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Phenalenone-Type Phytoalexins from *Musa acuminata* Synthesis of 4-Phenyl-phenalenones

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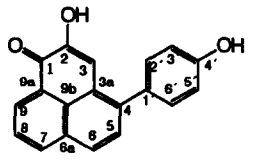
Abstract: Two new 4-phenyl-phenalenone-type phytoalexins (2, 3) have been isolated from rhizomes of *Musa acuminata* infected with the fungus *Fusarium oxysporum*. The structures of the new phytoalexins were elucidated on the basis of spectroscopic evidence, chemical correlation and synthesis from commercial phenalen-1-one. The chemical shift for all of the hydrogen and carbon atoms in the substances were unambiguously established by mono- and bidimensional, homo- and heteronuclear NMR experiments (^{1}H NMR,^{13}C NMR, COSY, HMQC and HMBC).In preliminary "in vitro" assays, the new phytoalexins have shown inhibitory activity on the growth of the germinative tube of *Fusarium oxysporum f. sp. cubense race 4*.

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, and several synthetic polyamines as well as some fungus-derived carbohydrates have been tested for their potential activity as phytoalexin inducers in species of *Pisum*[2,3] . Recently, we have described the isolation and characterisation[4] of irenolone (1), the first example of a new type of phytoalexins structurally based on a phenalenone skeleton, from *Musa paradisiaca* [*Musa acuminata* (AAA), Grand Nain], elicited by *Mycosphaerella fijiensis* (causal agent of Black Sigatoka), a pathogenic fungus which attacks banana leaves and greatly reduces their growth, or with kanamycin.

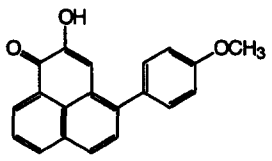
Several compounds with a phenalenone nucleus have been reported in plants and microorganisms[5]. The plant phenalenones isolated until now possess typically a side phenyl ring on C-9 and have been found in species of the *Haemodoraceae* plant Family, in the genera *Haemodorum, lachnanthes, Xiphidium, Wachendorfia and Anigozanthus,* while microbial phenalenones which do not have this side phenyl ring have been reported in *Hypomycetous (genera Penicillium, Fussicoccum, Giesmaniella and Verticilium)* and *Dyscomicetous* fungi. Nevertheless, no 4-phenylphenalenones had before been described either as natural or synthetic substances.

Now and from rhizomes of *Musa acuminata* Grand Nain, infected with *Fusarium oxysporum* a saprophytic pathogenic fungus which causes Panama disease in banana plants, we have isolated two new 4-phenylphenalenone-type phytoalexins, **2** and **3**.

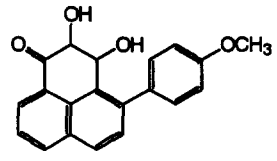
Eighty kilograms of rhizomes of banana plants which showed clear symptoms of Panama disease were used to extract the phytoalexins and the same weight of rhizomes from healthy plants was employed as control material. When compared on TLC, the extract of the material from infected plants showed the presence of eight coloured spots, of which the major one was observed, in a very low proportion, in the exctract of healthy plants as well, suggesting that it was induced, in this material, by cutting stress.The extract from mycelia of "in vitro" cultured *Fusarium oxysporum f. sp. cubense race 4* (causal agent of Panama disease), did not even show traces of such substances on TLC.



1



2



3

The major constituent of the infected rhizomes was identified as irenolone [4] (**1**) by comparison with an authentic sample. From the other substances, two of them were purified and their structures established as *follow:*

The less polar compound **2** had absorption maxima at 272, 313, 337 and 388 nm in the UV spectrum and bands for phenols (3317 cm^{-1}) in the IR spectrum. Mass spectrometry showed the molecular ion at m/z 302.09363 (corresponding to $C_{20}H_{14}O_3$ by HRMS). All of the hydrogen and carbon atoms in the molecule were discerned by homo- and heteronuclear NMR experiments (^1H NMR, ^{13}C NMR, COSY, HMQC and HMBC). The phenolic proton, interchangeable with deuterium oxide, appears at δ 4.81 in the ^1H NMR spectrum of **2**. A one-proton double doublet at δ 8.79 (J_1=1.3Hz, J_2=8.2Hz) which showed three-bond correlation with the carbonylic carbon atom C-1 was assigned to H-9, periplanar to the carbonyl group. Assignment of the chemical shifts for the H-8 and H-9 protons followed from the homonuclear COSY experiment.

