Irenolone and Emenolone: Two New Types of Phytoalexin from Musa paradisiaca

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Phenalenone-type phytoalexins were obtained for the first time from bananas and identified by COSY ¹H-¹H, HMQC and HMBC techniques, and X-ray analysis, among other methods. The aminoglycoside kanamycin was studied as a phytoalexin inducer in banana leaves and fruit and compared with the effects produced by the fungus *Mycosphaerella fidjiensis*. The phenyl side chain in banana phytoalexins is not in the same position as in other plant phenalenones and so an alternative biogenetic pathway has been proposed and is currently being verified. The specific response evoked by kanamycin opens the way to the study of the host–plant relationship from a new angle since more phytoalexin inducers can be designed on the basis of the chemical constituents of the microorganisms involved or on synthetic kanamycin analogues and used to improve the plants' defense mechanisms.

The phytoalexins are a group of natural products defined by their physiological rather than their structural features. These compounds are yielded *de novo* by some plant organs when provoked by physical, microbiological, or chemical agents,[1] and several synthetic polyamines and fungus-derived carbohydrates have been tested for their potential as phytoalexin inducers in species of *Pisum*.[2,3] The aminoglycoside kanamycin which has a carbohydrate nucleus and several amino groups has now been studied as a phytoalexin inducer in banana leaves and fruit. Phytoalexins have been detected in leaves infected with *Mycosphaerella fidjiensis*, a pathogenic fungus that attacks banana leaves and greatly reduces their growth.[4] This analysis was carried out when the disease was in its first stages because if it were well advanced, the extracts could already be contaminated by microbial metabolites or phytoalexin production slowed down or stopped entirely.

For the purposes of induction, leaves and green fruit (*Musa paradisiaca* vars. Grand Dwarf and Valery) were treated with aqueous kanamycin 10 mg/mL, Tween 20, 0.05% by the drop-diffusion method[1] and then extracted with ethyl acetate. After evaporation this extract was chromatographed on TLC plates, and two major compounds were detected. These compounds were not present in the extracts of leaves and fruit treated with water–Tween only. As the phytoalexins were present at very low concentrations in the exudates it was decided to obtain them from infected leaves and fruit peels treated with kanamycin.

The major constituent of the infected leaves was irenolone (1). This compound had a molecular formula $C_{19}H_{12}O_3$ (288.0786, HREIMS and ¹³C NMR). ¹H NMR spectra and a ¹H-¹H COSY experiment revealed the

Table I. Spectral Data of Irenolone (1)[a]

C/H no.	¹H	¹³C	long-range correlation
1	–	179.2 (s)	9, 3
2-OH	9.80 (br s)	150.5 (s)	–
3	7.09 (s)	113.1 (d)	9b, 2*, 1
3a	–	124.5 (s)	5
4	–	142.1 (s)	6,6', 2', 3
5	7.54 (d, 8.4)	129.7 (d)	–
6	8.02 (d, 8.4)	129.4 (d)	7
6a	–	130.8 (s)	8, 5
7	8.38 (d, 8.0)	135.9 (d)	9, 6
8	7.83 (t, 8.0)	126.7 (d)	–
9	8.58 (d, 8.0)	130.5 (d)	7
9a	–	128.5 (s)	8
9b	–	124.0 (s)	9, 7, 6, 3
1'	–	129.8 (s)	3', 5', 5
2', 6'	7.30 (d, 8.0)	131.5 (d)	2', 6', 4, 4'
3', 5'	6.95 (d, 8.0)	115.4 (d)	3', 5', 1', 1
4'-OH	9.60 (br s)	157.5 (s)	2', 6', 3', 5'

[a] Data were recorded in DMSO-d_6 at 100 MHz; long-range correlations represent two (*) or three-bond couplings (J = 7.0 or 10.0 Hz). Carbon multiplicities were determined by DEPT experiments.

presence of the following aromatic spin systems: AMX at δ 8.58 and 8.38 with a triplet at δ 7.83; AB, two doublets at δ 8.02 and 7.54 and finally, an AA'BB' of two doublets at δ 7.30 and 6.95 (Table I). There were also two exchangeable protons at δ 9.80 and 9.60 and a singlet at δ 7.09. In an HMBC experiment[5] the AA'BB' system at δ 7.30 and 6.95 was correlated with carbon signals at 131.5 (d, 2C) and 115.4 ppm (d, 2C) (Table I) displaying a *para*-substituted phenyl ring similar to the one found in many flavonoids. The high resolution mass spectra of irenolone showed loss of C≡O, CHO, and H_2O fragments, and peaks at m/z 202.0831 ($C_{16}H_{10}$) and 101.0392 (C_8H_5). These ions suggested the presence of neighboring dioxygenated groups since the first of these peaks originated from the fragment at m/z 260.0838 ($C_{18}H_{12}O_2$) with loss of $C_2H_2O_2$ (in which the carbonyl group was included). This group was presumed to be α,β-unsaturated and H-bonded from the appearance of an intense signal at 1633 cm⁻¹ in the IR spectrum.

