Chem. Pharm. Bull. 37(9) 2438—2440 (1989) Vol. 37, No. 9

Anti-plasmin Inhibitor. V.[1] Structures of Novel Dimeric Eckols Isolated from the Brown Alga *Ecklonia kurome* OKAMURA[2]

Yoshiyasu FUKUYAMA,*,[a] Mitsuaki KODAMA,[a] Iwao MIURA,[b] Ziunei KINZYO,[b,3] Hideo MORI,[b] Yasuo NAKAYAMA,[b] and Masayuki TAKAHASHI[b]

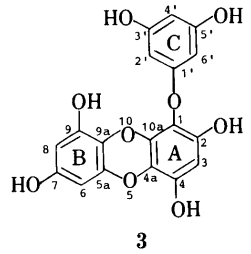
Faculty of Pharmaceutical Sciences, Tokushima Bunri University,[a] Yamashiro-cho, Tokushima 770, Japan and Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd.,[b] Kagasuno, Kawauchi-cho, Tokushima 771-01, Japan. *Received February 16, 1989*

6,6′-Bieckol (1), 2-*O*-(2,4,6-trihydroxyphenyl)-6,6′-bieckol (4), and 8,8′-bieckol (2), bispolyphenols with a dibenzo-1,4-dioxin skeleton, have been isolated as potent anti-plasmin inhibitors from the brown alga *Ecklonia kurome* OKAMURA. Their structures have been determined to be dimers of eckol linked at the C-6 or C-8 positions, through an aryl-aryl bond on the basis of spectral data. Their inhibitory actions on anti-plasmins (α_2-macroglobulin and α_2-plasmin inhibitor) and some proteases have been examined.

Keywords 6,6′-bieckol; 8,8′-bieckol; 2-*O*-(2,4,6-trihydroxyphenyl)-6,6′-bieckol; eckol; phlorotannin; *Ecklonia kurome*; negative NOE; anti-plasmin; α_2-macroglobulin; α_2-plasmin inhibitor

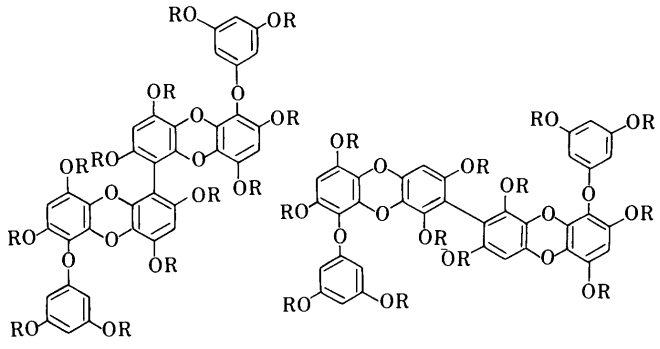
In the preceding papers,[4,5] we described the isolation and structure elucidation of eckol (**3**), 2-phloroeckol (**5**), and dieckol, belonging to a new type of phlorotannins,[6] from the brown alga *Ecklonia kurome* OKAMURA. Among them, in particular, eckol has been shown to be not only a potent and specific anti-plasmin inhibitor but also a possible lead compound for a new class of anti-thrombotic agents or potentiators of thrombolytic enzymes such as urokinase.[1] Our continuing search for more potent anti-plasmin inhibitors in the methanol extract of *E. kurome* has resulted in the isolation of novel dimeric eckols, named 6,6′-bieckol, 8,8′-bieckol,[7] and 2-*O*-(2,4,6-trihydroxyphenyl)-6,6′-bieckol, through bioassay-directed fractionation. In this paper, we deal with the structure elucidation of these new compounds and their properties as anti-plasmin inhibitors.

