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Simultaneous Operation of the Mevalonate and Non-mevalonate Pathways in the Biosynthesis of Isopentenyl diphosphate in *Streptomyces aeriouvifer*

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Abstract: Labeling experiments using [1,2-$^{13}C_2$]acetate and [U-$^{13}C_6$]glucose on menaquinone and naphterpin produced by *Streptomyces aeriouvifer* proved simultaneous operation of the mevalonate and non-mevalonate pathways for the formation of isopentenyl diphosphate in this organism.
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In contrast to the production of a wide variety of terpenoids and steroids by fungi[1], the genus *Streptomyces* rarely produces such metabolites. Biosynthetic studies performed with *Streptomyces* strains that produce terpenoidal and hemiterpenoidal metabolites including naphterpin[2] (Fig. 4), furaquinocins[3], napyradiomycins[4] and terpentecin[5] indicate involvement of the ubiquitous mevalonate pathway in the formation of these compounds.

Earlier biosynthetic studies conducted with the sesquiterpenoid pentalenolactone[6] produced by *Streptomyces* sp. have raised questions about the metabolite's biosynthetic mode of formation. The isolation of several biosynthetic intermediates possessing typical terpenoid structures such as pentalenene[7], pentalenic acid[8] and pentalenolactone H[8] suggested involvement of the usual mevalonate pathway to this sesquiterpene.

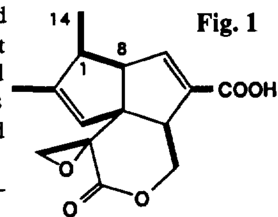


Fig. 1

However, the failure to incorporate ^{13}C-labeled acetic acid into pentalenolactone[6,9] and the simultaneous ^{13}C-labeling of three contiguous carbons (Fig. 1, C-1, 8 and 14) of pentalenolactone from the feeding of [U-$^{13}C_6$]glucose[6], used as an alternative ^{13}C-labeled precursor for ^{13}C-acetic acid was unexpected (bold lines indicate ^{13}C-^{13}C couplings). These labeling findings clearly indicate incompatibility with the classical mevalonate route to pentalenolactone.

Recently Rohmer[10] proposed a new biochemical pathway for the formation of the fundamental 5-carbon isoprenoid precursor unit, isopentenyl diphosphate (IPP), by condensation of glyceraldehyde 3-phosphate and pyruvate accompanied by decarboxylation and rearrangement. According to this pathway, C-1, 2 and 4, and C-3 and 5 of IPP are derived from glyceraldehyde 3-phosphate and pyruvate, respectively. It turned out that this non-mevalonate route to terpenoids is a ubiquitous pathway in some bacterial genera including the genus *Streptomyces*[10] and in the green alga *Scenedesmus obliquus*[11].

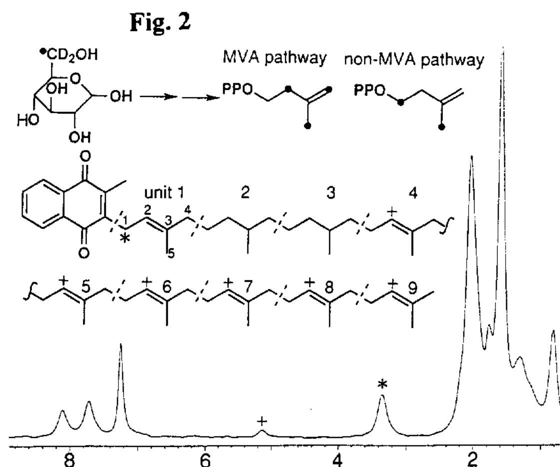
However, the experimental findings of incorporations of both acetate and mevalonate into the *Streptomyces* producing terpenoidal metabolites cited above[2-5] are contrary to the Rohmer proposal. These findings thus raise several unresolved issues in terpenoid biosynthesis in *Streptomyces*. These questions include the following; (1) Can organisms belonging to the *Streptomyces* be classified into two groups, one possessing the mevalonate pathway and the other the non-mevalonate pathway? (2) Do some *Streptomyces* species possess both pathways? (3) Can these two pathways operate independently of each other at different growth stages of the producing organisms that will result in the different labeling patters of the metabolites?

To address these unresolved issues we examined the biosynthesis of menaquinone (Fig. 2) in *Streptomyces aeriouvifer*. This organism also produces the hemiterpenoid naphterpin (Fig. 4) of known mevalonate and polyketide origin[2]. Menaquinone is a cell component common to all *Streptomyces* and its

structural feature is used as one of the important classification criteria of microorganisms[12].

