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Biosynthetic pathway of macrolactam polyketide antibiotic cremimycin

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ABSTRACT

Biosynthetic origin of macrolactam polyketide antibiotic cremimycin was investigated by feeding experiments with [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate, [1-^{13}C]propionate, succinate-d_4, and D-[6,6-$^{2}H_2$]glucose. NMR analysis of the resultant isotope-enriched cremimycins showed distinctive incorporation patterns, which suggested that the aglycon of cremimycin was constructed from two propionates and eleven acetates. Thus, 3-oxononanoate was proposed as a potential polyketide intermediate, that is, aminated to be the unique nitrogen-containing moiety of cremymicin. Further, characteristic propionate biosynthetic pathway in the cremimycin-producing strain was also described.

1. Introduction

Cremimycin is a 19-membered macrolactam antibiotic produced by *Streptomyces* sp. MJ635-86F5 and shows broad antibacterial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) (Fig. 1).[1] The structure of this compound was determined by a series of NMR spectrum analysis, showing that cremimycin has a 2,6-dideoxy-3-*O*-methyl-allose (cymarose) and a bicyclic 19-membered macrolactam ring. The same bicyclic lactam ring system is seen in hitachimycin, an anti-tumor antibiotic, except for the nitrogen-containing starter unit.[2] The starter unit of hitachimycin is reported to be β-phenyl-alanine by feeding experiment.[2] On the other hand, the origin of the carbon chain for the nitrogen-containing unit of cremimycin type of antibiotics including BE-14106,[3] ML-449,[4] and aureoverticillactam[5] was unclear, though 3-aminononanoate or its equivalent seemed to be a potential starter unit of cremimycin and its analogues (Fig. 1). To elucidate the origin of this unique nitrogen-containing unit of polyketide, we conducted feeding experiments with several stable isotope-labeled compounds in this study.

2. Results and discussion

The elongating units of cremimycin seemed to be derived from acetate–propionate by a standard polyketide pathway. Thus, standard supplementation culture with [1-^{13}C]acetate and [1-^{13}C] propionate was first carried out, and the labeled cremimycin was purified as reported. The ^{13}C-enrichment of cremimycin was determined by the signal intensity in the ^{13}C NMR data and the signal at δ_C 56.7 is defined as 1.0, since the *O*-CH_3 group is most likely derived from methionine by SAM-dependent methyltransferase (Table 1). When [1-^{13}C]acetate was administered to the culture of cremimycin-producing strain, the ^{13}C signals of C-1, C-3, C-5, C-7, C-9, C-11, C-13, C-15, C-17, C-19, C-21, C-23, and C-25 were enriched (Fig. 2 and Table 1). This result suggested that all the carbon source of cremimycin aglycon is derived from acetate. On the other hand, feeding experiment with [1-^{13}C]propionate showed highly efficient incorporation of two signals for C-5 and C-25. These results suggested that C-5, C-6, C-6–CH_3 and C-25, C-26, C-27 are derived from propionate, while C-5 and C-25 were also labeled by [1-^{13}C] acetate. In order to clarify these conflict results, [1,2-$^{13}C_2$]acetate was administered to the culture. As a result, intact incorporation of 11 acetate units for C-1/C-2 (65 Hz), C-3/C-4 (56 Hz), C-7/C-8 (44 Hz), C-9/C-10 (39 Hz), C-11/C-12 (64 Hz), C-13/C-14 (43 Hz), C-15/C-16 (39 Hz), C-17/C-18 (36 Hz), C-19/C-20 (40 Hz), C-21/C-22 (37 Hz), C-23/C-24 (33 Hz) were observed (Fig. 3). In addition, the signals of C-5, C-6, C-6–CH_3, C-25, C-26, and C-27 also showed satellite signals, indicating that acetate unit was incorporated into these positions. The detailed analysis including INADEQUATE spectrum of this sample clearly confirmed the incorporation pattern of C-5/C-6/C-6–CH_3 and C-25/C-26/C-27 from [1,2-$^{13}C_2$]acetate, in which all of carbon atoms were clearly coupled by one bond ^{13}C–^{13}C coupling as exemplified in Fig. 3. This result indicated that propionate extender unit is mainly biosynthesized from acetate via tricarboxylic acid (TCA) cycle as explain in below (Fig. 4).

