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Review

Enzymology of gallotannin and ellagitannin biosynthesis

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Abstract

Gallotannins and ellagitannins, the two subclasses of hydrolyzable tannins, are derivatives of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose. Enzyme studies with extracts from oak leaves (*Quercus robur*, syn. *Quercus pedunculata; Quercus rubra*) and from staghorn sumac (*Rhus typhina*) revealed that this pivotal intermediate is synthesized from β-glucogallin (1-*O*-galloyl-β-D-glucopyranose) by a series of strictly position-specific galloylation steps, affording so-called 'simple' gallotannins, i.e., mono- to pentagallyoylglucose esters. Besides its role as starter molecule, β-glucogallin was also recognized as the principal energy-rich acyl donor required in these transformations. Subsequent pathways to 'complex' gallotannins have recently been elucidated by the isolation of five different enzymes from sumac leaves that were purified to apparent homogeneity. They catalyzed the β-glucogallin-dependent galloylation of pentagallyoylglucose to a variety of hexa- and heptagalloylglucoses, plus several not yet characterized higher substituted analogous galloylglucoses. With respect to the biosynthesis of ellagitannins, postulates that had been formulated already decades ago were proven by the purification of a new laccase-like phenol oxidase from leaves of fringe cups (*Tellima grandiflora*) that regio- and stereospecifically oxidized pentagallyoylglucose to the monomeric ellagitannin, tellimagrandin II. This compound was further oxidized by a similar but different laccase-like oxidase to yield a dimeric ellagitannin, cornusiin E.

Keywords: *Quercus robur* (*Quercus pedunculata*); Fagaceae; Pedunculate oak; *Rhus typhina*; Anacardiaceae; Staghorn sumac; *Tellima grandiflora*; Saxifragaceae; Fringe cups; Biosynthesis; Enzymology; Hydrolyzable tannins; Gallotannins; Ellagitannins

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1. Introduction

Vegetable tannins, nowadays often called 'plant polyphenols' in order to provide a more adequate description of their heterogenous chemical structures and diverse reactions (cf. Haslam, 1989, 1998), have accompanied human life since its beginnings. As common and unavoidable components of food and beverages of plant origin, they attributed to their taste and palatability by their more or less pronounced astringency. Vegetable tannins were early recognized also as valuable chemicals for manifold technical processes. Among these, one of the oldest and even in our days still important procedures is tannery, i.e., the conversion of raw hides to durable leather, and it is that method that lent its name to this group of natural plant products. Plant tannins are also used in the production of dyes and inks or as versatile medical in traditional folk medicine, particularly in East Asia (cf. Haslam et al., 1989; Haslam, 1996). The antioxidant, antimicrobial, antiviral and antitumor characteristics, as well as many other medically important properties of plant tannins, are currently intensively investigated worldwide in many laboratories (for reviews related to these topics, see Gross et al., 1999). Over the past decades, enormous progress has been achieved in elucidating the complex structures of myriads of different plant tannins, which provided an excellent basis for subsequent studies on the biosynthesis of these compounds. Experiments on this challenging question, focusing on the biogenetic routes to hydrolyzable tannins (one subclass of plant tannins; for structure definitions, see Section 2), were performed in the authors' laboratory by enzyme studies with cell-free extracts from leaves of pedunculate oak (*Quercus robur*, syn. *Quercus pedunculata*) or red oak (*Quercus rubra*), staghorn sumac (*Rhus typhina*) and fringe cups (*Tellima grandiflora*). The results of these efforts have enabled us to describe now the principles and many details of the pathways leading to these natural products.

2. Structures and definitions

Before discussing the biogenetic routes to hydrolyzable plant tannins it appears helpful to insert a few comments on the structural principles and conventional definitions used in this field. According to the already classical definition of Freudenberg (1920), plant tannins are commonly divided into two subclasses: (i) condensed tannins (*syn*. proanthocyanidins) which are of flavonoid origin (and which are not the subject of this review), and (ii) hydrolyzable tannins that can be described as esters of gallic acid (**1**) with a polyol (typically β-D-glucose). The most simple member of this group, 1-*O*-galloyl-β-D-glucopyranose (β-glucogallin, **2**), has been identified more than a century ago as a natural product. Increasing substitution of this monoester leads to the fully galloylated glucose derivative, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (**3**). The various mono to penta-substituted esters are often classified as 'simple' galloylglucoses to allow their discrimination from 'complex' galloylglucoses, or gallotannins proper. This nomenclature, however, neglects the fact that even tetra- and particularly pentagalloylglucose display pronounced tanning potentials. 'Complex' gallotannins, in contrast, are the result of further galloylation reactions at the pentagalloylglucose (**3**) core by which high-molecular metabolites are formed that can contain up to 10, and occasionally even more, galloyl residues. A typical representative of this group, the hexagalloylglucose 2-*O*-digalloyl-1,3,4,6-tetra-*O*-galloyl-β-D-glucopyranose (**4**), is depicted in Fig. 1. The existence of one or more depsidic *meta*-digalloyl (**5**) moieties is characteristic of these gallotannins. Alternatively, pentagalloylglucose (**3**) can be subject to oxidation reactions that form linkages between suitably orientated galloyl residues to yield 3,4,5,3′,4′,5′-hexahydroxydiphenoyl (HHDP) moieties, thus giving rise to the second subclass of hydrolyzable tannins known as ellagitannins (cf. tellimagrandin II (**6**) in Fig. 1). Upon hydrolysis, bound HHDP residues are liberated as free hexahydroxydiphenic acid (**7**) which undergoes spontaneous conversion to the dilactone, ellagic acid (**8**). Interestingly, this artifact now serves as the name for this class of natural products. It should be emphasized that the stereochemistry of the central glucopyranose moiety determines which galloyl residues can undergo transformation to HHDP residues. The energetically preferred $^4C_1$-conformation (as in Fig. 1) allows 2,3- and 4,6-linkages of adjacent galloyl groups, while 1,6-, 3,6- and 2,4-*O*-HHDP bridges are obtained from the less stable $^1C_4$-conformer which thus enforce maintenance of this configuration (reviewed in detail by Haslam, 1989, 1998). Besides such intramolecular bonding, monomeric ellagitanins have also a strong tendency for subsequent intermolecular oxidative

