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A Polymer Model of Lignin (D.H.P.) ^{13}C Selectively Labelled at the Benzylic Positions: Synthesis and NMR Study

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SUMMARY:

D.H.P. polymer model of Lignin was synthesized by enzymatic dehydrogenation of coniferyl alcohol enriched to 90% in ^{13}C-γ on the benzylic position. On the ^{1}H NMR spectrum recorded at 250 MHz, of the ^{13}C labelled D.H.P., the study of the $J_{CH-\gamma}$ couplings, made possible a precise assignment of proton signals. The ^{13}C NMR spectra showed that the ^{13}C-γ carbon atom previously labelled in coniferyl alcohol was found in several sites in the polymer. Special NMR techniques like gate decoupling and selective proton irradiation were used together with the study of the chemical shifts, to make an assignment for the different ^{13}C-γ signals. C-γ in carbonyl compounds like vanillin and vanillic acid, C-γ vinylic atom like in coniferyl alcohol and cinnamaldehyde, C-γ atom like in β—5, β—β, β—O—4 dilignol units, and C-γ atom involved in C-γ benzyl ether bond in β—O—4 dimer were identified; the corresponding $J_{CH-\gamma}$ couplings of these C-γ carbon atoms were determined.

Introduction

In his tentative scheme illustrating the constitution of lignin, *Freudenberg*[1] establishes that the dehydrogenation of a mixture of *p*-hydroxycinnamic alcohols by phenol oxidases in the laboratory, leading to an amorphous polymer called D.H.P., is a close reproduction of the genuine process of lignification in wood. Quite every attempt in studying lignin structure confirms or refines *Freudenberg*'s proposal and, among them, of importance here is the work of *Nimz* et al. on ^{13}C NMR spectra of natural lignin[2] and synthetic lignin, D.H.P.[3].

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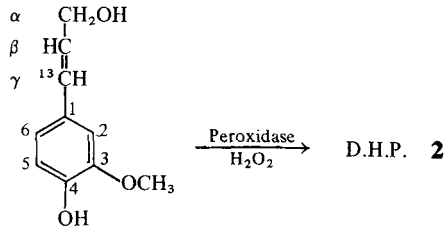
In a previous work[4], we studied ^1H NMR spectra of selectively deuterium labelled D.H.P. and used gel permeation chromatography (GPC) as a way to eliminate from D.H.P. fragments comparable in weight to monolignols or dilignols. Then, it seemed to us interesting to start an investigation by both ^1H NMR and ^{13}C NMR spectroscopy on a selectively ^{13}C labelled D.H.P. **2** synthetised from a sample of coniferyl alcohol*) **1** selectively ^{13}C labelled on the C-γ**) side chain atom:



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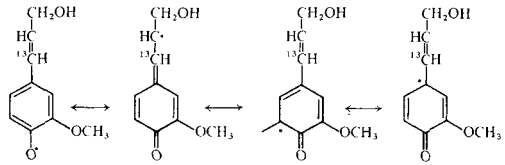
This labelling will give the possibilities to get the following informations:

– Identifying the carbon sites of the polymer which comes from the C-γ atom previously labelled in the monomer.

– Determination of the corresponding $J_{CH-\gamma}$ values by observation of the proton spectra and of the gate decoupled carbon spectra.

– Association of the δ_C to the δ_H shifts of carbon and proton directly bound by selective proton irradiation.

Our current knowledge[5] of the present status of lignin is for an important part based on the extensive study of the model dehydrogenation of coniferyl alcohol, and to some extent of *p*-coumaryl and sinapyl alcohols. The immediate result of enzymatic phenol oxidation of coniferyl alcohol is the formation of a free radical stabilized by mesomerism:

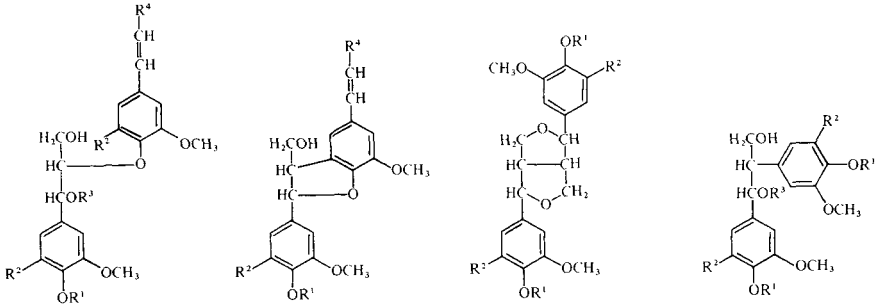


Ra **Rb** **Rc** **Rd**

*) IUPAC name: 3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol.

**) Nomenclature α, β, γ, in the C_6—C_3 unit is used according to the IUPAC rules A-13.3 and C-51.2 (1960).

This radical is prototype of many others that occur during lignification. Through various coupling possibilities of these mesomeric forms the final polymer is formed. Its formula is statistically given and the usually accepted formulation comes from the association through different linkages of around twenty guaiacyl-propane units[1]. Coupling of the mesomeric form **Rb** with the mesomeric forms **Ra, Rc, Rb, Rd,** gives a set of four principal types of dimers: β—O—4 or $\mathbf{D_1}$, β—5 or $\mathbf{D_2}$, β—β or $\mathbf{D_3}$, β—1 or $\mathbf{D_4}$. These designations are given according to the nature of the bond in which C-β is involved.



$\mathbf{D_1}$ $\mathbf{D_2}$ $\mathbf{D_3}$ $\mathbf{D_4}$

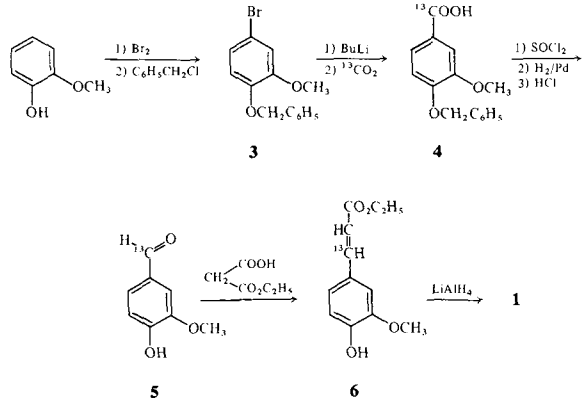
with R^1 = H, C′-γ or C′-β
R^2 = H, C′-β or C′-5
R^3 = H, C′-γ, C′-α, C′-4 or linkage with polysaccharides
R^4 = CH_2OH, CH_2OC'-γ, CHO

With their different substituents $\mathbf{D_1}$, $\mathbf{D_2}$, $\mathbf{D_3}$, $\mathbf{D_4}$ represent model compounds of the most important dimers isolated at the first step of the enzymatic dehydrogenation of coniferyl alcohol, and include the most representative bondings between the monomer units. For the discussion of the results, as far as C-γ and its proton directly bound are concerned, only these principal fragments will be considered.

