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Inactivation of *gilGT*, Encoding a C-Glycosyltransferase, and *gilOIII*, Encoding a P450 Enzyme, Allows the Details of the Late Biosynthetic Pathway to Gilvocarcin V to be Delineated

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Abstract

Resequencing of the gilGT gene, which encodes a putative glycosyltransferase (GT) that is 495 amino acids (aa) long, from the Streptomyces griseoflavus Gc3592 gilvocarcin V (GV) gene cluster, revealed that the previously reported gilGT indeed contains two genes. These are the larger gilGT, which encodes the C-glycosyltransferase GilGT (379 aa), and the smaller gilV gene, which encodes an enzyme of unknown function (116 aa). The gene gilV is located immediately upstream of gilGT in the GV gene cluster. In-frame deletion of gilGT created a mutant that accumulated defucogilvocarcin E (defuco-GE). The result proves the function of GilGT as a C-glycosyltransferase. Deletion of gilOIII, which is located immediately downstream of gilGT, led to a mutant that accumulated gilvocarcin E (GE). This confirms that the corresponding P450 enzyme, GilOIII, is involved in the vinyl-group formation of GV. Cross-feeding experiments in which GE, defuco-GE, and defucogilvocarcin V (defuco-GV) were fed to an early blocked mutant of the GV biosynthetic pathway, showed that neither GE nor any of the defuco- compounds was an intermediate of the pathway.

Keywords

antitumor agents; biosynthesis; cytochrome P450; gilvocarcin; glycosylation

Introduction

Post-polyketide synthase (PKS) modifying enzymes play an important role for both the diversification of biosynthetic pathways and the addition of structural elements that are crucial for the biological activity of natural products. Oxygenases (OXs) and glycosyltransferases (GTs) are of particular interest.[1–5] Gilvocarcin V (GV, Scheme 1), the principal product of *Streptomyces griseoflavus* Gc3592 and many other *Streptomyces* strains, and the most important member of the benzo[*d*]-naphtho[1,2-*b*]pyran-6-one C-glycoside anticancer antibiotics, contains a vinyl group side chain in the C8 position and a C-glycosidically linked α -fucofuranose. These are both critical for the unique mechanism of action of GV. GV has been found to mediate a unique cross-linking reaction between DNA and histone H3—a major component of the histone complex that plays an important role in DNA replication and packaging.[6–8] While the C8 vinyl group is essential for the covalent binding of GV to DNA

through a photo[2+2]cyclo-addition reaction with thymine residues,[9,10] the sugar moiety is likely to play a crucial role in GV's interaction with histone H3. Thus, the key to understanding the unique mechanism of action of gilvocarcin-type anticancer drugs, is the further investigation of the nature of the DNA–GV–histone H3 complex (Figure 1) that interrupts the DNA replication and repackaging process necessary for cell division. In this context, our laboratory plans to generate new gilvocarcin analogues through combinatorial biosynthesis, in which either the sugar moiety or the C8 side chain will be altered. To achieve this, it is important to study the GT responsible for the C-glycosidic linkage of GV's D-fucofuranose moiety as well as the enzymes that contribute to the C8 vinyl group formation of GV.

One possible scenario that could lead to GV's C8 vinyl group is 1''-hydroxylation of a precursor with an ethyl residue, followed by dehydration. Alternatively, hydroxylation of the terminal 2'' position or a direct dehydrogenation of this ethyl to a vinyl residue can be envisaged. The gilvocarcin gene cluster revealed four oxygenase-encoding genes.[11] Previous inactivation experiments indicated that oxygenases GilOI and GilOIV were involved in the oxidative rearrangement of homo-2,3-dehydroUWM6 to the unique coumarin-based aromatic core of gilvocarcins.[12] Genetic alignment analysis studies revealed that *gilOIII* encodes a cytochrome P450-dependent enzyme that is probably involved in the formation of the C8 vinyl group, and that *gilGT* most likely encodes the C-glycosyltransferase (C-GT).[11] Through gene inactivation and analysis of accumulated products of resulting mutants, we herein describe the unambiguous identification of both this C-GT and the unique enzyme involved in the vinyl-group formation of GV biosynthesis. The two new mutants of *Streptomyces lividans* TK24 (mutated cosG9B3) were generated by in-frame deletion of the *gilGT* and *gilOIII* genes by using a variant of the PCR-targeting system.[12] Furthermore, we also describe cross-feeding experiments with the mutant products. The overall results not only shed more light on the biosynthetic pathway of GV, but also pave the way for further investigation of these unique enzymes.

