ABSTRACT: Benzothiazoles are known to possess a number of biological activities and therefore are considered to be an important scaffold in the design and synthesis of pharmacophores. In this study, an improved synthesis method for novel fluorescent benzothiazole-based cyclic azacyanine (CAC) dyes bearing different electron-donating/withdrawing groups on their scaffold is presented. The improved method enabled us to increase the synthesis yield for the previously reported CACs. More importantly, it allowed us to synthesize new CAC dyes that were not synthesizable with the previously reported method. The synthesized dyes were characterized by 1H and 13C NMR spectroscopy, elemental analysis, and mass spectrometry and their optical (absorption and fluorescence) properties were investigated. All of the synthesized CACs were found to be displaying strong absorption within the range of 387 − 407 nm. The spectral shifts observed in the absorption and fluorescence measurements suggested that the spectroscopic and optical properties of CACs can be directly modulated by the nature of the electron-donating/withdrawing substituents. The fluorescence quantum yields (QYs) of the unsubstituted (parent CAC) and substituted CACs were also measured and compared. The fluorescence QY of CACs with electron-donating substituents (methoxy or ethoxy) was found to be at least four times higher than that of the parent CAC with no substitutions.

INTRODUCTION
The synthesis of novel heterocyclic cyanine dyes bearing various structural elements is of immense interest because of their potential mainly as fluorescent visualization and labeling agents. Cyanine dyes with their remarkable physicochemical characteristics including high photochemical stabilities, excellent molar absorption coefficients, narrow absorption bands, and high fluorescence intensities have found a wide range of applications in numerous fields such as single-molecule fluorescence studies, DNA labeling/detection, sensing of various biomolecules, cancer targeting, laser materials, and solar cells. Cyclic azacyanines (CACs) are nitrogen-containing heterocyclic benzothiazole/benzimidazole analogues which were first synthesized by Haddadin and co-workers as chloride-selective ion channel modulators. CACs are positively charged heterocyclic compounds with one or more nitrogen atoms and have recently emerged in a wide variety of fields (with respect to their spectral characteristics) including surface treatment of optical recording media, textile industry (as coloring agents), molecular recognition, biological applications, and biomedical imaging. Additionally, much effort has recently been put on the green/modified synthesis, spectral characterization, and other applications of numerous novel azacyanine dyes. Patra et al. investigated the spectroscopic and photophysical properties of three novel azacyanine dyes in various solvents to understand the solvent effect on the optical properties and energy states of CACs. They demonstrated that introduction of
electron-donating/withdrawing groups at specific positions on the azacyanine scaffold can lead to the modulation of its spectroscopic properties. An electron-donating (methoxy; $-\text{OCH}_3$) group had been shown to increase the fluorescence lifetime and influence the radiative process, whereas an electron-withdrawing (fluorine; $-\text{F}$) group had been shown to slightly increase the lifetime of the excited state and significantly enhance the radiative process with respect to the parent sulfur-containing CAC. Patra and co-workers also investigated the absorption and fluorescence properties of CACs both experimentally and theoretically. In line with their previous work, they also established the stabilization of the positive charge on the CAC ring by the electron-donating groups and, vice versa, the destabilization of the positive charge by the electron-withdrawing groups.\(^{20}\)

In a later study, Tasior et al.\(^{17}\) reported on the significant influence of electron-donating groups on the internal charge transfer and fluorescence quantum yield (QY) of V-shaped azacyanines. All these findings revealed that the photophysical properties of azacyanines can explicitly be controlled via the substitution of electron-donating/withdrawing groups. The same group also reported the synthesis and study of the optical properties of two bis(styryl)-azacyanines.\(^{21}\) In this case, the combination of high two-photon absorption cross sections and strong fluorescence QY resulted in good two-photon brightness, which opened up the possibility of the use of these dyes as bioimaging agents.

