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^{13}C NMR AND IR ANALYSES OF STRUCTURE, AGING AND BOTANICAL ORIGIN OF DOMINICAN AND MEXICAN AMBERS

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Key Word Index—*Hymenaea verrucosa*; *H. courbaril*; tropical Leguminosae; Dominican Republic amber; Mexican amber; resin; resin polymer; sesquiterpene hydrocarbons; solid state ^{13}C NMR; IR; labdane.

Abstract—^{13}C NMR analysis of Oligocene amber from the Dominican Republic, Oligo-Miocene amber from Mexico and trunk resins from certain extant species of *Hymenaea*, suggests a polylabdane structure for all and gives insights into structural changes during aging. Additionally, IR spectra, some aspects of the ^{13}C NMR spectra and paleobotanical data suggest a close relationship between Dominican amber and *H. verrucosa*. By contrast, spectral evidence shows that Mexican amber differs, not only from the former polymers, but from the *H. courbaril* polymer.

INTRODUCTION

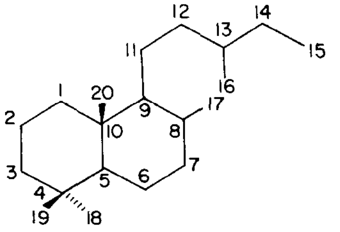
Terpenoid resins from both gymnosperms and angiosperms are usually chemically very durable and may become fossilized forming amber. This study is made in the context of a long-range investigation of the evolution of tropical resin-producing trees, initiated as a result of a survey of resins through space and geologic time which indicated the importance of tropical angiosperms as copious resin producers and the source of amber [1, 2]. Most previous studies focused on temperate zone conifer resins [1]. Interest has become directed both towards understanding the ecophysiological roles of these resins, in tropical ecosystems, and the changes in chemical constitution and processes that result during the hardening and aging of the resin, ultimately to form amber [1].

The amphi-Atlantic tropical leguminous genus *Hymenaea* (Caesalpinioideae, Detarieae) was selected as one of the model systems for this evolutionary study because of its copious resin production and resulting commercial value [2], its occurrence in a variety of tropical ecosystems and evidence that its resin had been fossilized as amber [1]. Although resin is synthesized in secretory tissues in all organs of *Hymenaea* [3] trunk resin in mature trees is secreted by epithelial cells lining pockets which are located in the cambial zone. Upon natural or man-made stress, resin collects in lysigenous cavities, produced by breakdown of resin secretory cells, and then exudes to the exterior of the trunk where it may collect in large quantities and harden. In the process it may serve ecological functions, such as engulfing small potential predators and protecting exposed surfaces from pathogens [1–3]. These hardened masses of resin, known in the trade as 'copal', have been noted for their toughness and durability. When they accumulate in sites of deposition they may, subsequently, form extensive deposits of amber [1].

Tertiary amber from Mexico, Colombia and Brazil has been identified by several criteria (including IR spectra) as having been produced by the genus *Hymenaea* [1]. In this study we compare the chemical constitution of Tertiary amber from the Dominican Republic (Oligocene; i.e. 20–35 million years old) and Mexico (Oligo-Miocene; i.e. 20–24 million years old) with resin of the African species of *Hymenaea* (*H. verrucosa* Gaertn.) and the Neotropical species (*H. courbaril* L.).

Hardened resin or amber may be divided into two fractions based on its solubility in organic solvents. The soluble portion of *Hymenaea* trunk resin is composed primarily of soluble polymer, diterpene acids having the labdane (**1**) and enantiolabdane skeletal structures [4–9]. Additionally, small quantities of sesquiterpenes have been isolated [5, 10].

