A Fluorescent Probe for Antioxidants

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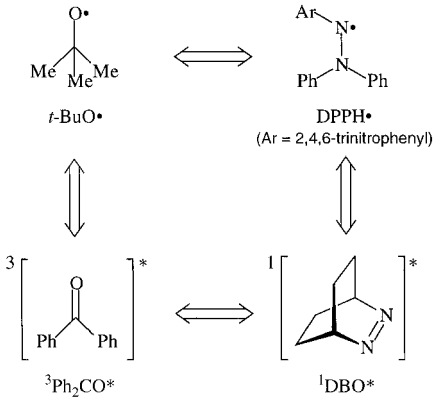
Abstract: The n,π^* singlet-excited azoalkane 2,3-diazabicyclo[2.2.2]-oct-2-ene (DBO) is efficiently quenched by the natural antioxidants α-tocopherol ($k_q = 5.3 \times 10^9$ M^{-1} s^{-1}), uric acid (3.4×10^9), ascorbic acid (2.05×10^9), and glutathione (0.82×10^9). This trend in reactivity is the same as that observed for alternative probes for antioxidants, e.g., triplet-excited benzophenone and the trichloromethylperoxyl radical. Since the quenching of singlet-excited DBO can be readily quantified by means of its long-lived (325 ns in aerated water) and strong fluorescence (λ_{max} ca. 425 nm), this azoalkane serves as the first fluorescent probe for the reactivity of antioxidants. The effects of pH and deuterium substitution and kinetic solvent effects were also examined. The data suggest the involvement of hydrogen atom transfer in the fluorescence quenching of DBO by antioxidants. The reaction efficiency for radical formation in the quenching of singlet-excited DBO by α-tocopherol was found to be one order of magnitude lower (only ca. 10%) than for triplet-excited benzophenone. This contrast is attributed to the variation in spin multiplicity, since reactions of singlet-excited states encounter additional deactivation channels. The potential of the fluorescent probe DBO in providing direct information on antioxidant activity in biological systems is evaluated.

Introduction

Antioxidants play a vital role in biology, polymer chemistry, and the food industry. By intercepting oxidizing species, predominantly reactive radicals, they prevent cellular damage and polymer or food degradation. Timely challenges in antioxidant research entail the assessment of spatial distributions of antioxidants in heterogeneous biological environments and the quantification of their absolute reaction kinetics.[1,2] With respect to the latter, the reactivity of *tert*-butoxyl (*t*-BuO•) or 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radicals (Scheme 1) toward antioxidants[3,4] has been studied by transient absorption spectroscopy. Alternatively, the reactivity of n,π^* triplet-excited ketones, mostly benzophenone (Ph_2CO), has been analyzed by laser flash photolysis to obtain direct information on antioxidant reactivity,[3,5] since it has long been recognized that n,π^*-excited states behave in a radical-like way and in their reactivity resemble simple alkoxyl radicals.[6−9]

We have now employed the n,π^* *singlet-excited* state of 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) as a model for a radical-like reactive species. This opens for the first time the opportunity to probe for antioxidant reactivity by means of *fluorescence*, both time-resolved and steady-state. The superior performance of fluorescence detection for sensing molecular events, for example, when compared to alternative transient absorption and EPR measurements, is well recognized. It comprises high sensitivity of detection down to the single molecule, high selectivity, for example, little interference from reaction products, subnanosecond temporal and submicrometer spatial resolution, ease of application and access, a great variety of experimental techniques, the possibility of noninvasive measurements, and more than a century of scientific experience.