The use of benzothiazole/benzimidazole derivatives as effective biological markers in DNA detection has also drawn considerable attention in recent years because of their positively charged planar structure.\(^{4}\) So far, several derivatives of these compounds had already been reported with improved biological activities and optical properties.\(^{22,23}\)

Accordingly, during the last years, the major focus of our research group has been the investigation of the interactions of azacyanines with different nucleic acid structures.\(^{14,15,24,25}\)

Among the azacyanines investigated, only 2 of them named azacyanine4 (Aza4, 2a) and azacyanine5 (Aza5, 2b) were benzothiazole derivatives, and both of them were found to be binding to the human telomeric sequence tel24 and poly(A) with high affinity.\(^{14,15}\) They were also recently evaluated for their potential as topoisomerase II inhibitors.\(^{26}\) However, one downside of the azacyanine synthesis via the previously reported method was the low yields. In addition, the reported procedure was not suitable for the synthesis of the derivatives. Therefore, in an attempt first to increase their yield of synthesis and second to broaden the repertoire of azacyanine dyes with different electron-donating and electron-withdrawing groups, we directed our efforts toward progressing the current synthesis method to obtain a series of azacyanine dyes as novel analogues of "Aza4".

Here, the synthesis of Aza4 derivatives with different electron-donating/withdrawing substituents in the benzothiazole moiety with moderate yields was reported and their optical properties were investigated. It is demonstrated that the presence of substituents on the CAC impacts their spectroscopic properties significantly. The fluorescence QY of CACs with electron-donating substituents (methoxy or ethoxy) is shown to be at least four times as high as that of the parent CAC (without any electron-donating/withdrawing substituent). Likewise, the fluorescence QY of CACs with the electron-withdrawing substituents was lower.

## RESULTS AND DISCUSSION

CACs azacyanine4 (Aza4, 2a) and azacyanine5 (Aza5, 2b) which were initially synthesized by Haddadin and coworkers\(^{26,27}\) were also synthesized by our research group several times to investigate their interactions with nucleic acids. During our synthesis, our yields were about 10 and 20% for Aza4 and Aza5, respectively.\(^{14,15}\) In order to enrich our repertoire and understanding of the effect of electron-donating and electron-withdrawing groups on the optical properties of CACs, we initially tried to synthesize CACs with different functional groups on the benzothiazole ring using the previously established synthesis method. However, our initial trials failed, especially with the CACs bearing electron-withdrawing groups or electron-donating groups at position 4. In some of our synthesis, even though we observed the formation of the product during the reaction through the color change monitoring and UV–vis measurements, we were not able to obtain the product afterward. Therefore, first, we began contemplating several synthesis possibilities, starting with the optimization of parameters such as the reaction temperature, reactant ratio, and solvent amount. Again, our efforts were in vain. Next, we started to explore different reaction conditions using different solvents and reagents. The solvent effect on the one-pot synthesis of the target CACs was tested using different pure solvents including dimethylformamide, tetrahydrofuran, acetonitrile, 1-butanol, 2-butanol, chloroform, isomyl alcohol, tolune, pyridine, dimethylacetamide, and their mixtures with 2-(2-ethoxy)ethanol (diethylene glycol monoethyl ether) at different ratios. Besides, bromofrom was individually tested instead of didiomethane (as the reagent) during the optimization of the synthesis. Compared to the use of pure organic solvents, the use of binary organic solvent mixtures slightly promoted the yield of Aza4 (parent CAC) and Aza5 but showed negligible influence on the success of the synthesis of other CACs with an electron-donating methyl group at position 6 or with the methoxy group at position 4. In addition, despite a relatively satisfactory yield based on the weigh-ins, more than one product was observed in the $^{13}$C and $^1$H NMR spectra. There was also a deviation in the results of the elemental analysis from the expected values. One of the products observed in NMR was confirmed to be the desired product via MS. However, recharacterization of the sample via NMR after several weeks revealed the decomposition of the desired product over time.

