

LATE STAGE OF BIOSYNTHESIS OF INTERMOLECULAR DIELS-ALDER TYPE ADDUCTS IN *MORUS ALBA* L. CELL CULTURES

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**Abstract** - The <sup>13</sup>C-enrichments of chalcone type Diels-Alder type adducts, kuwanons J (**1**), R (**3**), and V (**4**) resulting from administration experiment of [<sup>2-<sup>13</sup>C</sup>]acetate to *Morus alba* cell cultures as well as of 2-arylbenzofuran type adducts, chalcomoracin (**5**) and mulberrofuran E (**6**), revealed that major adducts (**1**) and (**5**) by the cell cultures are presumably derived from **4** and **6**, respectively, through hydroxylation reaction. Kuwanon V (**4**) and mulberrofuran E (**6**) were found to be primary adducts in the *M. alba* cell cultures.

*Morus alba* callus and suspension cultures induced from the seedlings or the leaves<sup>1</sup> produce characteristic mulberry Diels-Alder type adducts, kuwanons J<sup>2</sup> (**1**), Q<sup>2</sup> (**2**), R<sup>2</sup> (**3**), V<sup>2</sup> (**4**), chalcomoracin<sup>1,3</sup> (**5**), and mulberrofuran E<sup>4</sup> (**6**) (Figure 1). Among them, compounds (**1**) and (**5**) are major secondary metabolites in the cell cultures and the productivity by the cell cultures is estimated by about 100 - 1000 times more than that by the intact plant.<sup>1</sup> The biosynthetic studies of the mulberry adducts have been examined by employing the cell cultures through administration experiments of various exogenous substrates and putative precursors. Through administration experiments with <sup>13</sup>C-labeled acetates, kuwanon J (**1**) and chalcomoracin (**5**) have been found to be composed of two molecules of cinnamoylpolyketide intermediates.<sup>5</sup> Administration of precursory methoxychalcone to the cell cultures yielded several optically

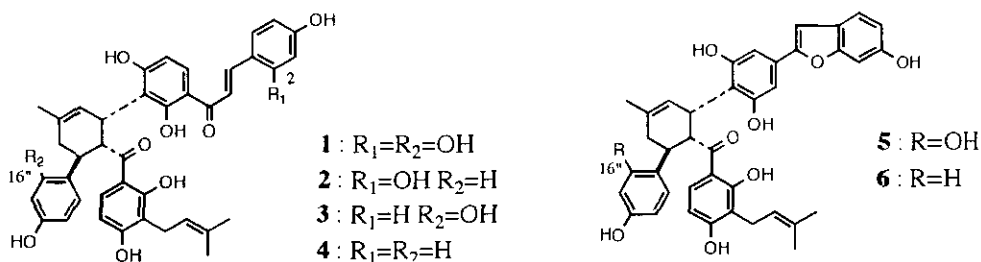


Figure 1 Diels-Alder type adducts of *Morus alba* callus cultures.

active Diels-Alder type metabolites corresponding to methyl ethers of usual Diels-Alder type adducts by the cell cultures.<sup>6</sup> Involvement of the precursory chalcone into the Diels-Alder type construction gave an evidence for biological intermolecular Diels-Alder type reaction in the *M. alba* cell cultures.<sup>6</sup> The other interesting finding was the biosynthesis of an isoprene unit for chalomoracin (5).<sup>7,8</sup> The isoprene unit is built up through junction of the glycolysis and the pentose-phosphate cycle<sup>7</sup> and participates in the Diels-Alder type cycloaddition reaction.<sup>8</sup> Our further studies of the biosynthesis of the Diels-Alder type adducts were focused on minor adducts, such as kuwanon V (3) and mulberrofuran E (6), in the *M. alba* cell cultures.

Minor Diels-Alder type adducts, kuwanons Q (2), R (3), V (4), and mulberrofuran E (6) are lacking one or two hydroxyl groups at specified positions of kuwanon J (1) and chalomoracin (5), respectively (Figure 1). From the administration experiments with precursory methoxychalcones, these adducts each are supposed to be independently biosynthesized through the Diels-Alder type reaction between two molecules of isoprenylphenols. On the other hand, in the feeding experiment with [2-<sup>13</sup>C]acetate, the <sup>13</sup>C-enrichment factor at the polyketide-derived aromatic rings of 1 and 5 was about 4 % and 17 %, respectively, in spite of both having the same chalcone molecule.<sup>9</sup> Such a large difference of the <sup>13</sup>C-enrichment between 1 and 5 may be attributable to different time schedule on the formation of these adducts in the cell cultures. In order to clarify the relationship among the major and the minor Diels-Alder type adducts in their biosyntheses, the <sup>13</sup>C-enrichment factors of other minor adducts from [2-<sup>13</sup>C]acetate were examined. This paper describes the late stage of biosynthesis of the Diels-Alder type adducts in the *M. alba* cell cultures.

