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# Selective Fluorescence Quenching of 2,3-Diazabicyclo[2.2.2]oct-2-ene by Nucleotides

**Cesar Marquez,[†] Uwe Pischel,[‡] and Werner M. Nau*,[†]**

*School of Engineering and Science, International University Bremen, Campus Ring 1, D-28759 Bremen, Germany, and REQUIMTE/Departamento de Química, Universidade do Porto, R. Campo Alegre, 4169-007 Porto, Portugal*

*w.nau@iu-bremen.de*

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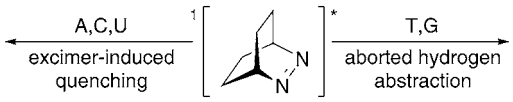
## ABSTRACT




**The fluorescence quenching of 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) by nucleotides has been studied. The quenching mechanism was analyzed on the basis of deuterium isotope effects, tendencies for exciplex formation, and the quenching efficiency in the presence of a molecular container (cucurbit[7]uril). Exciplex-induced quenching appears to prevail for adenosine, cytidine, and uridine, while hydrogen abstraction becomes competitive for thymidine and guanosine. Compared to other fluorescent probes, DBO responds very selectively to the type of nucleotide.**

The photochemical interactions of chromophores with nucleotides and DNA have been the focus of extensive recent research.[1] One aim is to develop "intelligent" and highly sensitive probes for detection and analysis.[2] Commonly, fluorescent probes are employed to achieve high sensitivity.[3] This high sensitivity can be further improved by employing time-resolved techniques along with so-called long-lifetime probes,[4] e.g., with luminescence lifetimes in the microsecond time range. This lifetime-based sensing bypasses problems related to background fluorescence since the latter is short-lived and can be readily removed by applying an electronic time gate. With respect to the "intelligence" of a fluorescent probe, an important goal is the differentiation of nucleotides,[1a] e.g., to obtain information on the precise intercalation site, and the differentiation of single- and double-stranded DNA.[5]

The azoalkane 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO or Fluorazophore-P) is a fluorophore with a weak n,$\pi^*$ absorption in the near-UV ($\lambda_{max}$ = 365 nm in water).[6] It fluoresces in water with a high quantum yield (ca. 20%) over a broad spectral range ($\lambda_{max}$ = 430 nm). DBO has distinct advantages[6] that have stimulated the study of its interaction with nucleotides. The fluorescence of DBO responds strongly to the chemical environment,[6] and DBO fulfills the requirements for a long-lifetime probe since its fluorescence lifetime is

[†] International University Bremen.
[‡] Universidade do Porto.

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exceedingly long, i.e., up to 1 $\mu$s in the gas phase and 325 ns in aerated water. In addition to these features, DBO has a small volume, good solubility in water, and high photostability, all of which favor its use as a fluorescent probe for biomolecules.

In this paper, we find that the fluorescence quenching of DBO displays a high selectivity toward different nucleotides, which has been previously only achieved for triplet-state probes.[1b] DBO combines the high selectivity of the latter with the advantages of fluorescence for detection.

The quenching rate constants ($k_q$) of DBO by the different ribonucleotides were determined by time-resolved fluorescence spectroscopy in $H_2O$ and $D_2O$ (Table 1, see the Supporting Information). The absolute quenching rate constants for the corresponding deoxyribonucleotides were the same, within error.

**Table 1.** Fluorescence Quenching Rate Constants of DBO by Nucleotides and Solvent Deuterium Isotope Effects

| | (d)GMP[b] | dTMP[c] | (d)UMP[b] | (d)CMP[b] | (d)AMP[b] |
|---|---|---|---|---|---|
| $k_q(H_2O)$[a] | 50 | 11 | 2.3 | 2.2 | 1.1[d] |
| IE[e] | 1.4 | 1.0 | 1.0 | 1.0 | 1.0 |

[a] $k_q/(10^7\ M^{-1}s^{-1})$; 10% error. [b] Studied in both the ribose and deoxyribose form. [c] Only studied in the deoxyribose form. [d] 20% error. [e] IE = $k_q(H_2O)/k_q(D_2O)$.

The fact that steady-state fluorescence quenching plots were linear and afforded the same quenching rate constants, within error, as the time-resolved measurement excludes a sizable association between DBO and the nucleotides. The same set of experiments demonstrates that the quenching is dynamic and not static in nature.

The predominant role of the nucleobase in the fluorescence quenching of the singlet-excited state of DBO was confirmed through measurements of model compounds lacking the D-ribose-5-phosphate part, namely adenine-9-acetic acid, cytosine-1-acetic acid, uracil-1-acetic acid, and thymine-1-acetic acid at pH 10 (see Table 4 of the Supporting Information).[7] The order of the quenching rate constants measured for these nucleobase derivatives resembled that measured for the nucleotides, except that the values were slightly larger (by ca. 20%), which may be indicative of a steric or diffusional effect exhibited by the sugar residue. The quenching by the nucleotides can therefore be assigned to a predominant interaction with the nucleobases.

