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Monogalactopyranosides of fluorescein and fluorescein methyl ester: synthesis, enzymatic hydrolysis by biotnylated β-galactosidase, and determination of translational diffusion coefficient

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ABSTRACT

Fluorescein monoglycosides (D-galactopyranoside (FMG) and D-glucopyranoside) and their methyl ester (MFMG) have been prepared from acetobromoglucose/galactose and fluorescein methyl ester in good yields. Enzymatic hydrolysis experiments (using biotinylated **β**-galactosidase) of the *galacto* derivatives have been performed and kinetic parameters were calculated. A 15–20 times increase of the fluorescence intensity has been observed during the hydrolysis. A linear increase of fluorescence has been noted at short time and low concentration of substrate, making these compounds useful and sensitive probes for galactosidases. The magnitude of the Michaelis–Menten constant (K_m) value for MFMG is higher than that of FMG suggesting a possible conformational change of the fluorogenic substrate. K_m value for biotinylated β-Gal with FMG is lower than that for the native enzyme. This observation indicates higher substrate affinity of the biotinylated enzyme in comparison to the native enzyme. Translational diffusion coefficients have been measured, for both fluorogenic substrates and both the products, employing fluorescence correlation spectroscopy. Translational diffusion coefficients for fluorogenic substrates and the enzymatic hydrolysis products have been measured to be similar, in the range of 3.5–4.5 $\times 10^{-10}$ m^2 s^{-1}. Thus an enhancement or retardation of the enzymatic kinetics due to difference in translational mobility of substrate and product is not that apparent.

1. Introduction

Fluorogenic glycosides are important probes for reporting glycosidase activities. In such compounds, a sugar blocks a chemical group necessary for fluorescence through a glycosidic linkage. Fluorescein is a suitable dye for that purpose and fulfills two requirements: (a) high fluorescence quantum yield; (b) a free phenol function that is necessary for fluorescence and can be blocked through an enzymatically labile glycosidic linkage. In this respect, fluorescein digalactoside (FDG) is a commercially available compound for galactosidase assays (Scheme 1). However, the fluorescence production from FDG needs two enzymatic steps: first step involves hydrolysis of FDG to give fluorescein mono galactoside (FMG)—a dark (or weakly-fluorescent) compound- and the second step to give fluorescein, the final fluorescent product. For *Escherichia coli* galactosidase, the first step is 12 times slower than the second: (k_a = 1.9 μmol min^{-1} mg^{-1}; k_b = 22.7 μmol min^{-1} mg^{-1})(Scheme 1).[1]

So, the rate of fluorescence increase from FDG was found to be very slow and not correlated with the β-galactosidase activity as a linear function at short time and low concentration (<<K_m) (Scheme 1). FMG will therefore be a far better fluorogenic compound than FDG for kinetic measurements.

In spite of more and more enzymatic assay studies, the structure–function relationship for enzymes at the single enzyme level is still not understood completely.[2–4] The major hurdle in this direction is the non availability of suitable fluorogenic probe having negligible emission and significantly higher signal to noise ratio of the highly fluorescent enzymatic hydrolysis product. Moreover, photostability of the fluorescent product is also a matter of concern.

We were interested in the enzymatic activity of a single immobilized enzyme, specially β-galactosidase, at the single molecule level, using a fluorogenic galactosyl reporter. A two step hydrolysis (of FDG) leading to the final fluorescent product dramatically limits the performance of this kind of experiment as the first hydrolysis product will diffuse out of the illuminating volume of the microscope. A single step hydrolysis reaction is needed and FMG could possibly be a suitable substrate for that purpose. Commercially

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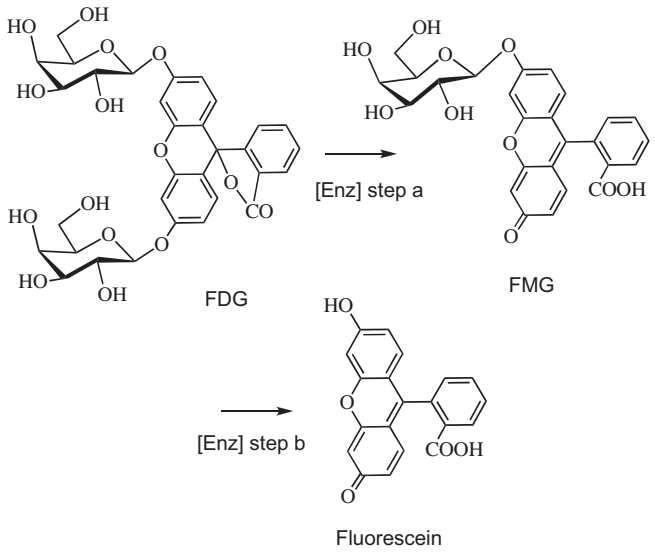


Scheme 1. Hydrolysis of FDG by galactosidase.

