TECHNISCHE UNIVERSITÄT WIEN
Vienna | Austria

DISSERTATION

Novel Strategies and Methods for the Development of Glycosidase Responsive Fluorogenic Probes

Ausgeführt zum Zwecke der Erlangung des akademischen Grades eines

Doktors der technischen Wissenschaften

unter der Leitung von

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Fakultät für Technische Chemie

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“The Universe is under no obligation to make sense to you”

Neil deGrasse Tyson

Abstract

Monitoring the quality of surface, industrial, waste and drinking water is a crucial aspect of environmental monitoring since the water quality directly influences the quality of life. An important part of water monitoring is the investigation of the bacterial load, which can be conducted by a variety of methods including enzyme assays of highly specific bacterial enzymes. The usage of artificial fluorogenic enzyme substrates (fluorogenic probes) for these assays is now commonly in place in laboratories worldwide. However, traditional methods of identifying bacteria can be time consuming as they involve incubation times up to 72 hours. This high incubation time can be problematic as often fast definitive answers are required on environmental health risks. Furthermore, incubation methods can be misleading as not all bacterial strains are equally represented. In the last decade quantitative real time fluorescence (QRTF) assays have been developed without the need of incubation. However, this technology also requests more sophisticated fluorogenic enzyme substrates, as commonly used substrates limit the capabilities of QRTF measurements.

Within this thesis, the problems of the most common commercially available substrate, 4-methylumbelliferyl-β-D-glucuronic acid during QRTF assays were analyzed. With the gained insight into the synthesis and purification of β-glucuronidase substrates a variety of different fluorogenic substrates were designed and successfully applied in QRTF enzyme assays. In these novel substrates, self immolative linkers were implemented to access new fluorophores and fluorescence resonance energy transfer (FRET) mechanisms. Furthermore, with the separated reaction and measurement (SRM) device a new approach for QRTF assays was designed.

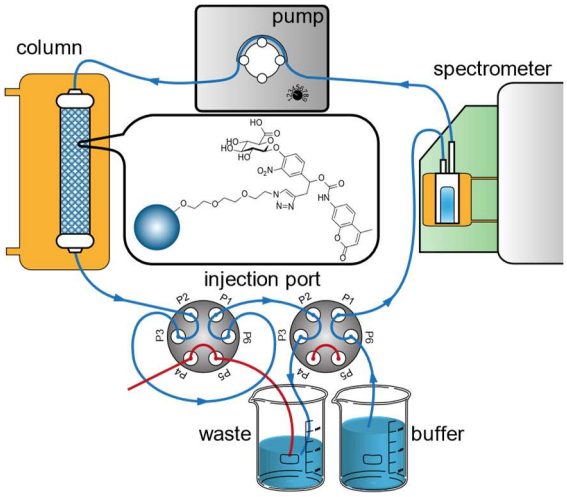


Figure 1. Schematic plan of the SRM QRTF device

In SRM assays the substrate is immobilized on a solid phase, after the enzymatic reaction the fluorophore is released and can be measured. Due to the separation of the substrate from the measurement unit, the background fluorescence is considerably diminished resulting in an increased sensitivity of the enzyme assay.

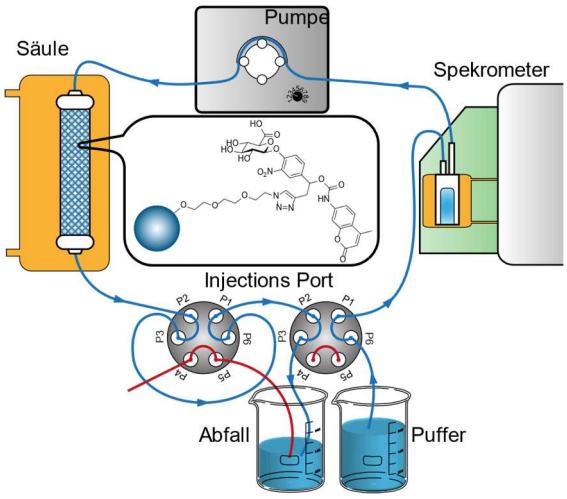
In summary, this work shows novel sensitive and fast strategies for the investigation of the bacterial load of biological systems.

Kurzfassung

Monitoring der Qualität von Oberflächen-, Industrie-, Abfall- und Trinkwasser ist ein entscheidender Aspekt der Umwelt-überwachung, da Wasser die Umwelt-qualität direkt beeinflusst. Ein wichtiger Teil dieses Monitorings ist die Untersuchung bakterieller Belastungen, welche mit einer Vielzahl von Methoden einschließlich spezifischer bakterieller Enzyme durchgeführt werden können. Für diese Assays ist die Verwendung von fluorogenen Enzym-substraten weltweit üblich. Klassische Methoden können jedoch aufgrund von bis zu 72 stündigen Inkubationszeiten zeitaufwendig sein und dadurch rasche und unmittelbare Reaktionen auf umwelt-bedingte Gesundheitsrisiken verzögern. Des Weiteren können inkubations-bedingt Bakterienstämme überrepräsentiert auftreten und dadurch Ergebnisse verfälscht werden.

Im letzten Jahrzehnt wurden deshalb Echtzeit Fluoreszenz Assays (QRTF) entwickelt, welche keine Inkubation der Proben benötigen. Um ihr Potential gänzlich ausschöpfen zu können, ist die Entwicklung neuer fluorogener Substrate notwendig.

Im Rahmen dieser Arbeit wurden zunächst die Probleme des am häufigsten verwendeten Substrates 4-Methylumbelli-eron Glucuronid während QRTF Assays untersucht. Die daraus erhaltenen Erfahrungswerte führten anschließend zur Herstellung und Applikation neuer Enzymsubstrate. In diesen Substraten wurden selbst-zerstörende Linker eingesetzt, um den Zugang zu neuen Fluorophoren und Fluoreszenz-Resonanzenergietransfer (FRET) Mechanismen zu ermöglichen. Es wurde ein neues Messgerät (SRM) entwickelt, das die Auslagerung der enzymatischen Reaktion aus der Messkammer ermöglicht.



Figur 1. Schematischer Plan des SRM QRTF Gerätes

Durch diese räumliche Trennung konnte das Hintergrundfluoreszenzsignal drastisch reduziert und dadurch die Sensitivität des Assays erheblich gesteigert werden.

Zusammengefasst entwickelt diese Arbeit innovative und schnelle Methoden für die Untersuchung bakterieller Belastung von biologischen Systemen.

Danksagung

Mein erster Dank gilt Univ.Prof. Dipl.-Ing. Dr. Johannes Fröhlich, der mir ermöglichte, meine Doktorarbeit in seiner Arbeitsgruppe realisieren zu können. Ebenso möchte ich meinem langjährigen Supervisor Dipl.-Ing. Dr. Hannes Mikula für die Betreuung und sein Vertrauen danken. Es ist mir ein Anliegen, mich weiters bei Ass.Prof. Dipl.-Ing. Dr. Christian Hametner und Dipl.-Ing. Dr. Ernst Horkel für ihre Unterstützung zu bedanken.

Hilfreichen waren für mich das familiäre Arbeitsklima und die fachlichen Beiträge meiner Laborkollegen und -kolleginnen Markus Schwarz, Dennis Svatunek, Stefan Kronister, Barbara Sohr, Philipp Skrinjar, Julia Weber und der restlichen Arbeitsgruppe. Herzlichen Dank an sie dafür.

Besonderer Dank gilt meinen Eltern Walter & Eva Lexmüller für ihr unerschütterliches Vertrauen und ihre bedingungslose Unterstützung. Meiner Schwester Teresa Lexmüller möchte ich führ ihre Ehrlichkeit und Motivation danken. Meinem während dieser Arbeit leider verstorbenen Großvater Ob. Ing. Franz Lexmüller, dessen ihm verwehrten Traum, ich durch diese Dissertation verwirklichen konnte, fühle ich mich besonders verpflichtet.

Speziell danken möchte ich meiner Freundin Susi Prinz für die Unterstützung bei der Arbeit und die Bereicherung meines Lebens in den gemeinsamen sechs Jahren.

General explanatory notes

Literature References

References to literature are distinguished as superscript Arabic numerals.

Substance Labeling

Compounds previously described in literature are sequentially numbered in bold Arabic numerals. Commercially available reagents used “as bought” were not numbered. Compounds unknown to literature that were prepared in the course of this thesis are numbered in bold roman numerals.

Nomenclature

The nomenclature of chemical compounds not previously described in the literature is based on the rules of Chemical Abstracts. Compounds known to literature, reagents or solvents might be described by simplified terms, common or trade names.

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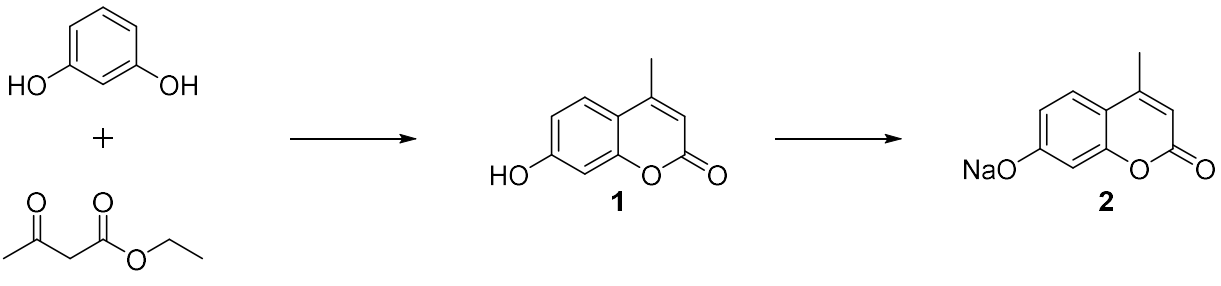
A) Synthetic Schemes

A.1.1 Synthesis of 4-Methylumbelliferyl-β-D-glucuronide & 4-Methylumbelliferyl-β-D-glucoside

A.1.1.1 Synthesis of 4-Methylumbelliferone



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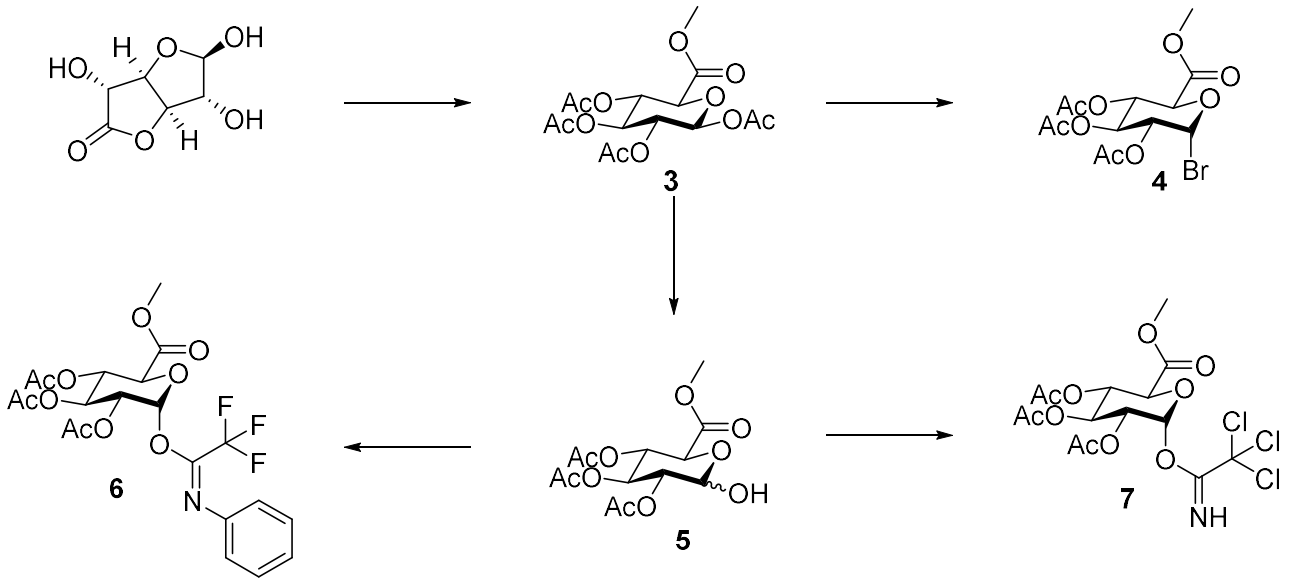

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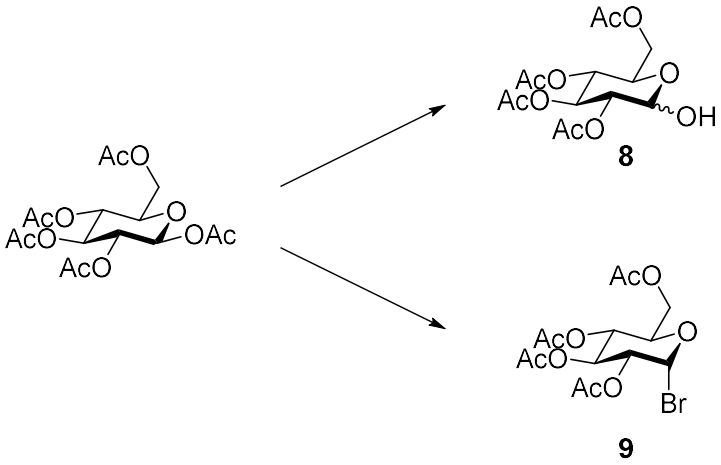
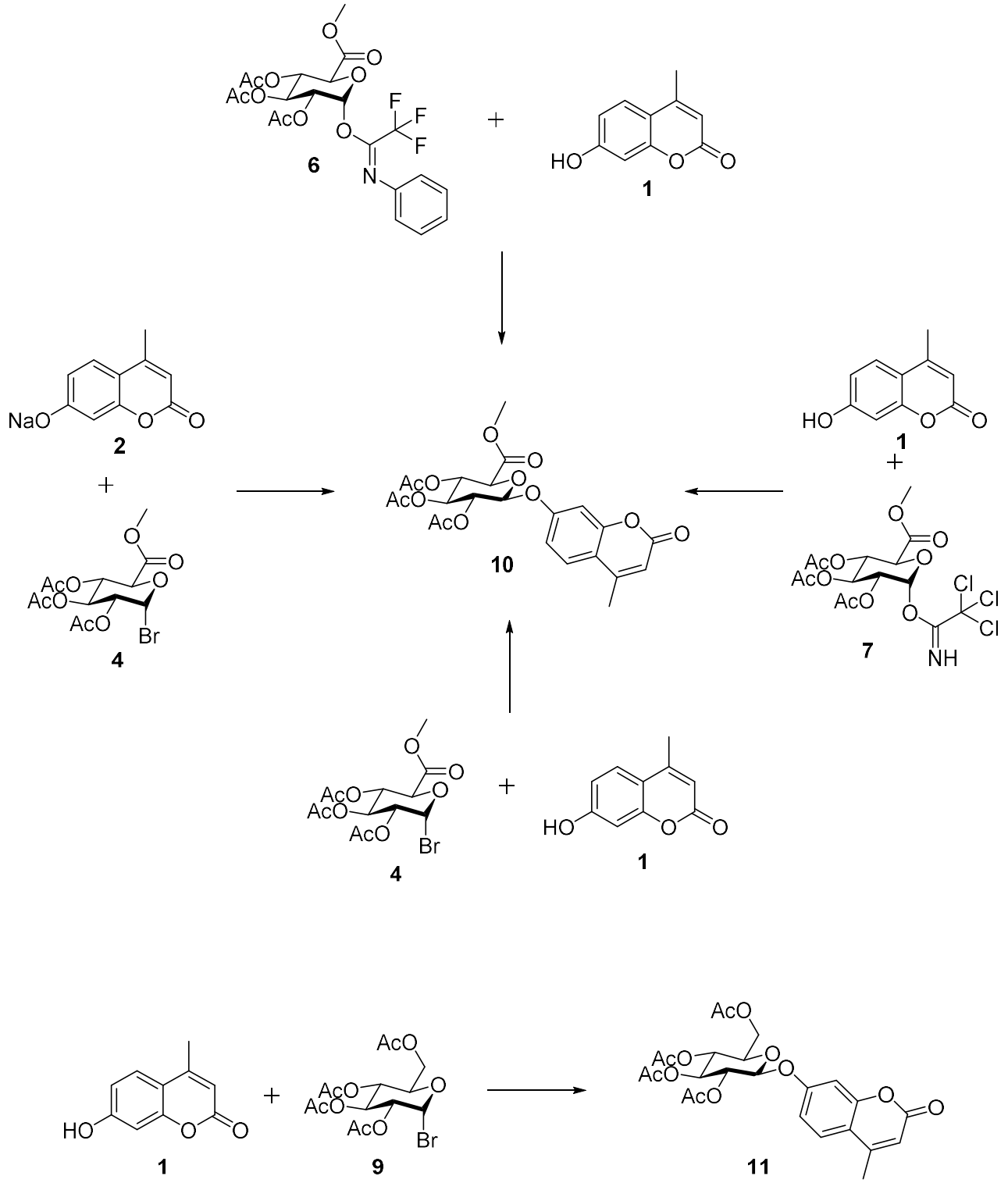
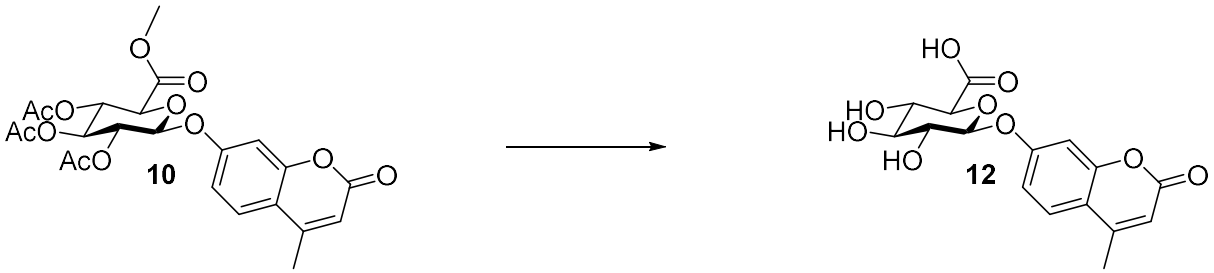
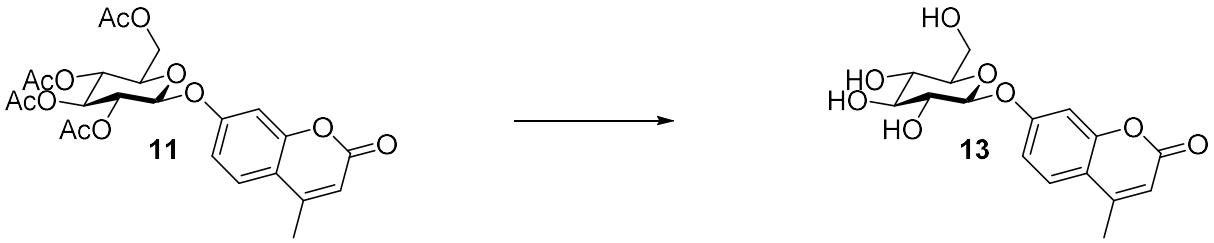


A.1.1.4 Chemical Glycosylation and Deprotection of 4-MUG

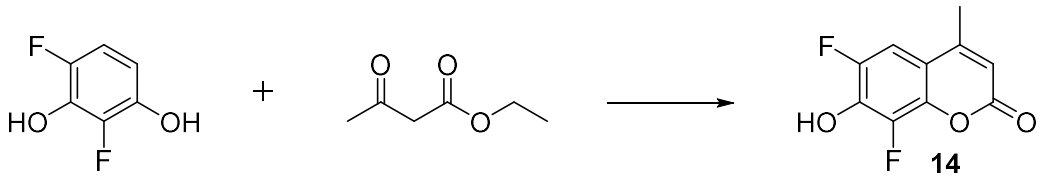


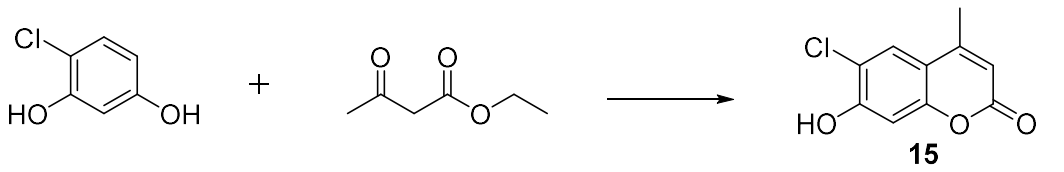


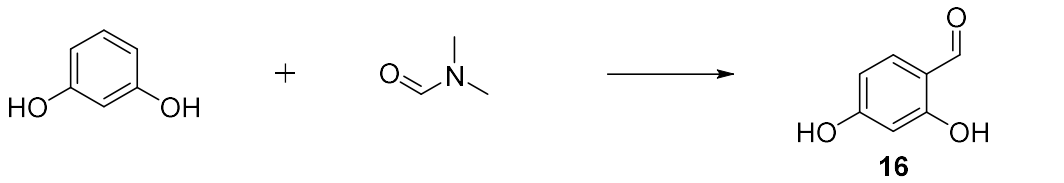


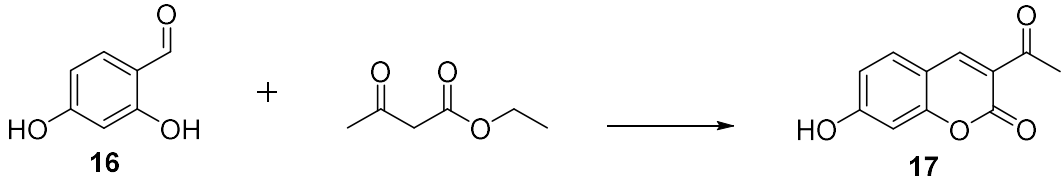
A.1.2 Synthesis of β-Glucuronidase Responsive Fluorogenic Probes

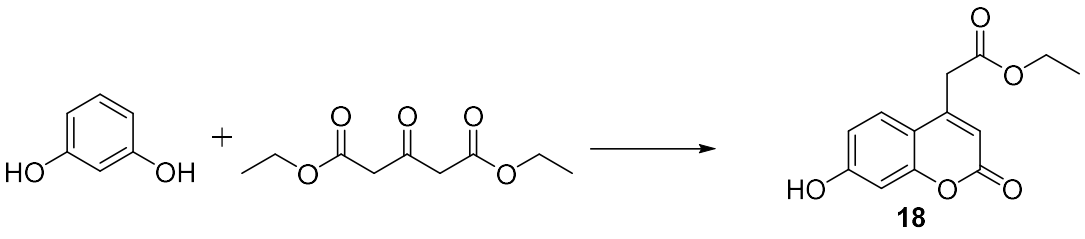
A.1.2.1 Synthesis of Fluorophores

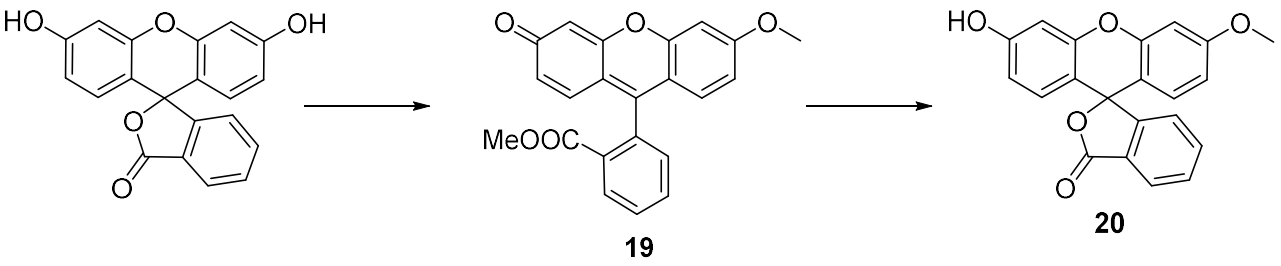












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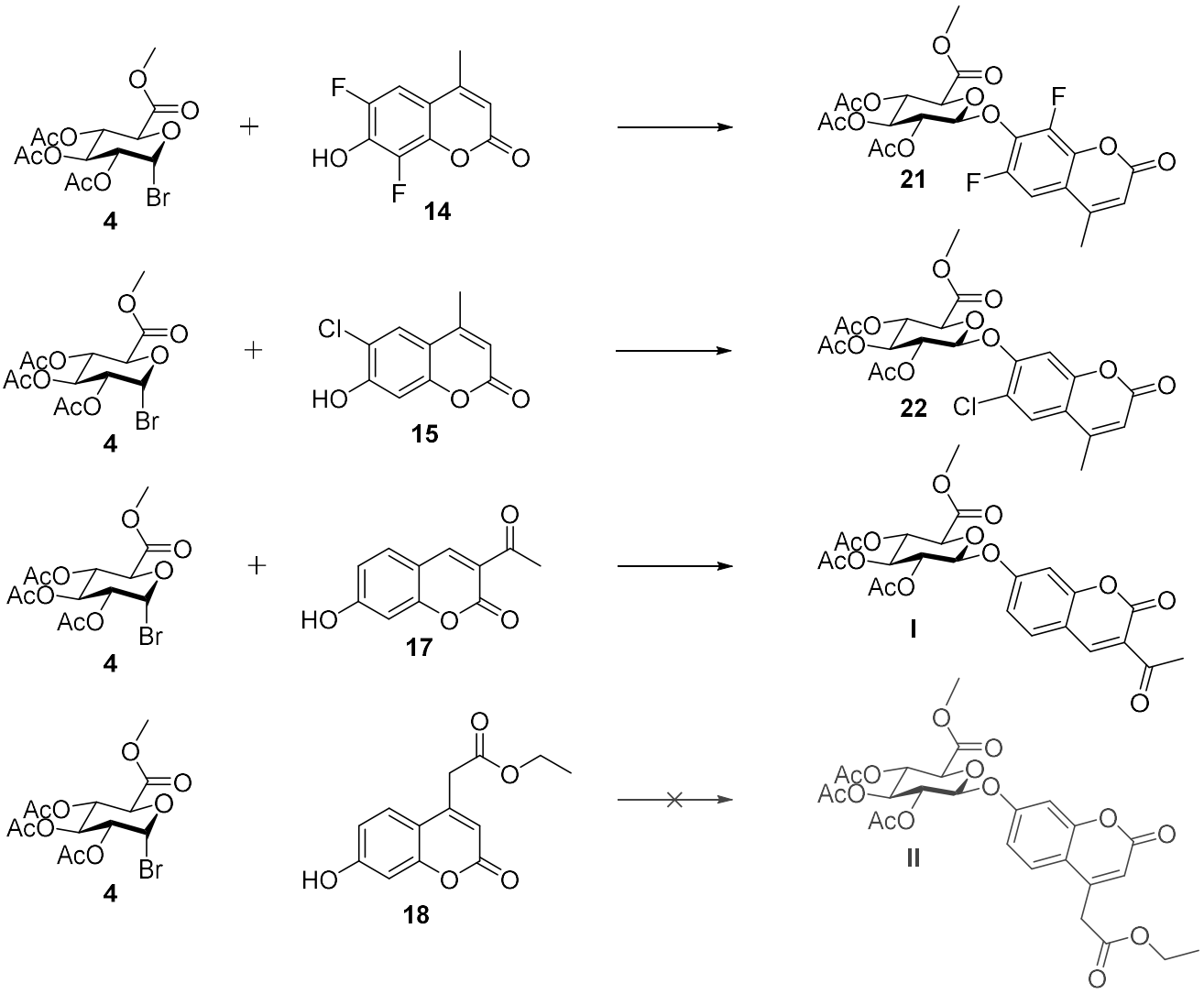

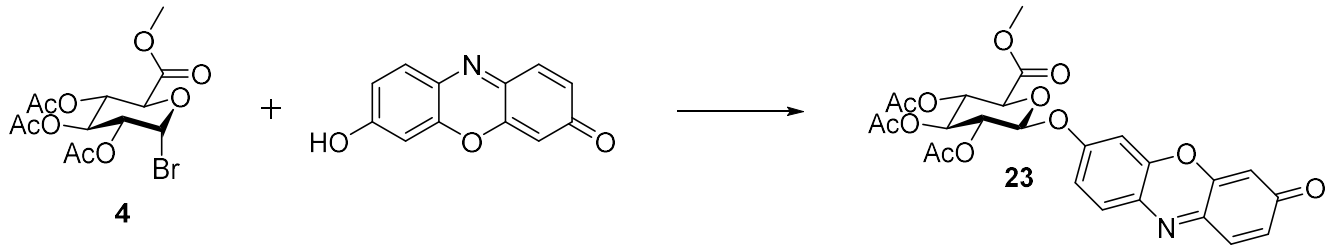

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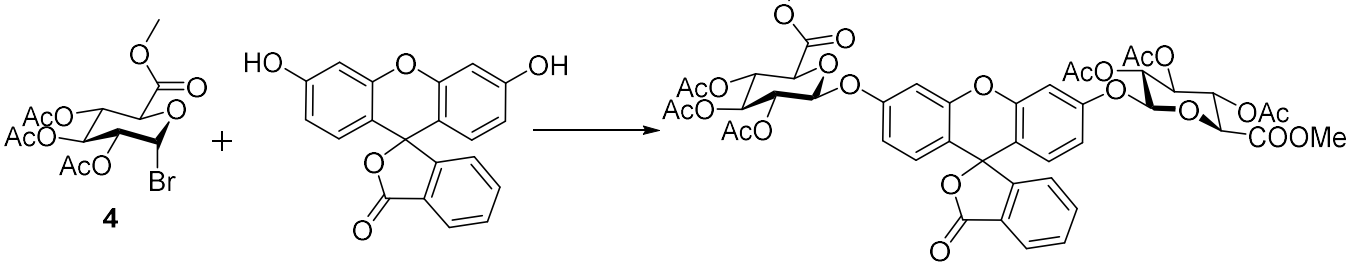

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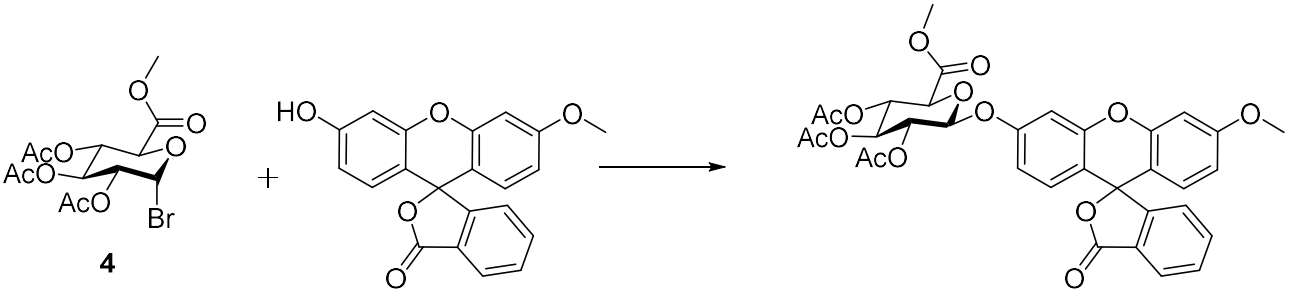

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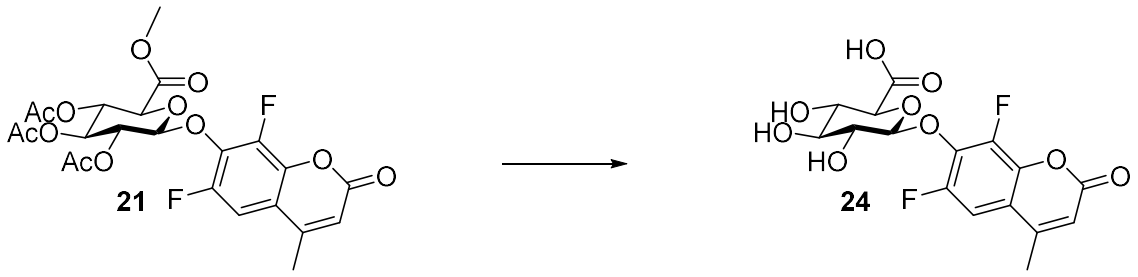

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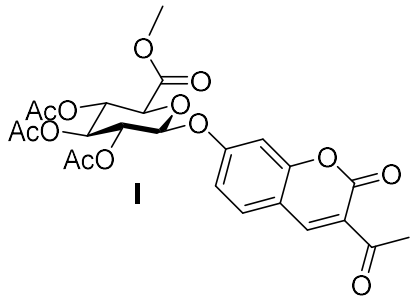

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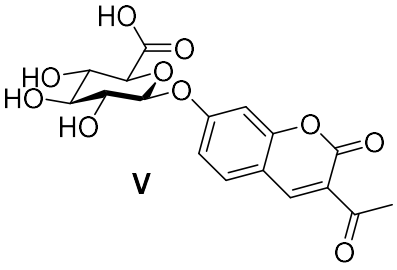

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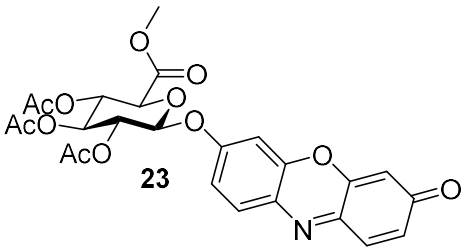

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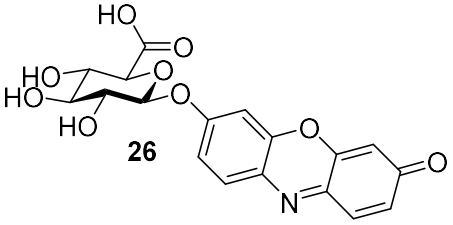

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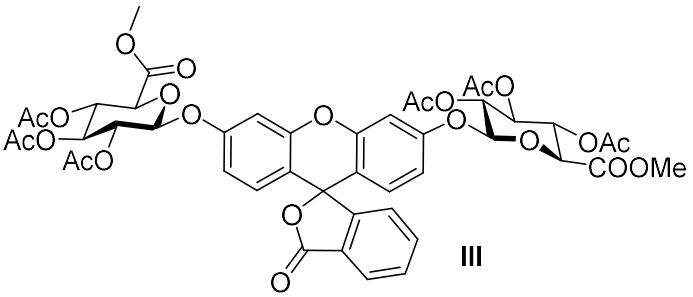

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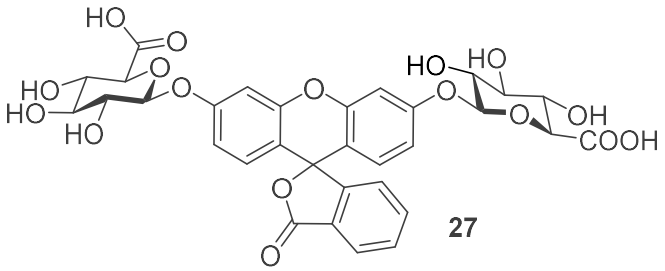

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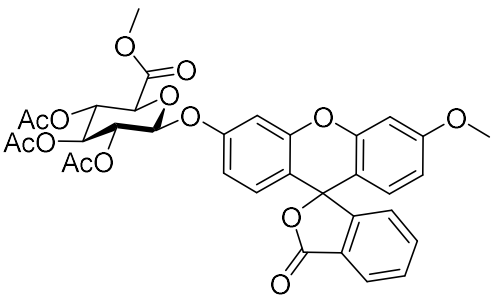

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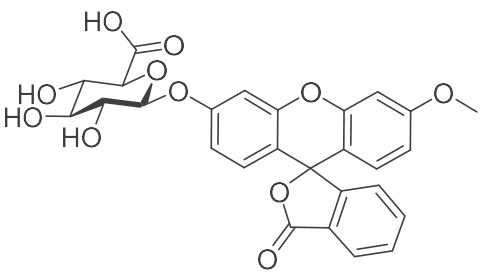
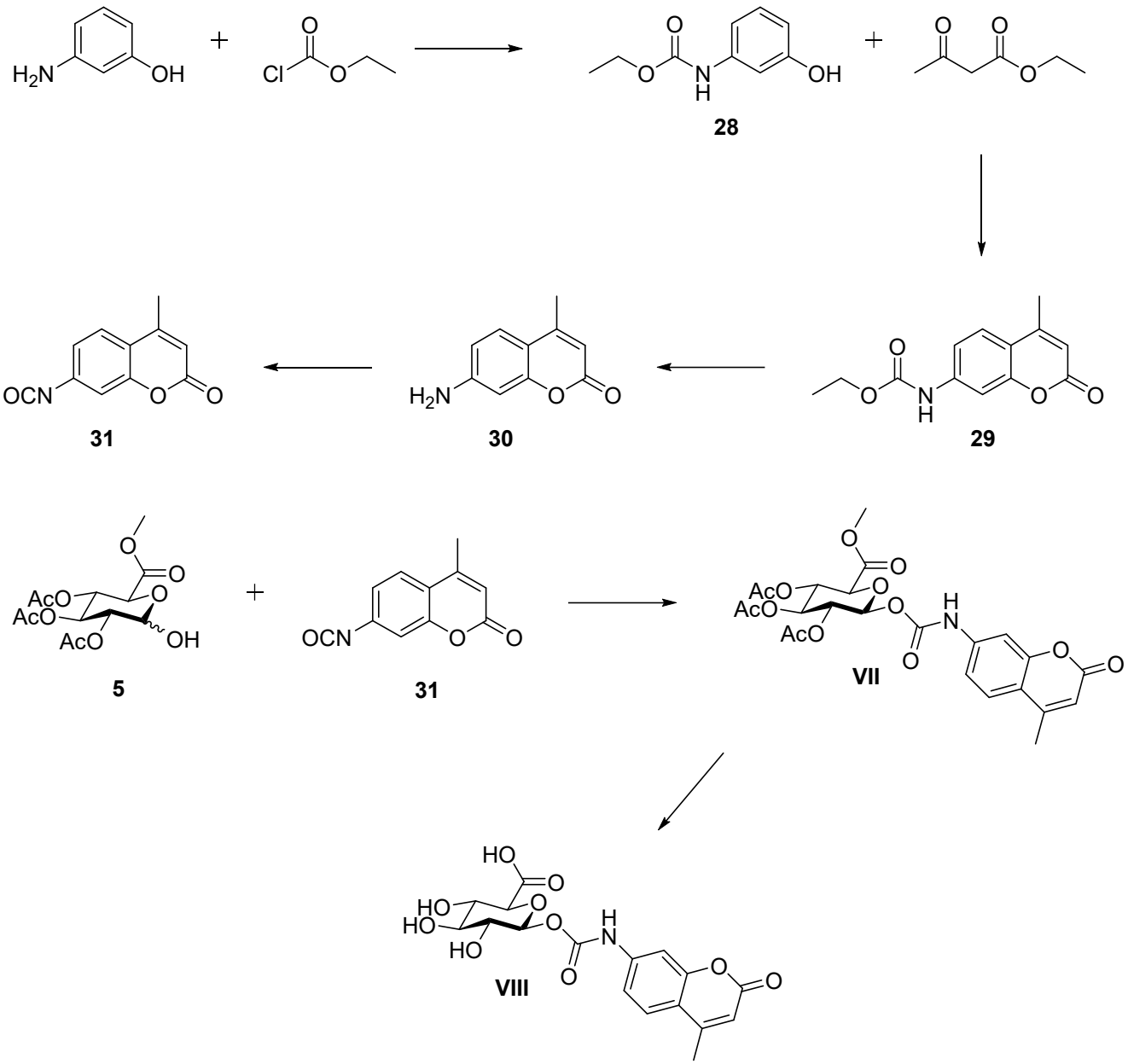
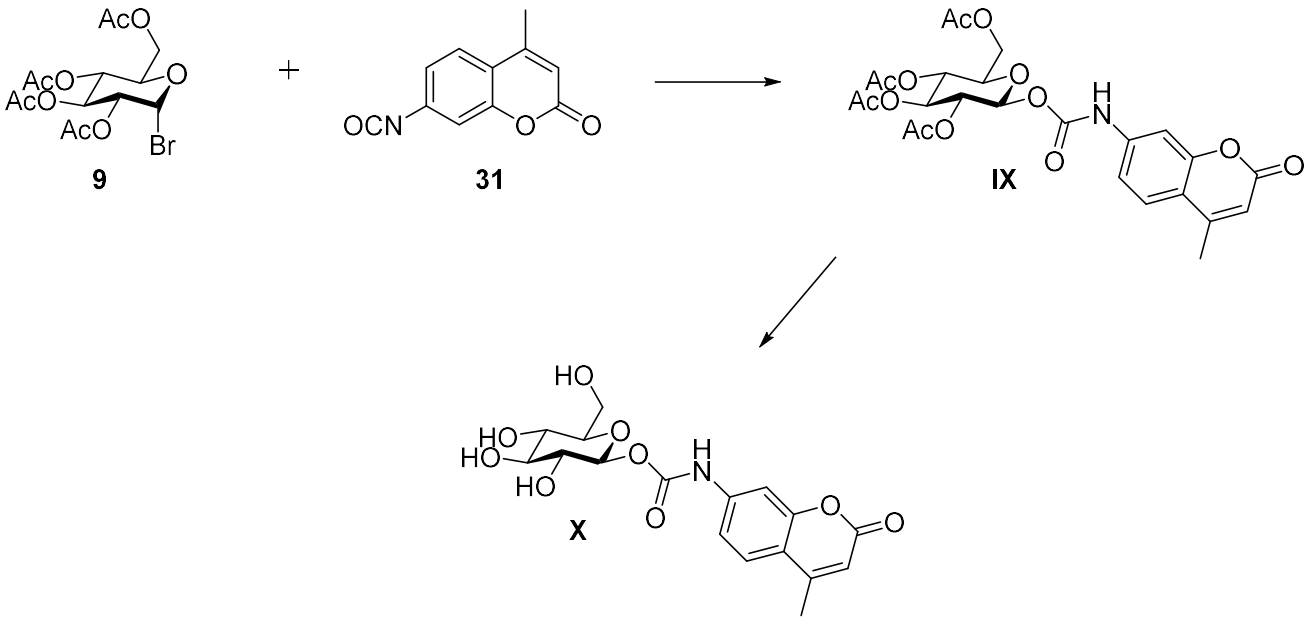


VI

A.1.3 Synthesis of Self-Immolative Nano Probes

A.1.3.1 Synthesis of Directly Conjugated Fluorogenic Carbamates and Carbonates

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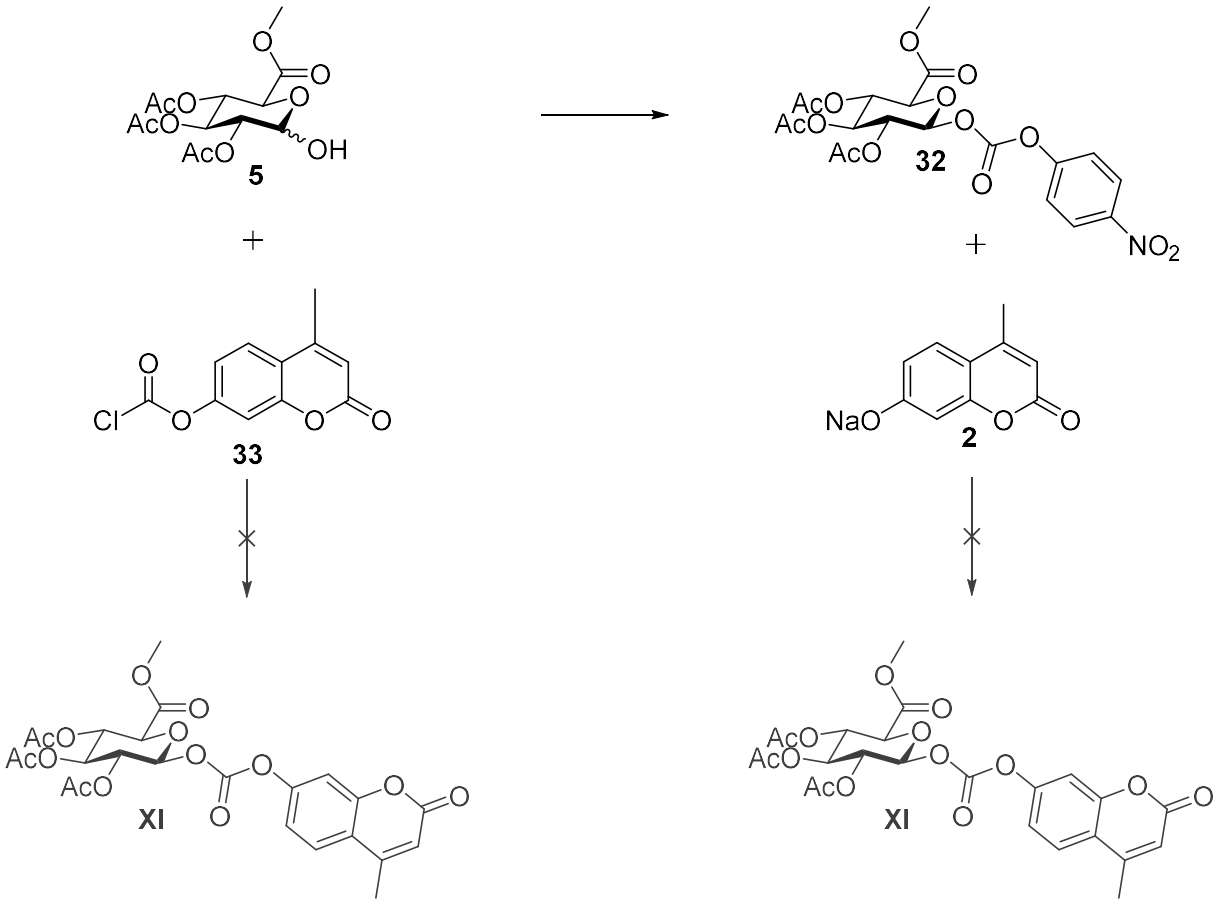
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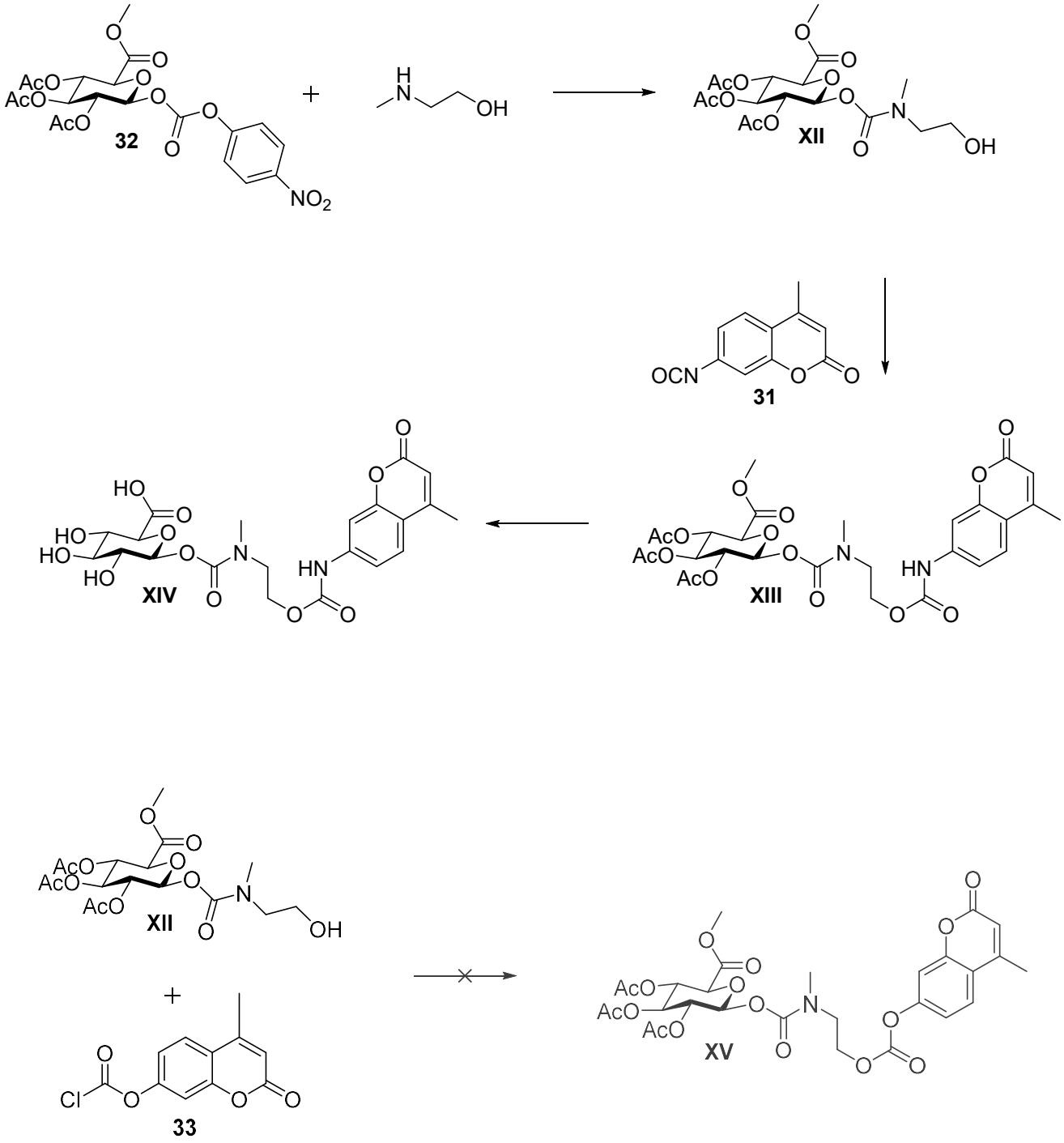
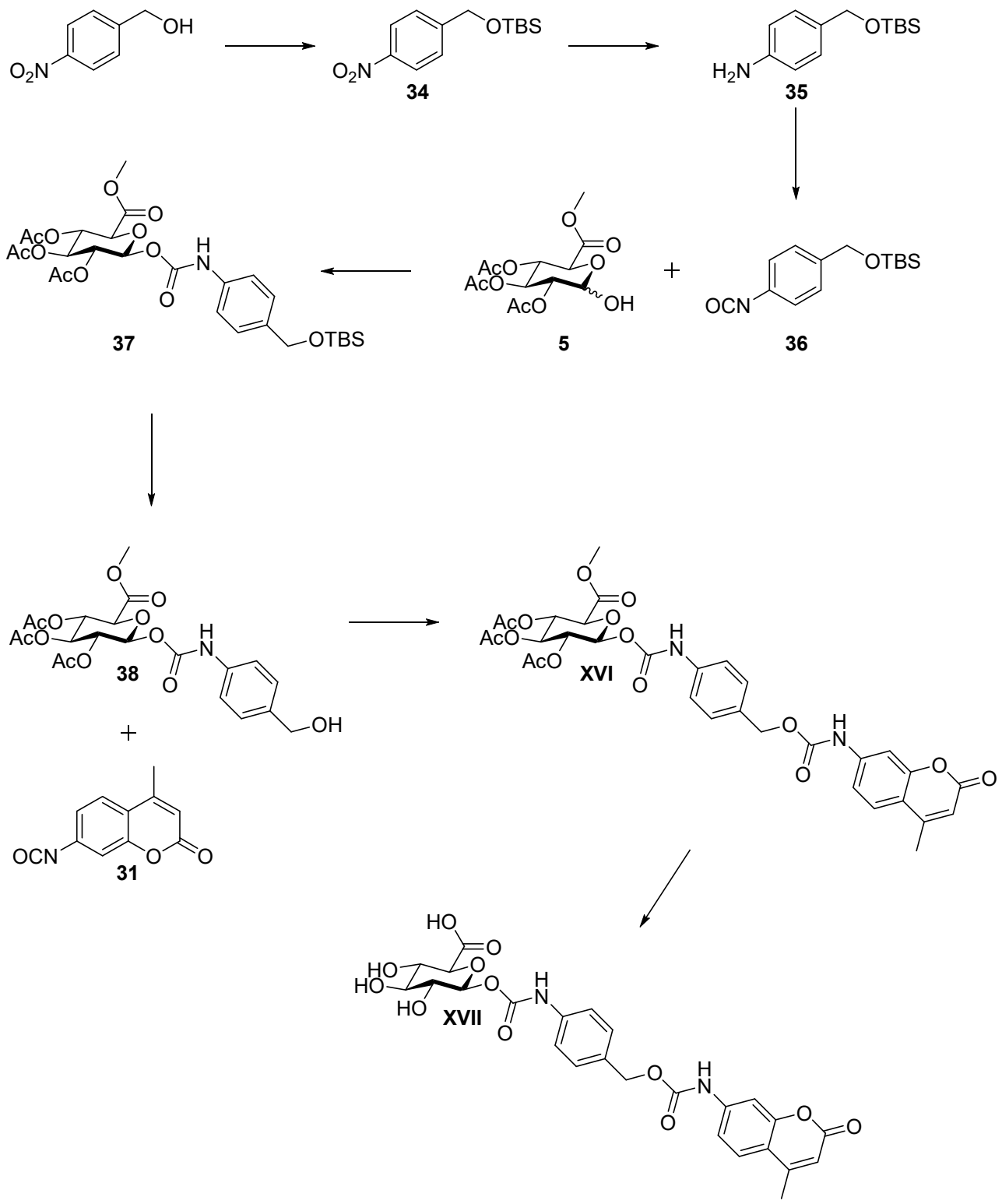
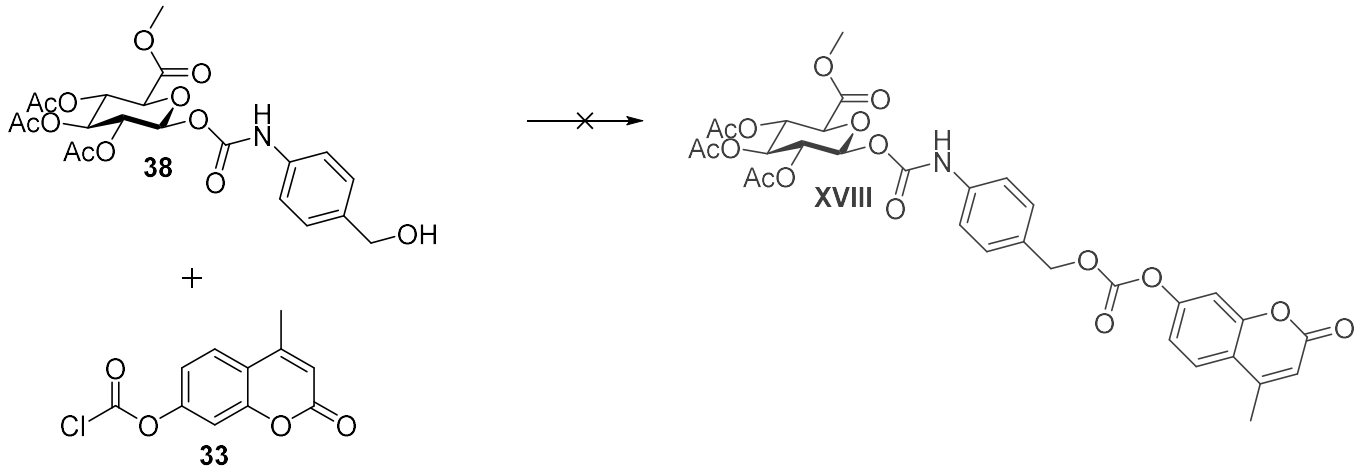
A.1.3.2 Synthesis of Self-Immolative Probes with Aliphatic Linkers



A.1.3.3 Synthesis of Self-Immolative Probes with Aromatic Linkers

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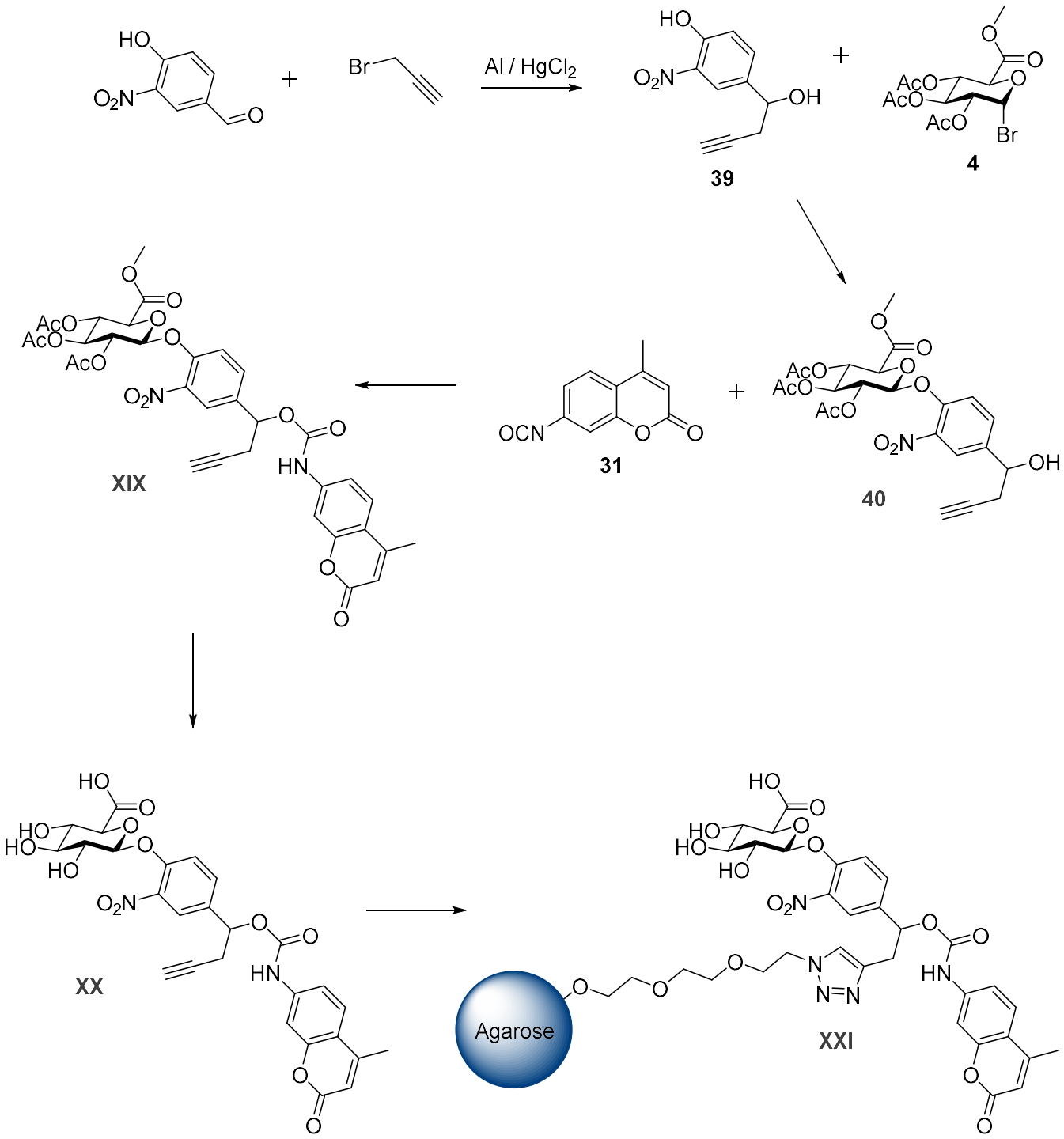
A.1.3.4 Synthesis of Immobilized Self-Immolative FRET Probes



B) General Introduction

B.1.1 Usage and Development of Artificial Enzyme Substrates

Since the role of enzymes, in almost all metabolic processes of a cell, was discovered in the end of the 19th century, it became a primary target of biochemistry to investigate their occurrence and behavior. This is possible by studying the substrates and products of the reactions they catalyze (Figure B 1), but "natural" enzyme substrates are generally hard to detect and often don't possess the desired enzyme affinity for assays.

