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Glucose-Based Molecular Rotors as Fluorescent Inhibitors and Probes of Glycogen Phosphorylase †

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Abstract: Herein, the synthesis and photochemical study of (*E*)-2-cyano-3-(6-(dimethylamino)naphthalen-2-yl)-*N*-(β-D-glucopyranosyl)acrylamide, a β-D-glucopyranosyl analogue of the widely used molecular rotor julolidine. The new compound is a fluorescent inhibitor of rabbit muscle glycogen phosphorylase with properties of a molecular rotor. Fluorescence measurements in solutions of increasing viscosity determined that the fluorescence intensity increases with the viscosity of the medium, indicating that the new compound exhibits molecular rotor characteristics. Although the compound fluoresces negligibly in aqueous buffer solution, in the presence of increasing amounts of rabbit muscle glycogen phosphorylase, we observed an increase of fluorescence intensity, attributed to the formation of and inhibitor:enzyme complex. *In-vitro* cellular studies were also undertaken, yielding promising preliminary results for the use of the new compound as a fluorescent probe.

Keywords: molecular rotor; glycogen phosphorylase; julolidine; fluorescent probes

1. Introduction

Glycogen phosphorylase (GP) is an enzyme which plays a key role in glucose homeostasis. The lack of proper GP regulation is involved in a number of pathological states such as type 2 diabetes [1,2] and cancer [3–6], while recent findings connect GP to Alzheimer's disease and a number of other neurological disorders [7,8]. The ubiquitous presence of GP has ascended the design and synthesis of GP activity-modulating agents into an exciting field in chemical research in recent years. Our team has been working on the synthesis and study of potential GP inhibitors, with the main aim the creation of new agents to be used against type 2 diabetes [9–11].

Molecular rotors are conditionally fluorescent compounds which contain two or more chromophores in a push-pull configuration. When the chromophores are forced to be aligned parallelly, the compounds fluoresce. If the system is free to rotate, it forms twisted intramolecular charge transfer (TICT) states, the excited state decays thermally and no fluorescence is observed (Figure 1). These fluorescence characteristics are strongly affected by particular microenvironments [12,13]. Therefore, molecular rotors show enhancement of their fluorescence with increasing viscosity of the surroundings, as well as during binding to protein targets in the cells. Indeed, compounds with molecular rotor function, such as α-cyanoacryl-derivatives of julolidine (Figure 1), have been applied as biosensors to determine the viscosity in various cellular microenvironments [14–16].

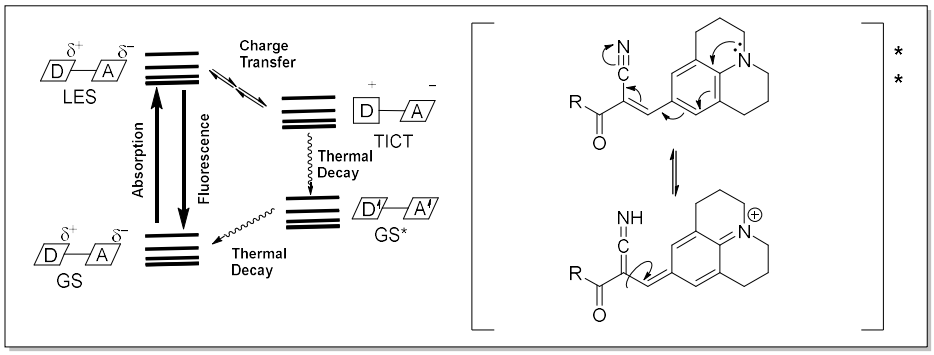


Figure 1. Mechanism of induced fluorescence in α-cyanoacryl-derivatives of julolidine via TICTs.

Compounds that exhibit selective fluorescence upon binding to the catalytic site of GP could be potentially useful fluorescent probes, allowing better understanding of glycogen metabolism by the means of fluorescence microscopy. Herein, the first attempts to extend our work to the synthesis of glucose-based GP ligands, with attached moieties acting as molecular rotors, are described.

