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MUSANOLONES: FOUR 9-PHENYLPHENALENONES FROM RHIZOMES OF *MUSA ACUMINATA*

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Key Word Index—*Musa acuminata*; Musaceae; banana; phytoalexins; musanolones; 9-phenylphenalenones; *Fusarium oxysporum*; Panama's disease.

Abstract—Four new phenalenone-type phytoalexins, named musanolones C–F, have been isolated from infected rhizomes of banana plants (*Musa acuminata*; AAA cultivar Grand Nain). These phytoalexins were biosynthesized *de novo* by the plants upon infection by *Fusarium oxysporum* f. sp. *cubense* race 4, Panama's disease causal agent. The structures of the new phytoalexins were elucidated using spectroscopic data and chemical correlations. Hydroxyanigorufone has been previously described as a constitutive natural product, from *Anigozanthos rufus*, but it was never described as a phytoalexin. The chemical shift for all of the hydrogen and carbon atoms in the musanolones C–F were unambiguously established by mono- and bi-dimensional, homo- and hetero-nuclear NMR experiments (^{1}HNMR, ^{13}CNMR, COSY, HMQC and HMBC). Preliminary *in vitro* assays of all the musanolones tested until now show a strong inhibitory activity on the growth of the germination tube of *F. oxysporum* f. sp. *cubense* race 4.

INTRODUCTION

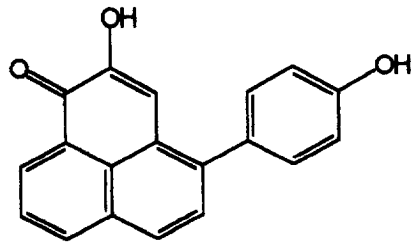
The phytoalexins are produced *de novo* by some plant organs when provoked by biotic, physical or chemical agents [1]; several synthetic polyamines as well as some fungus-derived carbohydrates have been tested as phytoalexin inducers in species of *Pisum* [2, 3]. Recently, we described the isolation and characterization [4] of irenolone **1**, the first example of a new type of phytoalexin structurally based on a phenalenone skeleton, from leaves and fruit peels of *Musa paradisiaca* [*M. acuminata* (AAA), Grand Nain], elicited by kanamycin or by *Mycosphaerella fijiensis* (causal agent of Black Sigatoka), a pathogenic fungus which attacks banana leaves and greatly reduces their growth. Structurally similar compounds, with a *p*-hydroxyphenyl substituent attached to the C-4 carbon atom of a perinaphthenone nucleus, were obtained [5] from rhizomes infected with the pathogenic fungus *Fusarium oxysporum* f. sp. *cubense* race 4, which causes the Panama's disease in banana plants.

To our knowledge, no 4-phenylphenalenones have ever been described either as natural or synthetic substances; nevertheless, microbial phenalenones lacking any side phenyl ring have been previously reported [6] in hypomycetous (*Penicillium*, *Fusicoccum*, *Giesmaniella* and *Verticilium*) as well as in dyscomicetous fungi. On the other hand, several plant phenalenones possessing a side phenyl ring on C-9 have been found [6, 7], as constitutive natural substances, in species of the Haemodoraceae, in the genera *Haemodorum*, *Lachnanthes*, *Xiphidium*, *Wachendorfia* and *Anigozanthos*. In all these cases, however, phenalenones have not been encountered as phytoalexins.

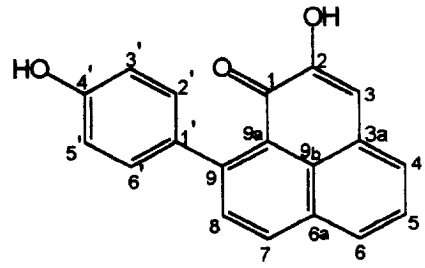
Now, from rhizomes of *M. acuminata* (AAA) Grand Nain infected with the pathogenic fungus *F. oxysporum* f. sp. *cubense* race 4, we have isolated and characterized four new 9-phenylphenalenone-type phytoalexins **2**–**6**, for which we propose the general name of musanolones. Recently, we also described the isolation and characterization [8], from the same source, of two intermediates with biosynthetic implications in *de novo* production of both 4-phenylphenalenone- and 9-phenylphenalenone-type phytoalexins.

