The Oxidative Ring Cleavage in Jadomycin Biosynthesis: A Multistep Oxygenation Cascade in a Biosynthetic Black Box

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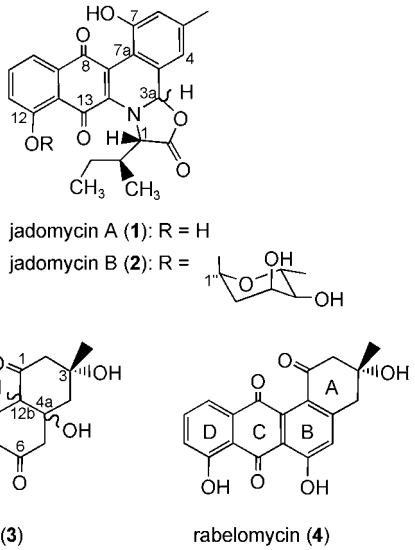
The antibiotic jadomycin B is derived from an angucycline intermediate that undergoes oxidative ring cleavage and the unique incorporation of L-isoleucine into its polyketide backbone. To elucidate the enzymes and substrates involved in this key oxygenation event, we have investigated a region of the *jad* gene cluster that is located immediately downstream of the previously identified oxygenase genes *jadF* and *jadG* and contains a third putative oxygenase gene, *jadH*, as well as a potential hydrolase gene, *jadK*. Inactivation of *jadG* and *jadH*, respectively, led to the accumulation of several shunt products and a novel potential pathway intermediate, named prejadomycin. Production of these angucyclines and the failure to generate a ring-cleavage product in various mutant strains illustrates the complex protein–protein interaction network within the oxygenase subcluster. Furthermore, these results demonstrate that both JadF and JadH display secondary dehydratase activities that contrary to their oxygenase activities, appear to be independent of the respective protein-complex binding partners.

The polyketide glycoside antibiotic jadomycin B (**2**)[1] and its aglycon jadomycin A (**1**) (Scheme 1) are produced by the soil bacterium *Streptomyces venezuelae* ISP5230 under stress conditions such as heat shock, phage infection, and particularly ethanol treatment.[2] The jadomycin family possesses a unique nitrogen-containing pentacyclic benz[*b*]oxazolophenanthridine backbone that has been shown by precursor-directed biosynthesis with various amino acids[3,4] as well as feeding experiments with ^{13}C-labeled acetate[5] to derive from the fusion of an L-amino acid, for example, L-isoleucine in the case of jadomycins A (**1**) and B (**2**), and a polyketide intermediate. Furthermore, accumulation of the well-known antibiotic rabelomycin (**4**)[6] upon inactivation of the putative oxygenase-encoding gene *jadF*,[7] as well as production of its unstable precursor UWM 6 (**3**) by heterologous expression of the *jad* polyketide synthase (PKS) genes (i.e. *jadABCDEI*, Figure 1) in *Streptomyces lividans*,[8a] established the angucycline origin of the jadomycin polyketide moiety. Yang et al.[7] proposed that an angucycline intermediate undergoes an oxidative cleavage in ring B and that subsequent reaction with L-isoleucine, a highly abundant component of the culture medium, yields the characteristic jadomycin scaffold. Consis-



Scheme 1. *Jadomycin antibiotic family members produced by* Streptomyces venezuelae *ISP5230[1,2,4] under standard culture conditions and angucyclinones previously implicated in their biosynthesis.[7,8]*

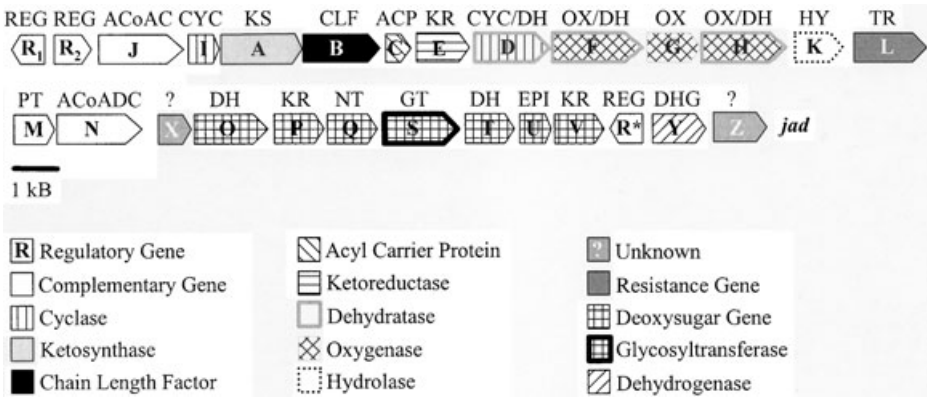


Figure 1. *Biosynthetic gene cluster of jadomycin B (**2**) in* Streptomyces venezuelae *ISP5230.*

