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Structural Characterisation of the Terminating Groups of some Polyprenyl Phosphates and of Rubber from the Lactarius *Mushroom*

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A series of trans- *and* cis-*allylic diphosphates were synthesized as model compounds of natural rubber and were analysed by NMR techniques. The* ^{1}*H-NMR spectra of terminal methylene protons of polyprenols linked to mono- and di-phosphates showed a triplet signal, centering around* δ *4.41–4.44 and* δ *4.46–4.49, respectively. The coupling constants of 5.0 Hz – 6.0 Hz and 6.0 Hz – 7.0 Hz were observed for* trans- *and* cis-*allylic diphosphate groups, respectively. In the* α*-terminal isoprene unit of polyprenols, the* cis- *and* trans-*allylic C-4 methylene protons resonate at* δ *4.081 and* δ *4.150, respectively. The* α *C-4 methylene carbon of mono- and di-phosphate groups showed a doublet signal around* δ *61–63 in the* ^{13}*C-NMR spectrum, the chemical shift value of which decreased with increase the chain-length of polyprenols. The* ^{31}*P-NMR spectra of mono- and di-phosphate groups showed a signal around* δ *1.5 and* δ *–7 to* δ *–8, respectively, independent of chain-length and configuration of the allylic compounds. A long-chain polyprenol from* Lactarius volemus, *composed of about 300 isoprene units, was phosphorylated to analyse the effect of chain length.*

Key words: polyprenyl phosphate, *trans*-polyisoprene, *cis*-polyisoprene, phosphorylation, ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR, *Lactarius volemus* mushroom

Rubber from *Hevea brasiliensis* consists of about 94% hydrocarbon and about 6% non-rubber components such as proteins, lipids and sugars, *etc.*[1]. These non-rubber components, especially proteins, have been presumed to be responsible for the outstanding properties of natural rubber. The early biochemical studies have postulated that the fundamental steps of natural rubber formation start from dimethylallyl diphosphates (DMADP) and proceed by successive chain extension to condensation with isopentenyl diphosphates (IDP) on the surface of latex particles[2]. We have carried out the structural characterisation

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of naturally occurring polyisoprenes using ^{1}H- and ^{13}C-NMR techniques to elucidate the steps of their biosynthesis, using polyprenols as model compounds[3]. The ^{13}C-NMR studies of rubbers from Goldenrod *(Solidago altissima)* and Sunflower *(Helianthus annuus)* revealed that there are two to three *trans*-isoprene units linked to the dimethylallyl-group[4–6]. The fundamental structure of rubber from mushroom *(Lactarius volemus)* was found to consist of a dimethylallyl group, which is known as the ω-terminal, two *trans*-isoprene units, a long sequence of *cis*-isoprene units and a hydroxyl or fatty acid ester terminal group, aligned in the order described[7]. On the other hand, *Hevea* rubber does not show the ^{13}C-NMR signals characteristic of the dimethylallyl group at ω-terminal and the hydroxy-group of α-terminal, although signals corresponding to small amounts of *trans*-isoprene units in the *trans-trans-* or dimethylallyl-*trans*-dyad sequences were observed[8].

Our previous work suggested that the possible initiating species in the biosynthesis of *cis*-1,4-polyisoprene are two- or three-*trans* oligoprenyl substances, *i.e.* farnesyl diphosphate (FDP, ω, *trans, trans*-DP), geranylgeranyl diphosphate (GGDP, ω, *trans, trans, trans*-DP)[8,9]. However, the terminating step of *Hevea* rubber biosynthesis remains unknown, due to the absence of terminal hydroxyl or ester groups, which can be commonly found in polyprenols and other rubbers from leaves of Goldenrod *(Solidago altissima)* and Sunflower *(Helianthus annuus)*[4,10]. It can be presumed that the rubber liberates a diphosphate group by the action of phosphatases present in the latex, to form a hydroxyl group in the terminating step, followed by allylic migration of the hydroxyl group and dehydration. Another possibility is that the termination reaction to form phospholipid group, which leads to the formation of a branching point in natural rubber[11].

In this paper, an attempt is made to study the structure of terminating end group of polyprenols, low $\overline{M}_W$ rubber from *Lactarius* mushroom and their diphosphate derivatives as a model of natural rubber, by ^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopies.

MATERIALS AND METHODS

Chemicals

Trans, trans-farnesol (C_{15} ω-t_2-OH), *trans, trans, trans*-geranylgeraniol (C_{20} ω-t_3-OH) and all *trans*-solanesol (C_{45} ω-t_8-OH) and betulaprenol-14, -15, -18 and -19 (C_n ω-t,t,C_{n-2}–OH, n = 14 ,15 and 19, respectively) were provided by Kuraray Co. Ltd., Japan. These were used without further purification. All of the reagents were reagent grades.

Sporophores of L. *volemus* mushroom were collected in Fukushima province, Japan. The fresh sporophores were cut into small pieces and soaked in ethanol to remove the non-rubber components, followed by extraction of rubber by toluene. The toluene extract was concentrated by rotary evaporation at 40°C and the rubber fraction was recovered by precipitation with methanol. The rubber was saponified by KOH solution in the usual way[12]. The number-average molecular-weight ($\overline{M}_n$) of this rubber was 2×10^4 mol/g, as determined by a Wescan Membrane Osmometer.

