were not detected at any stage of the reaction cascade between the initial substrate **1** and **5**. A common shunt product, **2**, detected in studies with related angucycline post-PKS enzymes (Rix et al., 2003; Künzel et al., 1999) and the *pga* pathway in vivo (Palmu et al., 2007), was not observed as a by-product in any of the reactions, nor was it accepted as a substrate by either of the enzymes. The deletion mutant, PgaMox, did not express any activity when coupled to the PgaE reaction, indicating that the reductase domain of PgaM is required to act before the oxygenase domain.

Gaudimycin C Formation Requires Uninterrupted Sequence of Reactions

In the optimized reaction conditions, with a 3-fold molar excess of PgaM over PgaE, conversion of **1** to **5** was quantitative

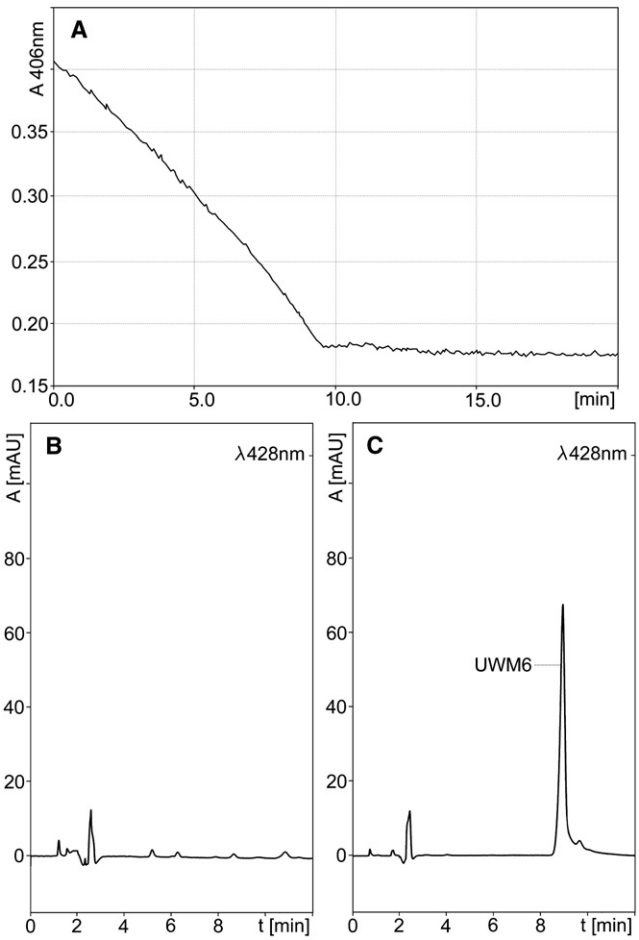

Figure 2. Monitoring of the PgaE Reaction in the Presence of 1, NADPH, and Molecular Oxygen

(A) Reaction followed spectrophotometrically at the substrate absorption maximum of 406 nm.

(B) HPLC analysis of extracted reaction products demonstrate that no compounds absorb at the Vis range.

(C) In the absence of PgaE, the substrate remains intact.

(Figure 4A). Failure to couple the successive biosynthetic steps induced intermediate degradation and resulted in the appearance of PgaE shunt products. First, the simultaneous presence of the two enzymes was a strict requirement for formation of **5**, and delayed introduction of PgaM during or at the end of the PgaE reaction caused a proportional reduction in the yield (Figures 4C–4D). Second, deviation from the optimal molar ratio between the enzymes by addition of an excess of PgaE had a corresponding effect (Figure 4E). Third, separation of PgaE and PgaM into compartments by using a semipermeable membrane also prevented formation of **5** (Figure 4F), although **1** and the minor products of the PgaE reaction were shown to diffuse freely through the membrane and to occupy both compartments. Despite the apparent need for close functional interaction between PgaE and PgaM, no signs of noncovalent complex formation could be detected in either glutaraldehyde cross-linking experiments or analytical HPLC gel filtration studies.

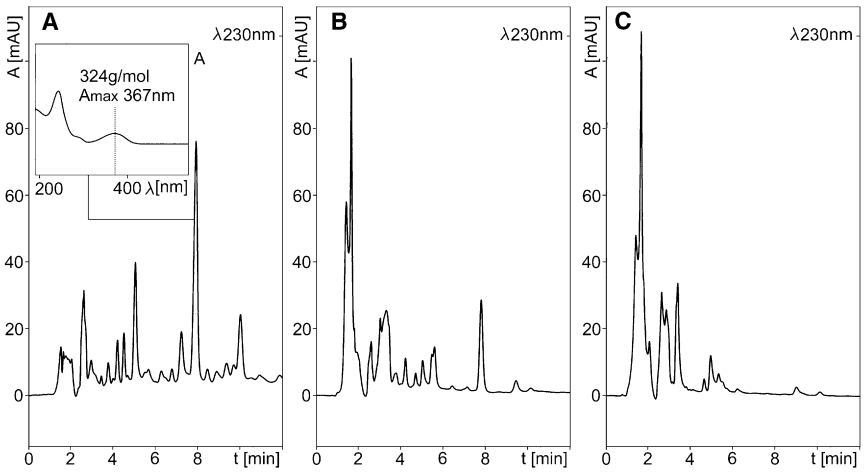

Figure 3. Degradation of the PgaE Reaction Product in Aqueous Solution

HPLC analysis of parallel reactions followed at three different time points after consumption of **1**.