RESULTS AND DISCUSSION

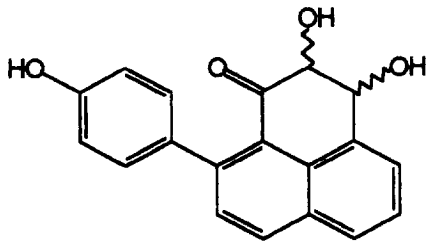
Rhizomes (80 kg) of banana plants which showed clear visual symptoms of Panama's disease were used to extract the phytoalexins; the same weight of rhizomes from healthy uninfected plants was employed as a control. When compared by TLC analysis, the extract of the material from infected plants showed the presence of eight coloured spots, of which the major one was also observed, although in very reduced intensity (traces), in the extract of healthy plants; this observation suggests



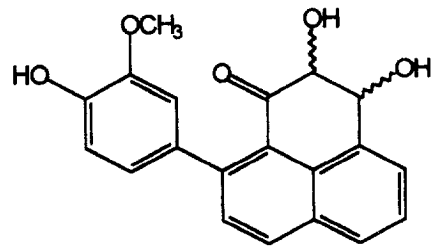
1 IRENOLONE



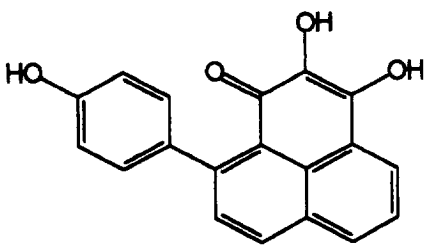
2 HYDROXYANIGORUFONE



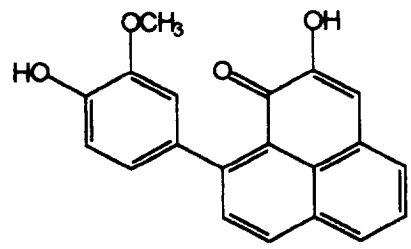
3 MUSANOLONE C



4 MUSANOLONE D



5 MUSANOLONE E



6 MUSANOLONE F

that in healthy plants it was induced by mechanical stress (cutting).

The major constituent of the infected rhizomes was identified as irenolone [4] (**1**) by comparison with an authentic sample. Of the other seven substances, five were purified, and their structures were established as indicated below.

Musanolone C (**3**) is a colourless compound with the molecular formula $C_{19}H_{14}O_4$, determined by HR mass spectrometry (*m/z* 306.0945, calc. 306.08921). The IR spectrum showed absorptions for hydroxyl (3360 cm^{-1}), and carbonyl (1694 cm^{-1}) groups. The ^1H NMR spectrum of musanolone C (Table 1) and the ^1H–^1H COSY experiment showed the presence of a phenalenone moiety in the molecule by the following spin systems: an AMX one at δ7.95 (*dd*, J = 1.4 and 8.4 Hz), δ7.60 (*t*, J = 7.9 Hz) and δ7.96 (*dd*, J = 1.4 and 8.4 Hz), and an AB system at δ8.16 (*d*, J = 8.3 Hz) and δ7.49 (*d*, J = 8.4 Hz). Besides, two double doublets with J_1 = 9.7 Hz; J_2 = 3.9 Hz) at δ4.96 (H-2) and δ4.70 (H-3) revealed other AB systems as was deduced from the COSY spectrum. In addition, two doublets at δ4.48 and δ5.21 (J = 3.9 Hz), which disappeared with D_2O addition were indicatives of a glycol. Finally, an AA′ BB′ system at δ7.20 (*d*, J = 8.5 Hz, 2H) and δ6.68 (*d*, J = 8.5, 2H) was attributed to the *p*-hydroxyphenyl group substituent. The ^{13}CNMR of **3** (Table 2) displayed two secondary carbon atoms bearing oxygen atoms at δ79.4 (*d*) and 75.8 (*d*), attached to protons at δ4.96 and 4.70, according to the HMQC experiment.

