

Isolation and Identification of Hexaketides from a Pigmented *Monosporascus cannonballus* Isolate

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Monosporascus cannonballus causes severe production losses to muskmelon and watermelon in the United States and other countries. Wild types of the fungus produce no pigments when grown on potato dextrose agar (PDA). After long-term storage on soil/oat hull mix, however, some isolates of the fungus produce yellow to brown pigments and no perithecia when grown on PDA. Five colored metabolites from pigmented cultures of *M. cannonballus* isolate TX923038 have now been identified. Two of these, monosporascone and dehydroxyarthrinone, have been isolated from other fungi, and three, demethylcerdarin, monosporascol A and azamonosporascone, have not previously been reported. The ¹H NMR and ¹³C NMR of all five compounds are reported.

KEYWORDS: *Monosporascus cannonballus*; hexaketide metabolites; fungal pathogen; monosporascone; dehydroxyarthrinone; demethylcerdarin; monosporascol A; azamonosporascone

INTRODUCTION

Monosporascus root rot/vine decline caused by *Monosporascus cannonballus* Pollack & Uecker is a serious disease of muskmelon and watermelon worldwide (1, 2). Crop losses caused by this ascomycetous fungus can reach 100% in individual fields (2–6). This fungus generally can be divided into two groups based on the amount of pigment in their cultures when they are grown on potato dextrose agar (PDA): non-pigmented (wild-type) isolates and yellow- to brown-pigmented isolates (2, 7). The yellow- to brown-pigmented isolates gradually exhibit other cultural degenerative changes such as reduced growth rate and eventual loss of perithecial and ascospore formation (2). These degenerative processes occur over time during semidormant storage and are not created as a result of sequential transfers. Pigmented isolates of *M. cannonballus* have been reported to harbor one or more double-stranded RNA (dsRNA) elements and to exhibit less virulence to muskmelon plants compared with wild-type isolates. The wild types, however, can also contain dsRNA elements (2, 8). Our interest in understanding the possible influence of the pigments on the virulence of the fungus led us to isolate and identify five colored metabolites.

MATERIALS AND METHODS

Fungal Isolates. A soil/oat hull mixture that contained 12 mL of a mixture of 99.5% artificial soil (Terra-Lite, Scotts-Sierra Horticultural Products Co., Marysville, OH), 0.5% ground oat hulls (Quaker Oats

Co., Chicago, IL), and 5 mL of deionized water was prepared. The moist soil/oat hull mixture was autoclaved for 1 h, kept at room temperature for 2 days, and then autoclaved for an additional 1 h. The *M. cannonballus* isolate TX923038 used in this study was collected as a wild-type isolate from muskmelon plant roots in the melon production area of southern Texas in 1992. A pure culture of the wild type was grown on PDA and then hyphal-tip transferred to a 16-mL glass screw-cap vial containing the prepared soil/oat hull mixture. The culture was stored in the dark at 22–25 °C for an extended period of time. In 1996, TX923038 was observed to be producing yellow to brown pigments. The biological characteristics of the pigmented isolate are described elsewhere (7). For the production and identification of the colored metabolites, a small amount of the fungal-colonized soil/oat hull mix was transferred to PDA. The fungus was allowed to grow for 4 days at 30 °C, 7 mm colonized agar disks (no. 5 cork borer) were removed, and then single disks were placed at the centers of 100 × 15 mm dishes each containing 20 mL of PDA. Under these conditions, the isolate produced first yellow then brown pigments when grown for 20–30 days at 30 °C. The PDA culture was extracted as described below.

