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INITIATION OF BIOSYNTHESIS IN *CIS* POLYISOPRENES

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Key Word Index—Polyisoprene; rubber; polyprenol; farnesyl diphosphate; geranylgeranyl diphosphate.

Abstract—The molecular species initiating rubber biosynthesis in leaves of *Solidago altissima* and *Helianthus annuus* and sporophores of *Lactarius volemus* have been analysed by structural characterization of the polyisoprenes by ^{13}C NMR spectroscopy. The alignment of *trans* and *cis* isoprene units in these *cis* polyisoprenes has been determined using polyprenol-16 [dimethylallyl-(*trans*)$_2$-(*cis*)$_{13}$-OH] and ficaprenol-11 [dimethylallyl-(*trans*)$_3$-(*cis*)$_7$-OH], respectively, as model compounds. The C-1 methylene carbon atoms of the *trans* isoprene units give rise to two and three signals at δ39.7–39.8 for polyprenol-16 and ficaprenol-11, respectively. These signals were assigned by comparison with the signal splitting pattern in solanesol-9 and spin-lattice relaxation times T_1 of the respective carbon atoms, also. Polyprenol-16 gives rise to a signal reflecting the presence of the *trans* unit in the *trans-trans-trans* sequence at about 5%. Similarly, ficaprenol-11 contains about 5% of the *trans-trans* sequence. Rubbers from *S. altissima* and *H. annuus* give rise to *trans* C-1 carbon signals, indicative of the presence of both two *trans* and three *trans* sequences in a ratio of 4:3 and 2:1, respectively. By contrast, only the dimethylallyl-(*trans*)$_2$-sequence was observed in the low M_r *cis* polyisoprene from sporophores of *L. volemus*. These findings demonstrate that the initiating species of rubber formation in the leaves are both *trans,trans*-farnesyl diphosphate (FDP) and *trans,trans,trans*-geranylgeranyl diphosphate (GGDP). Taking into account the presence of about 5% of the unexpected isomeric terminal sequences in these polyprenols, it is concluded that the primer selectivity of rubber transferase in the leaves is not highly specific with respect to the chain length of the allylic diphosphate initiator.

INTRODUCTION

On the basis of ^{1}H NMR studies polyprenols consisting of *cis* and *trans* isoprene units are classified into two groups, i.e. two-*trans*, poly-*cis* prenols and three-*trans*, poly-*cis* prenols [1]. Ficaprenols-10, -11 and -12 from *Ficus elastica* are typical members of a homologous series consisting of three *trans*, poly *cis* isoprene units [2]. The two *trans*, poly *cis* composition has been reported for betulaprenols-6 to -9 from *Betula verrucosa* [3], bacterial polyprenol-11 from *Lactobacillus plantatrum* [4] and dolichols from animals [5, 6]. On the other hand, the presence of 1.2–3 *trans* isoprene units has been suggested for polyprenols isolated from *Magnolia campbellii* [7]. Dolichol is a typical animal polyprenol consisting of two *trans*, poly-*cis* isoprene units. However, a small amount of a C_{55} dolichol containing three *trans* isoprene units has been isolated from pig liver. This was proposed to have arisen from plant polyprenols present in the diet [8]. These findings imply that the discrimination of two-*trans* type from three-*trans* type on the basis of the relative intensity of ^{1}H NMR signals for the *trans* and *cis* units is not an easy task. Some intermediate value for the number of *trans* units is often observed, even when careful quantitative ^{1}H NMR measurements are carried out.

^{13}C NMR spectrometry has been used to determine the arrangement of *trans* and *cis* isoprene units along the chain [9]. The alignment of dimethylallyl-(*trans*)$_3$-*cis*- units has been determined for ficaprenols [10] and that of dimethylallyl-(*trans*)$_2$-*cis*- units for polyprenols from *Ginkgo biloba* [11] and from Pinaceae [12], and for dolichol from pig liver [13]. This ^{13}C NMR method has been applied to the structural characterization of rubber. The presence of 2–3 *trans* isoprene units and a dimethylallyl group has been proved for rubbers from goldenrod (*Solidago altissima*) [14] and sunflower (*Helianthus annuus*) [15]. In these studies, the number of *trans* units was estimated on the basis of the intensity ratio of the ^{13}C NMR signals for the *trans* units and the dimethylallyl group. However, it is difficult to distinguish clearly between the two-*trans* type and the three-*trans* type, because accurate determination of the relative intensity from very small ^{13}C NMR signals is practically impossible. Therefore, the problem of the determination of the initiating species, whether it is *trans,trans*-farnesyl diphosphate (FDP) or *trans,trans,trans*-geranylgeranyl diphosphate (GGDP), has remained unsettled, although a *trans* allylic diphosphate has been confirmed to be a direct initiator for rubber formation in these rubbers.

