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# Coumarin-based, switchable fluorescent substrates for enzymatic bacterial detection

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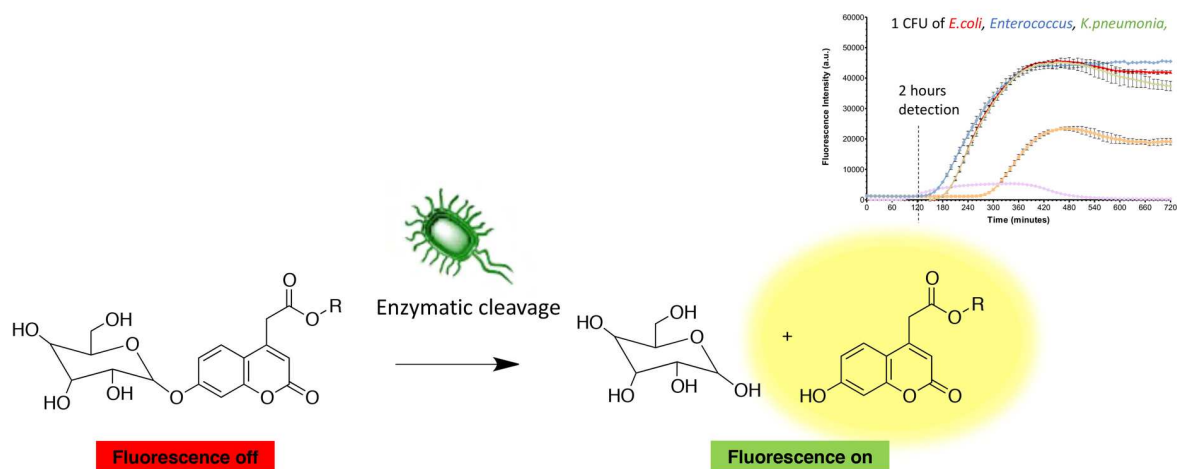
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## Abstract

Enzymatically-switchable fluorescent substrates, such as the commercially available 4-methylumbelliferones (4-MU) are used as standard indicators of enzymatic activity for the detection of various microorganisms and pathogens. However, a major disadvantage of 4-MU is its relatively high pKa leading to only partial dissociation of the fluorescent anion under the conditions where the enzymes are most effective (pH 6-6.5). Here we present a method for new, enzymatically-switchable, fluorescent substrates with improved photo-physico/chemical properties. The lead derivative, 4-AAU, shows excellent solubility in aqueous media (0.81mg/mL) when compared to 4-MU (0.16mg/mL), significantly improved quantum yield and wider dynamic range of its fluorescence properties. The corresponding bacterial substrate  $\beta$ -4-AAUG showed superior selectivity in the detection of clinically relevant amounts of *E. coli*, *Enterococcus* and *K. pneumonia* (1 CFU). The fluorescence intensity of  $\beta$ -4-AAUG was almost 5 times higher than that of the standard, the detection was possible in reasonably short time (~2.5 hours) and with excellent sensitivity.

32 **Graphical Abstract**

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41 **Introduction**  
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43 Infectious diseases cause millions of deaths and hospitalizations each year. Selective  
44 detection and identification of bacterial pathogens is therefore a scientific field that  
45 attracts significant interest from healthcare providers, industry and the general public.  
46 Despite the advances in micro-fabrication and nanotechnology [1], these infections are  
47 often misdiagnosed and there is an unacceptable delay in diagnosis [2].

48 Lab-on-a-chip devices could speed up and simplify several steps in this process. This,  
49 preferably portable, technology has promised it could provide an ideal solution to  
50 capture and identify specific bacterial species present in the sample medium [3,4].  
51 Many modern biosensors are fluorescence-based and they exploit switchable, 'on-off'  
52 fluorogenic probes as new tools for biological sensing and imaging [5]. These probes  
53 can provide specific and selective detection/labelling, producing low-background and  
54 high-contrast imaging [6]. From the commercially available 'on-off' fluorescent probes,  
55 coumarin-derivatives have been largely explored and have become very popular  
56 probes to detect metal ions, anions, small molecules [7,8], biological material  
57 (proteins, DNA, RNA etc.) [9,10] and enzymes [11-13]. The reference compound, 4-  
58 methyl-umbelliferone (**4-MU**), is readily available and its glycoside derivatives have  
59 proved efficient for detection of bacterial enzymes, such as  $\beta$ -galactosidase,  $\beta$ -

60 glucuronidase and  $\beta$ -glucosidase [14].  $\beta$ -glucosidase is produced by a wide array of  
61 microorganisms, such as exo-, endo- or ecto-enzymes [15] and belongs to the  
62 glycosidase family of metabolic enzymes, which are produced by heterotrophic  
63 bacteria.