Nimz et al. studying ^{13}C NMR spectra of milled-wood lignin (M.W.L.) of spruce and deciduous trees[2] and of D.H.P.[3], identified 35 carbon atoms of these polymers, by comparing their ^{13}C chemical shifts to those of 14 monomeric and 25 dimeric 4-hydroxyalkylbenzene model compounds of lignols and dilignols.

For the synthesis of selectively labelled coniferyl alcohol-^{13}C-γ (**1**) we followed the pathway used for ^{14}C labelling of this same alcohol[6] through

vanillin-carbonyl-^{13}C synthesis and usual malonic condensation followed by reduction[7].



From 5 g of commercial $Ba^{13}CO_3$ (ca. 90% ^{13}C enriched) the synthesis (cf. Exptl. Part) allows the preparation of about 920 mg coniferyl alcohol-^{13}C-γ (90% ^{13}C enriched as verified by mass spectrometry).

For the enzymatic polymerization of coniferyl alcohol according to *Freudenberg*'s „Zutropfverfahren" method, we used horseradish peroxydase as enzyme and hydrogen peroxide as hydrogen acceptor[8]. The purification is realised by gel permeation chromatography (GPC) on Sephadex Lh-20 in dioxane/water (9/1, v/v). GPC can be considered as a way to obtain fractions of lignin polymer, more homogeneous in weight. This filtration as we used it previously[4] for deuterium labelled D.H.P., has as its main purpose the elimination of low molecular weight fragments like monomers or dimers; furthermore the comparison of the ^{13}C spectra before and after gel filtration gives some additional information (see later). Fig. 1 gives the plot of absorbance at 280 nm of the different fractions versus the volume of eluant. Two samples were considered for the NMR study: fraction (I) and unfractionnated material, fraction (I)+(II).

Results and Discussion

^1H NMR spectra were recorded at 250 MHz using hexadeuterated dimethyl sulfoxide (DMSO-d_6) as solvent (10% w/w solutions). ^{13}C NMR spectra were recorded in the Fourier transform mode at 25,2 MHz and 62,8 MHz

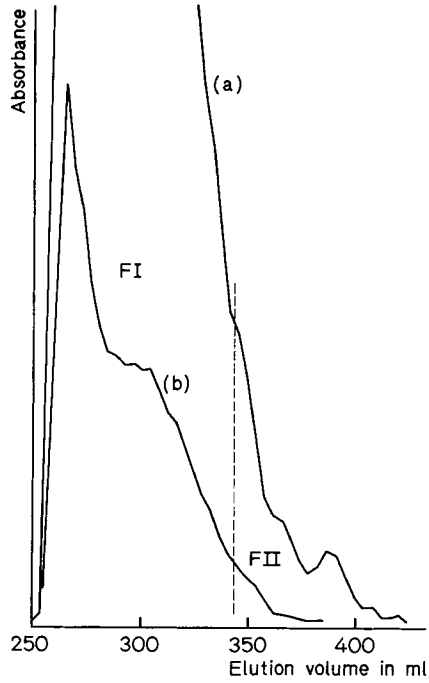


Fig. 1. GPC plot of absorbance at 280 nm versus effluent volume of 60 mg of D.H.P. **2**, on a LH-20 column in dioxane/water (9/1, v/v). (a): curve without dilution, (b): curve with dilution. Column: (3,1 cm × 120 cm). Fractions: 12 ml

in DMSO-d_6 or deuteroacetone/heavy water mixtures (9:1, v/v) (10% w/w solutions). The protons are noise decoupled (for further details s. Exptl. Part). Shifts values quoted are given in ppm downfield from TMS (^{1}H or ^{13}C).

^{1}H NMR spectra

A) Proton spectrum of ordinary D.H.P.

In the literature, ^{1}H NMR spectra of M.W.L. lignin or D.H.P. are recorded at 60 MHz or 100 MHz and deal most of the time with modified samples, acetylated for example, using $CHCl_3$ as solvent[9-10]. Some studies have been done at 60 MHz using DMSO as solvent on unmodified samples[11] or on methanol lignin[12].

On these spectra seven or eight chemical shift ranges have been determined, each of them characteristic of groups of protons present in lignin structure. On our ^{1}H NMR spectrum (Fig. 2) of an unmodified sample of filtrated

