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β-Alanyl aminopeptidase-activated fluorogenic probes for the rapid identification of *Pseudomonas aeruginosa* in clinical samples

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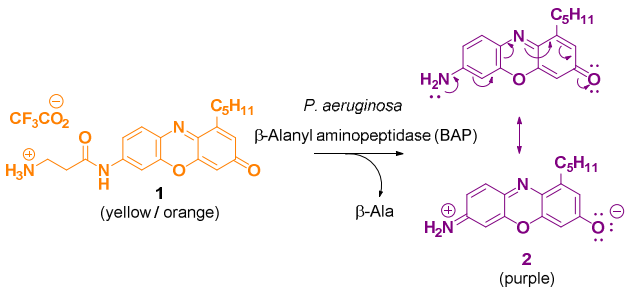
The fluorogenic self-immolative substrates 8 are specifically hydrolyzed by β-alanyl aminopeptidase, resulting in a 1,6-elimination and the release of the highly fluorescent hydroxycoumarins 6. 7-{4-(β-Alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin trifluoroacetate 8b has advantages over another fluorogenic substrate, 7-*N*-β-alanylamino-4-methylcoumarin 9, as it is retained by bacterial colonies in solid agar applications, and results in similar times to detection, stronger fluorescence intensities, and no decrease in signal over time in liquid media. Although 7-{4-(β-alanylamido)}benzyloxy-4-methylcoumarin trifluoroacetate 8a produces a weaker signal than substrate 8b, its use allowed better discrimination between the BAP producers *P. aeruginosa* (positive) and *S. marcescens* (negative).

Introduction

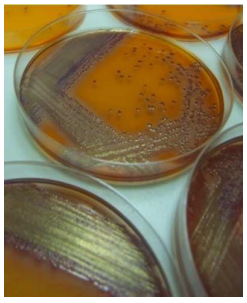
The Gram negative aerobic bacterium *Pseudomonas aeruginosa* is the 6th most common causative pathogen in healthcare-associated infections (HAI), being responsible for around 20% of hospital acquired diseases. In immunosuppressed patients, such as burns victims, and those with cancer or AIDS, as few as 10-100 *P. aeruginosa* cells can lead to gut colonisation.[1] *P. aeruginosa* is the major determinant of morbidity and mortality in cystic fibrosis patients, and 80% of adult patients harbour mucoid strains which even aggressive antibacterial therapy may be unable to eradicate.[2] It has been reported that the nosocomial mortality associated with *P. aeruginosa* bloodstream infections is greater than 20%, and is highest among patients who have received inappropriate initial empirical antimicrobial treatment, thus rapid directed antibiotic therapy is imperative.[3]

In addition to its simple nutritional requirements and ability to grow in the absence of oxygen and over a wide temperature range, *P. aeruginosa* has the largest and most complicated bacterial genome (6.3 M base pairs), which contains a large number of genes for the catabolism, transport, and efflux of many antibacterial drug classes, resulting in extensive antibacterial resistance.[4]

Early and rapid detection of *P. aeruginosa* is essential to prevent its transformation into the extremely hard to treat mucoid phenotype. Early identification would also help to elicit the maximum benefit from directed antibacterial therapy, involving combinations of an aminoglycoside with a β–lactam (penicillin or cephalosporin) or with a carbapenem and fluoroquinolone.



(a)



(b)

Figure 1. (a) The principle behind the chromogenic detection of *Pseudomonas aeruginosa* colonies and **(b)** the specific BAP-catalyzed hydrolysis of substrate **1**, liberating the purple 1-pentylresorufamine **2** (picture courtesy of Larissa Laine, Freeman Hospital, Newcastle upon Tyne, UK).