The organism was cultivated as reported previously[2]; [1-^{13}C]acetate and [1,2-$^{13}C_2$]acetate were separately fed 24 hrs after inoculation at the level of 1 mg/ml and the fermentation was contined for a further 48 hrs. Analysis of the purified and separately labeled menaquinones MK9(H_4)[13] by ^{13}C-NMR revealed an incorporation pattern compatible with the mevalonate pathway (incorporation rate 0.6-0.7%)[15] as was expected from the biosynthetic result on naphterpin[2]. Thus ^{13}C-^{13}C couplings were observed between C-1 and 2, and C-3 and 5 in each isoprene unit of the side chain of menaquinone labeled with [1,2-$^{13}C_2$]acetate, and the peaks corresponding to C-1 and 3 were enriched by feeding with [1-^{13}C]acetate.

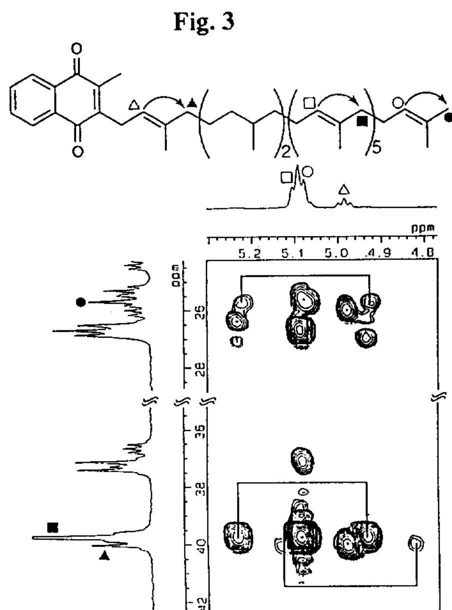
Fig. 2



However, when [6-$^2H_2$]glucose was added at an earlier fermentation stage (16 hrs inoculation), the isotope was incorporated into the C-1 position of unit 1 (3.36 ppm, shown by * in the ^{2}H-NMR spectrum of menaquinone[17], Fig. 2) which originated from C-1 of IPP. According to Rohmer[10], H-6 of glucose is incorporated into C-1 of IPP via the non-mevalonate pathway.

If IPP were formed from acetic acid via mevalonate, C-1 would be derived from the carboxyl group of acetic acid via mevalonate resulting in the complete loss of the deuterium. Thus these results together with the incorporation of ^{13}C-acetate into menaquinone suggest simultaneous operation of the mevalonate and non-mevalonate pathways for the formation of IPP in *S. aeriouvifer*. The weak ^{2}H-signal at 5.10 ppm (H-2 in units 4-9, shown by + in Fig. 2) implies the low incorporation of the isotope via acetate through the mevalonate pathway into menaquinone. The poor separation of ^{2}H-signals at ca. 2.0 ppm limited the information obtained by using ^{2}H-labeled glucose.

Fig. 3



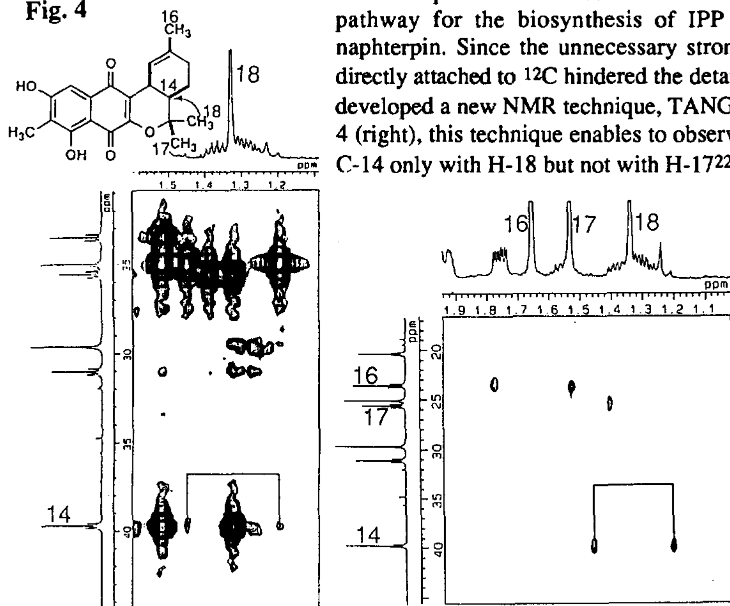
Analysis of menaquinone labeled with [U-$^{13}C_6$]glucose prepared in the same way as explained above (isotope at 1 mg/ml was added 16 hrs after initiation of the fermentation) gave confirmatory evidence for the operation of the non-mevalonate pathway. If IPP were formed via the Rohmer pathway in *S. aeriouvifer*, C-2 and C-4 would originate from the same glucose molecule[10], whereas these carbons would be labeled by two different acetic acid molecules metabolized from [U-$^{13}C_6$]glucose in the mevalonate pathway. Since this is the only difference in the ^{13}C-labeling patters between the two pathways, it is essential to detect a long-range ^{13}C-^{13}C coupling between C-2 and C-4 ($^2J_{C\text{-}C}$) to distinguish between these two pathways. However, such a long-range coupling is too small[18] to be observable by ^{13}C- NMR.

