INCORPORATION OF SHIKIMATE AND 4-(2'-CARBOXYPHENYL)-4-OXOBUTYRATE INTO PHYLLOQUINONE

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Key Word Index—Zea mays; Gramineae; maize; biosynthesis; phylloquinone.

Abstract—The patterns of incorporation of D-[G-14C]shikimate and variously labelled \(^{14}C\)-4-(2'-carboxyphenyl)-4-oxobutyrate into the naphthoquinone nucleus of phylloquinone by maize shoots have been investigated. The results show that (a) the alicyclic ring and C-7 of shikimate give rise to Ring A and either C-1 or C-4, and (b) the phenyl ring, \(^7\)-carboxy and C-4, and C-2 and -3 of 4-(2'-carboxyphenyl)-4-oxobutyrate give rise to Ring A, C-1 and -4 and C-2 and -3. Radioactivity from \(\alpha\)-[1-14C]naphthol, 1,4-[1,4-14C]naphthoquinone and [Me-14C]menadione is not incorporated into phylloquinone to any significant extent.

INTRODUCTION

It is now clear that the nuclei of bacterial menaquinones (6) and some shikimate-derived simple naphthoquinones (juglone and lawsone) and anthraquinones (alizarin, morindone and purpurin carboxylic acid) of plant origin have many biosynthetic features in common.\(^1\)\(^-\)\(^\)\(^16\) In each group of compounds Ring A and a Ring B carbonyl group, C-4 in the case of menaquinones and C-1 in the case of lawsone,\(^12\)\(^-\)\(^15\) are derived from the alicyclic ring and carboxylic carbon atom respectively of shikimate.\(^1\)\(^-\)\(^11\) Ring B is completed by a C\(_3\) unit provided by C-2, -3 and -4 of \(\alpha\)-oxoglutarate.\(^8\)\(^10\)\(^-\)\(^14\)\(^-\)\(^15\) The key biosynthetic step is believed to be the condensation of either shikimate (1) (favoured by Robins et al.\(^15\)) or chorismate (2) (favoured by Dansette and Azerad\(^15\)) with a C\(_4\) derivative of \(\alpha\)-oxoglutarate, possibly succinyl semialdehyde thiamine pyrophosphate (3),\(^15\) to form 4-(2'-carboxyphenyl)-4-oxobutyrate,\(^1\)\(^6\) which is then modified to form the appropriate quinone (Scheme

12 Grotzinger, E. and Campbell, I. M. (1972) Phytochemistry 11, 675.
In the case of menaquinones, for example, this would involve cyclisation, decarboxylation, isoprenylation and methylation (not necessarily in this order) (Scheme 1). So far there is no evidence to support the suggestion that 4-(2'-carboxyphenyl)-4-oxobutyrate is cyclized to form 1,4-dihydroxy-2-naphthoate (5). The oxidized form of the decarboxylation product of 1,4-dihydroxy-2-naphthoate, 1,4-naphthoquinone, and its methylated product, menadione (a possible menaquinone precursor) are incorporated into naphthoquinones by some, but not all, organisms; however, dilution studies and the requirement that in the final products C-1 and C-4 are biosynthetically asymmetrical make it unlikely that they are natural precursors.

Although the biosynthesis of the naphthoquinone nucleus of bacterial menaquinones has been actively studied, the formation of the nucleus of phyloquinone (7) (an analogue of the menaquinones) by plants has been neglected. The studies that have been carried out have shown that shikimate is incorporated into the naphthoquinone ring system, mevalonate is the precursor of the 3-phytyl group, and the S-methyl group of L-methionine gives rise to the 2-methyl group.

In this communication we report on the pattern of incorporation of shikimate and 4-(2'-carboxyphenyl)-4-oxobutyrate into the nucleus of phyloquinone by maize shoots. We also provide evidence that 2-naphthol, an aberrant precursor of menaquinones in some bacteri...
teria, \(^{18,19,23,24}\) naphthoquinone and menadione cannot be used in the biosynthesis of phylloquinone.

**RESULTS**

**Incorporation of D-[G-\(^{14}\)C]shikimic acid**

As expected, from previous studies, \(^{20}\) the incorporation of radioactivity from this substrate into phylloquinone by maize shoots was low (Table 1). The purified product, however, contained sufficient radioactivity (4770 dpm) for a meaningful degradation to be carried out by the procedure outlined in Scheme 2, pathway A.

