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Modulation of the Chemiluminescent Signal from N^{10}-(3-Sulfopropyl)-*N*-Sulfonylacridinium-9-carboxamides

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Abstract: Acridinium salts **3a–h** were synthesized from the corresponding sulfonamides **1a–h** and their chemiluminescence profiles were compared. The quantity of light emitted over the time studied did not correlate well with the pK_a of sulfonamide leaving group. Rather, steric factors contributed the most to modulating the light output from these compounds. The mesitylsulfonyl substituent of acridinium salt **3d** reduced the chemiluminescence signal by 20-fold relative to the reference acridinium salt **3a**.

Keywords: Acridines, Chemiluminescence, Sulfonamides

INTRODUCTION

Many years of research have been spent trying to maximize the sensitivity of chemiluminescent assays to reach the lowest possible detection limits. Indeed, chemiluminescent assays are now exquisitely sensitive.[1-3] In chemiluminescent immunodiagnostic assays, sensitivity beyond that of radioimmunoassay has been observed and several companies have introduced instruments to take advantage of this technology.[2]

Some of the most sensitive assays have used conjugates of acridinium salts as the chemiluminescent reagent. The initially described phenyl N^{10}-methyl-acridinium-9-carboxylates (R = CH_3, X = OPh)[4] suffered from poor stability[5] and the tendency to form a non-chemiluminescent pseudobase in the aqueous buffers used in immunoassays. These compounds were soon superceded by sterically hindered acridinium aryl esters[6] and N^{10}-methyl-*N*-sulfonylacridinium-9-carboxamides (R = CH_3, X = $NRSO_2R'$).[7-10] More recently, we have found it advantageous to replace the N^{10}-methyl group on the latter with the hydrophilic N^{10}-(3-sulfopropyl) group.[11] For the most sensitive assays we have explored the use of these chemiluminescent salts in an arborol labeling approach.[12]

While analytes (HIV, TSH, digoxin, etc.) with a sub-ng/mL clinical concentration require an assay with a low limit of detection and would benefit from these advances, other clinically relevant analytes that are present at a

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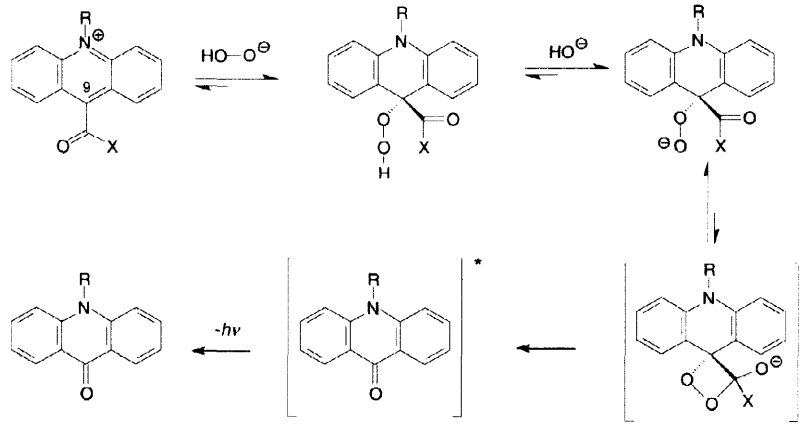
thousand-fold (μg/mL) or higher concentration (e.g. theophylline, phenytoin, phenobarbital) may not. In fact, when a clinical analyzer is designed with fixed reagent volumes and detection timing protocols that have been optimized for maximum sensitivity, assays for high concentration analytes often generate light that exceeds the linear range of the detector. Many solutions to this problem could be envisioned. For instance, one could just dilute the sample. This additional, and possibly manual, step would be undesirable in today's highly automated, high-throughput clinical laboratories. At least one commercial analyzer uses filters to attenuate the light.[13] We believed that a simpler solution could be achieved by modulating the signal of the acridinium salt chemically, based on the mechanism of the chemiluminescent reaction (Figure 1).

Figure 1. Proposed mechanism of acridinium salt chemiluminescence



The initial step in the chemiluminescent reaction is the addition of hydrogen peroxide anion to the 9-position of the acridinium salt.[14–16] This is followed by the intramolecular displacement of the leaving group **X** via the tetrahedral dioxetane intermediate that rapidly decomposes to give the acridone and light. In general it is known for reactions involving a tetrahedral intermediate that the leaving group can have an effect on the reaction kinetics by altering the electron density or steric environment of the reaction center.[17] This is indeed true for the reaction of acridinium salts with hydrogen peroxide. McCapra has reported that the pK_a of the leaving group in 10-methyl-acridinium-9-carboxylate salts had a marked effect on the luminescence efficiency of the chemiluminescent reaction.[18] Upon examination of a series of N^{10}-methyl-*N*-sulfonylacridinium-9-carboxamide chemiluminescent salts, no substituent effects on the chemiluminescence yield were observed.[8] However, the overall *rate* of the light output (i.e., the time in seconds for >90% of the light emission to occur) in this class of chemiluminescent acridinium salt was affected by the electronic properties of the sulfonamide leaving group. Substitution of electron donating groups on the sulfonamide leaving group slowed the rate of light generation, while electron withdrawing groups shortened the time course of the reaction. Likewise, incorporation of sterically demanding substituents on the sulfonamide leaving group decreased the rate of light output. In all these cases though, the total light output,

i.e., the luminescence efficiency of the reaction, remained essentially constant, unlike the 10-methyl-acridinium-9-carboxylate salts. Even so, the ability to control the quantity of light produced over a given time means that the observed signal on a clinical analyzer should be adjustable by matching the appropriate substituents on the sulfonamide leaving group with the signal integration timing employed on the analyzer to achieve the desired level of sensitivity.

While the previous study[8] presented a series of model N^{10}-methyl-*N*-sulfonylacridinium-9-carboxamide compounds with a range of chemiluminescence profiles, the compounds lacked the functionality necessary for conjugation to an analyte. Additionally, it was unclear whether or not similar results would be recorded for the more hydrophilic N^{10}-(3-sulfopropyl)acridinium inner salt labels. Earlier synthetic work on that series had indicated that introduction of the N^{10}-(3-sulfopropyl) group was very sensitive to the steric bulk of the sulfonamide substituent.[11] Thus, in the present study a series of sterically and electronically modified analogs of the recently introduced N^{10}-(3-sulfopropyl)-*N*-sulfonylacridinium-9-carboxamide chemiluminescent salt **3a**[11] were prepared and evaluated with the aim of providing chemiluminescent labels that might be more suitable for use with high concentration analytes.