A singlet proton at δ 7.34, which showed three-bond correlations with the carbonylic carbon atom C-1 and with the quaternary carbon atoms C-9b and C-4 in the HMBC experiment, was assigned to H-3. The H-5 and H-6 protons appear as clean doublets at δ 7.59 and δ 7.95 respectively and the protons on the substituent aromatic ring were observed as two equivalent aromatic AB systems at δ 7.07-7.42. In addition, the ^1H NMR spectrum showed the presence of a methoxy group as a three-proton singlet at δ 3.91.

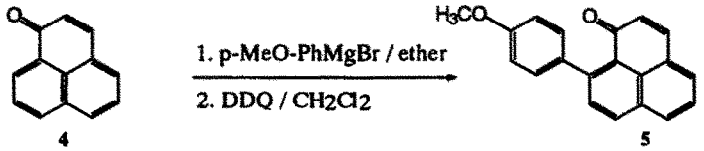
All the above data are in accordance with the structure of 4'-OMe-irenolone for this new phytoalexin **2**, which was chemically confirmed when treatment of it with boron tribromide in dichloromethane quantitatively gave irenolone (**1**).

The more polar compound **3**, which showed the same fragmentation pattern on MS as that of **2**, had absorption maxima at 276, 312, 340 and 386 nm in the UV spectrum and band for alcoholic groups (3391cm^{-1}) and carbonylic group (1725 cm^{-1}) in the IR spectrum. Mass spectrometry showed the molecular ion $[M]^+$ at m/z 320.10473 corresponding to $C_{20}H_{16}O_4$.The ^1H NMR spectrum of **3** was very similar to that of **2** except for the presence of an AB system as two one-proton double doublets at δ 4.74 (J_1=2.0Hz, J_2=3.3Hz) and δ 5.32 (J_1=0.9Hz, J_2=3.3Hz), assignable to a vicinal diol system, which was confirmed by the transformation of the above AB system in two one proton doublets and the disappearance of two hydroxylic one-proton doublets at

δ 2.98 and δ 4.25 when the ^{1}H NMR spectrum was run after adding deuterium oxide. The higher chemical shift for the H-9 proton (δ 8.25) in the ^{1}H NMR spectrum of **3** as compared with that for the same proton in the ^{1}H NMR spectrum of **2** (δ 8.79) suggested that the glycol system is situated on the C-2, C-3 bond in the former. The coupling constant $J_{2,3}$=3.3Hz, and the observed NOE effect between H-2 and H-3 in the ROESY experiment, established a cis disposition for the vicinal hydroxyl groups.

Chemical proof for the structure of cis-2,3-dihydroxy-4-(p-methoxyphenyl)-phenalen-1-one for **3** followed when treatment of it with aqueous sulphuric acid in acetone yielded a dehydration product with spectroscopic data identical with that of **2**.

Because of the novelty of 4-phenyl-phenalen-1-ones as natural or synthetic products and of the small quantities of this type of defensive substances made by the plant and the need to obtain enough material for biological assays, we accomplished a synthesis of **2** and **1** from commercial perinaphthenone.



4 **5**

Scheme 1

Because perinaphthenone easily undergoes Michael-type attack on C-9 with Grignard reagents[6], a good methodology to obtain 4-phenyl-phenalenones should be to prepare the corresponding 9-pheny-phenalenone system and then to interchange the carbonyl on C-1 and the double bond on C-2, C-3.

