Table 2. Selected ^{1}H-NMR assignments for steroidal ketals **1** and **2**.

H#	δ ($CDCl_3$, m, J in Hz)[a]	δ (C_6D_6, m, J in Hz)[b]	δ ($CDCl_3$, m, J in Hz)[a]	δ (C_6D_6, m, J in Hz)[b]
1	7.08 (d, J = 10)	6.34 (d, J = 10)	7.08 (d, J = 10)	6.40 (d, J = 10)
2	6.22 (dd, J = 10, 2)	6.27 (dd, J = 10, 2)	6.23 (dd, J = 10, 2)	6.29 (dd, J = 10, 2)
4	6.02 (m)	6.17 (m)	6.08 (m)	6.17 (m)
	3.69 (d, J = 12.5)	3.53 (d, J = 12.5)	3.71 (d, J = 12.5)	3.72 (dd, J = 12.5, 3)
18				
	3.40 (d, J = 12.5)	3.20 (d, J = 12.5)	3.41 (d, J = 12.5)	3.37 (dd, J = 12.5, 2)
19	1.20 (s)	0.66 (s)	1.21 (s)	0.67 (s)
20	2.15 (dq, J = 7, 2)	2.36 (dq, J = 7, 2)	2.05 (dq, J = 7, 2)	1.82 (m)
21	0.93 (d, J = 7)	0.95 (d, J = 7)	0.89 (d, J = 7)	0.94 (d, J = 7)
26[c]	1.38 (s)	1.49 (s)	1.33 (s)	1.43 (s)
27[c]	1.19 (s)	1.16 (s)	1.17 (s)	1.13 (s)

[a] 200 MHz. [b] 500 MHz. [c] Assignments may be reversed.

periments which were performed using polarization transfer pulses of 90° and 135°, obtaining in the first case only signals for -CH groups and in the other case positive signals for -CH and $-CH_3$ and negative ones for $-CH_2$ groups. Polarization transfer delays were adjusted to an average C-H coupling of 135 Hz. The shift correlations with polarization transfer via ^1J coupling[6] were carried out adjusting fixed delays to give maximum polarization for $J_{C-H} = 135$ Hz. The long range heteronuclear correlations[7] were performed with maximum polarization for $J_{C-H} = 8$ Hz, leading to ^2J and ^3J spots in the same spectrum.

Extraction and purification. Sinularia sp. was collected in November 1986 by dredging at −130 m in the vicinity of Guam. The frozen animal (ca 100 g wet) was blended in $CHCl_3$: acetone (1 : 1), the material was filtered and the solvents were removed in vacuo to leave 250 mg of crude extract. The extract was chromatographed over Sephadex LH-20, eluting with increasing proportions of CH_2Cl_2 in hexane. Fractions eluting with CH_2Cl_2 : hexane (3 : 1) were combined and further fractioned by Silica HPLC with 20 % EtOAc in isooctane. Final purification of ketals **1** and **2** was achieved by C-18 Silica reversed phase HPLC (15 % aqueous methanol) to yield **1** (15 mg, 6 %) and **2** (14 mg, 5.6 %) as viscous oils. Ketal **1** showed the following physical and spectral features: HRMS (EI) M^+, m/z = 410.2813, calc. for $C_{27}H_{38}O_3$, 410.2822 UV (MeOH) 244 nm (ε = 13,500), IR 1660, 1615 cm^{-1}. ^{1}H and ^{13}C-NMR data are reported in tables 1 and 2.

For ketal **2:** HRMS (EI) M^+ m/z = 410.2815, calc. for $C_{27}H_{38}O_3$, 410.2822, UV (MeOH) 244 nm (ε = 13,500), IR 1660, 1615 cm^{-1}. ^{1}H and ^{13}C-NMR data are reported in tables 1 and 2.

1 Kazlauskas, R., Murphy, P. T., Quinn, R. J., and Wells, R. J., Tetrahedron Lett. (1977) 4439.
2 Tatsuo, H., Tanaka, J., and Tachibaua, K., Tetrahedron Lett. (1981) 2777.
3 Higa, T., Tanaka, J., Tsukitani, Y., and Kikuchi, H., Chem. Lett. (1981) 1647.
4 Kashman, Y., and Carmely, S., Tetrahedron Lett. *21* (1980) 4939.
5 Tweit, R. C., and Kagawa, C. M., J. med. Chem. *7* (1964) 524.
6 Bax, A., and Morris, G., J. mag. Res. *42* (1981) 501.
7 Kessler, H., Griesinger, C., Zambsck, J., and Loosli, R., J. mag. Res. *57* (1984) 331.
8 Blunt, J. W., and Stothers, J. B., Org. mag. Res. *9* (1977) 439.