![Scheme 2. Chemical degradation of phylloquinone.](image)

The results obtained show that of the total radioactivity incorporated into the phylloquinone molecule 84% was present in Ring A and 16% was present in C-1 + C-4 or C-1 or C-4 (the degradation procedure does not distinguish between C-1 and C-4) (Table 2). In this part of the study, due to an oversight, the distribution of radioactivity between the

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sp. act (µCi/µmol)</th>
<th>Amount administered (µCi)</th>
<th>No. of maize shoots</th>
<th>Phylloquinone* (dpm/µmol)</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-[G-(^{14})C]Shikimate</td>
<td>1.86</td>
<td>10</td>
<td>200</td>
<td>9930</td>
<td>416</td>
</tr>
<tr>
<td>4-(2'-Carboxyphenyl)-4-oxo[(^{14})C]butyrate</td>
<td>0.5</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>4-(2'-Carboxyphenyl)-4-oxo[2,3-(^{14})C]butyrate</td>
<td>2.5</td>
<td>3.3</td>
<td>120</td>
<td>602258</td>
<td>9</td>
</tr>
<tr>
<td>4-(2'-Carboxyphenyl)-4-oxo[2,3'-(^{14})C]butyrate</td>
<td>0.5</td>
<td>1.8</td>
<td>190</td>
<td>85400</td>
<td>13</td>
</tr>
<tr>
<td>4-(2'-Carboxyphenyl)-4-oxo[2,3,4-(^{14})C]butyrate</td>
<td>1.03</td>
<td>9.6</td>
<td>180</td>
<td>102050</td>
<td>22</td>
</tr>
<tr>
<td>2-[1-(^{14})C]Naphthol</td>
<td>19.6</td>
<td>20</td>
<td>300</td>
<td>427</td>
<td>—</td>
</tr>
<tr>
<td>1,4-[(^{14})C]Naphthoquinone</td>
<td>19.6</td>
<td>20</td>
<td>300</td>
<td>40</td>
<td>—</td>
</tr>
<tr>
<td>[(^{14})C]Menadione</td>
<td>9.7</td>
<td>20</td>
<td>300</td>
<td>596</td>
<td>—</td>
</tr>
</tbody>
</table>

* The amount of phylloquinone fell in the range 0.4-0.8 µmol/100 shoots.


alicyclic ring and carboxyl carbon atom of the D-[G-14C]shikimic acid was not determined. However, other workers using D-[G-14C]shikimic acid samples from the same commercial source have quoted an average distribution ratio of 5:4:1, i.e. 15 6% of the radioactivity in the carboxyl carbon atom.

Table 2. Degradation of 14C-phylloquinones by the procedure outlined in scheme 2, pathway A

<table>
<thead>
<tr>
<th>Substrate</th>
<th>1-d-[G-14C]Shikimate expt. (dpm/μmol)</th>
<th>% Radioactivity in phylloquinone</th>
<th>4-(2-[Carboxyl-14C]carboxyphenyl)-4-oxo[2,3,4-14C]butyrate expt. (dpm/μmol)</th>
<th>% Radioactivity in phylloquinone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylloquinone</td>
<td>9930 (83)*</td>
<td>100</td>
<td>1020 (677)*</td>
<td>100</td>
</tr>
</tbody>
</table>
| Phthalic acid  
[- Ring A]  
+ C-1 and -4 | 82 (17) | 99 | 352 (81) | 52 |
| Anthranilic acid  
[= Ring A]  
+ (C-1 and -4)/2 | -- | -- | 39 | 25 |
| BaCO3  
[= (C-1 and -4)/2] | 1.4 | 8 | Phylloquinone-phthalic acid  
= C-2 and -3† | 48 |
| Ring A (by diiff.) | 84 | 2 × (Phthalic acid-  
anthranilic acid  
- C-1 and -4†) | 54 |

* Figures in parenthesis are the specific activity values after dilution with carrier material.
† Chemical degradation of phylloquinone labelled from 4-(2-carboxyphenyl)-4-oxo[2,3,4-14C]butyrate established that C-2 and -3 are equally labelled (Table 4).
‡ Since radioactivity was distributed equally between the carboxy group, C-2, -3 and -4 of the substrate and C-2 and -3 of phylloquinone (see previous footnote), it follows that C-1 and -4 are equally labelled.

