

Detection of biomacromolecules with fluorescent light-up probes*

Katja Faulhaber¹, Anton Granzhan², Heiko Ihmels^{2,‡}, and Giampietro Viola³

¹*Institute of Organic Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany;* ²*Organic Chemistry, University of Siegen, Adolf-Reichwein-Straße, D-57068 Siegen, Germany;* ³*Department of Pharmaceutical Sciences, University of Padova, via Marzolo 5, I-35131 Padova, Italy*

Abstract: The emission properties of selected benzo[*b*]quinolizinium (acridizinium) derivatives in the presence of double-stranded DNA and proteins are presented. Spectrophotometric studies and linear dichroism (LD) spectroscopic experiments reveal that benzo[*b*]quinolizinium derivatives bind to DNA, mainly by intercalation. In contrast to the 9-amino-benzo[*b*]quinolizinium, which exhibits a moderate emission quantum yield in water, the 6-aminobenzo[*b*]quinolizinium ion as well as *N*-phenyl-9-aminobenzo[*b*]quinolizinium derivatives are almost nonfluorescent. The low intrinsic fluorescence quantum yields of the latter compounds are caused by conformational changes in the excited state, as shown by a linear double-logarithmic plot of the emission quantum yield vs. the solvent viscosity. Most notably, the fluorescence intensities of these dyes increase significantly by a factor of 10 to 50 upon addition of double-stranded DNA or proteins such as human serum albumin (HSA) or chicken egg albumin (CEA). Thus, these compounds exhibit ideal properties to be used as DNA- or protein-sensitive light-up probes.

Keywords: photochemistry; organic chemistry; fluorescent probes; analytical chemistry; organic dyes.

INTRODUCTION

The detection and labeling of biomacromolecules by organic fluorescent probes is widely used in analytical chemistry, biochemistry, and medicine, mainly because of the high sensitivity of this method [1]. Recent developments in near-field microscopy and confocal microscopy techniques even allow the detection of single fluorescent molecules and probing of the microenvironment of the dye molecule. In particular, organic dyes offer the opportunity to tune the chemical and photophysical properties of fluorescent probes by a systematic variation of the substitution pattern, and thus provide access to an almost unlimited range of possible applications.

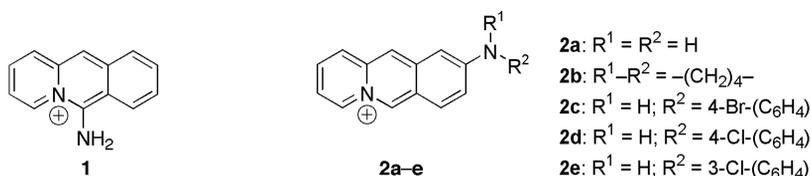
Fluorescent probes whose emission intensity increases upon association with DNA or proteins are useful tools in genomics and proteomics, because the binding event to the host molecule may be followed by the appearance of an intense fluorescence (“light-up probes”) [2]. For example, DNA staining by ethidium bromide is widely used to visualize DNA fragments in a gel [3]. Similarly, proteins,

*Paper based on a presentation at the XXIst IUPAC Symposium on Photochemistry, 2–7 April 2006, Kyoto, Japan. Other presentations are published in this issue, pp. 2193–2359.

[‡]Corresponding author

that are separated in polyacrylamide gel electrophoresis [4] or in microchip capillary electrophoresis [5], are commonly stained by fluorescent probes such as 1-anilino-8-naphthalenesulfonate (1,8-ANS) [6], Nile Red [7], or the SYPRO dye family [8]. Thus, several efficient DNA and protein stains are available. Nevertheless, studies toward a better understanding of the origin of the fluorescence response of a given fluorophore are scarcely addressed in the literature. For example, the mechanism of the fluorescence enhancement of ethidium bromide upon binding to DNA has been a matter of debate for more than 20 years [9]. Even less investigated is the mechanism of protein detection by light-up probes. Especially in the latter case, only few systematic strategies for the *rational design* of light-up probes or detailed analyses of structure–property relationships are published.