0014-4754/90/090980-03$1.50 + 0.20/0

Fijiensin, the first phytotoxin from *Mycosphaerella fijiensis*, the causative agent of Black Sigatoka disease

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Received 23 January 1990; accepted 14 March 1990

Summary. Fijiensin, a novel phytotoxic metabolite, was isolated from a culture of the fungus *Mycosphaerella fijiensis*, the causal agent of Black Sigatoka disease of banana. Fijiensin is phytotoxic on various banana cultivars, but not toward non-host plants. The structure of fijiensin was determined by X-ray analysis.
Key words. Fijiensin; *Mycosphaerella fijiensis;* Black Sigatoka; fungal phytotoxin.

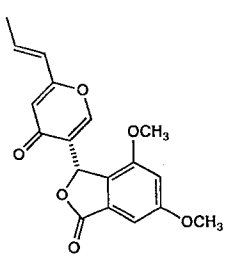
Bananas and plantains are important basic food crops on three continents, and they are valuable export products in many countries of Central and South America. These plants are currently being ravaged by the Sigatoka leaf spot disease complex. The disease complex involves three very closely related fungal pathogens: *Mycosphaerella*

musicola Leach ex Mulder, the cause of Yellow Sigatoka, first identified in Java in 1902; *M. fijiensis* Morelet, the cause of Black leaf streak, described in Fiji in 1964; and *M. fijiensis* var. *difformis* Mulder and Stover, the cause of Black Sigatoka discovered in Honduras in 1972[1,2]. Since there are no considerable morphological differences between *M. fijiensis* and *M. fijiensis* var. *difformis*, it is debatable whether to use the term Black Sigatoka or Black leaf streak; we prefer the former. During the last decade more virulent forms of Black Sigatoka have appeared and spread in the entirety of Latin America and the Caribbean Basin, Africa, parts of Asia, Australia and the Pacific Islands. The new forms are not only virulent on their traditional hosts, they also attack plantains which are not susceptible to Yellow Sigatoka, the previously dominant form of Sigatoka disease[3]. Thus, Black Sigatoka has replaced Yellow Sigatoka in range and agricultural importance.

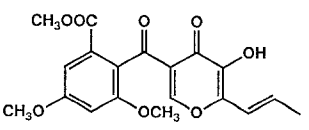
The epidemic of Black Sigatoka in Honduras in 1972–73, and subsequently in other countries, caused enormous losses in bananas and plantains. Estimated crop losses in different countries varied between 18–47% in gross yield and the loss of fruit due to premature ripening[4,5]. In Central America the cost for Black Sigatoka control accounts for 27% of the total outlay for commercial banana production, while control for all other diseases amounts to only 3–5% of the production cost[6]. Collectively, the annual costs for fungicides and their application amounts to 10's of millions of US dollars.

Symptoms of the disease include elongate necrotic lesions surrounded by zones of chlorosis, suggesting the involvement of one or more phytotoxins. Recently, a preliminary report based on a test of a crude extract of the pathogen suggested the presence of one or more host-specific phytotoxins[7]. Because of the enormous importance of this pathogen we became interested in its phytotoxins, which may ultimately be utilized as tools for discovering disease resistance in tissue cultures. Herein we report the chemical and biological properties of fijiensin (**1**), the first phytotoxin isolated from *M. fijiensis*.

M. fijiensis was provided by Dr. Krausz, FHIA, Honduras and was maintained on modified M-1-D medium containing inositol (5 g/l), thiamine (0.5 g/l), biotin (0.5 g/l) and coconut water (12 ml/l)[8]. *M. fijiensis*, when

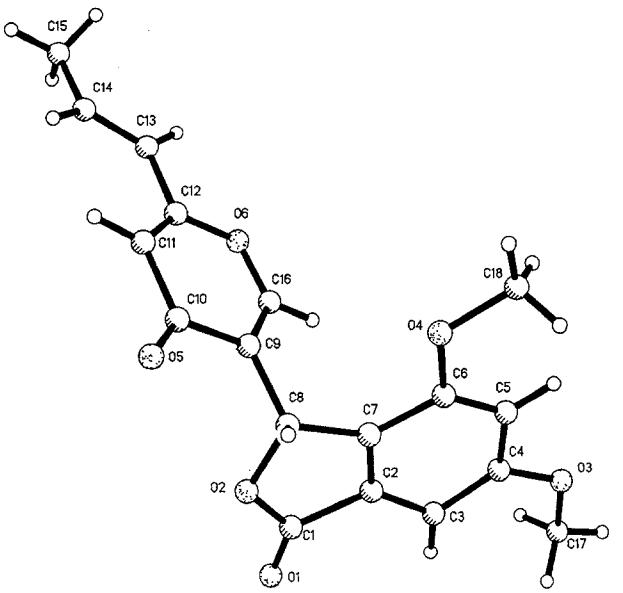


1



2

Structures **1** and **2**



A computer generated perspective drawing of the final X-ray model of fijiensin (**1**). No absolute configuration is implied.