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Chromogenic and fluorogenic enzyme substrates are widely used for the identification of bacteria due to their excellent sensitivities and specificities, ease of use and interpretation, cost effectiveness, and lack of requirement for instrumentation. We have previously reported the use of media containing 7- and 8-aminophenoxazinones for the detection of *P. aeruginosa*; the conversion of 7-*N*-β-alanyl-1-pentylresorufamine (7-*N*-β-alanyl-1-PRF) **1** to 1-PRF **2** is catalyzed by β-alanyl aminopeptidase (BAP), an enzyme which is specific to this organism, Figure 1.[5,6] Due to its excellent specificity and sensitivity, the medium containing 7-*N*-β-alanyl-1-PRF **1** now forms the basis of a commercial diagnostic test (chromID™ *P. aeruginosa*),[7] which can be used, for example, for the isolation of *P. aeruginosa* from the sputa of patients with cystic fibrosis.[8] One drawback with chromogenic media in general is the time taken for the generation of the coloured signal (usually 24-48 hours), while the 7-*N*-β-alanyl-1-pentylresorufamine **1** also imparts an orange background colour to the chromogenic medium, Figure 1b. A further drawback with the use of these substrates is the relatively lengthy synthetic route to appropriately substituted amino-substituted phenoxazinones. We sought to address these limitations in the current work through a convenient synthesis of fluorogenic substrates which target the same BAP recognition site as the chromogenic substrate **1**, and should result in greatly reduced times to detection due to their significantly enhanced detection sensitivities (detection methods based on fluorescence are more than 2 orders of magnitude more sensitive than those based upon chromogenic processes).[9]

Results and Discussion

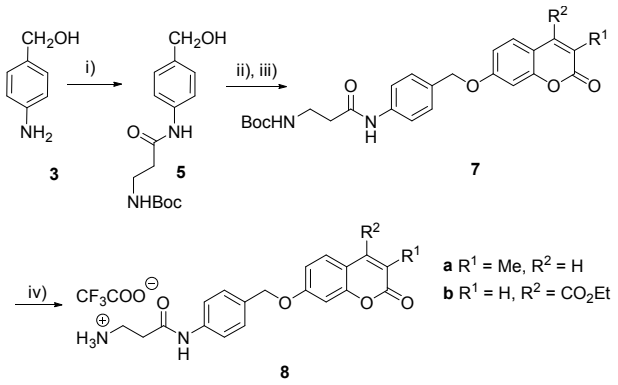


Figure 2. Synthesis of self-immolative BAP substrates **8**. Reagents and conditions: i) Boc-β-Ala **4**, DIPEA, HOBt, Et_3N, THF, 0 °C, N_2; ii) DIPEA, MsCl, DCM, 0 °C, N_2; iii) **6**, K_2CO_3, DCM; iv) TFA, DCM.

As can be seen in Figure 1, BAP activity usually liberates β-alanine and an amine. We have synthesised two novel fluorogenic substrates **8a,b** which would result in the release of a fluorescent phenol reporter upon BAP-catalyzed hydrolysis by linking β-alanine to a hydroxycoumarin **6** *via* a *para*-aminobenzylalcohol-based linker. One of the advantages of these substrates is their straightforward synthesis, which involves the initial coupling of Boc-β-alanine **4** with *para*-aminobenzylalcohol **3** to give alcohol **5**. The conversion of the primary hydroxyl group to a better leaving group (OMs or Cl), followed by coupling to the phenoxide form of the 7-hydroxycoumarin **6**, and deprotection of the Boc-β-Ala-linker-hydroxycoumarins **7**, gave the trifluoroacetate salts **8**, Figure 2.

The specific hydrolysis of these substrates at the β-alanine amide bond is followed by a 1,6-elimination and the self-immolative[10] loss of a *p*-aminobenzylidene fragment, resulting in the release of the 7-hydroxycoumarin derivatives **6**, Figure 3. These coumarins have pKa values of 7.8 (**6a**)[11] and 7.3 (**6b**),[‡] so the latter will exist to a greater extent in the phenoxide form at neutral pH, and thus exhibit stronger fluorescence.[12] It is this generation of a phenoxide **6** which results in the retention of the fluorescence within the bacterial colonies, Figure 4. As can be seen from Figure 4 and Table 1, the only microorganisms which generate the characteristic fluorescence when grown on an agar medium containing substrate **8b** are the known BAP producers, *P. aeruginosa*, *Burkholderia cepacia* and *Serratia marcescens,* Table 1. In contrast, the BAP-catalyzed hydrolysis of 7-*N*-β-alanylamino-4-methylcoumarin **9** generates 7-amino-4-methylcoumarin (7-AMC) **10**, which is not retained by bacterial colonies and spreads into the agar medium, thus complicating the detection of *P. aeruginosa* in the presence of other microorganisms.

Having demonstrated the specificity of an agar medium containing substrate **8b** for the detection of BAP producers, we next examined the behaviour of this substrate in liquid media in a microtiter plate arrangement. A range of *E. coli*, *P. aeruginosa* and *S. marcescens* strains were incubated with substrates **8a,b** and **9**.[§] Within the microplate reader arrangement, the chosen cell lines were incubated at a final microorganism concentration of 0.25 McFarland in Trypcase Soy Broth in the presence of the substrates at 50mg/L.