Preparation of Chemiluminescent Acridinium Salts (3a–h)

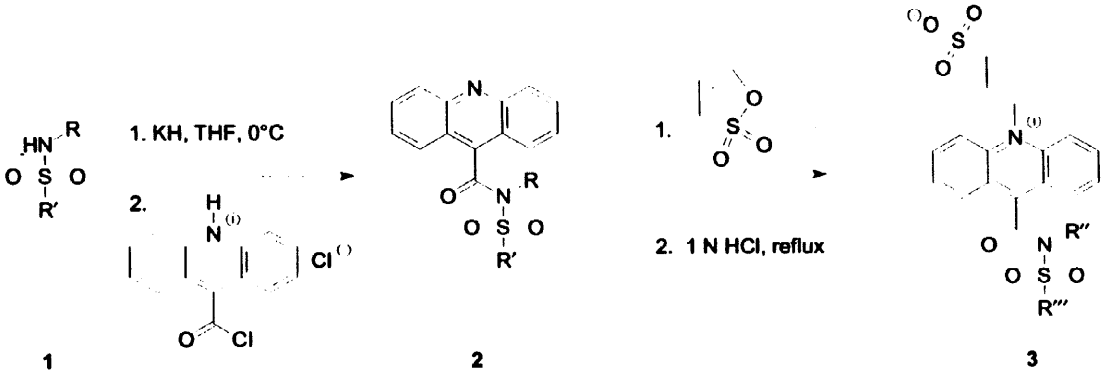
Sulfonamides **1a–h** were prepared straightforwardly from the corresponding amines and sulfonyl chlorides in dichloromethane. Acylation of the sulfonamide anions with acridinium acid chloride gave the *N*-sulfonylacridine-9-carboxamides **2a–h** (Scheme). With the exception of the *N*-methoxyphenyl examples **2f** and **2g**, the compounds were seen as mixtures of rotational isomers by ^{1}H NMR. In each case one rotamer was shielded relative to the other, consistent with a conformation that places either R or R′ over the shielding region of the acridine ring. Compounds **2a–d, h** were 1.2–1.5:1 mixtures in which the conformer with the shielded R-group was in slight excess. For compound **2e** containing the sterically demanding R′ isopropyl group, the ratio rose to 8 to 1. We had seen earlier that the ease of sulfopropylation of the N^{10}-acridine nitrogen could be influenced by the steric bulk of the sulfonamide substituent.[11] In this series though, all of the *N*-sulfonylacridine-9-carboxamides reacted with neat 1,3-propane sultone to give the desired N^{10}-(3-sulfopropyl)-*N*-sulfonylacridinium-9-carboxamide esters. Hydrolysis of the ester protecting groups in refluxing 1 N HCl gave the desired pure acridinium chemiluminescent labels **3a–h** (34–85%).

Chemiluminescence Evaluation

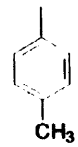
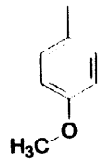
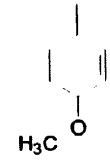
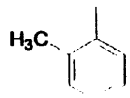
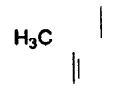
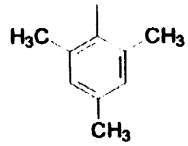
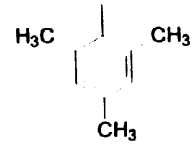
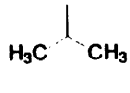
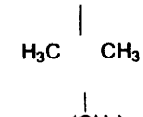
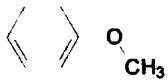
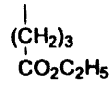
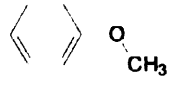
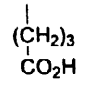
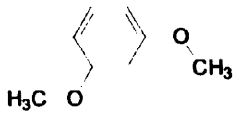
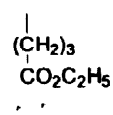
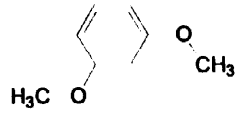
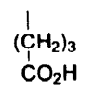
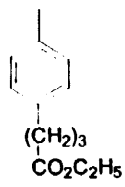
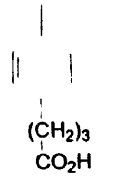
The electronic nature of the sulfonamide portion of the molecule can influence the rate of chemiluminescence from *N*-sulfonylacridinium-9-carboxamides.[8] Making that moiety more electron-donating would increase the electron density on the carbonyl reaction center, slowing the formation of the tetrahedral intermediate, and in turn the overall rate of light output. Conversely, making the sulfonamide more electron-withdrawing, would impart a greater positive charge to the carbonyl and speed the reaction. The pK_a of the sulfonamide is a good indication of

its expected influence on the rate of chemiluminescence. In the absence of steric effects, as the pK_a of the sulfonamide increases, the rate of chemiluminescence should decrease.

Scheme

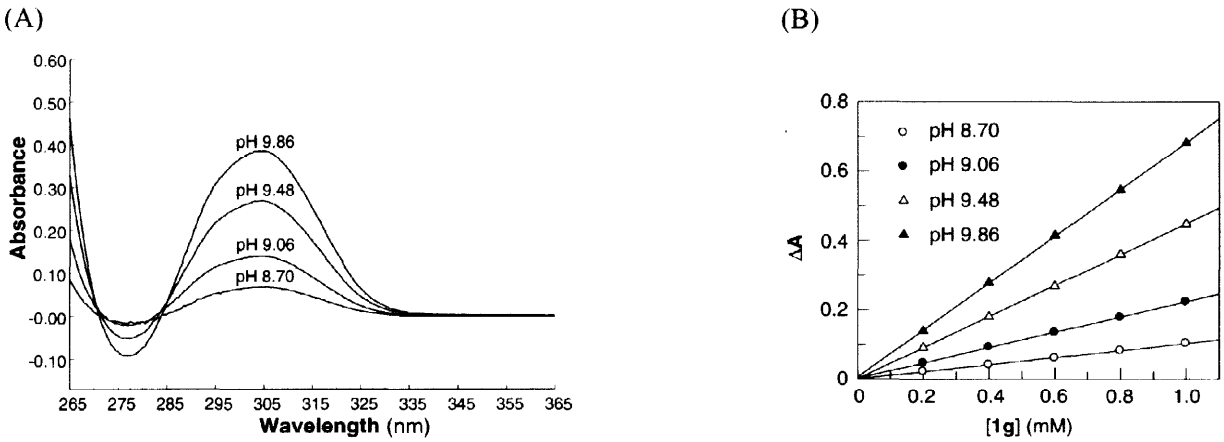


1 2 3

	R	R′	R″	R‴
a	$(CH_2)_3CO_2CH_3$		$(CH_2)_3CO_2H$	
b	$(CH_2)_3CO_2CH_3$		$(CH_2)_3CO_2H$	
c	$(CH_2)_3CO_2CH_3$		$(CH_2)_3CO_2H$	
d	$(CH_2)_3CO_2CH_3$		$(CH_2)_3CO_2H$	
e	$(CH_2)_3CO_2CH_3$		$(CH_2)_3CO_2H$	
f				
g				
h				

The pK_a of the sulfonamide esters **1a–h** were determined by the UV-visible spectral difference method of Soundararajan *et al.*[19] In this method, a reference solution was prepared such that the sulfonamide was in the un-ionized form (at least 2 pH units below the pK_a). Four sample solutions were then prepared at the same concentration, but at higher pHs as illustrated for compound **1f** (Figure 2A). The difference in absorbance (ΔA) between trough and the apex for each spectrum at a given pH was recorded.

