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Xanosporic acid, an intermediate in bacterial degradation of the fungal phototoxin cercosporin

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Abstract

The red fungal perylenequinone phototoxin cercosporin is oxidized by *Xanthomonas campestris* pv *zinniae* to a non-toxic, unstable green metabolite xanosporic acid, identified via its lactone as 1,12-bis(2′R-hydroxypropyl)-4,9-dihydroxy-6,7-methylenedioxy-11-methoxy-3-oxaperylen-10H-10-one-2-carboxylic acid. Xanosporolactone was isolated in approximately 2:1 ratio of M:P atropisomers.

Keywords: *Xanthomonas campestris* pv. *zinniae*; Bacterial detoxification; Perylenequinone; Phototoxin; Xanosporolactone; Xanosporic acid

1. Introduction

The red fungal phototoxin cercosporin (**1**), isolated from the soybean pathogen *Cercospora kikuchii* (Kuyama and Tamura, 1957), was the first of a large number of perylenequinones found in fungal plant pathogens (Weiss et al., 1987; Thomson, 1997). This metabolite is produced by *Cercospora* species pathogenic on a number of important crops: maize, rice, cotton, soybean, bean, pea, alfalfa, carrot, tobacco, coffee, and sugarbeet (Daub and Ehrenshaft, 2000; Assante et al., 1977). The gross structure of **1** was elucidated by Lousberg et al. (1971) and confirmed by Yamazaki and Ogawa (1972) who recognized that the chromophore was not fully planar due to the repulsion between the bulky hydroxypropyl substituents. The M-axial chirality of **1** was subsequently assigned by X-ray crystallography (Nasini et al., 1982). Its circular dichroism is dominated by the M-twist of the chromophore and has been used as a model to assign axial chirality to other fungal perylenequinones (Arnone et al., 1988, 1989). Cercosporin (**1**) is isomerized in refluxing toluene to the P-axial isomer isocercosporin (**2**) possessing a near mirror image CD spectrum.

Perylenequinone **1** is a well-studied photosensitizer of singlet oxygen ($^1O_2$) production (Daub, 1982). Being lipophilic, **1** has affinity for cell membranes where $^1O_2$ is produced on illumination. Oxidation of unsaturated acylglycerides damages cell membranes causing leakage of nutrients into the intercellular space where the fungal hyphae grow (Daub and Ehrenshaft, 2000). The role of this phototoxin in plant pathogenicity is supported by a correlation of relative damage to leaves in sunlight versus shade (Calpouzos and Stahlknecht, 1967) and by comparison of damage done to an illuminated soybean leaf inoculated with *C. kikuchii* versus that caused by a mutant that produces less than 2% of the phototoxin produced by the wild type (Upchurch et al., 1991). Cercosporin (**1**) is not significantly toxic in the absence of light. Other perylenequinones of fungal pathogens, such as the M-(+)-enantiomer of isocercosporin (**2**) (Tabuchi et al., 1991), the P-(−)-enantiomer of M-(+)-isophleichrome (**3**) (Arnone et al., 1988, 1989), P-(−)-phleichrome (**4**) (Yoshihara et al., 1975), cladochromes, calphostins (Kobayashi et al., 1989), elsinochromes

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(Arnone et al., 1993), hypocrellins (Kishi et al., 1991) and shiraiachromes (Wu et al., 1989; Mazzini et al., 2001), are believed to act similarly as photosensitizing pathogenicity factors of *Cladosporium* spp., *Elsinoe* spp., *Hypocrella bambusae, Shiraia bambusicola* and other plant pathogenic fungi (Daub and Ehrenshaft, 2000).

We have been investigating biodegradation of **1** as a possible strategy for control of cercospora diseases in crops (Mitchell et al., 2002). A survey of 244 bacterial isolates detected seven species of bacteria that degrade **1**. The ability to degrade **1** was particularly apparent in the plant pathogenic bacterium *Xanthomonas campestris* pathovar *zinniae* (32 of 32 isolates) and *X. campestris* pv. *pruni* (five of 23 isolates), while *X. campestris* pv. *campestris*, pv *pelargoniae* and pv *vesicatoria* did not. Evidence is presented here that the initial product of bacterial catabolism of **1** is labile xanosporic acid **5** which is readily lactonized to xanosporolactone **6**. We have previously shown that these two green transformation products are non-toxic to either a cercosporin-sensitive mutant of *C. kikuchii* or to tobacco leaf in light or dark (Mitchell et al., 2002).

2. Results

2.1. Xanosporic acid 5 and its lactone

Cercosporin (**1**) is completely catabolized by *X. campestris* pv *zinniae* in the dark within 60 h to a single green, water-soluble pigment, xanosporate (**5**) anion. The green metabolite is extractable into chloroform only after the alkaline culture medium (pH 8.5) is adjusted to pH 2.5 and is not back extractable into a pH 8.5 buffer, indicating that a carboxylic acid has been lactonized. Xanosporolactone **6** was purified by flash CC and TLC. The presence of a lactone was confirmed by IR absorption at 1721 cm^{-1} and by hydrolysis to water-soluble xanosporate **5** anion following brief exposure to 0.05 M NaOH. Longer exposure to alkali led to loss of green color and destruction of **5**. Both **5** and **6** are also rapidly degraded under normal laboratory lighting conditions, but are stable in the dark.

2.2. Spectroscopic analysis of xanosporolactone 6

The HRMS exact mass of 518.1232 agrees with an elemental composition of $C_{28}H_{22}O_{10}$ for xanosporolactone **6** (518.1213 required). This indicates a formal loss of CH_4 from cercosporin **1**, $C_{29}H_{26}O_{10}$, but ^1H NMR spectral analysis indicates loss of one methoxyl. These results can be expalained by an oxidative gain of one oxygen atom and loss of one methoxyl as methanol ($+O–CH_3OH = –CH_4$). Oxidation and loss of one methoxyl have apparently taken place on one side of the C_2 axis of symmetry of **1**, probably in the same methoxyl containing quinonoid ring. As a consequence of loss of the C_2 axis of symmetry, the NMR spectrum of **6** in DMSO-d_6 shows a near doubling of signals compared to that of **1** (Table 1). One set of signals correlates well with those of **1**, and a second set represents the other oxidized half of the xanosporolactone **6** molecule formerly bissected by the C_2 symmetry axis of **1**.