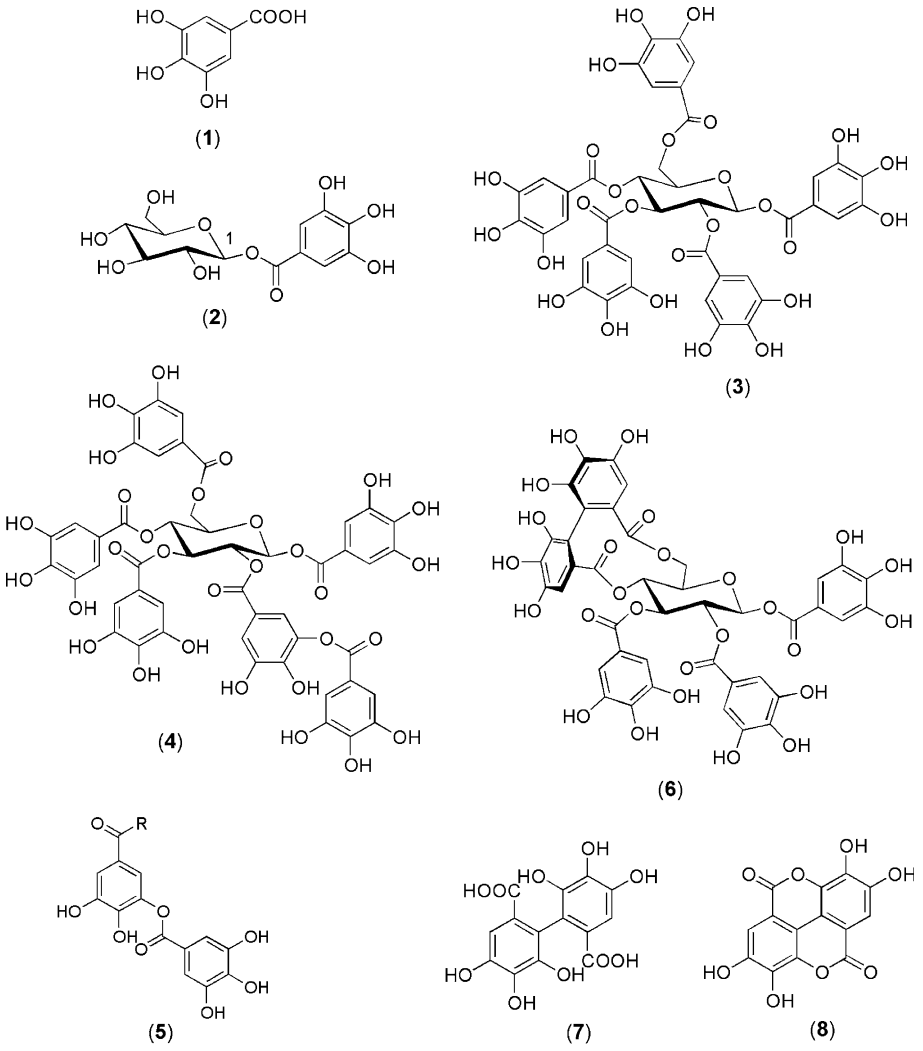

Fig. 1. Structural characteristics of hydrolyzable tannins. (**1**) Gallic acid, the principal phenolic unit of gallotannins and ellagitannins; (**2**) 1-*O*-galloyl-β-D-glucopyranose (β-glucogallin); (**3**) 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose, the immediate precursor of both gallotannins and ellagitannins; (**4**) 2-*O*-digalloyl-1,3,4,6-tetra-*O*-galloyl-β- D-glucopyranose, as an example of a hexagalloylated gallotannin; (**5**) *meta*-digalloyl residue, characteristic of 'complex' gallotannins; (**6**) tellimagrandin II, a typical monomeric ellagitannin; (**7**) 3,4,5,3′,4′,5′-hexahydroxydiphenic acid; (**8**) ellagic acid, the dilactone of (**7**).

processes that form various phenolic C–C and C–O linkages. A host of dimeric and oligomeric ellagitannins is produced by this means; already more than10 years ago, the existence of more than 150 ellagitannin dimers, trimers and tetramers has been reported (Okuda et al., 1993).

3. The pathway to pentagalloylglucose – biosynthesis of 'simple' galloylglucoses

It is the main purpose of this paper to review the biochemical transitions of pentagalloylglucose (**3**) to gallotannins and ellagitannins, respectively. The preceding pathway from gallic acid to pentagalloylglucose as the common and immediate precursor of the two classes of hydrolyzable tannins is therefore only briefly discussed here, with presentation of only some essential findings to provide a basis for the easier understanding of subsequent sections. More details on this subject have been published recently (Grundhöfer et al., 2001); for a comprehensive treatise, see Gross (1999). The origin of gallic acid (**1**), the starting compound in this reaction sequence, has been a matter of debate for decades. First conclusive results were obtained after feeding [^{13}C]glucose to the fungus *Phycomyces blakesleeanus* and to the dicotylodoneous plant *R. typhina.* NMR spectroscopy of the isotope distribution of gallic acid and aromatic amino acids isolated from these species, followed by interpretation of the isotopomer patterns of the metabolites by a 'retrobiosynthetic' approach, showed that gallic acid was derived from an early intermediate

of the shikimate pathway, most likely 5-dehydroshikimate (Werner et al., 1997). Quite recently, this interpretation was corroborated by measuring δ^{18}O-values of gallic acid from *R. typhina* leaves, indicating its formation by dehydrogenation of 5-dehydroshikimate and excluding alternative routes via phenylpropanoid C_6C_3-intermediates (Werner et al., 2004). Supporting evidence for this view was obtained by Ossipov et al. (2003) which reported the enzymatic reduction of 5-dehydroshikimate to gallate by enzyme extracts from leaves of *Betula pubescens*.

