JOC Article

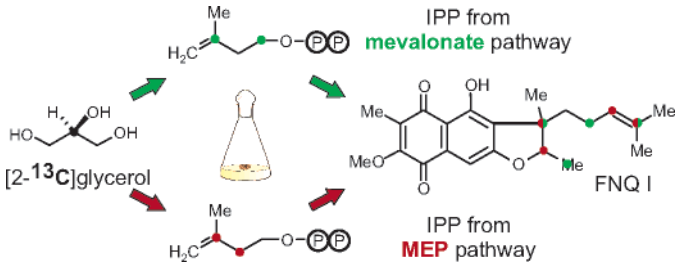
Biosynthesis of the Isoprenoid Moieties of Furanonaphthoquinone I and Endophenazine A in *Streptomyces cinnamonensis* DSM 1042

Gerhard Bringmann,*,† Yvonne Haagen,‡,§ Tobias A. M. Gulder,†,§ Tanja Gulder,†,§ and Lutz Heide*,‡

Institute of Organic Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany, and Pharmaceutical Biology, University of Tübingen, Auf der Morgenstelle 8, D-72076 Tübingen, Germany

bringman@chemie.uni-wuerzburg.de; heide@uni-tuebingen.de

Received February 19, 2007



Streptomyces cinnamonensis DSM 1042 produces the polyketide-isoprenoid compound furanonaphthoquinone I (FNQ I) and isoprenylated phenazines, predominantly endophenazine A. However, the recently identified biosynthetic gene cluster for these compounds only contains a single gene for a mevalonate pathway enzyme, that is, a putative mevalonate kinase gene. This is in strong contrast to all *Streptomyces* strains examined so far, where all six genes encoding the mevalonate pathway enzymes are clustered in a single operon of 6.8 kb and, thus, raised the question about the biosynthetic origin of the isoprenoid moieties of FNQ I and endophenazine A. In this study, we investigated the incorporation of [$^{13}C_2$]acetate and [2-^{13}C]glycerol into FNQ I and endophenazine A. The results unequivocally prove that the isoprenoid building blocks of both compounds are predominantly formed via the mevalonate pathway (approximately 80%) but that the MEP pathway (approximately 20%) contributes to the biosynthesis of these molecules, too. In actinomycetes, this is the first experimentally proven example of the utilization of both biosynthetic routes for the formation of one single secondary metabolite. The incorporation pattern of [2-^{13}C]glycerol was consistent with a "reverse" prenyl transfer, that is, with the formation of a C−C bond from C-3 of GPP to the polyketide nucleus of FNQ I.

Introduction

Streptomyces species are an abundant source of secondary metabolites, including compounds of isoprenoid and mixed isoprenoid/nonisoprenoid origin.[1] In animals and archaebacteria, only the mevalonate pathway is responsible for the formation of isoprenoid compounds.[1,2] In this pathway, isopentenyl diphosphate (IPP) is synthesized from acetyl coenzyme A via 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) and mevalonate. However, the existence of a mevalonate-independent pathway was demonstrated in several eubacteria, in apicomplexan protozoa, and in plants.[1−3] In this pathway, IPP is formed from pyruvate and glyceraldehyde-3-phosphate via 1-deoxy-D-xylulose-5-phosphate (DXP) and 2-*C*-methyl-D-erythritol 4-phosphate (MEP). Streptomycetes use the MEP pathway for the production of terpenoids of the primary metabolism, such as menaquinones,[1,4] and for the production of several secondary metabolites, such as pentalenolactone,[1,5] carquinostatin B,[6]

* To whom correspondence should be addressed. Phone: +49-931-888-5323 (G.B); +49-7071-29-78789 (L.H). Fax: +49-931-888-4755 (G.B); +49-7071-29-5250 (L.H.).
† Institute of Organic Chemistry, University of Würzburg.
‡ Pharmaceutical Biology, University of Tübingen.
§ These authors contributed equally to this work.

(1) Kuzuyama, T.; Seto, H. *Nat. Prod. Rep.* **2003**, *20*, 171−183.
(2) Eisenreich, W.; Bacher, A.; Arigoni, D.; Rohdich, F. *Cell Mol. Life Sci.* **2004**, *61*, 1401−1426.
(3) Rohmer, M.; Knani, M.; Simonin, P.; Sutter, B.; Sahm, H. *Biochem. J.* **1993**, *295* (Pt 2), 517−524.
(4) Seto, H.; Watanabe, H.; Furihata, K. *Tetrahedron Lett.* **1996**, *37*, 7979−7982.
(5) Cane, D. E.; Rosshi, T.; Pachlatko, J. P. *Tetrahedron Lett.* **1979**, *20*, 3639−3642.

10.1021/jo0703404 CCC: $37.00
Published on Web 05/03/2007

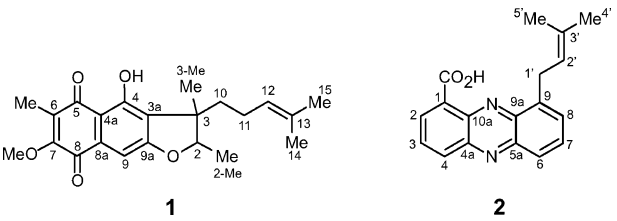
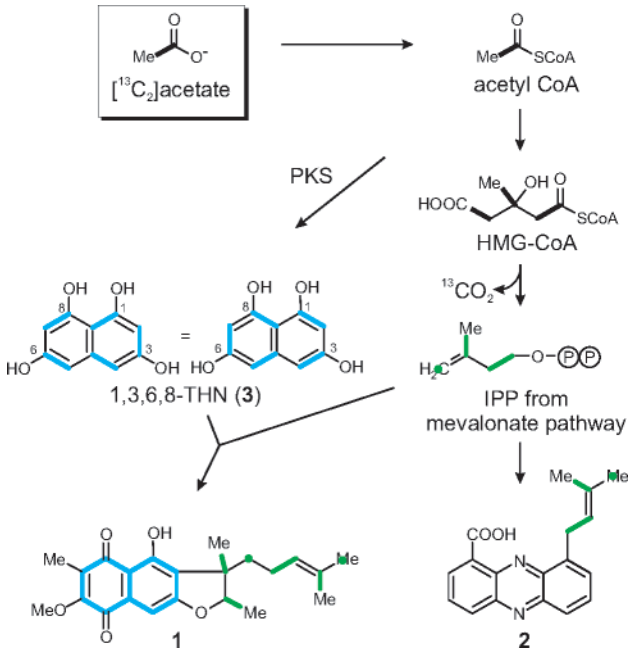