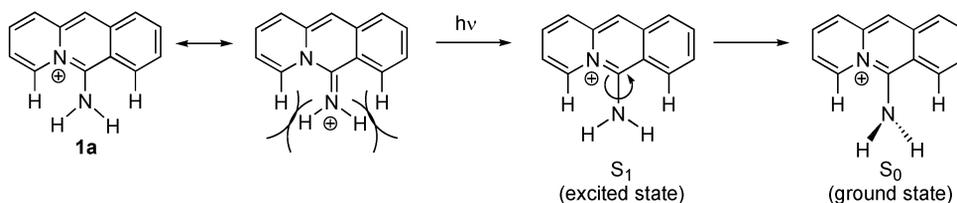
During our studies on the photophysical and photobiological properties of benzo[*b*]quinolizinium derivatives, we discovered that the 6-aminobenzo[*b*]quinolizinium (**1**) and the *N*-aryl-9-aminobenzo[*b*]quinolizinium derivatives **2c–e** exhibit a remarkable potential to be used as fluorescent light-up probes. In this contribution, we discuss the photophysical properties of **1** and **2c–e** and demonstrate that these dyes may be used as DNA and protein stains. For comparison, the properties of compounds **2a** and **2b** are presented to assess whether the *N*-aryl-substitution in **2c–e** is a general requirement to establish the light-up probe.



EMISSION PROPERTIES OF AMINO-SUBSTITUTED QUINOLIZINIUM DERIVATIVES

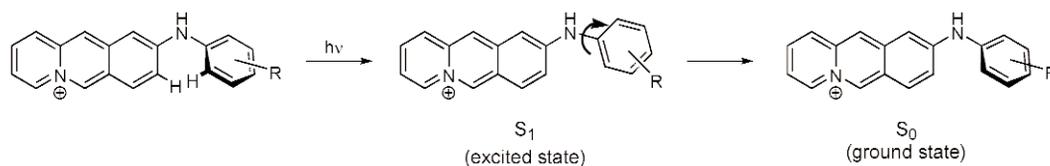
Aqueous solutions of the unsubstituted 9-aminobenzo[*b*]quinolizinium (**2a**) and the dialkyl-substituted derivative (**2b**) show a pronounced fluorescence (**2a**: $\lambda_{fl} = 507$ nm, $\phi_{fl} = 0.12$; **2b**: $\lambda_{fl} = 535$ nm, $\phi_{fl} = 0.36$) [10]. These fluorophores exhibit only a moderate solvatochromism. As a representative example, the emission maximum of the 9-aminobenzo[*b*]quinolizinium (**2a**) changes from $\lambda = 497$ nm in acetic acid to $\lambda = 516$ nm in dimethyl sulfoxide (DMSO). In contrast, the 6-aminobenzo[*b*]quinolizinium (**1**) and the *N*-aryl-substituted benzo[*b*]quinolizinium ions **2c–e** are almost non-fluorescent, i.e., in water as well as in organic solvents, the fluorescence quantum yields ϕ_{fl} are smaller than 0.01 [11,12].

In the case of the 6-aminobenzo[*b*]quinolizinium (**1**), the low fluorescence quantum yield is unlikely to be caused by specific interactions with the solvent, because the quantum yield is equally low in protic and aprotic solvents such as H_2O , alcohols, acetonitrile, DMSO, or dimethylformamide (DMF). In addition, the analysis of the structure of **1a** in the solid state reveals a C(6)–N bond length of 132 pm [13], which reflects a high degree of the double-bond character. This structural feature causes a significant steric repulsion between the amino functionality and the 4-H and 7-H proton. Thus, it is proposed that the radiationless deactivation of the excited state is due to bond lengthening and subsequent rotation around the exocyclic C(6)–N bond, which reduces the steric repulsion (Scheme 1).



Scheme 1 Radiationless deactivation of excited **1a** due to C–N rotation in the excited state.

The low intrinsic fluorescence quantum yields of the *N*-arylamino benzo[*b*]quinolizinium ions were proposed to be due to the rotation about the N–C_{phenyl} bond in the excited state (Scheme 2). Similar deactivation pathways have been demonstrated for a series of 2-*N*-arylamino-6-naphthalene-sulfonate derivatives [14].



