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# Structure of *cis*-polyisoprene from *Lactarius* mushrooms

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**Sporophores from five species of *Lactarius* mushrooms had a liquid rubber content of 0.1% to 7% based on the dry weight. Rubber from *L. volemus*, *L. chrysorrheus* and *L. hygrophoroides* was found to be a homologue of polyprenol being composed of dimethylallyl group, two *trans* isoprene units, 160–300 *cis* isoprene units, and terminal hydroxyl or ester group aligned in that order by $^{13}$C-NMR analysis. The ratio of fatty acid ester group to hydroxyl group was about 9/1 to 5/5. The number of both terminal groups and *trans* units decreased during aging of sporophores. Rubber from *L. piperatus*, *L. vellereus* and *L. subpiperatus* was found to be *cis* polyisoprene having very small quantities of both terminal groups and *trans* units. The biosynthesis of *cis* polyisoprene in *Lactarius* mushrooms was found to start from *trans*, *trans*-farnesyl pyrophosphate. The termination was assumed to occur by esterification of polyisoprenyl pyrophosphate. Occurrence of some chemical modifications on both terminal groups was presumed during aging of sporophores.**

Natural rubber from *Hevea brasiliensis* is composed of more than 5000 isoprene units exclusively in the *cis* configuration. A number of attempts have been made to elucidate the mechanism of rubber formation in *Hevea* tree. The biochemical studies in 1960s and 1970s provided some pieces of evidence on the chain elongation process; it occurs on the surface of existing rubber particles in latex by repeated addition of isopentenyl pyrophosphate. However, there have been no conclusive explanations on the site forming new rubber molecules and the mechanism controlling the molecular mass and geometric isomerism. The enzyme catalyzing chain elongation has received increasing attention in recent years. A new proposal assuming the presence of rubber elongation factor has been postulated to interpret the mechanism of formation of *cis* isoprene units. However, it has provided no confirmatory evidence on the initiation and termination mechanisms of rubber formation because the proposal was based on only enzymatic studies.

We have presumed that detailed structural studies provide direct evidence on the steps of rubber formation and mechanism controlling molecular mass and geometric isomerism. It was successful in the case of rubber from the leaves of Goldenrod and Sunflower [1, 2]. The fundamental structure of naturally occurring *cis* polyisoprene was found to consist of dimethylallyl group, two or three *trans* isoprene units, and a long sequence of *cis* isoprene units, and hydroxyl or ester terminal group aligned in that order [1–3]. The presence of terminal *trans* units in natural rubber was confirmed by $^{13}$C-NMR spectroscopy. However, the structure of both terminal groups was not clearly identified in the case of natural rubber and wild rubbers occurring as latex [3, 4].

Some species of fungal genera, mainly *Lactarius* species, were found to exude latex containing low molecular mass *cis* polyisoprene [5–8]. Rubber from mushroom is anticipated to be a good model to elucidate structural characteristics of rubbers occurring as latex. It may be

possible to analyze the structure of polyisoprene just after termination, since the life cycle of sporophores is usually one week. We have determined the structure of low molecular mass rubber from *L. volemus* and rubbers from five species of *Lactarius* mushrooms by $^{13}C$-NMR analysis [8, 9]. It has been disclosed that the fundamental structure of rubber from *L. volemus* is a high molecular mass homologue of polyprenol consisting of two-*trans* and poly-*cis* arrangement. However, both terminal groups have not been identified for rubbers from some *Lactarius* mushrooms.

In this paper we will show the detailed structure of rubber from several *Lactarius* mushrooms. The mechanism of initiation and termination reaction in rubber formation is postulated on the basis of structural evidence on both terminal groups and alignment of *trans* isoprene units. The occurrence of chemical modification on both terminal groups is discussed in connection with the effect of aging of sporophores.

