

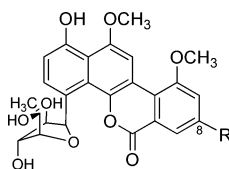
Roles of the Synergistic Reductive O-Methyltransferase GilM and of O-Methyltransferase GilMT in the Gilvocarcin Biosynthetic Pathway

Nidhi Tibrewal,[†] Theresa E. Downey,[†] Steven G. Van Lanen,[†] Ehesan Ul Sharif,[‡] George A. O'Doherty,[‡] and Jürgen Rohr^{*,†}[†]Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone Street, Lexington, Kentucky 40536-0596, United States[‡]Department of Chemistry and Chemical Biology, Northeastern University, 360 Huntington Avenue, Boston, Massachusetts 02115, United States

S Supporting Information

ABSTRACT: Two enzymes of the gilvocarcin biosynthetic pathway, GilMT and GilM, with unclear functions were investigated by *in vitro* studies using purified, recombinant enzymes along with synthetically prepared intermediates. The studies revealed GilMT as a typical S-adenosylmethionine (SAM) dependent O-methyltransferase, but GilM was identified as a pivotal enzyme in the pathway that exhibits dual functionality in that it catalyzes a reduction of a quinone intermediate to a hydroquinone, which goes hand-in-hand with a stabilizing O-methylation and a hemiacetal formation. GilM mediates its reductive catalysis through the aid of GilR that provides FADH₂ for the GilM reaction, through which FAD is regenerated for the next catalytic cycle. This unusual synergy eventually completes the biosynthesis of the polyketide-derived defuco-gilvocarcin chromophore.

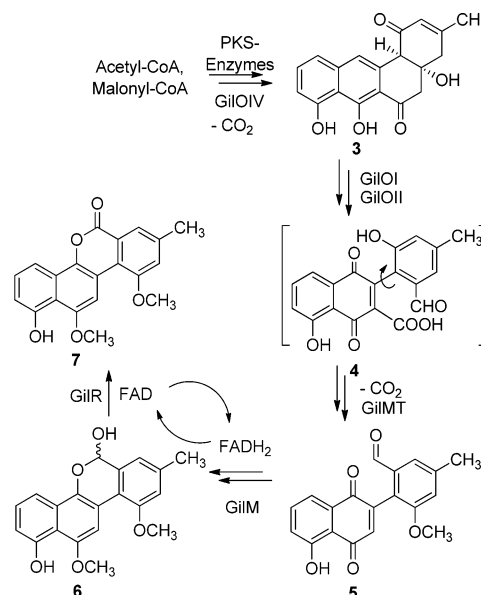
Gilvocarcin V (GV, **1**), produced by *Streptomyces griseoflavus* and various other *Streptomyces* strains, is the prototype of the rare group of polycyclic aromatic polyketides with benzo[*d*]naphtha[1,2-*b*]pyran-6-one chromophore, referred to as the gilvocarcin group. This group shows significant antitumor activities and remarkably low toxicity.^{1,2} Gilvocarcin V exhibits its light-induced anticancer activity by mediating cross-linking between DNA and histone H3.^{3,4} The vinyl group enhances the antitumor activity through formation of a photoinduced [2 + 2] cycloadduct with thymine residues of double stranded DNA,⁵ since the minor congeners gilvocarcin M (**2**) and gilvocarcin E, which lack the vinyl group, are considerably less active than GV.⁶ For the histone H3 interaction, the D-fucofuranose moiety is considered essential.



Gilvocarcin V (**1**): R = CH=CH₂
Gilvocarcin M (**2**): R = CH₃

The aromatic skeleton of the gilvocarcins is produced by a type II polyketide synthase (PKS),^{1,7} which initially yields an angucycline biosynthetic intermediate (e.g., **3** in Scheme 1). Intriguing oxidative post-PKS modifications then convert this

Scheme 1. Key Oxidative Rearrangement and Follow-up Sequence of Events Involving GilMT and GilM Reactions en route to Defuco-gilvocarcin M (7**)**



intermediate into the unique defuco-gilvocarcin chromophore typical for this class of anticancer drugs.⁸ The role of many of the post-PKS gene products have been assigned based on gene inactivation, cross-feeding and labeling studies,^{1,7–12} and most of the genes of the three known biosynthetic clusters¹³ of this group have been assigned to certain enzymatic activities with *gilM* (encoding an enzyme of unknown function) and *gilMT* (encoding a methyltransferase) being notable exceptions. Thus, the functions of their gene products, GilM and GilMT, remained obscure. Both the enzymes appear to be crucial for the biosynthesis of gilvocarcin and were proposed to be involved in O-methylation reactions. However, mutant strains created by deletion of these individual genes did not accumulate any isolable metabolites, and thus provided no clue regarding their potential substrates and hence structure of key biosynthetic intermediates.