Photochemical studies of *H. verrucosa* trunk resin suggest that UV radiation near 320 nm (within the natural solar envelope), possibly initiates radical polymerization of unsaturated constituents in the freshly exuded resin [11]. Studies on both *Agathis* trunk resin [12] and



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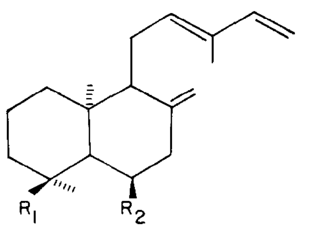

2

$R_1 = CO_2H$

$R_2 = OAc$

3

$R_2 = OH$

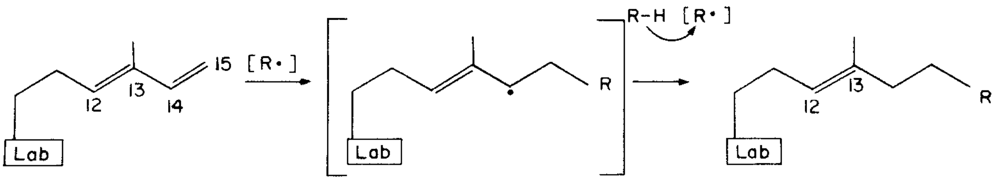

Fig. 1. Free radical attack on labdene monomer showing sterically preferred addition.

Hymenaea seed pod resin [Futton, J. and Langenheim, J. H., unpublished results] have further established that the predominant molecular species producing polymer in these resins are bicyclic labdene monomers which have a conjugated diene side chain and tend to polymerize via sterically preferred additions to the 14,15-unsaturation [13] (Fig. 1).

Upon exudation of the resin, the volatile components, such as sesquiterpene hydrocarbons which apparently function as solvents and natural plasticizers to control viscosity and flexibility, are gradually lost to produce hardened resin which contains up to 90% of polymer. Sesquiterpene hydrocarbons may have another important function in controlling, in part, the rate of photo-induced polymerization [Fulton, J. and Langenheim, J. H., unpublished results]. The effect of sesquiterpene hydrocarbon concentration on the hardening of the resin is evident in the neo-tropical species of *Copaifera* (a closely related genus to *Hymenaea*) which produce a comparatively thin 'oleo' resin composed primarily of sesquiterpenes and smaller amounts of diterpenoids [10]. This resin shows little or no tendency to polymerize, whereas resins from the African species of *Copaifera* contain predominantly diterpenoids and polymerize readily to form hardened masses similar to *Hymenaea* [10].

RESULTS AND DISCUSSION

IR studies of polymeric residues isolated from all specimens exhibit a broad hydroxy band, a carboxylic carbonyl band and olefinic absorption. With the exception of Mexican amber, which also shows relatively less intense olefinic absorption, the presence of an exocyclic methylene group is evident by absorption near 899 cm^{-1}.

The spectra of Dominican amber polymer and *H. verrucosa* polymer were essentially superimposable, exhibiting only relatively minor differences in the amplitude and shapes of certain bands. Purified polymer provides a better comparison in the fingerprint region than does whole resin and amber and, thus, a better basis for comparison and assessment of botanical relationships.

^{13}C NMR on solid samples, using magic angle spinning with cross-polarization (CP/MAS), confirmed a small number of carboxy and ester carbonyl carbons. No detectable ketone or aldehyde carbons ($> \delta 200$) were present in the resins or ambers [14–16]. The aliphatic region (Me, δ15–20; Me, CH_2, δ20–25; CH_2, δ25–35; CH_2CH, δ35–50) of the resin and amber spectra contained a major absorption centered near δ39. This corresponds to a C-7 labdane carbon. Signals in the δ50–70 region are found corresponding to carbons singly bonded to oxygen (e. g. CHOH, CH_2OH). The olefinic region (δ107–148) of the samples examined revealed a maximum of four resonances attributable to C-17 (107), C-12 (125), C-13 (140) and C-8 (148) of a labdene [17].

