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NAPHTHOQUINONES PRODUCED BY *FUSARIUM SOLANI* ISOLATED FROM CITRUS

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Key Word Index—*Fusarium solani*; Deuteromycetes; citrus blight; naphthoquinones; fungal toxicity.

Abstract—Eleven naphthoquinone pigments are described which were produced by *F. solani* isolates obtained from roots of diseased citrus trees. One of these pigments was shown to be the precursor for six of the isolated compounds.

INTRODUCTION

Fusarium solani (Mart.) Appel & Wr. Emend. Snyd. & Hans., a common soil fungus in citrus groves, can be readily isolated from vascular tissue of fibrous roots from citrus trees affected with blight. This disease has also been called young tree decline, sand hill decline or rough lemon decline, and has been observed to some degree on most citrus scion–rootstock combinations [1]. *F. solani* isolated from herbaceous crop plants has been shown to produce naphthoquinone pigments, some of which are toxic to plants [2]. Recently we found that isolates of this fungus from roots of blight-affected citrus trees produced toxins which may adversely affect plants [3]. We now report the isolation and identification of 11 naphthoquinones produced by *F. solani* obtained from roots of diseased citrus trees.

RESULTS AND DISCUSSION

Fifty single spore isolates of *F. solani*, obtained from fibrous roots of field grown citrus trees that exhibited blight symptoms were examined for production of naphthoquinones. Most of the isolates produced similar TLC patterns of pigments in about the same concentrations. However, six of the isolates differed markedly in type and/or amount of pigment produced. Because they were distinctively different, these six isolates were selected for production of naphthoquinones for isolation, identification and testing as potential phytotoxins. Two isolates of *F. solani* obtained from peas (*Pisum sativum*) and three from citrus trees in California that exhibited dry root rot symptoms were also tested. We developed eight solvent systems for survey and isolation of these pigments by TLC. Eleven naphthoquinones were isolated and identified (Table 1), two of which (**3**, **8**) had not been previously reported as metabolites of *F. solani*.

Based mainly on NMR and mass spectral data, compounds **1**–**3** are pyrans [4] and **1** and **2** were found to be the precursors to several of the compounds isolated (Fig. 1). Kurobane *et al.* [4] reported the NMR of **1** in pyridine-d_5 at 0° and of **2** in CD_2Cl_2 at 28° [4]. Their spectra were analysed with a Varian spin simulation program on a 620L data system to obtain the reported coupling constants. We confirmed their work by spin-spin decoupling.

In our study, the NMR spectra were determined in deuterochloroform at 270 MHz, and those listed in Table 2 are the observed spectra. The multiplets in **1** and **3** at δ 2.97 and 3.43 were eight line multiplets. Each multiplet appeared as four doublets. At δ 2.97 there were three evenly spaced doublets with a $J = 5.5$ Hz, the center doublet was split into a double doublet with a $J = 2.7$ Hz. At δ 3.43 there were three evenly spaced doublets with a $J = 3.5$ Hz, the center doublet was split into a double doublet with a $J = 1.4$ Hz. First order analysis could be applied to these four absorptions to obtain coupling constants. This was confirmed by decoupling of **1**. First-

Table 1. Naphthazarin toxins produced by *Fusarium solani*

1 Rel-(3*R*,4a*R*,10a*R*)-5,10-dioxo-3,4,4a,5,10,10a-hexahydro-7-methoxy-3-methyl-3,6,9-trihydroxy-1-*H*-naphtho(2,3-*c*) pyran
2 Rel-(3*R*,4a*R*,10a*S*)-5,10-dioxo-3,4,4a,5,10,10a-hexahydro-7-methoxy-3-methyl-3,6,9-trihydroxy-1-*H*-naphtho(2,3-*c*) pyran
3 Rel-(3*R*,4a*R*,10a*R*)-5,10-dioxo-3,4,4a,5,10,10a-hexahydro-3,7-dimethoxy-3-methyl-6,9-dihydroxy-1-H-naphtho(2,3-*c*) pyran
4 Fusarubin (oxyjavanicin)
5 Anhydrofusarubin
6 Javanicin (2-acetonyl-3-methyl-7-methoxy-naphthazarin)
7 Norjavanicin (2-acetonyl-7-methoxy-naphthazarin)
8 Methyl ether fusarubin
9 Marticin
10 Isomarticin
11 5,8-Dihydroxy-6-methoxy-3-methyl-2-aza-9, 10-anthracenedione
12 Ethyl ether fusarubin (synthetic product)