Results

Sequence analysis

Database comparison of the *gilGT* gene product revealed close similarity to GTs. The highest homologies were found with LanGT2 (38% amino-acid identity), a GT presumed to be involved in attaching the first D-olivose to landomycin aglycon,[13–15] and UrdGT2 (31% amino-acid identity), which is the C-GT of the urdamycin biosynthetic pathway.[16–18] The previously reported putative GilGT protein had 495 amino acids, which is approximately 120 amino acids longer than any other polyketide GT found so far.[11] Because several attempts to generate a *gilGT* mutant by in-frame deletion failed (data not shown), we resequenced the entire region of the gene cluster and found a sequencing error (see Supporting Information). Genetic alignment analysis of the revised sequence revealed that the previously reported *gilGT* gene is indeed composed of two genes: the *gilV* gene (GenBank accession no. AY233211, protein ID ABE03981.1; gene product of 116 amino acids with unknown function) and the real *gilGT* gene (GenBank accession no. AY233211, protein ID AAP69578.2; encodes a 379 amino-acid long GT; Figure 2). The start codon of *gilGT* overlaps with the stop codon of *gilV*, which indicates coupled transcription of the two genes.

The *gilOIII* gene is located immediately downstream of *gilGT*. Database searches revealed that GilOIII belongs to the superfamily of cytochrome-P450 enzymes. It carries a cysteine residue at position 346, which is strictly conserved in all cytochrome-P450 enzymes. This residue is part of the FGXGXXCXG motif, which has been implicated in anchoring the ferric protoporphyrin (heme) cofactor. By far the greatest homology (42% amino-acid identity) of this protein was found to be with an enzyme from *Nocardioides* sp., strain KP7 (*orf4* gene product), which is presumably involved in phenanthrene degradation.[19] Significant

homologies were also observed with EthB (34% amino-acid identity), a cytochrome-P450 monooxygenase from *Rhodococcus ruber* involved in the degradation of ethyl *tert*-butyl ether (ETBE), [20] and to a number of bacterial cytochrome-P450 enzymes (> 30% amino-acid identity), including oxygenases of macrolide antibiotic biosyntheses pathways, for example, EryF, [21] TylI, [22] PicK (PikC), [23,24] and OleP. [25] All the latter enzymes catalyze the hydroxylation of aliphatic macro-lide structures. EthB is also believed to hydroxylate ETBE to form a hemiacetal intermediate, which spontaneously decomposes into acetaldehyde and *tert*-butanol. [20]

Generation and characterization of *GilOIII*[−] and *GilGT*[−] mutants

Inactivation of *gilOIII* and *gilGT* in cosmid G9B3 (cosG9B3: contains the apramycin-resistance cassette and the entire GV gene cluster) was carried out by using a modification of a recently developed PCR-targeting method. [12,26] Coding sequences of *GilOIII* or *GilGT* were replaced by the chloramphenicol (CHL) resistance cassette through double crossover. Subsequently, the CHL-resistance gene was eliminated by using a helper plasmid that expressed FLP(flippase) recombinase. This enzyme acted on the FRT (FLP recognition target sites) sequences that flanked the resistance gene. The mutants were selected for apramycin (Apr) resistance and CHL-sensitivity and were then transformed into *S. lividans* TK24 (Figure 3). CHL-gene cassettes flanked by down- and upstream genes of *gilOIII* or *gilGT* were introduced into *E. coli* BW25113/pKD20, which contained cosG9B3. The mutated cosG9B3 with the CHL-resistance gene in place of *gilGT* or *gilOIII* was isolated from colonies resistant to both Apr and CHL and introduced into *E. coli* XL-Blue MRF⁺/pCP20. The mutation of *gilOIII* was confirmed by PCR analysis; while the original *gilOIII* PCR product from cosG9B3 produced a fragment of 1500 bp, the mutant with the CHL-resistance gene gave a PCR product of 1338 bp. After FLP-mediated excision of the disruption cassette, a PCR product of 390 bp was obtained (see Supporting Information). Similarly, PCR analysis of the in-frame deletion of *gilGT* was also carried out and 1464, 1362, and 314 bp fragments were generated from cosG9B3, the mutant with the CHL-resistance gene, and the subsequent mutant without the resistance cassette, respectively (see Supporting Information). Both mutant cosG9B3 derivatives (cosG9B3-GT[−] and cosG9B3-OIII[−]) obtained in this way were introduced into *S. lividans* TK24 by conjugation and Apr resistance colonies were selected for further secondary-metabolite analysis. This resulted in the *gilOIII*[−] and *gilGT*[−] mutants, *S. lividans* TK24 (cosG9B3-OIII[−]) and *S. lividans* TK24(cosG9B3-GT[−]), respectively.

