Metabolic Products of *Aspergillus terreus*. X. Biosynthesis of Asterriquinones

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The biosynthesis of asterriquinones in *Aspergillus terreus* var. *africanus* IFO 8835 has been elucidated. Enzymatic activities for the formation of demethyl asterriquinone-D (asterriquinone synthase), prenyltransferase and methyltransferase on asterriquinone were detected in a crude extract of mycelium. Demethyl asterriquinone is biosynthesized from indolepyruvic acid and then methylation of the hydroxyl of group of the quinone moiety occurs. Prenylation proceeds simultaneously at the C-7, C-2 and N-1 positions of the indole moiety.

Keywords asterriquinone; biosynthesis; *Asperigillus terreus*; asterriquinone synthase; prenyltransfease; methyltransferase

Three types of metabolites have been isolated from *Aspergillus terreus* var. *africanus* IFO 8835.[1] The first type consists of terrein, emodin and 3,6-dihydroxytoluquinone, which are biosynthesized *via* the polyketide route. The second group consists of α-oxo-β-(*p*-hydroxyphenyl)-γ-(*m*-3,3-dimethylallyl)-γ-methoxycarbonyl-γ-butyrolactone and aspulvinones, which are metabolized *via* the shikimate route through phenylalanine or tyrosin. The last group is analogous to asterriquinones (AQs), having a bisindolylbenzoquinone skeleton. Asterriquinones have antitumar activity.[2] This paper deals with the biosynthesis of asterriquinones.

A preliminary biosynthetic study of bisindolylbenzoquinone metabolites was done with asterriquinone, which is a metabolite of *A. terreus* IFO6123.[3] It was proved that asterriquinone is derived from tryptophan and mevalonate. Based on the chemical structure, it was assumed that the fundamental skeleton of 3,6-bis(3-indolyl)-2,5-hydroxy-1,4-benzoquinone (demethyl-AQ-D) or its quinol[4] is built initially from indolepyruvic acid, which might be formed by transamination of tryptophan, followed by methylation on the hydroxybenzoquinone moiety and prenylation on the indole moiety of demethyl AQ-D.

DL-[3-^{14}C]Tryptophan (1.17×10^8 dpm) was incorporated into all the AQs and AQ derivatives (I, II, III) as shown in Table I. The time course of the production of AQs is shown in Fig. 1. These results indicated that AQ-D is formed from tryptophan and is then prenylated to afford other AQs.

In order to elucidate the metabolic relationship among AQs, the administration of AQs to living cells was carried out. But, except for conversion of demethyl AQ-D to AQ-D (methylation), AQ-C-1 (methylation and prenylation) and AQ derivatives (I, II, III) (oxidation and cyclization), no incorporation of labelled AQ into other AQs could be detected. The reason for the low interconversion of administered compounds might be the low solubility of AQs in water and/or low ability of the added compound to permeate into fungi cells.

Next, a study using the cell-free extract was performed. The 7-d-old mycelia were crushed in a mortar with sea sand and polyvinylpolypyrrolidone (PVPP) in 0.01 M Tris buffer solution containing mercaptoethanol, magnesium chloride and phenylmethylsulfonyl fluoride (PMSF). the homogenate was centrifuged ($12000 \times g$, 20 min), the supernatante was fractionated with ammonium sulfate and the precipitate obtained by salting out was redissolved in 0.01 M Tris buffer and chromatographed on Sephadex G-25. The void volume fraction was referred to as the crude enzyme. The crude enzyme solution possessed: 1) the ability to condense two

TABLE I. Incorporation of DL-[3-^{14}C]Tryptophan to Asterriquinones and Their Derivatives

Metabolites	Amount (mg)	Specific radioactivity ($\times 10^7$ dpm/mM)	Incorporation ratio (%)
AQ-A-1	4	1.14	0.07
AQ-A-2	5	1.44	0.11
AQ-A-3	4	1.42	0.11
AQ-A-4	4	1.67	0.81
AQ-B-1	49	1.71	1.35
AQ-B-2	63	1.66	1.67
AQ-B-3	21	1.66	0.64
AQ-B-4	12	1.79	0.34
AQ-C-1	140	2.17	5.62
AQ-C-2	6	2.38	0.24
AQ-D	19	5.73	2.31
I	99	2.50	4.92
II	13	2.49	0.66
III	13	1.26	3.11

AQs were isolated from mycelia cultured on 2 l of medium.