FIGURE 1. Furanonaphthoquinone I (**1**) and endophenazine A (**2**) from *Streptomyces cinnamonensis* DSM 1042. Numbering of the FNQ I structure follows the suggestion by Sedmera et al.[21]

moenomycinA,[7] and novobiocin.[8,9] Some *Streptomyces* strains, however, possess in addition the mevalonate pathway, which appears to be used exclusively for the production of secondary metabolites in this bacterial genus.[1] Feeding experiments with [^{13}C]-labeled precursors have proven that the isoprenoid moieties, for example, of the secondary metabolites naphterpin,[4,10] furaquinocin,[11] napyradiomycin,[12] BE-40644,[13] and terpentecin[14] are synthesized via the mevalonate pathway. These results have recently been confirmed by genetic data. The biosynthetic gene clusters of furaquinocin, naphterpin, terpentecin, and BE-40644 were each shown to contain the gene coding for (a) HMG-CoA synthase, (b) HMG-CoA reductase, (c) mevalonate kinase, (d) phosphomevalonate kinase, (e) mevalonate diphosphate decarboxylase, and (f) type 2 IPP isomerase.[15−18] These six open reading frames (ORFs) were arranged in all clusters in an identical order (i.e., c-e-d-f-b-a) in a single operon, and the orthologous ORFs showed more than 70% identity with each other at the amino acid level. By heterologous expression, it was proven that these six ORFs direct the biosynthesis of IPP via the mevalonate pathway in streptomycetes, because the precursor acetoacetyl CoA is supplied by the primary metabolism in this genus.[19]

Streptomyces cinnamonensis DSM 1042 produces two classes of secondary metabolites with isoprenoid moieties: the mixed polyketide−isoprenoid compound furanonaphthoquinone I (FNQ I, **1**) and prenylated phenazines, predominantly endophenazine A (**2**, Figure 1). We have recently cloned and sequenced a contiguous 81.5 kb DNA region of this strain, which contains

SCHEME 1. Expected Incorporation of Label from [$^{13}C_2$]Acetate into FNQ 1 (1) and Endophenazine A (2) via the Mevalonate Pathway[a]



[a] ^{13}C-Labeled portions are shown in bold black, green (mevalonate pathway), and blue (polyketidic).

a gene cluster directing the biosynthesis of both FNQ I (**1**) and endophenazine A (**2**).[20] However, sequence analysis of the FNQ I−endophenazine A cluster revealed only a single gene for a mevalonate pathway enzyme, that is, a putative mevalonate kinase gene. Within a region of 47 kb upstream and 33 kb downstream of this gene, none of the other five genes of the mevalonate pathway was found. It was, therefore, tempting to speculate that the other mevalonate pathway genes may have been lost during the evolution of this cluster, leaving this strain to produce the isoprenoid moieties of **1** and **2** via the primary metabolic MEP pathway.

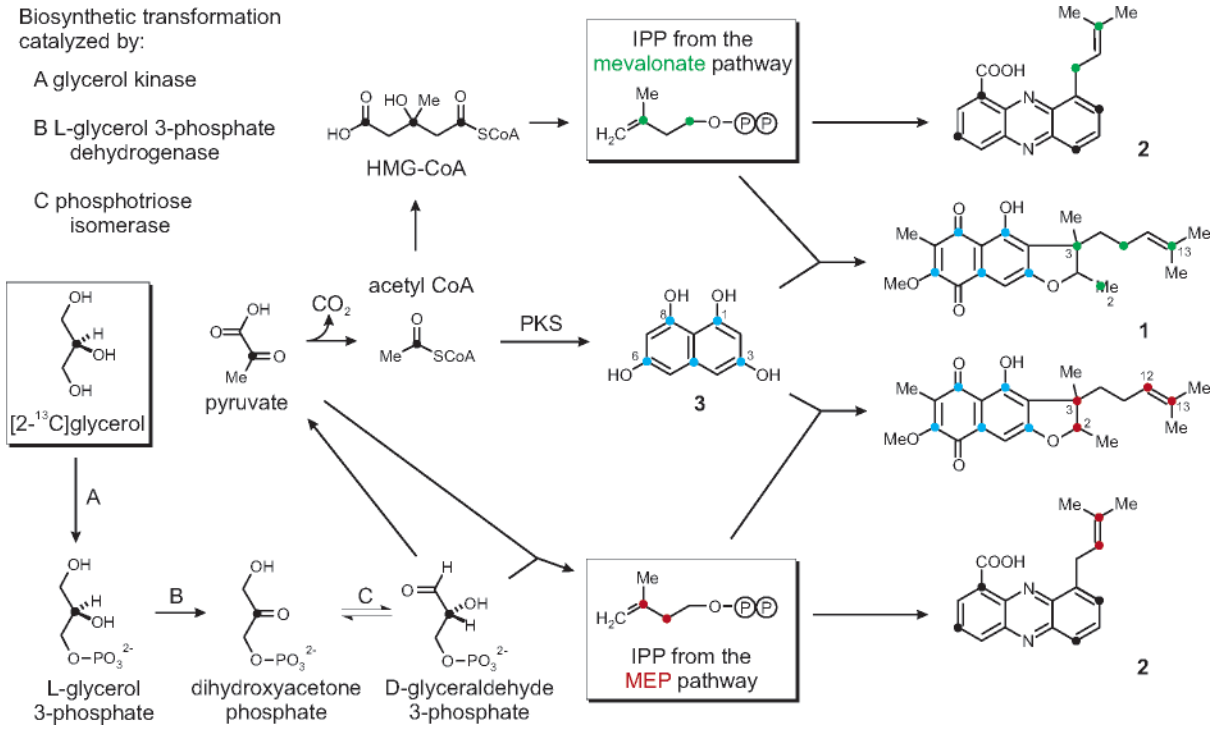
In the present study, we have investigated the biosynthetic origin of the furanonaphthoquinone **1** and endophenazine A (**2**) by feeding experiments with ^{13}C-labeled precursors and NMR analysis of the labeled secondary metabolites isolated by RP-HPLC.

