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NEAR-INFRARED DETECTION OF SINGLET MOLECULAR OXYGEN PRODUCED BY PHOTOSENSITIZATION WITH PROMAZINE AND CHLORPROMAZINE

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Abstract—A sensitive near-infrared detection system has been used to study the steady-state emission of $^1O_2$ at 1268 nm produced by promazine (PZ) and chlorpromazine (CPZ) during photo-illumination. Singlet molecular oxygen could be detected in a variety of ordinary and perdeuterated organic solvents, but was not detectable in water or deuterium oxide. The emission was enhanced in the perdeuterated organic solvents and could be eliminated by rigorous degassing or by addition of the singlet oxygen scavenger 2,3-dimethylfuran. Singlet oxygen could not be detected in any of the solvents during irradiation of the sulfoxides of PZ and CPZ. We conclude that in biological systems $^1O_2$ production is not a major pathway to phototoxicity for the sulfoxides, while for the parent phenothiazines the formation of $^1O_2$ is much more likely to be important in nonpolar environments such as cell membranes than in the aqueous parts of the cell.

INTRODUCTION

Phenothiazine drugs are widely used in medicine because of their tranquilizing, antihistaminic and anthelmintic properties. However, medical and veterinary patients undergoing treatment with phenothiazine derivatives can incur serious side effects, including cutaneous photosensitization (Fitzpatrick *et al.*, 1963) and ocular damage (Zelickson and Zeller, 1964; Johnson and Buffaloe, 1966; Siddall, 1965). Neither the mechanism of the therapeutic action of phenothiazines nor the origin of their side effects is completely understood. Free radical production appears to play an important role in photosensitization by phenothiazines (Chignell *et al.*, 1985), but experiments by Davies *et al.* (1978) suggest that $^1O_2$ is also formed: oxygen was consumed during irradiation of chlorpromazine (CPZ)[§] in 2-propanol in the presence of 2,5-dimethylfuran, a relatively specific acceptor of $^1O_2$, while DABCO inhibited oxygen consumption. Using an electron spin resonance (ESR) $^1O_2$ trapping technique, Saucin and Van de Vorst (1980) determined that $^1O_2$ may also be produced during near-UV irradiation of various phenothiazines in ethanol, including PZ and CPZ. However, Decuyper and coworkers (1983) concluded, on the basis of a different ESR technique and a chromatographic analysis of the oxidation products of cholesterol, that near-UV irradiation of several phenothiazine derivatives, including PZ and CPZ, does not produce $^1O_2$ in either ethanol or water. Only direct evidence for the production of $^1O_2$ by phenothiazines can unequivocally determine whether the phototoxicity of phenothiazine drugs can be ascribed, even in part, to $^1O_2$.

Metabolic and photochemical products of phenothiazine drugs may also contribute to the phototoxicity associated with their use (Chignell *et al.*, 1985). It has been suggested, for example, that the phenothiazine sulfoxide, a two-electron oxidation product of the parent drug, may be the metabolite that causes lens damage associated with the use of these drugs in therapy (Clare *et al.*, 1947; Carr, 1968; Buettner *et al.*, 1986). Yeung *et al.* (1983) have reported that more than half of the CPZ found in human plasma following administration of the drug is present as the sulfoxide. In previous papers (Motten *et al.*, 1985; Buettner *et al.*, 1986) we have identified the free radicals produced as a consequence of near-UV irradiation of PZ, CPZ, and their sulfoxides. In the present paper, we describe the results of a study of the 1268 nm luminescence of $^1O_2$ produced during near-UV irradiation of these drugs in a number of different solvents.

MATERIALS AND METHODS

The CPZ was from Sigma Chemical Co. (St. Louis, MO) and 2.5-dimethylfuran was from Aldrich Chemical Co. Promazine hydrochloride was a gift from Wyeth Laboratories (Philadelphia, PA). The free bases of PZ and CPZ were obtained by extracting an alkaline (1 *M* sodium

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§*Abbreviations*: CPZ, chlorpromazine; DABCO, diazabicyclo[2.2.2]octane; ESR, electron spin resonance; $^1O_2$, singlet ($^1\Delta_g$) molecular oxygen; PZ. promazine; UV, ultraviolet.