Table 1. ^{1}H NMR assignments for phenylphenalenones **1–6**

Proton	**2**	C (**3**)	D (**4**)	E (**5**)	F (**6**)	Irenolone **1**
1	—	—	—	—	—	—
2	—	4.96 (*dd*, 9.7, 3.9)	5.05 (*dd*, 2.8, 105)	—	—	—
3	7.15 (*s*)	4.70 (*dd*, 9.7, 3.9)	4.71 (*dd*, 4.0, 10.5)	—	7.15 (*s*)	7.09 (*s*)
4	7.85 (*dd*, 1.3, 8.3)	7.95 (*dd*, 1.4, 8.4)	7.86 (*d*, 8.3)	7.62 (*dd*, 1.3, 8.4)	7.75 (*dd*, 1.3, 8.2)	—
5	7.61 (*tbr*)	7.60 (*t*, 7.9)	7.67 (*t*, 8.3)	7.50 (*t*, 8.1)	7.61 (*t*, 8.0)	7.54 (*d*, 8.4)
6	7.93 (*dd*, 1.3, 8.3)	7.96 (*dd*, 1.4, 8.4)	8.01 (*d*, 8.3)	7.97 (*dd*, 1.3, 8.4)	7.95 (*dd*, 1.3, 8.2)	8.02 (*d*, 8.4)
7	8.23 (*d*, 8.3)	8.16 (*d*, 8.3)	8.09 (*d*, 8.5)	8.30 (*d*, 8.3)	8.22 (*d*, 8.3)	8.38 (*d*, 8.0)
8	7.62 (*d*, 8.3)	7.49 (*d*, 8.4)	7.52 (*d*, 8.5)	7.67 (*d*, 8.3)	7.63 (*d*, 8.3)	7.86 (*t*, 8.0)
9	—	—	—	—	—	8.58 (*d*, 8.0)
2′	7.31 (*dd*, 2.0, 8.5)	7.20 (*d*, 8.5)	6.85 (*d*, 1.9)	7.38 (*d*, 8.6)	6.91 (*d*, 1.9)	7.30 (*d*, 8.0)
3′	6.97 (*dd*, 2.0, 8.5)	6.68 (*d*, 8.5)	—	6.70 (*d*, 8.6)	—	6.95 (*d*, 8.0)
5′	6.97 (*dd*, 2.0, 8.5)	6.68 (*d*, 8.5)	7.01 (*d*, 8.0)	6.70 (*d*, 8.6)	7.06 (*dd*, 1.9, 8.2)	6.95 (*d*, 8.0)
6′	7.31 (*dd*, 2.0, 8.5)	7.20 (*d*, 8.5)	6.81 (*dd*, 1.9, 8.0)	7.38 (*d*, 8.6)	6.94 (*d*, 8.2)	7.30 (*d*, 8.0)
2-OH	*	4.48 (*d*, 3.9)	3.16 (*d*, 2.8)	**	*	9.80 (*sbr*)
3-OH	—	5.21 (*d*, 3.9)	4.16 (*d*, 4.0)	**	*	**
4′-OH	*	8.5 (*sbr*)	*	**	5.77 (*sbr*)	9.60 (*sbr*)
–OMe	—	—	3.91 (*s*)	—	3.90 (*s*)	—

Coupling constants (in parentheses, in Hz); spectra recorded in acetone-d_6.
*Signal was not observed.

