GILVOCARCINS, NEW ANTITUMOR ANTIBIOTICS

5. BIOSYNTHESIS OF GILVOCARCINS: INCORPORATION OF ^{13}C-LABELED COMPOUNDS INTO GILVOCARCIN AGLYCONES

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The biosynthesis of the aglycones of gilvocarcins V and M has been studied with ^{13}C-labeled precursors. The aglycones have been shown to be formed *via* the acetate pathway and a route for their biosynthesis is proposed which involves secondary addition of an alkyl group.

Streptomyces gilvotanareus nov. sp. produces the gilvocarcins, a complex of antibiotics showing antibacterial and antitumor activity against experimental tumors.[1~4]

The structure of gilvocarcins, which have a polycyclic aromatic skeleton with a *C*-glycoside attached, was elucidated from spectroscopic data,[2] and X-ray crystallographic analysis.[5] The same[6~9] or closely related compounds[10,11] were also isolated by other groups. These antibiotics are usually produced as a mixture of related homologues that consisted mainly of C-8-vinyl and C-8-methyl derivatives, except for ravidomycin.[10] The structure of gilvocarcins V and M is shown in Fig. 1.

It is of interest to study the biosynthesis of the gilvocarcin aglycone, especially the origin of the vinyl and methyl groups at C-8. Structural comparison of the gilvocarcin aglycones with other natural products suggests that they could be derived *via* the acetate pathway. Indeed, BROWN *et al.*[12] and CANHAM *et al.*[13,14] have proved that chartreusin aglycone is formed *via* the acetate pathway. From the structural similarity between the chartreusin and chrysomycin aglycones (the latter are identical to those of gilvocarcin), WEISS *et al.*[11] have proposed that the aglycones could similarly be formed *via* the acetate pathway. However, no experimental evidence was provided in their paper.

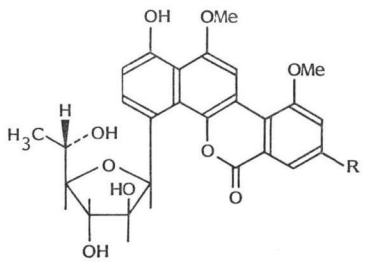
The present paper describes the labeling pattern of gilvocarcins enriched with [1-^{13}C] and [2-^{13}C]-acetate, and [2-^{13}C] and [3-^{13}C]propionate as determined by ^{13}C NMR spectroscopy, and a biosynthetic scheme for the gilvocarcin aglycones is proposed.

Materials and Methods

Microorganisms

Streptomyces gilvotanareus nov sp. strain F3[1] was used as the producer strain. *Bacillus subtilis*

Fig. 1. The structure of gilvocarcins.



Gilvocarcin V R = $CH=CH_2$
Gilvocarcin M R = CH_3
Gilvocarcin E R = CH_2CH_3

No. 10707 was used as an indicator strain for monitoring the amount of gilvocarcins in culture broths.

Fermentation

The seed cultures were prepared in large test tubes (10 ml of the medium per a 50-ml test tube) by inoculating with stock cultures maintained in a deep freezer (−70°C), and by incubating for 3 days at 28°C on a reciprocal shaker. A 5% vegetative seed was used to inoculate into 500-ml Sakaguchi flasks containing 100 ml of the production medium. The seed medium contained per liter: 10 g glycerol, 10 g peptone, 1 g yeast extract, and 1 g K_2HPO_4; after adjustment of pH to 7.0, 2 g $CaCO_3$ was added. The production medium contained per liter: 10 g glucose, 10 g soluble starch, 10 g Pharmamedia, 1 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$ and 3 g NaCl; after adjustment of pH to 7.0, 5 g $CaCO_3$ was added.

Monitoring of Production Cultures

Aliquots (3 ml) of production cultures were withdrawn at regular intervals. The pH was measured and 50 μl of the supernatant fluid was placed on paper discs which were assayed using *Bacillus subtilis* No. 10707 as an indicator strain. Total amounts of gilvocarcins were determined after extraction of 2 ml broth samples with 4 ml of ethyl acetate - acetone (1: 1). Aliquots (30 μl) of the solvent layer were spotted on silica gel thin-layer plate (silica gel 60 F_{254}, Merck). After developing the plates using the mixture of ethyl acetate - acetic acid (9: 1), gilvocarcins were assayed by measuring the absorption at 395 nm. Ratios of gilvocarcin V to gilvocarcin M in isolated samples were determined by HPLC according to WEISS *et al.*[11]

Addition of Labeled Compounds to Cultures

Labeled compounds were purchased from Merck Sharp & Dohme Canada Limited. Labeled compounds were fed as a solution in H_2O and sterilized by filtration through membrane filters (TM-2P, Toyo Roshi, Co. Ltd., Japan).