Complementation studies

Complementation of mutant *S. lividans* TK24(cosG9B3-GT[−]) was achieved with over-expression of the *gilGT* gene in this mutant by using plasmid pEM4*gilGT*. This led to a strain that mainly produced gilvocarcin E (GE; data not shown). While the glycosyltransferase activity was restored—as evidenced by the accumulation of GE in the above-mentioned *gilGT* complemented strain—the ethyl side chain of GE indicated a nonfunctional *GilOIII*. We suspected a polar effect to have been caused on *gilOIII* by the 81 bp FRT scar of the PCR-targeting system that had remained from the *gilGT* deletion experiment immediately upstream of *gilOIII*. Indeed, GV production was fully restored upon complementation of *S. lividans* TK24(cosG9B3-GT[−]) with both the *gilGT* and *gilOIII* genes (by using plasmid pEM4*gilGT-gilOIII*).

Identification of secondary metabolites accumulated by the mutant strains

GilOIII[−] mutant *S. lividans* TK24(cosG9B3-OIII[−]) accumulates GE as the major compound (5 mg mL^{−1}; Scheme 1) and gilvocarcin M (GM) as a minor product (0.5 mg mL^{−1}). The well-known compound GE was identified by comparison of its ¹H NMR, UV, and mass spectrum with literature data. [27] GE had a molecular mass of 496 g mol^{−1}, a yellow fluorescence under

UV light, and absorption maxima found in the UV spectrum at 245, 275, 307, and 384 nm, as reported by Balitz et al.[27] Compared with GV, GE's ^1H NMR spectrum showed a major difference in its C8 side chain, which displayed ethyl-group signals (q, $\delta_{1''\text{-H}} = 2.82$ (7.5); t, $\delta_{2''\text{-H}} = 1.29$ (7.5)) instead of the typical vinyl-group signals (Table 1). The minor compound GM (Scheme 1) had a UV spectrum identical to that of GE, but a mass of 482 g mol^{-1} , 14 amu less than defuco-GE, which is consistent with a C8 methyl group instead of an C8 ethyl group side chain. The GilGT $^-$ mutant, *S. lividans* TK24(cosG9B3-GT $^-$), accumulated defucogilvocarcin E (defuco-GE; 2 mg L^{-1}) as its major product and defucogilvocarcin M (defuco-GM; 0.2 mg L^{-1}) as a minor product. Defuco-GE also showed a yellow fluorescence under UV light and a similar UV spectrum as GE with absorption maxima at 243, 273, 306, and 375 nm; this indicates that the same chromophore is present as in GE. The atmospheric pressure chemical ionization (APCI) mass spectrum of defuco-GE (Scheme 1) revealed a molecular formula of $\text{C}_{21}\text{O}_5\text{H}_{18}$ (350 g mol^{-1}), which is consistent with GE without the sugar moiety. The ^1H NMR spectrum showed no sugar signals and exhibited an aromatic ABC system (2-H, 3-H, 4-H; Table 1) instead of the AB system found in GE, but was otherwise similar to that of GE. Also, the ^{13}C NMR data of defuco-GE widely matched those of the recently synthesized compound defuco-GM with the exception of the C8 side chain signals (Table 2). [28] The minor compound defuco-GM (Scheme 1) had a UV spectrum identical to that of defuco-GE, but a mass of 336 g mol^{-1} , 14 amu less than defuco-GE; this is consistent with a C8 methyl group instead of a C8 ethyl group side chain.

