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Synthesis and Spectroscopic and DNA-Binding Properties of Fluorogenic Acridine-Containing Cyanine Dyes

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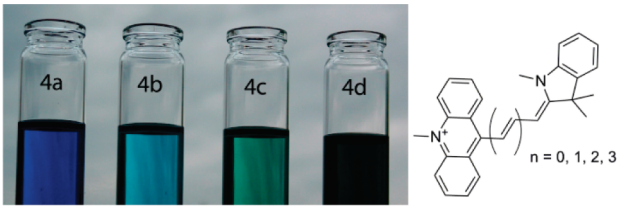

The synthesis of a new subclass of mono- and polymethine cyanine dyes that incorporate an acridinium moiety and that absorb in the orange to near-infrared region of the spectrum is reported. The mono-, tri-, and pentamethine dyes in particular exhibit promising fluorogenic properties. Their ability to aggregate in solution and to interact with B-DNA is also discussed.

Fluorescent molecules have been widely used as probes in chemical biology.[1] Depending on the nature of the biological process to be visualized or the molecular species that needs to be sensed it is possible to choose among a large variety of fluorescent probes that exhibit specific structural and spectroscopic properties. Of particular interest are molecules that can reversibly switch between a nonemissive state and an emissive state. Most representative examples are fluorogenic unsymmetrical cyanine dyes which become fluorescent upon interaction with specific nucleic acids or proteins,[2] and photochromic compounds which can undergo a reversible change of their optical properties under illumination.[3] Within the wide selection of fluorescent dyes available in the literature, those belonging to the Cyanine dye (Cy)[4] and Acridine[5] families have been extensively used as oligonucleotide labels or probes. Although those dyes can be covalently attached to a DNA strand for labeling purposes, of particular interest is their capacity to bind to DNA through noncovalent interactions either by intercalation between base pairs (acridines, monomethine cyanine dyes)[6] or aggregation into the minor groove of double-stranded DNA (trimethine and pentamethine cyanine dyes).[7] The family of cyanine dyes generically consists of a conjugated system based on a polymethine chain linking two nitrogen-containing heterocycles (most commonly indoles, benzothiazoles, benzoxazoles, or quinolines).[4] A well-known fluorogenic monomethine cyanine dye is Thiazole orange (TO), which is nonfluorescent when free in solution (due to rapid nonradiative deactivation to the ground state) but becomes highly fluorescent under conditions where motions of the polymethine chain are restricted (e.g., upon intercalation into DNA or when in viscous solvents).[6b,8] Similar properties are commonly observed with unsymmetrical polymethine cyanine dyes.[9] Here, we report a new family of fluorogenic and unsymmetrical mono-, tri-, penta-, and heptamethine cyanine dyes that incorporate one tricyclic *N*-methylacridinium heterocycle and cover a large spectral range from orange to near-infrared. The synthesis and spectroscopic properties of these dyes are described as well as their DNA binding properties.

Trimethine, pentamethine, and heptamethine cyanine dyes were synthesized by reaction of commercially available 2-methylene-1,3,3-trimethylindoline with the appropriate activated acridine hemicyanine (**3b**−**d**). The activated hemicyanines, or acridine "half-dyes", were obtained by reaction of 9,10-dimethylacridinium iodide **2**[10] with *N*,*N*-diphenylformamidine generated in situ, malonaldehyde bisphenylimine hydrochloride or glutaconaldehydedianil hydrochloride in a mixture of acetic anhydride, and either pyridine or triethylamine and used crude for the subsequent step of cyanine dye formation (Scheme 1).

For the synthesis of monomethine dyes, a different route was chosen starting from commercially available 10-methyl acridone **5**. Reaction of **5** with thionyl chloride afforded the corresponding 9-chloro-10-methylacridinium chloride **6**, which was subsequently reacted with 2-methylene-1,3,3-trimethylindolenin to produce the desired monomethine cyanine dye **4a** (Scheme 2).

