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Synthesis and Antifungal Activity of *Musa* Phytoalexins and Structural Analogs

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Abstract: Several perinaphthenone/phenylphenalenone compounds were synthesized to establish a relationship between structure and antifungal activity against *Mycosphaerella fijiensis*. Substitutions on the unsaturated carbonyl system or addition of a phenyl group reduced antibiotic activity.

Keywords: phenylphenalenones, synthesis, antifungal activity, *Mycosphaerella fijiensis*.

Introduction

Phytoalexins are natural antibiotic compounds proposed as fungicides or templates for production of new pesticides [1]. Recently, the search for novel antifungal compounds has received special attention as a result of an enhanced microbial resistance to current pesticides.

Banana plants are affected by the pathogenic fungi *Fusarium oxysporum* var. *cubensis* type 4 and *Mycosphaerella fijiensis*, causal agents of the diseases named Black Sigatoka and Panama Disease, respectively. These diseases can drastically reduce banana production by as much as ca. 20% [2]. Under colonization by these microorganisms or treatment of the leaves with kanamycin, banana plants produce two types of phytoalexins, 9-phenylphenalenones (also known as musanolones [3]) and 4-phenylphenalenones [4], The main aim of the present work was to synthesize several structurally-related compounds and to determine the relationship between phytoalexin structure and their antifungal effects against *M. fijiensis*.

Results and Discussion

The planned synthetic approach involved a 1,4-addition of a Grignard reagent to a perinaphthenone, followed by reduction with DDQ, epoxidation with ^{t}BuOOH and finally enolization induced by acid treatment (Figure 1).

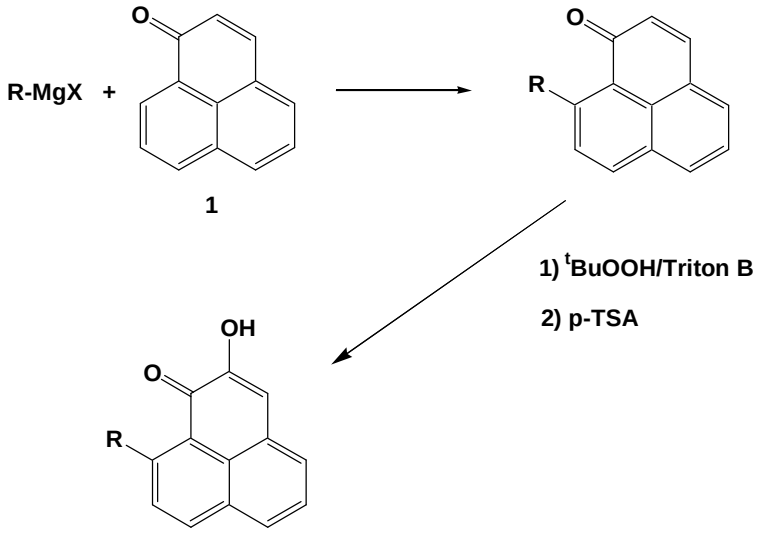
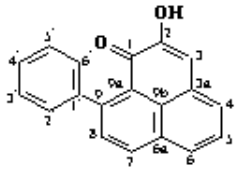
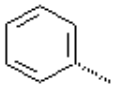
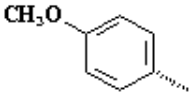
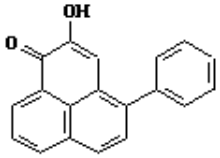
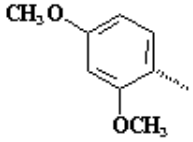
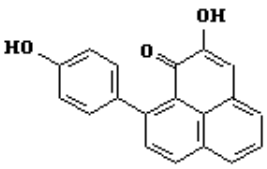
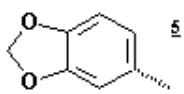
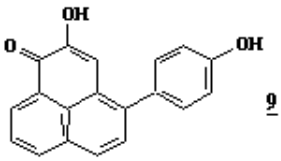


Figure 1. Synthesis of phenylphenalenones.