(A) Analysis of products extracted at the end of the reaction. The main fragment has an absorbtion maximum at 367 nm and molecular mass 324 g/mol.

(B) At 15 min after the depletion of 1, the main peak has decreased in size along with an increase of peaks with shorter retention times.

(C) After 30 min, the main peak has disappeared and the majority of the compounds detected elute within the first few minutes of the run.

Added Oxygens in Gaudimycin C Derive from O_2 and H_2O

Consumption of **1** by PgaE was inhibited when air was removed from the reaction mixture, and proceeded only after aerobic conditions were restored, as seen spectrophotometrically in a Thunberg cuvette. A parallel experiment to examine PgaM oxygen dependence could not be devised due to the lack of a stable substrate. A coupled reaction with both enzymes under $^{18}O_2$ atmosphere resulted in a 2 amu increase of all major fragmentation products detected by LC-MS in comparison with a reaction carried out in normal air (Table 1), corresponding to the incorporation of a single ^{18}O atom in the first aerobic step. Parallel experiments in ^{18}O-labeled water also resulted in a 2 amu shift in LC-MS peaks, demonstrating that another oxygen is introduced into **5** from H_2O (Table 1).

NMR Structural Analysis of Gaudimycin C

Preparative scale chemoenzymatic conversion of **1** by PgaE and PgaM yielded sufficient quantities of purified **5** for full structural analysis by NMR. The ^{1}H and ^{13}C NMR spectra (Table 2) of **5** were highly similar to those of **4**, which is the in vivo intermediate from the related *cab* pathway (Palmu et al., 2007). The COSY, HSQC, and HMBC correlations (see Figure S1 in the Supplemental Data available with this article online) confirmed that the benz[*a*]anthracene carbon skeleton and the position of hydroxyl groups at carbons 4a (δ 3.66), 6 (δ 4.25), 8 (δ 12.01) and 12b (δ 7.11) were the same in **4** and **5**. Differences were finally seen in stereochemistry at position 6 by 1D NOE experiments (Figure S1), which, in **5**, permitted the observation of NOEs between the hydroxyl proton at carbon 4a and the hydroxyl protons at positions 6 and 12b. In **4**, similar experiments revealed NOEs between the hydroxyl proton at position 4a and H-6 and the hydroxyl proton at carbon 12b (Palmu et al., 2007). These results proved **5** to be 6-hydroxy gaudimycin A and a diastereomer of **4**.

DISCUSSION

Several in vivo studies have indicated that reaction intermediates in early post-PKS tailoring steps of various angucycline biosynthetic pathways exhibit unusually high inherent reactivity. This forestalls direct approaches to analyze the individual catalytic events, as separation of consecutive steps readily induces shunt product formation through either nonenzymatic conversion and/or endogenous modification by the host (Palmu et al., 2007; Rix et al., 2003, 2005). Due to the requirement for simultaneous presence of all enzyme components to maintain the biosynthesis on the correct pathway, related reaction sequences have been referred to as "biosynthetic black boxes," and have been associated with the formation of putative post-PKS oligomeric protein complexes (Rix et al., 2005; Kharel et al., 2007). Here we report, to our knowledge, the first in vitro study of an isolated oxygenation cascade, providing biochemical insight into the order and nature of individual catalytic steps, with angucycline tailoring flavoproteins PgaE and PgaM from *Streptomyces* sp. PGA64 as model enzymes. PgaE and PgaM were shown to catalyze an array of reactions, converting the first stable intermediate of the pathway, **1**, into a novel metabolite, **5**. The results demonstrate to our knowledge, for the first time directly, that the early oxygenation steps in certain angucycline pathways are highly dependent on close functional coupling, and must proceed in an uninterrupted sequence to avoid intermediate breakdown. Although this may imply noncovalent association between the enzymes as suggested previously (Rix et al., 2005; Fedoryshyn et al., 2007), direct, size-based analytical methods did not show any evidence of complex formation. The NMR structure of **5** revealed the PgaE/PgaM reaction cascade to involve (1) hydroxylation of **1** at position C12 followed by C7,12 p-quinone formation, (2) dehydration between C2 and C3, (3) hydroxylation of C12b, and (4) stereospecific C6 ketoreduction. Together with ^{18}O incorporation studies, the results allowed us to postulate specific activities for each putative functional component involved.

