Coumarin-based, switchable fluorescent substrates for enzymatic bacterial detection

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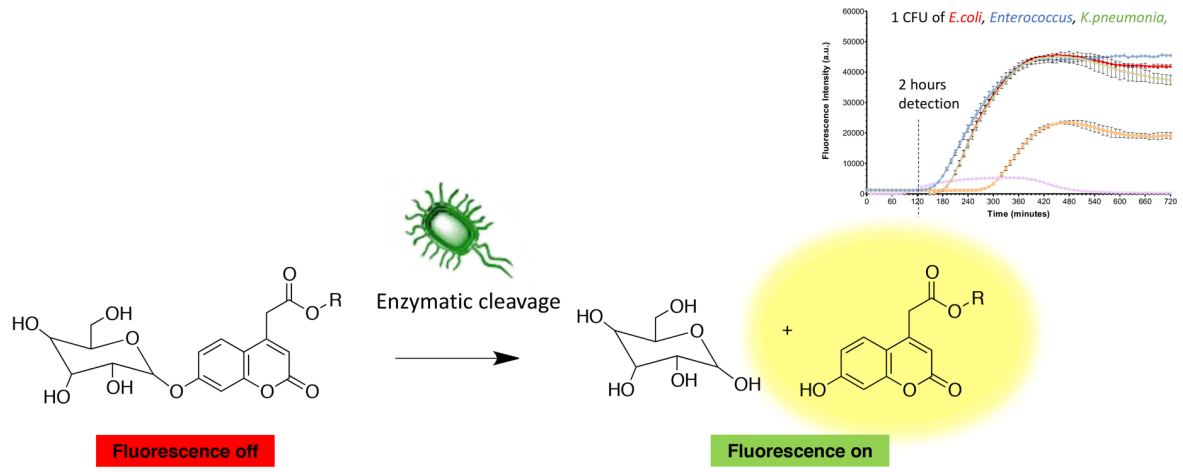
Abstract

Enzymatically-switchable fluorescent substrates, such as the commercially available 4-methyl umbelliferones (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 acqueous 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 β-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 β-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.

Graphical Abstract



Introduction

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

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

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

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

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

Results and Discussion

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

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

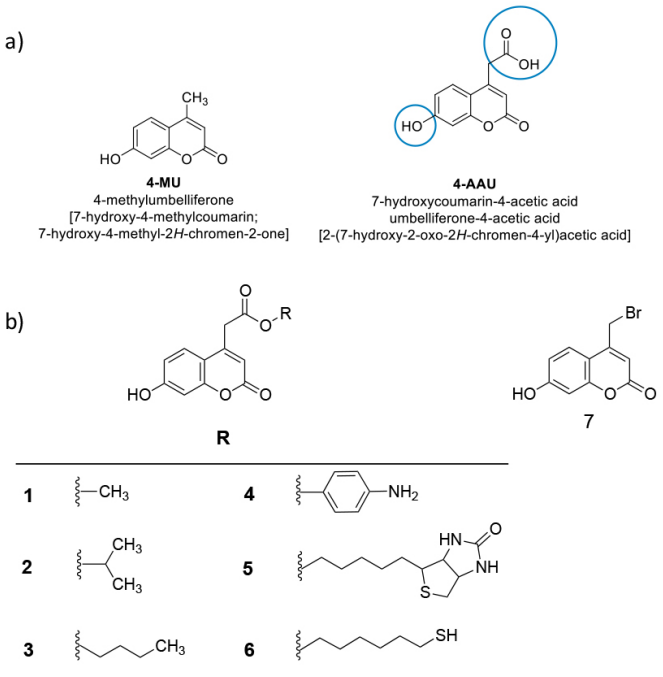


*Figure 1: a) Structures of **4-MU** and **4-AAU** with highlighted reactive sites in positions 4 and 7. b) 4-AAU was used as starting material for the synthesis of umbelliferone derivatives **1-6** except for compound 7*

