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Baeyer–Villiger C–C Bond Cleavage Reaction in Gilvocarcin and Jadomycin Biosynthesis

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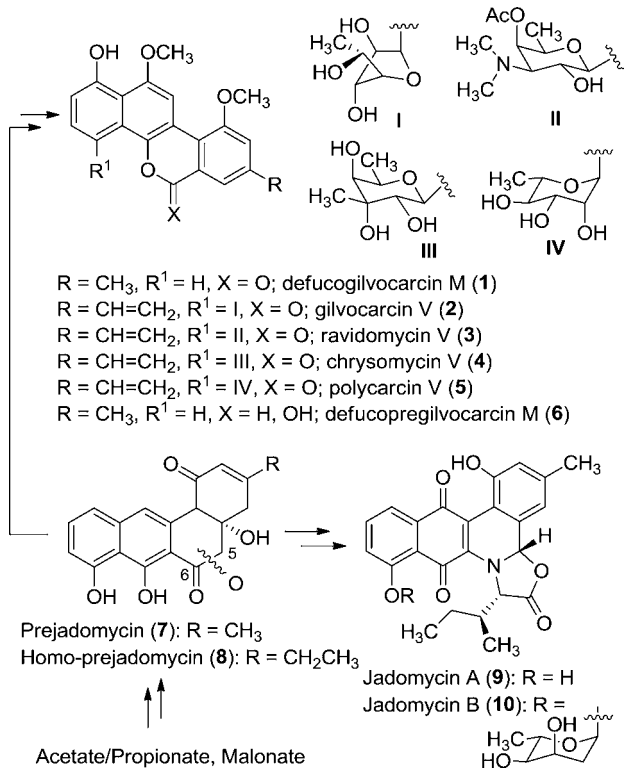
Supporting Information

ABSTRACT: GilOII has been unambiguously identified as the key enzyme performing the crucial C–C bond cleavage reaction responsible for the unique rearrangement of a benz[*a*]anthracene skeleton to the benzo[*d*]-naphthopyranone backbone typical of the gilvocarcin-type natural anticancer antibiotics. Further investigations of this enzyme led to the isolation of a hydroxyoxepinone intermediate, leading to important conclusions regarding the cleavage mechanism.

C–C bond cleavages initiate some of the most significant structural rearrangements in the biosynthesis of many natural products, through which numerous unique scaffolds are generated. Most of these cleavage reactions are also crucial for the biological activity of the natural products (e.g., mithramycin, aflatoxin). Thus, great attention has been devoted to identifying the relevant enzymes and cleavage mechanisms. One of the important examples is found during the biosynthesis of gilvocarcins. The gilvocarcins (e.g., **1**–**5**) are a group of natural anticancer agents produced by various *Streptomyces* species that are composed of a benzo[*d*]naphtho[1,2-*b*]pyran-6-one backbone decorated with a C-glycosidically linked sugar moiety. This group of natural products is well-known for their strong antitumor activities and unique mode of action.[1−5] Biosynthetically, the polyketide-derived backbone of the gilvocarcins is produced from acetate, propionate, and malonate subunits by the action of a type-II polyketide synthase (PKS). It has been proven that the early biosynthetic steps generate an angucyclinone intermediate [e.g., prejadomycin (**7**)/homoprejadomycin (**8**), dehydrorabelomycin **11**; Schemes 1 and 2) that subsequently undergoes a complex structural rearrangement via an oxidative C5–C6 bond cleavage to form the benzonaphthopyranone skeleton of the gilvocarcins.[1,6−8] Another intriguing group of natural products, the jadomycins (e.g., **9** and **10**), are believed to share the initial biosynthetic pathway, including the oxidative rearrangement reaction.[9] To date, however, it has not been unambiguously proven at which exact step and by which mechanism these oxidative rearrangements take place. In this work, we unambiguously confirmed that GilOII (JadG for the jadomycin pathway) is the sole enzyme responsible for this oxidative C–C bond cleavage, and on the basis of the structure of an isolated pivotal intermediate, we were also able to propose a mechanism for this reaction.

