CHEMBIOCHEM

Supporting Information

DOI: 10.1002/cbic.200900130

Supporting Information

for

GilR, an Unusual Lactone-Forming Enzyme Involved in Gilvocarcin Biosynthesis

Madan Kumar Kharel, Pallab Pahari, Hui Lian, and Jürgen Rohr*

Contents

Bacterial strains, culture conditions

Streptomyces strains *S. gresioflavus* Gö 3592, GilGT-minus mutant *S. lividans* TK24 (cosG9B3-GT^{-}), GilR-minus mutant *S. lividans* TK24 (cosG9B3-R^{-}) and *S. albundancus* AD819 were cultured on M2 agar or in SG liquid medium at 28℃ and 200 rpm.[1] *E. coli* strains were grown in Luria Broth (LB) or on agar at 37℃. Kanamycin sulfate (50 μg mL^{-1}) and apramycin sulfate (50 μg mL^{-1}) were supplemented to the media whenever necessary. Standard pregilvocarcin V was isolated from *S. lividans* TK24 (cosG9B3-R^{-}) mutant following the previously reported procedure.[1] The compound was generated in larger scale by chemical reduction of GV (described below). Chrysomycin A was isolated from *S. albanduncus* AD819. Spores of each *Streptomyces* strain were inoculated into 100 mL of SG medium in 500 mL Erlenmeyer flask, and the culture was grown for 3 days to prepare seed. The seed culture (3% by vol) was then taken for inoculation of production scale SG medium (5 L; 100 mL × 50 flasks) and grown for 7 days.

Isolation and purification of chrysomycin A, defucogilvocarcin V and gilvocarcin V

The fully grown culture was harvested by centrifugation (4000 *g*, 15 min). The cell pellets were resuspended in 400 mL of acetone, sonicated for 20 min and centrifuged to obtain acetone extraction fraction. The process was repeated twice, the acetone fractions were combined and the solvent was removed using rotator evaporator. Finally, the extract was resuspended in 200 mL of water and mixed with the culture broth. Reversed-phase silica (Rp_{18}, 200 g) was loaded in to a column (5 × 65 cm) and equlibrated with deionized water. The culture broth was passed through the column and the effluent was discarded. The column was washed with 1 L of 10% acetonitrile (in water). Elution was carried out using an increasing gradient of acetonitrile (20, 40, 60, 80 and 100%, 1 L each). HPLC/MS analyses revealed that chrysomycin A was the major compound in 40% and 60% fractions. Acetonitrile was removed from these fractions and the aqueous fractions were combined and dried in lyophilizer. The dried extract was dissolved in 30 mL dimethylsulfoxide (DMSO), filtered through 0.25 μM filter, and the filtrate was subjected to preparative HPLC separation. Fraction containing pure chrysomycin A was collected and dried. The purity and authenticity of the compound was confirmed through ^{1}H and ^{13}C NMR analyses. A linear gradient of acetonitrile and acidified water (solvent A = 0.1% formic acid in H_2O; solvent B = ace-

tonitrile; 0-15 min 25% B to 100% B; 16-24 min 100% B; 25-26 min 100% to 25% B; 27-29 min 25% B) was used to separate compounds. SunFire™ prepC_{18} column (19 × 150 mm, 5 µm) and Symmetry C_{18} (4.6 × 250 mm, 5 µm) columns were used for preparative and analytical scale separations, respectively. Flow rate was maintained to 10 mL min^{-1} and 1.0 mL min^{-1} for preparative and analytical scale separations, correspondingly. Micromass ZQ (Waters corporation) equipped with HPLC (Waters alliance 2695 model) and photodiode array detector (Waters 2996) were used to analyze the compounds. Atmospheric pressure chemical ionization (APCI) probe was used to detect molecular ions. Similar procedure was followed to isolate defucogilvocarcin V and gilvocarcin V.