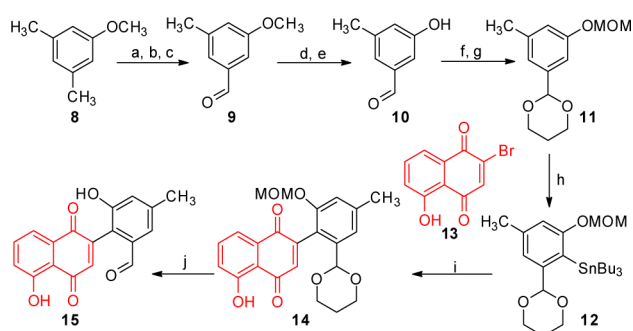
Received: May 25, 2012

Published: July 16, 2012



However, a recent combinatorial biosynthetic enzymology approach led to the enzymatic total synthesis of defucogilvocarcin M (7) from acetyl-CoA and malonyl-CoA, a model compound with the completed gilvocarcin chromophore. Moreover, systematic variations of the enzyme mixtures revealed a hypothetical pathway suggesting that 2-aryl-5-hydroxy-1,4-naphthoquinone-3-carboxylic acid (4) is likely the product of the critical oxidative C–C bond cleavage and thereby a key intermediate of gilvocarcin V. The results also suggested that only three more enzymes, namely GilM, GilMT and GilR, were necessary to finish the biosynthesis of 7. To confirm this hypothesis and fully enlighten the “biosynthetic black box”, a potential model substrate, 2-aryl-5-hydroxy-1,4-naphthoquinone (15) was synthesized and subsequently used to interrogate the function of GilM and GilMT (Scheme 2).

Scheme 2^a



^aReagents and Conditions: (a) NBS, dibenzoyl peroxide, CCl₄, reflux, 1h, 70% ; (b) NaHCO₃, H₂O, Me₂CO, reflux, 4 h, 86%; (c) PCC, silica gel, CH₂Cl₂, 1 h, quant.; (d) 1M BBr₃ in CH₂Cl₂, 2h; (e) 1:1 HCl/AcOH, reflux, 10h, 60%; (f) 1,3-propanediol, TsOH (cat.), toluene, reflux, 12 h, 75%; (g) chloromethyl methyl ether, iPr₂EtN, CH₂Cl₂, 40 °C, 24 h, 95%; (h) *n*-BuLi, *n*-Bu₃SnCl, hexane, 0 °C, 88%; (i) Pd₂(dba)₃·CHCl₃, PPh₃, CuI, THF, 75 °C, 12 h, 75%; (j) 2.4 M HCl in CH₃CN, 74%.

Synthesis of compound 15 began with commercially available dimethyl anisole (8).¹⁴ Bromination with *N*-bromo-succimide (NBS) and benzoyl peroxide provided mono bromo benzyl derivative. A sequential hydroxylation followed by oxidation with pyridinium chlorochromate (PCC) afforded 9 in 60% yield from 8.¹⁴ Removal of the methoxy group was achieved in 60% yield by treatment with BBr₃ at r.t., followed by acidic hydrolysis. Protection of the aldehyde as a cyclic acetal and the phenolic OH as methoxymethyl ether provided 11. An ortho-metalation with tributyltinchloride yielded the required stannane 12 in 88% yield.¹⁵ 2-Bromonaphthoquinone (13) was prepared from juglone using the reported protocol.¹⁶ A facile Stille coupling between 12 and 13 provided 14. 14 was then exposed to harsh acidic conditions (2.4 M HCl in CH₃CN) for 4 min to provide fully unprotected 2-aryl-1,4-naphthoquinone 15 in 74% yield.¹⁵

