Photochemistry of Phytoalexins Containing Phenalenone-like Chromophores: Photophysica and Singlet Oxygen Photosensitizing Properties of the Plant Oxoaporphine Alkaloid Oxoalgalaine

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ABSTRACT

Oxoalgalaine (OG) is an oxoaporphine alkaloid, which has been linked to plant defense mechanisms. It contains a phenalenone (PN)-like chromophore, which suggests a role as singlet oxygen (1O2) photosensitizer. Indeed, OG is able to photosensitize 1O2 with 100% efficiency in nonpolar environments, similar to PN. However, this efficiency decreases in polar and protic media, although 1O2 is formed in all environments ranging from benzene to water. OG is a rather inefficient 1O2 quencher (kq = 8 × 105 M−1 s−1) unlike the related alkaloids boldine and glaucine, for which an antioxidant role has been suggested. The results of this study contribute to the view that plant defense mediated by PN-like secondary metabolites may have a photochemical component.

INTRODUCTION

Oxoalgalaine (OG, Fig. 1) is an oxoaporphine alkaloid that has been isolated from overgrowths of plants belonging to different families such as Annonaceae (1,2), Lauraceae (3), Magnoliaceae (4), Fumariaceae (5,6), Menispermacae (7) and Papaveraceae (8,9). Especially interesting is the case of Magnoliaceae, in which OG is present in injured plants but absent in healthy ones (4). This finding led to propose that highly oxidized aporphines, such as oxoaporphines, its quaternary salts and 4,5-dioxoaporphines, might act as postinfectinal phytoxins, i.e., plant secondary metabolites biosynthesized de novo in response to an environmental aggression such as pathogen attack or in the case of OG, mechanical injury (10). The reduced form of OG, glaucine, is the main alkaloid found in these plants (4,11). Glaucine is oxidized easily to OG and other alkaloids such as corinnine and pontevedrine by chemical and photochemical processes, in particular by singlet oxygen (1O2) (Fig. 1) (8,12–14), which suggests that OG is formed in planta by (photo)chemical or enzymatic oxidation of glaucine. Nevertheless, to establish the role of OG as a phytoalexin, it is necessary to ascertain the process(es) in which plants might make use of OG for defense purposes. It had been suggested previously that the antimicrobial activity of a group of alkaloids related to OG could be related to their ability to act as 1O2 photosensitizer (15).

Recently, we have reported that banana plant phytoalexins elicited in response to fungal attack are able to photosensitize 1O2, thus supporting the view that the deleterious effects of photosensitized 1O2 against invading pathogens are part of the defense mechanisms of these plants (16). Interestingly, the structure of these phytoxins is similar to that of OG in that both contain a phenalenone (PN)-like skeleton, although in the latter case with an N atom in the ring. Other PN-like natural products are oxoaporphines and azasaporphines (Fig. 2). PN, also called perinaphthelenone, is a photosensitizer widely used in photochemistry and photobiology because of its close to unity and solvent-independent quantum yield of 1O2 production (Φ1O2) (17, 18). Φ1O2 = 1 is preserved even in the highly water-soluble 2-sulfonic acid derivative (19). Solvent insensitivity of Φ1O2 is a property unique to PN, other aromatic ketones showing strong solvent dependence of their ability to photosensitize 1O2 (20). We therefore decided to study the photosensitizing ability of OG and compare it with that of PN. This article reports on a photochemical investigation of this oxoaporphine alkaloid aimed at determining its ability to photosensitize 1O2 in different solvents, which may act as simple models of a diversity of biological environments.

MATERIALS AND METHODS

Chemicals. OG was synthesized from glaucine by lead tetraacetate oxidation as described elsewhere (21). Azulene, quinoline bisulfate and PN were purchased from Aldrich (Madrid, Spain) and the last was recrystallized from methanol. Zinc mesoporphyrin dimethyl ester (ZnMPDME) was a gift from Prof. F. R. Trull (22). Benzene, petroleum ether, toluene, acetone, methanol, butanol (SDS, Peppin, France), acetonitrile, 2-propanol, 2,4-methyl-1-pentanol (Aldrich), ethanol (Panrec, Barcelona, Spain) and dimethylsulfoxide (Fisca, Madrid, Spain) were of the highest quality available and used as received.

