# Multi-Oxygenase Complexes of the Gilvocarcin and Jadomycin Biosyntheses

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## Supporting Information

## Materials and Methods

### I. Microorganisms and culture conditions

*S. lividans* TK24 strains were cultured in 100 ml YEME medium in 250 mL baffled Erlenmeyer shaker flasks to prepare protoplast. Transformation of *Streptomyces* protoplast with plasmids was carried out according to standard protocols.[1, 2] Protoplasts were regenerated on R2YE agar medium. Transformation plates were overlaid with R3 soft agar (171 g/L sucrose, 10 g/L glucose, 4g/L peptone, 0.5 g/L potassium chloride, 8.1 g/L magnesium chloride hexahydrate, 2.2 g/L calcium chloride, 8.8 g/L agar) supplemented with appropriate antibiotics. For the analyses of the metabolites from the mutant strains, the strains were cultured in SG liquid medium (20 g/L glucose, 10 g/L soy peptone, 2 g/L calcium carbonate, 0.001 g/L cobalt-II-chloride, pH 7.2) for 4 days at 30 °C and 200 rpm. The antibiotics (thiostreptone 20 µg/mL and apramycin 50 µg/mL) were supplemented in the medium whenever necessary. For the jadomycin production studies, sterile L-isoleucine was added to SG-medium (0.3% final concentration) prior to the inoculation of the seed culture. For a solid culture, *S. lividans* strains were grown in M2-agar medium (4 g/L glucose, 10 g/L malt extract, 4 g/L yeast extract, 1g/L calcium carbonate, 15 g/L agar) at 37 °C. Seed cultures of *S. venezuelae* ISP5230 and *S. venezuelae* CH56 were carried out in MYM medium, whereas isoleucine-galactose (IG) medium as used to study the secondary metabolites as described previously.[3] *Escherichia coli* (*E. coli*) strains were cultured in liquid Luria Bertani (LB) medium and on agar plates supplemented with ampicillin (100 µg/mL), apramycin (50 µg/mL), chloramphenicol (25 µg/mL) and tetracycline (25 µg/mL) whenever necessary. DNA manipulations such as plasmid DNA isolation, purification, ligation, restriction analyses

etc. were carried out according to standard protocols.[1, 2] Bacterial strains used in this study are summarized in the table 1.

**Table 1.** Bacterial strains used in the experiment and their metabolites. For the chemical structures of the metabolites, see scheme 1 (below), for the plasmids used, see table 3 (below).

| **Bacterial Strains** | **Relevant characteristics** | **Metabolites produced** | **Reference/s** |
|---|---|---|---|
| *Streptomyces venezuelae* ISP5230 | Jadomycin-producing wild type | **21**, **22** | 4 |
| *Streptomyces venezuelae* CH56 | Jadomycin non-producing *jadA* (keto synthase α)-deletion mutant of *S. venezuelae* | - | 5, 6 |
| *Streptomyces lividans* TK24 | Expression host | - | 1 |
| *E. coli* BW25113 | Host for homologous recombination of the resistance cassette | | 7 |
| *E. coli* XL1Blue | Cloning host | | Stratagene |
| *E. coli* ET12567/pUZ008 | Conjugation host with a non transmissible plasmid | - | 1 |
| SLOIV-1 | *S. lividans* TK24 transformed with cosG9B3-OIV$^-$ | **3, 4** | 8 |
| SLOI-OII | *S. lividans* TK24 transformed with cosG9B3-OI$^-$-OII$^-$ | **5, 6, 12, 13** | This study |
| SLOIV-OII | *S. lividans* TK24 transformed with cosG9B3-OIV$^-$-OII$^-$ | **3, 4** | This study |
| SLOII | *S. lividans* TK24 transformed with cosG9B3-OII$^-$ | **8, 9** | This study |
| SLOII-1 | SLOII mutant transformed with pGilOII-1 | **31, 32, 33** | This study |
| SLU | *S. lividans* TK24 transformed with pWHM1238 | **3, 1** | analog to the construct described in 9 |
| SLF1 | *S. lividans* TK24 (cosG9B3-OIV$^-$) mutant transformed with pJadF1 | **31, 33, 3, 4** | This study |
| SLG1 | *S. lividans* TK24 (cosG9B3-OII$^-$) mutant transformed with pJadG1 | **8, 9** | This study |
| SLH1 | *S. lividans* TK24 (cosG9B3-OI$^-$) mutant transformed with pJadH1 | **31, 33, 3, 4, 27** | This study |
| SLF2 | *S. lividans* TK24 (cosG9B3-OI$^-$) mutant transformed with pJadF1 | **5, 6, 12, 13** | This study |
| SLR | *S. lividans* TK24 transformed with cosG9B3-R$^-$ | **24, 25, 26?** | This study |
| SLR1 | SLR transformed with cosG9B3-R$^-$ mutant transformed with pGilR | **31, 32, 33?** | This study |
| SLF2-1 | SLU transformed with pJadF2 | **5**, **12** | This study |
| SLG2 | SLU transformed with pJadG2 | **3**$^b$, **1**$^b$ | This study |
| SLH2 | SLU transformed with pJadH2 | **3** | This study |
| SLFG | SLU transformed with pJadFG | **5**, **12** | This study |

| | | | |
|---|---|---|---|
| SLFGH | SLU transformed with pJadFGH | **3**, **21**[a], **7, 12** | This study |
| SLOI | SLU transformed with pgilOI | **3** | This study |
| SLOII-2 | SLU transformed with pgilOII | **3**[b], **1**[b] | This study |
| SLOIV | SLU transformed with pgilOIV | **5**, **12** | This study |
| SLOIV-OII-1 | SLU transformed with pgilOIIOIV | **5, 12** | This study |
| SLOI-OIV | SLU transformed with pgilOIOIV | **3, 7** | This study |
| SLOIV-OII-OI | SLU transformed with pgilOIOIIOIV | **3**, **21**[a], **7, 12** | This study |

[a] L-isoleucine was added to the culture medium; [b] comparable to the production of SLU.

**II. Generation of single or multiple genes inactivated mutants**

Cosmid G9B3 which contains the entire gilvocarcin gene cluster was used for the *gilOII* and *gilR* inactivation experiments. The PCR targeting approach was adopted to delete *gilOII* and *gilR* from this cosmid following the modified protocol described previously.[8] The chloramphenicol (CHL) resistance gene flanked with FRT (flippase recognition target) and FLP (flippase) sites was used as a template to amplify inactivation cassettes. Inactivation cassettes for *gilOII* and *gilR* were amplified with the primer pairs GilOII_FRT_for and GilOII_FRT_rev, and GilR_FRT_for and GilR_FRT_rev respectively (see table 2). The underlined 39 nt sequence for *gilOII* represent homologous sequence to the immediate upstream and downstream region of the start and stop codons of the gene, respectively. Additional three nucleotides were included in the reverse primer to avoid the possible truncation of the downstream gene *gilR*. Similarly, the underlined 39 nucleotides in the *gilR* inactivation primers represent homologous nucleotides flanking the *gilR* gene. PCR was carried out to amplify the inactivation cassettes as described previously.[8] The cassettes were introduced into *E. coli* BW25113/pKD20 harboring cosG9B3 (apramycin (Am)-resistant) by electroporation. Am and CHL resistant colonies were obtained for both cases indicating the replacement of the genes with the inactivation cassettes. The replacement was further confirmed by PCR analysis of the products using control primers (GilOII_ctrl_for, GilOII_ctrl_rev,

GilR_ctrl_for, GilR_ctrl_rev). FLP-mediated excision of the chloramphenicol resistance gene cassettes was carried out following the previous protocol,[8] and the excision was confirmed by the 82 bp scar instead of the entire genes in the mutant cosmids cosG9B3-OII⁻ and cosG9B3-R⁻. Similarly, *gilOII* was deleted from cosG9B3-OI⁻ and cosG9B3-OIV⁻ mutant to generate cosG9B3-OI⁻-OII⁻ and cosG9B3-OIV⁻-OII⁻, respectively. The mutant cosmids were conjugally transferred in to *S. lividans* TK24 using *E. coli* ET12567/pUZ8002 creating SLOII, SLR, SLOI-OII and SLOIV-OII.

