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BIOSYNTHESIS OF PRIMIN AND MICONIDIN AND ITS DERIVATIVES

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Key Word Index—*Primula obconica*; primin; biosynthesis; 2-methoxy-6-*n*-alkyl-1,4-benzoquinones; 2-methoxy-6-*n*-alkylphenols.

Abstract—Numerous 2-methoxy-6-*n*-alkyl-1,4-benzoquinones with interesting biological activities have been found as constituents of plants and plant seeds. The most prominent is primin, the *n*-pentyl derivative, which is stored in trichomes on leaves and stems of *Primula obconica* and is responsible for primrose dermatitis. The biosynthesis of primin was studied by feeding experiments with radioactively labelled precursors and unlabelled homologues.

INTRODUCTION

2-Methoxy-6-*n*-alkyl-1,4-benzoquinones are found as secondary metabolites in various species of higher plants, and it has been shown that some of them possess interesting biological activities. Thus, primin (**1**, $n = 4$), isolated from surface extracts of *Primula obconica* [1, 2], has been known for a long time to be responsible for primrose dermatitis. Homologous compounds, differing only in length and degree of unsaturation of the side-chain, have been found in seeds of various Iridaceae [3–5] and rhizomes of *Iris missouriensis* [6] or *Ardisia japonica* [7], respectively. We are interested in the biosynthesis of these quinones and chose primin for our studies, since *P. obconica* is readily available and very easy to grow. The quinone **1** is stored in trichomes, which cover the entire, above-ground parts of the plant. The compound is easily extracted by soaking the leaves for a short while in an organic solvent. A thorough analysis of the extract showed the presence of several benzoquinones and phenols [8], which are certainly products of the same biosynthetic pathway. Besides primin (**1**) and its hydroquinone (**2**, $n = 4$, miconidin), first reported as constituent of some *Miconia* species [9], their *n*-heptyl homologues ($n = 6$) are also present in the extract [8]. In addition to several derivatives of the hydroquinone **2**, namely the methyl ethers **3** and **4** and the acetate **5**, the resorcinol **6** ($n = 4$, olivetol) and its methyl ether **7** ($n = 4$) have been found [8].

The alkyl side-chains of these compounds are most probably synthesized via the polyketide pathway, and for the formation of the pentyl-phenols or pentylquinones **1**–**7** ($n = 4$) the triketoacid **8** may be postulated as an intermediate. Cyclization of **8** would lead to 2,4-dihydroxy-6-*n*-pentylbenzoic acid (**9**), which may serve as an educt for the resorcinols, hydroquinones and bezoquinones (Scheme 1). In this paper, we report on our experiments to prove this proposed route for the biosynthesis of primin (**1**) and miconidin (**2**) and its derivatives.

RESULTS AND DISCUSSION

An aqueous solution of [2-^{3}H]acetate was injected into the stems of cut leaves of *P. obconica*, which were then kept in water. The progress of the incorporation was followed at regular intervals (24 hr). After extracting the surface of a leaf with Et_2O, the extract was treated with $FeCl_3$, to convert the hydroquinone **2** into primin (**1**). Subsequently, the quinone was isolated by preparative TLC on silica. The results are depicted in Fig. 1 and clearly show that the maximum incorporation (0.71% of the applied radioactivity) of radioactivity in **1** plus **2** is reached after 48 hr.

In the biosynthesis of polyketides starter molecules other than acetyl CoA are used by some pathways. Thus, in the biosynthesis of the related anacardic acids long chain fatty acids take over this role [10]. By analogy, hexanoic acid (**13**) may serve as the primer in the biosynthesis of primin. Elongation of the molecule by condensation with three malonyl CoA units would lead to the triketo acid **8**. We synthesized [2-^{3}H]hexanoic acid (**13**) by conventional methods and injected it into the stems of cut leaves of *P. obconica*. However, much less radioactivity (0.1%) than that found following the injection of [2-^{3}H]acetate was incorporated into primin, and the incorporation was considerably slower, reaching its maximum only after 72 hr (Fig. 1). The low yield and the

