Biosynthesis of Bacterial Menaquinones. Dissymmetry in the Naphthalenic Intermediate†

Ronald M. Baldwin, Clinton D. Snyder, and Henry Rapoport*

ABSTRACT: Biosynthesis of MK-9(II-H_2) in *Mycobacterium phlei* was found to involve only nonsymmetrical naphthalenic intermediates. This was established by feeding [7-^{14}C]-(−)-shikimic acid, synthesized *via* (3*R*,5*R*)-3,4,5-triacetoxycyclohexanone and H^{14}CN, to growing *M. phlei* cultures, and degrading the isolated MK-9(II-H_2) as follows. The side chain of the hydroquinone dimethyl ether was cleaved with osmium tetroxide–periodic acid, and the aldehyde thus formed was converted in three steps to 2-methyl-3-ethyl-1,4-naphthoquinone. Ozonolysis of this quinone, followed by hydrolysis and treatment with diazomethane, gave a 3:2 mixture of methyl *o*-pyruvoylbenzoate and methyl *o*-(2-oxobutyryl)-benzoate, separated by preparative gc. Hydrolysis of the quinoxaline derivative of each diketo ester gave a carboxylic acid which was decarboxylated in the one case to 2-methyl-3-phenylquinoxaline [containing C-1 of MK-9(II-H_2)] and in the other to 2-ethyl-3-phenylquinoxaline (containing C-4), retaining 3.8 and 105%, respectively, of the specific activity of the MK-9(II-H_2). Thus, the C-7 carbon of shikimic acid becomes the C-4 carbon in MK-9(II-H_2). A common scheme is proposed through which the biosynthesis of the shikimate-derived naphthoquinones and anthraquinones may be related.

The biosynthesis of bacterial menaquinones[1] (**9**) has been the subject of intensive study for some time. As a result of precursor feeding experiments, the biochemical sources of all the atoms in the molecule have been identified. Although much has been learned about the genesis of the ring system and the placement of the substituents, the precise nature of any intermediates containing the naphthalene skeleton remains in doubt.

Shikimic acid (**1**) has been shown to provide the aromatic ring and one of the quinone carbonyls (Cox and Gibson, 1966; Campbell *et al.*, 1971) (Scheme I). The remaining three carbons of the naphthalene nucleus arise from C-2, C-3, and C-4 of 2-oxoglutaric acid (**4**) with C-2 becoming the other quinone carbonyl (Campbell *et al.*, 1971). Incorporation of *o*-succinylbenzoic acid (**6**) (Dansette and Azerad, 1970) supports these findings and suggests that the condensation product of shikimic acid and 2-oxoglutaric acid is aromatized before cyclization to a naphthalenoid. What naphthalenic intermediates may be involved, however, is unclear. A number of naphthalene compounds have been tested, *i.e.*, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone (Campbell *et al.*, 1971), and α-naphthol (Leistner *et al.*, 1967; Brown *et al.*, 1968), with generally negative or ambiguous results. Demethylmenaquinone (**8**) has been implicated as the immediate precursor to menaquinone (**9**) by work on the methylating enzyme in *Mycobacterium phlei* (Samuel and Azerad, 1972); however, evidence has been reported (Ellis and Glover, 1968) that in *Escherichia coli*, DMK-8 is not the precursor of MK-8.

In order to shed light on the nature of the unknown naphthalenic intermediates represented by **7** in Scheme I, we decided to examine the possibility of a symmetrically substituted compound by means of the following experiment. Carboxy-labeled [7-^{14}C]shikimic acid would be given to a growing culture of *M. phlei* and the isolated menaquinone [MK-9-(II-H_2)[1]] would be chemically degraded in such a way as to allow determination of the extent of incorporation independently at C-1 and C-4. If biosynthesis were to proceed through a symmetrically substituted compound such as 1,4-naphthoquinone, then the specific activities of C-1 and C-4 would be equal; whereas, in the absence of such an intermediate, one or the other would be preferentially labeled.

A preliminary report of some of these results has been published (Baldwin *et al.*, 1973).

Materials and Methods

General. All reactions were run under a N_2 atomsphere unless otherwise indicated. Nuclear magnetic resonance (nmr) spectra were obtained in $CDCl_3$ containing Me_4Si. Column chromatographies were performed with Camag Kieselgel D-O using the indicated solvents. Radioactive samples were dissolved in a cocktail of 2,5-diphenyloxazole (5 g/l.) and dimethyl-1,4-bis[2-(5-phenyloxazolyl)]benzene (62 mg/l.) in toluene and counted on a Nuclear Chicago Mark I scintillation counter.

The procedures for growth of *M. phlei* ATCC 354 and isolation and purification of MK-9(II-H_2) have already been described (Snyder and Rapoport, 1970). [7-^{14}C]Shikimic acid was added in EtOH to the media at the time of inoculation.

Synthesis of [7-^{14}C]Shikimic Acid (**1**) *and [7-^{14}C]4-epi-Shikimic Acid* (**16**). (3*R*,5*R*)-3,4,5-Triacetoxycyclohexanone (**12**), prepared by the method of Snyder and Rapoport (1973) (544 mg, 2 mmol), was dissolved in a solution of HCN in 95% ethanol (0.10 N, 50 ml) and K^{14}CN (New England Nuclear, 3.5 mCi, 65 mg, 1 mmol) added. Immediately, the solution turned yellow and after an hour an aliquot of HCl–ethanol (1.0 N, 1.00 ml) was added to neutralize the KCN catalyst. Solvents were removed *in vacuo* and the H^{14}CN–ethanol mixture (2.16 mCi, 61%) recovered from a liquid nitrogen cooled trap. The crude cyanohydrin **13** (1.15 mCi, 33%) was treated with $POCl_3$–pyridine as described by Snyder and Rapoport (1973) to obtain a nitrile **14**/**15** mixture purified by column