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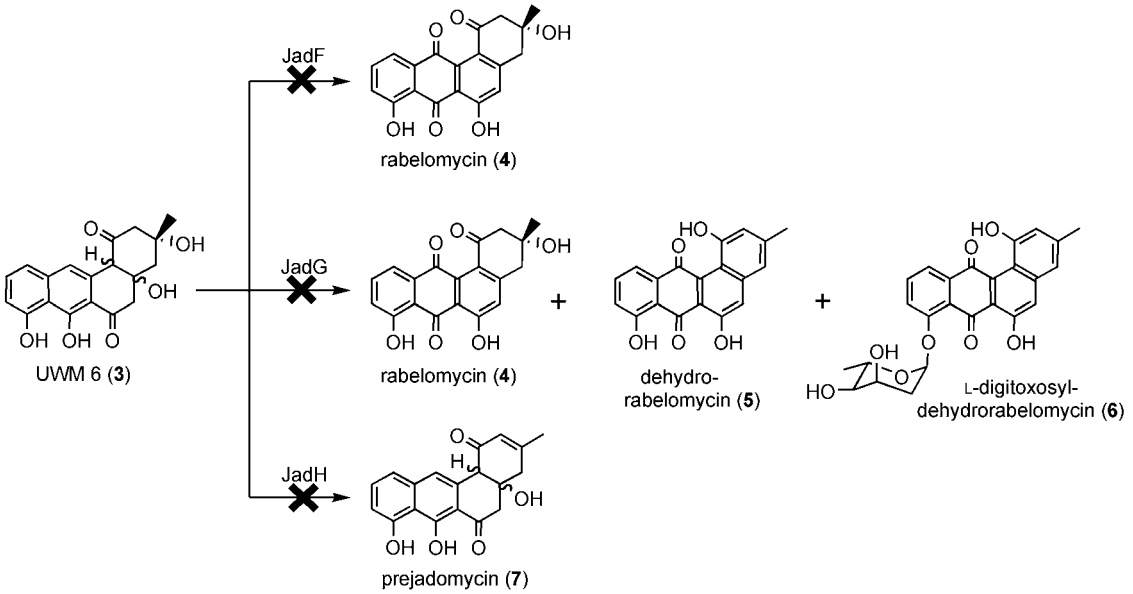
tent with this, the jadomycins belong to the intriguing subfamily of "atypical" angucyclines, which also includes kinamycin D,[9] vineomycin B_2,[10] and gilvocarcin V[11] and for which similar oxidative ring-scission reactions of the standard angucycline framework have been proposed. The oxidative processes observed in this class of polyketides and the respective oxygenase genes and enzymes form the basis for an extraordinary degree of structural diversity and are therefore particularly interesting for combinatorial biosynthetic approaches that aim to generate novel bioactive "unnatural" natural products by genetic engineering.

So far, the only gene from the *jad* cluster (Figure 1) that has been unambiguously identified to be involved in oxidative ring cleavage during jadomycin biosynthesis is the putative oxygenase gene *jadF*, which displays strong sequence homologies to FAD- and NADPH-dependent monooxygenases.[7] However, neither the catalytic mechanism nor the exact substrate of JadF have been described. Also, it was apparent from chemical considerations that another monooxygenase and possibly the putative anthrone oxygenase JadG would have to play important roles in the overall process as well.

The aim of this study was the investigation of the still poorly understood oxygenation cascade that paves the way for the unusual incorporation of the amino acid and the identification of the biosynthetic enzymes involved. Here we describe the identification of two new genes, *jadH* and *jadK*, from the jadomycin biosynthetic gene cluster and the isolation of several metabolites, among them two novel compounds, L-digitoxosyldehydrorabelomycin (**6**) and prejadomycin (**7**), which are generated by the inactivation of genes believed to be involved in this ring-cleavage event (Scheme 2). The results presented here shed additional light on the reaction sequence in question, which entails a set of three monooxygenases: JadF, JadG and the formerly unidentified JadH. Furthermore, the described metabolite spectrum unambiguously reveals the surprising bifunctional oxygenase-dehydratase nature of both JadF and JadH, thereby also illustrating the sequential character of the previously ignored aromatization process of ring A, which accompanies the ring-scission reactions.

Subcloning and sequence analysis of the 2.5 kb DNA fragment between the anthrone oxygenase-encoding *jadG* and the efflux pump-encoding *jadL*[12] uncovered two additional open reading frames, designated *jadH* and *jadK* (Figure 1).[12] The *jadK* gene is 166 nt downstream of *jadH* and encodes for a putative protein of 249 aa. Database searches showed some similarities between JadK and α,β-hydrolases. In contrast, the deduced sequence of the 543 aa JadH protein exhibited a close resemblance to FAD- and NADPH-dependent monooxygenases involved in polyketide biosynthesis—for example, to KinO2 of kinamycin (66% identity), UrdE of urdamycin A (54% identity),[13] SimA7 of simocyclinone (53% identity),[14] MtmOIV of mithramycin (41% identity),[15] GilOI of gilvocarcin V (36% identity),[11b] and its neighbor in the jadomycin B (**2**) biosynthesis pathway, JadF (44% identity).[7] Most significant is the presence of two sequence motifs for binding of FAD as a cofactor: these were the N-terminal $\beta\alpha\beta$ (Rossman)-fold with three conserved glycines that bind the adenosine moiety of FAD,[16] and a centrally located motif with a conserved aspartic acid residue involved in binding the flavin moiety (Figure 2).[17]

To elucidate the functions of JadH and JadG in jadomycin biosynthesis, each gene was insertionally inactivated in a double crossover experiment by employing an apramycin resistance cassette. Subsequent cultivation of the JadH-inactivated strain under conditions routinely used for jadomycin B (**2**) production[4] resulted in the formation of a single yellow compound, designated prejadomycin (**7**, Scheme 2), which displays a characteristic bright yellow fluorescence under UV light ($\lambda =$ 366 nm). Neither jadomycins, nor **4**, nor **3** were detected at any time by TLC or HPLC/MS analysis. The spectroscopic data of prejadomycin (**7**) closely resemble those reported for



Scheme 2. *Secondary metabolites accumulated in* S. venezuelae *ISP5230 upon inactivation of the early post-PKS tailoring genes jadF,[7] jadG, and jadH.*