64 However, 4-MU suffers from few notable disadvantages. Firstly, it has a relatively  
65 high pKa (7.8), [16] so the dissociation that yields the fluorescent anion is only partial  
66 at the pH values where enzymes (proteins) perform their functions. Therefore, when  
67 using 4-MU for enzymatic assay, addition of "stop buffer" is often needed at the end  
68 of the experiment in order to increase the fluorescent signal. However, this also  
69 quenches the enzymatic reaction. Secondly, 4-MU is relatively insoluble in aqueous  
70 solutions. This is undesirable considering the wide range of the fluorescent probe  
71 concentrations at which the enzyme activity is evaluated [17,18].

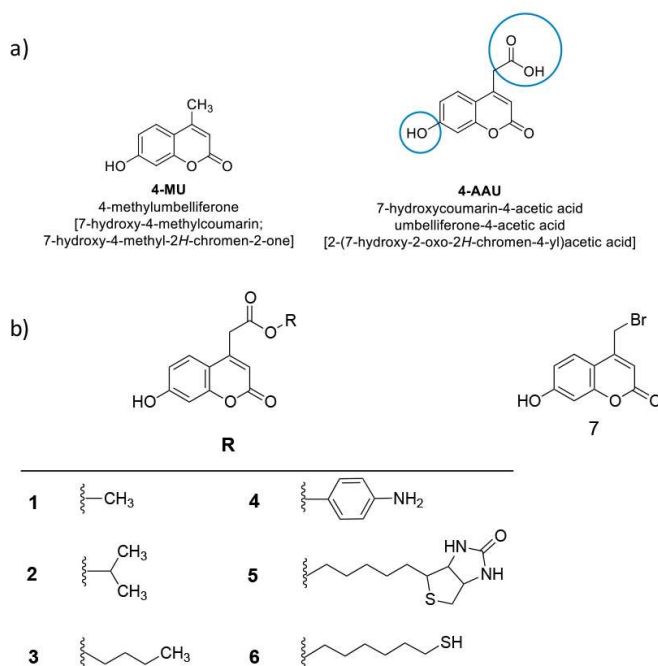
72 We have prepared seven, new umbelliferone derivatives and compared their photo-  
73 physico/chemical properties to 4-MU as the gold standard. The lead compound was  
74 then further modified to obtain the glycoside substrate, which was assessed for its  
75 ability to selectively detect various bacteria.

76

## 77 **Results and Discussion**

78 7-hydroxy-4-coumarin acetic acid (**4-AAU**) was used as starting material for the  
79 synthesis of the umbelliferone derivatives. 4-AAU has two obvious reactive sites: the  
80 phenolic group at position 7 and the carboxylic acid group at position 4 of the  
81 benzopyrone ring (*Figure 1a*). Because changes at position 4 do not alter the  
82 switchable properties of the compound, all derivatives have been synthesised by  
83 modifying the carboxyl group. The only exception is compound **7**, for which the  
84 umbelliferone ring was synthesised via Pechmann condensation between resorcinol  
85 with ethyl 4-bromoacetoacetate in 70% aqueous sulfuric acid (*Figure 1b*). Esters 1-4  
86 were prepared by Fisher-Speier esterification of 4-AAU with the respective alcohol. For  
87 the synthesis of compound **5**, D-biotin was firstly methylated and reduced to yield the  
88 terminal alcohol, which was then used for the esterification **4-AAU** using an EDC  
89 coupling reaction. The synthesis of compound **6** first involved dimerization of 6-  
90 mercaptoethanol through the formation of a disulphide bond. This di-ol derivative

91 was mixed with two equivalents of **4-AAU** and EDC to give the dimeric coumarin  
92 derivate.



93 *Figure 1: a) Structures of 4-MU and 4-AAU with highlighted reactive sites in positions 4 and 7. b) 4-AAU was used as starting material for the synthesis of umbelliferone derivatives 1-6 except for compound 7*

94

95 The disulfide bond was maintained until compound 6 was required, in order to avoid  
96 any possible intramolecular reaction during storage. When required 6 was produced  
97 by addition of dithiothreitol (DTT). All synthetic protocols and compound  
98 characterisation can be found in ESI.

99 The solubility and fluorescent properties of all compounds were evaluated and  
100 compared with those of 4-MU. In order to determine the optical properties of  
101 compounds synthesised, excitation/emission spectra were recorded for all compounds  
102 in water at a concentration of 100  $\mu$ M, as shown in *Figure S1a and S1b* (ESI). The  
103 absorbance peaks were broad and showed a slight red shift. Absorption and  
104 fluorescence spectra were recorded in water (pH 6) and the quantum yields were  
105 calculated for each compound using 4-MU as a reference (quantum yield 0.74 in water  
106 at pH 6 [18]). Derivatives **4-AAU**, **1**, **2**, **3** and **4** showed superior fluorescence properties,  
107 with their quantum yields being greater than 0.89 (*Fig. S1c, ESI*). Compounds **2**, **3**, **5**  
108 and **7** were poorly soluble in water, although they are more soluble than **4-MU** (*Fig.*  
109 *S1c, ESI*). Compounds **1** and **4**, were found to have the best solubility in water (0.81

110 and 0.73 mg/mL respectively), which is a significant improvement on **4-MU** (0.16  
111 mg/mL).