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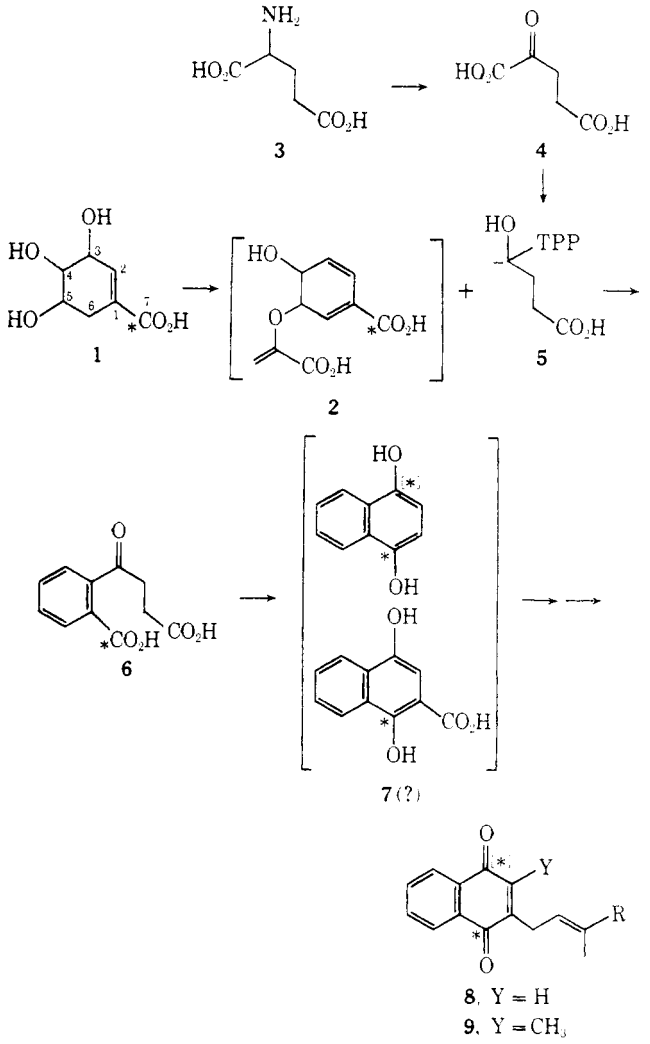
[1] For the systematic nomenclature of menaquinones see IUPAC-IUB Commission (1966).

SCHEME 1: Menaquinone Precursors.



chromatography (eluent, 15% acetonitrile–benzene) (170 mg, 30% mass yield; 0.61 mCi, 17% radio yield). The above cycle was repeated twice with the recovered $H^{14}CN$–ethanol and additional nitrile **14**/**15** (0.183 mCi, 9% radio yield and 0.081 mCi, 5% radio yield) obtained. The three preparations were then pooled and separated by gc (0.75 in. × 12 ft, 5% QF-1 on 60–80 Chromosorb W) to yield the purified isomer **14** (0.29 mCi) and isomer **15** (0.35 mCi). Shikimic acids **1** and **16** were then obtained by separate hydrolysis of nitriles **14** and **15**, respectively, as described by Snyder and Rapoport (1973): yield, **1**, 50%, 4% from $H^{14}CN$; **16**, 65%, 7% from $H^{14}CN$; specific activity, **1** and **16**, 0.50 Ci/mol. One spot was observed on an autoradiogram (cellulose plate developed in butanol–95% ethanol–borate buffer (25 mM $Na_2B_4O_7$), 1:1:1 for **1** (R_F 0.36) and **16** (R_F 0.51)). The physical characteristics of **1** and **16** otherwise agreed closely with nonlabeled samples as previously prepared (Snyder and Rapoport, 1973).

Degradation Sequence. Hydroquinone Dimethyl Ether of MK-9(II-H$_2$) (**17**). This compound was prepared by a published procedure (Bentley *et al.*, 1965), the product being purified by column chromatography with 2% ether–petroleum ether to give an 80% yield: nmr δ 0.80 (d, J = 6 Hz), 1.25 (s), 1.60 (s), 1.68 (s), 1.82 (s), 2.00 (s), 2.35 (s), 3.56 (d, J = 6 Hz), 3.84 (s), 3.86 (s), 5.13 (t, J = 6 Hz), 7.43 (m), 8.05 (m); uv (isooctane) 238 nm (ϵ 83,200), 285 (5530), 295 (5740), 326 (1200).

2-Methyl-1,4-dimethoxynaphthalene-3-acetaldehyde (**18**). This compound was prepared by cleavage of **17** with OsO_4 and HIO_4 as described by Campbell *et al.* (1971), but the aldehyde was isolated by extraction with EtOAc and purified by column chromatography with 6% ether–benzene to yield 60% of pure material after crystallization from ether: mp 105–106°; nmr δ 2.27 (s), 3.73 (s), 3.77 (s), 3.83 (d, J = 2 Hz), 7.35 (m), 9.64 (t, J = 2 Hz).

Anal. Calcd for $C_{15}H_{16}O_3$: C, 73.8; H, 6.6. Found: C, 73.8; H, 6.8.

2-Methyl-3-β-hydroxyethyl-1,4-dimethoxynaphthalene (**19**). 2-Methyl-1,4-dimethoxynaphthalene-3-acetaldehyde (**18**, 360 mg, 1.48 mmol) was dissolved with heating in 15 ml of 95% ethanol, $NaBH_4$ (190 mg, 5 mmol) was added, and stirring was continued for 30 min. Water (15 ml) was then added, the mixture was heated briefly at reflux, then extracted with 200 ml of ether, and the ether was dried over anhydrous $MgSO_4$ and evaporated. For the purpose of degradation, the alcohol was taken without further purification for the next step. The pure compound, mp 67–68°, was obtained by chromatography, eluting with 40% tetrahydrofuran–petroleum ether: nmr δ 2.36 (s, 3 H), 2.86 (s, 1 H), 3.06 (t, J = 6 Hz, 2 H), 3.78 (s), 3.83 (s), 3.78 (t, J = 6 Hz, 7 H), 7.39 (m, 2 H), 7.98 (m, 2 H).

Anal. Calcd for $C_{15}H_{18}O_3$: C, 73.1; H, 7.4. Found: C, 73.0; H, 7.3.

2-Methyl-3-ethyl-1,4-dimethoxynaphthalene (**20**). 2-Methyl-3-β-hydroxyethyl-1,4-dimethoxynaphthalene (**19**, 360 mg, 1.48 mmol) was dissolved in 6 ml of ether, then 1.13 ml (1.48 mmol) of 1.32 M *n*-butyllithium in hexane was added and the mixture was stirred 5 min. A 0.25 M solution of tosyl chloride in ether (6 ml, 1.50 mmol) was added, and after 5 min stirring, 6 ml (1.50 mmol) of 0.25 M lithium aluminum hydride suspension in ether was added and stirred for 2 hr. The mixture was partitioned between 200 ml of ether and 50 ml of 2 N H_2SO_4, and the ether phase was dried and evaporated. The residue was chromatographed with 5% ether–petroleum ether to yield 287 mg (84%) of the pure product as a colorless oil: nmr δ 1.23 (t, J = 7 Hz, 3 H), 2.42 (s, 3 H), 2.85 (q, J = 7 Hz, 2 H), 3.85 (s), 3.92 (s, 6 H), 7.42 (m, 2 H), 8.02 (m, 2 H); uv (isooctane) 215 nm (ϵ 36,800), 237 (73,500), 285 (5200), 295 (5400), 326 (960); mass spectrum *m/e* 230 (P), 215 (base).

