Structural Analyses of a Precursory Substance of Bitterness: New Polyisoprenepolyols Isolated from an Edible Mushroom (*Hypsizigus marmoreus*) by Fast Atom Bombardment Mass Spectrometry

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New polyisoprenepolyols (hypsiziprenol AA and BA) were isolated from an edible mushroom (*Hypsizigus marmoreus*). These polyols occur as a mixture of homologous polyisoprene derivatives with 40−70 carbon atoms. Analyses by FAB/MS in the positive and negative ion modes are complementary with each other in that the former provides information on the number of hydroxy groups present while the latter specifies the isoprenoid sequence, and thus become a powerful tool for analyzing the structures of polyisoprenepolyols. No polyisoprenepolyols obtained here were found to have antitumor activity on NCI-H292 and EL-4 cell lines.

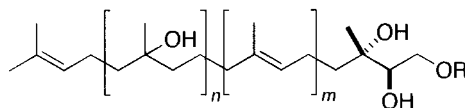
Keywords: *Polyisoprenepolyol; hypsiziprenol; mushroom; Hypsizigus marmoreus; FAB/MS*

INTRODUCTION

During the course of our search for functional molecules in edible fungi, we have isolated and characterized structures of sphingolipids (Sawabe et al., 1994, 1995a,b; Sawabe and Okamoto, 1996; Morita and Sawabe, 1998) and polyisoprenepolyols (Sawabe et al., 1996) in *Hypsizigus marmoreus (H. marmoreus;* Bunashimeji, a mushroom) by *B/E* constant linked scan fast atom bombardment (FAB) mass spectrometry.

As to polyisoprenepolyols in fungi, gymnopilins (Figure 1) were discovered independently by two groups almost at the same time in 1983 (Nozoe et al., 1983a; Aoyagi et al., 1983) from *Gymnopilus spectabilis* (*o*-waraitake, an hallucinogenic mushroom), as a bitter principle of the mushroom. However, all gymnoprenols A_9, A_{10}, A_{11} (Nozoe et al., 1983b, 1984a) (Figure 1), which have a free terminal hydroxy group instead of a half-ester of 3-hydroxy-3-methylglutaric acid, have no bitter taste. Recently, Nozoe (1991a,b) reported that these polyisoprenepolyols have an antitumor activity and also excitatory activity in central neurons. The stereochemistry of the 1,2,3-triol moieties of the polyol part (Nozoe et al., 1984b,c; Hanson 1984) and the β-carbon of the 3-hydroxy-3-methylglutaric acid group (Tanaka et al., 1992) were established as shown in Figure 1.

Previously, we isolated (Sawabe et al., 1996) hypsiziprenols A−C (Table 1, **1**−**3**) from *H. marmoreus* (Bunashimeji), which seem to be the precursor of gymnopilins. In this report, we describe a new type of polyisoprenepolyols (Table 1, **4** and **5**) found in *H. marmoreus*, which has vicinal hydroxy groups in addition to the hypsiziprenol structure. These polyisoprenepolyols were characterized by FAB mass spectrometry and tested for their antitumor activities with NCI-H292 and EL-4 cell lines.



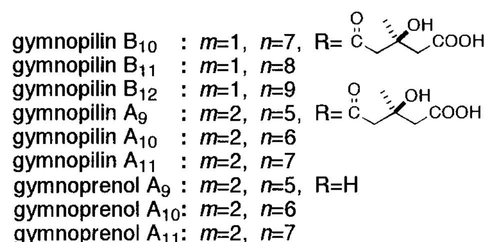


Figure 1. Structures of polyisoprenepolyols: gymnopilins and gymnoprenols.

MATERIALS AND METHODS

Isolation of Polyisoprenepolyols. Fresh fruiting bodies (1.4 kg) of *H. marmoreus* were kept in hot water at 96 °C for 20 min, chopped by a commercial blender, and homogenized after making up the total volume to 1.5 L with hot water. Cold ethanol (3.5 L) was added to the hot water solution, and the mixture was allowed to stand overnight in the dark. Then the hot water−ethanol extract (68.5 g) was treated with *n*-hexane and 1-butanol, successively. After removal of the solvent, the 1-butanol extract (4.7 g) was deposited on an Amberlite XAD-2 column (2.3 × 24 cm, Japan Organo Co., Ltd.), washed with 500 mL of water, and fractionated by successive elution with 20% methanol−water, 50% methanol−water, and methanol (500 mL each). The methanol eluate (0.65 g) was concentrated