Photophysical Measurements. Ground-state absorption spectra were recorded using a Varian Cary 4E spectrophotometer (Mulgrave, Australia). Steady-state fluorescence spectra were recorded using a Jobin-Yvon Spec Fluoromax-2 spectrophotometer (Edison, NJ). Transient absorption experiments in the ultraviolet-visible region were carried out using a home-built nanosecond laser flash photolysis system. In this instrument, the third harmonic of a Continuum Surelite I-10 Nd:YAG laser was used to irradiate the sample (5 ns pulsewidth, 1–10 mJ pulse−1). The sample absorbance changes were probed at right angles by a 75 W CW Xe lamp (PTI, Lawrenceville, NJ), and spectral discrimination was obtained using a PTI...
Figure 1. Oxidation pathways of glaucine.

101 monochromator. O₂ was monitored in time-resolved experiments via its phosphorescence at 1270 nm using a 77 K Ge detector (North Coast Scientific Corporation, Santa Rosa, CA). A model 100P (5017) monochromator with a 1050 nm cutoff silicon filter and a 1270 nm interference filter. For all these time-resolved experiments, the detector output was recorded using a LeCroy 9410 digital oscilloscope and interfaced to a personal computer for storage and analysis. To improve signal-to-noise ratios, data recorded from 10 to 100 independent laser pulses were generally averaged. Fluorescence lifetime decays were recorded with a FluoroTime 200 system (PicoQuant, Berlin, Germany). Data were processed using the FluorFit software (PicoQuant). Ultrafast transient absorption spectroscopy was performed as described previously (23).

For the determination of fluorescence quantum yields, Φ₂, quinine bisulfate in 1 N H₂SO₄ was used as standard, for which Φ₂ = 0.546 (24) and λₙs = 355 nm. It was routinely checked that the excitation spectra matched the absorption spectra in each solvent. The procedure for determining Φ₂ involved measuring the area under the fluorescence spectrum for a series of solutions of increasing absorbance and then plotting this area F vs the sample absorption factor (1 – 10⁻³). As deduced from the relationship F = ∫fλ(λ)dλ = (ελ/ελₙs)Φ₂λₙs[1 – 10⁻³], where λₙs and λₙ are the fluorescence and excitation wavelengths, respectively, ε is an instrumental factor, and n is the refractive index of the solvent, the slopes of the linear plots obtained yielded the ratio of the fluorescence quantum yields after correcting for refractive index differences. The Φ₂ values were determined by comparing the O₂ phosphorescence intensity produced by the compound with that produced by an optically matched solution of the reference compound (25), for which we assumed Φ₂ = 0.95 in all solvents (18,20). The photobleaching quantum yield (Φ₋₋₂O₂) was calculated from the changes in the absorption.

Figure 2. Molecular structure of PN, phenylphenalenones (R1 and R2 can vary between H, hydroxyl and methoxy, see Ref. 16), oxoaporphines, oxoisoaporphines and azaoxaporphines.

Figure 3. Steady-state absorption and emission spectra of OG in benzene (black), acetonitrile (red) and water (green). Inset: S₅→S₁ band for a series of solvents of increasing polarity. From left to right: benzene, acetonitrile, acrylonitrile, butanol, methanol and water.

A spectrum of a 55 μM solution upon pulsed laser irradiation (355 nm, 4 mJ pulse⁻¹) using Eq. 1:

A(λₙs) = A₀(λ₀) (1 – εₙs – ε₀) / [1 – (ε₀/εₙs)]

where A₀(λ₀) and A(λₙs) are the 347 nm absorbances at time zero and after absorption of n₀, εₙs and ε₀ are the molar absorption coefficients of OG and the photoproduct, respectively ε₀ is calculated at complete conversion assuming that 1 mol of OG gives 1 mol of photoprodu(I); f is the optical path and V the volume of the solution. The amount of absorbed photons is estimated as:

nₐ₀ = FᵣnpN /

where Fᵣ is the energy of each laser pulse, N is the number of pulses, N₀ is Avogadro’s constant, h is Planck’s constant, c is the speed of light, λₑₓ is the excitation wavelength and A(λₑₓ) is the absorbance at the excitation wavelength (355 nm). Only the initial points, for which it can be assumed that the incoming photons are mainly absorbed by OG and not by the photoprodu(I), are used for the calculation of Φ₋₋₂O₂.

