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

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Abstract - The ^{13}C-enrichments of chalcone type Diels-Alder type adducts, kuwanons J (**1**), R (**3**), and V (**4**) resulting from administration experiment of [2-^{13}C]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[1] produce characteristic mulberry Diels-Alder type adducts, kuwanons J[2] (**1**), Q[2] (**2**), R[2] (**3**), V[2] (**4**), chalcomoracin[1,3] (**5**), and mulberrofuran E[4] (**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.[1] 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 ^{13}C-labeled acetates, kuwanon J (**1**) and chalcomoracin (**5**) have been found to be composed of two molecules of cinnamoylpolyketide intermediates.[5] Administration of precursory methoxychalcone to the cell cultures yielded several optically

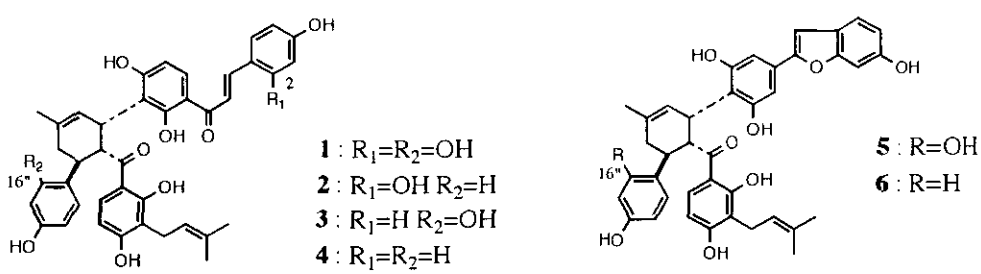


Figure 1 Diels-Alder type adducts of *Morus alba* callus cultures.

active Diels-Alder type metabolites corresponding to methyl ethers of usual Diels-Alder type adducts by the cell cultures.[6] 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.[6] The other interesting finding was the biosynthesis of an isoprene unit for chalcomoracin (**5**).[7,8] The isoprene unit is built up through junction of the glycolysis and the pentose-phosphate cycle[7] and participates in the Diels-Alder type cycloaddition reaction.[8] 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 chalcomoracin (**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-^{13}C]acetate, the ^{13}C-enrichment factor at the polyketide-derived aromatic rings of **1** and **5** was about 4 % and 17 %, respectivey, in spite of both having the same chalcone molecule.[9] Such a large difference of the ^{13}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 ^{13}C-enrichment factors of other minor adducts from [2-^{13}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-^{13}C]acetate (180 mg) was fed for seven days in the dark at 25 °C.[5] 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), chalcomoracin (**5**, 27 mg), and mulberrofuran E (**6**, 2 mg).

The ^{13}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 ^{13}C-enrichment factors of kuwanon J (**1**) and chalcomoracin (**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 ^{13}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 ^{13}C-enrichment factor was about 24 % (Chart 1c). Accordingly, in a series of chalcone-chalcone type adducts, the ^{13}C-enrichment factor was in inverse proportion to the number of hydroxyl group. Similar phenomenon was observed in chalcomoracin (**5**) and mulberrofuran E (**6**), in which the ^{13}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,[3] **7**) with 4 % of the ^{13}C-enrichment factor (Figure 3). Similarly, kuwanon V (**4**) is composed of two molecules of the chalcone derivative (**8**) with 24 % of the ^{13}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