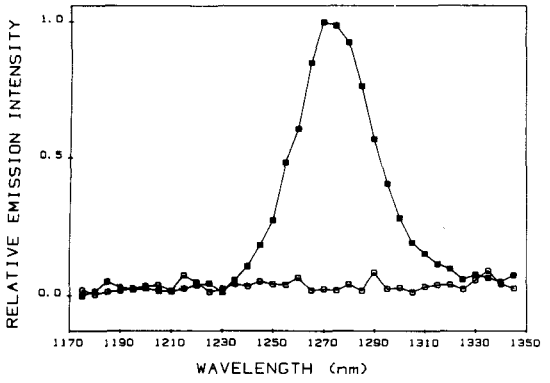

Figure 1. The infrared emission spectrum of UV-irradiated CPZ in benzene. Four freeze-thaw degassing cycles on a high vacuum line (5 μm Hg) eliminated (□–□) the 1268-nm emission band sensitized by chlorpromazine in aerated benzene (■–■). The light source ws a 150 W Xe lamp. Emission bandpass: 25 nm. Amplifier gain: 100. Spectra accumulated in 6 scans at 10-nm intervals averaging signal for 1 s/step. Chlorpromazine concentration was 0.2 m*M*.

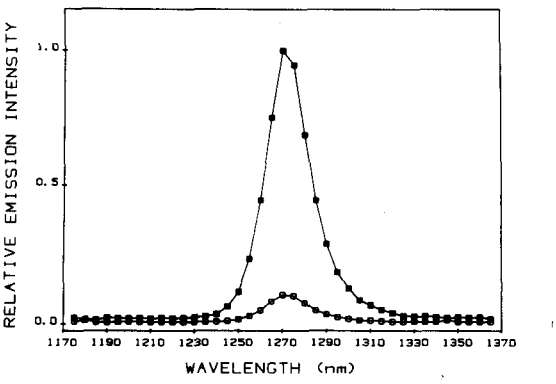

Figure 2. The effect of solvent perdeuteration on the infra-red emission spectrum of UV-irradiated CPZ in benzene. Substitution of perdeuterated benzene (■–■) for ordinary benzene (□–□) gave a 10-fold enhancement of the 1268-nm emission intensity sensitized by chorpromazine. The light source was a 200 W mercury lamp. Emission bandpass: 20 nm. Amplifier gain: 100. Spectra were obtained by scanning 4 times at 5-nm intervals averaging the signal for 1 s/step. No solvent emission was detected under the same conditions. Chlorpromazine concentration was 0.1 m*M*.

hydroxide) solution of the respective drug with cyclohexane. The free base was recovered from the organic extract by evaporation *in vacuo* and taken up in the appropriate solvent for the subsequent spectroscopic studies. The sulfoxide derivatives were synthesized as described previously (Buettner *et al.*, 1986). Reference samples of the CPZ and PZ sulfoxides were obtained from the Neurosciences Research Branch, National Institute of Mental Health (Rockville, MD) and Smith, Kline and French Labs (Philadelphia, PA) respectively. All solvents were of the highest grade available.

The singlet oxygen detector used in these experiments has been described elsewhere (Hall and Chignell, 1987). During the initial experiments (results shown in Fig. 1), a 150 W Xe arc lamp and a combination of Schott BG-24, KG-2 and KG-3 filters were used to irradiate samples. However, it was found that a 200 W Hg arc lamp gave a 10-fold greater light output at 300 nm, so the latter lamp was used to acquire the results shown in Fig. 2 and in Table 1. A liquid filter solution described by Wladimiroff (1966) containing 55% dimethylformamide, 0.42 *M* $NiCl_2$, 0.85 *M* $CoCl_2$ and 1 *M* HCl in water was used with the Hg arc lamp to pass UV light between 290 nm and 390 nm; the fluence rate at the point of entry to the rectangular cuvette was 12 kW m^{-2} as measured with a Yellow Springs Instrument Company (Yellow Springs, OH) model J225 radiometer. Ten centimeters of water were used to block infra-red radiation in the excitation light path and a Corion LL-950 filter was used to eliminate visible and ultraviolet light from the emission signal. The liquid filter was maintained under constant irradiation for an hour before and then during an entire experiment in order to maintain a constant filter transmittance.