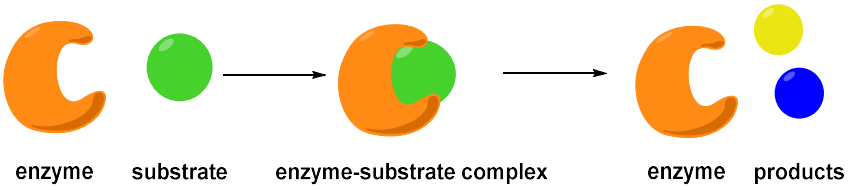


Fig. B 1. General principle of an enzyme catalyzed substrate cleavage reaction

These drawbacks have led to the research on artificial enzyme substrates, which are now primarily used to study enzyme kinetics, quantify enzymes or mark the locality of enzymes in tissue. Synthetic substrates (enzyme responsive probes) are specially designed for their field of application, for example they can contain chromogenic or fluorogenic moieties. When the enzyme cleaves such substrates the chromo- or fluorogenic moiety is set free (Figure B 9), which allows identification and quantification of enzymes at extremely low concentrations. Therefore, the production of these and other artificial enzyme substrates has increased rapidly over the last decades.[1,2] Some of this substrates have been adapted for microbiological uses, especially in bacterial diagnosis, since in the fields of medical diagnostics and environment monitoring, a rapid and precise way of quantifying bacteria is of utmost importance.[3,4] This can be achieved by utilizing assays for enzymes bacteria produce, which are often highly specific like β-glucuronidase (GUS) for *Escherichia coli* or β-glucosidase for *enterococci*. However approved methods like the multiple tube fermentation (MTF) or membrane filter method (MF) can be complicated, inaccurate and time consuming since they involve incubations up to 72 hours and many sequential tests. This causes problems, because too much time is required to obtain a definitive answer on environmental health risks.[5] Incubation methods can also be misleading since different bacterial strains often have different growth rates resulting in an overrepresentation of specific strains after incubation.[6] Therefore the research for novel substrates that can be used in fast "real-time" assays without the need for any incubation is of great interest.

B.1.2 Glycoside Hydrolases

Glycoside hydrolases (short glycosidases) are a family of enzymes that are very common in essentially all domains of life. They catalyze the hydrolysis of glycosidic bonds in polysaccharides like cellulose, starch and glycosaminoglycans (Figure B 2).[7,8]

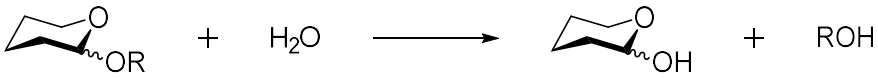


Fig. B 2. Hydrolysis of a glycosidic bond (R: organic substituent)

In prokaryotes they can be found as intra- and extracellular enzymes and are mostly involved in the nutrient acquisition. An important example of glycosidases in bacteria is β-galactosidase, an internal enzyme responsible for the cleavage of lactose and part of the lac operon in many enteric bacteria.[9] In eukaryotes glycosidases are found in many different parts of the organism like the lysosome, where they are responsible for the degradation of glycosaminoglycans. In the Golgi apparatus and the endoplasmic reticulum, they are involved in the processing of glycoproteins. In the salvia and gut they degrade lactose, starch and other oligo- and polysaccharides.

Glycosidases can be classified in different ways, the most common of which is the classification based on substrate specificity. This is the simplest approach but does not appropriately accommodate enzymes with several different substrates. Different methods of classification are on the basis of mode of action (endo: if the enzyme attacks within the polysaccharide, exo: if the enzyme attacks the termini of a polysaccharide) or on the basis of amino acid sequence similarities.[7]

B.1.3 β-Glucuronidase EC 3.2.1.31

β-Glucuronidase is responsible for the exo cleavage of glucuronide units from various polysaccharides and other glucuronide conjugates linked via the carbon-1 atom in β-configuration. The enzyme is widely spread in eukaryotes, typically associated with the lysosome where it is responsible for the breakdown of chondroitin sulfate, heparan sulfate and other glycosaminoglycans. In humans β-glucuronidase deficiency leads to sly syndrome (MPS VII), a mucopolysaccharide storage disease. [10]

In prokaryotes, Klian & Bülow observed that out of a collection of 633 strains of *Enterobacteriaceae* and *Vibrionaceae* only *Escherichia* strains possessed β-glucuronidase activity.[11] This has been confirmed in follow up studies, where out of 460 human, 105 cow and 55 horse *Escherichia coli* isolates 99.5% showed β-glucuronidase activity after 28 hours of incubation, whereas no non *Escherichia coli* isolate showed any β-glucuronidase activity.[12] Therefore, β-glucuronidase assays have become an important tool of environment monitoring, since *Escherichia coli* has been used as an indicator of fecal contamination for over 100 years.[12,13]

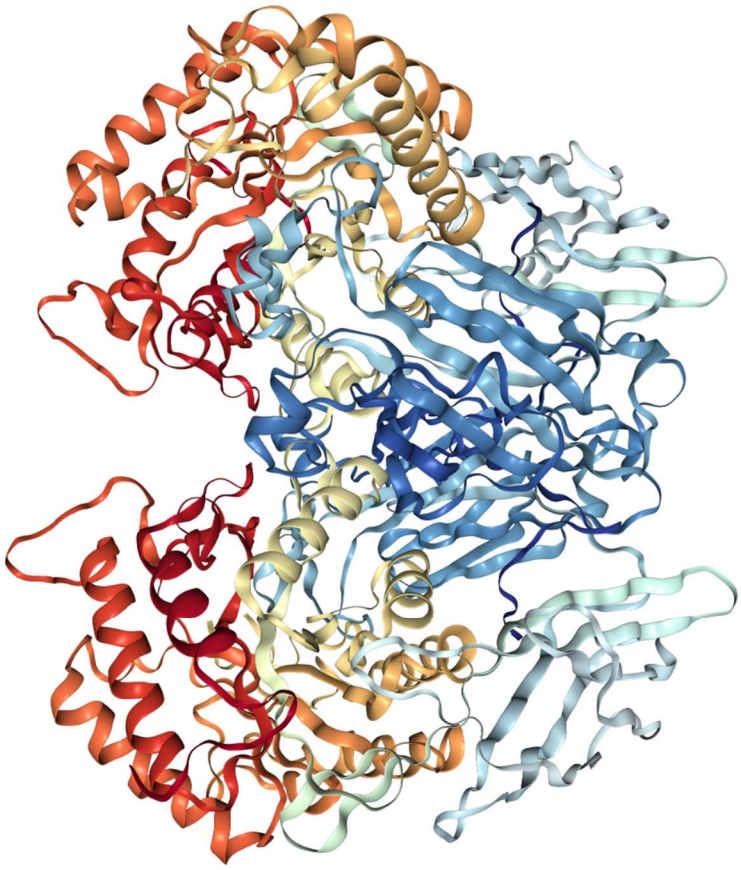


Fig. B 3. Crystal structure of *Escherichia coli* beta-glucuronidase.[14,15]

B.1.4 Quantitative Real-Time Fluorescence Measurement Technology

Like already mentioned in chapter B.1.1, today microbiological water monitoring is primarily based on cultivation methods.[16] These methods are not only time consuming[5] and tend to overrepresent specific bacterial strains[6], in the last decade it was also shown that bacteria in a viable but nonculturable state (VBNC) are a massive problem for these cultivation methods.[17] *Vienna Water Monitoring Solutions* has developed with the *Colimínder* (Figure B 4) a device that allows quantitative real-time fluorescence (QRTF) measurements of bacterial activity in water and is therefore capable to give more adequate estimations of pathogenic risks within minutes instead of days. Additionally, the *Colimínder* is almost fully automated and does not need highly skilled technical staff for routine analysis.

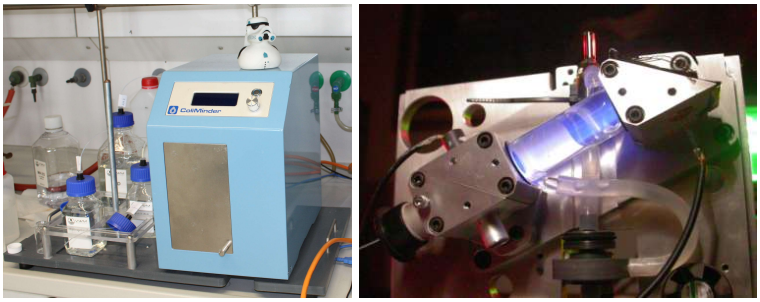


Fig. B 4. Prototype of the *Colimínder* & flow-through cuvette

Its center piece is the patented flow-through cuvette (Patent Nr. 510765; PCT/AT2011/000497) (Figure B 5), which allows a simultaneous real-time analysis of fluorescence intensity and transmission. With 4-methylumbelliferyl-β-D-glucuronic acid as substrate it is possible to detect 5 CFU (colony-forming units) *E. coli* / 100 ml within 10 minutes whereas cultivation methods have a detection limit of 30 CFU *E. coli* / 100 ml within 24 hours.[18]

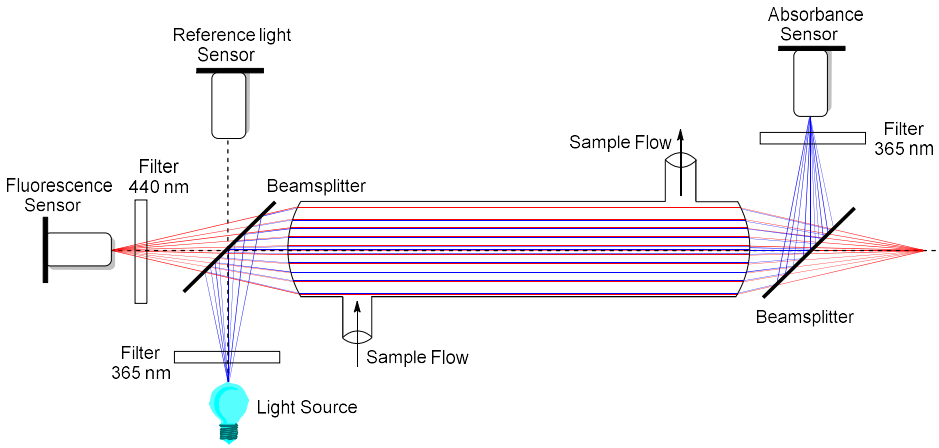


Fig. B 5. Schematic of flow-through cuvette application in QRTF analysis

Considering all its features the *Colimínder* is not only the ideal analytical instrument for sewage and industrial plants where high bacterial loads are common. It is also the perfect tool for environmental monitoring of surface waters, where it can detect the slight increase of fecal bacteria washed into streams after short periods of rainfall (Figure B 6).

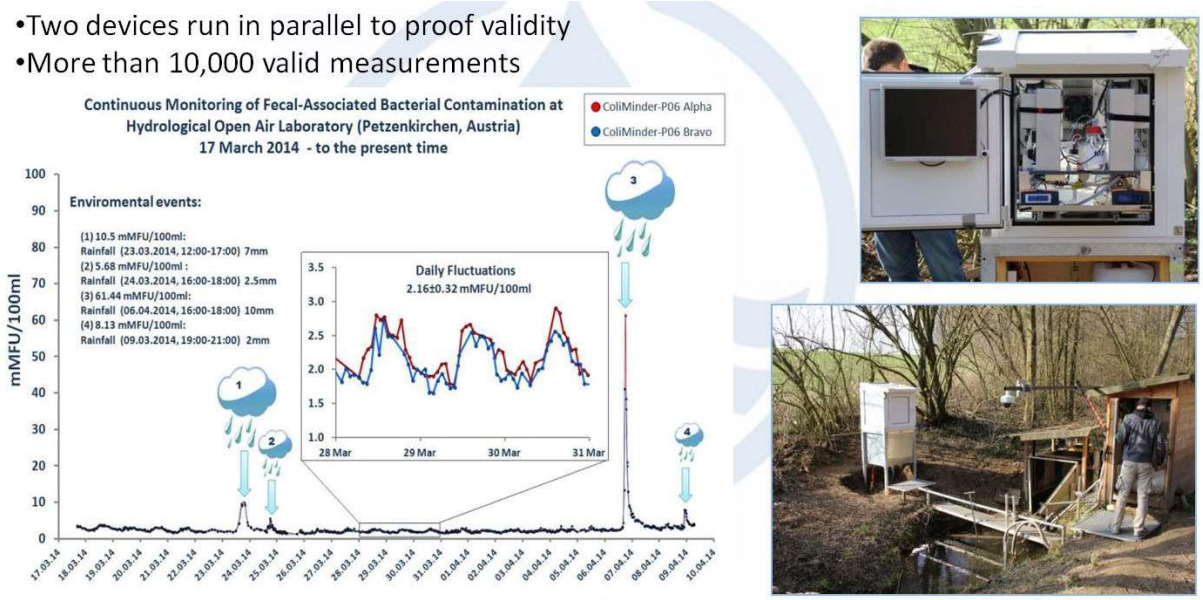


Fig. B 6. Continuous monitoring of fecal bacteria contamination at Hydrological Open-Air Laboratory (HOAL) (Pletzenkirchen, Austria) 17 March 2014 – 10 April 2014

Source: http://www.vienna-water-monitoring.com (01.03.2018)

The *Colimínder* is also a capable of measuring total microbial contamination at drinking water standards and has already been tested at disaster response exercises (Figure B 7).

Fig. 7. Testing of the Colimínder-Mobile Device at a disaster response exercise of the Austrian Armed Forces TÜPL Blumau-Neurißdorf - September 2014

Source: http://www.vienna-water-monitoring.com (01.03.2018)

B.1.5 Enzyme Responsive Chromo- and Fluorogenic Substrates

Like already mentioned before chromogenic or fluorogenic enzyme substrates are extremely versatile tools for the qualification and quantification of enzymes. Chromogenic substrates show a color change after the enzymatic cleavage (Figure B 8), this color change can be followed with the eye, or a photometer making these substrates suitable for low cost enzyme assays since to no expensive equipment is needed.[19]

Fig. B 8. Example of a low-cost E. coli assay using Resorufin β-D-Glucuronide[19]

Fluorogenic substrates themselves show little or no fluorescence, upon enzymatic cleavage the highly fluorescent agent is set free (Figure B 9), allowing quantification of extremely low enzyme concentrations, therefore many of these substrates are used routinely in hospital laboratories, food technology and in research.

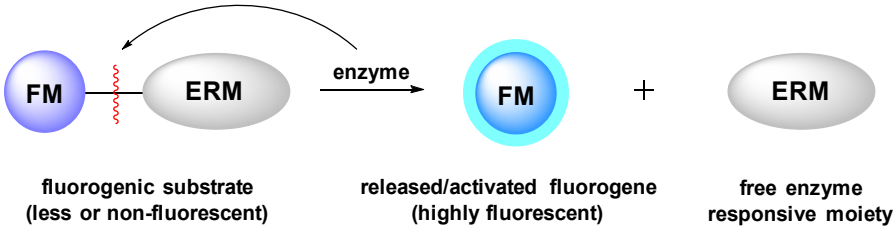
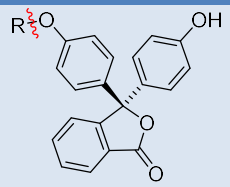
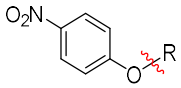
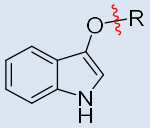
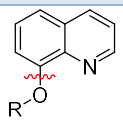
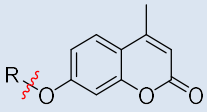
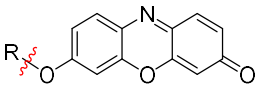
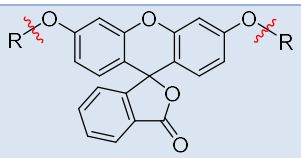


Fig. B 9. General principle of fluorogenic substrates for enzyme assays (FM: fluorogene/fluorogenic moiety, ERM: enzyme responsive moiety, e.g. carbohydrate, amino acid/peptide, phosphate, etc.)

Most of these probes consist of well-known and widely used chromo / fluorophores like, nitrophenols, indoxyls, coumarin- or fluorescein derivates. The most commonly used chromo/fluorogenic scaffolds are summarized in Table B 1.

Table B 1. Fluorogenic scaffolds/moieties applied for synthetic enzyme responsive substrates (Gal: galactoside, Glc: glucoside, GlcA: glucuronide, GlcNAc: N-acetylglucoside, BOC: *tert*-butyloxycarbonyl, R: enzyme specific cleavage site/group).

Fluorogenic moiety	Structure and cleavage site	Commonly used substrates
Phenolphthaleins		Phenolphthalein-β,D-Gal Phenolphthalein-β,D-GlcA Phenolphthalein-phosphate
p-Nitrophenols		p-Nitrophenyl-β,D-GlcA p-Nitrophenyl phosphate
Indoxyls		Indoxyl-β,D-Gal Indoxyl-β,D-GlcA
8-Hydroxyquinolines		8-Hydroxyquinolin-β,D-Gal 8-Hydroxyquinolin-β,D-GlcA
4-Methylumbelliferones		4-Methylumbelliferon -β,D-Gal 4-Methylumbelliferon -β,D-GlcA 4-Methylumbelliferon-phosphate
Phenoxazines (Resorufin)		Resorufin acetate Resorufin-β,D-Gal Resorufin-β,D-Glc
Fluorones (Fluorescein)		Fluorescein-di(β,D-Gal) Fluorescein-di(β,D-Glc)

The drawback of these substrates is their dependence on the pH value of the enzyme assay. The pH optimum of most glycosidases is acidic or neutral, but also the absorbance of commonly used chromophores is pH dependent and often not compatible with the optimum for the enzymes (Figure B 10). Fluorophores as well often have their maximal quantum efficiency far away from physiological pH values, as only their ionized forms (mostly deprotonated) show a strong fluorescence (Figure B 11). For most state of the art methods

this causes no problems since these assays are divided in different steps and only at the end of the assay during measurement the pH value is adjusted for a high signal intensity.

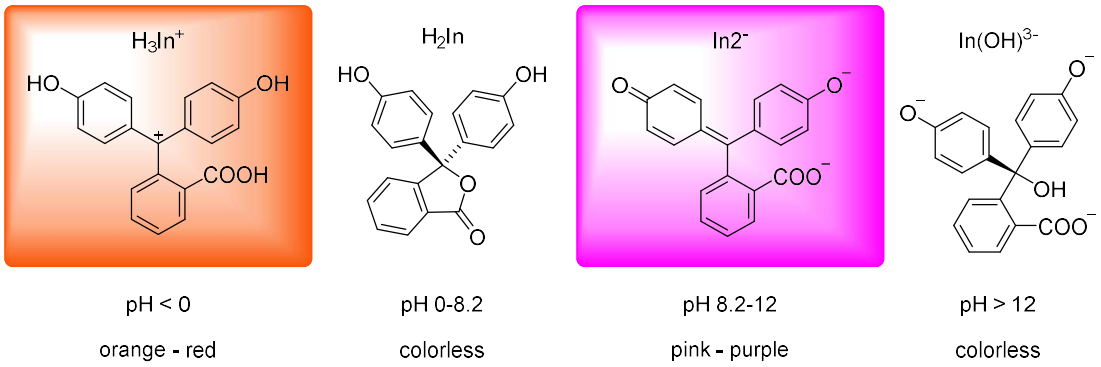


Fig. B 10. Different states & colors of phenolphthalein in aqueous solution.

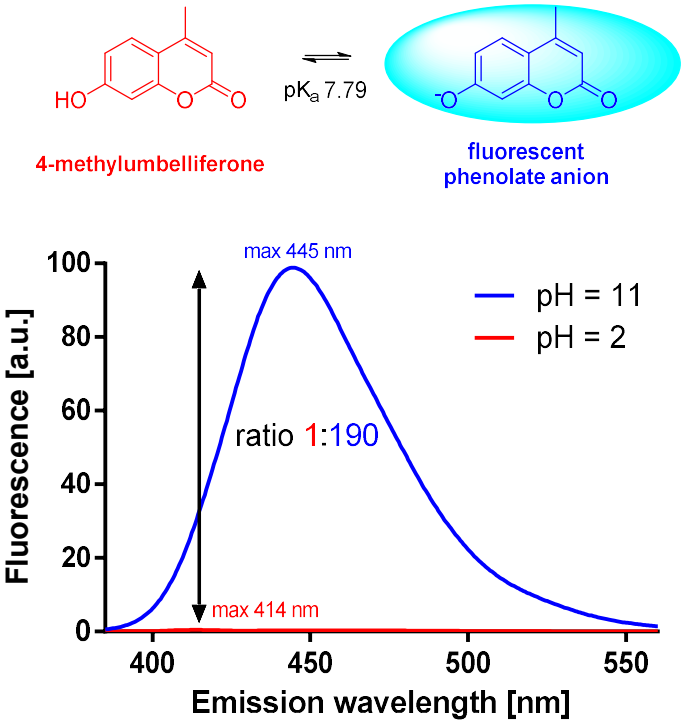


Fig. B 11. Comparison of the fluorescence of 4-methylumbelliferone and its phenolate anion at an excitation wavelength of 365 nm.

For real-time assays like the *Colimínder* approach (see B.1.4) this is major problem, because when measuring the working enzyme, conditions must not only be optimized for a high signal intensity but also for a good enzymatic conversion rate. By differing to far from the pH optimum of the enzyme not only the conversion rate of substrate gets worse, also inactivation or degradation of the enzyme sets in. This is can be shown on the example β-glucuronidase, which has its optimal pH value at 6.8.[20] When the pH value of the enzyme assay increases

significantly (pH > 8) not only the enzymatic conversion of the substrates gets slower. Also, the linearity range of the assay is much shorter, making the determination of enzyme kinetics difficult (Figure B 12).

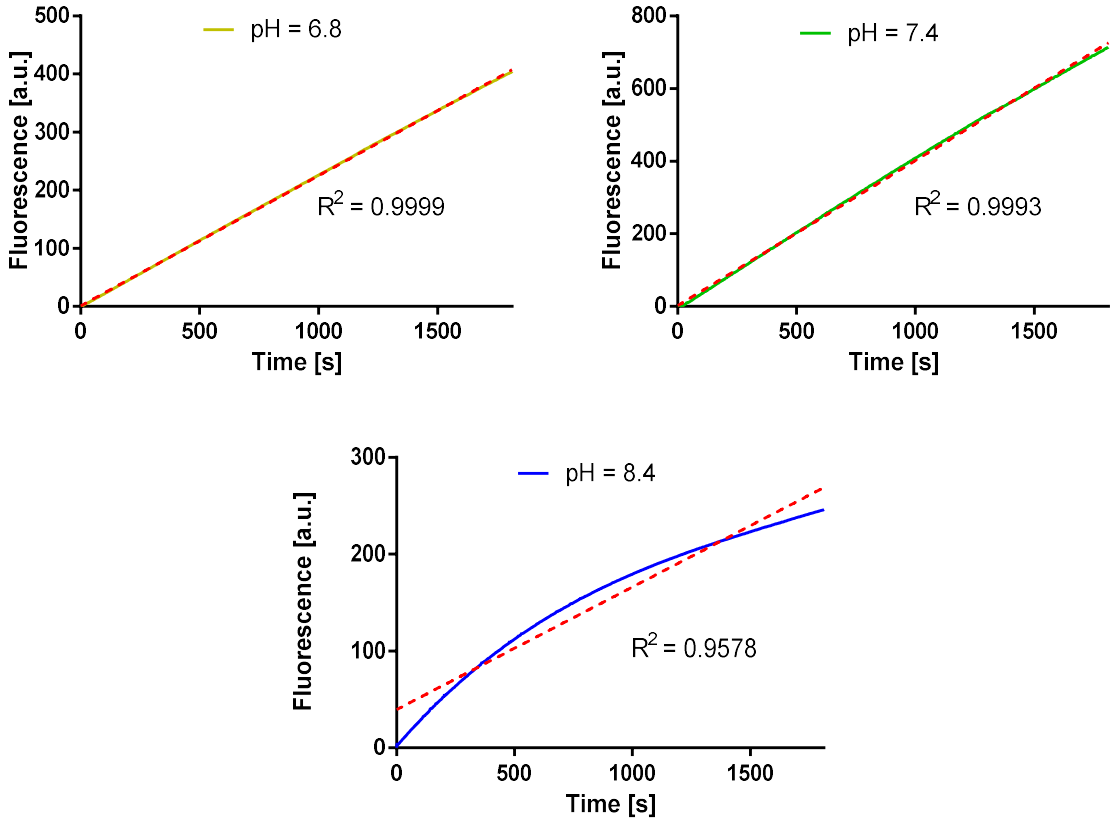


Fig. B 12. Conversion of 4-methylumbelliferyl-β-D-glucuronic acid (0.64 mM) by β-glucuronidase (25 U/l) at different pH conditions.

Therefore, the development of novel pH independent enzyme substrates is a main topic of this thesis.

B.1.6 Controlled Release via Self-Immolative Linkers

Most state of the art glycosidase responsive fluorogenic probes are designed following the classical prodrug approach. The drug, or in this case the fluorophore, is directly linked to the enzyme responsive moiety and set free upon enzymatic cleavage. This principle has been extensively explored for small molecules[21,22], but also has drawbacks for glycosidase probes. The fluorophore has to be directly linked via the glycosidic bond. Therefore, only fluorophores that change their fluorescence behavior (intensity or wavelength) via the deprotonation of

the alcoholic group that is directly linked to the glycoside, can be used for this classical approach (Figure B 13).

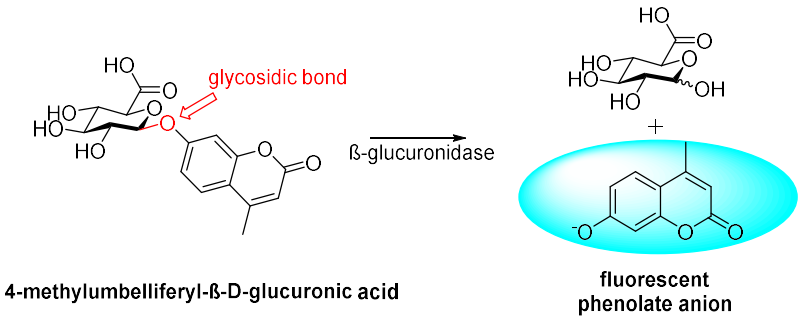


Fig. B 13. Example of a classical enzyme substrate, with the fluorophore directly linked to the glucuronide via the glycosidic bond.

By inserting a linker between the enzyme-labile bond and the fluorophore not only stability problems of commonly used enzyme substrates can be circumvented, also a wider range of fluorophores can be used. These linkers are specially designed to become labile upon enzymatic cleavage and disassemble rapidly (Figure B 14). This technology is now increasingly used in the development of new enzymatic probes and referred to as 'self immolative linkers'.[23]

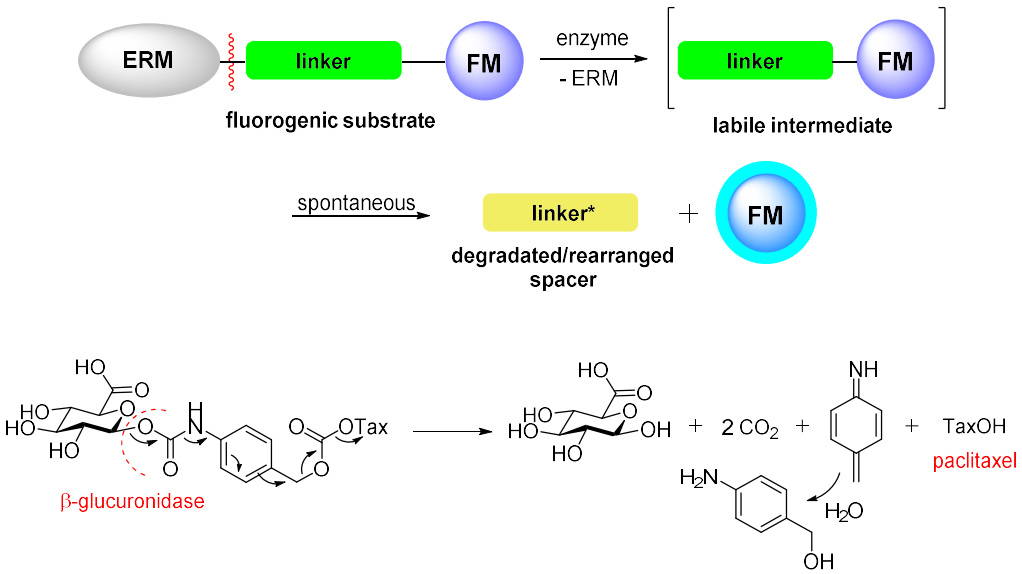


Fig. B 14. General principle of a self-immolative enzyme responsive probe & cleavage of a paclitaxel self-immolative prodrug.[24]

By using more complex linkers it is also possible to introduce new functionalities[25], increase solubility[26], binding the probes on solid phases or using different fluoresce turn on mechanisms like the FRET approach[27] described in B.1.7.

B.1.7 Förster Resonance Energy Transfer (FRET)

Förster resonance energy transfer, short FRET, is a physical mechanism describing an energy transfer between two chromophores. It is named after the German physical chemist Theodor Förster for his contributions in understanding this effect. A donor chromophore, in its exited state, can transfer energy nonradiatively to an acceptor chromophore as long as the two chromophores are linked by a covalent chain or within a non-covalent complex (Figure B 15).

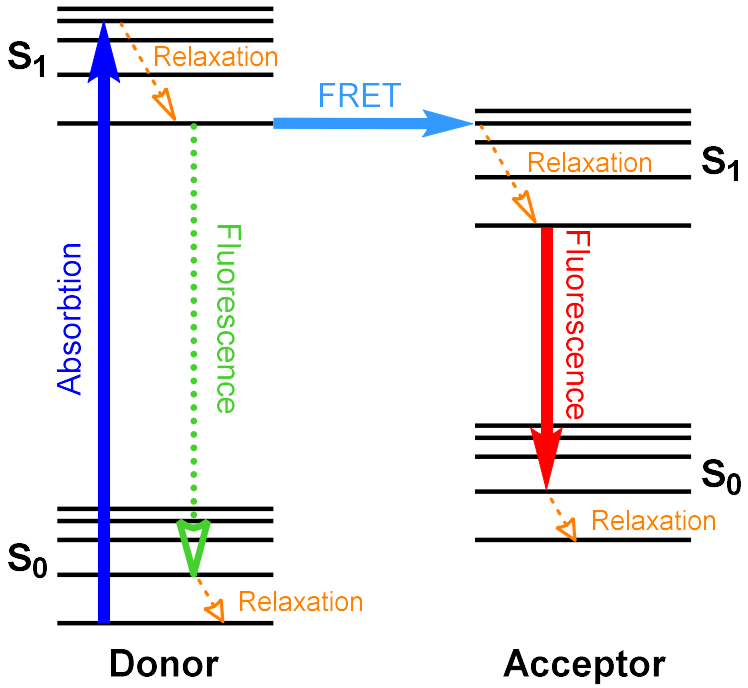


Fig. B 15. FRET in a Jablonski diagram

The efficiency of this transfer decreases with the sixth power of the distance between the donor and acceptor.[28] When both molecules are fluorescent, the fluorescence of the donor molecule is modified or quenched, upon cleavage the distance between the donor and acceptor increases and the donor fluorophore can be detected at its specific wavelength (Figure B 16). Therefore, FRET is one of the most efficient principles for detecting cleavage reactions[29] and an excellent method for the development of novel glycosidase responsive fluorogenic probes.[27]

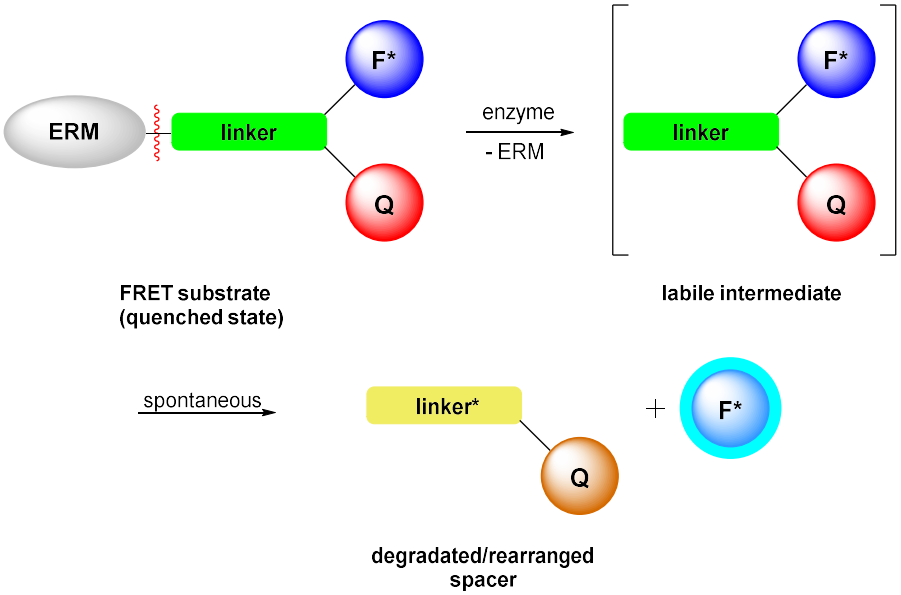


Fig. B 16. General principle of FRET substrates (F*: highly fluorescent moiety, ERM: enzyme responsive moiety, Q: quencher molecule).

C) Results and Discussion

Chapter 1

Analysis and Synthesis of 4-Methylumbelliferyl-β-D-glucuronic acid

C.1.1 Properties of 4-Methylumbelliferyl-β-D-glucuronic acid

4-Methylumbelliferyl-β-D-glucuronic acid short 4-MUG (Figure C 1) is today the most commonly used substrate for β-glucuronidase (GUS) assays. It is used as a fluorogenic enzyme substrate since the middle of the last century[30], its spectral properties are very well known and it is easily available from various vendors. Therefore, 4-MUG is ideal base substrate for the development of new quantitative real-time fluorescence (QRTF) measurement methods and understanding its advantages and drawbacks is of utmost importance for the development of new QRTF glycosidase responsive probes.

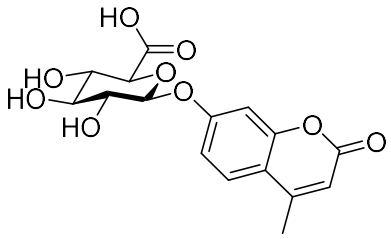


Fig. C 1. Structure of 4-methylumbelliferyl-β-D-glucuronic acid

The availability and the low price for an enzyme substrate can be easily explained by a simple retrosynthetic analysis (Figure C 2).

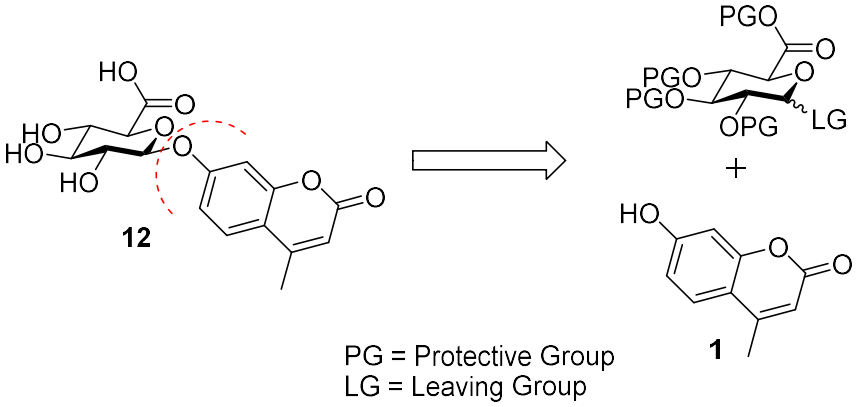


Fig. C 2. Retrosynthetic analysis-methylumbelliferyl-β-D-glucuronic acid

Already the first retrosynthetic step leads to a glycosylation of a glucuronyl donor and 4-methylumbelliferone which are both known to literature since the early 20th century.[31,32] Their precursors can be bought in large quantities making the availability and the modest price of 50 EUR per gram logical. As comparison other frequently used enzyme substrates like fluorescein di-β-D-glucuronide or resorufin glucuronide cost at least 150 EUR per milligram.

More importantly 4-methylumbelliferone has compared to other common fluorophores a large Stokes shift of 90 nm (Figure C 3). This is desirable, as less spectral overlap means less interference and a greater range of measurement can be used.

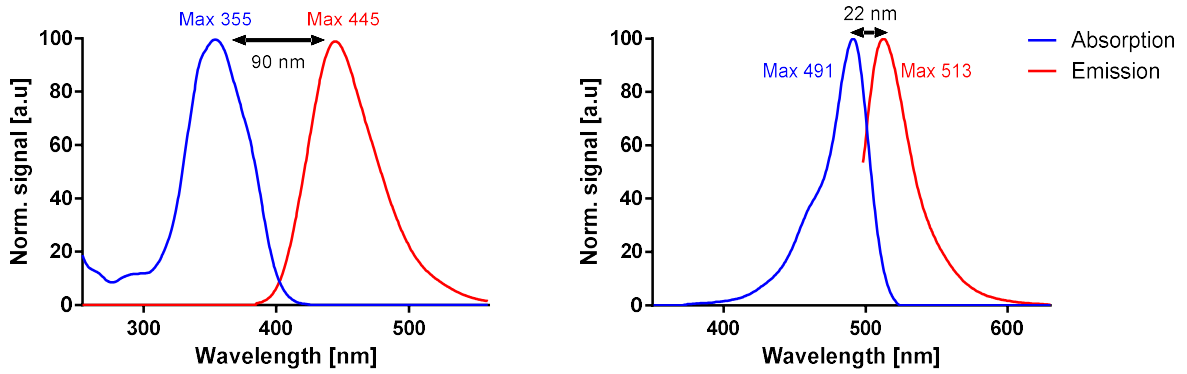


Fig. C 3. Stokes shifts of 4-Methylumbelliferone (left) and Fluorescein (right)

Another important factor for fluorogenic enzyme substrates is the "Fluorescence Turn On" ratio from the substrate to the free fluorophore. It determines the sensitivity of the enzyme substrate, a higher "Turn On" ratio directly results in better detection limits at lower concentrations or smaller time frames. At basic conditions (pH > 8) the "Turn On" ratio for 4-MUG is 4000 which is excellent, but at lower pH values (pH < 8) it starts to drop more than tenfold (Figure C 4).

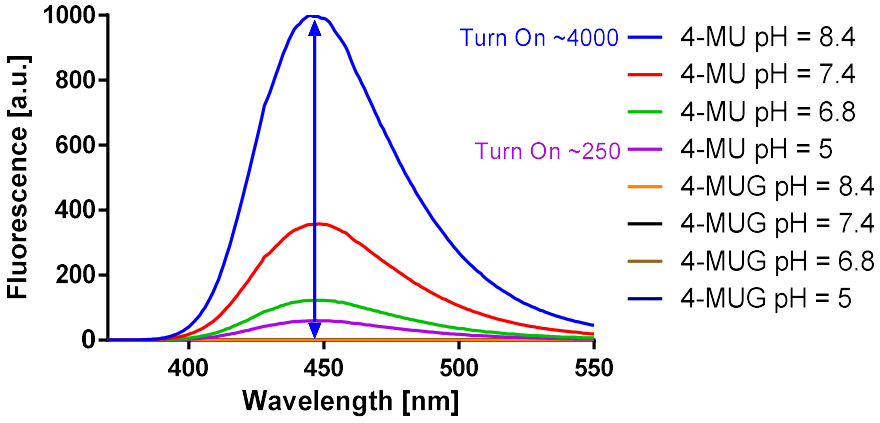


Fig. C 4. "Turn On" ratio of a commercial available 4-MUG substrate at 365 nm exc.

This effect was shortly described in chapter B.1.5. The major part of 4-methylumbelliferone´s fluorescence comes from its ionized phenolate form. The phenolic form, which is mostly present in neutral or acidic aqueous solutions, is not only far less fluorescent (Figure B 11,

chapter B.1.5), it also has fluorescence properties closer to 4-MUG (Figure C 5), resulting in a lower “Turn On” ratio and therefore decreased sensitivity for enzyme assays.

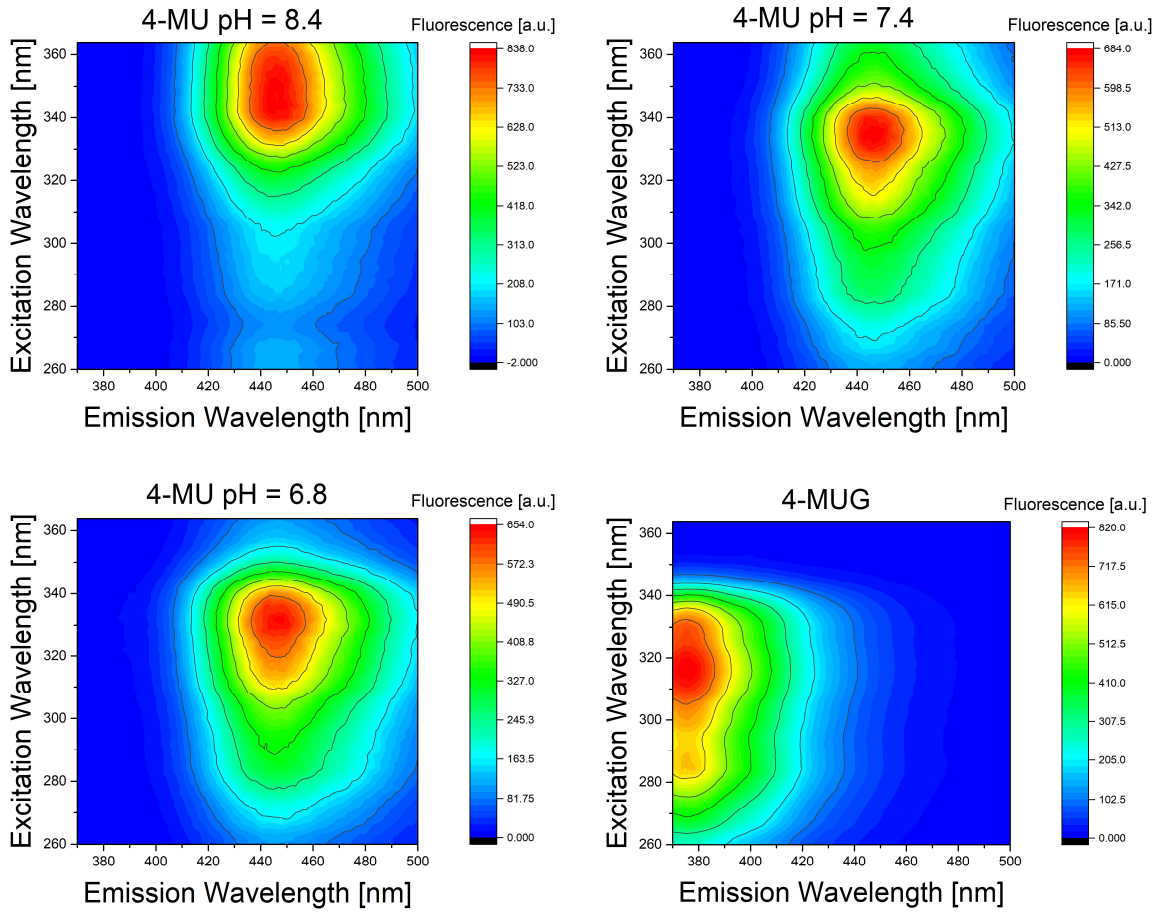


Fig. C 5. Fluorescence properties of 4-MUG & 4-MU in a pH range from 6.8 – 8.4

At a pH of 6.8, the optimum of the enzyme β-glucuronidase, the excitation maximum of 4-MU is at the same wavelength as the excitation maximum of 4-MUG. While this is no problem for standard β-glucuronidase assays since the enzymatic cleavage and fluorescence measurement are two different steps, for quantitative real-time fluorescence measurements on the *Colimínder* and other fluorescence spectrometers this causes sensitivity problems. Especially in the first minutes of the assay, when only small amounts of the substrate are cleaved by the enzyme, it is much harder to detect the cleavage product because of the interference with the initial fluorescence of the substrate. Therefore, compromises between optimal enzyme reactivity and fluorescence properties had to be taken, resulting in an optimal pH value of 8.4 for online measurements using 4-MUG as substrate. The standard operation procedure for QRTF measurements of Chapter 1 is described in chapter E.1.60.

C.1.2 Analysis of 4-Methylumbelliferyl-β-D-glucuronic acid

In addition to the known properties of 4-MUG substrates of chapter C.1.1 Vienna Water Monitoring observed big discrepancies between the quality of different commercially available substrates. On paper all commercial substrates had the same specifications, a water content of ≤ 16%, HPLC purity ≥ 98% and free methylumbelliferone impurities of < 0.06 %. Some of the substrates were very well suited for quantitative real-time fluorescence measurements, giving excellent detection limits down to 0.8 MFU (modified Fishman unit) β-Glucuronidase / 100 ml within 10 minutes. Other substrates could barely be used at all due to their not only up to 28 times higher initial fluorescence but also high non-enzymatic substrate degradation (Figure C 6).

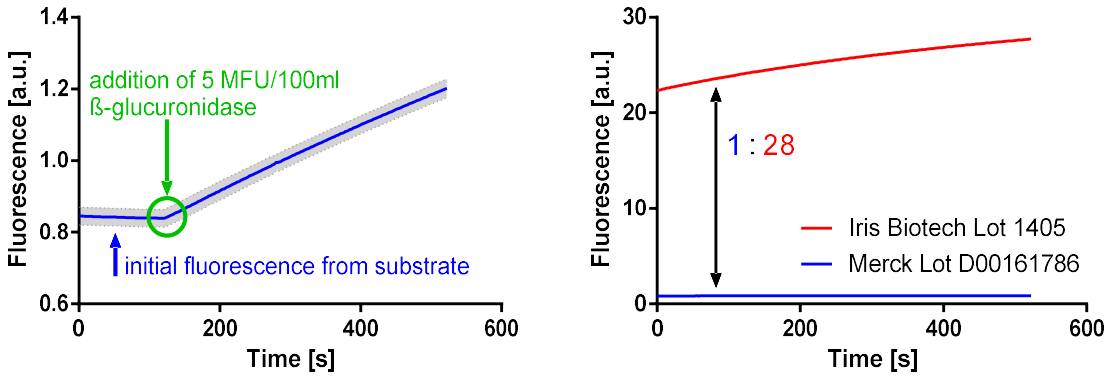


Fig. C 6. QRTF measurements of different 4-MUGs

An even bigger concern was that different batches of 4-MUG from the same supplier can differ greatly in its initial fluorescence and non-enzymatic degradation (Figure C 7).

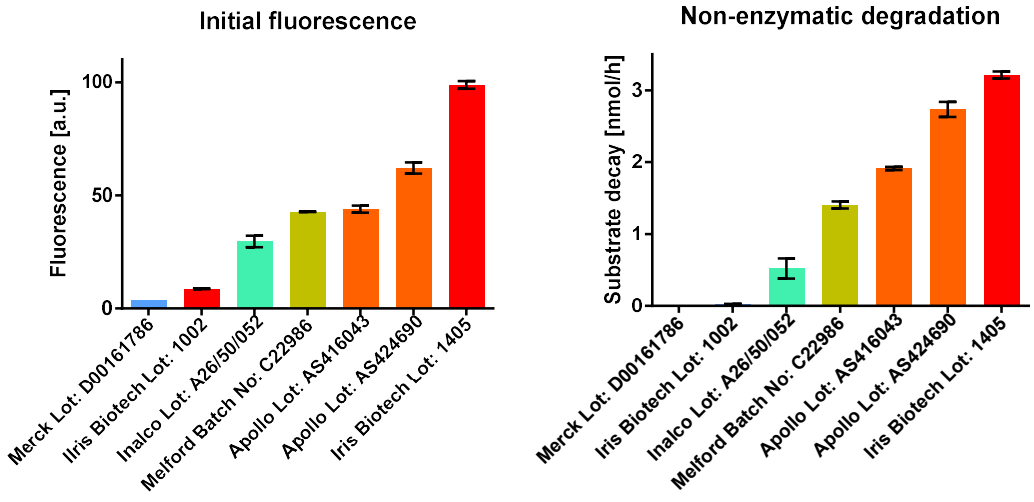


Fig. C 7. QRTF measurements of 4-MUGs from different suppliers & batches

The problem becomes even clearer, when comparing the non-enzymatic degradation with substrate conversion of the enzyme β-glucuronidase (Figure C 8). 4-MUG from Iris Biotech Lot: 1002 easily allows the detection of 5 MFU β-glucuronidase / 100 ml in a *Coliminder* assay. Lot: 1405 despite being the same product with identical specification sheets has a more than 10 times higher detection limit, making the detection of even 50 MFU β-Glucuronidase / 100 ml impossible.