Cloning, expression and purification of GilR

Pfu-polymerase (Stratagene) was used to amplify 1.5 kb nucleotide sequence of *gilR* from the cosmid G9B3 using a set of primers (GilR_for: 5'-CGC CAT ATG ACC GCT TCC GTA CCG CCG TTC ACG GTG-3'; GilR_rev: 5'-CCA GAA TTC TCA GAG TCC TAT GGA CAT GCT GTG-3'). The generated PCR product was cloned to Zero Blunt® TOPO® vector (Invitrogen), and the positive clones were sequenced to make sure that no mutation had been incorporated during amplification. GilR was recovered as NdeI-EcoRI fragments from the TOPO-gilR construct and ligated at the identical sites of pET28a(+) (Novagen) to generate a GilR expression construct. Routine procedures such as DNA extraction, ligation, restriction analyses, gel electrophoresis etc. were carried out following the standard protocol.[2] Thus generated GilR construct was transformed in to *E. coli* BL21(DE3) host. Single colony was inoculated in to 20 mL of LB liquid supplemented with kanamycin sulfate and grown the bacteria for 4 h to prepare a seed culture. A liter of LB (100 mL × 10 flasks) was inoculated with 10 mL of the seed and grown the culture at 37℃ until OD_{600} reached to 0.5. The culture was grown at 23℃ for 12 h following the IPTG (0.05 mM final concentration) addition. The cell pellets were collected by centrifugation (4000 *g*, 15 min) and were washed twice with 20 mL of lysis buffer (50 mM KH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.6). Disruption of the pellets was carried out using French press (Thermo electron corporation), and the crude soluble enzyme fraction was collected through centrifugation (17000 *g*). N-terminal His-tagged GilR was purified as an intense-yellow solution through immobilized metal affinity chromatography (IMAC) which is further desalted through profinia protein purification system (Bio-Rad). The concentration of the enzyme was determined using Bradford protein assay method,[3] and found to be

2.37 mg mL^{-1}. The purity of the protein (>95%) was estimated by SDS-PAGE analyses (Figure S1). The protein was also separated on native gradient PAGE (Invirogen) to determine its quaternary structure.

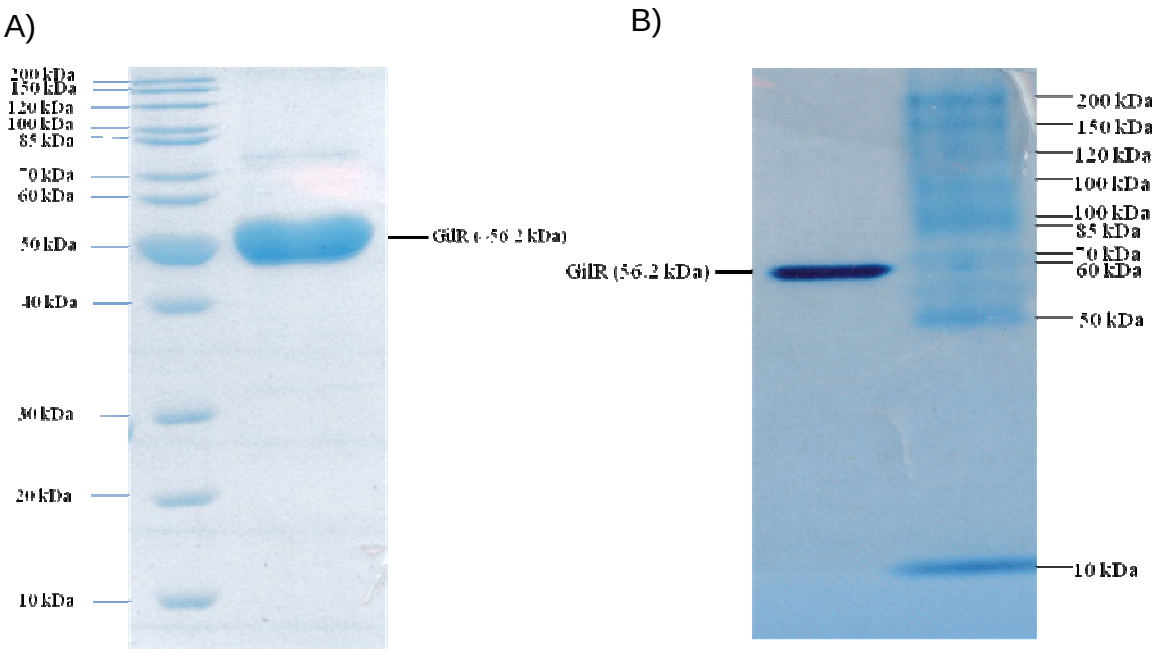


Figure S1. Separation of purified GilR at denaturating condition (SDS-PAGE, A) and at native condition (native gradient PAGE-B)

Cofactor analysis of GilR

UV spectrum of the purified enzyme was typical to that of FAD (Figure S2). To analyze the bound cofactor, the enzyme (2.37 mg) was boiled for 5 minutes and centrifuged (12000 *g*, 15 min). Interestingly, the supernatant was a clear solution, and yellow solid residue was noticed at the bottom of the tube. No UV-absorption was found for the supernatant but a typical of FAD spectrum found when the residue was dissolved in 100 µL of 10% SDS solution and analyzed in spectrophotometer. The protein was precipitated again with the addition of trichloroacetic acid (TCA, 400 µL) and then centrifuged to obtain the supernatant. The supernatant did not absorb UV again but the pellet fraction remained yellow. The results clearly indicated that FAD is covalently bound to the enzyme.