Figure 2. pK_a of **1f** by UV difference spectroscopy



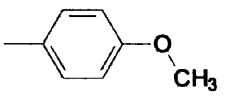
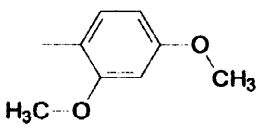
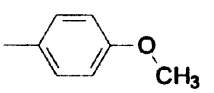
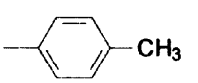
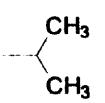
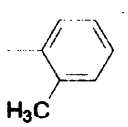
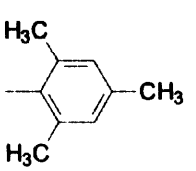
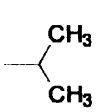
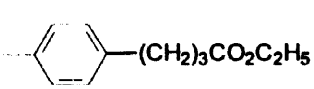
The experiment was repeated varying the concentration of sulfonamide. Plotting ΔA versus the concentration of the sulfonamide at each pH produced a series of lines (Figure 2B) from which the pK_a can be calculated using the formula

$$pK_a = -\log\left(\frac{S_1[H^+]_1 - S_2[H^+]_2}{S_2 - S_1}\right)$$

where S_1 and S_2 were the slopes of the plots at two different hydrogen ion concentrations, $[H^+]_1$ and $[H^+]_2$, respectively.

The pK_a values determined for sulfonamides **1a–h** are listed in ascending order in Table 1. The pK_a of sulfonamide **1e** could not be obtained by this method since it lacked a sufficient chromophore, but a value of 11.79 has been reported for a similar *N*-alkyl alkylsulfonamide (R = R′ = CH_3)[20] and has been shown for comparison. Since the reported pK_a values for most sulfonamides lie in the 5.5–12 range[21] and the sulfonamide (**1a**) portion of our standard chemiluminescent label (**3a**) was already at the high end of this range, only a limited choice of substituents were available for creating a less acidic sulfonamide derivative that might modulate the chemiluminescent signal observed from the corresponding acridinium salt.

Table 1. Structure and pK_a values of the sulfonamides **1a–h**.

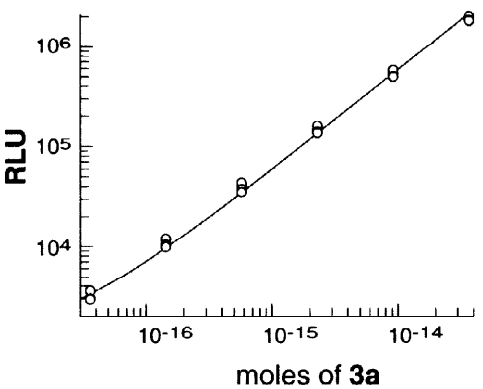
	R	R′	pK_a
1f		$-(CH_2)_3CO_2C_2H_5$	9.75 ± 0.14[b]
1g		$-(CH_2)_3CO_2C_2H_5$	10.20 ± 0.08[e]
1b	$-(CH_2)_3CO_2CH_3$		11.40 ± 0.06[a]
1a	$-(CH_2)_3CO_2CH_3$		11.58 ± 0.03[a]
1e	$-(CH_2)_3CO_2CH_3$		11.79 (est)
1c	$-(CH_2)_3CO_2CH_3$		11.87 ± 0.07[a]
1d	$-(CH_2)_3CO_2CH_3$		12.33 ± 0.06[d]
1h			12.37 ± 0.05[c]

*Averages of six independent determinations (six pairs of slope values).
a. 40 mM CHES/20% EtOH (pH 8.70, 9.06, 9.48, 9.86)
b. 40 mM CHES/20% EtOH (pH 9.06, 9.48, 9.86,10.22)
c. 40 mM CAPS/20% EtOH (pH 10.75, 11.22, 11.57, 11.84)
d. 40 mM CAPS/20% EtOH (pH 11.22, 11.57, 11.84, 12.34)
e. 40 mM CAPS/20% EtOH (pH 11.57, 11.84, 12.34, 12.62)

Only sulfonamides **1d** and **1h** had significantly higher ionization constants. Unfortunately, they also were among the most sterically hindered examples, thus making any straightforward correlation to the chemiluminescence rate of the corresponding acridinium salts based on the pK_a of the sulfonamide alone difficult. Compounds **1f** and **1g** had significantly lower pK_a values than the benchmark **1a**, and would have been expected to yield chemiluminescent acridinium salts with a more compressed chemiluminescence profile than **3a**.

The total light output in relative light units (RLU) per mole was determined for each acridinium salt **3a–h** (Table 2). A stock solution of acridinium salt was serially diluted in 10% aq DMF into the wells of a microtiter plate and the chemiluminescence measured. RLU versus moles of acridinium salt were plotted (Figure 3) to determine both the linear range of detection and the total light yield. There were only slight differences in total light output observed for the other acridinium salts. The two *N*-methoxyphenyl derived examples, **3f** and **3g** were somewhat lower, while mesitylene-derived **3d** was somewhat higher than the reference label **3a**. This similarity of total light yield is in agreement with the earlier model study.[8]

Figure 3. Log-log plot of total integrated emission intensity versus moles of compound **3a**. Linear regression analysis yields a slope of 5.7×10^{19} RLU/mole.



The overall rate of light production differed between the acridinium salts. Figure 4 depicts the chemiluminescence profiles recorded for the reference acridinium label **3a** and two progressively slower chemiluminescent salts **3e** and **3d**. For the purpose of further comparison under the conditions that might be expected in the fixed timing protocols of a clinical analyzer, the generated light from each acridinium salt was integrated over a fixed time period (0–3 s) and the results normalized to the standard label **3a** (Table 2).

Figure 4. Chemiluminescence profiles of **3a**, **3d**, and **3e** showing emission intensity as a function of time.

