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Microwave-assisted solvent-free synthesis and spectral properties of some dimethine cyanine dyes as fluorescent dyes for DNA detection

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ABSTRACT

A series of dimethine cyanine dyes were synthesized in a fast, efficient and high yield by the condensation of quaternary salts with 1*H*-indole-3-carbaldehyde in the presence of piperidine under solvent-free microwave irradiation. The products were identified by ^{1}H NMR, IR, UV–vis spectroscopy and elemental analysis. The absorption and fluorescence properties of the dyes in both the free state and DNA or BSA were investigated. Significant enhancement of the fluorescent quantum yield was observed for all the dyes in the presence of DNA, with one compound demonstrating excellent sensitivity as a fluorescent probe.

1. Introduction

Methine cyanine dyes present typical optical properties and act as the most important organic functional dyes in many processes of technological interest like sensitizers in photography [1], optical recording materials in laser disks [2], sensitizers in solar cell [3], etc. A very attractive additional feature of methine cyanine dyes is the affinity for biological structures, especially for DNA [4–6]. Fluorescence technology is now the most sensitive and easily available method to study intermolecular interactions and the transcriptional dynamics of the cell nucleus [7]. Therefore, these dyes are suggested to be used as fluorescent probes of DNA, for they exhibit a dramatic enhancement in fluorescence intensity upon binding to DNA [8–10]. They have been utilized in many fields, such as gel staining [11,12], DNA sequencing [13], and flow cytometry [14]. Besides, these dyes are the practical tool for cell imaging research, for they are nuclear membrane permeant, tolerated by living cells, and photostable [15,16]. Our previous efforts have been devoted to developing the synthesis and applications of cyanine and hemicyanine dyes [17–19].

Microwave irradiation presents a powerful tool toward organic reactions. Solvent-free microwave irradiation is well known as environmentally benign method, which offers several advantages including shorter reaction times, cleaner reaction profiles and simple experimental/product isolation procedures [20]. In this paper a microwave-assisted preparation of a series of dimethine cyanine dyes *via* condensation of quaternary salts having reactive methyl and 1*H*-indole-3-carbaldehyde without solvent was described in high yield. The approach provided an attractive and environmentally friendly pathway to several synthetically useful dimethine cyanine dyes. A further objective of this study was to investigate the spectral properties of prepared dimethine cyanine dyes both in free state and in the DNA/BSA presence.

2. Results and discussion

2.1. Synthesis of dyes

General scheme of the synthesis of dyes **3a**–**3g** is represented in Scheme 1. In all cases investigated, the dimethine cyanine dyes formation reactions proceeded efficiently with high to excellent yields in short reaction time (Table 1) and reactions proceeded well even when both the starting reactants were solids and the reaction temperature was maintained below the melting points of both components using the microwave-assisted strategy. A series of dimethine cyanine dyes were successfully synthesized with high yields 89–98% within 2–5 min when a mixture of 1 mmol of quaternary salts, 1 mmol of 1*H*-indole-3-carbaldehyde and a few drops of piperidine was subjected to microwave irradiation at 126–329 W under solvent-free conditions. The products could be easily purified by recrystallization from EtOH–H_2O. Besides, the waste disposal of solvents and excess chemicals in classical synthesis of dimethine cyanine dyes was avoided or minimized [21].

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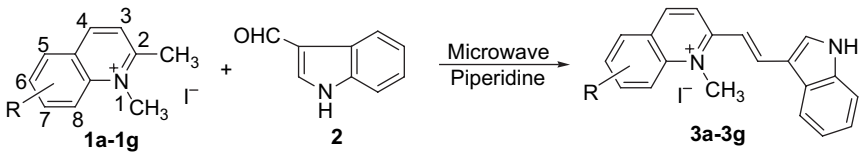

R: **a**, H; **b**, 6-CH_3; **c**, 6-Cl; **d**, 6-Br; **e**, 7-CH_3; **f**, 7-Cl; **g**, 7-Br

Scheme 1. Synthesis of dimethine cyanine dyes.

