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SEPARATION OF GERANYLGERANIOL ISOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND IDENTIFICATION BY ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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SUMMARY

The geometric isomers of geranylgeraniol were prepared by isomerization of *trans,trans,trans*-geranylgeraniol by UV irradiation in the presence of thiobenzoic acid. The resulting isomers were separated by high-performance liquid chromatography on styrene–divinylbenzene gel as the stationary phase. The mixture of eight isomers was separated into two fractions according to the geometric isomerism of the hydroxylated terminal unit using 2,2,4-trimethylpentane as the eluent. Each fraction was further separated into four fractions in methanol as the eluent. The isomers were identified by ^{13}C NMR spectroscopy. Pure *cis,cis,cis*-, *cis,trans,trans*-, *trans,cis,cis*-, *trans,cis,trans*- and *trans,trans,trans*-geranylgeraniols were obtained together with three other isomers in 75–87% purity.

INTRODUCTION

Geranylgeraniol (3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-ol) is a linear diterpene isolated from linseed oil[1] and the wood of *Cedrela tonna*[2]. The natural form exists as only one isomer comprising *trans,trans,trans* double bonds. It is widely recognized that *trans,trans,trans*-geranylgeranyl pyrophosphate plays a significant role as a precursor in the biosynthesis of cyclic diterpenes, carotenes, polyterpenoids and polyprenols. Although the other geometric isomers have never been isolated from natural sources, they would be expected to possess some activity in biological systems. The effects of saturation and chain length were determined for various polyprenyl phosphates employed as lipid acceptors for sugars[3–5]. However, very little evidence is available about the geometric isomerism of polyprenyl compounds. For example, undecaprenol phosphate consisting of two *trans*- and eight *cis*-isoprene units has been shown to act as a sugar carrier in the synthesis of peptidoglycan and polysaccharide in microorganisms, whereas the role of the isomeric undecaprenol containing three *trans*- and seven *cis*-isoprene units, which exists predominantly in the leaf tissues of angiosperms, has not yet been clarified[6]. It seems likely that a particular geometric arrangement offers advantages in certain biological situa-

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tions or a geometric isomer may simply result from biosynthesis sequences that provide no functional advantage to the final product. The synthesis of the geranylgeraniol isomers may provide a way to elucidate the biological significance of geometric isomerism in isoprenoid compounds.

Several methods have been proposed for the synthesis of linear terpenes. The *trans,trans,trans-* and *cis,trans,trans-*geranylgeraniols can be prepared by chain extension from *trans,trans*-farnesol[7]. Similarly, the other geranylgeraniol isomers may be synthesized using the other farnesol isomers as starting materials, although the desired products are always accompanied by geometric isomers. In a previous paper, we reported[8] the separation of the geometric isomers of farnesol by use of high-performance liuid chromatography (HPLC). In the present investigastion we undertook to prepare all of the geranylgeraniol isomers by separation of isomerized geranylgeraniol by means of preparative HPLC. The isomers were then characterized by ^{13}C NMR spectroscopy.

EXPERIMENTAL

Isomerization of geranylgeraniol

A solution of the geranylgeraniol isomer having the 2-*trans*,6-*trans*,10-*trans* configuration (I) (Kuraray Co.), abbreviated as t,t,t-GG, in benzene (2 g per 100 ml) was irradiated with a high-pressure mercury lamp in the presence of 0.01–0.15 g of thiobenzoic acid per 1 g of geranylgeraniol, for 7–10 h at 10°C under a nitrogen atmosphere and with magnetic stirring. The resulting solution was washed with aqueous 2 *M* sodium hydroxide to remove thiobenzoic acid. The 2-*cis*,6-*trans*,10-*trans* isomer (II) (Kuraray Co.), abbreviated as c,t,t-GG, was isomerized using the same procedure. The resulting geranylgeraniol isomers were separated by HPLC.

