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^{1}H and ^{13}C NMR Assignments for the Cyanine Dyes SYBR Safe and Thiazole Orange

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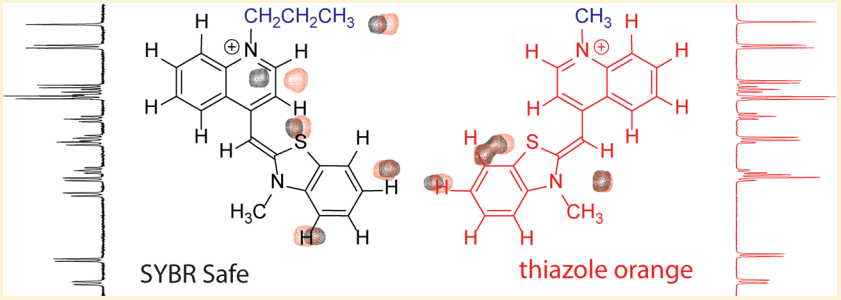
Supporting Information



ABSTRACT: Analysis of ^{1}H and ^{13}C NMR and mass spectral data for the fluorescent nucleic acid stain SYBR Safe indicates that it contains a cyanine-based cationic core structure identical to thiazole orange. The difference between these two compounds is the type of N-substitution on the quinolinium ring system (SYBR Safe, *n*-Pr; thiazole orange, Me). The ^{1}H and ^{13}C NMR resonances for both compounds were assigned on the basis of one- and two-dimensional (COSY, ROESY, HSQC, and HMBC) experiments. The preferred conformation of these compounds was computed by ab initio methods and found to be consistent with the NMR data.

Fluorescence plays a central role in nucleic acid detection, exemplified by cyanine-based "light up probes" whose emission can increase significantly (>1000) due to conformational restriction induced by intercalative or minor groove binding events.[1−5] In this paper, we report a structure elucidation of a commonly used fluorescent nucleic acid stain, SYBR Safe (Life Technologies/Invitrogen/Molecular Probes). This work was undertaken in the context of student-designed projects in an undergraduate NMR spectroscopy course.[6] Interest in this dye derived from its reported nontoxicity[7] and widespread use in biological and biochemical research.[8−11] In contrast with other fluorescent nucleic acid dyes such as ethidium bromide, SYBR Safe has been determined to be nongenotoxic and nonmutagenic in biological tests and is not classified as hazardous waste under United States federal regulations.

According to published information, SYBR Safe is one of several Life Technologies cyanine dyes for ultrasensitive nucleic acid detection, a product line which includes PicoGreen, SYBR Green I, and SYBR Gold.[12] While the chemical structure of SYBR Safe is not provided by the supplier nor with its Chemical Abstracts Service (CAS) entry (1030826-36-8), it became clear from preliminary NMR and LC-MS data obtained for our project, as well as details within the patent literature,[13] that the compound had a reasonable probability of being an *N*-propyl derivative of the cyanine dye thiazole orange. Once we assigned the ^{1}H and ^{13}C spectra of SYBR Safe, we wanted to confirm our structural assignment by comparison with thiazole orange NMR data. Despite its common use as a dye-based biological stain, a literature search revealed very little about the NMR assignments for thiazole orange, aside from one report listing the unassigned ^{1}H chemical shifts for the iodide salt.[14] To provide a valid comparison with SYBR Safe, we therefore undertook an NMR study of thiazole orange and report the ^{1}H and ^{13}C assignments here. Our study confirms the structural similarity of these two compounds, and we have determined that the dye component in SYBR Safe is (*Z*)-4-((3-methylbenzo[*d*]thiazol-2(3*H*)-ylidene)methyl)-1-propylquinolin-1-ium 4-methylbenzenesulfonate (**1**).

The putative SYBR Safe (**1**) and known thiazole orange (**2**) structures and numbering system are shown in Figure 1. Both structures are singly charged cyanine dyes consisting of a N-substituted quinolinium ring system conjugated at 4-C to a 3-methyl-2-methylene-2,3-dihydrobenzo[*d*]thiazole ("benzothiazole") system. The counterion in structures **1** and **2** is *p*-toluenesulfonate. Table 1 compiles the ^{1}H and ^{13}C data NMR obtained for compounds **1** and **2** dissolved in DMSO-d_6 at 25 °C.

