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Intrinsic factor-mediated modulation of cyanocobalamin–*N*-sulfonyl-acridinium-9-carboxamide chemiluminescence

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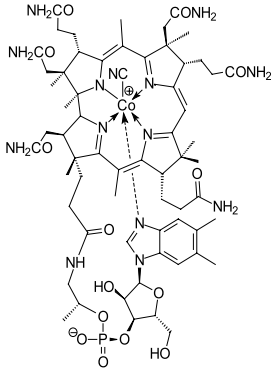
Abstract—Two cyanocobalamin–*N*-sulfonyl-acridinium-9-carboxamides with linkage through the N^{10} or 9-position were prepared from B_{12}-e-carboxylic acid. The noncovalent association of intrinsic factor with these ligands resulted in specific modulation of the associated chemiluminescence signal either by quenching or changing the emission profile. Either effect was sufficient to formulate a homogeneous assay to detect vitamin B_{12} in buffer.

Chemiluminescent *N*-sulfonyl-acridinium-9-carboxamide reporter groups have been successfully commercialized on high-throughput, automated in vitro diagnostic analyzers that utilize a heterogeneous assay format, that is, the bound and free labeled material was physically separated before the signal is generated.[1] We have recently reported that homogeneous chemiluminescent assays are possible using the same family of *N*-sulfonyl-acridinium-9-carboxamide reporter groups. Using the specific interaction of proteins as divergent as avidin and folate binding protein (FBP) with their complementary *N*-sulfonyl-acridinium-9-carboxamide-labeled ligands, assays for biotin[2] and folic acid[3] were demonstrated. Both assays relied on the quenching of the chemiluminescence signal from the labeled ligand by the specific binding protein, a phenomenon that was most efficient when the *N*-sulfonyl-acridinium-9-carboxamide–ligand linkage was via the N^{10}-position on the acridinium nucleus.

In this report we demonstrate that a noncovalent protein–ligand interaction between chemiluminescent *N*-sulfonyl-acridinium-9-carboxamide–cyanocobalamin ligands and intrinsic factor can result in specific modulation of the associated chemiluminescence signal either by quenching or by a change in the chemiluminescence emission profile. Either effect was sufficient to formulate a homogeneous assay to detect vitamin B_{12}.



1, Vitamin B_{12}

Cyanocobalamin (**1**, vitamin B_{12}) is routinely monitored for the diagnosis and clinical management of ailments (e.g., anemia, atherosclerosis, and neuropsychiatric disorders) arising from the impairment of the enzyme, methionine synthase.[4,5] Porcine intrinsic factor (IF), a gut-secreted 58–64 kDa glycoprotein[6,7] that binds B_{12} with high affinity (association constant $1.5–3.3\times10^{10}\,M^{-1}$),[7,8] has been used successfully in serum assays for B_{12}.[5,9–11]