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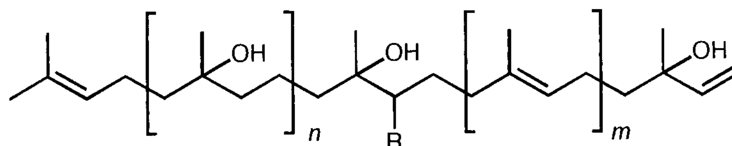
Table 1. Positive and Negative Ion FAB Data of Compounds 1a−5a



1a: *m*=1, *n*=4, R=H hypsiziprenol A_8
b: *m*=1, *n*=5, R=H hypsiziprenol A_9
c: *m*=1, *n*=7, R=H hypsiziprenol A_{11}
d: *m*=1, *n*=8, R=H hypsiziprenol A_{12}
e: *m*=1, *n*=9, R=H hypsiziprenol A_{13}
f: *m*=1, *n*=10, R=H hypsiziprenol A_{14}
2a: *m*=2, *n*=3, R=H hypsiziprenol B_8
b: *m*=2, *n*=4, R=H hypsiziprenol B_9
c: *m*=2, *n*=5, R=H hypsiziprenol B_{10}
d: *m*=2, *n*=7, R=H hypsiziprenol B_{12}
3a: *m*=3, *n*=3, R=H hypsiziprenol C_9
4a: *m*=1, *n*=4, R=OH hypsiziprenol AA_8
b: *m*=1, *n*=5, R=OH hypsiziprenol AA_9
c: *m*=1, *n*=8, R=OH hypsiziprenol AA_{12}
d: *m*=1, *n*=9, R=OH hypsiziprenol AA_{13}
e: *m*=1, *n*=10, R=OH hypsiziprenol AA_{14}
5a: *m*=2, *n*=5, R=OH hypsiziprenol BA_{10}

compd	deprotonated molecular ion[a] $[M-H]^-$	protonated molecular ion[b] $[M+H]^+$	$[MH-(H_2O)_n]^+$	$[MH-(H_2O)_{n+1}]^+$	structurally significant fragment ions[a]	fraction
1a[c]	651	653	545 ($n=6$)		297, 383, 469	2, 7
1b[c]	737	739	613 ($n=7$)		383, 469, 555	3, 4, 5, 6, 7
1c[c]	823	825	681 ($n=8$)		383, 469	8, 9
1d[c]	995	997	817 ($n=10$)		383, 469, 555	11, 12
1e[c]	1081	1083	885 ($n=11$)		383, 469, 555, 641, 727	11, 12, 13
1f[c]	1167	1169	953 ($n=12$)			13
2a[c]	633	635	545 ($n=5$)			1
2b[c]	719	721	613 ($n=6$)			2
2c[c]	805	807	681 ($n=7$)			3
2d[c]	977	979	817 ($n=9$)			9, 10
3a[c]	701	703	613 ($n=5$)			1
4a	667	669	561 ($n=6$)	543	383, 469	7, 8, 9
4b	753	755	629 ($n=7$)	611	383, 469	7, 8, 9
4c	1011	1013	833 ($n=10$)	815	383, 469, 555, 641, 727	14
4d	1097	1099	901 ($n=11$)	883	383, 469, 555, 641, 727	14
4e	1183	1185	969 ($n=12$)	951	383, 469, 555, 641, 727	14
5a	821	823	697 ($n=7$)	679	383, 469, 555	6, 7

[a] Negative ion mode. [b] Positive ion mode. [c] Previous work (Sawabe et al., 1996).

under vacuum, applied to a silica gel column (Wako gel C-300, Wako Pure Chemical Industries Ltd.), and chromatographed with chloroform−methanol (first with C:M = 9:1 and then with C:M = 5:1 v/v). The eluate was divided into 14 fractions (200 mL each), and each fraction (Fr.) was concentrated under vacuum. Yields (mg): Fr. 1, 16; Fr. 2, 54; Fr. 3, 84; Fr. 4, 48; Fr. 5, 22; Fr. 6, 18; Fr. 7, 4; Fr. 8, 4; Fr. 9, 2; Fr. 10, 2; Fr. 11, 94; Fr. 12, 58; Fr. 13, 18; Fr. 14, 86. The compounds in fractions 1−14 gave strong molecular ions in both the positive and negative ion mode MS. On the basis of the molecular mass information as well as subsequent TLC analyses, fractions 6 and 14 were found to contain only one major component; these fractions were analyzed by NMR.

Biological Activity Test of Polyisoprenepolyols. *(a) Cell Line and Culture Condition.* NCI-H292 human lung mucoepidermoid carcinoma and EL-4 mouse lymphoma cell line were used. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Biowhittaker) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in an atmosphere containing 5% CO_2 in air and 100% relative humidity. For subculture, adherent cells were detached using a 0.125% trypsin and 0.01% ethylenediamine tetraacetic acid (EDTA) mixture.