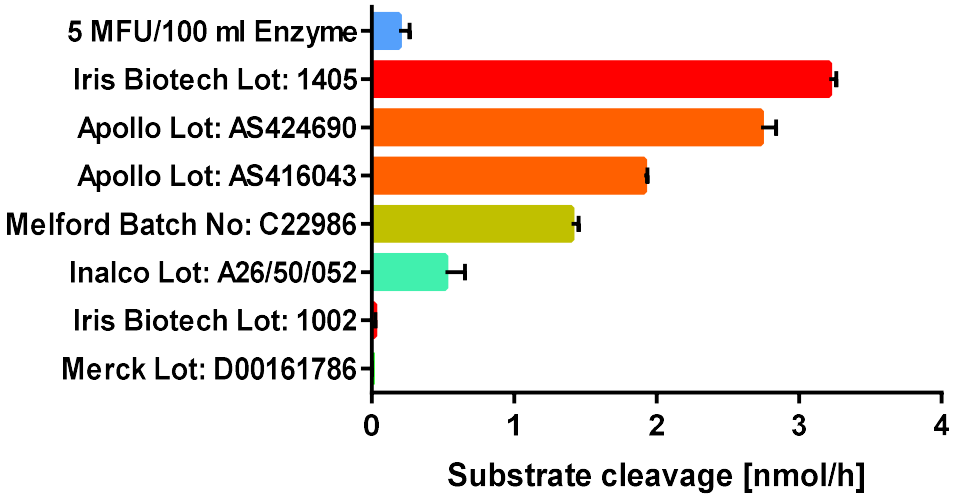


Fig. C 8. Substrate conversion rate of 5 MFU/100 ml from β-glucuronidase & rate of non-enzymatic degradation of commercial available substrates.

To explain the reason for these differences in substrate quality a series of HPLC / HRMS, NMR and water content analyses where conducted (Table C 1 & Figure C 9 & Figure C 10). All substrates surpassed their minimal product specifications having HPLC purities over 99 % and showing no impurities in NMR spectra. Karl Fischer titration confirmed the specified water content for each substrate.

Table C 1. HRMS data of 4-MUG confirmed in all substrates.

	Formula (M)	Ion	Meas. [m/z]	Pred. [m/z]	Diff [mDa]	Diff [ppm]
4-Methylumbelliferyl-β-D-glucuronic acid	$C_{16}H_{16}O_9$	$[M^-H]^-$	351.0715	351.0722	-0.7	-1.99

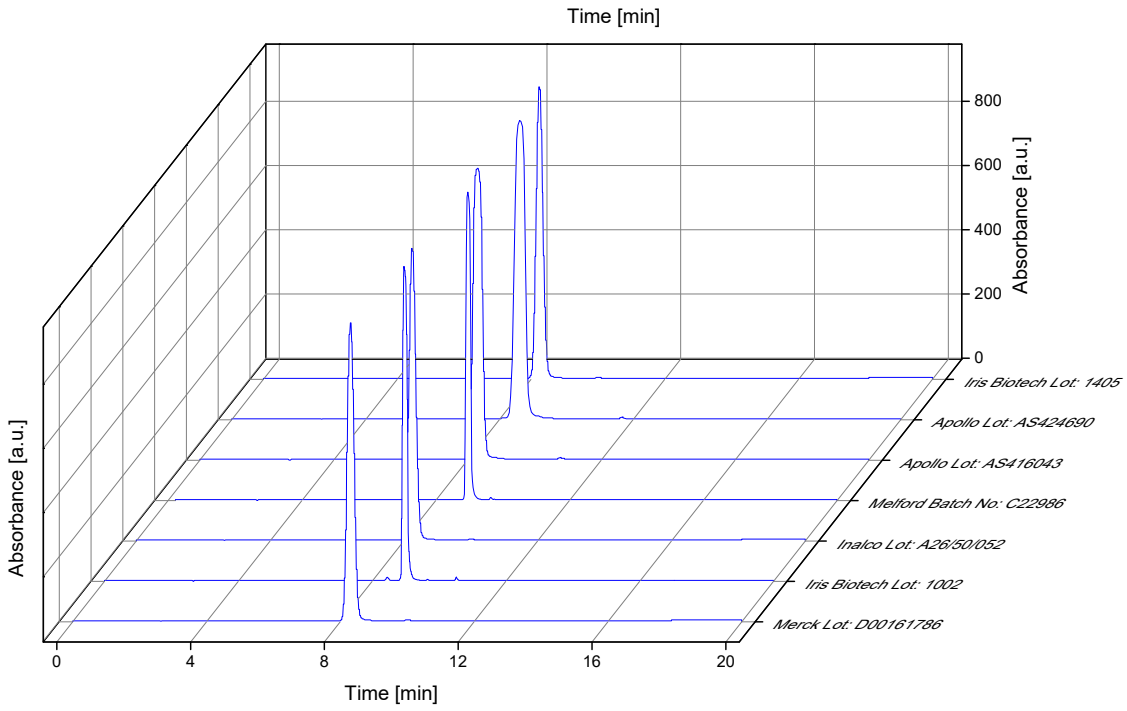


Fig. C 9. HPLC chromatograms of commercial available 4-MUG Substrates

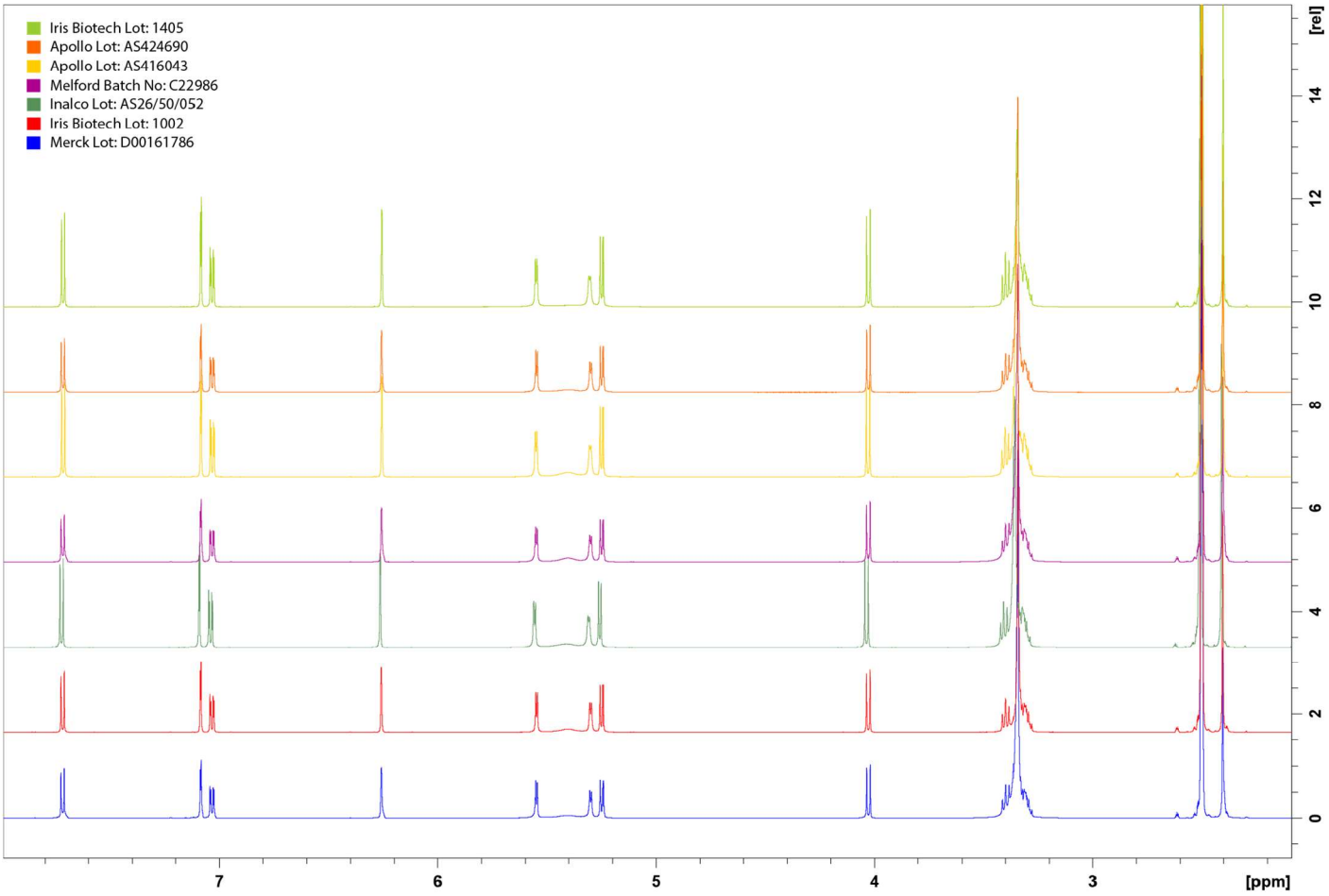


Fig. C 10. ^{1}H NMR spectra of commercial available 4-MUG substrates

Having found no traces of impurities or stabilizing components, a closer look on the degradation products of 4-methylumbelliferyl-β-D-glucuronic acid was taken. The most probable cause for a higher initial fluorescence is free 4-methylumbelliferone, which could also catalyze the degradation of the substrate.

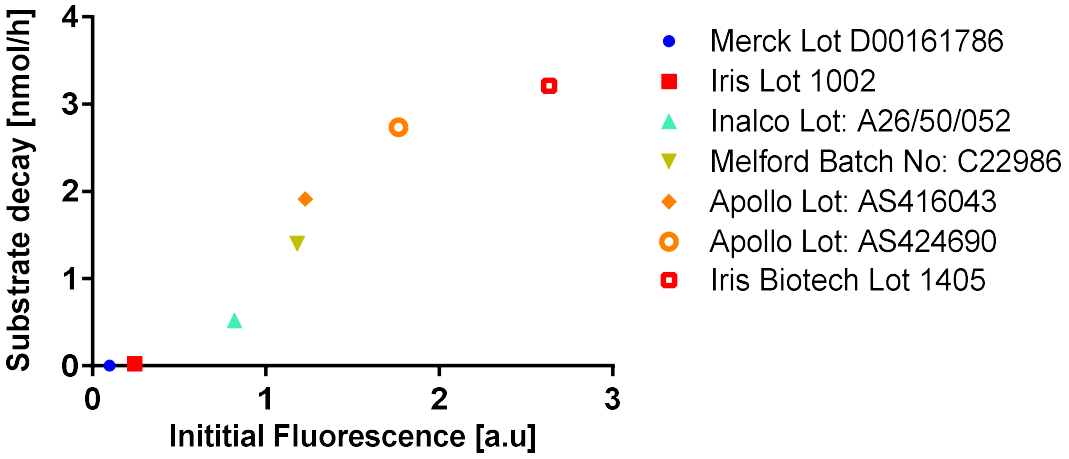


Fig. C 11. Correlation of initial fluorescence and non-enzymatic degradation of 4-MUG substrates.

Figure C 11 shows a clear a correlation between initial fluorescence and non-enzymatic degradation, but it could not be told for certain if the degradation products 4-MU and glucuronic acid are responsible for both effects. In order to investigate this hypothesis at first the amount of free 4-methylumbelliferone contained in 4-MUG substrates had to be determined via a calibration curve of 4-MU stock solutions (Figure C 12).

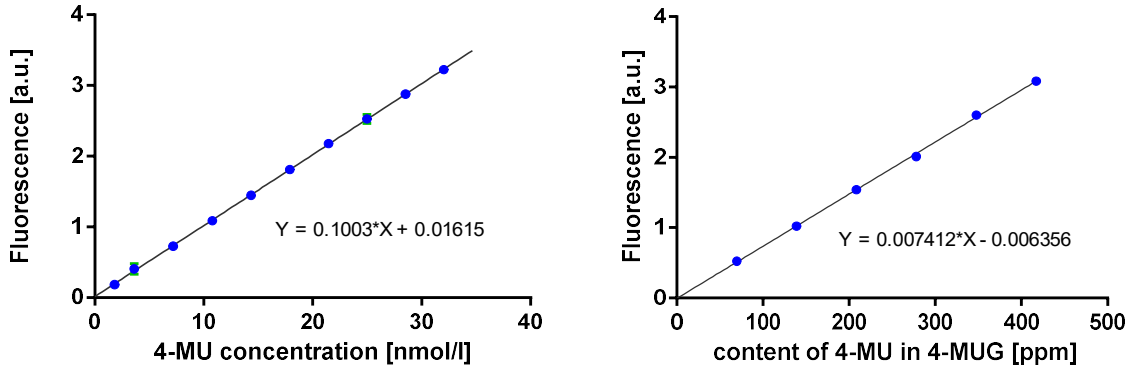


Fig. C 12. Calibration curves for 4-MU concentration and 4-MU content in 4-MUG

While varying quite a bit from 15 ppm (Merck Lot D00161786) to 375 ppm (Iris Biotech Lot: 1405), all substrates were within the product specifications of < 600 ppm free 4-MU. As a next step, a substrate with little non-enzymatic degradation (Merck Lot D00161786) was spiked

with glucuronic acid and 4-MU. Therefore, it would be detectable, if the cleavage products autocatalyze the degradation of the substrate. However, spiking the substrate with glucuronic acid had no effect and after spiking with 4-MU the non-enzymatic degradation even decreased (Figure C 13).

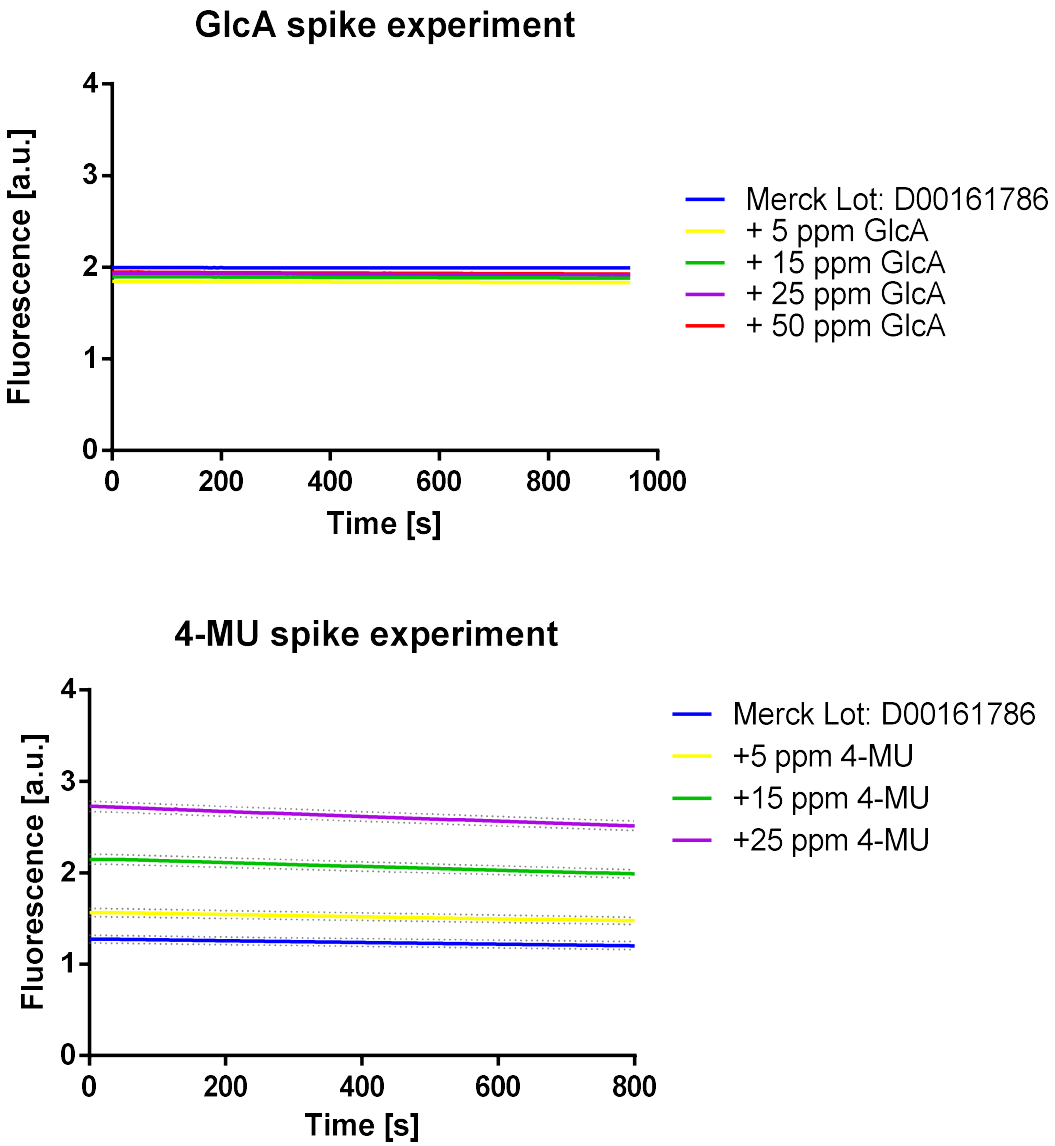


Fig. C13. Coliminder substrate spike experiments

This data can be explained by photobleaching of 4-MU in the experimental setup.[33] Over a measurement period of thousand seconds, 17 to 18 % of 4-MU fades by photochemical alteration of the dye. This effect results in a higher absolute decay of fluorophore with rising concentration and therefore a stronger decline of fluorescence intensity at the same experimental setup (Table C 2 & Figure C 14).

Table C 2. Photobleaching of 4-methylumbelliferone

4-MU concentration [nmol/]	Absolute decay [nmol/l]	Relative Decay [%]
1.80	0.31	17
3.61	0.65	17.9
7.20	1.22	17
10.78	1.95	18.1
14.35	2.60	18.1
17.91	3.30	18.4
21.46	3.78	17.6
24.99	4.32	17.3
28.52	4.96	17.4
32.03	5.54	17.3

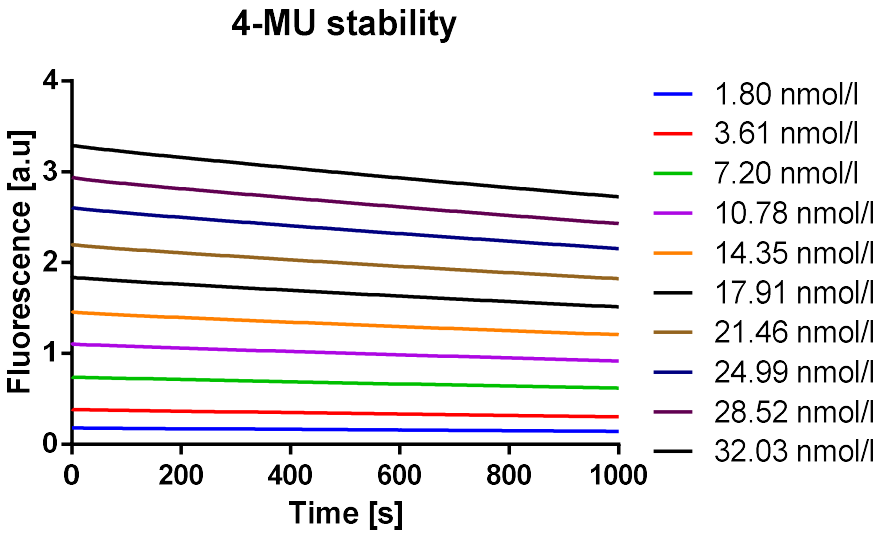


Fig. C 14. Photobleaching of 4-MU in the *Colimínder*

These experiments show that while free 4-MU is the most probable cause for a high initial fluorescence, neither 4-MU nor glucuronic acid are responsible for non-enzymatic degradation.

Another source for the high non-enzymatic degradation of substrates could be the contamination with β-glucuronidase or *Escherichia coli.* Although the presence of pure β-glucuronidase impurities in the substrates is highly unlikely, bacterial contamination could be possible especially since it is not mentioned in the product specifications if the substrates are sterilized.[34] To prove if such contaminations are responsible for non-enzymatic degradation, experiments with acetonitrile as β-glucuronidase inhibitor were conducted.

50 µl of acetonitrile was added to the measurement chamber before the addition of the substrate, at the end of the experiment 100 MFU β-glucuronidase / 100 ml was also added to prove the sufficient presence of inhibitor.

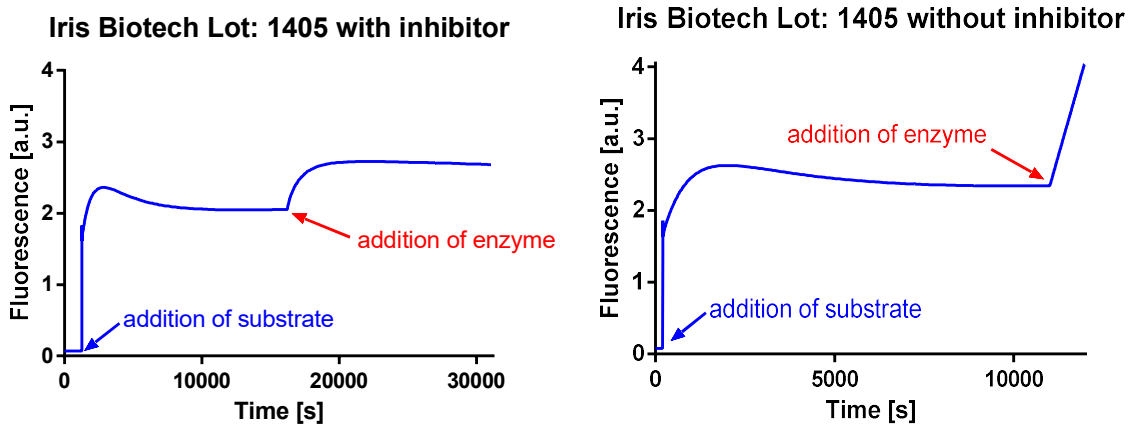


Fig. C 15. Inhibition experiments of 4-MUG (Iris Biotech Lot 1405)

As seen in Figure C 15 bacterial or enzymatic contamination is not responsible for non-enzymatic degradation, as the presence of inhibitor makes no difference. Additionally, this long-term experiment showed that the fluorescence of non-enzymatic degradation has a maximum followed by decline and stabilization. Even though this effect only occurred in substrates with non-enzymatic degradation, to exclude that contaminations of the experimental setup were responsible, another experiment was conducted. The substrate was added in two separate portions, if the contaminations are in the water source, buffers or the system itself this effect should only occur once. Since the effect appears after both substrate additions only the substrate itself can be responsible non-enzymatic degradation (Figure C 16).

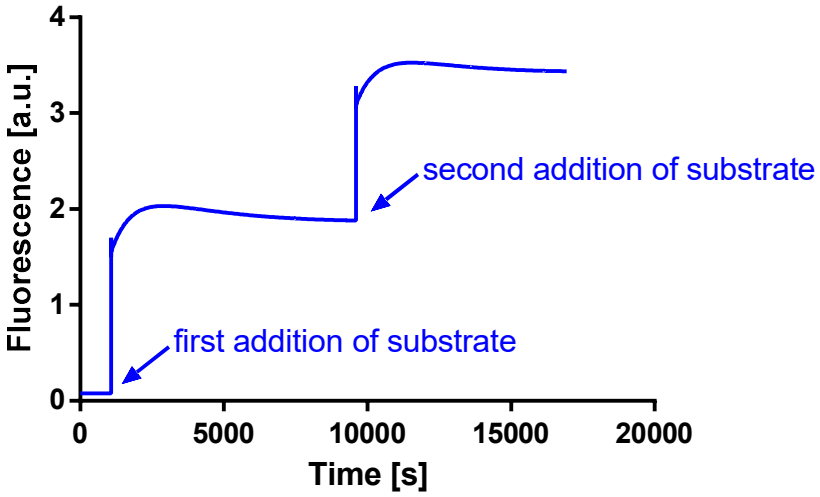


Fig. C16. Impurity source experiment of 4-MUG (Iris Biotech Lot 1405)

Another observed influence factor on non-enzymatic degradation is light exposure (Figure C 17).

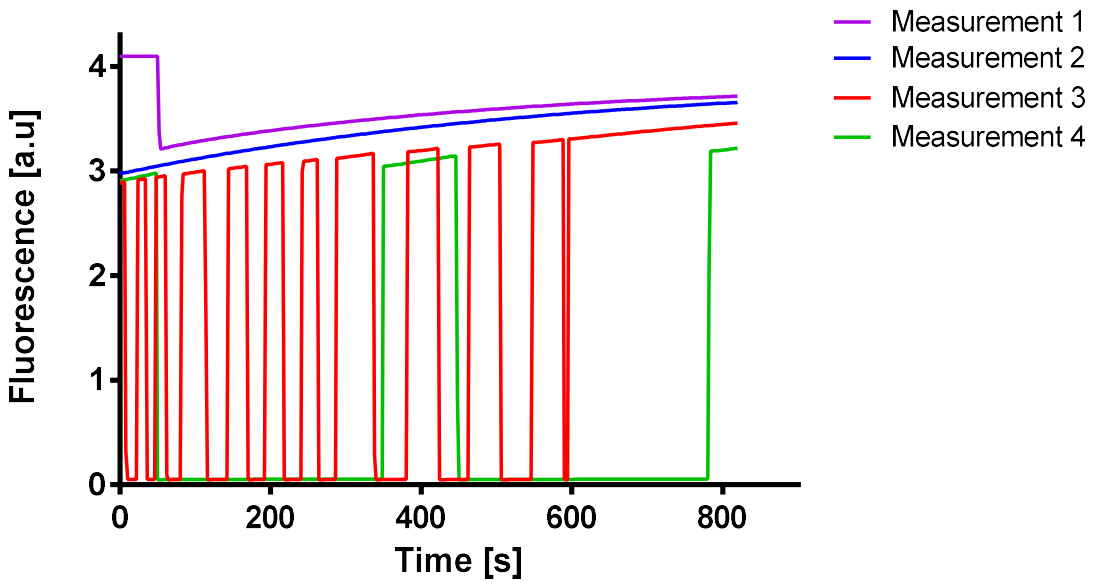


Fig. C 17. Light exposure experiment of 4-MUG (Iris Biotech Lot 1405)

For this experiment the light exposure of the substrate was altered during measurement. In measurement 1 the excitation level for the first 50 seconds was three times higher than in the standard setup. Measurement 2 was at standard excitation levels and for measurement 3 & 4 the lamp was turned off for periods of 20 sec (M3) or five minutes (M4). Figure C 17 shows that the non-enzymatic degradation is like photobleaching (Figure C 14), a photochemical effect triggered by the exposure to light. In fact, both effects compete with each other as can be seen in Figure C 18. At 100 % lamp excitation strength the initial fluorescence of 4-MUG (Iris Biotech Lot: 1002) declines due to photobleaching, but the same substrate shows non-enzymatic degradation at 50% excitation strength.

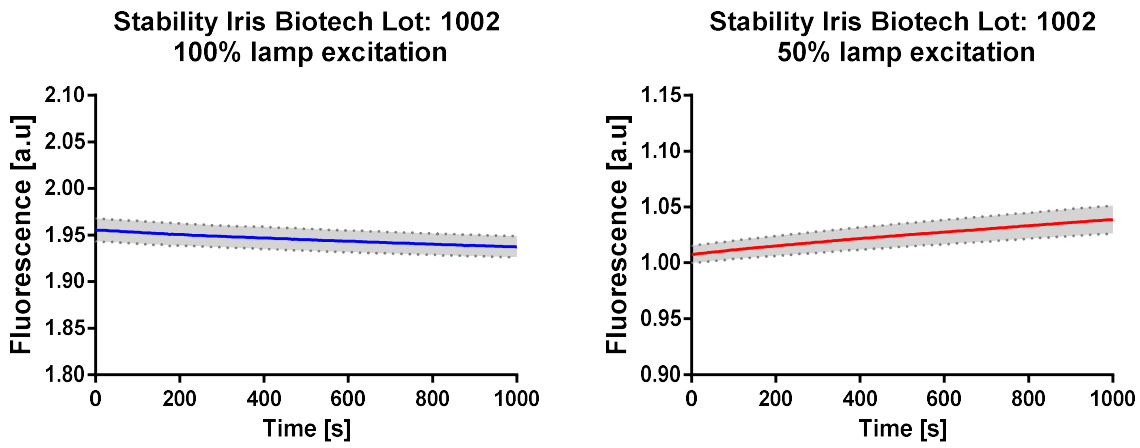


Fig. C18. Light exposure experiment of 4-MUG (Iris Biotech Lot 1405)

That the fluorescence signal stabilizes after certain amount of light exposure was very useful for stabilizing substrates with high non-enzymatic degradation. Therefore, stock solutions of substrates (0.22 mol/l) in DMSO/H_2O (9/1) were placed in an UV-chamber with an emission wavelength of 365 nm. After 30 minutes all substrates were stable and while having an increased initial fluorescence, they were still far better suitable for enzymatic assays (Figure C 19).

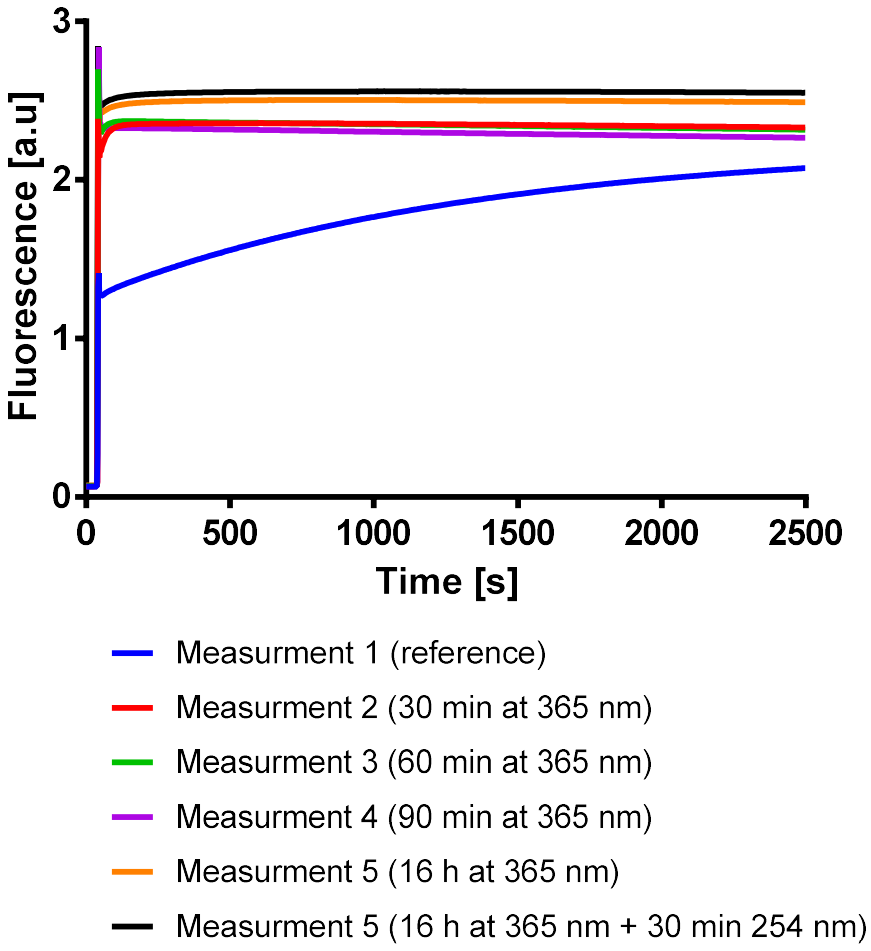
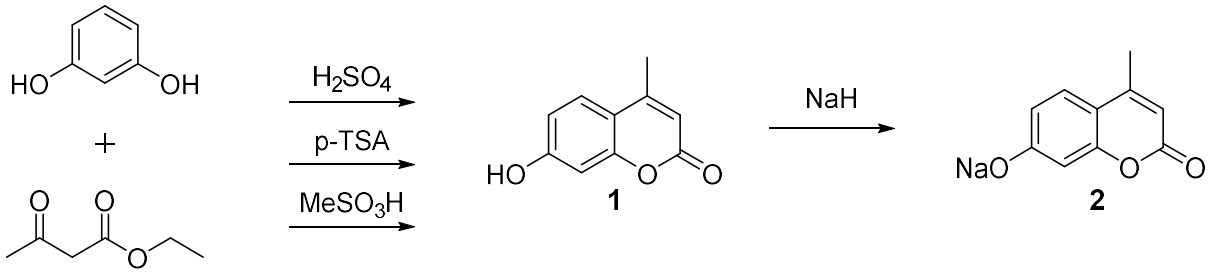


Fig. C19. Light exposure stabilization of 4-MUG (Melford Batch No: C22986)

C.1.3 Synthesis and Analysis of 4-MUG

To investigate if the impurities that are causing high non-enzymatic degradation originate from specific synthetic steps or workup methods, several different strategies for the synthesis of 4-MUG were applied.

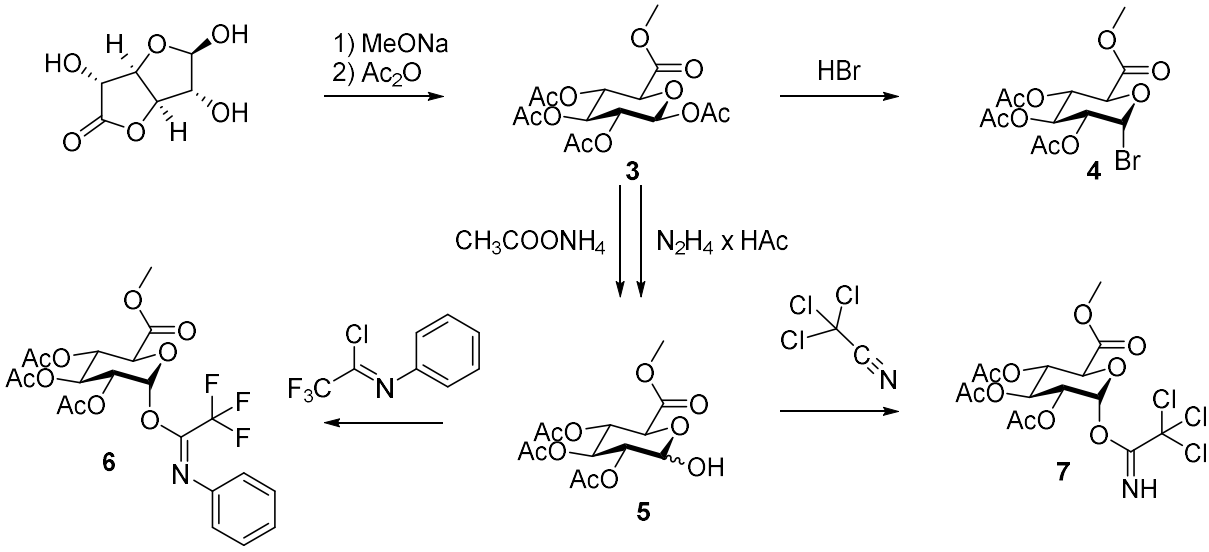
C.1.3.1 Synthesis of 4-Methylumbelliferone



Scheme C 1. Synthesis of 4-MU & 4-MU sodium salt

The first synthetic step, the preparation of 4-methylumbelliferone **1**, was accomplished via three different methods. The original method[32] of Hans von Pechmann using sulfuric acid (70 % in water) and a milder variant[35] using p-toluenesulfonic acid worked quite well giving yields up to 70 %. However, the third method[36] using methanesulfonic acid gave yields of 90 % and had by far the easiest workup. Therefore, it became the method of choice for the synthesis of 4-methylumbelliferone and its derivates. For phase transfer glycosylations also the 4-MU sodium salt **2** was prepared.[37]

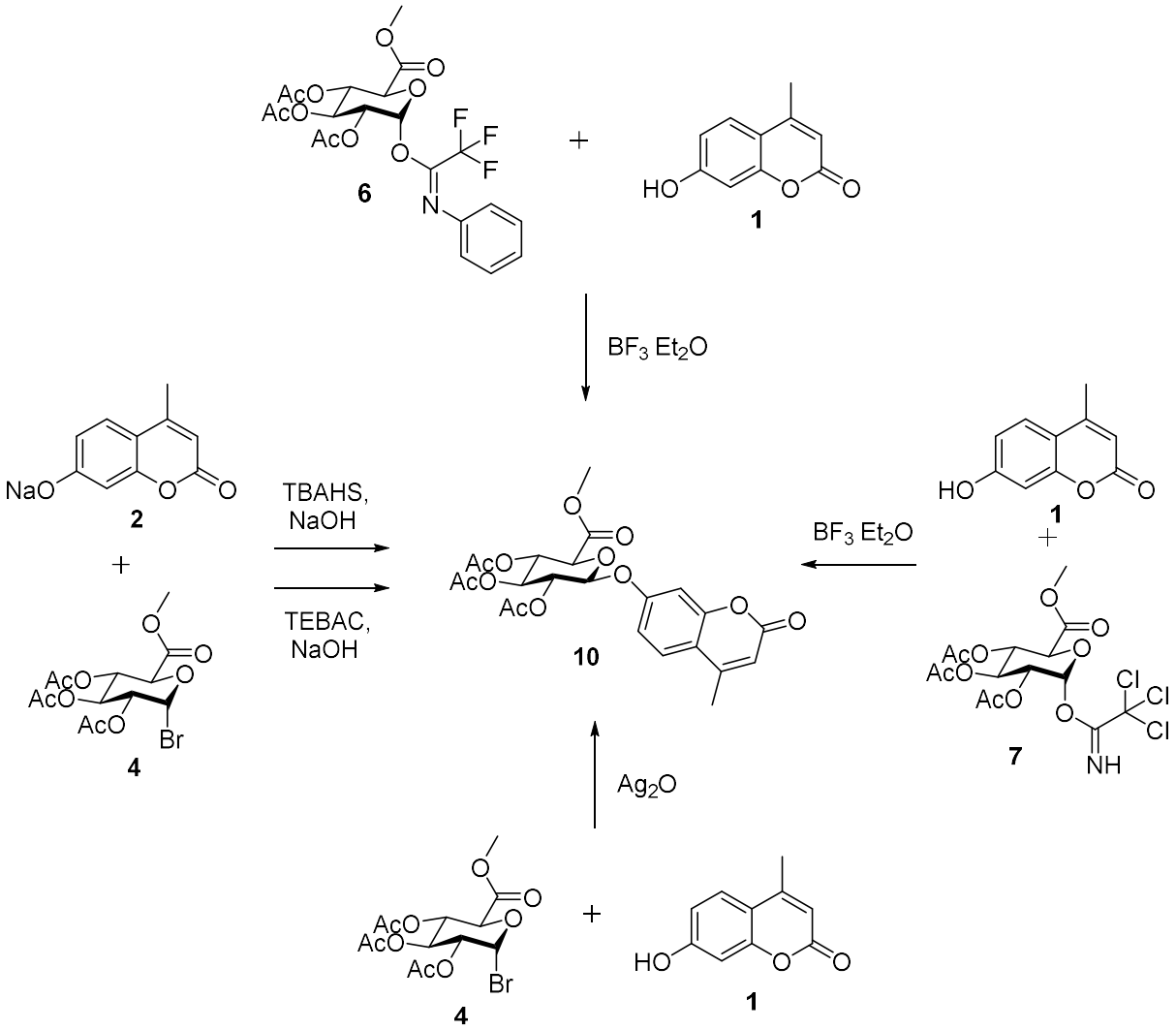
C.1.3.2 Preparation of Glucuronyl Donors



Scheme C 2. Preparation of glucuronyl donors

The synthesis of all three glucuronyl donors, needed for Koenigs-Knorr, Schmidt and phase transfer glycosylations, started from (+)-D-glucofuranurono-6,3-lacton. First, the lactone was opened to the fully acetylated methyl ester of glucuronic acid **3**. Compound **3** was then treated with hydrogen bromide (33 % in acetic acid) to obtain the α-bromide glucuronyl donor **4**. To obtain the imidate donors **6** & **7**, compound **3** was deprotected at the anomeric position using two different methods. For small approaches a protocol according to reference[38] with hydrazine acetate as reagent was used. Due to the high toxicity of hydrazine acetate a different protocol[39] was used for lager scales. The anomeric OH sugar **5** was then converted into the imidate donors **6** & **7** following the protocol in reference[40].

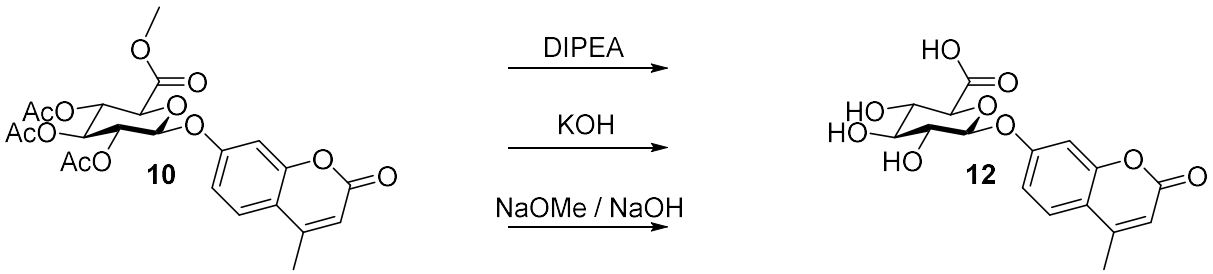
C.1.3.3 Chemical Glycosylation



Scheme C 3. 4-MUG glycosylation approaches

The chemical glycosylation was conducted with several different methods, since it is the most likely source for carbohydrate containing impurities such as α-glycosides. At first the well-known approach from Koenig & Knorr[31] with an α-bromide glucuronyl donor **4** and silver(I)oxide as promotor was used. After little optimization good yields up to 70 % with an excellent α to β glycoside ratio of over 50:1, in favor of the desired β-glycoside, were achieved. The second method was first described by Schmidt using imidate donors and Lewis acids as catalysts.[41] As donors were used α-2,2,2-trifluoro-N-phenylethanimidate glucuronide **6** and α-2,2,2-trichloroethanimidate glucuronide **7**. For both synthetic pathways boron trifluoride diethyl etherate proved to be the best Lewis acid catalysts giving excellent yields up to 80 %. However, compared to the Koenigs-Knorr method the alpha to beta ratio was worse only giving the desired β-glycoside in a ratio of 5:1 over the α -glycoside byproduct. The last used approach was basic phase transfer glycosylation.[42,43] These are most likely used in industrial synthesis since they are not water sensitive and use by far the cheapest reagents. As donor was again used an α-bromide glucuronide **4**, benzyltriethylammonium chloride (TEBAC) or tetrabutylammonium hydrogen sulfate (TBAHS) were used as phase transfer catalysts and sodium hydroxide was used as base in the two-phase system. The synthetic yields of these phase-transfer glycosylations were with up to 50 % not as good as with the other methods, but like the Koenigs-Knorr method the α to β glycoside ratio was excellent with a ratio of 50:1 in favor of the desired β-glycoside.

C.1.3.4 Deprotection of 4-Methylumbelliferyl-β-D-glucuronic acid



Scheme C 4. Deprotection of the acetyl groups and the methyl ester from 4-MUG

The last step towards the synthesis of 4-methylumbelliferyl-β-D-glucuronic acid **12** was the deprotection of the acetyl groups on position 2, 3 and 4 of the glucuronic acid and the cleavage of the methyl ester. Since the most commonly used approaches according to reference[44] and reference[42] both produced dehydrated side products, which were difficult to separate from

the desired 4-MUG **12**, a milder method[45] utilizing N,N-diisopropylethylamine (DIPEA) was preferably used giving 4-methylumbelliferyl-β-D-glucuronic acid **12** in a yield of 97 %.

C.1.4 Analysis of the Synthesized 4-MUG

After purification of the synthesized substrates via reversed phase MPLC (ACN/H_2O = 15 % ACN - 95% ACN), they were analyzed on the *Coliminder* and compared with the commercially available substrates (Figure C 20).

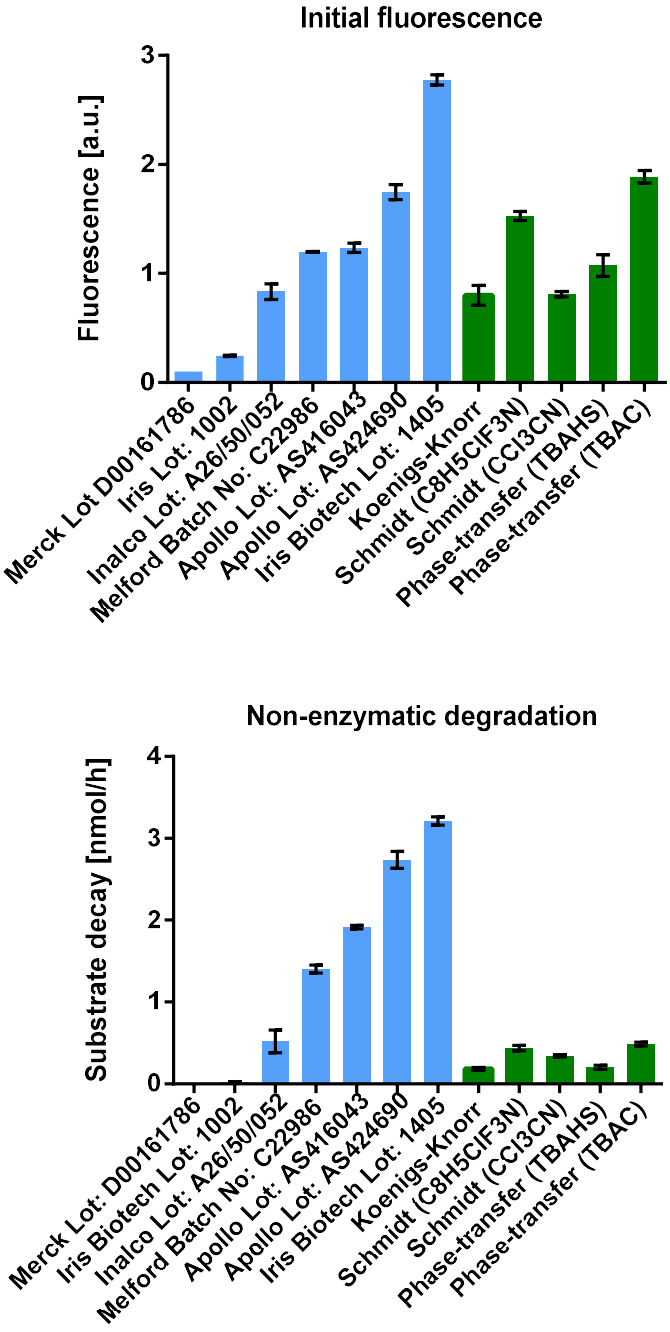


Fig. C 20. Initial fluorescence and non-enzymatic degradation of commercially available substrates compared with synthesized substrates

At the first glance all synthesized substrates seemed to have roughly the same properties as most commercial available substrates, but by looking at the non-enzymatic degradation it was noticed that synthesized substrates degrade far less during measurement (Figure C 21). While it has already been proven in chapter C.1.2 that initial fluorescence and non-enzymatic degradation are independent from each other, no commercially available substrate showed a gap between these two indicators as big as in all the synthesized substrates. Since this phenomenon occurred in all synthesized substrates it had to be related to the purification method rather than the synthetic approach. To investigate this effect substrates where purified via preparative reversed phase high-performance liquid chromatography (HPLC) and analyzed again in the *Coliminder*.

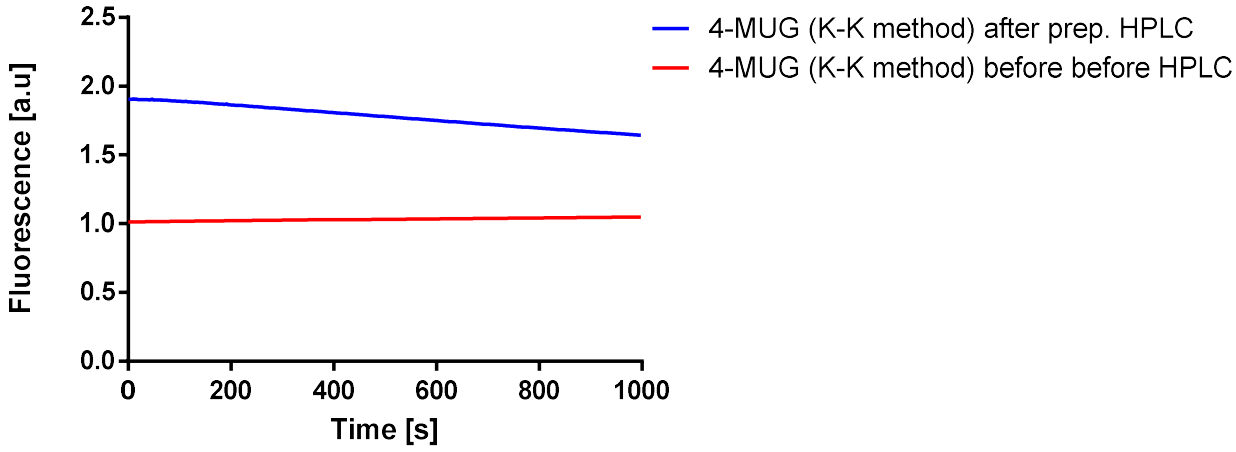


Fig. C 21. Stability measurements of synthesized 4-MUG (Koenigs-Knorr method) before and after preparative reversed phase HPLC

As seen in Figure C 21 the non-enzymatic degradation greatly decreases after preparative HPLC indicating that a chromatographically separable impurity is responsible for this effect. Also, the initial fluorescence was rising after HPLC, what can be simply explained by the time needed for workup. Unlike other purification methods reversed phase chromatography uses water as part of the mobile phase. Due to the higher boiling point of water compared to other commonly used solvents, the evaporation takes longer. This results in a longer thermal exposure and therefore a higher initial fluorescence.

To test this hypothesis the substrate was washed with diethyl ether and the solvent was then again evaporated. The thermal exposure time on the rotary evaporator was varied from zero

to twenty-five minutes, afterwards the initial fluorescence was measured on the *Coliminder* (Figure C 22).

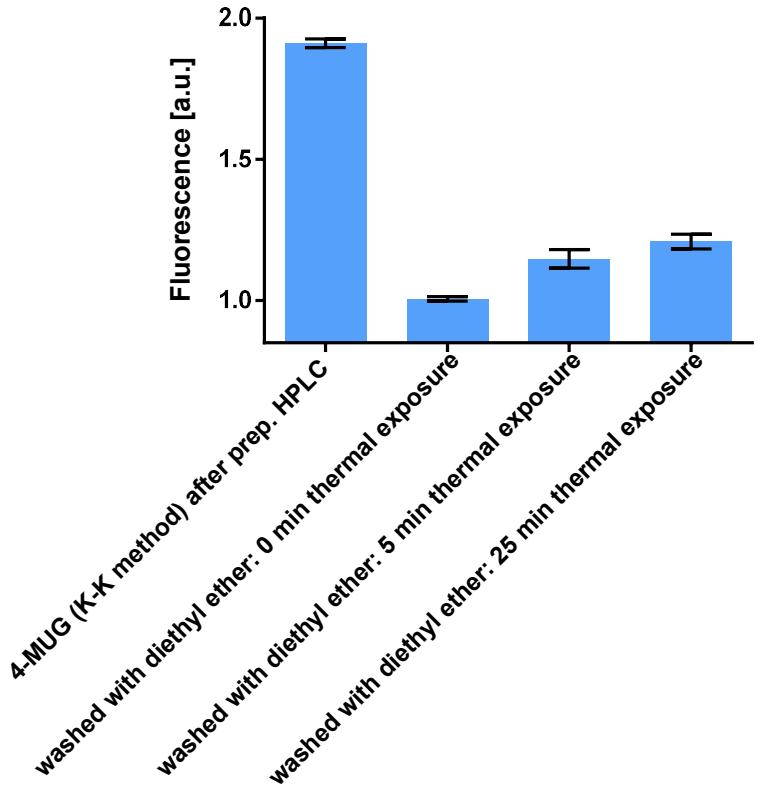
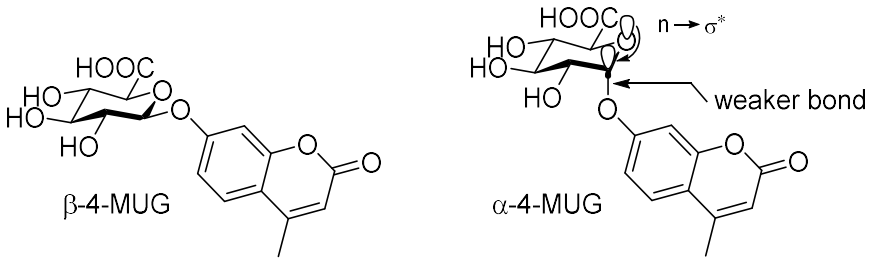


Fig. C 22. 4-Methylumbelliferyl-β-D-glucuronic acid workup time experiment

This experiment showed that the initial fluorescence is dependent on the workup time and explains the variance of the initial fluorescence of 4-methylumbelliferyl-β-D-glucuronic acid substrates.

C.1.4.1 Investigation of 4-Methylumbelliferyl-α-D-glucuronic acid

Another cause for the non-enzymatic degradation could be contamination with small amounts of α-4-MUG (Scheme C 5) a side product of chemical glycosylation (see chapter C.1.3.3).



Scheme C 5. Structure of β- and α-4MUG

Alpha glycosides are difficult to separate from the desired beta glycosides making them a likely contamination for the substrates. Due to the n→σ* interaction of the O1-atom and the anomeric center they can be less stable than the corresponding beta glycosides.[46] To test this theory the stability of two fractions of the same substrate from preparative HPLC purification were compared. While in fraction one of the 4-MUG substrate from the Schmidt glycosylation no α-4-MUG was present, fraction three contained 33 % α-4-MUG (Figure C 23).

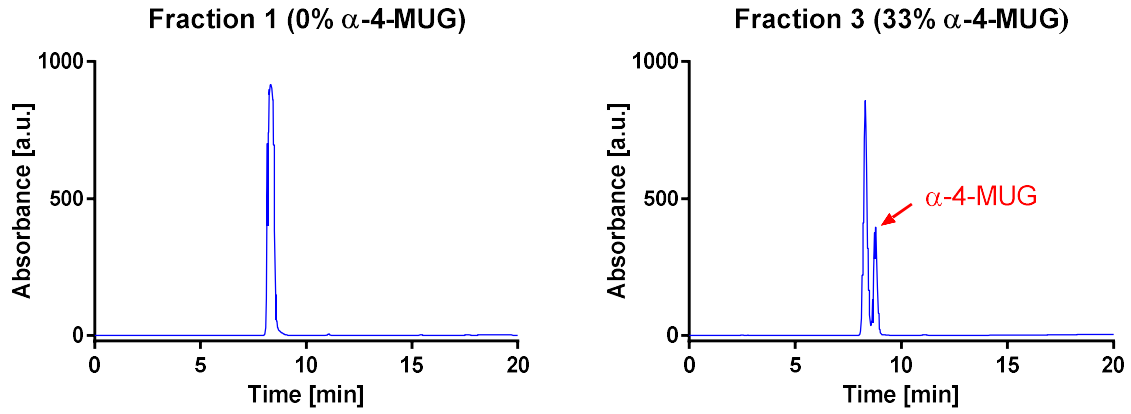


Fig. C 23. HPLC measurements of fractions from prep. HPLC purification of 4-MUG synthesized via the Schmidt glycosylation using a trichloroethanimidate glucuronyl donor.

