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Enzymatic Methylation and Structure-Activity-Relationship Studies on Polycarcin V, a Gilvocarcin-Type Antitumor Agent

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Abstract

Polycarcin V, a polyketide natural product of *Streptomyces polyformus*, was chosen to study structure-activity-relationships of the gilvocarcin group of antitumor antibiotics, because of a similar chemical structure and comparable bioactivity with gilvocarcin V, the principle compound of this group, and the feasibility of enzymatic modifications of its sugar moiety by auxiliary O-methyltransferases. Such enzymes were used to modify the interaction of the drug with histone H3, the biological target that interacts with the sugar moiety. Cytotoxicity assays revealed that a free 2'-OH group of the sugar moiety is essential to maintain the bioactivity of polycarcin V, apparently an important H-bond donor for the interaction with histone H3, while converting 3'-OH into an OCH₃ group improved the bioactivity. Bis-methylated polycarcin derivatives revealed weaker activity than the parent compound, indicating that at least two H-bond donors in the sugar are necessary for optimal binding.

Keywords

Gilvocarcin; Polycarcin; Combinatorial Enzymology; Chemical Biology; Methyl Transferases

Introduction

Gilvocarcin V (**1**, GV), a product of *Streptomyces griseoflavus* GÖ 3592 and other *Streptomyces* spp., is the most important member of the gilvocarcin-type aryl-*C*-glycosides, a subgroup of the angucycline family of antibiotics that share an oxidatively rearranged polyketide-derived benzo[*d*]naphtho[1,2-*b*]pyran-6-one core with *O*- or *C*-glycosidically linked sugars at various positions (Scheme 1).^[1] GV's remarkable antitumor and antibacterial activity *in vivo* with low toxicity is due to a unique, combined mechanism-of-action. First, a photochemical [2+2] cycloaddition of the vinyl side chain with thymine residues of DNA promoted by near-UV or visible blue light results in a covalent binding to

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DNA, which in turn leads to single-strand scissions.^[2] Besides, the bioactivity of GV is also attributed to protein-DNA association resulting from an additional interaction with histone H3, a core component of the histone complex, for which the sugar moiety, D-fucofuranose, was proposed to form H-bonds.^[3] The resulting tight interaction between the histone complex and DNA by these antibiotics leads to growth inhibition of cancer cells (Figure 1).^[3-4]

Despite intense studies, mainly by researchers of the US National Cancer Institute, a clinical application of GV was never achieved, to a great extent due to its poor solubility and the necessity of activation by light of ~ 400 nm wavelength, which limits tissue penetration, so that many scientists are trying to correct these drawbacks.^[1b, 5] While modest modifications on the polyketide-derived tetracyclic core can be accomplished through chemo-enzymatic methods, an alternative route for further diversification is through variations in the sugar moieties.^[4e, 5-6] Several chemical or enzymatic approaches to the synthesis of C-aryl glycosides and spiro-C-aryl glycosides exist, but so far none of them could be used to modify the sugar moiety of GV, D-fucofuranose, specifically without changing the polyketide-derived tetracyclic core.^[6a, 7] Even though different sugar moieties could be transferred to the tetracyclic core *in vivo* by using GilGT, the native glycosyltransferase of the gilvocarcin biosynthetic pathway, or other C-glycosyltransferases, such as RavGT and PlcGT, from the ravidomycin and polycarcin biosynthetic pathways, respectively, these variations did not improve GV, and SAR information regarding the interaction of the deoxysugar moiety and histone H3 was missing.^[1b]

Polycarcin V (**2**, PV) was first isolated from *Streptomyces polyformus* in 2008, and prior researches revealed that PV showed bioactivity comparable with GV (Scheme 1).^[1a, 8] PV offered the possibility of enzymatic *O*-methylation of the sugar moiety, since many L-rhamnose-*O*-methyltransferases are known. Unlike the rare sugar D-fucofuranose, an L-rhamnopyranose moiety, usually as mono-, di-, per-*O*-methylated derivatives are found commonly in polyketides, for example in the steffimycin, elloramycin and spinosyn pathways (Scheme 1).^[9] Thus, it was decided to interrogate the substrate flexibility of *O*-methyltransferases from such pathways to modify the sugar moiety of PV *in vitro*, and to synthesize mono- (**6-8**), di- (**9-11**), and per-methylated (**12**) PV derivatives (Scheme 2). It was expected that the methoxy groups might modify the interaction between the sugar moiety and histone H3 (Figure 1), thereby providing some SAR information.^[3] Since PV is the natural by-product of the GV producer *S. polyformus*, this compound could be easily produced to serve as a control standard. For these reasons we chose PV as an alternative to GV for initial SAR studies regarding the sugar moiety,^[8a] expecting some insight into the importance of the three hydroxyl groups of the sugar, which could be important to design better soluble GV-type analogues.

