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Towards novel biolabels: synthesis of a tagged highly fluorescent Schiff-base aluminium complex

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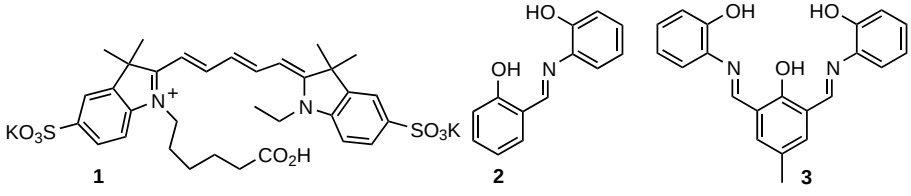
Abstract—Starting from 3-(4-hydroxyphenyl)propanoic acid, formylation *ortho* to the phenol and condensation with *ortho*-aminophenol gave a novel functionalised Schiff-base ligand. Formation of the *N*-hydroxysuccinimidyl ester and non-aqueous complexation with $AlEt_2Cl$ gave a novel fluorescent biolabel displaying a Stokes shift of 100 nm. The efficacy of the new system was demonstrated by its attachment to rabbit IgG.

As an alternative to the use of radioactive labelling, the detection and study of biomolecular materials increasingly requires attachment of a fluorescent organic dye via a covalent linker. This introduced, group significantly increases both the sensitivity and selectivity of detection due to characteristic excitation and emission wavelengths of the fluorophore.[1] Although many species such as Cy™3 **1**[2] have been successfully applied in this way, a feature of many such conjugated organic compounds is their relatively small Stokes shifts (15–20 nm for **1**) limiting the band width of both excitation and emission. Larger Stokes shifts would allow for the use of simple broad filtering systems enabling efficient light collection and an increase in sensitivity of detection. In seeking novel solutions to this requirement, we noted that many non-fluorescent organic ligands form fluophores on complexation with metal ions, providing selective and sensitive methods for metal detection.[3] In particular, the simple Schiff-bases **2**[4] and **3**[5] have been studied for the spectrofluorometric determination of aluminium ion concentrations in sub parts per million ranges, concentrations that equate with the use of fluorescent dyes in biological assays. Significantly, the complexes that form between **2** or **3** and Al^{3+} are reported to have Stokes shifts of $\sim$100 nm.

To verify that these candidate structures would provide the basis of new biolabels, we first confirmed their optical properties following complexation in ethanol with a tenfold excess of aluminium chloride in the presence of sodium acetate (Table 1).[4] Comparison of the two solutions revealed a significantly greater emission intensity for the complex generated from **2** compared to the species resulting from **3**, the former being



1 **2** **3**

Table 1. Fluorescent properties of ligand/Al complexes at 30 nM concentration in EtOH

Ligand	$\lambda_{exc.}$ (nm)	$\lambda_{em.}$ (nm)	Stokes shift (nm)	ε (M^{-1} cm^{-1})	τ_1 (ns)
2	413	513	100	14,000	5.05
3	433	539	106	20,000	1.16

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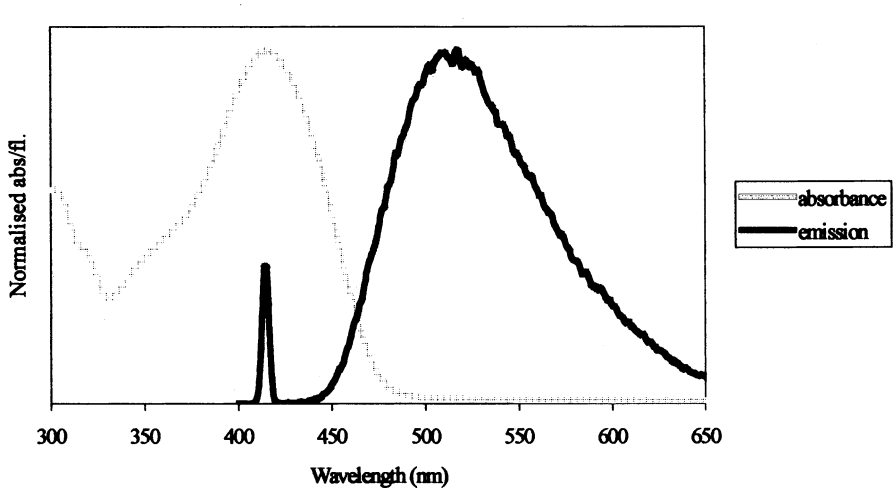


Figure 1. Absorption/emission profile of **2** complexed to aluminium.