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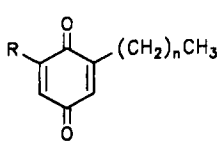
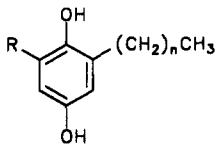
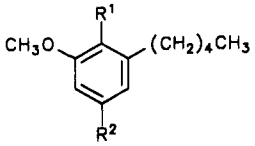


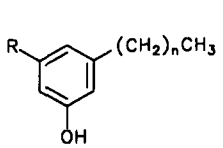
	R
1	OCH_3
14	OH



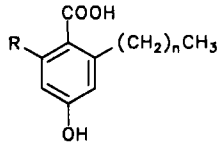
	R
2	OCH_3
15	OH



	R^1	R^2
3	OCH_3	OH
4	OH	OCH_3
5	$OCOCH_3$	OH

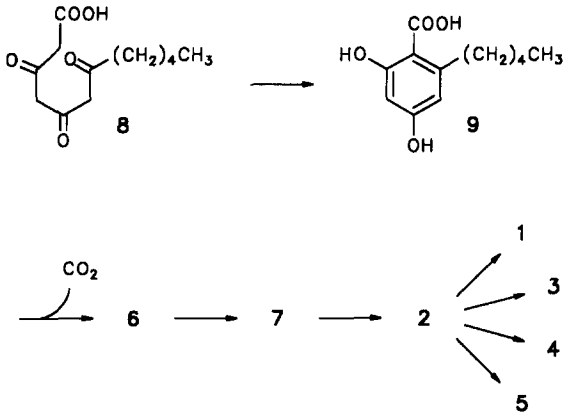


	R
6	OH
7	OCH_3



	R
9	OH
10	OCH_3

Scheme 3



Scheme 1. Proposed biosynthesis of primin and miconidin and its derivatives.

slow reaction may be explained by degradation of the acid and subsequent use of the resulting acetate for the *de novo* synthesis of the quinone **1**. Therefore, acetyl CoA is the most likely starter molecule, and after each of the first two condensation steps the β-keto group is reduced, to form hexanoic acid, which does not leave the condensing enzyme. Instead, further condensation reactions with three malonyl CoA units, without reduction of the keto groups, leads to formation of the triketoacid **8**. This result is confirmed by the fact that no hexanoic acid could be found in either part of the plant.

As pointed out, Claisen condensation of **8** should lead to the phenolic acid **9**, which presumably is rapidly decarboxylated to olivetol (**6**), as only the latter compound is present in the essential oils. Even with the aid of a synthetic reference, which was prepared by a modified method of Focella *et al.* [11] (Scheme 2), the acid **9** could not be detected. Nevertheless, this compound has to be considered as an intermediate in primin biosynthesis. As seen from the presence of homologues of **1** and **2** ($n = 4,6$), the enzymes, which are involved in this process, are capable in handling substrates of different chain length. To verify the course of the proposed reaction sequence from the ketoacid **8** to primin (**1**), we, therefore, decided to conduct feeding experiments with homologues of the natural substrates, bearing *n*-butyl or *n*-hexyl side-chains, since this would allow us to distinguish the synthetic substrates and their products from the natural products by GC or GC–MS without the need to prepare radioactively labelled compounds. We prepared the appropriate homologues ($n = 3,5$) of the naturally occurring phenols **6**, **7**, **9** and **10** by the routes shown in Scheme 2. The primin homologues with C_4 and C_6 side-chains, which were needed as reference samples, were prepared according to Bieber *et al.* [12]. Reduction of the quinones with $Na_2S_2O_4$ [9] gave the corresponding hydroquinones. Finally, the preparation of the derivatives **14** and **15** ($n = 3,4$) was achieved by ether cleavage of **2** ($n = 3,4$) with $SiCl_4$–NaI [13] and subsequent oxidation of the hydroquinones with $FeCl_3$ [9]. As for the acid **9**, comparison of the plant extracts with synthetic samples of **10**, **14** and **15** ($n = 4$) gave no indication of their natural occurrence.

