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Review

From lignins to tannins: Forty years of enzyme studies on the biosynthesis of phenolic compounds

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Abstract

In the early 1960s, enzyme studies increasingly began to replace the common 'feeding' experiments in which labeled tracers were applied to living plants or plant parts for elucidating metabolic pathways. This advanced technique allowed to gain much deeper insights into individual details of metabolic sequences, and particularly on the previously inaccessible role of activated 'energy-rich' intermediates. Based on the author's own experience for the past 40+ years in this field, principal findings and trends elucidating the pathways to lignin and lignin precursors, acyl amides and hydrolyzable tannins (gallotannins, ellagitannins) by enzyme studies are reported.

Keywords: Biosynthesis; Enzymology; Carboxyl-activated 'energy-rich' intermediates; Cinnamoyl-coenzyme A thioesters; Lignin; Piperine; Tannin

Contents

1. Introduction

For at least two decades, from the late 1940s to the 1960s, the so-called 'feeding' experiments with labeled

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precursors were the standard technique for analyzing metabolic pathways. In the early 1960s, enzyme studies began to gain increasing importance and later largely replaced these tracer experiments. Such enzyme studies allowed, at least in theory, achievement of deeper insights into previously inaccessible details of reaction steps and to understand the nature and role of activated 'energy-rich' intermediates that were proposed by thermodynamic considerations or by analogies to already known pathways. Often overlooked was the new chance of checking the plausibility of data against background reaction rates with 'blank' assays. Nevertheless, enzymology with higher plants was a largely frustrating endeavor in its infancy due to not yet developed plant-specific methods, plus the generally still meager availability of efficient and precise preparative and analytical methods. Fortunately, these obstacles were overcome in comparatively short time, to develop enzymology to the powerful and extensively used scientific tool that represents an indispensable standard method in our recent days for the examination of metabolic routes in higher plants.

It was my good fortune, as a graduating student, that my first contacts with the biochemistry of plant phenolics coincided with the onset of this decisive methodological transition. Moreover, I had the rare chance to stay active in this field over decades to personally experience the growing impact of enzymology in plant sciences, and I am also glad that I was able to contribute with some facets of my own research to this fascinating discipline. On the basis of that long lasting familiarity with the biosynthesis and metabolism of phenolic compounds, principal problems, findings and trends in this field over the past 40+ years will be reviewed here, addressing lignin precursors, phenolic amides and hydrolyzable tannins. Particular attention will be paid to the role of 'energy-rich' carboxyl-activated intermediates in these pathways.

2. Retrospect: plant biochemistry techniques in the early 1960s

For many years, 'feeding' experiments were the most common technique to analyze metabolic sequences. By this method, pathways were studied by administering radioactively labeled putative precursor compounds (typically containing ^{14}C or ^{3}H atoms, occasionally also ^{32}P or ^{35}S) to whole plants or plant parts. Next, investigated compounds were isolated from this plant material and purified. Determination of total radioactivity and, if required, of the specific position of labeled atoms in these molecules after their suitable degradation provided the basis for the construction of feasible metabolic routes. 'Double labeling' experiments with known ratios of ^{3}H and ^{14}C in the precursor (usually 10:1, to compensate for the low radiation energy of tritium) often gave additional valuable insights. This methodology had the advantage of requiring only commonly available chemistry equipment and know-how, plus some expertise in working with radioactive isotopes. Moreover, only rather simple separation methods and cheap analytical instrumentation were employed. Initially, paper chromatography was a major tool to separate and identify reaction products. The much more powerful thin layer chromatography (TLC) technique, utilizing silica gel coatings on inert solid supports, was brought to satisfying perfection only in the early 1960s (cf. the epochal books of Stahl, 1962; and Randerath, 1962 that soon became standard equipment of most laboratories). Commercially produced TLC plates were available only in the late 1960s; until then, personal skills were required to prepare TLC plates of consistent performance. Advanced, reliable separation techniques like high-performance liquid chromatography (HPLC) were not even thought in these days. Being developed in the mid-1970s and quickly improved with the development of new column packing materials (e.g. hydrophobic 'reversed phase' adsorbents) plus additional convenience of on-line detectors up to MS and NMR coupling, HPLC became an indispensable and efficient standard tool in all laboratories. Achieving the results described in Section 6 of this paper would have been clearly impossible without analytical and preparative HPLC.

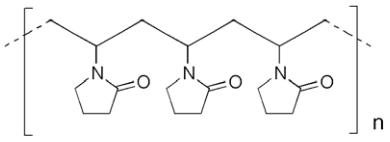
Radioactivity of 'spots' of precursors and products on chromatograms was usually detected by autoradiography, i.e. by exposing Roentgen-film sheets to the radioactivity of developed paper chromatograms. Position and intensity of spots on the film served as qualitative and semi-quantitative measures. Significant advantage was provided by radioactivity counting of eluted material in Geiger–Mueller counting tubes and particularly by liquid-scintillation counters available in the late 1960s.

Solid fundamental data to describe the principles of a multitude of pathways could be gained by this approach. However, the method did not allow feeding of labile or membrane-impermeable compounds and particularly suffered from the inherent inability to analyze reaction details, including the important role of 'energy-rich' activated intermediates. Another often discussed (but probably over-stressed) issue was the risk of microbial contamination in such experiments. In consequence, it was evident that biochemical routes could be *proposed* by feeding experiments, but could not be unequivocally *proven*.

In this situation, enzyme studies were thought to be an excellent tool to overcome these limitations. Serious practical problems, however, were encountered in the isolation of active enzymes from higher plants, e.g. (i) limited availability of metabolically specialized tissues or cells, (ii) rigid plant cell walls, requiring harsh homogenization methods, (iii) low protein contents of plant cells, causing meager recoveries of solubilized proteins and enzymes, and (iv) huge vacuoles in adult plant cells being overload with phenolics and organic acids known to inactivate and/or precipitate soluble enzymes. Also the available armament of separation techniques was fairly limited in these days, comprising only rather ineffective or drastic methods like gel adsorption or precipitation by ammonium sulphate, heat,

acid treatment or organic solvents. More advanced techniques utilizing column chromatography were just in their infancy. The principles of gel chromatography on Sephadex, for instance, were published only in 1959 by Swedish researchers (Porath and Flodin, 1959) and it took more time until commercial products were available and in common use. Similar limitations applied also to many other separation media designed for the purification of enzymes, like cation (e.g. carboxymethyl [CM]) or anion (diethylaminoethyl [DEAE]) ion exchange adsorbents. Due to such limitations, advantage in the enzymology of higher plant metabolism was meager in the early to mid-1960s, relevant studies remaining mostly confined to rather uncritical sources, e.g. spinach leaves characterized by comparatively soft tissues and the absence of troublesome phenolics.

Enormous progress, however, occurred in the late 1960s and early 1970s when advanced methods and new agents were introduced that allowed the efficient extraction of highly active enzymes from plant tissues. These new tools included, e.g. (i) homogenization of plant tissues in liquid nitrogen, allowing the mild, low-temperature disruption of cells under anaerobic conditions, (ii) use of borate buffers that form complexes with vicinal diols as typical constituents of enzyme-inhibiting plant phenols, or (iii) recognizing the essential importance of strict pH-monitoring and pH-readjusting of plant cell homogenates during their extraction (most plant tissues release incredible amounts of acids that would cause a perfect acid precipitation of all solubilized proteins). Addition of antioxidants like 2-mercaptoethanol or the more powerful agents dithioerythritol (DTE) or dithiothreitol (DTT, Cleland's reagent) provided additional benefits in many cases. Last, but not least, special credits have to be addressed to the fundamental paper of Loomis and Battaile (1966) for introducing insoluble polyvinylpyrrolidone (**1**, PVP, polyclar AT; structure in Fig. 1) into higher plant enzymology. This chemical, that originated from the American brewing industry as an efficient phenol-binding agent to reduce chill-haze in beers, proved to be an extremely powerful tool in the preparation of cell-free enzyme extracts from plants. It thus immediately became a still indispensable component of enzyme extraction mixtures.



