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Engineered Biosynthesis of Gilvocarcin Analogues with Altered Deoxyhexopyranose Moieties▽†

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A combinatorial biosynthetic approach was used to interrogate the donor substrate flexibility of GilGT, the glycosyltransferase involved in *C*-glycosylation during gilvocarcin biosynthesis. Complementation of gilvocarcin mutant *Streptomyces lividans* TK24 (cosG9B3-U⁻), in which the biosynthesis of the natural sugar donor substrate was compromised, with various deoxysugar plasmids led to the generation of six gilvocarcin analogues with altered saccharide moieties. Characterization of the isolated gilvocarcin derivatives revealed five new compounds, including 4-β-*C*-D-olivosyl-gilvocarcin V (D-olivosyl GV), 4-β-*C*-D-olivosyl-gilvocarcin M (D-olivosyl GM), 4-β-*C*-D-olivosyl-gilvocarcin E (D-olivosyl GE), 4-α-*C*-L-rhamnosyl-gilvocarcin M (polycarcin M), 4-α-*C*-L-rhamnosyl-gilvocarcin E (polycarcin E), and the recently characterized 4-α-*C*-L-rhamnosyl-gilvocarcin V (polycarcin V). Preliminary anticancer assays showed that D-olivosyl-gilvocarcin and polycarcin V exhibit antitumor activities comparable to that of their parent drug congener, gilvocarcin V, against human lung cancer (H460), murine lung cancer (LL/2), and breast cancer (MCF-7) cell lines. Our findings demonstrate GilGT to be a moderately flexible *C*-glycosyltransferase able to transfer both D- and L-hexopyranose moieties to the unique angucyclinone-derived benzo[D]naphtho[1,2*b*]pyran-6-one backbone of the gilvocarcins.

Gilvocarcin V (GV) (structure 2; see Fig. 1 for numbered structures), the principal product of *Streptomyces griseoflavus* Gö 3592 and other *Streptomyces* spp., is the most prominent member of a distinct class of antitumor antibiotics that share a polyketide-derived coumarin-based benzo[D]naphtho[1,2-*b*]pyran-6-one moiety. This small family of antitumor drugs is often referred to as gilvocarcin-type anticancer drugs (5, 10, 26, 41). Most of these natural products possess *C*-glycosidically linked 6-deoxy-D-hexose moieties in the 4-position, while variants within this group exist as either furanose (structures 1 to 6, structure 13) or pyranose sugars (structures 7 to 12 and 14 to 16) (5, 27, 38, 43, 45). Notably, BE-12406A (structure 14) and B (structure 15) (15, 25), as well as polycarcin V (structure 16) (16), are the only examples of 6-deoxy-L-sugars found in gilvocarcin-type compounds, with structures 14 and 15 representing the only *O*-glycosidically linked analogues reported so far.

GV's most likely mechanism of action is a photoactivated [2 + 2] cycloaddition of its vinyl side chain with thymine residues of DNA caused by near-UV or visible blue light which results in single-strand scissions leading to covalent binding with DNA (1, 3, 9, 14, 24). In addition, GV's activity is also attributed to a unique selective cross-linking of DNA and histone H3, a core component of the histone complex that plays an important role for DNA replication and transcription (2, 8, 22, 23, 30). The saccharide moiety, D-fucofuranose, of GV is essential for this activity, as it is believed to facilitate binding of histone H3 (17, 18).

Recently, we identified and characterized largely the deoxysugar biosynthetic pathway that leads to D-fucofuranose (structure 26), the sugar moiety of GV (18). It was possible to shut down the biosynthesis of gilvocarcin's natural sugar donor through the inactivation of *gilU*, the 4-ketoreductase involved in the biosynthesis of GV's deoxysugar moiety. The inactivation of *gilU* afforded a more active gilvocarcin analogue, 4′-hydroxy gilvocarcin V (structure 5, 4′-OH GV), illustrating an inherent substrate flexibility of GilGT, the glycosyltranferase responsible for *C*-glycosylation in GV biosynthesis (17, 18). Additionally, the improved bioactivity of 4′-OH GV over that of GV clearly demonstrated the importance of further investigations into modifying the glycosylation pattern of gilvocarcins.