Table 2. ^{13}C NMR spectral data for **2**, **3** and **6** (100 MHz, acetone-d_6, TMS int. standard)* and long-range correlations in the HMBC experiment for **3** and **6**

		^{13}C NMR		HMBC (H)	
Carbon	**2**	**3**	**6**	**3**	**6**
1	180.7 (*s*)	199.6 (*d*)	180.6 (*s*)	3	3
2	151.4 (*d*)	79.4 (*d*)	150.2 (*d*)	—	—
3	112.8 (*d*)	75.8 (*d*)	112.0 (*d*)	1, 4	—
3a	129.8 (*s*)	132.7 (*s*)	129.1 (*s*)	5	5
4	131.0 (*d*)	125.8 (*d*)	131.1 (*d*)	3, 6	3, 6
5	129.7 (*d*)	128.2 (*d*)	127.3 (*d*)	—	—
6	130.2 (*d*)	127.3 (*d*)	130.1 (*d*)	4, 7	7
6a	132.2 (*s*)	132.8 (*s*)	131.8 (*s*)	5, 8	5
7	136.2 (*d*)	136.0 (*d*)	135.9 (*d*)	6	6, 9
8	132.4 (*d*)	130.9 (*d*)	132.0 (*d*)	—	—
9	149.7 (*d*)	142.3 (*s*)	149.5 (*s*)	7	7
9a	124.5 (*s*)	127.1 (*s*)	123.9 (*s*)	8	8
9b	125.9 (*s*)	130.8 (*s*)	125.5 (*s*)	3, 4, 7	3, 4, 7
1′	134.2 (*s*)	132.9 (*s*)	134.5 (*s*)	3′, 5′, 9	5′, 8
2′	130.7 (*d*)	131.1 (*d*)	111.5 (*d*)	6′	6′
3′	115.7 (*d*)	115.7 (*d*)	146.8 (*s*)	5′	5′
4′	158.0 (*s*)	157.9 (*s*)	145.9 (*s*)	2′, 6′	6′
5′	115.7 (*d*)	115.7 (*d*)	114.8 (*d*)	3′	3′
6′	130.7 (*d*)	131.1 (*d*)	121.5 (*d*)	2′	2′
OMe	—	—	56.4 (*q*)	—	—

*Assignments were established by DEPT, HMQC and HMBC experiments.

A partial structure for **3** was established through HMQC and HMBC experiments simultaneously (Table 2). So, the carbonyl carbon atom, at δ199.6 showed long-range coupling with the signal at δ4.70 (H-3), which was correlated with two carbon atoms: one of them from the AMX system at δ125.8 (C-4) and the other with a quaternary carbon atom at δ130.8 (C-9b), which was in turn correlated with the proton at δ8.16 (H-7). These connectivities confirmed a phenalenone moiety with a 2,3-glycol system and a *p*-hydroxyphenyl group. Unfortunately, from the HMBC experiment we cannot assign the position of the latter, since linking at C-9 or at C-4 should generate similar spin systems in the ^{1}H NMR spectra. However, acid treatment of musanolone C (**3**) afforded hydroxyanigorufone (**2**), which was identified according to the comparison of the ^{1}H and ^{13}C NMR spectra [7]. Furthermore, the structure of (**2**) was confirmed by X-ray diffraction analysis [8].

Although it was not possible to assign the stereochemistry on C-2 and C-3, a relative spatial configuration between the corresponding protons can be deduced *trans*, due to the observed J value (9.7 Hz). Further evidence for the *trans*-axial conformation comes from the comparison between the J value obtained by spectroscopic data and the theoretical one ($J = 9.11$ Hz) obtained by molecular mechanic calculations (PC Model Program).

