

9-Aminoacridine peptide derivatives as versatile reporter systems for use in fluorescence lifetime assays†

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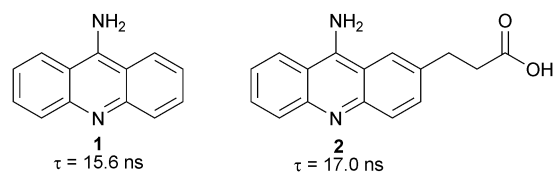
A novel long lifetime fluorescence reporter based on 9-aminoacridine was designed, the lifetime of which can be modulated in a defined manner when in proximity to a tryptophan residue enabling fluorescence lifetime based biochemical assays to be configured.

There is considerable interest in developing biochemical and cell-based assays that use fluorescence lifetime (FLT) as the reporting modality. Fluorescence lifetime is defined as the average time taken for the fluorophore to decay from the excited state to the ground state and, unlike fluorescence intensity, is generally independent of probe concentration and volume, and unaffected by auto-fluorescence, light scattering and inner filter effects.¹ Consequently, the inherent properties of this method should lead to more robust assays and offers key benefits over established approaches. In addition, this technique enables background interference from fluorescent compound libraries and cellular components to be minimised, leading to less false positives in drug screening applications.^{2,3} Such interfering fluorescent molecules typically have fluorescence lifetimes of less than 5 ns, and thus by using reporters with longer fluorescence lifetimes clear discrimination of signal from background can be achieved. However, many fluorophores currently used in biological applications have fluorescence lifetimes in the range 1–5 ns.^{4–7} Dye systems with fluorescence lifetimes greater than 5 ns include pyrene derivatives (>100 ns) and the ruthenium dyes (300–600 ns),⁸ but pyrene dyes are hydrophobic and tend to have poor solubility, whilst the lifetimes of ruthenium dyes are deemed too long for general HTS applications. Consequently, there is a drive to develop fluorophores with long fluorescence lifetimes, in the range 10–25 ns, to enable the broader benefits of fluorescence lifetime assays to be realised. Derivatives of acridone and quinacridone have been developed as fluorophores for fluorescence lifetime studies⁹ and a derivative of acridone (PureTime[®] 14) has recently been utilised in a fluorescence lifetime based biochemical assay,¹⁰ but there are no other

reports of fluorescent dyes with lifetimes in this range being used in such applications.

Thus we sought to develop novel fluorophores with lifetimes in the range 10–25 ns for use as general fluorescence lifetime reporters, and in particular, for the development of fluorescence lifetime assays for drug screening applications. In this regard, the fluorescent properties of 9-aminoacridine (9AA) **1** appeared particularly suited, as it has a reported lifetime of ~16 ns, a quantum yield approaching unity¹¹ and an excitation wavelength (400 nm)¹² compatible with most fluorescence spectrophotometers and plate-readers. As a result, we have focussed our efforts on developing this molecule as a reporter for fluorescence lifetime assays. Through systematic modification of the 9AA core we have generated a derivative of 9AA, 3-(9-aminoacridin-2-yl) propionic acid **2**, that is suitable for the labelling of peptides and proteins and which maintains the excellent fluorescence properties of the parent molecule. Importantly, we have found that the fluorescence lifetime of 9AA-labelled peptides can be modulated, in a defined manner, by placing an aromatic molecule in proximity to the fluorophore within the peptide sequence. This ability to modulate the fluorescence lifetime of 9AA-labelled substrates in a specific fashion has been used to develop a fluorescence lifetime assay for the protease Caspase-3.

In the first instance, the fluorescence properties of **1** were assessed in 10 mM phosphate buffered saline solution pH 7.4 (PBS) and it was shown to have an $E_{x,max}$ of 400 nm and $E_{m,max}$ of 429 and 454 nm, and the fluorescence lifetime was confirmed to be 15.6 ns (Scheme 1 and Fig. 1a, see Fig. S12, ESI† for absorption spectrum). Using the 9AA core as a starting point we undertook a systematic study to generate derivatives that were suitable for fluorescent labelling applications, and in particular, the fluorescent labelling of synthetic peptides and proteins. To facilitate ease-of-use, this new fluorophore should be compatible with the standard chemistries for labelling peptides and proteins and importantly, the fluorescence lifetime and brightness of the parent fluorophore



Scheme 1 Structure and fluorescence lifetime of 9-aminoacridine **1** and 3-(9-aminoacridin-2-yl)propionic acid **2**. Fluorescence lifetimes were measured in PBS with $\lambda_{ex} = 405$ nm.