After the *Morus alba* cells were suspended in sterilized water, sodium [2-<sup>13</sup>C]acetate (180 mg) was fed for seven days in the dark at 25 °C.<sup>5</sup> Separation and purification of the Diels-Alder type adducts from the lyophilized cells (4.9 g) by a combination of silica gel column chromatography, preparative TLC, and HPLC as previously reported afforded kuwanons J (1, 12 mg), R (3, 2 mg), V (4, 1 mg), chalomoracin (5, 27 mg), and mulberrofuran E (6, 2 mg).

The <sup>13</sup>C-NMR spectra of kuwanons J (1), R (3), and V (4) resulting from the experiment exhibited high incorporation of labeled acetate into the polyketide-derived aromatic rings of the adducts (Charts 1a - c). As described above, the <sup>13</sup>C-enrichment factors of kuwanon J (1) and chalomoracin (5) were about 4 % and 17 %, respectively. Kuwanon R (3), which is lacking one hydroxyl group at the C-2 of 1, showed high incorporation of the labeled acetate than that of 1, and the <sup>13</sup>C-enrichment factor was about 14 % (Chart 1b). Furthermore, in the case of kuwanon V (4), which is further lacking one hydroxyl group at the C-16'' of 3, the <sup>13</sup>C-enrichment factor was about 24 % (Chart 1c). Accordingly, in a series of chalcone-chalcone type adducts, the <sup>13</sup>C-enrichment factor was in inverse proportion to the number of hydroxyl group. Similar phenomenon was observed in chalomoracin (5) and mulberrofuran E (6), in which the <sup>13</sup>C-enrichment of 6 was about 22 % to 17% of 5 (Figure 2).

On the other hand, considering the results of the administration experiments with precursory methoxychalcones, kuwanon J (1) would be biosynthesized from two molecules of chalcone (= morachalcone A,<sup>3</sup> 7) with 4 % of the <sup>13</sup>C-enrichment factor (Figure 3). Similarly, kuwanon V (4) is composed of two molecules of the chalcone derivative (8) with 24 % of the <sup>13</sup>C-enrichment factor (Figure 3). In this point of view, kuwanon R (3) seems to be formed from two chalcone parts (7) and (8) each