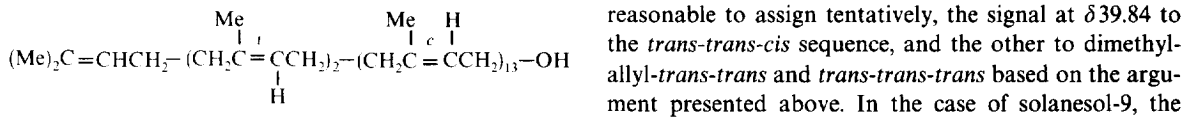
The initiation of new rubber molecules has been studied by the use of radioactive isopentenyl diphosphate (IDP) and washed rubber particles which were prepared by centrifugation or gel permeation techniques from latex [15]. The ^{13}C NMR data for rubber from goldenrod strongly suggest that farnesyl diphosphate or geranylgeranyl diphosphate acts as the initiating compound of rubber formation [14, 15]. Attempts have been made to clarify the initiating species which starts the addition of IDP in a *cis* configuration. It has been confirmed that the addition of FDP in the *trans* configuration has a stimulating effect on rubber formation in a system containing IDP and washed rubber particles from *H. brasiliensis* [17–19], *P. argentatum* [20, 21] and *Ficus elastica* [22]. Direct incorporation of [1-^{3}H]neryl disphosphate and [1-^{3}H]geranyl diphosphate was reported in the case of *H. brasiliensis* [23]. However, allylic diphosphates of chain length C_5–C_{20} also show the stimulating effect, the efficiency of which increases with increasing chain length of the allylic diphosphate, i.e. $C_5 < C_{10} < C_{15} < C_{20}$, rather than the geometric isomerism of the isoprene units [17, 23]. These findings suggest that the direct initiator is FDP and/or GGDP in the case of *H. brasiliensis*. Similarly, the use of C_5-, C_{10}-, C_{15}- and C_{10}-diphosphates as initiators has been reported for *in vitro* biosynthesis of rubber from IDP by washed rubber particles from *P. argentatum* [24]. It is remarkable in this study that these allylic diphosphates, including geranyl-DP and neryl-DP, have an equal activity in the polymerization reaction. This suggests that the rubber transferase from *P. argentatum* is able to use any short-chain allylic diphosphate as initiator independent of chain length or geometric isomerism. Although these biochemical studies indicate the possibility of using allylic diphosphates as an initiating species, they afford no direct evidence as to the true initiating molecule in *H. brasiliensis* and *P. argentatum*.

We have developed a new ^{13}C NMR method to characterize the structure of the *trans* isoprene units in the *cis* polyisoprene from the mushroom, *Lactarius volemus* [25]. The number of *trans* isoprene units linked to the dimethylallyl group in polymer chains provides conclusive evidence for the initiating species of rubber formation in *S. altissima* and *H. annuus*.

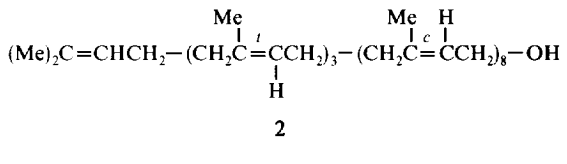
RESULTS AND DISCUSSION

Assignment of C-1 methylene carbons in trans *isoprene unit*

Polyprenol-16 (**1**), ficaprenol-12 (**2**) and solanesol-9 (**3**) are typical model compounds of (a) two-*trans*, poly-*cis* type, (b) three-*trans*, poly-*cis* type, and (c) all-*trans* type, respectively. The arrangement of *trans* and *cis* isoprene units being as shown in the formulae [10, 11].