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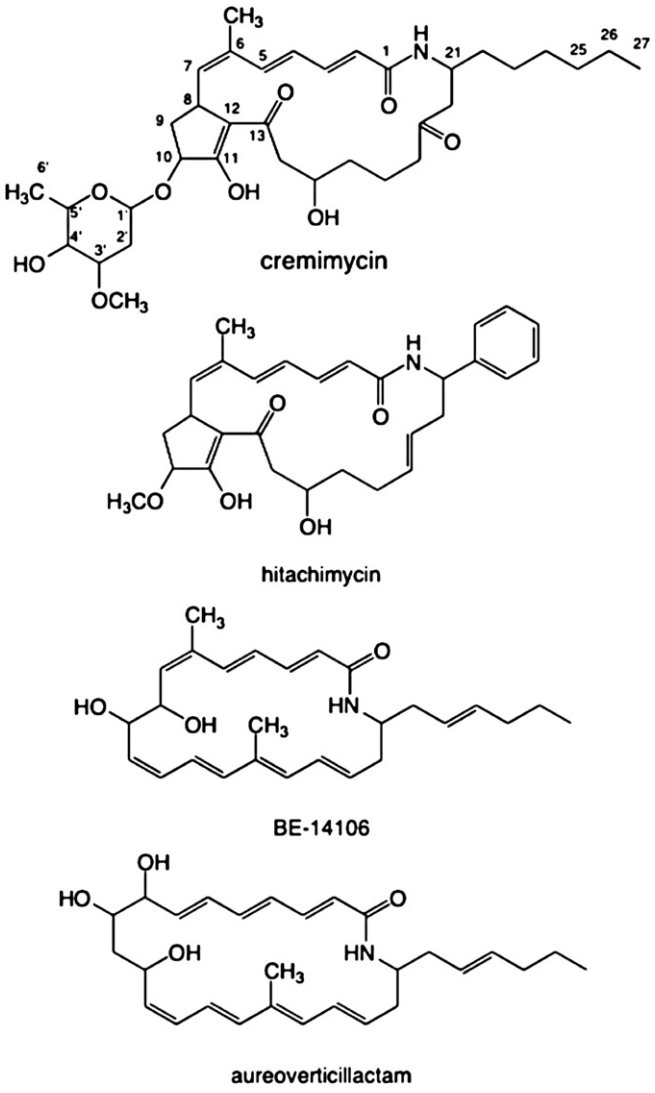

Fig. 1. Structures of cremimycin and its related macrolactam antibiotics.

In general, the propionate extender unit, methylmalonyl-CoA, in polyketide biosynthesis is derived from the following processes; (a) the carboxylation of propionyl-CoA, (b) the rearrangement of succinyl-CoA and epimerization, (c) the catabolism of valine, or (d) the multistep conversion of acetoacetyl-CoA through a crotonyl-CoA-dependent pathway.[6] Propionyl-CoA is the most frequent metabolic precursor of methylmalonyl-CoA, and it is formed as a product of the β-oxidation of odd- and branched-chain fatty acids or the catabolism of amino acids, such as isoleucine, methionine, and valine. Propionyl-CoA can also be formed by the incorporation of propionate from the culture followed by thioesterification with CoA by acyl-CoA ligase. This is the case that labeled propionate was highly incorporated into C-5 and C-25 of cremimycin when it was supplied.