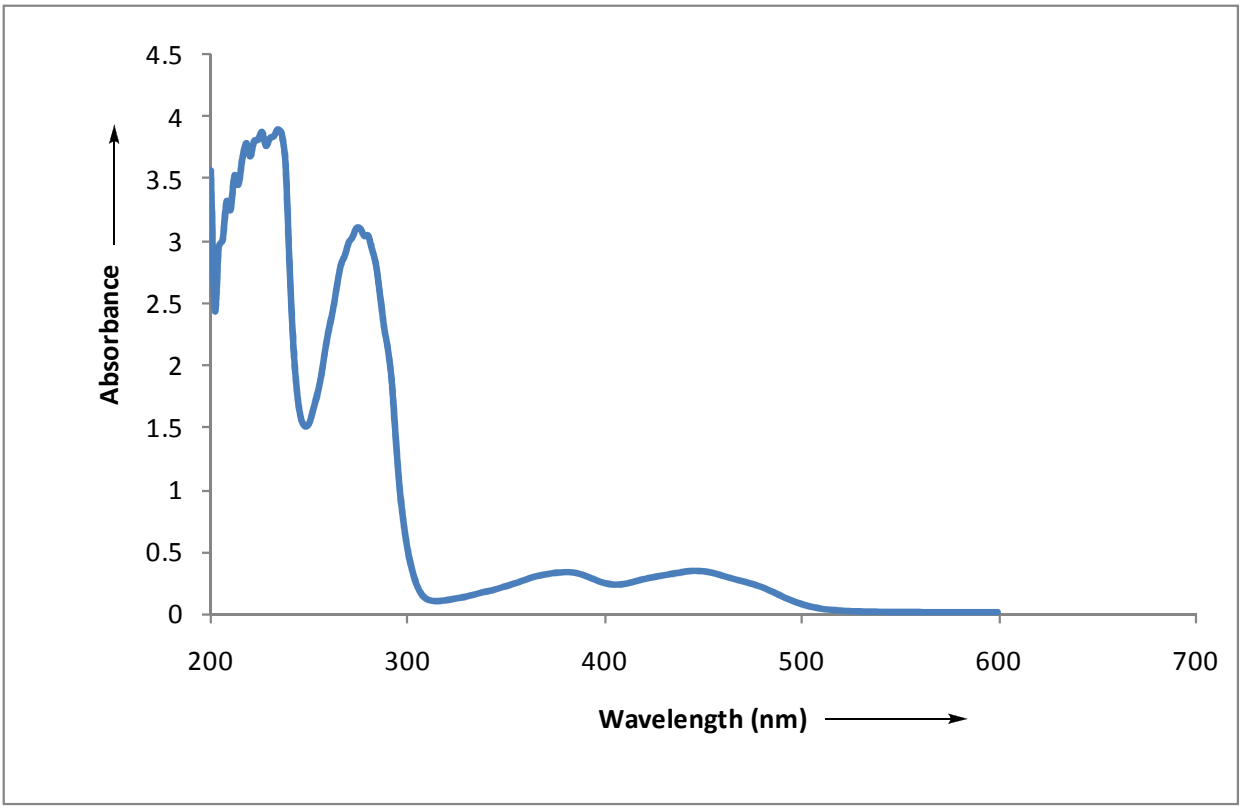


Figure S2. UV spectrum of the purified GilR (200 µL, 4.0 mg mL^{-1} concentration). Desalting buffer was used as a reference.

