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Biosynthesis of aspergiolide A, a novel antitumor compound by a marine-derived fungus *Aspergillus glaucus* via the polyketide pathway

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ABSTRACT

Aspergiolide A, a novel antitumor compound, was produced by a marine-derived filamentous fungus *Aspergillus glaucus*. The biosynthesis of it was unambiguously determined by feeding experiments using [1-^{13}C]sodium acetate, [2-^{13}C]sodium acetate, and [1,2-$^{13}C_2$]sodium acetate precursors followed by ^{13}C NMR spectroscopic investigation of the isolated products. Analysis of the patterns of ^{13}C-enrichment revealed that all 25 carbon atoms in skeleton of aspergiolide A were derived from labeled acetate. And among them, 12 carbon atoms were labeled from the carboxylic group of acetate, while the other 13 carbon atoms were labeled from the methylic group of acetate. Besides, the labeling pattern of [1,2-$^{13}C_2$]sodium acetate feeding experiment demonstrated that 12 intact acetate units were incorporated in aspergiolide A by polyketide pathway.

Polyketides are one of the largest and most structurally diverse classes of secondary metabolites produced by bacteria, fungi, and plants naturally.[1–3] Amongst these polyketides, aromatic polyketides afford some of the most common antibiotics and anticancer drugs that are currently in clinical use, such as antibiotics oxytetracycline, tetracenomycin, anthracyclines doxorubicin, and aclacinomycin.[4] They also play an important role for new drug discovery. Although aromatic polyketides are different from other polyketides by their characteristic polycyclic aromatic structures, they bear a resemblance to all fungal polyketides in their biosynthetic origin.[5] Their biosynthesis is accomplished by the catalytic reaction of polyketide synthases (PKSs), which catalyze repetitive Claisen condensations between an acyl-coenzyme A (CoA) starter and malonyl-CoA elongation units to yield a linear poly-β-ketone intermediate, followed by regiospecific reduction, aromatization, or cyclization to furnish the polycyclic aromatic structures.[2,4–6]

Aspergiolide A (Fig. 1), a novel anthraquinone derivative with naphtho[1,2,3-*de*]chromene-2,7-dione skeleton, has been isolated from cultures of a marine-derived fungus *Aspergillus glaucus*.[7a] In the study of the antitumor activity, aspergiolide A exhibited cytotoxicity against A-549, HL-60, BEL-7402, and P388 cell lines. Recently, the animal test also showed that aspergiolide A could inhibit the growth of tumor effectively in model mice (unpublished data). However, so far there was no experimental demonstration to explore its biosynthetic pathway. In our previous experiments, the amount of aspergiolide A in fermentation broth was found to significantly decrease in response to the addition of polyketide pathway specific inhibitors cerulenin and indoacetamide in the medium of *A. glaucus*, whereas the growth of cells was almost not effected (data not shown). This result indicated that the biosynthetic pathway of aspergiolide A was involved in polyketide pathway.

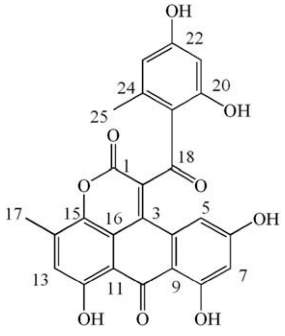

Figure 1. Structure of aspergiolide A.

In order to elucidate the biosynthetic origin of the carbon atoms in aspergiolide A, we performed preliminary studies with