Relative emission intensities were obtained by averaging signals at 1268 nm for 10 sec with the monochromator bandpass set at 40 nm. The emission intensity in benzene was found to be nearly linear with CPZ absorbance at the long-wavelength maximum of the absorption spectrum up to 0.4 absorbance units in a 10 mm^2 rectangular cuvette. Solvent backgrounds were subtracted in all cases. Very little degradation of the phenothiazine samples occurred in *n*-hexane, cyclohexane or benzene. However it was necessary to use a flow system (Hall and Chignell, 1987) in order to minimize loss of sample in the other solvents during irradiation. The excitation pathlength of the flow cell was 3 mm. About 20 mℓ of absolute ethanol sufficed to clear the flow system between measurements with nonmiscible solvents. The stability of the intensity readings was verified by frequent readings of a reference sample of CPZ in aerated cyclohexane. All samples had nearly equal absorbances (about 0.25 at the absorbance maximum in the flow cell).

RESULTS

The characteristic emission spectrum of $^1O_2$ was observed during irradiation of PZ or CPZ in a variety of solvents. Reproducible spectra could be obtained by irradiating the free bases of CPZ or PZ in benzene (Fig. 1) or cyclohexane; under the same conditions of irradiation, no emission was detected in cyclohexane alone and only a small emission was observed in benzene (< 1% of the phenothiazine sample emission). The $^1O_2$ emission was completely quenched by the addition of 10 m*M* 2,5-dimethylfuran, a relatively specific scavenger for $^1O_2$ (data not shown). Bubbling the solutions with oxygen slightly quenched the $^1O_2$ emission (≤ 5%). Bubbling with nitrogen could not completely eliminate the $^1O_2$ emission in the hydrocarbon solvents, probably as a result of the presence of residual oxygen. However, four freeze-thaw cycles on a high-vacuum line (mercury diffusion pump), operating at 5 μm Hg, completely eliminated the emission in solutions of CPZ or PZ in benzene (Fig. 1) or cyclohexane. Reaeration of the vacuum-degassed samples restored the $^1O_2$ emission spectrum.

Perdeuterated analogs of ordinary solvents generally enhance the signal intensity of singlet oxygen. (See Krasnovsky, 1981, for example.) The results in

Table 1. Relative emission quantum yields at 1268 nm

Solvent*	Photosensitizer		
	Chlorpromazine	Promazine	q†
Benzene	2.46‡	2.17	4.29
Benzene (vacuum de-gassed)	N.D.	ND	
Perdeuterated benzene	25.6	10.17	
Cyclohexane	1.0	0.96	1.0
Cyclohexane (oxygen-saturated)	0.95	0.91	
Cyclohexane (vacuum de-gassed)	N.D.	ND	
n-Hexane	1.18	1.06	
Dioxane	0.83	0.71	0.85
Ethanol	0.34	0.35	
Acetonitrile	0.34	0.72	1.60
Methanol	0.14	0.16	
Perdeuterated methanol	2.41	1.75	
Water	ND	ND	
Deuterium oxide	ND	ND	0.58

*Air-saturated unless otherwise noted.
†See text for definition of q.
‡All intensities are relative to CPZ in aerated cyclohexane.
ND No detectable signal.

Fig. 2 demonstrate the increase in emission intensity produced by replacing benzene with its perdeuterated analog. The spectra were obtained by irradiating solutions of C_6H_6 and C_6D_6 containing CPZ at the same absorbance (0.4 at 315 nm) in each solution.

The emission of $^1O_2$ sensitized by PZ or CPZ was also found in dioxane, acetonitrile, methanol and ethanol. Solutions of PZ and CPZ in these four solvents also showed a slight quenching of the $^1O_2$ signal when saturated with oxygen compared with that observed in aerated solutions, but nitrogen-saturated solutions gave no detectable emission. It appears, therefore, that low concentrations of oxygen were sufficient to maintain a steady-state singlet oxygen signal in the non-polar solvents but not in the polar solvents. The relative $^1O_2$ emission intensities sensitized by PZ and CPZ in the various solvents are summarized in Table 1. The flow cell apparatus helped to offset the photochemical degradation that occurred during sample irradiation so the emission intensity remained constant during irradiation. There was no significant degradation of PZ or CPZ in the organic solvents. However, in water and deuterium oxide, both compounds underwent extensive photochemical degradation without producing detectable levels of singlet oxygen, even when samples were pumped rapidly through the flow cell. The emission intensities were normalized to equal rates of photon absorbance as described in Hall and Chignell (1987). The results in Table 1 have not been corrected for trivial solvent absorbance of the emission because the transmittances of the solvents (other than water) used in these experiments were high and did not differ greatly at 1268 nm (Hall, unpublished results).