Liquid chromatography

HPLC was carried out using a JASCO Trirotar II as a high-pressure pump and a Waters R 401 differential refractometer as a detector. For analytical purposes, a 500 × 7.5 mm I.D. stainless-steel column was used at a flow-rate of 1 ml/min, and for preparative separations, a 600 × 21 mm I.D. column at a flow-rate of 6 ml/min. The columns were packed with a high-resolution styrene–divinylbenzene copolymer gel having an exclusion limit of 3000 in gel permeation chromatography. The gel was prepared by conventional suspension polymerization[9]. Analytical columns packed with silica gel (5 μm) or ODS-silica gel (5 μm) were also used.

Gas chromatography

Gas chromatography (GC) wass carried out using a Hitachi 163 gas chromatograph equipped with a 30-m glass capillary column coated with free fatty acid polyester (FFAP).

^{13}C NMR measurements

The ^{13}C NMR spectra were obtained at 50.1 MHz using a JEOL FX-200 spectrometer. Measurements were made at room temperature in deuterochloroform solution (about 5%, w/v). Chemical shifts were referred to tetramethylsilane added as an internal standard. The accuracy of the chemical shifts was ± 0.01 ppm.

RESULTS AND DISCUSSION

Preparation of geranylgeraniol isomers

Isomerized I showed four peaks in its gas chromatogram, corresponding to the $(cis)_3$, $(cis)_2$ $(trans)$, (cis) $(trans)_2$ and $(trans)_3$ isomers in order of increasing retention time. Fig. 1 shows the total amount of *trans* units in the mixture during the isomerization reaction. The amount levelled off after 2–7 h depending upon the amount of thiobenzoic acid. The final content of 62–64% *trans* units was presumed to be an equilibrium value, because the same value was obtained by isomerization starting from compound II. This equilibrium value is in good agreement with that found in the isomerization of *cis*- and *trans*-polyisoprenes using a similar procedure[10].

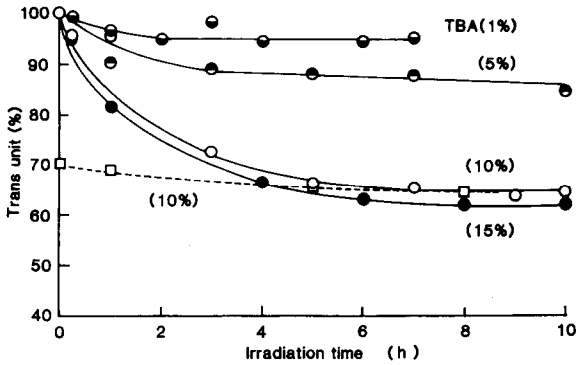


Fig. 1. The content of *trans*-isoprene units in isomerized geranylgeraniol from the t,t,t-GG isomer (——) and from the c,t,t-GG isomer (---). TBA = Thiobenzoic acid.

The observed relative intensities of these GLC peaks were in good agreement with the theoretical values obtained on the assumption of random isomerization of the isoprene units.

Separation of geranylgeraniol isomers by HPLC

The use of HPLC was proven to be effective in separating 2-*trans*,6-*trans*- and 2-*cis*,6-*trans*-farnesol on silica gel as the stationary phase[11]. We have demonstrated that a complete separation of the farnesol isomers can be achieved by HPLC using styrene–divinylbenzene gel as the stationary phase[8]. However, little is known about the separation of geranylgeraniol isomers. In order to find the most suitable separation conditions, the elution behaviour of isomers I and II was determined on silica gel, ODS-silica gel and styrene–divinylbenzene gel with various eluents.

Table I lists the observed elution volumes for compounds I and II. Columns packed with silica gel or ODS-silica gel yielded the same elution volume for both isomers independent of the eluent polarity. An unresolved peak was observed for isomerized I on the silica gel and ODS-silica gel columns. On the other hand, a marked difference in elution volumes was observed between I and II on styrene–divinylbenzene gel with 2,2,4-trimethylpentane, cyclohexane or methanol as an

TABLE I

ELUTION VOLUMES (ml) OF GERANYLGERANIOL ISOMERS

I = t,t,t-GG isomer; II = c,t,t-GG isomer.

