香川大学農学部学術報告　第76巻　1～6，2024

Biosynthetic pathway of dehydroanthrasesamone B and related anthraquinone derivatives in hairy roots of sesame（*Sesamum indicum*）

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Abstract

In order to investigate the biosynthesis of dehydroanthrasesamone B and its related anthraquinone derivatives in hairy roots of sesame（*Sesamum indicum* L.），^{2}H-labeled MPAQ was prepared and fed to the sesame hairy root cultures. Efficient conversion of fed MPAQ to（*Z*）-MPDEAQ was observed in this feeding experiment. However, the conversion of fed MPAQ to dehydroanthrasesamone B, anthrasesamone B and 2,3-epoxyanthrasesamone B was not observed. These results demonstrated that MPAQ is the actual intermediate for the production of（*Z*）-MPDEAQ and that（*Z*）-MPDEAQ is produced via dehydrogenation of the monoene side chain in MPAQ. Moreover, dehydroanthrasesamone B may be produced via dehydrogenation of the monoene side chain in anthrasesamone B, but not via hydroxylation of the anthraquinone ring in（*Z*）-MPDEAQ.

Key words：*Sesamum indicum*, hairy root culture, biosynthesis, dehydroanthrasesamone B

Introduction

Sesame（*Sesamum indicum* L., Pedaliaceae）is a cultivated species that is one of the most important crops throughout the world, and its seeds have been utilized for millennia as an oilseed and food material[1].

We have found that sesame roots and hairy roots produce several anthraquinone derivatives such as 2-（4-methylpent-3-en-1-yl）anthraquinone（MPAQ），2-［（*Z*）-4-methylpenta-1,3-dien-1-yl］anthraquinone（（*Z*）-MPDEAQ），anthrasesamone B and 2,3-epoxyanthrasesamone B（Fig. 1）[2-5]. These anthraquinone derivatives have the same carbon skeleton, which consists of a branched C_6 side chain at C-2 in the anthraquinone ring. Therefore, the biogenetic conversion of MPAQ to other anthraquinone derivatives was previously deduced from their chemical structures. In order to elucidate the intermediacy of MPAQ in the biosynthesis of their anthraquinone derivatives by the sesame hairy roots, the feeding experiment using ^{2}H-labeled MPAQ was carried out and demonstrated that MPAQ possessing the monoene side chain is the actual intermediate for the biosynthesis of（*Z*）-MPDEAQ possessing the diene side chain[6]. Contrary to our presumption, however, MPAQ was not converted to the anthraquinone derivatives possessing the hydroxylated anthraquinone ring such as anthrasesamone B and 2,3-epoxyanthrasesamone B, suggesting that these anthraquinone derivatives may be formed without passing through MPAQ[6]. In contrast, dehydroanthrasesamone B has the same side chain containing the conjugated diene moiety as that in（*Z*）-MPDEAQ and the same anthraquinone ring containing the hydroxy groups as that in anthrasesamone B[7]. The formation of dehydroanthrasesamone B via dehydrogenation of the monoene side chain in anthrasesamone B or via hydroxylation of the anthraquinone ring in（*Z*）-MPDEAQ was deduced from their chemical structures, but biosynthetic investigation has not been made. We therefore undertook the feeding experiment with ^{2}H-labeled MPAQ（Fig. 1）in order to obtain further evidence for the biosynthetic relationships among anthraquinone derivatives produced by sesame hairy roots.

Materials and methods

General

^{1}H and ^{13}C NMR spectra were measured with a JEOL JNM-ECA600 FT NMR spectrometer in $CDCl_3$ at 600 MHz for ^{1}H and at 150 MHz for ^{13}C. NMR chemical shifts were referenced to the solvent（δ_H 7.24, δ_C 77.0）. The deuterium content of individual hydrogen atoms was estimated by comparison of the proton signal integrals between the ^{2}H-labeled and unlabeled positions in each compound. Mass spectrum was obtained with a JEOL JMS-700 mass spectrometer. Silica gel（70–230 mesh, Nacalai Tesque, Japan）and Sephadex LH-20（GE

Healthcare Bio-Sciences AB, Sweden) were used for column chromatography. All experimental procedures were carried out in the dark or under as low light condition as possible, because some anthraquinone derivatives, in particular (*Z*)-MPDEAQ and dehydroanthrasesamone B, are unstable to light.