(**1**)

Fig. 1. Structure of polyvinylpyrrolidone (**1**, PVP, polyclar AT).

3. Biosynthesis of monolignols

One of the most challenging research problems in plant biochemistry of the 1960s and 1970s was the question how lignin, the cell wall impregnating polyphenolic macromolecule of vascular plants, was synthesized in vivo. Decades of research, with the German chemist Karl Freudenberg in Heidelberg in the front line, had led to well founded proposals on the structure of this complex macromolecule in conifers. This solid basis stimulated investigations on the biosynthesis of this natural product in many laboratories, e.g. those of S.A. Brown, T. Higuchi or A.C. Neish to mention just a few prominent names in this field. A fascinating summary of the state of the art in the late 1960s was published by Freudenberg and Neish (1968). Then developed, and still valid, theories favored phenylalanine-derived cinnamic acids as principal precursors supposed to be reduced to the corresponding alcohols known as monolignols, the monomeric units of polymeric lignin. Oxidation of these monolignols and their subsequent polymerization should finally yield the lignin macromolecule (Fig. 2).

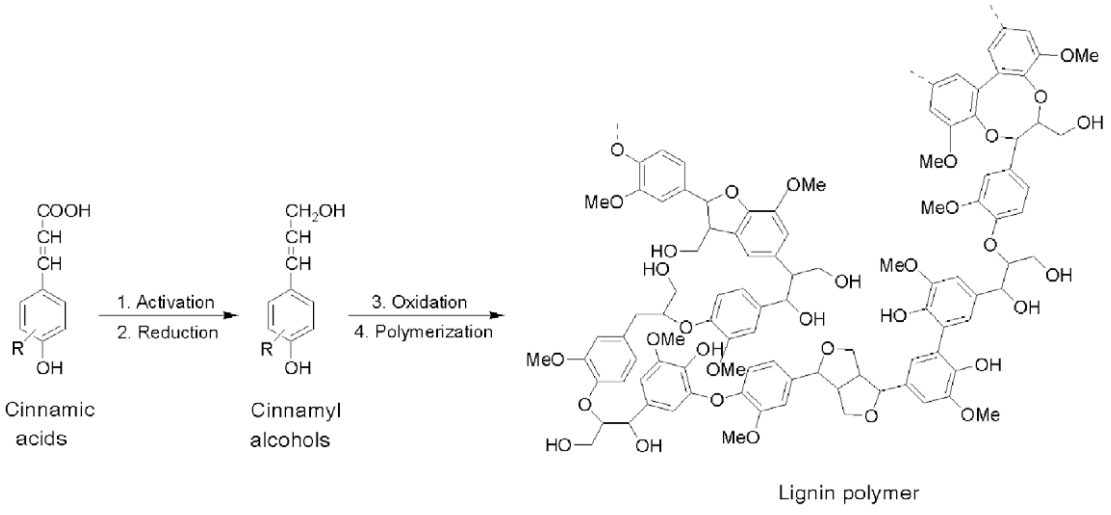


Fig. 2. Principal steps in the conversion of cinnamic acids to the lignin polymer. Lignin structure redrawn from Önnerud et al. (2002), depicting lignin according to the modern concept of a linear arrangement of lignol units. In the older literature, lignin is depicted as a 3-dimensionally cross-linked macromolecule.

An eminent question in this area regarded the nature of carboxyl-activated 'energy-rich' intermediates that had been postulated to be involved in these sequences as a result of thermodynamic considerations. By analogy to the already well understood metabolism of fatty acids, cinnamoyl-coenzyme A thioesters were considered as most promising candidates in the route to monolignols. As summarized in Fig. 3, such CoA esters were supposed to be involved also in many other pathways to numerous cinnamic acid derivatives, attributing cinnamoyl-CoA's a central role in the entire metabolism of phenolic compounds in higher plants.

3.1. Synthesis of cinnamoyl-coenzyme A thioesters

It is evident from the above considerations that availability of cinnamoyl-CoA esters, existing only in theory in the early 1960s, was an essential requirement for biosynthetic studies on the formation of monolignols. These thioesters were prepared and characterized for the first time by Gross and Zenk (1966), using an unspecific 'fatty acid activating enzyme' (FAAE) from beef liver mitochondria (Mahler et al., 1953; Zenk, 1960) that catalyzed a reaction requiring free acid, ATP and CoA as substrates to yield CoA esters together with AMP and diphosphate (PP_i) as by-products (the reaction is identical to that depicted in Fig. 7 for the analogous plant enzyme). With these CoA esters, the essential basis for later successful investigations on the transformation of cinnamic acids to cinnamyl alcohols in higher plants was laid (see Section 3.3). This enzymatic approach was particularly useful in small-scale preparations or to obtain labeled derivatives of high specific radioactivity. It is therefore still widely used until today, utilizing cinnamoyl-CoA ligases from various plants and microbial sources, including recombinant enzymes (Beuerle and Pichersky, 2002).

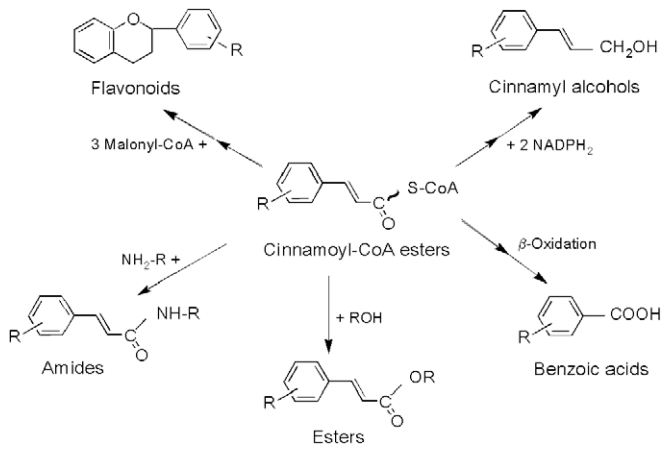


Fig. 3. The central role of cinnamoyl-coenzyme A thioesters in the metabolism of phenolic secondary plant products. This scheme was developed in the mid-1960s when all of the depicted reactions were plausible but still merely hypothetical; over the past decades, the correctness of these early assumptions was proven.

Increasing demand of large-scale preparations was answered by Stöckigt and Zenk (1975) by developing chemical methods for the synthesis of cinnamoyl-CoA esters. Cinnamoyl thiophenyl or *N*-hydroxysuccinimidyl derivatives were employed as intermediates for subsequent exchange reactions with free CoA-SH. The delicate free phenolic OH-groups of cinnamoyl moieties were blocked by *O*-glucosylation, and easy removal of this protecting group was afforded by treatment with β-glucosidase. The versatility of this chemical approach was documented by applying it to the synthesis of such different compounds as tropoyl-CoA (Gross and Koelen, 1980), galloyl-CoA (Gross, 1982) and piperoyl-CoA (**17**, Semler et al., 1987).