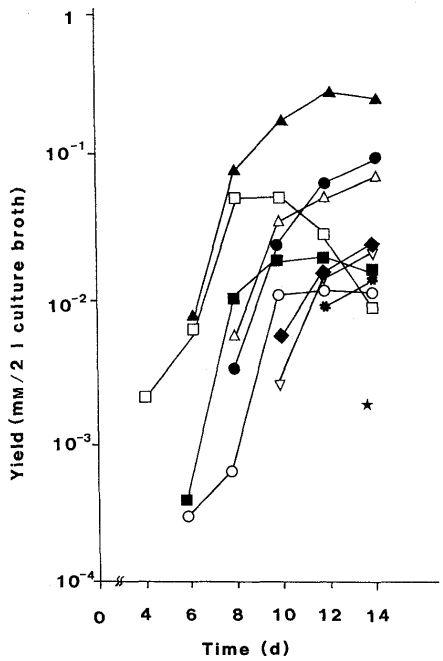

Fig. 1. Time Course of Production of Asterriquinones

AQ-A-1 (▽), AQ-A-2 (✱), AQ-A-3 (◆), AQ-A-4 (★), AQ-B-1 (△), AQ-B-2 (●), AQ-B-3 (■), AQ-C-1 (▲), AQ-C-2 (○), AQ-D (□).

molecules of indolypyruvic acid to bisindolylbenzoquinone in the presence of adenosine triphosphate (ATP) and magnesium chloride, 2) methylation activity on demethyl-AQ in the presence of *S*-adenosyl methionine (SAM), and 3) activity for prenylation of AQs in the presence of dimethylallylpyrophosphate (DMAPP).

Condensation of Indolepyruvic Acid [1-^{14}C]Indolepyruvic acid was incubated with the crude enzyme solution and ATP in 0.1 M Tris buffer (pH 8.2). The reaction was stopped by the addition of HCl and extracted with ether. The ether extract was treated with diazomethane so that clear separation of enzymatic products could be achieved on thin layer chromatography (TLC). A novel band was recognized in the middle zone on TLC compared to the heat-inactivated enzymatic system and its *Rf* value of corresponded to that of AQ-D. In the absence of ATP or magnesium chloride no enzymatic product was formed (Table II). The reaction was linear until 30 min and reached a maximum ratio at 1 h. The enzyme has an optimum pH at 8.2 and its K_m value was 0.91×10^{-4} M for indolepyruvic acid. Finally, the reaction was carried out on a large scale and the enzymatic product was purified by preparative TLC (PLC) without methylation. It was identical to demethyl AQ-D, which had not been isolated from a culture of *A. terreus* var. *africanus* IFO 8835.[5]

The biosynthesis of aromatic metabolites has attracted the attention of many researchers. Recently Yamamoto *et al.* reported novel formation of an aromatic compound[6] which might be formed by Diels–Alder reaction. But few of the enzymes concerned in the formation of aromatic metabolites have been isolated. One interesting aromatic polyketide synthase is known, namely 6-methylsalicylic acid synthase. This enzyme catalyzes the formation of 6-methylsalicylic acid from an acetyl-coenzyme A (CoA) and three malonyl-CoAs, and requires reduced nicotinamide adenine dinucleotide phosphate (NADPH),[7] but it has not been purified yet. Chalcone synthase and stilbene synthase have been purified from various plant species.[8] These enzyme catalyze the formation of aromatic metabolites from 4-coumaryl-CoA and malonyl-CoA.[8c] We detected an activity of asterriquinone synthase, but could not purify it.

Methylation of Demethyl-AQ-D Demethyl-AQ-D was methylated by the crude enzyme in the presence of SAM to give AQ-D (yield, 8%). Other demethyl-AQs (demethyl compounds of AQ-C-1, AQ-C-2 and AQ-B-1) also are methylated to afford the corresponding AQ (yields: AQ-C-1, 11%; AQ-C-1, 8%; AQ-B-1, 6%).

Prenylation Activity When AQ-D was treated with the crude enzyme solution in the presence of DMAPP, AQ-C-1, AQ-C-2 and AQ-B-3, mono-prenylated AQs, were recognized as prenylated products. AQ-C-1 gave further prenylated metabolites (AQ-B-1, -B-2, -B-4 and -A-3). AQ-C-2 was converted to mono-prenylated compounds (AQ-B-1 and AQ-B-2) and a small amount of di-prenylated products (AQ-A-2 and AQ-A-4). In this case, AQ-B-1, which was the prenylated product from AQ-C-2, was changed to AQ-A-2 and AQ-A-4, AQ-B-3 yielded to AQ-A-1 and AQ-A-3, and AQ-B-1 afforded AQ-A-2 and AQ-A-4. AQ-B-2, which is an isomer of AQ-B-1, did not change to any other AQ. AQ-B-4 changed to AQ-A-4. The results are summarized in Table III. In contrast, demethyl AQ-D changed to demethyl AQ-C-1 and AQ-B-3 in low yield compared to AQ-D, and other demethyl AQs did not yield the prenylated products. These results showed that in IFO 8835 strain, prenylation on the indole moiety of AQs mainly proceeded after the methylation of the two hydroxyl groups on the quinone moiety and occurred at three locations (C-2, C-7, N-1 of indole) simultaneously.