Scheme 1



Experimental Section

Materials. The azoalkane 2,3-diazabicylco[2.2.2]oct-2-ene (DBO) was synthesized according to the literature procedure.[10] It was purified by sublimation at reduced pressure and by subsequent 2-fold recrystallization from *n*-hexane. The commercial (Fluka) antioxidants ascorbic acid (AA), uric acid (UA), α-tocopherol (α-TOH), and the reduced form of glutathione (GSH) were used as received. Benzophenone (Fluka) was recrystallized from ethanol before use. Spectroscopic grade organic solvents and phosphate buffer (pH = 7) were also employed

(1) *Oxidative Stress, Oxidants and Antioxidants*; Sies, H., Ed.; Academic Press: New York, 1991.
(2) Washko, P. W.; Welch, R. W.; Dhariwal, K. R.; Wang, Y.; Levine, M. *Anal. Biochem.* **1992**, *204*, 1−14.
(3) Evans, C.; Scaiano, J. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1992**, *114*, 4589−4593.
(4) Valgimigli, L.; Banks, J. T.; Ingold, K. U.; Lusztyk, J. *J. Am. Chem. Soc.* **1995**, *117*, 9966−9971.
(5) Encinas, M. V.; Lissi, E. A.; Olea, A. F. *Photochem. Photobiol.* **1985**, *42*, 347−352.
(6) Walling, C.; Gibian, M. J. *J. Am. Chem. Soc.* **1965**, *87*, 3361−3364.
(7) Padwa, A. *Tetrahedron Lett.* **1964**, *5*, 3465−3468.
(8) Paul, H.; Small, R. D.; Scaiano, J. C. *J. Am. Chem. Soc.* **1978**, *100*, 8, 4520−4527.
(9) Griller, D.; Howard, J. A.; Marriott, P. R.; Scaiano, J. C. *J. Am. Chem. Soc.* **1981**, *103*, 619−623.
(10) Askani, R. *Chem. Ber.* **1965**, *98*, 2551−2555.

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(Fluka). Water was bidistilled quality. Deuterated AA and α-TOH were obtained by using D_2O and CH_3OD (Fluka) as solvents or cosolvents, respectively.

Time-Resolved Experiments. Samples were prepared by dissolving DBO (ca. 0.1−1 mM) or benzophenone (ca. 0.1 mM) and the appropriate amounts of antioxidants. The samples were kept at ambient temperature under air (for aqueous solutions), or they were degassed by two *freeze−pump−thaw* cycles (for organic solutions containing α-tocopherol). Homemade quartz cells (4 × 1 × 1 mm) with high-vacuum Teflon stopcocks were used for degassing.

A XeF excimer laser pulse from a Lambda Physics EMG 101 MSC or COMPex 205 laser (351 nm, fwhm ca. 20 ns, pulse energy 40−175 mJ) or an LTB MNL 202 nitrogen laser (337 nm, fwhm ca. 400 ps, pulse energy ca. 0.2 mJ) were used for excitation to obtain the time-resolved fluorescence and transient absorption decays. The fluorescence decays were monitored with a monochromator−photomultiplier setup at 420−500 nm, depending on signal intensity. The effect of uric acid on the transient absorption of triplet benzophenone in phosphate-buffered water ($\tau_0 = 28 \pm 2$ μs) was monitored at 600 nm to avoid overlap with the ketyl radical spectrum.[3]

The kinetic traces were registered by means of a transient digitizer and analyzed by nonlinear least-squares fitting of monoexponential functions. Steady-state fluorescence spectra and quenching was measured with a Spex Fluorolog fluorimeter ($\lambda_{exc} = 340-360$ nm). For the (Stern−Volmer) quenching plots, 4−6 concentrations of antioxidants were chosen up to a maximum quenching effect (Φ_0/Φ_q or τ_0/τ_q) of 3−4. An exception was UA, where the solubility range was limited to ca. 0.35 mM. Transient absorption spectra were recorded with a homemade OMA setup. UV spectra were obtained with a Hewlett-Packard 8452 Diode Array spectrophotometer (2-nm resolution) or with a Perkin-Elmer Lambda 19 spectrophotometer (0.1-nm resolution) from Varian.