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

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

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

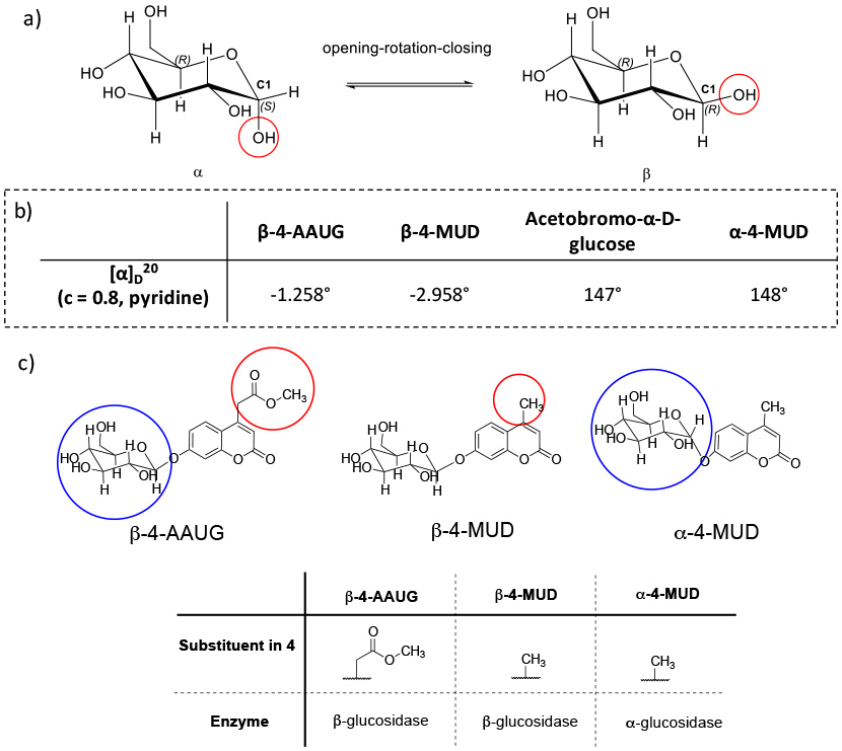
One limitation of **4-MU** is the high pK_a, due to which its fluorescence increases only at pH above 7. Therefore, the fluorescence properties of the synthesised compounds have been evaluated at a pH range between 4 and 8. As shown in ESI *(Figure S2)*, the fluorescent signal of all new derivatives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

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

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

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

The selectivity and sensitivity of **β-4-AAUG** for the β-glucosidase was then evaluated and compared with **α-4-MUD** and **β-4-MUD** as references. The optimal concentration range of the substrate in enzymatic assays was determined experimentally to be between 25 and 100 µM. When **β-4-AAUG** was treated with β-glucosidase, an increase of fluorescence, indicating the presence of the enzyme, was detected after a few minutes (Figure S4, ESI). The selectivity of the substrate for the specific enzyme was then evaluated by treating **β-4-AAUG** and **α-4-MUD** with α-glucosidase and then a cake enzyme (from baking powder) that contains a mixture of glucosidases *(Figure 3)*. As expected, a strong fluorescence signal was measured relatively quickly after addition of α-glucosidase to **α-4-MUD,** while a negligible signal was obtained for **β-4-AAUG** *(Figure 3a).*



	β-4-AAUG	β-4-MUD	Acetobromo-α-D-glucose	α-4-MUD
$[\alpha]_D^{20}$ (c = 0.8, pyridine)	-1.258°	-2.958°	147°	148°

	β-4-AAUG	β-4-MUD	α-4-MUD
Substituent in 4			
Enzyme	β-glucosidase	β-glucosidase	α-glucosidase

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

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

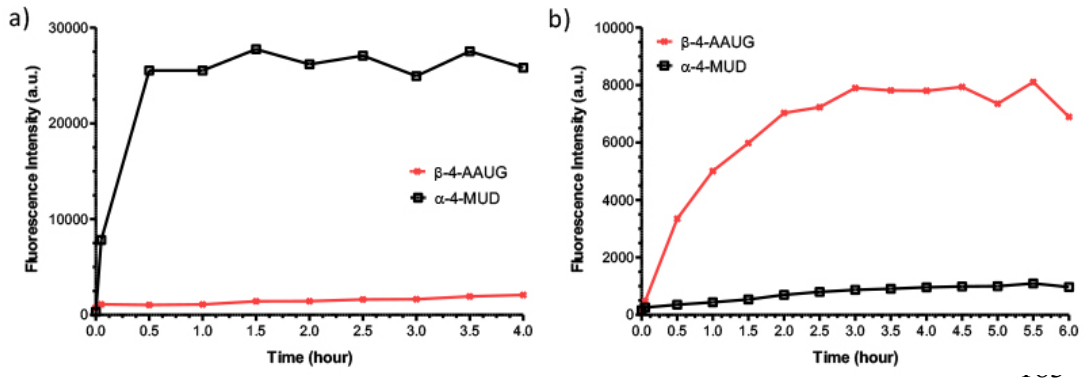


*Figure 3: a) **β-4-AAUG** and **α-4-MUD** were tested at concentration 0.1 mM in presence of α-glucosidase (250 µg/mL); and b) in the presence of 2.5 mg/mL of the 'cake enzyme'.*

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

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

The lower the K_m (Michaelis constant), the lower is the amount of substrate required to reach the saturation of the enzyme (V_{max}). Therefore, K_m is one of the key parameters used to indicate the affinity of the enzyme for the substrate. Similarly, the higher the K_m, the lower is the affinity of the protein for the substrate. By evaluating the V_{max} and K_m values, it was evident that the affinity of the β-glucosidase enzyme for **β-4-AAUG** is 5-times higher than for **β-4-MUD** in the concentration range 15 µM to 2 mM (Table 1).