Many enzymes catalyzing prenylation on indole have been reported; 1) an enzyme catalyzing transfer of the dimethylallyl group to cyclo-L-alanyl-L-tryptophanyl at position 2 of the indole moiety in *A. amsterodami*,[9] 2) the dimethylallyltransferase from *Penicillium cyclopein* which prenylates on 4 position of indole in cyclo-acetoacetyl-L-tryptophanyl,[10] 3) the dimethylallylpyrophosphate: tryptophan dimethylallyltransferase which is the first pathway-specific enzyme of ergot alkaloid biosynthesis in *Claviceps* sp.[11]

We have now found an enzymatic activity for the prenylation at N, C-2, and C-7 of indole. Other examples of prenylated bis-indolylbenzoquinones, asterriquinone,[3] cochliodinol,[12] neo-cochliodinol,[13] iso-cochliodinol[13] and hinnuliquinone,[14] have symmetrically prenylated indoles and hydroxy-quinonide structures. Nothing is yet known about the regulation of methylation and prenylation on AQs. It is also uncertain whether the prenyltransferase in strain IFO 8835 and other fungi which produce the asterriquinone analog, are the same or not.

A hypothetical biosynthetic pathway of asterriquinones in *A. terreus* var. *africanus* IFO 8835 based on the above results is shown in Fig. 2.

TABLE II. Effect of Co-Factor on the Formation of Demethyl AQ-D

Conditions	Demethyl AQ-D formation (%)
Complete	21
−ATP	0
+10 mM EDTA	2.3
−$MgCl_2$ + 1 mM EDTA	0

Complete assay conditions: 16 μM ATP, 2.5 μM [1-^{14}C]indolepyruvic acid, 10 mM $MgCl_2$ in 0.1 M Tris (pH 8.2). The activity was expressed as ^{14}C-incorporation ratio of [1-^{14}C]indolepyruvic acid to demethyl AQ-D.

TABLE III. Cell Free Prenylation of Asterriquinones

Starting AQ	Prenylated product
AQ-D	AQ-C-1 > AQ-B-3 > AQ-C-2
AQ-C-1	AQ-A-3 ≒ AQ-B-2 > AQ-B-1 > AQ-B-4
AQ-C-2	AQ-B-1 > AQ-B-2 ≫ AQ-A-2 ≒ AQ-A-4
AQ-B-3	AQ-A-1 > AQ-A-3
AQ-B-1	AQ-A-2 > AQ-A-4
AQ-B-2	None
AQ-B-4	AQ-A-4

Experimental

DL-[3-^{14}C]Tryptophan and [1-^{14}C]glycine were purchased from Amersham, England. Infrared (IR) spectra were measured with a JASCO A-202 apparatus. Ultraviolet (UV) spectra were recorded with a Hitachi 323 spectrometer. TLC (Merck, Silica gel 60 F254) was purchased from Merck. DMAPP was prepared as described before.[1] All other chemicals were of analytical grade. Protein was determined by the method of Lowry *et al.*[15]

Time Course of Production of AQs The cultivation of *A. terreus* var. *africanus* IFO 8835 and isolation of AQs were carried out according to reference 1. The mycelia from 10 Roux flasks (each 500 ml Roux flask