In addition to this complexity, the proton NMR spectrum of xanosporolactone **6** in DMSO clearly shows the presence of two very closely related isomers present in a 2:1 ratio. The proton NMR spectrum of the major isomer in DMSO-d_6 (Table 1) contains 22 hydrogen atoms: two C-methyl doublets, two sets of diastereotopic methylene hydrogens, one methoxyl, two non-equivalent oxymethine hydrogens, one hydroxyl coupled to one of the methine hydrogens, one set of diastereotopic methylenedioxy hydrogens, two non-equivalent aromatic hydrogens, one phenolic hydrogen in a broad peak at 10.6 ppm, and one strongly deshielded phenolic hydrogen bonded to a peri carbonyl at 14.52. All of these signals are duplicated for the minor isomer, isoxanosporolactone **7** and resolved from those of the major isomer with the exception of overlap of the methylenedioxy multiplets and of the broad phenolic signal at 10.6 ppm. Carbon chemical shifts were assigned from HMQC and HMBC correlation spectra. HMBC correlations to all of the chromophore carbon atoms and to the carboxylate carbon are shown in Fig. 1.

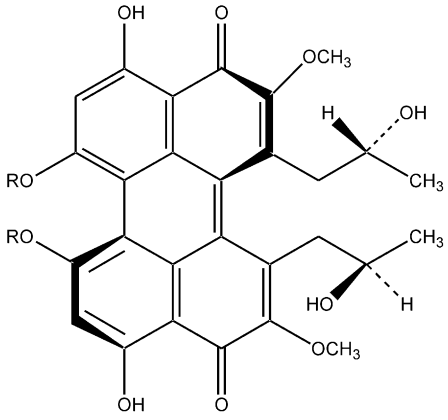
Table 1
Comparison of 500 mHz NMR spectra of cercosporin, **1**, and M-xanosporolactone, **6**

M-Cercosporin, **1** ($CDCl_3$)		M-Xanosporolactone, **6** (DMSO-d_6)	
Position	δH	δH	δC (HMQC)
2-OCH_3	4.01	None	None
4-OH	14.81	10.6, *br s*	
5	7.05	7.15, *s*	108.9
6,7-OCH_2O	5.71	5.69, *s*[a]	91.6
8	(7.05)[b]	6.82, *s*	106.0
9-OH	(14.81)[b]	14.52	
11-OCH3	(4.01)[b]	4.03, *s*	60.0
13	2.88	2.45, *dd*	41.8
	3.38	3.05, *dd*	
14	3.58	3.55, *m*	60.7
14-OH	1.6[c]	4.78, *d*	
15	0.62	0.46, *d*	22.4
16	(2.88)[b]	2.48, *dd*	33.8
17	(3.58)[b]	3.04, *dd*	75.3
17-OH	1.6[c]	None	
18	(0.62)[b]	1.35, *d*	19.6

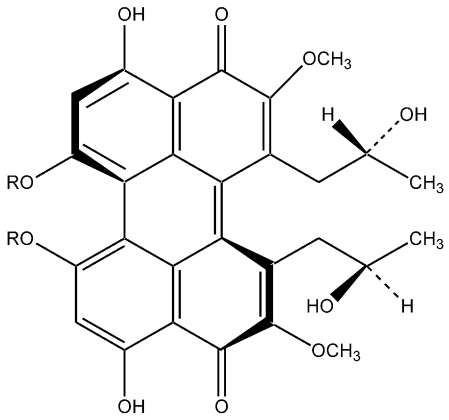
[a] Diasterotopic methylenedioxy hydrogens appear as unresolved singlet.

[b] For cercosporin the chemical shift of each of the second set of protons in parentheses is identical to one of the chemical shifts above by virtue of the C_2 axis of symmetry.

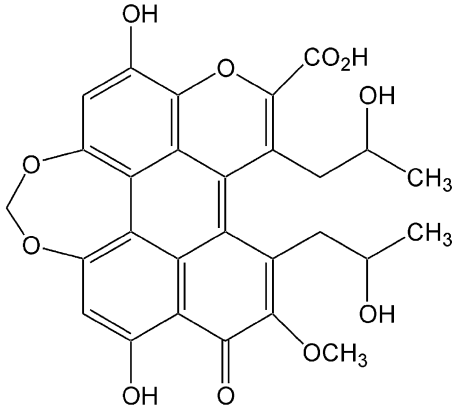
[c] Broad, exchangeable hydrogen signal.



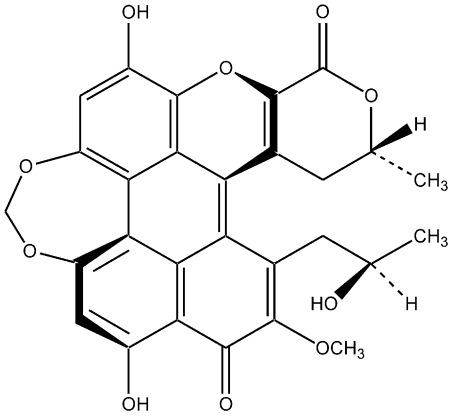
1, M-(+)-Cercosporin: R = R = CH_2
3, M-(+)-Isophleichrome, R = R = CH_3



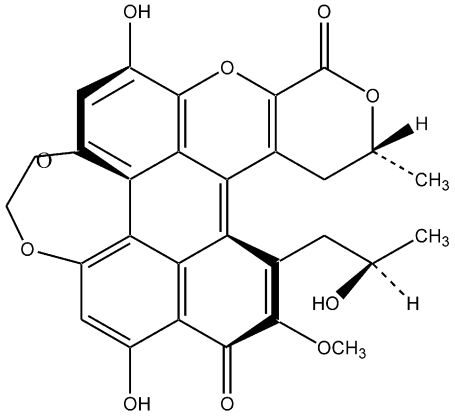
2, P-(-)-Isocercosporin, R = R = CH_2
4, P-(-)-Phleichrome: R = R = CH_3



