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# *N*-Aryl-9-amino-Substituted Acridizinium Derivatives as Fluorescent "Light-Up" Probes for DNA and Protein Detection

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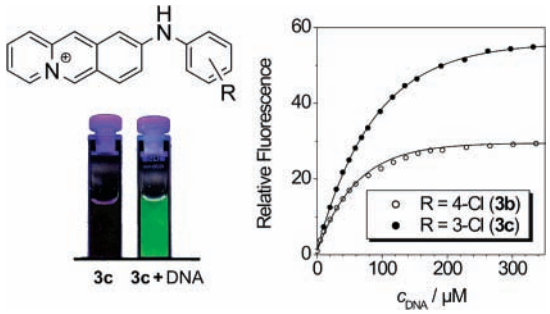
## ABSTRACT




***N*-Arylamino-substituted acridizinium (benzo[*b*]quinolizinium) derivatives are almost nonfluorescent in water or organic solvents; however, upon addition of calf thymus DNA or bovine serum albumin the fluorescence intensity increases by a factor of 10 to 50. Thus, these dyes exhibit ideal properties to be used as DNA- and protein-sensitive "light-up probes".**

The detection and labeling of biomacromolecules by fluorescence probes has developed into a powerful tool in analytical biochemistry and medicine. Fluorescence probes, such as cyanine dyes,[1] whose emission intensity increases significantly upon complex formation with DNA or proteins, are especially useful, because the binding event to the host molecule may be readily followed by the appearance of a strong fluorescence emission ("light-up probes").[2] This significant fluorescence enhancement upon DNA binding of fluorescence probes is often applied in analytical biochemistry, biology, and medicine. For example, DNA staining[3] by ethidium bromide is widely used to readily visualize DNA fragments in a gel.[4] The hybridization and renaturation kinetics of complementary DNA strands may also be monitored by a continuous fluorescence technique that is based on the emission enhancement of fluorescent dyes. Moreover, staining of proteins in 2D polyacrylamide gel electrophoresis[5] or in microchip capillary electrophoresis[6] by fluorescence probes such as 1-anilino-8-naphthalenesulfonate (1,8-ANS),[7] Nile Red,[8] or the SYPRO dye family[9] has become a useful method in proteomics. Especially in the latter case, only a few systematic strategies for the rational

(1) Armitage, B. *Top. Curr. Chem.* **2005**, *253*, 55−76.
(2) (a) Cosa, G.; Focsaneanu, K. S.; McLean, J. R. N.; McNamee, J. P.; Scaiano, J. C. *Photochem. Photobiol.* **2001**, *73*, 585−599. (b) Morozkin, E. S.; Laktionov, P. P.; Rykova, E. Y.; Vlassov, V. V. *Anal. Biochem.* **2003**, *322*, 48−50. (c) Lee, G.; Chen, C. H.; Chiu, L. A. *Cytometry* **1986**, *7*, 508−517.
(3) Prentø, P. *Biotech.* Histochem. **2001**, *76*, 137−161.
(4) Sharp, P. A.; Sugden, B.; Sambrook, J. *Biochemistry* **1973**, *12*, 3055−3063.
(5) (a) Gorg, A.; Obermaier, C.; Boguth, C.; Harder, A.; Scheibe, B.; Wildgruber, R.; Weiss, W. *Electrophoresis* **2000**, *21*, 1037−1053. (b) Andrews, A. T. *Electrophoresis*: *Theory, Techniques, and Biochemical and Clinical Applications*; Oxford University Press: Oxford, UK 1986.
(6) (a) Herr, A. E.; Singh, A. K. *Anal. Chem.* **2004**, *76*, 4727−4733. (b) Giordano, B. C.; Jin, L.; Couch, A. J.; Ferrance, J. P.; Landers, J. P. *Anal. Chem.* **2004**, *76*, 4705−4714. (c) Liu, Y.; Foote, R. S.; Jacobson, S. C.; Ramsey, R. S.; Ramsey, J. M. *Anal. Chem.* **2000**, *72*, 4608−4613.
(7) (a) Hartman, B. K.; Udenfriend, S. *Anal. Biochem.* **1969**, *30*, 391−394. (b) Horowith, P. M.; Bowman, S. *Anal. Biochem.* **1987**, *165*, 430−434.
(8) Daban, J.-R.; Bartolomé, S.; Samsó, M. *Anal. Biochem.* **1991**, *199*, 169−174.