grown on agar plates with a modified M-1-D/coconut water medium, commonly produced a dark gray colony which gradually turned pink-gray, and ultimately sectored at the margins into a black mycelial type. Segregation and separate reinoculation of the pink-gray or black mycelia gave rise to pure pink-gray and pure black colonies. The pink-gray colonies generally yield more fijiensin than the black colonies. The M-1-D/coconut water medium gave superior yields of toxin and fungal mycelium over other media tested (M-1-D with soybean broth, with banana leaf decoction, or with coconut extract). Use of the M-1-D/coconut medium also minimized the production of macromolecules, which can be a complication when using the other media[7].

For production of fungal metabolites, three plugs (1 cm^2) of agar containing the fungus were seeded into 800 ml of modified M-1-D/coconut water medium in a 2-l Erlenmeyer flask. The inoculated medium was incubated on a rotary shaker at 140 rpm for 28 days at 26 ± 1 °C under 12 h of light per day. At the termination of the incubation period an equal volume of methanol was added to the culture flask and it was allowed to stand overnight at 4 °C. The culture was then filtered through eight layers of cheesecloth and the filtrate reduced to one-third of its original volume under vacuum at 40 °C. The remaining solution was extracted three times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were washed once with water and concentrated to dryness under vacuum. A typical yield was 25 mg of ethyl acetate-soluble residue per liter of culture broth.

The various fractions were tested on detached banana leaves that were either the heart leaf or first leaf[9] using a leaf puncture method[10]. Test fractions were dissolved in 10% methanol/water and applied to the nicked leaf

surface in doses ranging from 50 to 200 µg (in a 5-µl drop) for crude samples, and 5–20 µg for pure fijiensin. In order to solubilize crystalline fijiensin it had to be first dissolved in chloroform, the chloroform evaporated, and the residue brought up in 10% methanol. Control solutions were 10% methanol/water, and a 10% methanol/water solution containing the residue from an ethyl acetate extract of uninoculated culture medium. No activity was detected in any of the controls. Treated leaves were incubated in a moist chamber for 72 h under intermittent light at 26 °C. Fractions were rated according to the relative area of necrosis.

The ethyl acetate extract, when subjected to preparative thin layer chromatography (TLC) on a 20 × 20 cm silica gel plate using 7:1 (v/v) chloroform/methanol as the eluant, yielded five fractions which showed activity in the leaf puncture assay. A bioactive band at R_f 0.80 was subjected to a second preparative TLC on silica using 1:2 (v/v) toluene/ethyl acetate as the eluant. A band at R_f 0.42 was further purified by HPLC on a C-18 column with 65:35 (v/v) acetonitrile/water to yield pure fijiensin. Typically, one liter of the pink-gray culture yielded 2 mg of fijiensin.

The high resolution mass spectrum [11] of fijiensin suggested the molecular formula $C_{18}H_{16}O_6$. The ^{13}C NMR spectrum confirmed the presence of eighteen carbon atoms, and showed all sixteen hydrogens were bonded directly to carbon atoms (DEPT). The terminal *trans*-propenyl side chain, the two *meta* aromatic protons, and the two methoxy groups are all readily discernable in the ^1H NMR spectrum. The ^1H NMR spectrum also showed three other protons which display no proton-proton coupling. The lack of long range proton-proton couplings precluded a straight forward solution of the fijiensin structure by spectroscopic methods.

Fortunately, fijiensin crystallized from a sample of the water washed ethyl acetate extract that had been concentrated and allowed to slowly evaporate at 4 °C, and we elected to examine the structure by single crystal X-ray analysis. Preliminary X-ray photographs displayed triclinic symmetry, and accurate lattice constants of $a = 4.9146(8)$, $b = 8.4256(11)$, $c = 19.108(3)$ Å, $\alpha = 86.442(12)°$, $\beta = 88.869(14)°$, and $\gamma = 86.922(12)°$ were obtained from moderate 2θ-values. Fijiensin belonged to space group P1 with two molecules of $C_{18}H_{16}O_6$ forming the asymmetric unit ($Z = 2$). All unique diffraction maxima with $2\theta \leqq 114°$ were collected using an ω-scan technique and graphite monochromated CuKα radiation (1.54184 Å). Of the 2435 reflections collected, 2103 (86%) were judged observed. The structure was solved by direct methods and refined by full-matrix least squares techniques with anisotropic heavy atoms and fixed isotropic hydrogens. The final crystallographic agreement factor is 6.53% [12].