Tetra-*n*-butyl ammonium dihydrogen phosphate (TNBDP) was prepared by mixing 1.2 g (10 mmol) of 85% phosphoric acid with 25 mL (10 mmol) 10 % (w/v) aqueous tetra-*n*-butyl ammonium hydroxide. The mixture was evaporated at 40°C to get a white residue and further dried by adding 1:1 v/v of acetonitrile and chloroform as a azeotropic co-solvent. The synthesized TNBDP was kept under vacuum at 25°C

Phosphorylation of Allylic Alcohols

Phosphorylation of the polyprenols was carried out according to the published method[14]. The allylic alcohol in dry trichloroacetonitrile (260 μmol/mL) was mixed with TNBDP in dried acetonitrile (340 μmol/mL). This mixture was stirred vigorously for 10 min under nitrogen atmosphere at 25°C, followed by evaporation to dryness in a rotary evaporator under reduced pressure at 25°C. The residue was dissolved in 2 mL of 1-butanol from a mixture of 1-butanol saturated with water (1:1, v/v), followed by washing with water phase from the same solution (2 mL × 10). The washed mixture was dried by addition of toluene/methanol (1:1, 3 mL × 3) to get complete dryness under reduced pressure at 25°C.

Phosphorylation of Saponified Rubber from *L. Volemus* Mushroom

A 2% w/v of rubber solution in chloroform was mixed with 1 mL of trichloroacetonitrile and chloroform (1:1) mixture, followed by mixing with TNBDP in chloroform (1.2 mmol/mL). The phosphorylation step was carried out as described above.

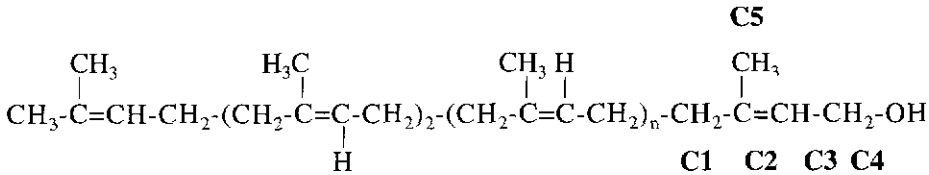
NMR Measurements

The ^{1}H- and ^{13}C-NMR measurements were made on a JEOL α-500 operating at 500 MHz and 125 MHz, respectively, on samples in $CDCl_3$ solution with TMS as an internal standard at 27°C, using a pulse repetition time of 7 s. The ^{31}P-NMR measurements were made on the same instrument operating at 202 MHz in $CDCl_3$ solution, with a 85% aqueous H_3PO_4 as an external standard at 27°C, using a pulse repetition time of 4 s.

RESULTS AND DISCUSSION

Phosphorylated allylic products stabilised in the form of tetra-*n*-butyl ammonium salts were subjected to NMR analysis. *Figure 1* shows the 500 MHz ^{1}H-NMR spectra of the phosphorylated products of *trans,trans,trans*-geranylgeraniol (GGOH) and betulaprenol (BOH). The latter is a typical two-*trans* and poly-*cis* composed of two *trans*-isoprene units at the initiating end linked to the 14–19 *cis*-isoprene units. Here, the proton and carbon atoms in the isoprene units of polyprenol, including both of the terminating groups are designated as shown in *Scheme 1*.

Two triplet signals centering at δ 4.429 and δ 4.484 are detected in the spectrum of phosphorylated GGOH. In the case of phosphorylated betulaprenol-18 (BDP-18), these signals shifted to a higher field, centering at δ 4.410 and δ 4.461. These triplet signals are assigned to the α C-4 methylene protons of mono- and di-phosphate terminating groups, respectively. The equivalent C-4 methylene protons together with the methine proton at