6,6′-Bieckol (**1**), mp >300 °C, exhibits a quasimolecular ion peak due to $(M^+ + Na)$ at *m/z* 765 and a molecular ion peak at *m/z* 742 in the field desorption mass spectrum (FDMS), giving the molecular formula $C_{36}H_{22}O_{18}$. The MS of **1** also showed a typical fragment peak at *m/z* 232 derived from the basic dibenzodioxin unit of eckol. The infrared (IR) spectrum of **1** revealed the presence of a hydroxy group (3300 cm^{-1}) and an aromatic nucleus (1600 cm^{-1}), but had not carbonyl absorption. The ^1H nuclear magnetic resonance (^1H-NMR) spectrum contained signals characteristic of five aromatic protons, an AB_2 system at δ 5.81 (1H, t, J=2.1 Hz) and 5.72 (2H, d, J=2.1 Hz), and two singlet signals at δ 6.05 and 6.12 as well as six phenolic hydroxy protons at δ 8.63, 9.07, 9.13, 9.15 (2H), and 9.27. The presence of six phenolic hydroxy groups was confirmed by the formation of a fully acetylated derivative (**1a**) (δ 1.98, 2.03, 2.05, 2.12, 2.27×2). These spectral feature indicated that **1** should be a symmetrical dimer of eckol formed by an aryl–aryl linkage. A comparison of the ^1H-NMR data of **1** with those of **3** (Table I) revealed that a pair of *meta* coupled signals (H-6, 8) existing in **3** was replaced with the singlet signal at δ 6.05 in **1**, and two phenolic hydroxy proton signals at δ 8.63 and 9.07 were found to appear at higher fields than the remaining ones. These spectral differences implied that **1** is dimerized at C-6 of eckol. This proposal was supported by the examination of the negative nuclear Overhauser effect (NOE).[8] Strong negative NOEs were observed for the aromatic resonance (H-3) at δ 6.12 (s)



3

Fig. 1



1 : R=H
1a : R=Ac

2 : R=H
2a : R=Ac

Fig. 2

TABLE I. ^1H-NMR Spectral Data[a] for **1**, **2**, **3**, and **4**

Proton	1	2	3	4	
3	6.12 s	6.17 s	6.16 s	5.80 s	6.10 s
6		5.97 s	5.82 d (2.7)[b]		
8	6.05 s		5.98 d (2.7)	6.04 s	6.07 s
2′,6′	5.72 d (2.1)	5.75 d (2.1)	5.75 d (2.2)	5.91 d (2.1)	5.75 d (2.1)
4′	5.81 t (2.1)	5.81 t (2.1)	5.83 t (2.2)	5.91 d (2.1)	5.75 d (2.1)
3″,5″				5.80 s	
2-OH	9.13 s	9.18 s	9.19 s		9.11 s
4-OH	9.07 s	9.46 s	9.48 s	9.04 s	9.07 s
7-OH	8.63 s	7.92 s	9.20 s	8.63 s	8.64 s
9-OH	9.27 s	8.78 s	9.53 s	9.23 s	9.26 s
3′,5′-OH	9.15 s	9.14 s	9.17 s	9.11 s	9.12 s
2″,6″-OH				9.02 s	
4″-OH				8.93 s	

a) In DMSO-d_6. *b*) *J*/Hz in parentheses.

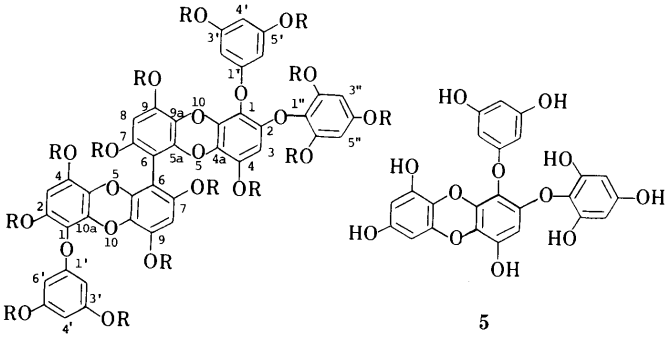
upon selective irradiations of the phenolic hydroxy protons at δ 9.07 and 9.13. In addition, irradiation of the highest-field located phenolic hydroxy proton at δ 8.63 led to a decrease in the intensity of the other aromatic signal at δ 6.05 (s), for which distinct negative NOE was also detected upon irradiation of the phenolic hydroxy proton at δ 9.27. These results indicated that the symmetric dimeric structure of **1** was formed through a direct aryl–aryl bond at C-6 in eckol, and the high-field shifts of the C_4-OH and C_7-OH proton signals (δ 9.07 and 8.63) could be reasonably rationalized as being due to a diamagnetic anisotropy effect caused by an aromatic ring linked at C-6. This structure was confirmed by the ^{13}C-NMR data of **1** (Table II). Namely, the chemical shifts for all the carbon signals in **1** were almost identical with those in eckol (**3**) except for the signals due to the carbons on the ring B. The quaternary carbon signal which had newly appeared at δ 99.8 could be assigned to C-6[9] and thus it could be rationalized as being due to an additive effect of an aromatic ring coupled at C-6 that the signals for C-5a, C-7, and C-9 were shifted to higher field than the corresponding ones in **3**. Thus, the structure of 6,6′-bieckol can be represented as **1**.