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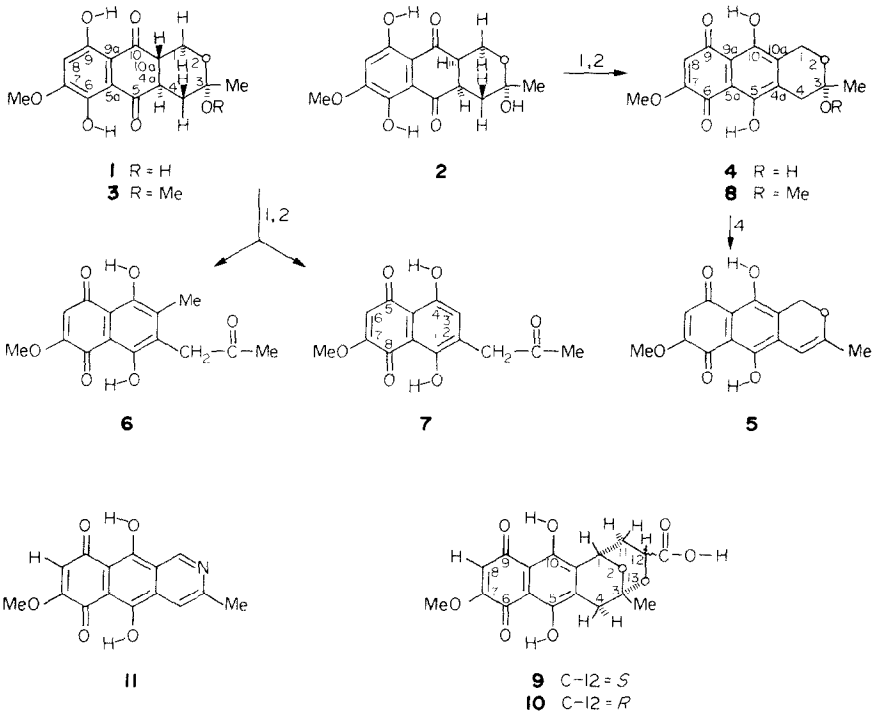

Fig. 1. Structures and pathway of formation of *Fusarium* naphthoquinones.

order analysis could not be applied to the multiplets in **2**. Decoupling of **1** at δ 3.43 caused the double doublets (*dd*) at δ 1.69 and δ 2.42 to appear as doublets (*d*) J = 13.5 Hz. The eight line multiplet at δ 2.97 was now a *dd* with J = 11.0 Hz (10a 1ax) and J = 5.4 Hz (10a-1eq). Decoupling at δ 4.22 gave a *d* at δ 2.97 J = 13.5 Hz (10a, 4a). Decoupling at δ 2.97 caused the seven peak multiplet at δ 4.22 to appear as two doublets at δ 4.15, J = 12.0 Hz (1ax-1eq) and at δ 4.25, J = 12.0 Hz (1eq-1ax). The eight line multiplet at δ 3.43 was now a *dd*, J = 11.0 Hz (4a, 4ax) and J = 3.5 Hz (4a, 4eq) after decoupling. The NMR and coupling constants were in agreement with the published data [4], except that in deuterochloroform the 1ax proton in **1**–**3** was upfield not downfield as shown in ref. [4]. This was confirmed by isolation of **3**, the methyl ether of **1**, which has also been synthesized from **1** [5]. The NMR of **3** showed the 1ax proton as a *dd* at δ 3.88 with overlap that caused the peaks to look like a triplet. The 1eq proton appeared as a *dd* at δ 4.22. This reversal for tetrahydropyrans was reported previously [6]. These three compounds, identified as dihydrofusarubins (**1**–**3**) were very unstable and subject to oxidation [4] with **3** being the most stable. Pure crystals of **2** and **3** were yellow; **1** yielded light red crystals, probably caused by the presence of a trace of **4**. All three appeared yellow on TLC plates under visible or UV light. Fusarubin (**4**) was removed from **1** by TLC using solvent system A.

