ANTITUMOR AGENTS FROM *STREPTOMYCES ANANDII*: GILVOCARCINS V, M AND E

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(Received for publication June 10, 1981)

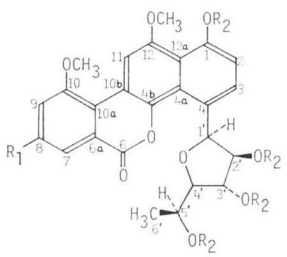
The isolation and structure elucidation of gilvocarcins V, M and E from *Streptomyces anandii* is reported. Solvent extraction and high performance liquid chromatography were employed along with NMR and X-ray crystallographic analysis. Gilvocarcins V and M showed antimicrobial activity while only gilvocarcin V demonstrated potential as an anti-tumor agent.

Very recently, TOMITA and coworkers[1,2] have reported the fermentation, isolation and structure elucidation of gilvocarcins V (**1**) and M (**2**). They note that **1** was previously described under the name of toromycin by HORII *et al.*[3] although the structure of toromycin was not elucidated. Recently we have isolated **1** and **2** and a related compound gilvocarcin E (**3**) from a culture of *Streptomyces anandii* subsp. *araffinosus* strain C-22437 (ATCC-31431).* The producing culture was isolated at Bristol Laboratories from a soil collected in Katpadi, Madras, India. We report here the isolation, structure elucidation and biological activities of compounds **1**~**3**.

Isolation of Gilvocarcins V, M and E

Purification of the complex extract from *Streptomyces anandii* (C-22437) has led to isolation of the gilvocarcins. The procedure is outlined in Scheme 1. The complex extract was obtained from methyl *iso*-butyl ketone whole broth extraction under slightly alkaline conditions (pH 9.5~10). In earlier

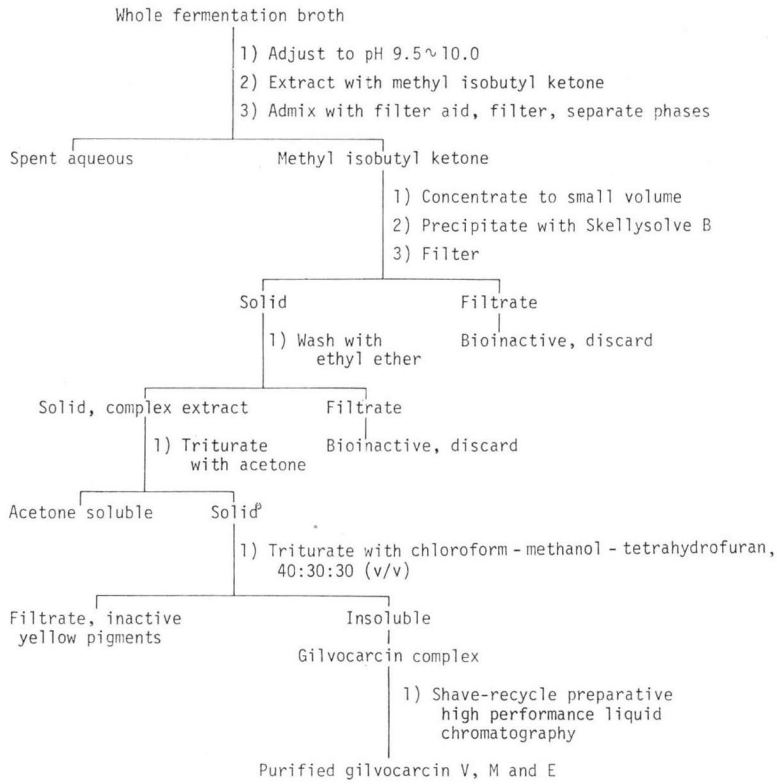
Fig. 1. Structures of gilvocarcins V(**1**), M(**2**) and E(**3**) and their peracetates **4**, **5**, **6**.



	R_1	R_2
1	$CH=CH_2$	H
2	CH_3	H
3	C_2H_5	H
4	$CH=CH_2$	Ac
5	CH_3	Ac
6	C_2H_5	Ac

* Compound **1** is 4-fucofuranosyl-1-hydroxy-10,12-dimethoxy-8-vinyl-6-*H*-benzo[d]-naphtho[1,2b]-pyran-6-one, compound **2** is 4-fucofuranosyl-1-hydroxy-10,12-dimethoxy-8-methyl-6-*H*-benzo[d]-naphtho[1,2b]-pyran-6-one and compound **3** is 4-fucofuranosyl-1-hydroxy-10,12-dimethoxy-8-ethyl-6-*H*-benzo[d]-naphtho[1,2b]-pyran-6-one.

Scheme 1. Gilvocarcin isolation, general flowchart.



extractions, ethyl acetate was used in the whole broth extraction. In subsequent extractions, methyl *iso*-butyl ketone was found to perform equally well and to have the additional property of lower volatility and consequently less loss during filtration of the mycelial mat. The solids obtained from methyl *iso*-butyl ketone extraction of whole broth were washed with ether to remove lard oils and antifoams used in the fermentation. This generally leaves an easily handled yellow solid for future operations.