In our initial attempt to probe the donor substrate flexibility of GilGT and advance the glycodiversity of gilvocarcin-type anticancer drugs, we complemented the aforementioned GilU mutant, *Streptomyces lividans* TK24 (cosG9B3-U⁻), with various deoxysugar plasmids directing the biosynthesis of neutral deoxysugars, namely, L-olivose (pLN2) (32), L-rhamnose (pRHAM) (33), L-mycarose (pFL942) (19), L-digitoxose (pLNBIV) (32), 4-keto-L-olivose (pKOLV), and D-oliose (pOLO). These plasmids are designed for producing specific activated deoxysugars through the combination of genes from one or more deoxysugar biosynthetic pathways, and they have been utilized in successfully altering the saccharide moieties of mithramycin, steffimycin, elloramycin, and rebeccamycin/staurosporin (4, 28, 31, 35, 36). We were particularly interested in the attachment of L-hexose sugars, as these are rarely found in gilvocarcin-type anticancer drugs. Of the six deoxysugar plasmids tested, complementation with pLN2 and

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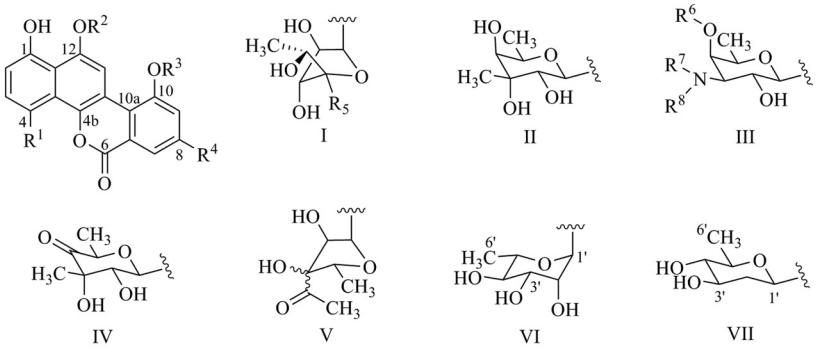

1: R^1=I; R^2,R^3,R^4=CH_3; R^5=H
2: R^1=I; R^2,R^3=CH_3; R^4=$CHCH_2$; R^5=H
3: R^1=I; R^2,R^3=CH_3; R^4=CH_2CH_3; R^5=H
4: R^1=I; R^2,R^3,R^4=CH_3; R^5=OH
5: R^1=I; R^2,R^3=CH_3; R^4=$CHCH_2$; R^5=OH
6: R^1=I; R^2,R^3=CH_3; R^4=CH_2CH_3; R^5=OH
7: R^1=II; R^2,R^3=CH_3; R^4=$CHCH_2$
8: R^1=III; R^2,R^3,R^7,R^8=CH_3, R^4=$CHCH_2$, R^6=$COCH_3$
9: R^1=III; R^2,R^3,R^7,R^8=CH_3, R^4=$CHCH_2$; R^6=H
10: R^1=III; R^2,R^3=CH_3, R^4=$CHCH_2$; R^6,R^8=H; R^7=$COCH_3$
11: R^1=III; R^2,R^3=CH_3, R^4=$CHCH_2$; R^8=H; R^6,R^7=$COCH_3$
12: R^1=IV; R^2,R^3=CH_3; R^4=$CHCH_2$
13: R^1=V; R^2,R^3=CH_3; R^4=$CHCH_2$
14: R^1=H; R^2=VI; R^3,R^4=CH_3
15: R^1,R^3=H; R^2=VI; R^4=CH_3
16: R^1=VI; R^2,R^3=CH_3; R^4=$CHCH_2$
17: R^1=VI; R^2,R^3=CH_3; R^4=CH_3
18: R^1=VI; R^2,R^3=CH_3; R^4=CH_2CH_3
19: R^1=VII; R^2,R^3,R^4=CH_3
20: R^1=VII; R^2,R^3=CH_3; R^4=$CHCH_2$
21: R^1=VII; R^2,R^3=CH_3; R^4=CH_2CH_3
22: R^1 =H; R^2, R^3 =CH_3; R^4=CH_3
23: R^1 =H; R^2, R^3 =CH_3; R^4=$CH{=}CH_2$
24: R^1 =H; R^2, R^3 =CH_3; R^4=CH_2CH_3

FIG. 1. Glycodiversity of gilvocarcin-type anticancer drugs. Gilvocarcins (structures 1 to 3), 4′-OH gilvocarcins (structures 4 to 6), chrysomycin V (structure 7), ravidomycin V (structure 8), deacetylravidomycin V (structure 9), FE35A (structure 10) and B (structure 11), Mer1020 dC (structure 12) and dD (structure 13), BE-12406A (structure 14) and B (structure 15), polycarcins (structures 16 to 18), D-olivosyl-gilvocarcins (structures 19 to 21), and defucogilvocarcins M, V, and E (structures 22 to 24).

pRHAM produced new gilvocarcin analogues with D-olivosyl and L-rhamnosyl moieties, respectively.

