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Perylenequinones Act as Broad-Spectrum Fungicides by Generating Reactive Oxygen Species both in the Dark and in the Light

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Natural perylenequinonoid pigments (PQPs) have long been known as excellent photosensitizers. Here it is reported that PQPs, when dispersed into a micellar system, are unexpectedly very effective in inhibiting the growth of various fungi in the dark and that light can enhance the activity further. In both cases, reactive oxygen species (ROS) play a crucial role. The unique ROS-based antifungal mechanism and the excellent physicochemical properties of PQPs promise the advent of a new generation of wide-spectrum agrofungicides.

KEYWORDS: Antifungal agents; reactive oxygen species; photosensitizer; perylenequinonoid pigments

INTRODUCTION

The desire for safer agrochemicals with lower environmental and mammalian toxicity is a major concern in modern society. Due to the continuing evolution of microbial resistance in medicine and agriculture, finding new antimicrobial compound is a key objective of modern life sciences. Naturally occurring perylenequinonoid pigments (PQPs), including hypocrellins A and B (HA and HB), cercosporin (CP), and elsinochromes A−C (EA, EB, and EC), hypericin (**Figure 1**), have long been known as excellent photosensitizers (*1*−*8*). They possess many attractive properties as potent photodynamic medicines, such as (i) high yields of reactive oxygen species (ROS), that is, superoxide anion radical ($O_2^{\bullet -}$) and singlet oxygen ($^1O_2$); (ii) low toxicity, for instance, natural products containing HA and HB have been used as folk medicine in China over hundreds of years; (iii) high metabolic rate in the human body; and (iv) high thermo- and photostabilities. Here we report that PQPs, when dispersed into a micellar system, are unexpectedly very effective in inhibiting the growth of various fungi in the dark and that light can enhance the activity further. In both cases, ROS play a crucial role. The unique ROS-based antifungal mechanism and the excellent physicochemical properties of PQPs not only promise the advent of a new generation of wide-spectrum agrofungicides but also open the way to new strategies for microbial control.

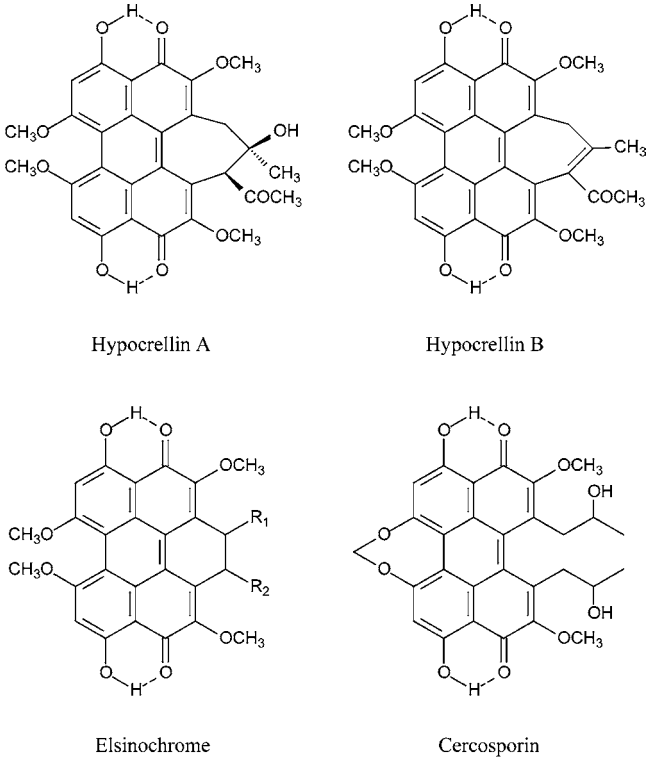

Figure 1. Molecular structures of some typical PQPs.

MATERIALS AND METHODS

Materials. PQPs employed in this study were extracted from the fermentation products of Ascomycetes *Hypomyces* (Fr) Tul. sp., a parasitic fungus growing in the southwestern mountains of Yunnan Province of the People's Republic of China, and were purified by column chromatography and recrystallization (*13*, *14*). The purity of PQPs is >98%.