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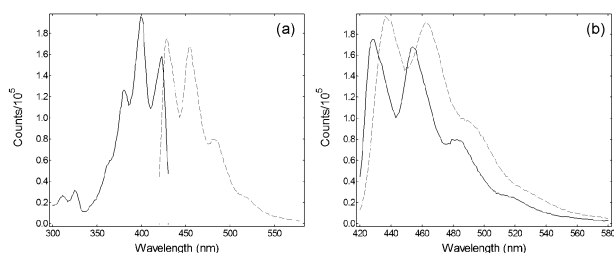
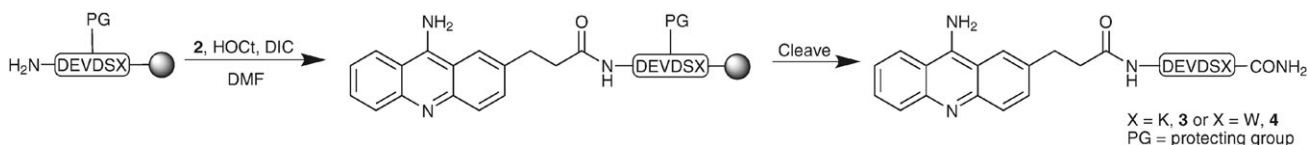


Fig. 1 (a) Excitation (solid) and emission (dash) spectra of **1** (500 nM) in PBS. (b) Emission spectra of **1** (solid) and **2** (dash) ($\lambda_{\text{ex}} = 405 \text{ nm}$).

should be maintained after conjugation to the target molecule. Initial attempts at derivatisation focussed on the 9-amino position but this route proved to be unsuccessful, as modifications at this position produced compounds that were considerably less bright than the free 9AA. Consequently, attention was diverted to the aromatic ring framework. It was shown that functionalisation with electron-withdrawing or electron-donating groups directly onto the aromatic ring had a significant effect on the fluorescence properties of the resultant fluorophore.¹³ These changes in the fluorescent properties upon derivatisation were difficult to predict *a priori*, and thus functionalisation with a methylene group was investigated as a more suitable route to the development of an appropriate fluorescent reporter. Indeed, it was shown that introduction of an alkyl ester group onto the ring structure at the 2-position provided a fluorophore with similar fluorescence properties to 9AA.¹³ This key finding provided a route for developing 9AA into an FLT-reporter suitable for biomolecular labelling and, hence, bioassay applications.

Carboxylic acid functionalities are often incorporated into fluorophores and probes to facilitate the fluorescent labelling of peptides and proteins, as such compounds can be used to label amino functionalities on the target sequence through amide bond formation. Thus, 3-(9-aminoacridin-2-yl)propionic acid (9AA-propionic acid) **2** was synthesised in 6 steps from commercially available starting materials (Fig. S1–S7, ESI†). The fluorescence properties of **2** were measured in PBS and directly compared to **1** and it was observed that the emission profile and intensity of the two derivatives were comparable (Fig. 1b) and the fluorescence lifetime increased from 15.6 ns to 17.0 ns (Scheme 1). Further studies revealed that the fluorescence lifetimes of **1** and **2** were independent of pH over the range 3 to 10 consistent with the $\text{p}K_{\text{a}}$ of the amino group being *circa* 10 (Fig. S13, ESI†).¹⁴ In addition, **2** was found to be readily soluble under aqueous buffered conditions making it particularly suitable for biological applications.

A generic methodology was then developed for the site-specific labelling of synthetic peptides and proteins with this fluorophore using solid-phase peptide synthesis (SPPS) (Scheme 2 and ESI†). This on resin process ensures



Scheme 2 Generic route for the preparation of 9AA-labelled peptides.

chemoselective reaction between the α -amino group of the protected resin-bound peptide and the carboxyl group of the 9AA derivative. The labelling is quantitative for both α -amino functionalities and ϵ -amino groups of lysine residues (data not shown). This approach was used to site specifically label the N^{α} -terminus of the peptide DEVDSK (Scheme 2), which is an excellent substrate for the protease Caspase-3.¹⁵ The peptide was assembled using Fmoc protocols for SPPS, and after labelling and cleavage, the peptide was purified to >98% purity by reverse phase HPLC (Fig. S8 and S9, ESI†). The fluorescence properties of the labelled peptide **3** were then evaluated in PBS and found to be comparable to those of free 9AA (Fig. 2a, Em_{max} 436 and 463 nm, $\tau = 17.7 \text{ ns}$). As a result, 9AA-propionic acid **2** has proven to be an excellent derivative of 9AA **1** for the labelling of biomolecules.

Key to the development of fluorescence lifetime bioassays is the ability to correlate changes in the fluorescence lifetime of the reporter to a specific biological event under study. One method of modulating the fluorescence intensity or fluorescence lifetime of a fluorophore within a polypeptide chain is through intramolecular interaction with proximal aromatic amino acids within the primary sequence.^{16,17} To investigate whether the fluorescence lifetime of 9AA-labelled peptides could be modulated in such a fashion, the lysine residue within the Caspase-3 substrate 9AA-DEVDSK **3** was replaced by tryptophan and the effect on the fluorescence properties of the peptide investigated. The dye labelled peptide, 9AA-DEVDSW **4**, was prepared in an analogous manner to the lysine analogue and obtained in >98% purity (Fig. S10 and S11, ESI†). It was observed that placement of tryptophan in close proximity to 9AA, five residues away within the peptide sequence, resulted in a dramatic decrease in the fluorescence lifetime of 9AA; from 17.7 ns for **3** to 7.6 ns for **4** (Fig. 2b). In addition, a concomitant decrease in the fluorescence intensity by 75% was also observed (Fig. 2a). The observation that tryptophan can be used to modulate the fluorescence lifetime of 9AA-labelled peptides in a specific fashion enables novel bioassays to be configured that use changes in fluorescence lifetime of 9AA as the reporting mechanism. We have utilised this phenomenon to develop a