2. Results and Discussion

Based on our previous experience on the synthesis of GP N^4-arylamine-substituted 1-(β-D-glucopyranosyl)pyrimidin-2-one inhibitors, we set out to design a *N*-glycoside incorporating a molecular rotor moiety. More specifically, it was decided to use the (*E*)-2-cyano-3-(6-(dimethylamino)naphthalen-2-yl)acryl- moiety [17,18], which has been shown to exhibit the desired molecular rotor characteristics, to be attached to β-D-glucopyranose by means of an amide bond. The proposed aglycon was expected to satisfactorily mimic the structural characteristics of molecules, previously synthesized by our group [9,10,19], necessary to strongly bind to the catalytic site of rabbit muscle glycogen phosphorylase (RMGP) (Figure 2). Furthermore, utilizing the dimethylaminonaphthyl- system was expected to lead to compounds with excitation and emission maxima falling within the region of the filter-sets commonly used in fluorescence microscopy for the observation of the mCherry fluorescent protein (450–600 nm for excitation and 600–700 nm for emission) [20]. The same chromophore system has also been utilized as a two-photon fluorescent sensor for near-infrared imaging in whole tissues [17,18].

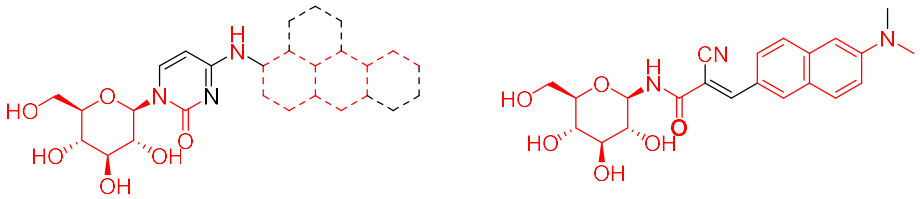
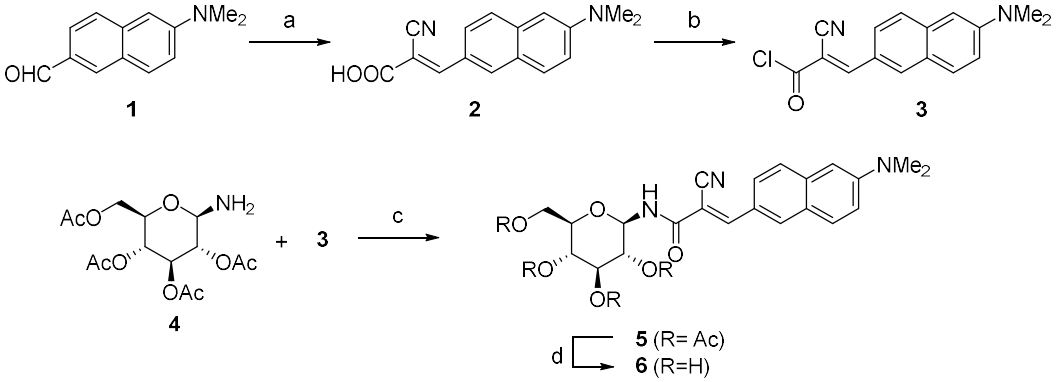


Figure 2. Structural comparison between the general framework of previously synthesized 4-arylamine-substituted 1-(β-D-glucopyranosyl)pyrimidin-2-one GP inhibitors (**left**) and the proposed compound (**right**).

The synthesis started by preparation of known 6-dimethylamino-2-naphtaldehyde (**1**) from commercially available β-naphthol, following previously published procedures [21,22]. Utilizing the Knoevenagel condensation with cyanoacetic acid [23], aldehyde **1** was transformed into the corresponding acid (**2**, Scheme 1), in 90% yield. It was then attempted to couple acid **2** with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamine (**4**) [24,25], initially by methodologies utilizing carbodiimide reagents (DCC and EDC), but this approach failed to produce the desired amide. Alternatively, transformation of **2** into the corresponding acid chloride **3**, by use of oxalyl chloride, was successful. The crude chloride **3** was then reacted with amine **4**, to yield the desired amide **5** as a component of an inseparable mixture. The crude mixture was directly submitted to deprotection,

using methanolic ammonia, to remove the acetyl groups. The final compound **6** was successfully isolated by column chromatography, albeit in moderate yield, over three steps, starting from acid **2**.