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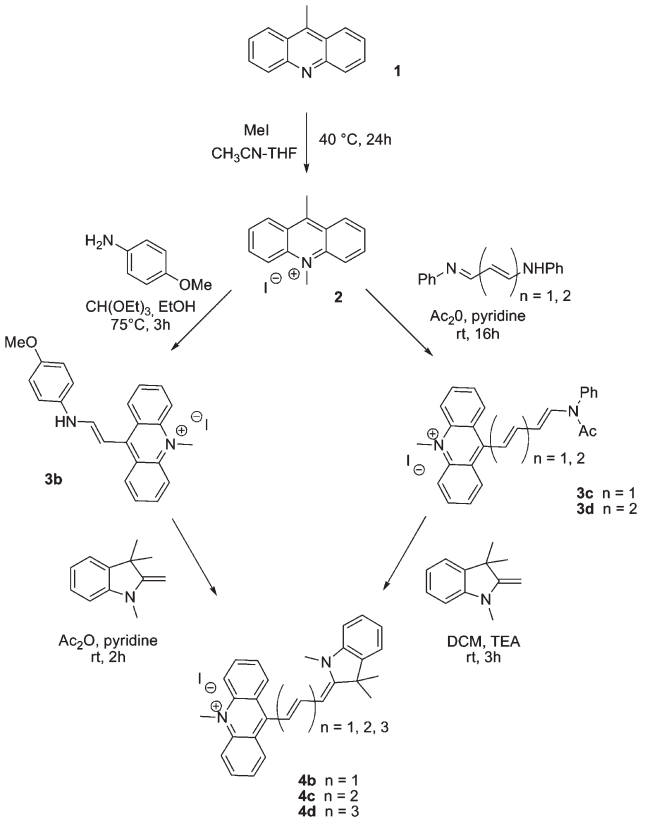
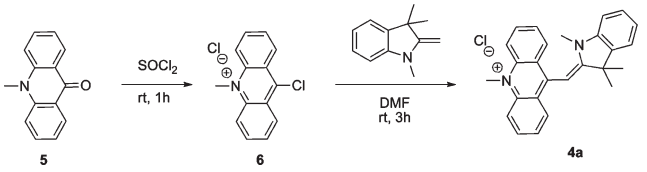
SCHEME 1. Synthetic Route to Trimethine (4b), Pentamethine (4c), and Heptamethine (4d) Acridine Cyanine Dyes



SCHEME 2. Synthetic Route to Monomethine Acridine Dye 4a



The spectroscopic properties of the four acridine cyanine dyes **4a**−**d** were then determined with absorption and fluorescence spectroscopy. Absorption coefficients and maximum absorption wavelengths were measured in methanol and in an aqueous buffer (50 mM TRIS·HCl pH 7.4 containing 100 mM KCl). The data are summarized in Table 1.

As commonly observed for cyanine dyes, the elongation of the polymethine bridge resulted in a significant red shift of the maximum absorption wavelength at a rate of ~100 nm per two methine groups added. Of high interest for future applications is the large spectral range, from orange to near-infrared, covered by this family of acridine-containing cyanine dyes. The contribution of the acridine moiety on the maximum absorption wavelength of the dye can be assessed by comparing the UV spectra of our dyes with those of similar unsymmetrical cyanine dyes lacking the acridine. For instance, the acridinium moiety induces a red shift of the λ_{abs} of 135 and 120 nm when compared with similar unsymmetrical pentamethine dyes in which the acridinium moiety has been substituted by a quinolinium or a benz[*e*]indolium, respectively.[11] Similar effects were observed within the trimethine family with red shifts of 80 and 110 nm, respectively.[11]

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TABLE 1. UV Absorption Properties of Dyes 4a−d (in their monomeric state)

	methanol		buffer[a]	
compd	λ_{abs} (nm)	ε ($M^{-1}\cdot cm^{-1}$)	λ_{abs} (nm)	ε ($M^{-1}\cdot cm^{-1}$)
4a	595	43690	592	26720
4b	677	62650	672	25630
4c	774 (700[b])	83300	771 (696[b])	–[c]
4d	872 (718[b])	–[c]	872 (668[b])	–[c]

[a]TRIS·HCl 50 mM + 100 mM KCl. [b]Maximum wavelength of H-aggregates. [c]Not following Beer's law.

