A sensitive and specific β-alanyl aminopeptidase-activated fluorogenic probe for the detection of *Pseudomonas aeruginosa*

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We report the synthesis of the sensitive and specific fluorogenic self-immolative substrate **8b**, which is hydrolzyed by β-alanyl aminopeptidase (BAP), resulting in a 1,6-elimination and the release of the highly fluorescent hydroxycoumarin **6b**. This fluorophore **6b** is retained within bacterial colonies, so has potential for the detection of *P. aeruginosa* (a BAP producer); it also has potential in liquid media due to the rapid and strong signal release from the substrate **8b**, and lack of self-quenching or photobleaching.

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 disease. In immunosuppressed patients, such as burns patients, and those with cancer or AIDS, as few as 10-100 *P. aeruginosa* cells can lead to gut colonization.[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 and to facilitate 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.

For the identification of bacteria, chromogenic and fluorogenic enzyme substrates are widely used 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 a medium containing 7-*N*-β-alanyl-1-pentylresorufamine (7-*N*-β-alanyl-1-PRF) **1** for the detection of *P. aeruginosa*; the conversion of this substrate **1** to 1-PRF **2** is catalyzed by β-alanyl aminopeptidase (BAP), which is specific to this organism, Figure 1.[5] Due to its excellent specificity and sensitivity, this medium now forms the basis of a commercial diagnostic test for this pathogen.[6] One drawback with chromogenic media in general is the time taken for the generation of the coloured signal (usually 24-48 hours) and we sought to address this in the current work through the enhanced detection sensitivities of fluorogenic substrates, which should result in greatly reduced times to detection.

Results and discussion

As can be seen in Figure 1, BAP activity usually liberates β-alanine and an amine; we have synthesized two novel fluorogenic substrates **8a,b** in which BAP activity would result in the release of a fluorescent phenol reporter, by linking β-alanine **4** to a hydroxycoumarin **6** *via* a *para*-aminobenzylalcohol-based linker. One of the

advantages of these substrates is their straightforward synthesis, which involves the coupling of Boc-β-alanine **4** with *para*-aminobenzylalcohol **3** to give alcohol **5**, conversion of the primary alcohol to a better leaving group (OMs or Cl) and coupling to the phenoxide form of the 7-hydroxycoumarin **6**, and, finally, Boc deprotection of the β-ala-linker-hydroxycoumarins **7** to give 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[7] 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**)[8] 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.[9] It is this generation of a phenol **6** which results in the retention of the fluorescence within the bacterial colonies, Figure 4a. 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* (spot 7), *Burkholderia cepacia* (spot 9) and *Serratia marcescens* (spot 5). 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* (1), *P. aeruginosa* (7) and *S. marcescens* (4) 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.

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 (see ESI). 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.

The relative fluorescent signal intensity originating from each well was recorded every 15 minutes for 24 hours 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. 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 2 *S. marcescens* strains; ATCC 264 and ATCC 43861) 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 ionization in the test wells). 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. On the other hand, unlike that from the hydrolysis of substrate **8b**, 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.

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 6. Even the time

taken to detection of the fluorescence generated by these substrates was comparable; in some cases the signal was slightly slower to develop for substrate **8b**, but only by a maximum of 10 cycles (2.5 hours) which, in a clinical setting, would not result in any significant deficiencies.

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.

Acknowledgements

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

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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Figures and captions

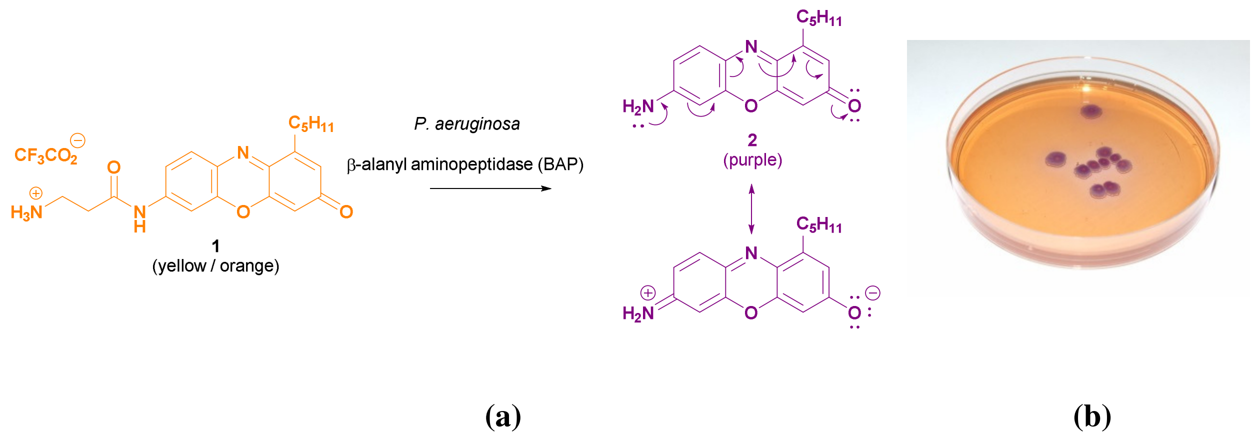


Figure 1. Diagram illustrating the principle behind the chromogenic detection of *Pseudomonas aeruginosa* colonies and the origin of the purple colour **(a)**, using chromID™ *P. aeruginosa* **(b)**.

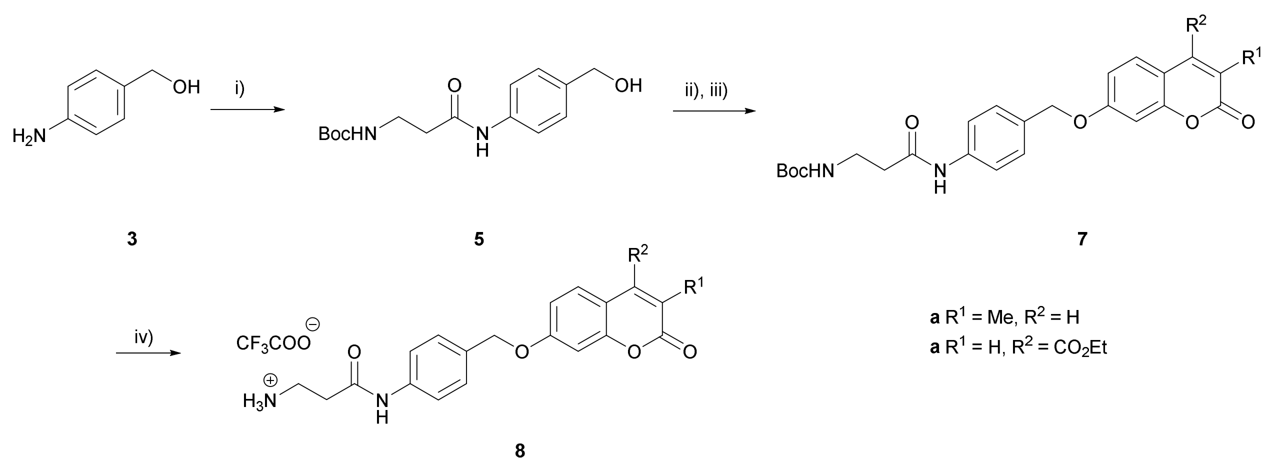


Figure 2. Synthesis of self-immolative BAP substrates **8**. Reagents and conditions: i) Boc-β-ala **4**, DIPEA, HOBt, Et_3N, THF, 0 °C, N_2; ii) DIPEA, MsCl, DCM, 0 °C, N_2; iii) **6**, K_2CO_3, DCM; iv) THF, DCM.

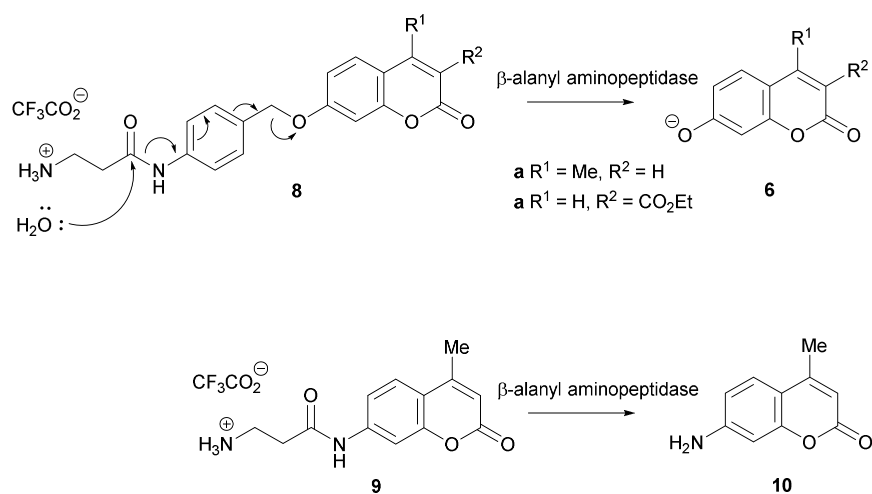
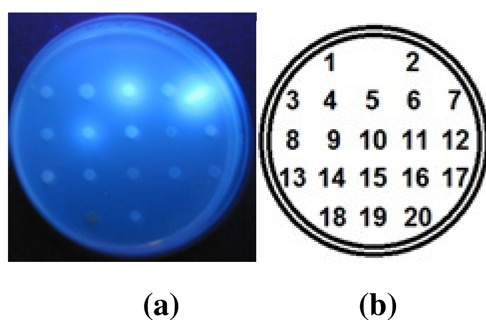


Figure 3. The principle behind the chromogenic detection of *Pseudomonas aeruginosa* colonies using the self-immolative β-alanyl aminopeptidase substrate **8** or 7-*N*-β-alanylamino-4-methylcoumarin **9** to generate the strongly fluorescent hydroxycoumarins **6** or 7-amino-4-methylcoumarin (7-AMC) **10**.

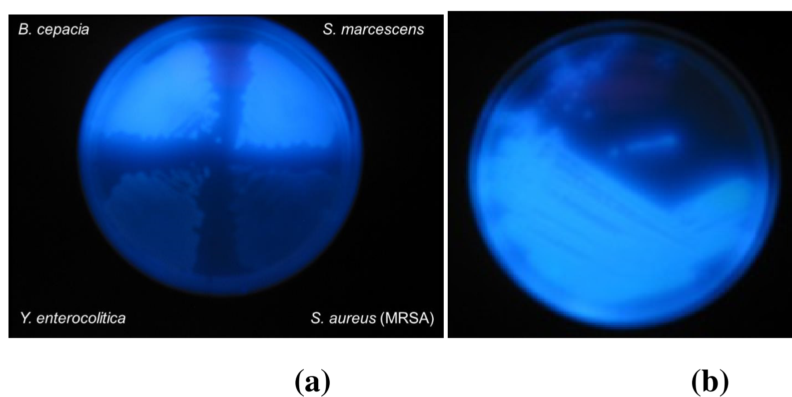


(a) **(b)**

Figure 4. Representative Gram positive, Gram negative, and yeast microorganisms grown on an agar medium containing substrate **8b**; fluorescence corresponds only to the known BAP producers, *Pseudomonas aeruginosa* (spot 7), *Burkholderia cepacia* (spot 9) and *Serratia marcescens* (spot 5).

Table 1. List of microorganisms inoculated onto incubation plates and corresponding spots (100,000 CFU/spot) in Figure 4.

Spot	Organism	Spot	Organism
1	*Escherichia coli*	**11**	*Steptococcus pyogenes*
2	*Klebsiella pneumoniae*	**12**	*Staphylococcus aureus* (MRSA)
3	*Providencia rettgeri*	**13**	*Staphylococcus aureus*
4	*Enterobacter cloacae*	**14**	*Staphylococcus epidermidis*
5	*Serratia marcescens*	**15**	*Listeria monocytogenes*
6	*Salmonella typhimurium*	**16**	*Enterococcus faecium*
7	*Pseudomonas aeruginosa*	**17**	*Enterococcus faecalis*
8	*Yersinia enterocolitica*	**18**	*Bacillus subtilis*
9	*Burkholderia cepacia*	**19**	*Candida albicans*
10	*Acinetobacter baumannii*	**20**	*Candida glabrata*



(a) **(b)**

Figure 5. Fluorescence generated on media containing substrate **8b** by BAP producers: **(a)** *Burkholderia cepacia* and *Serratia marcescens;* **(b)** *Pseudomonas aeruginosa*.