Keywords: Homogeneous chemiluminescent assays; Intrinsic factor; Vitamin B12; Cobalamin.
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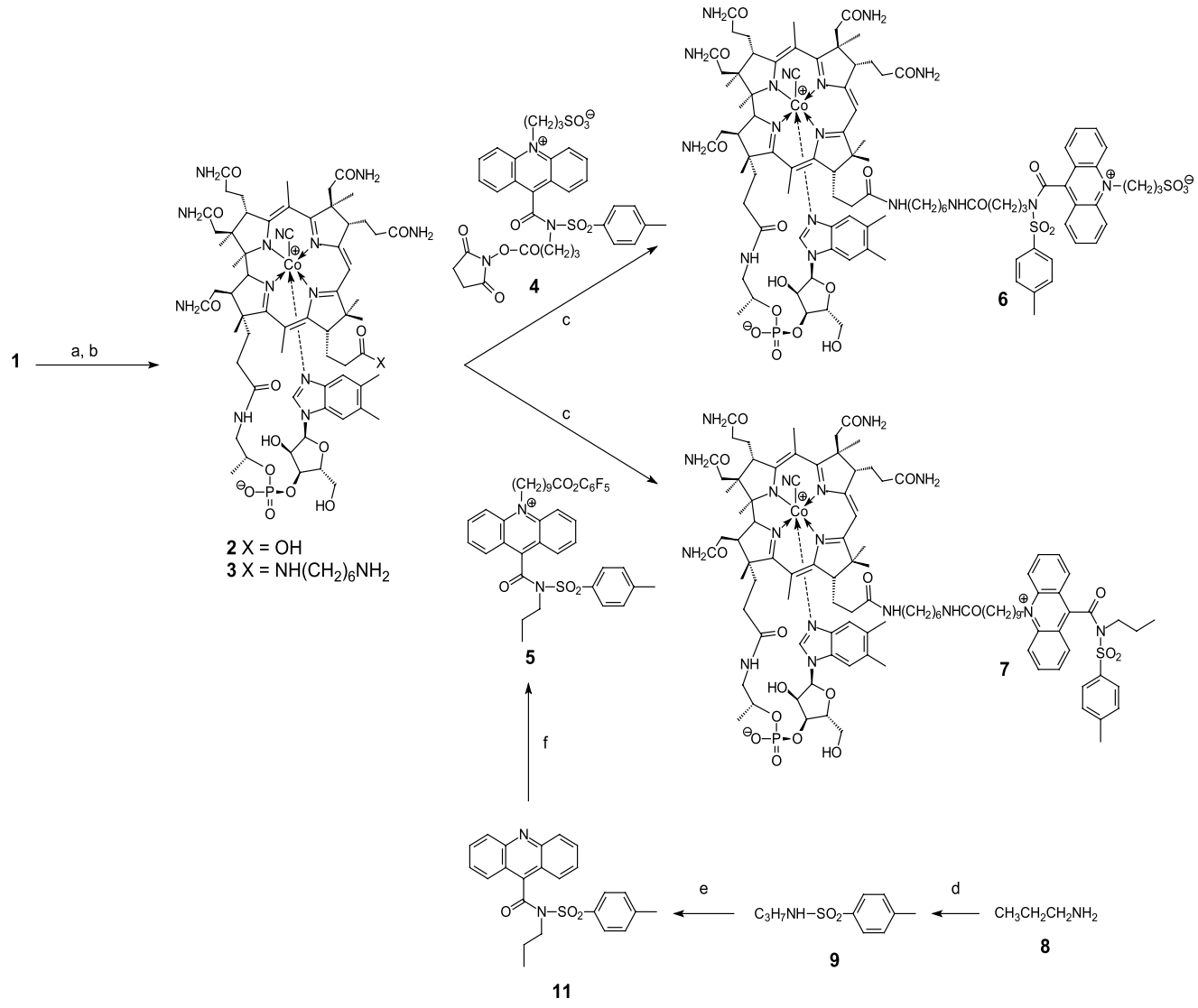

Scheme 1. Preparation of cyanocobalamin–*N*-sulfonyl-acridinium-9-carboxamide intrinsic factor ligands. Reagents and conditions: (a) 0.8 M aq H_3PO_4, 70 °C, 4 h, 10%; (b) $H_2N(CH_2)_6NH_2$, imidazole, EDAC, pH 4, 64%; (c) Et_3N, DMF, overnight, 60%; (d) tosyl chloride, Et_3N, 95%; (e) (i) K *t*-OBu/THF; (ii) acridine-9-carbonylchloride, **10**, 67%; (f) (i) $TfO(CH_2)_9CO_2CH_3$, 2,6-dibutyl-4-methylpyridine, CH_2Cl_2; (ii) aq HCl (1 N), reflux, 8 h; (iii) $CF_3CO_2C_6F_5$, pyridine, CH_2Cl_2, 15%.

Thus, for this study two cyanocobalamin–*N*-sulfonyl-acridinium-9-carboxamides with linkage through the N^{10} or 9-position were prepared from B_{12}-e-carboxylate (Scheme 1). Cyanocobalamin (**1**) was partially hydrolyzed in dilute phosphoric acid to afford the e-carboxylic acid **2**, then converted to the 6-aminohexyl amide **3**.[10] The acridinium-9-carboxamide active esters **4**[12] and **5** bearing linkers extending from the 9- and 10-positions, respectively, were conjugated to cyanocobalamin **3** to give the chemiluminescent cobalamin ligands for IF, **6** and **7**.

