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Synthesis and Evaluation of Glycosyl Luciferins

Daan Sondag,[a] Frank F. J. de Kleijne,[a] Sam Castermans,[a] Isa Chatzakis,[a] Mark van Geffen,[b] Cornelis van't Veer,[b] Waander L. van Heerde,[b, c, d] Thomas J. Boltje,*[a] and Floris P. J. T. Rutjes*[a]

Measuring glycosidase activity is important to monitor any aberrations in carbohydrate hydrolase activity, but also for the screening of potential glycosidase inhibitors. To this end, synthetic substrates are needed which provide an enzyme-dependent read-out upon hydrolysis by the glycosidase. Herein, we present two new routes for the synthesis of caged luminescent carbohydrates, which can be used for determining glycosidase activity with a luminescent reporter molecule. The substrates were validated with glycosidase and revealed a clear linear range and enzyme-dependent signal upon the *in situ* generation of the luciferin moiety from the corresponding nitrile precursors. Besides, we showed that these compounds could directly be synthesized from unprotected glycosyl-α-fluorides in a two-step procedure with yields up to 75%. The intermediate methyl imidate appeared a key intermediate which also reacted with D-cysteine to give the corresponding D-luciferin substrate rendering this a highly attractive method for synthesizing glycosyl luciferins in good yields.

Introduction

Glycosylation, the cell surface expression of carbohydrate structures on proteins and lipids, is found in all the domains of life. The glycosylation pattern of cells is produced by glycosyltransferases, detected by lectins and can be degraded by glycosidases.[1,2] The breakdown of glycans is achieved by glycosidase enzymes that are specific for their monosaccharide substrate and are expressed by the human host, bacteria and viruses. Aberrations in glycosidase expression and/or activity can be used to diagnose various pathologies. For example, in various cancer types, the overexpression of specific glycosidases can be used as a prodrug targeting strategy or as biomarker.[3–5] Furthermore, in infectious diseases such as influenza, glycosidase activity assays may be used as a diagnostic tool to detect upregulated neuraminidase activity.[6–8]

The monitoring of glycosidase activity for various diagnostic applications has been achieved by the preparation of synthetic substrates that produce a fluorescent, luminescent or chromogenic signal upon enzymatic conversion.[9,10] The use of luminescent glycosidase substrates could facilitate miniaturization of the assays since no external light source is required and may ultimately make point-of-care (POC) or fast clinical interceptions feasible.[11] To this end, various synthetic substrates have been developed in order to monitor enzymatic activity with a luminescent output, as also demonstrated for proteases such as in our recent work on the main protease (M^{Pro}) of SARS-CoV-2.[12–14]

The use of glycosyl luciferins has been explored by Goode and co-workers in 1990, demonstrating that the coupling of D-luciferin to a monosaccharide can be used to monitor glycosidase activity. Key in this approach is that the glycosyl luciferin is not a substrate for the luciferase and hence does not produce a light signal. Only when this substrate is hydrolyzed by a glycosidase, it liberates D-luciferin which is converted into a light signal that is proportional to the glycosidase activity upon the action of firefly luciferase (Figure 1A). However, the chemical lability of the luciferin moiety makes the synthesis of glycosyl luciferins synthetically challenging.[15] The luciferin moiety is known to be prone to oxidation to produce dehydroluciferin. In addition, the chiral center on luciferin is prone to racemization to afford L-luciferin. Both dehydroluciferin and L-luciferin are known to inhibit luciferase activity and interfere with the monitoring of glycosidase activity.[16,17] The D-luciferin moiety is typically constructed by the condensation of a benzothiazole-nitrile precursor with D-cysteine. However, due to the electrophilic nature of the nitrile moiety, it is prone to react with water to form the amide or methanol to afford the

[a] D. Sondag, F. F. J. de Kleijne, S. Castermans, I. Chatzakis, Dr. T. J. Boltje, Prof. Dr. F. P. J. T. Rutjes
Institute for Molecules and Materials
Radboud University
6525 AJ Nijmegen (The Netherlands)
E-mail: thomas.boltje@ru.nl
floris.rutjes@ru.nl
[b] Dr. M. van Geffen, Dr. C. van't Veer, Dr. W. L. van Heerde
Enzyre BV, Novio Tech Campus
Transistorweg 5-i, 6534 AT Nijmegen (The Netherlands)
[c] Dr. W. L. van Heerde
Department of Haematology
Radboud University Medical Centre
Nijmegen (The Netherlands)
[d] Dr. W. L. van Heerde
Haemophilia Treatment Centre
Nijmegen Eindhoven Maastricht (HTC-NEM)
(The Netherlands)