Scheme 1. (**a**)Piperidine, cyanoacetic acid in MeCN, 80 °C, 12 h, 90%, (**b**) oxalyl chloride, cat. DMF in DCM, r.t., 1 h, (**c**) 3, pyridine, r.t., 2 h (**d**) NH_3 in MeOH, 24 h. 35% over three steps.

Subsequently, the spectroscopic properties of compound **6** were studied in solution. In DMSO solution, compound **6** exhibits a λ_{max} at 427 nm (ε = 29,500 $cm^{-1}M^{-1}$). A red shift of D$\lambda \approx$ 10 nm, with increasing concentration from 1 to 75 μM, was observed. Excitation at the λ_{max} leads to emission as a broad peak, centered at 580 nm. The absorption spectra in solutions of diethylene glycol: glycerol (DEG:Gl) of increasing viscosity (100 % DEG, 50 % DEG:Gl, 100 % Gl, Figure 3) exhibit a $\lambda_{max} \approx$ 450 nm and a similar red shift is observed going from low to high viscosity. Excitation of the above solutions at the λ_{max} leads to variable fluorescence correlating well with solution viscosity. Specifically, a 60-fold increase in viscosity leads to 4-fold increase in fluorescence intensity, confirming that compound **6** exhibits the expected properties of a molecular rotor.

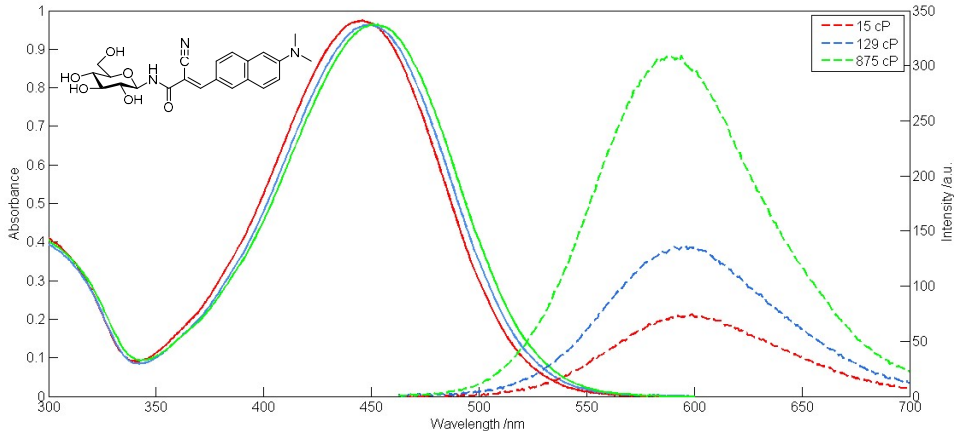


Figure 3. Absorption (**left**) and emission (**right**) spectra of 6 in relation to solution viscosity (Red: 100% Gl, μ = 15 cP; blue: 50% DEG:Gl, μ = 129 cP; green: 100% DEG, μ = 875 cP).

Having proven the molecular rotor behavior of **6**, the next step was to study its interaction with rabbit muscle glycogen phosphorylase (RMGP). A number of RMGP:**6** ratios ranging between 0–10 were used, to ensure maximal ligand binding to the enzyme. The experiments were performed in the suitable assay buffer (β-glycerophosphate 2.5 mM, 2-mercaptoethanol 2.5 mM, EDTA 0.05 mM) commonly used with RMGP. During the first set of experiments, the only visible effect was the flattening of the absorption spectrum of **6**, regardless of the enzyme's concentration. Absorption spectra of **6** under a variety of pH levels were examined, which ascertained the compound's stability at the pH of the assay buffer (6.8) used in the experiments with RMGP. Compound **6** exhibited the same absorption spectral characteristics in the 6.1–8.9 pH region. For this reason, we tried to attribute

the observed spectral changes of compound **6** in the assay buffer, to the buffer's constituents, as a source of possible interference. Structurally related α-cyanoacrylamide systems have been previously shown to exhibit a propensity to undergo 1,4 addition with thiols [26], which seems to be the case with **6**. Indeed, repeating the experiments in a modified assay buffer, excluding the usually added 2-mercaptoethanol, led to similar to the previously obtained spectra of **6** with a $\lambda_{max} \approx 450$ nm, which blue-shifted with increasing amounts of RMGP (Figure 4). At the maximum RMGP concentration (RMGP: **6** ratio of 10:1), fluorescence was enhanced 3 times compared to the fluorescence of free **6** in aqueous buffer solution (Figure 4).