Results and Discussion

In a first experiment, we fed [$^{13}C_2$]acetate to investigate its incorporation into the polyketide-derived naphthoquinone moiety of FNQ I (**1**) and into the isoprenoid-derived moieties of **1** and endophenazine A (**2**) by means of ^{13}C NMR spectroscopy and analysis of the ^{2}J-^{13}C-coupling constants. However, significant incorporation of labeled actetate into the isoprenoid moieties **1** and **2** is expected only via the mevalonate pathway (Scheme 1). We, therefore, wanted to carry out an additional feeding experiment with a precursor that would show significant ^{13}C incorporation into different positions of the isoprenoid moieties, depending on the utilization of either the mevalonate or the MEP pathway. In previous studies, [1-^{13}C]-D-glucose has frequently been used for this purpose.[8]

(6) Orihara, N.; Furihata, K.; Seto, H. *J. Antibiot. (Tokyo)* **1997**, *50*, 979−981.
(7) Endler, K.; Schuricht, U.; Hennig, L.; Welzel, P. *Tetrahedron Lett.* **1998**, 13−16.
(8) Li, S. M.; Hennig, S.; Heide, L. *Tetrahedron Lett.* **1998**, *39*, 2717−2720.
(9) Orihara, N.; Kuzuyama, T.; Takahashi, S.; Furihata, K.; Seto, H. *J. Antibiot. (Tokyo)* **1998**, *51*, 676−678.
(10) Shin-Ya, K.; Furihata, K.; Hayakawa, Y.; Seto, H. *Tetrahedron Lett.* **1990**, *31*, 6025−6026.
(11) Funayama, S.; Ishibashi, M.; Komiyama, K.; Omura, S. *J. Org. Chem.* **1990**, *55*, 1132−1133.
(12) Shiomi, K.; Iinuma, H.; Naganawa, H.; Isshiki, K.; Takeuchi, T.; Umezawa, H. *J. Antibiot. (Tokyo)* **1987**, *40*, 1740−1745.
(13) Seto, H.; Orihara, N.; Furihata, K. *Tetrahedron Lett.* **1998**, *39*, 9497−9500.
(14) Isshiki, K.; Tamamura, T.; Sawa, T.; Naganawa, H.; Takeuchi, T.; Umezawa, H. *J. Antibiot. (Tokyo)* **1986**, *39*, 1634−1635.
(15) Kawasaki, T.; Hayashi, Y.; Kuzuyama, T.; Furihata, K.; Itoh, N.; Seto, H.; Dairi, T. *J. Bacteriol.* **2006**, *188*, 1236−1244.
(16) Kawasaki, T.; Kuzuyama, T.; Furihata, K.; Itoh, N.; Seto, H.; Dairi, T. *J. Antibiot. (Tokyo)* **2003**, *56*, 957−966.
(17) Hamano, Y.; Dairi, T.; Yamamoto, M.; Kawasaki, T.; Kaneda, K.; Kuzuyama, T.; Itoh, N.; Seto, H. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1627−1635.
(18) Takagi, M.; Kuzuyama, T.; Takahashi, S.; Seto, H. *J. Bacteriol.* **2000**, *182*, 4153−4157.
(19) Dairi, T. *J. Antibiot. (Tokyo)* **2005**, *58*, 227−243.
(20) Haagen, Y.; Gluck, K.; Fay, K.; Kammerer, B.; Gust, B.; Heide, L. *ChemBioChem* **2006**, *7*, 2016−2027.

SCHEME 2. Expected Incorporation of Label from [2-^{13}C]Glycerol into 1 and 2 via the Mevalonate and the Methylerythritol Phosphate (MEP) Pathways[a]



[a] ^{13}C-Labeled portions are shown in bold black, green (mevalonate pathway), red (MEP pathway), and blue (polyketidic).

FNQ I (**1**) and endophenazine A (**2**) were produced by *S. cinnamonensis* DSM 1042 in amounts of 8 mg L^{-1} and 4 mg L^{-1}, respectively, using a production medium described by Sedmera et al.[21] This medium contains glycerol as a carbon source. We first tested whether addition of acetate or glucose would influence secondary metabolite formation. While the addition of sodium acetate (final concentration 1 g L^{-1}) did not affect the production, substitution of the original carbon source, glycerol (final concentration 5%), with D-glucose (final concentration 1%) dramatically reduced the formation of **1** and **2** (to 0.8 mg L^{-1} and 0.1 mg L^{-1}, respectively). Even just the addition of glucose (final concentration 1%) to the original medium (containing 5% glycerol) lowered FNQ I (**1**) and endophenazine A (**2**) production to 3 mg L^{-1} and 1 mg L^{-1}, respectively. Therefore, [1-^{13}C]D-glucose was not suitable for feeding as a precursor for our experiment. For this reason, labeled glycerol was used, instead.