The solids as obtained from the above fermentation extraction process contained what appeared to be a group of closely related yellow pigments, none of which have been shown to be antitumor active. These yellow pigments do show antibiotic properties and are the major substances present in the complex extract. Experience has demonstrated that separation of these impurities from the minor component of interest (*i.e.*, gilvocarcin) using silica gel chromatography is not possible because the pigments typically crystallize on the silica gel surface thereby leading to smearing effects which prevent any meaningful resolution. To remedy this problem, a simple organic solvent trituration scheme was developed which effects a gross separation of the yellow pigments. The trituration process was carried out in two steps each of which resulted in selective dissolution of impurities and hence separation from the gilvocarcins, which were insoluble throughout the process. The first step involved trituration of the complex extract with acetone. The insoluble solid from the acetone trituration contained additional yellow pigments along with gilvocarcin complex. The second phase of the process involved trituration with a ternary organic solvent system: chloroform - methanol - tetrahydrofuran 40: 30: 30 (v/v). The soluble fraction contained the yellow pigments. The insoluble fraction contained the enriched gilvocarcin complex.

Gilvocarcin V (**1**) was purified from the enriched mixture using preparative high performance liquid chromatography on silica gel. Gilvocarcin V exhibited both antimicrobial and antitumor activity.

Analytical HPLC, *Bacillus subtilis* zone of inhibition, and P388 mouse tumor assays were used throughout the separation processes to monitor relative levels of gilvocarcin V and co-produced substances.

Resolution of the mixture was accomplished through the use of high performance liquid chromatographic techniques employing silica gel columns. The analytical solvent system routinely used was 30: 70: 3, hexane - ethyl acetate - *iso*-propanol, along with 0.1 ~ 0.4 part water depending on the relative activity state of the silica gel surface in the column being used. If tailing effects were observed, then typically the water content of the solvent system was increased within the 0.1 ~ 0.4 part range to improve peak symmetry thereby permitting more quantitative analysis.

Preparative high performance liquid chromatographic separation of the gilvocarcin complex was possible only on a limited scale (200 ~ 300 mg) due to a relatively low solubility of the complex in the developing solvent system. The preparative solvent system was ethyl acetate - *iso*-propanol - water, 87: 13: 0.5 (v/v). Shave-recycle methodology was used to effect separation of **1** from **2**. The separation was achieved in two stages. Initial chromatography gave **1** enriched to 70 ~ 85% purity. Accumulated solids were rechromatographed to yield material of 95% purity.

In view of the poor solubility of **1** and **2** in solvents generally useful for preparative chromatography, an alternate procedure was attempted. The mixture was peracetylated and a preparative scale separation of the mixture attempted. The solubility of the acetylated compounds was greatly improved and separation of the acetates on larger scale proceeded smoothly. Saponification of gilvocarcin V tetraacetate (**4**) gave a 60% recovery of gilvocarcin V (**1**). In addition to gilvocarcin tetraacetate, gilvocarcin M tetraacetate (**5**) and gilvocarcin E tetraacetate (**6**) were isolated.

The fermentation production yield of compound **3** was very low. Consequently, a mixture of the peracetates of **1** and **2** was hydrogenated over 10% Pd/C. HPLC analysis of the product mixture showed complete disappearance of the peak for **4** with concomitant appearance of a new peak which cochromatographed with authentic **6** as isolated from the peracetylated gilvocarcin complex. This material also showed identical spectroscopic properties with **6**. Compound **6** was isolated from the mixture of **5** and **6** using preparative HPLC. Saponification of **6** gave **3** in modest yield which was purified using preparative HPLC. Compound **3** had identical chromatographic behavior with **1** in two HPLC systems.

Structure Elucidation

The structures of compounds **1** ~ **6** were determined using a combination of NMR spectroscopy and X-ray diffraction. Gilvocarcin V (**1**), had mp 220 ~ 230°C (dec),* MS: *m/z* 494, UV λ_{max}^{MeOH} (ε) 246 (34,400), 278 (28,750), 288 (31,300), 310 sh (14,200), 321 sh (13,000), 334 sh (10,800), 350 (8600), 396 nm (12,300) and IR ν_{max}^{KBr} 3390 (OH), 1700 (C=O) cm^{-1}. The elemental analysis was consistent with $C_{27}H_{26}O_9 \cdot H_2O$. The X-ray analysis confirmed a mole of water of hydration. The ^1H NMR spectrum of **1** (DMSO-d_6) was consistent with that reported earlier.[2] It showed 5 aromatic protons, a vinyl group, 5 methine protons attached to oxygen, a methyl group, two methoxy groups and 4 exchangeable protons.

Gilvocarcin M (**2**) had mp 241 ~ 243°C (dec), MS: *m/z* 482, UV λ_{max}^{MeOH} (ε) 244 (19,900), 267 (11,500), 275 (14,500), 304 (4800), 318 sh (4700), 329 (4400), 343 (3600), 385 nm (4500) and IR ν_{max}^{KBr} 3390 (OH), 1700, 1680 (C=O) cm^{-1}. The elemental analysis of **2** was consistent with $C_{26}H_{26}O_9 \cdot H_2O$. It was evi-

* All melting points are uncorrected and were determined in a Gallenkamp melting point apparatus.

Table 1. ^{1}H NMR data for **3**, **4** and **6**.[a]