The administration of the compounds was carried out as described for [2-^{3}H]acetate, except that due to their low solubility in H_2O, 2 mg of the test substrate was dissolved in 10 μl acetone for injection. Each experiment was repeated on 10 leaves. Again, surface extracts of the leaves were prepared at 24 hr intervals, chromatographed by preparative TLC, and the fractions analysed by GC–MS. Depending on the substrate, 2–10% of the

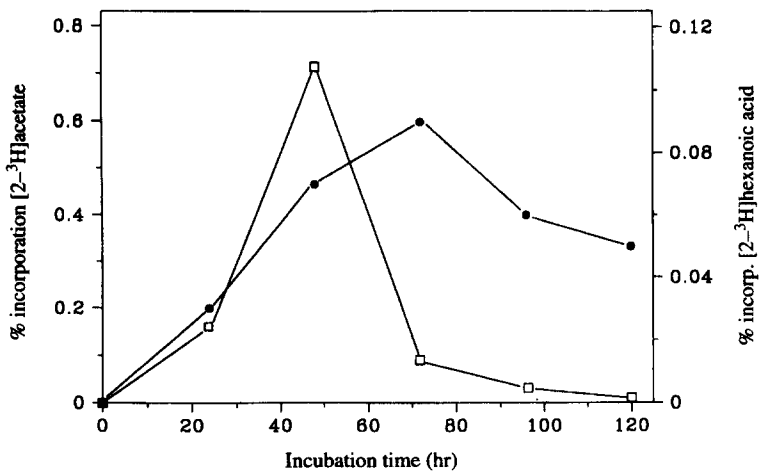
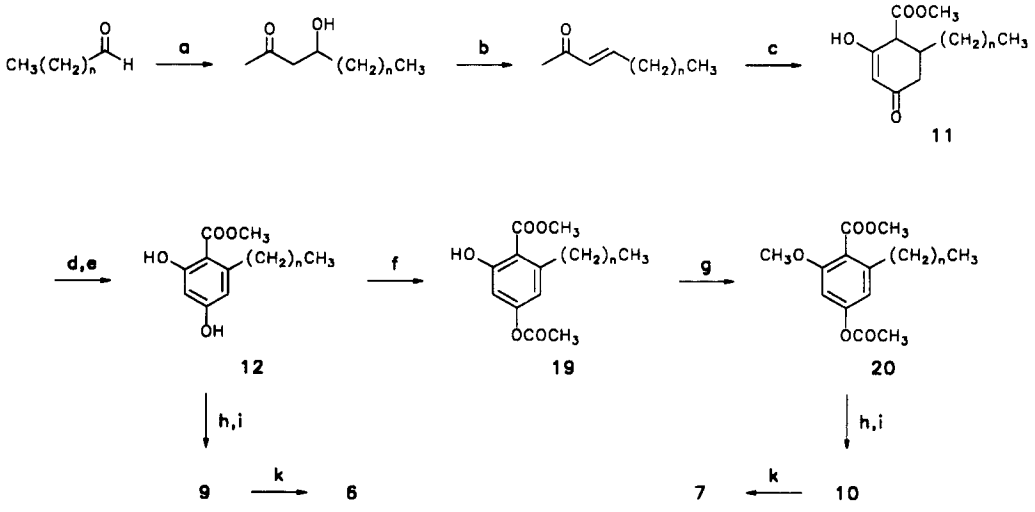


Fig. 1. Incorporation of ^3H into primin upon incubation of *P. obconica* leaves with □, [2-^{3}H]acetate and ●, [2-^{3}H]hexanoic acid (SD ±15%).

applied substances, or their derivatives, was found in these extracts. The greater amount was present in the water (40–50%) and $CHCl_3$–MeOH extracts of the stems (30–40%) or leaves (10–20%), respectively. The amount of substrates with butyl side-chain found in the surface extracts was about 10–15% higher than that of the corresponding hexyl homologues. Certainly, their transport is favoured due to their better solubility in H_2O. Except in the case of the experiments using **14** or **15** ($n = 3$), all of the substrates tested gave rise to the corresponding primin and miconidin homologues. The two products **1** and **2** ($n = 3,5$) were found in a 1:1 ratio and their maximum amount was obtained between 24 and 72 hr. The mean values of their yield in relation to the total of incubated substrate, found in the leaf surface extract during this time interval, are summarized in Table 1.

