Review

Chemistry and biosynthesis of isoprenylated flavonoids from Japanese mulberry tree

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Abstract: Many isoprenylated flavonoids have been isolated from Japanese mulberry tree (Moraceae). Among them, kuwanons G (**1**) and H (**2**) were the first isolated active substances exhibiting a hypotensive effect. These compounds are considered to be formed through an enzymatic Diels-Alder type reaction between an isoprenyl portion of an isoprenylphenol as the diene and an α, β-double bond of chalcone as the dienophile. The absolute configurations of these Diels-Alder type adducts were confirmed by three different methods. The stereochemistries of the adducts were consistent with those of ones in the Diels-Alder reaction involving *exo-* and *endo*-addition. Some strains of *Morus alba* callus tissues have a high productivity of mulberry Diels-Alder type adducts, such as chalcomoracin (**3**) and kuwanon J (**4**). The biosynthetic studies of the mulberry Diels-Alder type adducts have been carried out with the aid of the cell strain. Chalcomoracin (**3**) and kuwanon J (**4**) were proved to be enzymatic Diels-Alder type reaction products by the administration experiments with *O*-methylchalcone derivatives. Furthermore, for the isoprenoid biosynthesis of prenylflavonoids in *Morus alba* callus tissues by administration of [1,3-^{13}C$_2$]- and [2-^{13}C]-glycerol, a novel way through the junction of glycolysis and pentose-phosphate cycle was proved. Two independent isoprenoid biosynthetic pathways, that for sterols and that for isoprenoid-phenols, operate in the *Morus alba* cell cultures. The former is susceptible to compactin (ML-236) and the latter resists to compactin in the cell cultures, respectively.

Keywords: mulberry tree, mulberry flavonoids, Diels-Alder type reaction, biosynthesis

1. Introduction

Moraceae comprises a large family of sixty genera and nearly 1400 species, including important genera such as *Artocarpus*, *Morus*, and *Ficus*. The mulberry tree, typical plant of the genus *Morus*, has been widely cultivated in China, Korea, and Japan.[1] Its leaves are indispensable as food for silk worms. Many varieties of *Morus* are cultivated in Japan; these varieties are described as belonging to three species: *Morus alba* L. ("Karayamaguwa" in Japanese), *M. bombycis* Koidz. ("Yamaguwa" in Japanese), and *M. lhou* (ser.) Koidz. ("Roguwa" in Japanese).[2] In

addition, the root bark of the mulberry tree (Mori Cortex, *Morus alba* L. and other of genus *Morus*, "Sang-Bai-Pi" in Chinese, "Sohakuhi" in Japanese) has been used as a material of traditional Chinese medicine for an anti-inflammatory, diuretic, anti-tussive, expectorant, anti-pyretic purposes.[3,4] The earliest written reference to the use of Mori Cortex is contained in the "Shen Nong Ben Cao Jing" (Shin-nou-hon-zou-kyo in Japanese), the first Chinese dispensatory whose original anonymous volumes probably appeared by the end of the third century.[5,6] The crude drug (Sohakuhi) is used as a component in traditional Chinese medicinal prescriptions, such as "Wu Hu Tang (Goko-tou in Japanese) and "Mahuang Lianquiao Chixiaodou Tang "Maou-rensho-shakushozu-tou), which are applied clinically as a therapy for bronchitis and for nephritis, respectively.[7] On the other hand, a few pharmacological studies on the mulberry tree had demonstrated for a hypotensive effect of the

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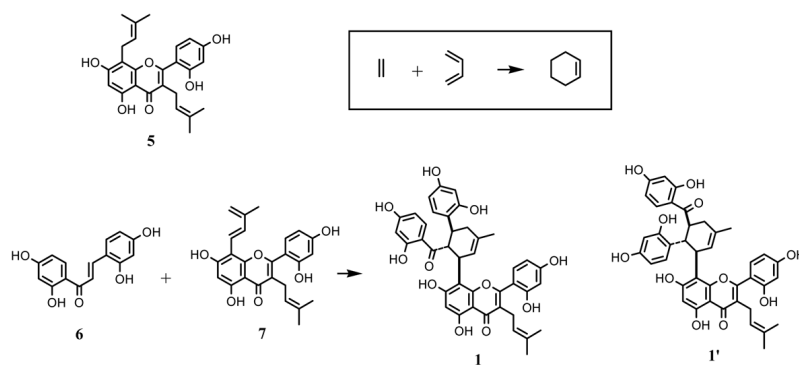
Fig. 1. Hypothetical formation of kuwanon G (**1**) and its isomer (**1'**) from **6** and **7** through the Diels-Alder reaction.

extract in rodents.[8),9)] Considering these reports, it was suggested that the hypotensive components were a mixture of unstable phenolic compounds. As for the components of *Morus* root bark, occurrence of triterpenoids, diglyceride, and piperidine alkaloids has been reported prior to the beginning of our work in 1966, where as the hypotensive constituents had not been identified.[10)] Our interests were focused on the phenolic constituents of the mulberry root bark. So we have studied phenolic compounds of mulberry root bark and related plants.[10)–18)] This article reviews the typical results of our chemical and biosynthetic studies for the isoprenylated flavonoids obtained from the Japanese mulberry tree.

2. Diels-Alder type adducts from Japanese mulberry tree

2.1. Hypotensive constituents, kuwanons G and H. Intravenous injection of the methanol extract of the root bark of mulberry tree, 1 mg–20 mg, showed a dose-dependent decrease in arterial blood pressure in pentobarbital-anesthetized rabbits.[18)] The extract was fractionated by several chromatographic methods to isolation of kuwanons G (**1**)[19)] and H (**2**).[20)] From the extract, **1** and **2** could be isolated in 0.2 percent and 0.13 percent yields, respectively. Intravenous injection of both compounds (0.1–3.0 mg/kg) showed an almost equally transient dose-dependent decrease in arterial blood pressure in anesthetized rabbits.[18)] Detailed analysis employed with pentobarbital-anesthetized pithed dogs suggested that mechanism on hypotensive actions of **1** and **2** mediated through peripheral system.[18)] Subsequently, several hypotensive phenolic constituents have been isolated from the mulberry root bark,[18)] which might have supported a hypothesis for hypo-

tensive components of the mulberry tree as mentioned above.

In parallel with our findings in the preceding paragraph, Hikino and co-workers isolated as two hypotensive compounds from *Morus* root bark which they named moracenin B[21)] and A,[22)] while Masamune and co-workers two prohibitins of mulberry shoot which they named albanins F and G.[23)] By direct comparison kuwanons G (**1**) was found to be identical with moracenin B and albanin F, and kuwanon H (**2**) with moracenin A and albanin G.[24)] Structure determination of these compounds were carried out by the three research groups independently. Finally, the three research groups proposed the structures (except absolute configurations), for kuwanons G (**1**) and H (**2**), respectively.[24),25)]