Anal. Calcd for $C_{15}H_{18}O_2$: C, 78.2; H, 7.9. Found: C, 78.2; H, 7.9.

2-Methyl-3-ethyl-1,4-naphthoquinone (**21**). 2-Methyl-3-ethyl-1,4-dimethoxynaphthalene (**20**) was oxidized with AgO as previously described (Snyder and Rapoport, 1972), to give, after chromatography with 10% ether–petroleum ether, a 90% yield of yellow crystals: mp 71.5–72° (lit. mp 72°; Fieser and Chang, 1942); nmr δ 1.12 (t, J = 7 Hz), 2.18 (s), 2.68 (q, J = 7 Hz), 7.66 (m), 8.08 (m); uv (isooctane) 242, 248, 260, 269, 325 nm; mass spectrum *m/e* 200 (P, base peak).

Ozonolysis of 2-Methyl-3-ethyl-1,4-naphthoquinone (**21**). Pure oxygen was led from a tank through a pressure-reducing valve to a magnesium perchlorate drying tower and then to the inlet of a Welsbach ozonizer, adjusted to 1 psi pressure, 1 cfh flow rate, and 100 V. The output was passed through a Dry Ice trap to the reaction vessel, which consisted of a small trap with a sintered glass dispersion tube, immersed in an ice bath, then through another Dry Ice trap to a Welsbach Model H80 ozone analyzer; a bypass was incorporated so that the reaction vessel could be introduced without interrupting the ozone flow which was measured by thiosulfate titration to be 0.5 mmol of O_3/min at a concentration of 52 mg/l. Methylene chloride was distilled from P_2O_5 just before use, and the ozonizer was allowed to stabilize for 2 hr. 2-Methyl-3-ethyl-1,4-naphthoquinone (**21**, 200 mg, 1.00 mmol) was dissolved

in 10 ml of methylene chloride in the reaction tube, which was swept with nitrogen and cooled in ice for 20 min. The vessel was then attached into the system and ozonized for 5 min. Nitrogen was passed through Drierite, then 50 ml of methylene chloride in a trap maintained in an ice bath, and finally through the reaction vessel for 60 min, or until the effluent did not turn a KI solution brown. The yellow solution was then transferred to a 50-ml round-bottomed flask with methylene chloride, swept with nitrogen, and let stand in the dark at room temperature for 24 hr. After solvent removal *in vacuo*, the residue (258 mg) was dissolved in dioxane (0.4 ml, distilled from Na), diluted with 0.6 ml of water, and heated with stirring under nitrogen for 30 min at 80°. Evaporation of solvents led to a thick oil (209 mg, 98%) which was dissolved in 2 ml of ether, cooled in an ice bath, treated with 12 ml (2.4 mmol) of 0.24 M diazomethane in ether (Arndt, 1943) at 0°, and immediately evaporated at 0° with a nitrogen stream. The residual bright yellow oil (178 mg) was subjected to preparative glpc (5% OV-17 on AW-DMCS Chrom W, 80–100, 10 ft × 1/4 in., at 180°, 120 ml/min) to yield *methyl o-pyruvoylbenzoate* (**24**), 74 mg, 37%, as yellow crystals: mp 34–35°; nmr δ 2.44 (s, 3 H), 3.77 (s, 3 H), 7.2–8.0 (m, 4 H); uv (isooctane) 218 nm (ϵ 13,500), 238 (6000), 280 (1430), 395 (70); ir (thin film) 1735 and 1705 cm^{-1}; mass spectrum *m/e* 206 (P), 163 (base).

Anal. Calcd for $C_{11}H_{10}O_4$: C, 64.1; H, 4.9. Found: C, 64.3; H, 4.9.

The yield of *methyl o-(2-oxobutyryl)benzoate* (**25**) was 40 mg (18%) as a yellow oil: nmr δ 1.03 (t, J = 7 Hz), 3.00 (q, J = 7 Hz), 3.84 (s), 7.2–8.0 (m); ir (thin film) 1735 and 1710 cm^{-1}; uv (isooctane) 218 nm (ϵ 13,400), 279 (1580), 395 (52); mass spectrum *m/e* 220 (P) and 163 (base).

Anal. Calcd for $C_{12}H_{12}O_4$: C, 65.4; H, 5.5. Found: C, 65.2; H, 5.5.

2-Methyl-3-(o-methoxycarbonylphenyl)quinoxaline (**26**). Methyl *o*-pyruvoylbenzoate (**24**, 73 mg, 0.35 mmol) was dissolved in 0.6 ml of absolute ethanol, then 77 mg (0.71 mmol) of *o*-phenylenediamine (sublimed at 100° (0.5 mm)) was added and the solution left under nitrogen for 18 hr at 25° in the dark. The solution was added to 15 ml of ethyl acetate, and the ethyl acetate phase was washed with 2 × 5 ml of 0.1 N HCl, 3 × 15 ml of water, and 2 × 15 ml of saturated sodium chloride solution, then dried over $MgSO_4$, and evaporated. The residue (97 mg) was chromatographed with 50% ether–benzene to give 81 mg (83%) of quinoxaline **26**: mp 92–93°; nmr δ 2.50 (s, 3 H), 3.60 (s, 3 H), 7.3–7.9 (m, 8 H), 7.9–8.3 (m, 3 H); ir (KBr disk) 1740 cm^{-1}; uv (EtOH) 239 nm (ϵ 38,600), 285 (5190), 318 (9850); mass spectrum *m/e* 278 (P).

Anal. Calcd for $C_{17}H_{14}N_2O_2$: C, 73.4; H, 5.1; N, 10.1. Found: C, 73.0; H, 4.9; N, 10.0.

2-Ethyl-3-(o-methoxycarbonylphenyl)quinoxaline (**27**) was prepared, following the procedure for quinoxaline **26**, from methyl *o*-(2-oxobutyryl)benzoate (**25**): yield, 70%; mp 81–82°; nmr δ 1.25 (t, J = 7 Hz, 3 H), 2.78 (q, J = 7 Hz, 2 H), 3.60 (s, 3 H), 7.3–8.3 (m, 8 H); ir (KBr disk) 1725 cm^{-1}; uv (EtOH) 239 nm (ϵ 38,400), 285 (4800), 318 (9400); mass spectrum *m/e* 292 (P) and 233 (base).

Anal. Calcd for $C_{18}H_{16}N_2O_2$: C, 74.0; H, 5.5; N, 9.6. Found: C, 74.2; H, 5.4; N, 9.5.