Results and Discussion

In vitro reactions with auxiliary *O*-methyltransferases

Three auxiliary *O*-methyltransferases (*O*-MTs), StfMII from the steffimycin producer *Streptomyces steffisburgensis* NRRL 3193, and ElmMII and ElmMIII from the elloramycin producer *Streptomyces olivaceus* Tü 2353, were used to methylate the L-rhamnopyranose

moiety of PV and produce 2'-*O*-methyl-polycarcin V (**6**), 3'-*O*-methyl-polycarcin V (**7**), 2', 3'-di-*O*-methyl-polycarcin V (**9**), 3', 4'-di-*O*-methyl-polycarcin V (**10**) (Scheme 2). Other *O*-MTs, e.g., from the spinosyn pathway failed to modify the L-rhamnopyranose moiety of PV, and all attempts failed to find an *O*-methyltransferase able to methylate 4'-OH without the presence of 3'-methoxy group (thus **8** and **11** could not be generated). The genes encoding these enzymes were constructed in the vector pET28a(+) to fuse with *N*-terminal His-Tag and over-expressed by *E. coli* BL21 (DE3). After being purified with IMAC column, all enzymes (40 μ M) and starter compounds (200 μ M) were incubated in a mixture containing 50 mM KH₂PO₄, 20 mM MgCl₂, and 2 mM *S*-adenosyl methionine (SAM) at 28 °C for 16 hrs. Low yields of the *in vitro* reactions were the major problem in preparing enough PV derivatives for bioassays. The instability of the enzymes usually led to premature reaction termination or even precipitation of both, enzymes and products. The maximum yield of every *O*-methylation step was about 20%, but the production of 2', 3'-di-*O*-methyl-polycarcin V (**9**) was unexpectedly low, just ~5%. We assumed that this phenomenon might be resulted from the tendency of GV-type compounds to self-assemble through intermolecular stacking in aqueous solution.^[10] The studies were further hampered since polycarcin V (**2**) is actually only a small by-product of the GV biosynthesis pathway of *S. polyformus*, and the average yield was ~0.5 mg L⁻¹, and required a multi-step purification process including liquid-liquid partition, normal phase silica gel chromatography, and semi-preparative HPLC (Figure 2). Various attempts to increase the productivity of polycarcin V (**2**) were met at best with moderate success. For example, media variations or adding supplementary scandium, a rare earth element that was shown to stimulate antibiotic production in *Streptomyces* spp. by 2 - 25 fold at low concentrations (10-100 μ M) to the culture broth, only moderately increased the average yield of PV. Because of these obstacles, we were not able to produce sufficient 2', 3', 4'-tri-*O*-methyl-polycarcin V (**12**) for cytotoxicity assays.^{[11],[12]}

Structure elucidation of the polycarcin V derivatives

The structures of **6**, **7**, **9**, and **10** were elucidated through NMR spectroscopy (Tables 1 and 2) and high-resolution mass spectrometry. HR-MS data directly revealed the occurrence of mono- and di-*O*-methylation while two-dimensional gHMBC spectra confirmed the methyl group/s to be transferred to the desired position/s of the sugar moiety. HR-EI-MS data of 2'-*O*-methyl-polycarcin V (**6**) and 3'-*O*-methyl-polycarcin V (**7**) were consistent with the molecular formula of C₂₈H₂₈O₉, indicating [M]⁺ *m/z* = 508.1717 and 508.1730 (calculated 508.1733) for **6** and **7**, respectively. Their ¹H and gHMBC spectra also showed one additional methoxy signal at 2.67 ppm and 3.48 ppm, which could be assigned as 2'- and 3'-methoxy group of the sugar moiety for **6** and **7**, respectively (Table 1 and 2). Similarly, for 3', 4'-di-*O*-methyl-polycarcin V (**10**), the presence of 3' and 4'-methoxy groups could be confirmed by both HR-EI-MS ([M]⁺ *m/z*, observed 522.1898, calculated 522.1890) and NMR data which indicating two additional methoxy signals at 3.55 and 3.62 ppm in ¹H spectrum and also their obvious correlations with 3' and 4'-proton in gHMBC spectrum (Tables 1 and 2). However, because of the poor synthetic yield of derivative **9** (<5%), we were unable to confirm its structure by 2D NMR and instead relied only on its ¹H NMR spectrum (Table 1) and mass spectrometry data to demonstrate the presence of 2' and 3'-methoxy groups. HR-ESI-MS data of **9** were consistent with a molecular formula of