A prep. TLC plate was spotted with 15 mg of material containing mainly **1** and **2** and the plate was developed twice in solvent system F. One major band contained **1** and **2** and after heating the plate at 85° for 3.5 hr, the major band was collected, eluted and the products separated by further TLC. From this band fusarubin (**4**), anhydrofusarubin (**5**) [5], javanicin (**6**) [7] and norjavanicin (**7**) [8], were isolated and identified, but **7** was present in trace amounts only. Both javanicins were unexpected products. In order to determine the possible source of **4** and **6** we extracted a culture filtrate with ethyl acetate until there was no further evidence of them. The extracted aqueous medium was then heated on a steam bath for 1 hr and re-extracted, whereupon, we found that **4** and **6** were present again. Next, a potassium bromide pellet of pure **1** was prepared and an IR spectrum was obtained showing a strong, sharp hydroxyl absorption at 3450 cm^{-1} due to the 3-hydroxyl. The phenol hydroxyls were not observed but the carbonyl absorption was strong at 1620 cm^{-1}. After placing the pellet in a vacuum oven at 160° for 30 min another spectrum was obtained and the 3-hydroxyl absorption was gone, a new carbonyl band was strong at 1715 cm^{-1} and the 1620 cm^{-1} carbonyl band had shifted to 1598 cm^{-1}. This spectrum was similar to that of javanicin (**6**). When the procedure was repeated with a sample of **2** with the temperature lowered to 140°, we again obtained the spectrum of javanicin (**6**). A sample of **1**, upon examination by TLC was relatively pure except for a minute quantity of **4**. A 4 mg sample of this material, placed in a test tube in a vacuum oven at 160° for 30 min, upon TLC contained **1** and **4**–**6** with **6** as the major product, thus, indicating **4**–**6** could form from **1**. Kurobane *et al.* [9] postulated that **6** is a co-metabolite from an intermediate compound in the formation of **1** and **2** rather than a conversion product. Our results, however, have shown that **6** can be a conversion product derived from **1**. Kurobane *et al.* also stated that **7** is a conversion product and our results agree with this conclusion.

For production of these compounds we have developed both still and shake cultures, using the same salt media in both procedures. The pH of both shake and still cultures at harvest was 2.4. By TLC analysis, compounds **4** and **6** were the major products in the still cultures with **1**, **2**, **5**, **7**

Table 2. ^{1}H NMR data of **1–12** (270 MHz, TMS as internal standard)

Compound	Me-3	H-4ax	H-4eq	H-10a	OH-3	H-4a	MeO-7	H-1ax	H-1eq	H-8	OH-6 and OH-9 OH-5 and OH-10	(1–3) (4,5,8–10,12)
1	1.54	1.69 *dd*	2.42 *dd*	2.97 *m*	2.02 *d*	3.43 *m*	3.97	4.22 *m*	4.22 *m*	6.68	12.05	12.22
	—	—	—	13.5 (4a)		13.5 (10*a*)	—	—	—	—	—	—
	—	13.5 (4eq)	13.5 (4ax)	11.0 (1ax)	2.4	11.0 (4ax)	—	—	—	—	—	—
	—	11.0 (4a)	3.5 (4a)	5.5 (1eq)		3.5 (4eq)	—	—	—	—	—	—
2	1.47	1.63 *t*	2.04 *dd*	2.99 *m*	2.02	3.62 *m*	3.98	4.06 *dd*	4.61 *d*	6.75	12.37	12.69
	—	13.0 (4eq)	13.0 (4ax)	—	—	—	—	11.5 (1eq)	11.5 (1ax)	—	—	—
	—	—	4.0 (4a)	—	—	—	—	3.0 (10a)	—	—	—	—
3	1.47	1.69 *dd*	2.42 *dd*	2.97 *m*	3.24 c	3.43 *m*	3.98	3.88 *dd*	4.22 *dd*	6.68	12.05	12.22
	—	—	—	13.5 (4a)	—	13.5 (10a)	—	—	—	—	—	—
	—	13.5 (4eq)	13.5 (4ax)	11.0 (1ax)	—	11.0 (4ax)	—	12.0 (1eq)	12.0 (1ax)	—	—	—
	—	11.0 (4a)	3.5 (4a)	5.5 (1eq)	—	3.5 (4eq)	—	11.0 (10a)	5.5 (10a)	—	—	—
4	1.67	2.77 *dd*	3.05 *d*	—	2.22 *d*	—	3.94	4.90	4.90	6.18	12.69	12.95
	—	18.0 (4eq)	18.0 (4ax)	—	2.0	—	—	—	—	—	—	—
	—	2.0	—	—	—	—	—	—	—	—	—	—
5	2.04	6.02f	—	—	—	—	3.94	5.24	5.24	6.15	12.70	13.09
6	2.28	2.22g	3.88h	—	—	—	3.91	—	—	6.18	12.82	13.21
7	—	2.31 g	3.81 h	7.18 r	—	—	3.93	—	—	6.18	12.55	12.59
8	1.55	2.66 *dt*	3.02 *dd*	—	3.33c	—	3.94	4.56 *dt*	4.88 *dd*	6.18	12.69	12.95
		18.0 (4eq)	18.0 (4ax)	—	—	—	—	18.0 (1eq)	18.0 (1ax)	—	—	—
		2.0	1.5	—	—	—	—	2.7	1.5	—	—	—
9	1.68	2.73 *d*	3.12 *d*	2.10 *mj*	2.86 *mi*	5.42 *dk*	3.94	—	4.58 *m*	6.18	12.53	12.93
	—	19.0 (4eq)	19.0 (4ax)	—	—	9.0 (11ax)	—	—	—	—	—	—
10	1.70	3.00 *d*	3.13 *d*	2.04 *mi*	2.30 *mj*	5.58 *dk*	—	—	4.31 *dd*	6.20	12.51	12.91
	—	19.0 (4eq)	19.0 (4ax)	—	—	5.0 (11ax)	—	—	12.0 (11eq)	—	—	—
	—	—	—	—	—	—	—	—	3.0 (11ax)	—	—	—
11	2.81	7.96n	—	—	—	—	4.02	9.50 p		6.75	13.10	13.50
12	1.55	2.67 *dt*	3.02 *dd*	1.16 *tu*	—	3.61 *qv*	3.94	4.53 *dt*	4.86 *dd*	6.18	12.69	12.97
	—	18.0 (1eq)	18.0 (1ax)	6.7	—	6.7	—	18.0 (1eq)	18.0 (1ax)	—	—	—
	—	2.3	1.4	—	—	—	—	2.7	1.0	—	—	—