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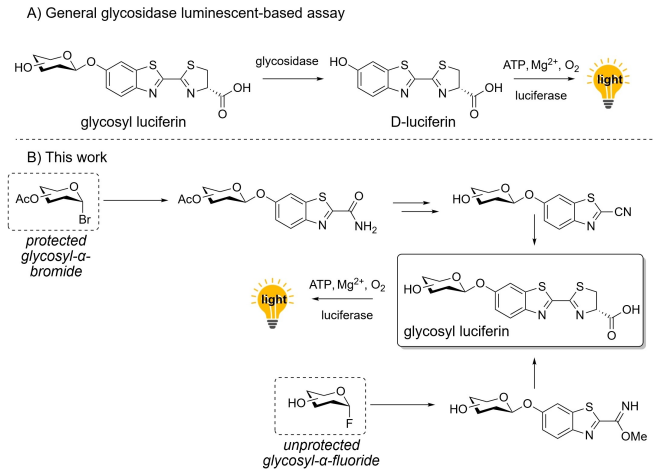


Figure 1. A) General principle of a glycosidase luminescence-based assay using glycosyl luciferins. The glycosidase cleaves D-luciferin from the caged monosaccharide substrate. The released D-luciferin is converted by luciferase in the presence of ATP and Mg^{2+} to produce a light signal proportional to the glycosidase activity. B) The two synthetic routes to prepare glycosyl luciferins from either protected glycosyl-α-bromides (top route) or unprotected glycosyl-α-fluorides (bottom route) and subsequent luciferin-induced generation of light.

corresponding imidate as previously observed by Amess *et al.*[15] Hence, there is a need for a robust synthetic scheme that can provide glycosyl luciferins in a high yield and purity.

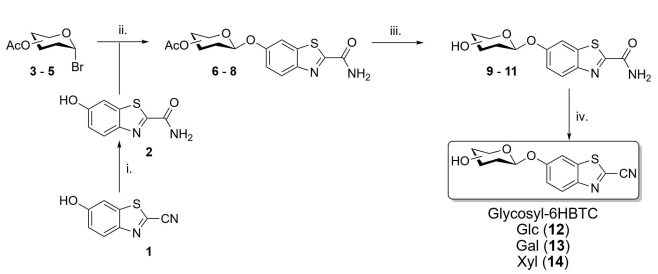
Herein, we present two robust routes for the synthesis of glycosyl luciferins. One route utilizes temporary hydration of the luciferin nitrile precursor leading to the less reactive, but more stable, amide and subsequent glycosylation with protected glycosyl α-bromides (Figure 1B). Alternatively, we demonstrated that unprotected α-glycosyl fluorides can be used to introduce the luciferin moiety, which under the base-promoted glycosylation conditions leads to the corresponding methyl imidate which, like the cyanide, also reacted with D-cysteine to give the corresponding D-luciferin moiety.

Results and Discussion

The first challenge while preparing luciferin-caged molecules, is the inherent low nucleophilicity and chemical instability of D-luciferin and its precursor 6-hydroxybenzo[*d*]thiazole-2-carbonitrile (**1**, 6-HBTC). Especially during glycosylation reactions and the subsequent deprotection of the glycoside substrates this instability can cause problems and induce byproduct formation. Amess *et al.* already proposed that the direct glycosylation of the luciferin moiety might be problematic,[14] due to the reactivity of D-luciferin and the risk of oxidation. Nevertheless, more recent routes have been reported employing direct glycosylation of D-luciferin, although generally an extensive purification was required.[7,18] The use of precursor **1** throughout the synthesis instead of D-luciferin is a viable alternative, however, during methanolysis of the acetyl protecting groups unwanted methyl imidate formation takes place.[15]