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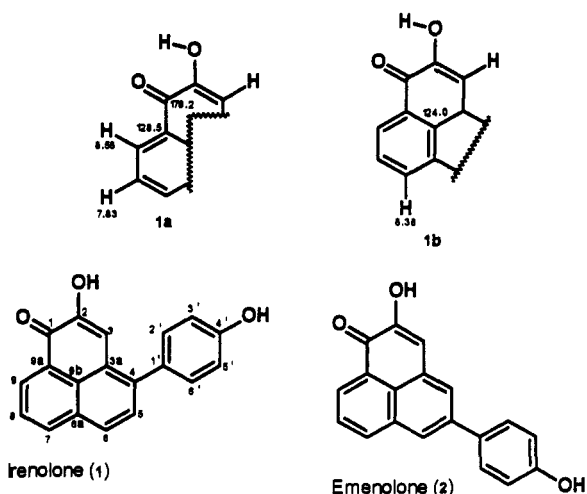(5) Bax, A.; Subramanian, S. J. *J. Magn. Reson.* 1986, 67, 656.

Figure 1. Structure of irenolone (1) and emenolone (2).

The DEPT spectra revealed ten CH groups and nine quaternary carbons including one carbonyl (179.2 ppm, s) and two oxygenated carbons (157.5 and 150.5 ppm). From the analysis of these spectra and the HREIMS data, irenolone (1) required 14 degrees of unsaturation and had 6 unidentified quaternary carbons, as has been mentioned above. Due to its complex structure, irenolone was identified by a combination of COSY ^{1}H–^{1}H, HMQC,[6] and HMBC experiments. The analysis began with the C=O shielded at 179.2 ppm since this group (C-1) showed correlation with the doublet at δ 8.58 and the singlet at δ 7.09. These protons are attached to C-9 (130.5 ppm) and C-3 (113.5) according to HMBC. The triplet at δ 7.83 (C-8, 126.7 ppm) which belongs to the AMX system is three bonds away from two quaternary carbons at 128.5 and 130.8 ppm. As an H-bonded carbonyl was observed, the partial structure shown in Figure 1 (1a) could be assembled. Both the doublet at δ 8.58 and the singlet at δ 7.09 were correlated with a quaternary carbon at 124.0 ppm and this carbon was also correlated with the doublet at δ 8.38 (C-7, 135.9 ppm). Structure 1a could thus be extended to that given in Figure 1 as 1b. The quaternary carbon at 124.0 ppm had a long-range coupling to the AB spin system doublet at δ 8.02 (C-6, 129.4 ppm) which, together with the singlet at δ 7.09, was coupled with another quaternary carbon at 142.1 ppm (C-4). The side phenyl group must be attached directly to this latter carbon. By starting from the signal at 142.1 ppm (Table I), the side phenyl ring carbons could be assigned and so C-2′ and C-6′ could be established at 131.5 ppm and then C-4′ at 157.5 ppm by means of the HMQC spectra. The C-3′ and C-5′ protons correlated with the carbon signal at 115.4 ppm displayed long-range coupling with a quaternary carbon at 129.8 ppm (C-1′). The C-5 assignment (129.7 ppm) could be deduced directly from HMQC, since C-6 was known to be at 129.4 ppm and the C-5 proton (δ 7.54) was correlated with a singlet at 124.5 ppm (C-3a). Finally, the chemical shift of C-2 was determined from the observation of a two-bond correlation with C-3.

The structure of irenolone (1) was deduced as a phenalenone system substituted on C-4 with a hydroxyphenyl ring as shown in Figure 1. It was confirmed by X-ray diffraction analysis which showed the phenyl group perpendicular to the phenalenone nucleus as was to be expected in such an aromatic system.

