Biosynthesis of Dynemicin A, a 3-Ene-1,5-diyne Antitumor Antibiotic

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Abstract: Biosynthetic studies of dynemicin A (DNM-A) were carried out by examining the incorporation of singly and doubly ^{13}C-labeled acetates, [*methyl*-^{13}C]methionine and [^{15}N,2-^{13}C]glycine, into DNM-A by cultures of *Micromonospora chersina* M956-1. The results show that the compound is biosynthesized from two heptaketide chains, which form the bicyclic enediyne core and the anthraquinone moiety, respectively; both are derived from seven head-to-tail coupled acetate units, while the carboxyl group is derived from one carbon of an acetate unit and the *O*-methyl group from methionine. Intact incorporation of glycine was not observed, but a carbon was incorporated into the *O*-methyl group. The related C_{15} enediyne ring skeletons in the esperamicin/calicheamicin class of antibiotics may be similarly derived as the enediyne core of dynemicin A, but the C_{14} cyclic carbonate/bicyclo[7.3.0]dodecadienediyne ring system of neocarzinostatin chromophore A appears to be biosynthesized via a somewhat different process.

Dynemicin A (DNM-A, **1**) is a potent antibacterial and antitumor antibiotic isolated from *Micromonospora chersina* M956-1 strain and has a striking hybrid structure combining the characteristics of both the enediyne and anthracycline classes of antibiotics.[1] The drug causes DNA strand breakage in vitro in a reaction that is stimulated by a variety of reducing agents.[2,3] The compound is the third member of a series of antibiotics that have an interesting bicyclo[7.3.1]enediyne substructure, which may be related biosynthetically to the cores of esperamicin (ESP)[4] and calicheamicin (CAL).[5] The neocarzinostatin chromophore A (NCS Chrom A), having a bicyclo[7,3,0]dienediyne core,[6] has also been classified in this family, and the biosynthetic origin of the carbon skeleton of NCS Chrom A has recently been reported.[7] However, thus far the biosynthesis of the ESP/CAL class of antibiotics has not been clarified. We now report on the full assignment of the ^{13}C NMR signals of **1** and the incorporation of various ^{13}C-labeled precursors into **1**. A possible biosynthetic scheme of **1** is proposed and, by analogy, a possible biosynthetic pathway of the related C_{15} enediyne ring skeleton in the ESP/CAL class of antibiotics is presented.

Results and Discussion

^{13}C NMR Assignments. ^{13}C NMR signals of DNM-A (**1**) measured in DMSO-d_6 were partially assigned previously,[1c] though many of the assignments were regarded as interchangeable. We, therefore, first established unambiguous assignments of the ^{13}C NMR signals of **1**, by combining ^1H-^{13}C COZY, INEPT, and heteronuclear multiple-bond [^1H-^{13}C] correlation spectroscopy (HMBC) as shown in Figure 2 and also by analyzing the incorporation patterns of [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates into **1**. Though the assignments of each of the four pairs of signals due to C13 and C20, C14 and C19, C15 and C18, and C16 and C17 were interchangeable on the basis of only on the NMR techniques, the incorporation patterns of labeled acetates allowed us to differentiate these signals. The ^{13}C NMR data and assignments thus obtained are summarized in Table I.

Incorporation of ^{13}C-Labeled Precursors. In order to establish the origins of carbon atoms in **1**, incorporation experiments with a variety of ^{13}C-labeled precursors were carried out with shaken cultures of *M. chersina* M956-1. Addition of Diaion HP-20, a nonionic highly porous resin, to the culture medium significantly increased the yield of **1** to 1 mg/mL. The resin was considered to adsorb excreted **1**, greatly reducing the contact of **1** with the producing organism, which is itself sensitive to this antibiotic.