The biosynthetic pathway may be deduced from the results of the feeding experiment: the closer the substrate to the end products the higher is their yield. Thus, after formation of the triketoacid **8**, cyclization and dehydration will lead to the benzoic acid **9**, which is decarboxylated to form the resorcinol **6**, Methylation of **6** will give the methyl ether **7**. An alternative pathway to compound **7**, which cannot be excluded from the experimental results, is the formation of the methyl ether **10** from the acid **9** and its subsequent decarboxylation. The final step is the oxidation of the phenol **7** to miconidin (**2**),



Scheme 2. Preparation of intermediates of primin biosynthesis ($n = 4$) and their homologues ($n = 3,5$). Reagents: (a) $CH_3(CH_2)_nCHO$, NaOH. (b) TosOH. (c) $CH_2(COOCH_3)_2$, NaOMe–MeOH. (d) Br_2–DMF, 0°. (e) Δ (70–80°), 24 hr. (f) Ac_2O–pyridine, 0°. (g) CH_3I–K_2CO_3, acetone. (h) NaOH–MeOH. (i) HCl. (k) MeOH–H_2O, Δ.

Table 1. Incubation of homologous precursors. Yields of primin homologues are reported in relation to the total amount of substrate transported to the leaf surface (SD ±31%)

Substrate	Yield of primin homologue (%)
9 ($n = 3$)	2.65
10 ($n = 5$)	3.00
10 ($n = 3$)	4.40
6 ($n = 5$)	5.25
6 ($n = 3$)	5.85
7 ($n = 5$)	6.25
7 ($n = 3$)	9.15

which is further oxidized to primin (**1**). The resorcinol **6** apparently is not oxidized to the triol **15** or in the sequence to the hydroxyquinone **14** prior to methylation, as the incubation of the latter two substances did not yield any miconidin or primin. The hydroquinone **2** also serves as an educt for the derivatives **3**, **4** and **5**, which are formed by methylation or acetylation. Long-chain (C_{15}–C_{19}) homologues of primin (**1**) and the phenols **6** and **7** have also been found in the seeds of various *Iris* species. Presumably, their biosynthesis follows the same route. However, this needs further study.

EXPERIMENTAL

General. GC: Shimadzu GC 8A, CC OV1 WCOT (15 m, 0.25 mm i.d.); Flash CC: silica 60 (32–63 μm, ICN, Eschwege, Germany); TLC: silica 60 F_{254} (Merck); GC–MS: Finnigan-MAT 4510 GC–MS (EI: 70 eV), CC OV1 (15 m, 0.25 mm i.d.); NMR: Bruker WH-300 (^{1}H: 300 MHz), Bruker AC 80E (^{1}H: 80 MHz); Liquid scintillation counting: Searle Delta 300; radioactive compounds or frs of interest were dissolved in 3 ml scintillation liquid (a soln of 6 g PPO (2,5-diphenyloxazol) and 0.4 g POPOP (1,4-bis(5-phenyl-2-oxazoyl)benzene) in 600 ml of toluene and 300 ml of Triton X-100). From the measured values the background was subtracted and the incorporated net radioactivity was calculated considering dilution of the sample and correction of the quenching. C^3H_3COONa (3.7 GBq mmol^{-1}) and NaB^3H_4 (259 GBq mmol^{-1}) were obtained from Amersham (Braunschweig, Germany).

