1.23 Lignans (Neolignans) and Allyl/Propenyl Phenols: Biogenesis, Structural Biology, and Biological/Human Health Considerations

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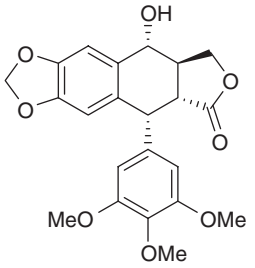
1.23.1 Introduction

Allyl-/propenylphenols are generally volatile substances intimately associated with essential oils of scents and flavors of flowers, herbs, and spices. Many are of great historical importance, for example, as spice constituents. Lignans (and neolignans) are also very important natural products of vast structural diversity, these being apparently found ubiquitously throughout the vascular plant kingdom. Many have roles in plant defense against herbivores/pathogens, and many contribute positively to desirable heartwood properties of various woody species, such as in enhancing durability, color, texture, and so on. Others are beneficial in foodstuffs and oils, such as in sesame, due in part to their antioxidant properties. Some are also medicinal, such as either the antiviral *Podophyllum* lignan, (−)-podophyllotoxin (**1b**, **Figure 1**), or its related derivatives widely used in conventional cancer chemotherapy.

The primary focus herein is on the advances made in dimeric/oligomeric lignan (neolignan) and allyl-/propenylphenol research since 1999.[1] Specifically, various developments made over the past decade are critically discussed, with regard to both metabolic pathways and the associated structural biology, as well as what is known of their corresponding biological/health-protecting properties (both established and potential). The addition of allyl-/propenylphenols is timely as the corresponding biosynthetic pathways are only now coming to light.[2–5] By contrast, the earlier treatise[1] in *Comprehensive Natural Products Chemistry* placed substantial emphasis on the pioneering work carried out in developing the lignan field, as well as giving a critical analysis of the accumulated knowledge that has made the more recent studies possible.

Podophyllum peltatum



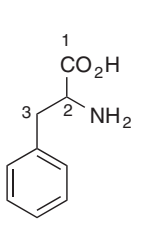
(**1b**) (–)-Podophyllotoxin

Figure 1 *Podophyllum peltatum* and (–)-podophyllotoxin (**1b**). The letters a and b in compound numbers throguhout the manuscript depict (+)- and (–)-enantiomeric forms, respectively. Photograph from Laurence Davin, Washington State University, USA.

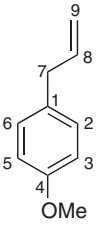
1.23.2 Definition and Nomenclature

1.23.2.1 Allyl-/Propenylphenols

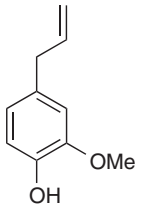
Allylphenols and propenylphenols are metabolites found in many plant species, whose core (C_6C_3) carbon skeleta comprise a C_3 side chain attached to an aromatic ring. They are products of the phenylpropanoid pathway (i.e., the so-called C_6C_3 natural products, which, in turn, are derived from the amino acid Phe (**2**)). They differ from most other phenylpropanoids (e.g., monolignols, hydroxycinnamaldehydes, hydroxycinnamic acids, and their downstream products) by lacking an oxygenated functionality at the terminal carbon (C9) of the side-chain. Additionally, they differ among themselves in the placement and/or absence of the double bond. The allylphenols have a C8–C9 terminal double bond (e.g., methylchavicol (**3**, estragole or 4-allylanisole) and eugenol (**4**)), whereas propenylphenols have it at C7–C8, conjugated with the aromatic ring (e.g., anethole (**5**) and isoeugenol (**6**)). In general, the carbon skeleton is intactly maintained as that originating from Phe (**2**),[2] except for the so-called pseudoisoeugenols (e.g., **7** or **8**). The latter results from a presumed NIH shift of the side-chain,[6] with some pseudoisoeugenols undergoing further functional modification such as epoxide formation.



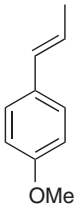
(**2**) Phenylalanine



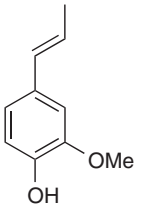
(**3**) Methylchavicol



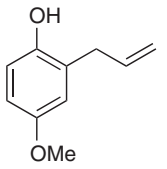
(**4**) Eugenol



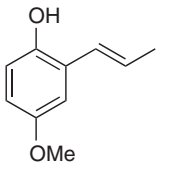
(**5**) Anethole



(**6**) Isoeugenol



(**7**) 2-Allyl-4-methoxyphenol

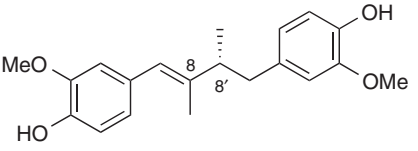


(**8**) Pseudoisoeugenol

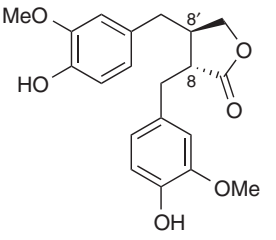
1.23.2.2 Inconsistencies in Current Nomenclature of Lignans and Neolignans

The structurally diverse lignans (and neolignans) encompass an extensive array of distinct carbon skeleta.[1] All are presumed derived from coupling of phenylpropanoid (C_6C_3) units to afford dimers or higher oligomers, linked through C–C and/or C–O interunit bonds. In contrast to the more abundant (by weight) polymeric lignins,[7–9] the lignans (and neolignans) do not have any known essential cell wall structural biopolymeric role(s) in the vascular apparatus.

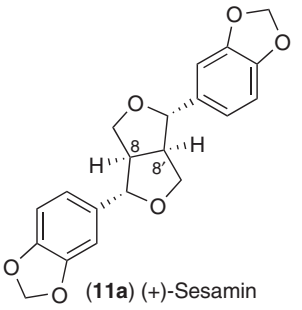
Since the late nineteenth or early twentieth century, numerous dimeric/oligomeric phenylpropanoids have been isolated from various plant species. A number of these were given trivial names indicative of botanical origin, for example, (−)-guaiaretic acid (**9b**) from *Guaiacum officinale* resin,[10–12] (−)-matairesinol (**10b**) from the Matai tree (*Podocarpus spicatus*),[13] (+)-sesamin (**11a**) from *Sesamum indicum*,[14,15] and (−)-olivil (**12b**) from the olive tree (*Olea europaea*).[16–18]



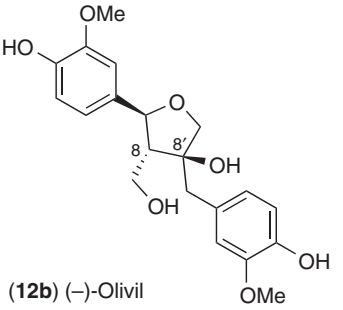
(**9b**) (–)-Guaiaretic acid



(**10b**) (–)-Matairesinol

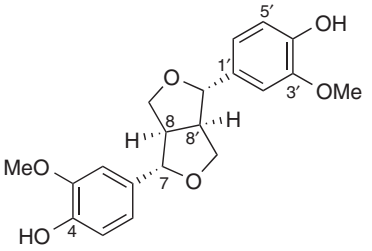


(**11a**) (+)-Sesamin

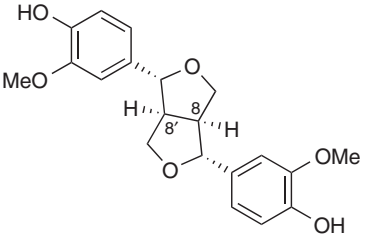


(**12b**) (–)-Olivil

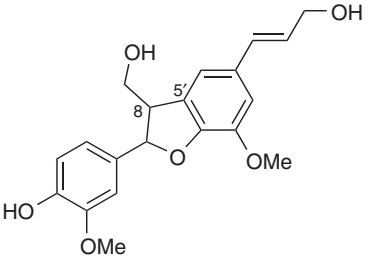
In 1937, Haworth[19] introduced the term lignane, later shortened to lignan, to define various dimeric phenylpropanoids that were considered to result from regiospecific (8–8′) coupling of two C_6C_3 molecules to afford different compounds such as (+)- and (−)-pinoresinols (**13a** and **13b**). A large number of other dimeric phenylpropanoids (and/or higher oligomers thereof) could not be classified according to this definition, and the term neolignan was thus introduced later by Gottlieb[20] to encompass other coupling modes, for example, the 8–5′-linked (±)-dehydrodiconiferyl alcohols (**14a/b**). This neolignan classification was subsequently further modified[21] to next include only phenylpropanoid dimers presumably derived from coupling of allyl-/propenylphenols (i.e., phenylpropanoid monomers lacking C9 oxygenation), such as magnolol (**15**) and (+)-conocarpan (**16a**). Curiously, current IUPAC lignan nomenclature[22] still utilizes the original definitions of both Haworth and Gottlieb in attempts to distinguish between lignans and neolignans, that is, based solely on the differences in interunit linkage type. Using such nomenclature, the lignans would thus be exclusively 8–8′-linked, whereas the neolignans would encompass all other linkage types. To add further to this complexity, the term oxyneolignan was also coined to define ether-linked structures, for example, isomagnolol (**17**).[23]



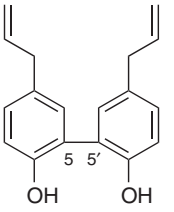
(**13a**) (+)-Pinoresinol



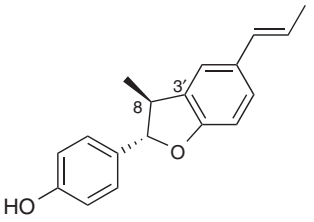
(**13b**) (–)-Pinoresinol



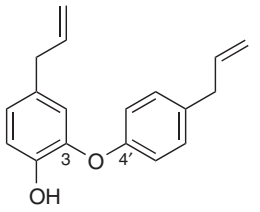
(**14a,b**) (±)-Dehydrodiconiferyl alcohols



(**15**) Magnolol



(**16a**) (+)-Conocarpan

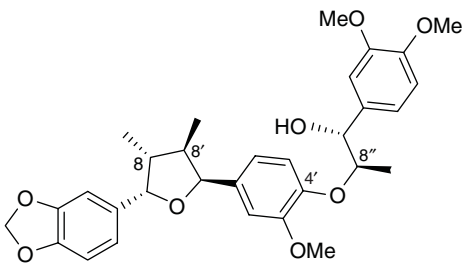


(**17**) Isomagnolol

In our view, however, the continued usage of the terms lignans, neolignans, and oxyneolignans results in serious nomenclature inconsistencies. Two examples serve to illustrate this point (**Figure 2**). The sesquilignan (−)-saucerneol D (**18b**) from *Saururus chinensis*[24] and the dilignan (+)-lappaol F (**19a**) from *Arctium lappa*[25,26] contain both lignan and neolignan interunit linkages according to the definitions of Haworth[19] and Gottlieb,[20] and are thus neither one nor the other.

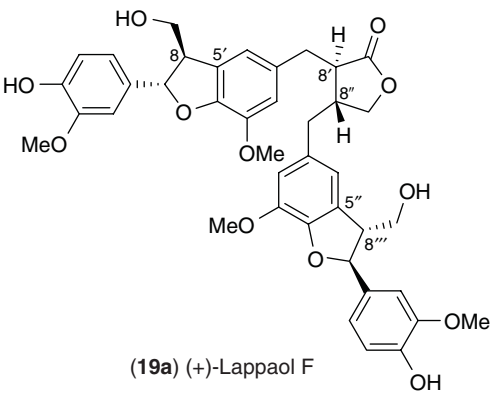
Therefore, it is continued[1] to propose that the original definition of 'lignan' be expanded to encompass all of the lignans, neolignans, and oxyneolignans, regardless of presumed monomeric composition (i.e., irrespective of whether the functionalities present are apparently either monolignol, hydroxycinnamaldehyde, hydroxycinnamic acid, or allyl-/propenylphenol-like). In this way, all of these natural products can be more conveniently characterized as lignans that would differ only through linkage type(s).

Saururus chinensis



(**18b**) (−)-Saucerneol D

Arctium lappa



(**19a**) (+)-Lappaol F

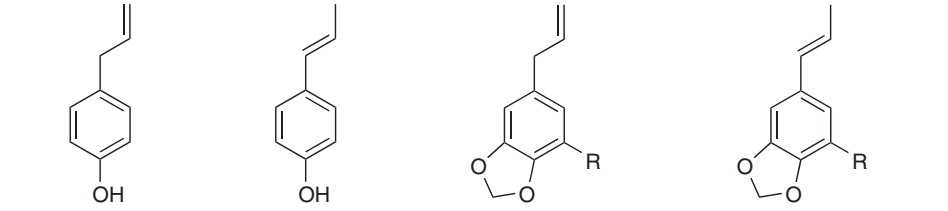
Figure 2 Sesquilignans. (−)-Saucerneol D (**18b**) from *Saururus chinensis* and (+)-lappaol F (**19a**) from *Arctium lappa*. Photograph of *S. chinensis* by Henri Moore, Washington State University USA. Photograph of *A. lappa* by Dr. Toshiaki Umezawa, Kyoto University, Japan.

1.23.3 Chemotaxonomical Diversity: Evolutionary Considerations

To provide an evolutionary and chemotaxonomical perspective of the occurrence of allyl-/propenylphenols and lignans, the three major land plant groups[27,28] are considered individually, as well as algae. The former include the bryophytes[27] (nonvascular land plants, including liverworts, hornworts, and mosses) (**Figure 3**); the pteridophytes[27] (seedless vascular land plants, i.e., lycophytes, horsetails, and all ferns) (**Figure 3**); and the spermatophytes[28] (seed-bearing vascular land plants: gymnosperms (cycads, conifers, ginkgos, and gnetophytes) and angiosperms, **Figure 4**).

1.23.3.1 Allyl-/Propenylphenols and Their Derivatives

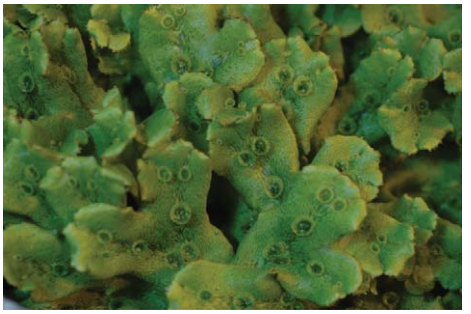
Allyl-/propenylphenols appear to have a fairly broad, albeit not uniform, chemotaxonomic distribution throughout the plant kingdom. In terms of structural diversity, the most commonly reported aromatic ring substitution patterns are those of hydroxylation/methoxylation. To our knowledge, there are no reports of any other heteroatoms (e.g., N, S) being present in allyl-/propenylphenols from plants. The C4 position is typically oxygenated, with further hydroxylation/methoxylation most frequently (but not exclusively) occurring at *ortho* positions (C3/C5) to the C4 oxygenated group. The most common plant allyl-/propenylphenols appear to be eugenol (**4**)/isoeugenol (**6**) and the *O*-methylated derivatives of chavicol (**20**)/*p*-anol (**21**), that is, methylchavicol (**3**)/anethole (**5**), based on current literature searches. Other aromatic ring modifications often reported include either the formation of methylenedioxy bridges, as in safrole (**22**)/isosafrole (**24**) and myristicin (**23**)/isomyristicin (**25**), or less commonly other hydroxylation/methoxylation substitution patterns, such as at C2 and C6 (*ortho* to the C_3 side-chain). These can be exemplified by allyl/propenyl-2,4,6-trimethoxybenzenes (**26**/**27**), 2-allyl-4,5-dimethoxyphenol (**28**), asarones (**29–31**), asaricin (**32**)/carpacin (**33**), croweacin (**34**)/isocroweacin (**35**), apiol (**36**)/isoapiol (**37**), dillapiol (**38**)/isodillapiol (**39**), and nothoapiol (**40**)/isonothoapiol (**41**). As previously mentioned above, the pseudoisoeugenols in *Pimpinella* spp. lack the *p*-oxygenation observed in these compound classes, having instead a 2,5-dioxy substitution as a consequence of side-chain migration,[6] for example, 2-allyl-4-methoxyphenol (**7**) and pseudoisoeugenol (**8**).



(**20**) R = H, Chavicol (**21**) R = H, *p*-Anol (**22**) R = H, Safrole (**23**) R = OMe, Myristicin (**24**) R = H, Isosafrole (**25**) R = OMe, Isomyristicin

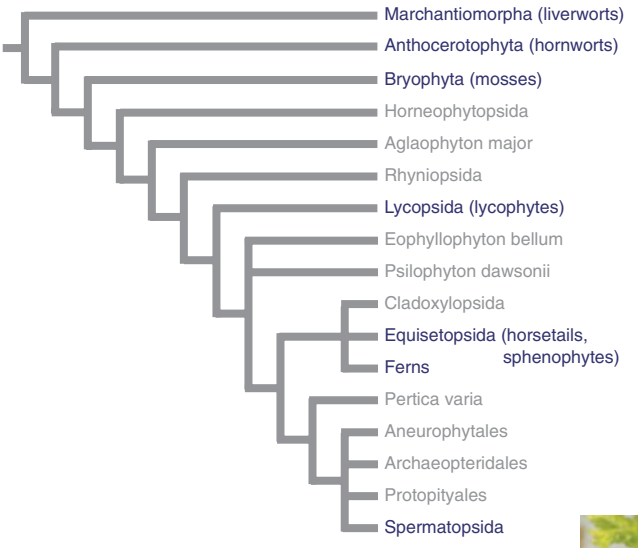
1.23.3.1.1 Algae

Sporadic, albeit somewhat preliminary, reports have been made of allyl-/propenylphenol occurrence in brown (*Spatoglossum variabile*),[29] red (*Jania rubens*[30] and *Hypnea musciformis*[31]), as well as green (*Enteromorpha compressa*,[31] *Caulerpa racemosa*,[32] and *Codium tomentosum*[32]) algae. The reported occurrence of very small amounts of apiol (**36**) and nothoapiol (**40**) in the brown alga (*S. variabile*)[29] growing on shoreline sublittoral rock near Karachi, Pakistan, would benefit from additional biochemical proof, preferably through the analysis of this alga grown under controlled laboratory growth conditions. Although the chemical identification in the study[29] is not in doubt, the possibility that apiol (**36**) and nothoapiol (**40**) may have been absorbed from other detritus at the shoreline or in the waters, or might have resulted from contaminant plant material being collected and analyzed at the same time, needs to be fully eliminated. A similar consideration can be given to preliminary reports of volatile constituents containing traces of eugenol (**4**) and isoeugenol (**6**) in the green (*E. compressa*) and red (*H. musciformis*) algae,[31] as well as that of anethole (**5**) in two green algae (*C. racemosa* and *C. tomentosum*)[32] and a red alga (*J. rubens*).[30]



Marchantia polymorpha (liverwort)

Anthoceros and Phaeoceros (hornworts)



Dicranum (moss)

Pteridium aquilinum (fern)

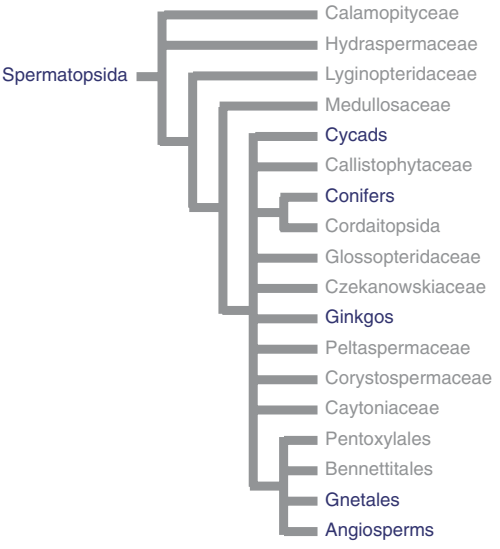
Equisetum hyemale (horsetail)

Selaginella kraussiana (lycophyte)

Figure 3 The tree of life: Embryophytes.[27] Extinct plant lineages are shown in gray, while extant lineages are in blue. Photographs of *Marchantia polymorpha, Dicranum* sp., *Selaginella kraussiana*, *Equisetum hyemale,* and *Pteridium aquilinum* by Henri Moore, Washington State University, USA. Photograph of *Anthoceros* and *Phaeoceros*, copyright Charles F. Delwiche, University of Maryland, USA.

Cycas revoluta (Cycad)

Araucaria araucana (Conifer)

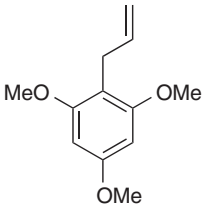


Ginkgo biloba

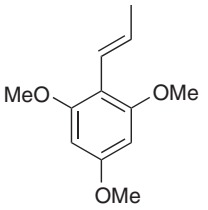
Larrea tridentata (Angiosperm)

Gnetum gnemon (Gnetales)

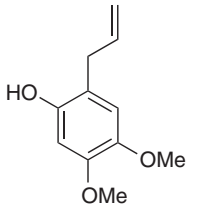
Figure 4 The tree of life: Spermatopsida.[28] Extinct plant lineages are shown in gray, while extant lineages are in blue. Photographs of *Cycas revoluta*, *Gnetum gnemon*, and *Larrea tridentata* by Henri Moore, Washington State University, USA, of *Araucaria araucana* (monkey puzzle tree) by Laurence Davin, Washington State University, USA, and of *Ginkgo biloba* by Daniel Vassãc, Washington State University, USA.



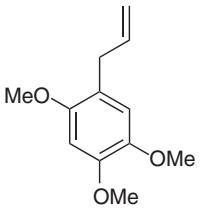
(**26**) Allyl-2,4,6-trimethoxybenzene



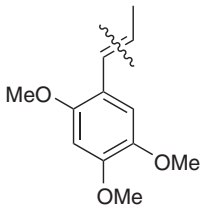
(**27**) Propenyl-2,4,6-trimethoxybenzene



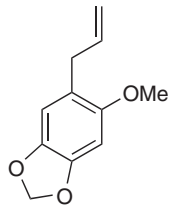
(**28**) 2-Allyl-4,5-dimethoxyphenol



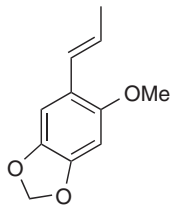
(**29**) γ-Asarone



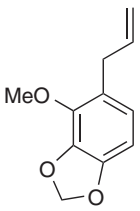
(**30**) α-Asarone (*trans*)
(**31**) β-Asarone (*cis*)



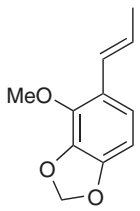
(**32**) Asaricin



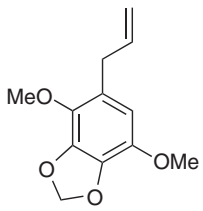
(**33**) Carpacin



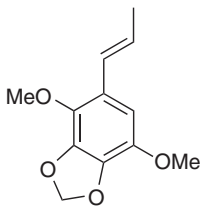
(**34**) Croweacin



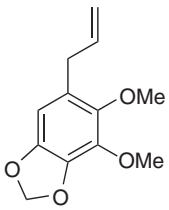
(**35**) Isocroweacin



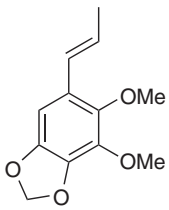
(**36**) Apiol



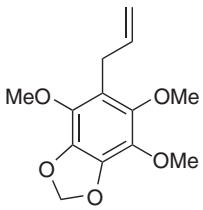
(**37**) Isoapiol



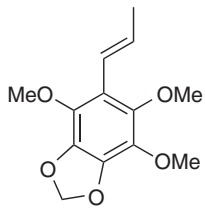
(**38**) Dillapiol



(**39**) Isodillapiol



(**40**) Nothoapiol



(**41**) Isonothoapiol

Their formation by algae is both of interest and of question because, to our knowledge, there is yet no compelling evidence of either algal genes or protein/enzymes encoding the full phenylpropanoid pathway to the monolignols and/or allyl-/propenylphenols as noted earlier (but see Chapter 6.01).[7] Currently, only phenylalanine ammonia lyase activity, the entry point in the phenylpropanoid pathway, has been described in one alga.[33] Furthermore, an *in silico* search for putative phenylpropanoid pathway genes in the recently sequenced green alga, *Chlamydomonas reinhardtii*,[34] was considered unsuccessful, that is, for those genes with homology to the corresponding gymnosperm/angiosperm genes, only fragments of low homology/identity were obtained (S.-J. Kim, unpublished results). This again, suggests, at least in this particular case, that the phenylpropanoid pathway was absent. Thus, if these allyl-/propenylphenol metabolites are indeed formed by such organisms (e.g., *C. racemosa*, *C. tomentosum*, *E. compressa*, *H. musciformis, J. rubens*, and *S. variabile*), this would be of considerable scientific and evolutionary significance. Accordingly, this needs to be confirmed further through more in-depth biochemical studies under controlled laboratory conditions.

1.23.3.1.2 Bryophytes: liverworts, hornworts, and mosses

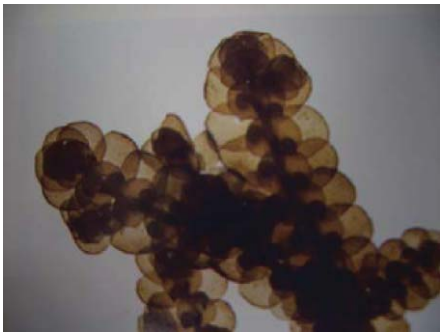
The liverworts (Marchantiophyta) are typically considered to be the most basal of the nonvascular land plants. They have the oldest fossil record among the bryophytes (see **Figure 3**) and apparently date back more than 475 My.[35] They are estimated to contain ~4500–5000 extant species.[36,37] By contrast, hornworts (Anthocerotophyta) contain the fewest species among the bryophytes (~300),[38] and their evolutionary relationship to vascular land plants appears to be uncertain. The hypothesis that hornworts are more basal than liverworts has been supported[39] as well as rejected[40] in recent years; their oldest fossil record is from the late Cretaceous (~70–90 Ma), long after when they are considered to have appeared.[41] The phytochemistry of hornworts is, however, largely unexplored. Mosses, on the contrary, are thought to have emerged up to ~360 Ma (early Carboniferous)[42] and are considered the largest group of bryophytes, totaling ~10 000 species.[43]

To our knowledge, the only few reports of monomeric allyl-/propenylphenols to date in bryophytes are from liverworts, and mostly come from a series of papers from the Asakawa laboratory from 1979 to 1985.[44–47] These studies described the GC–MS identification of eugenol (**4**) and/or methyleugenol (**42**) in five liverwort species, *Anthelia julacea*, *Conocephalum conicum*, *Frullania davurica*, *Marchesinia brachiata*, and *Marchesinia mackaii* (**Figure 5**). Additionally, γ-asarone (**29**) was reported in an unidentified Jamaican liverwort,[48] and, more recently, α-/β-/γ-asarones (**30/31/29**), (*E*/*Z*)-methylisoeugenols (**43/44**), and methyleugenol (**42**) were described in *M. mackaii* as well.[49] Interestingly, in the latter case, both allylphenols (**29**,**42**) and propenylphenols (**30**,**31**,**43**,**44**) were observed, with propenylphenols apparently being relatively more abundant.

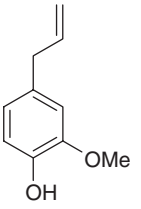
Some of the allylphenols were also reported as being major volatiles in the liverwort species investigated, for example, methyleugenol (**42**) in *M. mackaii*,[45] and were thus proposed to be able to possibly serve as

Anthelia julacea

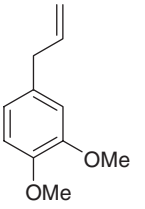
Conocephalum conicum



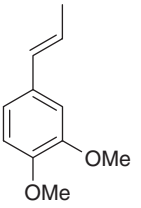
Frullania davurica



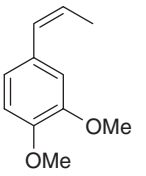
(**4**) Eugenol



(**42**) Methyleugenol



(**43**) (*E*)-Methylisoeugenol



(**44**) (*Z*)-Methylisoeugenol

Figure 5 Allyl-/propenylphenols in liverworts, *Anthelia julacea, Conocephalum conicum,* and *Frullania davurica*. Photographs from Professor Yoshinori Asakawa, Tokushima Bunri University, Japan.

chemotaxonomic markers.[47] These reports are of interest as they suggest a very early (evolutionary) elaboration of both the phenylpropanoid pathway and the allyl-/propenylphenol-forming enzymatic machinery in land plants. Conversely, neither hornworts nor mosses have been reported to accumulate monomeric allyl-/propenylphenols.

1.23.3.1.3 Pteridophytes: lycophytes, horsetails, and ferns

There are very few reports of monomeric allyl-/propenylphenols so far in other (vascular) basal plants. They apparently have not been detected in either lycophytes (the earliest extant vascular plant species, which are thought to have emerged ~420 Ma, in the late Silurian)[50] or ferns (see **Figure 3**). Anethole (**5**) has, however, been reported to occur in trace amounts (as identified by its retention index, mass spectrum, and coinjection with an authentic standard) in horsetail (*Equisetum arvense*, Equisetopsida).[51] As for the algae, this needs to be further biochemically established, that is, as to whether this is a true natural product formed by this species *in vivo* or a minor contaminant.

1.23.3.1.4 Spermatophytes: gymnosperms and angiosperms

1.23.3.1.4(i) Gymnosperms Cycads are often considered to be the most basal extant group of seed plants, with a fossil record dating back to the late Carboniferous (~300 Ma, **Figure 4**); nevertheless, the true evolutionary relationship between gymnosperm groups is at present still not certain.[52–54] There are ~300 species of cycads, distributed mainly through tropical and subtropical areas,[55] but their phytochemistry is not well explored. Nevertheless, the presence of both allyl- and propenylphenol derivatives in the volatiles from male and female cones of one cycad, *Cycas revoluta*,[56] appears to be on a firmer footing relative to the algae and horsetail reports above. Based on GC–MS analysis, together with comparison of the corresponding authentic standards, methylchavicol (**3**) was established as the primary component (~67–93%) of its volatiles, together with smaller amounts of anethole (**5**) and methyleugenol (**42**). To date, however, this is the only cycad known reportedly producing this class of natural products.

Conifers (Pinophyta) are thought to have emerged as early as the late Carboniferous (~310 Ma),[57,58] and now comprise ~630 extant species.[59] An initial review of the literature[8] suggested the absence of allyl-/propenylphenols proper in pine and hemlock (as well as poplar), except for numerous accounts of eugenol (**4**)/isoeugenol (**6**) being detected following pyrolysis GC–MS of presumed lignin-containing conifer tissues, for example, see Faix *et al.*[60] and Camarero *et al.*[61] Such moieties are considered generated by the pyrolysis of monolignol-derived metabolites, including polymeric lignins.

A recent, more in-depth, analysis using the specific structures for searching rather than compound names gave a somewhat different perspective. This resulted in the identification of a relatively small number of papers reporting complex mixtures of various essential oils and oleoresins obtained from either needles, bark, wood, or wounded stem tissue of nine pine (*Pinus*),[62–72] one spruce (*Picea*),[65] one Tasmanian conifer (*Lagarostrobos franklinii*),[73] and three juniper (*Juniperus*)[74] species. Buried within the tables in these studies, in addition to the plethora of mono-, sesqui-, and diterpenes, various allyl-/propenylphenols were detected in low to trace amounts, for example, methylchavicol (**3**) in seven of nine pine species examined,[62–64,66–70,72] with *Pinus taeda*[69] and *Pinus ponderosa*[72] also having methyleugenol (**42**). Three of these pines (*P. brutia*,[71] *P. contorta*,[62] and *P. sylvestris*[65]) as well as spruce (*Picea abies*)[65] reportedly also contained anethole (**5**), with methyleugenol (**42**) and (*E*)-methylisoeugenol (**43**) being detected in *P. brutia*[71] as well. In addition, the oil of *L. franklinii* wood was shown to contain **42**, **43**, and elemicin (**45**).[73] Anethole (**5**) was also detected in *J. brevifolia*, and (*E*)-methylisoeugenol (**43**) in *Juniperus formosana* and *Juniperus rigida*, whereas seven other juniper species apparently contained no allyl-/propenylphenols.[74] These data would thus suggest that both allyl- and propenylphenols can be present in these organisms. However, when the essential oil yields were taken into account, the overall amounts of these allyl-/propenylphenols were very low, that is, ranging from 0.002 to 0.06% of dry weight.

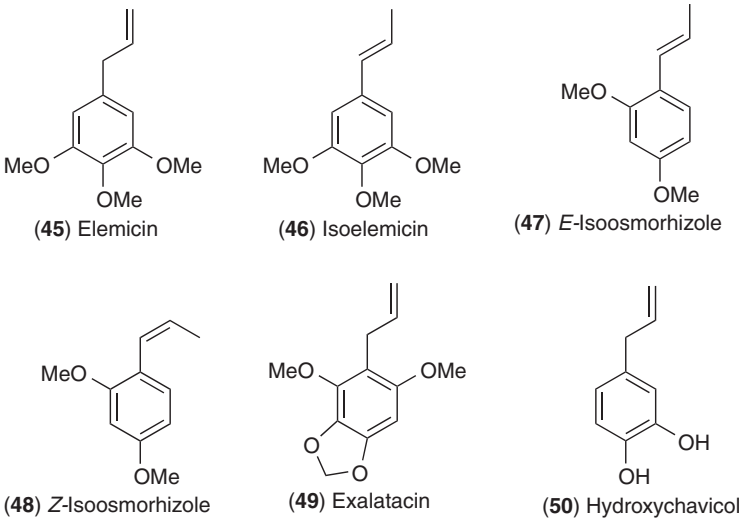
The detection of these substances, at trace to very low levels in the essential oil fractions, raises some interesting questions as to whether they are true gymnospermous natural products or, albeit less likely, result from other effects of an encroaching predator/pathogen, for example, through insect or pathogen attack with conversion of plant-derived monolignol-/lignin-derived components, resulting in release of trace amounts of

such substances. Such reports emphasize the need, however, for a full biochemical clarification to better understand the true chemotaxonomical significance of such observations.

Currently, there are no reports of allyl-/propenylphenols in either the Gnetales (which are thought to have emerged ~270 Ma)[75] or in *Ginkgo biloba* (the single remaining extant species of the Ginkgoaceae family, which is considered to have emerged at least ~170 Ma, see **Figure 4**).[76] Taken together, the evolutionary significance of this apparently scattered chemotaxonomy in the gymnosperms is, at present, not well understood.

1.23.3.1.4(ii) Angiosperms Angiosperms are the most widespread and diverse group of plants, having emerged ~125 Ma[77] (upper Jurassic, **Figure 4**) and are considered to comprise ~260 000 extant species.[78] They are divided into two major groups based on the number of seed embryonic leaves (cotyledons): the monocotyledons (~50–60 000 species, which contain most of the agriculturally cultivated plants) and the more abundant dicotyledons (~200 000 species). With a larger and better-studied number of species, the most frequent report of allyl-/propenylphenols and their derivatives are in the angiosperms. This includes the presence of allylphenols in various monocotyledons, such as eugenol (**4**)/methyleugenol (**42**) in *Lolium perenne* and *Bromus hordeaceus* (Poaceae) essential oils,[79] methyleugenol (**42**), 2-allyl-4,5-dimethoxyphenol (**28**), and γ-asarone (**29**) in the fragrance of the fruit fly orchid *Bulbophyllum cheiri*.[80] Other angiosperm orders where allyl-/propenylphenols have frequently been detected include the Apiales, Asterales, Lamiales, Laurales, Magnoliales, Malpighiales, Myrtales, Piperales, Rosales, and Sapindales. Allyl-/propenylphenols are often substantial contributors to the flavors of culinary spices, adding considerably to their taste/aroma; methylchavicol (**3**) and methyleugenol (**42**) are, for example, present as main components in the essential oil of tarragon (*Artemisia dracunculus*)[81,82] in the Asteraceae, whereas eugenol (**4**) constitutes up to 89% of the oil of cloves (*Syzygium aromaticum*, syn. *Eugenia aromaticum* or *Eugenia caryophyllata*, of the Myrtaceae) (**Figure 6**).[83–86]

The Piperaceae also accumulate a rich variety of specialized metabolites, including both allyl- and propenylphenols in several different chemotypes of *Piper marginatum*. These chemotypes reportedly accumulate as their main allyl-/propenylphenols either safrole (**22**), myristicin (**23**), (*E*)-isoosmorhizole (**47**), (*E*/*Z*)-isoosmorhizole (**47**/**48**), and anethole (**5**), or (α/β)-asarones (**30**/**31**) and exalatacin (**49**),[87] whereas the relatively uncommon hydroxychavicol (**50**) was found in *Piper betel*.[88] Myristicin (**23**) and elemicin (**45**) are also the main phenolic component of the oils of nutmeg (**Figure 6**) and mace (the seed and aril of fruits from *Myristica* spp., respectively), this being accompanied by methyleugenol (**42**), safrole (**22**), and (*E*)-methylisoeugenol (**43**).[89–93] Interestingly, although present in much smaller amounts, isoeugenol (**6**) and a methoxylated eugenol (of undetermined position) were also observed as glycosides.[94]



The allyl-/propenylphenols are perhaps best known, however, to accumulate (to various levels) in the herbs of the Apiaceae. This includes anethole (**5**) and methylchavicol (**3**) in fennel (*Foeniculum vulgare*)[95] and anise (*Pimpinella anisum*),[96] to more highly substituted metabolites such as isoelemicin (**46**) and asaricin (**32**) in

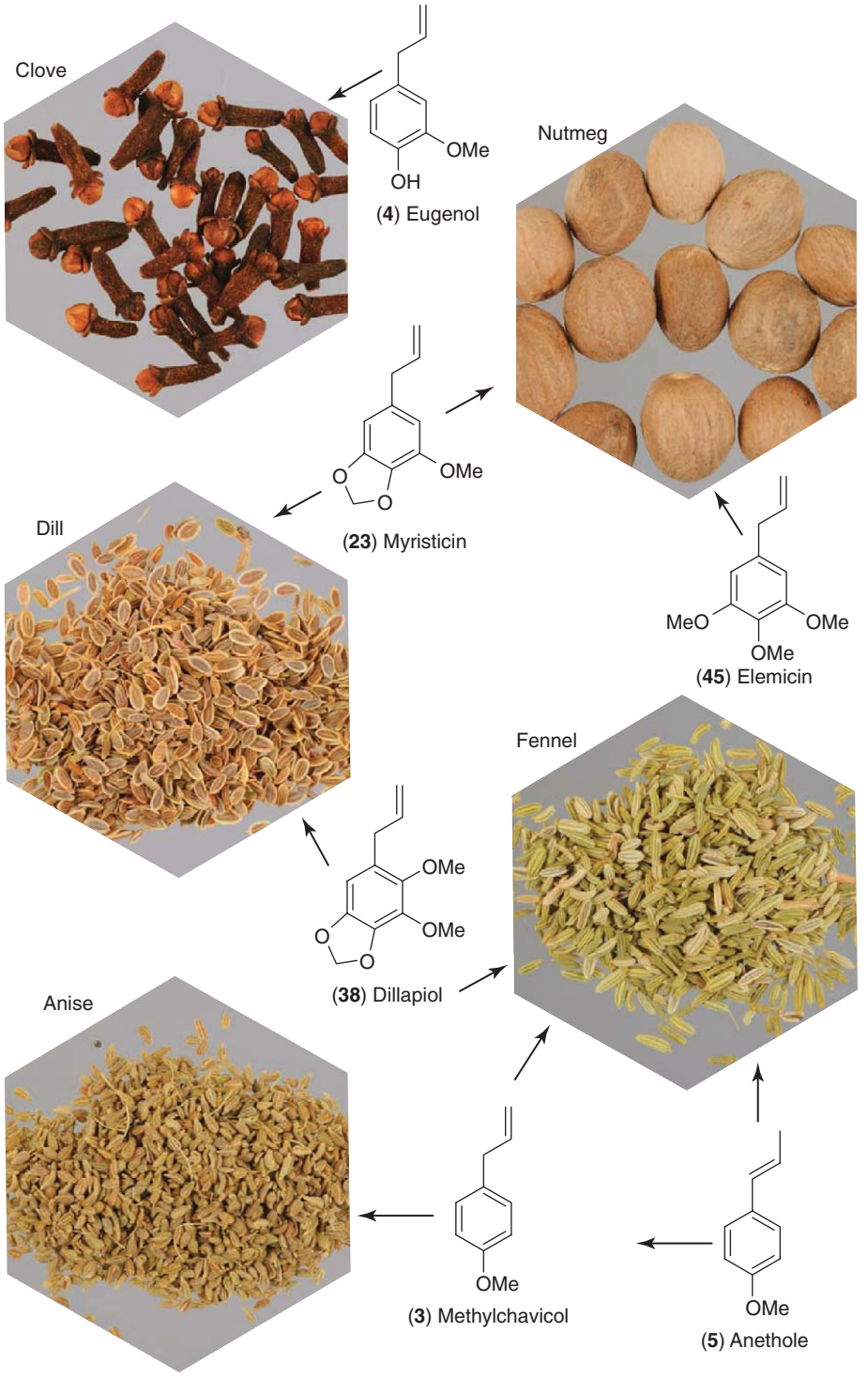


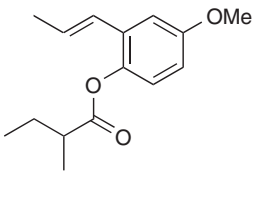
Figure 6 Aroma-contributing molecules in various spices. Photographs by Henri Moore, Washington State University, USA.

Ligusticum mutellina;[97] to myristicin (**23**) in dill (*Anethum graveolens*)[98] and parsley (*Petroselinum crispum*);[99] to apiol (**36**) in parsley[99] and celery (*Apium graveolens*);[100] and to dillapiol (**38**) in dill[98] and fennel[101] (**Figure 6**). The Lamiaceae species produce some of these natural products as well; for example, basil (*Ocimum basilicum*)

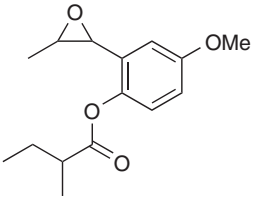
varieties accumulate methylchavicol (**3**), eugenol (**4**), and methyleugenol (**42**),[102,103] and different varieties of *Perilla frutescens* have been found to produce, among other compounds, methyleugenol (**42**), elemicin (**45**), isoelemicin (**46**), myristicin (**23**), dillapiol (**38**), and β-asarone (**31**).[104]

The pseudoisoeugenols, by contrast, appear to have a much more restricted chemotaxonomic distribution. They have been found mainly in the order Apiales, that is, in 22 *Pimpinella* species,[105,106] as well as in *Ferula szowitsiana*,[107] *Prangos pabularia*,[108] *Scaligeria tripartita*,[109] and *Tordylium ketenoglui*,[110] with *P. anisum* being the most extensively studied. Pseudoisoeugenols have also been reported to occur in *Hypericum perforatum*[111] (Clusiaceae) and *Origanum* × *adanense*[112] (Lamiaceae). Of the species examined so far, the most common pseudoisoeugenols reported are pseudoisoeugenyl-2-methylbutyrate (**51**), epoxypseudoisoeugenyl 2-methylbutyrate (**52**, **52a**, EPB), and epoxypseudoisoeugenyl tiglate (**53a/b**), respectively.

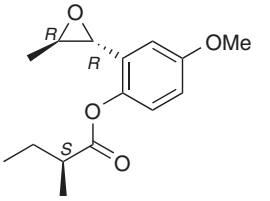
The formation of the epoxide functionality (e.g., in epoxypseudoisoeugenols **52–54**) gives rise to two stereochemical centers, which are, in most reports, not determined in terms of absolute configuration. Nevertheless, based on the relatively small number of structures with assigned configurations, the stereochemistry of the side-chain epoxide formation has been reported as *R,R* (e.g., **52a**), *S,S* (e.g., **53a**), and *S,R* (e.g., **53b**).[113,114] Interestingly, more than one stereoisomer has also been observed in some plant species. (For example, *P. major* reportedly contains both *S,S*- and *S,R*-epoxypseudoisoeugenyl tiglate (**53a** and **53b**), as well as *R,R*-epoxypseudoisoeugenyl 2-methylbutyrate (**52a**) in its roots, whereas *Pimpinella diversifolia* contains both the *R,R* and *S,S* epoxides of diesterified pseudoisoeugenols (**54a** and **54b**)).[113,114]



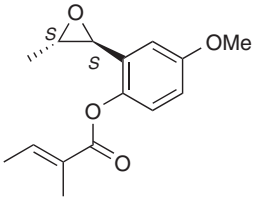
(**51**) Pseudoisoeugenyl 2-methylbutyrate



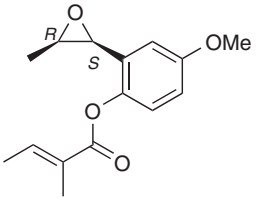
(**52**) Epoxypseudoisoeugenyl 2-methylbutyrate (EPB)



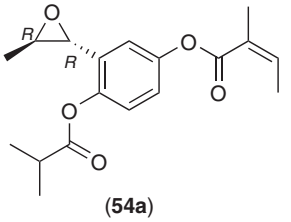
(**52a**) *trans*-Epoxypseudo-isoeugenyl 2-methylbutyrate



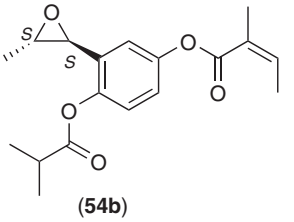
(**53a**) *trans*-Epoxypseudo-isoeugenyl tiglate



(**53b**) *cis*-Epoxypseudo-isoeugenyl tiglate



(**54a**)



(**54b**)

Taken together, the detection of allyl-/propenylphenols in the breadth of plant species examined so far raises interesting questions about how these pathways evolved, and whether these are examples of either convergent or divergent evolution (or both). If the provisional reports of algal allyl-/propenylphenols are correct, then elaboration of their biosynthetic pathways potentially preceded land plant colonization, with this being either sustained or reemerging in the liverworts and the other vascular plant species. However, this needs to be unequivocally established. The apparent lack of uniformity in allyl-/propenylphenol formation (and in known general phenylpropanoid metabolism as well) through extant plant lineages, though, might suggest a

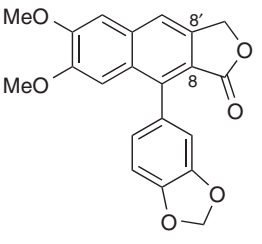
convergent evolutive process as perhaps more likely. That is, the formation of allyl-/propenylphenols might have appeared several times through plant evolution. These processes will only be better understood when the putative allyl-/propenylphenolic biosynthetic processes of algae, as well as those of nonvascular and early vascular plants are better studied, including that of phenylpropanoid pathway gene evolution.

1.23.3.2 Lignans

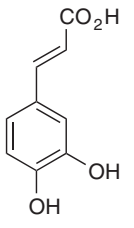
Lignans are known to be present in a large number of plant families, ranging from nonvascular plants such as the liverworts and hornworts, to ferns (in the pteridophytes), to the spermatophytes, but not, to our knowledge, in either the green algae or mosses. As discussed above (Section 1.23.3.1), with the exception of the entry step (phenylalanine ammonia lyase),[33] neither the enzymes nor the encoding genes of the phenylpropanoid pathway have been reported in algae. The sequenced genome of the moss, *Physcomitrella patens*,[115] on the contrary, appears to have genes of relatively high similarity (i.e., from 67 to ~86%) to most (if not all) of the corresponding *Arabidopsis thaliana* genes of the phenylpropanoid pathway proper,[8] from phenylalanine ammonia lyase to cinnamyl alcohol dehydrogenase. However, with the exception of 4-coumarate:CoA ligase,[116] their enzyme activities have not yet been established. To add to the possible complexity of the chemotaxonomy/structural diversity of lignans, a red alga-associated actinomycete bacterial species (*Nocardia* sp.) reportedly produces the 8–8′-linked arylnaphthalene lignan justicidin B (**55**),[117] whereas a biosynthetic pathway to form caffeic acid (**56**) was described in another actinomycete (*Saccharothrix espanaensis*).[118] The former is the first report of any lignan in bacteria and, although unprecedented, demands further biochemical confirmation. If correct, it would represent the most basal elaboration of a lignan-forming pathway from an evolutionary perspective.

1.23.3.2.1 Bryophytes: liverworts, hornworts, and mosses

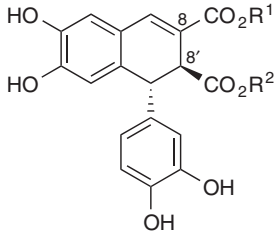
The chemotaxonomical comparison of basal plant lignan occurrence (i.e., in liverworts and hornworts) is of interest, since it possibly provides additional insight regarding the evolution of the phenylpropanoid pathway and of lignan biosynthesis. With one exception, the liverworts analyzed so far have been found to accumulate several 8–8′-linked arylnaphthalene and aryldihydronaphthalene lignans (or derivatives thereof), as well as norlignans. These can be provisionally envisaged as caffeic acid (**56**)-derived, for example, those from *Pellia epiphylla* (**57b–60b**),[119,120] *Lepidozia incurvata* (**57**), *Chiloscyphus polyanthos* (**57**),[121] *Lophocolea heterophylla* (**57b**),[122] *Jamesoniella autumnalis* (**61**, **62a–64a**),[122,123] *Lepicolea ochroleuca* (**65b–67b**),[124] *Bazzania trilobata* (**57b**, **68b**, trilobatins A–K (**69b**, **71b–80b**), **70b**, and the 5′–5″-dimer of **57b** (**81b**)),[125,126] as well as the putative norlignans from *L. incurvata* (**82**, **83**), *C. polyanthos* (**82**, **84**), *Jungermannia exsertifolia* (**82**, **85**),[121] *J. autumnalis* (**86**, **87**),[123] and *B. trilobata* (**82**).[125] Typically, they have aryldihydronaphthalene skeleta (e.g., **57–60**, **65**, **69–80**) with, in a few instances, the pendant aryl group having undergone fission at either the C2–C3 positions resulting in lactone formation as proposed by Tazaki *et al.* [123] (e.g., in **62–64**, **86**, **87**), or at C3–C4 in **68**. Interestingly, all of these metabolites contain (or contained) catechol groups, as well as having dicarboxylic acid (or ester-linked) moieties at carbons C9 and C9′, i.e., there is no apparent methylation of the phenolic groups in these species. A few structures also lack a terminal carboxyl group at C9, thereby providing entry into norlignans (e.g., **82–87**). Relative to our first chemotaxonomic analysis,[1] the lignans (**65–81**) present in *L. ochroleuca*[124] and *B. trilobata*,[125,126] as well as the norlignans **82–85**[121,125] represent new structures.



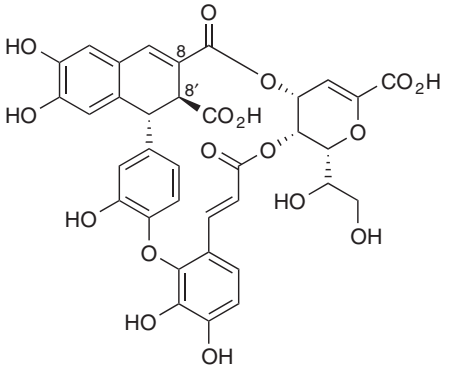
(**55**) Justicidin B



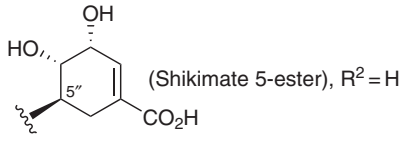
(**56**) Caffeic acid



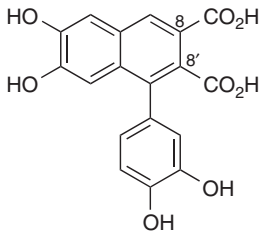
(**57b**) $R^1 = R^2 = H$, (–)-Epiphyllic acid
(**58b**) $R^1 = H$, $R^2 = Me$



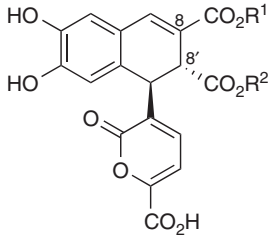
(**60b**) (–)-Pelliatin



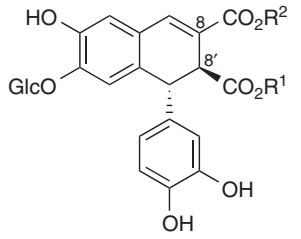
(**59b**) R^1 = (Shikimate 5-ester), R^2 = H



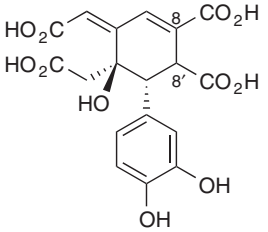
(**61**)



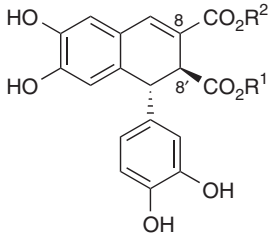
(**62a**) $R^1 = R^2$ = H, (+)-Jamesopyrone
(**63a**) R^1 = Me, R^2 = H
(**64a**) R^1 = H, R^2 = Me

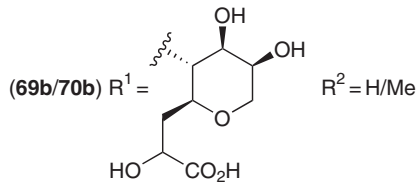


(**65b**) R^1 = Me, R^2 = H
(**66b**) R^1 = Shikimate 5′-ester, R^2 = H
(**67b**) $R^1 = R^2$ = Shikimate 5′-ester

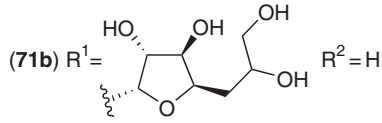


(**68b**) (–)-Bazzania acid

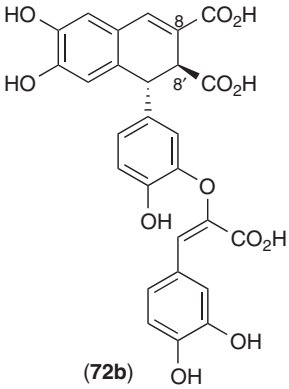




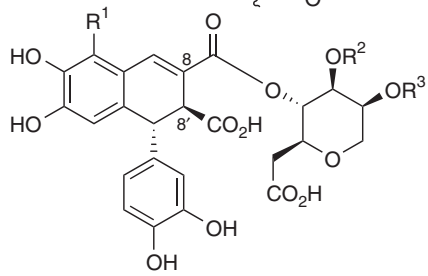
(**69b**/**70b**) R^1 = R^2 = H/Me



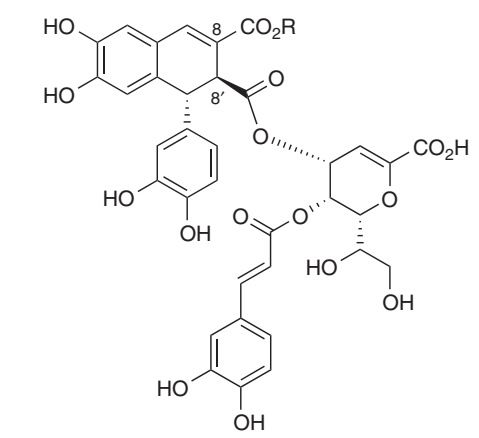
(**71b**) R^1 = R^2 = H

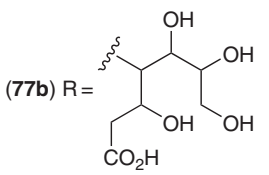


(**72b**)

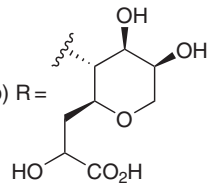


(**73b**) $R^1 = R^2 = R^3$ = H
(**74b**) R^1 = 5-Etherified epiphyllic acid, $R^2 = R^3$ = H
(**75b**) $R^1 = R^2$ = H, R^3 = 9-Esterified jamesopyrone
(**76b**) $R^1 = R^3$ = H, R^2 = 9-Esterified jamesopyrone

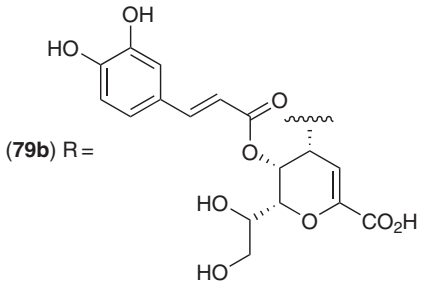




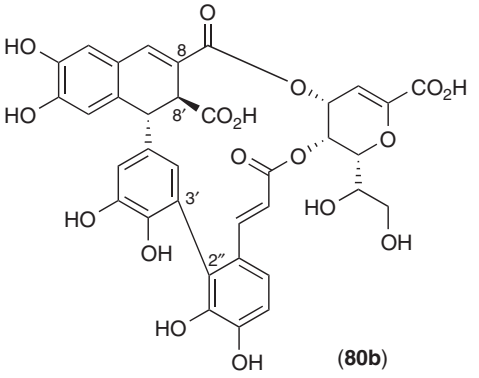
(**77b**) R =



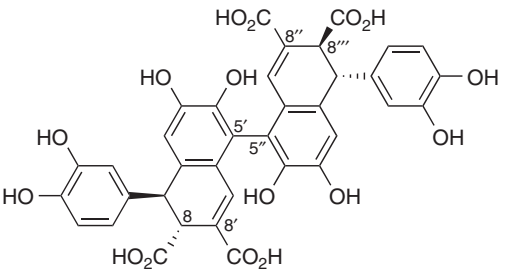
(**78b**) R =



(**79b**) R =



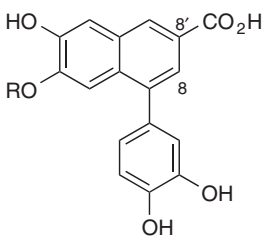
(**80b**)



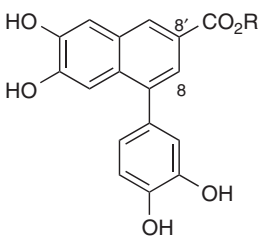
(**81b**)

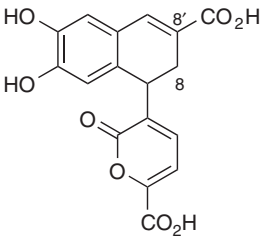
Of the lignans in different liverwort species, epiphyllic acid (**57**) and jamesopyrone (**62**) appear to be two of the most commonly found. Using chiral column chromatography, it was shown that enantiomerically pure (−)-epiphyllic acid (**57b**) accumulates in *Calypogeia azurea*, *L. heterophylla*, and *Aneura pinguis*, whereas the (+)-enantiomer **57a** is found in *Haplomitrium mnioides*, *J. autumnalis*, and *Marsupella emarginata*; (+)-jamesopyrone (**62a**) also accumulated in the latter two species. In other liverwort species, both enantiomers of epiphyllic acid are reported, with one being in enantiomeric excess (e.e.) over the other, that is, the (−)-enantiomer **57b** in >90% e.e. in *Bazzania yoshinagana* and *Heteroscyphus planus*, and the (+)-enantiomer **57a** in >80% e.e. in *Diplophyllum taxifolium*.[127]

Of particular interest also is the report of a presumed allyl-/propenylphenol-derived lignan, (−)-licarin A (**88b**) in *Jackiella javanica*.[128] This differs from the previously described liverwort allylphenols (Section 1.23.3.1.2) in having the pendant double bond conjugated with the aromatic ring. The appearance of a C9/C9′ deoxygenated lignan in this early land plant group is thus again of considerable evolutionary and biosynthetic interest, as it suggests further that pathway elaboration was an early feature in land plant colonization.

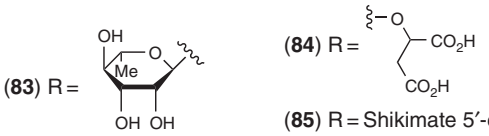


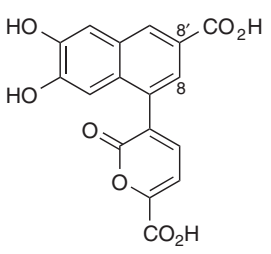
(**82**) R = H



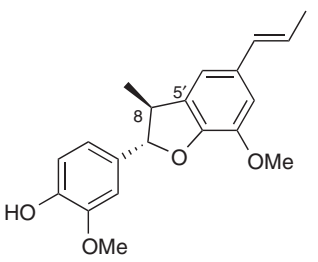


(**86**)



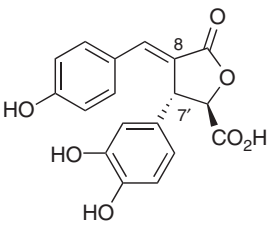


(**87**) Scapaniapyrone

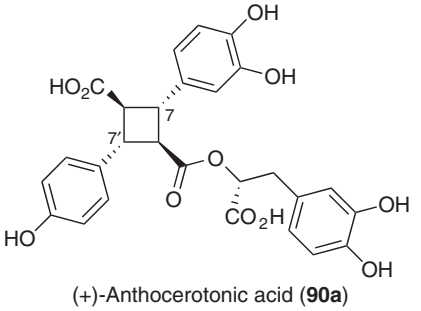


(**88b**) (–)-Licarin A

To our knowledge, the only reports of lignans in hornworts are those of the 8–7′-linked (+)-megacerotonic acid (**89a**) and the 8–7′-linked, 9′-*O*-esterified (+)-anthocerotonic acid (**90a**) present in the hornworts *Megaceros flagellaris*,[129,130] *Dendroceros japonicus*,[130] *Notothylas temperata*,[130] *Phaeoceros laevis*,[130] and *Anthoceros punctatus*,[129,130] respectively. Both are optically active, with the respective (+)-enantiomers being in excess. As before, for the majority of lignans reported in liverworts, these metabolites have highly oxygenated C9 and C9′ end-group moieties and catechol groups (lacking *O*-methylation). Although both lignans are 8–7′-linked, (+)-megacerotonic acid (**89a**) also has a lactone functionality, whereas (+)-anthocerotonic acid (**90a**) has a cyclobutane ring.



(+)-Megacerotonic acid (**89a**)

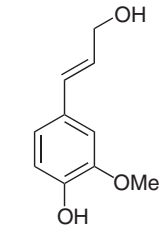


(+)-Anthocerotonic acid (**90a**)

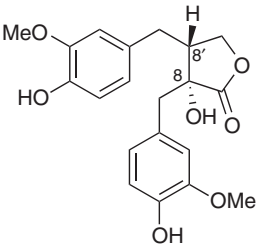
Mosses, on the contrary, have not been reported to accumulate lignans (or, as discussed above, allyl-/propenylphenols), thus differing from the other bryophytes. Their presumed absence in mosses is also of unknown overall significance in evolutionary terms, but underscores the differences in elaboration of various branches of phenylpropanoid metabolism during land plant colonization by early nonvascular plants.

1.23.3.2.2 Pteridophytes: lycophytes, horsetails, and ferns

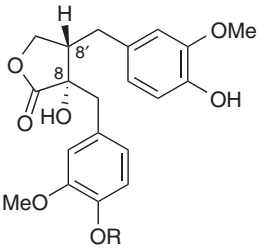
There appears to be very few reports of lignans in the basal vascular plant species so far, although there is considerable structural variety. To date, it is known that the lycophyte *Selaginella doederleinii* accumulates the presumed coniferyl alcohol (**91**)-derived (+)-matairesinol (**10a**), (+)-nortrachelogenin (**92a**, wikstromol) and the glucoside of (−)-nortrachelogenin (**92b**), (−)-nortracheloside (**93b**), as well as the presumed sinapyl alcohol (**94**)-derived 8–8′-linked (−)-*epi*-syringaresinol (**95b**, lirioresinol), and (−)-syringaresinol (**96b**, lirioresinol B).[131] These findings are in agreement with other studies that established the presence of sinapyl alcohol (**94**)-derived lignins in *Selaginella*.[132,133] Additionally, two syringyl-like lignan derivatives (i.e., both *O*-glycosylated and with side-chains containing one and two less carbons, respectively) were also reported to occur in two other *Selaginella* species, namely the 8–5′-linked tamariscinoside C (**97**) from *Selaginella tamariscina*[134] and (−)-moellenoside A (**98b**) from *Selaginella moellendorffii*.[135] In general, however, sinapyl alcohol (**94**) moieties are considered to have evolved mainly in the angiosperms.[136]



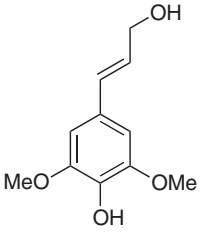
(**91**) Coniferyl alcohol



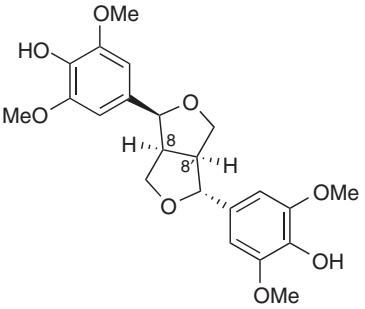
(**92a**) (+)-Nortrachelogenin
[= (+)-Wikstromol]



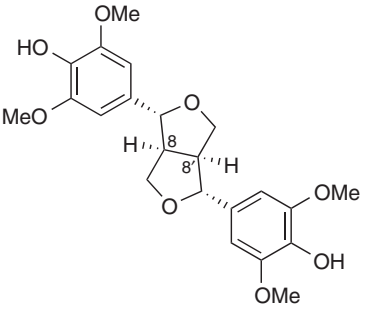
(**92b**) R = H, (–)-Nortrachelogenin
(**93b**) R = Glc, (–)-Nortracheloside



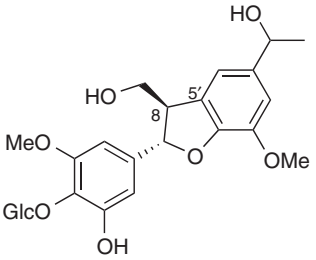
(**94**) Sinapyl alcohol



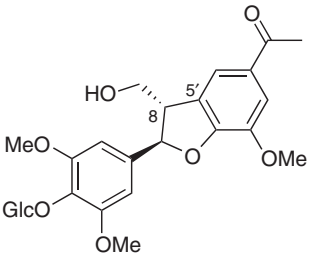
(**95b**) (–)-*epi*-Syringaresinol
[= (–)-Lirioresinol]



(**96b**) (–)-Syringaresinol
[= (–)-Lirioresinol B]



(**97**) Tamariscinoside C

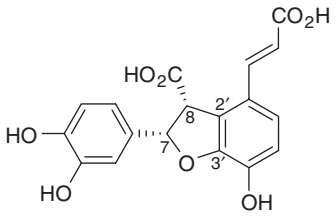


(**98b**) (–)-Moellenoside A

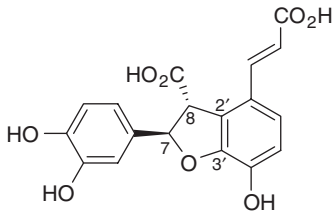
Some examples of reported lignans in fern species are the putatively hydroxycinnamic acid-derived metabolites. This includes the 8–2′-linked (−)-blechnic acid (**99b**) (and its C7 and C8 epimers, **100b** and **101b**, respectively) as well as its shikimate derivative (−)-brainic acid (**102b**), which have been isolated from several Blechnaceae fern species (**Figure 7**).[137,138] These lignans thus share some commonalities with those described aforehand in liverworts and hornworts, that is, with regard to the presence of catechol and carboxylic acid moieties. However, the 8–2′ linkage is quite distinct from the mostly 8–8′-linked aryldihydronaphthalenes in nonvascular lignans.