DNM-A (**1**) was purified by two-step column chromatography. The ^{13}C-labeling patterns after incorporation of [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates, L-[*methyl*-^{13}C]methionine, and [2-^{13}C,^{15}N]glycine were determined from the respective ^{13}C NMR spectra and are shown in Figure 4. The incorporation pattern of [1,2-$^{13}C_2$]acetate was confirmed by matching of $^1J_{cc}$ values as shown in Table I and Figure 3. The carboxyl group attached to C5 is unexpectedly derived from C2 of an acetate unit. Addition of [1,2,3-^{13}C]malonate to the culture of *M. chersina* induced overall ^{13}C-enrichment in **1**, but no ^{13}C-^{13}C coupling was observed between C5 and the carboxyl carbon, indicating that intact incorporation of a C_3 malonate unit at this point does not occur. Propionate and succinate were not specifically incorporated into **1**. Feeding of [^{15}N,2-^{13}C]glycine enhanced the intensity only of the *O*-methyl signal, which should be caused by one-carbon transfer from glycine to methionine, and neither ^{13}C-enrichiment at C22 nor N1-C22 coupling was observed. This indicates that glycine is not the precursor of the C21-C22-N unit. The origin of the N1 remains unknown.

The incorporation results summarized in Figure 4 demonstrate a remarkable feature in the labeling pattern at the two sets of vicinal carbons; both C5 and the carboxyl carbon are derived from C2 of acetate and both C8 and C9 from C1 of acetate. This indicates that the polyketide sequence of **1** should be biogenetically disconnected at these bonds. The experiment with doubly labeled

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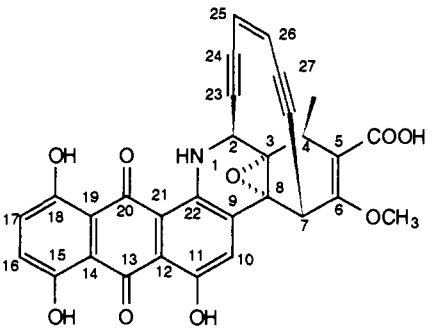
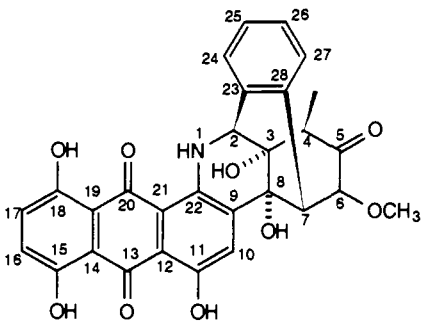
Table I. ^{13}C NMR Assignments of [1-^{13}C]- and [2-^{13}C]Acetate-Labeled DNM-A and $^1J_{cc}$ of [1,2-$^{13}C_2$]Acetate-Labeled Dynemicin A (in DMSO-d_6)

carbon	δ,[a] ppm	$^1J_{CH}$, Hz	$^1J_{cc}$, Hz	carbon	δ,[a] ppm	$^1J_{CH}$, Hz	$^1J_{cc}$, Hz
2	43.9 d (1)	154	68.4[b]	15	156.0 s (1)		64.8
3	70.2 s (2)		31.4[c]	16	127.0 d (2)	166	64.8
4-CH_3	18.5 q (2)	129	33.2	17	129.1 d (1)	165	64.7
4	35.5 d (1)	131	33.2	18	155.7 s (2)		64.7
5-COOH	167.4 s (2)			19	113.8 s (1)		55.5
5	114.6 s (2)		86.0	20	186.6 s (2)		55.5
6	153.3 s (1)		86.0	21	110.8 s (1)		66.5
6-OCH_3	57.8 q (M, G)	146		22	142.8 s (2)		66.5
7	31.4 d (2)	143	66.6[d]	23	98.0 s (2)		68.4[b]
8	63.2 s (1)		31.4[c]	24	89.6 s (1)		88.7
9	135.2 s (1)		65.7	25	124.4 d (2)	176	88.7
10	127.4 d (2)	163	65.7	26	124.2 d (1)	176	88.7
11	156.2 s (1)		64.7	27	88.9 s (2)		88.8
12	113.1 s (2)		64.7	28	99.4 s (1)		66.6[d]
13	189.0 s (1)		57.4				
14	113.1 s (2)		57.4				

[a] CH coupling multiplicity: s, singlet; d, doublet; q, quartet. In parentheses, ^{13}C enrichment from: 1, [1-^{13}C]acetate; 2, [2-^{13}C]acetate; G, [2-^{13}C]glycine; M, [*methyl*-^{13}C]methionine. [b-d] Mutual $^1J_{cc}$ coupling was observed.



Dynemicin A (1)



Dynemicin M (2)

Figure 1. Structure of dynemicins A and M.

