Biosynthesis of Nepodin (2-Acetyl-3-methylnaphthalene-1,8-diol) in *Rumex alpinus L.*

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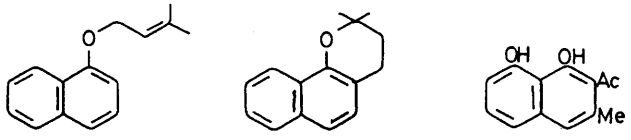
Labelling experiments are described which shed light on the late steps of the biosynthesis of an acetate-derived naphthol (2-acetyl-3-methylnaphthalene-1,8-diol) in *Rumex alpinus L.*

PRENYLATED α-naphthols [1-3] [*e.g.* (I) and (II)] and naphthalene-1,8-diols [4-7] [*e.g.* (III)] are naturally occurring constituents of higher plants. The biosynthetic pathways leading to compounds (I)—(III) are unknown



(I) (II) (III)

although the naphthols (I) and (II) have been implicated in the biosynthesis of shikimate-derived quinones.[2,8] Experiments on the biosynthesis of nepodin (III), which is associated with acetate-derived quinones,[9] are reported here.

RESULTS AND DISCUSSION

Radioactively labelled precursors (50 μCi in every case) were administered to cuttings (3 leaves) of *Rumex alpinus L.*; the material was extracted, and the nepodin (III) was purified to constant specific activity. The incorporation efficiency of [^{14}C]acetate and [^{14}C]malonate ranged from 0·5 to 1·0%. In one experiment (no. 7, Table 2) where [^{14}C]acetate along with a large excess of inactive malonate was fed, the incorporation efficiency was only 0·1%. The specific activity of purified nepodin ranged from 70,000 to 130,000 disint. min^{-1} μmol^{-1}. In experiment 7, however, the specific activity was 13,000 disint. min^{-1} μmol^{-1}. Nepodin was degraded as indicated in Scheme 1. The results of [1-^{14}C]- and [2-^{14}C]acetate feedings showed alternate labelling patterns (Tables 1 and 2, experiments 1 and 2). Acetate units are likely to be linked together by way of a 'starter' acetyl-CoA unit and malonyl-CoA to give nepodin after several further steps. These mechanisms are in agreement with previous reports on the biosynthesis of acetate-derived aromatic compounds (*e.g.* refs. 10—12). The primary reactions

TABLE 1

Specific activities (disint. min^{-1} μmol^{-1}) of nepodin (III) and its degradation products [(IX)—(XIV)] after [1-^{14}C]acetate and [2-^{14}C]acetate feeding. Figures in parentheses are specific activities (%) relative to nepodin (100%); those in square brackets are specific activities (%) predicted in degradation products if the polyacetate pathway operates

Experiment no.	Substrate	Nepodin (III)	(IX)	(X)	(XI)	(XII)	(XIII)	(XIV)
1	[1-^{14}C]Acetate	64·3	39·5	31·4	31·4	0·0	32·5	12·7
		(100·0)	(61·2)	(48·8)	(48·8)	(0·0)	(50·5)	(19·7)
			[66·7]	[50·0]	[50·0]	[0·0]	[50·0]	[16·7]
2	[2-^{14}C]Acetate	178·8	102·3	120·2	102·0	25·3	76·0	0·51
		(100·0)	(57·3)	(67·2)	(57·2)	(14·2)	(42·6)	(0·28)
			[57·2]	[57·2]	[57·2]	[14·3]	[42·9]	[0·0]