Cross-feeding experiments

GE, defuco-GE, and defucogilvocarcin V (defuco-GV) were fed (2 mg of each) to an early blocked mutant of GV biosynthesis, the GilOIV $^-$ mutant *S. lividans* TK24(cosG9B3-OIV $^-$). [12] We found that 72 h after inoculation, none of these metabolites could be converted to GV within 48 h (Scheme 2). Earlier experiments had shown that GilOIV catalyzes the first steps of the oxidative-rearrangement reaction cascade that converts an early angucyclinone to the unique benzo[d]naphtho[1,2-*b*]-pyran-6-one core that is typical for gilvocarcin antibiotics. Furthermore, the GilOIV $^-$ mutant still contained all other enzymes required for the conversion of intermediates accumulated through inactivation of later enzymes in the pathway to GV. [12]

Discussion

Based on genetic alignment GilOIII is closely related to EthB, which hydroxylates ETBE. Interestingly, EthB is not able to utilize methyl *tert*-butyl ether (MTBE) as a substrate; this indicates a clear preference for ethyl over methyl groups. Since GilOIII also resembles various other P450 oxygenases that hydroxylate aliphatic compounds, it is tempting to assume that GilOIII might be involved in the formation of the C8 vinyl group of GV by hydroxylating the ethyl side chain of a yet unknown gilvocarcin intermediate. On the other hand, the close resemblance of GilOIII to an oxygenase involved in the degradation of the aromatic hydrocarbon, phenanthrene, does not, from a mechanistic point of view, fit its proposed role as an aliphatic hydroxylase. This is because the phenanthrene-degrading enzyme encoded by *orf4* most likely hydroxylates an aromatic ring system. However, the overall structure of phenanthrene is very similar to the polyketide-derived “aglycon” portion of gilvocarcin, and the C-ring of phenanthrene, the proposed target of the *orf4* product, corresponds to the ethyl side chain of a hypothetical gilvocarcin intermediate. This suggests that the high homology of the *orf4* product with GilOIII could be caused by a similar substrate binding site and not by an analogous enzyme mechanism. The most frequently encountered function of cytochrome-P450 enzymes is as monooxygenases, which transfer one oxygen atom from O_2 to a substrate. However, it has also been found that some P450 enzymes catalyze very different reactions, including simple desaturation.[29,30] Thus the vinyl group of GV could be either generated

by a hydroxylation/elimination sequence, that is, most likely by two enzymes, or directly by a single desaturase. In both cases the reaction starts from an ethyl group of an unknown GV intermediate, possibly GE. The inactivation of *gilOIII* yielded GE, that is, a compound with an ethyl side chain. Thus, at this point we cannot distinguish between the two mechanistic alternatives. If the hydroxylation/elimination sequence is correct then a second enzyme or second-enzyme function is needed to catalyze a dehydration reaction. The deduced amino-acid sequence of *GilOIII* does not reveal such a second functionality. Furthermore, the *gilvocarcin* gene cluster did not reveal another gene candidate that encoded for such a dehydratase. However, the functions of the four products of *gilL*, *gilM*, *gilN*, and the newly discovered *gilV* still remain obscure.

The accumulation of GE upon inactivation of *gilOIII* provided direct evidence that *GilOIII* is the critical enzyme involved in the formation of the vinyl group of GV. However, cross-feeding GE to the early *GilOIV⁻* mutant showed that GE is not a biosynthetic intermediate of GV, but rather a shunt product. At this point, it is hard to determine the exact sequence of biosynthetic events within the context of the vinyl-group formation. This is because at least some of the enzymes that govern other post-PKS steps of GV biosynthesis, such as lactone formation, 10-*O*- and 12-*O*-methylation, and *C*-glycosylation, appear to be capable of converting intermediates with an ethyl instead of a vinyl group.