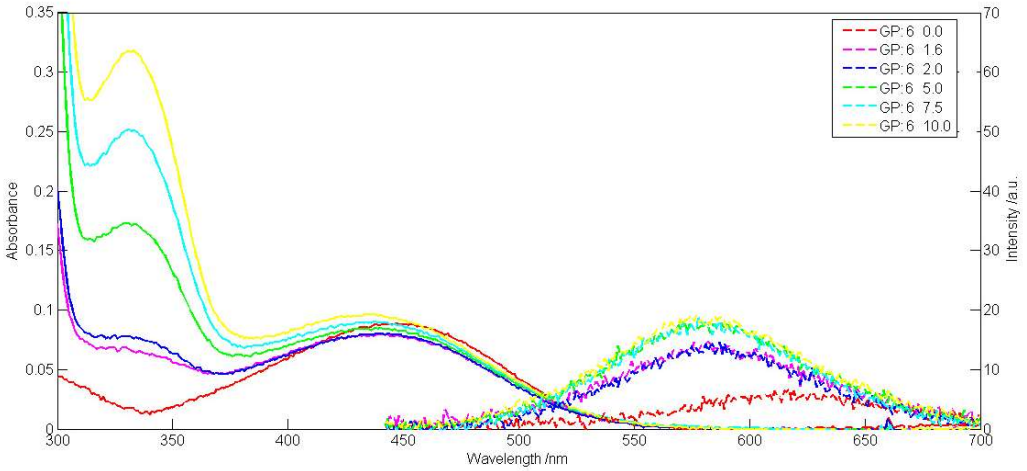


Figure 4. Absorption (**left**) and emission (**right**) spectra of RMGP: **6** complex in rising ratios.

The above experiments established that **6** behaves as a molecular rotor upon binding to the catalytic site of RMGP. The same fluorescence assays were repeated using bovine serum albumin to exclude possible generic interactions, as well as with hexokinase III, an enzyme which is a possible target for the binding of glucose-based molecules. In both cases there was no enhancement of fluorescence witnessed. While these results are preliminary, strongly indicate that **6** binds to RMGP in a target-specific fashion. There was a smaller enhancement of fluorescence in the presence of RMGP when compared to the one observed in solutions of increasing viscosity. This could be either due to weaker than expected binding to RMGP, or due to fluorescence quench by the residues lining up the catalytic site of RMGP. The exact reasons may be elucidated upon further kinetic and crystallographic studies.

In the final step of this study, some preliminary fluorescence microscopy experiments were performed. The A-431 and Hep2G cell lines were selected, since both are known to express high levels of the liver isoform of GP (PYGL) [27], localized in the cytoplasm, as well as the cell membrane [27]. The cells were incubated with 60 μM of **6** for 48 h and visualized under the microscope, using the mCherry filter set (Figure 5) [20].

In both cell lines, incubation with **6** led to the appearance of localized fluorescence (Figure 5), while no fluorescence was witnessed in the control cells. Further colocalization experiments utilizing **6**, in combination with immunocytochemistry methods, are in progress, to elucidate the exact nature of the observed fluorescence and ascertain the specific binding to GP in a cellular environment.

