# GILVOCARCINS, NEW ANTITUMOR ANTIBIOTICS

## 4. MODE OF ACTION

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The mode of action of gilvocarcins was studied. Gilvocarcins V, M and A possessed antibacterial activities decreasing in that order. Gilvocarcin V inhibited DNA synthesis in *Bacillus subtilis* through strong interaction with DNA and resulting cleavage. Gilvocarcin M showed interaction with DNA and a small change in DNA mobility upon electrophoresis in agarose gel, while gilvocarcin A showed no interaction with DNA, thus reflecting their relative biological activities.

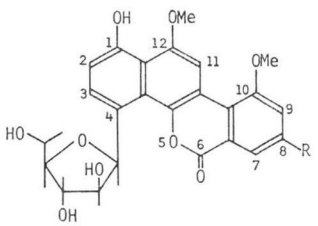
Gilvocarcins V and M are yellow crystalline antibiotics that were discovered in the culture broth of *Streptomyces gilvotanareus*[1] and later in the culture broth of *Streptomyces anandii*[2]. The structure of the antibiotics (Fig. 1) was elucidated by us[3,4] and was later confirmed by BALITZ *et al.*[2] Gilvocarcin V showed antibacterial activity and antitumor activity, while gilvocarcin M has weak antibacterial activity and practically no antitumor activity.[1,5] It has been suggested from studies by X-ray crystallography that gilvocarcin M has the molecular dimensions suitable for intercalating between the base pairs in DNA and gilvocarcin V has even stronger intercalation with DNA.[4] Thus we have initiated studies on the mode of action of gilvocarcins.

The present paper will describe antimicrobial activities and the mode of action of gilvocarcins.

### Antimicrobial Activities of Gilvocarcins

Gilvocarcins V and M were prepared as described in previous papers.[1,3] Gilvocarcin A (8-aldehyde isomer of gilvocarcin V) was prepared as follows. A suspension of gilvocarcin V (200 mg), dihydropyran (1 ml), and *p*-toluenesulfonic acid (10 mg) in dioxane (30 ml) was stirred for 19 hours at room temperature. After evaporation of solvent, the reaction product was dissolved in chloroform, washed with water, dried over sodium sulfate, and evaporated. The product was purified by silica gel column chromatography using chloroform as eluant to give 220 mg of tetrahydropyranyl gilvocarcin V (THP-gilvocarcin V). To a stirred solution of THP-gilvocarcin V (150 mg) in dioxane (15 ml) was added 15 mg of $OsO_4$ and the solution was stirred for 10 minutes. To the above reaction mixture was added water (1.5 ml) and then $NaIO_4$ (400 mg) in small portions during 10 minutes and the mixture was stirred for 2.5 hours. It was diluted with water and extracted with chloroform. The extract was washed with water, dried over sodium sulfate and evaporated to leave a syrup, which was purified on preparative silica gel

Fig. 1. Structure of gilvocarcins.




Gilvocarcin A: R = CHO
Gilvocarcin V: R = CH=$CH_2$
Gilvocarcin M: R = $CH_3$

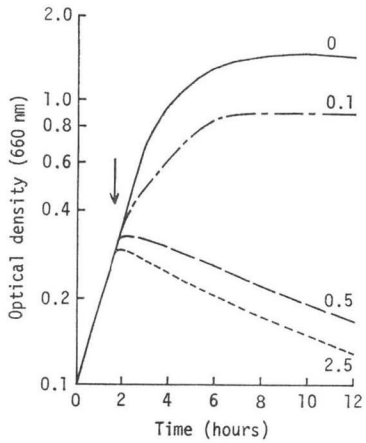
thin-layer chromatography using $CHCl_3$ - EtOAc (7 : 3) as developing solvent to afford 60 mg of THP-gilvocarcin A. A solution of THP-gilvocarcin A (30 mg) in dioxane (10 ml) and 2 N HCl (0.1 ml) was stirred for 6 hours at room temperature to give the crystalline products, which was recrystallized from $CHCl_3$-$Me_2CO$ to afford yellow powder of gilvocarcin A (10 mg). MS *m/z* 496 ($M^+$), 363, IR (KBr) 3390, 1720, 1700, 1605, 1373, 1240, 1148, 1130, 785 $cm^{-1}$, UV $\lambda_{max}^{EtOH}$ 244, 276, 292, 404 nm.