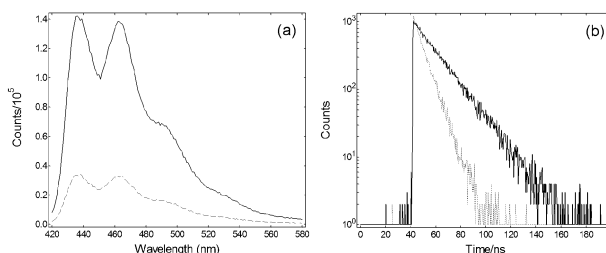


Fig. 2 (a) Fluorescence emission spectra and (b) fluorescence decay spectra of **3** (solid) and **4** (dash) ($\lambda_{\text{ex}} = 405 \text{ nm}$).

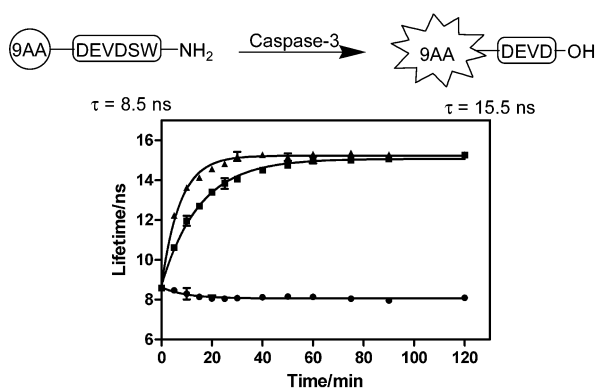


Fig. 3 Real-time monitoring of Caspase-3 mediated cleavage of 9AA-DEVDSW **4** in the absence of enzyme (●), 20 U enzyme (■) and 50 U enzyme (▲). Enzyme was added to a solution of **4** (1 μ M) in 20 mM HEPES buffer pH 7.4 containing 10% sucrose, 0.1% CHAPS, 100 mM NaCl, 1 mM EDTA, 10 mM DTT and the average fluorescence lifetime (at $\lambda_{\text{ex}} = 405$ nm) was measured at time intervals. Reactions were performed in triplicate and error bars show the standard deviation.

fluorescence lifetime assay for Caspase-3, a cysteine protease that plays an essential role in apoptosis.^{18,19} The peptide sequence DEVDSW is an efficient substrate for Caspase-3 (in house data), with cleavage occurring at the amide bond between the -Asp-Ser- residues.¹⁵ It was anticipated that cleavage of **4** by Caspase-3 should alleviate modulation by the tryptophan residue, resulting in a large increase in fluorescence lifetime. To investigate this strategy **4** was employed in a biochemical assay using recombinant Caspase-3 enzyme (Calbiochem). The assay was carried out using 1 μ M substrate **4** in 20 mM HEPES buffer pH 7.4 containing 10% sucrose, 0.1% CHAPS, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, in the presence of either 20 or 50 units of enzyme (100 μ l final volume). The assay mixture was analysed at time intervals using an Edinburgh Instruments NanoTaurus Fluorescence Lifetime Plate Reader. During the progress of the reaction a change in fluorescence lifetime of the reaction mixture was observed, from 8.5 ns to 15.5 ns, indicating that the cleavage of the substrate could be monitored in real time by measurement of the corresponding change in fluorescence lifetime (Fig. 3). The deviation in fluorescence lifetime from that stated previously was a consequence of the buffer system being utilised.

In conclusion, we have designed novel fluorophores based on 9-aminoacridine with long fluorescence lifetimes (17.0 ns), which are suitable for labelling biomolecules in a site-specific manner. Furthermore, the long fluorescence lifetime of the parent fluorophore is maintained after coupling to peptides and proteins, and the lifetime of the 9-aminoacridine labelled peptides can be modulated in a defined fashion through judicious placement of an aromatic moiety within the primary sequence. Consequently, these fluorophores are excellent reporters for fluorescence lifetime applications. To demonstrate their utility in the development of fluorescence lifetime assays, a homogenous assay for monitoring the activity of the

protease Caspase-3 was configured. This was readily achieved by specifically incorporating both 9-aminoacridine and a modulator of its fluorescence lifetime into a peptide substrate for the enzyme. Proteolytic cleavage of the substrate by Caspase-3 is accompanied by a large fluorescence lifetime change (increase), enabling the activity of the enzyme to be monitored in real time by direct measurement of the fluorescence lifetime of the reporter. It is envisaged that this approach should be generally applicable, enabling a variety of fluorescence lifetime protease assays to be developed in a facile manner. We are currently using 9-aminoacridine derivatives in a variety of fluorescence lifetime based applications, in particular, for the development of novel, homogeneous and cost effective assays for HTS applications.²⁰

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