To reduce the dilution of the isotope label, the glycerol content of the medium was lowered from 5% to 1%. This did not affect FNQ I (**1**) production (8 mg L^{-1}), but endophenazine A (**2**) production was diminished to 1.5 mg L^{-1}.

The achiral compound glycerol is metabolized by glycerol kinase and glycerol-3-phosphate dehydrogenase to give the chiral phosphotriose intermediates of the Embden−Meyerhof pathway (Scheme 2).[22] Depending on the specificity of the glycerol kinase of the respective organism in phosphorylating one or the other of the two enantiotopic "outer" OH groups, D-[1-^{13}C]glycerol may be converted to either [1-^{13}C] or [3-^{13}C]-phosphotrioses. Because this enantioselectivity had not yet been investigated in *S. cinnamonensis* DSM 1042, we used [2-^{13}C]-glycerol rather than [1-^{13}C]glycerol. Incorporation of this compound via the MEP or the mevalonate pathway should result in different, distinct labeling patterns, irrespective of the stereoselectivity of the involved glycerol kinase (Scheme 2).

Streptomyces cinnamonensis DSM 1042 was cultured as described in the Experimental Section. To 2 L of culture, a sterile solution of 2 g of sodium [$^{13}C_2$]acetate (adjusted to pH 7) or of 2 g of sterile [2-^{13}C]glycerol was added. After culturing for 5 days, the cells were harvested, extracted with methanol, and after addition of acetate buffer pH 4, **1** and **2** were extracted from the mixture with dichloromethane. HPLC analysis of the resulting crude extracts showed a total amount of 15 mg of **1** and 1.8 mg of **2** after feeding of sodium [$^{13}C_2$]acetate and a similar amount after administration of [2-^{13}C]glycerol (13 mg of **1** and 1.3 mg of **2**). Preparative RP-HPLC using a water−acetonitrile gradient gave the pure compounds (7 mg of **1** and 1 mg of **2** from the [$^{13}C_2$]acetate feeding; 6 mg of **1** and 0.8 mg of **2** from the [2-^{13}C]glycerol application).

An assignment of the ^{13}C NMR signals of FNQ I (**1**) has been published by Sedmera et al.[21] For most of the carbon atoms, this assignment could be unambiguously confirmed in the course of this study by analysis of the HMQC- and HMBC-NMR spectra of the metabolite derived from the pretests of the culturing conditions with the unlabeled precursors. However, our data suggested that Sedmera's assignment of the signals of C-4a and C-9 had to be reversed, as well as that of C-7 and C-9a. Both reversals were later on confirmed by the coupling constants observed after [$^{13}C_2$]acetate feeding.

After feeding of [$^{13}C_2$]acetate, the naphthoquinone portion of **1** was labeled in all ring positions as expected. Each carbon atom showed two direct C−C couplings interacting indepen-

(21) Sedmera, P.; PospÍšil, S.; Novák, J. *J. Nat. Prod.* **1991**, *54*, 870−872.

(22) Lin, E. C. *Annu. Rev. Microbiol.* **1976**, *30*, 535−578.

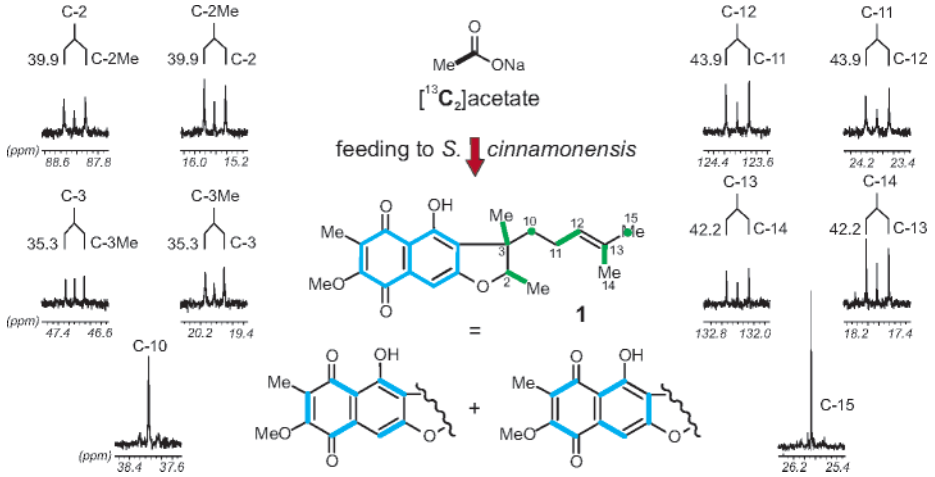


FIGURE 2. Labeling pattern of FNQ I (**1**) derived from the feeding of sodium [$^{13}C_2$]acetate to *Streptomyces cinnamonensis*. ^{13}C-labeled portions are shown in green (mevalonate pathway) and blue (polyketidic). ^2J coupling constants are given in Hz.