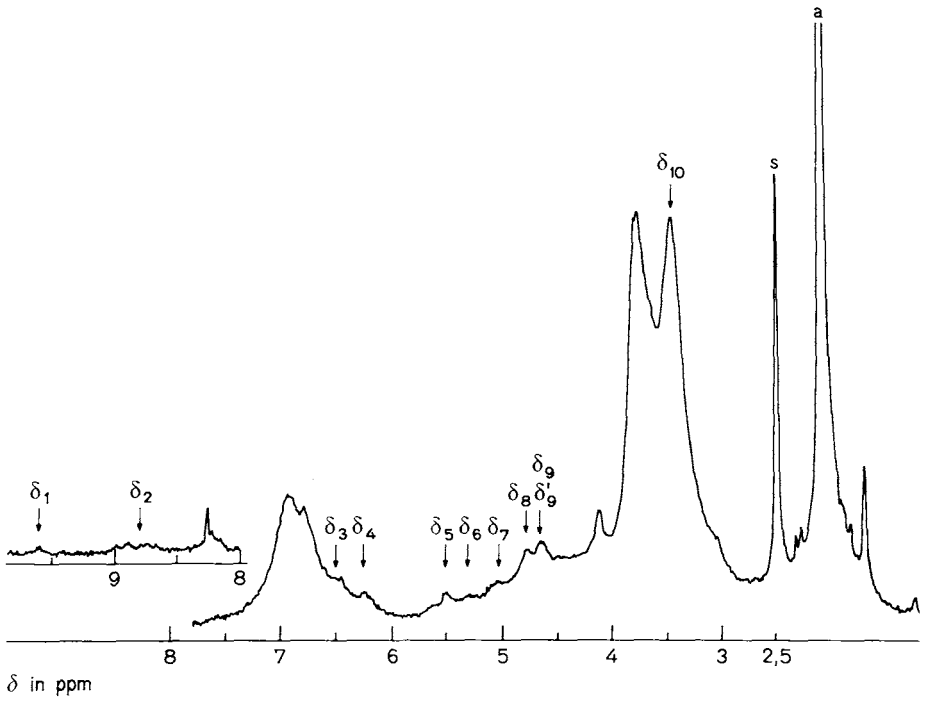


Fig. 2. ^{1}H NMR spectrum of ordinary filtrated D.H.P. (fraction I) recorded at 250 MHz in DMSO-d_6. "S" = solvent signal; "a" = acetone as internal standard. The arrows indicate centres of the protons signals and the corresponding chemical shifts are assigned as: $\delta_1 = 9{,}62$ ppm: aldehydic proton like in vanillin; $\delta_2 = 8{,}8$ ppm, $\delta_6 = 5{,}3$ ppm, $\delta_7 = 5$ ppm, $\delta_{10} \approx 3{,}4$ ppm: hydroxyl protons; $\delta_3 = 6{,}5$ ppm: γ vinyl proton; $\delta_4 = 6{,}25$ ppm: β vinyl proton; $\delta_5 = 5{,}5$ ppm: H-γ proton in β_5; $\delta_8 = 4{,}76$ ppm: H-γ proton in β—O—4; $\delta_9 = 4{,}64$ ppm: H-α proton in β—β; δ'_9 = H-β proton in β—O—4; $\beta = 4{,}1$ ppm: H-α methylene proton in —CH_2OR

D.H.P. recorded at 250 MHz in DMSO, in addition to the usual distribution of signals, we observe better resolved signals well separated enough to try to do a precise assignment for each of them. It is possible to consider three ranges:

– Range of hydroxyl protons: Fig. 3 shows proton spectra of ordinary D.H.P. (fraction I + II) before and after addition of a few drops of CF_3COOD; we chose spectra of unfractionated D.H.P. because of a more precise localisation of the proton signals, making easier the attribution of the corresponding

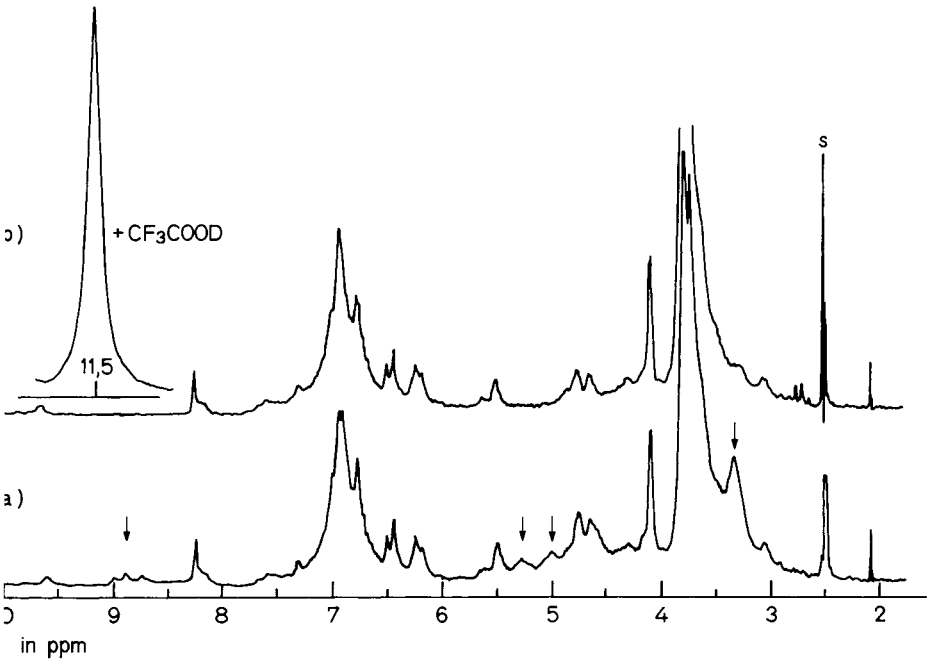


Fig. 3. ^{1}H NMR spectra of ordinary non filtrated D.H.P. (fraction I+II) recorded at 250 MHz (a) without and (b) with CF_3COOD. "S" solvent signal DMSO-d_6. TMS is used as internal standard. Shiftet hydroxyl signals are indicated with an arrow

signals on the spectrum of filtrated D.H.P. (Fig. 2). Being shifted to lower field by addition of CF_3COOD, signals at 8,9 ppm, 5,3 ppm, and 5 ppm are identified as hydroxyl protons. In addition, rapidly exchanging protons and hydroxyl groups of water, present in the sample, or in such hygroscopic solvents as DMSO, can be responsible for the signal at 3,35–3,45 ppm which is also shifted downfield and the intensity of which is variable depending of the samples. These four signals are indicated as δ_1, δ_6, δ_7, and δ_{10} on Fig. 2.

– Between 7,4 ppm and 6 ppm we previously demonstrated by selective deuteriation of the propane side chain of coniferyl alcohol[4] that δ_3 and δ_4 signals centered at 6,45 ppm and 6,20 ppm are respectively representing C-γ—H and C-β—H vinyl protons, thus locating more precisely aromatic protons between 7,4 ppm and 6,5 ppm.

– Between 6,0 and 3,9 ppm we observe the three signals δ_5, δ_8, δ_9 at 5,5 ppm, 4,76 ppm, and 4,64 ppm. The signal at 5,5 ppm is representative of a C-γ—H proton in phenyl-coumaran fragments like **D₂**. The assignment of the other signals will be done later on the ^{13}C labelled compound. The signal at 4,1 ppm has been previously identified by deuterium labelling as a C-α—H proton of methylenic type like in **D₁**, **D₂**, **D₃**, or **D₄** with $R^4 = CH_2OH$ or $—CH_2OC'$-γ.