PgaE Functions as UWM6 12-Hydroxylase

PgaE catalyzes the first step of the PgaE/PgaM reaction sequence with **1** as a substrate in the presence of air and NADPH. Demonstrated by the $^{18}O_2$ isotope incorporation studies and oxygen dependence, PgaE introduces a single oxygen atom from O_2 into the substrate. Comparison of the structure of **5** with that of **1** revealed positions C12 and C12b as the two possible

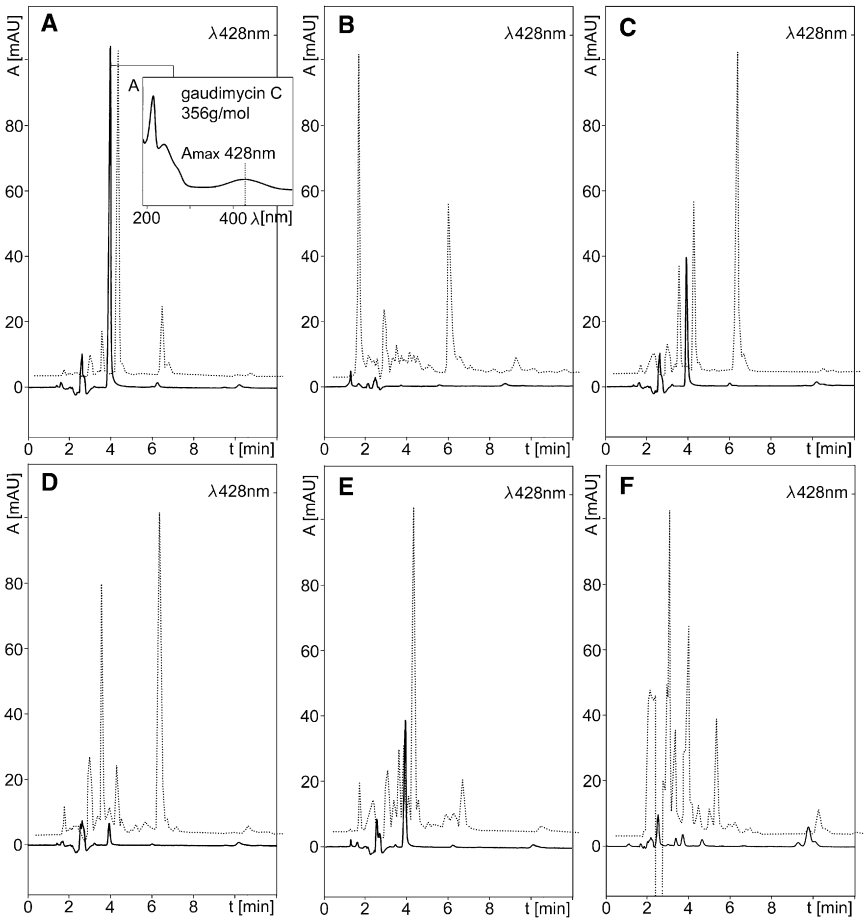

Figure 4. Comparison of Gaudimycin C, 5, Formation in Coupled PgaE and PgaM Reactions

Compound **5** is detected at 428 nm (solid line), whereas degradation products of the PgaE reaction are monitored at 230 nm (dashed line, relative scale).

(A) In an efficiently coupled reaction, PgaE and PgaM convert **1** to **5** in the presence of NADPH and molecular oxygen.

(B) Incubation of isolated products of the PgaE reaction with PgaM does not yield **5**.

(C) Addition of PgaM in a coupled reaction after over 50% of **1** has been consumed by PgaE results in a decreased yield of **5**, proportional to the remaining amount of substrate.

(D) Addition of PgaM at the end of the PgaE reaction produces a barely detectible amount of **5**.

(E) Decrease in formation of **5** can also be seen upon 100-fold increase of the relative amount of PgaE.

(F) Separation of the PgaE and PgaM reactions with a semipermeable membrane completely inhibits formation of **5**, although **1** and shunt products of the PgaE reaction pass through the membrane.

targets for PgaE-hydroxylation. This assessment, together with the observations that the in vivo reaction yields **2** (Palmu et al., 2007), which is not oxygenated at C12b, and that hydrogen peroxide is not formed in the reaction (Koskiniemi et al., 2007) verify PgaE as a **1** C12-hydroxylase (Figure 5). This is in agreement with the reported function of a close sequence homolog, UrdE (76% aa identity with PgaE), responsible for the corresponding reaction in urdamycin biosynthesis (Faust et al., 2000). The hydroxylation reaction yields a 7,12-dihydroquinone intermediate, which is conceivably readily oxidized to form the *p*-quinone structure observed in **5**. The oxidation may occur nonenzymatically, but, in the case of the *pga* system, requires enzyme stabilization to prevent further reactions that lead to a degradation pathway in aqueous solution.

