DUAL *p*-COUMAROYL CoA BIOSYNTHESIS IN *MORUS ALBA* CELL CULTURES

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Abstract - *Morus alba* callus and cell suspension cultures specifically produce chalcomoracin (**1**) and kuwanon J (**2**) both originated from cinnamoylpolyketide intermediate. Administration of [2-^{13}C]cinnamic acid *N*-acetylcysteamine thioester to the *M. alba* cell cultures revealed the cinnamoyl CoA intermediate to be a significant precursor. The present result, coupled with the parallel contribution of both L-phenylalanine and L-tyrosine to the same cinnamoyl part, gave a conclusive evidence for the dual route leading to *p*-coumaroyl CoA in the *M. alba* cell cultures.

Morus alba callus and cell suspension cultures specifically produce chalcones, 2-arylbenzofurans, and stilbenes with one or more isoprenyl units[1-3] and all the compounds are biosynthetically formed from cinnamoylpolyketide intermediate.[4,5] Among them, chalcomoracin (**1**)[1,4] and kuwanon J (**2**)[1] are the most characteristic components of the cell cultures (Figure 1). These compounds have been found to be biosynthesized through enzymatic intermolecular Diels-Alder type reaction that the isoprenyl portion of the isoprenylated 2-arylbenzofuran (or chalcone) acts as a diene and the α, β-double bond of the other chalcone functions as a dienophile.[6] On the other hand, simultaneous incorporation of both L-phenylalanine and L-tyrosine, amino acid precursors on the shikimate pathway, into the same cinnamoyl parts of chalcomoracin (**1**) suggested that the *M. alba* cell cultures operate the dual route leading to *p*-coumaroyl CoA by way of L-phenylalanine and L-tyrosine, respectively.[7,8] Such parallel contribution of the amino acid precursors to

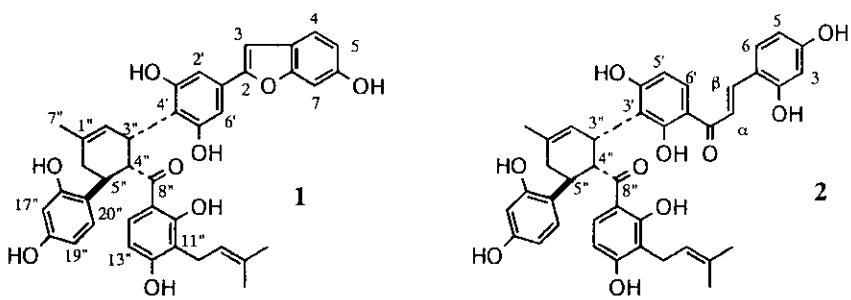

Figure 1 Characteristic components of *Morus alba* cell cultures

the same carbon framework is quite uncommon in higher plants, unlike mammalian cells operating direct hydroxylation of L-phenylalanine to L-tyrosine. In higher plants, the direct hydroxylation has been insignificantly found in *Salvia splendens*,[9] *Triticum vurgare*,[10] *Fagopyrum tataricum*,[10] and spinach leaves.[11] In order to corroborate the *p*-coumaroyl CoA biosynthesis in the *M. alba* cells, cinnamic acid *N*-acetylcysteamine thioester equivalent to cinnamoyl CoA intermediate was administered to the cell cultures. This paper deals with an evidence for the dual route leading to *p*-coumaroyl CoA in the shikimate pathway of the *M. alba* cell cultures.