In our search for a new more robust synthesis route to glycosyl luciferins, we hypothesized that the more stable amide derivative **2** could circumvent undesired imidate formation during the deprotection conditions. To this end, compound **2** was readily synthesized *via* acidic hydration of nitrile **1** in sulfuric acid at room temperature in quantitative yield (Scheme 1).[19] We prepared the acetylated glycosyl α-bromide donors **3** (glucoside), **4** (galactoside) and **5** (xyloside) according to known literature procedures with hydrogen bromide in acetic acid.[20] Subsequent glycosylation reactions of acceptor **2** with donors **3**–**5** were executed under phase transfer conditions in a water/chloroform mixture (1 : 1) with a catalytic amount of tetrabutylammonium bromide (TBAB) and potassium carbonate at 60 °C, to furnish the amide intermediates **6**–**8**. These glycosylation reactions provide moderate yields of the desired product, presumably due to the inherently unreactive character of phenol **2** and competing elimination reactions on the glycosyl bromide donors. Multiple attempts to improve the yield of the glycosylation reaction with stronger bases or different solvents were unsuccessful. Neither the *in situ* phenolate formation of **2** nor BF_3-mediated glycosylation of peracetylated glycosides proved to be higher yielding. Nevertheless, we proceeded with the acetyl deprotection of **6**–**8** with sodium methoxide in methanol to afford the glycosides **9**–**11**. We were able to reintroduce the cysteine reactive nitrile moiety via dehydration of the amides **9**–**11** with trifluoroacetic anhydride (TFAA) in pyridine in the presence of the free hydroxyl groups, to afford the luciferin precursors **12**–**14** in good yields.[21,22]

We envisioned that the reaction of D-cysteine with nitriles **12**–**14** could be executed *in situ* prior to the enzymatic assay to afford the glycosyl luciferins. In our hands, the nitrile-bearing substrates appeared to be more stable compared to the corresponding luciferins, making storage and use of these compounds more practical. In order to demonstrate that our probes could be used as substrates for determining glycosidase activity, we incubated **12**–**14** with D-cysteine hydrochloride and an equimolar amount of potassium carbonate prior to the addition of glycosidase (Figure 2A). This late-stage *in situ* luciferin generation reaction was also studied with an analytical HPLC assay, to prove that the cysteine condensation is complete within 20 minutes and solely yields the desired D-enantiomer (see Supporting Information). We subsequently



Scheme 1. Synthesis of the glycosyl luciferin precursor compounds **12**–**14** (glucose, galactose and xylose) via dehydration of the amide intermediates **9**–**11** starting from the glycosyl α-bromides **3**–**5** and amide **2**. i. H_2SO_4, 3 h, rt, quant. ii. TBAB (0.1 equiv), **2** (5 equiv), K_2CO_3 (1.2 equiv), $H_2O/CHCl_3$ (1 : 1), 60 °C, 20 h, 5–33 %. iii. NaOMe (cat), MeOH, rt, 56–82 %. iv. TFAA (20 equiv), pyr, 20 h, rt, 37–79 %.

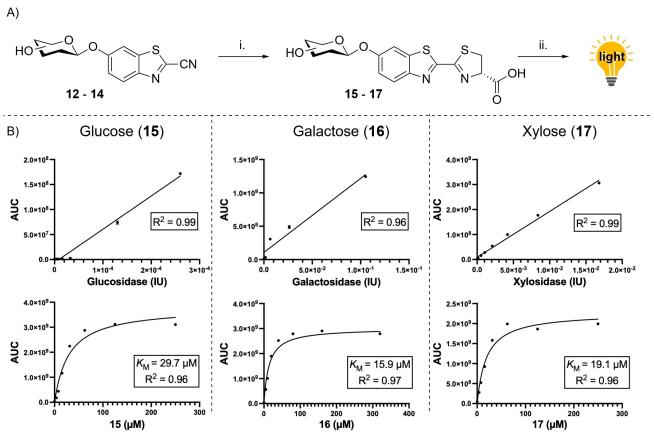


Figure 2. A) Reaction scheme for the luminescent glycosidase assay via *in situ* generation of the D-luciferin substrates **15**–**17**. i. D-cysteine·HCl (2 equiv), K_2CO_3 (2 equiv), H_2O (125 mM NaCl, 25 mM HEPES, 0.1 % w/w BSA, pH = 7.2), 1 h, 37 °C. ii. glycosidase (gluco-, galacto- or xylosidase), $MgCl_2$ (125 equiv), ATP (50 equiv), firefly luciferase (final conc. 0.147 mg/mL). B) The corresponding linear range of the luminescent assay (triplo) and Michaelis-Menten kinetic plots of substrates **12**–**13** (quintuple).

determined the enzymatic hydrolysis of the luciferin conjugates **15**–**17** upon reacting them with glycosidase in buffer and addition of luciferase, all performed in triplicate. This secondary readout allows for direct measurement of glycosidase activity, since glycosidase cleavage is known to be the rate limiting step in this experiment due to the excess of luciferase which has been previously optimized by our group for similar luminescent assays.[14,15, 23]