If the α-4-MUG would be responsible for the photo induced non-enzymatic degradation the stability of substrate fraction 3 should be inferior to fraction one, but as seen in Figure C 24 other than a higher initial fluorescence due to different workup times, there was no difference between the fractions.

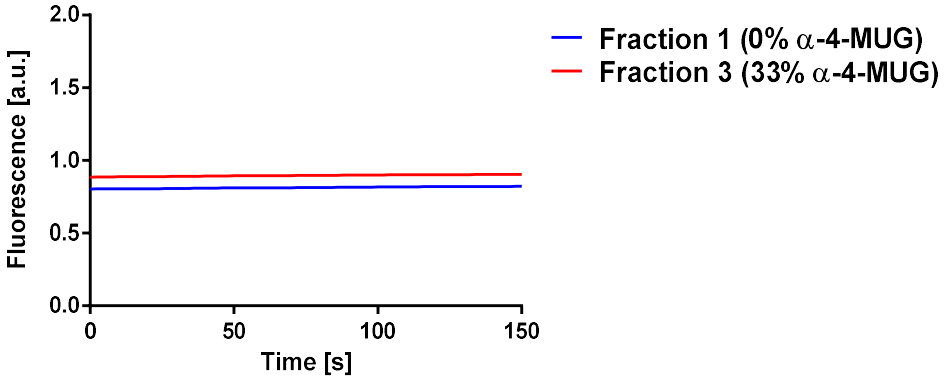


Fig. C 24. Stability measurements of the Schmidt glycosylation fractions from the prep. HPLC

Therefore, it can be concluded that contaminations of α-4-MUG are not responsible for the non-enzymatic degradation.

C.1.5 Substrate Purification

While the impurity causing the photo induced non-enzymatic degradation could not be found, the knowledge gained was then used for a multi-step purification of 4-MUG Iris Biotech Lot: 1405. The substrate was first purified via preparative HPLC (ACN/H_2O = 15 % ACN - 95% ACN) and then recrystallized from tetrahydrofuran, resulting in a highly improved initial fluorescence (5:1) and completely removing non-enzymatic degradation. The preparative HPLC purification was conducted for all prepared substrates of the following chapters.

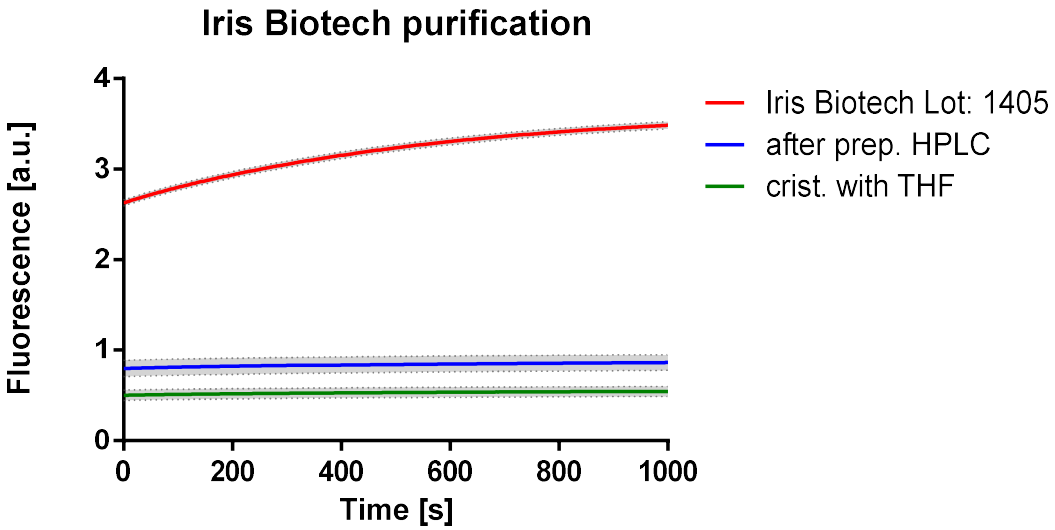


Fig. C 25. Substrate purification of Iris Biotech Lot: 1405

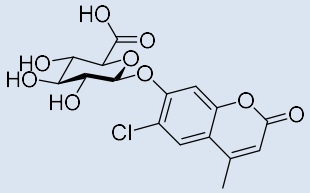
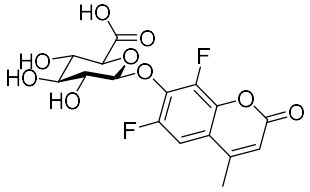
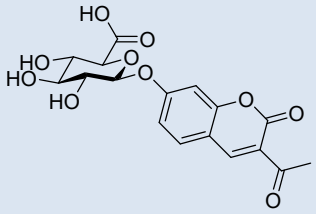
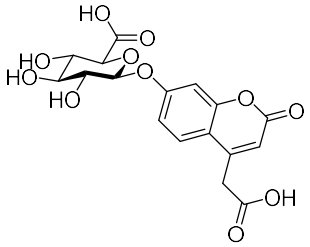
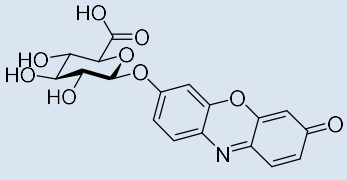
Chapter 2

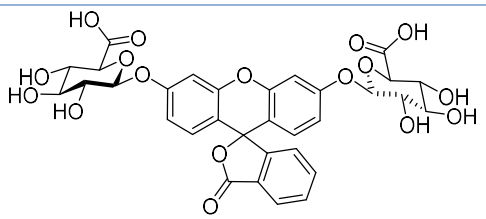
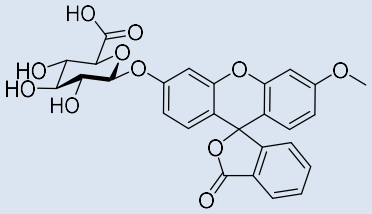
Synthesis and Analysis of β-Glucuronidase Responsive Fluorogenic Probes

C.2.1 Fluorogene Map

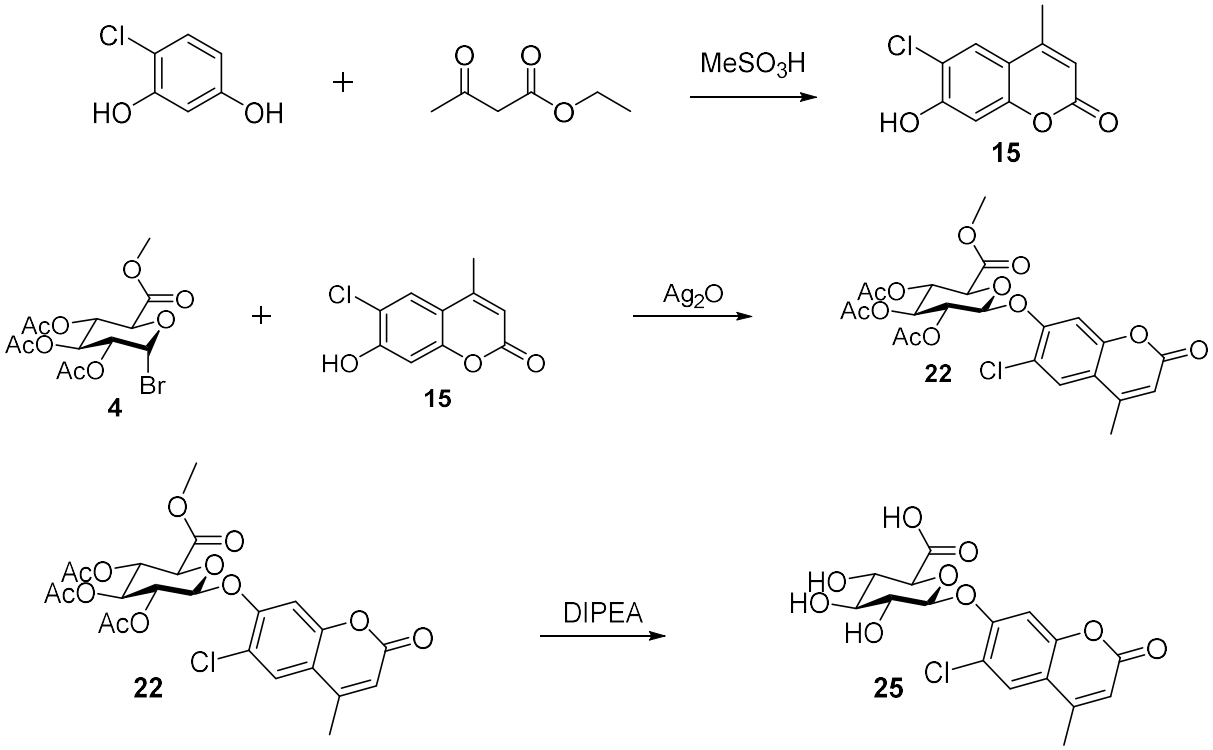
As already mentioned in chapter 1, 4-methylumbelliferone is due to its pK_a value (7.79) not an ideal fluorophore for quantitative real-time β-glucuronidase assays. Therefore, several β-glucuronidase responsive fluorogenic probes, based on commonly used fluorophores, were prepared using the optimized synthetic approaches und purification methods described in chapter 1. To keep the required effort reasonable the focus was set on the most promising scaffolds of 4-methylumbelliferone derivatives, fluorescein conjugates and resorufin (Table C 3).

Table C 3. Investigated β-glucuronidase responsive fluorogenic probes

Substrate Name (abbr.)	Structure
6-Chloro-4-methylumbelliferyl -β-D-glucuronic acid (6-Cl-4-MUG)	
6,8-Difluoro-4-methylumbelliferyl -β-D-glucuronic acid (DiFMUG)	
3-Acetylumbelliferyl-β-D-glucuronic acid (3-AcUG)	
4-Carboxymehtylumbelliferyl -β-D-glucuronic acid (4-CarbMUG)	
Resorufin-β-D-glucuronic acid (ReG)	

Fluorescein-bis[β-D-glucuronic acid] (Fl-DiG)	
Methoxyfluorescein- β-D-glucuronic acid (MeFlG)	

C.2.2 Synthesis of 6-Chloro-4-methylumbelliferyl-β-D-glucuronic acid



Scheme C 6. Synthesis of 6-Cl-4MUG **25**

The synthetic approach for 6-chloro-4-methylumbelliferyl-β-D-glucuronic acid **25** short 6-Cl-4-MUG was the same as for 4-MUG using the methanesulfonic acid protocol[36] and 4-chloro-resorcinol as reagent for the Pechmann condensation. The glycosylation was done via the Koenigs-Knorr method[47] and for the deprotection step, the diisopropylethylamine (DIPEA) method[45] was chosen.

C.2.3 Analysis of 6-Chloro-4-methylumbelliferyl-β-D-glucuronic acid

C.2.3.1 Fluorescence Properties

6-Chloro-4-methylumbelliferone short 6-Cl-4-MU is the logical development for quantitative real-time fluorescence measurements, as it is synthesized from easily available bulk chemicals like 4-methylumbelliferone and also has an excellent Stoke shift of 80 nm (Figure C 26).

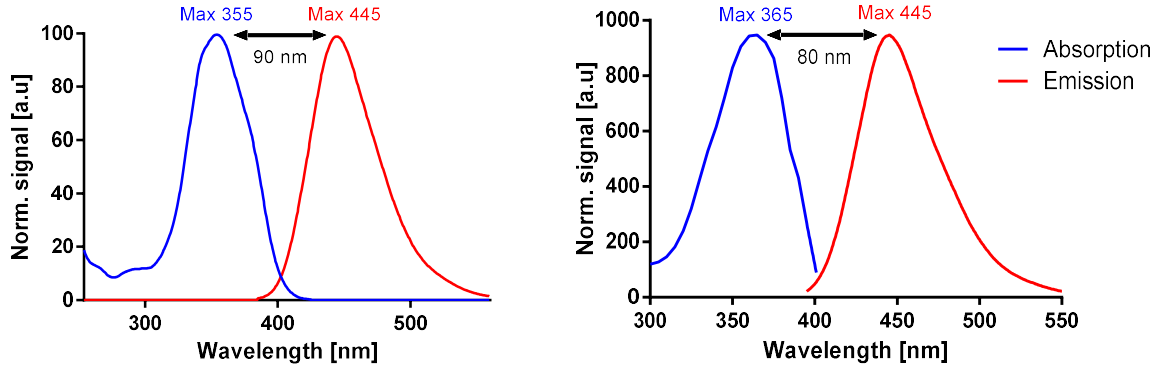


Fig. C 26. Stokes shifts of 4-methylumbelliferone (left) and 6-chloro-4-methylumbelliferone (right)

The main advantage of 6-Cl-4-MU over 4-MU is the lower pK_a value of 6.10 (compared to 7.79 of 4-MU), due to the electron withdrawing inductive effect of the chlorine, making 6-Cl-4-MU a brighter fluorophore than 4-MU at neutral or slightly acidic pH conditions (Figure C 27).[48]

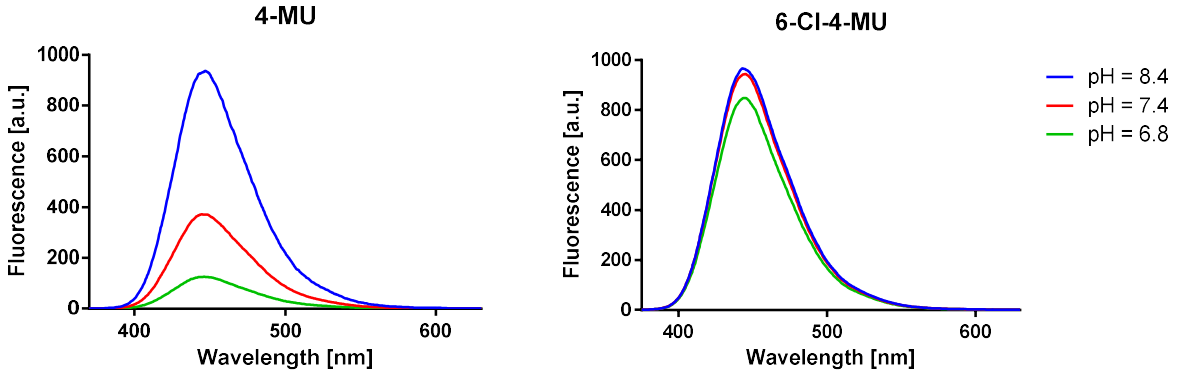


Fig. C 27. Emission spectra at pH 6.8, 7.4 and 8.4 of 4-methylumbelliferone (left) and 6-chloro-4-methylumbelliferone (right)

This also results in better "Turn On" ratios at the optimal pH range of β-glucuronidase. While the "Turn On" from 4-MUG to 4-MU drops from 4000 at pH 8.4 to 1000 at pH 6.8, the "Turn On" of 6-Cl-4-MUG to 6-Cl-4-MU is already higher at pH = 8.4 at 7000 and only drops to 6000 at pH 6.8. Another factor for the differences in the "Turn On" ratios between the two substrate fluorophore pairs are the maximal excitation wavelengths of the fluorophores. The maximal

excitation wavelength of 4-MU changes from 355 nm at pH 8.4 to 335 nm at pH 6.8 (Figure C 5 in chapter C.1.1). Since 4-MUG has its maximal excitation at 325 nm, inference between 4-MU and 4-MUG has a negative effect on the “Turn On” ratio. The maximal excitation wavelength of 6-Cl-4-MU stays constant over this pH range at 365 nm, causing no interference with 6-Cl-4-MUG that has its excitation maximum at 325 nm (Figure C 28).

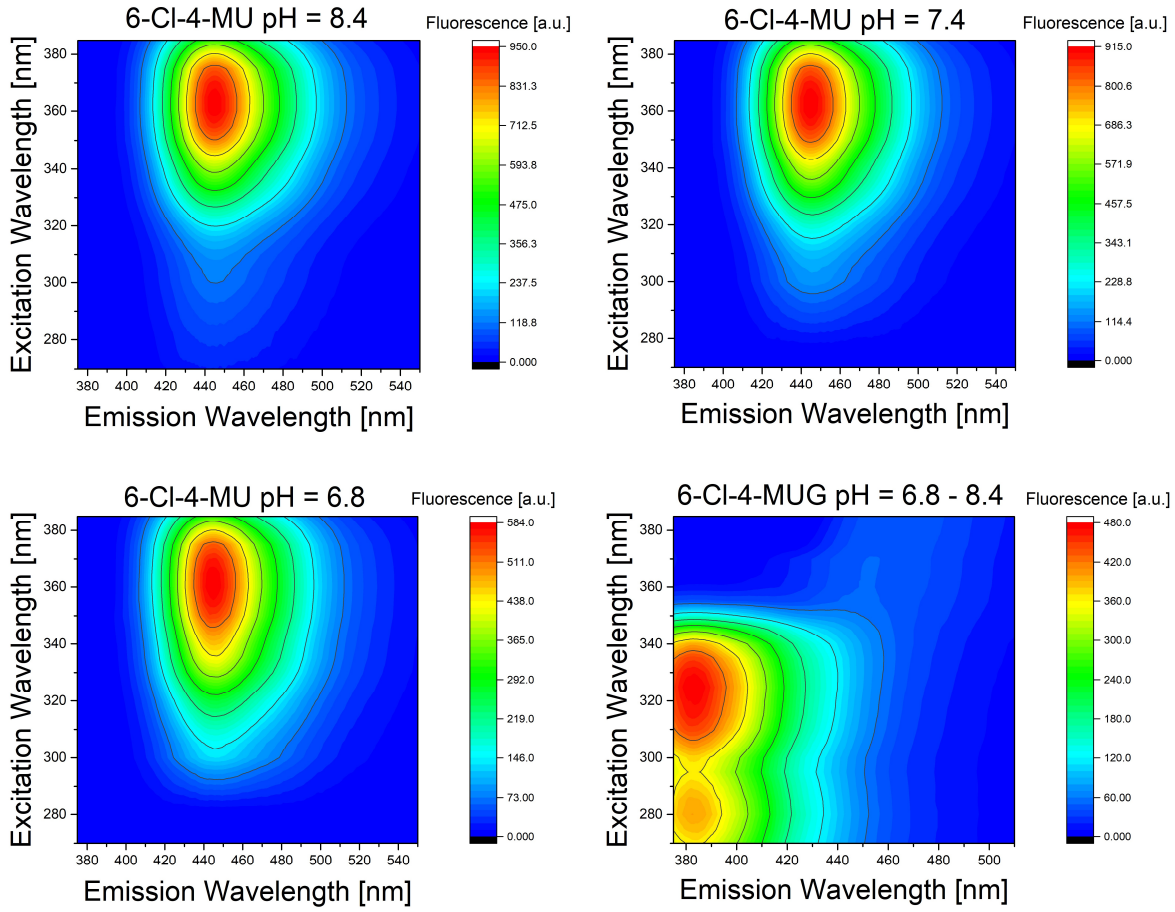


Fig. C 28. Fluorescence properties of 6-Cl-4-MU **15** & 6-Cl-4-MUG **25** in a pH range from 6.8 – 8.4

C.2.3.2 Stability and Enzyme Kinetics

In terms of stability at enzyme assay conditions 6-chloro-4-methylumbelliferyl-β-D-glucuronic acid is equal to 4-methylumbelliferone glucuronic acid substrates, showing only slight photobleaching and no signs of non-enzymatic degradation (Figure C 29).

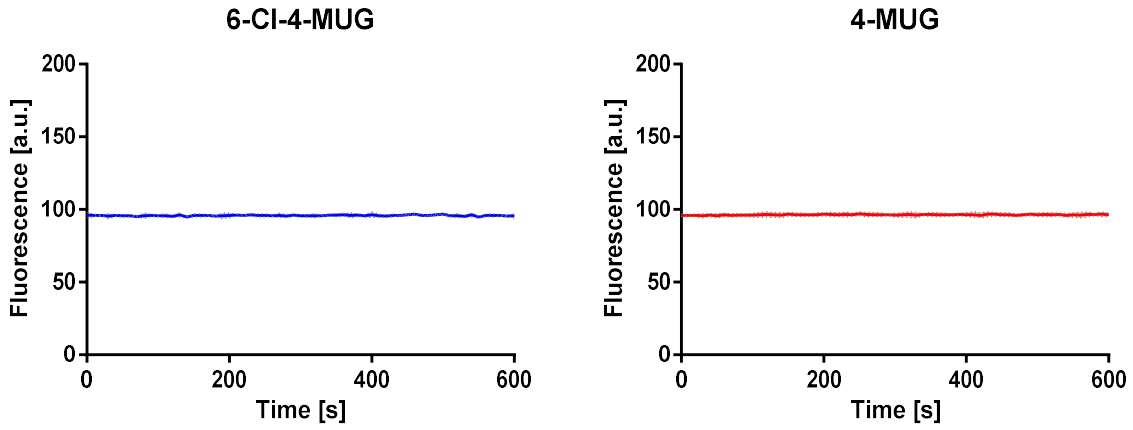


Fig. C 29. Stability tests of 6-Cl-4-MUG & 4-MUG (Iris Biotech Lot 1002) at 37°C and pH 6.8.

All enzyme assays were conducted at 37 °C, pH 6.8 and with the addition of 12.5 mU/ml β-glucuronidase. The standard operation procedure for all enzyme kinetic measurements of Chapter 2 is described in detail in chapter E.1.61.

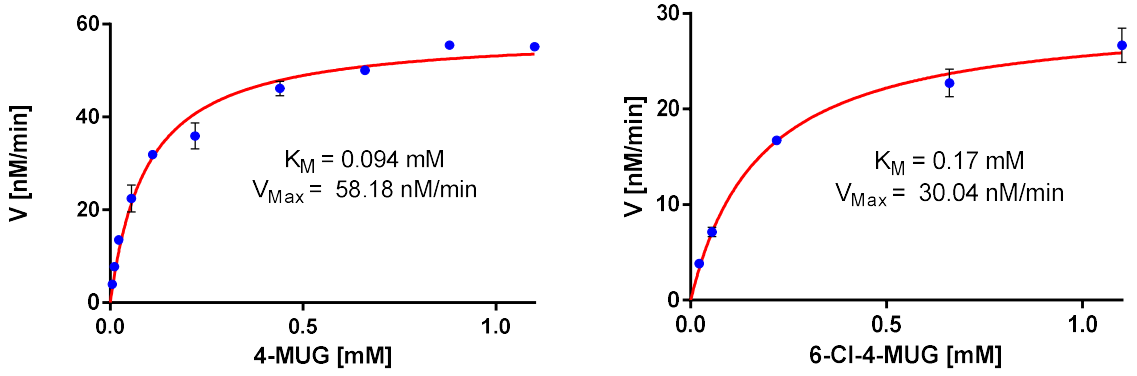
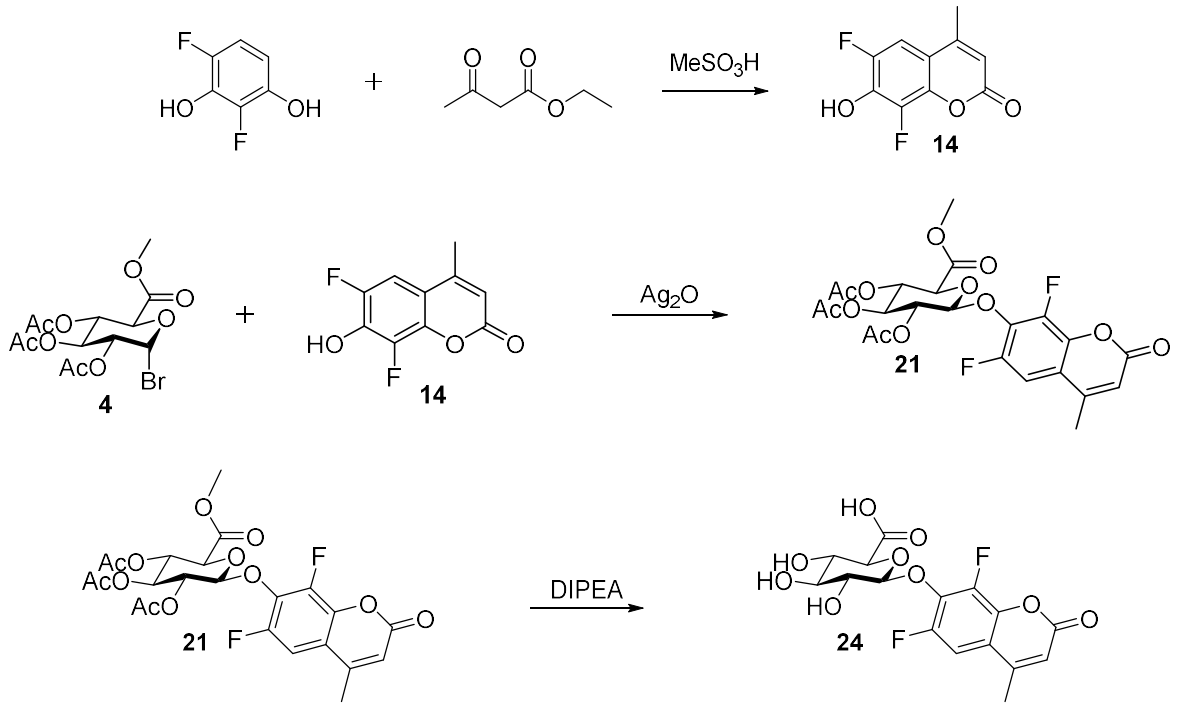


Fig. C 30. Michaelis-Menten enzyme kinetics of 4-MUG and 6-Cl-4-MUG

Although the data (Figure C 30) indicates that the kinetic parameters for 4-MUG (K_M = 0.094 mM, V_{Max} = 58.18 nM/min) are better than for 6-Cl-4-MUG (K_M = 0.17 mM, V_{Max} = 30.04 nM/min), the enzyme kinetics of 6-Cl-4-MUG are still excellent for an β-glucuronidase assay. Together the high "Turn On" ratio and good enzyme kinetics make 6-Cl-4-MUG a very suitable substrate for QRTF measurements, also shown by recently published data.[49]

C.2.4 Synthesis of 6,8-Difluoro-4-methylumbelliferyl-β-D-glucuronic acid



Scheme C 7. Synthesis of 6,8-difluoro-4-methylumbelliferyl-β-D-glucuronic acid **24**

6,8-Difluoro-4-methylumbelliferyl-β-D-glucuronic acid **24** short DiFMUG was synthesized using same approach as for the synthesis of 6-Cl-4-MUG. As first step 6,8-difluoro-4-methylumbelliferone **14** was formed via the Pechmann condensation of 2,4-difluororesorcinol and ethyl acetoacetate.[36] For the glucuronidation and following deprotection the optimized Koenigs-Knorr[47] and diisopropylethylamine (DIPEA)[45] methods were used.

C.2.5 Analysis of 6,8-Difluoro-4-methylumbelliferyl-β-D-glucuronic acid

C.2.5.1 Fluorescence Properties

Due to its two electron withdrawing fluorine atoms 6,8-difluoro-4-methylumbelliferone short DiFMU has an even lower pK_a (4.70)[50] than 6-Cl-4-MU (6.10) and its fluorescence properties are therefore completely pH independent in the used pH range of 6.8 to 8.4 (Figure C 30). Like all tested 4-MU derivates DiFMU also has large stoke shift of 90 nm making it a very interesting fluorophore for enzyme substrates (Figure C 31).[51]

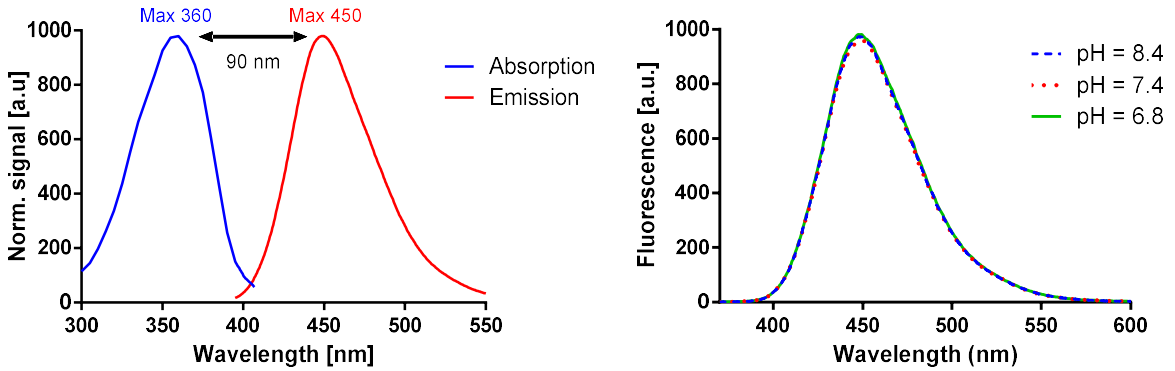


Fig. C 31. Stokes shift (left) and emission spectra at pH 6.8, 7.4 and 8.4 (right) of 6,8-difluoro-4-methylumbelliferone

Nevertheless, this pH independence does not result in a high "Turn On" ratio to the substrate, as DiFMUG & DiFMU have both the same maximum excitation (360 nm) and emission (450 nm) wavelength (Figure C 32). The resulting interference diminishes the "Turn On" ratio to 950 over the entire pH range from 6.8 to 8.4.

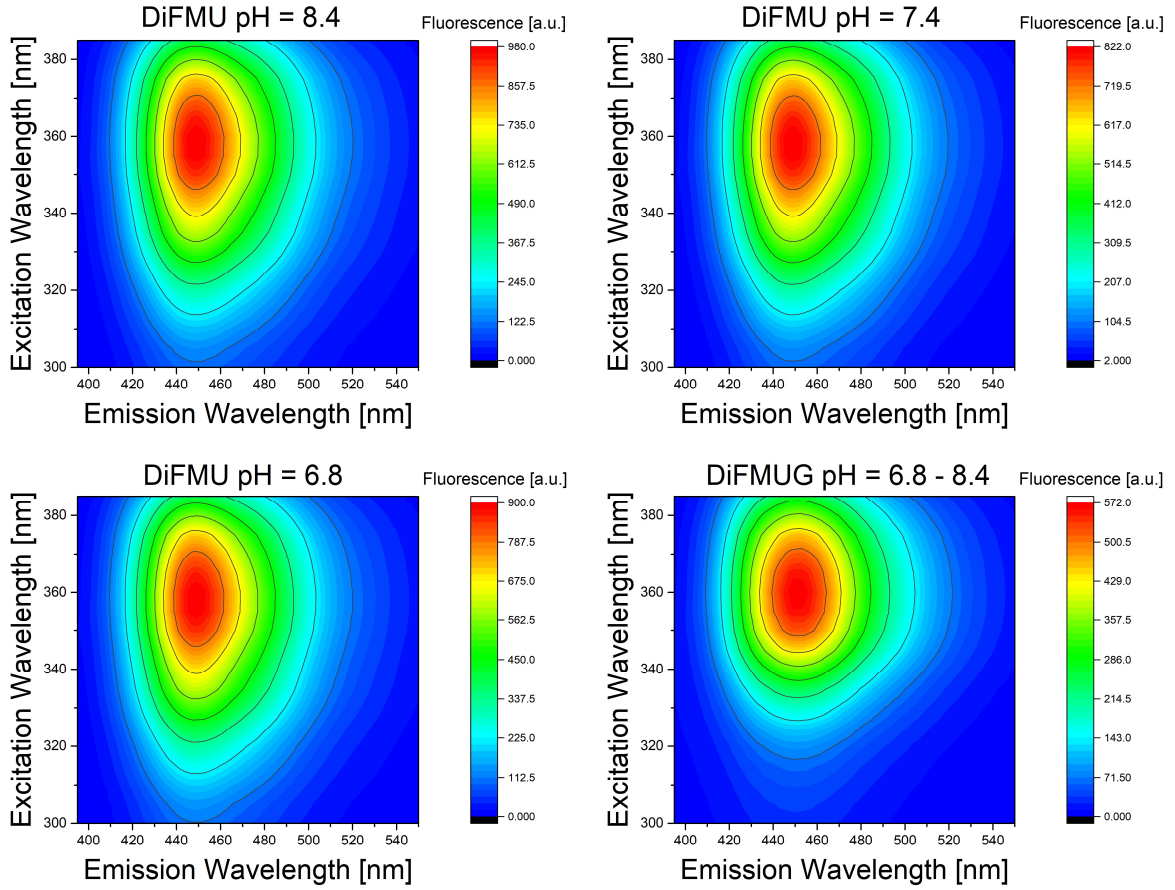


Fig. C 32. Fluorescence properties of DiFMU & DiFMUG in a pH range from 6.8 – 8.4

C.2.5.2 Stability and Enzyme Kinetics

The stability and kinetic parameters (K_M = 0.31 mM, V_{Max} = 29.04 nM/min) (Figure C 33) indicate that DiFMUG is a suitable substrate for QRTF measurements. However, compared to 6-Cl-4-MUG it has a decreased sensitivity due to the six times lower "Turn On" ratio at pH 6.8.

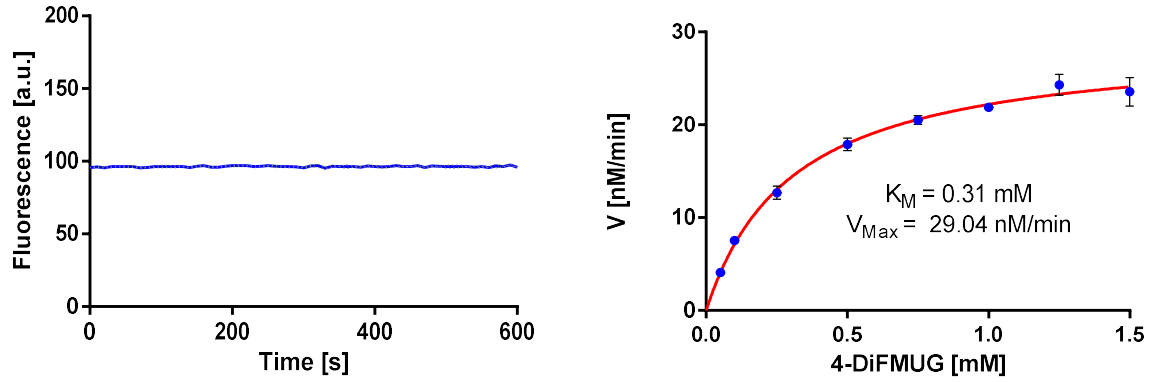
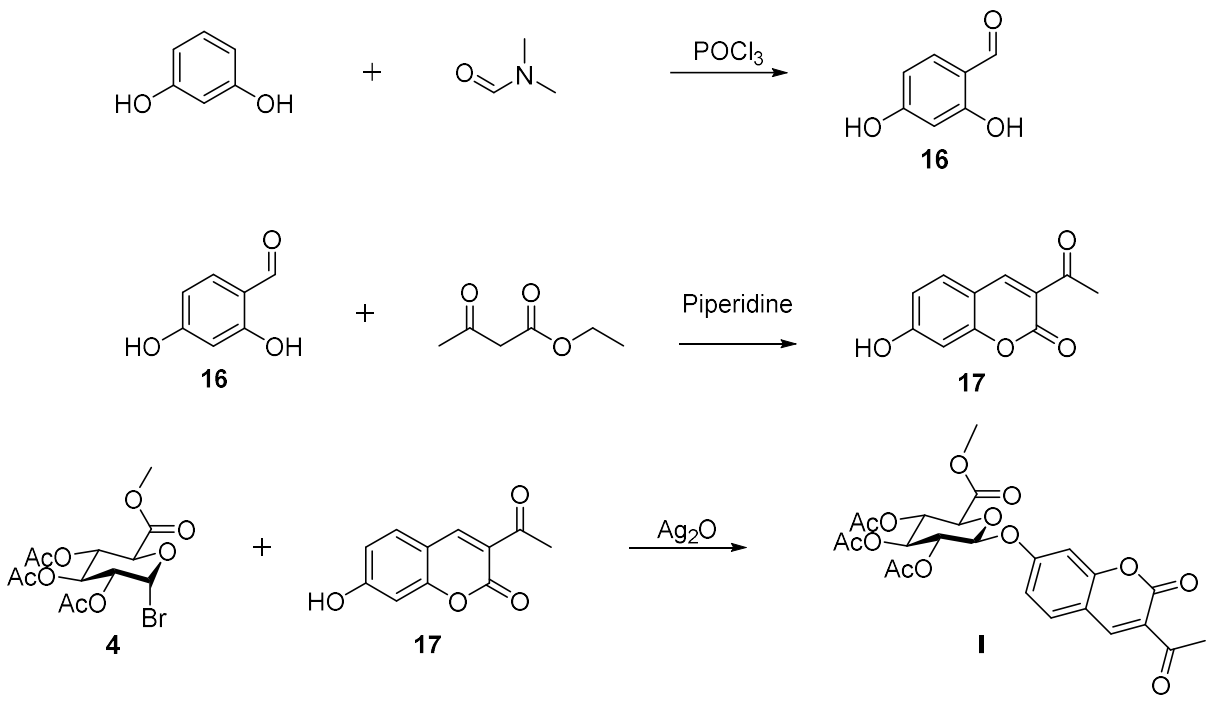
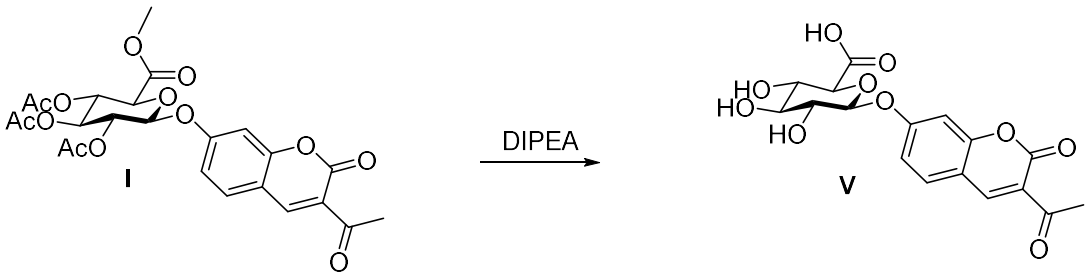


Fig. C 33 Stability tests (left) and Michaelis-Menten enzyme kinetics (right) of DiFMUG

C.2.6 Synthesis of 3-Acetylumbelliferyl-β-D-glucuronic acid



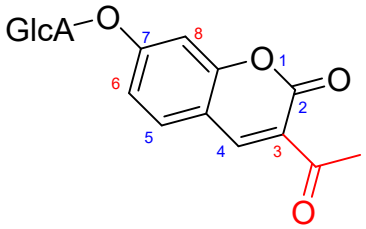


Scheme C 8. Synthesis of 3-acetylumbelliferyl-β-D-glucuronic acid **V**

As a first step 2,4-dihydroxybenzaldehyde **16** was formed via the Vilsmeier–Haack reaction. [52] For the synthesis of 3-acetylumbelliferone **17** short 3-AcU, a piperidine catalyzed condensation[53] was chosen, since 2,4-dihydroxybenzaldehyde wasn't stable under the reaction conditions of the Pechmann condensation. Glucuronidation and deprotection were then performed via the optimized Koenigs-Knorr[47] and diisopropylethylamine (DIPEA)[45] procedures.

C.2.7 Analysis of 3-Acetylumbelliferyl-β-D-glucuronic acid

During the kinetic measurements of the previous substrates, it was observed that introducing substituents with a *-I* effect at position 6 and 8 of the umbelliferone not only alters the pH dependence of the fluorophore, but also has a negative effect on enzyme kinetics of the β-glucuronidase assay (K_M 4-MUG 0.094 mM < K_M 6-Cl-4-MUG 0.17 mM < K_M DiFMUG 0.31 mM). By derivatizing the umbelliferone at position 3 (Scheme C 9) with an acyl group (*-M*), it was expected that the pH dependence of the fluorophore could also be enhanced, without the negative effects on the enzyme kinetics since the substituent is farther away from the cleavage site of the enzyme.



Scheme C 9. 3-Acetylumbelliferyl-β-D-glucuronic acid

C.2.7.1 Fluorescence Properties

As expected due to the lower pK_a (6.52)[54] compared to 4-MU (7.79), 3-AcU is less pH dependent than 4-MU over the pH range from 8.4 to 6.8. The stoke shift for 3-AcU is with 40

nm only half as big as for the other umbelliferyl derivates, but still large enough to perform the emission measurements at the maximum excitation wavelength.

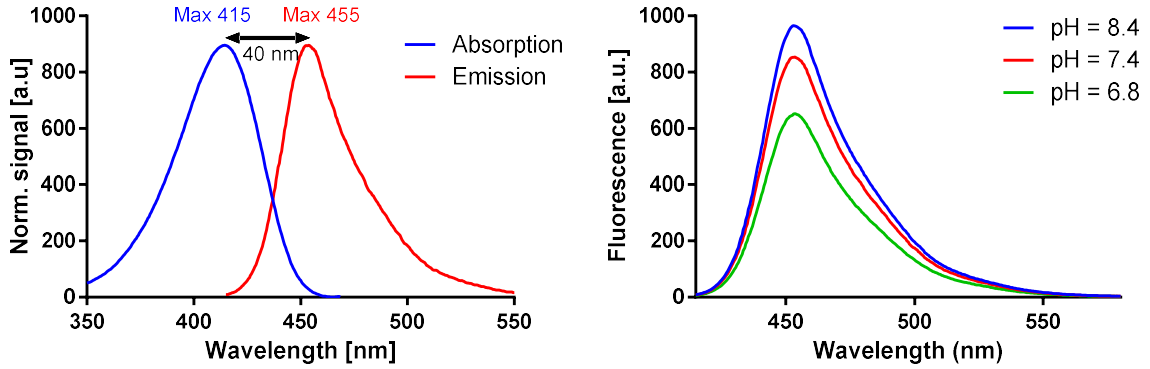


Fig. 34. Stokes shift (left) and emission spectra at pH 6.8, 7.4 and 8.4 (right) of 3-acylumbelliferone

However, 3-AcU and the substrate 3-AcUG also have the same excitation (415 nm) and emission (450 nm) maxima (Figure C 35) limiting the "Turn On" ratio to 800 at pH 6.8.

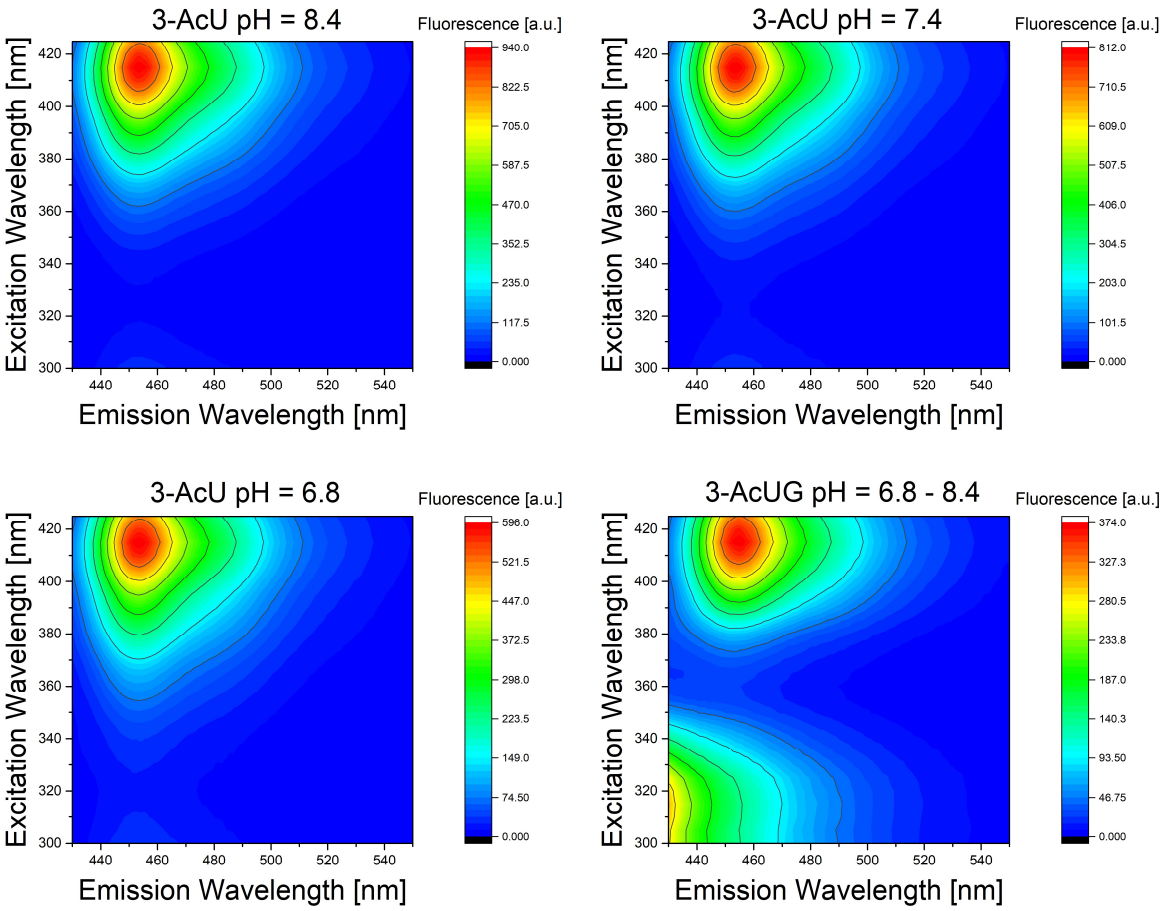


Fig. C 35. Fluorescence properties of 3-AcU & 3-AcUG in a pH range from 6.8 – 8.4

C.2.7.2 Stability and Enzyme Kinetics

Unexpectedly 3-AcUG´s enzyme kinetic parameters (K_M = 0.49 mM, V_{Max} = 41.39 nM/min) (Figure C 36) are worse than the enzyme kinetic parameters of 6-Cl-4-MUG and DiFMUG, indicating that substituents possessing a *-M* effect at position 3 of the umelliferone are not more favorable for enzyme kinetics than substituents possessing a *-I* effect at position 6 or 8.

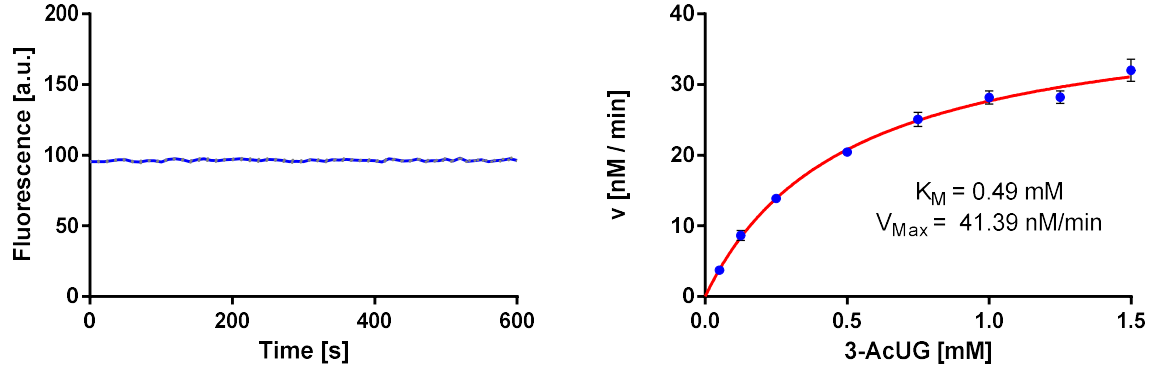
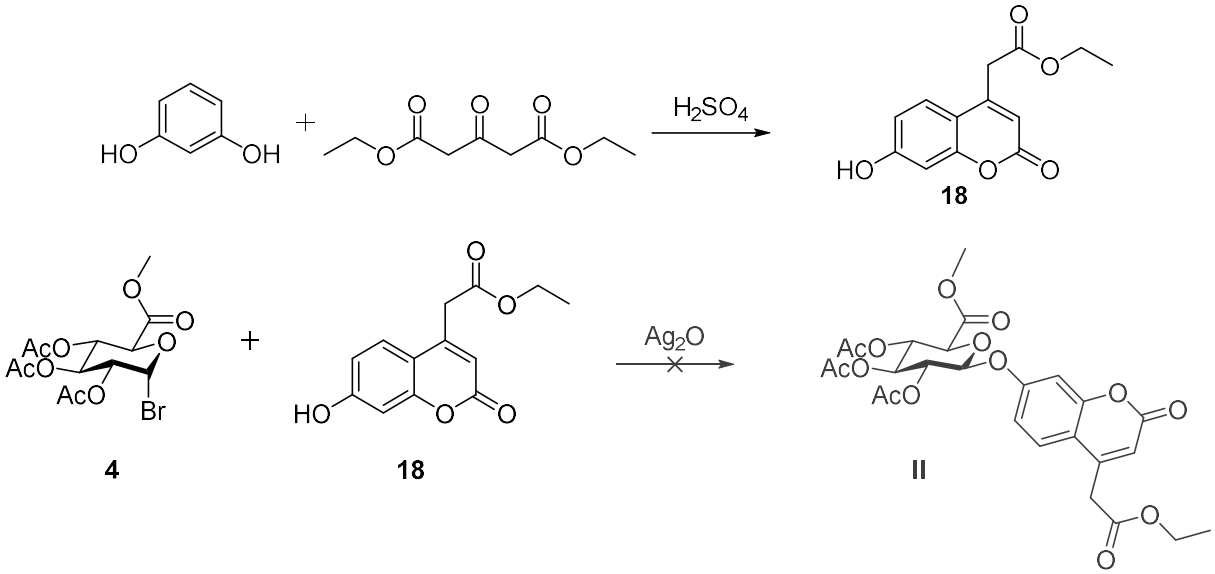
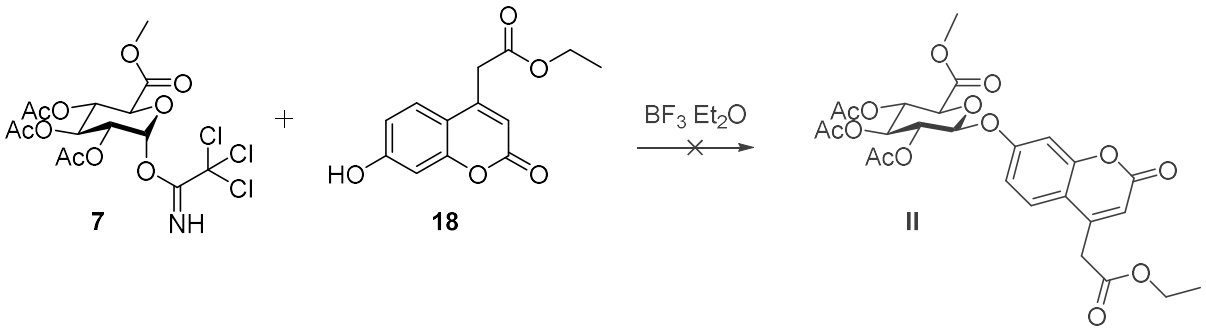


Fig. C 36. Stability tests (left) and Michaelis-Menten enzyme kinetics (right) of 3-AcUG

Considering the relatively low "Turn on" ratio, the smaller Stokes shift and slower enzyme kinetics than the other tested umelliferyl substrates. 3-acetylumbelliferyl-β-D-glucuronic acid is not the substrate of choice for QRTF measurements.

C.2.8 Synthesis of 4-(2-Ethoxy-2-oxoethyl)umbelliferyl-β-D-glucuronic acid





Scheme C 10. Synthesis of 4-(2-ethoxy-2-oxoethyl)umbelliferyl-β-D-glucuronic acid

Ethyl umbelliferone-4-acetate **18** was prepared following an optimized protocol[55] of the Pechmann condensation with β-ketoglutaric acid diethyl ester and resorcinol as reagents. For the glucuronidation step different procedures were tried, however none led to the formation of product. Since the fluorescence properties of ethyl umbelliferone-4-acetate also were not promising (chapter below), the synthesis of 4-(2-ethoxy-2-oxoethyl)umbelliferyl-β-D-glucuronic acid was set aside.

C.2.9 Analysis of Ethyl umbelliferone-4-acetate

Ethyl umbelliferone-4-acetate shows almost the same fluorogenic properties as 4-methylumbelliferone (Figure C 37 & C 38). The carboxylic acid seems to have no influence on the pH dependence of the fluorescence signal and since derivatization of the acid would not change the "Turn On" ratio of the umbelliferone, 4-(2-ethoxy-2-oxoethyl)umbelliferyl-β-D-glucuronic acid and its derivates would not be more suitable for QRTF measurements than 4-MUG.

