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Baeyer-Villiger C-C bond cleavage reaction in gilvocarcin and jadomycin biosynthesis

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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 for the gilvocarcin type natural anticancer antibiotics. Further investigations of this enzyme led to the isolation of a hydroxy-oxepinone intermediate which allowed important conclusions regarding the cleavage mechanism.

Keywords

C-C bond cleavage enzyme; Gilvocarcin; Jadomycin; biosynthesis; combinatorial biosynthetic enzymology

C-C bond cleavages initiate one 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 also (e.g., mithramycin, aflatoxin). Thus, great attention has been attracted to identify 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/homo-prejadomycin **7**/**8**, dehydrorabelomycin **11**; Schemes 1,2) which then 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

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

jadomycins (e.g., **9** and **10**), are believed to share the initial biosynthetic pathway including the oxidative rearrangement reaction.[9] However, so far it has not been unambiguously proven at which exact step and by which mechanism these oxidative rearrangements take place. Herein, we unambiguously confirmed that GilOII (JadG for the jadomycin pathway) is the sole enzyme responsible for this oxidative C-C bond cleavage, and –based on the structure of an isolated pivotal intermediate– we were also able to propose a mechanism for this reaction.

We have 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 were required to convert the angucyclinone intermediate dehydrorabelomycin (**11**) to **1**.[7]

With the function of GilR known,[10] the C-C bond cleavage was initially attributed to the rest of the enzymes, GilOII, GilMT and GilM alone or in combination. However, a much recent development has also revealed the functions and substrates of GilMT and GilM.[8] GilMT, an S-adenosylmethionine dependent *O*-methyltransferase, works on the intermediate **12**, plausibly derived from the oxidative cleavage of dehydrorabelomycin (**11**). A subsequent sequence of reactions catalyzed by the S-adenosylmethionine dependent reductive *O*-methyltansferase, 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 were needed. Thus, we interrogated reactions of dehydrorabelomycin (**11**) with individual enzymes and a cocktail of downstream enzymes. GilOII, GilMT, GilM and GilR were expressed in *E. 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 the gilvocarcin M as well as the jadomycin biosynthetic pathway, which was isolated from a Δ*gilOI* mutant strain *S. lividans* TK24 (cosG9B3–OI$^-$) following the reported procedure (see supporting information).[9]

As anticipated, the bi-functional enzyme GilM alone did not react with **11**. GilMT alone reacted with **11** unexpectedly, yielding three different compounds (**15** – **17**), the major product being dimethyl dehydrorabelomycin (**15**), and the other two minor products were identified as monomethylated dehydrorabelomycins **16** and **17** (Figure 1, trace D, Scheme 3). The yields confirmed our prior findings that GilMT normally works after the C-C bond cleavage on the aldehyde intermediate **12**.[8] Neither **15** nor **16** or **17** were converted to defucogilvocarcin M (**1**) when treated with a mixture of GilOII, GilM, GilMT and GilR, proving that the C-C-bond cleavage reaction requires an non-methylated substrate, while dehydrorabelomycin (**11**) with a cocktail of GilOII, GilMT and GilM did accumulate defuco-pregilvocarcin M (Figure 1, trace C).

The reaction of dehydrorabelomycin (**11**) with GilOII alone resulted in consumption of all the starting material but unexpectedly no product was seen. After careful screening of different co-factors (FMN, FAD, NADH, NADPH) and co-factor regeneration enzymes, we found the correct conditions by incubating **11** with GiOII, FAD and NADPH and adding *E. coli* flavin reductase Fre (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 running the reaction several times, circa 90 μg of **18** were isolated for NMR characterization. The ^{1}H NMR spectrum of the new compound showed a considerable

upfield shift of H-5, which also showed a coupling with a new proton signal at δ 3.56. The latter was exchangeable with D_2O. The complete spectral characterization along with HRMS revealed the compound to possess a tetracyclic core with a unique hydroxy-oxepinone ring B (**18**, Scheme 3).