Scheme 1

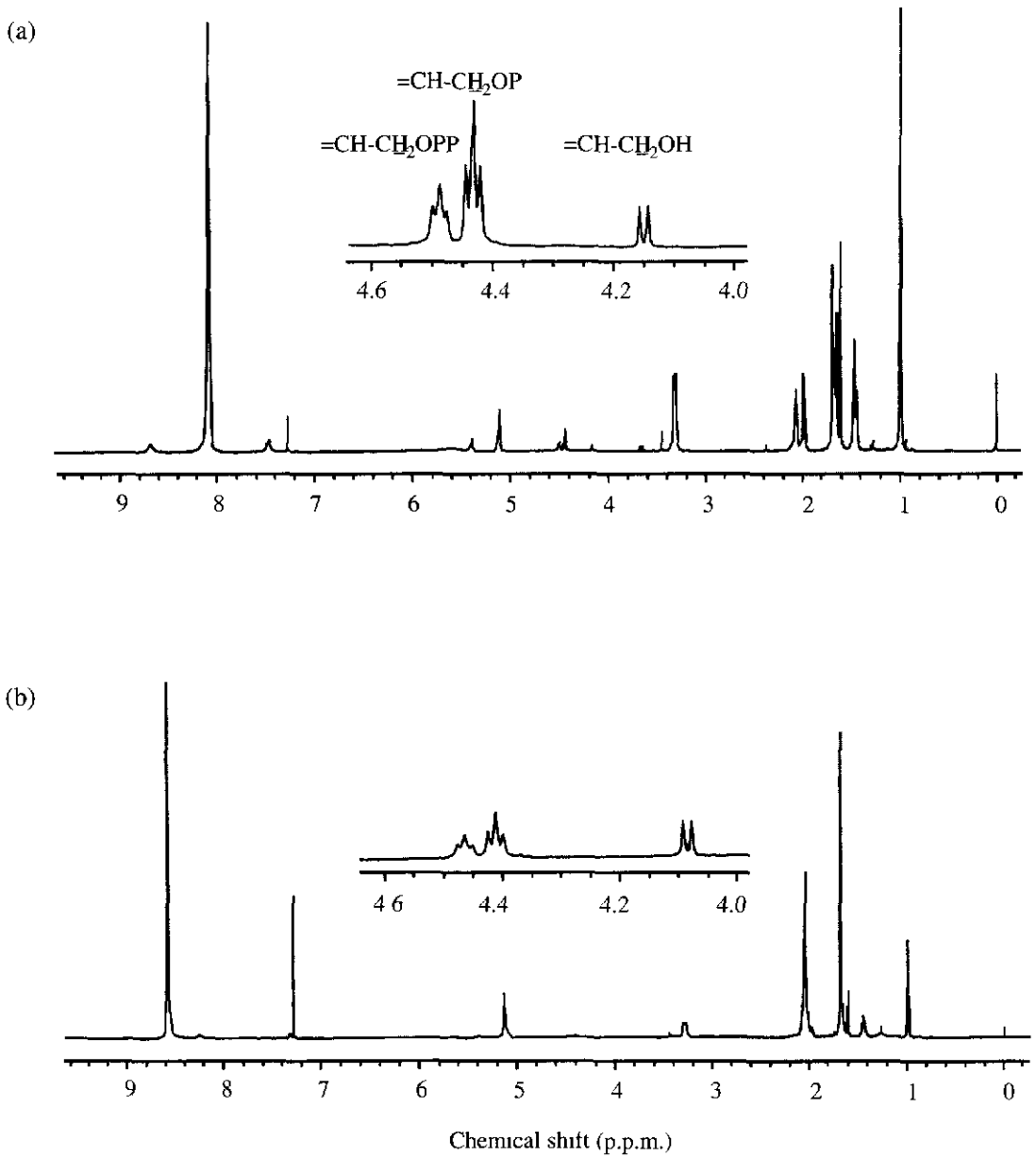


Figure 1. ^{1}H-NMR spectrum of phosphorylated (a) geranylgeraniol and (b) betulaprenol [BOH-18 (C_{90})].

C-3 and α ^{31}P nucleus in the terminating group form an A_2BX spin system. The methylene proton can give two doublets by spin-spin couplings between the ^{31}P nucleus and methine proton, which appears as a triplet when two of the lines overlap. The asymmetrical splitting pattern of the triplets of the methylene protons yields $^3J_{HCCH} = 5.9$ Hz and $^3J_{POCH} = 6.6$ Hz[14,15]. As shown in *Figure 1*, a doublet signal of the α C-4 methylene protons adjacent to the terminating hydroxyl group is also observed at δ 4.150 and δ 4.081 for GGOH and BOH, respectively.

The yield of phosphorylation can be estimated from the relative intensity of the signals of the α C-4 methylene protons linked to the phosphorylated and hydroxyl terminals in the ^1H-NMR spectra. *Figure 2* shows the relationship between the reaction time and yield of geranylgeranyl monophosphate (GGOP), geranylgeranyl diphosphate (GGOPP) and residual GGOH. The yield of the phosphorylation reaction reached about 90% after 10 min of the reaction time. If the reaction time was more than 15 min, the formation of by-products was observed. Therefore, in this