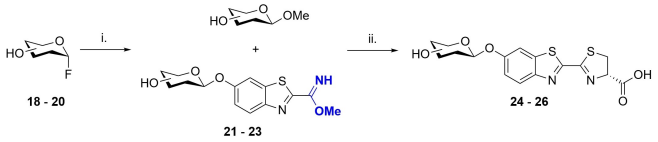
The released photons were measured with a luminometer and the area under the curve (AUC) and the subsequent linear range of our assay were determined (Figure 2B). All three substrates **15**–**17** revealed a clear linear range and K_m values in the low µmolar range (15–30 µM), similar to values found for fluorescent and chromogenic analogues in literature.[24,25] All control experiments without glycosidase, luciferase or D-cysteine indicated that our luminescent signal was glycosidase-dependent and that our assay was sufficiently robust.

Although we successfully demonstrated that the amide precursors are more stable, can be dehydrated to afford the corresponding nitrile, and that these could be used to generate glycosyl luciferins *in situ*, the overall synthesis yields were still moderate. To improve this, we explored the synthesis of glycosyl luciferins starting from unprotected glycosides, which would abrogate the need for a base-catalyzed deprotection and reduce overall reaction steps. Initial attempts with glucose and 2-chloro-1,3-dimethylimidazolinium chloride (DMC)-mediated glycosylation, as elegantly demonstrated by the Shoda group and Fairbanks and co-workers, were unsuccessful, as minimal conversion was observed and a large excess of donor **1** was required.[26–29] Next, we explored the use of α-glycosyl fluorides that were previously used by Miller and co-workers for the protecting group free *O*-glycosylation reactions of small molecules and peptides.[30,31] Initially, we aimed to couple nitrile **1** to the α-fluoride acceptors **18**–**20** (gluco-, galacto- and xyloside), which were either commercially available or synthesized from the corresponding peracetylated building blocks.[32] This protecting-group-free approach would save us synthetic steps and we hypothesized that the yield might also improve due to the relative stability of the α-fluorides towards hydrolysis as compared to the α-bromides. However, during the optimization of the *O*-glycosylation, we observed that the reaction in water did not give any conversion with nitrile **1** and glucoside **18**. Eventually, we found that the reaction in methanol/water (1 : 1) and calcium hydroxide as Ca^{2+} additive gave good conversions. Yet, upon isolation of the product, we observed undesired nitrile methanolysis to afford the corresponding methyl imidate **21**, presumably due to the alkaline glycosylation conditions.

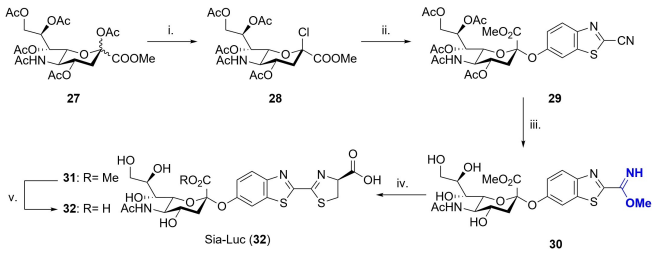
At first instance, we thought that methyl imidate **21** would be a dead-end intermediate for the luciferin synthesis. To our surprise, however, we found that **21** still reacted with D-cysteine to afford the corresponding D-luciferin. Although this exact reaction has never been reported in literature, we could find relevant examples of other methyl and ethyl imidates capable of reacting with a 1,2-aminothiol.[33–35] The glycosylation reactions of donor **1** with the α-fluoride acceptors **18**–**20** were executed and this afforded the methyl imidate intermediates **21**–**23** as an inseparable mixture of the product with the corresponding methyl-β-D-glycopyranoside. Subsequently, introduction of the thiazoline ring was performed upon reacting compounds **21**–**23** with D-cysteine and potassium carbonate in methanol to afford the pure glycosyl D-luciferins **24**–**26** after purification.