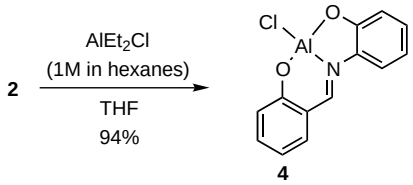
comparable to Cy™3 **1** over a range of concentrations (10^{-5}–10^{-8} M) (Fig. 1).

Although aluminium complexes of **2**, generated under similar conditions, have been isolated and characterised by X-ray crystallography,[6] the low yields and complex nature of this chemistry prompted our seeking an alternative non-aqueous complexation protocol. Thus, addition of a slight excess of diethyl aluminium chloride to **2** dissolved in dry THF resulted in ethane evolution and the formation of a bright yellow precipitate isolated in very high yield following evaporation of the solvent (Scheme 1). The core structure of this material **4** is based upon its homogeneity and the absence of ethyl groups and THF coordinated to aluminium, as revealed by ^{1}H NMR spectroscopy in dry DMSO-d_6.[7] Both the Lewis acidity of aluminium, and the reactivity of the metal–chlorine bond will result in the exact structure in solution being dependent upon the solvent(s) employed. However, the integrity of the O–N–O ligand derived from **2** maintains the desired optical properties, as confirmed following dissolution in EtOH ($\lambda_{exc.}=413$ nm, $\lambda_{em.}=517$ nm).

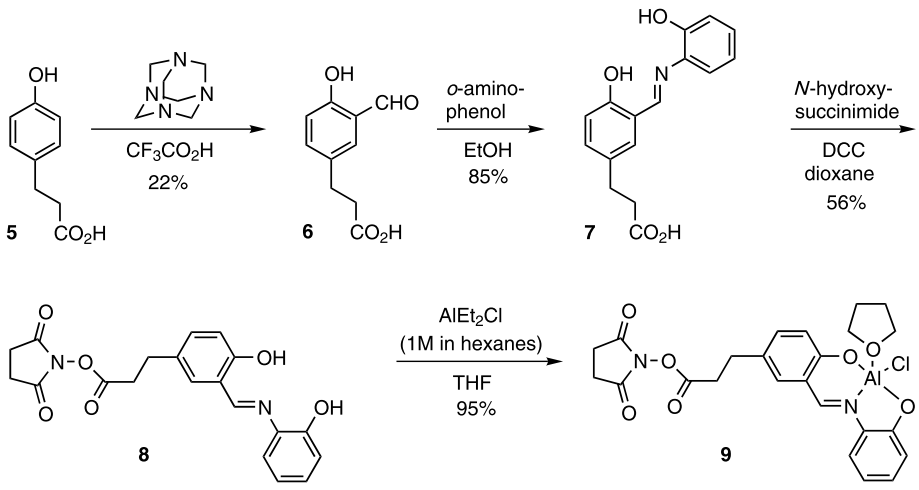
To use the complex resulting from **2** as a biolabel required the introduction of a suitably functionalised tag. As previously demonstrated with **1**, a carboxylic acid is attractive in this respect due to its ease of conversion into a *N*-hydroxysuccinimidyl ester, or related activated ester, promoting reactions with amine containing biomolecules under weakly basic buffered conditions.[8] Commercially available phenol **5** (Scheme 2) was therefore formylated with hexamethylenetetramine[9] to give a mixture of mono- and diformylated phenols. Careful separation by chromatography provided a modest yield of **6** from which Schiff-base **7** was generated with *ortho*-aminophenol by simply refluxing in ethanol. Subsequent DCC-mediated coupling with *N*-hydroxysuccinimide provided the activated ester **8** without interference from the two phenols. Application of the non-aqueous complexation protocol to this ligand again resulted in ethane evolution and formation of **9** as a very bright yellow solid in essentially quantitative yield. In this case, 1 equiv. of coordinated THF was observed by NMR spectroscopy.[10] Dissolution of a sample in EtOH revealed the now characteristic optical properties for these Schiff-base aluminium complexes ($\lambda_{exc.}=419$ nm, $\lambda_{em.}=518$ nm).