Maximal light absorbance of the thioester bond of cinnamoyl-CoA esters was in the long-wave UV region, ranging from ca. 311 to 355 nm according to the different substitution patterns of their aromatic rings (Gross and Zenk, 1966; Stöckigt and Zenk, 1975). As an example, the UV spectrum of feruloyl-CoA (**2**) is depicted in Fig. 4, displaying a thioester peak at 345 nm with the high ϵ-value of 19×10^6 in 1 molar solution. For comparison, aliphatic CoA esters absorb at 230–240 nm, benzoyl-CoA's around 260 nm, and piperoyl-CoA (**7**) (cf. Section 5) with two double-bonds in the C_5-side-chain has a maximum at 368 nm. This particular characteristic of cinnamoyl-CoA spectra allowed the facile and sensitive photometric monitoring of their in vitro formation or consumption without

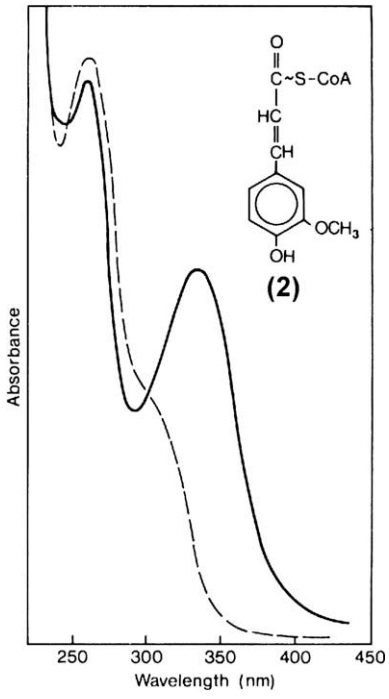


Fig. 4. UV spectrum of feruloyl-CoA (**2**). (—) Spectrum in 0.1 M phosphate buffer, pH 7.0; (--) spectrum after hydrolysis in 0.1 N NaOH and re-neutralization. The thioester linkage absorbs maximally at 345 nm. The peak at 260 nm corresponds to the adenine moiety of CoA. The shoulder at 320 nm in the hydrolysis spectrum reflects light absorption of the free acid.

significant interference with other components from plant extracts that usually absorb light in the 260–300 nm range.

3.2. Reduction of phenolic acids in fungi

Considering the insufficient technical armament in plant enzymology available at the early 1960s, model reactions with enzymes from fungi appeared promising to achieve deeper insights into the mechanisms involved in the reduction of aromatic acids. In this context, studies on the reduction of veratric acid (3,4-dimethoxybenzoic acid) to veratryl aldehyde and alcohol were performed with enzyme extracts from a wood degrading basidiomycete, *Polystictus* (*Trametes*) *versicolor* (Zenk and Gross, 1965). Due to insufficient growth rates of this organism, phenolic acid reducing enzyme systems from the ascomycete *Neurospora crassa* were intensively investigated. This organism produced a salicylate (2-hydroxybenoic acid) inducible enzyme that reduced benzoic (**3**) and cinnamic acids to the corresponding aldehydes which, in turn, were reduced to the alcohol level by a constitutive conventional alcohol dehydrogenase. It was found that the reduction of acids in fungi strictly depended on the presence of ATP and $NADPH_2$ as coenzymes but, in contrast to the working hypothesis, did not involve any intermediate CoA esters. Instead, the enzyme, arylaldehyde: NADP oxidoreductase, catalyzed a 2-step reaction via an enzyme-bound 'energy-rich' acyladenylate intermediate (Fig. 5). Omitting $NADPH_2$, this enzyme-acyl-AMP complex accumulated in the reaction mixture and could be isolated by gel-filtration; it yielded free aldehyde upon addition of $NADPH_2$ as reductant (Gross, 1969, 1971, 1972). The observed reaction mechanism appears to occur only sporadically and is perhaps confined to fungi. Besides the *N. crassa* system, an analogous conversion has been found in yeast for the reduction of 2-aminoadipate (Sagisaka and Shimura, 1962; Sinha and Bhattacharjee, 1971).

3.3. Monolignol biosynthesis in plants

After the above intermezzo with fungal systems, that had provided interesting insights but no real clues to solve the old monolignol biosynthesis issue, a new start to tackle this problem was begun in the early 1970s. At this time, many of the advanced techniques in plant enzymology discussed in Section 2 had been developed, thus promising better options now for this renewed approach. Indeed, as depicted in Fig. 6, it was possible with these optimized techniques to isolate three novel enzymes (cinnamate: CoA ligase, cinnamoyl-CoA reductase and cinnamyl alcohol reductase) from lignifying tissue of *Forsythia suspensa* (golden bells) that catalyzed the long sought transition of ferulic acid to coniferyl alcohol, the predominant monomeric unit of lignins (Gross et al., 1973). With these findings one of the "most important gaps remaining in our knowledge of lignification chemistry (Brown, 1966)" was at least partially filled.

The first enzyme of this sequence, hydroxycinnamate: CoA ligase, was characterized by Gross and Zenk (1974). In agreement with known mechanisms in CoA ester formation, the reaction catalyzed by this enzyme proceeded via an intermediate acyladenylate (Fig. 7) whose essential role was proven by studying cinnamate-dependent [^{32}P]PP_i-ATP exchange reactions and employing chemically synthesized [^{3}H]feruloyl-AMP as substrate. The subsequent enzyme of this series, NADPH-dependent cinnamoyl-CoA reductase, operates at an important branch point of general phenolic plant metabolism (cf. Section 3, Fig. 3) as this key enzyme (Fig. 8) directs metabolism to the

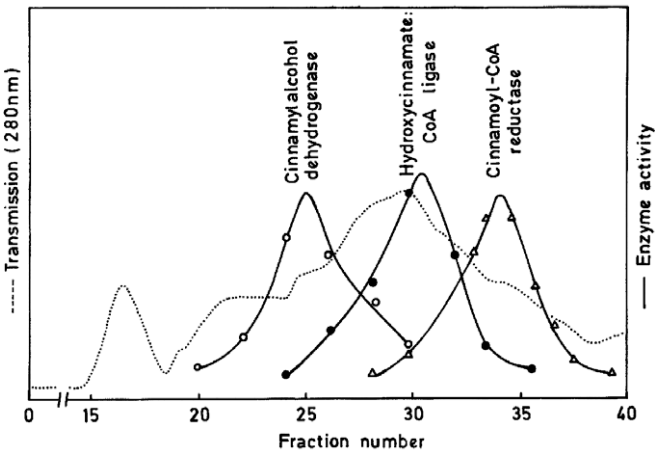


Fig. 6. Enzymes from lignifying stem tissue of *Forsythia suspensa* involved in the activation and reduction of cinnamic acids to monolignols. Separation was achieved by gel filtration on Sephadex G-200. Light transmission and enzyme activities in arbitrary units. From Gross (1977a).

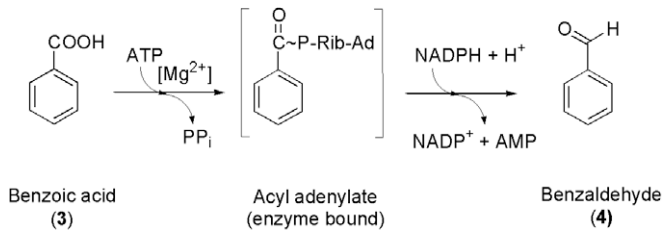


Fig. 5. Reduction of aromatic acids to aldehydes by arylaldehyde: NADP oxidoreductase from the ascomycete *Neurospora crassa*. Depicted is the conversion of benzoic acid (**3**) to benzaldehyde (**4**).

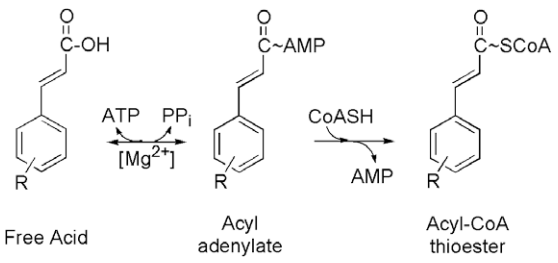


Fig. 7. Reaction catalyzed by hydroxycinnamate: CoA ligase from *Forsythia*. R = OH, OCH_3.