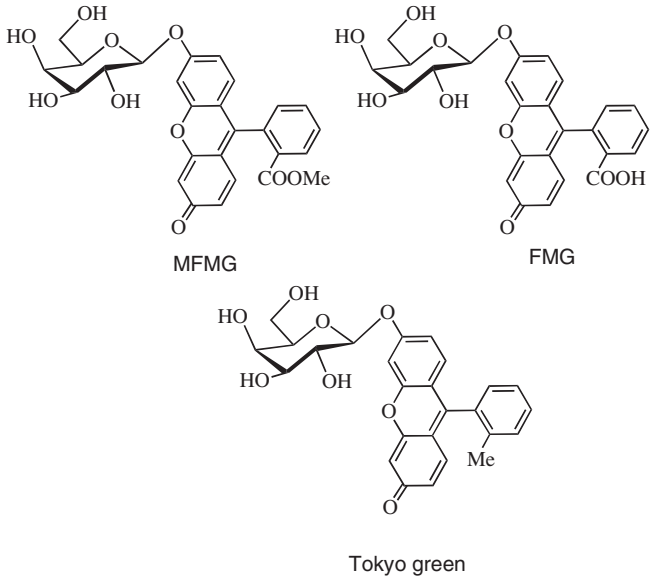


Figure 2. Chemical structures of MFMG, FMG and Tokyo green.

available alternative probes to FMG, resorufin-β-D-galactopyranoside, methylumbelliferyl-galactosides, and DDAO galactoside (9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside) have critical drawbacks.[5,6] Due to widely separated absorption profile of fluorogenic substrate and the enzymatic hydrolysis product two lasers need to be used in order to excite them. Either it is necessary to excite them in the UV region or significant overlap of the excitation and emission spectra of the enzymatic hydrolysis product reduces the sensitivity significantly.[5,6] Moreover, these dyes are not as bright as fluorescein.

In this manuscript we report detailed synthesis of Fluorescein monoglycosides (D-galactopyranoside and D-glucopyranoside) (FMG) and their methyl ester (MFMG). Characterization of all products has been made. We have employed biotinylated β-galactosidase instead of native enzyme as the former would be used for single molecule experiments. Michaelis–Menten constant (K_m) value for FMG and MFMG has been measured in order to verify whether there is any change because of chemical modification of the substrate. It is well-known that two steps of enzyme catalyzed reactions viz. (a) reversible formation of enzyme–substrate complex (ES) and (b) irreversible conversion of ES to product are determined partly by the diffusion constants of substrates. Thus we have measured the translational diffusion coefficients of both fluorogenic substrates and the products employing fluorescence correlation spectroscopy.

2. Results and discussion

2.1. Synthesis of the probes

We want to describe here the synthesis of fluorescein mono galactoside (FMG) and of their methyl ester (MFMG) and the kinetics of their enzymatic hydrolysis (Fig. 1). Strangely enough, FMG is not commercially available and no convenient synthesis of this compound is described in literature. Alternatively, Urano et al.[7] developed Tokyo green(s) (Fig. 2), mono galactosylated closely related compounds, prepared in several steps from a xanthone derivative.[8] They had shown that the presence of –COOH group on the pendant benzenic ring causes no change in the fluorescence

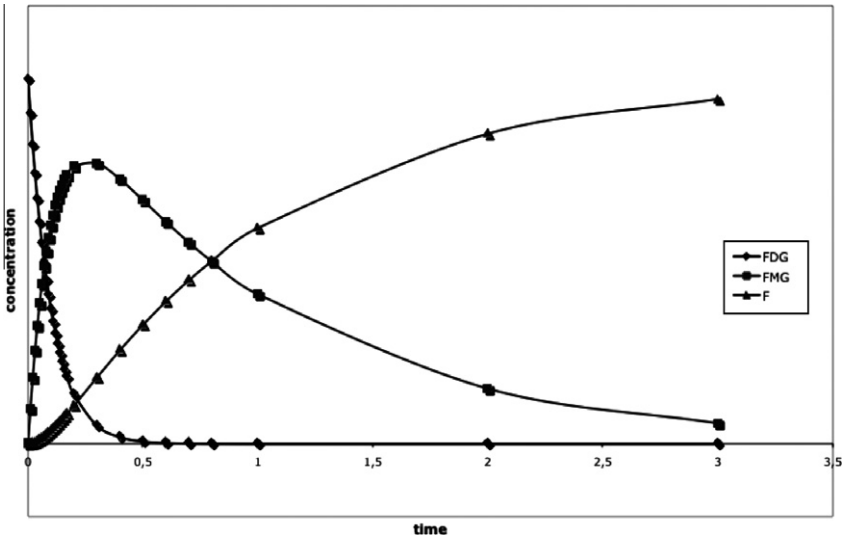
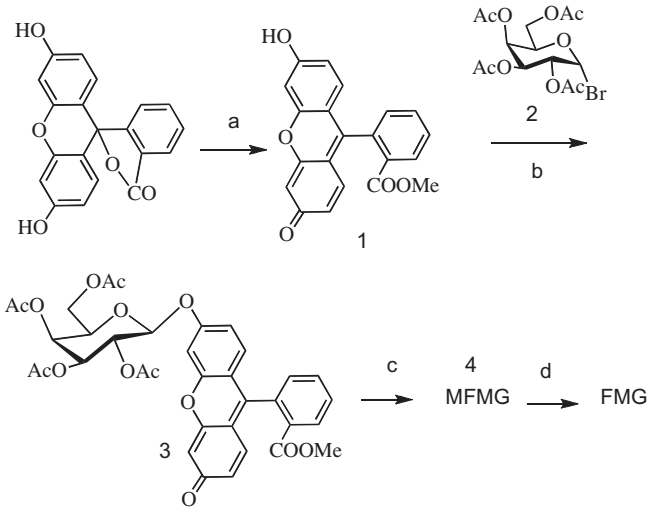
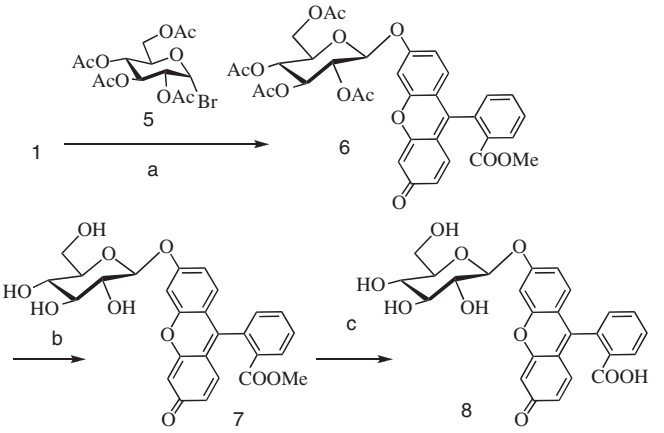


Figure 1. Kinetic profile of FDG → FMG → F sequence.