D_2O, 1,4-diazabicyclo[2.2.2]octane (DABCO), superoxide dismutase (SOD), and Triton X-100 were purchased from Sigma Chemical Co.

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Table 1. Comparison of EC_{50} Values (Micromolar) of HA and Hexaconazole To Inhibit Mycelial Growth for Five Fungi

fungus	HA		hexaconazole
	in dark	in light (3000 lx)	
Alternaria mali	2.44	1.90	10.80
Physalospora piricola	1.25	0.95	6.40
Glomerella cingulata	3.92	2.66	2.80
Fusarium oxysporum	1.26	1.10	18.82
Botrytis cinerea	1.36	0.40	1.03

(St. Louis, MO). Nitrotetrazolium blue chloride (NBT) was purchased from Shanghai Reagent Co., China. Other solvents were of analytical grade.

Hexaconazole [(*RS*)-2-(2,4-dichlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-hexa-2-ol] and the pathogenic fungus specimen were by courtesy of the National Pesticide Engineering Research Center, Nankai Univiersity.

Preparation of PQP Micellar Solution. First, Triton X-100 surfactant was dissolved in water to prepare the micellar solution. Second, PQP was dissolved by chloroform, and then chloroform was evaporated to give dispersed PQP, which was mixed with Triton X-100 micellar solution, and the mixture was stirred for ~40 min at 40 °C to give a transparent PQP micellar solution.

Superoxide Anion Yield Determination. Superoxide anion was chemically trapped by NBT, and the unique absorption band of reduced NBT at 560 nm indicates the formation of superoxide anion. In the present study, the absorption spectrum was measured in a UV−vis spectrophotometer (Unico UV-2102 PC, slot width = 2 nm). The absorbance at 560 nm was used to measure the relative superoxide anion yield.

Determination of Fungus-Inhibiting Rate. The routine method to evaluate the antifungal activity can be found elsewhere (*15, 16*). The PQP micellar solution was mixed with PSA culture medium (1:9, v/v). Fungi grown on PSA without PQP and any other reagents were used as a control group.

The fungus-inhibiting rate was calculated as

$$\text{inhibiting rate} = (\Phi_{\text{control}} - \Phi_{\text{exptl}})/\Phi_{\text{control}} \times 100\%$$

where Φ_{control} is the diameter of the fungus colony grown on the PSA medium without PQP and any other reagents and Φ_{exptl} is the diameter of the inhibition zone of the fungi after PQP and various reagents were added into the PSA medium.

All of the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

PQPs' photoactive antimicrobial activity was discovered in 1957 (*9*). However, little progress was made to improve their activity further and to bring them into the market (*10*, *11*). In an effort to measure PQPs' antifungal potential by the routine assays, we found by chance that PQPs were very effective in inhibiting the mycelial growth of tens of fungi in the dark in a dose-dependent manner. As indicated in **Table 1**, the in-dark 50% efficient concentration (EC_{50}) of HA is comparable with that of hexaconazole, a prevailing chemical fungicide. In addition, the activity can be efficiently enhanced by light (**Table 1**). As the activities of EA, EB, and EC are very similar to that of HA (**Table 2**), PQPs likely represent a new type of wide-spectrum fungicide and have great potential to overcome the existing resistance to currently used agrochemicals. However, this was missed during the past half-century. Because the side rings or chains of tested PQPs are much different, the similar antifungal activities imply that the common structure in PQPs, the perylenequinone ring, would be the active center for both action mechanisms.

