BIOSYNTHESIS OF CHALCOMORACIN AND KUWANON J, THE DIELS-ALDER TYPE ADDUCTS, IN *MORUS ALBA* L. CELL CULTURES

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Experiments with [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetates administered to *Morus alba* cell cultures revealed that both optically active Diels-Alder type adducts chalcomoracin (**1**) and kuwanon J (**2**) are formed through the condensation of two molecules of cinnamoylpolyketide-derived skeletons. The 2-arylbenzofuran skeleton of **1** is formed by a novel type cyclization of the cinnamoylpolyketide, followed by decarboxylation. The ^{13}C-labeling of [2-^{13}C]acetate was incorporated into the starter acetate carbons in the biosynthesis of the prenyl moieties of chalcomoracin (**1**), while that of [1-^{13}C]acetate was not incorporated into the prenyl moieties of **1**.

KEYWORDS *Morus alba*; Moraceae; cell culture; biosynthesis; chalcomoracin; kuwanon J; Diels-Alder type adduct; cinnamoylpolyketide; 2-arylbenzofuran; chalcone

Callus and cell suspension cultures of high pigment productivity have been obtained through the selection of callus tissues induced from the seedlings or the leaves of *Morus alba* L. The main phenolic pigments of the cell cultures are chalcomoracin (**1**)[1] and kuwanon J (**2**)[2] and their related compounds.[2,3] The structures of **1** and **2** suggest that the former is the Diels-Alder type adduct[4] from a prenylchalcone and a dehydroprenyl-2-arylbenzofuran, and the latter from a prenylchalcone and a dehydroprenylchalcone.

This paper describes the results of the administrations of sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetates to the mulberry cell cultures indicating the early stage of the biosynthesis of the optically active Diels-Alder type adducts.

Sodium [2-^{13}C]acetate (99.5 atom% ^{13}C, 175 mg) was administered to the *M. alba* cells suspended in sterilized water (500 ml). After shaking the suspension in the dark at 25 °C for 7 days, the cells were harvested and lyophilized. Extraction of the dry cells (4.9 g) with methanol followed by the usual work-up[2] yielded chalcomoracin (**1**, 27 mg) and kuwanon J (**2**, 12 mg). An analogous administration experiment with [1-^{13}C]-acetate (99.5 atom% ^{13}C, 175 mg) gave **1** (27 mg) and **2** (10 mg). As shown in the ^{13}C NMR spectra of **1**, both sodium [1-^{13}C] and [2-^{13}C]acetates were highly incorporated into the aromatic rings of **1** (Chart 1-a,b). Relatively high incorporations of both acetates were also observed in the case of **2** (Fig. 2 and Table II). The ^{13}C-labeling sites in kuwanon J (**2**) in the [1-^{13}C]acetate administration were the C-2', 4', 6', 10", 12", and 14" positions, while those in the [2-^{13}C]acetate administration were the C-1', 3', 5', 9", 11", and 13" positions (Fig. 2). The ^{13}C-labeling pattern in **2** indicating the incorporation of three successive acetate units into the rings B and C corroborates that kuwanon J (**2**) is composed of two cinnamoylpolyketide-derived chalcone skeletons. This indicates that the possibility of **2** as a member of the retrochalcone has been ruled out. The chalcone skeleton is considered to be formed through the deoxygenation[5] at C-5 of the cinnamoylpolyketide skeleton, followed by cyclization and aromatization.

On the other hand, the ^{13}C-labeling pattern of [1-^{13}C]- and [2-^{13}C]acetate-derived chalcomoracin (**1**) indicates that the chalcone moiety comes from a cinnamoylpolyketide skeleton (Chart 1-a,b). The ^{13}C-labeling in sodium [2-^{13}C]acetate was incorporated into the C-2', 4', and 6' positions in ring C of **1** (Chart 1a). This fact is compatible with the assumption that the 2-arylbenzofuran moiety is also formed by way of a C_6-C_3-C_6 cinnamoylpolyketide skeleton. The 2-arylbenzofuran moiety, however, is composed of a C_6-C_2-C_6 unit. The ^{13}C-labeling in sodium [1-^{13}C]acetate was found to be incorporated into the C-3' and 5' positions in ring C of **1**. This indicates that the 2-arylbenzofuran moiety is formed through the cyclization at C-3 and C-8 of the cinnamoylpolyketide skeleton, followed by decarboxylation (Fig. 3).