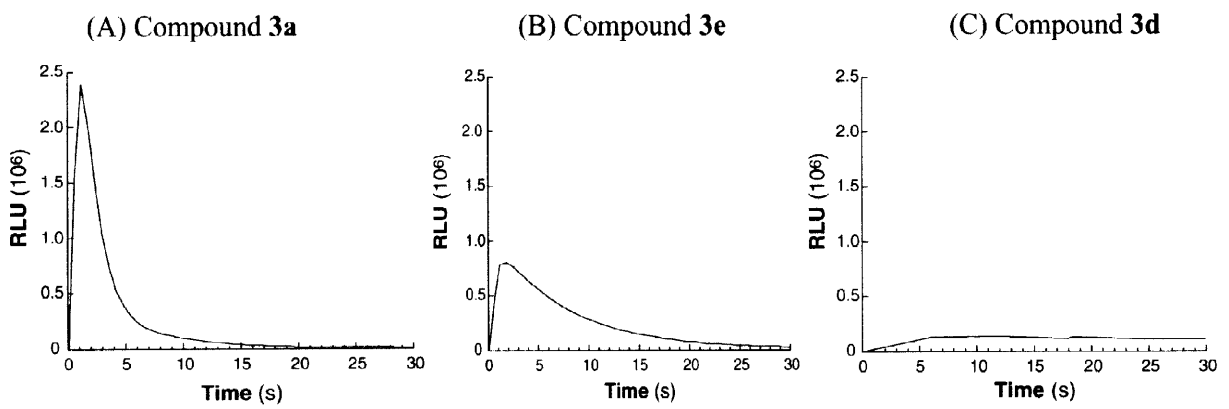
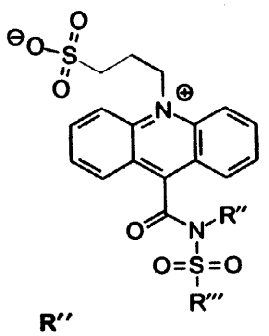
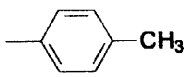
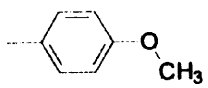
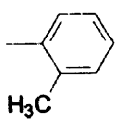
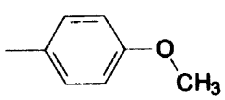
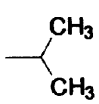
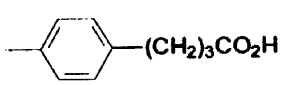
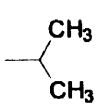
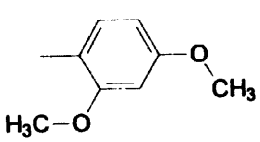
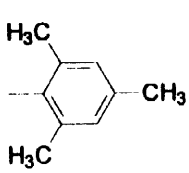


Table 2. Structure, total light output, and relative light output between 0–3 seconds of the chemiluminescent salts **3a–h**. Compounds are listed in descending order of relative light output setting the benchmark acridinium salt **3a** equal to 1.



	R″	R‴	Total RLU/mole (10^{19})	Relative RLU/mole (0-3 s)
3a	$-(CH_2)_3CO_2H$		5.7	1.00
3b	$-(CH_2)_3CO_2H$		5.7	0.99
3c	$-(CH_2)_3CO_2H$		5.2	0.85
3f		$-(CH_2)_3CO_2H$	3.6	0.75
3h			5.8	0.68
3e	$-(CH_2)_3CO_2H$		4.8	0.46
3g		$-(CH_2)_3CO_2H$	3.6	0.15
3d	$-(CH_2)_3CO_2H$		3.6	0.06

Compound **3b** had essentially the same performance as the standard under these conditions. Substituting a *p*-methoxy group in **3b** for a *p*-methyl group of **3a** did not introduce additional steric bulk and only changed the pK_a of the sulfonamide (**1b**) leaving group by –0.18 units. Shifting to an *o*-methyl substituent (**3c**) had a minimal effect on the pK_a of the leaving group (+0.3 units) and increased the steric hindrance closer to the reaction center with a resulting attenuation of the chemiluminescent signal to 85% of the standard. Introduction of a second *o*-methyl substituent (**3d**) further increased the pK_a of the leaving group by an additional +0.45 units, combining with the added steric hindrance to bring about a dramatic diminution of the chemiluminescent signal to only 6% of the standard. Based on the small difference in the pK_a between the arylsulfonamide **1a** and alkylsulfonamide **1e** leaving groups, little change in the chemiluminescence profiles of acridinium salts **3a** and **3e** would have been expected. However, the change from R‴ = *p*-tolyl to R‴ = *i*-propyl was sufficient to bring about a 54% reduction in signal. Introduction of branching in the R″-group also lowered the chemiluminescence response (**3a** vs. **3h**). The sulfonamide leaving group of compounds **3f** and **3g** have much lower pK_a values than the standard **3a**, but the rate of chemiluminescence emission was dominated by the increased steric demands of the R″-substituent (R″ = *p*-methoxyphenyl, *o,p*-dimethoxyphenyl vs. 3-carboxypropyl) and the observed signal was again attenuated.

In summary, the need for a chemiluminescent acridinium-based labeling reagent that is tuned for high concentration analytes has been addressed by altering the structure of the sulfonamide leaving group to slow the overall *rate* of chemiluminescence. When chemiluminescence is examined in a relatively short time frame (0–3 s), the result is a lower signal per mole of the label. By choosing the appropriate substituents, one can prepare a label with half or even one-tenth the signal of the benchmark acridinium chemiluminescent salt **3a** without altering the *N*′′′-(3-sulfopropyl)-9-acridone emitter or the total light yield. Evaluation of these attenuated chemiluminescent labels in a chemiluminescent immunoassay format is underway and will be reported in due course.

EXPERIMENTAL

All reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise noted. Acridinium chemiluminescent label 3a was prepared according to the literature.[11] Analytical HPLC was performed on a Waters system (Milford, MA) with UV detection at 254 nm using a Waters 3.9 × 300 mm μBondapak C18 column eluting with aqueous acetonitrile (v:v, CH_3CN/H_2O) containing 0.05% trifluoroacetic acid at a flow rate of 1 mL/min. ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded at on a Varian Gemini spectrometer (Palo Alto, CA). Chemical shifts are reported in ppm (δ) using tetramethylsilane (TMS) as the internal reference; coupling constants (*J*) are in Hz. Electrospray ionization mass spectrometry (ESI-MS) was carried out on a Perkin-Elmer (Norwalk, CT) Sciex API 100 Benchtop system employing the Turbo IonSpray™ ion source. Robertson Microlit Laboratories, Inc. (Madison, NJ) performed elemental analyses. IUPAC names were obtained using the ACD/ILab Web service version 3.5 at http://www.acdlabs.com/ilab/.

Preparation of Sulfonamides (1b–h): The amine component (50 mmol) and triethylamine (21 mL, 150 mmol) were added to dry dichloromethane (100 mL) and cooled to 0 °C. The sulfonyl chloride (50 mmol) in dry dichloromethane (50 mL) was added drop-wise to the stirred solution over 15 min under nitrogen. The reaction mixture was stirred at room temperature for 24 h, then evaporated to dryness under reduced pressure. The residue was redissolved in ethyl acetate (100 mL) and washed with water (100 mL), dilute hydrochloric acid (1 N, 100 mL), saturated sodium bicarbonate (100 mL) and brine (100 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The crude sulfonamide was either recrystallized from a mixture of ethyl acetate and hexanes or purified by column chromatography (silica gel, mixture of ethyl acetate and hexanes). For isopropylamine derivatives of sulfonamides, isopropylamine (6 eq) was used in excess both as a reactant and a base. No triethylamine was used.