2.2. Structures of Diels-Alder type adducts, kuwanon G. Kuwanon G (**1**), $[\alpha]_D − 534°$, had a molecular formula $C_{40}H_{36}O_{11}$ and gave positive Mg-HCl and Zn-HCl tests for characteristic of color reaction test of flavonoids, and characteristically had a large optical rotation. The UV spectrum was similar to that of kuwanon C (**5**) except for a shoulder at 280 nm, which suggested that **1** possesses kuwanon C (**5**) partial structure. From the chemical and spectroscopic evidence two possible plane structures were suggested for kuwanon G as structures **1** and **1'**.[10),19)] On the other hand, the Diels-Alder reaction is well known as a [4+2]cycloaddition of a conjugated diene and a dienophile to form a six-membered ring. For instance, the Diels-Alder reaction of ethylene (typical dienophile component) and butadiene (typical conjugated diene component) yields a cyclohexene called an adduct (Fig. 1). Considering these structures, kuwanon G seems to be formed through a Diels-Alder type reaction of a chal-
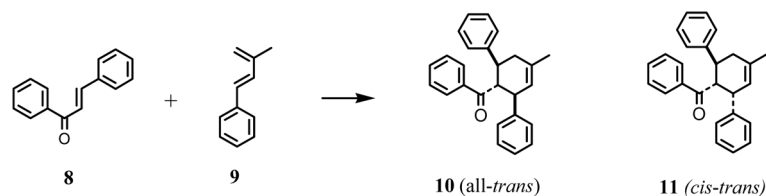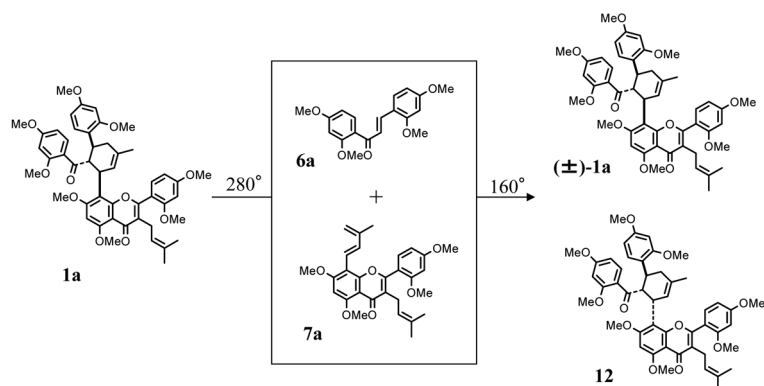
Fig. 2.   Model synthesis by Diels-Alder reaction.

Fig. 3.   Pyrolysis of kuwanon G octamethyl ether (**1a**) and reconstraction of (±)-**1a** through Diels-Alder reaction.

cone (**6**) and dehydrokuwanon C (**7**) or its equivalent. Furthermore, these two structures (**1** and **1'**) are isomers due to different regioselectivity of the Diels-Alder reaction (Fig. 1). An evidence of the regiochemistry for **1** was obtained by the following results: Diels-Alder reaction of *trans*-chalcone (**8**) and 3-methyl-1-phenyl-1,3-butadiene (**9**) gave two cycloadducts, one of which is all-*trans* type adduct (**10**) in relative configuration among three substituents on the methylcyclohexene ring and another is the *cis-trans* type adduct (**11**) in relative configuration. The X-ray analysis of these cycloproducts revealed that the regioselectivity in the [4 + 2] reaction was coincided with that estimated in the case of kuwanon G (**1**) (Fig. 2). Final proof of structure **1** for kuwanon G was obtained as the results of joint work between our group and that of Masamune.[24] Kuwanon G octamethyl ether (**1a**) was pyrolysed to give *trans*-chalcone tetramethyl ether (**6a**) and dehydrokuwanon C tetramethyl ether (**7a**). These structures were confirmed by chemical evidence. The Diels-Alder reaction of the fragmentation compounds, **6a** and **7a**, gave two [4 + 2]cycloadducts (Fig. 3). One of the adducts was identified with (±)-**1a** and the other was a *cis-trans* type cycloadduct (**12**) as was ob-

served in the case of the synthesis of the model compounds described above in Fig. 2. The structure of kuwanon G (**1**) has thus been established as depicted in Fig. 1.[10),17),24)] Kuwanon G (**1**) was optically active and considered to be formed through an enzymatic Diels-Alder type reaction of chalcone (**6**) as a dienophile and dehydrokuwanon C (**7**) as a diene.

2.3. Diels-Alder type adducts from moraceous plants. About fifty kinds of optically active Diels-Alder type adducts have been isolated from the moraceous plants. The mulberry Diels-Alder type adducts may be divided into the following four types on the basis of the phenol nuclei; a) adducts of a chalcone and a dehydroprenylflavone, e.g. kuwanons G (**1**),[19)] and H (**2**),[20)] b) adducts of a chalcone and dehydroprenylchalcone, e.g. kuwanons I (**13**)[26)] and J (**4**)[27),] c) adducts of a chalcone and dehydroprenyl-2-arylbenzofuran, e.g. mulberrofurans C (**14**)[28)] and J (**15**),[29)] d) adducts of a chalcone and a dehydroprenylstilbene, e.g. kuwanons X (**16**)[29)] and Y (**17**)[30)] (Fig. 4). It is interesting that a pair of isomers, such as **13** and **4** and others, could be isolated from the moraceous plant as in the case of the Diels-Alder reaction of a *trans*-chalcone (**8**) and a butadiene derivative (**9**) giving rise to a pair of isomers, all-*trans*
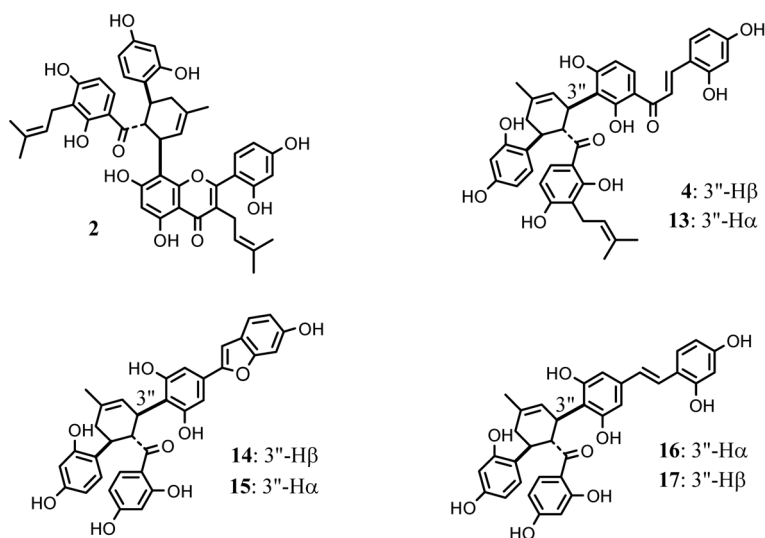
Fig. 4.   Typical Diels-Alder type adducts from *Morus* root bark.
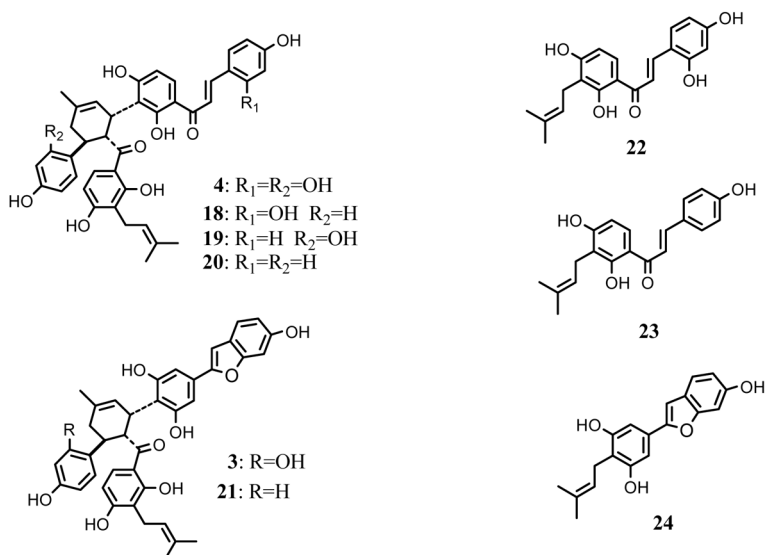
Fig. 5.   Phenolic components of *Morus alba* cell cultures.

and *cis-trans* type adducts.[10),17)] Taking these findings into account, the mulberry Diels-Alder type adducts are regarded biogenetically as [4 + 2]cyclo-adduct of a chalcone and dehydroprenylphenols.