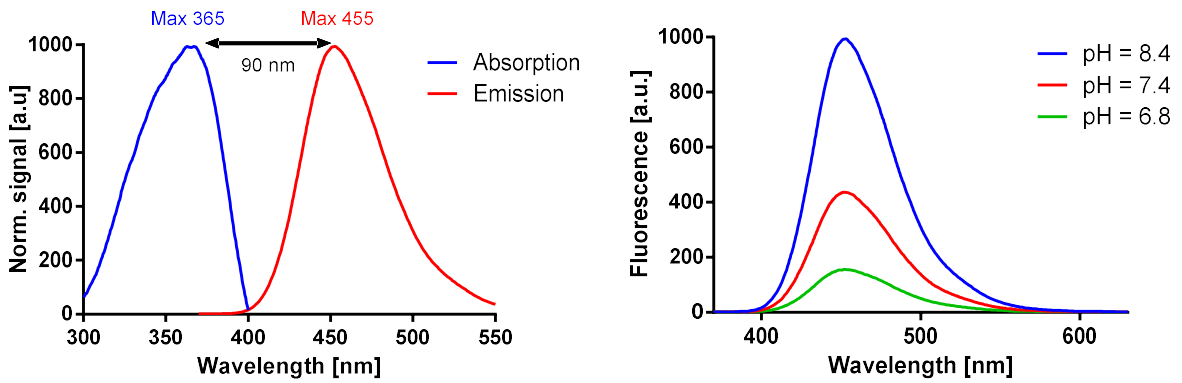


Fig. C 37. Stokes shift (left) and emission spectra at pH 6.8, 7.4 and 8.4 (right) of 4-ethylcarboxyumbelliferone

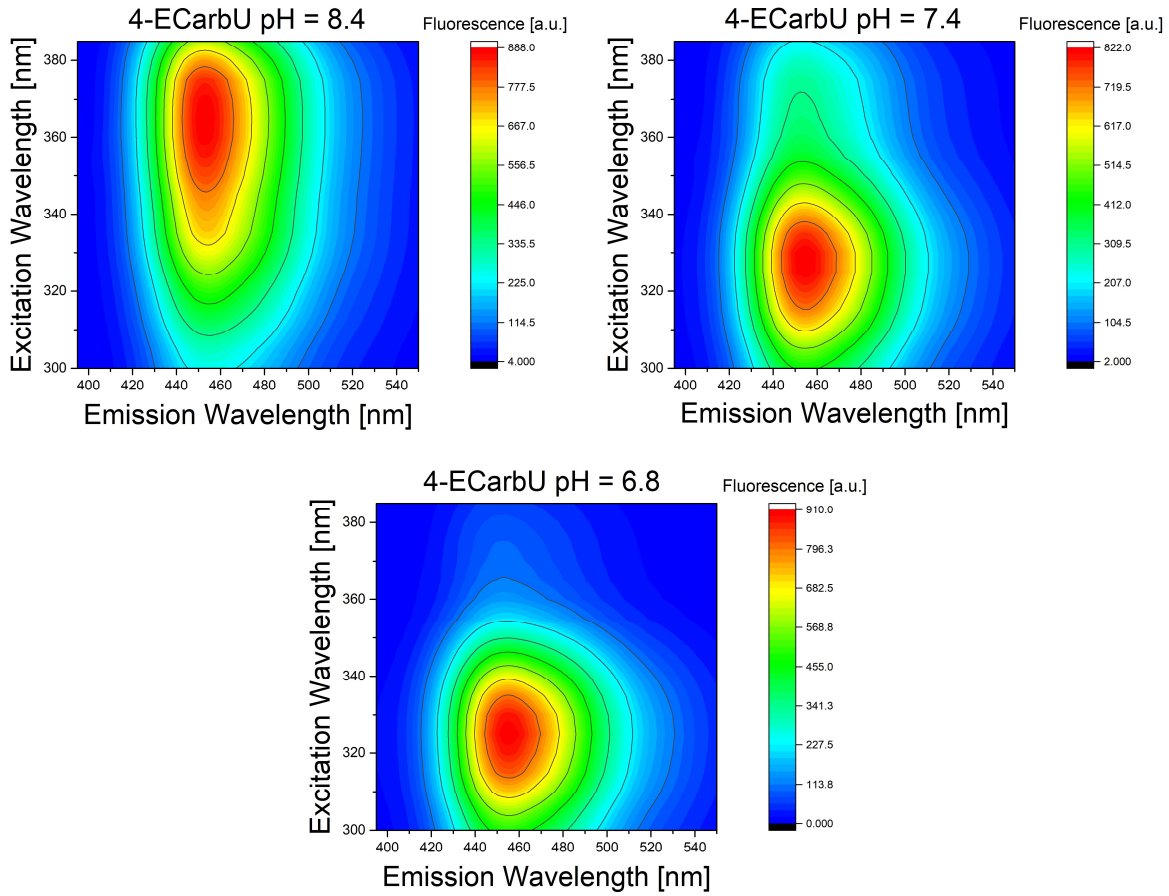
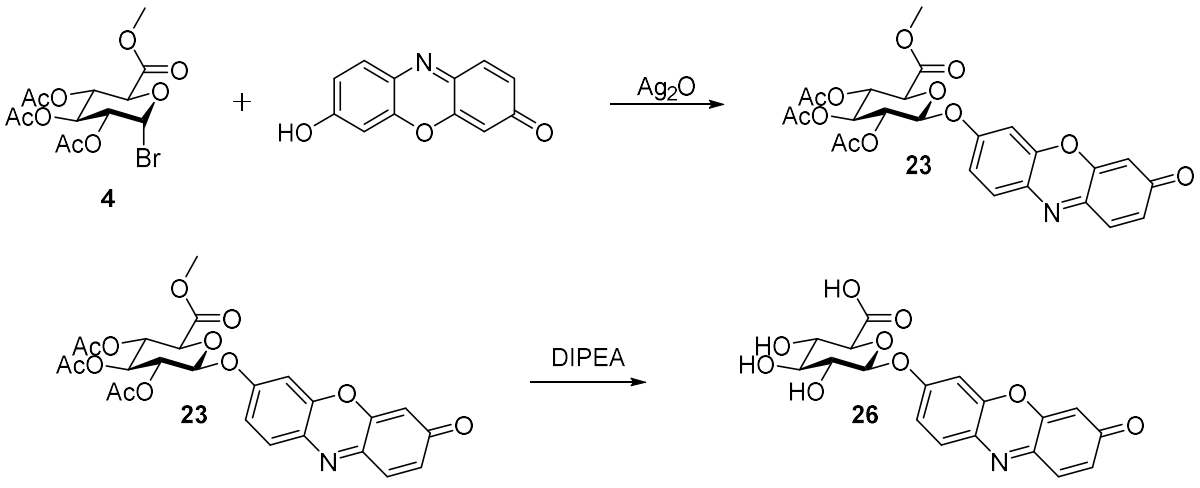


Fig. C 38. Fluorescence properties of 4-ECarbU in a pH range from 6.8 – 8.4

C.2.10 Synthesis of Resorufin-β-D-glucuronic acid



Scheme C 11. Synthesis of resorufin- β-D-glucuronic acid

The synthesis of resorufin-β-D-glucuronic acid **26** short ReG was accomplished by glucuronidation of commercially available resorufin following an optimized Koenigs-Knorr procedure,[47] followed by deprotection with diisopropylethylamine (DIPEA).[45]

C.2.11 Analysis of Resorufin-β-D-glucuronic acid

C.2.11.1 Fluorescence Properties

Due to its pK_a (5.8)[56] resorufin is almost pH independent over the tested pH range from 6.8 to 8.4, but compared to the umbelliferone fluorophores resorufin has a relatively small Stokes shift of only 10 nm (Figure C 39). This can cause measurement interference, especially when working with LED based spectrometers where the excitation wavelength is predetermined by the lamp.

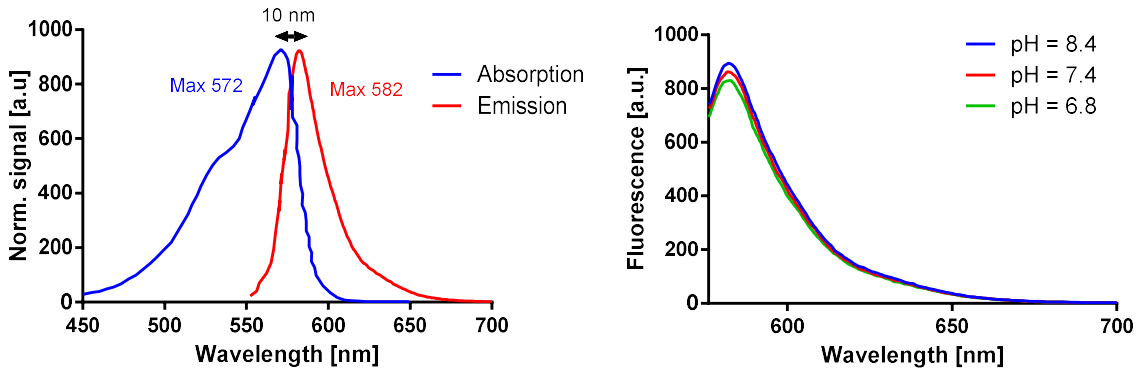
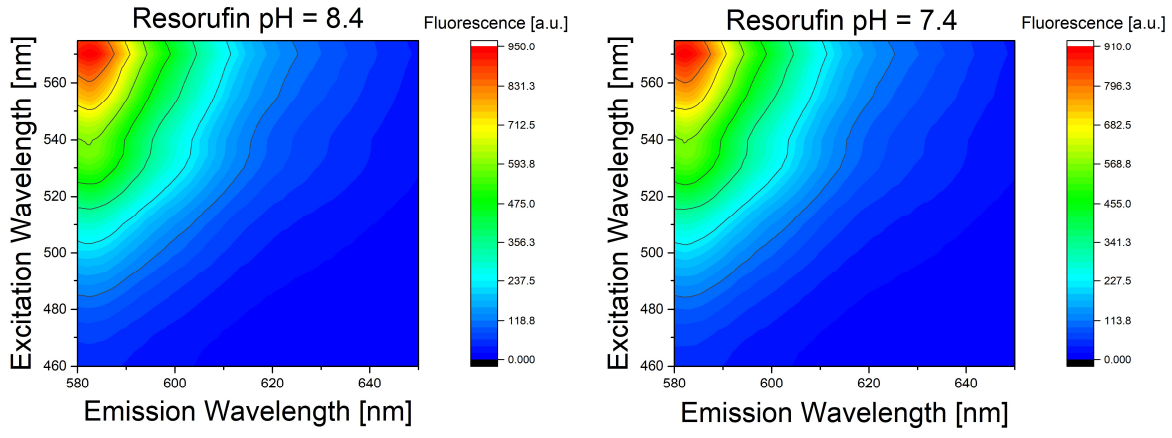


Fig. C 39. Stokes shift (left) and emission spectra at pH 6.8, 7.4 and 8.4 (right) of resorufin

Like already observed for DiFMUG & 3-AcUG, resorufin and its substrate ReG have the same excitation (572 nm) and emission (582 nm) maximum resulting in a “Turn On” ratio of 400 at pH 6.8 (Figure C 40).



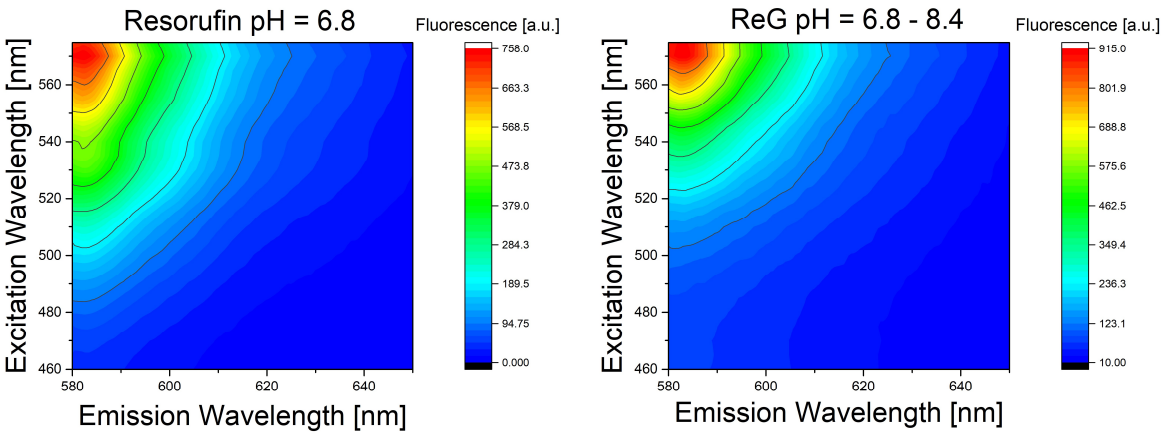


Fig. C 40. Fluorescence properties of resorufin & REG in a pH range from 6.8 – 8.4

C.2.11.2 Stability and Enzyme Kinetics

Resorufin-β-D-glucuronic acid shows very promising kinetic parameters (K_M = 0.12 mM, V_{Max} = 58.02 nM/min) towards β-glucuronidase (Figure C 41), but it has at pH 6.8 the worst "Turn On" ratio (400) of all substrates tested in this chapter. Hence resorufin-β-D-glucuronic acid is not the substrate of choice for single enzyme QRTF measurements, however it can be useful as complimentary substrate when testing multiple enzymes in the same assay since its fluorescence parameters are completely different from umbelliferone substrates.

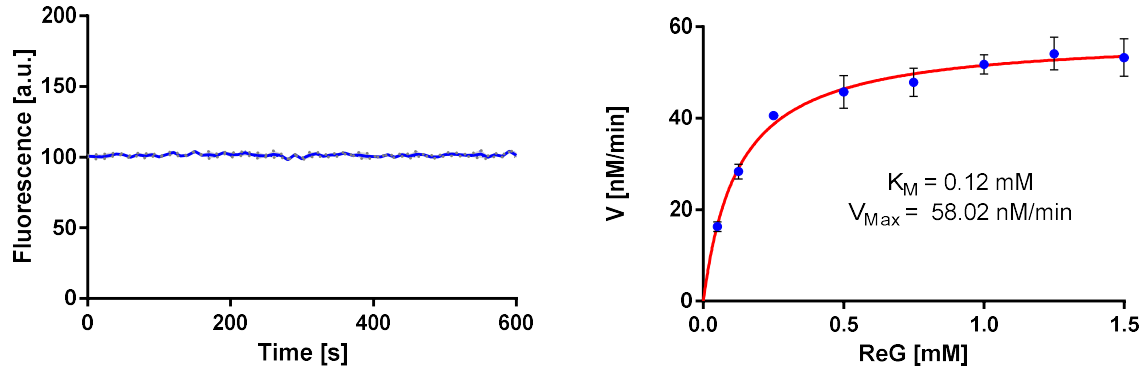
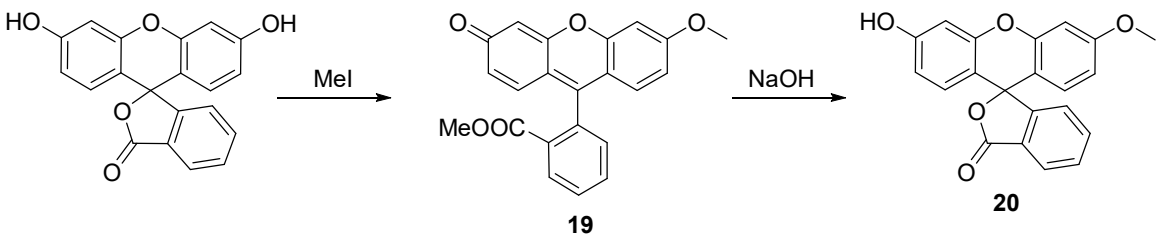


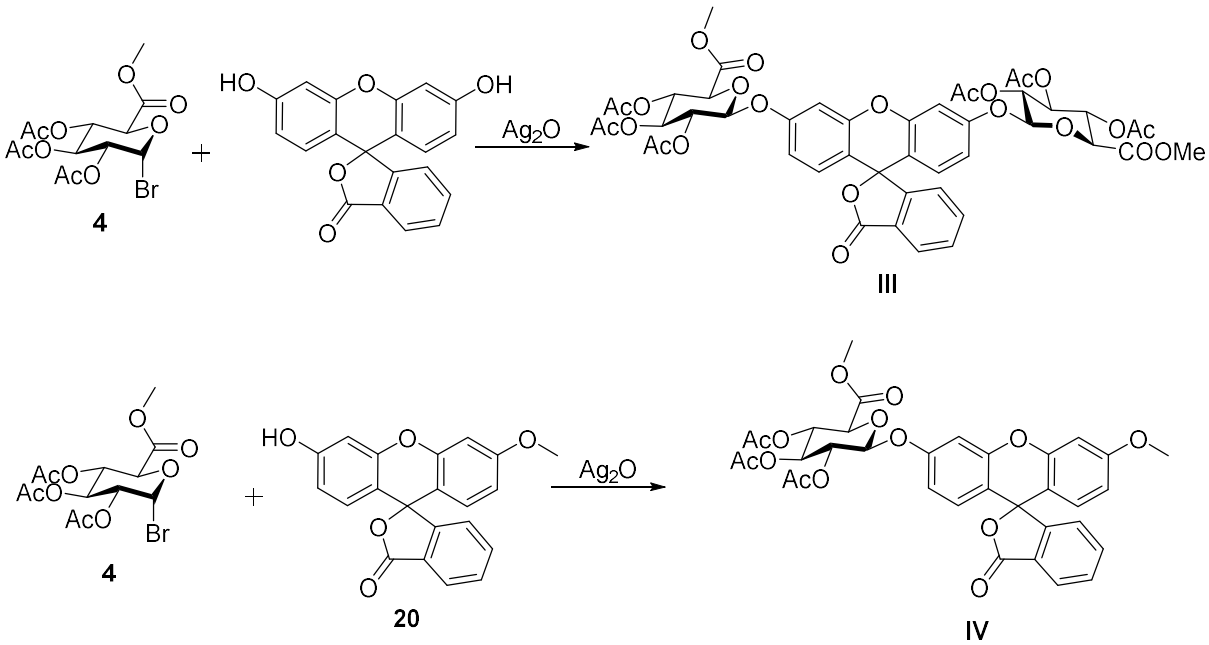
Fig. C 41. Stability tests (left) and Michaelis-Menten enzyme kinetics (right) of ReG

C.2.12 Synthesis of Fluorescein-bis[β-D-glucuronic acid] and Methoxyfluorescein-β-D-glucuronic acid



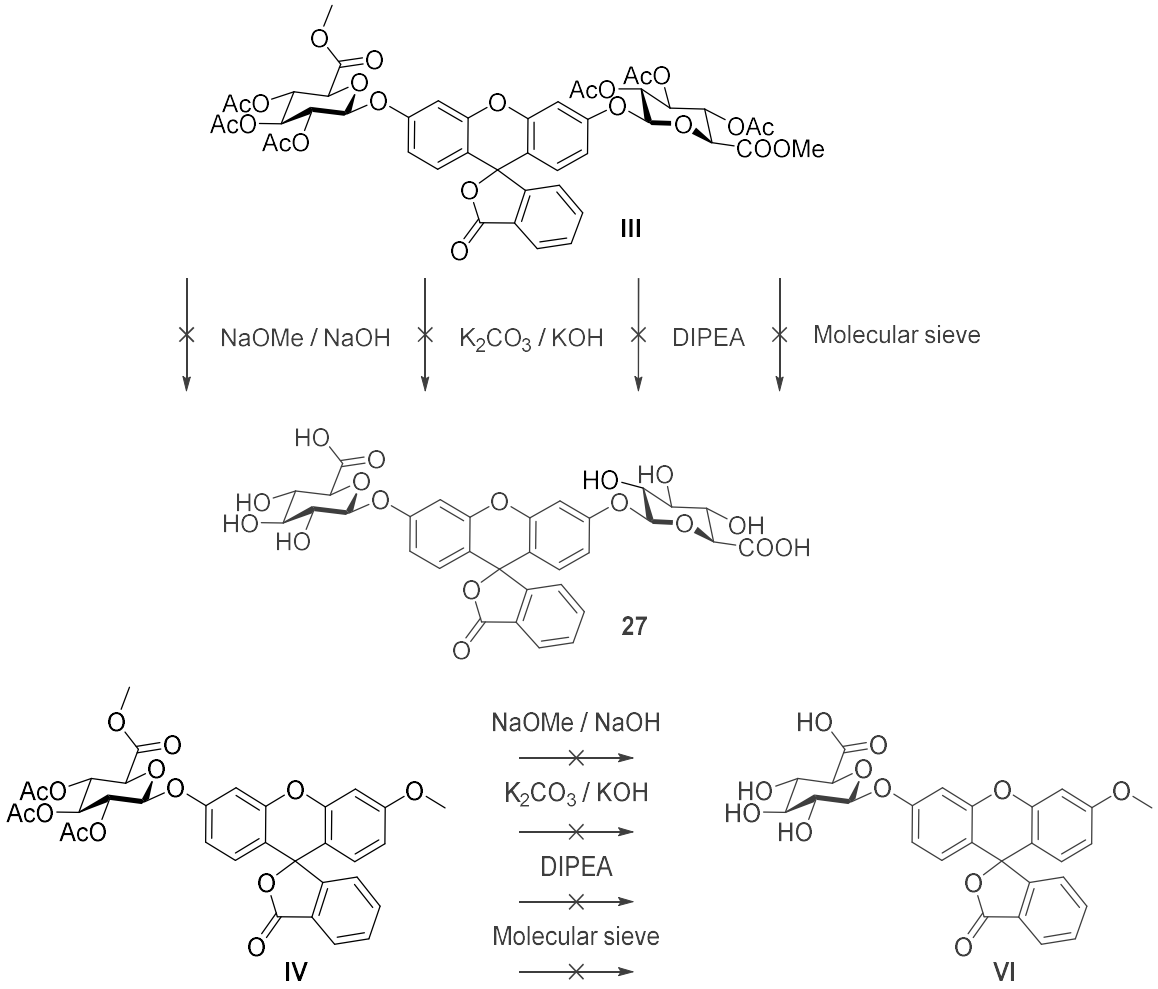
Scheme C 12. Synthesis of 3'-O-methylfluorescein

In difference to the other tested fluorophores, fluorescein has two potential glycosylation sites. For fluorescein-di-β-D-galactoside it is indicated in literature, that it is mainly directly hydrolyzed to fluorescein,[57] but as no such data was found for fluorescein-bis[β-D-glucuronic acid], the synthesis of a mono glucuronidated enzyme substrate was also attempted. For this substrate fluorescein was first methylated at the carboxyl and 3'-hydroxy group, followed by selective deprotection of the methyl ester to give 3'-O-methylfluorescein.[58]



Scheme C 13. Glucuronidation of fluorescein and 3'-O-methylfluorescein

Glucuronidation of fluorescein and 3'-O-methylfluorescein was achieved following the optimized Königs-Knorr protocol[47] as described in the previous chapters.



Scheme C 14. Synthesis of fluorescein-bis[β-D-glucuronic acid] and methoxyfluorescein-β-D-glucuronic acid

Unfortunately, the deprotection of both glucuronides **III** & **IV** could not be accomplished, even though several different synthetic protocols[42,45,59] were attempted.

C.2.13 Fluorescence Properties of Fluorescein and 3'-O-Methylfluorescein

Especially 3'-O-methylfluorescein is interesting for QRTF substrates (Figure C 42 & C 43), since its Stoke shift (57 nm) has more than twice the size of fluorescein's Stoke shift (22 nm). It is less pH dependent than fluorescein and its fluorescence properties are constant in the tested pH range from 6.8 to 8.4. However, since all synthetic approaches failed both substrates had to be set aside.

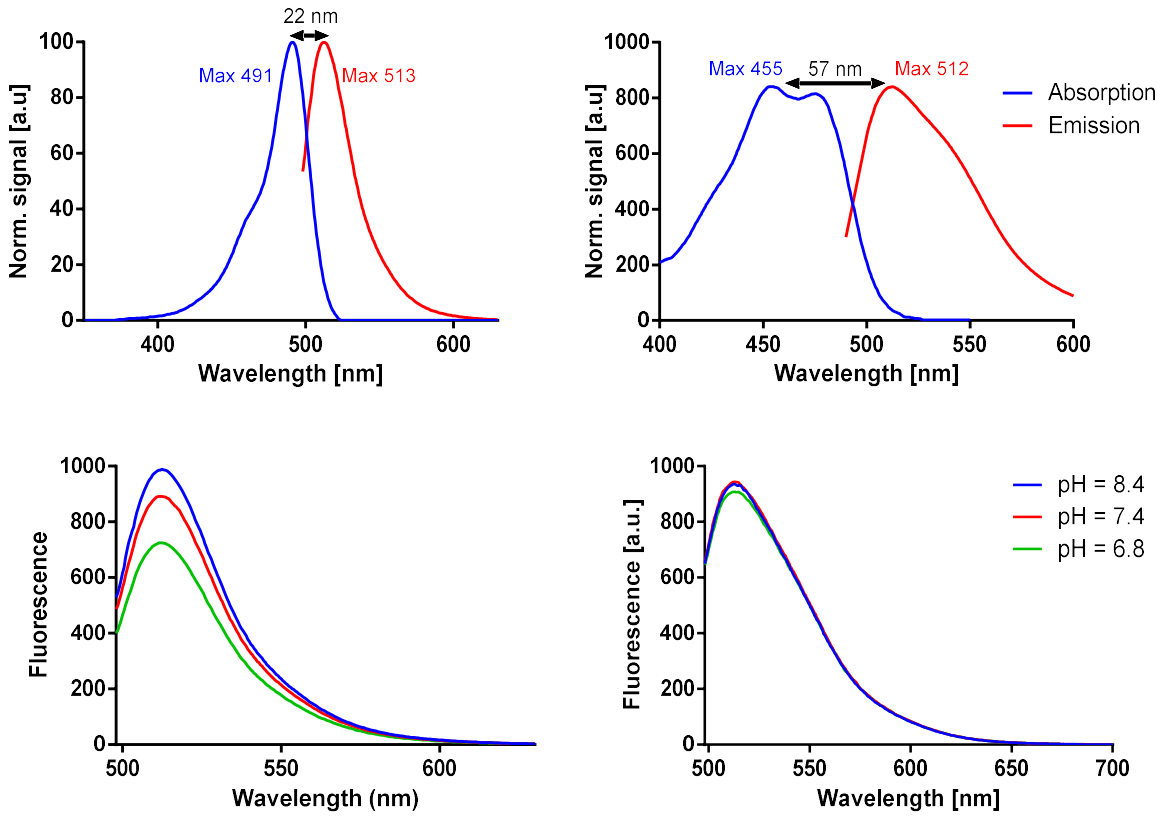


Fig. C 42. Stokes shift and pH dependence of fluorescein (left) and 3'-O-methylfluorescein (right)

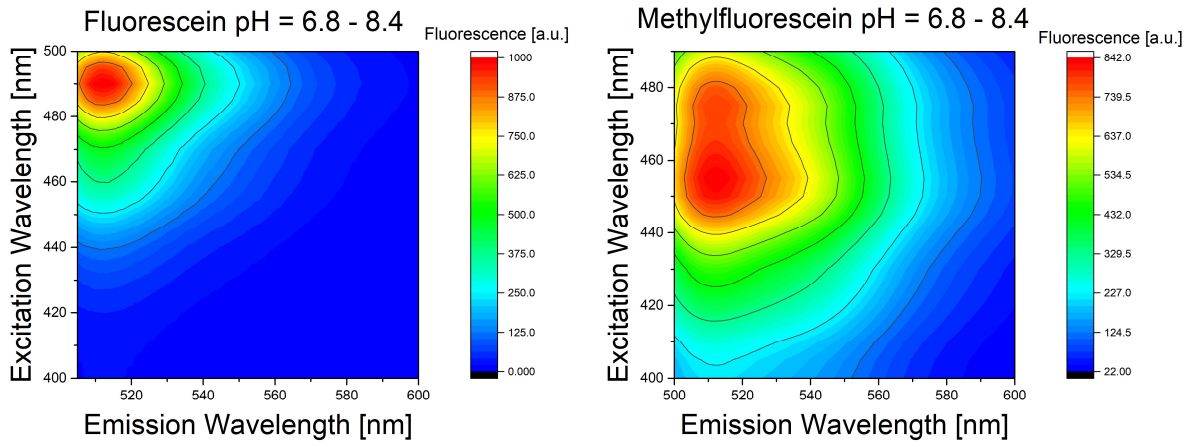


Fig. C 43. Fluorescence properties of fluorescein & 3'-O-methylfluorescein in a pH range from 6.8 – 8.4

Chapter 3

Synthesis and Analysis of Self Immolative β-Glucuronidase Responsive Probes

C.3.1 The Use of Self Immolative Linkers in Glycosidase Responsive Substrates

The advantages of self immolative linkers were already briefly discussed in chapter B.1.6. While substrate stability was no longer an issue after the purification protocol described in chapter C.1.5, the fact that only umbelliferone (**10** in Figure C 44), resorufin (**30** in Figure C 44) and fluorescein (**17** in Figure C 44) derivates are accessible for direct glycosylation, severely limits the scope of the approach taken in chapter 2. Even by introducing simple self immolative likers, a far greater variety of fluorophores is accessible. When using more complex linkers for the FRET and solid phase approaches described later in this and in the next chapter, almost the entire palette of fluorophores can be used.

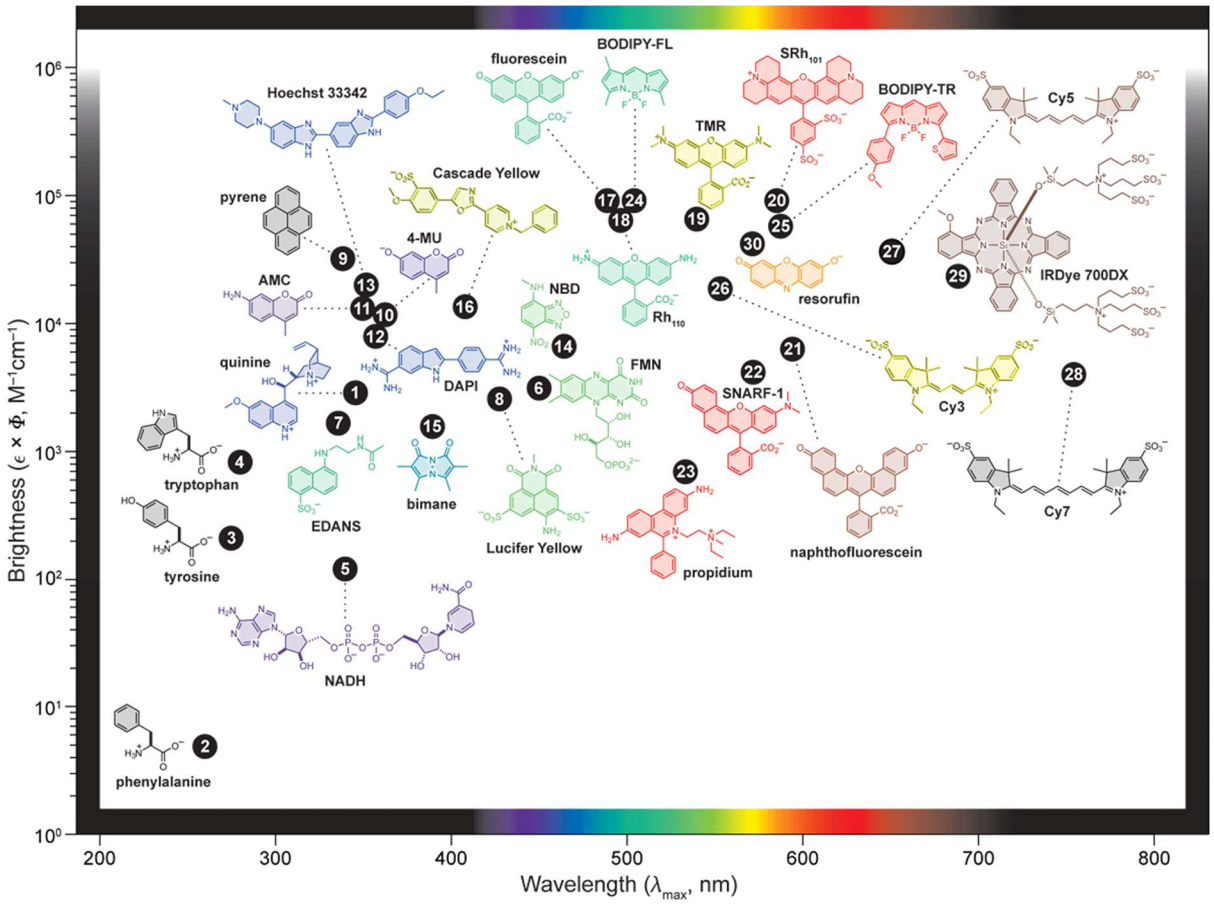


Fig. C 44. "Plot of fluorophore brightness (ε * ϕ, M^{-1} cm^{-1}) vs the wavelength of maximum absorption (λ, nm) for the major classes of fluorophores."[60]

C.3.2 Direct Carbamate and Carbonate Linkers

The first step was introducing carbamates and carbonates between the glucuronide and the fluorophore. These short linkers immolate to CO_2 upon enzymatic cleavage (Figure C 45) and were because of their simplicity the perfect starting point for fluorescence and enzymatic studies of self immolative fluorogenic probes.[23]

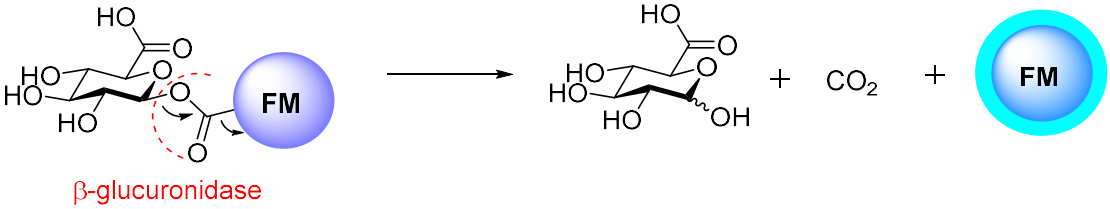
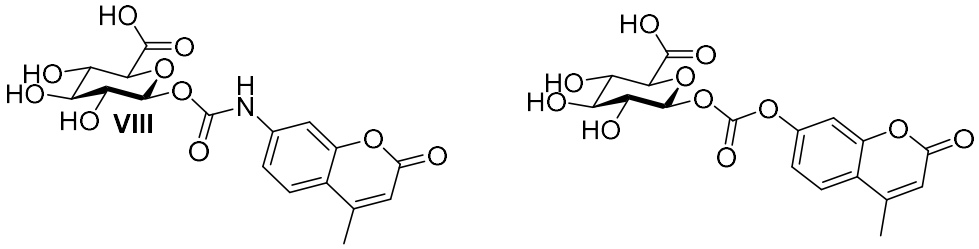


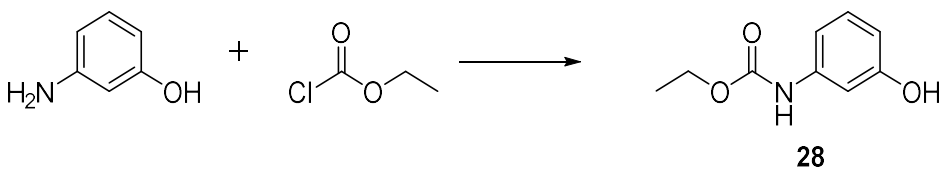
Fig. C 45. Self-immolation principle of carbonate and carbamate linkers (FM: fluorogene/fluorogenic moiety)

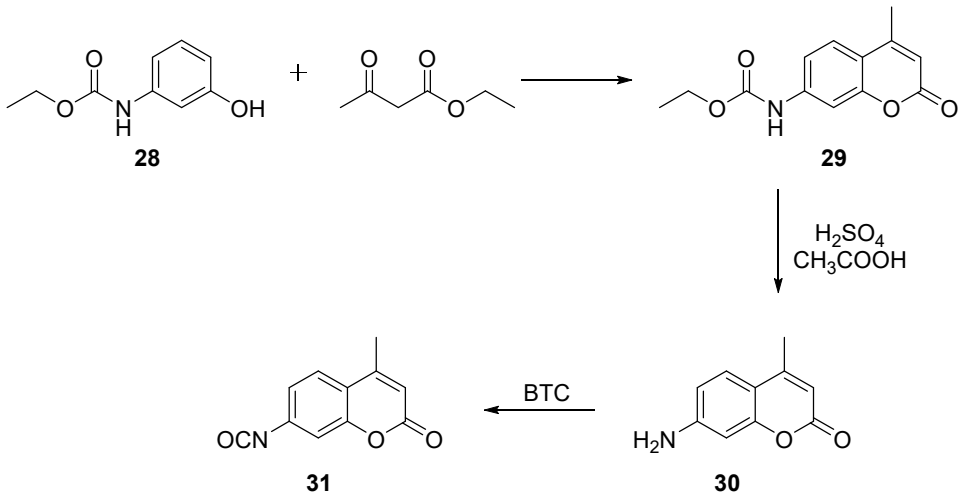
For the carbamate approach 7-amino-4-methylcumarin short 7-AMC was chosen, due to its analogisms to 4-MU and pH independence. For the carbonate approach 4-methyl-umbelliferone was chosen as fluorophore (Scheme C 15), because of the insight that could have been gained by the comparison of the carbonate substrate and 4-methylumbelliferyl-β-D-glucuronic acid.



Scheme C 15. [[[N-(4-Methyl-2-oxo-2H-1-benzopyran-7-yl)amino]carbonyl]oxy]-β-D-glucopyranosiduronic acid **VIII** short 7-AMC-Carb-GlcA (left) and [[[O-(4-methyl-2-oxo-2H-1-benzopyran-7-yl)oxy]carbonyl]oxy]-β-D-glucopyranosiduronic acid short 4-MU-Carb-GlcA (right)

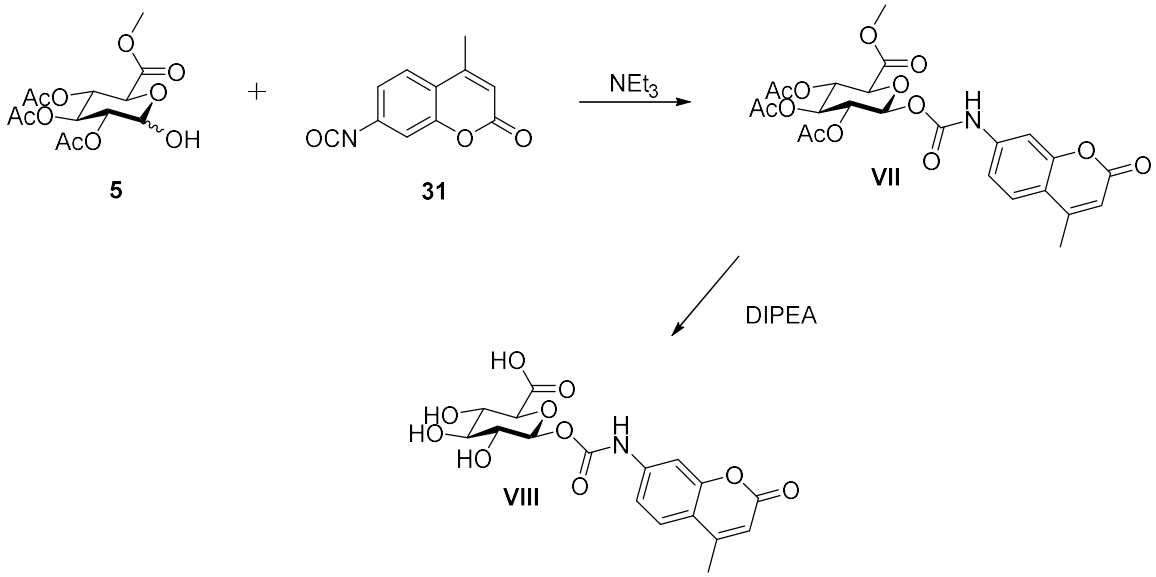
C.3.2.1 Synthesis of 7-AMC-Carb-GlcA (VIII)





Scheme C 16. Synthesis of 7-isocyanato-4-methylcoumarin

To obtain 7-AMC **30**, first (3-hydroxyphenyl)carbamic acid ethyl ester **28** was formed from aminophenol and ethyl chloroformate. The following Pechmann condensation led to the 7-AMC ethyl carbamate **29**, which was then deprotected by heating in a mixture of sulfuric and acetic acid.[61] 7-Isocyanato-4-methylcoumarin was synthesized by refluxing 7-AMC **30** with triphosgene in toluene, but in difference to the literature[62] it was not possible to isolate the isocyanate **31**. Therefore, the reaction mixture was used crude for next step.

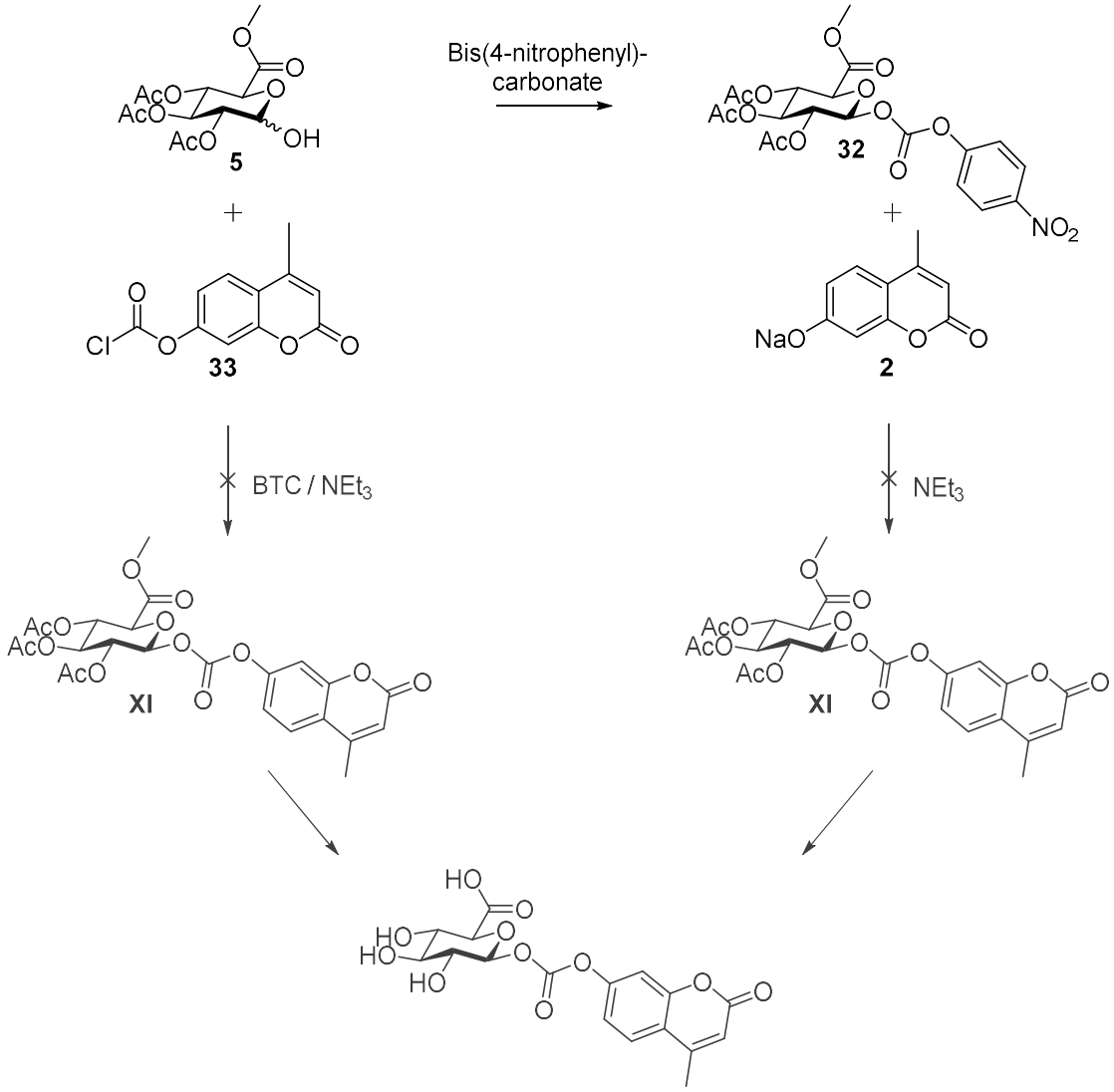


Scheme C 17. Synthesis of [[[N-(4-methyl-2-oxo-2H-1-benzopyran-7-yl)amino]carbonyl]oxy]-β-D-glucopyranosiduronic acid

The carbamate formation was then performed in the crude cooled reaction mixture of the isocyanate synthesis by addition of 2,3,4-triacetyl-glucopyranuronic acid, methyl ester **5** and triethylamine. Compound **VII** was obtained as pure β-glucuronide in a yield of 76%.[24] The

following deprotection only worked via the diisopropylethylamine (DIPEA) method[45] (95% yield), since 7-AMC-Carb-GlcA **VIII** decomposed by addition of sodium or potassium hydroxide.

C.3.2.2 Synthesis of 4-MU-Carb-GlcA



Scheme C 18. Synthesis of [[O-(4-methyl-2-oxo-2H-1-benzopyran-7-yl l)oxy]carbonyl] oxy]-β-D-glucopyranosiduronic acid

Although two different strategies for the carbonate formation were followed, compound **XI** could never be isolated. Literature also indicated that for the carbonate formation of 4-MU the chloroformate of the other reaction partner is necessary, which wasn't possible for the glucuronide **5**.

C.3.2.3 Analysis of 7-AMC-Carb-GlcA (VIII)
C.3.2.3.1 Fluorescence Properties

Like 4-MU, 7-AMC has a large stoke shift of 104 nm but is also completely pH independent in the used measurement range from pH 6.8 to 8.4 (Figure C 46).

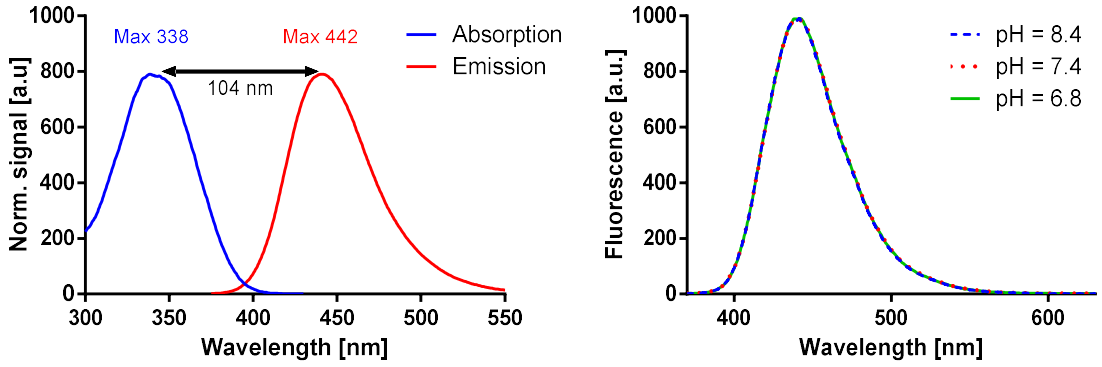


Fig. C 46. Stokes shift (left) and emission spectra at pH 6.8, 7.4 and 8.4 (right) of 7-AMC

Like already observed for some substrates in chapter C.2 the fluorescence properties of the substrate **VIII** overlap with the properties of the fluorophore resulting in an "Turn On" ratio of 1700 over the measured pH range (Figure C 47).

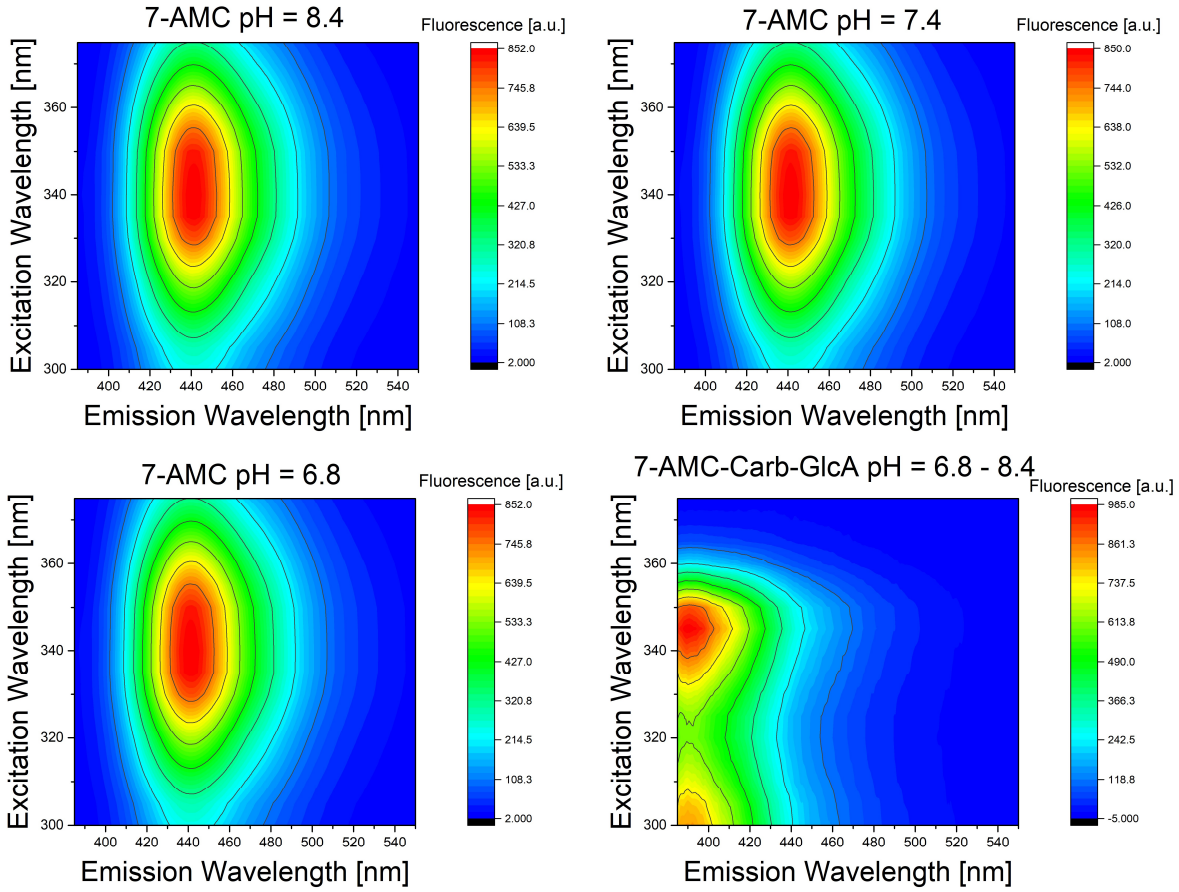


Fig. C 47. Fluorescence properties of 7-AMC & 7-AMC-Carb-GlcA **VIII** in a pH range from 6.8 – 8.4

C.3.2.3.2 Stability and Enzyme Kinetics

7-AMC-Carb-GlcA (K_M = 0.20 mM, V_{Max} = 34.76 nM/Min) shows excellent kinetic parameters (Figure C 48) even better than some umbelliferone derivates (DiFMUG & 3-AcUG), and out of all substrates tested in chapter C.2, only 6-Cl-4-MUG has a better "Turn On" ratio. Therefore, it is an adequate choice for QRTF measurements but more importantly it indicates that carbamate linkers have no negative effect on stability or enzyme kinetics.

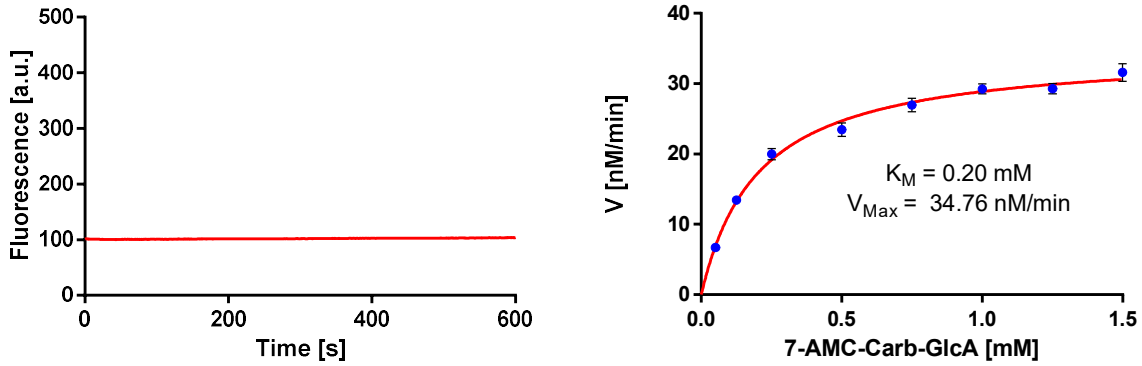


Fig. C 48. Stability tests (left) and Michaelis-Menten enzyme kinetics (right) of 7-AMC-Carb-GlcA **VIII**

C.3.3 Aliphatic Self Immolative Linkers

The next step in linker design was inserting 2-(methylamino)ethanol between the glucuronide and the fluorophore using either two carbamates or a carbamate and a carbonate. The resulting linker then immolates upon cleavage into two molecules of carbon dioxide and N-methyl-2-oxazolidone (Figure C 49).[21,24,63,64]

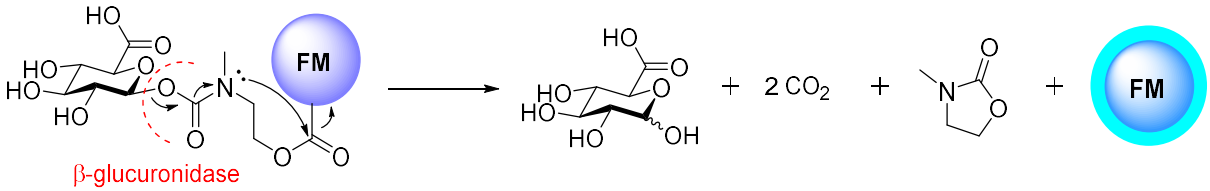


Fig. C 49. Self-immolation principle of aminoethanol carbamate linkers (FM: fluorogene/fluorogenic moiety)

When using different aminoethanols with more functional groups, this approach could also be applied for more sophisticated FRET or otherwise functionalized substrates (Figure C 50).