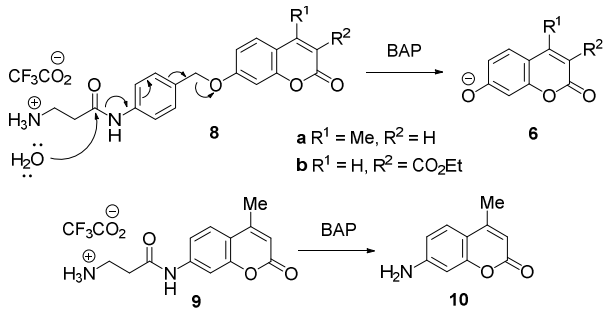


Figure 3. The principle behind the chromogenic detection of *Pseudomonas aeruginosa* colonies as a result of BAP-catalyzed hydrolysis of the self-immolative β-alanyl aminopeptidase substrate **8** or 7-*N*-β-alanylamino-4-methylcoumarin **9**, generating the strongly fluorescent hydroxycoumarins **6** or 7-amino-4-methylcoumarin (7-AMC) **10**.

To ensure satisfactory growth, the cell density within the wells was determined by measuring the absorption at 660 nm over 24 hours; no growth inhibition was observed in the presence of any of the substrates (Figure S2a-14a). After an initial lag phase of 10-20 cycles (1 cycle = 15 mins), presumably caused by stress, all strains grew and multiplied, with no significant differences between the wells in the absence, or presence, of the substrates.

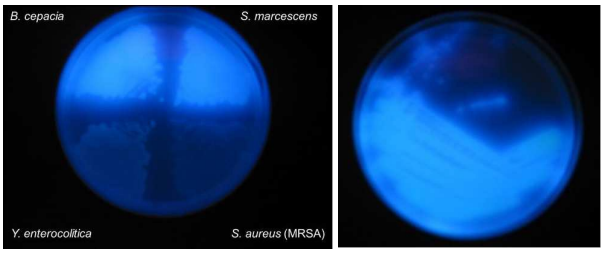
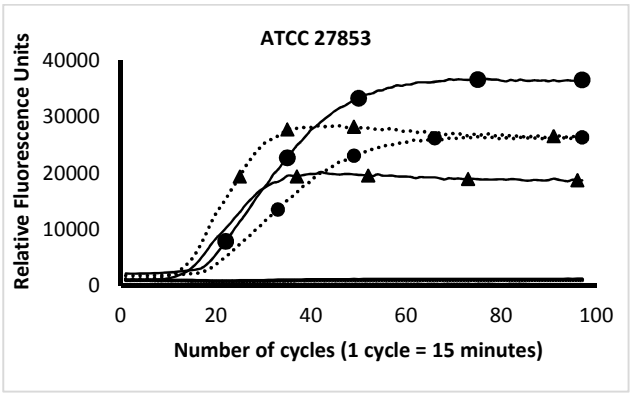


Figure 4. Fluorescence generated on media containing substrate **8b** only by BAP producers: **(a)** *B. cepacia* and *S. marcescens;* **(b)** *P. aeruginosa*.

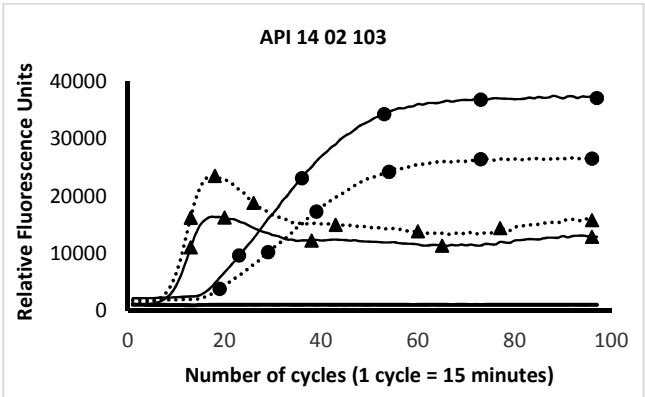
Table 1. Clinically relevant Gram positive, Gram negative, and yeast microorganisms (1.5×10^5 CFU/spot) grown for 18h on an agar medium containing substrate **8b** (NG, no growth; +, moderate growth or fluoresence, ++, strong growth or fluorescence).