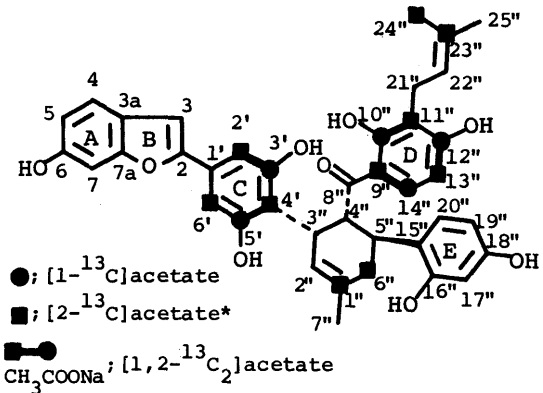
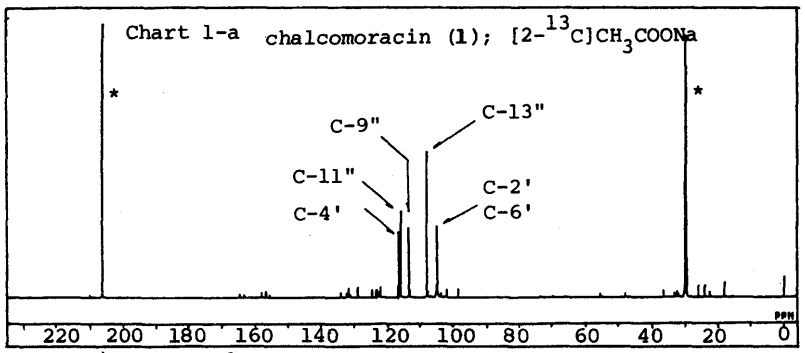


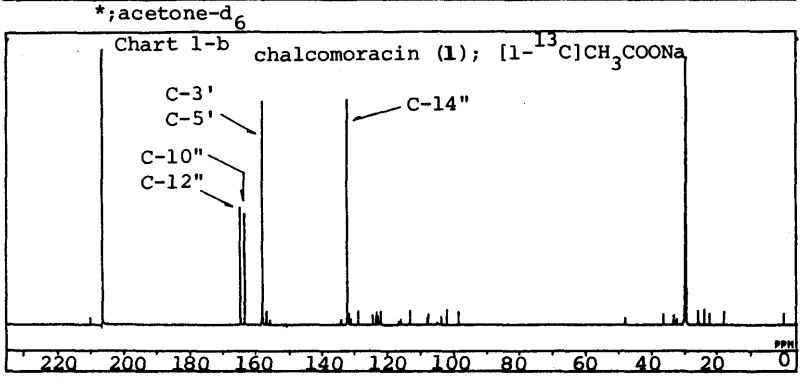
Fig. 1. ^{13}C-Labeling Pattern of **1**

*Enrichment factors: 2',4',6',9",11",13" (ca 18%); 1",6",23",24" (ca 0.4%).

Table I. ^{13}C-^{13}C Coupling Constants

C-2'– C-3'	J=60.2 Hz
C-4'– C-5'	J=66.8 Hz
C-9"– C-14"	J=58.7 Hz
C-12"– C-13"	J=60.2 Hz
C-10"– C-11"	J=69.7 Hz





