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COMPARATIVE EVALUATION OF PHOTODYNAMIC EFFICIENCY OF SOME NATURAL QUINONOID FUNGAL TOXINS

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Key Word Index—perylenequinones; metabolites; peroxyphleichrome; stemphyltoxin I; stemphyperylenol; altersolanol A; dothistromin; rubellin A; methyl oleate peroxidation.

Abstract—A scale of photodynamic efficiency of some quinonoid fungal metabolites measured *in vitro* as amount of hydroperoxides produced by methyl oleate ester peroxidation is presented and the behaviour of these metabolites is discussed. Cercosporin is the best photosensitizer of this group. Under prolonged irradiation times, peroxyphleichrome gave rise to two new photoderivatives, whose structures have been elucidated by NMR spectroscopy.

INTRODUCTION

In recent years considerable attention has been paid to the specific physiological role of the secondary metabolites produced by phytopathogenic fungi, the causal agents of necrotic diseases [1, 2]. Many of these natural substances have biological activity and exert their toxic effects at several steps of the cells metabolism, mainly producing irreversible damage to the transport systems of the cell membrane.

As a result of our studies on the isolation and chemical characterization of secondary metabolites from several fungal species [3], we had at our disposal a number of substances, some of which (cercosporin and phleichrome) were known to be active mainly as a result of their photodynamic action. This feature has attracted growing interest for its intriguing implication in host–pathogen relationships, since lipid peroxidation is a possible mechanism for the alteration of the cell membranes lipid phase observed in the formation of necrotic lesions [4]. Furthermore, studies on new photochemically active compounds has increased due to their possible use in the phototherapy of cancers [5].

Earlier investigations have shown that the main photosensitizing effect of some of the tested compounds [6, 7] resulted in the production of singlet oxygen. Subsequent damage to the substrate involves this oxygen in a reaction (Type II mechanism) [8], which in the case of unsaturated carbons gives rise to hydroperoxides [9]. In order to compare the different degree of photodynamic efficiency of our metabolites, we chose a quite homogeneous group of fungal toxins having an extended quinone system and performed a standardized reaction which utilized methyl oleate as the target substrate.

In this paper, we give a scale of photodynamic efficiency of some quinonoid fungal metabolites and derivatives on the basis of the amount of hydroperoxides produced in the presence of methyl oleate. In addition, we have determined the rate of hydroperoxide formation for a selected series of these metabolites. Finally, the isolation and characterization of the new derivatives **12** and **13** formed from photoexcited peroxyphleichrome (**4**) is reported.

RESULTS AND DISCUSSION

The metabolites tested and their origins are summarized in Table 1. The results from the average of four independent experiments for solutions absorbing all the incident light show that the production of hydroperoxides (%) gives rise to a scale of photodynamic efficiency roughly composed of three groups of compounds (Table 1): the first group includes cercosporin (**1**), elsinochrome A (**5**), peroxyphleichrome (**4**), phleichrome (**3**) and elsinochrome B (**6**) ($> 70\%$); the second includes stemphyltoxin I (**8**) and rubellin A (**11**) (*ca* 50%) and the third includes dimethylcercosporin (**2**), stemphyperylenol (**7**) and dothistromin (**10**) ($\leqslant 35\%$).

No hydroperoxides were produced when altersolanol A (**9**) was tested. Phleichrome and peroxyphleichrome afforded the photoproducts **12** and **13**, while stemphyltoxin I, dothistromin and dimethylcercosporin showed some photodecay after irradiation. None of the other substances tested gave rise to secondary compounds and were re-isolated at the end of the irradiation period.

The data in Table 1 suggest that a decrease in efficiency is roughly related to a decrease in overall aromaticity, i.e. the conjugated perylenequinones are more efficient than the reduced ones, and the extended anthraquinones more than the naphthoquinonoid derivatives.

