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Biosynthetic Precursors of Vitamin K as Growth Promoters for *Bacteroides melaninogenicus*

D. J. ROBINS, R. B. YEE, AND RONALD BENTLEY

Department of Biochemistry, Faculty of Arts and Sciences, and Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

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The growth of a vitamin K-requiring strain of *Bacteroides melaninogenicus* was promoted by some postulated and proven biosynthetic precursors of bacterial menaquinones, 1,4-dihydroxy-2-naphthoic acid, shikimic acid, chorismic acid, and 4(2'-carboxyphenyl)-4-oxobutyric acid. Growth of the organism with [2',4-$^{14}C_2$]-4(2'-carboxyphenyl)-4-oxobutyric acid as the vitamin K replacement gave rise to a mixture of radioactive menaquinone-9 and menaquinone-10; the dilution factor for this incorporation was 1.8.

For many strains of the fastidious anaerobe *Bacteroides melaninogenicus*, the following growth factors are required: peptides, hemin, and vitamin K (13, 20, 21). (The term vitamin K includes vitamin K_1 and the various vitamins of the K_2 series. By modern nomenclatural practice [11], these materials will be referred to, respectively, as phylloquinone and menaquinones. Terms such as "vitamin K requirement" will, however, be used when a general description is necessary.) In recent work, Lev and his colleagues (23) have shown that sodium succinate can replace the requirement for hemin in the presence of vitamin K, and succinate can also partially replace the vitamin K requirement when hemin is present. Vitamin K acts, at least in part, by stimulating biosynthesis of phosphosphingolipids, and radioactivity from labeled succinate is incorporated into ceramide phosphorylethanolamine and ceramide phosphorylglycerol (23, 24).

Of the naturally occurring forms of vitamin K, phylloquinone (Fig. 1A) was reported to be more effective than menaquinone (Fig. 1B), but this result was possibly due to a lower solubility of the menaquinone type (21). A number of other compounds have also been shown to replace the normal vitamin K requirement. With the exception of the previously described action of succinate, all of the effective growth promoters presently known are naphthalene compounds with the common structural feature of an oxygen function (quinone, hydroxyl, or carboxyl) at the 1 position (13, 21). Chemical substituents in the second ring impair utilization of the compound, and substances containing only a six-membered ring (e.g., benzoquinone, salicylic acid, phthalic acid, and 4-phenylbutyric acid) cannot replace the naphthalene system (13). Thus, ubiquinones (Fig. 1C), despite their many resemblances to menaquinones, fail to promote growth (12). Vitamin E (DL-α-tocopherol, Fig. 1D) was originally reported to have no stimulating effect (21), but later experiments have shown that both α-tocopherol and its quinone are effective as growth factors (26). These compounds, although essentially benzenoid in nature, have a second ring system; however, this second ring is heterocyclic and contains oxygen.

In view of our interest in menaquinone biosynthesis (6, 7, 27, 28), it was decided to attempt a quantitative study of the effect of a range of possible growth promoters for *B. melaninogenicus*, particularly those implicated in menaquinone biosynthesis. Interest was mainly centered on 1,4-dihydroxy-2-naphthoic acid, a postulated naphthalenoid biosynthetic intermediate for menaquinones (7), together with the known non-naphthalenoid precursors of bacterial menaquinones, shikimic acid (6, 8, 9), and 4(2'-carboxyphenyl)-4-oxobutyric acid (7, 10), which contain only a single carbocyclic ring of six atoms. The structures and postulated biosynthetic relationships of these compounds are shown in Fig. 2.

MATERIALS AND METHODS

A phylloquinone-dependent strain of *B. melaninogenicus* was obtained from M. Lev of the Albert Einstein College of Medicine, Bronx, N.Y. Stock cultures of the organism were maintained at room temperature in the lyophilized state.