**Table 2.** Primers used for amplifying DNA sequence for inactivation and expression experiments

| Name of Primer | Oligoneucleotide sequence |
|---|---|
| GilOII_FRT_for | 5'-CCCTCCGGGGCCGTCCGACACCTCATGGAAGGCCTCATG ATT CCG GGG ATC CGT CGA CC-3' |
| GilOII_FRT_rev | 5'-CCTCGCGGCCCACCGTGAACGGCGGTACGGAAGCGGTCACGA TGT AGG CTG GAG CTG CTT C-3' |
| GilOII_ctrl_for | 5'-*TCTAGA*CGTCCTCGTCGTCACCGAGGT-3' |
| GilOII_ctrl_rev | 5'-*GAATTC*AGTGCGACAGTTCGATGTACC-3' |
| GILR_FRT_for | 5'-GACCGTGTTCACCCAGTCGGTCGAGGGGTACGCGTCGTG ATT CCG GGG ATC CGT CGA CC-3' |
| GILR_FRT_rev | 5'-CTCCGTGCTGCCCGTTGGCATCGAGCGCCACTCCTCTCA TGT AGG CTG GAG CTG CTT C-3' |
| GILR_ctrl_for | 5'-*GAATTC*CGCACGCGGCAAGGCGCTGC-3' |
| GILR_ctrl_rev | 5'-*GAATTC*CTGGTAGAGCTTCTCGAAGGG-3' |
| JadF_comp_for | 5'-GCCA*TCTAGA*CTGCAGAGGGCAAGCGCTGACAT-3' |
| JadF_comp_rev | GATATCTA*GAATTC*GTCGGGGTTCAGAGGTGGG-3' |
| JadG_comp_for | 5'-TCTGAA*TCTAGA*CGAAGGACCGAGAACC-3 |
| JadG_comp_rev | 5'-TGGT*GAATTC*GCTCACTCCGCCGAGCGCGAGTG-3' |
| JadH_comp_for | 5'-TCGC*TCTAGA*CGGAGGAGGCGCCATGACCACCACCC-3' |
| JadH_comp_rev | 5'-CACTC*GAATTC*GTCGTCACCGGGCCGCGCC-3' |
| GilR-compF1 | 5'-*TCTAGA*GTCGGTCGAGGGGTACGCGTCGTG-3' |
| GilR-compF2 | 5'-*GAATTC*TGCCCGTTGGCATCGAGCGCCACTCCTC-3' |
| GilOIV_exp_for | 5'-GCCG*TCTAGA*AGAGGTGAGTGGGGACATG-3' |
| GilOIV_exp_rev | 5'-ACA*GAATTC*TCACCGCGTCACCTC-3' |
| GilOI_exp_for | 5'-AGCA*TCTAGA*GAGGCATGACGTTGCACGCC-3' |
| GilOI_exp_rev | 5'-TTCT*TCTAGA*TCTGGTCACGCGGCGGGCTG-3' |
| gilOII_exp_for | 5'-GACA*TCTAGA*GGAAGGCCTCATGCCGA-3' |
| GilOII_exp_rev | 5'-GTAC*TCTAGAATTC*ACGACGCGTACCCCTC-3' |

Restriction sites for cloning, and homologous sequences for inactivation primers are italicized and underlined, respectively.

**III. Construction of expression cassettes**

**IIIa. Construction of expression plasmids for the complementation of mutants**

A pair of primers (GilR_comp_for and GilR_comp_rev, table 2) was used to amplify the entire nucleotide sequence encoding GilR from the cosmid CosG9B3. The product was cloned into the TOPO vector (Invitrogen) and sequenced to make sure that no mutation had been introduced during PCR amplification. The TOPO clone was digested with *Xba*I and *Eco*RI to isolate the *gilR* region, which was then ligated at the identical sites of pEM4 to generate pGilR. Similarly, a *gilOII* overexpression plasmid (pGilOII-1) was constructed (see section **IIIc**).

PJV69A which contains 7.2 kb jadomycin biosynthetic gene cluster region (*jadDFGHK* and partial *jadL*) cloned in the pBluescript II KS(+) was a kind gift from Leo Vining (Dalhousie University, Halifax, Nova Scotia, Canada).[10] The plasmid was used as a template for the amplifications of *jadF*, *jadG* and *jadH*. The genes were amplified along with their putative ribosome binding sites (RBS). Artificial RBSs were created in primers for the genes which did not possess a distinct RBS. The nucleotide sequences encoding *jadF*, *jadG* and *jadH* were amplified with JadF_comp_for and JadF_comp_rev, JadG_comp_for, JadG_comp_rev, and JadH_comp_for and JadH_comp_rev primers, respectively (table 2). PCR was carried out using Taq polymerase (Clone Tech. Inc, USA) following the manufacturer's recommendations: 30 pmol of each primer was mixed with 100 ng of template DNA and 2 units of Taq-polymerase in a total reaction volume of 50 µl containing 2 mM of each dNTP, 10 µl of LA PCR buffer and GC-Melt buffer. Agarose gel electrophoresis and gel extraction kit (Qiagen) were used to purify the PCR products. The purified DNA was cloned into the TOPO-vector, and sequenced to confirm that no

mutation had been introduced during PCR amplifications. Each TOPO-clone was digested with *Xba*I and *Eco*RI, and the isolated fragments were ligated at the identical sites of *Streptomyces-E. coli* shuttle vector (pEM4) to generate pJadF1, pJadH1 and pJadG1.

**IIIb. Construction of jadomycin monooxygenase gene cassettes for the expression in *S. lividans*TK24/pWHM1238**

Since *jadG* and *jadF* were adjacent to each other, and were transcribing in the same direction, forward primer of *jadF* and reverse primer of *jadG* were used for the amplification of 2.24 kb *jadFG* region. The fragment was cloned into the TOPO vector for sequencing. The *Xba*I-*Eco*RI fragment containing *jadFG* genes was isolated from pJadFG-TOPO and cloned at the identical sites of pEM4 to generate pJadFG1. Finally, a *Hind*III-*Eco*RI fragment containing *jadF*, *jadG* and the *ermE** promoter was cloned at the identical sites of the *Streptomyces – E. coli* shuttle vector pKC1139 to generate pJadFG2. Similarly, *Eco*RI-*Hind*III fragments isolated from pJadH1, pJadF1 and pJadG1 were cloned into pKC1139 to generate pJadH2, pJadF2 and pJadG2, respectively. The 1.6 kb *jadH* fragment obtained through *Eco*RI digestion of pJadH-TOPO was ligated at the identical site of pJadFG2 to generate the final construct pJadFGH (Figure 1, below). The correct orientation of the gene was confirmed by restriction analyses of the cloned DNA.

**IIIc. Construction of gilvocarcin monooxygenase expression cassettes**

A *Hind*III-*EcoR*I fragment comprising the *ermE** promoter and the entire multiple cloning site of the pEM4 vector was transferred to pKC1139 to generate an expression plasmid pKC-ermE. All of the three candidate monooxygenases identified in the gilvocarcin biosynthetic gene cluster were amplified to generate the expression constructs. Genes g*ilOIV*, *gilOII* and *gilOI* were amplified with the GilOIV_exp_for and GilOIV_exp_rev, GilOII_exp_for and GilOII_exp_rev, and GilOI_exp_for and GilOI_exp_rev primers, respectively. The PCR products were cloned into the TOPO vector, and the inserts were sequenced. The nucleotide region for *gilOIV* and *gilOII* were isolated as *Xba*I-*Eco*RI fragments from their TOPO clones, and subsequently ligated at the identical sites of pKC-ermE vector generating pGilOIV and pGilOII, respectively. In addition, *gilOII* was also cloned into the *Xba*I-*EcoR*I site of pEM4 to generate pGilOII-1. Similarly, *gilOI* was isolated with *Xba*I from its TOPO clone (pGilOI-TOPO) and ligated at the identical site of pKC-ermE and pGilOIV constructs to generate pGilOI and pGilOIOIV, respectively. The correct orientation of the inserts was confirmed through the restriction analyses. To generate the pGilOIVOII construct, an *Xba*I fragment comprising the *gilOII* region was cloned at the identical site of pGilOIV. Finally, a plasmid containing all gilvocarcin monooxygenases (pGilOIOIIOIV) was generated by inserting *gilOII* (*Xba*I fragment) at the *Xba*I site of pGilOIOIV obtained through partial digestion (see Figure 1, below).