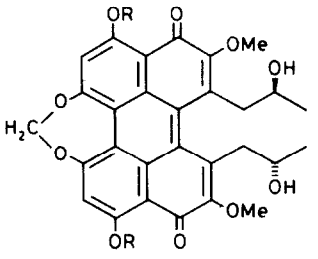
Furthermore, tautomeric equilibria have a fundamental role in lowering the energy of transition and in increasing the overall aromaticity: in fact the interruption of tautomerism in dimethylcercosporin due to the methylation of hydroxyl groups *peri* to the quinonoid system dramatically decreased its photodynamic property in comparison with that of **1**. Unfortunately, these

Table 1. Scale of efficiency of photodynamic production of hydroperoxides from methyl oleate by natural fungal toxins and some derivatives

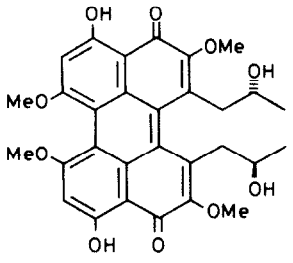
Toxin	Source	References	Hydroperoxide formation (%)
Cercosporin (**1**)	*Cercospora* spp.	[10]	80
Dimethylcercosporin (**2**)*		[10]	35
Phleichrome (**3**)	*Cladosporium phlei*	[11]	75
Peroxyphleichrome (**4**)*		[11]	80
Elsinochrome A(**5**)	*Elsinoe* spp.	[12]	85
Elsinochrome B(**6**)	*E.* spp.	[12]	75
Stemphyperylenol(**7**)	*Stemphylium botryosum v. lactucum*	[3]	10
Stemphyltoxin I(**8**)	*S. botryosum v. lactucum*	[3]	55
Altersolanol A (**9**)	*S. botryosum v. lactucum, Alternaria* spp.	[13]	0
Dothistromin (**10**)	*Mycosphaerella laricina*	[14]	10
Rubellin A(**11**)	*M. rubella*	[15]	50

All the values are ±1. The reactions were performed using a methyl cyanide solution of 30 μM quinone derivative and 6 mM methyl oleate, saturated with O_2.

*Derivatives.



1 R = H
2 R = Me



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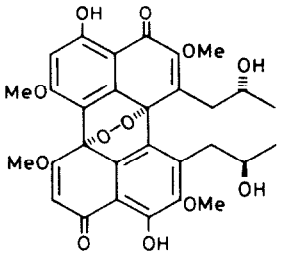

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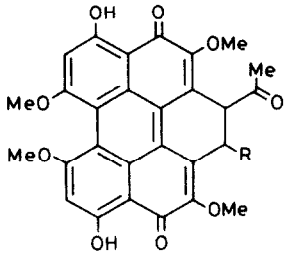
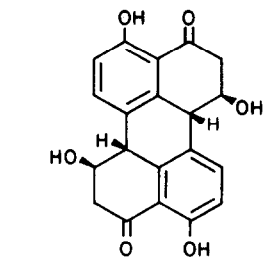


5 R = CO – Me
6 R = CHOH – Me

molecules do not exhibit appreciable phosphorescence at low temperature, but a partial confirmation of what is stated above is provided by the kind of transition implied for the quinone series, i.e. $n \rightarrow \pi^*$ in nature [16, 17] while the introduction of the hydroxyl groups in the right positions changes this transition to $\pi \rightarrow \pi^*$ [18, 19].

The photodynamic properties of some of the tested toxins having different efficiencies were measured for solutions of the same optical density. The rate of hydroperoxides formation (Table 2 and Fig. 1) was determined in comparison with cercosporin, which was shown to be the best photosensitizer of this group. The values obtained are in good agreement with those of Table 1, confirming the general trend.