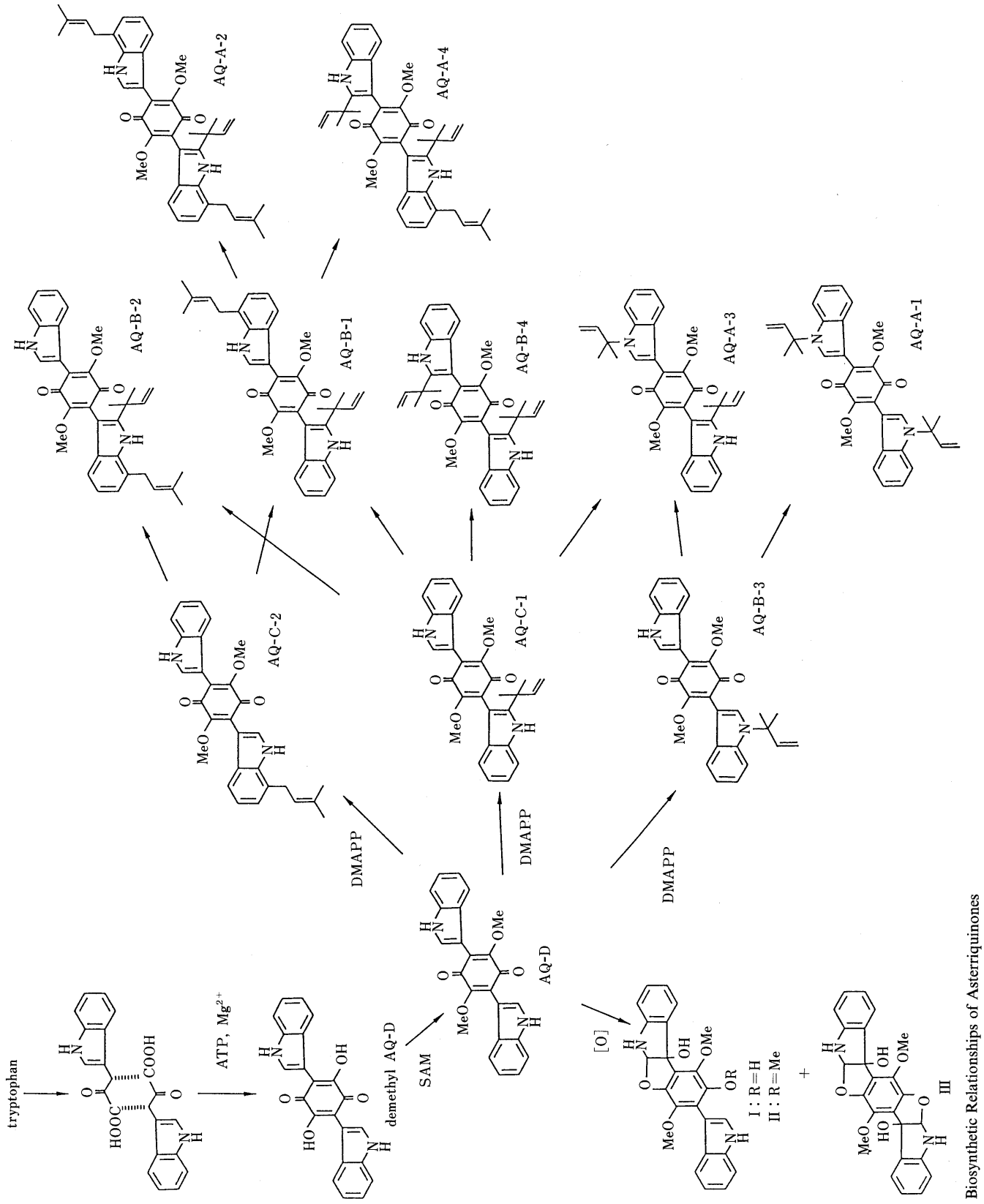

Fig. 2. The Biosynthetic Relationships of Asterriquinones

contained 200 ml of malt extract medium; 2 l of culture broth in total) were harvested and extracted with ether. The amount of AQs was calculated by measuring the absorption of isolated AQs at 340—650 nm using the molar extinction coefficient of each AQ.

Incorporation of DL-[3-^{14}C]Tryptophan into Metabolites ^{14}C-Tryptophan (1.17×10^8 dpm) was added to 7-d-old culture medium (1 l) and cultivated for a further 10 d. The mycelia (^{14}C-incorporation ratio; 76.4%) were extracted with ether and the extract (45.6%) was treated with 10% Na_2CO_3 to remove the acidic fraction (2.9%). The neutral fraction was chromatographed on a silica gel column and each metabolite was isolated and purified by recrystallization to constant specific activity. The radioactivity of the isolated compounds was measured with a liquid scintillation spectrometer in a dioxane scintillator after decolorizing with zinc and acetic acid, if necessary.

Incorporation of ^{14}C-Labelled Demethyl AQ-D The sodium salt of ^{14}C-labelled demethyl AQ-D (4.5 mg; 1.48×10^6 dpm) was dissolved in water and administered on the fourth day of culture. After cultivation for a further 10 d, mycelia were extracted with ether (13%), and then the ether extract was chromatographed on a silica gel column. Incorporations of radioactivity into AQ-D (4.4%), AQ-C-1 (0.2%) and metabolites I, II, III (total 4.1%) were observed.