Bacteria synthesize a wide variety of natural products that are subsequently tailored by GTs as one of the key step in their biosynthesis.[1,3–5] In the majority of cases, natural GTs catalyze the *O*-glycosyltransfer steps. Only a few *C*-GTs involved in the biosynthesis of natural products have been characterized so far, such as, the pathogen-associated *C*-GT IroB[31] or UrdGT2.[16–18] *C*-Glycosylated products are of particular interest as they are stable against glycosidase degradation.[32] We resequenced the putative *gilGT* gene which was believed to encode the *C*-GT involved in the biosynthesis of GV. The new sequence revealed that the previous hypothetical *gilGT* gene was composed of a new, shorter *gilGT* gene that encodes for the *C*-GT enzyme with only 379 amino acids, and a new shorter gene, named *gilV*, that codes for a small enzyme of 116 amino acids with unknown function. The two genes *gilGT* and *gilV* are translationally coupled, which might indicate that the *gilV* product is somehow involved the formation of GV's sugar moiety. A targeted in-frame deletion of *gilGT* has now been accomplished and confirmed by PCR analysis. The accumulation of defuco-GE upon inactivation of *gilGT* proves that *GilGT* is responsible for the transfer of the deoxysugar, probably D-fucofuranose, to a yet unknown acceptor substrate, possibly a precursor of defucogilvocarcin. The cross-feeding experiments showed that neither defuco-GV[33] nor defuco-GE is an intermediate of the GV pathway. Therefore, it is likely that the glycosyltransfer step occurs earlier during GV biosynthesis, possibly on a still to be identified intermediate.[34] Thus, the accumulation of the shunt-product defuco-GE upon inactivation of *GilGT* reveals that other post-PKS tailoring steps, for example, *O*-methylation steps, can also occur in the absence of the sugar moiety. That the *GilGT⁻* mutant accumulated defuco-GE and not defuco-GV, was explained by a polar effect on the downstream *gilOIII* gene, whose corresponding enzyme is responsible for vinyl-group formation. This downstream effect was caused by the PCR-targeting system used for the in-frame deletion of *gilGT*, since both *gilOIII* and *gilGT* were necessary to complement the *GilGT⁻* mutant to restore GV production. To our knowledge, such a polar effect caused by the scar of the PCR-targeting system has never been reported before. We believe that the deletion of *gilGT* might have caused the elimination of the *gilOIII* ribosomal binding site, which is suspected to be located within the *gilGT* sequence near the stop codon.

The exact mechanism of the *C*-glycosylation reaction catalyzed by *GilGT* is still elusive. Two different mechanisms can be discussed for the aromatic *C*-glycosylation: i) initial *O*-glycosylation of a phenolic hydroxyl group followed by a Fries-like rearrangement, or ii) a

direct aromatic substitution of a nucleophilic carbon. Typically, the nucleophilicity of such a carbon is enhanced by a phenolic-OH group in either the *ortho*- or *para*-position. The Fries-like rearrangement mechanism is supported by synthetic model reactions[35] and appears reasonable for a rearrangement to a carbon in the *ortho*- position. However, it is less favorable for enzymatic glycosylation of a carbon in the *para*- position to the activating phenol, such as required for IroB and GilGT reactions.[31] Not only the in vitro studies with IroB[31] but also biotransformation experiments with UrdGT2[18] seemed to disfavor the Fries-like rearrangement mechanism—even though the latter is an enzyme that catalyzes a C-glycosylation to a carbon in the *ortho*- position of an activating phenolic-OH group.

In summary, we inactivated *gilOIII*, which encodes the unique P450 enzyme GilOIII, and *gilGT*, which encodes the novel C-glycosyltransferase GilGT, and unambiguously assigned their functions through analysis of the accumulated metabolites of mutants. Cross-feeding experiments with the accumulated products of these mutants (GE, defuco-GE, and defuco-GV) revealed that none of these metabolites was an intermediate of GV biosynthesis, but rather they were shunt products. Thus, the true pathway intermediates and substrates of GilOIII and GilGT remain ambiguous and future work with the over-expressed, isolated enzymes might clarify their substrates and their exact role in GV biosynthesis. Scheme 3 summarizes an updated hypothetical biosynthetic sequence of GV biosynthesis and takes into consideration all knowledge available at this point.