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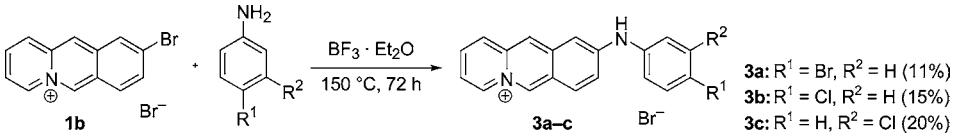
**Scheme 1.** Synthesis of *N*-Arylaminoacridizinium Derivatives




design of light-up probes are published, presumably because the structure of the SYPRO dyes is not available. Considering the need for efficient and selective light-up probes for the detection of biomacromolecules, the systematic exploration of novel classes of fluorescence probes, whose substitution pattern is easily varied, is still necessary, because the results of such studies may be used for the specific design of probes with new intriguing properties.

In this Letter we describe a new class of fluorescence probes on the basis of almost nonfluorescent *N*-arylamino-substituted acridizinium (benzo[*b*]quinolizinium) derivatives. We chose the 9-aminoacridizinium fluorophore because the parent system **1a** (Figure 1) exhibits a large Stokes' shift

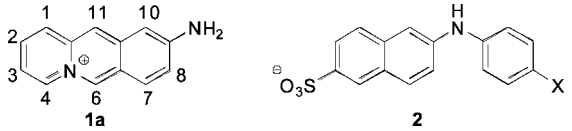



**Figure 1.**

(e.g. 122 nm in $H_2O$), appropriate fluorescence quantum yields (e.g., 0.12 in $H_2O$), and emission wavelength above 500 nm,[10] which may allow the detection of fluorescence without distortion by autofluorescence of the cell matrix.[11] Moreover, a large affinity for the DNA ($K = 10^4-10^5$ $M^{-1}$) and a preferential intercalative binding mode were demonstrated for acridizinium derivatives.[10b] We assumed that a substitution of the amino functionality with a phenyl group may constitute a system whose fluorescence may be quenched as in 2-*N*-arylamino-6-naphthalenesulfonates (**2**)[12] and thus provide low fluorescence quantum yield in an unbound state as required for light-up probes. The association with a biomacromolecule was proposed to suppress molecular motion in the excited state with an increase of fluorescence intensity.

The 9-aminoacridizinium derivatives **3a**−**c** were synthesized in low yield by the solvent-free nucleophilic substitution reaction of 9-bromoacridizinium bromide **1b**[13] with corresponding anilines (Scheme 1) and purified by several subsequent crystallizations. The structures of the acridizinium derivatives **3a**−**c** were confirmed by $^1H$ and $^{13}C$ NMR spectroscopy, mass-spectrometric data, and elemental analyses.

Solutions of derivatives **3a**−**c** show characteristic absorption spectra which resemble those of the parent compound, i.e., with a long-wavelength maximum at approximately $\lambda_{max} = 400$ nm (Table 1). The similarity of absorption spectra

**Table 1.** Absorption and Emission Properties of **3c** in Different Solvents

| solvent[a] | $\lambda_{abs}$/nm[b] | log $\epsilon$[c] | $\lambda_{em}$/nm[d] | $\phi_f$[e] |
|---|---|---|---|---|
| $H_2O$ | 397 | 4.33 | 550 | $2 \times 10^{-4}$ |
| $CH_3OH$ | 400 | 4.36 | 542 | $5 \times 10^{-4}$ |
| $CH_3CN$ | 397 | 4.36 | 536 | $6 \times 10^{-4}$ |
| DMSO | 402 | 4.33 | 552 | $1.8 \times 10^{-3}$ |
| $CH_2Cl_2$ | 413 | 4.29 | 527 | $5.9 \times 10^{-3}$ |
| $CHCl_3$ | 403 | 4.28 | 531 | $1.4 \times 10^{-2}$ |