TABLE 1. ^{13}C NMR Chemical Shifts and Enrichment Ratios of Furanonaphthoquinone I (2), Isolated after Feeding of [$^{13}C_2$]Acetate and [2-^{13}C]Glycerol[a]

	[$^{13}C_2$]acetate feeding			[2-^{13}C]glycerol feeding	
carbon	δ (ppm)	enrichment (%)	^2J (to C)	δ (ppm)	enrichment (%)
2	88.2	3.9[b]	39.9 (2-Me)	88.3	1.5
2-CH_3	15.6	3.9[b]	39.9 (2)	15.6	7.0
3	47.1	4.2[b]	35.3 (3-Me)	47.1	7.7
3-CH_3	19.9	4.2[b]	35.3 (3)	19.9	0.2
3a	128.1	[c]	61.1 (4); 75.1 (9a)	128.1	-0.4
4	161.4	[c]	61.1 (3a); 72.0 (4a)	161.4	11.6
4a	109.7	[c]	59.1 (5); 72.0 (4)	109.9	-0.4
5	184.1	[c]	51.9 (6); 59.1 (4a)	184.1	7.5
6	133.9	[c]	51.9 (5); 74.3 (7)	134.1	-0.2
6-CH_3	9.6			9.6	1.3
7	157.0	[c]	57.4 (8); 74.0 (6)	157.0	10.4
7-OCH_3	60.9			60.9	1.4
8	181.1	[c]	57.4 (7); 54.2 (8a)	181.3	0.4
8a	133.6	[c]	54.2 (8); 66.0 (9)	133.6	7.0
9	108.7	[c]	64.0 (9a); 65.4 (8a)	108.9	0.0
9a	156.7	[c]	63.7 (9); 75.1 (3a)	156.8	9.5
10	38.0	4.0		38.0	-0.1
11	23.9	4.1[b]	43.9	23.9	5.9
12	124.0	4.1[b]	43.9	124.0	1.5
13	132.3	3.8[b]	42.2	132.3	10.1
14	17.8	3.8[b]	42.2	17.8	0.1
15	25.9	4.1		25.9	-0.1

[a] The enrichment is given as atom% 13C, exceeding the natural abundance. [b] The average enrichment of the two carbons derived from a $^{13}C_2$ unit is shown. [c] The average enrichment of the 10 carbons of the polyketide-derived naphthoquinone moiety was 4.3%. The multiple coupling of the signals prevented a precise measurement for each position.

dently with both possible neighboring C atoms to an equally strong degree (Table 1). The observed coupling therefore corresponds to an overlay of the two imaginable C_2 labeling patterns of the polyketide moiety (see Figure 2). This C_2-wise randomization clearly proves that the biosynthesis of **1** involves a free symmetric intermediate, most likely 1,3,6,8-tetrahydroxynaphthalene (**3**, THN), the presumed product of the type III polyketide synthase Fnq6.[20] This intermediate is only later desymmetrized by oxidation, prenylation, *C*- and *O*-methylation. The result is in agreement with earlier studies on naphthoquinones derived from a type III polyketide synthase.[10−12]

Neither the C-6 methyl group directly linked to the naphthoquinone core nor the methoxy group at C-7 showed any additional ^{13}C labeling, suggesting that these substituents are installed by a secondary biosynthetic *C*- or *O*-methylation step, presumably by the *S*-adenosyl-methionine-dependent methyltransferases Fnq9 and Fnq27.[20]

The principal question of this study, however, was the origin of the isoprenoid moiety of FNQ I (**1**). By analysis of the ^2J-coupling constants of the carbon atoms of this part of the molecule (Table 1), the positions of two intact $^{13}C_2$ units and a ^{13}C singlet in each of the two isoprenoid building blocks were established as depicted in Figure 2. Thus, the $^{13}C_2$ units were localized at the positions C-2/C-2Me (^2J = 39.9 Hz), C-3/C-3Me (^2J = 35.3 Hz), C-11/C-12 (^2J = 43.9 Hz), and C-13/C-14 (^2J = 42.2 Hz), while ^{13}C singlets were identified at C-10 and C-15. The labeling pattern thus obtained was in agreement with a biosynthesis of the isoprenoids via the mevalonate pathway (Scheme 1).

To detect a possible additional contribution from the MEP pathway, the NMR spectrum of **1**, after feeding of [2-^{13}C]-

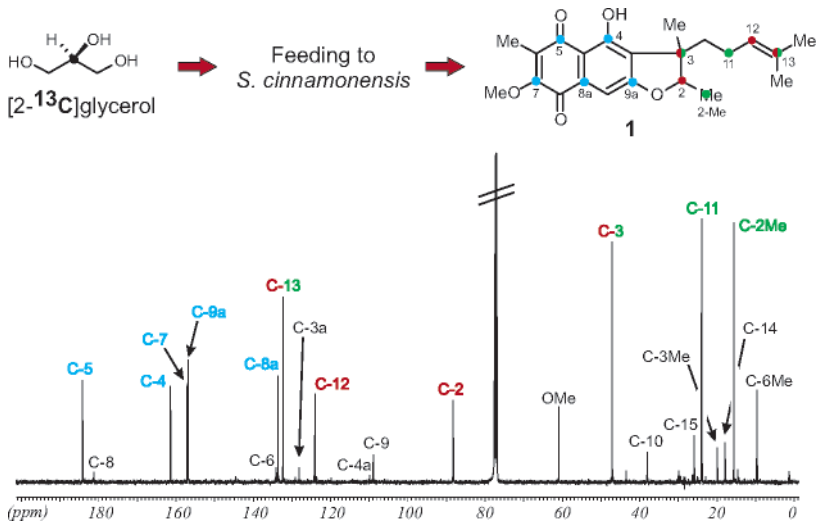


FIGURE 3. Labeling pattern of FNQ I (**1**) derived from feeding of [2-^{13}C]glycerol to *Streptomyces cinnamonensis*. ^{13}C-labeled carbons derived from the polyketide, mevalonate, and MEP pathways are shown in blue, green, and red, respectively.