The production of **18** clearly proved that GilOII was solely responsible for the key C-C bond cleavage. In the absence of FAD, NADPH or flavin reductase Fre, the assay failed to consume any starting material or to yield any product (Figure 2, trace B).

This proved that co-factor $FADH_2$ is absolutely necessary, here in-situ produced from FAD and NADPH by Fre, although the BLAST analysis showed that GilOII has no recognizable FAD-binding site. In fact, the enzyme resembled mostly co-factor free anthrone oxygenases, and we considered that GilOII might act similarly as recently proposed for the co-factor independent dioxygenase DpgC[11] involved in the biosynthesis of the dihydroxyphenylglyoxylate building block of glycoprotein antibiotics, as a possible alternative to the earlier proposed Baeyer-Villiger oxidation mechanism (Scheme 4, path B blue versus path A, 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 Baeyer-Villiger oxidation (Scheme 4, path A, green) was corroborated, while the dioxygenase mechanism involving a dioxetane intermediate (Scheme 4, path B, blue) could be refuted. The observation that the experiment without FAD also produced a small amount of the product **18** (Figure 2, trace C) was tracked to small quantities of FAD that were co-purified with Fre. The insufficient supply of FAD in the experiment monitored by trace C (Figure 2) also explained well the incomplete conversion of 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 defucogilvocarcin M (**1**) when it was incubated with a mixture of GilM, GilMT, and GilR. However, any combination of these three enzymes and suitable co-factors failed to convert **18** to **1**. Only when GilOII was added, **18** was converted to **1** (Figure 3). This clearly proves oxepinone **18** to be a pathway intermediate of the gilvocarcin biosynthesis. When compound **18** was incubated with GilOII alone, it was completely consumed and did not accumulate any product. This could be attributed to the unstable nature of the expected aldehyde (**12** or its carboxylated analogue). Overall, GilOII not only mediates C-5 hydroxylation and the following Baeyer-Villiger oxidation, but is also critical for ring opening.

Finally we wanted to investigate whether the closely related jadomycin biosynthesis also followed the same pathway regarding the C-C bond cleavage. It had recently been reported that dehydrorabelomycin (**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 co-factor 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 the substrate dehydrorabelomycin (**11**). Confirming our hypothesis all the substrate was converted into defucogilvocarcin M (**1**) (Figure 2, trace D). The results here demonstrated 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 dehydrorabelomycin (**11**) successfully produced hydroxyoxepinone **18** (Figure 2, trace E). Thus, the jadomycin biosynthesis shares the same C-C bond key cleavage reaction in its biosynthetic pathway as found for the gilvocarcin pathway.