1



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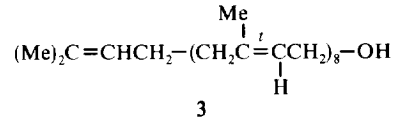
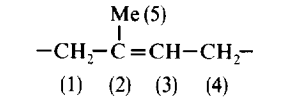


3

The numbering of the carbon atoms in both the *trans* and *cis* isoprene units and the dimethylallyl group is as follows:



The model compounds showed two to four ^{13}C NMR signals corresponding to the C-1 methylene carbon atoms of *trans* units (Fig. 1). The signal splitting patterns are expected to reflect the alignment of *trans* units longer than dyad sequences of isoprene units including the dimethylallyl group [25]. Triad sequences are expected to be the minimum length required to satisfy the signal splitting in solanesol-9 (Fig. 1a). Polyprenol-16 showed two signals resonating at δ39.79 and 39.81 with an intensity ratio of 1:1, which are assignable to the C-1 methylene carbon atoms in two *trans* isoprene units (Fig. 1b). Ficaprenol-12 showed two distinct signals resonating at δ39.80 and 39.84, intensity ratio 2:1 (Fig. 1c), the former was further split into two peaks when the measurements were made at 30° (Fig. 1d).

Detailed assignment of these signals was carried out by considering the spin-lattice relaxation times T_1 of the C-1 carbon atom in the *trans* units. The T_1 values were found to be 1.63 and 1.18 sec for the signals resonating at δ39.79 and 39.81 ppm in the case of polyprenol-16. It is usually observed in straight chain alcohols that the carbon atom shows an increase in the effective correlation time for molecular rotation toward the hydroxyl terminal of the molecule, resulting in shorter T_1 values for those carbon atoms near the hydroxyl terminal [26]. A similar tendency was observed for phytol [27]. If a similar relation holds for polyprenols, the signal having a longer T_1 value can thus be assigned to the *trans* unit adjacent to the dimethylallyl terminal unit, i.e. the T_1 value of the *trans* unit in the dimethylallyl-*trans-trans* sequence is longer than that of the *trans* unit in the *trans-trans-cis* sequence. Similarly, three signals in ficaprenol-12 showed T_1 values of 1.20, 1.58 and 0.97 sec in order of decreasing magnetic field. It is difficult to discuss the differences of the T_1 values for the two signals resonating around δ39.80, because of their incomplete separation, but it seems reasonable to assign tentatively, the signal at δ39.84 to the *trans-trans-cis* sequence, and the other to dimethylallyl-*trans-trans* and *trans-trans-trans* based on the argument presented above. In the case of solanesol-9, the signal resonating at δ39.64 is assigned to the *trans* unit

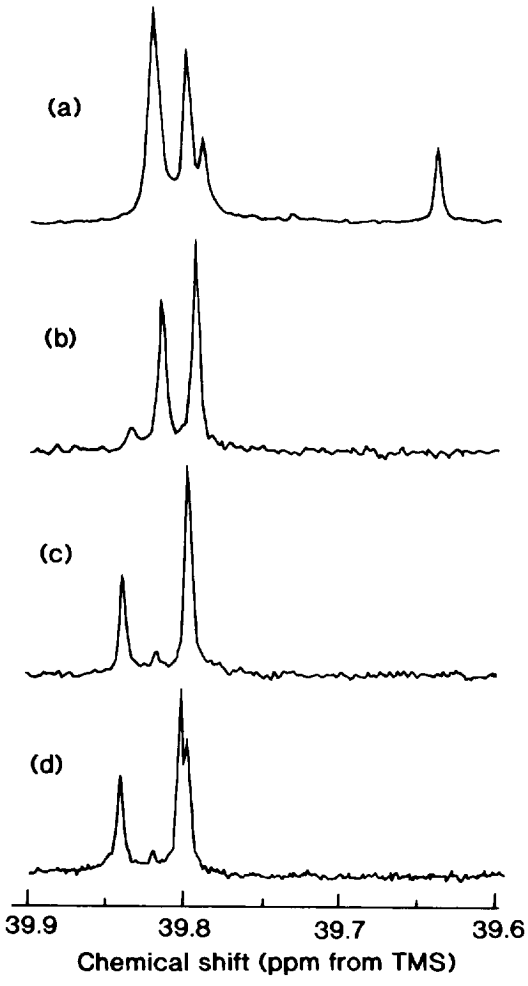


Fig. 1. C-1 methylene carbon signals of *trans* isoprene units in model compounds. (a) Solanesol-9, (b) polyprenol-16 and (c) ficaprenol-12 measured at 50° and (d) ficaprenol-12 measured at 30°.

with the hydroxyl terminal group, by comparison with the resonance of *trans,trans,trans*-geranylgeraniol [28]. The other signals were assigned tentatively by considering the T_1 values and their relative intensity (Table 1). It is remarkable that the assignments for these three model compounds are consistent with each other. The assignments were further confirmed by selective DEPT (Distortionless Enhancement of Polarization Transfer) measurements which will be described elsewhere.