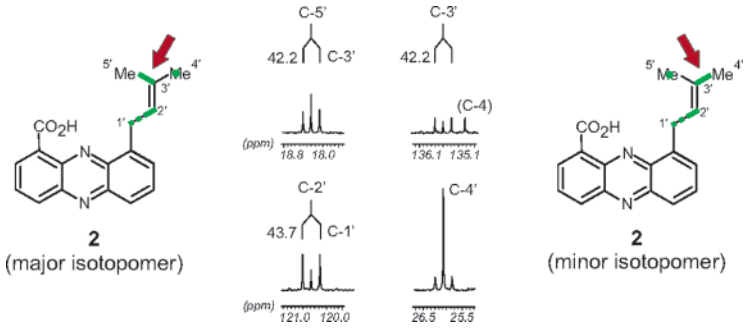


FIGURE 4. Labeling pattern of the prenyl side chain of endophenazine A (**2**) derived from feeding of sodium [$^{13}C_2$]acetate to *Streptomyces cinnamonensis*. ^{13}C-labeled portions are shown in green. The dotted line depicts the expected $^{13}C_2$ unit between C-2′ and C-1′, as suggested by the ^2J coupling of C-2′ (43.7 Hz).

glycerol was examined, as incorporation of [2-^{13}C]glycerol should lead to significantly different isotope patterns depending on the utilization of either the mevalonate pathway or the MEP pathway (Scheme 2).

Again, a good incorporation of the ^{13}C label into **1** was observed (Table 1). The acetogenic naphthoquinone portion of **1** was, as predicted, strongly labeled at positions C-4, C-5, C-7, C-8a, and C-9a (Figure 3). The analysis of the isoprenoid molecular portions revealed a strong enrichment at positions C-3 and C-13 (average 8.9%), which are expected to be labeled both via the mevalonate and via the MEP pathway, but also at positions C-2Me and C-11 (average 6.5%), which are labeled exclusively by incorporation via the mevalonate pathway. This confirmed, on the one hand, that the isoprenoid portion of the molecule was predominantly biosynthesized via the mevalonate pathway (Scheme 2) but, on the other hand, also showed an enrichment in positions C-2 and C-12 (average 1.5%), evidencing an additional contribution from the MEP pathway.

As described above, endophenazine A (**2**) was produced in smaller quantities than FNQ I (**1**). Nevertheless, the feeding experiment with [$^{13}C_2$]acetate allowed a clarification of the biosynthetic origin of the isoprenoid moiety of **2**. ^{13}C NMR spectroscopy of the isolated compound revealed a strong coupling of C-3′ to C-5′ (^2J = 42.2 Hz) and an interaction of C-2′ with C-1′ (^2J = 43.7 Hz, calculated from the satellites of C2′). The signal of C-1′ was not well resolved due to an overlap with impurity signals. The signal of C-4′ appeared, as expected, predominantly as a singlet. These data show that the isoprenoid moiety of **2** is again predominantly derived from the mevalonate pathway.

Unexpectedly, however, a weak coupling of C-4′ to C-3′ was observed, indicating a certain incorporation of a $^{13}C_2$ unit into position C-3′/C-4′, with formation of a minor isotopomeric **2** bearing the ^{13}C singlet at position C-5′ (Figure 4 and Table 2). A similar observation was made for C-10 and (to a smaller degree) for C-15 of **1** (Figure 2). This observation may be explained by a less-than-perfect stereocontrol of the DMAPP-IPP isomerase reaction. This has been observed in earlier feeding experiments in plants[23] and may be even more pronounced for the type II DMAPP-IPP isomerase of streptomycetes.[24]

The biosynthetic gene cluster of furanonaphthoquinone I (**1**), a compound of mixed polyketide/isoprenoid origin, has been found to contain only a single gene of the mevalonate pathway, that is, a putative mevalonate kinase gene.[20] This is in sharp contrast to all previously identified secondary metabolic gene clusters from streptomycetes containing mevalonate pathway genes. In all these clusters, the six genes that jointly direct the

(23) Arigoni, D.; Sagner, S.; Latzel, C.; Eisenreich, W.; Bacher, A.; Zenk, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10600−10605.
(24) Kaneda, K.; Kuzuyama, T.; Takagi, M.; Hayakawa, Y.; Seto, H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 932−937.

TABLE 2. ^{13}C NMR Chemical Shifts and Enrichment Ratios of Endophenazine A (2), Isolated after Feeding of [$^{13}C_2$]Acetate[a]

	[$^{13}C_2$]acetate feeding	
carbon	δ (ppm)	enrichment (%)
1	125.2	
1-COOH	166.3	
2	137.3	
3	130.4	
4	135.1	
4a	143.3	
5a	144.8	
6	128.3	
7	132.0	
8	131.8	
9	139.49	
9a	139.42[1]	
10a	139.39[1]	
1′	30.1	n/a[b]
2′	120.6	3.9
3′	135.7	2.7
4′	26.0	2.2
5′	18.3	3.2

[a] Enrichment ratios were calculated as described in Table 1. [b] The enrichment ratio at C-1′ was not measurable due to an overlap with impurity signals.

biosynthesis of IPP and DMAPP were found to be combined in a single operon of approximately 6.8 kb.[15,16,18,25] We, therefore, originally speculated that in the evolution of the FNQ I producer, the other five mevalonate pathway genes may have become lost and that IPP and DMAPP for FNQ I biosynthesis would now be supplied by the MEP pathway, which is used for primary metabolism in apparently all *Streptomyces* strains.[1] However, our present feeding study unequivocally confirms that the isoprenoid moiety of FNQ I (**1**) is predominantly derived from the mevalonate route. This was first proven by the incorporation pattern of [$^{13}C_2$]acetate, which was in perfect agreement with that expected for the mevalonate pathway (Figure 2 and Scheme 1). Because mevalonate kinase alone cannot direct the biosynthesis of IPP and DMAPP, the missing five genes of the mevalonate pathway must be situated at another locus of the genome. This is, thus, the first time that a gene cluster is identified in streptomycetes, which is responsible for the biosynthesis of a mevalonate-derived secondary metabolite and yet does not contain all genes of the mevalonate pathway. For the identification of gene clusters coding for isoprenoid secondary metabolites, screening of the DNA of *Streptomyces* strains with the highly conserved gene of HMG CoA reductase as a probe has been suggested to be a convenient method.[26] Our results show that this approach is not universally applicable, not even when the origin of the respective metabolites from the mevalonate pathway has been confirmed.

