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Biosynthesis of gallic acid in *Rhus typhina*: discrimination between alternative pathways from natural oxygen isotope abundance

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Abstract

The biosynthetic pathway of gallic acid in leaves of *Rhus typhina* is studied by oxygen isotope ratio mass spectrometry at natural oxygen isotope abundance. The observed δ^{18}O-values of gallic acid indicate an ^{18}O-enrichment of the phenolic oxygen atoms of more than 30‰ above that of the leaf water. This enrichment implies biogenetical equivalence with oxygen atoms of carbohydrates but not with oxygen atoms introduced by monooxygenase activation of molecular oxygen. It can be concluded that all phenolic oxygen atoms of gallic acid are retained from the carbohydrate-derived precursor 5-dehydroshikimate. This supports that gallic acid is synthesized entirely or predominantly by dehydrogenation of 5-dehydroshikimate.

Keywords: *Rhus typhina*; Anacardiaceae; Biosynthesis; Gallic acid; Gallotannins; Natural oxygen isotope abundance; Isotope ratio mass spectrometry; Carbon reduction

1. Introduction

Tannins are produced by a wide variety of plants (e.g., oak, acer, birch, tea, wine and sumac) where they play an important role in plant–plant, plant–animal, and plant–litter–soil interactions (for reviews see Kraus et al., 2003; Barman and Rai, 2000). More recently, galloyl derivatives were reported to have antineoplastic activity, probably due to their antioxidant properties (Gerhaeuser et al., 2003; Gomes et al., 2003; Kashiwada et al., 1992). Gallic acid (3,4,5-trihydroxybenzoic acid, **4**, Fig. 1) constitutes the basic building block of gallotannins. The detailed knowledge of the biosynthetic pathway of this compound which is still under discussion could provide the basis for the design of functional food with increased tannin content.

The biosynthesis of gallic acid has been studied for more than five decades. Experiments with [3-^{14}C]phenylalanine showed that the β-carbon atom of phenylalanine was incorporated, albeit only to less than 1%, into the carboxylic atom of gallic acid (Zenk, 1964; Dewick and Haslam, 1968). This implied that gallic acid is formed from phenylalanine (**1**) via caffeic acid or trihydroxycinnamic acid (route A, Fig. 1). Higher incorporation rates into gallic acid were found with [carboxy-^{14}C]shikimate as precursor suggesting that gallic acid can also be directly derived from an intermediate of the shikimate pathway, such as 5-dehydroshikimate (**2**) (route B) (Dewick and Haslam, 1968, 1969) or

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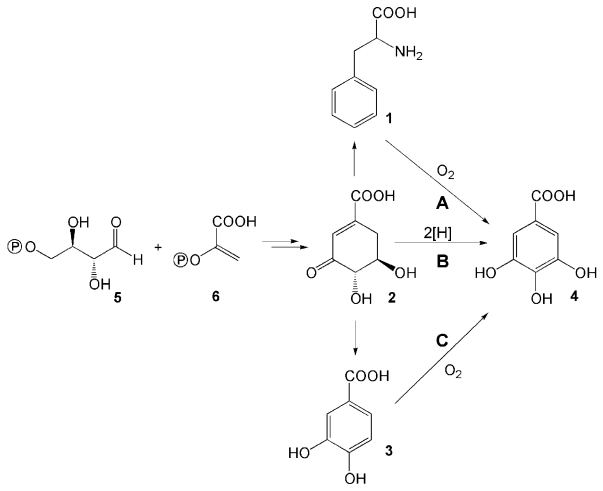

Fig. 1. Possible biosynthetic pathways of gallic acid formation. The data reported in this paper support that the vast majority of gallic acid is synthesised in *R. typhina* by route B. Minor contributions (<10%) by routes A and C cannot be excluded by the data in this paper.

protocatechuate (**3**) (route C) (Chandran and Frost, 2001; Kato et al., 1968). Even the existence of both pathways in certain plants has been proposed (Ishikura, 1975; Saijo, 1983).

