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The chemistry and biosynthesis of isoprenylated flavonoids from moraceous plants*

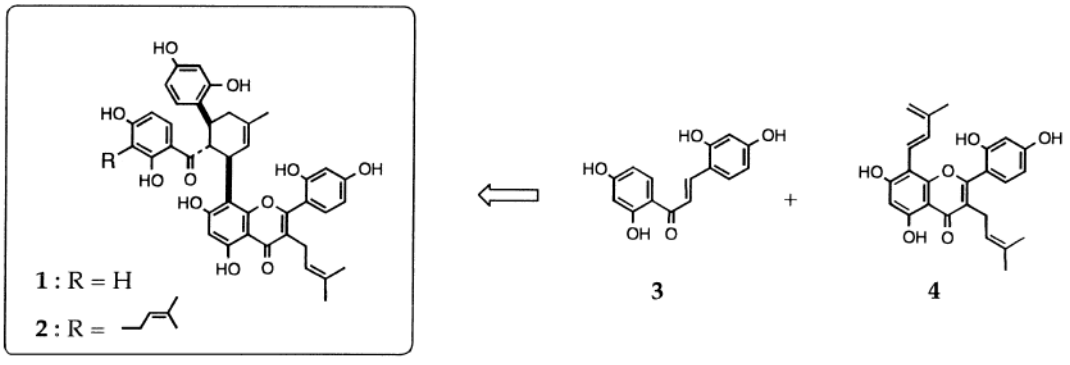
Taro Nomura†

Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan

Abstract: Many isoprenylated flavonoids have been isolated from the mulberry tree and related plants (Moraceae). Among them, kuwanons G (**1**) and H (**2**) were the first isolation of the active substance exhibiting the hypotensive effect from the Japanese *Morus* root bark. These compounds are considered to be formed through an enzymatic Diels–Alder reaction of a chalcone (**3**) and dehydrokuwanon C (**4**) or its equivalent. Since that time, about 40 kinds of Diels–Alder type adducts structurally similar to that of **1** have been isolated from the moraceous plants. Some strains of *Morus alba* callus tissues have a high productivity of mulberry Diels–Alder type adducts, such as chalcomoracin (**5**) and kuwanon J (**6**). The biosynthesis of mulberry Diels–Alder type adducts has been studied with the aid of the cell strains.

SURVEY OF ISOPRENYLATED FLAVONOIDS FROM MORACEOUS PLANTS

Mulberry tree, a typical plant of genus *Morus*, has been widely cultivated for its leaves which serve as indispensable food for silkworm. In the pharmacological field, the root bark of the mulberry tree has been used as a Chinese herbal medicine called 'Sang-Bai-Pi' (Japanese name 'Sohakuhi'), for an anti-phlogistic, diuretic, and expectorant [1]. A series of isoprenylated phenolic compounds could be isolated from the Japanese cultivated mulberry tree and Chinese crude drug 'Sang-Bai-Pi' [1–3]. Among them, kuwanons G (**1**) and H (**2**) were the first isolation of the active substances exhibiting the hypotensive effect from the Japanese *Morus* root bark [1]. Furthermore, the compound (**1**) is considered to be formed through an enzymatic Diels–Alder (D-A) type reaction of a chalcone (**3**) and dehydrokuwanon C (**4**) or its equivalent [1–3]. Since that time, about 40 kinds of D-A type adducts structurally similar to that of **1** have been isolated from moraceous plants [1–3]. Morusin (**7**), a flavove derivative, isolated from the root bark of *Morus alba*, as a main isoprenylated flavonoid, has a structure bearing an isoprenoid moiety at the C-3 position and a 2′,4′-dioxygenated pattern in the B ring [1]. These features are one of the characteristics of the isoprenylated flavonoids of *Morus* root bark (Scheme 1) [1].



Scheme 1

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† *Correspondence*: E-mail: nomura@phar.toho-u.ac.jp

Furthermore, from the Chinese crude drug 'Sang-Bai-Pi', the isoprenylated flavonoids such as sanggenon A (**8**), could be isolated [1]. Some of the flavonoids are the sanggenon A type flavanones, 2-isoprenyl-3-hydroxyflavanone having an ether linkage between C-3 and C-2′ positions [4]. Sanggenon C (**9**), one of the components of hypotensive constituents, seems to be a D-A type adduct of chalcone derivative and dehydroprenylphenol having a sanggenon A type partial structure [1,4].

