Isolation and Structure Elucidation of a New Metabolite Produced by *Aspergillus parasiticus*

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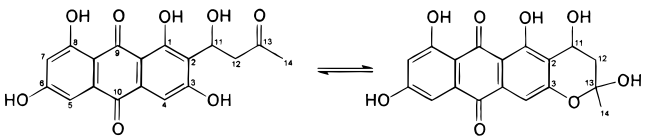
A new metabolite, 1,3,6,8-tetrahydroxy-2-(1′-hydroxy-3′-oxobutyl)-anthraquinone, designated as asparasone A (**1**), was isolated from an *Aspergillus parasiticus* mutant (ATCC 20979), and its structure was deduced from spectral data. Several wild-type isolates of *A. parasiticus* also produced **1**. Asparasone A (**1**) is structurally related to intermediates in the aflatoxin biosynthetic pathway.

Aflatoxins are carcinogenic secondary metabolites of *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare. Commodities such as corn, peanuts, and cottonseed are commonly contaminated with aflatoxins. Biochemical and genetic studies of aflatoxin biosynthesis have resulted in determination of the genes, enzymes, and precursors involved in the pathway.[1,2] The molecular transformations within the pathway have not been completely determined. A better understanding of aflatoxin biosynthesis may lead to new approaches for controlling aflatoxin contamination of agricultural produce.

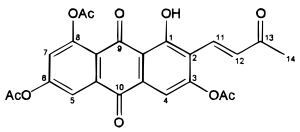
The purpose of this work was to isolate and identify a new pigment produced by mutant and wild-type strains of *A. parasiticus*. The pigment is structurally related to intermediates in the aflatoxin pathway.

The *A. parasiticus* mutant (ATCC 20979) was chosen for study because it produced high concentrations of an intensely colored pigment that possessed chemical and spectral properties similar to those of the versicolorins, which are known aflatoxin precursors.[3] Several chromatographic clean-up procedures and crystallizations resulted in the isolation of an orange crystalline compound designated as asparasone A (**1**). The molecular formula of **1** was deduced as $C_{18}H_{14}O_8$ based on HR-FABMS data, indicating 12 degrees of unsaturation. The UV and ^{1}H- and ^{13}C-NMR spectra established the presence of an anthraquinone moiety.[3] The NMR spectra, however, also demonstrated that **1** consisted of an equilibrium mixture of two isomers (**1** and **1a**, approximately 2:1 ratio) in either $(CD_3)_2CO$, CD_3OD, or DMSO-d_6. Its behavior in this respect was similar to that of versiconal hemiacetal acetate.[3] The molecular formula required that these anthraquinone isomers contain either an additional double bond or a saturated ring. The presence of a ketone group in the major isomer (**1**) was implied by a ^{13}C-NMR resonance at δ 206.4 (Table 1), although only a weak ketone carbonyl stretch was observed in the IR spectrum.

Because of its poor solubility in appropriate solvents, variable-temperature NMR studies of **1** were not feasible. Therefore, in order to simplify structure elucidation, **1** was acetylated. A triacetate (**2**) formed upon treatment of **1** with Ac_2O and was assigned a molecular formula of $C_{24}H_{18}O_{10}$ on the basis of HRFABMS. The ^{1}H-NMR spectrum of **2** (Table 2) indicated the presence of three acetyl groups and one strongly chelated phenolic OH group. The molecular formula of **2**, and its substitution pattern, as deduced from ^{1}H-NMR analysis and confirmed by analysis of HMBC data, required that it contain a C_4H_5O substituent and ^{1}H-, ^{13}C-, and 2D NMR analysis established the presence of an (*E*)-3′-oxobut-1′-enyl moiety.



1 **1a**



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A signal in the ^{1}H-NMR spectrum at δ 7.54 was assigned to H-4 on the basis of its HMBC correlation with the C-10 carbonyl resonance. The chelated OH group at C-1, H-4, and one of the *trans*-olefinic protons (H-12) all showed HMBC correlations with C-2, effectively placing the oxobutenyl substituent at C-2. Other HMBC correlations that further supported structure **2** and confirmed spectral assignments are given in Table 2. Compound **2** was therefore determined to be (*E*)-1-hydroxy-2-(3′-oxobut-1′-enyl)-3,6,8-triacetoxyanthraquinone.