Another major precursor of propionate extender unit is the primary metabolite succinyl-CoA. The most common source of succinyl-CoA is the TCA cycle. Succinyl-CoA is converted into propionate extender unit, (2*S*)-methylmalonyl-CoA, by methylmalonyl-CoA mutase, and methylmalonyl-CoA racemase. Thus, connection between acetate and propionate metabolism can be found in TCA cycle. When [1,2-$^{13}C_2$]acetate is administered, [3,4-$^{13}C_2$]succinyl-CoA is once formed via TCA cycle. Further, succinyl-CoA synthethase (or succinate thiokinase) in TCA cycle reversibly catalyzes interconversion between succinyl-CoA and

Table 1
^{13}C NMR assignment of cremimycin and incorporation of ^{13}C-labeled acetate and propionate into cremimycin as determined by ^{13}C NMR. nd; not determined due to signal overlapping

Position	δ	Relative intensity		
		[1-^{13}C] acetate	[1,2-$^{13}C_2$]acetate (multiplicity, ^{13}C–^{13}C coupling constant)	1-^{13}C propionate
1	167.1	30.7	27.3 (d, *J*=65 Hz)	2.9
2	123.1	1.9	18.5 (d, *J*=65 Hz)	2.3
3	141.5	21.7	13.6 (d, *J*=56 Hz)	1.7
4	126.7	3.4	21.7 (d, *J*=56 Hz)	2.7
5	136.6	10.9	11.8 (m)	81.8
6	130.5	4.8	14.2 (m)	2.9
6-CH_3	18.9	1.8	2.2 (m)	1.3
7	134.6	45.6	23.8 (d, *J*=44 Hz)	3.6
8	34.1	2.0	11.6 (d, *J*=44 Hz)	2.7
9	36.4	28.9	17.7 (d, *J*=39 Hz)	2.4
10	77.6	nd	12.3 (d, *J*=39 Hz)	1.8
11	186.1	46.3	23.8 (d, *J*=64 Hz)	3.3
12	111.8	5.0	33.0 (d, *J*=64 Hz)	3.7
13	194.6	40.0	45.0 (d, *J*=43 Hz)	3.0
14	45.6	2.1	16.2 (d, *J*=43 Hz)	2.0
15	67.5	22.9	15.8 (d, *J*=39 Hz)	2.1
16	36.6	1.8	18.1 (d, *J*=39 Hz)	2.5
17	19.2	8.7	4.6 (nd)	0.75
18	43.5	1.0	8.9 (d, *J*=36 Hz)	1.3
19	210.1	35.3	22.1 (d, *J*=40 Hz)	2.7
20	48.2	3.9	nd	1.9
21	47.6	16.6	nd	2.8
22	35.1	1.1	11.7 (d, *J*=37 Hz)	1.4
23	25.6	11.6	7.1 (d, *J*=33 Hz)	1.1
24	28.6	1.0	5.3 (d, *J*=33 Hz)	0.53
25	31.3	4.6	3.8 (d, *J*=35 Hz)	18.1
26	22.1	0.70	4.1 (d, *J*=34 Hz)	0.43
27	13.4	0.70	3.3 (d, *J*=35 Hz)	0.61
1′	97.9	3.2	2.5	4.8
2′	33.3	1.5	1.0	1.5
3′	77.1	nd	nd	nd
3′-OMe	56.7	1.0	1.0	1.0
4′	72.4	1.6	0.91	1.3
5′	70.4	1.3	1.1	1.4
6′	17.7	0.80	0.57	0.73