Esterification of gallic acid and glucose to yield β-glucogallin (1-*O*-galloyl-β-D-glucose, **2**), the first specific metabolite in the route to hydrolyzable tannins, was catalyzed by enzyme extracts from oak leaves with UDP-glucose serving as activated substrate (Gross, 1983a). These enzyme preparations were found to catalyze also the transformation of in situ formed β-glucogallin to di- and trigalloylglucoses whithout any further cofactors, indicating that β-glucogallin exerted a dual role, functioning not only as acyl acceptor but also as efficient acyl donor (Gross, 1983b). The latter would require that β-glucogallin had the then unexpected characteristic of an energy-rich 'activated' compound. This interpretation was supported by the finding of Mock and Strack (1993) that a related ester, 1-*O*-sinapoyl-β-D-glucose, had an unexpectedly high δ G_o of 35.7 kJ mol^{-1}, i.e., a value that is comparable to that of acyl-CoA thioesters.

Further studies with enzymes isolated from oak or sumac revealed that this reaction mechanism applied to the entire route from β-glucogallin (**2**) to pentagalloylglucose (**3**). As depicted in Fig. 2, substitution of glucose hydroxyls was not randomly distributed in these conversions but displayed an unexpected extreme specificity, thus constituting the metabolic sequence β-glucogallin (**2**) → 1,6-digalloylglucose (**9**, Schmidt et al., 1987) → 1,2,6-trigalloylglucose (**10**, Gross and Denzel, 1991) → 1,2,3,6-tetragalloylglucose (**11**, Hagenah and Gross, 1993) → 1,2,3,4,6-pentagalloylglucose (**3**, Cammann et al., 1989). Interestingly, an identical pattern has been reported for the chemical substitution of glucose in experiments with 1-benzyl or 1-methyl-β-D-glucopyranose which was explained as a combination of reactivity differences according to variations in chemical nature (primary vs. secondary hyroxyls), neighbor-activation effects and steric hindrance (Williams and Richardson, 1967; Reinefeld and Ahrens, 1971).

Most of the above enzymes were isolated from oak (*Q. robur*, *Q. rubra*), while sumac (*R. typhina*) was used only sporadically as an enzyme source. Supplementary experiments were carried out, however, which determined that the pathway to pentagalloylglucose was identical in both plant groups. Comparison of the data on the galloyltransferases involved in this metabolic route revealed a pronounced uniformity of their basic properties, e.g., their optimal reactivity and stability in slightly acidic media (pH 4–6), their low Q_{10} values

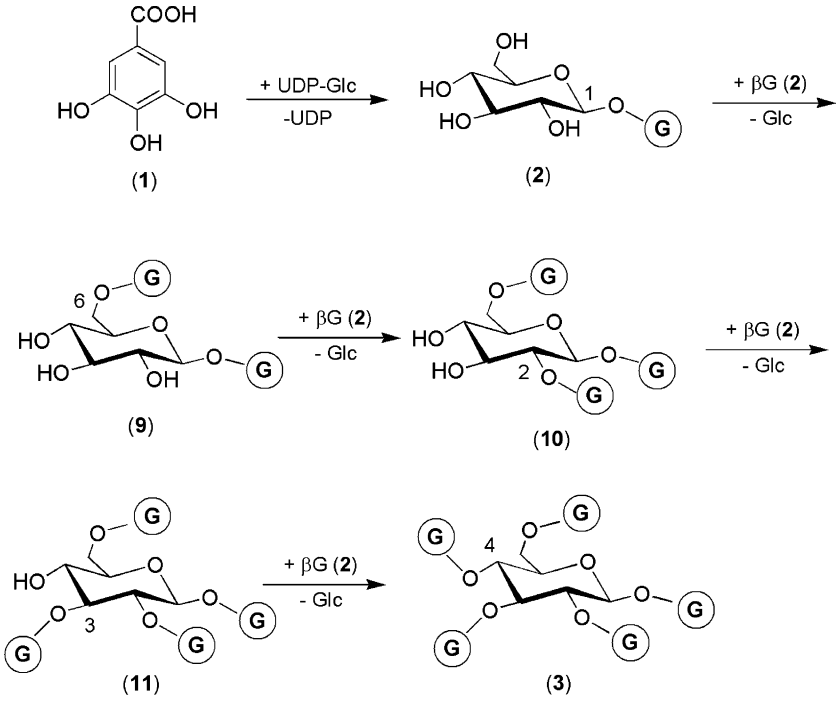

Fig. 2. Enzyme reactions catalyzing the pathway from gallic acid (**1**) to 1,2,3,4,6-pentagalloylglucose (**3**). (**2**) β-Glucogallin (1-*O*-galloyl-β-D-glucopyranose); (**9**) 1,6-di-*O*-galloyl-β-D-glucopyranose; (**10**) 1,2,6-tri-*O*-galloyl-β-D-glucopyranose; (**11**) 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose. G, galloyl residue.