The clearcut order of the quenching efficiency of DBO is G > T > U ≈ C > A, spanning more than 1 order of magnitude (ca. factor 40) between guanine and adenine. Comparison of the kinetic data with those for quenching of some other fluorescent dyes (Table 2) reveals that DBO shows a significantly improved selectivity. The quenching rate constants for the coumarines **1**−**4** described by Seidel et al.[1a] vary only by a factor of 4. Wenska described a similar behavior for the fluorescence quenching of *N*-methyl-1-naphthylcarbamate **5**.[8] While the selectivity of singlet-excited DBO toward nucleotides is remarkable for a fluorescent probe, it must be noted that there are some triplet states,[1b,9] which show also a high selectivity toward nucleotides, e.g., the ketones **6** and **7** (Table 2). However, one must recall that triplet states, unlike DBO, do not have the potential to serve as luminescent probes in water under air. This may present the major practical and conceptual advancement of the use of DBO.

**Table 2.** Rate Constants, $k_q/(10^7\ M^{-1}\ s^{-1})$, for Excited-State Quenching of Selected Fluorescent Dyes (from refs 1a and 8) and Triplet States (from ref 1b) by Nucleotides in Water

| excited state[a] | (d)GMP | (d)TMP | UMP | (d)CMP | (d)AMP |
|---|---|---|---|---|---|
| $^1$DBO* | 50 | 11 | 2.5 | 2.2 | 1.4 |
| $^1$**1*** | 250 | 170 | | 60 | 350 |
| $^1$**2*** | 530 | 250 | | 130 | 420 |
| $^1$**3*** | 470 | 140 | 120 | 230 | 390 |
| $^1$**4*** | 180 | 290 | 230 | 160 | |
| $^1$**5*** | 170 | | 190 | 170 | 130 |
| $^3$**6*** | 200 | 140 | 30 | 4 | 230 |
| $^3$**7*** | 130 | 1 | <5 | <5 | <5 |

[a] Chromophores labeled as follows: **1**, 7-methoxycoumarin; **2**, 7-methoxycoumarin-3-carboxylic acid ethyl ester; **3**, 3-cyano-7-methoxycoumarin; **4**, 7-amino-4-methylcoumarin; **5**, *N*-methyl-1-naphthylcarbamate; **6**, benzophenone; **7**, 3-methoxyacetophenone.

The major underlying reason for the improved selectivity lies in the lower quenching rate constants of DBO (Table 2), which ensure, resting on the reactivity−selectivity principle, an enhanced selectivity for the less reactive DBO. In fact, most fluorescent dyes **1**−**5** undergo quenching by nucleotides close to the diffusion-controlled limit. This fast quenching is a requirement to measure significant quenching of these dyes at practical nucleotide concentrations, because their singlet-excited states are shorter lived than DBO. A closer inspection of the quenching rate constants in Table 2 reveals further that adenosine is an efficient quencher of most excited states, but is least efficient in quenching DBO fluorescence. This suggests a change in the quenching mechanism, which may also contribute to the improved selectivity.

Fluorescence quenching of coumarines by nucleotides occurs by electron transfer.[1a] Fluorescence quenching of DBO is known to proceed either through hydrogen atom abstraction[6a−c] or exciplex formation[6e,10,11b] with subsequent rapid deactivation (energy transfer can be readily ruled out due to the low excitation energy of DBO). The two described quenching mechanisms of DBO are chemically inefficient, i.e., they display low photoproduct quantum yields. This is

(7) Note that the acetic acid residue does not contribute to the quenching since sodium acetate (up to 0.2 M) does not cause sizable fluorescence quenching of DBO.

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due to a mechanistic peculiarity, namely the occurrence of a conical intersection along the excited-state reaction pathway, which results in an "aborted" hydrogen or electron transfer.[6a,10] To confirm the low quantum yields, we have also determined this parameter representatively for the photoreaction of DBO with GMP, since guanine is the most reactive nucleobase. Expectedly, the quantum yield was found to be negligibly small (<1%, see the Supporting Information), which also discouraged the further search for intermediates by transient absorption or photoproducts. The low photoreaction quantum yield increases the photostability when used as fluorescent probe.

Exciplex formation requires partial charge transfer between the excited-state reactant and the nucleobase. To evaluate the degree of charge transfer, it is common to compare the driving force for a full electron transfer ($\Delta G°_{ET}$) as calculated according to Rehm and Weller (Table 3).

