

Mini Review

Diterpenoid Biopolymers: New Directions for Renewable Materials Engineering

Matthew L. Hillwig, Francis M. Mann, Reuben J. Peters

Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011

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ABSTRACT:

Most types of ambers are naturally occurring, relatively hard, durable resinite polymers derived from the exudates of trees. This resource has been coveted for thousands of years due to its numerous useful properties in industrial processes, beauty, and purported medicinal properties. Labdane diterpenoid-based ambers represent the most abundant and important resinites on earth. These resinites are a dwindling nonrenewable natural resource, so a new source of such materials needs to be established. Recent advances in sequencing technologies and biochemical engineering are rapidly accelerating the rate of identifying and assigning function to genes involved in terpenoid biosynthesis, as well as producing industrial-scale quantities of desired small-molecules in bacteria and yeast. This has provided new tools for engineering metabolic pathways capable of producing diterpenoid monomers that will enable the production of custom-tailored resinite-like polymers. Furthermore, this biosynthetic toolbox is continuously expanding, providing new possibilities for renewing dwindling stocks of naturally occurring resinite materials and engineering new materials for future applications. © 2010 Wiley Periodicals, Inc. *Biopolymers* 95: 71–76, 2011.

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INTRODUCTION

Amber resins have puzzled the scientific community for decades, yet they have now been characterized, categorized, and their biosynthetic origins unraveled by the hands of many researchers over recent decades. Although the definition of amber has varied over time, it is generally accepted as the fossilized exudates of plants. These resinous exudates are often used in defensive action against insects and herbivores, although such resins in some species of angiosperms have notable use as pollinator attractants.¹

Ambers have been historically difficult to describe due to changes in chemical components during the aging process. In whole form, they are typically quite insoluble, due to their large, interlinked carbonaceous molecular composition. Their complex polymeric nature made identification and characterization very difficult until the advent of such technologies as recombinant expression systems, solid-state NMR, and pyrolysis-GC-MS. Using these new technologies, categorization of amber was first attempted via their specific botanical origin, but this method was largely abandoned in favor of group-origin categorization as it was determined that previous extinction of the relevant species made categorization by botanical origin difficult, if not impossible. In the group-origin classification scheme, ambers were largely grouped into three categories: Baltic, Agathis, and Hymenaea.² As it became apparent that group-origin categorization lacked detail and precision, a chemically-based organizational scheme was proposed based on the polymer structure's monomeric constituents, and this has become the classification scheme with which all new amber resins are categorized.³ There are four classes of resins: Class I ambers are

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Correspondence to: Reuben J. Peters; e-mail: rjpeters@iastate.edu

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polymers of labdane-derived diterpenoids and are further divided into subclasses by specific constituents. Class II ambers are polymers of sesquiterpenoids with some triterpenoid components. Class III ambers are natural polystyrenes, and Class IV ambers are nonpolymeric sesquiterpenoids. This categorization can be applied to all characterized resins, but allows for further categories as may be required due to characterization of previously undefined ambers composed of novel resin-like monomers.

The desire to define amber stems from its biological and anthropological importance. Once prized for its supposed medicinal properties, amber jewelry has been found in anthropological sites along the Baltic sea corresponding to early hunter-gatherer colonization.¹ Amber trade is presumed to date back to at least 1000 B.C. and has influenced international relations in the Middle East, Far East, Europe, and Americas. Indeed, the use of naval stores resins for shipbuilding was important in the relationship between Britain and North America.¹ As much of the world reached the industrialized age, demand for amber became largely decorative instead of functional. Nevertheless, in the modern age amber has provided scientific investigators a source for preserved ancient species, fossils, and DNA that allow us to further our understand the ecosystems these resin producing trees lived in, up to millions of years ago. However, at the end of the 21st century amber mining slowed as many areas once prized for their amber deposits reported dwindling yields. The increasing threat of depletion for rubber, resinites and petroleum-based products has highlighted the requirement for bioengineered resources, which can be quickly and efficiently produced without depletion of dwindling natural resources.⁴

Characterization of the individual components and polymerizing cross-links has yielded information about the biosynthetic origins of amber, and it has become conceivable that resins similar to those found naturally can be engineered via modern scientific methods. Independently designed amber-like materials, produced via directed chemical and biological engineering, have the potential to replace many of the currently available natural materials, which also are limited in supply, as well as provide a source of new biomaterials. Here we review the chemistry of natural Class I resins, as well as the modern biochemical techniques that may allow for resinite materials engineering as a method for biorenewable materials preparation.