A computer generated perspective drawing of the final X-ray structure is shown in the figure. The absolute configuration was not determined by the X-ray analysis, so the enantiomer shown is arbitrary. The fijiensin skeleton has no precedent among naturally occurring compounds. There is however, one report describing a semisynthetic compound with the same skeleton. Merlini and coworkers [13] chemically transformed, in a single step, the fungal metabolite funicone (**2**) to 11-hydroxy-13,14-dihydrofijiensin. This ready conversion suggests a similar biogenetic origin for fijiensin and funicone.

Fijiensin showed specificity towards banana versus non-host plants at lower concentrations (5–10 µg/5 µl drop) by causing necrotic lesions only on banana leaves. With the exception of cucumber, which exhibits a little necrosis, fijiensin is not phytotoxic toward non-host plants at 20 µg (rice, sorghum, cotton, maize, barley). Fijiensin does not exhibit a differential reaction to various cultivars of bananas. The crude ethyl acetate extract is also specific towards banana at lower concentrations, but is non-specific at higher concentrations (200 µg). This may indicate the other phytotoxins present in the ethyl acetate extract are also host-specific.

Fijiensin production in liquid cultures could only be detected after 21–24 days of fungal growth. However, pathogenicity tests showed development of the initial speck stage of symptoms after 10–15 days on plants in the field [2]. This finding may suggest fijiensin plays a secondary role, instead of being a primary causative factor, in disease expression.

Acknowledgement. Thanks are due to Dr J. P. Krausz for providing a culture of *M. fijiensis*. RKU expresses his gratitude to the Government of India for a National Scholarship, NSF grant DMB-8607347, and an AID grant.

1 Meredith, D. S., Rev. Pl. Path. *49* (1970) 539.
2 Stover, R. H., Plant Dis. *64* (1980) 750.
3 Anonymous, in: Banana and Plantain Breeding Strategies. Eds G. J. Persley and E. A. DeLanghe. INIBAP ACIAR Proceedings, 1987.
4 Jaramillo, C. R., Banana and Plantain Breeding Strategies 1987, p. 39.
5 Chung, T. Y., and Jeger, M. J., Phytopathology *77* (1987) 1542.
6 Stover, R. H., and Simmonds, M. W., in: Banana. Longman, London 1987.
7 Molina, G. C., and Krausz, J. P., Plant Dis. *73* (1989) 142.
8 Pinkerton, R., and Strobel, G. A., Proc. natl Acad. Sci. USA *73* (1976) 4007.
9 Leach, R., Kingston, Jamaica. Government Printer 1946.
10 Sugawara, F., Strobel, G. A., Fisher, L. E., Van Duyne, G. D., and Clardy, J., Proc. natl Acad. Sci. USA *82* (1985) 8291.
11 Fijiensin, red needles, m.p. 207-8°. $[\alpha]_D$ −95° ($CHCl_3$, c = 0.001). IR ν ($CHCl_3$) 2980, 1760, 1660, 1650, 1610 cm^{-1}. UV λ_{max} nm (ε) 222 (3416), 258 (16 312), 273 (13 700), 284 (11 200), 295 (6950). ^1H NMR (400 MHz, $CDCl_3$) δ 7.41 (1H s), 6.96 (1H d, J = 1.9 Hz), 6.66 (1H d, J = 1.9 Hz), 6.59 (1H dq, J = 15.7, 6.8 Hz), 6.44 (1H s), 6.14 (1H s), 6.07 (1H dq, J = 15.6, 1.6 Hz), 3.89 (3H s), 3.78 (3H s), 1.90 (3H dd, J = 6.8, 1.6). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.3 s, 170.0 s, 163.0 s, 162.1 s, 154.9 s, 153.9 d, 135.9 d, 129.3 s, 127.7 s, 123.4 s, 123.1 d, 112.9 d, 105.1 d, 98.9 d, 73.6 d, 56.0 q, 55.8 q, 18.6 q. HRMS (EI) *m/z* 328.0936 daltons. LRMS *m/z* (rel. int.) 329 (17), 328 (100), 313 (14), 285 (40), 271 (23), 269 (42), 260 (41), 259 (22), 257 (10).
12 Archival crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK. Give a complete literature citation when ordering.
13 Merlini, L., Nasini, G., and Selva, A., Tetrahedron *26* (1970) 2739.

0014-4754/90/090982-03$1.50 + 0.20/0