[2-^{3}H]*Hexanoic acid* (**13**). *n*-Pentanal (100 mg, 1.16 mmol) was reduced with 37 MBq NaB^3H_4 following the procedure of Ref. [14] to give 33 MBq (90%) [1-^{3}H]pentanol. Bromination of the alcohol was achieved with 217 μl PBr_3 (2.32 mmol) in 1 ml Et_2O (24 hr). After workup, purification by CC on silica gel (pentane) gave 24 MBq (73%) of the bromide, which was dissolved in 1 ml DMSO. A soln of 85 mg (1.74 mmol) NaCN in 3 ml DMSO was added and the mixt. stirred for 24 hr at room temp. Subsequently, the Et_2O extract of the reaction mixt. was washed with satd brine, dried ($MgSO_4$) and evapd. The crude product was hydrolysed by heating to reflux with a soln of KOH (6.6 g) in 10 ml EtOH for 6 hr under N_2. After evapn of the solvent, the residue was acidified (pH 1–2) with 9M H_2SO_4. Extraction with Et_2O and CC on deactivated (10% H_2O) silica gel (petrol–Et_2O 9:1) gave 2.2 MBq **13**.

Syntheses of phenols and quinones. The phenols and quinones used as reference compounds or for incubation experiments were prepared by the methods outlined below for *n*-butyl derivatives.

2,4-*Dihydroxy*-6-n-*butylmethylbenzoate* (**12**). The preparation and bromination of 6 g (26.5 mmol) **11** followed the procedure of Ref. [11]. Dehydrobromination was achieved by heating the reaction mixture under N_2 to 70° (24 hr). The slurry was poured into 100 ml ice H_2O and extracted (3 ×) with 80 ml Et_2O. The combined organic layers were washed with satd brine, dried ($MgSO_4$) and evapd to give a red oil, which yielded after flash CC on silica gel (petrol–EtOAc, 17:3) 4.5 g (77%) of the ester **12**. ^{1}H NMR (80 MHz, $CDCl_3$): δ11.7 (*s*, 1H, OH), 6.21 (AB system, 2H, J_{AB} = 2.3 Hz, 3H, 5-H), 5.41 (*s*, 1H, OH), 3.9 (*s*, 3H, OCH_3), 2.8 (*t*, J = 6.5 Hz, 2H, Ar–CH_2), 1.7–1.1 (*m*, 4H, $2CH_2$), 0.92 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 224 $[M]^+$ (20), 192 (32), 182 (28), 150 (100), 122 (30).

2,4-*Dihydroxy*-6-n-*butylbenzoic acid* (**9**). The ester **12** (100 mg, 0.44 mmol) was hydrolyzed with a soln of 3.5 g NaOH in 10 ml H_2O/MeOH (3:2) for 24 hr at room temp. Upon cooling (0°) and dropwise addition of conc HCl the acid precipitated as a white solid. Extraction (2 ×) with 10 ml Et_2O and CC on deactivated (10% H_2O) silica gel with petrol–Et_2O) (1:1) yielded 40 mg (43%) **9**. ^{1}H NMR (80 MHz, acetone-d_6): δ6.3 (AB system, J_{AB} = 2.3 Hz, 2H, 3-H, 5-H), 2.95 (*t*, J = 6.6 Hz, 2H, Ar-CH_2), 1.2–1.8 (*m*, 4H, $2CH_2$), 0.92 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 210 $[M]^+$ (22), 192 (33), 168 (50), 150 (100), 124 (70).

2-*Hydroxy*-4-*acetyl*-6-n-*butyl-benzoic acid* (**19**). The methyl ester **12** (300 mg, 1.33 mmol) was dissolved in 10 ml dry pyridine and cooled to 0°. Within 1 hr a soln of 136 mg (1.33 mmol) acetanhydride, dissolved in 5 ml pyridine, was added dropwise. After 1 hr at room temp., 15 ml 2M HCl were added and the soln was extracted with 100 ml Et_2O. The organic layer was washed until neutral with satd brine, dried ($MgSO_4$) and evapd. CC on silica gel with petrol–Et_2O (9:1) gave 106 mg (30%) **19**. ^{1}H NMR (300 MHz, $CDCl_3$): δ6.55 (*s*, 2H, H-3, H-5), 3.85 (*s*, 3H, $COOCH_3$), 2.50 (*t*, J = 6.6 Hz, Ar-CH_2), 2.32 (*s*, 3H, $OCOCH_3$), 1.7–1.1 (*m*, 4H, $2CH_2$), 0.9 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 266 $[M]^+$ (8), 234 (12), 224 (40), 192 (20), 182 (100), 150 (80).