8,8′-Bieckol (**2**), mp >300 °C, failed to show a molecular ion peak in the MS, but its pertetramethylsilyl (TMS) derivative showed the highest ion peak at *m/z* 1606 in the electron impact mass spectrum (EIMS) and thereby the molecular formula $C_{32}H_{22}O_{18}$ was estimated coupled with the EIMS data of a fully acetylated derivative (**2a**). In fact, the ^{1}H- and ^{13}C-NMR spectra of **2** (Tables I and II) resembled those of **1** except for the chemical shift values due to ring B. This means that **2** is another symmetrical dimer of eckol. Among six phenolic proton signals, two proton signals wer observed at abnomally high field (δ 7.92 and 8.78), presumably owing to an anisotropy effect caused by an aromatic nucleus. The new singlet aromatic resonance (δ 5.97) showed strong negative NOE upon irradiation of the hydroxy signal at δ 7.92 at the highest field, whereas selective irradiation of the hydroxy signal at δ 8.78 had no effect on the aromatic proton signals. This evidence indicated that **2** should have the dimeric eckol structure linked at C-8 *via* an aryl–aryl bond. This structure was supported by the comparison of its ^{13}C-NMR data with those of **3** (Table II). The newly appeared quaternary carbon signal (δ 104.5) could be assigned to C-8,[9] and thereby the chemical shift values for C-5a (−1.2 ppm), C-7 (−1.2 ppm), and C-9 (−1.5 ppm) were well coincident with the corresponding ones of eckol, taking account of an additive effect derived from the benzene ring linked at C-8. Accordingly, the structure of 8.8′-bieckol was determined to be **2**.

2-*O*-(2,4,6-Trihydroxyphenyl)-6,6′-bieckol (**4**) has the molecular formula $C_{42}H_{26}O_{21}$ estimated from the quasi-molecular ion peak ($M^+ + Na$) at *m/z* 889 and the molecular ion peak at *m/z* 866 in the FDMS coupled with its ^{13}C-NMR spectral data. The IR (3300 cm^{-1}) and ^{1}H-NMR (Table I) spectra of **4** revealed the presence of fourteen phenolic hydroxy groups, which was verified by the formation of a tetradecaacetate (**4a**), and its ^{1}H-NMR spectrum contained signals due to twelve aromatic protons, *viz.*, a pair of AB_2 type system [δ 5.91 (2H, d, J=2.1 Hz) and 5.84 (1H, t, J=2.1 Hz); δ 5.75 (2H, d, J=2.1 Hz), 5.80 (1H, t, J=2.1 Hz)] and an A_2 type signal at δ 5.80 (2H, s) as well as four singlet signals [δ 5.80, 6.10, 6.04, and 6.07]. These spectral features suggested that **4** is an eckol-type phlorotannin made up from seven phloroglucinols. The ^{13}C-NMR data of **4** showed the appearance of thirty signals, which, however, should include twelve overlaps on the basis of the molecular formula. Having the number of carbons (forty-two) and the multiplicities of the carbon signals in mind, the ^{13}C-NMR data of **4** were rearranged into two columns (Table II). All the data for **4** in the right column were almost identical with those of 6,6′-bieckol (**1**), whereas the chemical shift values in the left column corresponded well to those of

TABLE II. ^{13}C-NMR Spectral Data (in DMSO-d_6) for **1**, **2**, **3**, and **4**

