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A latent green fluorescent styrylcoumarin probe for the selective growth and detection of Gram negative bacteria

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Dedicated to the memory of our good friend and colleague, Prof. Rosaleen J. Anderson.

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ABSTRACT

A novel, green fluorescent β-alanylstyrylcoumarin derivative was synthesized and evaluated for its performance as a fluorogenic enzyme substrate on a range of clinically relevant microorganisms. The substrate was selectively hydrolysed by β-alanyl aminopeptidase producing *P. aeruginosa* resulting in an on-to-off fluorescent signal. Growth inhibitory effect of the substrate was observed on Gram positive bacteria and yeasts. Meanwhile, Gram negative species, despite their extremely protective cell envelope, showed ready uptake and accumulation of the substrate within their healthy growing colonies displaying intense green fluorescence.

1. Introduction

Early-stage, low-cost and reliable detection and identification of pathogens in clinical settings is crucial in order to facilitate timely and informed decision-making to initiate appropriate therapy. Among the range of diagnostic methods which are available, chromogenic/fluorogenic culture media (exploiting specific bacterial enzyme activities) are still key components of clinical practice.[1] These techniques are based on the incubation of bacterial isolates in the presence of chromogenic/fluorogenic enzyme substrates which can undergo enzymatic hydrolysis, consequently releasing an optical signal when and only when the microorganism of interest is present. For example, the incubation of the multidrug-resistant respiratory pathogens (*Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Serratia marcescens*) in the presence of the yellow-coloured β-alanyl-pentylresorufamine (β-Ala-PRF) **1** (Scheme 1a), produces purple colonies due to the formation of PRF **2** as a result of the specific β-alanyl aminopeptidase (BAP) activity they produce.[2] Despite such colorimetric methods often being acknowledged as the gold standard, due to their excellent sensitivity and specificity, reliable detection of the colour change against the background requires 24–72 h. However, application of fluorogenic substrates can reduce this waiting time to 6–8 h due to the inherently more sensitive detection of fluorescence. For example, 7-hydroxy-4-methylcoumarin **4** was successfully converted into BAP substrate **3** (by the incorporation of a self-immolative linker) displaying blue fluorescence (λ_{em} 445 nm) upon hydrolysis by BAP within 6 h (Scheme 1b).[3] Meanwhile, blue to yellow shift of the substrate fluorescence before and after enzymatic hydrolysis was achieved by 2-(*N*-β-alanylamino)-10-benzylacridone.[4]

Coumarins are often used as fluorogenic core molecules for the detection of enzymatic processes due to their low toxicity and good cell-permeability as substrates, and their reasonable cell retention, allowing for localized signal release.[5,6] Moreover, sufficiently substituted coumarin-based substrates allow free enzymatic access to the targeting moiety (here, the β-alanyl fragment), which is essential for the hydrolysis and release of the signalling metabolic product.

Designing substrates with hydrolysis products that emit outside of the blueish autofluorescence region of some naturally occurring peptide moieties remains a desired outcome. To enable this desirable red-shift (away from the autofluorescence range) we have designed a coumarin-based fluorophore **9** containing an electron donor diethylamino group, to enhance intramolecular charge transfer (ICT) and a styryl moiety, in order to extend conjugation associated with coumarins. Herein, we report the synthesis (Scheme 2) and evaluation of novel, green fluorescent β-alanylstyrylcoumarin derivative **10** with a green emission wavelength of 519 nm targeting BAP.

2. Synthesis and characterization

The synthetic route to obtain substrate **10** combined a series of well-established methods (Scheme 2); for the synthesis of 7-

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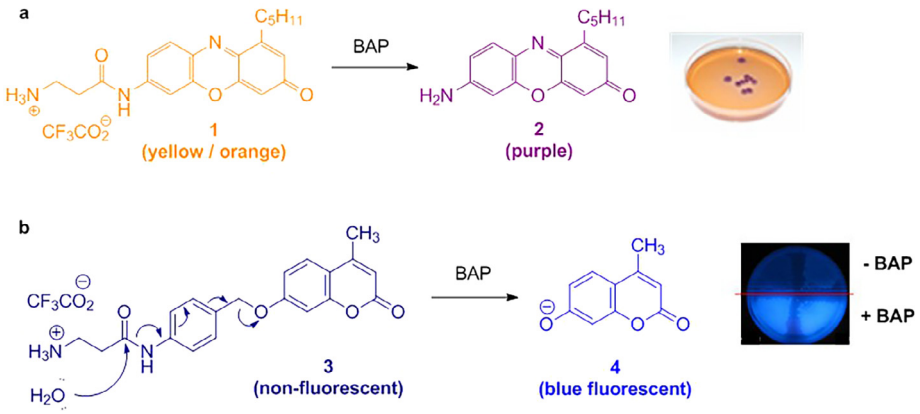
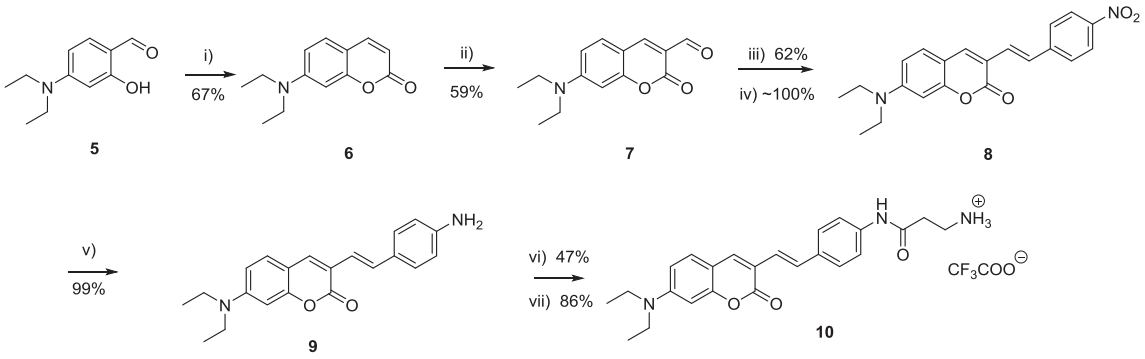
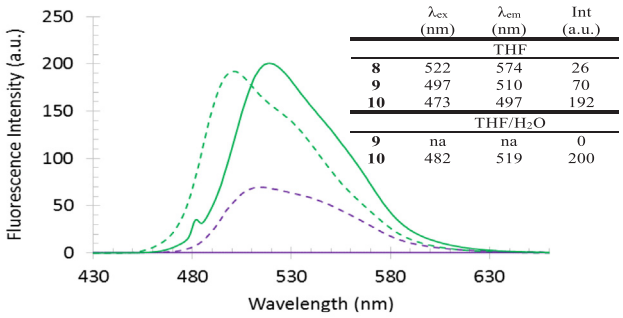