Several earlier studies on the biosynthetic origin of the isoprenoid compounds in *Streptomyces* strains used both single-labeled and double-labeled [^{13}C]acetate as a precusor.[11−13] Following the discovery of the MEP pathway,[1,2] feeding of acetate alone is no longer the method of choice for such studies, because a significant incorporation of this precursor is only achieved via the mevalonate pathway, while incorporation through the MEP pathway (via oxaloacetate) is low. To determine a possible contribution of the MEP pathway to FNQ I biosynthesis, we, therefore, carried out an additional feeding experiment with [2-^{13}C]glycerol as a labeled precursor. As depicted in Scheme 2, incorporation of this precursor via the MEP pathway will result in an enrichment in positions C-2 and C-12 of **1**, while incorporation via the mevalonate pathway should provide an enrichment at C-2Me and C-11. In addition, both pathways equally lead to an incorporation at C-3 and C-13. As a consequence, we found the highest enrichment (8.9%) for the latter positions (Table 1). An enrichment at C-2 and C-12 was also clearly observed (1.5%), though to a smaller extent than at C-2Me and C-11 (6.5%). Therefore, the relative contribution of the two pathways to FNQ I biosynthesis was estimated as 20% from the MEP pathway and 80% from the mevalonate pathway. It should be kept in mind, however, that the relative contribution of both pathways may have been influenced by the external precursor feeding.

An operation of both the mevalonate and the MEP pathway in a single *Actinomyces* strain has been reported in two previous studies.[4,13] Using [U-^{13}C]glucose and [1-^{13}C]glucose as the precursors, it was shown that the primary metabolic menaquinones in these strains were derived from the MEP pathway, while feeding of [^{13}C]acetate had revealed that the secondary metabolites naphterpin or BE-40644 were derived from the mevalonate pathway. Seto et al.[13] concluded that "the nonmevalonate pathway starts to operate at the early stage of the fermentation ... and its contibution is replaced by the mevalonate pathway when the production of secondary metabolites is switched on". This may largely be true for *Streptomyces cinnamonensis*, but our findings prove that at least for a certain time period part of the IPP for secondary metabolite production is supplied via the MEP pathway. Our present study is the first one to show incorporation of an isotope label via the two different isoprenoid pathways into a single secondary metabolite in actinomycetes. In plants, where the mevalonate pathway and the MEP pathway operate in parallel in different compartments of the cell, the contribution of both pathways to specific metabolites has been observed previously.[27−30]

Besides FNQ I (**1**), *Streptomyces cinnamonensis* DSM 1042 produces another secondary metabolite of mixed isoprenoid/nonisoprenoid origin, that is, endophenazine A (**2**, Figure 1). The genes for the biosynthesis of this metabolite are found directly adjacent to the biosynthetic gene cluster of **1**.[20] [$^{13}C_2$]-Acetate was incorporated into the isoprenoid moiety of **2** at a similar rate as into **1**, and the incorporation pattern was perfectly consistent with an incorporation via the mevalonate pathway (Figure 4 and Scheme 1). The low amount of endophenazine A (**2**) formed under the present culture conditions prevented an analysis of the incorporation pattern of [2-^{13}C]glycerol and, therefore, an estimation of the relative contribution of the MEP pathway to endophenazine biosynthesis.

Prenyltransferases usually form a bond between C-1 of the prenyl donor (e.g., DMAPP or GPP) and a carbon, oxygen, or nitrogen atom of the prenyl acceptor. The structure of **1** (Figure 1), by contrast, suggests that C-3, but not C-1, of GPP has been

(25) Hamano, Y.; Dairi, T.; Yamamoto, M.; Kawasaki, T.; Kaneda, K.; Kuzuyama, T.; Itoh, N.; Seto, H. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1627−1635.
(26) Kuzuyama, T.; Takahashi, S.; Dairi, T.; Seto, H. *J. Antibiot. (Tokyo)* **2002**, *55*, 919−923.
(27) Bartram, S.; Jux, A.; Gleixner, G.; Boland, W. *Phytochemistry* **2006**, *67*, 1661−1672.
(28) Kasahara, H.; Hanada, A.; Kuzuyama, T.; Takagi, M.; Kamiya, Y.; Yamaguchi, S. *J. Biol. Chem.* **2002**, *277*, 45188−45194.
(29) Jux, A.; Gleixner, G.; Boland, W. *Angew. Chem., Int. Ed.* **2001**, *40*, 2091−2094.
(30) Hampel, D.; Mosandl, A.; Wust, M. *Phytochemistry* **2005**, *66*, 305−311.

attached to the naphthoquinone nucleus. Structural rearrangements of terpenoid moieties have been reported to occur, for example, in the case of pentalenolactone.[2,31] The results of the present study prove that no such rearrangements occur in FNQ I biosynthesis. The observed incorporation pattern is consistent with the formation of a C−C bond between C-3 of GPP and C-3 of 1,3,6,8-tetrahydroxynaphthalene (THN, **3**, the putative product of the type III polyketide synthase Fnq6 of FNQ I biosynthesis), followed by an attack of C-3-OH of THN (**3**) at the C-2 of the geranyl moiety. Therefore, the prenyltransferase of FNQ I biosynthesis is likely to catalyze a "reverse" prenylation, similar to the recently identified enzyme FgaPT1 of fumigaclavin biosynthesis in *Aspergillus fumigatus* AF 293.[32] Because the incorporation pattern of [$^{13}C_2$]acetate into the polyketide moiety of FNQ I (**1**) proved the existence of a symmetric intermediate, prenylation cannot occur during but only after the formation of this symmetric intermediate (probably THN).