Kinetic study of GilR catalysis

A typical assay mixture (105 µL) composed of 0.1 mM substrate, 9.5 mM $Mgcl_2$, 50 mM phosphate buffer and 0.1-1 µM enzyme and 15% DMSO (final concentration) were incubated at 20-45 ℃ for 4-10 min to estimate the optimal assay condition for the kinetic analysis. The reaction was terminated through flash freezing at -80℃. The product was extracted twice with 300 µL of ethyl acetate, and the extract was combined and dried in vacuum. The extract was then dissolved in 100 µL of acetonitrile and filtered through 0.2 µM membrane. 70 µL of the filtrate was injected in HPLC/MS. Amount of product formation was estimated through plotting the product peak area in the standard calibration curve. For the kinetic analysis, 8 different substrate concentrations (4.76 to 714 µM final concentration) were incubated with enzyme (0.23 µM final concentration) for 5 minutes at 37 ℃. The res ulting data were fit to the Michaelis-Menten equation by nonlinear regression using GraphPad Prism 5.0 to determine the K_{cat} and K_m values. Three replicates of each sample were analyzed and the average was taken as a result. An identical protocol (described above) was followed to

analyze the products by HPLC/MS. Specific enzyme activities for various substrates were calculated from the amount of product formation (HPLC) at various time points.

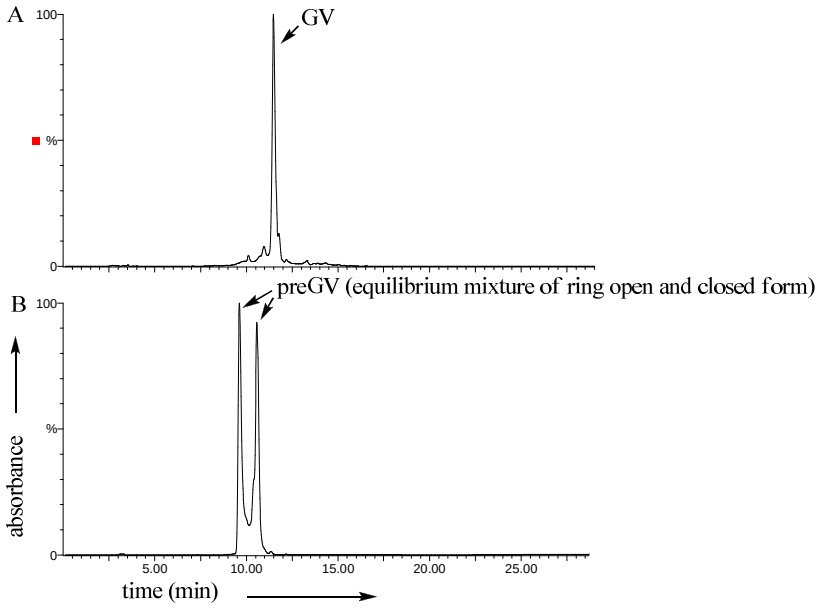


Figure S3. HPLC profile of enzyme assay mixtures: trace A; preGV (0.1 mM) incubated with GilR (1 μM) for 10 min, trace B; a control sample (without GilR) at the identical conditions.

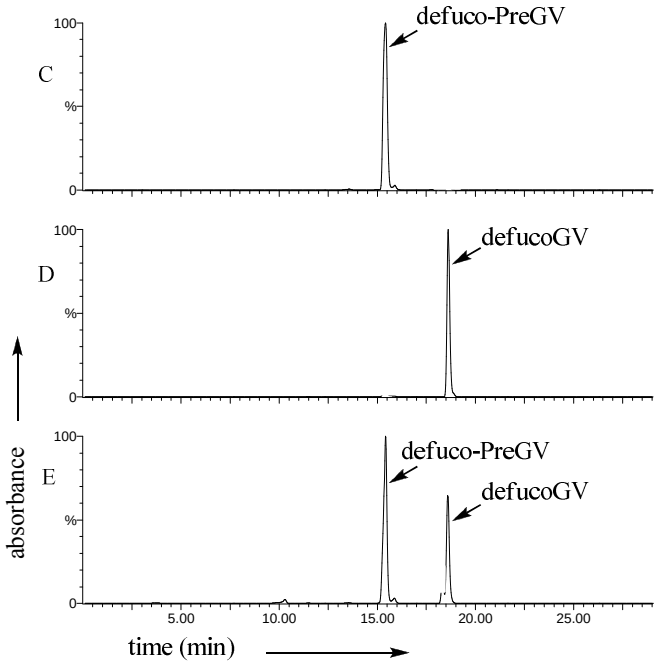


Figure S4. HPLC profile of enzyme assay mixtures incubated for 8 minutes: trace C; a control mixture without GilR, trace D; standard defucoGV, trace E; a reaction mixture (final concentration of 0.1 mM defucopreGV and 0.5 μM GilR).