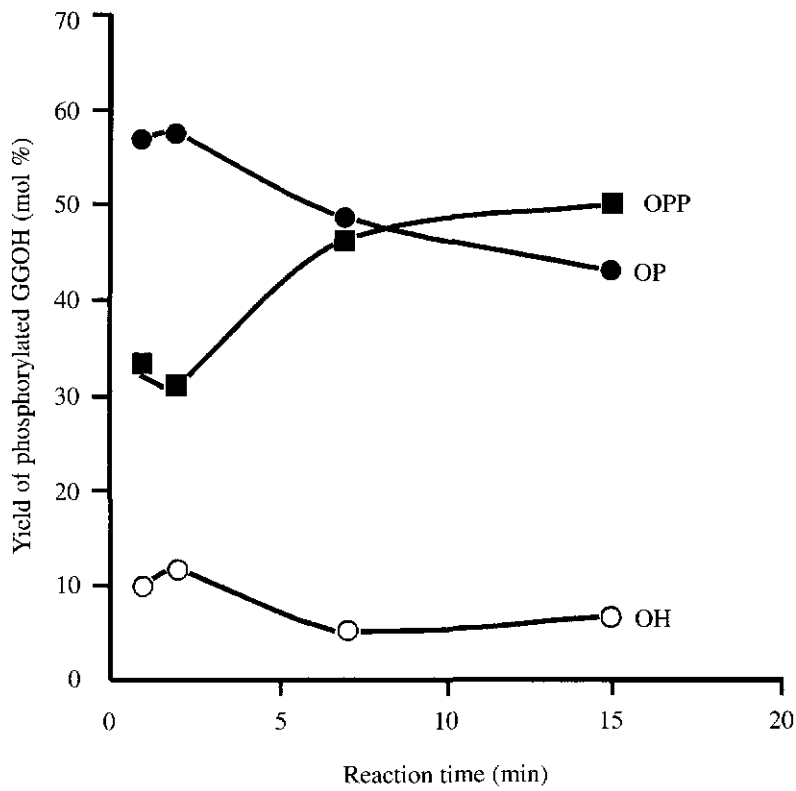


Figure 2. Relationship between reaction time and yield of phosphorylated GGOH.

work, the reaction time was set at 10 min and the yield of phosphorylation for all the samples was nearly 90%.

The triplet signals around δ 4.4 – δ 4.5 in the ^{1}H-NMR spectrum of phosphorylated BOH-18, as shown in *Figure 3 (a)*, were assigned by using ^{1}H-^{1}H homonucleus decoupling measurement. The complete decoupling of the methine proton at δ 5.391 in -C=CH-CH_2-OP or -C=CH-CH_2-OPP resulted in a clear change from the triplet to doublet signals centered at δ 4.458 and δ 4.407, respectively, as shown in *Figure 3 (b)*. This indicates that these triplet signals are due to C-4 methylene protons of a terminal isoprene unit linked with phosphate ester. Similarly by decoupling the C-3 methine proton, the doublet signal at δ 4.081 changed to a singlet at δ 4.080, as shown in *Figure 3 (c)*. showing that the doublet signal is assignable to a -CH_2-OH proton. These assignments were also confirmed by ^{1}H-^{1}H COSY NMR measurement.

Table 1 shows the chemical shifts of methylene protons obtained after homonucleus decoupling of the methine proton. These values represent the precise chemical shifts of methylene protons spin-coupled with ^{31}P in phosphate ester. The coupling constant (J_{POCH}) for polyprenyl monophosphate was found to be 5.5 Hz and 6.0 Hz – 6.5 Hz for *trans*-allylic compounds and *cis*-allylic compounds, respectively. In the case of a polyprenyl diphosphate, the triplet signal became broader due to the additional coupling from the second ^{31}P nucleus.

Figure 4 shows the relationship between the chemical shifts of C-4 methylene protons linked to hydroxyl, mono- and di-phosphate groups and the chain-length of model compounds. It is clear that these methylene proton signals of *trans*-allylic phosphate compounds resonate at lower field than those of *cis*-allylic phosphate compounds. The coupling constants in these triplet signals (J_{HCCH} and J_{POCH}) are in the range of 5.0 Hz – 6.0 Hz and 6.0 Hz – 6.5 Hz for *trans*- and *cis*-allylic phosphate compounds, respectively. The chemical shifts of C-4 methylene protons

TABLE 1. CHEMICAL SHIFT OF α C-4 METHYLENE PROTONS OF POLYPRENYL PHOSPHATES OBTAINED AFTER HOMONUCLEAR DECOUPLING FROM α C-3 METHINE PROTON

Sample	=CH-CH_2-OP (p.p.m.)	=CH-CH_2-OPP (p.p.m.)
FOH	4.436 (d, J=5.5 Hz)	4.493 (s)
GGOH	4.429 (d, J=5.5 Hz)	4.484 (d, J = 4.5 Hz)
SOH	4.442 (d, J=5.5 Hz)	4.496 (s)
BOH-14	4.413 (d, J=6.0 Hz)	4.467 (J = 6.0 Hz)
BOH-15	4.414 (d, J=6.5 Hz)	4.469 (d, J = 5.5 Hz)
BOH-18	4.407 (d, J=6.5 Hz)	4.459 (d, 4.5 Hz)
BOH-19	4.408 (d, J=6.5 Hz)	4.469 (d, J = 5.5 Hz)
Mushroom rubber	4.422 (d, J=6.5 Hz)	4.472 (d, J = 5.5 Hz)

-OP* : Monophosphate; -OPP# : Diphosphate; J : Coupling constant; d : Doublet;
SOH : Solanesol; FOH: Farnesol; s : singlet