Compounds were assayed for growth-promoting

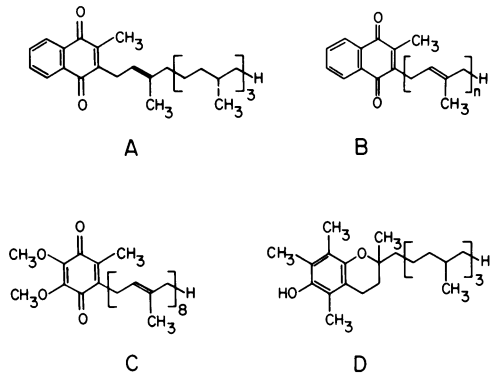

FIG. 1. *Compounds tested as growth promoters for B. melaninogenicus. (A) Phylloquinone, (B) menaquinone, (C) ubiquinone-8, (D)* DL-*α-tocopherol.*

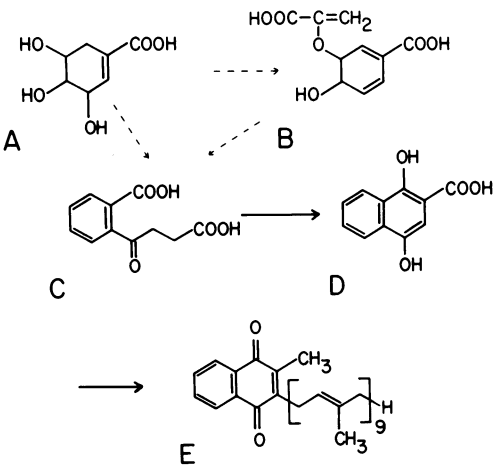

FIG. 2. *Relationship between the compounds tested as growth promoters for B. melaninogenicus and their role in menaquinone biosynthesis. (A) Shikimic acid, (B) chorismic acid, (C) 4(2'-carboxyphenyl)-4-oxobutyric acid, (D) 1,4-dihydroxy-2-naphthoic acid, (E) menaquinone-9.*

ability by a procedure similar to that used by Lev (21). The basal growth medium was composed of 3% Trypticase (BBL), 0.3% yeast extract (Difco), 0.5% NaCl, and 5×10^{-4}% hemin. The pH was adjusted to 7.4 with NaOH. The medium was distributed in 5-ml portions in culture tubes (18 by 100 mm) and autoclaved at 121 C for 15 min. Freshly autoclaved medium was used for all experiments. Solutions of the test compounds were made in water or ethanol, sterilized by filtration through 0.22-μm membrane filters (Millipore Corp.), and added to the basal medium to give final concentrations of 10^{-3} to 10^{-8} M. When the compounds were readily water soluble (for example, materials classified as being in group 4), concentrations up to 10^{-2} M were used. In cases where ethanol was present, the final concentration of ethanol was always $<2\%$. For routine growth, phylloquinone was added to the freshly autoclaved medium to give a final concentration of 10^{-6} M. Each tube of medium was inoculated with a loopful (about 0.01 ml) of a 2-day-old bacterial culture and incubated anaerobically in 90% H_2-10% CO_2 in Brewer jars at 37 C. Growth was estimated turbidimetrically by measuring the absorbance of the cultures with a Coleman Junior spectrophotometer model 6A at 690 nm. Control tubes without added hemin or phylloquinone, or both, exhibited no growth during the course of the experiments. Because most contaminating microorganisms are aerobes or facultative anaerobes, and many facultative anaerobic bacteria synthesize menaquinones, all tubes showing growth were routinely plated out on blood agar and grown both aerobically and anaerobically to test for contamination.

Chemical compounds. The following compounds were synthesized according to published procedures: 1,4-dihydroxy-2-naphthoic acid (17); 4(2'-carboxyphenyl)-4-oxobutyric acid (29); 1-hydroxy-2-naphthoic acid (4); and 6-methyl-1,4-naphthoquinone (3). Menaquinone-9 (Fig. 2E) and ubiquinone-8 (Fig. 1C) were extracted from *Streptomyces albus* and *Escherichia coli*, respectively (7). Lawsone (2-hydroxy-1,4-naphthoquinone) was a synthetic sample (14), and flaviolin (2,5,7-trihydroxy-1,4-naphthoquinone) was isolated from *Aspergillus niger* by E. McGovern in connection with other work, according to a modification of a published procedure (1). All other compounds were obtained from commercial sources.

