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

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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.

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

Keywords: Fluorescent enzyme substrate; Selective growth inhibitor; Gram negative detection; Styrylcoumarin

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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** (**Figure 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 hours. However, application of fluorogenic substrates can reduce this waiting time to 6-8 hours 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 hours (**Figure 1b**).[3]

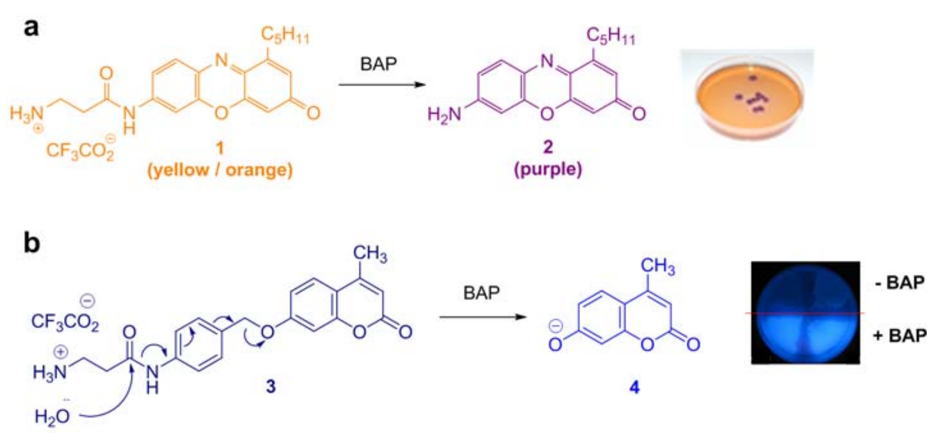
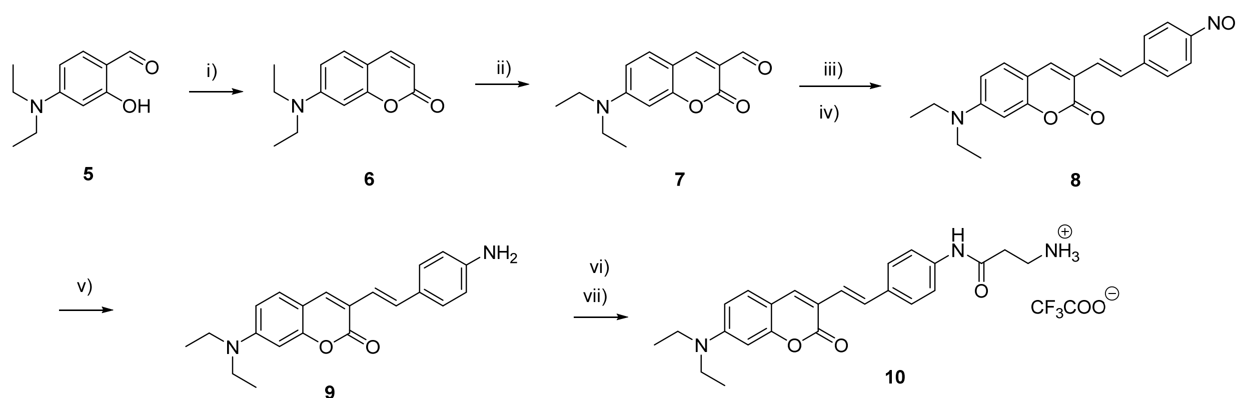


Figure 1. Detection of BAP producing *P. aeruginosa* using a) yellow coloured β-alanyl PRF **1** resulting in purple colonies upon enzymatic activity; b) non-fluorescent BAP substrate **3** resulting in blue fluorescent colonies upon hydrolysis by BAP.

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.[4, 5] 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.



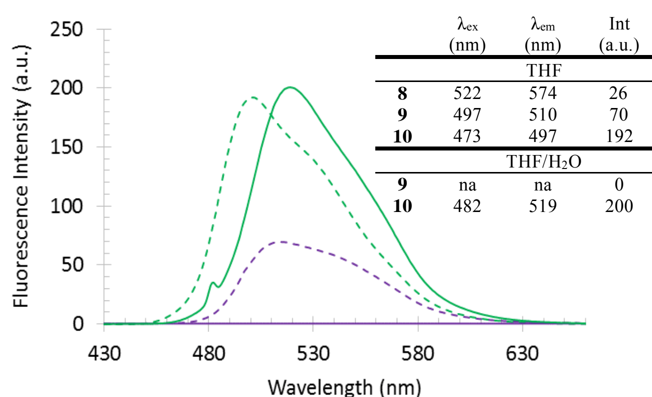
Scheme 2. Synthetic route for the preparation of substrate **10**. Reagents and conditions: i) methyl (triphenylphosphoranylidene)acetate, 180 °C, 1 hr, inert atm.; ii) DMF, $POCl_3$, 60 °C, 12 hrs, inert atmosphere; iii) 4-nitrobenzyltriphenylphosphonium bromide, CH_3OH, CH_3ONa, rt, 12 hrs; iv) iodine,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

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 1**) and evaluation of novel, green fluorescent β-alanylstyrylcoumarin derivative **10** with a green emission wavelength of 519 nm targeting BAP.

