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Sequential glucosylation determined by NMR in the biosynthesis of mulberroside D, a *cis*-oxyresveratrol diglucoside, in *Morus alba* L. cell cultures

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Abstract. Three new diglucosides, mulberrosides D, E, and F, were isolated from *Morus alba* cell cultures and their structures were established by spectroscopy. In the biosynthesis of mulberroside D, a cinnamoylpolyketide-derived *cis*-oxyresveratrol diglucoside in the cell cultures, the different ratio of ^{13}C-enrichment between two glucose moieties of mulberroside D, revealed by administering [U-$^{13}C_6$]-D-glucose and [1,3-$^{13}C_2$]glycerol to the cell cultures, suggested that two glucosylation steps in the biosynthesis of the glucoside occur with different time courses.
Key words. *Morus alba* cell cultures; oxyresveratrol; cinnamoylpolyketide; glucoside; biosynthesis; glucosylation.

Morus alba callus and cell suspension cultures biosynthesize cinnamoyl-polyketide-derived chalcones and 2-arylbenzofurans with a prenyl group in high yields [1]. Simultaneous incorporation of [1-^{13}C]-L-phenylalanine and [3-^{13}C]-L-tyrosine into the cinnamoyl moiety of the chalcone derivative revealed the contribution of the two amino acids in the shikimate pathway [2, 3]. The dimer of the prenylchalcone derivative such as kuwanon J [4] in *M. alba* cell cultures has been found to be formed through an intermolecular Diels-Alder type reaction [5]. Furthermore, it was found that two glucose catabolic pathways, the Embden-Meyerhof-Parnas pathway and the pentose phosphate cycle, participate in the biosynthesis of the the prenyl moiety [6, 7]. This paper describes the structural determination of three new diglucosides, mulberrosides D, E, and F isolated from *Morus alba* cell cultures, and the biosynthesis of mulberroside D in the cell cultures.

Materials and methods

The induction of *Morus alba* callus cultures from seedlings or mulberry leaves has been described previously [1]. *Morus alba* cells suspended in sterile water (1 l) were incubated for 7 days at 25 °C in the dark. After harvest and lyophilization, the lyophilized cells (3.2 g) were extracted with methanol (100 ml). The methanol solution was concentrated in vacuo to give a residue (0.65 g), which was extracted with acetone (50 ml) and the acetone-soluble and insoluble portions were separated. The acetone-insoluble portion (0.32 g) was subjected to high-pressure liquid chromatography (HPLC) with 70% methanol in water as solvent at a flow rate of 2 ml/min (column, Senshu Pak ODS-4251-N, 10 mm ∅ × 250 mm). The eluate was monitored at 286 nm using an ultraviolet (UV) detector (Senshu Scientific Co., SSC 3000-B), and three new components, mulberrosides D (**1**, retention time, 26 min, 8 mg), E (**2**, retention time, 22 min, 2 mg) and F (**3**, retention time, 33 min, 3 mg), along with known compound, mulberroside A (**4**, retention time, 18 min, 4 mg) [8], were obtained (fig. 1). The structures of these components were examined by spectroscopic methods. The fast-atom bombardment (FAB) mass spectra were measured with a JEOL JMS DX-303 double-focusing mass spectrometer with JMA-DA 5000 data system, and the nuclear magnetic resonance (NMR) data were recorded on a JEOL JNM EX-400 FTNMR spectrometer at ambient temperature using methanol-d_4 as a solvent and tetramethyl silane (TMS) as an internal standard. All the material isolated was dissolved in about 0.7 ml of the solvent for the NMR experiment. Complete assignments were performed by two dimensional (2D) NMR techniques including heteronuclear correlation. Optical rotations were recorded on a JASCO DIP-370 polarimeter.