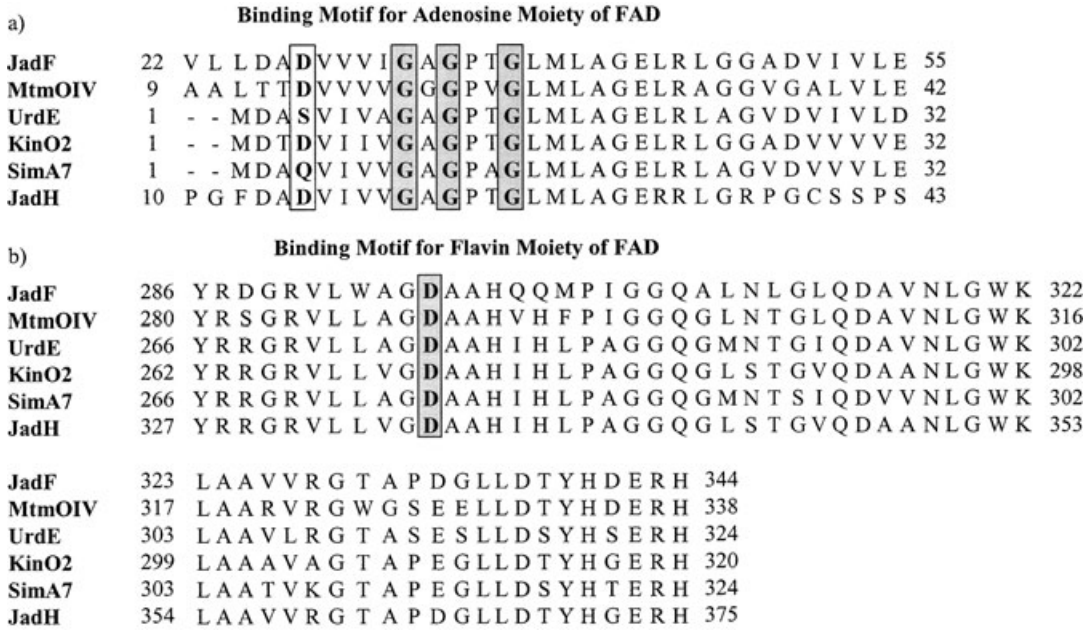


Figure 2. *Amino acid sequence binding motifs of FAD- and NADPH-dependent oxygenases. JadF, JadH (*S. venezuelae*); MtmOIV (*S. argillaceus*); UrdE (*S. fradiae*); KinO2 (*S. murayamaensis*); SimA7 (*S. antibioticus*). a) Binding motif for adenosine moiety of FAD displaying three conserved glycine residues (in gray boxes) that comprise a $\beta\alpha\beta$ or Rossman fold; an aspartic acid residue five amino acids upstream (white box) has been implicated with type-I BVMOs. b) Binding motif for flavin moiety of FAD with conserved aspartic acid residue (gray box).*

UWM 6 (**3**)[8] in having a UV absorption maximum at $\lambda =$ 405 nm. The deprotonated molecular ion peak at $m/z = 323$ in the negative-mode APCI-MS spectra indicated a molecular formula of $C_{19}H_{16}O_5$ and a molecular weight ($M_W = 324\ g\,mol^{-1}$) that is 18 amu lower than that of UWM 6 (**3**). The implication that prejadomycin (**7**) is formed by the loss of H_2O was supported by the NMR data (Table 1). The ^{1}H NMR spectrum of **7** showed characteristic signals at $\delta = 9.66$ and 16.05 for the 8- and 7-OH groups, respectively,[8] as well as a broad singlet at $\delta = 7.04$ for the aromatic 12-H proton. Similar signals were previously described for **3**.[8] Furthermore, a singlet at $\delta = 3.89$ showing an HSQC correlation to $\delta = 58.9$ could be assigned to the angular bridge 12b-methine proton. A major difference from the spectrum of **3** was the lack of signals for a 2-CH_2 group in ring A; instead **7** showed proton signals at $\delta = 5.96$ and 1.99 with ^{13}C values of $\delta = 126.1$ and 23.4, respectively. The interpretation of these signals as corresponding to an α,β-unsaturated ketone and an olefinic methyl group, respectively, was consistent with the observed COSY couplings and the downfield shift of C-3 to $\delta = 157.0$. Therefore, **7** was concluded to be an analogue of **3** in which ring A displays an α,β-unsaturated ketone group and therefore could also have been named 2,3-dehydro-UWM 6.

The cultivation of a disruption mutant of the putative anthrone-oxygenase gene *jadG*[7,12] and subsequent analysis of the crude product by TLC and HPLC/MS revealed the absence of any jadomycins. Instead, three compounds not present in the wild-type strain were observed (Scheme 2). Two of these were identified by their UV, MS, and ^{1}H NMR spectra as the well-known angucyclinones rabelomycin (**4**) and dehydrorabelomycin (**5**).[6] The third compound was of the same characteristic green color as dehydrorabelomycin (**5**) and consistently showed an almost identical UV spectrum. The sole difference was a hypsochromic shift of the absorption maximum found in **5** at $\lambda = 458$ nm to only 431 nm. A similar shift is generally ob-

Table 1. *^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for prejadomycin (**7**) in [D_6]acetone (relative to internal TMS, J in Hz).*