(b) Reagents. Test compounds, polyisoprenepolyols (fractions 4, 6, 12, and 14) were dissolved in dimethyl sulfoxide (DMSO) at 0.1 M. Positive antitumor compounds, mitomycin C (MMC) and actinomycin D (AMD), were dissolved in distilled water to give 10 mM. All compounds were stored at −80 °C. The antitumor activity (Jost et al., 1992; Geromichalos et al., 1996) was determined using XTT reagents, Cell Proliferation Kit II (Boehringer Mannheim). XTT reagents were freshly prepared before use. This assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by dehydrogenase activity in active mitochondria.

(c) Antitumor Activity. NCI-H292 and EL-4 cells were inoculated onto a 96-well microculture plate at a seeding density of 5000−10 000 cells per well. After inoculation, test compounds, MMC or AMD, were added to the well to give a final concentration of 1 μM. Cells were incubated for 72 h, and XTT reagents (final concentration 0.33 mg/mL) were added to each well. At 4 h after addition of XTT reagents, absorbance was measured with a spectrophotometric plate reader at 490/655 nm wavelength. Antitumor activities of the compound in test fraction were determined by measuring specific absorbance to quantify cell growth inhibition and calculated by the following equation: (test compounds OD/intact control OD) × 100.

Spectroscopy. *(a) FAB Mass Spectrometry.* MS was carried out with a JEOL JMS-HX 100 double-focusing mass spectrometer of *EB* geometry that was connected to a JEOL DA 5000 data system. The mass spectrometer was fitted with a high-field magnet, a FAB ion source, and a postaccelerating detector. The sample was dissolved in methanol (1 μg/μL), and 1 μL of the solution was added to the matrix (glycerol for both the positive ion and the negative ion modes) on the stainless steel FAB probe target. The sample was bombarded with a 6 keV xenon atom beam. The exact mass measurement by FAB/MS was carried out using a mixture of cesium iodide, sodium iodide, and glycerol (5:1:25, w/w/w) as mass calibrants. For linked scan analysis with constant *B/E*, collisional activation was performed in a collision chamber in the first field-free region using helium as the collision gas.

(b) NMR Spectroscopy. NMR spectra were recorded in deuterated methanol (compounds **4c**−**e**, fraction 14) and in

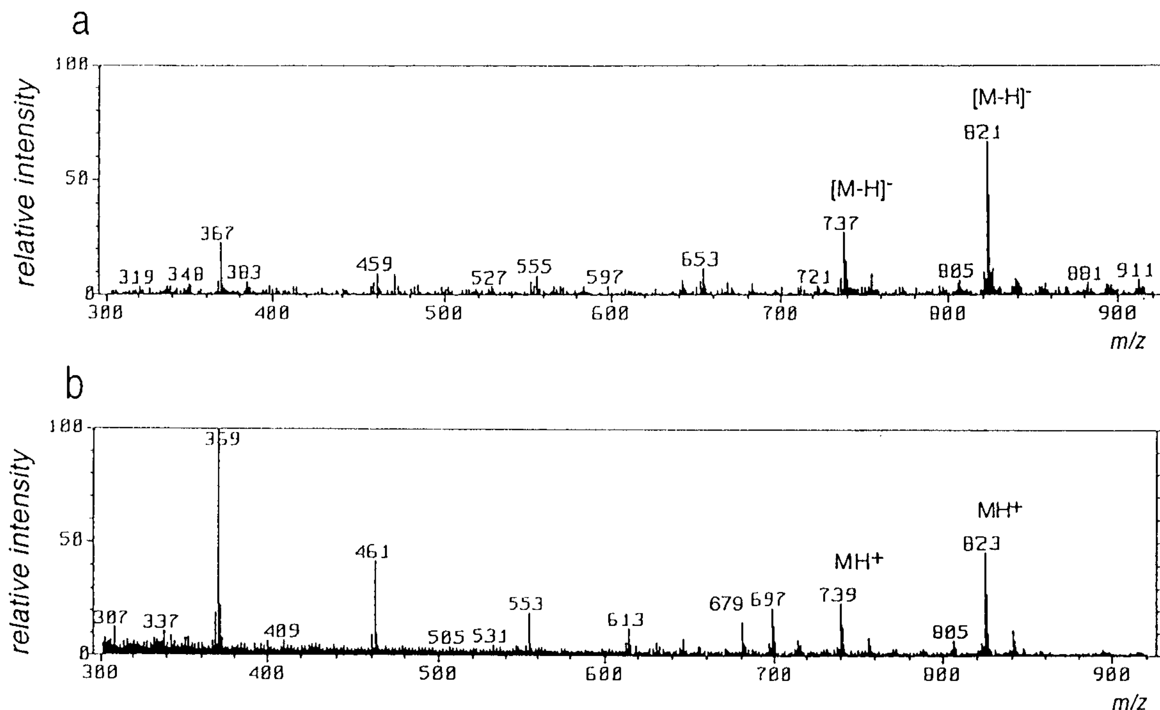


Figure 2. FAB mass spectra of fraction 6: (a) negative ion mode (matrix: glycerol); (b) positive ion mode (matrix: glycerol).

deuterated chloroform (compounds **1b**, **5a**, fraction 6) using a 270 MHz JEOL JNM-EX 270 spectrometer.