The main constituents of Class I resins are labdane-related diterpenoids, which are synthesized from the precursor molecule (*E,E,E*)-geranylgeranyl diphosphate (GGPP) by various diterpene synthases and cytochrome P450s (CYP) specific to each plant species (see Figure 1). Furthermore, this class of resins is divided into distinct subclasses based on monomer

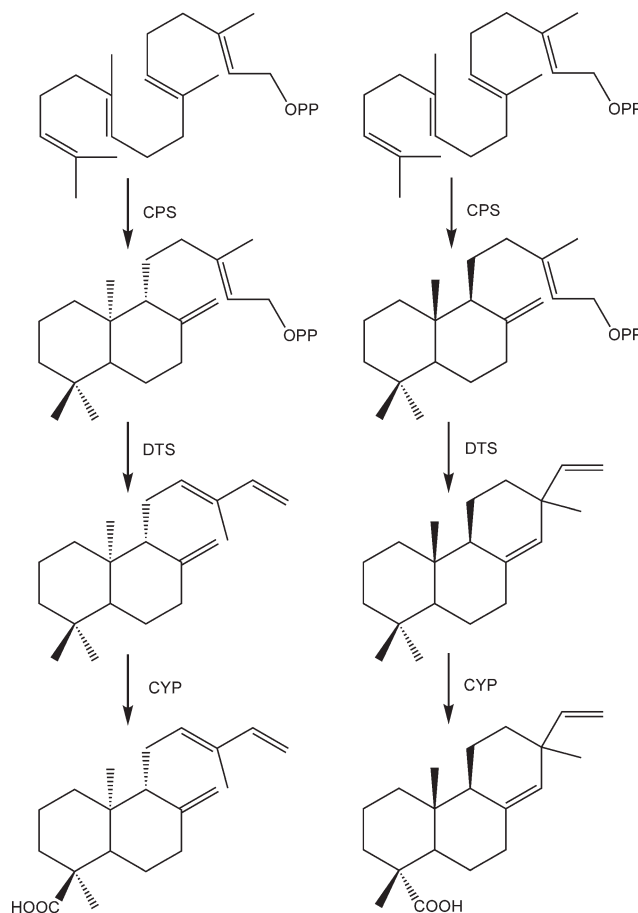


FIGURE 1 Production of diterpenoid monomers via enzymatic cyclization and functionalization of geranylgeranyl diphosphate. Geranylgeranyl diphosphate is cyclized by stereospecific copalyl diphosphate synthases (CPS) and Class I diterpene synthases (DTS) reactions to form labdane-related diterpenes. These can be further modified via cytochromes P450 (CYP), leading to formation of monomers suitable for subsequent polymerization.

configuration and the presence or absence of succinate within the resinite. Both Classes Ia and Ib are derived from polymers comprised of labdane monomers of the normal configuration, while Ic are derived from the *enantio* (ent) configuration.⁵ These two isomers originate from the stereoselective cyclization of GGPP to normal or ent configurations of labdadienyl/copalyl-diphosphate (CPP) by Class II diterpene cyclases. These bicyclic CPP units are then further modified, first by Class I diterpene synthases, and then CYP (see Figure 1), with the resulting monomers subsequently cross-linked into an amber matrix upon extrusion. In amber composed of labdane monomers with normal configuration (Ia and Ib), the presumed precursors are the normal labdatriene (bicyclic)-derived communic acid, and/or communol in some species, along with pimaradiene (tricyclic)-derived alcohols and acids, along with abietadiene (also tricyclic)-

derived diterpenoids that may be present as occlusion monomers (see Figure 2). In Class Ic (ent) amber, the presumed precursors are *ent*-labdatatriene-based ozic acid and ozol, along with *ent*-pimaradiene-derived alcohols and acids. Additionally, there are Class I ambers that contain both *ent* and normal configuration labdatatriene monomers in their occlusions. Interestingly, there are no ambers characterized to date that consist of *syn* configuration labdane monomers.⁶ This raises biochemical evolutionary questions, such as whether plant exudates diverged from *ent* to normal labdanes (or *visa versa*) due to selective pressure (e.g., the impact of monomer configuration on polymer structure and function), or did the formation of resinous exudates for defense arise multiple times with origins in biosynthetic processes with differing stereochemistry. It is possible there are ambers composed of *syn*-labdane-derived monomers, but which have not yet been recognized. Currently, monomer stereochemistry is analyzed by pyrolysis, which is used to breakup the amber polymer into simple small-molecule