The presence of a *trans* double bond in **2** that was clearly not present in **1** implied that the conditions employed for acetylation of **1** also induced dehydration. This was consistent with a 1′-hydroxy-3-oxobutyl group in **1** and suggested that the mixture of equilibrium isomers observed in the NMR spectra included 1,3,6,8-tetrahydroxy-2-(1′-hydroxy-3′-oxobutyl)anthraqui-

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Table 1. NMR Spectral Data for Compounds 1 and 1a in DMSO-d_6

	1			**1a**		
C/H no.	δ_H[a]	δ_C[b]	HMBC[c]	δ_H[a]	δ_C[b]	HMBC[c]
1		161.2,[d] s	H-11		161.2,[d] s	
1a		108.2, s	H-4		108.7, s	H-4
2		120.8, s	H-4, H-11, H-12a		117.5, s	H-4
3		163.1,[d] s	H-11		163.1,[d] s	
4	7.10, br s	108.7, d		6.90, s	109.2, d	
4a		134.8, s			132.9, s	
5	7.07, br s	108.7, d	H-7	7.07, br s	108.7, d	H-7
5a		134.8, s	H-5		134.8, s	
6		165.1, s	H-7		165.1, s	H-7
7	6.55, br s	108.0, d	H-5,[e] 8-OH[e]	6.55, br s	108.0, d	H-5,[e] 8-OH[e]
8		164.2, s	8-OH, H-7		164.2, s	8-OH, H-7
8a		108.2, s	H-5,[e] 8-OH[e]		108.2, s	H-5,[e] 8-OH[e]
9		188.7, s			188.7, s	
10		180.9, s	H-4, H-5		180.9, s	H-4, H-5
11	5.56, br d (5.0)	62.1, d	H-12a	4.90, br s	57.4, d	
12a	3.24, dd (5.0, 15.6)	48.6, t	H-11, H-14	2.38, m	37.9, t	H-14
12b	2.69, br d (15.6)	48.6, t	H-11, H-14	2.10, m	37.9, t	
13		206.4, s	H-11, H-12a, H-12b, H-14		99.2, s	H-14
14	2.38, s	30.5, q		1.56, s	27.8, q	
1-OH	12.74, br s			12.74, br s		
8-OH	12.08, br s			12.08, br s		

[a] (J) in Hz. [b] Multiplicity deduced by DEPT. [c] Protons showing long-range coupling to indicated carbon. [d] Interchangeable, although the literature supports assignments as indicated.[3] [e] These proton signals may correlate to either one or both of the indicated carbons.

Table 2. NMR Spectral Data for Compound 2 in $CDCl_3$

C/H no.	δ_H[a]	δ_C[b]	HMBC[c]	NOECY
1		163.9, s	H-11, 1-OH	
1a		114.2, s	H-4, 1-OH	
2		122.7, s	H-4, H-12, 1-OH	
3		155.3,[e] s	H-4, H-11	
4	7.54, s	114.1, d		
4a		132.9, s		
5	7.98, d (2.5)	119.0, d	H-7	
5a		136.0, s		
6		155.8,[e] s	H-5, H-7	
7	7.28, d (2.5)	123.6, d	H-5	
8		152.2, s	H-7	
8a		121.9, s	H-5	
9		186.6, s		
10		179.9, s	H-4, H-5	
11	7.59, d (16.6)	130.4, d		H-12, H-14, 1-OH
12	7.28, d (16.6)	133.6, d	H-11	H-11, H-14, 1-OH
13		198.6, s	H-11, H-12, H-14	
14	2.38, s	28.3, q		H-11, H-12
3-OCOCH_3	2.39,[d] s	21.0,[f] q		H-4
3-O*C*OCH$_3$		167.9,[g] s	3-OCOCH_3	
6-OCOCH_3	2.35,[d] s	21.07,[f] q		H-5, H-7
6-O*C*OCH$_3$		167.8,[g] s	6-OCOCH_3	
8-OCOCH_3	2.44,[d] s	21.11,[f] q		H-7
8-O*C*OCH$_3$		169.0,[g] s	8-OCOCH_3	
1-OH	13.80, s			H-11, H-12

[a] (J) in Hz. [b] Multiplicity deduced by DEPT. [c] Protons showing long-range correlations with indicated carbon. [d–g] Assignments bearing identical superscripts are interchangeable.

none. According to ^{1}H- and ^{13}C-NMR analysis, **1** indeed was the major equilibrium component. Three signals in the ^{1}H-NMR spectrum at δ 2.69, 3.24, and 5.56 were shown to be mutually coupled based on ^{1}H−^{1}H COSY data, with the signals at 2.69 and 3.24 representing geminal protons at a CH_2 unit. HMBC correlations of all three of these signals and a methyl singlet at δ 2.38 with δ_C 206.4, and between a methyl singlet at δ 2.38 and δ_C 206.4, confirmed the presence of a 1′-hydroxy-3′-oxobutyl moiety. Additionally, HMBC correlations of the oxymethine proton signal at δ 5.56 with C-1, C-2, and C-3 placed the OH group at the C-1′ position and the hydroxyoxobutyl moiety at C-2, as expected by analogy to **2**. Further assignments and HMBC correlations for compound **1** are given in Table 1.