Organism	Growth	Fluorescence
E. coli	NG	-
K. pneumoniae	NG	-
P. rettgeri	++	-
E. cloacae	++	-
S. marcescens	++	++ (blue)
S. typhimurium	++	-
P. aeruginosa	++	++ (blue)
Y. enterocolitica	++	-
B. cepacia	++	+ (blue)
A. baumannii	++	-
S. pyogenes	+	-
MRSA	+	-
S. aureus	+	-
S. epidermidis	+	-
L. monocytogenes	+	-
E. faecium	+	-
E. faecalis	+	-
B. subtilis	+	-
C. albicans	+	-
C. glabrata	+/-	-

The relative fluorescence signal intensity originating from each well was recorded over 24 hours, at 15 minute intervals, at two sets of excitation and emission wavelengths. Differences in the emission intensity were observed; β-Ala-7-AMC **9** gave more sensitive results at an excitation wavelength of 365 nm and an emission wavelength of 440 nm, while substrate **8b** exhibited greater sensitivity upon excitation at 375 nm and emission at 445 nm, for fluorophore **6a** the intensities were essentially identical at both sets of wavelengths, Figure 5 (and Figure S3-14). No fluorescence was observed in any of the control wells in the absence of strains and / or in the absence of any substrates. All β-alanyl aminopeptidase producers (apart from a *S. marcescens* strain; ATCC 264) displayed fluorescence during the course of these studies, while the control organism, *E. coli*, generated no fluorescence, as expected. Substrate **8a** was hydrolyzed by all BAP producers, but the fluorescence intensities were lower than for both substrates **8b** and **9** over the entire kinetic runs (presumably as a result of its higher pKa and so lower rate of ionisation in the test wells). One advantage of the use of this substrate (**8a**) is its discrimination between *P. aeruginosa* (positive) (Figure S4, S8-10, S12-14) and some *S. marcescens* (negative) (Figure S6, S7) strains. The fluorescence measurements at 365 nm (ex) / 440 nm (em) showed β-Ala-7-AMC **9** and substrate **8b** to be equally reliable in the detection of BAP activity (after 24 hours), with the emission intensities reaching the same levels by the end of the kinetics. The fluorescence signal from the hydrolysis of β-Ala-7-AMC **9** declined over the time period of the kinetics (presumably due to self-quenching/photobleaching), and this could lead to false negative results. There was no decline over time in the fluorescence resulting from the hydrolysis of substrate **8b**.



(a)



(b)

Figure 5. Kinetic data for the release of fluorophores **8b** (●) and **9** (▲) in liquid media in the presence of **(a)** *P. aeruginosa* ATCC 27853; **(b)** *P. aeruginosa* API 14 02 103 (bioMérieux strains collection). Fluorescence was recorded at 365 nm (ex) / 440 nm (em) (dotted line) and 375 nm (ex) / 445 nm (em) (solid line).

However, when the emission was detected at 445 nm after excitation at 375 nm, substrate **8b** resulted in significantly greater fluorescence intensities than those from β-Ala-7-AMC **9**, with the additional benefit of a more persistent signal (no self-quenching or photobleaching), Figure 5. Even the time to detection of the fluorescence generated by these substrates was comparable; the signal was slightly slower to develop for substrate **8b** in some cases, but only by a maximum of 10 cycles (2.5 hours), which would not result in any significant deficiencies in a clinical setting.

Experimental

Materials and Methods

All reagents and solvents were purchased from Sigma-Aldrich, Alfa Aesar, and ChemSupply, and used without any further purification or treatment. Compound **6b** was prepared as previously reported.[13] Thin layer chromatography was performed on Grace Reveleris® Silica Aluminum-backed TLC Plates (UV254). ^{1}H and ^{13}C NMR spectra were acquired on a Varian 400MR at 400 MHz and 100 MHz, respectively. Coupling constants (*J*) are in Hertz (Hz), chemical shifts (δ) are expressed in parts per million (ppm) and reported relative to residual solvent peaks. Low resolution mass spectra were obtained on TSQ Quantum Access Max (triple quadrupole) LCMS/MS in positive ion mode. High resolution mass spectra were obtained on a Bruker 7T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR), also in positive ion mode. Infrared spectra were recorded on Shimadzu FTIR-8400S and Shimadzu IRTracer-100 spectrometers. Elemental analyses were performed by the Campbell Microanalytical Laboratory at the University of Otago, NZ.