Table 1 lists the reaction conditions and yields for the dyes. The sequence of the reaction activity for 1*H*-indole-3-carbaldehyde and heterocyclic quaternary slats with various substituents was –Me > –H > –Br ≈ –Cl, and for the same substituent the condensation of 1*H*-indole-3-carbaldehyde and 6-substituted heterocyclic quaternary slats was easier than that of 7-substituted heterocyclic quaternary. In order to get optimized experiment conditions, the effect of microwave power and irradiation time on yield were examined. Table 2 lists the effect of microwave power and irradiation time on **3f**. The reaction yields were lower on 189/252 W, and the optimal microwave power was 329 W. It was also found that reaction yields increased with raise in irradiation time, but longer irradiation time caused too much heat leading to the thermal degradation of products.

2.2. Spectral properties of free dyes

Absorption maxima, fluorescence maxima, florescence quantum yields and extinction coefficient for the dyes are summarized in Table 3. Dyes **3a**–**3g** absorbed in the region 453.0–470.0 nm and had molar extinction coefficients of 1.1×10^4–$2.2 \times 10^4\ M^{-1}\ cm^{-1}$. They showed intense and broad absorption with high extinction coefficients in buffer. The dyes also exhibited fluorescence properties in buffer at room temperature. Their fluorescence maxima were located at 536.4–556.2 nm. Compared with the position of the excitation maximum for the dyes, the emission spectra were shifted to the red in the range of 82–88 nm (namely Stokes shift). For the dyes the fluorescence quantum yields were observed, being in the range of 0.0015–0.0032 in buffer. Compounds **3b** and **3e** had higher quantum yields than other dyes, suggesting that the σ–π hyperconjugation of Me made the conjugated-system became larger [22].

2.3. Spectral properties of the dyes in the presence of DNA

The detailed spectral properties of the DNA–dye solution were also measured (Table 4). Absorption maxima of the dyes in the presence of DNA were situated at 483–499 nm and showed a clear red shift (23–34 nm) relative to the corresponding maxima of free dyes in buffer. The intensity increase of the absorption bands was observed for all the dyes and the values of molar extinction coefficients were in the range from $1.9 \times 10^4\ M^{-1}\ cm^{-1}$ to $3.2 \times 10^4\ M^{-1}\ cm^{-1}$. Fig. 1 depicts the bathochromic shift (29 nm) and intensity increase ($\varepsilon^{DNA}/\varepsilon^{free} = 1.45$ times) for **3a** in the presence of DNA.

Table 1
The reaction conditions and yields for the dyes

Dye	R	Power (W)	Time (min)	Yield (%)
3a	H	189	2	95
3b	6-CH_3	126	4	94
3c	6-Cl	252	5	98
3d	6-Br	252	5	97
3e	7-CH_3	189	4	90
3f	7-Cl	329	4	90
3g	7-Br	329	4	89

The fluorescence spectra of the DNA–dyes showed a slight bathochromic shift of 4–12 nm, except for **3c** and **3e**, emission maxima of which were located at the same spectral region as the corresponding maxima of free dyes. Stokes shift values for the DNA–dyes were between 59 and 65 nm and became smaller than free dyes in buffer. Moreover, the fluorescence intensity increased for all the dyes in the presence of DNA. Compared with quantum yield of free dyes, the quantum yields of DNA–dyes increased up to 13.3 and 18.4 times for **3c** and **3d**, up to 24.3 and 27.6 times for **3f** and **3g**, up to 41 times for **3a** and **3b** and up to 68.8 times for **3e** (Fig. 2). Specially, **3e** and **3b** had higher quantum yields (0.200 and 0.1233, respectively) than other dyes.