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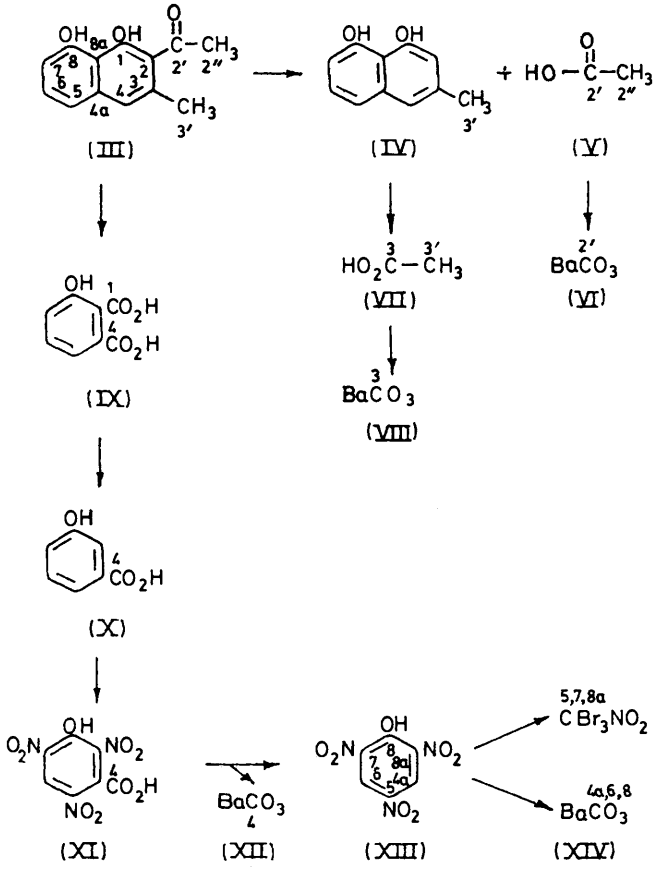
TABLE 2

Comparison of specific activities of the two potential acetate starter groups (columns 2 and 5) of nepodin. Figures in parentheses are specific activities (%) relative to nepodin (100%); those in square brackets are specific activities (%) predicted in degradation products if the polyacetate pathway operates

		Specific activities disint. min^{-1} μmol^{-1} of nepodin and degradation products					
		1	2	3	4	5	6
Experiment no.	Substrates	Nepodin (III)	Acetate (V) C-2′ + C-2″	$BaCO_3$ (VI) C-2′	Diol (IV)	Acetate (VII) C-3 + C-3′	$BaCO_3$ (VIII) C-3
1	[1-^{14}C]Acetate (50 μCi; 3·3 μmol)	80·7 (100)	13·4 (16·6) [16·7]	12·3 (15·2) [16·7]	69·8 (86·5) [83·3]	12·9 (16·0) [16·7]	10·8 (13·4) [16·7]
2	[2-^{14}C]Acetate (50 μCi; 3·3 μmol)	279 (100)	37·8 (13·6) [14·3]	2·0 (0·7) [0·0]	24·9 (89·3) [85·7]	42·5 (15·2) [14·3]	5·6 (2·1) [0·0]
3	[2-^{14}C]Acetate (50 μCi; 8·0 μmol) plus inactive malonate (20·5 μmol)	282 (100)	40·1 (14·2)		252 (89·3)	36·2 (12·8)	
4	[2-^{14}C]Acetate (50 μCi; 8·0 μmol) plus inactive malonate (205 μmol)	604 (100)	74·2 (12·25)		587 (97·1)	73·0 (12·1)	
5	[2-^{14}C]Acetate (50 μCi; 8·0 μmol) plus inactive malonate (2050 μmol)	82·1 (100)	12·9 (15·7)		72·9 (88·8)	10·1 (12·3)	
6	[2-^{14}C]Malonate (50 μCi; 8·0 μmol) plus inactive acetate (20·5 μ mol)	89·3 (100)	12·97 (14·50)		80·6 (90·2)	1·29 (1·44)	
7	[2-^{14}]Malonate (50 μCi; 8·0 μmol) plus inactive acetate (20·5 μmol)	953·0 (100)	158·0 (16·6)		856 (89·8)	49·0 (5·1)	



SCHEME 1 Degradation of nepodin

involved in the biosynthesis of nepodin are therefore clear from the data obtained from experiments 1 and 2.

Insight into the late steps of the biosynthesis of nepodin (III) (Scheme 2) was obtained from experiments 3—7 (Table 2). There are two possible starter acetyl-CoA groups, *viz.* C-2′ and -2″ (path *a*) or C-3 and -3′ (path *b*) of nepodin (III). Alternatively, both pairs of carbon atoms may be derived from starter acetyl-CoA groups. In this case the nepodin molecule would originate by condensation of two short polyketide units (path *c*). Finally, nepodin might arise by *C*-acetylation of 3-methylnaphthalene-1,8-diol (IV) (path d), which is a naturally occurring constituent.[13]

It is known that after [^{14}C]acetate feeding the starter acetyl-CoA group of acetate-derived compounds has a specific activity slightly higher than that of the C_2 units from which the remaining carbon skeleton is derived, because radioactivity enters the latter as malonyl-CoA and is thus further diluted.[14] No difference between the specific activities of the two potential starter groups, or between the specific activities of both potential groups and the average specific activity of the C_2 units from which nepodin is formed, was observed after [^{14}C]acetate feedings. This observation can be explained by the assumption that conversion of acetyl-CoA into malonyl-CoA is fast in comparison with nepodin biosynthesis.