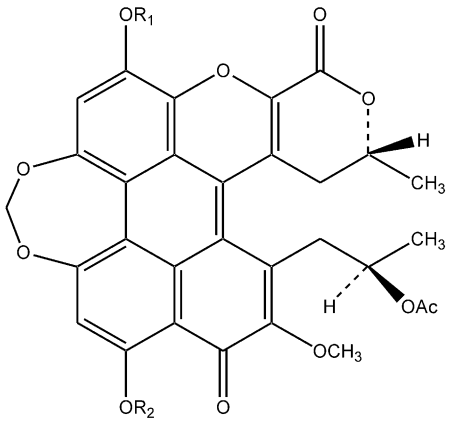
5, Xanosporic acid



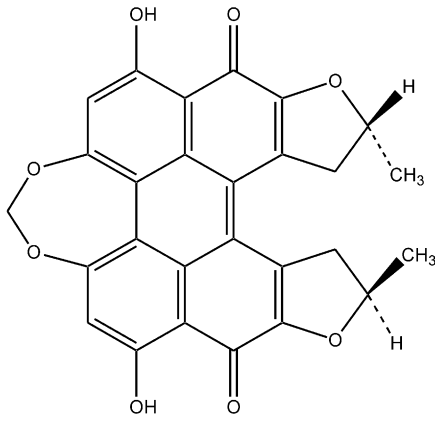
6, M-Xanosporolactone



7, P-isoxanosporolactone



8, R_1 = Ac, R_2 = H
9, R_1 = R_2 = A c
10, R_1 = R_2 = H
11, R_1 = H, R_2 = Ac



12, Bisnoranhydrocercosporin

The infrared spectrum of **6** and **7** affords evidence for the presence of two carbonyls at 1620 and 1721 cm^{-1} attributable to a strongly hydrogen-bonded chromophoric carbonyl and a six-membered lactone. The presence of the lactone is also supported by the 0.99 ppm deshielding of one hydroxypropyl methine-hydrogen and the acid-base extraction behavior of **5** and **6**. In **1** both phenolic hydroxyls are strongly hydrogen bonded to the peri carbonyls with the expected large chemical shift $\delta = 14.81$ ppm. In **6** only one of the phenolic hydroxyls is hydrogen bonded to a carbonyl ($\delta = 14.52$ ppm), while the second phenolic hydroxyl ($\delta = 10.6$, broad) is no longer adjacent to a peri-carbonyl. Given the spectral data and the fact that xanosporolactone **6** and isoxanosporolactone **7** are the products of bacterial catabolism of **1**, the only rational structures are the atropisomeric pair **6** and **7**.

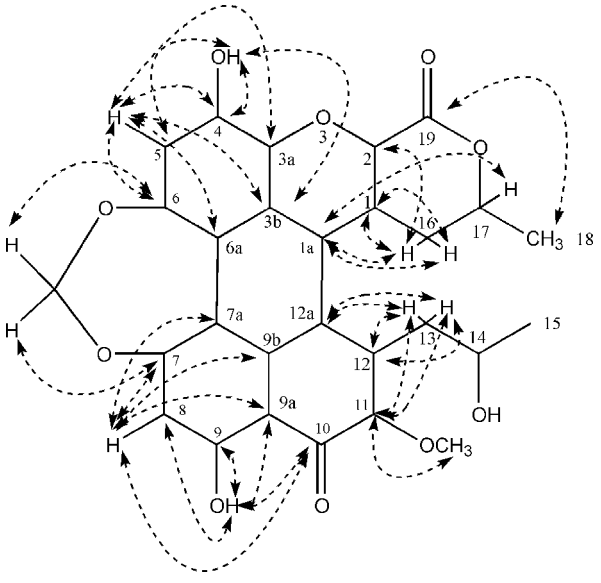


Fig. 1. Multiple bond proton-carbon correlations (HMBC) shown only for carbon atoms of the chromophore of the major isomer xanosporolactone **6**. All expected HMBC correlation to side chain carbon atoms were also observed but are omitted for simplicity.

2.3. *Xanosporolactone acetates **8–11***

Acetylation of xanosporolactone with pyridine and acetic anhydride produced four chromatographically resolvable acetates, in order of decreasing R_f: violet zone **8**, red **9**, green **10**, and violet **11**. Atropisomers of **10** and **11** were barely resolvable on analytical TLC, but resolution was insufficient for preparative separation. Atropisomers of **8** and **9** were unresolved, but NMR spectroscopic analysis showed that both atropisomers were present in approximately 2:1 ratio in all four of the acetates. MS and ^1H NMR spectral data for the major atropisomers are shown in Table 2. Monoacetate **10**, was acetylated only on the aliphatic hydroxyl and thus retained the green color of the unsubstituted chromophore. All four xanosporolactone **8–11** acetates show a

Table 2
Mass spectral and ^1H NMR characterization of xanosporolactone acetates

	8	**9**	**10**	**11**
Color	Violet	Red	Green	Violet
FABMS $\{M+H\}^+$	603	645	561	603
Acetate-CH_3 δH	1.98	1.97	1.96	1.95
	2.46	2.46		2.48
		2.49		
Acetoxymethine δH	4.86	4.88	4.88	4.83
Phenolic OH δH	–	–	8.7. *br*	8.2 *br*
Peri-OH δH	14.36	–	14.21	–

ca. 1 ppm downfield shift of the hydroxypropyl methine proton, indicating that all have an acetoxy propyl substituent. The structures are readily assignable from FAB–MS pseudomolecular ions and from the presence or absence of the two phenolic hydroxyls. If the four **8**–**11** acetates were left on the silica gel for 24–48 h, all zones turned green. Green eluates from all zones then comigrated with monoacetyl xanosporolactone **10** from the original green zone. However, acetates eluted immediately from silica gel were stable for at least several days in chloroform or acetone if stored in the dark.

3. Discussion

3.1. Axial helicity of fungal perylenequinones

The steric bulk of cercosporin (**1**) hydroxypropyl groups at C-1 and C-12 of the chromophore is buttressed by C-2 and C-11 methoxyl groups causing deviation of the perylenequinone chromophore from planarity. Equilibration of the atropisomers **1** and **2** in an approximately 1:1 ratio is achieved in 15 min in refluxing toluene (111 °C) (Yamazaki and Ogawa, 1972). Nasini et al. (1982) obtained isomerization rate constants over the temperature range 76–100 °C from which they derived values of $\Delta H^{\ddagger} = 20.0$ kcal/mol and $\Delta S^{\ddagger} = 20.8$ e.u. The unusually high entropy of activation and stability of the individual atropisomers was attributed to the necessity for simultaneous, coordinated rotation of both hydroxylpropyl side chains to allow them to pass each other during the reversal of the twist. By contrast, bisnoranhydrocercosporin **12**, lacking the buttressing effect of the C-2 and C-11 methoxyl groups and with both hydroxypropyl side chains pulled back into dihydrofuran rings, exists as a single compound whether prepared from **1** or **2** (Yamazaki and Ogawa, 1972). Detailed NMR studies reveal that **12** exists as a 79:21 mixture of (R,R)M and (R,R)P conformations showing aromatic proton NMR signal broadening below −40 °C (Scaglioni et al., 2001).