Materials. Thin-layer chromatography (TLC) was performed on 20 cm² glass plates coated with a 0.5 mm layer of 150 Å silica gel [Whatman International Ltd. (Maidstone, U.K.) and distributed by Fisher Scientific (Pittsburgh, PA)]. The developing solvent systems employed were: system 1, acetone/chloroform/formic acid (20:79:1); and system 2, methanol/toluene/formic acid (20:79:1). Mass spectra were obtained on a Hewlett-Packard (HP) 5989B mass spectrometer using a direct insertion probe heated to the temperature indicated. High-resolution MS were obtained on a Perkin-Elmer Sciex QStar hybrid quadrupole/TOF mass spectrometer using electrospray ionization in the solvent indicated. NMR spectra were obtained on Bruker Avance 300 or ARX-500 spectrometers. IR spectra were taken on a Nicolet FT/IR Magna 550 spectrometer. UV spectra were obtained on an HP 8453 spectrophotometer.

Extraction of Metabolites from *M. cannonballus* Culture. For the extraction of compounds 1–5, PDA cultures of *M. cannonballus*

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Table 1. ^1H NMR Chemical Shifts, Multiplicities, and Coupling Constants for Monosporascone (1), Monosporascol A (4), and Azamonsporascone (5)

position	1^a			4^b			5^b		
	δ	multiplicity	J (Hz)	δ	multiplicity	J (Hz)	δ	multiplicity	J (Hz)
H1	8.78	br s		7.87	d	1.47	7.68 ^c	d	1.36
H3	8.78	br s		8.31	d	1.47	7.67 ^c	d	1.36
H6	6.85	br d	2.25	6.42	d	2.46	6.66	d	2.60
H8	7.16	br d	2.25	6.88	d of d	2.46, 2.50	7.21	d	2.60
H9				5.75	br s				
C ₅ -OH	12.9	s		13.17	s		13.34	s	
C ₉ -OH				2.08	s				
OCH ₃	3.90	s		3.90	s		3.94	s	
N-H							11.7	s	

^a Spectrum was recorded in DMSO-*d*₆. ^b Spectrum was recorded in acetone-*d*₆. ^c Chemical shift values so designated in a column may be interchanged.

isolate TX923038, grown for 20–30 days, were harvested and chopped into small pieces (~1 cm²). Five hundred grams of the fungal PDA material was introduced into 1000 mL of 80% aqueous acetone in a 2-L flask. After 12 h at 4 °C, the extract was filtered through Miracloth (Calbiochem-Behring, La Jolla, CA) to remove the solid matter. The solid material was then re-extracted twice with acetone (1:1, w/v). The extracts were combined, and the acetone was removed under vacuum using an evaporator. The remaining aqueous residue was subsequently extracted three times with equal volumes of ethyl acetate. The ethyl acetate extract was concentrated to 15–50 mL and then stored at 4 °C until needed for further purification.

Isolation and Characterization of Metabolites. The concentrated crude extract from the TX923038 culture was subjected to TLC (systems 1 and 2). A number of compounds were observed on the developed TLC plates via their visible color and fluorescence under UV at 365 or 254 nm. Individual bands were scraped from the TLC plates with a razor blade, eluted from the silica gel with ethyl acetate, filtered through Whatman filter paper no. 5, and concentrated with an evaporator to ~5 mL. The two developing solvent systems were used successively until the target compounds were pure. Crystals of the compounds were obtained by reducing the volume of the solvent. Structures of the compounds were determined using NMR, IR, UV-visible, and mass spectroscopy.

Monosporascone (1) was obtained as yellow crystals; mp 205–215 °C (decomp); EI/MS (probe, 150 °C), m/z (% base peak) 244 (100), 215 (23), 214 (24); ^1H and ^{13}C NMR spectra are given in **Tables 1** and **3**, respectively. Elemental analysis found: C, 63.26%; H, 3.35%. Calcd for C₁₃H₈O₅: C, 63.92%; H 3.30%. Complete information is available in the Supporting Information.

Dehydroxyarthrinone (2) was obtained as orange crystals; mp 190–198 °C (decomp); EI/MS (probe, 200 °C), m/z (% base peak) 264 (100), 151 (22); [α]_D²⁵ +1.2° (c 0.083, MeOH) {lit. (10) [α]_D²⁵ +2.3° (c 0.0001 g/mL, MeOH)}; ^1H and ^{13}C NMR spectra are given in **Tables 2** and **3**, respectively. Complete information is available in the Supporting Information.

