J|A|C|S
COMMUNICATIONS

Published on Web 09/14/2004

# Oxidative Rearrangement Processes in the Biosynthesis of Gilvocarcin V

Tao Liu, Carsten Fischer, Claus Beninga, and Jürgen Rohr*

*Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 725 Rose Street, Lexington, Kentucky 40536-0082*

Received June 2, 2004; E-mail: jrohr2@uky.edu

Gilvocarcin V (GV, **1**), produced along with its minor congener gilvocarcin M (GM, **2**) by *Streptomyces griseoflavus* Gö 3592 and many other streptomycetes, is the most important member of the benzo[*d*]naphtho[1,2-*b*]pyran-6-one *C*-glycoside anticancer antibiotics, often referred to as gilvocarcin-type aryl-*C*-glycosides.[1] GV is a potent antitumor compound requiring only low concentrations and maintaining a low in vivo toxicity. Although the exact molecular mechanism responsible for the in vivo mode of action of GV is still unclear, it was found that **1** acts through UV light-induced DNA alkylation combined with topoisomerase II inhibition, for which the vinyl group is essential, because it promotes a [2+2] cycloaddition with DNA thymine residues.[2a,b] In addition, another unique antitumor mechanism appears to be promoted by GV, namely the selective cross-linking between DNA and the protein histone H3, which as an essential part of the histone complex plays an important role in DNA replication and transcription.[2c] This is discussed as a mechanism contributing to the better and more specific activity of GV compared with other DNA-binding antitumor drugs.

One of the key steps of gilvocarcin biosynthesis is an oxidative rearrangement, which ultimately generates the unique gilvocarcin chromophore. From incorporation experiments with $^{13}$C-labeled precursors, it was suggested that an angucyclinone intermediate, possibly **3**, is oxidatively opened between carbons 5 and 6, leading to dicarboxylic acid **4**, which then undergoes decarboxylation (loss of C-6) and lactonization between the C-5 carboxyl and 12-OH of a hydroquinone, resulting in the tetracyclic aromatic lactone characteristic for the gilvocarcins (Scheme 1).[3] To prove this hypothesis and to provide more details about this intriguing oxidative rearrangement cascade were the goals of the studies described in this Communication. Intriguing key questions were whether the oxidative rearrangement occurs prior or after the *C*-glycosylation step,[1,3] and whether this is catalyzed by only one or by more than one enzyme.

To study this oxidative rearrangement cascade, we performed (i) incorporation experiments with $^{18}$O-labeled precursors and (ii) targeted inactivation of two oxygenase-encoding genes. Although detailed studies on GV and related antibiotics were performed by Suzuki et al. and G. T. Carter et al.,[3] using singly and doubly labeled $^{13}$C-acetate and propionate precursors, none of these involved $^{18}$O-labeled precursors. First, we fed [1-$^{13}$C,$^{18}O_2$]acetate[4a] to growing cultures of our gilvocarcin producer *S. griseoflavus* Gö 3592,[1] and second, we grew this strain in an 1-L fermentor attached to a closed system apparatus in an $^{18}$O-enriched atmosphere.[4b] While the former experiment resulted in the enrichment of the three oxygens attached at C-1,C-10, and C-12 as expected (see Scheme 1), the latter experiment did not show any of the oxygen atoms to be enriched from $^{18}O_2$.[4b−d,5]

The recently published gilvocarcin gene cluster revealed four oxygenase-encoding genes. From sequence alignment studies, two of these genes encode most likely the enzymes responsible for the oxidative C−C bond cleavage, proposed as the key step for the