The large, antipodal circular dichroism (CD) of cercosporin **1**, and isocercosporin **2**, was an early indicator that these thermally interconvertible isomers differed in the handedness of the twist in the perylenequinone chromophore. Definitive evidence that the CD is dominated by the distortion of the chromophore and not by the 14R,17R-hydroxymethine chiral centers was obtained by demonstrating that the 4,9-di-*O*-methyl-14,17-diketo derivative of **1**, lacking any chiral centers, still retained a strong CD spectrum which is the mirror image of the 4,9-di-*O*-methyl-14-17-keto derivative of **2**. The absolute handedness of the chromophoric twist was assigned a M-chirality by X-ray crystallography based on knowledge that **1** has 14R,17R chiral centers (Nasini et al., 1982).

Correlation of the sign of the ellipticity of the CD spectrum of M-(+) cercosporin **1** and its thermally isomerized atropisomer P-(−)-isocercosporin **2**, with handedness of chromophoric twist, was used to assign M- or P-axial chirality to the fungal perylenequinone P-(−)-phleichrome **4** (Arnone et al., 1985), its thermal isomer M-(+)-isophleichrome **3** and the fungal quinones P-*ent*-isophleichrome (Arnone et al., 1988) and M-elsinochromes (Meille et al., 1989; Arnone et al., 1993). The P-axial chirality is also similarly established for the cladochromes and calphostins, all esters of P-*ent*-isophleichrome.

The atropisomers **6** and **7** are barely resolvable on analytical TLC. Preparative separation of these isomers has not been achieved. Therefore circular dichroism was measured on the natural 2:1 mixture of M:P atropisomers (Fig. 2). The molar circular dichroism of the positive CD extrema at 583, 467 and 376 nm and negative extrema at 340 and 289 nm is an order of magnitude less than that reported for twisted perylenequinones, probably due, in part, to the fact that the atropisomeric excess is only about 33%. No inference regarding absolute axial chirality of the dominant atropisomer can be drawn from the circular dichroism data because xanosporolactone **6** is the first example of the oxaperylenone chromophore.

3.2. Assignment of M-chirality to xanosporolactone **6** by NMR

The unoxidized quinonoid ring of **6** bears an unusually shielded hydroxypropyl C-methyl ($\delta = 0.46$) similar to that of **1** ($\delta = 0.55$). The unusual shielding of the C-methyl of **1**, but not that of **2,** is due to a side chain conformation imposed by the M-helicity that forces the methyl group of **1** into the shielding region of the perylenequinone π-system, while P-helicity moves the C-methyl away from the chromophore (Weiss et al., 1987). Investigation of other fungal perylenequinones reveals that in all examples 14R,17R,M- and 14S,17S,P-chirality forces the methyl groups of the hydroxypropyl side chains into the shielding zone while 14S,17S,M- and 14R,17R,P-chirality do not (Table 3). Neither isocercosporin **2** nor isoxanosporin **7** shows unusual shielding of the hydroxypropyl C-methyl. Therefore it is probable that the major isomer xanosporolactone **6** has the same helicity as 14R,17R,M-cercosporin **1** while 14R,17R,P-isocercosporin **2** and isoxanosporolactone **7** have the opposite helicity. An even more pronounced example of shielding afforded by the conformation imposed by 14R,17R,M-chirality (or enantiomeric 14S,17S,P-chirality) is found in the crinoid phenanthroperylenequinone gymnochrome C where the18-C-methyl doublet has a chemical shift of −0.07 ppm (DeRiccardis et al., 1991).

3.3. Bacterial catabolic pathway

Catabolism of **1** by *X. campestris* pv. *zinniae* may be initiated by cytochrome P-450 monooxygenase insertion

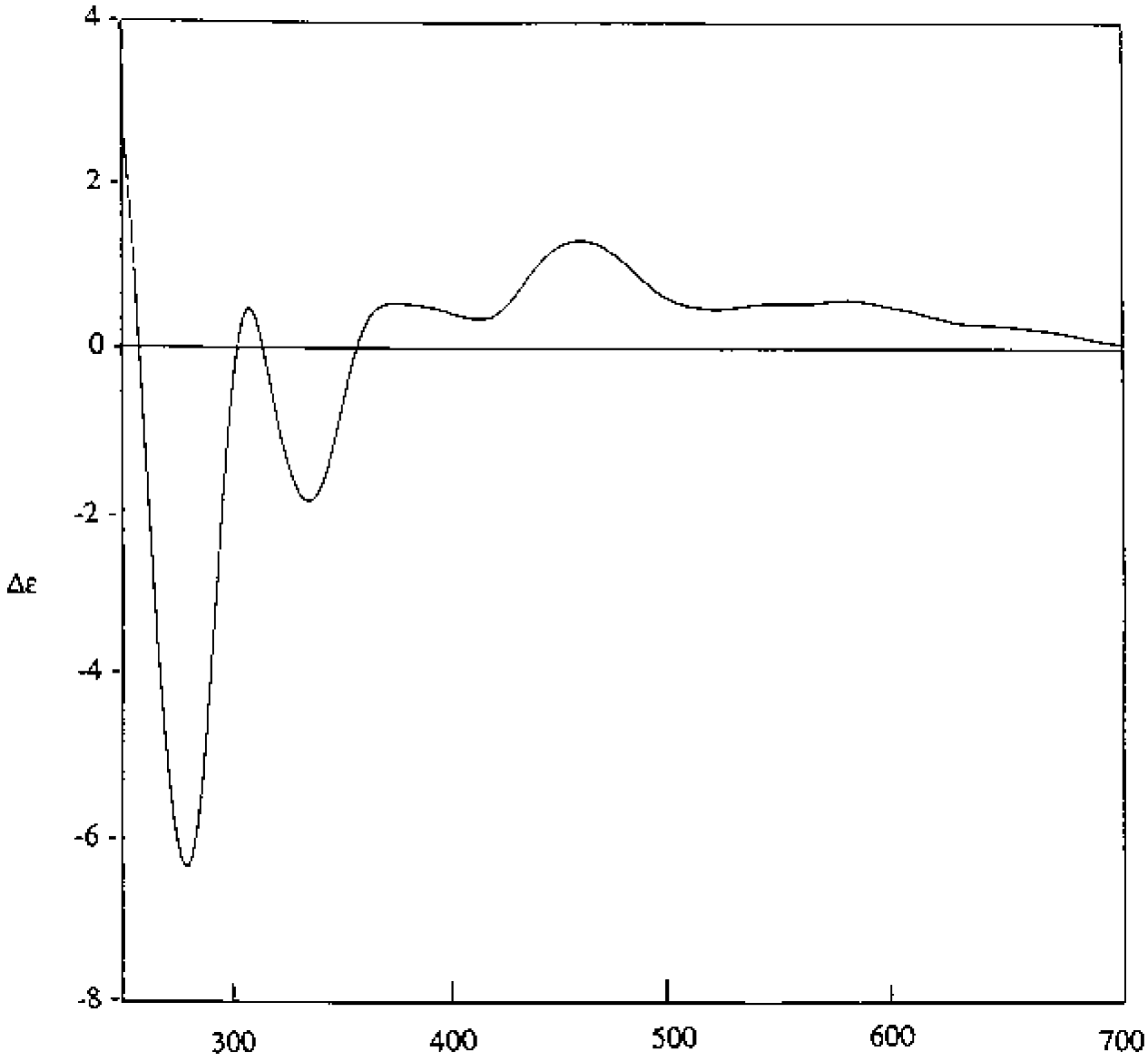


Fig. 2. Circular dichroism of a 2:1 mixture of M-xanosporolactone **6** and P-isoxanosporolactone **7** measured in MeOH solvent.