2.4. Spectral properties of the dyes in the presence of BSA

Absorption spectra maxima of the dyes in the presence of BSA were located at the same spectral region as corresponding free dyes, so did the emission maxima (Table 4). For BSA–dyes the intensity increase of the absorption bands was observed and the values of molar extinction coefficients were in the range from $2.1 \times 10^4\ M^{-1}\ cm^{-1}$ to $3.6 \times 10^4\ M^{-1}\ cm^{-1}$. The fluorescent quantum yields of BSA–dyes were 1.0–2.3 times that of free dyes.

3. Conclusion

A rapid and highly efficient method for the synthesis of dimethine cyanine dyes under solvent-free microwave irradiation by the condensation of quaternary salts with 1*H*-indole-3-carbaldehyde in the presence of piperidine was established. The absorption maxima of the free dyes were located at 453–471 nm, and that of the BSA–dyes were located at the same spectral region as the free dyes. However, the dyes in the presence of DNA were situated at 483–499 nm and showed a clear red shift (23–34 nm) relative to the free dyes. The fluorescence maxima of free dyes, DNA–dyes and BSA–dyes were basically located at 536–560 nm. The fluorescent quantum yields of the DNA–dyes and BSA–dyes increased up to 13–68 times and 1.0–2.3 times, respectively. The **3e** in the

Table 2
The effect of microwave power and irradiation time on **3f**

Power (W)	Time (min)	Yield (%)
189	2	25
189	3	30
189	4	31
189	5	35
189	6	33
252	2	20
252	3	39
252	4	65
252	5	54
252	6	33
329	1	38
329	2	45
329	3	51
329	4	90
329	5	76

Table 3
Spectral characteristics of dyes in buffer

Dye	In buffer				
	λ_{abs} (nm)	ε^{free} ($M^{-1}\,cm^{-1}$)	λ_{ex} (nm)	λ_{em} (nm)	Φ_F^{free} [a]
3a	458.0	2.2×10^4	458.0	540.6	0.0018
3b	453.0	1.3×10^4	453.0	536.4	0.0030
3c	470.0	1.1×10^4	470.0	556.2	0.0023
3d	465.0	1.6×10^4	465.0	549.8	0.0016
3e	458.0	1.3×10^4	458.0	544.2	0.0032
3f	470.0	1.7×10^4	470.0	553.8	0.0015
3g	465.0	1.6×10^4	465.0	552.4	0.0015

[a] The fluorescence quantum yields of the dyes were determined by the reference standard (rhodamine B $\Phi_F = 0.49$ in ethanol at 25 °C) [25].

presence of DNA/BSA demonstrated the largest quantum yields (0.2200/0.0073). These dyes could be proposed as fluorescent dyes for DNA detection.

4. Experimental

4.1. Preparation of the quinoline heterocycles and quaternary salts *1a–1g*

Quinoline heterocycles were prepared according to a modified literature procedure [23]. Quaternary salts **1a–1g** were synthesized when a mixture of 0.016 mmol quinoline heterocycles and 0.023 mmol methyl iodide in 10 mL ethanol was refluxed for 15 h. After cooling, the product was filtered and purified by recrystallization from EtOH [24].

4.2. Preparation of the dimethine cyanine dyes *3a–3g*

The condensation of quaternary salts having reactive methyl with 1*H*-indole-3-carbaldehyde was carried out in a Galanz microwave oven. A mixture of 1 mmol of quaternary salts, 1 mmol of 1*H*-indole-3-carbaldehyde and a few drops of piperidine was subjected to microwave irradiation at optimized power and time under solvent-free conditions. After cooling, the reaction mixture was recrystallized from EtOH–H_2O to afford pure dyes **3a–3g**. The details of reaction conditions and yields are provided in Table 1.

4.3. Measurements

Melting points were taken on a XT-4 micromelting apparatus and uncorrected. Elemental analysis were performed with Vario EL-III instrument. IR spectra in cm^{-1} were recorded on a Bruker Equinox-55 spectrometer. ^1H NMR spectra were recorded at 400 MHz on a Varian Inova-400 spectrometer and chemical shifts were reported relative to internal Me_4Si.