The SYBR Safe resonances were assigned with a standard set of 1D and 2D NMR techniques. The presence of an *N*-*n*-propyl residue was clearly evident from analysis of integrals and coupling patterns. Key ring ^{1}H chemical shifts were localized by Overhauser effects involving the exocyclic 3′-*N*-Me, 1-*α*-*N*-Pr, and 2a′-H resonances (Figure 1). The 1-*α*-*N*-Pr methylene protons were used to identify the quinolinium 8-H and 2-H

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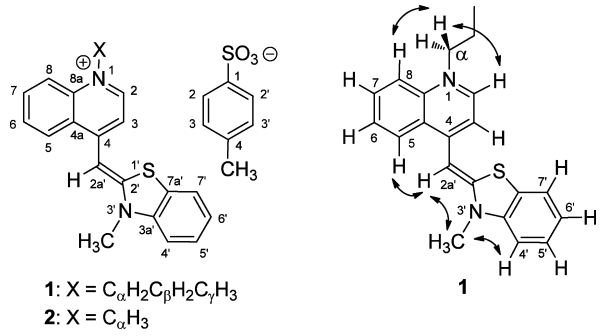

Figure 1. Left: Structures and numbering scheme for SYBR Safe (**1**), thiazole orange (**2**), and *p*-toluenesulfonate. Right: Key nuclear Overhauser interactions in SYBR Safe.

resonances, with the latter significantly deshielded (8.64 ppm) and scalar-coupled only to the 3-H resonance. The 2a′-H benzylidene hydrogen showed two Overhauser effects. One is to the 3′-CH_3 group, which suggests a *cis* relationship between these two groups. The other is to the most deshielded signal at 8.81 ppm, assigned to 5-H and confirmed on the basis of scalar and dipolar coupling connectivity via 6-H and 7-H to 8-H. The 2a′-H Overhauser data suggest a conformational bias in which the benzylidene hydrogen is proximal to H-5 and not H-3. This Overhauser effect is consistent with computed low-energy conformations of **1** (Figure 2). The benzothiazole 4′-H resonance was identified by its Overhauser interaction with the 3′-CH_3 group, and the remaining ring hydrogens (5′-H, 6′-H, and 7′-H) were identified by scalar and dipolar coupling interactions involving the 4′-H resonance.

In addition to the signal assignments already discussed, it was also necessary to account for a methyl group at 2.2 ppm and an aromatic AA′BB′ spin system at 7.10 and 7.47 ppm. These chemical shifts and couplings fit *p*-toluenesulfonate quite well. The presence of this anion is not surprising, as *p*-toluensulfonate esters are often used as alkylating agents in cyanine dye syntheses and this anion is explicitly mentioned in the patent preparation of **1**.[13]

With the ^{1}H assignments in hand, HSQC correlations were used to assign the C−$(H)_n$ carbon resonances. With the exception of one resonance, these assignments presented no difficulty. For the case of 6′-H, however, linear prediction in the F1 dimension proved necessary to correlate this resonance to the most downfield of three closely spaced ^{13}C resonances at 123.8, 124.2, and 124.4 ppm (details are provided in the Supporting Information).

The quaternary and sp^2-hybridized 2′-C, flanked by nitrogen and sulfur, was assigned (160.0 ppm) on the basis of its unique environment. The remaining quaternary carbons were assigned with HMBC correlations. In the quinolinium ring, the 1-α-N-CH_2 hydrogens showed ^3J correlations to 137.0 ppm (8a-C)

Table 1. ^{1}H and ^{13}C Signal Assignments for Compounds 1 and 2[a]