RESULTS AND DISCUSSION

OG is soluble in a wide range of solvents, from benzene to water, similar to the related molecule PN. Figure 3 shows its steady-state absorption and fluorescence spectra in a number of solvents. Clearly, there is a bathochromic shift upon increasing the solvent polarity, consistent with a π→π⁺ lowest excited singlet state. The singlet energies, calculated from the intercept of the normalized absorption and fluorescence spectra, are slightly lower than those of PN (Table 1).

Φ₂ values are very small and sensitive to the polarity and proticity of the solvent (Table 1, Fig. 4). Thus, it is highest in acetonitrile but decreases much more than other order of magnitude both going either to less

Table 1. Singlet-state properties of OG. Values for PN are given in brackets for comparison

<table>
<thead>
<tr>
<th></th>
<th>Benzene</th>
<th>Acetonitrile</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Φₛ (kJ mol⁻¹)</td>
<td>259 (265)</td>
<td>252 (265)</td>
<td>242 (269)</td>
</tr>
<tr>
<td>t₀ (fs)</td>
<td>0.004 (≤10⁻⁴)</td>
<td>0.06 (≤10⁻⁴)</td>
<td>0.002 (0.01)</td>
</tr>
<tr>
<td>t₅ (ps)</td>
<td>8</td>
<td>6700</td>
<td>&lt;10⁶</td>
</tr>
</tbody>
</table>

*In cyclohexane.
†From Ref. 18.
‡This study.
§20% error bars.
polar solvents (benzene) or to more protic solvents (water). A rationalization for these effects is given subsequently.

Similarly, the fluorescence decay in acetonitrile is described mainly (96%) by a 0.7 ns component (Fig. 5a), and its lifetime is strongly reduced in water as well as in benzene below the time window of the system in this study (≤0.1 ps). Ultrafast transient absorption spectroscopy was thus used to measure the singlet lifetime in benzene, whereas solubility precluded the measurement in water. Figure 5b shows the growth of the absorption at 500 nm, where two components with lifetimes ca. 500 fs and 8 ps can be observed clearly. Their dissimilar spectra demonstrate that we are observing two different processes, as is the case for the aromatic ketone benzophenone (26). The fast component is close to the 530 fs S2→S1 internal conversion in benzophenone and is therefore tentatively assigned to the same process. The second component is similarly close to the 9.8 ps S1→T1 intersystem crossing in benzophenone and is also assigned to this process.

Unlike PN, OG is not phosphorescent even in low-temperature glasses containing heavy atoms (27). Nevertheless, a transient species was detected using nanosecond laser flash photolysis whose spectrum matches that obtained after 40 ps in the ultrafast absorption experiments (Fig. 6). This transient decayed monoeXponentially with lifetime of 57 ± 5 ps in Ar-saturated benzene (inset, Fig. 6) and was quenched by O2 with kq = 2 × 109 M⁻¹ s⁻¹, confirming the assignment of the 8 ps growth to the formation of 1OG. The transient spectrum was unchanged in acetonitrile, which indicates that the triplet state is of the same orbital configuration in the two solvents. Some residual absorption was observed at some wavelengths in both solvents, revealing minor photoprodut formation under these conditions.

Because OG is not phosphorescent, triplet–triplet energy transfer experiments were used to estimate the triplet energy. Quenching of 1OG by azulene (Et = 163 kJ mol⁻¹ (28)) was observed with a rate constant of 5 × 10⁹ M⁻¹ s⁻¹ in benzene. On the other hand, triplet ZnMPDME (Et = 171 kJ mol⁻¹ (28)) could in turn be quenched by OG with a rate constant of 7 × 10⁸ M⁻¹ s⁻¹. These observations set the triplet energy of OG between those of azulene and ZnMPDME, slightly closer to the latter, according to the quenching rate constants. We propose a value of 168 ± 4 kJ mol⁻¹, which implies a singlet–triplet energy gap of ca. 90 kJ mol⁻¹, which is typical for ππ* states of aromatic compounds (28). For comparison, the triplet energy of PN lies at 185 kJ mol⁻¹ (18,27).