2-*Methoxy*-4-*acetyl*-6-n-*butyl methylbenzoate* (**20**). The methylation of 127 mg (0.47 mmol) **19** was achieved by following the procedure of Ref. [4]. After CC on silica gel with petrol–Et_2O (47:3), 92 mg (70%) **20** were obtained. ^{1}H NMR (80 MHz, $CDCl_3$): δ6.6 (AB system, J_{AB} = 2.3 Hz, 2H, H-3, H-5), 3.9 (*s*, 3H, OCH_3), 3.8 (*s*, 3H, OCH_3), 2.5 (*t*, J = 6.6 Hz, 2H, Ar-CH_2), 2.3 (*s*, 3H,

$COCH_3$), 1.8–1.1 (*m*, 4H, $2CH_2$), 0.9 (*t*, J = 5,8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 280 $[M]^+$ (3), 248 (20), 238 (40), 206 (20), 196 (30), 164 (100).

2-Methoxy-4-hydroxy-6-n-butyl-benzoic acid (**10**). Alkaline hydrolysis of **20** (100 mg, 0.35 mmol) was carried out as described for the preparation of **9**. After CC on deactivated (10% H_2O) silica gel with petrol–Et_2O (1:1) 21 mg (26%) **10** were isolated. ^1H NMR (80 MHz, $CDCl_3$): δ6.35 (*s*, 2H, H-3, H-5), 3.83 (*s*, 3H, OCH_3), 2.95 (*t*, J = 6.6 Hz, 2H, Ar-CH_2), 1.8–1.1 (*m*, 4H, $2CH_2$), 0.9 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 238 $[M]^+$ (5), 224 (20), 206 (35), 182 (30), 164 (100), 138 (53), 109 (45).

3-Methoxy-5-n-butylphenol (**7**). The benzoic acid **10** (20 mg, 0.11 mmol) was dissolved in H_2O–MeOH (1:1, 10 ml) and heated under N_2 to 60° for 5 hr. After evapn of the solvent, the residue was extracted with Et_2O. The organic layer was washed with satd brine, dried ($MgSO_4$), evaporated, and yielded after CC on silica gel with petrol–Et_2O (3:1) 15.4 mg (78%) **7**. ^1H NMR (80 MHz, $CDCl_3$): δ6.3 (*m*, 3H, aromatic H), 3.75 (*s*, 3H, OCH_3), 2.5 (*t*, J = 6.6 Hz, 2H, Ar-CH_2), 1.8–1.1 (*m*, 4H, $2CH_2$), 0.9 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.): 180 $[M]^+$ (10), 138 (100).

3-Hydroxy-5-n-butylphenol (**6**). The benzoic acid **9** (50 mg, 0.23 mmol) was decarboxylated as described for **7**, to give after CC on silica gel with petrol–Et_2O (4:1) 31 mg (80%) **6**. ^1H NMR (80 MHz, $CDCl_3$): δ6.25 (*m*, 3H, aromatic H), 2.5 (*t*, J = 6.6 Hz, 2H, Ar-CH_2), 1.8–1.1 (*m*, 4H, $2CH_2$), 0.9 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 166 $[M]^+$ (10), 124 (100).

2-Methoxy-6-n-butyl-1,4-benzoquinone (**1**). The quinone was prepared according to Ref. [12]. ^1H NMR (80 MHz, $CDCl_3$): δ6.45 (*m*, 1H, H-3), 5.85 (*d*, 1H, H-5), 3.79 (*s*, 3H, OCH_3), 2.40 (*t*, J = 6.8 Hz, 2H, Ar-CH_2), 1.7–1.1 (*m*, 4H, $2CH_2$), 0.95 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 194 $[M]^+$ (60), 179 (5), 153 (100), 124 (15), 69 (30).