In conclusion, we have shown that two previously believed co-factor free oxygenases, GilOII and JadG, are indeed $FADH_2$-depedent and are responsible for the critical C5-C6 bond cleavage of the benz[*a*]-anthracene skeleton of an-gucyclinone intermediate **11** and the subsequent rearrangements during the biosyntheses of gilvocarcin and jadomycin, respectively. These enzymes are not co-factor independent, despite misleading BLAST search results, in which these enzymes mostly resemble co-factor free anthrone oxygenases, such as TcmH (43% aa-identity with GilOII)[14] or AknX (34% aa-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/JadG catalyze a similar first reaction, namely a 'ortho-hydroquinone' formation, this is not further oxidized to an ortho-quinone, and requires $FADH_2$ instead for the H_2O-formation. Since the product FAD has to leave the active site, to be re-reduced to $FADH_2$ (in our experiment by Fre, in the *gil*-pathway presumably through GilH), the co-factor has to move freely and cannot have a tight binding site. The reaction is sequential requiring two $FADH_2$-equivalents, since the enzymes catalyze both, an initial 5- hydroxylation and the following Baeyer-Villiger 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 had been synthesized and proven to be an intermediate of both the gilvocarcin[8] and jadomycin[16] pathways. Baeyer-Villiger monooxygenases[17] were also suggested or proven to play key roles in the biosynthesis of other natural products, e.g., the pentalenolactones,[18] the aureolic acids,[19] BE7585A,[20] the aflatoxins,[21] and in addition play major roles in degradation processes, like the recently deciphered toxoflavin degradation. [22]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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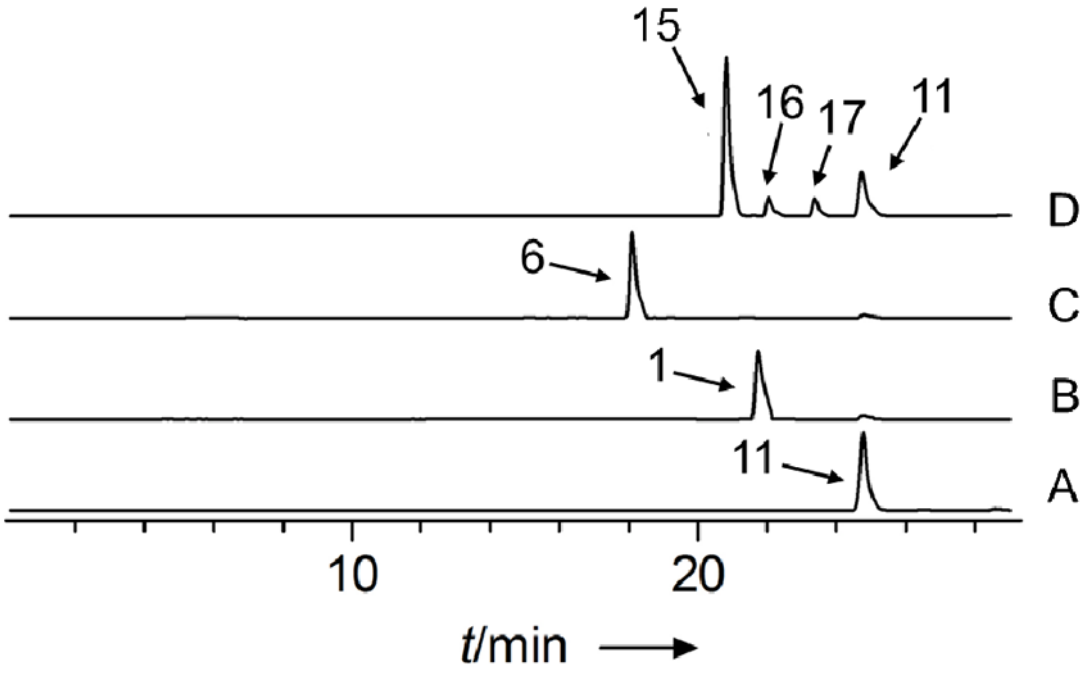


Figure 1.
HPLC traces of the enzymatic reactions: A) standard dehydrorabelomycin (**11**), B) **11** + GilOII + GilMT + GilM + GilR producing defucogilvocarcin M (**1**), C) **11** + GilOII + GilM + GilMT producing defucopregilvocarcin M (**6**), D) **11** + GilMT.

