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1,4-NAPHTHOQUINONE, AN INTERMEDIATE IN JUGLONE (5-HYDROXY-1,4-NAPHTHOQUINONE) BIOSYNTHESIS

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Key Word Index—*Juglans regia*; Juglandaceae; *Impatiens balsamina*; Balsaminaceae; biosynthesis; 1,4-naphthoquinone; juglone; lawsone.

Abstract—Evidence is presented which shows that 1,4-naphthoquinone, a new natural product, is involved in the biosynthesis of juglone in *Juglans regia*.

INTRODUCTION

Even though more than 120 naphthoquinones have been isolated from plants [1], 1,4-naphthoquinone (**1**) itself has been reported to occur in a plant (*Lawsonia inermis* L.) in one instance only. The evidence presented [2] was based on chromatographic data (one solvent system) and an UV spectrum between 260 and 360 nm. Although these observations are clearly insufficient to establish the natural occurrence of 1,4-naphthoquinone, they are in agreement with the fact that a ^{14}C-labelled 1,4-naphthoquinone had been incorporated into lawsone (2-hydroxy-1,4-naphthoquinone) (**2**) in *Impatiens balsamina* L. and into juglone (5-hydroxy-1,4-naphthoquinone) (**3**) in *Juglans regia* L. suggesting that 1,4-naphthoquinone (**1**) is a naturally occurring intermediate in quinone biosynthesis [3].

When the possible rôle of 1,4-naphthoquinone (**1**) in lawsone (**2**) and juglone (**3**) biosynthesis was further assessed by degradation of (**2**) and (**3**) after [^{14}C]-acetate [4] and ^{14}C-shikimic acid feeding [3] respectively, it became apparent that label from [2-^{14}C]-acetate was incorporated into lawsone (**2**) *non*symmetrically (Fig. 1) thus ruling out the intermediacy of a symmetrical compound like 1,4-naphthoquinone (**1**). Hence incorporation of [1,4-^{14}C]-naphthoquinone into lawsone (**2**) [3] was considered to be non-specific [4]. In contrast, label from shikimic acid was incorporated into juglone (**3**) symmetrically [3] (Fig. 1) an observation which had been confirmed by Leduc *et al.* [5]. Thus 1,4-naphthoquinone (**1**) is a possible intermediate in juglone (**3**) biosynthesis but it cannot be an intermediate in lawsone (**2**) biosynthesis. Evidence presented here shows that 1,4-naphthoquinone is a naturally occurring quinone and that it is biosynthesized from the same precursors as juglone (**3**) in *Juglans regia*.

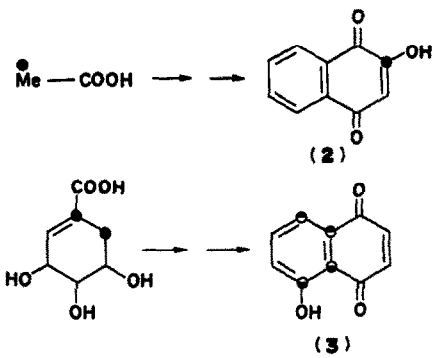

Fig. 1. Nonsymmetric incorporation of label from acetate (2-^{14}C) into lawsone (**2**) [4] and symmetric incorporation of label from shikimic acid (1,6-^{14}C) into juglone (**3**) [3,5].

