

Regiodependent Luminescence Quenching of Biotinylated *N*-Sulfonyl-acridinium-9-carboxamides by Avidin

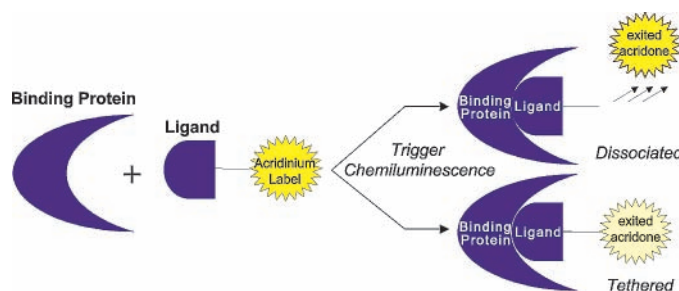
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ABSTRACT



Biotin was conjugated to chemiluminescent *N*-sulfonylacridinium-9-carboxamides at the N-10 or 9-position carboxamide. Upon binding to avidin, the light output of the N-10 derivative (**8**) was quenched up to 92% upon triggering with basic peroxide, while the 9-position carboxamide conjugate (**9**) was quenched only 33%. The utility of this effect was demonstrated in a model homogeneous chemiluminescence assay.

Chemiluminescence provides the most sensitive nonisotopic detection technology available today. One class of compounds extensively utilized in chemiluminescent detection formats are the ester and *N*-sulfonylamide derivatives of acridinium-9-carboxylic acid.^{1–3} Acridinium salts are highly desirable as labels for detection due in part to a low naturally occurring background signal in biological samples and the relatively high quantum yields of the resultant chemiluminescent reaction. Such properties permit detection of acridinium-labeled species over 5 orders of magnitude typically starting at attomole levels.

Numerous acridinium salt derivatives have been synthesized and evaluated as labels for detection. Acridinium salt

derivatives have been prepared in efforts to enhance the chemiluminescence quantum yield, to improve the stability or solubility of the labels, and to modulate the kinetics or color of the light generated in the chemiluminescent reaction.^{2,4–8} Historically, these acridinium salt derivatives have been synthesized with reactive functionalities incorporated into the ester or *N*-sulfonylamide moiety of the labels for coupling to ligands of interest. Synthesis of labels with reactive functionalities for coupling to ligands via other positions on the acridinium moiety have been explored less frequently.^{8–12}

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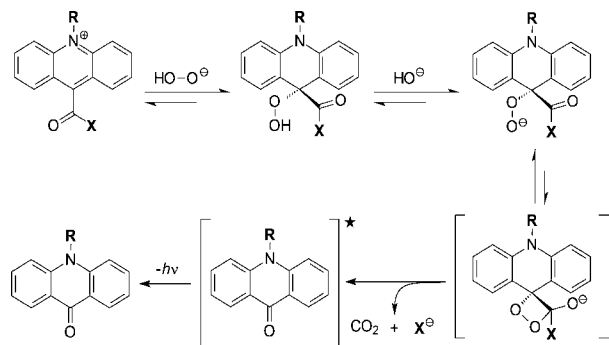
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The general mechanism for acridinium salt chemiluminescence is depicted in Scheme 1.^{7,13} Chemiluminescence

Scheme 1. Proposed Mechanism of Acridinium Salt Chemiluminescence



X = leaving group, eg. aryloxy or sulfonamide

is triggered by the addition of hydrogen peroxide anion to the 9-position of the acridinium salt, yielding an acridan hydroperoxide. Subsequent deprotonation of the hydroperoxide and attack at the carbonyl carbon provides a tetrahedral spirodioxetane intermediate. Decomposition of the unstable spirodioxetane intermediate results in formation of an excited-state acridone with concomitant elimination of CO₂ and a leaving group X. Light is generated upon return of the excited-state acridone to the ground state.