112 One limitation of **4-MU** is the high  $pK_a$ , due to which its fluorescence increases only  
113 at pH above 7. Therefore, the fluorescence properties of the synthesised compounds  
114 have been evaluated at a pH range between 4 and 8. As shown in ESI (*Figure S2*), the  
115 fluorescent signal of all new derivatives starts to increase between pH 6-6.5 rather than  
116 at pH 7-7.5 as it is in the case of **4-MU**. The best results were observed for compounds  
117 **1** and **2**: between pH 6 and 7, the fluorescence signal measured for **4-MU** was low  
118 (~1000 a.u.), while for compound **1** and **2**, the signal almost doubled between 6 to 6.5  
119 pH (from 4000 to 8000 a.u.). At pH 7.5, the **4-MU** signal rose to 3000, but at this pH  
120 the fluorescence signal for compound **1** was almost 7 times higher (20000 a.u.).

121 Besides the improvements in fluorescence signal and solubility, each derivative was  
122 also evaluated for its possible inhibitory effect on bacterial cell growth. 1 CFU of *E.coli*  
123 was treated with 500, 50 and 5  $\mu$ M of each substrate, incubated upon shaking at 37°C  
124 and the absorbance at 690 nm was recorded every 20 minutes. An increase in signal  
125 indicated an increase in turbidity, which showed that the bacteria were growing over  
126 time. From the results presented in ESI (*Figure S3*), it is evident that only compound **7**  
127 showed mild inhibitory effect on the bacterial growth at 500  $\mu$ M.

128 Considering its favourable fluorescent properties, solubility and toxicity, compound  
129 **1** was further exploited for the synthesis of the substrate to be used in bacterial  
130 enzyme detection. The glycosylic bond between **1** and the protected  $\alpha$ -glucose  
131 bromide was introduced by Williamson reaction to provide the desired substrate  **$\beta$ -4-**  
132 **AAUG** (full details in ESI). We originally attempted to use the  $\beta$ -glucose bromide to  
133 obtain the  $\beta$ -substrate but upon completion of this reaction, we observed an  
134 anomerisation of the  $\beta$ -glucose unit into its  $\alpha$ -anomer. This anomerisation is poorly  
135 described in the literature. It occurred either under the basic conditions of the  
136 Williamson and/or the deacetylation reaction, which lead to the opening form of the  
137 carbohydrate and the subsequent rotation of the asymmetric centre (the carbon in  
138 position 1). *Figure 2a* depicts the anomerisation from the  $\alpha$ -anomer to the  
139 corresponding  $\beta$ -anomer. The polarimetry values measured for the synthesised  
140 substrate  **$\beta$ -4-AAUG** and the commercial  **$\beta$ -4-MUD** were  $-1.258^\circ$  and  $-2.958^\circ$ ,

141 respectively, values that correspond to  $\beta$ -anomers (*Figure 2b*). The measured specific  
142 optical rotation of commercially available  $\alpha$ -4-MUD was  $+148^\circ$ , a value that was in  
143 agreement with that provided by the manufacturer. The anomerisation was surprising,  
144 as it is not often clearly stated in chemistry-related publications. However, some work  
145 found in literature states that  $\alpha$ -glucose was used as starting material for the synthesis  
146 of the substrate, but that it was then recognised by  $\beta$ -glucosidase [16,17]. The  
147 structure of the three substrates tested in the enzymatic and bacterial experiments are  
148 presented in *Figure 2c*.

149 The selectivity and sensitivity of  $\beta$ -4-AAUG for the  $\beta$ -glucosidase was then evaluated  
150 and compared with  $\alpha$ -4-MUD and  $\beta$ -4-MUD as references. The optimal concentration  
151 range of the substrate in enzymatic assays was determined experimentally to be  
152 between 25 and 100  $\mu$ M. When  $\beta$ -4-AAUG was treated with  $\beta$ -glucosidase, an increase  
153 of fluorescence, indicating the presence of the enzyme, was detected after a few  
154 minutes (*Figure S4*, ESI). The selectivity of the substrate for the specific enzyme was  
155 then evaluated by treating  $\beta$ -4-AAUG and  $\alpha$ -4-MUD with  $\alpha$ -glucosidase and then a cake  
156 enzyme (from baking powder) that contains a mixture of glucosidases (*Figure 3*). As  
157 expected, a strong fluorescence signal was measured relatively quickly after addition  
158 of  $\alpha$ -glucosidase to  $\alpha$ -4-MUD, while a negligible signal was obtained for  $\beta$ -4-AAUG  
159 (*Figure 3a*).