C₂₉H₃₁O₉, showing [M+H]⁺ *m/z* = 523.1961 (calculated 523.1968) and [M-H]⁻ *m/z* = 521.1809 (calculated 521.1812). The ¹H NMR spectrum showed four methoxy signals, and they could be assigned to two different groups based on the chemical shift values, one (δ_{H} = 4.26, and 4.27) representing the 10- and 12-methoxy groups of the polyketide-derived tetracyclic core, the other (δ_{H} = 3.17 and 3.50) the methoxy groups of the sugar moiety.

Cytotoxicity assays

The bioactivity of the new PV derivatives compared to the natural products GV (**1**) and PV (**2**) were assessed by cytotoxicity assays against two human lung cancer (A549 and H460), and two human colon cancer cell lines (HCT-116 and HT-29; Figure 3, Table 3). Docetaxel and SN-38 (active metabolite of irinotecan) were included to allow for comparisons of the analogues to standards of clinical treatment for lung and prostate cancers, respectively. The following order of activity (from high to low) was observed: 3'-OMe-PV (**7**) > PV (**2**) > 3', 4'-di-OMe-PV (**10**) > GV (**1**) > 2'-OMe-PV (**6**) > 2',3'-di-OMe-PV (**9**). Interestingly, the natural product with the L-rhamnose moiety (PV, **2**) was more active than GV (**1**, with D-fucofuranose moiety), possibly because the L-rhamnopyranose might better interact with histone H3 than the D-fucofuranose. Mono-methylation had different effects on the lead structure **2**, *O*-methylation of the 2'-position (2'-*O*-methyl-polycarcin V, **6**) led to decreased activity, while *O*-methylation of the 3'-position (3'-*O*-methyl-polycarcin V, **7**) increased the activity. This indicated the importance of 2'-OH of L-rhamnopyranose for target interaction, and the necessity of a H-bond donor group in 2'-position, while modifying 3'-OH with nonpolar functional groups (reducing 3'-O to a H-bond acceptor only) seems to improve the interaction between the L-rhamnopyranose sugar moiety and its biological target, presumably histone H3. In addition or alternatively, the bulkier 2'-OCH₃ group might cause steric problems, while a sterically more demanding 3'-residue has a positive effect, perhaps by slightly changing the positioning of the sugar moiety. Although we could not find an enzyme that could selectively *O*-methylate the 4'-position (to yield 4'-*O*-methyl-polycarcin V, **8**) for comparison, it is still reasonable to conclude that a less space-demanding 2'-OH with proton donor properties is most important for the binding of PV to histone H3 because the 4'-OMe of 3', 4'-di-*O*-methyl-polycarcin V (**10**) did not decrease the activity of this compound as much as observed in the other di-methylated polycarcin V, 2', 3'-di-*O*-methyl-polycarcin V (**9**), the weakest PV derivative. We initially hypothesized that two proton donors were required to stabilize the interaction between L-rhamnopyranose and histone H3. However, if this hypothesis were true, 3', 4'-di-*O*-methyl-polycarcin V (**10**) should be much less active than it actually was. Compound **10** is actually only a marginally weaker than PV. This suggests that the interaction between 2'-OH and histone H3 might be strong enough to overcome potential negative effects resulting from the elimination of other proton donor groups. Another possible explanation is that modifying 4'-OH with nonpolar groups could improve this interaction. According to the HPLC diagrams (Figure 2), the structural isomers GV, PV and 2'-*O*-methyl-polycarcin V (**6**), 3'-*O*-methyl-polycarcin V (**7**), respectively, have similar polarity indicated by very close HPLC retention times, while 2',3'-di-*O*-methyl-polycarcin V (**9**) is clearly more polar than 3', 4'-di-*O*-methyl-polycarcin V (**10**), because the HPLC retention time of **9** significantly differs from that of **10** (Figure 2). This information indicated that 2'-OH and 4'-OH also have different physical and chemical properties, which may contribute or lead to the observed effects on the bioactivity after their methylation.