2.4. Diels-Alder type adducts from the callus tissues of *Morus alba* L. One of our co-workers, the late Dr. Shinichi Ueda, Kyoto University, obtained pigment producing callus tissues of

Morus alba L. The callus tissues induced from seedlings were cultivated under specified conditions, and subjected to selection over a period of 9 years, giving rise to cell strains having a high-pigment productivity.[27)] From the extract of the callus tissues, six Diels-Alder type adducts, kuwanons J (**4**), Q (**18**),[31),32)] R (**19**),[31),32)] V (**20**),[31),32)] mulberrofuran E (**21**),[31),32)] and chalcomoracin (**3**),[27),33)] were isolated along with morachalcone A (**22**),[33)] isobava-chalcone (**23**)[34),*)] and moracin C (**24**)[35)] (Fig. 5). If we call morachalcone A (**22**) as A, isobavachalcone

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*) Isovabachalcone (**23**) has been isolated from *M. alba* cell cultures in our work (unpublished data).
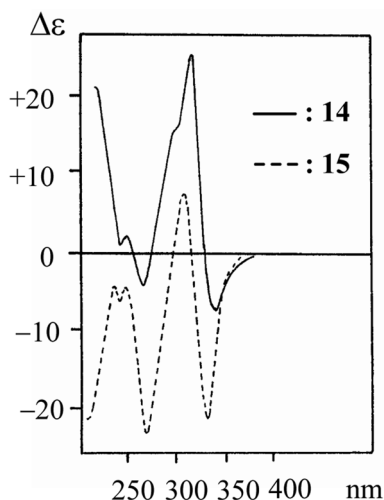
Fig. 6.   CD spectra of mulberrofurans C (**14**) and J (**15**).
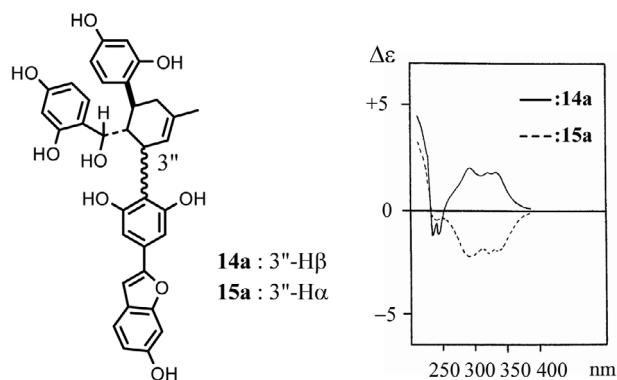

Fig. 7.   CD spectra of dihydromulberrofurans C (**14a**) and J (**15a**).

(**23**) as B and moracin C (**24**) as C, kuwanon J (**4**) is an adduct of dehydro-A with A (AA-type), kuwanon Q (**18**) is an adduct of dehydro-A with B (AB-type), kuwanon R (**19**) is an adduct of dehydro-B with A (BA-type), and kuwanon V (**20**) is an adduct of dehydro-B with B (BB-type). Furthermore, chalcomoracin (**3**) is an adduct of dehydro-C with A (CA-type), and mulberrofuran E (**21**) is an adduct of dehydro-C with B (CB-type). It is interesting that all possible combination of three monomers **22**, **23**, **24** could be isolated from *Morus alba* callus tissues. These results strongly suggest that kuwanons J (**4**), Q (**18**), R (**19**), V (**20**), mulberrofuran E (**21**), and chalcomoracin (**3**) isolated from the callus tissues are naturally occurring Diels-Alder type adducts.

3. Absolute configurations of mulberry Diels-Alder type adducts

The mulberry Diels-Alder type adducts could be divided into two groups, one of which is an all-*trans* type adduct and the other is a *cis-trans* type adduct. The all-*trans* type adduct seems to be formed by *exo*-addition in the Diels-Alder reaction of chalcone and dehydroprenylphenol, whereas the *cis-trans* type adduct seems to be formed by *endo*-addition. To confirm these points, we studied the absolute configuration of mulberry Diels-Alder type adducts by using following three different methods.[10]

3.1. Absolute configuration of mulberrofurans C and J by CD spectra.   In the CD spectra of the Diels-Alder type adducts, it is notable that the magnitude of $\Delta\varepsilon$ values in the spectra of mulberrofurans C (**14**) and J (**15**) is larger than any others in the spectra of the other compounds and that a strong split Cotton effect is observed in 280–350 nm region of both of these (Fig. 6). This property in the CD spectra seems to indicate exciton coupling.[36] As the absorption bands at 280–350 nm in the UV spectrum of **14** (or **15**) are supposed to be due to the 2,4-dihydroxylbenzoyl and 2-arylbenzofuran chromophores, one may suspect that the split Cotton effect originated from exciton coupling between the two chromophores. In order to ascertain whether this was so, **14** and **15** were reduced with LiAlH$_4$ to give the reduced products, **14a** and **15a**, respectively. In the CD spectra of **14a** and **15a**, the strong split Cotton effect, observed in **14** and **15**, disappeared and the magnitude of the $\Delta\varepsilon$ value decreased remarkably. These results clearly indicate that the strong split Cotton effect is due to exciton coupling of the 2,4-dihydroxybenzoyl and the 2-arylbenzofuran chromophores. Additionally, the CD spectra of **14a** and **15a** were mirror images of each other in the region 270–350 nm, as shown in Fig. 7. This suggests that the stereochemistries of **14** and **15** at the chiral center bearing the 2-arylbenzofuran chromophore are antipodal to each other (Fig. 7). Since both **14** and **15** exhibit a positive Cotton effect, the absolute configuration of mulberrofuran C is shown by the formula **14**, while that of mulberrofuran J is shown as formula **15** (Fig. 8).[10]

3.2. Absolute configuration of the chiral centers on the cyclohexene ring of kuwanon L. Kuwanon L (**25**) is regarded as a Diels-Alder type adduct of dehydroprenylflavanone and chalcone de-
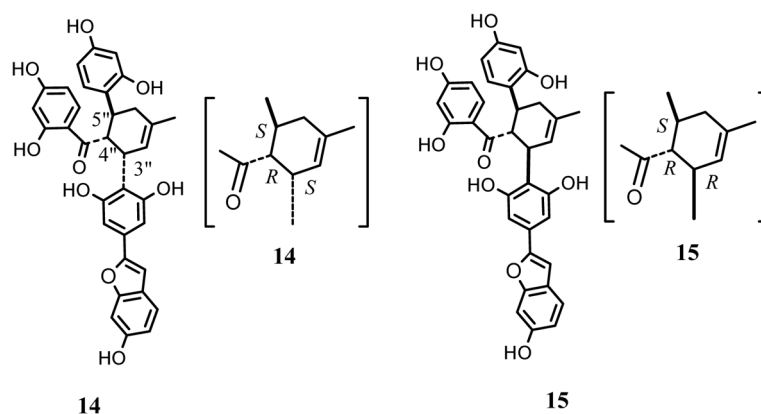
Fig. 8.   Absolute stereochemistries of mulberrofurans C (**14**) and J (**15**).
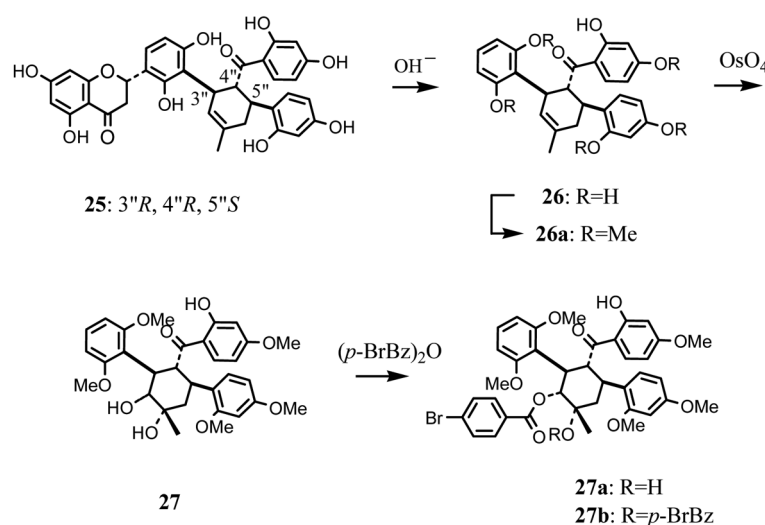

Fig. 9.   Determination of absolute stereochemistry of kuwanon L (**25**).

rivatives. The compound **25** treated with alkali gave a degradation product (**26**). Comparison of the ^{1}H-NMR and CD spectra of **26** with those of **25**, indicated that no epimerization of the chiral centers on the methylcyclohexene ring of **26** had occurred during treatment with alkali. Oxidation of the pentamethyl ether (**26a**) with osmium tetraoxide gave a *cis*-diol product (**27**) which furnished a mono-*p*-bromobenzoate (**27a**) and di-*p*-bromobenzoate (**27b**) (Fig. 9). The CD spectrum of **27b** exhibited a positive Cotton effect owing to exciton coupling between the two *p*-bromobenzoyl chromophores. Furthermore, the positive exciton coupling is shown clearly in the difference spectrum of **27a** and **27b**. Considering the possible conformation of the methylcyclohexane ring by using ^{1}H-NMR analysis, only one conformation can ac-

count for the positive Cotton effect owing to exciton coupling between two *p*-bromobenzoyl chromophores. From these results, absolute configuration of the three chiral centers of kuwanon L may be specified as 3''*R*, 4''*R*, 5''*S* (Fig. 9).[37] This result is in agreement with the result obtained from the CD spectra of mulberrofurans C (**14**) and J (**15**) described in the former section.