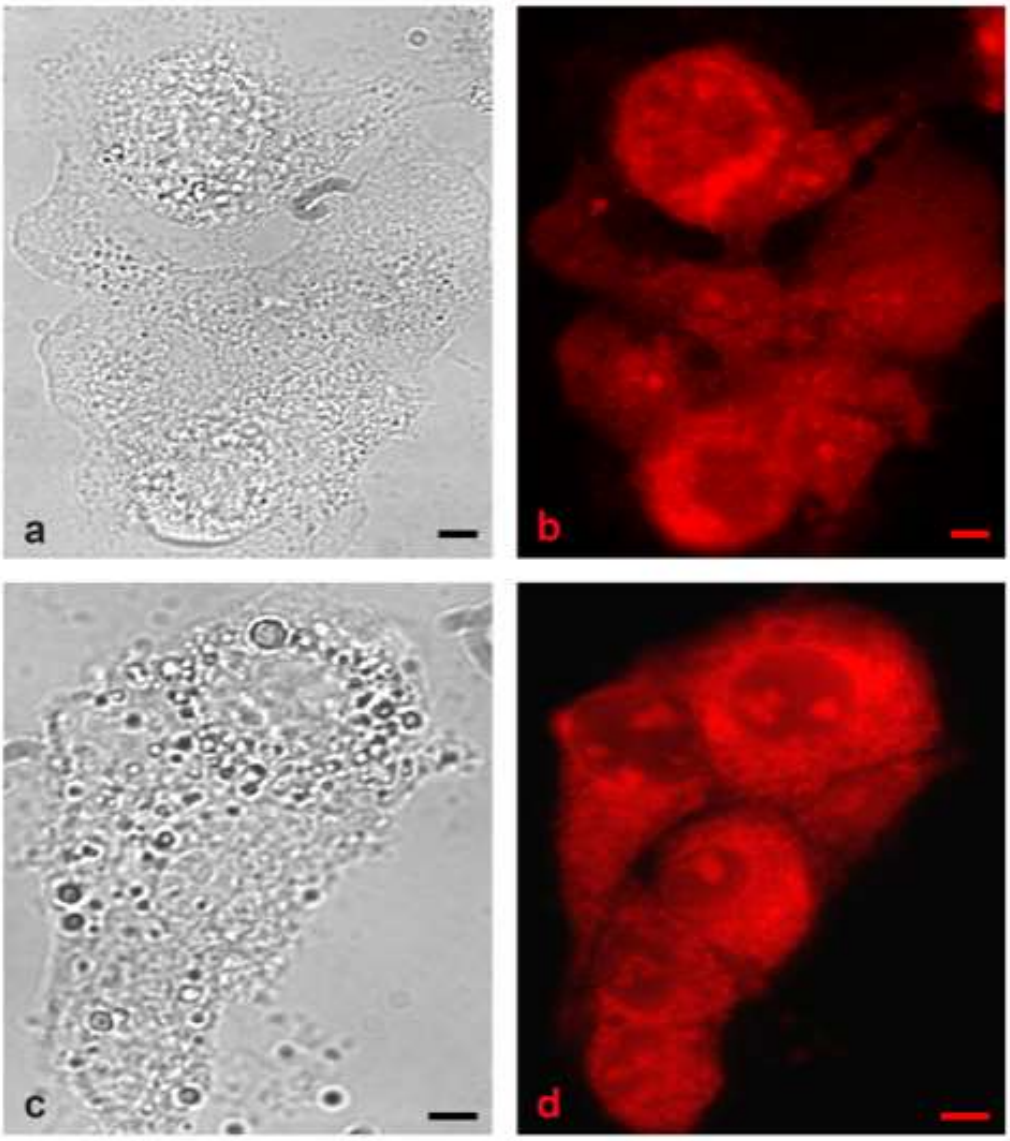


Figure 5. (**a**) A-431 cells under brightfield view, (**b**) A-431 cell fluorescence with mCherry filter set, (**c**) Hep2G cells under brightfield view and (**d**) Hep2G cells fluorescence with mCherry filter. Both cell lines were incubated with compound 60 μM of **6** for 48 h before acquisition of the above pictures.