of an oxygen atom between C-3 and C-3a (Fig. 3), expanding the A ring to a seven-membered unsaturated lactone. Hydrolysis of the A ring lactone may require an esterase. However, in the preparation of acetylated derivatives of xanosporolactone, the extreme ease of deacylation of the phenolic acyl groups was noted. Therefore an esterase may not be required for opening the A ring ester. Once the A-ring is opened, recyclization with the loss of the methoxyl group would probably be rapid and spontaneous. The final lactonization is, of course, an artifact of the isolation procedure. Therefore it is possible that only the oxygen insertion step requires an enzyme, the remaining steps being spontaneous. The insertion of an oxygen atom adjacent to a ketone carbonyl is the well-known Bayer–Villiger reaction of synthetic organic chemistry (Krow, 1993). Precedents for biological oxygen insertions into a carbocyclic ring adjacent to a carbonyl also exist (Casellas et al., 1997). For example, conversion of *O*-methylsterigmatocystin into aflatoxin G_1 and dihydro-*O*-methylsterigmatocystin into aflatoxin G_2 involves a gene having the sequence of a cytochrome P-450 monooxygenase. The cell-free reaction requires a microsomal fraction which would contain such a cytochrome P-450 monooxygenase and is inhibited by the cytochrome P-450 monooxygenase inhibitor SKF-525A (Yabe et al., 1999).

3.4. Loss of atropisomeric homogeneity

In the proposed catabolic pathway (Fig. 3) axial chirality derived from **1** would presumably be lost on hydrolytic cleavage of the seven-membered lactone. However, recyclization of the pyran ring to produce **5**, and subsequently isomers **6** and **7**, would reconstitute

Table 3
Correlation of atropisomers of perylenequinones and related metabolites with chemical shift of C-15 and C-18 methyl groups

Perylenequinones and related compounds	Atropisomer	C-14,17	δ CH_3	Source	Reference
(+)-Cercosporin, **1**	M	R,R	0.55[a]	Nat. prod.	Yamazaki and Ogawa (1972)
(−)-Phleichrome, **4**	P	S,S	0.53[a]	Nat. prod.	Arnone et al. (1985)
(−)-Isocercosporin, **2**	P	R,R	0.99	Isomerization	Yamazaki and Ogawa, (1972)
(+)-Isocercosporin	M	S,S	0.97	Nat. prod.	Tabuchi et al. (1991)
(+)-Isophleichrome, **3**	M	S,S	0.95	isomerization	Arnone et al. (1985)
(−)-Isophleichrome	P	R,R	0.95	Nat. prod.	Arnone, et al. (1988, 1989)
Xanosporolactone, **6**	M	R,R	0.46[a]	Nat. prod.	This work
Isoxanosporolactone, **7**	P	R,R	1.07	Nat. prod.	This work
Gymnochrome c[b]	P	S,S	−0.07[a]	Nat. prod.	DeRiccardis et al. (1991)

[a] Unusually shielded C-methyl groups.
[b] Phenanthroperylenequinone.