Previously, we have used this reaction to unambiguously confirm the structure of 4´-methoxy-irenolone, a natural phytoalexin [5]; in addition, aryl Grignard reagents have also been used for the synthesis of extended phenalenones [6]. Other compounds such as irenolone [4-(4'-hydroxy)phenylphenalenone)], and 4-phenylphenalenone were obtained from natural sources, as described elsewhere [1,5]. The results of the synthesis are summarized in Table 1; products derived from addition to the 2-en-1-one system were obtained in less than 5% yield.

Table 1. Structure and yield of phytoalexins and structural analogues.

Perinaphthenones, R:	Yield (%)	Phenylphenalenones	Yield (%)
H 1		 6	90
 2	85		
 3	65	 7	Natural (ca 10^{-5} %)
 4	75	 8	Natural (ca 10^{-5} %)
 5	86	 9	Natural (ca 10^{-5} %)

Activity was measured by the effect of the compounds on the weight of an established *Mycosphaerella fijiensis* colony (Figure 2). In this figure the results for each compound are represented by six bars (doses at 10.0, 50.0 and 100.0 ppm and measurements at 7, 12 and 15 days). When the antibiotic activity detected was very low (e.g. compound **3**), the corresponding bars are not displayed. Compound **7** was only tested at 50 and 100 ppm due to limited availability.

Perinaphthenone (*1H*-phenalen-1-one, **1**) was the most active antifungal compound assayed. Complete mycelial growth inhibition was observed at 50-100 ppm and its action remained over 15 days. This action was similar to benomyl, while 9-phenylperinaphthenone (**2**) showed slightly reduced activity. Additional substitution on the side phenyl group (compounds **3**, **4** and **5**) drastically reduces antibiotic properties. Natural phytoalexins (compounds **6**-**9**) were less effective when compared to the results obtained above, although 9-phenylphenalenone **6** exhibited significant activity during the first days of experimentation.

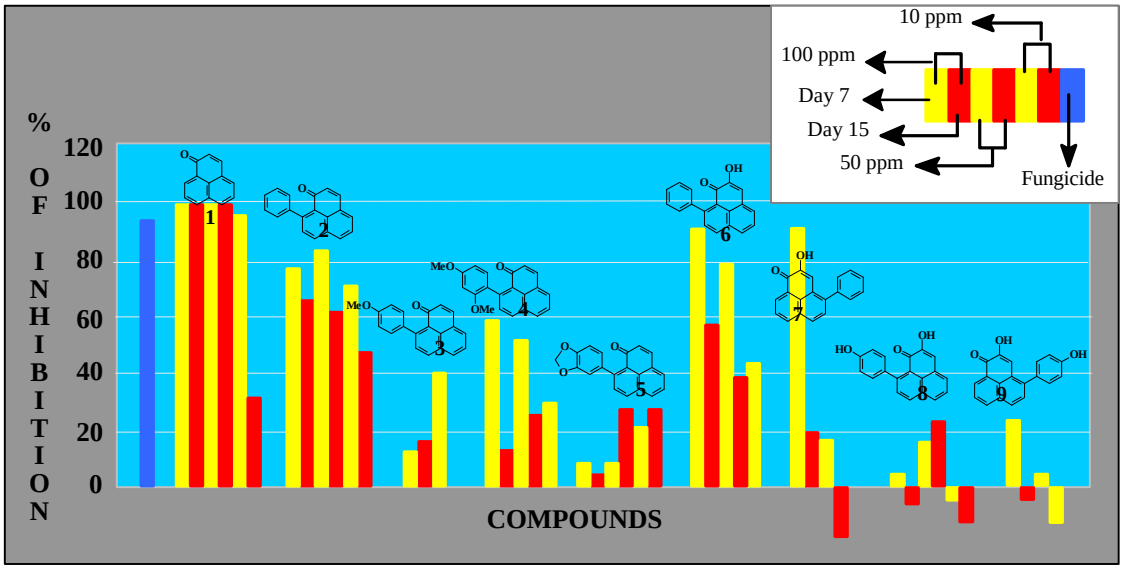


Figure 2. Antifungal activity on *M. fijiensis* growth (mycelial weight).