With a potential substrate in hand, both *gilM* and *gilMT* genes were cloned into pET28a and expressed in *E. coli* to yield soluble proteins that were purified to near homogeneity. Synthetic 15 when incubated with purified GilM produced a complex mixture (Figure 1, trace C). In contrast, HPLC-MS analysis of 15 with GilMT and *S*-adenosylmethionine (SAM) revealed a decrease in the amount of substrate and formation of a new product (Figure 1, trace E), while in the absence of SAM, no product was formed (Figure 1, trace D). The GilMT

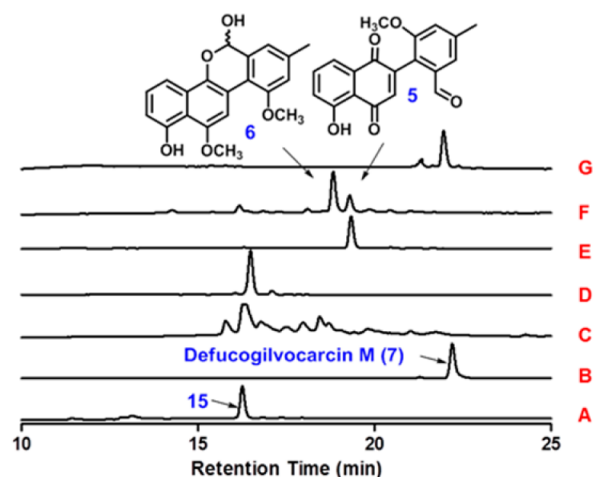


Figure 1. HPLC traces of the enzymatic reactions with 2-aryl-1,4-naphthoquinone (15). (A) Standard 2-aryl-1,4-naphthoquinone (15); (B) standard defuco-gilvocarcin M (7); (C) 15 + GilM; (D) 15 + GilMT; (E) 15 + GilMT + SAM; (F) 15 + GilMT + GilM + SAM; (G) 15 + GilMT + GilM + GilR + SAM.

product was isolated and characterized using NMR and HRMS. The analysis revealed the product as monomethylated derivative 5. Nuclear Overhauser enhancement (NOE) studies further confirmed that the hydroxyl group of the phenyl ring of substrate 15 was methylated.

When compound 15 was incubated with both GilM and GilMT along with SAM, a new product along with 5 accumulated (Figure 1, trace F). The new compound was identified as defuco-pregilvocarcin M (6) by NMR experiments (¹H NMR, HSQC). Adding GilR led to the accumulation of defuco-gilvocarcin M (7) (Figure 1, trace G), further confirming the structure of the GilM/MT product as 6. In total the results suggested that GilMT is a typical SAM-dependent *O*-methyltransferase, while GilM was responsible for the reduction of the quinone moiety necessary for the observed second *O*-methylation. BLAST (basic local alignment search tool) analysis^{17,18} revealed GilM to have low similarity (34–37% sequence identity) to nucleotidyl-*S*-transferases such as thiopurine-*S*-methyltransferases from *Rhodococcus equi* or *Mycobacterium avium* as well as to benzoquinone methyltransferases from *Mycobacterium tuberculosis* (33% sequence identity). The translated amino acid sequence of GilM contains VLDLGCGLG as residues 49–57, which appears to be a SAM binding motif (generally hh(D/E)hGXGXG, where h represents a hydrophobic residue). Thus, it remained unclear at this point whether GilMT or GilM catalyzes the second *O*-methylation. To solve this ambiguity, product 5 from GilMT reaction was used as substrate for GilM in the presence of SAM. The reaction accumulated 6, suggesting that GilM not only mediates reduction of the quinone but also catalyzes a second *O*-methylation. Surprisingly, the GilM reaction seemed to convert 5 to 6 even in absence of SAM, which prompted us to further investigate GilM for any bound SAM cofactor. The enzyme was boiled for 5 min and centrifuged (12000× *g*, 5 min). The supernatant when analyzed by HPLC showed UV-absorption at 260, typical of adenosine spectrum. A comparison with commercially available SAM verified that GilM copurifies with SAM, thereby solving the mystery of the methyl source (Supporting Information, Figure 1).