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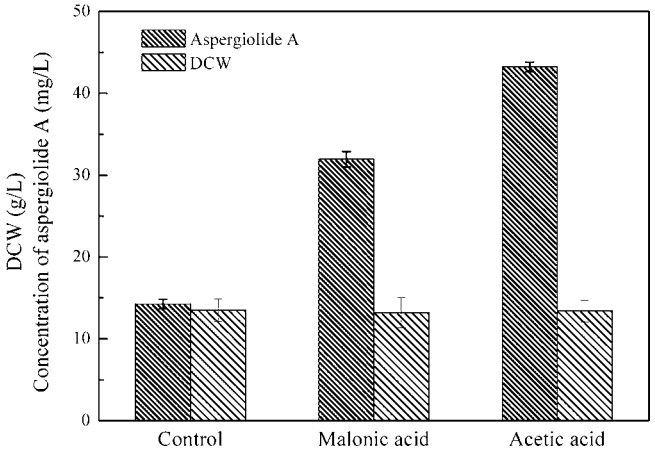

Figure 2. Effect of feeding with 6 mM malonic acid and acetic acid initiated at 48 h postinoculation, respectively, on aspergiolide A production and DCW.

unlabeled short chain fatty acids such as malonic acid and acetic acid to explore the biosynthetic precursor of aspergiolide A and the optimized conditions of feeding.[8] Prepared 0.2 M malonic acid and acetic acid stock liquors were added, respectively, to growing cultures of *A. glaucus* with different concentration gradients, and the addition was initiated at about 48 h postinoculation and was carried out every 24 h for three times. Analysis of the biomass and production of aspergiolide A in the fermentation broth indicated that addition of these putative precursors had no significant influence on dry cell weight (DCW), but had marked effects on aspergiolide A production. As shown in Figure 2, the addition of 6 mM malonic acid and acetic acid resulted in a 1.24-fold and 2.03-fold increase, respectively, in aspergiolide A production. Hence, it is reasonable to assume that malonic acid or acetic acid is the biosynthetic precursor of aspergiolide A in *A. glaucus*.

Based on these results, further labeling experiments were carried out with [1-$^{13}C_2$]sodium acetate, [2-$^{13}C_2$]sodium acetate, and [1,2-$^{13}C_2$]sodium acetate (99% ^{13}C abundance, purchased from Cambridge Isotope Laboratories, Inc. USA). Fifty milliliters of sterilized fermentation medium (soluble amylum 40 g, sucrose 40 g, maltose 30 g, peptone 2 g, yeast extract 1 g, soybean flour 0.5 g, monosodium glutamate 2 g, $MgSO_4$ 0.3 g, KH_2PO_4 0.5 g, dissolved in 1 L artificial sea water) were prepared in a 250-mL Erlenmeyer flask. And then, 8 mL of a inoculum was inoculated, followed by the incubation at 28 °C in a shaking incubator at 170 rpm for 7 days. The feeding with stable-isotope-labeled precursor was initiated at 48 h postinoculation and periodically carried out every 24 h afterwards. In the feeding process, 650 μL of a 0.2 M sterilized solution of [1-^{13}C]sodium acetate, [2-^{13}C]sodium acetate, and [1,2-$^{13}C_2$]sodium acetate was added, respectively, to each 60 mL culture every time and the final concentration of labeled sodium acetate was 6.5 mM after three additions. Separate feeding with unlabeled sodium acetate as cold precursor was also carried out in parallel for the control.

The cultures were harvested after 175 h, and then separated into mycelium and supernatant by absorbent gauze. The combined ethyl acetate extracts of mycelium (acetone extract, re-extraction with ethyl acetate) and supernatant (ethyl acetate extract) were concentrated to give a crude gum under reduced pressure. The crude gum was then isolated by column chromatography. The mixture was firstly applied to two columns of silica gel (200–300 mesh) which were developed by gradient elution with chloroform/methanol and petroleum ether/acetone. Each eluted fraction was collected and analyzed by TLC on silica gel using chloroform/methanol; 10:1 as mobile phase and a mixture of vanillin/H_2SO_4 as visualization reagent. The RF value of aspergiolide A was 0.52. Then the crude residue was further purified by column chromatography on gel Sephadex LH20 eluting with chloroform/methanol; 1:1 and pure methanol. For a better separation performance, a preparative reverse phase HPLC (C_{18}, eluted with 50% acetonitrile/water) was employed, to afford 13.2 mg, 19.6 mg, 18.5 mg of ^{13}C labeled aspergiolide A for [1-^{13}C]sodium acetate, [2-^{13}C]sodium acetate, and [1,2-$^{13}C_2$]sodium acetate feedings, respectively.

