Phytochemistry, 1978, Vol. 17, pp. 1739 1742 © Pergamon Press Ltd. Printed in England. 0031-9422/78/1001-1739 $02.00/0

AGLYCONES AND GLYCOSIDES OF OXYGENATED NAPHTHALENES AND A GLYCOSYLTRANSFERASE FROM *JUGLANS*

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(*Revised received* 20 *April* 1978)

Key Word Index—*Juglans*; Juglandaceae; walnut; 1,4-naphthoquinone; juglone; 4-hydroxy-1-naphthalenyl-β-D-glucoside; 4,8-dihydroxy-1-naphthalenyl-β-D-glucoside; *O*-glycosyltransferase; cell cultures.

Abstract—4-Hydroxy-1-naphthalenyl-β-D-glucopyranoside, a new natural product, as well as 1,4-naphthoquinone, juglone, and 4,8-dihydroxy-1-naphthalenyl-β-D-glucopyranoside have been isolated from fruits of different *Juglans* species. Callus and cell suspension cultures have been established from fruits of *J. major* and *J. microcarpa*. Cultural conditions have been found which trigger the formation of the metabolites listed above. A glycosyltransferase, which catalyses the glycosylation of benzohydroquinone as a model compound, has been isolated from leaves of *J. regia* and a callus culture of *J. major*.

INTRODUCTION

Among those quinones which are derived from shikimic acid and *o*-succinoylbenzoic acid [1–6], juglone (5-hydroxy-1,4-naphthoquinone, **2**) is unusual because it is synthesized within *Juglans regia* plants via at least one symmetrical intermediate [7–10]. In order to obtain more information on this pathway, we have undertaken a phytochemical analysis of fruits, callus and cell suspension cultures derived from different *Juglans* species.

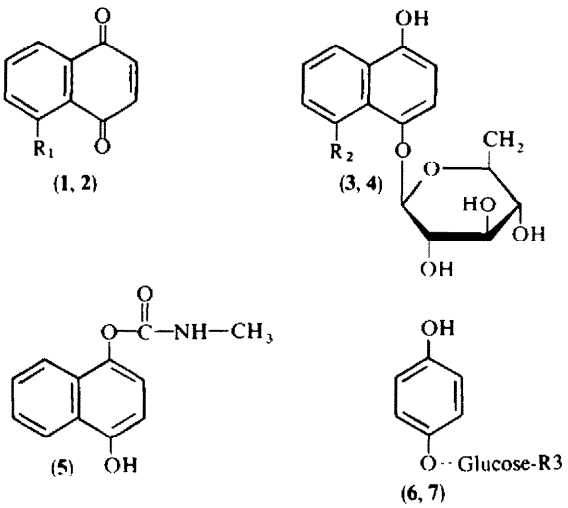
We have isolated from these plant materials 1,4-naphthoquinone (**1**), 4-hydroxy-1-naphthalenyl-β-D-glucopyranoside (**3**), juglone (**2**) and 4,8-dihydroxy-1-naphthalenyl-β-D-glucopyranoside (**4**) as well as an enzyme preparation catalysing the glycosylation of benzohydroquinone, using uridine diphosphate-D-glucose (UDPG) as a glucose donor.

RESULTS AND DISCUSSION

Whereas juglone (**2**) [11] and 4,8-dihydroxy-1-naphthalenyl-β-D-glucopyranoside (i.e. hydrojuglone glucoside) (**4**) [12, 13] are known constituents of *Juglans* fruits, we have recently reported on the detection and biosynthesis of 1,4-naphthoquinone (**1**) in *Juglans regia* plants [14]. The finding that 1,4-naphthoquinone is a natural product suggested that a glycoside of the corresponding hydroquinone would also be present in *Juglans* plants. This glycoside has been isolated, crystallized and identified (see Experimental) by comparison with a synthetic sample of 4-hydroxy-1-naphthalenyl-β-D-glucopyranoside (i.e. naphthoquinolglucoside). The isolated glucoside is easily hydrolysed by β-glucosidase. The glucose formed on hydrolysis can be oxidized by glucose oxidase yielding gluconic acid. This shows that the sugar is a β-D-glucose. The glucoside has therefore structure (**3**).