**Table 3.** Driving Force for Electron Transfer (in eV) between Singlet-Excited DBO and the Nucleobases[a]

| | G | T | U | C | A |
|---|---|---|---|---|---|
| $\Delta G°_{ET}$ ($N/N^{\bullet -}$) | 1.09 | 0.51 | 0.40 | 0.68 | 0.85 |
| $\Delta G°_{ET}$ ($N^{\bullet +}/N$) | 0.69 | 1.31 | 1.59 | 1.34 | 1.16 |

[a] The redox potentials for DBO were taken as $\Delta E_{p,ox} = 1.45$ V (vs SCE in acetonitrile) and $\Delta E_{p,red} = -2.8$ V (vs SCE in acetonitrile), cf. ref 11. The redox potentials of the nucleotides, in acetonitrile for oxidation and in dimethylformamide for reduction, were taken from ref 1a. The excitation energy of DBO was taken as 3.30 eV, and the Coulomb term was approximated as 0.06 eV in these solvents.

As can be seen, the driving force for electron transfer is significantly endergonic for both the reduction as well as the oxidation of the nucleobases. The thermodynamic data render electron transfer to or from DBO unlikely,[12] even if one allows for an additional driving force contribution due to a coupled proton transfer in the incipient nucleotide radical ion from or to water (as solvent).[1a,13−15] Wenska has placed an energetic contribution of 0.5 eV to this process,[8] which is presumably an upper limit.

Due to the endergonic electron-transfer energetics, only a partial electron transfer can take place in the quenching process with nucleobases. In the cases where the nucleobases serve as electron donors, this is likely to involve the formation of an n, $\pi^*$ exciplex, which we have described in detail for DBO,[6d,10,11b] as well as acetone,[16] with amines as donors. Exciplex-induced quenching depends, like electron transfer, on the electron-donor and electron-acceptor strengths. If one makes predictions exclusively on the basis of the calculated energetic data in Table 3, one expects the order U > T > C ≈ G > A for the ease of exciplex formation, which is the order of decreasingly favorable driving force and would involve a reduction for U, T, C, and A, and an oxidation for G. The experimental order of the quenching rate constants is G > T > U ≈ C > A, which indicates that G and T fall out of the expected order. For G and T hydrogen abstraction may compete with exciplex-induced quenching which may cause those nucleotides to react at particularly high rates.

To assess the participation of hydrogen abstraction, which is well-known for DBO and reflects the n,$\pi^*$ electronic configuration of its singlet-excited state, we have studied solvent deuterium isotope effects (IE values in Table 1). These are indicative for abstraction of solvent-exchangeable hydrogens, i.e., purine and pyrimidine N−H.[17] Only guanine, the fastest quencher, shows an experimentally significant (>10%) solvent isotope effect.

The absence of significant solvent deuterium isotope effects for the other nucleobases comes as a surprise since their quenching rate constants are lower, which usually allows isotope effects to become more pronounced as a consequence of the reactivity−selectivity principle.[18] It is therefore justified to conclude that hydrogen abstraction from the exchangeable N−H hydrogens operates predominantly for guanine. The rate of hydrogen abstraction from comparable bonds (N−H) should strongly depend on the bond strengths.[19] In fact, both the endocyclic and exocyclic N−H bonds of guanine have the lowest N−H homolytic bond dissociation energies, more than 2 kcal $mol^{-1}$ lower than for the other nucleobases according to UHF/AM1 calculations.[20] Accordingly, guanine should be most reactive toward hydrogen abstraction, which is consistent with the observed deuterium isotope effect and the enhanced quenching rate constant. The extreme position of guanine can therefore be rationalized.

For thymine, on the other hand, one must consider hydrogen abstraction from the labile α methyl hydrogens, which, however, are not subject to solvent exchange such that no solvent deuterium isotope effect is expected. This quenching mechanism has been documented for n,$\pi^*$ triplet-

(12) Note that electron transfer is the most common quenching mechanism for nucleobases: (a) Anderson, R. F.; Wright, G. A. *Phys. Chem. Chem. Phys.* **1999**, *1*, 4827−4831. (b) Saito, I.; Takayama, M.; Sugiyama, H.; Nakatani, K.; Tsuchida, A.; Yamamoto, M. *J. Am. Chem. Soc.* **1995**, *117*, 6406−6407. (c) Torimura, M.; Kurata, S.; Yamada, K.; Yokomaku, T.; Kamagata, Y.; Kanagawa, T.; Kurane, R. *Anal. Sci.* **2001**, *17*, 155−160.