Compounds 1b and 5a (Fraction 6). ^{1}H NMR ($CDCl_3$): δ 1.25 (3H, s), 1.27 (15H, s), 1.38 (3H, s), 1.43−1.59 (36H, br s), 1.71 (9H, s), 1.78 (3H, s), 2.08−2.20 (8H, m), 2.28−2.41 (2H, m), 4.08 (1H, t, J = 6.5 Hz), 5.16 (1H, dd, J = 10.5, 1.5 Hz), 5.17 (1H, t, J = 7 Hz), 5.22 (1H, t, J = 7 Hz), 5.31 (1H, dd, J = 17, 1.5 Hz), 5.48 (1H, t, J = 7 Hz), 6.01 (1H, dd, J = 17, 10.5 Hz) ppm.

Compounds 4c−e (Fraction 14). ^{1}H NMR (MeOD): δ 1.14 (30H, s), 1.25 (3H, s), 1.35 (3H, s), 1.40−1.47 (58H, br s), 1.50−1.60 (2H, m), 1.58 (3H, s), 1.62 (3H, s), 1.67 (3H, s), 1.95−2.12 (4H, m), 3.86 (1H, t, J = 6 Hz), 5.03 (1H, dd, J = 11, 1.5 Hz), 5.11 (1H, t, J = 7 Hz), 5.19 (1H, dd, J = 17, 1.5 Hz), 5.35 (1H, t, J = 7 Hz), 5.91 (1H, dd, J = 17, 11 Hz). ^{13}C NMR (MeOD): δ 11.23 (q), 17.75 (q), 19.41 (t), 23.32 (t), 23.70 (t), 25.95 (q), 26.95 (q), 27.67 (q), 30.20 (t), 38.85 (t), 42.66 (t), 43.07 (t), 43.29 (t), 43.45 (t), 73.13 (s), 73.33 (s), 73.47 (s), 73.58 (s), 73.80 (s), 79.35 (d), 112.13 (t), 125.84 (d), 127.60 (d), 132.02 (s), 138.09 (s), 146.25 (d) ppm.

RESULTS AND DISCUSSION

A crude mixture of polyisoprenepolyols was obtained from a hot water−ethanol extract of *H. marmoreus* by a procedure reported previously (Sawabe et al., 1996) and separated into 14 fractions by column chromatography over an Amberlite XAD-2 and silica gel. The positive and negative FAB/MS data of these fractions are summarized in Table 1. The FAB/MS data indicated that the polyols occur as a mixture of homologous polyisoprenes with 40−70 carbon atoms and that a new type of the isoprene chain is involved in association with some of these compounds. This new type of polyisoprenepolyol features a terminal olefin rather than a free terminal hydroxy group in gymnoprenol. We have named these new polyisoprenepolyols hypsiziprenols. Now, we have found another new type of polyisoprenepolyols in the same mushroom, which has vicinal hydroxy groups in addition to the hypsiziprenol structure. The structural elucidation is described in detail in the following section.

Table 2. Detection of the Exact Mass of Molecular Ion and Fragment Ions of Fractions 6 and 14

obsd mass (error, milli mass units)	expected mass	elemental formula	FAB mode	compd[a]
739.6452 (0.4)	739.6456	$C_{45}H_{87}O_7$	positive ion	**1b**
823.7027 (1.8)	823.7045	$C_{50}H_{95}O_8$	positive ion	**5a**
613.5712 (2.4)	613.5736	$C_{45}H_{73}$	positive ion	**1b**
697.6287 (−2.5)	697.6262	$C_{50}H_{81}O$	positive ion	**5a**
679.6182 (4.3)	679.6225	$C_{50}H_{79}$	positive ion	**5a**
1011.8439 (2.1)	1011.8460	$C_{60}H_{115}O_{11}$	negative ion	**4c**
1097.9171 (−1.1)	1097.9160	$C_{65}H_{125}O_{12}$	negative ion	**4d**
1183.9903 (5.7)	1183.9960	$C_{70}H_{135}O_{13}$	negative ion	**4e**
833.7540 (0.3)	833.7543	$C_{60}H_{97}O$	positive ion	**4c**
815.7434 (−2.9)	815.7405	$C_{60}H_{95}$	positive ion	**4c**
901.8166 (2.4)	901.8190	$C_{65}H_{105}O$	positive ion	**4d**
883.8060 (−1.1)	883.8049	$C_{65}H_{103}$	positive ion	**4d**
969.8791 (−3.2)	969.8759	$C_{70}H_{113}O$	positive ion	**4e**
951.8685 (2.7)	951.8712	$C_{70}H_{111}$	positive ion	**4e**

[a] Assignments of compounds in Table 1.