It has been reported that cercosporin acts only *via* singlet oxygen generation [20 and A. M. Braun, personal communication] and work is in progress to verify if hydroperoxidation induced by the other metabolites



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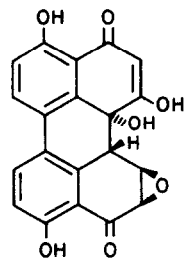

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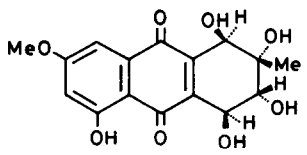

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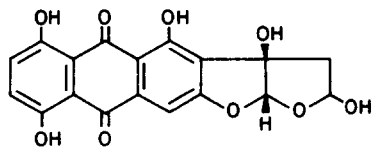

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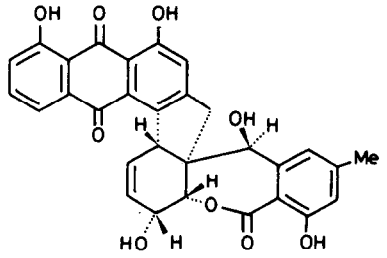

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Table 2. Rate of hydroperoxide formation from methyl oleate for some selected fungal toxins

Metabolite	Rate of hydroperoxidation relative to cercosporin
Cercosporin	1
Elsinochrome A	0.84
Phleichrome	0.80
Rubellin A	0.40
Dothistromin	0.08

Values of relative rates are ± 0.05.

takes a similar course or whether different mechanisms are involved. In a previous paper, a method for evaluating the superoxide formation by cercosporin was carried out in the presence of light and oxygen using methionine as reducing substrate and *p*-nitro blue tetrazolium chloride (NBT) [21]. Our attempts to confirm these data were unsuccessful: the easy photodecomposition of NBT itself leaves us with many doubts as to the validity of used method.

The last consideration is related to the physiopathological role played by these fungal toxins. If their production occurs during the disease process, they would account for the disruption of the cell membrane and then in part, for the formation of necrotic areas.

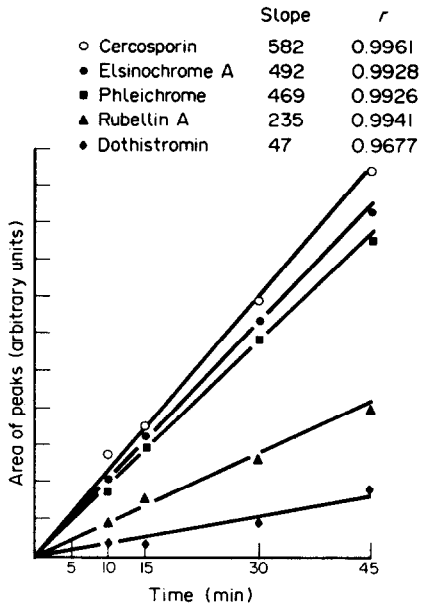
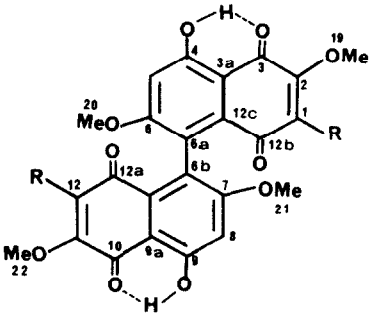


Fig. 1. Rate of methyl oleate hydroperoxide formation by selected fungal toxins.

Unlike the other perylenequinones studied, irradiation of phleichrome (**3**) gave, besides hydroperoxides, the corresponding endoperoxide (**4**) [11]. The latter, in turn, has photodynamic properties and under prolonged irradiation afforded compounds **12** and **13**, which have the same molecular formula (M^+582) as the parent compound (**4**). However, the ^1H and ^{13}C NMR spectra (see Tables 3 and 4) contained only half the expected resonances, this fact indicating a symmetrical dimeric structure for **12** and **13**.