The amounts of aglycones and glycosides isolated from different *Juglans* fruits are recorded in Table 1. The glycosides and aglycones were extracted in such a way as to avoid hydrolysis of the glucosides (see Experimental). The ratio between the yields of aglycones and glucosides isolated from different *Juglans* species varies from 0.02:1 to 20.8:1 (Table 1) and since all fruits have been extracted under identical conditions, this suggests that the aglycones isolated are not derived by hydrolysis of glucosides (**3**) and (**4**) during the extraction procedure but occur as such *in vivo*. Proof for this suggestion is presented in the accompanying paper [15].

We have also isolated naphthalenes (**1**) to (**4**) from callus and cell suspension cultures of two *Juglans* species. The



(**1**) 1,4-Naphthoquinone, R_1 = H
(**2**) Juglone (5-Hydroxy-1,4-naphthoquinone), R_1 = OH
(**3**) 4-Hydroxy-1-naphthalenyl-β-D-glucopyranoside, R_2 = H
(**4**) 4,8-Dihydroxy-1-naphthalenyl-β-D-glucopyranoside, R_2 = OH
(**5**) 4-Hydroxy-1-naphthyl *N*-methylcarbamate
(**6**) Arbutin, R_3 = H
(**7**) Gentiobioside, R_3 = Glucose

Table 1. Amounts of 1,4-naphthoquinone (1), juglone (2), 4-hydroxynaphthalenyl-β-D-glucopyranoside (3) and 4,8-dihydroxy-1-naphthalenyl-β-D-glucopyranoside (4) isolated from fruits of different *Juglans* species

Juglans Species	Number of fruits extracted	Fr. wt (g)	Dry wt (g)	Amount of (1) (mg/g dry wt)	(3) (mg/g dry wt)	Ratio (wt) aglycone/ glucoside	Amount of (2) (mg/g dry wt)	(4) (mg/g dry wt)	Ratio (wt) aglycone/ glucoside
J. regia	2	18	1.24	0.07	0.13	0.55:1	8.77	32.5	0.27:1
J. major	7	58	6.0	0.13	<0.007	—	10.80	0.52	20.8:1
J. mandschurica	6	72.2	4.9	—	<0.008	—	—	1.54	—
J. nigra	1	42.8	5.42	0.008	3.35	0.023:1	3.25	36.1	0.09:1

Table 2. Amounts of 1,4-naphthoquinone (1), juglone (2), 4-hydroxy-1-naphthalenyl-β-D-glucopyranoside (3), and 4,8-dihydroxy-1-naphthalenyl-β-D-glucopyranoside (4) isolated from callus cultures of two *Juglans* species. Components of the media are given in the Experimental, n.d. = not detected. + = trace

Juglans species	Medium	Hormones	Age of culture (d)	Dry wt (g)	Amount of (1) mg/g dry wt	(3) mg/g dry wt	Amount of (2) mg/g dry wt	(4) mg/g dry wt
J. microcarpa	A	2,4 D, NAA IAA, Kinetin	25	0.23	n.d.	n.d.	n.d.	n.d.
	B	2,4 D, NAA IAA, Kinetin	26	0.18	0.008	+	0.07	1.18
	C	NAA	20	0.26	0.36	6.08	2.14	26.73
	C	NAA	40	0.83	n.d.	n.d.	n.d.	n.d.
J. major	B	2,4 D, NAA IAA, Kinetin	39	0.67	n.d.	n.d.	n.d.	n.d.
	C	NAA	15	0.56	0.03	0.98	0.60	6.59
	C	NAA	60	0.95	n.d.	n.d.	n.d.	n.d.

media used (viz. media B and C, Table 2) have been previously employed for the production of quinones in cell suspension cultures of *Morinda citrifolia* [16, 17] and *Galium mollugo* [18]. Although quinones produced in these cultures and in *Juglans* plants are derived from *o*-succinoylbenzoic acid [5, 14, 16–19], compounds (1) to (4) were encountered in *Juglans* callus in significant amounts only when grown on medium C (Table 2). Naphthalene derivatives were not detected in callus cultures which were 40 or 60 days old (Table 2). Production of naphthalene derivatives in cell suspension cultures turned out to be rather poor although the components of the media were identical except that agar had been omitted.

The co-occurrence of naphthoquinones and glucosides of the corresponding hydroquinones suggested that a glucosyltransferase is present in *Juglans*. An effort was made to extract this glucosyltransferase. The enzyme preparation has been obtained from leaves of 6-month-old *Juglans regia* plants as described in the Experimental. Since activity of glucosyltransferases under *in vitro* conditions requires thiol reagents to be present [20,21], protein, UDPG-$U^{14}C$, and naphthohydroquinone or hydrojuglone were incubated in the presence of either mercaptoethanol, cysteine or glutathione. The thiol reagents, however, reacted with the quinones which were instantly formed by oxidation of the hydroquinones even when the incubation was carried out under N_2. No enzymatically formed (3) or (4) were formed under these conditions. The reaction of thiols with quinones is well known [22, 23].