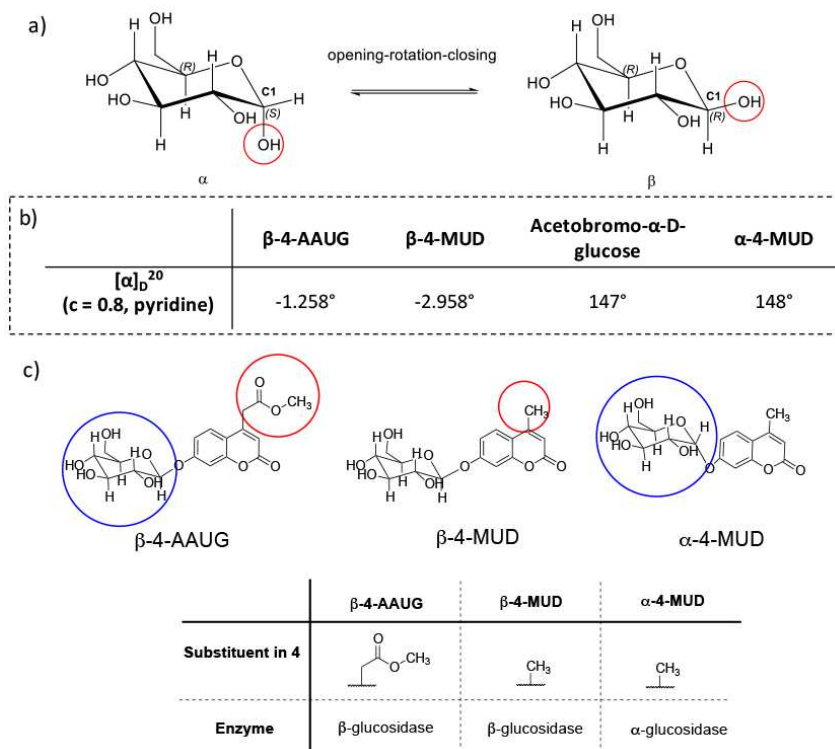
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Figure 2: a) In aldohexoses, the rotation occurs at C1 (asymmetric centre) of the open form of the glucose. b) Polarimetry measurements of the glucose-based compounds and c) structure of the three compounds tested in the enzymatic and bacterial assays

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168 A similar experiment was performed using the 'cake enzyme', purchased as baking  
 169 powder from a local shop. The results on Figure 3b show great selectivity of  **$\beta$ -4-AAUG**  
 170 for  $\beta$ -glucosidase and indicate that the main component of the baking mixture is,  
 171 indeed,  $\beta$ -glucosidase. The  $\alpha$ -type was present in lower amount since the fluorescent  
 172 signal increased only slightly using  **$\alpha$ -4-MUD**.

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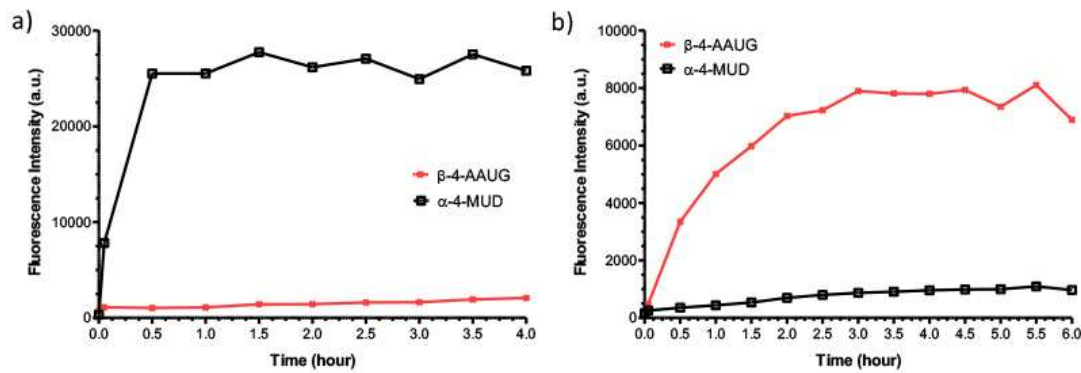


Figure 3: a)  $\beta$ -4-AAUG and  $\alpha$ -4-MUD were tested at concentration 0.1 mM in presence of  $\alpha$ -glucosidase (250  $\mu$ g/mL); and b) in the presence of 2.5 mg/mL of the 'cake enzyme'.