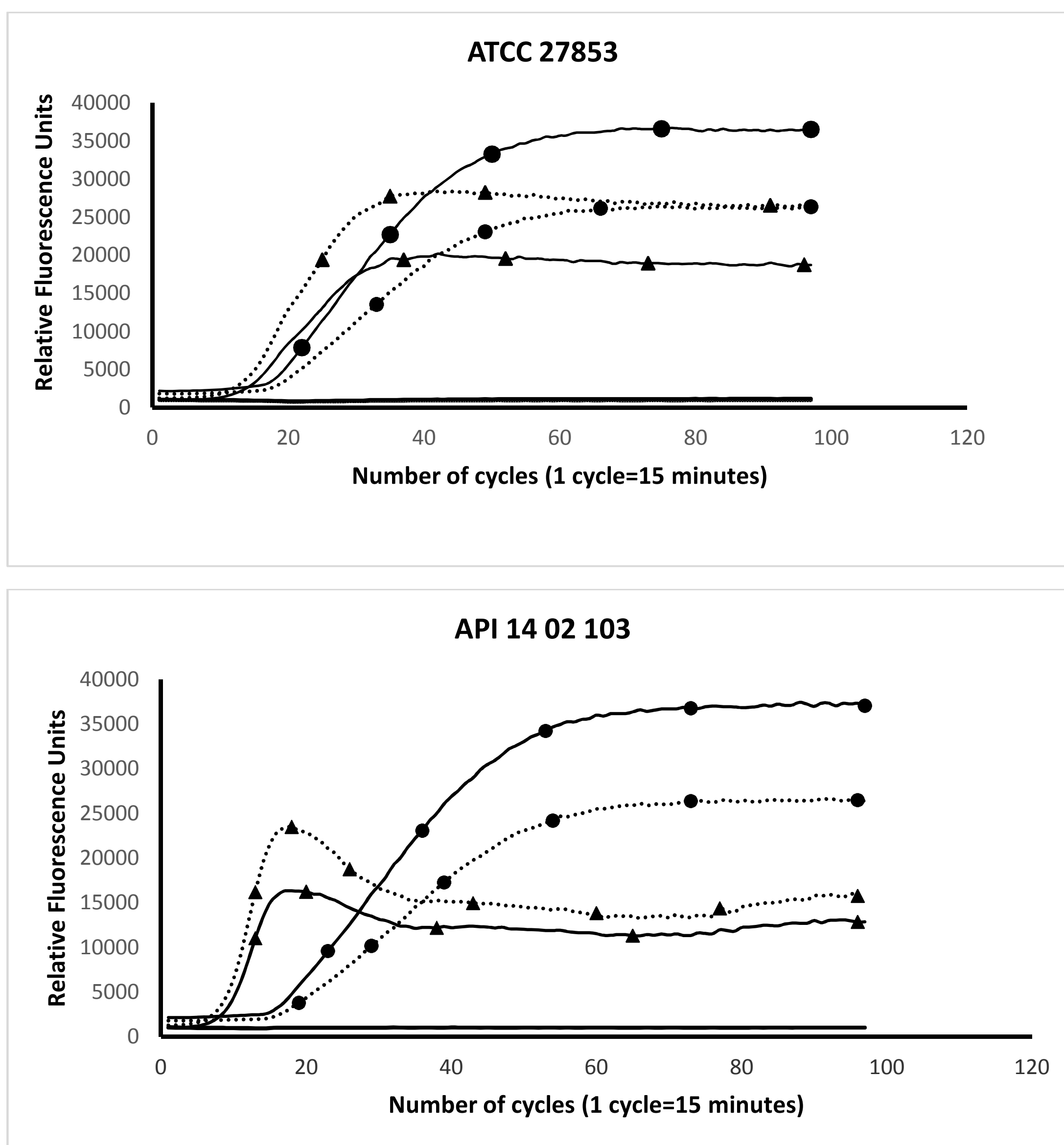


Figure 6. Kinetic data for the release of fluorophores **8b** (•) and **9** (▲) in liquid media in **(a)** *P. aeruginosa* ATCC 27853; **(b)** *P. aeruginosa* API 14 02 103 (bioMérieux strains collection). Fluorescence was recorded at 365 nm (ex) / 440 nm (em) (dotted line) and 375 nm (ex) / 445 nm (em) (solid line).

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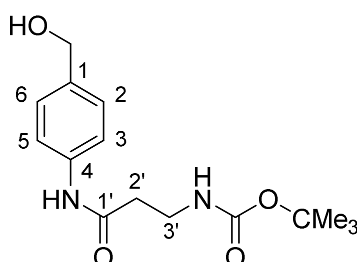
1. Chemistry

1.1 General

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.[1] 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.

1.2 4-(Boc-β-Alanylamido)benzyl alcohol 5

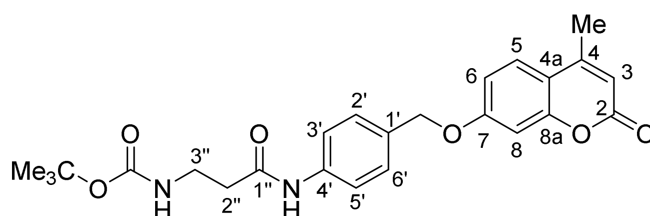


To a solution of 4-aminobenzyl alcohol **3** (1 g, 8 mmol) in anhydrous THF (150 mL), hydroxybenzotriazole hydrate (HOBt, 1.08 g, 8 mmol), Boc-β-alanine-OH **4** (1.51 g, 8 mmol), and EDAC hydrochloride (1.9 g, 10 mmol) were added sequentially, at 0 °C. Triethylamine (2.2 mL, 16 mmol) was then added 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), 1M 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**

[1] C. Ritter, N. Nett, C.G. Acevedo-Rocha, R. Lonsdale, K. Kräling, F. Dempwolff, S. Hoebenreich, P.L. Graumann, M.T. Reetz, E. Meggers; *Angew.Chem. Int. Ed.,* 2015, **54**,13440–13443.

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, C*Me*$_3$), 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, C*H$_2$*OH), 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^+).

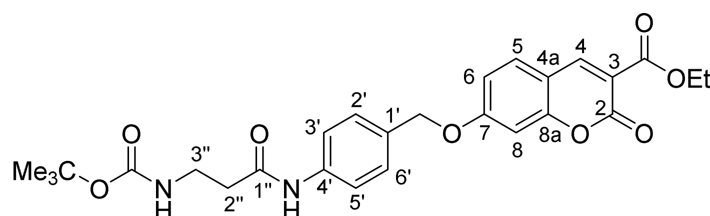
1.3 7-{4-(Boc-β-Alanylamido)}benzyloxy-4-methylcoumarin 7a



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 concn. 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 $C_{25}H_{28}N_2O_6Na$: 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 $(M\text{-}^t Boc)^+$.

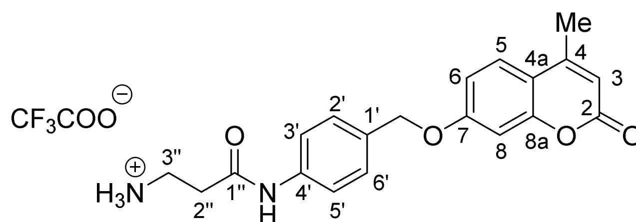
1.4 7-{4-(Boc-β-Alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin 7b



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-concn. 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 °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, C*H*$_3$$CH_2$), 2.47 (2H, t, J = 6.8 Hz, CH_2-2'), 3.21 (2H, q, J = 7.2 Hz, CH_2-3'), 4.27 (2H, q, J = 7.2 Hz, C*H*$_2$$CH_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,

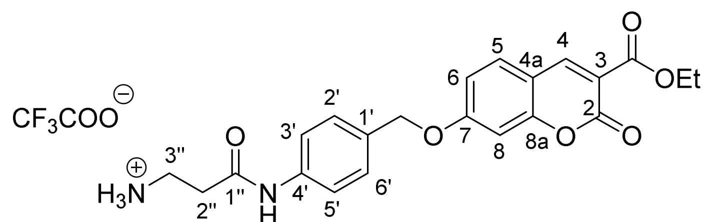
amide); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 14.6 (CH_3), 28.7 (CH_3, *CMe₃*), 36.9 (CH_2-3'), 37.2 (CH_2-2'), 61.4 (CH_2, O$CH_2$$CH_3$), 70.5 ($CH_2$, OCH_2), 78.1 (quat., *C*Me_3), 101.6 (CH, C-8), 111.9 (quat.), 113.9 (quat., C-8a), 114.3 (CH, C-6), 119.5 (2 × CH, C-3',5'), 129.2 (2 × CH, C-2',6'), 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 $(MH)^+$.

1.5 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. $C_{22}H_{21}F_3N_2O_6$•$0.5H_2O$ 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); ^1H 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 $(MH)^{+\cdot}$

1.6 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. $C_{24}H_{23}F_3N_2O_8$•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, O*CH*$_2$$CH_3$), 5.19 (2H, s, OCH_2), 7.05 (1H, dd, *J* = 8.8 and 2.4 Hz, H-6), 7.10 (1H, d, *J* = 2.4 Hz, H-8), 7.42 (2H, d, *J* = 8.4 Hz, H-3',5'), 7.62 (2H, d, *J* = 8.4 Hz, H-2',6'), 7.79 (3H, br, NH_3), 7.84 (1H, d, *J* = 8.8 Hz, H-5), 8.72 (1H, s, H-4), 10.22 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 14.6 (CH_3), 33.7 (CH_2-2'), 35.4 (CH_2-3'), 61.4 (CH_2, O*CH*$_2$$CH_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 $(MH)^+$.

2. Microbiological testing

2.1 Solid media

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. 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.

2.2 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 on GREINER 96 well plate (ref. 655 090) sealed with transparent GREINER viewseal (ref. 676 070).

Table S1. List of microorganisms inoculated onto 96-well plate for the evaluation of **8a,b** and **9**

Species	**bioMérieux strains collection number**	**ATCC / NCTC reference number**
E. coli	API 00 08 013	
P. aeruginosa	API 77 05 034	NCTC 10662
S. marcescens	API 04 04 009	
S. marcescens	API 75 08 042	ATCC 264
S. marcescens	API 95 05 151	ATCC 43861
P. aeruginosa	API 10 11 314	
P. aeruginosa	API 14 02 224	ATCC 27853
P. aeruginosa	API 10 06 028	ATCC 10145
S. marcescens	API 92 11 027	
P. aeruginosa	API 08 04 064	
P. aeruginosa	API 14 02 100	
P. aeruginosa	API 14 02 103	

*:API xx xx xxx = bioMérieux strains collection number

Method

For **8a,b** and **9**, a 10 µL solution of substrate (at 50g/L in *N*-methylpirrolidone) 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, 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.

Figure S1. Readings from wells in the absence of microorganisms containing no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

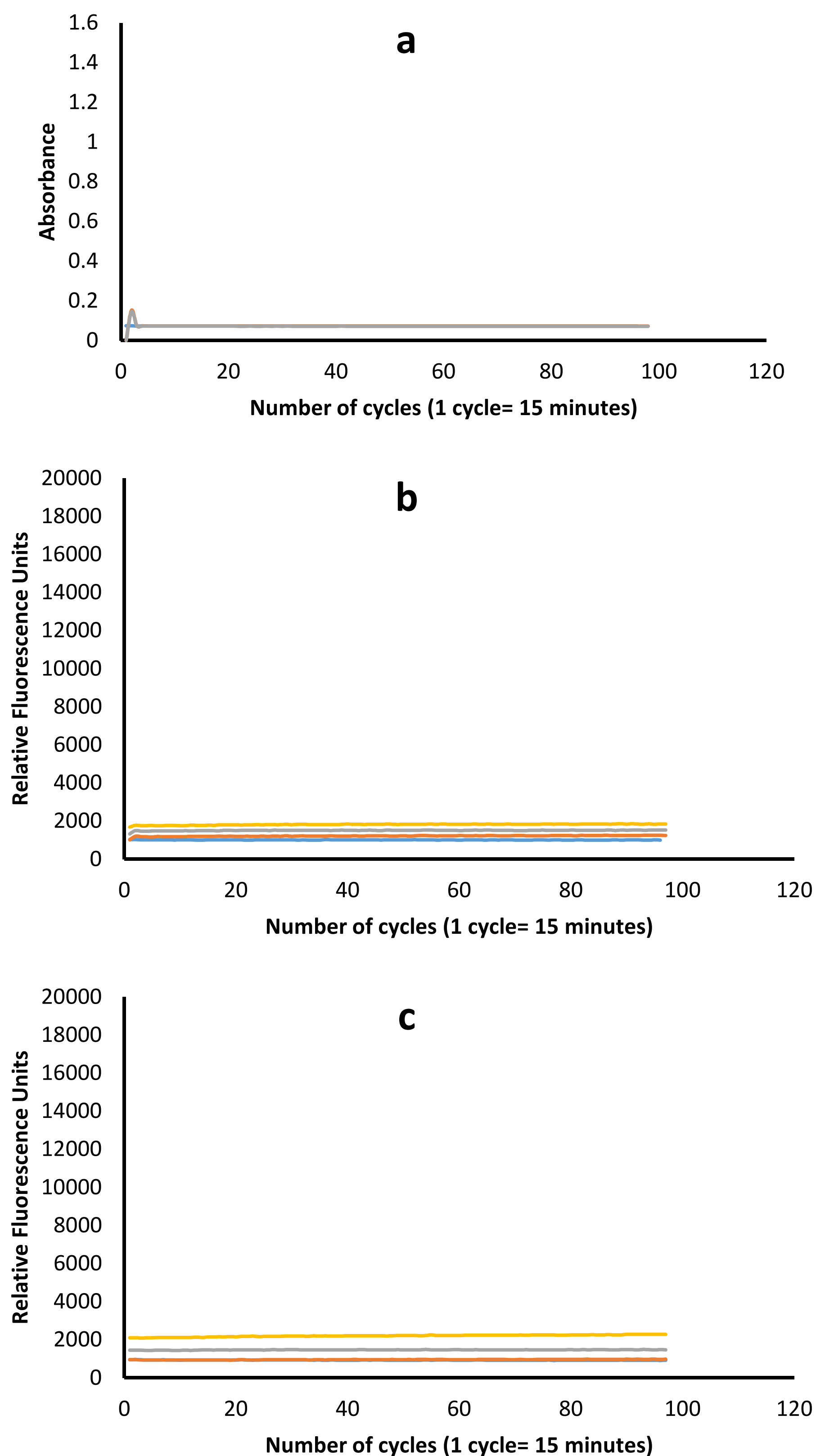


Figure S2. Readings from wells containing *E. coli* (API 00 08 013) in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