Synthetic Procedures

4-(Boc-β-Alanylamido)benzyl alcohol 5. To a solution of 4-aminobenzyl alcohol **3** (1 g, 8 mmol) in anhydrous THF (150 mL), hydroxybenzotriazole hydrate (HOBt, 1.08 g, 8 mmol), Boc-β-alanine-OH **4** (1.51 g, 8 mmol), and EDAC hydrochloride (1.9 g, 10 mmol) were added sequentially, at 0 °C. Triethylamine (2.2 mL, 16 mmol) was then added and the resulting solution was stirred at room temperature overnight. Further portions of triethylamine (2.2 mL) were added until completion of the reaction. The solvent was then removed, the residue was taken up in ethyl acetate (150 mL) and washed with water (150 mL), 1N HCl (50 mL), and then saturated aq. $NaHCO_3$ (50 mL). The organic layer was dried over Na_2SO_4 and concentrated under vacuum. Column chromatography, eluting with hexane : ethyl acetate (1:2), gave the desired product **5** as an off-white solid (1.45 g, 61%); mp 110-118 ^{o}C; (found C, 61.50; H, 7.68; N, 9.62. $C_{15}H_{22}N_2O_4$ requires C, 61.21; H, 7.53; N, 9.52 %); ν_{max}/cm^{-1} 3354 (NH), 3324 (NH), 1689 (amide I), 1528 (amide II); ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 1.37 (9H, s, CMe_3), 2.45 (2H, t, *J* = 7.2 Hz, CH_2-2'), 3.20 (2H, q, *J* = 7.2 Hz, CH_2-3'), 4.42 (2H, d, *J* = 5.2 Hz, CH_2OH), 5.08 (1H, t, *J*= 5.6 Hz, NH carbamate), 6.86 (1H, t, *J* = 5.2 Hz, OH), 7.22 (2H, d, *J* = 8.4 Hz, H-2,6), 7.53 (2H, d, *J* = 8.4 Hz, H-3,5), 9.88 (1H, s, NH amide); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 28.7 (3 × CH_3, CMe_3), 36.9 (CH_2), 37.1 (CH_2), 63.0 (CH_2OH), 78.0 (quat., *C*Me$_3$), 119.2 (2 × ArCH), 127.3 (2 × ArCH), 137.6 (quat.), 138.2 (quat.), 156.0 (quat., C=O), 169.6 (quat., C=O); MS (ESI) *m/z* 317 (MNa$^+$).

7-{4-(Boc-β-Alanylamido)}benzyloxy-4-methylcoumarin 7a. To a solution of *N*-(*N'-tert*-butoxycarbonyl-β-alanyl)-4-aminobenzyl alcohol **5** (1 g, 3.4 mmol) in dry DCM (40 mL) under an inert atmosphere at -10 °C, DIPEA (0.52 g, 4.0 mmol) was added, followed by the dropwise addition of methanesulfonyl chloride (0.423 g, 3.7 mmol). The resulting reaction mixture was stirred for 3 hours at 0 °C. After completion, the reaction was quenched by pouring into a mixture of ice and conc. HCl (100 mL) then the separated organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give an off-white oily solid, which was used without further purification in the next step. To a stirred solution of 4-methylumbelliferone **6a** (0.63 g, 3.6 mmol) and K_2CO_3 (1.15 g, 7 mmol) in dichloromethane, a solution of the mesylate (3.4 mmol) was added dropwise. The reaction mixture was stirred at room temperature overnight. After completion of the reaction, the organic layer was washed with water (5 × 25 mL), and brine (30 mL). The organic residues were purified by column chromatography on silica, eluting with hexane: ethyl acetate (1:2), to give 7-{4-(Boc-β-alanylamido)}benzyloxy-4-methylcoumarin **7a** as a white solid (0.66 g, 43%); mp 204.9-207.1 ^{o}C; (found C, 66.45; H, 6.37; N, 6.19. $C_{25}H_{28}N_2O_6$ requires C, 66.36; H, 6.24; N, 6.19 %); (found MNa$^+$, 475.1842. Calc. for $C_{25}H_{28}N_2O_6$Na: MNa, 475.1839); ν_{max}/cm^{-1} 2960, 2927, 2860 (NH), 1737 (C=O), 1670 (C=O), 1516 (NH), 1224 (C-O), 1149 (C-O); ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 1.37 (9H, s, *CMe$_3$*), 2.39 (3H, d, *J* = 1.2 Hz, CH_3), 2.47 (2H, t, *J* = 7.2 Hz, CH_2-2'), 3.21 (CH_2, q, *J*= 7.2 Hz, CH_2-3'), 5.15 (2H, s, OCH_2), 6.20 (1H, d, *J* = 1.2 Hz, H-3), 6.84 (1H, br, NH, carbamate), 7.01 (1H, dd, *J* = 8.8 and 2.8 Hz, H-6), 7.05 (1H, d, *J* = 2.8 Hz, H-8), 7.39 (2H, d, *J*= 8.8 Hz, 2 × ArH), 7.61 (2H, d, *J*= 8.8 Hz, 2 × ArH), 7.68 (1H, d, *J* = 8.8 Hz, H-5), 9.97 (1H, s, NH, amide); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 18.5 (CH_3), 28.7 (CH_3, *CMe$_3$*), 36.9 (CH_2-3'), 37.2 (CH_2-2'), 70.1 (CH_2, OCH_2), 78.1 (quat., *C*Me$_3$), 102.1 (CH, C-8), 111.6 (CH, C-3), 113.2 (CH, C-6), 113.7 (quat., C-4), 119.5 (2 × CH, C-3',5'), 126.9 (CH, C-5), 129.0 (2 × CH, C-2',6'), 131.1 (quat., C-4'), 139.5 (quat., C-1'), 153.8 (quat., C-4a), 155.1 (quat., C-8a), 155.9 (quat., C=O, carbamate), 160.55 (quat., C-2), 161.8 (quat., C-7), 169.9 (quat., C=O, amide); MS (ESI) *m/z* 475 (MNa)$^+$, 353 (M-^{t}Boc)$^+$.