In an attempt to avoid oxidation of the naphthoquinol and subsequent reaction with the thiol reagent, a mono-*O*-substituted naphthohydroquinone viz. (5) was used as substrate. After termination of the reaction, the incubation mixture was evaporated in a stream of N_2. The residue was submitted to conditions (1 N KOH, 2 min, 70°) which would hydrolyse the ester but not the glucoside linkage. (3), However, was not detectable.

This suggests that for the activity of the enzyme system described (*vide infra*) a quinol moiety is essential. This is in agreement with a previous observation [24]. Indeed incubation with benzohydroquinone and UDPG-$U^{14}C$ resulted in a radioactive substance which was identified as arbutin (6) (see Experimental). When the incubation mixture described in the Experimental was used, formation of arbutin (6) and decrease of UDPG-$U^{14}C$ was linear within 30 min after start of the reaction. Both curves levelled off after a two hr incubation. When increasing amounts of protein were incubated with benzohydroquinone and UDPG-$U^{14}C$, the reaction was linear until a concentration of 0.15 mg protein per 0.2 ml volume was reached. On further increase of protein concentration, the velocity of the reaction increased at a slower rate.

The temperature optimum observed was 30°. At 25° and 35° only 85% arbutin was formed under otherwise identical conditions. The pH-optimum (K-phosphate buffer) was rather broad (optimum at pH 7.0–7.5). The K_m value as determined by a Lineweaver–Burk plot was found to be 0.044 mM for UDPG. This is rather low when compared to other glucosyl transferases [24–32]. The K_m value for benzohydroquinone also proved to be low compared to other glucosyltransferases [24–32]. The K_m value for benzohydroquinone also proved to be low but still within the range of K_m values previously observed for other phenols [24–32]. The enzyme was inactive with glucosyl donors (ADPG, CDPG) other than UDPG. The enzyme was also inactive when mercaptoethanol was omitted from the incubation mixture.

When compared to the activity (100%) in the standard incubation mixture, inhibition of the enzyme was observed when gluconolactone (2 μmol/0.2 ml volume; 63% activity), 1,4-naphthoquinone (0.10 μmol/0.2 ml; 36% activity) or benzoquinone (0.17 μmol/0.2 ml; 34% activity) were included into the incubation mixture. When arbutin (**6**) was used as a substrate, the unchanged glucoside was reisolated from the incubation mixture. This means that neither a diglucoside (gentiogioside (**6**)) was formed nor did the enzyme preparation contain glucosidase activity. Glucosyltransferase activity was also detected in callus cultures of *Juglans major* (28-day-old). The specific activity of the enzyme preparation from this source was, however, much lower (5.8 pmol arbutin/30 min/mg protein) than in preparations obtained from intact leaves (500 pmol arbutin/30 min/mg protein).

UDP-glucose:benzohydroquinone *O*-glucosyltransferases have been previously detected in extracts from *Datura innoxia* suspension cultures [33], wheat germ [24], petals of *Impatiens balsamina* [20] and broad bean [34]. Glucosyltransferases acting on phenolic substrates other than benzohydroquinone have also been reported [21, 25–36].

EXPERIMENTAL

Plant material. Fruits of *Juglans regia* L. were collected in a private garden near Witten (F.R.G.). Fruits of *J. nigra* L. were obtained from the Stadtpark Bochum (F.R.G.). Fruits of *J. major* A. A. Heller, *J. mandschurica* Maxim, *J. microcarpa* Berland. and *J. cordiformis* Maxim were from the Botanical Garden Dortmund (F.R.G.).

Callus initiation. Fruits of the *Juglans* species mentioned above were sterilized in EtOH (96%) for 15 min, subsequently in NaOCl (5%) for 20 min and washed with sterile H_2O. Slices of the mesocarp were then put on an agar medium [37]. The medium contained 5 mg 2,4-D per l. medium. 10 mg 2,4-D per 1 l. medium proved to inhibit callus initiation. Calluses were obtained from fruits of *J. regia, J. major, J. cordiformis* and *J. nigra* but not from *J. mandschurica*. From the initial agar medium the calluses were either transferred to agar medium A [38], B (modified B 5 medium [16, 18, 39] containing 2,4-D, NAA, IAA and kinetin) or C (same but 1.86 mg NAA per l. as the only hormone).

