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# Studies on the Biosynthesis of Terpenoids Produced by Actinomycetes. Part 4[1]. Formation of BE-40644 by the Mevalonate and Nonmevalonate Pathways

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**Abstract.** *Actinoplanes* sp. A40644 biosynthesized a terpenoid, BE-406441, mainly by the mevalonate pathway, while its mycelial membrane component, menaquinone MK-9, was produced by the nonmevalonate pathway. These results imply that the nonmevalonate pathway is dominant at the early stage of the fermentation in this microorganism with the replacement by the mevalonate pathway at the later stage. 

**Keywords: BE-40644, biosynthesis, nonmevalonate pathway, terpenoid**

Although terpenoids are abundantly produced by many plants and fungi, only limited numbers of terpenoids of *Actinomycetes*-origin are described to date. Such terpenoids include naphterpin,[2] furaquinocins,[3] napyradiomycins[4] and terpentecins,[5] and are produced by *Streptomyces via* the classical mevalonate pathway. Very curiously, however, the biosynthesis of the sesquiterpene pentalenolactone which is produced by several kinds of *Streptomyces* species, is explained by operation of the nonmevalonate pathway[6] which has recently been proposed by Rohmer *et al.*[7] This new pathway has also been corroborated by our recent finding of a new enzyme named 1-deoxyxylulose reductoisomerase[8] which transformed the pivotal 1-deoxyxylulose to the first metabolite specific to this pathway, 2-*C*-methylerythritol.[9] This new enzyme has turned out to be a target of an antibiotic, fosmidomycin.[10] We have also proved that the terpenoid moieties of carquinostatin[11] and novobiocin,[1,12] an antioxidative metabolite of *Streptomyces exfoliatus* and an antibiotic produced by *Streptomyces niveus*, respectively, were synthesized by the nonmevalonate pathway. In contrast to these findings, we have proved that in the naphterpin-producing organism, *Streptomyces aeriouvifer*, the nonmevalonate pathway expressed at the beginning of the fermentation was replaced by the mevalonate pathway at the later stage of the fermentation.[13] This result strongly suggests that in addition to the nonmevalonate pathway, some *Streptomyces* species can utilize the mevalonate pathway as well.

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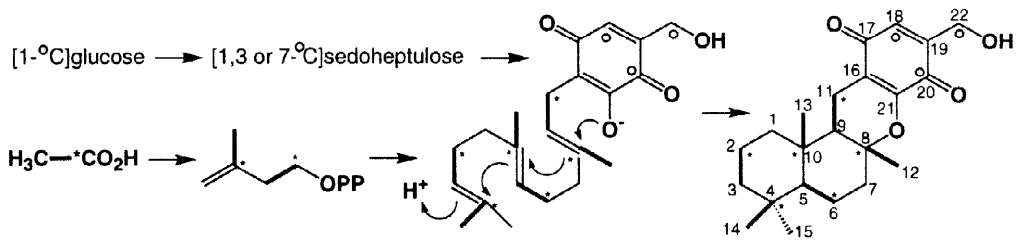
In order to pursue this interesting problem with Actinomycetes other than *Streptomyces*, we selected the next target BE-40644 (Fig. 1), a new human thioredoxin system inhibitor produced by *Actinoplanes* sp. A40644,[14] together with its primary metabolite, menaquinone which is produced from the beginning of the fermentation. Fermentation of *Actinoplanes* sp. and preparation of BE-40644 were done as reported previously. [14]

Fig. 1




The $^{13}$C-NMR spectrum of BE-40644 labeled with [1,2-$^{13}C_2$]acetate (diluted two folds by unlabeled acetate, added at 83 hr, 0.25 mg/ml, incubated at 27°C for a total of 9 days) showed six pairs of $^{13}$C-$^{13}$C couplings due to incorporation of the precursor into C-2 and 3 ($J$=34 Hz), C-4 and 14 (35 Hz), C-5 and 6 (35 Hz), C-8 and 12 (39 Hz), C-9 and 11 (34 Hz), and C-10 and 13 (35 Hz).[15] Use of [1$^{13}$-C]acetate (added at the concentration of 1 mg/ml) labeled the positions of C-2, C-4, C-6, C-8, C-10 and C-11 by roughly 5% (data not shown). These labeling patterns with carbons in the saturated ring system proved the operation of the mevalonate pathway for the formation of BE-40644 (Fig. 1).