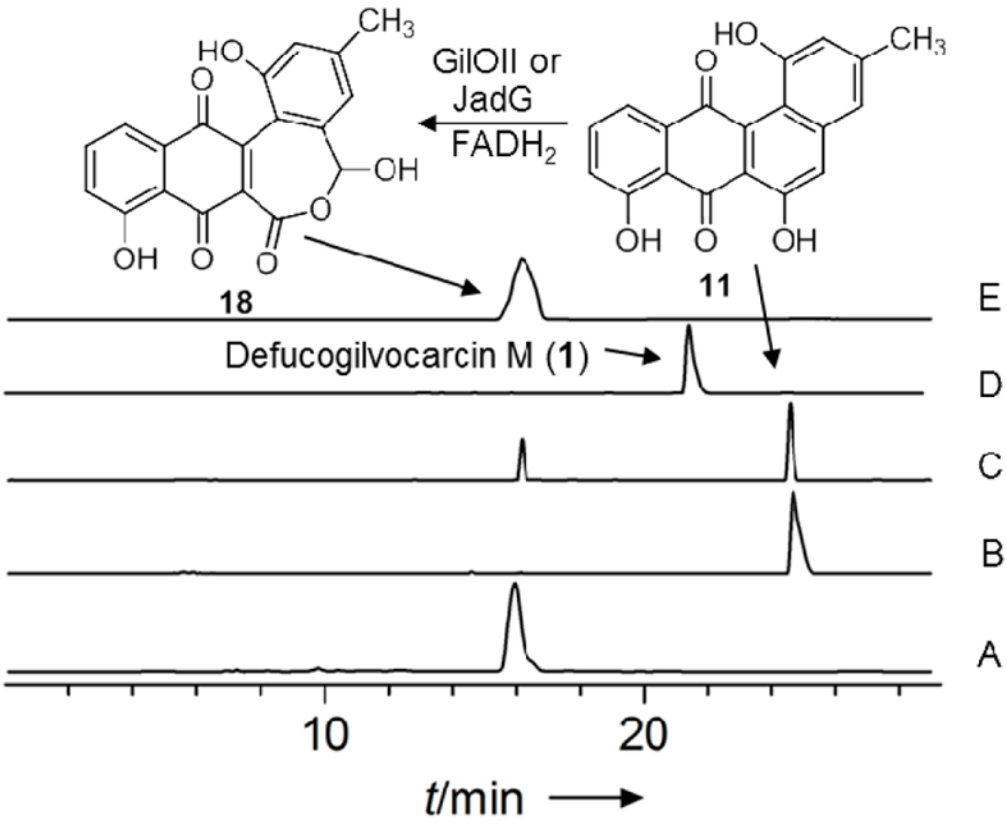


Figure 2.
HPLC traces of the enzymatic reactions: A) dehydrorabelomycin (**11**) + GilOII + NADPH + FAD + Fre, B) **11** + GilOII C) **11** + GilOII + NADPH + Fre (traces of **18** were formed due to traces of FAD co-purified with Fre), D) **11** + JadG + GilMT + GilM + GilR producing defucogilvocarcin M (**1**), E) **11** + JadG + NADPH + FAD + Fre.