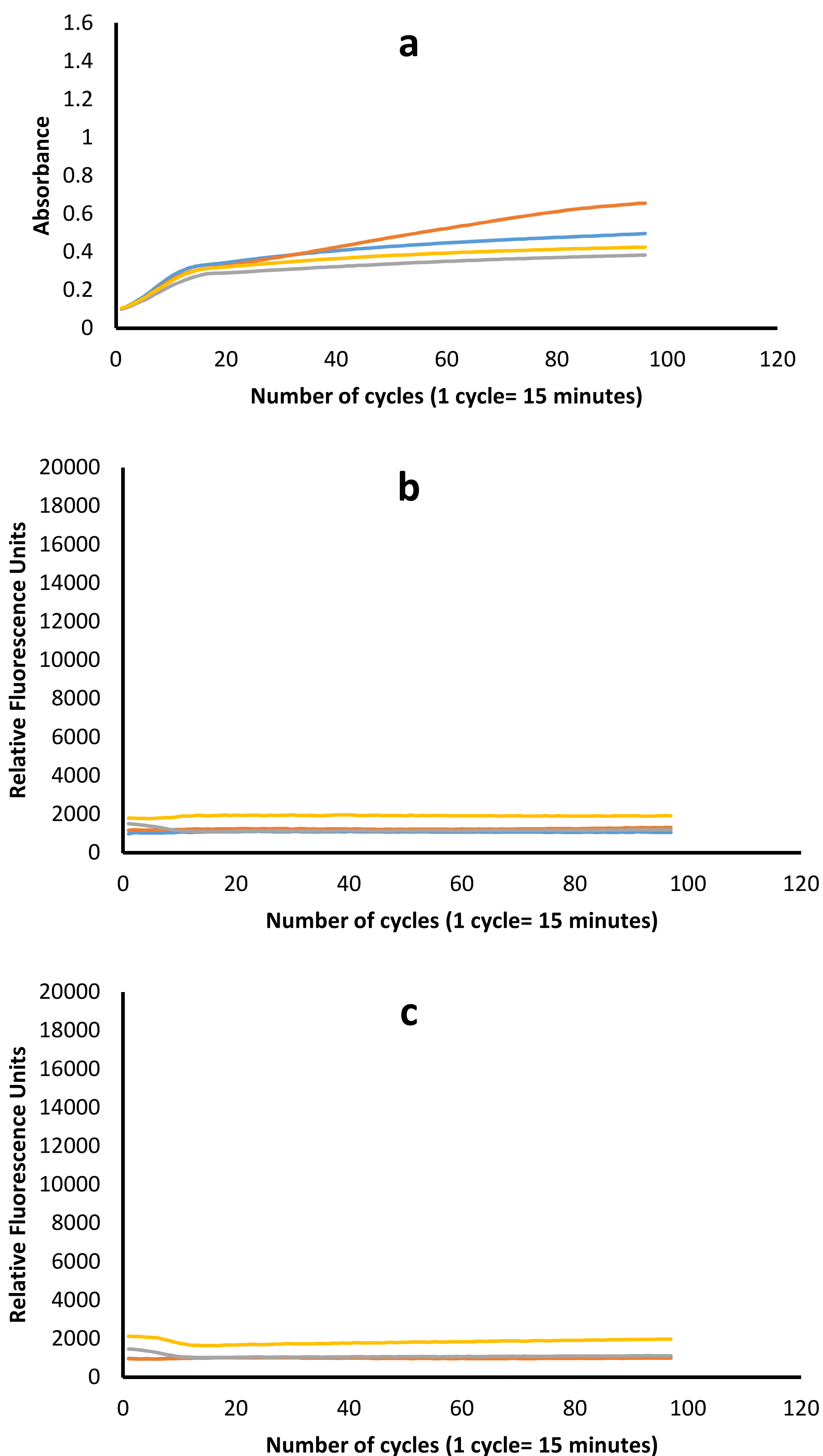
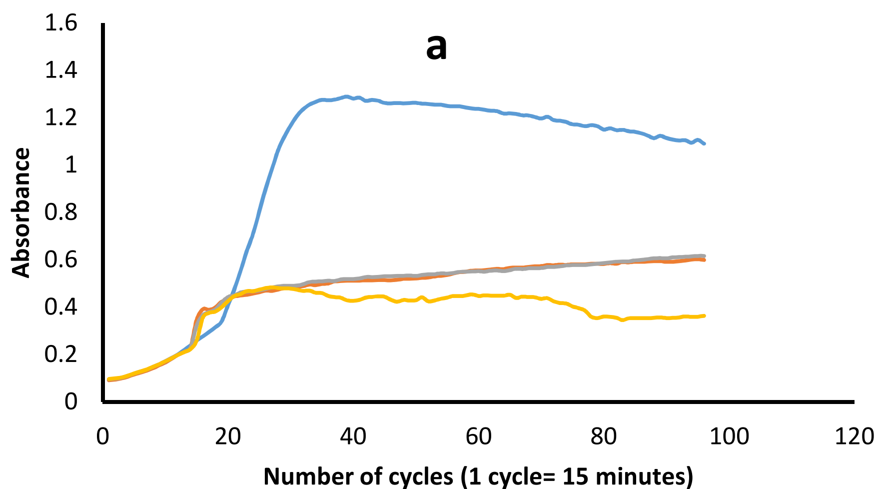
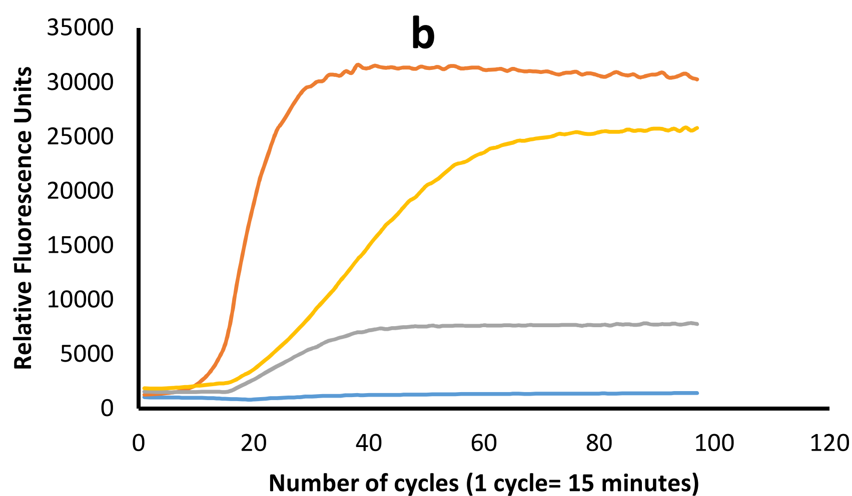


Figure S3. Readings from wells containing *P. aeruginosa* NCTC 10662 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.





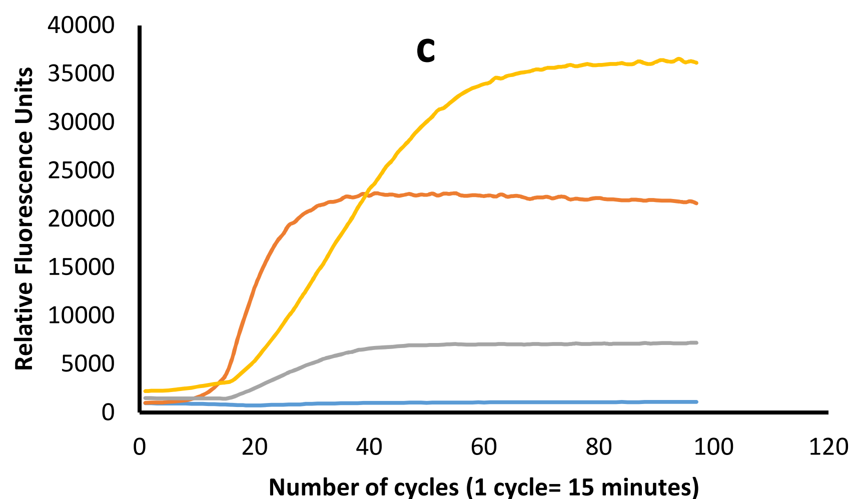


Figure S4. Readings from wells containing *S. marcescens* API 04 4 009 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

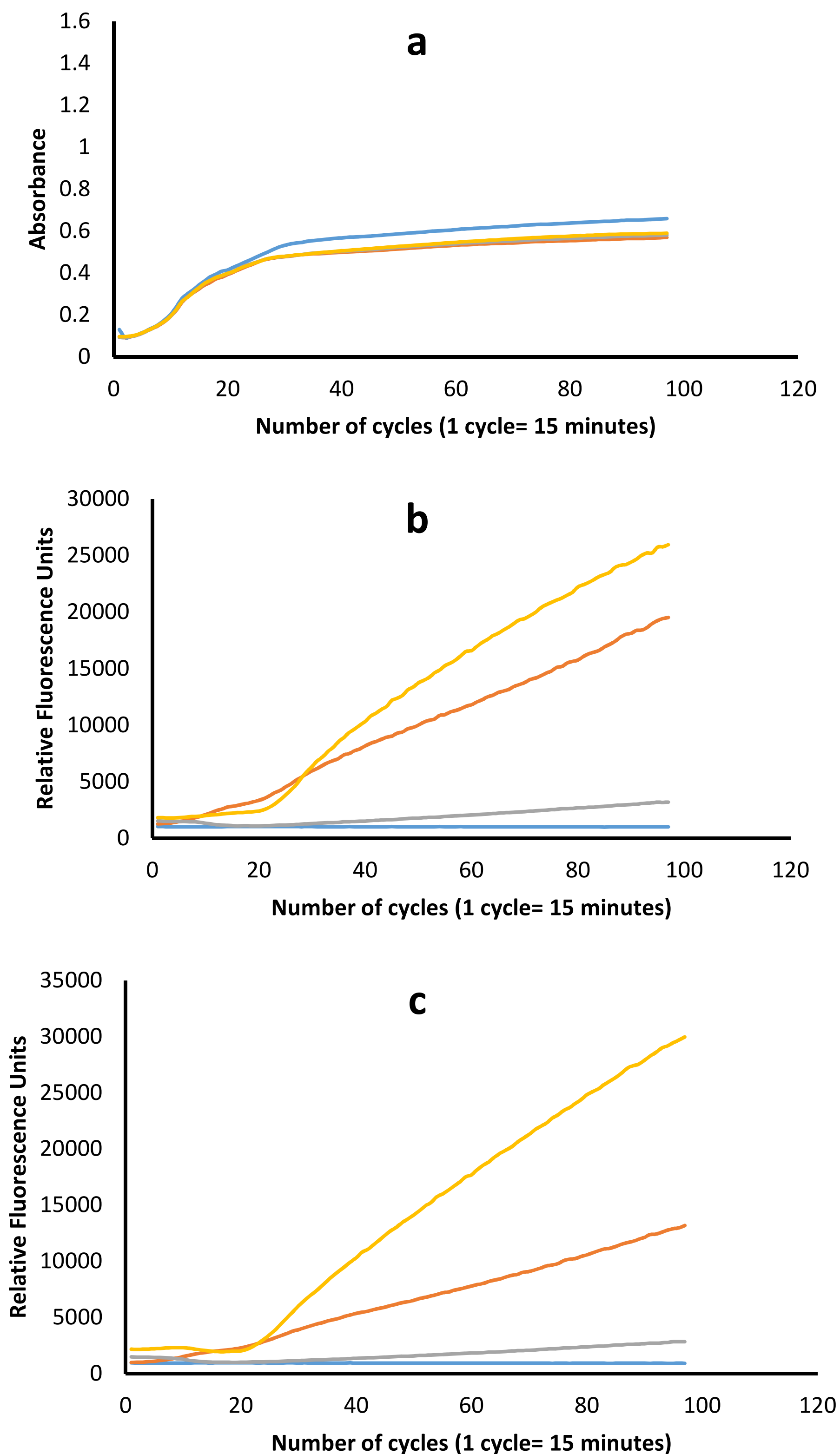
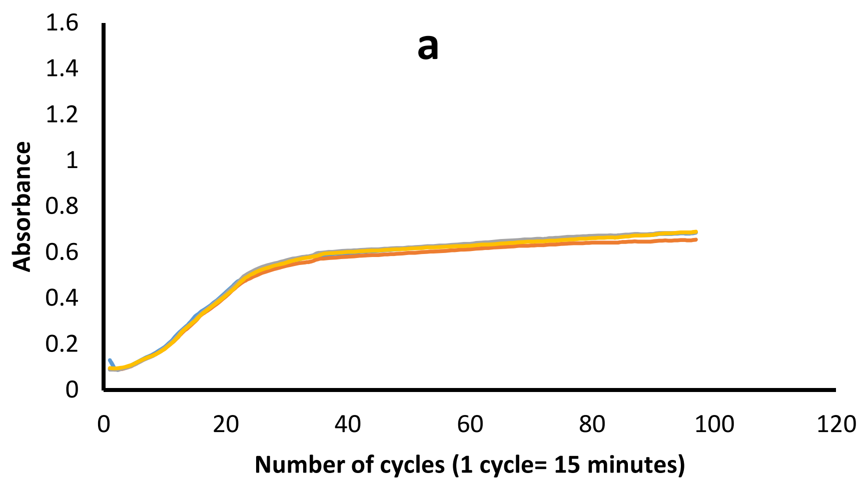
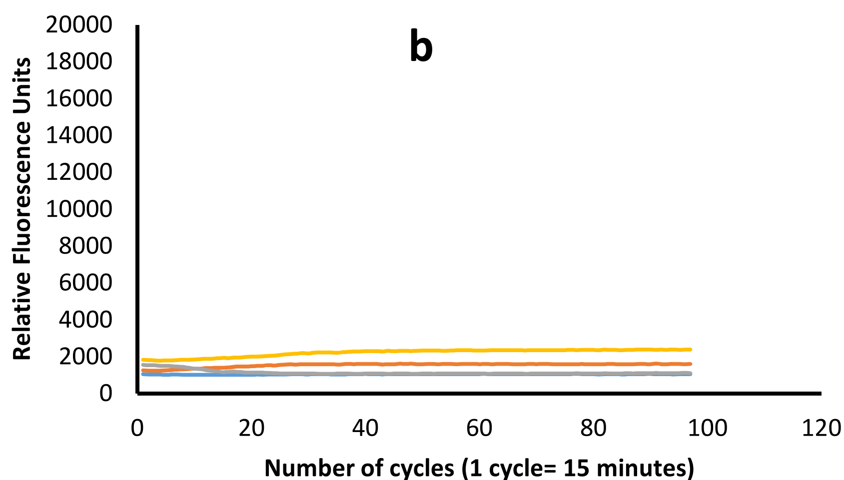


Figure S5. Readings from wells containing *S. marcescens* ATCC 264 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.