Other fern lignans can be presumed coniferyl alcohol (**91**)-derived,[1] that is, the two glucosides of (+)-dihydrodehydrodiconiferyl alcohol (**103a**) and (+)-lariciresinol (**105a**), namely, **104b** and **106b** from the fern *Pteris vittata* (Pteridaceae),[139] with these being 8–5′- and 8–8′-linked, respectively. In addition to their different linkage modes, these lignans are noteworthy in having a (saturated) propanol side-chain in **103a** and **104b**, as well as for the *O*-glucoside derivatization found in both **104b** and **106b**.

Didymochlaena truncatula (Dryopteridaceae) contains yet a third structural type, in terms of putative biosynthetic origin. In addition to (−)-nortrachelogenin ((−)-wikstromol (**92b**)) being present, this fern also contains the interesting 5–*O*–4′- and 5–*O*–2′-linked partially dearomatized lignans (+)-didymochlaenone A (**107**) and (−)-didymochlaenone B (**108**).[140] Both lignans are optically active, and both bear an allylphenol-like side-chain functionalization. These natural products are of particular interest since phenoxy radical–radical coupling has (provisionally) occurred at the C5 position, which presumably initially harbored a methoxyl group. Also noteworthy is the presence of methylenedioxy groups, which were previously considered to have evolved later in the gymnosperm lignans,[141] as well as the oxygenation at C2′ to form the conjugated enone moiety of **107** and **108** and the 5–*O*–2′ interunit linkage of **108**.

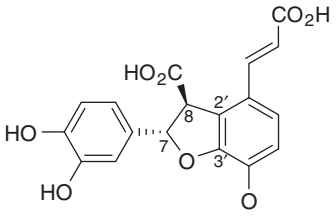


(**99b**) (–)-Blechnic acid

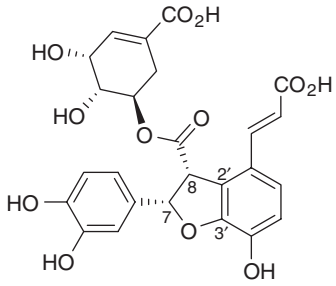


(**100b**) (–)-7-*epi*-Blechnic acid

Blechnum spicant

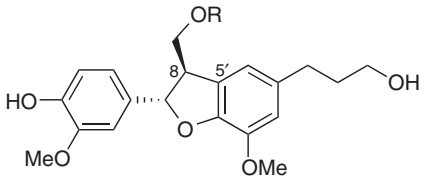


(**101b**) (–)-8-*epi*-Blechnic acid

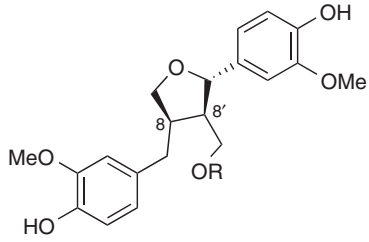


(**102b**) (–)-Brainic acid

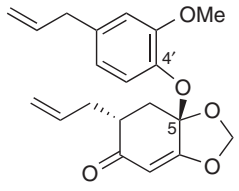
Figure 7 Lignans in *Blechnum spicant* (pteridophyte). Photograph by Henri Moore, Washington State University, USA.



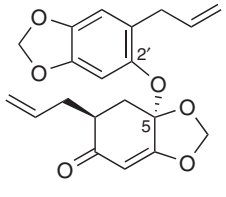
(**103a**) R = H, (+)-*trans*-Dihydrodehydrodiconiferyl alcohol
(**104b**) R = Glc, (–)-*trans*-Dihydrodehydrodiconiferyl alcohol-9-*O*-β-D-glucoside



(**105a**) R = H, (+)-Lariciresinol
(**106b**) R = Glc, (–)-Lariciresinol-9-*O*-β-D-glucoside



(**107**) (+)-Didymochlaenone A



(**108**) (–)-Didymochlaenone B

The fern lignans discussed above can thus be classified in three quite distinct groups based on their side-chain functionalities (i.e., whether containing carboxylic acid, alcohol, or allyl/propenyl moieties). Fern lignans thus appear to have an increased structural diversity relative to those present in earlier groups, including the degree of oxygenation of their side-chain moieties, the linkage modes between monomeric precursors, and glycosylation. No lignans have apparently yet been described in other early vascular plant groups, including the horsetails in which, as discussed before in Section 1.23.3.1.3, only the propenylphenol **5** has been reported to accumulate in trace amounts. Nevertheless, as discussed in the next section, this increased structural diversity trend becomes even more evident in gymnosperm and angiosperm lignans.

1.23.3.2.3 Spermatophytes: gymnosperms and angiosperms

1.23.3.2.3(i) Gymnosperms The distribution of various lignan/norlignan skeleta throughout the gymnosperms is not uniform in terms of structural types, with none so far reported in the cycads. As described earlier (Section 1.23.3.1.4), however, allyl-/propenylphenols have been found in at least one cycad, *C. revoluta*.[56]

The evolution of the different gymnosperm plant groups was, nevertheless, overall accompanied by a vast increase in the structural variety of both lignans and norlignans, with more than a hundred different structures now reported.[142] Although often particularly abundant in conifer heartwood[143] and knots,[144] they have also been isolated from trees at all developmental stages, and from all different tissues, such as bark, roots, needles, cones, and so on. Most lignans are 8–8′-linked,[142] giving rise to carbon skeleta classified as either furofurans (e.g., (−)-sesamin (**11b**) in *Juniperus thurifera*, **Figure 8**);[145] tetrahydrofurans (e.g., shonanin (**109**) in *Calocedrus formosana*[146] and *P. taeda* cell cultures);[147] dibenzylbutyrolactones (e.g., (−)-matairesinol (**10b**) in *P. spicatus*);[13] dibenzylbutanes (e.g., (−)-secoisolariciresinol (**110b**) in *P. spicatus*);[148,149] arylnaphthalenes (e.g., junaphthoic acid (**111**) in *Juniperus sabina*),[150] or aryltetrahydronaphthalenes (e.g., (−)-plicatic acid (**112b**) in *Thuja plicata*[151–153] and (−)-α-conidendrin (**113b**) in *Tsuga heterophylla*).[154,155] As aforementioned, some of these skeleta are also present in more basal plant groups, for example, arylnaphthalenes, which are abundant in liverworts, as well as furofurans and dibenzylbutyrolactones, which have been reported to occur in ferns.

Generally, gymnosperm lignans have either guaiacyl-like aryl groups, that is, coniferyl alcohol (**91**)-like, such as in shonanin (**109**), (−)-secoisolariciresinol (**110b**), and (−)-α-conidendrin (**113b**), or infrequently have 3,4-methylenedioxy bridges, such as with (−)-sesamin (**11b**). Interestingly, some species can accumulate lignans where the C5 carbon is hydroxylated (e.g., (−)-plicatic acid (**112b**) and its derivatives in *T. plicata*), with junaphthoic acid (**111**) having the analogous position additionally *O*-methylated. Although the majority of gymnosperm lignans have hydroxymethyl-like side-chain functionalities (i.e., either as alcohols/ethers or as products presumably derived from further metabolism, such as those affording lactones), there are also a few examples of methyl (i.e., C9- and/or C9′-deoxygenated) end groups in some conifer lignans, for example, junaphthoic acid (**111**) in *J. sabina*. Indeed, although it is also tempting to speculate that these C9- and/or C9′-deoxygenated lignans are allyl-/propenylphenol-derived, this needs to be established biochemically as other possibilities could exist.

Most 8–8′-linked lignans exist as dimers, although higher oligomers have been reported, with the largest documented so far being the (−)-plicatic acid (**112b**)-derived lignans of MW ∼10 000 Da in *T. plicata*.[156] As for the other plant classes described earlier, many of the conifer lignans are either optically pure or highly enriched in one enantiomeric form, for example, 7-hydroxymatairesinol (**114**) is present as ∼85% of the whole lignan mixture in *P. abies* knots, but where the (−)-7*S*- and (−)-7*R*-forms are in a ratio of 32:1.[157]

Lignans with 8–5′ and 8–*O*–4′ linkages are also frequently reported, for example, (±)-dehydrodiconiferyl alcohols (**14a/b**) and guaiacylglycerol 8–*O*–4′ coniferyl alcohol ether (**115**) in *P. taeda* cell cultures.[147] Other metabolic modifications often observed include those leading to saturated propanol side-chains, for example, (−)-dihydrodehydrodiconiferyl alcohol (**103b**) in *Juniperus chinensis*,[158]

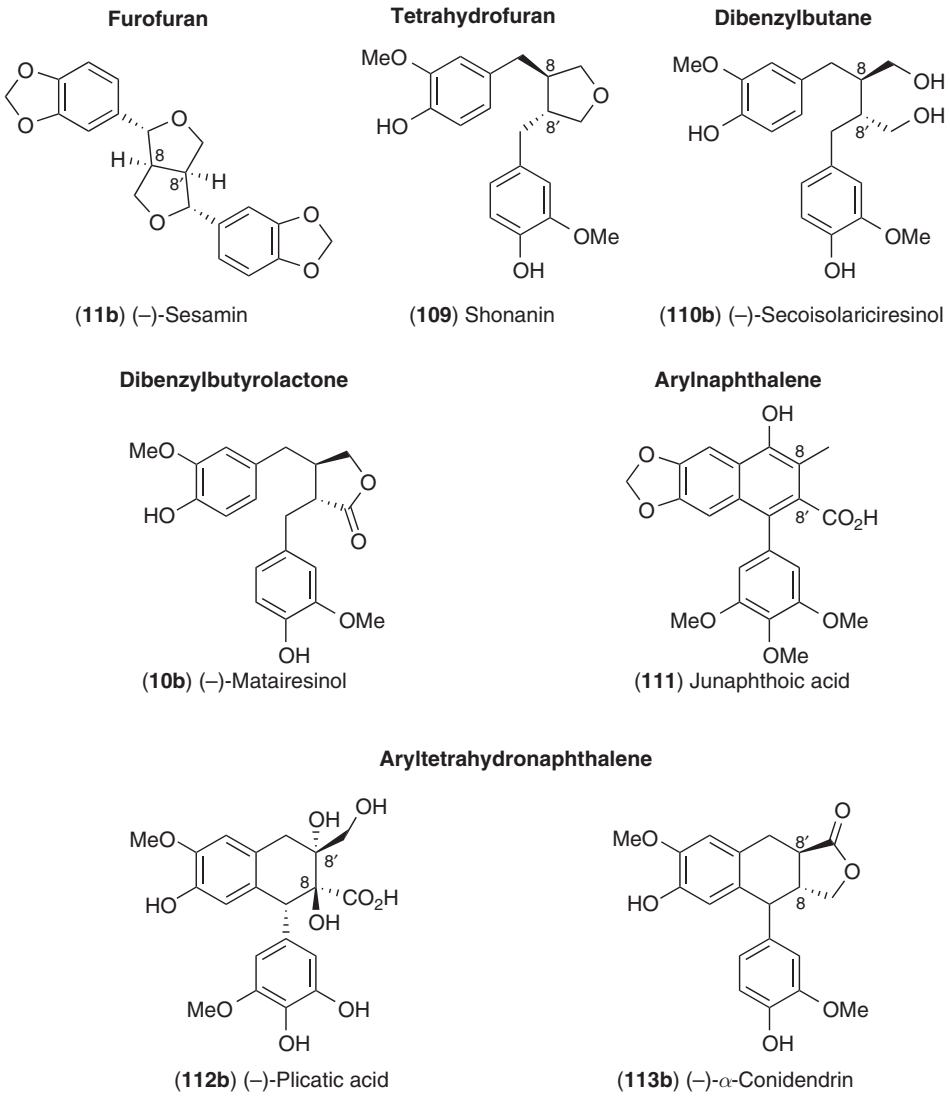
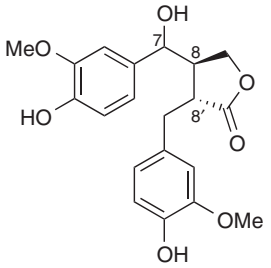
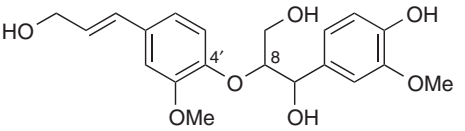


Figure 8 Different classes of 8–8′-linked lignans.

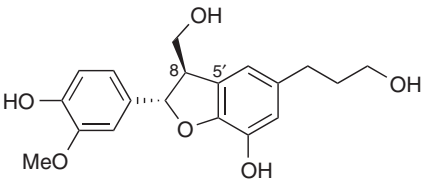
(+)-cedrusin (**116a**) in *Cedrus deodara*[159] and *T. heterophylla*,[155] as well as **117** in *J. chinensis*.[158] The additionally reduced (i.e., cleaved 7–*O*–4′ linkage) peracetylated lignan **118** is also found in *Cryptomeria japonica*.[160] Side-chain reduction apparently occurs on phenylpropanoid monomers as well, with dihydromonolignols such as dihydroconiferyl alcohol (**119**) and its *O*-glucoside (**120**) being present in *Picea glauca* knots.[161] The 8–5′-linked dihydrofuran lignans, (±)-dehydrodiconiferyl alcohols (**14a**/**b**), are thought to be central precursors of (±)-dihydrodehydrodiconiferyl alcohols (**103a**/**b**) and (±)-cedrusins (**116a**/**b**): *Pinus taeda* cell cultures have been shown to regiospecifically *O*-demethylate the C3′ of both dehydrodiconiferyl alcohols (**14a**/**b**) and dihydrodehydrodiconiferyl alcohols (**103a**/**b**).[1] In combination with side-chain reductions, these reactions afforded (±)-cedrusins (**116a**/**b**) as a product in these cell cultures.[1]



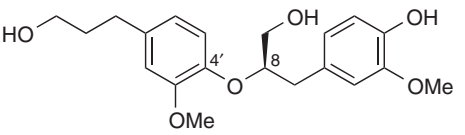
(**114a**) 7*R*, Hydroxymatairesinol
(**114b**) 7*S*, allo-Hydroxymatairesinol



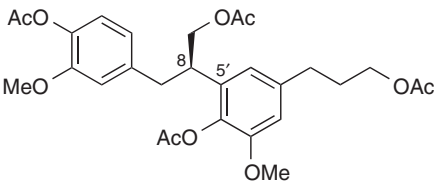
(**115**) Guaiacylglycerol 8-*O*-4′ coniferyl alcohol ether



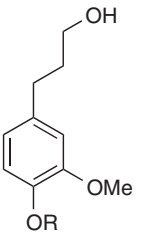
(**116a**) (+)-Cedrusin



117

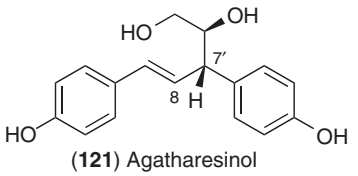


118

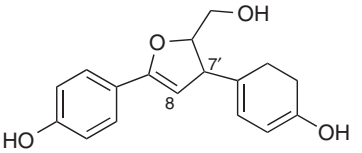


(**119**) R = H, Dihydroconiferyl alcohol
(**120**) R = Glc, Dihydroconiferyl alcohol glucoside

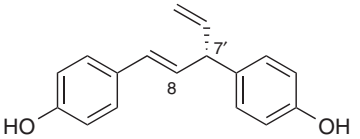
Loss of a terminal carbon at C9 affords norlignans.[142,162] Most of the true gymnosperm norlignans are 8–7′-linked dibenzylpentanes ($C_6C_5C_6$) and are notable components of the Cupressaceae, Araucariaceae, and Taxodiaceae, but are apparently absent from the Pinaceae, Podocarpaceae, and other conifers.[142,162] These are exemplified by agatharesinol (**121**) in *Agathis australis* (Araucariaceae),[163,164] *Sequoia sempervirens*,[165] and *Sequoiadendron gigantea*,[165] as well as (−)-cryptoresinol (**122b**)[166] and (−)-(*E*)-hinokiresinol (**123b**) in *C. japonica*.[167,168] They are structurally quite distinct from the arylnaphthalene norlignans found in the more basal plants mentioned earlier (Section 1.23.3.2.1). Although **121–123** only have monooxygenated phenyl groups, other 8–7′-linked dibenzylpentane norlignans can have catechol, dimethoxyaryl, and trimethoxyaryl moieties, for example, sequirin B (**124**) in *S. sempervirens*, and permethyl-sequirin E (**125**) and permethyl-sequirin G (**126**) in *S. gigantea*.[165] Other 8–7′-linked norlignans include the structurally rare spiro-lignan (−)-athrotaxin (**127b**) from *Athrotaxis selaginoides*[169] and *Metasequoia glyptostroboides*,[170] as well as the presumed product of (−)-hydroxyathrotaxin (**128b**) rearrangement, the cycloheptadiene lignan metasequirin B (**129**), from *M. glyptostroboides*.[171] Additional linkage modes present in conifer norlignans are less common, for example, sequirin D (**130**) in *S. sempervirens*[165] and the 7–7′-linked norlignan **131** in *Araucaria angustifolia*.[172]



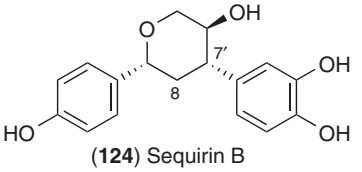
(**121**) Agatharesinol



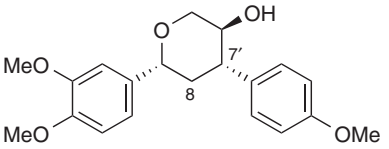
(**122b**) (–)-Cryptoresinol



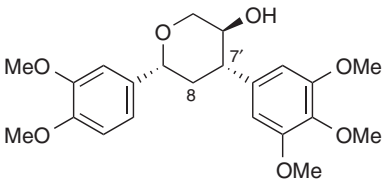
(**123b**) (–)-(*E*)-Hinokiresinol



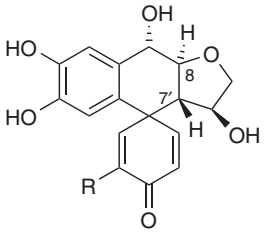
(**124**) Sequirin B



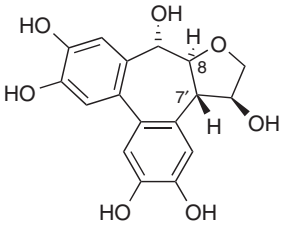
(**125**) Permethyl-sequirin E



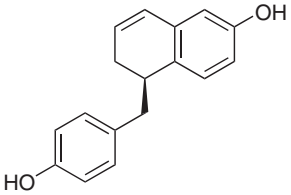
(**126**) Permethyl-sequirin G



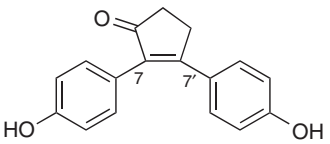
(**127b**) R = H, (–)-Athrotaxin
(**128b**) R = OH, (–)-Hydroxyathrotaxin



(**129**) Metasequirin B

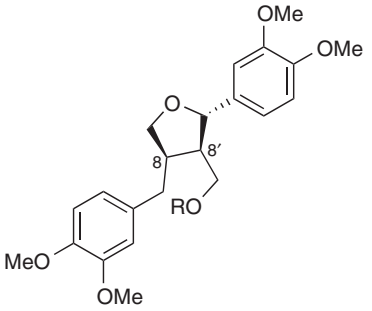


(**130**) Sequirin D

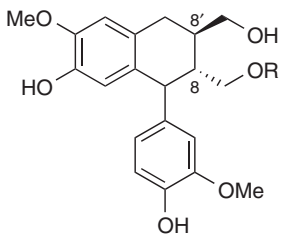


131

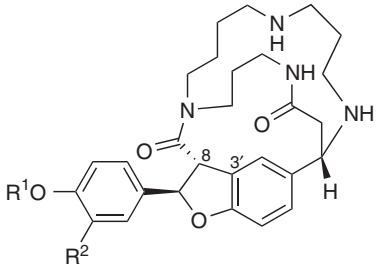
There are only few reports of lignans in Gnetales, most of which are of 8–8′-linked compounds, for example, (+)-syringaresinol (**96a**) in *Gnetum gnemon* roots;[173] (±)-syringaresinols (**96a**/**b**) in *Ephedra alata*;[174] (+)-lariciresinol (**105a**), (+)-isolariciresinol (**133a**), and their 9-acetate derivatives **132a** and **134a** in *Ephedra viridis*;[175] as well as the 8–3′-linked spermine diamides, ephedradines A (**135**), B (**136**), and C (**137**) in mao-kon, the crude drug from *Ephedra* roots.[176–178] The latter are rare examples of N-containing lignan compounds. *Ginkgo biloba*, on the contrary, has been reported to accumulate the 8–8′-linked (+)-sesamin (**11a**) in both its heartwood[179] and leaves.[180] As noted earlier, allyl-/propenylphenols have not been reported in either of the latter two gymnosperm families.



(**105a**) R = H, (+)-Lariciresinol
(**132a**) R = Ac, (+)-Lariciresinol 9-acetate



(**133a**) R = H, (+)-Isolariciresinol
(**134a**) R = Ac, (+)-Isolariciresinol 9-acetate



(**135**) $R^1 = R^2 = H$, Ephedradine A
(**136**) $R^1 = H$, $R^2 = OMe$, Ephedradine B
(**137**) $R^1 = Me$, $R^2 = OMe$, Ephedradine C

Additionally, in many highly valued woody species, nonstructural lignans and less frequently norlignans can be deposited in their heartwoods, for example, (−)-plicatic acid (**112**) and its derivatives, which can constitute up to 20% of its dry weight in *T. plicata*,[156] and which are in part responsible for the wood's durability and texture. Other heartwood-accumulating lignans/norlignans include 7-hydroxymatairesinol (**114**) and (−)-α-conidendrin (**113b**) in *T. heterophylla*,[154] and others such as **116**, **122–124** in *C. japonica*,[166,167] all of which significantly contribute to their heartwood properties. It is also of interest that all three of these species are long-living gymnosperms: *Thuja plicata*, native to the northwestern United States and southwestern Canada, and *C. japonica*, native to Japan and China, can have lifespans in excess of 3000 years, whereas *T. heterophylla*, a native to the west coast of North America, has specimens as old as 1200 years.

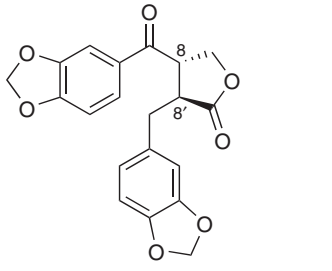
Therefore, the emergence of gymnosperms, in particular the conifers, apparently gave rise to a great increase in the number and variety of lignan and norlignan structures. Although the number of the reports so far may potentially result from a bias of phytochemical studies toward higher plants that are often important wood resources, the gymnosperm lignin structures are ostensibly structurally more varied, that is, in terms of linkage modes, oxygenation patterns of both aromatic groups and side chains, as well as further modifications such as those giving rise to dihydrophenylpropanoids and norlignans. This structural diversity is further expanded within the angiosperms, as described below.

1.23.3.2.3(ii) Angiosperms The emergence of the angiosperms was also accompanied by another massive increase in lignan and (less commonly) norlignan skeletal types, albeit with most of the common structures observed in the gymnosperms being retained in the angiosperms. Of the lignans/norlignans reported to date, however, most are found in the woody and nonwoody dicotyledons, with only a few examples in the monocotyledons. Many contain new skeletal types and many are again optically active.

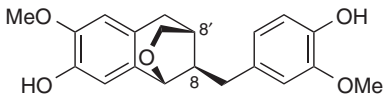
As for the gymnosperms, the terminal groups (C9/C9′) of the angiosperm lignans are frequently modified, that is, with these having lactone/ether (e.g., **138a**, (−)-padocin (**139b**)), hydroxymethyl (e.g., **139b**, (−)-balanophonin (**140b**)), aldehyde (e.g., **140b**), and carboxylic acid (e.g., 5–5′-dehydrodiferulic acid (**141**)) groups, as well as others lacking an oxygenated functionality at C9/C9′ (e.g., **142b–151**).[181,182] Note, however,

that 5–5′-dehydrodiferulic acid (**141**) can be viewed as a lignan artifact of sorts, since it is presumed to be derived from cell wall polymeric carbohydrates containing feruloyl moieties in close proximity (which can undergo radical–radical coupling), and indeed it is only released upon alkali treatment of cell walls.[183,184]

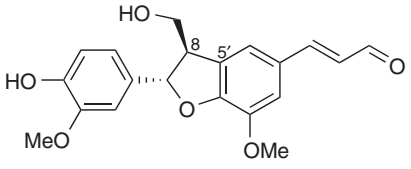
Although aromatic ring substitution patterns in many of the angiosperm lignans are guaiacyl-like (e.g.. **139b–141, 144b**, including those with methylenedioxy groups such as **138a, 146b**), numerous hydroxyphenyl (e.g., **150b**) and trihydroxylated phenyl ring(s) (e.g., **145a, 147b, 152a**) lignans have also been reported. Additional interesting features are those of relatively rare skeletal types, such as the 7–1′-linked (+)-peperomin A (**152a**),[185] the 1–2′-linked (−)-eupomatilone 6 (**153b**),[186] and the 7–1′-linked (+)-iso-ocobullenone (**154a**).[187]



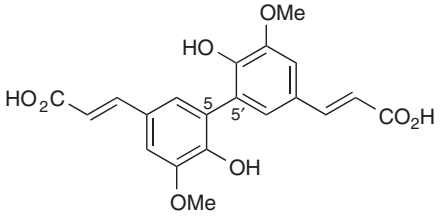
(+)-**138a**



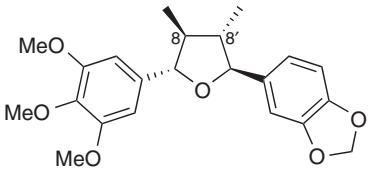
(**139b**) (–)-Padocin



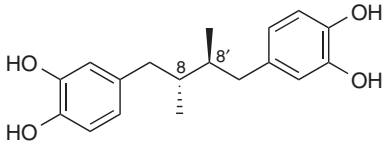
(**140b**) (–)-Balanophonin



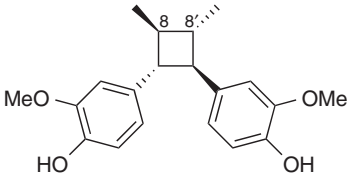
(**141**) 5–5′-Dehydrodiferulic acid



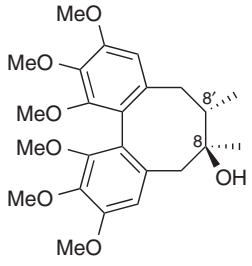
(–)-(**142b**)



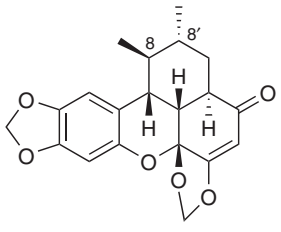
(**143**) Nordihydroguaiaretic acid (NDGA)



(**144b**) (–)-Endiandrin A



(**145a**) (+)-Schizandrin

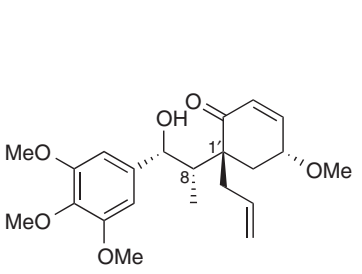


(**146b**) (–)-Sauchinone

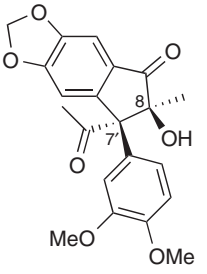
The 8–8′ linkage mode, however, overall remains the most frequent in terms of the number of examples of reported structures. These can be again classified as furofurans (e.g., (+)-sesamin (**11a**) from *S. indicum*);[14,15] tetrahydrofurans (e.g., (−)-olivil (**12b**) from *O. europaea*);[16–18] dibenzylbutanes (e.g., (−)-guaiaretic acid (**9b**) from *G. officinale*);[10–12] dibenzylbutyrolactones (e.g., (−)-matairesinol (**10b**) in *Forsythia intermedia*);[188] aryletrahydronaphthalene derivatives (e.g., (−)-podophyllotoxin (**1b**) from *Podophyllum* spp.);[189] dibenzocyclooctadienes (e.g., (+)-schizandrin (**145a**) from *Schizandra chinensis*);[190] cyclobutanes (e.g., (−)-endiandrin A

(**144b**) in *Endiandra anthropophagorum*);[191] as well as other more uncommon structures, such as the polycyclic (−)-sauchinone (**146b**) from *S. chinensis*[192] and (−)-padocin (**139b**) from *Haplophyllum cappadocicum*.[193]

Among other coupling modes reported in angiosperms, these include 8–5′- (e.g., **140b**);[194] 8–1′- (e.g., **147b**);[195] 8–7′- (e.g., **148**);[21] 7–1′- (e.g., **149**);[196] 8–*O*–4′- (e.g., **150b**);[197] and benzodioxocin- (e.g., **151**)[198] linked lignans. Such diverse skeletal types further underscore the varied biochemical processes operative in angiosperms, most of which still remain to be elucidated (see Section 1.23.4). Other structural features of interest are the presumed rearrangement products, such as **152a** and **153b**, formed through putative migration of aryl substituents, or that of **154a** through ring expansion.



(**147b**) (–)-Megaphone



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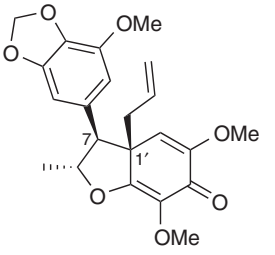
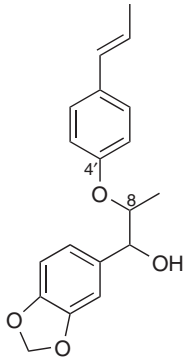
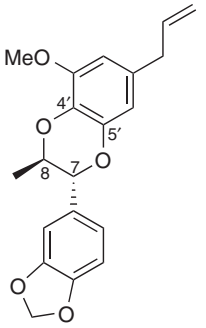


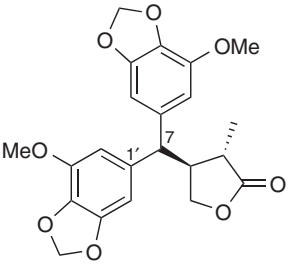
(**149**) Chrysophyllon 1A



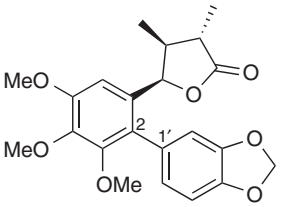
(–)-(**150b**)



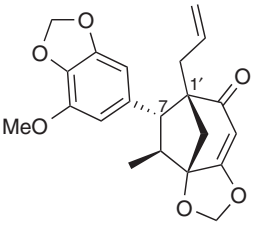
(**151**) Eusiderin B



(**152a**) (+)-Peperomin A

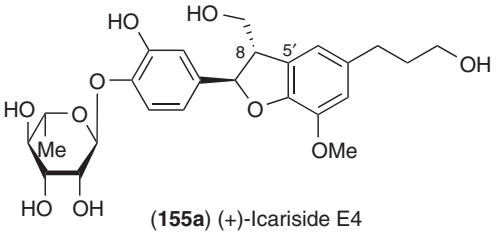


(**153b**) (–)-Eupomatilone 6

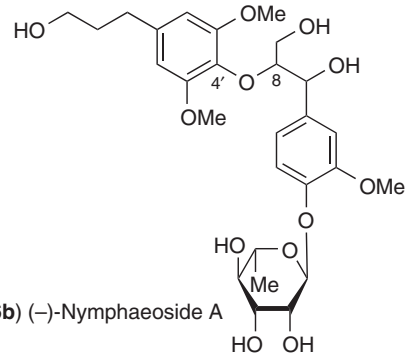


(**154a**) (+)-Iso-ocobullenone

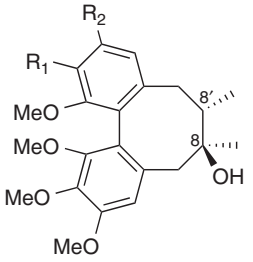
Additionally, the 8–5′-linked (+)-icariside E4 (**155a**) and the 8–*O*–4′-linked (−)-nymphaeoside A (**156b**) found in *Nymphaea odorata* have saturated side-chains.[199] This is a biochemical signature previously observed for some of the lignans in ferns and gymnosperms. The dibenzocyclooctadiene 8–8′-linked lignans present in *Sc. chinensis* (e.g., (+)-schizandrin (**145a**) and gomisins A–C (**157a–159b**))[190,200] are of an apparently unique skeletal type, however, presumably being formed from a linear 8–8′-linked diarylbutane lignan through aryl radical–radical coupling.



(**155a**) (+)-Icariside E4

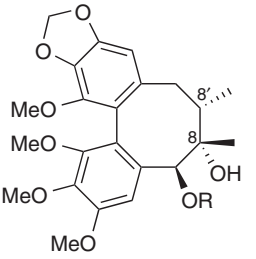


(**156b**) (–)-Nymphaeoside A



(**145a**) $R_1 = R_2 = OMe$, (+)-Schizandrin

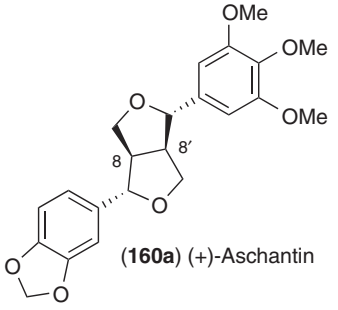
(**157a**) $R_1 + R_2 = OCH_2O$, (+)-Gomisin A



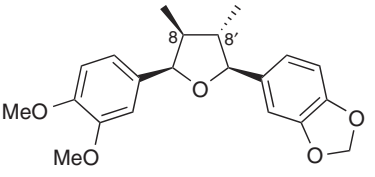
(**158b**) R =  (Angelate), (–)-Gomisin B

(**159b**) R = Benzoate, (–)-Gomisin C

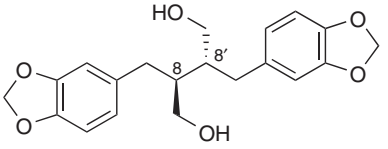
There are also more than 100 distinct lignans[201] isolated and characterized from the genus *Piper* alone, which contains ~700 species including *Piper nigrum*, the source of black and white peppers. Many of these lignans contain common structural motifs, such as the 8–8′-linked furofurans (e.g., (+)-aschantin (**160a**)), the tetrahydrofurans (e.g., (+)-calopiptin (**161a**)), and the diarylbutanes (both linear and cyclic, e.g., (−)-dihydrocubebin (**162b**) and heterotropan (**163**), respectively). However, there are also examples of other linkage types such as 8–3′ (e.g., (+)-conocarpan (**16a**) and schmiditin (**164**)), 8–1′ (e.g., (+)-burchellin (**165a**) and presumed rearrangement products, such as kadsurenin H (**166**)), as well as 8–*O*–4′ (e.g., polysyphorin (**167**)), and 8–2′/3′ (e.g., isofutoquinol A (**168**)).



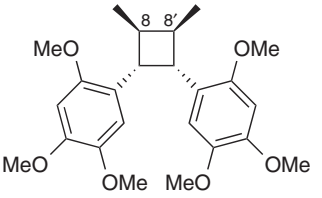
(**160a**) (+)-Aschantin



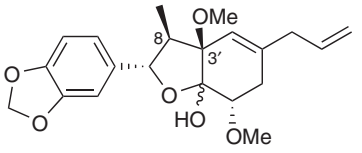
(**161a**) (+)-Calopiptin



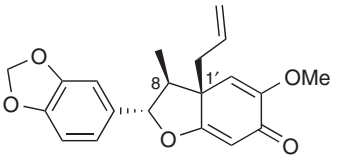
(**162b**) (–)-Dihydrocubebin



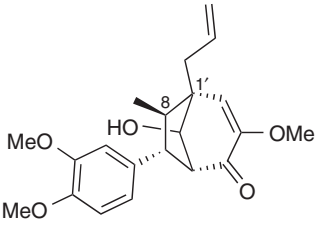
(**163**) Heterotropan



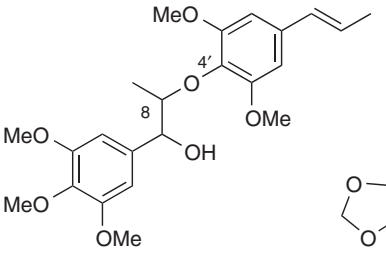
(**164**) Schmiditin



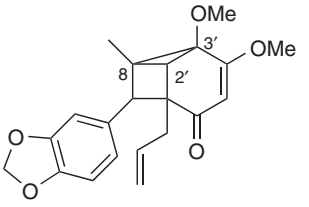
(**165a**) (+)-Burchellin



(**166**) Kadsurenin I

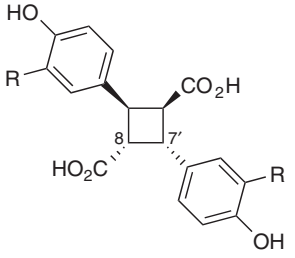


(**167**) Polysyphorin

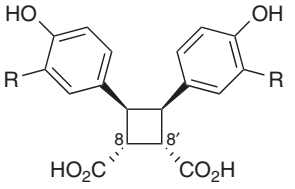


(**168**) Isofutoquinol A

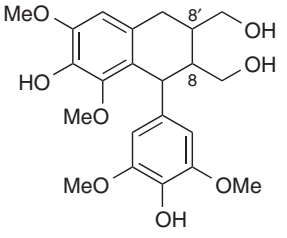
As noted before,[1] only a few monocotyledons are known to accumulate lignans, such as the aryl cyclobutanes, the dihydroxytruxillic/truxinic acids (**169**/**170**) in cereals and grasses.[202,203] Nevertheless, these, as well as **144b** and **163**, may not be necessarily formed through enzymatic catalysis, but perhaps instead as 2,2-photochemical adducts.[202–204] Bamboo (*Phyllostachys edulis*) stems, on the contrary, accumulate, in addition to the 8–8′-linked aryltetrahydronaphthalene lyoniresinol (**171**), the two dilignans phyllostadimers A (**172b**) and B (**173a**).[205] Although all three bamboo lignans contain syringyl-like aromatic moieties, the latter two are 8′–8″-linked dimers but containing the previously unknown 7′–*O*–9/8–*O*–9′ linkages.



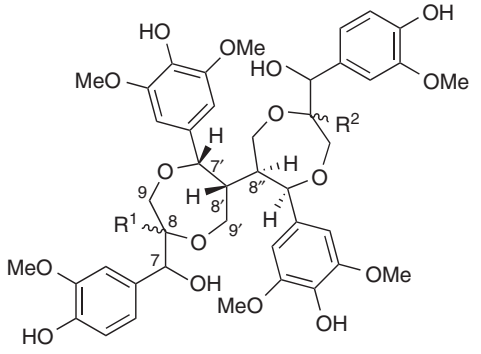
(**169**) R = H or OMe,
Dihydroxytruxillic acids



(**170**) R = H or OMe,
Dihydroxytruxinic acids



(**171**) Lyoniresinol



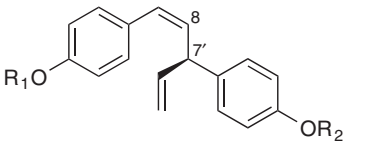
(**172b**) R1 = H, R2 = H, (–)-Phyllostadimer A

(**173a**) R1 = H, R2 = H, (+)-Phyllostadimer B

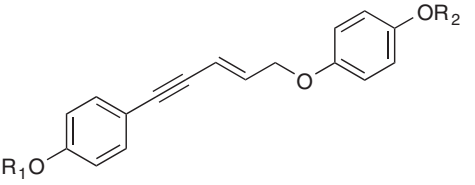
In contrast to the lignans, there are relatively few examples of natural products considered as norlignans in angiosperms. They are, however, most often found in the Asparagales, for example, the 8–7′-linked (+)-(*Z*)-hinokiresinol (**174a**) that occurs in *Asparagus* spp.,[206,207] and in its (−)-form (**174b**) in *Anemarrhena asphodeloides*,[208] as well as the glycosides **175**–**177** in *Hypoxis* spp., which are derived from the (+)-enantiomer

174a.[209,210] The rare acetylenic norlignans compounds, asparenydiol (**178**), asparenyol (**179**), and asparenyn (**180**) also occur in *Asparagus* spp.,[211] as well as rooperol (**181**) glycoside, hypoxoside (**182**) in *Hypoxis* spp.,[212] and nyasicoside (**183**) in *Curculigo capitulata*.[213] Of particular interest is the oxygen insertion forming metabolites **178**–**180**.

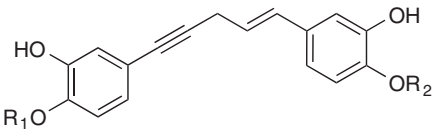
Additional presumed norlignans present in other orders are either 8–8′- or 8–3′-linked. These include the tetrahydrofuran cestrumoside (**184**) in *Cestrum diurnum* (Solanales),[214] the benzofuran machicendiol (**185**) in *Machilus glaucescens* (Laurales),[215] and the arylnaphthalene vitrofolal A (**186**) in *Vitex rotundifolia* (Lamiales).[216] Aromatic substitution patterns in the norlignans are again diverse, with hydroxyphenyl and guaiacyl moieties (and their *O*-methyl and methylenedioxy derivatives) apparently more common. Side-chain functionalities are also varied, being either hydroxymethyl, aldehydic, or deoxygenated.



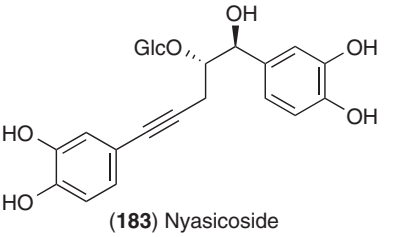
(**174a**) $R_1 = R_2 = H$, (+)-(*Z*)-Hinokiresinol
(**175**) $R_1 = H$, $R_2 = Glc$, (*Z*)-Mononyasine A
(**176**) $R_1 = Glc$, $R_2 = H$, (*Z*)-Mononyasine B
(**177**) $R_1 = R_2 = Glc$,(*Z*)-Nyasoside



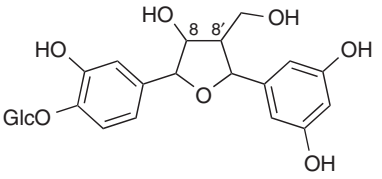
(**178**) $R_1 = R_2 = H$, Asparenydiol
(**179**) $R_1 = H$, $R_2 = CH_3$, Asparenyol
(**180**) $R_1 = R_2 = CH_3$, Asparenyn



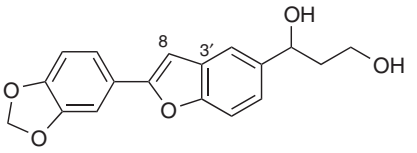
(**181**) $R_1 = R_2 = H$, Rooperol
(**182**) $R_1 = R_2 = Glc$, Hypoxoside



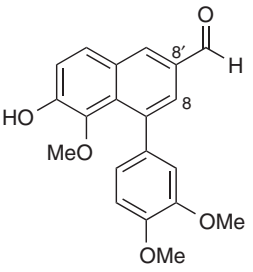
(**183**) Nyasicoside



(**184**) Cestrumoside



(**185**) Machicendiol



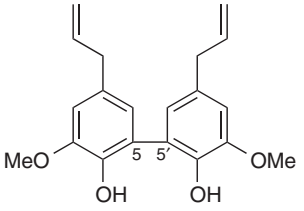
(**186**) Vitrofolal A

1.23.3.3 Evolution of Biochemical Pathways to Allyl-/Propenylphenols and Lignans: Observations on Co-occurrence

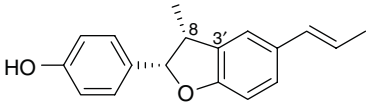
The diversity and distribution of lignans and norlignans from the most basal plants through to the spermatophytes (gymnosperms and angiosperms), allied to their possible absence in mosses, horsetails, and cycads, seem to indicate that their formation was a feature that may also have appeared multiple times during plant

evolution. That is, with their formation being part of a convergent, and not necessarily divergent, biochemical evolutionary process. This is consistent with the phylogenetic/evolutionary 'scattering' of the allyl-/propenylphenols discussed aforehand. Nevertheless, such considerations are at present merely speculative, and underscore the need for a much more thorough (bio)chemical understanding of lignan-forming processes through the diverse extant plant lineages.

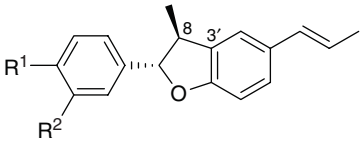
Interestingly, the relative levels of accumulation of lignans, norlignans, and their putative monomeric precursors vary markedly among species. In the case of allyl-/propenylphenols, for example, only a small amount of the lignan, dehydrodieugenol (**187**) (~0.0016% dry wt), accumulates in clove,[217] whereas there is up to ~89% of eugenol (**4**) in the essential oil.[86] *Piper regnellii*, by contrast, accumulates similar amounts (~1.5–1.6% dry wt) of both allyl-/propenylphenols (**23**, **36**, **38**) and the presumed allyl-/propenylphenol-derived lignans (**16a**, **188–193**, **Figure 9**).[218] On the contrary, the creosote bush (*Larrea tridentata*) apparently accumulates no significant amount of monomeric allyl-/propenylphenols, but yet can have ~10% nordihydroguaiaretic acid (NDGA) (**143**) (dry wt) as one of its lignans in the leaves.[219] Biochemical explanations for such variability need to be fully understood in future.



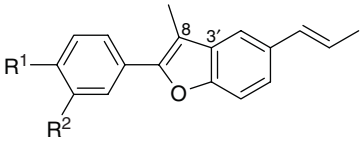
(**187**) Dehydrodieugenol



(**188b**) (–)-*epi*-Conocarpan



(**16a**) $R^1 = OH$, $R^2 = H$, (+)-Conocarpan
(**189a**) $R^1 = OH$, $R^2 = OMe$
(**190a**) $R^1 + R^2 = OCH_2O$, (+)-Regnelline



(**191**) $R^1 = OH$, $R^2 = H$, Eupomatenoid-6
(**192**) $R^1 = OH$, $R^2 = OMe$, Eupomatenoid-5
(**193**) $R^1 + R^2 = OCH_2O$, Eupomatenoid-3

Figure 9 Lignans in *Piper regnellii*. Photographs by Laurence B. Davin, Washington State University, USA.

1.23.4 Lignan Early Biosynthetic Steps: 8–8′ Phenylpropanoid Coupling

The previously discussed chemotaxonomical distribution of allyl-/propenylphenols, lignans, and norlignans resulted in two major observations: The first was that of the still incomplete understanding as to the extent of elaboration of the phenylpropanoid pathway throughout the plant kingdom, with provisional evidence suggesting some algae may have entire biochemical pathways to the allyl-/propenylphenols. The second was that of the enormous structural diversity of the lignans and norlignans, in terms of skeletal types occurring in the major land plant groups. Such observations and reports thus underscored the need to obtain a full biochemical understanding of the various proteins, enzymes, and genes involved in their specific pathways. Progress made so far in this is described below.

1.23.4.1 Discovery of the (+)-Pinoresinol-Forming Dirigent Protein and Encoding Gene

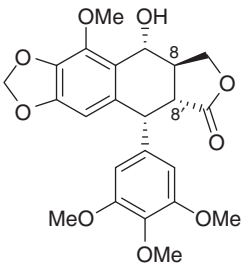
In the course of discovering how lignan formation occurs biochemically in different plant species, our earlier metabolic studies[220–226] established that pinoresinol (**13**) was the central precursor of various 8–8′-linked lignans, that is, leading to the formation of furofuran (e.g., sesamin (**11**)), tetrahydrofuran (e.g., lariciresinol (**105**)), dibenzylbutane (e.g., secoisolariciresinol (**110**)), dibenzylbutyrolactone (e.g., matairesinol (**10**)), and aryltetrahydronaphthalene (e.g., (−)-6-methoxypodophyllotoxin (**194b**, **Figure 10**) and (−)-plicatic acid (**112b**)), depending on the species. Additionally, secoisolariciresinol (**110**) and matairesinol (**10**) were deduced to be intermediates in the biosynthesis of the aryltetrahydronaphthalene (e.g., **194b**)[226] and, by extension, the arylnaphthalene lignans.

Forsythia shrubs (Oleaceae) were key to the discovery of many of these biochemical processes. *Forsythia* species accumulate various 8–8′-linked lignans in differing amounts: Specifically, *F. suspensa* contains (+)-pinoresinol (**13a**), (+)-phillyrin (**195a**), and (+)-phillygenin (**196a**),[227] whereas (−)-matairesinol (**10b**), (−)-arctigenin (**197b**), and (−)-arctiin (**198b**) are present in *Forsythia viridissima*,[227] with *F. intermedia* accumulating all of the above (**Figure 11**).[188]

The genus *Forsythia* was named after the Scottish botanist, William Forsyth (1737–1804). All but one of the known native species originates from northeastern Asia: Japan (*F. japonica*), Korea (*F. ovata*, *F. saxatilis*, *F. koreana,* and *F. densiflora*), and China (*F. giraldina*, *F. likiangensis*, *F. mandschurica*, *F. mira*, *F. suspensa,* and *F. viridissima*). One, *F. europaea*, is, however, endemic to Europe.[228] Other cultivars have been developed from these species, and *F. intermedia* was considered to be a hybrid between *F. suspensa* and *F. viridissima* based on the lignans isolated.[188] A recent phylogenetic study based on chloroplast DNA variation though suggests this not to be the case: *F. intermedia* does not group with either *F. suspensa* or *F. viridissima*, but forms a clade with *F. koreana*, *F. mandschurica*, and *F. saxatilis*.[228]

Initially, presumed *F. suspensa* stem cell wall residue preparations were demonstrated to engender stereoselective coupling of coniferyl alcohol (**91**) to afford (+)-pinoresinol (**13a**).[229] Subsequent solubilization and fractionation of the crude cell wall protein extract ultimately led to the isolation of a (+)-pinoresinol-forming dirigent protein (DP) named from the Latin *dirigere*, to guide or align.[230] This protein influenced the outcome of

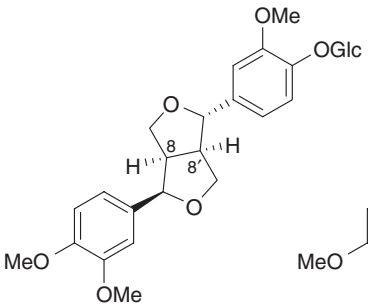
Linum flavum



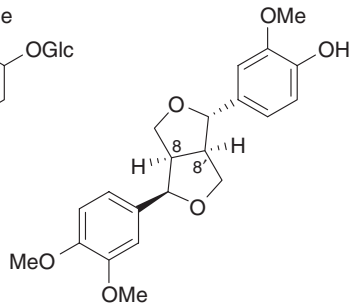
(**194b**) (–)-6-Methoxypodophyllotoxin

Figure 10 (–)-6-Methoxypodophyllotoxin (**194b**) in *Linum flavum*. Photograph by Laurence B. Davin, Washington State University, USA.

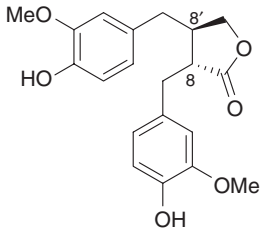
Forsythia intermedia



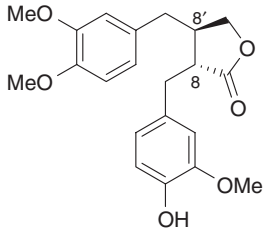
(**195a**) (+)-Phillyrin



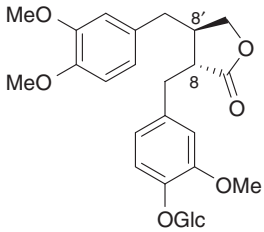
(**196a**) (+)-Phillygenin



(**10b**) (–)-Matairesinol



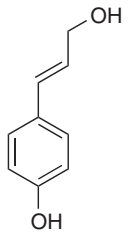
(**197b**) (–)-Arctigenin



(**198b**) (–)-Arctiin

Figure 11 Lignans in *Forsythia intermedia*. Photograph by Laurence B. Davin, Washington State University, USA.

coniferyl alcohol (**91**) radical–radical coupling reactions in the presence of an external one-electron oxidizing agent (such as laccase, ammonium peroxydisulfate, FMN). SDS–PAGE indicated a DP monomeric size of ~26–27 kDa.[230] In the absence of the DP, however, coniferyl alcohol (**91**) gave rise only to racemic mixtures of (±)-pinoresinols (**13a/b**), (±)-dehydrodiconiferyl alcohols (**14a/b**), and (±)-*erythro*/*threo* guaiacylglycerol 8–*O*–4′ coniferyl alcohol ethers (**115a/b**) due to nonregiospecific coupling. The (+)-pinoresinol-forming DP was next established to engender the formation of (+)-pinoresinol (**13a**) in a concentration-dependent manner, that is, higher DP concentrations *in vitro* led to a larger e.e. of (+)-pinoresinol (**13a**), instead of the other possible dimeric products.[231] Interestingly, the monomers used by the (+)-pinoresinol-forming DP did not appear to be seamlessly interchangeable, since neither *p*-coumaryl (**199**) nor sinapyl (**94**) alcohols afforded stereoselectively coupled products in *in vitro* assays.[230]



(**199**) *p*-Coumaryl alcohol

After amino acid sequencing, the corresponding (+)-pinoresinol-forming DP gene (*Fi_DP1*) was isolated from an *F. intermedia* cDNA library of young green stems using a PCR-amplified DNA probe. Analysis of the cloned gene (encoding an ~18.3-kDa predicted protein) suggested that the protein was glycosylated by posttranslational modification and contained a secretory system signal peptide.[232]

Heterologous expression of the corresponding Fi_DP1 protein was then next performed in a eukaryotic system (baculovirus-infected *Drosophila* cells) and, distinct from the native protein, three bands of DP

recombinant protein were visible by SDS–PAGE analysis. This indicated the presence of differentially glycosylated peptides, with these ranging from ~22 to 26 kDa.[232] Nevertheless, the recombinant DP was capable of engendering stereoselective coupling of coniferyl alcohol (**91**), in the presence of an one-electron oxidase/oxidant, to afford (+)-pinoresinol (**13a**).

1.23.4.2 Western Red Cedar Dirigent Proteins

As noted earlier, western red cedar (*T. plicata*) differentially accumulates various 8–8′-linked (−)-plicatic acid (**112b**)-derived (poly) lignans in its tissues, such as needles, stem, and bark, that are considered to be derived from (+)-pinoresinol (**13a**). This differential deposition of lignans in different tissues, which is particularly notable during the transition of its sapwood into heartwood, suggested the existence of distinct metabolic networks involving DPs in various tissues, that is, thereby orchestrating differential expression of the orthologous genes in monolignol radical–radical coupling and downstream metabolism. As a prelude to deciphering the biochemical pathway to (−)-plicatic acid (**112b**) in *T. plicata*, it was thus established that there were nine *DP* genes (*Tp_DP1–9*) having 72–99.5% identity to each other.[233] As for the *Forsythia* DP, they encoded proteins of 180–183 amino acids with each having a predicted molecular mass of ~20 kDa including the signal peptide. Several of these DPs (e.g., Tp_DP5 and Tp_DP8) were demonstrated *in vitro* to engender stereoselective coupling of coniferyl alcohol (**91**) to afford (+)-pinoresinol (**13a**), in the presence of an one-electron oxidase, indicative of the existence of a multigene family.[233]

1.23.4.3 Structural and Mechanistic Studies

The *Forsythia* DP exists apparently in dimeric form, based on the observations made using MALDI-TOF and ESI–MS, analytical ultracentrifugation, sedimentation velocity, and sedimentation equilibrium techniques.[234] It also had a propensity to further aggregate into ~12–18-mers, although this was prevented at increased NaCl concentrations. Additionally, application of circular dichroism (CD) demonstrated that the DP consisted mainly of β-sheet (35–42%) and loop (40–47%) secondary structures (**Figure 12(a)**), this being further supported by *in silico* modeling of the DP secondary structure (e.g., using PSIRED[235] and Folding@home (http://folding.stanford.edu)) (**Figure 12(b)**). Based on subsequent kinetic data and modeling, a steady-state kinetic model for the action of *F. intermedia* DP was proposed (**Figure 12(c)**), whereby the actual binding/coupling substrate was postulated to be a coniferyl alcohol (**91**)-derived free radical in solution ($CA^{\bullet}$).[231] In this proposed model, each DP monomer competes for binding of a $CA^{\bullet}$ with an apparent K_m of about 10 nmol l^{-1} relative to other diffusion-limited reactions (e.g., which would lead instead to the formation of racemic lignans). Upon binding of each $CA^{\bullet}$ to each monomeric DP (and possibly relative stabilization of the radical intermediate), the resulting proteinaceous dimeric complex is orientated in such a way that the two radicals approach each other from their *si–si* faces, thereby accounting for the stereoselectivity observed (**Figure 13(a)**).

1.23.4.4 Discovery of the (−)-Pinoresinol-Forming Dirigent Protein and Encoding Gene

Although *Forsythia* and *Thuja* spp. produce and utilize (+)-pinoresinol (**13a**), other species, for example, *Daphne tangutica*[236] and *A. thaliana*,[237] can accumulate the opposite (−)-enantiomer (**13b**) and/or downstream metabolites thereof. Indeed, *A. thaliana* contains a recently characterized DP homolog that preferentially forms (−)-pinoresinol (**13b**) from coniferyl alcohol (**91**) *in vitro*, again in the presence of a one-electron oxidase/oxidant (K. W. Kim, unpublished results). The corresponding *At_DP* gene encodes for a 21.4-kDa peptide (187 amino acids), and this finding now establishes the existence of distinct (+)- and (−)-pinoresinol (**13a** and **13b**)-forming DPs. Analogous to the *Forsythia* DP, generation of (−)-pinoresinol (**13b**) from coniferyl alcohol (**91**) is DP concentration-dependent. As before, the (−)-pinoresinol (**13b**)-forming DP does not affect substrate oxidation/coupling rates, and the protein lacks a catalytically active redox center. In this case, however, the two $CA^{\bullet}$ approach each other from their *re–re* faces (**Figure 13(b)**), thereby affording (−)-pinoresinol (**13b**) rather than the (+)-enantiomeric form (**13a**). It will next be of interest to

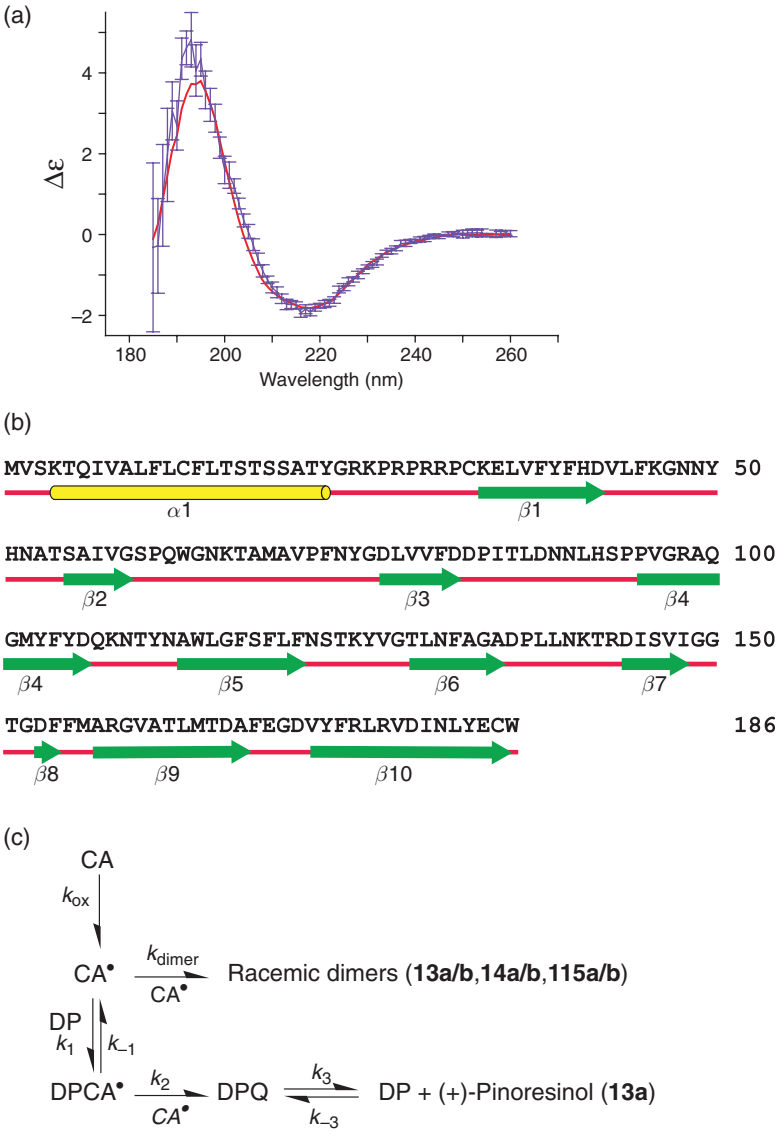


Figure 12 (a) CD spectrum of *F. intermedia* (+)-pinoresinol-forming DP. An expected fit (red) to the observed CD spectrum (purple) is shown for a protein with secondary structural components of 40–47% loop, 35–42% β-sheet, 9–14% turn, and 5–12% α-helix. Adapted with permission from S. C. Halls; N. G. Lewis *Biochemistry* **2002**, *41*, 9455–9461. Copyright 2002 American Chemical Society. (b) Predicted secondary structure of *F. intermedia* (+)-pinoresinol-forming DP using PSIRED server.[235] Yellow cylinder = α-helix, green arrow = β-strand and red bar = coil. (c) Proposed kinetic model for (+)-pinoresinol-forming DP.[231] Abbreviations: CA, coniferyl alcohol (**91**); CA•, coniferyl alcohol radical; DP, dirigent protein; DPCA•, dirigent protein–coniferyl alcohol radical complex; DPQ, dirigent protein quinone–methide intermediate complex; k_{ox}, rate constant of coniferyl alcohol (**91**) oxidation; k_1, rate constant of coniferyl alcohol radical (CA•) binding to DP, k_2, rate constant of second coniferyl alcohol radical (CA•) binding to the DPCA• complex; k_3, rate constant of release of (+)-pinoresinol (**13b**) from DP; k_{-1} and k_{-3} are the corresponding reverse rate constants to k_1 and k_3, respectively. Reproduced with permission from S. C. Halls; L. B. Davin; D. M. Kramer; N. G. Lewis, *Biochemistry* **2004**, *43*, 2587–2595. Copyright 2004 American Chemical Society.

establish fully the structural basis for the differential formation of (+)- and (−)-pinoresinols (**13a** and **13b**). (+) and (−)-Pinoresinol-forming DPs both share high amino acid sequence homology with each other (i.e., ~54% identity, ~70% similarity, not including the signal peptide), perhaps suggesting that only a few amino acids are involved in defining the different stereoselectivities in the active site(s).