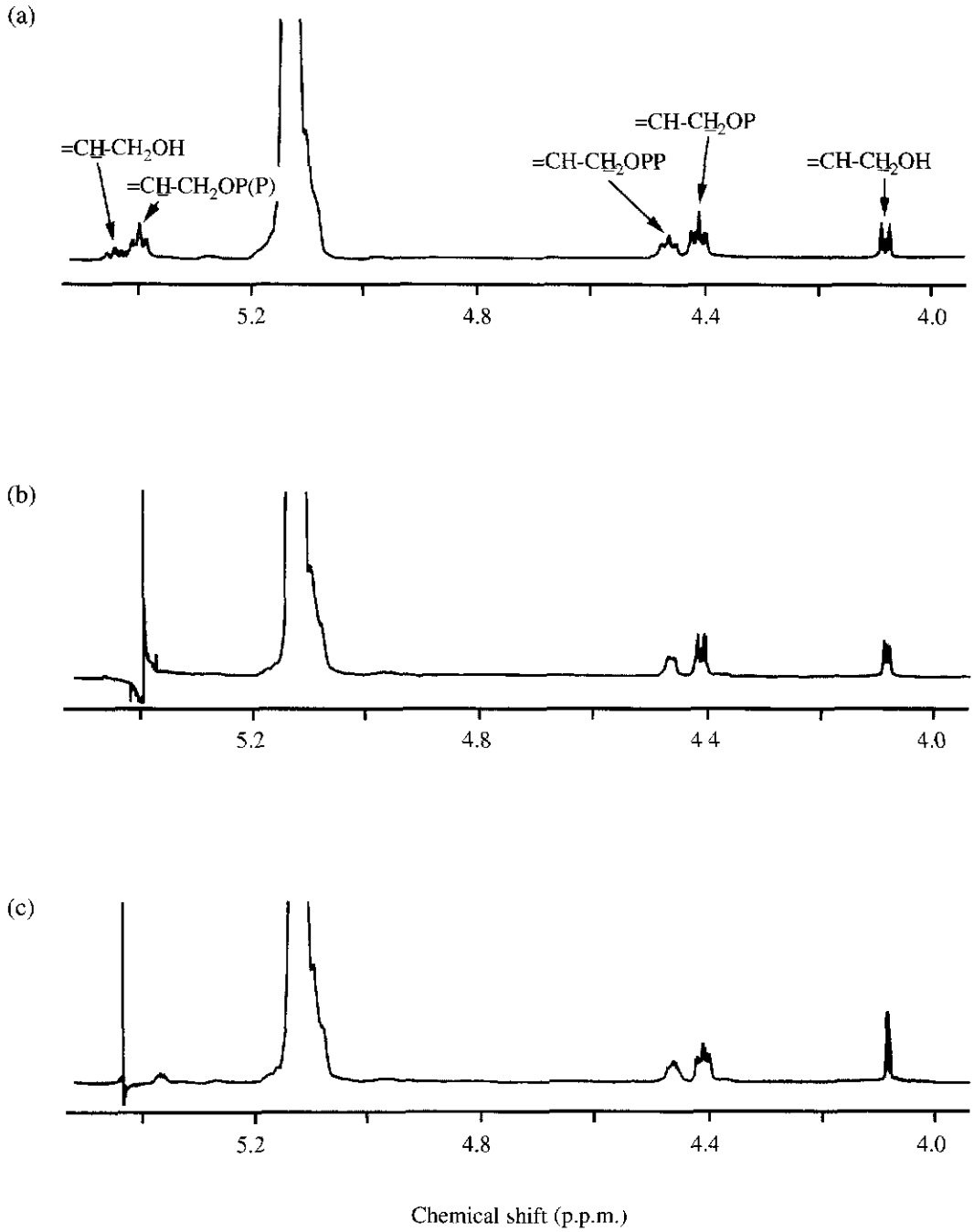


Figure 3. Methylene and methine ^{1}H-NMR spectra of (a) phosphorylated BOH, (b) and (c) after homonuclear decoupling at δ 5.391 and δ 5.481, respectively.

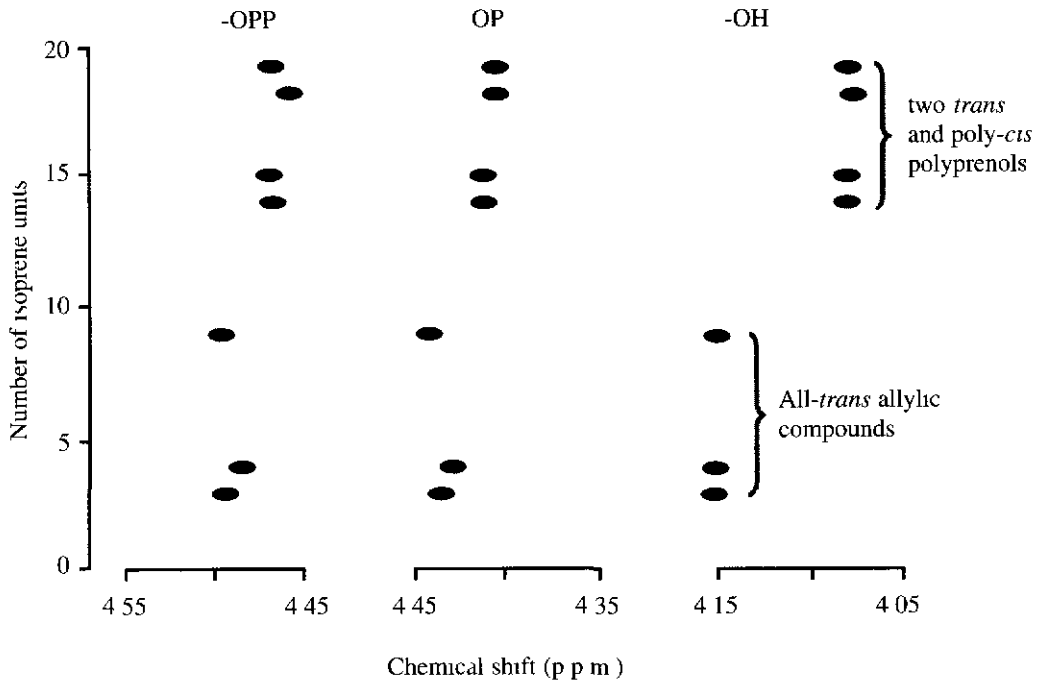


Figure 4 Relationship between the chain-length of allylic compounds and chemical shift of α C-4 methylene protons linked to hydroxyl, mono- and di-phosphate