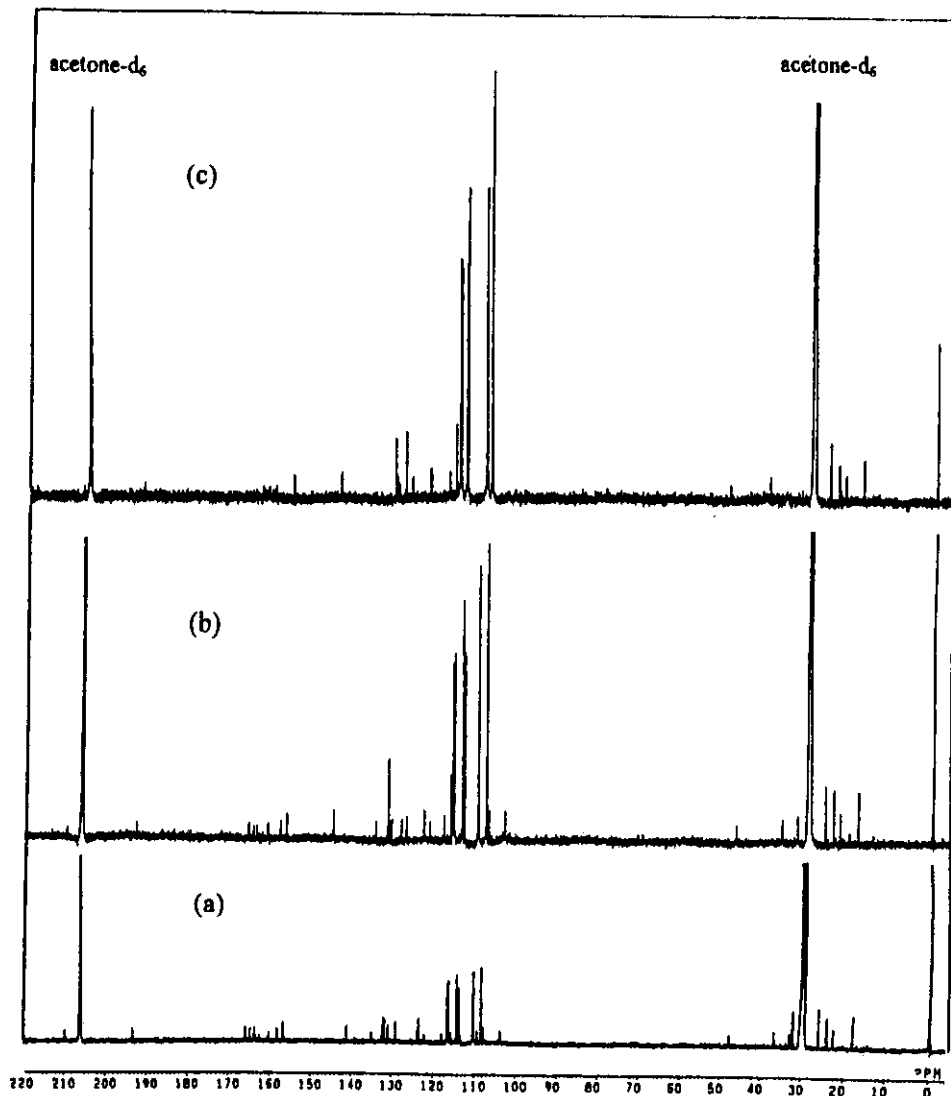


Chart 1  $^{13}\text{C}$ -NMR spectra of (a) kuwanon J (**1**), (b) kuwanon R (**3**), and (c) kuwanon V (**4**) resulting from the feeding experiment with  $[2\text{-}^{13}\text{C}]\text{acetate}$ .

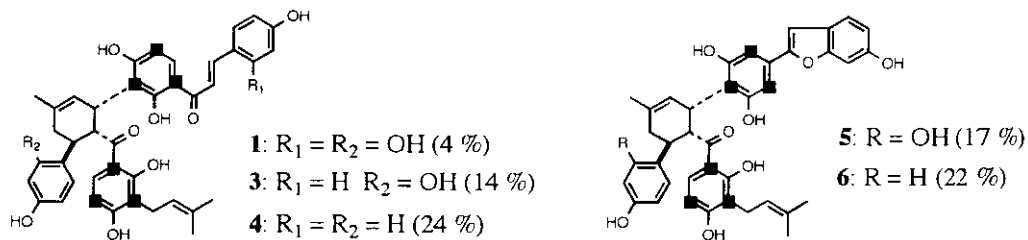


Figure 2 Enriched positions (■) with  $[2\text{-}^{13}\text{C}]\text{acetate}$ . Parenthes (%) denote enrichment factor.

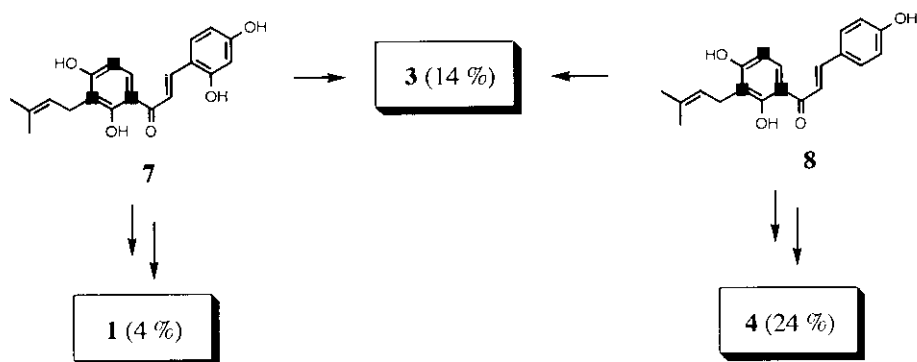


Figure 3 Hypothesis on the formation of 1, 3, and 4 from monomers (7) and (8).