## EXPERIMENTAL

Sporophores of *Lactarius volemus* and other five species of *Lactarius* were collected in Fukushima and Yamanashi (Japan), and were extracted with acetone followed by toluene. The toluene extract is concentrated and precipitated into methanol. The yield of rubber is given in Table 1. Rubber in latex was collected from grounded young and fresh sporophores of *L. volemus* by extraction with Triton X-100 followed by centrifugation and coagulation with methanol. The content of rubber was 6–10 (w/v%) of latex. Ficaprenol-11 and polyprenol-18 were obtained from silkworm feces and from leaves of *Ginkgo biloba*, respectively. The $^{13}C$-NMR spectra were obtained at 125 MHz, 100 MHz or 50 MHz with JEOL GSX-500, JEOL GX-400 or JEOL FX-200 in $CDCl_3$ solution at 50°C at a pulse repetition time of 7 s or 12 s for 45° pulse.

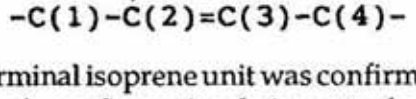
Table 1
*Content of rubber (per dry weight of sporophores) in Lactarius sporophores*

| Mushroom | Rubber content (%) |
|---|---|
| *L. volemus* | 1.1–7.1 |
| *L. hygrophoroides* | 2.2–4.7 |
| *L. chrysorrheus* | 0.31–0.53 |
| *L. piperatus* | 0.13–0.25 |
| *L. subpiperatus* | 0.42–0.71 |
| *L. vellereus* | 0.05 |

## RESULTS AND DISCUSSION

### Fundamental structure of rubber from *L. volemus*

The $^{13}C$-NMR spectrum of rubber from matured sporophores *L. volemus* showed small signals characteristic of dimethylallyl group (ω-terminal), *trans* units, and ester- and hydroxyl-terminal groups (α-terminal) as shown in Fig. 1. Here, the carbon atoms in isoprene units including both terminal units are designated as follows:




The terminal isoprene unit was confirmed to be in the *cis* configuration being attached to an ester group or hydroxyl group at the C-4 carbon as indicated by the presence of signals at 60.91 ppm (C-4 $CH_2OCOR$) and 59.13 ppm (C-4 $CH_2OH$). Signals characteristic of the fatty acid ester groups were detected at 14.04 ppm (-$CH_3$), 29–30 ppm (-$CH_2$), 34.47 ppm (=OOC$\underline{C}H_2$), 128–130 ppm (=CH), and 173.14 ppm (-OO$\underline{C}CH_2$). The relative intensity of ester group against hydroxyl group varied from about 9/1 to 5/5. Ester terminal group was the predominant in the case of matured sporophores.

The signals around 39.8 and 16.0 ppm were assigned to the C-1 $CH_2$ and C-5 $CH_3$ carbons, respectively, in the *trans* units. The chemical shift of the former signal indicates that the *trans* units are in the *trans-trans* and/or dimethylallyl-*trans* linkages [3, 10]. Both signals split into two peaks centered at 39.82 and 39.80 ppm for the C-1 $CH_2$ carbon and 16.03 and 16.02 ppm for the C-5 $CH_3$ carbon by measurement at 125 or 100 MHz as shown in Fig. 2. These splittings are assigned by comparison with those observed in the spectra of polyprenols as model compounds.

The C-1 $CH_2$ carbon of the *trans* units in polyprenol-18 consisting of two-*trans* and poly-*cis*