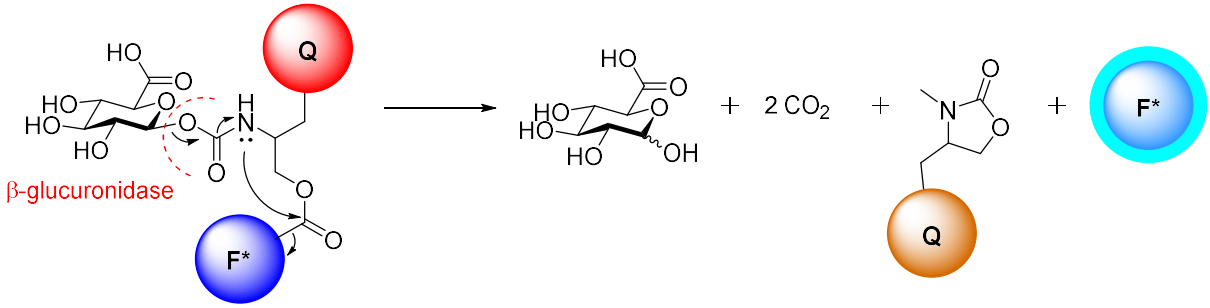
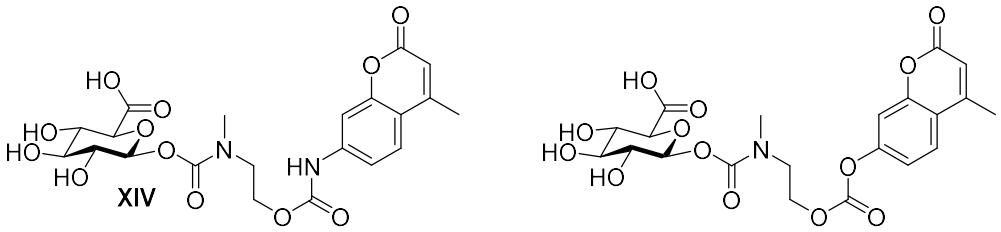


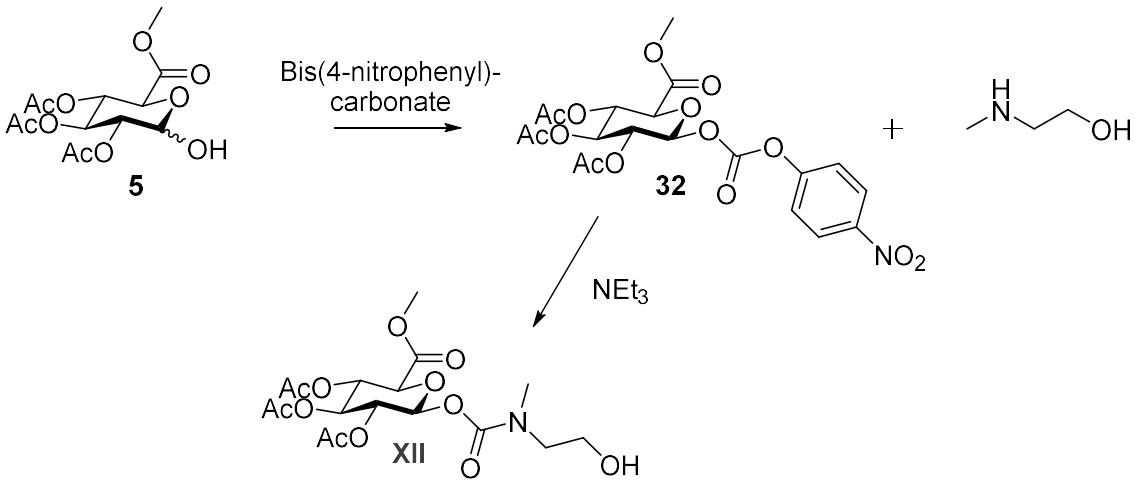
Fig. C 50. Self-immolation principle of aminoethanol FRET probes (F*: highly fluorescent moiety, Q: quencher molecule)

In order to obtain comparable data sets for the linker kinetics to the carbamate linker, 7-AMC was chosen as fluorophore for the double carbamate approach. For the carbonate approach 4-MU was chosen (Scheme C 19).



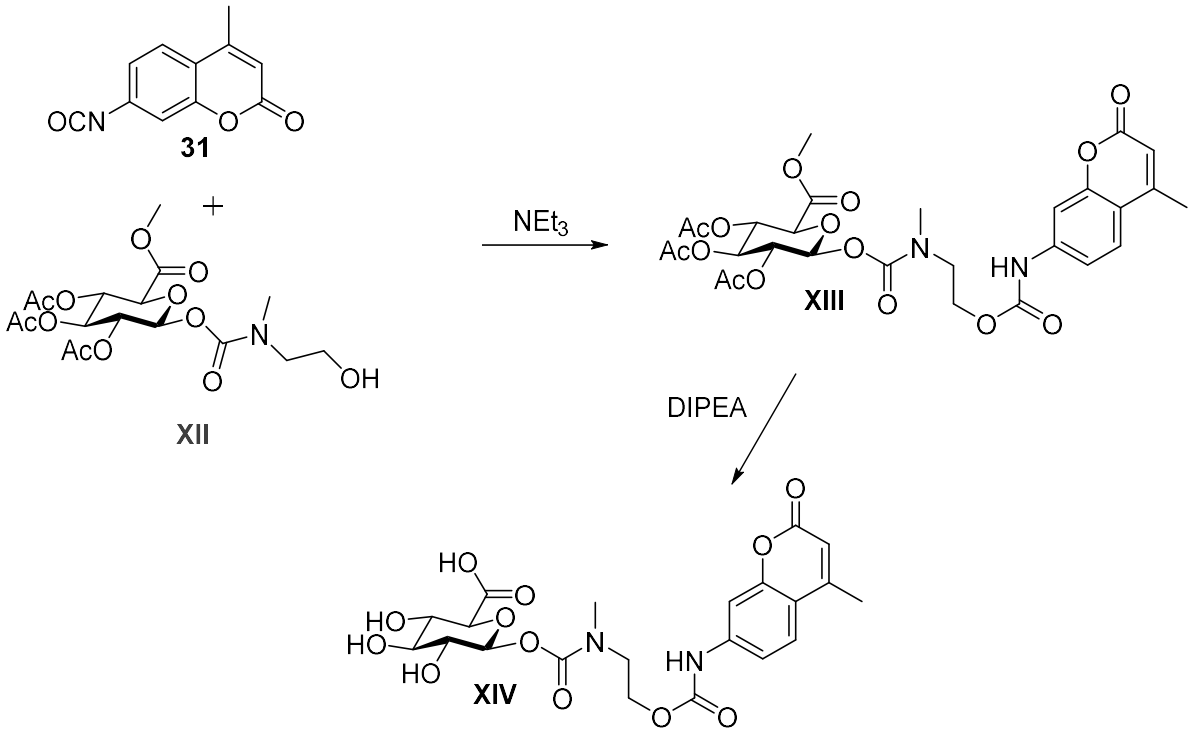
Scheme C 19. [[[N-[2-[[[N-(4-methyl-2-oxo-2H-1-benzopyran-7-yl)amino]carbonyl]oxy]ethyl]-N-methylamino]carbonyl]oxy]-β-D-glucopyranosiduronic acid **XIV** (left) short 7-AMC-AEt-GlcA and [[[N-[2-[[[O-(4-methyl-2-oxo-2H-1-benzopyran-7-yl)amino]carbonyl]oxy]ethyl]-N-methylamino]carbonyl]oxy]-β-D-glucopyranosiduronic acid (right) short 4-MU-AEt-GlcA

C.3.3.1 Synthesis of 7-AMC-AEt-GlcA (XIV)



Scheme C 20. Synthesis of [[[N-(2-hydroxyethyl)-N-methylamino]carbonyl] oxy]-2,3,4-tri-acetyl-β-D-glucopyranosiduronic acid, methyl ester **XII**

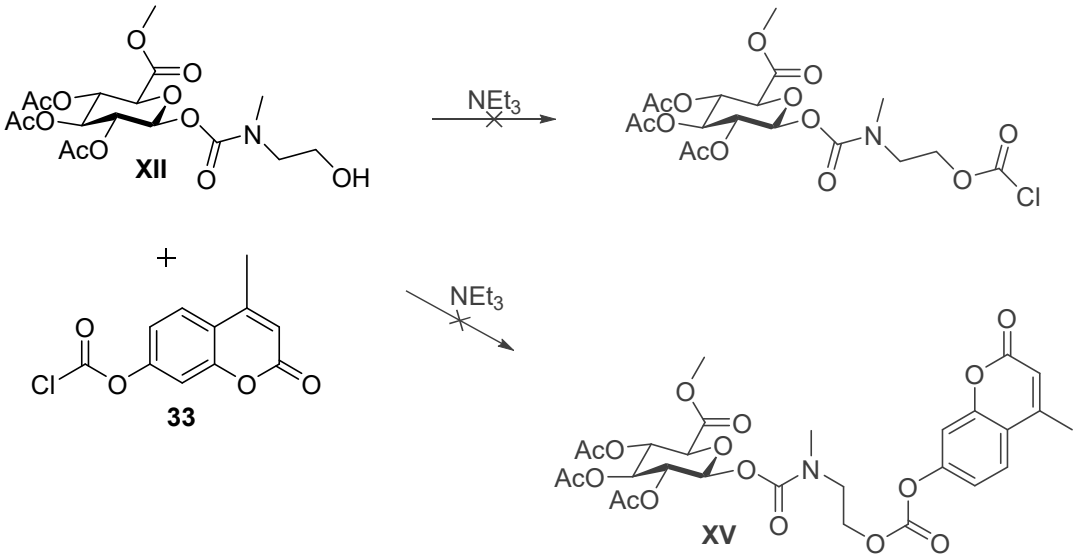
For the synthesis of compound **XII**, which was needed for both substrates, first the 4-nitrophenyl carbonate of compound **5** was prepared. The carbamate formation was then achieved by the addition of 2-(methylamino)ethanol and triethylamine.[45]



Scheme C 21. Synthesis of 7-AMC-AEt-GlcA **XIV**

The second carbamate formation was performed like previously described in chapter C.3.2.1, followed by deprotection after the diisopropylethylamine (DIPEA) protocol[45].

C.3.3.2 Synthesis of 4-MU-AEt-GlcA



Scheme C 22. Attempted synthesis of 4-MU-AEt-GlcA

Like already observed in the previous chapter the 4-MU chloroformate does not seem to be reactive enough to from the desired carbonate **XV** and since compound **XII** decomposed during the chloroformate synthesis, the carbonate approach for the aminoethanol linkers had to be abandoned.

C.3.3.3 Analysis of 7-AMC-AEt-GlcA (XIV)

C.3.3.3.1 Fluorescence Properties and Enzyme Kinetic Parameters

The fluorescence properties of 7-AMC were already described in chapter C.3.2.3.1. Also, the properties of 7-AMC-AEt-GlcA **XIV** are identical with 7-AMC-Carb-GlcA **VIII** resulting in an "Turn On" ratio of 1700 over the measured pH range (Figure C 51).

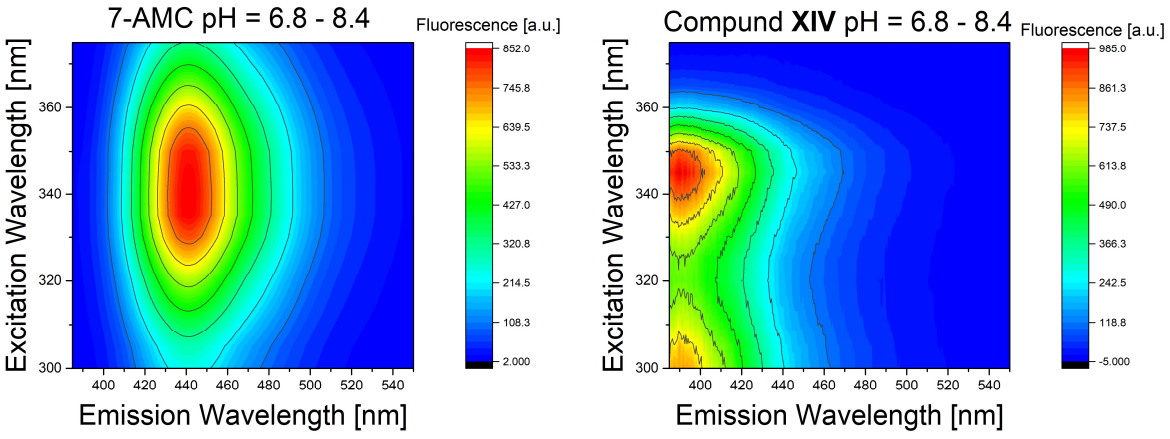


Fig. C 51. Fluorescence properties of 7-AMC & 7-AMC-Carb-GlcA **VIII** in a pH range from 6.8 – 8.4

However, enzyme assays showed that the linker did not immolate as expected, since compound **XIV** shows little interaction with β-glucuronidase compared to all other tested substrates. Upon addition of a 1000 times more enzyme (12.5 U/ml) than usual (12.5 mU/ml) the fluorescence intensity diminishes (Figure C 52), indicating that compound **XIV** immolates to the less fluorescent 7-AMC urea derivate (Scheme C 23) instead of N-methyl-2-oxazolidone and 7-AMC.

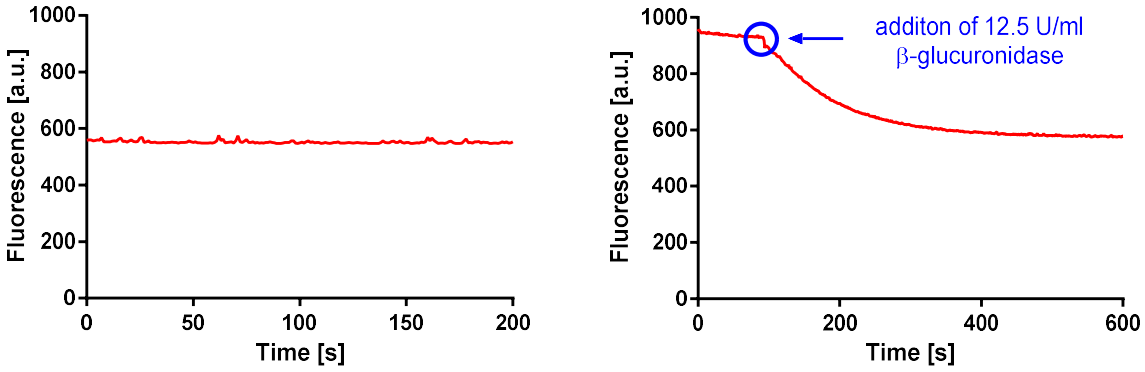
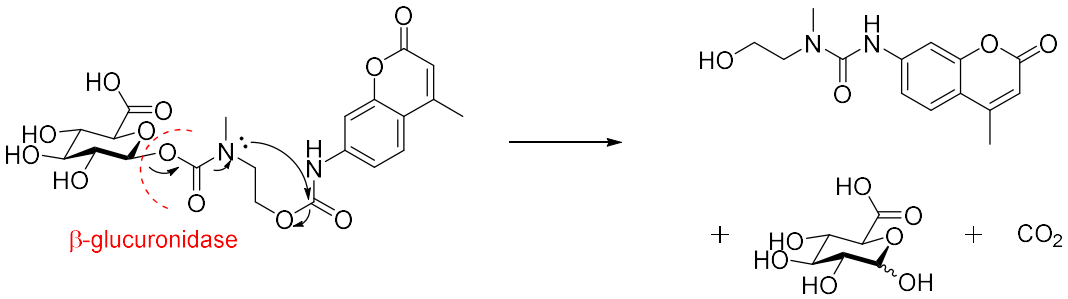


Fig. C 52. Stability test (left) and enzyme assay (right) of 7-AMC-AEt-GlcA **XIV** (0.25 mM)



Scheme C 23. Proposed immolation mechanism of compound **XIV**

C.3.4 Aromatic Self Immolative Linkers

In this approach *p*-aminobenzyl alcohol was used as linker between two carbamates or a carbamate and carbonate. Upon enzymatic cleavage the linker immolates to two molecules of carbon dioxide and an imine which than hydrolyzes back to *p*-aminobenzyl alcohol (Figure C 53).[24,65] Since this approach is not dependent on the formation of a cyclic system it should be better suited for 7-AMC as fluorophore.

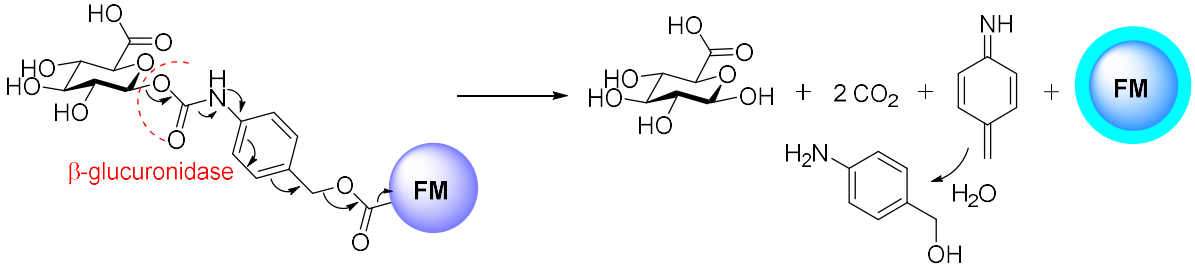


Fig. C 53. Self-immolation principle of the p-aminobenzyl alcohol linker (FM: fluorogene/fluorogenic moiety)

When using mandelic acid derivates as linkers[26], this strategy can also be applied for the synthesis of FRET probes like shown in Figure C 54. The linker and quencher for this particular probe was synthesized by Tobias Bauernfeind, the BODIPY derivate by Eleonora Hochreiner during their Bachelor theses.

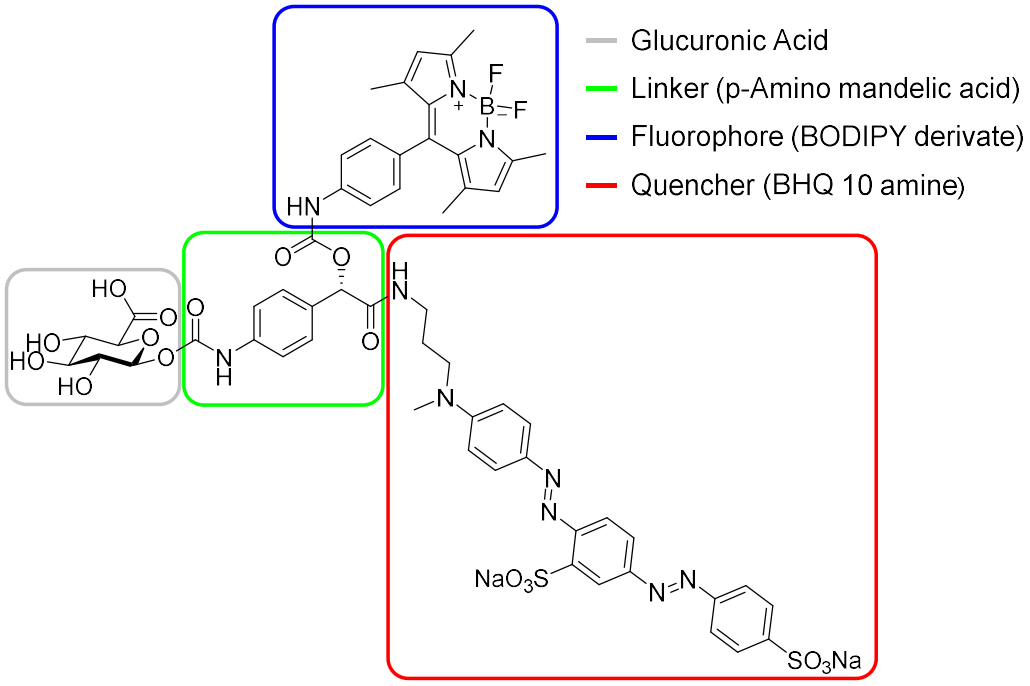
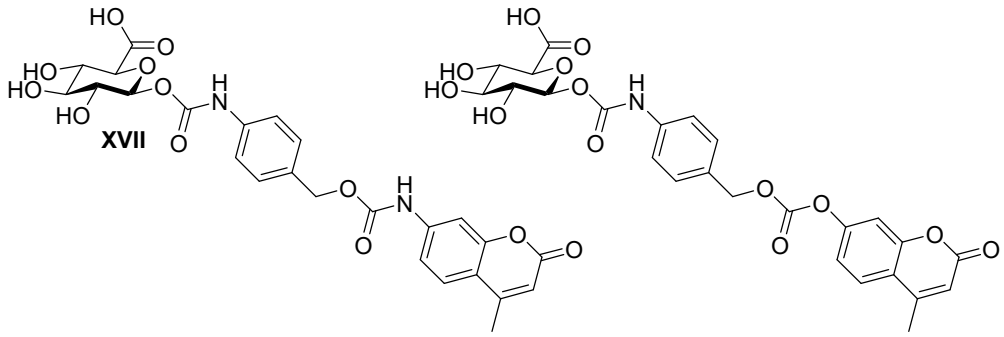


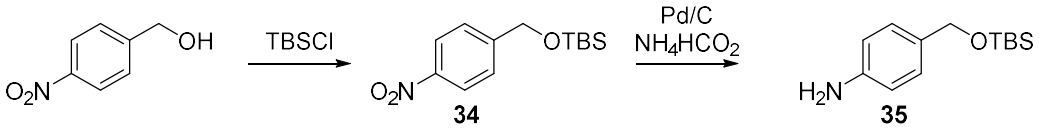
Fig. C 54. Proposed FRET Probe utilizing p-amino mandelic acid as linker.

For studies of enzyme and linker kinetics a *p*-aminobenzyl alcohol liker with 7-AMC and 4-MU as fluorophore was chosen (Scheme C 24).



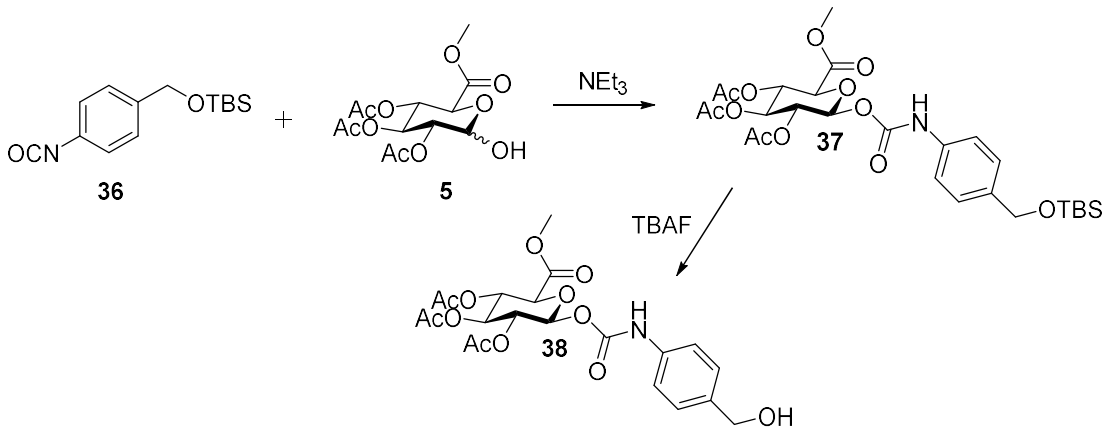
Scheme C 24. [[[[4-[[[[N-(4-Methyl-2-oxo-2H-1-benzopyran-7-yl)amino]carbonyl]oxy]methyl]phenyl]amino] carbonyl]oxy]-β-D-glucopyranosiduronic acid **XVII** short 7-AMC-*p*ABn-GlcA and [[[[4-[[[[O-(4-Methyl-2-oxo-2H-1-benzopyran-7-yl)amino]carbonyl]oxy]methyl]phenyl]amino]carbonyl]oxy]-β-D-glucopyranosiduronic acid short 4-MU-*p*ABn-GlcA

C.3.4.1 Synthesis of 7-AMC-pABn-GlcA (XVII)



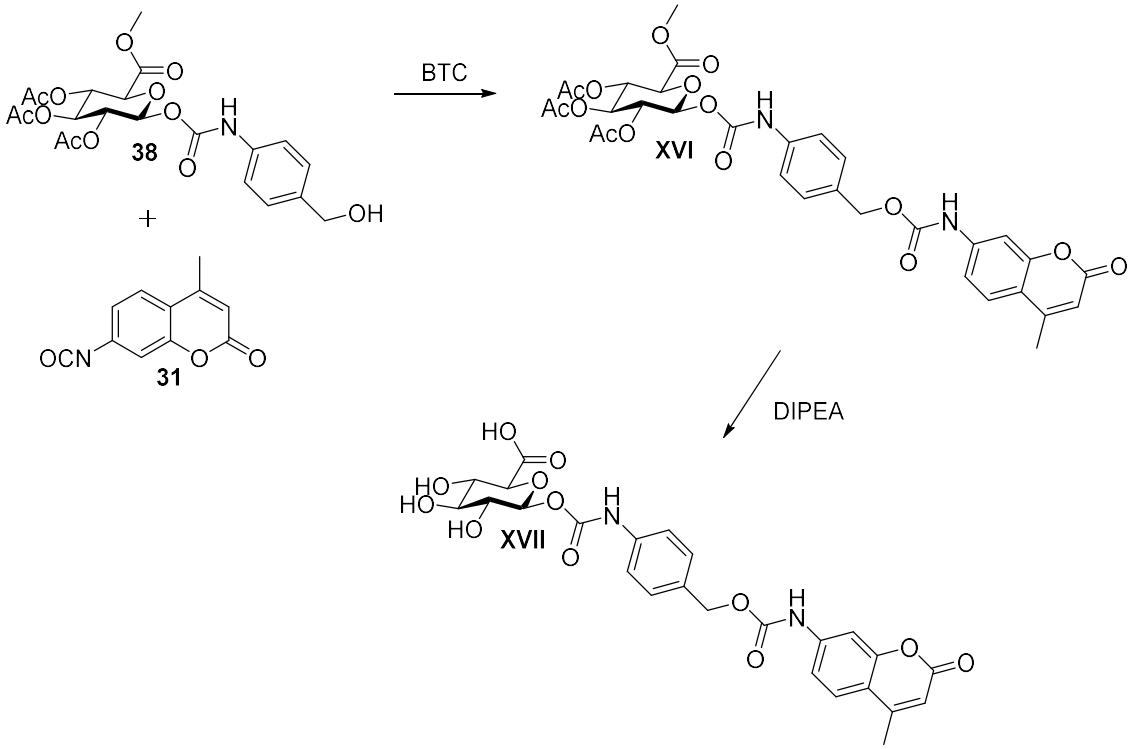
Scheme C 25. Synthesis of 4-[[[(1,1-dimethylethyl)dimethylsilyl]oxy]methyl]aniline

Since the amine of *p*-aminobenzyl alcohol was not reactive enough for the carbamate formation approach used in chapter C.3.3.1, the linker had to be synthesized from *p*-nitrobenzyl alcohol. In the first step the alcohol group was protected with tert-butyldimethylsilyl chloride (TBSCl) to avoid side reactions during the isocyanate synthesis. [24] The aromatic nitro group of compound **34** was than selectively reduced by hydrogen transfer to give the silyl protected *p*-aminobenzyl alcohol linker **35**.[66]



Scheme C 26. Synthesis of [[[[4-(hydroxymethyl)phenyl]amino]carbonyl]oxy]-2,3,4-tri-acetyl-β-D-glucopyranosiduronic acid, methyl ester **38**

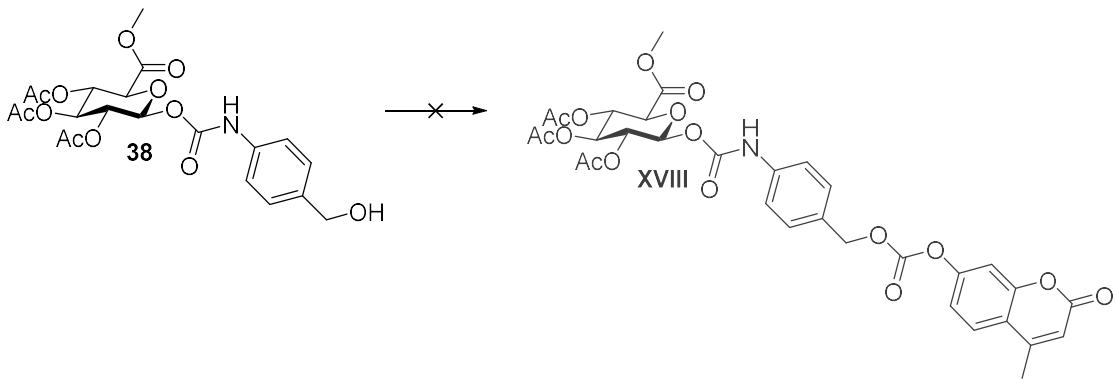
Compound **35** was then converted into the isocyanate **36** and the crude reaction mixture was used for the formation of the carbamate **37**.[24] The deprotection of the silyl ether was then performed with TBAF and acetic acid to afford compound **38**, which was used for the carbamate and carbonate route.



Scheme C 27. Synthesis of 7-AMC-pABn-GlcA (**XVII**)

The second carbamate formation was performed with 7-isocyanato-4-methylcoumarin **31** as described in chapter C.3.2.1, followed by deprotection with diisopropylethylamine[45] to afford 7-AMC-pABn-GlcA **XVII**.

C.3.4.2 Synthesis of 4-MU-pABn-GlcA (XVIII)



Scheme C 28. Attempted synthesis of 4-MU-pABn-GlcA

As expected from the results of the previous chapters, the carbonate formation to 4-MU-pABn-GlcA could not be achieved.

C.3.4.3 Analysis of 7-AMC-pABn-GlcA (XVII)

C.3.4.3.1 Fluorescence Properties and Enzyme Kinetic Parameters

Compared to the other 7-AMC substrates 7-AMC-Carb-GlcA **VIII** and 7-AMC-AEt-GlcA **XIV** the maximum excitation wavelength of 7-AMC-pABn-GlcA **XVII** is shifted by the *p*-aminobenzyl alcohol linker to 370 nm (Figure C 55). Since the maximum excitation wavelength of 7-AMC is at 442 nm, this shift results in a higher "Turn On" ratio for 7-AMC-pABn-GlcA **XVII** (2000).

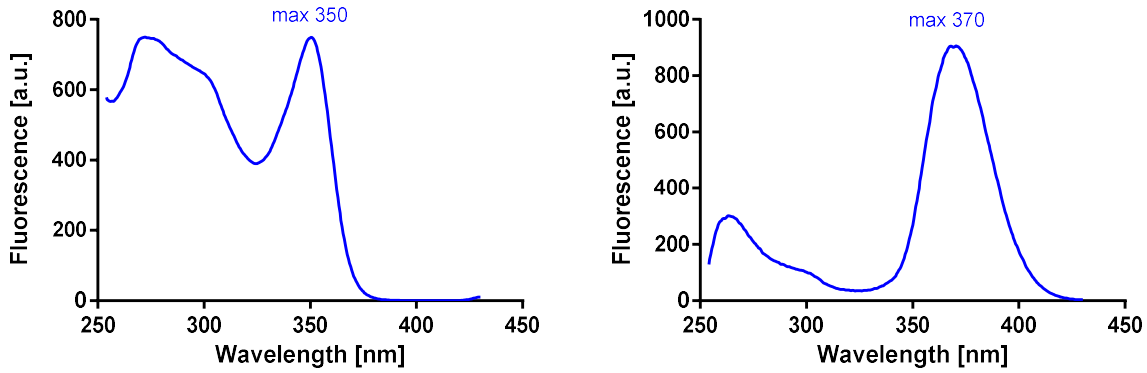


Fig. C 55. Excitation scan (445 nm Em. W.) of 7-AMC-Carb-GlcA **VIII** and 7-AMC-AEt-GlcA **XIV** (left) and 7-AMC-pABn-GlcA **XVII** (right)

However, 7-AMC-pABn-GlcA **XVII** shows little interaction with β-glucuronidase as is seen in Figure C 56. Upon addition from 12.5 U/ml enzyme an initial rise in fluorescence can be detected but even then, the measurement is not linear.

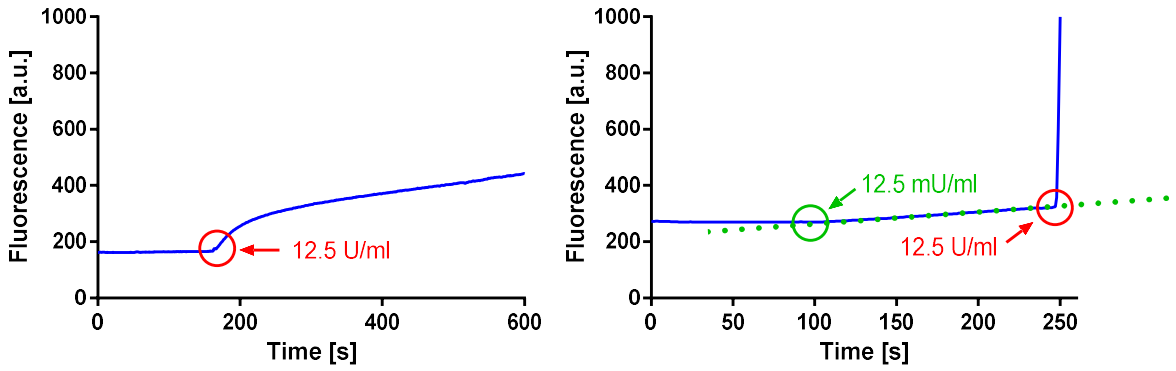


Fig. C 56. Enzyme assays of 7-AMC-pABn-GlcA **XVII** (0.25 mM, left) and 7-AMC-Carb-GlcA **VIII** (0.25 mM, right)

It is indicated in literature that the imine after enzymatic cleavage hydrolyzes not fast enough and could work as an inhibtor,[24,67] but an effect of this magnitude was not expected. Therefore, also the FRET substrates using *p*-aminomandelic acid as linker had to be set aside.

C.3.5 Modifiable Self Immolative FRET Probes

To avoid the inhibition problems of the *p*-aminobenzyl alcohol a different aromatic linker without the amine group was used next (Figure C 57). [25,68] Additionally, this linker also has an alkyne group for further modification and consists of a nitrophenol which can be used as a FRET partner for coumarin fluorophores.[69]

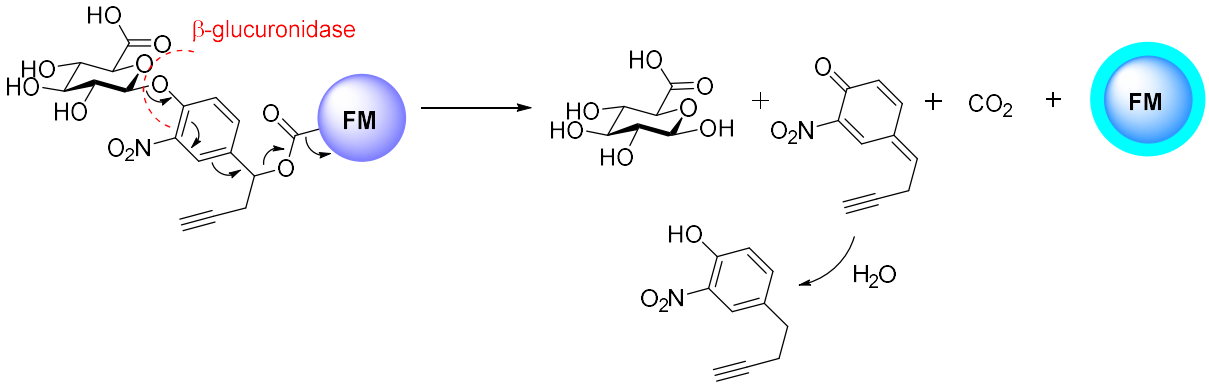
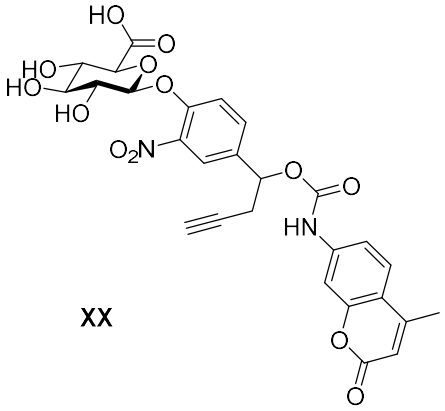


Fig. C 57. Self-immolation principle of the nitro phenolic linker (FM: fluorogene/fluorogenic moiety)

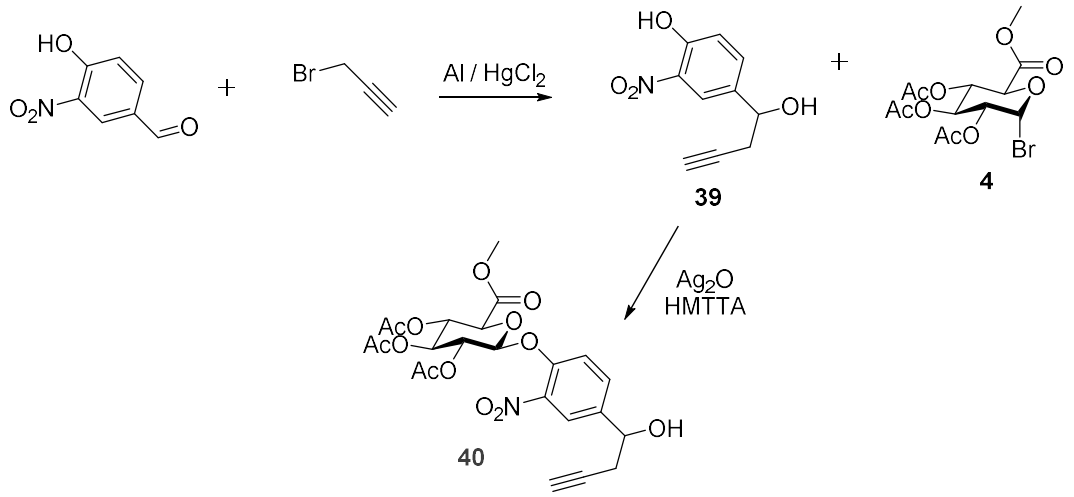
Since the carbamate formation of the benzylic alcohol group with 7-isocyanato-4-methylcoumarin **31** worked well for 7-AMC-pABn-GlcA XVII, 7-AMC was again chosen as fluorophore (Scheme C 29). The carbonate formation with 4-MU was not attempted, as there was no indication in the previous chapters that 4-MU carbamate substrates could be formed.



XX

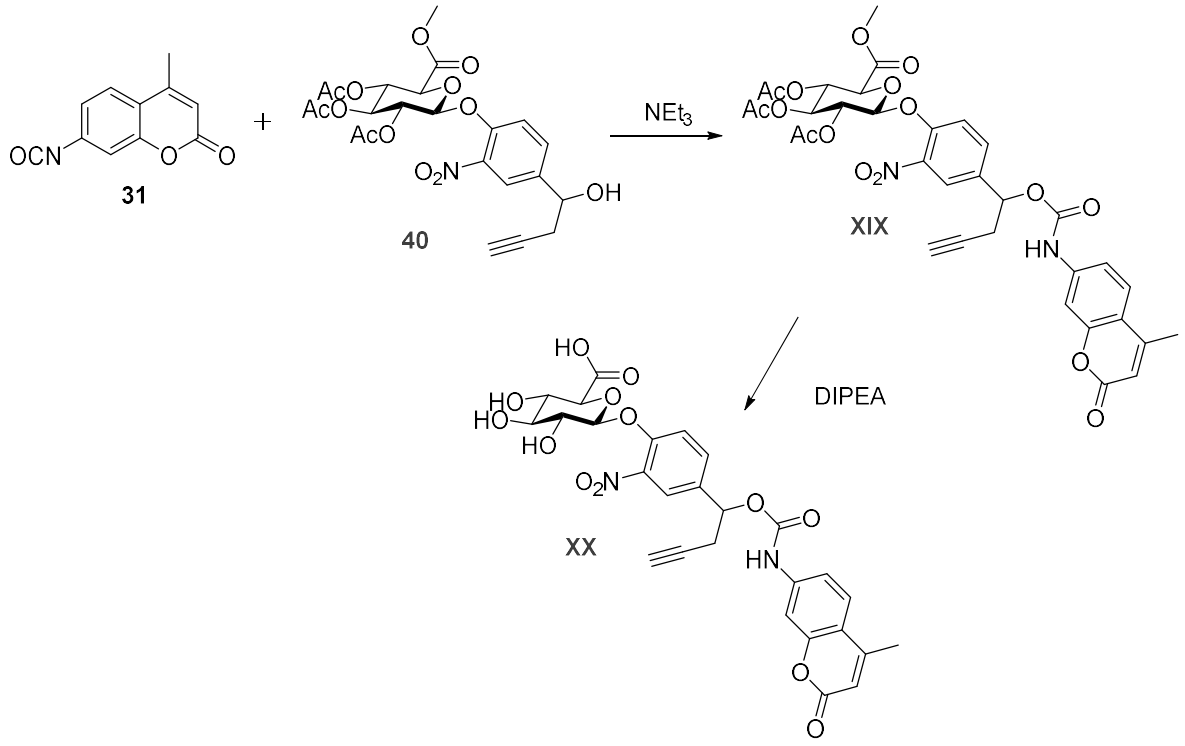
Scheme C 29 [[4-[1-[[[N-(4-methyl-2-oxo-2H-1-benzopyran-7-yl)amino]carbonyl]oxy]-3-butyn-1-yl]-2-nitrophenyl]oxy]-β-D-gluco-pyranosiduronic acid **XX** short 7-AMC-NitPh-GlcA

C.3.5.1 Synthesis of 7-AMC-NitPh-GlcA (XX)



Scheme C 30. Synthesis of [[4-(1-hydroxy-3-butyn-1-yl)-2-nitrophenyl]oxy]-2,3,4-triacetyl-β-D-glucopyranosiduronic acid, methyl ester **40**

For the synthesis of the linker **39**, 3-nitro-4-hydroxybenzaldehyde was reacted with propargyl bromide. The glucuronidation was then achieved following a modified Koenigs-Knorr procedure using 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTTA) to activate the phenol.[24]



Scheme C 31. Synthesis of 7-AMC-NitPh-GlcA **XX**

The carbamate formation was again achieved with 7-isocyanato-4-methylcoumarin **31** as described in chapter C.3.2.1, followed by deprotection with diisopropylethylamine (DIPEA) [45] to afford 7-AMC-NitPh-GlcA **XX**.

C.3.5.2 Analysis of 7-AMC-pABn-GlcA (XVII)

C.3.5.2.1 Fluorescence Properties

It was briefly mentioned before that the nitrophenol linker could quench the fluorescence of 7-AMC due to fluorescence resonance energy transfer (FRET).[69] Measurements indeed proved this FRET effect. At an excitation wavelength of 340 nm 7-AMC-NitPh-GlcA **XX** is more than ten times less fluorescent than 7-AMC-Carb-GlcA **VIII** (Figure C 58 & C 59).

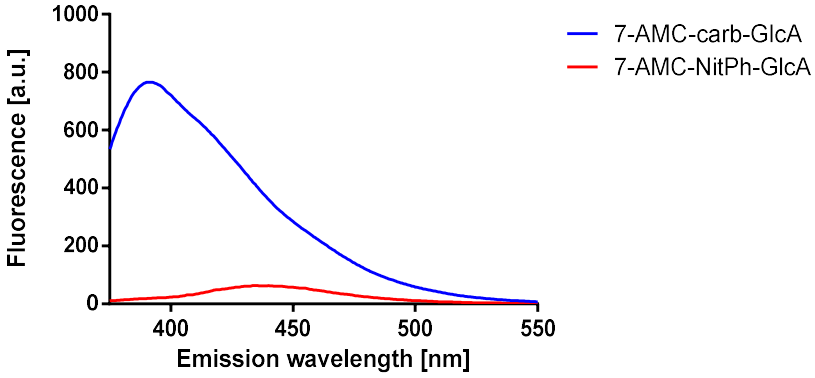


Fig. C 58. Emission scan of 7-AMC-Carb-GlcA VIII & 7-AMC-NitPh-GlcA **XX** (250 µM) at 365 nm exc. w.

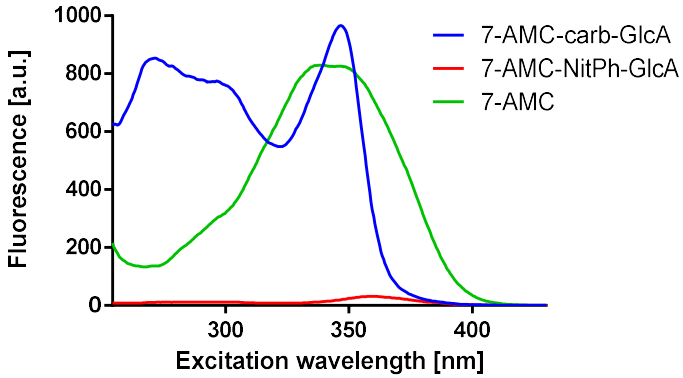


Fig. C 59. Excitation scan of 7-AMC-Carb-GlcA **VIII**, 7-AMC-NitPh-GlcA **XX** (250 µM) and 7-AMC (0.5 µM) at 445 nm em. w.

7-AMC-NitPh-GlcA **XX** has therefore at pH 6.8 with 17000 the best "Turn On" ratio measured during this thesis, that is almost three times higher than the "Turn On" ratio of 6-Cl-4-MUG (6000) (Figure C 60).

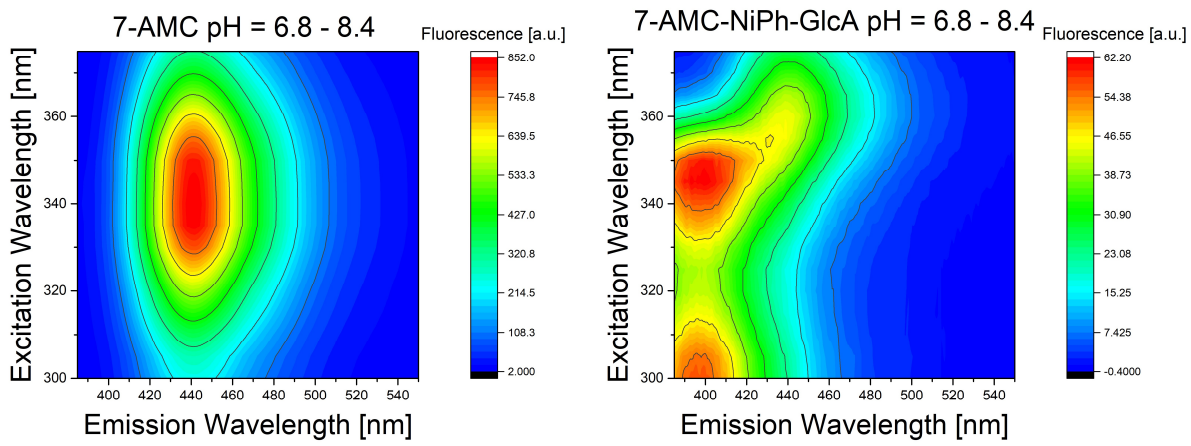


Fig. C 60. Fluorescence properties of 7-AMC & 7-AMC-NitPh-GlcA **XX** in a pH range from 6.8 – 8.4

C.3.5.2.2 Stability and Enzyme Kinetics

In terms of enzyme kinetic parameters (Figure C 61) 7-AMC-NitPh-GlcA **XX** (K_M = 0.21 mM, V_{Max} = 33.95 nM/Min) is at the same level as 7-AMC-Carb-GlcA **VIII** (K_M = 0.20 mM, V_{Max} = 34.76 nM/Min). Overall 7-AMC-NitPh-GlcA **XX** is the most promising substrate for QRTF measurements tested in this thesis. In difference to all other substrates it can still be modified at the alkyne group. Therefore, its properties can be further enhanced, and it can be used in advanced QRTF SRM measurements (chapter C.4).

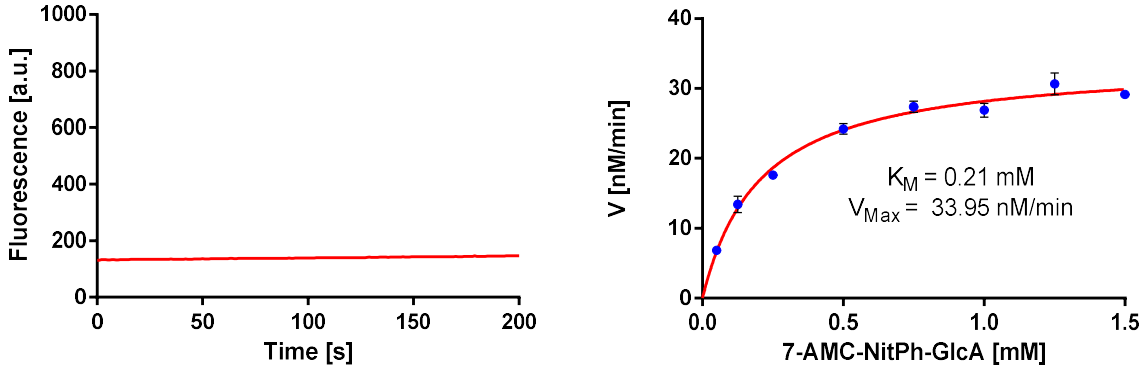


Fig. C 61. Stability tests (left) and Michaelis-Menten enzyme kinetics (right) of 7-AMC-NitPh-GlcA **XX**

Chapter 4

Quantitative Real Time Fluorescence Separated Reaction & Measurement Assay (QRTF-SRM)

C.4.1 Separation of Enzymatic Reaction & Fluorescence measurement

A major problem for all state of the art and quantitative real time fluorescence assays is that the sensitivity of the assay always relies greatly on the “Turn On” ratio from the substrate to the fluorophore. However, as shown in the previous chapters, this “Turn On” ratio cannot always be reliably predicted in substrate design. Furthermore, it is difficult to use several very potent fluorophores such as BODIPYs whose fluorescence does not change by linkage to an enzyme responsive moiety. In order to circumvent this problem a new device was designed where the enzymatic reaction and the fluorescence measurement are separated (Figure C 62) and therefore the entire palette of fluorophores can be used.

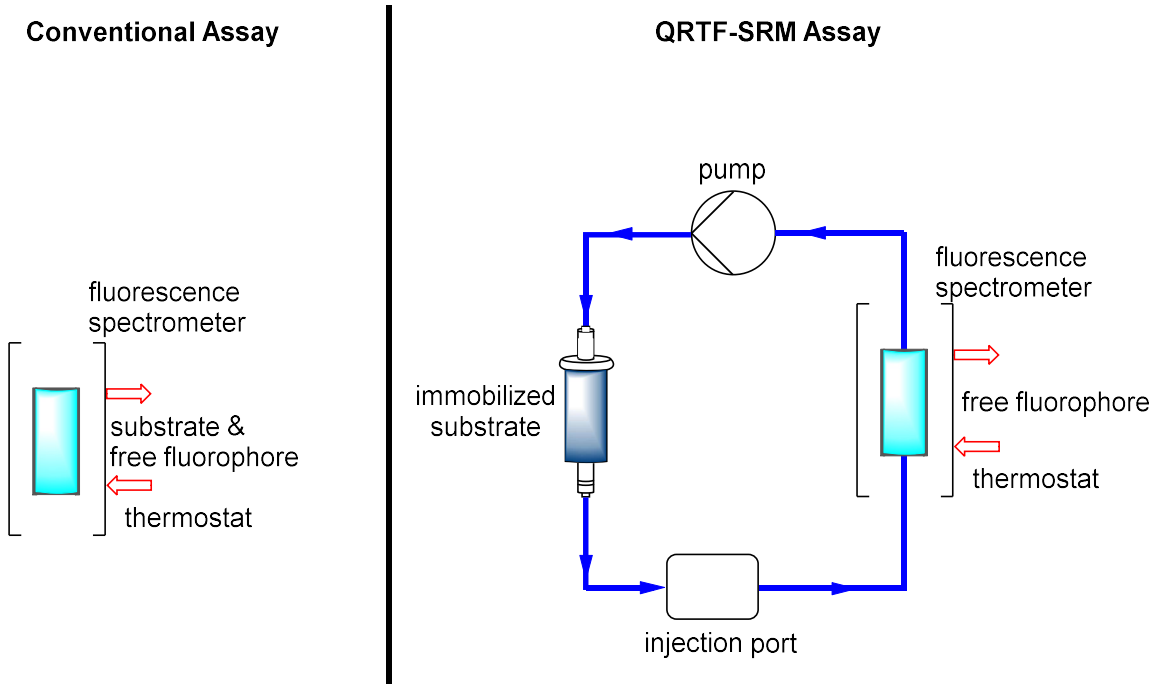
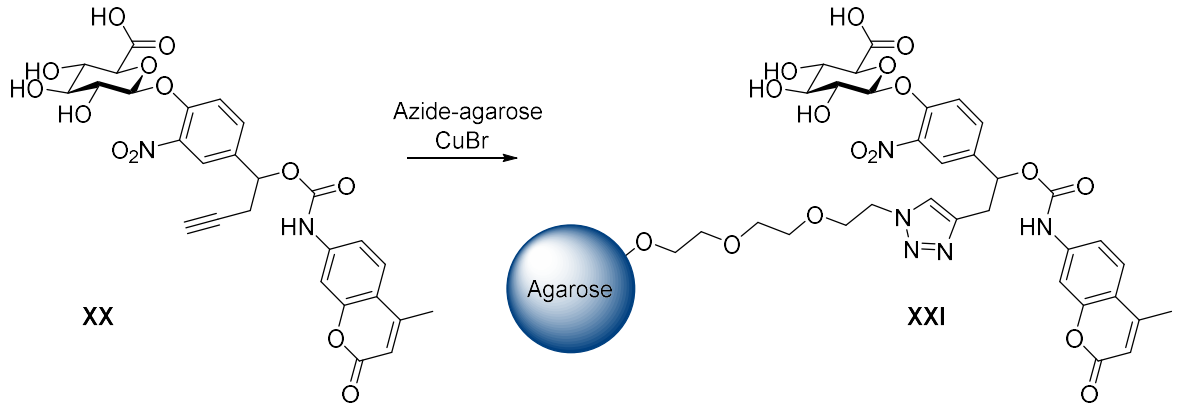


Fig. C 62. Conventional fluorescent enzyme assay versus QRTF SRM Assay

C.4.2 Substrate Immobilization

The most important step for this approach is the immobilization of the substrate on a solid phase without preventing the enzymatically cleaved fluorophore from freely moving to the measurement unit. This was achieved by binding 7-AMC-NitPh-GlcA **XX** via a copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) on azide-agarose (Scheme C 32).



Scheme C 32. Immobilization of 7-AMC-NitPh-GlcA **XX** on azide agarose.

Through the covalent bond to the agarose the substrate is completely immobilized on the column. Upon enzymatic cleavage and immolation of the linker, the fluorophore is set free and can flow with the buffer to the measurement unit (Figure C 63).

