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

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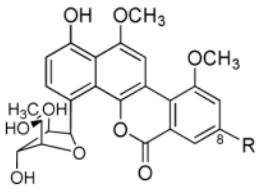
## Abstract

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 $FADH_2$ 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 chromphore.

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 gilvocarcin group. This group shows significant antitumor activities and remarkably low toxicity.[1,2] Gilvocarcin V exhibits its light-induced anticancer activity by mediating crosslinking between DNA and histone H3.[3,4] The vinyl group enhances the antitumor activity through formation of a photo-induced [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 = $CH{=}CH_2$
Gilvocarcin M (**2**): R = $CH_3$

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

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 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 clusters[13] 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 genes 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.

However, a recent combinatorial biosynthetic enzymology approach led to the enzymatic total synthesis of defuco-gilvocarcin 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.

Synthesis of compound **15** began with commercially available dimethyl anisole (**8**).[14] Bromination with NBS and benzoyl peroxide provided mono bromo benzyl derivative. A sequential hydroxylation followed by oxidation with PCC afforded **9** in 60% yield from **8**.[14] Removal of the methoxy group was achieved in 60% yield by treatment with $BBr_3$ at r.t., followed by acidic hydrolysis. Protection of the aldehyde as a cyclic acetal and the phenolic OH as methoxylmethyl 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 $CH_3CN$) for 4 min to provide fully unprotected 2-aryl-1,4-naphthaquinone **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 (Fig. 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 (Fig. 1, trace E), while in the absence of SAM, no product was formed (Fig. 1, trace D). The GilMT product was isolated and characterized using NMR and HRMS. The analysis revealed the product as mono-methylated 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 (Fig. 1, trace F). The new compound was identified as defuco-pregilvocarcin M (**6**) by NMR experiments ($^1$H NMR, HSQC). Adding GilR led to the accumulation of defuco-gilvocarcin M (**7**) (Fig. 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 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 minutes 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 co-purifies with SAM, thereby solving the mystery of the methyl source (Supporting information, Fig. 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 (Fig. 2). With stoichiometric enzyme quantities, the substrate was 80% converted into the product after 15 minutes (Fig. 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** (Fig. 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** (Fig. 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 (Fig. 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** (Fig. 3, trace C). In the absence of SAM, GilM and GilR accumulated a new peak (Fig. 3, trace D) that corresponds to demethyl-defuco-gilvocarcin M[20] (**17**, *m*/*z* 321 [M-H]$^-$). Adding SAM to the GilM-GilR reaction mixture led to the accumulation of **7** (Fig. 3, trace E). A 10-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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## ABBREVIATIONS

| | |
|---|---|
| **BLAST** | basic local alignment search tool |
| **NBS** | N-bromosuccimide |
| **PKS** | polyketide synthase |
| **SAM** | S-adenosylmethionine |
| **PCC** | pyridinium chlorochromate |