Table 2. Comparison of In-Light-Fungus-Inhibiting Rates of HA, EA, EB, and EC (16.5 μM/mL) on *A. mali*

	HA	EA	EB	EC
fungus-inhibiting rate (%)	71.8 ± 1.18	71.6 ± 0.59	72.7 ± 0.00	72.4 ± 1.41

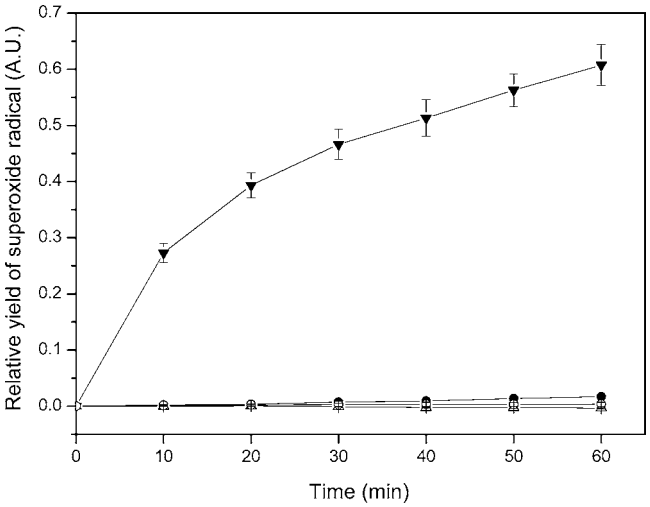

Figure 2. Relative $O_2^{\bullet-}$ yield of HA (50 μM) in the dark, trapped by NBT (56 μM): (○) acetone solution; (△) Trition X-100 micellar solution; (●) acetone solution with potato decoction (4:1 v/v); (▼) Trition X-100 micellar solution with potato decoction (4:1 v/v).

It is obviously very interesting to investigate why the present in-dark antimicrobial activities of PQPs are much higher than those previously reported (*9*−*11*). The only difference between the methodologies is that ethanol was used to dissolve the pigments in previous studies (*9*−*11*), whereas the Triton X-100 micelle is employed in the present research. Because the Triton X-100 micelle has no toxicity on the tested fungi, the enhanced activity of PQPs must result from a kind of collaborative effect between PQPs and Triton X-100. In view of the fact that a nonionic micelle could drastically lower the oxidation potential of dispersed PQPs (*6*) and reductants could induce PQPs to generate $O_2^{\bullet-}$ in the dark (*12*), it is speculated that the enhanced antifungal activity of PQPs results from the higher yield of $O_2^{\bullet-}$ in micellar system than in organic solution, with the presence of vitamin C and polyphenols (components of potato decoction in PSA culture medium) as reductants. This is verified by following chemical and biological experiments. As shown in **Figure 2**, there is no $O_2^{\bullet-}$ trapped by NBT in organic and micellar systems without reductant. However, whereas the potato decoction is added in both systems, $O_2^{\bullet-}$ is produced, and the micellar system is indeed much more efficient in bringing about $O_2^{\bullet-}$ than the organic solution. In addition, superoxide dismutase (SOD) suppresses the in-dark-fungus-inhibiting activity to a certain extent, and strong reductant (cysteine) enhances the activity clearly (**Figure 3a**,**b**), which provides solid evidence to support the assumption that $O_2^{\bullet-}$ is involved in the in-dark action mechanism of PQPs.

In view of the photosensitizing property of PQPs, their in-light-fungus-inhibiting mechanism should involve photogenerated $O_2^{\bullet-}$ and $^1O_2$. The role of the former ROS was confirmed by repeating the above procedure (**Figure 3a**,**b**), which meant that PQPs' activity would be controlled conveniently by regulating the dosage of reductants. The role of the latter ROS