components of the constituent monomers that are then separated by gas chromatography with mass spectrometry detection. While this method provides useful structural information, it does not allow for direct stereochemical assignment, since interpretation of the monomers is based on the retained configurations in the observed naphthalene derivatives released by the pyrolysis process. However, these only represent a portion of the original monomer unit, and leave the stereochemistry at C-9 obscure.^{5,6} Unfortunately, while solid-state NMR analysis provides useful information about functional groups within the polymer, it cannot resolve such questions regarding monomer configuration. Notably, synthesizing amber from predefined individual monomers would allow direct investigation of the effect of different stereochemical configuration on polymer structure, as well as comparison with currently available amber samples for classification purposes.

The enzymes responsible for diterpenoid resin acid biosynthesis were first characterized from the fir tree *Abies grandis*,^{7,8} with the single responsible diterpene synthase later identified and fully characterized.^{9,10} This led to further characterization of the diterpene synthases and CYPs responsible for resin biosynthesis in other conifers, particularly loblolly pine (*Pinus taeda*)^{11,12} and spruce (*Picea* spp.).^{13,14} Most recently, we have characterized a rice CYP responsible for similar oxidation of the C-19 methyl group in *syn*-pimara-7,15-diene and *syn*-stemodene to carboxylic acids, which are believed to be intermediates in rice phytoalexin biosynthesis, and may prove useful for engineering novel polymers.¹⁵ Furthermore, we have also identified a rice diterpene synthase that produces *syn*-labdatatriene,¹⁶ suggesting the possibility of producing *syn* resin acid counterparts to the normal and *ent* labdatatriene-derived communic and ozic acids (see Figure 2). These recent advances in identification of the responsible enzymatic genes provide the biosynthetic toolkit necessary to produce known resin acids, as well as new diterpenoid acids that provide an opportunity to synthesize polymers from labdane-related diterpenoid monomers of *syn* configuration, which may yield materials with potentially unique physical properties.

The biosynthesis of labdane-related diterpenes has been successfully accomplished via our previously described microbial metabolic engineering system,¹⁷ with recently reported optimization providing increased production levels.¹⁸ Furthermore, this system has proven amenable to the incorporation of CYP,¹⁹ including multifunctional oxidases that produce diterpenoid acids.^{15,20} Thus, our existing system is adequate for such small-scale synthesis of polymer materials, as it already has proven utility for the production of the multimilligram amounts necessary for structural characteri-

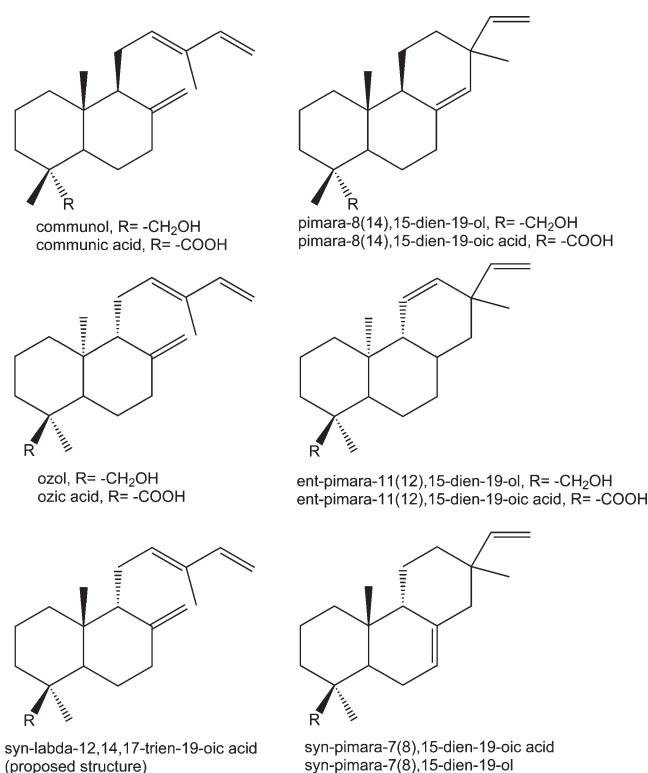


FIGURE 2 Common diterpenoid components of Class I amber resins. Communic and pimaradienyl derivatives are commonly found in Class Ia and Ib ambers, while labdane-derived structures of the *enantio* configuration (i.e., ozic and *ent*-pimaradienyl structures) are common components of Class Ic ambers. Resins containing labdanyl monomers of the *syn* conformation have not been reported, though recent characterization of *syn*-labdatatriene and *syn*-pimaradiene derivatives imply that such resins may exist.