B) Proton spectrum of C^{13} labelled D.H.P. **2**

Most of the C-γ carbon atoms involved in the proposed formula of lignin, are tertiary carbons (directly bound to one proton). When this carbon is enriched with C^{13} (ca. 90%), it is expected that the proton directly bound to it will give a doublet in ^1H NMR due to the $J_{CH-\gamma}$ coupling.

Fig. 4 shows spectra of (a) ^{13}C labelled D.H.P. **2** (fraction I), sample unmodified, of (b) the same sample with CF_3COOD, so to prevent hydroxyl signals to hide others signals, and of (c) a sample of ordinary D.H.P. (fraction I+II) with CF_3COOD. We expect that the coupling of the H-γ vinyl proton to the ^{13}C carbon will be of about 154 Hz, by analogy with the $J_{CH-\gamma}$ coupling in coniferyl alcohol-$^{13}C_\gamma$ itself; indeed, signal δ_3 at 6,45 ppm attributed to H-γ vinyl proton disappears except for a small shoulder attributed to the ^{12}CH-γ vinyl proton bound to the unlabelled fraction (10%) of the ^{12}C-γ carbon atom. The corresponding doublet due to the $J_{CH-\gamma}$ coupling appears underneath both the H-β vinyl δ_4 proton whose signal is degenerated and underneath the aromatic signal whose peak at 6,85 ppm increases.

In the range between 5,80 ppm and 4,30 ppm the three previous signals δ_5, δ_8, δ_9 at 5,5 ppm, 4,76 ppm, and 4,64 ppm are replaced by seven signals one of them δ'_9 staying at 4,64 ppm; we can associate six of them, two by two, in three doublets with respectively $J_{CH-\gamma} = 160$ Hz, 145 Hz, and 150 Hz (± 10 Hz). Signal δ_5 at 5,5 ppm is already known as being a C-γ—H proton in a β—5 fragment or phenyl-coumaran moiety **D₂**, the attribution is confirmed. Considering that in a molecule of pinoresinol (**D₃** with $R^1 = R^2 = H$) the C-γ—H proton gives a peak in NMR[11] centered at 4,60 ppm (in DMSO), we identify the δ_9 signal centered at 4,64 ppm as the signal of a H-γ proton of a pinoresinol fragment **D₃**. We assume that signal δ_8 belongs to a H-γ proton like in a β—O—4 fragment **D₁**: indeed the NMR signal of such a proton in a molecule of guaiacylglycerol β-coniferyl ether is reported[11]

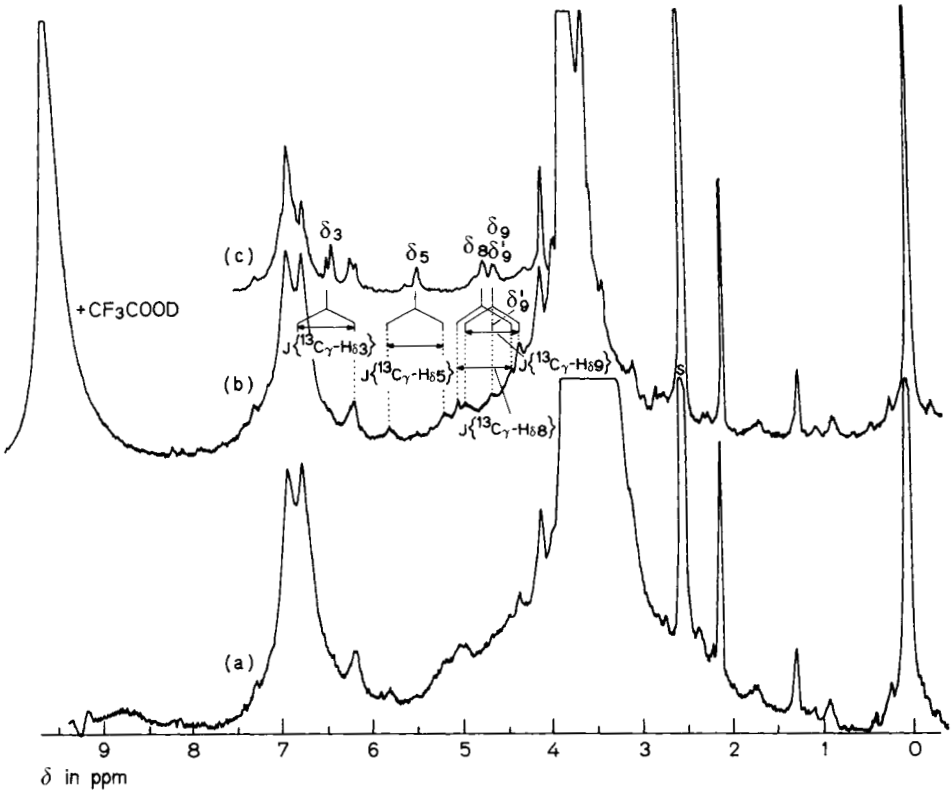


Fig. 4. ^{1}H NMR spectra (a) of D.H.P. **2** (filtrated sample (fraction I)), ^{13}C labelled at the benzylic positions, (b) of the same sample with CF_3COOD, and (c) of ordinary D.H.P. (fraction I + II) with CF_2COOD. "S" = solvent signal DMSO-d_6, TMS as internal standard. Study of protons directly bound to ^{13}C-γ carbon by considering the existence of $J_{CH-\gamma}$ couplings

in DMSO at 4,75 ppm; we note that usually this kind of proton gives a signal between 6,3 and 5,7 ppm in acetylated samples (D.H.P. or M.W.L.)[9-10] the acyl substitution on the H—C-γ—O oxygen atom shifting the proton to lower field.

For the δ'_9 signal at 4,64 ppm remaining unchanged as far as the shift is concerned, but decreasing in intensity, we can conclude that the corresponding proton was not directly bound to a ^{13}C-γ carbon atom, and considering the range of chemical shift, assume that it can be a H-β proton like in

a β—O—4 fragment, $\mathbf{D}_1$. On Fig. 2 we indicate signals δ_3, δ_4, δ_5, δ_8, δ_9, and δ'_9 the nature of which has been determined or confirmed by ^{13}C labelling.

^{13}C *NMR spectra*

A) Ordinary D.H.P.