Interrupted decoupling aided in the assignment of methyl functions and quaternary carbons. Figure 2 shows the ^{13}C spectra of *H. courbaril* and *H. verrucosa* resin, Dominican amber and Mexican amber with and without interrupted decoupling. The lower trace in each figure reveals remaining resonances due to quaternary and methyl carbons. The instrumental line-width, as judged from the ammonium oxalate peak, was δ1.0. The majority of peaks in all samples are wider than this indicating a superposition of peaks with nearly coincident chemical shifts. Considerable unresolved intensity in the δ20–30 range is reduced by delayed decoupling, indicating signals in this region arise mainly from protonated carbons. Assuming labdane precursors, intensity remaining in the delayed decoupling experiments in the δ19–22 range is probably due to C-20 methyls [15]. The intensity in the δ32–39 range could, likewise, be due to a quaternary carbon at C-10 and the C-18 methyl in the labdene skeleton. The resonance near δ46 is in the correct position for a quaternary carbon bearing a carbonyl (e.g. C-4).

The olefinic resonance near δ147 is probably due to C-8 of $\Delta^{8,17}$-labdene constituents while the resonances due to quaternary carbons near δ138–139 are in a region expected of C-13 in a Δ^{12}-labdene. Purified polymer spectra (Fig. 3A) were not substantially different from those of the parent resin (Fig. 3B).

Comparison of *H. verrucosa* resin (Fig. 2B) with *H. courbaril* resin (Fig. 2A) reveals that the resonance appearing near δ70 in *H. verrucosa* is not present in *H. courbaril*. The δ70 resonance is presumably due to polymer which was produced from polymerization of labdatriene monomers related to zanzibaric acid [18] (**2**) but having a 6-β-hydroxyl function (**3**), rather than a 6-β-acetoxy moiety of zanzibaric acid. Zanzibaric acid has been previously isolated from *H. verrucosa* pod resin [18].

Comparison of the ^{13}C NMR spectra of the *Hymenaea* resins (Fig. 3B, C) with those of the ambers (Fig. 3D, E) reveals a marked decrease in the amber spectra of the olefinic resonances near δ107, 125, 140 and 148 vis-a-vis signals in the δ15–60 region. The olefinic carbon signals may be assigned, respectively, to C-17, C-12, C-13 and C-8 of $\Delta^{8(17)}$, Δ^{12}-labdenes. The decrease in intensity of these signals coupled with a decrease in intensity of the signals due to allylic carbons near δ38 (C-7) and 56 C-9 would result from the consumption of the $\Delta^{8(17)}$ unsaturation with time. In comparing the ^{13}C spectra of the two ambers, it is obvious that in the Mexican amber the resonances associated with the $\Delta^{8(17)}$ linkage are almost absent and the resonance near δ57 (–CH–OH) is less prominent when compared to the other samples.

Lambert and Frye [20] have recently compared the ^{13}C (CP/MAS) spectra of several ambers. The Baltic amber (succinite) clearly showed four olefinic resonances in the δ107–148 region. Moravian amber (Walchowite) lacked the δ107 and most of the 148 resonances and gave signals in the 80 region. Burmese amber (Burmite) gave resonances in the δ70 region (C-6, β-OH?) while the 107 and 148