Specifically, the ^1H NMR spectra of each portion of **12** and **13** exhibited signals assigned to one chelated phenolic group, two methoxyl groups, one aromatic proton, and one CH_2 CHOHMe group, whereas the corresponding ^{13}C NMR spectra showed the presence of 10 resonances attributable to a pentasubstituted-1,4-naphthoquinone moiety, and five signals due to three methyl groups, two of them oxygen-bearing, one methylene group, and one oxygen-bearing sp^3-hybridized carbon atom.

Chemical shift criteria, in conjunction with the observed multiplicities in the coupled spectra and the ^1H-$\{^1H\}$ and ^{13}C-$\{^1H\}$ low-power selective decoupling experiments, permitted us to assign all of the resonances



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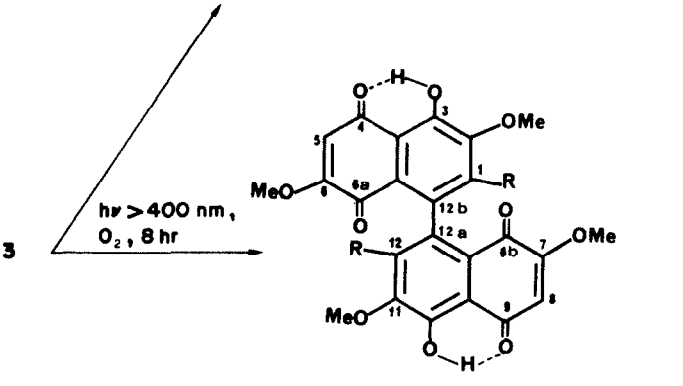

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R = CH_3 – CHOH – Me

Table 3. ^1H NMR data for compounds **12** and **13** in $CDCl_3$

H	12	13
5,8	6.65 *s*	6.03 *s*
13A,16A	2.60 *m*	2.80 *dd* (13.7,9.3)
13B,16B	2.56 *m*	2.17 *dd* (13.7,4.1)
14,17	3.80 *m*	3.57 *m*
15,18	1.13 *d* (6.2)*	0.99 *d* (6.2)
19,22	4.12 *s*	4.13 *s*
20,21	3.70 *s*	3.79 *s*
OH-3,OH-10		13.28 *s*
OH-4,OH-9	12.85 *s*	
OH-14,OH-17	2.40 *br*	2.52 *br*

* *J*(Hz) in parenthesis.

Table 4. ^{13}C NMR data for the compounds **12** and **13** in $CDCl_3$

C	**12** δ_C*/ppm	**12** J(CH)†/Hz	**13** δ_C*/ppm	**13** J(CH)†/Hz
1,12	133.29 *Sdt*	2.8(H-14),5.5(H_2-13)	137.41 *Sdt*	1.0(H-14),5.0(H_2-13)
2,11	158.02 *Stq*	4.8(H_2-13),3.4(H_3-19)	154.52 *Sm*	
3,10	184.49 *Ss(br)*	ca.1(H-5),ca.1(OH-4)	154.62 *Sdd*	1.0(H-5),4.8(OH-3)
3a,9a	109.15 *Sdd*	4.7(H-5), 5.0(OH-4)	114.99 *Sdd*	4.6(H-5),5.2(OH-3)
4,9	165.03 *Sdd*	4.3(H-5),5.4(OH-4)	191.13 *Sdd*	2.0(H-5),1.0(OH-3)
5,8	103.44 *Dd*	162(H-5),7.7(OH-4)	108.54 *Ds*	166(H-5)
6,7	164.57 *Sdq*	2.8(H-5),4.1(H_3-20)	161.21 *Sdq*	3.6(H-5),4.0(H_3-20)
6a,6b	122.85 *Sd*	6.5(H-5)	179.56 *Sd*	7.2(H-5)
12b,12a	185.98 *St*	4.6(H_2-13)	135.70 *Sdd*	6.0(H-13A),4.0(H-13B)
12c,9b	129.81 *Ss(br)*	ca.1(H-5)	122.98 *Ss(br)*	ca.1(H-5)
13,16	33.33 *Tm*	129(H_2-13)	37.84 *Tm*	129(H_2-13)
14,17	67.42 *Dm*	147(H-14)	66.34 *Dm*	146(H-14)
15,18	23.70 *Qm*	126(H_3-15)	23.92 *Qm*	126(H_3-15)
19,22	61.56 *Qs*	147(H_3-19)	61.12 *Qs*	146(H_3-19)
20,21	56.42 *Qs*	146(H_3-20)	56.68 *Qs*	147(H_3-20)