The PgaM Reductase Domain Catalyzes 2,3-Dehydration

The number of observed chemical changes in **5** confirmed the involvement of PgaM in multiple catalytic events, as postulated by the presence of two putative functional domains. One of these functions is 2,3-dehydratase activity (Figure 5), detected as the loss of the 3-hydroxyl group in both **5** and **3** (Palmu et al., 2007). In landomycin biosynthesis, 2,3-dehydration has been reported as one of the multiple in vivo functions of LanV (Mayer et al., 2005), which is homologous to the SDR domain of PgaM. Members of this SDR family commonly catalyze dehydrogenation reactions (Jörnvall et al., 1995), but some have been shown to act as dehydratases (Allard et al., 2002). The removal of the C3-hydroxyl group appears to be the first reaction catalyzed by PgaM, as the isolated oxygenase domain failed to exhibit any activity when coupled to the PgaE reaction in vitro.

The Oxygenase Domain of PgaM Is Responsible for 12b-Hydroxylation

The structure of **5** together with the deduced function of PgaE confirms that PgaM is also responsible for hydroxylation of position C12b. This is in agreement with the in vivo function assigned for the homologous gene product of *urdM* in urdamycin biosynthesis (Faust et al., 2000). Interestingly, there is an explicit difference between these reactions, as the C12b oxygen in urdamycin has been shown to derive from molecular oxygen (Udvarnoki et al., 1992), whereas, in the case of **5**, it clearly originates from water. This has direct mechanistic implications, which are discussed in detail in the final section of the Discussion.

The Loss of the 6-Hydroxyl Group In Vivo and 6-Ketoreduction In Vitro

PgaM is affiliated with yet another reaction, C6 ketoreduction of the angucyclic substrate, deduced by the presence of a hydroxyl group at this position in **5**. The reaction appears to be stereospecific, as the opposite orientation of the C6-OH groups is the only difference between this product and **4**, the corresponding in vivo product of the related *cab* pathway. This stereochemical difference can also be seen indirectly from the structure of the *pga* in vivo product, **3**. It has lost the C6-hydroxyl group still retained

Table 1. Analysis of Oxygen Incorporation in Coupled PgaE and PgaM Reactions Carried Out in $^{18}O_2$ and $H_2{}^{18}O$ by LC-MS

LC-MS Fragment	Peak Intensity ($^{18}O/^{16}O$)	In Air	In $^{18}O_2$ (100%)	In H_2O	In $H_2{}^{18}O$ (58%)
M + Na	381/379	0.16	12.97	0.06	2.66
M + 1	359/357	0.07	8.42	0.04	2.24
M − H_2O	341/339	0.06	12.86	0.09	2.07
M − $2H_2O$	323/321	0.05	2.05	0.09	2.46
M − 61 Da	295/293	0.08	3.13	0.13	2.27

The data demonstrate that one of the incorporated oxygens in **5** derives from molecular oxygen and the other from water. Efficiency of oxygen isotope incorporation is calculated as the ^{18}O:^{16}O ratio in reaction product fragments in LC-MS (rounded down for consistency). Fluctuations of values in the $^{18}O_2$ column are due to low ^{16}O-derived signals, which may fall between integration points.

Table 2. ^1H and ^{13}C NMR Data for Gaudimycin C, 5, in $CHCl_3$-*d1*

Position	^1H NMR	^{13}C NMR
1	—	196.3
2	5.92 (s)	122.4
3	—	161.2
4	2.93 (d)	43.1
	2.45 (d)	—
4a-OH	3.66 (s)	74.0
5	2.30 (dd)	34.7
	2.10 (dd)	—
6	4.94 (m)	61.0
6-OH	4.25 (d)	—
6a	—	144.1
7	—	188.5
7a	—	114.6
8	—	161.4
8-OH	12.01 (s)	—
9	7.35 (d)	125.7
10	7.70 (t)	137.0
11	7.73 (d)	120.5
11a	—	131.5
12	—	188.3
12a	—	138.4
12b-OH	7.11 (s)	78.4
13-CH_3	2.06 (s)	24.8

Assignments were made by using COSY, HSQC, and HMBC difference measurements. Carbons 7 and 12 were assigned based on the chemical shifts of the corresponding carbons in the related gaudimycins A and B (Palmu et al., 2007).

in **4** (Palmu et al., 2007), confirming that the C6-OH removal results from stereoselective endogenous modification by the host strain, *S. lividans* TK24. The *pga* product appears to be accepted as a substrate for the endogenous reactions, while the opposite stereoisomer of the *cab* pathway is left intact. Importantly, this confirms the C6 ketoreduction step to be enantioselective also in physiological surroundings, and not merely an effect caused by the reaction conditions in vitro. It is also noteworthy that all C12b-hydroxylated 12,7-quinone angucyclines appear to have undergone the C6 ketoreduction step, which may imply some mechanistic interconnection between the two catalytic events.