The sulfoxides of PZ and CPZ did not produce detectable levels of singlet oxygen in any of the solvents; nor did saturation of sulfoxide solutions with pure oxygen lead to observable singlet oxygen emission. Like the parent compounds, both sulfoxides were degraded rapidly in aqueous and deuterium oxide solutions but were relatively stable to irradiation in the organic solvents.

DISCUSSION

Using steady-state detection of its phosphorescence at 1268 nm, we have observed the production of $^1O_2$ by the free bases of PZ and CPZ in several different solvents during near-UV irradiation. The experimental emission spectrum was a single band centered at 1270 nm with full width at half height less than 40 nm. Furthermore, it could be completely eliminated through rigorous deaeration using the freeze-thaw technique under moderate vacuum conditions (5 μm Hg), or through the addition of 2,5-dimethylfuran, a relatively specific scavenger of singlet oxygen.

The fluorescence lifetimes of all four of the compounds we studied are less than 2.5 ns in water. Their fluorescence quantum yields are very low, their phosphorescence quantum yields are high at 77°K, and the energy differences between the fluorescence maxima and the phosphorescence maxima are less than 1 eV (Saucin and Van de Vorst, 1980; Hall, unpublished results). The energy lost through intersystem crossing in the photosensitizers is insuf-

ficient to sensitize the formation of $^1O_2$; thus, only triplet-state photosensitization should occur, consistent with the fact that the emission of $^1O_2$ shows only a weak dependence on the concentration of oxygen. Furthermore, PZ and CPZ appear to sensitize $^1O_2$ formation with about the same efficiency in each of the solvents, which suggests approximately equivalent intersystem crossing yields.

The intensity enhancements obtained with PZ and CPZ in the perdeuterated solvents were lower than expected. In perdeuterated benzene, C_6D_6, irradiation of CPZ produces a 10-fold enhancement in the $^1O_2$ emission intensity compared with that in ordinary benzene; the factor is approximately 17 between methanol and perdeuterated methanol, CD_3OD. In contrast, Rodgers (1983) found that the lifetimes of $^1O_2$ differed by a factor of 20 between C_6H_6 and C_6D_6 and by a factor of 22 between CH_3OH and CD_3OD. These results suggest that our photosensitizer concentrations (100 μM) were high enough to significantly quench the $^1O_2$ produced in perdeuterated solvents, where the lifetimes of $^1O_2$ are long (628 μs in C_6D_6 and 227 μs in CD_3OD; Rodgers, 1983). A difference in the quenching ability of the two photosensitizers might then account for the differences in enhancement produced by PZ and CPZ in the two pairs of solvents.

A moderate steady-state $^1O_2$ emission was detected during irradiation of PZ and CPZ in the non-polar solvents, while little or no emission was detected in the polar solvents. Self-quenching (singlet-singlet interactions) does not affect the steady-state $^1O_2$ emission at the levels of $^1O_2$ produced in these solvents (Hall and Chignell, 1987). Moreover, the differences in quantum yield reflected in Table 1 do not correlate with the slight trivial absorbance expected from the optical transmission spectra of the solvents used (Hall, unpublished results). Thus, as long as comparisons are made between samples containing the same photosensitizer and solvents having similar measured $^1O_2$ lifetimes, changes in the steady-state emission of $^1O_2$ should only reflect changes in the process of photosensitization itself.

Krasnovsky (1981) has concluded on the basis of a study of $^1O_2$ emission in acetone, water, deuterium oxide and carbon tetrachloride that the radiative lifetime of $^1O_2$ does not depend very greatly on the solvent in which the radiative process occurs (the radiative lifetime of $^1O_2$ is given as 4 ± 2 s). Gorman *et al.* (1987) concluded that the relative radiative rate constants of $^1O_2$ in benzene, cyclohexane, dioxane, acetonitrile and deuterium oxide are 13.3, 4.1, 3.0, 2.6 and 1.0 s^{-1}, respectively. Thus, it appears that the radiative rate constant of $^1O_2$ does not differ greatly among the various solvents studied (within the likely error involved in the measurements), with the exception of benzene, which stands out as a special case. Nevertheless, differences in the radiative rate constant of $^1O_2$ must be considered when $^1O_2$ emission intensities only vary within a small range of magnitude, as in the present study. In order to understand the effect of the radiative rate constant, it is useful to consider all the factors affecting the emission of $^1O_2$.