RESULTS AND DISCUSSION

Growth curves were obtained for each compound tested over a wide concentration range, all experiments being carried out in duplicate. Although the plots of absorbance against time were not exactly reproducible due to variable lag times, the shapes of the growth curves for a particular growth factor were superimposable within the limits of experimental error. The main variation caused by the variable lag periods was in the amount of growth observed in the first 24-h period with highly effective growth-stimulating compounds (i.e., those of group 1). To make a semi-quantitative comparison of the relative effectiveness of the various compounds tested, experience led to the choice of the extent of growth after 48 h as an appropriate assay, as was also used by Lev (21). The 48-h period usually coincided with maximal absorbance, as can be seen from the representative growth curves shown in Fig. 3. Excellent reproducibility of results was obtained under these conditions. Thus, in over 10 runs using phylloquinone, the absorbance at 48 h varied by less than 15% in all cases. When the growth at this time (as measured by absorbance) was plotted against the concentration of the compound under examination, five separate and distinct categories were apparent. The characteristics of these groups and examples of com-

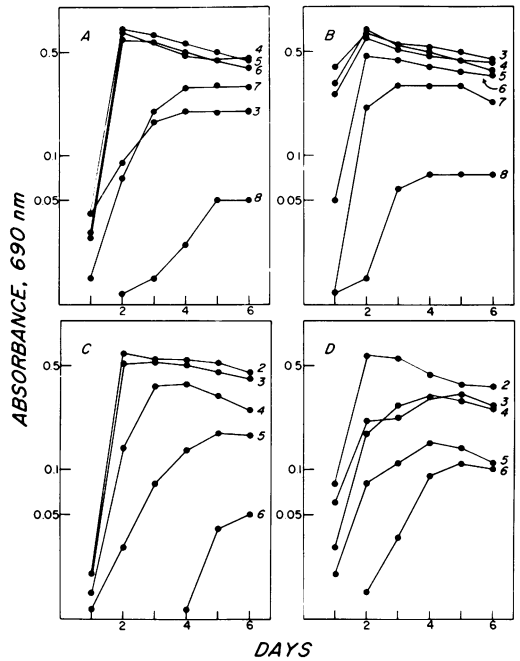

FIG. 3. *Growth curves for B. melaninogenicus in presence of phylloquinone and other promoters. (A) phylloquinone, (B) 1,4-dihydroxy-2-naphthoic acid, (C) shikimic acid, (D) 4(2'-carboxyphenyl)-4-oxobutyric acid. The italic numbers to the right of each plot are the negative logarithms of the concentration tested (2 = 10^{-2} M, etc.).*

pounds within the groups are summarized in Table 1.

No increase in optical density was detected in the assay tubes which contained compounds in group 5. These materials are, therefore, devoid of vitamin K-like activity, and growth does not occur. This "no-growth" condition corresponds to Lev's experience— "cultures inoculated into media containing blood but from which vitamin K had been omitted showed no growth" (23). It should be noted that Lev also reported that growth of this strain of *B. melaninogenicus* occurred, with the formation of elongated rods ("snakes"), to a limited extent in media not supplemented with vitamin K (22); large inoculations were used, and the cells were described as "vitamin K deficient." Under assay conditions similar to those employed in the present study, Lev also presented data showing no increase in absorbance in cultures grown with vitamin K-free medium (21).

The relationships between growth and concentration obtained for those substances in groups 1 to 4 (Table 1) are shown in Fig. 4. On a molar basis, within the limits of experimental error, there is no difference between the growth-promoting capabilities of phylloquinone, menaquinone-9, 2-methyl-1,4-naphthoquinone, 1,4-naphthoquinone, and 1,4-dihydroxy-2-naphthoic acid (Fig. 4A). These compounds, highly effective as growth promoters, are placed in group 1. The interrelationship of the first three compounds in *B. melaninogenicus* was demonstrated earlier by Martius and Leuzinger (25), who showed that when menaquinones are administered the isoprenoid side chain is removed; a new side chain is then added to the residual 2-methyl-1,4-naphthoquinone to form a mixture of menaquinones-9 and -10. In the present work the organism contained from 0.01 to 0.07 mg of menaquinone per g (dry weight) when grown in the presence of a variety of growth promoters. Preliminary evidence confirmed the presence of menaquinones-9 and -10, and a more detailed mass spectrometric analysis of these materials is in progress.