Synthesis of 4-hydroxy-1-naphthalenyl-β-D-glucopyranoside (**3**). The glucoside was prepared from naphthohydroquinone and acetobromglucose. Yield of the glucosides: 10%, mp 245–252° (dec) (Lit. 262–264°) [40].

Extraction of glucosides from J. nigra. 20 Fruits of *Juglans nigra* (693 g fr. wt, 79 g dry wt) were cut into pieces and kept at room temp. in HCl–EtOH (1 l., 0.2 N) for 5 hr. The suspension was filtered and the plant material submitted to further extraction in HCl–EtOH (0.2 N) for 18 hr at room temp. Finally the plant material was collected by filtration, ground in a mortar and extraction continued in HCl–EtOH for 8 hr. The pH of the combined extracts was adjusted to 4 by dropwise addition of NaOH (5 N). H_2O (400 ml) was added and the EtOH was evapd under red. pres. The aq. phase was extracted 3× with petrol (40–60°, 200 ml), 3× with C_6H_6 (200 ml), 3× with EtOAc (300 ml) and 4× with EtOAc (200 ml). The combined EtOAc extracts were dried, concd and petrol (40–60°) was added. A brownish ppt. (22.95 g) was formed. The ppt. was dissolved in EtOH (150 ml) and filtered through a column of Al_2O_3 (5 × 11 cm, Woelm, acidic). The column was washed with EtOH (96%, 250 ml) and EtOH (60%) until the effluent showed no UV-absorbance at 342 nm.

The combined EtOH was conc and extracted 5× with EtOAc (100 ml). The EtOAc was filtered through cotton and the glucoside fraction pptd by the addition of petrol (40–60°). The collected ppt. was chromatographed on a column of acetylated polyamide (5 × 29 cm). The column was eluted with H_2O (200 ml), MeOH (200 ml, 25%), MeOH (300 ml, 50%), MeOH (300 ml, 75%) and MeOH (96%). Fractions 36–39 (each containing 450 drops effluent) contained (**3**) and a minor amount of (**4**) whereas fractions 40–47 contained the bulk of (**4**). The latter was crystallized from EtOAc–petrol. Yield 1.17 g, mp 218° (Lit. 217°) [13].

Fractions 36–39 were combined, concd and again applied to a column (2.5 × 23 cm) of acetylated polyamide. The column was eluted with H_2O (100 ml) and MeOH (25%). (**3**) (21.1 mg) was eluted first and (**4**) (216 mg) subsequently. The former was further purified by TLC on acetylated polyamide (M + N) using H_2O, MeOH and CH_3COOH (40, 55, 5) as solvent (R_f 0.65) and on cellulose (CEL 300 M + N) using *iso* PrOH and H_2O (22.78) as solvent (R_f 0.67). (**3**) was crystallized from a mixture of EtOAc and petrol (40–60°). Yield 9.7 mg, mp 245–257° [Lit. 262–264°] [39].

Simultaneous extraction and quantitative determination of compounds (**1**)–(**4**). The plant material was extracted as described above. To the combined extracts (EtOH–HCl), H_2O (100 ml) was added and the solution conc under red. pres. The aq. layer was extracted ×3 with C_6H_6 (50 ml). The C_6H_6 was dried (Na_2SO_4) and (**1**) and (**2**) were determined quantitatively by GLC as previously described [14]. The aq. phase was extracted ×3 with EtOAc (5 × 50 ml). The organic phase was dried and concd and further purified as described above, however, the second polyamide column was omitted while for TLC the following systems instead of those mentioned above were used. Si gel G. 1. EtOAc–$CHCl_3$–HCOOH–H_2O (82:8:5:5). 2. $CHCl_3$–MeOH–HCOOH (85:13:2).

The identification of naphthoquinone has been previously described. The juglone (**2**) isolated was identical with published data [11]. (**4**) was shown to be identical with published data [12, 13] when mp, UV and products of hydrolysis were checked. The IR (ν_{max}^{KBr} 3390, 2935, 2910, 2880, 1632, 1611, 1520, 801, 735) and MS spectra (338 (M^+, 4%), 176 (100%), *m/e*) were also recorded.