The *M. alba* cells were suspended in sterile water, to which 10 % ethanol solution of [2-^{13}C]cinnamic acid (99.1 % atom ^{13}C, 70 mg) was added. After incubation of the cell suspension for 7 days at 25 °C in the dark, the cells were harvested and lyophilized. The lyophilized cells were extracted with methanol to afford methanol extract, and then the methanol extract was divided into acetone soluble and insoluble portions. The acetone soluble portion was subjected to column chromatography over silica gel with chloroform increasing amount of acetone as a mobile phase. Preparative thin-layer chromatography of the fraction eluted with chloroform - acetone (2 : 1) followed by HPLC (silica gel) afforded chalcomoracin (**1**) and kuwanon J (**2**). The ^{13}C NMR spectra of **1** and **2** obtained from the feeding experiment with [2-^{13}C]cinnamic acid showed no ^{13}C signal enhancement in both the compounds, indicating that the precursor was not involved in the shikimate pathway in the *Morus alba* cell cultures. Plausible explanation for the non-involvement of the exogenous precursor in the cell cultures may lie in the segregation of protein-bound intermediates from free intermediates.[12] On the other hand, several successful incorporations of biomimics for pivotal precursors, such as thioester derivative of *N*-acetylcysteamine, into end product have been reported in the biosynthetic studies of fatty acid,[13] macrolide antibiotics,[14,15] signal molecule in *Streptomyces* .[16] In an effort to verify the shikimate pathway in the *M. alba* cells, [2-^{13}C]cinnamic acid *N*-acetylcysteamine thioester (99.1 atom % ^{13}C, 40 mg) was administered to the cell cultures. Chalcomoracin (**1**, 24 mg) and kuwanon J (**2**, 4 mg) were obtained from this experiment. The ^{13}C NMR spectra of the resulting chalcomoracin (**1**) and kuwanon J (**2**) displayed that the thioester derivative was incorporated intact into the expected positions of **1** and **2** with 4 % and 1.5 % of the ^{13}C enrichment, respectively (Charts 1-a and -b, Figure 2). This result reinforced that both **1** and **2** are originated from two molecules of a cinnamoylpolyketide intermediate, one of which undergoes a Claisen-type condensation to give a chalcone skeleton and the other through an aldol-type condensation followed by decarboxylation to yield a 2-arylbenzofuran skeleton.[4] Furthermore, the result, coupled with well incorporation of L-phenylalanine into the cinnamoyl parts of **1** and **2**,[7,8] gave a confirmative evidence for the route from L-phenylalanine to *p*-coumaroyl CoA by way of cinnamoyl CoA in the *M. alba* cells. This is consistent with the report that L-phenylalanine is converted to *trans*-cinnamate followed by 4-hydroxylation reaction to give rise to *p*-coumarate by the action of cinnamate 4-hydroxylase in higher plants.[17] Thus, the route from L-phenylalanine to *p*-coumarate was confirmed. On the other hand, well incorporation[7,8] of L-tyrosine into the cinnamoyl parts of **1** and **2** neccesarily allowed to deduce the other route from L-tyrosine to *p*-coumarate in the *M. alba* cell cultures. The overlapping of the carbon framework from L-phenylalanine with that from L-tyrosine may be strictly restricted in higher plants, as has been observed in the biosynthesis of rosmarinic acid in *Mentha arvensa* and *Mentha piperita*.[18,19]

Our previous works[7,8] and the present study by NMR method revealed the dual route from L-phenylalanine