Carbon	1	2	3	4	5
1	123.9	123.5	123.5	122.8, 123.9	122.9
2	146.3	146.2	145.9	147.4, 146.5	147.6
3	98.0	98.4	98.4	96.1, 98.0	96.2
4	141.7	141.9	141.9	141.6, 141.7	141.5
4a	122.4	122.7	122.6	122.3, 122.3	122.6
5a	141.4	141.5	142.7	141.5, 141.5	142.0
6	99.8	94.0	94.0	99.9, 99.8	93.9
7	151.2	151.8	153.0	151.5, 151.4	153.0
8	98.1	104.5	98.7	98.1, 98.1	98.7
9	144.4	144.6	146.1	144.6, 144.6	146.0
9a	123.0	123.3	122.9	123.0, 123.0	122.7
10a	137.3	137.3	137.3	137.4, 137.4	137.3
1′	160.4	160.5	160.4	160.6, 160.5	160.4
2′,6′	94.0	94.1	94.0	94.3, 94.1	94.2
3′,5′	158.7	158.8	158.7	158.9, 158.9	158.6
4′	96.3	96.5	96.4	96.2, 96.2	96.4
1″				125.0	124.9
2″,6″				151.2	151.0
3″,5″				95.0	94.9
4″				154.8	154.7



4 : R=H
4a : R=Ac

5

Fig. 3

TABLE III. Inhibitory Activities (IC_{50}, μg/ml) of 6,6′-Bieckol (**1**), 8,8′-Bieckol (**2**), Eckol (**3**), and 2-*O*-(2,4,6-Trihydroxyphenyl)-6,6′-bieckol (**4**) on α_2-Macroglobulin (α-M), α_2-Plasmin Inhibitor (α-PI), and on Some Proteases[a]

Compound	α-M	α-PI	Plasmin	Trypsin	Thrombin
1	2.0	0.5	23	56	11
2	2.0	0.7	32	>100	32
3	2.5	1.6	>100	>100	13
4	1.9	0.7	13		

a) Assay was carried out according to the previously reported methods.[10]

2-phloroeckol (**5**)[4] isolated from the title plant except for C-5a (−0.5 ppm), C-6 (6 ppm), C-7 (−1.5 ppm), C-8 (−0.6 ppm), and C-9 (−1.4 ppm). These chemical shift differences between **4** and **5**, however, could be reasonably explained in terms of an electronic alteration of ring B caused by the linkage of an aromatic ring at C-6, which was found to be a quaternary carbon (δ 99.9 and 99.8) from the off-resonance ^{13}C-NMR spectrum. The evidence mentioned above indicated that **4** has a dimeric structure formed between eckol (**3**) and 2-phloroeckol (**5**) *via* an aryl–aryl linkage at C-6. Thus, 2-*O*-(2,4,6-trihydroxyphenyl)-6,6′-bieckol (**4**) was elucidated as 6,6′-bieckol having an extra unit of phloroglucinol linked at C-2 through an ether bond.

Although a number of phlorotannins having an aryl–aryl bond are known,[6] 6,6′-bieckol, 8,8′-bieckol, and 2-*O*-(2,4,6-trihydroxyphenyl)-6,6′-bieckol are the first example of them bearing a 1,4-dioxane ring linked by an aryl–aryl bond. Compounds **1**, **2**, and **4** exhibited inhibitory activities toward both α_2-macroglobulin (α-M) and α_2-plasmin inhibitor (α-PI) having potencies almost identical with those of eckol itself (Table III). However, these dimeric compounds inhibited the activity of plasmin, which is a key enzyme in fibrinolysis, at concentrations as low as IC_{50} = 23, 32, and 13 μg/ml, respectively. From the thrombolytic point of view, this feature is likely to be disadvantageous.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Ultraviolet (UV) spectra were recorded on a Hitachi 340 spectrophotometer. IR spectra were measured with a Jasco A-202 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained at 400 MHz (^{1}H-NMR) and 100.16 MHz (^{13}C-NMR), respectively, using a Bruker WH 400 spectrometer. Chemical shifts were expressed in δ (ppm) downfield from tetramethylsilane as an internal standard.