(1.8–2.0) and sensitivity against higher temperatures, their unusual cold tolerance, as expressed by residual reaction rates of 10–25% even at 0 °C, and their pronounced trend to unusually high molecular weights of about 260,000–450,000 daltons (only the β-glucogallin forming galloyltransferase with M_r 68,000 does not fit into this scheme).

4. Biosynthesis of gallotannins

The transition from 'simple' galloylglucoses to complex gallotannins is marked by the addition of further galloyl residues to 1,2,3,4,6-pentagalloylglucose (**3**) to yield their characteristic *meta*-depside groups (**5**). Formally, this process can be regarded as a continuation of the esterification reactions of the preceding steps; it must be emphasized, however, that gallic acid now combines with phenolic hydroxyls whose chemical properties are significantly different from those of the aliphatic OH-groups of glucose. It was thus interesting to discover that cell-free extracts from sumac (*R. typhina*) leaves catalyzed the acylation of pentagalloylglucose exactly according to the same reaction mechanism as described above, i.e., by utilizing β-glucogallin (**2**) as a specific galloyl donor. In these studies, sequential galloylation of pentagalloylglucose (**3**) to hexa-, hepta- and octagalloylglucoses was observed (Fig. 3; Hofmann and Gross, 1990). Additional evidence for the presumed gallotannin nature of the reaction products arose from degradation studies with purified products. Treatment with fungal tannase resulted in complete hydrolysis, leaving gallic acid (**1**) as the sole phenolic component. Methanolysis (which exclusively cleaves depside bonds) afforded 1,2,3,4,6-pentagalloylglucose (**3**) and methyl gallate in the expected molar ratios of 1:1, 1:2 and 1:3, respectively.

Closer analysis by normal-phase HPLC on silica gel showed that a full range of gallotannins with substitution degrees up to decagalloylglucoses had been formed in this experiment (Fig. 4), while reversed-phase (RP) HPLC revealed that each of these galloylglucose groups was a mixture of 3–4 isomers of the same substitution level. Conclusive proof of the proposed reactions was obtained when three hexagalloylglucoses and three heptagalloylglucoses were isolated from scaled-up enzyme assays by chromatography on Sephadex LH-20 and purified in mg quantities by semi-preparative RP-HPLC. Their structures, depicted in Fig. 6 (**4**–**9**), were unequivocally identified by ^{1}H and ^{13}C NMR spectrometry. Interestingly, the substitution pattern and the relative amounts of these in vitro reaction products from *R. typhina* closely resembled those of in vivo formed gallotannins in the related species *R. semialata* (Nishizawa et al., 1982). It appears to be a particular feature of 'Chinese gallotannin' from sumac that the C-1 and C-6 positions generally remain free of depside residues, in contrast to the gallotannins from *Q. infectoria* ('Turkish gallotannin'; Nishizawa et al., 1983a) or *Paeonia lactiflora* (Nishizawa et al., 1980, 1983b) where depside substituents are also found at C-6 of the glucose core.

It is evident from the above data that a multitude of different gallotannins is formed by one common, general reaction mode. The question arose whether these transformations were catalyzed by only one (or eventually a few) unspecific enzymes or if specific enzymes were involved in the individual biosynthetic steps. Investigations regarding this problem led to the isolation of three β-glucogallin-dependent galloyltransferases from crude leaf extracts of sumac (A, B and C, according to their elution pattern upon gel-filtration; Fig. 5) that preferentially acylated pentagalloylglucose and hexagalloylglucoses (Niemetz and Gross, 1998, 1999, 2001). These enzymes had been detected in screening

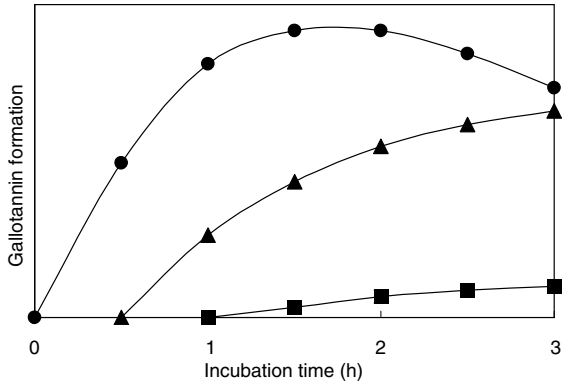

Fig. 3. Enzymatic synthesis of complex gallotannins by galloylation of 1,2,3,4,6-pentagalloylglucose (**3**) in the presence of β-glucogallin (**2**) with crude enzyme extracts from leaves of *Rhus typhina*. (●) Hexa-, (△) hepta-, (■) octagalloylglucoses.

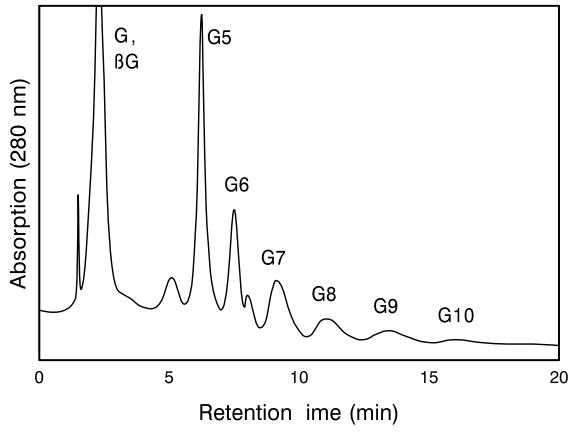

Fig. 4. Normal-phase HPLC (Si-60 silica-gel) of gallotannins formed from 1,2,3,4,6-pentagalloylglucose (**3**) with crude enzyme preparations from leaves of *Rhus typhina*. G, Gallic acid (**1**); βG, β-glucogallin (**2**); G5-G10, penta- to decagalloylglucoses.