Scheme 1. Representative Members of the Gilvocarcin and Jadomycin Groups of Natural Products



We recently reported the enzymatic total synthesis of defucogilvocarcin M (**1**), in which it was confirmed that only four enzymes, namely, oxygenase GilOII, methyltransferase GilMT, methyltransferase/reductase GilM, and oxidoreductase GilR, are needed to convert the angucyclinone intermediate **11** to **1**.[7]

With the function of GilR known,[10] the C–C bond cleavage was initially attributed to the remaining enzymes GilOII,

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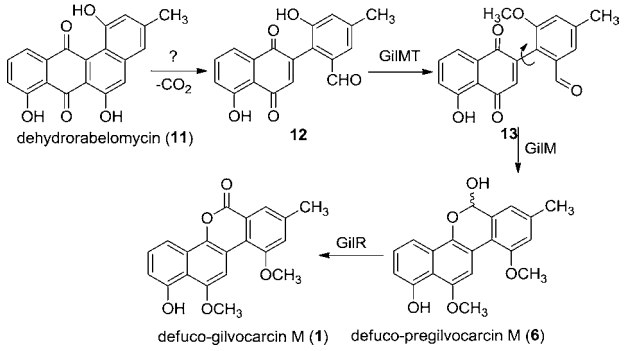
Scheme 2. Sequence of Events en Route to Defucogilvocarcin M (1)



GilMT, and GilM alone or in combination. However, a very recent development also revealed the functions and substrates of GilMT and GilM.[8] GilMT, an *S*-adenosylmethionine-dependent *O*-methyltransferase, works on intermediate **12**, which is plausibly derived from oxidative cleavage of **11**. A subsequent sequence of reactions catalyzed by GilM then generates the tetracyclic hemiacetal core, defucopregilvocarcin M (**6**), prompting us to impute the C−C bond cleavage reaction to GilOII. However, it remained unclear whether GilOII can catalyze the crucial C−C bond cleavage reaction alone or whether some support by one or more of the downstream enzymes is needed. Thus, we interrogated reactions of **11** with individual enzymes and a cocktail of downstream enzymes. GilOII, GilMT, GilM, and GilR were expressed in *Escherichia coli* and purified as His_6-tagged proteins following a procedure described earlier.[7] Substrate **11** was prepared enzymatically from prejadomycin (**14**), a proven intermediate of gilvocarcin M as well as the jadomycin biosynthetic pathway, which was isolated from a Δ*gilOI* mutant strain *Streptomyces lividans* TK24 (cosG9B3−OI$^-$) following the reported procedure (see the Supporting Information).[9]

As anticipated, the bifunctional enzyme GilM alone did not react with **11**. GilMT alone reacted with **11** unexpectedly, yielding three different compounds (**15**−**17**); the major product was dimethyldehydrorabelomycin (**15**), and the other two minor products were identified as monomethylated dehydrorabelomycins **16** and **17** (Figure 1, trace D; Scheme 3). The low yields confirmed our prior findings that GilMT normally works after the C−C bond cleavage on aldehyde intermediate **12**.[8] Neither **15**, **16**, nor **17** was converted to **1** when treated with a mixture of GilOII, GilM, GilMT, and GilR, proving that the C−C bond cleavage reaction requires a

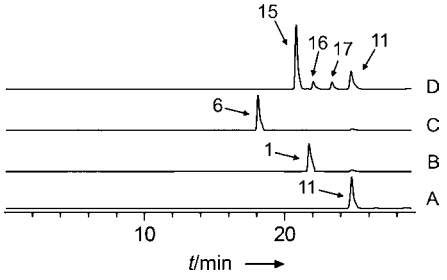
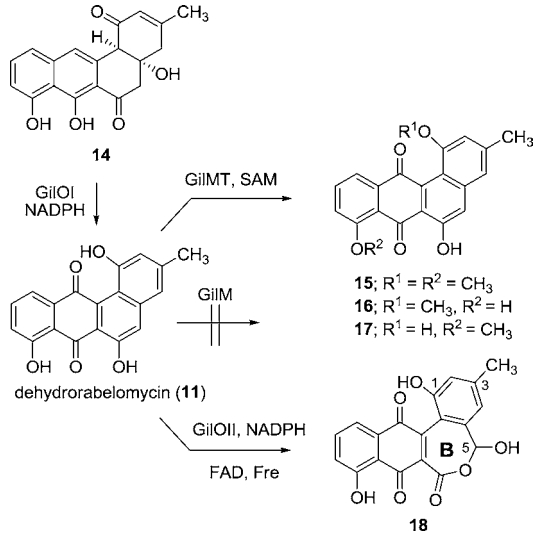


Figure 1. HPLC traces of the enzymatic reactions: (A) standard dehydrorabelomycin (**11**); (B) **11** + GilOII + GilMT + GilM + GilR, producing defucogilvocarcin M (**1**); (C) **11** + GilOII + GilM + GilMT, producing defucopregilvocarcin M (**6**); (D) **11** + GilMT.