Mechanisms Involved with 12b-Hydroxylation and 6-Ketoreduction

Flavoprotein monooxygenases homologous to the oxygenase domain of PgaM exclusively use molecular oxygen as a substrate, but certain reactions may proceed through an H_2O-assisted heterocycle ring cleavage to incorporate a water-derived oxygen into the product. These reactions, however, appear mechanistically incompatible with the PgaM-catalyzed steps in the biosynthesis of **5**. For example a Baeyer-Villiger reaction described for several types of flavoenzymes (Iwaki et al., 2006; Fraaije et al., 2005; Jones et al., 1993) may involve a step of lactone ring opening to introduce oxygen from water, but fails to explain the regeneration of the six-membered carbon ring A. The free carboxylic acid generated in the cleavage is not chemically a suitable substrate for known condensation mechanisms, such as aldol or Claisen. If the Baeyer-Villiger reaction proceeded via the rearrangement reaction proposed for urdamycin C12b monooxygenation (Rix et al., 2003), the incorporated oxygen would originate from O_2 and not from water, as observed here. Another candidate, an epoxidase reaction followed by opening of the oxirane ring by H_2O, has been discussed in the context of tetracenomycin 4a-hydroxylation (Rafanan et al., 2000) and urdamycin 12b-hydroxylation (Udvarnoki et al., 1992). This would, however, require the formation of a 4a/12b epoxysemiquinone structure, which does not seem feasible with the C4a tertiary carbon of the substrate being already hydroxylated and incapable of further oxidation. Other flavoprotein mechanisms, such as the aromatic monooxygenation catalyzed by the well-characterized p-hydroxybenzoate hydroxylase (Schreuder et al., 1989), cannot account for the H_2O-derived oxygen. In addition, none of the flavoprotein monooxygenase mechanisms explain the stereospecific C6 ketoreduction reaction, which occurs during gaudimycin C formation.

Intriguingly, a distinct group of FAD-dependent flavoproteins has been found to catalyze a sequence of reactions that closely resembles the C12b-hydroxylation and C6 ketoreduction steps in the biosynthesis of **5**. These enzymes hydroxylate various aromatic substrates in degradation of toxic phenol/benzo compounds (Cunane et al., 2000; Fraaije and van Berkel, 1997; Hopper and Cottrell, 2003), and bear no sequence similarity to PgaM. The reactions differ mechanistically from those of flavoprotein monooxygenases, and use oxidized flavin to generate a highly reactive QM intermediate, followed by incorporation of oxygen derived from water (Hopper and Cottrell, 2003; van den Heuvel et al., 2000). Ultimately, the reaction results in an intramolecular redox hydration involving parallel hydroxylation and stereospecific keto group reduction, as observed for **5**. Although the PgaM flavin redox cycle is presently unclear, the central concept of the catalytic mechanism, the redox conversion to form a highly polarized QM structure, has been described for for anthracyclines (Freccero, 2004), compounds closely related to angucyclines.