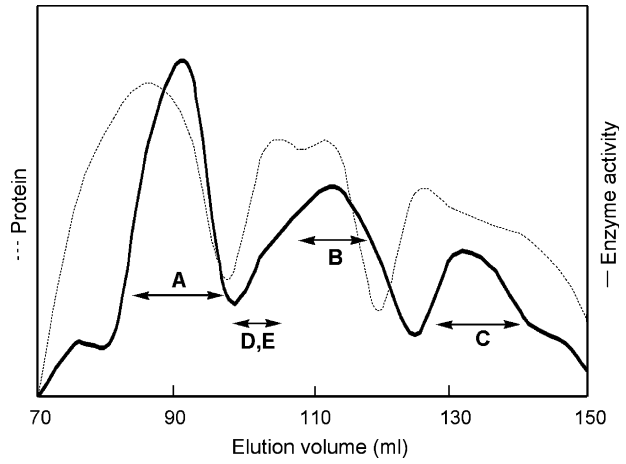

Fig. 5. Separation of gallotannin forming galloyltransferases from sumac leaves by gel-filtration on Sephacryl S-300. (—) Enzyme activity; (...) protein (UV 280 nm); both scales in arbitrary units. Fractions containing galloytransferases A–E were pooled as indicated by double arrows and further purified.

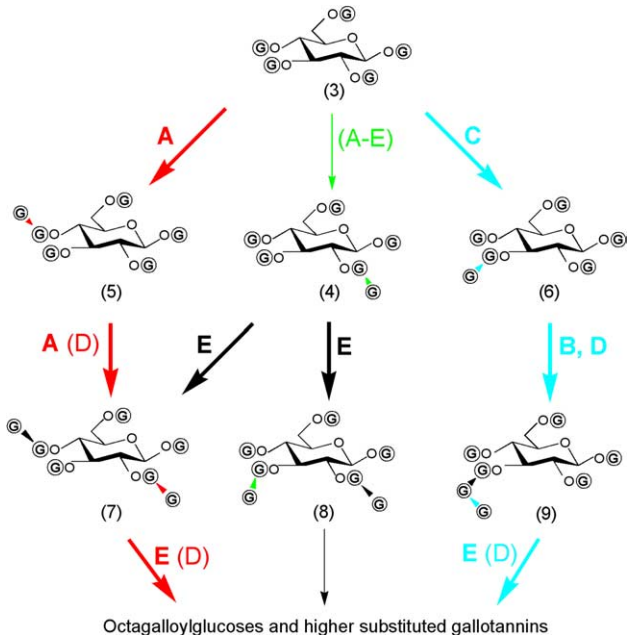

Fig. 6. Metabolic routes to gallotannins in staghorn sumac (*Rhus typhina*). Main reactions are marked by bold arrows, minor reactions by thin arrows. Enzymes: galloyltransferase A (Niemetz and Gross, 1999), galloyltransferase B (Niemetz and Gross, 2001), galloyltransferase C (Niemetz and Gross, 1998), galloyltransferases D, E (Fröhlich et al., 2002). Major enzyme activities are symbolized by bold letters, minor activities by plain letters in parentheses. Linkages to newly introduced galloyl groups are highlighted in the color of the enzyme catalyzing this step. G, Galloyl; G▲G, *meta*-digalloyl residue (**2**).

experiments with pentagalloylglucose (**3**) as standard acceptor substrate. However, substantial hints on the existence of additional enzymes were obtained in these investigations that pointed to the specific acylation of higher substituted gallotannin substrates. Screening experiments based on this observation with a hexagalloylglucose substrate (2-*O*-digalloyl-1,3,4,6-tetragalloyl-β-D-glucose **4**, cf. Fig. 6) led to the detection of two further galloyltransferases (named D and E) which displayed pronounced affinity towards hexa- and heptagalloylglucoses as substrates (Fröhlich et al., 2002).

All five enzymes could be highly purified from sumac leaves (apparent homogeneity was achieved for transferases A–C); some general properties are given in Table 1. Of particular interest were the substrate and product specificities of the galloyltransferases. It was found that none of the enzymes displayed high specificity but, on the basis of $v_{max}K_m^{-1}$ calculations, it was evident that each enzyme had a preferred substrate and also a major product. Combining the recently available data, it appears now appropriate to propose a scheme that illustrates the major trends in the formation of gallotannins in staghorn sumac (*R. typhina*) and that certainly applies also to the related species *R. semialata*.

As depicted in Fig. 6, several enzymes with different affinities towards penta-, hexa- and heptagalloylglucose substrates cooperate in producing the various gallotannins occurring in these plants. The few steps in that scheme which to date cannot be attributed to specific enzymes (for instance, the transition of pentagalloylglucose **3** to hexagalloylglucose **4**) could well be catalyzed in sufficient rates by the cooperation of several less active enzymes. However, it cannot be excluded that these missing specific galloyltransferases have not yet been discovered. To any event, the above expressed question, i.e., on the existence of one or a few unspecific galloyltransferase(s) vs. a greater number of more or less specific enzymes synthesizing gallotannins, appears to have been answered in favor of the second alternative.

Table 1
Comparison of gallotannin synthesizing β-glucogallin-dependent galloyltransferases (EC 2.3.1.-) from sumac leaves

Enzyme	MW (kDa)	Optimal pH	Main substrate	Main product
Transferase A	360	4.8	4-*O*-Digalloyl-1,2,3,6-tetra-*O*-GG (**5**)	2,4-Di-*O*-digalloyl-1,3,6-tri-*O*-GG (**7**)
Transferase B	260	5.6	3-*O*-Digalloyl-1,2,4,6-tetra-*O*-GG (**6**)	3-*O*-Trigalloyl-1,2,4,6-tetra-*O*-GG (**9**)
Transferase C	170	4.0–4.5	1,2,3,4,6-Penta-*O*-GG (**3**)	3-*O*-Digalloyl-1,2,4,6-tetra-*O*-GG (**6**)
Transferase D	300	6.0	3-*O*-Digalloyl-1,2,4,6-tetra-*O*-GG (**6**)	3-*O*-Trigalloyl-1,2,4,6-tetra-*O*-GG (**9**)
Transferase E	300	6.0	3-*O*-Trigalloyl-1,2,4,6-tetra-*O*-GG (**9**)	Octa-GG (unidentified substitution)

GG, galloylglucose.