ring system/position	^{1}H shifts		^{13}C shifts	
	1	**2**	**1**	**2**
quinolinium				
1-CH_2(H)α	4.57 (t, 7.3)	4.17 (s)	55.4	42.3
1-$CH_2\beta$	1.89 (sx, 7.3)		22.1	
1-$CH_3\gamma$	0.96 (t, 7.3)		10.6	
2	8.64 (d, 7.2)	8.61 (d, 7.1)	144.4	145.0
3	7.39 (d, 7.2)	7.36 (d, 7.1)	107.7	107.7
4			148.5	148.5
4a			124.2	123.9
5	8.81 (d, 8.4)	8.80 (d, 8.1)	125.7	125.4
6	7.76 (t, 7.5)	7.78 (td, 7.4, 1.0)	126.7	126.8
7	7.99 (t, 7.5)	8.02 (m)	133.2	133.1
8	8.17 (d, 8.5)	8.05 (m)	118.1	118.2
8a			137.0	137.9
benzothiazole				
2′			160.0	159.7
2a′	6.94 (br s)	6.93 (br s)	88.0	87.8
3′-CH_3	4.03 (s)	4.01 (s)	33.7	33.7
3a′			140.4	140.4
4′	7.80 (d, 8.2)	7.77 (d, 8.0)	112.9	112.8
5′	7.63 (td, 7.5, 1.0)	7.61 (td, 7.8, 1.0)	128.1	128.0
6′	7.43 (td, 7.5, 1.0)	7.41 (td, 7.6, 1.0)	124.4	124.3
7′	8.06 (dd, 7.9, 1.0)	8.04 (m)	122.8	122.8
7a′			123.8	123.7
p-toluenesulfonate				
1			145.8	145.8
2, 2′	7.47 (d, 8.0)	7.47 (d, 8.0)	125.4	125.4
3, 3′	7.10 (d, 8.0)	7.10 (d, 8.0)	127.9	127.9
4			137.4	137.4
4-CH_3	2.28 (s)	2.28 (s)	20.7	20.7

[a]Chemical shifts (in DMSO-d_6 at 25 °C) are reported in parts per million relative to TMS; ^{1}H multiplicities and coupling constants are reported in hertz.

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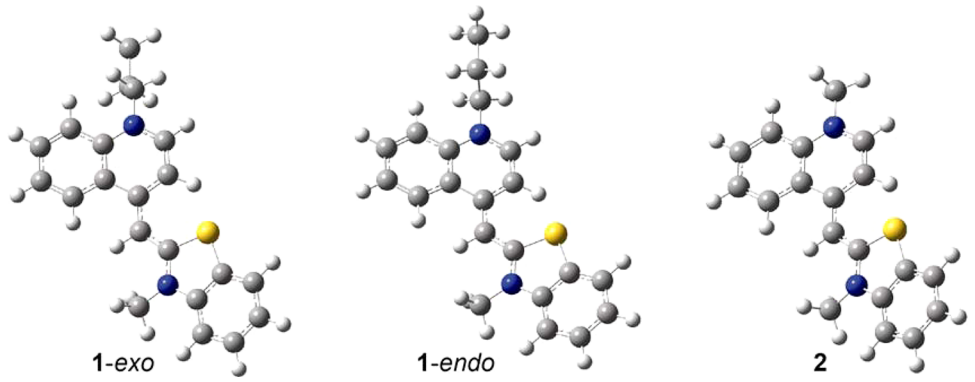

Figure 2. B3LYP/6-31G(d,p) computed low-energy structures of the cyanine dye component of **1** shown with the 1-*N*-Pr substituent in the *exo* and *endo* orientations. The computed low-energy conformation of thiazole orange (**2**) is shown at right.

and 144.4 ppm (2-C, assigned via HSQC). Likewise, this type of coupling provided correlations between the benzylidene 2a′-H resonance and 124.2 ppm (4a-C) and 107.7 ppm (3-C, assigned via HSQC). The two remaining benzothiazole quaternary carbons were assigned on the basis of their chemical shifts: 3a′-C at 140.4 ppm (C bound to N) as compared with 7a′-C at 123.8 ppm (C bound to S). These assignments were confirmed by the presence of ^3J HMBC connectivity between the 3′-*N*-CH_3 resonance and the signal at 140.4 ppm (3a′-C) and the 6′-H resonance and the signal at 123.8 ppm (7a′-C). The ^{13}C resonances for the *p*-toluenesulfonate anion were assigned on the basis of their chemical shifts and HSQC/HMBC data.

To determine the molecular mass of the cationic dye component in SYBR Safe, a sample was analyzed with liquid chromatography–mass spectrometry (LC–MS) operating in the positive ion mode. A single component, absorbing at 505 nm, produced a molecular ion at 333.1 *m*/*z* (calculated for **1**: 333.14). This result lent further support for our structural assignment.

We next assigned ^1H and ^{13}C spectra for thiazole orange (**2**), reasoning that **1** and **2** should share common NMR spectral characteristics. The thiazole orange resonance assignment process proceeded in a manner very similar to that described for **1**. The same pattern of Overhauser effects involving exocyclic resonances was observed. Namely, interactions between the 2a′-H resonance and the 3′-CH_3 and 5-H resonances are indicative of a preferred conformation similar to that computed for **1** (Figure 2). Relative to compound **1**, one difference in the aromatic ^1H spectrum of **2** is that the 7-H, 8-H, and 7′-H resonances are overlapped at 400 MHz. However, the HSQC experiment was used to assign these shifts (Table 1 and Figure 3). The HMBC experiment provided ^3J correlations necessary for completing the assignments of all quaternary carbons (details are provided in the Supporting Information).

