# BIOSYNTHESIS AND $^{13}C$ NMR ASSIGNMENT OF CYTOVARICIN, A NEUTRAL MACROLIDE ANTIBIOTIC†

TSUYOSHI KIHARA, MAKOTO UBUKATA, JUN UZAWA and KIYOSHI ISONO*

Antibiotics Laboratory, RIKEN, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01, Japan

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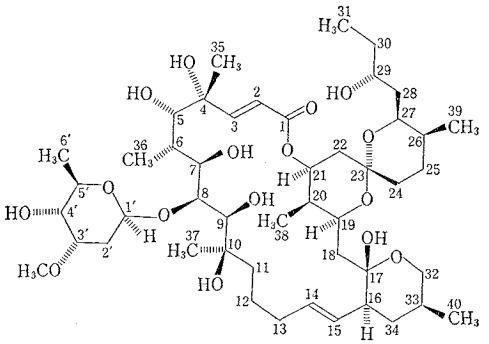
$^{13}C$ NMR analysis of $^{13}C$-labeled cytovaricin which was obtained by feeding sodium [1-$^{13}C$]-, [2-$^{13}C$]-, and [1,2-$^{13}C$]acetates, [1-$^{13}C$]- and [3-$^{13}C$]propionates, [1-$^{13}C$]isobutyrate and [*methyl*-$^{13}C$]methionine to cultures of *Streptomyces diastatochromogenes* showed that the aglycone of cytovaricin is derived from nine acetate units, six propionate units and one isobutyrate unit and the methoxy group at C-3′ of cymarose moiety is derived from the methionine-*S*-methyl group. The $^{13}C$ NMR spectra of $^{13}C$-labeled cytovaricins which were obtained from feeding experiments allowed the complete assignment of the $^{13}C$ NMR spectrum of cytovaricin.

Cytovaricin is a 22 membered neutral macrolide with a unique tetracyclic ring-system glycosylated with D-cymarose, which is produced by *Streptomyces diastatochromogenes*.[2] The absolute structure of cytovaricin (Fig. 1) was determined by X-ray analysis[3] and from the isolation of D-cymarose by acid hydrolysis.[4] Cytovaricin showed a potent inhibitory activity against Yoshida sarcoma cells in tissue culture. In this paper we report the results of studies employing $^{13}C$-labeled acetate, propionate, isobutyrate and methionine that helped elucidate the biosynthetic origins of cytovaricin. Assignments of the $^{13}C$ NMR signals of cytovaricin were mainly based on the analysis of a $^{13}C$-$^{13}C$ correlation spectroscopy (COSY) spectrum of cytovaricin labeled with [2-$^{13}C$]acetate, a $^{13}C$ NMR spectrum of cytovaricin labeled with [1,2-$^{13}C$]acetate and a $^{1}H$-$^{13}C$ heteronuclear COSY spectrum. Signals for all of the 47 carbons in cytovaricin were assigned.

Fig. 1. Absolute structure of cytovaricin.



## Materials and Methods

### Culture

*S. diastatochromogenes* sp. No. H-230 grown on starch - yeast agar was inoculated into 400-ml cylindrical flasks containing 60 ml of a medium composed of glucose 2%, soluble starch 1%, meat extract 0.1%, dry yeast 0.4%, soybean flour 2.5%, NaCl 0.2% and $K_2HPO_4$ 0.005%, and cultured at 27°C for 48 hours on a rotary shaker. Two ml of the seed culture was inoculated into each of several 400-ml cylindrical flasks containing 60 ml of the same medium. Fermentation was carried out on a rotary shaker at 27°C. After 36 hours of fermentation, a $^{13}C$-labeled precursor was added to the culture at a concentration of 0.1% (w/v), and fermentation was continued for an additional

† Outline of this study was presented (ref 1).

54 hours.

Chemicals

Sodium [1-$^{13}$C]acetate, sodium [2-$^{13}$C]acetate, sodium [1,2-$^{13}$C]acetate, sodium [1-$^{13}$C]propionate, sodium [3-$^{13}$C]propionate, sodium [1-$^{13}$C]isobutyrate and [*methyl*-$^{13}$C]methionine were purchased from MSD ISOTOPES.