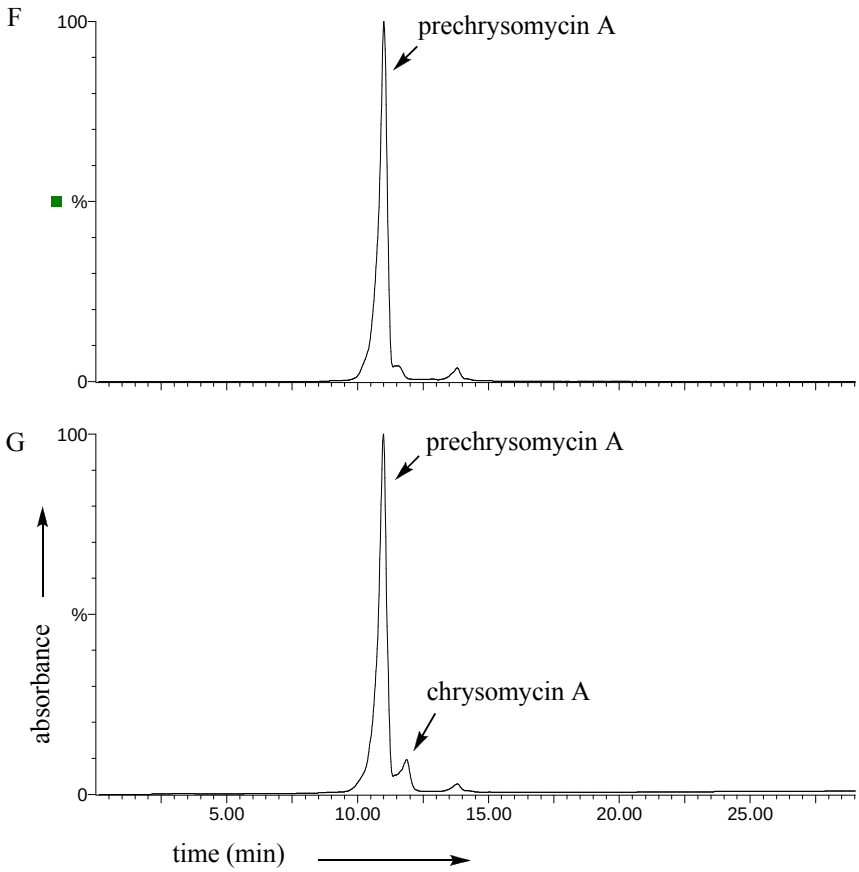


Figure S5. HPLC profile of enzyme assay mixtures after 1h incubation: trace F; a control sample without GilR, trace G; an assay mixture (final concentration of 0.1 mM prechrysomycin A and 1 μM GilR).

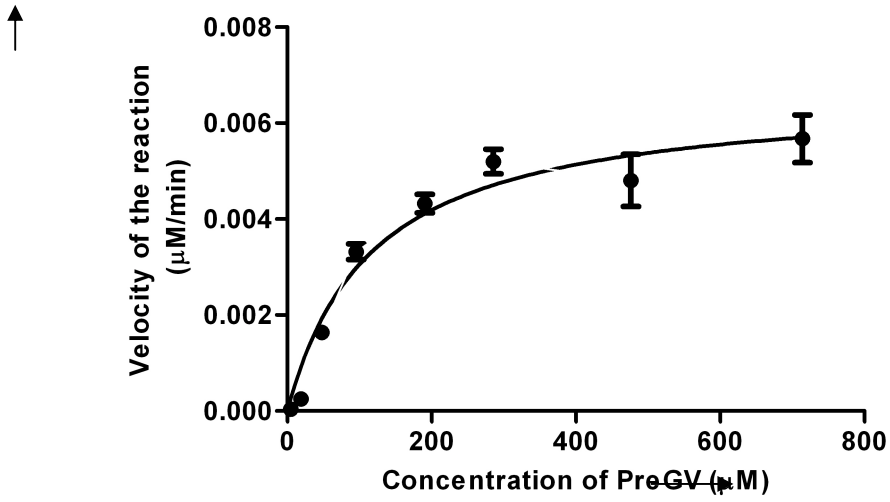


Figure S6. Kinetic profile of GilR with preGV.