Extraction and Purification Fresh whole plants (600 kg) of *Ecklonia Kurome* OKAMURA collected in Irino, Kochi prefecture, were immersed in methanol at room temperature for 6 d. The methanol was evaporated off *in vacuo* to give a gummy extract, which was partitioned between EtOAc and water. The course of purification was monitored by assay of the inhibitory activity on the action of α_2-macroglobulin against plasmin. The activity was concentrated into the EtOAc fraction (IC_{50} = 250 μg/ml). The EtOAc soluble portion (1.7 kg) mixed with Celite (3.4 kg) was dried under reduced pressure. The obtained solids were pulverized, packed into a glass column, and eluted successively with benzene (18 l), methylene chloride (36 l), ether (54 l), and methanol (20 l). The fraction (IC_{50} = 150 μg/ml, 552 g) eluted with ether was subjected to Sephadex LH-20 (3.5 kg) chromatography. Each fraction (2 l) eluted with acetone was collected, and the fourth fraction was evaporated *in vacuo* to yield eckol (**3**) (40 g) as crystals. The sixth fraction (150 g) was rechromatographed on Sephadex LH-20 (3.5 kg) with methanol (20 l) and the eluant was divided into six fractions. The fr. 1 was evaporated *in vacuo* to afford 6,6′-bieckol (**1**) (10 g) as crystals. The frs. 4—6 were rechromatographed on Sephadex LH-20 (3 kg) with methanol (20 l) to give 8,8′-bieckol (**2**) (10 g) as crystals and 2-*O*-(2,4,6-trihydroxyphenyl)-6,6′-bieckol (**4**) (1 g) as an amorphous material.

6,6′-Bieckol (1) Colorless prisms (from H_2O), mp >300 °C. FDMS *m/z*: 765 (M^++Na), 742 (M^+), 618 (M^+−124), 618, 600, 494, 232. UV λ_{max}^{MeOH} nm (ε): 233, 295 (11000). IR ν_{max}^{KBr} cm^{-1}: 3300, 1600, 1480. ^{1}H- and ^{13}C-NMR: see Tables I and II.

6,6′-Bieckol Dodecaacetate (1a) A mixture of **1** (100 mg), acetic anhydride (0.2 ml), and pyridine (0.5 ml) was allowed to stand at room temperature for 24 h. The reaction mixture was poured onto crushed ice and the precipitate formed was collected by filtration and recrystallized from ethanol to afford **1a** (120 mg) as colorless plates, mp >300 °C. EIMS *m/z* (rel. int.): 1204 (M^+−42, 16), 1162 (35), 1120 (46), 1078 (45), 1036 (17), 994 (14), 952 (10), 910 (13), 868 (5), 826 (2), 741 (3), 562 (15), 478 (12), 43 (100). IR ν_{max}^{KBr} cm^{-1}: 3075, 2925, 1760, 1600. ^{1}H-NMR ($CDCl_3$) δ: 1.98 (3H, s), 2.03 (3H, s), 2.05 (3H, s), 2.12 (3H, s), 2.27 (6H, s), 6.62 (1H, s), 6.64 (2H, d, J=2.2 Hz), 6.69 (1H, s), 6.72 (1H, t, J=2.2 Hz).

8,8′-Bieckol (2) Light brown prisms (from H_2O), mp >300 °C. EIMS of a TMS derivative *m/z* (rel. int.): 1606 (M^+, 25), 803 (24). UV λ_{max}^{MeOH} nm (ε): 231 (6300), 246, 295 (8800). IR ν_{max}^{KBr} cm^{-1}: 3250, 1595, 1465. ^{1}H- and ^{13}C-NMR: see Tables I and II.

8,8′-Bieckol Dodecaacetate (2a) A mixture of **2** (100 mg), acetic anhydride (1 ml), and pyridine (2 ml) was allowed to stand at room temperature overnight. The reaction mixture was poured onto crushed ice and the precipitate was collected by filtration and recrystallized from ethanol to yield **2a** (115 mg) as colorless plates, mp 230—232 °C. EIMS *m/z* (rel. int.): 1162 (M^+−84, 8), 1120 (12), 1078 (12), 1036 (10), 994 (6), 952 (3), 43 (100). IR ν_{max}^{KBr} cm^{-1}: 3075, 2925, 1760, 1595. ^{1}H-NMR ($CDCl_3$) δ: 1.76 (3H, s), 2.02 (3H, s), 2.12 (3H, s), 2.25 (6H, s), 2.34 (3H, s), 6.56 (2H, d, J=2.2 Hz), 6.66 (1H, s), 6.70 (1H, s), 6.70 (1H, t, J=2.2 Hz).