Synthesis and characterization

The synthetic route to obtain substrate **10** combined a series of well-established methods (**Scheme 1**); for the synthesis of 7-diethylaminocoumarin **6**, a Wittig reaction[6] between methyl (triphenylphosphoranylidene)acetate and 4-diethyl-aminosalicylaldehyde **5** resulted in a significantly higher yield in comparison to the conventional Knoevenagel condensation[7] (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.[7] 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[8] 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.).[4, 9] The approx. 1:1 mixture of (*Z*)- and (*E*)-7-diethylamino-3-(4-nitrostyryl)coumarin **8** obtained was converted into the sole (*E*)-isomer product *via* isomerization in the presence of iodine.[10] To enable attachment

of β-alanine (the *N*-terminal recognition site for BAP), reduction of the nitro group was carried out (using stannous chloride) to give amine **9**. This was followed by amide bond formation[11] and consequent Boc-deprotection to obtain BAP substrate **10** as a TFA salt.[12] This cationic form of the substrate is proven to facilitate water solubility and cell permeability.[13] *In vitro* fluorescence of the nitro **8**, amine **9**, and amide **10** derivatives were firstly recorded in THF at a concentration of 1 x 10^{-4} M (**Figure 2** and **Figure 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 intense green fluorescence, with an emission maximum of 519 nm (**Figure 2**). In general, the bright fluorescence of 7-dialkylamino-coumarins is a result of excited state ICT enhanced by the strongly electron donating dialkylamino group.[14] 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**.[15] 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 donating substituents, amides are much less electron donating than amines, hence they displayed rather different fluorescence properties. The above ICT process was described between the 7-diethylamino and coumarin carboxyl moieties. When the styryl-amino lone pair is occupied in the amide resonance structures of **10**, bright green fluorescence is displayed. However, in amine **9**, the freely available amino lone pair is presumed to reduce the probability of ICT, resulting in an 'isolated' diethylamino rotor, and allowing for non-radiative decay pathways (**Figure 3**).[5] Due to these observed properties, instead of the often-exploited fluorescence quenching by the amide bond-formation,[16] substrate **10** would provide 'on-to-off' signalling of BAP activity.



	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

Figure 3. 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).

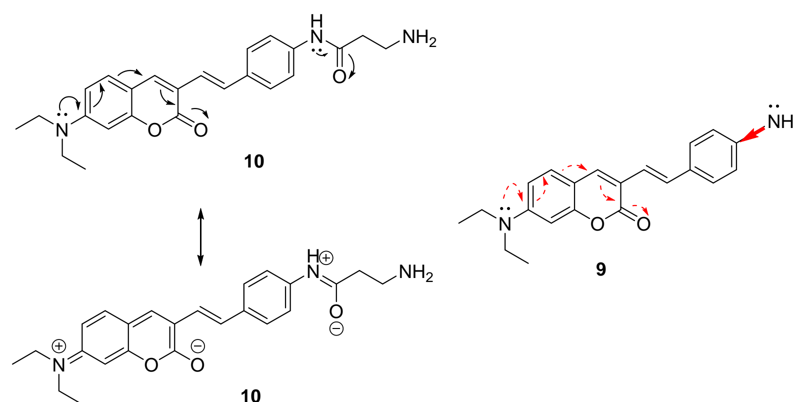


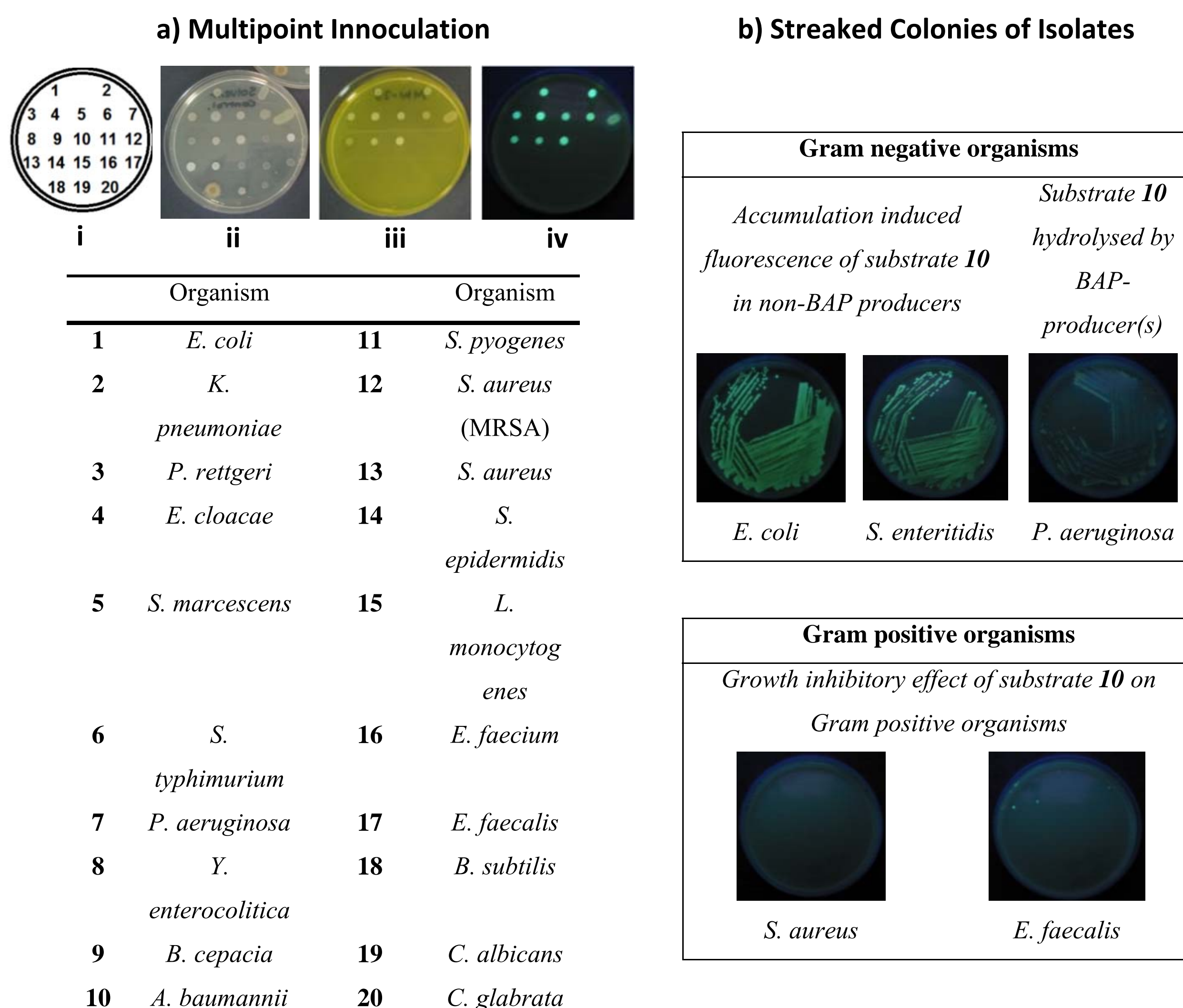
Figure 4. Proposed resonance structures resulting in ICT in substrate **10**, and inhibition of ICT in amine **9.**