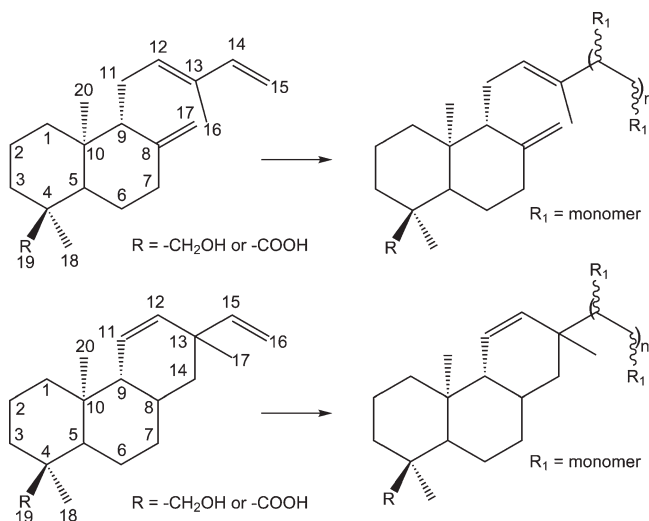


FIGURE 3 Polymerization of diterpenoid monomers for resin matrix formation. Bicyclic labdatrienes and tricyclic pimaradienes, along with the derived C-19 carboxylic acids, are common components of amber resins. These are likely cross-linked via polymerization of the labdatriene isoprenyl group (C14-C15) or the pimaradiene ethylene group (C15-C16).

zation of the products of novel diterpene synthases and CYP.^{15,16,19,21,22} In addition, the feasibility of biochemical/metabolic engineering of microbes for large-scale terpenoid production has already been demonstrated. In particular, Amyris Biotechnologies, cofounded by Prof. Jay Keasling, has reported producing industrial scale quantities of the sesquiterpenoid artemisinic acid, a precursor to the antimalarial drug artemisinin, using microbial fermentation.²³ These impressive production levels are largely based on improvements in supply of general terpenoid/isoprenoid precursors, which could then provide a source for industrial scale production of any terpenoid. Of interest here is biosynthesis of diterpenoid monomers in quantities sufficient for more elaborate polymer chemistry studies, followed by possible physical materials engineering characterization, and real-world studies of industrialization feasibility.

While the polymerization process involved in amber formation has not been fully elucidated, some information has been determined regarding the polymerizing cross-links of labdane-derived resin acids, specifically communic acid.^{6,24} It is clear that the terminal C14-C15 double bond on the extended isoprenyl side chain of communic acid is the favored site for such cross-linking,^{6,24} while for the related pimaric acids, the C15-C16 double bond on the ethylene side chain serves the same purpose (see Figure 3). The formation of these resin polymers is known to occur simply by exposure to UV-radiation and oxygen following the exudation of resin from conifer wound sites. This suggests that this particular radical-based polymerization

does not necessarily need to occur in a gas phase, as is common practice for synthetic petroleum-based polymer production, and such a solution phase process allows for a more energy-efficient means of polymer creation. In addition, metabolic engineering is a practical and efficient means for obtaining large quantities of labdane-related diterpenoid monomers that are not naturally abundant in large quantities, such as syn configuration labdane monomers. Together, this provides a potential route for the production of designer polymers via the biosynthesis of known and novel monomers, which could then be polymerized either homogeneously or from heterogeneous mixtures of defined composition (both monomer constituents and ratio). Initial cross-linking via UV radiation-induced radical formation could then be followed by extensive pressure application experimentation to create more durable cross-linked and defunctionalized polymers, mimicking the underground geological process of natural amber formation.^{25,26}