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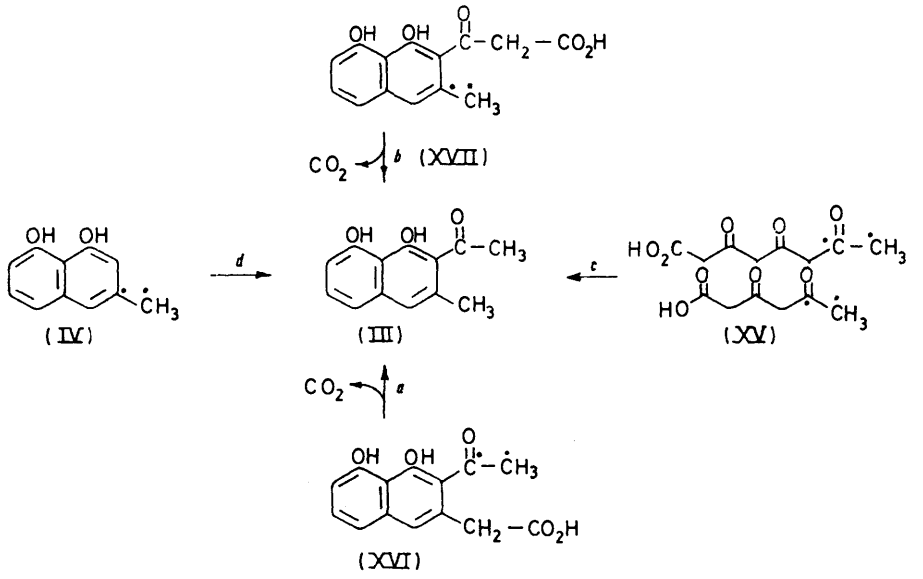
Therefore, [^{14}C]acetate and increasing amounts of inactive malonate were fed (experiments 3—5, Table 2). It was hoped that the malonate would be activated to give malonyl-CoA and thus dilute radioactivity entering that part of nepodin which is not derived from the starter acetyl-CoA group(s). However, in this case no significant difference was observed in the specific activities of the C_2 units of which nepodin is composed. It may be that substrate inhibition of the malonate-activating system prevents malonyl-CoA formation. Alternatively formation of malonyl-CoA from inactive malonate might be much slower than formation of [^{14}C]malonyl-CoA from [^{14}C]acetyl-CoA. In both cases the specific activities of [^{14}C]acetyl-CoA and [^{14}C]-malonyl-CoA would be (almost) equal.

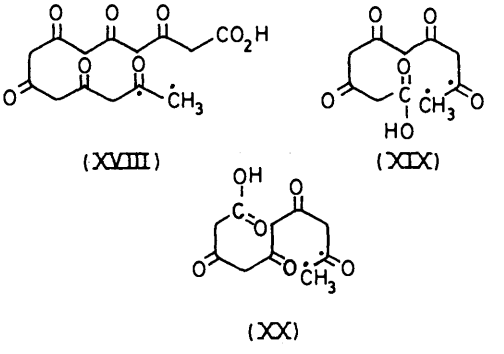
Further experiments, however (nos. 6 and 7, Table 2) showed that C-3 and -3′ are derived from the starter acetyl-CoA unit. When [2-^{14}C]malonate and inactive acetate were fed, a drop in the specific activity of a starter acetyl group was expected. Indeed C-3 and -3′, but not C-2′ and -2″ had a specific activity much lower than the other C_2 units of nepodin. Thus path *b* is likely to operate. However it is also possible that decarboxylation of (XVII) (Scheme 2) takes place at a pre-aromatic stage. These results show that nepodin is derived from one rather than two polyketide chains as implicated in the biosynthesis of sclerin.[15] The folding mechanism of the polyketide chain forming nepodin (III) should operate as depicted in (XVIII). Other folding mechanisms [(XIX) and (XX)] which have been suggested for the formation of naphthalene-1,8-diols [15] do not operate in nepodin biosynthesis.