Biological evaluation

Substrate **10** was incorporated into both solid agar-based and liquid growth media for their incubation with microorganisms. The agar plates were subjected to *in vivo* evaluation on both multispot inoculation and individually streaked clinical isolates of commonly encountered pathogens. For the multispot inoculation, 10 Gram negative (spots no. 1-10), 8 Gram positive bacteria (spots no. 11-18), and 2 yeasts (spots no. 19-20) (**Figure 4a**) were selected as representative cell lines for the clinical evaluation. Colonies of Gram negative *E. coli*, *S. enteritidis*, *P. aeruginosa*, and Gram positive *S. aureus*, and *E. faecalis* were streaked on individual plates (**Figure 4b**, **Figure S2**). There are two apparent effects of the incorporation of substrate **10** into the growth media; the growth of all Gram positive bacteria and yeast species was inhibited, while the Gram negative species formed colonies (**Figure 4a-iii**, **Table S1**), and secondly, Gram negative cell lines displayed intense green fluorescence as a result of accumulating substrate **10** within the colonies (**Figure 4a-iv**). Moreover, as was suggested by the *in vitro* fluorescence studies in aqueous solution (**Figure 2**), BAP producing *P. aeruginosa* displayed non-fluorescent colonies due to the hydrolysis of substrate **10** to non-fluorescent metabolic product **9** (**Figure 4b**). This change provides a ready visual differentiation between BAP and non-BAP producing Gram negative species.

Preliminary results in liquid growth media confirmed these results described above; the pathogens were the Gram negative non-BAP producing *E. coli*, BAP producing *S. marcescens*, *B. cepacia*, and *P. aeruginosa*, as well as Gram positive *S. aureus*. The pathogens were incubated, in the absence and presence, of substrate **10** in a well plate for 20 hours before determining optical densities (OD) by measuring the increase in absorbance intensity at 690 nm, and the fluorescence intensity increase (λ_{ex} 365 nm, λ_{em} 460 nm) (**Table S2**, **Figure S3**). OD values confirmed the growth inhibitory effect of the substrate on *S. aureus*, along with weak suppression of OD of Gram negative *S. marcescens* and *P. aeruginosa* (**Figure S3a**). Substrate **10** exhibited

a background fluorescence as measured in the blank control wells containing no microorganisms (**Figure S3b**). Non-BAP producing *E. coli*, and BAP producing *S. marcescens* displayed increased green fluorescence within the time frame of the test. Although *S. marcescens* and *B. cepacia* express BAP, its activity is significantly lower than that of *P. aeruginosa*.[17] Substrate **10** is thus virtually unhydrolysed by *S. marcescens*, and partly hydrolysed by *B. cepacia* within the time frame of this study. The expression of BAP by *P. aeruginosa* is apparent through the quenched fluorescence of substrate **10** *via* its hydrolysis to non-fluorescent metabolic product **9**. Wells inoculated with *S. aureus* displayed equal fluorescence intensity to that of the blank wells, confirming that there was no growth of these Gram positive microorganisms.