Musanolone F (**6**), $C_{20}H_{14}O_4$ (*m/z* 318.08894, calc 318.089219), was obtained as a red powder; many features in its IR and ^{13}C NMR indicated that it is closely related in structure to musanolone C. The ^1H NMR spectrum (Table 1) displayed an AMX spin system: δ7.75 (H-4), 7.61 (H-5) and 7.95 (H-6), and an AB system at δ8.22 (H-7) and 7.63 (H-8) assignable to the phenalenone nucleus. Besides, another AMX spin at δ6.91 (H-2′), 7.06 (H-5′) and 6.94 (H-6′) and a singlet signal at δ3.90 for a methoxyl group were observed, indicating the trisubstituted nature of the side-phenyl substituent.

The complete structure of (**6**) was obtained by the HMBC experiment (Table 2). This revealed cross-correlation for sequential signals: a ketone group (δ180.6, C-1) with H-3 (δ7.15), which correlated with an sp^2 carbon (δ131.1, C-4) and with an *sp* carbon (δ125.5, C-9b). The last one gave a cross-peak with a downfield proton at δ8.22 (H-7), which displayed long-range coupling with a quaternary carbon atom at δ149.5 (C-9). A cross-correlation between C-9 and a signal from the AMX system (δ6.91, H-2′) was also observed, indicating that the phenyl group substituent must be linked to C-9. The location of the remaining oxygen functional groups, one hydroxyl and the methoxyl group, was determined through a similar analysis (Table 2).

Musanolone E (**5**) is an orange compound with IR absorption bands at 3361 cm^{-1} (phenolic OH) and 1610 cm^{-1} (α, β-unsaturated ketone). It gives in low resolution mass spectrometry the higher fragment at *m/z* 303 $[M-1]^+$, indicating a decrease of two mass units in comparison with musanolone C. The ^1H NMR spectrum (Table 1) shows signals for three spin systems: AMX, AB and AA′BB′, and when compared with that of hydroxyanigorufone (**2**), the main observed difference was the lack of the singlet signal corresponding to the H-3 proton. Taking into account all these facts, the structure of **5** was assigned as 2,3-didehydro-musanolone-C.

Musanolone D (**4**) is an amorphous powder, $C_{20}H_{16}O_5$ (*m/z* 336.10040, calc. 336.09977). The ^1H NMR spectrum exhibited AMX and AB spin systems with chemical shifts very similar to that observed for the protons 4, 5, 6, 7 and 8 in the ^1H NMR spectrum of musanolone C in addition to another aromatic ABX system and a methoxyl group, suggesting a trisubstituted side-phenyl as in musanolone F (**6**). Other observed signals at δ4.71 (*dd*, $J = 4.0$ and 10.5 Hz, H-3) and 5.05 (*dd*, $J = 2.8$ and 10.5 Hz, H-2) and two exchangeable hydroxyl protons at δ3.16 (1H, *d*, $J = 2.8$ Hz) and 4.16 (1H, *d*, $J = 4.0$ Hz) were indicative of an 2,3-glycol in the molecule. Musanolone E treated with sulphuric acid quantitatively yielded musanolone F.

Analysis by molecular mechanic calculations (PC Model program) indicated a minimal energy value for a β conformation of the hydroxyl group in C-2, with respect to perinaphthenone moiety; the calculated coupling constant for that conformation was in accordance with the experimental value, indicating a H-2, H-3 trans relationship.

EXPERIMENTAL

Instruments. NMR: Bruker AMX400 (100 MHz) and WP200SY (50 MHz); MS (HR): VG-Micromass ZAB- 2F at 70 eV; MS (LR): HP 5995 at 70 eV; IR: Perkin-Elmer 1600 (FTIR). All the NMR spectra were run in $CDCl_3$ using TMS as int. standard.

Plant material. Rhizomes of Panama's disease infected 8–9 month old plants and of healthy plants of the same age were collected at the CITA experimental station in Tenerife, Canary Islands.