The absolute quantum yield of $^1O_2$ phosphorescence (as a function of photons absorbed by the photosensitizer) for triplet-state sensitized $^1O_2$ production can be written as follows:

$$\phi_{em} = \phi_T S_\Delta \frac{k_r}{k_r + k_{nr}} = \phi_\Delta \frac{k_r}{k_r + k_{nr}} \qquad (1)$$

where ϕ_T is the quantum yield for the intersystem crossing between the singlet and triplet excited states of the photosensitizer, S_Δ is the fraction of triplet states of the photosensitizer leading to $^1O_2$ excited states, ϕ_Δ is the quantum yield for $^1O_2$ production, and k_r and k_{nr} are, respectively, the radiative and non-radiative rate constants for $^1O_2$ in a given solvent. The last equation can be simplified by noting that the non-radiative rate constant is much larger than the radiative rate constant in all the solvents under study; thus, k_{nr} is approximately equal to $1/\tau$ where τ is the excited-state lifetime of $^1O_2$ in a given solvent (Rodgers, 1983):

$$\phi_{em} \approx \phi_\Delta k_r \tau \qquad (2)$$

In order to distinguish solvent effects on the production of $^1O_2$ in the case of the phenothiazine derivates examined in this paper, we can also calculate the radiative probability of $^1O_2$, ϕ, in a given solvent:

$$\phi' \equiv \frac{\phi_{em}}{\phi_\Delta} \approx k_r \tau \qquad (3)$$

Finally, the relative emission probability of $^1O_2$ under conditions of equivalent $^1O_2$ production can be defined as follows:

$$q \equiv \frac{\phi}{\phi(\text{ref})} \approx \frac{k_r}{k_r(\text{ref})} \cdot \frac{\tau}{\tau(\text{ref})} \qquad (4)$$

where ϕ(ref) and τ(ref) are ϕ as defined in Eq. (3) and the singlet-state lifetime of $^1O_2$ respectively, in a reference solvent (cyclohexane in the present study). Clearly, q is fixed by the specific rate constants for de-excitation of $^1O_2$ in a given solvent. Combining Eqs. 2 and 4 and including appropriate subscripts, we obtain:

$$\frac{\phi_\Delta}{\phi_\Delta(\text{ref})} \approx \frac{\phi_{em}}{\phi_{em}(\text{ref})} \frac{1}{q} \qquad (5)$$

Thus, the relative emission intensities obtained for $^1O_2$ in different solvents (Table 1), divided by q (where q can be calculated), immediately reveal differences in efficiency of $^1O_2$ production with respect to the reference solvent. Such information does not, by itself, indicate what photophysical or photochemical process affects $^1O_2$ production. How-

ever, other information about the photosensitizer excited states can be used to assert probable mechanisms of reaction.

In Table 1, we present the calculated values of q for the solvents for which excited-state lifetimes (Rodgers, 1983) and information about k_r are available (*vide supra*). Although the solvents differ markedly with regard to dielectric constant and hydrogen-bonding properties, only in benzene does q differ by more than a factor of 2 with reference to cyclohexane.

The relative $^1O_2$ emission yield is close to the calculated value of q in dioxane, indicating that $^1O_2$ production by PZ and CPZ is equally efficient in cyclohexane and dioxane. On the other hand, $^1O_2$ production sensitized by PZ and CPZ in benzene and acetonitrile appears to be lower than that in cyclohexane. Finally, $^1O_2$ production in deuterium oxide could not be observed even though the value of q calculated for deuterium oxide demonstrates explicitly that efficient production of $^1O_2$ by PZ and CPZ in deuterium oxide would have resulted in easily detectable levels of $^1O_2$ phosphorescence. We can surmise on the basis of the small differences in q between deuterium oxide and the other solvents, as well as Krasnovsky's results (1981), that the yields of $^1O_2$ production in methanol and ethanol are also low compared with those in cyclohexane. Thus, we conclude that the emission of $^1O_2$ in deuterium oxide and water was not observed because production of $^1O_2$ is not favored in hydroxylic solvents. It remains to propose alternative explanations for the lower yields of $^1O_2$ production.