The absorption spectra of the prepared dyes were examined at room temperature in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and recorded using1 cm quartz cells on a Shimadzu UV-1700 UV–vis spectrometer. Fluorescence measurements were carried out at room temperature on a Hitachi F-4500 spectrofluorimeter in TE buffer in 1 cm quartz cells. Excitation maximum was fixed by 3D-fluorescence spectra.

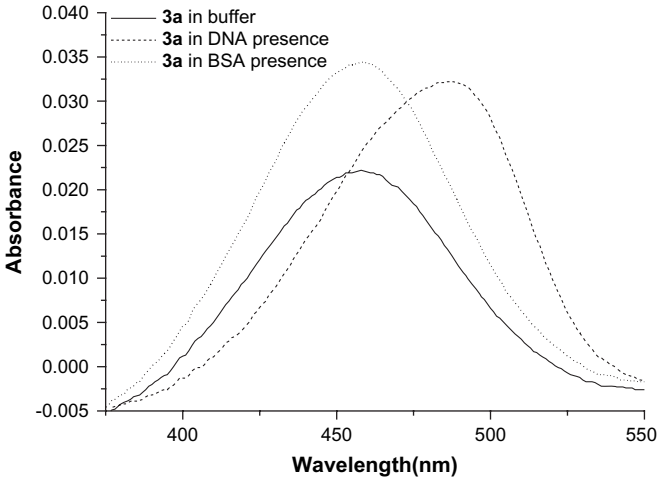

Fig. 1. Absorption spectra of **3a** in buffer, in the presence of DNA or BSA.

4.4. Preparation of stock solutions

The 5×10^{-4} M dye stock solutions were prepared by dissolving the dye in DMSO and further diluted with TE buffer (pH 7.5) to result in 5×10^{-5} M stock solutions of dye containing 10% DMSO. Stock solution of DNA/BSA was prepared by dissolving DNA/BSA in TE buffer. The concentrations of DNA and BSA in stock solutions were 8×10^{-5} M base pairs (bp) for DNA and 0.5 mg/mL for BSA.

4.5. Preparation of working solutions

Working solutions of all the dyes, DNA and BSA were prepared by further dilutions in TE buffer. Working solutions of DNA–dye complexes were prepared by mixing an aliquot of the dye stock solution and an aliquot of DNA stock solution in buffer. The concentrations of dye, DNA and BSA in working solutions were equal to 1×10^{-6} M, 1.6×10^{-5} M bp and 0.1 mg/mL, respectively. All the working solutions contain 0.2% DMSO. Thus, the dye to DNA concentration ratio is one dye molecule to 16 DNA base pairs. All working solutions were prepared immediately before the experiment.

4.6. Structural confirmations

4.6.1. 3-[2-(1-Methyl-quinolium-2-yl)-vinyl]-1H-indole iodide (*3a*)

Brown crystal, m.p.: 264–265 °C. ^1H NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.48 (s, 3H, N^+CH_3), 7.31–7.33 (m, 2H, ArH, CH=CH), 7.56–7.60 (m, 2H, ArH), 7.85 (t, 1H, ArH), 8.09 (t, 1H, ArH), 8.21–8.27 (m, 2H, ArH), 8.40 (s, 1H, pyrrole-H), 8.46 (d, $J = 9.2$ Hz, 1H, ArH),