Concerning the effects on spore germination, perinaphthenone (**1**) at 50-100 ppm, was a more active antibiotic than the commercial fungicides tested (benomyl, propioconazole, tridermorph), as shown in Figure 3. The other compounds, including the phytoalexins, displayed only moderate effects.

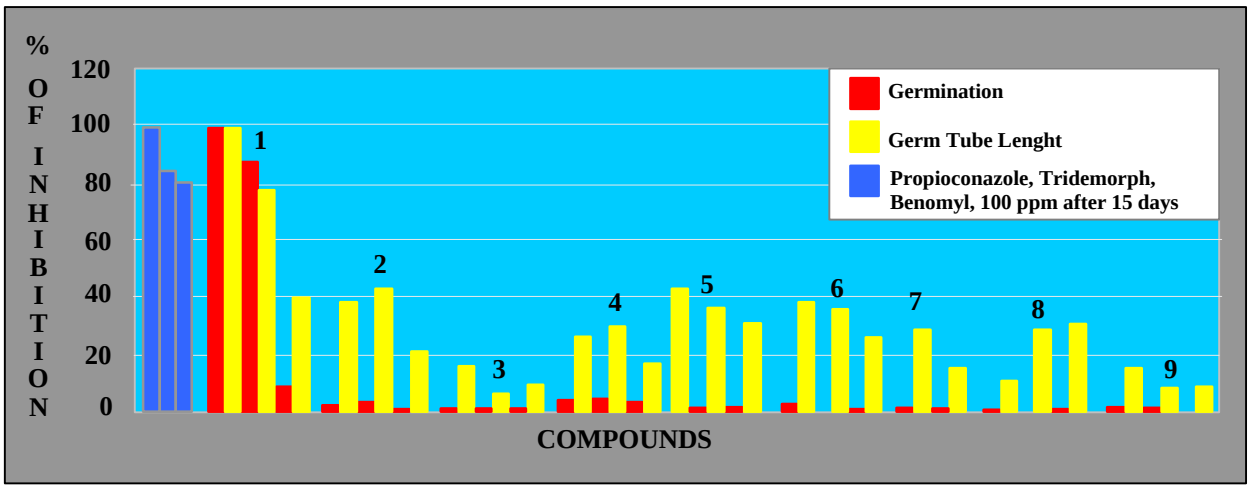


Figure 3. Antifungal activity on *M. fijiensis* spores.

These results indicate the important antifungal properties of synthetic substances **1** and **2**. Although perinaphthenone (**1**) is an efficient singlet oxygen sensitizer [7], very limited information is available about its mode of action. Formation of singlet oxygen and damage to biomolecules (DNA, proteins, fatty acids etc) could be an explanation (Figure 4).