A new type of polyisoprenepolyols is found in fractions 6 (**5a**) and 14 (**4c−e**), which seemed to contain a single component as determined by TLC and NMR analyses.

Structural Elucidation of Polyisoprenepolyols 1b and 5a. Positive and negative ion FAB mass spectra of fraction 6 are shown in Figure 2a,b), respectively. The molecular weights can be determined from intense peaks of [M − H]$^-$ at *m/z* 737 and 821 in Figure 2a. The positive ion mode FAB mass spectrum of fraction 6 (Figure 1b) indicated the presence of protonated molecules at *m/z* 739 and 823 with characteristic ion peaks at *m/z* 613, 679, and 697. The exact masses of these molecular ions determined by the positive ion mode are listed in Table 2. The *B/E* constant linked scan spectra of deprotonated molecules and protonated molecules were measured for the purpose of confirming each characteristic ion in detail. The structure of the compound having a peak at *m/z* 737 in the negative ion spectrum (Figure 2a) was assigned by *B/E* constant linked scan spectra of deprotonated molecule at *m/z* 737 in the negative ion mode (Figure 3c) and that of

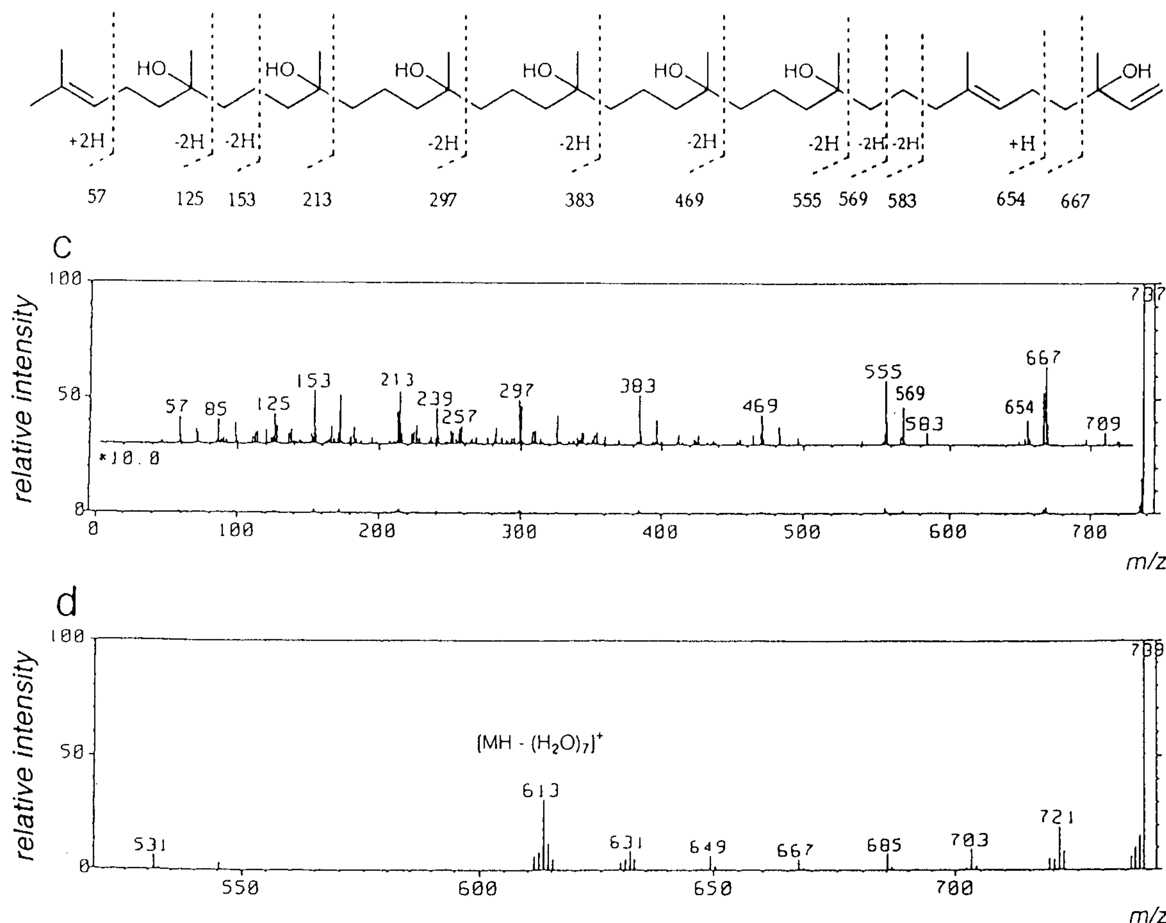


Figure 3. *B/E* constant linked scan spectrum of fraction 6: (c) deprotonated molecule at *m/z* 737 in negative ion mode; (d) protonated molecule at *m/z* 739 in positive ion mode.