2-Methoxy-4-hydroxy-6-n-butylphenol (**2**). The hydroquinone **2** was obtained by reduction of **1** with $Na_2S_2O_4$ as described in Ref. [9]. ^1H NMR (80 MHz, $CDCl_3$): δ6.25 (AB system, 2H, H-3, H-5, J_{AB} = 2.6 Hz), 5.2 (*s*, 1H, OH), 4.4 (*s*, 1H, OH), 3.8 (*s*, 3H, OCH_3), 2.55 (*t*, J = 6.6 Hz, 2H, Ar-CH_2), 1.8–1.1, 4H, $2CH_2$), 0.95 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 196 $[M]^+$ (70), 167 (5), 154 (100), 139 (20), 125 (18), 69 (20).

2,4-Dihydroxy-6-n-butylphenol (**15**). Demethylation of **2** (n = 3), as described in Ref. [13], gave the dihydroxyphenol **15**. ^1H NMR (80 MHz, acetone-d_6): δ7.6 (br *s*, 1H, OH), 6.2 (AB system, 2H, J_{AB} = 2.6 Hz, H-2, H-4), 3.3 (br *s*, OH), 2.5 (*t*, J = 6.8 Hz, 2H, Ar-CH_2), 1.8–1.1 (*m*, 4H, $2CH_2$), 0.9 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 182 $[M]^+$ (30), 140 (100).

2-Hydroxy-6-n-butyl-1,4-benzoquinone (**14**). Oxidation of **15** with $FeCl_3$ according to Ref. [9] gave the quinone **14**. ^1H NMR (80 MHz, acetone-d_6): δ6.5 (*m*, 1H, H-5), 5.9 (*d*, 1H, H-3), 2.5 (*t*, J = 6.8 Hz, 2H, Ar–CH_2), 1.8–1.1 (*m*, 4H, $2CH_2$), 0.9 (*t*, J = 5.8 Hz, 3H, CH_3); EIMS: *m/z* (rel. int.) 180 $[M]^+$ (35), 139 (100).

Plant material. Plants of *P. obconica* were obtained from local markets in February 1992.

Incubations. C^3H_3COONa. For each experiment an aq. soln (10 μl), containing 0.01% unlabelled acetate and 18.5×10^3 Bq [2-^{3}H]-acetate, was injected into the stem of a leaf. The experiment was repeated three times for each incubation period (24, 48, 72, 96, 120 hr). After the appropriate incubation time, the leaves were separated from the stems and their surface extracted by soaking for 15 min in Et_2O. These extracts contained 2–4 ($\pm$0.5)% of the applied radioactivity. After evapn of the solvent, the residue was oxidized with $FeCl_3$ [9] and primin was isolated by TLC with petrol–EtOAc (4:1). The results are shown in Fig. 1.

[2-^{3}H]hexanoic acid. An Me_2CO soln (10 μl), containing 0.01% of unlabelled hexanoic acid and 185×10^3 Bq **13**, was injected into the stem of a leaf. The incubation and analysis of the results followed the procedure described for [2-^{3}H]acetate. 1–2 ($\pm$0.3)% of the injected radioactivity was found in the surface extract of the leaves. Its incorporation into primin is shown in Fig. 1.

Aromatic substrates. For each incubation period (24, 48, 72, 96 and 120 hr, respectively), 10 leaves (weighing 1.3–2 g each) were cut off above the ground. The substrate (2 mg) was injected as a 20% soln in Me_2CO into the stems of the leaves. Subsequently, the leaves were allowed to stand in water for the duration of the incubation. After the appropriate incubation time, the leaves were extracted as described above. The soln was dried ($MgSO_4$), the solvent evapd, and the residue was analysed by GC–MS, to determine the amount of substrate on the leaf surface. Subsequently, the extract was separated by TLC with petrol–EtOAc (4:1). The silica gel layer was cut into 5 frs, which were eluted with Et_2O and analysed by GC–MS. The primin and miconidin homologues were identified by comparison with synthetic references. Each incubation experiment was repeated five times.

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