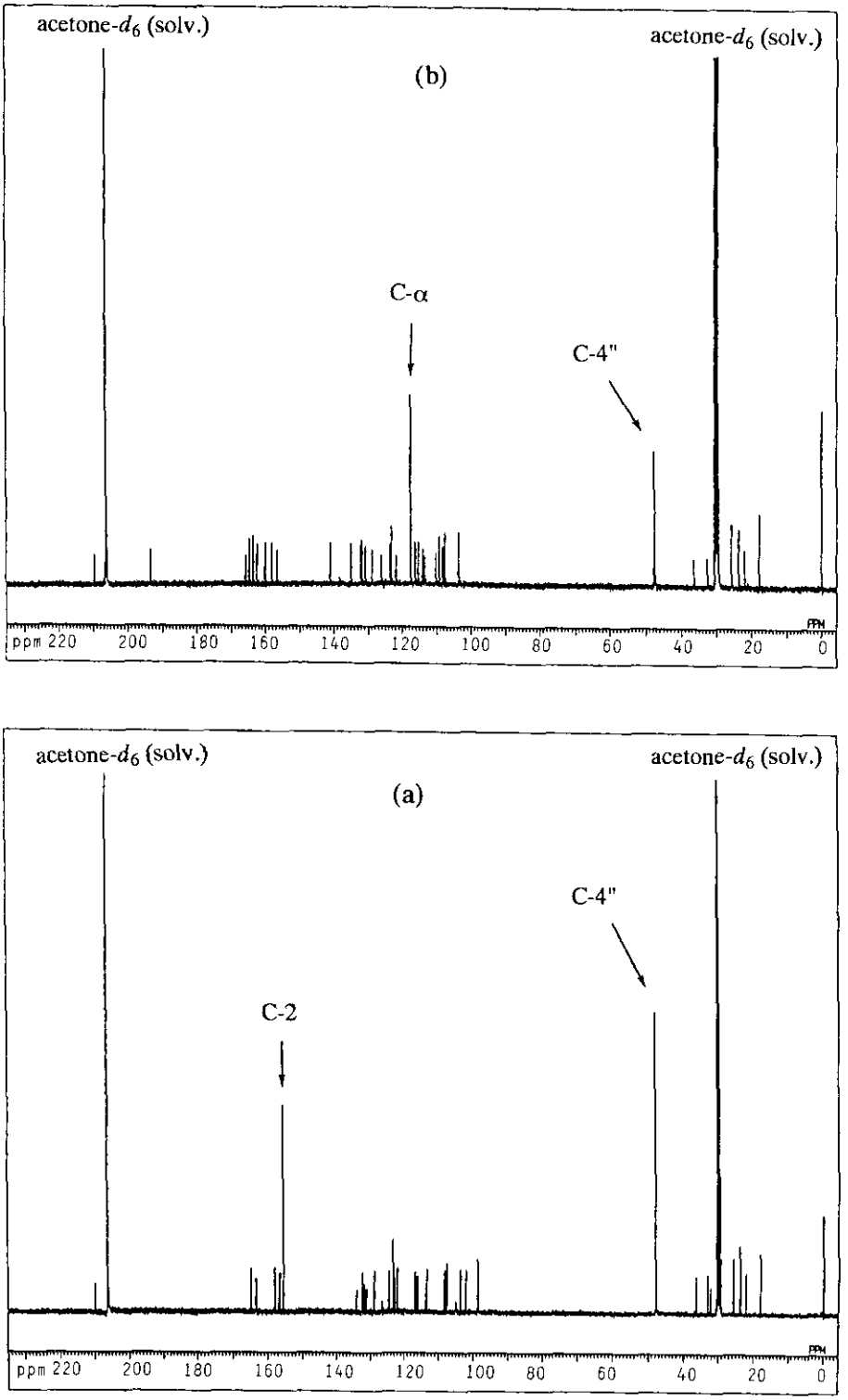

Chart 1 ^{13}C-NMR spectra of (a) **1** and (b) **2** resulting from the feeding experiment with [2-^{13}C]cinnamic acid *N*-acetylcysteamine thioester.

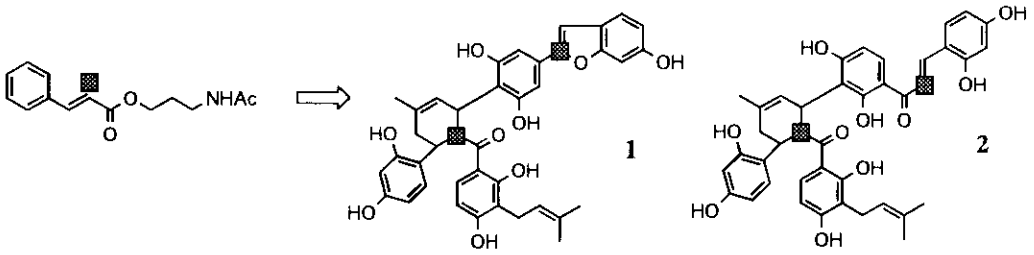

Figure 2 Incorporation of [2-^{13}C]cinnamic acid *N*-acetyl cysteamine thioester into **1** and **2**