Experimental Section

Bacterial strains, cosmid, and culture conditions—Cosmid cosG9B3 was derived from plasmid pOJ446 and contained the entire gilvocarcin gene cluster. *S. lividans* TK24 was routinely cultured on M2 agar plates (1.5% agar, 0.4% glucose, 1% malt extract, 0.4% yeast extract, and 0.1% CaCO₃) until sporulation. The spores were stored in glycerol (20%) –80°C and used for conjugation. 2 × YT broth (pH 7.0) containing tryptone (1.6%), yeast extract (1%), and NaCl (0.5%) was used for conjugation.

E. coli XL1-Blue MRF' (Stratagene) was used for propagation of plasmids and cosmids and was grown in liquid (220 rpm) or on solid Luria–Bertani medium (pH 7.0) that contained tryptone (1%), yeast extract (0.5%), NaCl (1%), and agar (1.5%), at 37°C.

The Redirect® technology kit containing *E. coli* strains ET12567, ET12567/pUZ8002, BW25113, pKD20, pIJ790, and pCP20 was a gift from Plant Bioscience Ltd. (Norwich, UK).

Apr (50 µg mL⁻¹), CHL (25 µg mL⁻¹), nalidixic acid (25 µg mL⁻¹), carbenicillin (100 µg mL⁻¹), and kanamycin (50 µg mL⁻¹) were used for selection of recombinant strains.

DNA isolation, manipulation, and cloning—Standard procedures for DNA isolation and manipulation were performed according to standard protocols.[36] Isolation of DNA fragments from agarose gels and purification of PCR products were carried out by using the QIAquick® gel extraction kit by following the manufacturer's instructions. Isolation of the mutated derivatives of cosG9B3 was carried out by using ion-exchange columns (Nucleobond AX kits, Macherey–Nagel, PA, USA) according to the manufacturer's protocol.

Inactivation of *gilOIII* and *gilGT* by PCR-targeting system—To generate these mutants, the disruption gene cassettes were amplified by using the CHL-resistance gene in combination with primer pairs GilOIII_FRT_for (5'-TGATCGAAGACCTCCTCGCGCGAAAGGATGAAGCGGTGATTCCGGGGATCCGT CGACC-3') and GilOIII_FRT_rev (5'-CCGTCGATCCTGACCACCGTCACGTCCTCGACGGGCTCATGTAGGCTGGAGCTG CTTC-3') or GilGT_FRT_for (5'-

ACGCGACAAGGACCGGTCACGGGAGGGCGCCGCGTGAAGATTCCGGGGATCCG TCGACC-3') and *GilGT_FRT_rev* (5'-TCGAGGTGGGGGATCCTCGATGTGGAGATCACCGCTTCATGTAGGCTGGAGCTG CTTC-3'). For *gilOIII*, the underlined letters in the forward and reverse primers represent homologous extensions to DNA regions immediately up- and downstream of *gilOIII*, respectively, that include the putative start and stop codons. Underlined letters for the *gilGT* forward primer represent a homologous extension upstream of the gene and include the start codon, together with an additional 3 nucleotides in order to prevent truncating upstream genes. Underlined letters for the *gilGT* reverse primer represent a homologous extension immediately downstream of the gene and include the putative stop codon. The cassettes were introduced into *E. coli* BW25113/pKD20 which contained *cosG9B3* (Apr resistant); this cosmid includes the entire bio-synthetic gene cluster of GV. The disrupted *cosG9B3* with the CHL-resistance gene was introduced into *E. coli* XL-Blue MRF'/pcp20 in order to remove the resistance gene itself. Apr-resistant, CHL-sensitive colonies were identified by replica plating and verified by PCR analysis by using primer pairs *GilOIII_Ctrl_forw* (5'-AGGAGATGCTCGGGGATCCGTCC-3') and *GilOIII_Ctrl_rev* (5'-GACCGGATCAACCGCTCCACCTGC-3'), or *GilGT_Ctrl_forw* (5'-GATGGACAACTACCTGGACCTG-3') and *GilGT_Ctrl_rev* (5'-TGCAGGGCCGCGTACACCTCGT-3'). The mutated *cosG9B3gilGT⁻* or *cosG9B3gilOIII⁻* were introduced into *S. lividans* TK24 by conjugation from *E. coli* ET12567 that carry the nontransmissible pUZ8002, by using standard protocols.[37]