The 9-(p-methoxyphenyl)-phenalen-1-one **5** was obtained in good yield by treatment of commercial perinaphthenone **4** with p-methoxyphenylmagnesium bromide and then dehydrogenation of the resultant enone with DDQ (2,3-dichloro-5,6-dicyano-p-benzoquinone), (Scheme 1).

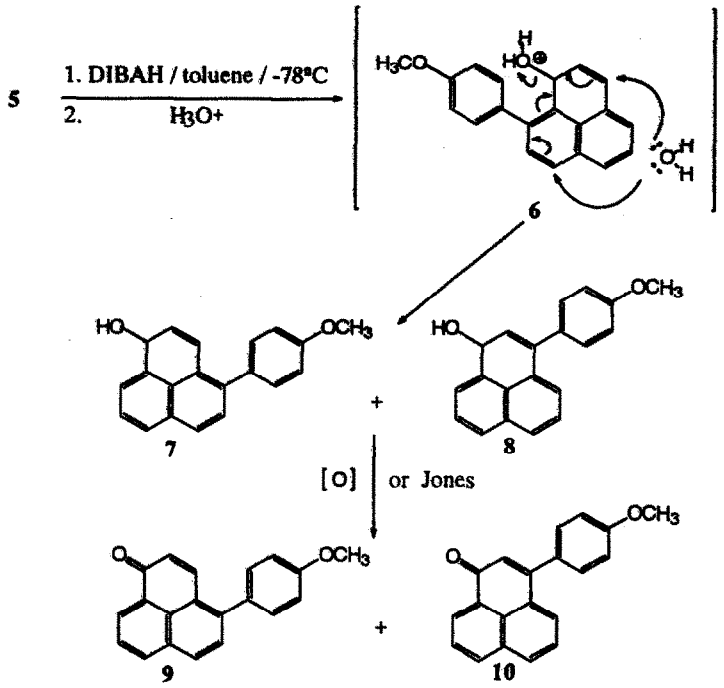
All attempts to transpose the enone functionality in **5** using known methodologies (Wharton rearrangement[7] or dialkylative enone transposition[8]) were unsuccessful.

Nevertheless, when 9-(p-methoxyphenyl)-phenalen-1-one **5** was reduced with DIBAH we observed that, on silica gel TLC, the initial product of the reaction slowly decomposed to a mixture of two stable, intensely coloured substances, which after separation by flash chromatography from the expected allylic alcohol, were characterised from their spectroscopic data to be : 4-(p-methoxyphenyl)-phenalen-1-one (**9**) (the major one), and 3-(p-methoxyphenyl)-phenalen-1-one (**10**) (the minor one).

The ^{1}H NMR spectra of both **9** and **10** show the typical C-9 proton, periplanar to the C-1 carbonyl group, as a double doublet at δ 8.68, the main difference between the two ^{1}H NMR spectra being the chemical shift and multiplicity of the H-2 proton, which appears as a clean doublet at δ 6.78 (J=9.7Hz) coupled with the H-3 proton (δ 7.82) in the ^{1}H NMR spectrum of **9**, while it can be seen as a one-proton singlet at δ 6.70 in that of **10**.

It appears to be clear that both **9** and **10** have been formed from the original allylic alcohol **6** by acid-catalysed transposition to the allylic alcohols **7** and **8**, followed by aerial oxidation as indicated in Scheme 1. This transposition must be thermodynamically favoured because of the lack in **7** and **8** of the steric interaction between the quasi-periplanar hydroxyl group at C-1 and the large p-methoxyphenyl group at C-9 which exists

in **6**. On the other hand, the electronic density map for the latter, obtained by molecular mechanic calculations using the PCMODEL program, indicated that at the carbon atoms C-3 and C-7 approximately the same electronic density exists.