7-{4-(Boc-β-Alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin 7b. *N*-(*N'-tert*-Butoxycarbonyl-β-alanyl)-4-aminobenzyl alcohol **5** (0.71 g, 2.4 mmol) was dissolved in dry dichloromethane (20 mL) under an inert atmosphere and cooled in an acetone-ice bath. DIPEA (0.37 g, 2.88 mmol) was added, followed by the dropwise addition of methanesulfonyl chloride (0.33 g, 2.88 mmol) and the reaction mixture was stirred for 2 hours at 0 °C, then poured onto an ice-conc. HCl mixture (50 mL). After separation, the organic layer was added to a stirred solution of **6b** (0.4 g, 1.71 mmol) and K_2CO_3 (1.41 g, 8.55 mmol) in dichloromethane. The reaction mixture was stirred at room temperature overnight and after completion of the reaction, the organic layer was washed with water (3 × 15 mL). The resulting organic layer was dried over Na_2SO_4 and evaporated under vacuum. The solid residue obtained was recrystallised from ethyl acetate to give 7-{4-(Boc-β-alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin **7b** as a pale yellow solid (0.42 g, 48 %); mp 196.8-199.6 ^{o}C; (found C, 63.52; H, 5.92; N, 5.49. $C_{27}H_{30}N_2O_8$

requires C, 63.26; H, 5.90; N, 5.41 %); (found: MNa^+, 533.1896. Calc. for $C_{27}H_{30}N_2O_8Na$: MNa, 533.1894); ν_{max}/cm^{-1} 2922 (NH), 2850 (NH), 1743 (C=O), 1683 (C=O), 1602 (NH), 1521 (NH), 1373, 1217 (C-O), 1176 (C-O); ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 1.30 (3H, t, J = 7.2 Hz, CH_3CH_2), 2.47 (2H, t, J = 6.8 Hz, CH_2-2'), 3.21 (2H, q, J = 7.2 Hz, CH_2-3'), 4.27 (2H, q, J = 7.2 Hz, CH_2CH_3), 5.18 (2H, s, OCH_2), 6.84 (1H, br, NH, carbamate), 7.06 (1H, dd, J = 8.8 and 2.8 Hz, H-6), 7.10 (1H, d, J = 2.8 Hz, H-8), 7.40 (2H, d, J = 8.8 Hz, H-3',5'), 7.62 (2H, d, J = 8.8 Hz, H-2',6'), 7.84 (1H, d, J = 8.8 Hz, H-5), 8.71 (1H, s, H-4), 10.00 (1H, s, NH, amide); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 14.6 (CH_3), 28.7 (CH_3, CMe_3), 36.9 (CH_2-3'), 37.2 (CH_2-2'), 61.4 (CH_2, OCH_2CH_3), 70.5 (CH_2, OCH_2), 78.1 (quat., CMe_3), 101.6 (CH, C-8), 111.9 (quat.), 113.9 (quat., C-8a), 114.3 (CH, C-6), 119.5 (2 × CH, C-3',5'), 129.2 (2 × CH, C-2',6'), 130.8 (quat., C-1'), 132.1 (CH, C-5), 139.7 (quat., C-4'), 149.6 (CH, C-4), 155.9 (quat., C=O, carbamate), 156.7 (quat., C-3), 157.3 (quat., C-4a), 163.3 (quat., C=O, CO_2Et), 164.2 (quat., C-7), 169.9 (quat., C=O, amide); MS (ESI) m/z 533.5 $(MNa)^+$, 511 $(MH)^+$.