Transient Yields. The relative yield of the α-tocopheroxyl (α-TO•) radical resulting from reaction of α-TOH with triplet-excited benzophenone or singlet-excited DBO was quantified from its relative transient absorbance ($\lambda_{max} = 425$ nm) after all quenching was complete (end-OD). Optically matched (OD = 0.40) solutions of DBO and benzophenone in benzene were prepared and the same amount of α-TOH (8 mM) was added subsequently, sufficient to quench ≥95% of the DBO singlets and benzophenone triplets. The end-ODs were measured upon 351-nm laser excitation of the degassed solutions at identical pulse energy and the ratios were taken as the relative yields of the α-TO• radical. Measurements at different pulse energies (up to 25 mJ/cm^2) confirmed the absence of significant nonlinear effects, for example, due to multiphoton absorptions. The end-OD obtained for benzophenone was not corrected for the minor absorption of the ketyl radical at 425 nm.

Quantum Yield Measurements. The quantum yield for reaction of DBO with AA in aerated phosphate-buffer (pH = 7) was determined under conditions of quantitative (≥95%) quenching of singlet-excited DBO. For this purpose, DBO solutions (ca. 12 mM) with an absorbance of ca. 0.6 at the excitation wavelength of 351 nm were prepared, and AA (30−40 mM) was added. Solutions of DBO in aerated water [quantum yield of decomposition (Φ_r) ≈ 1%, ϵ^{364}(max) = 48 ± 3 M^{-1} cm^{-1}, $\epsilon^{351} = 40 \pm 2$ M^{-1}cm^{-1}, this work] or of 2,3-diazabicyclo[2.2.1]-hept-2-ene in *n*-hexane [$\Phi_r = 1.0$,[11] ϵ^{341}(max) = 420 M^{-1} cm^{-1},[12] ϵ^{351} = 12 ± 3 M^{-1} cm^{-1} (lit.:[13] 14 M^{-1} cm^{-1})] served as reference (actinometer). The decay of the absorbance (A) at the excitation wavelength of 351 nm was monitored as a function of incident laser pulses (≡ irradiation time) at constant pulse energy. Plots of $\log([10^{A_0} - 1]/[10^{A} - 1])$ versus irradiation time, that is, the photochemical conversion normalized to conditions of partial rather than total light absorbance,[14] were linear at low conversions, where the interference from competitive absorption of photoproducts was minor. The ratio of the slopes of the logarithmic plots (after correction for the known values of Φ_r and ϵ^{351})[14] provided values of 2% (for DBO as reference) and 4% (for DBH as reference) for the reaction quantum yields of DBO with AA.

Results and Discussion

The measurement of the concentrations of antioxidants and their depletion, as well as of their absolute reactivity, is of significant importance for a variety of medical, biological, and industrial purposes. One possible approach toward the development of alternative probes for antioxidants is to employ fluorescence as a highly sensitive and also otherwise advantageous detection technique. In the following, we have evaluated the potential of the azoalkane DBO to serve as such a fluorescent probe for antioxidants.

Conceptual Approach. The azoalkane DBO is strongly fluorescent (ca. 20% quantum yield in water).[15] Due to the n,π^* electronic configuration and the long lifetime (up to 1 μs) of singlet-excited DBO, it can undergo bimolecular reactions with hydrogen donors.[16] Since antioxidants are well-known to act as such, they should also serve as quenchers of DBO fluorescence. The amount of quenching, quantified through experimental fluorescence quantum yields (Φ) or fluorescence lifetimes (τ) in the absence (Φ_0 and τ_0) or presence (Φ_q and τ_q) of antioxidants, should then be proportional to the concentration of the antioxidant and its reaction rate (k_q) according to the Stern−Volmer expression (eq 1). One of the latter quantities

$$\Phi_0/\Phi_q = \tau_0/\tau_q = 1 + \tau_0 k_q[\text{antioxidant}] \qquad (1)$$

can be determined if the other is known, thus providing the desirable information on the concentration or reactivity of the antioxidants. In other words, the fluorescence lifetime of DBO or the intensity of its fluorescence should constitute a direct measure for the concentration and reactivity of dissolved antioxidants.