Isolation of $^{13}$C-Labeled Cytovaricin

Each fermentation broth (180 ml, pH 7.6) was filtered and the filtrate was extracted with ethyl acetate and the mycelium cake was extracted with 80% acetone. The acetone extract was concentrated *in vacuo* to give an aqueous solution, which was then extracted with ethyl acetate. Both the ethyl acetate extracts were combined and concentrated *in vacuo* to dryness. The residue was chromatographed on a silica gel column with chloroform - methanol (30 : 1). The eluate was monitored with a reverse phase HPLC system (Nucleosil $5C_{18}$ column with methanol - water (87 : 13) as solvent) and the fractions containing cytovaricin were combined and concentrated. A colorless powder of cytovaricin was obtained from methanol - pentane. From each 180 ml of the culture broth supplemented with a $^{13}$C-labeled precursor, 10~20 mg of purified $^{13}$C-labeled cytovaricin was obtained.

NMR Spectroscopy

$^{13}$C NMR spectra were measured on a Jeol JNM-FX400 spectrometer at 100.7 MHz. Cytovaricins were dissolved in $CD_2Cl_2$ and TMS was used as an internal reference. The spectral width was 20 KHz and 32 K data points were recorded giving maximum spectral accuracy of 1.25 Hz. $^1$H-$^{13}$C COSY and $^{13}$C-$^{13}$C COSY spectrum were recorded with a Jeol JNM-GX400 spectrometer operating at 100.535 MHz for $^{13}$C and at 399.784 MHz for $^1$H.

## Results and Discussion

### Incorporation of $^{13}$C-Labeled Precursors into Cytovaricin

In the $^{13}$C NMR spectrum from the labeling experiment with [1-$^{13}$C]acetate, carbon enhancement was observed on one carbonyl (132.95 ppm), two ketal and hemiketal carbons (98.62 and 97.33 ppm), three oxygen-bearing carbons (77.25, 69.75 and 67.69 ppm) and two methine carbons (37.74 and 33.35 ppm). Enrichment ratios are shown in Table 1. These results indicate that nine acetate units were incorporated into cytovaricin.

In the $^{13}$C NMR spectrum from the labeling experiment with [2-$^{13}$C]acetate, enhancement was observed at two olefinic carbons (133.06 and 119.67 ppm), one oxygen-bearing methine carbon (85.77 ppm), one methine carbon (51.40 ppm) and five methylene carbons (42.05, 40.01, 35.57, 29.38 and 22.31 ppm). Enrichment ratios are shown in Table 1. Other carbons (except for the nine carbons described above) were also enriched by [2-$^{13}$C]acetate. These results indicate randomization of [2-$^{13}$C]acetate into [1,2,3-$^{13}$C]propionate presumably *via* conversion of acetate to propionate by the multiple passage through the Krebs' cycle and the action of methylmalonyl Co-A mutase as already observed in cationomycin biosynthesis.[5] Multiple recycling through Krebs' cycle was demonstrated in showdomycin biosynthesis from [2-$^{14}$C]acetate[6,7] and polyoxamic acid biosynthesis from [2-$^{14}$C]-acetate and [5-$^{14}$C]glutamate.[8]

In the $^{13}$C NMR spectrum from the labeling experiment with [1-$^{13}$C]propionate, enhancement was observed at one olefinic carbon (150.20 ppm), four oxygen-bearing carbons (79.38, 74.20, 68.61 and 66.66 ppm), and one methine carbon (27.20 ppm).