3. Materials and Methods

All reagents, solvents, cell lines, cell culture media and reagents were purchased from commercial sources and used without further purification. All reactions were carried out under an argon atmosphere on a magnetic stirrer and monitored by thin-layer chromatography. Compounds were purified by flash chromatography on silica gel 40–60 μm, 60Å. NMR measurements were performed with a Varian Mercury 200 Nuclear Magnetic Resonance Spectrometer (at 200 MHz for ^{1}H and at 50 MHz for ^{13}C). Chemical shifts are given in ppm and were referenced on residual solvent peaks. Coupling constants were measured in Hz. High-Resolution Mass Spectrometry experiments were carried out in a Q-TOF Bruker MaXis Impact HR-Mass Spectrometer. 6-dimethylamino-2-naphthaldehyde (**1**) [21,22] and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamine (**4**) [24,25] were synthesized by previously reported procedures.

Absorption spectra were acquired on a Shimadzu UV-Vis-NIR 3600 spectrophotometer, and excitation spectra were acquired on a Shimadzu RF-5301PC spectrofluorometer, utilizing a matched set of dual-path cuvettes. Britton Robinson universal buffers (BRB) were used for variable pH

experiments and were prepared as described [28]. RMGP was isolated from rabbit skeletal muscle and its potency was assayed according to previously established protocols [29,30]. Measurements involving RMGP were performed in aqueous buffers ("assay buffer"), consisting of β-glycerophosphate (2.5 mM), 2-mercaptoethanol (2.5 mM) and EDTA (0.05 mM) for the first set of experiments, and the same buffer was used without inclusion of 2-mercaptoethanol for the second (successful) set of experiments described.

For the fluorescence microscopy experiments, both HepG2 and A431 cell lines were used. Cells were cultured as monolayers in DMEM medium supplemented with 10% (*v*/*v*) Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin and were grown in 60mm tissue culture dishes in a humidified 5% CO2 atmosphere at 37 °C. Cells in their log phase of growth were harvested, counted and seeded (4 × 104 cells/well in 250μL DMEM containing 10% FBS) in 8-well plates (μ-Slide 8 well, IBIDI GMBH). After 24h of incubation to allow cell attachment, the synthetic phosphorylase inhibitor (**6**) is added to a final concentration of 60 μM and cells were incubated for 48 h before observation. Two replicates were set up in each experiment.

Before observing the cells on a Zeiss Axio Observer Z1 inverted microscope equipped with proper filters and the ZEN Blue software, the culture medium was discarded and attached cells were washed twice with isotonic PBS solution. For visualization of **6**, a 538-562 nm band pass excitation filter was used, and the fluorescence was observed in the range of 570-640 nm, through the red mCherry filter.

(*E*)-2-Cyano-3-(6-dimethylamino)-2-naphthylacrylic acid (2) To a solution of 6-dimethylamino-2-naphthaldehyde (**1**) (409 mg, 2.05 mmol) in 4.2 mL of anhydrous MeCN, were added cyanoacetic acid (266 mg, 3.1 mmol) and piperidine (230 μL, 2.3 mmol). The mixture was refluxed for 16 h, decanted into a 1:1 1M HCl- solid water ice mixture. The settling solid was filtered, washed three times with deionized water and dried under reduced pressure, to yield 491 mg (90%) of spectroscopically pure **2** as a brown powder. **^{1}H NMR (200 MHz, DMSO-d_6)** δ 8.30 (d, *J* = 2.3 Hz, 1H), 8.28 (s, 1H), 8.07 (dd, *J* = 8.9, 1.8 Hz, 1H), 7.80 (d, *J* = 9.2 Hz, 1H), 7.72 (d, *J* = 8.9 Hz, 1H), 7.26 (dd, *J* = 9.1, 2.5 Hz, 1H), 6.94 (d, *J* = 2.4 Hz, 1H), 3.08 (s, 6H). **^{13}C NMR (200 MHz, DMSO-d^6)** δ 164.2, 154.4, 150.6, 137.3, 135.2, 130.7, 126.7, 124.9, 124.8, 124.4, 117.2, 116.6, 104.9, 98.8, 39.9 (2C). **HRMS** [M-H]$^-$: Calcd for $C_{16}H_{13}N_2O_2^-$ 265.0983; Found 265.0965.