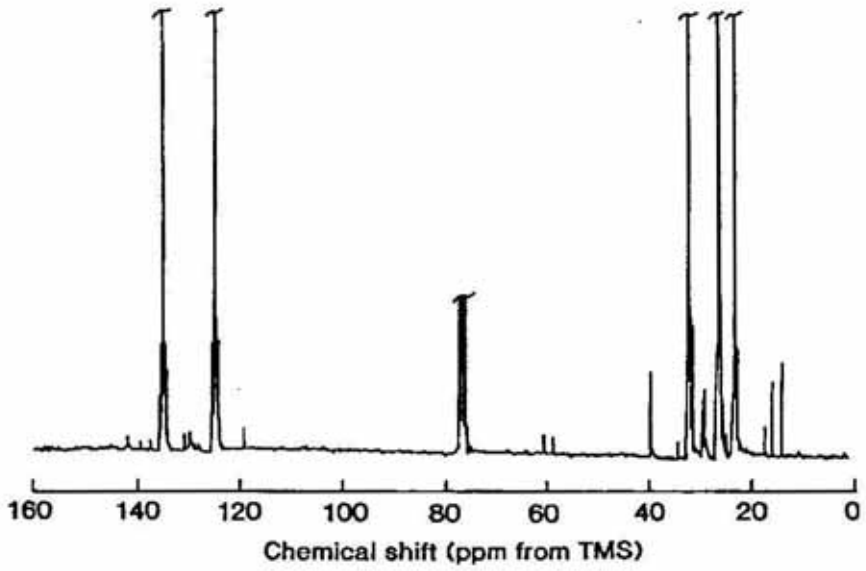



*Fig. 1. $^{13}C$-NMR spectrum of L. volemus rubber observed at 50 MHz.*

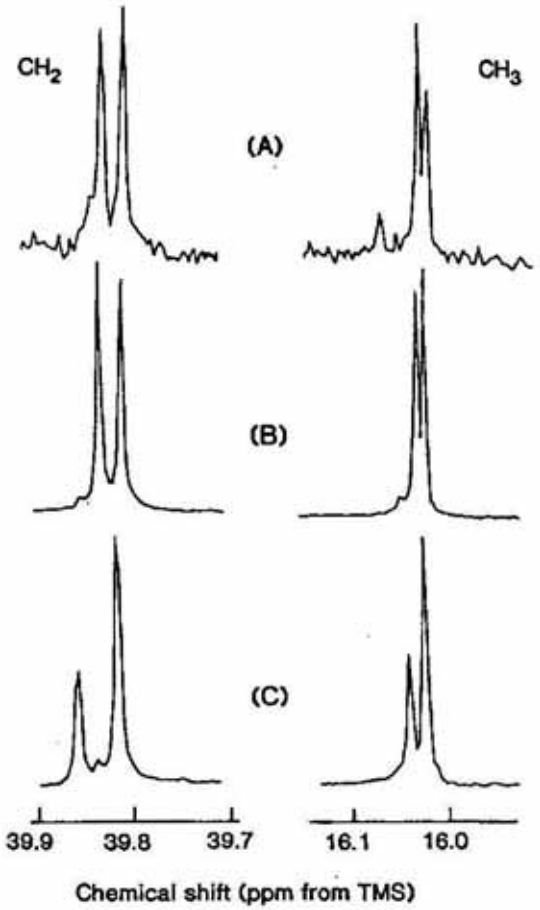



*Fig. 2. Signal splitting of C-1 $CH_2$ and C-5 $CH_3$ carbon atom of trans units observed at 125 MHz; (A) rubber from L. volemus, (B) polyprenol-18, and (C) ficaprenol-11.*

showed signals at 39.83 and 39.81 ppm and the C-5 $CH_3$ carbon at 16.03 and 16.02 ppm, both approximately in equal intensities. On the other hand, the corresponding carbons in ficaprenol-11 consisting of three-*trans* and poly-*cis* resonated at 39.85 and 39.81 ppm (C-1 $CH_2$) and 16.05 and 16.03 ppm (C-5 $CH_3$) in about 1:2 intensity ratio. The intensity ratio of these splittings and spin-lattice relaxation time $T_1$ indicate that the signal at a low-field is tentatively assignable to the carbon of the *trans* unit in the *trans-trans* linkage and the signal at a high-field to that in the dimethylallyl-*trans* linkage for C-1 $CH_2$ carbon atom. The detailed assignment of these signals will be given in a subsequent paper. The chemical shifts and intensity ratios of these signals in *L. volemus* rubber are in good agreement with those of polyprenol-18 [8]. This finding clearly indicates that rubber from *L. volemus* contains two *trans* units in the dimethylallyl-*trans-trans-* sequence at the terminal.