[1,3-$^{13}C_2$]glycerol (99 atom % ^{13}C) and [U-$^{13}C_6$]-D-glucose (99 atom % ^{13}C) were purchased from Isotec Inc. (USA). [1,3-$^{13}C_2$]glycerol (99 atom % ^{13}C) was administered to the *M. alba* cell cultures as follows. The *M. alba* cells were suspended in sterilized water (1 l) to which a 10% ethanol solution (10 ml) of [1,3-$^{13}C_2$]glycerol (99 atom % ^{13}C, 132.7 mg) was added. After incubation of the cell suspension for 7 days at 25 °C in the dark, the cells were harvested and lyophilized. Mulberroside D (**1**) was prepared from the lyophilized cells in an analogous way as described above; 8 mg was obtained from the lyophilized cells (3.05 g). An analogous feeding experiment with [U-$^{13}C_6$]-D-glucose (99 atom % ^{13}C, 170 mg) was carried

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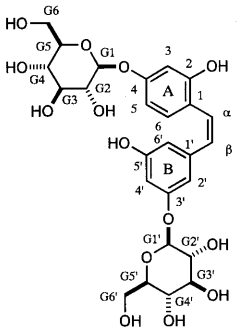

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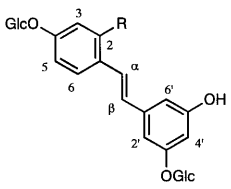
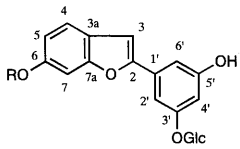


2 : R = H
4 : R = OH



3 : R = Glc
5 : R = H

Figure 1. Diglucosides from *Morus alba* cell cultures.

out under the same conditions as with [1,3-$^{13}C_2$]glycerol. Mulberroside D (20 mg) was obtained from the lyophilized cells (5.4 g). Incorporation of the precursor into mulberroside D (**1**) was estimated by comparing the ^{13}C signal intensity of **1** from the feeding experiment with that of naturally occurring **1**.

Results and discussion

Mulberroside D (**1**), $[\alpha]_D^{22}$ − 76°, was obtained as a white amorphous powder and its molecular formula was determined to be $C_{26}H_{33}O_{14}$ {$(M+H)^+$, m/z 569.1864} from the high-resolution FABMS spectrum. The ^1H NMR spectrum of **1** (δ ppm) showed the proton signals due to the oxyresveratrol moiety [δ 7.01 (1H, d, $J=8$ Hz, C-6-H), 6.59 (1H, d, $J=2$ Hz, C-3-H), 6.45 (1H, dd, $J=2$ and 8 Hz, C-5-H), 6.52 (1H, br d, $J=2$ Hz, C-2′-H), 6.38 (1H, br d, $J=2$ Hz, C-6′-H), 6.34 (1H, t, $J=2$ Hz, C-4′-H), 6.55 (1H, d, $J=12$ Hz, C-α-H), 6.40 (1H, d, $J=12$ Hz, C-β-H)] and two glycosyl moieties [δ 4.88 (1H, d, $J=7$ Hz, G1-H), 4.56 (1H, d, $J=7$ Hz, G1′-H), 3.10–3.95 (12 H, m)]. These data suggested **1** to be an oxyresveratrol diglycoside like mulberroside A (**4**). Assignments of the protons in the ^1H NMR spectrum as well as the carbons in the ^{13}C NMR spectrum (table 1) were performed by 2D ^1H-^1H correlation spectroscopy (COSY) and 2D ^{13}C-^1H COSY. The 2D nuclear Overhauser effect shift correlation spectroscopy (NOESY) demonstrated the correlation between one of two anomeric protons at δ 4.56 ppm (G1′-H) and two aromatic protons at δ 6.34 (C-4′-H) and 6.52 ppm (C-2′-H), as well as between the other anomeric proton at δ 4.88 ppm (G1-H) and two aromatic protons at δ 6.45 (C-5-H) and 6.59 (C-3-H) ppm, indicating that the two glycosyl residues are linked with different aromatic rings. The value of the coupling constant ($J=12$ Hz) between two olefinic protons at δ 6.55 and 6.40 ppm, less than that between the relevant protons of mulberroside A (**4**, $J=16$ Hz), indicated that mulberroside D is a geometric isomer of **4**. The ^{13}C-NMR spectrum also supported this suggestion (table 1). Furthermore, photo-irradiation of the methanol solution of **4** with a 100 W high-pressure mercury lamp gives mulberroside D in 90% yield. Thus the molecular structure of mulberroside D was represented by the formula **1**.