^{13}C NMR spectra were recorded in DMSO-d_6 on a JEOL Eclips-600 spectrometer operating at 150.9 MHz and using TMS as internal standard (see Supplementary data). ^{1}H-decoupled ^{13}C NMR spectra of samples from labeling experiments with labeled acetates and unlabeled acetate (natural ^{13}C abundance, 1.1% ^{13}C) were recorded under the same conditions. ^{13}C NMR chemical shift assignment of aspergiolide A was as same as reported previously.[7b] The abundance of each carbon atom was determined from the ^{13}C-signal intensities in the one-dimensional spectrum. The enrichment ratio of individual carbon atom was calculated by comparison of ^{13}C signal intensity between ^{13}C-labeled aspergiolide A and unlabeled, and these values of enrichment ratios were then normalized using enrichment ratio of the C-19 for [1-^{13}C]sodium acetate labeling and C-1 for [2-^{13}C]sodium acetate labeling, respectively, as described by Kubota et al.[9]

The results of these labeling experiments are summarized in Table 1, and high levels of enrichments were observed at C-1, C-4, C-6, C-8, C-10, C-12, C-14, C-16, C-20, C-22, and C-24 (bold type in Table 1) in [1-^{13}C]sodium acetate-labeled aspergiolide A. These enrichments correspond to high levels of enrichment for C-2, C-3, C-5, C-7, C-11, C-13, C-15, C-19, C-21, and C-25 (bold type

Table 1
^{13}C NMR analysis of aspergiolide A after feeding with [1-^{13}C]sodium acetate, [2-^{13}C]sodium acetate, and [1,2-$^{13}C_2$]sodium acetate

Carbon no.	Chemical shift δ (ppm)	Enrichment ratio[a]		J_{cc}[c] (Hz)
		[1-^{13}C] AcONa	[2-^{13}C] AcONa	
1	158.9	**2.68**[b]	1.00	71.39(2)
2	132.4	0.70	**2.63**	Nd[d]
3	130.8	0.70	**1.81**	Nd
4	133.9	**2.80**	1.04	55.27 (3)
5	110.9	1.38	**2.17**	64.48 (6)
6	164.8	**3.39**	1.31	64.48 (5)
7	105.4	1.33	**2.05**	69.10 (8)
8	165.7	**3.11**	1.22	69.10 (7)
9	107.9	0.67	1.06	59.87 (10)
10	187.7	**3.28**	1.34	59.87 (9)
11	108.1	1.31	**1.71**	57.58 (12)
12	159.2	**2.60**	1.15	57.58 (11)
13	122.2	1.29	**1.81**	S[e]
14	136.3	**1.97**	1.22	43.76 (17)
15	142.6	1.35	**2.27**	64.46 (16)
16	116.0	**2.92**	1.10	64.46 (15)
17	16.0	0.69	1.32	43.76 (14)
18	190.8	1.09	0.42	Nd
19	115.0	1.00	**1.84**	62.17 (18)
20	162.9	**1.95**	0.75	Nd
21	100.7	1.28	**2.11**	66.79 (20)
22	162.9	**1.95**	0.26	Nd
23	112.0	0.85	1.32	62.17 (22)
24	145.3	**2.05**	0.63	41.43 (25)
25	23.2	1.38	**2.15**	41.43 (24)

[a] ^{13}C signal intensity of each peak in the labeled aspergiolide A divided by that of the corresponding signal in the unlabeled, normalized to give an enrichment ratio of aspergiolide A for unriched peak (C-19 for [1-^{13}C]sodium acetate labeling and C-1 for [2-^{13}C]sodium acetate labeling).