Proton	3[b]	4[c]	6[b]
C1–OH	9.74 (s, exchangeable)	—	—
C11–H	8.49 (s)	8.55 (s)	8.55 (s)
C3–H	8.08 (d, 8.4 Hz)	8.10 (d, 8.3 Hz)	8.02 (d, 8.2 Hz)
C2–H	6.95 (d, 8.4 Hz)	7.20 (d, 8.3 Hz)	7.31 (d, 8.2 Hz)
C7–H	7.83 (d, 1.6 Hz)	8.15 (d, 2.0 Hz)	7.84 (d, 1.5 Hz)
C9–H	7.53 (d, 1.6 Hz)	7.38 (d, 2.0 Hz)	7.55 (d, 1.5 Hz)
C1′–H	6.22 (d, 5.5 Hz)	6.60 (d, 3.3 Hz)	6.41 (d, 3.2 Hz)
C2′–H	4.64 (m)	6.21 (dd, 3.3+1 Hz)	6.08 (dd, 0.8+3.2 Hz)
C3′–H	3.90 (m)	5.23 (dd, 4+1 Hz)	5.09 (dd, 3.9+0.8 Hz)
C4′–H	3.52 (dd, 6.5+4 Hz)	4.21 (dd, 4+6.5 Hz)	4.20 (dd, 4.0+6.3 Hz)
C5′–H	3.90 (m)	5.42 (dq, 6.5+6.5 Hz)	5.30 (dq, 6.5+6.3 Hz)
C6′–H_3	1.25 (d)	1.47 (d, 6.5 Hz)	1.43 (d, 6.5 Hz)
C2′–OH	4.49 (d, 7.0 Hz)	—	—
C3′–OH	5.08 (d, 5.6 Hz)	—	—
C5′–OH	4.83 (d, 5.2 Hz)	—	—
C10–OCH_3	4.16 (s)	4.16 (s)	4.17 (s)
C12–OCH_3	4.16 (s)	4.03 (s)	4.03 (s)
C8–R	1.38 (t, 7.0 Hz)	6.85 (dd, 11+17.5 Hz)	1.36 (t, 7.2 Hz)
	2.84 (q, 7.0 Hz)	5.96 (d, 17.5 Hz)	2.85 (q, 7.2 Hz)
	—	5.48 (d, 11 Hz)	—
C2′–OAc	—	1.58 (s)	1.54 (s)
C3′–OAc	—	2.19 (s)	2.18 (s)
C5′–OAc	—	2.36 (s)	2.32 (s)
C1–OAc	—	2.44 (s)	2.42 (s)

a. At 100 MHz in ppm with TMS as internal standard. b. DMSO-d_6. c. $CDCl_3$.

dent from the ^{1}H NMR spectrum of **2** that compounds **1** and **2** differed only in the substitution of the vinyl group in **1** for a methyl.

From the peracetylated complex, we isolated the peracetates of gilvocarcins V and M (*i.e.* compounds **4** and **5**). In addition a low yield of a third peracetate (**6**) was isolated. The ^{1}H NMR spectrum of **6** was similar to that of compounds **4** and **5** with the exception that the vinyl group of **4** and methyl group of **5** were substituted by an ethyl group in **6**. Conversion of **4** to **6** by hydrogenation confirmed that the ethyl and vinyl functions in **4** and **6** were on the same carbon of the nucleus. Hydrolysis of **6** gave **3** the NMR spectrum of which was consistent with **3** being a homolog of **2**. The ^{1}H NMR spectra of compounds **3**, **4** and **6** are recorded in Table 1 while the ^{13}C NMR spectra are in Table 2. The spectra of **1**, **2** and **5** were reported by TOMITA *et al.*[2]

A number of attempts were made to grow crystals of **1**, **2**, **4** and **5** for X-ray analysis. This resulted in crystals too small for analysis. We observed that a mixture of **1** and **2** gave larger crystals than either compound alone. Inasmuch as the ^{1}H and ^{13}C NMR spectra of **1** and **2** indicated that the compounds were very similar to one another, we decided to proceed with analysis of the mixed sample.[8~10] Since then, a referee informed us that HIRAYAMA and coworkers[11] have published the crystal and molecular structure of gilvocarcin M (**2**).

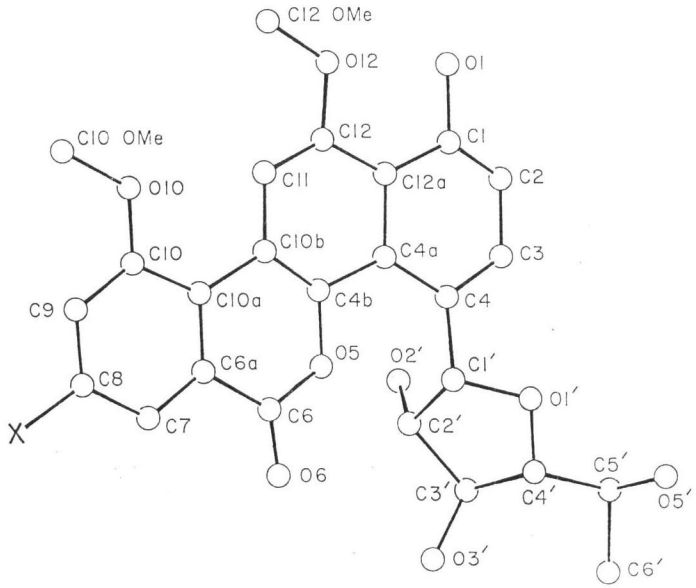
Fig. 2 is a computer generated perspective drawing of the final X-ray model of gilvocarcin V (**1**) and M (**2**). The group represented as X is a 0.75 to 0.25 mixture of CH_3 and vinyl (see Experimental). The

Table 2. ^{13}C NMR data for **3**, **4** and **6**.