Eluent	*Polystyrene gel**		*ODS-silica gel***		*Silica gel***	
	I	*II*	*I*	*II*	*I*	*II*
2,2,4-Trimethylpentane	79.0	71.0	19.5	19.3	28.4	28.4
Hexane	–	–	–	–	26.0	26.0
Cyclohexane	40.8	38.4	18.0	17.3	–	–
Diisopropyl ether	33.1	33.6	–	–	–	–
Chloroform	14.5	14.5	–	–	–	–
Tetrahydrofuran	–	–	13.5	13.3	16.6	16.4
Acetone	34.6	34.6	–	–	–	–
Acetonitrile	70.7	69.9	38.6	34.1	18.3	18.0
Methanol	91.0	83.5	20.2	19.5	16.6	16.5

* Measured on a 500 × 10 mm I.D. column at a flow-rate of 1 ml/min.
** Measured on a 500 × 7.5 mm I.D. column at a flow-rate of 1 ml/min.

eluent. This elution behaviour is in accord with that of farnesol isomers, and indicates that the separation of geranylgeraniol isomers proceeds according to two types of mechanisms; one reflects the number of *cis* units in the isomers in methanol as eluent, and the other reflects the geometric isomerism of the hydroxylated terminal (α-terminal) units in 2,2,4-trimethylpentane or cyclohexane as eluent, as in the case of farnesol isomers[8].

Fig. 2 shows the chromatograms of isomerized I in 2,2,4-trimethylpentane and in methanol as eluent. The first and second peaks in 2,2,4-trimethylpentane were tentatively assigned to the isomers having the *cis* α-terminal unit (c,c,c-, c,t,c-, c,c,t- and c,t,t-GG) and those having the *trans* α-terminal unit (t,t,t-, t,t,c-, t,c,t- and t,c,c-GG), respectively. In methanol as eluent the isomers were separated into four peaks, which were tentatively assigned to the $(cis)_3$, $(cis)_2$ $(trans)$, (cis) $(trans)_2$ and $(trans)_3$ isomers in order of increasing elution volume.

The preparative separation of the isomers was carried out under similar conditions. With a sample of 250 mg in 1 ml of solution, fractions A and B were obtained by recycling three times in 2,2,4-trimethylpentane as eluent, as shown in Fig. 3a. The

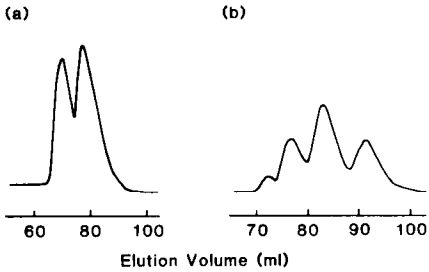


Fig. 2. Liquid chromatograms of isomerized geranylgeraniol in 2,2,4-trimethylpentane (a) and in methanol (b) as eluent.

purity of both fractions was confirmed to be 100% by comparison of the ^{13}C NMR signals characteristic of *cis* and *trans* α-terminal units. Fractions A and B were then subjected to further chromatography in methanol as eluent as shown in Fig. 3b and c. The fraction A was separated into three peaks after three cycles. After removing the first and the last fractions, A-1 and A-4, the central peak was recycled 23 times and separated into two fractions, A-2 and A-3. Similarly, fraction B was separated into four fractions, B-1 to B-4. According to the results mentioned above, fractions A-1, A-4, B-1 and B-4 can be unequivocally assigned to c,c,c-, c,t,t-, t,c,c- and t,t,t-GG, respectively. However, it is difficult to identify fractions A-2, A-3, B-2 and B-3 only from their elution behaviours. The fractions A-2 and A-3 correspond to either c,c,t-GG or c,t,c-GG, and B-2 and B-3 to either t,c,t-GG or t,t,c-GG. These fractions were identified on the basis of the ^{13}C NMR analysis.