Methyl 4-{[(4-methoxyphenyl)sulfonyl]amino}butanoate (1b) was obtained as a white solid after recrystallization from 50% ethyl acetate in hexanes. Yield: 4.85 g (84%). mp: 70–72 °C. ^{1}H NMR δ 7.80 (2 H, m), 6.98 (2 H, m), 5.11 (1 H, t, J = 6), 3.87 (3 H, s), 3.65 (3 H, s), 2.97 (2 H, q, J = 7), 2.36 (2 H, t, J = 7), 1.78 (2 H, p, J = 7). ^{13}C NMR δ 173.7, 162.9, 131.4, 129.2, 114.2, 55.5, 51.6, 42.3, 30.7, 24.4. ESI-MS *m/z*: 310.3 $(M + Na)^+$, 288.0 $(M + H)^+$. Anal. Calcd for $C_{12}H_{17}NO_5S$: C 50.16, H 5.96, N 4.87, S 11.16; Found: C 50.38, H 6.01, N 4.76, S 11.07.

Methyl 4-{[(2-methylphenyl)sulfonyl]amino}butanoate (1c) was isolated as a colorless oil after column chromatography (25% ethyl acetate in hexanes). Yield: 8.51 g (63%). ^{1}H NMR δ 7.99 (1 H, m), 7.52 (1 H, m), 7.37 (2 H, m), 4.81 (1 H, t, J = 6), 3.69 (3 H, s), 3.04 (2 H, q, J = 7), 2.69 (3 H, s), 2.39 (2 H, t, J = 7), 1.84 (2 H, p, J = 7). ^{13}C NMR δ 173.7, 137.8, 137.0, 132.8, 132.6, 129.5, 126.2, 51.7, 42.3, 30.8, 24.6, 20.1. ESI-MS *m/z*: 293.9 $(M + Na)^+$, 272.0 $(M + H)^+$. Anal. Calcd. for $C_{12}H_{17}NO_4S$: C 53.12, H 6.32, N 5.16, S 11.82; Found: C 52.88, H 6.26, N 5.16, S 11.64.

Methyl 4-[(mesitylsulfonyl)amino]butanoate (1d) was obtained as a white solid after recrystallization from 10% ethyl acetate in hexanes. Yield 12.5 g (83.5%). mp: 84–86 °C. ^{1}H NMR δ 6.93 (2 H, s), 5.06 (1 H, t, J = 6), 3.61 (3 H, s), 2.91 (2 H, q, J = 7), 2.60 (6 H, s), 2.31 (2 H, t, J = 7), 2.27 (3 H, s), 1.76 (2 H, m). ^{13}C NMR δ 173.7, 142.1, 139.0, 133.5, 131.9, 51.6, 41.6, 30.7, 24.4, 22.7, 20.7. ESI-MS *m/z*: 322.3 $(M + Na)^+$, 300.2 $(M + H)^+$. Anal. Calcd. for $C_{14}H_{21}NO_4S$: C 56.17, H 7.07, N 4.68, S 10.71; Found: C 56.31, H 7.05, N 4.55, S 10.81.

Methyl 4-[(isopropylsulfonyl)amino]butanoate (1e) was isolated as a colorless oil after column chromatography (25% ethyl acetate in hexanes). Yield: 8.1 g (73 %). ^{1}H NMR δ 4.44 (1 H, t, J = 6), 3.69 (3 H, s), 3.23–3.11 (3 H, m), 2.44 (2 H, t, J = 7), 1.89 (2 H, p, J = 7), 1.37 (6 H, d, J = 7). ^{13}C NMR δ 173.7, 53.3, 51.7,

42.9, 30.8, 25.6, 16.5. ESI-MS *m/z*: 246.0 $(M + Na)^+$, 224.0 $(M + H)^+$. Anal. Calcd. for $C_8H_{17}NO_4S$: C 43.03, H 7.67, N 6.27, S 14.36; Found: C 43.14, H 7.60, N 6.18, S 14.16.

Ethyl 4-[(4-methoxyanilino)sulfonyl]butanoate (1f) was obtained as an off-white solid after column chromatography (35% ethyl acetate in hexanes). Yield: 18%. ^{1}H NMR δ 7.22 (2 H, d, J = 9), 6.88 (2 H, d, J = 9), 6.74 (1 H, br s), 4.12 (2 H, q, J = 7), 3.80 (3 H, s), 3.12 (2 H, m), 2.48 (2 H, t, J = 7), 2.14 (2 H, m), 1.24 (3 H, t, J = 7). ^{13}C NMR δ 172.6, 158.0, 129.1, 124.7, 114.8, 60.7, 55.4, 50.0, 32.0, 18.9, 14.0. ESI-MS *m/z*: 324.3 $(M + Na)^+$, 302.1 $(M + H)^+$. Anal. Calcd. for $C_{13}H_{19}NO_5S$: C 51.81, H 6.35, N 4.65, S 10.64; Found: C 52.10, H 6.36, N 4.67, S 10.64.

Ethyl 4-[(2,4-dimethoxyanilino)sulfonyl]butanoate (1g) was isolated as an oil obtained after column chromatography (35% ethyl acetate in hexanes). Yield: 37%. ^{1}H NMR δ 7.39 (1 H, d, J = 9), 6.64 (1 H, br s), 6.50 (1 H, d, J = 3), 6.46 (1 H, dd, J = 3 and 9), 4.10 (2 H, q, J = 7), 3.86 (3 H, s), 3.79 (3 H, s), 3.04 (2 H, m), 2.43 (2 H, t, J = 7), 2.11 (2 H, m), 1.22 (3 H, t, J = 7). ^{13}C NMR δ 172.2, 158.5, 151.7, 124.2, 118.5, 104.4, 98.8, 60.3, 55.5, 55.3, 49.7, 32.0, 18.7, 13.8. Anal. Calcd. for $C_{14}H_{21}NO_6S$: C 50.74, H 6.39, N 4.23, S 9.67; Found: C 50.50, H 6.40, N 4.18, S 9.62.

Ethyl 4-{4-[(isopropylamino)sulfonyl]phenyl}butanoate (1h) was isolated as a colorless oil after column chromatography (20% ethyl acetate in hexanes). Yield 2.10 g (43%). ^{1}H NMR δ 7.81 (2 H, m), 7.33 (2 H, m), 4.59 (1 H, m), 4.14 (2 H, q, J = 7), 3.46 (2 H, m), 2.73 (2 H, t, J = 8), 2.33 (2 H, t, J = 7), 1.98 (2H, m), 1.27 (2 H, t, J = 7), 1.08 (6 H, d, J = 6). ^{13}C NMR δ 173.3, 146.7, 138.8, 129.2, 127.2, 60.4, 46.0, 34.8, 33.4, 26.0, 23.6, 14.1. ESI-MS *m/z*: 336.1 $(M + Na)^+$, 314.0 $(M + H)^+$. Anal. Calcd. for $C_{15}H_{23}NO_4S$: C 57.49, H 7.40, N 4.47, S 10.23; Found: C 57.33, H 7.35, N 4.31, S 10.05.