3.3. Absolute configuration of mulberrofurans C by X-ray crystallographic analysis. Takasugi *et al.* isolated a phytoalexin from diseased mulberry tree and designated it as chalcomoracin (**3**).[33] On the other hand, we isolated mulberrofurans C (**14**) and G (**28**)[38] as hypotensive components. Absolute configuration of **14** was confirmed from the following results. The ketalized Diels-Alder
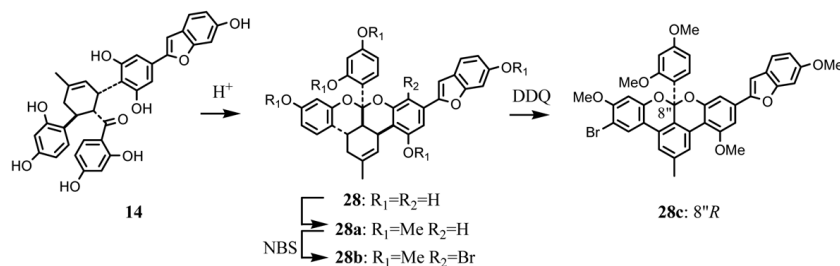
Fig. 10.   Leading process to an aromatized compound (28c) from mulberrofuran C (14) *via* mulberrofuran G (28).
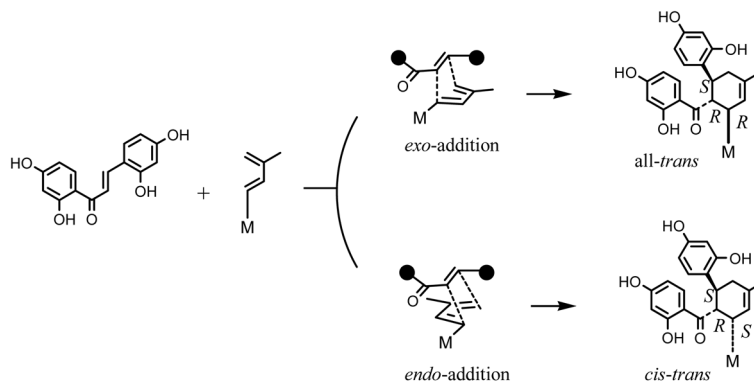

Fig. 11.   Absolute configuration of mulberry Diels-Alder type adducts.

type adduct **28** could be derived stereospecifically from the original adduct (**14**) under acidic conditions, as described in Fig. 10.[38)] The relative configurations of the four chiral centers of mulberrofuran G pentamethyl ether (**28a**) were confirmed by X-ray crystallographic analysis.[39)] Monobromomulberrofuran G pentamethyl ether (**28b**) was derived from **28a** by treatment with *N*-bromosuccinimide (NBS), and **28b** was converted to an aromatized compound **28c** through dehydrogenation by 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (Fig. 10). The X-ray crystallographic analysis of **28c** revealed that the absolute configuration of the chiral center at C-8'' is *R*.[40)] As the correlation between **28c** and **14** through **28a** was confirmed, the absolute configuration of **14** was determined to be 3''*S*, 4''*R*, 5''*S*.[40)] Furthermore, as the stereochemistry of mulberrofuran J (**15**) at the C-3'' position was determined to be antipodal to that of mulberrofuran C (**14**) by the CD spectra of related compounds, the absolute configuration of **15** then expressed as 3''*R*, 4''*R*, 5''*S* (Fig. 8). Regarding optical rotation values of mulberrofuran C (**14**), [α]$_D$ + 153°, and mulberrofuran J (**15**), [α]$_D$ − 341°, the sign of optical rotation depends on the stereochemistry of the C-3'' position. Meanwhile, other

all-*trans* type adducts showed minus values as in **15**, while *cis-trans* type adducts showed plus values as in **14**.[39)] Namely, the absolute configuration of the all-*trans* type adducts is the same as that of **15**, while that of the *cis-trans* type adducts is the same as that of **14**. Absolute configurations of the mulberry Diels-Alder type adducts were thus determined, and the adducts having all-*trans* relative configuration are *exo*-addition products in the Diels-Alder reaction, whereas the *cis-trans* type adducts are formed through *endo*-addition (Fig. 11).[40)]

4. Biosynthesis of mulberry Diels-Alder type adducts

As described in section 2.4, some cell strains of *Morus alba* callus tissues have a high productivity of the mulberry Diels-Alder type adducts. The yield of major adducts are 100∼1000 times more than those of the intact plant (Fig. 5).[27),31),32)] The biosynthesis of the mulberry Diels-Alder type adducts has been studied with the aid of the cell strains.[13),14),15)]

4.1. Administration experiment with ¹³C-labeled acetate to the *Morus alba* cell cultures. Administration of [1-¹³C]-, [2-¹³C]-, or [1,2-¹³C₂]-acetates to the *Morus alba* cell cultures revealed that
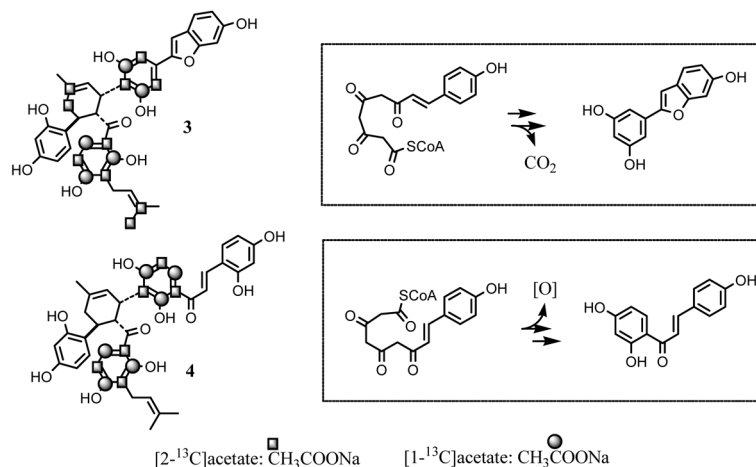
Fig. 12. ^{13}C-Labeling patterns of **3** and **4** from [1-^{13}C]- and [2-^{13}C]acetate.
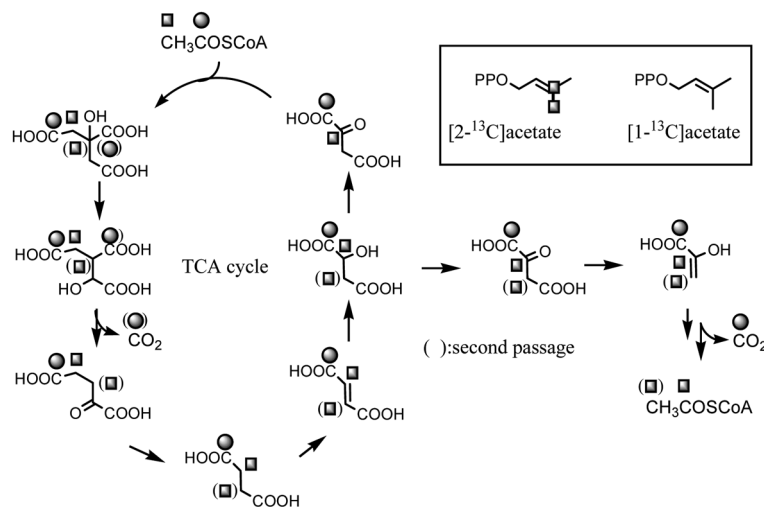