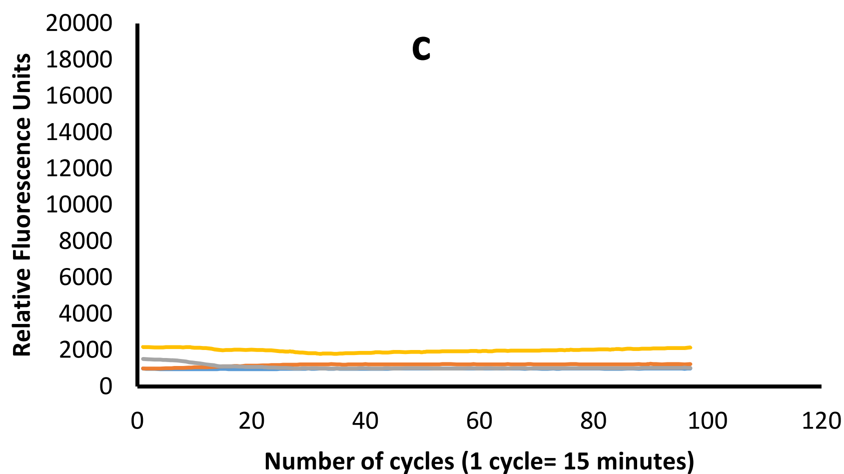


Figure S6. Readings from wells containing *S. marcescens* ATCC 43861 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

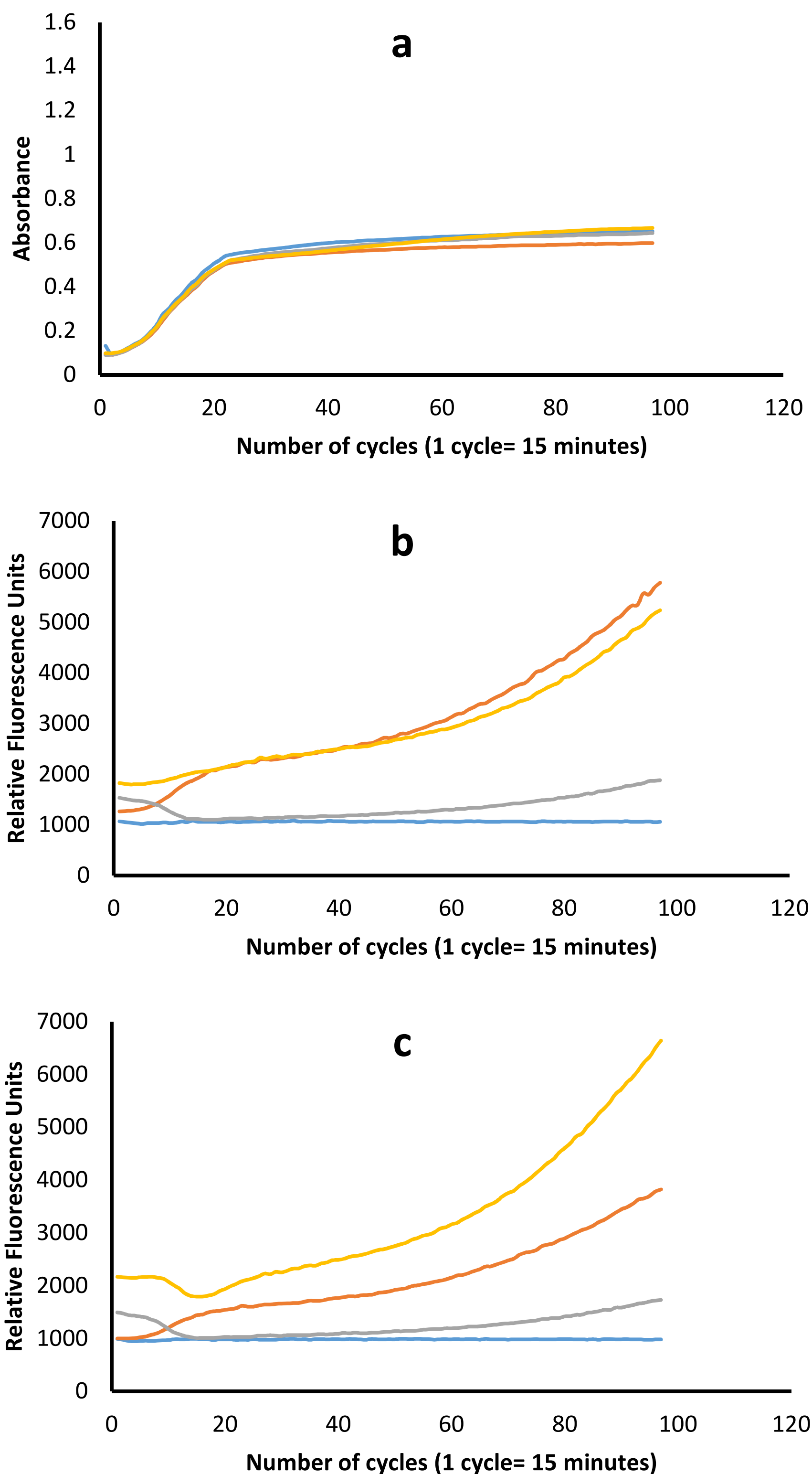


Figure S7. Readings from wells containing *P. aeruginosa* API 10 11 314 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

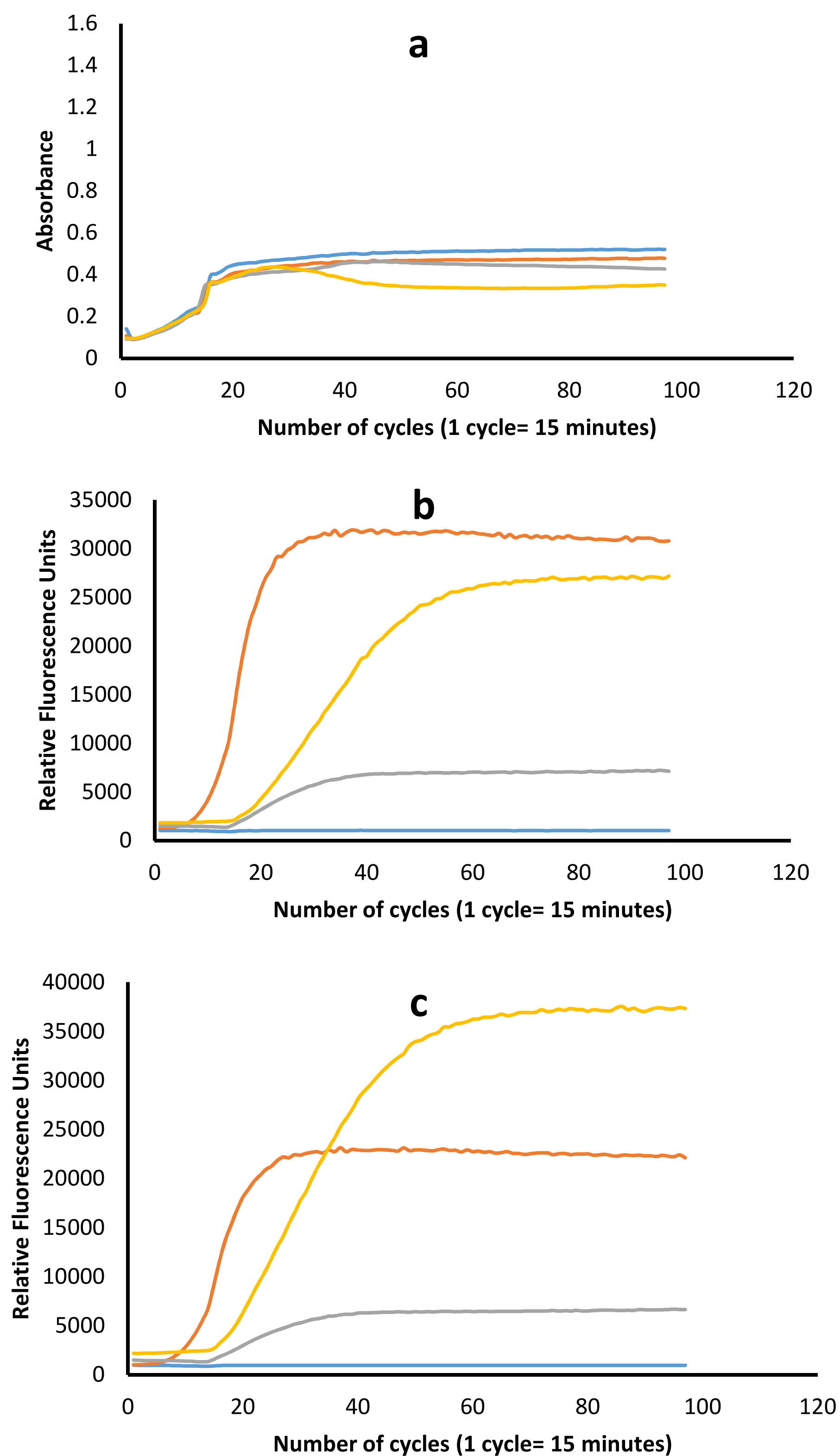


Figure S8. Readings from wells containing *P. aeruginosa* ATCC 27853 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

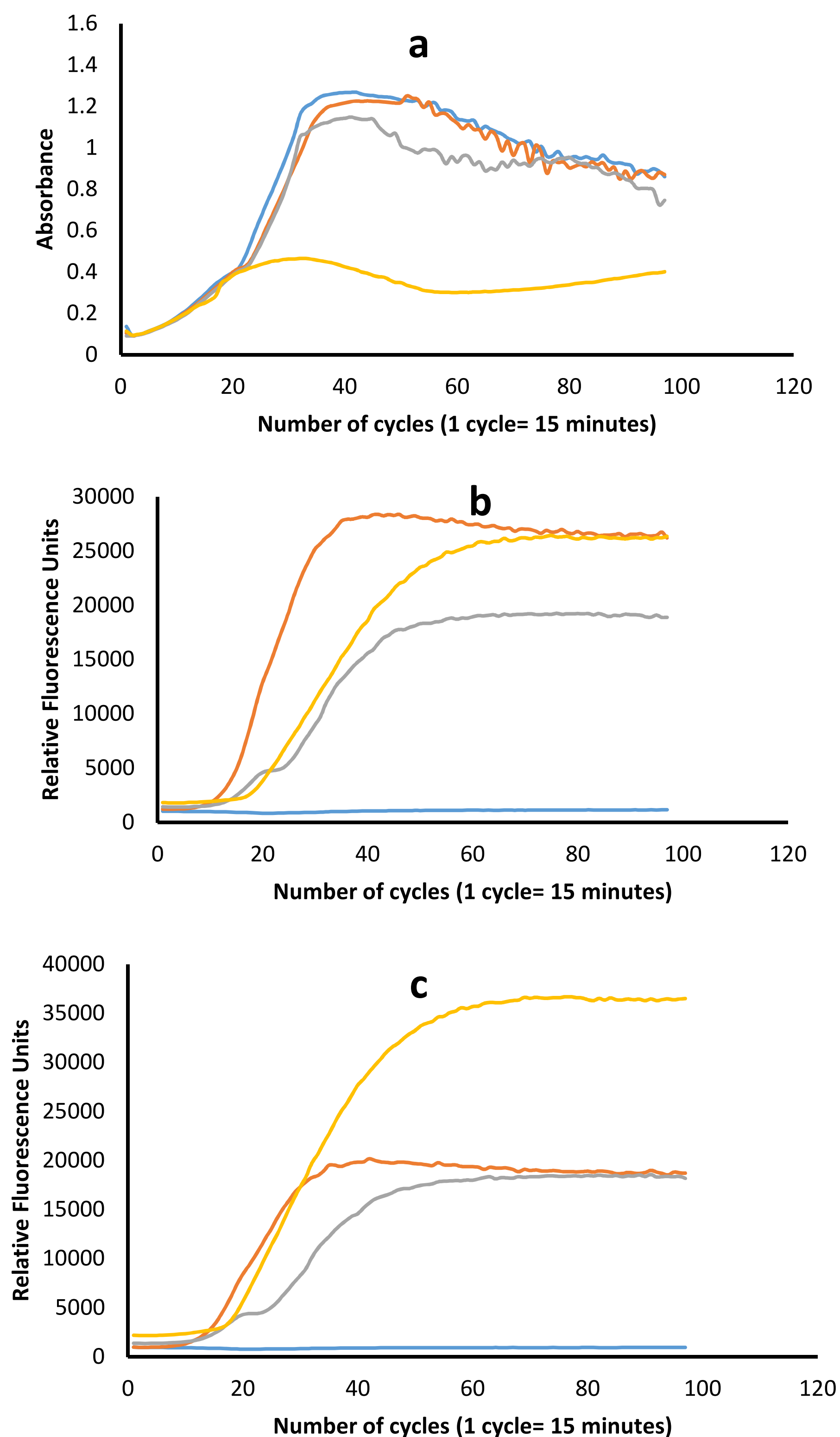


Figure S9. Readings from wells containing *P. aeruginosa* ATCC 10145 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