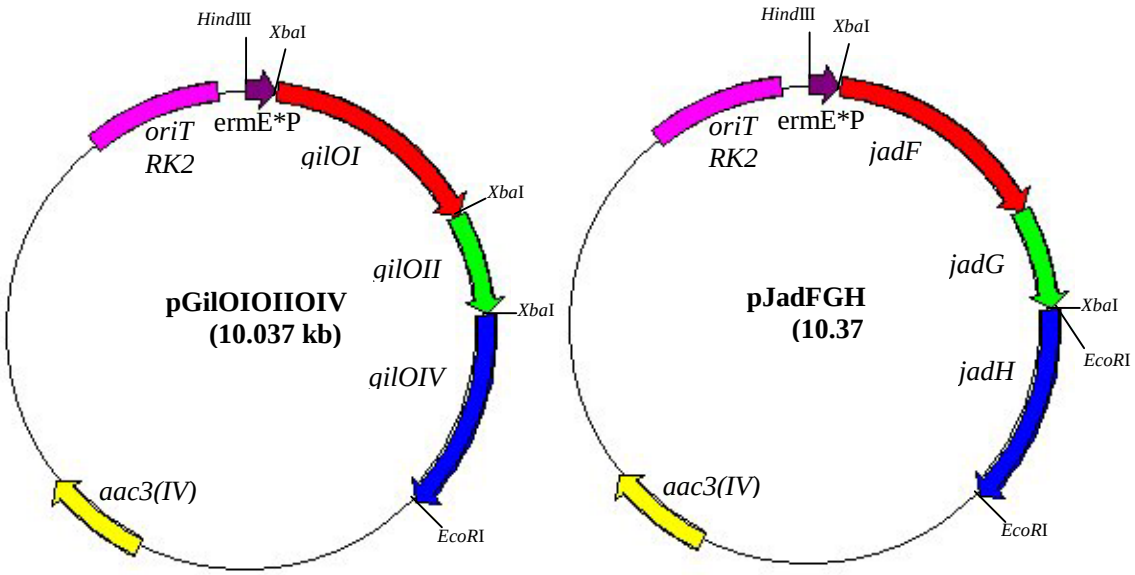



**Figure 1.** Map of pGilOIOIIIOIV and pJadFGH.

**Table 3.** Plasmids constructed and/or used in this study

| Plasmids/Cosmids | Relevant Characteristics | References |
|---|---|---|
| Zero Blunt® TOPO® | Vector for cloning PCR products | Invitrogen |
| pEM4 | *E. coli* - *Streptomyces* shuttle plasmid with *ermE** promoter, $amp^R$, $tsr^R$ | [11] |
| pKC1139 | *E. coli*- *Streptomyces* shuttle plasmid, $amp^R$ | 1 |
| pWHM1238 | Contains genes the jadomycin PKS and cyclase genes *jadJIABCED* cloned behind the *ermE** promoter (for UWM6 **1** generation) | 9 |
| pKC-ermE | *E. coli*- *Streptomyces* shuttle plasmid with *ermE** promoter | This study |
| pJadF-TOPO | *jadF* cloned into TOPO vector | This study |
| pJadH-TOPO | *jadH* cloned into TOPO vector | This study |
| pJadG-TOPO | *jadG* cloned into TOPO vector | This study |
| pJadFG-TOPO | *jadF* and JadG cloned into TOPO vector | This study |
| pJadF1 | *jadF* cloned into pEM4 | This study |
| pJadF2 | *ermE** promotor and *jadF* cloned into pKC1139 | This study |
| pJadH1 | *jadH* cloned into pEM4 | This study |
| pJadH2 | *ermE** promotor and *jadH* cloned into pKC1139 | This study |
| pJadG1 | *jadG* cloned into pEM4 | This study |
| pJadG2 | *ermE** promotor and *jadG* cloned into pKC1139 | This study |
| pJadFG1 | *ermE** promoter, *jadF* and *jadG* cloned into pEM4 | This study |
| pJadFG2 | *ermE** promoter, *jadF* and *jadG* cloned into pKC1139 | This study |
| pJadFGH | *ermE** promoter, *jadF, jadG* and *jadH* cloned into pEM4 | This study |
| PGilOI-TOPO | *gilOI* cloned into TOPO vector | This study |
| pGILOII-TOPO | *gilOII* cloned in TOPO vector | This study |
| pGILOIV-TOPO | *gilOIV* cloned into TOPO vector | This study |

| | | |
|---|---|---|
| pGilOII | *ermE** promoter, *gilOII* cloned into pKC1139 | This study |
| pGilOII-1 | *ermE** promoter, *gilOII* cloned into pEM4 | This study |
| pGilOIV | *ermE** promotor and *gilOI* cloned into pKC1139 | This study |
| pGilOI | *ermE** promotor and *gilOI* cloned into pKC1139 | This study |
| pGilOIOIV | *ermE** promoter, *gilOI* and *gilOIV* cloned into pKC1139 | This study |
| pGilOIOIIOIV | *ermE** promoter, *gilOI* , *gilOII* and *gilOIV* cloned into pKC1139 | This study |
| cosG9B3-OIV$^-$ | *gilIV* deletion mutant of cosG9B3 | 8 |
| cosG9B3 | A cosmid with the complete gilvocarcin biosynthetic gene cluster | [12] |
| cosG9B3-OII$^-$ | *gilOII* deletion mutant of cosG9B3 | This study |
| cosG9B3-OI$^-$-OII$^-$ | *gilOII* and *gilOI* deleted mutant of cosG9B3 | This study |
| cosG9B3-OIV$^-$-OII$^-$ | *gilOII* and *gilOIV* deleted mutant of cosG9B3 | This study |
| cosG9B3-R$^-$ | *gilR* deletion mutant of cosG9B3 | This study |
| pGilR | *gilR* expression plasmid (*gilR* cloned into pEM4) | This study |

tsr$^R$, thiostrepton resistance cassette; amp$^R$, ampicilillin resistance cassette

## IV. Complementation of gilvocarcin-monooxygenase deletion mutants with jadomycin monooxygenase expression constructs

Protoplast of *S. lividans* TK24 (cosG9B3-OI$^-$) mutant was transformed with pJadH1 and pJadF1. Similarly, *S. lividans* TK24 (cosG9B3-OIV$^-$) and *S. lividans* TK24 (cosG9B3-OII$^-$) mutants were transformed with pJadF1 and pJadG1, respectively following a similar protocol. Several thiostrepton- and apramycin- resistant colonies were observed on an R2YE agar plate after 70 hrs of overlay with soft agar. A colony from each transformation plate was inoculated in 100 mL of SG medium, and cultured at 28 ºC and 200 rpm for 72 hrs. This prepared pre-culture (3 mL) was taken as seed for the inoculation of 100 mL medium of the same composition under identical conditions. The culture was harvested after 120 hrs. The culture broth was extracted with the equal volume of ethyl acetate for two times. The extracts were dried in vacuo, dissolved in methanol and analyzed by HPLC or HPLC-MS. Upon complementation of the *S. lividans* TK24 (cosG9B3-OI$^-$) mutant with pJadH1, gilvocarcin productio0n was restored (Figure 2, below).