protonated molecule at *m/z* 739 in the positive ion mode (Figure 3d) to be hypsiziprenol A_9 (Table 1, **1b**), which had been isolated from the same mushroom in our previous study (Sawabe et al., 1996). The *B/E* constant linked scan spectrum of the deprotonated molecule showed a peak at *m/z* 821 in the negative ion FAB mass spectrum (Figure 4e) and the results coincide with the proposed structures of **5a** in Table 1. The spectrum of deprotonated molecule at *m/z* 821 in Figure 4e indicated the fragment ions at *m/z* 751, 733, 681, 653, 555, 469, 383, 325, 213, and 167. The isoprenoid sequence in **5a** was thus confirmed by the *B/E* constant linked scan method. The *B/E* constant linked scan spectrum of the protonated molecule at *m/z* 823 in positive ion FAB mass spectrum clearly elucidated the successive loss of water molecules as shown in Figure 4f. HR-FAB/MS analyses of the peaks at *m/z* 697 and 679 (Table 2) suggested the elemental composition $C_{50}H_{81}O$ and $C_{50}H_{79}$, which corresponds to $[MH - (H_2O)_7]^+$ and $[MH - (H_2O)_8]^+$, respectively.

On the basis of the evidence of positive and negative ion FAB mass spectrometry and NMR spectroscopy, the structure of polyisoprenepolyol (MW 822) of fraction 6 was determined to be that shown in Table 1 (**5a**), which is here named as hypsiziprenol BA_{10}.

Furthermore, as for the structural characteristics of polyisoprenepolyols having vicinal hydroxy groups by FAB/MS, two dehydration ion peaks are observed.

Structural Elucidation of Polyisoprenepolyols 4c–e. Positive and negative ion FAB mass spectra of fraction 14 are shown in Figure 5a,b. The molecular weights can be determined from intense peaks of $[M - H]^-$ at *m/z* 1011, 1097, and 1183 in Figure 5a. The exact masses of these molecular ions determined by the negative ion mode are listed in Table 2. The ^{13}C NMR analysis of fraction 14 exhibited prominent signals at δ19.41 (t), 26.95 (q), 43.45 (t), and 73.47 (s), indicating the presence of polyisoprenoid partial structures, together with many weak signals, suggesting the presence of two trisubstituted double bonds [δ 125.84 (d), 127.60 (d), 132.02 (s), and 138.09 (s)] and terminal olefinic carbons [δ 112.13 (t) and 146.25 (d)], similar to the compounds reported in the previous paper (Sawabe et al., 1996). Thus, the loss of 86 u moieties giving rise to peaks at *m/z* 813, 727, 641, 555, 469, and 383 was rationalized as loss of $C_5H_{10}O$ units from isoprenoid partial structures. The *B/E* constant linked scan spectrum of the deprotonated molecular ion at *m/z* 1011, 1097, and 1183 in the negative ion FAB mass spectra and the results presented above supported the proposed structures of **4c–e** in Table 1. Moreover, the exact masses of the ions at *m/z* 833 and 815, *m/z* 901 and 883, and *m/z* 969 and 951 in the positive ion mode (Table 2) indicate the elemental formulas of $C_{60}H_{97}O$ and $C_{60}H_{95}$, $C_{65}H_{105}O$ and $C_{65}H_{103}$, and $C_{70}H_{113}O$ and $C_{70}H_{111}$, respectively. The characteristic fragment ions at *m/z* 833 and 815, *m/z* 901 and 883, and *m/z* 969 and 951 in the positive ion spectrum of fraction 14 were thus assigned to be $[MH - (H_2O)_{10}]^+$ for an ion of *m/z* 833 and $[MH - (H_2O)_{11}]^+$ for an ion of *m/z* 815, $[M'H - (H_2O)_{11}]^+$ for an ion of *m/z* 901 and $[M'H - (H_2O)_{12}]^+$ for an ion of *m/z* 883, and $[M''H - (H_2O)_{12}]^+$ for an ion of *m/z* 969 and