Table 3. Incorporation of 4-(2'-carboxyphenyl)-4-oxo[2,3,4-14C]butyrate (2.5 μCi/μmol) into phylloquinone by 7-day-old etiolated and green maize shoots

<table>
<thead>
<tr>
<th>Shoots Type</th>
<th>Conditions of incubation</th>
<th>Substrate (μCi)</th>
<th>Phylloquinone (dpm)</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>Time (hr)</td>
<td>Illumination</td>
<td>(μmol)</td>
<td>(dpm)</td>
</tr>
<tr>
<td>Etiolated</td>
<td>50</td>
<td>18</td>
<td>+</td>
<td>1-5</td>
</tr>
<tr>
<td>Etiolated</td>
<td>50</td>
<td>18</td>
<td>-</td>
<td>1-5</td>
</tr>
<tr>
<td>Etiolated</td>
<td>50</td>
<td>18</td>
<td>+</td>
<td>1-5</td>
</tr>
</tbody>
</table>

* In this incubation the specific activities (dpm/μmol) of plastoquinone-9 and 4-demethylsterols were found to be 3567 and 320 respectively.

Incorporation of 4-(2'-carboxyphenyl)-4-oxobutyric acid

To test the ability of maize to utilize this acid for phylloquinone biosynthesis, 4-(2'-carboxyphenyl)-4-oxo[2,3,4-14C]butyric acid was administered to etiolated maize shoots kept in the dark and to etiolated and green maize shoots kept in the light (Table 3). It was found that, (a) the acid was incorporated with little dilution into phylloquinone in the three experimental systems used (Tables 1 and 3), and (b) the amount of acid incorporated was more or less directly proportional to the terminal pool size of the phylloquinone (Table 3). It is of note that both the plastoquinone-9 and 4-demethylsterols isolated in the course of this experiment were found to be radioactive (Table 3), indicating that some degradative metabolism of the acid had taken place.
In view of the above findings it was decided for the rest of the study to use etiolated shoots exposed to light, since this system gave a good incorporation of radioactivity into phylloquinone and the purification procedures were not hampered by the presence of large quantities of carotenoids.

To determine the manner of the incorporation of this acid into phylloquinone, three species of $^{14}$C-4-(2'-carboxyphenyl)-4-oxobutyric acid were administered to maize shoots. In the experiment in which 4-(2'-carboxyphenyl)-4-oxo-$^{[1-14}$C]butyric acid was administered no radioactivity was incorporated into the phylloquinone molecule (Table 1), thus establishing that the 1-carboxy group is lost. In the remaining two experiments, the phylloquinone, as expected, was radioactive (Table 1). Chemical degradation of the phylloquinone sample labelled with radioactivity from 4-(2'-carboxyphenyl)-4-oxo-$^{[2,3-14}$C]butyric acid by the procedures outlined in Scheme 2, pathways B and C, showed that the greater part of the radioactivity in the molecule was equally distributed between C-2 and -3 (Table 4). The phylloquinone sample labelled from 4-(2'-carboxy-$^{[14}$C]carboxyphenyl)-4-oxo-$^{[2,3-14}$C]butyric acid was not subjected to a full degradation procedure, since the degradation just described had established that the $^{[2,3-14}$C]butyrate portion of the substrate labels C-2 and -3. Instead it was subjected to the partial degradation outlined in Scheme 2, pathway A: the results of which, when considered in conjunction with the previous degradation, demonstrated that the radioactivity was distributed equally between C-1, -2, -3 and -4 (Table 2).

**Table 4. Degradation of $^{14}$C-phylloquinone labelled from 4-(2'-carboxyphenyl)-4-oxo-$^{[2,3-14}$C]butyrate by the procedures outlined in Scheme 2, pathways B and C**

<table>
<thead>
<tr>
<th>Procedure B:</th>
<th>(dpm/μmol)</th>
<th>% Radioactivity in phylloquinone</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-Phylloquinone + carrier</td>
<td>4875</td>
<td>100</td>
</tr>
<tr>
<td>Naphthalene acetic acid</td>
<td>4490</td>
<td>92</td>
</tr>
<tr>
<td>Phthalic acid</td>
<td>195</td>
<td>4</td>
</tr>
<tr>
<td>Malonic acid</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Naphthalene acetic acid-phthalic acid = C-2 and -3</td>
<td>88</td>
<td>41 (27950 dpm)†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Procedure C:</th>
<th>(dpm/μmol)</th>
<th>% Radioactivity in phylloquinone</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-Phylloquinone + carrier</td>
<td>2380 (58950 dpm)</td>
<td>100</td>
</tr>
<tr>
<td>Acetic acid (= C-2)</td>
<td></td>
<td>47</td>
</tr>
<tr>
<td>Naphthalene acetic acid-acetic acid = C-3</td>
<td>41</td>
<td>41</td>
</tr>
</tbody>
</table>

* Insufficient for purification.
† Schmidt-degradation showed all the radioactivity to be present in the carboxyl group, i.e. C-2 of phylloquinone.