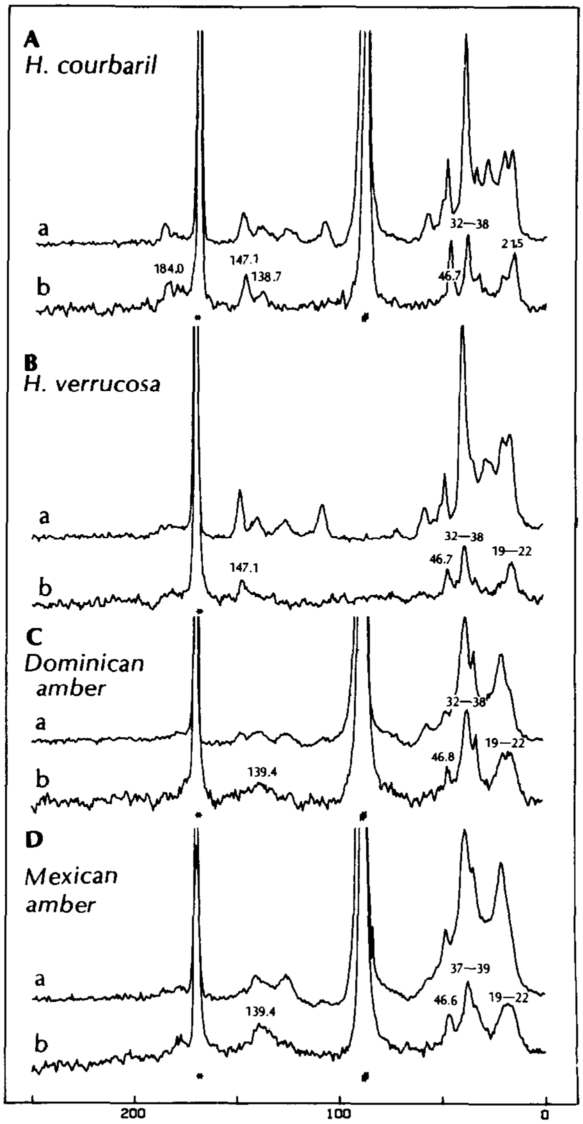

Fig. 2. ^{13}C NMR spectra of resins and ambers. (A) ^{13}C spectrum of *H. courbaril* resin. (a) Upper trace, normal spectrum; (b) lower trace, protonated carbons suppressed by delayed decoupling; * peak from added ammonium oxalate; # peak from delrin spinner. Chemical shift scale in δ-values (ppm) with respect to TMS. (B) ^{13}C spectrum of *H. verrucosa* resin, as in (A). (C) ^{13}C spectrum of Dominican amber, as in (A). (D) ^{13}C spectrum of Mexican amber, as in (A).

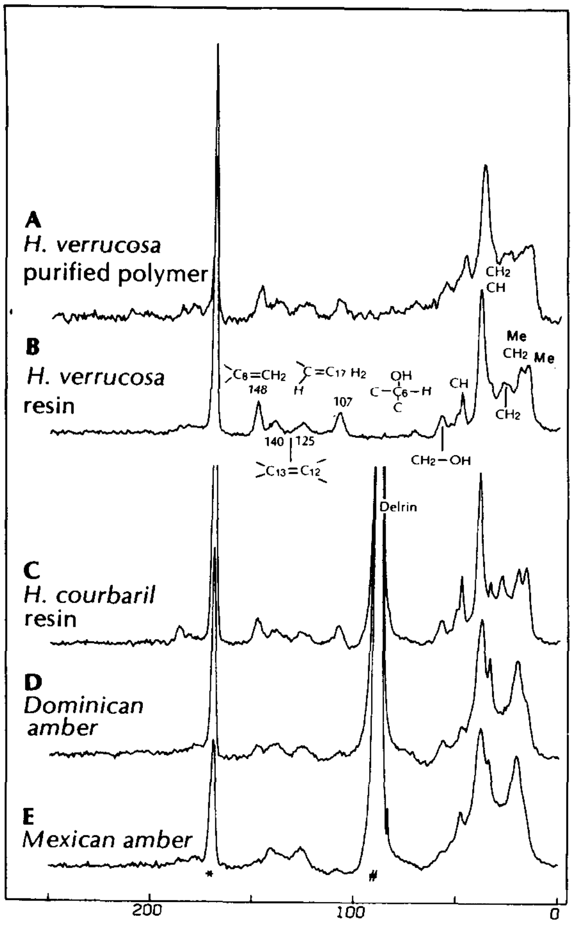

Fig. 3. ^{13}C NMR spectra of polymers, resins and ambers. Superimposed ^{13}C spectra of (A) *H. verrucosa* purified polymer; (B) *H. verrucosa* resin; (C) *H. courbaril* resin; (D) Dominican amber; (E) Mexican amber.

resonances were decreased in intensity relative to the 125–137 resonances.