Scheme 2 Radiationless deactivation of excited **2c–e** due to N–Ar rotation in the excited state.

This assumption is further supported by the marked dependence of the fluorescence quantum yield on the viscosity of the solvent. Thus, in glycerol–water mixtures the fluorescence quantum yield of, e.g., **2e**, increases significantly with the solvent viscosity, i.e., with increasing glycerol content (Fig. 1A). Moreover, a double-logarithmic plot of the quantum yield vs. the viscosity is linear ($r^2 = 0.991$, Fig. 1B), which provides a supporting evidence that a change of the conformation in the excited state is a crucial factor that causes the low intrinsic emission quantum yield [15].

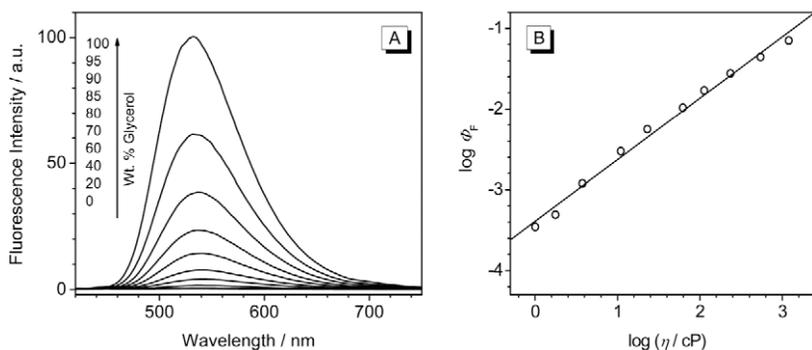


Fig. 1 (A) Fluorescence emission spectra of derivative **2e** in glycerol–water mixtures of varied viscosity; excitation wavelength $\lambda_{\text{ex}} = 390$ nm. (B) Viscosity dependence of the fluorescence quantum yield of **2e**.

INTERACTION WITH NUCLEIC ACIDS

The interactions of the benzo[*b*]quinolizinium derivatives **1** and **2** with DNA were determined by spectrophotometric titrations and “flow” linear dichroism (LD) spectroscopy. Such as the parent benzo[*b*]quinolizinium and derivatives thereof [16], the association of the derivatives **1** and **2** with DNA is clearly indicated by a red-shift of the absorption maximum of the dye, along with a significant hypochromism (see Fig. 2A for a representative example) [11,12]. The Scatchard plots resulting from the spectrophotometric titrations were used to determine the binding constants, which range from 10^4 to 10^5 M⁻¹. In addition, LD spectroscopic investigations in a flow cell reveal an intercalative binding mode, as indicated by the negative LD signal in the long-wavelength absorption of the benzo[*b*]quinolizinium derivatives (Fig. 2B). Moreover, the LD signals are almost wavelength-independent, indicating that the intercalative binding mode is adopted almost exclusively under these conditions. Nevertheless, the photometric titrations, in particular the loss of isosbestic points, indicate the contribution of other binding modes such as groove binding or π stacking along the DNA backbone to the overall binding at higher DNA-to-dye ratios.

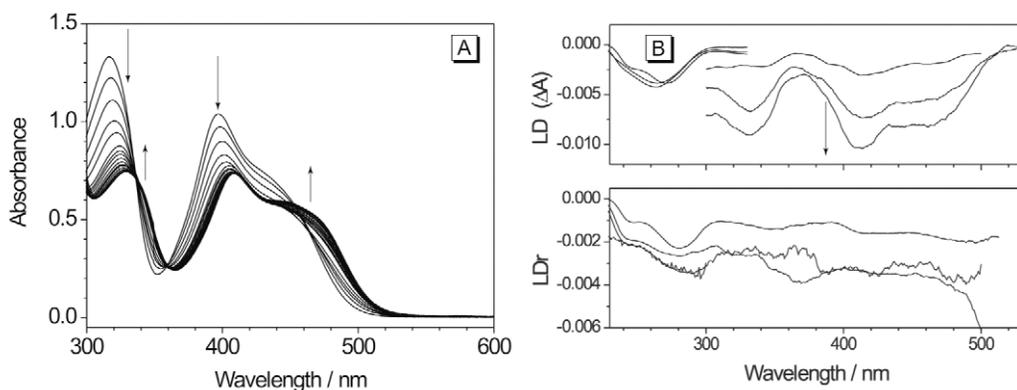


Fig. 2 (A) Spectrophotometric titrations of ct DNA to compound **2d** in aqueous phosphate buffer. (B) Flow LD (upper panel) and reduced LD (lower panel) spectra of **2d**-DNA complexes at ligand-to-DNA ratios of 0.04, 0.08, and 0.2.