The minor metabolite was emenolone (2); its ^{1}H NMR greatly resembled that of irenolone: AMX [δ 8.21 (d, J = 8.3 Hz), 7.72 (t, J = 6.1 Hz) 7.93 (d, J = 8.3 Hz)], AA′BB′ [δ 7.30 (d, J = 8.5 Hz), 6.96 (d, J = 8.5 Hz)], and AB [δ 7.63 (d, J = 2.4 Hz), 7.59 (d, J = 2.4 Hz)] plus one exchangeable proton at δ 7.09. No C-4′-OH was observed and it was possibly obscured by the solvent signal. Emenolone (2) had the molecular ion at m/z 288.0787 ($C_{19}H_{12}O_3$) in HREIMS (as did irenolone) and prominent peaks at m/z 271.0759 ($C_{19}H_{11}O_2$) and m/z 260.0837 ($C_{18}H_{12}O_2$). Emenolone would thus seem to be an irenolone isomer and its structure could be established from its coupling constant since the AB system showed a typical *meta* value (2.4 Hz), while in irenolone the same value was 8.5 Hz, characteristic of *ortho* coupling. Therefore emenolone (2) is suggested to be 4′-hydroxy-5-phenylphenalenone.

A third compound ("U") was isolated from kanamycin-treated leaves but structural elucidation proved difficult due to the small amount obtained. However, a similar aromatic spin system to that of irenolone (1) seemed to be present.

Several compounds with a phenalenone nucleus have been reported in plants and microorganisms.[7] The plant phenalenones possess a side phenyl ring on C-9 and have been found in species of the Haemodoraceae plant family, in the genera *Haemodorum, Lachnanthes, Xiphidium, Wachendorfia*, and *Anigozanthus* while microbial phenalenones which do not have this side phenyl ring have been reported in Hypomycetous (genera *Penicillium, Fusicoccum, Giesmaniella*, and *Verticillium*) and Dyscomycetous fungi. The biogenetic pathway to the latter phenalenones has been established beyond doubt as originating from acetate[8] but experiments were unclear with regard to the plant phenalenones as the mass spectra and precursor-labeled experiments diverged.[9] A mixed pathway was therefore proposed: two phenylalanine (or tyrosine) units were coupled with one acetate unit through a Diels–Alder reaction.[10] However, it is clear that the biogenetic pattern of banana phytoalexins with a side *p*-hydroxyphenyl ring at C-4 (or C-5) could not be produced with the same precursors and type of reaction from which Haemodoraceae pigments are formed. An alternative pathway is being tested in our laboratory using banana tissue cultures and ^{13}C acetate.

The presence in bananas of phenalenone-type phytoalexins is reported here for the first time although other classes of phytoalexins had been isolated previously, principally flavonoids, diterpenes, sesquiterpenes, stilbenes, coumarins, alkaloids, etc.[11] The production of several compounds in plant material treated with kanamycin undoubtedly indicated that these substances are plant metabolites although there were very much influenced by the inducer agent which means that the response is specific. As a consequence phytopathology and the study of the host–plant relationship can be viewed in a new way since further phytoalexin inducers can be designed based on the microorganimsms' chemical components or on synthetic kanamycin analogues.

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Experimental Section

Spectral Analysis and General Procedures. ^{1}H NMR chemical shifts were taken in DMSO-d_6 and CDCl$_3$ with TMS as internal standard. The ^{1}H NMR were run at 400 MHz and the ^{13}C at 100 MHz. ^{13}C NMR were taken in DMSO-d_6. The heteronuclear [1]H–[13]C connectivities were determined by a proton-detected HMQC experiment, and methine and quarternary carbons were distinguished by DEPT experiments and ^{1}H–^{1}H COSY experiments. UV spectra were recorded in MeOH and MS were determined in EI mode. TLC was carried out on silica (0.25 or 0.50 mm) using diethyl ether/hexane (3:1 v/v); plates were developed with H$_2$SO$_4$ and heated to 110 °C. The extraction solvents were distilled before use.

Induction Conditions. Healthy and infected (*Mycosphaerella fidjiens*) leaves of *Musa paradisiaca* (varieties Valery and Grand Dwarf) were obtained from the Instituto Colombiano Agropecuario, already defatted with hexane-impregnated cotton. Aqueous kanamycin (10 mg/mL and Tween 20, 0.05%) was applied to 10 healthy leaves by the drop-diffusion method. After 48 h, the solution (1.5 L) was recovered and extracted with EtOAc, dried, and evaporated *in vacuo*.