Scheme 3. Enzymatic Reactions of 11 with GilMT and GilOII (Fre = *E. coli* Flavin Reductase)



nonmethylated substrate, while **11** with a cocktail of GilOII, GilMT, and GilM did accumulate **6** (Figure 1, trace C).

The reaction of **11** with GilOII alone resulted in consumption of all the starting material, but unexpectedly, no product was seen. After careful screening of different cofactors (FMN, FAD, NADH, NADPH) and cofactor regeneration enzymes, we found the correct conditions by incubating **11** with GiOII, FAD, and NADPH and adding *E. coli* flavin reductase (Fre), which is known to regenerate $FADH_2$ from FAD using NADPH, and an NADPH-regeneration system containing glucose-6-phosphate and glucose-6-phosphate dehydrogenase that maintained a constant supply of NADPH in the reaction.

Under these conditions, a new peak (**18**) was observable at a wavelength of 420 nm (Figure 2, trace A). After the reaction was run several times, ca. 90 μg of **18** was isolated for NMR characterization. The ^{1}H NMR spectrum of the new compound showed a considerable upfield shift of H5, which also showed a

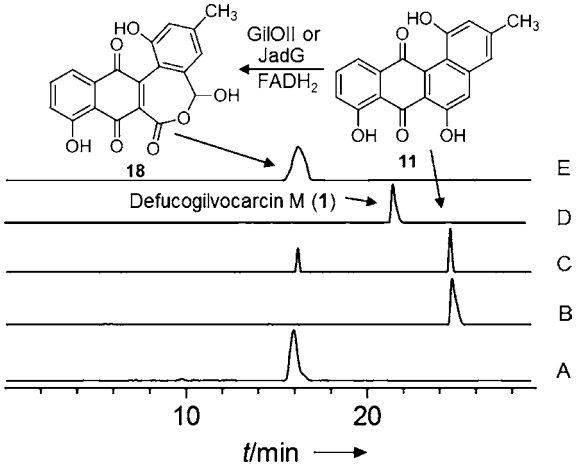


Figure 2. HPLC traces of the enzymatic reactions: (A) **11** + GilOII + NADPH + FAD + Fre; (B) **11** + GilOII; (C) **11** + GilOII + NADPH + Fre (traces of **18** were formed as a result of traces of FAD copurified with Fre); (D) **11** + JadG + GilMT + GilM + GilR, producing **1**; (E) **11** + JadG + NADPH + FAD + Fre.