Demethylcerdarin (3) was obtained as orange crystals; mp 216–221 °C (decomp); IR (KBr), ν_{max} 1655, 1618, 1570, 1477, 1458, 1446, 1425, 1393, 1355, 1333, 1303, 1262, 1210, 1173, 1155, 1081, 1042, 1026, 1004, 953, 920, 890 cm⁻¹; UV (MeOH), λ_{max} (log ϵ) 217 (4.40), 267 (4.07), 436 (3.57) nm; EI/MS (probe, 150 °C), m/z (% base peak) 264 (100), 246 (15), 244 (30), 218 (17), 216 (15), 206 (27), 205 (16), 203 (19), 190 (21), 189 (19), 186 (12), 179 (18), 179 (18), 178 (14), 177 (32), 175 (16), 163 (30), 162 (10), 161 (24), 160 (47), 153 (15), 152 (25), 151 (82), 150 (11), 147 (26), 145 (15), 135 (14), 132 (11), 131 (10), 125 (16), 123 (12), 122 (12), 121 (10), 118 (16), 117 (14), 111 (14), 103 (11), 102 (16), 95 (15), 94 (10), 91 (11), 90 (14), 89 (30), 87 (11), 79 (12), 77 (23), 78 (12), 75 (12), 74 (11), 73 (22), 69 (42), 66 (12), 65 (16), 64 (11), 63 (32), 62 (16), 60 (20), 55 (17), 53 (26), 52 (10), 51 (26); HR/ESI/TOF-MS (MeOH), m/z 263.0557 [calcd for C₁₃H₁₁O₆: 263.0556]; [α]_D²⁵ -69.4° (c 0.014, MeOH); ^1H and ^{13}C NMR spectra are given in **Tables 2** and **3**, respectively.

Monosporascol A (4) was obtained as orange crystals; mp 165–168 °C; IR (KBr), ν_{max} 1613, 1594, 1572, 1544, 1473, 1437, 1370, 1282, 1272, 1238, 1210, 1194, 1140, 1128, 1060, 1037, 1005, 874,

Table 2. ^1H NMR Chemical Shifts, Multiplicities, and Coupling Constants for Dehydroxyarthrinone (2) and Demethylcerdarin (3)

position	2^a			3^a		
	δ	multiplicity	J (Hz)	δ	multiplicity	J (Hz)
H1	4.17	d	10.3	6.46	d	3.6
H1	4.29	d	10.3			
H3	4.11	d	11.1	4.88	d	16.6
H3	4.30	d	11.1	5.06	d/d	3.6, 16.6
H6	6.42	d	2.4	6.78	d	2.4
H8	6.50	d	2.4	7.10	d	2.4
H9	5.23	bs				
C ₅ -OH	11.64	s		12.07	bs	
C ₉ -OH	2.10	s				
OCH ₃	3.84	s		3.99	s	

^a Spectrum was recorded in acetone-*d*₆.

834, 814, 756, 681, 624, 593 cm⁻¹; UV (MeOH), λ_{max} (log ϵ) 202 (4.23), 222 (4.14), 300 (4.06), 335 (3.94) nm; EI/MS (probe, 240 °C), m/z (%) 246 (100), 245 (23), 244 (76), 230 (31), 229 (21), 215 (27), 214 (23), 201 (16), 189 (14), 187 (19), 186 (24), 175 (11); HR/ESI/TOF-MS (LiCl/MeOH), m/z 253.0685 [calcd for C₁₃H₁₀LiO₅: 253.0688]; [α]_D²⁵ -70.6° (c 0.085, MeOH); ^1H and ^{13}C NMR spectra are given in **Tables 1** and **3**, respectively. We propose the trivial name monosporascol A for this compound.