The *trans-cis* and *cis-cis* linkages can be differentiated by the characteristic C-1 $CH_2$ signal of the *cis* unit at 32.11 and 32.24 ppm, respectively [3, 10]. The relative intensity of both signals in polyprenol-18 and ficaprenol-11 supports the validity of the assignment. In the spectrum of rubber from *L. volemus*, a small signal at 32.10 ppm is clearly separated from the strong signal at 32.34 ppm, showing the presence of the *cis* unit in the *trans-cis* linkage. These findings provide direct evidence that *L.*

Table 2
*Assignment of $^{13}$C-NMR signals in L. volemus rubber*

| Carbon atom | | Chemical shift (ppm from TMS) |
|---|---|---|
| ester -OCOR | | 173.14 |
| α C-2 >C= | -OCOR | 142.17 |
| | -OH | 139.84 |
| *cis* C-2 >C= | | 135.25 |
| ω C-2 >C= | ω-*trans* | 131.14 |
| *cis* C-3 =CH- | | 125.18 |
| α C-3 =CH- | -OCOR | 119.68 |
| | -OH | 124.34 |
| α C-4 -$CH_2$- | -OCOR | 60.91 |
| | -OH | 59.13 |
| *trans* C-1 -$CH_2$- | *trans*-*trans* | 39.83 |
| | ω-*trans* | 39.81 |
| ester $CH_2$ | | 29.2-29.7 |
| *cis* C-4 -$CH_2$- | | 26.52 |
| ω C-1 -$CH_2$- | | 25.63 |
| *cis* C-5 -$CH_3$ | | 23.40 |
| ω C-5 -$CH_3$ | | 17.66 |
| *trans* C-5 -$CH_3$ | ω-*trans* | 16.03 |
| | *trans*-*trans* | 16.02 |
| ester -$CH_3$ | | 14.04, 14.01 |

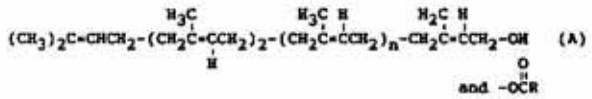
ω-terminal $(CH_3)_2C{=}CH{-}CH_2{-}$

α-terminal $-CH_2-\overset{CH_3}{\overset{|}{C}}{=}CH-CH_2-OH$ or -OCOR

*volemus* rubber consists of the dimethylallyl-(*trans*)$_2$-(*cis*)$_n$- sequence [8]. Assignment of $^{13}$C-NMR signals is listed in Table 2.

The number of *cis* units in rubber was estimated to be 260 from the intensity ratio between the *cis* C-1 $CH_2$ and *trans* C-1 $CH_2$ carbon signals, which was in fairly good agreement with the degree of polymerization of 300 estimated from GPC-LALLS measurement [8]. The number of dimethylallyl group and termi... ester group plus hydroxyl group was found to be 1.08 and 0.92, respectively, on the assump-

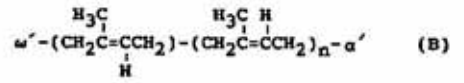
tion of two *trans* units per molecule. On the basis of these findings, the structure of rubber from *L. volemus* is determined as follows:



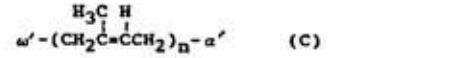
(A)

### Structure of rubber from other *Lactarius* mushrooms

Rubber from *L. chrysorrheus* exhibited the $^{13}C$-NMR spectrum similar to that of *L. volemus* rubber and was found to be a structure (A) with the sequence length of *cis* units of 160 [8]. Similar structure was found for rubber from *L. hygrophoroides*. On the other hand, rubber from *L. vellereus* showed the signals from *cis* units and very small signals due to *trans* units, while the signals characteristic of both terminal units were not detected and the structure is expressed in (B), where ω′ and α′ are unidentified terminal groups. The absence of dimethylallyl group and α-terminal groups is also observed in the case of natural rubber from *Hevea brasiliensis* [3, 4]. The structure (C) corresponding to wild rubbers occurring as latex was the case of rubbers from *L. subpiperatus* and *L. piperatus*, because signals due to *trans* units and both terminal groups were not detected in the spectra [9].