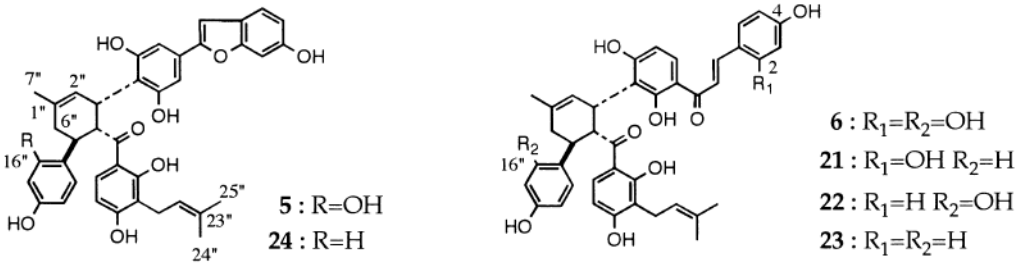
On the other hand, the plants of *Artocarpus* species have been used as traditional folk medicine. Many kinds of isoprenylated flavonoids have been isolated from *Artocarpus* species by Venkataraman's group, our group, and other several groups [5]. The flavonoids, except some ones, have a structure bearing an isoprenoid side chain at the C-3 position of flavone skeleton, and the B ring has a 2, 4, 5,-trioxygenated pattern, such as artonin E (**10**) [5]. In addition to the feature, some of the flavones, such as artobiloxanthone (**11**) has a unique structure having the C–C linkage between an isoprenoid side chain at the C-3 position and the 6′-carbon of the B ring of flavone skeleton [5].

As described above, kuwanons G (**1**) and H (**2**) caused decrease of arterial blood pressure in a dose-dependent and reversible manner at the dose of between 0.1 and 3 mg/kg i.v. in pentobarbital-anesthetized rabbit [1]. Other D-A type adducts, such as mulberrofurans C (**12**), G (**13**), and F (**14**) as well as sanggenons C (**9**) and D (**15**), showed the hypotensive actions in rodents [1]. Furthermore, kuwanon H (**2**) was the most potent of nonpeptide bombesin receptor antagonist that had been reported [6]. Morusin (**7**) was reported as an anti-tumor promoter [7]. Kuwanon E (**16**) showed the potent testosterone 5α-reductase inhibitory activity (IC_{50} 6.9×10^{-7} M) [8]. Some of the isoprenylated flavonoids from *Artocarpus* species showed some kinds of biological activities, such as inhibitory effect on mouse TNF-α release and 5-lipoxygenase, cytotoxicity, anti-bacterial activity against cariogenic bacteria. Particularly, artonin E (**10**) was the potent inhibitor of arachidonate 5-lipoxygenase and mouse TNF-α release, and showed cytotoxic activities against mouse L-1210 and colon 38, which was stronger than TFU [5].

STRUCTURES OF THE MULBERRY DIELS–ALDER TYPE ADDUCTS

In the case of kuwanon G (**1**), dehydrokuwanon C (**4**) can be regarded as a diene and a chalcone derivative (**3**) as a dienophile. As a model reaction, D-A reaction of *trans*-chalcone (**17**) and 3-methyl-1-phenylbutadiene (**18**) was carried out to give two cycloproducts, one of which is all-*trans* (**19**) in relative configuration among three substituents on the methylcyclohexene ring and another is *cis-trans* (**20**) in relative configuration [1,9]. The structure of **1** was confirmed by the chemical and spectroscopic evidence as well as by the result of model reaction [1,9].

On the other hand, the callus tissues of *M. alba* induced from the seedlings or the leaves were cultured, giving rise to cell strains having a high-pigment productivity. From the callus tissues, six D-A type adducts, chalcomoracin (**5**), kuwanons J (**6**), Q (**21**), R (**22**), V (**23**) and mulberrofuran E (**24**), were isolated along with morachalcones A (**25**) and B (**26**), and moracin C (**27**) [1,2]. It is interesting that all possible combinations of these monomers (**25**, **26**, **27**) could be isolated (Scheme 2).