184

185 Furthermore, the different sensitivity between the substrates  $\beta$ -4-AAUG and  $\beta$ -4-MUD  
 186 in detecting the activity of the specific enzyme,  $\beta$ -glucosidase, was evaluated by  
 187 comparing parameters determined from the Michaelis-Menten equation, one of the  
 188 best-known models for enzyme kinetics. In this model, the kinetic rate of an  
 189 enzymatically-catalysed reaction is related to the concentration of the substrate used,  
 190 as shown in the equation below:

$$191 \quad v = \frac{V_{max} [S]}{K_m + [S]}$$

192 The lower the  $K_m$  (Michaelis constant), the lower is the amount of substrate required  
 193 to reach the saturation of the enzyme ( $V_{max}$ ). Therefore,  $K_m$  is one of the key  
 194 parameters used to indicate the affinity of the enzyme for the substrate. Similarly, the  
 195 higher the  $K_m$ , the lower is the affinity of the protein for the substrate. By evaluating  
 196 the  $V_{max}$  and  $K_m$  values, it was evident that the affinity of the  $\beta$ -glucosidase enzyme for  
 197  $\beta$ -4-AAUG is 5-times higher than for  $\beta$ -4-MUD in the concentration range 15  $\mu$ M to 2  
 198 mM (Table 1).

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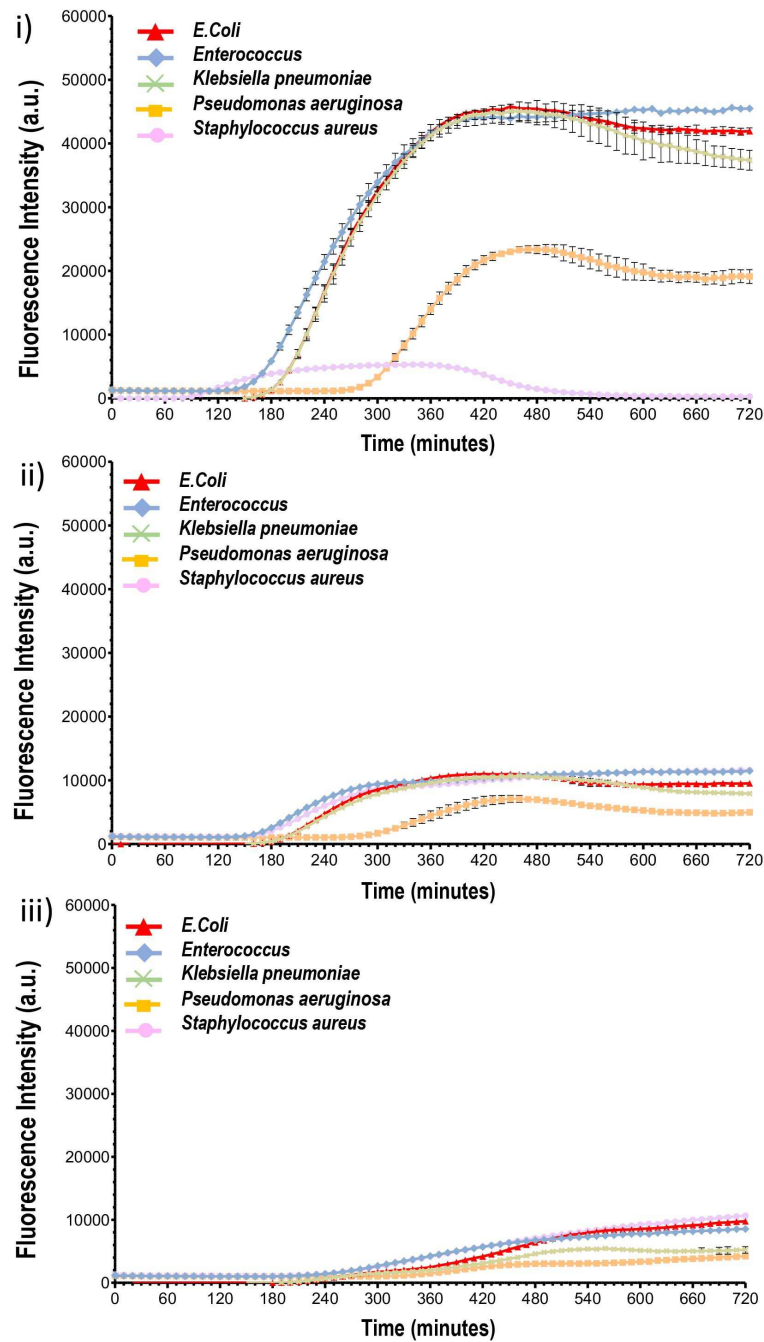
Table 1: Values calculated for  $\beta$ -4-AAUG and  $\beta$ -4-MUD using the Michaelis-Menten equation.