The minor component in the equilibrium mixture was determined to be a hemiketal (**1a**). Three minor signals in the ^{1}H-NMR spectrum of the mixture, at δ 2.10, 2.38, and 4.90 (H-12b, H-12a, and H-11, respectively), were shown to be mutually coupled to each other by ^{1}H−^{1}H COSY analysis, while the minor methyl group signal at δ 1.56 showed HMBC correlations to C-12 and the dioxygenated carbon signal at δ_C 99.2 (C-13). Two strongly chelated phenolic OH signals in the ^{1}H-NMR spectrum of this closed-ring component indicated that it was the linear hemiketal **1a**, as opposed to the angular analogue, which would exhibit cyclization at the C-1 OH rather than the C-3 OH. A strongly chelated hydroxyl group at C-1 would presumably be less readily available for hemiketal formation. This conclusion was supported by treatment of compound **1** with Ac_2O, which failed to yield a product acetylated at the C-1 OH. Other spectral assignments and HMBC correlations supporting structure **1a** are given in Table 1.

To study the dynamics of **1** production by the *A. parasiticus* mutant (ATCC 20979) and to investigate the possible production of this new metabolite by wild-type isolates of *A. parasiticus*, a simple and fast HPLC method for simultaneous detection and quantitation of **1** and the versicolorins in synthetic medium was developed. Extraction of pigments from the isolates with $CHCl_3$ followed by a purification procedure yielded a clean fraction of the pigments suitable for direct HPLC analysis. Partition HPLC was performed on Si gel using n-C_7H_{16}−$(CH_3)_2CHOH$−H_2O−CH_3COOH (1100:300:20:3, v/v) as a mobile phase. Partition HPLC was demonstrated to be more effective than adsorptive chromatography. Figure 1a demonstrates a baseline separation of some selected versicolorin standards. The HPLC mobile phase had high UV-transparency, which permitted the use of a photodiode array detector in 220−600 nm range for confirmation and quantitative determination of versicolorins produced by the fungi.