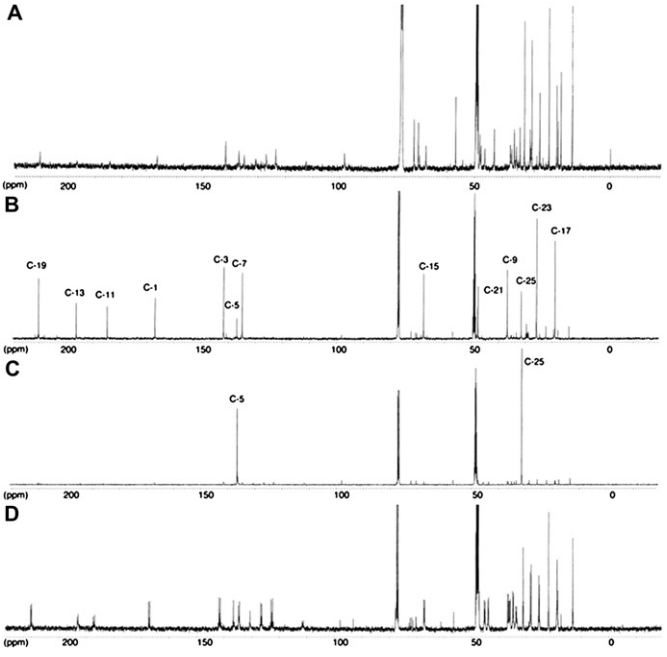

Fig. 2. ^{13}C NMR spectra of (A) non-labeled, (B) [1-^{13}C]acetate labeled, (C) [1-^{13}C]propionate labeled, and (D) [1,2-$^{13}C_2$]acetate labeled cremimycin (125 MHz, in $CDCl_3$/CD_3OD).

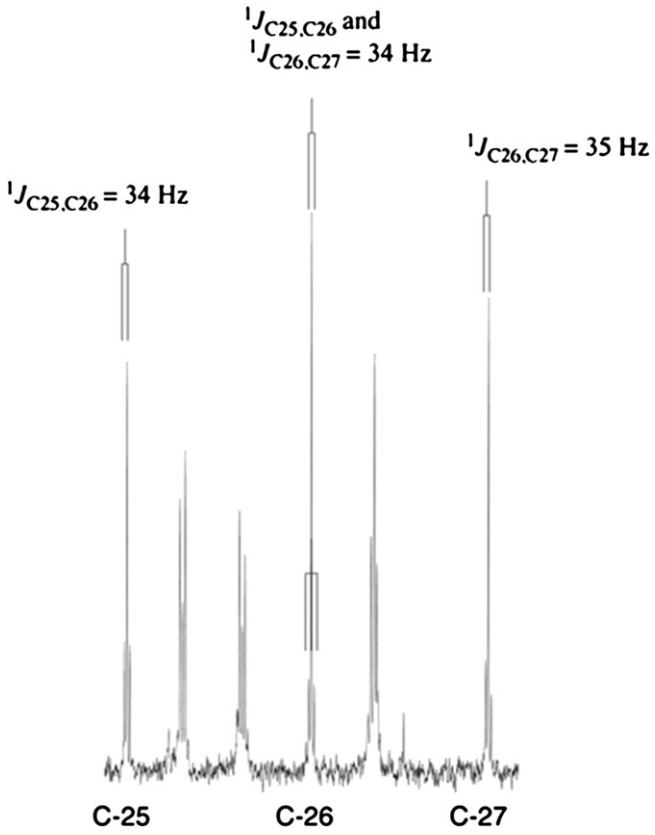

Fig. 3. Partial ^{13}C NMR spectrum of [1,2-$^{13}C_2$]acetate labeled cremimycin (125 MHz, in $CDCl_3/CD_3OD$).

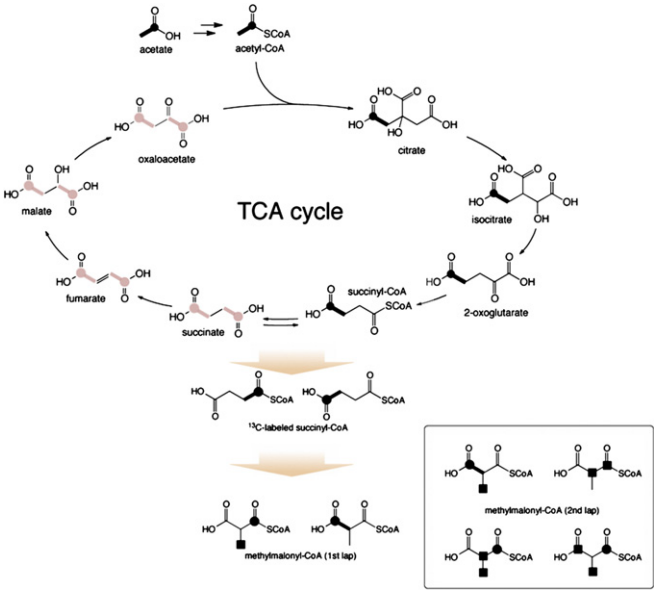

Fig. 4. Deduced methylmalonyl-CoA biosynthesis from acetate via TCA cycle.