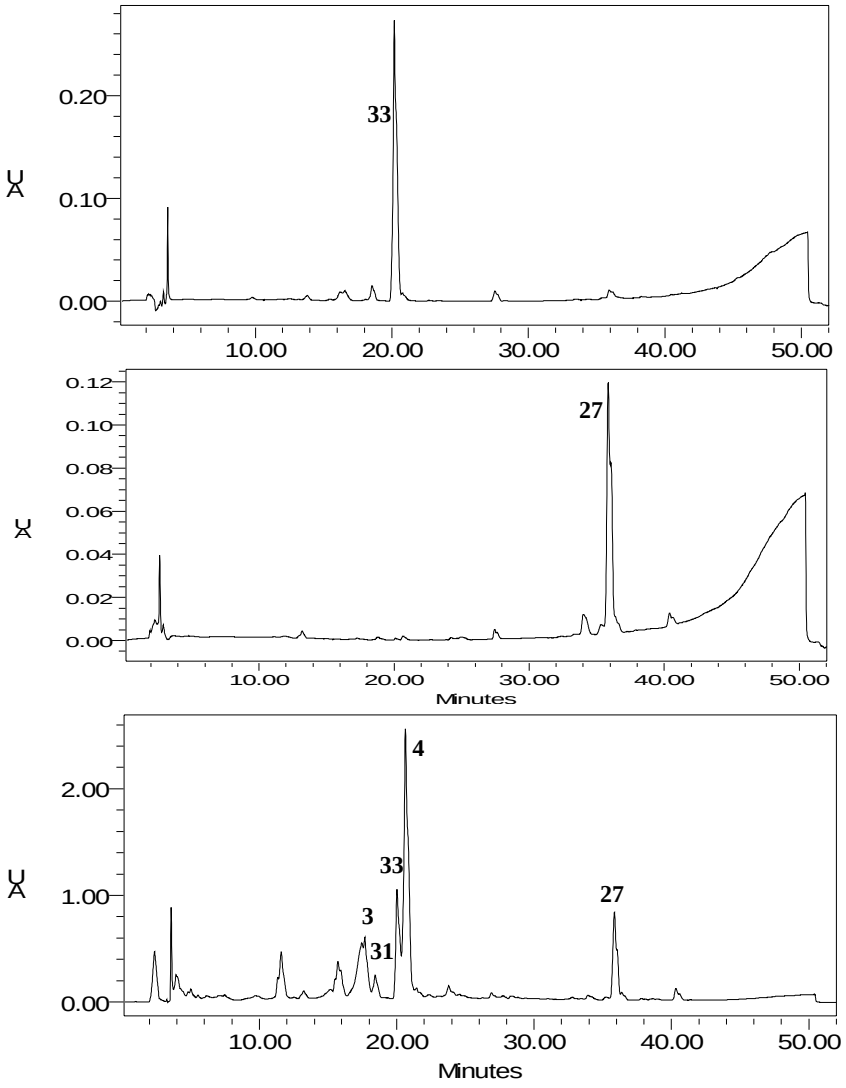



**Figure 2.** HPLC analysis of metabolites from the *gilOI*-minus mutant complemented with *jadH* (SLH1, see section **IX-e** for HPLC conditions). The chromatograms **I** and **II** represent standard gilvocarcin V (**33**) and defuco-gilvocarcin V (**27**) whereas **III** represents the metabolites of SLH1. The peaks represent the metabolites homorabelomycin (**4**)**,** rabelomycin (**3**)**,** defuco-gilvocarcin V (**27**), gilvocarcin M (**31**) and gilvocarcin V (**33**). For chemical structures, see scheme 1 (below).

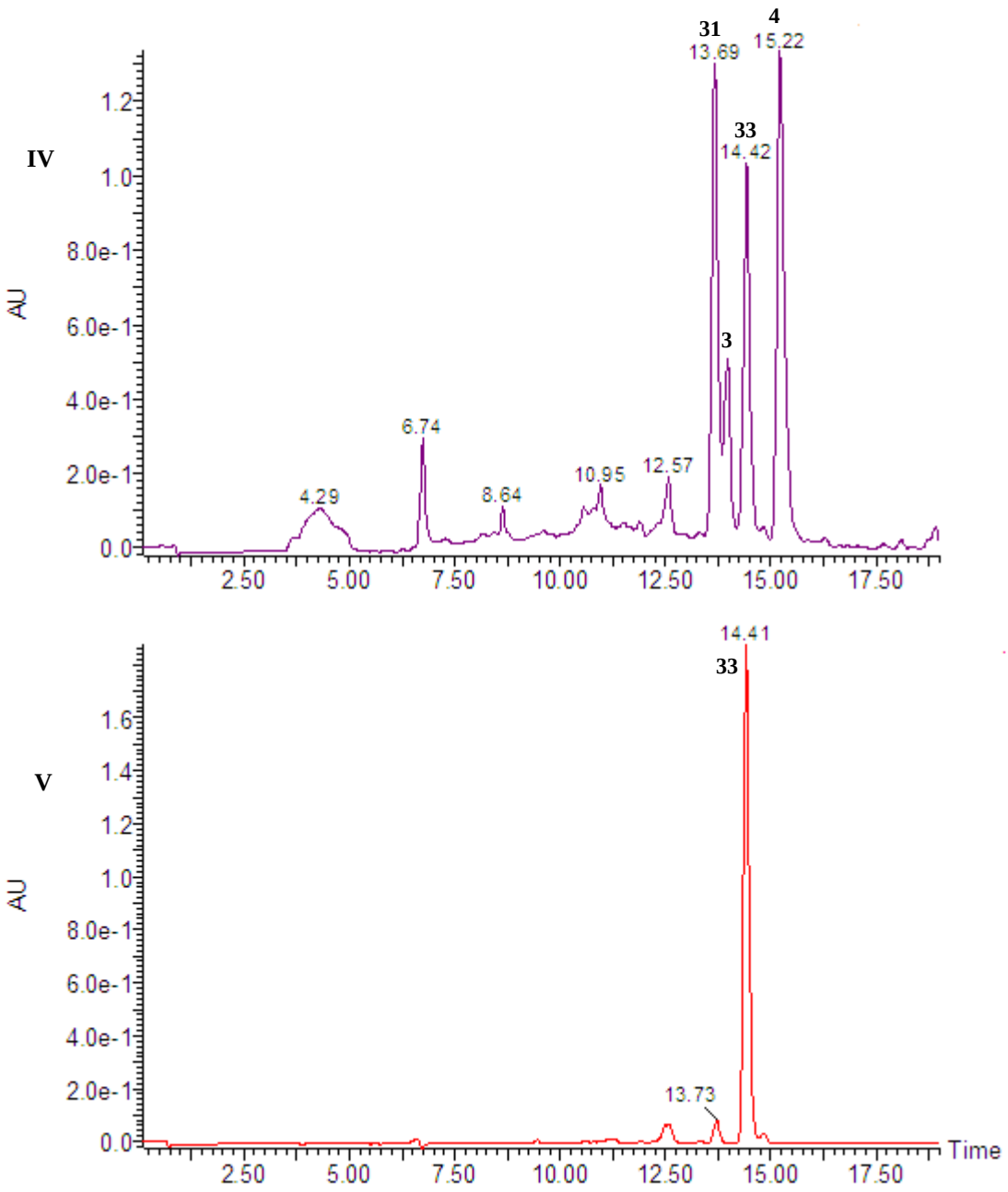



**Figure 3.** HPLC-MS analysis of metabolites from the *S. lividans* TK24 (cosG9B3-OIV⁻) mutant transformed with pJadF1 (SLF1, see section **IX-d** for HPLC conditions). The chromatograms **IV** and **V** represent metabolites of SLF1 and standard gilvocarcin V (**33**), respectively. The peaks representing the metabolites rabelomycin (**3**), homorabelomycin (**4**), gilvocarcin M (**31**), and gilvocarcin V (**33**) (scheme 1). The peaks were identified through their retention times, UV- and MS spectra.