There is a high degree of similarity between the ^1H and ^{13}C resonances in SYBR Safe and thiazole orange, particularly among the ^{13}C shifts (Table 1 and Figure 3). The ^1H and ^{13}C shifts compare very favorably even within the quinolinium ring structure, although some deviations should be expected on the basis of differences in N-substitution. The ^1H and ^{13}C chemical shifts for the *p*-toluenesulfonate anion resonances are virtually identical in the two samples. As this is the known anionic component in the thiazole orange formulation, it is reasonable

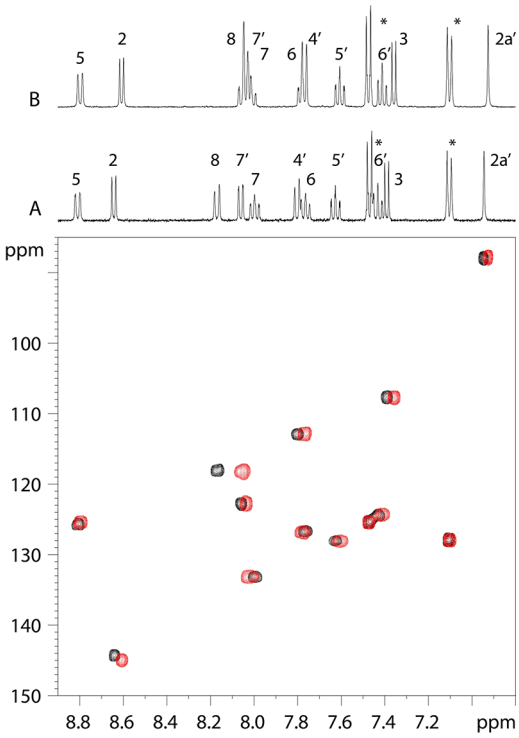

Figure 3. ^1H NMR (400 MHz) spectra for aromatic and benzylidene resonances in (A) SYBR Safe **1** and (B) thiazole orange **2**. Two-dimensional HSQC plot showing aromatic C–H correlations for compounds **1** (black) and **2** (red). The peaks labeled with an asterisk are assigned to the *p*-toluenesulfonate anion.

to conclude that it also serves as the anionic species in the formulation of SYBR Safe used in our NMR study.

A compound with structure **1** (CAS 167504-51-0) has been referenced twice in Chemical Abstracts. The first is a 1995 article from a Becton Dickinson/Molecular Probes group that examined its use in flow cytometric studies of multi-drug resistance in various cell lines.[15] The other is the 2005 Molecular Probes patent describing a number of tests indicative of the safety of **1** as well as it applicability in nucleic acid detection.[13] Although the 2005 patent does not explicitly refer to the compound as SYBR Safe, toxicological data in the disclosure are reproduced verbatim in the web-available mutagenicity and environmental safety assessment for SYBR Safe.[7] Absent our structural analysis, this fact would reasonably

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inform a user of SYBR Safe as to the material's primary molecular identity.

The use of thiazole orange as a nucleic acid stain also emerged from the Becton Dickinson Laboratories. The compound was given its name in conjunction with a 1986 report describing its use in reticulocyte (immature blood cells) analyses.[16] The structural similarities between **1** and **2** might suggest similar toxicity profiles, but the 2005 patent reported a key difference. Namely, compound **1** tested negative in three out of three mammalian carcinogen screens, whereas ethidium bromide and thiazole orange each tested positive in one, a transformation test utilizing hamster embryo cells. It was also reported that compound **1** produced fewer mutations than ethidium bromide in the Ames test. In comparison with ethidium bromide, thiazole orange was determined to be 3−4 times less mutagenic whereas compound **1** was found to be 4−5 times less mutagenic.[13] These comparative tests, together with favorable acute toxicity data (rat) and aquatic toxicity data (fathead minnow), provide the basis for the manufacturer's assertions of product safety and ease of handling—factors which have contributed to its increased use within the research community.