	Organism		Organism
1	*E. coli*	**11**	*S. pyogenes*
2	*K. pneumoniae*	**12**	*S. aureus* (MRSA)
3	*P. rettgeri*	**13**	*S. aureus*
4	*E. cloacae*	**14**	*S. epidermidis*
5	*S. marcescens*	**15**	*L. monocytogenes*
6	*S. typhimurium*	**16**	*E. faecium*
7	*P. aeruginosa*	**17**	*E. faecalis*
8	*Y. enterocolitica*	**18**	*B. subtilis*
9	*B. cepacia*	**19**	*C. albicans*
10	*A. baumannii*	**20**	*C. glabrata*

Figure 5. a) Multispot inoculation Columbia agar plates with isolates after 20 hours of incubation. i) Numbered spots correlating to species named in the table and ii) corresponding microorganisms in the absence of substrate **10**; iii) in the presence of substrate **10** (50 mg/L) under VIS light, and iv) under 365 nm light source; b) Streaked Columbia agar plates containing substrate **10** (50 mg/L) under UV light (365 nm) after 20 hours incubation in the presence of (from top left to bottom right): Gram negative non-BAP producing *E. coli* and *S. enteritidis*, BAP producing *P. aeruginosa*, and Gram positive *S. aureus* (inhibited) and *E. faecalis* (inhibited).

Although substrate **10** was metabolized by the targeted enzyme, BAP, it displayed on-to-off rather than the more desirable off-to-on fluorescence. However, the combination of selective growth (enrichment) of Gram negative organisms, and the display of intense green fluorescence upon accumulation by viable colonies provides substrate **10** with a highly sought after dual role. These attributes exhibited by the same enzyme substrate can assist the every-day clinical task of isolating and identifying multi-drug resistant Gram negative pathogens. Furthermore, Gram negative bacteria exhibit greater resistance to most known antibiotics due to a combination of the effect of their complex outer membrane providing low permeability, and their multidrug efflux pumps deterring the intracellular accumulation of drugs.[18] Thus, the combination of cellular uptake and accumulation of substrate **10** by Gram negative species can be exploited as a lead towards identifying molecular structural requirements for both intracellular uptake and retention by Gram negative species.

Conclusion

In conclusion, a novel β-alanylstyrylcoumarin aminopeptidase substrate **10** was synthesized and characterized. Substrate **10** displayed intense green fluorescence (outside the inherent autofluorescence region) upon localization within Gram negative bacterial colonies, while inhibited the growth of all tested Gram positive bacteria and yeasts. Thus, this compound has the potential to be exploited as a selective enrichment agent and a detection tool at the same time. Selective detection of BAP activity (specific to *P. aeruginosa*) was achieved *via* fluorescence quenching, instead of the more desired off-to-on signal generation. More significantly, the substrate was taken up and retained by Gram negative colonies. This is an essential requirement for both diagnostic enzyme substrates for the localization of the detection signal, and for therapeutic agents to reach and act on their intracellular targets. Additionally, exchange of the β-alanine targeting moiety for other amino acids, or the modification of the fluorescent coumarin allow for the design of customized agents for the detection, selective growth, or advanced cellular uptake of other microorganisms.

Experimental

Synthetic chemistry

All solvents and reagents were obtained from Sigma-Aldrich (Castle Hill, Sydney, Australia) and used without any further purification or treatment. The THF for the fluorescence measurements specifically contained no fluorescent stabilizers. Thin layer chromatography was performed on silica gel plates from Grace Davison Discovery Sciences (US). Column chromatography was performed on a Grace Reveleris X2. ^{1}H and ^{13}C NMR spectra were obtained on a Varian 400MR at 400 MHz and 100 MHz, respectively. Coupling constants (J) are in Hertz (Hz), and chemical shifts (δ) are expressed in parts per million (ppm) and

reported relative to residual solvent peaks. Melting points were recorded on a Stuart Scientific SMP 10. Infrared spectra were obtained on Shimadzu FTIR IR Tracer-100 and Shimadzu FTIR-8400S spectrophotometers.

Excitation and emission spectra were obtained on a Shimadzu RF-5301PC spectrophotometer. Microwave reactions were performed on CEM Discover. Low resolution mass spectra were obtained on Thermo Scientific TSQ Quantum Access Max LCMS/MS & TLX1 Turboflow Chromatography System in positive ion mode. High resolution mass spectra were generated on Bruker 7T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR) in positive ion mode.

*Synthesis of 7-diethylaminocoumarin **6***

Method A: 4-Diethylaminosalicylaldehyde **5** (0.773 g, 4 mmol) was dissolved in ethanol (30 mL), then 1,8-diazabicycloundec-7-ene (DBU) (1.5 mL) and diethylmalonate (1.5 mL) were added. The mixture was stirred for 6 hours at 80 °C. The ethanol was removed under vacuum and glacial acetic acid (20 mL) and concentrated HCl (37%) (20 mL) were added to the reaction, which was stirred overnight at 75 °C. The reaction mixture was cooled to room temperature and poured into ice water (50 mL). Aq. NaOH solution (5 mol/L) was added dropwise to the mixture to adjust the pH of the solution to 5. The precipitate formed was filtered, and washed with water to give a carboxylic acid intermediate (0.314 g, 36 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.25 (6H, t, J = 7.2 Hz, 2 × CH_3), 3.48 (4H, q, J = 7.2 Hz, 2 × CH_2), 6.51 (1H, d, J = 2.4 Hz, Ar-H), 6.70 (1H, dd, J = 8.8, 2.4 Hz, Ar-H), 7.44 (1H, d, J = 8.8 Hz, Ar-H), 8.64 (1H, s, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.4, 45.3, 96.8, 105.6, 108.6, 110.9, 131.9, 150.3, 153.8, 158.0, 164.5, 165.5; LRMS (ESI) *m/z* 262 (MH^+). This carboxylic acid intermediate (0.314 g, 1.45 mmol) was then further reacted with glacial acetic acid (5 mL) and HCl (5 mL) at 120 °C overnight. The reaction was cooled to room temperature and poured over ice water (50 mL). Aq. NaOH solution (5 mol/L) was added to the mixture dropwise to adjust pH of the solution to 5. The precipitate formed was filtered, and washed with water to give product **6** as a dark brown solid (0.158 g, 18 %).[7]