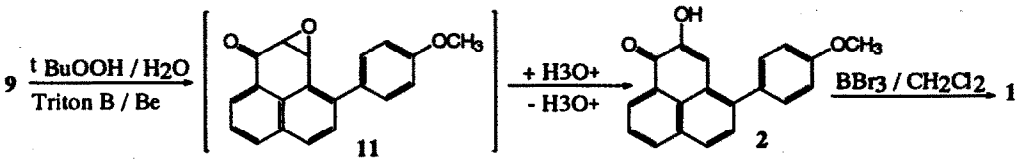
Scheme 2

The allylic alcohols **7** and **8** had practically the same R_f values in different systems and could not be separated. The ^1H NMR clearly showed this mixture to contain **7** (70 %) and **8** (30 %), with the distinctive signals corresponding to the methoxy groups which can be seen as a pair of three proton singlets at δ 3.89 and δ 3.88 respectively, and the protons geminal to the allylic alcoholic groups, which appear as two broad singlets at δ 4.12 and δ 4.19, respectively.

When the mixture of allylic alcohols **7** and **8** obtained as the main products by reduction of **5** with DIBAH was stirred in acetone with Jones reagent it quantitatively yielded a mixture of **9** (70%) and **10** (30%) which were separated and purified by silica gel chromatography.

The treatment of 4-(p-methoxyphenyl)-phenalen-1-one **9** dissolved in benzene with aqueous t-butylhydroperoxide and Triton B (methanolic benzyltrimethylammonium hydroxide) at room temperature for 24 hours (Scheme 3) gave, after acidic workup, directly and quantitatively **2** with spectral data identical with those of the natural product. The intermediate epoxide **11**, or the corresponding 2,3-diol could not be isolated. Demethylation of **2** with boron tribromide, quantitatively yield irenolone (**1**).

The above procedure constitutes a simple and direct route to the new 2-hydroxy-4-phenyl-phenalen-1-one system present in some of the phytoalexins isolated from *Musa acuminata*.



Scheme3

When assayed on "in vitro" cultures of *Fusarium oxysporum f. sp. cubense race 4* (causal agent of Panama disease) on solid support both the isolated phytoalexins were shown to inhibit growth of the germinative tubes. Complete results of these assays will be published elsewhere.

EXPERIMENTAL

General

^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX400 and WP200SY spectrometers. IR spectra were taken on a Perkin-Elmer 1600 (FTIR) spectrophotometer and UV spectra on a Perkin Elmer 550SE instrument. High resolution mass spectra were run on a VG-Micromass ZAB-2F at 70 eV.

Plant material

Rhizomes of Panama disease-infected plants and healthy plants were collected at the CITA experimental station in Tenerife.

Extraction and isolation

Freshly collected rhizomes of infected plants (80 kg) were immediately chopped and pressed to eliminate the water which was collected and immediately extracted with $CHCl_3$. The residual material was kept under maceration with EtOH (96 %) (20 l) at room temperature for 5 days. The filtered ethanolic extract was evaporated in a rotavapor to 1/4 of its volume and repeatedly extracted with $CHCl_3$. The mixed chlorophorm extracts were evaporated to dryness to give 9.8 g of crude material, which was percolated through a silica gel column (300 g) using successively n-hexane, n-hexane-EtOAc (1:1), EtOAc and MeOH (1.5 l each).

The n-hexane-EtOAc (1:1) washing was evaporated to dryness to yield 5.4 g of semicrude material which was submitted to chromatographic separation on Sephadex LH-20 (500 g), using as eluent n-hexane: $CHCl_3$: MeOH (2:1:1). 57 Fractions of 40 ml were collected.

The rhizomes from healthy plants (80 kg) were treated in exactly the same way.

The above fractions 11-14 (0.59 g) were repeatedly chromatographed on silica gel (63-200 μ , Merck) using as eluent mixtures of n-hexane - EtOAc and the substances **1-3** were purified by preparative TLC (on precoated 0.25 mm silica gel plates from G. Schleicher & Schull). By increasing order of polarity: **1** (24 mg); **2** (17.0 mg) and **3** (3.2 mg).