^{13}C NMR spectra, when obtained by the CP/MAS method, provide useful structural information regarding the polymerized components of resins and ambers and changes that occur in these complex materials through time. Compelling paleobotanical data (floral remains enclosed in the amber [Hueber, F. and Langenheim, J. H., unpublished results]), IR spectral evidence and some aspects of the ^{13}C NMR spectra suggest that the Dominican amber was produced by a species very closely related to the African *H. verrucosa* from the primitive section *Trachylobium* of *Hymenaea* [20]. Both ^{13}C NMR and IR spectral evidence indicate differences between the Dominican and Mexican ambers. They further establish that Mexican amber and *H. courbaril* polymer are not as similar as are the Dominican amber and *H. verrucosa* resin.

EXPERIMENTAL

Resin and amber samples. H. verrucosa resin (JHL No. 5521) was from rain forest trees in Kwale District, Kenya, Africa. *H. courbaril* resin was from Amazonian rain-forest trees near Santarém, Brazil (JHL No. 5621). Oligocene amber was from the Dominican Republic (JHL No. 5825) and Oligo-Miocene amber was from Chiapas, Mexico (JHL No. B-7046).*

Isolation of polymer from amber and resin samples. Resin and amber were pulverized and exhaustively extracted for 72 hr with EtOAc. The insoluble gel-like mass remaining was evaporated *in vacuo* to remove residual solvent.

^{13}C NMR conditions. ^{13}C NMR spectra were obtained at 15.08 MHz using ^{1}H cross-polarization [21] and magic angle spinning

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*Specimens documented in these collections are deposited at the University of California, Berkeley and Santa Cruz.

[22] on a modified TT-14 spectrometer. ^1H and ^{13}C fields of 40 or 70 kHz were used, with no apparent difference in spectra. An Andrew-type magic angle spinner was used, with a spinning rate of 2.5 kHz. Non-protonated carbons were identified by delaying decoupling for 60 μsec after cross-polarization. This suppressed signals from protonated carbons [23]. All spectra were taken with a single contact Hartmann–Hahn cross-polarization of 2 m sec. Due to the low filling factor of the probe, 1–4 × 10^4 contacts were averaged for each sample. NMR samples were prepared by grinding the sample to a fine powder, mixing with *ca* 10% by vol. of ammonium oxalate and pressing into the spinner. The ammonium oxalate served both as a chemical shift standard and to achieve correct adjustment of the magic angle. Thus, all spectra show a large peak at δ169.8 from ammonium oxalate. Some spectra, which were run in delrin spinners show a peak at δ90 from the spinner material, others which were run in Kel-F spinners do not show this peak.

^{13}C *NMR*. *H. verrucosa* resin. δ16.3, 19.7, 26.3, 28.2, 34.8, 39.5, 48.1, 58.2, 71.4, 108.2, 126.3, 140.1, 148.4, 182.8, 187.3.

Dominican amber. δ16.3, 20.5, 33.9, 38.2, 47.7, 57.1, 107.6, 125.1, 138.0, 147.1.

H. courbaril resin. δ16.5, 20.2, 28.3, 34.0, 39.6, 48.2, 49.4, 58.0, 108.3, 123.3, 127.8, 140.4, 148.7, 186.7.

Mexican amber. δ20.6, 34.3, 38.1, 46.7, 108.7, 126.2, 140.9.

IR. *H. verrucosa* resin polymer. Colorless powder ν_{max}^{KBr} cm^{-1}: 3400 (OH) broad, 2914, 1714 (carboxyl carbonyl), 1643 (olefinic), 1444, 1384, 1241, 1054, 899 (exocyclic methylene), 710, 649, 617.

Dominican amber polymer. ν_{max}^{KBr} cm^{-1} 3343 (OH broad, 2914, 1718, 1643, 1251, 1054, 899.

H. courbaril resin polymer. Colorless powder. ν_{max}^{KBr} cm^{-1}: 3400 (OH) broad, 2940, 1710 (carboxyl carbonyl), 1640 (olefinic), 1440, 1385, 1265, 1167, 1121, 925, 885, 795.

Mexican amber polymer. Powder; ν_{max}^{KBr} cm^{-1}: 3450 (OH) broad, 2930, 1725 (carboxyl carbonyl), 1650 (olefinic), 1455, 1365, 1238, 1145, 1030.

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