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Multi-oxygenase Complexes of the Gilvocarcin and Jadomycin Biosyntheses

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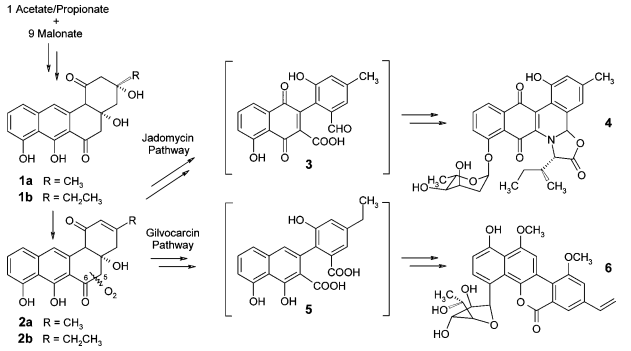
The biosyntheses of the gilvocarcins (e.g., gilvocarcin V, **6**, Scheme 1) and jadomycins (e.g., jadomycin B, **4**) are both dominated by oxidative rearrangement cascades that lead from simple polyketide-derived angucyclinones to the unique tetracyclic benzo[*d*]naphtho[1,2-*b*]pyran-6-one and pentacyclic benz[*b*]oxazolophenanthridine backbones, respectively, which are typical and essential for these anticancer antibiotics. Previous work showed that UWM6 (**1a**) and prejadomycin (2,3-dehydro-UWM6, **2a**) or their homologues **1b** and **2b** are the last isolable intermediates of these pathways and also serve as intermediates of various other angucycline group antibiotics.[1−4] The immediately following multistep oxygenation cascades, which include the key rearrangements of the jadomycin and gilvocarcin biosyntheses, remained widely obscure and were referred to as a biosynthetic black box.[2] For the jadomycin and the gilvocarcin pathways, two similar but clearly different products of the oxidative 5,6-bond cleavage of **2** were suggested, namely a phenyl-naphthoquinone decorated with an acid and an aldehyde function (**3**) in the former and a phenyl-naphtholdiacid (**5**) for the latter.[2,4] The reactive aldehyde function in **3** appeared essential for the nonenzymatic incorporation of L-isoleucine into the **4** pathway,[5−7] while an acid function in **5** seemed to be necessary for the lactone formation in the **6** pathway.[4] In this Communication, we present results of our further examination of these key reaction cascades which suggest that jadomycin and gilvocarcin biosyntheses diverge later in the respective pathways than previously assumed, and that the 5,6-bond cleavage necessitates the formation of multi-oxygenase complexes consisting of at least three and four enzymes, respectively.

From previous work and alignment studies,[2−4,8−10] we concluded that the oxygenases JadF, JadG, and JadH participate in the oxidative rearrangement of the jadomycin pathway, while their counterparts GilOIV, GilOII, and GilOI catalyze similar reactions in the gilvocarcin pathway. JadF/H and GilOIV/OI were suggested to prepare and perform the oxidative 5,6-bond cleavage, for which a Baeyer−Villiger oxidation is likely to serve as an essential key step.[11,12] JadG and GilOII showed high similarities to cofactor-free monooxygenases such as DnrG or TcmH, which catalyze anthrone oxidation reactions.[13−16] For the gilvocarcin pathway, we also considered GilR in this study, a putative oxidoreductase that has strong similarities with an FAD/FMN-dependent dehydrogenase from the steffimycin biosynthesis and an oxidoreductase from the spinosad biosynthesis.[17,18] However, the exact role of any of these enzymes remained unclear in the gilvocarcin pathway as well as in any of the other pathways that require a GilR homologue.