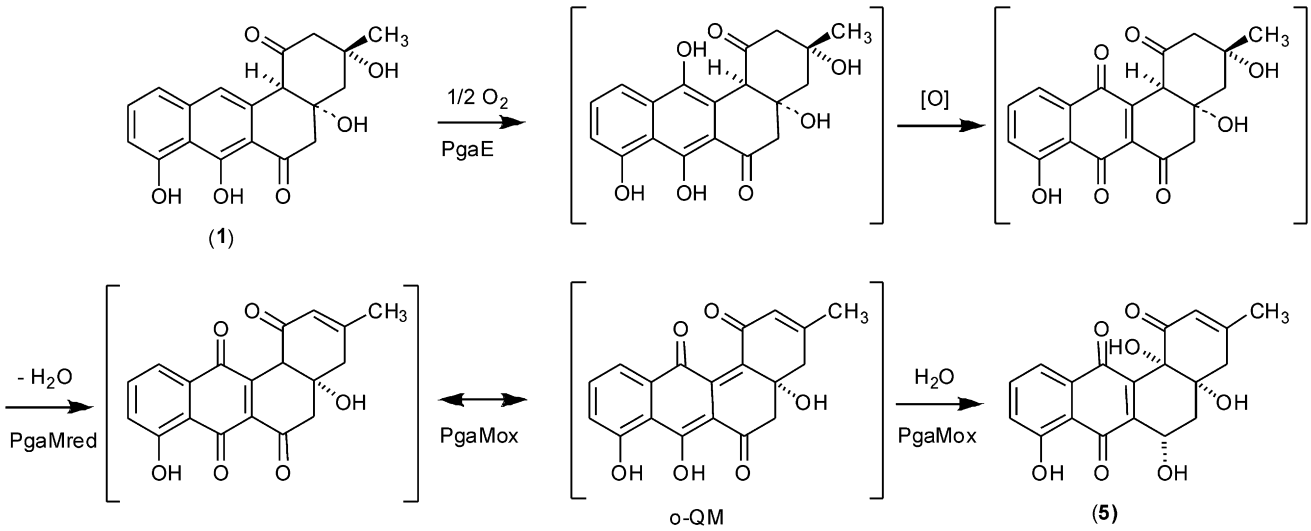

Figure 5. Model for Gaudimycin C Biosynthesis

In the first step, PgaE catalyzes hydroxylation of UWM6 at position 12, which is followed by quinone formation. The first reaction of PgaM is catalyzed by the SDR domain (PgaMred), resulting in 2,3-dehydration. Finally, the oxygenase domain of PgaM (PgaMox) catalyzes C12b-hydroxylation and C6 ketoreduction by the QM mechanism. This is initiated by enzyme-induced tautomerization of the substrate to form a highly polarized o-QM. The conjugated intermediate is readily hydrated to **5** in an intramolecular redox reaction involving enzyme-stabilized nucleophilic addition of water at C12b and reduction of C6.

All chemical changes associated with the PgaM oxygenase domain in formation of **5** can be explained by the model presented in Figure 5. The reaction initiates by substrate activation in an enzyme-induced tautomerization to generate a highly conjugated angucycline o-QM intermediate, a structure analogous to the anthracycline QM species (Freccero, 2004). The keto group adjacent to the reacting C12b facilitates the α-proton removal to yield this tautomer. Once formed, the QM intermediate is expected to readily undergo hydration in the presence of water, in this case by stereospecific nucleophilic attack of an enzyme-aligned H_2O molecule (van den Heuvel et al., 2000) to introduce the hydroxyl group at C12b. Characteristically, the hydroxylation event is accompanied by simultaneous ketoreduction of the substrate (Cunane et al., 2000; Hopper and Cottrell, 2003; Freccero, 2004). Assisted by π electron delocalization across the conjugated ring structure and specifically positioned hydrogen donor in the enzyme active site, this leads to the C6 ketoreduction of the angucycline to yield **5**.

Validation of the proposed mechanism by kinetic means (Fraaije and van Berkel, 1997) is unfortunately not feasible due to the number of interlinked enzymatic reactions and simultaneous presence of several intermediates in the PgaE/PgaM reaction. For these reasons, verification of the model can most likely be obtained only through structure/function studies currently in progress in our laboratory. Previously, several different enzymes involved in aromatic polyketide biosynthesis have been associated with atypical catalytic mechanisms (Sultana et al., 2004; Jansson et al., 2005; Sciara et al., 2003). Characteristically, these mechanisms employ the reactive chemical nature of the highly conjugated polyaromatic substrate, which allows stabilization through charge distribution over the intermediate ring structure (Fetzner, 2002; Schneider, 2005). The results presented here suggest that the reaction mechanism of PgaM follows this paradigm.

SIGNIFICANCE

Angucyclines are a well-defined group of aromatic polyketides that have been shown to exhibit antimicrobial and antitumor activities. Thus far, the biosynthesis of these compounds has been studied mainly in vivo, where tailoring reactions in particular have raised wide interest due to their potential use in combinatorial biosynthesis applications. These studies have confirmed the curious existence of so-called biosynthetic black boxes, defined to describe multistep cascades of nonseparable reactions.

In this in vitro study, we have isolated an early post-PKS oxygenation cascade to gain insight into the catalytic steps involved. PgaE and PgaM, which are representatives of flavoenzymes typically associated with these reactions, were shown to catalyze multiple sequential reactions and to convert UWM6 into a novel metabolite, gaudimycin C. The catalytic sequence required close synchronization between the successive steps to prevent intermediate degradation and shunt product formation, and consisted of C12-hydroxylation followed by 12,7-quinone formation, C2,3-dehydration, and C12b-hydroxylation, together with stereospecific C6 ketoreduction.

Another key finding of the study was that the C12b oxygen of gaudimycin C derives from water. It demonstrates that the oxygenase domain of PgaM catalyzes a novel type of hydroxylation reaction and that it is not a flavoprotein monooxygenase, despite its significant sequence similarity to known members of the family. Furthermore, the results indicate that the C12b-hydroxylation might be mechanistically connected to C6 ketoreduction and that the reaction cascade may proceed through a quinone methide intermediate.