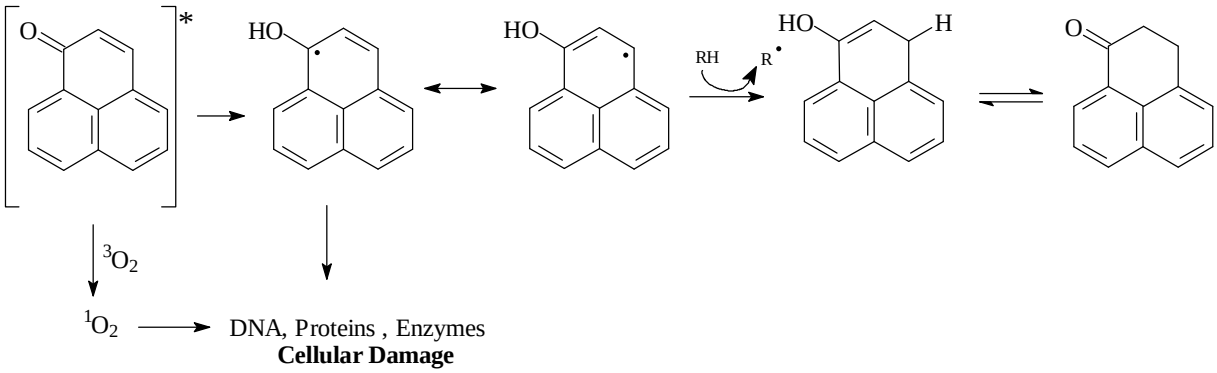


Figure 4. Singlet oxygen and biological effects by perinaphthenone.

However, more complex biochemical interactions could be involved, since the growth inhibition profile observed is very diferent from the spore germination one: e.g. 9-phenyl-perinaphthenone (**2**) is powerful as an inhibitor whereas in germination it is practically inactive. Although it has been reported that a hydroxyphenyl group in the phenylphenalenone moiety is essential for antibiotic activity against *Colletotrichum musa* [9], this action seems to be dependent on other factors, especially molecular shape and the inductive effect of substituents.

Perinaphthenone, with its planar tricyclic system, may act as a DNA intercalating agent. On the other hand, steric influence effects are displayed in the reduced activity of phenylperinaphthenone **2** (Figures 2 and 3). Some derivatives (compounds **3**-**5**) do not exhibit significant antifungal activity, probably due to modifications in their insertion ability or a lack of stabilization of potential free radicals on the side ring due to inductive effects of the substituents. Other natural products such as hypericin and perylenequinone displayed an activity related to presence of free radicals [8] and oxygen activation. Finally, the natural phytoalexins assayed (compounds **6**-**9**) displayed low antifungal activity, probably due to poor solubilization and incorporation to the agar media. Further investigations concerning the *in vivo* effects of this kind of phenalenones are ongoing, since they represent a new type of antifungal compounds which display long lasting antibiotic activity.

Experimental

General

400 MHz ^{1}H and 100 MHz ^{13}C NMR spectra were recorded for $CDCl_3$ solutions on in a Bruker 400 Avance instrument. MS were registered on a VG Micromass ZAB-2F at 70 eV; IR on a Perkin Elmer 1600 (FTIR) instrument. Perinaphthenone (**1**) was obtained from a commercial source (Aldrich). All anhydrous reactions were carried out under an argon atmosphere using freshly dried solvents.

General synthetic procedures

A solution of **1** (0.47g, 0.003 mol) in THF (5 mL) was added to the appropriate Grignard reagent (0.004 mol; prepared from the respective bromide and Mg in THF (10 ml)). After stirring for 20 min at -70°C the reaction mixture was allowed to reach room temperature and then was quenched with an aqueous saturated solution of NH_4Cl. The aqueous phase was extracted with dichloromethane and the combined organic extracts were washed with brine, dried over Na_2SO_4 and evaporated to dryness. The crude material dissolved in CH_2Cl_2 was treated with DDQ (1.0 eq), refluxed for 3h and then allowed to reach room temperature. After water addition the CH_2Cl_2 phase was separated and purified by HPLC (hexane-EtOAc 99:1)

The preparation of the 2-hydroxyphenylphenalenone type compounds was carried out as follows: a solution of the compound (3 mmol, obtained as above) in benzene (15 mL) was cooled at 0°C and treated with Triton B (3 mmol, benzene) and t-butylhydroperoxide (3 mmol) for 3h. The reaction was quenched with NH_4Cl, the organic phase was separated, dried and evaporated. The residue (2.5 mmol) was dissolved in diethyl ether and several crystals of p-toluenesulfonic acid were added. After workup the final product was purified by HPLC, affording deep-yellow solids.