J (Hz); unmarked signals are singlets; *d*, doublet; *m*, multiplet; *q*, quartet; *t*, triplet; c, MeO-3; f, H–C=C-4; g, Me–$\overset{O}{\overset{\|}{C}}$; h, $\overset{O}{\overset{\|}{C}}$–$CH_2$; i, H-11ax; j, H-11eq; k, H-12ax, n, H-4; p, H-1; r, H-3; u, Me–CH_2; v, Me–CH_2.

and **8** as minor components, while **1** and **2** were the major products in most shake cultures with **3**, **4**, **6** and **9**–**11** as minor components. Two isolates produced large quantities of fusarubin, even though the medium remained quite acidic (pH < 3). Kurobane *et al.* [9] stated that fusarubin is a conversion product occurring when media containing **1** and **2** become basic. However, our results show that some strains of *F. solani* can produce fusarubin as a true metabolite under acidic conditions.

Figure 1 shows the proposed pathway for formation of 6 of the 11 compounds isolated in this study. As previously shown, **1** and **2** can be easily oxidized to **4** [4, 9] and we have shown also that they can easily be converted to **6**. The structures of **4** and **6** were originally verified by synthesis [10, 11] but NMR data were only reported for **6**, and ours is identical. In the attempted synthesis of **4** only a derivative of it was obtained. Chilton reported a partial NMR spectrum of **4** in pyridine-d_5 and a partial spectrum of **5** and **7** in deuterochloroform [8]. We are presenting the complete NMR spectra as shown in Table 2. Compound **4** is easily converted to **5** by acid or to **8** in the presence of acid and methanol [5].