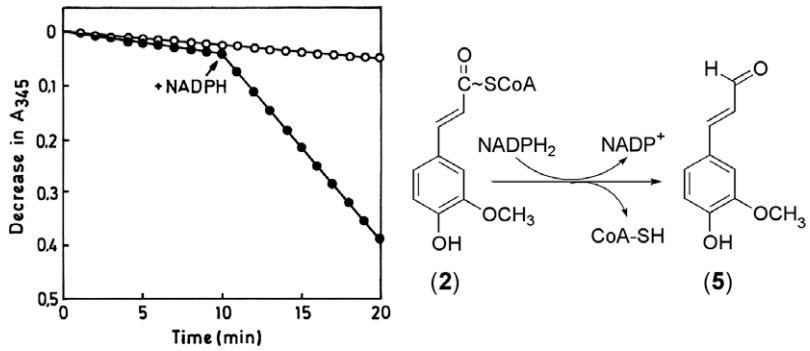


Fig. 8. Cinnamoyl-CoA: NADP oxidoreductase from *Forsythia*. Left panel (from Gross et al., 1973): photometric detection of enzymatic reduction of feruloyl-CoA (**2**). (–●–) Complete reaction mixture (containing tris-buffer, Mg^{2+}, DTE, feruloyl-CoA; NADPH added as indicated); (–○–) control (feruloyl-CoA **2** omitted, but NADPH throughout the reaction). Right panel: reaction catalyzed by the enzyme, shown with feruloyl-CoA (**2**) as substrate affording coniferyl aldehyde (**5**).

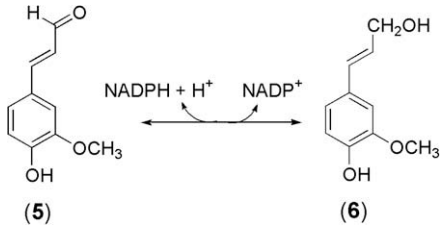


Fig. 9. Reaction catalyzed by cinnamyl alcohol dehydrogenase, shown for coniferyl aldehyde (**5**) and coniferyl alcohol (**6**) as substrates.

specific pathway towards monolignols and their derivatives. Principal characteristics of cinnamoyl-CoA reductase were published by Gross and Kreiten (1975). Also the final enzyme in this sequence, cinnamyl alcohol dehydrogenase (Fig. 9), proved to be strictly specific for NADP(H) as coenzyme and displayed pronounced preference for 3- or 4-substituted cinnamyl substrates (Mansell et al., 1974).

Detection of the above mentioned dehydrogenases that catalyze the first steps in the route to lignin, the second most abundant natural product on earth, stimulated a multitude of subsequent studies in this area until today. Not only scientific curiosity but also far-reaching commercial aspects, e.g. manipulating lignin contents of industrially relevant trees, were addressed in these efforts. Multiple isoforms of these enzymes were detected (cf., e.g., Grima-Pettenati et al., 1994), and cloning and expression experiments have been conducted to further characterize these important biocatalysts (e.g., Lacombe et al., 1997). Established polymorphism of genes and enzymes provoked general considerations on the significance and role of these reductases in different metabolic routes (e.g., Boudet et al., 2004; Li et al., 2005).

4. Oxidative polymerization of monolignols to lignins

Decades ago, Karl Freudenberg determined an average elementary composition for spruce lignin of $C_9H_{7.15}O_2$-$(H_2O)_{0.4}(OCH_3)_{0.92}$ (Freudenberg and Neish, 1968). This formula closely resembles that of coniferyl aldehyde (**5**), $C_9H_7O_2(OCH_3)$, indicating that the lignin of conifers roughly corresponds to the multiple of a slightly oxidized coniferyl alcohol (**6**) unit. In other words: lignins are polymerized oxidation products of monolignols. Several oxidizing enzymes have been discussed as natural agents for this process but the system peroxidase/H_2O_2 was, and still is, the most favored one among these. Accordingly, a one-electron oxidation of monolignols gives corresponding free radicals that stabilize in several mesomeric forms and couple together to yield dilignols, oligolignols and finally polymeric lignin derivatives. In contrast to any other natural product, such a mechanism would depend only on the initial oxidizing starter enzyme to afford polymers in a random process without any further enzymatic control. Considering marked differences between natural and artificial lignins, however, this traditional view has been recently questioned, discussing the possible participation of regulatory proteins in these coupling reactions (cf., eg., Gang et al., 1999; Burlat et al., 2001).

Peroxidase has been postulated as the exclusive oxidizing agent in lignifying trees (Harkin and Obst, 1973). The occurrence of this enzyme in actively lignifying cell walls of sclerenchyma fibers and of young xylem in *Forsythia* has been demonstrated by staining experiments (cf. Fig. 10; Gross, unpublished), while lignin mainly occurred in the fully differentiated xylem. There was only a small zone of xylem cells, being just in the earliest phases of lignification, in which both stains overlapped (mature, fully lignified xylem cells are dead and should thus display no enzyme activities). Given the participation of peroxidase in lignification, questions about the origin of H_2O_2 in lignifying cells became important. Based on the finding that horseradish cell walls were able to produce H_2O_2 at the expense of NAD(P)H, a search for wall-associated oxidoreductases was undertaken with tissue slices and isolated cell wall suspensions from horseradish and *Forsythia* xylem that revealed malate dehydrogenase as sole candidate for providing reduced nucleotide coenzyme (Gross, 1977b; Gross and Janse, 1977). Detailed studies led to the

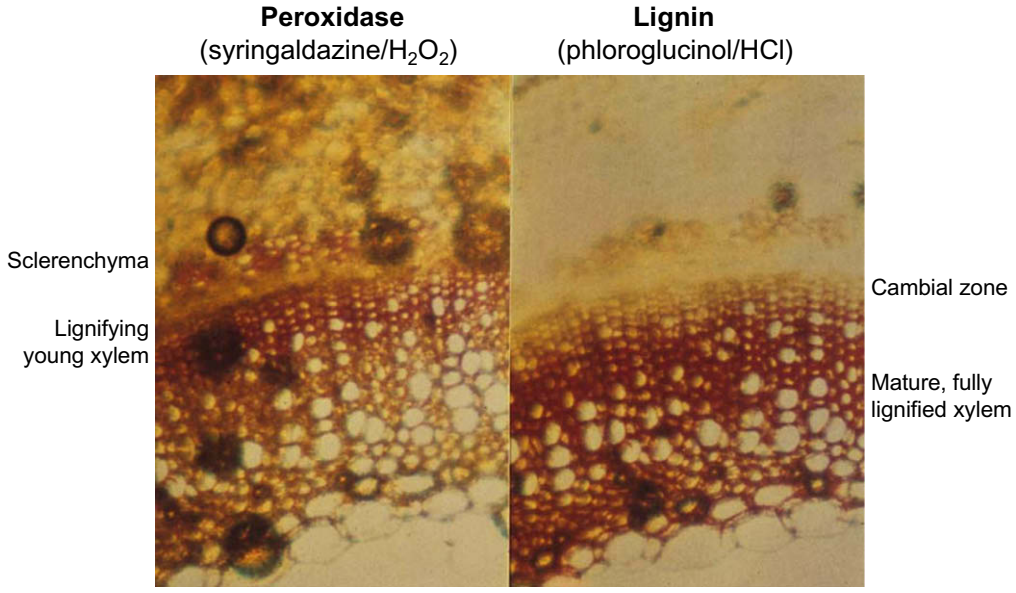


Fig. 10. Detection of peroxidase activity and lignin deposition in *Forsythia*. Left panel: a semi-thin cross section through a young stem was stained for the presence of peroxidase according to Harkin and Obst (1973). Red coloration shows activity of this enzyme in cell walls of sclerenchyma fibers and particularly in young xylem cell walls in the initial phases of lignification, with decreasing activity from the cambium towards older tissue. Right panel: the same cross section was subsequently treated by the standard color test for lignin, revealing an inverse staining pattern that ranged from just maturing, slightly lignified cells located a few rows below the cambial zone to the heavily stained walls of fully lignified (dying or already dead) xylem cells.

proposal of a scheme (Fig. 11) that involved reaction cycles reaching from a plasmalemma-associated malate-oxaloacetate shuttle to the H_2O_2 finally required for lignification (cf. the reviews of Gross, 1979, 1980). This concept, based on the idea that lignifying cell walls were the site of H_2O_2 generation, offered several advantages. For instance, the problems related to the transport of toxic H_2O_2 from the cytoplasm to the cell wall would be eliminated. Also regulation of the polymerization process would be facilitated. Assuming that the preferred monophenols required in the generation of H_2O_2 (coniferyl alcohol **6**, in particular) are the same as those to be polymerized in the cell wall then the operation of the entire sequence would simply depend on the presence of these lignin precursors.