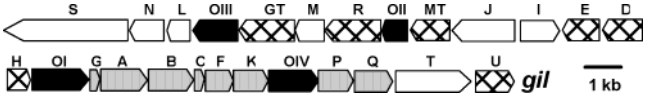
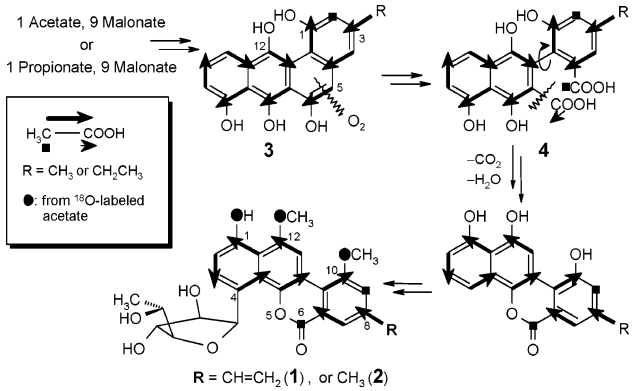



***Figure 1.*** Biosynthetic gene cluster of the gilvocarcins. Oxygenases are shown in black, polyketide synthase (PKS) genes in gray, genes encoding the deoxysugar biosynthesis, glycosyl transfer, and other post-PKS modifications with a diagonal grid pattern, and other genes (regulatory, of unknown function etc.) in white.

***Scheme 1.*** Suggested Oxidative Opening of Angucyclinone **3** and Follow-Up Reactions Leading to the Gilvocarcins Based on Incorporation Experiments



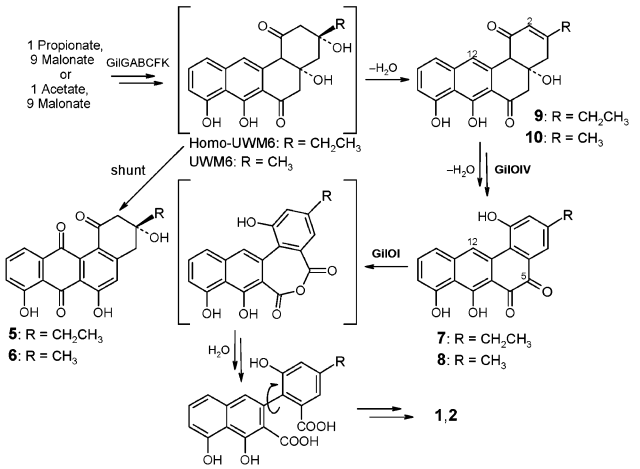

gilvocarcin biosynthesis.[1a] Both of their deduced amino acid sequences show high similarity to JadF and JadH, oxygenases proposed to be responsible for a similar step in the related jadomycin pathway.[6a−c] Thus, we wanted to inactivate these two genes, and to isolate accumulated products of the resulting mutant strains. Because the gilvocarcin producer *S. griseoflavus* showed a remarkable resistance toward any kind of gene manipulation, mutants of *Streptomyces lividans* TK24 (cosG9B3) needed to be generated. Previous experiments proved that it was possible to produce gilvocarcin V (ca. 25 mg/L) and gilvocarcin M (ca. 3 mg/L) with the heterologous host *S. lividans* TK 24, after a cosmid harboring the entire gilvocarcin gene cluster (cosG9B3) was expressed.[1a]Two new mutants of *S. lividans* (cosG9B3) were generated by replacing the *gilOI* and *gilOIV* genes, respectively, with the chloramphenicol resistance cassette CHL-*oriT*, using a variant of a recently described PCR-targeting gene replacement strategy.[7] This way, two mutants of cosG9B3 were generated in *Escherichia coli*, namely cosG9B3−OI$^-$ and cosG9B3−OIV$^-$, respectively, which then were transformed into *S. lividans* TK24 through conjugal gene transfer, creating *S. lividans* (cosG9B3−OI$^-$) and *S. lividans* (cosG9B3−OIV$^-$), respectively. The switch from the typically used apramycin-resistance cassette[7] to the CHL-resistance cassette was necessary because cosG9B3 already contains an apramycin-resistance cassette.[1a] The new two mutants were grown under standard conditions, and their spectrum of secondary metabolites was analyzed by HPLC-MS. The *gilOIV*-minus mutant *S. lividans* (cosG9B3−OIV$^-$)