Generation of gene expression constructs—The *gilGT* or *gilGT + OIII* genes were obtained from *cosG9B3* by using PCR. Primers used for amplification of *gilGT* were 5'-CCGTCTAGAGGGAGGGCGCCGCATGAAGGCC-3' and 5'-CTGAATTCCGTCACGTCCTCGACGGGC-3'. For the amplification of *gilGT + OIII*, the same forward primer was used together with 5'-TGGGGAATTCTCGATGTGATCACCGCT-3' as reverse primer. Sites for *XbaI* and *EcoRI* are underlined on the forward and reverse primers, respectively. The purified PCR products *gilGT* or *gilGT + OIII* were digested with *XbaI* and *EcoRI* and cloned into the over-expression vector pEM4 which contains the strong promoter *ermEp**. [38] The resulting vectors, pEM4*gilGT* or pEM4*gilGT + gilOIII*, which contained a thiostrepton-resistance gene for selection in *Streptomyces*, were used for complementation of the *gilGT* mutant.

Above two vectors were introduced into the *gilGT* mutant by using PEG-mediated protoplast transformation.[18] Thiostrepton and Apr-resistant colonies were selected for product analysis.

Analysis, isolation, and characterization of secondary metabolites in *S. lividans* TK24(mutated *cosG9B3*) strains—All *S. lividans* TK24(mutated *cosG9B3*) strains were cultured in SG medium (20 g L⁻¹ glucose, 10 g L⁻¹ soy peptone, 2 g L⁻¹ CaCO₃, 1 mg L⁻¹ CoCl₂; pH 7.2 prior to sterilization) supplemented with Apr (50 mg L⁻¹). This preculture was grown for 1 day at 30°C and 220 rpm and used to inoculate the main culture of the same composition, which was harvested after 4 days of shaking as above. The culture broth was extracted three times with equal volumes of ethyl acetate. Extracts were dried in vacuo, dissolved in methanol, and examined by HPLC-MS.

Purification was achieved by semipreparative HPLC. HPLC-MS was performed on a Waters Alliance 2695 system with Waters 2996 photodiode array detector and a Micromass ZQ 2000 mass spectrometer equipped with an APCI ionization probe (solvent A = 0.1% formic acid in H₂O; solvent B = acetonitrile; flow rate = 0.5 mL min⁻¹; 0–6 min 75% A and 25% to 100% B (linear gradient), 7.5–10 min 75% A and 25% B). Semipreparative HPLC was run on a Waters Delta 600 instrument with a Waters 996 photodiode array detector (solvent A = H₂O; solvent B = acetonitrile; 0–2 min 100% A and 0% B, 2–4 min 100% to 60% A and 40% B (linear

gradient), 4–30 min 60% A and 40% B to 45% A and 55% B (linear gradient), 30–32 min 45% A and 55% to 100% B (linear gradient), 32–36 min 100% B, 36–38 min 100% A (linear gradient), 38–52 min 100% A). The columns used for HPLC were Waters Symmetry C18, 4.6 × 50 mm, particle size 5 µm (HPLC-MS), and Waters Symmetry Prep™ C18, 19 × 150 mm, particle size 5 µm (semiprep HPLC).

Purified compounds were characterized by mass spectrometry, UV, and NMR spectroscopy.