Table 4
Spectral characteristics of dyes in DNA and BSA presence

Dye	In DNA presence					In BSA presence					$\Phi_F^{DNA}/\Phi_F^{free}$	$\Phi_F^{BSA}/\Phi_F^{free}$
	λ_{abs} (nm)	ε^{DNA} ($M^{-1}\,cm^{-1}$)	λ_{ex} (nm)	λ_{em} (nm)	Φ_F^{DNA}	λ_{abs} (nm)	ε^{BSA} ($M^{-1}\,cm^{-1}$)	λ_{ex} (nm)	λ_{em} (nm)	Φ_F^{BSA}		
3a	487.0	3.2×10^4	487.0	546.6	0.0750	458.0	3.4×10^4	458.0	542.8	0.0023	41.7	1.3
3b	483.0	2.7×10^4	483.0	548.8	0.1233	454.0	2.8×10^4	454.0	536.6	0.0033	41.1	1.1
3c	493.0	1.9×10^4	493.0	557.6	0.0307	470.0	2.1×10^4	470.0	556.4	0.0023	13.3	1.0
3d	495.0	2.5×10^4	495.0	559.4	0.0295	470.0	2.6×10^4	470.0	556.4	0.0021	18.4	1.3
3e	486.0	2.2×10^4	486.0	545.6	0.2200	458.0	2.5×10^4	458.0	544.2	0.0073	68.8	2.3
3f	499.0	3.0×10^4	499.0	558.6	0.0414	468.0	3.1×10^4	468.0	555.2	0.0020	27.6	1.3
3g	499.0	2.7×10^4	499.0	560.4	0.0364	471.0	3.6×10^4	471.0	553.8	0.0015	24.3	1.0

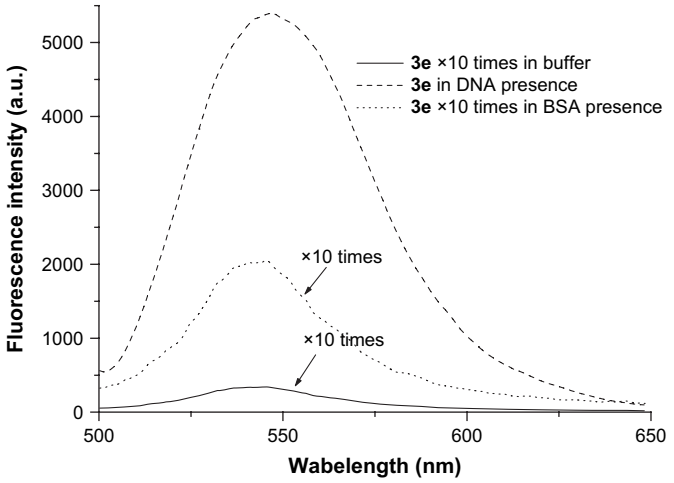

Fig. 2. Fluorescence spectra of **3e** in buffer, in the presence of DNA or BSA.

8.60–8.68 (m, 2H, ArH, CH═CH), 8.84 (d, $J = 9.2$ Hz, 1H, ArH), 12.34 (s, 1H, NH). IR (KBr) υ: 3447 (m, $\upsilon_{=NH}$), 3133 ($\upsilon_{=C-H}$), 1592, 1568 ($\upsilon_{C=C}$), 1508, 1463, 1427 (s, $\upsilon_{C=C}$, $\upsilon_{C=N}$), 1354, 1337, 1310, 1229, 1166, 1130 (δ_{CH}), 958, 747 (m, $\delta_{=CH}$) cm^{-1}. Anal. Calcd. for $C_{20}H_{17}N_2I = 412.27$: C, 58.27; H, 4.16; N, 6.79. Found: C, 58.09; H, 4.11; N, 6.63.

*4.6.2. 3-[2-(1,6-Dimethyl-quinolium-2-yl)-vinyl]-1H-indole iodide (**3b**)*

Brown crystal, m.p.: 262–263 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 2.57 (s, 3H, CH_3), 4.46 (s, 3H, N^+CH_3), 7.30–7.32 (m, 2H, ArH), 7.54–7.57 (m, 2H, ArH, CH═CH), 7.94 (d, $J = 9.2$ Hz, 1H, ArH), 8.03 (s, 1H, pyrrole-H), 8.20–8.22 (m, 1H, ArH), 8.36 (d, $J = 9.6$ Hz, 2H, ArH), 8.55–8.61 (m, 2H, ArH, CH═CH), 8.74 (d, $J = 9.2$ Hz, 1H, ArH), 12.28 (s, 1H, NH). IR (KBr) υ: 3441 (m, $\upsilon_{=NH}$), 3064 ($\upsilon_{=C-H}$), 1597, 1568 ($\upsilon_{C=C}$), 1511, 1477, 1426 (s, $\upsilon_{C=C}$, $\upsilon_{C=N}$), 1346, 1310, 1236, 1168, 1123 (δ_{CH}), 960, 948, 873, 840 (s, $\upsilon_{=CH}$), 805, 747 (m, $\delta_{=CH}$) cm^{-1}. Anal. Calcd. for $C_{21}H_{19}N_2I = 426.30$: C, 59.15; H, 4.46; N, 6.57. Found: C, 59.11; H, 4.23; N, 6.47.