Experimental Test. In the present, exploratory study we have used the relationship in eq 1 to measure the absolute reactivity of DBO toward several antioxidants. Indeed, addition of the natural antioxidants, glutathione (GSH),[5] ascorbic acid (AA),[17,18] uric acid (UA),[19−21] and α-tocopherol (α-TOH),[3,5,22] to a solution of DBO (ca. 0.1−1 mM) caused a shortening of the fluorescence lifetime and a concomitant decrease of the fluorescence intensity of DBO (Figure 1). Plots of τ_0/τ_q for 4−6 different antioxidant concentrations according to eq 1 were linear (Figure 2) and provided the fluorescence quenching rate constants of DBO (Table 1). Steady-state fluorescence measurements (Φ_0/Φ_q) gave consistent results.

The data for DBO are compared in Table 1 with the known rate constants for triplet benzophenone (the value for UA stems from the present study), the *tert*-butoxyl and the trichloromethylperoxyl radicals (*t*-BuO• and Cl_3COO•). The latter are examples of reactive alkoxyl and peroxyl radicals. The former

(11) Clark, W. D. K.; Steel, C. *J. Am. Chem. Soc.* **1971**, *93*, 6347−6355.
(12) Mirbach, M. J.; Liu, K.-C.; Mirbach, M. F.; Cherry, W. R.; Turro, N. J.; Engel, P. S. *J. Am. Chem. Soc.* **1978**, *100*, 5122−5129.
(13) Adam, W.; Moorthy, J. N.; Nau, W. M.; Scaiano, J. C. *J. Am. Chem. Soc.* **1997**, *119*, 5550−5555.
(14) Adam, W.; Fragale, G.; Klapstein, D.; Nau, W. M.; Wirz, J. *J. Am. Chem. Soc.* **1995**, *117*, 12578−12592.
(15) Nau, W. M.; Greiner, G.; Rau, H.; Wall, J. Unpublished results.
(16) Adam, W.; Moorthy, J. N.; Nau, W. M.; Scaiano, J. C. *J. Org. Chem.* **1997**, *62*, 8082−8090.
(17) Wayner, D. D. M.; Burton, G. W.; Ingold, K. U. *Biochim. Biophys. Acta* **1986**, *884*, 119−123.
(18) Frei, B.; England, L.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6377−6381.
(19) Ames, B. N.; Cathcart, R.; Schwiers, E.; Hochstein, P. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6858−6862.
(20) Maples, K. R.; Mason, R. P. *J. Biol. Chem.* **1987**, *263*, 1709−1712.
(21) Simic, M. G.; Jovanovic, S. V. *J. Am. Chem. Soc.* **1989**, *111*, 5778−5782.
(22) Burton, G. W.; Ingold, K. U. *Acc. Chem. Res.* **1986**, *19*, 194−201.

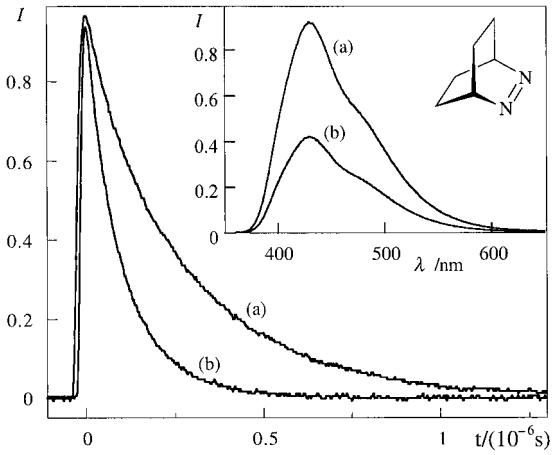


Figure 1. Time-resolved fluorescence decays (λ_{mon} = 450 nm) and steady-state fluorescence spectra (λ_{exc} = 340 nm) of DBO (0.1 mM) in aerated water (phosphate-buffered, pH = 7) in the absence (a) and presence (b) of 1.8 mM ascorbic acid.