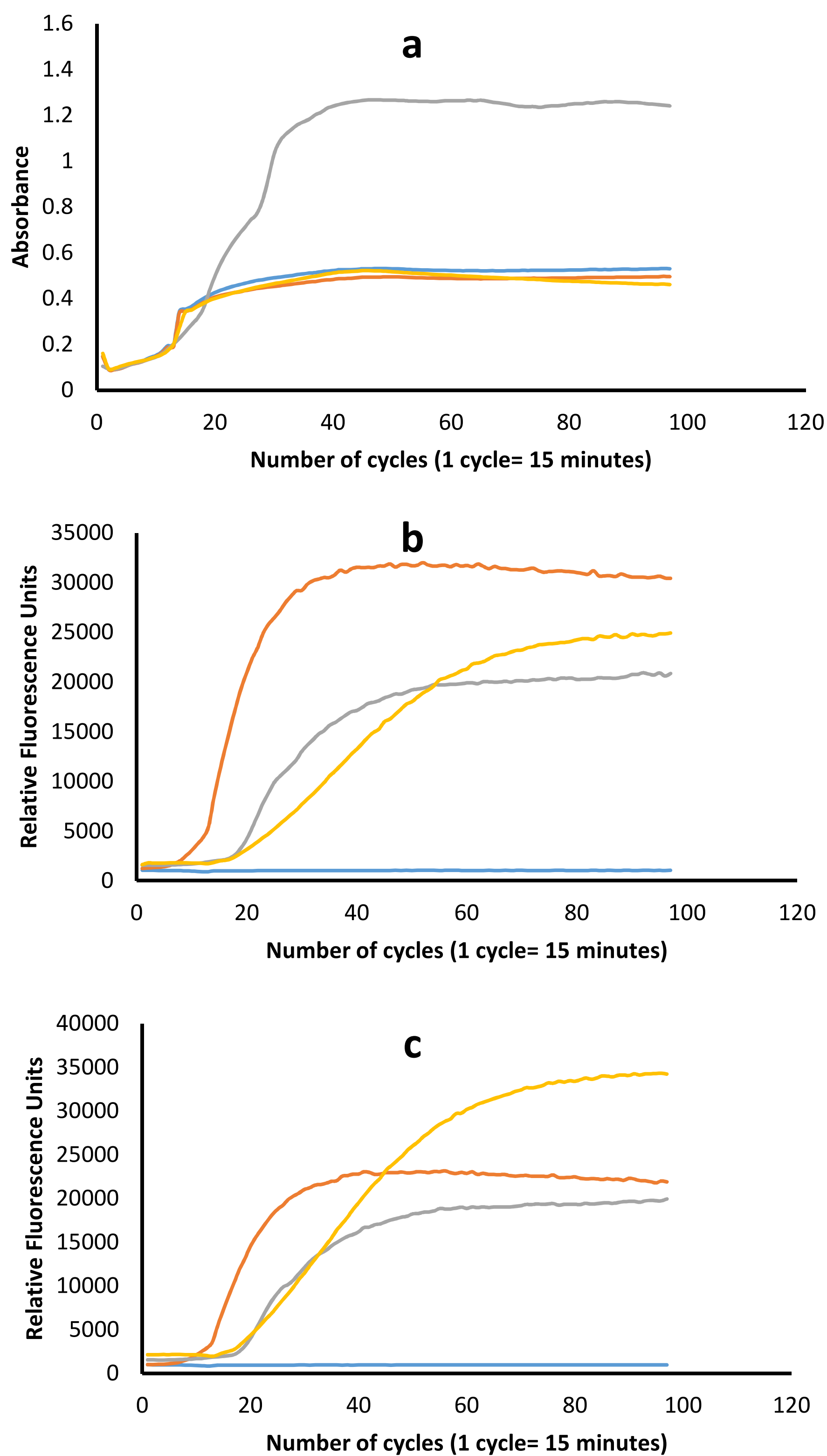


Figure S10. Readings from wells containing *S. marcescens* API 92 11 027 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

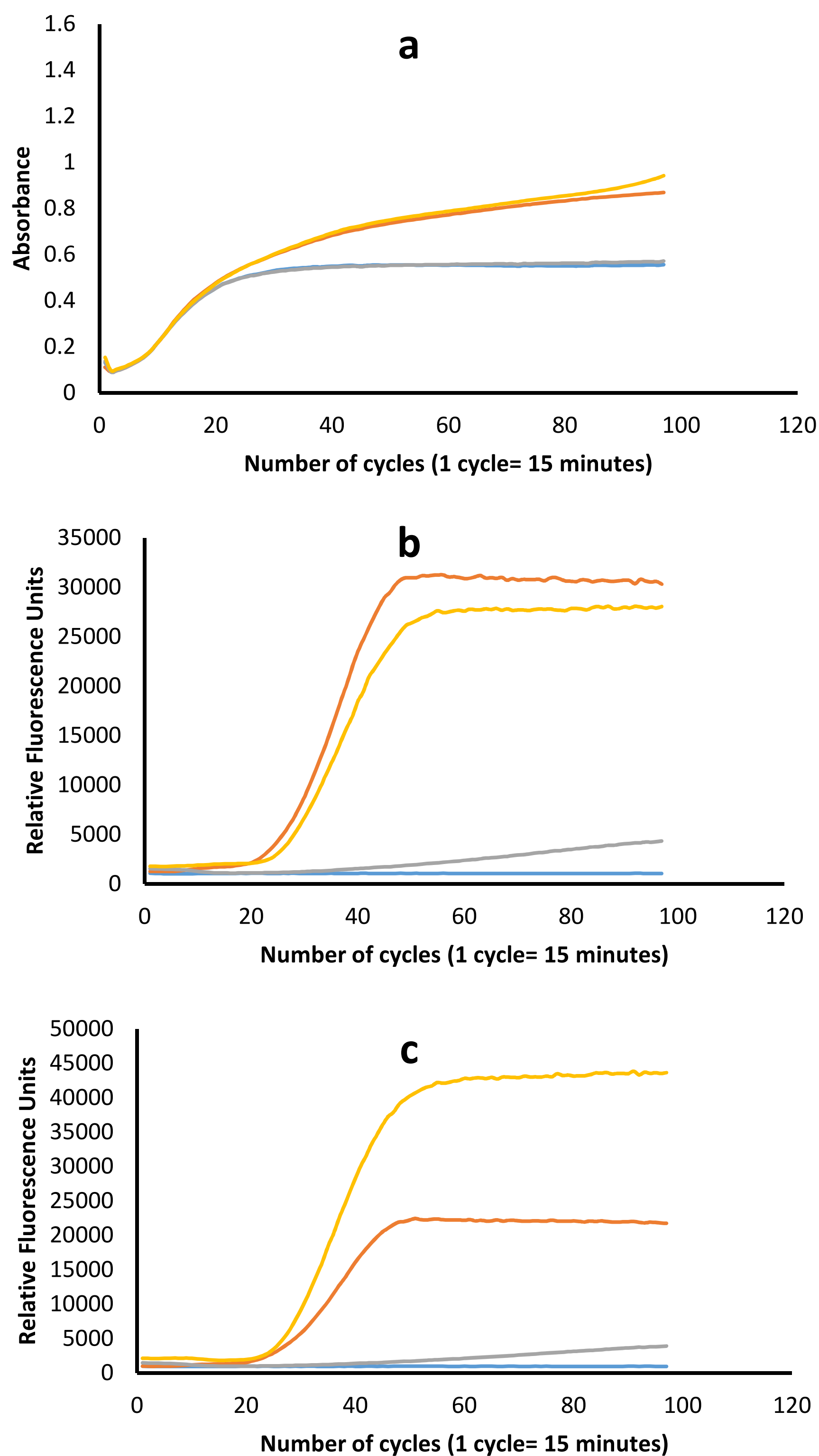


Figure S11. Readings from wells containing *P. aeruginosa* API 08 04 064 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

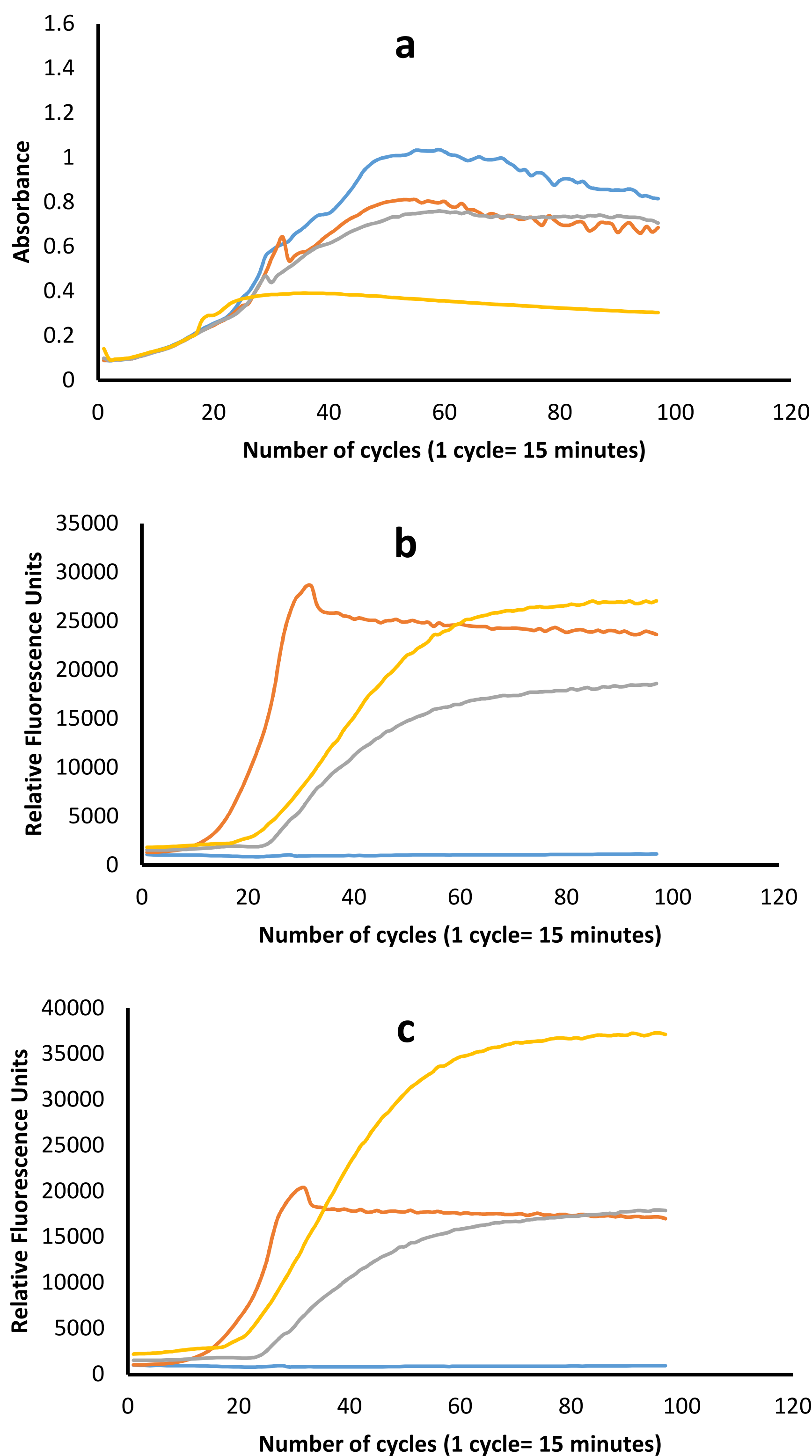


Figure S12. Readings from wells containing *P. aeruginosa* API 14 02 100 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