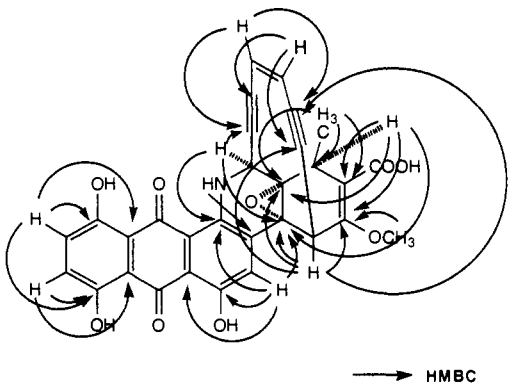


Figure 2. Heteronuclear multiple-bond ^1H–^{13}C correlation.

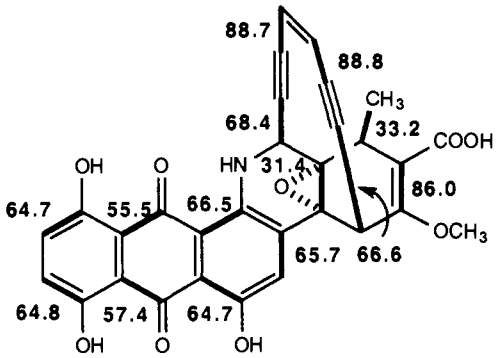
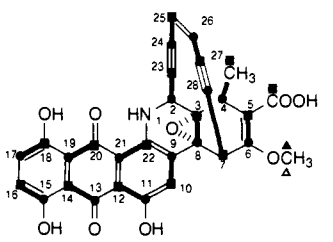


Figure 3. ^{13}C–^{13}C Couplings of [1,2-$^{13}C_2$]acetate-labeled dynemicin A.

[1,2-^{13}C]acetate to establish the carbon–carbon connectivities strongly suggested that the respective C_{14} bicyclo[7.3.1]enediyne and anthraquinone moieties are biosynthesized separately as two different heptaketide chains derived from seven head-to-tail coupled acetate units and should be connected at a later stage.

From inspection of the established carbon–carbon connectivities in **1**, a possible precursor of the bicyclo[7.3.1]enediyne substructure is assumed to be a C_{14} chain such as **5**, **6** (in Scheme I), or their biogenetic analogue, which can be connected with the anthraquinone moiety through a Friedel–Crafts type of acylation reaction to form the C8–C9 bond and can also cyclize to the bicyclo structure through two condensation reactions to form the C3–C4 and C7–C8 bonds. Introduction of an additional acetate unit at C5 followed by oxidative degradation to form the carboxyl group and O-methylation should occur at some point. Biosynthesis of anthraquinones from heptaketide precursors is so far unknown. Though a heptacarbonyl acid **3** is tentatively drawn as a possible precursor, the location of the starter acetate unit is not defined yet. Moreover, an enediyne chain such as **5** is also conceivable as a precursor of the anthraquinone moiety.

In the light of the present data on DNM-A (**1**), the biosynthesis of the C_{15} enediyne skeleton of the ESP/CAL class of antibiotics may be explained similarly to that of **1** (shown in Scheme II), with an octaketide **7** or its biogenetic analogue as the common precursor. A formal loss of a two-carbon unit from the carboxylate