On the basis of the foregoing experiments path *d* can also be ruled out. If nepodin were formed by *C*-acetylation of (IV), the specific activity of C-2′ and -2″ should drop in experiments 6 and 7. Such was not the case. It is likely that, contrary to path *d*, deacetylation of nepodin yields the diol (IV). Tentative identification of (IV) by co-chromatography with an authentic sample led us to believe that this naphthol occurs in *Rumex alpinus*. Application of a sample of [G-^{14}C]nepodin (916,000 disint. min^{-1}) led to a radioactive compound (900 disint. min^{-1}) which after co-chromatography and co-crystallization with authentic (IV) remained radioactive, whereas chrysophanol (1,8-dihydroxy-3-methylanthraquinone) was inactive. However the occurrence of nepodin deacetylation needs further corroboration.



(XVII) (IV) (III) (XV) (XVI)

SCHEME 2 Hypothetical pathways leading to nepodin; closed circles refer to the starter actyl-CoA group for each possible pathway



(XVIII) (XIX) (XX)

EXPERIMENTAL

Plant Material and Feeding Techniques.—*Rumex alpinus L.* and *Rumex patientia* were obtained from the Botanical Garden, Bochum, West Germany. The plant material was grown outdoors. Tracers were fed to cuttings of *Rumex alpinus* (three young expanding leaves) for 24 h. [G-^{14}C]Nepodin was applied to the leaves in a 0·05% solution of Tween 20.

Preparation of [G-^{14}C]*Nepodin.* Three leaves of *Rumex alpinus* were exposed to an atmosphere containing [^{14}C]-carbon dioxide (500 μCi) in a closed glass container (200

[15] T. Tokoroyama and T. Kubota, *J. Chem. Soc.* (*C*), 1971, 2703.

ml). The leaves were illuminated for 3 h, then nepodin was isolated and purified as described later. After chromatography on silica gel G [benzene–ethyl acetate (3 : 1); nepodin R_F 0·9] further purification was carried out, also on silica gel [light petroleum (b.p. 60—80°)–ethyl acetate–acetic acid (75 : 24 : 1); nepodin R_F 0·55]; yield 916,500 disint. min^{-1}.

Large-scale Isolation of Nepodin from Rumex patientia.—Half a root of an adult plant was macerated and mixed with an equal volume of $CaSO_4$ granules. The mixture was extracted for several days (Soxhlet) with light petroleum (b.p. 60—80°). The extract was shaken 3 times with aqueous Na_2CO_3 (5%; 600 ml) and separated from the organic phase. The carbonate solution was acidified (10N-HCl; 30 ml) and the precipitated crude nepodin was filtered off, washed with water, and dried. The material (5 g) was sublimed at 10 mmHg and 140—150°, and crystallized from light petroleum (b.p. 60—80°); m.p. 164—165°.

Isolation and Purification of Nepodin after Administration of [^{14}C]*Acetate to Leaves of* Rumex alpinus.—*Rumex alpinus* leaves were ground with solid CO_2 and extracted under nitrogen in boiling water for 0·5 h. The extract was filtered and acidified (HCl; final concentration 1N). The acidic solution was refluxed under nitrogen (30 min), cooled to room temperature, and extracted 3 times with peroxide-free ether. The ethereal solution was washed, dried, and evaporated. The residue was chromatographed on silica gel [benzene–ethyl acetate (3 : 1); nepodin R_F 0·9]. Nepodin was eluted with light petroleum (b.p. 60—80°) containing a small amount of methanol. The u.v. spectrum of nepodin thus isolated was identical with that published.[16] Authentic nepodin (from *Rumex patentia*; 120 mg) was dissolved in the eluate, which was then evaporated, and the residue was recrystallized from light petroleum (b.p. 60—80°); yield 90—100 mg.