Incorporation studies with ^{13}C-labelled glucose in sumac (*Rhus typhina*) showed that the carboxylic group in the bulk amount of analysed gallic acid (>95%) is biosynthetically equivalent to the carboxylic group of an early shikimate intermediate and not to the β-carbon atom of phenylalanine (Werner et al., 1997). It has therefore been concluded that, in the major route, the committed precursor of gallic acid is an intermediate of the shikimate pathway prior to prephenate or arogenate rather than a phenylpropanoid compound. Thus, following earlier proposals (Dewick and Haslam, 1968, 1969), the dehydrogenation of 5-dehydroshikimate (**2**) has been suggested as the most probable route of gallic acid formation (route B, Fig. 1). However, the data could not rule out a more complex pathway, e.g., via dehydration of 5-dehydroshikimate affording protocatechuic acid followed by a monooxygenase reaction (route C). More recently, a dehydrogenase has partially been purified from *Betula pubescens* catalyzing the conversion of 5-dehydroshikimate into gallic acid (Ossipov et al., 2003). However, in light of the reported K_m value of 0.49 mM for 5-dehydroshikimate, it remains unclear whether 5-dehydroshikimate is the physiological substrate for this enzyme.

It has been shown recently (Schmidt et al., 2001) that oxygen atoms in natural compounds are mainly originating from three primary sources, H_2O, CO_2, and O_2, giving rise to two pools with different δ^{18}O-values (H_2O and O_2) from where oxygen atoms are incorporated into organic molecules with specific thermodynamic and kinetic isotope effects, respectively. The oxygen atoms derived from CO_2 are very intimately associated with (leaf) water by an oxygen isotope exchange reaction between carbonyl or carboxyl groups (products from CO_2) and water leading relatively rapidly to an oxygen isotope equilibrium. Capitalizing on this fact, positional ^{18}O-abundances in defined positions of organic compounds can be explained or even predicted (Schmidt et al., 2001). Using this principle, for example, the origin or the synthetic route of compounds of unknown provenience have been deduced from their ^{18}O-patterns (Fronza et al., 2002). It should be noted that the method is well qualified to assess the origins of oxygen atoms in metabolites from plants grown under natural conditions. Due to the inherent error limitations of the method, contributions of minor pathways (<10%) cannot be determined accurately.

In this paper, we have analyzed the natural ^{18}O/^{16}O-ratio of gallic acid from *R. typhina*. Preliminary results have been reported as note added in proof in Schmidt et al. (2001).

2. Results and discussion

In any of the discussed pathways the biosynthesis of gallic acid is fundamentally based on the shikimate pathway starting from phosphenol pyruvate (**6**) and erythrose 4-phosphate (**5**) (Fig. 1). In the direct route, 5-dehydroshikimate (**2**) is dehydrogenated preserving oxygen functions from the precursors **5** and **6**. The phenolic oxygen atoms in gallic acid are then biosynthetically equivalent to the oxygen atoms at the positions 1, 2, and 3 of erythrose 4-phosphate (**5**) (cf. Fig. 1).

On the basis of the published ^{18}O/^{16}O ratios of oxygen atoms in carbohydrates of plants (Schmidt et al., 2001), their δ^{18}O-values are predicted, taking into account a thermodynamic isotope effect, to be about +30‰ above that of cellular water present during the biosynthesis. If, on the other hand, gallic acid were synthesised via phenylalanine (**1**) or protocatechuate (**3**), all or at least one of the phenolic hydroxyl groups in gallic acid must have been introduced by a monooxygenase reaction with atmospheric O_2 as oxygen source leading, due to an implied kinetic oxygen isotope effect, to a δ^{18}O-value of about +6‰. As the isolation procedure of gallic acid involves hydrolysis, the δ^{18}O-value of both oxygen atoms of the carboxyl group will be determined by an isotope equilibration with the hydrolysis water implying an ^{18}O-enrichment of +19‰ towards this oxygen source (Schmidt et al., 2001).

Under these premises, young leaves (length, 1–3 cm) of *R. typhina* were collected in the morning and the evening of a hot summer day. Leaf water was obtained by lyophilization of plant material. After acidic hydrolysis of the residual cell mass, gallic acid was extracted and purified by HPLC and recrystallization. The purity of

the compound was estimated as >98% by ^{1}H and ^{13}C NMR spectroscopy (data not shown). Average $\delta^{18}O$-values of gallic acid, leaf water and the water used for acid hydrolysis during work-up were determined by isotope ratio mass spectrometry (IRMS) (Table 1).