On the basis of public domain patent information and our NMR and mass spectral data, coupled with a comparison with the assigned thiazole orange NMR data, we conclude that the dye species in SYBR Safe is an *N*-Pr variant of thiazole orange with *p*-toluenesulfonate as the counterion (i.e., structure **1**).

EXPERIMENTAL SECTION

Sample Preparation. SYBR Safe (Life Technologies/Invitrogen/Molecular Probes, catalog number S33102, lot 1105019) was obtained as a DMSO solution (400 μL, 10 000× concentrate). The DMSO was removed by lyophilization, replaced with 0.7 mL of DMSO-d_6, and lyophilized a second time. The residue was dissolved in 0.7 mL of DMSO-d_6 and transferred to a 5 mm NMR tube. Tetramethylsilane (TMS) vapor was added by briefly holding the uncapped NMR tube under the lip of a TMS container held at a 45° angle. The sample was then thoroughly mixed. Five milligrams of thiazole orange (Anaspec ultrapure grade, catalog number 83227) was dissolved in DMSO-d_6, and the sample was transferred to a 5 mm NMR tube. TMS vapor was added using the technique described above.

NMR Spectra. NMR spectra were obtained on a 400 MHz NMR spectrometer equipped with a 5 mm dual ^{1}H/^{13}C Z-gradient probe whose temperature was maintained at 25 °C. One-dimensional ^{1}H and ^{13}C spectra were acquired with standard pulse sequences and parameters. Details for the 2D experiments are as follows:

Gradient-enhanced 2D COSY experiment.[17] The cosygpqf pulse program was used with the following acquisition parameters: F2 and F1 sweep widths, 10.21 ppm. F2 and F1 digital resolution, 1.99 Hz/pt. 128 FIDs were recorded, each consisting of 8 scans and 2048 data points (AQ = 0.251 s). A recycle delay of 2.0 s was employed. Processing parameters: unshifted sinusoidal apodization was applied in both dimensions prior to the Fourier transformation.

2D ROESY experiment.[18] The roesyph.2 pulse program was used with the following acquisition parameters: F2 and F1 sweep widths, 10.21 ppm. F2 and F1 digital resolution, 1.99 Hz/pt. 256 FIDs were recorded, each consisting of 16 scans and 2048 data points (AQ = 0.251 s). A 400 ms spin lock consisted of 2702 cycles of phase-shifted pairs of 74 μs 180° pulses. A recycle delay of 2.0 s was employed. Processing parameters: $\pi/2$ shifted sinusoidal apodization was applied in both dimensions prior to the Fourier transformation.

Gradient-enhanced 2D ^{1}H−^{13}C HSQC experiment.[19] The hsqcetgp pulse program was used with the following acquisition parameters: F2 sweep width, 10.07 ppm, F1 sweep width, 165.66 ppm. F2 digital resolution, 3.94 Hz/pt, F1 digital resolution, 130.21 Hz/pt. 128 FIDs were recorded, each consisting of 128 scans and 1024 data points (AQ = 0.127 s). The basic evolution period was set to 1.725 ms ($1/4J$ = 145 Hz). A recycle delay of 1.0 s was employed. Processing parameters: Zero-filling was applied eight times in F1 to achieve a digital resolution of 16 Hz/pt. The $\pi/2$ shifted sinusoidal apodization was applied in both dimensions prior to the Fourier transformation. Forward linear prediction (32 or 64 LP coefficients) was used in the F1 dimension.

Gradient-enhanced 2D ^{1}H−^{13}C HMBC experiment.[20] The hmbcgplpndqf pulse program was used with the following acquisition parameters: F2 sweep width, 13.27 ppm, F1 sweep width, 221.85 ppm. F2 digital resolution, 5.18 Hz/pt, F1 digital resolution, 174.36 Hz/pt. 128 FIDs recorded, each consisting of 256 scans and 1024 data points (AQ = 0.097 s). The basic evolution period was set to optimally detect C−H couplings of 8 Hz and the low-pass filter was set to minimize 145 Hz C−H couplings. A recycle delay (D1) of 1.8 s was employed. Processing parameters: Unshifted sinusoidal apodization was applied in both dimensions prior to the Fourier transformation. Forward linear prediction (32 or 64 coefficients) was used in the F1 dimension.

Computational Chemistry. The calculations reported here used Gaussian 09.[21]

ASSOCIATED CONTENT

Supporting Information

Spectroscopic data and computed Cartesian coordinates for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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