7-{4-(β-Alanylamido)}benzyloxy-4-methylcoumarin trifluoroacetate 8a. To a solution of 7-{4-(Boc-β-alanylamido)}benzyloxy-4-methylcoumarin **7a** (0.2 g, 0.46 mmol) in methanol, trifluoroacetic acid was added in two portions (2× 5 mL). Upon completion of the reaction the excess TFA was removed *via* repeated co-evaporation with methanol (5 × 10 mL). The resulting residue was triturated with cold diethyl ether to give 7-{4-(β-alanylamido)}benzyloxy-4-methylcoumarin trifluoroacetate **8a** as a white crystalline solid (0.123 g, 56%); mp 200.0-202.8 °C; (found C, 55.88; H, 4.54; N, 5.90. $C_{22}H_{21}F_3N_2O_6 \bullet 0.5H_2O$ requires C, 55.58; H, 4.66; N, 5.89 %); (found: MH^+, 353.1500. Calc. for $C_{20}H_{21}N_2O_4$: MH, 353.1496); ν_{max}/cm^{-1} 3055-2968 ($^+NH_3$), 1695 (C=O), 1662 (C=O), 1606 (NH), 1516 (NH), 1199 (C-O), 1188 (C-O); ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 2.40 (3H, s, CH_3), 2.70 (2H, t, J = 6.4 Hz, CH_2-2'), 3.09 (2H, t, J= 6.4 Hz, CH_2-3'), 5.17 (2H, s, OCH_2), 6.22 (1H, s, H-3), 7.02 (1H, d, J = 8.8 Hz, H-6), 7.06 (1H, d, J = 2.4 Hz, H-8), 7.42 (2H, d, J = 8.4 Hz, H-3',5'), 7.62 (2H, d, J = 8.4 Hz, H-2',6'), 7.67 (3H, br, NH_3), 7.69 (1H, d, J = 8.8 Hz, H-5), 10.20 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 18.6 (CH_3), 33.7 (CH_2), 35.4 (CH_2), 70.0 (OCH_2), 102.1 (CH, C-8), 111.7 (CH, C-3), 113.2 (CH, C-6), 113.7 (quat., C-4), 119.6 (2 × CH, C-3',5'), 126.9 (CH, C-5), 129.1 (2 × CH, C-2',6'), 131.5 (quat., C-4'), 139.2 (quat., C-1'), 153.9 (quat., C-4a), 155.1 (quat., C-8a), 160.6 (quat., C-2), 161.8 (quat., C-7), 168.9 (quat., C=O, amide); MS (ESI) m/z 353 $(MH)^+$.