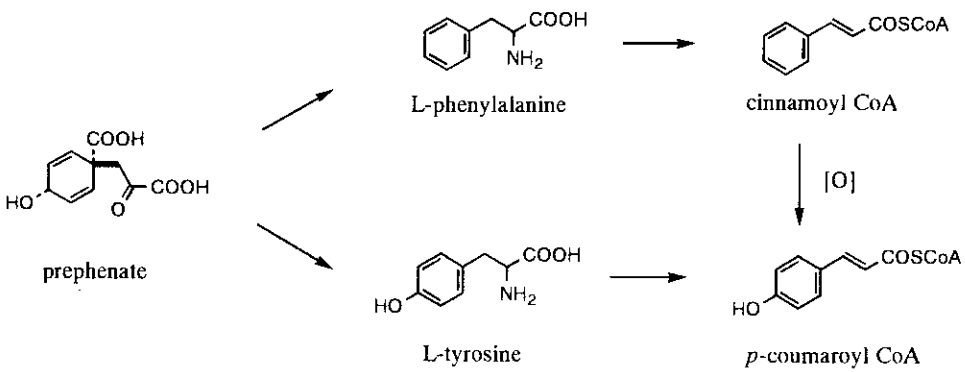

Figure 3 Dual route to *p*-coumarate in *Morus alba* L. cell cultures

and L-tyrosine to *p*-coumaroyl CoA in the flavonoid biosynthesis of *M. alba* cell cultures (Figure 3). Furthermore, it is noteworthy that the metabolic work of the shikimate pathway requires not free acid, but the biomimic *N*-acetylcysteamine thioester, as according with the segregation theory.[12]

EXPERIMENTAL

Morus alba callus and suspension cultures were derived from the seedlings or the leaves of the mulberry tree under specific condition, as described previously.[1] NMR spectral data were recorded on a JEOL JNM EX-400 FTNMR spectrometer at ambient temperature using acetone-d_6 as a solvent and TMS as an internal standard. HPLC was carried out using SSC 3100-J Flow System (Senshu Scientific Co., Tokyo, Japan) equiped with a UV detector (SSC 3000-B).

Preparation of [2-^{13}C]Cinnamic Acid

A mixture of [2-^{13}C]malonic acid (0.5 g, 99.1 atom % ^{13}C, Isotec, Inc. U.S.A.), benzaldehyde (1 g) and a catalytic amount of pyperidine in a solution of pyridine (20 mL) was kept at 80 - 90°C for 6 h. The reaction mixture was poured to water and then acidification with conc. hydrochloric acid allowed to precipitate crude

[2-^{13}C]cinnamic acid. The crude acid was recrystallized from H_2O - methanol to give pure compound (99.1 atom % ^{13}C) in 80 % yield. mp 130 - 131 °C.

Preparation of [2-^{13}C]Cinnamic acid N-Acetylcysteamine Thioester

To a solution of [2-^{13}C]cinnamic acid (0.14 g) in CH_3CN (20 mL), 1,1-carbonyldiimidazol (0.2 g) was added. After stirring up for 1.5 h at 0 °C, *N*-acetylcysteamine (0.3 g) was added to the solution at 0 °C below, and then further stirring up for 2.5 h. After usual work-up, the reaction mixture was subjected to column chromatography over silica gel with ether as a mobile phase followed by recrystallization to afford [2-^{13}C]cinnamic acid *N*-acetylcysteamine (40 mg). mp 104 - 106 °C.

Administration experiment of [2-^{13}C]Cinnamic Acid

The *M. alba* cells were suspended in sterile water (1 L), to which 10 % ethanol solution (10 mL) of [2-^{13}C]cinnamic acid (70 mg) was added. After incubation of the cell suspension for 7 days at 25 °C in the dark, the cells were harvested and lyophilized. The lyophilized cells (3.2 g) were extracted with methanol (150 mL) to afford the extract (256 mg), and then the extract extract was divided into acetone soluble and insoluble portions. The acetone soluble portion (154 mg) was subjected to column chromatography over silica gel (60 g) with chloroform increasing amount of acetone as a mobile phase. Preparative thin-layer chromatography of the fraction eluted with chloroform - acetone (2 : 1) followed by HPLC (column, silica gel, Senshu Pak Silica 4251-N, 10 mmϕ x 250 mm, solvent, ether) to afford chalcomoracin (**1**, retention time 28 min, 30 mg) and kuwanon J (**2**, retention time 32 min, 2 mg).

Administration experiment of [2-^{13}C]Cinnamic Acid N-Acetylcysteamine Thioester

Administration experiment of [2-^{13}C]cinnamic acid *N*-acetylcysteamine thioester(99.1 atom % ^{13}C, 40 mg) to the cell cultures was carried out in analogous way of the above experiment. Chalcomoracin (**1**, 24 mg) and kuwanon J (**2**, 4 mg) were obtained from this experiment.

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