Fig. 13.   Formation of reorganized [1,2-^{13}C$_2$]acetate from exogenous ^{13}C-labeled acetate through the TCA cycle and the labeling patterns of isoprenyl units of **3**.

both kuwanon J (**4**) and chalcomoracin (**3**) are composed of two molecules of cinnamoylpolyketide skeletons.[40] Namely, the compound **4** is regarded as a dimer of isoprenylated chalcones, whereas **3** is composed of isoprenylated 2-arylbenzofuran and isoprenylated chalcone (Fig. 12). From the labeling patterns, the chalcone skeleton seems to be originated through the Claisen-type condensation of cinnamoylpolyketide and the 2-arylbenzofuran skeleton through the aldol-type condensation (Fig. 12). Administration of ^{13}C-labeled acetate to the cell cultures resulted in the highly ^{13}C enriched aromatic carbons of **3** (about 17 percent enrichment from the ^{13}C-NMR spectrum), whereas two isoprenyl units of **3**

were labeled lesser extent (about 0.4 percent enrichment). In addition, on the basis of ^{13}C-^{13}C spin-spin coupling in the ^{13}C-NMR spectrum, the labeling of [2-^{13}C]acetate takes place in the contiguous carbons at the starter acetate unit with regard to the mevalonate biosynthesis. On the other hand, incorporation of [1-^{13}C]acetate was not found in the isoprenyl units.[41] These findings suggest the participation of the tricarboxylic acid (TCA) cycle to the biosynthesis of the isoprenyl units of **3**. In the experiment with [2-^{13}C]acetate, contiguous ^{13}C atoms can be derived from the two methyl groups of the intact acetate administered by way of at least two passages through the TCA cycle. Accordingly, the acetate incorpo-
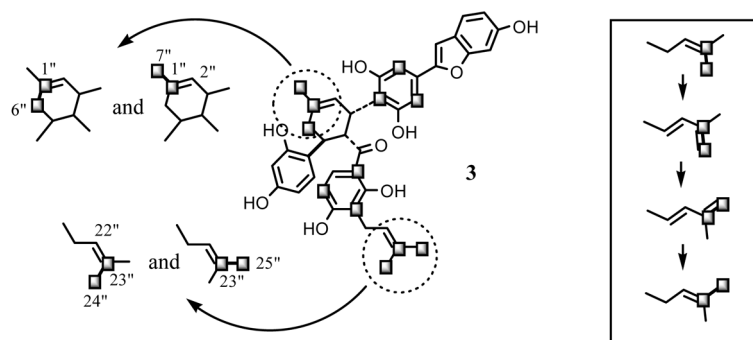
Fig. 14.   Two independent ^{13}C-labeling patterns at the isoprenyl units of **3** and the transfer of the ^{13}C-labeling from *cis*-methyl carbon to *trans*-methyl carbon through the diene formation.
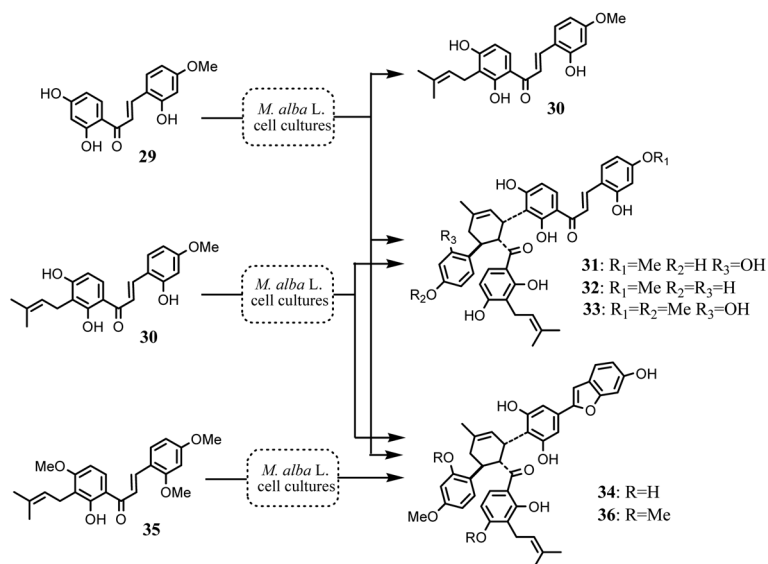

Fig. 15.   Aberrant metabolism of *O*-methylated precursory chalcones in the *Morus alba* cell cultures.

rated into the isoprenyl units of **3** was not the intact acetate administered, but [1,2-^{13}C]acetate reorganized from the methyl group of the intact acetate through the TCA cycle (Fig. 13).[41] This hypothesis was reinforced by the administration experiment with [2-^{13}C]acetate in a pulsed manner.[42] This result enable us to disclose the satellite peaks based on the ^{13}C-^{13}C spin-spin coupling between the carbons at C-25'' and 23'' as well as that between the carbons at C-7'' and C-1'', in addition to the ^{13}C-^{13}C spin coupling between C-23'' and C-24'' and that between C-6'' and C-1''. No satellite peaks were observed at C-22'' or C-2'' (Fig. 14). This result suggests that the three carbons were contiguously enriched with ^{13}C atoms. However, this assumption was ruled out

from the coupling patterns of the central carbons at C-1'' and C-23'' in the ^{13}C-NMR spectrum. These central carbons appeared as doublet signals. If the ^{13}C-labelings continuously related in sequence, the central carbons must appear as the doublet of doublet signals. The appearance of the doublet signal indicates that the central carbon is independently coupled with the two adjacent methyl carbons. The independent ^{13}C-labeling pattern at the isoprenyl group might be explained as transfer of ^{13}C-labeling from *cis*-methyl to *trans*-methyl through the diene formation. Furthermore, the phenomenon of the ^{13}C enrichment of the third acetate units found at C-7'' and C-25'', in spite of the lack of ^{13}C-labeling at both second acetate unit, can be explained by the
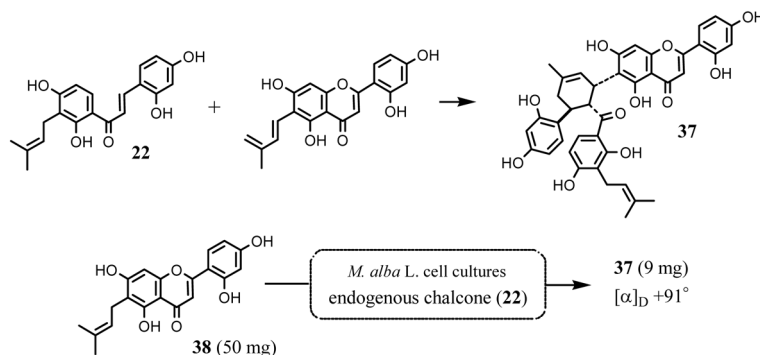
Fig. 16.   Formation of artonin I (**37**) by Diels-Alder reaction and bioconversion of artocarpesin (**38**) to artonin I (**37**) with the aid of the *Morus alba* cell cultures.

isomerization between the two 3,3-dimethylallyl and 3-methylbutadienyl groups (Fig. 14). It is note-worthy that the isomerization takes place not only at the prenyl group participating in the intermolecu-lar Diels-Alder type reaction, but also in the other isoprenyl group that remains intact. This finding gave confirmative evidence on the formation of the diene structure at the isoprenyl portion for the Diels-Alder type cyclization reaction. Thus the ad-ministration experiment with ^{13}C-labeled acetate re-vealed that the Diels-Alder type adducts kuwanon J (**4**) and chalcomoracin (**3**) are presumably biosynthe-sized through the [4 + 2]cyclization reaction between two molecules, cinnamoylpolyketide-derived skeleton and mevalonate.