In the $^{13}$C NMR spectrum from the labeling experiment with [3-$^{13}$C]propionate, enhancement was observed at six methyl carbons (28.85, 22.62, 11.40, 10.47, 6.34 and 5.83 ppm). Enrichment

Table 1. $^{13}C$ chemical shifts and isotopic incorporations into cytovaricin.

| Carbon No. | $^{13}C$ shift (ppm)[a] | Enrichment ratio(s)[b] | Precursor(s) |
|---|---|---|---|
| 1 | 165.28 | 2.8 | [1-$^{13}$C]Acetate |
| 3 | 150.20 | 18.9, 4.5 | [1-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 14 | 133.06 | 5.6 | [2-$^{13}$C]Acetate |
| 15 | 132.95 | 8.7 | [1-$^{13}$C]Acetate |
| 2 | 119.67 | 8.0 | [2-$^{13}$C]Acetate |
| 1′ | 100.25 | | |
| 23 | 98.62 | 4.5 | [1-$^{13}$C]Acetate |
| 17 | 97.33 | 4.5 | [1-$^{13}$C]Acetate |
| 8 | 85.77 | 4.4 | [2-$^{13}$C]Acetate |
| 5 | 79.38 | 9.1, 2.2 | [1-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 3′ | 77.47 | | |
| 27 | 77.25 | 7.6 | [1-$^{13}$C]Acetate |
| 10 | 75.94 | | |
| 4 | 75.07 | | |
| 9 | 74.20 | 13.5, 2.5 | [1-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 4′ | 72.43 | | |
| 5′ | 72.22 | | |
| 21 | 69.75 | 8.1 | [1-$^{13}$C]Acetate |
| 29 | 68.61 | 11.0, 2.2 | [1-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 7 | 67.69 | 6.6 | [1-$^{13}$C]Acetate |
| 19 | 66.66 | 12.1, 2.4 | [1-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 32 | 66.52 | | |
| $OCH_3$ | 57.46 | 35.0 | [*Methyl*-$^{13}$C]methionine |
| 16 | 51.40 | 4.7 | [2-$^{13}$C]Acetate |
| 28 | 42.05 | 4.8 | [2-$^{13}$C]Acetate |
| 18 | 40.01 | 5.1 | [2-$^{13}$C]Acetate |
| 11 | 37.74 | 7.2 | [1-$^{13}$C]Acetate |
| 6 | 35.98 | | |
| 22 | 35.57 | 5.0 | [2-$^{13}$C]Acetate |
| 20 | 34.73 | | |
| 2′ | 34.15 | | |
| 34 | 33.98 | 17.4 | [1-$^{13}$C]Isobutyrate |
| 13 | 33.35 | 6.8 | [1-$^{13}$C]Acetate |
| 26 | 31.19 | | |
| 33 | 30.93 | | |
| 30 | 30.61 | | |
| 24 | 29.38 | 4.5 | [2-$^{13}$C]Acetate |
| 35 | 28.85 | 14.4, 3.0 | [3-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 25 | 27.20 | 11.6, 2.5 | [1-$^{13}$C]Propionate. [2-$^{13}$C]acetate |
| 37 | 22.62 | 14.3. 2.8 | [3-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 12 | 22.31 | 4.8 | [2-$^{13}$C]Acetate |
| 6′ | 18.20 | | |
| 40 | 17.21 | | |
| 39 | 11.40 | 12.5, 2.3 | [3-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 31 | 10.47 | 14.1, 1.9 | [3-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 38 | 6.34 | 16.1, 2.7 | [3-$^{13}$C]Propionate, [2-$^{13}$C]acetate |
| 36 | 5.83 | 16.4, 2.9 | [3-$^{13}$C]Propionate, [2-$^{13}$C]acetate |

[a] 100.7 MHz $^{13}C$ NMR spectrum in $CD_2Cl_2$ with TMS reference at 0.00 ppm.

[b] Ratio of carbon signal intensities for enriched and natural abundance samples.

ratios are shown in Table 1. These results show that six propionate units were incorporated into cytovaricin.

The four carbons of the side chain, namely C-32, C-33, C-34 and C-40 were not labeled with acetate or propionate. We observed that [*U*-$^{14}$C]valine was efficiently incorporated into cytovaricin but [1-$^{14}$C]valine was not (data not shown). [1-$^{13}$C]Isobutyrate was used as a $^{13}$C-labeled precursor and high incorporation of this compound into cytovaricin was obtained. In the $^{13}$C NMR spectrum from this experiment, enhancement was observed at the methylene carbon (33.98 ppm) with an enrichment ratio of 17.4 (Table 1).