These results indicated that one should be careful and should take such a possibility into consideration, especially with the synthesis of those with functional groups at position 4. As a result, among all the tested binary solvent mixtures and/or reagents, the best result was achieved in 2-(2-ethoxy)ethanol/pyridine (4:1 v/v) using CH$_2$I$_2$ as a reagent, which enhanced the yields of Aza4 and Aza5 CACs (more than 10%) with the desired characteristics and physiochemical properties.

However, our attempts to synthesize CACs with the electron-withdrawing substituents also led to time-dependent decomposition of the products. Subsequently, repeating the synthesis under an acidic medium in our synthetic route under the optimized conditions using glacial acetic acid was found to greatly promote the yield of all CACs. In other words, all of the desired CAC derivatives were synthesized successfully using a binary solvent mixture [2-(2-ethoxy)ethanol/pyridine: 4:1 v/v] and under mildly acidic conditions (Table 1). Spectral data
for the synthesized CACs were monitored weekly/monthly and no decomposition was observed.

Table 1. Spectral Data and the Yield for the Synthesized CAC Dyes (2a–2g)

| synthesized CAC | R₁ | R₂ | yield (%) | λ<sub>max</sub> (nm) | ε (M<sup>−1</sup> cm<sup>−1</sup>) ± SD<sup>b</sup> |
|----------------|----|----|-----------|---------------------|------------------------|
| 2a             | H  | H  | 41        | 387                 | 26,000 ± 2600          |
| 2b             | H  | OCH₃ | 44       | 407                 | 27,600 ± 2000          |
| 2c             | OCH₃ | H  | 27        | 400                 | 21,800 ± 1900          |
| 2d             | H  | CH₃  | 45        | 392                 | 31,000 ± 1100          |
| 2e             | H  | OCH₂CH₃ | 31      | 408                 | 26,600 ± 2800          |
| 2f             | H  | F    | 34        | 387                 | 29,500 ± 1700          |
| 2g             | H  | Cl   | 60        | 390                 | 31,600 ± 2500          |

<sup>a</sup> Measured in DMSO. <sup>b</sup> SD: standard deviation.

Azacyanine4 (2a), azacyanine5 (2b), and the other new CAC dyes (2c–2g) were synthesized through a simple one-step path, as depicted in Scheme 1. A proposed plausible mechanism for the CAC synthesis using the parent compound (2a) as the model is depicted in Scheme 2. All of the synthesized compounds were characterized by NMR spectroscopy (¹H and ¹³C), elemental analysis, and mass spectrometry (details are shown in the Materials and Instrumentation section). The yields of the synthesized CACs are listed in Table 1. Clearly, all of the synthesized compounds exhibited relatively satisfactory results in terms of the product yields. The highest product yield was observed for the compound 2g (60%).

The presence of electron-donating groups is possibly leading to a more stable state with lower energy by donating electron density to the π system of the CACs and stabilizing the positive charge on the nitrogen atom. Accordingly, among the electron-donating groups, only the alkyl substituent bound to position 6 of 1d (starting material of 2d) cannot donate the lone-pair electrons to the π system. However, it could still increase the electron density of the ring system, as evident from 45% product yield of 2d. In the case of the compound 2c, the methoxy group attached to position 4 is believed not able to stabilize the ring system as effectively as the other electron-donating groups, and therefore, it exhibits the lowest product yield (27%). Besides, the position 4 is believed to be creating steric hindrance for the dimerization reaction (can also be observed from the proposed reaction mechanism) and less stability resulting in a lower yield. This can be considered as the main reason for the fact that our efforts for the synthesis of

Scheme 1. Path to Synthesize CAC Dyes<sup>a</sup>

<sup>a</sup> Reagents and conditions for all of the synthesized azacyanines: 2-(2-ethoxy)ethanol/pyridine (4:1 v/v) under slightly acidic conditions, diiodomethane/200 °C.

Scheme 2. Proposed Reaction Mechanism for the Formation of Parent CAC (Aza4, 2a).
the CAC derivative with the methyl substituent at position 4 failed to yield the final product.