*Capital letters refer to the pattern resulting from directly bonded (C, H) coupling [^1J(CH)] and lower case letters to that from (C, H) couplings over more than one bond [$^{>1}J$(CH)].

†Coupling constants refer to the upper portion of the symmetrical dimeric molecules.

and to establish as **12** and **13** the structures of the two dimeric compounds.

In compound **12** the resonances at δ_c 185.98 and 184.49 were immediately assigned to the carbonyl carbon atoms C-12b and C-3, since the former appeared as a triplet, the observed 4.6 Hz splittings being removed by irradiation of the benzylic H_2-13, whilst the latter was a broadened singlet. In addition, irradiation of the 13-methylene protons caused the signals at δ_c 133.29 (C-1) and 155.02 (C-2) to change to a doublet and a quartet respectively, the residual couplings being the three-bond couplings with H-14 and H_3-19.

Irradiation of H-5 caused the double doublets at δ_c109.15 (C-3a) and 165.03 (C-4), the double quartet at δ_c164.57 (C-6), and the doublet at δ_c122.85 (C-6a) to simplify, the remaining couplings being, respectively $^3J_{C\text{-}3a,OH\text{-}4}=5.0$, $^2J_{C\text{-}4,OH\text{-}4}=5.4$ and $^3J_{C\text{-}6,H_3\text{-}20}=4.1$ Hz. Finally, the unassigned aromatic broad singlet at δ_c129.81 was attributed to C-12c.

On the basis of the evidence presented, the two portions of the molecule must be linked through the C-6a, C-6b bond. Similar decoupling experiments, the results of which are reported in Table 4, enabled us to propose structure **13** for the other dimeric compound. In particular, the ^{13}C signals at δ_c191.13,179.56 and 154.62 were readily assigned to the carbonyl carbon atoms C-4 and C-6a, and to C-3 since C-4 and C-6a presented (C,H) couplings of 2.0 and 7.2 Hz, respectively, with H-5, and C-3 was coupled to OH-3 via a two-bond coupling of 4.8 Hz.

Since the two OMe and the CH_2 CHOHMe groups are located at C-1, C-2 and C-6, it follows that the two portions of compound **13** must be connected through the C-12a, C-12b bond. Formation of **12** and **13** from **4** is consistent with the rupture of the peroxidic bond [22, 23] followed by a formation of a common biradical intermediate. Subsequent breaking of the C-C bond at either 12a–12b or 6a–6b and the formation of an unstable oxete intermediate would lead to the formation of the isolated compounds.

EXPERIMENTAL

Mps: uncorr. UV: 95% EtOH; Flash chromatography: Merck silica gel (0.040–0.063 mm): TLC; Merck HF_{254} silica gel; MS: 70 eV; ^1H (300.13 MHz) and ^{13}C (75.47 MHz) NMR: $CDCl_3$, TMS as int. standard.

Fungal toxins and derivatives were obtained from agar cultures of suitable fungal strains or from chemical reactions (see Table 1 refs) and were of chromatographic purity. Me oleate was supplied from Sigma Chemicals Co. and solvents were of analytical purity.