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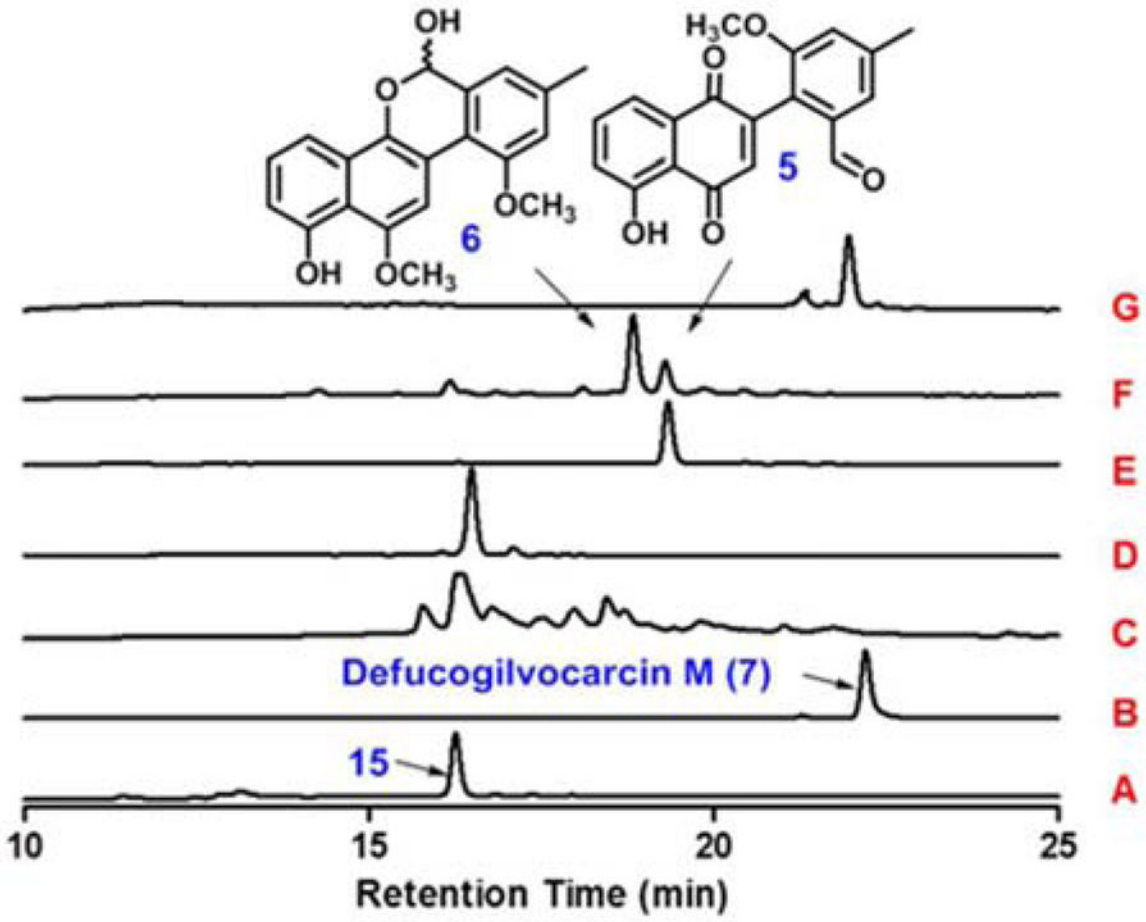



**Figure 1.**

HPLC traces of the enzymatic reactions with 2-aryl-1,4-naphthaquinone (**15**): (A) standard 2-aryl-1,4-naphthaquinone (**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.