*4.6.3. 3-[2-(6-Chloro-1-methyl-quinolium-2-yl)-vinyl]-1H-indole iodide (**3c**)*

Brown crystal, m.p.: 244–245 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.44 (s, 3H, N^+CH_3), 7.31–7.34 (m, 2H, ArH), 7.53–7.58 (m, 2H, ArH, CH═CH), 8.10 (d, $J = 9.6$ Hz, 1H, ArH), 8.20–8.22 (m, 1H, ArH), 8.37–8.38 (m, 1H, ArH), 8.41 (s, 1H, pyrrole-H), 8.46 (d, $J = 9.2$ Hz, 1H, ArH), 8.63–8.73 (m, 3H, ArH, CH═CH), 12.36 (s, 1H, NH). IR (KBr) υ: 3435 (m, $\upsilon_{=NH}$), 3171 ($\upsilon_{=C-H}$), 1598, 1568 ($\upsilon_{C=C}$), 1502, 1429 (s, $\upsilon_{C=C}$, $\upsilon_{C=N}$), 1389, 1649, 1308, 1225, 1174, 1135, 1094 (δ_{CH}), 960, 885, 808, 748 (m, $\delta_{=CH}$) cm^{-1}. Anal. Calcd. for $C_{20}H_{16}ClN_2I = 446.72$: C, 53.77; H, 3.61; N, 6.27. Found: C, 53.91; H, 3.57; N, 5.99.

*4.6.4. 3-[2-(6-Bromo-1-methyl-quinolium-2-yl)-vinyl]-1H-indole iodide (**3a**)*

Brown crystal, m.p.: 252–253 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.43 (s, 3H, N^+CH_3), 7.31–7.33 (m, 2H, ArH), 7.51–7.57 (m, 2H, ArH, CH═CH), 8.19–8.21 (m, 2H, ArH), 8.37 (d, $J = 10$ Hz, 1H, ArH), 8.42 (s, 1H, pyrrole-H), 8.51 (s, 1H, ArH), 8.62–8.66 (m, 2H, ArH, CH═CH), 8.69–8.71 (m, 1H, ArH), 12.40 (s, 1H, NH). IR (KBr) υ: 3411 (m, $\upsilon_{=NH}$), 3153 ($\upsilon_{=C-H}$), 1595, 1566 ($\upsilon_{C=C}$), 1499, 1428 (s, $\upsilon_{C=C}$, $\upsilon_{C=N}$), 1387, 1347, 1306, 1223, 1172, 1131, 1082 (δ_{CH}), 958, 878, 807, 746 (m, $\delta_{=CH}$) cm^{-1}. Anal. Calcd. for $C_{20}H_{16}BrN_2I = 491.17$: C, 48.91; H, 3.28; N, 5.70. Found: C, 48.98; H, 3.19; N, 5.44.