of the hydroxyl terminal isoprene unit were in the region of δ 4 150 and δ 4 081 for the *cis* and *trans* configurations respectively

The low molecular weight rubber obtained from *L volemus* mushroom was used as a high molecular weight model compound of natural rubber As shown in *Figure 5 (a)*, the rubber obtained from *Lactarius* mushrooms contains mainly fatty acid ester terminal groups[7] The rubber was saponified to convert all the terminating groups to hydroxyl *[Figure 5 (b)]* and phosphorylated as in the case of polyprenols Two triplet signals centered at δ 4 422 and δ 4 472 are assigned to C-4 methylene protons linked with mono- and di-phosphate esters, respectively, as shown in *Figure 5 (c)* In spite of the fact that the mushroom rubber is composed of about 300 isoprene units, the chemical shift and coupling constant [J_{POCH}] of the phosphorylated rubber from mushroom are found to be close to those of the phosphorylated BOH This indicates that the coupling constant J_{POCH} of 6 0 Hz – 6 5 Hz is independent of chain-length but dependent only on the configuration of the terminal isoprene unit

The ^{31}P-NMR spectrum of phosphorylated BOH-18 is shown in *Figure 6* A doublet signal centered at δ 1 563 is assigned to ^{31}P in monophosphate form and the signals at δ 8 051 and δ 8 382 are due to the α- and β-^{31}P in diphosphate forms, respectively The chemical shifts of the other polyprenyl phosphates are