MATERIALS AND METHODS

Microorganisms and culture conditions. All complementation experiments were carried out in the heterologous host *S. lividans* TK24 (13). The mutant cosmid cosG9B3-U$^-$ was introduced into *S. lividans* TK24 through conjugal transfer according to standard protocols (13), producing *S. lividans* TK24 (cosG9B3-U$^-$). Conjugation was carried out on MS agar (13) supplemented with 10 mM $MgCl_2$ and overlaid with nalidixic acid with appropriate antibiotics after 18 h. Exconjugates were grown on solid M2 medium (4 g/liter glucose, 10 g/liter malt extract, 4 g/liter yeast extract, 1g/liter $CaCO_3$, 15 g/liter agar) supplemented with appropriate antibiotics. *S. lividans* TK24 (cosG9B3-U$^-$) was transformed via protoplast transformation with deoxysugar plasmids (pLN2, pRHAM, pLNBIV, pFL942, pKOLO, and pOLO) according to standard protocols (13). Protoplasts were regenerated on R2YE agar medium and overlaid after 18 h with R3 soft agar (171 g/liter sucrose, 10 g/liter glucose, 4 g/liter peptone, 0.5 g/liter K_2SO_4, 8.1 g/liter $MgCl_2 \cdot 6H_2O$, 2.2 g/liter $CaCl_2$, 8.8 g/liter agar) supplemented with appropriate antibiotics (13). Regenerated protoplasts were transferred to solid M2 agar supplemented with appropriate antibiotics. Initially, each strain was grown in 250-ml baffled Erlenmeyer flasks containing 100 ml of liquid SG medium (20 g/liter glucose, 10 g/liter soy peptone, 2 g/liter $CaCO_3$, 0.001 g/liter cobalt-II chloride, pH 7.2) with appropriate antibiotics and screened for gilvocarcin analogue production using high-performance liquid chromatography–mass spectrometry (HPLC-MS) as described previously (11). *Escherichia coli* XL1-Blue (Stratagene) was used as the subcloning host and was grown at 37°C in lysogeny broth medium, while *E. coli* ET12567/pUZ8002 (21, 29) was used for conjugation. When antibiotics were required for strain selection, 50 μg/ml of kanamycin, 100 μg/ml of ampicillin, 50 μg/ml of apramycin, and 25 μg/ml of thiostrepton were used.

DNA manipulation and PCR amplification. Plasmid DNA isolations were carried out using the GeneJet plasmid miniprep kit (Fermentas). All restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other DNA manipulations were performed according to standard protocols (37). PCR was used to amplify *mtmU* from *Streptomyces argillaceus* ATCC 12596 (mithramycin producer) using the specific oligonucleotide primer mtmU_F (5′-ACTAGTAGAAGGAGCGCCGTGCCCG) (SpeI site is underlined). PCR conditions were as follows: 50 ng of template DNA was mixed with 50 ng of each primer with 1.25 units of Native *PFU* DNA polymerase (Stratagene) in a total volume of 50 μl containing 0.4 mM each deoxynucleoside triphosphate (dNTP), 2 mM $MgCl_2$, and 5 μl dimethyl sulfoxide (DMSO). The normal PCR thermocycler conditions were 60 s at 98°C followed by 30 cycles of 60 s at 98°C, 45 s at 65°C, and 90 s at 76°C and then 10 min at 76°C. The PCR product was gel purified using a QIAquick gel extraction kit (Qiagen), subcloned into PCR-Blunt II-TOPO (Invitrogen), and sequenced (SeqWright, Houston, TX).

Plasmid constructs. Deoxysugar plasmid pKOLV was constructed by the deletion of *oleU*, as a SpeI-NheI fragment, from pLN2 and then self-ligated (Table 1). To produce pOLO, *oleU* from pLN2 was replaced with *mtmU* as a SpeI-NheI fragment.