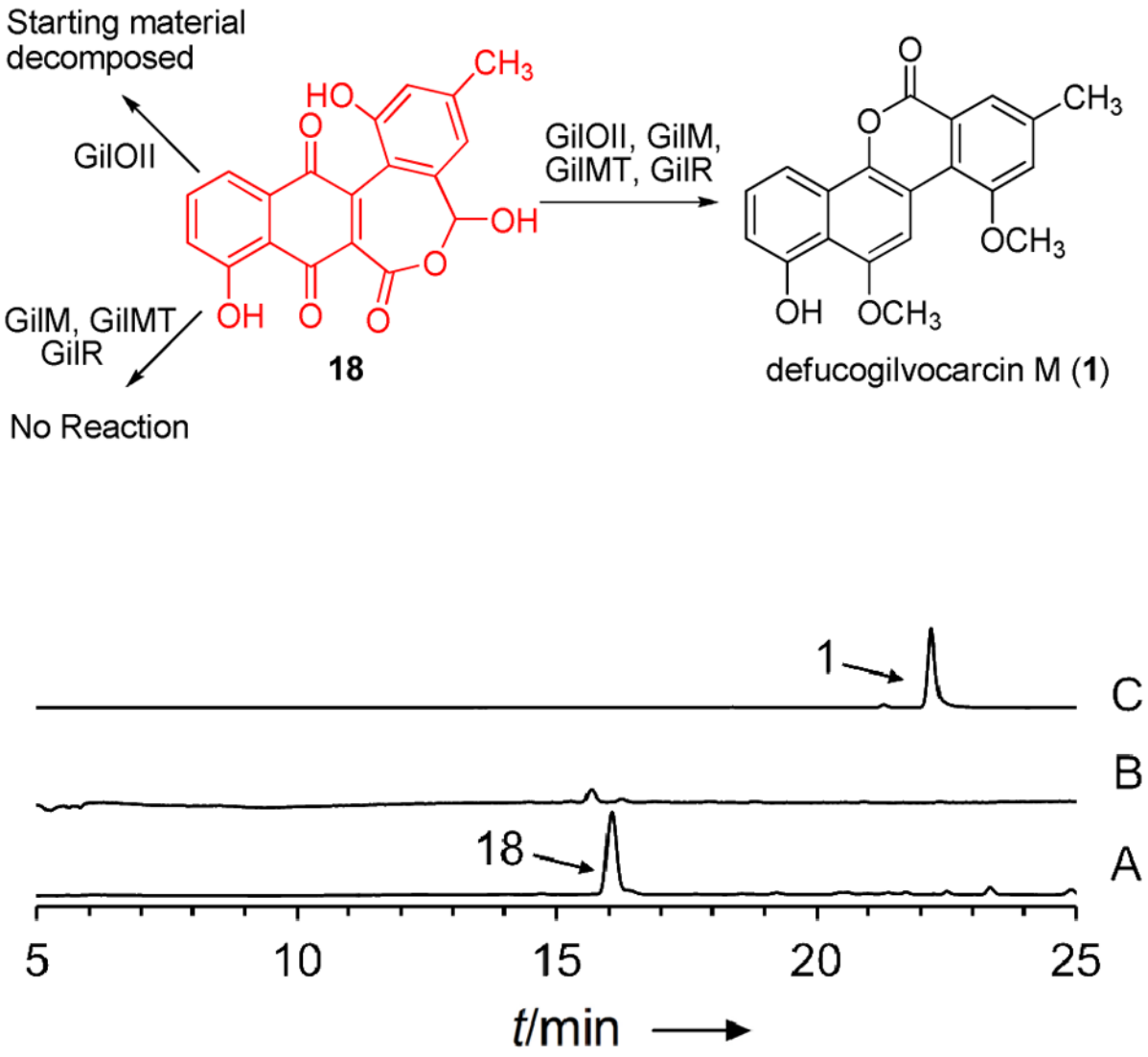
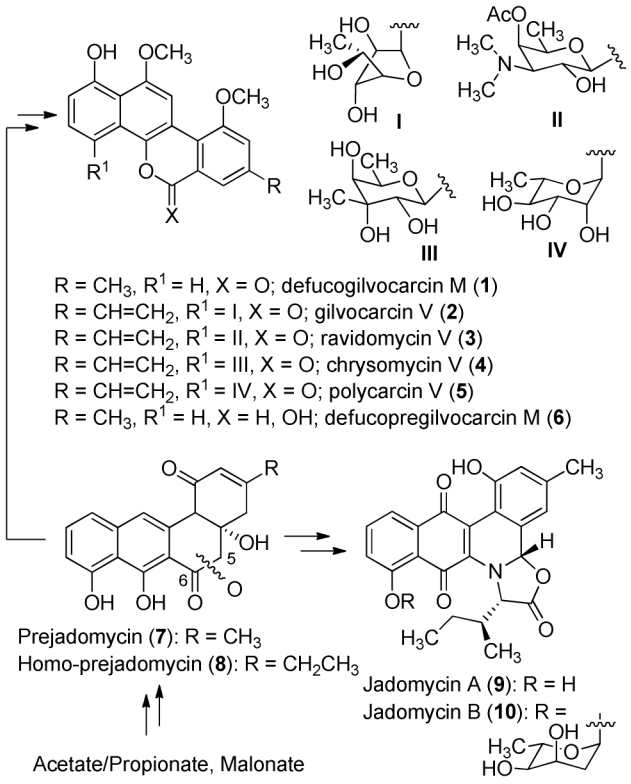


Figure 3.