Comparing the ^{13}C spectrum of an ordinary D.H.P., Fig. 5(a), with *Nimz*'s spectrum of a „Zutropfverfahren" D.H.P.[3] we can confirm the assignment of the different signals; we notice they are shifted upfield of about 0,5 ppm to 1,5 ppm when the samples are in DMSO-d_6 instead of perdeuteroaceton/heavy water (9/1, v/v).

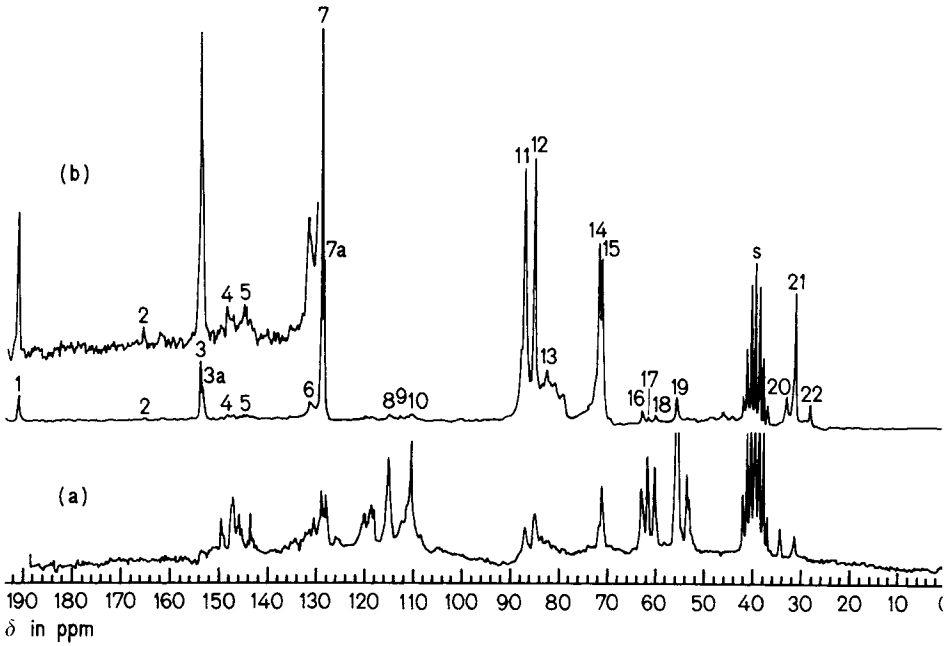


Fig. 5. ^{13}C NMR spectra (a) of ordinary D.H.P. and (b) of ^{13}C labelled D.H.P. **2** (fraction I+II for both samples). Spectra are proton totally decoupled, recorded at 25,2 MHz in DMSO-d_6 with TMS as internal standard. Vertical scale has been increased to enhance signals (2), (4), and (5).

B) ^{13}C-labelled D.H.P. **2**

On the corresponding ^{13}C spectrum, Fig. 5(b), we observe 9 sharp peaks (1), (3), (3a), (7), (7a), (11), (12), (14), (15), and 2 broad signals centered at 83 ppm (13) and 132 ppm (6) and, in addition, we notice the weak ^{13}C signals (2), (4), (5), (8), (9), (10), (16), (17), (18), (19), whose chemical shifts correspond to those of some carbon signals of the naturally enriched D.H.P., Fig. 5(a); by comparison of both spectra of Fig. 5 we can first assign signal (19) at 55,7 ppm to (—OMe) signal, signal (18) at 60,2 ppm, (17) at 61,6 ppm, (16) at 62,8 ppm to C-α carbon, respectively, in $\mathbf{D_1}$, in coniferyl alcohol, and in $\mathbf{D_2}$ and $\mathbf{D_4}$; signals (10) at 110,6 ppm to carbon C-2, signal (9) at 115 ppm to carbon C-5, signal (8) at 118,6 ppm to carbon C-6, signal (5) at 144,5 ppm to C-4 or C-4′ in $\mathbf{D_2}$, signal (4) at 147 ppm to C-3. Signal (19) has the highest intensity among the other signals belonging to the ordinary D.H.P. After this first assignment, it is possible to conclude that the other signals of spectrum Fig. 5(b) belong to the labelled site coming from the C-γ atom previously enriched in the starting monomer **1**. Signal (21) is due to the C-γ methylenic carbon of traces of dihydroconiferyl alcohol present in **1**.

For doing the assignment of the different signals we used two special NMR techniques, gate decoupling and proton selective irradiation, together with the comparison of ^{13}C chemical shifts with those already known of mono lignols, dilignols[13], and D.H.P.

Gate decoupling technique (cf. Exptl. Part): This experiment allows the existence of a ^{13}C—H coupling and its exact measurement without loosing the Overhauser effect. On Fig. 6(a) by gate decoupling experiment, we observe that signals (1), (3), (3a), (7), (7a), (11), (12), (14), and (15), give a doublet (s. Tab. 1 for the $J_{CH-\gamma}$ values). We can conclude that these carbons are tertiary carbons (bound only to one hydrogen) and that signal (21) which gives a triplet after gate decoupling is a secondary carbon (bound to two hydrogens). The effect of the gate decoupling on signals (20) and (22) are partially or totally hidden by the triplet given by signal (21); nevertheless, we can assume that at least (22) gives a triplet. Such a signal as signal (2) at 165 ppm staying unchanged after a gate decoupling experiment can be considered as a quaternary carbon (carbon with no hydrogen bound to it) and due to its chemical shift range could correspond to a γ carbonyl group like in vanillic acid. The values of the couplings given by this method particularly confirm, with a better precision (3 Hz) that is twice the resolution, the $J_{CH-\gamma}$ values of δ_3, δ_5, δ_8, and δ_9 signal protons shown on the ^{1}H