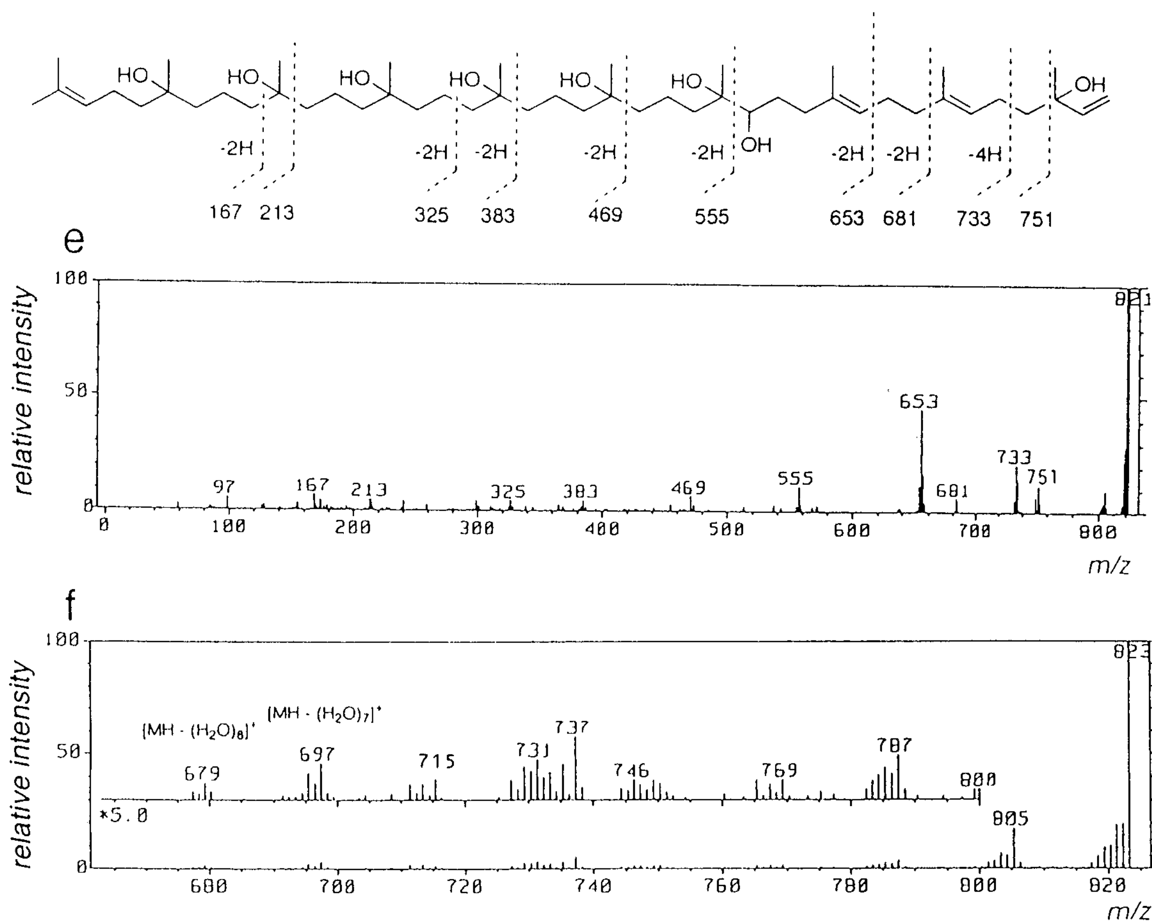


Figure 4. *B/E* constant linked scan spectrum of fraction 6: (e) deprotonated molecule at *m/z* 821 in negative ion mode; (f) protonated molecule at *m/z* 823 in positive ion mode.