Fig. 2

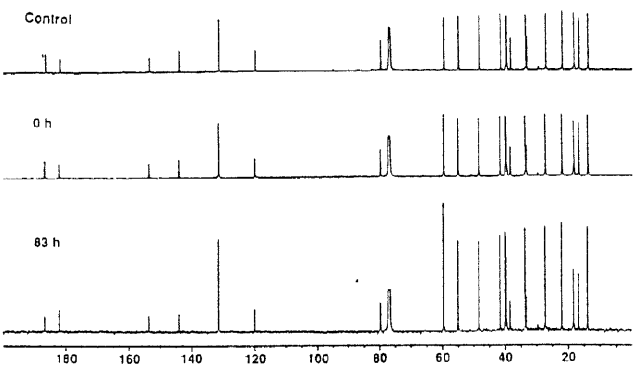



Fig. 3

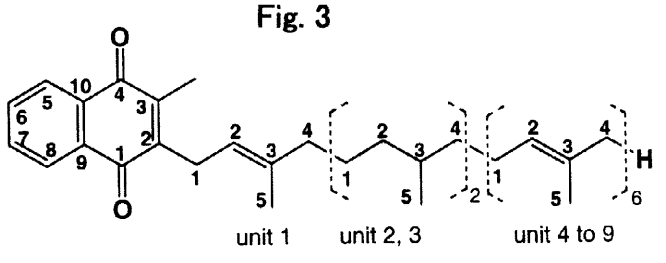
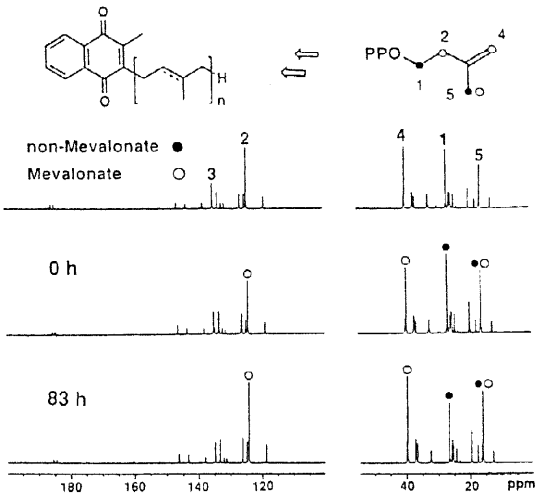



Fig. 4




In the next experiment, [1-$^{13}$C]glucose (1 mg/ml) was separately fed to cultures at 0 and 83 hr after the inoculation of the microorganism, and the fermentation was continued at 27°C for a total of 9 days. The production of BE-40644 started at about 50 hr after initiation of the cultivation and reached plateau at about 9 days, while the growth of *Actinoplanes* sp. A40644 became maximum at about 3 - 4 days. When added at 0 hr, [1-$^{13}$C]glucose was not incorporated

into BE-40644, while the precursor added at 83 hr enhanced by 1.8 to 2.0 times C-1, 3, 5, 7, 9, 12, 13, 14, and 15[16] which were assumed to be derived from C-2, 4 and 5 of IPP (Fig. 2). This labeling pattern is explained by incorporation of [1-$^{13}$C]glucose *via* [2-$^{13}$C]acetate to mevalonate which is produced at the later stage of the fermentation. These findings clearly show that BE-40644 is mainly synthesized by the mevalonate pathway.[17] Incorporation of $^{13}$C into C-18, 20 and 22 may be explained by involvement of [1,3 or 7-$^{13}$C]sedoheptulose 7-phosphate which is formed from [1-$^{13}$C]glucose *via* the pentose phosphate cycle. The cyclization of the heptulose may proceed in a similar manner as reported for the biosynthesis of the $mC_7$ unit in rifamycin.[18]

On the other hand, the primary metabolite menaquinone, which was identified as MK-9($H_4$)[19] (Fig. 3), was mainly produced by the nonmevalonate pathway when [1-$^{13}$C]glucose was added at 0 hr. As shown in Fig. 4, strong signals due to C-1 (overlapping of unit 5 to unit 9, marked with ●) at 26.70 ppm (enrichment ratio, X1.4) and C-5 (unit 4 to unit 8, marked with ○ and ●) at 16.03 ppm (X1.5) are enriched by [1-$^{13}$C]glucose, but C-2 (unit 5 to unit 8, marked with ○) at 124.28 ppm (X1.0), C-3 (unit 5 to unit 8) at 134.91 ppm (X1.0) and C-4 (unit 4 to unit 8, marked with ○) at 39.73 ppm (X1.1) were not labeled. These results imply that IPP is biosynthesized mainly by the nonmevalonate pathway.[7] Although C-5 of unit 1 to unit 9 could be enriched by both pathways, non-enrichment of C-2, 3 and 4 signals indicates that C-5 is presumably derived *via* the nonmevalonate pathway.