Scheme 1. Detection of BAP producing *P. aeruginosa* using a) yellow coloured β-alanyl PRF **1** resulting in purple colonies upon enzymatic activity; b) non-fluorescent BAP substrate **3** resulting in blue fluorescent colonies upon hydrolysis by BAP.



Scheme 2. Synthetic route for the preparation of substrate **10**. Reagents and conditions: i) methyl (triphenylphosphoranylidene)acetate, 180 °C, 1 hr, inert atm.; ii) DMF, $POCl_3$, 60 °C, 12 hrs, inert atmosphere; iii) 4-nitrobenzyltriphenylphosphonium bromide, CH_3OH, CH_3ONa, rt, 12 hrs; iv) iodine, chloroform, rt, 12 hrs; v) $SnCl_2{\cdot}2H_2O$, EtOAc, 85 °C, 12 hrs. vi) Boc-β-alanine anhydride, DCM, DIPEA, 35 °C, 72 hrs. vii) TFA, DCM, 0 °C, 3 hrs.

diethylaminocoumarin **6**, a Wittig reaction[7] between methyl (triphenylphosphoranylidene)acetate and 4-diethylaminosalicylaldehyde **5** resulted in a significantly higher yield in comparison to the conventional Knoevenagel condensation[8] (see Section 5.1.1). Introduction of the formyl functionality onto position 3 of the coumarin ring (**7**) involved a Vilsmeier-Haack formylation *via* a previously reported procedure.[8] A *para*-nitrostyryl moiety was then introduced into coumarin **7** for the dual purpose of extending the conjugation and facilitating the covalent attachment of the β-alanine enzyme targeting moiety. To achieve this, a Wittig reaction[9] using *p*-nitrobenzyl triphenylphosphonium ylide gave higher yield of **8** than the condensation between *p*-nitrophenylacetic acid and coumarin **7** (see Section 5.1.2).[5,10] The approx. 1:0.8 mixture of (*E*)- and (*Z*)-7-diethylamino-3-(4′-nitrostyryl) coumarin **8** obtained was converted into the sole (*E*)-isomer product *via* isomerization in the presence of iodine.[11] To enable attachment of β-alanine (the *N*-terminal recognition site for BAP), reduction of the nitro group was carried out (using stannous chloride dihydrate) to give amine **9**. This was followed by amide bond formation[12] and consequent Boc-deprotection to obtain BAP substrate **10** as a TFA salt.[13] This cationic form of the substrate is proven to facilitate water solubility and cell permeability.[14] In vitro fluorescence of the nitro **8**, amine **9**, and amide **10** derivatives were firstly recorded in THF at a concentration of 1×10^{-4} M (Fig. 1 and Fig. S1). A change in the substituent at the *para*-styryl position, from the strongly electron withdrawing nitro group in **8** to the electron donating amine in **9** and amide in **10**, resulted in the blue shift of the emission wavelength from 574 to 510 nm and 497 nm, respectively. A significant increase in emission intensity was also observed from $-NO_2$ **8** < $-NH_2$ **9** < amide **10**. In aqueous THF 1:1 mixtures fluorescence emission of both nitrostyrylcoumarin **8** and amino derivative **9** were quenched, while substrate **10** displayed an



	λ_{ex} (nm)	λ_{em} (nm)	Int (a.u.)
		THF	
8	522	574	26
9	497	510	70
10	473	497	192
		THF/H_2O	
9	na	na	0
10	482	519	200

Fig. 1. Emission of amine **9** (purple) in THF (dotted line) (λ_{ex}: 497 nm), and in THF:water 1:1 (solid line); Emission of substrate **10** (green) in THF (dotted line) (λ_{ex}: 473 nm), and THF:water 1:1 (solid line) (λ_{ex}: 482 nm).

intense green fluorescence, with an emission maximum of 519 nm (Fig. 1). In general, the bright fluorescence of 7-dialkylamino-coumarins is a result of excited state ICT enhanced by the strongly electron donating dialkylamino group.[15] However, especially in polar solvents, when a strongly electron withdrawing group (such as nitro) is conjugated via position 3 of the coumarin ring, the enhanced push–pull effect of the EDG-EWG substituents stabilizes this charge separation in the excited state, thus opening up the possibility of non-radiative twisted intramolecular charge transfer (TICT) decay and consequently resulting in the weakening of the fluorescence emission intensity, as is observed in the case of the nitro derivative **8**.[16] The strength of the push–pull effect across the coumarin ring has been reported to narrow the HOMO-LUMO gap, thus resulting in a red shift of the emission wavelength. Although, amine **9** and substrate **10** both have electron