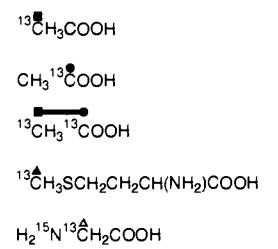
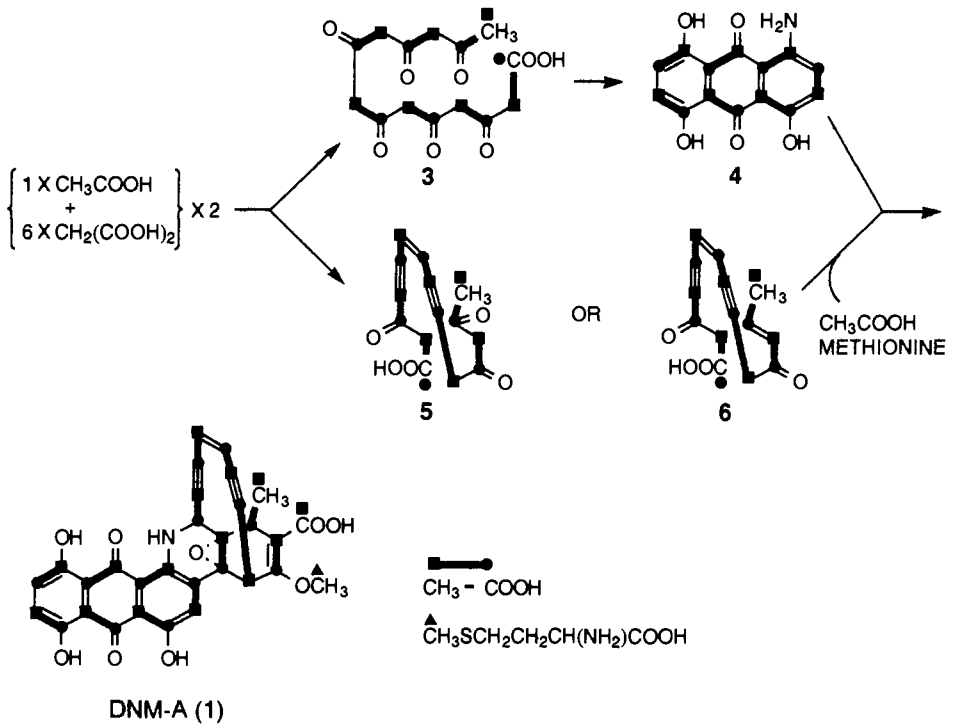


Figure 4. Incorporation patterns of ^{13}C-labeled precursors.

Scheme I



end (path b) leads to **5**, and loss of one carbon from either end (path a or c) results in the ESP/CAL bicyclic core, **8a**, **8b**, **8c**, or **8d**. The stereochemistry of **5** and **8** in Scheme II accords with the established absolute stereochemistry of ESP/CAL cores[8] and with the conventional structure of **1**. A recent report on a model study for the dynemicin–DNA complex predicted that this enantiomer (2*S*,7*R*) of **1** should be the correct absolute stereochemical structure.[9] The four possible pathways leading to **8a–d** can be easily differentiated by analyzing incorporation patterns of 1,2-^{13}C doubly labeled acetate into the ESP/CAL core. The precursors such as **5** and **7** could be derived from the oleate–crepenynate pathway as discussed for NCS Chrom A biosynthesis.[7] This pathway, however, has so far been shown to operate only in higher plants and fungi.[10]

It should be noted that the incorporation patterns of [1,2-$^{13}C_2$]acetate into **1** established here and that into NCS Chrom A[7] evidently differ in the labeling patterns of diynes. Namely, in DNM-A, the two carbons composing the respective yne moieties derive from separate acetate units, whereas the corresponding carbons in NCS Chrom A derive from the same acetate units. It appears that the biosynthetic pathway of NCS Chrom A is somewhat different from that of the bicyclodiynene substructure in **1**.

Several compounds such as dynemicins M (**2**),[1b] O, P, and Q,[11] which have an oxo group at C5 (type 2) in place of the carboxyl group (type 1), were isolated from the culture broth of *M. chersina* M956-1. The biogenetic relationship between the type 1 and type 2 compounds is intriguing with respect to the functional group at C5. Type 2 compounds may be formed from type 1 compounds by oxidative decarboxylation, but it is also possible that the latter compounds are directly derived from some heptaketide precursor(s) without introduction of an acetate unit.

Conclusion

In summary, we have fully assigned the ^{13}C NMR signals and the carbon–carbon connectivities of the DNM-A structure. The bicyclic enediyne skeleton and anthraquinone moiety were shown to be biosynthesized from two separate heptaketides consisting of seven head-to-tail coupled acetate units. We propose a scheme for the biosynthesis of the bicyclic core in DNM-A via a heptaketide precursor such as **5** or **6**; such a scheme can be extended to the biosynthesis of the ESP/CAL enediyne core as well, as illustrated in Scheme II. The heptaketide **3** is tentatively suggested as a possible precursor of the anthraquinone moiety, though a C_{14} enediyne chain such as **5** or **6** could also be the precursor. In this regard, it would be informative to determine the starter acetate unit of the heptaketide composing the anthraquinone moiety.

Experimental Section

Materials. More than 90% enriched stable isotope precursors were purchased as follows: sodium [1-^{13}C]acetate and sodium [3-^{13}C]-propionate from MSD, sodium [2-^{13}C]acetate, [1-^{13}C]propionic acid, [2-^{13}C,^{15}N]glycine, and [1,4-$^{13}C_2$]succinic acid from ICON, sodium [1,2-$^{13}C_2$]acetate and [1,2,3-$^{13}C_3$]malonic acid from ISOTEC, and L-[*methyl*-^{13}C]methionine from CEA.