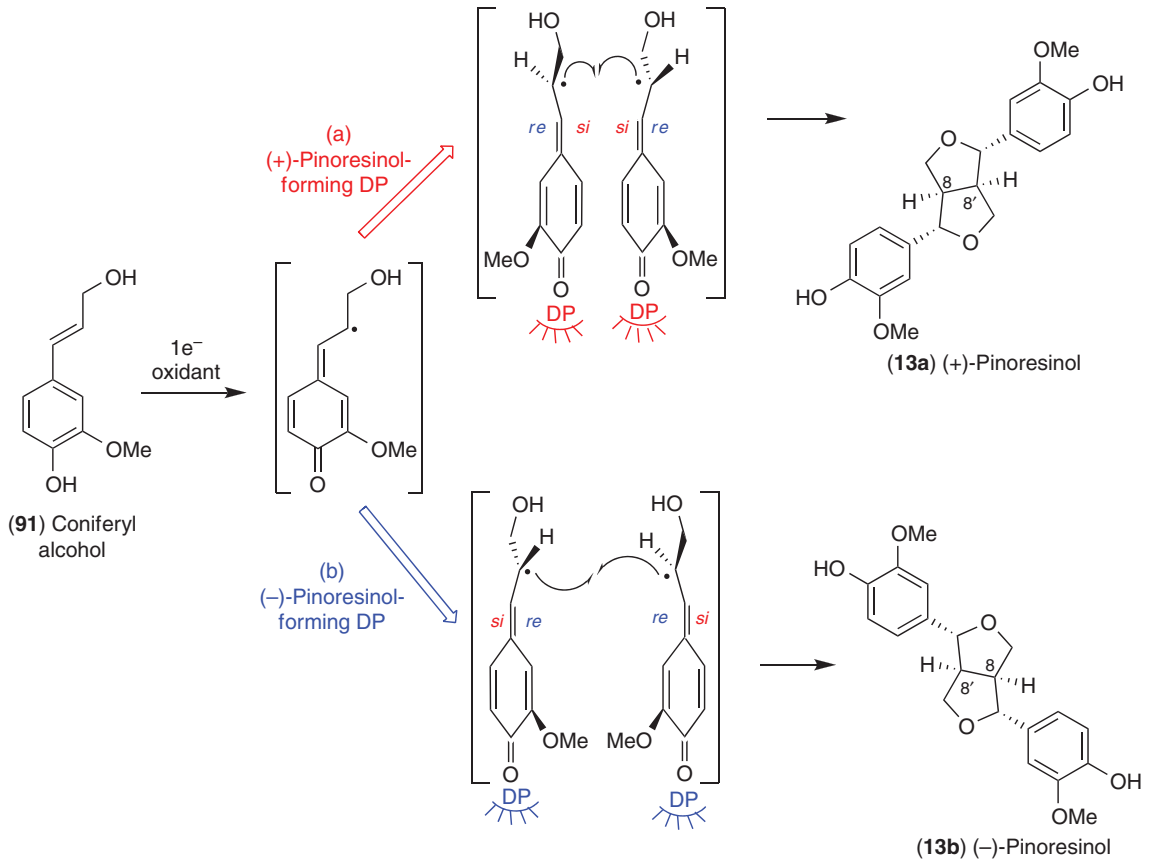


Figure 13 Stereoselective coupling of coniferyl alcohol (**91**) in presence of (a) (+)-pinoresinol- and (b) (–)-pinoresinol-forming dirigent proteins (DPs).

1.23.4.5 Dirigent Protein Tissue Localization and Metabolic Networks

1.23.4.5.1 mRNA tissue localization

Using *in situ* mRNA hybridization techniques, tissue-specific expression of the (+)-pinoresinol-forming DP gene was examined in *F. intermedia*.[238,239] Tissue-printing hybridization of fresh cross-sections of stems, petioles, and roots indicated that DP mRNAs were expressed in the vascular cambium regions of all tissues examined. *In situ* hybridization further confirmed the presence of DP mRNAs in the stem vascular cambium regions (**Figures 14(a)** and **14(c)**), as well as in the ray parenchyma cells adjacent to lignified tracheary elements in the youngest development stage examined (first internode, **Figure 14(b)**), that is, indicating sites of their lignan biosynthetic processes.[239]

Localization of DP gene transcripts was also investigated in western red cedar (**Figure 15**) using a single generic riboprobe to localize mRNA transcripts for all nine DP isovariants[240] (see Section 1.23.4.2). In sapwood, DP transcripts were detected in radial ray parenchyma cells (**Figure 15(c)**) and in vascular cambium; they were also detected in developing cells of cork cambium (**Figure 15(a)**).[240] Of particular interest, no hybridization was observed in the heartwood region under the conditions employed. By contrast, using the same technique, 18S rRNA transcripts (control) were detected in radial parenchyma cells of apparently preformed heartwood, as well as in all the other tissues where the DP was detected (not shown). Taken together, this was a most interesting finding. It demonstrated that the radial ray parenchyma cells were directly involved in the heartwood biosynthetic processes, which ultimately afforded the (–)-plicatic acid (**112b**)-derived lignans, and that other yet unknown biochemical processes were still occurring in the heartwood itself.

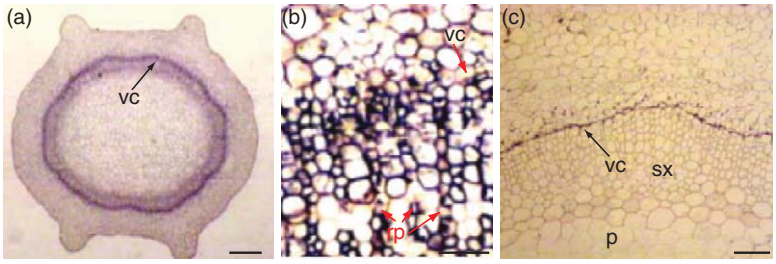


Figure 14 *In situ* hybridization of *Forsythia intermedia* DP mRNA with digoxigenin-labeled riboprobes in the first internode (a and b) and mature 10th internode (c). p, pith; rp, radial parenchyma; sx, secondary xylem; vc, vascular cambium. Bars: 150 μm (a), 25 μm (b), and 50 μm (c). Reproduced from V. Burlat; M. Kwon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2001**, *57*, 883–897. Copyright 2001, with permission from Elsevier.

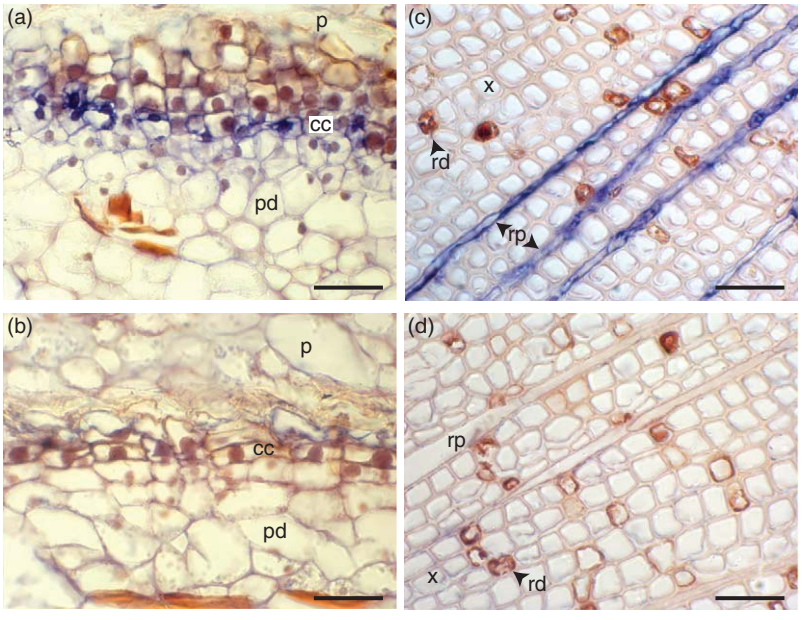


Figure 15 Detection of dirigent protein gene expression in young stem tissues of western red cedar by *in situ* hybridization. Hybridization of antisense probe for dirigent protein was detected in transverse sections by the blue color reaction in cells of cork cambium (a) and radial parenchyma (c) in young stem tissue (sapwood). A RNA probe of the sense strand of the dirigent transcript was used as a negative control (b, d). Abbreviations: cc, cork cambium; p, phellem; pd, phelloderm; rd, resin deposits; rp, radial parenchyma; x, xylem. Bars: 30 μm (a–d). Reproduced from A. M. Patten; L. B. Davin; N. G. Lewis, *Phytochemistry* **2008**, *69*, 3032–3037. Copyright 2008, with permission from Elsevier.

Heartwood tissue properties and the metabolites therein also provide generally a means of readily distinguishing between various woody plants. Thus, this ray parenchyma involvement provides additional insight into how this massive extrusion process occurs, and which is partially responsible for the often metabolite-specific heartwood formation. This may be of particular utility in understanding how the complex biochemical process involved in heartwood generation can be biotechnologically manipulated.

1.23.4.5.2 Dirigent protein tissue localization and proposed proteins harboring arrays of dirigent sites

Dirigent protein and proteins containing presumed arrays of dirigent sites (monolignol radical-binding sites) were also localized at the tissue and subcellular levels in *F. intermedia* using polyclonal antibodies raised against

the DP.[239,241] Overall, the patterns were quite similar to those for DP mRNA localization.[239] In the stems, labeling was localized to the vascular cambium region and young developing xylem, as well as in the cortex outer layers. As stem maturation proceeded, however, the label became restricted to the vascular cambium region (cambium and secondary phloem). Labeling was also mainly restricted to the cambial layers, secondary phloem, and the developing xylem in mature petioles, and to the pericycle layers/vascular tissues of the stele in the roots.

These observations were indicative of the cell/tissue types involved in lignan biosynthesis. Interestingly, high-resolution transmission electron microscopy also showed that in the stems, labeling was associated with the S1 sublayer and compound middle lamella of vessels, ray cells, and fibers, as well as to a lesser extent in their S3 sublayer. This latter set of observations is provisionally considered as indicative of detection of initiation sites for lignification, that is, sites harboring proteins containing arrays of presumed dirigent sites (see Chapters 5.01–5.21).

1.23.4.5.3 Proposed dirigent protein metabolic networks

There is additional evidence for the presence of DP metabolic networks in various plant species, such as western red cedar, spruce (*Picea* sp.), and *Arabidopsis*. This is contemplated even though only a relatively small number of DPs so far in their multigene families appear to be involved in stereoselective coupling to afford either (+)- or (−)-pinoresinols (**13a** or **13b**).

1.23.4.5.3(i) Western red cedar For western red cedar, the overall patterns of temporal and spatial expression of the nine DP isovariants (*Tp_DP1–9*) discussed previously above (Section 1.23.4.2) were analyzed by real-time (RT)-PCR and promoter analysis using the β-glucuronidase (GUS)-reporter gene in *Arabidopsis*.[242] Each DP ortholog was expressed differentially in individual organs, tissues, and cells at all stages of plant growth and development, indicative of the presence of a metabolic gene network. For example, *Tp_DP5* was only associated with the hypocotyl–root transition zone and the developing shoot meristem at 7–12 days old (**Figure 16(a)**), with *Tp_DP8* being strongly expressed throughout the vasculature (**Figure 16(b)**). In contrast, *Tp_DP2* was trichome- and root-specific (**Figures 16(c)** and **16(d)**). Differential expression patterns were also more pronounced in the reproductive tissues. For instance, *Tp_DP1* and *Tp_DP8* had distinct expression profiles in the flowers, that is intense GUS staining in the stamen filament, none in the anther (*Tp_DP1*,

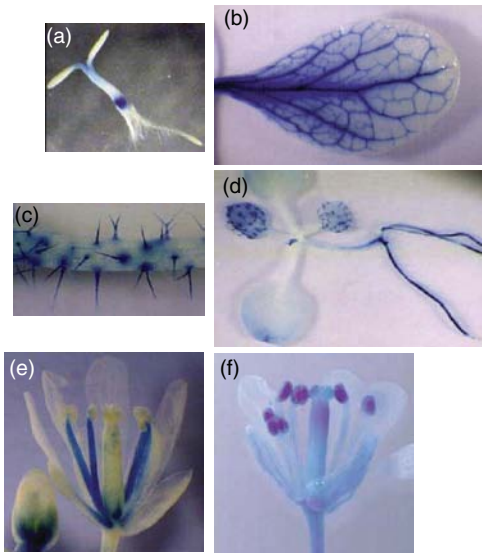


Figure 16 Histochemical localization of GUS activity in selected transgenic *Arabidopsis* plant lines containing various western red cedar *DP* promoter::*GUS* fusions.[242] (a) *TpDP5*gp::*GUS*. (b) *TpDP8*gp::*GUS*. (c and d) *TpDP2*gp::*GUS*. (e) *TpDP1*gp::*GUS*. (f) *TpDP8*gp::*GUS*. Reproduced from M. K. Kim; J.-H. Jeon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2002**, *61*, 311–322. Copyright 2002, with permission from Elsevier.

Figure 16(e)), whereas *Tp_DP8* expression was weak in the stamen filament and strong in the pollen grain (**Figure 16(f)**), as well as in the silique valves (lignifying area, not shown).

As noted earlier, both Tp_DP5 and Tp_DP8 were able to help confer stereoselective coupling of coniferyl alcohol (**91**) to afford (+)-pinoresinol (**13a**), a precursor of (−)-plicatic acid (**112b**). Taken together, these data thus further suggest the existence of distinct metabolic networks involved in the regulation of lignan deposition in this species.

1.23.4.5.3(ii) **Picea *species*** Additional support for the existence of DP multigene families was established from the analysis of both expressed sequence tags (ESTs) and full-length cDNAs from three spruce species (*Picea sitchensis*, *P. glauca*, and *P. glauca* × *engelmannii*), which resulted in the detection of 35 DP and DP-like cDNAs.[243,244] Identities between their predicted amino acid sequences ranged from 99.5 to 17.6%, with predicted molecular masses from ∼17.4 to 21.7 kDa. Phylogenetic analyses showed that they clustered in three subfamilies (DP-a, DP-b, and DP-f), with each gene differentially expressed throughout the tissues analyzed (i.e., shoots, roots, cortex, phloem, cambium, xylem, and embryos). Although the DP-a group also clusters with known (+)-pinoresinol-forming DPs, both these and the others (DP-b and DP-f) currently have no established biochemical functions in all three species.

Nevertheless, a 16.7 k cDNA microarray with 30 ESTs representing at least 22 distinct DP/DP-like genes was used to examine expression profiles under several stress conditions. These included, among others, methyl jasmonate application, wounding, and weevil (*Pissodes strobi*) stem-boring herbivory attacks (alone and in combination), as well as in wood and apex development. Interestingly, upon analysis of the expression profiles, most of the DP/DP-like array elements clustered in accordance with their phylogenetic subfamilies (i.e., DP-a, DP-b, and DP-f). DP-a genes were apparently strongly induced in bark upon either wounding or weevil herbivory (with moderate to weak induction after other treatments). DP-b genes, on the contrary, were induced only weakly (if at all) upon stress treatments. By contrast, DP-f genes gave a more scattered induction/downregulation pattern upon stress treatments, perhaps indicative of more specialized individual functions. The transcript profiles so obtained thus suggest that spruce DP/DP-like genes, especially those from the DP-a subfamily, could play a significant role in constitutive and induced phenolic defense mechanisms against stem-boring insects. Others were speculated to be involved in defense against either pathogens and/or defoliating herbivores, or wounding, or in the formation of compression wood, or perhaps associated with tissue development; however, as indicated above, all of their precise biochemical roles and physiological functions speculated above[243,244] await elucidation.

1.23.4.5.3(iii) **Arabidopsis** There are also 16 isovariants of dirigent proteins harboring consensus regions involved in monolignol radical (and possibly other natural products) binding sites in *A. thaliana*. To date, three are known to be able to engender stereoselective coupling of coniferyl alcohol (**91**) *in vitro* in this species. Nevertheless, all 16 *Arabidopsis* isovariants were cloned (At5g42510, At5g42500, At5g49040, At2g21110, At1g64160, At4g23690, At3g13650, At3g13662, At2g39430, At2g28670, At1g22900, At4g11180, At4g11190, At4g11210, At4g38700, At4g13580), with corresponding promoters isolated and used to obtain transformed *GUS-DIR*/*GFP-DIR Arabidopsis* lines (K. W. Kim, unpublished results). The results obtained are again suggestive of the presence of a comprehensive (cell- and tissue-specific) network with each dirigent gene (homolog) having an unique pattern of expression 'in the vascular apparatus'. This, therefore, provides further potential insight into the presence of (partially overlapping) metabolic networks controlling various aspects of phenoxy radical coupling. For example, eleven of the sixteen genes (see *At_DP5*, *8*, and *10*, **Figures 17(a)**–**17(e)**) were expressed in the lignifying leaf vasculature, two at the base of lignifying leaf trichomes (see *At_DP3*, **Figures 17(f)** and **17(g)**), four in the lignifying leaf hydathodes (vasculature) (see *At_DP10*, **Figures 17(h)** and **17(i)**), twelve in the lignifying abscission zone of the siliques (e.g., *At_DP2*, **Figure 17(j)**), and twelve were differentially expressed in lignifying regions of the stem vasculature (i.e., protoxylem, vascular cambium, xylary and interfascicular fibers, etc (see *At_DP1*, *8*, *12*, and *13*, **Figures 17(k)**–**17(n)**)). These findings were thus in good agreement with those previously obtained using the DP nine-membered multigene found in western red cedar[242] (discussed above).

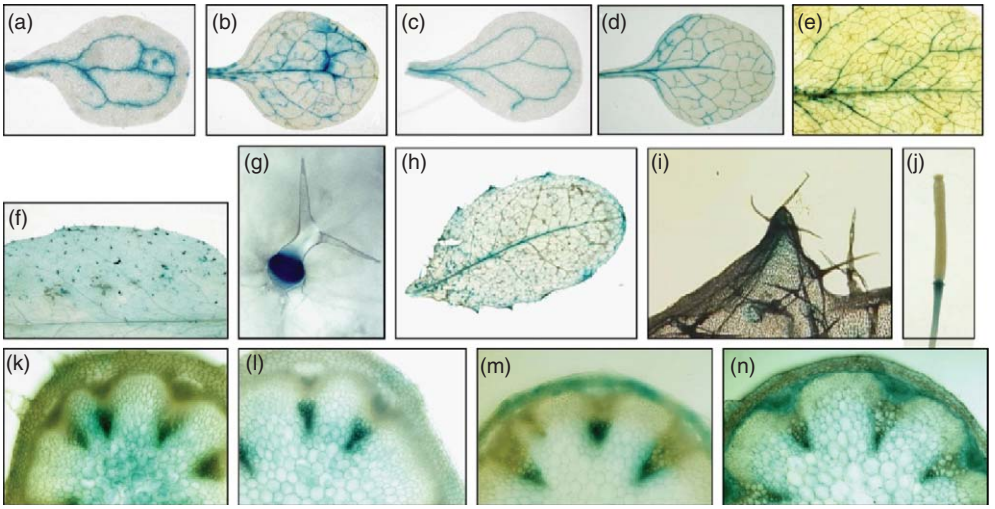


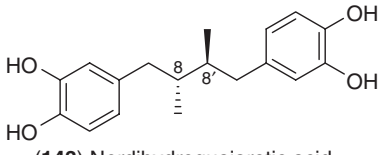
Figure 17 Selected patterns of expression of the 16-membered DP isovariant family using the GUS reporter gene. Cotyledons and leaf tissues at 3 weeks growth for At_DP5 (a, b) and At_DP10 (c, d) and leaf tissues at 4 weeks development for At_DP8 (e). Labeling is seen at the base of the trichomes for At_DP3 (f, g), in the leaf hydathodes for At_DP10 (h, i), in the silique abscission zone for At_DP2 (j) at 5 weeks growth, as well as in the lignifying regions of the stem vasculature for At_DP1 (k), 8 (l), 12 (m), and 13 (n) (Kye-Won Kim, unpublished results).

1.23.4.6 Other Examples of 8–8′ Phenylpropanoid Coupling: Hydroxycinnamic Acid and Allyl-/Propenylphenol-Derived Lignans in Liverworts and the Creosote Bush

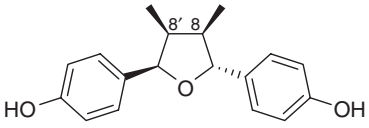
Examples of putative DPs engendering the formation of other types of 8–8′-linked lignans have also been suggested. These include the liverworts, *J. autumnalis* and *L. heterophylla*, whose intact cell cultures were shown to be able to metabolize [8-^{2}H]-caffeic acid (**56**) into either (+)-epiphyllic acid (**57a**) or its (−)-enantiomer (**57b**),[122] respectively, depending on the species. In *J. autumnalis*, the (+)-[8-^{2}H]-epiphyllic acid (**57a**) so formed can then be further metabolized to afford (+)-jamesopyrone (**62a**) and scapaniapyrone (**87**).[122] In either species, the corresponding chiral products that accumulate (i.e., (+)-jamesopyrone (**62a**) in *J. autumnalis* and (−)-epiphyllic acid (**57b**) in *L. heterophylla*) were enantiomerically pure, as determined by chiral HPLC analyses. On the contrary, incubation of caffeic acid (**56**) and H_2O_2 with cell-free extracts of both liverworts *in vitro* led only to the formation of racemic epiphyllic acid (**57a**/**b**). Thus, although the process controlling the proposed stereoselective coupling has not yet been detected; it is provisionally considered to involve DP control.[122] However, this still remains to be established.

As indicated above, 8–8′-regiospecific coupling also exclusively occurs during the formation of the 9–9′-deoxygenated lignans in the creosote bush (*L. tridentata*, Zygophyllaceae, **Figure 18**). This is a desert shrub of 1–3 m height, which is of increasing interest as a medicinal plant (discussed in Section 1.23.10.2). It has also long been used in Native American traditional medicine for treating more than 50 different ailments, including kidney and gall-bladder stones. Its most abundant lignan, NDGA (**143**), accumulates up to ∼5–10% of the leaves' dry weight, and is considered to be the creosote bush's main bioactive principle.[245] Many other 9–9′-deoxygenated lignans have also been detected in this species, the structurally simplest being the tetrahydrofurans (−)-larreatricin (**200b**), (−)-8′-*epi*-larreatricin (**201b**), *meso*-3,3′-didemethoxynectandrin B (**202**), and (±)-3,3′-didemethoxyverrucosins (**203a**/**b**).[246,247] Aryltetrahydronaphthalene lignans, such as norisoguaiacin (**204**), are also present, and apparently have the same C8/C8′ configurations as larreatricin (**200**).

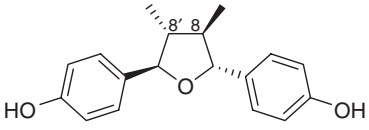
The 8–8′-linked lignans are thought to be derived from allyl-/propenylphenol-coupling products, for example, from *p*-anol (**21**) to afford initially (±)-larreatricins (**200a**/**b**) and its possible tetrahydrofuran ring diastereoisomers (**201**–**203**) with subsequent downstream metabolism then occurring (to generate NDGA (**143**), etc.). However, *p*-anol (**21**), when incubated *in vitro* with oxidases/laccases, generates a range of nonregiospecific coupling products. This, by contrast, does not occur *in vivo* in *Larrea*, suggesting the involvement of DP (or DP-like) proteinaceous control.



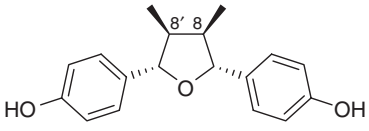
(**143**) Nordihydroguaiaretic acid



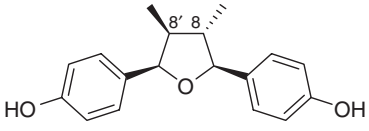
(**200b**) (–)-Larreatricin



(**201b**) (–)-8′-*epi*-Larreatricin

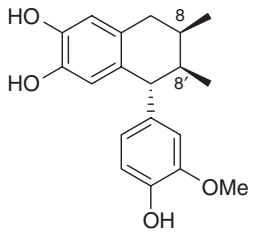


(**202**) *meso*-3,3′-Didemethoxynectandrin B



(**203a/b**) (±)-3,3′-Didemethoxyverrucosins

Larrea tridentata



(**204**) Norisoguaiacin

Figure 18 Various lignans in the creosote bush (*Larrea tridentata*). Photograph by Henri Moore, Washington State University, USA.

1.23.5 Downstream Lignan Metabolism

1.23.5.1 Furofuran Lignans in Sesame

1.23.5.1.1 Methylenedioxy bridge formation

Sesame (*S. indicum*), in addition to coconut, is one of the two oldest oilseed plants used by humanity,[248] with records dating back to about 6000 years.[249] The seed is highly valued as a source of oil, as well as for its antioxidant lignans.[250–252] There are contradictory reports though on the sesame plant origin: According to De Candolle,[253] it originated from the Sunda Isles in the Malay archipelago, following which it was introduced into India and the Euphrates valley to Egypt 2000 or 3000 years ago. Other records also suggest that it originated in the savanna of Central Africa and then spread to Egypt, India, the Middle East, and China.[249]

Sesame seed lignans are all 8–8′-linked and can contain either one or two methylenedioxy bridges (**Figures 19** and **20**). Sesame lignans can also be readily separated into those that are lipid-soluble, including (+)-sesamin (**11a**), (+)-piperitol (**205a**), and (+)-sesamolin (**210a**),[250,251] and those that are water-soluble, for example, (+)-sesaminol 2-*O*-mono- (**207a**), 2-*O*-di- (**208a**), and 2-*O*-tri- (**209a**) glucosides,[254] respectively. Of these, however, **11a** and **209a** are the most abundant lipid- and water-soluble lignans.[255,256] (+)-Sesamolin (**210a**) is of additional interest because of the unusual acetal oxygen insertion between the furanofuran group and the aryl moiety.

Sesame seedpods develop at different stages along the stems, with the oldest being closest to the base (**Figure 20**); the biosynthesis of its lignans is also developmentally regulated with seedpod development.[1,225] For example, at different developmental stages of the pods of 8-week-old plants, two interesting observations

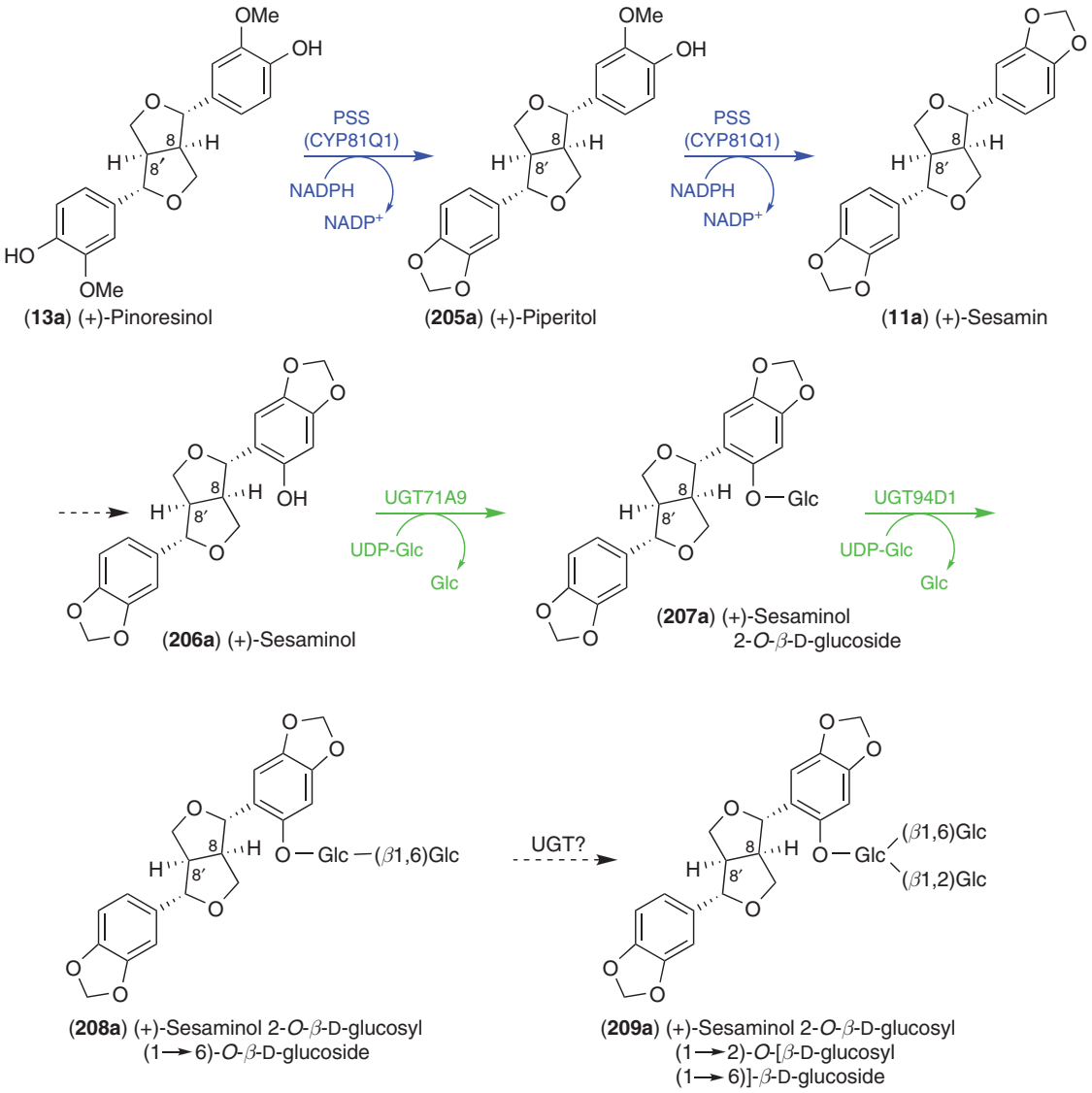
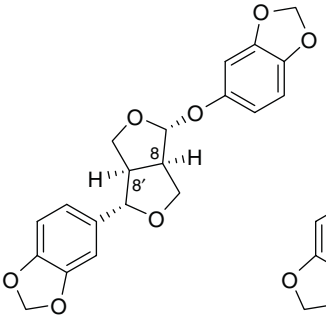


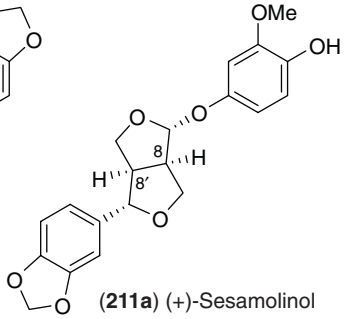
Figure 19 Proposed biosynthetic pathway to sesame (*Sesamum indicum*) lignans.

were previously made.[225] First, when (±)-[3,3′-$O^{14}CH_3$]-pinoresinols (**13a/b**) were administered to intact seeds, only the (+)-antipode (**13a**) was metabolized into the sesame lignans **11a**, **205a**, and **210a**, but not the corresponding enantiomer **13b** (**Figure 19**). Second, the relative efficacy of the incorporation of (+)-[3,3′-$O^{14}CH_3$]-pinoresinol (**13a**) into **11a**, **205a**, and **210a** varied with seed maturation stage.[225]

These findings were extended using microsomal preparations from the first and second stages of seedpod maturation. When incubated with (±)-[3,3′-$O^{14}CH_3$] pinoresinols (**13a/b**), only (+)-[3-$O^{14}CH_2$, 3′-$O^{14}CH_3$]-piperitol (**205a**) was formed when NADPH (1 mmol l^{-1}) was present. The corresponding (−)-enantiomer (**205b**), however, was not biosynthesized. This O_2-requiring, NADPH-dependent, cytochrome P-450 was thus subsequently named (+)-piperitol synthase.[225] Interestingly, although (+)-sesamin (**11a**) was not formed under the assay conditions used, incubation of (+)-piperitol (**205a**) as above resulted in its formation.[1] This, therefore, suggested the involvement of a second cytochrome P-450. In any event, these data established that both methylenedioxy bridges of sesame seed lignans resulted from cytochrome P-450-catalyzed transformations,[225] as had already been noted in alkaloid biosynthesis.[257]



(**210a**) (+)-Sesamolin



(**211a**) (+)-Sesamolinol

Sesamum indicum

Figure 20 (+)-Sesamolin (**210a**), (+)-sesamolinol (**211a**), and sesame (*S. indicum*). Photographs by Laurence B. Davin, Washington State University, USA.

Having thus established the overall enzymology and enantiospecificity of these transformations, subsequent cloning of a gene named *CYP81Q1* from a cDNA library obtained from sesame seed actively synthesizing sesamin (**11a**)[258] confirmed and further extended our original findings.[225] The corresponding protein, CYP81Q1, was heterologously expressed in yeast, with the resulting microsomal preparation individually incubated with (+)-pinoresinol (**13a**) and (+)-piperitol (**205a**) in the presence of NADPH. (Apparently, the corresponding (−)-enantiomers **13b** and **205b** were not, however, tested.) In agreement with our findings, (+)-sesamin (**11a**) was formed upon incubation with (+)-piperitol (**205a**), although it was also formed when (+)-pinoresinol (**13a**) was used as a substrate (**Figure 19**). This suggested that the CYP81Q1 was bi- and not monofunctional, and the name was expanded to (+)-piperitol/(+)-sesamin synthase (PSS) to indicate the bifunctional nature. Again, these data apparently confirmed our findings[225] of the enantiospecificity of methylenedioxy bridge formation and that a cytochrome P-450 was involved.

Identification of the reaction products was confirmed by LC–MS with m/z of 374 ($M + NH_4^+$) and 372 ($M + NH_4^+$) for **205a** and **11a**, respectively. Apparent K_m values were determined, using the yeast microsomal fraction, these being 10.2 and 11.7 μmol l^{-1} for (+)-pinoresinol (**13a**) and (+)-piperitol (**205a**), respectively. No other kinetic data were, however, reported. (+)-Sesamolinol (**211a**) was also not converted into (+)-sesamolin (**210a**) when incubated with this microsomal preparation,[258] perhaps suggesting the involvement of another cytochrome P-450 for the formation of its methylenedioxy bridge.

Two other *CYP81Q1* homologous genes, *CYP81Q2* and *CYP81Q3*, were also isolated from the related species, *Sesamum radiatum* and *Sesamum alatum*, respectively. These were of interest since the seeds of the former accumulate (+)-sesamin (**11a**), whereas those of the latter do not. After individual heterologous expression in yeast, microsomal preparations were again obtained. (+)-Piperitol (**205a**) and (+)-sesamin (**11a**) were formed when (+)-pinoresinol (**13a**) and NADPH were incubated with CYP81Q2, but not when CYP81Q3 was used.[258] This finding provisionally explains the absence of (+)-sesamin (**11a**) in *S. alatum*.

The report that CYP81Q1 apparently catalyzes dual methylenedioxy bridge formation is, however, in contrast to other cytochrome P-450's in alkaloid metabolism. The latter apparently only catalyze a single methylenedioxy bridge-forming reaction,[257,259] for example, the recently characterized CYP719A2 converted

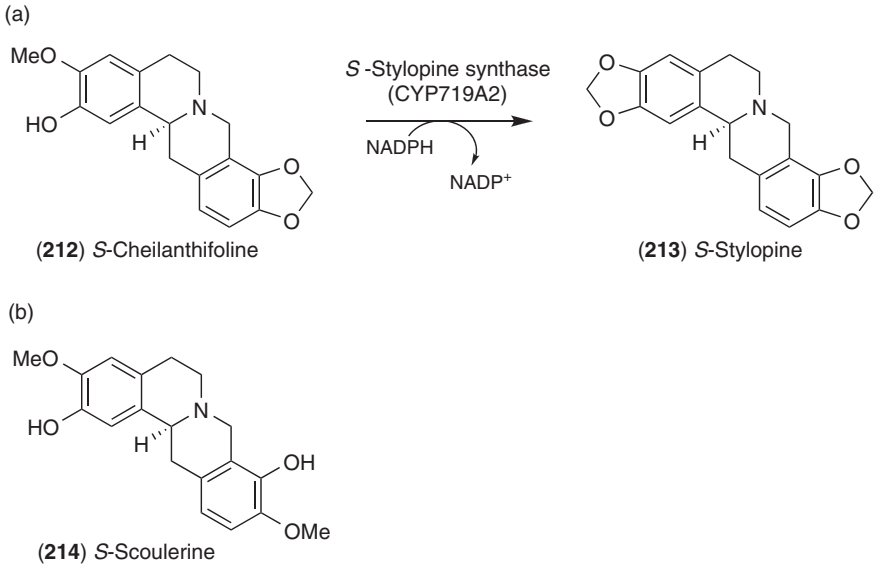


Figure 21 Methylenedioxy bridge formation in isoquinoline alkaloids. (a) Formation of (*S*)-stylopine (**213**) from (*S*) cheilanthifoline (**212**) catalyzed by (*S*)-stylopine synthase, a cytochrome P-450. (b) (*S*)-Scoulerine (**214**) did not serve as a substrate with (*S*)-stylopine synthase in *in vitro* assays.

S-cheilanthifoline (**212**) into *S*-stylopine (**213**) (**Figure 21(a)**), but not *S*-scoulerine (**214, Figure 21(b)**) into *S*-cheilanthifoline (**212**)[259] as demonstrated using crude microsomal preparations from *Eschscholzia californica*.[257]

It will be of interest in the future to resolve the ternary structure of this cytochrome P-450. This will hopefully provide further insight into the basis of its catalytic mechanism, and how substrate specificity is controlled, that is, including as to whether (+)-piperitol (**205a**) is first synthesized then released (or not) from the CYP81Q1 active site. If the former occurs (i.e., with product released from the active site), this may explain why the previous studies only detected (+)-piperitol (**205a**) formation when (+)-pinoresinol (**13a**) was incubated with a sesame seed microsomal preparation. That is, the released (+)-piperitol (**205a**) might have been too low in concentration in the assays to compete with the relatively large amounts of (+)-pinoresinol (**13a**) present under saturating conditions.

1.23.5.1.2 Glucosylation

UDP-glucose glucosyltransferases (UGT) presumed to be involved in the formation of (+)-sesaminol 2-*O*-triglucoside (**209a**), the most abundant water-soluble lignan in sesame seeds,[255,256] have also been characterized.[260] They were obtained by screening the sesame seed cDNA library with probes containing a well-conserved UGT sequence. After two rounds of screening, 10 clones were obtained, with each heterologously expressed in *Escherichia coli* as His-tag fusion proteins This resulted in the characterization of UGT71A9 and UGT94D1, which catalyzed the conversion of (+)-sesaminol (**206a**) into (+)-sesaminol 2-*O*-β-D-glucoside (**207a**), and the latter into (+)-sesaminol 2-*O*-β-D-glucosyl (1 → 6)-*O*-β-D-glucoside (**208a**) (**Figure 19**). In both cases, LC–MS confirmed identity of the reaction products: In the reaction catalyzed by UGT71A9, the product had a m/z 555.1450 $[M + Na]^+$ indicating that one glucose had been added to (+)-sesaminol (**206a**), which has a calculated mass of m/z 393.1 $[M + Na]^+$. In the reaction catalyzed by UGT94D1, by contrast, the enzymatically formed product **208a** had a m/z 717.2000 $[M + Na]^+$. The identity of both products was confirmed by ^1H, ^{13}C, and 2D-NMR spectroscopic analyses.

Kinetic parameters were determined for both glucosyltransferases. For UGT71A9, apparent K_m values were 6.32 and 41.0 μmol l^{-1} for (+)-sesaminol (**206a**) and UDP-glucose, respectively, with a k_{cat}/K_m value of 196 000 $mol^{-1}\,l\,s^{-1}$ (for **206a**). For UGT94D1, K_m values were 77.0 and 228.0 μmol l^{-1} for (+)-sesaminol

2-*O*-β-D-glucoside (**207a**) and UDP-glucose, respectively, but with a much lower k_{cat}/K_m of 11 700 mol^{-1} l s^{-1} (for **207a**). The UGT catalyzing the final $\beta 1 \rightarrow 2$ glucosylation to form (+)-sesaminol 2-*O*-triglucoside (**209a**, **Figure 19**) from **208a** was, however, not identified.

As for *CYP81Q1*, two homologs of *UGT71A9*, *UGT71A8*, and *UGT71A10* were cloned by RT-PCR from *S. alatum* and *S. radiatum*, respectively. The encoding proteins showed 98 and 91% similarity to UGT71A9. When the corresponding recombinant proteins were heterologously expressed in *E. coli*, both were able to enzymatically convert (+)-sesaminol (**206b**) into (+)-sesaminol 2-*O*-β-D-glucoside (**207a**), suggesting that this glucosyltransferase is conserved in the *Sesame* genome.

1.23.5.1.3 Oxygen insertion

The insertion of oxygen to form the acetal bridge between the furanofuran group and the aryl moiety during (+)-sesamolin (**210a**) formation still remains to be clarified,[261] as the precise sequence of oxygenation and methylenedioxy bridge formation is unknown. Interestingly, (+)-pinoresinol (**13a**), (+)-piperitol (**205a**), or, less likely, (+)-sesamin (**11a**) could all potentially serve as substrates for the oxygen insertion step. However, radiolabeled precursor administration experiments[225,262] did not resolve among these possibilities, and the enzyme participating in acetal bridge formation has not yet been identified.

(+)-Sesamolin (**210a**) is known to be rearranged into (+)-sesaminol (**206a**), however, during sesame oil processing.[252] This reaction is acid-catalyzed, with the acetal bridge being cleaved to afford the presumed intermediate oxonium ion and sesamol (**215**), which can then undergo nucleophilic attack (**Figure 22(a)**). In the presence of even a trace amount of water, however, both samin (**216**) and sesamol (**215**) are instead formed (**Figure 22(b)**).[252] Although the enzymatic formation of (+)-sesamolin (**210a**) has not yet been characterized, a possible mechanism for acetal bridge formation might involve rearrangement of (+)-sesaminol (**206a**) to afford (+)-sesamolin (**210a**) (**Figure 23**).

1.23.5.2 Pinoresinol/Lariciresinol Reductases and Pinoresinol Reductase

Discovery of genes encoding either (+)- or (−)-pinoresinol-forming DPs began to bring the much needed clarification to hitherto enigmatic differences in optical activities of various lignans from different plant species. This section thus focuses next on the discovery of pinoresinol/lariciresinol reductases (PLRs),[263,264] which catalyze conversions of pinoresinol (**13**) into either lariciresinol (**105**), secoisolariciresinol (**110**), or both. This includes discussion of their quite distinct enantiospecificities in various plant species, as well as progress made toward establishment of the operative biochemical mechanisms and the presumed involvement of enzyme-bound intermediary quinone methides. These studies also led to the discovery of related reductases, the 'provisionally' annotated phenylcoumaran benzylic ether reductases[265] (PCBERs, see Section 1.23.7.2), as well as the biochemical mechanisms/3D structures of PCBER and the related isoflavone reductases (IFRs).[266]

Partly due to serendipity, other PLR-related genes were also isolated in our investigations in 1999, but which instead encoded enzymes affording entry into the allyl-/propenylphenol pathways, for example, chavicol/eugenol synthases (CES).[2–4,267,268] Deduction of the allyl-/propenylphenol biochemical pathway came, however, from both delineation of PLR biochemical mechanisms and mechanistic considerations of substrate-to-product relationships in norlignan (*E*/*Z*-hinokiresinol (**123**/**174**)) biosynthesis (see Section 1.23.8). As far as PLRs proper are concerned though, most work has been carried out with *Forsythia*, western red cedar, *Linum* sp., and *A. thaliana* as summarized below.

1.23.5.2.1 Forsythia PLR: discovery of (+)-pinoresinol/lariciresinol reductase

The first-known PLR, discovered in *F. intermedia*,[263,269] catalyzes the sequential NADPH-dependent enantioselective conversion of (+)-pinoresinol (**13a**) into (+)-lariciresinol (**105a**), and then the latter into (−)-secoisolariciresinol (**110b**), respectively (**Figure 24**).[269] It was purified to apparent homogeneity and its measured kinetic parameters demonstrated that it efficiently reduced both substrates with apparent K_m, V_{max}, and k_{cat}/K_m values for **13a** and **105a** of 27/121 µmol l^{-1}, 4.5/7.0 pkat μg^{-1} protein, and 5800/

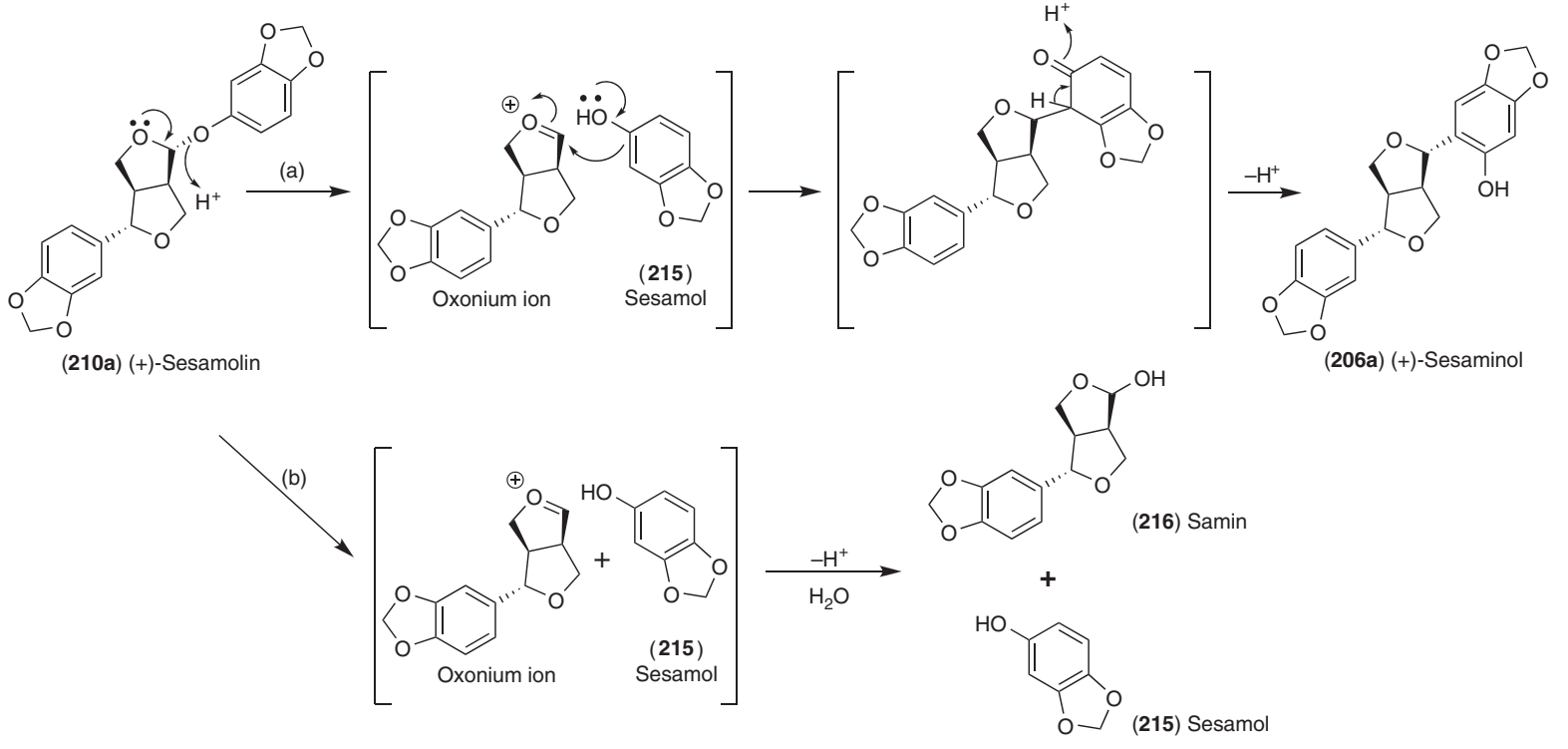


Figure 22 Proposed mechanism for the formation of (a) (+)-sesaminol (**206a**) via (+)-sesamolin (**210a**) rearrangement during sesame oil processing and (b) sesamol (**215**) and samin (**216**) from (+)-sesamolin (**210a**) in presence of H_2O.

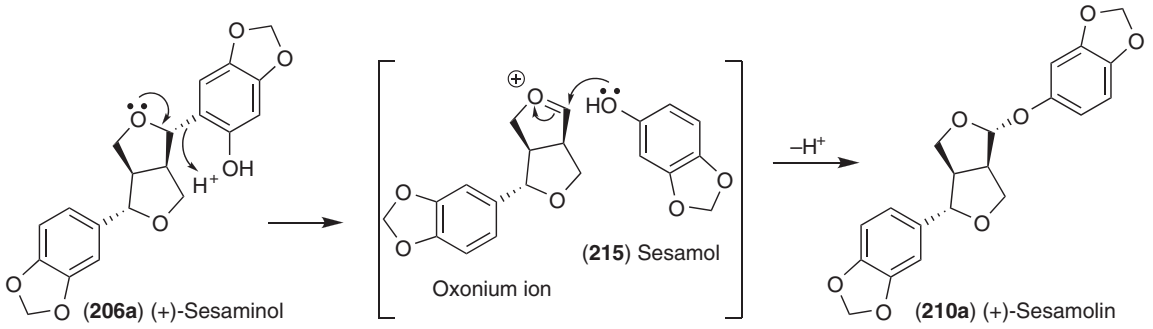


Figure 23 Putative rearrangement resulting in oxygen insertion to afford (+)-sesamolin (**210a**) from (+)-sesaminol (**206a**).

2000 mol^{-1} l s^{-1},[263] respectively. Kinetic data were thus reasonably consistent with enzyme turnover data for others in the general phenylpropanoid pathway to the monolignols **91**, **94**, and **199**.[7] The corresponding cDNA PLR_Fi1 was subsequently cloned,[263] with this encoding a polypeptide of 312 amino acids having a calculated molecular mass of 34.9 kDa.

1.23.5.2.2 Gymnosperm PLR/PLR homologs: discovery of PLR/PLR homologs of differing PLR and pinoresinol reductase enantiospecificities

PLR and/or PLR homolog cDNAs were also isolated from both western red cedar and western hemlock, that is, *PLR_Tp1–PLR_Tp4* and *PLR_Th1/PLR_Th2*, respectively, with the corresponding proteins having ~52–61% identity and ~66–79% similarity to PLR_Fi1.[264]

When heterologously expressed in *E. coli*, the western red cedar *PLR_Tp2* as well as the *PLR_Tp1* homolog (sharing >70% identity) gave recombinant proteins of very different enantiospecificities. As for the *Forsythia* PLR_Fi1, PLR_Tp2 efficiently converted (+)-pinoresinol (**13a**) into (−)-secoisolariciresinol (**110b**) using (+)-lariciresinol (**105a**). However, interestingly, PLR_Tp2 was also able to slowly reduce (−)-pinoresinol (**13b**) into (−)-lariciresinol (**105b**), albeit with the latter not further metabolized (**Figure 24**). This was indicative of distinct PLR and pinoresinol reductase (PR) activities, depending on the enantiomer present *in vitro*.

In contrast, the homolog PLR_Tp1 less efficiently catalyzed the NADPH-dependent conversion of (−)-pinoresinol (**13b**) into (−)-lariciresinol (**105b**), as well as (−)-lariciresinol (**105b**) into (+)-secoisolariciresinol (**110a**). (+)-Pinoresinol (**13a**) was also slowly converted into (+)-**105a**, but this was also not metabolized further (**Figure 24**). Moreover, kinetic analyses carried out using **13a**, **13b**, **105a**, and **105b** individually indicated that PLR_Tp2 was overall ~150-fold more catalytically efficient relative to the PLR_Tp1 homolog.[270] This was consistent with the proposed role of PLR_Tp2 in (−)-plicatic acid (**112b**) biosynthesis.

1.23.5.2.3 Linum species PLR: additional discovery of genes encoding (−)-PLR activity

The genus *Linum* in the family Linaceae consists of about 230 species,[271] which can be divided into six sections based on morphological characters:[272] *Linum*, *Dasylinum*, *Linastrum*, *Cathartolinum*, *Syllinum*, and *Cliococca*. Of the *Linum* species, flax (*L. usitatissimum*, 'the most useful' in Latin[273]) is the most common, which is grown for both its fibers (linen) and its seeds (oil and lignans). Indeed, it was apparently one of the first domesticated plants where the cultivation most likely began in the Fertile Crescent within the valleys of the Tigris and Euphrates some 8000 years ago.[274]

Flaxseed contains various ester-linked oligomers of the 8–8′-linked secoisolariciresinol diglucoside (SDG, **217**, **Figure 25**), which are covalently attached to hydroxymethyl glutaryl moieties (HMG, **218**),[275,276] such as the lignan **219**. SDG (**217**)/HMG (**218**)-containing flaxseed lignans have become somewhat better understood structurally in recent years, with several new features described. Although the macromolecular backbone is composed mainly of SDG (**217**) moieties ester-linked to HMG (**218**) molecules as indicated above,[275,276] the flavonoid herbacetin diglucoside (HDG, **220**) has been reported as a backbone component (estimated to be

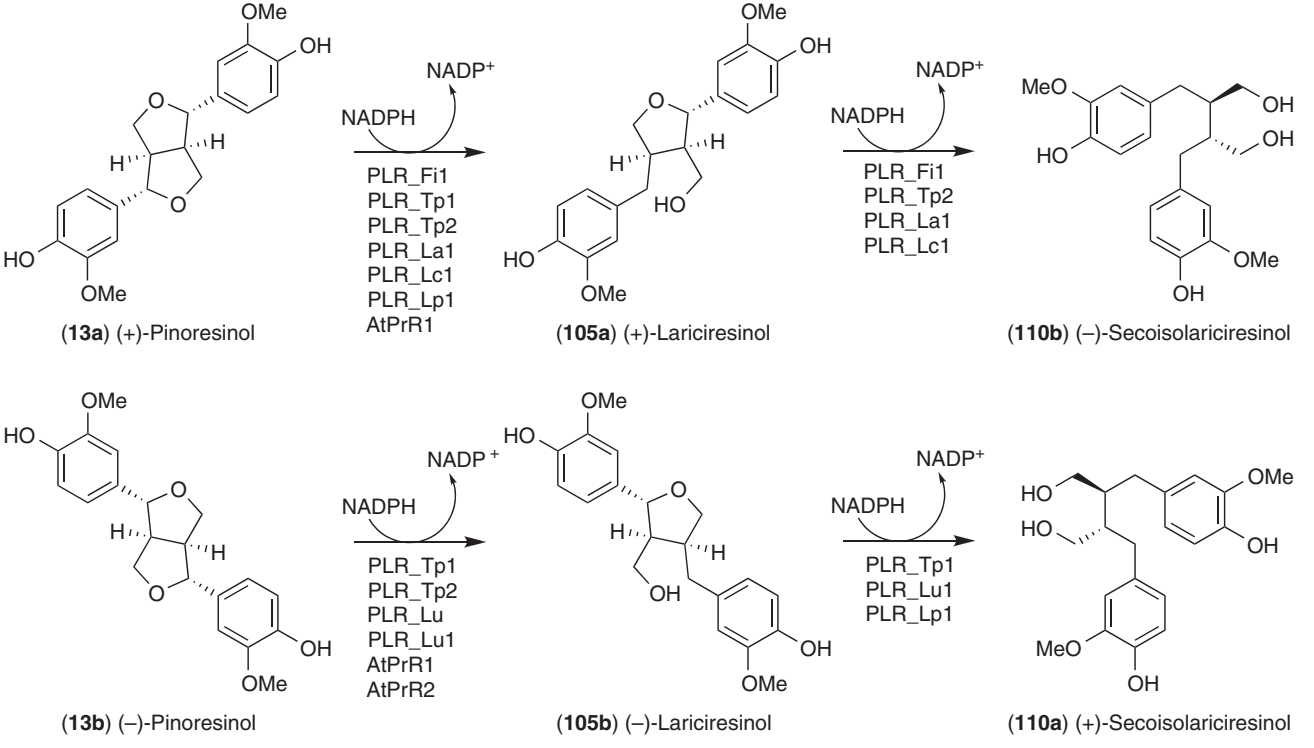
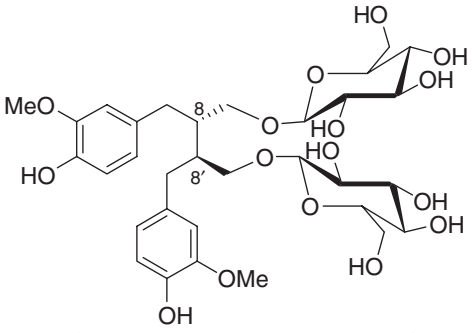
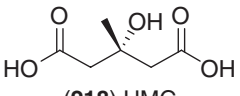


Figure 24 Reactions catalyzed by pinoresinol/lariciresinol reductases (PLRs), pinoresinol reductases (PRs), and homologs thereof. At, Arabidopsis thaliana; Fi, Forsythia intermedia; La, Linum album; Lc, L. corymbulosum; Lp, L. perenne; Lu, L. usitatissimum; Tp, Thuja plicata.

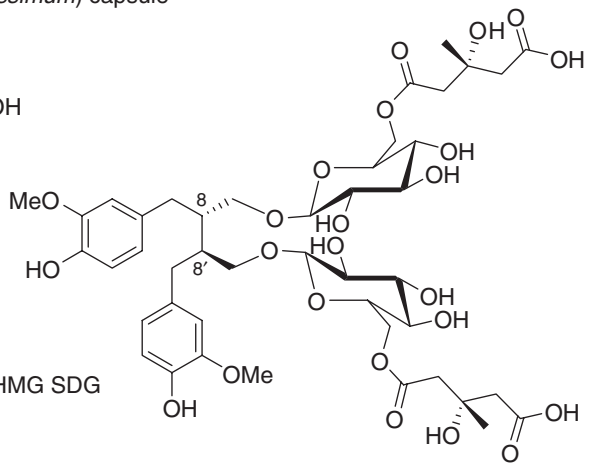
Flax (*Linum usitatissimum*) capsule



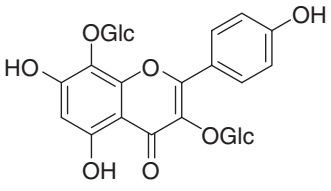
(**217**) Secoisolariciresinol diglucoside (SDG)



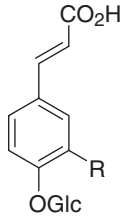
(**218**) HMG



(**219**) 6a,6a′ di-HMG SDG



(**220**) Herbacetin diglucoside (HDG)

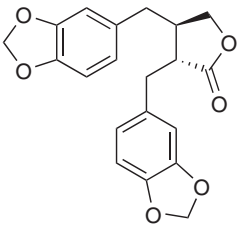


(**221**) R = H, *p*-Coumaric acid glucoside
(**222**) R = OMe, Ferulic acid glucoside

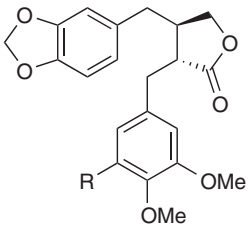
Figure 25 Various phytochemical constituents in flax (*Linum usitatissimum*). Photograph by Laurence B. Davin, Washington State University, USA.

~10 × lower in amount than that of SDG (**217**)).[277] The average flaxseed lignan 'macromolecule' was also recently provisionally estimated to contain ~3–5[276,278] (but varying between 1 and 7)[278] SDG (**217**) and/or HDG (**220**) backbone units. Both *p*-coumaric and ferulic acid glucosides (**221, 222**) are considered possibly to be linked as well,[279] but apparently as terminal (i.e., end) groups, with a negative correlation being noted between their amounts and the average length of the macromolecular chain.[278] The SDG (**217**) released from these conjugates upon alkali treatment mainly contains the (+)-secoisolariciresinol (**110a**) enantiomer, with the ratio of (+) (**110a**) to (−) (**110b**) being ~99:1.[275]

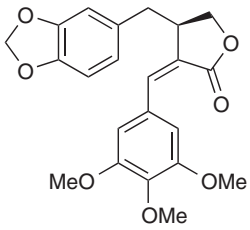
Interestingly, at full flower development, flax floral tissues reportedly also accumulate other 8–8′ dibenzylbutyrolactone lignans, such as (−)-hinokinin (**223b**), (−)-bursehernin (**224b**), (−)-yatein (**225b**), (−)-*E*-anhydropodorhizol (**226b**), (−)-matairesinol dimethyl ether (**227b**), and (−)-thujaplicatin trimethyl ether (**228b**).[280,281]



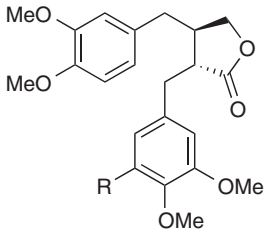
(**223b**) (–)-Hinokinin



(**224b**) R = H, (–)-Bursehernin
(**225b**) R = OMe, (–)-Yatein



(**226b**) (–)-*E*-Anhydropodorhizol



(**227b**) R = H, (–)-Matairesinol dimethyl ether
(**228b**) R = OMe, (–)-Thujaplicatin trimethyl ether

In terms of enzymology, PLRs of differing enantiospecificities have been very preliminarily characterized in four *Linum* species, that is, *L. usitatissimum* (PLR_Lu[282–284] and PLR_Lu1[285]), *L. album* (PLR_La1),[285] *L. corymbulosum* (PLR_Lc1),[286] and *L. perenne* (PLR_Lp1);[287] these are of ~60–74% identity and ~78–87% similarity to PLR_Fi1, respectively. Of these, PLR_Lu[282–284] and PLR_Lu1[285] were individually expressed heterologously in *E. coli.* and both catalyzed the conversion of (−)-pinoresinol (**13b**) into (−)-lariciresinol (**105b**) and, in one instance, of the latter into (+)-secoisolariciresinol (**110a**) as well. These data thus begin to provide a biochemical explanation for the predominance of the (+)-secoisolariciresinol (**110a**) enantiomer in flaxseed.

The other recombinant PLRs examined from *L. album* (PLR_La1)[285] and *L. corymbulosum* (PLR_Lc1)[286] apparently, also converted (+)-pinoresinol (**13a**) into (−)-secoisolariciresinol (**110b**), as described above for the *Forsythia* (PLR_Fi1) and western red cedar (PLR_Tp2) PLRs (**Figure 24**). These *Linum* PLRs, however, have not been subjected to detailed kinetic parameter characterization, and thus their relative enzymatic efficacies are as yet unknown. Nevertheless, the reported enantiospecific properties of the PLRs are consistent with the known optical activities of the isolated lignans: *L. album* accumulates (−)-podophyllotoxin (**1b**), (−)-6-methoxypodophyllotoxin (**194b**), and their derivatives thereof,[288] whereas (−)-hinokinin (**223b**)[289] is found in *L. corymbulosum.*

On the contrary, a report of a PLR from *L. perenne*, which accumulates justicidin B (**55**),[290] gave contradictory findings.[287] In assays carried out with (±)-pinoresinols (**13a/b**) in the presence of NADPH and increasing amounts of the recombinant PLR_Lp1, the enzyme reportedly preferentially utilized (+)-pinoresinol (**13a**) for the first reduction and (−)-lariciresinol (**105b**) for the next[287] (**Figure 24**). Such preliminary studies need, however, to be followed up with more conclusive and comprehensive kinetic parameter determinations. Accordingly, to establish the actual substrate specificities, pure enantiomeric substrates need to be used, that is, **13a**, **13b**, **105a**, and **105b**, rather than using racemic mixtures.

1.23.5.2.4 Arabidopsis *PLR homologs: Pinoresinol reductases*

Following β-glucosidase treatment of wild-type *A. thaliana* roots, small amounts of (−)-lariciresinol (**105b**) in ~88% e.e. were released.[237] To begin to provide clarification to this enantiomeric preponderance, two *Arabidopsis* PLR cDNAs (*AtPrR1* and *AtPrR2*) were obtained, whose corresponding proteins had ~59% identity and 75–78% similarity to PLR_Fi1. When incubated with (±)-pinoresinols (**13a/b**) *in vitro*, the recombinant AtPrR2 generated (−)-lariciresinol (**105b**) in 96% e.e., whereas AtPrR1 afforded both enantiomers **105a/b** with ~6% e.e. of the (+)-antipode **105a** (**Figure 24**). Interestingly, AtPrR1 was more catalytically efficient in *in vitro* assays, with a k_{cat}/K_m ~11–16 times higher for (±)-pinoresinols (**13a/b**) (12 800 and 19 000 $mol^{-1}\,l\,s^{-1}$ for **13a** and **13b**, respectively) than that of AtPrR2 for (−)-pinoresinol (**13b**) (1170 $mol^{-1}\,l\,s^{-1}$). On the contrary, the

predominance of (−)-lariciresinol (**105b**) *in vivo* was more in apparent agreement with the catalytic properties of AtPrR2.

To better understand the role of each gene in the formation of (−)-lariciresinol (**105b**), T-DNA insertion mutants were obtained, with homozygous lines further selected (i.e., *atprr1-1* and *atprr1-2* for *AtPrR1*, and *atprr2* for *AtPrR2*). A double mutant, *atprr1-1 atprr2*, was also generated. There was a significant increase (1.6–1.8×) in lariciresinol (**105**) content in the roots of the single mutants as compared to that of the wild-type line, with (−)-lariciresinol (**105b**) from *atprr1* and *atprr2* mutants having an e.e. of 94–96 and 82%, respectively. By contrast, lariciresinol (**105**) was not detectable in the double mutant *atprr1-1 atprr2*. Instead, it accumulated a relatively large amount of (−)-pinoresinol (**13b**) in 74% e.e. (~11 times WT levels of (±)-lariciresinols (**105a/b**)), this being in agreement with the preponderance of the upstream DP-mediated (−)-pinoresinol (**13b**) formation as previously discussed (Section 1.23.4.4).

1.23.5.2.5 Tissue localization of PLRs and PRs

Various studies have begun to establish tissue-specific spatial and temporal patterns of gene expression of both PLRs and PRs. These findings have also provided the required comparative insight with those of pinoresinol-forming DPs as well.