RESULTS AND DISCUSSION

In a typical experiment a young *Juglans regia* plant was kept in contact with an aqueous solution of 4-(2′-carboxyphenyl)-4-oxobutyrate-[2-^{14}C] (**4b**) (Fig. 2), an established precursor of naphtho- and anthraquinones [6]. After 25 hr the plant was cut into pieces and extracted with ether as described previously [3]. The eth-

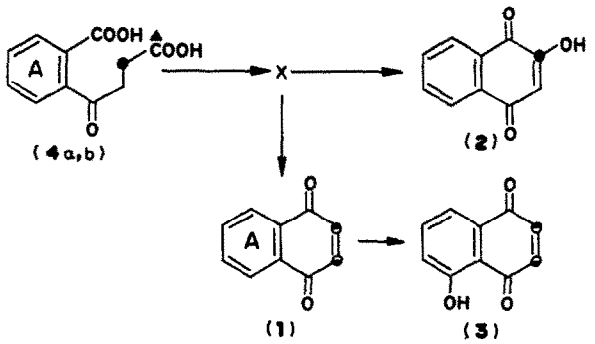

Fig. 2. Biosynthetic relation between 1,4-naphthoquinone (**1**) and its hydroxylated derivatives (**2**,**3**). ▲ indicates label in 4-(2′-carboxyphenyl)-4-oxobutyrate-1-^{14}C (**4a**), ● indicates label in 4-(2′-carboxyphenyl)-4-oxobutyrate-2-^{14}C (**4b**).

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Table 1. Incorporation of 4-(2'-carboxyphenyl)-4-oxobutyrate and 1,4-dihydroxy-2-naphthoic acid into quinones (1), (2) and (3)

Plant	Exp.	Total act. (μCi)	Sp. act. (μCi/μMol)	Position of label	Incorp. (%) into (2) or (3)	Incorp. (%) into (1)
		4-(2'-carboxyphenyl)-4-oxobutyrate (**4a,b**)				
Juglans regia	1	5	1	$2^{14}C$ (**4b**)	juglone 34·1	1·1
Juglans regia	2	5	1	$1^{14}C$ (**4a**)	juglone 0·02	0·0
Impatiens balsamina	3	5	1	$2^{14}C$ (**4b**)	lawsone 5·0	0·0
		1,4-dihydroxy-2-naphthoic acid				
Juglans regia	4	0·1	0·08	$1,4^{14}C$	juglone 6·0	1·2
Impatiens balsamina	5	0·1	0·08	$1,4^{14}C$	lawsone 17·1	0·0

ereal extract was submitted to TLC. The silica gel corresponding in R_f to an authentic sample of 1,4-naphthoquinone was scraped off and eluted with methanol. The eluate was concentrated and injected into a gas chromatograph which was connected to a radiodetector. Simultaneous mass- and radioactivity recording revealed a radioactive substance which eluted from the column with the same retention time as an authentic sample of 1,4-naphthoquinone. This experiment was repeated using a different column. Again a mass-peak corresponding in retention time not only to authentic naphthoquinone but also coinciding with a radioactivity peak appeared. When the same extract was injected into a GC–MS apparatus the peak in question had a fragmentation pattern which was identical with the fragmentation pattern of an authentic sample of 1,4-naphthoquinone. Further corroboration for the occurrence of naphthoquinone in *Juglans regia* was sought. To the ethereal extract of *Juglans regia* plants to which 4-(2'-carboxyphenyl)-4-oxobutyrate-[2-^{14}C] (**4b**) had been applied for 25 hr, carrier naphthoquinone was added. The naphthoquinone was reisolated and purified to constant specific activity (incorporation 1·1%, Table 1). The dinitrophenylhydrazone of 1,4-naphthoquinone was prepared and re-crystallized four times from acetone–H_2O. The specific activity remained constant (10·6; 9·3; 9·9; 10·0 dpm/μMol). Phthalic acid obtained by oxidation of naphthoquinone after 4-(2'-carboxyphenyl)-4-oxobutyrate-[2-^{14}C] feeding was expected to be inactive (Fig. 2). This was in fact observed (Table 2, Exp. 1). When 4-(2'-carboxyphenyl)-4-oxobutyrate-[1-^{14}C] (**4a**) was fed to a *Juglans* plant juglone (**3**) and the reisolated naphthoquinone were inactive (Table 1). The results of experiments 1 and 2 (Tables 1 and 2) are in agreement with a specific incorporation of 4-(2'-carboxyphenyl)-4-oxobutyrate into 1,4-naphthoquinone (**1**) (Fig. 2).