Carbon	3[a,c]	4[a,d]	6[b,d]	Carbon	3[a,c]	4[a,d]	6[b,d]
1	151.8	146.0	146.2	8–R_1	15.0q	116.4t	15.2q
2	111.7	120.4	120.1	—	28.0t	135.3d	28.9t
3	129.0	127.6	127.4	10–OCH_3	(56.4)[e]	56.1	56.2
4	125.9	129.9	129.7	12–OCH_3	(56.6)[e]	56.1	56.2
4a	123.7	124.1	124.1	1′	80.8	81.5	81.5
4b	142.0	141.7	141.5	2′	78.7	77.9	77.9
6	159.7	160.0	160.1	3′	78.8	78.9	78.8
6a	121.3	122.5	122.0	4′	85.9	83.4	83.3
7	118.0	120.1	121.1	5′	66.4	69.8	69.7
8	146.3	138.8	145.8	6′–CH_3	20.1	16.4	16.4
9	119.9	113.9	117.1	1–CO	—	169.8	169.6
10	157.1	157.4	157.2	2′–CO	—	168.5	168.3
10a	121.9	122.2	122.3	3′–CO	—	170.5	170.2
10b	113.0	114.4	114.5	5′–CO	—	170.5	170.2
11	101.7	104.7	104.8	1–$COCH_3$	—	21.3	21.3
12	152.6	150.9	150.8	2′–$COCH_3$	—	20.1	20.1
12a	114.8	119.9	119.6	3′–$COCH_3$	—	(21.0)[e]	(21.0)[e]
				5′–$COCH_3$	—	(20.9)[e]	(20.9)[e]

a) δ in ppm 90.13 MHz with TMS as internal standard. b) δ in ppm 25.2 MHz with TMS as internal standard. c) DMSO-d_6. d) $CDCl_3$. e) Assignments reversible.

Fig. 2. A computer generated perspective drawing of the final X-ray model of gilvocarcin. Hydrogens are omitted for clarity and the absolute configuration shown is based on the assumption of D-fucose. The group represented as X is both $-CH_3$ and $-CH=CH_2$ (see text).



18-membered aromatic ring system is very nearly planar with a maximum deviation of 0.11 Å for C8 from the best least-squares plane. The absolute configuration was not determined by the X-ray experiment and the enantiomer shown was selected on the assumption that the sugar was D-fucose.

The conformation of the furanose ring is best described as an envelope with O1′ forming the flap. This atom (O1′) is 0.5 Å from the plane of the four furanose carbons. The configurations at the chiral carbons (assuming D-fucose) are C1′(*R*), C2′(*R*), C3′(*R*), C4′(*S*) and C5′(*R*).

There are several H-bonds in the unit cell. Atom O2′ forms hydrogen bonds to O6 and O3′ in the X+1 symmetry position of length 2.70 Å and 2.77 Å respectively. The water of hydration which is disordered between two sites forms a bond of 2.82 Å with O3′.

Biological Effects

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of gilvocarcin V and M fractions for a variety of microorganisms was determined by using the standard 2-fold dilution procedure.[4] The results, shown in Table 3, indicate that both fractions are inhibitory to Gram-positive bacteria with little effect on Gram-negative bacteria and fungi. The largest differential in effect involved the strain of *Proteus vulgaris* tested which was inhibited by gilvocarcin V at 2 μg/ml whereas gilvocarcin M inhibited growth at about 30 times that concentration.

Induction of Lysogenic Bacteria

Gilvocarcin V was tested for its ability to induce bacteriophage production in the lysogenic strain of *Escherichia coli* W1709 (λ) using the methods of PRICE, *et al.*[5] There was no evidence of induction at levels up to 1.6 μg/ml which was toxic to the host cells.

Antitumor Effects

Tests for inhibition of P388 leukemia in mice were performed using procedures previously reported[6] (Table 4). Dose response titrations were run using both a day 1 only and day 1, 5 and 9 injection schedules. Tumor inhibition was observed at or above 8 mg/kg/injection with little evidence of schedule dependency.

Commentary on Nomenclature

In view of their origin, compounds **1**, **2** and **3** were initially referred to as anandimycins A, B and C respectively. As there is a multiplicity of names for these compounds due to their independent discovery by three separate groups, we suggest that in the future they be referred to as toromycins on the basis of priority of the paper by HATANO *et al.*[3] Thus toromycin A=gilvocarcin V=anandimycin A, toromy-

Table 3. Minimum inhibitory concentration (μg/ml).

Organism	Code No.	Gilvocarcin V (1)	Gilvocarcin M (2)	Organisms	Code No.	Gilvocarcin V (1)	Gilvocarcin M (2)
Streptococcus pneumoniae	9585	0.13	0.25	*Pseudomonas aeruginosa*	9843A	>63	>63
〃 *pyogenes*	9604	0.13	0.5	*Trichophyton rubrum*	A22789	32	>32
Staphylococcus aureus	9535	0.06	0.13	〃 *mentagrophytes*	A9870	32	>32
〃 *aureus* (Pen-Res)	9606	0.25	0.5	*Microsporum canis*	A9872	32	32
Streptococcus faecalis	20688	0.016	0.13	〃 *canis*	A22494	>32	>32
Escherichia coli	15119	>63	>63	*Candida albicans*	9540	>32	>32
〃 *coli*	203410-1	>63	>63	〃 *albicans*	15049	>32	>32
Klebsiella pneumoniae	15130	>63	>63	〃 *albicans*	15050	>32	>32
Proteus vulgaris	21559	2	>63	〃 *tropicalis*	15051	>32	>32
Serratia marcescens	20019	>63	>63	〃 *krusei*	15052	>32	>32
Enterococcus cloacae	9659	>63	>63				

Table 4. Effect of gilvocarcin V on P388 leukemia (Exp. 6747).