Method B: A mixture of 4-diethylaminosalicylaldehyde **5** (1 g, 5.17 mmol) and methyl (triphenylphosphoranylidene)acetate (2 g, 5.96 mmol) was heated at 180 °C under nitrogen for 1 hour. The mixture was cooled to room temperature. On the obtained solid mixture column chromatography was performed using hexane : ethyl acetate (10 to 15% ethyl acetate) to give product **6** as a light orange solid (0.749 g, 67 %).[6] Mp 87-88 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.20 (6H, t, J=7.2 Hz, 2 × CH_3), 3.40 (4H, q, J=7.2 Hz, 2 × CH_2), 6.02 (1H, d, J=9.6 Hz, Ar-H3), 6.48 (1H, d, J=2.4 Hz, Ar-H8), 6.56 (1H, dd, J=8.8, 2.4 Hz, Ar-H6), 7.23 (1H, d, J=8.8 Hz, Ar-H5), 7.52 (1H, d, J=9.6 Hz, Ar-H4); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.4 (2 × CH_3), 44.8 (2 × CH_2), 97.6 (CH), 108.3 (quat.), 108.6 (CH), 109.2 (CH), 128.7 (CH), 143.7 (CH), 150.7 (quat.), 156.7 (quat.), 162.3 (C=O); IR (υ_{max}/cm^{-1}): 1701 (C=O); LRMS (ESI) *m/z* 218 (MH^+); HRMS (ESI) *m/z* found: MNa^+, 240.0997; Calcd. for $C_{13}H_{15}NO_2Na^+$, 240.0995.

*Synthesis of 7-diethylaminocoumarin-3-carbaldehyde **7***

Anhydrous DMF (6 mL) was added dropwise to $POCl_3$ (6 mL) in an ice water bath and the mixture was stirred for 30 minutes under nitrogen. 7-Diethylaminocoumarin **6** (1.5 g, 6.91 mmol) was dissolved in DMF (15 mL) and the solution was then added dropwise to the above solution. The mixture was stirred at 60 °C overnight under nitrogen, and poured into ice water(50 mL). Aq. NaOH solution (5 mol/L) was added dropwise to adjust the pH of the mixture to about 6, resulting in a precipitate which was filtered to give product **7** as an orange solid (0.993 g, 59 %).[7] Mp 162-164 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.25 (6H, t, *J*=7.2 Hz, 2 × CH_3), 3.47 (4H, q, *J*=7.2 Hz, 2 × CH_2), 6.48 (1H, d, *J*=2.4 Hz, Ar-H8), 6.63 (1H, dd, *J*=8.8, 2.4 Hz, Ar-H6), 7.40 (1H, d, *J*=8.8 Hz, Ar-H5), 8.24 (1H, s, Ar-H4), 10.12 (1H, s, CHO); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.4 (2 × CH_3), 45.3 (2 × CH_2), 97.2 (CH), 108.3 (quat.), 110.2 (CH), 114.4 (quat.), 132.5 (CH), 145.3 (CH), 153.4 (quat.), 158.9 (CH), 161.9 (C=O), 187.9 (CHO); IR (υ_{max}/cm^{-1}): 1701 (C=O), 1670 (CHO); LRMS (ESI) *m/z* 246 (MH^+); HRMS (ESI) *m/z* found: MNa^+, 268.0946; Calcd. for $C_{14}H_{15}NO_3Na^+$, 268.0944.

*Synthesis of (E)-7-diethylamino-3-(4-nitrostyryl)coumarin **8***

Method A: A mixture of 7-diethylaminocoumarin-3-aldehyde **7** (245 mg, 1 mmol), 2-(4′-nitrophenyl)acetic acid (207 mg, 1.15 mmol) and piperidine (196 mg, 2.3 mmol) was stirred for 1.5 hours at 140 °C under reflux. The reaction mixture was cooled to room temperature then ethanol (15 mL) was added and sonicated for 10 minutes. The mixture was then stirred overnight and the precipitate was filtered under vacuum, washed with ethanol (3 mL) and hexane (3 mL) resulting in coumarin **8** as a dark red solid (7 mg, 2 %).[4]