It is possible that the efficiency with which ground-state oxygen quenches the photosensitizer triplet state changes from solvent to solvent independently of changes in the rates of other excited-state reactions. Such a change would depend on the viscosity and dielectric constant of the particular solvent as well as the effects of the solvent on the concentration of ground-state oxygen and on the triplet-state energy of the photosensitizers (Gijzeman *et al.*, 1973). However, it appears that oxygen quenches the triplet states of PZ and CPZ very efficiently in the hydrocarbon solvents, because careful de-gassing was required to completely eliminate the $^1O_2$ emission signal. At the same time, in none of the solvents were we able to significantly change the $^1O_2$ phosphorescence by saturating with oxygen, which further suggests that the triplet states of PZ and CPZ were efficiently quenched by oxygen in *all* the aerated (organic) solvents in our study. Gijzeman *et al.* (1973) concluded that aromatic hydrocarbons that sensitize $^1O_2$ production *via* high-energy triplet states produce higher yields of $^1O_2$ in more polar solvents. Since PZ and CPZ also sensitize $^1O_2$ production *via* high energy triplet states, it does not seem likely that changes in oxygen quenching can account for the differences in $^1O_2$ production observed.

Other studies have demonstrated that photoionization of PZ and CPZ occurs in aqueous and ethanolic solutions, and that the yield of photoionization is much larger in aqueous solution (Navaratnam *et al.*, 1978; Moore and Tamat, 1979). The fluorescence quantum yield, which is less than 0.05, only changed by a factor of 2 in the same study, so it is unlikely that the singlet-to-triplet intersystem crossing yield changes significantly (*vide supra*). While the exact nature of the photolysis of PZ and CPZ is not clear (Motten *et al.*, 1985), it seems reasonable to assume that excited-state reactions compete with $^1O_2$ production. We include here direct reactions with ground-state oxygen not involving free $^1O_2$. Moreover, the assumption that PZ and CPZ undergo photochemical reactions in hydroxylic solvents also accounts for the loss of photosensitizer observed in those solvents during irradiation in the $^1O_2$ spectrometer. We conclude, therefore, that solvent-dependent changes in the triplet-state photochemistry of PZ and CPZ are the strongest determinants of changes in the yields of $^1O_2$ emission sensitized by these drugs.

Our results are consistent with the observations of Davies *et al.* (1976), Moore (1977) and Saucin and Van de Vorst (1980) that phenothiazines in general, and PZ and CPZ in particular, produce singlet oxygen in nonpolar and alcoholic solutions. On the other hand, the low intensity of the singlet oxygen emission in alcoholic solutions and its absence in aqueous solutions suggests that production of singlet oxygen is at best a reaction of secondary biological importance in the aqueous phase. Since PZ and CPZ produce $^1O_2$ most efficiently in non-polar media, phenothiazines imbedded in membranes (Forrest *et al.*, 1984) may be more likely to produce singlet oxygen *in vivo*. Further work with micellar and liposomal systems may show how membrane binding affects singlet oxygen formation.

The sulfoxides of PZ and CPZ did not produce detectable levels of $^1O_2$ in any of the solvents we studied; thus, the results appear to exclude $^1O_2$ production by phenothiazine sulfoxides as a biologically important pathway to photodamage in living cells. In a previous paper (Buettner *et al.*, 1987), we have proposed that the sulfoxides of PZ and CPZ may contribute to the phototoxicity associated with phenothiazine therapy through the production of another active oxygen species, the hydroxyl radical.

We have detected $^1O_2$ in ethanol while Decuyper *et al.* (1983) did not. They have estimated the sensitivity of their cholesterol product analysis to be 1.6 μmol of the oxygen-cholesterol adduct per mole of singlet oxygen produced. Although it is difficult to compare their results directly with our own (since one method is integrative and the other is dynamic), we have calculated that our instrument can detect rates of photon emission from $^1O_2$ as low as 50 fmol

s^{-1} in a 0.5 mℓ cuvette (Hall and Chignell, 1987). Certainly, the sensitivity of the spectroscopic method is high enough to permit detection of singlet oxygen production by PZ and CPZ in alcohols. Nevertheless, singlet oxygen production by PZ and CPZ in water, if it occurs, remains below the present detection limit.

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