**V. Expression of gilvocarcin and jadomycin monooxygenases in the UWM6 producing strain *S. lividans* TK24 (pWHM1238) (= SLU strain).**

Protoplasts of *S. lividans* TK24 (pWHM1238), SLU, was transformed with different expression plasmids, delineated in table 3 (above), and apramycin and thiostrepton resistant colonies were taken to study their metabolites by HPLC-MS (Figure 4).

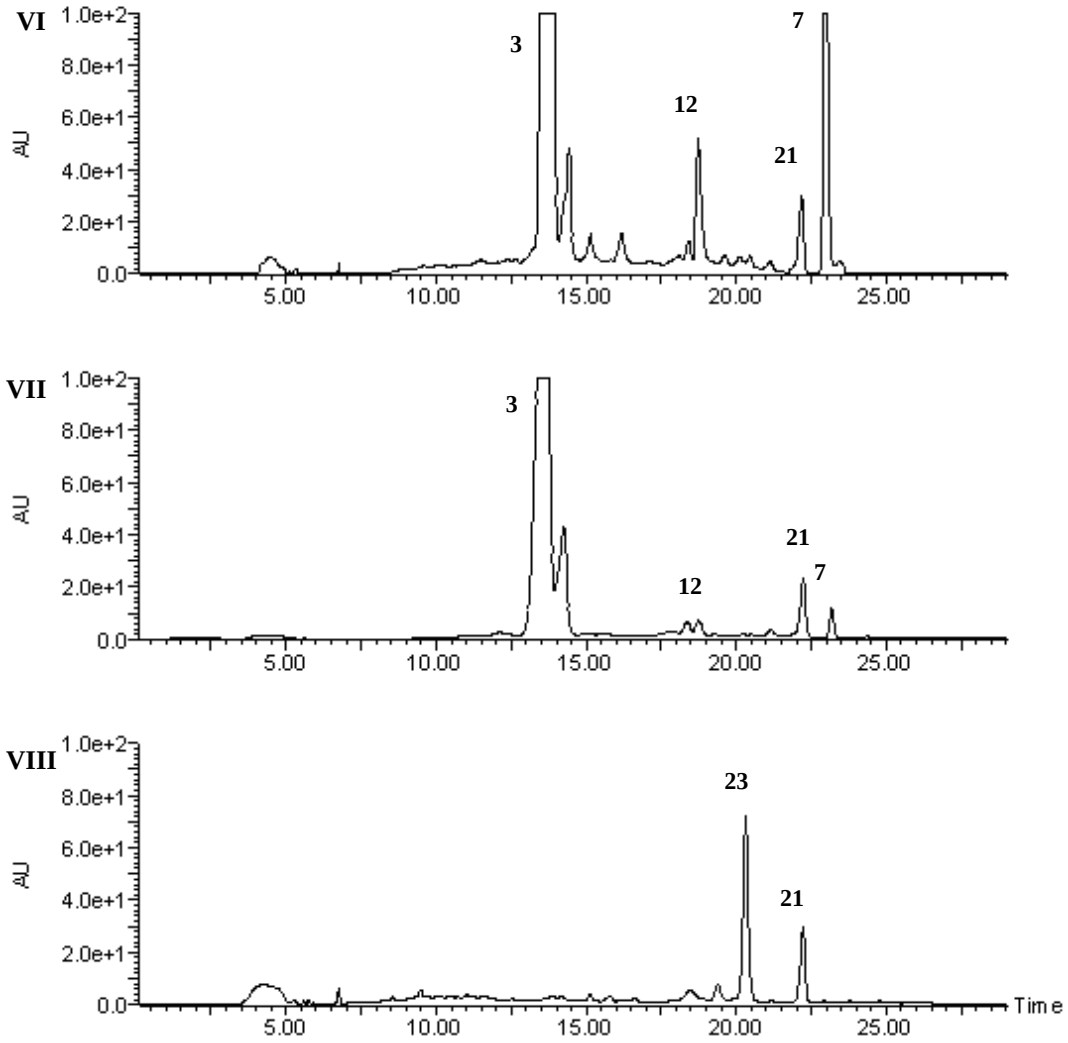



**Figure 4.** HPLC-MS (conditions, see **IX-d**) analyses of metabolites from strains SLOIV-OII-OI (**VI**, see table 1), SLFGH (**VII**, see table 1) in comparison to jadomycin producer *S. venezuelae* ISP5230 (**VIII**; jadomycin A **21**; jadomycin B **23**). The culture media of all three stains were supplemented with L-isoleucine. **3** = rabelomycin; **7** = dehydrorabelomycin, **12** = pregilvocarcin M–orthoquinone.

To study the relative productions of prejadomycin (**5**) and pregilvocarcin-M-*o*-qoinone (**12**), i.e. the conversion of **5** to **12**, by the SLF2-1 strain (see table 1), an aliquot (10 mL) of the culture was withdrawn at various culture intervals and extracted for three times with equal volumes of ethyl acetate. The extract was dried in vacuo, re-suspended in 0.5 ml of methanol. The extract was filtered through a Whatmann membrane filter (0.25 µm pore size), and 70 µL of each sample was injected for HPLC-MS analysis. The relative production was determined by measuring the peak area (at 254 nm) of each compound in the sample (Figure 5).

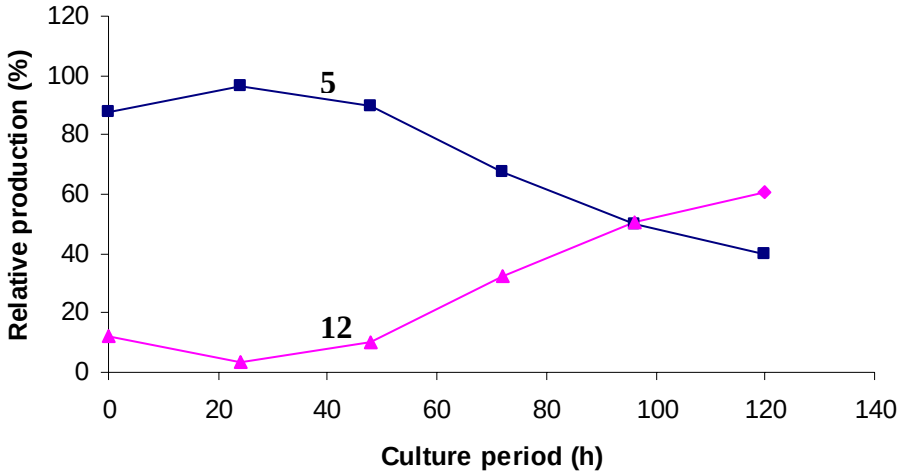



**Figure 5.** Conversion of prejadomycin (**5**) into pregilvocarcin-M-*o*-quinone (**12**) in SLF2-1.

**VI. Isolation and characterization of pregilvocarcins (28 and 30) from the SLR mutant.**

A seed culture of the SLR strain was grown at 30 ºC in 300 mL of apramycin supplemented SG medium for three days. 4 L of the main culture were inoculated with

the fully grown seed, and incubated for 4 days. The culture broth was separated from the mycelia through the centrifugation (4000×g, 30 min). The cell pellets were suspended in 500 mL of acetone-water (80:20) solution, sonicated for 15 min, and centrifuged to collect the extract. The organic phase was removed and the aqueous fraction was combined with the culture broth. The crude aqueous extract was loaded on a preparative reverse phase column (22 cm x 4 cm), and washed with 20%~90% (gradient) of acetonitrile-water solution. Two major components, pregilvocarcin M (**28**) and pregilvocarcin V (**30**), were eluted with 40% to 60% acetonitrile and subsequently concentrated to obtain crude pregilvocarcins. This crude extract was dissolved in methanol, acidified to pH 5, and further subjected to preparative HPLC purification, to obtain pure **28** and **30**, respectively (see section **IX-a** for HPLC conditions).