Material	Treatment schedule	Dose, i.p. mg/kg/injection	MST days	Effect MST % T/C	AWG g Day 4	Survivors Day 5
Gilvocarcin V (Lot S654-G96)	day 1	64	14.5	161	−0.3	6 / 6
		32	13.0	144	−0.6	6 / 6
		16	11.0	122	−0.6	6 / 6
		8	11.5	128	−0.2	6 / 6
		4	10.0	111	−0.9	6 / 6
		2	10.0	111	−0.2	5 / 5
		1	9.0	100	−0.1	6 / 6
		0.5	9.0	100	−0.1	6 / 6
	days 1, 5, 9	16	13.0	144	0	5 / 5
		8	13.5	150	+0.2	6 / 6
		4	10.5	117	+0.7	6 / 6
		2	9.0	100	+0.2	6 / 6
		1	9.0	100	+0.6	6 / 6
		0.5	9.0	100	−0.1	6 / 6
Control		Saline	9.0	—	0	10 / 10

Tumor inoculum: 10^6 ascites cells implanted i.p.
Host: CFD_1 ♀ mice.
Toxicity: <4/6 mice alive on day 5.
Evaluation: MST=media survival time.
Effect: % T/C=(MST treated/MST control)×100.
Criteria: % T/C≥125 considered significant antitumor activity.

cin B=gilvocarcin M=anandimycin B, and toromycin C=gilvocarcin E=anandimycin C. The name anandimycin is retained here for reference by any biological papers arising from studies on materials submitted by us to the Division of Cancer Treatment, National Institute of Health.

Experimental

Production of Gilvocarcins with Strain C-22437

A. Shake-flask Fermentation: *Streptomyces anandii* subsp. *araffinosus* strain C-22437 was maintained and transferred in test tubes on agar slants of yeast extract-malt medium consisting of 4 g glucose, 4 g yeast extract, 10 g malt extract and 20 g agar made up to one liter with distilled water. With each transfer, the agar slant culture was incubated for seven days at 27°C. To prepare an inoculum for the production phase, the surface growth from a slant culture was transferred to a 500 ml Erlenmeyer flask containing 100 ml of sterile medium consisting of 30 g glucose, 30 g soy flour and 3 g $CaCO_3$ made up to one liter with distilled water. This vegetative culture was incubated at 27°C for 48 hours on a Gyrotory tier shaker (Model G-53, New Brunswick Scientific Co., Inc.) set at 230 rev./minute describing a circle with a 5.1 cm diameter. Four ml of vegetative culture was transferred to a 500 ml Erlenmeyer flask containing 100 ml of sterile production medium consisting of 50 g glucose, 20 g soy flour, 5 g Fermo 30 yeast (Yeast Products, Inc., Paterson, NJ), 5 g washed brewers' yeast, 5 g Maggi yeast extract (The Nestle Co., White Plains, NY), 0.25 g $(NH_4)_2SO_4$ and 3 g $CaCO_3$ made up to one liter with distilled water. The production culture was incubated at 27°C on a shaker such as that used for the vegetative culture set at 210 rev./minute for 240 hours at which time the culture contained gilvocarcin.

B. Tank Fermentation: For production of gilvocarcin in a tank fermentor 380 liters of vegetative culture was transferred to a tank containing 3,030 liters of medium consisting of 50 g glucose, 20 g soy flour, 5 g Fermo 30 yeast, 5 g yeast extract, 5 g washed brewers' yeast, 0.25 g $(NH_4)_2SO_4$ and 3 g $CaCO_3$ made up to one liter with tap water. Temperature was maintained at 27°C; the air flow rate

was 1,400 liters/minute. Polypropylene glycol was used to control foaming. The tank fermentation was terminated after 82 hours of incubation for isolation of gilvocarcin.

Isolation of Complex Extract from Fermentation of *Streptomyces anandii* Strain C-22437

A. Small Scale Isolation: Whole fermentation broth, 8 liters, was adjusted from pH 7.9 to pH 9.5 with dilute aqueous sodium hydroxide and stirred vigorously with an equal volume of methyl *iso*-butyl ketone for 20~30 minutes. The mixture was admixed with inert diatomaceous earth filter aid and filtered on a mat formed of the same material under vacuum suction. The phases in the filtrate were separated, and the aqueous phase, except for a small sample reserved for bioassays, was discarded. The organic phase was evaporated under vacuum to a small volume (50~100 ml) and diluted with Skellysolve B* to precipitate a dark yellow solid which was dried *in vacuo* to give 3.5 g of complex extract.

B. Large Scale Isolation: Whole fermentation broth (3,000 liters) at pH 8.0 was admixed with inert diatomaceous earth filter aid and filtered under vacuum. The filtrate was chilled at 0~10°C. The mycelia/filter aid cake was extracted by recirculating 1,000 liters of acetone through the mat filter under vacuum. The acetone extract was concentrated to an aqueous residue under reduced pressure which was combined with the chilled filtrate, adjusted to pH 9.5~10.0 with 10% sodium hydroxide, and stirred vigorously with a half volume of methyl *iso*-butyl ketone. The phases were separated using continuous centrifugation (Centrico Inst. Corp., Model MEM-1256 centrifuge). The pH of the aqueous layer was checked and readjusted to pH 9.5~10.0 if necessary. Reextraction with an additional half volume of methyl *iso*-butyl ketone was carried out as above. The spent aqueous was then discarded and the methyl *iso*-butyl ketone extracts combined. Vacuum concentration at 0~10°C to a near oil and dilution with 10~20 volumes of heptane or Skellysolve B gave an orange-yellow tacky solid which weighed approximately 800 g. The latter was washed with 10 liters of diethyl ether to remove approximately 100 g of inactive oily material. The resultant orange-yellow solid was dried *in vacuo* to yield approximately 700 g of complex extract.