4.2. Administration experiment with *O*-methylchalcone derivative. Final confirmation of the biosynthesis of the mulberry Diels-Alder type adducts was obtained by an administration experi-ment with *O*-methylchalcone derivative to the *Morus alba* cell cultures.[43] *O*-Methylated chalcone or *O*-methylated Diels-Alder type adducts have not been detected in the cell culture. Administration of *O*-methylated chalcone (**29**) to the cell cultures yielded the metabolites **30**, **31**, **32**, **33**, and **34** (Fig. 15). The formation of the chalcone (**30**) from **29** in the tissue cultures indicated that an isoprenylation takes place after the completion of chalcone skeleton. The metabolites **31**, **32**, **33** and **34** revealed that the pre-cursory chalcone (**29**) was incorporated intact into the Diels-Alder adducts. An analogous experiment employing synthesized **30** yielded the same Diels-Alder type metabolites **31**, **32**, **33** and **34** (Fig. 15). Administration of tri-*O*-methylated chalcone (**35**) afforded the Diels-Alder type metabolite **36**. These

results suggest that one molecule of isoprenylated chalcone is recognized to a dienophile at the α, β-double bond, while another molecule of the chalcone acts as a diene at the isoprenyl portion. Furthermore, the Diels-Alder metabolites from the precursory chal-cone **29**, **30** and **35** were all optically active, having the same stereochemistries as those of kuwanon J (**4**) and chalcomoracin (**3**). These results revealed that **3** and **4** have been proved to be enzymatic Diels-Alder reaction products. Artonin I (**37**) isolated from an Indonesian moraceous plant, *Artocarpus hetero-phyllus*, was considered to be formed through the Diels-Alder type reaction of a chalcone derivative, morachalcone A (**22**) and artocarpesin (**38**), as pre-cursors.[44),45)] Both **37** and **38** are not inevitably de-tected in *Morus alba* cell cultures. We attempted the synthesis of natural Diels-Alder type adduct, artonin I (**37**), with the aid of an enzyme system of *Morus alba* cell cultures. Administration of artocar-pesin (**38**) to the cell cultures resulted unusual me-tabolite being identical with **37** (Fig. 16).[46] This is the first example of the elucidation of the structure of an organic natural product by application of an enzymatic synthesis of the target substance with the aid of the cell cultures of the related plants.

4.3. Confirmation of the stage of the Diels-Alder reaction in the cell cultures. To confirm the stage of the Diels-Alder reaction in the cell cultures, the hydroxylation process of the ad-ducts was studied.[47] The incorporation of [2-13C] acetate into chalcomoracin (**3**) (about 17 percent, calculated on the basis of the 13C signal intensity against the natural abundance in the 13C-NMR spec-trum) is higher than that into kuwanon J (**4**) (about 4 percent). While **3** has the same chalcone part as **4**,
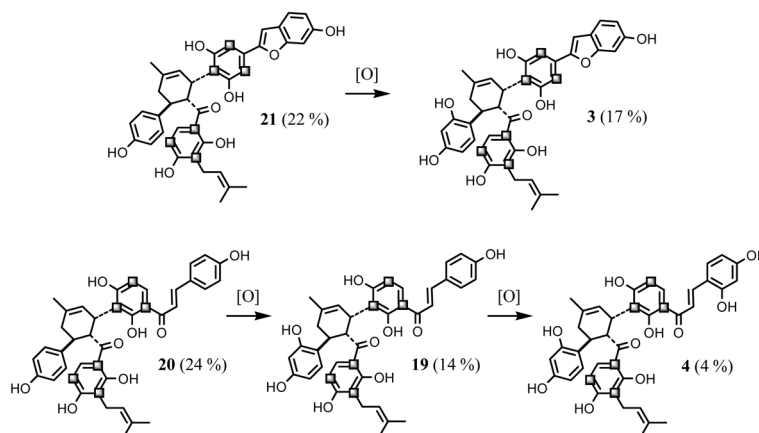
Fig. 17. ^{13}C-Enrichment of the Diels-Alder type adducts in administration experiment of [2-^{13}C]acetate and late stage of the biosynthesis of the adducts in the *Morus alba* cell cultures.

the ^{13}C-enrichment of the two compounds is very different. We examined the ^{13}C-enrichment of the Diels-Alder type adducts in the cell cultures by the feeding experiment of [2-^{13}C]acetate. The enrichment factors of these compounds are described in Fig. 17. From these results, kuwanon J (**4**) consists of two molecules of morachalcone A (**22**) with 4 percent of ^{13}C-enrichment. Similarly, kuwanon V (**20**) is composed of two molecules of isobavachalcone (**23**) with 24 percent of ^{13}C-enrichment. In the case of kuwanon R (**19**), the actual ^{13}C-enrichment was about 14 percent, and the ^{13}C-enrichment of the upper unit of **19** is the same as that of the lower unit. If **19** is biosynthesized through the respective Diels-Alder type reaction of two molecules of the chalcone derivatives **22** and **23**, the agreement of the ^{13}C-enrichment between the upper and lower units of **19** seem to be unlikely. From these results, it could be indicated that successive increases of the hydroxyl groups diminish the ^{13}C-enrichment. If kuwanon V (**20**) is initially biosynthesized followed by successive hydroxylation reaction to form **19** and then **4**, the ^{13}C-enrichment of the two chalcone parts must always be the same number in every adducts. The major adducts, kuwanon J (**4**) and chalcomoracin (**3**), in the cell cultures are presumably derived from kuwanon V (**20**) and mulberrofuran E (**21**) as "primer" of the Diels-Alder type adducts, respectively (Fig. 17).

4.4. Biosynthesis of the cinnamoyl moiety of chalcomoracin and kuwanon J. As described above, administration experiment with ^{13}C-labeled

acetates revealed that both the mulberry chalcone and 2-arylbenzofuran skeletons originate from cinnamoylpolyketide. In order to confirm the biosynthesis of the cinnamoyl moiety derived from shikimate *via* aromatic amino acid, phenylalanine or tyrosine, further experiments administering phenylalanine and tyrosine to the *Morus alba* cell cultures were carried out.[48] The ^{13}C-enrichments of a pair of carbons, C-1' and C-8'', of chalcomoracin (**3**) originating from [1-^{13}C]-L-phenylalanine and another pair of carbons, C-3 and C-5'', originating from [3-^{13}C]-L-tyrosine, were 17 and 4 %, respectively. Similarly, the incorporation of both amino acids to kuwanon J (**4**) was also observed as was found in **3**. Both L-phenylalanine and L-tyrosine, intermediates on the shikimate pathway, are thus precursory to the mulberry chalcone and 2-arylbenzofuran skeletons.[49] This finding, however, raised the question of whether or not L-phenylalanine is converted to L-tyrosine by direct hydroxylation in the *Morus alba* cell cultures. The direct conversion of L-phenylalanine to L-tyrosine has been well established in mammal cells,[50] whereas, in higher plants, two independent pathways leading to these amino acids operate.[51] The direct conversion has only been reported in the case of an enzyme system isolated from spinach leaves.[52] Further investigation of simultaneous administration of [1-^{13}C]-L-phenylalanine and [3-^{13}C]-L-tyrosine to the cell cultures revealed the parallel participation leading to the cinnamoyl moieties of **3** and **4** in *Morus alba* cell cultures. The ^{13}C-labeling pattern of the simultaneous administration experiment is described in Fig. 18. Thus
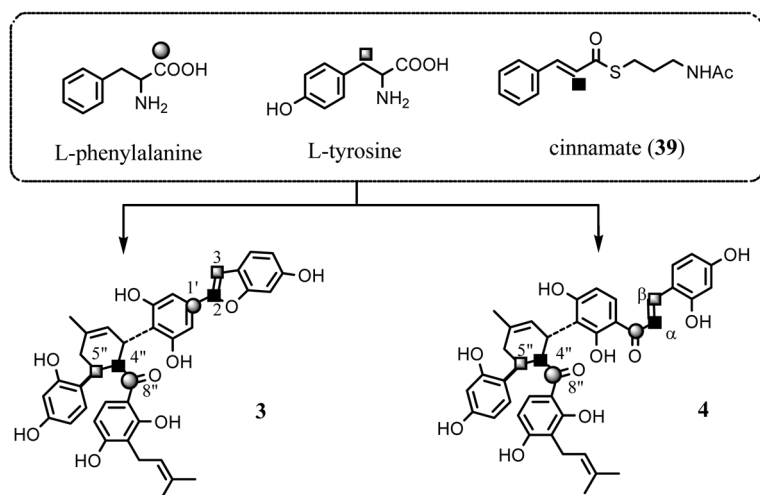
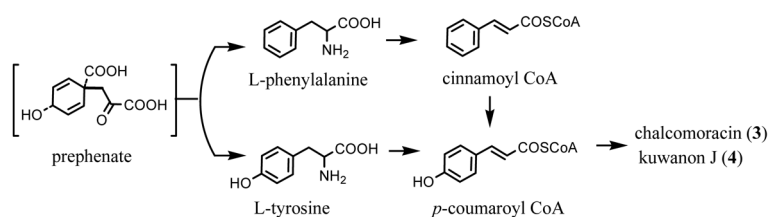Fig. 18.   Parallel contribution of L-phenylalanine and L-tyrosine to the biosynthesis of **3** and **4**.