Scheme 2. Synthesis steps of MFMG and FMG. Reagents and conditions: (a) litt: H_2SO_4, MeOH reflux; (b) Cs_2CO_3, DMF, 5 h, rt, 79%; (c) MeONa, MeOH, 2 h, rt, 47%; (d) NaOH, water, 1 h, rt, 53%.



Scheme 4. Reagents and conditions: (a) Cs_2CO_3, DMF, 5 h, rt, 72%; (b) MeONa, MeOH, 2 h, rt, 56%; (c) NaOH, water, 1 h, rt, 69%.

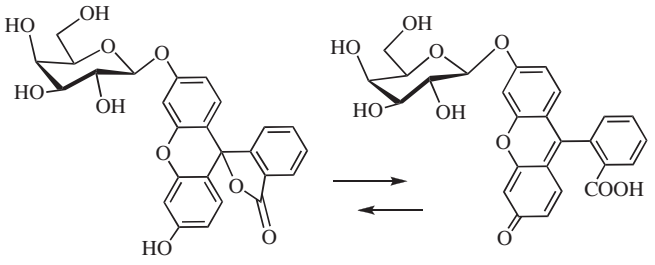
property of the fluorophore. Rather, it causes the benzene ring to remain in an orthogonal plane with respect to the xanthenes plane and thus maintains a high fluorescence quantum yield.[7] The methyl ester of FMG (MFGM) (Fig. 2) is thus expected to have similar photo-physical properties but may cause a difference in the kinetics of the enzymatic hydrolysis

The synthesis of FMG is depicted in Scheme 2. After unsuccessful[9] direct galactosylation of fluorescein, we decided to protect the acid function as a methyl ester and therefore fluorescein methyl ester (**1**) was prepared using a method first published in 1901 by Feuerstein and Dutoit[10] and recently improved by Adamczyk et al.[11] by reacting fluorescein with refluxing methanol in the presence of sulfuric acid. The reaction of this ester with acetobromogalactose **2** in dimethylformamide (DMF) in the presence of cesium carbonate gave the key glycoside **3** in good yield 79% (Scheme 2).

Compound **3** is a mixture of two atropisomers, due to restricted rotation around the central bound.

The treatment of **3** with sodium methylate in dry methanol gave the methyl ester **4** (MFMG), a neutral compound analogous to Tokyo greens. The β selectivity was controlled by the participating group and confirmed in ^{1}H NMR (H-1 5.13 ppm $J_{1,2}$ = 7.8 Hz) and ^{13}C NMR (C-1 100.3 ppm). Saponification of **4** with an aqueous solution of sodium hydroxide gave FMG. FMG is in fact a mixture of two compounds in equilibrium, as seen in NMR. In DMSO, the less polar cyclized form is favored (Scheme 3).

In a similar way, *gluco* derivatives (**7** and **8**) were prepared from **1** and acetobromoglucose (**5**) in comparable yields (see Section 4 and Scheme 4). Compound **8** was previously prepared by enzymatic glucosylation of fluorescein with a plant cell culture (*Rauwolfia serpentina*).[12]



Scheme 3. Equilibrium between open and lactonic form of FMG.

2.2. Enzymatic hydrolysis of galacto derivatives: FMG and MFMG

We wanted to study first the hydrolysis of FMG and MFMG by a β-galactosidase in solution by the formation of the and fluorescent products (fluorescein and methylated fluorescein) and to evaluate the kinetics parameters. The galactosidase used is a genetically engineered biotinylated β-Gal (β-Gal from Thermo Scientific/Pierce Protein Research Products) as the latter will be used in our future experiments in order to probe its enzymatic activities at the single molecule level. This preliminary study would allow us to check the substrate purity and to estimate the alteration of the activity of the biotinylated enzyme.

A literature report suggests that for FMG fluorescence intensity follows a strict linear relation with substrate concentration within 1 μM.[13] We have kept the concentration of the substrate within this limit. The initial concentration of the substrate was kept low enough (absorbance at around 0.05) in order to avoid concentration related problems.