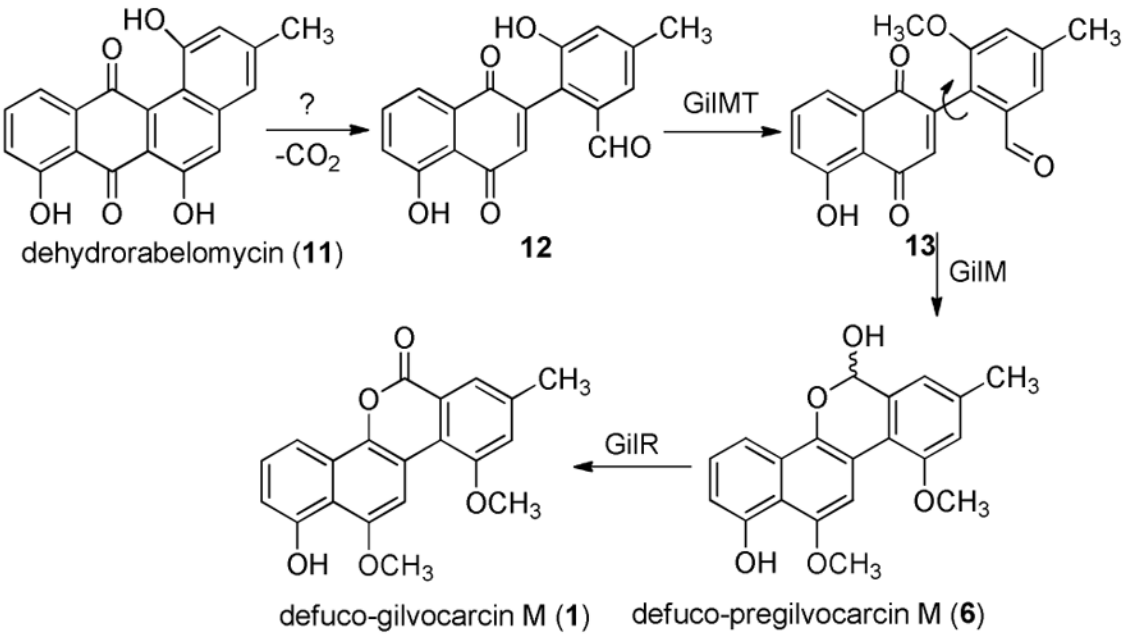
Top: In vitro reactions of **18** with different combinations of downstream enzymes; GilOII, GilM, GilMT and GilR.

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.