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(17) Proton exchange of the 8-C−H occurs only on a time scale of weeks: (a) Benevides, J. M.; Lemeur, D.; Thomas, G. J., Jr. *Biopolymers* **1984**, *23*, 1011−1024. (b) Thomas, G. J., Jr.; Livramento, J. *Biochemistry* **1975**, *14*, 5210−5218. (c) Elvidge, J. A.; Jones, J. R.; O'Brien, C.; Evans, E. A.; Sheppard, H. C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 174−176. (d) Gamble, R. C.; Schimmel, P. R. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 1356−1360. $^1$H NMR experiments demonstrated that this rate is not enhanced in the presence of DBO (this work).

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(20) Calculations were performed within Hyperchem 6.01 (Hypercube, Inc., Gainesville, FL).

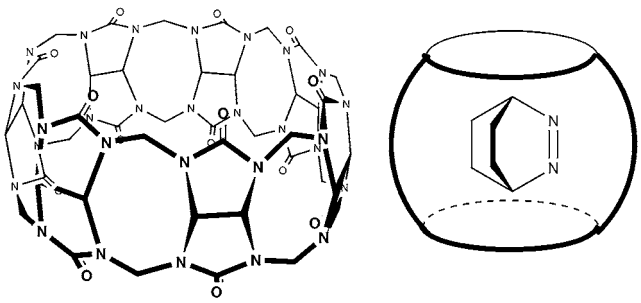
excited benzophenone (from thymine)[21] as well as for DBO (from toluene).[6c] Only hydrogen abstraction from the α methyl group of thymine can account for the large difference between thymine and uracil and the observed order, i.e., T > U ≈ C > A. The latter cannot be reconciled alone by means of the energetics for electron transfer, which should govern exciplex formation (Table 3).

We conclude, based on the observed deuterium isotope effect for guanine and the enhanced quenching rate for thymine, that besides exciplex formation hydrogen abstraction competes in the quenching process for these two most efficient quenchers. The absence of significant solvent deuterium isotope effects for the other bases, on the other hand, demonstrates that the N−H bonds of the nucleobases are not involved to a sizable extent. We presume that exciplex-induced quenching is mainly operative for these nucleobases. This means that the quenching mechanism varies in dependence on the nucleobase, which has also been pointed out by Wood and Redmond for triplet states.[1b,13]

For future applications, including studies of oligonucleotide flexibility analogous to our recent studies on polypeptides,[22] it is essential to provide compelling *experimental* evidence that the fluorescence quenching of DBO by nucleotides requires indeed a close molecular contact, either in the form of a hydrogen-transfer transition state or an exciplex, and does not occur through-space or through-solvent. The latter applies for other quenching mechanisms, in particular fluorescence resonance energy transfer and electron transfer.

It is possible to include DBO into the cavity of cucurbit[7]uril (CB7)[23] and, thus, to form quantitatively ($K$ ca. $4 \times 10^5$ $M^{-1}$) a supramolecular host−guest complex (CB7•DBO),[6d] which excludes an intimate contact between the (excited) chromophore and external additives. In essence, CB7 acts as a protective shield toward the attack by external quenchers. This supramolecular inclusion prevents excited-state hydrogen abstraction or exciplex formation, while maintaining the possibility, perhaps at a reduced rate, of through-space electron or energy transfer over a moderate distance (ca. 5−8 Å) through the walls of the supramolecular cage.[24]

None of the nucleotides, which are efficient quenchers of free singlet-excited DBO, caused any quenching of the long-lived excited CB7•DBO complex even at 0.2 M nucleotide concentrations. At first glance, this is surprising since the fluorescence lifetime of the CB7•DBO complex (950 ns in aerated 0.2 M $Na_2SO_4$ solution) is much longer, as a consequence of the shielding from quenching by water and oxygen, than for DBO in aerated water (325 ns), thus providing an improved dynamic range to observe quenching. The absence of quenching suggests, for all cases studied (dGMP, dAMP, dTMP, dCMP, and UMP), quenching rate constants $< 5 \times 10^5$ $M^{-1}$ $s^{-1}$. This demonstrates experimentally that the quenching of DBO requires indeed close molecular contact (hydrogen transfer, exciplex formation). Note that the nucleotides themselves are not included by CB7. Incidentally, it should be noted that the comparison of the quenching rate constants of DBO in the absence and presence of the molecular container provides a novel and unique photophysical tool to establish the quenching mechanisms of this excited state.



CB7 CB7•DBO

In summary, the exceptionally long fluorescence lifetime of DBO even in water under air ($\tau_0 = 325$ ns), the convenient access to derivatives, its high photostability in the absence and presence of quenchers, the high selectivity, in particular toward the two purine nucleotides, and the necessity of contact to induce fluorescence quenching characterize DBO as a useful fluorophore for future work with nucleotides and as a potential *intelligent* fluorescent probe.

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**Supporting Information Available:** Experimental and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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