Scheme 2

Absolute stereochemistries of the D-A type adducts were confirmed by CD spectroscopic evidence and by X-ray crystallographic analysis [1]. From these results, the adducts having all-*trans* relative configuration are *exo*-addition products in D-A reaction, while the *cis-trans* type adducts are formed through the *endo*-addition [2,3].

BIOSYNTHESIS OF THE MULBERRY DIELS–ALDER TYPE ADDUCTS BY USING MORUS ALBA CELL CULTURES

Examination of biosynthesis of the mulberry D-A type adducts is very important, because of no evidence for the biological intermolecular D-A reaction until today. The biosynthesis of the mulberry D-A type adducts has been studied with the aid of the *M. alba* cell cultures. Administration experiments of [1-^{13}C]- and [2-^{13}C]acetate revealed that chalcomoracin (**5**) and kuwann J (**6**) are composed of two molecules of cinnnamoylpolyketide skeletons [2]. From the labeling patterns, the chalcone skeleton seems to be originated through the Claisen type condensation of the cinnamoylpolyketide and the 2-arylbenzofuran skeleton aldol type condensation. Administration of [2–^{13}C]acetate to the cell cultures resulted that two isoprenyl units of **5** were labeled to a lesser extent (about 0.4%) than the aromatic carbons (about 17%). On the basis of ^{13}C–^{13}C spin-spin coupling, the labeling of [2-^{13}C]acetate takes place in the contiguous carbons at the starter acetate unit [2]. These findings suggest that the participation of TCA cycle to the biosynthesis of the isoprenyl unit of **5**. The 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. This hypothesis was reinforced by the administration experiment with [2-^{13}C]acetate in a palsed manner [2,3]. This result enable us to disclose the satellite peaks based on the ^{13}C–^{13}C spin-spin coupling between the carbons at C-24″ and C-23″ as well as the between the carbons at C-6″ and C-1″, in addition to the coupling between C-25″ and C-23″ and that between C-7″ and C-1″. Furthermore, the central carbons, C-1″ and C-23″, appeared as the doublet signals. These results indicate that the central carbons independently coupled with the two adjacent methyl carbons. This 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 a diene formation. These results give confirmative evidence on the formation of diene structure at the prenyl moieties of the chalcone for the D-A type cyclization [2,3,10].