Averaged $\delta^{18}O$-values for the three phenolic oxygen atoms can be calculated from the measured average $\delta^{18}O$-value of gallic acid and that of the carboxyl group (which can be inferred from the $\delta^{18}O$-value of the water used for hydrolysis and the corresponding isotope shift (+19‰, see above).

As we do not know, whether the synthesis of gallic acid takes place at all during the night, we cannot explain why the product isolated from leaves collected in the morning with relatively ^{18}O-depleted water is more enriched in ^{18}O than that isolated in the evening after a hot day implying ^{18}O-enrichment in the leaf water (cf. Table 1). We have therefore used for the calculation of the $\delta^{18}O$-values of the phenolic OH-groups the mean of the experimental global $\delta^{18}O$-values of gallic acid (+21.5‰) as well as of the water (−2.1‰). The following equation has been used.

$$5 * \delta^{18}O[‰]_{\text{average in gallic acid}} = 2 * (\delta^{18}O[‰]_{\text{hydrolysis water}} + 19) + 3 * \left(\delta^{18}O[‰]_{\text{phenolic groups in gallic acid}}\right)$$

$$5 * 21.5 = 2 * (-10.4 + 19) + 3 * \left(\delta^{18}O[‰]_{\text{phenolic groups in gallic acid}}\right)$$

$$\delta^{18}O[‰]_{\text{phenolic groups in gallic acid}} = +30.2$$

This $\delta^{18}O$-value indicates an ^{18}O-enrichment of the hydroxyl groups of gallic acid of 32.3‰ above that of the leaf water [+30.2–(−2.1)], a value at the upper limit of carbohydrates and even slightly exceeding those known so far for immediate descendents from carbohydrates (Schmidt et al., 2001).

Assuming that one of the hydroxyl groups of gallic acid is introduced from molecular oxygen by a monooxygenase reaction ($\delta^{18}O$-value, +6‰) (route C in Fig. 1), a corresponding calculation would yield an average $\delta^{18}O$-value of +42.3‰ (44.4‰ above that of leaf water) for the two others carbohydrate derived oxygen atoms. Values of this order of magnitude have so far never been described for naturally occurring compounds (Schmidt et al., 2001). Only recently, in studies on the heterotrophic synthesis of cellulose from starch by wheat seeds, Sternberg et al. (2003) reported for oxygen at C-2 of the intermediate glucose a $\delta^{18}O$-value of +49‰ above that of water. On the other side, taking into account the generally found mean enrichment of ~+30‰ above that of water for carbohydrates, this exceptional positional value would demand corresponding depletions at other positions. Nevertheless, even if one presumes, although none of the oxygen atoms of erythrose 4-phosphate is originating from the oxygen atom at C-2 of glucose, a corresponding extreme enrichment in one of the oxygen atoms of the tetrose and +6‰ for its second one, still +39.8‰ would result for the third oxygen atom. Such extreme ^{18}O-enrichments for two out of three oxygen positions in a carbohydrate have no precedents and are not plausible (Schmidt et al., 2001).

This supports the hypothesis that all three phenolic OH-groups in the vast majority (>90%) of gallic acid from *R. typhina* originate from erythrose 4-phosphate (**5**) (route B in Fig. 1) and implies that the direct precursor of gallic acid is 5-dehydroshikimate (**2**) and not protocatechuate (**3**). Indeed, a labile enzyme catalyzing the NADP-dependent conversion of 5-dehydroshikimate into gallic acid has been recently purified from birch (Ossipov et al., 2003). It remains open whether orthologs of this enzyme are associated with gallic acid formation in sumac and other plants.

Table 1
Average $\delta^{18}O$-values of gallic acid from different sources, of leaf water, and of water used for the hydrolysis during work-up

Sample and origin	$\delta^{18}O[‰]_{V\text{-}SMOW}$
Gallic acid, commercial, unknown	+17.7 ± 0.1
Gallic acid, *R. typhina*, 2001	+21.2 ± 0.7
Gallic acid, *R. typhina*, 2003 (1)	+22.4 ± 0.2
Leaf water, *R. typhina*, 2003 (1)	−4.5
Gallic acid, *R. typhina*, 2003 (2)	+21.0 ± 0.2
Leaf water, *R. typhina*, 2003 (2)	+0.3
Water used for hydrolysis	−10.4

(1) Collection in the morning. (2) Collection in the evening.