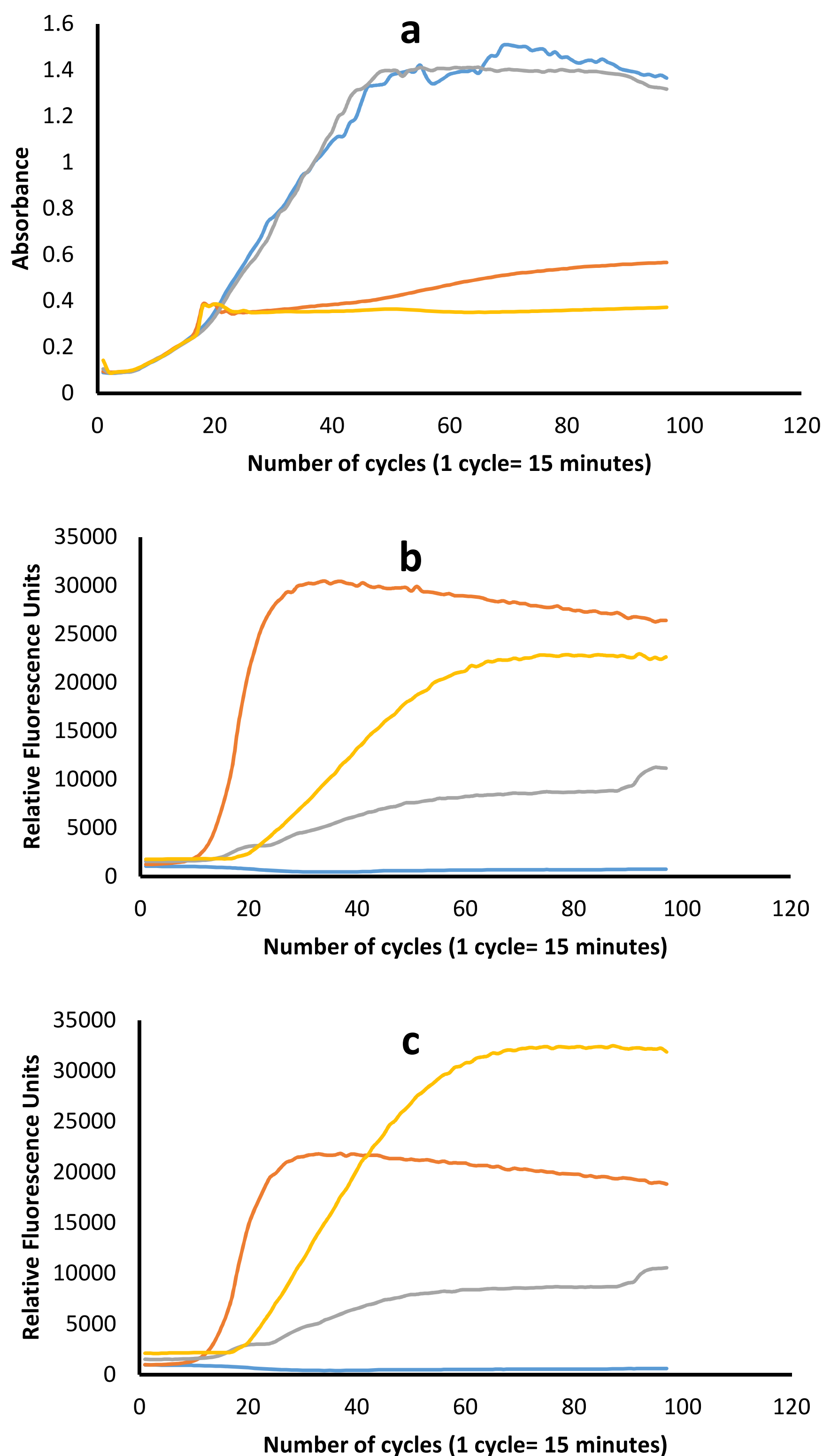
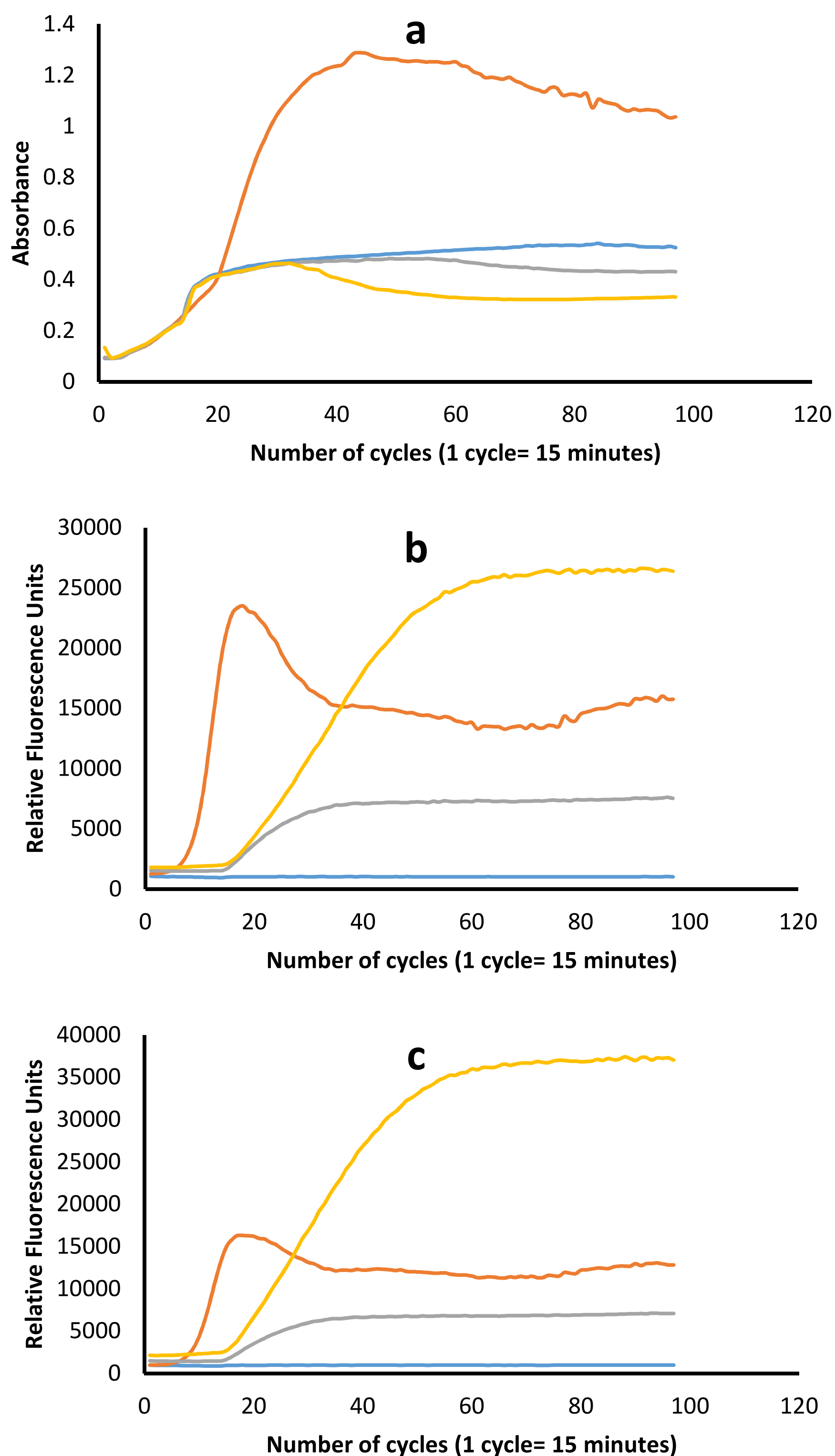
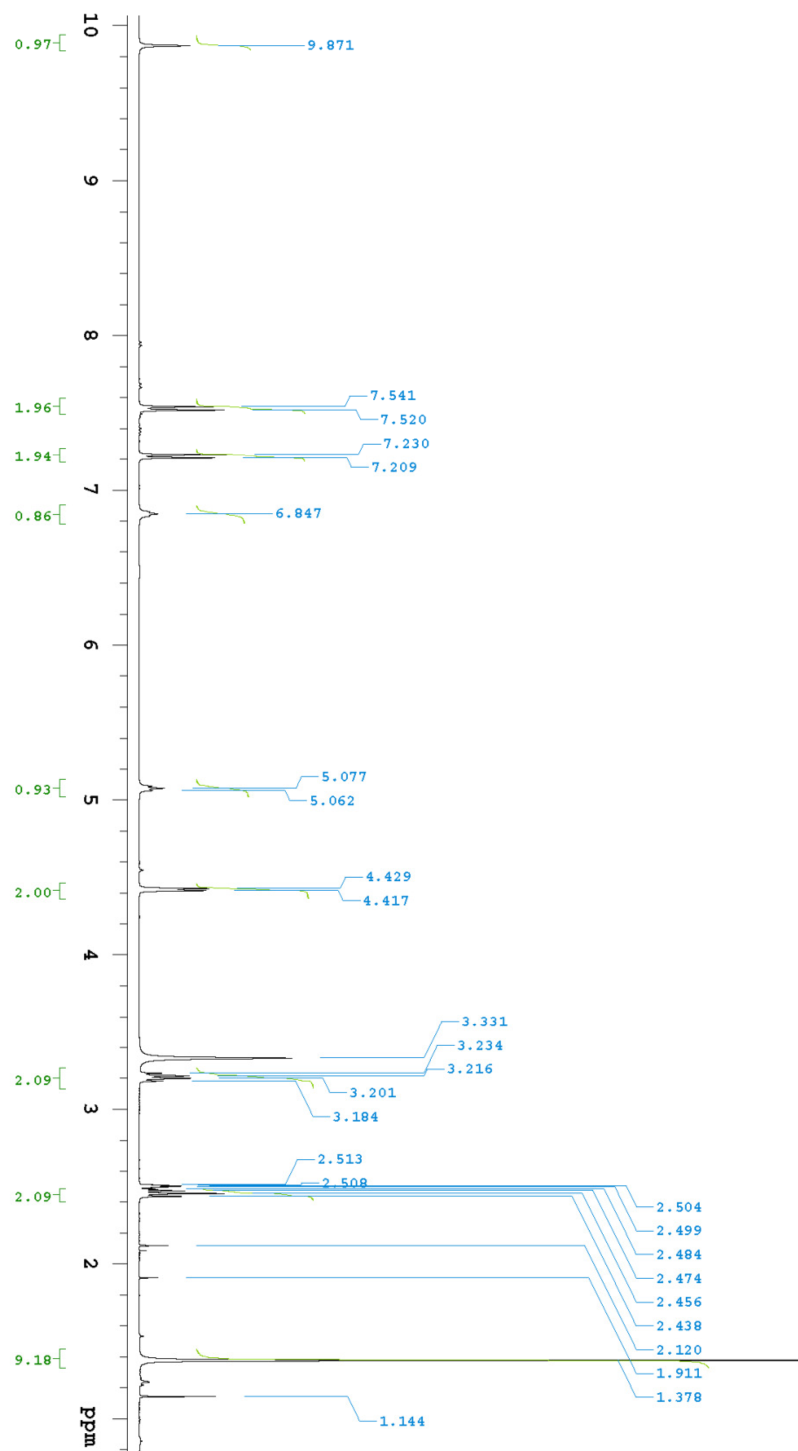


Figure S13. Readings from wells containing *P. aeruginosa* API 14 02 103 in the presence of no substrate (blue line), substrate **8a** (grey line), substrate **8b** (yellow line) or substrate **9** (orange line), respectively; **a)** absorbance at 660 nm; **b)** fluorescent signal intensities at 365 / 440 nm; **c)** fluorescent intensities at 375 / 445 nm.