(*E*)-2-Cyano-3-(6-(dimethylamino)naphthalen-2-yl)-*N*-(β-D-glucopyranosyl)acrylamide (**6**) A solution of acid **2** (267 mg, 1.00 mmol) in 2 mL of anhydrous DCM was cooled in an ice bath. A drop of DMF and oxalyl chloride (180 μL, 2.09 mmol) were added dropwise. The mixture was brought to room temperature and stirred for a further 2 h. Volatiles were removed under reduced pressure. The residue was dissolved in 5 mL of anhydrous DCM and added dropwise to an ice-cooled solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamine (**4**) (315 mg, 0.91 mmol) in 2 mL of anhydrous pyridine. The mixture was brought to room temperature and left to stir for 1 h, after which the reaction mixture was quenched by the addition of 1 mL of deionized water. The mixture was concentrated under reduced pressure, taken up in EtOAc and the organic portion was washed successively with 5% Na_2CO_3 (1 × 15 mL), deionized water (1 × 15 mL) and brine (1 × 10 mL), separated, dried over anhydrous sodium sulfate, filtered through a silica plug and concentrated. To the residue was added 7M methanolic ammonia solution (7.5 mL). After 24 h, the mixture was concentrated and purified by column chromatography to yield 150 mg of **6** (35% from **2**) as a bright orange powder. **^{1}H NMR (200 MHz, DMSO-d6)** δ 8.81 (d, J = 8.7 Hz, 1H), 8.23 (s, 2H), 8.02 (dd, J = 8.6, 1.2 Hz, 1H), 7.85 (d, J = 9.2 Hz, 1H), 7.76 (d, J = 8.9 Hz, 1H), 7.29 (dd, J = 9.3, 2.4 Hz, 1H), 6.98 (d, J = 2.4 Hz, 1H), 5.09 – 5.01 (m, 2H), 4.95 (d, J = 4.9 Hz, 1H), 4.87 (t, J = 8.6 Hz, 1H), 4.56 (t, J = 5.7 Hz, 1H), 3.68 (dd, J = 11.7, 5.7 Hz, 1H), 3.46 (dd, J = 11.4, 5.3 Hz, 1H), 3.31 – 3.12 (m, 4H), 3.09 (s, 6H). **^{13}C NMR (50 MHz, DMSO-d6)** δ 163.0, 151.2, 150.7, 137.2, 134.0, 130.7, 126.9, 125.1 (2C), 124.8, 117.4, 116.9, 105.1, 102.2, 80.6, 78.9, 77.3, 72.1, 70.0, 61.0, 40.1 (2C). **HRMS [M+Na]$^+$**: Calcd for $C_{22}H_{25}N_3NaO_6^+$ 450.1641; Found 450.1637.

4. Conclusions

The first reported glucose derivative, (*E*)-2-cyano-3-(6-(dimethylamino)naphthalen-2-yl)-*N*-(β-D-glucopyranosyl)acrylamide (**6**), bearing a molecular rotor moiety has been synthesized and studied. The new compound was shown to exhibit molecular rotor fluorescence characteristics in viscous solutions, as well as in complex with RMGP. Incubation of A-431 and Hep2G cancer cells led to the appearance of localized fluorescence in the red region, possibly indicating a selective binding of the new compound **6** to GP, present in the cell. These studies correspond to a proof-of-concept, that a RMGP inhibitor with molecular rotor properties is feasible. Further studies are underway to develop further carbohydrate-based molecules with molecular rotor functionality, improved chemical stability and strong binding to the catalytic site of GP. The aim is to access compounds with high fluorescence quantum yield and to finetune both the absorption and emission spectral values by varying substitution on the aromatic system. This will allow to cover a range of fluorescence filter-sets commonly used in fluorescence microscopy, as well as to extend applications to two-photon excitation, in order to probe living tissue.

Author Contributions: Conceptualization, T.G.; methodology, K.F.M. and M.M.; investigation, K.F.M., P.P.; writing—original draft preparation, T.G. and K.F.M.; writing—review and editing, T.G. and K.F.M.; visualization, T.G., P.P., K.F.M. and M.M.; supervision, T.G. and P.P.; project administration, T.G. and P.P.; funding acquisition, T.G. and K.F.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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