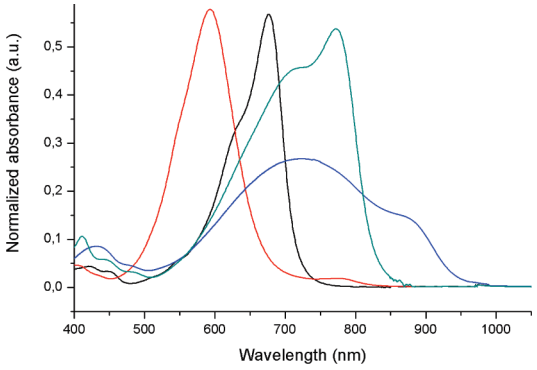

FIGURE 1. UV absorption spectra of **4a** (red), **4b** (black), **4c** (green), and **4d** (blue) taken in MeOH.

Typically, the visible absorption spectrum of a cyanine dye shows two close maxima: the band of longest wavelength is the most intense while the intensity and resolution of the vibronic shoulder at shorter wavelength differ from dye to dye. In solution, cyanine dyes can spontaneously assemble to form dimers or also polymer-type aggregates which can be composed of up to thousands of molecules. These aggregates can be of H- or J-type depending on whether the dyes assemble in a face-to-face or end-to-end manner.[12]

The absorption spectra of mono- and trimethine dyes **4a** and **4b** (Figure 1) were typical of nonaggregated cyanine dyes and similar in shape when taken in either methanol or buffer, thus suggesting the existence of the dyes in solution mainly under their monomeric form. This was no longer the case for the pentamethine dye **4c** for which a broadband at shorter wavelength (compared to that of the monomeric dye) and corresponding to the formation of H-aggregates[12] became apparent in methanol and predominant in buffer. This aggregation phenomenom was even more pronounced for the heptamethine dye **4d**, which was mainly present in solution as an H-aggregate (characterized by a very broad absorption band around 600−800 nm) in both methanol and buffer. In the conditions of our experiments, however, i.e., in aqueous or organic solvent at dye concentrations up to 200 μM, no J-aggregate was ever observed by absorption

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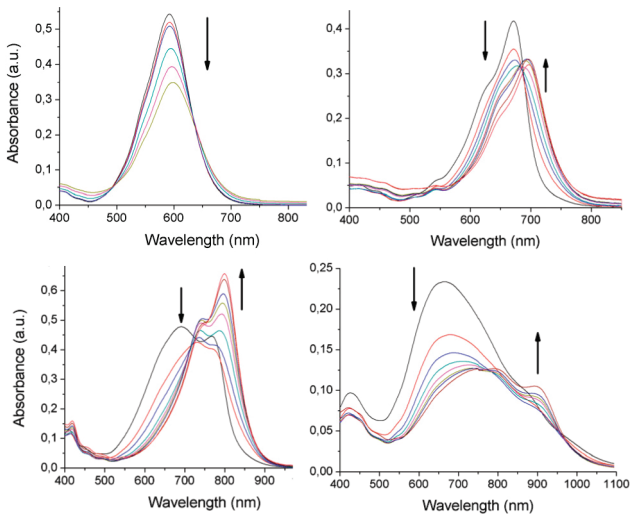

FIGURE 2. UV−vis spectra recorded during titration of CT-dsDNA into an aqueous solution of **4a** (top left), **4b** (top right), **4c** (bottom left), and **4d** (bottom right). CT DNA concentration varies from 0 to 300 μM (CT DNA concentration is given in base pairs and is estimated assuming an extinction coefficient at 260 nm of 12 824 $M^{-1}\cdot cm^{-1}$).

spectroscopy. All together, these observations are consistent with the data accumulated in the literature, although mainly on symmetrical cyanine dyes, which demonstrated that in aqueous solution, (i) the tendency of cyanine dyes to aggregate increases with the length of the polymethine chain (i.e., as hydrophobicity and polarizability increase) and (ii) unsubstituted dyes favor H-aggregation rather than J-aggregation.[12b]

Next, the ground state interaction between cyanine dyes **4a**−**d** and double-stranded DNA was investigated. Titration experiments were carried out by adding increasing amounts of Calf-Thymus (CT) DNA to a 20 μM solution of acridine dye (in TRIS·HCl buffer 50 mM also containing 100 mM KCl). Interestingly, different behaviors were obtained with each dye that can be interpreted in terms of different DNA binding mode. For monomethine cyanine dye **4a**, a decrease in intensity of the UV band was observed when increasing the DNA concentration, which was accompanied by a very small (4 nm) red shift (Figure 2). For trimethine dye **4b**, a similar decrease in intensity was observed along with a significant red shift of 24 nm. Although the bathochromic shifts observed for compounds **4a** and **4b** upon binding to DNA were suggesting intercalation of the dyes between base pairs, viscosity experiments showed no DNA lengthening upon addition of either dye, suggestive of a nonintercalative or heterogeneous mode of binding (see the Supporting Information).