3. Experimental

3.1. Plant accession

Young leaves (length, 1–3 cm) were cut from a local tree of *R. typhina*. The plant material was stored for 2 weeks at −30 °C until work-up.

3.2. Isolation of leaf water

Leaves of *R. typhina* (30 g, fresh weight) were lyophilized. Water was collected from the cold trap as ice. The ice was molten and the water was used for ^{18}O-determination according to standard methods (Koziet et al., 1995).

3.3. Isolation of gallic acid

Leaves of *R. typhina* (30 g, fresh weight) were triturated with liquid nitrogen. The plant biomass was then treated with 1 M sulphuric acid (100 ml) at 98 °C for

6 h. The solution was filtered and was then continuously extracted with 300 ml of diethyl ether for 12 h. The organic phase was extracted twice with 75 ml of saturated sodium bicarbonate. The aqueous solution was adjusted to pH 1 by the addition of concentrated sulphuric acid and was again extracted continuously with 150 ml of diethyl ether. The organic phase was concentrated to 5 ml under reduced pressure. The solution was applied to a column of silica gel (Merck, 2.5×39 cm). The column was developed with a mixture of diethyl ether/ethyl acetate/formic acid (5:4:1, v/v). Gallic acid was eluted at a retention volume of 140 ml. The fraction was concentrated to dryness under reduced pressure. Gallic acid was crystallized from hot water (2 ml). The crystals were collected and dried under reduced pressure (yield, 30 mg).

3.4. $\delta^{18}O$-Value analysis by IRMS

The determination of the $\delta^{18}O$-values [1] of the gallic acid samples was performed in a "carbon reduction" system consisting of an autosampler (AS 128, CE Instruments, Rodano, Italy), a high temperature elemental analyser (TC/EA, Finnigan MAT, Bremen, Germany), and an isotope ratio mass spectrometer (IRMS Delta^{plus} XL, Finnigan MAT), coupled *via* a home-built interface ("ConFlo III") (Werner et al., 1999). The TC/EA system resembles the one described by Kornexl et al. (1999a) with a few modifications (reaction glassy carbon tube filled solely with glassy carbon grit, any quartz wool replaced by Ag-wool, operation temp. 1450 °C) (Werner, 2003).

The samples and the laboratory reference standards (2 batches of different benzoic acid lab standards with ^{18}O-values of +23.06‰ and +71.40‰) were applied in solid samples silver capsules (Lüdi, purchased from IVA, Meerbusch, Germany). The $\delta^{18}O$-value (difference) of both benzoic acid lab standards was confirmed by an independent method (off-line decarboxylation; Santrock and Hayes, 1985) by A. Schimmelmann (Indiana University, Bloomington, USA; Brand, Schimmelmann) (Werner to be published). The standardisation scheme of the carbon reduction measurements (analogous to that described by Kornexl et al., 1999b) included the required SLAP/V-SMOW normalisation (Coplen, 1988), and the measurement strategy and calculation of $\delta^{18}O$-values was analogous to that described by Werner and Brand (2001).

[1] $\delta^{18}O[‰]_{V\text{-}SMOW} = \left[\frac{(^{18}O/^{16}O)_{Sample}}{(^{18}O/^{16}O)_{V\text{-}SMOW}} - 1\right] * 1000$. The international standard for oxygen isotope ratio measurements is: V-SMOW, Vienna Standard Mean Ocean Water with an $^{18}O/^{16}O$ ratio of $(2005.20 \pm 0.45) * 10^{-6}$, according to international agreement.

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References

Barman, K., Rai, S.N., 2000. Role of tannins in plant–animal relationship – a review. Indian J. Dairy Sci. 53, 390–410.

Chandran, S.S., Frost, J.W., 2001. Aromatic inhibitors of dehydroquinate synthase: synthesis, evaluation and implications for gallic acid biosynthesis. Bioorg. Med. Chem. Lett. 11, 1493–1496.

Coplen, T.B., 1988. Normalization of oxygen and hydrogen isotope data. Chem. Geol. (Isot. Geosci. Sect.) 72, 293–297.

Dewick, P.M., Haslam, E., 1968. Biosynthesis of gallic acid and caffeic acid. Chem. Comm., 673–675.

Dewick, P.M., Haslam, E., 1969. Phenol biosynthesis in higher plants; gallic acid. Biochem. J. 113, 537–542.