Recently Inouye *et al.* [7,8] isolated several non-aromatic naphthalenes from *Catalpa ovata* G. Don. Assuming that these compounds are likely to be unstable, we considered that the naphthoquinone isolated from *Juglans* might be an artifact derived by oxidation of a pre-aromatic naphthalene. Using the extraction and purification procedure employed by Inouye, however, we were still able to detect 1,4-naphthoquinone (**1**) in extracts of *Juglans regia*.

Likewise 1,4-naphthoquinone also seems to occur in *Juglans cordiformis* Maxim: an ethereal extract of leaves of this plant showed a peak coinciding on GLC with a peak of a sample of authentic naphthoquinone. (**1**) would be present in this plant at a concentration of 4 μg/g dry wt whereas in *Juglans regia* the amount is 110 μg/g dry wt of the aerial parts. The conclusion that (**1**) is an intermediate in the biosynthesis of (**3**) in *Juglans regia* is therefore based on the observation that 1,4-naphthoquinone is a naturally occurring constituent of this plant and that it is derived from the same precursor as is juglone (**3**) *viz.* from 4-(2'-carboxyphenyl)-4-oxobutyrate (**4ab**) (Fig. 2). Moreover 1,4-naphthoquinone is formed within the plant at a time when juglone is also synthesized from (**4b**) and a radioactive sample of

Table 2. Specific activities (dpm/μMol) of labelled quinones obtained from experiments 1, 4 and 5 (Table 1) and their degradation products

Exp.	Quinone	Phthalic or 3-OH phthalic acid	3-OH Benzoic or anthranilic acid
1	Naphthoquinone 85·9 (100)	phthalic acid 0·8 (0·9) [0·0]	—
4	juglone 5·6 (100)	3-OH phthalic acid 5·0 (89·3) [100]	3-OH benzoic acid 2·8 (50·0) [50·0]
	Naphthoquinone 19·6 (100)	phthalic acid 25·4 (129) [100]	anthranilic acid 10·0 (51·0) [50·0]
5	lawsone 44·7 (100)	phthalic acid 45·8 (103) [100]	anthranilic acid 23·8 (53·2) [50·0]

* Figures in parentheses are sp. act. (%) relative to quinones (100%); those in square brackets are sp. act. (%) predicted in degradation products if 4-(2'-carboxyphenyl)-4-oxobutyrate-2^{14}C or $1,4^{14}$C-1,4-dihydroxy-2-naphthoic acid are incorporated specifically and non-randomly.

1,4-naphthoquinone had been incorporated into juglone (3) specifically and non-randomly [3]. According to the hypothetical pathway leading to lawsone (2) in *Impatiens balsamina*, (1) should not occur in this plant (*vide supra*). Indeed GLC analysis of this plant showed that it contains, if at all, less than 0·4 μg 1,4-naphthoquinone per gram dry wt. Reisolation of 1,4-naphthoquinone carrier material which was added to a crude extract of *Impatiens* plants infused with an aqueous solution of 4-(2'-carboxyphenyl)-4-oxobutyrate-[2-^{14}C] for 24 hr led to inactive 1,4-naphthoquinone whereas lawsone (2) which had been isolated from the same plant was highly radioactive (Table 1, Exp. 3). An attempt to detect naphthoquinone in *Lawsonia inermis* was also unsuccessful, despite previous claims [2].