It should be mentioned that research in this area is still a major objective in many laboratories. Recent reports, just to quote a few selected publications, cover a diverse array dealing with, e.g., enzyme studies on peroxidase isoenzymes (Marjamaa et al., 2006), the functions of wall-associated malate dehydrogenase, peroxidase and laccase (Karkonen et al., 2003), analyses of pathways to lignins (Nose et al., 1995), or molecular biological cloning experiments (Gabaldon et al., 2005).

5. Biosynthesis of phenolic amides

As depicted in Fig. 3, cinnamoyl-CoA esters have been proposed long ago as activated precursors of phenolic amides (Gross and Zenk, 1966). First proof of this hypothesis at the enzyme level was presented by Bird and Smith (1983) that reported the condensation of agmatine (the biogenic amide of arginine) and *p*-coumaroyl-CoA with cell-free extracts from barley seedlings to yield *p*-coumaroylagmatine; dimers of this compound, known as hordatines, possess antifungal properties. Formation of a related amide, feruloyltyramine (supposed to participate in hypersensitive reactions in higher plants), was catalyzed by *N*-feruloyl-CoA transferase from *Nicotiana tabacum* (Negrel and Martin, 1984).

Of higher relevance to humans' daily life is the structurally related compound piperine (**9**), the pungent principle of black pepper (*Piper nigrum* and related species) that should not be confused with another amide, capsaicin, the principal hot component of red peppers like paprika or chili (for a detailed review on the structures and sensory qualities of pepper ingredients see Freist, 1991). It had been suggested already decades ago (Geissman and Crout, 1969) that piperine (**9**) should originate from the condensation of the *N*-heterocycle piperidine (**8**) with the 'activated' coenzyme A ester of piperic acid, an analogue of cinnamic acids characterized by a phenolic 3,4-methylene dioxy group and a pentadienoic side chain (cf. Fig. 12). The correctness of this assumption could be proven upon incubation of chemically synthesized piperoyl-CoA (**7**, Semler et al., 1987) and piperidine (**8**) with an enzyme preparation from stems of black pepper plants (Fig. 12; Geisler and Gross, 1990). This rather unusual enzyme source was chosen because mature, fully differentiated stem tissues displayed remarkably high contents of piperine (**9**, ca. 0.2% dry weight) that exceeded those found in leaves and roots by a factor of more than 10, reaching the contents of commercially available white or black pepper seeds of lower quality (Semler and Gross, 1988).

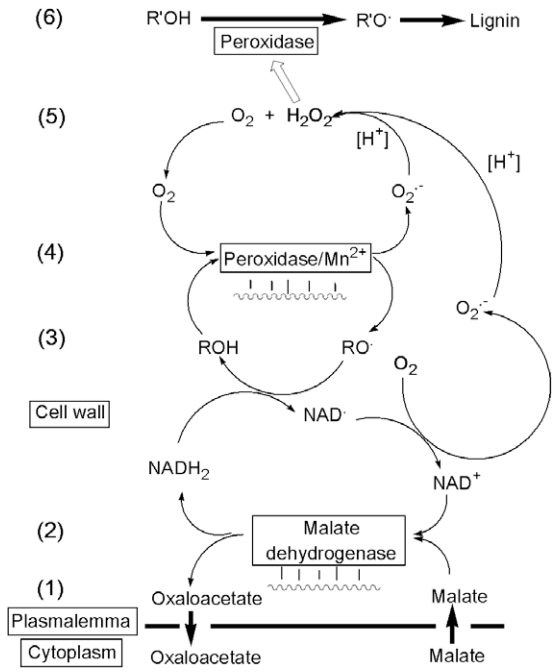


Fig. 11. Proposed scheme of reactions generating hydrogen peroxide by wall bound/associated malate dehydrogenase and peroxidase in plant cell walls (Gross et al., 1977). In summary, malate is oxidized by O_2 to yield oxaloacetate and H_2O_2 that can be utilized for the formation of monolignol radicals to initiate lignin formation. Explanations: (1) well documented malate-oxaloacetate shuttles transport reducing equivalents across the plasmalemma; (2) bound malate dehydrogenase (MDH) in lignifying cell walls of *Forsythia* (Gross and Janse, 1977) differs from other MDH's (references in Gross, 1980); (3) most efficient monophenol (ROH) stimulation of H_2O_2 formation in cell walls was effected by coniferyl alcohol (**6**, Gross et al., 1977); (4) high concentrations of Mn in wood have been reported (e.g., Shortle, 1970; Önnerud et al., 2002); (5) H_2O_2 production via the superoxide radical ($O_2^{\cdot-}$) cycle is driven by O_2, ROH and wall-bound peroxidase (Gross et al., 1977); (6) in summary, H_2O_2 formation largely depends on the availability of monolignols in cell walls.

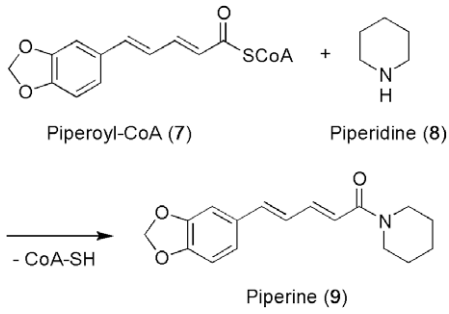


Fig. 12. Biosynthesis of piperine (**9**), the pungent principle of black pepper (*Piper nigrum*) and related plant species.

6. Biosynthesis of hydrolyzable tannins

Growing increasingly weary of lignin related research, a new challenge was found in the early 1980s by studying the biosynthesis of gallotannins as another group of plant polyphenols that had been widely unexplored until those days. In retrospect, it was a rather daring endeavor to tackle this question just by enzymatic means as these compounds strongly bind to proteins, thus precipitating and inactivating enzymes. Fortunately, such complications were considered only years later when it was already obvious that the gallotannin synthesizing enzyme systems actually did work in vitro, even in the presence of such extremely potent agents like pentagalloylglucose. In contrast, availability of the many different tannin compounds required as substrates or references remained a permanent major concern in these investigations. None of these delicate chemicals was, and still is, commercially available but had to be obtained by isolation from natural sources and by chemical or enzymatic synthesis. Purification of these often very similar compounds was another challenge, a problem that was solved by low-pressure chromatography on Sephadex LH-20 and particularly by preparative reversed-phase HPLC. Specific questions were encountered when the usually minimal amounts of reaction products in scaled-up enzyme assays had to be isolated in 5–10 mg quantities that were required for their exact identification, including ^1H and ^{13}C NMR, FAB-MS, MALDI-TOF MS and CD-spectroscopy. Procedures for the preparation and analysis of 'simple' galloylglucoses have been compiled in an earlier review by Gross (1993). It should be emphasized that the nature of the reaction products of all of the enzymes mentioned below has been scrupulously checked by such methods.

Before going into the details of biogenetic routes, a few general remarks appear appropriate. According to the classical definition of Freudenberg (1920), plant tannins are divided into the two classes of (i) condensed tannins (*syn.* proanthocyanidins) which are of flavonoid origin and are not addressed in this review, and (ii) hydrolyzable tannins that can be described as esters of gallic acid (**10**) with a central polyol, typically β-D-glucopyranose. 1,2,3,4,6-Pentagalloylglucose (**12**) requires particular attention among these derivatives, as it represents an important branch point in their biosynthesis. Addition of further galloyl residues to this molecule leads to the gallotannin subclass that is characterized by *meta*-digalloyl groups (**13**), while oxidation of pentagalloylglucose (**12**) affords ellagitannins, a subclass that is characterized by hexahydroxydiphenoyl (**14**, HHDP) residues. Principal reactions and structures of these compounds are summarized in Fig. 13.