Extraction and isolation. Freshly collected rhizomes of infected and healthy (uninfected) plants (80 kg each) were immediately chopped and pressed to eliminate the water (8 l), which was collected and immediately extracted with $CHCl_3$ (3 × 500 ml). The residual material was kept under maceration with EtOH (96 %) (20 l) at room temp. for 5 days. The filtered EtOH extract was evapd in a rotavapor to 1/4 of its vol. (being 90% water) and extracted with $CHCl_3$ (3 × 500 ml). The combined $CHCl_3$ extracts were evapd to dryness to give 9.8 g crude material, which was dissolved in the minimum vol. of $CHCl_3$ and placed at the head of a 40 × 9.5 cm, silica gel (300 g) column; the solvent was allowed to evaporate and elution was carried out with, successively, *n*-hexane, *n*-hexane–EtOAc (1:1), EtOAc and MeOH (1.5 l each). The phytoalexins were detected by TLC.

The *n*-hexane–EtOAc (1:1) washing containing phytoalexins was evapd to dryness to yield 5.4 g semi-crude material which was dissolved in the minimum vol. of the elution mixt., and then submitted to sepn on a 50 × 7.5 cm, Sephadex LH-20 column (500 g), previously equilibrated with *n*-hexane–$CHCl_3$–MEOH (2:1:1), using as eluent the same mixt. 57 frs of 40 ml were collected. From the above, frs 5–10 (0.71 g) were repeatedly chromatrographed on silica gel (63–200 μm, Merck) using mixt of *n*-hexane–EtOAc as eluent, and compounds **1–5** were purified by prep. TLC (on precoated 0.25 mm silica gel plates G. Schleicher & Schull, using as solvent a mixt. of *n*-hexane, EtOAc, $CHCl_3$, C_6H_6, Me_2CO and CH_2Cl_2. Hydroxyanigorufone (**2**) (14 mg); musanolone C (**3**) (2.1 mg); musanolone D (**4**) (1.7 mg); musanolone E (**5**) (1.4 mg) and musanolone F (**6**) (5.7 mg).

Musanolone C (**3**). Yellowish solid; mp 258–261°; HREIMS: $[M]^+$ at *m/z* 306.0945 (calc. for $C_{19}H_{14}O_4$, 306.08921); UV (EtOH) λ_{max} nm: 265, 280, 323, 377. ^1H NMR (see Table 1). ^{13}C NMR (see Table 2). MS: *m/z* (rel. int. %): 306 $[M]^+$ (38), 287 (52), 271 (178), 259 (81), 231 (33), 213 (10), 202 (35), 189 (23), 81 (22), 60 (100), 55 (23).

Musanolone D (**4**). Solid; mp 248–250°; HREIMS: $[M]^+$ at *m/z* 336.10040 (calc. for $C_{20}H_{16}O_5$, 336.09977);

UV (EtOH) λ_{max} nm: 266, 352, 384. ^{1}H NMR (see Table 1). MS: *m/z* (rel. int. %): 336 $[M]^+$ (88), 322 (42), 304 (39), 303 (67), 287 (81), 275 (60), 257 (52), 229 (36), 189 (100),163 (42), 101 (30), 57 (53).

Musanolone E (**5**) (*2,3-didehydro-musanolone-C*). Orange solid; mp 273–276° UV (EtOH) λ_{max} nm: 276, 362. ^{1}H NMR (see Table 1). MS: *m/z* (rel. int. %): 303 $[M-1]^+$ (6), 285 (14), 279 (18), 259 (31), 213 (9), 111 (30), 98 (11), 88 (4), 57 (100), 55 (88).

Musanolone F (**6**). Red solid; mp 182–183°; HREIMS: $[M]^+$ at *m/z* 318.08894 (calc. for $C_{20}H_{14}O_4$, 318.08921); UV (EtOH) λ_{max} nm: 270, 324, 356, 364; ^{1}H NMR (see Table 1). ^{13}C NMR (see Table 2). MS: *m/z* (rel. int. %): 318 $[M]^+$ (86), 317 (100), 303 (55), 301 (37), 287 (44), 275 (31), 258 (14), 229 (10), 163 (12), 129 (25), 100 (10), 94 (14).

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