2-*O*-(2,4,6-Trihydroxyphenyl)-6,6′-bieckol (4) Colorless amorphous. FDMS *m/z*: 889 (M^++Na), 866 (M^+). λ_{max}^{MeOH} nm (ε): 234 (sh), 244 (sh), 295 (11000). IR ν_{max}^{KBr} cm^{-1}: 3300, 1605, 1460, 1250, 1140, 1080, 1000, 810. ^{1}H- and ^{13}C-NMR: see Tables I and II.

2-*O*-(2,4,6-Trihydroxyphenyl)-6,6′-bieckol Tetradecaacetate (4a) A mixture of **4** (100 mg), acetic anhydride (0.6 ml), and pyridine (1 ml) was allowed to stand at room temperature for 20 h. The reaction mixture was poured onto crushed ice and then extracted with ether. The ether layer was washed with 2 N HCl and brine, and dried over $MgSO_4$. Evaporation of the solvent afforded **4a** (110 mg) as an amorphous powder. EIMS *m/z*: 1454 (M^+), 1412, 1370, 1328, 1286, 1244, 1202, 1160. ^{1}H-NMR ($CDCl_3$) δ: 1.93 (3H, s), 1.97 (3H, s), 2.01 (6H, s), 2.04 (6H, s), 2.05 (3H, s), 2.06 (3H, s), 2.12 (3H, s), 2.26 (3H, s), 2.27 (12H, s), 6.25 (1H, s), 6.60 (1H, s), 6.64 (2H, d, J=2.2 Hz), 6.67 (2H, s), 6.65 (2H, d, J=2.2 Hz), 6.71 (4H, t, J=2.2 Hz), 6.88 (2H, s).

Anti-α_2-macroglobulin Activity α-M (17 μg) was preincubated with a test substance at 37 °C for 20 min and then residual activity of α-M was determined using plasmin (0.6 unit) or trypsin (5 μg) as a protease by the caseinolytic method.[10]

Anti-α_2-plasmin Inhibitor Activity α-PI (3 μg) was preincubated with a test substance at 37 °C for 20 min and then 0.05 unit of plasmin in 0.1 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 25% glycerin (v/v) was added. The mixture was incubated at 37 °C for 30 s, and then 0.1 ml of 3 mM S-2251 was added. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 0.1 ml of 50% acetic acid and the absorbance of the reaction mixture at 405 nm was determined. The percentage inhibition was calculated as follows: $[(a-b)/(c-b)] \times 100$, where a is the absorbance with α-PI and test substance, b is that with α-PI but without test substance, and c is that without α-PI and test substance.[5]

Acknowledgments We thank Prof. M. Ochi, Kochi University for his helpful discussion on the structure determination, and are indebted to Dr. M. Nishikawa and Mr. M. Inoue for the identification and collection of *E. kurome*.

References and Notes

1) Part IV: Y. Nakayama, M. Takahashi, Y. Fukuyama, and Z. Kinzyo, *Agric. Biol. Chem.*, in press.
2) A part of this study was presented at the 26th Symposium on the Chemistry of Natural Products, Kyoto, October 1983.
3) Present address: *Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Ooehonmachi, Kumamoto 862, Japan.*
4) Y. Fukuyama, I. Miura, Z. Kinzyo, H. Mori, M. Kido, Y. Nakayama, M. Takahashi, and H. Ochi, *Chem. Lett.*, **1985**, 739.
5) Y. Fukuyama, M. Kodama, I. Miura, Z. Kinzyo, M. Kido, H. Mori, Y. Nakayama, and M. Takahashi, *Chem, Pharm. Bull.*, **37**, 349 (1989).
6) K.-W. Glombitza, "Marine Natural Products Chemistry," ed. by D. J. Faulkner and W. H. Fenical, Plenum Press, New York, 1971, p. 191.
7) 6,6′-Bieckol and 8,8′-bieckol were isolated as their peracetylated derivatives from *Eisenia arborea*. K.-W. Glombitza and G. Gerstberger, *Phytochemistry*, **24**, 543 (1985).
8) J. R. Kalman and D. H. Williams, *J. Am. Chem. Soc.*, **102**, 906 (1980).
9) Assignments for all the carbon signals were confirmed by LSPD (long-range selective proton decoupling).
10) Y. Nakayama, K. Matsui, A. Kato, and M. Takahashi, *Agric. Biol. Chem.*, **49**, 993 (1985).