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A Novel Reagent for Labelling Macromolecules with Intensely Luminescent Lanthanide Complexes

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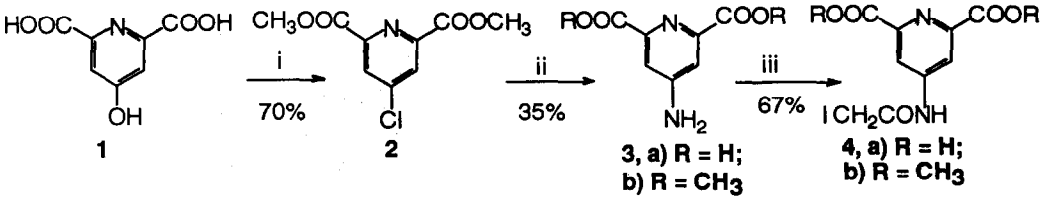
Abstract: A novel bifunctional chelator, 4-iodoacetamidodipicolinicacid, has been synthesized in three steps starting with chelidamic acid, and used to prepare protein conjugates that form stable and intensely luminescent complexes with Tb(III) and Eu(III). The excited state lifetime of Tb(III) complexes with this reagent was 1.25 ms, permitting its use for ultrasensitive time-resolved luminescence assays.

Because of the costs, hazards, short half-lives, and intrinsically limited sensitivity of radionuclides, there has been a growing need for nonradioactive reagents to label macromolecules for use in immunoassays, nucleic acid hybridization, and other biotechnology applications.[1] Bifunctional chelating agents[2] have been attractive as tools for attaching the highly luminescent lanthanide ions, Tb(III) and Eu(III), to macromolecules, because of the highly efficient background rejection obtained by time-gated measurements of emission from the millisecond-duration lanthanide excited states.[3] Unfortunately, lanthanide complexes require energy transfer from a strongly ultraviolet light-absorbing organic ligand to the bound metal ion for efficient excitation, and available bifunctional chelating agents have been only weak enhancers of Tb(III) and Eu(III) emission.[4] In contrast, dipicolinic acid (pyridine, 2,6, dicarboxylicacid) is a very efficient sensitizer of lanthanide luminescence.[5]

To prepare a bifunctional chelating agent that incorporates the efficiently sensitizing dipicolinic acid moiety into a reagent that can be easily used for modifying macromolecules, we have prepared 4-iodoacetamidodipicolinicacid, dimethylester (**4b**) from commercially available chelidamic acid, **1**, according



i) $PhOCl_2$, 130°C, 1 h; Dry MeOH; ii) 30% NH_3 (aq.), 150 °C, bomb[6] MeOH/HCl refl. 24 h
iii) $(ICH_2CO)_2O$, H_2SO_4 (cat)/60 °C/1 h[7]

Scheme 1. Synthesis of IADP

to scheme 1. Dimethyl esters **3b** and **4b** were purified by silica gel chromatography using a benzene/ethyl acetate solvent system, after attempts to isolate the free acids by ion exchange chromatography were

unsuccessful. The esterified reagent **4b** is rapidly converted under mildly alkaline conditions to the highly water soluble carboxylate form (*e.g.* dipotassium salt of **4a**) and forms complexes with Tb(III) and Eu(III) that show very efficient sensitization of luminescence. The excitation spectrum (Figure 1) is characteristic of the absorbance of the ligand (**4a**) rather than that of the metal ion, indicative of energy transfer from excited state(s) of the pyridine system to the bound Tb(III).

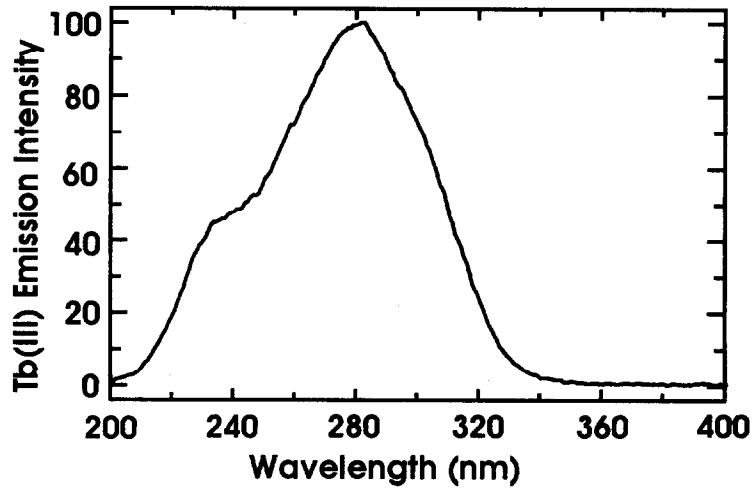


Figure 1. Excitation spectrum of energy transfer-induced Tb(III) luminescence from the $Tb(IADP)_3$ complex.