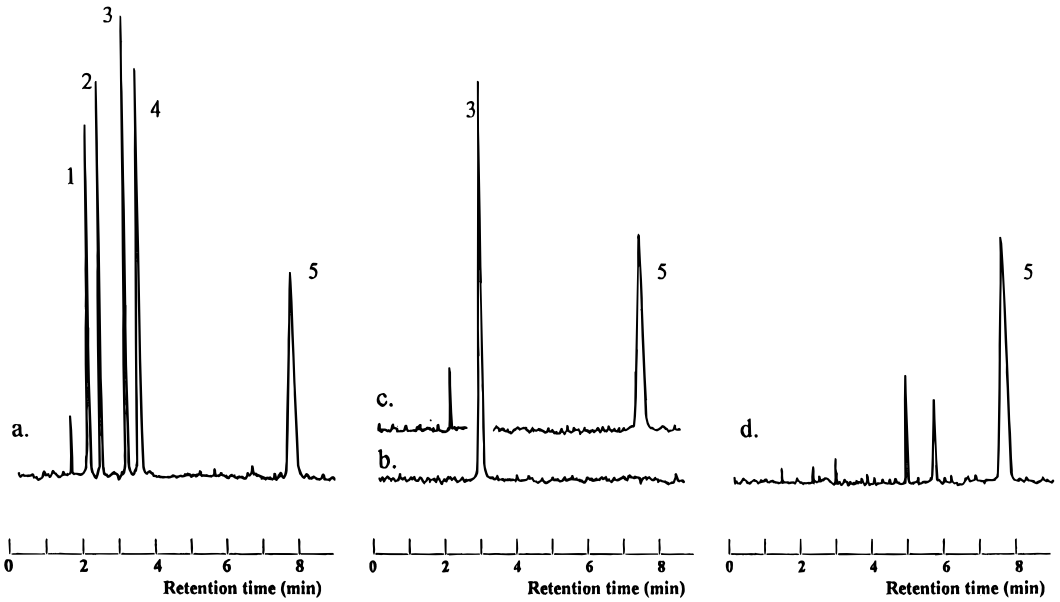

Figure 1. HPLC chromatograms. (**a**) Standards of the versicolorins; 1, norsolorinic acid; 2, averufin; 3, versicolorin A; 4, averantin; 5, asparasone A. (**b**) *A. parasiticus* mutant (ATCC 20979) extract after 3 days of incubation. (**c**) *A. parasiticus* mutant (ATCC 20979) extract after 5 days of incubation. (**d**) Extract of aflatoxin-producing isolate of *A. parasiticus* (NPL P19) after 5 days of incubation (the concentration of asparasone A was 160 μg/mL).

The study of the dynamics of pigment production by the *A. parasiticus* mutant (ATCC 20979) gave the following results. The strain produced versicolorin A (Ver A) as the only detectable anthraquinone metabolite after 3 days of incubation (Figure 1b). At 4 days, the concentration of Ver A remained almost unchanged, and **1** was first detected, while after 5 days Ver A disappeared and asparasone A was the major anthraquinone metabolite (Figure 1c). The relationship between Ver A and **1** production by ATCC 20979 is not understood. The wild-type *A. parasiticus* isolate (ATCC 62882), from which ATCC 20979 was derived, did not produce **1** under the same conditions.

For analysis of pigment production by other wild-type isolates of *A. parasiticus,* isolates from a peanut field in southwestern Georgia were selected based on genetic differences indicated by vegetative incompatibility.[4] Preliminary studies showed that some wild-type isolates of *A. parasiticus* (both aflatoxin producers and nonproducers from the culture collection at the National Peanut Research Laboratory) formed **1** as one of the major metabolites. Figure 1d gives an example of asparasone A production by an aflatoxin-producing isolate of *A. parasiticus* (NPL P19) after 5 days of incubation.

The proposed significance of **1** as a possible intermediate in the aflatoxin biosynthetic pathway is based on its chemical structure (similar to other known biosynthetic precursors of the aflatoxins and sterigmatocystins), as well as its production by wild-type isolates of *A. parasiticus*. Further biochemical work is necessary to confirm or disprove its role in the biosynthesis of aflatoxins.

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-NMR data were obtained at 360 and 90.7 MHz, respectively, on a Bruker WM-360 spectrometer. Residual solvent signals were used as internal references. All 2D NMR experiments were recorded at 600 MHz on a Bruker AMX-600 spectrometer. HMBC and HMQC experiments were optimized for $^nJ_{CH} = 8.3$ Hz and $^1J_{CH} = 150.2$ Hz, respectively. HRFABMS data were obtained using a VG ZAB-HF instrument.

The HPLC system consisted of a Waters Model 510 pump, Waters Model 490E programmable multiwavelength detector, or Shimadzu Model SPD-M10A photodiode array detector, Waters Model 730 data module, and a Beckman Ultrasphere-Si column 250 × 4.6 mm i.d. 5-μm particles. All solvents used for HPLC were HPLC grade. The mobile phase consisted of: n-C_7H_{16}−$(CH_3)_2CHOH$−H_2O−CH_3COOH (1100:300:20:3, v/v), with a flow rate of 1.5 mL/min. The column was equilibrated with the mobile phase at the above flow rate within 2 h. After elucidation of its structure and confirmation of its purity, **1** was used as an HPLC standard. Compound **1** and the versicolorins concentrations in culture extracts were determined by reference to peak areas of the pure standards.

Organism and Culture Conditions. The mutant *A. parasiticus* (ATCC 20979) that produced asparasone A (**1**) was obtained after UV (254 nm) irradiation of wild-type *A. parasiticus* ATCC 62882 that accumulated *o*-methylsterigmatocystin, an immediate biosynthetic precursor to aflatoxin B_1. The mutant strain was maintained on Czapek agar slants and was grown in static culture for 14 days at 30−32 °C in 2.8-L Fernbach flasks (80 total) containing 200 mL of liquid medium consisting of 150 g sucrose, 40 g glucose, 10 g soytone, and 20 g yeast extract per 1 L of H_2O. The pH of the medium was adjusted to 4.8 with HCl. For the purpose of comparative analysis of strains for production of **1**, cultures were incubated at 30 °C in 1-dram vials containing 1 mL of the same medium adjusted to pH 5.9.