[a] In order of their decreasing $E_T(30)$ values. [b] Long-wavelength absorption maximum, in nm, $c(\mathbf{3}) = 50$ $\mu$M. [c] Extinction coefficient, $cm^{-1}$ $M^{-1}$. [d] Emission maximum, nm, $c(\mathbf{3}) = 10$ $\mu$M; excitation wavelength $\lambda_{ex} = 390$ nm. [e] Fluorescence quantum yield, measured relative to Coumarin 153 ($\phi_f = 0.38$ in EtOH).[15]

of aryl-substituted derivatives with **1a** may be rationalized by the assumption that the phenyl substituents do not interact electronically with the 9-aminoacridizinium chromophore in the ground state. Similarly to **2**, derivatives **3a**−**c** exhibit just weak solvatochromism with respect to absorption properties; only in halogenated solvents a bathochromic shift is observed, especially pronounced in dichloromethane, where the main absorption maximum shifts for 15−20 nm compared to solvents such as water or acetonitrile.[14] All three compounds exhibit weak fluorescence in chloroform solution (**3a**: $\phi_f = 4.5 \times 10^{-3}$; **3b**: $\phi_f = 3.6 \times 10^{-3}$; **3c**: $\phi_f = 1.4 \times 10^{-2}$); in all other solvents investigated, including water, they are virtually nonfluorescent. However, with increasing viscosity of the solvent the fluorescence quantum yield of **3b** and **3c** increases significantly (cf. Supporting Information), which supports the assumption that the low fluorescence intensity of such compounds is the result of conformational changes in the excited state.

(9) (a) Berggren, K.; Chernokalskya, E.; Steinberg, T. H.; Kemper, C.; Lopez, M. F.; Diwu, Z.; Haugland, R. P.; Patton, W. F. *Electrophoresis* **2000**, *21*, 2509−2521. (b) Steinberg, T. H.; Jones, L. J.; Haugland, R. P.; Singer, V. L. *Anal. Biochem.* **1996**, *239*, 223−237.

(10) (a) Ihmels, H.; Faulhaber, K.; Engels, B.; Lennartz, C. *Chem. Eur. J.* **2000**, *6*, 2854−2864. (b) Ihmels, H.; Faulhaber, K.; Sturm, C.; Bringmann, G.; Messer, K.; Gabellini, N.; Vedaldi, D.; Viola, G. *Photochem. Photobiol.* **2001**, *74*, 505−511.

(11) Patonay, G.; Antoine, M. D. *Anal. Chem.* **1991**, *63*, 321A−327A.

(12) Kosower, E. M. *Acc. Chem. Res.* **1982**, *15*, 259−266.

(13) Bradsher, C. K.; Sherer, J. P.; Parham, J. H. *J. Chem. Eng. Data* **1965**, *10*, 180−183.

(14) Such an influence of $CH_2Cl_2$ is occasionally observed for cationic dyes, see e.g.: Jager, W. F.; Kudasheva, D.; Neckers, D. C. *Macromolecules* **1996**, *29*, 7351−7255.

The interactions of the acridizinium derivatives **3a**−**c** with DNA were investigated by spectrophotometric and spectrofluorimetric titrations with calf thymus DNA (ct DNA) in aqueous phosphate buffer (Figure 2). Photometric titration

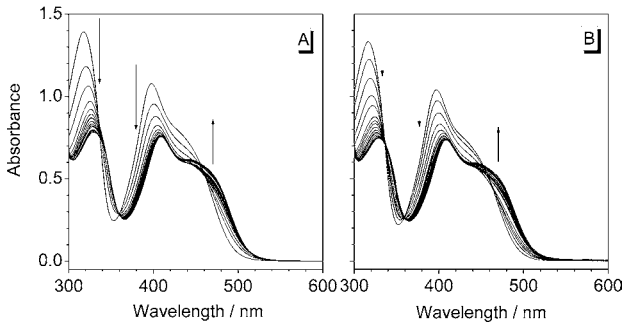



**Figure 2.** Spectrophotometric titrations of ct DNA to **3a** (A) and **3b** (B) at a dye concentration of 50 $\mu$M. Arrows indicate changes in the absorption spectra upon addition of the DNA (0−0.3 mM).

at a dye concentration of 50 $\mu$M turned out to be problematic, since at low DNA-to-dye ratios (1−4) a colored fibrous DNA−dye complex precipitated from the solution. Nevertheless, upon further addition of DNA (at least 6 equiv with respect to the dye concentration) the precipitate partly dissolves. This precipitate was not observed, either, when the amount of DNA, which corresponds to a final DNA-to-dye ratio of at least four, was added in one portion and the solution was thoroughly shaken immediately. At lower dye concentration (10 $\mu$M) the complex did not precipitate at any DNA-to-dye ratios.