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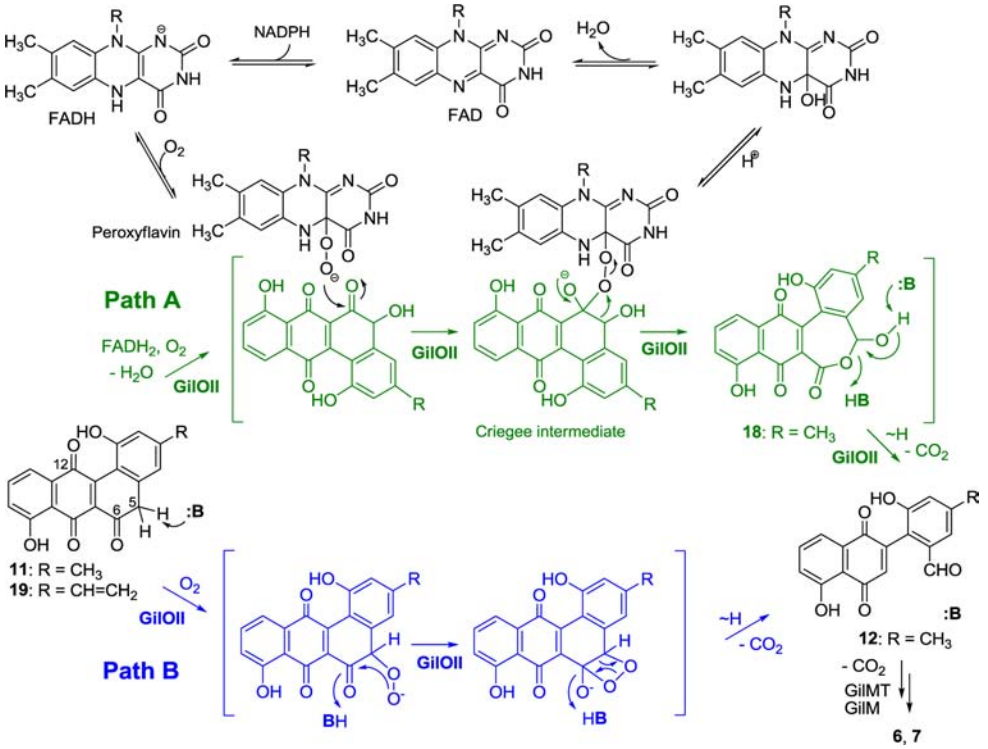
Scheme 4. Mechanistic Alternatives for the Key C−C Bond Cleavage Reaction



coupling with a new proton signal at δ 3.56. The latter was exchangeable with D_2O. The complete spectral characterization along with high-resolution mass spectrometry revealed **18** to possess a tetracyclic core with a unique hydroxyoxepinone ring B (Scheme 3). The production of **18** clearly proved that GilOII is solely responsible for the key C−C bond cleavage. In the absence of FAD, NADPH, or Fre, the assay failed to consume any starting material or to yield any product (Figure 2, trace B).

This proved that cofactor $FADH_2$ (here produced in situ from FAD and NADPH by Fre) is absolutely necessary, although the BLAST analysis showed that GilOII has no recognizable FAD-binding site. In fact, the enzyme resembles mostly cofactor-free anthrone oxygenases, and we considered that GilOII might act by a mechanism similar to that recently proposed for the cofactor-independent dioxygenase DpgC,[11] which is involved in the biosynthesis of the dihydroxyphenylglyoxylate building block of glycoprotein antibiotics, as a possible alternative to the earlier-proposed Baeyer−Villiger (BV) oxidation mechanism [Scheme 4, path B (in blue) vs path A (in green)]. The above-described GilOII reaction and the isolated compound **18** were also critical for solving this mechanistic ambiguity. The stepwise mechanism, 5-hydroxylation followed by BV oxidation (Scheme 4, path A) was corroborated, while the dioxygenase mechanism involving a dioxetane intermediate (Scheme 4, path B) could be refuted. The observation that the experiment without FAD also produced a small amount of **18** (Figure 2, trace C) was tracked to small quantities of FAD that were copurified with Fre. The insufficient supply of FAD in the experiment monitored by trace C (Figure 2) also explained well the incomplete conversion of the starting material **11** to **18**.

Next, we wanted to verify that compound **18** is a true intermediate of the pathway and not a shunt product. We monitored conversion of **18** to **1** when it was incubated with a mixture of GilM, GilMT, and GilR. However, any combination of these three enzymes and suitable cofactors failed to convert **18** to **1**. Only upon addition of GilOII was **18** converted to **1** (Figure 3). This clearly proves that oxepinone **18** is a pathway intermediate of the gilvocarcin biosynthesis. When **18** was incubated with GilOII alone, it was completely consumed, but no product accumulated. This could be attributed to the unstable nature of the expected aldehyde (**12** or its carboxylated analogue). Overall, GilOII not only mediates C5