Production, isolation, and purification of gilvocarcin analogues. Strains producing novel gilvocarcin analogues were grown as seed cultures in 250-ml baffled Erlenmeyer flasks containing 100 ml liquid SG medium, as described above, and allowed to grow at 28°C for 48 h at 250 rpm. The seed cultures of *S. lividans* TK24 (cosG9B3-U$^-$/pLN2) and *S. lividans* TK24 (cosG9B3-U$^-$/pRHAM) were used to inoculate 60 liters and 80 liters of liquid SG medium, respectively. After 5 days

TABLE 1. Plasmid constructs generated in this work

Plasmid	Genes	Description
pKOLO	*oleV oleW oleY oleL oleS oleE*	pLN2 was digested with SpeI and NheI and then religated
pOLO	*oleV oleW mtmU oleY oleL oleS oleE*	pLN2 was digested with SpeI and NheI and replaced with *mtmU* with the same flanking restriction sites

TABLE 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for D-olivosyl-gilvocarcin M (structure 19) DMSO-d_6

Position	δ_H (J in Hz)	δ_C, mult.[d]
1		152.9, C
1-OH	9.69, s	
2	6.90, d (8.5)	111.9, CH
3	7.71, d (8.0)	129.4, CH
4		126.6, C
4a		122.3, C
4b		141.0, C
6		159.3, C
6a		121.1, C
7	7.58, s	120.4, CH
8		140.2, C
8-CH_3	2.39, s	21.1, CH_3
9	7.20, s	118.5, CH
10		156.6, C
10-OCH_3	3.96, s	56.0, CH_3
10a		121.0, C
10b		113.4, C
11	8.22, s	101.6, CH
12		151.6, C
12-OCH_3	4.02, s	56.2, CH_3
12a		114.6, C
1′	5.63, d (10.5)	75.0, CH
2′-H_a	1.30, dd (11.5, 11.5)[a]	43.2, CH_2
2′-H_e	2.36, m[c]	
2′-OH		
3′	3.80, m	72.0, CH
3′-OH	4.73, d (4.5)[b]	
4′	2.91, dd (9.0, 8.5)[a]	77.8, CH
4′-OH	4.96, d (5.0)[b]	
5′	3.49, m	76.0, CH
6′-H_3	1.27, d (6.0)	18.7, CH_3
1″		
2″-H_e		
2″-H_z		

[a] Coupling constants calculated after D_2O exchange.
[b] Signal lost after D_2O exchange.
[c] Signal partially obscured.
[d] mult., multiplicity.

TABLE 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for D-olivosyl-gilvocarcin V (structure 20) DMSO-d_6

Position	δ_H (J in Hz)	δ_C, mult.[c]
1		153.0, C
1-OH	9.75, s	
2	6.96, d (8.5)	112.4, CH
3	7.76, d (8.5)	129.6, CH
4		126.9, C
4a		122.8, C
4b		141.7, C
6		159.4, C
6a		122.5, C
7	7.97, s	119.2, CH
8		138.9, C
8-CH_3		
9	7.71, s	114.6, CH
10		157.4, C
10-OCH_3	4.15, s	56.3, CH_3
10a		122.2, C
10b		113.4, C
11	8.45, s	101.8, CH
12		151.9, C
12-OCH_3	4.10, s	56.7, CH_3
12a		115.0, C
1′	5.73, d (11.0)	75.0, CH
2′-H_a	1.30, dd (11.5, 11.5)[a]	43.1, CH_2
2′-H_e	2.33, m	
2′-OH		
3′	3.81, m	72.0, CH
3′-OH	4.73, d (5.0)[b]	
4′	2.89, dd (9.0, 8.5)[a]	77.8, CH
4′-OH	4.94, d (5.0)[b]	
5′	3.47, m	76.0, CH
6′-H_3	1.26, d (6.0)	18.6, CH_3
1″	6.93, dd (17.5, 11.0)	135.2, CH
2″-H_e	6.14, d (18.0)	122.0, CH_2
2″-H_z	5.50, d (11.0)	

[a] Coupling constants calculated after D_2O exchange.
[b] Signal lost after D_2O exchange.
[c] mult., multiplicity.

of fermentation at 28°C with reciprocal shaking (250 rpm), the flasks were removed and combined with celite (100 g/liter) and filtered. The mycelial cake was extracted with acetone by sonication and again filtered. The acetone was removed under vacuum, and the aqueous portion was combined with the culture broth filtrate and passed through C_{18} reverse-phase silica. The column was eluted with 1-liter fractions of 0, 25, 50, 75, and 100% methanol in water. Fractions containing gilvocarcin analogues were dried and lyophilized. Further purification was obtained through high-performance liquid chromatography (HPLC) according to previously described procedures (11).