The chemiluminescence of **6** and **7** was first evaluated in the absence of IF. Serial dilutions of the two ligands were prepared in buffer (10 mM borate, 2 mM EDTA, 0.1% Tween-20, pH 7.3) from initial stock solutions (concentrations calculated based on the A_{550} nm for vitamin B_{12}, $\varepsilon_{550} = 8739\ M^{-1}\ cm^{-1}$). As shown in Figure 1, signal generated from the two compounds over a 30 s integration period differed by an order of magnitude, unlike the analogous biotin[2] and folate[3] conjugates previously described. The integrated signal for compound **6**, where the cyanocobalamin was attached at the 9-position carboxamide, was 5.19×10^{19} RLU/mol, while the signal from compound **7**, with the label attached at the N^{10}-position,was only 4.9×10^{18} RLU/mol, that is, the signal is quenched by over 90%. This result is consistent with the mechanism of the chemiluminescent reaction (Fig. 2), in which the acridone chemiluminophore is released on reaction with peroxide and base from the cyanocobalamin in **6** but remains tethered in compound **7**. Analogous quenching of tethered fluorophores by cyanocobalamin has been reported.[13–17]

Upon examination of the chemiluminescence profile of each ligand further differences were noted. The emission from the 9-position carboxamide ligand **6** (Fig. 3a, red trace) was noticeably faster (2 s), than the 10-position

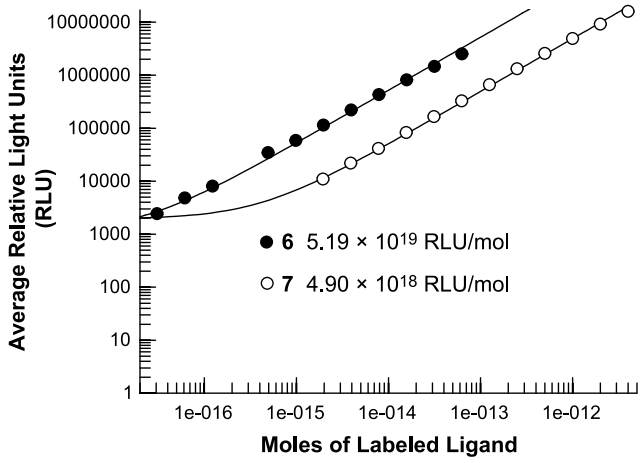

Figure 1. Chemiluminescence of ligands **6** and **7**. Aliquots (50 μL) of each ligand (10 mM borate, 2 mM EDTA, 0.1% Tween-20, pH 7.5) were triggered (200 μL 0.18 N NaOH, 0.7% H_2O_2, 1% Triton X-100, 0.05% diethylenetriaminepentaacetic acid) in a microplate luminometer (MicroLumat Plus, Perkin–Elmer). Data points represent the average integrated RLUs of duplicate values obtained over a 30 s window. The data was fit using linear regression analysis with proportional weighting in Grafit (Erithacus Software).

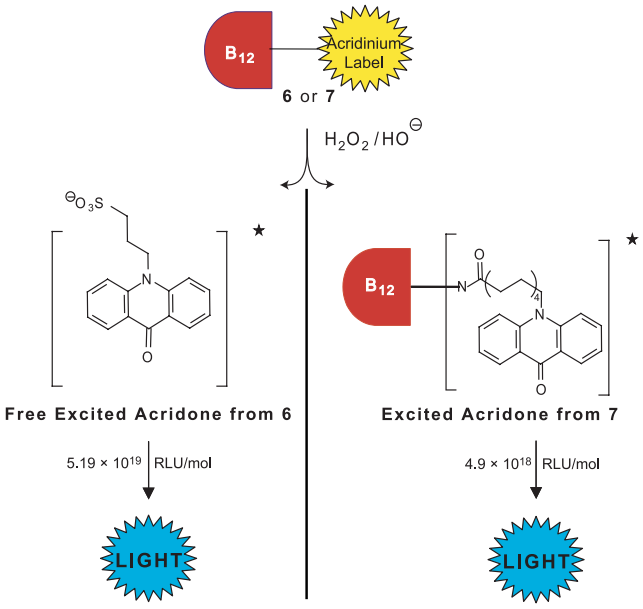

Figure 2. Excited state acridones generated from **6** and **7**.