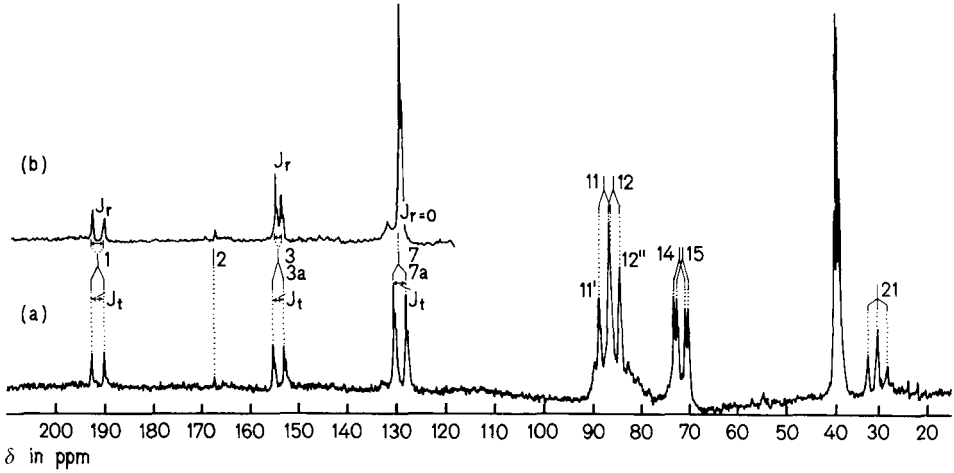


Fig. 6. (a) ^{13}C gate decoupled spectrum of D.H.P. **2** with determination of the $J_{t,CH-}$ total couplings of signals (1), (3), (3a), (7), (7a), (11), (12), (14), (15), and (21); signal (2) being not coupled. (b) Part of a ^{13}C selectively proton decoupled spectrum of D.H.P. **2** showing the residual coupling J_r for signal (1), (3), and (3a), and total decoupling $J_t = 0$ for carbons (7) and (7a), the proton irradiation being done at $\nu_H = 1615$ Hz/TMS. Spectra are recorded at 62,8 MHz on a CAMECA 250; solvent is used as internal standard

NMR spectrum of D.H.P. **2** [Fig. 4(b)]. Due to the overlapping of signals in Fig. 6(a), values of J_{CH-} coupling for (11) and (12) have to be calculated from δ_{11} and δ_{12} and the δ values of the best defined component of the doublet of each signal, that is (11') and (12'').

Selective proton irradiation: This technique allows to associate proton and carbon directly bound together. The proton spectrum has to be sufficiently first order to allow a precise selective irradiation so do we need a high frequency spectrometer (250 MHz for the proton, 62,8 MHz for the ^{13}C). The existence of a spectrometer (CAMECA 250 MHz) with a double tuned probe makes this experiment easier and more precise. Let us take as an example a compound having only one carbon ^{13}C (A) strongly coupled to only one proton (X)[14]. If X is irradiated with a weak power of irradiation, for example, 100 mG*) for a J_{CH-} coupling of 150 Hz, the observed ^{13}C spectrum is a doublet with a splitting depending of the frequency and of

*) SI-unit: 1 G = 10^{-4} Vs m^{-2}.

the intensity power of irradiation. The residual $J_{CH-\gamma}$ coupling J_{rX} can be approximately calculated by the formula[15]:

$$J_{rX} = \frac{J\Delta\omega}{\gamma H_2}$$

(with J = total coupling, J_{rX} = residual coupling with (X), $\Delta\omega = \nu_{rX} - \nu_2$, and γH_2 = irradiation field (mG))

If the irradiation is done exactly for the resonance frequency of the coupled proton, $\nu_{rX} = \nu_2$, then $J_{rX} = 0$ and the ^{13}C carbon signal becomes a singlet. On Fig. 6(b) we give a example of a selective proton irradiation made at $\nu H = 1615$ Hz/TMS: signals (7) and (7a) remain unchanged; all other signals are partially decoupled, but we can nevertheless notice that signal (1) coupled with a residual coupling of 150 Hz is less decoupled than signals (3), (3a) with a residual coupling of 80 Hz; $\Delta\omega$ in the first case being greater than in the second one. In the case of several independent ^{13}C—^{1}H systems like the different tertiary or secondary ^{13}C carbon atoms of D.H.P. **2** (^{13}C selectively enriched when there is sufficient space between the proton signals >40 Hz) an experiment of selective irradiation makes possible the assignment of the ^{13}C atom coupled to the proton whose chemical shift corresponds to the irradiation frequency. The results of an experiment of gate decoupling and of 7 selective irradiations are given in Tab. 1. We can make some additionnal remarks:

- Signals (3) and (3a) are both concerned and with equal intensity by the same selective proton irradiation; they have the same $J_{CH-\gamma}$ coupling under gate decoupling experiment, so the irradiated protons bound to them are of the same type.

- For signals (7) and (7a) we reach the same conclusion and, in addition, we notice that gel filtration brings a decreasing in the intensity of these two signals. In ^{13}C NMR there is no accurate correlation between the number of carbons comprising a peak and the integrated area of the peak, because of lattice relaxation time and variable nuclear Overhauser enhancement; nevertheless, we can appreciate the relative intensity of the peaks and estimate that the decreasing of signal (7) versus signals (11), (12), (14), (15) varies from 22% to 34% ± 1%. On the other hand ratio (7):(7a) is 2,28 in non filtrated D.H.P. and becomes 2,05 after filtration; we have a confirmation of elimination by GPC of fragments having vinylic propane side chains, and we can conclude that signal (7) represents such fragments with a smaller

Tab. 1. Results of NMR investigation on D.H.P., polymer model of Lignin

Values of $J_{CH-\gamma}$ couplings determined by gate decoupling technique	Frequency ν_H of irradiation in Hz/TMS and nature of the selectively irradiated proton	Selectively decoupled carbon signal signal no.	δ in ppm (from TMS)
$J_{C(1)H-\gamma}=172$ Hz	2420 Hz Aldehydic proton like in vanillin	(1)	190,80
$J_{C(3,3')H-\gamma}=153$ Hz	1830 Hz Vinylic H-γ proton like in cinnamaldehyde	(3) (3a)	154,05 153,61
$J_{C(7,7')H-\gamma}=152$ Hz	1615 Hz Vinylic H-γ proton like in coniferyl alcohol	(7) (7a)	128,82 128,40
$J_{C(11)H-\gamma}=158$ Hz[a]	1405 Hz H-γ proton in phenyl coumaran fragment (β—5)	(11)	86,91
$J_{C(14)H-\gamma}=144$ Hz	1210 Hz H-γ proton[b] in (β—O—4) and (β—1) fragments	(14)	71,04
$J_{C(15)H-\gamma}=142$ Hz		(15)	71,6
$J_{C(12)H-\gamma}=146$ Hz[c]	1120 Hz H-γ proton[d] in pinoresinol fragment (β—β)	(12)	85

[a] $J_{C(11)H-\gamma}=2\cdot(\delta_{11}-\delta_{11'})$.
[b] Real ν_H of the irradiated proton 1190 Hz (cf. text).
[c] $J_{C(12)H-\gamma}=2\cdot(\delta_{12}-\delta_{12''})$.
[d] Real ν_H of the irradiated proton 1160 Hz (cf. text).

molecular weight than fragments represented by signal (7a), these later fragments being less easily eliminated by GPC. Filtration does not affect significantly the intensities of the other signals.