Consistent with the formation of the triplet state of OG and its energy value, sensitization of 1O2 was observed. Remarkably, in nonpolar solvents, the efficiency of this process is close to unity, similar to PN. This is unprecedented to the best of our knowledge for a natural alkaloid (29), and given the fact that OG is involved in plant defense (4), one might speculate that its role may have a photochemical component, i.e., OG may act as a phototoxic phytoalexin as is the case for the related phenylphenolones (16). However, in biological media, there are different microenviron-ments with largely varying polarity. To assess the effect of such environmental factor, we measured 1OG in solvents of different polarities (Et [30]). As shown in Fig. 7, 1OG decreases upon increasing solvent polarity.

Nonpolar solvents favor intersystem crossing to triplet OG, whereas in polar nonprotic solvents, intersystem crossing is less efficient, increasing 1OG and reducing 1OG. We note at this point that the sum of 1OG and 1OG does not equal 1, thus indicating that internal conversion pathways are operating as well. This can be rationalized in terms of the solvent effects on the energy of the excited states as in other ketones such as fluorone (31). Specifically, we propose that in nonpolar solvents, intersystem crossing is fast because it occurs from S1(ππ*) to T2(ππ*), with subsequent relaxation to T1(ππ*). Increasing solvent polarity destabilizes T2(ππ*), eventually rising it above S1(ππ*) with the concomitant decrease in the intersystem-crossing rate constant. This model is supported by the following observations: (i) the lowest excited singlet state is ππ* in all solvents as demonstrated by the bathochromic shift observed in both the absorption and fluorescence spectrum; (ii) the lowest triplet state is of the same electronic configuration in benzene and acetonitrile as demonstrated by the invariability of the triplet

Figure 5. (a) Fluorescence decay of OG in acetonitrile at 510 nm; (b) absorbance growth (500 nm) in benzene. Inset: transient spectra at 9.0 and 40 ps (see text).
absorption spectrum with the triplet lifetime upon solvent change; (iii) the lowest triplet state is of \( \pi^* \) configuration in benzene—and hence in acetonitrile—as demonstrated by the quantum yield of \( ^1O_2 \) approaching unity in this solvent. \( \pi^* \) triplets show much lower \( ^1O_2 \) yields, typically 0.2-0.4 (32); (iv) the rate constant for inter-system crossing in benzene, estimated as \( k_{isc} = \Phi_{t/tr} = \Phi_{t/tr} = 1.3 \times 10^{-11} \text{s}^{-1} \), is unusually large for inter-system crossing between states of the same electronic configuration but is typical for \( \pi^*(\pi^*) \rightarrow \pi^*(\pi^*) \) transitions, indicating that a \( \pi^* \) triplet lies below the lowest \( \pi^* \) singlet (33) and (v) in contrast, the rate constant for inter-system crossing in acetonitrile, \( ca. 1.0 \times 10^{-9} \text{s}^{-1} \), is typical for \( \pi^*(\pi^*) \rightarrow \pi^*(\pi^*) \) transitions, indicating that the \( \pi^* \) triplet in this polar solvent lies above the lowest \( \pi^* \) singlet.

Protic solvents cause an additional decrease in \( \Phi_0 \). This is best exemplified by the case of 2,4-dimethyl-3-pentanol whose \( \Phi_0 \) is significantly lower than expected for a solvent of its \( E_T^N \) value (Fig. 7). As shown for several other aromatic ketones, pro ont solvents form hydrogen bonds with the carbonyl group, which act as efficient accepting modes of radiationless deactivation, thus enhancing internal conversion (34). It is particularly revealing that the decrease in \( \Phi_0 \) in these solvents is not compensated by an increase in \( \Phi_\lambda \), which in fact also decreases, (e.g., in water, Table 1). This clearly confirms that internal conversion plays a major role in these solvents.

\[ E_T^N \]

\[ \Phi_0 \]

\[ \Phi_\lambda \]

\[ \Phi_0 \]

\[ \Phi_\lambda \]

Thus, the solvent effects on \( \Phi_0 \) (Table 1) can be accounted for by the model above. Although the decrease in the intersystem-crossing rate constant upon increasing the solvent polarity leads to an enhancement in \( \Phi_0 \), the increase in the internal conversion rate constant upon increasing the solvent proticity causes \( \Phi_\lambda \) to decrease. These opposite trends explain that the least polar and the most polar and protic solvents (benzene and water, respectively) have similar \( \Phi_0 \) values, whereas intermediate solvents such as acetonitrile show substantially higher \( \Phi_0 \).