Mulberroside E (**2**), $[\alpha]_D^{22}$ − 75°, was obtained as a white amorphous powder and its molecular formula was determined to be $C_{26}H_{32}O_{13}Na$ {$(M+Na)^+$, m/z 575.1743} by the high-resolution FABMS spectrum. The signal pattern of **2** in the ^1H NMR spectrum was closely similar to that of **4**, except the A_2B_2 type of signal in the A ring of **2**, indicating that **2** is a resveratrol diglucoside [resveratrol moiety: δ 7.46, 7.08 (each 2H, d, $J=8$ Hz, C-2 and 6-H, C-3 and C-5-H), 6.81, 6.63 (each 1H, br d, $J=2$ Hz, C-6′ and 2′-H), 6.46 (1H, t, $J=2$ Hz, C-4′-H), 7.06, 6.93 (each 1H, d, $J=16$ Hz, C-α and β-H); two glucosyl moieties: δ 4.89, 4.92 (each 1H, d, $J=7$ Hz), 3.30–3.95 (12H, m)]. The value of the coupling constant ($J=16$ Hz) between two olefinic protons at δ 7.06 and 6.93 ppm indicated that **2** has a *trans*-stilbene skeleton. The 2D NOESY spectrum indicated the correlation between one of two anomeric protons at δ 4.89 ppm (G1′-H) and two aromatic protons at δ 6.46 (C-4′-H) and 6.81 ppm (C-2′-H) as well as between the other anomeric proton at δ 4.92 (G1-H) and two aromatic protons at δ 7.08 ppm (C-3 and 5-H), indicating that the two glucose residues are linked with different aromatic rings. The ^{13}C NMR spectrum of **2** supported the formula **2** for the structure of mulberroside E (table 1).

Mulberroside F (**3**), $C_{26}H_{30}O_{14}$, $[\alpha]_D^{22}$ − 75°, is a diglucoside of a 2-arylbenzofuran derivative. The molecular formula was determined by the FABMS {$(M+Na)^+$, m/z 589.1510}. The ^1H NMR spectrum of **3** disclosed the presence of a 2-arylbenzofuran moiety [δ 7.46 (1H, d, $J=8$ Hz, C-4-H), 7.32 (1H, br d, $J=2$ Hz, C-7-H), 7.05 (1H, dd, $J=2$ and 8 Hz, C-5-H), 7.05 (1H, s,

Table 1. ^{13}C-NMR chemical shifts (ppm).

Solvent a; CD_3OD c; DMSO-d_6
b, The multiplicities by ^{13}C-^{13}C spin-spin coupling were observed in [U-$^{13}C_6$]-D-glucose-derived **1**