1.23.5.2.5(i)* In situ *hybridization of* Forsythia *PLR: Comparison with DP gene expression PLR_Fi1 localization in *F. intermedia* tissues was carried out using *in situ* hybridization.[291] As for the (+)-pinoresinol-forming DP, the strongest signal intensity was observed in the stems, as compared to leaves, petioles, and roots. For the stem tissues, the early development stage (first internode) gave labeling in the vascular cambium and primary phloem, respectively (**Figure 26(a)**), then later (i.e., from second to twentieth internode) in both ray parenchyma cells (**Figure 26(b)**) and vessels adjacent to ray parenchyma (twentieth internode, **Figure 26(c)**), in addition to the vascular cambium. For the roots, petioles, and leaves, PLR gene expression was only associated with meristematic tissues.[291]

When compared with (+)-pinoresinol-forming DP gene expression in *F. intermedia*, both PLR and DP mRNA expression were overall co-localized in the cambial regions (**Figures 14** and **26**). The DP mRNAs were also observed in ray parenchyma cells of the first stem internode stage (**Figure 14(b)**), whereas, by contrast, PLR mRNAs, were noted in these same cell types at later stages (**Figures 26(b)** and **26(c)**).[238,239] In western red cedar, however, the (+)-pinoresinol-forming DP gene expression was evident in both cambial regions, as well as ray parenchyma cells (**Figures 15(a)** and **15(c)**). Taken together, both the PLR and the DP thus appear to be restricted to the same cell types, although whether there are more subtle temporal differences in their individual gene expression patterns cannot be gauged at this time.

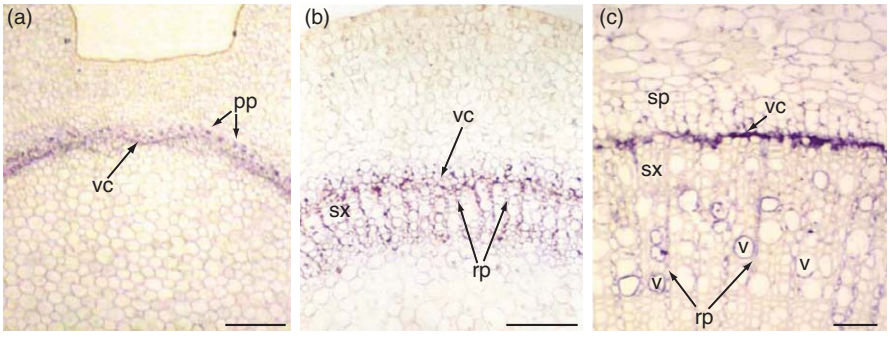


Figure 26 PLR_Fi1 mRNA accumulation in *F. intermedia* stems at different developmental stages as shown by *in situ* hybridization. (a) First stem internode. (b) Second stem internode. (c) Twentieth stem internode. PLR_Fi1 mRNA strongly accumulated in the vascular cambium regions (vc) (a–c), the ray parenchyma cells (rp) (b and c) and in the vessels (v) adjacent to the ray parenchyma cells (c). Abbreviations: pp, primary phloem; rp, radial parenchyma; sp, secondary phloem; sx, secondary xylem; v, vessel; vc, vascular cambium. Bars: 100 μm (a), 60 μm (b), and 300 μm (c). Reproduced from M. Kwon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2001**, *57*, 899–914. Copyright 2001, with permission from Elsevier.

1.23.5.2.5(ii) Flax PLR gene expression Spatiotemporal expression of PLR in flax was also investigated using both semiquantitative RT-PCR and GUS reporter gene (using the *PLR_Lu* promoter) strategies.[292] The RT-PCR analyses indicated that PLR_Lu expression occurred in the seed coats at all five developmental stages (10, 16, 20, 24, and 35 days after flowering, respectively), with the highest level being observed at stage 3 of seed maturation. GUS staining was carried out on both vegetative and reproductive organs. As expected, strong tissue-specific expression occurred in developing seeds, but not in stems, leaves, and roots (which do not accumulate SDG-derived lignans to any considerable extent). Gene expression was mainly localized to the seed coat (**Figure 27**, seeds shown at stage 3 of maturation), but was not detectable in the embryo (**Figures 27(b)** and **27(c)**). The seed coat is the known site of accumulation of SDG-HMG lignans, such as **219**.[293]

1.23.5.2.5(iii) AtPrR localization in Arabidopsis Quantitative RT-PCR analyses have also been used to study expression levels of both *AtPrR1* and *2* genes in *Arabidopsis*, with both reportedly expressed at similar levels in the root tissues, and to a lesser extent, in stems.[237]

1.23.5.2.6 Structural biology studies: PLR and PLR homolog

Comprehensive structural biology studies of both PLR and a PLR homolog have provided the much needed insight into their overall catalytic mechanisms, as well as that of the PLR-related proteins (PCBERs, IFRs, and CESs, discussed later). The PLR and PLR homolog studies include establishing stereospecificity of hydride transfer resulting in an inversion of product configuration; evidence for presumed involvement of enzyme-bound quinone methide intermediates; and progress made toward an understanding of the biochemical basis for distinct enantiospecificities.

1.23.5.2.6(i) Stereospecificity of hydride transfer with resulting inversion of product configuration *Forsythia* PLR is a type A reductase, as established when using [4*R*-^{3}H]- and [4*S*-^{3}H]-NADPH as cofactor; only the *pro-R* hydrogen on the nicotinamide ring of NADPH was abstracted and transferred to form both (+)-lariciresinol (**105a**) and (−)-secoisolariciresinol (**110b**), respectively (**Figure 28**). Moreover, the incoming hydride also took up the *pro-R* position in the corresponding products. For example, when (±)-pinoresinols (**13a/b**) and (±)-lariciresinols (**105a/b**) were incubated with the *Forsythia* PLR in the presence of [4*R*-^{2}H]-NADPH, the enzymatic products were established to be (+)-[7′*R*-^{2}H]-lariciresinol (**105a**) and (−)-[7*R*,7′*R*-^{2}H]-secoisolariciresinol (**110b**), respectively. Furthermore, using (±)-[7,7′-^{2}H$_2$]-pinoresinols (**13a/b**) and [7,7′-^{2}H$_3$]-lariciresinols (**105a/b**) as substrates and unlabeled NADPH, NMR spectroscopic analyses of the resulting enzymatic products **105a** and **110b** also demonstrated that the incoming hydride took up the *pro-R* position in the corresponding substrate (**Figures 29(b)** and **29(d)**; the corresponding ^{1}H-NMR spectra of unlabelled **105** and **110** are shown for comparison in **Figures 29(a)** and **29(c)**). From these observations, it was demonstrated that an 'inversion' of configuration had occurred at C7 for (+)-lariciresinol (**105a**) and at C7/C7′ for (−)-secoisolariciresinol (**110b**), with the presumed intermediate(s) being the enzyme-bound quinone methide(s) (**Figure 28**).[269]

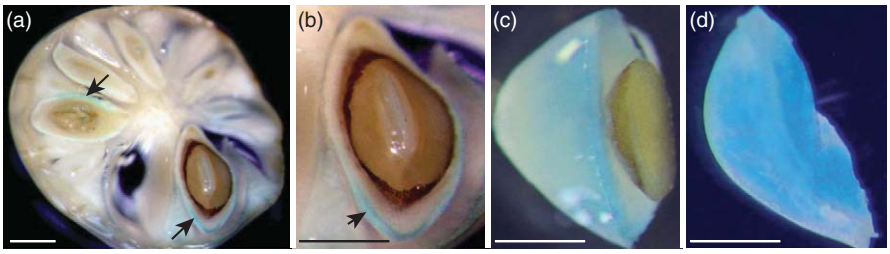


Figure 27 Histochemical localization of GUS in flax (*L. usitatissimum*) containing the *PLR_Lu* promoter. Cross-sections of: (a) Flaxseed capsule. (b and c) Seed showing seed-coat stained and unstained embryo. (d) Seed-coat. Bars represent 1 mm. Reproduced with permission from C. Hano; I. Martin; O. Fliniaux; B. Legrand; L. Gutierrez; R. R. J. Arroo; F. Mesnard; F. Lamblin; E. Lainé, *Planta* **2006**, *224*, 1291–1301. **Figure 4n–4q**. Copyright 2006.

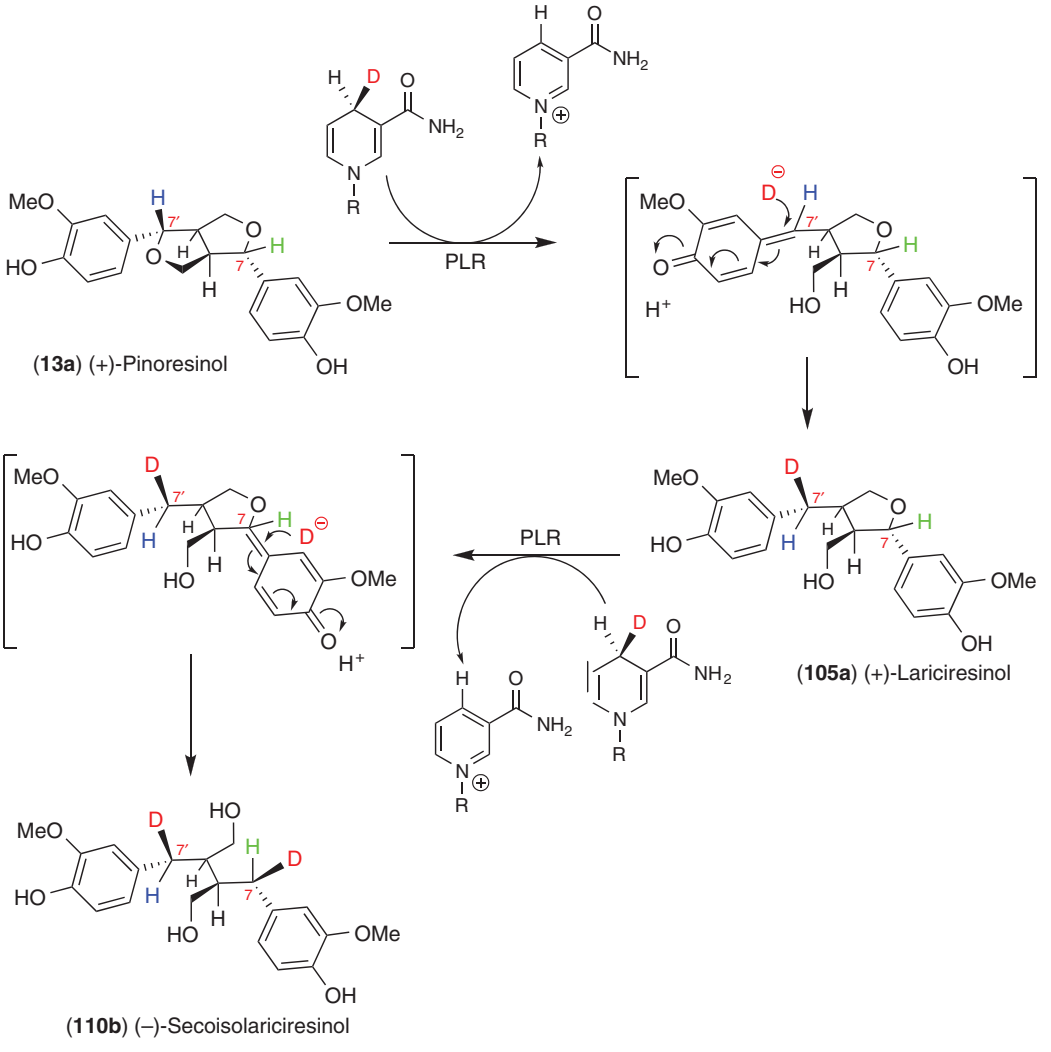


Figure 28 Stereospecificity of hydride transfer during *F. intermedia* PLR catalysis resulting in proposed formation of quinone methide intermediate from (+)-pinoresinol (**13a**) and inversion of product configuration for (+)-lariciresinol (**105a**) and (–)-secoisolariciresinol (**110b**). Adapted from A. Chu; A. Dinkova; L. B. Davin; D. L. Bedgar; N. G. Lewis, *J. Biol. Chem.* **1993**, *268*, 27026–27033.

1.23.5.2.6(ii) Structural biology/substrate versatility studies Based on sequence similarities (and, as discussed later, structural features as well), PLRs are classified within the somewhat diverse short-chain dehydrogenase/reductase (SDR) superfamily.[266] In general, most SDRs are about 250 amino acid residues in length, and share many structural similarities, for example, a conserved N-terminal nucleotide cofactor-binding sequence motif (GXXGXG, the so-called Rossman fold), and a conserved catalytic Lys residue. SDRs, in general, are able to process a wide range of substrates and may have as little as only 15–30% sequence identity. However, typically, their tertiary structures/folding patterns are quite similar.[294]

Of those involved in phenylpropanoid metabolism, the *T. plicata* PLR (PLR_Tp1) homolog (apo-form), as well as the modeled PLR_Tp2, were the first to have their X-ray crystal structures determined (at 2.5 Å resolution for PLR_Tp1, **Figure 30(a)**).[266] Both are dimers as demonstrated by sedimentation equilibrium experiments (with PLR_Tp1), which gave an apparent molecular mass of 69.9 ± 0.4 kDa, whereas MALDI-TOF analysis gave a distinct monomer at 35 096 *m*/*z*, as well as a broad less intense dimer centered at ~70 099 *m*/*z*.[266] PLR_Tp2 and the PLR_Tp1 homologs have two functional domains, a conserved nucleotide cofactor-binding N-terminal domain and a presumed substrate-binding C-terminal domain, with a deep cavity between

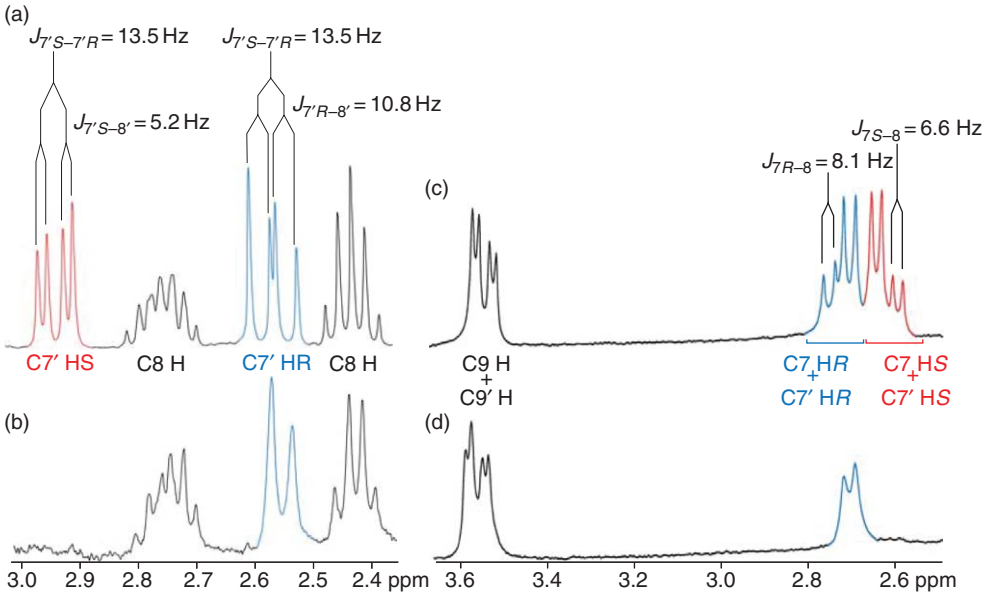
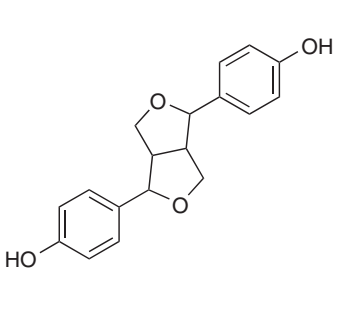


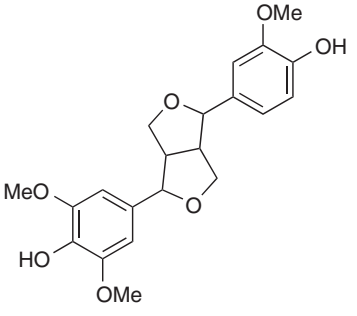
Figure 29 Partial ^{1}H NMR spectra of lariciresinol (**105**) and secoisolariciresinol (**110**) showing regions for C7′/C8/C8 and C7/C7′/C9 proton resonances, respectively. (a) Synthetic (±)-lariciresinols (**105a/b**). (b) Enzymatically synthesized (+)-[7,7′ *S*-$^{2}H_2$]lariciresinol (**105a**) obtained following incubation of PLR_Fi1 with (±)-[7,7′ $^{2}H_2$]pinoresinols (**13a/b**). (c) Synthetic (±)-secoisolariciresinols (**110a/b**). (d) Enzymatically synthesized (−)-[7,7′ *S*-$^{2}H_3$]secoisolariciresinol (**110a**) obtained following incubation of PLR_Fi1 with (±)-[7,7′ *S*-$^{2}H_3$]lariciresinols (**105a/b**). Redrawn from A. Chu; A. Dinkova; L. B. Davin; D. L. Bedgar; N. G. Lewis, *J. Biol. Chem.* **1993**, *268*, 27026–27033.

them. The highly conserved catalytic lysine residue (Lys138) was present in the active site and presumed to be involved in catalysis, this being further established by site-directed mutagenesis,[266] where, for example, the K138A mutant of PLR_Tp1 had its PLR activity abolished.

Substrate/cofactor docking *in silico* suggested that Lys138 was in close proximity to the phenolic group of the substrate. Lys138 was thus considered to act as a general base to initiate catalysis, abstracting the phenolic proton of pinoresinol (**13**) to facilitate the formation of the putative quinone methide intermediate[269] (**Figure 31(a)**). Hydride addition (from NADPH) at C7 then occurs to afford the first product, lariciresinol (**105**), which is envisaged to leave the active site, and then bind again through the phenolic moiety (attached to the remaining furan group), adjacent to Lys138. A second round of catalysis can then proceed, with the presumed intermediacy of a second quinone methide as before, and hydride addition at C7′ affording secoisolariciresinol (**110**). Quinone methide intermediacy was further supported by substrate versatility studies, where ligballinol (**238**), medioresinol (**239**), and syringaresinol (**96**) were processed as substrates, whereas sesamin (**11**) was not; that is, there was an apparent requirement for a free phenolic group in the substrate thereby enabling quinone methide formation as had been proposed earlier in 1993.[269]



(**238**) Ligballinol



(**239**) Medioresinol

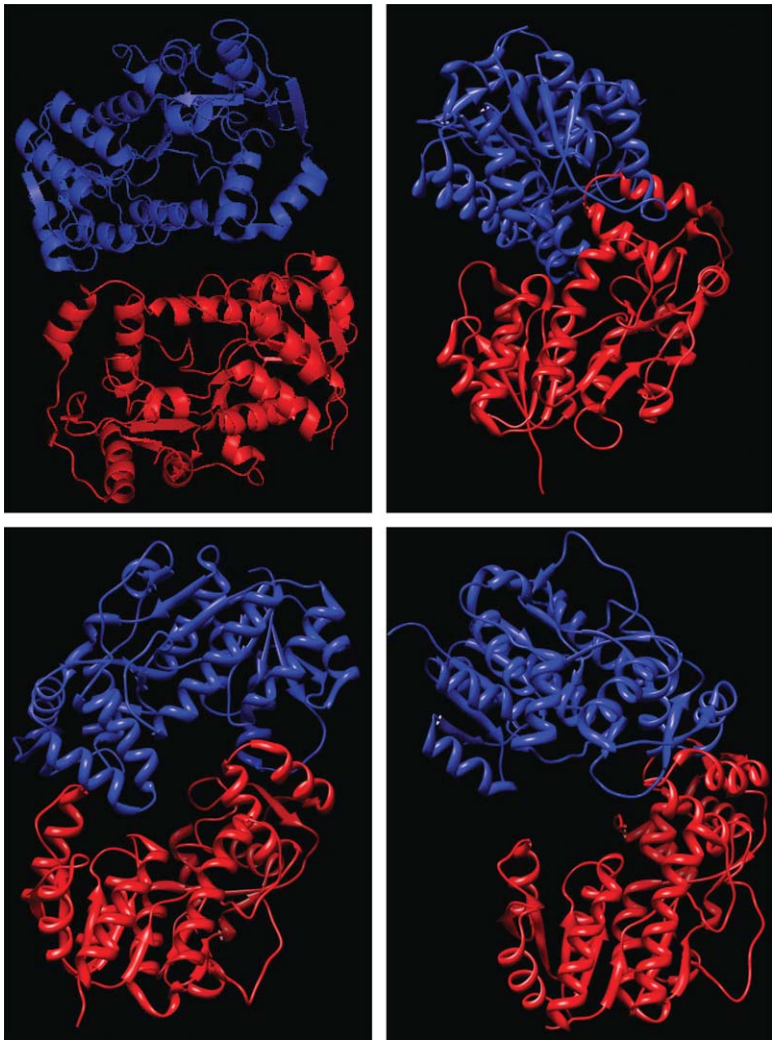


Figure 30 Schematic outlines of the dimeric forms of (a) *Thuja plicata* PLR_Tp1, (b) *Pinus taeda* PCBER_Pt1, (c) *Medicago sativa* IFR_Ms1, and (d) *Ocimum basilicum* EGS depicted in ribbon form by alignment of their twofold axes.

1.23.5.2.6(iii) PLR and PLR homolog enantiospecificity In general, PLR_Tp2 and PLR_Tp1 display opposite preferences with regard to substrate enantiomeric forms. From comparison of both structures, various potential (candidate) residues present in the corresponding active sites were considered as possibly involved in stipulation of the distinct enantiospecificities observed, that is, Phe164, Ser167, Val268, and Leu272 in PLR_Tp1, and Leu164, Gly167, Gly267, and Phe271 in PLR_Tp2. To investigate the question of differential enantiospecificity, site-directed mutagenesis of some of these residues (in both enzymes) was performed, with mutants obtained indeed displaying altered overall enantiospecificities.[270] Interestingly, the G267V PLR_Tp2 mutant protein was almost inactive (<5% of wild-type PLR_Tp2 activity) with regard to its activity toward (+)- and (−)-pinoresinols (**13a** and **13b**), whereas the V268G PLR_Tp1 mutant protein had its enantiospecificity almost completely reversed. However, this was achieved mainly by abolishing the ability to process (−)-pinoresinol (**13b**). Thus, the overall underlying reasons for distinct enantiospecificities are beginning to be understood.

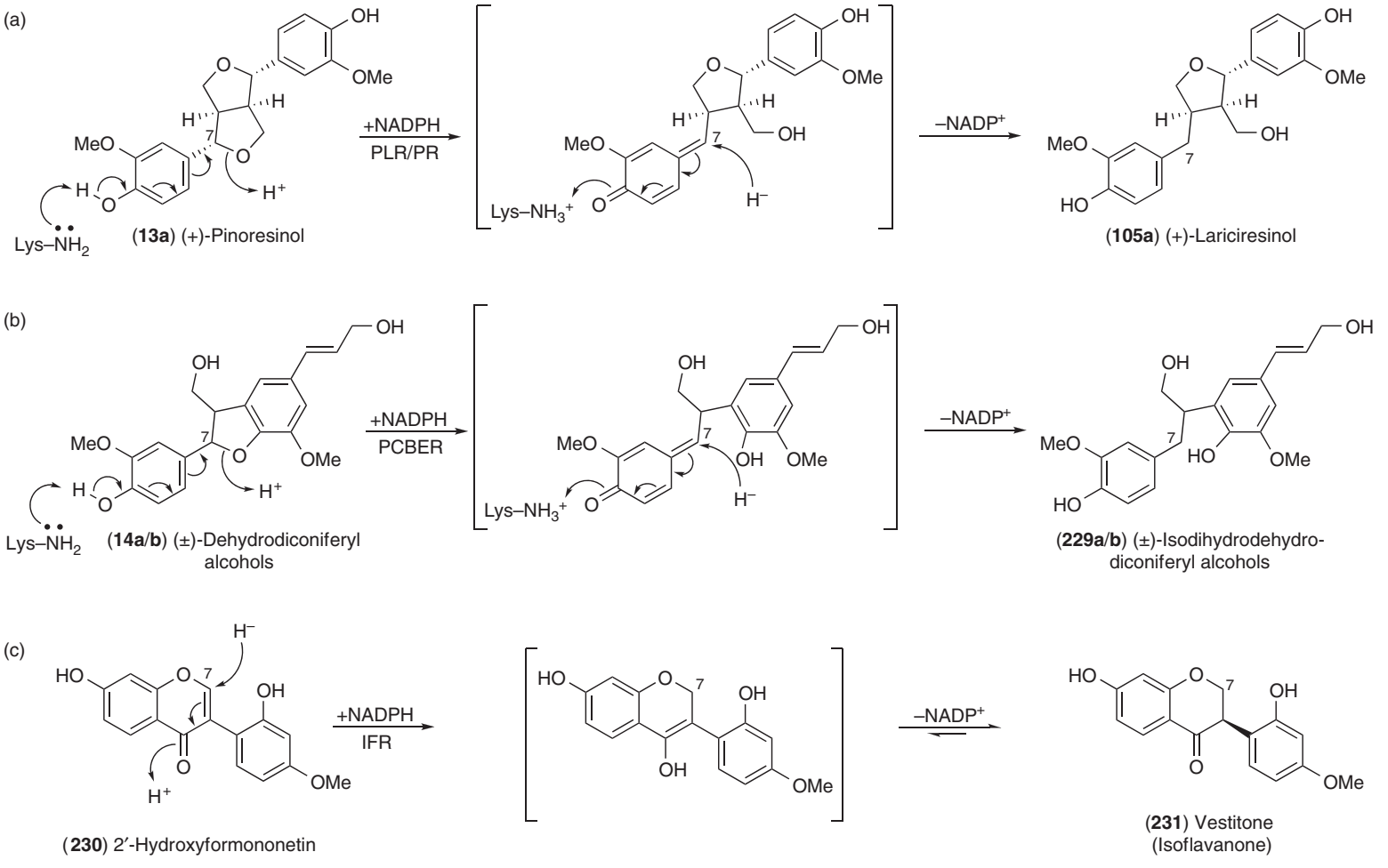


Figure 31 (Continued)

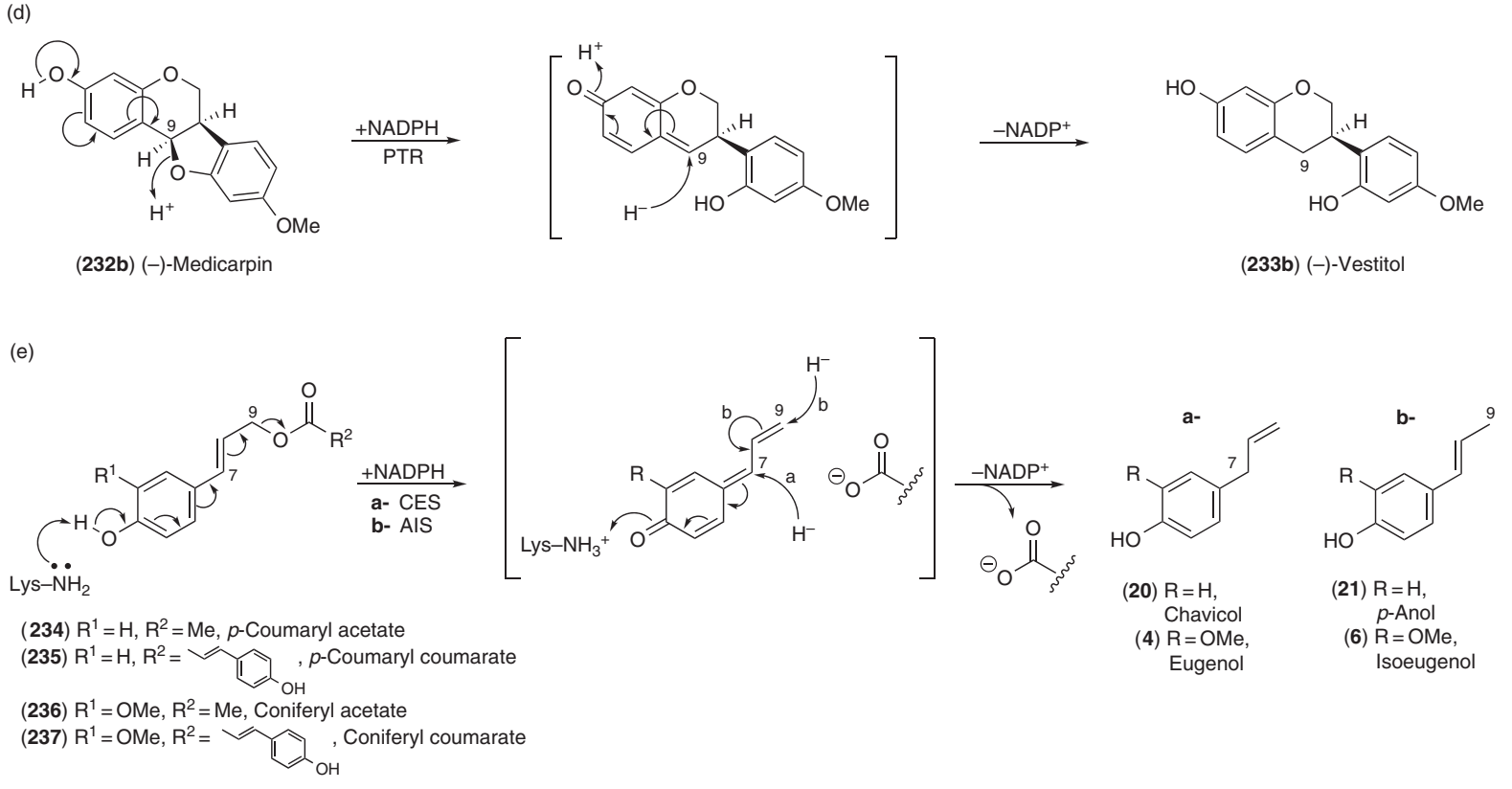


Figure 31 Proposed reaction mechanisms for (a) pinoresinol–lariciresinol reductases/pinoresinol reductases, PLR/PR, (b) phenylcoumaran benzylic ether reductase, PCBER, (c) isoflavone reductase, IFR, (d) pterocarpan reductase, PTR, and (e) chavicol eugenol synthase/anol isoeugenol synthase, CES/AIS.

1.23.5.3 Secoisolariciresinol Dehydrogenase

In many plant species, secoisolariciresinol dehydrogenase (SDH) catalyzes the conversion of secoisolariciresinol (**110**) to matairesinol (**10**). The latter is, in turn, a central intermediate to numerous 8–8′-linked lignans, such as the aforementioned (−)-podophyllotoxin (**1b**) and (−)-plicatic acid (**112b**).

1.23.5.3.1 Discovery of SDH and encoding gene

Using *F. intermedia* cell-free extracts, radiotracer/stable isotope studies initially established that (−)-secoisolariciresinol (**110b**) was enantiospecifically converted into (−)-matairesinol (**10b**) in the presence of NAD^+;[220,222] the (+)-enantiomer (**110a**) was not utilized. This was unambiguously established by the incubation of both (±)-[9,9′-^{3}H]- and (±)-[Ar-^{2}H]-secoisolariciresinols (**110a/b**) to afford (−)-[9-^{3}H]- and (−)-[Ar-^{2}H]-matairesinol (**10b**), respectively. The resulting secoisolariciresinol dehydrogenase (SDH) was also purified (>6000-fold) from *F. intermedia* stems, the encoding gene (*SDH_Fi321*) cloned, and fully functional recombinant protein expressed in *E. coli*;[295] the corresponding 831-bp cDNA encoded a 277-amino acid protein. Using recombinant SDH_Fi321, catalysis was observed to occur via intermediacy of (−)-lactol (**240b**) (**Figure 32**), indicative of the bifunctional nature of the SDH-catalyzed transformation. Interestingly, however, (−)-lactol (**240b**) intermediacy was not detected though when assaying the native *F. intermedia* SDH.

Additionally, an SDH gene (*SDH_Pp7*) was isolated from the (−)-podophyllotoxin (**1b**)-containing species, *Podophyllum peltatum*. Following the heterologous recombinant protein expression of the presumed native SDH in *E. coli*, and purification to apparent homogeneity, the kinetic parameters (K_m and V_{max} values) were determined using (−)-lactol (**240b**) as the substrate. The apparent K_m, V_{max}, and k_{cat}/K_m values of 160.2, 118 pkat μg^{-1} protein, and 23 600 mol^{-1} l s^{-1} obtained were also consistent with kinetic data for the general phenylpropanoid pathway enzymes to the monolignols.[7]

NMR spectroscopic analyses were also employed to establish the stereospecificity of hydride transfer from (−)-secoisolariciresinol (**110b**) to [4-^{2}H]-NAD^+, and whether this occurred at C-4 of either the *pro-R* position, the *pro-S* position, or both.[296] To investigate this further, enzymatic syntheses of both [4*R*-^{2}H]- and [4*S*-^{2}H]-NADH were carried out, as well as that of [4-^{2}H]-NAD^+.[296] Analysis of [4*R*-^{2}H]- and [4*S*-^{2}H]-NADH by ^{1}H (**Figures 33(b)** and **33(c)**) and heteronuclear multiple-quantum correlation (HMQC)-NMR spectroscopy, and comparison with natural-abundance NADH (**Figure 33(a)**), enabled the 4*S*-proton to be assigned to δ 2.65 ppm and the 4*R*-proton to δ 2.77 ppm.

Further NMR studies of the [4-^{2}H]-NADH generated during dehydrogenation of (−)-secoisolariciresinol (**110b**) by SDH established the stereochemistry of hydride transfer. Following incubation of (±)-secoisolariciresinols (**110a/b**) with partially purified SDH in the presence of [4-^{2}H]-NAD^+, the enzymatically generated (deuterated) NADH was purified and analyzed by ^{1}H- (**Figure 33(d)**) and HMQC- (not shown) NMR spectroscopy. The broad doublet signal observed at δ 2.63 ppm ($\mathcal{J}_{4S-5}$ 3.0 Hz, **Figure 33(d)**), in addition to lack of geminal coupling, established the deuterium to be in the 4*R* position. Thus, the hydride abstracted from (−)-secoisolariciresinol (**110b**) and/or (−)-lactol (**240b**) was added to the *pro-S* position of [4-^{2}H]-NAD^+, affording [4*R*-^{2}H]-NADH, thereby establishing SDH as a type B dehydrogenase.

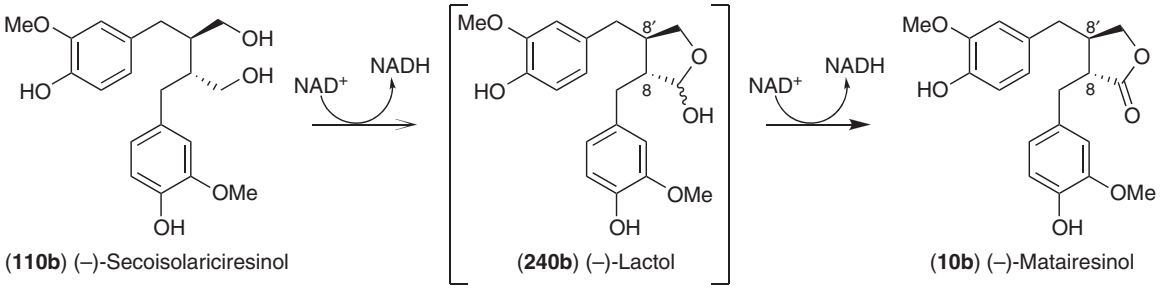


Figure 32 Reaction catalyzed by secoisolariciresinol dehydrogenase (SDH).

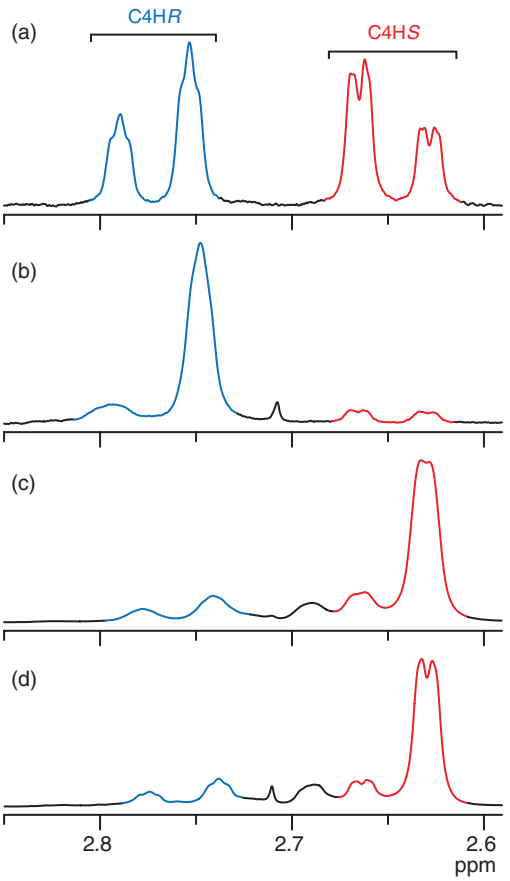


Figure 33 Partial ^{1}H NMR spectra of NADH showing spectroscopic regions for 4*S* and 4*R* protons at C4 of the dihydropyridine ring. (a) Natural-abundance NADH. (b) [4*S*-^{2}H]-NADH. (c) [4*R*-^{2}H]-NADH. (d) Enzymatically synthesized [4*R*-^{2}H]-NADH with SDH_Pp7 following incubation with (±)-secoisolariciresinols (**110a/b**) and [4-^{2}H]-NAD^{+}. Reproduced from S. G. A. Moinuddin; B. Youn; D. L. Bedgar; M. A. Costa; G. L. Helms; C. Kang; L. B. Davin; N. G. Lewis, *Org. Biomol. Chem.* **2006**, *4*, 808–816. Copyright 2006, with permission from the Royal Society of Chemistry.

1.23.5.3.2 Structural biology studies

SDH belongs to the short-chain dehydrogenase (SDR) superfamily. *P. peltatum* SDH (SDH_Pp7) crystal structures were obtained in apo-form, as well as their binary and ternary complexes using NAD^{+} and NAD^{+}/(−)-matairesinol (**10b**), at 1.6, 2.8, and 2.0 Å resolution, respectively.[297] In solution, SDH exists as a homotetramer (as determined by size-exclusion chromatography and multiangle laser light scattering), with each subunit having a single α,β domain structure. In its active site, the SDR-conserved Ser153–Tyr167–Lys171 (the so-called catalytic triad) can apparently function, in combination with the ribose 2′-OH of the cofactor, as a putative proton-relay system (**Figures 34(a)–34(c)**). These residues are considered responsible for cofactor binding, with Tyr167 also placed in close proximity to the alcohol functionality of (−)-secoisolariciresinol (**110b**) upon binding. Participation of the proposed catalytic triad was further supported by site-directed mutagenesis of residues Tyr167 and Lys171 to Ala, which abolished catalytic activity, and Ser153 to Ala, which displayed modest activity.[296]

The entropically favored NAD^{+} binding is provisionally considered to be further stabilized by hydrogen bonding between the cofactor ribose 2′- and 3′-OH groups and Tyr167 and Lys171, respectively (**Figure 34(b)**). This, in turn, would favor Tyr167 deprotonation to afford the corresponding phenolate (which is further stabilized by the neighboring Ser153 hydroxyl group). Tyr167 is thus considered to function

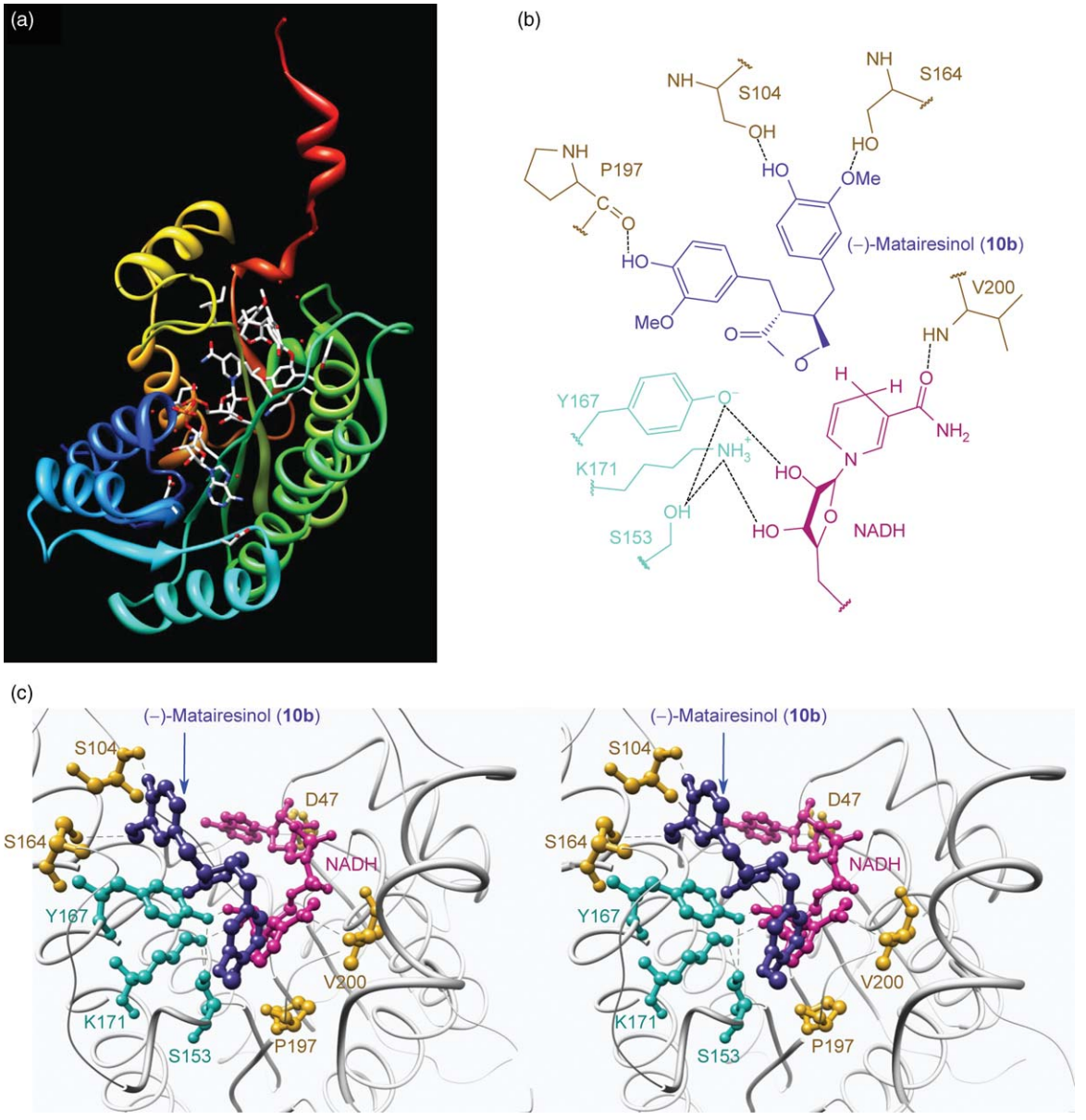


Figure 34 (a) Ternary complex of SDH_Pp7 with NAD^+ and (−)-matairesinol (**10b**). (b) The Tyr167 phenolic and Lys171-protonated amino groups are hydrogen-bonded to 2′- and 3′-OH of NADH, respectively. (c) Structures of the substrate-binding pocket of SDH_Pp7 in ternary complex as a stereoview. The catalyt are shown in blue triad residues, Ser153, Tyr167, and Lys171.

as a general catalytic base, abstracting (or perhaps more accurately influencing the local electronic environment of) the alcohol proton of (−)-secoisolariciresinol (**110b**), thereby facilitating intramolecular cyclization to form (−)-lactol (**240b**), via concerted transfer of the C9-hydride to the *pro-S* position of NAD^+. The NADH thus formed is released from the active site, with a second round of catalysis involving a similar process with (−)-lactol (**240b**), resulting in formation of the lactone product, (−)-matairesinol (**10b**), with concomitant hydride transfer to NAD^+ as before.

1.23.5.4 Creosote Bush Lignan Metabolism: Enantiospecific Polyphenol Oxidase

As summarized earlier, the creosote bush (*L. tridentata*) accumulates only 8–8′-linked lignans, such as NDGA (**143**). The latter is presumed to form via regiospecific 8–8′ coupling of the monomeric precursors, affording larreatricin (**200**) and/or other coupled forms (**201–203**) as possible biosynthetic pathway intermediates. In this context, (+)-larreatricin (**200a**) (but not its (−)-antipode (**200b**)) was enantiospecifically hydroxylated *in vitro* by an *L. tridentata* polyphenol oxidase (PPO, **Figure 35**),[298] this being in agreement with the observed 92% e.e. of (−)-larreatricin (**200b**) *in planta*.[247] Additional reductive steps can thus be envisaged to afford NDGA (**143**).

The native protein catalyzing C3′/C3 hydroxylation was purified (~1700-fold) to apparent homogeneity to afford, via SDS–PAGE analysis, an ~43-kDa protein band. Using the purified PPO from *L. tridentata* twigs, hydroxylation at C3′ was favored, affording (+)-3′-hydroxylarreatricin (**241a**) in an ~7:1 ratio relative to the C3-hydroxylated (+)-3-hydroxylarreatricin (**242a**).[298] Peptide fragments from trypsin digestion of the isolated protein were analyzed by microcapillary reversed-phase-HPLC nanoelectrospray tandem MS (μLC-MS/MS), and gave sequences homologous to conserved PPO motifs. The corresponding gene was obtained from a cDNA library, which encoded an ~66-kDa protein. The higher predicted molecular weight (66 kDa vs ~43 kDa) was indicative of posttranslational processing, a feature common to PPOs, this being further confirmed by comparison of deduced and observed amino acid sequences. Of particular note was the presence of five histidine residues, His188, 197, 319, 323, and 353, which are considered involved in the binding of the copper atoms. Larreatricin-3′-hydroxylase is the first characterized example of an enantiospecific PPO.

1.23.5.5 Additional (Preliminary) Studies Toward Justicidin B, Hinokinin, and Podophyllotoxin/6-Methoxypodophyllotoxin Biosynthesis

1.23.5.5.1 Justicidin B

The achiral justicidin B (**55**) described earlier was first reported in *Justicia procumbens*,[299] and is also found in a number of *Linum* species.[290,300–306] To begin to establish whether *L. perenne* PLR (PLR_Lp1, see Section 1.23.5.2.3) was involved in justicidin B (**55**) biosynthesis, shoot cultures were transformed with *Agrobacterium rhizogenes* containing a vector with an RNAi construct for the silencing of the *PLR_Lp1* gene.[287] From the hairy roots later obtained (six independent lines and control lines with and without empty constructs), justicidin B (**55**) levels were reduced to ~24% of control levels, this presumably resulting from the *PLR_Lp1* mRNA levels being partly suppressed. These data are thus provisionally consistent with our earlier projections[1] that pinoresinol (**13**) serves as a common obligatory intermediate to arylnaphthalenes and so on, via the action of PLR and other downstream enzymes.

1.23.5.5.2 Hinokinin

(−)-Hinokinin (**223b**), which can accumulate in *L. corymbulosum* suspension cultures, differs from (−)-matairesinol (**10b**) via two methylenedioxy bridge additions. Bayindir *et al.*[286] proposed two possible pathways for its

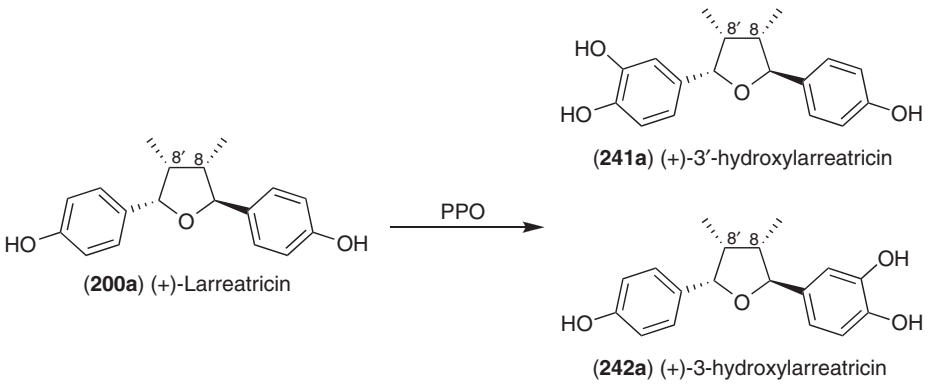


Figure 35 Reaction catalyzed by enantiospecific PPO, larreatricin-3′-hydroxylase, from the creosote bush.

formation: One from secoisolariciresinol (**110**) with further modification, and a second (more unlikely one) from sesamin (**11**) involving a reductase catalyzing an analogous conversion to that of PLR. *L. corymbulosum* shoots were transformed with *A. rhizogenes* harboring the construct (*plr-Lc1*-ihpRNAi) for silencing of *PLR_Lc1* (see Section 1.23.5.2.3) to generate hairy root cultures as above. The resulting transformed cultures were unable to produce (−)-hinokinin (**223b**). No metabolic profiling was, however, reported to indicate whether any upstream metabolites accumulated, such as (+)-pinoresinol (**13a**). Nevertheless, these data were provisionally consistent with the common intermediacy of PLR and pinoresinol (**13**) for the biosynthesis of dibenzylbutyrolactones, such as **223b**, as previously proposed.[1]

1.23.5.5.3 Podophyllotoxin/6-Methoxypodophyllotoxin

As indicated in the previous sections, (−)-podophyllotoxin (**1b**) is found in *Podophyllum* species, and with its derivative, (−)-6-methoxypodophyllotoxin (**194b**), is also present in some *Linum* species. Using *Linum flavum* roots that accumulate **194b**, the pathway from (+)-pinoresinol (**13a**) to (−)-matairesinol (**10b**) was shown to be operative.[226] It was also established, using mass spectral analyses, that following the uptake of matairesinol (**10**) by *L. flavum* root tissue, and its further metabolism for 6 h, conversion to 7-hydroxymatairesinol (**114**) occurred.[226] Moreover, when [7-^{3}H]-7-hydroxymatairesinol (**114**) was also administered to *L. flavum* roots, and allowed to be metabolized for 6 h, the radiolabel was incorporated into (−)-6-methoxypodophyllotoxin (**194b**), suggesting **114** as a pathway intermediate.

In an apparent contrast to the above results,[226] it has been suggested that (−)-deoxypodophyllotoxin (**243b**) might serve instead as a common precursor to both (−)-podophyllotoxin (**1b**) and (−)-6-methoxypodophyllotoxin (**194b**), respectively.[307] A presumed deoxypodophyllotoxin 6-hydroxylase was preliminarily characterized from a crude microsomal preparation obtained from *L. flavum* cell suspension cultures: It was able to catalyze the formation of (−)-β-peltatin A (**244b**) from (−)-deoxypodophyllotoxin (**243b**),[308] with apparent K_m values of 20 and 36 μmol l^{-1} for **243b** and NADPH, respectively (as determined from crude microsomal preparations). In addition, another crude microsomal preparation from *L. album* cell suspension culture was able to convert (−)-deoxypodophyllotoxin (**243b**) into (−)-β-peltatin A (**244b**) in the presence of

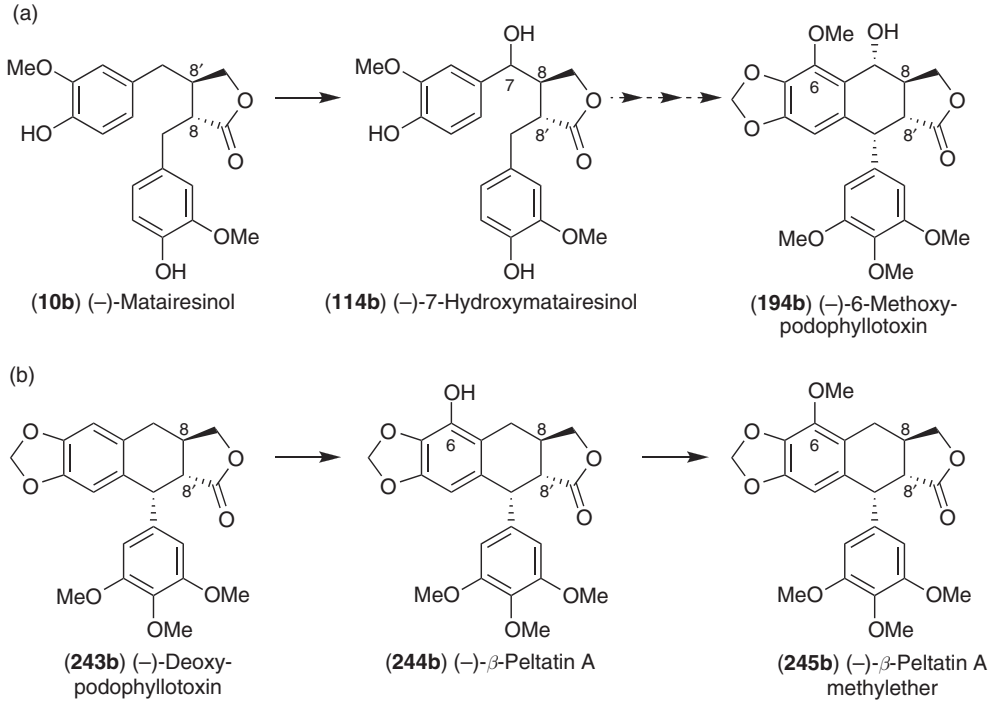


Figure 36 Proposed pathways to (−)-6-methoxypodophyllotoxin (**194b**) from (−)-matairesinol (**10b**) via (a) (−)-7-hydroxymatairesinol (**114b**)[226] or (b) (−)-deoxypodophyllotoxin (**243b**).[288,308]

NADPH (with apparent K_m values of 3 and 41 μmol l^{-1} for **243b** and NADPH, respectively).[288] No other kinetic data were, however, obtained.

In any event, the complete pathway to **1b**/**194b** still remains to be elucidated, that is, in terms of the various hydroxylations, *O*-methylation, and methylenedioxy bridge-forming steps, as well as for aryltetrahydronaphthalene ring formation.

Additionally, a presumed *β*-peltatin 6-*O*-methyltransferase was preliminarily detected in cell suspension cultures of *Linum nodiflorum*, which converted *β*-peltatin A (**244b**) into *β*-peltatin A methylether (**245b**). Apparent K_m values were estimated (using proteins recovered after $(NH_4)_2SO_4$ precipitation) as 40 and 15 μmol l^{-1} for **244b** and *S*-adenosyl methionine, respectively. Neither matairesinol (**10**), nor pinoresinol (**13**), nor podophyllotoxin (**1**), apparently served as substrates.[307] To date, however, none of these enzymes has been purified and/or the encoding genes cloned.

1.23.6 Other Phenylpropanoid Coupling Modes: 8–2′, 8–3′ (8–5′), and 8–*O*–4′-Linked Lignans

Although many additional coupling modes in lignans are evident (see Section 1.23.3.2) from the different skeletal types and optical activities reported, most studies have thus far focused mainly on the formation of the 8–8′-linked lignans. Nevertheless, the generation of other optically active skeletal types can be envisaged to occur through DP-assisted coupling of two radical intermediates, although whether they are derived from monolignols, allylphenols, or hydroxycinnamic acids, and/or combinations thereof to generate different linkage modes is currently unknown.

Interestingly, DP participation has been reported as involved in the formation of the toxic chiral (atropisomeric) (+)-gossypol (**247a**),[309] a terpenoid derived from the coupling of the achiral precursor hemigossypol (**246**) in moco cotton (*Gossypium hirsutum* L. var. *marie galante*) flower petals. The optical activity in this case results from restricted rotation around the C–C biphenyl linkage (**Figure 37**). When laccase/O_2 were added to partially purified and presumed DP-enriched fractions from flower petals, the e.e. of product formation increased to ~65% (from being 59% without laccase/O_2), with the overall amounts of (+)-gossypol (**247a**) also increased. This contrasts with one-electron oxidation of hemigossypol (**246**) *in vitro*, which produces only the racemic products (±)-**247a/b**. These data, therefore, suggest involvement of DPs beyond the phenylpropanoid pathway, albeit still utilizing phenolic substrates.

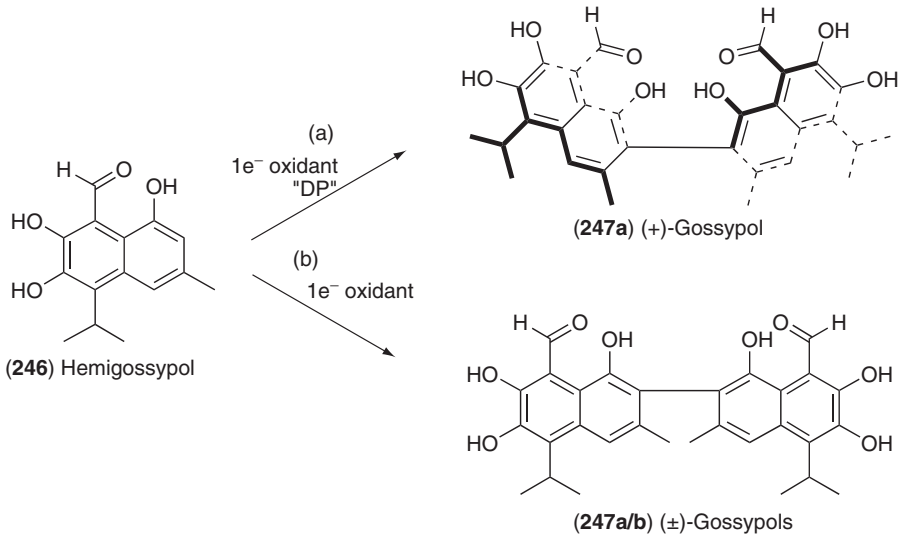


Figure 37 Stereoselective and non-stereoselective formation of gossypol (**247**) from hemigossypol (**246**). (a) Stereoselective: presence of an $1e^-$ oxidant and a putative dirigent protein (DP) in cotton flower petals to afford (+)-gossypol (**247a**).[309] (b) Non-stereoselective: presence of $1e^-$ oxidant affording (±)-gossypols (**247a/b**).

Some of the other coupling modes described earlier in the chemotaxonomical analysis of lignans have been preliminarily investigated from a biochemical perspective, as summarized below.

1.23.6.1 8–2′ Coupling

The optically active 8–2′-linked lignans, (−)-(*Z*/*E*)-blechnic acids (**248b**/**99b**), and its derivative (−)-*E*-brainic acid (**102b**), in the fern *Blechnum spicant* were demonstrated to be derived from labeled [U-^{14}C]-, [1-^{13}C]-, [2-^{13}C]-, and [3-^{13}C]-Phe (**2**), as well as [9-^{14}C]-cinnamic (**249**), [8-^{14}C]- and [8-^{13}C]-*p*-coumaric (**250**), and [8-^{13}C]-caffeic (**56**) acids *in vivo*.[138] Based on the incorporation data obtained, (−)-*Z*-blechnic acid (**248b**) was apparently the initial coupling product (**Figure 38**), with further metabolism affording both (−)-*E*-blechnic acid (**99b**) and (−)-*E*-brainic acid (**102b**). The proteins/enzymes involved in these transformations, as well as the corresponding encoding genes, have not yet been reported. One possible is that they involve either DP- or DP-like-mediated conversions.

1.23.6.2 8–3′ (8–5′) Coupling

In *Piper regnellii*, the 8–3′-linked lignans (+)-conocarpan (**16a**) and (−)-*epi*-conocarpan (**188b**), as well as fully conjugated derivatives such as **191**, accumulate in the leaf and root tissues.[310] Of these, (+)-conocarpan (**16a**) formation was envisaged in preliminary studies to result through 8–3′ regio- and stereoselective coupling of

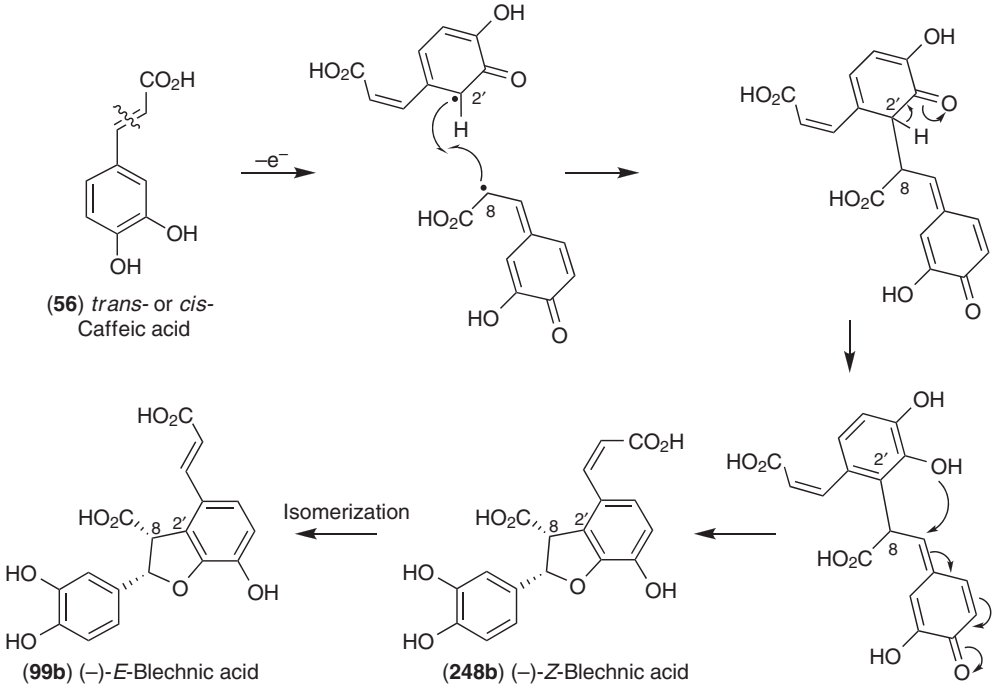
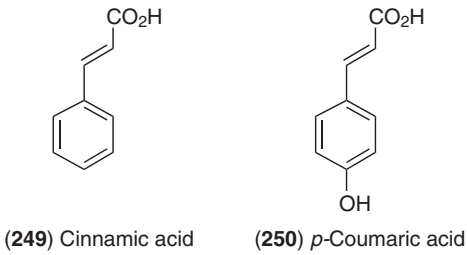


Figure 38 Putative biosynthetic pathway to 8–2′-linked (−)-*E*-blechnic acid (**99b**) in *Blechnum spicant*.[138]



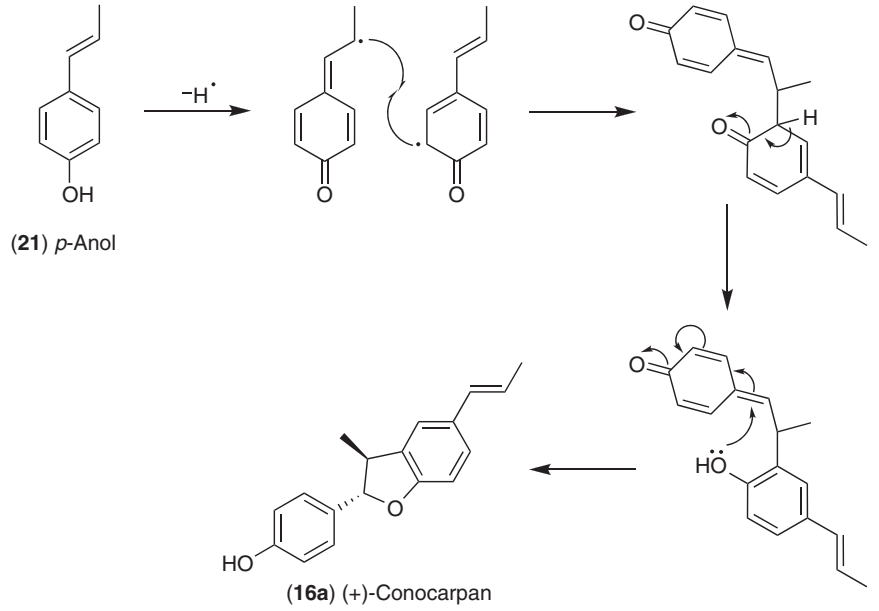


Figure 39 Putative biosynthetic pathway to 8–3′-linked (+)-conocarpan (**16a**) in *Piper regnellii*.

two *p*-anol (**21**) moieties (**Figure 39**). While of interest, these preliminary studies have not yet established the nature of the proteins and enzymes involved. Again, how this either compares or differs with the above DP-mediated processes will be important to determine.

1.23.6.3 8–*O*–4′ Coupling

Eucommia ulmoides reportedly produces diastereoselectively coupled 8–*O*–4′-linked lignans, with the stereochemistries at positions 7 and 8 being of particular interest, in terms of how they are controlled. For example, coniferyl alcohol (**91**) was preferentially converted into the optically active 8–*O*–4′-linked (+)-*erythro*- and (−)-*threo*-guaiacylglycerol coniferyl alcohol ethers (**115**, in an ∼3:2 ratio, **Figure 40**) using *E. ulmoides* cell-free extracts containing a presumed insoluble cell wall residue preparation in the presence of H_2O_2.[311] Similarly, sinapyl alcohol (**94**) was apparently also metabolized to preferentially afford both (+)-*erythro*- and (−)-*threo*-syringylglycerol sinapyl alcohol ethers (**252**), in an ∼10:1 ratio.[312] Furthermore, young excised *E. ulmoides* shoots offered both coniferyl (**91**) and sinapyl (**94**) alcohols formed guaiacylglycerol sinapyl alcohol ether (**251**) in a ∼2.4:1 *erythro*/*threo* ratio.[313] The biochemical factor(s) controlling diastereoselective coupling, including involvement of possible DP protein(s), await further clarification.