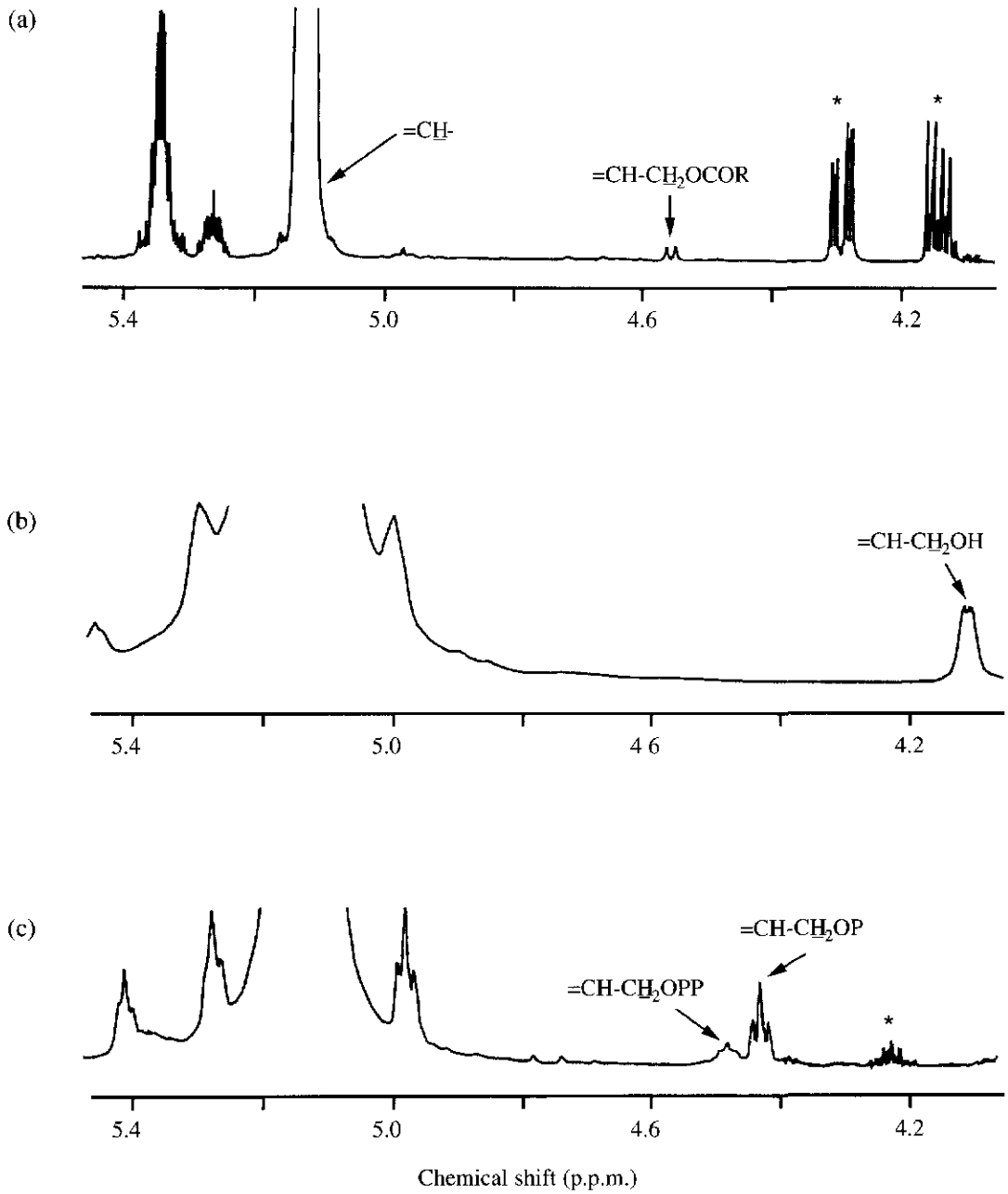


Figure 5. ^{1}H-NMR spectra of the rubber from Lactarius volemus *mushrooms: (a) original rubber; (b) saponified rubber and (c) phosphorylated rubber.*

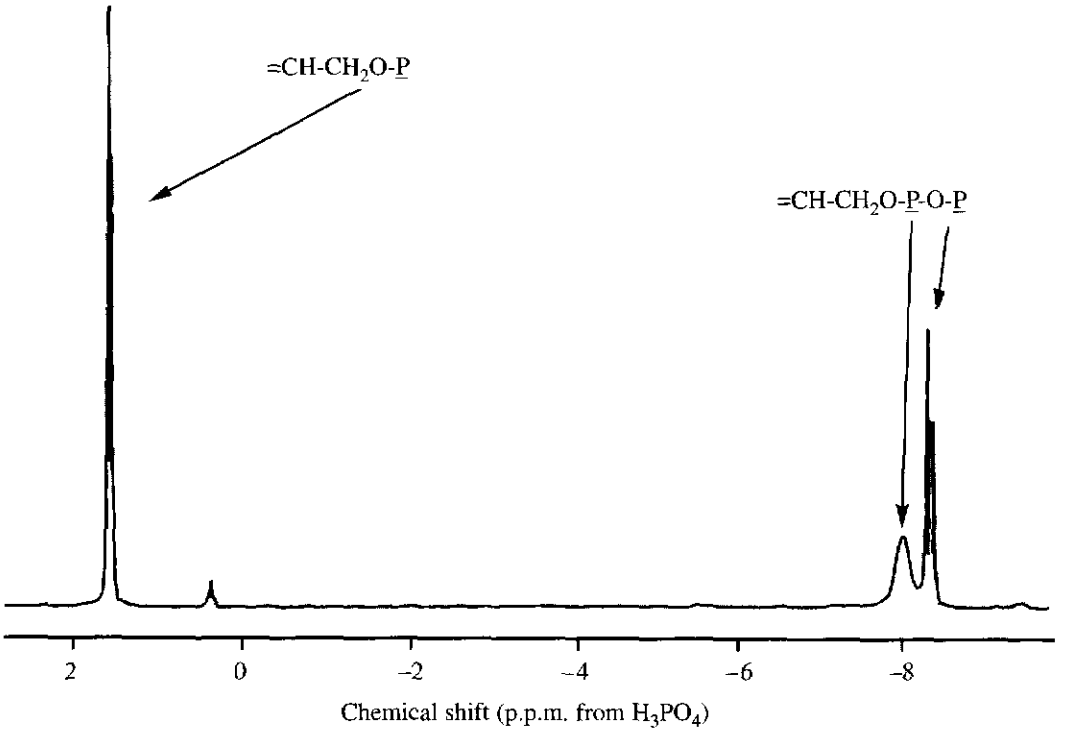


Figure 6. ^{31}P-NMR spectrum of phosphorylated BOH-18 (C_{90}).

given in *Table 2*. It is clear that all the chemical shifts are independent of chain-length and configuration of isoprene unit linked to the phosphate group in these model compounds of natural rubber. The doublet signal of the allylic monophosphate in the ^{31}P-NMR spectrum can be explained as due to the coupling from the adjacent ^{13}C nucleus. In the case of the allylic diphosphate, the α-^{31}P signal was broader. This may be due to the coupling from ^{13}C and β-^{31}P nuclei. A doublet signal of β-^{31}P is derived from coupling between a- and β-^{31}P nuclei.

Figure 7 shows the ^{13}C-NMR spectrum of phosphorylated BOH-18. The α C-4 methylene carbon linked to mono- and di-phosphates showed two doublet signals, centering at δ 61.35 (J_{COP} = 5.1 Hz) and δ 62.05 (J_{COP} = 5.1 Hz), respectively. The ^{13}C-^{1}H COSY spectrum of phosphorylated BOH-18 showed that the doublet signals are due to the α C-4 methylene carbon linked to mono- and di-phosphates. The doublet splitting implies the presence of a coupling between ^{13}C and ^{31}P nuclei. A singlet signal observed at δ 58.91 is assigned to the α C-4 methylene carbon linked to the hydroxyl group.