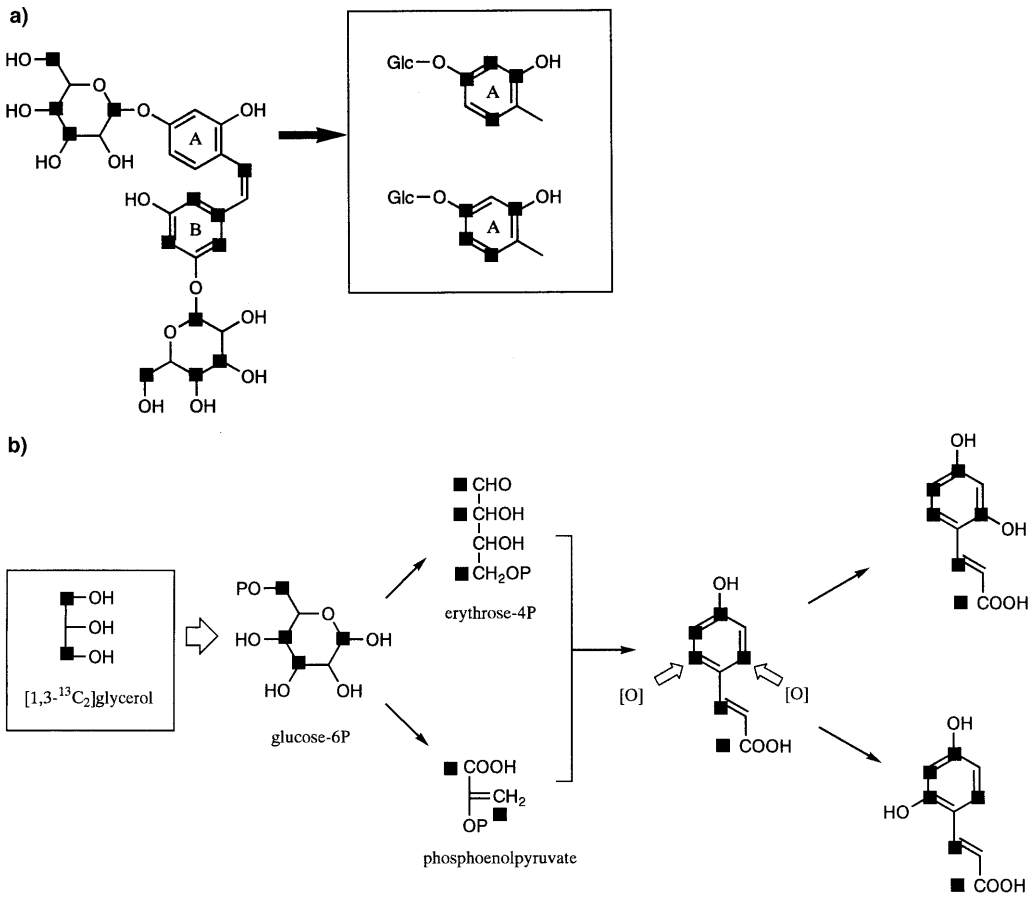


Figure 2. (*a*) ^{13}C-labelling pattern of [1,3-$^{13}C_2$]glycerol-derived **1**. (*b*) Two independent ^{13}C-labelling patterns in the shikimate-derived aromatic ring caused by hydroxylation at two different isotopic carbons.

C-3-H), 6.96, 7.11 (each 1H, br s, C-2′ and 6′-H), 6.56 (1H, t, $J = 2$ Hz, C-4′-H)] and two glycosyl moieties [δ 4.93, 4.95 (each 1H, d, $J = 7$ Hz), 3.30–3.95 (12H, m)]. The 2D NOESY spectrum demonstrated the correlation between one of two anomeric protons at δ 4.93 ppm (G1′-H) and two aromatic protons at δ 6.56 (C-4′-H) and 7.11 ppm (C-2′-H) as well as between the other anomeric proton at δ 4.95 ppm (G1-H) and two aromatic protons at δ 7.05 (C-5-H) and 7.32 ppm (C-7-H), indicating that the two glycosyl residues are linked with different aromatic rings. Comparison of the ^{13}C NMR spectrum of **3** with that of moracin M-3′-O-β-glucopyranoside (**5**) [9] revealed that **3** is moracin M-6, 3′-di-O-β-glucopyranoside (**3**) (table 1). Thus the structures of the three new glucosides in the *M. alba* cell cultures were established by spectroscopic evidence.

On the other hand, the biosynthetic intermediate of both the chalcone and the 2-arylbenzofuran skeletons has been found to be a cinnamoyl-polyketide [10], while that of the stilbene skeleton in the cell cultures is not yet clear. In order to clarify the biosynthetic route of the stilbene derivative in the *M. alba* cell cultures, mulberrosides D (**1**) resulting from [1,3-$^{13}C_2$]glycerol and [U-$^{13}C_6$]-D-glucose were examined by ^{13}C NMR spectroscopy.

The ^{13}C-NMR spectrum of mulberroside D (**1**) obtained from feeding with [1,3-$^{13}C_2$]glycerol (99% atom ^{13}C) revealed the precursor of the stilbene skeleton to be a cinnamoylpolyketide as in the case of the chalcone and the 2-arylbenzofuran skeletons (figs 2a and 2b). The ^{13}C-labelling pattern of the stilbene skeleton of **1** was the same as that of the 2-arylbenzofuran skeleton in an analogous experiment [6, 7]. The ^{13}C-labelling pattern of the A ring demonstrated that the aromatic ring is formed through the shikimate pathway. However, two independent ^{13}C-labelling patterns are observed in the aromatic ring (fig. 2a). This can be attributed to oxidation at two different isotopic carbons. Namely, oxidation at the two different isotopic carbons gives rise to the formation of two independent ^{13}C-labelling patterns (fig. 2b). The ^{13}C-labelling pattern of the B ring disclosed the aromatic ring to originate from a triketide intermediate. Thus the biosynthesis of the stilbene skeleton in *M. alba* cell cultures was confirmed to be formed through the aldol-type condensation of a cinnamoyl-polyketide followed by decarboxylation (fig. 3) [10]. On the other hand, four enhanced signals in both the glucose moieties of **1** demonstrated intact incorporation of two molecules of [1,3-$^{13}C_2$]glycerols into the moiety. This result indicates that [1,3-$^{13}C_2$]glycerol was trans-