Upon addition of DNA to buffered solutions of **3a**−**c**, a significant decrease of the absorbance and a red shift (10−12 nm) of the corresponding long-wavelength absorption maxima were observed. Simultaneously new weak shoulders at longer wavelengths and one or more isosbestic points were found. These observations already indicate a binding interaction between the dyes and DNA, and the isosbestic points reveal that almost exclusively one binding mode takes place. For the evaluation of the DNA-binding constants and binding-site sizes, the data from spectrophotometric titrations were represented as Scatchard plots and fitted to the neighbor-exclusion model of McGhee and von Hippel.[16] The binding constants of the compounds investigated are in the $10^4$ $M^{-1}$ range within experimental error (Table 2). The derivatives **3a** and **3b** have unusually low values of the binding site size (2.0−2.3). Presumably, like the parent 9-aminoacridizinium,[10b] **3a** and **3b** form aggregates in the presence of DNA, which stack along the DNA backbone, as indicated by partial loss of the long-wavelength isosbestic points during titration.

Upon addition of DNA to an aqueous solution of **3a**, the fluorescence intensity increases by a factor of about 2.3, but

**Table 2.** DNA-Binding Properties of the Acridizinium Derivatives **3a**−**c**

| | $\lambda_{abs}$/nm[a] | | $\lambda_{em}$/nm[b] | | | | |
|---|---|---|---|---|---|---|---|
| | free | bound | free | bound | % H[c] | $K$[d]/$10^4$ $M^{-1}$ | $n$[e] |
| **3a** | 398 | 410 | 508 | 558 | 70.1 | 3.3 ± 0.2 | 2.0 ± 0.1 |
| **3b** | 397 | 409 | 513 | 555 | 71.1 | 2.9 ± 0.2 | 2.3 ± 0.1 |
| **3c** | 397 | 407 | 542 | 539 | 68.1 | *f* | *f* |

[a] Absorption wavelength, in nm, that corresponds to the free and bound dye absorption maxima. [b] Emission maxima, in nm, for the free and bound dye. [d] Percent hypochromicity, calculated from the absorbance at the long-wavelength maxima. [d] Binding constant (in nucleic bases). [e] Binding-site size (in nucleic bases), determined from fitting the Scatchard plots to the McGhee−von Hippel model. [f] Not determined due to precipitation of DNA−dye complex during titration.

remains rather low; at the same time the emission maximum shifted bathochromically for 50 nm (Figure 3A, Table 2).

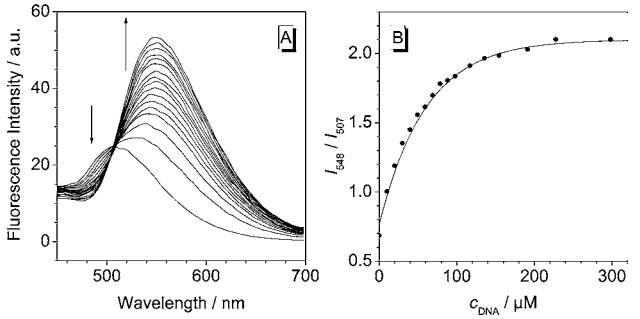



**Figure 3.** (A) Spectrofluorimetric titration of ct DNA (0−0.3 mM) to compound **3a** (10 $\mu$M in aqueous phosphate buffer); excitation wavelength $\lambda_{ex}$ = 360 nm. (B) Ratiometric plot for titration of ct DNA to **3a**.

This development of a weak yellow emission may be observed by the naked eye. Notably this difference of the emission maxima allows a ratiometric detection of DNA (Figure 3B).

The DNA-induced fluorescence enhancement is much more pronounced in the case of the derivatives **3b** and **3c**. The very weak fluorescence of these compounds in aqueous buffer solution increases by factors of approximately 30 (**3b**) and 50 (**3c**) upon addition of ct DNA (Figure 4). Presumably, association of the acridizinium moiety into the DNA helix reduces the possibility of free rotation about the $N^9$-aryl bond, which prevents the nonradiative decay from the excited state. Smaller fluorescence enhancement of **3a** compared to **3b**,**c** may be attributed to the internal heavy atom effect in **3a**, which reduces the quantum yield of the fluorescence by intersystem crossing, as was found in structurally similar 2-*N*-arylamino-6-naphthalenesulfonate systems.[17]

The fluorescence enhancement of **3b**,**c** upon DNA binding is significantly larger than the one observed with conven-

(15) Jones, G.; Jackson, W. R.; Choi, C. Y.; Bergmark, W. R. *J. Phys. Chem.* **1985**, *89*, 294−300.