The *in vitro* activities against various bacteria were determined by the agar dilution method at pH 7.0 using a medium consisting of 3 g Tryptone, 3 g meat extract, 1 g yeast extract, 1 g glucose and 16 g of agar per liter of tap water. As we have previously reported[1], gilvocarcin V had strong activity against *Staphylococcus aureus* and *Bacillus subtilis*, moderate activity against *Escherichia coli*, while no activity was observed against other Gram-negative bacteria tested, and gilvocarcin M showed greatly reduced activity against Gram-positive bacteria and no activity against Gram-negative bacteria. Gilvocarcin A was devoid of antibacterial activity at 100 $\mu$g/ml except for *Staphylococcus aureus*.

The effect of gilvocarcin V on the growth of *Bacillus subtilis* is shown in Fig. 2. The growth was inhibited at 0.1 $\mu$g per ml and increasing concentrations showed more suppression of growth. At 0.5 $\mu$g per ml, lysis of cells was observed indicating that gilvocarcin V acts as a bactericidal antibiotics.

Fig. 2. Effect of gilvocarcin V on the growth of *Bacillus subtilis*.

Gilvocarcin V was added at the time indicated by the arrow and the numbers in the figure indicate amounts of the drug added ($\mu$g/ml). The medium consisted of 0.2g $MgSO_4 \cdot 7H_2O$, 2g citric acid, 10g $K_2HPO_4$, 3.5 g $NaNH_4HPO_4 \cdot 4H_2O$, 5 g glucose, 1 g Casamino acids, 2 g yeast extract, 50 mg tryptophan and 50 mg arginine per liter of tap water (pH 7.0 prior to sterilization). Growth was automatically recorded with the Bio-photorecorder (Toyo Kagaku Sangyo, Japan) at 37°C.




Effect of Gilvocarcin V on the Syntheses of Macromolecules in *Bacillus subtilis*

Gilvocarcin V exhibited a rather narrow antibacterial spectrum. However, its activities against experimental tumors are marked.[5] Thus it is of interest to study the effect of gilvocarcin V on the syntheses of macromolecules. The effects of gilvocarcin V on the syntheses of DNA, RNA and protein in growing cells of *B. subtilis* are presented in Fig. 3. The syntheses of DNA, RNA and protein were followed by measuring the incorporation of labeled [methyl-$^3$H]thymidine, [2-$^{14}$C]uracil and [4,5-$^3$H]-L-leucine into acid-insoluble precipitates. After the addition of radioactive precursors, 0.5 ml samples were removed at intervals and poured into 2.5 ml of ice-cold 5% trichloroacetic acid and placed for one hour in the ice bath. They were filtered through HA Millipore filters (0.45 $\mu$) and washed with 15 ml of cold 5% trichloroacetic acid. The filters were dried and counted in vials containing toluene scintillation fluid consisted of 4 g 2,5-diphenyloxazole and 0.1 g 2,2′-*p*-phenylene-bis-(5-phenyloxazole) per liter of toluene.

Inhibition of protein synthesis was slight and detected only after 20 minutes at the concentration of 0.25 $\mu$g per ml. However, RNA synthesis was inhibited in 5 to 10 minutes, although the inhibition was not extensive as DNA synthesis. DNA synthesis was blocked completely at 5 minutes and even at the

Fig. 3. Effects of gilvocarcin V on macromolecular syntheses in *Bacillus subtilis*.

(A) Incorporation of [$^3$H]thymidine into the acid-insoluble fraction.