Azamonsporascone (5) was obtained as yellow crystals; mp 279–283 °C; IR (KBr), ν_{max} 3404, 1627, 1593, 1531, 1446, 1389, 1368, 1336, 1274, 1263, 1217, 971 cm⁻¹; UV (MeOH), λ_{max} (log ϵ) 396 (3.94), 272 (3.85), 250 (3.84), 223 (3.92) nm; EI/MS (probe, 240 °C), m/z (%) 243 (100), 242 (14), 214 (25), 213 (19), 200 (10), 185 (24), 177 (16), 159 (10), 151 (13), 121 (13); HR/ESI/TOF-MS (MeOH), m/z 244.0607 [calcd for C₁₃H₁₀NO₄: 244.0610]; ^1H and ^{13}C NMR spectra are given in **Tables 1** and **3**, respectively. We propose the trivial name azamonsporascone for this compound.

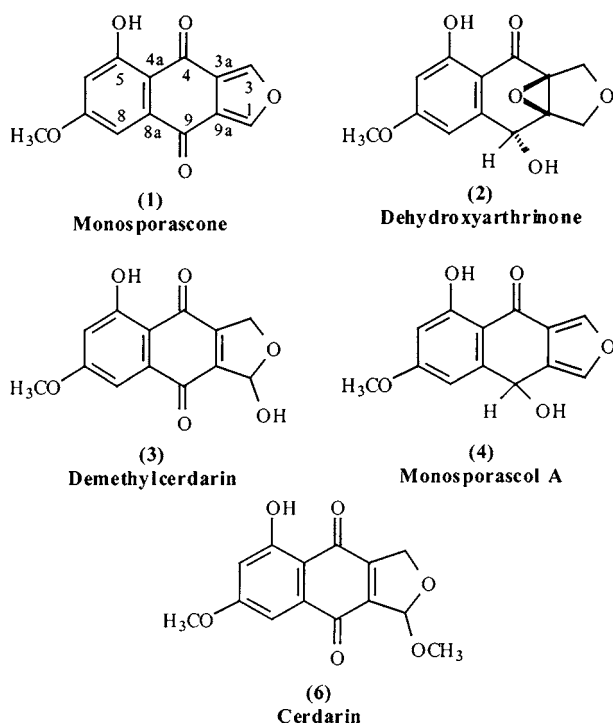
RESULTS AND DISCUSSION

Identification of Monosporascone (1). After TLC purification, this compound was obtained as yellow crystals. It exhibited yellow fluorescence under both UV 365 and 254 nm light. Mass spectrometric analysis, utilizing a direct insertion probe, provided a parent ion at m/z 244, which together with elemental analysis indicated a formula of C₁₃H₈O₅. The ^1H and ^{13}C NMR spectra provided a pattern of peaks that were found in all of the subsequent compounds, specifically, the appearance of a methoxy group, a hydroxyl group chelated to a carbonyl group, and a pair of *meta*-aromatic protons. In the case of compound **1** (**Tables 1** and **3**), two protons characteristic of positions 1 and 4 in furan were also evident together with the corresponding peaks in the ^{13}C NMR spectrum. A literature search found that the spectroscopic characteristics of compound **1** matched those reported by Fujimoto et al. (9) for 5-hydroxy-7-methoxy-4,9-

Table 3. ^{13}C NMR Chemical Shifts and Multiplicities of Monosporascone (1), Dehydroxyarthrinone (2), Demethylcerdarin (3), Monosporascol A (4), and Azamonosporascone (5)