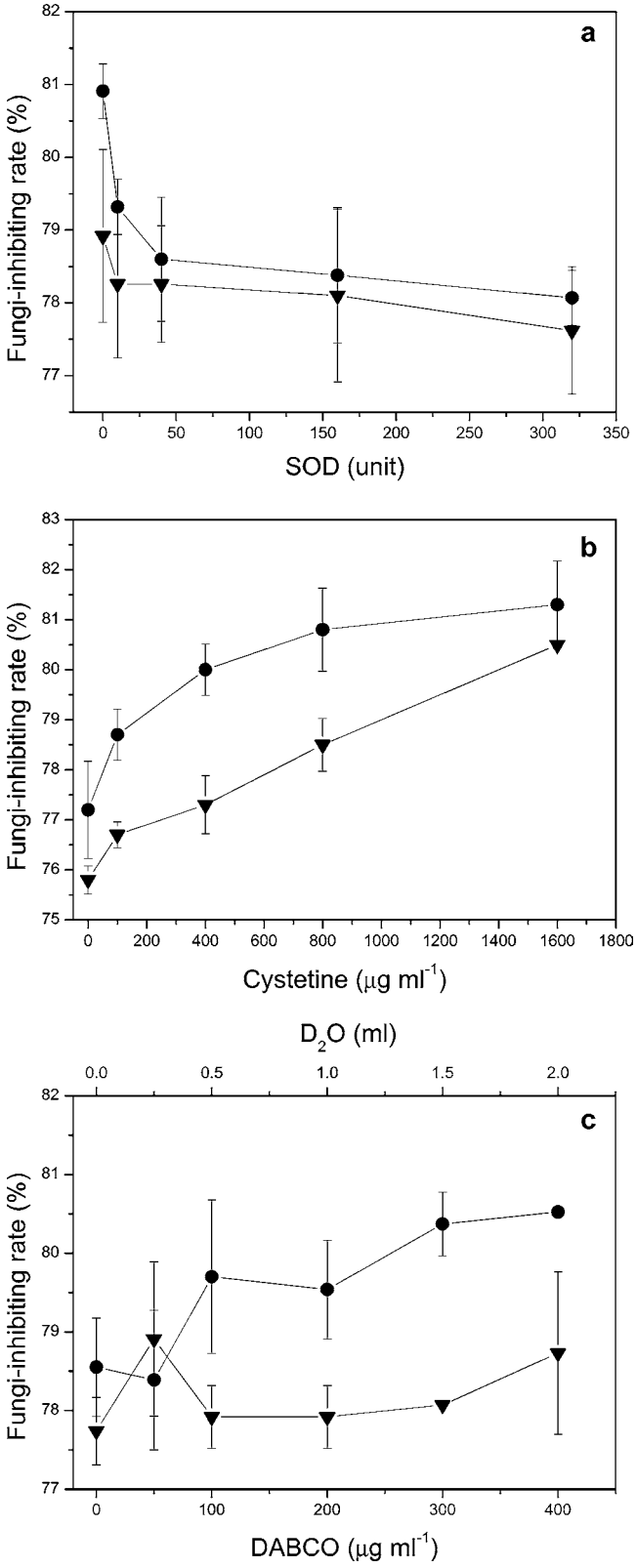

Figure 3. Role of ROS in antifungal activity of HA: (**a**) SOD effect on antifungal activity of HA in the dark (▼) and in light (●, 3000 lx); (**b**) cysteine effect on antifungal activity of HA in the dark (▼) and in light (●, 3000 lx); (**c**) D_2O (●) and DABCO (▼) effects on antifungal activity of HA in light (3000 lx). *Alternaria mali* was employed in the test. All of the experiments were carried out in triplicate.

was explored by adding D_2O or DABCO in the culture medium, which were known to prolong the lifetime of $^1O_2$ or scavenge $^1O_2$, respectively. Although the activity of HA was little influenced by DABCO, it was evidently enhanced by D_2O (**Figure 3c**). Hence, $^1O_2$ should also be involved in the in-light antifungal process.

In summary, the excellent properties of PQPs meet almost all of the requirements for an ideal agrofungicide. Moreover, the unique ROS-based antifungal mode not only results in PQPs' broad fungus-inhibiting spectrum but also has the advantage of multiplying action targets on the microbe and, thus, is promising to delay the resistance of fungi. The potential of PQPs as agrofungicides has been verified further by a field test that HA, EA, and reductants dispersed in Triton X-100 micelle (25−33 μM) are very effective in controlling watermelon leaf spot and pumpkin, balsam pear, and grape downy mildew without damage on foliage. As the mass production of PQPs, for example, HA, HB, EA, EB, and EC, is feasible by means of fermentation (*13*, *14*), our serendipitous discovery likely opens the door to a new generation of agrofungicides and to new strategies for microbial control as well.

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