The finding demonstrates that two-*trans* and poly-*cis* polyprenols can be differentiated from three-*trans* and poly-*cis* on the basis of the marked separation of the *trans* C-1 methylene carbon signals. It is noteworthy that ficaprenol-12 and polyprenol-16 show a small signal with a relative intensity of 5% against the inherent signal corresponding to the two-*trans* type and three-*trans* type, respectively, even after extensive purification. This is confirmatory evidence that even these typical polyprenols are a mixture of geometric isomers.

Table 1. Assignment of *trans* C-1 methylene carbon signals in model compounds

	Chemical shift†		
Assignment*	Polyprenol-16	Ficaprenol-12	Solanesol-9
t-t-OH (t_8)			39.64 (1.26)
t-t-OH (t_7)			39.79 (0.88)
ω-t-t (t_1)	39.79 (1.63)	39.80 (1.20, 1.58)	
ω-t-t-t (t_2)			39.80 (1.15)
ω-t-t-c	39.81 (1.18)		
t-t-t (t_3-t_6)			39.82 (0.71)
t-t-t-c		39.84 (0.97)	

**Trans* and *cis* isoprene units and the dimethylallyl group designated as t, c and ω, respectively. Subscripts indicate the number of *trans* isoprene units attached to the dimethylallyl group.

†Values in parentheses indicate the spin-lattice relaxation time T_1 in sec.

Number of trans *units in the rubber molecule*

Rubbers from *S. altissima* and *H. annuus* showed five major ^{13}C NMR signals at δ23.41, 26.51, 32.31, 125.16 and 135.24 derived from the resonances of C-5, C-4, C-1, C-3 and C-2 atoms, respectively, in the *cis* isoprene units. Small signals characteristic of *trans* isoprene units were observed at δ16.00 (C-5) and 39.80 (C-1), and that from the dimethylallyl group at δ17.66, which was assigned to the methyl carbon atom in the *trans* configuration [28] (Fig. 2). The C-1 methylene carbon atoms in the *trans* units gave rise to three peaks resonating at δ39.79, 39.81 and 39.84 for both samples when the measurements were made at 100 MHz (Fig. 3a and b). The chemical shifts of these peaks are in good agreement with those of the 1:1 mixture of polyprenol-16 and ficaprenol-12 (Fig. 3c). This clearly indicates that the signal at δ39.79 is assignable to the overlap of dimethylallyl-*trans-trans* in two-*trans* chains, and dimethylallyl-*trans-trans* and *trans-trans-trans*, in three-*trans* chains. Similarly, the signals at δ39.81 and 39.84 are assigned to *trans-trans-cis* in two-*trans* chains and three-*trans* chains, respectively.

The ratios of two-*trans* and three-*trans* chains in the rubbers from *S. altissima* were found to be 67:33, 63:37 and 60:40, and that from *H. annuus* to be 60:40, from the relative intensity of these signals.

Low M_r rubber from sporophores of *Lactarius volemus* showed the C-1 methylene carbon signals due to the *trans* isoprene units at δ39.79 and 39.81 (Fig. 4). This pattern is in good agreement with that of polyprenol-16, showing the presence of *trans* isoprene units in the dimethylallyl-*trans-trans* sequence. It is noteworthy that an additional signal due to the three-*trans* sequence was not observed, even when the measurement was made at 125 MHz [25].

The ^1H NMR spectrum of model compounds shows splitting of the methyl proton signals reflecting triad sequences of *trans* isoprene units [29]. Three-*trans* and two-*trans* isomers can be differentiated by characteristic signals: (a) three-*trans* shows a signal from the *trans-trans-trans* sequence at δ16.24 in addition to that from dimeth-