Further adding to the diversity of engineered resins is the possibility of introducing occlusion monomers into these designer polymers, much like those residing within natural resins.⁶ For example, an ent polymer may contain occluded monomers with configurations such as ent, normal or both, similar to those found naturally in exudates from modern gymnosperms.²⁷ Abietanes, pimaranes, totaranes, and kauranes (see Figure 4) are all common labdane-related diterpenoid monomeric occlusion material in most Class I ambers with normal and ent configurations.⁵ Doping engineered labdane biopolymers with carefully selected nonpolymerizing

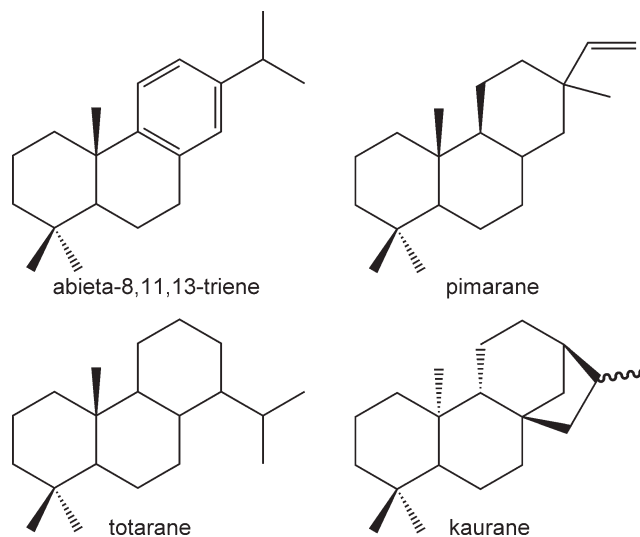


FIGURE 4 Common occlusion monomers in Class I amber resins. Incorporation of occlusion monomers in amber resins changes the physical properties of the resin. Abieta-8,11,13-triene, pimarane, totarane, and kaurane are all common occlusion monomers in Class I resins. The addition of occlusion monomers to synthetically derived resins may alter the physical properties of the resin, thus creating novel compounds fine-tuned for each application.



FIGURE 5 Caribbean ambers are prized for their light refraction properties. The unique and rare Dominican and Caribbean ambers are prized for their light refracting properties (e.g., the sample shown here under different lighting or with differing backgrounds). Synthetic production of these ambers may be useful for optical applications such as providing raw materials for light-refracting or harvesting equipment.

labdane-related monomers could provide a powerful tool for fine-tuning the properties of the polymeric material, such as hardness, color, transparency, or susceptibility to further oxidation. For example, similar to the addition of phthalates to petroleum-based plastics, adding more or less conformationally constrained labdane occlusion (or cross-linking) monomers should modulate the rigidity of the resulting polymeric material. Furthermore, the refractive index of an optically active polymer may be fine tuned by adding occlusion materials of specific configuration. Current engineering systems could be utilized to biosynthesize specific polymers directed by information acquired by analysis of amber to answer evolutionary questions and to recreate specific properties already observed in ambers. One example of such an application would be reproduction of the ent Caribbean ambers (see Figure 5), such as the Dominican blue ambers that have unique fluorescence and light-refracting properties.²⁸ These unique ambers are in limited natural supply and have yet to be found from alternative, more abundant sources, making them prime targets for such resinite biomaterial engineering. The ability to synthesize significant quantities of a resinite

polymer with similar properties could prove useful for optical applications.

In conclusion, decades of elegant biochemical work on terpenoid-derived amber biosynthesis have provided a solid foundation for future researchers and engineers to build upon. With the advent of next-generation sequencing technologies enabling in-depth analysis of the transcriptomes of nonmodel organisms (i.e., those lacking a sequenced genome), the number and variety of genes responsible for terpenoid biosynthetic machinery will continue to expand. Such studies provide potential sources for monomer diversity and, hence, elaborate polymer chemistry, via the ever-increasing number of characterized genes for such diterpenoid resin biosynthesis. This is especially true of the CYP genes responsible for production of the alcohols and acids that provide a wealth of oxidative products from each diterpene precursor. Even relatively simple diterpenoids, such as labdatriene-derived acids, provide great potential for production of high value designer synthetic materials. Production of such monomer feedstock via metabolic engineering represents a method of making resinite formation a renewable resource with significant potential for the

engineering of novel resinite polymers for applications in materials engineering.

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