(B)



(C)

### Structural changes during aging of sporophores in *L. volemus*

Rubbers from matured and withered sporophores were found to contain both terminal groups less than the theoretical value [9]. The effect of aging of sporophores on the structure of rubber was simulated by taking advantage of withering of sporophores by storage in refrigerator. Fresh sporophores of *L. volemus* were stored in refrigerator for 51 days after collection and rubber was extracted from the sporophores. Assuming that two *trans* units are present per molecule, the relative intensities of both terminal groups and *cis* units were determined for two sets of fresh and withered sporophores as listed in Table 3.

By GPC measurements no significant difference in the degree of polymerization was observed for rubbers from fresh sporophores and withered ones, while considerable increase of *cis* units was observed after storage of both sets of samples. If the number of *cis* units is proportional to the degree of polymerization as determined by GPC, the number of *trans* unit is anticipated to decrease from 2 to 1.2–1.3 and decrease of 30% is estimated for both terminal groups as shown by the values in parentheses in Table 3. These findings suggest that the decrease of both terminal groups is accompanied with the decrease of *trans* units. It is remarkable that the number of both terminal groups was found to be 0.65 to 0.86 in the fresh and matured sporophores.

Table 3

*Number of both terminal groups and trans units in L. volemus rubber*

| Sample[1] | $\overline{M}_w/\overline{M}_n$ | $\overline{DP}_n$ | Number of isoprene units[2] | | | | |
|---|---|---|---|---|---|---|---|
| | (GPC) | (GPC) | ω | *trans* | *cis* | α | |
| | | | | | | ester | –OH |
| Fresh A | 2.0 | 280 | 0.86 | 2 | 250 | 0.51 | 0.14 |
| Storage A | 2.9 | 210 | 1.1 (0.63) | 2 (1.2) | 330 (190) | 0.49 (0.28) | 0.36 (0.20) |
| Fresh B | 3.9 | 300 | 0.67 | 2 | 210 | 0.44 | 0.17 |
| Storage B | 4.7 | 300 | 0.73 (0.46) | 2 (1.3) | 330 (210) | 0.36 (0.23) | 0.31 (0.20) |

[1]Fresh and Storage indicate rubbers obtained from fresh sporophores and withered ones after storing for 51 days in refrigerator, respectively.

[2]Values in parenthesis are calculated assuming that the degree of polymerization is proportional to that estimated by GPC.

The deviation from the theoretical value suggests the decrease of both terminal groups even rubber from fresh sporophores. However, the reliability of the analysis depends on the accuracy of $^{13}$C-NMR measurements of intensity ratios between the signals due to both terminal groups and *trans* units. The number of both terminal groups shown in Table 3 is anticipated to include the errors inherent in the quantitative analysis by $^{13}$C-NMR method, i.e., effect of spin-lattice relaxation time $T_1$ and Nuclear Overhauser Effect. The former was minimized in these experiments by the measurement with pulse repetition time of 12 s for 45° pulse by considering $T_1$ values of the signals. The latter was estimated to be 10–15% by the measurement of the NOE values of ficaprenol-11 as a model; 2.62, 2.62 and 2.22 for the signals resonated at 59.1, 39.8 and 16.0 ppm, respectively. Detailed analysis of the number of both terminal groups is made by $^1$H-NMR measurements for these rubbers, which will be mentioned in a subsequent paper. Taking into account these facts it is reasonable to presume a marked tendency in Table 3 for the decrease of both terminal groups and *trans* units during aging of sporophores.

The number of both terminal groups was analyzed for rubber obtained directly from latex and that present in residual sporophores. Rubber obtained from latex of young and fresh sporophores of *L. volemus* showed the presence of 1.1 dimethylallyl group and 0.43 ester terminal and 0.43 hydroxyl terminal groups on the assumption of two *trans* units per chain. On the other hand, rubber obtained by solvent extraction from residue exhibited the presence of 0.37 dimethylallyl group and 0.42 ester terminal but no hydroxyl terminal groups. This suggests that rubber in latex has the structure just after polymerization while that not exuded as latex has the structure after modification of both terminal groups. This also implies that hydroxyl terminal group is derived from pyrophosphate terminal groups and the termination of polymerization occurs by esterification with fatty acids.