FLUORIMETRIC DETECTION OF DNA AND SELECTED PROTEINS

The emission quantum yield of 6-aminobenzo[*b*]quinolizinium (**1**) changes in the presence of DNA (Fig. 3); however, the emission intensity increases only slightly ($I/I_0 = 2-3$) upon addition of calf thymus DNA (ct DNA) or [poly(dG-dC)-poly(dG-dC)]. In contrast, a much higher emission quantum yield ($I/I_0 \approx 33$) was observed upon addition of [poly(dA-dT)-poly(dA-dT)] (Fig. 3) [11].

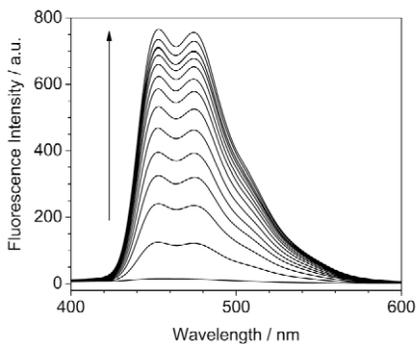


Fig. 3 Spectrofluorimetric titration of [poly(dA-dT)-poly(dA-dT)] to 6-aminobenzo[*b*]quinolizinium (**1**) in aqueous phosphate buffer. The arrow indicates the change of the emission intensity with increasing DNA concentration [17].

The increase of the emission intensity upon DNA addition may be explained by a significant suppression of the C(6)-NH₂ bond rotation within the intercalation binding site of the DNA, and thus the radiationless deactivation of the excited state. The significantly smaller increase of the emission intensity in GC-rich DNA, however, shows that other processes in the excited state need to be considered. In this particular case, fluorescence quenching by an electron-transfer (ET) reaction between the excited dye and the DNA is responsible for the lower emission quantum yield in GC-rich binding sites. The reduction potential of the excited **1** was estimated to be slightly smaller than 2.0 V in isotropic acetonitrile solution [11], while the oxidation potentials of guanine and adenine in acetonitrile are 1.47 and 1.94, respectively [18]. Although these data do not necessarily reflect the actual redox potentials in the binding site, it may be proposed that the photoinduced ET between excited aminoacridizinium **1** and gua-

nine is exergonic, whereas the ET reaction with adenine is energetically disfavored. Thus, in ct DNA and [poly(dG-dC)-poly(dG-dC)], two effects with opposite results take place: (a) the emission intensity increases due to conformational restriction and (b) the emission intensity decreases due to ET with the guanine bases. Since the latter effect is not possible in AT-rich regions, a much higher fluorescence is observed therein. The weak emission enhancement in the presence of ct DNA is likely due to the higher binding affinity of **1** toward GC base pairs.

The addition of DNA to *N*-arylamino benzo[*b*]quinolizinium derivatives **2c–e** leads also to an increased emission intensity of these dyes [12], whereas the fluorescence of **2a** and **2b** is significantly quenched in the presence of DNA [10]. Notably, the fluorescence quantum yield of the intercalated dyes depends on the substitution pattern. Thus, the emission intensity of the 4-bromo-substituted derivative **2c** only increases by a factor of about 2 upon addition of DNA (Fig. 4A), whereas the emission intensity of the chloro-substituted derivatives **2d** and **2e** increases by a factor of 30 (**2d**) and 50 (**2e**) in the presence of DNA (Fig. 4B). The latter fluorescence enhancements are large enough to be applied in DNA detection (e.g., in gel electrophoresis). For comparison, the emission intensity of ethidium bromide (i.e., one of the commonly used DNA stains) increases only by a factor of up to 10 in the presence of DNA [1a]. It should be mentioned, however, that several DNA stains on the basis of monomethine dyes, such as PicoGreen and dimeric dyes YOYO and TOTO exhibit much more pronounced emission enhancements ($I/I_0 > 1000$) [1a].