DNM-A Production. *M. chersina* M956-1 strain was grown on slants containing soluble starch 0.5%, glucose 0.5%, fish meat extract 0.1%, Bacto yeast extract (Difco) 0.1%, NZ-case 0.2%, NaCl 0.2%, $CaCO_3$ 0.1%, and agar 1.5% in distilled water, adjusted to pH 7.0, which were incubated at 28 °C and stored in tubes sealed with screw caps at 4 °C. Prior to DNM-A production, *M. chersina* was precultivated in a 500-mL Sakaguchi flask containing 100 mL of a medium consisting of soluble starch 3%, lactose 1%, white fish meal (Hokusui) 1%, $CaSO_4 \cdot 2H_2O$ 0.6%, and $CaCO_3$ 0.5% in distilled water, adjusted to pH 7.0 before sterilization. The culture was shaken reciprocally at 200 rpm at 30 °C for 240 h. Fifty milliliters of this culture broth was inoculated into each of three Erlenmeyer flasks of 5-L capacity containing 1 L of medium consisting of soluble starch 0.4%, Bact yeast extract (Difco) 0.4%, Bact malt extract (Difco) 1.0%, NaI 0.00025%, and Diaion HP-20 (Mitsubishi kasei) 3.0% in distilled water, at pH 7.4 before sterilization, and shaken with rotatory shaker at 28 °C for 240 h. Maximum yield of DNM-A under this

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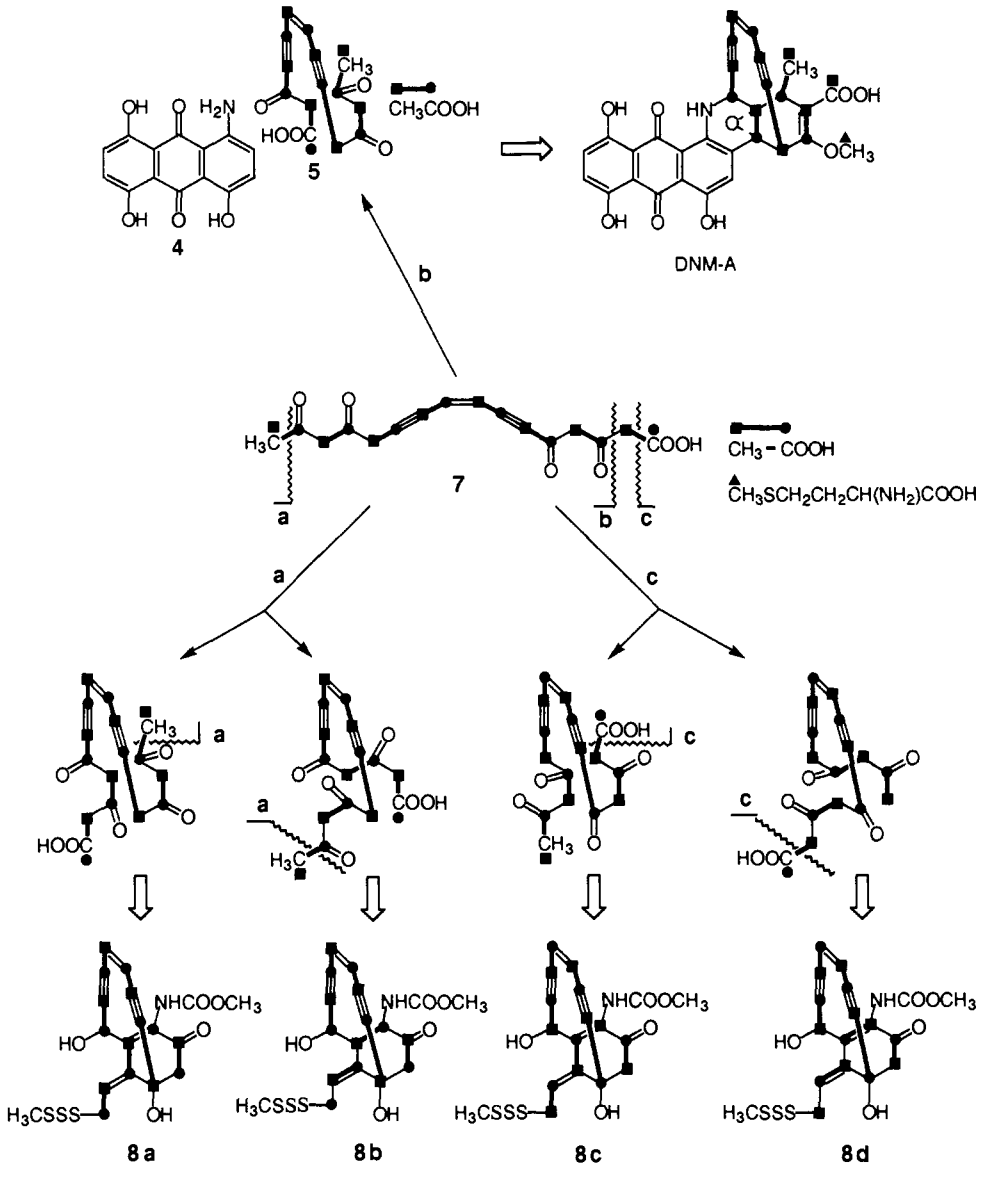
Scheme II. Proposed Biosynthetic Pathways of the Esperamicin/Calicheamicin Class of Antibiotics