It had been suggested that 4-(2'-carboxyphenyl)-4-oxobutyrate (4) is incorporated into juglone (3) and lawsone (2) via 1,4-dihydroxy-2-naphthoic acid [6,9], a compound which has been isolated from the culture filtrate of an *E. coli* mutant unable to produce Vitamin K_2 [10]. The possible position (X) of 1,4-dihydroxy-2-naphthoic acid in the biosynthetic pathway is shown in Fig. 2. We have prepared this compound by carboxylation of [1,4-^{14}C]-naphthohydroquinone on a semimicroscale (sp. act. 180000 dpm/μmol). Subsequently the acid was applied to *Impatiens balsamina* and *Juglans regia* plants in an aqueous solution containing traces of EtOH. Incorporation rates of 6% (juglone) and 17% (lawsone) were recorded (Table 1). When 1,4-naphthoquinone carrier material was re-isolated from extracts of *Juglans* and *Impatiens* plants after these feedings, the naphthoquinone from *Impatiens* was inactive whereas 1,4-naphthoquinone from *Juglans* was radioactive (Table 1). Labelled quinones obtained after feeding [1,4-^{14}C]-1,4-dihydroxy-2-naphthoic acid were degraded to phthalic and anthranilic acid [11] (lawsone (2) and 1,4-naphthoquinone (1) or to 3-hydroxyphthalic acid and 3-hydroxybenzoic acid [3] (juglone (3)). The data show that [1,4-^{14}C]-dihydroxy-2-naphthoic acid was incorporated specifically and non-randomly (Table 2). Thus it can be assumed that *Juglans regia* is able to decarboxylate externally applied 1,4-dihydroxy-2-naphthoic acid whereas *Impatiens balsamina* plants are likely to hydroxylate and decarboxylate the externally applied acid simultaneously according to a mechanism which has been postulated to be involved in benzoquinone biosynthesis [12]. The data presented are not sufficient, however, to postulate the intermediacy of this acid in lawsone (2) and juglone (3) biosynthesis because the enzymes which catalyze the last steps on the pathways may be able [9] to produce the end products from compounds that are not *in vivo* intermediates.

EXPERIMENTAL

Plant material. Juglans regia plants (4–7-month-old) and *Impatiens balsamina* (6-week-old) were grown from seeds in the greenhouse. Leaves from *Juglans cordiformis* (6-yr-old) were obtained from the Botanical Garden, Bochum, W. Germany and branches from *Lawsonia inermis* (3-yr-old) were taken from a plant grown in the greenhouse.

Isolation of juglone and 1,4-naphthoquinone from Juglans regia. In each case TLC was carried out on acid washed Si gel plates (0·5 mm thickness). Plant material was extracted as described previously[3]. When plant material was intended to be analyzed by GLC, GC–MS or GLC-radiodetector the crude extract was prepurified on TLC using C_6H_6 as a solvent (1,4-naphthoquinone R_f 0·41). The Si gel corresponding in R_f to an authentic sample of 1,4-naphthoquinone was scraped off, eluted with MeOH, the eluate was concentrated and used for analysis. When 1,4-naphthoquinone carrier material was reisolated after applications of radioactive (4a) or (4b) to the plants, 1,4-naphthoquinone (2 mg) was added to the crude extract and juglone and 1,4-naphthoquinone separated using C_6H_6 as a solvent (juglone R_f 0·50, 1,4-naphthoquinone R_f 0·41). Juglone was further purified by TLC using C_6H_6-petrol (2:3) (juglone R_f 0·23). 1,4-Naphthoquinone was further purified by TLC using the same solvent (1,4-naphthoquinone R_f 0·18) and finally C_6H_6-HOAc (4:1) (1,4-naphthoquinone R_f 0·78).