The marticins (**9** and **10**) have been reported to be quite toxic to tomatoes and peas [2] and small quantities of these compounds were produced by several of our *Fusarium solani* isolates. We isolated more of **9** than **10**, because when cleaning up on TLC, **10** is converted to **9**. This had previously been reported by Kern and Naef-Roth [12]. The marticins are diasteromeric at C-12. The ring that contains C-12 is a 1,3-dioxane. Conformational analysis of substituted 1,3-dioxanes has shown that stabilizing the ring at the 2- or 5-position affects the signals of certain ring and methyl substituent hydrogens in positions corresponding to the 4-position in cyclohexane [13, 14]. In the marticins the 2- and 4-positions of the dioxane ring are the C-4 and C-1 bridgehead carbons. The NMR spectrum of **9** shows the H-11 (a–e) to have a chemical shift of 0.76 ppm and the H-4ae to have a shift of 0.39 ppm. In **10** the chemical shift for H-11 (a–e) is 0.26 ppm and H-4(a–e) is 0.13 ppm. NMR resonance experiments on acetals [15] show that in 2,4,6-substituted dioxanes, r-2-Me-*cis*-4-*trans*-6-substitution, the chemical shift at C-5 is greatest. In the marticins the C-11 is equal to the C-5 position of the dioxane ring. In **9** the dioxane ring would be predominately in the chair form with the carbonyl group in the equatorial position *cis* to Me-4 and the hydrogen axial with substitution at C-1 *trans*. This configuration has the least strain and steric hindrance. In **10** the dioxane ring would be predominately in the boat form with the carbonyl group in an equatorial position *trans* to Me-4 and the hydrogen axial with substitution at C-1 *trans*. Compound **9** has the *S*-configuration at C-12 while **10** has the *R*-configuration. Both **9** and **10** gave identical mass spectral cracking patterns and a molecular ion of 376. A strong carbonyl absorption in the IR at 1720 cm^{-1} was observed for both, but the minor isomer, **10**, showed a strong sharp hydroxyl at 3430 cm^{-1} and then broad tailing typical of an acid. The hydroxyl region of **9** is broad and diffuse at 3600–2800 cm^{-1}.

Compound **12** was reported as a metabolite of *F. solani* [16] and later reported to be a possible artifact [8]. We synthesized **12** from **4** for use as a TLC marker but this compound was not observed in extracts from any of our isolates. The anthracenedione **11** was found in several of the isolates which had been grown as shake cultures, but not in the still cultures. This compound has been previously isolated from *F. bostrycoides* and was named bostrycoidin [17]. This compound was also found along with **1**, **2**, **4** and **5** in shake cultures of both our *F. solani* isolates from *Pisum sativum*. Compound **11** was also isolated by Kurobane *et al.* [9], who reported it was only "produced under conditions where fusarubin accumulated". Although we did not vary the carbon–nitrogen ratio in our medium, four of our isolates produced **11** under acidic conditions. At this point it is not clear whether **11** is a metabolite, a conversion product, or both. All three *F. solani* cultures isolated from California citrus produced naphthoquinones **1**, **2**, **4** and **5**, and several other unidentified trace components.

Table 3 lists the UV and visible spectra of all compounds isolated, and Table 4 shows the R_f's in four solvent systems. These data would readily help in a preliminary identification of compounds from different *F. solani* isolates.

Using cultures obtained from blighted citrus trees, we have isolated and identified most of the known naphthoquinones produced by *F. solani*. Many of the isolated and identified compounds appear to be present in most of the isolates, at least in trace quantities. Two of our isolates did not produce any naphthoquinone compounds. Depending on the specific isolate and the culture conditions the major products varied between **1**, **2**, **4** and **6**. Compound **6** was produced in moderate amounts in still cultures that were grown for 2 weeks and in trace quantities in shake cultures. Compound **7** was only found in trace amounts in still cultures. Compounds **1** and **2** were the major products in shake cultures that were harvested after 3 or 4 days and also they were minor products in still cultures. Compounds **3** and **7**–**10** were only produced in trace quantities by any of our isolates. We are presently producing larger quantities of some of these compounds for testing their toxicity on citrus.

EXPERIMENTAL

TLC solvent systems. A, C_6H_6–nitromethane–HOAc (75:25:2); B, $CHCl_3$–EtOAc–hexane–HOAc (10:5:5:0.3); C, hexane Me_2CO–HOAc (15:5:0.3); D, C_6H_6–EtOAc–*iso*-

Table 3. UV and visible spectra in ethanol

Compound	λ_{max} (nm)	Compound	λ_{max} (nm)
1	214,245,277,304,394	**7**	225,299,477,503,540
2	212,245,277,304,394	**8**	228,306,475,499,534
3	210,245,277,304,394	**9**	229,304,474,501,536
4	227,305,475,499,536	**10**	229,304,474,501,536
5	207,234,292,544,578	**11**	253,322,476,500,530
6	227,305,478,504,541	**12**	227,305,474,499,534

Table 4. Thin layer chromatography data (R_f 15 cm)