Synthesis of ^{2}H-labeled MPAQ

A solution of ammonium cerium (IV) sulfate dihydrate (7.59 g) in 2 M H_2SO_4 (100 mL) was added to [$^{2}H_8$]naphthalene (273 mg, 99 atom% ^{2}H, Sigma-Aldrich, USA) in MeCN (80 mL) and 2 M H_2SO_4 (20 mL)[8]. The solution was stirred overnight at room temperature. The reaction mixture was poured into H_2O (200 mL) and treated with EtOAc (50 mL × 4). The EtOAc solution obtained was washed with saturated NaCl (100 mL × 2), dried over Na_2SO_4 and concentrated to dryness under reduced pressure to afford [$^{2}H_6$]-1,4-naphthoquinone (315 mg, 96% yield).

[5,6,7,8-$^{2}H_4$]MPAQ was synthesized according to the method used for the synthesis of unlabeled MPAQ[3, 9]. A solution of [$^{2}H_6$]-1,4-naphthoquinone (151 mg) and β-myrcene (277 mg, 72% purity, Nacalai Tesque) in dry toluene (10 mL) was allowed to stand for 5 days at 60℃. A solution of 1 M KOH in MeOH (5 mL) was added to the reaction mixture at room temperature, and the solution was stirred vigorously for 90 min without sealing. The reaction mixture was poured into H_2O (50 mL) and treated with EtOAc (25 mL × 3). The EtOAc solution obtained was washed with saturated NaCl (40 mL × 2) and concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel column by elution with EtOAc-hexane (5:95, v/v). The fractions containing ^{2}H-labeled MPAQ were combined and further purified by recrystallization from EtOAc-hexane to give ^{2}H-labeled MPAQ (210 mg, 78% yield). ^{1}H NMR ($CDCl_3$): δ_H 1.52 (3H, br.s, H-6'), 1.66 (3H, br.s, H-5'), 2.36 (2H, br.dt, *J* = 7.2, 7.7 Hz, H-2'), 2.79 (2H, t-like, J = 7.7 Hz, H-1'), 5.13 (1H, tqq, *J* = 7.2, 1.4, 1.4 Hz, H-3'), 7.58 (1H, dd, *J* = 7.9, 1.8 Hz, H-3), 8.10 (1H, d, *J* = 1.8 Hz, H-1), 8.20 (1H, d, *J* = 7.9 Hz, H-4). Two residual ^{1}H signals were observed at ^{2}H-labeled positions (<1 atom% ^{1}H): δ_H 7.77 (H-6 and H-7), 8.29 (H-5 and H-8). ^{13}C NMR ($CDCl_3$): δ_C 17.7 (C-6'), 25.7 (C-5'), 29.4 (C-2'), 36.3 (C-1'), 122.7 (C-3'), 127.0 (C-1), 127.4 (C-4), 131.5 (C-4a), 133.1 (C-4'), 133.5 (C-9a), 133.6 (C-8a), 133.6 (C-10a), 134.5 (C-3), 149.6 (C-2), 183.1 (C-10), 183.5 (C-9). Four ^{13}C signals at ^{2}H-labeled positions (C-5, C-6, C-7 and C-8) were not observed. HR-EIMS *m/z* (M^+): calculated for $C_{20}{}^{1}H_{14}{}^{2}H_4O_2$, 294.1558; found, 294.1567.

Plant material

Hairy roots of *S. indicum* were induced by directly infecting axenic seedlings with *Agrobacterium rhizogenes* ATCC 15834[10]. The established hairy root clone (SI-16) were maintained in a phytohormone-free Gamborg B5 liquid medium[11] containing 2% glucose (pH 6.1) at 25℃ in the dark on a rotary shaker at 70 rpm at intervals of 14–20 days.