The chemical shifts of the α C-4 methylene carbon of phosphorylated solanesol, BOH-15, BOH-18 and rubber from *Lactarius* mushroom are given in *Table 3*. It is clear that the chemical shift shows a shift trend to a higher field with an increase in chain-length, but the

TABLE 2. CHEMICAL SHIFT OF POLYPRENYL PHOSPHATE IN ^{31}P-NMR SPECTRUM

Sample	=CH-CH_2-OP (p.p.m.)	=CH-CH_2-OPP (p.p.m.)
FOH	1.728	α : –8.253 (b) β : –8.566 (d, J = 18.63 Hz)
GGOH	1.544	α : –7.866 (b) β : –8.244 (d, J = 18.63 Hz)
SOH	1.636	α : –7.720 (b) β : –7.996 (d, J = 14.98 Hz)
BOH-14	1.618	α : –8.480 (b) β : –8.566 (d, J = 14.98 Hz)
BOH-15	1.672	α : –8.070 (b) β : –8.226 (d, J = 18.63 Hz)
BOH-18	1.581	α : –8.051 (b) β : –8.382 (d, J = 14.78 Hz)
BOH-19	1.618	α : –8.473 (b) β : –8.657 (d, J = 14.98 Hz)

b : broad; d : doublet; J : coupling constant

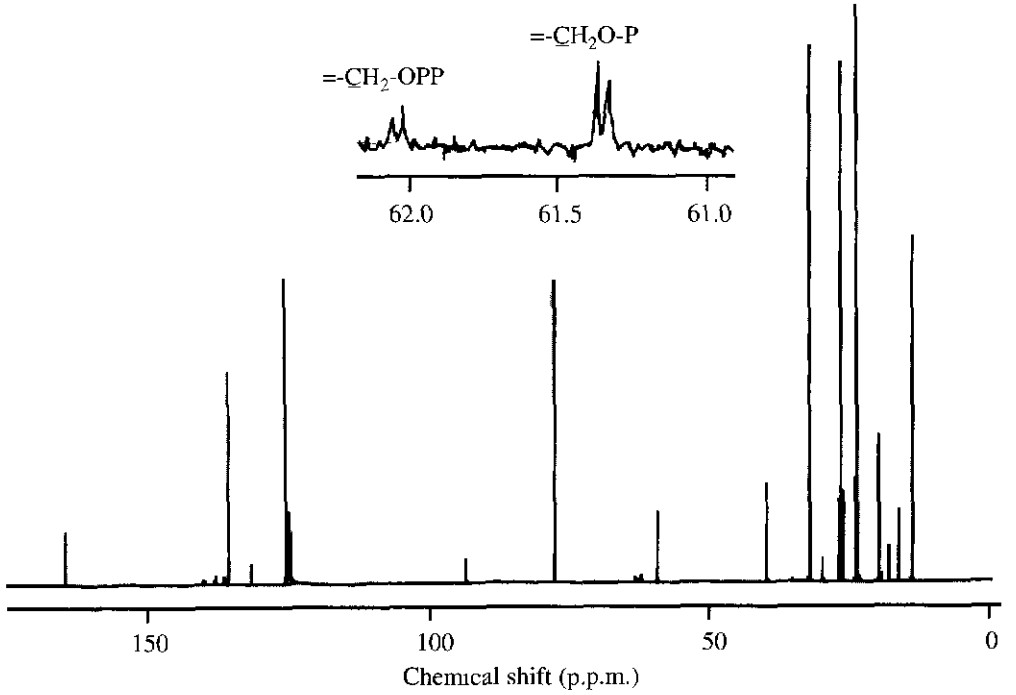


Figure 7. ^{31}C-NMR spectrum of phosphorylated BOH-18 (C_{90}).

TABLE 3. ^{13}C-NMR CHEMICAL SHIFT OF THE α C-4 METHYLENE CARBON OF POLYPRENYL PHOSPHATES

Methylene protons linked to	*SOH*	BOH-15	BOH-18	Mushroom Rubber
-OH	58.693 (s)	58.841 (s)	58.907 (s)	59.147 (s)
-OP*	61.680 (d, J = 5.1 Hz)	61.581 (d, J = 5.1 Hz)	61.347 (d, J = 5.1 Hz)	61.100 (d, J = 5.1 Hz)
-OPP#	62.404 (J = 5.1 Hz)	62.165 (J = 5.1 Hz)	62.046 (J = 5.1 Hz)	62.453 (J = 5.1 Hz)

-OP* : Monophosphate; -OPP# : Diphosphate; J : Coupling constant
d : Doublet; s : Singlet

coupling constant is independent of the chain-length. On the contrary, the chemical shift of α C-4 methylene linked to a hydroxyl group shows a shift trend to lower field as the chain-length increases.

The characterisation of polyprenyl phosphates provides some important information on the terminal unit of *trans*- and *cis*-polyisoprenes, and may help to clarify the structure of the terminal group of natural rubber.

CONCLUSION

NMR analysis of allylic phosphate compounds and phosphorylated rubber from Lactarius mushroom was carried out for model compounds of natural rubber. The ^{1}H- and ^{13}C- and ^{31}P-NMR spectra of polyprenyl phosphates provided information on the chemical shift and splitting of the signals due to the terminal phosphorylated *cis*-isoprene unit. This was confirmed further by the use of phosphorylated rubber from *Lactarius* mushroom.

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