While pentamethine cyanine dyes typically aggregate into the minor groove of double-stranded DNA, results obtained with compound **4c** indicate a different binding mode. While **4c** existed predominantly as an H-aggregate in aqueous buffer, addition of increasing amounts of CT DNA led to the appearance of a new band, red-shifted (29 nm) and of increasing intensity with respect to the initial band for the monomeric dye in solution. This corresponds to a complete disassembly of the H-aggregate upon binding to DNA. Comparable effect was observed with heptamethine dye **4d**

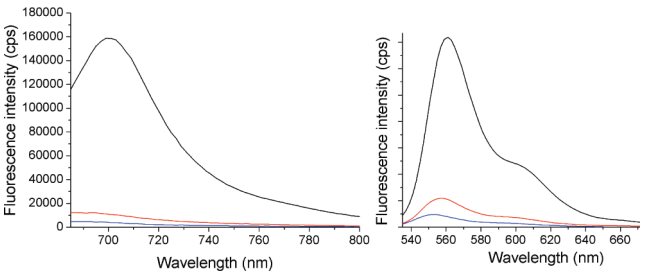

FIGURE 3. Fluorescence emission spectra (λ_{exc} = 670 nm) of **4b** (5 μM, left) and **4a** (5 μM, right) in solution in buffer (blue), methanol (red), and 85% glycerol (black).

upon binding to CT DNA, i.e., partial disappearance of the H-aggregate at the expense of the monomeric dye.

Finally, the fluorescence properties of dyes **4a**−**d** were examined in methanol, buffer, and 85% glycerol on a Jobin Yvon Fluorolog 3.22 instrument. In buffer, monomethine cyanine dye **4a** showed a maximum emission band around 552 nm with a shoulder of weaker intensity around 594 nm. As commonly observed for monomethine cyanine dyes, compound **4a** exhibits fluorogenic behavior. Its fluorescence is very low in aqueous buffer and methanol (Φ_f = 0.20 and 0.23%, respectively) while it increases significantly (ca. 25-fold, Φ_f = 5.6%) when measured in 85% glycerol (Figure 3). This is due at least in part to a restriction in conformational freedom of the monomethine bridge. A similar fluorogenic property was observed for dyes **4b**,**c**. While almost no fluorescence was observed when exciting trimethine dye **4b** around its maximum absorption wavelength (λ_{exc} = 670 nm) in buffer, a moderate emission was detected in methanol (Φ_f = 0.10%), which significantly increased (ca. 15-fold) when measured in 85% glycerol (Figure 3). An analogous effect, although of weaker amplitude, was observed with the pentamethine dye **4c** for which a moderate emission of fluorescence around 810 nm (λ_{exc} = 770 nm) was detectable in glycerol solution only. Unfortunately, no fluorescence was ever obtained when exciting heptamethine dye **4d** around its maximum absorption wavelength (λ_{exc} = 870 nm), even in glycerol solution.

It is noteworthy that dyes **4b**−**d** also share two common fluorescence emission maxima at shorter wavelengths (i.e., 552 and 654 nm upon excitation at 520 and 620 nm, respectively). These could be assigned to the formation after excitation of locally excited (LE) states that could also result in excited state intramolecular charge transfer (ICT).[13]

Fluorescence titration experiments were carried out by adding increasing concentrations of CT DNA into a solution of acridine dye **4a** or **4b** (5 μM in buffer). For monomethine dye **4a**, the addition of CT DNA resulted in a significant decrease of the fluorescence intensity, which is consistent with a nonintercalative binding mode (i.e., no restriction of conformational freedom upon binding to DNA), likely due to steric hindrances preventing the dye from adopting a planar conformation (see the Supporting Information).[9] Interestingly, a modest (ca. 3-fold, see the Supporting Information) fluorescence enhancement was observed for trimethine dye **4b** upon addition of CT DNA, which was accompanied by a 23 nm red shift of the emission maximum. Although the fluorescence intensity remains very moderate

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compared to that obtained in glycerol, it demonstrates the fluorogenic properties of **4b** upon binding to B-DNA.