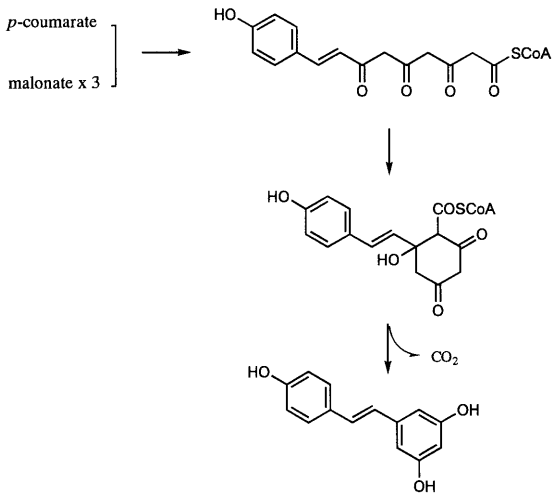


Figure 3. Biosynthesis of the stilbene derivative in the *M. alba* cell cultures.

formed into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, which converted into fructose 1,6-diphosphate to give glucose 1-phosphate for the glycosylation.

In an analogous experiment with [U-$^{13}C_6$]-D-glucose (99 atom % ^{13}C), the ^{13}C-labelling pattern of **1** on the basis of the ^{13}C-^{13}C coupling constant showed the biosynthesis of the stilbene skeleton comprising the shikimate pathway and the malonate pathway (table 1 and fig. 4). Two independent ^{13}C-labelling patterns in the shikimate-derived aromatic ring are due to hydroxylation at two different isotopic carbons, as was observed in the experiment with [1,3-$^{13}C_2$]glycerol. Furthermore, the ^{13}C-labelling of the erythrose 4-phosphate moiety composing the shikimate-derived aromatic ring revealed that about 50% of the C-1 aldehyde carbon and the penultimate carbon are disconnected (fig. 4). This disconnectivity in the erythrose 4-phosphate could arise through glucose metabolism via two triose phosphates, glyceraldehyde 3-phosphate and dihydroxyacetone 3-phosphate. Both trioses formed from [U-$^{13}C_6$]-D-glucose participate in the formation of sedoheptulose 7-phosphate, which gives erythrose 4-phosphate with the C-3 + C-1 type of ^{13}C-labelling [6, 7]. Two independent ^{13}C-labelling patterns in the triketide-derived aromatic ring can be attributed to glucosylation at two different isotopic positions. On the other hand, the ^{13}C-labellings in both the glucose moieties also showed two independent patterns, in one of which intact glucose is administered and the other glucose is reconstructed from triose. The 4- and 4′-carbon signals of the glucose moieties are accompanied with satellite peaks based on ^{13}C-^{13}C spin-spin coupling (fig. 5). In the satellite peaks, a double doublet type of satellite peak comes from intact [U-$^{13}C_6$]-D-glucose administered, and a doublet type of satellite peak originates from the glucose reconstructed from the two trioses which are metabolites of [U-$^{13}C_6$]-D-glucose. The formation of the latter type of glucose is compatible with the result in the case of [1,3-$^{13}C_2$]glycerol. The abundance ratio of these glucoses is about 1:1 from the intensities of satellite peaks due to ^{13}C-^{13}C spin-spin coupling. Thus, at least two pathways might be involved in the formation of glucose 1-phosphate for the glucosylation in the *Morus alba* cell cultures. Furthermore, the satellite peaks at the 4- and 6-carbons were clearly more intense than those at the 4′- and 6′-carbons (fig. 5), showing that the ^{13}C-incorporation from the precursor into the glucose moiety attached to the A ring was higher than that of one attached to the B ring. This result revealed that the two steps of the glucosylations in the formation of mulberroside D (**1**) occur with different time courses, first in the A ring and next in the B ring. Similar results were also observed in the ^{13}C NMR spectrum of [1,3-$^{13}C_2$]glycerol-derived **1**. This is the first example of