$^{13}$C NMR chemical shifts of cytovaricin labeled with [1,2-$^{13}$C]acetate and their $^{13}$C-$^{13}$C coupling constants ($J_{cc}$) are shown in Table 2. These results confirm that nine pairs of acetate units were incorporated into cytovaricin.

In the $^{13}$C NMR spectrum from the [*methyl*-$^{13}$C]methionine feeding experiment, enhancement was observed at one methoxy carbon (57.46 ppm) with an enrichment ratio of 35.0.

## Biosynthesis of Cytovaricin

The data above show that the aglycone of cytovaricin is derived from nine acetate units and six propionate units and one isobutyrate unit. The methyl carbon of the methoxy group at C-3′ of the cymarose moiety is derived from methionine-methyl (Fig. 2).

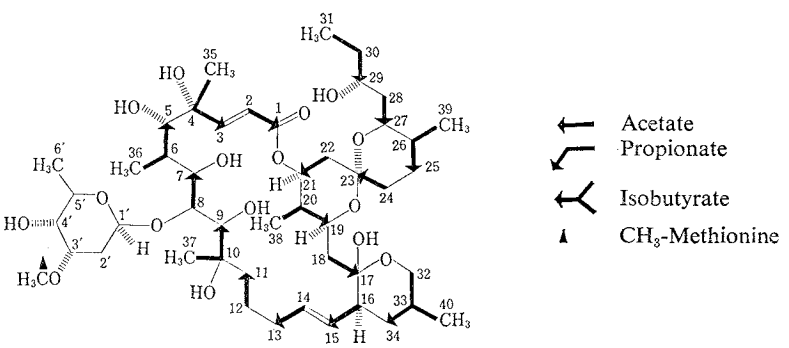
The hydroxyisobutyrate side chain is a unique feature in the cytovaricin structure, since there is no precedent for this type of biological precursor. Efficient incorporation of [1-$^{13}$C]isobutyrate indicates the following three biosynthetic possibilities: 1) The basic skeleton of cytovaricin is formed by a single polyketide chain from nine acetate and six propionate units followed by introduction of an isobutyl group into C-16, 2) the skeleton is formed from condensation of two polyketide chains (between C-16 and C-17), one of which starts with isobutyric acid, 3)

Table 2. $^{13}$C chemical shift and $J_{cc}$ of cytovaricin labeled with [1,2-$^{13}$C]acetate.

| Carbon No. | $^{13}$C shift (ppm)[a] | $J_{cc}$ (Hz) |
|---|---|---|
| 1 | 165.28 | 76.25 |
| 2 | 119.67 | 76.25 |
| 7 | 67.69 | 41.50 |
| 8 | 85.77 | 41.50 |
| 11 | 37.74 | 36.51 |
| 12 | 22.31 | 36.51 |
| 13 | 33.35 | 42.75 |
| 14 | 133.06 | 42.75 |
| 15 | 132.95 | 45.65 |
| 16 | 51.40 | 45.65 |
| 17 | 97.33 | 47.75 |
| 18 | 40.01 | 47.75 |
| 21 | 69.75 | 39.01 |
| 22 | 35.57 | 39.01 |
| 23 | 98.62 | 45.01 |
| 24 | 29.38 | 45.01 |
| 27 | 77.25 | 41.55 |
| 28 | 42.05 | 41.55 |

[a] 100.7 MHz $^{13}$C NMR spectrum in $CD_2Cl_2$ with TMS reference at 0.00 ppm.

Fig. 2. Biosynthetic origin of cytovaricin.



homoisovalerate is formed first from isobutyrate and acetate units, then incorporated into the polyketide chain together with eight acetate and six propionate units. Since the enrichment of C-15 and C-16 is at the same level with other carbons derived from acetate, possibility 3 does not appear to be likely. The efficient incorporation of [*U*-$^{14}$C]valine suggests that valine is metabolized to 2-ketoisovalerate which is then converted to isobutyryl Co-A.[9] The latter compound may be introduced into C-16 by an aldol type condensation. Clearly, further studies should be carried out for an unequivocal determination of this matter.