Feeding of ^{2}H-labeled MPAQ and isolation of anthraquinone derivatives

The hairy roots (about 0.3 g fresh weight) were cultured in

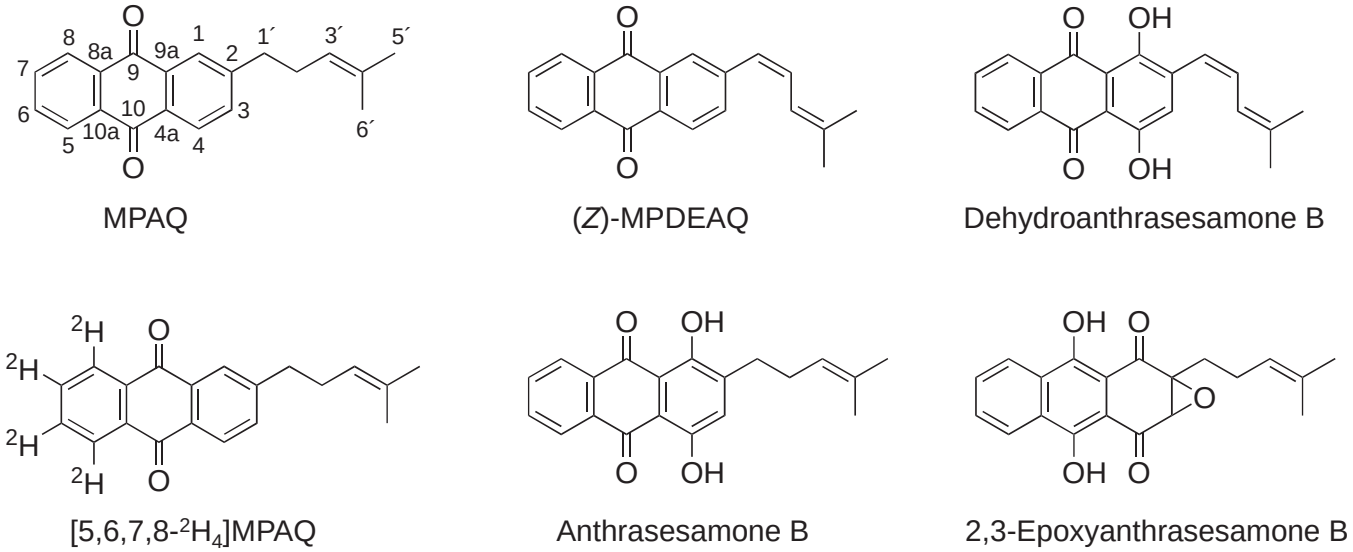

Fig. 1 Structures of MPAQ, (*Z*)-MPDEAQ, anthrasesamone B, 2,3-epoxyanthrasesamone B and dehydroanthrasesamone B.

a 100-mL conical flask containing 50 mL of the Gamborg B5 liquid medium supplemented with 2% sucrose at 25℃ in the dark at 70 rpm. Twenty flasks (50 mL×20) containing hairy roots that had been grown for 14 days were used for this experiment. The old medium was removed from each flask, and the hairy roots remaining in the flask were washed with sterile H_2O (25 mL×2). The fresh B5 medium (50 mL) supplemented with 0.4% sucrose was added to each flask. ^{2}H-labeled MPAQ (1 mg) dissolved in EtOH (0.12 mL) was then fed to each flask containing the fresh medium and washed hairy roots. The hairy roots were cultured under same condition for further 14 days with ^{2}H-labeled MPAQ.

The hairy roots and secreted metabolites were separated from the culture medium by being passed through filter paper (No. 2, Advantec, Japan). The harvested hairy roots and filter papers were lyophilized and then sonicated for 30 min in MeOH (125 mL). This extraction procedure was conducted three times. The combined MeOH solution was evaporated to dryness. The MeOH extract was then partitioned between CH_2Cl_2 (100 mL×3) and H_2O (100 mL) to give a CH_2Cl_2-soluble fraction (294 mg). This fraction was subjected to silica gel column chromatography, using stepwise elution with 0%, 5% and 10% acetone-hexane containing 0.1% AcOH (v/v/v). The 5% acetone fraction (60 mg) was purified by Sephadex LH-20 column chromatography eluted with MeOH-CH_2Cl_2 (1:1, v/v). The fractions containing the metabolites (30 mg) were further purified by preparative reversed-phase HPLC in the following conditions: column, Cosmosil 5C_{18}-AR-II, 250×10 mm i.d. (Nacalai Tesque); mobile phase, MeOH-AcOH (100:0.2, v/v); flow rate, 1.5 mL/min; detection, 254 nm; temperature; 28–30℃. Each peak in the HPLC separation was collected and concentrated to afford dehydroanthrasesamone B (0.5 mg) together with MPAQ (10 mg), anthrasesamone B (0.5 mg), 2,3-epoxyanthrasesamone B (2 mg) and (*Z*)-MPDEAQ (5 mg). Identification of the isolated anthraquinone derivatives was confirmed by comparing their ^{1}H NMR spectral data with those previously reported[2–5] (data not shown), as well as by their chromatographic behaviors in the HPLC.