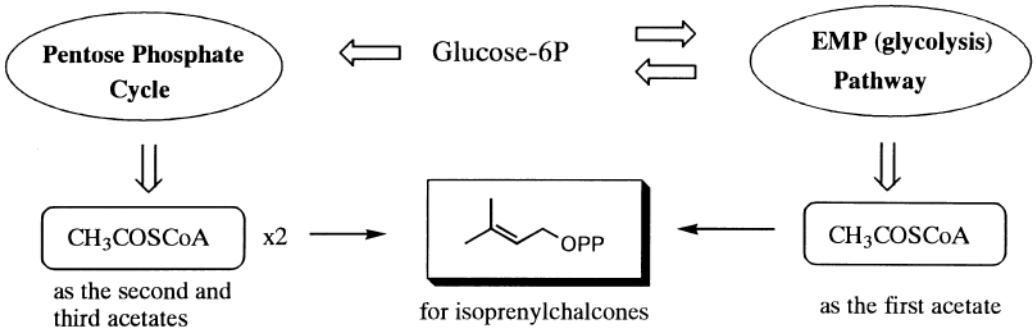
Final confirmation of the biosynthesis of the mulberry D-A type adducts was performed by the administration experiment with *O*-methylchalcone (**28**) as well as *O*-methylprenylchalcone (**29**) derivatives to the *M. alba* cell cultures [2,3,11]. Six kinds of the metabolites (**30**–**35**) were isolated, one (**30**) of which was a prenylated compound **28**. The other five metabolites were *O*-methylated derivatives of D-A type adducts from the tissue cultures. Furthermore, the D-A type metabolites (**31**–**35**) from the precursory chalcones were all optically active, having the same stereochemistries as those of **5** and **6**. These results revealed that chalcomoracin (**5**) and kuwanon J (**6**) have been proved to be enzymatic D-A type reaction products. From the above described administration experiments with precursory methoxychalcones [11], these adducts each are supposed to be independently biosynthesized through the D-A type reaction between two molecules of isoprenylphenols. On the other hand, in the feeding experiment with [2-^{13}C]acetate, the ^{13}C-enrichment between chalcomoracin (**5**) and kuwnon J (**6**) were about 17% and 4%, respectively, in spite of both having the same chalcone molecule. So we examined the ^{13}C-enrichment of the D-A type adducts in the cell cultures by the feeding experiment using [2-^{13}C]acetate [12]. Kuwanon R (**22**), which is lacking one hydroxyl group at the C-2 of **6**, showed the ^{13}C-enrichment of about 14%. Furthermore, in the case of kuwanon V (**23**), which is further lacking one hydroxyl group at the C-16″ of **22**, the ^{13}C-enrichment was about 24%. The ^{13}C-enrichment was inverse proportion to the number of hydroxyl group. Similar phenomenon was observed in **5** and mulberrofuran E (**24**), in which the ^{13}C-enrichment of **24** was about 22% to 17% of **5**. In the chalcone-chalcone type D-A type adducts, **6**, **22**, and **23**, the two chalcones composing one molecule of the adduct are always enriched with the same degrees of the ^{13}C. A possible explanation on this fact is that foremost biosynthesis of lesser hydoxylated adduct, **23**, in the cell cultures followed by successive hydroxylation lead to the formation of **22** and then **6**. On the other hand, in the 2-arylbenzofuran type adducts, **5** would be formed by the hydroxylation at the C-16″ of **24** primarily biosynthesized in the cell cultures.

ISOPRENOID BIOSYNTHESIS OF ISOPRENYLATED FLAVONOIDS IN *MORUS ALBA* CELL CULTURES

Studies with isoprenoid biosynthesis were carried out by administering *d,l*-[2-^{13}C]mevalonolactone or L-[2-^{13}C]leucine to the *M. alba* cell cultures [2,10]. In both experiments, no ^{13}C-incorporation was observed at the isoprenyl carbons of chalcomoracin (**5**). Furthermore, β-sitosterol (**36**), co-occurring in the cell cultures, was the next target for examination of the isoprenoid biosynthesis. The ^{13}C-labeling of **36** with

[1-^{13}C]- or [2-^{13}C]acetate was in accordance with Ruzicka's biogenetic rule [10,13]. *d,l*-[2-^{13}C]Mevalono-lactone was incorporated into the expected positions of **36** [10].

For further examination of the biosynthesis of **5**, D-[U-^{13}C]glucose was administered to the cell cultures [14]. The labeling patterns at the isoprenyl moieties of **5** seemed to be derived from the mevalonate pathway. However, administration of [1,3-$^{13}C_2$]- and [2-^{13}C]glycerols gave the unexpected labeling patterns on the isoprenyl moieties of **5** [14]. These patterns excluded the participation of the TCA cycle to the isoprene biosynthesis [14]. The ^{13}C-labeling was incorporated into the starter acetate units as expected. On the other hand, the ^{13}C-labeling pattern in the second and the third acetate units for the mevalonate biosynthesis was reversed. This reversal phenomenon is explainable when the pentose-phosphate cycle takes part in the biosynthesis. Regarding the three acetate units constituting the hemiterpene moieties of **5**, the starter acetate unit comes from the glycolytic pathway, while the second and the third acetate units come from the pentose-phosphate cycle. As described above, the mulberry cell cultures operate two independent isoprene biosynthesis. One is conventional for **36**, the other is a novel way for isoprenylflavonoids through the junction of the glycolysis and the pentose-phosphate cycle [14]. This assumption was supported by the response of the two pathways in the cell cultures to compactin, competitive inhibitor of HMG-CoA reductase (Scheme 3) [15].



Scheme 3

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