*Table 1: Values calculated for **β-4-AAUG** and **β-4-MUD** using the Michaelis-Menten equation.*

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

The catalytic efficiency parameter (k_{cat}/K_m), an indicator of how efficiently the enzyme converts a substrate into the product, is also more than 4-times higher for **β-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 µM of **β-4-AAUG, α-4-MUD** and **β-4-MUD**. As seen in *Figure 4a*, 50 µM of **β-4-AAUG** can be used to detect 1 CFU of *E.coli, Enterococcus and K.Pneumoniae* in about 2.5-3 hours of incubation. **β-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. aereus* 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 µM of **β-4-MUD** and **α-4-MUD**. While **β-4-MUD** and **α-4-MUD** cannot discriminate between different bacterial species with high selectivity or sensitivity, **β-4-AAUG** can detect *E. coli, Enterococcus* and *K. pneumoniae* after 2.5 – 3h. The maximum signal that was reached by using **β-4-AAUG** was almost 5-fold higher than that reached with **β-4-MUD.** Interestingly, **β-4-MUD** appeared to be better at detecting *S. aureus* compared with **β-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).

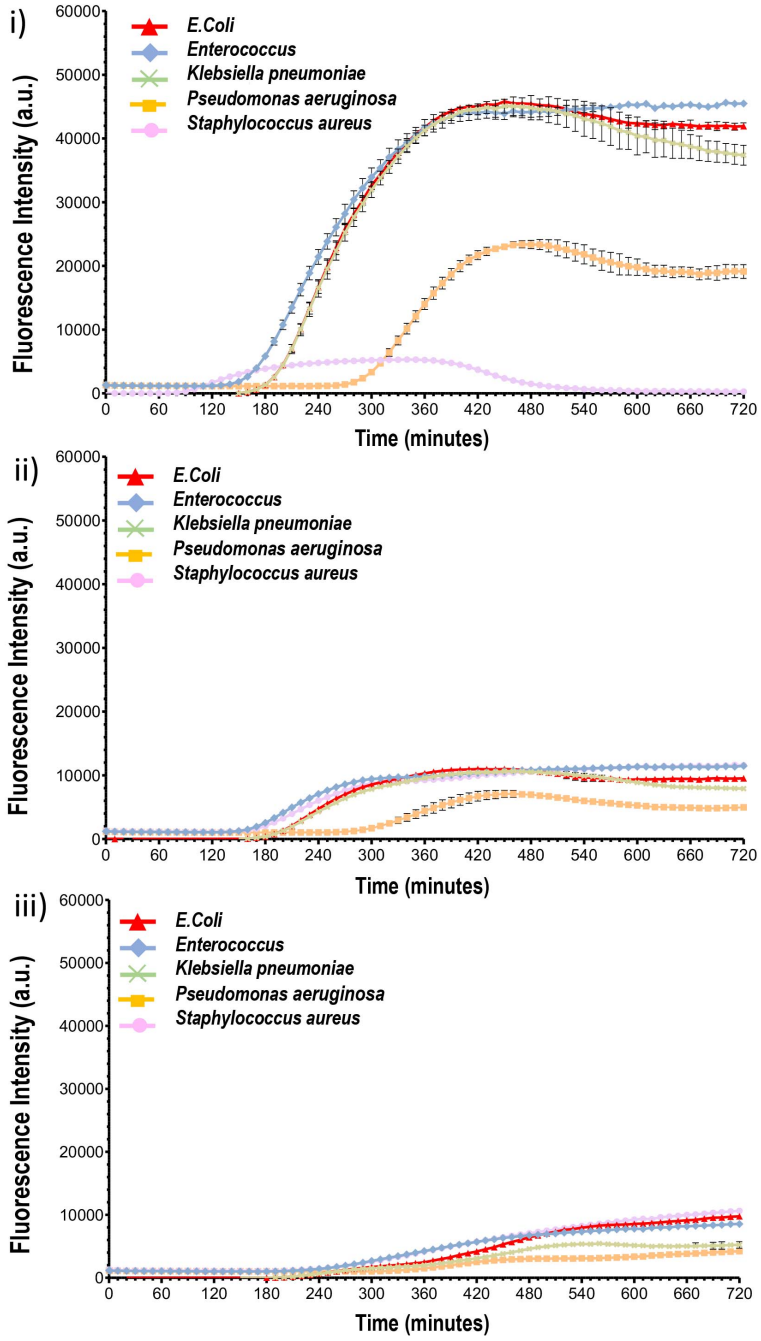


Figure 4: i) 50 μM of β-4-AAUG ii) of β-4-MUD and iii) of α-4-MUD were treated with 1 CFU of each type of bacteria

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

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

Conclusion

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

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