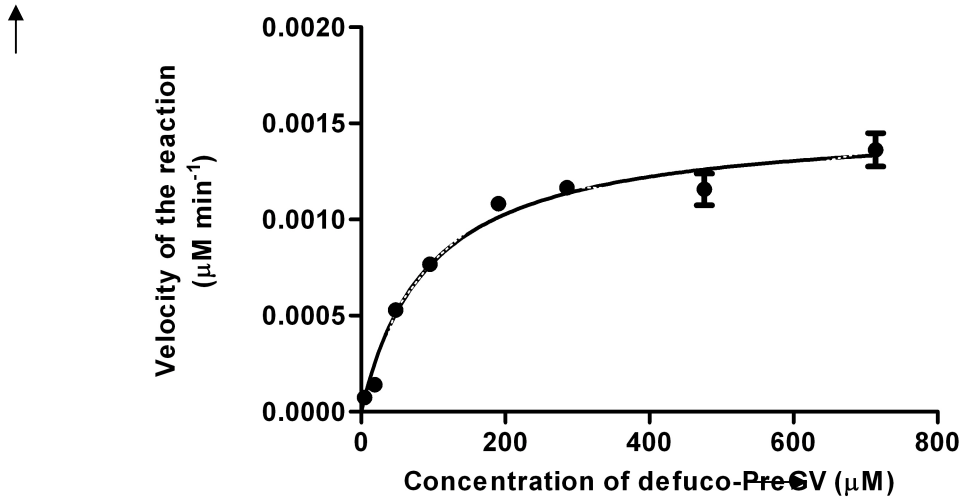
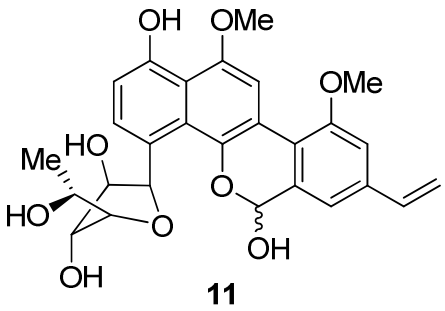


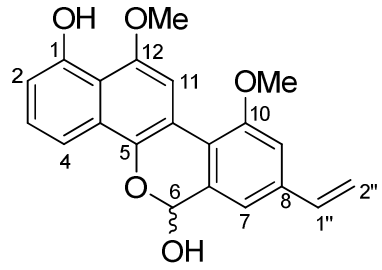
Figure S7. Kinetic profile of GilR with defuco-PreGV.

Preparation of pregilvocarcinV (PreGV, 11), defuco-preGV (12) and prechrysomycin A (13):



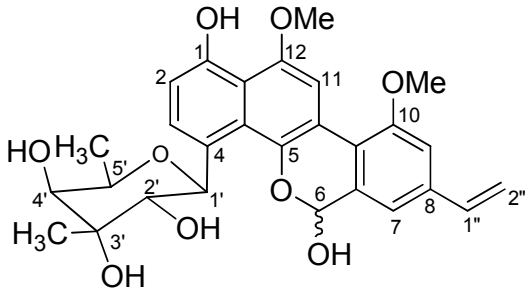
11

Pregilvocarcin V (11). A solution of DIBALH (1 M in hexane, 0.4 mL, 0.4 mM) was added dropwise to a stirred solution of gilvocarcin V (**1**; 20 mg, 0.04 mM) in THF (7 mL) at -78 ℃. After 1 h ethyl acetate (10 mL) was added to quench the reaction. Solvent was removed in vacuum and the mixture was purified through reversed-phase HPLC (Symmetry Prep C_{18}7μm column, 19 × 150 mm) using linear gradient of acetonitrile and water to afford **11** (14 mg, 70%) as colorless solid. The structure was confirmed through the comparison of NMR and MS data with the standard compound isolated from the GilR-minus mutant.[1]