1.23.7 Allylic (Phenylpropenal) Double Bond Reductases and Phenylcoumaran Benzylic Ether Reductases

1.23.7.1 Allylic (Phenylpropenal) Double Bond Reductases: Biosynthesis of Dihydrolignans and Dihydromonolignols

As far as current chemotaxonomical information permits, the evolutionarily earliest known example of dihydrolignans (i.e., lignans with saturated side-chains) is in the ferns (e.g., **103a** and **104b** in *P. vittata*[139]), with this structural motif sporadically reoccurring in the gymnosperms and angiosperms. Metabolites with reduced side-chains now encompass not only 8–5′-linked lignans, but those that are 8–*O*–4′-linked (e.g., **116** and **117**) as well as various monomeric phenylpropanoids. That is, *P. glauca* shoots and galls accumulate, among other phenolics, dihydrodehydrodiconiferyl alcohol (**103**) and its demethylated derivative **116**, dihydroconiferyl alcohol (**119**), dihydro-*p*-coumaric acid (**253**), and dihydroferulic acid (**255**), as well as the

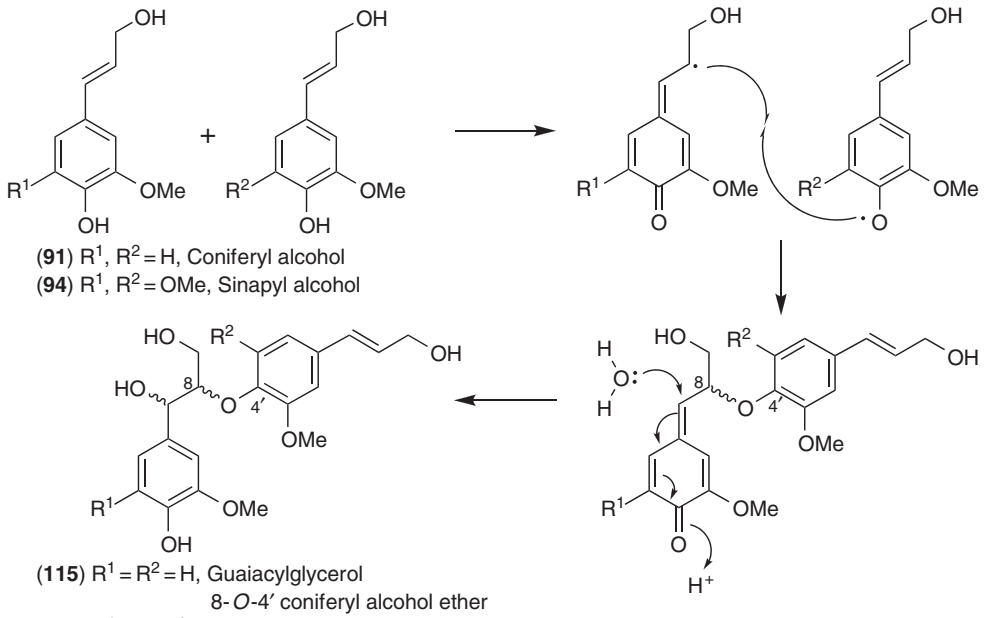
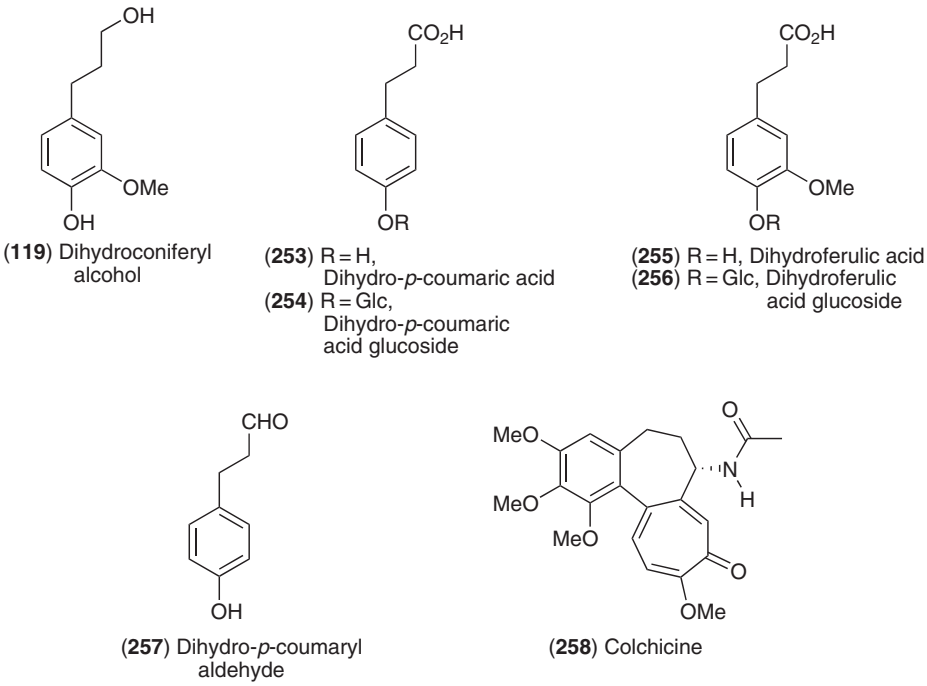


Figure 40 Putative diastereoselective 8–*O*–4′-coupling in *Eucommia ulmoides* affording lignans **115**, **251**, and **252**.

corresponding glucosides **254**/**256**.[161] While the main function of these natural products appear to be in plant defense (see Section 1.23.10.2), dihydro-*p*-coumaryl aldehyde (**257**) was also proposed as a precursor of the phenethylisoquinoline alkaloid, colchicine (**258**), in *Colchicum byzantinum* and *Colchicum autumnale*.[314]



*1.23.7.1.1 Discovery of allylic (phenylpropenal) double bond reductases and gene cloning: Loblolly pine (*Pinus taeda*)*

Biosynthetic studies to dihydrolignans and dihydromonolignols began with observations made using *P. taeda* cell cultures elicited with an 8% sucrose solution. Under these conditions, which result in phenylpropanoid pathway induction, dihydrodehydrodiconiferyl alcohol (**103**) was isolated from the complex mixture of soluble phenolics accumulating in the cell bathing medium.[147] Using a partially purified protein preparation from this source, the NADPH-dependent conversion of dehydrodiconiferyl alcohol (**14**) into dihydrodehydrodiconiferyl alcohol (**103**) was demonstrated.[315,316] Ultimately, three predominant coeluting protein bands were separated using SDS–PAGE, with trypsin digestion and peptide sequencing leading to the identification of cinnamyl alcohol dehydrogenase (CAD), the last enzymatic step involved in monolignol biosynthesis, a fructose-bisphosphate aldolase, and an ~39-kDa protein of undetermined function.

The full-length cDNA clone for the latter was obtained, which encoded an ~38.7-kDa NADPH-dependent protein that was subsequently expressed in *E. coli*, albeit mainly in an insoluble form. The reductase was, however, unable to catalyze the NADPH-dependent side-chain reduction of either dehydrodiconiferyl (**14**) or coniferyl (**91**) alcohols directly. Based on the earlier protein assays, which contained this protein and CAD, it was both deduced and then established using recombinant protein that the actual substrates for the reductive transformations in the *P. taeda* cell cultures were dehydrodiconiferyl aldehyde (**259**) and coniferyl aldehyde (**262**), respectively, to afford **260** and **263** (**Figure 41**). The gene encoding this protein was thus named *PtPPDBR* (*P. taeda* phenylpropenal double bond reductase).

This finding thus apparently differed from another preliminary report indicating that coniferyl aldehyde (**262**) could be converted *in vitro* into coniferyl (**91**) and dihydroconiferyl (**119**) alcohols, in the presence of NADPH, using a microsomal preparation from developing xylem of *Pinus strobus*.[317] That study instead suggested, based on the data obtained, the involvement of a cytochrome P-450.

Based on the data above, the functionally operative enzyme isolated from the *P. taeda* cell culture was an NADPH-dependent phenylpropenal double bond reductase (PtPPDBR).[315,316] Comprehensive kinetic parameters could not be measured, however, due to poor expression levels of recombinant PtPPDBR. Such data still need to be obtained, though, in order to assess its relative efficacy in the overall phenylpropanoid pathway.

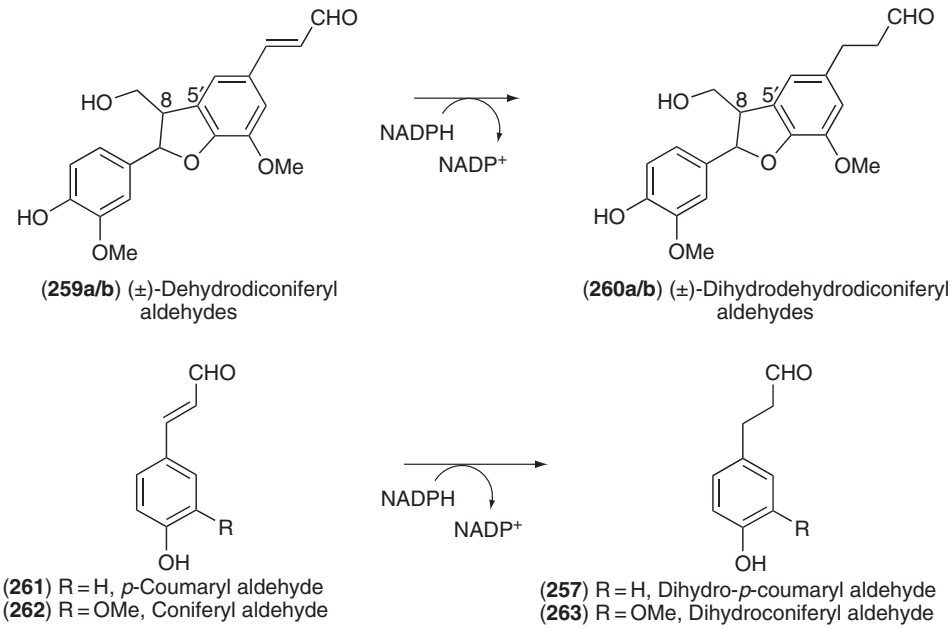


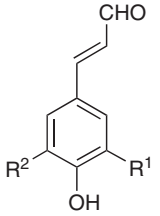
Figure 41 Reactions catalyzed *in vitro* by allylic double bond reductases from loblolly pine (*Pinus taeda*) and *Arabidopsis thaliana*.

1.23.7.1.2 mRNA tissue localization of PtPPDBR in loblolly pine

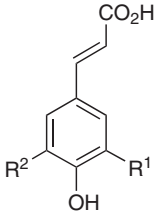
Pinus taeda PPDBR mRNAs were also localized in various stem tissues using *in situ* hybridization.[316] These were detected in the vascular cambium, radial parenchyma cells of the xylem, and axial parenchyma cells of the cortex of young (just below the apical meristem, **Figure 42(a)**) and more mature *P. taeda* stems (**Figures 42(b)** and **42(c)**). Localization to the vascular cambium and radial parenchyma cells thus correlated well with mRNA localization of DP in *F. intermedia* and *T. plicata*, as well as that of PLR in *F. intermedia*. That is, the PtPPDBR mRNA localization co-occurs in cell types known to be involved in lignan biosynthesis.

1.23.7.1.3 Allylic double bond reductase homologs: eleven-membered multigene family in Arabidopsis

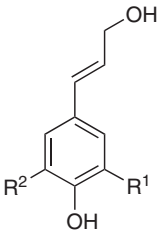
Based on sequence comparisons of PtPPDBR with homologs in the *A. thaliana* genome, a very provisional annotation of an 11-membered alkenal double bond reductase (AtDBR) multigene family was made,[318] based on an ~63/43% amino acid similarity/identity to PtPPDBR. It was, therefore, of interest to clone each of the encoding genes, in order to obtain the corresponding recombinant proteins in *E. coli* and assess their potential phenylpropenal reductase activities. Of the 11 recombinant proteins (obtained as His-tag fusion proteins), AtDBR1, 8, and 9 were catalytically competent to reduce substrates such as *p*-coumaryl (**261**) and coniferyl (**262**) aldehydes, to the corresponding (propyl) single bond derivatives, **257** and **263**, with AtDBR1 and 9 also able to reduce caffeyl aldehyde (**264**) to a lesser extent. Of these, AtDBR1 was further characterized with its kinetic parameters determined for **261** and **262**: The K_m, k_{cat}, and k_{cat}/K_m values were 0.53/0.41 mmol l^{-1}, 2.82/0.43 s^{-1}, and 5360/1060 mol^{-1} l s^{-1}, respectively. None of the other tested phenylpropanoid aldehydes (5-hydroxyconiferyl (**265**) and sinapyl (**266**) aldehydes), carboxylic acids (**56**, **249**, **250**, **267**–**269**), alcohols (**91**, **94**, **199**, **270**, **271**), or dehydrodiconiferyl aldehyde (**259**) served as substrates for side-chain reduction *in vitro*.



(**261**) R^1, R^2 = H, *p*-Coumaryl aldehyde
(**264**) R^1 = OH, R^2 = H, Caffeyl aldehyde
(**262**) R^1 = OMe, R^2 = H, Coniferyl aldehyde
(**265**) R^1 = OMe, R^2 = OH, 5-OH Coniferyl aldehyde
(**266**) R^1, R^2 = OMe, Sinapyl aldehyde



(**250**) R^1, R^2 = H, *p*-Coumaric acid
(**56**) R^1 = OH, R^2 = H, Caffeic acid
(**267**) R^1 = OMe, R^2 = H, Ferulic acid
(**268**) R^1 = OMe, R^2 = OH, 5-OH Ferulic acid
(**269**) R^1, R^2 = OMe, Sinapic acid



(**199**) R^1, R^2 = H, *p*-Coumaryl alcohol
(**270**) R^1 = OH, R^2 = H, Caffeyl alcohol
(**91**) R^1 = OMe, R^2 = H, Coniferyl alcohol
(**271**) R^1 = OMe, R^2 = OH, 5-OH Coniferyl alcohol
(**94**) R^1, R^2 = OMe, Sinapyl alcohol

Following the PtPPDBR work, AtDBR1 was later reported as able to convert 4-hydroxy-(2*E*)-nonenal (4-HNE, **272**) into 4-hydroxynonanal (4-HNA, **273**, **Figure 43**) *in vitro*.[320] However, in our hands, this occured with much lower enzymatic efficacy (K_m of 0.28 mmol l^{-1}, k_{cat} of 0.16 s^{-1}, and k_{cat}/K_m of 620 mol^{-1} l s^{-1}) relative to *p*-coumaryl aldehyde (**261**).[318] 4-HNE (**272**) was considered as a potential substrate, since

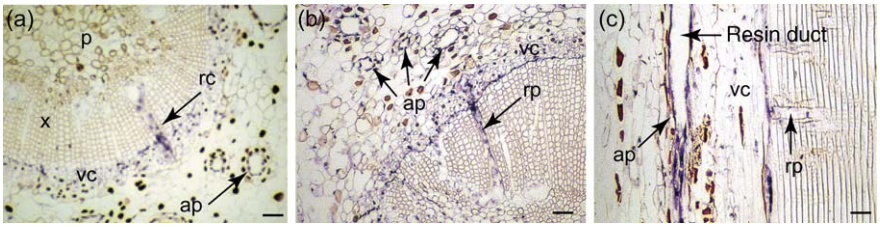


Figure 42 PtPPDBR gene expression localization in *P. taeda* stems at different developmental stages. (a) Transversal cross-section of meristematic region; (b and c) transversal (b) and longitudinal (c) cross sections of a young stem. Abbreviations: ap, axial parenchyma cells; p, pith; rp, radial parenchyma cells; vc, vascular cambium. Bars: 50 μm. Reproduced from H. Kasahara; Y. Jiao; D. L. Bedgar; S.-J. Kim; A. M. Patten; Z.-Q. Xia; L. B. Davin; N. G. Lewis, *Phytochemistry* **2006**, *67*, 1765–1780. Copyright 2006, with permission from Elsevier.

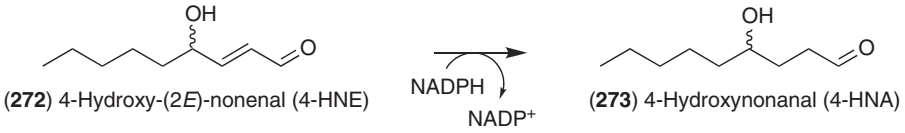


Figure 43 Reaction catalyzed by AtDBR using HNE (**272**) as substrate.

it is a well-characterized lipid peroxidation product that can induce apoptosis; reduction leads to 4-HNA (**273**), which is thought to participate in 4-HNE (**272**) detoxification *in vivo*. It is important to emphasize that, while enzymatically catalyzed conversions have been demonstrated *in vitro*, the true physiological substrate(s) of AtDBR1 in *Arabidopsis* and its role *in vivo* remain uncertain. Indeed, a similar situation currently holds for all members of this multigene family in *Arabidopsis*.

In summary, both PtPPDBR and AtDBR1 were demonstrated *in vitro* to be able to catalyze the addition of an NADPH-derived hydride to the (side chain) double bond of a conjugated propenal (e.g., **261** and **262**), but not of the monolignols (e.g. **199**, **91**). Other related double bond reductases have also been described with similar structural motif requirements. For example, *Artemisia annua* artemisinic aldehyde reductase can reduce the aldehyde-conjugated Δ11(13) double bond of artemisinic aldehyde (**274**) into **275**, but not that of artemisinic alcohol (**276**, **Figure 44(b)**),[321] and (+)-pulegone reductase from *Mentha piperita* (amino acid identities of 42% to PtPPDBR and 63% to AtDBR1) can convert **277** into **278** and **279**[322] (**Figure 44(b)**). The reported enzymes thus have similar biochemical functions, in terms of requiring the substrate to have a conjugated enone system.

1.23.7.1.4 Structural biology studies: Arabidopsis *DBR1*

AtDBR1 is a homodimer in solution, as determined by size-exclusion chromatography, as well as by static and dynamic multiangle light scattering.[318] It is a member of the zinc-independent family of medium-chain dehydrogenases/reductases (MDR), with a nucleotide-binding Gly-rich motif. Its closest structural homolog in the protein database is presently 12-hydroxydehydrogenase/15-oxo-prostaglandin 13-reductase (12-HD/PGR) from guinea pig.

Recombinant AtDBR1 was studied to establish its catalytic mode of action. In this context, crystal structures at 2.5 – 2.8 Å resolution were obtained in apo-form, as well as binary and ternary complexes with $NADP^+$ in the absence and presence of either potential substrates, *p*-coumaryl aldehyde (**261**, **Figure 45**) or 4-HNE (**272**), respectively.

Mechanistically, the highly conserved residue (Tyr260 in AtDBR1) is considered hydrogen-bonded to the aldehyde oxygen of the substrate, and is thus thought to act as a general acid during catalysis. The proposed catalytic mechanisms for AtDBR1[318] (**Figures 46(a)** and **46(b)**), and by extension PtPPDBR, as well as the related HD/PGR are thus very similar.[323] For AtDBR1, an enolate/carbocation intermediate can be envisaged to be generated via double bond migration, followed by the addition of the hydride from NADPH to the carbocation at either C7 from *p*-coumaryl aldehyde (**261**, **Figure 46(a)**) or C3 from 4-

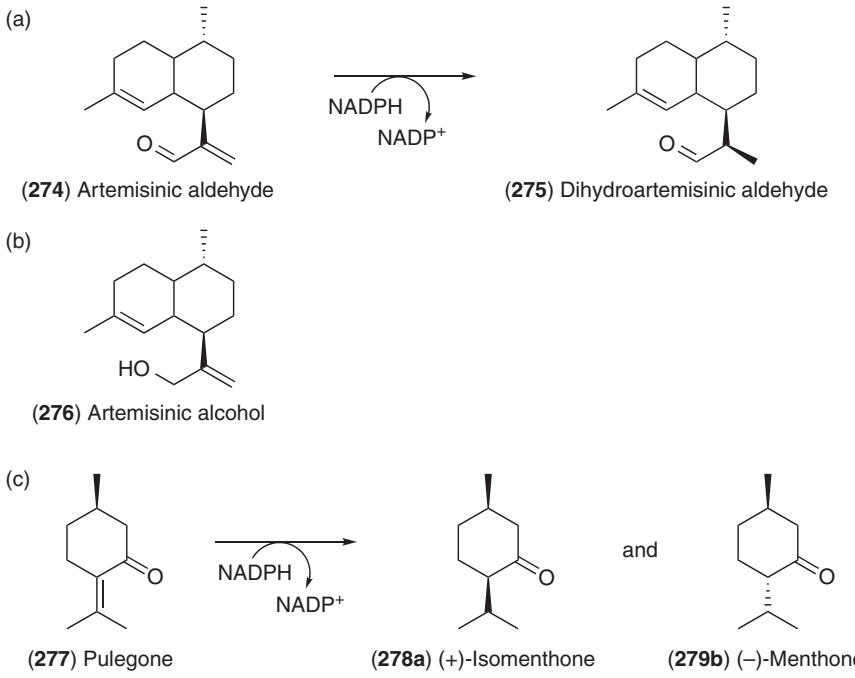


Figure 44 Reactions catalyzed by other NADPH-dependent double bond reductases *in vitro*. (a) Artemisinic aldehyde reductase from *Artemisia annua*. (b) Structure of artemisinic alcohol (**276**). (c) (+)-Pulegone reductase from *Mentha piperita*.

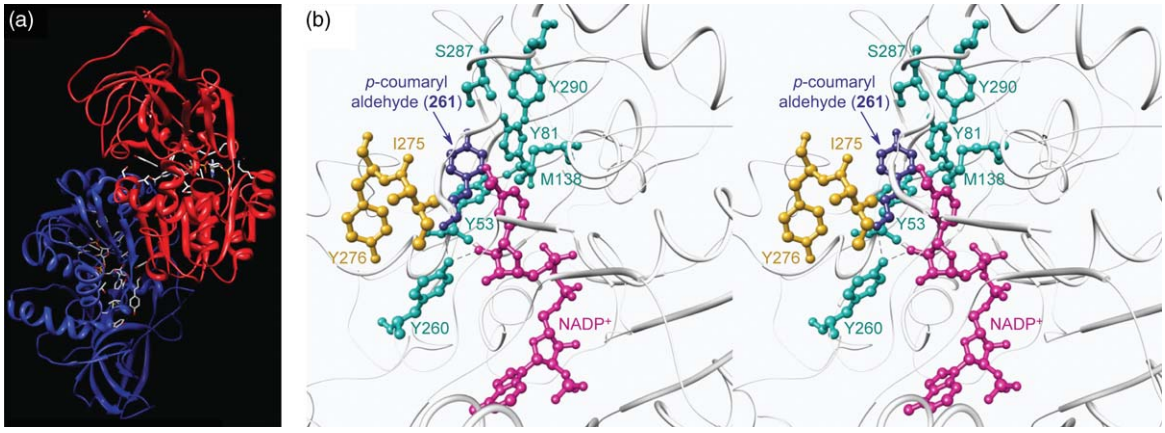


Figure 45 (a) Crystal structure of AtDBR1 ternary complex. (b) Stereoview of the substrate-binding pocket of AtDBR1 (viewed from the bulk solvent) in the ternary complex with $NADP^+$ and *p*-coumaryl aldehyde (**261**). The participating residues in the binding substrate and cofactor are shown with their residue position numbers. The residues are depicted in light blue and yellow to represent their belonging to two different subunits.

HNE (**272**, **Figure 46(b)**). Ultimately, the adjacent carbon (C8 in *p*-coumaryl aldehyde (**261**) or C2 in 4-HNE (**272**)) is protonated by solvent with re-formation of the aldehydic moiety. An alternative concerted mechanism (**Figures 46(c)** and **46(d)**), however, cannot be ruled out. Hydride addition (to either C7 of *p*-coumaryl aldehyde (**261**) or C3 of 4-HNE (**272**)) could lead directly to double bond migration and protonation of the carbonyl oxygen with H^+ from Tyr260, to form an enol as product, which would then undergo tautomerism to yield the aldehydes **257** or **273**.

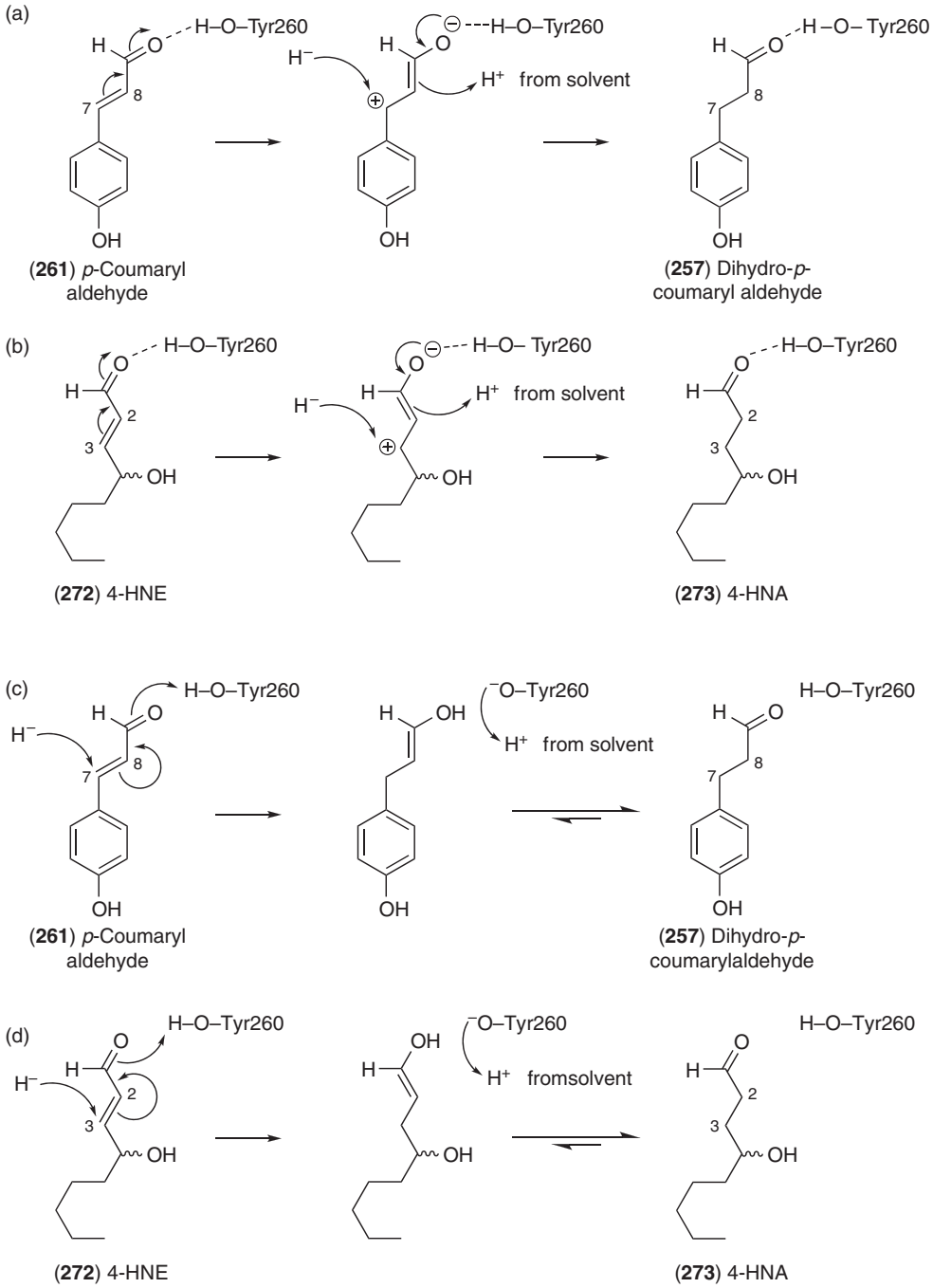


Figure 46 Proposed mechanisms for AtDBR1-catalyzed double bond reduction of *p*-coumaryl aldehyde (**261**) (a, c) and 4-HNE (**272**) (b, d). (a), (b) Hydride addition to an enolate carbocation intermediate Reproduced from B. Youn; S.-J. Kim; S. G. A. Moinuddin; C. Lee; D. L. Bedgar; A. R. Harper; L. B. Davin; G. Lewis; C. Kang, *J. Biol. Chem.* **2006**, *281*, 40076–40088. (c), (d) Concerted hydride addition to give the enol form of the product, with subsequent tautomerization to afford the final aldehyde.

In summary, while these data provide good insight into the overall catalytic mechanism of allylic double bond reductases, it must be emphasized that the actual physiological roles of the AtDBR multigene family (11 members) still remain to be fully established.

1.23.7.2 PLR Homologs: Phenylcoumaran Benzylic Ether Reductases, Isoflavone Reductases, and Pterocarpan Reductases

8–5′-Linked lignans (e.g., dihydrodehydrodiconiferyl alcohol triacetate (**280**, **Figure 47(a)**)) can co-occur with the phenylcoumaran benzylic ether-reduced homolog, tetrahydrodehydrodiconiferyl alcohol tetraacetate (**118**) in the needles of the gymnosperm *C. japonica*.[160] Reduction of the 7–*O*–4′ interunit linkage can thus be envisaged to occur through a similar biochemical process to that of PLR, that is, via presumed quinone methide intermediacy with hydride attack at C7.

Using *Forsythia* PLR cDNA as a probe, a PLR homolog was obtained from a *P. taeda* cell suspension cDNA library, this having ~45% amino acid identity to the *Forsythia* PLR_Fi1. Its full-length cDNA (PCBER_Pt1), which encoded for a 33.6-kDa protein was obtained, with the corresponding recombinant native protein expressed in *E. coli*.[265] This was demonstrated to catalyze *in vitro* the NADPH-dependent reduction of the racemic phenylcoumaran lignans, (±)-dehydrodiconiferyl alcohols (**14a**/**b**), and (±)-dihydrodehydrodiconiferyl alcohols (**103a**/**b**), into the corresponding racemic 7–*O*–4′-reduced products, (±)-isodihydrodehydrodiconiferyl alcohols (IDDC, **229a**/**b**), and (±)-tetrahydrodehydrodiconiferyl alcohols (TDDC, **281a**/**b**) (**Figure 47(b)**). The protein was thus 'provisionally' named phenylcoumaran benzylic ether reductase (PCBER), to reflect the mode of

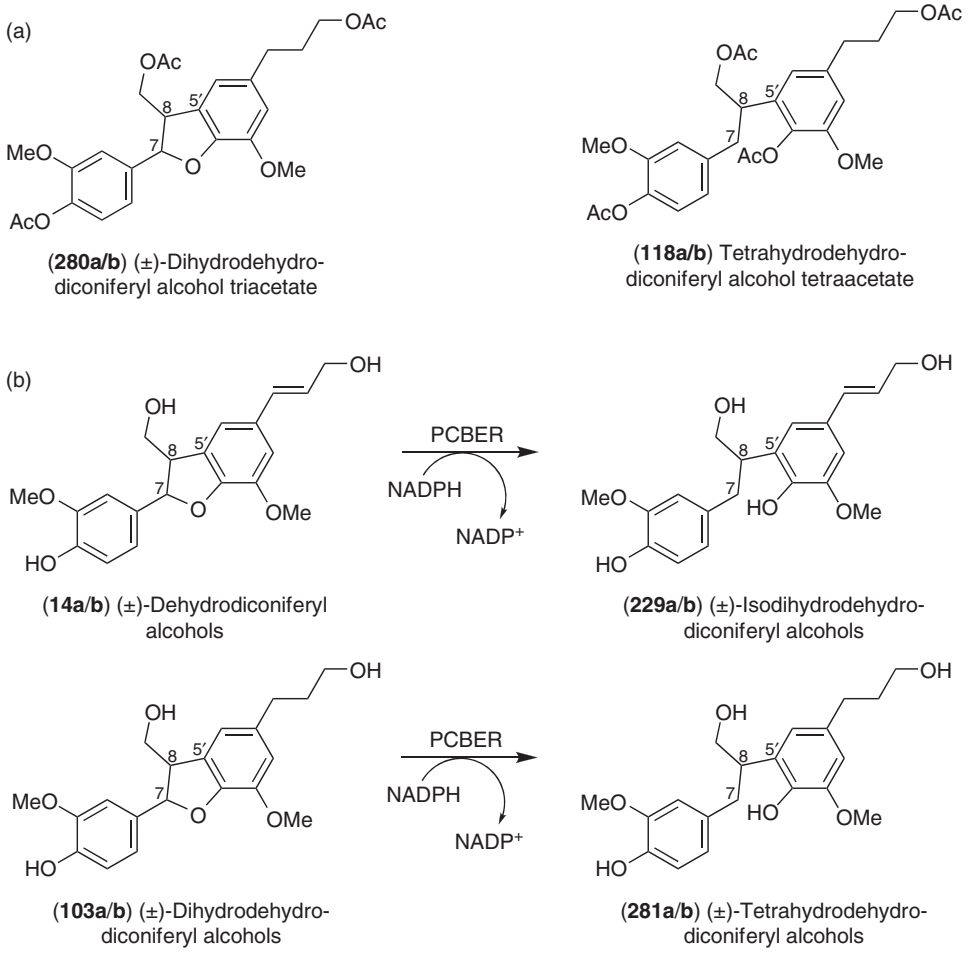


Figure 47 (a) (±)-Dihydrodehydrodiconiferyl alcohol triacetate (**280a/b**) and (±)-tetrahydrodehydrodiconiferyl alcohol tetraacetates (**118a/b**) from *Cryptomeria japonica* needles. (b) Reactions catalyzed by *Pinus taeda* phenylcoumaran benzylic ether reductase, PCBER.

catalysis detected, and was established to be a type A reductase (as for PLR and IFR). Interestingly, the recombinant PCBER was able to reduce both enantiomers of the respective substrates tested into their racemic products, thereby differing from PLR in terms of being regioselective, but not enantiospecific.[265] Two homologs (*PCBER_Cj79* and *PCBER_Cj80*) were also obtained from a *C. japonica* leaf cDNA library using *PCBER_Pt1* as a probe, these having ~83 and 87% similarity and ~72 and 80% identity to PCBER_Pt1, respectively (H. Kasahara, unpublished results).

While the recombinant PCBER product formation matched that present in *P. taeda* cell suspension cultures and *C. japonica*, the kinetic parameters obtained made the physiological function or functions tentative as repeatedly emphasized.[8,265] That is, kinetic parameters using (±)-dehydrodiconiferyl alcohols (**14a**/**b**) and (±)-dihydrodehydrodiconiferyl alcohols (**103a**/**b**) as potential substrates were very low for PCBER_Pt1 with K_m, V_{max}, and k_{cat}/K_m values of 0.61/1.95 mmol l^{-1}, 0.029/0.016 pkat μg^{-1} protein, and 1.59/0.27 $mol^{-1}\,l\,s^{-1}$ respectively. PLR substrates, (±)-pinoresinols (**13a**/**b**), however, were not efficiently processed by PCBER from either the *P. taeda* source or when using another PCBER homolog from *Populus trichocarpa*.[265]

1.23.7.2.1 Tissue localization

Localization of the putatively annotated PCBER was carried out at the tissue and cellular levels using polyclonal antibodies raised against both the *P. taeda*[291] and *P. trichocarpa*[324] enzymes. In *P. taeda* seedlings, it was apparently localized in the vascular cambium/differentiating secondary xylem regions of young developing shoots (tip of seedlings, **Figure 48(a)**), and in fully differentiated axial and radial parenchyma cells. It was also detected in the vascular cambium of mature woody stems (base of seedling, **Figure 48(b)**).[291] In *P. trichocarpa*, the putative PCBER was also localized in differentiating xylem, in young differentiating fibers and ray parenchyma cells both in juvenile (greenhouse-grown poplar) and mature (field-grown tree) wood.[324] Interestingly, it was considered to be the most abundant protein in *P. trichocarpa* secondary xylem, as demonstrated by two-dimensional gel electrophoresis.[325]

These data were thus again provisionally consistent with a role for the putatively annotated PCBER in lignan deposition, although other possibilities could not be ruled out. As repeatedly emphasized,[8,265] other substrates and functions for PCBERs also need to be considered. In this respect, there is the possibility that PCBERs might have multiple catalytic functions (see Section 1.23.9).

1.23.7.2.2 Structural biology studies of PLR homologs: PCBER, IFR, and pterocarpan reductases

Availability of recombinant PCBER provided an opportunity to consider its catalytic mechanism, as well as that of the related IFR. A PCBER_Pt1 crystal structure was thus obtained in apo-form (at 2.2 Å resolution)

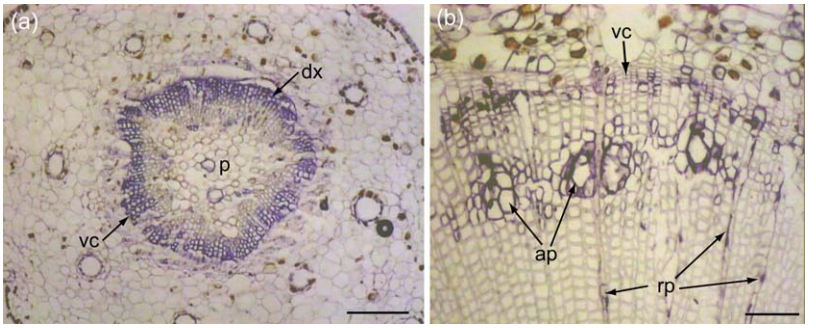


Figure 48 PCBER localization in *P. taeda* (1-year-old) seedlings. (a) PCBER in developing xylem regions of young green shoots. (b) PCBER was mainly present in axial and radial parenchyma cells, as well as in vascular cambium regions of basal stem sections. Abbreviations: ap, axial parenchyma; dx, developing xylem; p, pith; rp, radial parenchyma; vc, vascular cambium. Bars: 70 μm (a) and 400 μm (b). Reproduced from M. Kwon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2001**, *57*, 899–914. Copyright 2001, with permission from Elsevier.

(Figure 30(b)), and not unexpectedly was similar to those of PLR_Tp2 and PLR_Tp1 (Figure 30(a)): PCBER_Pt1 also contained two domains separated by a cleft, with a conserved nucleotide cofactor-binding Rossman fold motif. As before, the highly conserved Lys134 residue was present in the active site, indicative of its conserved role during catalysis and the intermediacy of a presumed quinone methide (Figure 31(b)).

Another related protein, the *Medicago sativa* isoflavone reductase (IFR_Ms1) had high sequence homology to both PLR_Tp1 and PCBER_Pt1 (67/72% similarity and 44/55% identity). X-ray crystal structures from both were used to model, *in silico*, the 3D IFR_Ms1 structure.[266] Interestingly, energy minimization did not lead to significant changes from initial structural coordinates, with the extra residues of IFR falling into disordered loop regions. The structural features for PLR_Tp1 and PCBER_Pt1 were also evident in IFR_Ms1, for example, a Rossman fold motif, as well as the conserved Lys144 residue in the active site. The X-ray structure has also been confirmed by others (Figure 30(c)).[326]

The proposed IFR mechanism does not, however, involve a quinone methide intermediate via abstraction of a phenolic proton (as with PLR and PCBER). On the other hand, the conjugated enone system in the substrate (e.g., **230**) can undergo hydride addition, presumably to give an enol product, which then affords the more stable ketone tautomer (**231**) in solution (Figure 31(c)).

Interestingly, however, the isoflavonoid (−)-medicarpin (**232b**) can undergo a NADPH-dependent reduction to afford (−)-vestitol (**233b**) (Figure 31(d)). This reaction is catalyzed by pterocarpan reductase (PTR), and is considered to proceed via a quinone methide intermediate (Figure 31(d)).[327] All of the above reductases (PLRs, PCBERs, IFRs, and PTRs), together with leucoanthocyanidin reductases (LACRs), apparently form a phylogenetically and structurally related family of enzymes.

1.23.8 Norlignan Biosynthesis

While C8-decarboxylated norlignans, such as compounds **82**–**87** (Section 1.23.3.2.1), are found in bryophytes, most norlignans reported so far are from gymnosperms and angiosperms. For example, the gymnosperm *C. japonica* accumulates various 8–7′-linked norlignans, such as (*E*)-hinokiresinol (**123**), agatharesinol (**121**), cryptoresinol (**122**), and sequirin B (**124**).[142] The angiosperm asparagus, *Asparagus officinalis*, on the contrary, biosynthesizes the isomeric (*Z*)-hinokiresinol (**174**), as well as the presumed norlignan derivatives, the acetylenic alkyl–phenyl ethers, asparenydiol (**178**) and its *O*-methylated derivatives, asparenyol (**179**) and asparenyn (**180**).[207,328]

1.23.8.1 Hinokiresinol: Discovery of Biochemical Pathway, Encoding Genes, and Enzymes

The biosynthesis of (*E*/*Z*)-hinokiresinols (**123**/**174**) is now quite well understood, beginning with findings from radiolabeled precursor experiments using cell-free protein extracts from both *A. officinalis* and *C. japonica*.[168,207,329,330] Initially, the hinokiresinol isomers (**123**/**174**) were considered derived from two non-identical phenylpropanoid moieties, *p*-coumaryl alcohol (**199**) and a *p*-coumaroyl derivative (i.e., CoA ester **282**),[329,330] with this later being extended to *p*-coumaryl coumarate (**235**) as the presumed precursor.[168] Following consideration of possible biosynthetic mechanisms from **235** to (*Z*)-hinokiresinol (**174**) (Figure 49) initially from our laboratory[2] and then the Umezawa laboratory,[207] it was envisaged that a somewhat analogous process to allyl-/propenylphenols might be occurring (see Section 1.23.9.1).

Following initial precursor administration experiments, hinokiresinol synthase (HRS) was later purified from *A. officinalis* elicitor-treated cells.[207] After a six-step column chromatographic protocol, native PAGE suggested that it had been purified to apparent homogeneity, although SDS–PAGE gave two bands at ∼21 and 23 kDa, respectively. Peptidase treatment, followed by HPLC purification and amino acid sequencing of the fragments obtained, suggested that both proteins were similar, these being named HRSα and HRSβ.

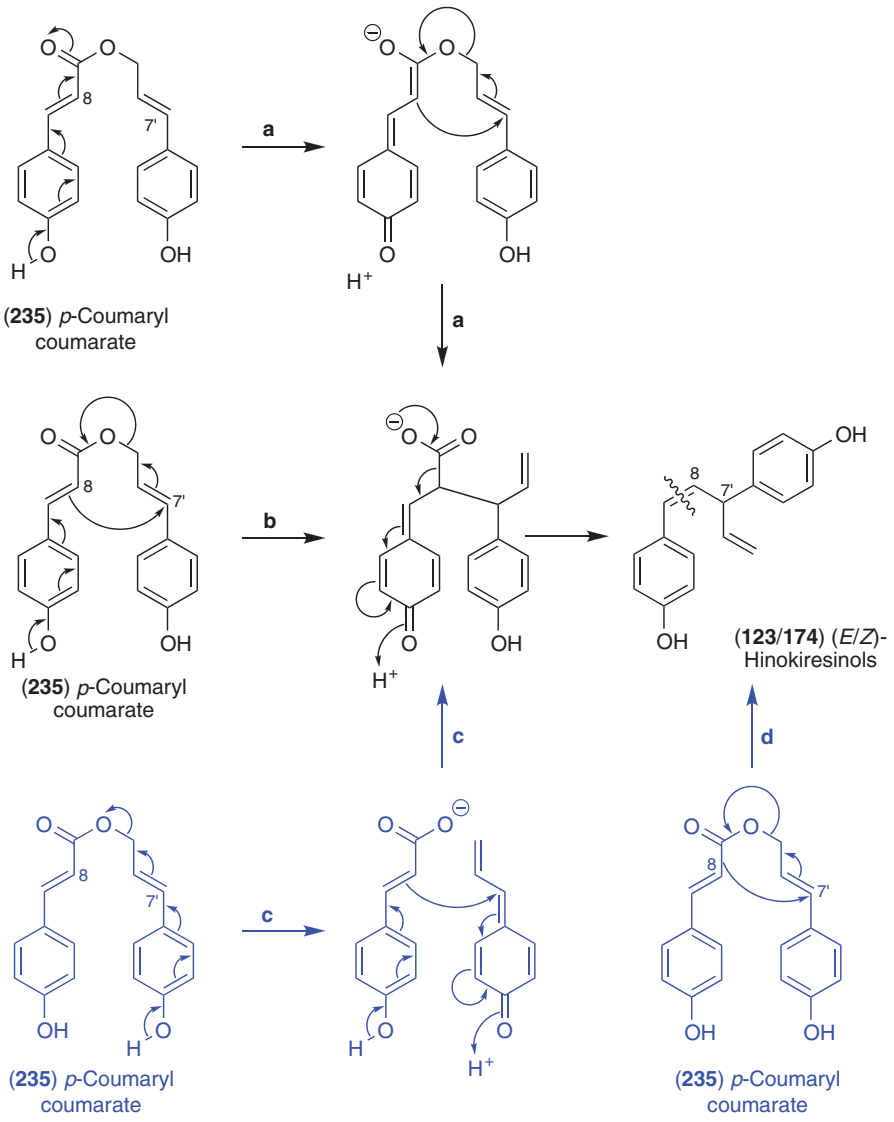
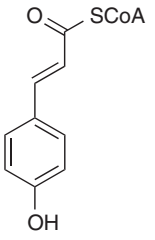


Figure 49 Proposed mechanisms for the formation of (*E*/*Z*)-hinokiresinols (**123**/**174**) from *p*-coumaryl coumarate (**235**) (Vassão *et al.*[2] in blue, Suzuki *et al*.[207] in black). Both (a) ester enolate Claisen rearrangement[207] and (b) two-step intramolecular rearrangement[207] involve the formation of a quinone methide in the *p*-coumarate moiety of the substrate, while route (**c**) involves ester link breakage with the formation of a quinone methide in the *p*-coumaryl (alcohol) moiety, followed by bimolecular coupling[2] to give the same *p*-coumarate quinone methide intermediate as (a) and (b). In all the three (a–c) proposed mechanisms, the *p*-coumarate quinone methide (center) undergoes decarboxylation with re-aromatization to give either (*E*/*Z*)-hinokiresinols (**123**/**174**). Route (d) involves direct intramolecular rearrangement, with concerted decarboxylation and C7′–C8 bond formation without intermediacy of a quinone methide (i.e., no participation of the phenolic rings).[2]



(**282**) *p*-Coumaroyl CoA

The corresponding sequenced fragments were then used to obtain full-length cDNAs, with these encoding ~20.4- and 19.8-kDa proteins for HRSα and HRSβ, respectively. *In silico* searches indicated that several homologs in literature databases were annotated as either phloem protein 2 (PP2) or PP2-like, but with no known physiological/biochemical function(s). Nevertheless, these form a gene superfamily of which HRSs is part of a clade: HRSα and HRSβ were also ~49% identical to each other based on amino acid sequence, and the corresponding fully functional recombinant proteins were expressed in *E. coli* as His-tag proteins.

Interestingly, recombinant HRSα and HRSβ when individually assayed with *p*-coumaryl coumarate (**235**), afforded (*E*)-hinokiresinol (**123**). By contrast, *A. officinalis* accumulated the corresponding *Z*-isomer **174**, and the native HRS preparation also catalyzed the formation of this isomer as well. The (*Z*)-isomer was, however, produced when both HRSα and HRSβ isoforms were assayed together in an 1:1 ratio. These data, together with gel filtration analysis, suggested that *A. officinalis* HRS was a heterodimer of HRSα and HRSβ. Kinetic parameters for the HRS heterodimer gave an apparent K_m of 0.44 μmol l^{-1}, a V_{max} of ~0.075 pkat μg^{-1} protein, a k_{cat} of ~1.5×10^{-3} s^{-1}, and a k_{cat}/K_m of ~3400 mol l^{-1} s^{-1} for *p*-coumaryl coumarate (**235**) (considering one active site per monomer). The kinetic data were thus also consistent with those of monolignol-related pathway enzymes as indicated earlier.

From a mechanistic perspective, HRS catalysis (**Figure 49**) involves decarboxylation and 8–7′ bond formation, and requires no additional cofactors. Four mechanisms have now been proposed as possible: an ester enolate Claisen rearrangement (**Figure 49(a)**); either a concerted (**Figure 49(d)**) or a two-step (**Figure 49(b)**) molecular rearrangement; and/or an ester cleavage followed by bimolecular coupling (**Figure 49(c)**).[2,207] In three of these mechanisms (**Figures 49(a)**–**49(c)**), a putative quinone methide intermediate is considered to be generated.

1.23.8.2 Agatharesinol

Agatharesinol (**121**) is a norlignan closely related structurally to (*E*)-hinokiresinol (**123**), differing only by the addition of two hydroxyl groups to the pendant olefinic moiety. It is constitutively found in *C. japonica* heartwood, but its formation can also be induced in sapwood tissue, since sapwood from freshly felled trees, when allowed to stand at room temperature, slowly generates agatharesinol (**121**).

Somewhat surprisingly, agatharesinol (**121**) biosynthesis has been reported as not involving (*E*)-hinokiresinol (**123**) as an intermediate,[331] although this was not actually proven in these studies. Using the norlignan-inducible sapwood tissue, administration of [ring-^{2}H]-Phe (**2**), [ring-^{13}C]-(*E*)-cinnamic acid (**249**), [9-$^2H_2$]-*p*-coumaryl alcohol (**199**), and [9-$^2H_2$]-(*E*)-hinokiresinol (**123**) gave somewhat conflicting results.[331,332] While [ring-^{2}H]-Phe (**2**) and [ring-^{13}C]-(*E*)-cinnamic acid (**249**) were intactly incorporated into agatharesinol (**121**), [9-$^2H_2$]-*p*-coumaryl alcohol (**199**) was only converted into [9′-$^2H_2$]-(*E*)-hinokiresinol (**123**) but not agatharesinol (**121**). On the other hand, uptake of [7-^{2}H]-(*E*)-hinokiresinol (**123**) did not afford deuterium-labeled agatharesinol (**121**), prompting these researchers to conclude that a different biosynthetic pathway to (*E*)-hinokiresinol (**123**) must be operative.

This conclusion cannot, however, be made from such data: For example, both [9-$^2H_2$]-*p*-coumaryl alcohol (**199**) and [7-^{2}H]-(*E*)-hinokiresinol (**123**) may simply not have been efficiently translocated to the site(s) of agatharesinol (**121**) biosynthesis, whereas both [ring-^{2}H]-Phe (**2**) and [ring-^{13}C]-(*E*)-cinnamic acid (**249**) were.

Interestingly, immunolocalization of agatharesinol (**121**) was examined in *C. japonica* heartwood tissue,[333] using bovine serum albumin-linked agatharesinol (**121**) to raise polyclonal antibodies from rabbits.[334] The antiserum so obtained detected agatharesinol (**121**) in ray parenchyma cells of heartwood, as well as in inner walls of some of the adjacent tracheid cells[333] (**Figure 50**). This finding was the first immunolocalization study of a heartwood (norlignan) compound, even though it is well known that lignans accumulate in such cell types.

1.23.8.3 Acetylenic Norlignans

The biosynthetic pathways to the more unusual acetylenic norlignans found in *A. officinalis* and *Hypoxis* spp. are less well understood, although preliminary radiolabeling precursor studies have been performed. *Hypoxis* corms

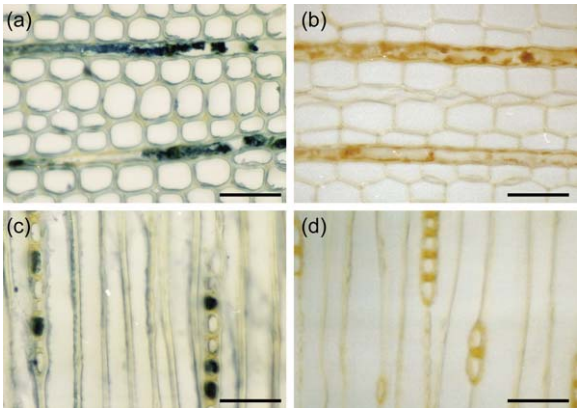


Figure 50 Immunolocalization of agatharesinol (**121**) in *Cryptomeria japonica*. (a), (b) Cross-section of heartwood tissue treated with (a) antiagatharesinol antiserum and (b) pre-immune serum. (c) (d) Tangential section of heartwood tissue treated with (c) antiagatharesinol antiserum and (d) pre-immune serum. Reproduced from T. Nagasaki; S. Yasuda; T. Imai, *Phytochemistry* **2002**, *60*, 461–466. Copyright 2002, with permission from Elsevier.

were established to be the main sites of hypoxoside (**182**) biosynthesis[335] with whole plants able to incorporate radiolabeled Phe (**2**), cinnamic acid (**249**), *p*-coumaric acid (**250**), caffeic acid (**56**), and acetate into hypoxoside (**182**) – albeit with different efficacies.[336,337] Detailed incorporation studies with stable isotope-labeled precursors in *A. officinalis* also established that both aromatic rings (as well as the butyne moiety) of asparenyn (**180**), asparenyol (**179**), and asparenydiol (**178**) were shikimic acid-derived through Phe (**2**).[211,338,339] A preliminary biosynthetic sequence for their formation was proposed (but without experimental confirmation) to involve a hinokiresinol (**123**/**174**)-like skeleton and a spiro intermediate.[211] To date, no enzymes or genes have yet been reported for their formation.

1.23.9 Allyl-/Propenylphenol Biosynthesis

As indicated earlier (Section 1.23.3.1), allyl-/propenylphenol biosynthetic pathways are present in a wide range of plant species from algae (at least provisionally), to the hornworts and liverworts, to the ferns, gymnosperms, and angiosperms. The apparently sporadic distribution of their occurrence, however, raises the possibility that their biochemical pathway or pathways may have evolved independently at different times. This section now addresses deduction of the biochemical pathway(s) to this most interesting class of monomeric – and frequently dimeric – natural products, and some of the twists and turns that occurred.

As previously discussed, one potential complication, at least from a chemotaxonomical perspective, is that monomeric allyl-/propenylphenols are often found in near trace amounts. Indeed, such occurrences are generally only reported in tabular form as very minor constituents of complex mixtures of terpenoids and the like. This is a complication because such reports are currently more difficult to trace in the literature when documented in this way. Nevertheless, the recent discovery of their biochemical pathways raises another interesting issue. This is in terms of some of the enzymes involved being rather substrate versatile, and thus perhaps able to participate in apparently unrelated (multiple) biochemical pathways. This potentially adds yet another level of complexity to this branch of plant metabolism.

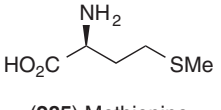
1.23.9.1 Deduction of Allyl-/Propenylphenol (Monomeric and Dimeric) Biosynthetic Pathways

Over the past few years, the biosynthetic route leading to allyl-/propenylphenols *in planta* has been fully elucidated.[2–5] This settled a question that had remained unresolved for more or less five decades of enquiry, including whether the C9 (terminal) carbon was lost or not.

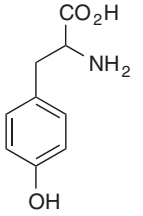
Interest in allyl/propenyl biosynthetic pathways can be traced back to the chemical studies by Birch and Slaytor in 1956.[340] These researchers very briefly reported the chemical conversion of 3,4-methylenedioxycinnamyl alcohol (**283**) into a mixture of safrole (**22**) and isosafrole (**24**) (~60% yield in an ~55:45 ratio), by action of $LiAlH_4$ in boiling ether in the presence of $AlCl_3$ (Lewis acid) (**Figure 51(a)**). This conversion was presumed to occur via generation of a resonance-stabilized allylic carbocation intermediate following loss of the terminal hydroxyl group, with concomitant reduction via hydride addition. A similar conversion did not occur, however, with cinnamyl alcohol (**284**, **Figure 51(b)**), suggesting to these researchers that a *para*-oxygenated aromatic ring moiety (although not necessarily a free phenol) was needed for the displacement of the terminal hydroxyl group. A somewhat analogous generation of a resonance-stabilized carbocation intermediate was thus initially postulated for the corresponding biosynthetic processes.

1.23.9.1.1 Radiolabel tracer studies: controversy over intact incorporation of monolignol pathway intermediates and scientific judgment?

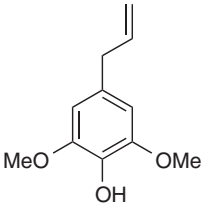
The first biosynthetic studies toward gaining an understanding of allyl-/propenylphenol biosynthesis were reported in the early 1960s, with Kaneko[341–344] demonstrating, via a combination of radiolabeling precursor administration and chemical degradation approaches, that [2-^{14}C]-Phe (**2**), [9-^{14}C]-*p*-coumaric acid (**250**), and [Me-^{14}C]-Met (**285**) were incorporated into anethole (**5**) in fennel (*F. vulgare*). Further demonstration of phenylpropanoid pathway intermediates being involved was also reported by others in banana[345] and basil,[346] respectively. Specifically, [9-^{14}C]-caffeic acid (**56**) and [1-^{14}C]-Phe (**2**) were incorporated into eugenol (**4**), methyleugenol (**42**), 5-methoxyeugenol (**287**), and elemicin (**45**) in banana disks. Administration of double-labeled [9-^{14}C, OC^{3}H$_3$]-ferulic acid glucoside (**288**), as well as [9-^{14}C, OC^{3}H$_3$]- and [9-^{14}C, ^{3}H$_2$OH]-coniferyl alcohol glucoside (**289**) in basil also gave ^{3}H-/^{14}C-labeled eugenol (**4**) and methyleugenol (**42**) with only minor changes in ^{3}H:^{14}C ratios between administered substrates and products. Confirmation of the C9 label in the allylphenols **4** and **42** was determined by degradation studies. These data were thus consistent with retention of the intact phenylpropanoid skeleton into allyl-/propenylphenols.



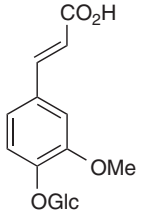
(**285**) Methionine



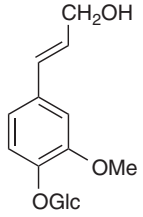
(**286**) Tyrosine



(**287**) 5-Methoxyeugenol



(**288**) Ferulic acid glucoside



(**289**) Coniferyl alcohol glucoside

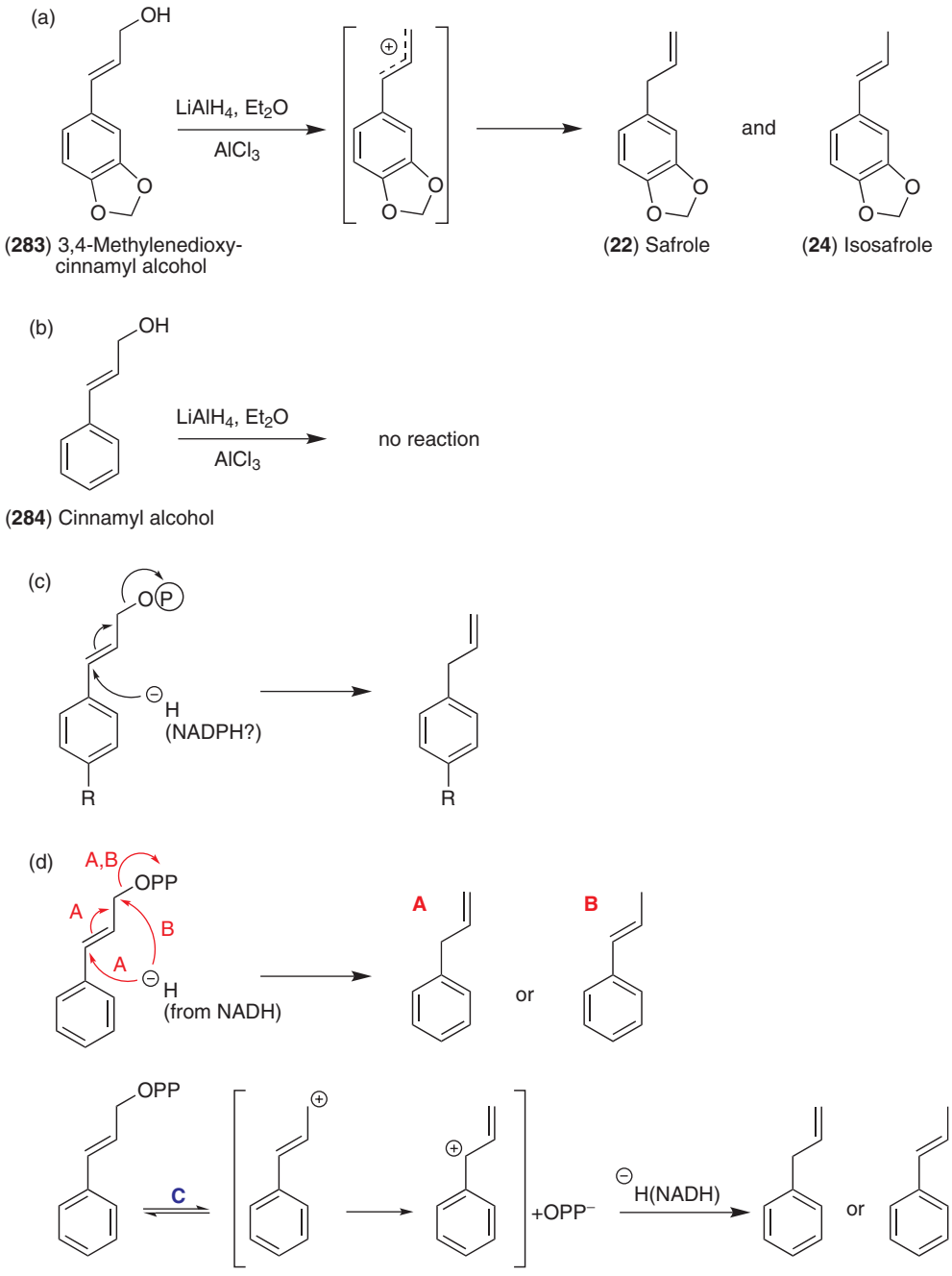


Figure 51 Proposed chemical and biochemical mechanisms leading to allyl-/propenylphenols. (a), (b) Chemical formation of (a) safrole (**22**) and isosafrole (**24**) from 3,4-methylenedioxycinnamyl alcohol (**283**), in presence of $LiAlH_4$ and $AlCl_3$, and (b) lack of reaction when cinnamyl alcohol (**284**) was used, as reported by Birch.[340] (c) Birch's refined biochemical mechanism for the formation of allylphenols (R = not specified), with nucleophilic (allylic) displacement of a phosphorylated group by H^- (presumed derived from NADPH).[347] (d) Biochemical mechanisms proposed by Geissman and Crout,[348] via either allylic (A) or direct (B) nucleophilic displacement of pyrophosphate by H^- (from NADH) to afford the corresponding allyl- or propenylphenol, or (C) through a S_N1 mechanism and delocalized carbocation intermediate with hydride reduction.

In further postulates to account for allyl/propenyl formation, Birch in 1963[347] next proposed a mechanistic rationale for displacement of the terminal hydroxyl group (**Figure 51(c)**). Based partly on comparison with other metabolic pathways, the terminal hydroxyl group was now anticipated to require activation for more

facile displacement. This consideration was based on parallels to terpenoid biochemistry, that is, involving the formation and metabolism of isopentenyl pyrophosphates. Accordingly, a monolignol-derived phosphate ester derivative was envisaged, with allylic addition of a hydride leading to displacement thereby generating the corresponding allylphenol. Geissman and Crout[348] also later proposed a nucleophilic addition (of a hydride from NADH) to a (hydroxy)cinnamyl pyrophosphate, via either direct or allylic displacement of the pyrophosphate group, or alternatively via an S_N1 mechanism with the formation and reduction of a delocalized allylic carbocation (**Figure 51(d)**).

Conversely, there were also various accounts of C9 carbon loss occurring during the formation of eugenol (**4**) in both basil[349,350] and cinnamon (*Cinnamon zeylanicum*).[351] That is, during the 1970s, administration of double-labeled Phe (**2**) (nonspecifically tritiated, as well as containing either 1-, 2-, or 3-^{14}C) was carried out using basil, with the corresponding radiolabeled forms of eugenol (**4**) individually isolated. A much higher ^{3}H:^{14}C radiolabel ratio (~20 times than that of the ^{3}H:^{14}C precursor) was reported for eugenol (**4**) when the ^{14}C label was in the carboxylic acid group of Phe (**2**). This suggested the loss of the terminal C9 carbon during metabolism of Phe (**2**) into eugenol (**4**). Additionally, in cinnamon, the radiolabel from [1-^{14}C]-Phe (**2**) was reportedly poorly incorporated into eugenol (**4**, 0.003%), relative to marginally better incorporations with either [2-^{14}C]- or [3-^{14}C]-Phe (**2**) (0.047 and 0.098%, respectively). [Me-^{14}C]-Met (**285**) also allegedly led to eugenol (**4**, 0.1% incorporation) radiolabeled at the C9 position (based on chemical degradation), suggesting a methionine (**285**) origin for this particular carbon.

It must be emphasized that most of these radiotracer studies generally suffered from low incorporations, at best 0.5% and most often much less than 0.1%. Such low incorporation data (i.e., <0.1%) are, however, generally suspect. Nevertheless, in spite of the rather flimsy radiochemical data in support of C9 loss, this pathway was still being considered by some researchers as late as 2001,[352] that is, whereby an additional carbon (presumed to be derived from *S*-adenosyl methionine, SAM) was considered covalently attached to a *p*-coumaric acid (**250**) moiety to form cyclopropyl intermediate **290** (**Figure 52**). This putative intermediate was then envisaged to undergo C9 decarboxylation with ring opening to directly afford the allyl/propenyl side chains of chavicol (**20**) and *p*-anol (**21**). This, however, seemed an unlikely possibility from a biochemical standpoint. Indeed, the very weak evidence for C9 loss can be considered as a question of scientific judgment, when compared to the evidence for intact incorporation.