Cross-feeding experiments—For the feeding of GE, defuco-GV,[33] and defuco-GE to *S. lividans* TK24(cosG9B3-OIV[−]) cultures were prepared by using SG medium inoculated with spores of *S. lividans* TK24(cosG9B3-OIV[−]) in Erlenmeyer flasks (250 mL) containing media (100 mL). Cultures were then incubated in an orbital shaker for 120 h at 30°C and 250 rpm. GE, defuco-GE, and defuco-GV (2 mg each) were fed to the growing cultures 72 h after inoculation of spores. The cultures were grown for another 48 h after feeding. The HPLC control sample was taken before and just after the addition of the compounds as well as 48 h after feeding. All the peaks of the chromatograms were identified by HPLC-UV and HPLC-MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Structures of gilvocarcin V (GV), gilvocarcin M (GM), gilvocarcin E (GE), defucogilvocarcin V (defuco-GV), defucogilvocarcin E (defuco-GE), and defucogilvocarcin M (defuco-GM).

Figure 1.
Proposed cross-linking of DNA and histone H3 by GV; A: adenine; T: thymine.

Figure 2.

Biosynthetic gene cluster of GV. Oxygenases are shown in black and PKS genes in gray; genes encoding deoxysugar biosynthesis, glycosyl transfer, and other post-PKS modifications are represented with a diagonal grid pattern; other genes (regulatory or of unknown function) are in white.

Figure 3.

Application of the Redirect PCR-targeting system in cosG9B3 for the inactivation of *gilOIII*. FRT: FLP(flippase) recognition target; CHL: chloramphenicol-resistance gene; primer 1: *GilOIII_FRT_rev*; primer 2: *GilOIII_FRT_forw*; GT: glycosyltransferase-encoding gene; L: gene of unknown function (see also Figure 2). After an in-frame deletion a 81 bp long FRT scar remains.

Scheme 2.

Cross-feeding of products accumulated upon inactivation of *gilOIII* and *gilGT*, to an early mutant of the gilvocarcin-biosynthetic pathway (*GilOIV*⁻ mutant *S. lividans* TK24(cosG9B3-*OIV*⁻)). The experiments showed that neither of these accumulated products were intermediates of the pathway. The same mutant was shown previously to convert intermediate pregilvocarcin-*o*-quinone (Scheme 3) into GV.[12]

Scheme 3.

Proposed biosynthetic pathway to GV and shunt pathways to GE and defuco-GV after inactivation of GilOIII and GilGT, respectively. Homo-UWM6 and structures labeled with one letter (W–Z) are proposed main pathway intermediates; Y' is a proposed shunt pathway intermediate. All other compounds are fully characterized intermediates or shunt products. Defuco-GE branches off from Y' (not shown).

Table 1

¹H NMR (300 MHz, TMS) of GE and defuco-GE in [D₆]DMSO relative to TMS, multiplicity (*J* in Hertz).

position	δ [ppm]	
	GE	defuco-GE
1-OH	s, 9.70	s, 9.46
2	d, 6.93 (8.4)	dd, 6.93 (0.8, 8.8)
3	d, 8.06 (8.4)	t, 7.48 (8.8)
4	—	dd, 7.86 (0.8, 8.8)
7	d, 7.80 (2)	d, 7.83 (1.2)
9	d, 7.53 (2)	d, 7.44 (1.2)
10-OCH ₃	s, 4.13	s, 4.12
11	s, 8.47	s, 8.41
12-OCH ₃	s, 4.12	s, 4.12
1''-H	q, 2.82 (7.5)	q, 2.81 (7.6)
2''-H	t, 1.29 (7.5)	t, 1.29 (7.6)
1'	d, 6.19 (5.1)	—
2'	m, 4.67	—
2'-OH	br s, 4.53	—
3'-H	m, 3.82–3.90	—
3'-OH	br s, 5.12	—
4'-H	dd, 3.51 (3.9, 6)	—
5'-H	m, 3.82–3.90	—
5'-OH	br s, 4.84	—
6'-CH ₃	s, 1.24	—

Table 2¹³C NMR (75 MHz) data for defuco-GE in CDCl₃.

carbon	¹³ C δ [ppm]	carbon	¹³ C δ [ppm]
1	154.2	2''	15.4
2	112.6	9	113.5
3	128.5	10	157.1
4	121.6	10-OCH ₃	56.4
4a	126.3	10a	122.0
4b	146.2	10b	113.1
6	161.4	11	101.8
6a	123.2	12	152.5
7	121.6	12-OCH ₃	56.2
8	141.3	12a	114.7
1''	22.9	–	–