position	1 ^a		2 ^b		3 ^b		4 ^b		5 ^b	
	δ	multiplicity	δ	multiplicity	δ	multiplicity	δ	multiplicity	δ	multiplicity
C1	147.4	d	66.2	t	101.6	d	142.6	t	123.7 ^c	d
C3	147.7	d	66.1	t	71.0	t	145.9	t	124.1 ^c	d
C3a	122.0	s	64.5	s	149.6	s	122.3	s	122.7 ^d	s
C4	183.6	s	194.1	s	185.7	s	185.8	s	186.2	s
C4a	111.5	s	106.6	s	110.7	s	110.7	s	112.5	s
C5	165.2	s	165.4	s	165.1	s	166.6	s	166.2	s
C6	106.1	d	101.4	d	106.4	d	100.7	d	105.8	d
C7	165.8	s	167.0	s	166.8	s	167.2	s	166.4	s
C8	107.2	d	110.3	d	108.7	d	108.5	d	107.6	d
C8a	137.0	s	143.7	s	134.7	s	149.4	s	138.8	s
C9	177.7	s	65.3	d	181.6	s	62.1	d	179.3	s
C9a	121.9	s	69.4	s	145.2	s	127.3	s	123.0 ^d	s
OCH ₃	56.2	q	55.9	q	56.6	q	56.21	q	56.4	q

^a Spectrum was recorded in DMSO-*d*₆. ^b Spectrum was recorded in acetone-*d*₆. ^{c,d} Chemical shift values so designated in a column may be interchanged.

**Figure 1.** Structures of metabolites isolated from *M. cannonballus*.

dioxonaphtho[2,3-*c*]furan (**Figure 1**), which had been isolated from the ascomycete *Gelasinospira pseudoreticulata*. HMBC and HMQC 2D NMR experiments confirmed this structure. We propose the trivial name of monosporascone for this compound.

Identification of Dehydroxyarthrinone (2). Compound **2** was obtained as orange crystals. Mass spectrometric analysis, using a direction insertion probe, provided a parent ion at m/z 264. Similarities were immediately noted between the ^1H NMR and ^{13}C NMR spectra of monosporascone (**1**) and compound **2**. Specifically, the appearances of a methoxy group at δ 3.84, a hydroxyl group at δ 11.64, and *meta*-aromatic protons at δ 6.42 and 6.50 in the ^1H NMR spectrum were similar to those of monosporascone. There was also a carbonyl group indicated in the ^{13}C NMR spectrum (δ 194.1), but it was at lower field than that normally observed for quinones. Furthermore, an HMBC experiment showed that the proton on C-8 was coupled to a secondary oxygenated carbon at δ 65.3, whereas the proton on this carbon was similarly coupled to C-8. Four other nonaromatic carbons were evident in the ^{13}C NMR spectrum.

On the basis of the molecular weight provided by the mass spectrum, a search of the literature was made for possible structures. The spectroscopic data of dehydroxyarthrinone reported by Whyte et al. (10) were in good agreement with that of compound **2** (**Figure 1**). HMBC and HMQC NMR experiments confirmed this structure and provided the assignments given in **Tables 2** and **3**.

Identification of Demethylcerdarin (3). Compound **3** was obtained as orange crystals. The EI/MS spectrum of compound **3** was very similar to that observed for monosporascone (**1**), including a parent ion at m/z 244. However, CI/MS gave a parent ion at m/z 263 ($M + 1$), which indicated compound **3** had decomposed during the acquisition of the EI/MS with the loss of water to give monosporascone (**1**) or a compound closely related to it. The HR/ESI/TOF-MS analysis indicated a molecular formula of $\text{C}_{13}\text{H}_{10}\text{O}_6$, which differs from that of monosporascone (**1**) by the addition of H_2O . The ^{13}C NMR results (**Table 3**) were in fairly good agreement with those reported by Whyte et al. (10) for cerdarin (**6**). The exceptions were for carbons 1, 9a, and 3a, which appeared at δ 108.1, 150.9, and 143.5 in cerdarin, respectively. Cerdarin (**6**) also has an additional peak at δ 55.4 due to the presence of a methyl ether. All other peaks in the ^{13}C NMR spectrum agreed with those of cerdarin (**6**), differing by 1.1 ppm or less. 2-D NMR experiments, HMBC, and HMQC established that the compound was 1,3-dihydro-1,5-dihydroxy-7-methoxynaphtho[2,3-*c*]furan-4,9-dione (**Figure 1**), for which we propose the trivial name demethylcerdarin.