Finally, the cytotoxicity assays revealed an interesting phenomenon – the colon cancer HT-29 cell line is relatively more resistant against these gilvocarcin-type drugs than all other tested cell lines.

In conclusion, using suitable enzymes we generated four polycarcin V derivatives with different combinations of methoxy groups at the sugar moiety for SAR studies. These derivatives may prove useful for further development of new gilvocarcin-type aryl-*C*-glycosides. Despite the set-back that derivatives with only 4'-methoxy or 2',4'-dimethoxy patterns (**8**, **11**) were unavailable, we still obtained important information from the highly consistent data of the cytotoxic assays against four different cancer cell lines. Carbohydrate methyltransferases were also used to diversify indolocarbazole antitumor antibiotic glycosides and insecticides of the spinosyn class. [9b], [13] Such diversifications can also be readily expanded by using recently developed variants of SAM methyltransferases into transferases of other alkyl groups. [14]

The spatial demands and H-donor/H-acceptor properties of the sugar moiety of gilvocarcin-type aryl-*C*-glycosides likely play an important role in their binding to histone H3, and it is indeed possible to produce more active derivatives from the natural products by modifying the sugar moieties. Moreover, we noticed somewhat unexpectedly that the new mono- and di-methylated-PV derivatives are partly and completely, respectively, soluble in nonpolar solvents. Their solubility is still far from more desirable water solubility but an improvement compared to the natural products GV and PV, which are only soluble in DMSO. This is the first report of specific enzymatic modification on the sugar moiety of gilvocarcin-type aryl-*C*-glycosides, which provides not only SAR information for further drug discovery and development, but is also another good example of the “combinatorial biosynthetic enzymology” approach in pharmaceutical sciences.

Experimental Section

General experimental conditions

All operations were performed under ambient atmosphere. All organic solvents, such as chloroform, dichloromethane, ethyl acetate, and methanol, were purchased from Fisher Scientific Co. (Hampton, NH, USA). All chemicals used for the preparation of culture broth or reaction solution were obtained from Fisher Scientific Co. or Acros Organics Co. (Belgium), except *S*-adenosyl methionine (SAM), which was purchased from Nature Made Nutritional Products Co. (Northridge, CA, USA). The water used in this research was distilled and further purified with Millipore water purification system (Millipore Co., Billerica, MA, USA). Analytical thin layer chromatography (TLC) was conducted on silica gel 60-F₂₅₄ from EMD Chemicals Inc. (Darmstadt, Germany), and TLC plates were visualized under UV light at 254 nm. The silica gel (ultra pure, 40-60 μm, 60 Å) used for the flash column chromatography was purchased from Acros Organics Co.

Escherichia coli strains XL1 Blue (Stratagene, La Jolla, CA, USA) and BL21 (DE3) (EMD 4 Biosciences) were used as the hosts for general DNA cloning and over-expression, respectively. Vectors pET28a(+) (Novagen, Darmstadt, Germany) was used for protein overproduction. Cultivation, DNA cloning, and transformation in *E. coli* were carried out

with standard protocols. The wild type *Streptomyces polyformus* was obtained from Dr. C. Hertweck and Dr. I. Sattler at the Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knöll-Institute (HKI), Beutenbergstr. 11a, D-07745, Jena, Germany.

¹H, gHSQC, and gHMBC spectra were recorded using Varian Inova 500 spectrometer at a magnetic field strength of B₀ 11.74 T (Varian, Inc., Palo Alto, CA, USA). Chemical shifts are quoted in parts per million (ppm) relative to TMS. *J* values are recorded in Hz. All D-containing solvents, such as DMSO-*d*₆, chloroform-*d*₁, methanol-*d*₄, and acetone-*d*₆, were purchased from Cambridge Isotope Laboratories Inc. (Boston, MA, USA) or Sigma-Aldrich Co. (St. Louis, MO, USA). A photodiode array detector (Waters 2996) along with a Micromass ZQ 2000 mass spectrometer (Waters Corporation) equipped with and electrospray ionization (ESI) probe was used to detect the molecular ions and identify the compounds (Waters Co., Milford, MA, USA).