Position	^{1}H NMR	Multiplicity	^{13}C NMR	HMBC	COSY
1			204.3		
2	5.96	s	126.1		3-CH_3, 4β
3			157.0		
3-CH_3	1.99	s (br)	23.4	2	
4α	3.06	d (16.5)	40.9		
β	2.64	d (16.5)			2
4a			74.0		
4a-OH	4.84[a]	s (br)			
5α	3.60	m	47.1		
5β	2.78	m			
6			200.3		
6a			110.3[b]		
7			168.7		
7-OH	16.05 [a]	s (br)			
7a			112.5		
8			159.0		
8-OH	9.66 [a]	s		7a,8	
9	6.82	d (7.5)	112.0	7a,8,11	10, 11
10	7.53	dd (7.5,7.5)	133.6	8,11a	9, 11
11	7.25	d (7.5)	119.5	7a,9,11a	9, 10, 12
11a			140.2		
12	7.04	s (br)	113.6	7	11
12a			133.6[b]		
12b	3.89	s (br)	58.9		

[a] Exchangeable with D_2O. [b] Assignments interchangeable.

served in the jadomycin family after glycosylation of the aglycon at the phenolic hydroxyl group of ring D.[4b] The positive-mode APCI-MS displayed a protonated molecular ion at $m/z=$ 451; this indicated a molecular weight of $M_W=450\ g\,mol^{-1}$ and a molecular formula of $C_{25}H_{22}O_8$. In addition, the mass spectrum shows a strong fragment peak at $m/z=321$ corresponding to the loss of a dideoxysugar. The identification of this compound as L-digitoxosyl-dehydrorabelomycin (**6**) was supported by the NMR data (Table 2), which are very similar to

Table 2. *^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for L-digitoxosyldehydrorabelomycin (**6**) in [D_6]acetone (relative to internal TMS, J in Hz).*

Position	^{1}H NMR	Multiplicity	HSQC	COSY
1-OH	9.99[a]	s		
2	7.20	s (br)	119.1	3-CH_3, 4
3-CH_3	2.41	s	21.3	2,4
4	6.86	d (1.5)	116.8	3-CH_3, 2
5	7.68	s	122.2	
6-OH	12.48[a]	s		
9	7.92	m[b]	121.5	10, 11
10	7.93	m[b]	n.o.[c]	9, 11
11	7.77	m[b]	n.o.[c]	9, 10
1′	6.13	d (3)	95.3	2′$_{ax}$, 2′$_{eq}$
2′$_{ax}$	2.29	ddd (15,3,3)	35.2	1′, 3′
2′$_{eq}$	2.43	ddd (15,3,1)		1′, 3′
3′	4.02	m	66.3	2′$_{ax}$, 2′$_{eq}$, 4′
4′	3.21	dd (10,3)	n.o.[c]	3′, 5′
5′	3.85	dq (10,6)	65.9	4′, 5′-CH_3
5′-CH_3	1.16	d (6)	17.5	5′

[a] Exchangeable with D_2O. [b] Higher order effect. [c] Not observed.

those of dehydrorabelomycin (**5**), the most significant difference being the lack of the 8-OH signal at $\delta=11.74$. Instead, **6** displays an anomeric proton as an additional doublet at $\delta=$ 6.13 with a characteristic HSQC correlation to $\delta=95.3$. Further analysis and comparison of the remaining ^{1}H and ^{13}C NMR signals of the sugar moiety to jadomycin B (**2**) and other glycosylated jadomycin family members[4a] confirm this dideoxysugar to be L-digitoxose.

In order to obtain a PKS mutant that can serve as a host for feeding experiments, the ketosynthase gene *jadA* was disrupted in *S. venezuelae* VS662, an inactivation mutant of the regulatory gene *jadR$_2$*,[18a] to create a double mutant with overexpressed levels of post-PKS tailoring enzymes. As expected, standard cultivation of this strain did not produce any jadomycins or related compounds as determined by TLC and HPLC/MS. This mutant was subsequently utilized for feeding experiments with the potential pathway intermediates **4** and **7**. Analysis of the crude extracts by HPLC/MS showed the complete conversion of **7** after 12 h to jadomycin A (**1**), whereas, in the case of **4**, no jadomycins could be detected under the same conditions.