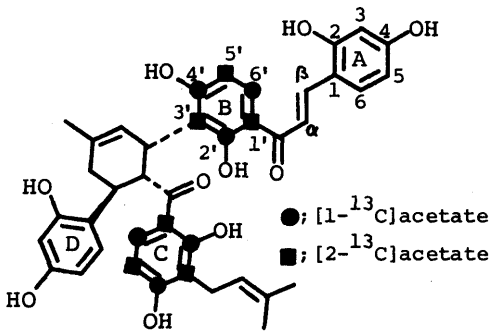


Fig. 2. ^{13}C-Labeling Pattern of **2**

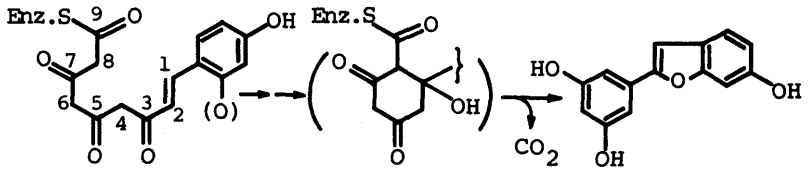


Fig. 3. Biosynthetic Pathway of 2-Arylbenzofuran Skeleton

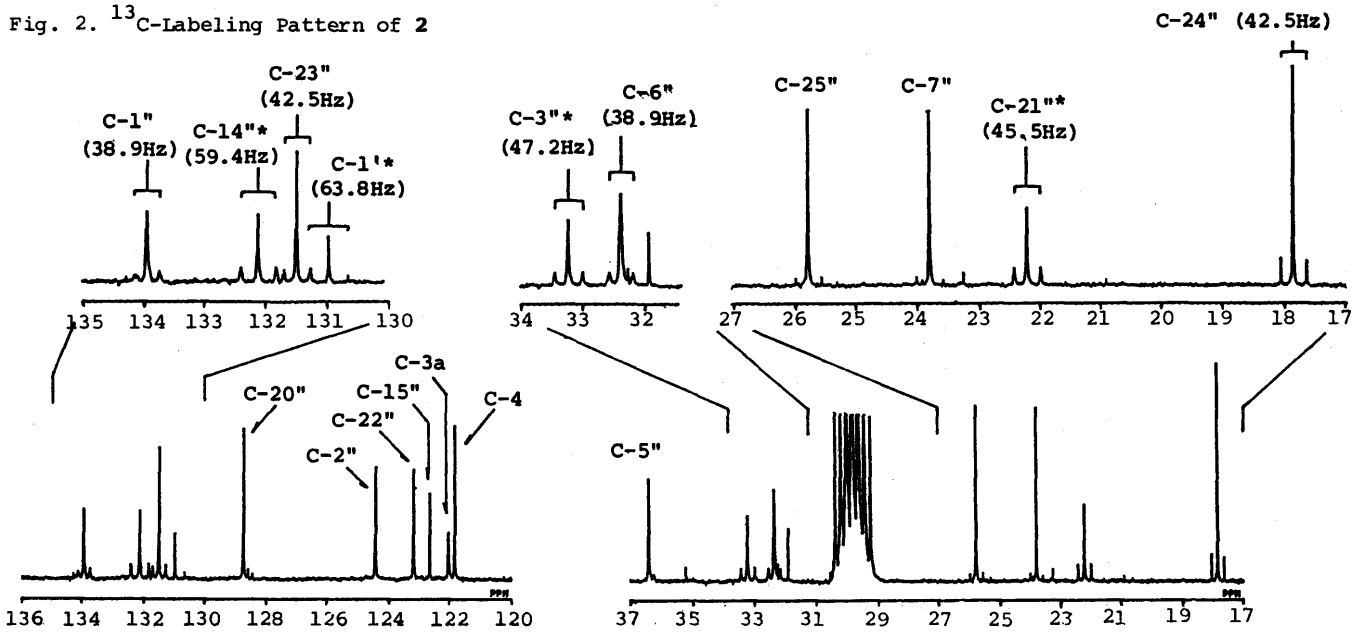


Chart 2. The ^{13}C NMR Signals in the Region of Two Prenyl Units of [2-^{13}C]Acetate-derived **1**

* The splitting carbon signals of C-21", C-3", C-1', and C-14" is due to coupling with highly ^{13}C-enriched carbons at C-11", C-4', C-2'(6'), and C-9"(13"), respectively.

Table II. ^{13}C-NMR Chemical Shifts of **1** and **2** in Acetone-d_6 (*; [1-^{13}C] and **; [2-^{13}C]Acetates)