*4.6.5. 3-[2-(1,7-Dimethyl-quinolium-2-yl)-vinyl]-1H-indole iodide (**3e**)*

Brown crystal, m.p.: 279–280 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 2.66 (s, 3H, CH_3), 4.45 (s, 3H, N^+CH_3), 7.30–7.32 (m, 2H, ArH), 7.53–7.57 (m, 2H, ArH, CH═CH), 7.69 (d, $J = 8$ Hz, 1H, ArH), 8.13–8.27 (d, $J = 8$ Hz, 1H, ArH), 8.20–8.22 (m, 1H, ArH), 8.28 (s, 1H, pyrrole-H), 8.36 (s, 1H, ArH), 8.51 (d, $J = 9.2$ Hz, 1H, ArH), 8.58 (d, $J = 16$ Hz, 1H, CH═CH), 8.77 (d, $J = 8.8$ Hz, 1H, ArH), 12.39 (s, 1H, NH). IR (KBr) υ: 3437 (m, $\upsilon_{=NH}$), 3129 ($\upsilon_{=C-H}$), 1594, 1568 ($\upsilon_{C=C}$), 1504, 1424 (s, $\upsilon_{C=C}$, $\upsilon_{C=N}$), 1345, 1311, 1235, 1174, 1108 (δ_{CH}), 963, 833, 739 (m, $\delta_{=CH}$) cm^{-1}. Anal. Calcd. for $C_{21}H_{19}N_2I = 426.30$: C, 59.15; H, 4.46; N, 6.57. Found: C, 59.00; H, 4.27; N, 6.43.

*4.6.6. 3-[2-(7-Chloro-1-methyl quinolium-2-yl)-vinyl]-1H-indole iodide (**3f**)*

Brown crystal, m.p.: 260–261 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.43 (s, 3H, N^+CH_3), 7.31–7.34 (m, 2H, ArH), 7.55 (d, $J = 16$ Hz, 2H, ArH, CH═CH), 7.90 (d, $J = 8$ Hz, 1H, ArH), 8.21–8.27 (m, 2H, ArH), 8.42 (s, 1H, pyrrole-H), 8.58–8.62 (m, 2H, ArH), 8.69 (d, $J = 16$ Hz, 1H, CH═CH), 8.79 (d, $J = 9.2$ Hz, 1H, ArH), 12.39 (s, 1H, NH). IR (KBr) υ: 3435 (m, $\upsilon_{=NH}$), 3118 ($\upsilon_{=C-H}$), 1591, 1568 ($\upsilon_{C=C}$), 1504, 1459, 1430 (s, $\upsilon_{C=C}$, $\upsilon_{C=N}$), 1349, 1309, 1226, 1178, 1133 (δ_{CH}), 963, 922, 845, 745 (m, $\delta_{=CH}$) cm^{-1}. Anal. Calcd. for $C_{20}H_{16}ClN_2I = 446.72$: C, 53.77; H, 3.61; N, 6.27. Found: C, 54.02; H, 3.40; N, 6.24.

*4.6.7. 3-[2-(7-Bromo-1-methyl quinolium-2-yl)-vinyl]-1H-indole iodide (**3g**)*

Brown crystal, m.p.: 266–267 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.43 (s, 3H, N^+CH_3), 7.31–7.33 (m, 2H, ArH), 7.55 (d, $J = 16$ Hz, 2H, ArH, CH═CH), 8.2 (d, $J = 8$ Hz, 1H, ArH), 8.17 (d, $J = 8$ Hz, 1H, ArH), 8.22–8.24 (m, 1H, ArH), 8.44 (s, 1H, pyrrole-H), 8.63 (d, $J = 9.2$ Hz, 1H, ArH), 8.68–8.72 (m, 2H, ArH, CH = CH), 8.79 (d, $J = 9.2$ Hz, 1H, ArH), 12.41 (s, 1H, NH). IR (KBr) υ: 3459 (m, $\upsilon_{=NH}$), 3128 ($\upsilon_{=C-H}$), 1592, 1568 ($\upsilon_{C=C}$), 1501, 1458, 1432 (s, $\upsilon_{C=C}$, $\upsilon_{C=N}$), 1350, 1308, 1224, 1177, 1133, 1084 (δ_{CH}), 960, 842, 746 (m, $\delta_{=CH}$) cm^{-1}. Anal. Calcd. for $C_{20}H_{16}BrN_2I = 491.17$: C, 48.91; H, 3.28; N, 5.70. Found: C, 49.10; H, 3.36; N, 5.46.

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