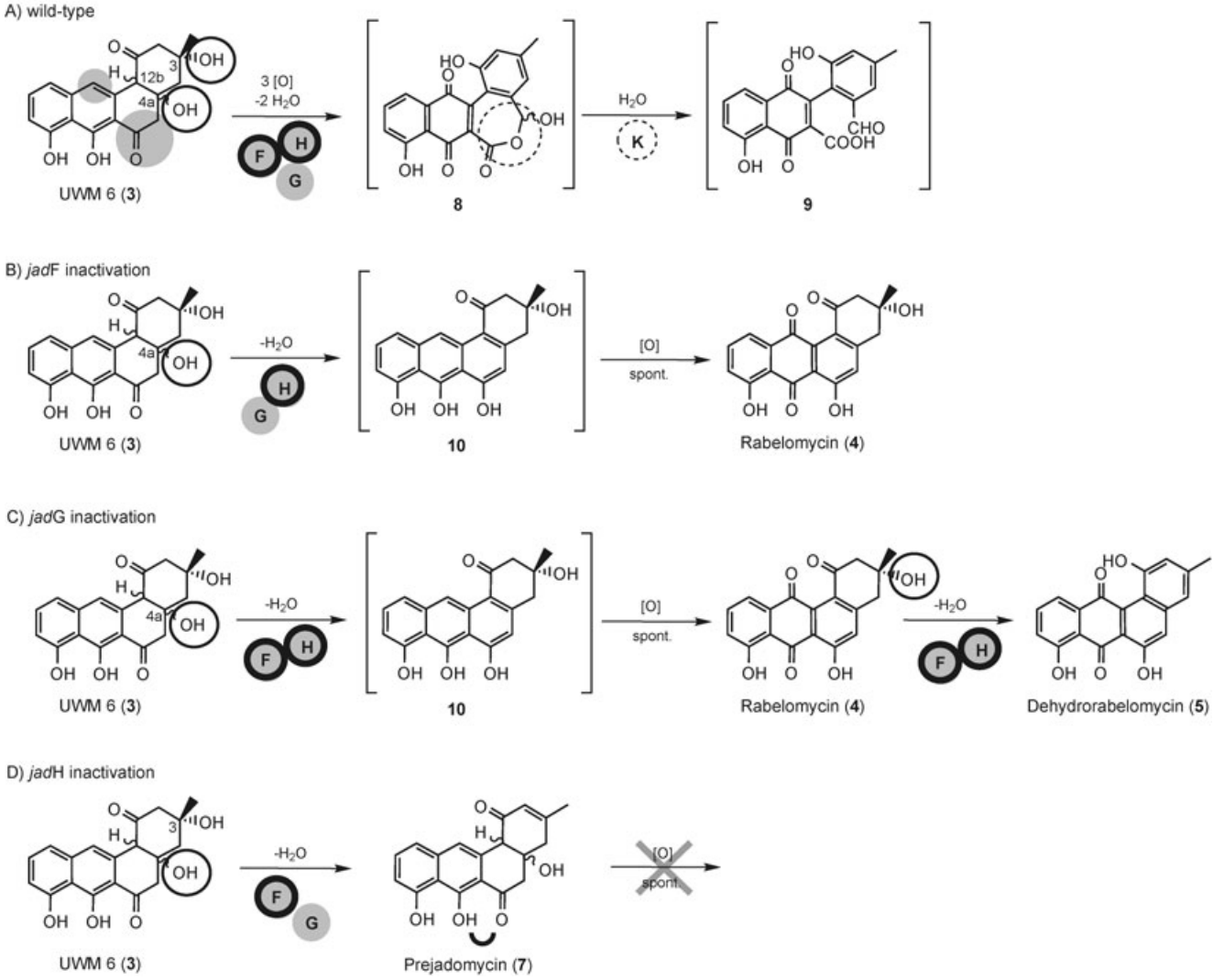
The identification of *jadH* and *jadK* closes an important gap in the jadomycin gene cluster, thus complementing also the understanding of early post-PKS tailoring steps in jadomycin B (**2**) biosynthesis. Particularly intriguing, although the significance of this still remains obscure, is the close arrangement of all three *jad* monooxygenase genes within the whole *jad* biosynthetic gene cluster. Such a subcluster of oxygenase genes is rare, and only recently has a similar set of interactive oxygenases been suggested to catalyze the key oxidative C–C-bond cleavage in gilvocarcin V biosynthesis.[11c]

In order to obtain a detailed picture of the concrete function of the enzymes, we successfully inactivated both *jadH* and *jadG* and identified the secondary-metabolite spectrum of the corresponding mutant strains (Scheme 2). The disruption of *jadF* was described previously.[7] However, all of the obtained metabolites still possessed an intact angucycline backbone, that is, the expected oxidation in ring B did not occur in any of these cases. To explain this surprising result, we first considered the possibility of having introduced polar effects through the genetic manipulation experiments we performed. Since polar effects only affect downstream genes, we were able to rule out such an effect on *jadF* as a consequence of *jadG* and *jadH* inactivation. Disruption of *jadF*, on the other hand, could theoretically cause a polar effect on *jadG* and *jadH*, but in this case we successfully restored jadomycin production in the ΔF mutant described by Yang et al.[7] by plasmid-borne overexpression of JadF[18b] thereby ruling out the possibility of an effect on any downstream genes.

In our opinion, the observed phenomenon can only be plausibly explained by assuming a functional oligomeric protein complex—mirroring here the subcluster organization on the genetic level—that includes all three enzymes JadF, G, and H, as illustrated in Scheme 3. Consequently, deletion of one of these enzyme units would stop essential protein–protein interactions within this complex and render it inactive with regard to the oxygenase activities. Similarly vital protein–protein interactions within a polyketide biosynthetic-enzyme complex have been discussed in several other cases,[19,20] particularly the oxytetracycline biosynthesis,[20b] and also were recently suggested for the oxygenation processes in gilvocarcin V biosynthesis.[11c]

In this context it is important to note that the formation of the *p*-quinone moieties of compounds **4**–**6** is *not* due to the putative anthrone oxygenase activity of JadG, but the result of spontaneous air oxidation of the proposed unstable precursor **10** (Scheme 3 B, C), or by out-of-context behavior of JadH (if present).[21] This is most apparent from the analysis of the accumulation products of the *jadG* inactivation mutant, which lacks the JadG enzyme, but can still produce *p*-quinoid compounds. Also, JadG is unable to catalyze quinone formation in the ΔH mutant strain, and we did not observe air oxidation of the accumulated anthrone prejadomycin (**7**) due to the sufficiently stabilizing effect (compared to **10**) of the intramolecular hydrogen bond between the 7-OH and 6-carbonyl groups (Scheme 3 D). Furthermore, this underlying **3** degradation pathway towards **4** has already been observed by Kulowski et al.[8a] as well as by Metsä-Ketelä et al.[8b,c]

One important observation from the *jadF* inactivation mutant is the fact that **4** is indeed the only angucycline produced, that is, not a trace of **3** is detected, as confirmed by careful reinvestigation of this mutant and analysis of the crude product by TLC and HPLC/MS. In contrast, during the spontaneous degradation process of **3** to **4**, significant amounts of **3**



Scheme 3. *Protein–protein interactions within the gene products of the jad oxygenase subcluster governing the oxygenase activities (gray circles) of JadF, JadG, and JadH. The secondary dehydratase activities (black rings) of JadF and JadH, however, are independent of these interactions. The activity of the putative hydrolase JadK is depicted with a dotted ring.*

can still be detected and isolated.[8] In the ΔF mutant strain (as well as in the ΔG mutant), however, the conversion is complete; this means that some other factor must come into play in addition. It is unlikely that this is due to different cytoplasmic pHs between *S. lividans*—the host strain used for **3** production[8]—and *S. venezuelae*, since this would have to affect the ΔH mutant in the same way and consequently cause the same metabolite spectrum.