Chalcomoracin (**1**)						Kuwanon J (**2**)					
C-2	156.74	C-1"	134.00**	C-15"	122.67	C-1	115.35	C-6'	130.69*	C-14"	132.10*
C-3	101.86	C-2"	124.42	C-16"	156.42	C-2	159.93	C-1"	134.90	C-15"	121.89
C-3a	122.09	C-3"	33.28	C-17"	103.62	C-3	103.73	C-2"	123.52	C-16"	156.50
C-4	121.83	C-4"	47.89	C-18"	157.92	C-4	162.31	C-3"	32.67	C-17"	103.70
C-5	113.16	C-5"	36.45	C-19"	107.60	C-5	109.23	C-4"	47.49	C-18"	158.01
C-6	156.59	C-6"	32.44**	C-20"	128.77	C-6	131.78	C-5"	36.43	C-19"	107.65
C-7	98.42	C-7"	23.80	C-21"	22.23	C=O	193.41	C-6"	32.53	C-20"	128.77
C-7a	155.49	C-8"	209.81	C-22"	123.20	C-α	117.56	C-7"	23.81	C-21"	22.21
C-1'	130.99	C-9"	113.60**	C-23"	131.50**	C-β	141.00	C-8"	209.62	C-22"	123.21
C-2'	104.95**	C-10"	164.68*	C-24"	17.86**	C-1'	114.06**	C-9"	113.53**	C-23"	131.50
C-3'	157.84*	C-11"	115.95**	C-25"	25.79	C-2'	163.58*	C-10"	164.64*	C-24"	17.86
C-4'	116.62**	C-12"	163.19*			C-3'	116.29**	C-11"	115.95**	C-25"	25.81
C-5'	157.84*	C-13"	108.12**			C-4'	165.73*	C-12"	163.32*		
C-6'	104.95**	C-14"	132.13*			C-5'	110.09**	C-13"	108.23**		

Sodium [1,2-$^{13}C_2$]acetate (93 atom% ^{13}C, 90 mg) diluted with non-labeled sodium acetate (90 mg) was administered to M. alba cells suspended in sterilized water (500 ml). After a conventional work-up, **1** (20 mg) and **2** (4 mg) were obtained. Examination of the ^{13}C NMR spectrum of [1,2-$^{13}C_2$]acetate-derived **1** indicating the ^{13}C-^{13}C spin-spin coupling constants (Table I) revealed the ^{13}C-labeling pattern on the aromatic rings, as shown in Fig. 1. Accordingly, it was concluded that the 2-arylbenzofuran moiety was biosynthesized as depicted in Fig. 3. Whereas, the chalcone moiety (C-4",5",8"–20") was formed through the same pathway as that of **2**. It is thus evident that the optically active Diels-Alder type adducts, chalcomoracin (**1**) and kuwanon J (**2**) in M. alba cell cultures are composed of two cinnamoylpolyketide-derived skeletons.

It is noteworthy that two types of cyclization reactions operate in the biosynthesis of chalcomoracin (**1**). Of the two cinnamoylpolyketide-derived moieties, the 2-arylbenzofuran skeleton is formed through the aldol-type condensation at C-3 and C-8 of the precursor followed by decarboxylation, while the chalcone skeleton is formed through the Claisen-type condensation at C-4 and C-9. The present study has thus established the novel biosynthetic pathway leading to the 2-arylbenzofuran skeleton occurring in mulberry constituents.

In the ^{13}C NMR spectrum of sodium [2-^{13}C]acetate-derived chalcomoracin (**1**), spin-spin coupling was observed between C-23" and C-24" (J=42.5 Hz) as well as between C-6" and C-1" (J=38.9 Hz) (Chart 2). As the C-6" and C-24" positions correspond to the cis methyls of the dimethylallyl pyrophosphate, it is considered that the ^{13}C-labeling has taken place at the starter acetate carbons on the biosynthesis of acetoacetyl CoA leading to the prenyl unit. Partial transfer of the ^{13}C-labeling of [2-^{13}C]acetate to the carboxyl carbon may be due to the participation of the tricarboxylic acid (TCA) cycle.[6] On the other hand, the carboxyl carbon labeling from [1-^{13}C]acetate was not incorporated into the prenyl unit, presumably due to decarboxylation through the TCA cycle.[7] These results imply that the first acetate incorporated into the prenyl unit of **1** was not the intact acetate administered to the cell cultures, but was that reformed from the original methyl group of the acetate and one carbon unit joined afterward.

Unlike the polyketide-derived aromatic carbons labeled distinctly with ^{13}C, both prenyl units at C-11" and C-4' are labeled in a different way to a lesser extent. These labeling patterns clearly demonstrated the mixed biosynthesis of mulberry prenylchalcones from different origins. From the above described results, chalcomoracin (**1**) could be a cycloaddition product of the α,β-double bond of the chalcone as a dienophile and the prenyl portion at C-4' of the 2-arylbenzofuran skeleton as a diene. Kuwanon J (**2**) comprising the same type of the cycloaddition structure as that of **1** is also considered to be a dimer of a cinnamoylpolyketide-derived chalcone bearing a prenyl unit.

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