10.1021/ja0467521 CCC: $27.50 

accumulated one major and one minor product, with molecular masses of 352 and 338 g/mol, respectively, which are both considerably smaller than GV (**1**) and GM (**2**), but show the same mass difference of 14 g/mol, indicating two homologues. Subsequent isolation and structure determination by NMR spectroscopy and MS revealed the minor compound to be the known angucyclinone rabelomycin (**6**) and the major compound to be its homologue, homorabelomycin (**5**). The *gilOI*-minus mutant *S. lividans* (cosG9B3−OI$^-$) accumulated four new compounds, one major and one minor purple compound (MW 334 and 320, respectively) and two yellow compounds (MW 338 and 324, respectively). The structure determination revealed two sets of homologue pairs, the purple pregilvocarcin-*o*-quinones, **7** and **8**, with **7** being the major product (for yields see Supporting Information), and the yellow 2,3-dehydro-UWM6[6c] (**10**) along with its homologue **9**.

The accumulation of **5** and **6** upon inactivation of oxygenase GilOIV parallels the findings of Yang et al. in context with the jadomycin biosynthesis, where the inactivation of oxygenase JadF led to the accumulation of **6**.[6a] This proves that GilOIV is involved in the cleavage reaction, and that the gilvocarcins are biosynthesized via an angucyclinone intermediate. The accumulation of compounds **7**−**10** upon inactivation of GilOI reveals that this enzyme also takes part in the oxidative ring cleavage reaction, and therefore links two oxygenases to this biosynthetic key reaction cascade. Moreover, the fact that two of these metabolites bear an oxygen in the 5-position suggests that the GilOI reaction follows the GilOIV reaction, and that GilOI is ultimately responsible for the C−C bond-breakage step. Compounds **9** and **10** are presumably immediate precursors of **7** and **8** and might be accumulated due to feedback inhibition of GilOIV. Evidence for this sequence of events came also from cross-feeding experiments, in which the principal product of *S. lividans* (cosG9B3−OI$^-$), **7**, was fed to *S. lividans* (cosG9B3−OIV$^-$) and consequently was completely converted into gilvocarcin V (**1**). In contrast, feeding of **5** to *S. lividans* (cosG9B3−OI$^-$) yielded neither **1** nor any other conversion product, suggesting that the block caused by the GilOIV inactivation is earlier than the one caused by the GilOI inactivation. In contrast to the product pair **5**/**6**, none of the products of the GilOI$^-$ mutant contain an oxygen in the 12-position. Thus, homorabelomycin (**5**) and rabelomycin (**6**) must be considered shunt products rather than intermediates, whose 12-oxygenation either is caused by GilOII[1a] or occurs spontaneously. In conclusion, the results of all experiments described here can be summarized in a novel hypothesis for the gilvocarcin biosynthesis (Scheme 2). This suggests for the gilvocarcin biosynthesis that (i) the oxidative cleavage occurs prior to the *C*-glycosylation, (ii) two enzymes, GilOIV and GilOI, cooperate in a sequential action to achieve the C−C bond cleavage, with GilOIV acting prior to GilOI, and (iii) the C−C bond cleavage happens earlier than the other oxygenation processes of **1** biosynthesis, i.e., 12-oxygenation and vinyl group formation. Although dioxygenases are often responsible for the oxidative breakage of aromatic rings,[6a] the accumulation of intermediates **7**/**8** indicates a stepwise action of two monooxygenases. For the latter, we propose an unprecedented Baeyer−Villiger-type oxygenation of an *o*-quinone into an anhydride, which then is opened to a dicarboxylic acid different from the originally proposed **4**. Future experiments with the isolated overexpressed enzymes GilOI and GilOIV might shed further light onto this intriguing sequence of oxidative events,