Method B: A mixture of 7-diethylaminocoumarin-3-aldehyde **7** (50 mg, 0.204 mmol), 2-(4-nitrophenyl)acetic acid (40.9 mg, 0.226 mmol), imidazole (21 mg, 0.308 mmol), piperidine (26.2 mg, 0.308 mmol) and ethylene glycol (0.6 mL) was stirred under microwave irradiation at i) 150W, 160 °C) or ii) 300W, 160 °C for 30 minutes. Both reaction mixtures were cooled to room temperature and acidified with HCl (1 mol/L) to adjust the pH to 5. The aqueous solution was then extracted with ethyl acetate (2- mL), which was separated and dried over sodium sulfate. The organic layer was evaporated, ethanol (10 mL) was added and sonicated for 90 minutes to form a precipitate, which was then filtered under vacuum, washed with ethanol (3 mL) and hexane (3 mL) resulting in a dark red solid **8** (5 mg, 7 %).[9]

Method C: *Synthesis of 4-nitrobenzyltriphenylphosphonium bromide:* 4-Nitrobenzyl bromide (3 g, 13.89 mmol) was dissolved in toluene (50 mL), then triphenylphosphine (3.643 g, 13.89 mmol) was added and the mixture was heated at 120 °C overnight. The product was filtered under vacuum, washed with hexane (50 mL) to give a fine white powdered solid (6.333 g, 13.18 mmol, 95 %).[19] Mp 273-274 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.99 (2H, d, *J*=15.6 Hz, Ar-H), 7.45 (2H, dd, *J*=7.2, 2.8 Hz, Ar-H), 7.56-7.84 (17H, m, Ar-H); LRMS (ESI) *m/z* 398 (M^+); HRMS (ESI) *m/z* found: M^+, 398.1306; Calcd. for $C_{25}H_{21}NO_2P^+$, 398.1304.

Synthesis of (E)-7-diethylamino-3-(4-nitrostyryl)coumarin ***8****:* In a round bottom flask fitted with drying tube, 7-diethylaminocoumarin-3-aldehyde **7** (0.8 g, 3.2 mmol) and 4-nitrobenzyltriphenylphosphonium bromide (1.872 g, 3.92 mmol) were dissolved in dry methanol (80 mL) and then CH_3ONa (0.96 g, 17.6 mmol) was added. The mixture was stirred at room temperature overnight and was then poured into ice water (160 mL), and stirred for 2 hours at room temperature. The solid was filtered under vacuum and triturated with ethanol (3 × 100 mL) resulting in a mixture of the (*E)* and (*Z)* isomers of **8** (0.743 g, 62.5%). This mixture of (*E)* and (*Z)*-**8** (743 mg, 2.036 mmol) was dissolved in chloroform (60 mL) in a round bottom flask fitted with drying tube then iodine (103.43 mg, 0.204 mmol) was added and the reaction mixture was stirred at room temperature overnight. The solution was washed thoroughly with saturated sodium metabisulfite solution (5 × 20 mL). The organic layer was dried over sodium sulfate and evaporated under reduced pressure to give the product (*E)*-**8** as a dark red solid (743 mg, 100 %).[8, 10] Mp 259-260 °C (lit. 253-255 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.23 (6H, t, *J*=7.2 Hz, 2 × CH_3), 3.44 (4H, q, *J*=7.2 Hz, 2 × CH_2), 6.51 (1H, d, *J*=2.4 Hz, Ar-H8), 6.62 (1H, dd, *J*=8.8, 2.4 Hz, Ar-H6), 7.19 (1H, d, *J*=16 Hz, CH_A or CH_B), 7.31 (1H, d, *J*=8.8 Hz, Ar-H5), 7.59 (1H, d, *J*=16 Hz, CH_A or CH_B), 7.61 (2H, d, *J*=8.8 Hz, Ar-H2′,6′), 7.71 (1H, s, Ar-H4), 8.19 (2H, d, *J*=8.8 Hz, Ar-H3′,5′); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.5 (2 × CH_3), 45.0 (2 × CH_2), 97.2 (CH), 108.9 (quat.), 109.4 (CH), 116.4 (quat.), 124.1 (CH-2′,6′ or CH-3′,5′), 126.8 (CH-2′,6′ or CH-3′,5′), 127.6 (CH), 128.0 (CH), 129.3 (CH), 140.5 (CH), 144.4 (CH), 146.5 (quat.), 151.0 (quat.), 156.0 (quat.), 161.0 (C=O); IR (υ_{max}/cm^{-1}): 1705 (C=O), 1616 (C=C), 1519 (NO_2), 1331 (NO_2); LRMS (ESI) *m/z* 365 (MH^+); HRMS (ESI) *m/z* found: M^+, 364.1421 and MH^+, 365.1500; Calcd. for $C_{21}H_{20}N_2O_4$, 364.1418 and for $C_{21}H_{21}N_2O_4^+$, 365.1451.