To further analyze the reaction sequence catalyzed by GilM, compound **5** was incubated with GilM and the reaction was studied at different time points (Figure 2). With stoichiometric

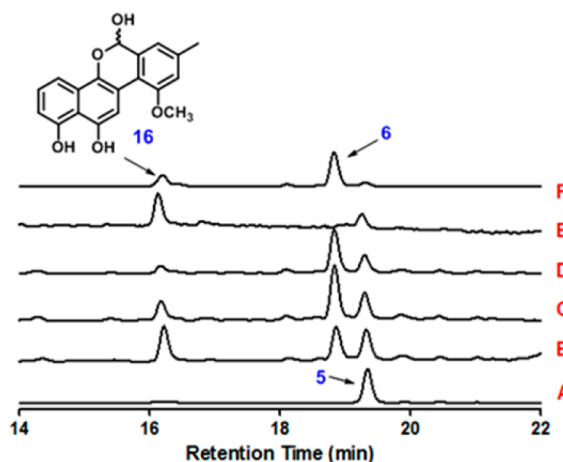


Figure 2. HPLC traces of the enzymatic reactions with **5**. (A) Standard **5**; (B) **5** + GilM + SAM + 5 min; (C) **5** + GilM + SAM + 10 min; (D) **5** + GilM + SAM + 15 min; (E) intermediate (**16**) isolated from HPLC (mixture of closed and open form); (F) **16** + GilM.

enzyme quantities, the substrate was 80% converted into the product after 15 min (Figure 2, trace D). When the reaction was analyzed by reversed phase HPLC-MS in 3–5 min intervals, a new peak (**16**) appeared with shorter retention time than the overall product **6** (Figure 2). Although NMR analysis of **16** was impossible due to its instability, LC-MS analysis suggested it to be demethyl-defuco-pregilvocarcin M (m/z 323 [$M - H$] $^-$). To prove that **16** was an intermediate en route to **6** and not a shunt product, **16** was incubated with GilM, and, as anticipated, was rapidly converted to **6** (Figure 2, trace F). Overall, the results showed that GilM catalyzes a sequence of reactions: (i) quinone reduction, (ii) hemiacetal formation and (iii) O-methylation, to construct the tetracyclic core of the gilvocarcins.

The only remaining question was the regeneration of GilM after reduction of the quinone. Interestingly, the respective GilM-activity in the biosynthetic pathways of other structurally related compounds, chrysomycin A and ravidomycin V, is encoded on the same gene as the oxidoreductase GilR-activity,¹³ while in gilvocarcin pathway, *gilM* and *gilR* are two separate genes.¹ This prompted us to propose that GilM could be working in conjunction with GilR, an FAD dependent oxidoreductase that catalyzes the very last step in gilvocarcin biosynthesis by converting pregilvocarcin to gilvocarcin (**2**), but also was shown to convert sugar free defuco-pregilvocarcins.^{9,19} We hypothesized that GilM utilizes the reduced flavin generated in the GilR reaction to reduce the quinone thereby regenerating oxidized flavin for the next catalytic cycle. To validate this hypothesis, compound **5** was incubated with GilM and GilR (Figure 3). The assistance of GilR for the reducing capabilities of GilM was established by measuring the amounts of the products formed in the reactions. Catalytic amounts of GilM alone accumulated mostly starting material along with minor production of **6** (Figure 3, trace C). In the absence of SAM, GilM and GilR accumulated a new peak (Figure 3, trace D) that corresponds to demethyl-defuco-gilvocarcin M²⁰ (**17**, m/z 321 [$M - H$] $^-$). Adding SAM to the GilM-GilR reaction mixture led to the accumulation of **7** (Figure 3, trace E). A 10-

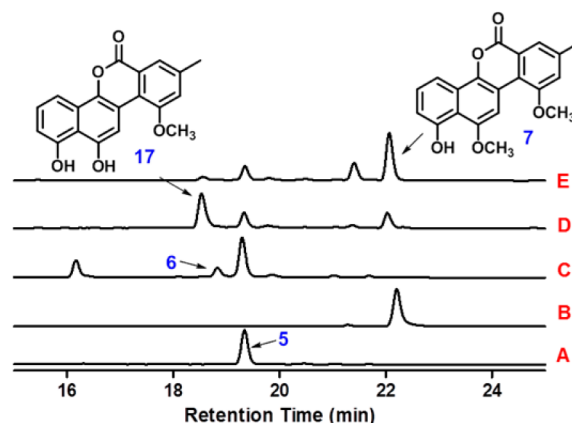


Figure 3. HPLC traces of the enzymatic reactions with **5**. (A) Standard **5**; (B) standard defuco-gilvocarcin M (**7**); (C) **5** + GilM; (D) **5** + GilM + GilR; (E) **5** + GilM + GilR + SAM.

fold increase in the formation of **7** in the GilM-GilR reaction versus using GilM alone confirmed that GilM works synergistically with GilR.