This two-step protecting-group-free synthetic procedure allows for the rapid synthesis of caged luminescent glycosyl substrates. We improved and shortened our initial dehydration method and showed that the methyl imidates were a key intermediate to react with D-cysteine to provide the pure D-luciferin-caged glycosyl substrates **24**–**26** (Scheme 2). We significantly improved the yield with this new method (28–85 % over two steps) as compared to the initial dehydration route (< 5 % over four steps)

To further extend the scope of our synthesis method, we aimed to synthesize a luminescent probe for measuring sialidase activity. Sialic acid is a nine-carbon carbohydrate which plays a key role in immune regulation, viral infection and various types of cancer, making sialidase an exceptionally attractive diagnostic target.[36,37] To this end, a concise method for the synthesis of luminescent carbohydrate substrates is needed. The synthesis of Sia-Luc (**32**, Scheme 3) has been described in literature, but is inefficient by the need for



Scheme 2. Synthesis of the glycosyl luciferins **24**–**26** from the unprotected α-fluorides **18**–**20**. The methyl imidate intermediates **21**–**23** were obtained as inseparable mixtures with the corresponding methyl-β-D-glycopyranoside which could be converted into the pure luciferins. i. **21**–**23** (3 equiv), **1** (1 equiv), $CaOH_2$ (3 equiv), MeOH/H_2O (3 : 1), 16 h, rt. ii. D-cysteine·HCl (2 equiv), K_2CO_3 (2 equiv), MeOH, 16 h, rt, 28–75 % over two steps.



Scheme 3. Synthesis of sialic acid luciferin substrate **32** via imidate formation. i. AcCl (56 equiv), MeOH (30 equiv), DCM, 17 h, rt. ii. **1** (1.2 equiv), K_2CO_3 (1.7 equiv), MeCN, 16 h, rt. iii. NaOMe (cat), MeOH, rt, 50% over three steps. iv. D-cysteine·HCl (2 equiv), K_2CO_3 (2 equiv), MeOH, 16 h, rt, 85%. v. NaOH (3 equiv), H_2O, 1.5 h, rt, 55%.

extensive purification as a result of the byproducts that are formed during the direct glycosylation approach.[7,18] We commenced our synthesis with the fully protected sialic acid **27**, which was converted into α-chloride **28** by *in situ* hydrochloride formation with acetyl chloride in methanol.[38,39] Compound **28** was directly used without further purification for the *O*-glycosylation with nitrile **1** and potassium carbonate in acetonitrile, to provide **29** which was directly used in the next reaction step. Deprotection of the acetyl protecting groups furnished product methyl imidate **30** in 50% yield over three steps. Methyl imidate **30** was reacted with D-cysteine to give **31** in a good yield of 85%. Hydrolysis of the methyl ester of **31** was successful with sodium hydroxide to give final product Sia-Luc (**32**) in 55% yield, which is a glycosyl caged luminescent probe for measuring sialidase activity.

Having substrate **32** in hand, we continued to validate the compound for measuring sialidase activity (Figure 3). We determined the linear range upon reacting **32** with neuraminidase and found K_m is 40.5 μM, which is similar to values reported in literature for fluorescent sialic acid analogues.[40] These results indicate that our method can most likely be extended to any other more complex saccharide and that our synthetic methodology is fairly robust.

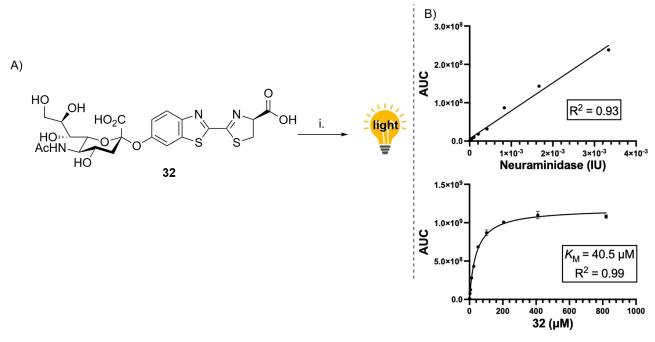


Figure 3. A) Reaction scheme for the luminescent neuraminidase assay via the hydrolysis of D-luciferin from substrate **32**. i. neuraminidase, $MgCl_2$ (125 equiv), ATP (50 equiv), firefly luciferase (final conc. 0.147 mg/mL). B) The respective linear range of the luminescent assay (triplo) and Michaelis-Menten kinetic plot of substrate **32** (quintuple).