2-Methyl-3-(o-carboxyphenyl)quinoxaline (**28**). Quinoxaline ester **26** (75 mg, 0.27 mmol) was dissolved in 3.5 ml (0.7 mmol) of ethanolic 0.2 N KOH and left at room temperature under N_2 for 3 days. The ethanol was evaporated, the residue was dissolved in 1 ml of H_2O, 10 ml of 0.1 N HCl was added, and the mixture was extracted with 3 × 15 ml of ethyl acetate. The combined extracts were washed with 2 × 15 ml of H_2O and 2 × 15 ml of saturated NaCl solution, dried over $MgSO_4$, and evaporated to give 76 mg of residue. Chromatography with ether–benzene–HOAc (60:40:1) gave pure quinoxaline acid **28** in 99% yield: mp 191–192° (lit. mp 184°; Bernatek, 1960); nmr δ 2.35 (s, 3 H), 7.3–7.8 (m, 5 H), 7.8–8.2 (3 H), 11.2 (s, 1 H); ir (KBr disk) 2650–2350, 1700 cm^{-1}; uv (EtOH) 239 nm (ϵ 34,200), 284 (4800), 318 (9350); mass spectrum *m/e* 264 (P).

Anal. Calcd for $C_{16}H_{12}N_2O_2$: C, 72.7; H, 4.6; N, 10.6. Found: C, 73.0; H, 4.3; N, 10.4.

2-Ethyl-3-(o-carboxyphenyl)quinoxaline (**29**) was prepared in a similar fashion by hydrolysis of quinoxaline ester **27**: 81% yield; mp 211–212° dec; nmr ($CDCl_3$/CD_3OD) δ 1.20 (t, J = 7 Hz, 3 H), 2.76 (q, J = 7 Hz, 2 H), 7.3–8.3 (m, 8 H); ir (KBr disk) 2780–2300, 1710 cm^{-1}; uv (EtOH) 239 nm (ϵ 32,000), 284 (4800), 318 (8700); mass spectrum *m/e* 278 (P) and 233 (base).

Anal. Calcd for $C_{17}H_{14}N_2O_2$: C, 73.4; H, 5.1; N, 10.1. Found: C, 73.3; H, 5.0; N, 10.0.

2-Methyl-3-phenylquinoxaline (**30**). Quinoxaline acid **28** (75 mg, 0.27 mmol) was dissolved in 2 ml of THF, 38 mg of copper chromite (prepared according to Lazier (1943)) was added, and the mixture was heated at reflux for 90 min. The solvent was evaporated and the residue was heated at 225° for 10 hr. The oil (35 mg) which distilled to a cold finger was chromatographed with hexane–ether–acetic acid (80:10:10) to yield 30 mg (48%) of 2-methyl-3-phenylquinoxaline (**30**): mp 55.8–56.2° (lit. mp 57°; von Auwers (1917)); nmr δ 2.75 (s, 3 H), 7.4–7.8 (m, 7 H), 8.05 (m, 2 H); uv (isooctane) 239 nm (ϵ 30,400) and 323 (10,000); mass spectrum *m/e* 220 (P).

2-Ethyl-3-phenylquinoxaline (**31**) was prepared in a similar fashion by decarboxylation of quinoxaline acid **29**: yield 37%: mp 41–43°; nmr δ 1.32 (t, J = 7 Hz, 3 H), 3.06 (q, J = 7 Hz, 2 H), 7.4–8.2 (m, 9 H); mass spectrum *m/e* 234 (base, P).

Anal. Calcd for $C_{16}H_{14}N_2$: C, 82.0; H, 6.0; N, 12.0. Found: C, 81.9; H, 6.0; N, 11.9.

2-(α-Hydroxyethyl)-3-(o-carboxyphenyl)quinoxaline lactone (**36**) was obtained in 8% yield from column chromatography of the product from decarboxylation of **29**: mp 209–210°; mass spectrum *m/e* 276 (P) and 232 (base); nmr δ 2.03 (d, J = 6 Hz), 5.6 (q, J = 6 Hz), 7.3–8.2 (m).

Anal. Calcd for $C_{17}H_{12}O_2N_2$: C, 73.9; H, 4.4; N, 10.1. Found: C, 73.8; H, 4.5; N, 10.0.

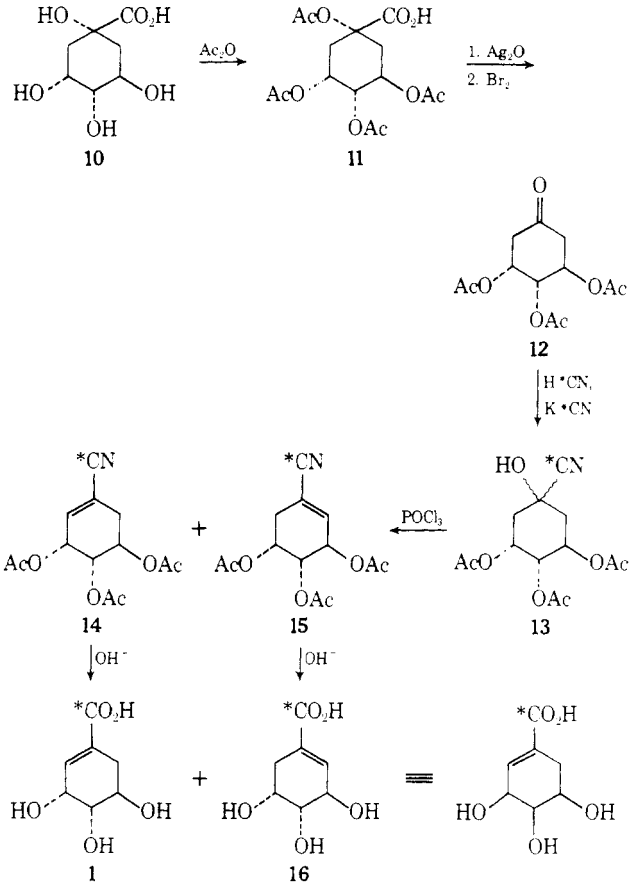
Results

Chemical Studies. Synthesis of [7-^{14}C]Shikimic Acid (**1**) *and [7-^{14}C]4-epi-Shikimic Acid* (**16**). The reported syntheses of shikimic acid (McCrindle *et al.*, 1960; Smissman *et al.*, 1962; Grewe and Hinrichs, 1964; Grewe and Kersten, 1967) proceeded in low yield from costly labeled precursors and resulted in racemic shikimic acid. When our work was begun, details for an enzymatic synthesis of optically active [7-^{14}C]shikimic acid had not yet been published (Scharf and Zenk, 1971) and we wished to develop a synthetic procedure which might be more generally applicable.