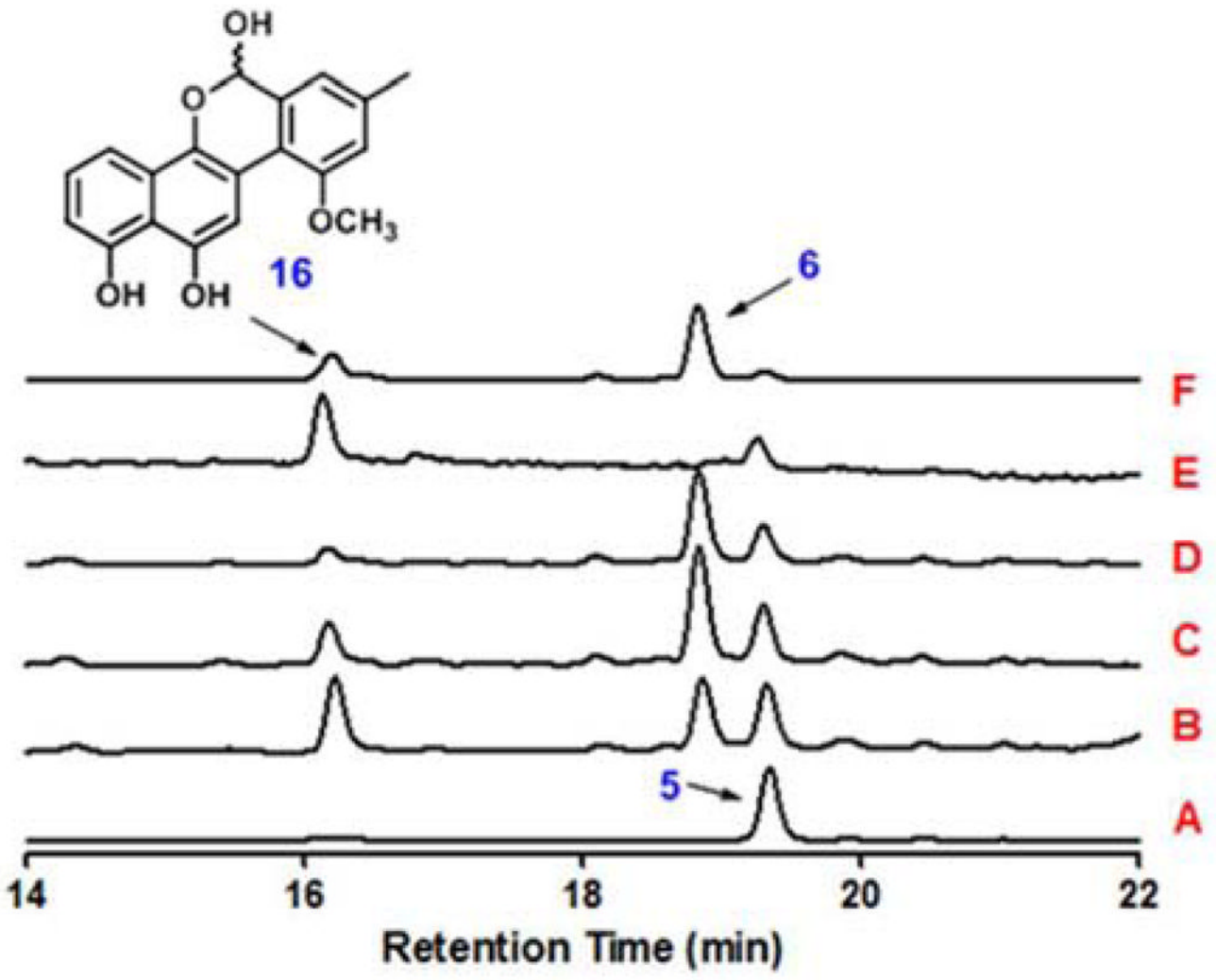



**Figure 2.**
HPLC traces of the enzymatic reactions with **5**: (A) standard **5**; (B) **5** + GilM + SAM + 5 min; (C) **5** + GilM + SAM + 10 min; (D) **5** + GilM + SAM + 15 min; (E) intermediate (**16**) isolated from HPLC (mixture of closed and open form); (F) **16** + GilM.