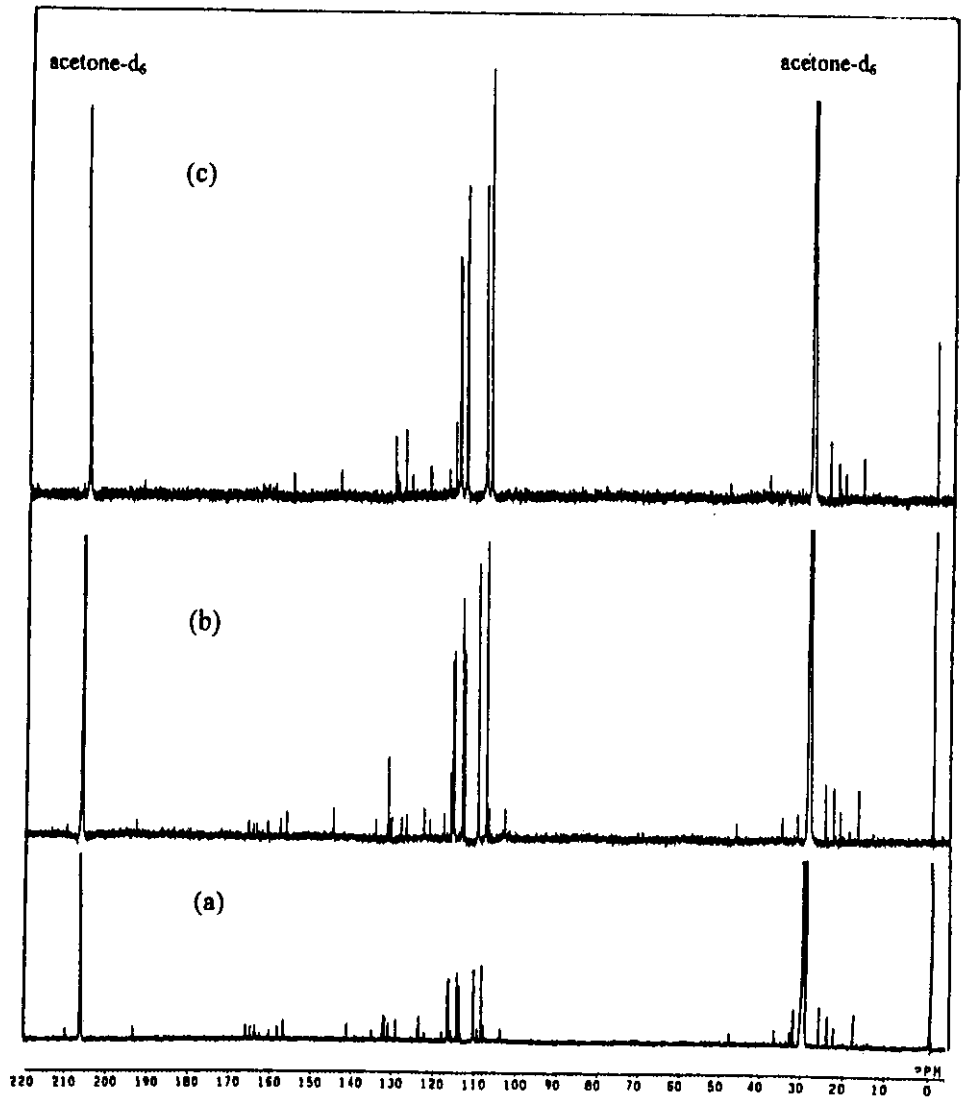
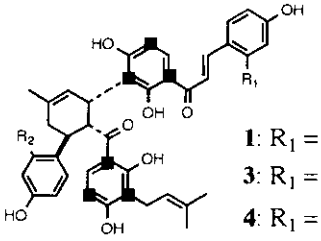
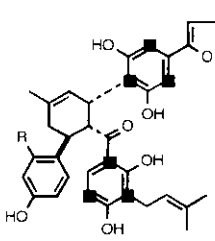


Chart 1 ^{13}C-NMR spectra of (a) kuwanon J (**1**), (b) kuwanon R (**3**), and (c) kuwanon V (**4**) resulting from the feeding experiment with [2-^{13}C]acetate.