Time based kinetic studies of enzymatic assays for both substrates are depicted in Figures 3 and 4. We have used a very dilute solution of FMG and MFMG (a few hundred nM to 1 μM) in order to reduce the concentration quenching due to high concentration. The absorption spectra show a 40 nm red shift of the absorption maximum on going from substrate to the product for both substrates. For 1 μM substrate concentration, a 15–20 times enhancement of the fluorescence intensity was observed at the emission maximum. As can be seen from the above Figure 4, during first 50–100 s the curve is clearly linear for all four substrate concentrations ([S]) and the initial velocity (v) of the enzymatic reaction was calculated from this time period. These data were used for the Lineweaver–Burk plot (LB plot) of 1/v versus 1/[S]. The Michaelis–Menten constant (K_m) values were obtained for both FMG and MFMG from these LB plots. To the best of our knowledge there is no report for K_m values of MFMG. K_m value obtained for MFMG is 11.85 μM. K_m values for FMG using native β-Gal are reported to be 117.6 μM and 96.6 μM.[13] In our experiment using biotinylated β-Gal, we have obtained a K_m value for the same substrate of 2.35 μM. So, the substrate concentration is well below the K_m value. Lower K_m value for biotinylated β-Gal indicates higher substrate affinity in comparison to native enzyme. This result, although it is new, however, it is not unprecedented. A similar result was reported for the same enzyme (native and biotinylated β-Gal) with a different substrate (*o*-nitrophenyl-β-D-galacto pyranoside).[14] Another important observation

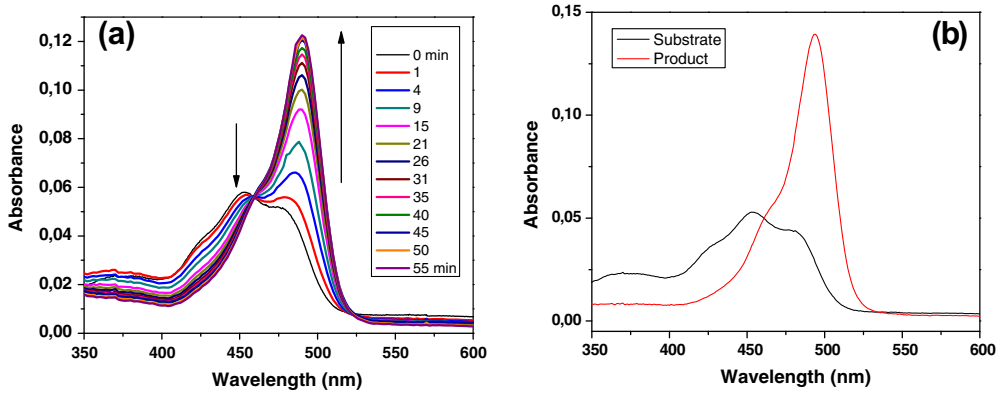


Figure 3. Time dependent change of the absorption spectra for FMG (a) and MFMG (b) upon enzymatic hydrolysis. In case of MFMG only initial and final spectra are shown for clarity. A clear isosbestic (both in (a) and (b)) at around 460 nm confirms the mechanism.

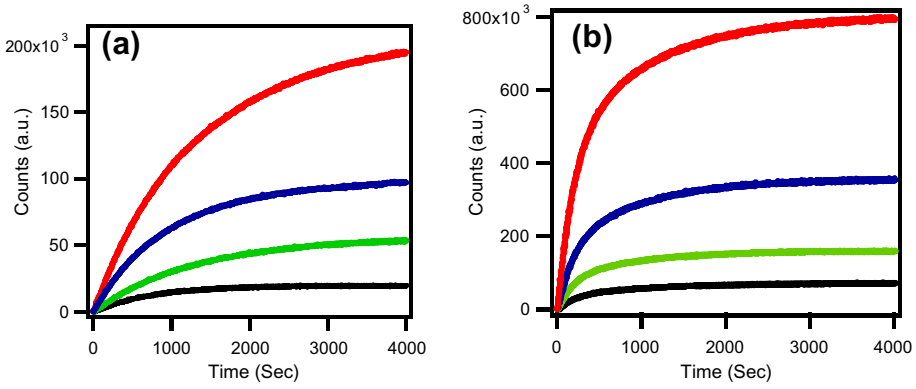


Figure 4. Time dependent fluorescence (monitored at 511 nm) measurement plot for different concentrations of FMG (a) and MFMG (b) with a fixed β-Gal concentration. (red: 1 μM, blue: 500 nM, Green: 200nM, black: 100 nM) (excitation at 488 nm, emission monitored at 511 nm).

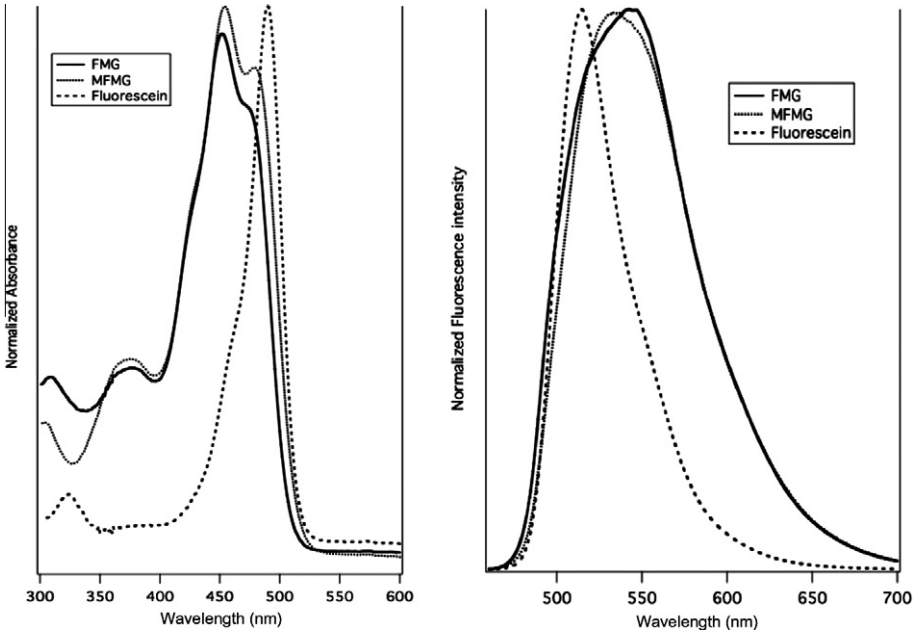


Figure 5. Absorption and emission spectra of FMG, MFMG and fluorescein: (a) Normalized absorption spectra. (Concentration ca. 10^{-5} M^{-1} in 100 mM phosphate buffer, pH 7.4. (b) Normalized emission spectra with an excitation at 450 nm (concentration ca. 10^{-6} M^{-1} in 100 mM phosphate buffer, pH 7.4).