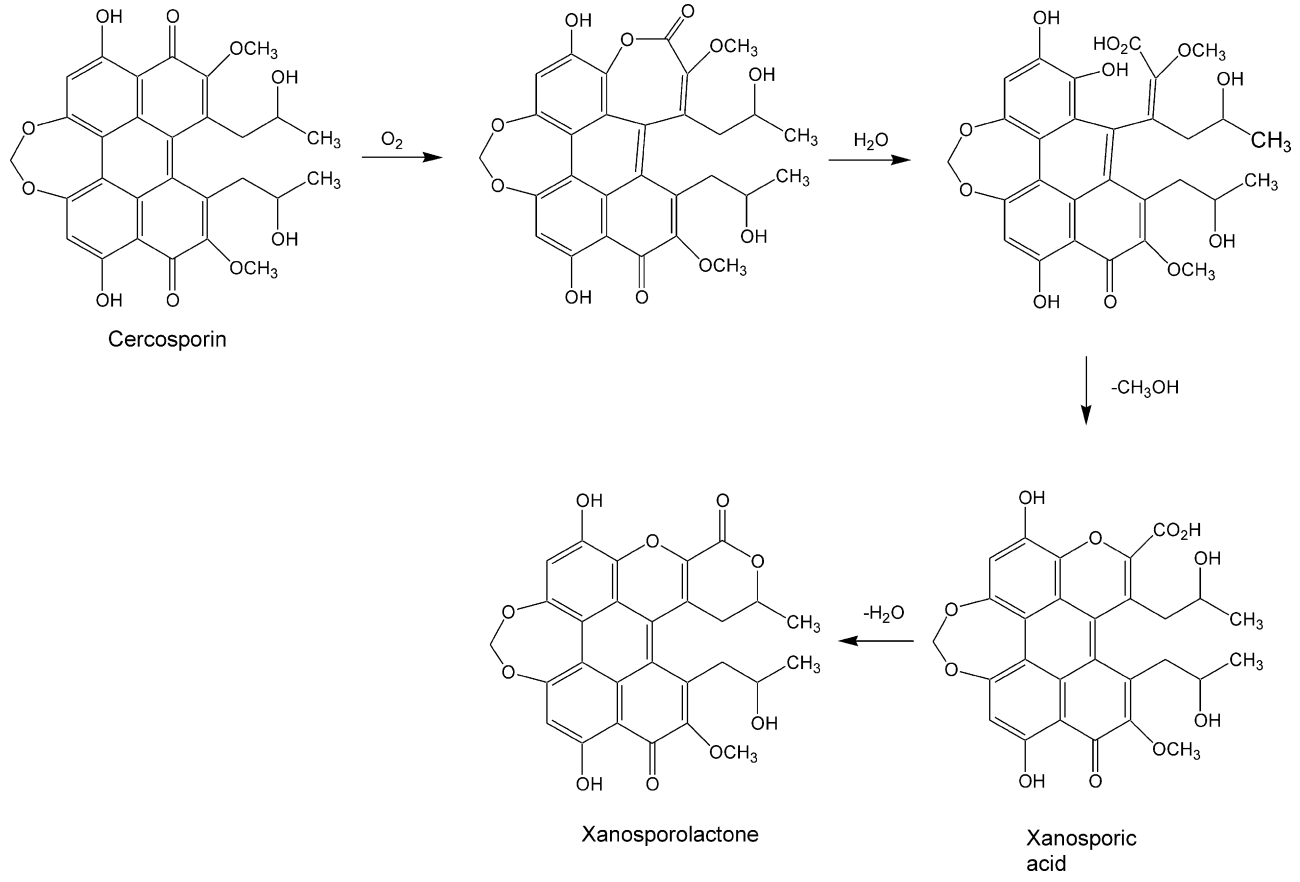


Fig. 3. Proposed pathway of oxidative transformation of cercosporin **1** into xanosporic acid **5** and xanosporolactone **6**.

the oxo-analog of the quinonoid ring disrupted by oxidation and hydrolysis. The closure of the pyran ring in place of the quinonoid ring of **1** reestablishes similar steric interactions to those that stabilize the twist in **1**. The 68:32 M-xanosporolactone: P-isoxanosporolactone ratio may be the result of either kinetic control of pyran ring closure or atropisomeric equilibration after closure of the pyran ring. The ratio of C-methyl signal areas of M-xanosporolactone: P-isoxanosporolactone was essentially unchanged (65:35) after heating in toluene at 111 °C for 70 min. By comparison, **1** is isomerized to a 50:50 equilibrium mixture of cercosporin (**1**):isocercosporin (**2**) after heating 15 min in refluxing toluene. The failure to detect any change in the isomer ratio of the xanosporolactones may be because the barrier to isomerization is low, and they are already equilibrated

before isolation, or because the barrier is much higher than that for isomerization of **1**. A higher barrier seems less likely because one of the bulky hydroxypropyl groups of **1** is pulled back in **6** and **7** by formation of the 6-membered lactone ring. Incorporation of both hydroxypropyl group of **1** into fused dihydrofuran rings in bisnoranhydrocercosporin, **12** reduces steric bulk of the hydroxypropyl substituents and abolishes detectable twist isomerism in **12** (Yamazaki and Ogawa, 1972; Scaglioni et al., 2001).

4. Experimental

4.1. Instrumentation

All of the NMR experiments were performed on a Bruker AVANCE 500 MHz spectrometer (1996) with Oxford narrow bore magnet (1989). The NMR probe was tuned to ^{1}H frequency 500.128 MHz. Homonuclear ^{1}H and heteronuclear ^{1}H–^{13} C correlated NMR methods applied to study the structure of xanosporolactone were (^{1}H–^{1}H)-Correlation Spectroscopy (COSY), heteronuclear multiple quantum coherence experiment (HMQC, ^{1}H–^{13}C one bond correlation), heteronuclear multiple bond correlation (HMBC, ^{1}H-^{13}C long range correlation). All spectra were acquired at 298 K. $CDCl_3$ and DMSO-d_6 were used as solvents. TMS was used as internal standard. The standard instrumental parameters for acquisition of the gradient two-dimensional 2D COSY, 2D HMQC and 2D HMBC were used. Data were processed with Bruker software XWINMR 2.6 and standard processing parameters.

High resolution mass spectra were measured with direct probe fast atom bombardment (FAB) on a JEOL HX110HF mass spectrometer in the Mass Spectrometry Laboratory for Biotechnology Research, Department of Chemistry, North Carolina State University. Circular dichroism was measured on a JASCO 600 CD spectrometer.

4.2. Cercosporin production

Cercosporin (**1**) was isolated from *C. kikuchii* grown on potato dextrose agar as previously described (Daub, 1982). The red pigment was extracted from mycelia on dried agar with EtOAc and crystallized from $CHCl_3$–hexane.

4.3. Preparation of xanosporolactones *6* and *7* from cercosporin (*1*)

X. campestris pv *zinniae* strain XCZ-3 was maintained by weekly transfer onto Luria agar. Bacterial oxidation of **1** to **5** was carried out in the dark at 26 °C in 1 l, of Luria broth in 2-l Fernbach flasks rotated at 150 rpm for aeration. Cercosporin **1** (25 mg) was dissolved in minimal 0.025 M NaOH solution and added dropwise to 1 l of medium with vigorous swirling. The green anion of cercosporin **1** immediately turned red. Buffering capacity maintained the uninoculated medium at pH 7.0. Each flask was inoculated with one sixth of a fresh petri plate culture of *X. campestris*. The Fernbach flasks were incubated in the dark at 25–28 °C and 150 rpm to provide aeration. After 36 h the red culture medium began turning brown. At about 48 h the medium was green. Green medium was harvested between 48 and 60 h. At that time the yield of **5** from **1** was 99% determined by measuring the optical density of a butanolic extract at $\lambda_{max} = 580$ nm. After that time the green color began to fade.

The pH 8.5 culture medium was centrifuged to remove bacteria, extracted 2× with $CHCl_3$ to remove any residual **1**, adjusted to pH 2 and again extracted 2×with $CHCl_3$ to remove **6** and **7** formed from **5** at pH 2. The crude mixture of lactones **6** and **7**, recovered from the $CHCl_3$ extract, was further purified on a silica gel (Whatman, 70–230 mesh) flash column using hexane:EtOAc:MeOH 43:55:2 for elution followed by prep. TLC (hexane:EtOAc:MeOH 59:34:7). The isolation yield of unresolved amorphous atropisomers **6** and **7** was 60% of **1** supplied in the bacterial growth medium. Purity of the amorphous, mixed xanosporolactone atropisomers was verified by Si-gel (C_6H_{12}:EtOAc:-MeOH 59:34:7), which barely resolves the atropisomers **6** (R_f 0.44) and **7** (R_f 0.47), The analytical TLC was developed with phosphomolybdic acid to check purity of the xanosporolactone.