	$V_{max}$	$K_m$	$k_{cat}$	$K_{cat}/k_m$
$\beta$ -4-AAUG	0.022	804	$4.5 \times 10^{-4}$	$5.6 \times 10^{-7}$
$\beta$ -4-MUD	0.027	4032	$5.4 \times 10^{-4}$	$1.3 \times 10^{-7}$

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The catalytic efficiency parameter ( $k_{\text{cat}}/K_m$ ), an indicator of how efficiently the enzyme converts a substrate into the product, is also more than 4-times higher for  **$\beta$ -4-AAUG** (Figure S5, ESI).

Finally, the efficacy of **1** in the detection of 1 CFU of bacteria (*E. coli*, *Enterococcus*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*) was evaluated using 50  $\mu\text{M}$  of  **$\beta$ -4-AAUG**,  **$\alpha$ -4-MUD** and  **$\beta$ -4-MUD**. As seen in *Figure 4a*, 50  $\mu\text{M}$  of  **$\beta$ -4-AAUG** can be used to detect 1 CFU of *E.coli*, *Enterococcus* and *K.Pneumoniae* in about 2.5-3 hours of incubation.  **$\beta$ -4-AAUG** appeared to be less sensitive to the presence of *P. aeruginosa*, given that an increase of signal was observed only after 5 hours. The low signal detected in presence of *S. aureus* limits the possibility of using this substrate for the detection of this type of bacterium. *Figures 4b and 4c* present the data for the detection of the same concentration of the five different types of bacteria using 50  $\mu\text{M}$  of  **$\beta$ -4-MUD** and  **$\alpha$ -4-MUD**. While  **$\beta$ -4-MUD** and  **$\alpha$ -4-MUD** cannot discriminate between different bacterial species with high selectivity or sensitivity,  **$\beta$ -4-AAUG** can detect *E. coli*, *Enterococcus* and *K. pneumoniae* after 2.5 – 3h. The maximum signal that was reached by using  **$\beta$ -4-AAUG** was almost 5-fold higher than that reached with  **$\beta$ -4-MUD**. Interestingly,  **$\beta$ -4-MUD** appeared to be better at detecting *S. aureus* compared with  **$\beta$ -4-AAUG**, which indicates that the fluorophore does partially play some role in the enzyme-substrate affinity. Similar data was obtained by treating the bacterial samples at concentrations of 2.5 CFU (*Table S1A, ESI*) and 150 CFU (*Table S1B, ESI*). Further details concerning the bacterial experiments can be found in ESI (Figures S6-S8).



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Figure 4: i) 50  $\mu$ M of  $\beta$ -4-AAUG ii) of  $\beta$ -4-MUD and iii) of  $\alpha$ -4-MUD were treated with 1 CFU of each type of bacteria

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236 Two conclusions can be drawn from these experiments: i)  $\beta$ -4-AAUG remains the  
 237 superior fluorescent probe for the detection of *E. coli*, *Enterococcus* and *K. pneumoniae*  
 238 and ii) the speed of the detection does not appear to be proportional to the increased  
 239 concentration of the bacteria in the samples. The increase in fluorescence was

240 recorded only approximately 1 hour earlier, despite the fact that the bacterial samples  
241 were 60 times more concentrated (Table S1).

242

## 243 **Conclusion**

244 Overall, all synthesised compounds, with the exception of compound **7**, were very  
245 suitable analogues for the development of switchable probes. Their fluorescence  
246 increases between pH 6 and 6.5, conditions at which enzymatic and bacterial assays  
247 are normally performed. Considering the  $\Phi$  values and the solubility tests, compounds  
248 **1** and **4** showed improved photo-physico/chemical properties compared to the 4-MU  
249 standard. Compound **1** was further glycosylated to give the substrate  **$\beta$ -4-AAUG**.  
250 During this reaction, we observed an anomerisation of the glucose unit, a phenomenon  
251 rarely (if ever) reported before.  **$\beta$ -4-AAUG** showed to be selective for the enzyme  $\beta$ -  
252 glucosidase, with no increase in fluorescent signal observed when it was treated with  
253  $\alpha$ -glucosidase. The enzyme  $\beta$ -glucosidase also showed to have higher affinity towards  
254  **$\beta$ -4-AAUG** than to  **$\beta$ -4-MUD**, as confirmed by the lower  $K_m$  and higher  $K_{cat}/K_m$  values.  
255 In addition, the fluorescence intensity of  **$\beta$ -4-AAUG** was almost 5 times higher than  
256 that of  **$\beta$ -4-MUD**, leading to a better signal-to-noise ratio. As a general conclusion, our  
257 work indicates that the activity of  $\beta$ -glucosidase and its detection is closely related not  
258 only to the carbohydrate (recognition element) but also to the structure of the  
259 fluorophore (transducer element), which is in agreement with the literature [19,20].  
260 The development of new generations of switchable fluorophores, such as those  
261 synthesised here, will pave the way for less laborious and time-consuming, more  
262 accurate, robust and reliable detection of selected species of bacteria. We believe that  
263 our results will motivate scientists to develop faster, reliable and low-cost systems for  
264 the selective detection of whole bacteria, ideally without resorting to identifying its  
265 metabolites, growing cultures or using PCR to identify the DNA signatures [21],  
266 processes typically requiring from 48 to 72h.