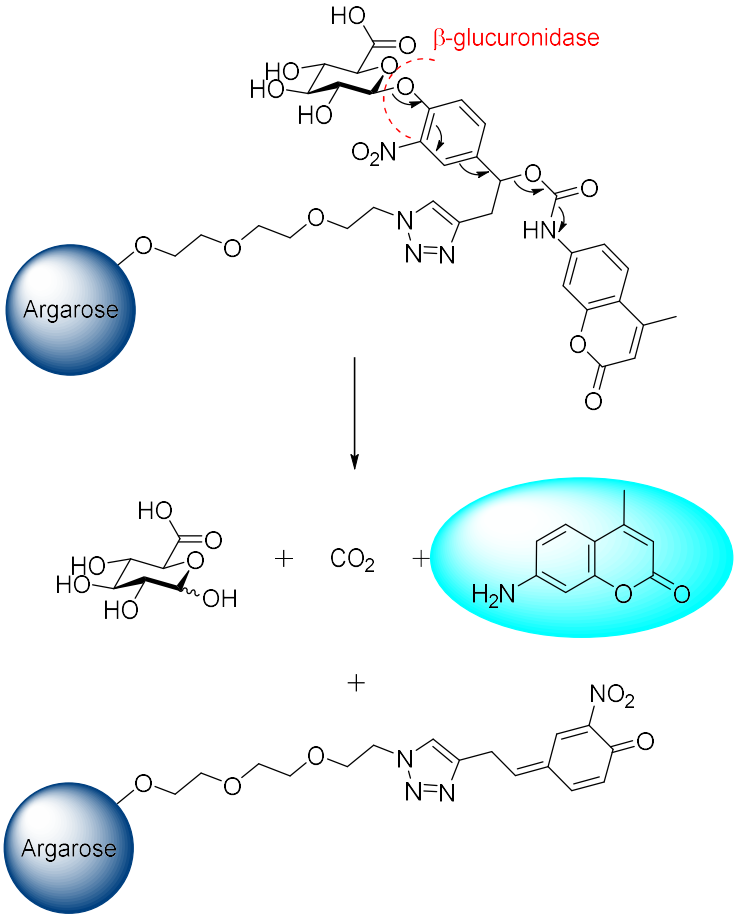


Fig. C 63. Self-immolation of the nitro phenolic linker & release of 7-AMC

Another benefit of this strategy is that the substrate is concentrated in a considerably smaller volume than in a classic enzyme assay. While the entire volume of the QRTF SRM device

(2.31 ml) is a little bit bigger than the volume of all other enzyme assays which were conducted in this thesis (2ml), the substrate is concentrated in the solid phase which has only a volume of 0.1 ml. Since the absolute substrate concentration of both assays is the same (0.5 µmol) the concentration in the solid phase is 20 times higher than in the conventional assay.
Additionally, one solid phase can be used for several enzyme assays as in a ten-minute β-glucuronidase assay only about ten ppm of the available substrate is enzymatically cleaved. In a conventional enzyme assay the excess substrate cannot be separated from the enzyme and must be discarded. The solid phase can be regenerated by elution of the enzyme and therefore be used for multiple consecutive enzyme assays.

C.4.3 QRTF SRM Device Design

The separated reaction and measurement device consists of six major parts:

1. Measurement unit
2. Column
3. Pumping system
4. Injection port
5. Thermostat
6. Tubing System

C.4.3.1 Measurement Unit, Column and Pumping System

As measurement unit a LS 55 fluorescence spectrometer from Perkin Elmer with a Perkin Elmer “semi-micro flow through cell” was used. An Ominfit® EZ glass column was chosen for the loading of the solid phase and the pumping system consisted of a peristaltic pump from VWR with Tygon® S3 tubings (i.d. 2.06 mm).

C.4.3.2 Injection Port

The most difficult part was designing a working injection port. Through this injection port the sample had to be administered without disrupting the measurement or bringing air into the system. The first approach was using a six-way valve, since HPLC systems in which these valves are primarily used have the same requirements for sample injection. The valve was equipped

with a sample loop and connected via tubings to the column and measurement unit (Figure C 64).

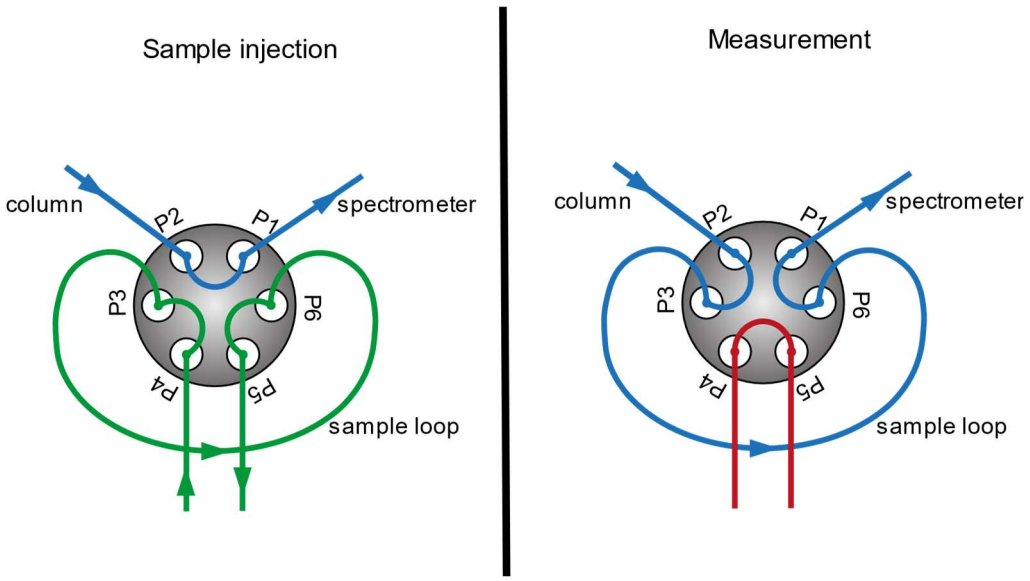
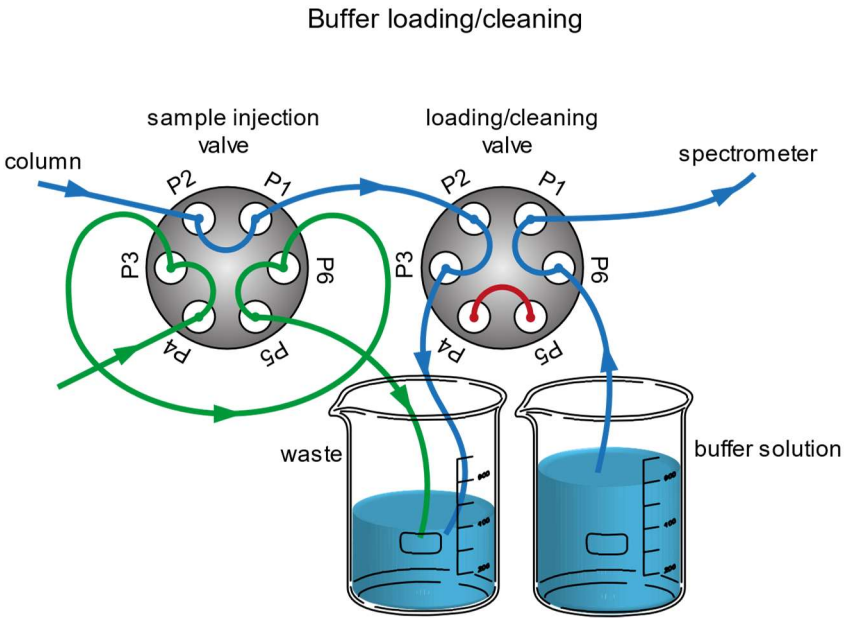


Fig. C 64. Six-way valve injection port

When the six-way valve is in injection mode the sample can be loaded while the SRM device is conditioning (left). Upon switching the valve to measurement mode, the sample is flushed into the device and the enzyme assay starts (right).

A different problem that could not be solved by this approach was the loading of buffer solution and cleaning of the system. For these steps the tubing had to be disconnected and placed in solvent reservoirs, afterwards the tubings had to be reconnected under air-exclusion. In order to make the system more reliable and easier to use, a second six-way valve was implemented in the SRM device (Figure C 65).



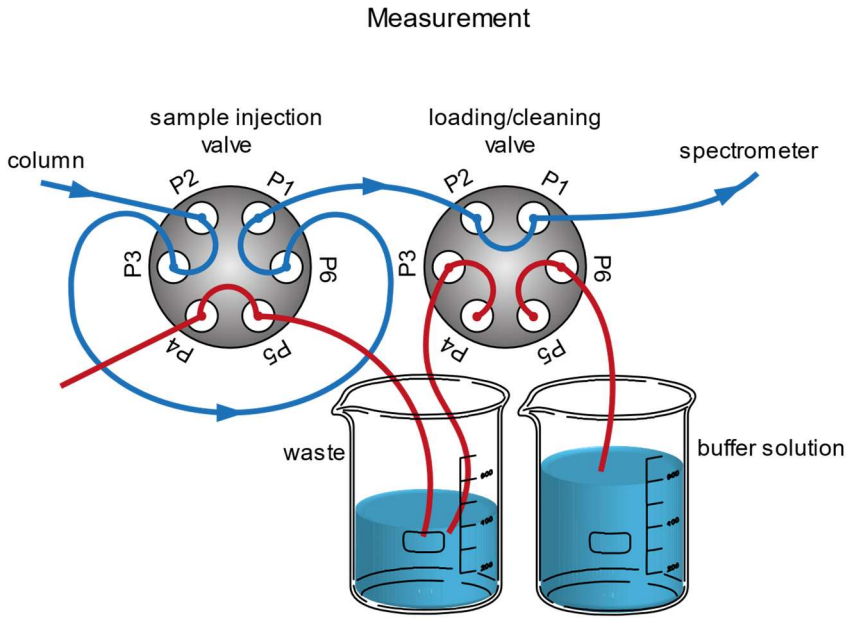


Fig. C 65. Double valve injection and solvent loading port

In loading/cleaning position the entire volume of the SRM device can be replaced under air-exclusion, when switching back in measurement position the system is again closed. Therefore, the system can be operated without dismantling it for cleaning purposes between measurements.

C.4.3.3 Thermostat and Tubing System

Since all enzyme assays were conducted at 37 °C, a thermostat system had to be used for the SRM device. As the injection port and the peristaltic pump could not be placed in a thermostat, two separate thermostats where used. In the first thermostat the column and tubing were placed, the second thermostat contained the measurement cell.

For the tubing system a total of 1.5 meters of peek tubings (o.d. 1/16 inch, i.d. 0.04 inch) with 10-32 UNF fittings was used (Figure C 66).

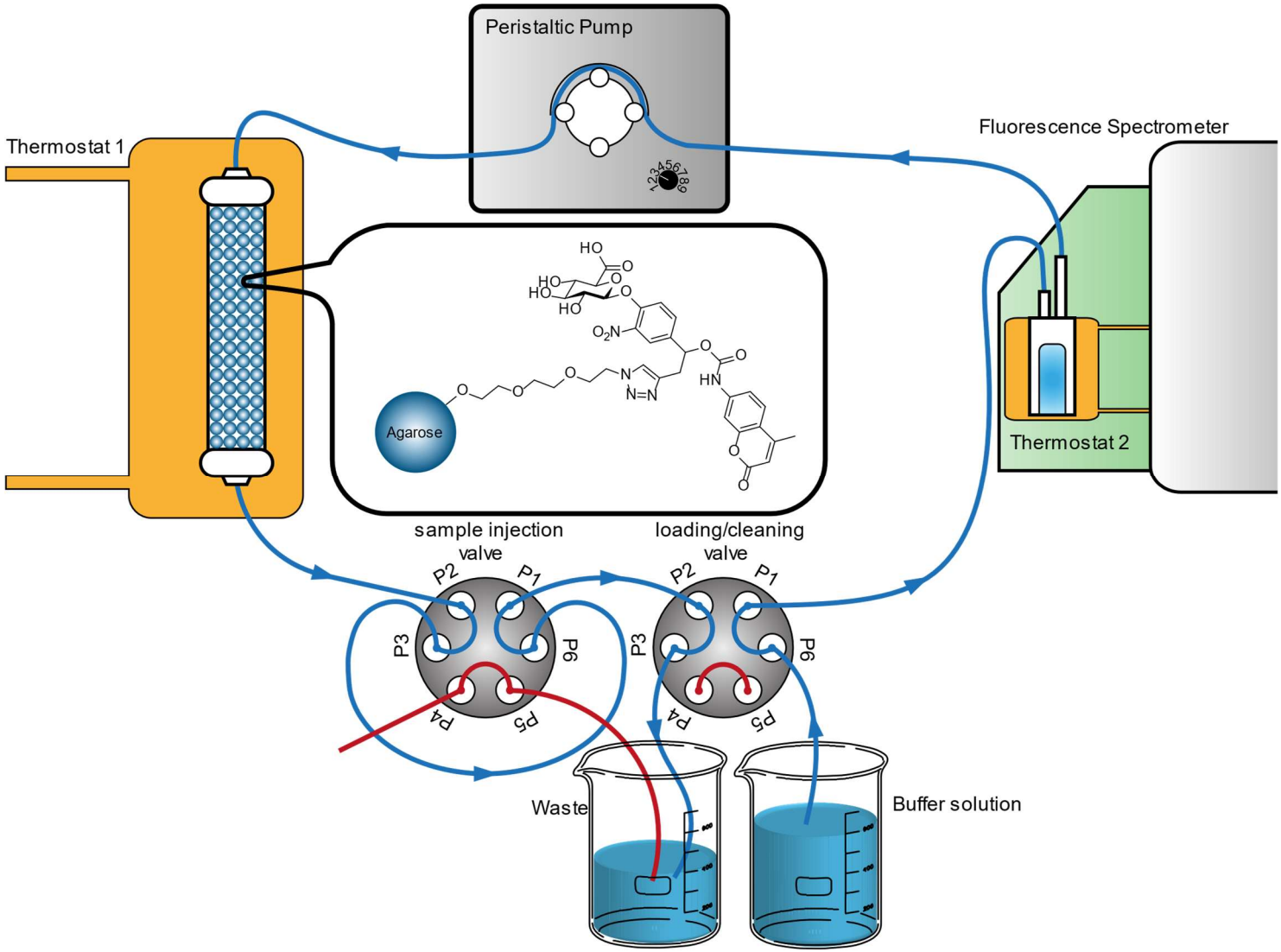


Fig. C 66. Schematic plan of the quantitative real time fluorescence separated reaction and measurement system during buffer loading.

C.4.4 QRTF-SRM System Tests

All measurements described in this chapter were conducted in a 75mM phosphate buffer at pH 6.8 and 37 °C. At first the fluorescence signal of the 3x3 mm semi-micro flow cell was compared with the signal of a standard 10x10 mm cell. As expected due to the reduced light path the fluorescence intensity in the semi-micro flow cell is almost one third lower (Figure C 67).

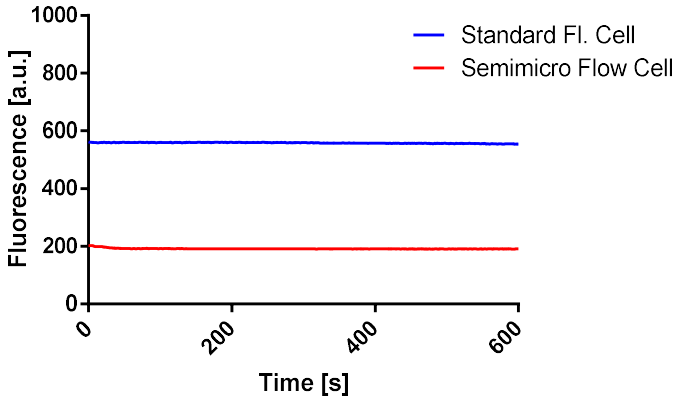


Fig. C 67. Fluorescence intensity of 1 µM 7-AMC

A different effect recognized in this first measurements was that the distribution of fluorophore in the SRM system took longer than in stirred 10x10 mm fluorescence cells (Figure C 68). This could have been avoided by addition of a mixing chamber after the sample loop, but since stabilization was only a matter of time and keeping the system volume small was more important this effect was accepted.

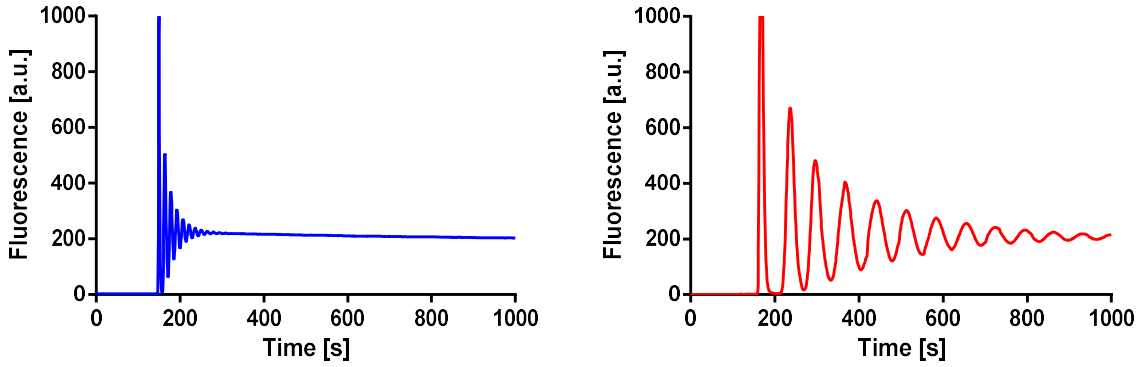


Fig. C 68. Distribution of 1 µM 7-AMC in the SRM system without agarose (left) with 0.1 ml agarose (right)

When adding 0.1 ml agarose in the column the distribution took even longer (Figure C 66). This can be explained by the longer pathway of the system when the column is filled with solid phase. Additionally, the peristaltic pump did not prove to be ideal for this system, as the solvent flow was cut in half (from 8 ml/min to 4 ml/min) after loading the system with the agarose solid phase. The next step was comparing enzyme assays of 7-AMC-NitPh-GlcA **XX** (0.25 mM) conducted without solid phase loaded in the column, with enzyme assays of 7-AMC-NitPh-GlcA **XX** (0.25 mM) with 0.1 ml of agarose as solid phase loaded in the column (Figure C 69).

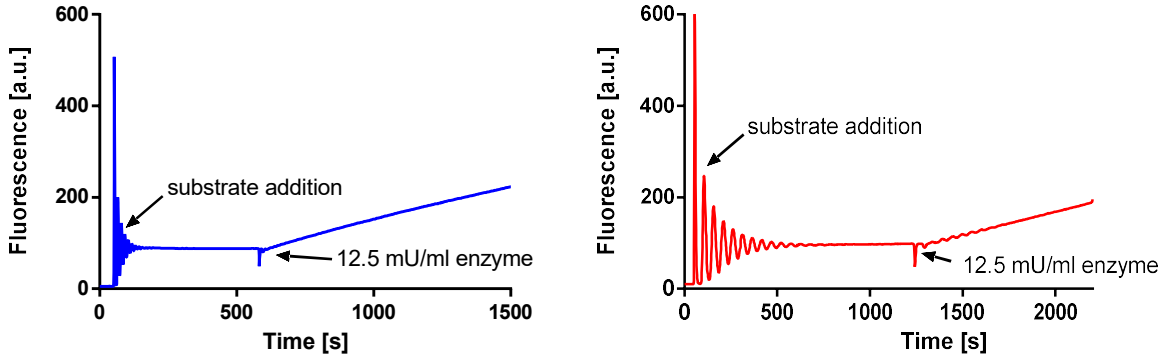


Fig. C 69. Enzyme assay of 7-AMC-NitPh-GlcA **XX** (c = 250 µM) without solid phase (left) & with agarose as solid phase (right)

In these assays the distribution also took also longer when column was filled with agarose (Figure C 69), nonetheless the slope of the enzymatic cleavage is unchanged by addition of agarose as solid phase (Figure C 70). Therefore, it can be concluded that the solid phase has no effect on enzyme kinetics and neither the enzyme nor the fluorophore interact with agarose.

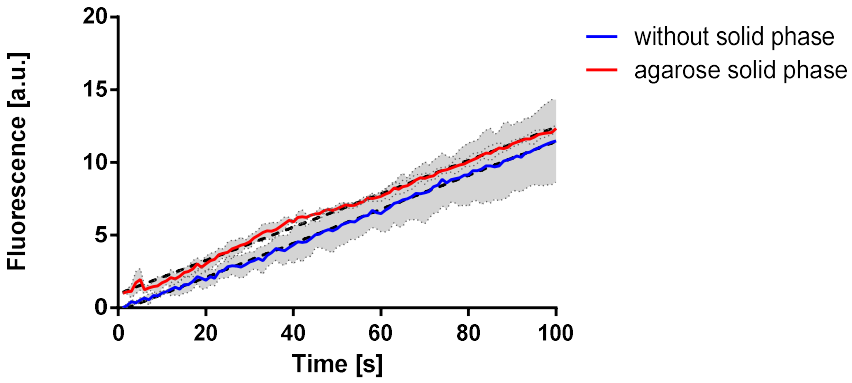


Fig. C 70. Enzymatic cleavage of 7-AMC-NitPh-GlcA **XX** without solid phase & with agarose as solid phase

C.4.5 QRTF-SRM Measurements

For the QRTF-SRM measurements 0.1 ml of agarose with 0.5 – 2 µmol immobilized substrate was used. All measurements were conducted with a 75 mM phosphate buffer at pH 6.8 and 37 °C.

First the initial fluorescence of 0.25 mM 7-AMC-NitPh-GlcA **XX** injected in the system was compared to 0.22-0.87 mM 7-AMC-NitPh-GlcA **XX** immobilized on agarose (Figure C 71).

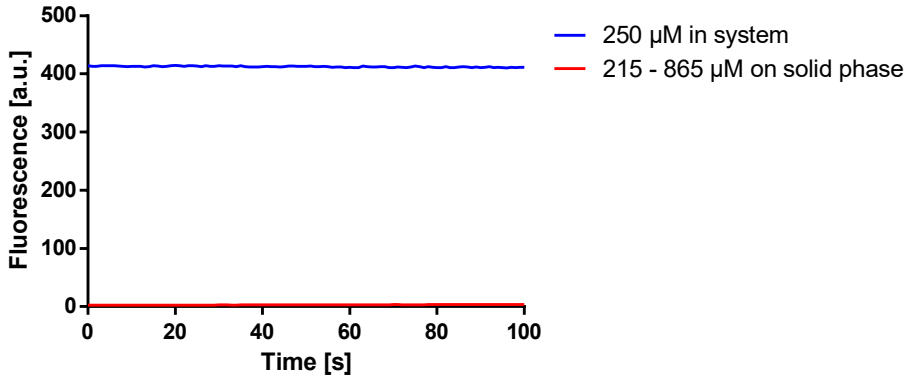


Fig. C 71. Initial fluorescence and stability of 7-AMC-NitPh-GlcA **XX** in the system (250 µM) and immobilized on agarose (215 – 865 µM)

The initial fluorescence is improved approximately 100 times by binding the substrate on the solid phase giving the SRM approach with 7-AMC-NitPh-GlcA **XX** a "Turn On" ratio of 1.5 million compared to 7-AMC. That also is a more than 1000 times higher "Turn On" ratio than commercially used 4-MUG possesses in QRTF measurements at pH 6.8 and theoretically highly improves assay sensitivity.

However, enzymatic cleavage from the solid phase caused some problems as the immobilized substrate needs a 560 times higher β-glucuronidase concentration to achieve the same enzymatic cleavage rate than the free 7-AMC-NitPh-GlcA **XX**.

To test if the enzymatic reaction takes part on the solid phase, or if only substrate that has broken off from the solid phase is available for the enzyme, further tests were conducted. First the SRM system with immobilized substrate was conditioned at enzyme assay parameters (75 mM phosphate buffer pH 6.8 and 37°C) and circulated for one hour. Then the buffer solution of the SRM system was flushed into a 10x10 mm fluorescence cell and an enzyme assay was conducted with 7 U/ml β-glucuronidase. The SRM system was then loaded with a new buffer

solution (75 mM phosphate buffer pH 6.8) and a SRM enzyme assay was conducted (Figure C 72).

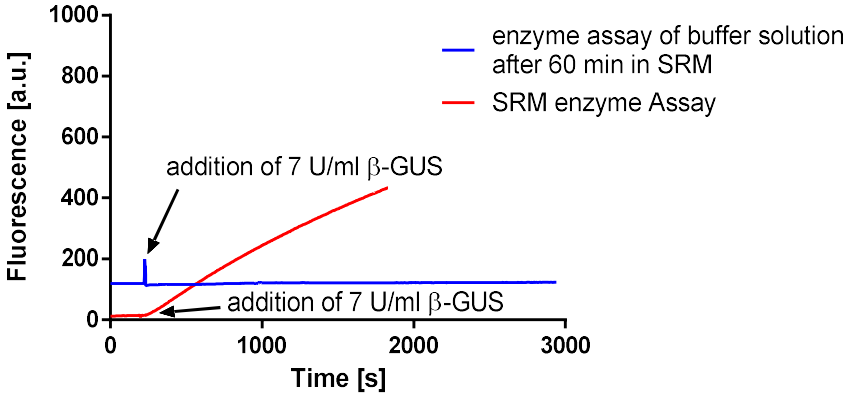


Fig. C 72. Enzyme assays of buffer solution after 60 min in SRM and SRM assay

These measurements proved that enzymatic cleavage takes place on the solid phase and no intact substrate brakes off the solid phase.

At last the reusability of the solid phase was tested, therefore multiple consecutive enzyme assays were conducted, between the assays the system was flushed for 30 min with buffer soliton (120 ml 75 mM phosphate buffer). Also, different enzyme concentrations of 7, 14 and 70 U/ml β-glucuronidase were used (Figure C 73).

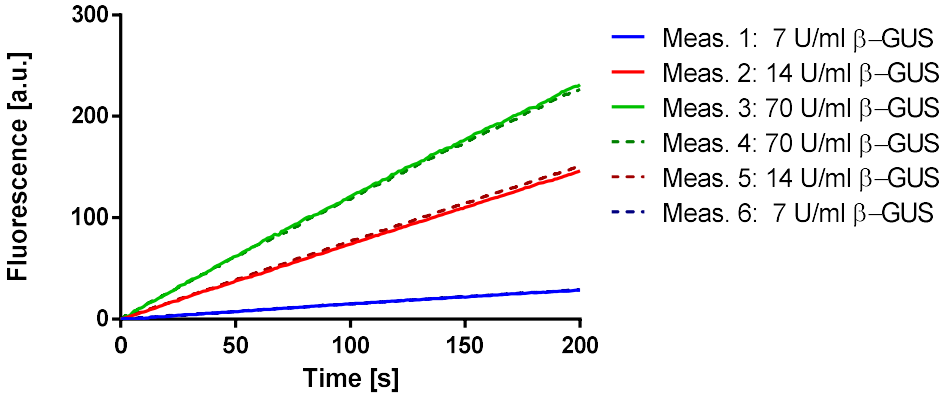


Fig. C 73. Consecutive β-glucuronidase QRTF SRM assays

These assays prove the reusability of the immobilized substrate on the solid phase as the enzymatic cleavage rate from the last measurement does not differ from the first measurement. Also, the enzymatic cleavage gets faster by the addition of more enzyme.

Overall these measurements showed a proof of concept and the potential of the QRTF SRM device, although substrate and system optimizations are required.

D) Conclusion & Outlook

Conclusion

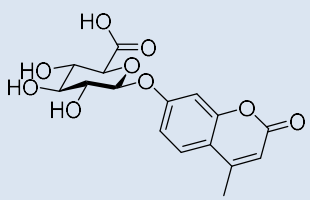
D.1.1 Synthesis and Purification

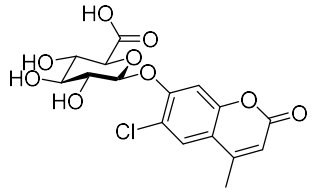
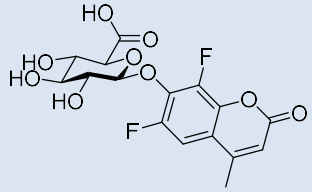
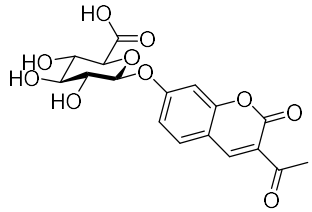
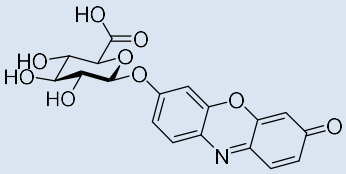
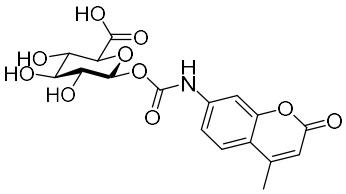
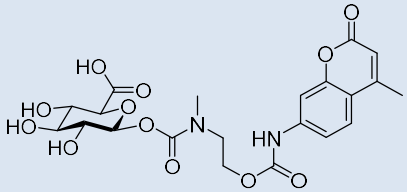
The problems of commercially available 4-methylumbelliferone glucuronide for quantitative real time fluorescence β-glucuronidase assays could be identified and solved by preparative HPLC chromatography and recrystallization. Also, synthetic pathways for fluorogenic β-glucuronidase responsive substrates were optimized and then used for the successful synthesis of three different coumarin-based and resorufin β-glucuronidase substrates.
Additionally, four different strategies were successfully developed for the implementation of self immolative linkers into novel β-glucuronidase substrates. These strategies resulted in the synthesis of a FRET based and modifiable substrate, that was applied in the first reported quantitative real time fluorescence separated reaction and measurement assay.

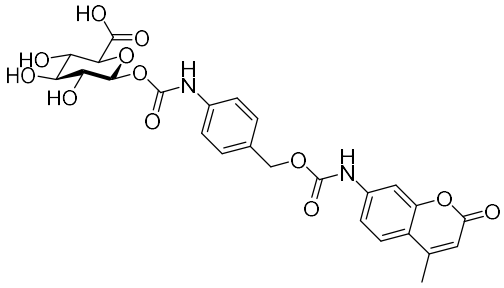
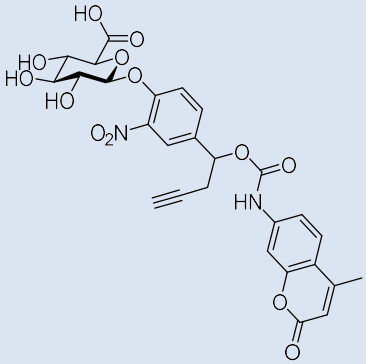
D.1.2 Fluorescence Properties and Enzyme Kinetics of the Substrates

Out of all substrates synthesized and tested in this thesis, only 7-AMC-AEt-GlcA **XIV** and 7-AMC-pABn-GlcA **XVII** are not suitable for QRTF β-glucuronidase assays. All other substrates possess comparable enzyme kinetic parameters and good fluorescence properties. Especially 7-AMC-NitPh-GlcA **XX** stands out as it has a seventeen times higher "Turn On" ratio at pH 6.8 than the commercially available 4-MUG, making it a very interesting option for the development of new QRTF assays.

Table D 1. Summarized maximal "Turn On" ratios and enzyme kinetic parameters of all tested substrates.

Substrate	max "Turn On" ratio pH			Enzyme kinetics 12.5 mU/ml β-GUS	
	6.8	7.4	8.4	K_M mM	V_{max} nM/min
4-MUG (12) 	1000	2000	4000	0.094	58.18

Substrate	max "Turn On" ratio pH			Enzyme kinetics 12.5 mU/ml β-GUS	
	6.8	7.4	8.4	K_M mM	V_{max} nM/min
6-Cl-4-MUG (25) 	6000	7000	7000	0.17	30.04
DiFMUG (24) 	1000	1000	1000	0.31	29.04
3-AcMUG (V) 	600	800	900	0.49	41.39
ReG (26) 	400	500	500	0.12	58.02
7-AMC-Carb-GlcA (VIII) 	1700	1700	1700	0.2	34.76
7-AMC-AEt-GlcA (XIV) 	1700	1700	1700	-	-

Substrate	max "Turn On" ratio pH			Enzyme kinetics 12.5 mU/ml β-GUS	
	6.8	7.4	8.4	K_M mM	V_{max} nM/min
7-AMC-pABn-GlcA (XVII) 	2000	2000	2000	-	-
7-AMC-NitPh-GlcA (XX) 	17000	17000	17000	0.21	33.95

D.1.3 Separated Reaction and Measurement

Furthermore, a device for real time separated enzymatic reaction and fluorescence measurement was designed. The proof of concept was achieved by immobilizing 7-AMC-NitPh-GlcA **XX** on azide-agarose via copper(I)-catalyzed alkyne-azide cycloaddition, followed by enzymatic cleavage directly from the solid phase. These assays also showed the potential of SRM measurements, as the initial fluorescence in the SRM device for 7-AMC-NitPh-GlcA **XX** is hundred times lower than in a classical enzyme assay setup.

Outlook

Although the proof of concept for the SRM device was achieved some optimization is still required.

The first step would be replacing the peristaltic pump with a LC or HPLC pump as these pumps are better suited for maintaining a constant flow in a system with a solid phase loaded column. Also, enzyme kinetics on the solid phase could be further improved by optimizing the linkage to the solid phase and flow optimization.

Furthermore, with the insight gained by the synthesis of the self immolative substrates, the development of new substrates containing different fluorophores for the SRM approach is possible. Additionally, the nitrophenol linker could be derivatized over the alkyne for example by adding PEG chains to improve water solubility (Figure D 1).

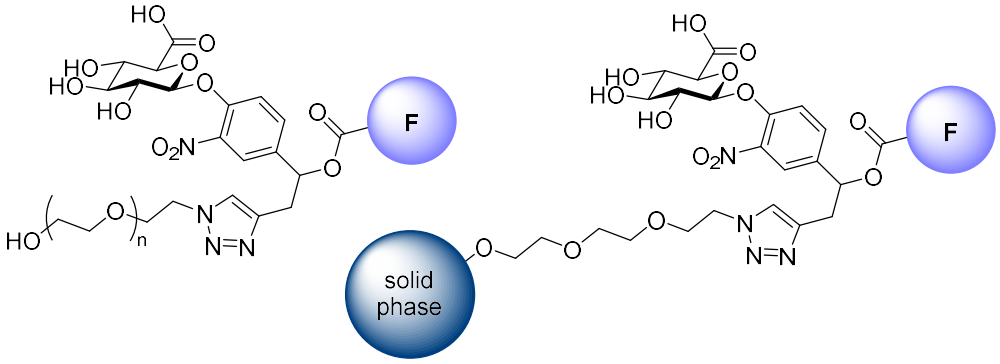


Fig. D 1. Possible further substrate modifications (F = Fluorogenic moiety or highly fluorescent fluorophore)

E) Experimental Part

Materials and Methods

E.1.1 Reactants and Solvents

Unless noted otherwise, all reagents were purchased from commercial suppliers and used without further purification. Dichloromethane, diethyl ether, methanol, tetrahydrofuran and toluene intended for water-free reactions were purified on PURESOLV-columns (Innovative Technology Inc.). Acetone (Sigma Aldrich), acetic acid (Sigma Aldrich), DMF (Sigma Aldrich) and ethanol (Merck) were used as received. All other solvents were distilled prior to use.

E.1.2 Chromatographic Methods

Thin layer chromatography was performed on TLC alumina & glass plates:

- Merck, TLC Silica gel 60 F_{254}
- Merck, HPTLC Silica gel 60 F_{254}, with concentrating zone 10 x 2.5 cm
- Merck, HPTLC RP 18, with concentrating zone 20 x 2.5 cm

Column chromatography was performed on the following systems:

- Büchi Sepacore Flash System (2 x Büchi Pump Module C-605, Büchi Pump Manager C-615, Büchi UV Photometer C-635, Büchi Fraction Collector C-660)
- Shimadzu Prep HPLC System (2 x Shimadzu LC–8A Pump Module, Shimadzu CBM-20A HPLC System Controller, Shimadzu SPD-20A Prominence HPLC UV-Vis Detector, Shimadzu FRC-10A Fraction Collector)
- Grace (now Büchi) Reveleris ® PREP System

Unless noted otherwise silica gel from Merck (40-63 µm) and distilled or HPLC grade solvents were used.

HPLC experiments were conducted on the following systems:

- Thermo Finnigan Surveyor System (Thermo Finnigan Surveyor LC Pump Manager Plus, Thermo Finnigan Surveyor Autosampler Plus, Thermo Finnigan Surveyor PDA Plus)
- Agilent 1XXX Series System with Bruker ion trap mass spectrometer (Agilent 1200 Series G1367B HiP ALS Autosampler, Agilent 1100 Series G1311A Quat Pump, Agilent 1100 Series G1379A Degasser, Agilent 1200 Series G1316B TCCSL, Agilent 1260 Infinity G1315D DAD, Bruker Esquire HCT Ion Trap MS)

- Shimadzu Nexera UHPLC/MS System (2x Nexera LC-30AD Solvent Delivery Unit, Shimadzu SIL-30AC UHPLC Autosampler, Shimadzu DGU-20A3R/DGU-20A5R HPLC Degassing Unit, Shimadzu CBM-20A HPLC System Controller, Shimadzu CTO-20AC Prominence HPLC Column Oven, Shimadzu SPD-20A Prominence HPLC UV-Vis Detector, Shimadzu RF-20Axs Fluorescence Detector, Shimadzu LCMS-2020 Single Quadrupole Liquid Chromatograph Mass Spectrometer (LC/MS))

GC/MS experiments were run on the following systems:

- Thermo Finnigan GC 8000 Top gas chromatograph on a BGB5 column (l=30 m, d_i=0,32 mm, 1 µm coating thickness) coupled to a Voyager Quadrupol mass spectrometer (electron ionization).
- Thermo TRACE 1300 gas chromatograph also on a BGB5 column (l=30 m, d_i=0,32 mm, 1 µm coating thickness) coupled to a Thermo ISQ LT Single Quadrupole mass spectrometer (electron ionization).

E.1.3 High Resolution Mass Spectrometry

HRMS experiments were performed on the following systems:

- LCMS-IT-TOF Mass Spectrometer (2x Prominence LC-20AD Solvent Delivery Unit, Shimadzu SIL-20AC HPLC Autosampler, Shimadzu DGU-20A3R/DGU-20A5R HPLC Degassing Unit, Shimadzu CBM-20A HPLC System Controller, Shimadzu CTO-20AC Prominence HPLC Column Oven, Shimadzu SPD-20A Prominence HPLC UV-Vis Detector, Shimadzu LCMS-IT-TOF Mass Spectrometer).
- Focus hybrid quadrupole-orbitrap (Thermo Ultimate 3000 system, CTC PAL autosampler, Thermo Q Exactive Focus).

E.1.4 NMR Spectroscopy

NMR spectra were recorded on a Bruker AC 200 (200 MHz), Bruker Avance UltraShield 400 (400 MHz) and an Bruker Avance III HD 600 (600 MHz) spectrometer. Chemical shifts are reported in ppm using tetramethylsilane as internal standard. Calibration was performed *via* solvent residual peaks. The following abbreviations were used: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, br s = broad signal.

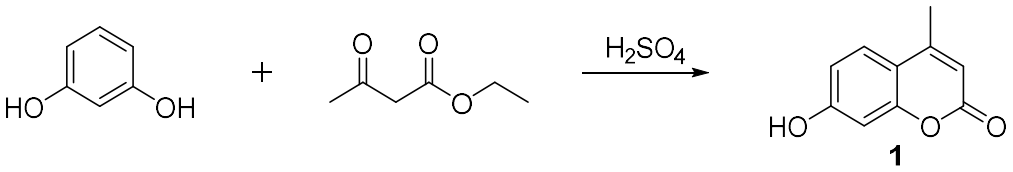
E.1.5 Spectrometers

For QRTF assays of chapter C.1 a Colimindер® of VWM was used. All other fluorescence measurements where conducted on a LS-55 spectrometer from Perkin Elmer. Absorption measurements were conducted on a Nanodrop One© from ThermoFischer Scientific.

Synthesis of 4-Methylumbelliferyl-β-D-glucuronide

E.1.6 4-Methylumbelliferone (1)

E.1.6.1 Method A

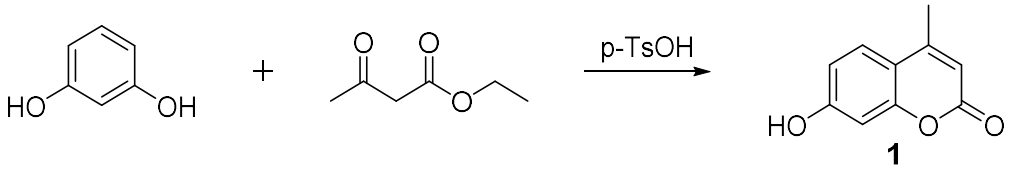


Molecular Weight: 110.11 Molecular Weight: 130.14 Molecular Weight: 176.17

Procedure according to Koenigs *et al.*[32] Resorcinol (2.20 g, 20 mmol, 1.0 eq), ethyl acetoacetate (3.90 g, 30 mmol, 1.5 eq) and the sulfuric acid (10 ml, 70% in water) were stirred at room temperature for 24 hours. Afterwards the solution was poured into ice water (50 ml), the yellowish solid was filtrated and washed with cold water. The solid was dissolved in 2 M NaOH at 80 °C and then precipitated again by addition of concentrated HCl. After cooling to 4°C the product was filtrated and washed with cold water and EtOAc. After drying, compound **1** was obtained as off white solid.

Yield = 2.45g (70 % of theory).

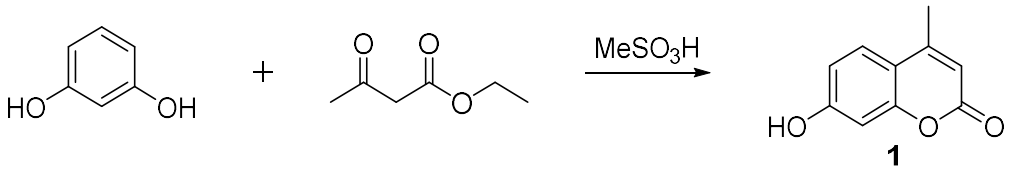
E.1.6.2 Method B



Molecular Weight: 110.11 Molecular Weight: 130.14 Molecular Weight: 176.17

Procedure according to Kumar *et al.*[35] Resorcinol (1.10 g, 10 mmol, 1.0 eq), ethyl acetoacetate (1.43 g, 11mmol, 1.1 eq) and p-TsOH (0.38 g, 2 mmol) were solved in dry toluene (80 ml) and then refluxed with a water separator for 3 hours. Afterwards the reaction was diluted with EtOAc (200 ml) and washed 2 times with saturated $NaHCO_3$ solution. The aqueous phase was extracted with EtOAc two times and the combined organic phases were dried over Na_2SO_4. The crude product was purified via flash chromatography (PE/EtOAc = 5:1 - 2:1). After evaporating the solvents under reduced pressure, compound **1** was obtained as white solid.
Yield =1.16 g (66 % of theory)

E.1.6.3 Method C

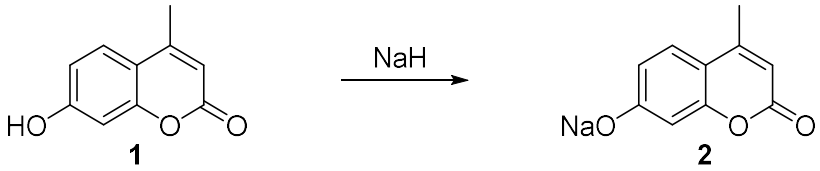


Molecular Weight: 110.11 Molecular Weight: 130.14 Molecular Weight: 176.17

Procedure according to Hedberg *et al.*[36] Resorcinol (1.10 g, 10 mmol, 1.0 eq) and ethyl acetoacetate (1.43 g, 11 mmol, 1.1 eq) were placed in a vial and cooled to 0°C. $MeSO_3H$ (16.5 ml, 251 mmol, 25.1 eq) was added dropwise while keeping the temperature of the reaction at 0°C. The reaction was stirred for another 30 minutes at 0°C and then for 24 hours at room temperature. Afterwards the reaction was again cooled to 0°C and quenched with cold water (10 ml). The precipitate was collected, washed with cold water (3x5 ml) and cold Et_2O (2x5 ml) and then dried under reduced pressure to give compound **1** as white solid.
Yield =1.16 g (90 % of theory)

HR-ESI-IT-TOF $[M+H]^+$ m/z calcd. 177.0546 for $C_{10}H_9O_3^+$, found 177.0543.
^1H NMR (200 MHz, $CDCl_3$): δ = 10.51 (s, 1H), 7.58 (d, J=8.61 Hz, 1H), 6.79 (dd, J=8.80, 2.35 Hz, 1H), 6.70 (d, J=2.35 Hz, 1H), 6.11 (s, 1 H), 2.35 (s, 3H) ppm
^{13}C NMR (50 MHz, $CDCl_3$): δ = 161.56 (s, 1C), 160.70 (s, 1C), 155.25 (s, 1C), 153.94 (s, 1C), 127.02 (d, 1C), 113.26 (d, 1C), 112.43 (s, 1C), 110.67 (d, 1C), 102.59 (d, 1C), 18.52 (q, 1C) ppm

E.1.7 Synthesis of 4-Methylumbelliferone sodium salt (2)



Molecular Weight: 176.17 Molecular Weight: 198.15

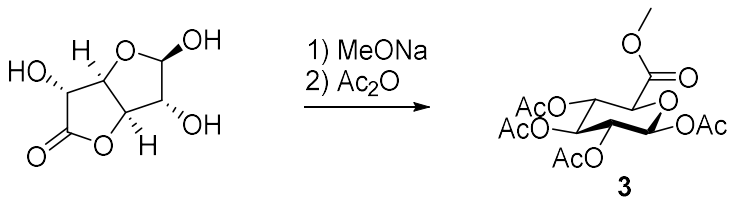
Procedure referred to Schwanenberg *et al.*[37] 4-Methylumbelliferone **1** (1.00 g, 5.7 mmol, 1.0 eq) was dissolved in THF and pure NaH (136 mg, 5.7 mmol, 1 eq) was slowly added. After stirring for one hour the solvent was removed under reduced pressure, leaving the desired compound **2** as a yellow solid.

Yield =1.02 g (90 % of theory)

^{1}H NMR (200 MHz, DMSO-d_6): δ = 7.08 (d, J=8.94 Hz, 1H), 6.05 (dd, J=8.91, 2.11 Hz, 1H), 5.79 (d, J=2.22 Hz, 1H), 5.36 (s, 1H), 2.17 (s, 3H) ppm

^{13}C NMR (50 MHz, DMSO-d_6): δ = 174.76 (s, 1C), 159.79 (s, 1C), 155.39 (s, 1C), 151.07 (s, 1C), 122.52 (d, 1C), 116.82 (d, 1C), 100.95 (s, 1C), 100.65 (d, 1C), 97.99 (d, C), 15.70 (q, 1C) ppm

E.1.8 1,2,3,4-Tetraacetyl-β-D-glucopyranuronic acid, methyl ester (3)



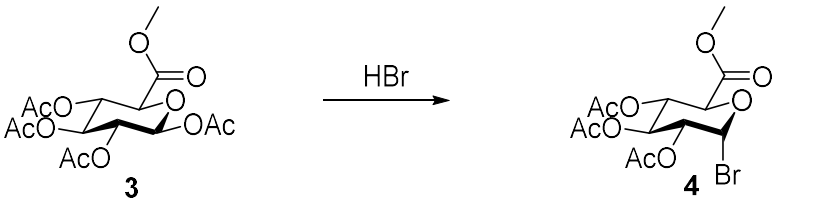
Molecular Weight: 176.12 Molecular Weight: 376.31

Procedure according to Bollenback *et al.*[70] (+)-D-Glucofuranurono-6,3-lacton (50 g, 284 mmol, 1 eq) and sodium methoxide (285 mg, 5.3 mmol, 0.02 eq) were suspended in methanol (300 ml) and the reaction was stirred at room temperature overnight. Then the solvent was removed under reduced pressure and the residue was solved in Ac_2O (400ml) while stirring for 16 hours. Afterwards the solution was cooled to 0°C and perchloric acid (3 ml in 100ml

Ac_2O) was slowly added. After stirring for 2 hours the mixture was put in the freezer (-20°C) for 16 hours, then the residue was filtered off and washed with Et_2O to give the pure β product **3** as white solid. Yield = 43 g β anomer (40 % of theory) and 27 g mix of lactone and both anomers

^{1}H NMR (200 MHz, $CDCl_3$): δ = 5.74 (d, J = 7.6 Hz, 1H), 5.36 - 5.04 (m, 3H), 4.16 (d, J = 9.4 Hz, 1H), 3.71 (s, 3H), 2.09 (s, 3H), 2.01 (s, 6H), 2.00 (s, 3H) ppm
^{13}C NMR (50 MHz, $CDCl_3$): δ = 170.0 (s, 1C), 169.6 (s, 1C), 169.3 (s, 1C), 169.0 (s, 1C), 166.9 (s, 1C), 91.5 (d, 1C), 73.1 (d, 1C), 71.9 (d, 1C), 70.3 (d, 1C), 69.0 (d, 1C), 53.2 (q, 1C), 20.9 (q, 1C), 20.71 (q, 1C), 20.68 (q, 1C), 20.6 (q, 1C) ppm

E.1.9 1-Bromo-1-deoxy-2,3,4-triacetyl-α-D-glucopyranuronic acid, methyl ester (4)



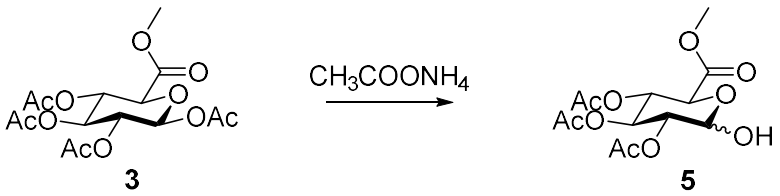
3 Molecular Weight: 376.31 — **4** Molecular Weight: 397.17

Procedure according to Bollenback *et al.*[70] Compound **3** (6 g, 16 mmol, 1 eq) was suspended in HBr (30% in acetic acid) (30 ml) and stirred at room temperature until the reaction was complete (TLC control). The solvent was evaporated, and the residue solved in $CHCl_3$ (250 ml). The reaction was washed with $NaHCO_3$ and water, the organic phase was dried over Na_2SO_4 and the solvent evaporated under reduced pressure. The crude product was recrystallized from EtOH (15 ml) and dried in vacuum to give compound **4** as a white solid.
Yield = 4.71 g (74 % of theory)

^{1}H NMR (200 MHz, $CDCl_3$): δ = 6.62 (d, J = 4.1 Hz, 1H), 5.58 (t, J = 9.8 Hz, 1H), 5.21 (t, J = 9.9 Hz, 1H), 4.83 (dd, J = 10.0, 4.1 Hz, 1H), 4.55 (d, J = 10.0 Hz, 1H), 3.73 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H) ppm

^{13}C NMR (50 MHz, $CDCl_3$): δ = 169.80 (s, 1C), 169.78 (s, 1C), 169.6 (s, 1C), 166.8 (s, 1C), 85.5 (d, 1C), 72.2 (d, 1C), 70.4 (d, 1C), 69.4 (d, 1C), 68.6 (d, 1C), 53.3 (q, 1C), 20.7 (q, 2C), 20.6 (q, 1C) ppm

E.1.10 2,3,4-Triacetyl-glucopyranuronic acid, methyl ester (5)



3 Molecular Weight: 376.31 **5** Molecular Weight: 334.28

Procedure according to Chittaboina *et al.*[39] Compound **3** (10 g, 26.6 mmol, 1 eq) was solved in DMF (30 ml) and ammonium acetate (4.1 g, 53.2 mmol, 2 eq) was added. After stirring for 24 h the reaction was diluted with EtOAc and washed with water. The aqueous phase was extracted two times with EtOAc, the combined organic phases where washed with $NaHCO_3$ solution and brine. Afterwards the solvent was dried over $NaSO_4$ and removed under reduced pressure. The crude product was purified via flash chromatography (PE/EtOAc = 2:1 - 1:2) to give compound **5** as colorless oil.

Yield = 7.39 g (83 % of theory)

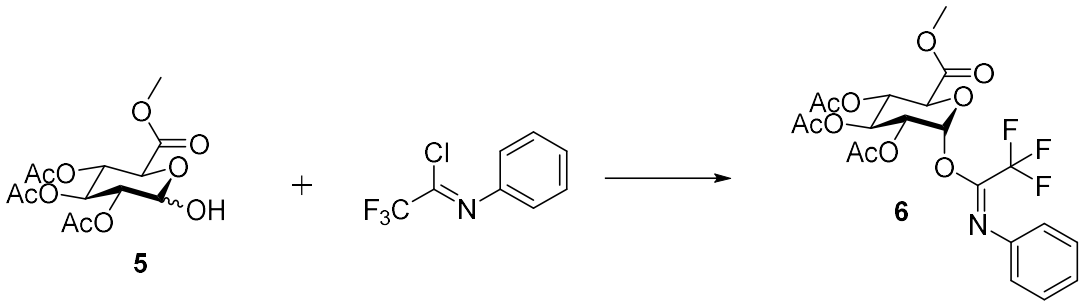
^{1}H NMR (400 MHz, $CDCl_3$): **α-5**: δ = 5.56 (t, J = 9.8 Hz, 1H), 5.53 (d, J = 3.5 Hz, 1H), 5.17 (t, J = 9.7 Hz, 1H), 4.89 (dd, J = 10.1, 3.7 Hz, 1H), 4.58 (d, J = 10.2 Hz, 1H), 3.73 (s, 3H), 2.07 (s, 3H), 2.023 (s, 3H), 2.018 (s, 3H) ppm

β-5: δ = 5.29 (t, J = 9.4 Hz, 1H), 5.21 (t, J = 9.5 Hz, 1H), 4.91 (t, J = 8.3 Hz, 1H), 4.80 (d, J = 7.9 Hz, 1H), 4.10 (d, J = 9.7 Hz, 1H), 3.74 (s, 3H), 2.07 (s, 3H), 2.023 (s, 3H), 2.018 (s, 3H) ppm

^{13}C NMR (100 MHz, $CDCl_3$): **α-5**: δ = 170.4 (s, 1C), 170.2 (s, 1C), 169.9 (s, 1C), 168.6 (s, 1C), 90.4 (d, 1C), 70.9 (d, 1C), 69.7 (d, 1C), 69.2 (d, 1C), 68.2 (d, 1C), 53.1 (q, 1C), 20.9 (q, 2C), 20.7 (q, 1C) ppm

β-5: δ = 170.8 (s, 1C), 170.2 (s, 1C), 169.8 (s, 1C), 167.8 (s, 1C), 95.7 (d, 1C), 73.1 (s, 1C), 72.8 (d, 1C), 71.7 (d, 1C), 69.6 (d, 1C), 53.2 (q, 1C), 20.9 (q, 1C), 20.8 (q, 1C), 20.7 (q, 1C) ppm

E.1.11 2,3,4-Triacetyl-1-(2,2,2-trifluoro-N-phenylethanimidyl)-α-D-glucopyranuronic acid, methyl ester (6)

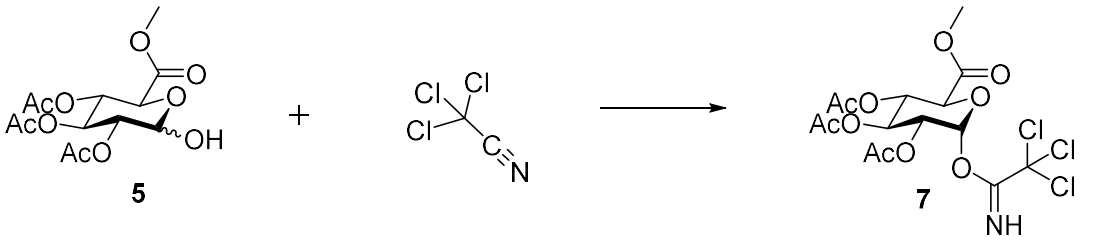


Molecular Weight: 334.28 | Molecular Weight: 207.58 | Molecular Weight: 505.40

Procedure according to Nakajima *et al.*[40] Compound **5** (3.5 g, 10 mmol, 1 eq) was solved in dichloromethane (50 ml), K_2CO_3 (2.76 g, 20 mmol, 2 eq) and N-phenyltrifluoroacetimidoyl chloride (4.35 g, 20 mmol, 2 eq) were added. The reaction was stirred at room temperature for one hour, then it was filtrated, and the solvent evaporated. The crude product was purified via flash chromatography (PE/EtOAc = 5:1 – 3:1) to give compound **6** as a white solid.
Yield = 4.81 g (91 % of theory)

^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.24 (t, J = 6.0 Hz, 2 H), 7.07 (d, J = 6.6 Hz, 1 H), 6.78, 6.72 (2 d, J = 7.4 Hz, 2 H), 6.57, 5.88 (2 br s, 1 H), 5.54, 5.25-5.10 (t + m, J = 9.5 Hz, 3 H), 4.40, 4.08 (d + br s, J = 9.6 Hz, 1 H), 3.69 (s, 3 H), 2.00, 1.99, 1.97 (3 s, 9 H) ppm
^{13}C NMR (50 MHz, $CDCl_3$): δ = 169.9 (s, 1C), 169.3 (s, 1C), 169.0 (s, 1C), 166.9 (s, 1C), 142.8 (s, 1C), 128.8 (d, 2C) , 124.7 (d, 1C), 119.2 (d, 2C), 94.0 (d, 1C), 73.0 (d, 1C), 71.0 (d, 1C) , 70.0 (d, 1C), 68.4 (d, 1C), 53.0 (q, 1C), 20.6 (q, 1C), 20.5 (q, 1C), 20.4 (q, 1C) ppm

E.1.12 2,3,4-Triacetyl-1-(2,2,2-trichloroethanimidyl)-α-D-glucopyranuronic acid, methyl ester (7)



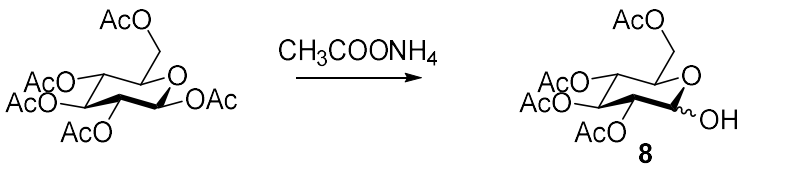
Molecular Weight: 334.28 Molecular Weight: 144.38 Molecular Weight: 478.66

Procedure according to Nakajima *et al.*[40] Compound **5** (1.5 g, 4.5 mmol, 1 eq), K_2CO_3 (0.94 g, 6.8 mmol, 1.5 eq) and molecular sieve (MS 3 Å, 0.5 g) were suspended in dichloromethane (10 ml) and stirred at 0°C for 30 min. Trichloroacetonitrile (1.95 g, 13.5 mmol, 3 eq.) solved in 5 ml dichloromethane was added and the reaction was stirred for another 5 hours at 0°C. Afterwards the mixture was filtered and the filtrate was washed with sodium pyrosulfite solution (2 x 25 ml, 5% in water). The organic solvents were evaporated and the crude product was purified via flash chromatography (PE/EtOAc = 5:1).