we would like to mention here is that the replacement of –COOH group with the –COOMe group does not alter the absorption or emission maxima significantly but alters the K_m values. This perhaps indicates that the replacement of –COOH with –COOMe causes

significant change in the molecular conformation of the fluorogenic substrate (presence of a negative charge or coexistence of the cyclic lactone for FMG).

Absorption and emission spectra of the enzyme substrates (FMG and FMMG) and fluorescein are shown in Figure 5. Although, there are a red shift in absorption and a blue shift in emission, on going from substrate to the product, there is significant overlap of the spectra both in absorption and in fluorescence. Neither selective excitation of fluorescein nor a selective observation of fluorescein is thus possible to increase the sensitivity. Although these two galactosides (FMG and MFMG) can be useful tools for galactosidases assays at the ensemble level (a 15–20-fold enhancement of fluorescence intensity), however, for single molecule fluorescence experiments these probes are not quite suitable, because these experiments demand a negligible background signal and significantly higher signal to noise ratio. The research for new probes in this direction is currently underway.

2.3. Measurement of diffusion coefficient

It is well-known that the rate coefficient, $k(t)$, for enzyme–substrate binding is time dependent due to the influence of diffusion. Two steps of the enzyme-catalyzed reaction that is (i) reversible formation of the enzyme–substrate complex (ES), and (ii) irreversible conversion of the substrate in ES to product, are controlled by the diffusion rates of the substrate and the product. Hence, we thought it will be worth measuring the diffusion coefficient of both the products and both the substrates under identical experimental condition employing fluorescence correlation spectroscopic technique. The result is shown in Figure 6 below.

As can be seen from Figure 6 and Table 1, the diffusion behavior is quite similar for both substrates as well as both products. This is an important observation as for both substrate–product pair the diffusion coefficients are similar, so enhancement or retardation of the enzymatic hydrolysis due to difference of diffusion coefficient of substrate and/or product is not that much apparent.

3. Conclusion

We have synthesized two fluorogenic probes for the kinetic studies of β-galactosidases. Both the substrate–product pairs exhibit similar absorption and fluorescence maxima, suggesting that methyl substitution at the carboxyl group does not alter photochemical behavior. Both the substrates show strict linearity of fluorescence increase with time. K_m value for MFMG is higher than that of FMG suggesting a possible conformational change of the fluorogenic substrate. Magnitude of the Michaelis–Menten constant (K_m) for biotinylated β-Gal with FMG is lower than that for the native enzyme. This observation indicates higher substrate affinity of the biotinylated enzyme in comparison to the native enzyme. Similar translational diffusion coefficients have been obtained for both substrates and both the products. Thus an enhancement or retardation of the enzymatic kinetics due to difference in translational mobility of substrate and product is not that apparent.

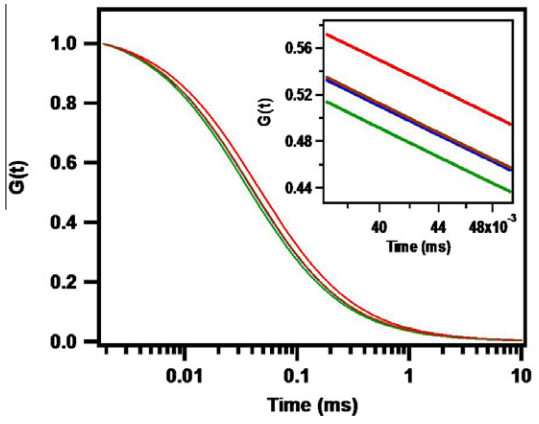
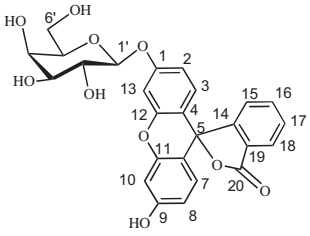


Figure 6. Diffusion behavior of Fluorescein (green), 1 (MF) (blue), FMG (brown), and MFMG (red). For clarity a blown up part of the graph has been shown as inset (details of the experimental condition is in Section 4).

4. Experimental

4.1. General procedures

All compounds were homogeneous by TLC analysis and had spectral properties consistent with their assigned structures. Optical rotations were measured with a Perkin–Elmer Model 241 digital polarimeter at 22 ± 3 °C. Compound purity was checked by TLC on Silica Gel 60 F_{254} (E. Merck). Column chromatography was performed on Silica Gel 60 (E. Merck) using cyclohexane (Cyhex), dichloromethane (DCM) and Ethyl acetate. ^{1}H NMR spectra were recorded with Brüker AM 250, AM 400 instruments



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4.2. Synthesis of substrates