Xanosporolactone **6**/**7** UV (MeOH) λ_{max}, nm (log ε), 445 (3.5), 580 (3.3). CD (JASCO-600, MeOH) λ nm (Δε): 279 (−6.3), 306 (+0.5), 333 (−1.8), 369 (+0.5), 410 (+0.2), 458 (+1.3), 581 sh (+0.5); IR (KBr) ν_{max}, cm^{-1}: 1721 (lactone CO). HR-FAB–MS [M$^+$] *m*/*z* 518.1232 (calc. for $C_{28}H_{22}O_{10}$, 518.1213). For ^{1}H NMR and HMQC carbon assignments, see Table 1; ^{13}C NMR chemical shifts from HMQC: 109.2 (C-7a), 109.2, (C-6a), 109.9 (C-9a), 116.1 (C-9b), 120.5 (C-12a), 124.8 (C-1a), 133.6 (C-3b), 136.0 (C-12), 140.6 (C-1), 141.7 (C-3a), 141.9 (C-4), 150.0 (C-6),150.4 (C-11), 158.8 (C-2), 159.3 (C-19), 162.8 (C-7), 163.5 (C-9), 181.4 (C-10).

4.4. Xanosporolactone acetates *8–11*

Purified xanosporolactone (20 mg) was acetylated with pyridine-acetic anhydride. Excess acetic anhydride was destroyed with ice water. The acetates were extracted into $CHCl_3$ which was back extracted several times with water to remove pyridine. Analytical TLC indicated the presence of several acetates which were separated on preparative SiO_2 TLC developed with hexane:acetone 6:4.

4.4.1. 4,14-di-O-Acetylxanosporolactone, ***8***

Amorphous, violet solid, 5 mg, TLC (Si gel, C_6H_{12}:Me_2CO 6:4) R_f 0.68; FAB–MS, *m*-nitrobenzoic acid, $[M+H]^+ = m/z$ 603; ^1H NMR (500 MHz, $CDCl_3$): δ 0.63 (*d*, H-15), 1.49 (*d*, H-18), 1,98 (*s*, 14-acetoxy), 2.47 (*dd*, H-16a), 2.48 (*s*, 4-acetoxy), 2.50 (*dd*, H-13a), 3.02 (*dd*, H-16b), 3.63 (*dd*, H-13b), 4.47 (*m*, H-17), 4.86 (*m*, H-14), 5.62 and 5.66 (*d*, 6,7-OCH_2O), 6.91 (*s*, H-8), 7,28 (*s*, H-5), 14.36 (*s*, 9-OH); ^{13}C NMR chemical shifts (HMBC, HMQC, $CDCl_3$): δ 19.5 (C-15), 20.7 (4-acetoxy CH_3), 20.9 (C-18), 21.33 (14-acetoxy CH_3), 34.9 (C-16), 38.5 (C-13), 70.1 (C-14), 76.0 (C-17), 92.3 (6,7-OCH_2O–), 109.9 (C-9a), 115.3 (C-5), 116.3 (C-6a), 122.4 (C-12a), 125.3 (C-1a), 131.6 (C-12), 134.6 (C-3b), 137.6 (C-3a), 140.1 (C-1), 150.5 (C-6), 152.0 (C-11), 152.1 (C-4), 164.4 (C-7), 108.1 (C-8), 165.8 (C-9), 168.5 (4-acetoxy CO), 170.2 (14-acetoxy CO).

4.4.2. 4,9,14-tri-O-Acetylxanosporolactone, ***9***

Amorphous, red solid, 7 mg. TLC (Si gel, C_6H_{12}:Me_2CO 6:4) R_f 0.64; FAB–MS, *m*-nitrobenzoic acid, $[M+H]^+$ 645. ^1H NMR (500 MHz, $CDCl_3$): δ 0.72 (*d*, H-15), 1.46 (*d*, H-18), 1.97 (*s*, 14-acetoxy), 2.39 (*dd*, H-16a), 2.46 (*s*, 9-acetoxy), 2.49 (*s*, 4-acetoxy), 2.51 (*dd*, H-13a), 2.95 (*dd*, H-16b), 3.48 (*dd*, H-13b), 4.05 (*s*, 11-OCH_3), 4.48 (*m*, H-17), 4.89 (*m*, H-14), 5.58 and 5.69 (*d*, 6,7-OCH_2O), 7.05 (*s*, H-8), 7.25 (*s*, H-5).

4.4.3. 14-O-Acetylxanosporolactone, ***10***

Amorphous, green solid, 5 mg. TLC (Si gel, C_6H_{12}:Me_2CO 6:4) R_f 0.49;. UV (MeOH) λ_{max}, nm: 268, 447, 577; FAB–MS, *m*-nitrobenzoic acid $[M+H]^+$ 561. ^1H NMR spectral data (500 MHz, $CDCl_3$): δ 0.69 (*d*, H-15), 1.47 (*d*, H-18), 1.96 (*s*, 14-acetoxy), 2.45 (*dd*, H-16a) 2.50 (*dd*, H-13a), 3.06 (*dd*, H-16b), 3.64 (*dd*, H-13b), 4.19 (*s*, 11-OCH_3), 4.54 (*m*, H-17), 4.88 (*m*, H-14), 5.58 and 5.64 (*d*, 6,7-OCH_2O), 6.86 (*s*, H-8), 7.16 (*s*, H-5), 8.7 (*br s*, 4-OH), 14.21 (*s*, 9-OH); ^{13}C chemical shifts (HMBC, HMQC, $CDCl_3$): δ 18.2 (C-15), 20.1 (C-18), 21.3 (14-acetoxy CH_3), 34.7 (C-16), 38.6 (C-13), 61.0 (11-OCH_3), 70.3 (C-14), 76.9 (C-17), 92.1 (6,7-OCH_2O), 108.1 (C-8), 110.4 (C-5), 151.7 (C-6), 152.3 (C-11), 164.4 (C-7), 165.6 (C-9), 170.5 (14- acetoxy CO).

4.4.4. 9,14-di-O-Acetylxanosporolactone, ***11***

Violet, amorphous solid, 2 mg. TLC (Si gel, C_6H_{12}:Me_2CO 6:4) R_f 0.42; UV (MeOH) λ_{max}, nm: 262, 401, 535 nm. FAB–MS, *m*-nitrobenzoic acid, $[M+H]^+ = m/z$ 603; ^1H NMR spectral data (500 MHz, $CDCl_3$): δ 0.75 (*d*, H-15), 1.48 (*d*, H-18), 1.95 (*s*, 14-acetoxy), 2.34 (*dd*, H-13a), 2.47 (*dd*, H-16a), 2.48 (*s*, 9-acetoxy), 2.96 (*dd*, H-16b), 3.48 (*dd*, H-13b), 4.04 (*s*, 11-OCH_3), 4.54 (*m*, H-17), 4.83 (*m*, H-14), 5.52 and 5.65 (*d*, 6,7-OCH_2O), 6.99 (*s*, H-8), 7.09 (*s*, H-5), 8.2 (*br s*, 4-OH). ^{13}C NMR spectral data (HMBC, HMQC, $CDCl_3$): δ 19.4 (C-15), 20.3 (C-18), 21.3 (9-acetoxy CH_3), 21.2 (14-acetoxy CH_3), 32.8 (C-16), 37.8 (C-13), 60.3 (11-OCH_3), 70.2 (C-14), 76.7 (C-17), 92.2 (7-OCH_2O), 109.7 (C-5), 114.6 (C-8) 152.1 (C-6), 152.4 (C-11), 162.0 (C-7), 169.3 (9-acetoxy CO), 169.6 (14-acetoxy CO).