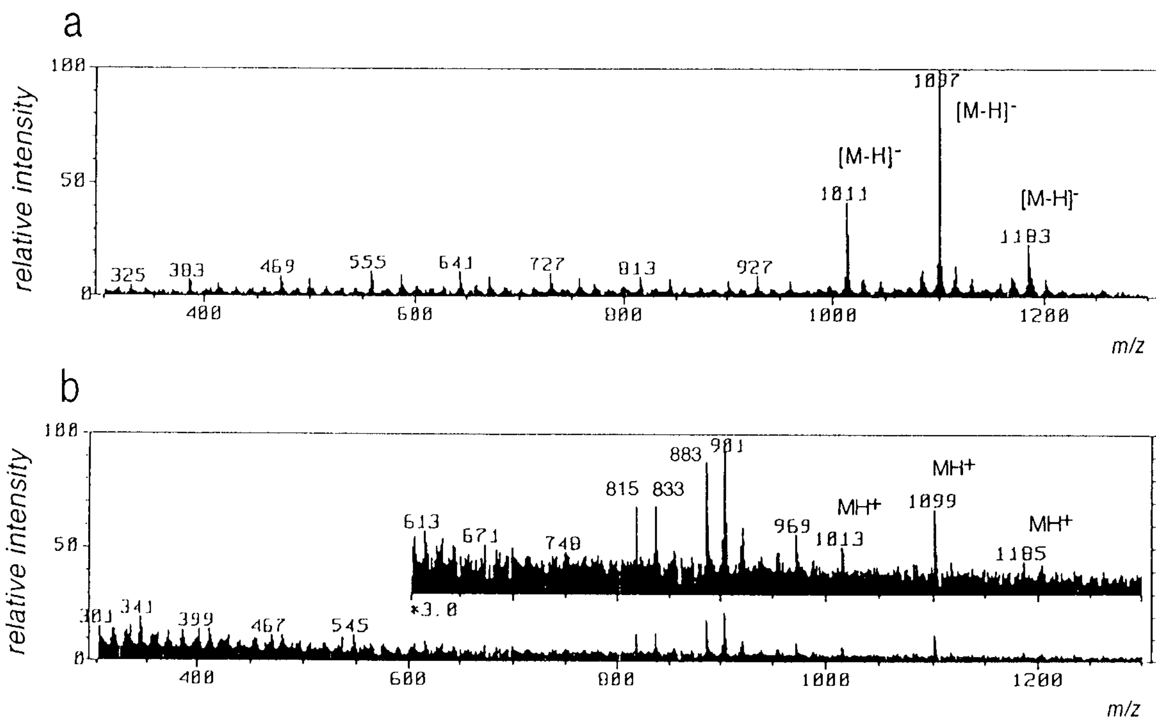


Figure 5. FAB mass spectra of fraction 14: (a) negative ion mode (matrix: glycerol); (b) positive ion mode (matrix: glycerol).

$[M''H - (H_2O)_{13}]^+$ for an ion of *m/z* 951, respectively. From these couples of dehydration ion peaks, these compounds are suggested to be polyisoprenepolyols having vicinal hydroxy groups.

On the basis of the evidence of positive and negative ion FAB mass spectrometry and NMR spectroscopy, the structures of these polyisoprenepolyols (MW 1012, 1098, and 1184) of fraction 14 were determined to be those

Table 3. Antitumor Activity of Polyisoprenpolyols As Determined with NCI-H292 and EL-4 Cell Lines

compd	% of intact control NCI-H292	EL-4
fraction 4	91.3 ± 1.9	100.5 ± 3.2
fraction 6	92.9 ± 1.4	100.3 ± 0.9
fraction 12	95.8 ± 1.7	98.5 ± 0.6
fraction 14	97.8 ± 1.4	95.7 ± 1.8
actinomycin D	2.6 ± 0.2	4.1 ± 0.5
mitomycin C	35.7 ± 2.9	35.5 ± 0.9

[a] Each datum represents the mean value ($n = 3$) ± SE of these experiments.

shown in Table 1 (**4c**−**e**), which are here named as hypsiziprenols AA_{12}, AA_{13}, and AA_{14}, respectively.

Antitumor Activities on NCI-H292 and EL-4 Cell Lines. The antitumor activity of polyisoprenepolyols was examined by using NCI-H292 human lung mucoepidermoid carcinoma and EL-4 mouse lymphoma cell lines. Since it is known that gymnopilins have an antitumor activity (Nozoe 1991a,b), fraction 4 (hypsiziprenol A_9, **1b**), fraction 6 (a mixture of hypsiziprenol A_9 (**1b**) and hypsiziprenol BA_{10} (**5a**)), fraction 12 (a mixture of hypsiziprenol A_{12} (**1d**) and hypsiziprenol A_{13} (**1e**)), and fraction 14 (a mixture of hypsiziprenol AA_{12} (**4c**), hypsiziprenol AA_{13} (**4d**), and hypsiziprenol A_{14} (**4e**)) obtained here were subjected to the tests for antitumor activity. However, these fractions had no significant antitumor activity (Table 3).

CONCLUSIONS

FAB mass spectrometry is a powerful tool for analyzing the structure of polyisoprenepolyols, because it provides data on the number of isoprenoid units present, which NMR analyses could not. The FAB/MS data indicate that the polyols occur as homologous polyisoprenes with different numbers of carbon atoms, some of which have a new type of the isoprene chain. The characteristic fragment ions of $[MH - (H_2O)_n]^+$ and $[MH - (H_2O)_{n+1}]^+$ in the positive ion mode are found to be very useful for the structural determination of polyisoprenepolyols such as hypsiziprenols (Table 1, **1**−**5**) in the analyses by FAB mass spectrometry, because the number of hydroxy groups present can be estimated from ion peaks. The isoprenoid sequences in polyisoprenepolyols are clearly determined by the *B/E* constant linked scan method.

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