The primary new observation, to which we attach considerable significance, is that excellent growth of *B. melaninogenicus* occurs in the presence of low concentrations of 1,4-dihy-

TABLE 1. *Classification of compounds tested according to growth-promoting capabilities for B. melaninogenicus*

Group	Characteristics	Compounds
1	Highly effective as growth promoters at 10^{-5} M, but inhibitors at $\geq 10^{-3}$ M	Phylloquinone and menaquinone-9 (no inhibition at high concentrations); 2-methyl-1,4-naphthoquinone; 1,4-naphthoquinone; 1,4-dihydroxy-2-naphthoic acid.
2	Moderately effective as growth promoters at 10^{-5} to 10^{-4} M, but inhibitors at $\geq 10^{-3}$ M	Lawsone; 1-hydroxy-2-naphthoic acid; 6-methyl-1,4-naphthoquinone; DL-α-tocopherol and its quinone
3	Weakly effective as a growth promoter at 10^{-5} M, but inhibitor at $\geq 10^{-3}$ M	1-Naphthol
4	Highly effective as growth promoters but only at high concentrations of $\geq 10^{-3}$ M	Shikimic acid; chorismic acid; 4(2'-carboxyphenyl)-4-oxobutyric acid
5	Unable to promote growth over the entire range of 10^{-2} to 10^{-8} M	Benzoic acid; phthalic acid; flaviolin; ubiquinone-8

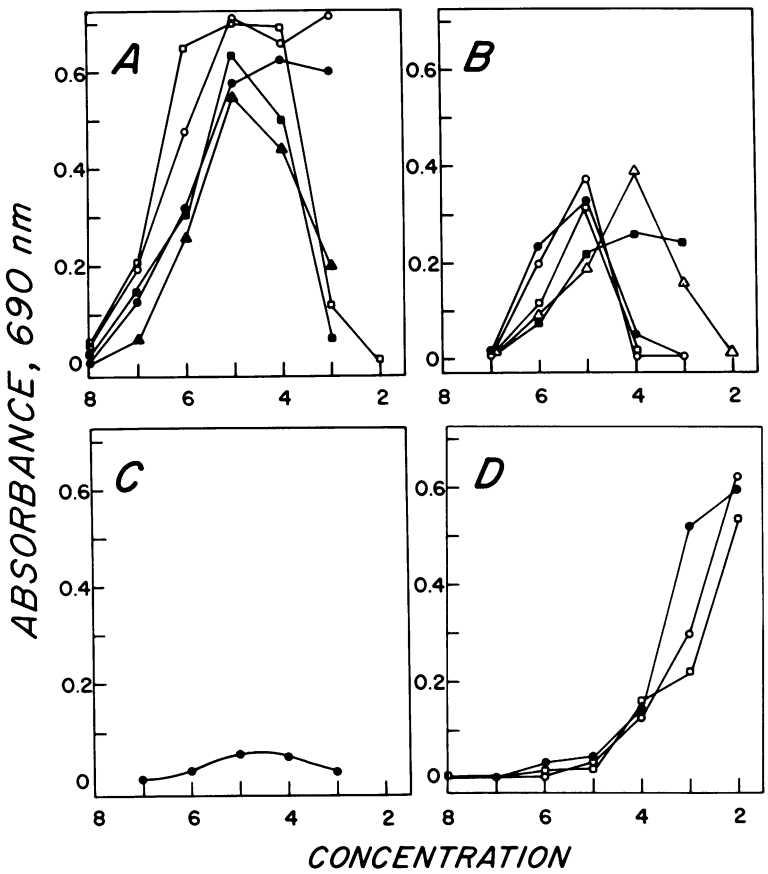

FIG. 4. *Relationship between growth of B. melaninogenicus and concentration of vitamin K replacements. On the abscissa are plotted the negative logarithms of the concentrations of the various compounds tested. (A) Group 1 compounds:* ○, *phylloquinone;* ●, *menaquinone-9;* □, *1,4-dihydroxy-2-naphthoic acid;* ■, *2-methyl-1,4-naphthoquinone;* ▲, *1,4-naphthoquinone. (B) Group 2 compounds:* ○, DL-α-*tocopherol;* ●, DL-α-*tocopherol quinone;* □, *6-methyl-1,4-naphthoquinone;* ■, *lawsone;* △, *1-hydroxy-2-naphthoic acid. (C) Group 3 compound:* ●, *1-naphthol. (D) Group 4 compounds:* ○, *chorismic acid;* ●, *shikimic acid;* □, *4(2'-carboxyphenyl)-4-oxobutyric acid. With the following compounds,* 10^{-2} *M concentrations could not be used since they were highly colored: 1-naphthol, lawsone, 1,4-naphthoquinone, 2-methylnaphthoquinone, and the tocopherols.*

droxy-2-naphthoic acid (Fig. 4A); this replacement, therefore, is also placed in group 1 (Table 1) and is the only nonquinonoid compound in this group. It is of interest because it has been suggested as a late biosynthetic intermediate in the formation of bacterial menaquinones (Fig. 2) (7, 28) and of simpler plant naphthoquinones.