When the cell density of *B. subtilis* in the medium shown in Fig. 2 reached $OD_{660nm}=0.1$, [methyl-$^3$H]thymidine (1 $\mu$Ci/ml) was added. After the addition of the drug and the radioactive precursor at time 0, 0.5 ml samples were removed and treated as described in the text. All the incubation were carried out at 37°C with shaking. Numbers in Figures indicate amounts of the drug added.

(B) Incorporation of [$^{14}$C]uracil into the acid-insoluble fraction.

Experimental procedures were the same as those described in (A) except that [2-$^{14}$C]uracil (0.05 $\mu$Ci/ml) was added.

(C) Incorporation of [$^3$H]leucine into the acid-insoluble fraction.

Experimental procedures were the same as those described in (A) except that [4,5-$^3$H]-L-leucine (0.2 $\mu$Ci/ml) was added.

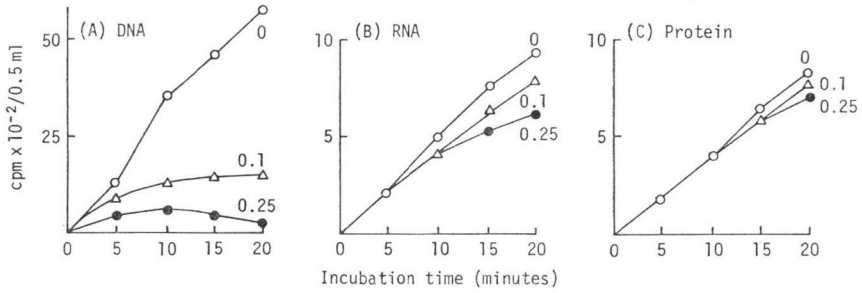
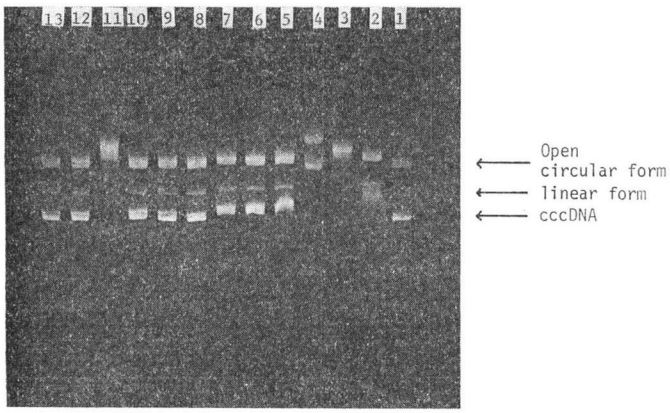



Fig. 4. The effect of increasing concentrations of gilvocarcins on PM2 DNA as shown by agarose gel electrophoresis.

(1) Control, PM2 DNA, (2)~(4) 0.001 mM, 0.01 mM, 0.1 mM gilvocarcin V, (5)~(7) 0.001 mM, 0.01 mM, 0.1 mM gilvocarcin M, (8)~(10) 0.001 mM, 0.01 mM, 0.1 mM gilvocarcin A, (11) 0.001 mM gilvocarcin V+1 mM $NaBH_4$, (12) 0.001 mM gilvocarcin M+1 mM $NaBH_4$, (13) 0.001 mM gilvocarcin A+1 mM $NaBH_4$.




concentration of 0.1 $\mu$g per ml, where inhibition of the growth was slight (Fig. 3), the synthesis was severely inhibited. These results indicate that gilvocarcin V primarily inhibits DNA synthesis and subsequently affected RNA synthesis, without affecting protein synthesis.

## Interaction of Gilvocarcins with DNA Molecules

As shown in the foregoing section gilvocarcin V was found to be a potent inhibitor of DNA synthesis at the concentrations without protein and RNA syntheses. Thus interaction of gilvocarcins to DNA was examined further in order to find how the antibiotics affect DNA molecules.