Conclusions

In conclusion, we have developed two new routes for the synthesis of glycosyl luciferins. Applying temporary hydration of the nitrile precursor of luciferin appeared to be an appealing method in order to eliminate the unwanted byproducts that were formed from the nitrile during the glycosylation and subsequent deprotection reactions. We demonstrated that the *in situ* formation of the D-luciferin moiety, prior to the enzymatic assay, is an attractive synthetic approach, especially since the nitrile moiety seems to be more stable thereby facilitating storage and presumably a longer shelf-life. The gluco-, galacto- and xylosidase-substrates appeared excellent substrates for determining glycosidase activity and the linear range and Michaelis-Menten kinetics were determined, providing an overall robust and enzyme-dependent assay. Although in the initial synthesis route the overall yields appeared to be moderate, we also demonstrated that unprotected glycosyl-α-fluorides can be used to generate the glycosyl D-luciferins in good yields in two single steps. The methyl imidate intermediates that were generated upon the glycosylation reactions were initially hypothesized to be a dead-end product, however, to our delight we found that these intermediates still reacted with D-cysteine to provide the corresponding D-luciferin-caged glycosyl. Besides, we showed that this methodology could also be used for more complex carbohydrates, by synthesizing a sialidase probe that was validated giving an enzyme-dependent signal. We feel that the newly developed chemistry in this approach might benefit and stimulate the synthesis of novel luminescent caged carbohydrate substrates in the near future with considerable opportunities for diagnostics, for inhibitor design or for potential POC applications.

Experimental

Linear titration experiments substrates 12–14: Substrate **12**, **13**, or **14** (final conc. 20 μM), D-cysteine·HCl (final conc. 40 μM), K_2CO_3 (final conc. 40 μM) and buffer (25 mM HEPES, 125 mM NaCl, 0.5% BSA, pH = 7.2) were added to a well with a total volume of 23 μL. After incubation for 1 h at 37 °C, 2 μL of corresponding glycosidase (different concentrations) was added and 5 μL of detection mix containing ATP (final conc. 1000 μM), $MgCl_2$ (final conc. 5.0 mM), firefly luciferase (Quantilum®, Promega, final conc. 0.147 mg/mL) and buffer were added. The luminescence was recorded in Relative Light Units (RLU) for 90 minutes at rt under continuous shaking at 60 rpm with an integration time of 1000 ms per well. Measurements were executed in triplo and the Area Under the Curve (AUC) was calculated using Graphpad Prism (version 9.0) and fitted with a linear regression. For each glycosidase, the optimal concentration was determined which was used in the Michaelis-Menten kinetic experiments.

Michaelis-Menten kinetics experiments substrates 12–14: Substrate **12**, **13**, or **14** (different concentrations), D-cysteine·HCl (2 equiv), K_2CO_3 (2 equiv) and buffer (25 mM HEPES, 125 mM NaCl, 0.5% BSA, pH = 7.2) were added to a well with a total volume of 23 μL. After incubation for 1 h at 37 °C, 2 μL of corresponding glycosidase was added and 5 μL of detection mix containing ATP (final conc. 1000 μM), $MgCl_2$ (final conc. 5.0 mM), firefly luciferase (Quantilum®, Promega, final conc. 0.147 mg/mL) and buffer were

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Linear titration experiment substrate 32: Substrate **32** (final conc. 20 μM), and buffer (25 mM HEPES, 125 mM NaCl, 0.5% BSA, pH = 7.2) were added to a well with a total volume of 23 μL. 2 μL of corresponding glycosidase (different concentrations) was added and 5 μL of detection mix containing ATP (final conc. 1000 μM), $MgCl_2$ (final conc. 5.0 mM), firefly luciferase (Quantilum®, Promega, final conc. 0.147 mg/mL) and buffer were added. The luminescence was recorded in Relative Light Units (RLU) for 90 minutes at rt under continuous shaking at 60 rpm with an integration time of 1000 ms per well Measurements were executed in triplo and the Area Under the Curve (AUC) was calculated using Graphpad Prism (version 9.0) and fitted with a linear regression.

Michaelis-Menten kinetics experiment substrate 32: Substrate **32** (different concentrations) and buffer (25 mM HEPES, 125 mM NaCl, 0.5% BSA, pH = 7.2) were added to a well with a total volume of 23 μL. 2 μL of neuraminidase was added and 5 μL of detection mix containing ATP (final conc. 1000 μM), $MgCl_2$ (final conc. 5.0 mM), firefly luciferase (Quantilum®, Promega, final conc. 0.147 mg/mL) and buffer were added. The luminescence was recorded in Relative Light Units (RLU) for 90 minutes at rt under continuous shaking at 60 rpm with an integration time of 1000 ms per well. Measurements were executed in quintuple and the Area Under the Curve (AUC) was calculated using Graphpad Prism (version 9.0) and the data was fitted with the nonlinear regression (curve fit)-Michaelis Menten algorithm.

Supporting Information

The authors have cited additional references within the Supporting Information.[41–43]

Acknowledgements

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Glycosidase assay · luminescence · enzyme · luciferin · sialic acid

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