Filtered out mycelia of "in vitro" cultured *Fusarium oxysporum. sp. cubense race 4* and the culture medium were both extracted with ethanol, and the combined alcoholic extracts were evaporated to dryness. This extract did not show the presence of phenyl-phenalenones on TLC.

***2-Hydroxy-4-(p-methoxyphenyl)-phenalen-1-one*(2).** Bright red needles; m.p. 222-223 ºC; HREIMS: $[M]^+$ at m/z 302.09363 (calc. for $C_{20}H_{14}O_3$, 302.09429); IR ($CHCl_3$): ν_{max}/ cm^{-1}: 3317, 1619, 1557, 1511, 1412, 1346, 1259, 1219, 1174, 1067; UV(EtOH) λ_{max} /nm: 272, 313, 337, 388; ^1H NMR δ: 3.91(3H s, OCH_3), 7.07(2H, dd, J_1=2.1Hz, J_2=8.6Hz, H-3' and H-5'), 7.34(1H, s, H-3), 7.42(2H, dd, J_1=2.1Hz, J_2=8.6Hz, H-2' and H-6'), 7.59(1H, d, J=8.5Hz, H-5), 7.81(1H, t, J=8.5Hz, H-8), 7.95(1H, d, J=8.5Hz, H-6), 8.28(1H, dd, J_1=1.3Hz, J_2=8.2Hz, H-7), 8.79(1H, dd, J_1=1.3Hz, J_2=8.2Hz, H-9); ^{13}C NMR($CDCl_3$) ppm: 55.81(q, OCH_3), 113.16(d, C-3), 114.43(d, C-3' and C-5'), 125.32(s, C-9b), 125.33(s, 3a), 126.85(d, C-8), 128.06(s, C-9a), 130.08(d, C-5), 130.36(d, C-6), 130.98(s, C-6a), 131.94(d, C-9), 131.98(d, C-2' and C-6'), 132.29(s, C-1'), 136.89(d, C-7), 144.24(d, C-4), 149.79(s, C-2), 160.04(s, C-4'), 180.27(s, C-1); MS: m/z (rel. int. %): 302$[M]^+$ (100), 287(26), 284(43), 271(57), 259(25), 241(17), 213(21), 202(70), 187(10), 88(11). Treatment of **2** with boron tribromide in dichloromethane at room temperature for 15 min quantitatively yield irenolone (**1**).

***cis-2,3-Dihydroxy-4-(p-methoxyphenyl)-phenalen-1-one* (3).** Colourless needles; m.p. 175-177 ºC; $[\alpha]_D^{25}$= +40.9º(0.203, $CHCl_3$); HREIMS: $[M]^+$ at m/z 320.10473 (calc. for $C_{20}H_{16}O_4$, 320.10486); IR ($CHCl_3$): ν_{max}/cm^{-1}: 3391, 3005, 1725, 1607, 1514, 1249, 1219, 1176, 1017; UV(EtOH) λ_{max} /nm: 267, 312, 340, 386; ^1H NMR δ: 2.98(1H, d, J=0.9Hz, 3-OH), 4.25(1H, d, J= 2.0Hz, 2-OH), 3.90(3H, s, OCH_3), 4.74(1H, dd, J_1=2.0Hz, J_2=3.3Hz, H-3), 5.32(1H, dd, J_1=0.9Hz, J_2=3.3Hz, H-2), 7.03(2H, d, J=8.7Hz, H-3' and H-5'), 7.58(2H, d, J=8.7Hz, H-2' and H-6'), 7.61(1H, d, J=8.7Hz, H5), 7.65(1H, t, J=7.4Hz, H-8), 7.98 (1H, d, J=8.7Hz, H-6), 8.18(1H, dd, J_1=1.3Hz, J_2=7.4 Hz, H7), 8.25(1H, dd, J_1=1.3Hz, J_2=7.4Hz, H-9); MS: m/z(rel. int. %): 302$[M-18]^+$ (42), 287(11), 284(17), 271(17), 259(11), 241(5), 213(19), 202(100), 187(37), 88(9). When a solution of **3** in acetone was stirred with aqueous sulphuric acid (20%) for 1h, it quantitatively yielded **2**.