However, direct evidence in support of this suggestion is, so far, lacking. For example, efforts to use 1,4-dihydroxy-2-naphthoic acid to dilute the radioactivity from labeled 4(2'-carboxyphenyl)-4-oxobutyric acid into lawsone in *Impatiens balsamina* were not successful (10). Furthermore, 1,4-dihydroxy-2-naphthoic acid has not been detected in extracts of *I. balsamina* by the combined gas-liquid chromatography-mass spectrometry technique (Grotzinger and Campbell, unpublished observation). Although these experimental observations with *B. melaninogenicus* do not prove that 1,4-dihydroxy-2-naphthoic acid is a biosynthetic precursor, they do provide the first tentative evidence for its participation in the biosynthesis of bacterial menaquinones.

Even though 1,4-naphthoquinone was shown to promote good growth of *B. melaninogenicus* (Fig. 4A), its significance as a true menaquinone precursor is still in doubt. 1,4-Naphthoquinone is a symmetrical compound, and there is uncertainty at the moment whether bacterial menaquinones are biosynthesized via a symmetrical or a non-symmetrical intermediate. It has been demonstrated that the symmet-

rical 1,4-naphthoquinone is a precursor of juglone (18). On the other hand, 1,4-naphthoquinone is not thought to be a true intermediate of the biosynthetically related compounds, alizarin (1,2-dihydroxyanthraquinone) and lawsone, since the biosynthesis of these quinones involves a non-symmetrical intermediate (14, 19).

In the present work, 1-naphthol was observed to have only a very slight effect in promoting the growth of *B. melaninogenicus* (Fig. 4C). It was placed in a separate group 3 of our classification (Table 1) and was the least effective of all the naphthalene derivatives tested, with the exception of flaviolin. 1-Naphthol is postulated by several authors to be involved in menaquinone biosynthesis (15, 16, 18), whereas others maintain that it is not an obligatory intermediate (7). These new observations tend to eliminate 1-naphthol as a direct intermediate in the biosynthesis of bacterial menaquinones.

Three compounds containing only a single ring of six carbon atoms, shikimic acid, chorismic acid, and 4(2'-carboxyphenyl)-4-oxobutyric acid, were equally effective in promoting growth of *B. melaninogenicus* (Fig. 4D) when used at relatively high concentrations; they are classified in group 4 (Table 1). Of these compounds, shikimic acid (6, 8, 9) and 4(2'-carboxyphenyl)-4-oxobutyric acid (7, 10) have been demonstrated by direct tracer experiments to be precursors of bacterial menaquinones, whereas other evidence implicates chorismic acid as well (9, 10).

These three substances are the first monocyclic compounds shown to have a growth-promoting effect on *B. melaninogenicus*. The specificity of the organism is evident since both benzoic and phthalic acids, placed in group 5 (Table 1), were not effective as growth promoters. The ability of shikimate to replace the vitamin K requirement suggests that this bacterium is primarily deficient in the pathways for the biosynthesis of benzenoid aromatics. The growth medium is complex, and benzenoid aromatic amino acids are supplied by trypticase and possibly yeast extract. However, in *Mycobacterium phlei* phenylalanine does not contribute carbon to menaquinone biosynthesis, presumably since the shikimate → phenylalanine pathway is not reversible (7). If this is also the case in *B. melaninogenicus*, the need for vitamin K, or for a precursor in the pathway from shikimate to vitamin K, can be rationalized readily.