Isolation of lawsone and reisolation of 1,4-naphthoquinone from Impatiens. The aerial parts of *Impatiens balsamina* plants were cut into pieces and refluxed in EtOH (80%, 100 ml) in the presence of a sample of 1,4-naphthoquinone (2 mg). The extract was filtered and extraction repeated for 30 min. The combined extracts were concentrated, some H_2O was added and extracted with petrol repeatedly. To the aq. phase, HCl (2 N, 50 ml) was added and the extract refluxed for 1 hr. Hydrolysate was extracted three times with Et_2O. Lawsone was isolated from the Et_2O using TLC in C_6H_6–HCO_2Et–HCO_2H, 75:24:1 (R_f 0·47); C_6H_6–dioxane–HOAc, 70:25:4 (R_f 0·82); $CHCl_3$–HOAc, 9:1 (R_f 0·82). 1,4-Naphthoquinone was isolated from the petrol fraction by TLC using C_6H_6–HCO_2Et–HCO_2H, 75:24:1 (R_f 0·81) and C_6H_6 (R_f 0·41). When extracts of *Impatiens balsamina* and *Lawsonia inermis* were checked for the presence of naturally occurring 1,4-naphthoquinone by GLC they were worked up as described for *Juglans regia* (*vide supra*).

Dinitrophenylhydrazone of 1,4-naphthoquinone. This derivative was prepared using standard procedures. The product consisted mainly of the monohydrazone (mp 278°; Lit. 278° [13]) but also of a minor amount of the bishydrazone. They were separable by high vacuum sublimation at 220°. On MS the bishydrazone showed a MW peak consistent with its suggested structure. MP 298° (Lit. 298° [13]).

GLC analysis. The gas chromatograph was equipped with a FID. Column: 2, 10 m × 2 mm i.d. packed with 3% SE – 30. Carrier gas: N_2 at a rate of 75 ml/min. Fractions were injected at 80° (Column oven) and then the temp. was raised to 180° at a rate of 10° per min. (1,4-naphthoquinone R_t 6·5 min.).

Radio gas chromatography. A Perkin-Elmer RCG 170 radiodetector connected to a Varian Aerograph 1800 equipped with a stream splitter and a FID were used. Fractions eluted from the GLC were continuously combusted to CO_2 and counted in a 10 ml counting chamber with 4π geometry. The conditions given above were used except that N_2 was replaced by He. Alternatively a 1,20 m × 2 mm i.d. column packed with 5% QF – 1, Chrom WAW DMCS was used (1,4-naphthoquinone R_t 7·0 min.).

GC–MS. A Varian MAT 111 equipped with an all glass system and a column (1·50 m × 2 mm i.d.) packed with 3% SE – 30 on Supelcoport was used. Carrier gas: He at a rate of 25 ml/min. The temp. was programmed as given above. The MS was set to 80 eV. The fragmentation pattern of 1,4-naphthoquinone was identical with the fragmentation pattern of an authentic sample of 1,4-naphthoquinone and the data given by Bowie *et al.* [14].

Degradation of 1,4-naphthoquinone to phthalic acid. This was carried out as described for the oxidative degradation of alizarin [15].

Synthesis of [1,4-^{14}C]-1,4-dihydroxy-2-naphthoic acid. [1,4-^{14}C]-1,4-Naphthoquinone was prepared from [1-^{14}C]-α-naphthol (250 μCi) as described previously [11]. [1,4-^{14}C]-1,4-Naphthoquinone was dissolved in Et_2O and the Et_2O was shaken with a solution of $Na_2S_2O_4$ (5 g/20 ml H_2O) until the Et_2O was decolourized. The ether was evaporated under a stream of N_2 in a thick walled tube. The naphthohydroquinone was dried by applying vacuum to the tube. Naphthohydroquinone (320 mg) and $NaHCO_3$ (210 mg) were added

and thoroughly mixed. An equal vol glycerol was added and the contents stirred again The tube was then evacuated and sealed, and kept at 115° for 6 hr and the tube opened and washed with a 3% soln of $NaHCO_3$. The soln was filtered through cotton and the filtrate washed 4× with Et_2O. The aq. soln was acidified (HCl) and extracted with Et_2O. The Et_2O was washed, dried and evaporated. Residue was recrystallized 2× from HOAc mp 200° dec. (Lit. 201–202°, 186° [16]), yield 2%. The product was chromatographically homogeneous and identical with a sample prepared according to [16].

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