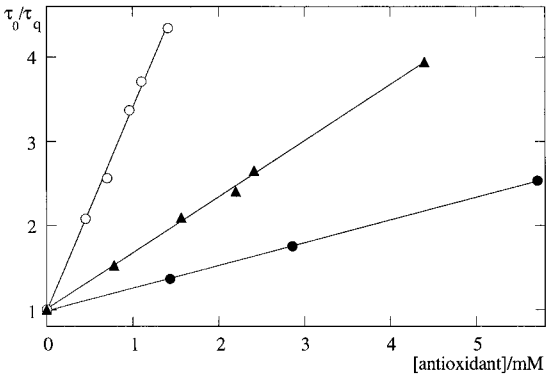


Figure 2. Fluorescence quenching plots of DBO by antioxidants according to eq 1: α-tocopherol in benzene (○) and ascorbic acid (▲) and reduced glutathione (●) in water at neutral pH.

is traditionally quoted for comparison with triplet-excited ketones,[3,8,9,23] whereas the latter is one of the few organic peroxyl radicals for which rate data are available for all examined natural antioxidants.[24]

The comparison of the *absolute* reactivity reveals an excellent agreement between singlet-excited DBO and the triplet state of benzophenone (Table 1).[25] The rate constants follow the order α-TOH > UA > AA > GSH. Moreover, although the absolute reactivity of the Cl_3COO• radical is about 1 order of magnitude lower than that of singlet-excited DBO (Table 1), the general trend, that is, α-TOH > UA > AA > GSH, remains the same as for the excited states. The more pronounced drop in the reactivity of glutathione toward Cl_3COO• can be accounted for in terms of the reactivity−selectivity principle.

The agreement between the reactivity of singlet-excited DBO and triplet-excited benzophenone may be surprising at first glance, since different chromophores and reactive sites (oxygen in benzophenone versus nitrogen in DBO) are involved. Apparently, the common n,π^* configuration of DBO and benzophenone appears to dictate the overall radical-like reactivity toward antioxidants. This photochemical situation is entirely analogous to radical chemistry, where the *nitrogen-centered* DPPH• radical serves to complement the results obtained for *oxygen-centered* radicals such as *t*-BuO•.[4,26] In this train of thought, we consider DBO as a structural equivalent of DPPH•, whereas triplet benzophenone serves as a model for the *t*-BuO• radical. The proposed analogy is presented in Scheme 1.

While the overall consistency of the relative experimental rate constants (Table 1) provides an important prerequisite for the practical use of DBO as a fluorescent probe for antioxidants, there are a number of additional advantages. These comprise good solubility of DBO in practically all solvents, high photostability ($\Phi_r \approx 1\%$ in water), relatively slow oxygen quenching (2.1×10^9 $M^{-1}s^{-1}$ in water), and the absence of intersystem crossing.[27] The long wavelength absorption (λ_{max} ca. 370 nm)[27] for selective laser excitation between 320 and 380 nm combined with the broad fluorescence spectrum, which extends far into the visible range (Figure 1),[15,27] constitute also useful features. Of course, the exceptionally long fluorescence lifetime (e.g., 325 ns in *aerated* water) is the most important feature of DBO. On one hand, it allows for intermolecular quenching effects at practically relevant concentrations of antioxidants (μM−mM range). On the other hand, it results in an enhanced selectivity in time-resolved experiments, since very few compounds display fluorescence in this time regime. This means that fluorescent impurities, for example, in biological systems, do not interfere with the measurement of DBO fluorescence kinetics. For comparison, the fluorescence of ketones is too short-lived and too weak to serve for such purposes.[28]

Mechanistic Aspects. Most experiments were run in aerated phosphate-buffered water at neutral pH, except for α-TOH, for which we have examined kinetic solvent effects in degassed benzene, benzene−methanol, and acetonitrile−water. The rate constant for quenching of DBO fluorescence by α-TOH decreased significantly in the presence of protic solvents, as was previously observed for triplet benzophenone, the *t*-BuO• (Table 1), and several other radicals.[3] This decrease is typically attributed to protection of the reactive OH-bond in α-TOH by hydrogen-bonding. The less pronounced effect for triplet-excited ketones compared to *t*-BuO• has been accounted for in terms of counteracting charge-transfer interactions in the former case.[3] The same argument can be employed for the DBO data (cf. discussion on Selectivity Aspects below).