1.23.9.1.2 Intermediacy of monolignol esters in allyl-/propenylphenol biosynthesis: clues from norlignans?

Our long-term interests in allyl-/propenylphenol biosynthesis encompass how the formation of both the monomeric and dimeric natural products occurs. Accordingly, particular attention was given to plant species such as basil, creosote bush, and various *Piper* spp. These distinct plant species can differentially harbor allyl-/propenylphenols in either monomeric, dimeric, or both forms, and thus provide the means to establish any parallels and differences for biosynthesis of the monomers and dimers. The biosynthetic pathway findings made to date are summarized below, beginning with basil, which typically accumulates the monomeric derivatives, methylchavicol (**3**) and eugenol (**4**).

The Thai basil variety was chosen for biosynthetic studies as it largely accumulates methylchavicol (**3**) in its glandular trichomes;[2] basil is not known, however, to accumulate any lignans in those cell types. Various labeled

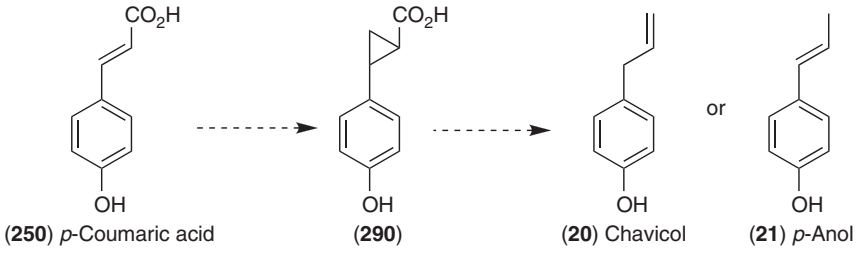


Figure 52 Putative biochemical pathway to chavicol (**20**)/*p*-anol (**21**) from *p*-coumaric acid (**250**) via the unusual cyclopropyl intermediate (**290**) by Gang *et al*.[352] (No evidence for pathway exists.)

precursor administration/incorporation experiments were thus performed using young apical basil leaves,[2] which we had established to be the most biosynthetically active in terms of producing methylchavicol (**3**). Specifically, [U-^{14}C]-Phe (**2**), [8-^{14}C]-cinnamic acid (**249**), and [9-^{3}H]-*p*-coumaryl alcohol (**199**), but not [U-^{14}C]-Tyr (**286**), were metabolized into methylchavicol (**3**) *in vivo*, albeit to differing levels of efficacy (~1–4% by 24 h). These data thus again eliminated the notion of a need for C9 loss, such as via a cyclopropyl intermediate (**290**, **Figure 52**), from further consideration as had been previously proposed.[352] Using cell-free extracts, however, *p*-coumaryl alcohol (**199**) (either unlabeled or [9-^{3}H]-labeled) was not directly converted to afford chavicol (**20**) *in vitro*, suggesting that additional enzymatic steps and/or then unknown cofactors were needed.

Three routes for *p*-coumaryl alcohol (**199**) transformation were next concurrently considered as being possibly viable (**Figure 53**): (a) side-chain double bond reduction initially to afford dihydro-*p*-coumaryl alcohol (**291**) (i.e., as for the double bond reductase described above in Section 1.23.7.1), followed by dehydration; (b) *O*-methylation of the phenolic group to afford *p*-methoxyl cinnamyl alcohol (**292**), with this possibly preceding deoxygenation (as proposed in Kaneko[344]); and (c) activation of the side-chain hydroxyl group to afford a *p*-coumaryl alcohol (**199**) derivative bearing a better leaving group. Routes a and b were eliminated following *in vivo* precursor administration experiments, as well as using *in vitro* incubations with crude cell-free enzyme preparations.[2]

Potentially activated forms of the monolignol side-chain hydroxyl group were next examined using cell-free extracts *in vitro*, for example, by coincubation of monolignols with ATP, GTP, UTP, Glc-6-phosphate, and/or glutathione, but with no success. At about the same time, however, the monolignol ester *p*-coumaryl coumarate (**235**) was demonstrated to serve as substrate in norlignan biosynthesis, to afford *E*- and *Z*-hinokiresinols (**123** and **174**) in *A. officinalis* and *C. japonica*,[168,329,330] respectively (Section 1.23.8.1). Although this conversion apparently took place in the absence of any external cofactor, no biochemical/organic chemical mechanism was proposed. Therefore, a possible mechanism[2] was considered (**Figure 54(a)**) to explain the formation of **123**/**174**, which, in turn, led to the formulation of another mechanism for allyl-/propenylphenol production (**Figure 54(b)**).

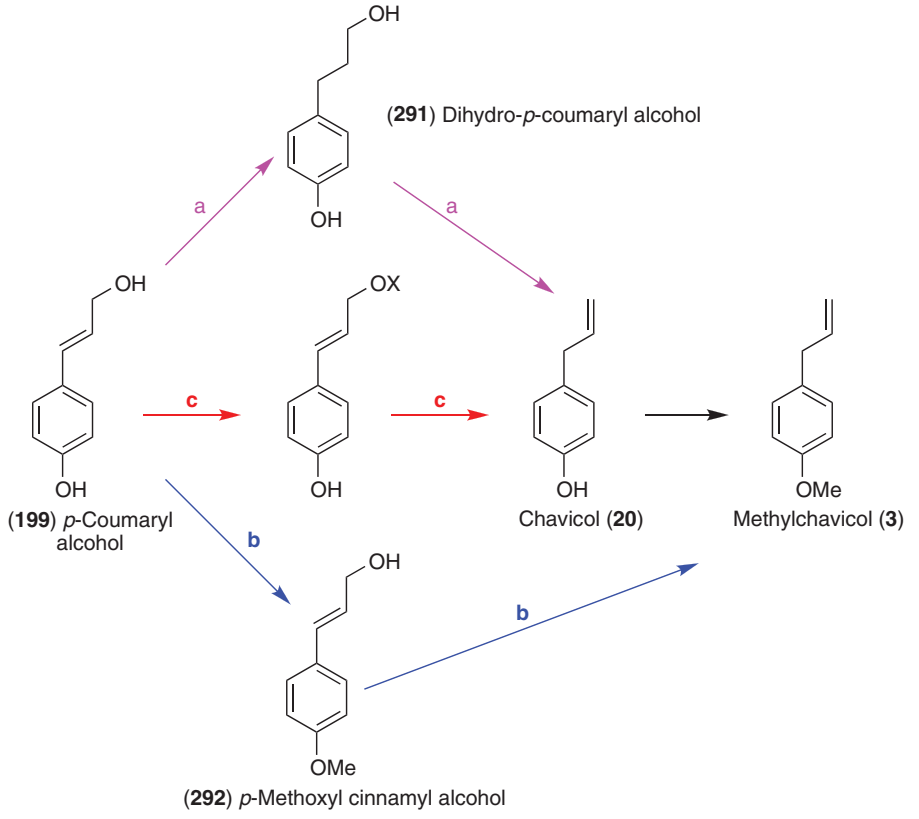


Figure 53 Possible biosynthetic pathways to chavicol (**20**) and methylchavicol (**3**). (X = facile leaving group.)

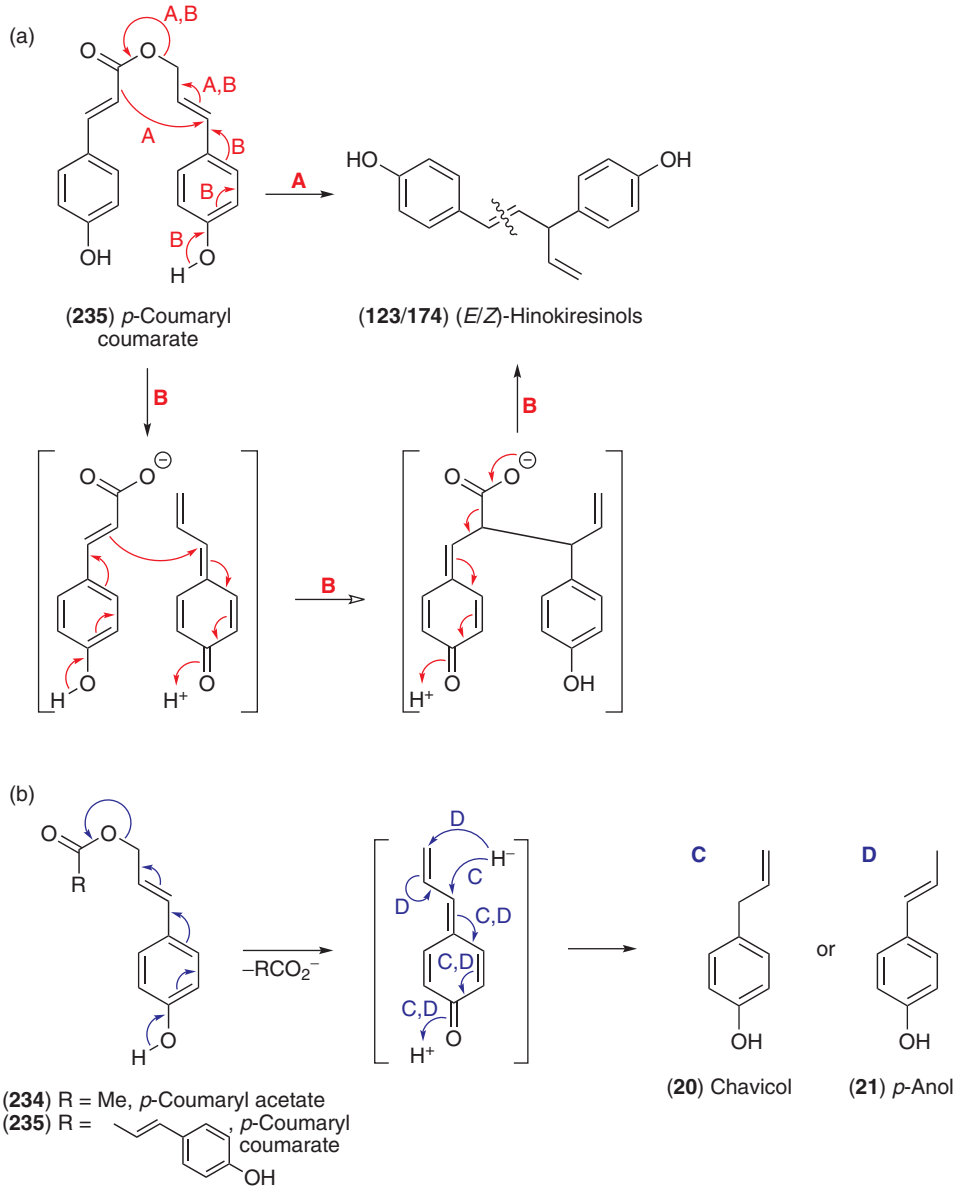


Figure 54 Possible mechanisms for conversion of *p*-coumaryl esters into (a) (*E*/*Z*)-hinokiresinols (**123**/**174**) and (b) chavicol (**20**)/*p*-anol (**21**). (A) Concerted; (B) ester cleavage, followed by cyclization, decarboxylation, and rearomatization; (C) and (D) ester displacement, putative quinone methide formation with subsequent reduction by hydride (from NAD(P)H), and rearomatization to form (C) chavicol (**20**) and (D) *p*-anol (**21**). In (C) and (D), the acid moiety may be interchangeable.

That is, hydride addition to a putative activated monolignol (e.g., *p*-coumaryl coumarate (**235**)) bound in the enzyme active site could potentially lead to C9 deoxygenation through ester displacement (e.g., to afford chavicol (**20**) and *p*-anol (**21**), respectively, and/or homologs thereof) (**Figure 54(b)**). In this way, the (*p*-coumarate) ester functionality might, therefore, serve as a good leaving group, perhaps being displaced by an incoming hydride through nucleophilic addition directly at either C7 or C9, or through an intermediary quinone methide, as for PLR (**Figure 31(a)**), to form the isomeric allyl-/propenylphenols. Such a process would, however, also require a reductive step, for example, with NADH/NADPH as cofactor.

Crude cell-free protein extracts from basil glandular trichomes were thus next demonstrated to be able to convert *p*-coumaryl coumarate (**235**) into chavicol (**20**) *in vitro*, in the presence of either NADPH or NADH.[2]

Additional experiments[3] (Vassão, unpublished results) established that acetylated derivatives (*p*-coumaryl acetate (**234**), coniferyl acetate (**236**)) were also utilized as substrates to afford chavicol (**20**) and eugenol (**4**), respectively, that is, thereby demonstrating, at a minimum, the substrate versatile nature of these transformations. Analogous processes occurred in the creosote bush,[4] as discussed below using the very same substrates (**234**–**236**) (**Figure 31**). These data, therefore, resolved the nature of the biochemical pathway to this class of molecules. Taken together, the findings demonstrated that allyl-/propenylphenol formation occurred through a two-step process: Monolignol activation by acylation, and ester displacement by means of an NAD(P)H-dependent conversion, presumably involves quinone methide intermediacy, as for PLR, PCBER, and so on.[2] The studies did not, however, unambiguously clarify the precise chemical identity of the ester-cleaved moiety (leaving group).

1.23.9.2 Allyl-/Propenylphenol Synthases

1.23.9.2.1 Bifunctional chavicol/eugenol and p*-anol/isoeugenol synthases (CES and AIS): The twists and turns to biochemical clarity*

Subsequent identification of genes encoding chavicol/eugenol and *p*-anol/isoeugenol synthases (CES and AIS) was somewhat facilitated by recognition that suppression of a transcription factor (*ODORANT1*) in petunia upregulated expression of a PLR/PCBER/IFR homolog of unknown biochemical function.[353] A full-length sequence for this homolog was obtained from a petunia petal EST collection, and encoded an ~36 kDa peptide with amino acid sequence similarities/identities of ~59/40% to that of PLR_Tp2 from *T. plicata*, ~62/42% to a PLR homolog isolated from the creosote bush in 1999, ~61/41% to the *P. taeda* PCBER, as well as ~59/37% to an IFR from *M. sativa*.[3]

A creosote bush PLR homolog, isolated from a cDNA library obtained from leaves actively producing allyl-/propenylphenol-derived lignans, also encoded a protein of ~35 kDa, but whose biochemical function was not initially unambiguously established when first isolated in 1999. Additionally full-length cDNA for a basil homolog was obtained (based on the petunia PLR/PCBER/IFR homolog above) using data from a basil trichome EST database, and which encoded a 35.6 kDa protein.[3]

Escherichia coli cells were then next transformed to obtain the corresponding petunia and basil recombinant proteins, in addition to that already available for the creosote bush PLR homolog. Each was examined for its ability to afford allyl- or propenylphenols when incubated with either *p*-coumaryl or coniferyl acetate/*p*-coumarate esters (**234**–**236**).[2–4]

1.23.9.2.1(i) Creosote bush chavicol/eugenol synthase (CES) Although this ultimately represented the second open literature report of a functional chavicol/eugenol synthase (CES), for the benefit of the reader, this is described first. As discussed earlier in Section 1.23.5.4, it is of interest that the creosote bush massively accumulates numerous lignans, all 8–8′-linked, such as NDGA (**143**). However, there are no reports of other skeletal types, including 8–5′- or 8–*O*–4′-linked lignans from this species. Nor is there any 'substantial' accumulation of monomeric allyl-/propenylphenols, such as chavicol (**20**)/eugenol (**4**) and *p*-anol (**21**)/isoeugenol (**6**). As indicated earlier though, monomeric allyl-/propenylphenols often occur in trace amounts in different plant species and thus may go long undetected from a chemotaxonomical perspective.

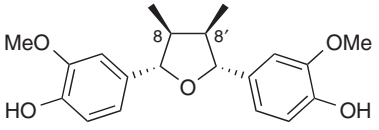
The creosote bush PLR homolog efficiently converted, in the presence of NADH/NADPH, all of the esters assayed to afford either chavicol (**20**) or eugenol (**4**), respectively, and thus became the first substrate versatile chavicol/eugenol synthase (CES) to be characterized. It efficiently converted *p*-coumaryl coumarate (**235**), *p*-coumaryl acetate (**234**), and coniferyl acetate (**236**); apparent K_m values of 210, 350, and 290 $\mu mol\,l^{-1}$, V_{max} values of 75, 200, and 190 $pkat\,\mu g^{-1}$ protein, and k_{cat}/K_m of 12 000, 19 500, and 22 000 $mol^{-1}\,l\,s^{-1}$, were obtained for substrates **235**, **234**, and **236**, respectively (**Table 1**). These data were thus comparable to the catalytic efficacies noted earlier for PLR, and for other monolignol pathway enzymes.

On the contrary, the creosote bush CES showed at best marginal activity toward a number of other potential substrates, including pinoresinol (**13**), larreatricin (**200**), nectandrin B (**293**), licarin A (**88**), dehydrodiconiferyl alcohol (**14**), as well as the isoflavones genistein (**294**) and biochanin A (**295**), as

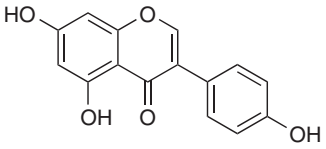
Table 1 Reported kinetic properties of CES and AIS

Enzyme function	*Trivial name*	*Source*	*Substrate*	K_m (μM)	V_{max} (*pkat* μg^{-1} *prot.*)	k_{cat} (s^{-1})	k_{cat}/K_m ($mol^{-1}\ l\ s^{-1}$)
CES	LtCES1[4]	Creosote bush	**234**	350	200	6.8	19 500
CES	LtCES1[4]	Creosote bush	**235**	210	75	2.55	12 000
CES	LtCES1[4]	Creosote bush	**236**	290	190	6.46	22 000
CES	ObEGS1[3]	Basil	**236**	5100	20	0.7	160
CES	CbEGS1[354]	*Clarkia breweri*	**236**	93	7.3	0.26	2 800
CES	CbEGS2[354]	C. *breweri*	**236**	311	6.9	0.25	800
CES	PhEGS1[354]	Petunia	**236**	245	18.4	0.6	2 400
AIS	PhIGS1[3]	Petunia	**236**	1600	7	0.3	136
AIS	PhIGS1[354]	Petunia	**236**	226	35.7	1.3	5 700
AIS	CbIGS1[354]	C. *breweri*	**236**	212	27.6	0.99	4 700
AIS	PaAIS1[356]	Anise	**236**	230	28.2	1.02	4 440
AIS	PaAIS1[356]	Anise	**234**	135	1.9	0.07	520

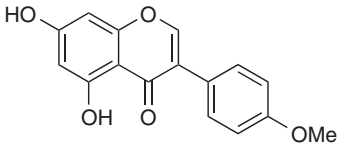
previously reported.[4] These data therefore demonstrated that the creosote bush CES (LtCES1) was unable to function as an efficacious PLR, PCBER, or IFR.[4] Surprisingly, a later report[354] stated that the creosote bush CES had not been assayed for PCBER activity. These researchers[354] were in error with such assertions, as this had been comprehensively examined and described,[4] with compelling evidence obtained only for CES activity (see above).



(**293**) Nectandrin B



(**294**) Genistein



(**295**) Biochanin A

In addition to CES, the creosote bush contains a *p*-anol/isoeugenol synthase, AIS (LtAIS1) with ~63/43% similarity/identity to LtCES1, which is capable of efficiently converting *p*-coumaryl (**234**, **235**)/coniferyl (**236**) esters to afford *p*-anol(**21**)/isoeugenol (**6**), respectively (S.-J. Kim, unpublished results). This demonstrated that this organism harbors genes encoding proteins capable of forming both allyl- and propenylphenols.

1.23.9.2.1(ii) **Piper regnellii** This *Piper* species biosynthesizes both allylphenols (e.g., myristicin (**23**), apiol (**36**), and dillapiol (**38**)), as well as presumed propenylphenol-derived lignans, such as (+)-conocarpan (**16a**), (−)-*epi*-conocarpan (**188b**), and eupomatenoid-6 (**191**).[218] It was, therefore, of interest to also investigate whether this organism was capable of biosynthesizing allyl- and/or propenylphenols in either monomeric or dimeric form, or both. As indicated below, this has also been established: For example, two recombinant *P. regnellii* enzymes, PrCES1 and PrCES2, with ~82/71% similarity/identity to LtCES1, are able to efficiently catalyze monomeric allylphenol formation efficiently *in vitro* (S.-J. Kim, unpublished results), thereby providing the needed insight into the biochemical processes affording each class of metabolites (monomers and dimers) within this organism.

1.23.9.2.1(iii) Basil and petunia chavicol/eugenol and* p*-anol/isoeugenol synthases (CES and IGS): Kinetic parameter inconsistencies The initial report[3] on basil and petunia CES and AIS was unfortunately both ultimately misleading and, in parts, in error. The basil enzyme was mistakenly restricted to being a eugenol synthase (ObEGS1) and the petunia enzyme as an isoeugenol synthase (PhIGS1).[3] This was mistakenly stated even though the earlier basil work[2] had already established that chavicol (**20**) formation could result from the incubation of cell-free extracts with either *p*-coumaryl acetate (**234**) or *p*-coumaryl coumarate (**235**) and either NADPH or NADH. It is more accurate to describe these enzymes as being, (at a minimum, substrate-versatile) as for the creosote bush CES.[4]

More problematic in the original account[3] was the kinetic data reported for recombinant CES/AIS from basil/petunia. That is, the kinetic values obtained for coniferyl acetate (**236**) were indicative of very poor efficacies: These had apparent K_m values of 5.1 and 1.6 mmol l^{-1}, V_{max} of ~20 and 7 pkat μg^{-1} protein, and k_{cat}/K_m of 160 and 136 $mol^{-1}\,l\,s^{-1}$ for the basil (ObEGS1) and petunia (PhIGS1) enzymes, respectively (**Table 1**). The overall k_{cat}/K_m values for **236** were therefore lower by nearly 2 orders of magnitude than those of the creosote bush CES (LtCES1), that is, 22 000 $mol^{-1}\,l\,s^{-1}$ for the latter *versus* 160 and 136 $mol^{-1}\,l\,s^{-1}$ for the basil and petunia enzymes. Although cognizant at the time of the report[3] that the basil/petunia kinetic data were not compelling (relative to PLR, etc.), this was provisionally rationalized due to uncertainty of the true identity of the actual ester form being used *in vivo* (i.e., *p*-coumarate, acetate, or some other ester).

The petunia PhIGS1 kinetic measurements were ultimately corrected by the same researchers[354] upon our prompting (see **Table 1**). The corrected data, without explanation/clarification and embedded within other reports, were now somewhat closer to the creosote bush CES values (i.e., k_{cat}/K_m of 5700 vs 22 000 $mol^{-1}\,l\,s^{-1}$, but still being of lower efficacy by a factor of ~4). This k_{cat}/K_m of 5700 $mol^{-1}\,l\,s^{-1}$ was, however, in stark contrast to the earlier petunia report of ~136 $mol^{-1}\,l\,s^{-1}$. Surprisingly, the corrected petunia data had a K_m of 0.23 mmol l^{-1} (down by a factor of 7) and a V_{max} increased by a factor of ~5. Additionally, the K_m for the basil CES was also corrected to 0.57 mmol l^{-1} (also being reduced by nearly an order of magnitude). Kinetic data for other compounds, such as *p*-coumaryl acetate (**234**) and *p*-coumaryl coumarate (**235**), were apparently not measured, even though the basil CES (ObEGS1), in our hands, was apparently able to utilize both to form chavicol (**20**) with an efficiency somewhat comparable to that toward coniferyl acetate (**236**) (D. G. Vassão, unpublished data). These data thus contradict later claims[355] that the basil CES was of 'limited ability' in terms of being able to use *p*-coumaryl acetate (**234**) as a potential substrate, since no comparable substrate effects were observed in our work.

1.23.9.2.1(iv) Other species Other CES/AIS homologs able to carry out monolignol ester reductions *in vitro* to give either allylphenols or propenylphenols have also been preliminarily characterized in *Clarkia breweri* (CbIGS1, CbEGS1, CbEGS2),[354] petunia (PhEGS1),[354] and anise (*P. anisum*, PaAIS1).[356] Kinetic data for the *Clarkia* CES homologs (the so-called CbEGS1 and CbEGS2) and a putative petunia CES were again lower than those of the creosote bush CES when using coniferyl acetate (**236**) as substrate, that is, with k_{cat}/K_m values of factors from ~5 to more than an order of magnitude lower (**Table 1**). Our nomenclature of CES and AIS[4] has, however, finally begun to be adopted, that is, for the presumed *Pimpinella* AIS (PaAIS1).[356] This protein, though, also has a slow turnover relative to the creosote bush CES (**Table 1**).

1.23.9.2.2 Chemotaxonomy, kinetic properties, and homology comparisons of CES/AIS with PCBER, PLR, IFR (-like) annotations in the plant kingdom: caveats on incomplete analyses

The recent explosion in genomics has often facilitated the correct annotation of gene function, as well as providing some additional insights into the existence of putative homologs (e.g., PLR-like, PCBER-like, etc.), based on sequence comparisons and predictions.

On the contrary, as repeatedly emphasized by ourselves, such database annotations and phylogenetic classifications must be treated with great care and caution as such analyses still represent the application of inexact science. For instance, 17 genes were previously annotated as cinnamyl alcohol dehydrogenase (CAD) and/or CAD-like in The *A. thaliana* Information Resource (TAIR) database. However, a detailed *in silico* analysis established that eight of these had very low homology to *bona fide* CADs, and that they also lacked the Zn catalytic center and Zn-binding signatures of CADs. Of the remaining nine NADPH-dependent proteins,

only six were catalytically competent to reduce the cinnamyl aldehydes **261**, **262**, **264**–**266** to the corresponding alcohols. Of those, only AtCAD4 and AtCAD5 were catalytically the most active,[357] with both having this physiological function *in vivo*.[358] Another example is the Klee *et al.*[359] reinterpretation of the presumed function of a CAD homolog in tobacco (CAD1),[360] which was apparently instead a gene involved in phenylethanol (**296**) biosynthesis, as shown using tomato, etc. In short, many researchers are currently relying too heavily on computationally derived phylogenetic analyses and on homology comparisons, both of which can often be limited in scope and/or give misleading indications.

By contrast, the following are required to establish function: demonstration of comparable *in vitro* enzymatic efficacies relative to the *bona fide* enzyme in question; colocalization of biochemical pathway enzyme and substrates/products *in planta*; demonstration (e.g., by overexpression and/or RNAi inhibition/gene expression suppression) that the pathway in question can be enhanced or suppressed (perhaps involving more than one member of a multigene family). Unfortunately, this is not often done, leading to significant levels of confusion and inaccuracies in the literature, such as in the aforementioned reports.[354,356]

Some additional examples will suffice for the need for circumspection, these being drawn from either CES/AIS and/or PCBER, PLR, and IFR (homolog) comparisons.

1.23.9.2.2(i) **Piper regnellii** This species, as aforementioned, produces both allylphenols and 8–8′-linked phenylpropene-type dimeric lignans, that is, myristicin (**23**), apiol (**36**), and dillapiol (**38**), as well as (+)-conocarpan (**16a**), (−)-*epi*-conocarpan (**188b**), and eupomatenoid-6 (**191**).[218] The *P. regnellii* CES (PrCES1 and PrCES2) amino acid sequences have ∼82% similarity and ∼71% identity to that of the creosote bush CES (LtCES1), and both currently fall within the same clade (**Figure 55**), as do several PCBER and PCBER-like genes from other plant species. This additionally puts into question the true physiological role(s) of PCBER homologs in some of these organisms, as we have repeatedly emphasized.[8,265] However, *P. regnellii* does not contain, to our knowledge, any 8–3′-/8–5′-linked lignans that have undergone 7–*O*–4′ interunit linkage reduction. *P. regnellii* CES1 and CES2 are thus unlikely to have both CES and PCBER functions *in vivo*, and presumably only serve as a CES. This again underscores the limitations of only using phylogenetic analyses to attempt to gauge potential biochemical function(s).

1.23.9.2.2(ii) ***Creosote bush*** This species, as also aforementioned, essentially exclusively accumulates 8–8′-linked lignans, but apparently not others with, for example, 8–5′ linkages, and so on. Interestingly, both CES (LtCES1 and LtCES2) and AIS (LtAIS1) genes are present in this plant, whose corresponding proteins are of ∼63/43% similarity/identity to each other. Their presence, therefore, also provides a biochemical explanation for both allyl- and propenylphenol formation in this species. Additionally, even though both CES genes cluster relatively close to various PCBER/PCBER homologs (**Figure 55**), only marginal (if any) PCBER activity could be detected for LtCES1 under the assay conditions tested.

Interestingly, the creosote bush AIS (LtAIS1) amino acid sequence also has ∼83/68% and ∼78/61% similarity/identity to those of petunia IGS (PhIGS1) and anise AIS (PaAIS1), respectively, and all group together separately as a subclade (**Figure 55**). Segregation as such may, however, not reflect independent evolution, as the creosote bush CES and AIS also fall into different subclades. Many more *bona fide* plant AIS and CES are thus needed to consider evolutionary processes than currently available.

1.23.9.2.2(iii) ***Basil*** This organism produces chavicol (**20**) and eugenol (**4**) in its glandular trichomes. The gene encoding the basil CES (ObEGS1) currently falls into a subclade with the petunia AIS (PhlGS1), as well as the creosote bush AIS (LtAIS1), and the *Clarkia* CES/AIS (CbEGS1 and CblGS1) genes. It segregates, however, from the creosote bush CES (LtCES1 and LtCES2) (**Figure 55**). More information is thus also required before any meaningful explanation can be made with regard to biochemical pathway evolution, that is, whether the evolutionary processes leading to CES activity in different plants were of either convergent or divergent nature.

1.23.9.2.2(iv) **Pinus taeda** Interestingly, according to the phylogenetic depiction in **Figure 55**, the PtPCBER falls into a separate clade from CES and/or AIS gene families. As indicated earlier, however, PtPCBER was isolated from a cell line harboring 8–5′-linked lignans, such as **14**, **103**, and **116**.[147] Although cognizant of its low kinetic parameters, PtPCBER is, nevertheless, expressed in the vascular cambium/ray

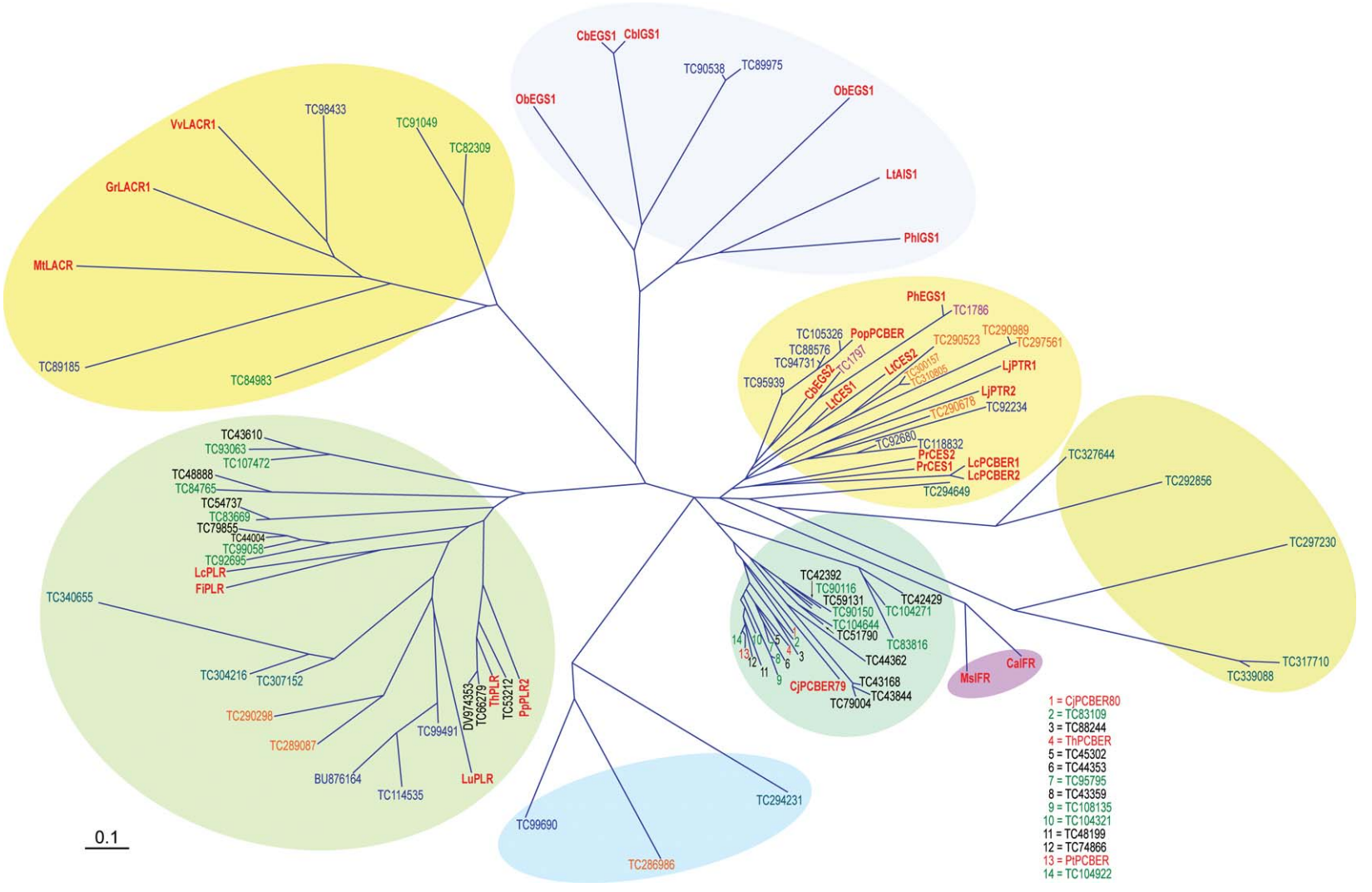


Figure 55 Current phylogenetic dendrogram of tentative consensus sequences of allyl-/prophenylphenol synthases, CES/AIS (*Arabidopsis* (orange), petunia (fushia), rice (light blue), poplar (dark blue), pine (green) and spruce (black)) and *Petunia hybrida* AIS (PhIGS1, DQ372813) and CES (PhEGS1, EF467241), *Ocimum basilicum* CES (ObEGS1, DQ372812), *Clarkia breweri* AIS (CbIGS1, EF467238), CES (CbEGS1 (EF467239) and CbEGS2 (EF467240)), *Larrea tridentata* CES (LtCES1 and LtCES2) and AIS (LtAIS1), and *Piper regnellii* CES (PrCES1 and PrCES2), as well as *Cicer arietinum* isoflavone reductase IFR (CaIFR, Q00016), *Medicago sativa* IFR (MsIFR, CAA1106), *Lotus japonicus* pterocarpan reductase (LjPTR1 and LjPTR2, AB265589 and AB265590), *Gossypium raimondii* leucoantocyanidine reductase (GrLACR, CAI56324), *Medicago truncatula* LACR (MtCAI56327), *Vitis vinifera* LACR (VtCAI26309), *Pinus taeda* PCBER (PtPCBER, AAC32591), *Populus trichocarpa* PCBER (PopPCBER, CAA06706), *Tsuga heterophylla* PCBER (ThPCBER, AAF64177), *Linum strictum* PCBERs (LsPCBER1 (ACA60729) and PCBER2 (ACA60730)) and *Forsythia intermedia* PLR (FiPLR, AAC49608), *Linum usitatissimum* PLR (LuPLR, CAH60858), *Thuja plicata* PLR (TpPLR2, AF242504) and *T. heterophylla* PLR (ThPLR, AAF64184) (red). Amino acid sequences were aligned using ClustalW. To reconstruct phylogenetic tree, maximum likelihood was carried out using Phylip 3.68.

parenchyma cells,[291] this also being consistent with a lignan-related metabolic function. It needs to be established though as to whether this protein is bifunctional, that is, having CES and/or AIS properties *in vitro* and *in vivo*, as well as the PCBER properties already documented. As noted earlier, members of the Pinaceae can also contain very minor amounts of allyl-/propenylphenols in their essential oils, so this (dual) possibility must be considered.

1.23.9.2.2(v) **Cryptomeria japonica** This organism contains at least two PCBER genes of ~85 and 72–80% similarity and identity to that of the PtPCBER. *Cryptomeria japonica* is known to biosynthesize various 8–5′-linked lignans, including one with a reduced 7–*O*–4′ interunit linkage, the tetra-acylated derivative **118**.[160] There are, therefore, PCBER-like enzymatic transformations also occurring in this species. On the contrary, there is currently no chemotaxonomic evidence for the presence of allyl-/propenylphenols in *C. japonica*. It can be considered that the formation of tetra-acetylated lignans, such as **118**, may also involve intermediacy of monolignol esters (e.g., **236**). If correct, then the occurrence of the tetra-acetylated derivative is indicative of only PCBER-like activities.

1.23.9.2.2(vi) ***Petunia CES*** It was quite surprising that a petunia CES (PhEGS1) was considered for its potential/efficacy as a PCBER,[354] particularly because of the following: (1) 8–5′-linked lignans with 7–*O*–4′ interunit linkage-reduced metabolites have never been reported chemotaxonomically from this species; and (2) the gene family is not part of the clades containing PCBER and/or PCBER-like genes. Nevertheless, although the petunia CES (PhEGS1) had marginal PCBER activity, it is well known that many enzyme classes display varying levels of enzymatic activities with a variety of substrates that are of no physiological relevance. Thus, the significance of comparing the petunia CES with PtPCBER was questionable, particularly since petunia apparently does not biosynthesize both classes of metabolites.

1.23.9.2.2(vii) **Clarkia *CES*** The CbEGS2 protein was also considered as a potential PCBER,[354] which in this case provisionally falls into a CES and PCBER-like subclade (**Figure 55**). Its CES activity was, however, more than an order of magnitude lower than that of the creosote bush CES (LtCES1), that is, indicative of fairly low catalytic activity overall. Again, though, the relevance of testing this protein for PCBER activity was questionable, as, to our knowledge, *Clarkia* flowers are not known to produce 7–*O*–4′-reduced 8–5′-linked lignans.

1.23.9.2.3 CES (AIS) structural and mechanistic studies: comparison to PLRs, PCBERs, and IFRs

The closest homologs to the allyl-/propenylphenol synthases are the aforementioned, comprehensively studied, PLRs, [263,264,266,269] PCBERs,[265,266] and IFRs.[361,362] All, as previously indicated, are members of the short-chain dehydrogenase/reductase (SDR) family. Of these, the PLRs and PCBERs were mainly discovered and characterized during the 1990s. Accordingly, determination of PLR and PCBER X-ray crystal structures, as well as that of the modeled IFR,[266] provided incisive and key insights into CES/AIS catalytic function.[4]

In this regard, PLRs, PCBERs, IFRs, and CES/AIS all catalyze the NADPH-dependent hydride transfer to carbons derived from a phenylpropanoid side-chain within their respective phenolic substrates. As for this enzyme class, the creosote bush CES (LtCES1) has a nucleotide-binding Rossman motif (residues 11–17 (GxxGxxG)), as well as the corresponding conserved catalytic Lys residue (Lys133 in LtCES1). The latter residue was mutagenized to afford the recombinant K133A mutant, which lacked catalytic activity, thereby providing further support that Lys133 functioned as a general base during catalysis.[4] Based on our previous studies, Tyr15 and Ile16 were also predicted to interact with the NADPH pyrophosphate group, whereas Phe155 was envisaged to be stacked against the NADPH nicotinamide group.

Taken together, these data further supported the involvement of a quinone methide as the enzyme-bound intermediate in the CES catalytic mechanism[4] (**Figure 31(e)**), as had already been proposed for PLR, PCBER, and PTR (**Figures 31(a), 31(b), and 31(d)**). In the LtCES1 catalytic mechanism, the Lys133 residue can be considered to abstract the phenolic proton from the substrate, thereby facilitating the formation of the putative quinone methide intermediate, with loss of the corresponding ester functionality as a leaving group. Addition of the incoming NAD(P)H hydride to C7 of the intermediate for CES, with concomitant rearomatization of the phenolic ring, affords the corresponding allylphenol

products. Analogously, hydride addition to C9 of the putative quinone methide intermediate in AIS results in the formation of the conjugated propenylphenol product. Note, however, that in LtCES1, various substrates (**234**–**236**) were efficiently converted into chavicol (**20**) and eugenol (**4**), indicative of its substrate versatility.[4]

An X-ray crystal structure was also recently reported for the basil CES (ObEGS1), which confirmed our findings. The basil CES was obtained in its apo-form (**Figure 30(d)**) at ~1.8 Å, as well as complexes with either $NADP^+$, NADPH, or $NADP^+$ in combination with the inhibitor EMDF ((7*S*,8*S*)-ethyl-(7,8-methylene)-dihydroferulate, **297**).[355] However, the putative (but still unproven) 'true' substrate coniferyl acetate (**236**) could not be stably incorporated into the crystals or readily modeled into the active site of the basil CES (ObEGS1). Nevertheless, these data thus provided additional confirmation to the above earlier study[4] of CES catalysis.

The structures so reported, as fully anticipated, closely resembled those previously described by ourselves[266] for PLR, PCBER, and IFR (**Figures 30(a)**–**30(c)**), as well as CES.[4] That is, the basil CES had a Rossman fold motif at residues 14–20 (GXXGXXG), relative to that previously reported for LtCES1 at residues 11–17; the Tyr and Ile residues interacting with the pyrophosphate group of NADPH were at residues 18/19, whereas the earlier study for LtCES1 had these at 15/16. Other confirmatory findings included Phe154 (Phe155 in LtCES1), stacking against the NADPH nicotinamide group, as well as that of Lys132 (Lys133 in LtCES1) for general base catalysis. In further confirmation of our findings,[4] mutation of Lys132 residue to afford K132A resulted in the abolition of enzymatic activity. The X-ray data therefore confirmed our prior results from site-directed mutagenesis and modeling, as well as several other aspects of overall structure prediction.

Furthermore, given that PLRs, PCBERs, CESs, and AISs display considerable homology, and have a similar catalytic mechanism involving transfer of a hydride onto a phenylpropanoid-derived side chain, it should not be too surprising to observe some degree of substrate versatility (degeneracy) among each of the enzymes with regard to potential substrates *in vitro*. This would not be unexpected, as the monolignol esters **234**–**236** are either smaller or of similar size than, for example, PLR substrates, and also share some common structural features (e.g., a free phenolic group). That is, both sets of substrates can potentially bind to the enzyme active site in a similar orientation and presumably undergo similar C7 hydride addition. Following the same rationale, one can therefore expect that similar versatility may be displayed by some of the other PLR, PCBER, and IFR reductases. Conversely, CESs might not necessarily be able to utilize the larger (and less conformationally flexible) lignans as substrates, as their active sites might present greater steric hindrance to efficient binding.

1.23.9.2.4 Allyl-/propenylphenol downstream metabolism

Although allyl-/propenylphenols are present both as monomers, dimers, or both in many plant species, most downstream metabolism for the monomers largely duplicates that of other conversions already discussed for lignan biogenesis. For the monomers, downstream reactions can also precede both accumulation and emission of the typically volatile compounds. In this regard, floral emission to the atmosphere of volatiles in petunia is thought to occur passively, based on their physiological concentrations and boiling points.[363] The most commonly observed metabolic derivatization is *O*-methylation of the phenolic groups, catalyzed by the SAM-dependent *O*-methyltransferases (OMTs): for example, allyl-/propenylphenol OMTs have been isolated from *C. breweri*,[364,365] and more recently from anise[356] and basil.[366] Both enzymes were shown to have strong substrate preferences for either chavicol (**20**) or eugenol (**4**), with these activities being interchanged by mutations in single amino acid residues (F260S in chavicol OMT, S261F in eugenol OMT).[367]

Other ring substitution patterns can be effectuated by the action of specific P-450 enzymes, as previously noted for the sesame lignans[225] (Section 1.23.5.1.1). These include, for the monomers, a preliminary description of the formation of the methylenedioxy bridge in safrole (**22**) from eugenol (**4**) in *Illicium parviflorum* by action of a cytochrome P-450.[368] Other derivatization reactions include phenol conjugation, for example, *O*-acylation.

The most usual phenol ring substitution patterns in both the general phenylpropanoid pathway and the allyl-/propenylphenols are the same (i.e., where carbons 3, 4, and 5 bear H, OH, or OMe substituents, whereas carbons 2 and 6 are unsubstituted). Other more unusual patterns are present in some plant species, as indicated in the chemotaxonomical analysis section. For the latter, these conversions can result through additional (i.e., C2 and/or C6) hydroxylation/*O*-methylation of existing ring substructures deriving from the phenylpropanoid pathway.

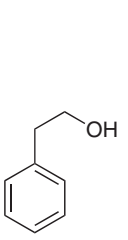
On the contrary, some unusual propenylphenols from *Pimpinella* spp. have the so-called pseudoisoeugenol carbon skeletons, for example, **8**, in which the ring substitution patterns (1-propenyl-2-hydroxy-5-methoxybenzene) deviate from the typical 4-hydroxylated patterns with the three carbon side-chain at C1. Pseudoisoeugenol biosynthesis was initially studied in the late 1980s using isotope labeling/tracer incorporation studies,[369,370] with *P. anisum* cell cultures. Demonstration of an NIH shift of the propenyl side chain during hydroxylation of anethole (**5**) to afford the pseudoisoeugenol skeleton was the later demonstrated.[6,371] However, the enzyme responsible for side-chain migration, as well as the remaining enzymatic steps involved in further derivatization (e.g., epoxidation and acylation), remain still to be described.

1.23.9.3 Monolignol Acyltransferases: Incomplete Characterization and Substrate Degeneracy

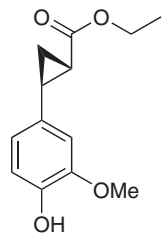
After monolignol esters were identified as substrates for allyl-/propenylphenol synthases, an *in silico* analysis of a petunia petal EST database led to the identification of a cDNA, by the Clark laboratory,[372] which encoded a protein homologous to several known BAHD acyltransferases. These enzymes constitute a class of acyltransferases named for the first four enzymes characterized in this class:[373] benzyl alcohol *O*-acetyltransferase (BEAT), anthocyanin *O*-hydroxycinnamoyl transferase (AHCT), anthranilate *N*-hydroxycinnamoyl/benzoyltransferase (HCBT), and deacetylvindoline 4-*O*-acetyltransferase (DAT). BAHD acyltransferases are known to be involved in the biosyntheses of several aroma and flavor compounds, although amino acid identities among homologous enzymes can vary enormously, with the petunia BAHD homolog being only 22–26% identical to benzyl alcohol/phenylethanol benzoyl transferase (BPBT) from petunia, BEAT from *C. breweri*, and a rose (*Rosa hybrida*) alcohol acyltransferase (AAT1), respectively.

The corresponding putative acyltransferase gene was found to be expressed primarily in petal limbs, with a circadian rhythm profile correlating well with floral volatile emission (i.e., highest amount of transcripts were observed at night). Additionally, RNAi-induced gene silencing led to a decrease in accumulation and emission of isoeugenol (**6**) (along with decreases in levels of 2-phenylethanol (**296**), phenylacetaldehyde (**298**), phenylethyl acetate (**299**), phenylethyl benzoate (**300**), and benzyl acetate (**301**)), as well as an approximately sevenfold increase in the amounts of coniferyl aldehyde (**262**). These effects, however, encompass several different pathways.

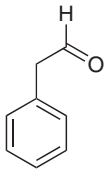
Nevertheless, the full-length cDNA was heterologously expressed in *E. coli*, with the recombinant protein assayed for acyltransferase activity *in vitro*. At pH 7.5, it efficiently acetylated coniferyl alcohol (**91**) as its preferred substrate among the compounds tested (with apparent K_m of 27.5 μmol l^{-1}, k_{cat} of 0.81 s^{-1}, and k_{cat}/K_m of 29 450 mol^{-1} l s^{-1}). Other test substrates such as cinnamyl alcohol (**284**), sinapyl alcohol (**94**), octanol (**302**), and geraniol (**303**) were processed ~2–4 times slower, with activities 30–60 times lower being observed using *p*-coumaryl alcohol (**199**) and 2-phenylethanol (**296**) at the same substrate concentrations. At pH 6.0, the enzyme had an ~2-fold higher K_m for coniferyl alcohol (**91**) (56.5 μmol l^{-1}), but with a ~2.5-fold higher k_{cat} (2.05 s^{-1}), thereby resulting in apparently a similar overall catalytic efficiency (k_{cat}/K_m of 36 280 mol^{-1} l s^{-1}). The enzyme was thus named acetyl-CoA:coniferyl alcohol acetyltransferase (PhCAAT), although it clearly shows extensive substrate versatility. By contrast, a basil ObCAAT enzyme has been preliminary reported to acetylate *p*-coumaryl alcohol (**199**) as its preferred substrate *in vitro*, while also using caffeyl alcohol (**270**) and coniferyl alcohol (**91**) to afford *p*-coumaryl acetate (**234**), caffeyl acetate (**304**), and coniferyl acetate (**236**), respectively.[374]



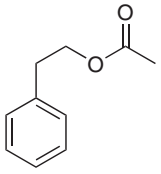
(**296**) 2-Phenylethanol



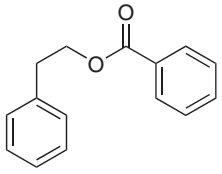
(**297**) EMDF [(7S,8*S*)-ethyl-(7,8-methylene)-dihydroferulate]



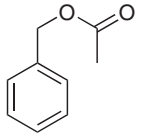
(**298**) Phenylacetaldehyde



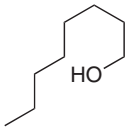
(**299**) Phenylethyl acetate



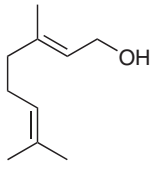
(**300**) Phenylethyl benzoate



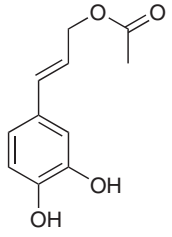
(**301**) Benzyl acetate



(**302**) Octanol



(**303**) Geraniol



(**304**) Caffeyl acetate

Although initial studies[2,3] were not conclusive about the true identity of the monolignol esters involved in regiospecific reduction (i.e., regarding their acyl moieties), subsequent studies[354,356] have essentially only tested coniferyl esters (e.g., **234**) for allyl-/propenylphenol synthesis. However, petunia also accumulates and emits benzoyl esters and phenylethanol derivatives. Using RNAi for the so-called PhCAAT, their levels were also adversely affected as noted above. Surprisingly, benzoyl-CoA and other potential substrates were apparently not tested with the petunia PhCAAT that is now implicated in allyl-/propenylphenol biosynthesis. Nor were other putative substrates, such as *p*-coumaroyl- and feruloyl-CoAs examined either. Alternate acyl donors were apparently also not tested during the preliminary characterization of the basil acyltransferase.

By contrast, an *in vitro* biochemical characterization of an acyltransferase from *L. tridentata* (LtCAAT1, having ~71/57% amino acid similarity/identity to PhCAAT) is currently in progress in our laboratory, and indicates that both acetyl-CoA and benzoyl-CoA can also serve as acyl donors with all the monolignols tested (S.-J. Kim, unpublished results); that is, LtCAAT1 apparently forms, at least *in vitro*, different classes of monolignol esters. Additionally, the acyltransferases appear to efficiently process different monolignols as acyl acceptors, for example, *p*-coumaryl (**199**), caffeyl (**270**), coniferyl (**91**), 5-hydroxyconiferyl (**271**), and sinapyl (**94**) alcohols. Given the observed substrate versatilities being noted, it is thus considered that the depiction of a 'true' *in vivo* substrate (e.g., coniferyl alcohol (**91**) and acetyl-CoA) lacks experimental rigor.

1.23.10 Biological Properties *in Planta* and in Human Usage

Allyl-/propenylphenols, lignans, and to a lesser extent, norlignans are of increasing scientific interest, not only for their utility by humans, but also for their important roles *in planta*. These are now considered in terms of those that have established properties and uses, followed by those whose bioactivities are of a more preliminary

character. In many of the latter cases, these represent promising avenues of research. On the contrary, more often than naught their potentially interesting pharmacological findings are seldom pursued further. In other instances, some of their properties are only effective at very high dose levels/concentrations that would generally preclude utility as medicinals. Such limitations in current natural compound pharmacological studies should thus be kept in mind during the coming subsections.

1.23.10.1 Allyl-/Propenylphenols

1.23.10.1.1 Antimicrobial properties

The most common allylphenol in Nature, eugenol (**4**), has been long used in cloves for its antimicrobial properties, both in food preparations and as a constituent of dental resins. Indeed, for millennia, humanity has used allyl-/propenylphenol-containing spices to protect foods against microbial spoilage. This is because spices were, in addition to salting and drying, the main means of food preservation, especially in warmer climates (i.e., in tropical and equatorial regions), where bacterial growth and contamination are frequently more favored.[375] Furthermore, plants growing in such warmer/more humid climates are typically challenged with numerous and diverse microbial pathogens, and the formation of their chemical defense compounds (e.g., allyl-/propenylphenols, terpenoids, alkaloids) presumably helps withstand such attacks. Other allyl-/propenylphenols (e.g., methylchavicol (**3**) and anethole (**5**)) also have antimicrobial properties, and plants accumulating them (e.g., tarragon, anise, and fennel) are cultivated and used as herbs and spices. Humanity has long treasured the flavors and fragrances of spices, perhaps culturally linking their presence to such beneficial qualities and which result in their uses as food seasonings to the current time. In many societies, however, they have lost much of their relevance as food preservatives *per se*.

Centuries ago, spices were brought to Europe, where they gained large economic importance and became expensive and desirable trade items. Indeed, eugenol (**4**)-rich cloves, which grow in tropical climates (until recently, almost exclusively in the so-called 'Spice Islands' in Indonesia), subsequently became important for their antimicrobial and analgesic properties. Cloves were one of the main spices (along with cinnamon (*Cinnamomum verum*, syn. *C. zeylanicum*) and black pepper (*P. nigrum*)) imported by Europeans since Roman times. The Aksumite Empire (based in nowadays Ethiopia) controlled the main trading routes between the Mediterranean and India, through Egypt and the Red and Arabian Seas, until its decline with the rise of Islam during the seventh century, when these trade routes were effectively blocked. Eventually, trade was reestablished and controlled by Arabs until the fifteenth century, when the rise of the Turk Ottoman Empire again interrupted trade by land. This led to the 'age of exploration' and the creation of early maritime spice trade routes by Europeans around the African coast in the fifteenth/sixteenth centuries. This, in turn, resulted in successive periods of maritime domination by different nations, that is, where Portugal established several coastal colonies during the sixteenth century, many of which were later under Dutch, French, and British control during the rise of their own colonial periods. The development of these routes led to the 'discovery' and colonization of the American continent by the Europeans. Nowadays, Indonesia produces again most of the world's clove supply, after a decrease in production in the past decades in Zanzibar and Pemba (Tanzania), where they had been introduced.

Eugenol (**4**) is thought to exert its antimicrobial effects by affecting either glucose uptake/metabolism[376] or cellular membrane shape/integrity.[377,378] Nevertheless, its antimicrobial effects are apparently attained only at relatively high concentrations. Reported minimal inhibitory concentration (MIC) values against bacteria typically are ~1–5 mmol l^{-1} (e.g., against *Staphylococcus mutans*,[379] *E. coli*, and *Staphylococcus aureus*,[380] as well as *Bacillus subtilis* and *Listeria innocua*[381]); these values are more than 1000 times higher than those of the conventional antibacterial nisin (1 and 2 μmol l^{-1}, respectively).

Although such antibacterial effects are observed only at relatively high eugenol (**4**) concentrations, such functions are presumably attained *in planta*. Some volatile oil components accumulate in very high concentrations, for example, basil contains ~10 mg eugenol (**4**) per gram (fresh weight).[352] Accordingly, utilization of such spices during food preparation can potentially lead to sufficiently high concentrations for antibacterial effect, for example, millimolar eugenol (**4**) concentrations can result from the use of a few hundred milligram cloves, as it comprises ~15% of the dry weight.

In addition to the effects on bacteria, eugenol (**4**) reportedly acts as a fungicidal/fungistatic agent, albeit with relatively high MIC values ranging between 1 and 5 mmol l^{-1} against *Trametes versicolor* (white rot fungus),

Coniophora puteana (brown rot fungus),[382] *Drechslera sorokiniana* (a root rot fungus), *Colletotrichum graminicola* (antrachinose pathogen), *Fusarium solani* (a plant and human fungal pathogen), and *Macrophomina phaseolina* (charcoal rot fungus).[383] It also affects the growth of the yeast *Saccharomyces cerevisiae* with an MIC of 1.8 mmol l^{-1}, where it was demonstrated to cause membrane damage/leakage.[384] Interestingly, membrane damage was of enough utility to allow for eugenol (**4**) use in cell lysis during extraction of genomic DNA from *S. cerevisiae* and *Candida albicans*, instead of SDS/zymolyase. Additionally, *in vivo* studies using oral *C. albicans* infection in immunosuppressed rats reported an MIC of 12 mmol l^{-1} for eugenol (**4**), whereas that for the reference treatment nystatin was 5.8 μmol l^{-1}, or ~2000 times lower.[385] Again, as discussed above for the antibacterial effects of eugenol (**4**), such dosage levels/concentrations are too high for consideration as a potential drug. Nevertheless, beneficial effects may be achieved following food preparation and ingestion.

1.23.10.1.2 Anesthetic properties

In addition to being a useful antimicrobial, eugenol (**4**) is a topical anesthetic, and combination of these properties led to its use in dental polymers as pulp-capping agents.[386–389] It has also been reported to be an efficient anesthetic in fish (as well as rats), facilitating capture, transport/handling, and also surgical procedures, although it becomes lethal at higher doses.[388,390,391] In rainbow trout, eugenol (**4**) concentrations of 50 mg l^{-1} result in short-term anesthesia (<5 min), with 'some deaths' observed at 100 mg ml^{-1}.[392] Cloves are also added to clove ('kretek') cigarettes, originally as a means of clove oil delivery directly to the lungs, to help combat chest pains.[393] The anesthetic activities of eugenol (**4**) are thought to derive from its effects on Na^+ and Ca^{2+} ion channels, inhibiting voltage-gated currents/ion potentials,[394–396] although its effect on GABA receptors may also be involved.[392]

1.23.10.1.3 Other reported activities

Other reports of eugenol (**4**) biological/medicinal properties in the scientific literature are generally more preliminary, and in need of further exploration from a potential medicinal perspective. For example, it is preliminarily reported as having antioxidant, antitumor, antiviral, antileishmanial, anthelmintic, and hypotensive properties in humans/animals.[86,397–399] It has also been shown to modulate ion channel activities as described above[400–403] and inhibit a number of enzymes, including monoamine oxidase,[404] 5-lipoxygenase,[405] and inducible nitric oxide synthase,[406] with reported reduction of NF-κB activity.[407] A number of enzymes are also apparently activated by eugenol (**4**), these including p38 MAP kinases[408] and UDP-glucuronyl transferases.[409] Additionally, micromolar concentrations of eugenol (**4**) have been reported to induce apoptosis in HL-60 leukemia cells (40 μmol l^{-1})[410] and also in mast cells, whereas at higher concentration (700 μmol l^{-1}) it leads to the translocation of phospho-Ser15-p53 into mitochondria and its interaction with Bcl-2 and Bcl-xL.[411] Eugenol (**4**) (at 0.5–2.5 μmol l^{-1} concentration) also inhibits melanoma growth by inhibition of E2F1 transcription.[412]

Other reported allyl-/propenylphenol biological activities include the anti-inflammatory action of anethole (**5**) through inhibition of tumor necrosis factor (TNF)-mediated signaling,[413] for example, via NF-κB activation, the same mechanism proposed for the anti-inflammatory activity of isoeugenol (**6**).[414] Safrole (**22**) and isosafrole (**24**) reportedly protect liver tissue from CCl_4 carcinogenicity by inhibition of its CYP2E1-dependent activation.[415] On the contrary, hydroxychavicol (**50**) potentializes the deleterious effects of benzopyrene in cigarette smoke leading to oral squamous cell carcinoma,[416] and asaricin (**32**) can kill moth larvae at submilligram quantities,[417] the latter being potentially useful as a pesticide and fungicide.[418,419]

1.23.10.1.4 *Effects* in planta

Besides the antimicrobial effects described above, allyl-/propenylphenols have other reported roles *in planta*. Methyleugenol (**42**) is present in a number of floral scents, and is the major attractive component of *Bactrocera* fruit fly pollinators in the orchid *B. cheiri*. It is present in amounts of ~400 μg per flower, about 10 times more than the other allyl-/propenylphenols present.[80] Indeed, methyleugenol (**42**) is commercially used in insect lures and traps, thus reducing the use of conventional insecticides, for example, against Oriental fruit flies (*Bactrocera dorsalis*) in Hawaii.[420–424]

1.23.10.1.5 Mutagenicity

Allyl-/propenylphenols have also long been studied for possible mutagenic potential, as they are typical components of several prepared foods and some can be carcinogenic in test animals at very high doses.

FEMA acceptable/safe daily intakes for methylchavicol (**3**) and methyleugenol (**42**) have been estimated at 1–10 mg kg^{-1} body weight per day, which is 100–1000 times the average human dietary consumption.[425] In low doses, such as those typically found within normal diets, they are metabolized *in vivo* to form harmless products through, for example, demethylation, glucuronidation, and epoxidation/epoxide hydrolysis.[426] In laboratory experiments utilizing much larger doses (100–2000 mg kg^{-1} day^{-1}), allyl-/propenylphenols were also metabolized through P-450-catalyzed C7 side-chain oxidation, followed by the action of sulfotransferases.[427–431] The sulfooxyalkenyl phenol derivatives thus formed were found to be less stable in aqueous media and generated, upon sulfate loss, carbocations that reacted with proteins and DNA to afford covalent adducts. This, however, perhaps accounts for their mutagenic/tumorigenic potential at very high doses.[431–433] Another possibility for their reported toxicity at very high doses may be the reaction of their quinone methide derivatives with basic amino acids (i.e., Lys and Arg).[434] Indeed, such protein adducts have been detected in rat hepatocytes,[435] and more stable carbonium ions/quinone methides have been found to be more genotoxic.[436] Nevertheless, dosages utilized in these studies are abnormally high and orders of magnitude higher than those typically observed from dietary intake.

1.23.10.1.6 Potential future uses as commodity chemicals/biofuels

Allyl-/propenylphenols are now also being considered as potentially useful petrochemical substitutes.[5] Aromatic hydrocarbons (e.g., ethylbenzene) are already present in automotive and aviation fuels, and contribute favorably to their combustion characteristics. Most allyl-/propenylphenols are liquid at room temperature, and release relatively high energy upon combustion (i.e., they have relatively high carbon to oxygen ratios, with higher energy densities especially when compared to ethanol). Another important potential niche for these compounds as petrochemical substitutes is in polymer industries. More than 10% of the current petroleum uses are for nonenergy purposes, including synthesis of polymers and polymer intermediates such as styrene; these facets of the current petroleum crisis are not addressed by any of the main proposed solutions, such as cellulosic ethanol and biodiesel. Allylphenols can be readily converted into the corresponding propenylphenols through alkaline isomerization, and the resulting propenylphenols are structurally substituted/functionalized styrenes. These can undergo similar polymerization reactions to afford polymer chains of relatively high molecular weight, depending on reaction conditions, or may be further refined into other useful products. Therefore, such properties create the exciting possibility for their consideration as (bio)fuels/fuel additives and/or polymer intermediates, if technologies for production at the commodity level become available and economically viable. Additionally, the remaining biomass, if partly reduced in lignin contents, without adversely affecting physiological properties *in planta*, could facilitate cellulose fermentation for ethanol production and also wood processing in pulp/paper industries.

1.23.10.2 Lignans

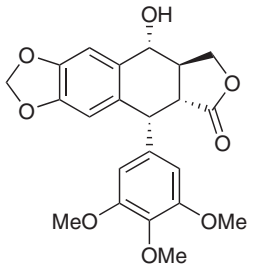
Several lignans have either achieved continued success as pharmaceuticals and/or are showing considerable promise in cancer treatment. Others have been implicated in beneficial effects for human health either in various foodstuffs or in traditional medicine; for example, *S. chinensis*, a plant used in Traditional Chinese Medicine (TCM) for its liver-protecting properties, produces the lignin, gomisin A (**157**),[437–439] whereas secoisolariciresinol diglucoside (SDG, **217**) present in flaxseed (*L. usitatissimum*) has been proposed to prevent onset of various cancers[284] and to modulate fat metabolism,[440] and NDGA (**143**) is thought to be the main bioactive component of *Larrea* species used in traditional Native American medicine.[245] On the contrary, there are many recent but preliminary bioactivity reports of phytochemical studies of lignans of purported medicinal value. In general, these have either not been pursued in more detailed studies, or whose 'efficacious' activities are only achieved at very high concentrations (dosages). These properties are discussed in more detail in the following subsections.