condition was estimated by HPLC to be 10 mg/L.

Isolation Procedure. The whole fermentation broth (3 L) was extracted with an equal volume of ethyl acetate. The organic layer was concentrated and the crude extract was chromatographed on a Toyopearl HW-40F (Toso) column (32 × 250 mm) eluted with methanol. Reddish purple fractions were collected and concentrated to dryness. The crude mixture was further separated by preparative HPLC using an ODS column (10 × 250 mm) eluted with an aqueous acetonitrile gradient (40–50%) at 4 mL/min. Pure DNM-A was isolated in a yield of ca. 3 mg/L.

Biosynthetic ^{13}C-Labeling of DNM-A. Biosynthetically ^{13}C-labeled DNM-A was prepared by adding a labeled precursor core (at 0-h cultivation) or twice (at 0- and 24-h cultivation) after filtration–sterilization of the sample solution, adjusted to pH 7.0. The incorporation patterns and rates were determined by ^{13}C NMR spectroscopy, JEOL GSX-500. ^{13}C-Enriched DNM-A samples (ca. 3 mg each) were dissolved in 0.5 mL of DMSO-d_6 (Merck). Broad-band ^{1}H decoupled, INADEQUATE, and HMBC ^{13}C NMR spectra were obtained on these solutions in 5-mm tubes at probe temperatures of 27 and 70 °C.

[^{13}C]Acetate Incorporation. As a precursor, 200 mg/L of sodium [1-^{13}C]- or [2-^{13}C]acetate or 400 mg/L of sodium [1,2-$^{13}C_2$]acetate was added twice (at 0- and 24-h cultivation) to each of three flasks containing 1 L of culture medium, and incubation was conducted for 10 days. Peak intensity enhancements at the specific carbon signals were 2- to 4-fold.

L-[*methyl*-^{13}C]Methionine Incorporation. To each of three flasks containing 1 L of culture medium, 200 mg of ^{13}C-labeled methionine was added at 0-h cultivation, and incubation was conducted for 10 days. Peak intensity enhancement of the methoxy methyl signal was ca. 20-fold. Addition of more than 400 mg/L of methionine inhibited DNM-A production.

[2-^{13}C,^{15}N]Glycine Incorporation. The labeled glycine (126 mg) was added to each of three flasks containing 1 L of culture medium twice at 0- and 24-h cultivation, and incubation was conducted for 10 days. Peak intensity enhacement of the methoxy methyl signal was ca. 5-fold.

[1,2,3-^{13}C]Malonate Incorporation. As a precursor, 150 mg of sodium [1,2,3-$^{13}C_3$]malonate was added to three flasks containing 1 L of culture medium twice at 0- and 24-h cultivation. ^{13}C-Enrichments of all the carbons except the methoxy methyl carbon were observed, but the incorporation rates were about half those of acetates.

Other Precursors. Likewise, 300 mg/L each of [1-^{13}C]- and [3-^{13}C]propionate and [1,4-$^{13}C_2$]succinic acid were added to the culture, but they were not incorporated specifically into **1**.

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