Our approach to [7-^{14}C]shikimic acid (Scheme II) elaborated a reported sequence (Grewe and Vangermain, 1965) with modifications because of the unexpected presence of nitrile **15** from which [7-^{14}C]-4-*epi*-shikimic acid (**16**) could be obtained.[2] The advantage of this approach is that by utilizing

[2] A detailed examination of the stereochemical aspects of the sequence presented in Scheme II has been performed (Snyder and Rapoport, 1973).

SCHEME II: Synthesis of [7-^{14}C]Shikimic Acid (**1**) and [7-^{14}C]-4-*epi*-Shikimic Acid (**16**).



quinic acid (**10**) as a starting material the stereochemistry of the future trihydroxycyclohexene system is predetermined assuming no racemization during the sequence. The latter assumption has been verified in that shikimic acid with total retention of optical activity has been obtained from the overall process (Bestmann and Heid, 1971).

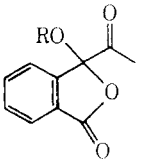
In order to maximize the radio yield of **1**, particular attention was given to the conversions of triacetoxycyclohexanone **12** to cyanohydrin **13**. Originally a large molar excess (*ca.* 15-fold) of cyanide had been utilized; in a radioactive experiment this would result in significant dilution. Therefore, as a measure of the excess cyanide actually necessary to effect this conversion, the dissociation constant of cyanohydrin **13** was determined (Benkeser and Bennett, 1958). While cyclohexanone gave a K_D of 5.2×10^{-3} M (El-Abbady, 1956; Wheeler and Zabicky, 1958; Benkeser and Bennett, 1958), the dissociation constant of cyanohydrin **13** (1.1×10^{-1} M) was considerably greater so that an excess of cyanide is clearly necessary for an optimal yield. For example, a 3:1 ratio of HCN to ketone **12** would give a theoretical cyanohydrin yield of only 45% (based upon ketone, 15% based upon HCN) whereas a 20-fold excess of HCN would achieve an 86% yield. Since maximizing specific activity was of importance, it was decided to use the lower concentration of HCN and accept the lower yield and then to recycle the H^{14}CN–ethanol mixture (purified by distillation) for use in the formation of additional [7-^{14}C]-**13**. The labeled cyanohydrin obtained from several cycles was then dehydrated with $POCl_3$–pyridine to obtain a [7-^{14}C]nitrile **14**/**15** mixture which was separated by preparative gc. Hydrolysis of nitrile **14** led to [7-^{14}C]shikimic acid (**1**) in 4% overall radio yield from HCN while the corresponding [7-^{14}C]-4-*epi*-shikimic acid (**16**) was obtained from nitrile **15**. The purity and homogeneity of these two acids were further determined by autoradiography using a cellulose–borate buffer system known to resolve **1** and **16** (Snyder and Rapoport, 1973).

Unsymmetrical Degradation. There was no published degradation of the menaquinone nucleus which allowed independent counting of C-1 and C-4. A suitable means to accomplish this was found in ozonolysis. Although the ozonolysis of 1,4-naphthoquinone has been reported (Bernatek, 1958), this reaction has seen little application. 1,4-Naphthoquinones undergo "anomalous ozonolysis," which inserts an oxygen between one of the original quinone carbonyls and the ketone functions derived from the double bond, resulting in precisely the type of differentiation of oxidation states of C-1 and C-4 demanded for an unsymmetrical degradation. It should then be possible to remove by decarboxylation that carbon present as a carboxylic acid.

Initially the perhydro derivative MK-9(H_{18}) (Gale *et al.*, 1963), in which the side chain double bonds have been reduced, was intended as the substrate for ozonolysis; however, low and variable yields were experienced with ozonolysis of this compound. The native quinone therefore was reduced to the simplest system retaining nonsymmetry, methyl-3-ethyl-1,4-naphthoquinone (**21**), which has the advantage that both C-1 and C-4 activity can be determined in similar compounds. First MK-9(II-H_2) was converted to the hydroquinone dimethyl ether **17**. The side chain then was cleaved by OsO_4–HIO_4 oxidation to give aldehyde **18** in 60% yield. In addition there was formed a small amount of 2-methyl-3-formyl-1,4-dimethoxynaphthalene (**32**), which was shown to result from periodate oxidation of **18**.

The alcohol obtained quantitatively from sodium borohydride reduction of **18** was converted by sequential treatment in ether with butyllithium, *p*-toluenesulfonyl chloride, and lithium aluminum hydride, to 2-methyl-3-ethyl-1,4-dimethoxynaphthalene (**20**). Finally, oxidative demethylation with argentic oxide gave the desired quinone for ozonolysis, **21**.

Ozonolysis of **21** in methylene chloride at 0°, followed by rearrangement at 25°, produced a 3:2 mixture of diketoanhydrides **22** and **23**. Although these could be characterized spectrally, chromatography on silica gel hydrolyzed them to the corresponding acids. The mixture was therefore hydrolyzed in aqueous dioxane to a mixture of *o*-pyruvylbenzoic acid γ-lactol (**33**; Bernatek, 1960) and the ethyl homolog. The



33, R = H
34, R = CH_3

acids are stable to heat and storage but decompose partly on column chromatography or preparative glpc. Furthermore, the quinoxaline acids **28** and **29**, obtained directly from the acids by reaction with *o*-phenylenediamine hydrochloride in aqueous ethanol, could not be efficiently separated.

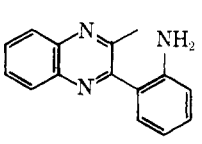
The diketo acids were therefore converted with diazomethane to the diketo esters **24** and **25**, which were readily separated by preparative glpc. The isomeric pseudo-ester structure **34** is ruled out by the reaction with *o*-phenylenediamine in absolute ethanol to give the quinoxaline ester **26**, which was also prepared by diazomethane treatment of quinoxaline acid **28**. Further, a characteristic uv band at 395

TABLE I: Incorporation of [7-^{14}C]Shikimic Acids into MK-9(II-H_2) in *M. phlei*.

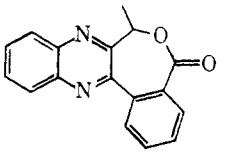
Expt	Precursor	Amount of Shikimate Fed mg	Amount of Shikimate Fed μCi	Incorporation into Bacteria (%)	Incorporation into MK-9(II-H_2) dpm/mmol	Incorporation into MK-9(II-H_2) %
1	[7-^{14}C]Shikimic acid	91	31.8	1.03	1.84×10^6	0.31
2	[7-^{14}C]Shikimic acid	92	35.4	0.62	3.10×10^6	0.30
3	[7-^{14}C]-4-*epi*-Shikimic acid	8.2	23.7	0.27	1.74×10^4	2.5×10^{-3}

nm and the ir absorptions (1735 and 1705 cm^{-1}) are consistent with the diketo structure (Grove and Willis, 1951). The quinoxaline esters thus obtained were simply hydrolyzed to the acids **28** and **29**.