a symmetry molecule, succinate. Thus, two types of ^{13}C-enriched succinyl-CoA, [1,2-$^{13}C_2$]succinyl-CoA, and [3,4-$^{13}C_2$]succinyl-CoA, are generated from [1,2-$^{13}C_2$]acetate through this process. These ^{13}C-enriched succinyl-CoA isotopomers could be converted into methylmalonyl-CoA by methylmalonyl-CoA mutase and methylmalonyl-CoA racemase as mentioned above. In addition, four types of ^{13}C-enriched methylmalonyl-CoA could be obtained when [1,2-$^{13}C_2$]acetate run the TCA cycle twice. Therefore, the resultant six types of methylmalonyl-CoA isotopomers might cause the incorporation pattern found in the ^{13}C NMR and INADEQUATE spectra of the labeled cremimycin (Fig. 4).

In order to confirm methylmalonyl-CoA formation from succinate, succinate-d_4 was supplied to the cremimycin-producing strain. As anticipated, ^2H NMR spectrum of the resultant cremimycin clearly showed deuterium signals at 0.8, 1.1, and 1.8 ppm, which correspond to positions at H-27, H-26, and 6-CH_3, respectively (Fig. 5). Hence, the present study verified that propionate extender unit of cremimycin was derived from not only propionyl-CoA, but succinate via TCA cycle.

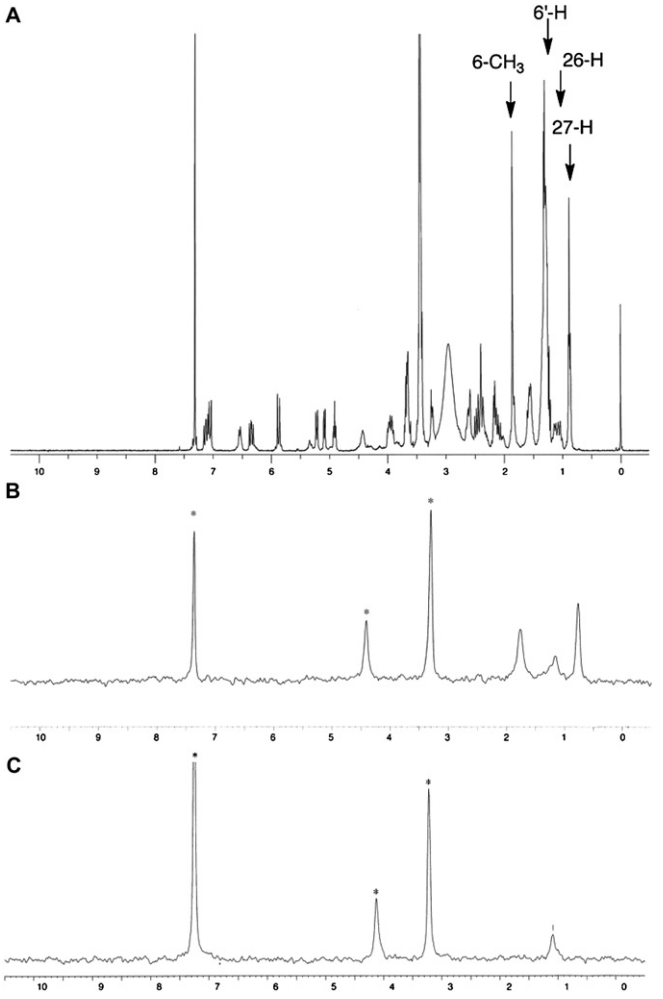

Fig. 5. ^2H NMR spectrum (500 MHz, in $CDCl_3/CD_3OD$) of (A) non-labeled cremimycin and ^2H NMR spectra (77 MHz, in $CHCl_3/CH_3OH$) of (B) succinate-d_4 labeled, and (C) D-[6,6-$^2H_2$]glucose labeled cremimycin. Asterisks are solvent signals.