267

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269 scholarship

270 **References**

- 271 [1] W. Lee, D. Kwon, W. Choi, G. Y. Jung, A. K. Au, A. Folch and S. Jeon, 3D-printed  
272 microfluidic device for the detection of pathogenic bacteria using size-based  
273 separation in helical channel with trapezoid cross-section, *Sci. Rep.*, 5 (2015) 7717,  
274 <http://dx.doi.org/10.1038/srep07717>.  
275
- 276 [2] A. Ahmed, J. V. Rushworth, N. A. Hirst and P. A. Millner, Biosensors for Whole-Cell  
277 Bacterial Detection, *Clin. Microbiol. Rev.*, 27 (2014) 631-646,  
278 <http://dx.doi.org/10.1128/CMR.00120-13>.
- 279
- 280 [3] H. Yan, Y. Zhu, Y. Zhang, L. Wang, J. Chen, Y. Lu, Y. Xu and W. Xing, Multiplex  
281 detection of bacteria on an integrated centrifugal disk using bead-beating lysis and  
282 loop-mediated amplification, *Sci. Rep.*, 7 (2017) 1460,  
283 <http://dx.doi.org/10.1038/s41598-017-01415-x>  
284
- 285 [4] J. Mairhofer, K. Roppert and P. Ertl, Microfluidic Systems for Pathogen Sensing: A  
286 Review, *Sensors*, 9 (2009) 4804-4823, <http://dx.doi.org/10.3390/s90604804>.  
287
- 288 [5] N. Sanvicens, C. Pastells, N. Pascual and M. P. Marco, *Nanoparticle-based biosensors*  
289 *for detection of pathogenic bacteria*, *Trends Anal. Chem.*, 28 (2009) 1243-1252,  
290 <http://dx.doi.org/10.1016/j.trac.2009.08.002>.  
291
- 292 [6] H. Lai, Y. Xiao, S. Yan, F. Tian, C. Zhong, Y. Liu, X. Weng and X. Zhou, Symmetric  
293 cyanovinylyl-pyridinium triphenylamine: a novel fluorescent switch-on probe for an  
294 antiparallel G-quadruplex, *Analyst*, 139 (2014) 1834-1838, [http://dx.doi.org/](http://dx.doi.org/10.1039/C3AN02269B)  
295 [10.1039/C3AN02269B](http://dx.doi.org/10.1039/C3AN02269B).  
296
- 297 [7] D. Gong, Y. Tian, C. Yang, A. Iqbal, Z. Wang, W. Liu, W. Qin, X. Zhu and H. Guo, A  
298 fluorescence enhancement probe based on BODIPY for the discrimination of cysteine  
299 from homocysteine and glutathione, *Biosens. Bioelectron.*, 85 (2016) 178-183,  
300 <http://dx.doi.org/10.1016/j.bios.2016.05.013>.  
301
- 302 [8] K. Xu, X. Liu, B. Tang, G. Yang, Y. Yang and L. An, Design of a phosphinate-based  
303 fluorescent probe for superoxide detection in mouse peritoneal macrophages, *Chem.*  
304 *Eur. J.*, 13 (2007) 1411-1416, <http://dx.doi.org/10.1002/chem.200600497>.  
305
- 306 [9] L. Wang, L. Yuan, X. Zeng, J. Peng, Y. Ni, J. C. Er, W. Xu, B. K. Agrawalla, D. Su, B. Kim  
307 and Y.-T. Chang, A Multisite-Binding Switchable Fluorescent Probe for Monitoring  
308 Mitochondrial ATP Level Fluctuation in Live Cells, *Angew. Chem. Int. Ed.*, 55 (2016)  
309 1773-1776, <http://dx.doi.org/10.1002/anie.201510003>.  
310
- 311 [10] H. Kwon, K. Lee and H.-J. Kim, An aryl-thioether substituted nitrobenzothiadiazole  
312 probe for the selective detection of cysteine and homocysteine, *Chem. Commun.*, 47  
313 (2011) 1773-1775, <http://dx.doi.org/10.1039/C5CC01071C>.  
314
- 315 [11] J.-A. Richard, M. Massonneau, P.-Y. Renard and A. Romieu, 7-  
316 Hydroxycoumarin-Hemicyanine Hybrids: A New Class of Far-Red Emitting