Irradiation. The apparatus used for irradiation was a Rayonet RPR-100 (Southern New England UV Co.) equipped with 16 fluorescent lamps (Sylvania F8T5/CW and F8T5/D) and a merry-go-round. All reactions were carried out at 25°. Irradiations were preformed in two different ways: (i) 20 ml of MeCN containing the test quinone (30 μm) and Me oleate (6 mM) were placed in a Pyrex glass tube and O_2 bubbled through for the 90 min of the reaction time. Under these conditions all the incident light was absorbed by the soln. Controls were run and no detectable amount of hydroperoxides was found either for N_2 saturated solns or for O_2 saturated solns stored in the dark or irradiated in the absence of a photosensitizer. (ii) MeCN containing compounds **1**, **3**, **5**, **10** or **11** (concentrations sufficient to give an A of 0.976) and Me oleate (6 mM) was bubbled with O_2 and irradiated through a $CuSO_4$ light filter. Samples were taken at regular intervals and hydroperoxide formation determined by one of the followed methods. No significant change of A was found at the end of the irradiation time. A typical plot of these determinations is shown in Fig. 1.

Detection of hydroperoxides. Formation of hydroperoxides was (i) detected by analytical HPLC: reversed-phase column of LiChrosorb RP-18 (5 μm), MeCN-H_2O (3:1), flow rate 1 ml/min, monitored at 210 nm, and (ii) followed by means of a photodensitometer Camag TLC/HPTLC Scanner. The same vol. of each soln was transferred to a TLC plate by a micrometer syringe (2 μl on a TLC Merck HF_{254} silica gel) and the chromatogram developed in hexane–EtOAc (6:1). Spots were visualized by spraying with phosphomolybdic acid–H_2SO_4 and heating at 80° for 5 min. The size and intensity of the spots were measured by photodensitometery at 589 nm.

Isolation and characterization of the products formed on irradiation of peroxyphleichrome (**4**). Compound **3** (10 mg) dissolved in 10 ml MeCN was put in a glass tube and the soln saturated with O_2; 8 tubes containing this soln were placed in the photoreactor and irradiated with visible light. At the first step (light irradiation at > 400 nm, 2 hr), photo-oxidation of the pigment occurred to give peroxyphleichrome **4**. After prolonged irradiation (8 hr), compound **4** was transformed to more polar products which themselves were photosensitizers. At the time indicated, the reaction was stopped, the solns in the tubes bulked and after evaporation of the solvent the residue chromatographed on silica gel (CH_2Cl_2 – MeOH, 15:1). The major fractions, containing the mixture of the isomers, were purified by PLC with C_6H_6 – Et_2O – HCO_2H (100:100:1) to give pure substances with R_f 0.38 (**12**) and 0.2 (**13**). Compound **12** (20 mg), orange amorphous powder, mp 94–96°; $[\alpha]_D$ –270.6°; ($CHCl_3$ *c* 0.1); (Found C,61.5; H,5.1. $C_{30}H_{30}O_{12}$ requires C,61.85; H,5.19 %); UV λ_{max} nm: 220, 270, 305 and 445 (ε 54250, 25000, 22500 and 10750); IR ν_{max}^{KBr} cm^{-1}: 3420 (OH) and 1630 (conj. CO); MS *m/z*: 582 $[M]^+$, 564 $[M-18]^+$, 538, 519, and 504. Compound **13** (25 mg), mp 83–85°; $[\alpha]_D$ + 138° (MEOH; *c* 0.1); (Found C, 61.7; H,5.1. $C_{30}H_{30}O_{12}$ requires C, 61.5; H, 5.19); UV λ_{max} nm: 223, 248, 270, 300 and 435 (ε 47650, 26500, 20600, 20000, and 10000); IR ν_{max}^{KBr} cm^{-1}: 3410 (OH) and 1630 (conj. CO); MS *m/z*: 582 $[M]^+$ 564 $[M-18]^+$, 538, 504 and 478. The ^1H and ^{13}C NMR data of **12** and **13** are reported in Tables 3 and 4.

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