In many cases, the polyketide producing organisms are known to adjust the metabolic source of methylmalonyl-CoA according to the growth conditions. As to erythromycin biosynthesis in *Saccharopolyspora erythraea*, either labeled propionate or succinate was incorporated into macrolide aglycon depending on the growth conditions.[7,8] Further, incorporation of succinate into propionate extender unit was observed in several polyketide antibiotics.[9,10] However, to our knowledge, it might be unusual that acetate was highly incorporated through succinate from TCA cycle into propionate unit.

Finally, D-[6,6-$^2H_2$]glucose was added to the culture. ^2H NMR spectrum of resultant cremimycin showed the signal at 1.1 ppm, corresponding C-6′ position of cymarose moiety (Fig. 5). This showed that the origin of cymarose is D-glucose and it could be proposed that cymarose is biosynthesized from gulucose-1-

phosphate by a series of enzymatic reactions of TDP-glucose synthase, TDP-glucose 4,6-dehydratase, TDP-4-keto-6-deoxy-glucose 2,3-dehydratase, TDP-3,4-diketo-2,6-dideoxy-glucose 3-ketoreductase, and TDP-4-diketo-2,6-dideoxy-glucose 4-ketoreductase, in the similar manner of digitoxose biosynthesis.[11] Methylation of 3′-hydroxyl group could be occurred after the attachment of cremimycin aglycon as in the case of *O*-methylated sugar.[12,13] Overall, it was found that macrolactam skeleton of cremimycin is constructed with 11 acetates and 2 propionates in a head to tail manner (Fig. 6). During the polyketide chain elongation, a nitrogen atom must be incorporated at C-21, presumably by transamination or reductive amination of a plausible intermediate 3-oxononanoate. In the biosynthesis of structurally related macrolactam antibiotics, BE-14106 and ML-449, it is proposed that the nitrogen atom of the starter unit is derived from glycine by a FAD-dependent glycine oxidase encoded in the gene cluster.[2,3] Similar scenario can be applied for the cremimycin starter biosynthesis. Our incorporation study clearly supported the β-carbonyl group of elongated polyketide chain is aminated. Thus, the nitrogen incorporation mechanism is intriguing issue how nitrogen atom is carried to react with the carbonyl group.

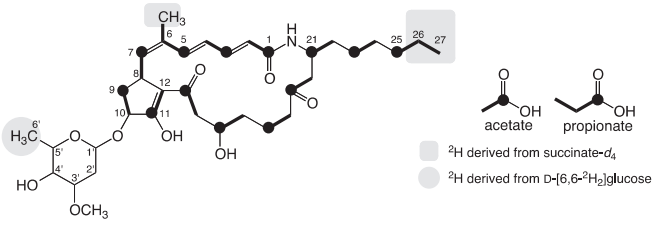

Fig. 6. Precursor–product relationship of cremimycin.

In contrast, only few insights were obtained about the mechanism of bicyclic macrolactam ring formation. Incorporation pattern of [1,2-$^{13}C_2$]acetate showed that three acetates were condensed in a standard manner of polyketide synthase from C-7 to C-12. This incorporation pattern is the same as that of hitachimycin,[2] and these two compounds have same oxygen functionality bound to C-10, which must not come from acetate carboxyl group in the normal polyketide biosynthesis. Therefore, this oxygen atom seemed to be attached by P450 monooxygenase or other oxygenase. Thus, it is proposed that the bicyclic ring formation occurs in conjugation with the oxidation of C-10 carbon before glycosylation (in the cremimycin biosynthesis) or methylation (in the hitachimycin biosynthesis).