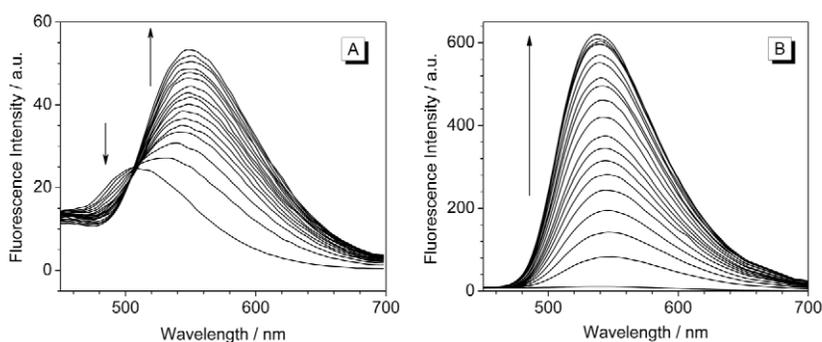


Fig. 4 Spectrofluorimetric titrations of ct DNA to compounds **2c** (A) and **2e** (B) in aqueous phosphate buffer.

The smaller fluorescence enhancement of **2c** compared to the one of **2d** and **2e** may be attributed to the internal heavy atom effect in **2c**, which reduces the quantum yield of the fluorescence by intersystem crossing, such as in resembling 2-*N*-arylamino-6-naphthalenesulfonate systems [19]. Although this enhancement of the emission intensity is too small to be used for DNA staining, the red-shift of the emission maximum offers an opportunity for a *ratiometric* DNA detection.

The propensity of the fluorescent probe **2e** to stain proteins was studied with selected proteins, namely bovine serum albumin (BSA), human serum albumin (HSA), and chicken egg white albumin (CEA). Since the main application of protein detection is the staining in electrophoresis gels, the experiments were performed in the presence of an anionic surfactant (sodium dodecyl sulfate, SDS). The latter forms micelles with protein guest molecules and thus provides sufficient mobility of the proteins within the gel matrix. The fluorescence of **2e** increases by a factor of approximately 20 upon addition of the proteins (Fig. 5A). Notably, at low protein concentrations (0–50 $\mu\text{g mL}^{-1}$), the relationship between the fluorescence intensity of **2e** and the protein concentration is linear (Fig. 5B), which may allow the quantitative fluorimetric detection of the proteins in solution within this concentration range.

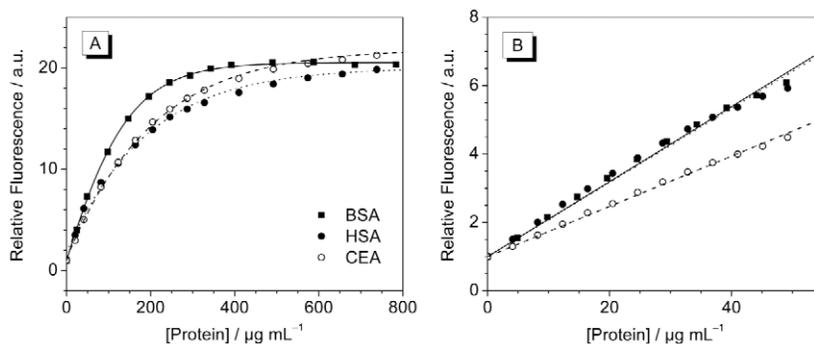


Fig. 5 (A) Fluorescence enhancement upon titration of proteins (BSA: squares and solid lines; HSA: filled circles and dashed lines; CEA: empty circles and dotted lines) to compound **2e** (10 μM in aqueous phosphate buffer in the presence of 0.05 % SDS). (B) Linear regions of titration curves.