NMR analysis. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data (Tables 2 and 3; see Table S7 in the supplemental material) were recorded on a Varian Vnmr 500 spectrometer, operating on 500 MHz (^{1}H) and 125 MHz (^{13}C), respectively, using the indicated deuterated solvents.

RESULTS

***In vivo* coexpression of *S. lividans* TK24 (cosG9B3-U$^-$) and pLN2.** The well-established deoxysugar plasmid pLN2 is responsible for producing the TDP-activated 2,6-dideoxy-hexopyranose, TDP-L-olivose (structure 29) (32). The biosynthesis of TDP-L-olivose involves two unique 2,6-dideoxy-4-ketosugar intermediates, one in D- and the other in L-configuration, which can further be reduced to TDP-D- or TDP-L-olivose. In addition, the ketosugars themselves may also serve as acceptable donor substrates for GilGT (Fig. 2), as was shown previously for the 4-keto-D-fucofuranose (18).

Fermentation of the recombinant mutant strain *S. lividans* TK24 (cosG9B3-U$^-$/pLN2) accumulated three new peaks, structures 19, 20, and 21 (Fig. 3b). Large-scale fermentation and preparative HPLC were used to isolate the new compounds, which were structurally characterized through NMR spectroscopy and mass spectrometry. A 60-liter fermentation of *S. lividans* TK24 (cosG9B3-U$^-$/pLN2) afforded 4 mg of the major peak, D-olivosyl-gilvocarcin M (structure 19; ~0.07 mg/liter). MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) high-resolution mass spectrometry of structure 19 gave m/z = 466.1590 ($[M+H]^+$ $C_{26}H_{25}O_8$, requires 466.1549). The ^{1}H and ^{13}C NMR of structure 19 clearly showed the presence of the gilvocarcin chromophore; however, new sugar signals were observed (Tables 2 and 3). We expected the sugar to be L-olivose; however, two-dimensional (2D) NMR (correlation spectroscopy [COSY], nuclear Overhauser enhancement spectroscopy [NOESY], heteronuclear multiple-bond correlation [HMBC], and heteronuclear single quantum correlation [HSQC]) experiments confirmed the sugar residue to be D-olivose (Fig. 4A). From HMBC correlations, we found clear evidence of *C*-glycosidic attachment at the C-4 position

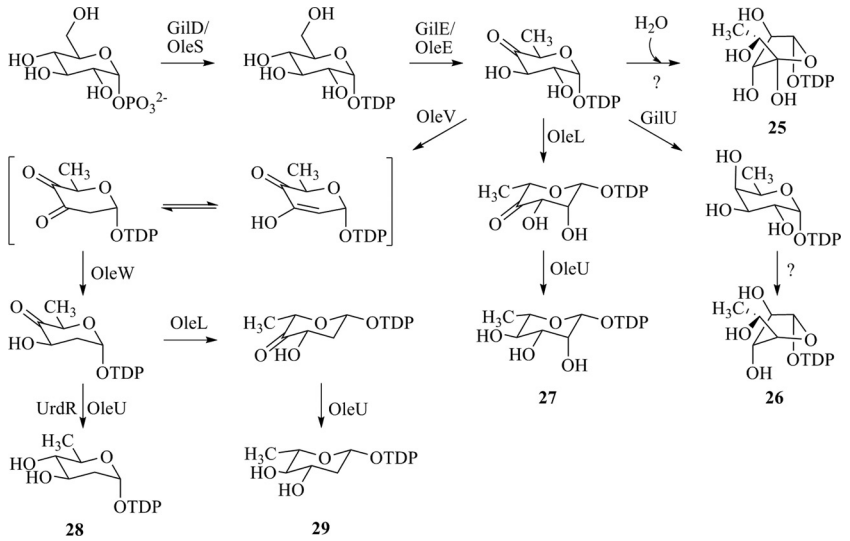

FIG. 2. Biosynthesis of TDP-4′-OH-D-fucofuranose (structure 25), TDP-D-fucofuranose (structure 26), TDP-L-rhamnose (structure 27), TDP-D-olivose (structure 28), and TDP-L-olivose (structure 29).