The mechanism in Scheme 1 illustrates that the site of ligand attachment to an acridinium salt may impart a subtle but potentially very important characteristic to the label. Acridinium salt derivatives linked to ligands of interest through the ester or sulfonamide moiety result in generation of an excited-state acridone that is released upon triggering of the chemiluminescent reaction (Scheme 2). From a sensitivity standpoint, such a process is desirable, as the light-

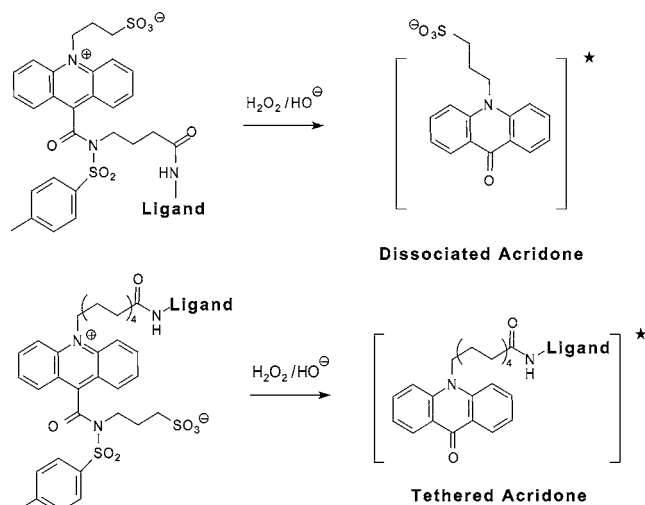
emitting species is dissociated from potential collisional quenching entities (i.e., proteins). In contrast, a derivative linked to ligands of interest through the acridinium ring results in generation of an excited-state acridone that remains tethered to the ligand upon chemiluminescence triggering. Tethered acridinium light-emitting species have the potential to undergo collisional quenching or to serve as donors in energy transfer processes.²

Nonacridinium salt-based reagents have been described in luminescence assays that rely on quenching or energy transfer. Specifically, assays for biotin have been developed utilizing the bioluminescent photoprotein aequorin.^{14–19} Bioluminescence resonance energy transfer (BRET) has recently been demonstrated to be useful for studies of protein–protein interactions.^{20–22} Similarly, chemiluminescence resonance energy transfer assays utilizing aminobutylethylisoluminol (ABEI) as the luminescent donor and fluorescein as the acceptor have been successfully developed for several biological ligands of interest.^{23–27}

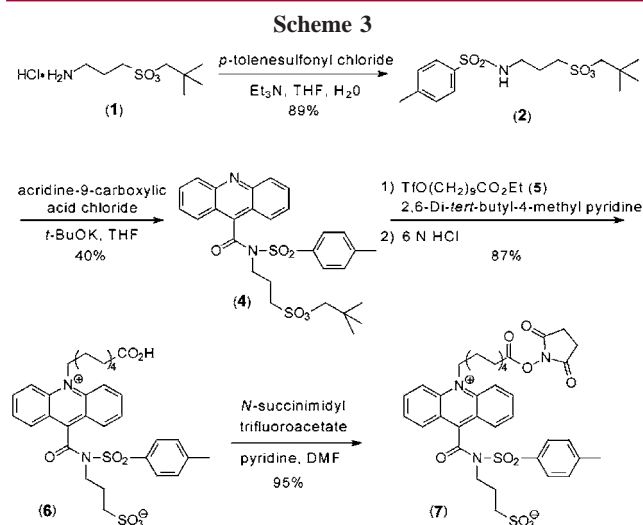
Here we describe the synthesis of an *N*¹⁰-carboxyalkyl-functionalized acridinium label. The *N*¹⁰-carboxyalkyl acridinium label was prepared for direct comparison to an analogous acridinium label with the reactive functionality for coupling to ligands of interest incorporated into the sulfonamide moiety. The labels were coupled to a derivative of biotin, generating model tethered and releasable acridone-generating tracers. The resulting biotin tracers were evaluated in terms of their chemiluminescent properties in buffer alone and in the presence of the binding protein avidin.