Ascorbic acid (pK_1 = 4.04 and pK_2 = 11.4)[29] is known to act as a poor electron donor in its neutral form (at pH = 1−2) but as a potent one in the anionic form (at neutral pH).[30,31] Hence, the drop in the reaction rate by nearly 2 orders of magnitude for Cl_3COO• upon lowering the pH from 7 to 1 has been taken as evidence for an electron-transfer reaction with the ascorbate anion (followed or coupled with proton transfer).[30] The fact that singlet-excited DBO displays only a factor of 2 difference upon a similar lowering of the pH (Table 1) could be related to a change in the reaction mechanism in favor of a

(23) Das, P. K.; Encinas, M. V.; Steenken, S.; Scaiano, J. C. *J. Am. Chem. Soc.* **1981**, *103*, 4162−4166.
(24) Neta, P.; Hule, R. E.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1990**, *19*, 413−513.
(25) Interestingly, the agreement for the natural antioxidants appears to be better than for the previously examined "synthetic" hydrogen donors (ref 16), although the solvents were not identical in this study.
(26) Valgimigli, L.; Lucarni, M.; Pedulli, G. F.; Ingold, K. U. *J. Am. Chem. Soc.* **1997**, *119*, 8095−8096.
(27) Solomon, B. S.; Thomas, T. F.; Steel, C. *J. Am. Chem. Soc.* **1968**, *90*, 2249−2258.
(28) Nau, W. M.; Cozens, F. L.; Scaiano, J. C. *J. Am. Chem. Soc.* **1996**, *118*, 2275−2282.
(29) Serjeant, E. P.; Dempsey, B. *Ionization Constants of Organic Acids in Aqueous Solution, IUPAC Chemical Data Series*; Pergamon: Oxford, 1979; Vol. 23.
(30) Neta, P.; Huie, R. E.; Maruthamuthu, P.; Steenken, S. *J. Phys. Chem.* **1989**, *93*, 7654−7659.
(31) Bisby, R. H.; Parker, A. W. *Arch. Biochem. Biophys.* **1995**, *317*, 170−178.

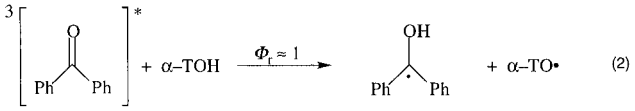
Table 1. Rate Constants (k_q) and Deuterium Isotope Effects [in brackets] for the Quenching of Reactive Intermediates by Natural Antioxidants

antioxidant	solvent	$k_q/10^9$ M^{-1} s^{-1} [k_q(H)/k_q(D)]			
		^{1}DBO*[a]	^{3}Ph$_2$CO*[b]	*t*-BuO•[c]	Cl$_3$COO•[d]
glutathione (GSH)	water (pH = 7)	0.82	0.67		0.003
ascorbic acid (AA)	water (pH = 7)	2.05 [1.07]	1.2		0.11−0.13
	water (pH = 2)	1.18 [1.25]			0.0031[e]
uric acid (UA)	water (pH = 7)	3.4	3.3		0.30−0.32
α-tocopherol (α-TOH)	benzene	5.3	5.1 [1.09]	3.8 [1.31]	0.5
	benzene−MeOH (1.3 M)	3.8 [1.08]	3.7		
	acetonitrile−H$_2$O (2 M)	3.05 [1.16]		0.66 [2.69]	

[a] Error in data is 5%. [b] Data from refs 3, 5 except for UA (this work, 10% error). [c] Data from ref 3. [d] In water/2-propanol ≈ 1/1 mixtures or in water (only GSH) from refs 21, 24, 30. [e] Measured at pH = 1.

direct hydrogen atom abstraction from the heteroallylic oxygen. The small but significant deuterium isotope effects measured for DBO (both for AA and α-TOH, Table 1) support also a direct hydrogen abstraction, since the observed deuterium isotope effects between 1−1.25 are characteristic for hydrogen abstractions close to diffusion control.[3,28] Larger deuterium isotope effects on hydrogen abstractions from ascorbate have only been observed for slower reactions, for example, with α-tocopheroxyl radicals (α-TO•).[31,32] Note that the deuterium isotope effects (and the formation of the expected radicals, see below) provide direct evidence that hydrogen transfer is involved in the quenching process of singlet-excited DBO by antioxidants.