Microorganisms and culture conditions

Both GV and PV were purified from the wild type *Streptomyces polyformus* which was first inoculated on solid M2 medium (4 g/liter glucose, 10 g/liter malt extract, 4 g/liter yeast extract, 1 g/liter CaCO₃, and 20 g/liter agar) and then transferred to SG liquid media (20 g/liter glucose, 10 g/liter soytone, 2 g/liter CaCO₃, 1 mg/liter cobalt chloride, 175 μM Sc, pH 7.2) for incubation at 28 °C for 7 days. No antibiotics were added when culturing wild type *S. polyformus*. *Escherichia coli* XL1 Blue and BL21 (DE3) were both grown in Lysogeny Broth (LB) supplemented appropriate antibiotics.

Purification of gilvocarcin V and polycarcin V

The spores of the wild type *S. polyformus* on solid M2 medium were used to inoculate overall 100 L of liquid SG medium (= 5 batches of each 20 L) in 250-ml baffled Erlenmeyer flasks (100 mL SG per flask). After 7 days of fermentation at 28 °C with reciprocal shaking (250 rpm), the culture broth of SG media was centrifuged at 4000 rpm for 25 min to collect cell pellets that were extracted with methanol by sonication. Methanol was then removed from the supernatant under vacuum, and all residues were re-suspended in the aqueous portion of SG media before the extraction with ethyl acetate. After removing ethyl acetate with rotary evaporator, the extract was passed through normal-phase silica gel column and eluted with 1-liter fractions of 0, 4, and 5% methanol in dichloromethane. Fractions containing gilvocarcins and polycarcins were dried under vacuum and further purified through semi-preparative high-performance liquid chromatography (Waters HPLC system, consisting of a controller, a 2487 Dual λ Absorbance Detector, a 1525EF Binary HPLC Pump, and a column: SunFire™ Prep. C18 5 μm 10 × 250 mm; 32.5 min runs at a flow rate of 2.5 mL/min, UV monitoring absorbance at 254 and 360 nm). Gradient used: solvent A: water with 2% formic acid, solvent B: acetonitrile. Solvent B was increased from 25% to 65% (min 0 to 10), then from 65% to 100% (min 10 to 21), then was decreased back to 25% within 3 minutes, and kept at 25% for the last 8.5 min.

Plasmid amplification and protein purification

The genes encoding StfMII, ElmMII, and ElmMIII were constructed in the vector pET28a(+) purchased from Novagen for gene over-expression/protein overproduction. All proteins contain an *N*-terminal 6 × His tag and could be purified with immobilized metal affinity chromatography (IMAC). After amplifying the DNA in *E. coli* XL1 Blue, plasmid isolation were carried out with GeneJet plasmid miniprep kit (Fermentas, Waltham, MA, USA) and then used to transform *E. coli* BL21 (DE3) competent cells for protein overproduction. 1% volume of seed *E. coli* BL21 (DE3) culture with desired plasmid was inoculated into 1 liter of LB supplemented with 50 µg/mL kanamycin (final concentration) and then incubated at 37 °C until OD₆₀₀ value reached 0.4~0.6. The addition of 100 µM β-D-1-thiogalactopyranoside (IPTG, final concentration) induced protein over-production, and target proteins were obtained after overnight incubation at 18 °C. *E. coli* BL21 (DE3) cell pellets were collected by centrifugation (4000 rpm for 25 min) at room temperature, washed twice with 20 mL lysis buffer (50 mM KH₂PO₄, 300 mM KCl, 10 mM imidazole, pH 8.0), re-suspended in 30 mL lysis buffer for high-pressure French press, and finally centrifuged at 16,500g for 35 min at 4 °C to remove cell debris. The supernatant containing target proteins was loaded on IMAC column (TALON affinity resin, Clontech Laboratories Inc.) that had been equilibrated with 10-fold volume lysis buffer in advance. Impurities and target proteins were eluted by lysis buffer and elution buffer (50 mM KH₂PO₄, 300 mM KCl, 250 mM imidazole, pH 8.0), respectively. After concentrating the elution with Millipore Amicon 30K ultracentrifugal filters, all proteins were desalted with reaction buffer (50 mM KH₂PO₄, 150 mM NaCl, 30% glycerol, pH 7.5) and used immediately for *in vitro* reactions. Protein concentrations were determined with Bradford reagent while their sizes were confirmed with sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses.^[15] The measured sizes of purified proteins were in agreement with the calculated ones (Figure S1).