Fig. 19.   Dual *p*-coumaroyl CoA bioisynthesis in *Morus alba* cell cultures.

L-phenylalanine and L-tyrosine are simultaneously incorporated into the isoprenylchalcone derivatives chalcomoracin (**3**) and kuwanon J (**4**) in *Morus alba* cell cultures (Fig. 19). Furthermore, the predominant contribution of [1-^{13}C]-L-phenylalanine over that of [3-^{13}C]-L-tyrosine in the biosynthesis of isoprenyl-chalcone derivatives in *Morus alba* cell cultures, thus direct conversion of L-phenylalanine to L-tyrosine is unlikely (Fig. 19). To confirm this assumption, administration experiment of [2-^{13}C]-cinnamoyl thio-ester derivative (**39**) to the cell cultures was carried out. The cinnamoyl moiety was incorporated intact into the shikimate-derived moieties of **3** and **4** (Fig. 18).[53] This result is the first example confirming parallel contribution of L-phenylalanine and L-tyrosine to the biosynthesis shikimate-derived metabolites in higher plants.

4.5. Biosynthesis of the isoprenyl unit of chalcomoracin. As described in section 4.1, the acetate incorporated into the isoprenyl units of chalcomoracin (**3**) was reconstructed acetate from the methyl group of exogenous acetate through the TCA cycle. On the basis of this novel finding, further studies with respect to the biosynthesis of the iso-prenyl unit of **3** were carried out by administering [2-^{13}C]-*dl*-mevalonate or [2-^{13}C]-L-leucine, the candi-dates for isoprenyl precursor, to the *Morus alba* cell cultures.[41] In the case of [2-^{13}C]-L-leucine, the ^{13}C-NMR spectrum of **3**, isolated from the cell cultures, indicates that the isoprenyl signals were not enriched with ^{13}C from [2-^{13}C]-L-leucine, whereas polyketide-derived aromatic carbons were enriched. The label-ing pattern from [2-^{13}C]-L-leucine was the same as that from [1-^{13}C]acetate, but the level of ^{13}C-enrich-ment from L-leucine was about one-fifth of that from [1-^{13}C]acetate. This result indicated the [2-^{13}C]-L-leucine was metabolized in *Morus alba* cell cultures to [1-^{13}C]acetyl CoA, which subsequently partici-pations in triketide-synthesis (Fig. 20).[42] Such a fate for L-leucine has been reported in the case of sesquiterpene paniculide biosynthesis in *Andrographis paniculata* tissue cultures.[54] *Morus alba* cell cultures also yielded β-sitosterol (**40**),[27] which is a good target for the examination of isoprenoid biosyn-
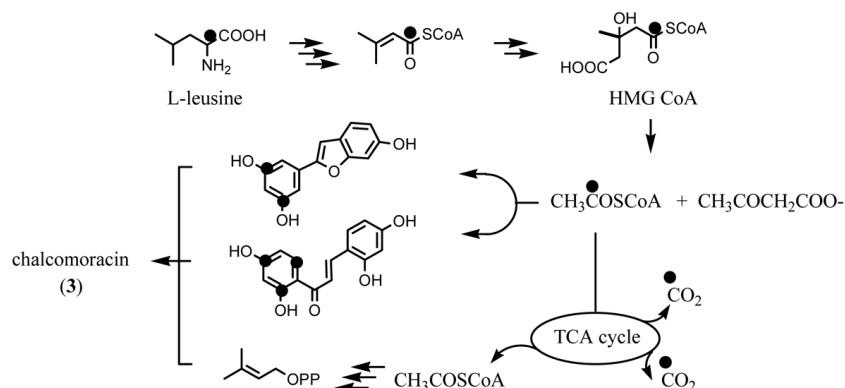
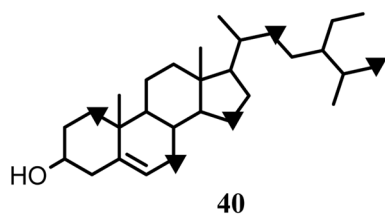Fig. 20.   The fate of L-leusine in *Morus alba* cell cultures.

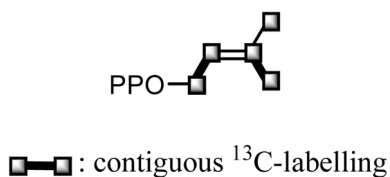Fig. 21. ^{13}C-Labeling pattern of β-sitosterol with [2-^{13}C]mevalonate (▼).

▣━▣ : contiguous ^{13}C-labelling

Fig. 22. ^{13}C-Labeling pattern on the IPP unit for **3** from [U-^{13}C]-D-glucose.

thesis from isoprenyl precursors in the cell cultures. Administration experiments of [1-^{13}C]-, [2-^{13}C]-, or [1,2-^{13}C$_2$]acetate to the cell cultures gave **40** along with **3** and **4** all labeled with ^{13}C. The ^{13}C-labeling pattern was in accordance with Ruzicka's biogenetic isoprene rule as was verified in the case of **40** in tissue cultures of *Rabdosia japonica*.[55] Accordingly, the exogenous acetates were incorporated into the isoprenyl unit of **40**. [2-^{13}C]-*dl*-Mevalonate was not incorporated into the isoprenyl moieties of chalcomoracin (**3**) in *Morus alba* cell cultures. On the contrary, it was incorporated into the expected positions of **40** (Fig. 21). These results suggest that the non-incorporation of mevalonate into the isoprenyl

moieties of **3** is not due to the permeability of the precursor. Thus the incorporation manner of the precursors, including acetate, into the isoprenyl units of **3** is different from that observed in **40**. It is most likely that at least two independent isoprenoid biosynthetic pathways, that for sterols and that for isoprenylphenols, operate in the *Morus alba* cell cultures.