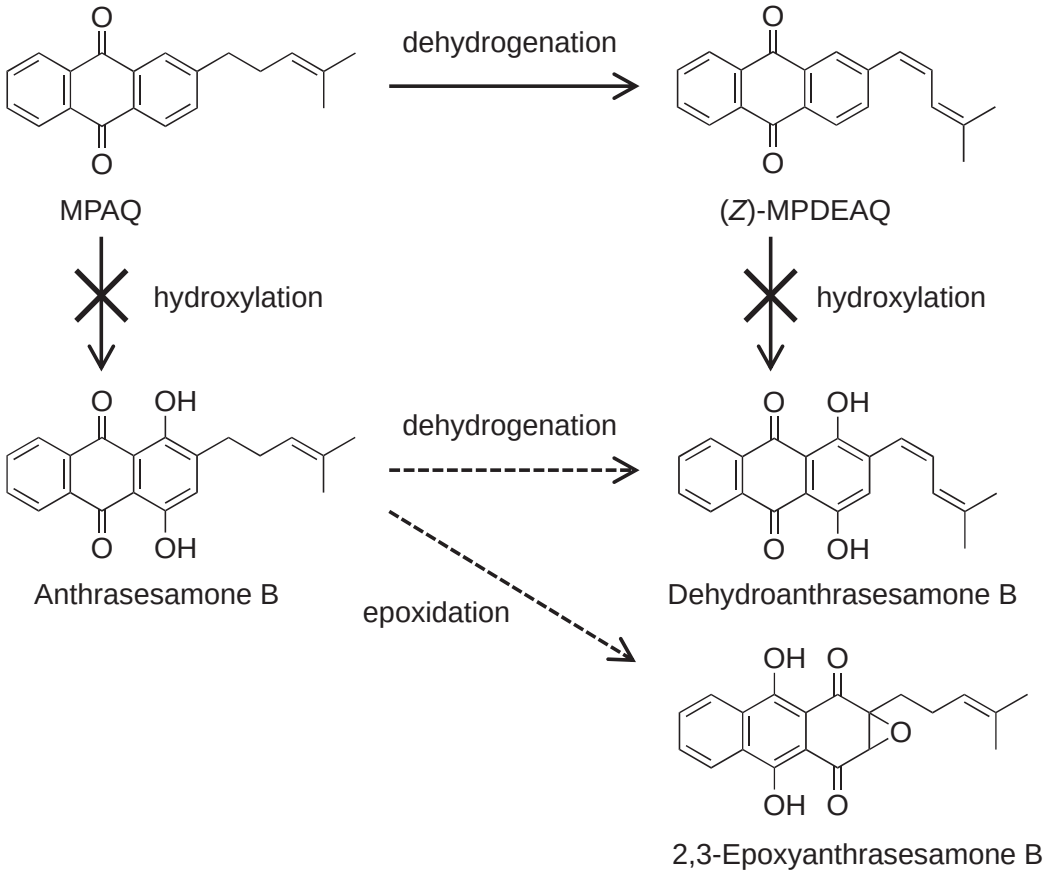

Fig. 2 Proposed biosynthetic pathway of dehydroanthrasesamone B and its structurally related anthraquinone derivatives in sesame hairy roots.

Results and discussion

^{2}H-labeled MPAQ (Fig. 1) was synthesized by the Diels-Alder cycloaddition reaction of ^{2}H-labeled 1,4-naphthoquinone and β-myrcene and subsequent aromatization of the adduct[3, 9]. ^{2}H-labeled MPAQ (>99 atom% ^{2}H) was fed to a two-week-old hairy root culture of sesame. After two weeks of incubation with ^{2}H-labeled MPAQ, anthraquinone derivatives including dehydroanthrasesamone B were extracted and isolated as described the Materials and methods section. In order to confirm whether fed ^{2}H-labeled MPAQ serves as the substrate for the isolated anthraquinone derivatives, the ^{1}H-NMR spectra of these isolated anthraquinone derivatives were measured.