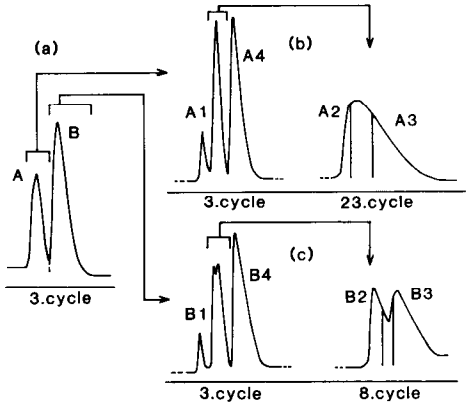
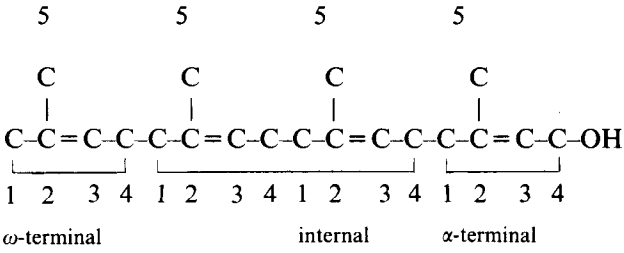


Fig. 3. Preparative HPLC separation of isomerized geranylgeraniol in 2,2,4-trimethylpentane (a) and in methanol (b and c) as eluent.

^{13}C NMR analysis of geranylgeraniol isomers

The ^{13}C NMR spectra of the isomers were assigned by considering the corresponding assignments for geraniol, nerol and farnesol isomers and solanesol[12], and the spin-lattice relaxation times, T_1[13]. The aliphatic and olefinic carbon atoms in the isomers showed signals characteristic of the alignment of the *cis* and *trans* units as well as of the geometric isomerism of the internal and α-terminal units. The chemical shifts and assignments of the signals are listed in Tables II–IV.

The carbon atoms are designated as follows by considering the similarity of the chemical and steric environments of the corresponding carbons in repeating isoprene units:



The geometric isomerism of the internal and α-terminal units can be determined from the chemical shifts of the methyl carbon signals listed in Table II. The C-4 methylene carbon atom in the *trans* and *cis* α-terminal units showed characteristic signals at 59.4 and 59.1 ppm, respectively as listed in Table III.

TABLE II

CHEMICAL SHIFTS OF METHYL CARBON SIGNALS FOR GERANYLGERANIOL ISOMERS

The abbreviations t and c correspond to *trans* and *cis* units, respectively.: (*cis*) and (*trans*) correspond to the methyl carbon of the ω-terminal unit in *E* and *Z* configurations, respectively.

Fraction	*Isomer*	*ω-Terminal*		*Internal*		*α-Terminal*	
		(cis)	*(trans)*	*cis*	*trans*	*cis*	*trans*
A-1	c,c,c-GG	25.71	17.64	23.39		23.44	
A-2	c,c,t-GG	25.68	17.68	23.37	15.98	23.45	
A-3	c,t,c-GG	25.71	17.63	23.37	16.00	23.43	
A-4	c,t,t-GG	25.68	17.67		16.01	23.44	
B-1	t,c,c-GG	25.70	17.64	23.40			16.28
B-2	t,c,t-GG	25.69	17.69	23.40	16.00		16.30
B-3	t,t,c-GG	25.70	17.64	23.38	16.03		16.29
B-4	t,t,t-GG	25.68	17.68		16.02		16.28

The C-1 methylene carbon atom in the *trans* and *cis* units showed signals around 40 and 32 ppm, respectively, reflecting the geometric isomerism of the unit linked to the C-1 carbon atom, *i.e.*, 39.6 ppm [*trans-trans*(α)], 39.7–39.8 ppm (*ω-trans* and *trans-trans*), 39.9 ppm [*cis-trans*(α)], 40.0 ppm [*cis-trans*(α)], 32.0–32.1 ppm [*ω-cis*, *trans-cis*, and *trans-cis*(α)] and 32.3–33.4 ppm [*cis-cis* and *cis-cis*(α)]. It was observed that the ω-terminal unit has the same shielding effect on the subsequent C-1 methylene carbon atom as does an internal *trans* unit[12]. The fractions A-2, A-3, B-2 and B-3 were unequivocally identified on the basis of these signal assignments.

The C-2 carbon atom in the ω-terminal unit in the *ω-trans* linkage gave a signal around 131.3 ppm, while that in the *ω-cis* linkage showed a signal around 131.5 ppm as listed in Table IV. These signals reflecting the sequence structure of the isoprene units were used to determine the geranylgeraniol isomers. The other olefinic carbons and C-4 methylene carbons showed complicated signals reflecting the diad or triad sequences of the isoprene units. The relationship between the chemical shifts and the structure of the isoprene units has been observed for various linear isoprenoid compounds, independent of their molecular weights[12].