Solvent Trituration of the Complex

A. Small Scale Purification: Crude complex extract (15 g) as extracted from whole broth was triturated with 1.5 liters acetone using magnetic stirring and sonication aids. The thoroughly triturated solution/suspension was filtered under vacuum using a suitable medium porosity sintered glass filter. The soluble fraction was evaporated to dryness to give 7.6 g of dark tannish-red solid. The original insoluble fraction (7.4 g) was a yellow solid which by analysis contained a yellow pigment and a mixture of gilvocarcin V, M and E. Secondary processing of the 7.4 g yellow solid involved trituration with 1 liter of chloroform - methanol - tetrahydrofuran, 40: 30: 30 (v/v). Magnetic stirring and sonication procedures were used as with the acetone trituration. The resulting solution/suspension was filtered using a medium porosity sintered glass filter. Evaporation of the filtrate yielded 4.6 g of yellow pigment with only trace amounts of gilvocarcin complex. The insoluble fraction, which was greenish-yellow in color, weighed 2.8 g, and was shown by HPLC analysis to contain enriched gilvocarcin complex.

B. Larger Scale Purification: Crude complex extract (700 g), as extracted from whole broth obtained from a 3,028-liter scale tank fermentation, was triturated vigorously with two 5-liter portions of acetone using air driven mechanical overhead stirring devices. The resulting solution/suspension was filtered under vacuum using a large Buchner funnel. The combined filtrates were evaporated to dryness yielding 518 g of solid. The insoluble yellow solid, which weighed 120 g, was subsequently triturated with two 4 liter portions of chloroform - methanol - tetrahydrofuran, 40: 30: 30 (v/v). The combined filtrates were evaporated to dryness to yield 73.2 g of yellow pigment solids. The final insoluble greenish-yellow solid weighed 46.5 g, HPLC analysis of which confirmed the presence of gilvocarcins.

TLC Definition of Gilvocarcin Complex in Crudes

The complex extract was evaluated in several different TLC systems. Eventually a chloroform - methanol - water, 90: 10: 0.25 (v/v), solvent system was observed to afford the best resolution of the systems tried. At least 20 different components were resolved. The gilvocarcin complex was a single

* Skellysolve B is a commercially available petroleum solvent (Skelly Oil Co.) comprising isomeric hexanes and having a boiling point of 60~68°C. The main component of Skellysolve B is *n*-hexane.

spot, distinctly characterized by a bright yellow fluorescence under long wave light, appearing from Rf 0.4~0.5 depending on the brand of silica plates. This property facilitated the initial discovery and development of isolation procedures.

HPLC Separation of Gilvocarcin Complex

A. Analytical HPLC System: The HPLC apparatus used in this procedure consisted of a Waters Assoc. Model M-6000A Solvent Delivery System, a Waters Assoc. Model U6K injector with 2 ml loop, a μ-Porasil prepacked column (Waters Assoc., 3.9 mm ID×30 cm, 10 μparticle size, surface area 400 m^2/g), a Waters Assoc. Model 440 UV detector, a Schoeffel Model SF770 spectroflow monitor detector, and a Heath/Schlumberger strip chart recorder.

The solvent system developed for optimum analytical separation of gilvocarcin complex was hexane - ethyl acetate - *iso*-propanol - water, 30: 70: 3: 0.1 (v/v). The following separation parameters were observed:

$$k'_V=1.35, \quad k'_M=1.90, \quad \alpha_{V,M}=1.4$$

Variability was noted between different columns and in the same column over time with respect to silica activity, resulting in k' variability and, frequently, in adverse tailing affects. It was determined that when adverse tailing occurred, increasing the water concentration of the solvent system (between the range of 0.1~0.4 parts) served as a convenient remedy. The water level in the solvent system was kept to a minimum, however, in order to maximize the lifespan of ideal column activity state after which extensive regeneration procedures are necessary to re-activate the silica gel surface.

The column effluents were monitored at 254 nm or 365 nm. The flow rate was typically 3.0~4.0 ml/minute. Samples of 1~50 μg were injected. Relatively enriched gilvocarcin solids were dissolved in 10 percent methanol in tetrahydrofuran to approximately 1 mg/ml concentration for HPLC assay.

B. Preparative HPLC System: The column chamber of a Prep LC/System 500 apparatus (Waters Assoc., Inc., Milford, Mass.) was loaded with two PrepPak-500 silica gel columns and placed under a radial pressure of 40 atm. When a new PrepPak was being used for the first time, deactivation of the silica gel surface with a water containing solvent system was carried out. The deactivation procedure involved pumping a 5-liter volume of solvent through the columns at a flow rate of 200 ml/minute, wasting the first 2 liters and then recirculating the remaining 3 liters for 30 minutes. In this method the deactivation solvent system was ethyl acetate - methanol - water, 90: 10: 1 (v/v), which also was used as a routine post-chromatography column wash solvent.

The chromatographic solvent system employed in this method was ethyl acetate - *iso*-propanol - water, 87: 13: 0.5 (v/v). Typically, 20 liters of this solvent mixture was prepared for one chromatographic process. Equilibration to this solvent system was effected by passing 5 liters through the Prep-Pak columns to waste and was evidenced by a stable refractive index baseline at a relative response setting of 20.

Load sample preparation involved saturation of 500 ml of chromatographic solvent with enriched gilvocarcin mixture using sonication dispersal of the solid. Typical weight loads ranged from 100 mg to 300 mg. The maximum load weight achievable (within this range) was inversely related to the purity of the mixture. For example, 2.3 g of gilvocarcin-rich solid from the solvent trituration enrichment process was triturated with 500 ml of ethyl acetate - *iso*-propanol - water, 87: 13: 0.5 (v/v) with sonication. Filtration yielded 2.2 g of insolubles. The filtrate (100 mg) was pumped into the equilibrated Prep LC/System 500 *via* one of the solvent inlet ports, following which elution was begun from the main solvent reservoir through the other inlet. The effluent stream from the stream splitter, which diverts 1~2% of the main column effluent for detection purposes, was monitored using a UV detector (Schoeffel Instrument Corp., Spectroflow SF770) with the variable wavelength accessory set at 400 nm. The built in refractive index detector was not a useful monitor of this chromatography presumably due to insensitivity.