5. Biosynthesis of ellagitannins

In contrast to the rather limited distribution of gallotannins in nature, ellagitannins are typical constituents of many plant families. As briefly discussed in Section 2, these compounds display an enormous structural variability due to the manifold possible sites for the linkage of HHDP (**7**) residues with the glucose moiety, and particularly by their strong tendency to form dimeric and oligomeric derivatives. This structural diversity, together with their recently recognized role as promising chemotherapeutic agents (cf., e.g., Okuda et al., 1989a), has attracted the interest of chemists for decades, culminating in recent successful efforts on the extreme challenge of synthesizing ellagitannins by chemical means (for a recent comprehensive review, see Khanbabaee and van Ree, 2001).

Progress in the study of the biosynthesis of ellagitannins was significantly slower. Already in the 1930s it was postulated that the HHDP (**7**) residues of ellagitannins originated from the dehydrogenation of gallic acid esters (Erdtman, 1935). Twenty years later, Schmidt and Mayer (1956) explicitly proposed 1,2,3,4,6-pentagalloylglucose (**3**) as the immediate precursor of ellagitannins that should be produced by oxidative biaryl coupling of neighboring galloyl groups, a view that was corroborated and refined later by Haslam and coworkers (cf., e.g., Haslam, 1989). Many attempts to unravel the mechanism of ellagitannin biosynthesis have been carried out in the past by experiments with chemical oxidants (e.g., O_2, Fe^{3+}) or by studies with the enzymes laccase and peroxidase, using gallic acid, methyl gallate, β-glucogallin, 3,6-digalloylglucose or pentagalloylglucose as substrates. Only free ellagic acid (**8**), however, was detected in these experiments while the formation of true ellagitannins, characterized by glucose-bound HHDP residues, was never observed (references in Gross, 1999).

We concluded that inadequate analytical techniques represented the decisive obstacle in such investigations, that suffered from minimal enzyme reaction rates yielding numerous structurally closely related reaction products and unspecific by-products, and particularly from the inherent risk of contamination with in vivo formed ellagitannins that had been transferred into the enzyme assays by complexation with protein due to their tanning potential. Such problems are eliminated by using radioactively labeled compounds, a technique that dramatically increases both the sensitivity and specificity of test systems. We therefore produced [*U*-^{14}C]pentagalloylglucose by photoassimilation of $^{14}CO_2$ in leaves of sumac (Rausch and Gross, 1996). This compound was used as standard substrate in an extended screening program for enzymes that formed reaction products liberating [^{14}C]ellagic acid (**8**) upon hydrolysis, thus providing a general probe for the in vitro synthesis of ellagitannins of widely differing structures.

By this strategy we were able to discover a novel soluble enzyme in leaves of *T. grandiflora* (fringe cups, Saxifragaceae), a weed that is known as a rich source of ellagitannins. As depicted in Fig. 7, a partially purified enzyme preparation was found to catalyze the conversion of [*U*-^{14}C]pentagalloylglucose to several radioactively labeled products, while no evidence for the formation of ellagic acid (**8**) was obtained. The most prominent peak among the reaction products co-eluted with authentic tellimagrandin II (**6**), thus providing the long sought first hint on the in vitro synthesis of a compound that traditionally has been proposed as the primary metabolite in the biosynthesis of $^4C_1$-pentagalloylglucose derived ellagitannins (Niemetz et al., 2001). Scaled-up enzyme assay mixtures with enzyme that had been purified to apparent homogeneity revealed the formation of a single reaction product that was unequivocally proven to be tellimagrandin II (**6**) by chemical degradation, negative FAB-MS and ^{1}H and ^{13}C NMR spectrometry (Niemetz and Gross, 2003a). Circular dichroism (CD) spectroscopy demonstrated that the HHDP residue of the enzyme reaction product had (*S*)-stereoconfiguration, which well correlated with published data for the naturally occurring compound (Gupta et al., 1982; Okuda et al., 1989b) and for synthetically obtained material (Feldman and Sahasrabudhe, 1999).

Analysis of side-products encountered in the above studies, obtained with crude enzyme preparations from *T. grandiflora* leaves, revealed the existence of a higher molecular weight compound that was later identified as cornusiin E (**10**, cf. Fig. 9; structure elucidated by Hatano et al., 1989), i.e., a dimeric ellagitannin that

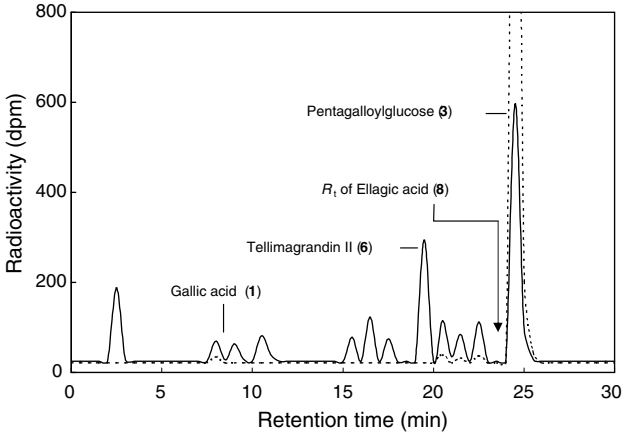

Fig. 7. Reversed-phase HPLC of the in vitro oxidation of [*U*-^{14}C]pentagalloylglucose (**3**) to the monomeric ellagitannin, tellimagrandin II (**6**), by a crude enzyme preparation from *Tellima grandiflora* leaves. (—) Enzyme assay; (...) control with acid-denatured enzyme. Gallic acid (**1**) was formed as a minor hydrolytically formed by-product. Note that no radioactivity was found at the retention time (R_t) of ellagic acid (**8**). For further details, see text.