The high solubility of **9** in DMSO provides a convenient means of introducing this species into an aqueous environment. Any resulting hydrolysis of the aluminium–chlorine bond is likely to increase water solubility without affecting the integrity of the activated ester. To test the potential of **9** as a biolabel, a stock solution (1 mg/50 μl) was prepared in dry DMSO and added with vigorous mixing to rabbit IgG present in concentrations of 20, 10 and 5 mg/ml of 0.1 M sodium bicarbonate buffer (pH 9.4). Unconjugated dye was separated from the labelled protein by gel permeation chromatography using water as eluant. Proteins labelled with **9** were fluorescent (Fig. 2); proteins labelled with **8** under the same conditions gave no fluorescence emission. Furthermore, addition of anti-rabbit IgG to the labelled protein resulted in immuno precipitation with retention of fluorescence, confirming the retention in protein functionality after labelling.

In summary, we have demonstrated that a Schiff-base ligand containing an activated ester can be readily synthesised. In combination with high yielding non-aqueous aluminium complexation, these procedures provide a novel fluorescent biolabel displaying a large Stokes shift.



Scheme 1.



Scheme 2.

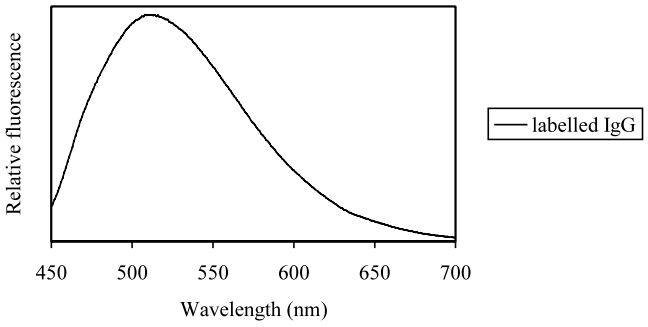


Figure 2. Emission profile of rabbit IgG labelled with **9**.

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7. **4**: ν_{max} (KBr) 1621 cm^{-1} (C=N); ^{1}H NMR (400 MHz, DMSO-d_6) 6.68 (1H, m, Ar*H*), 6.78 (3H, m, Ar*H*), 7.14 (1H, t *J* 7.6, Ar*H*); 7.39 (1H, t *J* 7.5, Ar*H*); 7.55 (1H, d *J* 7.5, Ar*H*); 7.71 (1H, d *J* 8.0, Ar*H*); 9.03 (1H, s, -N=C*H*); ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6) 115.93 (Ar), 118.40 (Ar), 118.68 (Ar), 119.32 (Ar), 122.04 (Ar), 123.16 (Ar), 131.67 (Ar), 134.62 (Ar), 135.77 (Ar), 138.84 (Ar), 158.27 (Ar), 160.45 (Ar), 166.14 (-N=*C*-); *m*/*z* (APCI of sample in DMSO) 595.6 (100%, [**4**$_2$·DMSO–Cl]$^+$).
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10. **9**: ν_{max} (KBr) 1735 (C=O), 1625 cm^{-1} (C=N); ^{1}H NMR (400 MHz, DMSO-d_6) 1.70 (4H, m, -OCH$_2$C*H*$_2$-), 2.78 (4H, s, (C*H*$_2$)$_2$), 2.81 (2H, t *J* 7.2, C*H*$_2$), 2.93 (2H, t *J* 7.20, C*H*$_2$), 3.54 (4H, m, -OC*H*$_2$CH$_2$-), 6.57 (1H, t *J* 7.6, Ar*H*); 6.61 (1H, d *J* 8.3, Ar*H*), 6.63 (1H, d *J* 7.4, Ar*H*), 7.00 (1H, t *J* 7.7, Ar*H*); 7.20 (1H, d *J* 6.4, Ar*H*), 7.31 (1H, s, Ar*H*), 7.53 (1H, d *J* 8.0, Ar*H*), 8.80 (1H, s, -N=C*H*); ^{13}C{^{1}H} NMR (63 MHz, DMSO-d_6) 22.5 (-*C*H$_2$-), 25.7 (-CO-(*C*H$_2$)$_2$CO-), 29.2 (-*C*H$_2$-), 31.4 (-*C*H$_2$-), 32.4 (-*C*H$_2$-), 115.0 (Ar), 115.8 (Ar), 117.9 (Ar), 120.0 (Ar), 121.6 (Ar), 126.7 (Ar), 130.2 (Ar), 133.4 (Ar), 133.8 (Ar), 135.3 (Ar), 156.0 (Ar), 159.7 (Ar), 163.8 (-N=*C*-), 168.8 (*C*=O), 170.5 (-*C*O(CH$_2$)$_2$*C*O-); *m*/*z* (FAB-MNOBA) 631 (16%, **9**+MNOBA–HCl), 281 (100%).