**Incorporation of $^{14}$C-naphthalenic compounds**

Over the past four years various colleagues of one of the authors (D.R.T) have administered a range of $^{14}$C-naphthalenic compounds to maize shoots.$^{19,25}$ To complete this report their findings are presented in Table 1. They show that none of the compounds tested, α-naphthol (an aberrant precursor in some organisms), naphthoquinone and menadione, was incorporated to a significant extent. It is of note that, in agreement with the experience of workers using other organisms,$^{26}$ large amounts of radioactivity were found to be associated with the naphthoquinone-containing fractions making the purification of


the phylloquinone extremely difficult. Indeed, it may be that the inclusion of extra purification steps would have resulted in non-radioactive phylloquinone samples.

**DISCUSSION**

Whistance et al.\(^{20}\) reported that shikimate is a precursor of the naphthoquinone nucleus of phylloquinone. In this investigation we have extended this observation and shown that shikimate is either a precursor of Ring A and one of the carboxyl groups of Ring B or a precursor of Ring A and a contributor to both carboxyl groups of Ring B (Table 2). It was not possible, since a G-\(^{14}\)C substrate was used, to establish the absolute relationships between the carbon atoms of shikimate and the nucleus of phylloquinone. However, in view of the fact that the ratio of \(^{14}\)C in the ring and carboxyl group of shikimate (54:1) was similar to the ratio of \(^{14}\)C in Ring B and the carboxyl groups of phylloquinone (51:1), it is reasonable to assume that shikimate is incorporated as a C\(_6\)-C\(_1\) unit: the alicyclic ring giving rise to Ring A and the carboxyl group giving rise to either C-1 or. as in menaquinone biosynthesis,\(^{12}\) C-4.

The source of the C\(_3\) unit required to complete Ring B was not investigated. There seems no reason for doubt, however, that as in the case of menaquinones it will be derived from C-2, -3 and -4 of \(\alpha\)-oxoglutarate.\(^{9,10,14,15}\)

In keeping with the findings of other workers studying the biosynthesis of shikimate-derived naphthoquinones,\(^{9,11,16}\) 4-(2'-carboxyphenyl)-4-oxobutyrate was found to be a most effective precursor of the naphthoquinone ring system of phylloquinone (Table 1). Chemical degradation of \(^{14}\)C-phylloquinone samples labelled from different species of \(^{14}\)C-4-(2'-carboxyphenyl)-4-oxobutyrate, established that the 1-carboxyl group of 4-(2'-carboxyphenyl)-4-oxobutyrate is lost and that the phenyl ring, the 4-carboxyl and 2'-carboxyl groups, and C-1 and -3 of this compound give rise to Ring A. C-1 and -4, and C-2 and -3 respectively of phylloquinone.

None of the naphthalenic compounds tested, \(\alpha\)-naphthol, 1.4-naphthoquinone and menadione, was incorporated into phylloquinone to a significant extent. These findings were not unexpected, since there is a wide variation in the abilities of plants and bacteria to incorporate these compounds into shikimate-derived naphthoquinones.\(^{6,7,10,17-19}\) Indeed, in the light of recent studies,\(^{7,12,13}\) the poor incorporation of 1.4-naphthoquinone and menadione is probably an indication that they are not natural precursors.

The above findings provide the first evidence that the pathway for the biosynthesis of the naphthoquinone nucleus of phylloquinone is similar to the pathways for the biosynthesis of bacterial menaquinones and some shikimate-derived simple naphthoquinones and anthraquinones of plant origin.

**EXPERIMENTAL**

Radiochemicals [2,3-\(^{14}\)C]Succinic acid (22 mCi/mmol), [1.4-\(^{14}\)C]sucinic acid (21 mCi/mmol), \(\beta\)-[\(-^{14}\)C]naphthol (19.6 mCi/mmol) and [\(-^{14}\)C]menadione (9.7 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. \(\alpha\)-[\(-^{14}\)C]Shikimic acid (186 mCi/mmol) and [\(-^{14}\)C]phthalic anhydride (105 mCi/mmol) were purchased from NEN Chemicals GmbH, Dreieich, West Germany. 4-(2'-Carboxyphenyl)-4-oxo[\(-^{14}\)C]butyric (9.5 mCi/mmol), 4-(2'-carboxyphenyl)-4-oxo[2,3-\(^{14}\)C]butyric (0.5 and 2.5 mCi/mmol) and 4-(2'-carboxy-\(^{14}\)C)carboxy-phenyl)-4-oxo[4-\(^{14}\)C]butyric (1.56 mCi/mmol) acids were synthesized from the appropriate mixtures of labelled and unlabelled species of succinic acid and phthalic anhydride by the method of Roser\(^{27}\) as modified by Leitner.\(^{10}\) 4-[\(-^{14}\)C]Carboxy-[\(-^{14}\)C]Carboxyphenyl]-4-oxo-

Incorporation of shikimate

[2,3,4-14C]butyric acid (1.03 mCi/mmol) was prepared by mixing 4-(2-[carboxy-14C]carboxy-phenyl)-4-oxo[4-
14C]butyric acid with 4-(2-carboxyphenyl)-4-oxo[2,3-14C]butyric acid.