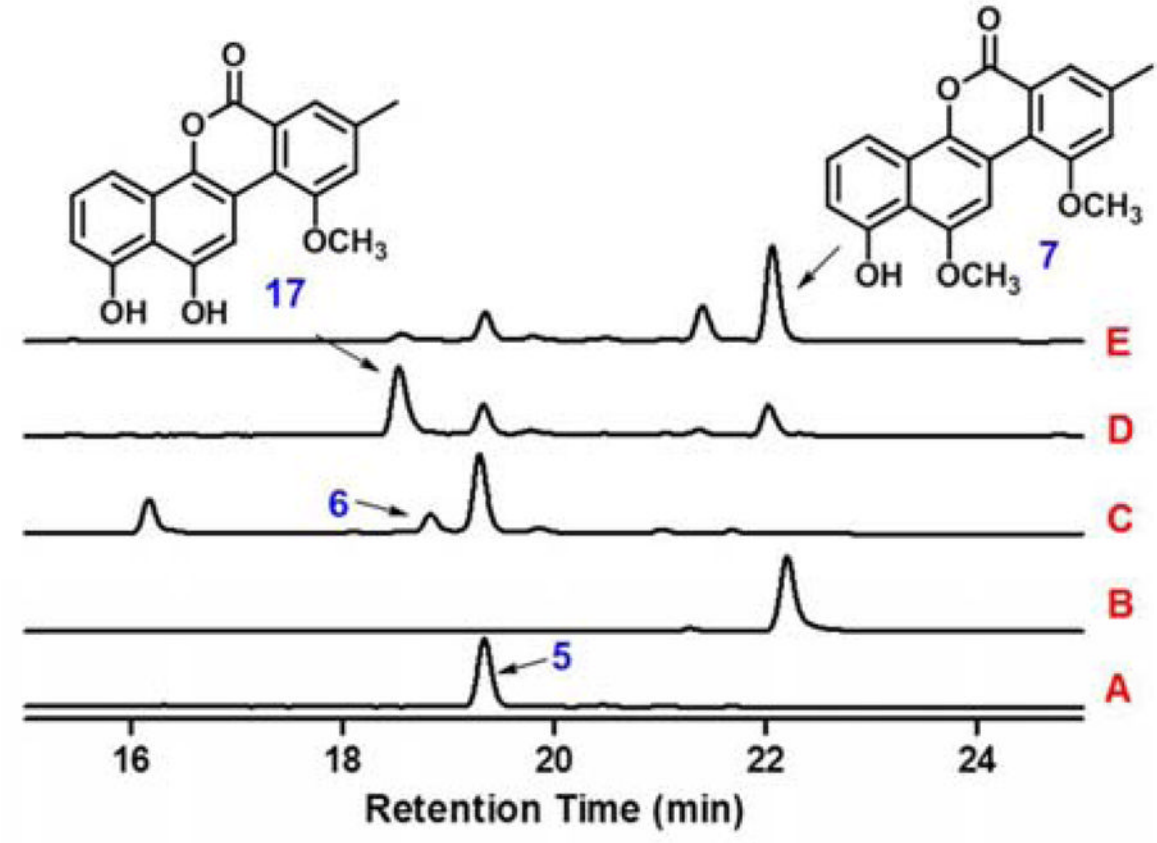
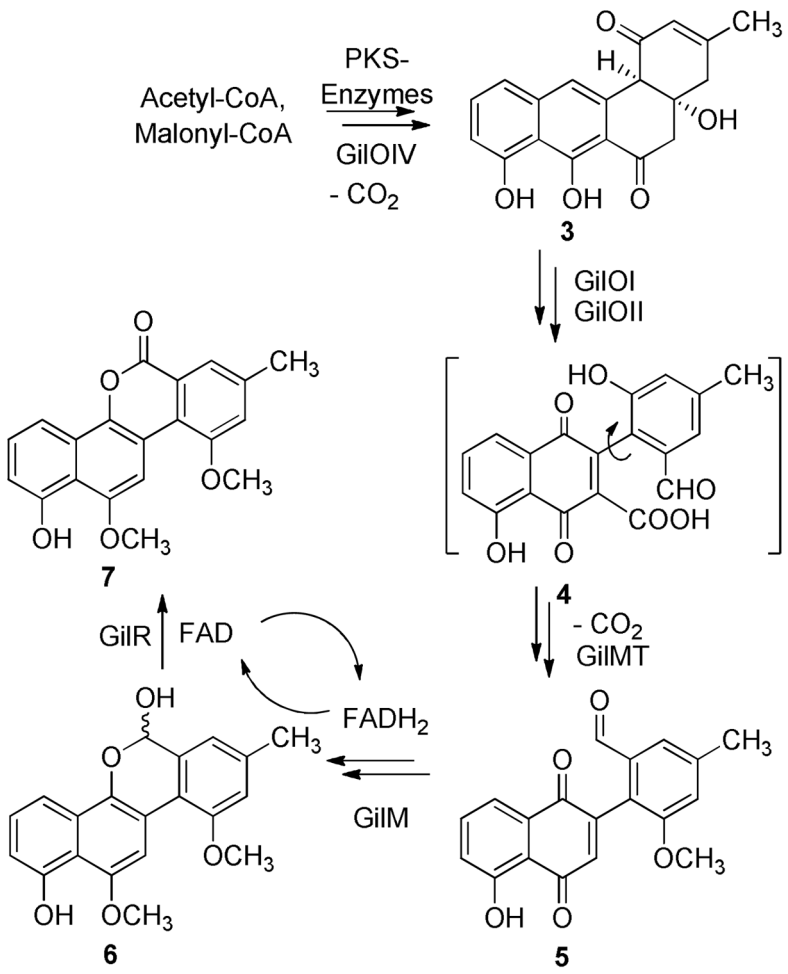
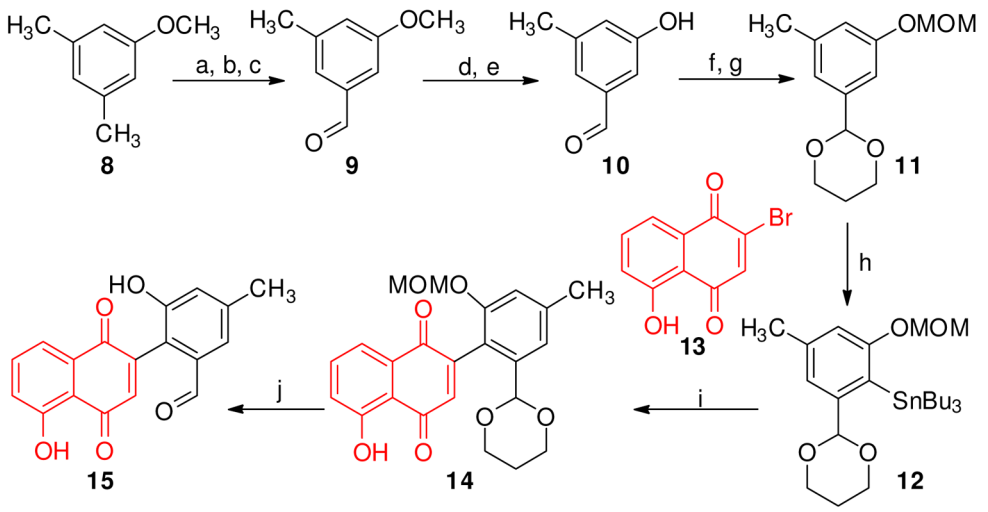