The *N*¹⁰-carboxyalkyl-functionalized acridinium-9-carboxamide label **6** that results in a tethered acridone species upon chemiluminescence triggering was synthesized as depicted

Scheme 2



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in Scheme 3. Specifically, sulfonamide **2** was prepared in 89% yield from 3-aminopropylsulfonate derivative **1** and *p*-toluenesulfonyl chloride in THF. Acylation of the sulfonamide anion with acridinium-9-carboxylic acid chloride gave the protected *N*-sulfonylacridine-9-carboxamide **4** in 40% overall yield. Alkylation of the N-10 position on the acridine ring with triflate-activated ethyl 10-hydroxydecanoate (**5**) and acidic hydrolysis provided the desired acridinium-9-carboxamide label **6** in 87% yield. The *N*-hydroxysuccinimidyl active ester of acridinium sulfonamide **6** was subsequently prepared in $\geq 95\%$ yield using *N*-succinimidyl trifluoroacetate. The sulfonamide-functionalized acridinium-9-carboxamide label that results in release of an excited state acridone species upon chemiluminescence triggering was prepared following previously described procedures.²⁸

The structures of the N-10 and 9-position biotin tracers utilized in model studies described here are depicted in Figure

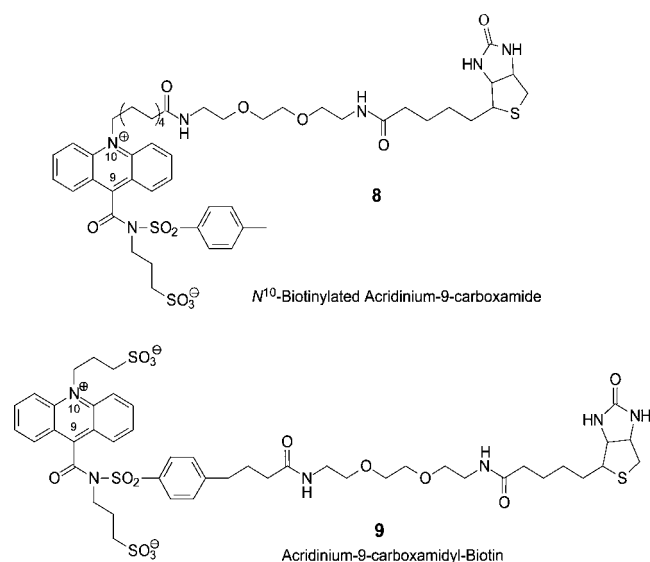


Figure 1. Structures of N-10-tethered and 9-position biotin tracers.

1. The tethered acridone-generating tracer **8** was synthesized by coupling of active ester **7** to biotin-PEO-amine. Similarly, the dissociated acridone-generating tracer **9** was from biotin-PEO-amine and the previously described sulfonamide-derivatized acridinium active ester.²⁸

The biotin tracers **8** and **9** were directly compared in terms of their chemiluminescence profiles and light output in assay buffer (10 mM borate, 2 mM EDTA, 0.1% Tween-20, pH 7.3). Both tracers exhibited flash-type luminescence profiles with all light being generated within 5 s after addition of the chemiluminescence trigger (Figure 2). The overall

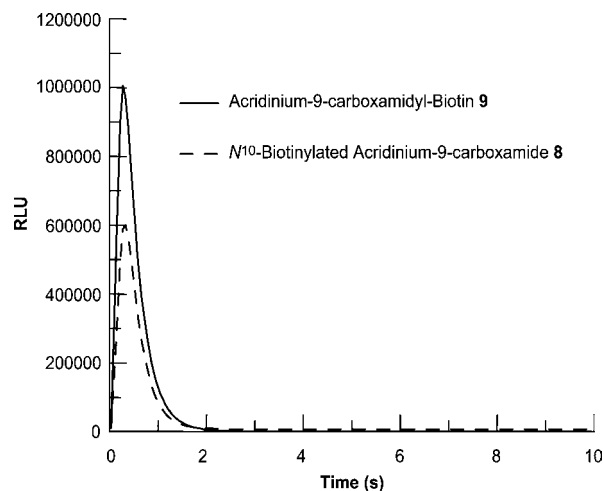


Figure 2. Representative luminescence profiles of the N-10-tethered and 9-position biotin tracers. Luminescence profiles were obtained by triggering 25 μL aliquots of tracer (~ 0.6 nM) in 10 mM borate, 2 mM EDTA, 0.1% Tween-20, pH 7.3 using a microplate luminometer (MicroLumat Plus, Perkin-Elmer). Chemiluminescence trigger solution: 200 μL 0.18 N NaOH, 0.7% H_2O_2 , 1% Triton X-100, 0.05% diethylenetriaminepentacetic acid.

chemiluminescent output from the two tracers was also of the same magnitude. The N-10 tracer yielded $\sim 2.3 \times 10^{19}$ counts/mol. The 9-position tracer yielded $\sim 4.2 \times 10^{19}$ counts/mol.