Preparation of *N*-sulfonylacridine-9-carboxamides (2b–h). A suspension of potassium hydride in mineral oil (35 wt. %) (3.90 g, 34 mmol) was washed with anhydrous THF (20 mL × 3) under argon then suspended in anhydrous THF (124 mL). The mixture was cooled to between –10 and –15 °C in an ethylene glycol/Dry Ice bath. A solution of the sulfonamide (16.7 mmol) in anhydrous THF (42 mL) was added drop-wise over 15 min. The internal reaction temperature was monitored by an immersed thermometer and was kept below –10 °C during the addition. Stirring was continued for 75 min under argon. Acridinium-9-carbonylchloride hydrochloride (4.6 g, 16.5 mmol) was added in one portion and the reaction mixture was allowed to warm to ambient temperature. After 16 h the reaction mixture was diluted with of chloroform (500 mL) and washed with water (250 mL × 2). The organic phase was separated, dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The crude material was purified by column chromatography (30 g silica gel/mmol of sulfonamide eluting with a mixture of ethyl acetate and hexanes). Like fractions were combined and evaporated under reduced pressure.

Methyl 4-{(9-acridinylcarbonyl)[(4-methoxyphenyl)sulfonyl]amino}butanoate (2b) was obtained as an off-white solid after column chromatography (50% ethyl acetate in hexanes). Yield: 2.43 g (59.4%). ^{1}H NMR δ 8.40–6.80 (10 H, m), 7.15 and 6.46 (2 H, d, *J* = 9, ratio: 1.2/1), 4.32 and 3.52 (2 H, t, *J* = 8, ratio: 1/1.5), 3.97 and 3.78 (3 H, s, ratio; 1.2/1), 3.75 and 3.25 (3 H, s, ratio: 1/1.2), 2.64 and 1.93 (2 H, t, *J* = 7, ratio: 1/1.2), 2.43 and 1.72 (2 H, m, ratio: 1/1.2). ^{13}C NMR δ 168.4, 148.7, 131.8, 130.8, 130.3, 130.2, 129.9, 129.8, 127.9,126.7, 125.2, 124.1, 122.5, 121.7, 114.2, 114.0, 55.8, 55.6, 51.8, 51.3, 47.8, 46.3, 31.4, 30.5, 26.0, 24.8. ESI-MS *m/z*: 541.7 (M + Na)$^+$, 519.3 (M + H)$^+$. Anal. Calcd. for $C_{26}H_{24}N_2O_6S$: C 63.40, H 4.91, N 5.69, S 6.51; Found: C 63.41, H 4.79, N 5.50, S 6.44.

Methyl 4-{(9-acridinylcarbonyl)[(2-methylphenyl)sulfonyl]amino}butanoate (2c) was obtained as an off-white solid after column chromatography (50% ethyl acetate in hexanes). ^{1}H NMR δ 8.42–7.34 (10 H, m), 6.96 and 6.42 (1 H, t, *J* = 8, ratio: 1/1), 6.82 and 6.21 (1 H, d, *J* = 8, ratio: 1.2/1), 4.45 and 3.57 (2 H, t, *J* = 8, ratio: 1/1.1), 3.78 and 3.25 (3 H, s, ratio: 1/1), 2.83 and 2.42 (3 H, s), 2.68 and 2.00 (2 H, t, *J* = 7, ratio: 1/1.1), 2.51 and 1.75 (2 H, m). ^{13}C NMR δ 173.2, 172.1, 168.5, 148.7, 148.2, 138.2, 137.6, 134.4, 133.6, 132.6, 132.4, 132.3, 130.8, 130.3, 130.2, 129.6, 129.5, 127.8, 126.7, 126.6, 125.7, 125.0, 124.0, 122.6, 121.7, 51.8, 51.3, 48.0, 46.3, 37.3, 31.5, 30.6, 26.1, 24.9, 20.7, 20.2, 19.1.). ESI-MS *m/z*: 477.1 (M + H)$^+$. Anal. Calcd. for $C_{26}H_{24}N_2O_5S$: C 65.53, H 5.08, N 5.88, S 6.73; Found: C 65.78, H 4.80, N 5.57, S 6.47.

Methyl 4-[(9-acridinylcarbonyl)(mesitylsulfonyl)amino]butanoate (2d) was obtained as an off-white solid after column chromatography (35% ethyl acetate in hexanes). Yield 71%.. ^{1}H NMR δ 8.26 and 8.09 (2 H, d, *J* = 9, ratio: 1.4/1), 7.90–7.30 (8 H, m), 7.11 and 6.22 (2 H, s, ratio: 1.4/1), 4.46 and 3.52 (2 H, s, ratio: 1/1.5), 3.78 and 3.24 (3 H, s, ratio: 1/1.4), four peaks: 2.83, 2.37, 1.97 and 1.74 (9 H, s), 2.69 and 2.50 (2 H, m), 2.01 and 1.78 (2 H, m). ^{13}C NMR δ 173.2, 172.1169.0, 168.6, 148.7, 148.4, 144..2, 143.6, 141.1, 139.7, 137.9, 133.7, 132.3, 132.1, 131.6, 130.8, 130.3, 130.0, 129.5, 127.7, 126.5, 125.5, 124.3, 122.4, 121.7, 51.8, 51.3, 47.9, 46.1, 31.6, 30.7, 26.0, 24.8, 22.8, 21.7, 21.1, 20.6. ESI-MS *m/z*: 505.7 (M + H)$^+$. Anal. Calcd. for $C_{28}H_{28}N_2O_5S$: C 66.65, H 5.59, N 5.55, S 6.35; Found: C 66.61, H 5.32, N 5.38, S 6.25.

Methyl 4-[(9-acridinylcarbonyl)(isopropylsulfonyl)amino]butanoate (2e) was obtained as a white solid after column chromatography (40% ethyl acetate in hexanes). Yield: 1.23 g (51%). ^{1}H NMR δ 8.34–8.24 (2 H, m), 7.94–7.76 (4 H, m), 7.20–7.56 (2 H, m), 4.61 (1 H, m), 3.87 and 3.40 (2 H, t, *J* = 7, ratio: 1/8), 3.77 and 3.21 (3 H, s, ratio: 1/8), 2.59 and 1.84 (2 H, t, *J* = 8, ratio: 1/5), 2.15 and 1.42 (2 H, m, ratio: 1/8), 1.67 and 1.41 (6 H, d, *J* = 7, ratio: 7/1). ^{13}C NMR δ 171.9, 170.4, 148.7, 137.7, 130.9, 130.4, 130.1, 128.1, 124.1, 121.7, 56.3, 51.3, 47.5, 30.4, 25.8, 16.2, 15.5. ESI-MS *m/z*: 429.1 (M + H)$^+$. Anal. Calcd. for $C_{22}H_{24}N_2O_5S$: C 61.67, H 5.65, N 6.54, S 7.48; Found: C 61.43, H 5.40, N 6.37, S 7.47.