Instead, some enzymatic influence of the remaining enzymes from the oxygenase complex has to be responsible for the different metabolite patterns observed in the three inactivation mutants. Closer scrutiny reveals that the differences between the obtained metabolites lie in—besides the formation of the quinone moiety already discussed—the presence or lack of the 3- and 4a-OH groups and, accordingly, in the degree of aromatization. Thus, it becomes apparent that the 3-OH group is only cleaved off in the presence of JadF, as shown clearly for the prejadomycin structure, whereas the 4a-OH group is only lost when JadH is present (Scheme 3). Independent evidence for the 4a,12b-dehydratase activity of JadH comes from the complete conversion of prejadomycin (**7**) to dehydrorabelomycin (**5**) by overexpressed and purified JadH protein.[18b,21] Again, the intermittent formation of **10** is accompanied by air oxidation and quinone formation. As a result, the combined secondary 3- and 4a-dehydratase activities of JadF and JadH, respectively, are responsible for the aromatization of ring A in jadomycin biosynthesis, a process that has been largely unnoticed so far. Curiously, these dehydratase activities are *not* dependent on the correct protein–protein interactions within the oxygenase complex, like the oxygenase activities that are performed by the same enzymes. Whether this phenomenon is based on the existence of a second active site on each protein (unlikely) or on an activity of the same active site that is insensitive to conformational changes brought about by complex formation, remains to be elucidated by X-ray crystallography and/or mutagenesis studies.

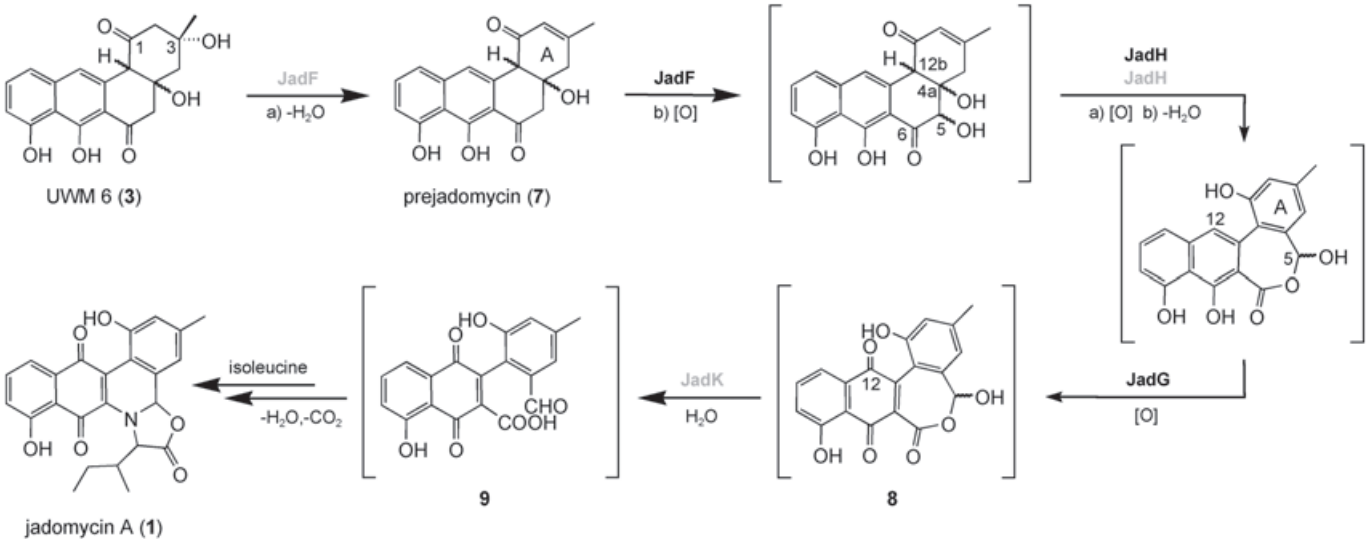
Even though we were able to elucidate the aromatization process of ring A and complement the *jad* gene cluster, the information gained by these experiments with regard to the oxygenation cascade, that is, the order, catalytic mechanisms, and substrates of the involved oxygenases, is unfortunately scarce. However, some careful assumptions can be made nonetheless. The isolation of prejadomycin (**7**) suggests that the first oxygenation step already occurs on the early "anthrone stage" versus a later "quinone stage", that is, that a UWM 6-like compound is the substrate for the first oxygenase rather than a rabelomycin-like compound. Experimental evidence for this hypothesis is also provided by the successful conversion of **7** to jadomycins in a PKS inactivation mutant compared to **4**. This indicates that **7** might be a true biosynthetic pathway intermediate whereas **4** most likely represents a shunt product.