Looking back to the early 1980s when the investigations summarized below were started, studies on the chemistry of tannins were a comparatively uncommon facet and biochemical approaches could almost be classified as exotic. Meanwhile, a real 'run' in this field is apparent that originated from new insights into the phytochemical basis of ancient East-Asian ethnopharmacology. Their recently recognized antioxidant, antimicrobial, antiviral and antitumor characteristics, together with many other health related aspects of plant tannins, are now intensively investigated worldwide by in vitro and in vivo studies in many laboratories.

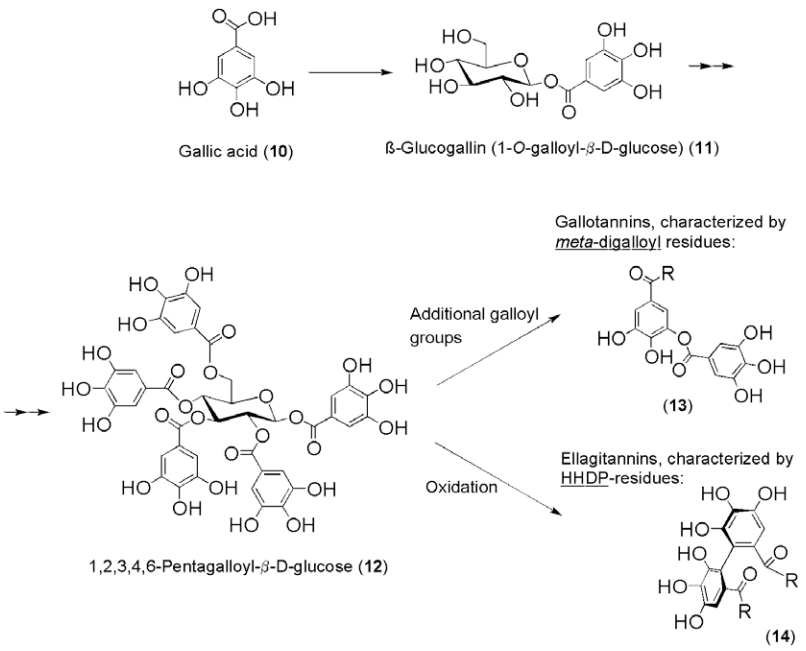


Fig. 13. Principal steps in the biosynthesis of gallotannins and ellagitannins. HHDP (**14**), 3,4,5,3′,4′,5′-hexahydroxydiphenoyl.

6.1. 'Simple' galloylglucoses

Mono to penta-substituted galloylglucoses are often referred to as 'simple' galloylglucoses to discriminate them from 'complex' galloylglucoses, the gallotannins proper. This nomenclature, however, disregards the fact that their higher substituted members are true tannins; pentagalloylglucose (**12**), for instance, is one of the most potent tanning agents known in nature due to its molecular size and shape. The reactions in the pathway from gallic acid to pentagalloylglucose (**12**) in oak and sumac are depicted in Fig. 14. Esterification of gallic acid (**10**) and glucose to yield β-glucogallin (**11**), the first specific intermediate in the route to hydrolyzable tannins, was initially thought to proceed via the galloyl-CoA thioester, an assumption that, however, never could be proven. Instead, enzyme extracts from oak leaves utilized UDP-glucose as 'activated' reaction partner that reacted with the free acid (Gross, 1983a). Surprisingly, these enzyme preparations also catalyzed further transformations with in situ generated β-glucogallin (**11**) and in the absence of any other cofactors, to yield di- and trigalloylglucoses. Consequently, β-glucogallin (**11**) must have acted as both acyl acceptor and acyl donor molecule (Gross, 1983b). The latter role would require that β-glucogallin (**11**) had the unexpected function of an energy-rich 'activated' compound, an interpretation that was supported by results of Mock and Strack (1993) that determined a $\Delta G_0'$ of 35.7 kJ mol^{-1} for the structurally related ester, 1-*O*-sinapoyl-β-D-glucose, a value that is comparable to that of the well known acyl-CoA esters.

β-Glucogallin (**11**) was identified as the principal acyl donor in the conversion of mono- to pentagalloylglucoses (cf. steps 2–5 in Fig. 14) by numerous detailed studies in the years following these basic findings. All enzymes involved in this sequence have been isolated and characterized (for references, see the recent review of Niemetz and Gross, 2005). In these reactions, glucose hydroxyls were not substituted in a random pattern. Instead, an

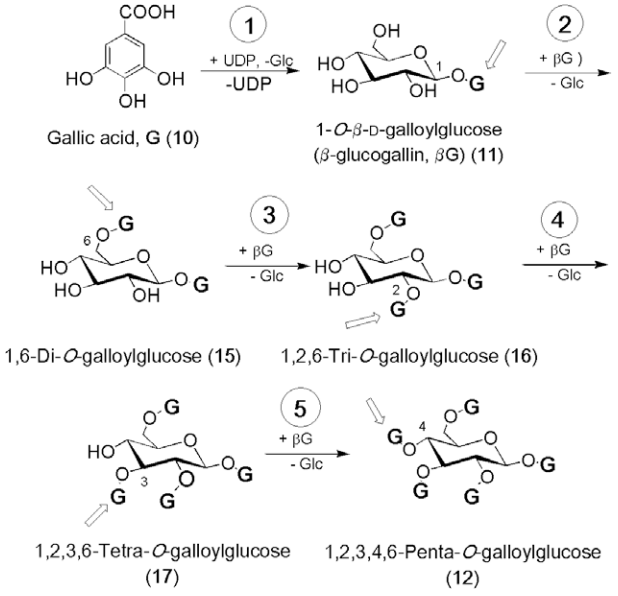


Fig. 14. Enzyme reactions catalyzing the pathway from gallic acid (**10**) to 1,2,3,4,6-pentagalloylglucose (**12**) in pedunculate oak (*Quercus robur*, *Q. pedunculata*). Arrows indicate the position of each newly introduced galloyl residue. Step 1 in this sequence is a glucosyl transferase reaction utilizing UDP-glucose as energy-rich glucosyl donor. Steps 2–5 are galloyl transferase reactions that require β-glucogallin (**11**) as activated acyl donor. In step 2, this compound thus has a dual role as both galloyl donor and acceptor, a reaction that occasionally has been described as 'disproportionation'. The depicted reactions have also been found in sumac (*Rhus typhina*). Modified from Niemetz and Gross (2005).

unexpected high position specificity was displayed in these conversions, a finding that could be explained by a combination of reactivity differences due to variations in chemical nature, neighbor-activating effects and steric hindrance (cf. Williams and Richardson, 1967; Reinefeld and Ahrens, 1971).

6.2. Gallotannins

The transformation of 'simple' galloylglucoses to 'real' gallotannins occurs by substitution of pentagalloylglucose (**12**) with additional galloyl residues, affording complex polygalloylglucoses that usually contain up to 12 galloyl groups, occasionally even more. Five of these galloyl residues are esterified with the aliphatic hydroxyls of the glucose core (see above), while the remnant ones are attached via chemically quite distinct phenolic OH-groups to form the characteristic *meta*-depsides of gallotannins. Studies with crude enzyme extracts from leaves of sumac (*Rhus typhina*), a plant that is characterized by high gallotannin concentrations, revealed however, that such polygalloylglucoses were produced in a manner quite identical to the mode reported above for 'simple' galloylglucose esters, i.e., by utilizing β-glucogallin (**11**) as general galloyl donor. As depicted in Fig. 15, a sequential formation of hexa-, hepta- and octagalloylglucoses, at the expense of the pentagalloylglucose substrate, must have occurred in this system (Hofmann and Gross, 1990).