Identification of Monosporascol A (4). Compound **4** was obtained as orange crystals. Its EI/MS had a parent ion at m/z 246. The molecular formula was established as $\text{C}_{13}\text{H}_{10}\text{O}_5$ by HR/ESI/TOF-MS, and it was optically active. As with the other compounds, the ^1H NMR showed the characteristic absorptions due to a hydroxyl proton hydrogen bonded to a carbonyl group (δ 13.17), a methoxy group (δ 3.90), and *meta*-substituted aromatic protons at δ 6.42 and 6.88 (**Table 1**). These chemical shifts are similar to those of the C-6 and C-8 protons in monosporascone (**1**). A second hydroxyl group was noted at δ 2.08. Two other protons with chemical shifts similar to those of C-1 and C-3 in monosporascone were also noted at δ 7.87 and 8.31, respectively. The remaining proton appeared at δ 5.75. A COSY experiment showed that the C-1 and C-3 protons were coupled as expected and that the C-1 proton was further coupled to the proton at δ 5.75 (C-9). These chemical shifts and couplings indicated that compound **4** had the structure indicated (**Figure 1**). An HMBC experiment confirmed this structure. The

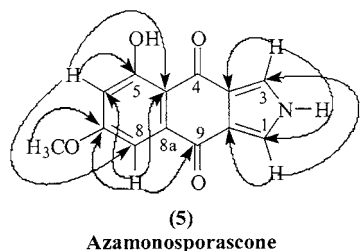


Figure 2. HMBC correlations for azamonosporascone (5).

strong coupling of the proton on C-9 to C-8, C-9a and C-8a, and the weak couplings to C-3a, C-4a and C-1 were particularly relevant to the establishment of the structure and to the assignments of chemical shifts (Tables 1 and 3). We propose the trivial name monosporascol A for this compound. The absolute configuration at C-9 was not established.

Identification of Azamonosporascone (5). Compound 5 was obtained as yellow crystals. Its EI/MS gave a parent ion at m/z 243. The HR/FAB/MS indicated a molecular formula of $C_{13}H_{10}NO_4$ for the parent + 1 peak; thus, a molecular formula of $C_{13}H_9NO_4$ was established. The 1H NMR results (Table 1) showed similarities to those of monosporascone (1). That is, the characteristic absorptions due to a hydroxyl proton hydrogen bonded to a carbonyl group (δ 13.34), a methoxy group (δ 3.94), and *meta*-substituted aromatic protons at δ 6.66 and 7.21 were observed. An N-H absorption was noted at δ 11.7. The remaining protons at δ 7.68 and 7.67 were in reasonably good agreement with those found in 5-methoxy-2*H*-benz[*f*]isoindole-4,9-dione (i.e., δ 7.60 and 7.50) (11). An HMBC experiment confirmed that compound 5 was azamonosporascone, 8-hydroxy-7-methoxy-2*H*-benz[*f*]isoindole-4,9-dione. The structure and HMBC correlations are shown in Figure 2. The 1H and ^{13}C NMR chemical shift assignments for C-1, C-3, C-3a and C-9a are tentative and based on those previously assigned for 5-methoxy-2*H*-benz[*f*]isoindole-4,9-dione (11).

In this study, the five colored hexaketides described above were isolated and identified from metabolites produced by the *M. cannonballus* isolate TX923038. The same compounds have been detected in other yellow to brown isolates of the fungus when grown on PDA (7). Only a trace of monosporascone (1) was found in PDA cultures of some wild-type isolates of *M. cannonballus* (7). The possible effect of these compounds on virulence by wild types of *M. cannonballus* requires additional study.

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Supporting Information Available: Complete IR, UV, and mass spectral information for monosporascone (1) and dehydroxyarthrinone (2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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