Since 4(2'-carboxyphenyl)-4-oxobutyric acid is the only benzenoid aromatic compound so far identified as a menaquinone precursor, the incorporation of radioactivity from a labeled sample of this compound into the menaquinone component of *B. melaninogenicus* was investigated. For this purpose, 119.06 mg of [2',4-$^{14}C_2$] 4(2'-carboxyphenyl)-4-oxobutyric acid, which had been prepared from [7-^{14}C]-phthalic anhydride, as described by Dansette and Azerad (10), was added to 5 liters of growth medium. The sample had a specific activity of 15,100 disintegrations per min per μmol and was present at a concentration of 10^{-4} M. After inoculation with *B. melaninogenicus* and growth for 4 days, the cells (44.5 g wet weight) were worked up for menaquinone as previously described (7). There was obtained 0.2 mg of a mixture of menaquinone-9 (molecular weight 785) and menaquinone-10 (molecular weight 852) in the ratio of 2:1, respectively (ratio determined by mass spectrometry). The total radioactivity in the well-purified menaquinone was 2,060 dpm, i.e., 8,320 disintegrations per min per μmol, assuming an average molecular weight of 807. The incorporation was, therefore, 0.9% of the added radioactivity. Of particular significance is the fact that there was very little dilution of the radioactivity in this incorporation; the dilution factor (specific activity of precursor divided by specific activity of product) was 15,100/8,320 = 1.8. Thus, in a conventional tracer experiment, 4(2'-carboxyphenyl)-4-oxobutyric acid functioned as an excellent precursor of the menaquinones of *B. melaninogenicus*.

All of the other compounds tested which showed moderate growth promoting effects (Fig. 4B; group 2 of Table 1) fall into the category of naphthalene derivatives with an oxygen function in the 1-position, confirming and extending the original findings (13, 21). The exceptions to this classification are the tocopherols tested. These are benzenoid compounds, albeit with a second heterocyclic ring, and would not be expected a priori to function as growth promoters. Their mode of action is uncertain, especially since the structurally related ubiquinone (Fig. 1C) fails to stimulate growth of *B. melaninogenicus*.

Although some of the compounds examined in this work were strongly hydrophobic and had a low solubility in water, this fact did not seem to be a major impediment to their ability to stimulate growth. The use of ethanol to prepare the initial solution did not create any problem since excellent growth was obtained, for example, with phylloquinone. No increased growth was observed when Tween 80 was added in attempts to improve the solubility of phylloquinone, 1-naphthol, and lawsone. With at least two of the group 5 compounds (benzoic and phthalic acids), water solubility was not a

problem. Although a problem in membrane transport might also be postulated, it would presumably be as severe for 4(2'-carboxyphenyl)-4-oxobutyric acid as for benzoic or phthalic acids. Thus, it seems reasonable to argue that group 5 compounds are truly not biosynthetic intermediates.

The role of succinate in *B. melaninogenicus* is not yet completely clear. Cultures without vitamin K, but with blood and the addition of succinate, grow slowly to about 70% of the weight of vitamin K-supplemented cultures. These cells can be maintained in serial subculture from small inocula (23). However, they are regarded as essentially vitamin K-requiring cells; they show an increased growth rate on subculture into vitamin K-containing medium, have the elongated (snake-like) morphology of vitamin-K deficient cells, and lack the odor and mucoid nature of normal cells grown with vitamin K and heme (23). Radioactivity from [2,3-^{14}C]succinate is incorporated into ceramide phosphorylethanolamine and ceramide phosphorylglycerol of *B. melaninogenicus*, although its location in these rather complex molecules (ceramide phosphorylethanolamine with a C_{18} fatty acid at the amide linkage of sphing-4-enine contains, for example, 38 carbons per molecule) is not known (24). This incorporation of radioactivity is stimulated by the presence of vitamin K (24). On balance it must be concluded that these studies do not directly implicate succinate in vitamin K biosynthesis.

Thus, the only nonaromatic compounds which function as true replacements for vitamin K appear to be shikimic acid and chorismic acid. The only benzenoid aromatic compound which replaces vitamin K is 4(2'-carboxyphenyl)-4-oxobutyric acid; this compound, in addition, has been shown to contribute radioactive carbon to the menaquinones of *B. melaninogenicus*. Among non-quinonoid naphthalene compounds, 1,4-dihydroxy-2-naphthoic acid is as effective as phylloquinone or menaquinone in replacing vitamin K, and is considerably more effective than 1-hydroxy-2-naphthoic acid. All of the materials noted here (except 1-hydroxy-2-naphthoic acid) have been implicated in vitamin K biosynthesis in bacterial or plant systems where the vitamin is not required as a growth supplement. Tentatively, we ascribe the effectiveness of 1,4-dihydroxy-2-naphthoic acid to its direct participation in menaquinone biosynthesis in *B. melaninogenicus*.

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