When the labeled glucose was added at 83 hr, C-2 and 4 (and presumably C-5 as well) of the $C_5$ units were enriched by ca. 1.7 to 1.8 times with C-1 being negligibly enriched. These results reveal that the nonmevalonate pathway starts to operate at the early stage of the fermentation in *Actinoplanes* sp. A40644 and that its contribution is replaced by the mevalonate pathway when the production of secondary metabolites is switched on.

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15. $^{13}$C-NMR spectra were taken in $CDCl_3$ at 125 MHz.
16. The following $^{13}$C-NMR assignment of BE-40644 labeled with [1-$^{13}$C]glucose (carbon number, enrichment ratio) was based on the previous paper[14] except for the exchange of C-14 and 15. The signal intensity of C-8 at 79.75 ppm was normalized to 1.0. 13.81 (C-13, 2.0), 16.70 (C-11, 1.2), 18.17 (C-6, 1.1), 18.32 (C-2, 1.1), 21.88 (C-14, 2.0), 27.20 (C-12, 2.0), 33.16 (C-4, 1.1), 33.66 (C-15, 2.0), 38.44 (C-10, 1.0), 39.65 (C-7, 2.1), 39.93 (C-1, 2.0), 41.63 (C-3, 1.8), 48.41 (C-9, 1.7), 55.07 (C-5, 1.8), 59.63 (C-22, 2.6), 79.75 (C-8, 1.0), 119.97 (C-16, 1.1), 131.66 (C-18, 1.8), 144.12 (C-19, 0.9), 153.62 (C-21, 1.2), 182.08 (C-20, 1.7), 186.67 (C-17, 0.9).
17. Analysis of the BE-40644 sample prepared by addition of [1-$^{13}$C]glucose at 58 hr showed a small contribution of the nonmevalonate pathway (data not shown).
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19. The $^{13}$C-NMR assignment of menaquinone MK-9 ($H_4$) labeled with [1-$^{13}$C]glucose (carbon number, enrichment ratio at 0 h and 83 hr) was based on the previous data[13]. The signal intensity of C-3 of the unit 3 at 32.47 ppm was normalized to 1.0. 12.69 (3-Me; 1.1, 1.3), 16.03 (C-5 of unit 4~8; 1.5, 2.0), 16.31 (C-5 of unit 1; 1.7, 2.3), 17.69 (C-5 of unit 9; 1.4, 2.2), 19.65 (C-5 of unit 2; 1.7, 2.0), 19.69 (C-5 of unit 3; 1.7, 2.0), 24.41 (C-1 of unit 3; 1.5, 1.3), 25.31 (C-1 of unit 2; 1.5, 1.5), 25.50 (C-1 of unit 4; 1.5, 1.3), 25.69 (C-4 of unit 9; 1.0, 1.7), 26.70 (X4) (C-1 of unit 5~8; 1.4, 1.2), 26.78 (C-1 of unit 9; 1.4, 1.5), 32.47 (C-3 of unit 3; 1.0, 1.0), 32.65 (C-3 of unit 2; 1.0, 1.0), 36.68 (C-2 of unit 2; 1.1, 1.9), 37.13 (C-4 of unit 3; 1.1, 1.8), 37.29 (C-2 of unit 3; 1.1, 1.7), 37.40 (C-4 of unit 2; 1.2, 1.8), 39.73 (C-4 of units 4~8; 1.1, 1.7), 40.04 (C-4 of unit 1; 1.2, 1.9), 118.81 (C-2 of unit 1; 1.0, 1.8), 124.28 (X4) (C-2 of units 5~8; 0.9, 1.6), 124.41 (C-2 of unit 9; 1.1, 1.5), 124.96 (C-2 of unit 4; 1.0, 1.7), 126.19 and 126.31 (C-5 and C-8; 1.5, 1.0, and 2.1, 1.0), 131.25 (C-3 of unit 9; 0.9, 1.1), 132.18 and 132.22 (C-9 and C-10; 1.2, 1.1 and 1.1, 1.1), 132.28 and 133.33 (C-6 and C-7; 1.5, 1.7 and 1.2, 1.7), 134.59 (C-3 of unit 4; 0.9, 1.0), 134.91 (X4) (C-3 of units 5~8; 0.9, 1.0), 137.99 (C-3 of unit 1; 1.0, 1.1), 143.36 (C-3; 1.6, 2.2), 146.24 (C-2; 1.8, 1.8), 184.56 (C-1; 0.9, 1.0), 185.50 (C-4; 0.9, 1.0).