A potential substrate structure such as **3** or **7** has a carbonyl function in the position of ring B that is to be cleaved. Therefore, a Baeyer–Villiger-type mechanism can be proposed for the C–C-bond cleavage (Scheme 4),[22] for which numerous examples exist in biology, most prominently cyclohexanone monooxygenase from baker's yeast.[23] This hypothesis is supported by further analysis of the deduced amino acid sequences of JadF and JadH. As already discussed, both enzymes display very clear sequence motifs for FAD- and NADPH-dependent monooxygenases (Figure 2). Both proteins also show a characteristic aspartic acid residue located five amino acids upstream of the $\beta\alpha\beta$-fold (Figure 2a). Intriguingly, this aspartate moiety has been previously implicated with type I (FAD- and NADPH-dependent) Baeyer–Villiger-monooxygenases (BVMOs),[24] and for example, the sequence of the proven BVMO MtmOIV from the mithramycin biosynthetic pathway in *S. argillaceus*[15] reveals the very same aspartate residue as well.

The hypothesis of a Baeyer–Villiger-type oxygenation mechanism is also consistent with the finding of the putative hydrolase gene *jadK*, whose gene product would be able to assist in the hydrolytic ring opening of the proposed lactone-like Baeyer–Villiger reaction product **8**. Hydrolysis of **8** would yield the ring-opened aldehyde **9**, which we previously hypothesized as the target molecule for the following amino acid attack.[4] A similar Baeyer–Villiger/hydrolysis mechanism as proposed in Scheme 4 might also occur in the biosynthesis of gilvocarcin V[11c] and of the gibberellin family of plant growth hormones as part of the reaction sequence catalyzed by GA 20 oxidases, which has been shown to involve lactone and aldehyde intermediates.[25]

A final interesting aspect regards the third metabolite produced by the *jadG* inactivation mutant, L-digitoxosyldehydrorabelomycin (**6**). Whereas the formation of **4** and **5** can be attributed to the dehydratase activities of JadF and JadH, **6** also contains the 2,6-dideoxysugar L-digitoxose linked to the phenolic hydroxyl group of ring D, as it is found in all jadomycins, as well. This suggests that the glycosyltransferase responsible for the sugar attachment to the jadomycin aglycon, JadS,[26] displays a remarkable flexibility towards its polyketide substrate, particularly considering the significant structural difference as a result of the missing isoleucine moiety. However, since no glycosylated product is detected in the *jadF* inactivation mutant, which provides **4**, one can deduce that JadS shows a strict requirement for not just the phenolic hydroxyl group of ring D, but also for an aromatized ring A.

Through identification of the two early post-PKS genes *jadH* and *jadK* we were able to close an important gap in the jadomycin biosynthetic gene cluster and link the PKS with later tailoring genes responsible for sugar biosynthesis and attachment. Interestingly, the three monooxygenase encoding genes *jadF*, *jadG*, and *jadH* are arranged in a unique subcluster organization. Be it coincidental or not, the inactivation experiments described above show that the respective gene products have to be arranged in an oligomeric protein complex displaying essential functional interactions as well. Accordingly, deletion of one of these enzymes causes the loss of its complex partners' oxygenase functions. This phenomenon unfortunately makes



Scheme 4. *Proposed pathway for the oxygenation cascade responsible for the cleavage of ring B during the biosynthesis of the jadomycin antibiotics (bold black indicates oxygenase activity, gray indicates dehydratase activity).*

further investigation of the specific functions of any of these three oxygenases in the bacteria strain extremely difficult, thus presenting a "biosynthetic black box" that prevents us from gaining insight into these processes, which we know govern the crucial C–C-bond cleavage and ring opening in jadomycin B (**2**) biosynthesis. However, in the course of these investigations, we unexpectedly identified the secondary 2,3- and 4a,12b-dehydratase functions of the flavoproteins JadF and JadH, respectively, which are responsible for the previously unexamined aromatization of ring A. Apparently, these additional enzyme functions are *not*, like the oxygenase activities, dependent on the correct protein complex formation, but are more rugged and insensitive to the presence of the neighboring proteins. The functional interrelationships within this protein complex, or the lack thereof, make the JadF/G/H cluster a highly interesting, albeit extremely challenging, target for structural studies based on X-ray crystallography and site-directed mutagenesis, which potentially will reveal the protein-binding interfaces, active sites, and the amino acid residues involved.