Phage PM2 DNA was purchased from Boeringer Mannheim, Germany and showed three bands on agarose gel electrophoresis. According to AAIJ and BORST,[6] the fastest moving band corresponded to the native form of covalently closed circular (ccc) DNA, the intermediate the linear form of the DNA and the most slowly moving one the open circular form as illustrated in Fig. 4, lane 1. The reaction mixture of PM2 DNA and gilvocarcins consisted of 0.3 $\mu$g PM2 DNA and various amounts of gilvocarcins in 20 mM tris-HCl buffer (pH 8.0). Reaction was carried out for 1 hour at 37°C and stopped by the addition of bromophenol blue and sucrose to make 0.01% and 10%, respectively. Reaction was followed by agarose gel (0.8%) electrophoresis as described by YAGI *et al.*[7]

As shown in Fig. 4, 0.001 mM of gilvocarcin V caused changes in the mobility of DNA (lane 2) and 0.01 mM of gilvocarcin V caused single strand scission in addition to intercalation with DNA (lane 3 and 4). In the presence of reducing agent (1 mM $NaBH_4$) gilvocarcin V revealed enhanced activity in strand scission of the cccDNA, namely 0.001 mM gilvocarcin V in the presence of 1 mM $NaBH_4$ caused equivalent scission of the cccDNA by 0.01 mM gilvocarcin V alone (lane 11).

Gilvocarcin M also caused changes in the mobility of DNA (lane 5~7), but strand scission was not observed even in the presence of a reducing agent (1 mM $NaBH_4$) (lane 12).

Gilvocarcin A did not cause changes in the mobility of PM2 DNA even in the presence of a reducing agent (lane 8~10, 13) and $Fe^{2+}$ (data not shown).

The above data correlate well with those of biological activities as shown in the foregoing sections and shows the importance of substituents at 8 position to their biological activity, in contrast to expectation from the molecular dimensions of gilvocarcins. Specifically the formyl group at 8 position would be of similar bulkiness to the vinyl group and less bulkiness than the methyl group, but no intercalation of gilvocarcin A with PM2 DNA was observed while gilvocarcin M and gilvocarcin V showed intercalation with DNA.

The present studies showed that gilvocarcins M and V are a new member of DNA-intercalating antitumor antibiotics and gilvocarcin V has DNA-cleaving activity as well.

Added in Proof

After submitting this paper, the similar results were obtained by T. T. WEI *et al.* (J. Antibiotics 35(4): 545~548, 1982).

## References


1) NAKANO, H.; Y. MATSUDA, K. ITO, S. OHKUBO, M. MORIMOTO & F. TOMITA: Gilvocarcins, new antitumor antibiotics. 1. Taxonomy, fermentation, isolation and biological activities. J. Antibiotics 34: 266~270, 1981
2) BALITZ, D. M.; F. A. O'HERRON, J. BUSH, D. M. VYAS, D. E. NETTLETON, R. E. GRULICH, W. T. BRADNER & T. W. DOYLE: Antitumor agents from *Streptomyces anandii*. J. Antibiotics 34: 1544~1555, 1981
3) TAKAHASHI, K.; M. YOSHIDA, F. TOMITA & K. SHIRAHATA: Gilvocarcins, new antitumor antibiotics. 2. Structure elucidation. J. Antibiotics 34: 271~275, 1981
4) HIRAYAMA, N.; K. TAKAHASHI, K. SHIRAHATA, Y. OHASHI & Y. SASADA: Crystal and molecular structure of antibiotic gilvocarcin M. Bull. Chem. Soc. Jap. 54: 1338~1342, 1981
5) MORIMOTO, M.; S. OHKUBO, F. TOMITA & H. MARUMO: Gilvocarcins, new antitumor antibiotics. 3. Antitumor activity. J. Antibiotics 34: 701~707, 1981
6) AAIJ, C. & P. BORST: The gel electrophoresis of DNA. Biochem. Biophys. Acta 269: 192~200, 1972
7) YAGI, M.; T. NISHIMURA, H. SUZUKI & N. TANAKA: Chartreusin, an antitumor glycoside antibiotic, induces DNA strand scission. Biochem. Biophys. Res. Commun. 98: 642~647, 1981