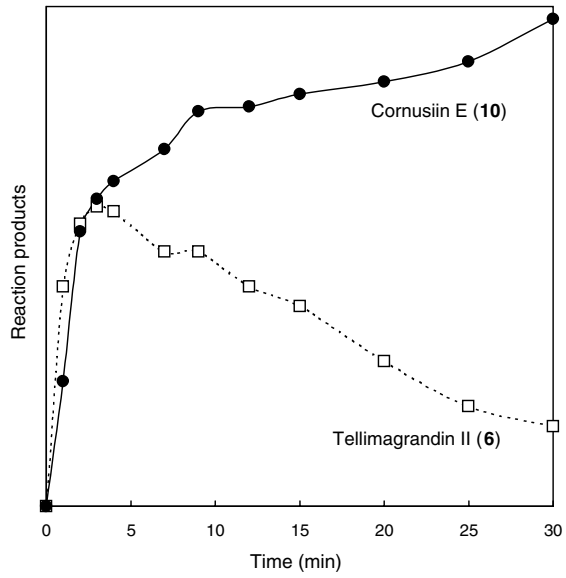

Fig. 8. Time course of the in vitro oxidation of pentagalloylglucose (**3**) by crude enzyme extracts from *Tellima grandiflora* to the monomeric ellagitannin, tellimagrandin II (**6**) (...□...), and subsequent dimerization of this intermediate to cornusiin E (**10**) (—●—).

must have resulted from the oxidative condensation of two molecules of tellimagrandin II (**6**). Analysis of this phenomenon in a time course experiment with [*U*-^{14}C]pentagalloylglucose corroborated the postulated intermediacy of tellimagrandin II. As depicted in Fig. 8, the pentagalloyllgucose (**3**) substrate was rapidly oxidized to monomeric tellimagrandin II (**6**). This product, however, could not accumulate because it was almost simultaneously transformed to dimeric cornusiin E (**10**). After ca. 3 min, net synthesis was observed only for this end product, at the expense of intermediate tellimagrandin II (Niemetz et al., 2003). The structure of cornusiin E (**10**), isolated from scaled-up assays with pure enzyme, was proven by MALDI-TOF analysis and ^{1}H and ^{13}C NMR spectrometry. Total acid hydrolysates of the reaction product revealed gallic, ellagic and valoneic acid (i.e., the triaryl bridge linking the two glucosyl moieties; cf. **10** in Fig. 9) as phenolic constituents, while sanguisorbic acid (an isomer of valoneic acid that occurs in several other ellagitannins) was not detected. (*S*)-Configuration of both the HHDP and valoneoyl residue was proven by CD spectroscopy, establishing the