1.23.10.2.1 Lignans in cancer chemotherapy and cancer prevention

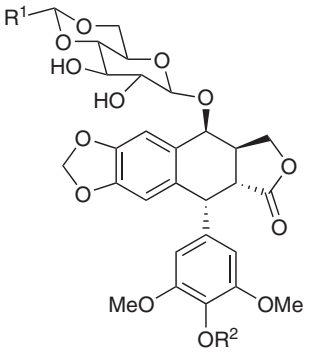
1.23.10.2.1(i) Podophyllotoxin and derivatives Perhaps the most important use of a lignan for medicinal purposes is that of the (−)-podophyllotoxin (**1b**) derivatives in chemotherapy. In this regard, (−)-podophyllotoxin (**1b**) was the first use of a lignan as a lead compound in the semisynthesis of antitumoral agents. However, it proved too toxic for successful use on its own as a therapeutic agent. Derivatives such as etoposide (**305**) and teniposide

(**306**) are now extensively used instead in treatment of various cancers (e.g., testicular, ovarian, brain, breast, and small- and large-cell lung cancers, and lymphomas),[441,442] usually in combination with other antitumoral drugs. By itself, (−)-podophyllotoxin (**1b**) inhibits microtubule assembly, thus leading to cell cycle arrest, whereas its derivatives such as etoposide (**305**) and teniposide (**306**) act on DNA topoisomerase II.[442] This enhanced efficacy, among other structural differences, has been correlated with the presence of bulkier *epi*-C-4 equatorial substituents (e.g., glycosides).[443,444] Such inhibitors stabilize a covalent complex between transient double-stranded DNA breaks and DNA topoisomerase II (which occur normally during DNA recombination and replication), inhibiting DNA strand religation and thus leading to lasting DNA damage.[445]

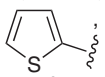
Development of (−)-podophyllotoxin (**1b**) analogs, such as **305** and **306**, still required either large solution volumes or addition of solubilizers, such as polysorbate 80 and polyethylene glycol, for administration of larger doses thereby limiting therapeutic use. Through extensive derivatization studies, however, the etoposide (**305**) C-4′ phosphate derivative etopophos (**307**) was later developed as a more water-soluble prodrug, as it is converted into etoposide (**305**) *in vivo* by action of endogenous phosphatases. As a result, etopophos (**307**) is now a preferred drug for clinical administration, having been approved for intravenous use by the FDA in 1996.[446] Other promising analogs include the epimeric picropodophyllin (**308**), an inhibitor of insulin-like growth factor-1 receptor that inhibits vascular endothelial growth factor (VEGF) secretion and neovascularization, as well as uveal melanoma growth.[447,448] Another derivative of interest is the dual topoisomerase inhibitor tafluposide (F 11782, **309**),[449–451] which is currently under phase I clinical safety studies as a chemotherapeutic agent.[452] Likewise, other semisynthetic analogs have been developed with lower cytotoxicity ID_{50} values, for example, the 4′-*O*-demethyl diamine derivative **310**, with ID_{50} of 0.027 μmol l^{-1} (compared to 0.2 μmol l^{-1} for etoposide (**305**)).[444]



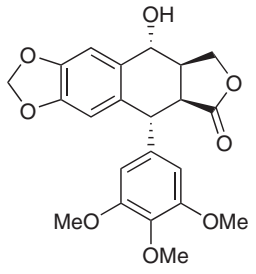
(**1b**) (–)-Podophyllotoxin



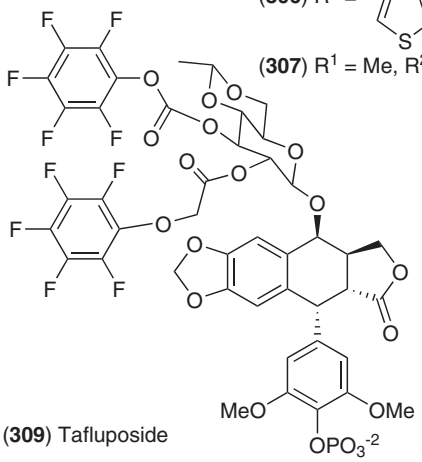
(**305**) R^1 = Me, R^2 = H, Etoposide

(**306**) R^1 =  , R^2 = H, Teniposide

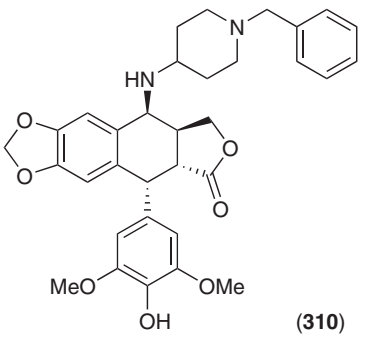
(**307**) R^1 = Me, R^2 = PO_3^{-2}, Etopophos



(**308**) Picropodophyllin



(**309**) Tafluposide



(**310**)

As the therapeutic uses of these compounds continue to grow, new sources have been sought to account for the increased demand; US etoposide (**305**) sales tripled in 1995 and have risen more than 10% annually in the

following years.[441] (−)-Podophyllotoxin (**1b**) is normally extracted from podophyllin, the resin of *Podophyllum* spp. (e.g., *P. emodi*, syn. *P. hexandrum*, and *P. peltatum*) rhizome. By comparison, its content in *P. peltatum* is only 0.25% of dry weight, whereas *P. emodi* accumulates up to 4.3% of dry weight. However, with glucosidase action prior to extraction, (−)-podophyllotoxin (**1b**) yields can be increased up to 5.2% of dry weight of *P. peltatum* leaves and rhizomes.[453]

Synthetically prepared alternatives for their production are disfavored due to typically low yields, resulting from the large number of steps and the challenges in obtaining the correct enantiomeric forms.[441] *In vitro* plant cultures are also currently unable to generate (−)-podophyllotoxin (**1b**) at a low enough cost, with among the highest amounts obtained being 130 mg l^{-1} in 10 days.[454] According to Verpoorte *et al.*[455] productions rates of 300 mg l^{-1} within 14 days would still result in prices 10 times higher than those obtained through traditional production methods, and even elicitation or precursor administration methods may not lower prices enough for biotechnological production of (−)-podophyllotoxin (**1b**) or derivatives.[454] Therefore, *in vitro* production protocols must be considerably improved before traditional extraction methods are economically surpassed. These efforts perhaps may be facilitated by the ongoing elucidation of (−)-podophyllotoxin (**1b**) biosynthetic pathway steps.

1.23.10.2.1(ii) Nordihydroguaiaretic acid and derivatives Another lignan whose derivatives are gaining increased interest as chemotherapeutic agents is NDGA (**143**). Prior to this, it was used as an antioxidant and as an additive in rubber production. The actual bioactive entity in chemotherapy is not completely established, with NDGA (**143**) perhaps serving in the capacity of a prodrug. This is being considered since NDGA (**143**) was recently shown to auto-oxidize in aqueous media (with a reaction half-life of ~3 h at pH 7.4)[456] to form a schizandrin-like dibenzocyclooctadiene lignan (**311**); the latter is perhaps the actual bioactive agent responsible for the biological activities discussed below. NDGA (**143**) has also been shown to undergo auto-oxidation to form quinones that are reactive toward thiols, thus leading to GSH depletion by adduct formation, this being another of its potential mechanisms of action.[457]

In recent years, NDGA (**143**) has become a promising potential pharmaceutical due to the anticancer properties reported for itself and some of its derivatives, and it has also been licensed for treatment of actinic keratosis (Actinex, Chemex Pharmaceuticals, Denver, CO).[245,458] Its reported anticancer properties on varied cancer cell lines may result from a combination of different activities. Examples include arresting cells on G1 phase (at least partially by reactivation of p16 INK4a, a gene that undergoes methylation in some cancers),[459] activation of TRAIL-induced apoptosis,[460] and inhibition of IGF signaling[461–464] as well as of lipoxygenase[462,465] and topoisomerase II alpha[466] activities. Among cancer cell lines that NDGA (**143**) has shown promising activity against are colorectal tumors (IC_{50} 1.9 μmol l^{-1}),[467] breast cancers,[468] adenocarcinomas,[469] and neuroblastomas (blocking growth at 15–30 μmol l^{-1}).[463] M4N (Terameprocol, **312**), the tetra-*O*-methylated derivative of NDGA (**143**), is also active against melanomas with similar IC_{50} values (1–20 μmol l^{-1}),[470] and is particularly suitable for intratumoral injection due to its low aqueous solubility, as it remains concentrated at the injection site.[471] Indeed, M4N (**312**) is currently undergoing phase I/II NIH trials[452] as a promising potential treatment against treatment-refractory solid tumors.[472,473] Another derivative, tetra-*O*-acetyl NDGA (**313**), has also been reported to be effective against adenocarcinomas.[469] As more structural studies are performed with these lignans, as for the podophyllotoxin (**1**) derivatives described above, NDGA (**143**) or derivatives of potentially higher efficacy may find similar extensive pharmacological applications as chemotherapeutic drugs.

1.23.10.2.1(iii) Enterolignans and cancer prevention Other classes of lignans showing some potential as cancer-preventative drugs/nutraceuticals are next discussed, albeit with the caveat that much more detailed studies are still necessary. These include the so-called 'mammalian' or entero-lignans, enterodiol (**316**) and enterolactone (**317**), which are produced by colonic microflora. These can be formed from many plant lignans and their glucosides. Both possess weakly estrogenic and antiestrogenic activities on different cell lines *in vitro*, and have been proposed to protect against the growth of some human cancers.[474,475] Higher levels of these lignans *in vivo* are found after intake of various foods, most prominently flaxseed (*L. usitatissimum*).[476] They have also been implicated in the antitumor activities of wheat bran on colon cancer cells,[477] of rye bran on diabetes,[478] and of flaxseed on atherosclerosis-related processes.[479]

Epidemiological studies suggest a protective effect of enterolignans against lower digestive tract cancers. These compounds were reported to inhibit growth of prostate cancer cell lines *in vitro*,[480–482] and significant inverse associations were observed between serum concentrations of enterolignans (but not of genistein, daidzein or equol) and prostate cancer.[483] Different *in vivo* studies reported protective effects on colorectal adenomas (considered to be cancer precursors)[484] but no effect on colorectal cancers.[485] Similarly, enterolignans were reported to have no correlation with either adenoma formation in mice[486] or lower myocardial infarction risk,[487] and a positive correlation was observed between enterodiol (**316**) plasma levels and risk of premalignant lesions of the cervix.[488] Enterolactone (**317**), on the contrary, was reported to reduce growth and metastasis of hepatomas in rats.[489]

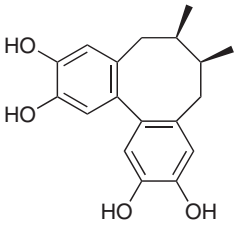
Using *in vitro* experiments with varied cell lines, enterolignans were found to decrease the activities of aromatase and 17β-hydroxysteroid dehydrogenase in MCF-7 breast cancer cells, thereby reducing cell proliferation,[490] whereas other researchers found a proliferation-stimulatory estrogenic effect.[491] Other studies have indicated that enterolactone (**317**) differentially modulates estrogen receptors α and β signaling, which could account for these discrepancies.[492,493]

Although the study of such hormonal effects is inherently difficult, most epidemiological studies correlate higher levels of plasma enterolactone (**317**) with reduced breast cancer risk and/or metastasis,[494–498] with some studies narrowing the beneficial effects to particular woman or cancer genotypes.[495,499] Again, these effects are not universally observed and/or accepted, and it has been reported that a flaxseed diet during gestation or lactation can increase the susceptibility of rat offspring to mammary tumorigenesis.[500] From these results, it is evident that the metabolism, mechanism of action, and potential of these compounds must be better understood before their potential as either cancer-preventative nutraceuticals or medicinal products are fully established.

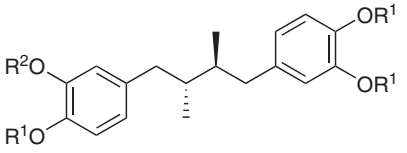
The main *in vivo* precursors of enterodiol (**316**) and enterolactone (**317**) are considered to be various plant lignans, such as matairesinol (**10**), secoisolariciresinol (**110**), as well as sesamin (**11**), pinoresinol (**13**), syringaresinol (**96**), arctigenin (**197**), 7-hydroxymatairesinol (**114**), lariciresinol (**105**), and isolariciresinol (**133**), as well as glucosides thereof, for example, secoisolariciresinol diglucoside (SDG, **217**) from flax.[474,475,501–503] The biosynthetic steps from these precursors is thought to involve deglycosylation (when necessary, e.g., from SDG (**217**)), followed, apparently as a necessary first step, by *O*-demethylation of one of the methoxyl groups of, for example, **110**. Ring dehydroxylation then probably occurs by loss of aromaticity and dehydration.[284,474,475,504,505] Additionally, precursor lignans such as pinoresinol (**13**) can be reduced to afford lariciresinol (**105**) and secoisolariciresinol (**110**) *in vivo*, whereas enterodiol (**316**) can be oxidized to afford enterolactone (**317**). All these reactions have been reported to be performed by strains of phylogenetically diverse colonic bacteria, for example, *Bacteroides*, *Eubacterium*, *Clostridium*, *Peptostreptococcus*, *Enterococcus*, *Eggerthella*, and *Ruminococcus* spp.[506–510] The enterolignans **316**/**317** thus formed are taken up and further metabolized by colon epithelial cells (and also perhaps by liver enzymes), affording the corresponding sulfates and glucuronides.[511] Indeed, it is mainly those metabolites that are later secreted in urine and bile, whereas the free enterolignans are only found in feces and (together with their conjugated derivatives) in plasma.[474,475] Typically, these lignans appear in plasma 8–10 h after intake, and have a mean elimination half-life of ~4–12 h,[512] with considerable inter-individual variations in the levels of lignans observed both in plasma and in secreted fluids, probably due to differences in gut microflora. Interestingly, while most lignans found in plants are optically active (including the (+)-secoisolariciresinol (**110**) moiety of SDG (**217**) in flaxseed),[275] the enterolactone (**317**) found in urine is racemic;[475] the reaction(s) potentially leading to racemization are not yet understood, and these observations may thus suggest a lignin origin as well.

These lignans have been reported to exert varied other activities *in vivo*, including protection against oxidants,[513] lowering diet-induced fat accumulation in mice,[440] lowering of plasma cholesterol and glucose concentrations in high-cholesterol subjects,[514] alleviating lower urinary tract symptoms in subjects with benign prostatic hyperplasia,[515] and also aiding in glycemic control in type-2 diabetic patients.[516]

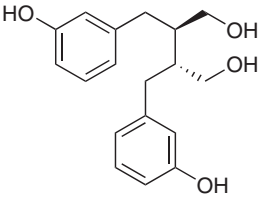
1.23.10.2.1(iv) Other lignans and norlignans with anticancer potential Some other lignans have also been reported to suppress either tumor growth or tumorigenicity of known carcinogens *in vitro*, but these results are still very preliminary. For instance, several recent studies have suggested that honokiol (**318**) from *Magnolia officinalis* is a promising potential chemotherapeutic agent for treatment of several cancers (e.g., colorectal and ovarian), based on antioxidant, proapoptotic, and antiangiogenic activities both *in vivo* and *in vitro* against several cancer cell lines.[517–524] Although clinical studies are still lacking, honokiol (**318**) was considered nearly as effective an antitumoral agent against colorectal carcinoma cells as the common chemotherapeutic drug adriamycin.[517]



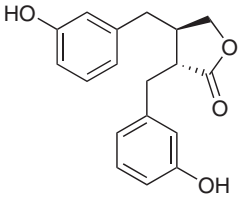
(**311**)



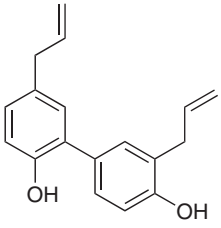
(**143**) $R^1 = R^2 =$ H, Nordihydroguaiaretic acid
(**312**) $R^1 = R^2 =$ Me, Tetra-*O*-methyl NDGA (M4N)
(**313**) $R^1 = R^2 =$ Ac, Tetra-*O*-acetyl NDGA
(**314**) $R^1 = R^2 =$ Gly, Tetra-*O*-glycyl NDGA
(**315**) R^1 = H, R^2 = Me, 3-*O*-methyl NDGA



(**316**) Enterodiol



(**317**) Enterolactone



(**318**) Honokiol

The unusual acetylenic norlignan hypoxoside (**182**) from African potato (*Hypoxis hemerocallidea*), along with its aglycone rooperol (**181**), may also have good potential as pharmaceuticals.[525–527] The latter (**181**) has been reported as an antioxidant and a cancer-cell growth inhibitor at concentrations of 2–10 μg ml^{-1}. Its glucoside (**182**) can also serve as a nontoxic prodrug (up to 100 μg ml^{-1}) that is deconjugated *in vivo* into its aglycone form (**181**),[528] but circulates mainly as phase II metabolites (glucuronides and sulfates).[212,525–527,529] Furthermore, *H. hemerocallidea* is possibly the best-known medicinal plant in South Africa, whose hypoxoside (**182**)-rich extracts are used for the treatment of urinary diseases, prostate hypertrophy, and cancer.[530] Although promising, the reported bioactivities for these African potato norlignans are still quite preliminary, and will require numerous additional *in vitro* and *in vivo* toxicity studies and structure–activity analyses before their potential as medicinal compounds can be better understood.

1.23.10.2.2 Antiviral lignans

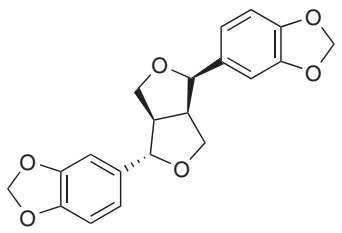
Another set of interesting lignan biological activities either currently used or being considered for medicinal purposes is that of antivirals. In addition to the previously discussed antitumor activities, (−)-podophyllotoxin (**1b**) and its derivatives also inhibit viral replication, for example, against measles, human papilloma HPv, cytomegalovirus, and herpes simplex type I viruses, being used medicinally against genital herpes.[442] NDGA (**143**) and derivatives also have antiviral activities, particularly against HIV and papillomavirus, although with relatively high concentrations being necessary. NDGA (**143**) has an IC_{50} of 25 μmol l^{-1} against HIV, while its tetra-*O*-methylated derivative (M4N, **312**) has an IC_{50} of 11 μmol l^{-1};[531] NDGA (**143**) and M4N (**312**) were also found to inhibit HIV-1 replication by suppressing proviral and HIV Tat-transactivated transcription.[531] The tetra-*O*-glycyl NDGA derivative (**314**) has an IC_{50} values of 12 μmol l^{-1} against HIV[532] and 3–5 μmol l^{-1} against herpes simplex virus-1.[533] Tetra-*O*-acetyl NDGA (**313**) and 3-*O*-methyl NDGA (**315**) are also effective against human papillomavirus and induce apoptosis in cervical tumor cells in *in vitro* assays at low micromolar concentrations,[534,535] whereas M4N (**312**) has shown promising results in Phase I/II trials as a vaginal ointment in treatment of HPV-linked cervical cancers.[536,537]

1.23.10.2.3 Nutraceutical lignans: sesame

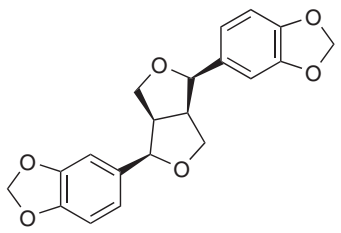
Many lignans have reported antioxidant properties, which in large part can be ascribed to the presence of free phenolic groups within their structures. Nevertheless, sesame represents perhaps one of the best-studied dietary lignan sources, as the lignans in both its seed and oil have beneficial properties for humans.

Sesame seed is consumed either raw or roasted, and its oil is extracted from either raw or roasted seed as well, leading to products with different sensory properties. South Korea has the highest *per capita* consumption (~6–7 g day^{-1}), with the Japanese consuming about 3 g day^{-1}, mainly as sesame oil.[249] Sesame oil is considered one of the best vegetable oils for deep-frying, as it is more resistant to high-temperature oxidation than soybean and canola oils, and also helps increase the durability of fried foodstuffs. Those antioxidant properties are thought to derive mainly from the presence of lignans in sesame and sesame oil, and the antioxidant potential of sesame oil apparently increases after roasting.

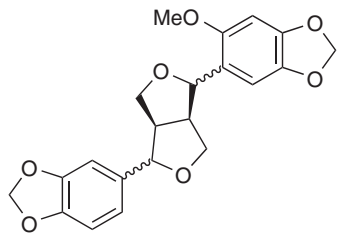
The most abundant sesame phenylpropanoids characterized are (+)-sesamin (**11a**), sesamolin (**210**), sesaminol (**206**), and sesamolinol (**211**) as previously discussed, together with *epi*-sesamin (**319**), diasesamin (**320**), sesangolin (**321**), 2-episesalatin (**322**), simpleoxide aglycone (**323**), and (+)-pinoresinol (**13a**) (from which the majority of sesame lignans are thought to be derived). Mono-, di-, and tri-glucosides of those lignans are also present.[249] Their levels vary among different *Sesamum* species, with sesamin (**11a**) and sesamolin (**210**) contents in sesame oil being typically 0.4% and 0.3%, respectively (with up to 2.4% (+)-sesamin (**11a**) in the oil of *S. radiatum*).[249]



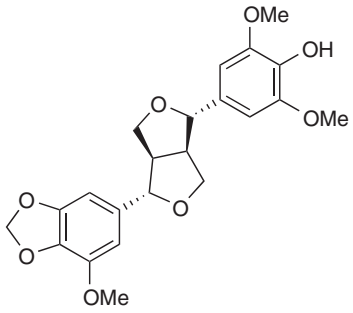
(**319**) Episesamin



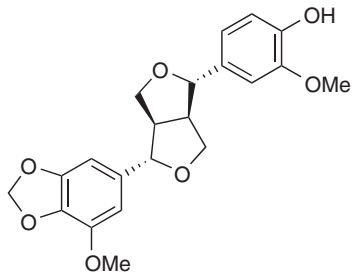
(**320**) Diasesamin



(**321**) Sesangolin



(**322**) 2-Episesalatin



(**323**) Simpleoxide aglycone

Sesaminol (**206**) is thought to be the main antioxidant lignan present in sesame oil, and its available levels are thought to increase (concomitant to sesamolin (**210**) level decrease) upon roasting, oil processing, or in the case of whole sesame seed or sesame meal, through hydrolysis of sesaminol (**206**) glucosides. (+)-Sesamin (**11a**) has no free phenol group and shows weak or no antioxidant effects; however, it is thought to be metabolized *in vivo* to afford the aforementioned enterolignans, for example, **313** and **314**.[503]

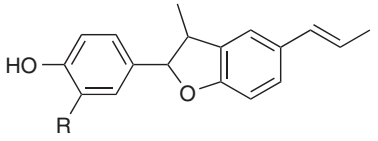
In addition to their antioxidant properties, sesame lignans affect tocopherol/vitamin E metabolism, increasing tocopherol levels in plasma, brain, and liver (two- to four-fold in the latter, where these lignans inhibit tocopherol metabolism).[249,538] Fatty acid metabolism is also affected by sesame lignans in microorganisms and animals, for example, sesamin (**11**) and other sesame lignans inhibit $\Delta 5$ desaturase activity in the fungus *Mortierella alpina* and rats, with IC_{50} values for sesamin (**11**) of 5.6 and 72 μmol l^{-1}, respectively (other fatty acid desaturases were not inhibited to the same extent).[249,539] Sesame lignans were also shown to increase hepatic fatty acid oxidation and decrease hepatic fatty acid synthesis in rats, which leads to decreased levels of serum fatty acids.[540,541] A similar trend was observed regarding cholesterol metabolism, with sesamin (**11**) in combination with α-tocopherol lowering the concentration of serum and liver cholesterol in rats and humans,

thus indicating a preventive role against atherosclerosis.[542] Additionally, sesamin (**11**) was reported to have potential beneficial effects on hypertension[543] and to increase the rate of alcohol metabolism in rats and humans.[249]

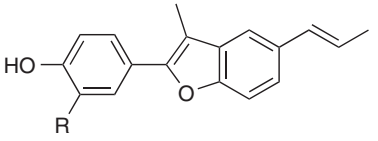
It is thus considered that sesame seed lignans can exert a number of different potentially beneficial effects in humans and other animals, with mechanisms for their actions having been proposed in some cases. Some of these activities are apparently specific to sesame lignans, while others can be potentially ascribed to their downstream metabolic products, for example, enterolignans such as **313** and **314**. Further detailed *in vivo* studies regarding these different effects and the true active principles must still be performed. Nevertheless, sesame seed continues to gain well-deserved attention as a nutraceutical dietary component.

1.23.10.2.4 Antichagasic lignans

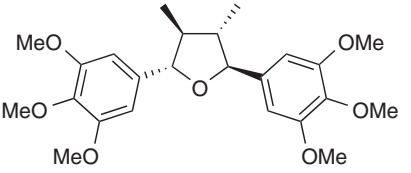
Some lignans have shown somewhat promising *in vitro* activities in cases where currently available treatments are not efficient (e.g., requiring high drug concentrations), thus potentially serving as useful lead compounds for the development of pharmaceutical compounds. For example, eupomatenoids (**191**, **192**), their possible *in vivo* precursors conocarpan (**16**) and **324**, as well as grandisin (**325**), have been isolated from a few plant species including *Conocarpus erectus*,[544] *P. regnellii*,[218] and *Piper solmsianum*,[545] and are active against some tropical parasites and insect larvae.[546] More importantly, though, they have shown activity against epimastigote forms of *Trypanosoma cruzi*, the causative agent of Chagas' disease, with IC_{50} values of 3–9 $\mu g\,ml^{-1}$, and being ~20% more inhibitory to *T. cruzi* growth than the typical medicaments benznidazole (which is less effective during the chronic part of the disease) and gentian violet (which has an IC_{50} value of 28 $\mu g\,ml^{-1}$).[547–549] Eupomatenoid-5 (**192**) also acts on the amastigote (intracellular) forms of *T. cruzi* at similar concentrations. Nevertheless, while these compounds may have some promising potential applications in Chagas' disease treatment, further pharmacological studies are lacking regarding their toxicities or activities of semisynthetic derivatives.



(**16**) R = H, Conocarpan
(**324**) R = OMe



(**191**) R = H, Eupomatenoid-6
(**192**) R = OMe, Eupomatenoid-5



(**325b**) (–)-Grandisin

1.23.10.2.5 Properties in planta

As discussed in detail before,[1] functional assignment of lignans (as well as most other specialized metabolites) *in planta* is very sparse and often circumstantial. Nevertheless, in most described cases, their functions seem to be mainly defensive, for example, antimicrobial, antioxidant, antifeedant, and allelopathic. For instance, in agreement with a putative defense application, the levels of dihydromonolignols (e.g., **291**, **119**) apparently increase upon aphid attack (e.g., *Adelges abietis* attack on *P. glauca*).[161] In other cases, they may serve to attract microbes or insects, sometimes with deleterious effects. One example is olivil (**12**), which was isolated from the resin of olive trees in Europe. Olive trees were later introduced in Japan in 1908, and olivil (**12**) has been recently found to act as a feeding stimulant to a species of weevils in Japan. These weevils, which originally used other Oleaceae plants (e.g., *Ligustrum japonicum* and *Ligustrum obtusifolium*) for their diet, have since changed their host preferences to the olive tree. These so-called 'olive weevils' now constitute the most serious pest of the olive tree in Japan,[550] indicating the delicate balance in Nature that can exist.

Strong antioxidant lignans are present in some of the most valuable plant oils, for example, sesame seed oil, where they are thought to prevent degradation and help stabilize these oils at high cooking temperatures. NDGA (**143**), as mentioned before, is also a very strong antioxidant and is found in large amounts in the creosote bush (*L. tridentata*), perhaps helping its survival in high-temperature desert areas. Of course, such distinctive properties are employed for human benefit, leading to the use of sesame oil in cooking, and of NDGA (**143**) as a stabilizer in polymer and rubber applications.

Some lignans are also reported to possess allelopathic properties. NDGA (**143**) is thought to be the main contributor to the phytotoxic effects of *L. tridentata*, which is often present as the single plant species in large colonized areas; indeed, early uses of this plant included sprinkling over train beds to stop weed growth.[551] Interestingly, on the contrary, dihydroconiferyl alcohol (**119**) was studied during the 1970s as a plant growth regulator, inducing hypocotyl growth in several plant species.[552–556] More recently, both dihydroconiferyl alcohol (**119**) and dihydro-*p*-coumaryl alcohol (**291**) contents were noted to be lower in the extractive contents of *Pinus laricio* after prescribed burnings;[557] dihydroconiferyl alcohol (**119**) is also found in pine weevil (*Hylobius abietis*) feces deposited to close holes bored for egg deposition, where it reportedly functions as an antifeedant to other pine weevils signaling the presence of the egg cavities under the trunk surface.[558]

As another example, the previously mentioned, *G. officinale* (ironwood, palo-santo, *lignum vitae*), a member of the Zygophyllaceae (which also includes the creosote bush, *L. tridentata*), accumulates in its heartwood a resin containing the 8–8′-linked 9–9′-deoxygenated lignan (−)-guaiaretic acid (**9b**). Its massive deposition is thought to contribute to the exceptional durability of this wood, one of the densest and hardest among commercial timbers. Indeed, the presence of large amounts of resin results in water resistance and 'self-lubricating' properties, which have made it ideal for use in ship construction. Other past uses of its wood have included manufacturing of bowling balls, mallets, and bearings.[559,560] Lignans contributing to heartwood durability and texture in other plant species include (−)-plicatic acid (**112**) and its derivatives in *T. plicata*,[156] 7-hydroxymatairesinol (**114**) and (−)-α-conidendrin (**113b**) in *T. heterophylla*,[154] and (nor)lignans such as **116**, **122**–**124** in *C. japonica*,[166,167] as discussed before in Section 1.23.3.2.3.

Acknowledgments

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Dedicated to Professor David G. I. Kingston on the occasion of his 65th birthday.

References

1. N. G. Lewis; L. B. Davin, Lignans: Biosynthesis and Function. In *Comprehensive Natural Products Chemistry Vol.1: Polyketides and Other Secondary Metabolites, Including Fatty Acids and Their Derivatives*; Sir D. H. R. Barton, K. Nakanishi, O. Meth-Cohn, Eds.; Elsevier: Oxford, UK, 1999; pp 639–712.
2. D. G. Vassão; D. R. Gang; T. Koeduka; B. Jackson; E. Pichersky; L. B. Davin; N. G. Lewis, *Org. Biomol. Chem.* **2006**, *4*, 2733–2744.
3. T. Koeduka; E. Fridman; D. R. Gang; D. G. Vassão; B. L. Jackson; C. M. Kish; I. Orlova; S. M. Spassova; N. G. Lewis; J. P. Noel; T. J. Baiga; N. Dudareva; E. Pichersky, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 10128–10133.
4. D. G. Vassão; S.-J. Kim; J. K. Milhollan; D. Eichinger; L. B. Davin; N. G. Lewis, *Arch. Biochem. Biophys.* **2007**, *465*, 209–218.
5. D. G. Vassão; L. B. Davin; N. G. Lewis, Metabolic Engineering of Plant Allyl/Propenyl Phenol and Lignin Pathways: Future Potential for Biofuels/Bioenergy, Polymer Intermediates, and Specialty Chemicals? In *Advances in Plant Biochemistry and Molecular Biology*; H. J. Bohnert, H. Nguyen, N. G. Lewis, Eds.; Elsevier: Oxford, UK, 2008; Vol. 1, Bioengineering and Molecular Biology of Plant Pathways, pp 385–428.
6. R. Martin; J. Reichling, *Phytochemistry* **1992**, *31*, 511–514.
7. N. G. Lewis; L. B. Davin; S. Sarkanen, The Nature and Function of Lignins. In *Comprehensive Natural Products Chemistry*; Sir D. H. R. Barton, K. Nakanishi, O. Meth-Cohn, Eds.; Elsevier: Oxford, UK, 1999; Vol. 3, pp 617–745.
8. L. B. Davin; M. Jourdes; A. M. Patten; K.-W. Kim; D. G. Vassão; N. G. Lewis, *Nat. Prod. Rep.* **2008**, *25*, 1015–1090.
9. L. B. Davin; A. M. Patten; M. Jourdes; N. G. Lewis, Lignins: A Twenty-First Century Challenge. In *Biomass Recalcitrance. Deconstructing the Plant Cell Wall for Bioenergy*; M. E. Himmel, Ed.; Blackwell Publishing: Oxford, UK, 2008; pp 213–305.
10. O. Doebner; E. Lücker, *Archiv. Pharm.* **1896**, *234*, 590–610.
11. G. Schroeter; L. Lichtenstadt; D. Irineu, *Chem. Ber.* **1918**, *51*, 1587–1613.
12. R. D. Haworth; C. R. Mavin; G. Sheldrick, *J. Chem. Soc.* **1934**, 1423–1429.
13. T. H. Easterfield; J. Bee, *J. Chem. Soc.* **1910**, *97*, 1028–1032.
14. V. Villavecchia; G. Fabris, *Ann. Lab. chim. centr. delle Gabelle* **1896**, *3*, 13–26.
15. J. Böeseken; W. D. Cohen, *Biochem. Z.* **1928**, *201*, 454–463.
16. B. L. Vanzetti, *Gazz. Chim. Ital.* **1929**, *59*, 373–378.

17. B. L. Vanzetti; P. Dreyfuss, *Gazz. Chim. Ital.* **1934**, *64*, 381–399.
18. D. C. Ayres; S. E. Mhasalkar, *J. Chem. Soc.* **1965**, 3586–3589.
19. R. D. Haworth, *Ann. Rep. Progr. Chem.* **1937**, *33*, 266–279.
20. O. R. Gottlieb, *Phytochemistry* **1972**, *11*, 1537–1570.
21. O. R. Gottlieb; M. Yoshida, Lignans. In *Natural Products of Woody Plants – Chemical Extraneous to the Lignocellulosic Cell Wall*; J. Wand, C. H. Kirk, Eds.; Springer Verlag: Berlin, 1989; pp 439–511.
22. G. P. Moss, *Pure Appl. Chem.* **2000**, *72*, 1493–1523.
23. F. S. El-Feraly; S. F. Cheatham; R. L. Breedlove, *J. Nat. Prod.* **1983**, *46*, 493–498.
24. B. Y. Hwang; J.-H. Lee; J. B. Nam; Y.-S. Hong; J. J. Lee, *Phytochemistry* **2003**, *64*, 765–771.
25. A. Ichihara; S. Kanai; Y. Nakamura; S. Sakamura, *Tetrahedron Lett.* **1978**, *19*, 3035–3038.
26. A. Ichihara; Y. Nakamura; H. Kawagishi; S. Sakamura, *Tetrahedron Lett.* **1979**, *20*, 3735–3736.
27. P. Kenrick; P. Crane, *Embryophytes. Land Plants. Version 01 January 1996 (temporary). http://tolweb.org/Embryophytes/20582/1996.01.01 in The Tree of Life Web Project*, http://tolweb.org/, 1996.
28. P. Crane, *Spermatopsida. Seed Plants. Version 01 January 1996 (temporary). http://tolweb.org/Spermatopsida/20622/1996.01.01 in The Tree of Life Web Project*, http://tolweb.org/, 1996.
29. Atta-ur-Rahman; M. I. Choudhary; S. Hayat; A. M. Khan; A. Ahmad; S. Malik, *Phytochemistry* **1999**, *52*, 495–499.
30. N. E. Awad, *Bull. Fac. Pharm. (Cairo Univ.)* **2002**, *40*, 169–174.
31. M. Y. Moussa, *Bull. Fac. Pharm. (Cairo Univ.)* **2003**, *41*, 139–144.
32. N. E. Awad, *Bull. Fac. Pharm. (Cairo Univ.)* **2002**, *40*, 233–243.
33. K. Bouarab; F. Adas; E. Gaquerel; B. Kloareg; J.-P. Salaün; P. Potin, *Plant Physiol.* **2004**, *135*, 1838–1848.
34. S. S. Merchant; S. E. Prochnik; O. Vallon; E. H. Harris; S. J. Karpowicz; G. B. Witman; A. Terry; A. Salamov; L. K. Fritz-Laylin; L. Maréchal-Drouard; W. F. Marshall; L.-H. Qu; D. R. Nelson; A. A. Sanderfoot; M. H. Spalding; V. V. Kapitonov; Q. Ren; P. Ferris; E. Lindquist; H. Shapiro; S. M. Lucas; J. Grimwood; J. Schmutz; P. Cardol; H. Cerutti; G. Chanfreau; C.-L. Chen; V. Cognat; M. T. Croft; R. Dent; S. Dutcher; E. Fernandez; H. Fukuzawa; D. Gonzalez-Ballester; D. Gonzalez-Halphen; A. Hallmann; M. Hanikenne; M. Hippler; W. Inwood; K. Jabbari; M. Kalanon; R. Kuras; P. A. Lefebvre; S. D. Lemaire; A. V. Lobanov; M. Lohr; A. Manuell; I. Meier; L. Mets; M. Mittag; T. Mittelmeier; J. V. Moroney; J. Moseley; C. Napoli; A. M. Nedelcu; K. Niyogi; S. V. Novoselov; I. T. Paulsen; G. Pazour; S. Purton; J.-P. Ral; D. M. Riano-Pachon; W. Riekhof; L. Rymarquis; M. Schroda; D. Stern; J. Umen; R. Willows; N. Wilson; S. L. Zimmer; J. Allmer; J. Balk; K. Bisova; C.-J. Chen; M. Elias; K. Gendler; C. Hauser; M. R. Lamb; H. Ledford; J. C. Long; J. Minagawa; M. D. Page; J. Pan; W. Pootakham; S. Roje; A. Rose; E. Stahlberg; A. M. Terauchi; P. Yang; S. Ball; C. Bowler; C. L. Dieckmann; V. N. Gladyshev; P. Green; R. Jorgensen; S. Mayfield; B. Mueller-Roeber; S. Rajamani; R. T. Sayre; P. Brokstein; I. Dubchak; D. Goodstein; L. Hornick; Y. W. Huang; J. Jhaveri; Y. Luo; D. Martinez; A. Ngau Wing Chi; B. Otillar; A. Poliakov; A. Porter; L. Szajkowski; G. Werner; K. Zhou; I. V. Grigoriev; D. S. Rokhsar; A. R. Grossman, *Science* **2007**, *318*, 245–251.
35. C. H. Wellman; P. L. Osterloff; U. Mohiuddin, *Nature* **2003**, *425*, 282–285.
36. B. Crandall-Stotler; R. E. Stotler, Morphology and Classification of the Marchantiophyta. In *Bryophyte Biology*; A. J. Shaw, B. Goffinet, Eds.; Cambridge University Press: Cambridge, UK, 2000; pp 21–70.
37. L. L. Forrest; E. C. Davis; D. G. Long; B. J. Crandall-Stotler; A. Clark; M. L. Hollingsworth, *The Bryologist* **2006**, *109*, 303–334.
38. R. J. Duff; J. C. Villarreal; D. C. Cargill; K. S. Renzaglia, *The Bryologist* **2007**, *110*, 214–243.
39. D. L. Nickrent; C. L. Parkinson; J. D. Palmer; R. J. Duff, *Mol. Biol. Evol.* **2000**, *17*, 1885–1895.
40. Y.-L. Qiu; L. Li; B. Wang; Z. Chen; V. Knoop; M. Groth-Malonek; O. Dombrovska; J. Lee; L. Kent; J. Rest; G. F. Estabrook; T. A. Hendry; D. W. Taylor; C. M. Testa; M. Ambros; B. Crandall-Stotler; R. J. Duff; M. Stech; W. Frey; D. Quandt; C. C. Davis, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 15511–15516.
41. F. Němejc; B. Pacltová, *Palaeobotanist* **1974**, *21*, 23–26.
42. B. A. Thomas, *Ann. Bot.* **1972**, *36*, 155–161.
43. A. V. Troitsky; M. S. Ignatov; V. K. Bobrova; I. A. Milyutina, *Biochemistry (Moscow)* **2007**, *72*, 1368–1376.
44. Y. Asakawa; N. Tokunaga; M. Toyota; T. Takemoto; C. Suire, *J. Hattori Bot. Lab.* **1979**, *45*, 395–407.
45. Y. Asakawa; N. Tokunaga; T. Takemoto; S. Hattori; M. Mizutani; C. Suire, *J. Hattori Bot. Lab.* **1980**, *47*, 153–164.
46. S. R. Gradstein; R. Matsuda; Y. Asakawa, *J. Hattori Bot. Lab.* **1981**, *50*, 231–248.
47. S. R. Gradstein; R. Matsuda; Y. Asakawa, *Nova Hedwigia* **1985**, *80*, 63–86.
48. J. D. Connolly, Recent Advances in the Chemistry of the Hepaticae. In *Natural Products Chemistry*; R. I. Zalewski, J. J. Skolik, Eds.; Elsevier Science Publishers: Amsterdam, 1985; pp 3–14.
49. A. C. Figueiredo; M. Sim-Sim; J. G. Barroso; L. G; Pedro; P. A. G. Santos; S. S. Fontinha; J. Schripsema; S. G. Deans; J. J. C. Scheffer, *J. Essent. Oil Res.* **2002**, *14*, 439–442.
50. R. B. Rickards, *Geol. Mag.* **2000**, *137*, 207–209.
51. N. Radulović; G. Stojanović; R. Palić, *Phytother. Res.* **2006**, *20*, 85–88.
52. S.-M. Chaw; A. Zharkikh; H.-M. Sung; T.-C. Lau; W.-H. Li, *Mol. Biol. Evol.* **1997**, *14*, 56–68.
53. S.-M. Chaw; C. L. Parkinson; Y. Cheng; T. M. Vincent; J. D. Palmer, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 4086–4091.
54. S.-M. Chaw; T. W. Walters; C.-C. Chang; S.-H. Hu; S.-H. Chen, *Mol. Phylogen. Evol.* **2005**, *37*, 214–234.
55. K. D. Hill; D. W. Stevenson; R. Osborne, *Bot. Rev.* **2004**, *70*, 274–298.
56. H. Azuma; M. Kono, *J. Plant Res.* **2006**, *119*, 671–676.
57. A. Scott, *Nature* **1974**, *251*, 707–708.
58. A. C. Scott; W. G. Chaloner, *Proc. R. Soc. London Ser. B* **1983**, *220*, 163–182.
59. F. A. Bisby; Y. R. Roskov; T. M. Orrell; D. Nicolson; L. E. Paglinawan; N. Bailly; P. M. Kirk; T. Bourgoin; J. van Hertum, Species 2000 & ITIS Catalogue of Life: 2008 Annual Checklist. Digital resource at www.catalogueoflife.org/annual-checklist/2008/. Reading, U.K.
60. O. Faix; D. Meier; I. Grobe, *J. Anal. Appl. Pyrolysis* **1987**, *11*, 403–416.
61. S. Camarero; P. Bocchini; G. C. Galletti; A. T. Martínez, *Rapid Commun. Mass Spectrom.* **1999**, *13*, 630–636.
62. D. M. Shrimpton, *Bi-Monthly Research Notes – Canada, Forestry Service* **1974**, *30*, 12.
63. K. Snajberk; E. Zavarin, *Phytochemistry* **1975**, *14*, 2025–2028.

64. S. Shatar; R. P. Adams, *J. Essent. Oil Res.* **1996**, *8*, 549–552.
65. A. Wibe; A.-K. Borg-Karlson; T. Norin; H. Mustaparta, *J. Comp. Physiol. A* **1997**, *180*, 585–595.
66. S. Rezzi; A. Muselli; A. Bighelli; J. Casanova, *Rivista Italiana EPPOS* **1998**, 766–772.
67. G. D. L. Boyd. The composition of terpenes and 4-allylanisole in loblolly southern yellow pine oleoresin and their potential impact on host selection by the female southern pine beetle. Ph.D. Thesis. Mississippi State University, MS, USA, 1998.
68. J. Velásquez; M. E. Toro; O. Encinas; L. Rojas; A. Usubillaga, *Flavour Fragr. J.* **2000**, *15*, 432–433.
69. E. M. Pettersson; B. T. Sullivan; P. Anderson; C. W. Berisford; G. Birgersson, *J. Chem. Ecol.* **2000**, *26*, 2507–2525.
70. M. Krauze-Baranowska; M. Mardarowicz; M. Wiwart; L. Poblocka; M. Dynowska, *Z. Naturforsch. C* **2002**, *57*, 478–482.
71. M. W. Ghosn; N. A. Saliba; S. Y. Talhouk, *J. Essent. Oil Res.* **2006**, *18*, 445–447.
72. K. Kurose; D. Okamura; M. Yatagai, *Flavour Fragr. J.* **2007**, *22*, 10–20.
73. J. J. Brophy; R. J. Goldsack; A. C. Rozefelds, *J. Essent. Oil Res.* **2003**, *15*, 217–220.
74. R. P. Adams, *Biochem. Syst. Ecol.* **1998**, *26*, 637–645.
75. Z.-Q. Wang, *Ann. Bot.* **2004**, *94*, 281–288.
76. Z. Zhou; S. Zheng, *Nature* **2003**, *423*, 821–822.
77. G. Sun; Q. Ji; D. L. Dilcher; S. Zheng; K. C. Nixon; X. Wang, *Science* **2002**, *296*, 899–904.
78. R. F. Thorne, *Taxon* **2002**, *51*, 511–512.
79. J. Kałużna-Czaplińska, *Acta Chromat.* **2007**, *19*, 279–282.
80. R. Nishida; K.-H. Tan; S.-L. Wee; A. K.-W. Hee; Y.-C. Toong, *Biochem. Syst. Ecol.* **2004**, *32*, 245–252.
81. G. M. Nano; A. Martelli; P. Sancin, *Rivista Italiana Essenze, Profumi, Piante Officinali, Aromi, Saponi, Cosmetici, Aerosol* **1966**, *48*, 409–412.
82. O. Vostrowsky; K. Michaelis; H. Ihm; R. Zintl; K. Knobloch, *Z. Lebensm.-Unters. Forsch.* **1981**, *173*, 365–367.
83. W. Lenz, *Zeit. anal. Chem.* **1894**, *33*, 193–200.
84. H. Thoms, *Arch. Pharm.* **1903**, *241*, 592–603.
85. A. K. Srivastava; S. K. Srivastava; K. V. Syamsundar, *Flavour Fragr. J.* **2005**, *20*, 51–53.
86. K. Chaieb; H. Hajlaoui; T. Zmantar; A. B. Kahla-Nakbi; M. Rouabhia; K. Mahdouani; A. Bakhrouf, *Phytother. Res.* **2007**, *21*, 501–506.
87. E. H. A. Andrade; L. M. M. Carreira; M. H. L. da Silva; J. D. da Silva; C. N. Bastos; P. J. C. Sousa; E. F. Guimarães; J. G. S. Maia, *Chem. Biodivers.* **2008**, *5*, 197–208.
88. A. J. Amonkar; M. Nagabhushan; A. V. D'Souza; S. V. Bhide, *Food Chem. Toxicol.* **1986**, *24*, 1321–1324.
89. F. W. Semmler, *Ber.* **1890**, *23*, 1803–1810.
90. F. B. Power; A. H. Salway, *J. Chem. Soc.* **1907**, *91*, 2037–2058.
91. A. W. Archer, *J. Chromatogr. A* **1988**, *438*, 117–121.
92. D. Ehlers; J. Kirchhoff; D. Gerard; K.-W. Quirin, *Int. J. Food Sci. Tech.* **1998**, *33*, 215–223.
93. G. R. Mallavarapu; S. Ramesh, *J. Med. Aromat. Plant Sci.* **1998**, *20*, 746–748.
94. M. Jukić; O. Politeo; M. Miloš, *Croat. Chem. Acta* **2006**, *79*, 209–214.
95. M. Marotti; R. Piccaglia, *J. Essent. Oil Res.* **1992**, *4*, 569–576.
96. A. Orav; A. Raal; E. Arak, *Nat. Prod. Res. Part A* **2008**, *22*, 227–232.
97. R. Spitaler; E.-P. Ellmerer-Müller; C. Zidorn; H. Stuppner, *Sci. Pharm.* **2002**, *70*, 101–109.
98. E. P. Lichtenstein; T. T. Liang; K. R. Schulz; H. K. Schnoes; G. T. Carter, *J. Agric. Food Chem.* **1974**, *22*, 658–664.
99. A. J. MacLeod; C. H. Snyder; G. Subramanian, *Phytochemistry* **1985**, *24*, 2623–2627.
100. A. J. MacLeod; G. MacLeod; G. Subramanian, *Phytochemistry* **1988**, *27*, 373–375.
101. L. Tóth, *Planta Med.* **1967**, *15*, 157–172.
102. Y.-R. Naves, *Perfum. Essent. Oil Rec.* **1950**, *41*, 286–287.
103. R. J. Grayer; G. C. Kite; F. J. Goldstone; S. E. Bryan; A. Paton; E. Putievsky, *Phytochemistry* **1996**, *43*, 1033–1039.
104. M. Nitta; H. Kobayashi; M. Ohnishi-Kameyama; T. Nagamine; M. Yoshida, *Biochem. Syst. Ecol.* **2006**, *34*, 25–37.
105. N. Tabanca; A. W. Douglas; E. Bedir; F. E. Dayan; N. Kirimer; K. H. C. Baser; Z. Aytac; I. A. Khan; B. E. Scheffler, *Plant Genet. Resour.* **2005**, *3*, 149–169.
106. K. H. C. Baser; N. Tabanca; N. Kirimer; E. Bedir; I. A. Khan; D. E. Wedge, *Pure Appl. Chem.* **2007**, *79*, 539–556.
107. G. Özek; T. Özek; G. Iscan; K. H. C. Baser; A. Duran; E. Hamzaoglu, *J. Essent. Oil Res.* **2008**, *20*, 186–190.
108. O. Özek; T. Özek; G. Iscan; K. H. C. Baser; E. Hamzaoglu; A. Duran, *S. Afr. J. Bot.* **2007**, *73*, 563–569.
109. N. Tabanca; B. Demirci; K. H. C. Baser; E. Mincsovics; S. I. Khan; M. R. Jacob; D. E. Wedge, *J. Chromatog. B* **2007**, *850*, 221–229.
110. A. Tosun; M. Kürkçüoglu; K. H. C. Baser; H. Duman, *J. Essent. Oil Res.* **2007**, *19*, 153–154.
111. A. Smelcerovic; M. Spiteller; A. P. Ligon; Z. Smelcerovic; N. Raabe, *Biochem. Syst. Ecol.* **2007**, *35*, 99–113.
112. K. H. C. Baser; H. Duman; Z. Aytaç, *J. Essent. Oil Res.* **2000**, *12*, 475–477.
113. I. Bohn; K.-H. Kubeczka; W. Schultze, *Planta Med.* **1989**, *55*, 489–490.
114. V. Dev; C. S. Mathela; A. B. Melkani; N. M. Pope; N. S. Sturm; A. T. Bottini, *Phytochemistry* **1989**, *28*, 1531–1532.
115. S. A. Rensing; D. Lang; A. D. Zimmer; A. Terry; A. Salamov; H. Shapiro; T. Nishiyama; P.-F. Perroud; E. A. Lindquist; Y. Kamisugi; T. Tanahashi; K. Sakakibara; T. Fujita; K. Oishi; T. Shin-I; Y. Kuroki; A. Toyoda; Y. Suzuki; S.-I. Hashimoto; K. Yamaguchi; S. Sugano; Y. Kohara; A. Fujiyama; A. Anterola; S. Aoki; N. Ashton; W. B. Barbazuk; E. Barker; J. L. Bennetzen; R. Blankenship; S. H. Cho; S. K. Dutcher; M. Estelle; J. A. Fawcett; H. Gundlach; K. Hanada; A. Heyl; K. A. Hicks; J. Hughes; M. Lohr; K. Mayer; A. Melkozernov; T. Murata; D. R. Nelson; B. Pils; M. Prigge; B. Reiss; T. Renner; S. Rombauts; P. J. Rushton; A. Sanderfoot; G. Schween; S.-H. Shiu; K. Stueber; F. L. Theodoulou; H. Tu; Y. Van de Peer; P. J. Verrier; E. Waters; A. Wood; L. Yang; D. Cove; A. C. Cuming; M. Hasebe; S. Lucas; B. D. Mishler; R. Reski; I. V. Grigoriev; R. S. Quatrano; J. L. Boore, *Science* **2008**, *319*, 64–69.
116. M. V. Silber; H. Meimberg; J. Ebel, *Phytochemistry* **2008**, *69*, 2449–2456.
117. M. M. A. El-Gendy; U. W. Hawas; M. Jaspars, *J. Antibiot.* **2008**, *61*, 379–386.
118. M. Berner; D. Krug; C. Bihlmaier; A. Vente; R. Müller; A. Bechthold, *J. Bacteriol.* **2006**, *188*, 2666–2673.
119. F. Cullmann; K.-P. Adam; H. Becker, *Phytochemistry* **1993**, *34*, 831–834.

120. F. Cullmann; K.-P. Adam; J. Zapp; H. Becker, *Phytochemistry* **1996**, *41*, 611–615.
121. F. Cullmann; A. Schmidt; F. Schuld; M. L. Trennheuser; H. Becker, *Phytochemistry* **1999**, *52*, 1647–1650.
122. H. Tazaki; T. Hayashida; F. Ishikawa; D. Taguchi; T. Takasawa; K. Nabeta, *Tetrahedron Lett.* **1999**, *40*, 101–104.
123. H. Tazaki; K.-P. Adam; H. Becker, *Phytochemistry* **1995**, *40*, 1671–1675.
124. F. Cullmann; H. Becker, *Phytochemistry* **1999**, *52*, 1651–1656.
125. U. Martini; J. Zapp; H. Becker, *Phytochemistry* **1998**, *49*, 1139–1146.
126. J. M. Scher; J. Zapp; H. Becker, *Phytochemistry* **2003**, *62*, 769–777.
127. H. Tazaki, *Curr. Top. Phytochem.* **2002**, *5*, 141–151.
128. F. Nagashima; M. Toyota; Y. Asakawa, *Phytochemistry* **1990**, *29*, 2169–2174.
129. R. Takeda; J. Hasegawa; M. Shinozaki, *Tetrahedron Lett.* **1990**, *31*, 4159–4162.
130. R. Takeda; J. Hasegawa; K. Sinozaki, Phenolic compounds from Anthocerotae. In *Bryophytes. Their Chemistry and Chemical Taxonomy*; H. D. Zinsmeister, R. Mues, Eds.; Clarendon Press: Oxford, UK, 1990; pp 201–207.
131. R. C. Lin; A.-L. Skaltsounis; E. Seguin; F. Tillequin; M. Koch, *Planta Med.* **1994**, *60*, 168–170.
132. E. White; G. H. N. Towers, *Phytochemistry* **1967**, *6*, 663–667.
133. Z. Jin; Y. Matsumoto; T. Tange; T. Akiyama; M. Higuchi; T. Ishii; K. Iiyama, *J. Wood Sci.* **2005**, *51*, 424–426.
134. X.-k. Zheng; S.-p. Shi; Y.-f. Bi; W.-s. Feng; J.-f. Wang; J.-z. Niu, *Yaoxue Xuebao (Acta Pharmaceutica Sinica)* **2004**, *39*, 719–721.
135. X. K. Zheng; K. K. Li; Y. Z. Wang; W. S. Feng, *Chin. Chem. Lett.* **2008**, *19*, 79–81.
136. J.-K. Weng; X. Li; J. Stout; C. Chapple, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 7887–7892.
137. H. Wada; T. Kido; N. Tanaka; T. Murakami; Y. Saiki; C.-M. Chen, *Chem. Pharm. Bull.* **1992**, *40*, 2099–2101.
138. L. B. Davin; C.-Z. Wang; G. L. Helms; N. G. Lewis, *Phytochemistry* **2003**, *62*, 501–511.
139. T. Satake; T. Murakami; Y. Saiki; C.-M. Chen, *Chem. Pharm. Bull.* **1978**, *26*, 1619–1622.
140. S. Cao; M. M. Radwan; A. Norris; J. S. Miller; F. Ratovoson; A. Mamisoa; R. Andriantsiferana; V. E. Rasamison; S. Rakotonandrasana; D. G. I. Kingston, *J. Nat. Prod.* **2006**, *69*, 284–286.
141. N. G. Lewis; M. J. Kato; N. Lopes; L. B. Davin, Lignans: Diversity, Biosynthesis and Function. In *Chemistry of the Amazon: Biodiversity, Natural Products, and Environmental Issues*; R. S. Seidl, O. R. Gottlieb, M. A. C. Kaplan, Eds.; ACS Symposium Series: Washington, DC, USA, 1995; Vol. 588, pp 135–167.
142. M. A. Castro; M. Gordaliza; J. M. Miguel Del Corral; A. San Feliciano, *Phytochemistry* **1996**, *41*, 995–1011.
143. D. R. Gang; M. Fujita; L. B. Davin; N. G. Lewis, The ''Abnormal Lignins'': Mapping Heartwood Formation Through the Lignan Biosynthetic Pathway. In *Lignin and Lignan Biosynthesis*; N. G. Lewis, S. Sarkanen, Eds.; ACS Symposium Series: Washington, DC, USA, 1998; Vol. 697, pp 389–421.
144. B. Holmbom; C. Eckerman; P. Eklund; J. Hemming; L. Nisula; M. Reunanen; R. Sjöholm; A. Sundberg; K. Sundberg; S. Willför, *Phytochem. Rev.* **2003**, *2*, 331–340.
145. A. San Feliciano; M. Medarde; J. L. Lopez; P. Puebla; J. M. Miguel Del Corral; A. F. Barrero, *Phytochemistry* **1989**, *28*, 2863–2866.
146. J.-M. Fang; K.-C. Hsu; Y.-S. Cheng, *Phytochemistry* **1989**, *28*, 3553–3555.
147. M. Nose; M. A. Bernards; M. Furlan; J. Zajicek; T. L. Eberhardt; N. G. Lewis, *Phytochemistry* **1995**, *39*, 71–79.
148. L. H. Briggs; R. C. Cambie; J. L. Hoare, *Tetrahedron* **1959**, *7*, 262–269.
149. L. H. Briggs; R. C. Cambie; J. L. Hoare, *Tetrahedron Lett.* **1959**, *1*, 14–15.
150. A. San Feliciano; J. M. Miguel Del Corral; M. Gordaliza; A. Castro, *Phytochemistry* **1991**, *30*, 3483–3485.
151. J. A. F. Gardner; G. M. Barton; H. MacLean, *Can. J. Chem.* **1959**, *37*, 1703–1709.
152. J. A. F. Gardner; B. F. MacDonald; H. MacLean, *Can. J. Chem.* **1960**, *38*, 2387–2394.
153. J. A. F. Gardner; E. P. Swan; S. A. Sutherland; H. MacLean, *Can. J. Chem.* **1966**, *44*, 52–58.
154. R. L. Krahmer; R. W. Hemingway; W. E. Hillis, *Wood Sci.Technol.* **1970**, *4*, 122–139.
155. F. Kawamura; H. Ohashi; S. Kawai; F. Teratani; Y. Kai, *Mokuzai Gakkaishi* **1996**, *42*, 301–307.
156. C. I. Johansson; J. N. Saddler; R. P. Beatson, *Holzforschung* **2000**, *54*, 246–254.
157. H. Hovelstad; I. Leirset; K. Oyaas; A. Fiksdahl, *Molecules* **2006**, *11*, 103–114.
158. J.-M. Fang; C.-K. Lee; Y.-S. Cheng, *Phytochemistry* **1992**, *31*, 3659–3661.
159. P. K. Agrawal; S. K. Agarwal; R. P. Rastogi, *Phytochemistry* **1980**, *19*, 1260–1261.
160. W.-C. Su; J.-M. Fang; Y.-S. Cheng, *Phytochemistry* **1995**, *40*, 563–566.
161. C. Kraus; G. Spiteller, *Phytochemistry* **1997**, *44*, 59–67.
162. S. Suzuki; T. Umezawa, *J. Wood Sci.* **2007**, *53*, 273–284.
163. C. R. Enzell; B. R. Thomas, *Acta Chem. Scand.* **1965**, *19*, 913–919.
164. C. R. Enzell; Y. Hirose; B. R. Thomas, *Tetrahedron Lett.* **1967**, *8*, 793–798.
165. P. Henley-Smith; D. A. Whiting, *Phytochemistry* **1976**, *15*, 1285–1287.
166. K. Takahashi; M. Yasue; K. Ogiyama, *Phytochemistry* **1988**, *27*, 1550–1552.
167. K. Takahashi; K. Ogiyama, *Mokuzai Gakkaishi* **1986**, *32*, 457–461.
168. S. Suzuki; M. Yamamura; M. Shimada; T. Umezawa, *J. Chem. Soc. Chem. Commun.* **2004**, 2838–2839.
169. P. Daniels; H. Erdtman; K. Nishimura; T. Novin; P. Kierkegaard; A.-M. Pilotti, *J. Chem. Soc. Chem. Commun.* **1972**, 246–247.
170. A. Enoki; S. Takahama; K. Kitao, *Mokuzai Gakkaishi* **1977**, *23*, 579–586.
171. A. Enoki; S. Takahama; K. Kitao, *Mokuzai Gakkaishi* **1977**, *23*, 587–593.
172. H. Ohashi; S. Kawai; Y. Sakurai; M. Yasue, *Phytochemistry* **1992**, *31*, 1371–1373.
173. I. Iliya; Z. Ali; T. Tanaka; M. Iinuma; M. Furusawa; K.-i. Nakaya; J. Murata; D. Darnaedi; N. Matsuura; M. Ubukata, *Phytochemistry* **2003**, *62*, 601–606.
174. M. A. M. Nawwar; H. H. Barakat; J. Buddrus; M. Linscheid, *Phytochemistry* **1985**, *24*, 878–879.
175. S. V. Pullela; S. Takamatsu; S. I. Khan; I. A. Khan, *Planta Med.* **2005**, *71*, 789–791.
176. M. Tamada; K. Endo; H. Hikino, *Heterocycles* **1979**, *12*, 783–786.
177. M. Tamada; K. Endo; H. Hikino; C. Kabuto, *Tetrahedron Lett.* **1979**, *20*, 873–876.
178. C. Konno; M. Tamada; K. Endo; H. Hikino, *Heterocycles* **1980**, *14*, 295–298.
179. T. Kariyone; H. Kimura; I. Nakamura, *Yakugaku Zasshi* **1958**, *78*, 1152–1155.