On the other hand, the presence of electron-withdrawing groups is leading to a less-stable structure by decreasing the electron density on the aromatic ring.30 They are neither able to stabilize the positive charge on the nitrogen nor contribute to the conjugation of the \( \pi \) system. Therefore, the presence of electron-withdrawing functional groups could decrease the reactivity of the molecule. However, in this case, \(-\text{Cl}\) and \(-\text{F}\) as electron-withdrawing substituents (which are exceptions of electron-withdrawing groups with an ability to donate their lone pairs to the \( \pi \) system of aromatic rings31) can still induce electron density and enhance the reactivity. Hereby, the product yield of the 2f and 2g compounds (34 and 60%, respectively) was in an acceptable range and close to the yield of the parent CAC with no functional group (2a).

Once the synthesis of CACs was determined to be successful via \(^1\text{H}\) and \(^13\text{C}\) NMR, elemental analysis, and mass spectroscopy, their optical properties were investigated using UV–vis absorption and fluorescence spectroscopy techniques. All the synthesized CAC dyes (2a–2g) were found to be displaying intense and broad absorptions \((\varepsilon > 20,000 \text{ M}^{-1}\text{cm}^{-1})\) in the visible region \((\lambda_{\text{max}}: 387–408 \text{ nm}, \text{Table 1})\). As seen, the maximum absorption wavelength for the synthesized CACs rises in the order 2a = 2f < 2g < 2d < 2c < 2b < 2e, probably as a consequence of the presence of the electron-withdrawing \(-\text{F}\) and \(-\text{Cl}\) and electron-donating \(-\text{CH}_3\), \(-\text{OCH}_3\), and \(-\text{OCH}_2\text{CH}_3\) substituents at the specific positions on the synthesized CAC scaffold in relation to 2a (CAC with no functional group).

Figure 1 depicts the normalized UV–vis absorption, fluorescence emission, and excitation spectra of the synthesized CAC dyes at the same concentration [30.0 \( \mu \text{M}, \text{in dimethyl sulfoxide (DMSO)}\)] at room temperature. Based on the results, Aza4 (2a, Figure 1A) as the parent compound (without any electron-donating/withdrawing substituent) showed a broad absorption spectrum between 330 and 420 nm \((S_0\rightarrow S_1\) transition) with two observable vibronic bands at around 387 and 396 nm. Also, a maximum emission intensity at 446 nm was observed (Table 2) in its fluorescence emission spectrum. The presence of the electron-donating \(-\text{OCH}_3\) group as in 2b and 2c, \(-\text{CH}_3\), \(-\text{OCH}_3\text{CH}_3\) as in 2d and 2e, respectively) and an electron-withdrawing group \(-\text{Cl}\) as in 2g) resulted in a red shift in the absorption spectra of these synthesized CACs. The maximum absorbance \((\text{max} \lambda_{\text{abs/\text{nm}}})\) and maximum emission \((\text{max} \lambda_{\text{em/\text{nm}}})\) wavelengths as well as

Table 2. Optical and Photophysical Properties of the Synthesized CAC Compounds (2a–2g)

| synthesized CAC | \(\lambda_{\text{abs max}}\) (nm) | \(\lambda_{\text{em max}}\) (nm) | Stokes’ shift (cm\(^{-1}\)) | fwhm (nm)\(^a\) | \(\Phi_F\) (%)\(^b\) |
|-----------------|------------------|------------------|-----------------|-----------------|-------------|
| 2a              | 387              | 446              | 3418            | 63              | 17.0        |
| 2b              | 407              | 478              | 3650            | 70              | 70.0        |
| 2c              | 400              | 474              | 3903            | 72              | 26.4        |
| 2d              | 392              | 458              | 3676            | 63              | 47.5        |
| 2e              | 408              | 486              | 3934            | 70              | 71.0        |
| 2f              | 387              | 447              | 3468            | 64              | 13.4        |
| 2g              | 390              | 456              | 3711            | 64              | 17.7        |

\(^a\)fwhm: full width at half-maximum for the fluorescence band.\(^b\)Measured in DMSO.
the Stokes' shifts (in cm\(^{-1}\)) of the synthesized CAC dyes are tabulated in Table 2.