12

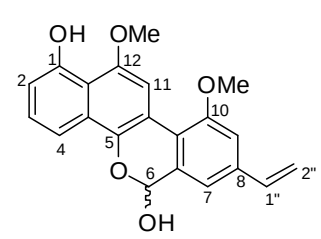
Defuco-pregilvocarcin (12). A solution of DIBALH (1 M in hexane, 0.4 mL, 0.4 mM) was added drop wise to a stirred solution of defuco-gilvocarcin V (**9**; 30 mg, 0.09 mM) in THF (10 mL) at -78 ℃. After 1 h ethyl acetate (10 mL) was added to quench the reaction. Solvent was removed in vacuum and the mixture was purified through reversed-phase HPLC (Symmetry Prep C_{18}7µm column, 19 × 150 mm) using linear gradient of acetonitrile and water to afford **12** (22 mg, 73%) as light yellow solid. ^{1}H NMR ([D_6]DMSO, 500 MHz) δ 9.48 (s, 1H, 1 -OH), 8.03 (s, 1H, H11), 7.72 (d, *J* = 8.0 Hz, 1H, H4), 7.37 (t, *J* = 8.0 Hz, 1H, H3), 7.32 (d, *J* = 5.5 Hz, 1H, 6 -OH), 7.28 (s, 1H, H9), 7.19 (s, 1H, H7), 6.82 (d, *J* = 8.0 Hz, 1H, H2), 6.80 (dd, *J* = 17.0, 11.0 Hz, 1H, H1˝), 6.37 (d, *J* = 4.0 Hz, 1H, H6), 5.98 (d, *J* = 17.0 Hz, 1H, H2˝$_E$), 5.35 (d, *J* = 11.0 Hz, 1H, H2˝$_Z$), 4.06 (s, 3H, -OMe), 4.02 (s, 3H, OMe); ^{13}CNMR ([D_6]DMSO, 125 MHz) δ 156.2, 153.8, 149.3, 140.6, 137.8, 136.2, 134.8, 128.1, 127.2, 116.4, 116.3, 115.0, 114.3, 113.1,111.0, 109.9, 103.6, 92.3, 56.2, 56.1.



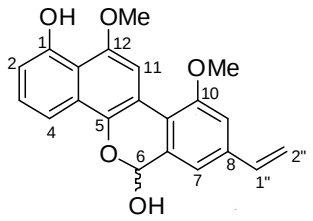
13

Prechrysomycin (13). A solution of DIBALH (1 M in hexane, 0.4 mL, 0.4 mM) was added drop wise to a stirred solution of chrysomycin A (**10**; 20 mg, 0.04 mM) in THF (7 mL) at -78 ℃. After 1 h ethyl acetate (10 mL) w as added to quench the reaction. Solvent was removed in vacuum and the mixture was purified through reversed-phase HPLC (Symmetry Prep C_{18}7µm column, 19 × 150 mm) using linear gradient of acetonitrile and water to afford **13** (11 mg, 55%) as colorless solid. It exists as 1:1 mixture of two diastereomers. ^{1}HNMR ([D_6]DMSO, 500 MHz) δ 9.81 & 9.78 (2 s, 1H,

-OH, D_2O exchangeable), 8.00 & 7.94 (2 s, 1H, H11), 7.75 & 7.73 (2 d, *J* = 8.5 Hz, 1H, H3), 7.30 & 7.08 (2 d, *J* = 6.5 Hz, 1H, -OH, D_2O exchangeable), 7.27 (2 s, 1H, H9), 7.18 (2 s, 1H, H7), 6.83 (2 d, *J* = 8.5 Hz, 1H, H2), 6.80 (2 dd, *J* = 17, 11 Hz, 1H, H1˝), 6.46 & 6.33 (2 d, *J* = 9.0 Hz, 1H, H1´), 6.28 (2 d, *J* = 5.0 Hz, 1H, H6), 5.99 (2 d, *J* = 18.0 Hz, 1H, $H2''_E$), 5.36 (2 d, *J* = 11.0 Hz, 1H, $H2''_Z$), 4.67 (d, *J* = 8.0 Hz, 1H, -OH, D_2O exchangeable), 4.67 & 4.51 (2 s, 1H, -OH, D_2O exchangeable), 4.25 & 4.20 (2 q, *J* = 7.0 Hz, 1H, H5´), 4.06 (2 s, 3H, -OMe), 4.01 & 4.00 (2 s, 3H, -OMe), 3.97 & 3.61 (2 d, *J* = 8.0 Hz, 1H, -OH, D_2O exchangeable), 3.38 & 3.28 (2 d, *J* = 9.5 Hz, 1H, H2´), 3.13 (2 d, *J* = 8.0 Hz, 1H, H4´), 1.25 & 1.21 (2 s, 3H, 3´-OMe), 1.13 & 1.08 (2 d, *J* = 7.0 Hz, 3H, 5´-OMe); ^{13}CNMR ([D_6]DMSO, 125 MHz) δ (156.2, 156.1), (153.2, 153.0), (149.8, 149.7), (142.5, 142.2), (137.9, 137.8), (136.3, 136.2), (135.6, 134.5), (130.2, 129.9), (127.9, 127.3), (127.2, 127.0), 117.0, (116.8, 116.6), (116.1, 115.8), (115.3, 115.2), (114.7, 114.6), (111.0, 110.9), (110.1, 109.9), (103.7, 103.6), (92.5, 91.8), (76.5, 76.4), (76.1, 75.8), 74.2, (73.5, 73.2), (71.1, 71.0), 56.4, 56.1, (23.5, 23.4), 17.2.