On the basis of these ^{13}C NMR assignments, the purity of the fractions A-1 to A-4 and B-1 to B-4 were determined by considering the relative intensities of the signals characteristic of each isomer as listed in Table V. The results show that the pure c,c,c-, c,t,t-, t,c,c-, t,c,t- and t,t,t-GG isomers can be obtained by this preparative HPLC method. The purity of the other three isomers was found to be 74.7–87.4%, which is expected to be improved by injecting smaller samples or by further recycling.

TABLE III

CHEMICAL SHIFTS OF METHYLENE CARBONS IN GERANYLGERANIOL ISOMERS

Fraction	*Isomer*	*C-1 (CH_2) (internal, α-terminal)*						*C-4 (CH_2)*				
		*trans**				*cis**		*ω-Terminal*	*Internal*		*α-Terminal*	
		$C\underline{T}$	$C\underline{T}_\alpha$	$T\underline{T}$ $\omega\underline{T}$	$T\underline{T}_\alpha$	$C\underline{C}_\alpha$ $C\underline{C}$	$T\underline{C}_\alpha$ $T\underline{C}$ $\omega\underline{C}$				*trans*	*cis*
A-1	c,c,c-GG					32.24	31.94	26.68	26.40	26.34		59.04
A-2	c,c,t-GG			39.75		32.27	31.94	26.74	26.58	26.35		59.03
A-3	c,t,c-GG	39.99					31.99	26.64	26.54	26.55		59.06
A-4	c,t,t-GG			39.70			32.02	26.79	26.60	26.47		59.00
				39.73								
B-1	t,c,c-GG		39.84			32.29	31.98	26.70	26.39	26.22	59.42	
B-2	t,c,t-GG		39.85	39.77			31.99	26.75	26.53	26.22	59.42	
B-3	t,t,c-GG	40.00			39.57		32.02	26.65	26.55	26.35	59.41	
B-4	t,t,t-GG			39.75	39.60			26.82	26.68	26.39	59.35	

* The signals reflect diad sequences of *cis* and *trans* units. The observed unit is underlined.

TABLE IV

CHEMICAL SHIFTS OF OLEFINIC CARBONS IN GERANYLGERANIOL ISOMERS

Fraction	*Isomer*	*C-2 (=CH)*						*C-3 (=C)*			
		*ω-Terminal**		*Internal*		*α-Terminal*					
		ωC	*ωT*			*cis*	*trans*				
A-1	c,c,c-GG	131.50		136.45	135.11	139.91		124.88	124.53	124.47	124.31
A-2	c,c,t-GG		131.51	136.29	135.23	140.03		124.48	124.48	124.36	124.05
A-3	c,t,c-GG	131.51		136.27	135.22	139.91		124.92	124.47	124.37	123.61
A-4	c,t,t-GG		131.23	135.04	135.96	139.87		124.45	124.41	124.12	123.62
B-1	t,c,c-GG	131.53		135.37	135.50		139.76	124.99	124.60	124.35	123.41
B-2	t,c,t-GG		131.32	135.22	135.60		139.80	124.56	124.38	124.13	123.41
B-3	t,t,c-GG	131.50		135.37			139.76	125.02	124.41	123.83	123.40
B-4	t,t,t-GG		131.22	134.96	135.38		139.64	124.45	124.23	123.85	123.48

* Abbreviations as in Table III.

TABLE V

PURITY (%) OF GERANYLGERANIOL ISOMERS

Fraction	*c,c,c-GG*	*c,c,t-GG*	*c,t,c-GG*	*c,t,t-GG*	*t,c,c-GG*	*t,c,t-GG*	*t,t,c-GG*	*t,t,t-GG*
A-1	100	0	0	0				
A-2	0	87.4	12.6	0				
A-3	0	25.3	74.7	0				
A-4	0	0	0	100				
B-1					100	100	0	0
B-2					0	100	0	0
B-3					0	17.9	82.1	0
B-4					0	0	0	100

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