[b] The numbers in bold type indicate ^{13}C-enriched atoms from ^{13}C-labeled acetate.

[c] Detected with the ^{13}C-enriched sample from the feeding experiment with [1,2-$^{13}C_2$]sodium acetate. Coupled carbon atoms are in parentheses.

[d] Nd, not determined due to line broadening or signal overlapping.

[e] Singlet.

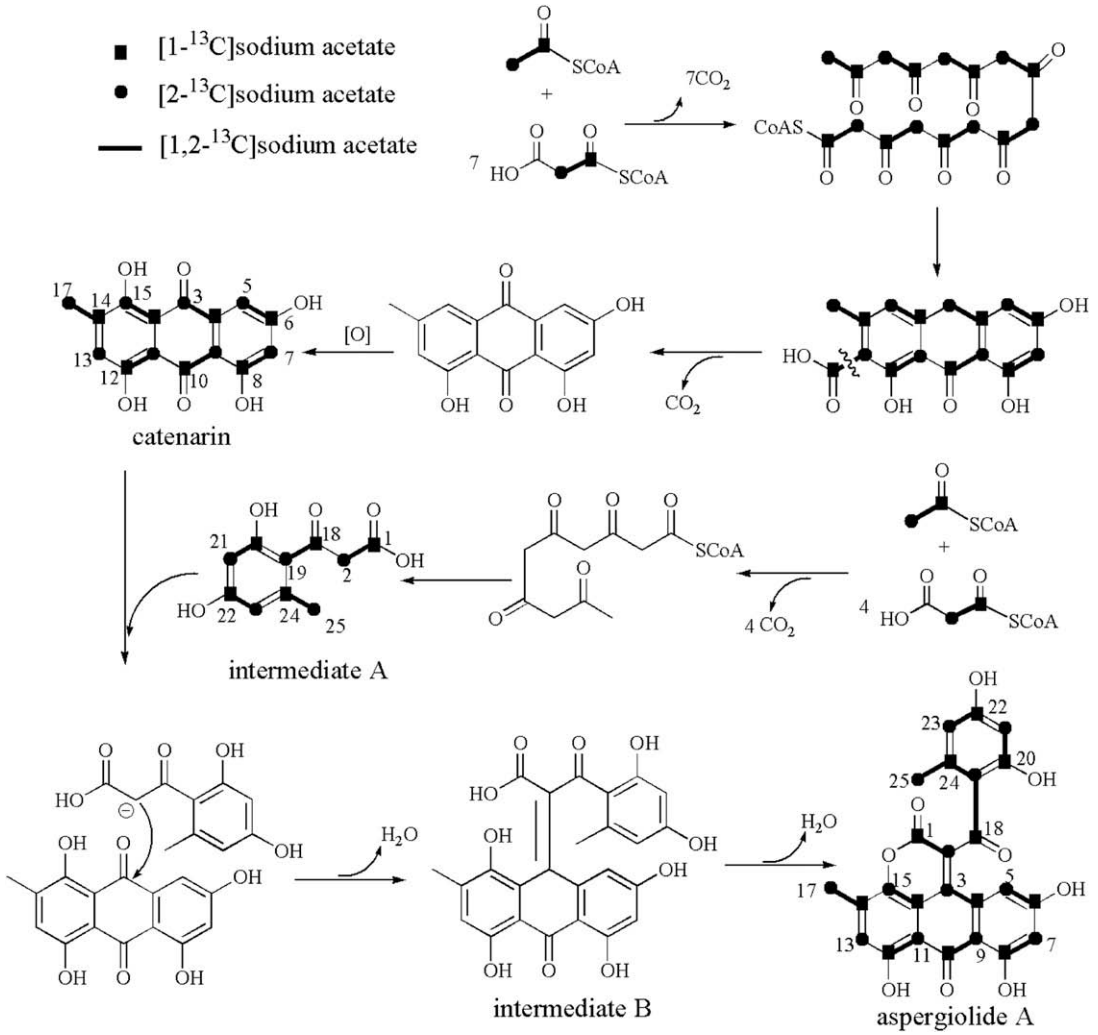

Figure 3. The biosynthetic pathway of aspergiolide A based on the labeling experiments using [1-^{13}C]sodium acetate (labeled by ■), [2-^{13}C]sodium acetate (labeled by ●), and [1,2-$^{13}C_2$]sodium acetate (^{13}C labeled isotopomers with directly adjacent ^{13}C atoms were indicated by bold lines).