Synthesis of (E)-3-(4-aminostyryl)-7-(diethylamino) coumarin ***9***

(*E*)-7-Diethylamino-3-(4-nitrostyryl)coumarin **8** (200 mg, 0.549 mmol) and $SnCl_2{\cdot}H_2O$ (1.23 g, 5.49 mmol) in ethyl acetate (10 mL) was refluxed overnight. Saturated Na_2CO_3 was added to adjust the pH to 10. Ethyl acetate (20 mL) was added to the mixture which was then filtered through celite, and washed with ethyl acetate (60 mL). The organic layer was dried over sodium sulfate and evaporated under vacuum resulting in the product **9** as a dark orange solid (182 mg, 99 %). [4] Mp 188-192 °C (lit. 166-168 °C); ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ: 1.13 (6H, t, *J*=7.2 Hz, 2 × CH_3), 3.44 (4H, q, *J*=7.2 Hz, 2 × CH_2), 5.33 (2H, broad s, NH_2), 6.54 (1H, d, *J*=2.4 Hz, Ar-H8), 6.57 (2H, d, *J*=8.8 Hz, Ar-H3′,5′), 6.71 (1H, dd, *J*=8.8, 2.4 Hz, Ar-H6), 6.79 (1H, d, *J*=16.4 Hz, CH_A or CH_B), 7.23 (2H, d, *J*=8.4 Hz, Ar-H2′,6′), 7.29 (1H, d, *J*=16.4 Hz, CH_A or CH_B), 7.43 (1H, d, *J*=9.2 Hz, Ar-H5), 7.93 (1H, s, Ar-H4); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ: 13.0, 44.7, 97.0, 109.3, 109.9, 114.6, 117.9, 118.0, 125.6, 128.1, 129.6, 130.8, 137.3, 149.5, 150.5, 155.4, 161.2; IR (υ_{max}/cm^{-1}): 3456 (NH_2), 3360 (NH_2), 1686 (C=O), 1605 (C=C); LRMS (ESI) *m/z* 335 (M^+); HRMS (ESI) *m/z* found: M^+, 334.1677 and MH^+, 335.1756; Calcd. for $C_{21}H_{22}N_2O_2$, 334.1676 and for $C_{21}H_{23}N_2O_2^+$, 335.1754.

Synthesis of (E)-3-(4-β-alanylstyryl)-7-(diethylamino) coumarin trifluoroacetate ***10***

Boc-β-alanine (508.97 mg, 2.69 mmol) was added to dicyclohexylcarbodiimide (610.53 mg, 2.959 mmol) dissolved in DCM (10 mL) and stirred in an ice bath for 3 hours. The reaction mixture was filtered and the filtrate was added into a mixture of (*E*)-3-(4-aminostyryl)-7-(diethylamino)coumarin **9** (300 mg, 0.897 mmol) and diisopropyl ethyl amine (DIPEA) (197.08 mg, 1.525 mmol) in DCM (20 mL). The mixture was heated at 35 °C over 72 hours. Aqueous HCl (1 mol/L, 10 mL) was added and the organic layer was washed with saturated $NaHCO_3$ solution (10 mL) then dried over sodium sulfate and filtered under reduced pressure to give a yellow solid (214 mg, 47 %).[11] Mp 216-218 °C; ^1H NMR (400 MHz, DMSO-*d6*) δ: 1.14 (6H, t, *J*= 7.2 Hz, 2 × CH_3), 1.39 (9H, s, 3 × CH_3), 2.47-2.52 (2H, m, CH_2-C or CH_2-D), 3.23 (2H, m, *J*=6.8, 6.0 Hz, CH_2-C or CH_2-D), 3.45 (4H, q, *J*=7.2 Hz, 2 × CH_2), 6.56 (1H, d, *J*=2.4 Hz, Ar-H8), 6.73 (1H, dd, *J*=6.8, 2.4 Hz, Ar-H6), 6.86 (1H, t, *J*=5.2 Hz, NH), 7.02 (1H, d, *J*=16.4 Hz, CH_A or CH_B), 7.42 (1H, d, *J*=16.4 Hz, CH_A or CH_B), 7.45-7.48 (3H, m, 3 × Ar-H), 7.61 (2H, d, *J*=8.8 Hz, Ar-H3′,5′), 8.03 (1H, s, Ar-H4), 10.01 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-*d6*) δ: 13.0, 28.9, 37.1, 37.4, 44.7, 78.2, 96.9, 109.1, 110.0, 117.0, 119.9, 122.5, 127.3, 129.5, 129.9, 132.8, 139.4, 150.9, 155.8, 156.2, 161.0, 170.0; IR (υ_{max}/cm^{-1}): 1709 (C=O), 1667 (C=O), 1616 (C=C), 1593 (amide I), 1508 (amide II); LRMS (ESI) *m/z* 506 (MH^+); HRMS (ESI) *m/z* found: M^+, 505.2573 and MH^+, 506.2607; Calcd. for $C_{29}H_{35}N_3O_5$, 505.2571 and for $C_{29}H_{36}N_3O_5^+$, 506.2605.