($^2J_{C\text{-}H}$ coupling between 1′-H and C-4), while the typical $^4C_1$-conformation of D-sugars was confirmed through NOESY correlations between 1′-H, 3′-H, and 5′-H and between 2′-H_a and 4′-H (Fig. 4B). Assuming GilGT belongs to the GT-1 family of inverting glycosyltransferases (6, 7, 42), the use of TDP-D-olivose as a donor substrate should result in a glycosidic bond with β-D-configuration, as evident from the large coupling constant of C-1′ (δ 5.63, J = 10.5 Hz).

The remaining minor compounds were identified as D-olivosyl-gilvocarcin V (structure 20; ~0.05 mg/liter) and D-olivosyl-gilvocarcin E (structure 21; ~0.01 mg/liter). As before, structural elucidation of structure 20 was carried out through NMR (Table 3) and mass spectrometry (high-resolution mass spectrometry [HRMS] [MALDI-TOF+] m/z = 478.1490; $[M+H]^+$ $C_{27}H_{25}O_8$, requires 478.1549). Due to the poor production yield of structure 21, we were unable to confirm its structure through NMR and instead relied only on mass spectrometry data (atmospheric pressure chemical ionization [APCI] m/z = 480; $[M+H]^+$ $C_{27}H_{27}O_8$, requires 480.1706) to propose the presence of D-olivosyl-gilvocarcin E. This postulation is indirectly supported by the fact that gilvocarcins (structures 1 to 3) are naturally produced as three congeners differing only in their side chains attached at C-8 (39). This is also illustrated in the host strains' ability to produce compounds 4 to 6, as seen in Fig. 3a.

***In vivo* coexpression of *S. lividans* TK24 (cosG9B3-U$^-$) and pRHAM.** The deoxysugar plasmid pRHAM is responsible for producing the TDP-activated 6-deoxy-hexopyranose, TDP-L-rhamnose (structure 27) (33). In comparison with pLN2, pRHAM lacks the genes responsible for 2-deoxygenation (*oleV* and *oleW*) and therefore leads to only a single L-4-ketosugar intermediate. Since all gilvocarcin-type compounds discovered thus far contain 2-hydroxy groups in their sugar moieties, pRHAM encoding a TDP-6-deoxyhexopyranose was considered a more conservative attempt to modify the gilvocarcin

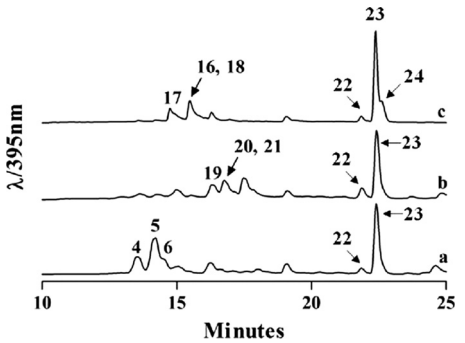

FIG. 3. HPLC trace of *S. lividans* TK24 (cosG9B3-U$^-$) (a), *S. lividans* TK24 (cosG9B3-U$^-$/pLN2) (b), and *S. lividans* TK24 (cosG9B3-U$^-$/pRHAM) (c).

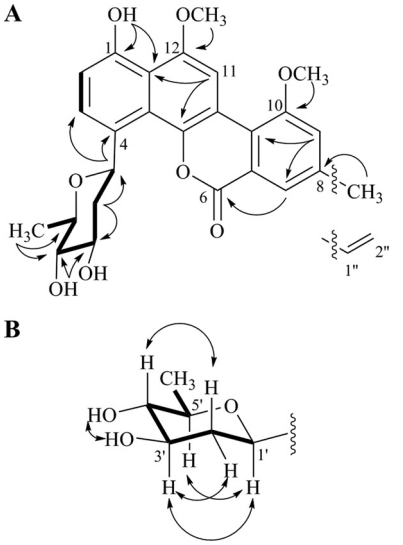

FIG. 4. 2D NMR results for D-olivosyl-gilvocarcin M highlighting COSY (thick line) and selected HMBC (→) (A) as well as NOESY (↔) (B) correlations.

sugar moiety. It was only after preliminary structural characterization of structure 17 that polycarcin V (structure 16) was reported as a natural secondary metabolite of *Streptomyces polyformus* (16).