Extraction and Purification of Gilvocarcins

The fermentation was continued for 3 days after feeding the labeled compounds. Whole broths were extracted twice with one volume of a mixture of ethyl acetate - acetone (2: 1). The solvent layer was washed twice with H_2O and dried over Na_2SO_4 and then concentrated. The residue was crystallized from ethyl acetate - acetone and this procedure was repeated twice to obtain pure gilvocarcins.

General

The ^{13}C NMR spectra were obtained on a Jeol FX-100. Intensity of absorption on TLC plates was measured with a Shimadzu CS-900 chromatoscanner. HPLC was carried out using Jasco Triorotar-II.

Results

In order to find the most suitable time to feed labeled compounds, cultures used were monitored for pH, residual sugar, and amount of gilvocarcins. The fermentation was found to proceed as described in the previous paper[1] and the most suitable time for feeding labeled compounds was found to be 48 hours.

Sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [2-^{13}C]propionate, and sodium [2-^{13}C]propionate were administered to cultures at 48 hours and the fermentation was continued for a further 3 days.

Gilvocarcins were isolated as described in Materials and Methods. Gilvocarcins thus obtained were a mixture of gilvocarcin V and gilvocarcin M varying in ratio from 7: 1 to 10: 1 depending on the experiment. As described in the later section, the small amounts of M did not disturb the results and no further purification was attempted. The amounts of gilvocarcin obtained are described in Table 1.

Sodium [1-^{13}C]acetate was incorporated into aromatic ring system, but almost negligibly into the sugar moiety. The ^{13}C NMR spectrum showed enrichment for eight carbons, C-1, C-3, C-4a, C-6a, C-8, C-10, C-10b, and C-12. Sodium [2-^{13}C]acetate was incorporated into aromatic ring and side chains.

Table 1. Amounts of gilvocarcins produced from various precursors.

Precursors	Amounts of labeled compounds fed (mg)	Amounts of broth obtained (ml)	Gilvocarcins obtained (mg)	V/M[a]
[1-^{13}C]Acetate	200	200	20	7:1
[2-^{13}C]Acetate	100	200	18	10:1
[2-^{13}C]Propionate	100	200	25	8:1
[3-^{13}C]Propionate	50	100	10	10:1

a) Ratio of gilvocarcin V to gilvocarcin M.

Table 2. The ^{13}C NMR assignments of gilvocarcin aglycones and enrichment of labeled precursors.

Carbon No.	δ[a]	I_e/I_u[b]			
		[1-^{13}C]Acetate	[2-^{13}C]Acetate	[2-^{13}C]Propionate	[3-^{13}C]Propionate
1	152.5	9.0	1.0	1.1	1.2
2	111.7	0.9	5.6	1.1	1.4
3	128.7	13.7	1.3	1.2	1.6
4	125.7	0.7	3.7	1.1	1.1
4a	123.3	5.7	0.9	1.1	1.0
4b	141.9	0.5	3.0	1.0	1.0
6	159.3	0.7	3.3	1.1	1.0
6a	121.6	7.8	0.9	1.0	0.9
7	118.7	0.7	4.8	1.0	1.3
8	138.1	3.5	1.8	1.1	1.3
9	113.8	0.7	4.7	1.0	1.4
10	156.8	7.6	0.8	0.9	0.9
10a	122.5	0.8	3.0	0.9	0.9
10b	112.5	4.8	1.0	0.9	0.9
11	100.9	0.7	6.5	1.1	1.5
12	151.3	6.7	1.0	1.0	1.1
12a	114.5	0.8	3.4	1.2	1.0
Vinyl-α-C	135.0	0.8	3.5	6.0	1.8
Vinyl-β-C	116.5	1.0	4.0	1.3	9.7
10-OMe	56.2[c]	0.9	1.0	0.8	0.7
12-OMe	55.7[c]	1.0	1.0	0.8	0.7
8-Me	21.0	1.0	7.5	1.5	1.0

a) Chemical shifts in ppm are downfield from Me_4Si in DMSO-d_6.
b) $I_{enriched}/I_{unenriched}$ measured under identical conditions.
c) Assignments may be reversed.

The ^{13}C NMR showed enrichment for nine aromatic carbons, C-2, C-4, C-4b, C-6, C-7, C-9, C-10a, C-11, and C-12a, and for two carbons of vinyl group at C-8 of gilvocarcin V and methyl group at C-8 of gilvocarcin M.