Conclusion

The biosynthetic gene cluster of furanonaphthoquinone I (**1**) does not contain all six genes that are necessary for IPP and DMAPP biosynthesis via the mevalonate pathway. Nevertheless, the present feeding study clearly demonstrates that the mevalonate pathway is the dominant route for the biosynthesis of the isoprenoid portion of the FNQ I molecule, providing appoximately 80% of the isoprenoid precursors. Therefore, the missing five mevalonate pathway genes are expected to reside at one or more other loci of the genome of the producer strain *Streptomyces cinnamonensis* DSM 1042. This is the first proof of a gene cluster for a mevalonate-derived secondary metabolite in *Streptomyces* that does not contain a complete set of genes for the mevalonate pathway.

Using [2-^{13}C]glycerol as a labeled precursor, we were able to prove that also the MEP pathway contributes to the biosynthesis of the isoprenoid portion of **1**, providing approximately 20% of the isoprenoid precursors. This is the first example in actinomycetes that a simultaneous incorporation of an isotope label via the two different isoprenoid pathways into a single secondary metabolite is shown.

The incorporation pattern suggested that FNQ I biosynthesis involves a symmetric polyketide intermediate, probably 1,3,6,8-tetrahydroxynaphthalene (**3**), and that C-3, rather than C-1, of the geranyl side chain is attached to the naphthalene nucleus in a "reverse" prenylation reaction.

Experimental Section

***Streptomyces cinnamonensis* DSM 1042** was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig, Germany, and grown in liquid YMG medium or on solid HA medium.[33] For production of secondary metabolites, the medium described by Sedmera et al.[21] was modified as follows: soy flour (0.3%), NaCl (0.25%), $CaCO_3$ (0.15%), $FeSO_4 \times 7H_2O$ (0.015%), and glycerol (1%) in tap water at pH 7. Sodium [$^{13}C_2$]acetate and [2-^{13}C]glycerol were obtained from Cambridge Isotope Laboratories, Andover, U.S.A.

Feeding of Sodium [$^{13}C_2$]Acetate or [2-^{13}C]Glycerol and Isolation of FNQ I (1) and Endophenazine A (2). *Streptomyces cinnamonensis* DSM 1042 was precultured for 48 h in liquid YMG medium (50 mL) at 30 °C and 190 rpm. Preculture (2.5 mL) was used for inoculating of modified production (50 mL) medium. Sterile sodium [$^{13}C_2$]acetate (50 mg) dissolved in water (adjusted to pH 7) or sterile [2-^{13}C]glycerol (50 mg) diluted in water was added to each flask. A total of 40 flasks (total culture volume 2 L) were cultured in each experiment on a rotary shaker at 30 °C and 190 rpm for 120 h.

For isolation of **1** and **2**, the culture (2 L) was centrifuged with 3500 *g* for 10 min, and the cells were extracted with methanol (400 mL) in the ultrasonic bath for 5 min. The extract was mixed in a separating funnel with sodium acetate buffer (400 mL; 1 M, pH 4) and extracted twice with dichloromethane (250 mL). The combined organic phases were concentrated (to 100 mL), washed twice with aqueous acetic acid (50 mL; 0.1%), and dried over sodium sulfate. After evaporation of the solvent, the two secondary metabolites were isolated by preparative HPLC using a Waters Symmetry C18 column (300 mm × 19 mm) at a flow rate of 12 mL min^{-1} with a linear gradient from 80 to 100% in 10 min (solvent A, water/phosphoric acid 999:1; solvent B, acetonitrile/phosphoric acid 999:1). FNQ I (**1**), was eluted after 8.3 min, while endophenazine A (**2**) had a retention time of 10.2 min. After distillative removal of the acetonitrile of the fractions derived from the chromatographic purification step, the remaining aqueous phases were each exhaustively extracted with ethylacetate. The organic layers were dried using $MgSO_4$, and the solvent was evaporated in vacuo, thus leading to 7 mg of **1** and 1 mg of **2** from the feeding experiments with labeled acetate and to 6 mg of **1** and 0.8 mg of **2** from the feeding experiments with [2-^{13}C]glycerol. The NMR spectroscopic data and the physical properties (UV, EIMS) of FNQ I (**1**) and endophenazine A (**2**) thus isolated were in agreement with those previously published.[21,34]

Acknowledgment. This work was supported by the DFG (SPP 1152, "Evolution of Metabolic Diversity") and the Fonds der Chemischen Industrie (fellowship to T.G. and research funds).

Supporting Information Available: Calculations of the enrichment ratios, spectroscopic methods, and ^{1}H and ^{13}C NMR spectra of unlabeled and labeled (including enlarged sections of the respective most important regions) FNQ I (**1**) and endophenazine A (**2**) can be obtained in the supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0703404

(31) Cane, D. E.; Rossi, T.; Tillman, A. M.; Pachlatko, J. P. *J. Am. Chem. Soc.* **1981**, *103*, 1838−1843.

(32) Unsold, I. A.; Li, S.-M. *ChemBioChem* **2006**, *7*, 158−164.

(33) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*, 2nd ed.; John Innes Foundation: Norwich, U.K., 2000.

(34) Krastel, P.; Zeeck, A.; Gebhardt, K.; Fiedler, H.-P.; Rheinheimer, J. *J. Antibiot. (Tokyo)* **2002**, *55*, 801−806.