Fronza, G., Fuganti, C., Schmidt, H.-L., Werner, R.A., 2002. The $\delta^{18}O$-value of the p-OH group of L-tyrosine permits the assignment of its origin to plant or animal sources. Eur. Food Res. Technol. 215, 55–58.

Gerhaeuser, C., Alt, A.P., Klimo, K., Knauft, J., Frank, N., Becker, H., 2003. Isolation and potential cancer chemopreventive activities of phenolic compounds of beer. Phytochemistry Rev. 1, 369–377.

Gomes, C.A., Girao da Cruz, T., Andrade, J.L., Milhazes, N., Borges, F., Marques, M.P.M., 2003. Anticancer activity of phenolic acids of natural or synthetic origin: a structure–activity study. J. Med. Chem. 46, 5395–5401.

Ishikura, N., 1975. Incorporation rate of carbon-14-labeled shikimic acid and carbon-14-labeled phenylalanine into gallic acid in *Rhus* and *Acer* leaves. Experientia 31, 1407–1408.

Kashiwada, Y., Nonaka, G., Nishoka, I., Chang, J.J., Lee, K.H., 1992. Antitumor agents, 129. Tannins and related compounds as selective cytotoxic agents. J. Nat. Prod. 55, 1033–1043.

Kato, N., Shiroya, M., Yoshida, S., Hasegawa, M., 1968. Biosynthesis of gallic acid by a homogenate of the leaves of *Pelargonium inquinans*. Shokubutsugaku Zasshi 81, 506–507.

Kornexl, B.E., Gehre, M., Höfling, R., Werner, R.A., 1999a. On-line $\delta^{18}O$ measurement of organic and inorganic substances. Rapid Comm. Mass Spectrom. 13, 1685–1693.

Kornexl, B.E., Werner, R.A., Gehre, M., 1999b. Standardization for oxygen isotope ratio measurement – still an unsolved problem. Rapid Comm. Mass Spectrom. 13, 1248–1251.

Koziet, J., Rossmann, A., Martin, G.J., Johnson, P., 1995. Determination of the oxygen-18 and deuterium content in fruit and vegetable juice water. An European inter-laboratory comparison study. Anal. Chim. Acta 302, 29–37.

Kraus, T.E.C., Dahlgren, R.A., Zasoski, R.J., 2003. Tannins in nutrient dynamics of forest ecosystems – a review. Plant Soil 256, 41–66.

Ossipov, V., Salminen, J.-P., Ossipova, S., Haukioja, E., Pihlaja, K., 2003. Gallic acid and hydrolysable tannins are formed in birch leaves from an intermediate compound of the shikimate pathway. Biochem. Sys. Ecol. 31, 3–16.

Saijo, R., 1983. Pathway of gallic acid biosynthesis and its esterification with catechins in young tea shoots. Agricult. Biol. Chem. 47, 455–460.

Santrock, J., Hayes, J.M., 1985. Pyrolytic decarboxylation of aromatic acids as a means of isotopic analysis. Anal. Chem. 57, 1441–1443.

Schmidt, H.-L., Werner, R.A., Roßmann, A., 2001. ^{18}O Pattern and biosynthesis of natural products. Phytochemistry 58, 9–32.

Lobo Sternberg, Leonel da Silveira, da, S.L., Anderson, W.T., Morrison, K., 2003. Separating soil and leaf water ^{18}O isotopic signals in plant stem cellulose. Geochim. Geophys. Acta 67, 2561–2566.

Werner, I., Bacher, A., Eisenreich, W., 1997. Formation of gallic acid in plants and fungi. A retrobiosynthetic study with ^{13}C-labeled glucose. J. Biol. Chem. 272, 25474–25482.

Werner, R.A., Bruch, B.A., Brand, W.A., 1999. ConFlo III – an interface for high precision $\delta^{13}C$ and $\delta^{15}N$ analysis with an extended dynamic range. Rapid Comm. Mass Spectrom. 13, 1237–1241.

Werner, R.A., Brand, W.A., 2001. Referencing strategies and techniques in stable isotope ratio analysis. Rapid Comm. Mass Spectrom. 15, 501–519.

Werner, R.A., 2003. The online $^{18}O/^{16}O$ analysis: development and application. Isot. Environ. Health Stud. 39, 85–104.

Zenk, M.H., 1964. Biosynthesis of gallic acid. Z. Naturforsch. 19b, 83–84.