In vitro reaction conditions and purification of the products

All *in vitro* reactions were carried out in the same condition: 500 µL aliquot containing 50 mM KH₂PO₄ (pH 7.4), 20 mM MgCl₂, 2 mM *S*-adenosyl methionine (SAM), 200 µM starter compound, 40 µM methyltransferase, and ddH₂O (doubly distilled water). The reaction solutions were incubated at 28 °C for at least 16 hrs and then extracted with ethyl acetate three times before further purification by semi-preparative HPLC with the same program, as described above. All compounds submitted to cytotoxic assays were at least 95% pure obtained by one more purification with analytical HPLC and confirmed with LC-MS. (Waters HPLC system, consisting of a controller, a 2996 photodiode array detector, and a 2695 Separations Module, Micromass ZQ, and a column: Symmetry® C18 5 µm 4.6 × 250 mm column; 29 min runs at a flow rate of 0.5 mL/min). Gradient used: solvent A: water, solvent B: HPLC-grade acetonitrile. Solvent B was increased from 25% to 100% (min 0 to 15), kept at 100% for 9 min, before it was decreased back to 25% within 2 minutes (min 24 to 26), and kept at 25% for the last 3 min.

Nuclear Magnetic Resonance and Mass Spectrometry

¹H NMR, gHMBC, and gHSQC spectra of PV derivatives, **6**, **7**, **9**, and **10**, were recorded on a Varian Inova 500 spectrometer in either 5% DMSO-*d*₆ in 95% methanol-*d*₄, acetone-*d*₆,

chloroform-*d*₁ (Tables 1 and 2) MS analysis was carried out by MS facilities at University of Kentucky.

Cytotoxicity Assays

H460 and A549 cells were obtained from ATCC (Manassas, VA). HCT-116 and HT-29 were a gift from Dr. Qing-Bai She at the University of Kentucky. Docetaxel and SN-38 were commercially available from LC-Laboratories and Tocris, respectively. Powdered RPMI-1640 media, McCoy's 5A media, resazurine sodium salt and molecular-grade DMSO were from Sigma-Aldrich. The panel of polycarcin analogues was tested for their cytotoxic activity in human lung and colon cancer cell lines at concentrations ranging from 0.1 to 10,000 nM. All drug solutions were prepared in amber tubes and, all drug treatments were carried out under red light. Lung cancer cells (H460 and A549) were grown in RPMI-1640 (pH 7.4) with 10% FBS (v/v) and 1% streptomycin/penicillin (v/v), whereas the colon cancer cell lines (HCT-116 and HT-29) were cultured in McCoy's 5A (pH 7.4) containing the same supplements. Cells were seeded into 96-well plates at densities yielding exponential growth over a 96 hr experimental time period, specifically at 1,000 (H460), 2,000 (A549 and HCT-116) or 5,000 (HT-29) cells/well in 100 μ L of the appropriate media. The cells were allowed to adhere at 37 °C, and 5% CO₂ for 24 hr. The following day concentrated drug solutions in 1.1% DMSO and 98.9% RPMI-1640 were prepared by serial dilution of 10 mg/mL drug stocks in DMSO and were added to the cells. Cell viability was assessed in a subset of wells following drug/diluent addition and at the end of treatment in all cells (10 μ L of 1 mM resazurine in PBS, pH 7.4). Resazurine was allowed to react for 3 hrs at 37 °C (5% CO₂) and fluorescence (590 nm emission/560 excitation) was measured with a spectrophotometer. GI₅₀ parameters and best-fit lines were obtained by nonlinear regression analysis using a dose-response-inhibition equation in Prism 5.04 (GraphPad Prism).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Gilvocarcin V (**1**), polycarcin V (**2**), and some natural products containing methylated L-rhamnoses in the structures – steffimycin (**3**), elloramycin A (**4**), and spinosyn A (**5**).