– We cannot differenciate signals (14) and (15) by selective proton irradiation; nevertheless, considering the chemical shift range, if (14) represents a C-γ atom of a β—O—4 fragment, we could think of signal (15) as corresponding to a C-γ atom of a β—1 fragment, but without supplementary proof supporting

this hypothesis we would rather assign signals (14) and (15) as suggested by a referee according to a recent work[16] on acetylated D.H.P., to diastereomeric forms of β—O—4 dilignol units.

– Since the space between proton signals at 4,76 ppm and 4,64 ppm Fig. 4(c) is about 32 Hz, it is difficult to selectively irradiate one signal. Nevertheless, (cf. Exptl. Part) we succeeded in irradiating at $\nu_H = 1120$ Hz (4,48 ppm) one proton group leaving only signal (12) decoupled. Decoupling of group (14–15) without decoupling (12) remained ambiguous: with irradiation at $\nu_H = 1210$ Hz (4,84 ppm) decoupling of (14–15) is performed, eventhough splitting of (12) is not entirely realised. This case gives an idea of the limitation of the method according to the distance between protons under consideration. The association by this technique of the δ_H and δ_C of proton and carbon directly bound made possible the assignments collected in Tab. 1.

Assignment of the broad signals (6) and (13)

Decoupling techniques cannot be used efficiently with poorly resolved signals. For the assignment of broad signal (6) centered at 132 ppm a possible explanation was that it could represent C-γ vinylic carbon belonging to a more cross-linked part of the molecule, meanwhile sharp signals (7) and (7a) would represent C-γ vinylic carbons belonging to more linear and flexible part of the molecule, that would explain the non-equivalence of the chemical shifts different of about 3 ppm and the broadness of the signals, like in the case of denatured and native proteins[17]; an alternative explanation would be to assign signal (6) to the C-γ atom in vinylic groups other than cinnamyl alcohols, for instance like stilbenes, although a selective proton irradiation made at the resonance frequency of *trans*-stilbene protons $\nu_H = 1775$ Hz leaves only signal (6) unchanged. The broadness of the signal is too important to really make the difference between a total decoupling or a small partial coupling.

For broad signal (13) between 79 ppm and 83 ppm the appearance of more or less well resolved signals in it, and the large difference in the chemical shifts values compared with those of the neighbouring sharp signals (11), (12), (14), and (15) prevent us to make the same hypothesis as above. But considering the different C-γ-carbon atoms responsible for signals in this range we noticed[18] that in a β—O—4 fragment, when the rest R_3 is a C'-4 substituant, that is an aromatic carbon of guaiacyl propane unit,

the corresponding $\delta^{13}C$ shifted to lower field of about 7,1 ppm and reaches a value of 78,2 ppm instead of 71,1 ppm when $R_3 = H$ in the same $\mathbf{D_1}$ fragment. On the other hand, in the β—1 compound $\delta^{13}C$-γ is supposed to be around 75 ppm (in aceton/water, 9/1) if $R_3 = H$, it will be shifted to 82,1 ppm if $R_3 = C$-4 with a small additionnal shift upfield if the spectrum is taken in DMSO. According to the usual proposed formula of D.H.P. and according to the study of the products of degradation and the Rydholm-diagram[19], about 35% of β—O—4 and β—1 dilignols have an aromatic substituent at the oxygen atom of the H—C-γ—O—4 fragment. So we can assume that these moieties are responsible for part of the broad signal between 83 ppm and 79 ppm.

Conclusion

We have studied by NMR a sample of D.H.P. synthetised from selectively labelled coniferyl alcohol-^{13}C-γ. The study of ^1H NMR spectra allowed:

– determination of some chemical shifts with satisfactory precision;

– confirmation of the presence of H-γ and H-β vinylic protons, the proportion of which decreased after gel filtration;

– identification of a H-β proton like in β—O—4 fragment, previously hidden before labelling by a H-γ signal.

The ^{13}C NMR spectra showed the different labelled sites coming from the labelled ^{13}C-γ atom of the initial monomer. The corresponding signals appear both like broad and sharp peaks. For the assignment of the sharp signals we used special NMR techniques like gate decoupling and proton selective irradiation all together with the observation of the chemical shifts. This study made possible the identification of:

– C-γ carbon present in the three principal dimers, β—5, β—β, and β—O—4;

– C-γ vinylic carbon like in coniferyl alcohol and like in cinnamaldehyde fragments;

– C-γ carbon of carbonyl type like in vanillin and in a weak proportion like in vanillic acid.

The broad signal between 79 and 89 ppm allowed us to identify:

– C-γ atom of β—O—4 dilignol unit, involved in benzyl ether bond.

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Experimental Part

Synthesis of coniferyl alcohol-^{13}C-γ (**1**)

4-Bromoguaiacol benzyl ether (**3**): Refluxing[20] for 8 h 27 g (0,13 mol) of 4-bromoguaiacol, 15,66 g (0,12 mol) of benzyl chloride, and 17,04 g (0,17 mol) of potassium carbonate in 23 ml of acetone yielded, after two recrystallisations in ethanol, 16,5 g (42%) of white needles of **3**; mp 60–60,3 °C.