ligand **7** (20 s) (Fig. 3b, red trace). The broader chemiluminescence profile exhibited by **7**, was more typical of acridinium-9-carboxamides that are sterically hindered about the 9-position,[18,19] or triggered under non-optimum conditions (e.g. low peroxide anion concentration). In the case of the N^{10} and 9-position biotin[2] and folic acid[3] acridinium derivatives, equivalent flash chemiluminescent profiles were observed regardless of the point of attachment to the ligand.

When examined in the presence of intrinsic factor (5× molar excess), the maximal intensity of the signal from both ligands **6** and **7** was diminished. The

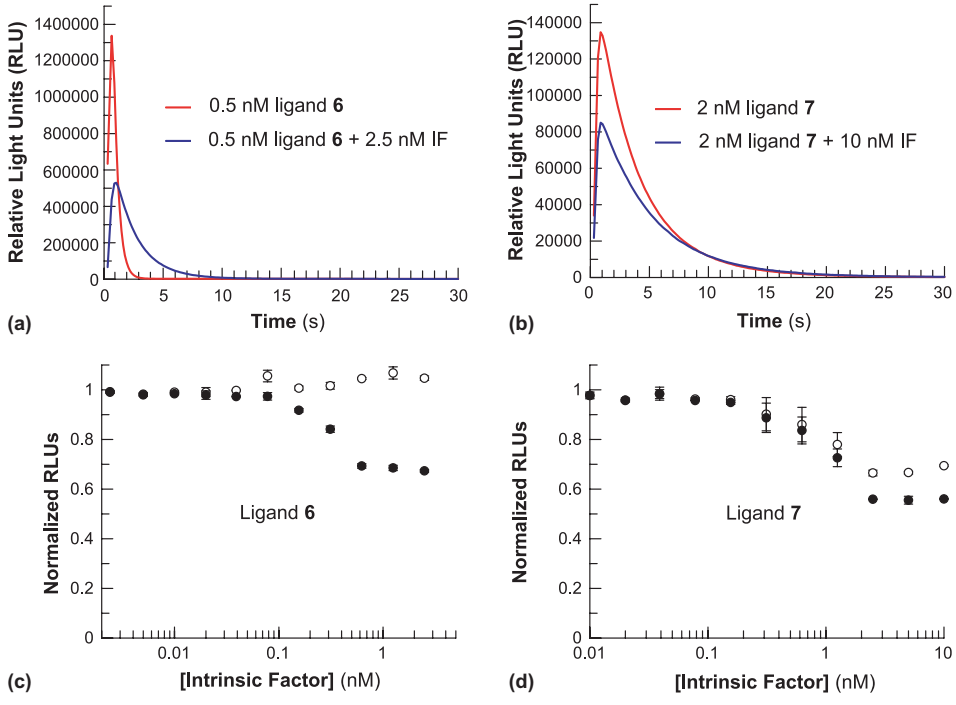

Figure 3. Effect of added IF on the chemiluminescence of ligands **6** and **7**. Aliquots (50 μL) of each ligand/IF solution (10 mM borate, 2 mM EDTA, 0.1% Tween-20, pH 7.5) were triggered (200 μL 0.18 N NaOH, 0.7% H_2O_2, 1% Triton X-100, 0.05% diethylenetriaminepentaacetic acid) in a microplate luminometer (MicroLumat Plus, Perkin–Elmer) and the signal recorded for 30 s. (a) Chemiluminescence profile of **6** w/wo IF. (b) Chemiluminescence profile of **7** w/wo IF. (c) Plots of normalized chemiluminescent response for **6** [0.5 nM, 10 s (○) and 2 s (●) integration)] versus IF (0.0024–2.5 nM). (d) Plots of normalized chemiluminescent response for **7** [2 nM, 10 s (○) and 2 s (●) integration)] versus IF (0.01–10 nM).