Isolation of Chrysophanol and 3-Methylnaphthalene-1,8-diol (IV) *after* [G-^{14}C]*Nepodin Administration.*—Chrysophanol was isolated by the same procedure as described for nepodin; however the nitrogen atmosphere was replaced by air to permit conversion of chrysophanol anthrone into the corresponding anthraquinone. The ethereal extract of the hydrolysate was evaporated and the residue chromatographed on silica gel G [light petroleum–ethyl acetate–acetic acid (75 : 24 : 1); nepodin R_F 0·55; chrysophanol R_F 0·69; 3-methylnaphthalene-1,8-diol (IV) R_F 0·3]. The diol (IV) (tentatively identified; see later) and chrysophanol were eluted with chloroform. Chrysophanol turned out to be inactive after addition of carrier material and recrystallization from acetic acid. The diol (IV) was rechromatographed on silica gel G [benzene–acetic acid (8 : 2); (IV) R_F 0·59]. It was eluted, carrier was added, and the product was recrystallized from water. Its radioactivity was then determined.

Tentative Identification of 3-Methylnaphthalene—1,8-diol. When *Rumex alpinus* leaves were extracted as described for nepodin, a t.l.c. band was observed which darkened on exposure to air and light. The same behaviour was observed for an authentic sample of the diol (IV) obtained by deacetylation of nepodin. The two samples had identical R_F values in the solvent systems given above. Material obtained by elution of the t.l.c. band from a plant which had been fed with [G-^{14}C]nepodin co-crystallized with the authentic diol (IV), without loss of radioactivity. Compound (IV) was detectable during the summer but not during early spring.

Degradation of Nepodin (*Scheme* 1). *Isolation of C-2′ and -2″* (cf. *Ref.* 16).—Nepodin (III) (100 mg), potassium hydroxide (150 mg), sodium hydroxide (150 mg), and water (0·3 ml) were heated (200—210°) under a stream of nitrogen for 15 min. When the mixture had cooled, it was diluted with water (3 ml), cooled in an ice–bath, and acidified (conc. H_2SO_4). The precipitated diol (IV) was removed by centrifugation and resuspended in water. Centrifugation was repeated and from the combined supernatants acetic acid (C-2′ and -2″) was separated by steam distillation and estimated by titration (0·1N-NaOH; phenolphthalein); yield 300 μmol. The solution was then concentrated to 1 ml, a drop of ammonia (conc.) was added and the solution was heated on a water-bath (60°). Aqueous silver nitrate (50%) was added dropwise until no further precipitation was observed. The suspension was stirred for 5 min at 60° and centrifuged. The supernatant was removed and the solid resuspended. Centrifugation was repeated and to the combined supernatants a twofold volume of acetone was added. Crystallization of silver acetate took place at −4°. The crystals were collected and dried, and their specific activity determined; yield 40 mg.

Decarboxylation of silver acetate to give C-2′ of nepodin as barium carbonate was carried out by the method of Phares.[17]

Kuhn–Roth Oxidation of the Diol (IV).—The precipitate obtained after deacetylation of nepodin was suspended in dilute acid (see above) and extracted into ether. The extract was washed, dried, and evaporated. The residue was chromatographed on silica gel [benzene–tetrahydrofuran (3 : 1); (IV) R_F 0·75]. The naphthol was eluted with methanol; its u.v. spectrum was identical with that reported.[16] A sample of the eluate was withdrawn for determination of radioactivity and for quantitative determination of the naphthol by u.v. spectroscopy; yield 250 μmol. The eluate was evaporated and the residue submitted to Kuhn–Roth oxidation.[18] The acetic acid (C-3 and -3′) formed was isolated by steam distillation and the silver acetate was decarboxylated giving C-3 of nepodin.

Degradation of Nepodin (III) *to 3-Hydroxyphthalic Acid* (IX) *and the Products* (X)—(XIV) (*Scheme* 1).—Nepodin (50 mg) was oxidized to hydroxyphthalic acid by the procedure previously described for the oxidation of juglone [19] and chrysophanol.[11] Further degradation was carried out as described by Gatenbeck.[20]

Determination of Radioactivity.—Activities were determined by liquid scintillation counting (Berthold Frieseke Liquid Scintillation Counter, Betaszint model 5000). Barium [^{14}C]carbonate was counted in a suspension of Liquifluor and thixotropic gel powder. Counting efficiency was determined by using radioactive barium carbonate of known specific activity. Radioactive silver acetate and nepodin were subjected to combustion before counting.[21]

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