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

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Abstract - The 13C-enrichments of chalcone type Diels-Alder type adducts, kuwanons J (1), R (3), and V (4) resulting from administration experiment of [2-13C]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 leaves1 produce characteristic mulberry Diels-Alder type adducts, kuwanons J2 (1), Q2 (2), R2 (3), V2 (4), chalcomoracin1,3 (5), and mulberrofuran E4 (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 13C-labeled acetates, kuwanon J (1) and chalcomoracin (5) have been found to be composed of two molecules of cinnamoylpyroketide intermediates.5 Administration of precursory methoxychalcone to the cell cultures yielded several optically

![Figure 1 Diels-Alder type adducts of Morus alba callus cultures.](image-url)
active Diels-Alder type metabolites corresponding to methyl ethers of usual Diels-Alder type adducts by the cell cultures.\textsuperscript{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.\textsuperscript{5} The other interesting finding was the biosynthesis of an isoprene unit for chalcomoracin (5).\textsuperscript{7,8} The isoprene unit is built up through junction of the glycolysis and the pentose-phosphate cycle\textsuperscript{7} and participates in the Diels-Alder type cycloaddition reaction.\textsuperscript{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-\textsuperscript{13}C]acetate, the \textsuperscript{13}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.\textsuperscript{9} Such a large difference of the \textsuperscript{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 \textsuperscript{13}C-enrichment factors of other minor adducts from [2-\textsuperscript{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-\textsuperscript{13}C]acetate (180 mg) was fed for seven days in the dark at 25 °C.\textsuperscript{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 \textsuperscript{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 \textsuperscript{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 \textsuperscript{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 \textsuperscript{13}C-enrichment factor was about 24 \% (Chart 1c). Accordingly, in a series of chalcone-chalcone type adducts, the \textsuperscript{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 \textsuperscript{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\textsuperscript{3,7} 7) with 4 \% of the \textsuperscript{13}C-enrichment factor (Figure 3). Similarly, kuwanon V (4) is composed of two molecules of the chalcone derivative (8) with 24 \% of the \textsuperscript{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.

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