Sodium [3-^{13}C]propionate was only incorporated into the β-carbon of the vinyl group of gilvocarcin V, while sodium [2-^{13}C]propionate was only incorporated into the α-carbon of the vinyl group of gilvocarcin V.

Fig. 2. The labeling pattern of gilvocarcin V and M aglycones.

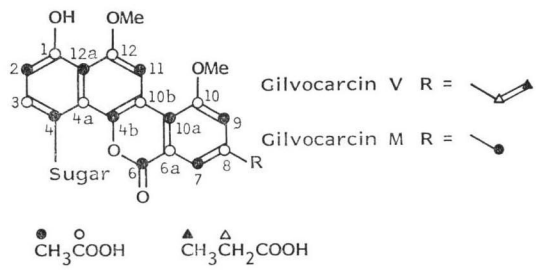
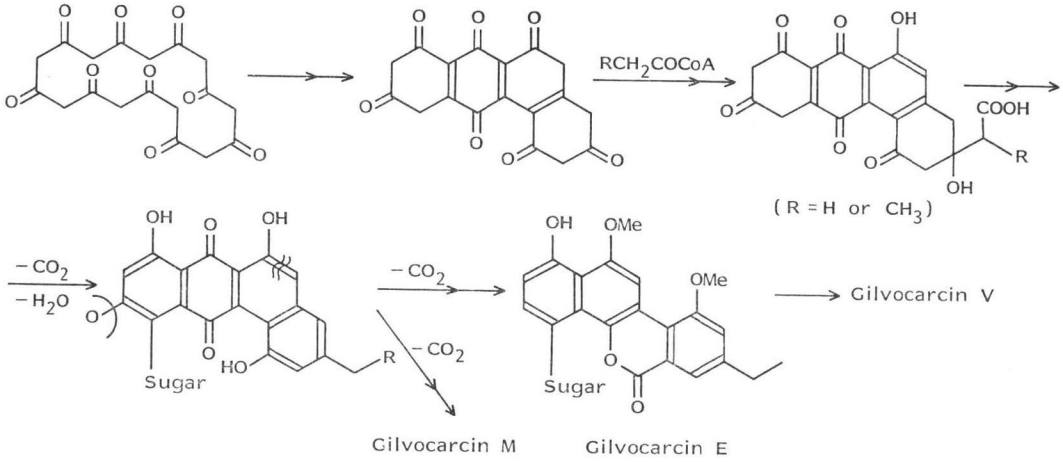


Fig. 3. Possible biosynthetic pathway of gilvocarcins.



These results are summarized in Table 2.

Discussion

The results of feeding experiments can be interpreted according to the case of chartreusin proved by BROWN *et al.*[12] and CANHAM *et al.*[13,14] These data clearly indicated the acetate pathway as the route of biosynthesis for the gilvocarcin V and M aglycones. However, the aglycones cannot be formed from one chain directly, oxidative cleavage and recyclization would have to occur as shown in Fig. 3 and these data could not establish where initiation and termination of the polyketide precursor occurred.

The origin of the vinyl group of gilvocarcin V can be concluded as propionate from the results of feeding [2-^{13}C] and [3-^{13}C]propionate that showed specific enrichment for the vinyl carbons. This conclusion is not inconsistent with the results of feeding [2-^{13}C]acetate that showed almost same degree of enrichment for two carbons of vinyl group of gilvocarcin V. A scrambling of C-2 acetate can easily be explained by the well-known mechanism of recycling of acetate through the TCA cycle and synthesis of propionate from acetate through succinate and methylmalonate.[15,16] These data also suggest that the vinyl group is added secondarily to the aromatic ring system, since no scheme could be devised for the synthesis of the side chain following the polyketide pathway that was consistent with our data. Similarly it is plausible to assume that the secondary addition of propionate to C-8 followed by decarboxylation will afford gilvocarcin E[10] which through dehydrogenation of the ethyl group will give rise to gilvocarcin V. In the same way the origin of the methyl group of gilvocarcin M can be suggested as acetate added secondarily to the aromatic ring system as shown in Table 2. Thus, the secondary addition of acetate to C-8 followed by decarboxylation would afford gilvocarcin M. The secondary addition of an alkyl group is assumed to occur before aromatization of gilvocarcin aglycones as shown in Fig. 3, although the actual step of the side chain addition has not been proved as yet.

Based on the results presented above, it was concluded that the gilvocarcin aglycones were synthesized *via* the acetate pathway with the secondary addition of an alkyl group to C-8. It is proposed that gilvocarcin V is synthesized from gilvocarcin E by dehydrogenation. The labeling pattern for gilvocarcin V and M aglycones are shown in Fig. 2 and a possible biosynthetic pathway for the gilvocarcins is shown in Fig. 3.

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