Experimental Section

Construction of jadH disruption mutant: A 6.0 kb *Bam*HI fragment from *S. venezuelae* containing *jadFGHK* was cloned in pHJL400 to obtain pJV77A. To construct a *jadH* disruption plasmid, pJV77A was digested with *Eco*RI and *Mlu*I in order to remove an *Eco*RI/*Mlu*I fragment. The remaining fragment, consisting of a 3.7 kb *Bam*HI/*Mlu*I insert, and the vector were subsequently end-filled to create blunt ends and religated to generate plasmid pJV91. Plasmid pJV91 was digested with *Nco*I (internal to *jadH*) and ligated with an apramycin resistance gene with *Nco*I ends. Two recombinant plasmids, pJV92A and pJV92B, in which the apramycin resistance gene was inserted in opposite orientations, were obtained. Both plasmids were used to transform *S. venezuelae* as described before.[7] Transformants (VS667) were obtained with pJV92B. Selection for apramycin resistance and thiostrepton sensitivity gave *jadH* disruption mutants VS668. The apramycin-resistance gene in pJV92B and VS668 is oriented in the opposite direction to that of *jadFGHK*. Therefore, it is assumed to have blocked the transcription of all downstream genes, that is, *jadKLMNX*. Another *jadH* deletional mutant that maintains normal transcription of downstream genes was also obtained, but is not discussed in this paper. Hybridization experiments with pJV92B as a probe against *S. venezuelae* and VS668 genomic DNA digested with *Xho*I confirmed the insertion of the apramycin-resistance gene in *jadH*: the wild-type DNA gave a single hybridizing band at 7.3 kb, while that of VS668 showed two hybridizing bands at about 4.2 kb. VS668 (*jadH*-disrupted mutant) was cultivated under typical jadomycin-production conditions described elsewhere.[4] The culture was harvested by mixing the mycelium with Celite and subsequent filtration in vacuo. The mycelium cake was resuspended in acetone, sonicated, and filtered again. The filtrate was concentrated in vacuo to remove organic solvent, and combined with the culture filtrate for extraction with ethyl acetate. Analysis of the crude ethyl acetate extract by TLC (chloroform/methanol 9:1, $R_f = 0.51$) and HPLC/MS (Waters Symmetry C_{18}, 4.6×50 mm; solvent A = 0.1% formic acid in H_2O; solvent B = acetonitrile; flow rate = 0.5 mL min^{-1}; 0–6 min 25% B to 100% B, 6–7 min 100% B, 7–7.5 min 100% B to 25% B, 7.5–10 min 25% B) showed one major yellow metabolite, which was purified by silica gel column chromatography (chloroform/methanol 9:1), semipreparative HPLC (Waters SymmetryPrep C_{18}, 19×150 mm; isocratic conditions: 45% acetonitrile, 55% H_2O with 0.1% TFA; flow rate = 10 mL min^{-1}), and Sephadex LH-20 (methanol) to yield 3 mg L^{-1} of prejadomycin (**7**) $C_{19}H_{16}O_5$ (M_W = 324 g mol^{-1}). ^{1}H and ^{13}C NMR: see Table 1; IR (Methanol): $\tilde{\nu}$ = 3537 (br, s), 2926 (sh, s), 2855 (sh, m), 1725 (sh, m), 1650 (sh, s), 1630 (sh, s), 1590 (br, s), 1535 (sh, m), 1457 (sh, m), 1383 (sh, m), 1328, 1226 (sh, m), 1165, 1105, 845 cm^{-1}; UV/Vis (methanol): λ_{max} (ε) = 405 (4100), 320 (1700), 306 (2700), 294 (2400), 266 (19200), 224 nm (16900); negative-mode APCI-MS: m/z = 323 (100) $[M-H]^-$; positive-mode ESI-MS: m/z = 325 (100) $[M+H]^+$; high resolution: calcd for $C_{19}H_{17}O_5$: 325.107; found: 325.106.

In-frame deletion of jadG: A 7.1 kb *Xho*I fragment (contains *jadDFGHK* and part of *jadL*) was cloned in pBluescript II KS(+) to give pJV69A. A 3015 bp fragment and a 2042 bp *Pst*I/*Apa*LI fragment from pJV69A were ligated with *Pst*I-digested pHJL400 to generate pHK400G. This was reisolated from the DNA methylase-deficient strain, *E. coli* ET12567, and transformed into *S. venezuelae* protoplasts. Transformants were propagated on MYM plate without thiostrepton for three generations. From hundreds of single colonies, six thiostrepton-sensitive clones were identified. Two (designated CH63) produced yellow compounds instead of orange jadomycin B when fermented in D-galactose-L-isoleucine medium. PCR analyses performed on pHK400G, genomic DNAs of the two CH63 strains and wild-type *S. venezuelae* with primers: PgF (5′-GACTCGACGCCGTCCT-3′) and PgR (5′-GGAGGTGACGAGGGAG-3′) clearly indicated a 1.0 kb fragment was present in samples of pHK400G and CH63s; while a sample of wild-type *S. venezuelae* DNA gave a 1.16 kb fragment. This confirmed that a 159 bp *Apa*LI fragment internal to *jadG* was deleted in the two CH63 strains.

The jadG inactivation mutant was cultivated as described above. Analysis of the crude product by HPLC/MS (see above) revealed three metabolites: rabelomycin[6] (t_R = 4.93 min), dehydrorabelomycin[6] (t_R = 8.44 min), and L-digitoxosyl-dehydrorabelomycin (**6**; t_R = 6.52 min). Purification was achieved by silica-gel (chloroform/methanol 20:1) and Sephadex LH20 (methylene chloride) column chromatography yielding 1 mg L^{-1} of L-digitoxosyl-dehydrorabelomycin (**6**) $C_{25}H_{22}O_8$ (M_W = 450 g mol^{-1}). ^{1}H and ^{13}C NMR: see Table 2; UV/Vis (acetonitrile): λ_{max} = 431, 325, 268, 234 nm; positive-mode APCI-MS: m/z = 451 (15) $[M+H]^+$, 321 (100) $[M+H-C_6H_{10}O_3]^+$.

Construction of jadA disruption mutant: Two 1.5 kb fragments flanking *jadA* were obtained by PCR with primer pairs P1 and P2. For the fragment upstream of *jadA*, P1F 5′-CCCAAGCTTGCAGTGCCTGGCCGACCA-3′ and P1R 5′-GGAATTCCATATGTCACGCGTTCGCCTCCCA-3′ were used; for the fragment downstream of *jadA*, P2F 5′-GGAATTCCATATGAGCGCGTCCGTGGTG-3′ and P2R 5′-CGGAATTCAGGCGGCGGCGACGGC-3′ were used. After digestion with appropriate enzymes, the two fragments were inserted into *Hind*III/*Eco*RI-digested pHJL400 to generate pHK400A. This was used to transform protoplasts of VS662a. Transformants were propagated on an MYM plate without thiostrepton selection for three generations, and spores from the nonselective plates were plated out and picked for sensitivity to thiostrepton. Single colonies identified were examined for loss of jadomycin production in liquid culture and deletion of *jadA* by PCR by using P3F (5′-GGCCACCCGCTTCTACAAC-3′) as the forward and P3R (5′-CGAAGGTGGAGCCGTATCC-3′) as the reverse primer.

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