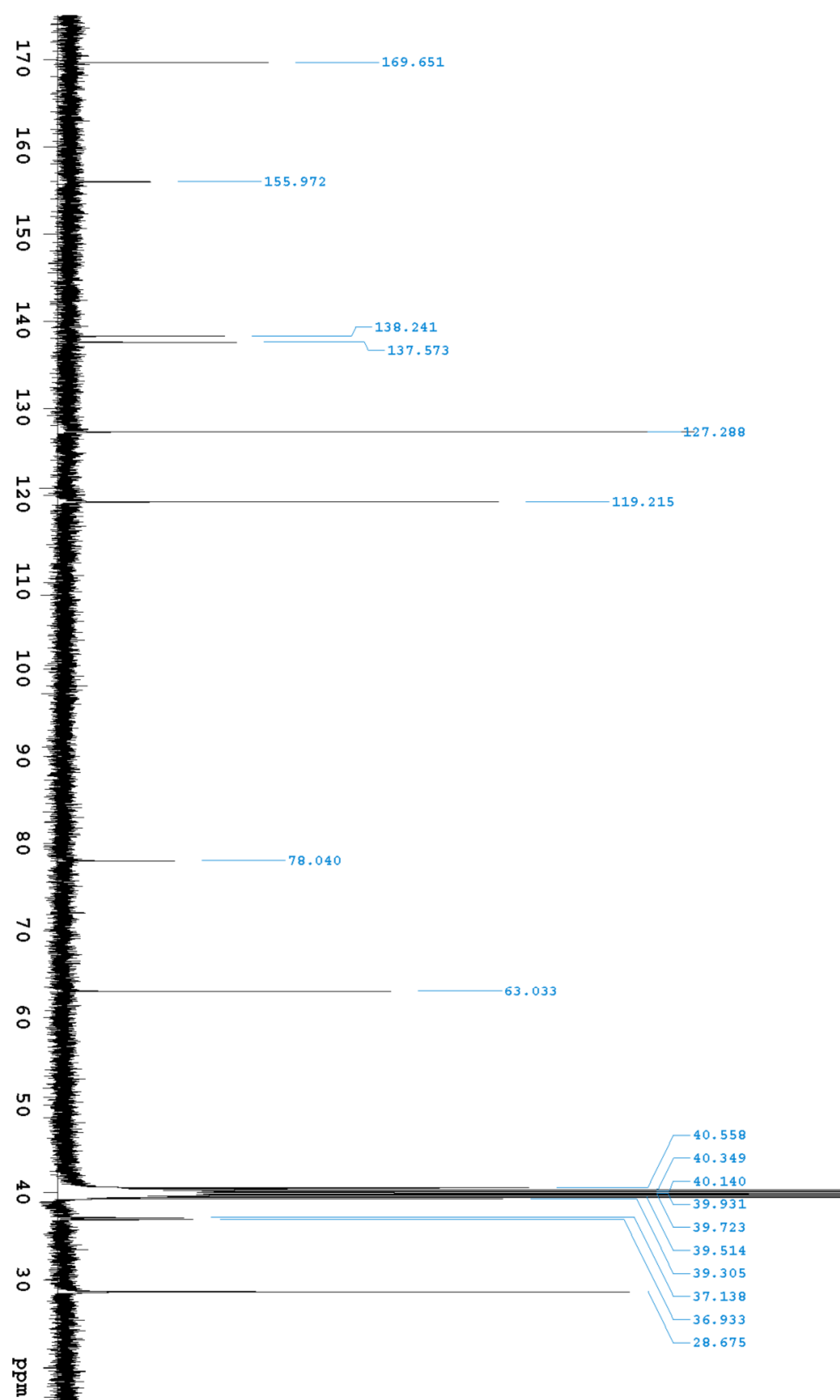


3. NMR Spectra

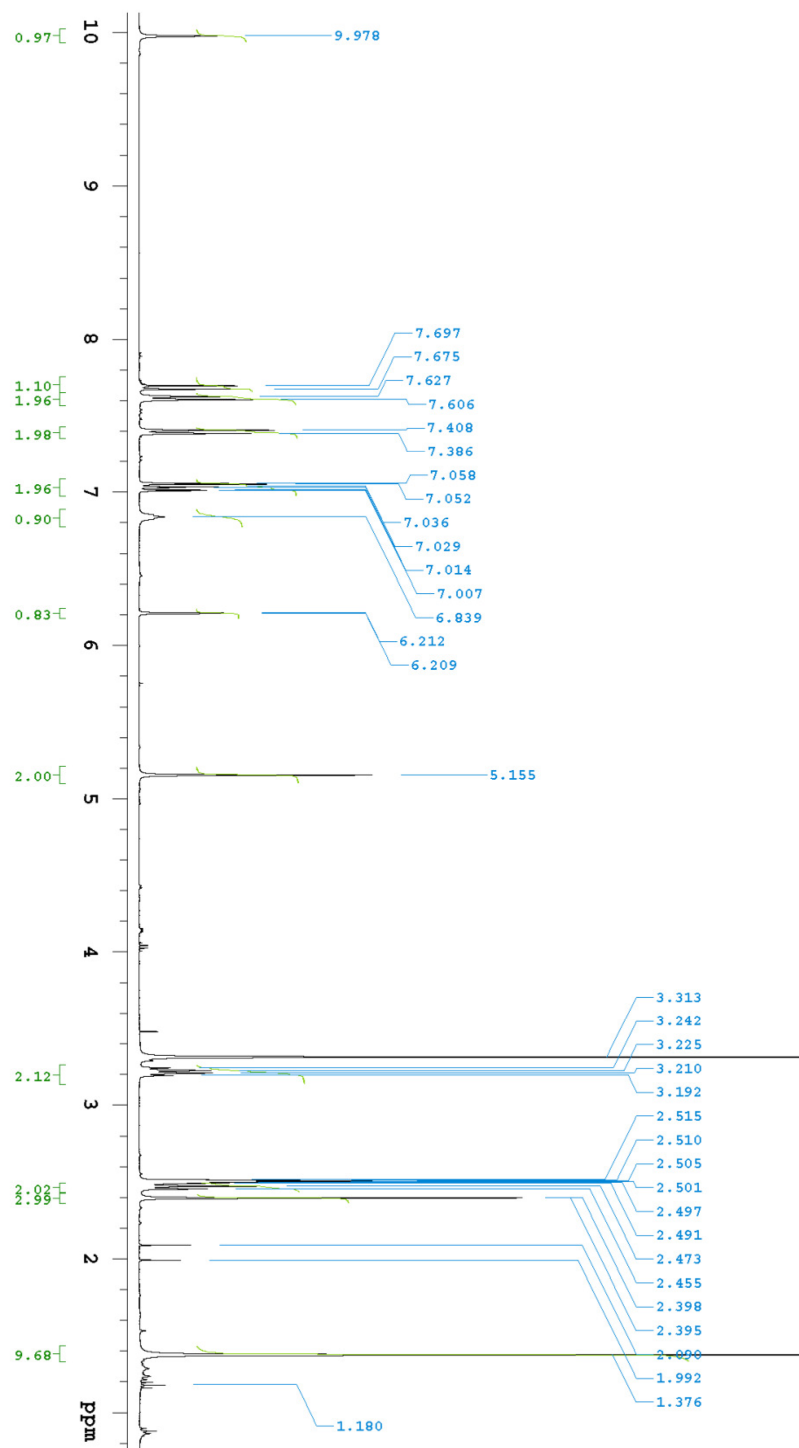
3.1 4-(Boc-β-Alanylamido)benzyl alcohol 5 ^{1}H NMR



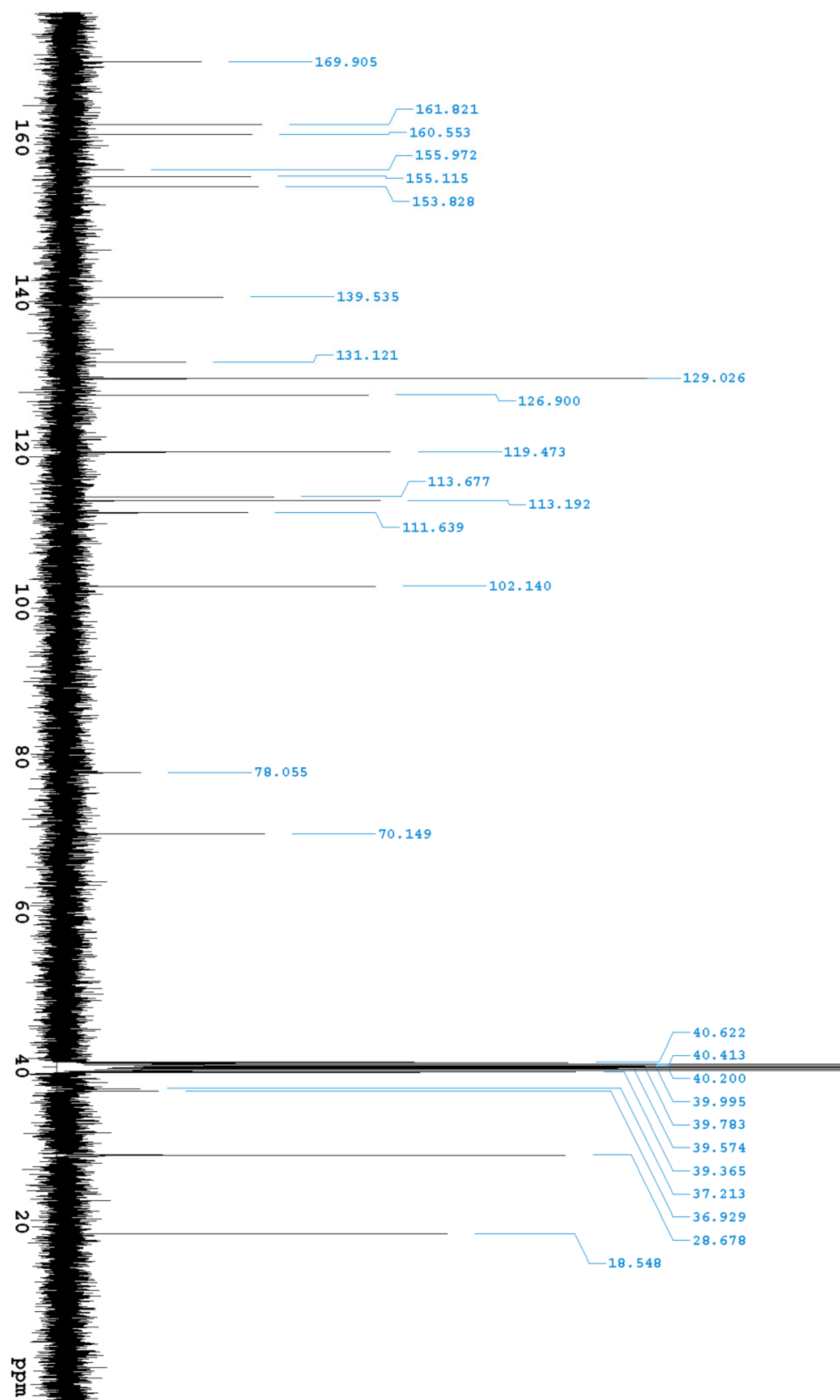
3.2 4-(Boc-β-Alanylamido)benzyl alcohol 5 ^{13}C NMR



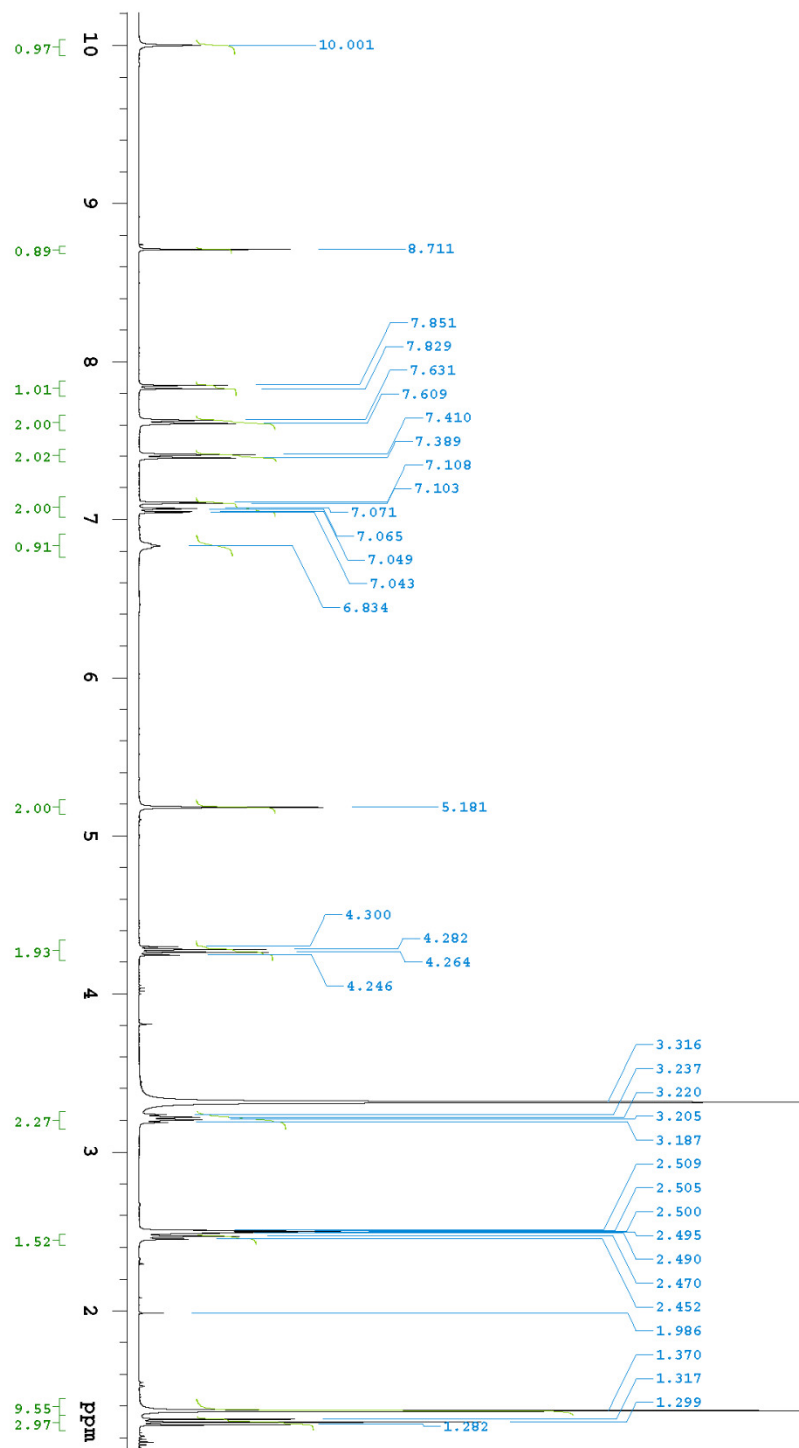
3.3 7-{4-(Boc-β-Alanylamido)}benzyloxy-4-methylcoumarin 7a ^{1}H NMR



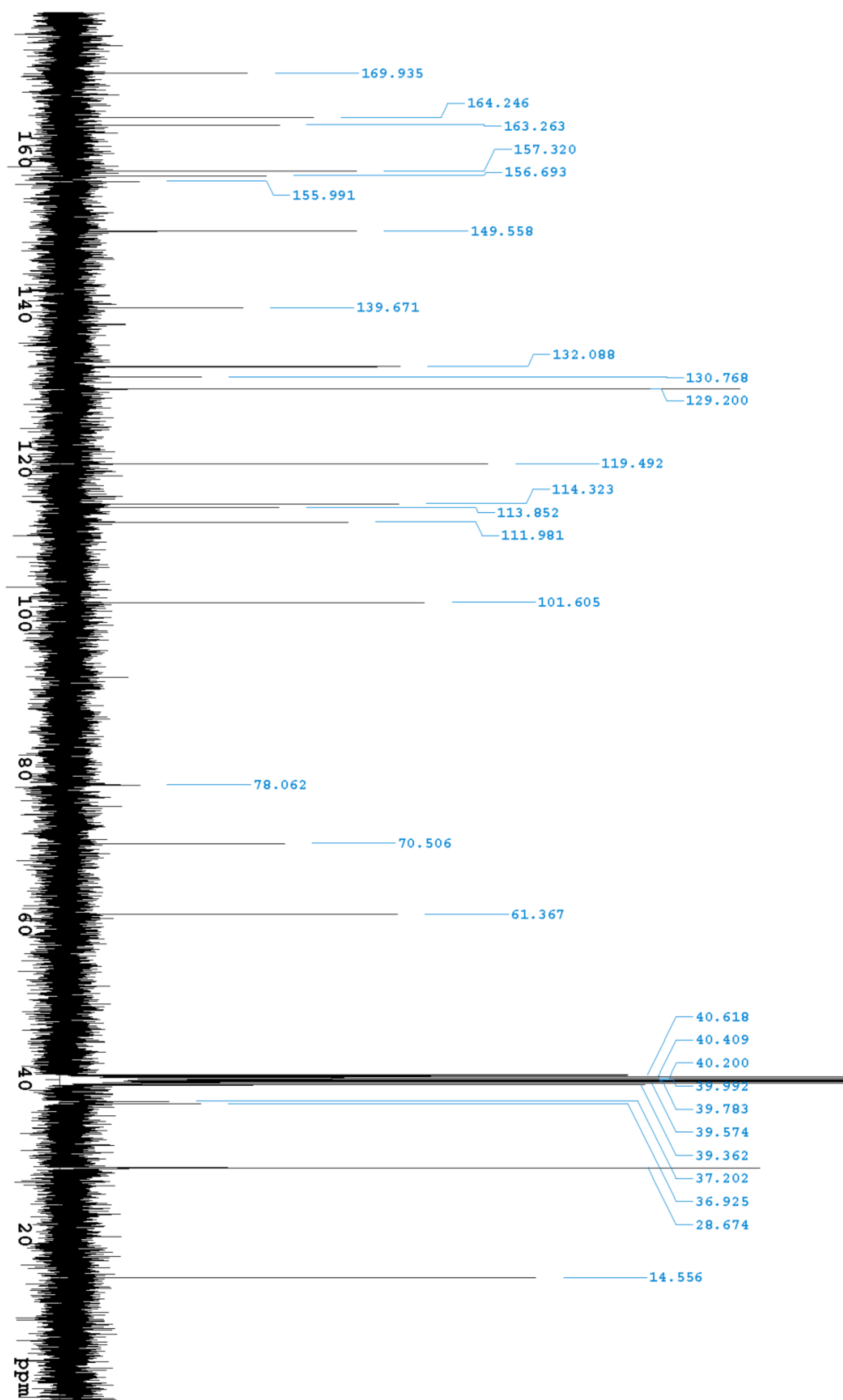
3.4 7-{4-(Boc-β-Alanylamido)}benzyloxy-4-methylcoumarin 7a ^{13}C NMR