**Biosynthesis of rubber in *Lactarius* mushrooms**

The structure of both terminal groups and alignment of the *trans* units give conclusive evidence for the initiation step of rubber formation in mushroom. As illustrated in Fig. 3, the polymerization is presumed to start from *trans, trans*-farnesyl pyrophosphate and proceeds by successive condensation of isopentenyl pyrophosphate to form isoprene units exclusively in the *cis* configuration. The termination process is assumed to occur by esterification of polyisoprenyl pyrophosphate (PIP-PP) or hydrolysis of PIP-PP followed by esterification with fatty acids.

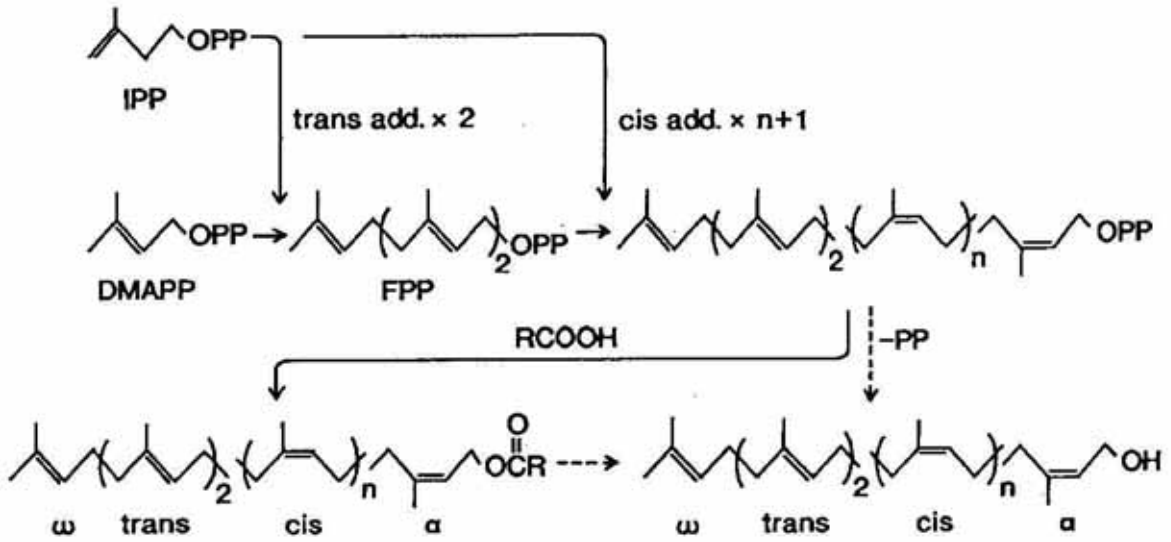



*Fig. 3. Presumed biosynthesis mechanism of cis polyisoprene in Lactarius mushroom.*

It is remarkable that the number of dimethylallyl group and ester and hydroxyl terminal groups decreases during aging of sporophores. This implies that a chemical or biochemical modification, such as oxidation or cyclization, occurs at the terminal. It is expected that the reaction proceeds to the isoprene unit linked to dimethylallyl group resulting in the decrease of the number of *trans* units. In the case of natural rubber occurring as latex, ester or hydroxyl terminal group and dimethylallyl group were not observed even rubber samples from fresh latex, although the presence of *trans* units was confirmed [2, 3]. These findings suggest that the biosynthesis mechanism of *cis* polyisoprene in *Hevea* tree is fundamentally the same as that in nonlaticiferous cells and in mushroom. The absence of both terminal groups in *Hevea* rubber implies the occurrence of modifications as in the case of mushroom at the terminal during the storage of latex in *Hevea* tree.

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