As can be seen, 2b and 2e displayed a similar spectral behavior (Figure 1B and E). In both, the maximum absorption was around 407 and 408 nm, respectively, and a transition from S\(_0\) to the two different vibrational energy levels of S\(_1\) was observed. Moreover, the highest red shift compared to the maximum \(\lambda_{\text{abs}}\) of Aza4 (2a) was observed in 2e. This observation is probably due to the effect of the "ethoxy" substituent as the strong activating electron-donating group capable of donating the lone-pair electrons to the \(\pi\) system of adjacent carbons and increasing the electron density on the fused ring system by resonance effect accompanied by the delocalization of the electrons. In the absorption spectrum of 2c, the peak intensity of the transition from S\(_0\) to the higher vibrational level of S\(_1\) has sharply decreased and two separate peaks were present around 330 nm (the absorption maximum for the S\(_0\)-S\(_1\) transition in the 300–350 region) and 400 nm, respectively. Evidently, in this case, the spectral region for the S\(_0\)-S\(_1\) transition was different from those obtained for the other synthesized CACs which might be attributed to the presence of an electron-donating functional group (methoxy) at position 4. This also shows that the same substituent exerted very different effects in different positions. As mentioned previously, the synthesis of compounds with functional groups at position 4 was unsuccessful compared to the synthesis of compounds with functional groups at position 6. Figure 2, displaying the UV–vis absorption spectra of the synthesized CAC dyes at 30.0 \(\mu\)M concentration in DMSO at room temperature.

![Figure 2](https://dx.doi.org/10.1021/acsomega.0c02202)

Figure 2. UV–vis absorption spectra of the synthesized CAC dyes at 30.0 \(\mu\)M concentration in DMSO at room temperature.

- The Stokes' shifts (in cm\(^{-1}\)) of the synthesized CAC dyes are tabulated in Table 2.
- As can be seen, 2b and 2e displayed a similar spectral behavior (Figure 1B and E). In both, the maximum absorption was around 407 and 408 nm, respectively, and a transition from S\(_0\) to the two different vibrational energy levels of S\(_1\) was observed. Moreover, the highest red shift compared to the maximum \(\lambda_{\text{abs}}\) of Aza4 (2a) was observed in 2e. This observation is probably due to the effect of the "ethoxy" substituent as the strong activating electron-donating group capable of donating the lone-pair electrons to the \(\pi\) system of adjacent carbons and increasing the electron density on the fused ring system by resonance effect accompanied by the delocalization of the electrons. In the absorption spectrum of 2c, the peak intensity of the transition from S\(_0\) to the higher vibrational level of S\(_1\) has sharply decreased and two separate peaks were present around 330 nm (the absorption maximum for the S\(_0\)-S\(_1\) transition in the 300–350 region) and 400 nm, respectively. Evidently, in this case, the spectral region for the S\(_0\)-S\(_1\) transition was different from those obtained for the other synthesized CACs which might be attributed to the presence of an electron-donating functional group (methoxy) at position 4. This also shows that the same substituent exerted very different effects in different positions. As mentioned previously, the synthesis of compounds with functional groups at position 4 was unsuccessful compared to the synthesis of compounds with functional groups at position 6.
withdrawing/donating groups. The electron-donating groups enhance it. Among the synthesized CACs, 2e and 2b are anticipated to be the best potential candidates for the optical detection and imaging platforms based on their high QYs. An analogous platform was recently developed utilizing 2b by our group.41