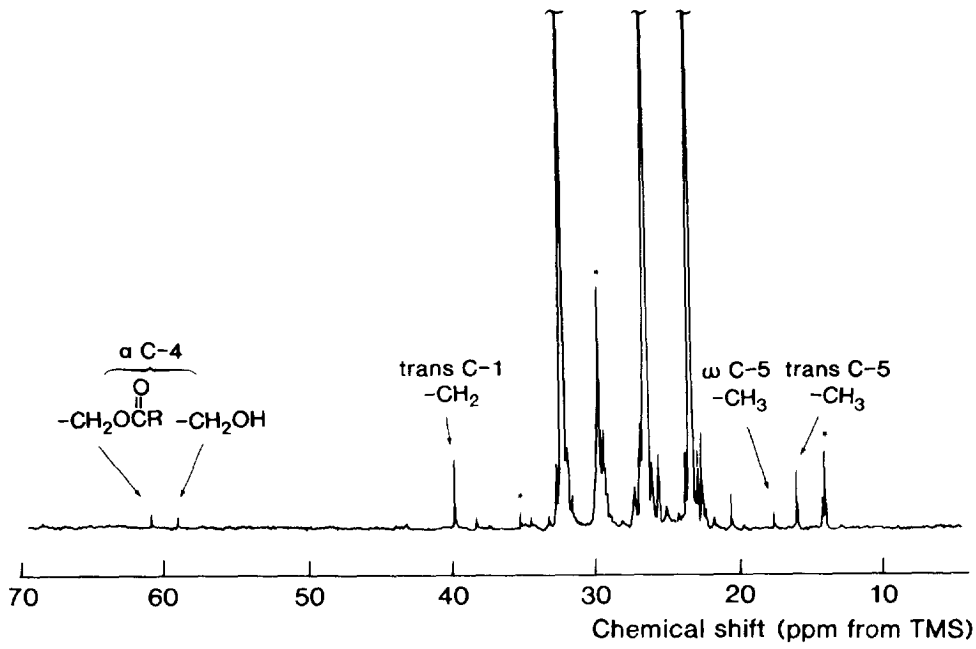


Fig. 2. Aliphatic carbon spectrum of rubber from *H. annuus*. Signals due to fatty acid groups are indicated by an asterisk.

ylallyl-*trans-trans* sequence at δ1.621, and (b) the *trans* unit in the *trans-trans-cis* sequence resonates at δ1.639 and 1.644 for two-*trans* and three-*trans* isomers. The rubber samples gave methyl proton signals similar to those of model compounds, although a slight drift of chemical shifts was observed due to a viscosity and/or concentration effect. Broad peaks observed around δ1.62 and 1.64 imply that both samples contain two-*trans* and three-*trans* isomers.

Initiating species of cis *polyisoprenes*

The structural evidence thus obtained clearly indicates that the initiating species for rubber formation are *trans,trans*-FDP and *trans,trans,trans*-GGDP in the case of rubbers from leaves of *H. annuus* and *S. altissima*. It is possible to interpret this in two ways, i.e. (a) a low specificity of rubber transferase with respect to the chain length of the *trans* allylic diphosphate primers or (b) the presence of two types of rubber transferase, one utilizing FDP the other GGDP. The former is consistent with the findings that C_5–C_{20} allylic diphosphates are used equally well as initiators in the case of the rubber transferase from *P. argentatum* [24] and a slight difference of the stimulating effect is observed between FDP and GGDP in the case of rubber transferase from *H. brasiliensis*. In addition, the rubbers used in the present study show a typical unimodal M_r distribution, which implies that these are not a mixture of two types of rubber molecules formed separately.

Rubber occurring as latex from *L. volemus* was found to be a typical high M_r homologue of a two-*trans* polyprenol. The absence of three-*trans* molecules seems to be related to the presence of a prenyl transferase forming GGDP in latex.

EXPERIMENTAL

Materials. Leaves from goldenrod (*Solidago altissima*) and sunflower (*Helianthus annuus*) growing in Tokyo, Japan, were collected from September to October and August 1992, respectively. Fresh leaves were cut into small pieces, crushed in a blender, and extracted with EtOH followed by hexane in a Soxhlet extractor. After centrifugation, the hexane extract was concd in a rotary evaporator. The rubber fraction was pptd by pouring the hexane soln into EtOH, and then purified several times by reprecipitation from hexane soln with EtOH. The yield of rubber based on dried leaves was 1–3 and 1%, for goldenrod and sunflower, respectively. Sporophores of *Lactarius volemus* were collected in August 1993 in Fukushima, Japan. The fresh young sporophores were cut into small pieces and extracted with Me_2CO followed by extraction with hexane ×3. The rubber fraction was obtained from the toluene soln and was purified in a similar way to that from goldenrod and sunflower. The M_rs of these rubbers were determined by GPC and vapour pressure osmometric measurements (Table 2).