Compound	Solvent system			
	A	B	C	D
1	0.21	0.27	0.21	0.38
2	0.18	0.21	0.17	0.33
3	0.43	0.57	0.35	0.63
4	0.23	0.29	0.23	0.39
5	0.54	0.57	0.37	0.61
6	0.36	0.45	0.30	0.51
7	0.33	0.35	0.28	0.41
8	0.43	0.50	0.36	0.55
9	0.12	0.13	0.10	0.17
10	0.11	0.12	0.09	0.15
11	0.31	0.38	0.35	0.43
12	0.49	0.51	0.38	0.57

PrOH–HOAc (145:50:5:1); E, C_6H_6–hexane–Me_2CO (9:9:2) or (3:6:1); F, C_6H_6–Me_2CO (17:3); G, hexane–Me_2CO–HOAc (15:5:0.1); H, C_6H_6–EtOAc–*iso*-PrOH–HOAc (16:3:1:0.1). Systems A–D were used with 250 μm Si gel GF plates for survey of extracts. Systems E–H were used with 1 mm Si gel HF-60 plates for isolation.

Samples. Isolates of *F. solani* were obtained from fibrous roots of Florida citrus trees with obvious blight symptoms and from roots of California citrus trees with dry root rot symptoms. Isolates from pea roots were obtained from Dr. John M. Kraft, ARS-USDA, Prosser, Washington. These isolates were grown at 27° in a previously described mineral salts–glucose liquid medium [3]. Shake cultures were incubated for 3–4 days on a reciprocal shaker at 150 rpm, while still cultures were grown for 14 days without shaking. Cultures were filtered through cheesecloth, and the filtrate extracted twice with EtOAc.

^{1}H NMR 270 MHz, TMS as int. standard in $CDCl_3$ and MS were obtained through the Chemistry Department of Florida State University. Mps are uncorr. Rel-(3*R*, 4a*R*, 10a*R*)-5,10-dioxo-3, 4, 4a, 5, 10, 10a-hexahydro-7-methoxy-3-methyl-3, 6, 9-trihydroxy-1-H-naphtho(2, 3-*c*)pyran (**1**). MS *m/z* 308: pale red crystals 158–160° (EtOAc) or (CH_2Cl_2–hexane), 153–154° [4]. Rel-(3*R*, 4a*R*, 10a*S*)-5, 10-dioxo-3, 4, 4a, 5, 10, 10a-hexahydro-7-methoxy-3-methyl-3,6,9-trihydroxy-1-*H-naphtho*(2,3*c*)pyran (**2**). MS *m/z* 308: yellow crystals 126–129° MeOH, 117–118° [4]. Rel-(3*R*,4a*R*,10a*R*)-5,10-dioxo-3,4, 4a, 5, 10, 10a-hexahydro-3,7-dimethoxy-3-methyl-6, 9-dihydroxy-1-*H*-naphtho(2,3*c*)pyran (**3**). MS *m/z* 322: yellow crystals 176–180° MeOH. Fusarubin-(oxyjavinicin) (**4**). MS *m/z* 306: red crystals 196–198° C_6H_6, 195–197° [18], charring at *ca* 200° but mp 218° [5], 210° dec. [8]. Anhydrofusarubin (**5**). MS *m/z* 288: blue-black crystals 197–198° C_6H_6–CH_2Cl_2, 204° [5]. 2-Acetonyl-3-methoxy-7-methoxy-naphthazarin (javanicin) (**6**). MS *m/z* 290: red needles 207–208° C_6H_6, 207–208° [11]. 2-Acetonyl-7-methoxy-naphthazarin (norjavanicin) (**7**). MS *m/z* 276: red needles 195–200° CH_2Cl_2–MeOH, 200–204° [8]. Methyl ether of fusarubin (**8**). MS *m/z* 320: red crystals 188–190° EtOAc, 190° [5]. Marticin (**9**) MS *m/z* 376: red crystals 180–182° C_6H_6, 200–201° [12]. Isomarticin (**10**) MS *m/z* 376: red crystals 160–163° MeOH, 168–169° [12]. 5,8-Dihydroxy-6-methoxy-3-methyl-2-aza-9,10-anthracenedione (bostrycoidin) (**11**). MS *m/z* 285: red crystals 231–235° C_6H_6–CH_2Cl_2. Ethyl ether of fusarubin (**12**). MS *m/z* 334. Red needles 197–198° C_6H_6–CH_2Cl_2, 185–195° [15].

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