■ CONCLUSIONS

In conclusion, an improved synthesis method based on the use of the binary solvent mixture, 2-(2-ethoxy)ethanol/pyridine (4:1 v/v) in a slightly acidic medium, for the synthesis of CAC dyes was developed in this study. The improved method enabled the synthesis of novel CACs with different electron-donating/withdrawing groups in their benzothiazole ring. It was found that introducing electron-donating/withdrawing substituents onto the benzothiazole scaffold was affecting their synthesis and spectroscopic/optical properties. Compared to the other synthesized CACs, compound 2c exhibited the lowest product yield probably because of the methoxy group being located at position 4. Except for the compound 2f, a red shift was observed in the absorption spectra for all the CACs whether they are bearing an electron-donating or electron-withdrawing group. However, the synthesized CACs with electron-donating groups displayed greater red shifts than the CACs with electron-withdrawing groups. The highest red shift was observed in 2e because of the effect of the ethoxy substituent. The compounds 2f and 2g exhibited lesser red shifts than 2e and 2b. Despite the fact that 2f possesses two evident electron-withdrawing groups (2,8-F), its absorption spectrum was quite similar to the spectrum of 2a and did not exhibit any obvious red shift. The compound 2c also displayed a similar red shift to 2g. However, its spectrum was completely different than the spectrum of all other CACs more likely because of the functional group being at position 4.

In terms of the fluorescence spectra, all the synthesized CAC dyes, except 2f, showed intense and broad emission and substitution-dependent shifts in their emission maxima. The type of the substituent also had a notable impact on the QYs. The compounds with electron-donating groups had higher QYs in general where the highest fluorescence QY was observed for the compound 2e. More importantly, its QY was relatively high compared to the QYs of other CACs and cyanine dyes reported. All the observations confirm that the compounds 2e and 2b can be plausible candidates for optical imaging applications. More importantly, the improved synthesis method reported here opens up new possibilities in the synthesis of new CAC dyes.

■ MATERIALS AND INSTRUMENTATION

2-Aminobenzothiazole (1a, 97%), 2-(2-ethoxy)ethanol (99.0%), perylene (≥99%), bromoform (CHBr3, 99%), and dichloromethane (≥99%) were obtained from Sigma-Aldrich (St. Louis, USA), 2-Amino-6-methoxybenzothiazole (1b, 98%) and 2-amino-4-methoxybenzothiazole (1c, 97%) were purchased from Acros Organics (New Jersey, USA). 2-Amino-6-methylbenzothiazole (1d, 99%), 2-amino-6-ethoxybenzothiazole (1e, 99%), and 2-amino-6-fluorobenzothiazole (1f, 98%) were supplied by Alfa Aesar (Kandel, Germany). 2-Amino-6-chlorobenzothiazole (1g, 98%) was purchased from TCI (Tokyo Chemical Industry, Tokyo, Japan). DMSO (99.8%) and cyclohexane (99.5%) were obtained from AppliChem GmbH (Darmstadt, Germany). Deuterated dimethyl sulfoxide (DMSO-d6) for NMR spectroscopy, pyridine (spectroscopic

Figure 4. Digital photograph of the synthesized CAC dyes taken under a 365 nm UV lamp.
Also, both solvents were checked for background absorbance values; all would be kept between 0.02 and 0.1. Concentration of the samples were plotted and the slopes of the synthesized CACs and the standard sample was 410 nm, respectively. The spectral data were collected using a Cary Eclipse spectrophotometer (Agilent Technologies, USA). Elemental analysis (C, H, N, and S) was performed on a CE Instruments (Milford, MA, USA). To use this absorption spectra for the synthesized CACs at room temperature. Fluorescence intensity measurements were performed at room temperature using an Agilent Cary Eclipse spectrophotometer (Agilent Technologies, USA). The excitation and emission slit widths were set at 2.5 and 5.0 nm, respectively. The spectral data were collected using ChemStation software and data processing/analysis was performed using IGOR Pro software.

**General Information. Molar Extinction Coefficients (ε) and Fluorescence QY Measurements**. The molar extinction coefficients (ε) of all the synthesized derivatives were calculated with the well-established approaches using the absorption wavelengths (λ_max) of the as-synthesized CAC standard solutions. 