The ^{1}H NMR spectrum of isolated (*Z*)-MPDEAQ showed that two proton signals at 7.78 ppm (H-6 and H-7) and 8.30 ppm (H-5 and H-8) had considerably decreased intensities in comparison with other signals at the unlabeled positions. The estimated content of deuterium atoms at the ^{2}H-labeled positions in isolated (*Z*)-MPDEAQ was 90 %, indicating that (*Z*)-MPDEAQ was efficiently converted from exogenously fed ^{2}H-labeled MPAQ rather than endogenously produced MPAQ. On the other hand, the conversion of ^{2}H-labeled MPAQ to anthrasesamone B and 2,3-epoxyanthrasesamone B was not observed in the ^{1}H NMR spectra of isolated anthrasesamone B and 2,3-epoxyanthrasesamone B, indicating that exogenously fed ^{2}H-labeled MPAQ was not utilized for the production of these hydroxylated anthraquinone derivatives. Incidentally, dilution of fed ^{2}H-labeled MPAQ by endogenously produced MPAQ was excluded, because recovered MPAQ was still satisfactorily enriched with the deuterium atoms (90%). These findings in the present study were similar to the previous results[6].

Dehydroanthrasesamone B has the same side chain containing the conjugated diene moiety as that in (*Z*)-MPDEAQ and the same anthraquinone ring containing the hydroxy groups as that in anthrasesamone B[7]. In the ^{1}H NMR spectrum of isolated dehydroanthrasesamone B, the conversion of ^{2}H-labeled MPAQ to dehydroanthrasesamone B was not observed. The fact indicates that dehydroanthrasesamone B was not produced from MPAQ. Moreover, this finding also suggests that dehydroanthrasesamone B was not produced from (*Z*)-MPDEAQ, because (*Z*)-MPDEAQ obtained in this study was satisfactorily enriched with the deuterium atoms (90%) as described above.

On the basis of the above findings, we presumed the biosynthetic pathway of dehydroanthrasesamone B and its structurally related anthraquinone derivatives to be as shown in Fig. 2. Dehydrogenation of the monoene side chain in MPAQ to form the diene side chain certainly leads to the production of (*Z*)-MPDEAQ. In contrast, dehydroanthrasesamone B may be produced via dehydrogenation of the monoene side chain in anthrasesamone B, but not via hydroxylation of the anthraquinone ring in (*Z*)-MPDEAQ. In addition, 2,3-epoxyanthrasesamone B may be produced via epoxidation of the anthraquinone ring in anthrasesamone B. The difference in the biosynthetic pathways among anthraquinone derivatives produced by sesame hairy roots, in particular the identification of the intermediates for the production of hydroxylated anthraquinone derivatives such as anthrasesamone B, 2,3-epoxyanthrasesamone B and dehydroanthrasesamone B, is an interesting topic under investigation.

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ゴマ毛状根において生産されているデヒドロアントラセサモンBと関連するアントラキノン系化合物の生合成経路

古本敏夫・佐藤龍太

要　約

ゴマは，その根や毛状根（培養根）において共通の炭素骨格を有するアントラキノン系化合物（アントラセサモン類）を生産していることが知られている．これらのアントラキノン系化合物のうち，デヒドロアントラセサモンBは，(Z)-MPDEAQと同じジエン側鎖とアントラセサモンBと同じヒドロキシ化されたアントラキノン環を有することから，その生合成経路に興味が持たれた．本研究では，デヒドロアントラセサモンBと他のアントラキノン系化合物との生合成的関連を明らかにするために，重水素標識したMPAQのゴマ毛状根培養への投与実験を行った．ゴマ毛状根による投与したMPAQからの（Z)-MPDEAQへの変換は確認されたが，アントラセサモンBとそのエポキシ体への変換は確認されなかった．また，デヒドロアントラセサモンBへの変換も確認されなかった．以上の結果から，デヒドロアントラセサモンBは，(Z)-MPDEAQのアントラキノン環がヒドロキシ化されることにより生合成されているのではなく，アントラセサモンBのモノエン側鎖が脱水素化されることにより生合成されていることが示唆された．