The complex was put through 8~10 shave-recycle passes. A shave-recycle method involves increasing the effective silica gel column length through the employment of continuous multiple pass chromatography of the gilvocarcin V, M band. This allows one to achieve maximum resolution between gilvocarcins V and M. NETTLETON[7] provides a review of the shave-recycle methodology. After the shave-

recycle process, fractions were assayed using analytical HPLC. The work-up of composites involved evaporation to dryness under reduced pressure. The following is a description of the composites and weight recovered from the chromatographic process.

Composite fractions	Weight	Analytical HPLC summary
f1	31.4 mg	≥90 area percent gilvocarcin V
f2	26.2 mg	Gilvocarcin mixture
f3	35.4 mg	Gilvocarcin M rich

A total of 93 mg of solid was recovered, a 93% weight recovery.

Initial shave-recycle chromatography typically processed gilvocarcins V and M to at least 80~90 area percent purity as estimated from analytical HPLC. Solids of this purity were accumulated and processed a final time, using the shave-recycle method, to obtain **1** and **2** of at least 95% purity.

Gilvocarcin V (**1**): Calcd. for $C_{27}H_{26}O_9 \cdot H_2O$: C, 63.3; H, 5.5
Found: C, 63.2; H, 5.2
Gilvocarcin M (**2**): Calcd. for $C_{26}H_{26}O_9 \cdot H_2O$: C, 62.3; H, 5.6
Found: C, 61.8; H, 5.4

Peracetylation of Gilvocarcin Complex

To a suspension of the complex (4.0 g) in pyridine (400 ml), acetic anhydride (46 ml) was added and the suspension was stirred for 20 hours at ambient temperature. The reaction mixture was concentrated *in vacuo* to a 75 ml volume, diluted with water, and extracted with dichloromethane. The dichloromethane layer was dried over sodium sulfate and evaporated *in vacuo* to an oil. Skellysolve B was added to the oily residue to effect a fine yellow solid precipitate (4.5 g) which was collected using vacuum filtration.

Isolation of Peracetates

A. Isolation of Gilvocarcin V Tetraacetate (**4**) and Gilvocarcin M Tetraacetate (**5**): The shave-recycle silica gel preparative HPLC separation technique used to resolve the complex was applied to the peracetate mixture using a toluene - dichloromethane - acetone, 41: 55: 4 (v/v), solvent system. The improved solubility of the peracetates permitted chromatographic load masses to be at least 50 fold larger than possible in the separation of the underivatized complex.

Purification of the crude peracetate complex (4.5 g) yielded 0.9 g 80 area percent compound **4** and 0.7 g 80 area percent compound **5**. Final shave-recycle chromatography was carried out on these enriched solids to obtain purity levels of at least 95 area percent.

Compound **4** had mp 180~181°C, MS: *m/z* 662, UV λ_{max}^{MeOH} (ε) 245 (28,000), 278 sh (26,100), 285 (27,700), 308 sh (11,800), 323 (10,500), 344 (10,000), 346 sh (8750), 383 nm (11,500) and IR ν_{max}^{KBr} 1748 (C=O) cm^{-1}.

Elemental analysis: Calcd. for $C_{35}H_{34}O_{13}$: C, 63.4; H, 5.2
Found: C, 62.0; H, 5.2

Compound **5** had mp 241~243°C (dec), MS: *m/z* 650, UV λ_{max}^{MeOH} (ε) 243 (40,700), 266 (26,300), 275 (37,400), 323 (10,800), 366 (11,100), 374 nm (10,500) and IR ν_{max}^{KBr} 1750, 1730 (C=O) cm^{-1}.

Elemental analysis: Calcd. for $C_{34}H_{34}O_{13}$: C, 62.8; H, 5.3
Found: C, 61.2; H, 5.1

B. Isolation of Gilvocarcin E Tetraacetate (**6**): Acetylation of an enriched gilvocarcin V fraction (18 mg) obtained from preparative chromatography yielded a mixture of compounds **4** and **6**. HPLC analysis of the mixture indicated that the relative peak area ratio of **4** to **6** was 90 to 1. Semipreparative* HPLC was carried out on the acetylation residue (21 mg) using a hexane - ethyl acetate - acetone, 75: 15: 10 (v/v), solvent system to yield 0.1 mg 95 area percent compound **6**.

Reduction of Gilvocarcin Peracetate Complex

The peracetate complex (208 mg) was dissolved in 30 ml absolute ethanol and 100 ml ethyl acetate.

* For semipreparative HPLC, a 50 cm×1 cm ID stainless steel column packed with Partisil-20 (Whatman, Inc.) was employed.

To the resulting solution was added 12 drops concentrated HCl followed by 400 mg 10% Pd/C. Hydrogenation was then carried out at 2.81 kg/cm^2 for 18 hours after which the reaction mixture was filtered and the filtrate evaporated to yield a yellow solid (170 mg).

Isolation of Gilvocarcin E Tetraacetate (**6**) from Hydrogenation

Analytical HPLC assay of the hydrogenation product showed complete conversion of compound **4** to compound **6**. Compound **5** was unchanged. The reduced gilvocarcin tetraacetate complex (170 mg) was chromatographed using the silica gel preparative HPLC, toluene - dichloromethane - acetone, 41: 55: 4 (v/v), system to yield 63 mg $\geq$98 area percent compound **6**.