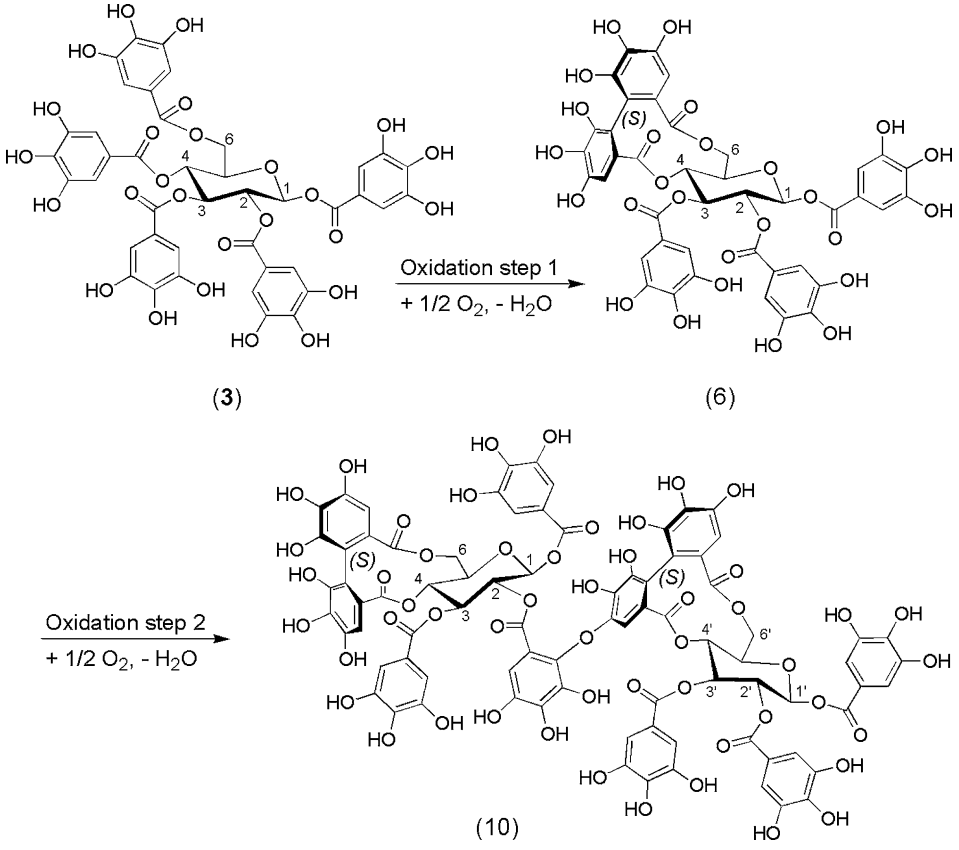

Fig. 9. Pathway from 1,2,3,4,6-pentagalloylglucose (**3**) to the dimeric ellagitannin, cornusiin E, (**10**) in *Tellima grandiflora* (fringe cups). In the first oxidation step, pentagalloylglucose is specifically oxidized to the monomeric ellagitannin, tellimagrandin II (**6**) by a laccase-type phenol oxidase. In the subsequent oxidation step 2, this monomeric ellagitannin is oxidatively dimerized to cornusiin E (**11**) under the catalysis of a second, different laccase. Both ellagitannins are typical constituents of *Tellima grandiflora*.

identity of in vivo and in vitro formed cornusiin E (**10**). This compound is characterized by a 2,4′,6′-linked valoneoyl bridge (cf. Fig. 9). A related dimer, rugosin D, which is characterized by a 1,4′,6′-bridge (Hatano et al., 1990) has been claimed to occur in *T. grandiflora* (Haslam, 1989). The results of our studies, based on enzyme studies and phytochemical analyses, do not lend any support to the existence of rugosin-type ellagitannin dimers in *T. grandiflora*. Instead, it must be concluded that metabolic activities in this plant are specifically directed towards the biogenesis of cornusiin-type (i.e., 2,4′,6′-linked) ellagitannin dimers (Niemetz et al., 2003).

As already mentioned, both enzyme activities involved in the route from pentagalloylglucose to the dimeric ellagitannin, cornusiin E, have been purified to apparent homogeneity (Niemetz and Gross, 2003a,b). Determination of their basic properties, summarized in Table 2, revealed significant differences that verify that the two enzymes catalyze similar reactions but are physically different.

Finally, investigations on the nature of the enzymes catalyzing the above reported transformations should be briefly discussed. Both oxidative steps (pentagalloylglucose → tellimagrandin II → cornusiin E) were found to be oxygen dependent but did not require further cofactors. Hydrogen peroxide was a strong inhibitor, suggesting that the enzymes were no peroxidases. Carbon monoxide, in contrast, had virtually no effect; together with the absence of any requirement for NADPH and the lacking association with the microsome fraction it was concluded that these enzymes did not belong to the cytochrome P450-depending monooxygenases but were a member of the vast class of oxygen dependent (poly)phenol oxidases. This enzyme class is commonly divided into (i) *o*-diphenol: O_2 oxidoreductases (EC 1.10.3.1) which are known under several trivial names (e.g., *o*-diphenol oxidase, catecholase, etc.) and that also include the 'tyrosinases' (EC 1.14.18.1), and (ii) the related *p*-diphenol: O_2 oxidoreductases (EC 1.10.3.2). The latter group is known as 'laccases' which widely occur in fungi, while they have been characterized less frequently in higher plants (Mayer and Staples, 2002). One approach to discriminate between these two enzyme groups is the use of selective inhibitors. Experiments with various inorganic chemicals, phenylacrylic acids, chelators, detergents and sulfur compounds (for details, see Niemetz and Gross, 2003a,b) lead to the conclusion that both the pentagalloylglucose oxidizing enzyme (trivial name: pentagalloylglucose: O_2 oxidoreductase) and its tellimagrandin II oxidizing counterpart (tellimagrandin II: O_2 oxidoreductase) belong to the laccase (EC 1.10.3.2) subgroup of phenol oxidases. The reactions catalyzed by these two enzymes are depicted in Fig. 9.

Table 2
Properties of pentagalloylglucose (**3**) and tellimagrandin II (**6**) oxidizing laccases (EC 1.10.3.2) from fringe cups (*Tellima grandiflora*)

	Pentagalloylglucose: O_2 oxidoreductase[a]	Tellimagrandin II: O_2 oxidoreductase[b]
pH Optimum	4.5	5.2
Temperature optimum (°C)	45	22
Activation energy (kJ mol^{-1})	55	116
Q_{10}	2.0	5.5
Isoelectric point	6.3	5.2
Molecular weight, native (kDa)	60	160
Molecular weight, subunits (kDa)	60	40
K_m (μM)	80	8.5
v_{max} (μmol s^{-1})	75	44

[a] Reaction product: tellimagrandin II (**6**).
[b] Reaction product: cornusiin E (**10**).

6. Conclusions and perspectives

The results of numerous enzyme studies that have been conducted in the authors' laboratory over the past two decades enable us now to draw a rather detailed picture that illustrates the metabolic routes from gallic acid (**1**), the principal phenolic moiety, to 1,2,3,4,6-pentagalloylglucose (**3**) and to the tannins that are derived from this important precursor. In accordance with old hypotheses that had been formulated on the mere basis of structural considerations, the central role of this compound in the biosynthesis of gallotannins and ellagitannins has been proven unequivocally. With respect to gallotannins, which are characterized by a rather simple and uniform biogenetic pattern, much data is available now to explain many details of their biogenetic relationships; it thus appears inappropriate to date to address further extensive efforts to this facet. Regarding ellagitannins, the current situation is less comfortable despite the fact that two important biogenetic steps could be elucidated recently. This has been a very promising beginning, but too many significantly differing structural principles have been recognized for this class of natural products as that their biogenetic relations could be explained only on the basis of the limited evidence available to date. Besides biochemical efforts to find enzymes catalyzing the synthesis of other linkage types and of oligomeric ellagitannins, it appears important to put more emphasis also on physiologically orientated aspects in future research, e.g., on seasonal variation of metabolite concentrations and enzyme activities.

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