- 317 Fluorogenic Dyes, *Org. Lett.*, 10 (2008) 4175-4178,  
318 <http://dx.doi.org/10.1021/ol801582w>.  
319
- 320 [12] Y. Meyer, J.-A. Richard, M. Massonneau, P.-Y. Renard and A. Romieu, Development  
321 of a New Nonpeptidic Self-Immolative Spacer. Application to the Design of Protease  
322 Sensing Fluorogenic Probes, *Org. Lett.*, 10 (2008) 1517-1520,  
323 <http://dx.doi.org/10.1021/ol800198f>.  
324
- 325 [13] H.-C. Huang, K.-L. Wang, S.-T. Huang, H.-Y. Lin and C.-M. Lin, Development of a  
326 sensitive long-wavelength fluorogenic probe for nitroreductase: a new fluorimetric  
327 indicator for analyte determination by dehydrogenase-coupled biosensors, *Biosens.*  
328 *Bioelectron.*, 26 (2011) 3511-3516, <http://dx.doi.org/10.1016/j.bios.2011.01.036>.  
329
- 330 [14] J. D. Perry, A. L. James, K. A. Morris, M. Oliver, K. F. Chilvers, R. H. Reed and F. K.  
331 Gould, Evaluation of novel fluorogenic substrates for the detection of glycosidases in  
332 *Escherichia coli* and enterococci, *J. Appl. Microbiol.*, 101 (2006) 977-985,  
333 <http://dx.doi.org/10.1111/j.1365-2672.2006.03018.x>.  
334
- 335 [15] V. Veena, P. Poornima, R. Parvatham, K. Sivapriyadharsini and K. Kalaiselvi, Isolation  
336 and characterization of  $\beta$ -glucosidase producing bacteria from different sources, *Afr.*  
337 *J. Biotechnol.*, 10 (2011) 14907-14912, <http://dx.doi.org/10.5897/AJB09.314>.  
338
- 339 [16] N. Baggett, M. A. Case, P. R. Darby and C. J. Gray, Action of almond  $\beta$ -d-glucosidase  
340 on fluorogenic substrates derived from 4-substituted 7-hydroxycoumarins, *Enz.*  
341 *Microb. Technol.*, 15 (1993) 742-748, [http://dx.doi.org/10.1016/0141-](http://dx.doi.org/10.1016/0141-0229(93)90004-L)  
342 [0229\(93\)90004-L](http://dx.doi.org/10.1016/0141-0229(93)90004-L).  
343
- 344 [17] C. Malet, J. L. Viladot, A. Ochoa, B. Gallégo, C. Brosa and A. Planas, Synthesis of 4-  
345 methylumbelliferyl-beta-D-glucan oligosaccharides as specific chromophoric  
346 substrates of (1 $\rightarrow$ 3), (1 $\rightarrow$ 4)-beta-D-glucan 4-glucanohydrolases., *Carbohydr. Res.*,  
347 274 (1995) 285-301, [http://dx.doi.org/10.1016/0008-6215\(95\)00102-Y](http://dx.doi.org/10.1016/0008-6215(95)00102-Y).  
348
- 349 [18] K. F. Chilvers, J. D. Perry, A. L. James and R. H. Reed, Synthesis and evaluation of  
350 novel fluorogenic substrates for the detection of bacterial beta-galactosidase, *J.*  
351 *Appl. Microbiol.*, 91 (2001) 1118-1130, [http://dx.doi.org/10.1046/j.1365-](http://dx.doi.org/10.1046/j.1365-2672.2001.01484.x)  
352 [2672.2001.01484.x](http://dx.doi.org/10.1046/j.1365-2672.2001.01484.x).  
353
- 354 [19] K. J. Panosian and S. C. Edberg, Rapid identification of *Streptococcus bovis* by using  
355 combination constitutive enzyme substrate hydrolyses, *J. Clin. Microbiol.*, 27 (1989)  
356 1719-1722, PMID: PMC267660.  
357
- 358 [20] X. Wei, Q. Wu, J. Zhang, Y. Zhang, W. Guo, M. Chen, Q. Gu, Z. Cai and M. Lu,  
359 Synthesis of precipitating chromogenic/fluorogenic  $\beta$ -glucosidase/ $\beta$ -galactosidase  
360 substrates by a new method and their application in the visual detection of  
361 foodborne pathogenic bacteria, *Chem. Commun.*, 53 (2017) 103-106,  
362 <http://dx.doi.org/10.1039/C6CC07522C>.  
363

364 [21] M. E. E. Alahi and S. C. Mukhopadhyay, Detection Methodologies for Pathogen and  
365 Toxins: A Review, *Sensors*, 17 (2017) 1-20, <http://dx.doi.org/10.3390/s17081885>.

366

367