in Table 1) in [2-^{13}C]sodium acetate-labeled aspergiolide A. Although C-18 and C-9, C-17, C-23 did not show high levels of enrichment as extensive as those observed at the other labeled carbons in each labeling experiment, C-18 is derived from carboxyl carbon of [1-^{13}C]sodium acetate while C-9, C-17, C-23 are derived from methyl carbon of [2-^{13}C]sodium acetate according to the principle of polyketide biosynthesis and the labeling pattern of [1,2-$^{13}C_2$]sodium acetate-labeled aspergiolide A. Thus, all the 25 carbons contained in aspergiolide A were implied to be derived from acetates.

In the spectrum of aspergiolide A labeled from [1,2-$^{13}C_2$]sodium acetate, 19 of the 25 carbon atoms appeared as distinct triplets flanked by two strong satellite signals because the incorporation of intact acetate units and C-13 was a distinct singlet. The pair of methyl and carboxyl carbons of the intact acetate unit incorporated into aspergiolide A can be readily identified by their similar J_{C-C} values, thus, the couplings found were following pairs: C-5/C-6, C-7/C-8, C-9/C-10, C-11/C-12, C-14/C-17, C-15/C-16, C-24/C-25 (reported in Table 1). Due to line broadening or signal overlapping, the splitting of C-2, C-3, C-18, C-20, C-22 was immersed in the complex and could not be determined, however, the ^{13}C–^{13}C coupling of intact [1,2-$^{13}C_2$]acetate units stood still at these positions. It could be deduced that C-23 appears with a satellite doublet (J = 62.17 Hz) and since it cannot be coupled to C-24 (whose coupling to C-25 was established), it must be coupled to C-22, although the splitting of C-22 could not be determined due to signal overlapping. For the same reason, C-2, C-3, C-18, and C-20 must be successively coupled to C-1, C-4, C-19, and C-21. Therefore, the observed ^{13}C–^{13}C couplings together with the distinct singlet for C-13 unequivocally indicated the incorporation of 12 intact acetate-derived C_2 units in aspergiolide A.

After careful analysis of the preliminary experiments and the ^{13}C NMR spectrum of labeled aspergiolide A in conjunction with the principle of polyketide biosynthesis,[10] the postulated biosynthetic pathway of aspergiolide A can be deduced (Fig. 3), which conforms to a classical acetate-polymalonate pathway as most aromatic polyketide pathways in which acyl CoA precursors take place as a sequence of decarboxylative condensation. In conclusion, it was clear that aspergiolide A was biosynthesized from two intact acetate starter units and 11 malonate derived C_2 extender units, and hence two intermediates were formed. One is an anthraquinone intermediate, catenarin,[11] which was also isolated from *A. glaucus*, and the other is the supposed intermediate A which was formed by one acetyl-CoA unit and four malonyl-CoA units. The aldol condensation then took place between catenarin and intermediate A to form a putative intermediate B. Then, the intermediate B further underwent intramolecular esterification between the carboxyl and the hydroxyl groups to synthesize aspergiolide A. Lovastatin, an cholesterol-low-

ering drug, was also synthesized by a similar pattern in fungus *Aspergillus terreus*.[12,13] To confirm this conclusion, the cloning and characterization of the corresponding polyketide synthase gene cluster for aspergiolide A are underway in our laboratory.

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Supplementary data

Supplementary data (^{13}C NMR spectra data of aspergiolide A) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.12.094.

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