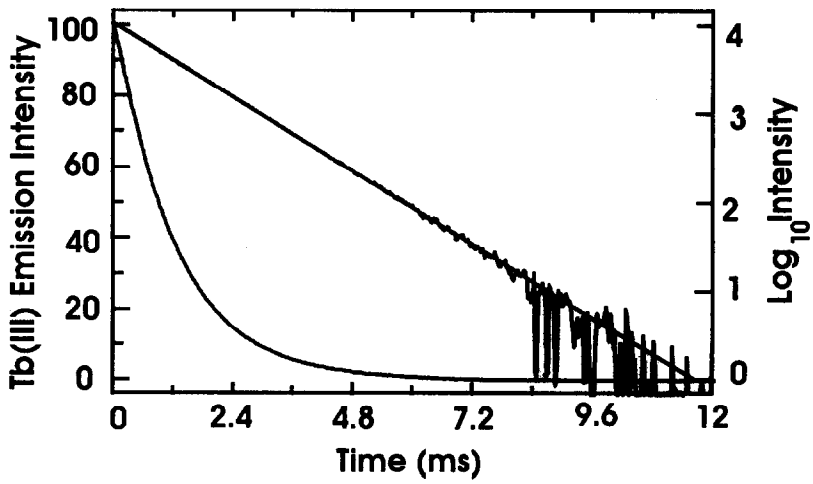
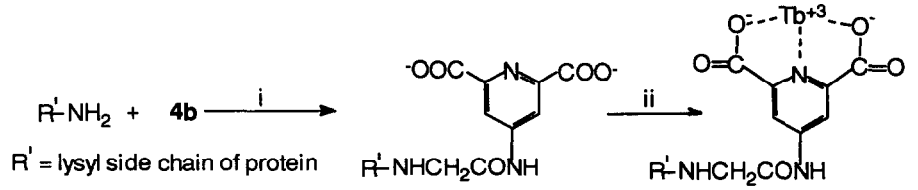


Figure 2. Millisecond luminescence decay of Tb(III) complexed with IADP. The response of a 3:1 complex of IADP and Tb(III) to a pulse of laser light (275 nm) is shown. The theoretical curve shown corresponds to a single exponential decay with an excited state lifetime of 1.25 ms.

Similar results were obtained with Eu(III). Lifetime measurements using a chopped argon ion laser beam (275 nm) for excitation[8] revealed a single excited state lifetime of 1.25 ms (Figure 2). This long lifetime allows for efficient rejection of background signals due to scattering and nanosecond scale fluorescence[3].

Protein Labelling. Goat immunoglobulin G (IgG, Sigma, 2mg) was reacted with **4b** (2 mg) in 700 µl of 0.1 M potassium borate, pH 9 at room temperature for 24 hrs (Scheme 2). The protein conjugate was



i) 0.1 M potassium borate, pH 9, 24 h, centrifugal ultrafiltration; ii) $TbCl_3$ (1 mM). Tb(III) may bind as many as three dipicolinate moieties.

Scheme 2. Preparation of Tb(III) Complex with IADP-Labelled Protein.

washed free of reagent by four washes in a Centricon-30 ultrafiltration apparatus (Amicon), and concentrated in the same apparatus to 8-10 mg/ml. Labelling was detected by immobilizing the protein conjugate on nitrocellulose sheets in a slot-blot vacuum filtration apparatus (BioRad) and staining with $TbCl_3$ (1 mM). The resultant bright green Tb(III) luminescence was detected under ultraviolet illumination (254 nm mercury lamp). To confirm formation of a stable covalent conjugate, the protein conjugate was analyzed by

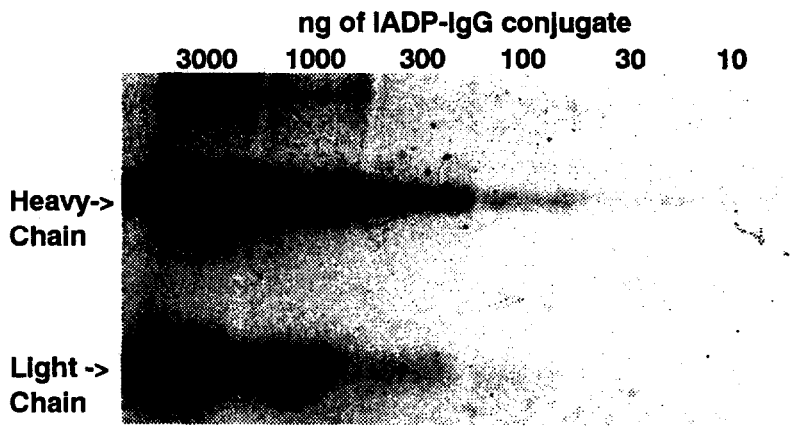


Figure 3. Photograph of Tb(III) luminescence from a gel containing electrophoretically resolved IADP-modified IgG heavy and light chains stained with $TbCl_3$. The gel was photographed using ultraviolet illumination from a trans-illuminator; a digitized image of the negative is shown. The indicated amounts of IADP-modified IgG were added to each lane of the gel.