Ethyl 4-{[(9-acridinylcarbonyl)-4-methoxyanilino]sulfonyl}butanoate (2f) was obtained as a brown solid after column chromatography (35%–50% ethyl acetate in hexanes). Yield: 1.03 g (68%). ^{1}H NMR δ 8.17 (2

H, d, J = 9), 8.13 (2 H, d, J = 9), 7.77 (2 H, m), 7.67 (2 H, m), 6.98 (2 H, d, J = 9), 6.29 (2 H, d, J = 9), 4.23 (2 H, q, J = 7), 4.16 (2 H, m), 3.46 (3 H, s), 2.66 (2 H, t, J = 7), 2.45 (2 H, m), 1.31 (3 H, t, J = 7). ^{13}C NMR δ 172.2, 169.6, 160.3, 148.2, 138.3, 130.5, 130.4, 129.9, 127.5, 126.1, 124.6, 121.5, 113.8, 60.9, 55.0, 54.3, 31.9, 25.8, 19.0, 14.1. ESI-MS *m/z*: 507.3 $(M + H)^+$. Anal. Calcd. for $C_{27}H_{26}N_2O_6S$: C 64.02, H 5.17, N 5.53, S 6.33; Found: C 63.69, H 5.13, N 5.44, S 6.19.

Ethyl 4-{[(9-acridinylcarbonyl)-2,4-dimethoxyanilino]sulfonyl}butanoate (2g) was isolated as a brown oil after column chromatography (40%–60% ethyl acetate in hexanes). Yield: 894 mg (55.5%). ^{1}H NMR δ 8.30–7.50 (8 H, m), 6.89 (1 H, d, J = 9), 5.91 (1 H, d, J = 2.5), 5.84 (1 H, dd, J = 9 and 2.5), 4.40–4.10 (4 H, m), 3.53 (3 H, s), 3.48 (3 H, s), 2.68 (2 H, m), 2.60–2.40 (2 H, m), 1.31 (3 H, t, J = 7). ^{13}C NMR δ 172.4, 170.2, 161.9, 156.3, 148.4, 148.2, 1338.6, 132.2, 130.5, 130.3, 129.9, 129.6, 127.4, 125.9, 125.7, 124.8, 121.8, 115.5, 60.7, 55.1, 55.0, 54.7, 32.3, 18.5, 14.1. ESI-MS *m/z*: 537.5 $(M + H)^+$. Anal. Calcd. for $C_{28}H_{28}N_2O_7S$: C 62.67, H 5.26, N 5.22, S 5.97; Found: C 62.43, H 5.48, N 4.92, S 6.29.

Ethyl 4-(4-{[(9-acridinylcarbonyl)(isopropyl)amino]sulfonyl}phenyl)butanoate (2h) was obtained as an off-white solid after column chromatography (35% ethyl acetate in hexanes). Yield: 0.87 g (72%). ^{1}H NMR δ 8.60–6.90 (12 H, m), 4.90 and 3.74 (2 H, m, ratio: 1/1.7), 4.15 (2 H, q, J = 7), 2.83 and 2.59 (2 H, t, J = 8, ratio: 1.5/1), 2.37 and 2.30 (2 H, t, J = 7), 2.04 and 1.88 (2 H, m), 1.81 and 1.46 (6 H, d, J = 7), 1.27 (3 H, t, J = 7). ^{13}C NMR δ 173.2, 169.0, 168.4, 148.7, 148.5, 140.4, 138.7, 137.5, 135.7, 130.8, 130.3, 130.2, 129.8, 127.7, 127.5, 126.6, 125.0, 124.1, 122.4, 121.5, 60.4, 56.1, 54.3, 35.0, 34.7, 33.3, 25.9, 25.8, 22.0, 20.4, 14.1. ESI-MS *m/z*: 477.1 $(M + H)^+$.

Preparation of N^{10}-(3-sulfopropyl)-*N*-sulfonylacridinium-9-carboxamide Inner Salts (3b–h). (A) *Sulfopropylation of acridine derivatives.* *N*-Sulfonylacridine-9-carboxamides (**2b–h**) were heated at 120–125 °C with 1,3-propane sultone (1000 mol %) in an oil bath under argon for 5 h, then allowed to cool to ambient temperature. The yellow mixture was dissolved in a small amount of chloroform/methanol (1:1), added to silica gel (5 to 10 g/mmol of acridine) and evaporated under reduced pressure. The silica gel was loaded on a silica gel gravity column (40 g/mmol of acridine) and eluted with dichloromethane, followed by 5% methanol in dichloromethane until all the unreacted starting material was removed. The column was finally eluted with 15% methanol in dichloromethane to obtain a mixture of all the fluorescent yellow acridinium compounds. Fractions were combined and evaporated under reduced pressure. The mixture was not characterized and was used directly in the following step. (B) *Hydrolysis of sulfopropylated mixture.* The mixture of sulfopropylated acridinium compounds was heated at reflux in 1 N hydrochloric acid on an oil bath for 4.25 h. A yellow homogeneous solution was obtained. The solution was cooled to room temperature and the desired acridinium acid precipitated out of the reaction mixture. The yellow crystals were collected by filtration and dried in vacuo.

3-[9-({(3-carboxypropyl)[(4-methoxyphenyl)sulfonyl]amino}carbonyl)-10-acridiniumyl]-1-propanesulfonate inner salt (3b). Yield: 590 mg (47%). ^{1}H NMR (DMSO-d_6) δ 9.02 (2 H, m), 8.43 (2 H, m), 8.26–6.78 (8 H, m), 5.67 (2 H, m), 3.96 and 3.80 (3 H, s, ratio: 1/1.3), 4.30–1.40 (10 H, m). ESI-MS *m/z*: 623.7 (M + Na)$^+$, 601.5 (M + H)$^+$. Analytical HPLC [40:60]: 4.25 min, 98.3% . Anal. Calcd. for $C_{28}H_{28}N_2O_9S_2$: C 55.99; H 4.70; N 4.66; S 10.67. Found: C 55.86; H 4.71; N 4.51; S 10.46.

3-[9-({(3-carboxypropyl)[(2-methylphenyl)sulfonyl]amino}carbonyl)-10-acridiniumyl]-1-propanesulfonate inner salt (3c). Yield: 2.54 g (48%). ^{1}H NMR (DMSO-d_6) δ 9.05 and 8.94 (2 H, d, J = 9, ratio: 1.1/1), 8.49 and 8.37 (2 H, t, J = 8), 8.31–7.60 (6 H, m), 7.28 and 6.78 (1 H, t, J = 8, ratio: 1/1), 7.16 and 6.62 (1 H, d, J = 8, ratio: 1.1/1), 5.66 (2 H, m), 4.36 and 3.52 (2 H, m, ratio: 1/1.2), 2.75 and 2.43 (3 H, s), 2.90–1.40 (8 H, m). ESI-MS *m/z*: 607.7 (M + Na)$^+$, 585.7 (M + H)$^+$. Analytical HPLC [40:60]: 4.39 min, 97.5%. Anal. Calcd. for $C_{28}H_{28}N_2O_8S_2$: C 57.52; H 4.83; N 4.79; S 10.97. Found: C 57.35; H 4.68; N 4.63; S 10.39.