Synthesis of 4-Phenyl-Phenalen-1-ones

9-(p-methoxyphenyl)-Phenalen-1-one (5). Perinaphthenone (4) (Aldrich) (1.48 g, 8.25 mmol), dissolved in dry benzene (150 ml) was slowly added to a solution of p-methoxyphenylmagnesium bromide (11.85 mmol) in ether (100 ml). The mixture of reaction was refluxed under argon atmosphere for 3h, poured into a HCl solution (5 %) and extracted with benzene. The Na_2SO_4 dried extract was filtered and evaporated to dryness.

The crude material dissolved in CH_2Cl_2 was treated with DDQ (1 equivalent) and refluxed for 3h. The product was purified by silica gel column chromatography using n-hexane-AcOEt (9:1) as eluent to give 1.6 g (66% yield) of **5**, which crystallised from benzene /n-hexane as yellow needles: m.p. 176-178ºC; IR ($CHCl_3$): ν_{max} /cm^{-1}: 3036, 1639, 1608, 1555, 1516, 1492, 1289, 1241, 1174, 1127, 1028, 854; ^{1}HNMR δ: 3.89(3H, s, OCH_3), 6.62(1H, d, J=9.7Hz, H-2), 7.01(2H, dd, J_1=2.0Hz, J_2=8.6Hz, H-3' and H-5'), 7.36(2H, dd, J_1=2.0Hz, J_2=8.6Hz, H-2' and H-6'), 7.59(1H, d, J=8.3Hz, H-8), 7.62(1H, t, J=7.8Hz, H-5), 7.68(1H, d, J=9.7Hz, H-3), 7.78(1H, dd, J_1=1.3Hz, J_2=8.3Hz, H-4), 8.03(1H, dd, J_1=1.3Hz, J_2=8.3Hz, H-6), 8.23(1H, d,

J=8.3 Hz, H-7); MS: m/z (rel. int. %): 286 $[M]^+$ (18), 242(37), 212(100), 199(18), 163(10), 128(24), 87(24), 77(37), 63 (46).

4-(p-methoxyphenyl)-Phenalen-1-ol **(7) and** ***3-(p-methoxyphenyl)-phenalen-1-ol*** **(8).** A solution of 9-(p-methoxyphenyl)-phenalen-1-one (**5**) (50 mg, 0.18 mmol) in dry toluene (15 ml) was cooled at -78°C and treated, under argon atmosphere, with DIBAH (1.0M in toluene)(175 µl). When the reaction was completed (4 h) it was quenched with saturated NH_4Cl solution, washed with water (3 x 10 ml), dried on anhydrous Mg SO_4 and the solvent evaporated under vacuum. The crude product of the reaction showed on TLC, after developing with oleum, two dark-blue spots with practically identical Rfs. When this material was submitted to preparative TLC, it was observed that two more polar intensely red-brown spots, visible without being developed, had been formed from the original dark-blue ones. Cutting off the frames and extraction with ethyl acetate gave a mixture of **7** and **8** (corresponding to the dark-blue spots) (40 mg); ^1H NMR δ: 3.88(70%) and 3.89(30%)(s, 2 x OCH3), 4.12(70%) and 4.19(30%) (bs, 2 x H-1), 6.02 and 6.61(3H, H-2 and H-3 from **7** and H-2 from **8**), 7.03(m), 7.30(m) and 7.64(m); **9** (7mg) and **10** (3mg) characterised from their spectroscopic data and by formation from **7** and **8** as follows :