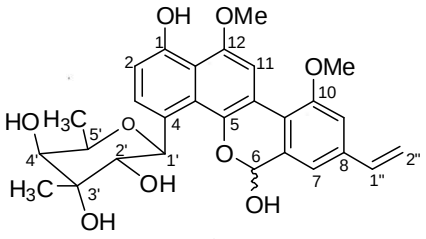


12


OH
OMe
1
12
2
11
OMe
10
5
4
O
6
8
2"
7
1"
OH

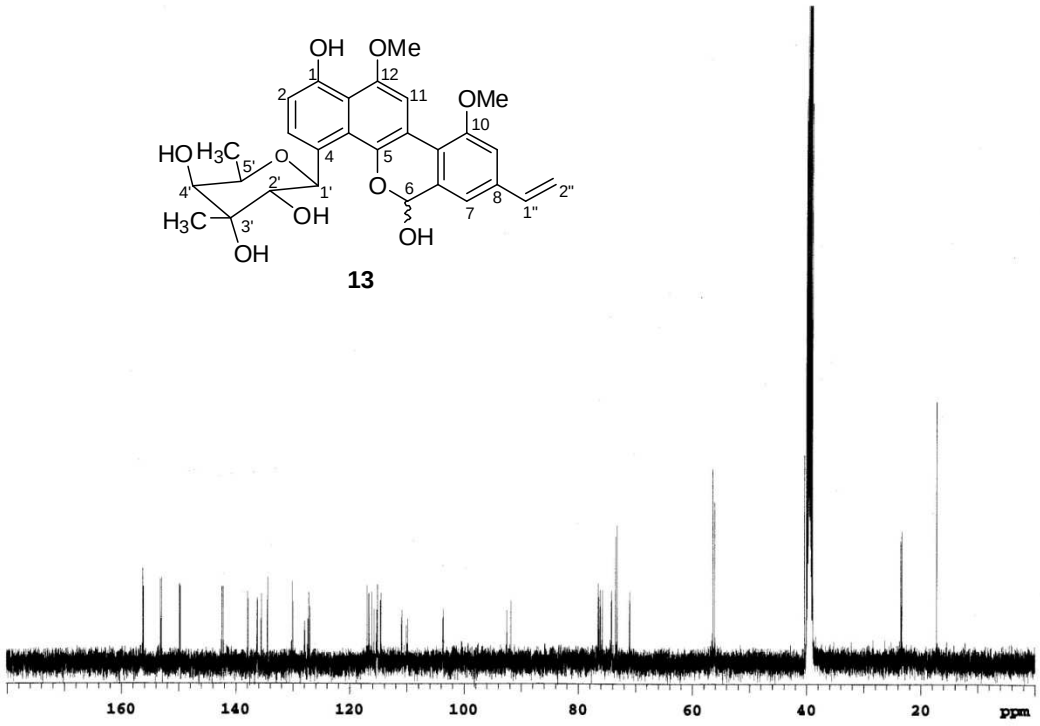
12

200
180
160
140
120
100
80
60
40
20
ppm


OH
OMe
1
12
2
11
OMe
10
HO
H3C
5'
O
4
5
4'
2'
1'
O
6
8
2"
7
1"
H3C
3'
OH
OH
OH

13

11
10
9
8
7
6
5
4
3
2
1
ppm
2.92
3.07
2.91
4.31
2.73
1.90
6.01
1.49
4.39
2.94
2.74
4.40
1.44
1.43
1.40
0.84
9.06
8.48
1.28
1.37
3.44
2.99
6.56
10.67
9.78



13

References

[1] M. K. Kharel, L. L. Zhu, T. Liu, J. Rohr, *J. Am. Chem. Soc.* **2007**, *129*, 3780.

[2] J. Sambrook, D. W. Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, **2001**.

[3] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248.