Satisfactory spectroscopic data were obtained for all compounds.

Spectral data

*Perinaphthenone (**1**)*

^{1}H NMR: δ 8.57 (dd, J = 1.1 and 7.4 Hz, 1H, H-9), 8.13 (dd, J= 0.8 and 8.0 Hz, 1H, H-7), 7.95 (d, J= 8.5 Hz, 1H, H-6), 7.72 (d, J= 7.6 Hz ,1H, H-8), 7.72 (d, J= 7.6 Hz,1H, H-4), 7.68 (d, J= 9.8 Hz,1H, H-3), 7.53 (dd, J= 7.6 and 8.2 Hz, 1H, H-5), 6.68 (d, J= 9.8 Hz ,1H, H-3); ^{13}C NMR: δ 185.16 (C-1), 141.38 (C-3), 134.51 (C-3a), 131.67 (C-9a), 131.55 (C-9), 131.08 (C-4), 129.80 (C-6a), 128.96 (C-7), 128.73 (C-6), 127.29 (C-5), 126.99 (C-8), 126.60 (C-9a), 126.23 (C-2)

*9-Phenylperinaphthenone (**2**)*

^{1}H NMR: δ 8.18 (d, J= 8.3 Hz, 1H, H-7), 8.05 (d, J= 8.2 Hz, 1H, H-6), 7.79 (d, J= 7.0 Hz, 1H, H-4), 7.70 (d, J= 9.7 Hz, 1H, H-3), 7.62 (dd, J= 7.1 and 8.2 Hz, 1H, H-5), 7.61 (d, J= 8.3 Hz, 1H, H-8), 7.45-7.34 (m, 5H, H-2',3',4',5',6'), 6.62 (d, J= 9.7 Hz, 1H, H-2); ^{13}C NMR: δ 186.13 (C-1), 148.07 (C-9), 143.32 (C-1'), 140.75 (C-3), 134.09 (C-7), 132.18 (C-9a), 132.11 (C-6), 132.01 (C-8), 131.76 (C-4), 130.94 (C-2), 128.94 (C-3a), 128.81 (C-9b), 128.67 (C-3',5'), 128.27 (C-6'), 127.55 (C-2',4'), 126.76 (C-6a), 126.48 (C-5).

*9-(p-Methoxyphenyl)perinaphthenone (**3**)*

^{1}H NMR: δ 8.15 (d, J= 8.3 Hz, 1H, H-7), 8.03 (d, J= 8.2 Hz, 1H, H-6), 7.76 (d, J= 6.4 Hz, 1H, H-4), 7.68 (d, J= 9.7 Hz, 1H, H-3), 7.62 (dd, J= 7.0 Hz and 7.9 Hz, 1H, H-5), 7.61 (d, J= 8.3 Hz , 1H, H-8), 7.34 (dd, J = 2.1 and 6.6 Hz, 2H, H-3',5'), 7.03 (dd, J= 2.1 and 6.6 Hz, 2H, H-2',6'), 6.62 (d, J= 9.7 Hz, 1H, H-2), 3.90 (s, 3H, -OCH_3); ^{13}C NMR: δ 186.26 (C-1), 159.51 (C-4'), 147.96 (C-9), 140.64 (C-3), 135.36 (C-1'), 134.08 (C-7), 132.38 (C-6), 132.11 (C-8), 132.01 (C-9a), 131.72 (C-4), 131.07 (C-

2), 129.86 (C-2',6'), 128.95 (C-3a), 128.86 (C-9b), 126.59 (C-5), 126.41 (C-6a), 114.23 (C-3',5'), 55.66 (-OCH_3)