Oxidation of **7** ***and*** **8.** A solution of **7** and **8** (35 mg) in acetone was treated with Jones reagent (200 ml) and stirred at room temperature. After 15 min the reaction was completed. Usual workup and separation on preparative TLC gave *4-(p-methoxyphenyl)-phenalen-1-one* (**9**) (24 mg): Orange needles, m.p. 128 -131 °C; IR($CHCl_3$): ν_{max}/cm^{-1}: 3036, 1637, 1606, 1555, 1516, 1460, 1389, 1247, 1178, 1117, 1025, 838; ^1H NMR($CDCl_3$) δ: 3.92 (3H, s, OCH_3), 6.78(1H, d, J=9.7Hz, H-2), 7.01(2H, d, J=8.6Hz, H-3' and H-5'), 7.42 (2H, d, J= 8.6Hz , H-2' and H-6'), 7.48 (1H, d, J=8.3Hz, H-8), 7.72(1H, d, J=8.5Hz, H-5), 7.78(1H, d, J=8.5Hz, H-6), 7.82(1H, d, J=9.7Hz, H-3), 8.16(1H, dd, J_1=1.3Hz, J_2=8.3Hz, H-7), 8.68(1H, dd, J_1=1.3Hz, J_2=8.3Hz, H-9); MS: m/z (rel. int. %): 286 $[M]^+$ (97), 271(22), 243(52), 213(100), 163(13), 150(21), 87(13), 75(10), 63(18); and *3-(p-methoxyphenyl)-phenalen-1-one* (**10**): dark yellow needles, m.p. 139-140 °C; IR ($CHCl_3$): ν_{max}/cm^{-1}: 3055, 2836, 1634, 1606, 1570, 1509, 1461, 1402, 1289, 1338, 1288, 1248, 1178 1028; ^1H NMR ($CDCl_3$) δ: 3.90(3H, s, OCH_3), 6.70(1H, s, H-2), 7.04(2H, d, J=8.6Hz, H-3' and H-5'), 7.43(2H, d, J=8.6Hz , H2' and H-6'), 7.54(1H, t, J=8.0Hz, H-5), 7.78(1H, d, J=8.5Hz, H4), 7.80(1H, t, J=8.0Hz, H-8), 8.03(1H, dd, J_1=1.2Hz, J_2=8.3Hz, H-6), 8.22(1H, dd, J_1=1.2Hz, J_2=8.3Hz, H-7), 8.68(1H, dd, J_1=1.2Hz, J_2=8.3Hz, H-9); ^{13}C NMR($CDCl_3$) ppm: 55.39(q, OCH_3), 113.93(d, C-3' and C-5'), 126.25(s, C-5), 126.92(d, C-8), 128.05(s,C-6a), 128.32(s,C-9b), 128.60(d,C-2), 129.58(s,C-9a), 130.13(d, C-9), 130.20(s, C-1'), 130.54(d, C-2' and C-6'), 131.57(d, C-4), 132.06 (d, C-6), 132.47(s, C-3a), 135.03(d, C-7), 153.51(s, C-3), 160.10(s, C4'), 184.94(s, C-1); MS: m/z (rel. int. %): 286$[M]^+$ (41), 258(22), 215(100), 189(37), 150(68), 63(25).

2-Hydroxy-4-(p-methoxyphenyl)-phenalen-1-one **(2).** To a 0°C cooled solution of 4-(p-methoxyphenyl)-phenalen-1-one (**9**) (20 mg) in benzene (10 ml), t-butyl-hydroperoxide (70 % in water) (22 µl) and benzyltrimethylammonium hydroxide (40 % in MeOH) (22 µl), were successively added and the reaction mixture was stirred at room temperature, repeating the addition of reagents two more times every three hours. The reaction was left to stand overnight and then washed with water. The solvent was evaporated and the crude material, dissolved in CH_2Cl_2, was treated with p-toluenesulphonic acid (15 mg) and stirred for 2h.

Separation of the product on preparative TLC gave **2** (19.8 mg) (94 % yield) with spectroscopic data identical with that of the natural product. Demethylation of **2** with BBr_3 in CH_2Cl_2 quantitatively yielded irenolone (**1**).

ACKNOWLEDGEMENTS

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