Overall, these results described here allowed us to fully prove the post-PKS reaction sequence of the gilvocarcin biosynthetic pathway as shown in Scheme 1. The fact that the easy to synthesize compound **15** is an intermediate of the gilvocarcin pathway opens up the future possibilities of generating gilvocarcin analogues through chemo-enzymatic synthesis or mutasynthesis,^{21,22} thus coupling the power of chemical synthesis with metabolic engineering.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, kinetic studies of GilM and GilMT, enzymatic total synthesis of **6**, and NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

jrohr2@email.uky.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by NIH grants CA 102102 and CA 091901 to J.R. We thank Ms. Manjula Sunkara as well as Drs. J. Goodman and Andrew Morris for the mass spectra.

■ REFERENCES

- (1) Fischer, C.; Lipata, F.; Rohr, J. *J. Am. Chem. Soc.* **2003**, *125*, 7818.
- (2) Nakano, H.; Matsuda, Y.; Ito, K.; Ohkubo, S.; Morimoto, M.; Tomita, F. *J. Antibiot.* **1981**, *34*, 266.
- (3) Matsumoto, A.; Fujiwara, Y.; Elespuru, R. K.; Hanawalt, P. C. *Photochem. Photobiol.* **1994**, *60*, 225.
- (4) Matsumoto, A.; Hanawalt, P. C. *Cancer Res.* **2000**, *60*, 3921.
- (5) McGee, L. R.; Misra, R. J. *Am. Chem. Soc.* **1990**, *112*, 2386.
- (6) Elespuru, R. K.; Gonda, S. K. *Science* **1984**, *223*, 69.
- (7) Shepherd, M. D.; Kharel, M. K.; Zhu, L. L.; Van Lanen, S. G.; Rohr, J. *Org. Biomol. Chem.* **2010**, *8*, 3851.
- (8) Pahari, P.; Kharel, M. K.; Shepherd, M. D.; Van Lanen, S. G.; Rohr, J. *Angew. Chem., Int. Ed.* **2012**, *51*, 1216.
- (9) Kharel, M. K.; Pahari, P.; Lian, H.; Rohr, J. *ChemBioChem* **2009**, *10*, 1305.

- (10) Kharel, M. K.; Zhu, L.; Liu, T.; Rohr, J. *J. Am. Chem. Soc.* **2007**, *129*, 3780.
- (11) Liu, T.; Fischer, C.; Beninga, C.; Rohr, J. *J. Am. Chem. Soc.* **2004**, *126*, 12262.
- (12) Liu, T.; Kharel, M. K.; Fischer, C.; McCormick, A.; Rohr, J. *ChemBioChem* **2006**, *7*, 1070.
- (13) Kharel, M. K.; Nybo, S. E.; Shepherd, M. D.; Rohr, J. *ChemBioChem* **2010**, *11*, 523.
- (14) Srikrishna, A.; Ravikumar, P. C. *Synthesis-Stuttgart* **2007**, 65.
- (15) Shan, M.; Sharif, E. U.; O'Doherty, G. A. *Angew.Chem., Int. Ed.* **2010**, *49*, 9492.
- (16) Kitani, Y.; Morita, A.; Kumamoto, T.; Ishikawa, T. *Helv. Chim. Acta* **2002**, *33*, 1186.
- (17) Altschul, S. F.; Lipman, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5509.
- (18) Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J. H.; Zhang, Z.; Miller, W.; Lipman, D. J. *Nucleic Acids Res.* **1997**, *25*, 3389.
- (19) Noinaj, N.; Bosserman, M. A.; Schickli, M. A.; Piszczek, G.; Kharel, M. K.; Pahari, P.; Buchanan, S. K.; Rohr, J. *J. Biol. Chem.* **2011**, *286*, 23533.
- (20) de Frutos, Ó.; Atienza, C.; Echavarren, A. M. *Eur. J. Org. Chem.* **2001**, 163–171.
- (21) Kirschning, A.; Hahn, F. *Angew. Chem., Int. Ed.* **2012**, *51*, 4012.
- (22) Taft, F.; Harmrolfs, K.; Nickeleit, I.; Heutling, A.; Kiene, M.; Malek, N.; Sasse, F.; Kirschning, A. *Chem.—Eur. J.* **2012**, *18*, 880.