Figure 1. Proposed *in vivo* mechanism of gilvocarcin V (1) resulting from associations to DNA (red) and histone H3 (blue).

Scheme 2.

Enzymatic synthesis strategy of mono- (**6-8**), di- (**9-11**), and permethylated (**12**) polycarcin V derivatives.

Figure 2.

HPLC traces of gilvocarin V and polycarcins. a) GV, **1** (RT = 15.52 min, AUC = 312,942); b) PV, **2** (RT = 15.71 min, AUC = 464,750); c) 2'-*O*-methyl-polycarcin V, **6** (RT = 17.00 min, AUC = 429,135); d) 3'-*O*-methyl-polycarcin V, **7** (RT = 17.03 min, AUC = 278,817); e) 2', 3'-di-*O*-methyl-polycarcin V, **9** (RT = 19.04 min, AUC = 61,550); f) 3', 4'-di-*O*-methyl-polycarcin V, **10** (RT = 20.85 min, AUC = 129,134). A_{254} : absorbance at 254 nm. RT: retention time. AUC: area under curve.

Figure 3.

Cytotoxicity assays of GV (1), PV (2), and PV derivatives (6, 7, 9, 10) against two human lung cancer cell lines (A549 and H460) and two human colon cancer cell lines (HCT-116 and HT-29).

Table 1

¹H NMR data (500 MHz) for **6**, **7**, **9**, **10**

Compound	2'- <i>O</i> -methyl PV, 6 ^a	3'- <i>O</i> -methyl PV, 7 ^a	2', 3'-di- <i>O</i> -methyl PV, 9 ^b	3', 4'-di- <i>O</i> -methyl PV, 10 ^c
Position	δ_{H} (J in Hz)			
1-OH			9.79, s	9.71, s
2	6.89, d (8.0)	6.88, d (8.0)	6.98, d (8.5)	7.05, d (8.5)
3	7.83, d (8.5)	7.82, d (8.0)	7.97, d (8.5)	7.96, d (8.5)
7	7.83, brs	7.82, brs	8.09, brs	8.09, brs
9	7.40, brs	7.37, brs	7.78, brs	7.37, brs
10-OCH ₃	4.08, s	4.04, s	4.27, s	4.12, s
11	8.26, s	8.23, s	8.65, s	8.45, s
12-OCH ₃	4.06, s	4.04, s	4.26, s	4.11, s
1'	5.81, brs	5.72, brs	5.87, brs	5.92, brs
2'	3.79, d (3.0)	4.48, d (2.5)	3.96, d (3.5)	4.65, d (3.0)
2'-OCH ₃	2.67, s	N/A	3.17, s	N/A
3'	4.03, dd (3.0, 9.5)	3.78, dd (3.0, 9.0)	3.77, dd (3.0, 9.5)	3.94, dd (3.0, 9.5)
3'-OCH ₃	N/A	3.48, s	3.50, s	3.55, s
4'	3.41, t (9.5)	3.57, t (9.0)	3.56, m (4.3, 9.0, 9.0)	3.25, t (9.5)
4'-OCH ₃	N/A	N/A	N/A	3.62, s
5'	3.56, dd (6.0, 9.5)	3.60, dd (6.5, 9.5)	3.47, dd (5.5, 9.0)	3.57~3.62, m
6'	1.47, d (6.0)	1.47, d (5.5)	1.36, d (5.5)	1.43, d (6.5)
1''	6.78, dd (11.0, 17.5)	6.76, dd (11.5, 18.0)	6.97, dd (12.3, 16.3)	6.80, dd (11.0, 18.0)
2''	5.45, d (10.5)	5.43, d (11.0)	5.49, d (11.0)	5.45, d (10.5)
	5.98, d (17.5)	5.96, d (18.0)	6.14, d (18.0)	5.95, d (17.5)

^aThe solvent used for NMR experiments was 5% DMSO-*d*₆ in 95% methanol-*d*₄.^bThe solvent used for NMR experiments was acetone-*d*₆.^cThe solvent used for NMR experiments was chloroform-*d*₁.