4.2.1. Galacto series

4.2.1.1. Fluorescein methyl ester mono (2,3,4,6-tetra-*O*-acetyl β-D-galactopyranoside) (3). A mixture of **1** (450 mg, 1.292 mmol, 1 equiv), Cs_2CO_3 (4.21 g, 12.92 mmol, 10 equiv) and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**2**) (4.50 g, 10.72 mmol, 8.3 equiv) in dry DMF (150 mL) was stirred for 5 h at room temperature. The precipitate was filtered off and the filtrate was concentrated under reduced pressure. The residue obtained was diluted in water and extracted three times with DCM. The organic layers were combined and washed with water and saturated NaCl, dried over $MgSO_4$, and evaporated. The residue was chromatographed on silica gel with Cyhex/AcOEt 50:50 and DCM/MeOH 97/3 to give **3** as an orange powder (694 mg, 79%, two atropoisomers). R_f: 0.31 (DCM/MeOH 100:3) α_D^{25} +19 (*c* 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.28 (d, 1H, J = 7.8 Hz), 7.76 (m, 1H), 7.70 (t, 1H, J = 7.8 Hz), 7.33(d, 1H, J = 5.7 Hz), 7.13 (m, 1H), 6.91 (dd, 1H, J = 0.87 Hz, J = 8.8 Hz), 6.86 (dd, 1H, J = 4.0 Hz, J = 9.7 Hz), 6.81 (m, 1H), 6.56 (dd, 1H, J = 1.8 Hz, J = 9.7 Hz), 6.44 (t, 1H, J = 2.0 Hz), 5.54 (m, 2H), 5.18 (m, 2H), 4.21 (m, 3H), 3.68 (2s, 3H), 2.1 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 186.2, 170.8, 170.5, 170.4, 169.7, 165.9, 160.7, 159.2, 154.0, 149.9, 134.9, 133.3, 131.6, 130.9, 130.8, 130.7, 130.2, 129.4, 118.9, 117.0, 115.2, 114.7, 106.3, 104.2, 103.8, 99.2, 72.0, 71.1, 68.7, 67.2, 61.9, 52.9, 21.2, 21.1, 21.0, 21.0. MS-ESI-HRMS m/z $[M+H]^+$ calculated for $C_{35}H_{33}O_{14}$: 677.18648, found: 677.18711

4.2.1.2. Fluorescein methyl ester mono (β-D-galactopyranoside) (4 MGFM). To a solution of **3** (380 mg, 0.562 mmol, 1 equiv) in anhydrous MeOH (3 mL) was added sodium (13 mg, 0.56 mmol,

Table 1
Diffusion coefficient of both fluorogenic substrates (FMG and MFMG) and their enzymatic reaction products (fluorescein, methylated fluorescein)

Probe	Diffusion Coefficient ($\times 10^{-10}$ m^2 s^{-1})	Reference
Fluorescein (F)	4.36	[15]
Fluorescein methyl ester (MF)	4.05	This work
FMG	4.03	This work
MFMG (**4**)	3.45	This work

1 equiv). The mixture was stirred for 2 h at room temperature, neutralized with Amberlite IR120-H+ resin, filtered (the resin was rinsed with MeOH). The solution was concentrated under reduced pressure and the residue was chromatographed on silica gel with DCM/MeOH 90:10 to give **4** as a red solid (180 mg, 47%, two atropoisomers). R_f: 0.22 (DCM/MeOH 90:10) α_D^{25} −82 (*c* 1, MeOH). ^{1}H NMR (400 MHz, MeOD) δ (ppm): 8.30 (2 dd, 1H, *J* = 7.8 Hz, *J* = 1.6 Hz), 7.88 (2t, 1H, *J* = 7.1 Hz), 7.50 (2t, 1H, *J* = 7.3 Hz), 7.46 (d, 1H, *J* = 7.5 Hz), 7.40 (t, 1H, *J* = 2.3 Hz), 7.08 (m, 2H), 7.04 (d, 1H, *J* = 2.1 Hz), 6.60 (2d, 1H, *J* = 2.1 Hz, *J* = 3.1 Hz), 6.50 (2d, 1H, *J* = 1.9 Hz), 5.13 (2d, 1H, *J* = 7.8 Hz, H′1), 3.96 (d, 1H, *J* = 3.3 Hz), 3.84 (m, 3H), 3.67 (2dd, 1H, *J* = 9.6 Hz, *J* = 3.3 Hz), 3.64 (2s, 3H). ^{13}C NMR (100 MHz, MeOD) δ (ppm): 186.3, 166.0, 163.2, 160.4, 154.8, 154.7, 134.3, 133.1, 131.6, 131.2, 130.8, 130.4, 130.3, 129.7, 128.6, 117.6, 116.1, 115.5, 104.5, 103.6, 101.3, 76.3, 73.7, 70.9, 69.1, 61.4, 51.9. MS-ESI-HRMS *m*/*z* [M+H]$^+$ calculated for $C_{27}H_{25}O_{10}$: 509.14422, found: 509.14384.