Isolation of 1. The combined content of the above Fernbach flasks was extracted with $CHCl_3$ (substrate−$CHCl_3$ ratio 1:3) three times with a high-speed blender (Ultra-Turrax, model SD-45; Janke & Kunkel), then filtered under low vacuum and evaporated under re-

duced pressure. For isolation of **1**, a Buchner-type filtering funnel (Fisher Buchner-type with fritted disk; 2000 mL capacity, 125 mm i.d.; E. Merck TLC Si gel 60H, mean particle size, 15 μm; layer height, 85 mm) was used as a column for preparative chromatography. The column was first eluted under low vacuum with *n*-C_6H_{14}, then with a gradient of *n*- C_6H_{14}–$(CH_3)_2CO$ from 6:1 (v/v) to 100% $(CH_3)_2CO$. The solvents for column chromatography were reagent grade. The eluates from the column were analyzed by TLC (TLC plates Kieselgel 60, 10 × 10 cm; E. Merck); using the HPLC mobile phase described below. Developed TLC plates were placed into a chamber saturated with NH_3 for about 20 s. Fractions containing a yellow pigment that turned purple on the TLC plates after exposure to NH_3 and had identical R_f values were combined and evaporated on a rotary evaporator (Rotavapor-R; Brinkman) to give an amorphous solid. The pigment-containing fraction was further purified by chromatography on Si gel under the same conditions described above. After evaporation of the solvent, the resulting dry solid residue was recrystallized twice from EtOAc–*n*-C_6H_{14}, yielding 1.76 g of orange microcrystalline powder of **1**.

Extraction of Versicolorins. After incubation, the fungal cultures were extracted by adding 1 mL of $CHCl_3$ to each vial, chopping the mycelium with a spatula, and vortexing for 30 sec. After separation of the layers, an aliquot of the $CHCl_3$ extract (200–400 μL) was transferred into a 1-dram vial with a microsyringe, and 1 mL of $CHCl_3$ was added. The versicolorins from the $CHCl_3$ layer were reextracted with 1 mL of 5% NH_4OH in H_2O by vortexing the vial for 15–20 s. The NH_4OH–H_2O layer turned purple when high concentrations of versicolorins were present. After separation, the $CHCl_3$ layer was collected with a Pasteur pipette and discarded. One mL of $CHCl_3$ and 1 mL of 10% HCl solution in H_2O were added to the remaining NH_4OH–H_2O extract followed by vortexing for 15–20 s. After separation, the H_2O layer was strongly acidic. The $CHCl_3$ layer was then transferred into a clean 1-dram vial and evaporated with N_2 stream in a 40 °C heated block. The residue was dissolved in 1.5 mL of the HPLC mobile phase and sonicated in an ultrasonic bath for 10–15 s. Of the filtrate, 20–100 μL were injected into the HPLC system.

Compound 1: orange microcrystalline powder; $[\alpha]_D$ 0° (*c* 0.002 g/mL, MeOH); UV (MeOH) 226 (ϵ 19 000), 266 (11 000), 294 (18 000), 315sh (7200), 456 nm (6500), (+NaOH) 214 (ϵ 19 000), 232 (15 000), 262 (10 000), 312 (29 000), 382 (6400), 398 (6000), 541 nm (5900); IR (KBr) 1700, 1620, 1570 cm^{-1}; ^{1}H-NMR, ^{13}C-NMR, and HMBC data, see Table 1; HRFABMS, obsd 359.0748, calcd for $C_{18}H_{14}O_8$ + H, 359.0767.

Compound 2: orange powder [obtained through acetylation of **1** with Ac_2O in $CHCl_3$–pyridine at 25 °C; purified by preparative TLC on Si gel using *n*-C_6H_{14}–$(CH_3)_2CO$ (3:1, v/v) as a mobile phase; crystallized from *n*-C_6H_{14}–EtOAc]; $[\alpha]_D$ 0° (*c* 0.002 g/mL, MeOH); UV (MeOH) 237 (ϵ 7800), 273 (20 000), 424 nm (5600), (+NaOH) 220 (ϵ 23 000), 301 (17 000), 375 (19 000), 534 (2600) nm; IR (CH_2Cl_2) 3541, 1778, 1675, 1635, 1601 cm^{-1}; ^{1}H-NMR, ^{13}C-NMR, HMBC, and NOECY data, see Table 2; HRFABMS, obsd 467.0998, calcd for $C_{24}H_{18}O_{10}$ + H, 467.0978.

References and Notes

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