***Scheme 2.*** Proposed Stepwise Oxidative C−C Bond Breakage of Gilvocarcin Biosynthesis[a]




[a] Compounds **5** and **6** accumulated upon inactivation of oxygenase GilOIV, compounds **7**−**10** upon inactivation of oxygenase GilOI.

which has close parallels in the biosyntheses of both the jadomycins[6a−c] and the kinamycins.[6d]

**Acknowledgment.** Dedicated to Heinz G. Floss on the occasion of his 70th birthday. This work was supported by the Kentucky Lung Cancer Research Program and in part by the NIH (CA 91901). We thank Dr. B. Gust (John Innes Centre, Norwich, England) and Plant Biosciences Ltd., Norwich, England, for the template plasmids and strains used for the PCR-targeting inactivation experiments.

**Supporting Information Available:** NMR and other physicochemical data for homorabelomycin (**5**), pregilvocarcin-*o*-quinone (**7**), and 2,3-dehydrohomo-UWM 6 (**9**); experimental details of the incorporation experiments, and of the new variant of the targeted gene inactivation experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) (a) Fischer, C.; Lipata, L.; Rohr, J. *J. Am. Chem. Soc.* **2003**, *125*, 7818−7819. (b) Hirayama, N.; Takahashi, K.; Shirahata, K.; Ohashi, Y.; Sasada, Y. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 1338−1342. (c) Krohn, K.; Rohr, J. *Top. Curr. Chem.* **1997**, *188*, 127−195 and references cited therein.

(2) (a) Knobler, R. M.; Radlwimmer, F. B.; Lane, M. J. *Nucleic Acids Res.***1992**, *20*, 4553−4557. (b) Oyola, R.; Arce, R.; Alegria, A. E.; Garcia, C. *Photochem. Photobiol.* **1997**, *65*, 802−810 and references cited therein. (c) Matsumoto, A.; Hanawalt, P. C. *Cancer Res.* **2000**, *60*, 3921−3926.

(3) (a) Takahashi, K.; Tomita, F. *J. Antibiot.* **1983**, *36*, 1531−1535. (b) Carter, G. T.; Fantini, A. A.; James, J. C.; Borders, D. B.; White, R. J. *J. Antibiot.* **1985**, *38*, 242−248.

(4) (a) Boyer, P. D.; Koeppe, O. J.; Luchsinger, W. W. *J. Am. Chem. Soc.* **1956**, *78*, 356−357. (b) Udvarnoki, G.; Henkel, T.; Machinek, M.; Rohr, J. *J. Org. Chem.* **1992**, *57*, 1275−1276. (c) Gould, S. J.; Cheng, X.-C. *Tetrahedron* **1993**, *49*, 11135−11144. (d) Gould, S. J.; Cheng, X.-C.; Melville, C. *J. Am. Chem. Soc.* **1994**, *116*, 1800−1804.

(5) Enrichment of O-5 and O-6 was expected from $^{18}O_2$, but not found, possibly due to exchange with water combined with insufficient $^{18}O$ label. It also proved impossible in other pathways to label O-12 (becomes O-5 in **1**) of an angucyclinone with $^{18}O_2$.[4b−d]

(6) (a) Yang, K.; Han, L.; Ayer, S. W.; Vining, L. C. *Microbiology* **1996**, *142*, 123−132. (b) Wang, L.; White, R. L.; Vining, L. C. *Microbiology* **2002**, *148*, 1091−1103. (c) Kulowski, K.; Wendt-Pienkowski, E.; Han, L.; Yang, K.; Vining, L. C.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 1786−1794. (d) Cone, M. C.; Melville, C. R.; Gore, M. P.; Gould, S. J. *J. Org. Chem.* **1993**, *58*, 1058−1061.

(7) Gust, B.; Challis, G. L.; Fowler, K.; Kieser, T.; Chater, K. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1541−1546

JA0467521