Table 2

 $^{13}\text{C}^a$ and gHMBC data (500 MHz) for 6, 7, 10

Compound	2'-O-methyl PV, 6 ^b		3'-O-methyl PV, 7 ^b		3', 4'-di-O-methyl PV, 10 ^c	
Position	δ_c , mult. ^d	gHMBC correlation	δ_c , mult.	gHMBC correlation	δ_c , mult.	gHMBC correlation
1	154.8, C	3-H	153.5, C	3-H	153.8, C	3-H
2	113.3, CH		113.1, CH		114.0, CH	
3	130.1, CH	1'-H	130.6, CH	1'-H	130.5, CH	
4	127.1, C	2-H, 1'-H	126.0, C	2-H, 1'-H	110.6, C	2'-H
4a	123.7, C	3-H	123.1, C	3-H	125.7, C	1'-H
4b	143.0, C	11-H	141.9, C	11-H	142.8, C	11-H
6	161.1, C	7-H	160.1, C	7-H	160.1, C	
6a	122.3, C		122.3, C		122.3, C	
7	120.4, CH	9-H	120.3, CH	9-H	120.3, CH	
8	140.3, C	2''-H	139.2, C	2''-H	139.2, C	
9	115.2, CH	7-H	115.2, CH	7-H	115.5, CH	
10	158.8, C	10-OCH ₃	157.5, C	10-OCH ₃	157.5, C	10-OCH ₃
10-OCH ₃	56.8, CH ₃		56.8, CH ₃		57.6, CH ₃	
10a	124.1, C	7-H, 9-H, 11-H	123.0, C	7-H, 9-H, 11-H	123.0, C	
10b	113.4, C		113.4, C		113.4, C	
11	102.8, CH		102.6, CH		103.5, CH	
12	153.4, C	11-H, 12-OCH ₃	152.5, C	11-H, 12-OCH ₃	157.5, C	12-OCH ₃
12-OCH ₃	56.6, CH ₃		56.5, CH ₃		57.6, CH ₃	
12a	116.2, C	2-H, 11-H	115.2, C	2-H, 11-H	115.9, C	11-H
1'	78.4, CH	3-H	78.9, CH	3-H	78.4, CH	
2'	84.9, CH	2'-OCH ₃	69.3, CH		70.2, CH	
2'-OCH ₃	61.7, CH ₃	2'-H	N/A	N/A	N/A	N/A
3'	76.1, CH	2'-H, 4'-H	85.4, CH	3'-OCH ₃	85.7, CH	3'-OCH ₃
3'-OCH ₃	N/A	N/A	56.9, CH ₃		58.3, CH ₃	
4'	74.9, CH	2'-H, 6'-H	73.3, CH	2'-H, 5'-H, 6'-H	83.5, CH	4'-OCH ₃ , 6'-H
4'-OCH ₃	N/A	N/A	N/A	N/A	62.1, CH ₃	
5'	77.8, CH	1'-H, 6'-H	78.0, CH	1'-H, 6'-H	75.1, CH	6'-H
6'	18.7, CH ₃	4'-H, 5'-H	18.6, CH ₃		19.6, CH ₃	
1''	136.5, CH	7-H, 9-H	136.4, CH	7-H, 9-H	136.5, CH	
2''	117.0, CH ₂		116.9, CH ₂		118.0, CH ₂	

^a ^{13}C NMR data was inferred from gHSQC and gHMBC spectra.^bThe solvent used for NMR experiments was 5% DMSO-*d*₆ in 95% methanol-*d*₄.^cThe solvent used for NMR experiments was chloroform-*d*₁.^dmultiplicity

Table 3

Cytotoxic activities (GI₅₀) of various gilvocarcin/polycarcin derivatives in comparison with two established anticancer drugs against human lung and colon cancer cell lines

Compound	Cytotoxicity (GI ₅₀ , nM)			
	A549	H460	HCT-116	HT-29
Docetaxel ^a	1.9	3.3		
SN-38 ^b			4.4	37.4
GV (1)	510.7	287.6	193.6	596.8
PV (2)	281.3	129.4	70.4	203.5
2'-OMe-PV (6)	789.5	452.9	526.6	1140.0
3'-OMe-PV (7)	77.2	33.4	25.3	135.3
2',3'-di-OMe-PV (9)	5.0×10^8	2.6×10^8	6.4×10^8	7.6×10^8
3',4'-di-OMe-PV (10)	269.0	273.5	167.4	459.5

^a used as control for human lung cancer cell lines A549 and H460;

^b used as control for human colon cancer cell lines HCT-116 and HT-29