Preparation of Crude Enzyme All steps were carried out at 4 °C. A buffer solution containing 10 mM each of mercaptoethanol and magnesium chloride, and 10% glycerin was used in all experiments. The 7-d-old mycelia, were washed thoroughly with 0.01 M Tris buffer (pH 7.0) and dried until damp with filter paper. The mycelia (40 g) were ground in a mortar with sea sand (40 g), PVPP (40 g) and 10 mM Tris buffer (120 ml). The homogenate was centrifuged at $12000 \times g$ for 20 min, and the resulting supernatant was fractionated at 30—60% saturation with ammonium sulfate. The precipitate was collected by centrifugation and redissolved in a minimum volume of Tris buffer. The solution was chromatographed on a column of Sephadex G-25 (30×1.4 cm) with Tris buffer. The protein fraction was referred to the crude enzyme.

In the case of the extract of the prenylation enzyme, ammonium sulfate fractionation was done in the range of 30—75% saturation and 0.05 M Tris buffer (pH 7.5) was used.

Synthesis of [1-^{14}C]Indolepyruvic Acid[16] Indolealdehyde and [^{14}C]hydantoin, which was synthesized from [1-^{14}C]glycine and KOCN, were condensed to yield ^{14}C-indolehydantoin. Hydrolysis of ^{14}C-indolehydantoin afforded [1-^{14}C]indolepyruvic acid (total yield; 40%, specific radio activity; 2.71×10^5 dpm/mg).

Assay of Enzymatic Reactions The Condensation Reaction: [1-^{14}C]-indolepyruvic acid (2.5 μM) was dissolved in dimethyl sulfoxide (DMSO) (0.1 ml) and the solution was diluted with 0.1 M Tris buffer (pH 8.2; 2 ml). To this solution, ATP (16 μM) and the enzyme solution (4 ml) were added. After incubation at 30°C for 3 h, the reaction mixture was acidified with HCl and extracted with ether. This extract was treated with CH_2N_2 and the reaction mixture was separated by TLC (benzene–ethyl acetate, 3 : 1, v/v). The products were detected by means of a TLC radioscanner.

The Methylation Reaction: A solution of ^{14}C-labelled demethyl AQ-D (final concentration 4 μM) in 1% Na_2CO_3 was mixed with 0.015 M Tris buffer (pH 7.0) and SAM (14 μM) and then the crude enzyme solution (10 ml) was added and the mixture was incubated at 30°C for 3 h. The reaction was stopped with HCl. The product was extracted with ether and separated with TLC.

The Prenylation Reaction: An AQ or its demethyl derivative (final concentration 1 mM) was dissolved in DMSO (0.2 ml) and diluted with 0.05 M Tris buffer (pH 8.2, 2 ml). To the solution, DMAPP (final concentration 20 μM), KF (final concentration 19 μM) and crude enzyme (5 ml) were added. After incubation at 30°C for 3 h, the reaction mixture was acidified with HCl and extracted with ether. In the case of the demethyl derivative used as the substrate, this extract was treated with ethereal CH_2N_2. The ether was evaporated off and the residue was subjected to TLC. The enzyme blank contained heat-inactivated enzyme. The enzymatically prenylated compounds were identified by comparing their *Rf* values with those of authentic samples.

Enzymatic Condensation Product of Indolepyruvate A solution of indolepyruvic acid (40 mg) in DMSO (4 ml) was mixed with 0.1 M Tris buffer (pH 8.2; 40 ml) and 0.1 M ATP (neutralized with 0.1 N NaOH) (3 ml), then the crude enzyme mixture (180 ml prepared from 300 g mycelia) was added, and the mixture was incubated at 30°C for 3 h. The mixture was extracted with ether ans subjected to oxalic acid-treated silica-gel preparative TLC (benzene–ethyl acetate, 3 : 1, v/v). The red-purple product was isolated and identified as demethyl AQ-D by IR spectroscopy.

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4) Since two AQ-quinols and three dimethyl AQ-quinol derivatives were isolated (ref. 1), AQs are also metabolized in the quinol form. The quinols were auto-oxidized during the extraction and chromatographic procedures, so the amounts of isolated quinones were the sum of biosynthetically accumulated quinones and auto-oxidized quinols.
5) In IFO 8835 strain the methylation ability is very high, so the pool of demethyl AQ-D in cells may be small and this may be why this compounds has not been isolated.
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