The obtained (*E*)-3-(4-*tert*-butoxycarbonylamino-propanamidostyryl)-7-(diethylamino)coumarin (150 mg, 0.297 mmol) was dissolved in DCM (10 mL) on an ice bath. A mixture of trifluoroacetic acid (2 mL) and DCM (2 mL) was added dropwise. The reaction was then allowed to warm to room temperature and stirred for 3 hours. The reaction mixture was concentrated *in vacuo* then methanol (2 × 10 mL) and the mixture was concentrated *in vacuo* (for the removal of residual TFA). The reaction mixture was triturated with diethyl ether twice and the solid was filtered and washed with diethyl ether (20 mL) resulting in product **10** as an orange solid (132 mg, 86 %).[12] Mp 222-226 °C; ^1H NMR (400 MHz, DMSO-*d6*) δ: 1.14 (6H, t, *J*= 7.2 Hz, 2 × CH_3), 2.72 (2H, t, *J*= 6.8 Hz, CH_2-C or CH_2-D), 3.11 (2H, t, *J*=6.8 Hz, CH_2-C or CH_2-D), 3.45 (4H, q, *J*=7.2 Hz, 2 × CH_2), 6.56 (1H, d, *J*=2.4 Hz, Ar-H8), 6.74 (1H, dd, *J*=8.8, 2.4 Hz, Ar-H6), 7.03 (1H, d, *J*=16.4 Hz, CH_A or CH_B), 7.41-7.51 (4H, m, CH_A or CH_B and 3 × Ar-H), 7.62 (2H, d, *J*=8.8 Hz, Ar-H3′,5′), 7.76 (3H, broad s, NH_3^+), 8.03 (1H, s, Ar-H4), 10.23 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-*d6*) δ: 12.8, 33.8, 35.4, 44.6, 96.8, 108.9, 109.9, 116.8, 119.8, 122.6, 127.3, 129.2, 129.8, 133.0, 138.8, 139.3, 150.8, 155.6, 160.8, 168.8; IR (υ_{max}/cm^{-1}): 3325 (NH_3^+), 1697 (C=O), 1670 (C=C), 1585 (amide I), 1508 (amide II); LRMS (ESI) *m/z* 406 (M^+); HRMS (ESI) *m/z* found: M^+, 406.2126; Calcd. for $C_{24}H_{28}N_3O_3^+$, 406.2125.

***In vitro* fluorescence properties**

The concentration of stock solution for (*E*)-3-(4′-nitrostyryl)-7-(diethylamino)coumarin **8**, (*E*)-3-(4′-β-alanylstyryl)-7-(diethylamino)coumarin trifluoroacetate **10** and (*E*)-3-(4′-aminostyryl)-7-(diethylamino)coumarin **9** was 2 × 10^{-4} mol/L in THF. 2 mL of THF, or 2 mL water was added to 2 mL of

the stock solution of each compound, respectively. The slit width of fluorescence spectrophotometer was set at 3 mm.

Biological evaluation

Solid agar media

Preparation of culture media containing substrate. Columbia agar was prepared as follows; 41 g of Columbia agar (Oxoid Basingstoke, UK) was added to deionized water and the volume was made up to 1 L. The medium was sterilized by autoclaving at 116 °C for 20 minutes and left to cool at 50 °C. 2 mg of the substrate to be tested was initially dissolved in 100 μL of *N*-methylpyrrolidone and this was added to Columbia agar (made up to 20 mL), then poured into sterile Petri dishes to give a final concentration of 100 mg/L for the substrates. Columbia agar incorporating an equivalent concentration of *N*-methylpyrrolidone was used as a growth control.

Microbial suspension preparation. Microbial reference strains were obtained from either the National Collection of Type Cultures (NCTC) or the National Collection of Pathogenic Fungi (NCPF) which are both located at the Central Public Health England Laboratory, Colindale, UK or the American Type Culture Collection (ATCC), Manassas, USA. The 20 test microorganisms were maintained on Columbia agar.

Multipoint inoculation. Colonies of each microbial strain were harvested using a loop from overnight cultures on Columbia agar. These were suspended in sterile deionized water to a suspension equivalent to 0.5 McFarland units using a densitometer. 100 μL of this suspension was pipetted into the corresponding wells of a multipoint inoculation device. Each set of plates received 1 μL of bacterial suspension, giving 1.5×10^5 organisms per spot on each inoculation. Twenty strains were inoculated per plate and the plates were incubated for 18 hours in air at 37 °C.

Activity determination. After incubation, the activity of the microorganisms with the test substrates was determined by observing the plates under UV irradiation at 365 nm and comparing with the substrate-free control.

Liquid media

For the evaluation of substrates **8**, **9**, and **10** in liquid media, N-methyl-2-pyrrolidinone (NMP) was purchased from ACROS ORGANICS. Trypcase Soy Broth (TSB BIOMERIEUX ref. 42 100) and Suspension Medium BIOMERIEUX (ref. 70 640) were used. Microplate reader TECAN Infinite M-200 was used to record fluorescence (365/460 setting) and absorption (at 690 nm for organism density). After a 20 hour period of incubation of the assessed strains (Table S2 and S3) on GREINER 96 well plate (ref. 655 090) sealed with transparent GREINER viewseal (ref. 676 070). For **8**, **9** and **10**, a 10 μL solution of substrate (at 50g/L in NMP) was suspended in 5 mL of TSB (final concentration 100 mg/L). Every well was filled with 100 μL of specified solution of substrate and 100 μL of bacterial suspension at 0.5 McFarland for a final

concentration of 0.25 McFarland (about 7.5 × 10^7 bacteria/mL). For control, in the absence of enzyme substrates the wells were filled with 100 µL TSB and 100 µL of bacterial suspension at 0.5 McFarland. For negative control (blank), the wells were filled with 100 µL TSB in the absence or presence of any of the substrates and 100 µL Suspension Medium. The absorption (at 690 nm for microbial growth) and relative fluorescent intensities (at λ_{ex}= 365 nm / λ_{em}= 460 nm) for enzymatic activity were recorded after a period of 20 hours.

Acknowledgments

PWG, DEH, and JDP would like to thank the National Health and Medical Research Council (NHMRC) for funding.

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