**Pregilvocarcin V (30)**

Yield = 3-5 mg/L; Retention time = 13.54 min (section **IX-d**).

Mol. Wt. = 496 g/mol ($C_{27}H_{28}O_9$; high resolution-FAB MS, $M^+$ = 496.1719; calculated for $C_{27}H_{28}O_9$: 496.1733).

UV (from HPLC - diode array): $\lambda_{max}$ 212 nm (95.82%), 289 nm (100%), 379 nm (39.62%), 390 nm (37.91%).

NMR data: see table 4 (below)

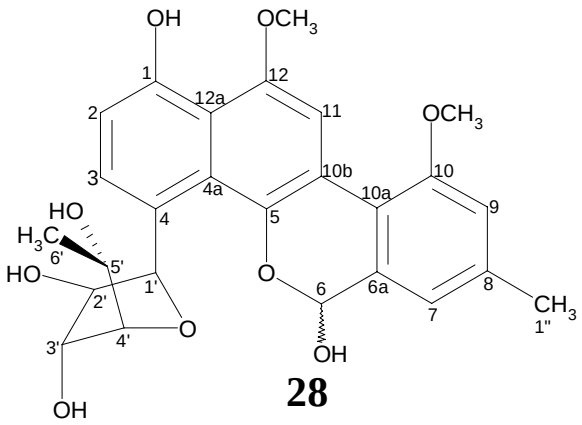
**Pregilvocarcin M (28)**

Yield = 2-3 mg/L; Retention time = 12.94 min (section **IX- d**).

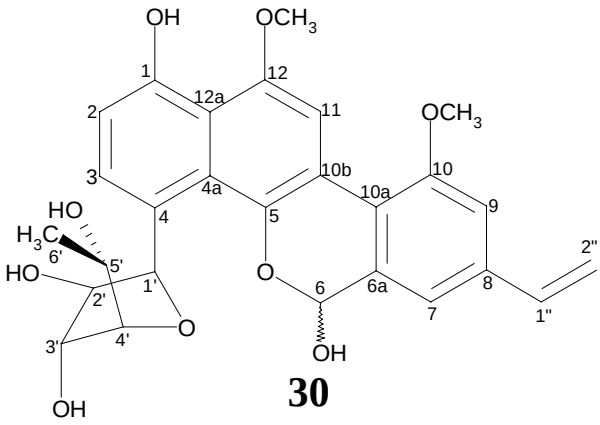
Mol. Wt. = 484 g/mol ($C_{26}H_{28}O_9$; high resolution-FAB MS, $M^+$ = 484.1720; calculated for $C_{26}H_{28}O_9$: 484.1733

UV (from HPLC-diode array): $\lambda_{max}$ 225 nm (81.10%), 270 nm (100%), 360.79 nm (37.91%), 378.79 nm (40.20%).

NMR data: see table 4 (below).




**28**




**30**

**Table 4.** NMR data of pregilvocarcin V (**30**) and pregilvocarcin M (**28**) in $d_6$-DMSO, δ in ppm relative to internal TMS (for $^1$H NMR: multiplicity and coupling constants *J* in Hz are given in parentheses). Only the signals of the major diastereomers are given.

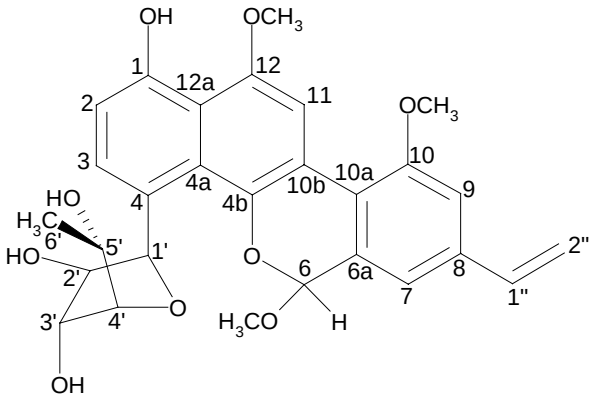
| 30 | | | 28 | 30 | | | | 28 |
|---|---|---|---|---|---|---|---|---|
| Position | $^{1}H^{a}$ | gCOSY | $^{1}H^{a}$ | Position | $^{13}C^{b}$ | HSQC | CIGAR-HMBC | $^{13}C^{b}$ |
| 1-OH | 9.71 (br, s) | | 9.70 (br, s) | C-1 | 152.7 | | C-1, C-2, C-12a | 152.5 |
| 2-H | 6.79 (d, 8.0) | 3-H | 6.76 (d, 8.0) | C-2 | 110.3 | 2-H | C-1, C-3, C-4, C-12a | 110.1 |
| 3-H | 7.86 (d, 8.0) | 2-H | 7.84 (d, 8.0) | C-3 | 127.6 | 3-H | C-1, C-4a, C-1’ | 127.5 |
| 6-H | 6.30 (d, 6.4) | 7-H | 6.23 (d, 6.4) | C-4 | 125.4 | | C-5, C-7, C-10a | 125.1 |
| 6-OH | 4.42 (d, 6.4) | | 4.30 (d, 6.4) | C-4a | 125.9 | | | 125.8 |
| 7-H | 7.19 (d, 2.0) | 6-H | 6.87 (d, 1.5) | C-5 | 142.1 | | C-6, C-9, C-1”, C-2”, C-10, C-10a | 141.5 |
| 1”-H | 6.81 (dd 18.0, 10.0) | 2”-$H_2$ | 2.39 (s) | C-6 | 91.5 | 6-H | C-7, C-8, C-9, C-2” | 91.6 |
| 2”-$H_E$ | 5.99 (d, 18.0) | 1”-H | - | C-6a | 134.6 | | C-8, C-1” | 134.2 |
| 2”-$H_Z$ | 5.35 (d, 10.0) | 1”-H | - | C-7 | 115.8 | 7-H | C-8, C-1” | 116.0 |
| 9-H | 7.27 (d, 2.0) | | 7.02 (d, 1.5) | C-8 | 137.7 | | C-7, C-8, C-10, C-1”, C-10a, | 138.5 |
| 10-$OCH_3$ | 4.01 (s) | | 3.96 (s) | C-1” | 136.2 | 1”-H | C-10 | 30.7 |
| 11-H | 8.01 (br, s) | 12-$OCH_3$ | 8.00 (br, d) | C-2” | 115.0 | 2”-$H_E$, 2”-$H_Z$ | C-5, C-12, C-10a, C-10b, C-12a | |
| 12-$OCH_3$ | 4.07 (s) | 11-H | 4.06 (s) | C-9 | 109.9 | 9-H | C-12 | 109.8 |
| 1’-H | 6.39 (d, 3.6) | | 6.38 (d, 3.2) | C-10 | 156.1 | | C-3, C-4a | 155.6 |
| 2’-H | 4.42 (m) | 3’-H | 4.41 (m) | 10-O$\underline{C}H_3$ | 56.1 | 10-$OCH_3$ | C-3’, C-1’ | |
| 2’-OH | 3.92 (br, s) | | 3.86 (s) | C-10a | 115.7 | | | 114.6 |
| 3’-H | 4.20 (m) | 2’-H | 4.17 (m) | C-10b | 116.6 | | C-4’ | 118.2 |
| 3’-OH | 5.14 (d, 3.6) | | 5.12 (d, 4.0) | C-11 | 103.9 | 11-H | | 103.9 |
| 4’-H | 3.62 (dd, 3.6, 3.2) | | 3.60 (dd, 3.6, 3.2) | C-12 | 149.6 | | C-3’ | 149.4 |
| 5’-H | 3.89 (m) | 6’-$H_3$ | 3.90 (m) | 12-O$\underline{C}H_3$ | 56.5 | 12-$OCH_3$ | C-4’, C-6’ | |
| 5’-OH | 5.01 (d, 4.4) | | 5.00 (d, 4.4) | C-12a | 115.1 | | | 113.1 |
| 6’-$H_3$ | 1.24 (d, 6.4) | 5-H | 1.24 (d, 6.4) | C-1’ | 81.2 | 1’-H | C-4’, C-5’, C-6’ | 81.0 |
| | | | | C-2’ | 78.7 | 2’-H | | 78.7 |
| | | | | C-3’ | 79.7 | 3’-H | | 79.7 |
| | | | | C-4’ | 88.0 | 4’-H | | 88.0 |

[a] recorded at 400 MHz; [b] recorded at 100.6 MHz.