A number of conventional decarboxylation methods failed with this system due to the sensitivity of the alkyl substituent on the quinoxaline ring toward oxidation. Thus, treatment of **28** with bromine and mercuric oxide, or bromine on the silver salt, or thionyl chloride at room temperature resulted in halogenation of the aromatic methyl. A modified Schmidt reaction (Phares, 1951) was successful, but gave amine **35** in only 10% yield.



35



36

Use of various copper compounds (*i.e.*, cuprous and cupric oxide, copper chromite) in quinoline or HMPA at 200–225° did indeed yield the decarboxylated material, 2-methyl-3-phenylquinoxaline (**30**), but invariably contaminated with impurities that were extremely difficult to remove. This problem was solved by heating a solid mixture of carboxylic acid and copper chromite at 225°, from which the decarboxylated product sublimed. This procedure was successful with both homologs; however, in decarboxylation of the ethyl compound (**29**), a by-product quinoxaline cosublimed whose mass spectrum and nmr are consistent with the lactone structure **36**.

The overall yield of the degradation sequence outlined above from MK-9(II-H_2) was 5.5% for 2-methyl- and 1.7% for 2-ethyl-3-phenylquinoxaline.

Biological Studies. Incorporation of [7-^{14}C]Shikimic Acid. Since the incorporation of shikimic acid into MK-9(II-H_2) in *M. phlei* is low (Campbell *et al.*, 1967; Guerin *et al.*, 1970) and because of the need for high activity in the quinone for the purpose of degradation, the conditions for the feeding experiments were examined to optimize the radioactive yield of labeled menaquinone. It was found that addition of carrier shikimic acid (10^{-4} M) increased the incorporation from 0.02–0.04% to 0.1–0.5% using either [G-^{14}C]- or [7-^{14}C]shikimic acid. In addition the dilution of specific activity was *less;* the quinone's specific activity was 0.5–1.1% that of the shikimic acid compared to 0.0008–0.001% when no carrier was added.

After feeding [7-^{14}C]shikimic acid to *M. phlei* for 3 days (Table I), the media was assayed for shikimic acid by isotope dilution, which showed that 75% of the activity in the media was shikimic acid (expt 2 only).

Incorporation of [7-^{14}C]-4-epi-Shikimic Acid. In a test of the specificity of the *M. phlei* enzyme system to tolerate slight changes in shikimic acid structure, the [7-^{14}C]-4-*epi*-shikimic acid (**16**) prepared above was fed to the bacteria (Table I, expt 3). Incorporation into the quinone was very low; also, the other cellular constituents were virtually unlabeled, indicating that this epimer of shikimic acid cannot be used by *M. phlei*.

Degradation of Radioactive MK-9(II-H_2). The radioactive quinone isolated from *M. phlei* fed with [7-^{14}C]shikimic acid was degraded according to Scheme III. The results in Table II show that the ethylquinoxaline **31**, which contains C-4, retains the specific activity of the starting quinone, while the methylquinoxaline **30** (containing C-1) is essentially inactive. The source of the low activity associated with **30** was further investigated by diluting the material from experiment 2 with carrier and subjecting it to column chromatography, recrystallization, and sublimation. The specific activity of the final product, when corrected for dilution, was identical. The most obvious candidate for a possible radioactive impurity is the ethylquinoxaline **31**, and this was specifically excluded by glpc, tlc, and mass spectroscopy.

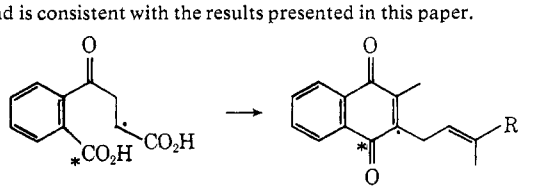
Discussion

The results from this experiment clearly demonstrate that the carboxyl carbon of shikimic acid becomes C-4 in MK-9(II-H_2), and that therefore symmetrically substituted naphthalene compounds such as 1,4-naphthoquinone are not intermediates in the normal biosynthetic pathway in *M. phlei*. The small amount of activity in the C-1 fragment (**30**) may be due to (a) metabolism of shikimic acid and resynthesis to place label at C-1, C-2, C-2′, and/or the aromatic ring, (b) minor intervention of a symmetrical intermediate, or (c) minor duality in the biosynthetic path by which the alkyl substituents are attached in the reverse order.

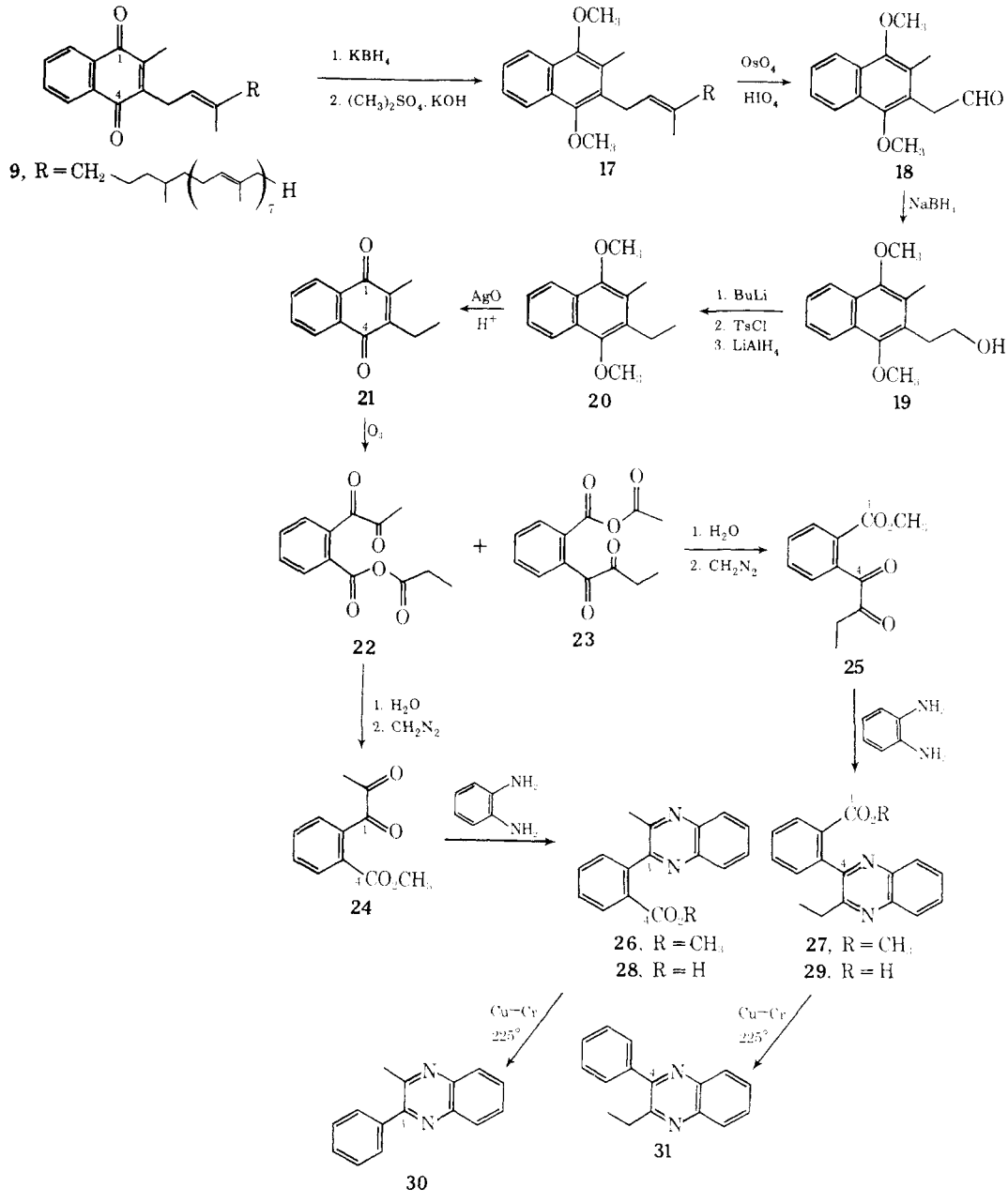
In comparing these data to what is known about the symmetry of other shikimate-derived naphthoquinones[3] (Scheme IV), the carboxyl of shikimic acid becomes C-1 in 2-hydroxy-1,4-naphthoquinone, lawsone (Grotzinger and Campbell, 1972), and biosynthesis does not proceed through a symmetrical intermediate. In the case of 5-hydroxy-1,4-naphthoquinone (juglone) however, the label is equally distributed between C-1 and C-4 (Leistner and Zenk, 1968) indicating a symmetrical intermediate, probably 1,4-naphthoquinone, since this compound is well incorporated.