Closer analysis by normal-phase HPLC (Fig. 16a) showed that a wide range of gallotannins up to at least decagalloylglucoses had been formed in this experiment. Subsequent reversed-phase HPLC (Fig. 16b) revealed that each of these apparently uniform peaks comprised a mixture of 3–4 isomers of the same substitution level. It is evident from this data that numerous different gallotannins are formed according to one common reaction mechanism, rising questions on the number and specificity of the enzyme(s) involved in these transformations. Screening experiments aiming at this question, that were based on analyzing galloyltransferase activities after separation by gel-filtration chromatography and employing penta- and hexagalloylglucose substrates, afforded the existence of five such enzymes (A–E in Fig. 17) in sumac leaves until today. All these enzymes could be highly purified (for relevant references, see Niemetz and Gross, 2005), and it was found that none of these displayed high substrate or product specificity. However, it was evident on the basis of $v_{max} \times K_m^{-1}$ calculations that each enzyme catalyzed only a few preferred reactions. Combining the recently available data, a metabolic scheme was developed that illustrates the major trends in the formation of hexa- and heptagalloylglucoses in sumac (Fig. 17). In spite of serious attempts, it was impossible to gather additional information on the biosynthesis of higher substituted gallotannins. Momentarily available HPLC separation techniques allowed only partial separation of octagalloylglucoses required as substrates and reference compounds. Moreover, the fatal tendency of these compounds for intramolecular acyl migration, as observed in these experiments, appears to be a feature that will prevent any further studies in this direction.

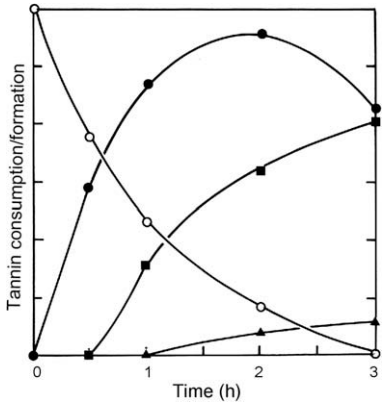


Fig. 15. In vitro synthesis of gallotannins by galloyl transfer from β-glucogallin (**11**) to 1,2,3,4,6-pentagalloylglucose (**12**) with enzyme extracts from leaves of *Rhus typhina*. (–○–) Penta-, (–●–) hexa-, (–■–) hepta-, (–▲–) octagalloylglucose.

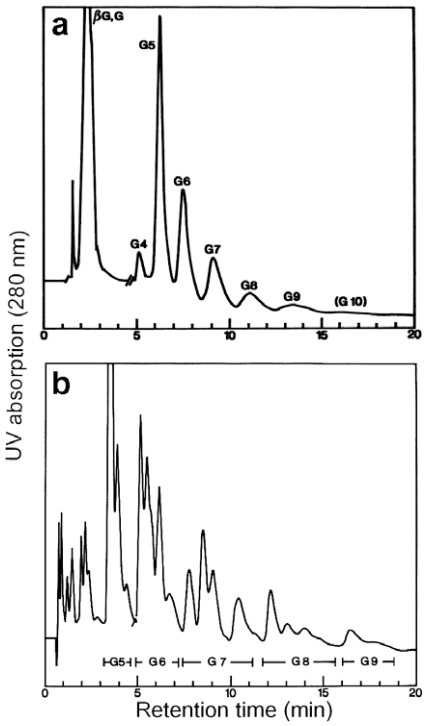


Fig. 16. HPLC analysis of gallotannins in reaction mixtures with crude extracts from *Rhus typhina* and pentagalloylglucose (**12**) and β-glucogallin (**11**) as enzyme substrates. Panel A: normal phase HPLC on Si-60 silica gel; Panel B: reversed phase (C18) HPLC. G, Gallic acid (**10**); βG, β-glucogallin (**11**); G4-G10, tetra- to decagalloylglucoses.

6.3. Ellagitannins

In contrast to the limited occurrence of gallotannins in nature, ellagitannins are typical constituents of many

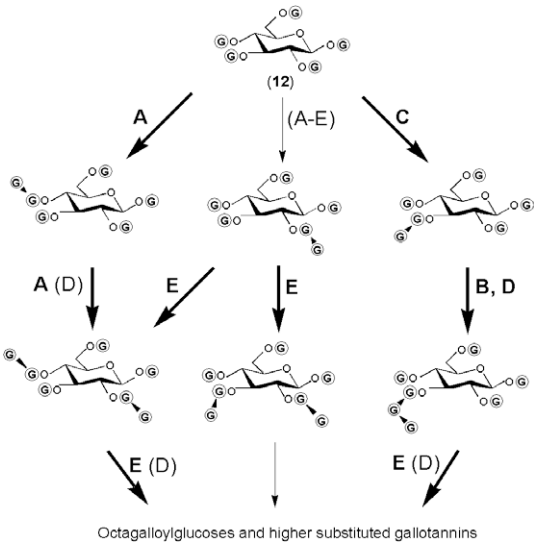


Fig. 17. Biosynthesis of gallotannins: metabolic routes from pentagalloylglucose (**12**) to hexa- and heptagalloylglucoses in *Rhus typhina* (sumac). Main reactions are marked by bold arrows, minor reactions by thin arrows. For characteristics and references to galloyltransferases A–E, see the review article of Niemetz and Gross (2005). Major enzyme activities are symbolized by bold letters, minor activities by plain letters in parentheses. (Ⓖ), Galloyl (**10**); (Ⓖ)▶(Ⓖ), *meta*-digalloyl (**13**) residue.

plant families where they are found in an enormous structural variety, a feature that is due to their pronounced tendency to form dimeric and oligomeric derivatives. The structural characteristics of this interesting group of natural products have attracted chemists many decades ago and also stimulated considerations about their biogenesis. Already in the mid-1930s it was postulated that the hexahydroxydiphenoyl (**14**, HHDP) groups of ellagitannins originated from the dehydrogenation of galloyl esters (Erdtman, 1935), a view that was corroborated and refined later in several laboratories, with particular emphasis on the role of pentagalloylglucose (**12**) as immediate precursor of these compounds. Many experiments to unravel the mechanism of ellagitannin biosynthesis have been carried out in the past, employing chemical oxidants (e.g., O_2, Fe^{3+}) or enzyme systems with phenolase, laccase and peroxidase. Free ellagic acid (the dilactone of released HHDP **14** units) was observed occasionally in these studies while true ellagitannins, characterized by glucose-bound HHDP groups, were never found (references in Gross, 1999).

Also in my laboratory, only negative results were initially encountered in intensive research on the in vitro oxidation of pentagalloylglucose. It was finally concluded that inadequate analytical techniques represented the decisive problem in these investigations that apparently suffered from a combination of only minimal enzyme reaction rates, possible formation of numerous similar reaction products and by-products that could not be identified by HPLC, and of uncertainties to what extent the isolated compounds were contaminated with in vivo formed ellagitannins. The necessity of a completely new approach to this question was thus evident. The idea was to eliminate these problems by working with [U-^{14}C]pentagalloylglucose (**12**) as substrate, thus dramatically enhancing both the sensitivity and specificity of test systems. This radiochemical was prepared by photoassimilation of several mCi of $^{14}CO_2$ in sumac leaves (Rausch and Gross, 1996). Given the in vitro formation of ellagitannins of any structural configuration, subsequent hydrolysis of labeled products would release [^{14}C]ellagic acid that served as sole, general probe for numerous, but principally identical oxidative reactions.

Extended screening programs based on this method finally led to the discovery of a novel enzyme activity in leaves of *Tellima grandiflora* (fringe cups), a weed known as a rich source of ellagitannins, that catalyzed the oxidation of [U-^{14}C]pentagalloylglucose (**12**) to several radioactively labeled products. The most prominent compound among these afforded ^{14}C-labeled glucose, gallic acid (**10**) and ellagic acid upon hydrolysis and was finally identified as the monomeric ellagitannin, tellimagrandin II (Fig. 18). The long sought first hint on the in vitro biosynthesis of an ellagitannin had thus been provided in this experiment (Niemetz et al., 2001).