Compound **6** had mp 207~208°C, MS: *m/z* 664, UV λ_{max}^{MeOH} (ε) 244 (23,000), 266 (14,900), 276 (21,500), 300 (5500), 323 (6000), 336 (6300), 374 nm (5800) and IR ν_{max}^{KBr} 1755, 1727 (C=O) cm^{-1}.

Hydrolysis of Gilvocarcin V Tetraacetate (**4**)

Compound **4** (134 mg) was dissolved in dichloromethane - methanol, 1: 2 (150 ml), followed by an addition of 1.2 ml (6 eq.) 1 N methanolic NaOH. The hydrolysis reaction was monitored using the analytical HPLC assay which indicated complete reaction after 2 hours at ambient temperature. The reaction mixture was immediately diluted with dichloromethane (200 ml) and washed with equal volumes of water until the pH of the water layer was neutral. The dichloromethane layer was then dried over Na_2SO_4 and concentrated *in vacuo* to dryness yielding compound **1** (60 mg, 60% yield) of 80 area percent purity by HPLC assay.

Hydrolysis of Gilvocarcin E Tetraacetate (**6**)

Compound **6** (40.5 mg) was dissolved in dichloromethane - methanol, 1: 1 (10 ml). To this solution was added 0.37 ml (6 eq.) 1 N methanolic NaOH. Rapid hydrolysis was observed using the analytical HPLC assay. After 17 minutes at ambient temperature, the reaction mixture was immediately diluted with dichloromethane (125 ml) and washed with water. The dichloromethane layer was dried over Na_2SO_4 and evaporated *in vacuo* to dryness to yield a yellow residue (16.7 mg). Compound **3** (6.0 mg) of >98 area percent purity was obtained from semi-preparative HPLC purification of the residue using a dichloromethane - tetrahydrofuran - methanol - water, 75: 25: 1: 0.4 solvent system.

Gilvocarcin E (**3**) had mp 210~212°C, UV λ_{max}^{MeOH} 243, 265, 274, 305, 316 sh, 327, 343 sh, 382 nm and IR ν_{max}^{KBr} 3390 (OH), 1695 (C=O) cm^{-1}.

X-Ray Crystallographic Determination of Gilvocarcin

Most attempts to grow single crystals of **1** and **2** resulted in crystals that were too small to give a measurable X-ray scattering pattern. The cause of this difficulty appears to be the relatively low solubility of gilvocarcin in solvents that will give crystals. One sample of **1** and its major contaminant, **2**, gave relatively large crystals from acetone. While recognizing the possibility that these would be "mixed" crystals, we elected to carry out the analysis on this sample. Preliminary X-ray photographs showed monoclinic symmetry and accurate lattice parameters, determined by a least-squares fit of fifteen diffractometer measured 2θ-values were a=4.954 (1), b=16.046 (2), c=15.167 (2) Å and β=92.02 (1)°. If the crystals had been pure gilvocarcin V hydrate, the density would have been a reasonable 1.45 g/cc for one molecule of $C_{27}H_{26}O_9 \cdot H_2O$ in the asymmetric unit. The limited amount of sample precluded a careful density measurement. A total of 1689 unique diffraction maxima with $2\theta \leq 114°$ were collected using 1° ω-scans and graphite monochromated Cuk-α (1.54178 Å) radiation. After correction for Lorentz, polarization and background effects, 1092 (65%) were judged observed.

The phase problem for gilvocarcin was solved only with some difficulty. Most E-syntheses generated by the MULTAN 78 system of programs showed large hexagonal arrays of atoms resembling chicken wire. We felt that the hexagonal motif and planar orientation of this fragment were probably correct and elected to modify the E's with an oriented but translationally mobile 18 atom fragment.[8] E-syntheses following phase development using this approach were uninterpretable and not even a sensible planar fragment appeared. When we reduced the modifying fragment to a 10 atom naphthalene piece, the E-synthesis from the highest combined figure of merit and lowest NQEST clearly showed the entire ring structure of gilvocarcin.[9] Most atomic features were developed from an F-synthesis calculated after partial least-squares refinement.[10]

One troublesome aspect of the structure refinement was the existence of a composite image of **1** and **2**. An F-synthesis showed one peak linked to C8 at a distance of 1.60 Å. This peak was initially modeled as a methyl in the least squares refinement and a subsequent F-synthesis showed two nearby major peaks which were consistent with a vinyl group. The final structure was modeled with occupancy factors of 0.75 and 0.25 for methyl and vinyl respectively. This ratio was suggested by the integration of the ^{1}H NMR spectrum of the sample used to grow crystals. This ratio need not be observed in the crystal but it seemed a reasonable starting point, and the successful refinement suggests that they are very similar if not identical. The final unweighted residual computed from all of the observed data, anisotropic nonhydrogen atoms and isotropic hydrogens, is 0.07. Tables of final fractional coordinates, bond distances and bond angles have been deposited with the Cambridge Crystallographic Data Centre and are also available from J. CLARDY.

Acknowledgements

We wish to thank M. MISIEK for antimicrobial assays and V. Z. ROSSOMANO for the lysogenic induction test. Mr. JOHN MOSELEY and Miss CHERYL HOOD gave technical assistance during the isolation work. This work was supported in part by Contract NO1-CM-07299 with the Division of Cancer Treatment, National Institutes of Health, U.S.P.H.S., D.H.H.S. EA wishes to thank the NSF for a predoctoral fellowship and some support was provided by CA-24487 (JC).

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