From the synthetically prepared (**3**), the IR spectrum was taken: IR ν_{max}^{KBr} 3300, 2950, 2940, 2915, 2885, 1628, 1593, 1510, 1469, 820, 782, 769, 757. Both the isolated and the synthetic material were identical with respect to the following criteria: Mmp was undepressed. The R_f values were identical when 10 different TLC systems were used. On spraying with thymol–H_2SO_4, vanillin–H_2SO_4 and $FeCl_3$, the colour reactions of both were identical. Acid hydrolysis yielded 1,4-naphthohydroquinone and glucose in a 1:0.96 ratio. Enzymatic hydrolysis and simultaneous oxidation by glucose oxidase yielded (**1**) and gluconic acid. UV: λ_{max} (0.1 N HCl in EtOH) 328, 317 (ε = 5550), 309, 240 nm. When EtONa was added to an ethanolic soln of the glucoside a bathochromic shift of 25 nm between 309 and 328 nm was observed. MS: 322 (M^+, 5%), 160 (100%), *m/e*. NMR: (CD_3OD/TMS = 0) δ: 3.0–5.2 (*m*); 6.70 (*d*, 1H); 7.09 (*d*, 1H); 7.43 (*m*, 2H); 8.12 (*m*, 1H); 8.32 (*m*, 1H). When both the isolated and synthetic materials were chromatographed separately and successively on columns of Al_2O_3 and acetylated polyamide (see above) the IR spectrum (but not UV, NMR or MS spectrum) changed slight for unexplained reasons; IR: ν_{max}^{KBr} 3300, 2977, 1632, 1596, 1450, 816, 803, 760.

Enzyme extraction. All steps were carried out at 4°. Leaves (10 g) from plants (6 months old) of *Juglans regia* were homogenized with sand in the presence of Polyclar AT (3.5 g) and K-Pi buffer (20 ml, 0.05 M, pH 7.0) plus mercaptoethanol (20 mM) in a mortar. During homogenation further buffer (20 ml) was added dropwise. The homogenate was filtered through cheese cloth and the filtrate stirred with Dowex (5 g, 1X2, Cl^-) for 5 min. After centrifugation (20000 *g*, 20 min) and filtration through glass wool, the filtrate was submitted to $(NH_4)_2SO_4$ fractionation (20–60% satn). The ppt was collected by centrifugation, dissolved in the buffer mentioned above (1 ml) and dialysed by centrifugation through Sephadex G 25 as described by Kohl [41]. The half-life of the enzyme so obtained was 1 month at +4°. Protein was determined according to Kunitz [42]. Crystalline bovine serum albumin was used as a standard.

Enzyme assay. The incubation mixture consisted of UDPG-U^{14}C (0.15 nmol, 100000 dpm), 1,4-benzohydroquinone (0.15

μmol) (or 0.15 nmol UDPG and 0.15 μmol 1,4-benzohydroquinone-2,3,5,6^{14}C), K-Pi-buffer (7.5 μmol, pH 7.0) mercaptoethanol (3 μmol), EtOH (35 nmol) and protein (0.14 mg) in a total vol. of 200 μl. The mixture was incubated at 30° for 30 min and at the end of the incubation heated on a H_2O bath (100°, 3 min). The soln was evapd in a stream of N_2 on a water bath (70°) and the residue dissolved. The soln was applied to a TLC plate (cellulose) which was developed in *n*BuOH–CH_3COOH–H_2O (2:1:1). The radioactive arbutin zone was detected with a Berthold–Frieseke scan recorder.

Identification of the product. The radioactive zone associated with arbutin (*vide supra*) cochromatographed with an authentic sample of arbutin in 5 solvent systems on Si gel and cellulose plates. The same observations were made when ^{14}C-benzohydroquinone and UDPG had been incubated instead of UDPG-U^{14}C and benzohydroquinone. On enzymic (β-glycosidase) and acid hydrolysis, either radioactive benzohydroquinone and glucose or inactive benzohydroquinone and ^{14}C-labelled glucose were formed. Both kinds of arbutin were eluted from the TLC plates and recrystallized with authentic material. The sp. act. remained constant when recrystallized repeatedly from EtOAc–petrol (40–60°).

Acknowledgements—We thank Mr Lawo from the Stadtpark Bochum (FRG) and Dr Bünnemann from the Botanical Garden Dortmund (FRG) for the fruits of the *Juglans* species mentioned above. The technical assistance of Frau M. Winnemöller and Frau A. Puklowski and the support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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