The fruit pulp from green bananas (45 kg) was scooped out and 50 mL of the same solution of kanamycin as above was put into the cavity. After 48 h, the solution (9 L) was removed and the peels (15 kg) were ground with 70% EtOH, concentrated to $^1/_4$ of the original volume, extracted with EtOAc, and evaporated until dryness. Leaves infected with *M. fidjiensis* (first stages, 24 kg) were milled in the same way as the fruit, and the EtOAc extract was concentrated. A control experiment was carried out with healthy leaves and fruit treated only with water–Tween 20.

Phytoalexin Detection. EtOAc extracts from equal amounts of induced and control material were chromatographed on silica gel plates using diethyl ether/hexane (3:1). Several compounds were detected as red spots. Irenolone (1) was found in fruit peels (2 mg), infected leaves (20 mg), and the extract of kanamycin-treated leaves (2 mg); emenolone (2) was found in infected leaves (5 mg) and in trace form in fruit peels and the kanamycin-treated leaf extract. A third compound ("U") with an unknown structure was isolated from the kanamycin-induced extract (1 mg) and as traces in infected leaves and fruit peels.

Isolation. The phytoalexins were purified by preparative TLC using diethyl ether/hexane.

Irenolone (1): red needles from CHCl$_3$–MeOH; mp 285 °C; IR (KBr) 3300, 1633, 1606, 1583, 1550, 1417, 1349, 1273, 1221, 1170, 1064, 879, 814, 764 cm^{-1}; UV (MeOH) 225 sh (4.1), 271 (4.2), 325 (3.8), 350 (3.9), 390 (3.6); ^{1}H NMR, see Table I; ^{13}C NMR, see Table I; EIMS m/z (rel. inten) 288 [M$^+$] (100%), 271 (86), 260 (20), 242 (64), 231 (54), 202 (98), 101 (43), 73 (62), 63 (90); HREIMS, found 288.0786, calcd for C$_{19}$H$_{12}$O$_3$, 288.0783; X-ray diffraction C$_{19}$H$_{12}$O$_3$, monoclinic, $P2_1/n$, $a = 11.019(4)$ Å, $b = 6.901(4)$ Å, $c = 18.417(7)$ Å, $B = 106.91(4)$ Å, $V = 1339.8$ Å^3, 4Z $= 4$, $D_x = 1.43$ g/cm^3, $\mu = 7.42$ cm^{-1}, Cu Kα, red crystals, size 0.16 × 0.16 × 0.4 mm. Siemens AED diffractometer graphite monochromator Cu Kα (1.5418 Å) radiation and ω–θ scan mode; correction for intensity variation of check reflection (2%) 2567 data collected, 1567 unique and 1301 observed $(I > 3\sigma(I))$; intensities were corrected for Lp effects as usual. Solved by direct methods, weighted full-matrix refinement, all non-hydrogen atoms anisotropic, hydrogen atoms refined with fixed isotropic U equal to U_{eq} of the corresponding carbon atom; $R = 0.081$, $R_w = 0.078$. The largest peak in the final difference map was 0.294 e/Å^3, number of refined parameters 235, ratio data/parameters, 6.

Emenolone (2): red gum; IR (film) 3389m, 1633, 1606, 1580, 1545, 1462, 1360, 1284, 1222, 938, 860, 812, 760 cm^{-1}; UV (MeOH) 245 (3.8), 259 (4.2), 319 (3.9), 363 (3.8); ^{1}H NMR (CDCl$_3$) δ 8.21 (1H, d, $J = 8.3$ Hz), 7.93 (1H, d, $J = 8.3$ Hz), 7.72 (1H, t, $J = 6.1$ Hz), 7.63 (1H, d, $J = 2.4$ Hz), 7.59 (1H, d, $J = 2.3$ Hz), 7.30 (2H, d, $J = 8.5$ Hz), 7.14 (1H, s), 7.09 (1H, s), 6.96 (2H, d, $J = 8.5$ Hz); EIMS m/z (rel inten) 288 [M$^+$] (36%), 287 (52), 271 (10), 259 (5), 202 (16), 149 (60), 101 (12), 98 (28), 84 (38), 77 (52), 71 (56), 57 (100); HREIMS, found 288.0787, calcd for C$_{19}$H$_{12}$O$_3$, 288.0783.