For ε measurements, first, each of the synthesized CAC derivatives was weighed at different amounts (5.0, 10.0, 15.0, 20.0, and 25.0 mg) and diluted to 50.0 mL with DMSO. Second, five different stock solutions were prepared for each CAC and each stock solution was diluted to different dilution ratios. Their UV–vis absorption values were measured at their maximum absorption wavelength. Afterward, the graphs of the absorbance versus the concentration of the samples were plotted and the slopes of the five plots of the synthesized compounds were averaged to obtain their molar extinction coefficients (ε) as assessed by Beer–Lambert law (Beers law).

The fluorescence QYs of the synthesized CACs (in DMSO) were measured under dilute conditions with a relative optical method using perylene (QY = 0.94) in cyclohexane as a reference standard. To avoid concentration effects including internal reflections, self-quenching, and secondary inner filter effect (reabsorption of the emission), the sample/standard concentration ranges were carefully adjusted such that the absorbance values would all be kept between 0.02 and 0.1. Also, both solvents were checked for background fluorescence prior to the experiments. The excitation wavelength used for the synthesized CACs and the standard sample was 410 nm, and the fluorescence QYs were determined using the following equation:

\[ \Phi_{ST} = \Phi_{ST} \frac{(\text{Grad}_{ST})}{(\eta_{ST})^2} \]

where Φ is the fluorescence QY (the subscripts ST and x denote the standard and the synthesized CAC, respectively).

Grad and Grad_{ST} are the measured integrated emission intensity of the CAC and the standard sample, respectively, and η is the refractive index of the solvents.

**General Procedure for the Synthesis of CACs.** The general one-step procedure for the synthesis of “Aza4” and other CAC dyes as their analogues were as follows:

A total of 1.0 g of the starting material was taken in a dry round-bottom flask equipped with a magnetic stirrer. A mixture of 5.0 mL of pyridine and 20.0 mL of 2-(2-ethoxyethoxy) ethanol was added to it and stirred carefully at room temperature to get a clear solution. Next, 600.0 μL of glacial acetic acid was added to the reaction mixture and the solution was heated to 200 °C. Afterward, diiodomethane (2.0 g, 600.0 μL, 7.5 mmol) was immediately injected into the above-mentioned mixture and refluxed at 200 °C for 15 min under a gentle flow of nitrogen. Upon completion of the reaction, the mixture was allowed to cool down to room temperature. Finally, the precipitated solid was filtered off and washed thoroughly (several times) with pure acetone and dichloromethane and dried at 65 °C overnight. The final obtained products were recrystallized from methanol (20% v/v; MeOH–water) and characterized. Because of its lower polarity (in relation to alcohols) and the good solubility of the CACs, DMSO was preferred as a solvent to measure the molar extinction coefficients. The stock solutions (1 mM) of the synthesized CACs were prepared in DMSO and the working solutions were prepared after dilution with DMSO to 10.0 and 30.0 μM during recording the fluorescence and absorption spectra, respectively.
Azacyanine-6-methyl: (3,9-Dimethyl-13H-benzo[4,5]-thiazolo[3,2-b]benzo[4,5]thiazolo[2,3-d][1,3,5]triazin-12-(2H). The product was obtained as a pale-yellow solid compound; 1H NMR (400 MHz, DMSO-d6): δ 2.50 (s, 6H), δ 6.5945 (s, 2H), δ 6.7667, 7.6645, 7.643 (dd, 2H), 7.8267, 7.8059 (d, 2H), 8.04 (s, 2H); 13C NMR (125 MHz, DMSO-d6): δ 20.8858, 38.847, 39.054, 39.263, 39.472, 39.680, 39.890, 40.097, 112.729, 112.921, 123.885, 124.009, 129.607, 134.021, 136.727; HRMS (m/z): calculated for [M + H]+ 324.629; found, 324.629; elemental analysis experimental: calculated for C14H12N2S2: C, 45.24%; H, 3.13%; N, 9.31%; S, 14.21%. Found: C, 44.52%; H, 3.32%; N, 9.52%; S, 14.18%. Yield: 45%; Φ: 0.475, λem = 458 nm; UV–vis (DMSO): ε = 31,000 M−1 cm−1, λmax = 392 nm.