7-{4-(β-Alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin trifluoroacetate 8b. To a solution of **7b** (100 mg, 0.196 mmol) in DCM (10 mL) trifluoroacetic acid (2 × 4 mL) was added. Upon completion of the reaction the excess the volatiles were removed *via* repeated co-evaporation with methanol (5 × 10 mL). The resulting residue triturated with cold diethyl ether to give 7-[4-(β-alanylamido)]benzyloxy-3-ethoxycarbonylcoumarin trifluoroacetate **8b** as a white crystalline solid (0.102 g, 99 %); mp 196.4-198.3 °C; (found C, 52.81; H, 4.20; N, 5.20. $C_{24}H_{23}F_3N_2O_8 \bullet H_2O$ requires C, 53.14; H, 4.65; N, 5.16 %); (found: MH+, 411.1554. Calc. for $C_{22}H_{23}N_2O_6$: MH, 411.1551); ν_{max}/cm^{-1} 3122-2983 ($^+NH_3$), 1749 (C=O), 1683 (C=O), 1670 (C=O), 1602 (NH), 1541 (NH), 1508, 1197 (C-O), 1182 (C-O); ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 1.29 (3H, t, J = 7.2 Hz, CH_3), 2.70 (2H, t, J= 6.8 Hz, CH_2-2'), 3.09 (2H, t, J = 6.8 Hz, CH_2-3'), 4.26 (2H, q, J = 7.2 Hz, OCH_2CH_3), 5.19 (2H, s, OCH_2), 7.05 (1H, dd, J = 8.8 and 2.4 Hz, H-6), 7.10 (1H, d, J = 2.4 Hz, H-8), 7.42 (2H, d, J = 8.4 Hz, H-3',5'), 7.62 (2H, d, J = 8.4 Hz, H-2',6'), 7.79 (3H, br, NH_3), 7.84 (1H, d, J = 8.8 Hz, H-5), 8.72 (1H, s, H-4), 10.22 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 14.6 (CH_3), 33.7 (CH_2-2'), 35.4 (CH_2-3'), 61.4 (CH_2, OCH_2CH_3), 70.4 (CH_2, OCH_2), 101.6 (CH, C-8), 112.0 (quat.), 113.9 (quat., C-8a), 114.3 (CH, C-6), 119.6 (2 × CH, C-3',5'), 129.3 (2 × CH, C-2',6'), 131.1 (quat., C-1'), 132.1 (CH, C-5), 139.3 (quat., C-4'), 149.6 (CH, C-4), 156.7 (quat., C-3), 157.3 (quat., C-4a), 163.3 (quat., C=O, COOEt), 164.2 (quat., C-7), 168.9 (quat., C=O, amide); MS (ESI) m/z 411 $(MH)^+$.

Microbiological testing

Preparation of culture media containing substrates 8a and 8b. Columbia agar was prepared as follows; 41 g of Columbia agar (Oxoid Basingstoke, UK) was added to deionised water and the volume was made up to 1 L. The medium was sterilised by autoclaving at 116 °C for 20 minutes and left to cool at 50 °C. 2 mg of each substrate **8a,b** 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.[14] 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 deionised 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 **8a,b** and **9** in liquid media, **9** was purchased from Glycosynth (UK), *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 (365/440 nm and 375/445 nm settings) and absorption (at 660 nm for organism

density) during a 24 hour period of incubation of the 12 assessed strains (Table S1) on GREINER 96 well plate (ref. 655 090) sealed with transparent GREINER viewseal (ref. 676 070). For **8a,b** and **9**, a 10 µL solution of substrate (at 50g/L in *N*-methylpyrrolidone) was suspended in 5 mL of TSB (final concentration 100 mg/L).[14] 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, the wells were filled with 100 µL TSB with or in the absence or presence of any of the substrates and 100 µL Suspension Medium.

The absorption (at 660 nm for microbial growth) and relative fluorescent intensities at the respective settings of λ_{ex}= 365 nm / λ_{em}= 440 nm and λ_{ex}= 375 nm / λ_{em}= 445 nm) for enzymatic activity were recorded over a period of 24 hours in 96 × 15 minute cycles.

Conclusions

In summary, the fluorogenic substrate **8b**, which consists of β-alanine attached *via* a self-immolative *p*-aminobenzylalcohol unit to 2-carbethoxy-7-hydroxycoumarin **6b**, is a specific and sensitive probe for the detection of BAP producers, in particular the opportunistic Gram negative ESKAPE pathogen *P. aeruginosa*. The synthetic route to this substrate is simpler and more efficient than that to the chromogenic substrate **1** and a medium containing this fluorogenic substrate has the potential for more rapid detection of *P. aeruginosa* than chromogenic media. This substrate **8b** also has advantages over another fluorogenic substrate, 7-*N*-β-alanylamino-4-methylcoumarin **9**, as it is retained by bacterial colonies in solid agar applications, and results in similar times to detection, stronger fluorescence intensities, and no decrease in signal over time in liquid media. Although substrate **8a** produces a weaker signal than substrate **8b**, its use allowed better discrimination between the BAP producers *P. aeruginosa* (positive) and *S. marcescens* (negative).

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Notes and references

‡ Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2016 ACD/Labs).

§ Vitek MS and Vitek2 GN controls were run in parallel; β-alanine-*p*-nitroanilide was hydrolyzed by all *P. aeruginosa* and *S. marcenscens* species after 24 hours incubation (see ESI).

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β-Alanyl aminopeptidase-activated fluorogenic probes for the rapid identification of *Pseudomonas aeruginosa* in clinical samples

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