## Pregilvocarcin V methylacetal

To facilitate the structure elucidation, 3 mg of pregilvocarcin V were stirred in 10 mL methanol at room temperature, and one drop 37% HCl was added. The compound

converted completely into its methyl acetal, predominantly into one diastereomer, yield: 2.8 mg




$^{1}$H NMR ($d_6$-acetone, TMS, 400 MHz): δ 9.77 (s, 1-OH*), 8.13 (s, 11-H), 8.02 (d, *J*= 8 Hz, 3-H), 7.32 (d, *J*= 1.6 Hz, 9-H), 7.24 (d, *J* = 1.6 Hz, 7-H), 6.83 (dd, *J* = 17.6, 10.8 Hz, 1”-H), 6.80 (d, *J* = 8 Hz, 2-H), 6.59 (d, *J* = 2.8 Hz, 1’-H), 6.10 (s, 6-H), 5.97 (dd, *J* = 17.6, 0.4 Hz, 2”-$H_E$), 5.33 (dd, *J* = 10.8, 0.4 Hz, 2”-$H_Z$), 4.46 (d, 3.2, 3’-OH*), 4.36 (d, 4.0, 5’-OH*), 4.32 (ddd, *J* = 8, 2.8, 1.2 Hz, 2’-H), 4.17 (s, 12-OC$\underline{H}_3$), 4.16 (ddd, *J* = 3.2, 2.0, 1.2 Hz, 3’-H), 4.08 (s, 10-OC$\underline{H}_3$), 4.06 (dq, *J* = 6.4, 3.4 Hz, 5’-H), 3.84 (dd, *J* = 3.4, 2.0 Hz, 4’-H), 3.85 (d, *J* = 8.0 Hz, 2’-OH*), 3.48 (s, 6-OC$\underline{H}_3$), 1.38 (d, *J* = 6.4 Hz, 6’-$H_3$) ppm. The asterisk indicates $D_2O$-exchangeable protons.

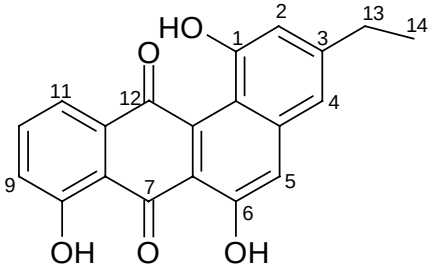
$^{13}$C NMR ($d_6$-acetone, TMS, 100.6 MHz): δ 20.9 (C-6'), 56.6 and 56.7 (10-O$\underline{C}H_3$ and 12-O$\underline{C}H_3$), 57.1 (6-O$\underline{C}H_3$), 69.0 (C-5’), 80.2 (C-2’), 81.3 (C-3’), 83.1 (C-1’), 90.0 (C-4’), 100.0 (C-6), 105.3 (C-11), 111.3 (C-9), 111.9 (C-2), 115.0 (C-2”), 116.8 (C-12a), 117.6 (C-7), 117.7 (C-10a), 118.1 (C-10b), 125.4 (C-4), 126.8 (C-4a), 129.4 (C-3), 133.9 (C-

6a), 137.3 (C-1”), 139.4 (C-8), 142.8 (C-4b), 151.5 (C-12), 154.9 (C-1), 157.8 (C-10) ppm.

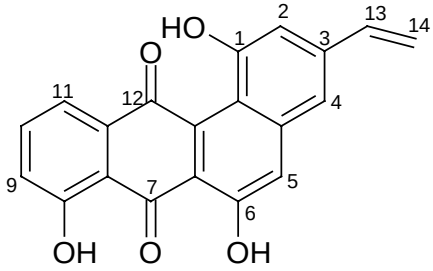
Diagnostic CIGAR-HMBC couplings were observed between 6-H and C-4b, C-7, C-10a, 6-O<u>C</u>H$_3$, between 6-OC<u>H</u>$_3$ and C-6, and between 7-H and C-6, C-9, C-10a, C-1”. Another diagnostic $^4J_{H\text{-}H}$ coupling was observed between 6-H and 7-H.

**VII. Isolation and characterization of dehydrorabelomycins (8 and 9) from SLOII mutant**

The SLOII mutant strain was cultured and harvested under identical conditions as described for the SLR strain above. The culture broth (4 L) was extracted three times with equal volumes of ethyl acetate. The cell pellet fraction was also extracted with acetone. Both fractions were concentrated, combined and dried. A methanol solution of the combined extracts was used for the preparative HPLC separation, to isolate homo-dehydrorabelomycin (**8**) and vinyl-dehydrorabelomycin (**9**).




**8**




**9**

**Homo-dehydrorabelomycin E (8)**

Yield: 1 mg/L, retention time = 24.46 min (HPLC conditions, see section **IX-e**).

Mol. Wt. = 334 g/mol ($C_{20}H_{14}O_5$), APCI-MS, 335 (positive mode, $M+H^+$).

UV (from HPLC-diode array): $\lambda_{max}$ 237.79 nm (100%), 269.79 nm (58%), 320.79 nm (53.71%), 457.79 nm (25.82%).

NMR data: see table 5 (below).

**Vinyl-dehydrorabelomycin V (9)**

Yield: 1 mg/L, retention time = 23.56 min (section **IX-e**)

Mol. Wt. = 332 g/mol ($C_{20}H_{12}O_5$), APCI-MS, 333 (positive mode, $M+H^+$).

UV (from HPLC-diode array): $\lambda_{max}$ 245 (100%), 277 (58%), 337 (48%) and 463(25%).

**Table 5.** NMR data of homo-dehydrorabelomycin (**8**) and vinyl-dehydrorabelomycin (**9**) in $CDCl_3$, δ in ppm relative to internal TMS.

| | $^1$H signals | | $^{13}$C signals |
|---|---|---|---|
| | **8**[a] | **9**[a] | **9**[b] |
| Position | δ/ppm (multiplicity, *J*/Hz) | δ/ppm (multiplicity, *J*/Hz) | δ/ppm |
| 1 | -OH*, 10.29 (s) | -OH*, 10.30 (s) | 154.8 |
| 2 | 6.98 (d, 1.2) | 7.18 (d, 2.0) | 117.8 |
| 3 | | | 140.8 |
| 4 | 7.09 (d, 1.2) | 7.23 (d, 2.0) | 119.0 |
| 4a | | | 141.7 |
| 5 | 7.68 (s) | 7.68 (s) | 125.4 |
| 6 | -OH*, 12.11 (s) | -OH*, 11.74 (s) | 156.9 |
| 6a | | | 119.8 |
| 7 | | | 193.1 |
| 7a | | | 114.9 |
| 8 | -OH*, 11.76 (s) | -OH*, 12.15 (s) | 162.0 |
| 9 | 7.84 (d, 8.1) | 7.84 (dd, 7.6, 1.2) | 124.8 |
| 10 | 7.71 (t, 8.0) | 7.71 (dt, 1.2, 7.6) | 137.9 |
| 11 | 7.32 (d, 8.0) | 7.32 (dd, 7.6, 1.2) | 121.8 |
| 11a | | | 133.0 |
| 12 | | | 189.2 |
| 12a | | | 135.0 |
| 12b | | | 120.1 |
| 13 | 2.74 (q, 8.1) | 6.75 (dd, 17.2, 10.8) | 135.4 |
| 14 | 1.31 (t, 8.1) | | 113.3 |
| 14-$H_E$ | | 5.95 (d, 17.2), | |
| 14-$H_Z$ | | 5.46 (d, 10.8) | |

[a] recorded at 400 MHz; [b] recorded at 100.6 MHz; * exchangeable with $D_2O$

**VIII. Cross-Feeding/Bioconversion experiments**

The substrates for cross-feeding experiments (**5**, **6**, **8**, **9, 12** and **30**) were isolated from the mutant strains described in table 1. 1 mg of each substrate was dissolved in 200 µl of DMSO, and fed to the 100 ml culture of a 3-day grown mutant strains. After 48 hours, the metabolites were extracted with ethyl acetate and analyzed by HPLC-MS, as described above (section **IV**). The results of the bioconversion studies are summarized in the table 6.