**Figure 3.**
HPLC traces of the enzymatic reactions with **5**: (A) standard **5**; (B) standard defuco-gilvocarcin M (**7**); (C) **5** + GilM; (D) **5** + GilM + GilR; (E) **5** + GilM + GilR + SAM.




**Scheme 1.**

Key oxidative rearrangement and follow-up sequence of events involving GilMT and GilM reactions en route to defuco-gilvocarcin M (**7**)




**Scheme 2. Reagents and Conditions**

(a) NBS, dibenzoyl peroxide, $CCl_4$, reflux, 1h, 70%; (b) $NaHCO_3$, $H_2O$, $Me_2CO$, reflux, 4h, 86%; (c) PCC, silica gel, $CH_2Cl_2$, 1h, quant.; (d) 1M $BBr_3$ in $CH_2Cl_2$, 2h; (e) 1:1 HCl:AcOH, reflux, 10h, 60%; (f) 1,3-propanediol, TsOH (cat.), toluene, reflux, 12h, 75%; (g) chloro-methyl methyl ether, $iPr_2EtN$, $CH_2Cl_2$, 40 °C, 24h, 95%; (h) *n*-BuLi, *n*-$Bu_3SnCl$, hexane, 0°C, 88%; (i) $Pd_2(dba)_3$•$CHCl_3$, $PPh_3$, CuI, THF, 75 °C, 12h, 75%; (j) 2.4 M HCl in $CH_3CN$, 74%.