## Assignments of $^{13}$C NMR Signals of Cytovaricin

$^{13}$C NMR spectra of cytovaricin enriched by $^{13}$C-labeled precursors, made it possible to assign all the $^{13}$C signals of cytovaricin.

The C-1 carbon is the only ester carbon in cytovaricin and the lowest field signal at 165.28 ppm was assigned to C-1. The signal at 150.20 ppm was assigned to C-3 because it is the only propionate-C-1 derived olefinic carbon in cytovaricin. There are two acetate-C-2 derived olefinic carbons, namely C-2 and C-14 (signals at 119.67 and 133.06 ppm). As shown in Table 2, the C-1 signal at 165.28 ppm is coupled with C-2 at 119.67 ppm thus, the signal at 133.06 ppm was assigned to C-14. A signal at 132.95 ppm was assigned to C-15 which is the only acetate-C-1 derived olefinic carbon in cytovaricin. The C-1′ carbon was assigned a chemical shift of 100.25 ppm since C-1′ of cymarose is the only acetal carbon in cytovaricin.

There are ketal and hemiketal carbons, C-23 and C-17 respectively (signals at 98.62 and 97.33 ppm) in cytovaricin. Methanolysis[4] of cytovaricin gave methyl cymaroside and an aglycone (field desorption mass spectra (FD-MS) $M^+$ *m/z* 738). $^1$H and $^{13}$C NMR of the aglycone revealed that elimination of the OH on C-17 was accompanied by formation of a double bond between C-16 and C-17. Furthermore, the signal at 97.33 ppm in cytovaricin had disappeared but the signal at 98.62 ppm remained virtually unchanged. Therefore, the signal of the hemiketal carbon at 97.33 ppm was assigned to C-17 and the signal of a ketal carbon at 98.62 ppm was assigned to C-23. The chemical shift at 85.77 ppm was assigned to C-8 because the C-8 carbon is the only acetate-C-2 derived methine carbon bearing an oxygen in cytovaricin.

The patterns of carbon-carbon correlation observed by cross-peaks between the coupled carbons in the $^{13}$C-$^{13}$C COSY spectrum of cytovaricin labeled with [2-$^{13}$C]acetate are shown in Table 3 (spectrum not shown). Not only *intraunit* coupling (C-31—C-30—C-29, C-36—C-6—C-5 *etc.*) but also *interunit* coupling (C-5—C-4, C-19—C-18 *etc.*) were observed in the $^{13}$C-$^{13}$C COSY spectrum of cytovaricin labeled with [2-$^{13}$C]acetate. This may be due to randomization of [2-$^{13}$C]acetate aforementioned, followed by incorporation of [1,2,3-$^{13}$C]propionate into cytovaricin. As shown in Table 3, the carbon-carbon correlation from C-2 to C-36 is: C-2—C-3—C-4—C-5—C-6—C-36. Signals of C-2 and C-3 were already assigned to 119.67 and 150.20 ppm. Therefore, C-4, C-5, C-6 and C-36 were assigned as shown in Table 1.

Table 3. Carbon-carbon correlation patterns observed in $^{13}$C-$^{13}$C COSY spectrum of cytovaricin labeled with [2-$^{13}$C]acetate.

| |
|---|
| C-36—C-6—C-5—C-4—C-3—C-2 |
| C-31—C-30—C-29—C-28 |
| C-35—C-4—C-3—C-2 |
| C-38—C-20—C-19—C-18 |
| C-39—C-26—C-25 |
| C-37—C-10 |
| C-8—C-9 |

From the $^1$H-$^{13}$C heteronuclear COSY spectrum of cytovaricin, 1′-H was assigned a chemical shift of 4.88 ppm by $^1$H, $^{13}$C correlation. In the $^1$H-$^1$H homonuclear COSY spectrum, the

couplings between 1′-H (4.88 ppm), 2′-H (2.35 ppm), 3′-H (3.64 ppm), 4′-H (3.22 ppm), 5′-H (3.76 ppm) and 6′-H (1.28 ppm) were observed by cross-peaks, thus permitting the assignment of the signals. From the $^{1}H$-$^{13}C$ heteronuclear COSY spectrum of cytovaricin, the corresponding signals of C-1′, C-2′, C-3′, C-4′, C-5′ and C-6′, were assigned as shown in Table 1 by $^{1}H$, $^{13}C$ correlation.