Fermentation of the recombinant mutant strain *S. lividans* TK24 (cosG9B3-U$^-$/pRHAM) accumulated three new peaks, structures 16, 17, and 18 (Fig. 3c). As before, large-scale fermentation and preparative HPLC were used to isolate all new compounds which were then characterized through NMR and mass spectrometry. Due to poor yields, an 80-liter fermentation of *S. lividans* TK24 (cosG9B3-U$^-$/pRHAM) was needed to isolate 4 mg of the major peak, L-rhamnosyl-gilvocarcin M (= polycarcin M; structure 17; ~0.05 mg/liter). To simplify future discussions on the growing collection of gilvocarcin analogues, structures 17 and 18 will be referred to as polycarcin M and polycarcin E, as they are congeners of the recently reported polycarcin V (16). HRMS (MALDI-TOF+) of structure 17 gave m/z = 482.1540 ($[M+H]^+$ $C_{27}H_{27}O_8$, requires 482.1499), which was in agreement with the attachment of L-rhamnose. The structure was confirmed by ^1H and ^{13}C NMR (see Fig. S5 and S6 and Table S7 in the supplemental material), as well as various 2D NMR experiments (COSY, NOESY, HMBC, and HSQC). The sugar moiety, L-rhamnose, was further confirmed through comparisons of reported NMR data from polycarcin V (16).

The remaining minor compounds were identified as polycarcin V (L-rhamnosyl-gilvocarcin; structure 16; ~0.03 mg/liter) and polycarcin E (L-rhamnosyl-gilvocarcin E; structure 18; ~0.01 mg/liter). The ^1H NMR, ^{13}C NMR, and HRMS (MALDI-TOF+) of structure 16 matched those previously reported for polycarcin V (16). Due to the limited quantity of structure 18 isolated (<1 mg) from large fermentations, we relied on APCI mass spectrometry (m/z = 496; $[M+H]^+$ $C_{27}H_{27}O_8$, requires 496.1655) to propose its structure.

The host strain *S. lividans* TK24 (cosG9B3-U$^-$) as well as the two recombinant strains *S. lividans* TK24 (cosG9B3-U$^-$/pLN2) and *S. lividans* TK24 (cosG9B3-U$^-$/pRHAM) also produced significant amounts of the previously described (17) sugar-free defucogilvocarcins, defucogilvocarcin M (structure 22), defucogilvocarcin V (structure 23), and defucogilvocarcin E (structure 24) (Fig. 3).

***In vivo* coexpression of *S. lividans* TK24 (cosG9B3-U$^-$) and pLNBIV, pFL942, pKOLV, and pOLO.** The deoxysugar plasmids pLNBIV and pFL942 have been shown to direct biosynthesis toward L-digitoxose and L-mycarose, respectively (19, 32). When these plasmids were used to complement *S. lividans* TK24 (cosG9B3-U$^-$), no new metabolites were observed. This indicates that GilGT is unable to transfer either L-digitoxose or L-mycarose to the gilvocarcin backbone. Similarly, the newly constructed plasmids pKOLV (4-keto-L-olivose) and pOLO (D-oliose) did not yield new products. We anticipated that the removal of *oleU* (4-ketoreductase) from pLN2 would result in 4-keto-L-olivose while the replacement of *oleU* with *mtmU* (4-ketoreductase that forms the axial 4-OH in D-oliose biosynthesis) would result in D-oliose. The products of plasmids pKOLV and pOLO have not yet been verified; therefore, we cannot claim with certainty that the expected sugars were produced. However, based on their derivation from pLN2, a well-established deoxysugar plasmid, we hypothesize that GilGT cannot utilize 4-keto-L-olivose or D-oliose.

TABLE 4. Anticancer activity assays[a] of gilvocarcins

Gilvocarcin	Anticancer activity (% T/C at 100 μM)[b]		
	H460	MCF-7	LL/2
Gilvocarcin V (structure 2)	5.5 ± 0.3	4.5 ± 1.0	1.4 ± 0.4
4′-OH-gilvocarcin V (structure 5)	2.6 ± 0.5	7.0 ± 2.5	1.4 ± 0.5
Polycarcin V (structure 16)	2.3 ± 1.0	8.9 ± 0.4	4.8 ± 5.3
D-Olivosyl-gilvocarcin V (structure 20)	1.2 ± 0.2	2.5 ± 0.4	3.4 ± 2.1

[a] Sulforhodamine B assay (34, 40); the numbers represent the mean results for three assays.