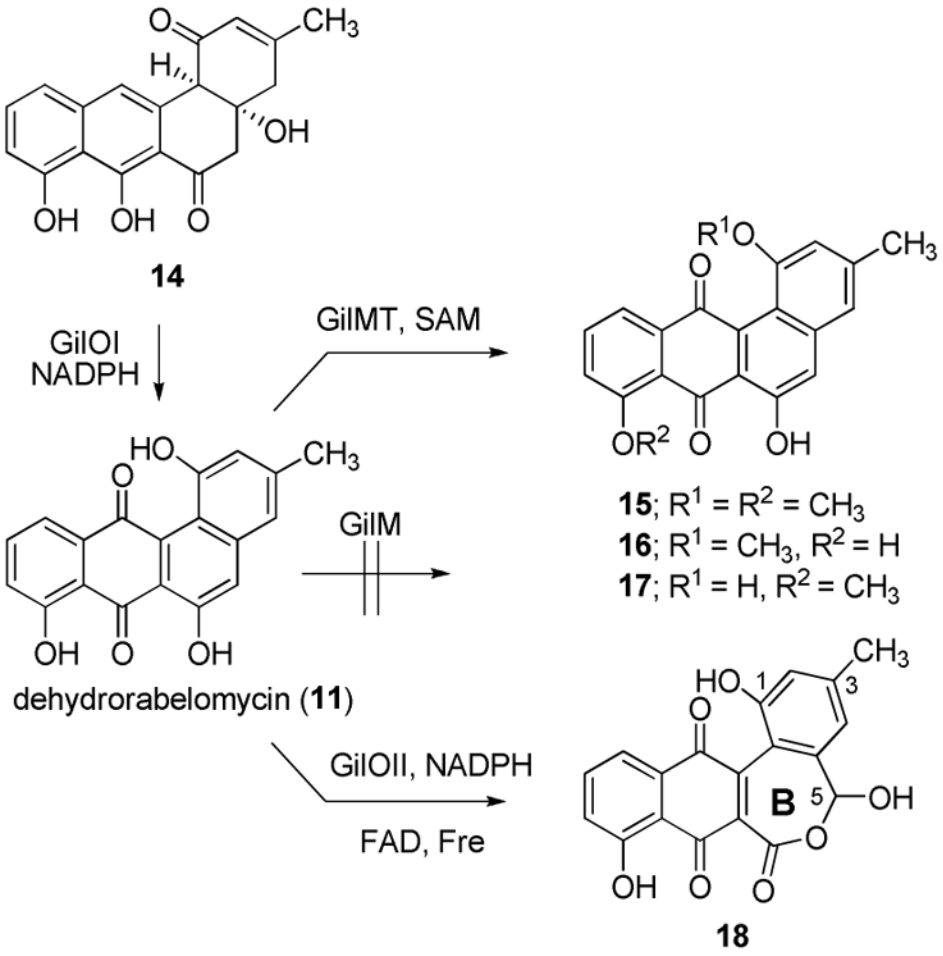


Scheme 1.

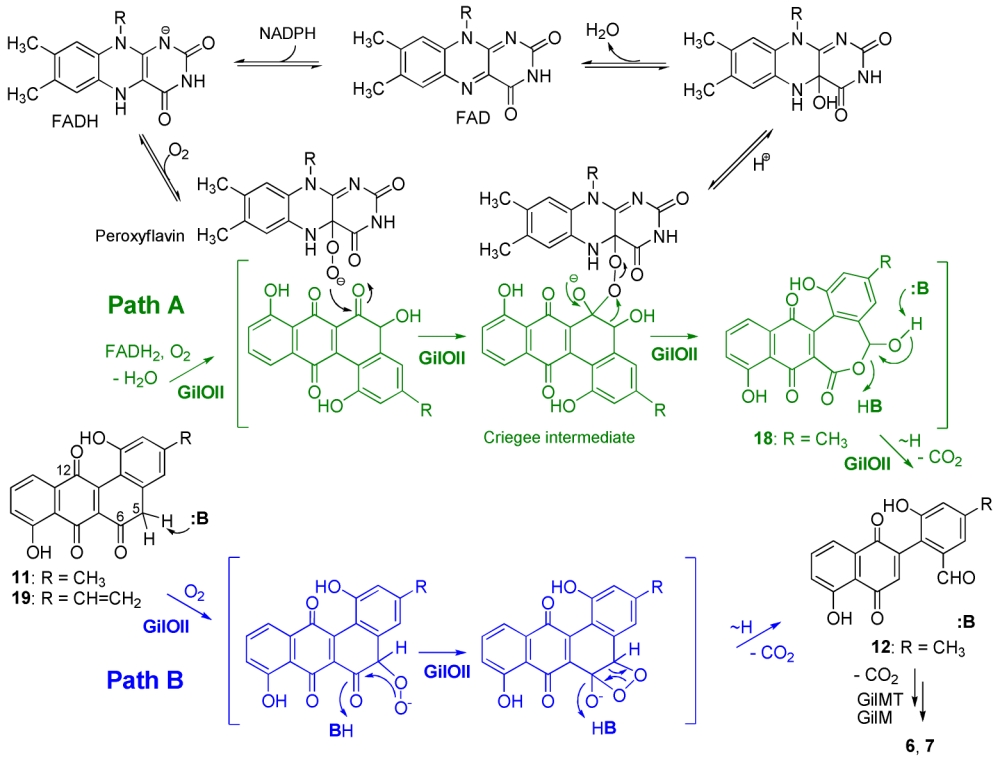
Representative members of the gilvocarcin and jadomycin groups of natural products.



Scheme 2.
Sequence of events en route to defucogilvocarcin M.



Scheme 3.
Enzymatic reaction of dehydrorabelomycin (**11**) with GilMT and GilOII; Fre: *E. coli* flavin reductase.



Scheme 4.

Mechanistic alternatives for the key C-C bond cleavage reaction