4.2.1.3. Fluorescein mono (β-D-galactopyranoside) (FMG). Compound **4** (62 mg, 0.122 mmol, 1 equiv) was dissolved in H_2O (2 mL). aq NaOH 1 M (240 μL, 0.24 mmol, 2 equiv) was added and the mixture was stirred for 1 h at room temperature, neutralized with aq HCl 1 M (0.244 mmol, 244 μL, 2 equiv), and concentrated under reduced pressure. The residue was chromatographed on silica gel with AcOEt/MeOH/H_2O 70:5:3 to give a yellow powder (31 mg, 53%). R_f: 0.38 (AcOEt/MeOH/$_{H2}$O 90:5:3) α_D^{25} +20 (*c* 1, MeOH). ^{1}H NMR (400 MHz, DMSO) δ (ppm): 8.00 (d,1H, H18, *J* = 7.2 Hz), 7.79 (t, 1H, H16, *J* = 7.3 Hz), 7.73 (t, 1H, H17, *J* = 6.8 Hz), 7.29 (d, 1H, H15, *J* = 6.3 Hz), 7.00 (s, 1H, H13), 6.77 (d, 1H, H2, *J* = 9.0 Hz), 6.68 (m, 2H, H3, H10), 6.58 (s, 2H, H7, H8), 4.93 (m, 1H, H1′), 3.59 (m, 6H, H2′,H3′,H4′,H5′, H6′), 2.08 (s, 4H, OH saccharides). ^{13}C NMR (100 MHz, DMSO) δ (ppm): 168.9 (C20), 159.8 (C9), 159.2 (C1), 152.6 (C14), 152.0 (C11), 135.9 (C16), 130.4 (C17), 129.3 (C7), 129.1 (C3), 126.2 (C19), 124.9 (C18), 124.0 (C15), 113.5 (C2), 113.1 (C8), 112.4 (C4), 109.6 (C6), 103.4 (C13), 102.4 (C10), 100.9 (C1′), 82.8 (C5), 75.62 (C5′), 73.2 (C3′), 70.1 (C4′), 68.1 (C2′), 60.4 (C6′). MS-ESI-HRMS *m*/*z* [M+H]$^+$ calculated for $C_{26}H_{23}O_{10}$: 495.12857, found: 495.12839.

4.2.2. Gluco series

Similar experiments starting from aceto-bromoglucose.

4.2.2.1. Fluorescein methyl ester mono (2,3,4,6-tetra-*O*-acetyl β-D-glucopyranoside) (6). A mixture of **1** (200 mg, 0.5742 mmol, 1 equiv), Cs_2CO_3 (1.87 g, 5.742 mmol, 10 equiv), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (2.00 g, 4.766 mmol, 8.3 equiv) in dry DMF (70 mL) was stirred for 5 h at room temperature. The inorganic precipitate was filtered off and the filtrate was concentrated under reduced pressure. The residue obtained was diluted in water and extracted three times with DCM. The organic layers were combined and washed with water and saturated aq NaCl, dried over $MgSO_4$, and evaporated. The residue was chromatographed on silica gel with Cyhex/AcOEt 50:50 and DCM/MeOH 100/3 to give **6** as an orange powder (281 mg, 72% two atropoisomers). R_f: 0.31 (DCM/MeOH 100:3) α_D^{25} 2 (*c* 1, $CHCl_3$). ^{1}H NMR (400 MHz, CDCl3) δ (ppm): 8.26 (d,1H, *J* = 7.6 Hz), 7.77 (m, 1H, H), 7.69 (t, 1H, *J* = 7.6 Hz), 7.31 (m, 1H), 7.11 (2d, 1H, *J* = 2.3 Hz), 6.90 (d,1H, *J* = 8.9 Hz), 6.85 (2d, 1H, *J* = 9.7 Hz), 6.78 (m, 1H), 6.54 (dd, 1H, *J* = 1.9 Hz, *J* = 9.7 Hz), 6.42 (2d, 1H, *J* = 2.7 Hz), 5.26 (m, 4H), 4.25 (m, 2H), 4.00 (m, 1H), 3.67 (2s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 186.2, 170.9, 170.5, 170.5, 169.8, 165.8, 160.7, 159.2, 154.0, 150.0, 133.3, 131.6, 130.9, 130.7, 130.6, 130.2, 129.3, 118.9, 117.0, 115.0, 114.7, 106.2, 104.2, 103.9, 98.5, 72.9, 71.3, 68.5, 67.2, 62.3, 52.9, 21.2, 21.1, 21.0, 21.0. MS-ESI-HRMS *m*/*z* [M+H]$^+$ calculated for $C_{35}H_{33}O_{14}$: 677.18648, found: 677.18758.

4.2.2.2. Fluorescein methyl ester mono (β-D-glucopyranoside) (7). To a solution of **6** (1.202 g, 1.778 mmol, 1 equiv) in anhydrous MeOH (30 mL) was added sodium (41 mg, 1.778 mmol, 1 equiv). The mixture was stirred for 2 h at room temperature, neutralized with Amberlyst IR120-H$^+$ resin, (the resin was rinsed with MeOH), and concentrated under reduced pressure and the residue was chromatographed on silica gel with DCM/MeOH 90:10 to give **7** as a red solid (503 mg, 56%). R_f: 0.19 (DCM/MeOH 90:10) α_D^{25} −98 (*c* 1, MeOH). ^{1}H NMR (400 MHz, MeOD) δ (ppm): 8.33 (d, 1H, *J* = 7.8 Hz), 7.88 (2t, 1H, *J* = 6.9 Hz), 7.81 (2t, 1H, *J* = 7.1 Hz), 7.46 (d, 1H, *J* = 7.5 Hz), 7.39 (s, 1H), 7.07 (m, 3H), 7.04 (d, 1H, *J* = 2.3 Hz), 6.60 (2dd, 1H, *J* = 2.6 Hz, *J* = 9.6 Hz), 6.51 (d, 1H, *J* = 2.0 Hz), 5.17 (m, 1H), 3.96 (d, 1H, *J* = 12.1 Hz), 3.74 (m, 1H), 3.64 (2s, 3H), 3.53 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 187.4, 167.0, 164.2, 161.4, 155.9, 155.7, 135.4, 134.3, 132.6, 132.3, 131.9, 130.8, 129.7, 118.7, 117.3, 116.5, 116.4 105.7, 104.9, 104.7, 101.7, 101.6, 78.4, 77.8, 74.7, 71.2, 62.4, 53.9. MS-ESI-HRMS *m*/*z* [M+H]$^+$ calculated for $C_{27}H_{25}O_{10}$: 509.14422, found: 509.14481.