4.6. Origin of the acetate units composing the isoprenyl units of chalcomoracin in *Morus alba* cell cultures.   We have examined the isoprenoid biosynthesis of chalcomoracin (**3**) in more detail through administration of [U-^{13}C]-D-glucose to the cell cultures provided **3** labeled with ^{13}C.[56] The ^{13}C-labeling patterns in the two isoprenyl moieties appeared to be in accordance with expected pattern based on a conventional mevalonate biosynthesis *via* 3-hydroxy-3-methylglutaryl CoA (HMG CoA) arising from three acetate units (Fig. 22). Continuous ^{13}C-labeling of [U-^{13}C]-D-glucose, however made it impossible to locate each glucosyl carbon which participates in the formation of the isoprenyl moieties.[56] Further administration of [1,3-^{13}C$_2$]- and [2-^{13}C]glycerol to the cell cultures revealed a unique ^{13}C-labeling pattern in **3**, as shown in Fig. 23, which suggests a novel isoprenyl (hemiterpene) biosynthesis.[56] In the case of formation on an acetate unit from exogenous glycerol by way of glycolysis *via* GAP and DHAP, [1,3-^{13}C$_2$]- and [2-^{13}C]glycerol are converted to [2-^{13}C]acetate and [1-^{13}C]acetate, respectively. The experiment with [1,3-^{13}C$_2$]glycerol revealed the expected enrichment at the carbon atoms in the starter acetate unit for mevalonate biosynthesis, but the ^{13}C-labelings in the second and third acetate units were reversed. A similar phenomenon was also
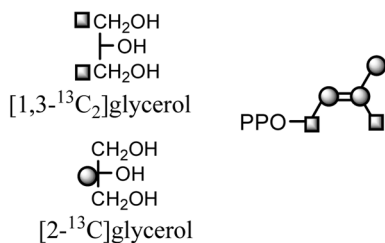
Fig. 23. ^{13}C-Labeling pattern on the IPP unit for **3** from ^{13}C-labeled glycerols.

observed in the experiment with [2-^{13}C]glycerol (Fig. 23). Reversal of ^{13}C-labeling at the second acetate and third acetate carbons in both experiments implies participation of the pentose-phosphate cycle in the biosynthesis of isoprenyl units. Both GAP and DHAP derived from [1,3-[13]C$_2$]- or [2-^{13}C]glycerol result in the formation of fructose-1,6-diphosphate, which enter into the pentose-phosphate cycle *via* glucose-6-phosphate and then phosphogluconate. The resulting sedoheptulose-7-phosphate provides erythrose-4-phosphate along with GAP with reversed ^{13}C-labeling compared to the initial GAP. Acetyl-CoA derived from the resultant GAP is incorporated into the isoprenyl unit of chalcomoracin (**3**) as the second and third acetate units. With regard to the origin of the acetate units participating in the isoprenyl unit biosynthesis for **3**, it was concluded that the starter acetate unit for mevalonate synthesis is of glycolytic (Emden-Meyerhof-Parnas, EMP) pathway origin, while the second and third acetate units originate from the pentose-phosphate cycle

(Fig. 24). Each step in the mevalonate biosynthesis for **3** thus strictly requires that the acetate units have different origins in the cell cultures.[56] On the other hand, Rohmer *et al.*, in studies of bacterial polyterpenoids, proposed a novel biosynthesis of the isoprene unit.[57] The labeling patterns at the isoprene units of **3** by administering ^{13}C-labeled substrates such as acetate and glycerol were inconsistent with Rohmer's novel pathway. This result provides the first example demonstrating chimeric biosynthesis of the isoprene unit by two different pathways in a higher plant.

4.7. Response of two isoprenoid biosynthetic pathways to compactin in *Morus alba* cell cultures. Further studies of the two isoprenoid biosynthetic pathways in *Morus alba* cell cultures were carried out by response to compactin,[58] a competitive inhibitor of HMG CoA reductase isolated from *Penicillium citrinum*[59] or *P. brevicompactum*.[60] Responses of the two above-mentioned isoprenoid biosynthetic pathways to compactin were examined by administering [2-^{13}C]acetate along with compactin to *Morus alba* cell cultures, followed by examination of ^{13}C-labeling in chalcomoracin (**3**) and β-sitosterol (**40**). The ^{13}C-NMR spectrum of **40** indicated no ^{13}C-enriched signals, contrary to the regular signal enhancements at the specified position of **40** observed in the previous feeding experiment with [2-^{13}C]acetate.[42] This finding implies that the biosynthesis of mevalonate by way of HMG CoA was inhibited by the action of compactin. On the other hand, the ^{13}C-NMR spectrum of **3** indicates the ^{13}C-labeling only at the two starter acetate-
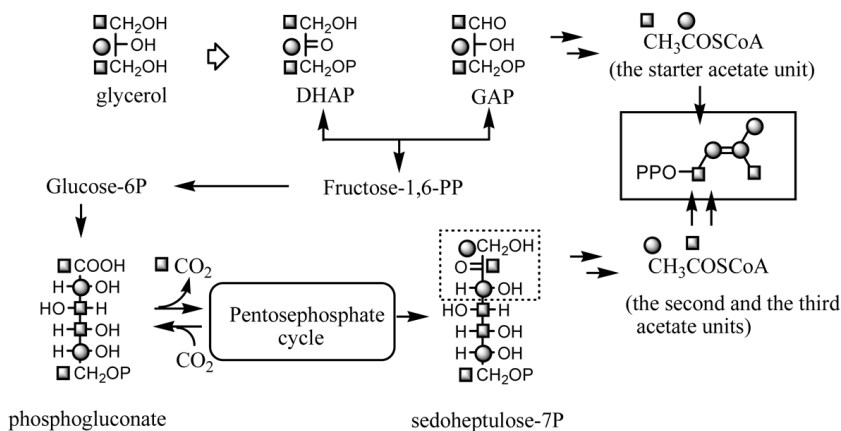

Fig. 24. IPP biosynthesis in *Morus alba* callus through junction of the glycolysis and the pentose-phosphate cycle.
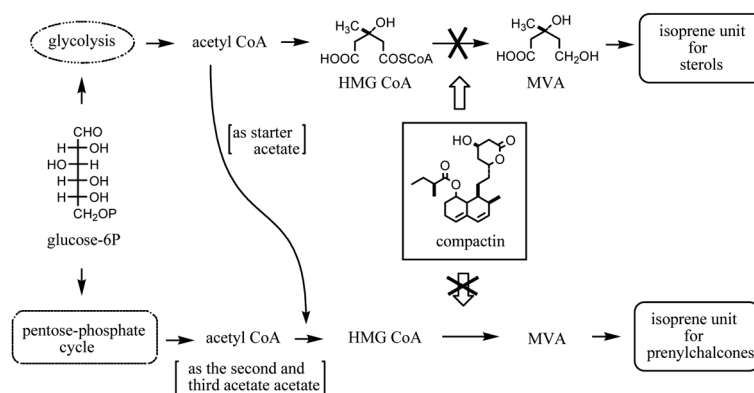
Fig. 25.   Compactin-susceptible and -resisitant mevalonate pathway in *Morus alba* cell cultures.

derived successive carbons of both isoprenyl units in addition to the two aromatic rings.[41],[42] The inhibitory action of compactin against HMG CoA reductase could thus not affect the mevalonate biosynthesis for isoprenylchalcone chalcomoracin (**3**). This work thus indicates the occurrence of at least two mevalonate biosynthetic pathways, one of which is susceptible to compactin and the other resistant to compactin in *Morus alba* cell cultures (Fig. 25).[58]

Acknowledgements

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Profile

Taro Nomura was born in 1935 in Gumma Prefecture (Japan). In 1959, he graduated from the Faculty of Pharmaceutical Sciences, Toho University, and in the same year entered the graduate school of Hokkaido University. He studied the natural product chemistry in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Hokkaido University. In 1964, he received his Ph. D. degree from Hokkaido University under the direction of the late Professor Hiroshi Mitsuhashi. He returned to Toho University as a lecture-ship in 1965. He was promoted to an Associate Professor in 1973, and to a Professor in 1979. He was Dean, Faculty of Pharmaceutical Sciences, Toho University in 1991–1994. After retirement from Toho University in 2001, he became a Professor emeritus of Toho University. He was a Professor (Organic Chemistry) of the Department of Kampo, Nihon Pharmaceutical University in 2004–2008. His research interests were the chemical studies of bioactive compounds isolated from the medicinal plants. He studied the chemistry, biosynthesis, and bioactivities of the phenolic compounds isolated from the moraceous plants and licorice. He received "The PSJ Award for Divisional Scientific Contributions 2000" from the Pharmaceutical Society of Japan. Part of his research works were summarized in the following two review articles. (1) Nomura, T. (1988) Phenolic Compounds of the Mulberry Tree and Related Plants. Progress in the Chemistry of Organic Natural Products, Vol. 53. Springer, Wien, pp. 87–201. (2) Nomura, T. and Fukai, T. (1998) Phenolic Constituents of Licorice (*Glycyrrhiza* species). Progress in the Chemistry of Organic Natural Products. Vol. 73. Springer, Wien, pp. 1–158.