4-Benzylvanillic Acid-Carbonyl-^{13}C (**4**): The apparatus used[6] consisted of a high vacuum manifold, filled with dry nitrogen. The carbonation flask attached to it was charged with 6 g (20 mmol) of **3** in 80 cm^3 of anhydrous diethyl ether, then was frozen in an acetone/dry ice bath; 22 cm^3 of a 1,04 M solution of butyllithium in ether (10% excess) was added and the mixture was allowed to warm up to about −10 °C. After being refrozen with liquid nitrogen, the system was evacuated to a pressure of 0,008 torr and the carbonation was effected by dropping sulfuric acid onto 3 g of $Ba^{13}CO_3$. When all the $^{13}CO_2$ was evolved the flask was warmed to −10 °C and stirring continued for 10 min. Nitrogen was then admitted to the system and 5 ml of water and 5 ml of concentrated hydrochloric acid were added. The organic material was extracted with diethyl ether, treated with 1 M sodium hydroxide, and the alkaline solution was acidified. Yield of **4**: 2,850 g (72% based on barium carbonate). The acid is purified by chromatography on silica (eluant: 20 vol.-% ether – 80 vol.-% chloroform). Isotopic labelling was confirmed by mass spectroscopy: 91% ± 0,2%.

Vanillin-carbonyl-^{13}C (**5**) *(4-hydroxy-3-methoxybenzaldehyde-carbonyl-^{13}C)*: To a solution of 1,936 g (6 mmol) of the chloride of **4** (4-benzylvanillic chloride) in 10,5 cm^3 of C.P. xylene was added 206 mg of palladium sulfate catalyst poisoned by 20 microliters of quinoline-sulfur poison. The Rosenmund[6] reduction is carried out at reflux temperature with vigorous stirring while passing a stream of hydrogen. The evolution of hydrogen chloride is followed by passing the effluent gas through 100 cm^3 of water plus phenolphthalein indicator and adding small increments of 0,2 M sodium hydroxide to keep the solution alkaline. The reduction is completed after ca. 6 h after which 13 cm^3 of alkaline solution were added. After elimination of the catalyst and of xylene the product is treated with a mixture of 10 cm^3 of ethanol and 5 cm^3 of concentrated hydrochloric acid and kept at reflux for 1 h. The extraction is performed in a Soxhlet apparatus for 18 h with diethyl ether. The product is purified by chromatography (80 vol.-% $CHCl_3$ – 20 vol-% C_6H_6). Yield: 0,55 g (60%). Isotopic labelling: 90,9% ± 0,4%, NMR: $^1J(^{13}C_{ald}—H_{ald}) = 172{,}8$ Hz.

Coniferyl alcohol-^{13}C-γ (**1**): By condensation of 5,5 cm^3 (≈ 32 mmol) of malonic acid monoethyl ester with 5 g (32 mmol) of **5** in pyridine with three drops of aniline and piperidine, at 55 °C for 20 h we obtained 5,6 g of ethyl ferulate [ethyl 3-(4-hydroxy-3-methoxyphenyl)propenoate] (**6**), chromatographied on silica with $CHCl_3$ as eluent. Reduction of **6**[21] with $LiAlH_4$ in the usual way allows obtaining **1** purified by chromatography on silica (eluent 50 vol.-% ether – 50 vol.-% C_6H_6). Yield: (61%). Isotopic labelling: 90,4% ± 0,4%; NMR: $J_{CH-\gamma} = 152$ Hz.

D.H.P. **2**

To 100 ml of a water solution containing 2 mg of peroxydase (horseradish, 200 units per mg (N.B.C.)) dissolved in 3 ml of citrate buffer (pH = 5,5) are dropped simultaneously 100 ml of a 0,025% (w/v) solution of H_2O_2 (0,08 mmol) and 200 mg of coniferyl alcohol (**1**) (1 mmol) dissolved in 100 ml of water and 1,5 ml of acetone. The reaction mixture is stirred at room temperature. The same operation is repeated after 24 h and 48 h with the same quantities and then after 48h with half the quantities. Then for three days, we add every day 0,5 mg of peroxidase dissolved in 2 ml of citrate buffer and drop 160 ml of H_2O_2 solution. After distillation of 420 ml of water and centrifugation, the product is resuspended in 70 ml of water and liophylised. We obtained 400 mg (80%) of D.H.P. **2**.

60 mg of a sample of D.H.P. **2** was filtrated on GPC column of LH-20 column (3,1 cm × 120 cm) in dioxane/water (9/1, by vol.). Fractions of 12 ml were collected at a flow rate of 12 ml/h and their absorbance recorded at 280 nm on a Beckman spectrophotometer.

Fractions 21–48 were gathered and evaporated to a volume of 10 ml. By pouring this solution in 25 ml of water we precipitated the D.H.P. After final lyophilisation 58,5 mg of D.H.P. were overed.

NMR spectra

^1H NMR spectra are taken of 12% w/w solutions of the D.H.P. in DMSO-d_6 by means of a Camera 250 spectrometer operating at 250 MHz for the proton.

^{13}C NMR spectra on the Varian XL-100 spectrometer were taken of 10 to 12% w/w solutions of D.H.P. in DMSO-d_6. The spectrometer is operating at 25,2 MHz using the Fourier Transform (F.T.) technique employing a spectral width of 5000 Hz and 8 K of data storage which gives a resolution of 1,25 Hz per point; it is locked to the CD_3 groups of DMSO. TMS is used as internal reference. 385 blocks of 100 transients were accumulated in the long averaging mode for samples of ordinary D.H.P.; for labelled D.H.P., transients were accumulated without using the blocks system.

^{13}C NMR spectra on Cameca 250 operating at 62,8 MHz were recorded using the F.T. technique with a spectral width of 12500 Hz and 16 K of data storage (resolution 1,52 Hz per point) without lock, using DMSO-d_6 as internal reference.

Gate decoupling experiment on Cameca 250: the proton noise decoupler was pulse modulated in such a way that the decoupler is gated off, only during the pulse and the acquisition time, while during the remainder of the experience is gated on; considering the fact that the Nuclear Overhauser Effect (N.O.E.) is a slow effect and decoupling a fast one, when decoupler is off, we get the $J_{CH-?}$ coupling and still keep advantage of the N.O.E. established during the preceding pulse delay.

Proton selective irradiation experiment is conducted on a Cameca 250 spectrometer equipped with a double tuned probe which can be directly switched from carbon to proton observation and thus allows an easy selection of the proton irradiation frequency.

For selective irradiation of proton δ_9 at 4,64 ppm (1 160 Hz) we irradiate at $\nu_H = 1\,120$ Hz to be sure not to irradiate the proton at 4,76 ppm (1 190 Hz).

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