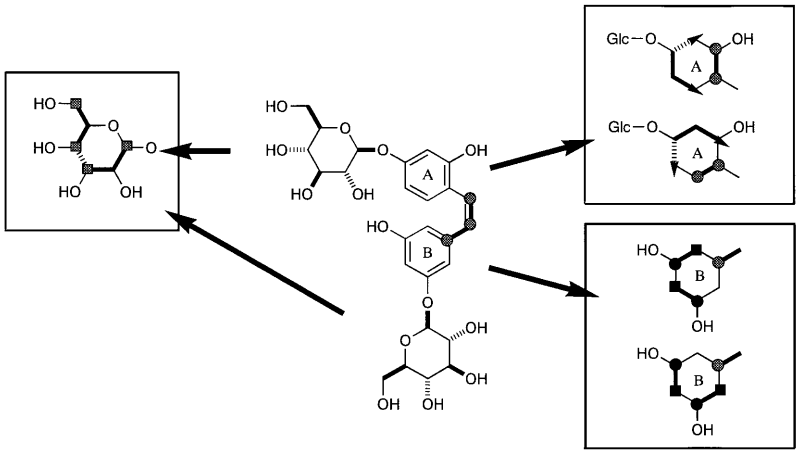


Figure 4. ^{13}-C-labelling pattern of [U-$^{13}C_6$]-D-glucose-derived **1**. : D-glucose (containing a 50% $C_3 + C_3$ type of glucose). ( ): pyruvate unit. : acetate unit. : erythrose unit (containing a 50% $C_3 + C_3$ type of erythrose).

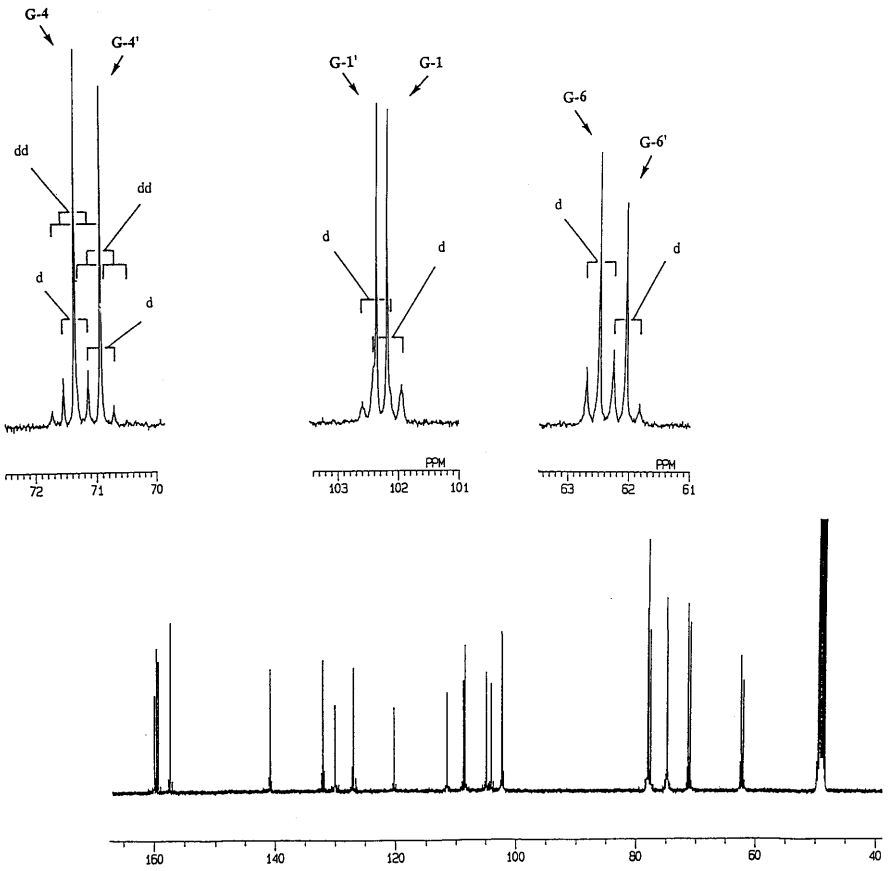


Figure 5. ^{13}C-NMR spectrum of [U-$^{13}C_6$]-D-glucose-derived **1**.

determining the order of glycosylations from ^{13}C-enrichment using a ^{13}C-labelled precursor. Thus mulberroside D (**1**) has been found to be biosynthesized through the aldol-type condensation of cinnamoyl-polyketide followed by the two steps of glucosylations with different time courses.

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