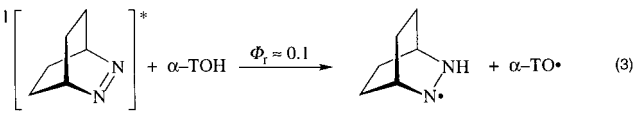
A contrast in the behavior of singlet-excited DBO and triplet-excited benzophenone was noted when the *efficiency* of their reaction with antioxidants was examined. The quenching of triplet benzophenone by α-TOH in eq 2 produces the benzophe-



(2)

none ketyl radical with unit efficiency (the exact value, 88 ± 4%, was considered to be insignificantly different from unity within error).[3] The transient absorption of the ketyl radical derived from hydrogen abstraction (λ_{max} = 545 nm) was monitored in this laser flash photolysis experiment.

Since the hydrazinyl radical derived from hydrogen abstraction by singlet-excited DBO does not absorb within the accessible range,[16] we have employed the transient absorption of the α-TO• radical as a probe (λ_{max} = 425 nm).[3,33] Indeed, this radical is formed upon quenching of singlet-excited DBO by α-TOH, as was confirmed by comparison of the transient absorption spectra. Taking the efficiency of the reaction in eq 2 as unity,[3] we obtained a value of 8 ± 2% for the reaction efficiency between singlet-excited DBO and α-TOH in benzene (eq 3), that is, the reaction efficiency is about 1 order of



(3)

magnitude lower than that for triplet benzophenone.

The reduced efficiency of radical formation for DBO (cf. eq 2 versus eq 3) must be a consequence of its singlet multiplicity, since the hydrogen transfer to singlet-excited states may encounter two additional channels of deactivation. These comprise a crossing (conical intersection) with the ground-state

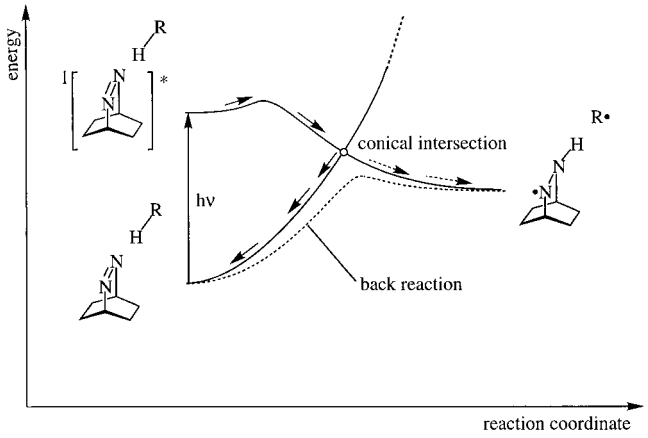


Figure 3. Reaction coordinate for hydrogen atom abstraction of n,π* singlet-excited DBO from antioxidants (H−R). Shown are possible pathways for deactivation through a conical intersection or through back reaction of the (caged) singlet radical pair.

surface and the spin-allowed back-reaction of the caged radical pair (Figure 3).[28,34] The reduced reaction efficiency for radical formation renders DBO relatively photostable in solution, even in the presence of antioxidants. For example, the decomposition quantum yield of DBO in the presence of sufficient ascorbic acid to quench >95% of the excited singlets was determined as only 3 ± 1%. This results in a very slow depletion of DBO upon irradiation, which may facilitate practical applications.