Ficaprenol-11 and polyprenol-16 were isolated from silkworm faeces and from needles of *Pinus thunbergii*, respectively, in the usual way, and were fractionated by RP-HPLC. Solanesol-9 was supplied by Kuraray Co. Japan and was used without further purification.

Measurements. The ^{1}H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, with a Jeol GX-400 NMR spectrometer using TMS as int. standard. The conditions for ^{1}H and ^{13}C NMR measurements are listed in Table 3. The signal resolution was 0.0032 ppm for ^{13}C NMR and 0.00045 or 0.00091 ppm for ^{1}H NMR measurements. GPC measurements were made using two GPC columns packed with polystyrene gel using $CHCl_3$

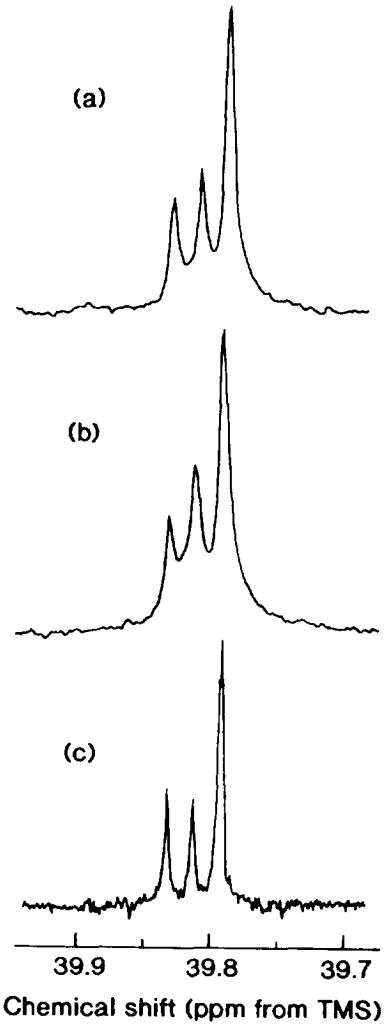


Fig. 3. C-1 methylene carbon signals of *trans* isoprene units in rubber from leaves of (a) *S. altissima* and (b) *H. annuus*, and (c) a 1/1 mixture of polyprenol-16 and ficaprenol-12.

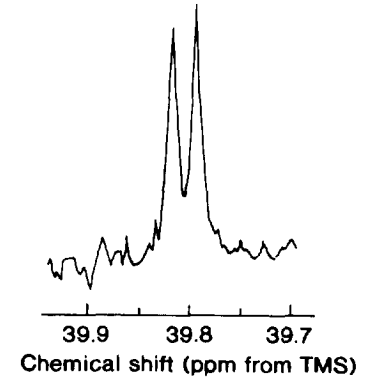


Fig. 4. C-1 methylene carbon signals of *trans* isoprene units of rubber from sporophores of *L. volemus*.

as eluent. M_r calibration was performed with commercially obtained standard polystyrenes. Number average M_r was determined by means of a Hitachi 117 Vapor Pressure Osmometer.

Table 2. M_r and M_r distribution of rubbers from *S. altissima*, *H. annuus* and *L. volemus*

	GPC measurement*		VPO measurement†
Sample	$\bar{M}_n \times 10^{-4}$	$\bar{M}_w \times 10^{-4}$	$\bar{M}_n \times 10^{-4}$
S. altissima	2.4	9.6	1.8
S. altissima	2.2	8.4	
H. annuus	1.4	4.8	1.0
L. volemus	2.1	6.9	1.2

*Number average M_r ($\bar{M}_n$) and weight average M_r ($\bar{M}_w$) estimated by GPC using standard polystyrenes.

†Number average M_r ($\bar{M}_n$) determined by vapour pressure osmometric measurement.

Table 3. Conditions used for ^{13}C and ^{1}H NMR measurements

	^{13}C NMR	^{1}H NMR
Sample concentration	10% w/v	3% w/v
Solvent	$CDCl_3$	C_6D_6
Temp	50° or 30°	50°
Sample tube	10 mm o.d.	5 mm o.d.
Point	128 K	32 or 64 K
Pulse width	45 deg	45 deg
Pulse interval	4–6 sec	4 sec
No. of scans		
(model compound)	100	200
(rubber)	10 000–20 000	1000
Frequency	100.40 MHz	399.65 MHz

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