(16) McGhee, J. D.; von Hippel, P. H. *J. Mol. Biol.* **1974**, *86*, 469−489.

(17) Kosower, E. M.; Dodiuk, H. *J. Phys. Chem.* **1978**, *82*, 2012−2015.

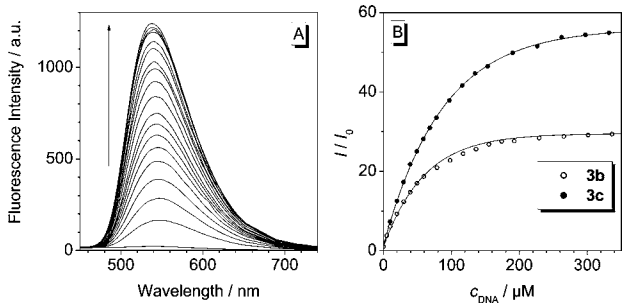



**Figure 4.** (A) Spectrofluorimetric titration of ct DNA (0−0.3 mM) to compound **3c** (10 $\mu$M in aqueous phosphate buffer); excitation wavelength $\lambda_{ex}$ = 335 nm. (B) Fluorescence enhancement plots for titrations of ct DNA to derivatives **3b** and **3c**.

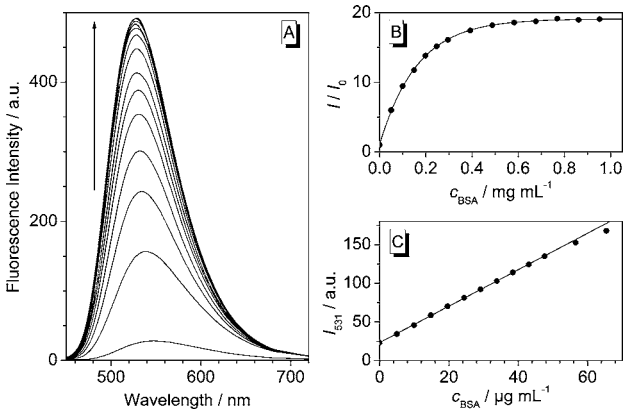



**Figure 5.** (A) Fluorimetric titration of BSA to a solution of **3c** (10 $\mu$M in aqueous phosphate buffer containig 0.05% SDS). (B) Binding isotherm of BSA to **3c**. (C) Linear region of the binding isotherm, allowing direct determination of the protein concentration.

tional DNA stains ethidium bromide ($I_{max}/I_0 \approx 10$) and Hoechst 33258 ($I_{max}/I_0 \approx 30$).[2a] Similarly to compound **3a**, the emission maximum (555 nm) is considerably red-shifted with the respect to the emission of the unbound compound.

Preliminary experiments also reveal a drastic increase of emission quantum yield of **3c** upon addition of proteins, such as bovine serum albumin (BSA). Thus, the fluorescence of **3c** increases by a factor of approximately 20 upon addition of BSA in the presence of an anionic surfactant (sodium dodecyl sulfate, SDS) (Figure 5). Notably, the plot of the fluorescence intensity versus BSA concentration is linear in a rather broad range (0−50 $\mu$g mL$^{-1}$) of BSA concentrations, the correlation coefficient being 0.999 (Figure 5C), thus allowing direct fluorimetric detection of the protein concentration.

In summary, we synthesized novel fluorescence probes whose interaction with DNA and proteins may be monitored by absorption and emission spectroscopy. The dyes **3a**−**c** offer promising properties for DNA detection. Thus, **3a** may allow a ratiometric detection of DNA, and the intrinsically weak fluorescence of **3b** and **3c** increases significantly upon association to DNA, so that these compounds have ideal properties to be used as DNA-sensitive "light-up probes". Most notably, the substitution pattern of the acridizinium fluorophore may be varied in a broad range, which allows detailed studies of structure−property relationships. Thus, further detailed studies on the mechanism of the fluorescence enhancement upon complex formation and on the application in DNA and protein detection are presently under way.

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**Supporting Information Available:** Full synthetic details and spectroscopic data for the new compounds; solvatochromic properties of **3a**−**c**; Scatchard plots and numerical fits for DNA titrations; and viscosity dependence of fluorescence quantum yield of **3b**,**c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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