In summary, we have developed the first family of acridine-containing fluorogenic cyanine dyes that cover a broad spectral range from orange to near-infrared. The introduction of an acridine moiety accounts for a red shift of the maximum absorption wavelength of ca. 100−120 nm when compared to the corresponding quinoline dyes. Of particular interest are (i) trimethine dye **4b**, which emits maximally at 697 nm under conditions of restricted mobility only, and (ii) pentamethine **4c**, which absorbs in the near-infrared region of the spectrum (770 nm). Cyanine dyes from this family could find valuable application as fluorogenic sensors (e.g., viscosity sensors) and their versatile synthesis should allow the development of optimized dyes with increased fluorogenic properties.

Experimental Section

Preparation of Compound 4b. A mixture of 9,10-dimethylacridinium iodide[10] (200 mg, 0.59 mmol), 4-methoxyaniline (150 mg, 1.18 mmol), and triethyl orthoformate (200 μL, 1.18 mmol) in ethanol (3 mL) was refluxed at 75 °C for 3 h. The reaction mixture was cooled to room temperature and then poured into Et_2O (50 mL). A solid precipitated, which was collected by filtration and washed with Et_2O. Crude hemicyanine **3b** was obtained as a dark solid and was used directly for the next step without further purification. The crude product **3b** obtained above was taken in a mixture of acetic anhydride (250 μL) and pyridine (5 mL). 2-Methylene-1,3,3-trimethylindoline (120 mg, 0.70 mmol) was added and the resulting mixture was stirred at room temperature for 2 h. Et_2O (50 mL) was added, thus leading to the formation of a dark blue solid that was filtered off solution and washed with Et_2O. Purification by silica gel chromatography (CH_2Cl_2, then CH_2Cl_2/MeOH 98:2) afforded the trimethine cyanine dye **4b** as dark blue solid (192 mg, 63%). ^{1}H NMR (CD_3OD, 400 MHz) δ 1.69 (s, 6H), 3.84 (s, 3H), 4.10 (s, 3H), 6.90 (d, 1H, J = 12 Hz), 7.40 (t, 1H, J = 8 Hz), 7.48−7.54 (m, 4H), 7.59 (d, 1H, J = 8 Hz), 7.86−7.94 (m, 3H), 8.27 (d, 1H, J = 8 Hz), 8.36 (t, 1H, J = 12 Hz). ^{13}C NMR (CD_3OD, 100 MHz) δ 26.6, 31.0, 34.8, 49.8, 108.1, 111.7, 115.3, 118.4, 122.1, 122.2, 122.9, 126.3, 127.0, 128.7, 133.4, 140.6, 141.5, 142.6, 151.7, 154.3, 176.6. HRMS (ESI) m/z calcd for $C_{28}H_{27}N_2$ [M^+] 391.217, found 391.217.