Selectivity Aspects. While the fluorescence of DBO is efficiently quenched by a number of antioxidants (Table 1), it must be considered that other additives may also serve as quenchers. This limitation is often encountered in photochemical applications which rely on quenching effects. For DBO, two additional quenching mechanisms are of prominent interest, singlet energy transfer and charge transfer. The possibility of singlet energy transfer is readily addressed since only compounds with lower singlet energies will be able to quench DBO in this manner. Practically, since the absorption maximum of DBO lies at ca. 370 nm, only colored compounds in sufficiently high concentration will act as singlet energy acceptors. For comparison, energy-transfer-induced quenching of triplet-excited benzophenone occurs with compounds which possess lower *triplet* energies than benzophenone. Since the triplet energies of many compounds lie below that of benzophenone, and those of many others are not even known accurately,[35] complications

(32) Njus, D.; Kelley, P. M. *FEBS Lett.* **1991**, *284*, 147−151.
(33) Packer, J. E.; Slater, T. F.; Willson, R. L. *Nature* **1979**, *278*, 737−738.
(34) Nau, W. M.; Greiner, G.; Wall, J.; Rau, H.; Olivucci, M.; Robb, M. A. *Angew. Chem. Int. Ed.* **1998**, *37*, 98−101.
(35) These facts are due to the small singlet−triplet energy gaps of ketones compared to other, mostly aromatic compounds and due to the general difficulties in determining accurate triplet energies, cf. Turro, N. J. *Modern Molecular Photochemistry*; University Science Books: Mill Valley, 1991.

due to energy transfer should be more severe or at least more uncertain for benzophenone than for DBO.

The probability of charge-transfer to excited states can be semiquantitatively assessed by means of the Rehm−Weller equation ($\Delta G_{et} = 23.1 \times [E_{ox} - E_{red}] - E^*$), where the oxidation potential of the additive (E_{ox}) is kept constant. Accordingly, charge-transfer is favored (negative ΔG_{et}) when the excited-state energy (E^*) is high and when the reduction potential (E_{red}) of the excited state is small. Although the singlet excitation energy of DBO (76 kcal mol^{-1}) is higher than for triplet benzophenone (69 kcal mol^{-1}),[36] this effect is practically counterbalanced by the low reduction potentials of azoalkanes ($E_{red} \leq -2.0$ V).[37] These lie at least 0.2 V lower (corresponding to ca. 5 kcal mol^{-1}) than for benzophenone ($E_{red} \leq -1.8$ V).[37] Clearly, while complications due to charge-transfer-induced quenching present a general drawback of excited-state probes as opposed to "true" radical probes,[3,4,24] it is important to know that for DBO such effects should not be dramatically larger than for triplet benzophenone.

Conclusions

In summary, singlet-excited DBO may serve as a useful, alternative fluorescent probe for antioxidants by providing direct experimental information on the absolute reactivity or concentration of antioxidants, for example, in biological systems. This information is complementary to the known kinetic probes, which rely on transient absorption techniques,[3,4] but is clearly distinct from the established TRAP, ORAC, or ECL-HRP assays for antioxidants,[38−40] which assess their *capacity* under conditions of complete consumption. We note that the fluorescent probe DBO could serve to approach the ultimate challenge of monitoring the spatial distributions and reactions of antioxidants in heterogeneous biological systems, since the fluorescent properties of DBO allow spatial resolution by common filters of fluorescence microscopes. The possibility for measurement in aerated aqueous solution, the relatively high photostability even in the presence of antioxidants, and the general advantages of fluorescence as a detection technique render the further development and practical application of DBO and derivatives as fluorescent probes for antioxidants promising.

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(36) Nau, W. M.; Scaiano, J. C. *J. Phys. Chem.* **1996**, *100*, 11360−11367.

(37) Adam, W.; Moorthy, J. N.; Nau, W. M.; Scaiano, J. C. *J. Am. Chem. Soc.* **1997**, *119*, 6749−6756.

(38) Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Barclay, L. R. C.; Locke, S. J. *Biochim. Biophys. Acta* **1987**, *924*, 408−419.

(39) Whitehead, T. P.; Thorpe, G. H. G.; Maxwell, S. R. J. *Anal. Chim. Acta* **1992**, *266*, 265−277.

(40) Cao, G.; Alessio, H.; Cutler, R. G. *Free Radical Biol. Med.* **1993**, *14*, 303−311.