The tracers exhibited markedly different chemiluminescent properties relative to each other in the presence of avidin. Specifically, each biotinylated acridinium tracer (0.5 nM) was individually mixed with several concentrations of avidin (8–8000 pM) in assay buffer, and the solutions were incubated for 45 min. A chemiluminescent response from each mixture was subsequently obtained from 25 μL of each mixture as described above. Plots of the normalized relative light units (RLU) obtained versus avidin concentration are shown in Figure 3. The data show that acridinium chemiluminescence from a biotin tracer can be quenched by avidin in a concentration-dependent manner. Significantly, the

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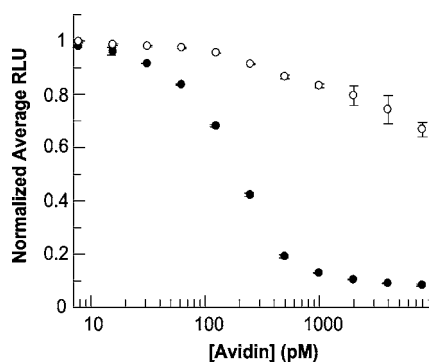


Figure 3. Plot of normalized chemiluminescent response for tethered (**8**) (●) and releasable (**9**) biotin tracers (○) vs avidin concentration. Data points represent the average of duplicate values.

chemiluminescent output derived from biotin tracer **8** resulting in generation of a tethered excited-state acridone upon luminescent triggering is quenched up to ~92% upon titration with avidin. In contrast, biotin tracer **9**, which results in generation of a dissociated excited state acridone upon luminescent triggering, is quenched only ~33% upon titration with avidin. Identical studies utilizing biotinylated acridiniums **8** and **9** and bovine serum albumin (BSA) were also conducted as controls. Chemiluminescent outputs from these controls over the entire BSA concentration range tested (8–8000 pM) did not vary significantly (less than 1%). The results demonstrate that the quenching phenomenon is due to a specific binding interaction.

The avidin-specific modulation of acridinium chemiluminescence was further demonstrated in a model homogeneous assay. Thus, a fixed concentration of biotinylated tracer **8** (0.5 nM, tethered biotin) was added to several concentrations of the water-soluble biotin derivative biotin-PEO-amine (0.06–16 nM) in assay buffer. The biotin-containing mix-

tures were subsequently added to a fixed concentration of avidin also in assay buffer. The solutions were allowed to incubate for 45 min, and 25 μL of each sample was triggered as above.

The dose–response curve obtained is depicted in Figure 4. The average % quenching value reported in Figure 4 is

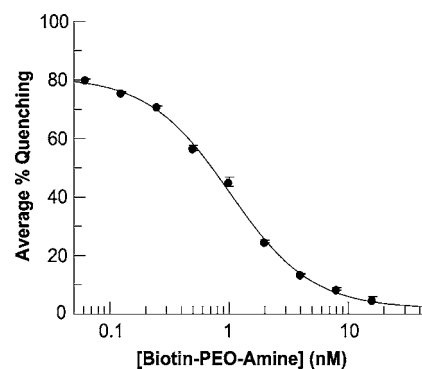


Figure 4. Dose–response curve generated for biotin-PEO-amine. Data points represent the average of triplicate values. The curve represents the best nonlinear fit of the data using a four-parameter logistic.

relative to the chemiluminescent response obtained for biotinylated tracer **8** in buffer alone. As expected, acridinium chemiluminescence quenching due to association of avidin with the biotinylated tracer decreases with increasing concentrations of the biotin analyte.

Investigation of the generality of this chemiluminescence-quenching phenomenon in other assay formats is ongoing.

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