Preparation of Compound 4c. To a suspension of 9,10-dimethylacridinium iodide (200 mg, 0.60 mmol) in acetic anhydride (15 mL) were successively added malonaldehyde bis(phenylimine) hydrochloride (155 mg, 0.60 mmol) and pyridine (1 mL). After the mixture was stirred at room temperature for 8 h, Et_2O (50 mL) was added and the resulting brown solid was filtered off and washed extensively with Et_2O. The activated hemicyanine **3c** was then collected by extraction in CH_2Cl_2 (100 mL) and obtained as an orange solid after evaporation of the solvent under vacuum. To a solution of crude **3c** (130 mg) in CH_2Cl_2 (25 mL) was added 2-methylene-1,3,3-trimethylindoline (103 mg, 0.60 mmol) and triethylamine (1 mL). The reaction mixture was then stirred at room temperature and in the dark for 3 h. Et_2O (100 mL) was added and the dark blue solid was collected by filtration and washed with Et_2O. The crude material was purified by silica gel chromatography (CH_2Cl_2, then CH_2Cl_2/MeOH 95:5) to give **4c** as a blue/green solid (235 mg, 72%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.75 (s, 6H), 3.89 (s, 3H), 4.03 (s, 3H), 7.12 (d, 2H, J = 8 Hz), 7.27−7.33 (m, 3H), 7.38 (t, 1H, J = 8 Hz), 7.44−7.50 (m, 4H), 7.67 (t, 2H, J = 8 Hz), 7.76 (t, 2H, J = 12 Hz), 7.95 (t, 1H, J = 12 Hz), 8.04 (d, 2H, J = 8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.8, 27.8, 34.5, 35.6, 50.4, 109.9, 112.4, 114.5, 121.7, 122.5, 123.2, 127.2, 132.2, 132.2, 140.8, 141.9, 142.5, 146.6, 153.0, 154.0, 176.4, 207.1. HRMS (ESI) m/z calcd for $C_{30}H_{29}N_2$ [M^+] 417.233, found 417.239.

Preparation of Compound 4d. Compound **4d** was synthesized following the same protocol as for compound **4c** but starting from 9,10-dimethylacridinium iodide and glutaconaldehydedianil hydrochloride. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.75 (s, 6H), 3.73 (s, 3H), 4.30 (s, 3H), 6.78 (t, 1H, J = 12 Hz), 6.81 (d, 1H, J = 8 Hz), 7.07 (t, 1H, J = 12 Hz), 7.13 (d, 1H, J = 12 Hz), 7.24 (t, 2H, J = 8 Hz), 7.30 (d, 2H, J = 8 Hz), 7.40−7.56 (m, 8H), 7.82 (d, 2H, J = 8 Hz), 7.97 (t, 1H, J = 12 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.5, 34.6, 35.0, 50.7, 110.7, 112.7, 113.9, 121.9, 122.3, 122.4, 122.4, 123.5, 126.7, 127.5, 129.2, 130.3, 131.0, 131.9, 140.8, 142.0, 142.1, 142.1, 146.9, 153.3, 153.6, 177.1. HRMS (ESI) m/z calcd for $C_{32}H_{31}N_2$ [M^+] 443.248, found 443.250.

Preparation of Compound 4a. A solution of 10-methylacridone (100 mg, 0.48 mmol) in thionyl chloride (2 mL) was stirred at room temperature for 1 h. Excess $SOCl_2$ was then removed under reduced pressure to afford the desired 9-chloro-10-methylacridinium chloride intermediate **6** as an orange solid. To a solution of **6** in anhydrous DMF (1 mL) was added 2-methylene-1,3,3-trimethylindoline (124 mg, 0.72 mmol). The reaction mixture was then stirred at room temperature for 3 h and the product was extracted with CH_2Cl_2 and washed with water. The organic layer was dried ($MgSO_4$) and the solvent was evaporated off under reduced pressure. The crude material was finally purified by silica gel chromatography (CH_2Cl_2, then CH_2Cl_2/MeOH 97:3) to give **4a** as a dark blue solid (125 mg, 65%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.82 (s, 6H), 2.66 (s, 3H), 4.76 (s, 3H), 6.58 (s, 1H), 7.03 (d, 1H, J = 8 Hz), 7.28 (t, 1H, J = 8 Hz), 7.41 (t, 1H, J = 8 Hz), 7.45 (d, 1H, J = 8 Hz), 7.61 (t, 2H, J = 8 Hz), 8.14 (t, 2H, J = 8 Hz), 8.19 (d, 2H, J = 8 Hz), 8.46 (d, 2H, J = 8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.6, 36.5, 38.3, 50.0, 87.9, 110.6, 118.6, 122.5, 123.7, 124.7, 125.6, 136.6, 138.5, 140.4, 144.2, 157.4, 174.8. HRMS (ESI) m/z calcd for $C_{26}H_{25}N_2$ [M^+] 365.201, found 365.203.

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Supporting Information Available: Experimental details, characterization of compounds **4a**−**d**, and spectroscopic (Ultraviolet, fluorescence) data. This material is available free of charge via the Internet at http://pubs.acs.org.