In order to overcome this (low sensitivity) problem, we employed HMBC[19] which was modified to observe direct ^{13}C-^{1}H couplings by removal of the low-pass J-filter (modified HMBC). If we assume that C-2 and C-4 of unit 1 in menaquinone (see Fig. 2) are simultaneously labeled with ^{13}C in the same molecule, then the protons appended to C-2 will be split to give satellite peaks due to ^{13}C-^{1}H coupling.

Therefore, the cross peaks between H-2 and C-4 will be observed with these satellite peaks in the modified HMBC spectrum of menaquinone labeled with [U-$^{13}C_6$]glucose. As illustrated in Fig. 3, H-2s in unit 1 (4.985 ppm), unit 9 (5.08 ppm) and units 4-8 (5.09 ppm) (indicated by △, ○ and □) show long-range couplings to C-4s in unit 1 (40.01 ppm), unit 9 (25.69 ppm) and units 4-8 (39.72 ppm) (indicated by ▲, ● and ■), respectively. These NMR spectral data prove that C-2 and C-4 in each isoprene unit originated from the same glucose molecule via the Rohmer pathway. Units 2 and 3 could not be analyzed in this manner due to overlapping signals.

The modified HMBC spectrum of naphterpin labeled with [U-$^{13}C_6$]glucose isolated from the same fermentation showed long-range coupling between H-18 (1.34 ppm) and C-14 (39.7 ppm) due to incorporation of [U-$^{13}C_6$]glucose via the Rohmer pathway (Fig. 4, left). However, the very weak intensity of the cross peaks[20] indicates a smaller contribution of the non-mevalonate pathway for the biosynthesis of IPP utilized for the formation of naphterpin. Since the unnecessary strong central peaks due to protons directly attached to ^{12}C hindered the detailed analysis of other signals, we developed a new NMR technique, TANGO-HMBC[21]. As shown in Fig. 4 (right), this technique enables to observe the presence of cross peaks of C-14 only with H-18 but not with H-17[22].

Fig. 4



In view of the formation of naphterpin at a somewhat later stage of the fermentation, the difference in the contribution of the two pathways may be explained by the operation of the non-mevalonate pathway at the initial growth stage (formation of the primary metabolite, menaquinone), and its replacement by the mevalonate pathway at a later growth stage (formation of the secondary metabolite, naphterpin). Suppression of the production of naphterpin by pravastatin, an inhibitor of HMG-CoA reductase, without affecting the growth of the producing organism (data not shown) is compatible with this explanation.

It should be emphasized that this is the first report of the simultaneous operation of the mevalonate and non-mevalonate pathways in the same organism without organelles[23]; operations of the pathways being dependent on the growth stage of the producing organism.

TANGO-HMBC which permits the observation of 1,3-long-range ^{13}C-^{13}C couplings, is a useful technique especially for studying the 1,2-migration of a methyl group in the biosynthesis of terpenoids and steroids using [1,2-$^{13}C_2$]acetate as a precursor.

Acknowledgements

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   12.69 (3-Me), 15.95 and 16.01 (X4) (C-5 of units 4 ~ 8), 16.29 (C-5 of unit 1), 17.68 (C-5 of unit 9), 19.63 (C-5 of unit 3), 19.68 (C-5 of unit 2), 24.40 (C-1 of unit 3), 25.28 (C-1 of unit 2), 25.47 (C-1 of unit 4), 25.69 (C-4 of unit 9), 25.98 (C-1 of unit 1), 26.68 (X4) and 26.75 (C-1 of units 5 ~ 9), 32.44 (C-3 of unit 3), 32.63 (C-3 of unit 2), 36.65 (C-2 of unit 2), 37.10 (C-4 of unit 3), 37.26 (C-2 of unit 3), 37.37 (C-4 of unit 2), 39.72 (X5) (C-4 of units 4 ~ 8), 40.01 (C-4 of unit 1), 118.77 (C-2 of unit 1), 124.24 (X4)(C-2 of units 5 ~ 8), 124.38 (C-2 of unit 9), 124.93 (C-2 of unit 4), 126.18 and 126.30 (C-5 and 8), 131.24 (C-3 of unit 9), 132.16 and 132.20 (C-9 and 10), 133.27 and 133.33 (C-6 and 7), 134.56 and 134.90 (X4) (C-3 of units 4 ~ 8), 137.96 (C-3 of unit 1), 143.33 (C-3), 146.19 (C-2), 184.53 (C-1), 185.47 (C-4). ^{13}C-^{13}C couplings were observed with a sample labeled with [1,2-$^{13}C_2$]acetate between C-1 and C-2, and C-3 and C-5 of units 2 and 3 ($J_{C\text{-}C}$ = 35 Hz), between C-1 and C-2 of units 4 ~ 9 ($J_{C\text{-}C}$ = 44 Hz), and between C-1 and C-2 and C-3 and C-5 of units 1 and 4 ~ 9 ($J_{C\text{-}C}$ =42 Hz).
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