electrophoresis in a denaturing polyacrylamide gel containing sodium dodecylsulfate.[9] When the gel was stained with $TbCl_3$ and illuminated with UV light, luminescence was observed in both the heavy chain and light chain bands (fig 2). Similar enhancement of Tb(III) luminescence was not observed with unlabelled IgG. The complexation of Tb(III) was very stable, and Tb(III) could not be washed out of the gel even after several days of continuous soaking in EDTA. The $TbCl_3$.stained protein could not be transferred from the gel to nitrocellulose under standard conditions for Western electrophoretic transfer.[10] The most likely explanation for these results is crosslinking in the gel of the labelled immunoglobulin chains by Tb(III) ions, each of which is known to be capable of coordinating as many as three dipicolinate groups.[11,3]

These results show that IADP can be used to prepare protein conjugates that form stable and brightly luminescent Tb(III) complexes whose millisecond excited state lifetimes can be employed in ultrasensitive delayed luminescence immunoassays. The simplicity and good yield of our synthetic procedure makes this reagent much more convenient to prepare than many previously described bifunctional chelators containing lanthanide-sensitizing aromatic rings.

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REFERENCES AND NOTES

1. Kricka, L.J. *Non Isotopic DNA Probe Techniques;* Academic Press: New York, 1992; pp 3-28. Barnard, G. J. R.; Williams, J. L.; Paton, A. C.; Shah, H. P. Time-Resolved Fluoroimmunoassay. In *Complementary Immunoassays;* Collins, W.P. Ed.; John Wiley & Sons Ltd.: London, 1988; pp 149-167.
2. Meares, C.F. and Wensel, T. G. *Accts. Chem. Res.* **1984** *17,* 202-209.
3. Soini, E.; Lovgren, T. *CRC Crit. Rev. Anal. Chem.* **1987,** *18,* 105-154.
4. Abusaleh, A.; Meares, C. F.; *Photochem. Photobiol.* **1984,** *39,*763-769.
5. Barela, T. D.; Sherry, A. D.; *Anal. Biochem.* **1976,** *76,* 351-357.
6. Large amounts (50-60 g) of **3a**, R= H, can be obtained using the procedure reported by: Bradshaw, J.S.; Maas, G.E.; Lamb, J.D.; Izatt, R.M.; Christensen, J.J. *J. Am. Chem. Soc.* **1980,** *102,* 408-415.
7. **H NMR ($CDCl_3$-MeOH-d_4)**: 8,50 (s, 2H, Ar-H); 4,00 (s, 6H, 2X $COOCH_3$); 3,89 (s, 2H, ICH_2CO); **^{13}C NMR (MeOH-d_4)**: 170.26 (COOCH3), 166.12 (CONH); 150.43 (CCCH3); 149.55 (Ar-C_4); 118.14 (Ar-$4C_{2,3,5,6}$); 53.39 2X OCH_3); -1.64 (I$\underline{C}H_2$). **GC/MS:** Position of the peak (Relative intensity): 379 $(M+H)^+$; (18); 378 (10); 348 (12); 323 (32); 321 (32); 320 (66); 288 (20); 194 (28); 145 (30); 142 (68); 141 (40); 127 (68); 120 (36); 91 (40); 28 (58); and 18 (100, base peak). **FAB MS:** 379 $(M+H)^+$.
8. Lamture J. B. , Zhou, Z., Suresh Kumar,A. and Wensel T. G. ***Biophys. J.*, 1991,** *159,* 191a; a full paper will be published elsewhere.
9. Laemmli, U. K , *Nature* **1970** *227,* 680-685.
10. Harlow, E.; Lane, D. *Antibodies A Laboratory Manual;* Cold Spring Harbor Laboratory; Cold Spring Harbor. 1988; pp. 471-510.
11. Grenthe, I. *J. Amer. Chem. Soc.* **1961**, 83, 360-364; Miller, T. L., Senktor, I. S. *Anal. Chem.,* **1982,** *54,* 2022-2025..

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