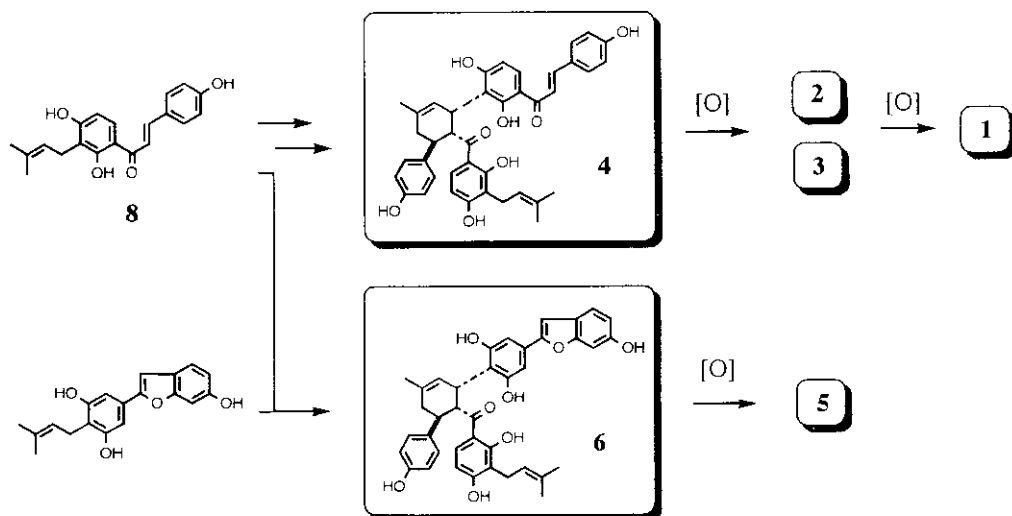


Figure 4 Late stage of the biosynthesis of the Diels-Alder type adducts in *M. alba* cell cultures.

having 4 % and 24 % of  $^{13}\text{C}$ -enrichment factors, respectively. However, the  $^{13}\text{C}$ -NMR spectrum of kuwanon R (3) indicated that both chalcone parts (7) and (8) have the same  $^{13}\text{C}$ -enrichment factor (14%). The agreement of the  $^{13}\text{C}$ -enrichment factor of 7 with that of 8 in kuwanon R (3) was unexplainable result, if the Diels-Alder type adducts each are independently formed through the Diels-Alder type cycloaddition reaction (Figure 3). The most important point, however, was that, in the chalcone-chalcone type Diels-Alder type adducts (1, 3, and 4), the two chalcones forming one molecule of the adduct are always enriched with the same degrees of the  $^{13}\text{C}$ . In addition to the fact, the  $^{13}\text{C}$ -enrichment of the adduct was diluted as increasing the number of hydroxyl group. A possible explanation on this fact is that foremost biosynthesis of lesser hydroxylated adduct (4) in the cell cultures followed by successive hydroxylation reactions of 4 to form kuwanon R (3) and then kuwanon J (1) (Figure 4). Thus, the  $^{13}\text{C}$ -enrichments of the diene and dienophile parts must be always the same degree in every adducts. On the other hand, in the

series of the 2-arylbenzofuran type adducts, chalcomoracin (**5**) and mulberrofuran E (**6**), the relationship between the  $^{13}\text{C}$ -enrichment and the number of hydroxyl group was the same as that in the series of chalcone-chalcone type adducts. Furthermore, the  $^{13}\text{C}$ -enrichment of the 2-arylbenzofuran moiety of **6** was larger than that of **5**, inspite of the same structure. This fact also suggested that chalcomoracin (**5**) is formed by the hydroxylation at the C-16" position of mulberrofuran E (**6**) primarily biosynthesized in the cell cultures (Figure 4).

It was thus concluded that the major adducts (**1** and **5**) in the *M. alba* cell cultures are presumably derived through the hydroxylation of **4** and **6**, respectively, which are primary adducts in the cell cultures (Figure 4). Present study revealed the late stage of the biosynthesis for the intermolecular Diels-Alder type adducts in the *M. alba* cell cultures.

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#### REFERENCES AND NOTES

† Dedicated to the late Dr. S. Ueda.

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9.  $^{13}\text{C}$ -Enrichment factor was calculated by the  $^{13}\text{C}$  signal intensity of the compound resulting from the feeding experiment to that in natural abundance.

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