EXPERIMENTAL PROCEDURES

Expression Constructs

The plasmid, pBHBΔ, used for PgaE expression was modified from a commercial vector, pBAD/HisB (Invitrogen), by shortening the region between N-terminal polyhistidine tail and the multiple cloning site as described elsewhere (Kallio et al., 2006). The entire 1476 bp coding region of *PgaE* was cloned from pTOPO-pgaE (Palmu et al., 2007) into pBHBΔ as an EcoRI fragment. The N-terminal end (319 bp BglII /*Bsi*WI) was then replaced with a modified PgaE PCR fragment to remove the original ATG start codon and extra 5″ nucleotides from the first cloning step. The amplification primers used to generate this fragment were 5′-AGATCTGACGCTGCCGTGATTGTCGT-3′ and 5′-ACTGTTCCA GGTGGGTCTCGGTCAC-3′. The final construct, pBHBΔ_PgaE, contained a 5′ 7-histidine coding region and two additional codons encoding for arginine and serine preceding the PgaE ORF. The primary PgaM vector, pBHBΔ_PgaM, was constructed accordingly. EcoRI fragment of pTOPO-pgaM (Palmu et al., 2007) was subcloned into pBHBΔ, after which the PgaM N-terminal end was replaced with a PCR product to remove the original start codon and to add a BglII site. The PCR primers used to generate this fragment were 5′-AGTTTCCAGCCGAGGTTGACG-3′ and 5′-AGATCTAGCGAGGACCA CACCGAGAC-3′. A PgaM *Streptomyces* expression plasmid was constructed by ligating the entire coding region and *E. coli* RBS from pBHBΔ_PgaM (*Hin*dIII/BamHI) into pIJE486 under the control of constitutively active *ermE*p promoter (Ylihonko et al., 1996). Finally, a deletion mutant construct was designed to produce the PgaM N-terminal oxygenase domain alone in *E. coli* as a His-tag fusion. This was done by substituting the C-terminal half of PgaM (PstI/*Hin*dIII) in pBHBΔ_PgaM with a PCR fragment, which contains the end of PgaM oxygenase domain followed by a termination codon, as described elsewhere (Kallio et al., 2007). The primers used to generate the fragment were 5′-GGCCAGCCCTCAACCTCGG-3′ and 5′-TATAAGCTTTTAGAACCAGCGGCG CAGC.

Expression and Purification of Proteins

PgaE was overexpressed in *E. coli* TOP10 strain as a 500 aa-long fusion protein containing the 490 aa native polypeptide and an N-terminal tag, AHHHHHHHRS. The protein was produced in a 5 l fermentor batch culture in 2× YT (yeast extract/tryptone) at room temperature (18 hr) and purified to homogeneity in one nickel affinity chromatography step (5 ml HisTrap; Amersham Biosciences). Purity was visualized by SDS PAGE gel electrophoresis (Pharmacia PhastSystem), and the exact mass verified by protein mass spectrometry (TOF MS ES+). PgaE remained apparently stable for months when stored at −20°C (0.01 M Na_3PO_4 [pH 7.4], 0.25 M NaCl, ~ 0.125 M imidazole, and 50% glycerol). PgaM was overexpressed in *Streptomyces* TK24 in parallel 250 ml TSB batches in 1 l Erlenmayer flasks at 30°C for 3 days. The protein was purified in one-step nickel affinity chromatography (5 ml HisTrap), carried out in successive, consecutive loops to avoid protein precipitation induced by column overloading. Elution was followed by an immediate buffer exchange gel filtration step to remove destabilizing imidazole in the buffer. PgaM remained stable for months at −20°C (0.01 M Na_3PO_4 [pH 7.4], 0.08 M NaCl, 25 mM imidazole, and 50% glycerol). PgaMox deletion mutant lacking the C-terminal reductase domain was produced as N-terminal His-tag fusion and purified by nickel affinity chromatography and gel filtration, as described elsewhere (Kallio et al., 2007). Enzyme concentrations were measured by the Bradford dye binding method (Bradford, 1976).

Preparation of the Substrate

UWM6 (**1**) was produced in *Streptomyces* TK24/pMC6BD (Metsä-Ketelä et al., 2003) with parallel 250 ml batches of E1 medium (Ylihonko et al., 1994) with XAD-7 absorbent material (20 g/l) in a 7 day incubation at 30°C/250 rpm. Purification was done by isopropanol gradient elution from XAD, H_2O/chloroform extractions to remove hydrophilic bulk impurities, and selective methanol solubilization to eliminate more hydrophobic compounds. Preparative RP-HPLC was used as a polishing step (Merck-Hitachi HPLC system, LiChroCART 250-10/RP-18 10 μm particle size column, detection at 400 nm, 250 μl injection, 2.5 ml/min flow, gradient 15% acetonitrile + 0.1% HCOOH > acetonitrile + 0.1% HCOOH, elution at ~27 min). In vivo by-product **2** was extracted from the preparate of **1** at this step.