4.2.2.3. Fluorescein mono (β-D-glucopyranoside) (8). Compound **7** (96 mg, 0.1890 mmol, 1 equiv) was dissolved in H_2O (2 mL). NaOH 1 M (378 μL, 0.3780 mmol, 2 equiv) wad added and the mixture was stirred for 1 h at room temperature, neutralized with HCl 1 M (378 μL, 0.3780 mmol, 2 equiv), and concentrated under reduced pressure. The residue was chromatographed on silica gel with AcOEt/MeOH/H_2O 90:5:3 to give **8** as a yellow powder (64 mg, 69%). R_f: 0.38 (AcOEt/MeOH/H_2O 70:5:3) α_D^{25} −22 (*c* 1, $CHCl_3$). ^{1}H NMR (400 MHz, DMSO) δ (ppm): 8.02 (d, 1H, H_{18}, *J* = 6.9 Hz), 7.81 (m, 1H, H16), 7.74 (t, 1H, H17, *J* = 6.8 Hz), 7.30 (d, 1H, H15, *J* = 6.5 Hz), 7.03 (s, 1H, H13), 6.80 (d, 1H, H2, *J* = 8.6 Hz), 6.69 (m, 2H, H3, H10), 6.59 (s, 2H, H7, H8), 5.00 (d,1H, H1′, *J* = 6.2 Hz), 3.71 (m, 1H, H6′a), 3.24 (m, 5H, H2′, H3′H4′, H5′, H6′b). 13C NMR (100 MHz, DMSO) δ (ppm): 168.4 (C20), 160.1 (C9), 159.2 (C1), 152.0 (C14), 151.9 (C11), 151.8 (C12), 135.9 (C16), 130.4 (C17), 129.4 (C7), 129.2 (C3), 126.4 (C19), 125.00 (C18), 124.3 (C15), 113.5 (C2), 113.3 (C8), 112.6 (C4), 109.6 (C6), 103.6 (C13), 102.5 (C10), 100.3 (C1′), 83.5 (C5), 77.3 (C5′), 76.7 (C3′), 73.4 (C2′), 69.9 (C4′), 60.9 (C6′). MS-ESI-HRMS *m*/*z* [M+H]$^+$ calculated for $C_{26}H_{23}O_{10}$: 495.12857, found: 495.12919 (in agreement with lit data)

4.3. Absorption and fluorescence experiments

Absorption and florescence measurements were performed in Uvikon-940 spectrophotometer (Zurich, Switzerland) and LPS 220 spectrofluorometer (PTI, NJ) respectively. The temperature was maintained using circulating baths (Polystat 34-R2 Fischer Bioblock Scientific, France) and the temperature was directly measured using a type K Thermocouple connected to ST-610B digital Pyrometer (Stafford Instruments, UK). For fluorescence based experiments 488 nm light was used for excitation and the emission was monitored at 511 nm. For each experiment, 750 μL of different concentrations of substrate was allowed to be cleaved by β-Gal

(concn <<1 U/mL). Concentrations of substrate and enzyme were kept in the regime where initial kinetic velocity was proportional to both substrate and enzyme concentration. All experiments were performed at 25 °C. All enzymatic assays were performed in phosphate buffer (100 mM) at pH 7.4 in the presence of 1 mM $MgCl_2$ and 110 mM mercaptoethanol.

4.4. Fluorescence correlation spectroscopy experiments

Fluorescence correlation spectroscopy (FCS) was used to calculate the translational diffusion coefficient of the fluorogenic substrates as well as products. For excitation of the fluorophore, 488 nm laser (Ar-ion, Spectra Physics) was used. Excitation laser as well as fluorescence photons were passed through Olympus UPlan Apo 60× 1.20 W infinity corrected objective. To collect the fluorescence photons 488 nm long pass filter (Semrock) was used. After passing through the longpass filter the fluorescence photons were coupled to optical fibers (FG200 LCR multimode fiber, Thorlabs) and were detected with two avalanche photodiodes (SPCM-AQR-14, Perkin–Elmer) coupled to an ALV-6000 correlator (ALV GmbH). The incident powers at the sample were measured with a NOVA II powermeter (Laser Measurement Instruments). All the series of experiments reported in the present work were performed in a regime of laser powers (3–5 mW) in which fluorescein exhibits a linear dependence of the intensity of fluorescence emission on the illumination power. The focussed beam was assumed to adopt a 3D-Gaussian excitation profile characterized by Eq. 1.

$$I = I_0 \exp\left[-\frac{2(x^2+y^2)}{\omega_{xy}^2} - \frac{2z^2}{\omega_z^2}\right] \quad (1)$$

where, ω_z and ω_{xy} are the sizes of the beam waist in the direction of propagation of the laser beam and in the perpendicular direction respectively. FCS autocorrelation curve was fitted successfully with the following expression (Eq. 2) of the freely diffusing species:

$$G(\tau) = \frac{1}{\bar{N}}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\frac{\tau}{\omega^2\tau_D}\right)^{-1/2} \quad (2)$$

where $\bar{N}$ is the average number of fluorescein molecules contained in the illuminated volume. $\tau_D = \omega^2_{xy}/8D$ is the diffusion time through the beam waist, and $\omega = \omega_z/\omega_{xy}$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.05.017.

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