*9-(2',4'-Dimethoxyphenyl)-perinaphthenone (***4***)*

^{1}H NMR: δ 8.19 (d, J= 8.4 Hz, H-7), 8.05 (d, J= 8.0 Hz, H-6), 7.78 (d, J= 6.8 Hz, H-4), 7.70 (d, J= 9.6 Hz, H-3), 7.62 (dd, J= 7.2 and 8.4 Hz, H-5), 7.60 (d, J= 8.4 Hz, H-8), 7.16 (d, J= 8.0 Hz, H-6'), 6.66 (dd, J= 2.4 and 9.7 Hz, H-5'), 6.62 (d, J= 9.6 Hz ,H-2), 6.58 (d, J= 2.4 Hz, H-3'), 3.92 (s, -O$C\underline{H}_3$), 3.73 (s, -O$C\underline{H}_3$); ^{13}C NMR: δ 185.66 C-1, 160.49 C-2', 157.52 C-4', 143.46 C-9, 140.07 C-3, 133.67 C-7, 132.12 C-6', 131.68 C-6, 131.57 C-8, 130.98 C-9a, 130.28 C-4, 129.28 C-2, 128.39 C-3a, 128.22 C-9b, 126.96 C-5, 126.02 C-1', 104.57 C-5', 98.77 C-3', 55.57-O$\underline{C}H_3$, 55.35-O$\underline{C}H_3$.

*9-(3',4'-Methylenedioxy)perinaphtenone (***5***)*

^{1}H NMR: δ 8.15 (d, J= 8.3 Hz, 1H, H-7), 8.03 (d, J= 8.1 Hz, 1H, H-6), 7.78 (d, J= 6.8 Hz, 1H, H-4), 7.69 (d, J= 9.7 Hz, 1H, H-3), 7.63 (dd, J= 7.2 Hz and 8.1 Hz, 1H, H-5), 7.60 (d, J= 8.3 Hz, 1H, H-8), 6.93 (d, J= 7.8 Hz, 1H, H-6'), 6.88 (s, 1H, H-2'), 6.86 (d, J= 7.8 Hz, 1H, H-5'), 6.62 (d, J= 9.7 Hz, 1H, H-2), 6.04 (s, 2H, -O-CH_2-O-); ^{13}C NMR : δ 186.12 (C-1), 148.02 (C-4'), 147.67 (C-3'), 147.45 (C-9), 140.68 (C-3), 137.03 (C-1'), 134.09 (C-7), 132.25 (C-6'), 132.15 (C-6), 132.11 (C-8), 131.97 (C-9a), 130.97 (C-4), 130.22 (C-2), 128.87 (C-3a), 126.87 (C-9b), 126.58 (C-5), 126.11 (C-6a), 109.46 (C-5'), 108.82 (C-2'), 101.46 (O-CH_2-O-)

Antifungal activity

Inhibition of mycelial growth [10]

Mycosphaerella fijiensis was initially isolated from naturally infected banana plantations located in Uraba (Colombia); cultures were routinely grown in PDA medium. Sterile distilled water suspensions of conidia were obtained by washing the surface of the sporulating colony growing in PDA at 26°C; these conidia were dispersed in PDA medium in petri dishes and incubated at 26°C for 4 days. Some of these colonies were placed in petri dishes with PDA medium containing different concentrations of the compounds to be assayed or with EtOH only. After incubation in the dark at 26°C, radial mycelial growth was measured after 7, 9, 12 and 15 days of incubation. The inhibition of fungal growth was estimated as a percentage of the control values. In addition, colonies were recovered, agar traces were eliminated and the mycelia were weighed.

Inhibition of spore germination

Ascospores discharged from infected banana leaves were used [11]; germination and length of the germ tubes were established microscopically in 50-150 ascospores. Appropriate controls of fungal cultures without compounds and controls with or without 1% EtOH were run. The following reference compounds were used to determine the threshold of detection: propiconazole, tridemorph and benomyl. Experiments were done in quadruplicate, and data were analyzed by one-way analysis of variance (ANOVA)

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Sample Availability: Available from MDPI.