180. E. Bedir; I. I. Tatli; R. A. Khan; J. Zhao; S. Takamatsu; L. A. Walker; P. Goldman; I. A. Khan, *J. Agric. Food Chem.* **2002**, *50*, 3150–3155.
181. R. S. Ward, *Nat. Prod. Rep.* **1997**, *14*, 43–74.
182. R. S. Ward, *Nat. Prod. Rep.* **1999**, *16*, 75–96.
183. R. D. Hartley; E. C. Jones, *Phytochemistry* **1976**, *15*, 1157–1160.
184. H. U. Markwalder; H. Neukom, *Phytochemistry* **1976**, *15*, 836–837.
185. C.-M. Chen; F.-Y. Jan; M.-T. Chen; T.-J. Lee, *Heterocycles* **1989**, *29*, 411–414.
186. A. R. Carroll; W. C. Taylor, *Aust. J. Chem.* **1991**, *44*, 1615–1626.
187. S. E. Drewes; M. M. Horn; B. M. Sehlapelo; N. Ramesar; J. S. Field; R. S. Shaw; P. Sandor, *Phytochemistry* **1995**, *38*, 1505–1508.
188. S. Nishibe; A. Sakushima; S. Kitagawa; B. Klimek; R. Benecke; H. Thieme, *Shoyakugaku Zasshi* **1988**, *42*, 324–328.
189. J. L. Hartwell; A. W. Schrecker, *J. Am. Chem. Soc.* **1951**, *73*, 2909–2916.
190. Y. Ikeya; H. Taguchi; I. Yosioka; H. Kobayashi, *Chem. Pharm. Bull.* **1979**, *27*, 1383–1394.
191. R. A. Davis; A. R. Carroll; S. Duffy; V. M. Avery; G. P. Guymer; P. I. Forster; R. J. Quinn, *J. Nat. Prod.* **2007**, *70*, 1118–1121.
192. E.-C. Wang; M.-H. Shih; M.-C. Liu; M.-T. Chen; G.-H. Lee, *Heterocycles* **1996**, *43*, 969–976.
193. B. Goezler; B. Kivcak; G. Arar; T. Goezler; M. Hesse, *Heterocycles* **1994**, *39*, 243–250.
194. M. Haruna; T. Koube; K. Ito; H. Murata, *Chem. Pharm. Bull.* **1982**, *30*, 1525–1527.
195. S. M. Kupchan; K. L. Stevens; E. A. Rohlfing; B. R. Sickles; A. T. Sneden; R. W. Miller; R. F. Bryan, *J. Org. Chem.* **1978**, *43*, 586–590.
196. M. N. Lopes; M. S. Da Silva; J. M. Barbosa; Z. S. Ferreira; M. Yoshida; O. R. Gottlieb, *Phytochemistry* **1986**, *25*, 2609–2612.
197. N. H. Anh; H. Ripperger; A. Porzel; T. V. Sung; G. Adam, *Phytochemistry* **1997**, *46*, 569–571.
198. R. Braz Filho; M. G. De Carvalho; O. R. Gottlieb; J. G. S. Maia; M. L. Da Silva, *Phytochemistry* **1981**, *20*, 2049–2050.
199. Z. Zhang; H. N. ElSohly; X.-C. Li; S. I. Khan; S. E. Broedel, Jr.; R. E. Raulli; R. L. Cihlar; C. Burandt; L. A. Walker, *J. Nat. Prod.* **2003**, *66*, 548–550.
200. H. Taguchi; Y. Ikeya, *Chem. Pharm. Bull*. **1977**, *25*, 264–366.
201. V. S. Parmar; S. C. Jain; K. S. Bisht; R. Jain; P. Taneja; A. Jha; O. D. Tyagi; A. K. Prasad; J. Wengel; C. E. Olsen; P. M. Boll, *Phytochemistry* **1997**, *46*, 597–673.
202. R. D. Hartley; C. W. Ford, Phenolic Constituents of Plant Cell Walls and Wall Biodegradability. In *Plant Cell Wall Polymers. Biogenesis and Biodegradation*; N. G. Lewis, M. G. Paice, Eds.; ACS Symposium Series: Washington, DC, USA, 1989; Vol. 399, 137–145.
203. C. W. Ford; R. D. Hartley, *J. Sci. Food Agric.* **1990**, *50*, 29–43.
204. S. Tachibana; K. Ohkubo; G. H. N. Towers, *Phytochemistry* **1992**, *31*, 81–83.
205. A. Suga; Y. Takaishi; S. Goto; T. Munakata; I. Yamauchi; K. Kogure, *Phytochemistry* **2003**, *64*, 991–996.
206. W.-Y. Tsui; G. D. Brown, *Phytochemistry* **1996**, *43*, 1413–1415.
207. S. Suzuki; M. Yamamura; T. Hattori; T. Nakatsubo; T. Umezawa, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 21008–21013.
208. E. Minami; M. Taki; S. Takaishi; Y. Iijima; S. Tsutsumi; T. Akiyama, *Chem. Pharm. Bull.* **2000**, *48*, 389–392.
209. I. Messana; J. D. Msonthi; Y. De Vicente; G. Multari; C. Galeffi, *Phytochemistry* **1989**, *28*, 2807–2809.
210. G. B. Marini-Bettolo; M. Nicoletti; I. Messana; C. Galeffi; J. D. Msonthi; W. A. Chapya, *Tetrahedron* **1985**, *41*, 665–670.
211. K. Terada; W. Kamisako, *Biol. Pharm. Bull.* **1999**, *22*, 561–566.
212. G. B. Marini Bettolo; M. Patamia; M. Nicoletti; C. Galeffi; I. Messana, *Tetrahedron* **1982**, *38*, 1683–1687.
213. W.-L. Chang; C.-H. Chen; S.-S. Lee, *J. Nat. Prod.* **1999**, *62*, 734–739.
214. K. M. Mohamed; M. A. Fouad; K. Matsunami; M. S. Kamel; H. Otsuka, *ARKIVOC* **2007**, 63–70.
215. B. Talapatra; T. Ray; S. K. Talapatra, *Indian J. Chem. Sect B* **1976**, *14B*, 613–614.
216. K. Kawazoe; A. Yutani; Y. Takaishi, *Phytochemistry* **1999**, *52*, 1657–1659.
217. M. Miyazawa; M. Hisama, *J. Agric. Food Chem.* **2003**, *51*, 6413–6422.
218. P. J. C. Benevides; P. Sartorelli; M. J. Kato, *Phytochemistry* **1999**, *52*, 339–343.
219. T. J. Mabry; D. R. DiFeo, Jr.; M. Sakakibara; C. F. Bohnstedt, Jr.; D. Seigler, The Natural Products Chemistry of *Larrea*. In *Creosote Bush – Biology and Chemistry of Larrea in New World Deserts*; T. J. Mabry, J. H. Hunziker, D. R. DiFeo, Jr., Eds.; Dowden, Hutchinson & Ross, Inc.: Stroudsburg, PA, USA, 1977; pp 115–134.
220. T. Umezawa; L. B. Davin; N. G. Lewis, *Biochem. Biophys. Res. Commun.* **1990**, *171*, 1008–1014.
221. T. Umezawa; L. B. Davin; E. Yamamoto; D. G. I. Kingston; N. G. Lewis, *J. Chem. Soc. Chem. Commun.* **1990**, 1405–1408.
222. T. Umezawa; L. B. Davin; N. G. Lewis, *J. Biol. Chem.* **1991**, *266*, 10210–10217.
223. T. Katayama; L. B. Davin; N. G. Lewis, *Phytochemistry* **1992**, *31*, 3875–3881.
224. T. Katayama; L. B. Davin; A. Chu; N. G. Lewis, *Phytochemistry* **1993**, *33*, 581–591.
225. Y. Jiao; L. B. Davin; N. G. Lewis, *Phytochemistry* **1998**, *49*, 387–394.
226. Z.-Q. Xia; M. A. Costa; J. Proctor; L. B. Davin; N. G. Lewis, *Phytochemistry* **2000**, *55*, 537–549.
227. M. Chiba; S. Hisada; S. Nishibe, *Shoyakugaku Zasshi* **1978**, *32*, 194–197.
228. K.-J. Kim, *Plant Syst. Evol.* **1999**, *218*, 113–123.
229. L. B. Davin; D. L. Bedgar; T. Katayama; N. G. Lewis, *Phytochemistry* **1992**, *31*, 3869–3874.
230. L. B. Davin; H.-B. Wang; A. L. Crowell; D. L. Bedgar; D. M. Martin; S. Sarkanen; N. G. Lewis, *Science* **1997**, *275*, 362–367.
231. S. C. Halls; L. B. Davin; D. M. Kramer; N. G. Lewis, *Biochemistry* **2004**, *43*, 2587–2595.
232. D. R. Gang; M. A. Costa; M. Fujita; A. T. Dinkova-Kostova; H.-B. Wang; V. Burlat; W. Martin; S. Sarkanen; L. B. Davin; N. G. Lewis, *Chem. Biol.* **1999**, *6*, 143–151.
233. M. K. Kim; J.-H. Jeon; M. Fujita; L. B. Davin; N. G. Lewis, *Plant Mol. Biol.* **2002**, *49*, 199–214.
234. S. C. Halls; N. G. Lewis, *Biochemistry* **2002**, *41*, 9455–9461.
235. D. Frishman; P. Argos, *Protein Eng.* **1996**, *9*, 133–142.
236. L. Zhuang; O. Seligmann; K. Jurcic; H. Wagner, *Planta Med.* **1982**, *45*, 172–176.
237. T. Nakatsubo; M. Mizutani; S. Suzuki; T. Hattori; T. Umezawa, *J. Biol. Chem.* **2008**, *283*, 15550–15557.

238. M. Kwon; V. Burlat; L. B. Davin; N. G. Lewis, Localization of Dirigent Protein Involved in Lignan Biosynthesis: Implications for Lignification at the Tissue and Subcellular Level. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; G. G. Gross, R. W. Hemingway, T. Yoshida, Eds.; Kluwer Academic/Plenum Publishers: New York, NY, USA, 1999; pp 393–411.
239. V. Burlat; M. Kwon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2001**, *57*, 883–897.
240. A. M. Patten; L. B. Davin; N. G. Lewis, *Phytochemistry* **2008**, *69*, 3032–3037.
241. L. B. Davin; N. G. Lewis, *Plant Physiol.* **2000**, *123*, 453–461.
242. M. K. Kim; J.-H. Jeon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2002**, *61*, 311–322.
243. S. Ralph; J.-Y. Park; J. Bohlmann; S. D. Mansfield, *Plant Mol. Biol.* **2006**, *60*, 21–40.
244. S. G. Ralph; S. Jancsik; J. Bohlmann, *Phytochemistry* **2007**, *68*, 1975–1991.
245. S. Arteaga; A. Andrade-Cetto; R. Cárdenas, *J. Ethnopharmacol.* **2005**, *98*, 231–239.
246. C. Konno; Z.-Z. Lu; H.-Z. Xue; C. A. J. Erdelmeier; D. Meksuriyen; C.-T. Che; G. A. Cordell; D. D. Soejarto; D. P. Waller; H. H. S. Fong, *J. Nat. Prod.* **1990**, *53*, 396–406.
247. S. G. A. Moinuddin; S. Hishiyama; M.-H. Cho; L. B. Davin; N. G. Lewis, *Org. Biomol. Chem.* **2003**, *1*, 2307–2313.
248. P. Budowski; K. S. Markley, *Chem. Rev.* **1951**, *48*, 125–151.
249. M. Namiki, *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 651–673.
250. Y. Fukuda; T. Osawa; M. Namiki; T. Ozaki, *Agric. Biol. Chem.* **1985**, *49*, 301–306.
251. T. Osawa; M. Nagata; M. Namiki; Y. Fukuda, *Agric. Biol. Chem.* **1985**, *49*, 3351–3352.
252. Y. Fukuda; M. Nagata; T. Osawa; M. Namiki, *J. Amer. Oil Chem. Soc.* **1986**, *63*, 1027–1031.
253. A. De Candolle, *Origin of Cultivated Plants*; D. Appleton and Company: New York, NY, 1902.
254. H. Katsuzaki; S. Kawakishi; T. Osawa, *Phytochemistry* **1994**, *35*, 773–776.
255. S. N. Ryu; C.-T. Ho; T. Osawa, *J. Food Lipids* **1998**, *5*, 17–28.
256. A. A. Moazzami; R. E. Andersson; A. Kamal-Eldin, *J. Agric. Food Chem.* **2006**, *54*, 633–638.
257. W. Bauer; M. H. Zenk, *Phytochemistry* **1991**, *30*, 2953–2961.
258. E. Ono; M. Nakai; Y. Fukui; N. Tomimori; M. Fukuchi-Mizutani; M. Saito; H. Satake; T. Tanaka; M. Katsuta; T. Umezawa; Y. Tanaka, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 10116–10121.
259. N. Ikezawa; K. Iwasa; F. Sato, *FEBS J.* **2007**, *274*, 1019–1035.
260. A. Noguchi; Y. Fukui; A. Iuchi-Okada; S. Kakutani; H. Satake; T. Iwashita; M. Nakao; T. Umezawa; E. Ono, *Plant J.* **2008**, *54*, 415–427.
261. P. A. Marchand; M. J. Kato; N. G. Lewis, *J. Nat. Prod.* **1997**, *60*, 1189–1192.
262. M. J. Kato; A. Chu; L. B. Davin; N. G. Lewis, *Phytochemistry* **1998**, *47*, 583–591.
263. A. T. Dinkova-Kostova; D. R. Gang; L. B. Davin; D. L. Bedgar; A. Chu; N. G. Lewis, *J. Biol. Chem.* **1996**, *271*, 29473–29482.
264. M. Fujita; D. R. Gang; L. B. Davin; N. G. Lewis, *J. Biol. Chem.* **1999**, *274*, 618–627.
265. D. R. Gang; H. Kasahara; Z.-Q. Xia; K. Vander Mijnsbrugge; G. Bauw; W. Boerjan; M. Van Montagu; L. B. Davin; N. G. Lewis, *J. Biol. Chem.* **1999**, *274*, 7516–7527.
266. T. Min; H. Kasahara; D. L. Bedgar; B. Youn; P. K. Lawrence; D. R. Gang; S. C. Halls; H. Park; J. L. Hilsenbeck; L. B. Davin; N. G. Lewis; C. Kang, *J. Biol. Chem.* **2003**, *278*, 50714–50723.
267. N. G. Lewis; L. B. Davin; A. T. Dinkova-Kostova; J. D. Ford; M. Fujita; D. R. Gang; S. Sarkanen. Recombinant Pinoresinol/Lariciresinol Reductase, Recombinant Dirigent Protein, and Methods of Use. U.S. Patent 6,210,942, 3 April, 2001.
268. N. G. Lewis; L. B. Davin; S.-J. Kim; D. G. Vassão; A. M. Patten; D. Eichinger. Genes encoding chavicol/eugenol synthase form the creosote bush (*Larrea tridentata).* PCT Application no. PCT/US2007/069911, filed 29 May, 2007.
269. A. Chu; A. Dinkova; L. B. Davin; D. L. Bedgar; N. G. Lewis, *J. Biol. Chem.* **1993**, *268*, 27026–27033.
270. K.-W. Kim; S. G. A. Moinuddin; L. B. Davin; N. G. Lewis **2010**, Manuscript in preparation.
271. M. Hickey, *100 Families of Flowering Plants*, 2nd ed.; University Press: Cambridge, UK, 1988.
272. A. Diederichsen; K. Richards, Cultivated Flax and the Genus *Linum* L. Taxonomy and Germplasm Conservation. In *Flax. The Genus Linum*; A. D. Muir, N. D. Westcott, Eds.; Taylor & Francis: New York, NY, USA, 2003; pp 22–54.
273. P. P. Kolodziejczyk; P. Fedec, Processing Flaxseed for Human Consumption. In *Flaxseed in Human Nutrition*; S. C. Cunnane, L. U. Thompson, Eds.; AOCS Press: Champaign, IL, USA, 1995; pp 261–280.
274. D. Zohary; M. Hopf, Oil and Fibre Crops. In *Domestication of Plants in the Old World. The Origin and Spread of Cultivated Plants in West Asia, Europe and the Nile Valley*; 2nd ed., D. Zohary, M. Hopf, Eds.; Clarendon Press: Oxford, UK, 1993; pp 118–133.
275. J. D. Ford; K.-S. Huang; H.-B. Wang; L. B. Davin; N. G. Lewis, *J. Nat. Prod.* **2001**, *64*, 1388–1397.
276. A. Kamal-Eldin; N. Peerlkamp; P. Johnsson; R. Andersson; R. E. Andersson; L. N. Lundgren; P. Åman, *Phytochemistry* **2001**, *58*, 587–590.
277. K. Struijs; J.-P. Vincken; R. Verhoef; W. H. M. van Oostveen-van Casteren; A. G. J. Voragen; H. Gruppen, *Phytochemistry* **2007**, *68*, 1227–1235.
278. K. Struijs; J.-P. Vincken; T. G. Doeswijk; A. G. J. Voragen; H. Gruppen, *Phytochemistry* **2009**, *70*, 262–269.
279. K. Struijs; J.-P. Vincken; R. Verhoef; A. G. J. Voragen; H. Gruppen, *Phytochemistry* **2008**, *69*, 1250–1260.
280. T. J. Schmidt; S. Hemmati; E. Fuss; A. W. Alfermann, *Phytochem. Anal.* **2006**, *17*, 299–311.
281. T. J. Schmidt; A. W. Alfermann; E. Fuss, *Rapid Commun. Mass Spectrom* **2008**, *22*, 3642–3650.
282. J. D. Ford. Cancer Chemopreventive Flax Seed Lignans: Delineating the Metabolic Pathway(s) to the SDG-HMG Ester-Linked Polymers. Ph.D. Thesis, Washington State University, WA, USA, 2001.
283. L. B. Davin; N. G. Lewis, *Phytochem. Rev.* **2003**, *2*, 257–288.
284. K. H. Teoh; J. D. Ford; M.-R. Kim; L. B. Davin; N. G. Lewis, Delineating the Metabolic Pathway(s) to Secoisolariciresinol Diglucoside Hydroxymethyl Glutarate Oligomers in Flaxseed (*Linum usitatissimum)*. In *Flaxseed in Human Nutrition*, 2nd ed; L. U. Thompson, S. C. Cunnane, Eds.; AOCS Press: Champaign, IL, USA, 2003; pp 41–62.
285. C. B. I. von Heimendahl; K. M. Schäfer; P. Eklund; R. Sjöholm; T. J. Schmidt; E. Fuss, *Phytochemistry* **2005**, *66*, 1254–1263.
286. Ü. Bayindir; A. W. Alfermann; E. Fuss, *Plant J.* **2008**, *55*, 810–820.
287. S. Hemmati; T. J. Schmidt; E. Fuss, *FEBS Lett.* **2007**, *581*, 603–610.
288. K. Federolf; A. W. Alfermann; E. Fuss, *Phytochemistry* **2007**, *68*, 1397–1406.

289. A. Mohagheghzadeh; T. J. Schmidt; Ü. Bayindir; E. Fuss; I. Mehregan; A. W. Alfermann, *Planta Med.* **2006**, *72*, 1165–1167.
290. T. J. Schmidt; S. Vößing; M. Klaes; S. Grimme, *Planta Med.* **2007**, *73*, 1574–1580.
291. M. Kwon; L. B. Davin; N. G. Lewis, *Phytochemistry* **2001**, *57*, 899–914.
292. C. Hano; I. Martin; O. Fliniaux; B. Legrand; L. Gutierrez; R. R. J. Arroo; F. Mesnard; F. Lamblin; E. Lainé, *Planta* **2006**, *224*, 1291–1301.
293. B. Madhusudhan; D. Wiesenborn; K. Tostenson; J. Schwarz; J. Gillespie, *Lebensm.-Wiss. u.-Technol.* **2000**, *33*, 268–275.
294. H. Jörnvall; J.-O. Höög; B. Persson, *FEBS Lett.* **1999**, *445*, 261–264.
295. Z.-Q. Xia; M. A. Costa; H. C. Pélissier; L. B. Davin; N. G. Lewis, *J. Biol. Chem.* **2001**, *276*, 12614–12623.
296. S. G. A. Moinuddin; B. Youn; D. L. Bedgar; M. A. Costa; G. L. Helms; C. Kang; L. B. Davin; N. G. Lewis, *Org. Biomol. Chem.* **2006**, *4*, 808–816.
297. B. Youn; S. G. A. Moinuddin; L. B. Davin; N. G. Lewis; C. Kang, *J. Biol. Chem.* **2005**, *280*, 12917–12926.
298. M.-H. Cho; S. G. A. Moinuddin; G. L. Helms; S. Hishiyama; D. Eichinger; L. B. Davin; N. G. Lewis, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 10641–10646.
299. M. Okigawa; T. Maeda; N. Kawano, *Tetrahedron* **1970**, *26*, 4301–4305.
300. A. Mohagheghzadeh; T. J. Schmidt; A. W. Alfermann, *J. Nat. Prod.* **2002**, *65*, 69–71.
301. H. R. Vardapetyan; A. B. Kirakosyan; A. A. Oganesyan; A. R. Penesyan; W. A. Alfermann, *Russ. J. Plant Physiol.* **2003**, *50*, 297–300.
302. A. Koulman; B. Konuklugil, *Biochem. Syst. Ecol.* **2004**, *32*, 91–93.
303. N. Vasilev; I. Ionkova, *Pharm. Biol.* **2005**, *43*, 509–511.
304. N. P. Vasilev; I. Ionkova, *Fitoterapia* **2005**, *76*, 50–53.
305. N. Vasilev; Elfahmi; R. Bos; O. Kayser; G. Momekov; S. Konstantinov; I. Ionkova, *J. Nat. Prod.* **2006**, *69*, 1014–1017.
306. B. Konuklugil; I. Ionkova; N. Vasilev; T. J. Schmidt; J. Windhövel; E. Fuss; A. W. Alfermann, *Nat. Prod. Res.* **2007**, *21*, 1–6.
307. K. Kranz; M. Petersen, *Phytochemistry* **2003**, *64*, 453–458.
308. G. A. Molog; U. Empt; S. Kuhlmann; W. van Uden; N. Pras; A. W. Alfermann; M. Petersen, *Planta* **2001**, *214*, 288–294.
309. J. Liu; R. D. Stipanovic; A. A. Bell; L. S. Puckhaber; C. W. Magill, *Phytochemistry* **2008**, *69*, 3038–3042.
310. P. Sartorelli; P. J. C. Benevides; R. M. Ellensohn; M. V. A. F. Rocha; P. R. H. Moreno; M. J. Kato, *Plant Sci.* **2001**, *161*, 1083–1088.
311. T. Katayama; Y. Kado, *J. Wood Sci.* **1998**, *44*, 244–246.
312. N. Lourith; T. Katayama; K. Ishikawa; T. Suzuki, *J. Wood Sci.* **2005**, *51*, 379–386.
313. N. Lourith; T. Katayama; T. Suzuki, *J. Wood Sci.* **2005**, *51*, 370–378.
314. R. B. Herbert; A. E. Kattah; E. Knagg, *Tetrahedron* **1990**, *46*, 7119–7138.
315. H. Kasahara; L. B. Davin; N. G. Lewis. Aryl Propenal Double Bond Reductase. U.S. Patent 6,703,229, 9 March, 2004.
316. H. Kasahara; Y. Jiao; D. L. Bedgar; S.-J. Kim; A. M. Patten; Z.-Q. Xia; L. B. Davin; N. G. Lewis, *Phytochemistry* **2006**, *67*, 1765–1780.
317. R. A. Savidge; H. Förster, *Phytochemistry* **2001**, *57*, 1095–1103.
318. B. Youn; S.-J. Kim; S. G. A. Moinuddin; C. Lee; D. L. Bedgar; A. R. Harper; L. B. Davin; N. G. Lewis; C. Kang, *J. Biol. Chem.* **2006**, *281*, 40076–40088.
319. J. Mano; Y. Torii; S.-i. Hayashi; K. Takimoto; K. Matsui; K. Nakamura; D. Inzé; E. Babiychuk; S. Kushnir; K. Asada, *Plant Cell Physiol.* **2002**, *43*, 1445–1455.
320. J. Mano; E. Belles-Boix; E. Babiychuk; D. Inzé; Y. Torii; E. Hiraoka; K. Takimoto; L. Slooten; K. Asada; S. Kushnir, *Plant Physiol.* **2005**, *139*, 1773–1783.
321. Y. Zhang; K. H. Teoh; D. W. Reed; L. Maes; A. Goossens; D. J. H. Olson; A. R. S. Ross; P. S. Covello, *J. Biol. Chem.* **2008**, *283*, 21501–21508.
322. K. L. Ringer; M. E. McConkey; E. M. Davis; G. W. Rushing; R. Croteau, *Arch. Biochem. Biophys.* **2003**, *418*, 80–92.
323. T. Hori; T. Yokomizo; H. Ago; M. Sugahara; G. Ueno; M. Yamamoto; T. Kumasaka; T. Shimizu; M. Miyano, *J. Biol. Chem.* **2004**, *279*, 22615–22623.
324. K. Vander Mijnsbrugge; H. Beeckman; R. De Rycke; M. Van Montagu; G. Engler; W. Boerjan, *Planta* **2000**, *211*, 502–509.
325. K. Vander Mijnsbrugge; H. Meyermans; M. Van Montagu; G. Bauw; W. Boerjan, *Planta* **2000**, *210*, 589–598.
326. X. Wang; X. He; J. Lin; H. Shao; Z. Chang; R. A. Dixon, *J. Mol. Biol.* **2006**, *358*, 1341–1352.
327. T. Akashi; S. Koshimizu; T. Aoki; S.-i. Ayabe, *FEBS Lett.* **2006**, *580*, 5666–5670.
328. K. Terada; C. Honda; K. Suwa; S. Takeyama; H. Oku; W. Kamisako, *Chem. Pharm. Bull.* **1995**, *43*, 564–566.
329. S. Suzuki; T. Umezawa; M. Shimada, *J. Chem. Soc. Perkin Trans. 1* **2001**, 3252–3257.
330. S. Suzuki; T. Nakatsubo; T. Umezawa; M. Shimada, *J. Chem. Soc. Chem. Commun.* **2002**, 1088–1089.
331. T. Imai; M. Nomura; Y. Matsushita; K. Fukushima, *J. Plant Physiol.* **2006**, *163*, 1221–1228.
332. T. Imai; M. Nomura; K. Fukushima, *J. Plant Physiol.* **2006**, *163*, 483–487.
333. T. Nagasaki; S. Yasuda; T. Imai, *Phytochemistry* **2002**, *60*, 461–466.
334. T. Nagasaki; S. Yasuda; T. Imai, *Phytochemistry* **2001**, *58*, 833–840.
335. A. D. Bayley; J. van Staden, *Plant Physiol. Biochem.* **1990**, *28*, 691–695.
336. A. D. Bayley; J. van Staden, *Plant Physiol. Biochem.* **1990**, *28*, 697–702.
337. A. D. Bayley; J. van Staden, *Plant Physiol. Biochem.* **1991**, *29*, 403–408.
338. K. Terada; C. Honda; S. Takeyama; K. Suwa; W. Kamisako, *Biol. Pharm. Bull.* **1995**, *18*, 1472–1475.
339. K. Terada; K. Suwa; S. Takeyama; C. Honda; W. Kamisako, *Biol. Pharm. Bull.* **1996**, *19*, 748–751.
340. A. J. Birch; M. Slaytor, *Chem. Ind.* **1956**, 1524.
341. K. Kaneko, *Chem. Pharm. Bull.* **1960**, *8*, 611–614.
342. K. Kaneko, *Chem. Pharm. Bull.* **1960**, *8*, 875–879.
343. K. Kaneko, *Chem. Pharm. Bull.* **1961**, *9*, 108–109.
344. K. Kaneko, *Chem. Pharm. Bull.* **1962**, *10*, 1085–1087.
345. R. Tressl; F. Drawert, *J. Agric. Food Chem.* **1973**, *21*, 560–565.
346. M. Klischies; J. Stöckigt; M. H. Zenk, *J. Chem. Soc. Chem. Commun.* **1975**, 879–880.

347. A. J. Birch, Biosynthetic Pathways. In *Chemical Plant Taxonomy*; T. Swain, Ed.; Academic Press: London, UK, 1963; pp 141–166.
348. T. A. Geissman; D. H. G. Crout, Compounds Derived from Shikimic Acid. In *Organic Chemistry of Secondary Plant Metabolism*; T. A. Geissman, D. H. G. Crout, Eds.; Freeman, Cooper & Company: San Francisco, CA, USA, 1969; pp 136–166.
349. L. Canonica; P. Manitto; D. Monti; A. M. Sanchez, *J. Chem. Soc. Chem. Commun.* **1971**, 1108–1109.
350. P. Manitto; D. Monti; P. Gramatica, *J. Chem. Soc. Perkin Trans. 1* **1974**, 1727–1731.
351. U. M. Senanayake; R. B. H. Wills; T. H. Lee, *Phytochemistry* **1977**, *16*, 2032–2033.
352. D. R. Gang; J. Wang; N. Dudareva; K. H. Nam; J. E. Simon; E. Lewinsohn; E. Pichersky, *Plant Physiol.* **2001**, *125*, 539–555.
353. J. C. Verdonk; M. A. Haring; A. J. van Tunen; R. C. Schuurink, *Plant Cell* **2005**, *17*, 1612–1624.
354. T. Koeduka; G. V. Louie; I. Orlova; C. M. Kish; M. Ibdah; C. G. Wilkerson; M. E. Bowman; T. J. Baiga; J. P. Noel; N. Dudareva; E. Pichersky, *Plant J.* **2008**, *54*, 362–374.
355. G. V. Louie; T. J. Baiga; M. E. Bowman; T. Koeduka; J. H. Taylor; S. M. Spassova; E. Pichersky; J. P. Noel, *PLoS ONE* **2007**, *2* (10), e993.
356. T. Koeduka; T. J. Baiga; J. P. Noel; E. Pichersky, *Plant Physiol.* **2009**, *149*, 384–394.
357. S.-J. Kim; M.-R. Kim; D. L. Bedgar; S. G. A. Moinuddin; C. L. Cardenas; L. B. Davin; C. Kang; N. G. Lewis, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 1455–1460.
358. M. Jourdes; C. L. Cardenas; D. D. Laskar; S. G. A. Moinuddin; L. B. Davin; N. G. Lewis, *Phytochemistry* **2007**, *68*, 1932–1956.
359. D. M. Tieman; H. M. Loucas; J. Y. Kim; D. G. Clark; H. J. Klee, *Phytochemistry* **2007**, *68*, 2660–2669.
360. I. Damiani; K. Morreel; S. Danoun; G. Goeminne; N. Yahiaoui; C. Marque; J. Kopka; E. Messens; D. Goffner; W. Boerjan; A.-M. Boudet; S. Rochange, *Plant Mol. Biol.* **2005**, *59*, 753–769.
361. N. L. Paiva; R. Edwards; Y. Sun; G. Hrazdina; R. A. Dixon, *Plant Mol. Biol.* **1991**, *17*, 653–667.
362. N. L. Paiva; Y. Sun; R. A. Dixon; H. D. VanEtten; G. Hrazdina, *Arch. Biochem. Biophys.* **1994**, *312*, 501–510.
363. N. Oyama-Okubo; T. Ando; N. Watanabe; E. Marchesi; K. Uchida; M. Nakayama, *Biosci. Biotechnol. Biochem.* **2005**, *69*, 773–777.
364. J. Wang; N. Dudareva; S. Bhakta; R. A. Raguso; E. Pichersky, *Plant Physiol.* **1997**, *114*, 213–221.
365. J. Wang; E. Pichersky, *Arch. Biochem. Biophys.* **1998**, *349*, 153–160.
366. E. Lewinsohn; I. Ziv-Raz; N. Dudai; Y. Tadmor; E. Lastochkin; O. Larkov; D. Chaimovitsh; U. Ravid; E. Putievsky; E. Pichersky; Y. Shoham, *Plant Sci.* **2000**, *160*, 27–35.
367. D. R. Gang; N. Lavid; C. Zubieta; F. Chen; T. Beuerle; E. Lewinsohn; J. P. Noel; E. Pichersky, *Plant Cell* **2002**, *14*, 505–519.
368. M. Varbanova; I. Orlova; N. Dudareva; D. Reichhard; E. Pichersky. A cytochrome P450 enzyme from *Illicium parviflorum* capable of catalyzing the formation of a methylenedioxy bridge on eugenol to produce safrole. Phytochemical Society of North America Annual Meeting Abstract, St. Louis, MO, 2007. (http://www.psna-online.org/newsletters/PSNAN 461.web.pdf)
369. R. Martin; G. Schilling; J. Reichling, *Z. Naturforsch. C. Biosci.* **1988**, *43*, 328–336.
370. J. Reichling; R. Martin; U. Thron, *Z. Naturforsch. C. Biosci.* **1988**, *43*, 42–46.
371. J. Reichling; R. Martin, *Pharm. Ztg. Wiss.* **1991**, *4*, 225–231.
372. R. Dexter; A. Qualley; C. M. Kish; C. J. Ma; T. Koeduka; D. A. Nagegowda; N. Dudareva; E. Pichersky; D. Clark, *Plant J.* **2007**, *49*, 265–275.
373. B. St-Pierre; V. De Luca, Evolution of Acyltransferase Genes: Origin and Diversification of the BAHD Superfamily of Acyltransferases Involved in Secondary Metabolism. In *Recent Advances in Phytochemistry*, Elsevier: Oxford, UK, 2000; Vol. 34, Evolution of Metabolic Pathways, J. T. Romeo, R., Ibrahim, L. Varin, V. De Luca, Eds.; pp 285–315.
374. B. Harrison; D. R. Gang Characterization of coniferyl acetate acetyl transferase from sweet basil (Ocimum basilicum). In *The 19th Rocky Mountain Regional Meeting*, Tucson, AZ, 2006 (http://acs/confex.com/acs/rmrm06/tech program/P3334 HTM).
375. P. W. Sherman; G. A. Hash, *Evol. Human Behav.* **2001**, *22*, 147–163.
376. A. O. Gill; R. A. Holley, *Appl. Envir. Microbiol.* **2004**, *70*, 5750–5755.
377. K. Rhayour; T. Bouchiki; A. Tantaoui-Elaraki; K. Sendide; A. Remmal, *J. Essent. Oil Res.* **2003**, *15*, 356–362.
378. A. O. Gill; R. A. Holley, *Int. J. Food Microbiol.* **2006**, *108*, 1–9.
379. M. Greenberg; M. Dodds; M. Tian, *J. Agric. Food. Chem.* **2008**, *56*, 11151–11156.
380. S. E. Walsh; J.-Y. Maillard; A. D. Russell; C. E. Catrenich; D. L. Charbonneau; R. G. Bartolo, *J. Appl. Microbiol.* **2003**, *94*, 240–247.
381. N. A. Olasupo; D. J. Fitzgerald; A. Narbad; M. J. Gasson, *J. Food Prot.* **2004**, *67*, 596–600.
382. K. Voda; B. Boh; M. Vrtačnik; F. Pohleven, *Int. Biodet. Biodegr.* **2003**, *51*, 51–59.
383. U. Dev; C. Devakumar; J. Mohan; P. C. Agarwal, *J. Essent. Oil Res.* **2004**, *16*, 496–499.
384. S. Bennis; F. Chami; N. Chami; T. Bouchikhi; A. Remmal, *Lett. Appl. Microbiol.* **2004**, *38*, 454–458.
385. N. Chami; F. Chami; S. Bennis; J. Trouillas; A. Remmal, *Braz. J. Infect. Dis.* **2004**, *8*, 217–226.
386. E. W. Skinner, *The Science of Dental Materials*; W.B. Saunders Company: Philadelphia, PA, USA, 1940.
387. J. E. Weinberg; J. L. Rabinowitz; M. Zanger; A. R. Gennaro, *J. Dent. Res.* **1972**, *51*, 1055–1061.
388. J. Grush; D. L. G. Noakes; R. D. Moccia, *Zebrafish* **2004**, *1*, 46–53.
389. S. A. Guénette; P. Hélie; F. Beaudry; P. Vachon, *Vet. Anaesth. Analg.* **2007**, *34*, 164–170.
390. K. K. Sladky; C. R. Swanson; M. K. Stoskopf; M. R. Loomis; G. A. Lewbart, *Am. J. Vet. Res.* **2001**, *62*, 337–342.
391. S. A. Guénette; F. Béaudry; J. F. Marier; P. Vachon, *J. Vet. Pharmacol. Therap.* **2006**, *29*, 265–270.
392. S. A. Guénette; F. C. Uhland; P. Hélie; F. Beaudry; P. Vachon, *Aquaculture* **2007**, *266*, 262–265.
393. M. Hanusz, A Century of Kretek. In *Smoke: A Global History of Smoking*; S. L. Gilman, Z. Xun, Eds.; Reaktion Books: London, UK, 2004; pp 140–143.
394. M. H. Lee; K.-Y. Yeon; C.-K. Park; H.-Y. Li; Z. Fang; M. S. Kim; S.-Y. Choi; S. J. Lee; S. Lee; K. Park; J.-H. Lee; J. S. Kim; S. B. Oh, *J. Dent. Res.* **2005**, *84*, 848–851.
395. C.-K. Park; H. Y. Li; K.-Y. Yeon; S. J. Jung; S.-Y. Choi; S. J. Lee; S. Lee; K. Park; J. S. Kim; S. B. Oh, *J. Dent. Res.* **2006**, *85*, 900–904.
396. J. S. Cho; T. H. Kim; J.-M. Lim; J.-H. Song, *Brain Res.* **2008**, *1243*, 53–62.
397. L. M. Pessoa; S. M. Morais; C. M. L. Bevilaqua; J. H. S. Luciano, *Vet. Parasitol.* **2002**, *109*, 59–63.

398. L. F. L. Interaminense; J. H. Leal-Cardoso; P. J. C. Magalhaes; G. P. Duarte; S. Lahlou, *Planta Med.* **2005**, *71*, 376–378.
399. T. Ueda-Nakamura; R. R. Mendonça-Filho; J. A. Morgado-Díaz; P. K. Maza; B. P. D. Filho; D. A. G. Cortez; D. S. Alviano; M. D. S. S. Rosa; A. H. C. S. Lopes; C. S. Alviano; C. V. Nakamura, *Parasitol. Int.* **2006**, *55*, 99–105.
400. H. Xu; M. Delling; J. C. Jun; D. E. Clapham, *Nat. Neurosci.* **2006**, *9*, 628–635.
401. L. F. L. Interaminense; D. M. Jucá; P. J. C. Magalhães; J. H. Leal-Cardoso; G. P. Duarte; S. Lahlou, *Fundam. Clin. Pharmacol.* **2007**, *21*, 497–506.
402. H. Y. Li; C.-K. Park; S. J. Jung; S.-Y. Choi; S. J. Lee; K. Park; J. S. Kim; S. B. Oh, *J. Dent. Res.* **2007**, *86*, 898–902.
403. G. Chung; J. N. Rhee; S. J. Jung; J. S. Kim; S. B. Oh, *J. Dent. Res.* **2008**, *87*, 137–141.
404. G. Tao; Y. Irie; D.-J. Li; W. M. Keung, *Bioorg. Med. Chem.* **2005**, *13*, 4777–4788.
405. H. Raghavenra; B. T. Diwakr; B. R. Lokesh; K. A. Naidu, *Prostaglandins Leukot. Essent. Fatty Acids* **2006**, *74*, 23–27.
406. W. Li; R. Tsubouchi; S. Qiao; M. Haneda; K. Murakami; M. Yoshino, *Biomed. Res.* **2006**, *27*, 69–74.
407. B. B. Aggarwal; S. Shishodia, *Biochem. Pharm.* **2006**, *71*, 1397–1421.
408. A. S. L. Chan; H. Pang; E. C. H. Yip; Y. K. Tam; Y. H. Wong, *Planta Med.* **2005**, *71*, 634–639.
409. H. Yokota; H. Hashimoto; M. Motoya; A. Yuasa, *Biochem. Pharmacol.* **1988**, *37*, 799–802.
410. C.-B. Yoo; K.-T. Han; K.-S. Cho; J. Ha; H.-J. Park; J.-H. Nam; U.-H. Kil; K.-T. Lee, *Cancer Lett.* **2005**, *225*, 41–52.
411. B. S. Park; Y. S. Song; S. B. Yee; B. G. Lee; S. Y. Seo; Y. C. Park; J. M. Kim; H. M. Kim; Y. H. Yoo, *Apoptosis* **2005**, *10*, 193–200.
412. R. Ghosh; N. Nadiminty; J. E. Fitzpatrick; W. L. Alworth; T. J. Slaga; A. P. Kumar, *J. Biol. Chem.* **2005**, *280*, 5812–5819.
413. G. B. N. Chainy; S. K. Manna; M. M. Chaturvedi; B. B. Aggarwal, *Oncogene* **2000**, *19*, 2943–2950.
414. C. Y. Choi; K.-R. Park; J.-H. Lee; Y. J. Jeon; K.-H. Liu; S. Oh; D.-E. Kim; S. S. Yea, *Eur. J. Pharmacol.* **2007**, *576*, 151–159.
415. Z. S. Zhao; P. J. O'Brien, *Toxicol. Appl. Pharmacol.* **1996**, *140*, 411–421.
416. D.-W. Tang; K.-W. Chang; C.-W. Chi; T.-Y. Liu, *Toxicol. Lett.* **2004**, *152*, 235–243.
417. J. Zhang; G. Feng; Z.-Q. Ma; J.-T. Feng; X. Zhang, *Kunchong Xuebao* **2007**, *50*, 574–577.
418. F. Zhang; Q. Xu; S. Fu; X. Ma; H. Xiao; X. Liang, *Flav. Fragr. J.* **2005**, *20*, 318–320.
419. Z. Ma; J. He; Z. Jiang; Z. Wang; J. Feng; X. Zhang, Application of asaricin in control of plant pathogenic bacteria-induced plant diseases. China Patent Number 101027990, 2007.
420. C. H. Chuah; H. S. Yong; S. H. Goh, *Biochem. Syst. Ecol.* **1997**, *25*, 391–393.
421. E. Jang; D. McInnis; R. Vargas; R. Mau, Area-wide integrated pest management (IPM) of fruit flies in Hawaiian fruits and vegetables. http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=167070
422. T. E. Shelly; E. Pahio; J. Edu, *Fla. Entomol.* **2004**, *87*, 481–486.
423. U. S. E. P. Agency. Pesticides: Regulating Pesticides. Methyl Eugenol (ME). http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factsheet_203900.htm
424. D. M. Suckling; E. B. Jang; L. A. Carvalho; J. T. Nagata; E. L. Schneider; A. M. El-Sayed, *J. Chem. Ecol.* **2007**, *33*, 1494–1504.
425. R. L. Smith; T. B. Adams; J. Doull; V. J. Feron; J. I. Goodman; L. J. Marnett; P. S. Portoghese; W. J. Waddell; B. M. Wagner; A. E. Rogers; J. Caldwell; I. G. Sipes, *Food Chem. Toxicol.* **2002**, *40*, 851–870.
426. I. U. Fischer; G. E. von Unruh; H. J. Dengler, *Xenobiotica* **1990**, *20*, 209–222.
427. I. Gardner; H. Wakazono; P. Bergin; I. de Waziers; P. Beaune; J. G. Kenna; J. Caldwell, *Carcinogenesis* **1997**, *18*, 1775–1783.
428. S. M. F. Jeurissen; J. J. P. Bogaards; H. M. Awad; M. G. Boersma; W. Brand; Y. C. Fiamegos; T. A. van Beek; G. M. Alink; E. J. R. Sudhölter; N. H. P. Cnubben; I. M. C. M. Rietjens, *Chem. Res. Toxicol.* **2004**, *17*, 1245–1250.
429. I. M. C. M. Rietjens; M. G. Boersma; H. van der Woude; S. M. F. Jeurissen; M. E. Schutte; G. M. Alink, *Mutat. Res.* **2005**, *574*, 124–138.
430. S. M. F. Jeurissen; J. J. P. Bogaards; M. G. Boersma; J. P. F. ter Horst; H. M. Awad; Y. C. Fiamegos; T. A. van Beek; G. M. Alink; E. J. R. Sudhölter; N. H. P. Cnubben; I. M. C. M. Rietjens, *Chem. Res. Toxicol.* **2006**, *19*, 111–116.
431. S. M. F. Jeurissen; A. Punt; M. G. Boersma; J. J. P. Bogaards; Y. C. Fiamegos; B. Schilter; P. J. van Bladeren; N. H. P. Cnubben; I. M. C. M. Rietjens, *Chem. Res. Toxicol.* **2007**, *20*, 798–806.
432. H. Wakazono; I. Gardner; E. Eliasson; M. W. H. Coughtrie; J. G. Kenna; J. Caldwell, *Chem. Res. Toxicol.* **1998**, *11*, 863–872.
433. G.-D. Zhou; B. Moorthy; J. Bi; K. C. Donnelly; K. Randerath, *Envir. Mol. Mutag.* **2007**, *48*, 715–721.
434. M. H. G. Medeiros; P. Di Mascio; A. P. Pinto; R. R. Vargas; E. J. H. Bechara, *Free Radical Res.* **1996**, *25*, 5–12.
435. I. Gardner; P. Bergin; P. Stening; J. G. Kenna; J. Caldwell, *Chem. Res. Toxicol.* **1996**, *9*, 713–721.
436. R.-S. Tsai; P.-A. Carrupt; B. Testa; J. Caldwell, *Chem. Res. Toxicol.* **1994**, *7*, 73–76.
437. S. Yamada; Y. Murawaki; H. Kawasaki, *Biochem. Pharmacol.* **1993**, *46*, 1081–1085.
438. M. Nomura; Y. Ohtaki; T. Hida; T. Aizawa; H. Wakita; K.-I. Miyamoto, *Anticancer Res.* **1994**, *14*, 1967–1972.
439. S.-H. Kim; Y. S. Kim; S. S. Kang; K. Bae; T. M. Hung; S.-M. Lee, *J. Pharmacol. Sci.* **2008**, *106*, 225–233.
440. S. Fukumitsu; K. Aida; N. Ueno; S. Ozawa; Y. Takahashi; M. Kobori, *Br. J. Nutr.* **2008**, *100*, 669–676.
441. C. Canel; R. M. Moraes; F. E. Dayan; D. Ferreira, *Phytochemistry* **2000**, *54*, 115–120.
442. M. Gordaliza; P. A. García; J. M. Miguel del Corral; M. A. Castro; M. A. Gómez-Zurita, *Toxicon* **2004**, *44*, 441–459.
443. S. Apers; A. Vlietinck; L. Pieters, *Phytochem. Rev.* **2003**, *2*, 201–217.
444. V. Srivastava; A. S. Negi; J. K. Kumar; M. M. Gupta; S. P. S. Khanuja, *Bioorg. Med. Chem.* **2005**, *13*, 5892–5908.
445. A. Montecucco; G. Biamonti, *Cancer Lett.* **2007**, *252*, 9–18.
446. K. R. Hande, *Eur. J. Cancer* **1998**, *34*, 1514–1521.
447. M. A. Economou; S. Andersson; D. Vasilcanu; C. All-Ericsson; E. Menu; A. Girnita; L. Girnita; M. Axelson; S. Seregard; O. Larsson, *Invest. Ophthalmol. Vis. Sci.* **2008**, *49*, 2337–2342.
448. M. A. Economou; J. Wu; D. Vasilcanu; L. Rosengren; C. All-Ericsson; I. van der Ploeg; E. Menu; L. Girnita; M. Axelson; O. Larsson; S. Seregard; A. Kvanta, *Invest. Ophthalmol. Vis. Sci.* **2008**, *49*, 2620–2626.
449. J.-M. Barret; A. Kruczynski; C. Etiévant; B. T. Hill, *Cancer Chemother. Pharmacol.* **2002**, *49*, 479–486.
450. J. M. Sargent; A. W. Elgie; C. J. Williamson; B. T. Hill, *Anti-Cancer Drugs* **2003**, *14*, 467–473.
451. J. Kluza; R. Mazinghien; H. Irwin; J. A. Hartley; C. Bailly, *Anti-Cancer Drugs* **2006**, *17*, 155–164.
452. M. S. Butler, *Nat. Prod. Rep.* **2008**, *25*, 475–516.
453. C. Canel; F. E. Dayan; M. Ganzera; I. A. Khan; A. Rimando; C. L. Burandt, Jr.; R. M. Moraes, *Planta Med.* **2001**, *67*, 97–99.
454. E. Fuss, *Phytochem. Rev.* **2003**, *2*, 307–320.

455. R. Verpoorte; R. van der Heijden; H. J. G. ten Hoopen; J. Memelink, *Biotechnol. Lett.* **1999**, *21*, 467–479.
456. J. L. Billinsky; E. S. Krol, *J. Nat. Prod.* **2008**, *71*, 1612–1615.
457. J. L. Billinsky; M. R. Marcoux; E. S. Krol, *Chem. Res. Toxicol.* **2007**, *20*, 1352–1358.
458. J. D. Lambert; R. T. Dorr; B. N. Timmermann, *Pharm. Biol.* **2004**, *42*, 149–158.
459. Y. Cui; C. Lu; L. Liu; D. Sun; N. Yao; S. Tan; S. Bai; X. Ma, *Life Sci.* **2008**, *82*, 247–255.
460. T. Yoshida; T. Shiraishi; M. Horinaka; S. Nakata; T. Yasuda; A. E. Goda; M. Wakada; Y. Mizutani; T. Miki; A. Nishikawa; T. Sakai, *Cancer Sci.* **2007**, *98*, 1417–1423.
461. J. F. Youngren; K. Gable; C. Penaranda; B. A. Maddux; M. Zavodovskaya; M. Lobo; M. Campbell; J. Kerner; I. D. Goldfine, *Breast Cancer Res. Treat.* **2005**, *94*, 37–46.
462. J. E. Blecha; M. O. Anderson; J. M. Chow; C. C. Guevarra; C. Pender; C. Penaranda; M. Zavodovskaya; J. F. Youngren; C. E. Berkman, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4026–4029.
463. G. E. Meyer; L. Chesler; D. Liu; K. Gable; B. A. Maddux; D. D. Goldenberg; J. F. Youngren; I. D. Goldfine; W. A. Weiss; K. K. Matthay; S. M. Rosenthal, *J. Cell. Biochem.* **2007**, *102*, 1529–1541.
464. M. Zavodovskaya; M. J. Campbell; B. A. Maddux; L. Shiry; G. Allan; L. Hodges; P. Kushner; J. A. Kerner; J. F. Youngren; I. D. Goldfine, *J. Cell. Biochem.* **2008**, *103*, 624–635.
465. M. West; M. Mhatre; A. Ceballos; R. A. Floyd; P. Grammas; S. P. Gabbita; L. Hamdheydari; T. Mai; S. Mou; Q. N. Pye; C. Stewart; S. West; K. S. Williamson; F. Zemlan; K. Hensley, *J. Neurochem.* **2004**, *91*, 133–143.
466. R. K. Mandraju; A. K. Kondapi, *Arch. Biochem. Biophys.* **2007**, *461*, 40–49.
467. B. Hausott; H. Greger; B. Marian, *J. Cancer Res. Clin. Oncol.* **2003**, *129*, 569–576.
468. D. L. Rowe; T. Ozbay; L. M. Bender; R. Nahta, *Mol. Cancer Ther.* **2008**, *7*, 1900–1908.
469. C. Plaza; M. Pavani; M. Faundez; J. D. Maya; A. Morello; M. I. Becker; A. De Ioannes; M. A. Cumsille; J. Ferreira, *In Vivo* **2008**, *22*, 353–361.
470. J. D. Lambert; R. O. Meyers; B. N. Timmermann; R. T. Dorr, *Cancer Lett.* **2001**, *171*, 47–56.
471. J. D. Heller; J. Kuo; T. C. Wu; W. M. Kast; R. C. C. Huang, *Cancer Res.* **2001**, *61*, 5499–5504.
472. R. A. Lopez; A. B. Goodman; M. Rhodes; J. A. L. Blomberg; J. Heller, *Anti-Cancer Drugs* **2007**, *18*, 933–939.
473. P. Smolewski, *IDrugs* **2008**, *11*, 204–214.
474. B. Raffaelli; A. Hoikkala; E. Leppälä; K. Wähälä, *J. Chromatog. B* **2002**, *777*, 29–43.
475. L.-Q. Wang, *J. Chromatog. B* **2002**, *777*, 289–309.
476. L. U. Thompson; P. Robb; M. Serraino; F. Cheung, *Nutr. Cancer* **1991**, *16*, 43–52.
477. H. Qu; R. L. Madl; D. J. Takemoto; R. C. Baybutt; W. Wang, *J. Nutr.* **2005**, *135*, 598–602.
478. G. Hallmans; J.-X. Zhang; E. Lundin; P. Stattin; A. Johansson; I. Johansson; K. Hultén; A. Winkvist; P. Lenner; P. Åman; H. Adlercreutz, *Proc. Nutr. Soc.* **2003**, *62*, 193–199.
479. D. Fuchs; R. Piller; J. Linseisen; H. Daniel; U. Wenzel, *Proteomics* **2007**, *7*, 3278–3288.
480. X. Lin; B. R. Switzer; W. Demark-Wahnefried, *Anticancer Res.* **2001**, *21*, 3995–3999.
481. N. Danbara; T. Yuri; M. Tsujita-Kyutoku; R. Tsukamoto; N. Uehara; A. Tsubura, *Anticancer Res.* **2005**, *25*, 2269–2276.
482. M. J. McCann; C. I. R. Gill; T. Linton; D. Berrar; H. McGlynn; I. R. Rowland, *Mol. Nutr. Food Res.* **2008**, *52*, 567–580.
483. C. L. Heald; M. R. Ritchie; C. Bolton-Smith; M. S. Morton; F. E. Alexander, *Br. J. Nutr.* **2007**, *98*, 388–396.
484. A. Kuijsten; I. C. W. Arts; P. C. H. Hollman; P. van't Veer; E. Kampman, *Cancer Epidemiol. Biomarkers Prev.* **2006**, *15*, 1132–1136.
485. A. Kuijsten; P. C. H. Hollman; H. C. Boshuizen; M. N. C. P. Buijsman; P. van 't Veer; F. J. Kok; I. C. W. Arts; H. B. Bueno-de-Mesquita, *Am. J. Epidemiol.* **2008**, *167*, 734–742.
486. S. Oikarinen; S.-M. Heinonen; T. Nurmi; H. Adlercreutz; M. Mutanen, *Eur. J. Nutr.* **2005**, *44*, 273–280.
487. A. Kuijsten; H. B. Bueno-de-Mesquita; J. M. A. Boer; I. C. W. Arts; F. J. Kok; P. van't Veer; P. C. H. Hollman, *Atherosclerosis* **2009**, *203*, 145–152.
488. B. Y. Hernandez; K. McDuffie; A. A. Franke; J. Killeen; M. T. Goodman, *Nutr. Cancer* **2004**, *49*, 109–124.
489. D. Miura; N. M. Saarinen; Y. Miura; R. Santti; K. Yagasaki, *Nutr. Cancer* **2007**, *58*, 49–59.
490. J. D. Brooks; L. U. Thompson, *J. Steroid Biochem. Mol. Biol.* **2005**, *94*, 461–467.
491. M. Cosentino; F. Marino; M. Ferrari; E. Rasini; R. Bombelli; A. Luini; M. Legnaro; M. G. Delle Canne; M. Luzzani; F. Crema; S. Paracchini; S. Lecchini, *Pharmacol. Res.* **2007**, *56*, 140–147.
492. P. Penttinen; J. Jaehrling; A. E. Damdimopoulos; J. Inzunza; J. G. Lemmen; P. van der Saag; K. Pettersson; G. Gauglitz; S. Mäkelä; I. Pongratz, *Endocrinology* **2007**, *148*, 4875–4886.
493. C. Carreau; G. Flouriot; C. Bennetau-Pelissero; M. Potier, *J. Steroid Biochem. Mol. Biol.* **2008**, *110*, 176–185.
494. J. Chen; L. U. Thompson, *Breast Cancer Res. Treat.* **2003**, *80*, 163–170.
495. R. Piller; J. Chang-Claude; J. Linseisen, *Eur. J. Cancer Prev.* **2006**, *15*, 225–232.
496. N. M. Saarinen; A. Wärri; M. Airio; A. Smeds; S. Mäkelä, *Mol. Nutr. Food Res.* **2007**, *51*, 857–866.
497. M. S. Touillaud; A. C. M. Thiébaut; A. Fournier; M. Niravong; M.-C. Boutron-Ruault; F. Clavel-Chapelon, *J. Natl. Cancer Inst.* **2007**, *99*, 475–486.
498. K. A. Power; L. U. Thompson, *Mol. Nutr. Food Res.* **2007**, *51*, 845–856.
499. A. Olsen; K. E. B. Knudsen; B. L. Thomsen; S. Loft; C. Stripp; K. Overvad; S. Møller; A. Tjønneland, *Cancer Epidemiol. Biomarkers Prev.* **2004**, *13*, 2084–2089.
500. G. Khan; P. Penttinen; A. Cabanes; A. Foxworth; A. Chezek; K. Mastropole; B. Yu; A. Smeds; T. Halttunen; C. Good; S. Mäkelä; L. Hilakivi-Clarke, *Reprod. Toxicol.* **2007**, *23*, 397–406.
501. S. Heinonen; T. Nurmi; K. Liukkonen; K. Poutanen; K. Wähälä; T. Deyama; S. Nishibe; H. Adlercreutz, *J. Agric. Food Chem.* **2001**, *49*, 3178–3186.
502. A. N. Begum; C. Nicolle; I. Mila; C. Lapierre; K. Nagano; K. Fukushima; S.-M. Heinonen; H. Adlercreutz; C. Rémésy; A. Scalbert, *J. Nutr.* **2004**, *134*, 120–127.
503. J. L. Peñalvo; S.-M. Heinonen; A.-M. Aura; H. Adlercreutz, *J. Nutr.* **2005**, *135*, 1056–1062.
504. L.-Q. Wang; M. R. Meselhy; Y. Li; G.-W. Qin; M. Hattori, *Chem. Pharm. Bull.* **2000**, *48*, 1606–1610.

505. E. Eeckhaut; K. Struijs; S. Possemiers; J.-P. Vincken; D. De Keukeleire; W. Verstraete, *J. Agric. Food Chem.* **2008**, *56*, 4806–4812.
506. L.-H. Xie; T. Akao; K. Hamasaki; T. Deyama; M. Hattori, *Chem. Pharm. Bull.* **2003**, *51*, 508–515.
507. T. Clavel; G. Henderson; W. Engst; J. Doré; M. Blaut, *FEMS Microbiol. Ecol.* **2006**, *55*, 471–478.
508. J.-S. Jin; N. Kakiuchi; M. Hattori, *Biol. Pharm. Bull.* **2007**, *30*, 2204–2206.
509. J.-S. Jin; Y.-F. Zhao; N. Nakamura; T. Akao; N. Kakiuchi; M. Hattori, *Biol. Pharm. Bull.* **2007**, *30*, 904–911.
510. J.-S. Jin; Y.-F. Zhao; N. Nakamura; T. Akao; N. Kakiuchi; B.-S. Min; M. Hattori, *Biol. Pharm. Bull.* **2007**, *30*, 2113–2119.
511. G. H. E. Jansen; I. C. W. Arts; M. W. F. Nielen; M. Müller; P. C. H. Hollman; J. Keijer, *Arch. Biochem. Biophys.* **2005**, *435*, 74–82.
512. A. Kuijsten; I. C. W. Arts; T. B. Vree; P. C. H. Hollman, *J. Nutr.* **2005**, *135*, 795–801.
513. C. Hu; Y. V. Yuan; D. D. Kitts, *Food Chem. Toxicol.* **2007**, *45*, 2219–2227.
514. W. Zhang; X. Wang; Y. Liu; H. Tian; B. Flickinger; M. W. Empie; S. Z. Sun, *Br. J. Nutr.* **2008**, *99*, 1301–1309.
515. W. Zhang; X. Wang; Y. Liu; H. Tian; B. Flickinger; M. W. Empie; S. Z. Sun, *J. Med. Food* **2008**, *11*, 207–214.
516. A. Pan; J. Sun; Y. Chen; X. Ye; H. Li; Z. Yu; Y. Wang; W. Gu; X. Zhang; X. Chen; W. Demark-Wahnefried; Y. Liu; X. Lin, *PLoS ONE* **2007**, *2*, e1148.
517. F. Chen; T. Wang; Y.-F. Wu; Y. Gu; X.-L. Xu; S. Zheng; X. Hu, *World J. Gastroenterol.* **2004**, *10*, 3459–3463.
518. S. Dikalov; T. Losik; J. L. Arbiser, *Biochem. Pharmacol.* **2008**, *76*, 589–596.
519. A. Garcia; Y. Zheng; C. Zhao; A. Toschi; J. Fan; N. Shraibman; H. A. Brown; D. Bar-Sagi; D. A. Foster; J. L. Arbiser, *Clin. Cancer Res.* **2008**, *14*, 4267–4274.
520. E.-R. Hahm; J. A. Arlotti; S. W. Marynowski; S. V. Singh, *Clin. Cancer Res.* **2008**, *14*, 1248–1257.
521. S.-K. Lee; H.-N. Kim; Y.-R. Kang; C. W. Lee; H.-M. Kim; D. C. Han; J. Shin; K. Bae; B.-M. Kwon, *Bioorg. Med. Chem.* **2008**, *16*, 8397–8402.
522. H. Liu; C. Zang; A. Emde; M. D. Planas-Silva; M. Rosche; A. Kühnl; C.-O. Schulz; E. Elstner; K. Possinger; J. Eucker, *Eur. J. Pharmacol.* **2008**, *591*, 43–51.
523. H. Luo; Q. Zhong; L.-j. Chen; X.-r. Qi; A.-f. Fu; H.-s. Yang; F. Yang; H.-g. Lin; Y.-q. Wei; X. Zhao, *J. Cancer Res. Clin. Oncol.* **2008**, *134*, 937–945.
524. S. M. Raja; S. Chen; P. Yue; T. M. Acker; B. Lefkove; J. L. Arbiser; F. R. Khuri; S.-Y. Sun, *Mol. Cancer Ther.* **2008**, *7*, 2212–2223.
525. C. F. Albrecht; E. J. Theron; P. B. Kruger, *S. Afr. Med. J.* **1995**, *85*, 853–860.
526. C. F. Albrecht; P. B. Kruger; B. J. Smit; M. Freestone; L. Gouws; R. Miller; P. P. van Jaarsveld, *S. Afr. Med. J.* **1995**, *85*, 861–865.
527. V. D. P. Nair; A. Dairam; A. Agbonon; J. T. Arnason; B. C. Foster; I. Kanfer, *J. Agric. Food Chem.* **2007**, *55*, 1707–1711.
528. E. J. Theron; C. F. Albrecht; P. B. Kruger; K. Jenkins; M. J. van der Merwe, *In Vitro Cell. Dev. Biol.* **1994**, *30A*, 115–119.
529. V. D. P. Nair; I. Kanfer, *Phytochem. Anal.* **2007**, *18*, 475–483.
530. P. Vinesi; M. Serafini; M. Nicoletti; L. Spanò; P. Betto, *J. Nat. Prod.* **1990**, *53*, 196–199.
531. J. R. Hwu; W. N. Tseng; J. Gnabre; P. Giza; R. C. C. Huang, *J. Med. Chem.* **1998**, *41*, 2994–3000.
532. R. C. C. Huang; Y. Li; P. E. Giza; J. N. Gnabre; I. S. Abd-Elazem; K. Y. King; J. R. Hwu, *Antivir. Res.* **2003**, *58*, 57–64.
533. R. Park; P. E. Giza; D. E. Mold; R. C. C. Huang, *Antivir. Res.* **2003**, *58*, 35–45.
534. J. Craigo; M. Callahan; R. C. C. Huang; A. L. DeLucia, *Antivir. Res.* **2000**, *47*, 19–28.
535. K. L. Allen; D. R. Tschantz; K. S. Awad; W. P. Lynch; A. L. DeLucia, *Mol. Carcinog.* **2007**, *46*, 564–575.
536. N. Khanna; R. Dalby; M. Tan; S. Arnold; J. Stern; N. Frazer, *Gyn. Oncol.* **2007**, *107*, 554–562.
537. N. Khanna; R. Dalby; A. Connor; A. Church; J. Stern; N. Frazer, *Sex. Transm. Dis.* **2008**, *35*, 577–582.
538. K. Yamashita, *J. Clin. Biochem. Nutr.* **2004**, *35*, 17–28.
539. S. Shimizu; K. Akimoto; Y. Shinmen; H. Kawashima; M. Sugano; H. Yamada, *Lipids* **1991**, *26*, 512–516.
540. T. Ide; L. Ashakumary; Y. Takahashi; M. Kushiro; N. Fukuda; M. Sugano, *Biochim. Biophys. Acta* **2001**, *1534*, 1–13.
541. T. Ide; D. D. Hong; P. Ranasinghe; Y. Takahashi; M. Kushiro; M. Sugano, *Biochim. Biophys. Acta* **2004**, *1682*, 80–91.
542. F. Hirata; K. Fujita; Y. Ishikura; K. Hosoda; T. Ishikawa; H. Nakamura, *Atherosclerosis* **1996**, *122*, 135–136.
543. C.-C. Lee; P.-R. Chen; S. Lin; S.-C. Tsai; B.-W. Wang; W.-W. Chen; C. E. Tsai; K.-G. Shyu, *J. Hypertens.* **2004**, *22*, 2329–2338.
544. T. Hayashi; R. H. Thomson, *Phytochemistry* **1975**, *14*, 1085–1087.
545. R. C. C. Martins; L. R. Latorre; P. Sartorelli; M. J. Kato, *Phytochemistry* **2000**, *55*, 843–846.
546. D. C. Chauret; C. B. Bernard; J. T. Arnason; T. Durst; H. G. Krishnamurty; P. Sanchez-Vindas; N. Moreno; L. S. Roman; L. Poveda, *J. Nat. Prod.* **1996**, *59*, 152–155.
547. R. C. C. Martins; J. H. G. Lago; S. Albuquerque; M. J. Kato, *Phytochemistry* **2003**, *64*, 667–670.
548. A. A. a Silva Filho; S. Albuquerque; M. L. A. Silva; M. N. Eberlin; D. M. Tomazela; J. K. Bastos, *J. Nat. Prod.* **2004**, *67*, 42–45.
549. P. S. Luize; T. Ueda-Nakamura; B. P. D. Filho; D. A. G. Cortez; C. V. Nakamura, *Biol. Pharm. Bull.* **2006**, *29*, 2126–2130.
550. E. Kadowaki; Y. Yoshida; T. Nitoda; N. Baba; S. Nakajima, *Biosci. Biotech. Biochem.* **2003**, *67*, 415–419.
551. S. D. Elakovich; K. L. Stevens, *J. Chem. Ecol.* **1985**, *11*, 27–33.
552. N. Sakurai; K. Shibata; S. Kamisaka, *Plant Cell Physiol.* **1974**, *15*, 709–716.
553. K. Shibata; T. Kubota; S. Kamisaka, *Plant Cell Physiol.* **1974**, *15*, 191–194.
554. N. Sakurai; K. Shibata; S. Kamisaka, *Plant Cell Physiol.* **1975**, *16*, 845–855.
555. K. Shibata; T. Kubota; S. Kamisaka, *Plant Cell Physiol.* **1975**, *16*, 871–877.
556. S. Kamisaka; K. Shibata, *Plant Cell Physiol.* **1977**, *18*, 1057–1066.
557. M. Cannac; V. Pasqualini; S. Greff; C. Fernandez; L. Ferrat, *Molecules* **2007**, *12*, 1614–1622.
558. A.-K. Borg-Karlson; G. Nordlander; A. Mudalige; H. Nordenhem; C. R. Unelius, *J. Chem. Ecol.* **2006**, *32*, 943–957.
559. W. A. Whistler, *Tropical Ornamentals: A Guide*; Timber Press: Portland, OR, USA, 2000.
560. J. K. Francis, *Guaiacum officinale* L. In *Tropical Tree Seed Manual*; J. A. Vozzo, Ed.; USDA Forest Service: Washington, DC, USA, 2002; pp 483–485.

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Kye-Won Kim graduated in 2001 with a Ph.D. degree from Kyungpook National University (under the supervision of Professor Jong-Guk Kim). Her studies focused on the biosynthesis/molecular biology of capsaicinoid formation in *Capsicum annuum* and of factors involved in the metabolic regulation of capsaicinoids. She joined Professor Lewis' group as a postdoctoral fellow in 2001, and has worked largely on the functional characterization of genes/proteins involved in both lignan and lignin biosynthesis in plants. There is an emphasis in her work on dirigent proteins, which mediate phenoxy radical coupling, as well as how enantiospecificity of pinoresinol/lariciresinol reductase is achieved.

Laurence B. Davin received her B.Sc. (1983) and Ph.D. (1987) degrees in plant biochemistry/physiology from the Université Paul Sabatier (Toulouse, France). Dr. Davin next studied glucosinolate biogenesis with Ted Underhill in the National Research Council of Canada's Plant Biotechnology Institute as a postdoctoral fellow. Since 1989, she has turned her attention to lignan, lignin, and allyl/propenyl phenol biosynthesis at both the Virginia Polytechnic Institute and State University (Blacksburg, VA) and the Institute of Biological Chemistry (Washington State University). Dr. Davin is a member of the Editorial Board for Recent Advances in Phytochemistry.

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