1,4-[1,4-14C]Naphthoquinone (19.6 mCi/mmol) was prepared from α-[1-14C]naphthol by the method of
Guérin et al.7

Exposure of maize shoots to 14C-substrates. Shoots of etiolated 7-day-old maize seedlings (Zea mays var. South
African White Horse Tooth; grown in the manner described by Threlfell and Whistance8) were excised at
the node and the cut ends dipped into an aqueous solution of the radiosubstrate (30 ml/100 shoots). They were
then exposed to continuous illumination (800 lm/ft2) for 24 hr at 28°C. In the last series of experiments the 14C-
naphthalenic compounds, because of their poor solubilities in H2O, were administered in aq. 1% dimethylsul-
phoxide.

Extraction and purification of phylloquinone, plastoquinone-9 and 4-demethylsterols. The extraction and sub-
sequent purification of these compounds was carried out as described by Whistance and Threlfall.8,9

Chemical degradation of phylloquinone. The 14C-phylloquinone samples were degraded by procedures that
have been well documented.9,28–30 The choice of the procedure used for any given sample was determined by
the nature of the expected labelling pattern. (a) In the first procedure (Scheme 2, pathway A) the 14C-phyllo-
quinone sample was mixed with 54 μmol of phylloquinone and oxidized with KMnO4.30 The 14C-phthalic acid
formed in this reaction was isolated, assayed for radioactivity, diluted by the addition of 100 μmol of phthalic
acid and converted to anthranilic acid by the Schmidt-degradation. Three modifications made to the published
procedure were, (i) phthalic acid (Rf 0.5) was purified by TLC on silica gel H in EtOH-NH3-H2O (100:12:27),
(ii) anthranilic acid (Rf, 0.3) was purified by TLC on silica gel G in CHCl3-HOAc (19:1), and (iii) BaCO3 formed
in the Schmidt-degradation was assayed for 14C-activity by suspension in a thixotropic gel prepared by adding
700 mg of Cab-O-Sil to 20 ml of our standard scintillation fluid. (b) In the second procedure (Scheme 3, pathway
B) a mixture of 14C-phylloquinone and 220 μmol phylloquinone was reductively acetylated and then degraded
to 1,4-diacetoxy-2-methyl-3-naphthaleneacetic acid by treatment with, (i) osmium tetroxide-periodic acid in diox-
ane, and (ii) neutral KMnO4.9 The next step in this procedure is alkaline H2O2 oxidation of the naphthalene
acetic acid to phthalic acid and malonic acid. In our hands, however, the yield of malonic acid was so low as
to preclude its purification and subsequent assay for 14C-activity. (c) In the third procedure (Scheme 3, pathway
C) 14C-phylloquinone was mixed with 25 μmol phylloquinone and the radioactivity in C-2 determined by a com-
bination of a Kuhn–Roth oxidation and a Schmidt-degradation.8,9

Spectrophotometric determination of phylloquinone, plastoquinone-9, phthalic acid, anthranilic acid and 1,4-di-
acetoxy-2-methyl-3-naphthaleneacetic acid. Phylloquinone and plastoquinone-9 were assayed by the procedures
described by Threlfall and Whistance.8,9 Phthalic acid (λmax 285 nm, in EtOH), anthranilic acid (λmax 335 nm
in aq. 80% MeOH) and naphthalene acetic acid (λmax 286 nm, in EtOH) were assayed using ε values of 1928
(determined experimentally), 40029 and 47409 respectively.

Radioassay. Samples were assayed for radioactivity in a Beckman Liquid Scintillation Spectrometer. Lipid
samples were taken up in 10 ml toluene containing 0.05 g 2,4-diphenyloxazole and 0.003 g 1,4-bis-(4-methyl-5-
phenyloxazol-2-yl)-benzene. Sodium acetate was dissolved in H2O and a 0.2 ml sample added to 10 ml toluene-
methoxyethanol (10:3) containing 0.046 g 2,5-diphenyloxazole, 0.0023 g 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)
benzene and 0.00077 g naphthalene. All counts were corrected for background and instrument efficiency.

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