\[ \text{OG is a moderate} \quad ^1O_2 \quad \text{quencher, with overall} \quad \text{(physical} \quad \text{and chemical)} \quad \text{rate constant} \quad k_q = 8 \times 10^5 \text{M}^{-1} \text{s}^{-1} \quad \text{in perdeuterated benzene (Fig. 8).} \quad \text{For comparison,} \quad k_q = 3.2 \times 10^5 \text{M}^{-1} \text{s}^{-1} \quad \text{for PN in perfluorodecalin (35) and} \quad k_q = 0.5-68 \times 10^7 \text{M}^{-1} \text{s}^{-1} \quad \text{for its putative biological precursor glaucine depending on the solvent (14).} \quad \text{These rate constants are significantly larger than expected for molecules quenching by electronic-to-vibrational deactivation (36).} \quad \text{The excess is likely caused by charge-transfer-induced quenching, as already demonstrated for glaucine (14).} \]

Photobleaching was observed with quantum yield, \( \Phi_{OG} = 7 \times 10^{-2} \) for a 55 \( \mu \text{M} \) solution of OG in air-saturated benzene (Fig. 9). As \( \Phi_{OG} \) depends on the concentration of OG, a more meaningful description of OG’s photodegradation is given by the reactive rate constant \( k_r \). Assuming that photodegradation is mainly caused by

\[ \text{Figure 7.} \quad ^1O_2 \quad \text{quantum yields vs solvent polarity (} E_T^N \text{) for nonprotic (\bullet) and protic (\circ) solvents. Tol = toluene, Ben = benzene, DMA = dimethylacetamide, ACN = acetonitrile, MPOH = 2,4-dimethyl-3-pentanol, ACR = acrylonitrile, BuOH = butanol, iPrOH = 2-propanol, EtOH = ethanol, MeOH = methanol.} \]

\[ \text{Figure 8.} \quad \text{Normalized} \quad ^1O_2 \quad \text{signals from perdeuterated benzene solutions containing increasing [OG]. Inset: Stern–Volmer plot for the deactivation of} \quad ^1O_2 \quad \text{by ground-state OG.} \]

\[ \text{Figure 9.} \quad \text{Photobleaching of OG upon laser irradiation in air-saturated benzene. Inset: linear regression of the absorbance at 347 nm vs \( \Delta A_{347} \) according to Eq. 1.} \]
reaction with $^1\text{O}_2$, which is the major reactive species in the system ($\Phi_A = 1$), $k_5$ can be calculated from Eq. 3:

$$\Phi_{\text{OG}} = \Phi_A \frac{k_5[\text{OG}]}{k_5 + k_{\text{cr}}}$$

(3)

where $k_5$ is the rate constant of natural deactivation of $^1\text{O}_2$ in the solvent. Using $k_5 = 3.3 \times 10^7 \text{ s}^{-1}$ and $[\text{OG}] = 55 \mu\text{M}$, $k_{\text{cr}}$ is calculated as $4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, which accounts for 50% of the overall quenching of $^1\text{O}_2$ by OG.

As stated above, its putative biological precursor glaucine is a considerably more efficient quencher, which has led to the suggestion that glaucine and its analogue boldine act as antioxidants in plants (14). A similar role for OG is unlikely, given its much lower $^1\text{O}_2$ quenching ability. Furthermore, our finding that OG is such a good $^1\text{O}_2$ photosensitizer suggests, Interestingly, a protonoid role of glaucine because OG is one of its oxidation products.

In conclusion, we have shown that another PN-like phototoxin, the oxoaporphine alkaloid OG, is able to photosensitize $^1\text{O}_2$ similar to the phenylethamamines found in infected banana plants. If its photosensitizing ability were confirmed in vivo, it would seem that a picture is emerging that suggests a particular role for the PN chromophore in the realm of light-assisted plant defense. It will be interesting to investigate whether other PN-like secondary metabolites such as oxoaporphines, oxoisoaporphines and azaoaporphines do play similar role in other plants.

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