To compare the jadomycin with the gilvocarcin enzymes, we used our previously generated oxygenase-inactivation mutants[4,19] along with newly generated single and double mutants and carried out various gene complementation experiments (for the complete set of experiments, see Table 1 in the Supporting Information). These studies revealed that JadH and JadF were interchangeable with GilOI and GilOIV, respectively. For instance, the *gilOI*-minus

Scheme 1. Key Oxidative Cleavage Steps of the Jadomycin and Gilvocarcin Biosyntheses, with the Previously Suggested Different Hypothetical Intermediates **3** and **5** Resulting from the Oxidative 5,6 Bond Cleavage



mutant *Streptomyces lividans* (cosG9B3-OI$^-$) could be complemented with JadH (but not with JadF or JadG) to reconstitute **6** biosynthesis, and gilvocarcin biosynthesis could only be restored in the *gilOIV*-minus mutant *S. lividans* (cosG9B3-OIV$^-$) through complementation with *jadF*. However, *jadG* was not able to replace *gilOII*, pointing out somewhat different roles of these closely related enzymes in both pathways.

The newly generated *gilOII*- and *gilR*-minus mutant strains accumulated intriguing metabolites, which were isolated and their structures investigated with NMR and mass spectrometry (for physicochemical data of the new compounds and their derivatives, see Supporting Information). The *gilOII*-minus mutant *S. lividans* (cosG9B3-OII$^-$) accumulated vinyl dehydrorabelomycin (**8a**, Scheme 2) as its major metabolite, an angucyclinone shunt product, which proves that GilOII partakes as an essential component of the C−C bond cleavage process within the oxidative rearrangement cascade of the gilvocarcin pathway. This was not unexpected, since the *jadG* inactivation had also yielded angucycline and angucyclinone shunt products, suggesting a similar important role in the oxidative rearrangement of the jadomycin pathway.[2] However, the vinyl group in molecule **8a** reveals that the GilOIII reaction, which establishes the vinyl group, occurs much earlier in the pathway than expected from the accumulation of gilvocarcin E (**11**) in the *gilOIII*-minus mutant. We now assume that the GilOIII-catalyzed reaction occurs as soon as the aromatization of the angucyclic ring A is complete or shortly afterward (see Scheme 2). The *gilR*-minus mutant *S. lividans* (cosG9B3-R$^-$) accumulated pregilvocarcin V (**15**) as its major metabolite. Compound **15** appeared as a mixture of two diastereomeric compounds. Isolating each one of these from and re-injecting it into the HPLC showed the same mixture of two compounds in the same ratio, indicating that these two compounds were in equilibrium. Once a hemiacetal was suspected from the NMR data of the mixture, stirring in acidic methanol yielded the corresponding methylacetals, which were separated and characterized by NMR (Supporting Information), leading to the unambiguous

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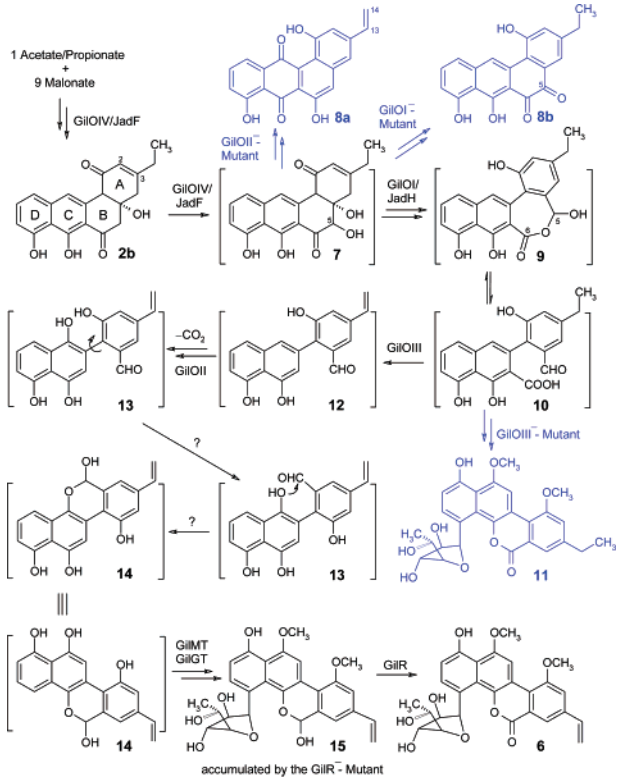
Scheme 2. Revised Gilvocarcin Biosynthetic Pathway (Shunt Products in Blue)[a]