Azacyanine-6-ethoxy: (3,9-Diethoxy-13H-benzo[4,5]-thiazolo[3,2-b]benzo[4,5]thiazolo[2,3-d][1,3,5]triazin-12-(2H). The product was obtained as a yellow solid compound; 1H NMR (400 MHz, DMSO-d6): δ 1.3723, 1.3898, 1.3723 (t, 6H), δ 4.1503, 150.3, 4.1327, 4.1152 (q, 4H), δ 6.5531 (s, 2H), δ 7.4235, 7.4289, 7.4126, 7.4063 (dd, 2H), δ 7.804, 7.7918 (d, 2H), δ 7.848, 7.8417 (d, 2H); 13C NMR (125 MHz, DMSO-d6): δ 14.467, 38.881, 39.080, 39.298, 39.507, 39.715, 40.113, 60.468, 64.167, 108.773, 113.998, 116.638, 124.383, 129.807, 157.114, 164.540; HRMS (m/z): calculated for [M + H]+, 384.0840; found, 384.0829; elemental analysis experimental: calculated for C14H17O2N2S2: C, 46.90%; H, 3.78%; N, 8.77%; S, 13.38%. Found: C, 43.98%; H, 3.74%; N, 8.37%; S, 13.36%. Yield: 31%; Φ: 0.710, λem = 486 nm; UV–vis (DMSO): ε = 26,600 M−1 cm−1, λmax = 408 nm.

Azacyanine-6-fluoro: (3,9-Difluoro-13H-benzo[4,5]-thiazolo[3,2-b]benzo[4,5]thiazolo[2,3-d][1,3,5]triazin-12-(2H). The product was obtained as a creamy yellow solid compound; 1H NMR (400 MHz, DMSO-d6): δ 6.645 (s, 2H), δ 7.826, 7.820, 7.803, 7.797, 7.778, 7.814 (dt, 2H), δ 7.963, 7.953, 7.941, 7.930 (m, 2H), δ 8.204, 8.198, 8.183, 8.177 (dd, 2H); 13C NMR (125 MHz, DMSO-d6): δ 60.944, 111.424, 111.726, 114.712, 114.813, 116.642, 116.910, 125.585, 125.719, 132.992, 158.662, 161.090, 166.704; HRMS (m/z): calculated for [M + H]+, 332.0115; found, 332.0128; elemental analysis experimental: calculated for C14H11F2N2S2: C, 39.23%; H, 1.76%; N, 9.15%; S, 13.96%. Found: C, 39.11%; H, 1.97%; N, 9.08%; S, 14.08%. Yield: 34%; Φ: 0.134, λem = 447 nm; UV–vis (DMSO): ε = 29,450 M−1 cm−1, λmax = 387 nm.

Azacyanine-6-chloro: (3,9-Dichloro-13H-benzo[4,5]-thiazolo[3,2-b]benzo[4,5]thiazolo[2,3-d][1,3,5]triazin-12-(2H). The product was obtained as a yellowish-brown solid compound; 1H NMR (400 MHz, DMSO-d6): δ 6.638 (s, 2H), δ 7.9195, 7.8975 (d, 2H), δ 7.987, 7.981, 7.645, 7.643 (dd, 2H), δ 7.391, 8.386 (d, 2H); 13C NMR (125 MHz, DMSO-d6): δ 60.502, 114.580, 124.068, 125.743, 129.069, 130.935, 134.994, 166.946; HRMS (m/z): calculated for [M + H]+, 363.953; found, 363.954; elemental analysis experimental: calculated for C14H11Cl2N2S2: C, 36.60%; H, 1.64%; N, 8.54%; S, 13.03%. Found: C, 35.74%; H, 1.82%; N, 9.49%; S, 12.71%. Yield: 60%; Φ: 0.177, λem = 456 nm; UV–vis (DMSO): ε = 31,600 M−1 cm−1, λmax = 390 nm.

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