The crucial question in menaquinone biosynthesis is now the identity of the naphthalenic intermediates. Incorporation

[3] Independent testing for an unsymmetrical intermediate in *M. phlei* has recently come to our attention, demonstrating that *o*-succinylbenzoic acid, labeled differently in separate experiments, was incorporated as shown below (personal communication from P. Dansette, Jan 1973). This is strong evidence for the specific involvement of *o*-succinylbenzoic acid and is consistent with the results presented in this paper.



SCHEME III: Degradation of Menaquinone-9(II-H_2) (9).



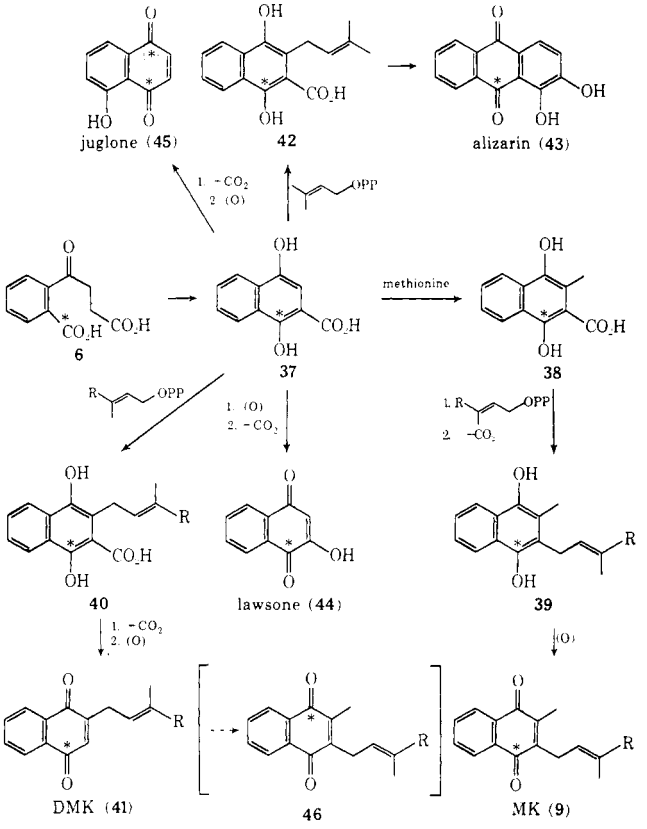
experiments with a number of potential naphthalenoid precursors have failed to unearth a definite intermediate. The case of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone deserves special comment. Both are incorporated into the menaquinone produced by two organisms, *Bacteroides melaninogenicus* (*Fusiformis nigrescens*) (Martius and Leuzinger, 1964), a heterotroph requiring naphthoquinones for growth, and by *Aerobacter aerogenes* 170-44 (Guerin *et al.*, 1970). In the latter case, 1,4-naphthoquinone was incorporated 20 times more efficiently, indicating a more direct involvement in biosynthesis. 2-Methyl-1,4-naphthoquinone was also incorporated into menaquinone in *Staphylococcus aureus* (Hammond and White, 1969), an organism which normally produces 2-methyl-1,4-naphthoquinone. On the other hand, neither quinone is incorporated into menaquinone in wild type *E. coli* or *M. phlei* (Campbell *et al.*, 1971); in these experiments labeled precursor was recovered from within the cells, so that cell penetration is not a difficulty. It would appear, therefore, that neither 1,4-naphthoquinone nor 2-methyl-1,4-naphthoquinone is an obligatory intermediate in the normal biosynthesis of menaquinone, but under certain cir-

TABLE II: Specific Activity of Degradation Products of MK-9(II-H_2).

Compd	Specific Activity[a] in dpm/mmole × 10^3 (% of **17**)	
	Expt 1	Expt 2
9	13.7 (90)	36 (100)
17	15.3 (100)	36 (100)
21	14.9 (97)	36.6 (102)
26	16.6 (108)	39.5 (110)
27	15.7 (103)	39.1 (110)
30 (C-1)	0.6 (3.9)	1.3 (3.6)
31 (C-4)	16.5 (108)	36.5 (102)

[a] Specific activities are corrected for dilution.

SCHEME IV: Proposed Biosynthesis of Some Shikimate-Derived Quinones.



cumstances (*e.g.*, mutant or heterotrophic organisms, or precursor added in excess) some organisms can be induced to accept them for conversion to menaquinone by an aberrant route.