More detailed investigations following this principal finding revealed the existence of a soluble, O_2-dependent laccase-type enzyme that oxidized pentagalloylglucose (**12**) exclusively to tellimagrandin II (**18**). Circular dichroism (CD) spectroscopy demonstrated that the newly formed HHDP moiety (**14**) of this ellagitannin had the (*S*)-stereoconfiguration known for the in vivo formed natural product (Fig. 19; Niemetz and Gross, 2003a). Such a stereoselective 4,6-(*S*)-coupling of two adjacent galloyl groups could well be expected from energetic and molecular modeling-based considerations that clearly favored this reaction with the energetically preferred

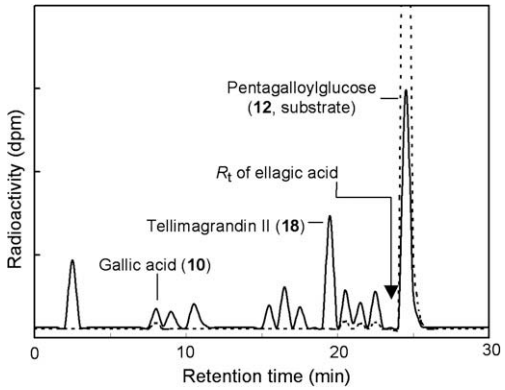


Fig. 18. Reversed-phase HPLC of reaction products after oxidation of [U-^{14}C]pentagalloylglucose (**12**) by a crude enzyme preparation from *Tellima grandiflora* (fringe cups) leaves, demonstrating the in vitro synthesis of an ellagitannin, tellimagrandin II (**18**). (–) Enzyme assay; (···) control with acid-denatured enzyme. Gallic acid (**10**) was detected as a minor by-product, probably as a result of hydrolytic side reactions. Note that no radioactivity coincided with the retention time of ellagic acid, a possible degradation product of ellagitannins. Modified from Niemetz et al. (2001).

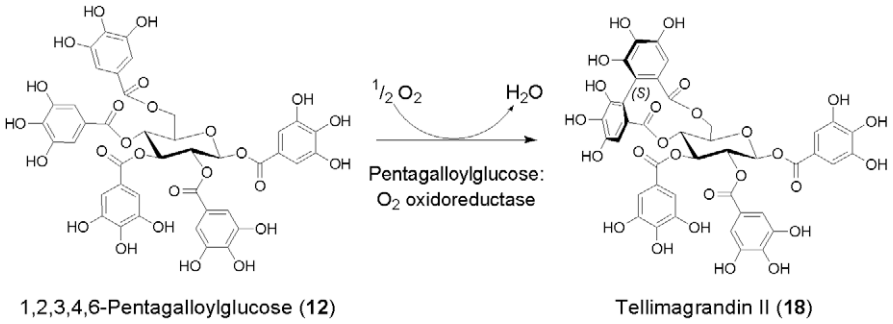


Fig. 19. Oxidation of 1,2,3,4,6-pentagalloylglucose (**12**) to the monomeric ellagitannin, tellimagrandin II (**18**), by a phenol oxidase from leaves of *Tellima grandiflora* (fringe cups).

$^4C_1$-configuration of the β-glucopyranose core. The same situation also applies to the 2,3-(*S*)-coupling reaction, while the energetically less favorable $^1C_4$-glucopyranose-based 2,4- and 3,6-coupling leads to (*R*)-HHDP derivatives (for recent review articles discussing these aspects in more detail, see, e.g., Feldman et al., 1999; Khanbabaee and van Ree, 2001). It is an open question to date, however, to what extent these fundamental chemical characteristics are actually governing enzyme catalyzed galloyl coupling reactions that might involve some kind of phenoxy radical coupling control mechanism (cf. Davin and Lewis, 2005).

Closer analysis of the formation of some side-products obtained with crude enzyme extracts from *T. grandiflora* leaves revealed the existence of a new enzyme activity that efficiently transformed tellimagrandin II (**18**) to a higher molecular weight compound that was later identified as cornusiin E (**19**), a dimeric ellagitannin that apparently resulted from the oxidative coupling of two tellimagrandin II (**18**) moieties (Fig. 20). It was shown that the reaction mechanism of this enzyme was identical to that reported above for the tellimagrandin II (**18**) synthesizing enzyme. However, both enzymes clearly differed in their physical and kinetic properties. (*S*)-Configuration of both the HHDP (**14**) and the tri-arylic valoneoyl bridge of in vitro formed cornusiin E (**19**) was proven by CD spectroscopy, establishing the identity of in vitro and in vivo generated products (Niemetz and Gross, 2003b; Niemetz et al., 2003).

7. Concluding remarks

It has been my privilege and pleasure to contribute for more than four decades to our recent knowledge of phenolic metabolism in plants. Enzymology was the predominating tool in these studies, with particular emphasis on the essential role of 'energy-rich' intermediates. Progress over this period was intimately associated with worldwide perfecting enzymological techniques, equally regarding preparative and analytical techniques. These new, advanced methods were the prerequisite for expanding the basic knowledge provided by former 'feeding experiments' to the recent detailed description of biogenetic pathways and their impact on describing and explaining physiological and ecological phenomena. Recently, we are experiencing a similar profound transition, molecular biology becoming now the predominant technique that, however, will be most beneficial by not simply replacing traditional biochemical approaches but adding it's own inherent potentials. Anyway, it is easy to predict that a new generation of scientists is going to reach new horizons in the permanent struggle of getting more exciting insights into the origin, metabolic fate and general purpose of plant phenolics.

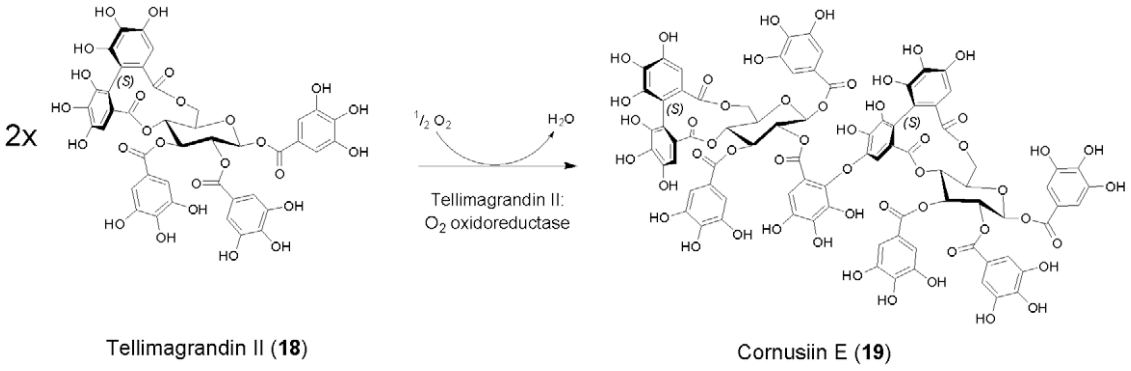


Fig. 20. Oxidative dimerization of the monomeric ellagitannin, tellimagrandin II (**18**), to cornusiin E (**19**) by a phenol oxidase from *Tellima grandiflora* (fringe cups).

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Georg G. Gross was professor for plant physiology and plant biochemistry at the department "Molecular Botany" of the University of Ulm, Germany. After being active as researcher and teacher for more than 40 years, he retired in the fall of 2006. He was specialized in investigating the enzymology of phenolic natural products, with particular emphasis on energy-rich 'activated' acyl derivatives. After many years devoted to questions related to the formation of lignins and various alkaloids, his research interest focused on the biosynthesis of gallotannins and ellagitannins. Over the past 20+ years, principles and details of the biogenesis of these complex polyphenols have been elucidated in his laboratory by the extensive application of enzyme studies. His research activities, being documented in more than 80 original papers and review articles, were honoured in 2006 with the "5th Tannin Conference Award".