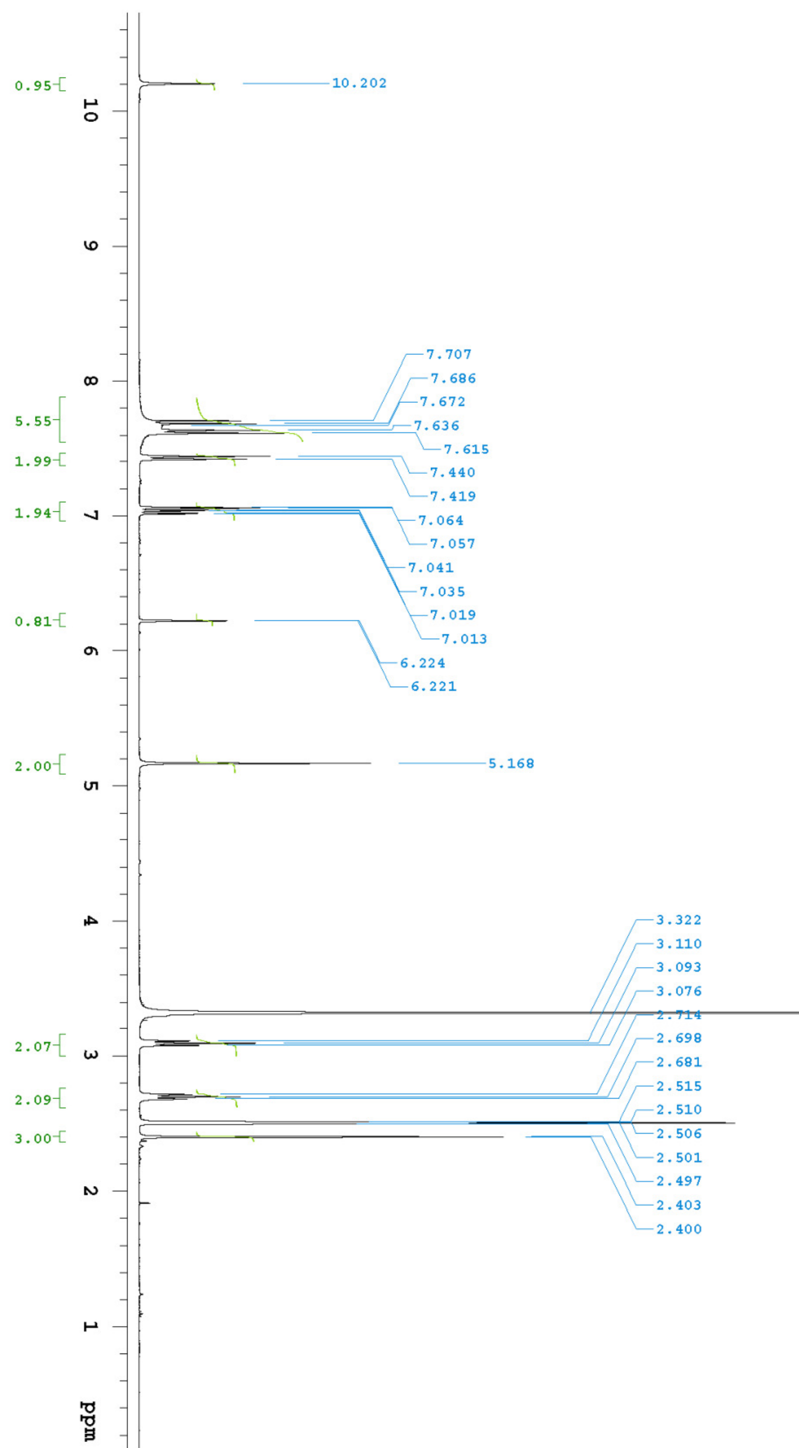
3.5 7-{4-(Boc-β-Alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin 7b ^{1}H NMR



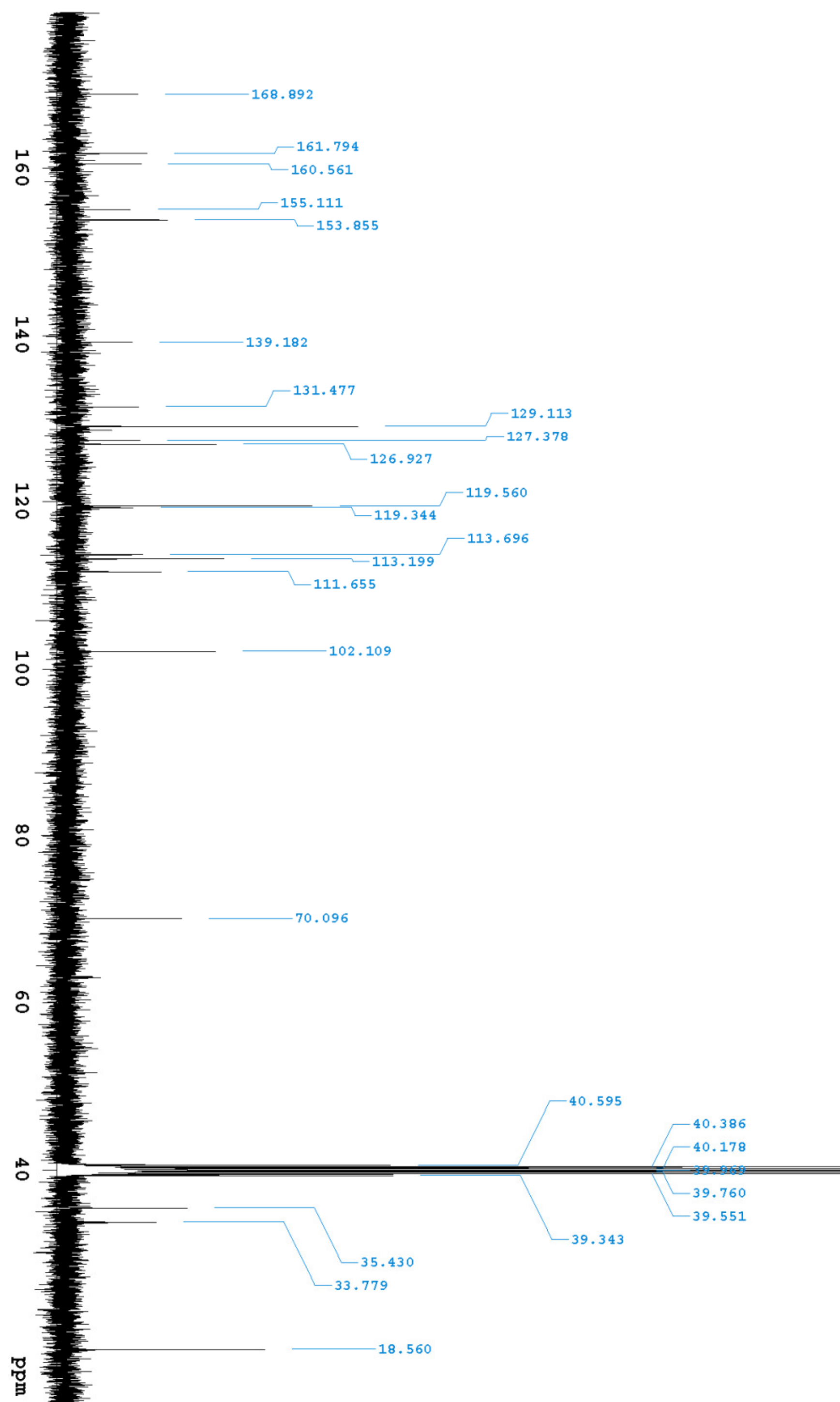
3.6 7-{4-(Boc-β-Alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin 7b ^{13}C NMR



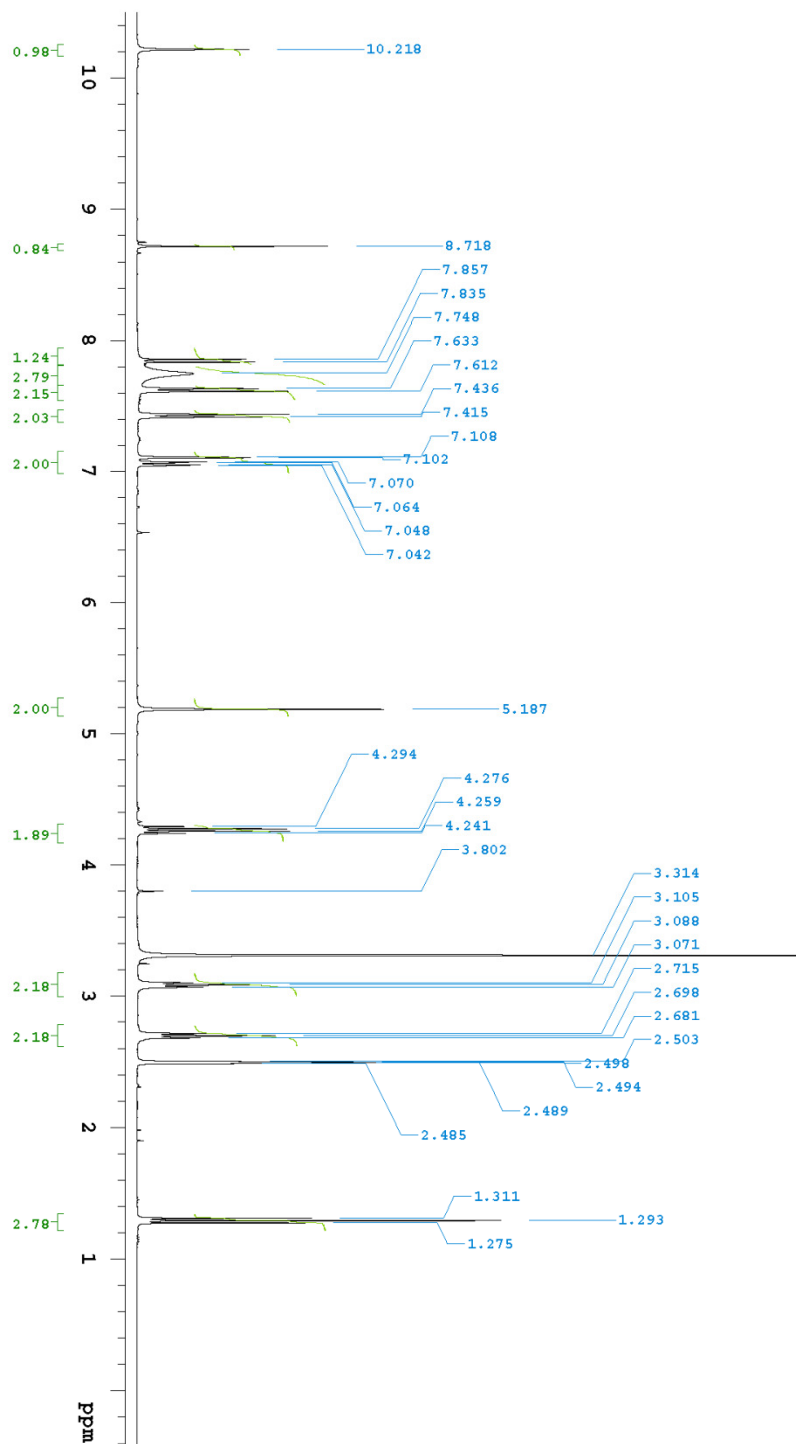
3.7 7-{4-(β-Alanylamido)}benzyloxy-4-methylcoumarin trifluoroacetate 8a ^{1}H NMR



3.8 7-{4-(β-Alanylamido)}benzyloxy-4-methylcoumarin trifluoroacetate 8a ^{13}C NMR



3.9 7-{4-(β-Alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin trifluoroacetate 8b
^{1}H NMR



3.10 7-{4-(β-Alanylamido)}benzyloxy-3-ethoxycarbonylcoumarin trifluoroacetate 8b
^{13}C NMR