Enzyme Assays

PgaE in vitro activity was measured spectrophotometrically as a depletion of **1** at its absorption maximum at 406 nm. The reactions were carried out in 1 ml volume of 100 mM potassium phosphate buffer [pH 7.5], containing a total of 6% methanol, 0.2 mM NADPH, 50–75 nM PgaE, and ~17.5 μM **1**. Coupled PgaE/PgaM reactions were prepared in microcentrifuge tubes (RT, gentle mixing for 10–30 min) in equivalent conditions with threefold molar excess of PgaM over PgaE. Reaction compounds were isolated in quantitative chloroform extractions, or by using analytical scale solid-phase extraction columns (DSC-18; Supelco). Samples were solubilized/eluted in methanol and analyzed with HPLC (Shimadzu VP series HPLC chromatography system with a diode array detector, Merck RP-18 column, isocratic 40% acetonitrile). Parallel reactions were quantified and compared on the basis of peak areas at 406 or 428 nm, whereas degradation intermediates were detected at 230 nm.

^{18}O Incorporation and Compartmentalized Reactions

Anaerobic reactions and conversions in $^{18}O_2$ gas (Isotec; Sigma-Aldrich) were carried out in a Thunberg cuvette, which allowed complete oxygen removal with a combined vacuum (10 min) and repeated N_2 rinses before mixing the reaction components. ^{18}O water reactions were prepared in a total volume of 300 μl, in which 2:3 of the reaction buffer was replaced by H_2O ^{18}O isotope (Aldrich Chemical Co.). Reactions were monitored spectrophotometrically and products extracted quantitatively with acidic ethyl acetate for analysis by HPLC-ESI-MS. The compartmentalized PgaE/PgaM reactions were carried out in a 12 ml test tube with a cellulose acetate dialysis membrane (~30 mm outer diameter) with a 12–14 kDa MWCO to separate the enzymes; **1** was added on the side of PgaM to demonstrate its ability to permeate through the membrane. The reaction volumes were scaled up to 10 ml and incubation times increased up to 60 min. Isolation and analysis of the compounds were carried out as described above.

Preparation and Purification of Gaudimycin C for NMR

Chemoenzymatic conversion of **1** to **5** was carried out in 65 parallel 10 ml reactions in conditions equivalent to the analytical-scale PgaE/PgaM reactions. Complete reactions were pooled and the compounds extracted with chloroform, and **5** was purified in two steps. First, a silica chromatography step (Merck silica gel 60, 0.04–0.063 mm), with elution from hexane with increasing chloroform (to 100%) and methanol gradient (to 2%). Second, preparative HPLC (Phenomenex Luna phenyl-hexyl 100, 250 × 10 mm/10 μm column, 5%–100% acetonitrile gradient, detection at 420 nm, elution at around 50% acetonitrile) with the same equipment used for purification of **1**. The product, monitored by HPLC, remained unchanged throughout the extraction steps and in storage in methanol.

LC-MS and NMR Measurements

HPLC-ESI-MS analysis was performed with a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ion-spray interface. The HPLC system consisted of two Perkin-Elmer Series 200 micro pumps (Perkin-Elmer, Norwalk, CT) with a Series 200 autosampler and 785A UV/VIS detector. Separation of compounds was carried out in the same conditions and column as described for analytical HPLC. The ^{18}O incorporation data presented in Table 1 were calculated from intensities of ion fragments by dividing the signal of ^{18}O ionization fragments with the corresponding ^{16}O fragment signal in each run. NMR spectra were obtained with a Bruker Avance DRX500 spectrometer. The NMR measurements were made in chloroform-d_1 at 233K and the ^1H and ^{13}C NMR chemical shifts were referenced to tetramethylsilane. The number of scans for 1H, selective 1D-NOESY, COSY, HSQC, and HMBC were 64, 256, 16, 64, and 160, respectively, due to the dilute sample. The decoupled ^{13}C spectrum was run with 34,800 scans to reach reasonable signal-to-noise ratio.

Analysis of Complex Formation

Analytical gel filtration was performed with the HPLC system described for compound analysis, with a Tosoh Bioscience TSKgel SuperSW 3000 column (4.6 × 300 mm, 4 μm) in isocratic conditions (0.1 M Na_2SO_4 in 0.1 M phosphate buffer [pH 7.6]) and 0.2 ml/min flow rate. The HMW gel filtration calibration kit (Amersham Biosciences) was used as a molecular size ladder. Cross-linking experiments between PgaE and PgaM preparates were carried out by

glutaraldehyde treatment of combined enzyme dilutions followed by SDS PAGE analysis, as described elsewhere (Baykov et al., 1995).

SUPPLEMENTAL DATA

Supplemental Data, including one additional figure, can be found with this article online at http://www.chembiol.com/cgi/content/full/15/2/157/DC1/.

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