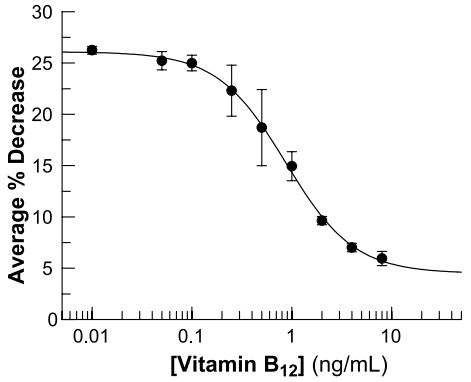

Figure 4. Dose–response curve generated for vitamin B_{12}. Solutions of B_{12}, ligand **6** (0.5 nM) and IF (0.5 nM) in buffer (10 mM borate, 2 mM EDTA, 0.1% Tween-20, pH 7.4) were incubated for 1 h. Aliquots (25 μL) were then triggered [200 μL 0.18 N NaOH, 0.7% H_2O_2, 1% Triton-X100, 0.05% diethylenetriaminepentaacetic acid, MicroLumat Plus (Perkin–Elmer)]. The chemiluminescent response from each sample was subsequently integrated over a 2 s window and normalized relative to the integrated value obtained for **6** alone in buffer. Data points represent the average of triplicate values. The curve represents the best nonlinear fit of the data using a four-parameter logistic.

chemiluminescence emission profile of compound **6** (Fig. 3a, blue trace) was broadened, but the overall integrated signal was undiminished. The chemiluminescence emission profile of **7** (Fig. 3b, blue trace) was unchanged, but the overall integrated signal was further decreased.

The effects on the chemiluminescent response of ligands **6** and **7** upon binding to intrinsic factor were dose-dependent. For the acridinium-derivatized ligand **6**, the effect was only apparent when the data from the first 2 s of the emission were considered (Fig. 3c, •). Under these conditions, the signal decreased by 32%. The concentration discrimination disappeared when the signal integration was extended to 10 s (Fig. 3c, ○). In contrast, the data integration window (2 or 10 s) had little effect on the dose-dependent response of ligand **7** to intrinsic factor; in either case, the chemiluminescence was quenched by 33–44% (Fig. 3d) relative to the unbound ligand.

In this regard, the cyanocobalamin–N^{10}-acridinium-9-carboxamide ligand (**7**)/intrinsic factor interaction was similar to that observed in the biotin/avidin and folic acid/FBP systems, that is, chemiluminescence from the excited-state acridone was specifically quenched in the noncovalent ligand/protein complex. Moreover, the observed protein-mediated quenching was in addition to that induced by the attachment of the excited-state acridone to cyanocobalamin. As in the previous examples, a homogeneous assay for B_{12} (**1**) based on this quenching phenomenon was possible using this reagent combination (data not shown).

The interaction of the cyanocobalamin–acridinium-9-carboxamide ligand (**6**) with intrinsic factor suggested that a homogeneous assay would be possible based on the difference in the emission profile of **6** upon binding. Thus, a fixed concentration of ligand **6** (0.5 nM) was added to several concentrations of vitamin B_{12} (0.01–8 ng/mL). The mixtures were incubated with intrinsic factor (0.5 nM) for 1 h. Twenty five-μL aliquots of each solution were then triggered and the chemiluminescent response from each sample was subsequently integrated over a 2 s window and normalized relative to the integrated value obtained for tracer alone in buffer. The dose–response curve obtained is depicted in Figure 4.

In summary, the chemiluminescence efficiency of acridinium-9-carboxamide–cyanocobalamin conjugates was affected by the regiochemistry of the linkage between the components. N^{10}-acridinium-9-carboxamide–cyanocobalamin conjugate **7** was quenched by over 90% and the chemiluminescence emission profile was broadened relative to the 9-position conjugate **6**. The chemiluminescence signal from ligand **7** was further quenched upon specific binding to intrinsic factor, while interaction of **6** with intrinsic factor resulted in a broadening of its chemiluminescence emission profile. The IF–specific modulation of the chemiluminescence signal from these ligands, whether from quenching or from changes in the emission profile, was sufficient to demonstrate a homogeneous assay to detect vitamin B_{12}.

We continue to explore other binding protein/ligand-acridinium-9-carboxamide pairs to further define the general scope of this technology.

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