3-(9-{[(3-carboxypropyl)(mesitylsulfonyl)amino]carbonyl}-10-acridiniumyl)-1-propanesulfonate inner salt (3d). Yield: 692 mg (55%). ^{1}H NMR (DMSO-d_6) δ 9.05 and 8.92 (2 H, d, J = 9, ratio: 1.4/1), 8.50 and 8.41 (2 H, t, J = 8), 8.13–7.78 (4 H, m), 7.31 and 6.46 (2 H, s, ratio: 1.3/1), 5.69 and 5.59 (2 H, m, 1.3/1), 4.39 and 3.47 (2 H, m, ratio: 1/1.4), four singlets: 2.74, 2.37, 2.00 and 1.76 (9 H, s), 3.00–1.40 (8 H, m). ESI-MS *m/z*: 613.7 (M + H)$^+$. Analytical HPLC [50:50]: 4.16 min, 95.6%. Anal. Calcd. for $C_{30}H_{32}N_2O_8S_2$: C 58.81; H 5.26; N 4.57; S 10.46. Found: C 58.88; H 5.20; N 4.51; S 10.78.

3-(9-{[(3-carboxypropyl)(isopropylsulfonyl)amino]carbonyl}-10-acridiniumyl)-1-propanesulfonate inner salt (3e). Yield: 818 mg (85%). ^{1}H NMR (DMSO-d_6) δ 9.12–8.98 (2 H, m), 8.66–8.42 (2 H, m), 8.36–8.02 (4 H, m), 5.78–5.60 (2 H, m), 4.54–1.00 (11 H, m), 1.56 and 1.12 (6 H, d, J = 7, ratio: 2.2/1). ESI-MS *m/z*: 559.6 (M + Na)$^+$, 537.5 (M + H)$^+$. Analytical HPLC [35:65]: 4.17 min, 97.2%. Anal. Calcd. for $C_{24}H_{28}N_2O_8S_2$: C 53.72; H 5.26; N 5.22; S 11.95. Found: C 53.68; H 5.27; N 5.09; S 11.93.

3-[9-({[(3-carboxypropyl)sulfonyl]-4-methoxyanilino}carbonyl)-10-acridiniumyl]-1-propanesulfonate inner salt (3f). Yield: 392 mg (65%). ^{1}H NMR (DMSO-d_6) δ 8.93 (2 H, d, J = 9), 8.56 (2 H, d, J =9), 8.46 (2 H, t, J = 8), 8.15 (2 H, m), 7.15 (2 H, d, J = 9), 6.49 (2 H, d, J = 9), 5.53s (2 H, m), 4.31 (2 H, m), 3.46 (3 H, s), 2.76 (2 H, m), 2.60 (2 H, t, J = 7), 2.50–2.00 (4 H, m). ESI-MS *m/z*: 601.5 (M + H)$^+$. Analytical HPLC [40:60]: 3.84 min, 98.3%. Anal. Calcd. for $C_{28}H_{28}N_2O_9S_2$: C 55.99; H 4.70; N 4.66; S 10.67. Found: C 56.02; H 4.64; N 4.51; S 10.70.

3-[9-({[(3-carboxypropyl)sulfonyl]-2,4-dimethoxyanilino}carbonyl)-10-acridiniumyl]-1-propanesulfonate inner salt (3g). Red crystals. Yield: 187 mg (34%). ^{1}H NMR (DMSO-d_6) δ 9.10–8.00 (8 H, m), 7.84 and 7.05 (1 H, d, J = 9, ratio: 1/5.2), 6.93 and 6.11 (1 H, d, J = 2.5, ratio: 1/5.2), 6.80 and 6.02 (1 H, m), 5.52 (2 H, m), 4.40–4.10 (2 H, m), 3.47 (3 H, s), 3.39 (3 H, s), 2.90–2.70 (2 H, m), 2.59 (2 H, t, J = 7), 2.30–2.10 (4 H,

m). ESI-MS *m/z*: 631.7 $(M + H)^+$. Analytical HPLC [40:60]: 3.93 min, 98.9%. Anal. Calcd. for $C_{29}H_{30}N_2O_{10}S_2.1/2H_2O$: C 54.45; H 4.88; N 4.38; S 10.02. Found: C 54.44; H 4.80; N 4.27; S 9.98.

3-(9-{[{[4-(3-carboxypropyl)phenyl]sulfonyl}(isopropyl)amino]carbonyl}-10-acridiniumyl)-1-propanesulfonate inner salt (3h). Yield: 353 mg (44%). ^{1}H NMR (DMSO-d_6) δ 9.03 (2 H, d, J = 9), 8.45 (2 H, m), 8.24–7.24 (8 H, m), 5.68 (2 H, m), 4.77 and 3.91 (1 H, m, ratio: 1.8/1), 2.91–2.19 (8 H, m), 1.88 and 1.77 (2 H, m, ratio: 2/1), 1.67 and 1.38 (6 H, d, ratio: 2.2/1). ESI-MS *m/z*: 635.7 $(M + Na)^+$, 613.7 $(M + H)^+$. Analytical HPLC [40:60]: 5.24 min, 95.3%. Anal. Calcd. for $C_{30}H_{32}N_2O_8S_2$: C 58.81; H 5.26; N 4.57; S 10.46. Found: C 58.75; H 5.29; N 4.37; S 10.45.

pK_a Determination of Sulfonamide Derivatives. pK_a Values for sulfonamide derivatives were calculated from acid ionization constants (K_a) determined by the UV-visible spectral difference method of Soundararajan *et al.*[19] Ultraviolet-visible difference spectra were recorded on a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). Difference spectra were obtained for five different concentrations of each sulfonamide (0.2–1 mM) at four different pH values in 40 mM buffer/20% EtOH between 230 and 400 nm. Final solution pH measurements were made on an Orion pH meter (model 420A) equipped with a Ross 8103 semi-micro combination glass electrode (Orion, Boston, MA).

Chemiluminescence Analysis. In a typical experiment, a stock solution of an acridinium derivative (concentration determined gravimetrically) was diluted serially 1:4 into 10% DMF/H_2O across the row of a 96 well, white, polystyrene microtiter plate (Wallac, Inc.; Gaithersburg, MD). Chemiluminescence was triggered well by well using a solution of sodium peroxide (0.18 N NaOH, 0.7 % H_2O_2, 1 % Triton X-100) and measured using a microplate chemiluminometer (LB96V2; Wallac, Inc.; Gaithersburg, MD). Total light emission was integrated immediately following the injection of the trigger solution. Neither DMF nor H_2O was found to contribute any significant background chemiluminescence. RLU/mole were determined via linear regression from a plot of relative light units in counts versus moles of acridinium derivative.

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