CONCLUDING REMARKS

We demonstrated that the 6-aminobenzo[*b*]quinolizinium fluorophore as well as *N*-arylamino-substituted benzo[*b*]quinolizinium derivatives exhibit a very low intrinsic fluorescence quantum yield because of conformational changes in the excited state. Upon association with biomacromolecules such as DNA or proteins, however, the fluorescence intensity of these compounds increases significantly. Considering the straightforward derivatization, especially of the *N*-aryl-9-aminobenzo[*b*]quinolizinium derivatives, these fluorophores represent a promising platform for the design of a series of light-up probes whose photophysical properties may be studied in detail. Such studies should gain more knowledge about the basic mode of action of light-up probes and allow the intentional construction of even more efficient and selective fluorescent stains.

ACKNOWLEDGMENTS

This work was generously financed by the Deutsche Forschungsgemeinschaft, the Deutscher Akademischer Austauschdienst (Vigoni-Programme), and the Fonds der Chemischen Industrie.

REFERENCES

- (a) G. Cosa, K. S. Focsaneanu, J. R. N. McLean, J. P. McNamee, J. S. Scaiano. *Photochem. Photobiol.* **73**, 585 (2001); (b) E. S. Morozkin, P. P. Laktionov, E. Y. Rykova, V. V. Vlassov. *Anal. Biochem.* **322**, 48 (2003); (c) G. Lee, C. H. Chen, L. A. Chiu. *Cytometry* **7**, 508 (1986).
- P. Prentø. *Biotech. Histochem.* **76**, 137 (2001).
- P. A. Sharp, B. Sugden, J. Sambrook. *Biochemistry* **12**, 3055 (1973).
- L. R. Williams. *Biotech. Histochem.* **76**, 127 (2001).
- (a) A. E. Herr, A. K. Singh. *Anal. Chem.* **76**, 4727 (2004); (b) B. C. Giordano, L. Jin, A. J. Couch, J. P. Ferrance, J. P. Landers. *Anal. Chem.* **76**, 4705 (2004).
- (a) B. K. Hartman, S. Udenfriend. *Anal. Biochem.* **30**, 391 (1969); (b) P. M. Horowitz, S. Bowman. *Anal. Biochem.* **165**, 430 (1987).
- J.-R. Daban, S. Bartolomé, M. Samsó. *Anal. Biochem.* **199**, 169 (1991).
- (a) K. Berggren, E. Chernokalskya, T. H. Steinberg, C. Kemper, M. F. Lopez, Z. Diwu, R. P. Haugland, W. F. Patton. *Electrophoresis* **21**, 2509 (2000); (b) T. H. Steinberg, L. J. Jones, R. P. Haugland, V. L. Singer. *Anal. Biochem.* **239**, 223 (1996).
- N. Luedtke, Q. Liu, Y. Tor. *Chem. Eur. J.* **11**, 495 (2005).
- H. Ihmels, K. Faulhaber, B. Engels, C. Lennartz. *Chem. Eur. J.* **6**, 2854 (2000).

11. H. Ihmels, K. Faulhaber, K. Wissel, G. Viola, D. Vedaldi. *Org. Biomol. Chem.* **1**, 2999 (2003).
12. A. Granzhan, H. Ihmels. *Org. Lett.* **7**, 5119 (2005).
13. CCDC reference number 216672. See <<http://www.rsc.org/suppdata/ob/b3/b305439j/>> for crystallographic data in CIF or other electronic format.
14. E. M. Kosower. *Acc. Chem. Res.* **15**, 259 (1982).
15. T. Förster, G. Hoffmann. *Z. Physik. Chem., NF* **75**, 63 (1971).
16. H. Ihmels, K. Faulhaber, D. Vedaldi, F. Dall'Acqua, G. Viola. *Photochem. Photobiol.* **81**, 1107 (2005).
17. K. Faulhaber. Dissertation (Dr. rer. nat.), University of Würzburg, Germany (2003). Available at: <<http://opus.bibliothek.uni-wuerzburg.de/opus/volltexte/2003/679/>>.
18. C. A. M. Seidel, A. Schulz, M. H. M. Sauer. *J. Phys. Chem.* **100**, 5541 (1996).
19. E. M. Kosower, H. Dodiuk. *J. Phys. Chem.* **82**, 2012 (1978).