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References

Arnone, A., Camarda, L., Nasini, G., 1985. Secondary mould metabolites. Part 13. Fungal perylenequinones: phleichrome, isophleichrome and their endoperoxides. Journal of the Chemical Society, Perkin Transactions 1, 1387–1392.

Arnone, A., Assante, G., Di Modugno, V., Merlini, L., Nasini, G., 1988. Perylenequinones from cucumber seedlings infected with *Cladosporium cucumerinum*. Phytochemistry 27, 1675–1678.

Arnone, A., Assante, G., Merlini, L., Nasini, G., 1989. Structure and stereochemistry of cladochrome D and E, novel perylenequinone pigments. Gazzetta Chimica Italiana 119, 557–559.

Arnone, A., Merlini, L., Mondelli, R., Nasini, G., Ragg, E., Scaglioni, L., 1993. Structure, conformational analysis and absolute configuration of the perylenequinone pigments elsinochromes B_1, B_2, C_1 and C_2. Gazzetta Chimica Italiana 123, 121–126.

Assante, G., Locci, R., Camarda, L., Merlini, L., Nasini, G., 1977. Screening of the genus *Cercospora* for secondary metabolites. Phytochemistry 16, 243–247.

Calpouzos, L., Stallknecht, G.F., 1967. Symptoms of *Cercospora* leaf spot of sugar beets influenced by light intensity. Phytopathology 57, 799–800.

Casellas, M., Grifoll, M., Bayona, J.M., Solanas, A.M., 1997. New metabolites in the degradation of fluorene by *Arthrobacter* sp. strain F101. Applied and Environmental Bacteriology 63, 819–826.

Daub, M.E., 1982. Cercosporin. A photosensitizing toxin from *Cercospora* spp. Phytopathology 72, 370–374.

Daub, M.E., Ehrenschaft, M., 2000. The photoactivated *Cercospora* toxin cercosporin: contributions to plant disease and fundamental biology. Annual Review of Phytopathology 38, 461–490.

DeRiccardis, F., Iorizzi, M., Minale, L., Riccio, R., Richer de Forges, B., Debitus, C., 1991. The Gymnochromes: novel brominated phenanthroperylenequinone pigments from the stalked crinoid *Gymnocrinus richeri*. Journal of Organic Chemistry 56, 6781–6787.

Kishi, T., Tahara, S., Taniguchi, N., Tsuda, M., Tanaka, C., Takahashi, S., 1991. New perylenequinones from *Shiraia bambusicola*. Planta Medica 57, 376–379.

Kobayashi, E., Nakano, H., Morimoto, M., Tamaoki, T., 1989. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochemical Biophysical Research Communications 159, 548–553.

Krow, G.R., 1993. The Baeyer Villiger Reaction. Organic Reactions 43, 251–798.

Kuyama, S., Tamura, T., 1957. Cercosporin. II. Physical and chemical properties of cercosporin and its derivatives. Journal of the American Chemical Society 79, 5726–5729.

Lousberg, J. J. Ch., Weiss, U., Saleminck, C.A., Arnone, A., Merlini,L., Nasini, G., 1971. The structure of cercosporin, a naturally occurring quinone. Chemical Communications 1463–1164.

Mazzini, S., Merlini, L., Mondelli, R., Scaglioni, L., 2001. Conformation and tautomerism of hypocrellins. Revised structure of shiraiachrome A. Journal of the Chemical Society, Perkin Transactions 2, 409–416.

Meille, S., Malpezzi, L., Allegra, G., Nasini, G., 1989. Structure of elsinochrome A: a perylenequinone metabolite. Acta Crystallographica C45, 628–632.

Mitchell, T.K., Chilton, W.S., Daub, M.E., 2002. Biodegradation of the polyketide toxin cercosporin. Applied and Enivronmental Microbiology 68, 4173–4181.

Nasini, G., Merlini, L., Andreetti, G.D., Bocelli, G., Sgarabotto, P., 1982. Stereochemistry of cercosporin. Tetrahedron 38, 2787–2796.

Scaglioni, L., Mazzini, S., Mondelli, Merlini, L., Ragg, E., Nasini, G., 2001. Conformational and thermodynamical study of some helical perylenequinones. Journal of the Chemical Society, Perkin Transactions 2, 2276–2286.

Tabuchi, H., Tajimi, A., Ichihara, A., 1991. (+)-Isophleichrome, a phytotoxic compound isolated from *Scolecotrichum graminis* Fuckel. Agricultural and Biological Chemistry 55, 2675–2676.

Thomson, R.H., 1997. Naturally Occurring Quinones IV. Chapman and Hall, London. pp. 553–566.

Upchurch, R.G., Walker, D.L., Rollins, J.A., Ehrenshaft, M., Daub, M.E., 1991. Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. Applied and Environmental Microbiolog 57, 2940–2945.

Weiss, U., Merlini, L., Nasini, G., 1987. Naturally Occurring Perylenequinones. Progress in the Chemistry of Natural Products 52, 43.

Wu, H., Lao, X.-F., Wang, Q.-W., Lu, R.R., Shen, C., Zhang, F., Liu, M., Jia, L., 1989. The Shiraiachromes, novel fungal perylenequinone pigments from *Shiraia bambusicola*. Journal of Natural Products 52, 448–951.

Yabe, K., Nakamura, M., Hamasaki, T., 1999. Enzymatic formation of G-group aflatoxins and biosynthetic relationship between G- and B-group aflatoxins. Applied and Environmental Microbiology 65, 3867–3872.

Yamazaki, S., Ogawa, T., 1972. The chemistry and stereochemistry of cercosporin. Agricultural and Biological Chemistry 36, 1707–1718.

Yoshihara, T., Shimanuki, T., Araki, T., Sakamura, S., 1975. Phleichrome, a new phytotoxic compound produced by *Cladosporium phlei*. Agricultural and Biological Chemistry 39, 1683–1684.