Since 31-H is the only triplet-methyl in the $^{1}H$ NMR spectrum of cytovaricin, it was assigned a chemical shift of 0.96 ppm. From $^{1}H$-$^{13}C$ heteronuclear COSY spectrum, C-31 was assigned at 10.47 ppm by $^{1}H$, $^{13}C$ correlation. Because C-31 had already been assigned a signal at 10.47 ppm, C-30, C-29 and C-28 were assigned as shown in Table 1 from the carbon-carbon correlation C-31—C-30—C-29—C-28 (Table 3). A signal of C-28 at 42.05 ppm is coupled with C-27 at 77.25 ppm (Table 2).

There are two quaternary carbons bearing one oxygen, namely, C-10 and C-4 (75.94 and 75.07 ppm) in cytovaricin. Since a chemical shift of 75.07 ppm was already given to C-4, the signal at 75.94 ppm was assigned to C-10. There is a carbon-carbon correlation between C-8 at 85.77 ppm (already assigned) and C-9 at 74.20 ppm. Further, the signal of C-8 is coupled with C-7 at 67.69 ppm (Table 2).

There are three acetate-C-1 derived carbons bearing one oxygen, namely, C-7, C-21 and C-27 (signals at 67.69, 69.75 and 77.25 ppm) in cytovaricin. Signals at 77.25 and 67.69 ppm were already assigned to C-27 and C-7 respectively, therefore, the residual signal at 69.75 ppm was assigned to C-21. A signal of C-17 at 97.33 (already assigned) is coupled with C-18 at 40.01 ppm. Because the carbon-carbon correlation is shown in Table 3 as; C-18—C-19—C-20—C-38, the following C-19, C-20 and C-38 were assigned as shown in Table 1.

The only methylene carbon bearing one oxygen is assigned to C-32 (66.52 ppm) and a signal at 57.46 ppm was assigned to the methoxy carbon. There are two acetate-C-1 derived methine carbons, namely, C-11 and C-13 (signals at 37.74 and 33.35 ppm). The signal of C-14 at 133.06 (already assigned) is coupled with C-13 at 33.35 ppm (Table 2). Therefore, the residual signal at 37.74 ppm was given to C-11.

The signal of C-21 at 69.75 ppm (already assigned) is coupled with C-22 at 35.57 ppm. The signal at 33.98 ppm was assigned to C-34 because this carbon is the only isobutyrate-C-1 derived methylene carbon in cytovaricin. A signal at 27.20 ppm was assigned to C-25 because C-25 carbon is the only propionate-C-1 derived methylene carbon in cytovaricin.

As shown in Table 3, the carbon-carbon correlation is: C-39—C-26—C-25. Because C-25 was already given a signal at 27.20 ppm., C-26 and C-39 were assigned chemical shifts of 31.19 ppm and 11.40 ppm, respectively (Table 1).

There are four carbons which were not labeled with $^{13}C$-labeled acetate and propionate, namely C-32, C-33, C-34 and C-40 (signals at 66.52, 30.93, 33.98 and 17.21 ppm, respectively) in a cytovaricin aglycone. C-32 at 66.52 ppm and C-34 at 33.98 ppm were already assigned. The residual C-33 and C-40 should be methine and methyl carbons, thus assigned to signals at 30.93 and 17.21 ppm, respectively.

The carbon-carbon correlation C-37—C-10 (Table 3) made it possible to assign C-37 as the signal at 22.62 ppm, because C-10 was already given an assignment of 75.94 ppm. The signal of C-11 at 37.74 (already assigned) is then coupled to C-12 at 22.31 ppm.

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