[b] % T/C indicates the percentage of surviving treated cancer cells over untreated control cells; % T/C < 32 at 100 μM is considered active, according to NCI standards (34, 40).

Bioactivity study. Preliminary anticancer assays against human lung (H460), human breast (MCF-7), and murine lung (LL/2) cancer cell lines using the sulforhodamine B assay (34, 40) indicated that structure 16 has anticancer activity that is comparable to those of structures 2 and 5; however, structure 20 showed slightly better anticancer activity toward cancer cell lines H460 and MCF-7 than structures 2 and 5 (Table 4). Additionally, dose-dependent curves showed that structures 16 and 20 have 50% growth-inhibitory (GI_{50}) values comparable to (H460) or higher than (MCF-7 and LL/2) that of parent congener structure 2. It should be noted that in all cell lines structure 5 received the lowest GI_{50} values (data not shown).

DISCUSSION

The bioactivity of glycosylated natural products produced by actinomycetes, in many cases, can be attributed to their saccharide moiety. Recently, glycodiversification studies have garnered much attention, with the overall goal of generating more bioactive compounds or compounds with improved pharmacological properties (20, 42, 44). In this study, we chose to probe the donor substrate flexibility of GilGT, the glycosyltransferase involved in gilvocarcin biosynthesis. The recent generation of *S. lividans* TK24 (cosG9B3-U$^-$) provided a suitable model host for probing GilGT flexibility, as cosG9B3-U$^-$ lacks the genes required for the production of the natural D-fucofuranose moiety of the natural gilvocarcins. By expressing deoxysugar plasmids in *S. lividans* TK24 (cosG9B3-U$^-$), we were able to supply GilGT with alternative donor substrates for glycosylation.

The complementation of *S. lividans* TK24 (cosG9B3-U$^-$) with pLN2 and pRHAM resulted in the production of five novel compounds, including D-olivosyl-gilvocarcins M, V, and E as well as polycarcins M and E. The recently reported polycarcin V was isolated as a natural metabolite from *S. polyformus*; however, its side chain derivatives were not reported (16). Here we report the engineered production of polycarcin V as well as its methyl and ethyl side chain congeners.

The engineered D-olivosyl-gilvocarcins discussed here are the first examples of gilvocarcin-type anticancer drugs with a neutral multiply deoxygenated sugar moiety. The finding that D- and not L-olivose was the attached sugar moiety of these compounds was unexpected and surprising, since pLN2 encodes the biosynthetic pathway to TDP-L-olivose. We assume either that the 4-ketoreductase OleU, encoded by pLN2, can

act on the intermediate TDP-4-keto-6-deoxy-D-olivose or that the latter was reduced by a ketoreductase naturally present in *S. lividans* before it could be converted to 4-keto-6-deoxy-L-olivose by the 3,5 epimerase OleL. Originally, pLN2 was tested with the flexible glycosyltransferase ElmGT from the elloramycin biosynthetic pathway, which resulted in the exclusive production of L-olivosyl-tetracenomycin C (32). In the presence of ElmGT, a GT which naturally transfers L-rhamnose, the biosynthetic pathway favored the production of NDP-L-olivose. However, in the gilvocarcin pathway it is clear that L-olivose cannot be utilized by GilGT, which naturally transfers a D-sugar, and instead it scavenges the limited amount of D-olivose produced by pLN2, generating a small flux toward D-olivose and, therefore, isolable amounts of D-olivosyl-gilvocarcins.

The results presented here provide further examples of successful glycodiversification efforts by utilizing deoxysugar plasmids and relying on the (here limited) flexibility of a glycosyltransferase. We have established GilGT as being a moderately flexible glycosyltransferase able to accept its yet-undetermined natural donor substrate D-fucofuranose or D-fucopyranose as well as D-olivose and L-rhamnose. Additionally, this work demonstrates how substrate preference can shift the biosynthetic flux toward an unexpected outcome, further highlighting the intricacies of engineered biosynthetic approaches to produce new natural product analogues. The fact that several other deoxysugar plasmids failed to trigger the production of new gilvocarcins, however, shows that this method of glycodiversification has its limits when being applied to the gilvocarcin biosynthetic pathway. Besides the limited flexibility of GilGT, the apparently narrow substrate specificity of the terminal enzyme of the gilvocarcin pathway, oxidoreductase GilR (12), may cause problems, and we are currently further investigating this gatekeeper enzyme with the long-term goal to reengineer it toward broader substrate specificity.

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