**Table 6.** Bioconversion experiments

| Strain | Compound fed | Compound identified by LC/MS |
|---|---|---|
| *Streptomyces venezuelae* CH56 | **6** | **23, 4, 13** |
| *Streptomyces venezuelae* CH56 | **5** | **21, 22** |
| *Streptomyces venezuelae* CH56 | **12** | No conversion |
| SLOI | **12** | No conversion |
| SLOIOIV | **12** | No conversion |
| SLOIOIIOIV | **12** | No conversion |
| SLF2-1 | **12** | No conversion |
| SLH2 | **12** | No conversion |
| SLOIV-1 | **30** | **33** |
| SLOIV-1 | **8** | No conversion |
| SLOIV-1 | **9** | No conversion |
| *S. lividans* TK24 (reference) | **6** | No conversion |

**IX.** HPLC conditions: Gradients of acetonitrile and water were used to isolate and analyze the metabolites. Various separation programs were used in these experiments, and are described below.

**a) HPLC separation of 28 and 30:** SunFire™ prep$C_{18}$ column (19 × 50 mm); solvent A = 0.1% formic acid in $H_2O$; solvent B = acetonitrile; flow rate = 10 mL/min; 0-4 min 25% B to 40% B; 5-24 min 40% to 50% B; 25-26 min 50% to 100% B; 27-29 min 100% B; 30-31 min 100%B to 25% B, 32-37 min 25% B.

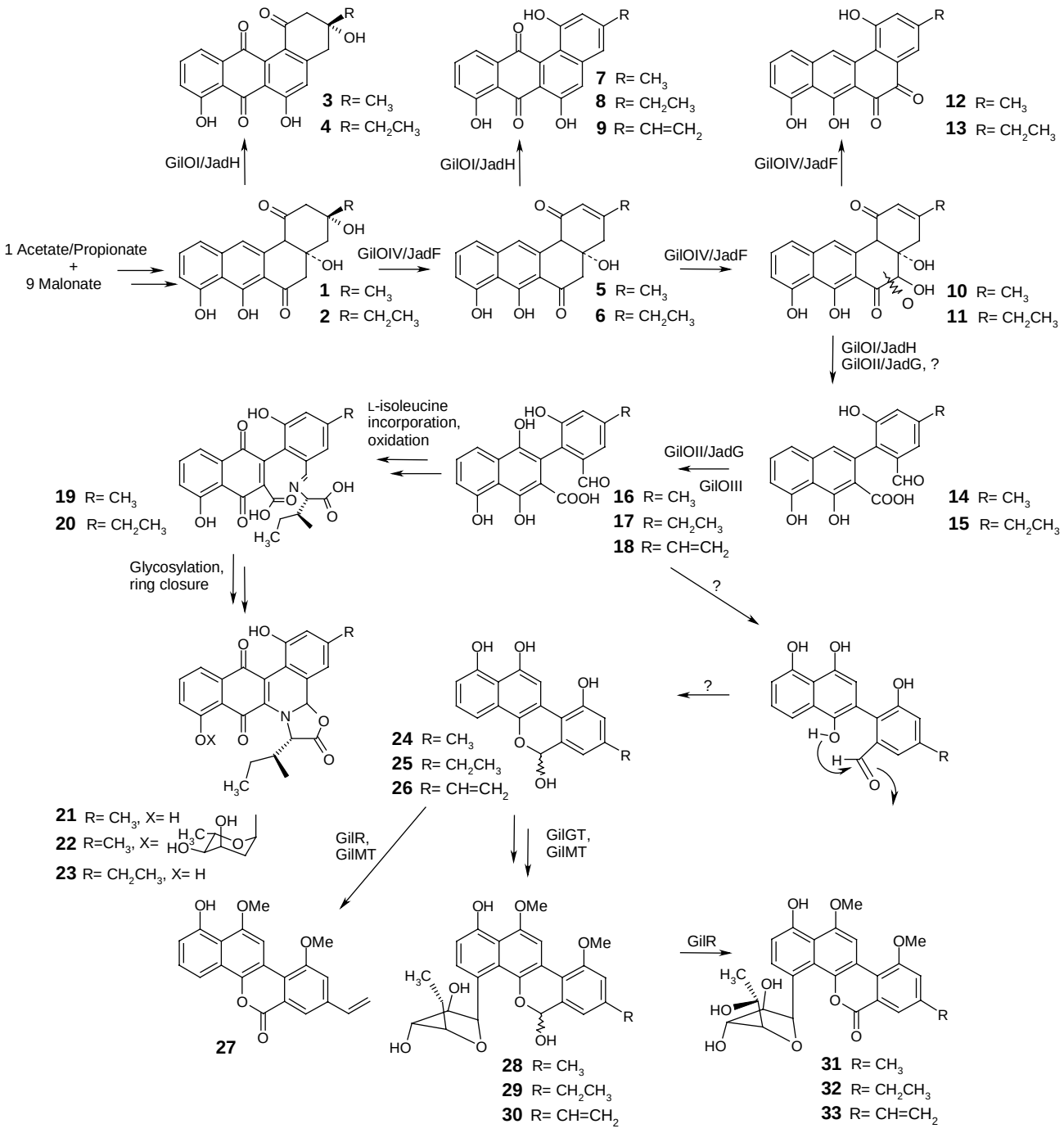
**b) HPLC separation of 8 and 9:** SunFire™ prep$C_{18}$ column (19 × 50 mm); solvent A = 0.1% formic acid in $H_2O$; solvent B = acetonitrile; flow rate = 10 mL/min; 0-5 min 70% B to 92% B; 6-15 min 92% B; 15-16 min 92% to 50% B; 17-20 min 50% B.

**c) Analytical HPLC:** Waters Symmetry® $C_{18}$ (4.6 × 250 mm); solvent A = 0.1% formic acid in $H_2O$; solvent B = acetonitrile; flow rate = 0.5 mL/min; 0-1 min 0% B, 2-19 min 0% to 50% B; 21-35 min 50%-100% B, 36-40 min 100% B; 41-45 min 100% to 0% B; 46-49 min, 0% B.

**d) Analytical (HPLC-MS)-P1:** Waters Symmetry® $C_{18}$ (4.6 × 250 mm); solvent A = 0.1% formic acid in $H_2O$; solvent B = acetonitrile; flow rate = 0.5 mL/min; 0-15 min 25% to 100% B; 16-19 min 100% B; 20-21 min 100% to 25% B, 22-23 min 25% B. Photodiode array; UV:200-600 nm; APCI-MS (+ve and –ve modes) : 200-1200 amu.

**e) Analytical (HPLC-MS)-P2:** Waters Symmetry® $C_{18}$ (4.6 × 250 mm); solvent A = 0.1% formic acid in $H_2O$; solvent B = acetonitrile; flow rate = 0.5 mL/min; 0-15 min

25% B to 100% B; 16-24 min 100% B; 25-26 min 100% B to 25% B, 27-29 min 25% B. Photodiode array; UV: 200-600 nm; APCI-MS (+ve and –ve modes) : 200-1200 amu.




**Scheme 1.** Proposed jadomycin and gilvocarcin biosynthetic pathways. The question marks represent yet unknown enzymes involved in the respective biosynthetic steps.

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