[a] Oxygenases GilOIV and GilOI can be substituted by the jadomycin pathway using enzymes JadF and JadH, respectively. GilOIV/GilOI/GilOII and a fourth, so far unidentified enzyme (catalyzing steps marked with a "?") form a multienzyme complex. The aldehyde function of the suggested C−C splitting product **10** is indirectly "visible" in the hemicacetal function found in intermediate **15**.

elucidation of structure **15**. The accumulation of hemiacetal **15** as a result of inactivating *gilR* shows that GilR acts as a dehydrogenase oxidizing hemicacetal **15** to the corresponding lactone, gilvocarcin V (**6**, Scheme 2). In addition, GilR might also directly or indirectly regenerate the cofactor-free GilOII, while the regeneration of the cofactor-free JadG in the jadomycin pathway (and its analogues of the daunorubicin and tetracenomycin pathways) is achieved through establishing their final quinone moiety (see **3** and **4**) from a hydroquinone intermediate. Crossfeeding experiments using the *gilOIV*-minus mutant[19] showed **15** to be clearly an intermediate of the gilvocarcin pathway, while **8a** is not. This suggests that the GilR reaction is the final step in gilvocarcin biosynthesis, following even the tailoring O-methylation and C-glycosylation reactions. The result is in good agreement with previous crossfeeding studies that showed that the "aglycon" defucogilvocarcin V is not an intermediate of the gilvocarcin pathway.[19]

Finally, we complemented a minimal gene set, establishing the formation of intermediate UWM6 (**1a**), accumulated on plasmid pWHM1238.[1] In these experiments, *S. lividans* TK24 was transformed with pWHM1238 as well as with various other plasmids containing oxygenase genes from either the jadomycin or the gilvocarcin pathways (see Table 1, Supporting Information). The experiments revealed that the multi-oxygenase complexes Jad-F-G-H (on plasmid pJadFGH) or Gil-OI-OII-OIV (on pGilOIOIIOIV) could complement the UWM6 genes to reconstitute jadomycin A (the aglycon of **4**) production when isoleucine was added to the culture medium. However, these multi-oxygenase complexes could not reconstitute the typical backbone of the gilvocarcins, which shows that there is an enzyme missing that is most likely an essential component of the multi-oxygenase complex of the gilvocarcin pathway. This enzyme is probably responsible for both the rotation of the phenyl residue in **13** and the immediately following formation of hemiacetal **14** (Scheme 2). Because the C−C bond cleavage occurs in the absence of either GilOIII (→gilvocarcin E **11**) or GilR (→pregilvocarcin V **15**), we have to exclude these two enzymes from partaking in the multi-oxygenase complex responsible for the 5,6 bond cleavage. However, the gilvocarcin cluster offers four candidate genes encoding enzymes with so far unknown function (*gilN*, *gilL*, *gilV*, and *gilM*), which we will explore in the near future.

In summary, our studies shed some light on the enigmatic oxidative processes in jadomycin and gilvocarcin biosyntheses. The studies revealed that the oxidative rearrangement cascades in both pathways are catalyzed by multi-oxygenase complexes and follow essentially identical paths up to the C−C bond cleavage product **10**, thus diverging much later than previously appreciated. Furthermore, GilR was identified as hemiacetal dehydrogenase, an enzyme catalyzing a lactone formation in a novel way.

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Supporting Information Available: Listing and experimental details of all gene complementation experiments, isolation and physicochemical properties of compounds **8a** and **15**, derivatization of **15**, and crossfeeding experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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