Demethylmenaquinone has been implicated as a menaquinone precursor by the existence of an enzyme system in *M. phlei* capable of methylating demethylmenaquinones of specific structure (Samuel and Azerad, 1972). However, in intact *E. coli* the biosynthetic relationship between DMK-8 and MK-8 appears confused. Using ^{14}C-labeled shikimate as precursor, the specific activity of MK-8 was found to be greater than that of the DMK-8 (Ellis and Glover, 1968). Conversely, with *o*-succinylbenzoic acid as the labeled precursor, DMK-8 had a greater specific activity than MK-8 (Dansette and Azerad, 1970). These apparently contradictory results may reflect multiple pathways or precursor influence.

α-Naphthol has received an inordinate amount of attention since the report of specific incorporation into MK-7 in *Bacillus megaterium* (Leistner *et al.*, 1967). Subsequent work has shown, however, that exhaustive purification of menaquinone removes essentially all the activity (Brown *et al.*, 1968; Guerin *et al.*, 1970), and that the decomposition products are difficult to separate from menaquinone; this was verified on the same strain of bacteria as in the original work.

We have investigated the stability of α-naphthol by incubating [1-^{14}C]-α-naphthol under typical growth conditions and analyzing for remaining α-naphthol by reverse isotope dilution. α-Naphthol was totally destroyed (0.06% survived) after 44 hr at 37° in the sterile media used for growing *M. phlei*, although it was stable (96% survival) in distilled water. In view of this proven instability of α-naphthol, incorporation data in an experiment where most of the activity remaining in the media after growth is not accounted for must be considered suspect. Finally, α-naphthol as a menaquinone precursor is inconsistent with the observation that both quinone oxygens are derived from water and not from molecular oxygen, as would be required for an aromatic hydroxylation (Snyder and Rapoport, 1970). Thus, there appears to be no case for α-naphthol as a menaquinone precursor.

There is no necessity that all shikimate-derived naphthoquinones arise through the same biosynthetic pathway, nor even that the same sequence be used by different organisms for a given quinone. Inconsistencies in the literature may thus simply be a result of the multiplicity of biosynthetic routes available. However, it is possible to propose a hypothesis which provides a unifying framework for the biosynthesis of shikimate-derived naphthoquinones and anthraquinones. This is presented in Scheme IV. 1,4-Dihydroxy-2-naphthoic acid (**37**), the product of a Dieckmann-type cyclization of *o*-succinylbenzoic acid (**6**), is an attractive candidate for the key intermediate (Robins *et al.*, 1970), from which the various shikimate derived aromatic quinones arise. Support for this postulate also is provided by the recent finding that **37** promoted growth of a phylloquinone-dependent strain of *B. melaninogenicus* (Robins *et al.*, 1973).

Thus, the first subsequent step in menaquinone synthesis would be alkylation at the unsubstituted C-2. Methylation would give the methylnaphthoic acid **38** (or its quinone), which could be prenylated and decarboxylated (without the necessary intermediacy of MK-O) to give the menaquinone structure. In demethylmenaquinone biosynthesis, alkylation of **37** at unsubstituted C-2 would still be the first step, but with the prenylating agent, to yield **40**, which on decarboxylation and oxidation lead to DMK (**41**), in those organisms which produce it. This could be tested by determination of the labeling pattern of the MK and DMK in such a case. The scheme predicts the prenyl group to be meta to the labeled carbon (using [7-^{14}C]shikimic acid) in DMK, as compared to ortho in MK. Thus the DMK would have the labeling pattern showing in **41**, while the labeling pattern of MK would be that of **9**, as found in the present work. Also, to the extent that any MK is biosynthesized from DMK, this scheme predicts the labeling pattern of **46**.

Biosynthesis of anthraquinones would then lead through a similar intermediate (*i.e.*, **42**), which after transformation to the anthraquinone (*e.g.*, alizarin, **43**) produces the observed labeling pattern (Leistner, 1973). Oxidative decarboxylation of **37** produces lawsone (**44**) with the appropriate label (Grotzinger and Campbell, 1972), whereas juglone (**45**) is explained as arising by initial decarboxylation to the symmetrical 1,4-naphthoquinone, followed by oxidation at the 5 position to give quinone with equal label at each carbonyl (Leistner and Zenk, 1968).

Based on the single assumption of initial alkylation, whether it be methylation or prenylation, at the unsubstituted C-2, the biosynthesis of an entire subgroup of quinones from a number of organisms can be rationalized by this common scheme. An attractive feature of this hypothesis is that it leads to consequences and predictions subject to experimental test. Of course, the postulated commonality is not necessary, and different biosynthetic pathways may exist in different organisms.

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Biosynthesis of Head-to-Head Terpenes. Carbonium Ion Rearrangements Which Lead to Head-to-Head Terpenes†

C. Dale Poulter,* Oliver J. Muscio, and Robyn J. Goodfellow

ABSTRACT: Hydrolysis of (1*S*,2*R*)-2-[*trans*-2′-(2′′-methylpropenyl)cyclopropyl]propan-2-yl *p*-nitrobenzoate and (1*S*,3*R*)-*trans*-2,2-dimethyl-3-(2′-methylpropenyl)cyclobutyl *p*-toluenesulfonate gave 2-[*trans*-2′-(2′′-methylpropenyl)cyclopropyl]-propan-2-ol, *trans*-2,7-dimethyl-3,6-octadien-2-ol and (*S*)-2,7-dimethyl-2,6-octadien-4-ol. The three alcohols were also obtained by hydrolysis of *trans*-2,7-dimethyl-3,6-octadien-2-yl 3,5-dinitrobenzoate. The chemical properties of the carbonium ion intermediates are discussed in terms of the product and stereochemical studies. Biosynthesis of head-to-head terpenes is compared to the chemical results and a biosynthetic mechanism is presented.

Synthesis of the higher terpenes in the sterol and carotenoid classes requires head-to-head condensation of two head-to-tail polyprenyl pyrophosphates. In squalene (sterol) synthesis the overall process is reductive, while in phytoene (carotenoid) synthesis it is not[1] (see Scheme I). With the discovery of cyclopropylcarbinyl pyrophosphates as intermediates in these two pathways (Epstein and Rilling, 1970; Altman *et al.*, 1972), the transformations can be considered in terms of two distinct steps. The first is the formation of the intermediate **2** by stereospecific insertion of C_1 of a molecule of **1** into the C_2–C_3 double bond of a second **1** (Popjak *et al.*, 1973). The second is the stereospecific rearrangement of the cylopropylcarbinyl intermediates to squalene (**3**) or phytoene (**4**) by rupture of the $C_{1'}$–$C_{2'}$ and $C_{1'}$–$C_{3'}$ cyclopropane bonds followed by bonding

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[1] This aspect has been discussed by Gregonis and Rilling (1974). We will treat phytoene as the first isolatable intermediate; however, an extension of the arguments for squalene to lycopersene is trivial, assuming identical stereochemistry.