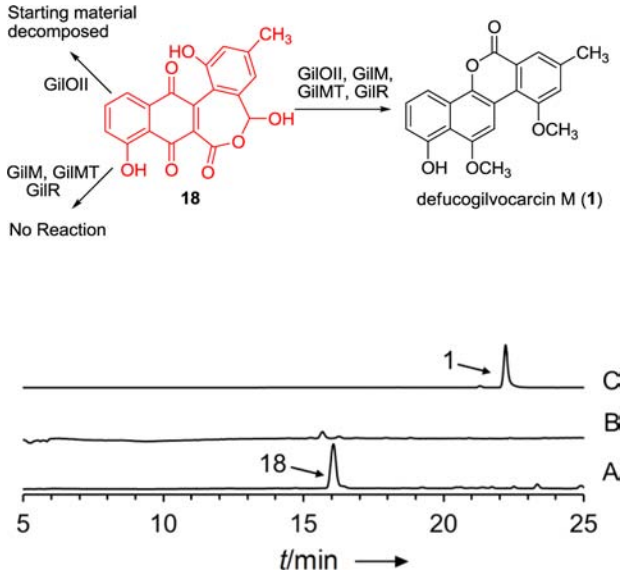


Figure 3. (top) In vitro reactions of **18** with different combinations of downstream enzymes; GilOII, GilM, GilMT and GilR. (bottom) HPLC traces of the enzymatic reactions: (A) **18** + GilMT + GilM + GilR + Fre + FAD + NADPH; (B) **18** + GilOII; (C) **18** + GilOII + GilMT + GilM + GilR + Fre + FAD + NADPH.

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hydroxylation and the following BV oxidation but is also critical for ring opening.

Finally, we wanted to investigate whether the closely related jadomycin biosynthesis also follows the same pathway regarding the C−C bond cleavage. It had recently been reported that **11** is also a biosynthetic intermediate for jadomycin A.[12,13] Among the three oxygenases reported in the biosynthetic pathway of jadomycin, JadG (also a seemingly cofactor-free anthrone oxygenase) showed the highest similarity with GilOII (51.5%). Thus, JadG was expressed in *E. coli*, purified to near-homogeneity, and incubated with a mixture of GilM, GilMT, GilR, and substrate **11**. All of the substrate was converted into **1** (Figure 2, trace D), confirming our hypothesis. The results here demonstrate the functional equivalence of GilOII and JadG, although earlier cross-complementation experiments showed that swapping of *gilOII* with *jadG* in the *gilOII*-deleted mutant failed to restore gilvocarcin production.[9] Adding JadG to a mixture of NADPH, FAD, Fre, and **11** successfully produced hydroxyoxepinone **18** (Figure 2, trace E). Thus, the jadomycin biosynthesis shares the same key C−C bond cleavage reaction in its biosynthetic pathway as found for the gilvocarcin pathway.

In conclusion, we have shown that two oxygenases previously believed to be cofactor-free, GilOII and JadG, are indeed $FADH_2$-dependent and are responsible for the critical C5−C6 bond cleavage of the benz[*a*]anthracene skeleton of angucyclinone intermediate **11** and the subsequent rearrangements during the biosyntheses of gilvocarcin and jadomycin, respectively. These enzymes are not cofactor-independent, despite misleading BLAST search results indicating that these enzymes mostly resemble cofactor-free anthrone oxygenases such as TcmH (43% amino acid identity with GilOII)[14] and AknX (34% amino acid identity with GilOII).[15] Anthrone oxygenases require a second oxidation (dehydrogenation) of the immediate hydroquinone to the corresponding quinone to produce the necessary two H atoms for the formation of the second product, a molecule of H_2O. Although GilOII and JadG catalyze a similar first reaction, namely, formation of an "orthohydroquinone", this is not further oxidized to an orthoquinone and requires $FADH_2$ instead for the H_2O formation. Since the product FAD has to leave the active site to be rereduced to $FADH_2$ (in our experiment by Fre and in the *gil* pathway presumably by GilH), the cofactor has to move freely and cannot have a tight binding site. The reaction is sequential and requires 2 equiv of $FADH_2$ since the enzymes catalyze both an initial 5-hydroxylation and the following BV oxidation, which initiates the scaffold rearrangements in these pathways. The work described here closes the gaps of the gilvocarcin and jadomycin biosyntheses, since oxepinone **18** opens under decarboxylation into aldehyde **12**, which has been synthesized and proven to be an intermediate of both the gilvocarcin[8] and jadomycin[16] pathways. BV monooxygenases[17] have also been suggested or proven to play key roles in the biosynthesis of other natural products, such as the pentalenolactones,[18] the aureolic acids,[19] BE7585A,[20] and the aflatoxins,[21] and in addition play major roles in degradation processes such as the recently deciphered toxoflavin degradation.[22]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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