In summary, the precursor–product relationship in the biosynthesis of polyketide macrolactam antibiotic cremimycin has been elucidated. The elongating units of the aglycon formation involves 11 acetates and 2 propionates according to the standard polyketide pathway, and the starter unit is the most likely to be 3-aminononanoate, which is derived from 3-oxononanoate. Further, characteristic propionate metabolic pathway in the cremimycin-producing strain was clarified. To understand details of cremimycin biosynthesis, genetic as well as enzymological studies are currently underway.

3. Materials and methods

3.1. General procedures

^1H, ^2H, ^{13}C NMR, and INDEQUATE spectra were recorded in a mixture of $CDCl_3/CD_3OD$ (^1H, ^{13}C, and INADEQUATE) or $CHCl_3/CH_3OH$ (^2H) with a JEOL Lambda 400, a JEOL AL 400 or a JEOL ECX 500 or a Brucker DRX 500 spectrometer. Spectra were calibrated to residual solvent signals with resonances at $\delta_{H/C}$=3.31/49.0 ppm (CD_3OD) or $\delta_{H/C}$=7.26/77.2 ppm ($CDCl_3$) and δ_H=0 ppm (TMS). [1-^{13}C]CH_3COONa, [1,2-$^{13}C_2$]CH_3COONa, [1-^{13}C]CH_3CH_2COOH, and succinate-d_4 were purchased from Sigma–Aldrich. D-[6,6-$^2H_2$]Glucose was synthesized according to the previous report.[14]

3.2. Production and purification of cremimycin

Cultivation of *Streptomyces* sp. MJ635-86F5 for the production of cremimycin was carried out in a medium (9.0 g/L of oatmeal, 1.5 g/L of malt extract, 0.9 g/L of yeast extract, 0.12 g/L of $MgSO_4\cdot 7H_2O$, 1.5 g/L of $CaCO_3$, 0.3 g/L of NaCl, 9.0 g/L of soluble starch, 11 g/L of MOPS, and supplemented with 10 g/L of D-glucose) adjusted to pH 7.0 with 2 M of NaOH. Fermentations were performed in 500 mL of Erlenmeyer flask with 100 mL of medium. The production cultures were inoculated with 5% (vol/vol) of the preculture with a 0.5× modified tryptone soya broth (TSB) medium (0.85 g/L of tryptone, 0.15 g/L of soytone, 0.25 g/L of NaCl, 0.125 g/L of KH_2PO_4, and 2 g/L of D-glucose) (3 days, 200 rpm). Fermentations were run for 3 weeks at 27 °C with agitation (200 rpm). Resultant mycelia was extracted with $CHCl_3/CH_3OH$ (1:1) and filtered. The extract was concentrated in vacuo. Resultant material was dissolved in CH_3OH, and then extracted with a mixture of hexane/CH_3OH (1:1). The lower layer was collected and concentrated. The material containing cremimycin was dissolved in a mixed solvent of $CHCl_3/CH_3OH$ (4:1) then washed with distilled water three times and brine. The organic layer was collected and concentrated by evaporation. The residue was dissolved in a mixed solvent of $CHCl_3/CH_3OH$ (4:1) and precipitated by adding hexane. The precipitate was collected by centrifugation and washed twice with hexane. Resultant faint pink powder was further purified by the gel filtration (LH-20, $CHCl_3/CH_3OH$=4:1).

3.3. Administration of stable isotope-labeled substrates

Preculture and culture of cremimycin-producing strain were carried out by the same procedure as described above. After 4 days of the main culture, each stable isotope-labeled substrate, ([1-^{13}C]CH_3COONa, [1,2-$^{13}C_2$]CH_3COONa, [1-^{13}C]CH_3CH_2COOH, succinate-d_4, D-[6,6-$^2H_2$]glucose) was added at the final concentration of 0.5%. Resultant isotope-labeled cremimycin was isolated in the same manner described above.

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Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.tet.2011.08.073. These data include MOL files and InChiKeys of the most important compounds described in this article.

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