1: $R_1 = R_2 = OH$ (4 %)
3: $R_1 = H$ $R_2 = OH$ (14 %)
4: $R_1 = R_2 = H$ (24 %)



5: R = OH (17 %)
6: R = H (22 %)

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

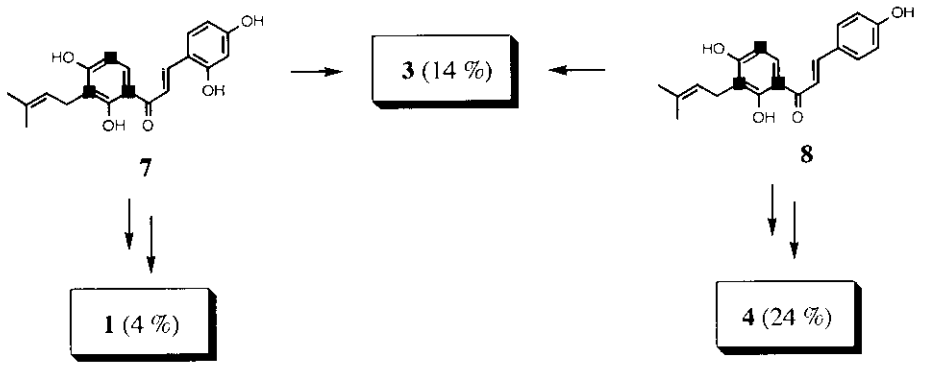


Figure 3 Hypothesis on the formation of **1**, **3**, and **4** from monomers (**7**) and (**8**).

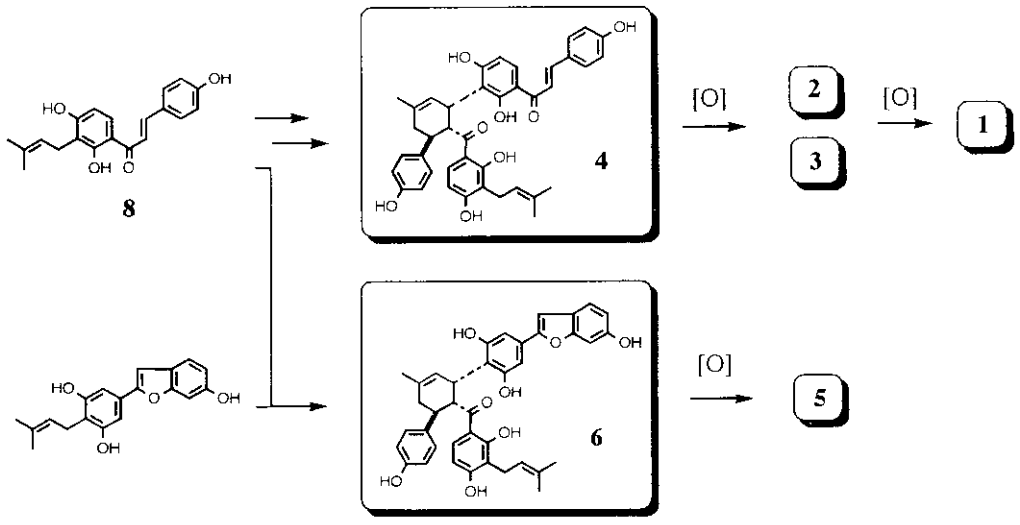


Figure 4 Late stage of the biosynthesis of the Diels- Alder type adducts in *M. alba* cell cultures.

having 4 % and 24 % of ^{13}C-enrichment factors, respectively. However, the ^{13}C-NMR spectrum of kuwanon R (**3**) indicated that both chalcone parts (**7**) and (**8**) have the same ^{13}C-enrichment factor (14%). The agreement of the ^{13}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}C. In addition to the fact, the ^{13}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}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}C-enrichment and the number of hydroxyl group was the same as that in the series of chalcone-chalcone type adducts. Furthermore, the ^{13}C-enrichment of the 2-arylbenzofuran moiety of **6** was lager 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}C-Enrichment factor was calculated by the ^{13}C signal intensity of the compound resulting from the feeding experiment to that in natural abundance.

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