^{1}H NMR (400 MHz, acetone-d_6): δ = 9.59 (s, 1H), 6.63 (d, J = 3.5 Hz, 1H), 5.62 (t, J = 9.9 Hz, 1H), 5.26 (t, J = 9.8 Hz, 1H), 5.20 (dd, J = 10.1, 3.7 Hz, 1H), 4.48 (d, J = 10.2 Hz, 1H), 3.70 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H) ppm

^{13}C NMR (100 MHz, acetone-d_6): δ = 170.4 (s, 1C), 170.2 (s, 1C), 170.0 (s, 1C), 167.8 (s, 1C), 160.3 (s, 1C), 93.5 (d, 1C), 87.7 (s, 1C), 71.5 (d, 1C), 70.3 (d, 1C), 69.9 (d, 1C), 69.6 (d, 1C), 53.2 (q, 1C), 20.6 (q, 1C), 20.5 (q, 1C), 20.4 (q, 1C) ppm

E.1.13 2,3,4,6-Tetraacetyl-D-glucopyranose (8)



Molecular Weight: 390.34 Molecular Weight: 348.30

Procedure according to Chittaboina *et al.*[39] β-D-Glucopyranose, 1,2,3,4,6-pentaacetate (5 g, 13 mmol, 1 eq) and ammonium acetate (1.2 g, 15.5 mmol, 1.2 eq) were solved in DMF (15 ml) and stirred for 16 hours. Afterwards the reaction was diluted with EtOAc and washed with water. The aqueous phase was extracted two times with EtOAc, the combined organic phases where washed with $NaHCO_3$ solution and brine. The solvent was dried over $NaSO_4$ and removed under reduced pressure. The crude product was purified via flash chromatography (PE/EtOAc = 2:1 - 1:2) to give compound **8** as colorless oil.

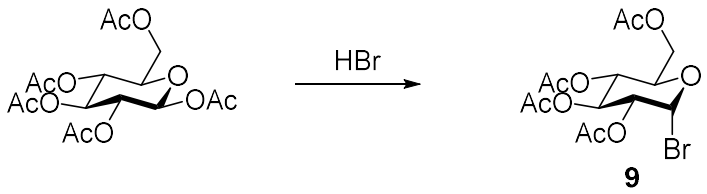
Yield = 4.00 g (89 % of theory)

^{1}H NMR (200 MHz, $CDCl_3$): δ = 5.53 (t, J = 9.9 Hz, 1H), 5.45 (d, J = 3.7 Hz, 1H-α), 5.07 (t, J = 9.78, 1H), 4.89 (dd, J = 10.3, 3.6, 1H), 4.74 (d, J = 8.02, 1H-ß), 4.33 - 4.02 (m, 3H), 3.57 (br s, 1H), 2.08 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H) ppm

^{13}C NMR (50 MHz, $CDCl_3$): **α-8**: δ = 171.03 (s, 1C), 170.36 (s, 2C), 169.81 (s, 1C), 90.08 (d, 1C), 71.22 (d, 1C), 70.0 (d, 1C), 68.59 (d, 1C), 67.08 (d, 1C), 62.06 (t, 1C), 20.77 (q, 2C), 20.68 (q, 1C), 20.64 (q, 1C) ppm

ß-8: δ = 171.52 (s, 1C), 170.94 (s, 1C), 170.67 (s, 1C), 169.64 (s, 1C), 95.47 (d, 1C), 73.11 (d, 1C), 72.47 (d, 1C), 72.01 (d, 1C), 68.45 (d, 1C), 61.69 (t, 1C), 21.10 (q, 2C), 21.00 (q, 1C), 20.88 (q, 1C) ppm

E.1.14 2,3,4,6-Tetraacetyl-α-D-glucopyranosyl bromide (9)



9

Molecular Weight: 390.34 Molecular Weight: 411.20

Procedure according to Bollenback *et al.*[70] HBr (30% in acetic acid) (20 ml) was cooled to 0°C and β-D-glucopyranose, 1,2,3,4,6-pentaacetate (10 g, 25.6 mmol, 1 eq) was added in small portions. The reaction was stirred for 2 hours (TLC control) while warming up to room temperature. Afterwards the reaction was quenched with ice water and extracted three times with dichloromethane. The combined organic phases were washed with $NaHCO_3$ and water, were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was recrystallized from EtOH (15 ml) and dried in vacuum to give compound **9** as a white solid.

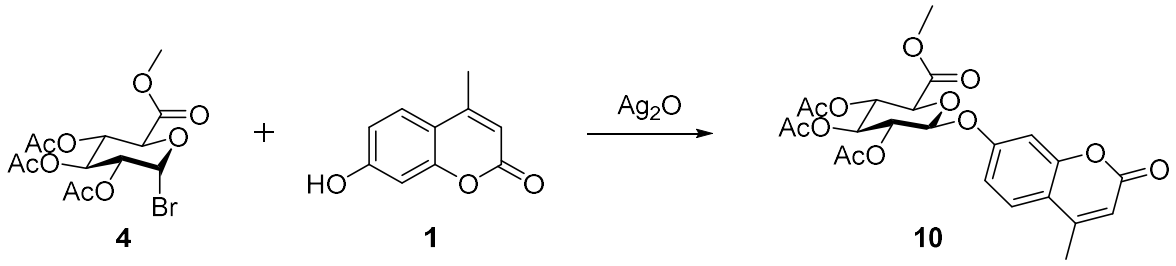
Yield = 8.52 g (81 % of theory)

^{1}H NMR (200 MHz, $CDCl_3$): δ = 6.61 (d, J = 3.9 Hz, 1H), 5.56 (t, J = 9.8 Hz, 1H), 5.16 (t, J = 9.8 Hz, 1H), 4.83 (dd, J = 10.0, 4.1, 1H), 4.39 - 4.23 (m, 2H), 4.18 - 4.06 (m, 1H), 2.10 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H) ppm

^{13}C NMR (50 MHz, $CDCl_3$): δ = 170.55 (s, 1C), 169.90 (s, 1C), 169.84 (s, 1C), 169.51 (s, 1C), 86.61 (d, 1C), 72.17 (d, 1C), 70.63 (d, 1C), 70.19 (d, 1C), 67.20 (d, 1C), 61.20 (t, 1C), 20.64 (q, 1C), 20.63 (q, 1C), 20,60 (q, 1C), 20.53 (q, 1C) ppm

E.1.15 4-Methylumbelliferyl-2,3,4-triacetyl-β-D-glucopyranuronic acid methyl ester (10)

E.1.15.1 Method A

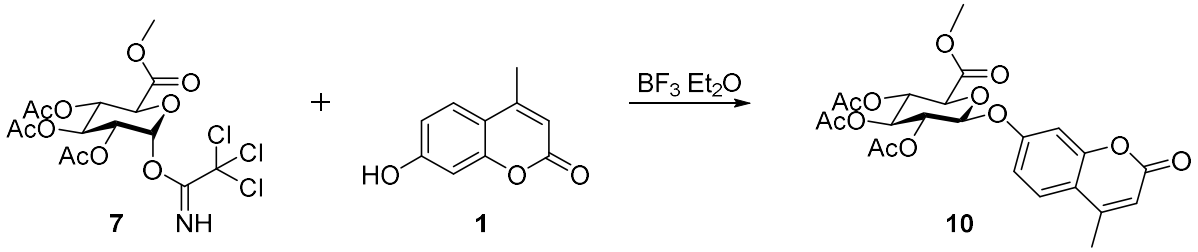


Molecular Weight: 397.17 Molecular Weight: 176.17 Molecular Weight: 492.43

Procedure according to Kwan *et al.*[47]. Compound **1** (1.06 g, 6 mmol, 1 eq), compound **4** (4.77 g, 12 mmol, 2 eq) and molecular sieve (powder 3 Å, 500 mg) were dispersed in abs. acetonitrile (35 ml) and stirred for 2 hours at room temperature. Afterwards Ag_2O (1.39 g, 6 mmol, 1 eq) was added and the reaction was stirred for 48 hours (TLC control). The reaction mixture was filtrated over celite and washed with dichloromethane. The solvent was removed under reduced pressure and the crude product purified via flash chromatography (PE/EtOAc = 3:1 – 1:2) to give compound **10** as a white solid.

Yield = 2 g (68 % of theory)

E.1.15.2 Method B



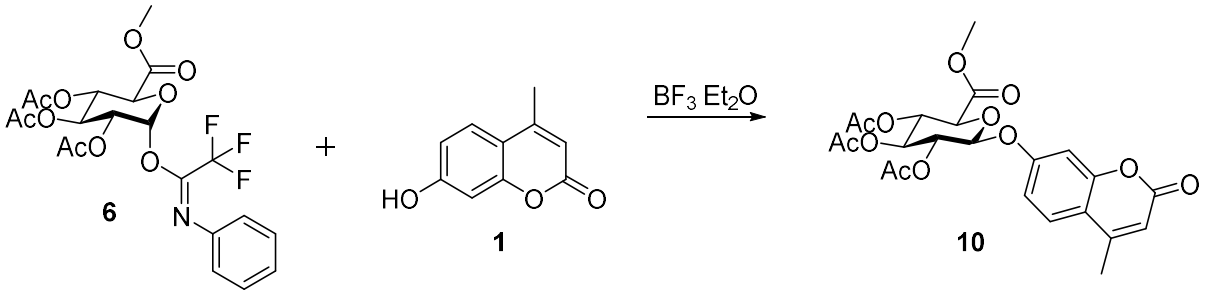
Molecular Weight: 478.66 Molecular Weight: 176.17 Molecular Weight: 492.43

Procedure according to Demchenko.[71] A suspension of **1** (50 mg, 0.28 mmol, 1.1 eq), **7** (120 mg, 0.25 mmol, 1 eq) and molecular sieve (250 mg, 3 A powder) was stirred at ambient

temperature for one hour. Then the reaction was cooled to -15°C, BF_3xEt_2O (0.016 ml, 0.13 mmol, 0.5 eq) was added slowly and the suspension was stirred for 30 minutes at -15°C. Afterwards the reaction was allowed to warm to room temperature and was stirred for another 20 hours. The mixture was filtrated over celite, washed with sat. Na_2CO_3 solution and was dried over Na_2SO_4. After removal off the solvent the crude product was purified using flash chromatography (PE/EtOAc = 3:1 – 1:2) to give compound **10** as a white solid.

Yield = 60 mg (49 % of theory)

E.1.15.3 Method C

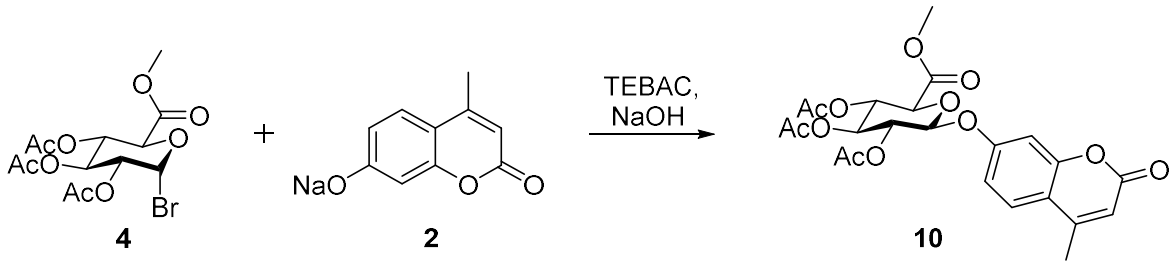


Molecular Weight: 505.40 Molecular Weight: 176.17 Molecular Weight: 492.43

Procedure referred to Demchenko.[71] A suspension of **1** (50 mg, 0.28 mmol, 1.1 eq), **6** (130 mg, 0.25 mmol, 1 eq) and molecular sieve (250 mg, 3 A powder) was stirred at ambient temperature for one hour. Then the reaction was cooled to -15°C, BF_3xEt_2O (0.016 ml, 0.13 mmol, 0.5 eq) was added slowly and the suspension was stirred for 30 minutes at -15°C. Afterwards the reaction was allowed to warm to room temperature and was stirred for another 20 hours. The mixture was filtrated over celite, washed with sat. Na_2CO_3 solution and was dried over Na_2SO_4. After removal off the solvent the crude product was purified using flash chromatography (PE/EtOAc = 3:1 – 1:2) to give compound **10** as a white solid.

Yield = 101 mg (79 % of theory)

E.1.15.4 Method D

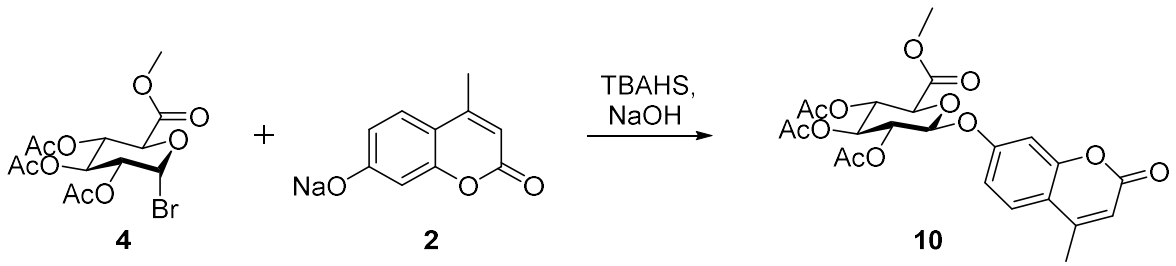


Molecular Weight: 397.17 Molecular Weight: 198.15 Molecular Weight: 492.43

Procedure according to Park *et al.*[42] Compound **4** (500 mg, 1.3 mmol, 1 eq), compound **2** (296 mg, 1.5 mmol, 1.2 eq) and benzyltriethylammonium chloride (467 mg, 2.0 mmol, 1.5 eq) were dissolved in chloroform (5 ml) and 5 ml of a 0.1 M NaOH solution was added. After heating for 5 hours, the reaction mixture was diluted with chloroform and the organic layer was washed with 0.1 M NaOH three times to remove unreacted 4-methylumbelliferone. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (PE/EtOAc = 3:1 – 1:2) to give compound **10** as white solid.

Yield = 365 mg (57 % of theory)

E.1.15.5 Method E



Molecular Weight: 397.17 Molecular Weight: 198.15 Molecular Weight: 492.43

Procedure according to Chen *et al.*[43]To a mixture of compound **4** (1.79 g, 4.5 mmol, 1.5 eq) and tetrabutylammonium hydrogen sulfate (1.53 g, 4.5 mmol, 1.5 eq) in dichloromethane was added a solution of compound **2** (0.53 g, 3 mmol, 1 eq) in 5% NaOH (9 ml, 11.13 mmol, 3.75 eq). The reaction mixture was stirred overnight at room temperature, diluted with

dichloromethane (150 mL), washed with 1 M NaOH (2 × 60 ml), water (75ml) and brine (75 ml). The organic layer was dried over Na_2SO_4, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography (PE/EtOAc = 3:1 – 1:2) to give compound **10** as white solid.

Yield = 635 mg (43 % of theory)

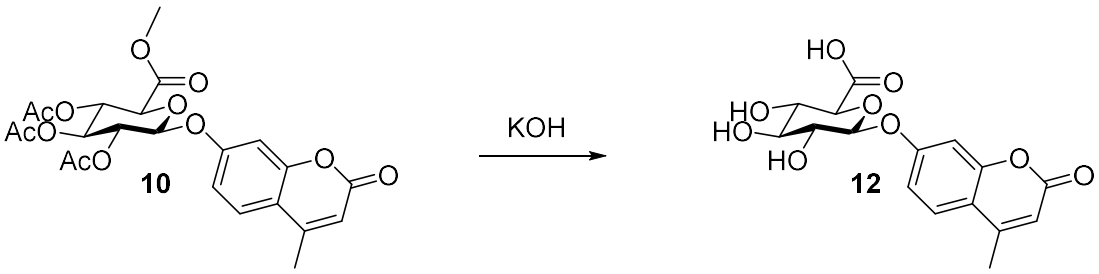
HR-ESI-IT-TOF $[M+H]^+$ m/z calcd. 177.0546 for $C_{10}H_9O_3^+$, found 177.0543.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74 (d, J = 8.8 Hz, 1H), 7.07 (d, J = 2.4 Hz, 1 H), 7.00 (dd, J = 8.8, 2.5 Hz, 1H), 6.29 (d, J = 1.2 Hz, 1H), 5.82 (d, J = 7.8 Hz, 1 H), 5.46 (t, J = 9.6 Hz, 1 H), 5.15 (dd, J = 9.7, 7.8, 1 H), 5.10 (t, J = 9.7 Hz, 1H), 4.78 (d, J = 10.0 Hz, 1H), 3.64 (s, 3 H), 2.40 (d, J = 1.0, 3 H), 2.02 (s, 6 H), 2.00 (s, 3 H) ppm

^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.49 (s, 1C), 169.27 (s, 1C), 168.99 (s, 1C), 166.93 (s, 1C), 159.80 (s, 1C), 158.57, (s, 1C), 154.27 (s, 1C), 153.08 (s, 1C), 126.88 (d, 1C), 115.05 (s, 1C), 113.10 (d, 1C), 112.32 (d, 1C), 103.17 (d, 1C), 96.34 (d, 1C), 71.02 (d, 1C), 71.00 (d, 1C), 70.27 (d, 1C), 68.78 (d, 1C), 52.59 (d, 1C), 20.25 (q, 2C), 20.18 (q, 1C), 18.07 (q, 1C) ppm

E.1.16 4-Methylumbelliferyl-β-D-glucuronide (12)

E.1.16.1 Method A



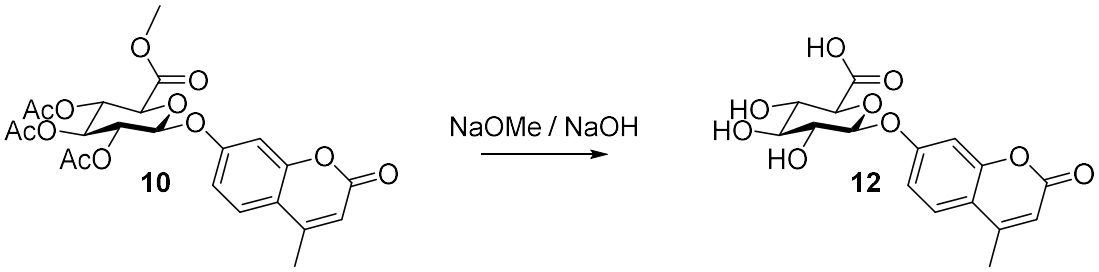
Molecular Weight: 492.43 Molecular Weight: 352.30

Procedure according to Mikula *et al.*[44] Compound **10** (80 mg, 0.16 mmol, 1 eq) was dissolved in THF/H_2O (4/1, 8 ml) and 1M KOH (1.6 ml, 1.6 mmol, 10 eq) was added. The reaction was stirred for 4 hours at room temperature, then the pH value was set to 3 and the mixture was extracted with ethyl acetate five times. The combined organic phases where washed with brine, dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude

product was purified by C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **12** as a white solid.

Yield = 20 mg (60 % of theory)

E.1.16.2 Method B

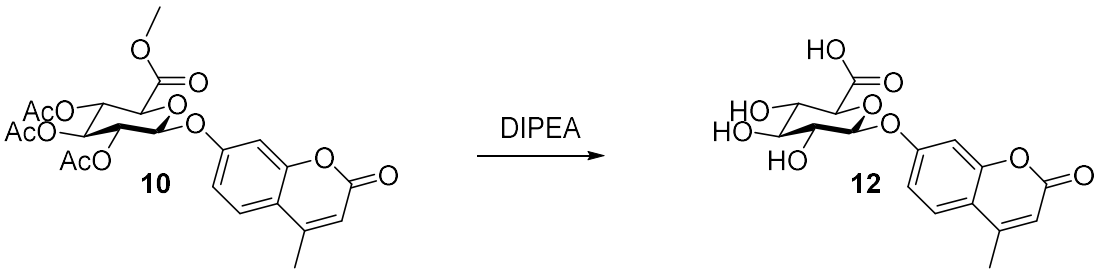


Molecular Weight: 492.43 Molecular Weight: 352.30

Procedure referred to Park *et al.*[42] Compound **10** (50 mg, 0.1 mmol, 1 eq) and 50 mM NaOMe in methanol (200 µl, 0.01 mmol, 0.1 eq) were stirred in 2 ml methanol for 1h. The solvent was removed under reduced pressure, THF/H_2O (1/1, 2 ml) and LiOH were added and the suspension was stirred for another hour. Afterwards the solution was neutralized with ion exchange residue (Allawit H^+ form), then the residue was washed several times with MeOH and the solvent was removed under reduced pressure. Purification by C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) gave compound **12** as a white solid.

Yield = 18 mg (51 % of theory)

E.1.16.3 Method C



Molecular Weight: 492.43 Molecular Weight: 352.30

Procedure according to Bunnelle *et al.*[45] Compound **10** (100 mg, 0.2 mmol, 1 eq) was solved in MeOH/H_2O (2/1, 6 ml) and cooled to 0°C. Ethyldiisopropylamine (0.6 ml, 3.6 mmol, 18 eq) was added and the reaction was stirred for 8 h while it warmed up to room temperature. The solvent was removed under reduced pressure and the residue purified by C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **12** as a white solid.

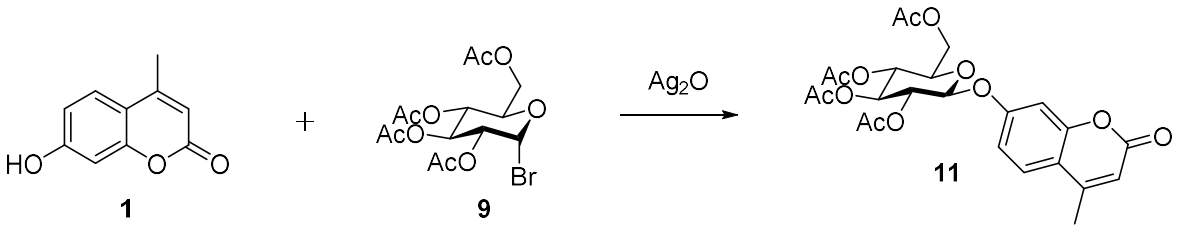
Yield = 68 mg (97 % of theory)

HR-ESI-IT-TOF $[M-H]^-$ m/z calcd. 351.0722 for $C_{16}H_{15}O_9^-$, found 351.0715.

^{1}H NMR (600 MHz, DMSO-d_6): δ = 7.72 (d, J = 8.8 Hz, 1H), 7.09 (d, J = 2.5 Hz, 1H), 7.04 (dd, J = 2.5, 8.8 Hz, 1H), 6.26 (s, 1H), 5.56 (d, J = 4.7 Hz, 1H), 5.31 (d, J = 4.2 Hz, 1H), 5.26 (d, J = 7.4 Hz, 1H), 4.04 (d, J = 9.7 Hz, 1H), 3.41 (t, J = 9.2 Hz, 1H), 2.41 (s, 3H) ppm

^{13}C NMR (150 MHz, DMSO-d_6) δ = 170.53 (s, 1C), 160.52 (s, 1C), 160.10 (s, 1C), 154.85 (s, 1C), 153.78 (s, 1C), 127.03 (s, 1C), 114.71 (d, 1C), 113.68 (d, 1C), 112.28 (d, 1C), 103.47 (d, 1C), 99.71 (d, 1C), 76.15 (d, 1C), 75.77 (d, 1C), 73.26 (d, 1C), 71.71 (d, 1C), 18.60 (q, 1C) ppm

E.1.17 4-Methylumbelliferyl-2,3,4,6-tetraacetyl-β-D-glucopyranoside (11)



Molecular Weight: 176.17 | Molecular Weight: 411.20 | Molecular Weight: 506.46

Procedure according to Kwan *et al.*[47] Compound **1** (1.06 g, 6 mmol, 1 eq), compound **9** (4.77 g, 12 mmol, 2 eq) and molecular sieve (powder 3 Å, 500 mg) were dispersed in abs. acetonitrile (35 ml) and stirred for one hour at room temperature. Afterwards Ag_2O (1.39 g, 6 mmol, 1 eq) was added and the reaction was stirred for 24 hours (TLC control). The reaction mixture was filtrated over celite and washed with dichloromethane. The solvent was removed under reduced pressure and the crude product purified via flash chromatography (PE/EtOAc = 3:1 – 1:2) to give compound **11** as a white solid.

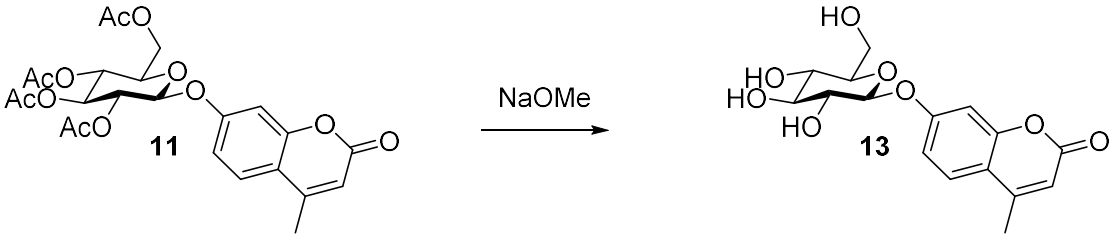
Yield = 2.47 g (81 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.73 (d, J = 8.8 Hz, 1H), 7.05 (d, J = 2.4 Hz, 1H), 6.99 (dd, J = 2.4, 8.8 Hz, 1H), 6.28 (s, 1H), 5.73 (d, J = 8.0 Hz, 1H), 5.41 (t, J = 9.6 Hz, 1H), 5.11 (dd, J = 8.0, 9.7 Hz, 1H), 5.03 (t, J = 9.7 Hz, 1H), 4.36 – 4.31 (m, 1H), 4.22 – 4.17 (m, 2H), 2.41 (s, 3H), 2.02 (s, 9H), 1.98 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.94 (s, 1C), 169.60 (s, 1C), 169.32 (s, 1C), 169.09 (s, 1C), 159.84 (s, 1C), 158.76 (s, 1C), 154.28 (s, 1C), 153.15 (s, 1C), 126.76 (d, 1C), 114.90 (s, 1C), 113.32 (d, 1C), 112.25 (d, 1C), 103.23 (d, 1C), 96.54 (d, 1C), 71.89 (s, 1C), 71.05 (s, 1C), 70.50 (s, 1C), 67.94 (s, 1C), 61.62 (s, 1C), 20.41 (s, 1C), 20.38 (s, 1C), 20.31 (s, 1C), 20.26 (s, 1C), 18.11 (s, 1C) ppm

E.1.18 4-Methylumbelliferyl β-D-glucopyranoside (13)

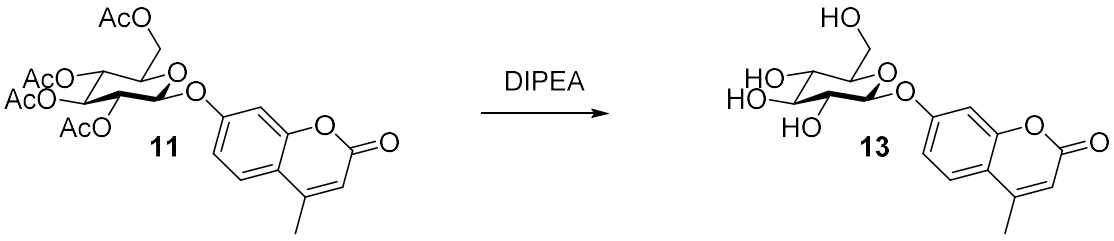
E.1.18.1 Method A



Molecular Weight: 506.46 Molecular Weight: 338.31

Procedure referred to Magro *et al.*[19] Compound **11** (300 mg, 0.6 mmol, 1 eq) was solved in methanol (15 ml) and NaOMe solution 4.4 M in methanol (1.4 ml, 6 mmol, 10 eq) was added. The reaction was monitored via LCMS which indicated completion after 30 min. Afterwards the solvent was removed under reduced pressure and the crude product was purified via C18 flash chromatography (ACN/H_2O = 5 % ACN – 95% ACN), to give compound **13** as a white solid.
Yield = 50 mg (25 % of theory)

E.1.18.2 Method B



Molecular Weight: 506.46 Molecular Weight: 338.31

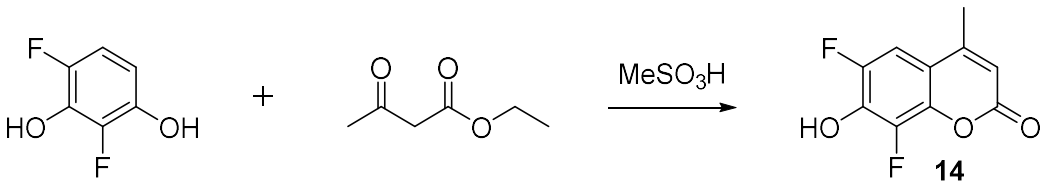
Procedure according to Bunnelle *et al.*[45] Compound **11** (100 mg, 0.2 mmol, 1 eq) was solved in MeOH (10 ml) and cooled to 0°C. Ethyldiisopropylamine (0.34 ml, 2 mmol, 10 eq) was added and the reaction was stirred for 8 h while it warmed up to room temperature. The solvent was removed under reduced pressure and the by the residue purified by C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **13** as a white solid.
Yield = 67 mg (99 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.70 (d, J = 9.2 Hz, 1H), 7.03 (m, 2H), 6.25 (s, 1H), 5.39 (d, J = 4.8 Hz, 1H), 5.12 (d, J = 4.7 Hz, 1H), 5.04 (m, 2H), 4.59 (t, J = 5.6 Hz, 1H), 3.70 (m, 1H), 3.45 (m, 2H), 3.27 (m, 2H), 3.16 (m, 1H), 1.93 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 160.63 (s, 1C), 160.57 (s, 1C), 154.87 (s, 1C), 153.80 (s, 1C), 126.88 (d, 1C), 114.55 (s, 1C), 113.86 (d, 1C), 112.27 (d, 1C), 103.69 (d, 1C), 100.48 (d, 1C), 77.62 (d, 1C), 76.96 (d, 1C), 73.60 (d, 1C), 70.11 (d, 1C), 61.12 (t, 1C), 18.60 (q, 1C) ppm

Synthesis of β-glucuronidase responsive fluorogenic probes

E.1.19 6,8-Difluoro-4-methylumbelliferone (14)



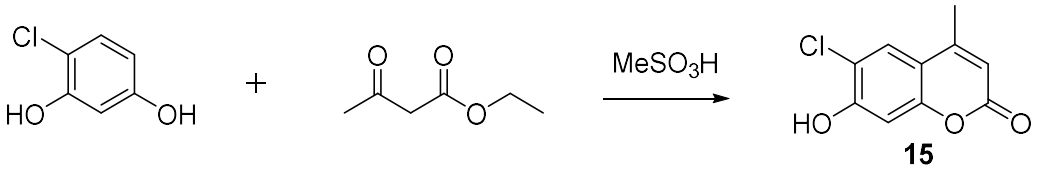
Molecular Weight: 146.09 Molecular Weight: 130.14 Molecular Weight: 212.15

Procedure according to Hedberg *et al.*[36] 2,4-Difluororesorcinol (100 mg, 0.77 mmol, 1.0 eq) and ethyl acetoacetate (111 mg, 0.85 mmol, 1.1 eq) were placed in a vial and cooled to 0°C. $MeSO_3H$ (1.26 ml, 19.32 mmol, 25.1 eq) was added dropwise while keeping the temperature of the reaction at 0°C. The reaction was stirred for another 30 minutes at 0°C and then for 24 hours at room temperature. Afterwards the reaction was again cooled to 0°C and quenched with cold water (10 ml). The precipitate was collected, washed with cold water (3x5 ml) and cold Et_2O (2x5 ml) and then dried under reduced pressure to give compound **14** as white solid. Yield =121 mg (74 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 11.47 (s, 1H), 7.49 (q, J = 4.5 Hz, 1H), 6.31 (s, 1H), 2.37 (s, 1H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 158.74 (s, 1C), 153.17 (t, J = 2.7 Hz, 1C), 148.42 (dd, J = 5.0, 239.4 Hz, 1C), 139.34 (dd, J = 2.2, 9.7 Hz, 1C), 139.27 (dd, J = 6.7, 244.1 Hz, 1C), 137.50 (dd, J = 12.9, 18.1 Hz, 1C), 112.33 (s, 1C), 110.94 (d, J = 9.0 Hz, 1C), 106.36 (dd, J = 3.2, 21.4 Hz, 1C), 18.21 (s, 1C) ppm

E.1.20 6-Chloro-4-methylumbelliferone (15)

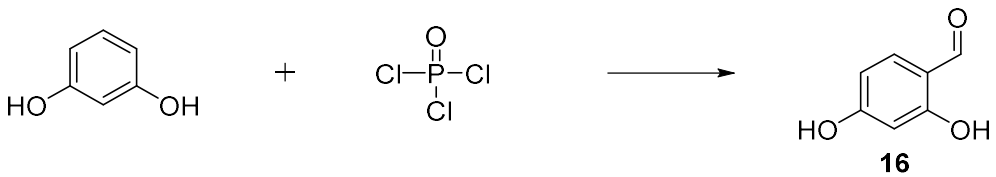


Molecular Weight: 144.55 Molecular Weight: 130.14 Molecular Weight: 210.61

Procedure according to Hedberg *et al.*[36] 4-Chlororesorcinol (1 g, 6.92 mmol, 1.0 eq) and ethyl acetoacetate (0.99 g, 7.61 mmol, 1.1 eq) were placed in a vial and cooled to 0°C. $MeSO_3H$ (12 ml, 26.17 mmol, 25.1 eq) was added dropwise while keeping the temperature of the reaction at 0°C. The reaction was stirred for another 30 minutes at 0°C and then for 24 hours at room temperature. Afterwards the reaction was again cooled to 0°C and quenched with cold water (10 ml). The precipitate was collected, washed with cold water (3x5 ml) and cold Et_2O (2x5 ml) and then dried under reduced pressure to give compound **15** as white solid.
Yield = 1.17 g (81 % of theory)

^{1}H NMR (200 MHz, DMSO-d_6) δ = 7.67 (s, 1H), 6.85 (s, 1H), 6.15 (s, 1H), 2.34 (s, 3H) ppm
^{13}C NMR (50 MHz, DMSO-d_6) δ = 159.83 (s, 1C), 156.25 (s, 1C), 152.97 (s, 1C), 152.75 (s, 1C), 125.97 (d, 1C), 116.85 (s, 1C), 112.78 (s, 1C), 111.31 (d, 1C), 103.30 (d, 1C), 18.08 (q, 1C) ppm

E.1.21 2,4-Dihydroxybenzaldehyde (16)



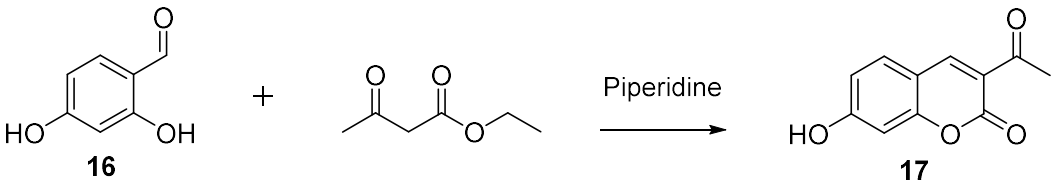
Molecular Weight: 110.11 Molecular Weight: 153.32 Molecular Weight: 138.12

Procedure according to Vilsmeier *et al.*[72] Resorcinol was solved in DMF (40 ml) and cooled to 0°C. Afterwards phosphoryl chloride was added at 0°C and the solution was than stirred at 85°C for 16 hours. The mixture was poured into ice water (200 ml) and extracted with CH_2Cl_2 (3 x 100 ml) and EtOAc (100ml). The combined organic phases where dried over Na_2SO_4 and

the solvent removed under reduced pressure. The crude product was purified with flash chromatography (PE/EtOAc = 6:1 – 2:1) to give compound **16** as yellowish solid.

^{1}H NMR (200 MHz, DMSO-d_6) δ = 10.78 (br s, 2H), 9.90 (s, 1H), 7.52 (d, J = 8.5 Hz, 1H), 6.39 (dd, J = 2.0, 8.5 Hz, 1H), 6.32 (d, J = 2.0 Hz, 1H) ppm
^{13}C NMR (50 MHz, DMSO-d_6) δ = 191.19 (d, 1C), 165.23 (s, 1C), 163.31 (s, 1C), 133.04 (d, 1C), 115.22 (s, 1C), 108.69 (d, 1C), 102.25 (d, 1C) ppm

E.1.22 3-Acetylumbelliferone (17)

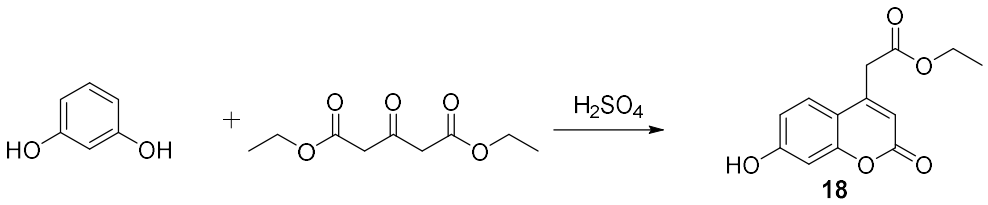


Molecular Weight: 138.12 Molecular Weight: 130.14 Molecular Weight: 204.18

Procedure according to Starčević *et al.*[53]. Compound **16** (2 g, 14.4 mmol, 1 eq) and ethyl acetoacetate (2.59 ml, 20.02 mmol, 1.4 eq) were dissolved in absolute ethanol (50 ml), piperidine (0.03 ml, 0.29 mmol, 0.02 eq) was added and the reaction was refluxed for 4 h. The product dropped out of the solution after cooling, it was washed with cold ethanol and dried in vacuum to give compound **17** as yellow solid.

^{1}H NMR (200 MHz, DMSO-d6) δ = 8.58 (s, 1H), 7.78 (d, J = 8.6 Hz, 1H), 6.84 (dd, J = 2.2, 8.6 Hz, 1H), 6.74 (d, J = 2.1 Hz, 1H), 2.54 (s, 3H) ppm
^{13}C NMR (50 MHz, DMSO-d6) δ = 194.67 (s, 1C), 164.32 (s, 1C), 159.09 (s, 1C), 157.30 (s, 1C), 147.88 (d, 1C), 132.69 (d, 1C), 119.14 (s, 1C), 114.28 (d, 1C), 110.79 (s, 1C), 101.80 (d, 1C), 30.11 (q, 1C) ppm

E.1.23 Ethyl umbelliferone-4-acetate (18)



18

Molecular Weight: 110.11 Molecular Weight: 202.21 Molecular Weight: 248.23

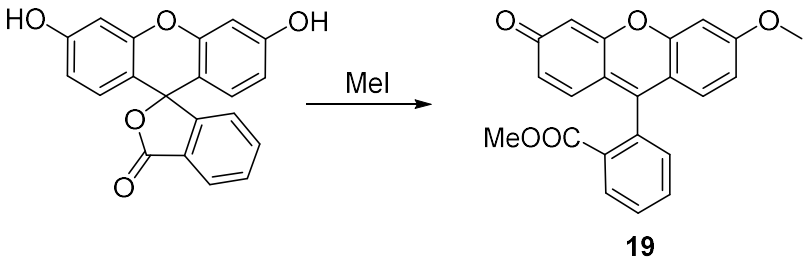
Procedure according to Pisani *et al.*[55] Resorcinol (10 g, 90 mmol, 1 eq), diethyl 1,3-acetone-dicarboxylate (20.1 g, 99 mmol, 1.1 eq) and 1 ml of 96% sulfuric acid were stirred at 120°C for 1 hour. The oily residue obtained, was crystallized from absolute ethanol and dried in vacuum to give compound **18** as off white solid.

Yield = 9.1 g (41 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.58 (s, 1H), 7.50 (d, J = 8.7 Hz, 1H), 6.79 (dd, J = 2.3, 8.7 Hz, 1H), 6.73 (d, J = 2.2 Hz, 1H), 6.23 (s, 1H), 4.10 (q, J = 7.1 Hz, 2H), 3.91 (s, 2H), 1.17 (t, J = 7.1 Hz, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.16 (s, 1C), 161.28 (s, 1C), 160.14 (s, 1C), 155.04 (s, 1C), 149.59 (s, 1C), 126.68 (d, 1C), 113.02 (d, 1C), 112.11 (d, 1C), 111.21 (s, 1C), 102.35 (d, 1C), 60.87 (t, 1C), 36.89 (t, 1C), 13.96 (q, 1C) ppm

E.1.24 Fluorescein, methyl ester, methyl ether (19)



19

Molecular Weight: 332.31 Molecular Weight: 360.37

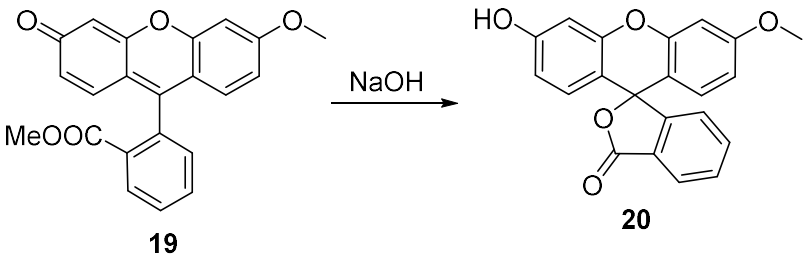
Procedure according to Mugherli *et al.*[58] Fluorescein (3.32 g, 10.0 mmol, 1 eq) and K_2CO_3 (2.07 g, 15.0 mmol, 1.5 eq) were dispersed in DMF (10 ml) and MeI (2.13 g, 15 mmol, 1.5 eq) was added dropwise ad room temperature. After stirring for 24 hours, the reaction was diluted with water and extracted three times with ethyl acetate. The combined organic phases were washed with 1 M $NaHCO_3$ solution and brine, dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified via flash chromatography (PE/EtOAc = 1:1 – 0:1) to give compound **19** as orange solid.

Yield = 2.50 g (69 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.21 (dd, J = 1.1, 7.9 Hz, 1H), 7.87 (dt, J = 1.3, 7.5 Hz, 1H), 7.78 (dt, J = 1.3, 7.7 Hz, 1H), 7.50 (dd, J = 1.0, 7.6 Hz, 1H), 7.23 (d, J = 2.3 Hz, 1H), 6.90 -6.79 (m, 3H), 6.38 (dd, J = 1.9, 9.7 Hz, 1H), 6.24 (d, J = 1.9 Hz, 1H), 3.91 (s, 3H), 3.58 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 183.82 (s, 1C), 165.16 (s, 1C), 163.87 (s, 1C), 158.34 (s, 1C), 153.55 (s, 1C), 150.03 (s, 1C), 133.88 (s, 1C), 133.18 (d, 1C), 130.68 (d, 2C), 130.34 (d, 1C), 130.02 (d, 1C), 129.48 (s, 1C), 129.35 (d, 1C), 128.82 (d, 1C), 116.62 (s, 1C), 114.27 (s, 1C), 113.54 (d, 1C), 104.56 (d, 1C), 100.56 (d, 1C), 56.26 (q, 1C), 52.28 (q, 1C) ppm

E.1.25 3'-O-Methylfluorescein (20)



19 **20**

Molecular Weight: 360.37 Molecular Weight: 346.34

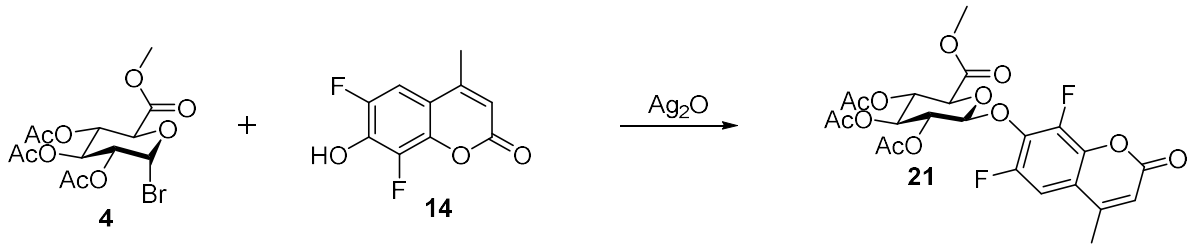
Procedure according to Mugherli *et al.*[58] Compound **19** (1.8 g, 5 mmol, 1 eq) was solved in methanol (20 ml) and 10 % NaOH in water (10 ml, 25 mmol, 5 eq) was added. The reaction was stirred at room temperature for 21 hours, then the solvent was evaporated under reduced pressure and the residue solved in water (20 ml). The aqueous solution was acidified to pH 5 with conc. HCl and extracted with ethyl acetate seven times (7 x 20 ml). The combined organic phases were washed with brine and dried over Na_2SO_4 and the solvent was removed under reduced pressure. Compound **20** was obtained as a red solid that needed no further purification.

Yield = 1.43 g (83 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.00 (d, J = 7.6 Hz, 1H), 7.79 (dt, J = 1.1, 7.5 Hz, 1H), 7.71 (dt, J = 0.7, 7.4 Hz, 1H), 7.27 (d, J = 7.7 Hz, 1H), 6.93 (d, J = 2.4 Hz, 1H), 6.71 – 6.64 (m, 3H), 6.58 (d, J = 1.2 Hz, 2H), 3.81 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 168.65 (s, 1C), 161.01 (s, 1C), 159.55 (s, 1C), 152.48 (s, 1C), 151.87 (s, 1C), 151.76 (s, 1C), 135.63 (d, 1C), 130.12 (d, 1C), 129.08 (d, 1C), 128.93 (d, 1C), 126.03 (s, 1C), 124.65 (d, 1C), 123.98 (d, 1C), 112.78 (d, 1C), 111.87 (d, 1C), 110.98 (s, 1C), 109.43 (s, 1C), 102.20 (d, 1C), 100.77 (d, 1C), 82.70 (s, 1C), 55.63 (q, 1C) ppm

E.1.26 6,8-Difluoro-4-methylumbelliferyl-2,3,4-triacetyl-β-D-glucopyranuronic acid, methyl ester (21)



Molecular Weight: 397.17 Molecular Weight: 212.15 Molecular Weight: 528.41

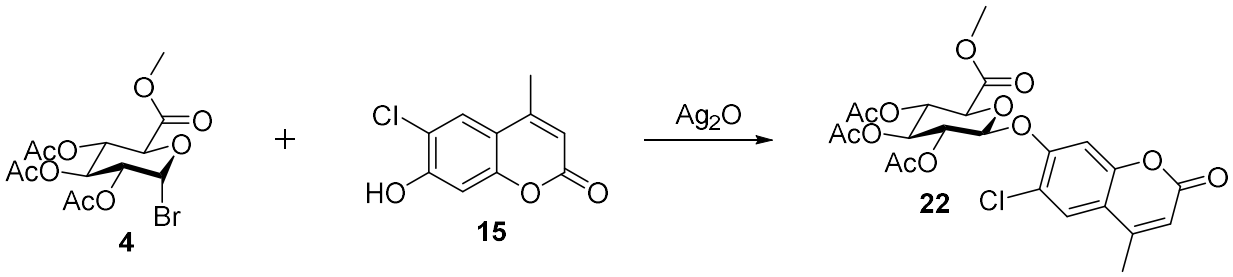
Procedure according to Kwan *et al.*[47] Compound **14** (50 mg, 0.24 mmol, 1 eq), compound **4** (191 mg, 0.48 mmol, 2 eq) and molecular sieve (powder 3 Å, 50 mg) were dispersed in abs. acetonitrile (3 ml) and stirred for one hour at room temperature. Afterwards Ag_2O (56 mg, 0.24 mmol, 1 eq) was added and the reaction was stirred for 24 hours (TLC control). The reaction mixture was filtrated over celite and washed with dichloromethane. The solvent was removed under reduced pressure and the crude product purified via flash chromatography (PE/EtOAc = 3:1 – 1:2) to give compound **21** as a colorless oil.

Yield = 100 mg (79 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.66 (dd, J = 1.9, 11.0 Hz, 1H), 6.50 (d, J = 1.0 Hz, 1H), 5.58 (d, J = 7.9 Hz, 1H), 5.50 (t, J = 9.6 Hz, 1H), 5.18 (dd, J = 7.9, 9.6 Hz, 1H), 5.09 (t, J = 9.7 Hz, 1H), 4.55 (d, J = 9.9 Hz, 1H), 3.62 (s, 3H), 2.40 (d, J = 1.1 Hz, 3H), 2.07 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 170.10 (s, 1C), 169.75 (s, 1C), 169.48 (s, 1C), 167.25 (s, 1C), 158.15 (s, 1C), 152.46 (t, J = 2.7 Hz, 1C), 151.05 (dd, J = 2.39, 245.3 Hz, 1C), 142.86 (dd, J = 4.8, 252.2 Hz, 1C), 139.31 (dd, J = 2.6, 9.7 Hz, 1C), 134.9 (dd, J = 11.6, 16.6 Hz, 1C), 177.61 (d, J = 9.2 Hz, 1C), 115.33 (s, 1C), 107.77 (dd, J = 3.3, 20.2 Hz, 1C), 71.21 (s, 1C), 69.35 (s, 1C), 60.22(s, 1C), 53.11 (s, 1C), 21.19 (s, 1C), 20.76 (s, 1C), 20.63 (s, 1C), 18.71 (s, 1C) 14.53 (s, 1C) ppm

E.1.27 6-Chloro-4-methylumbelliferyl-2,3,4-triacetyl-β-D-glucopyranuronic acid, methyl ester (22)



Molecular Weight: 397.17 Molecular Weight: 210.61 Molecular Weight: 526.88

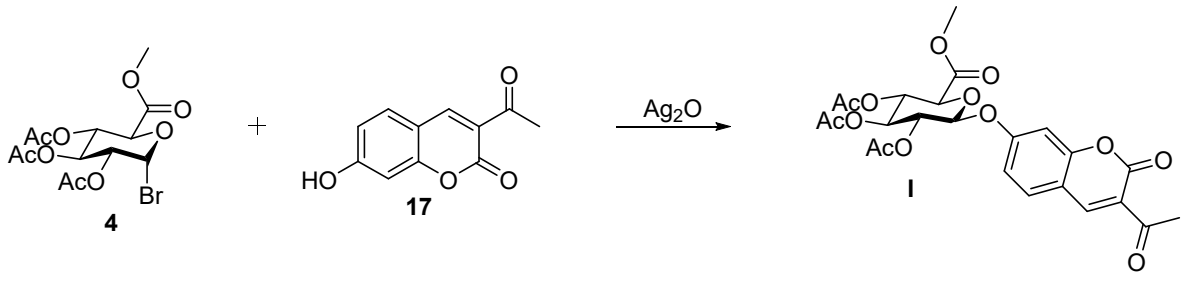
Procedure according to Kwan *et al.*[47] Compound **15** (500 mg, 2.36 mmol, 1 eq), compound **4** (1.87 g, 4.72 mmol, 2 eq) and molecular sieve (powder 3 Å, 1 g) were dispersed in abs. acetonitrile (30 ml) and stirred for one hour at rt. Afterwards Ag_2O (546 mg, 2.36 mmol, 1.5 eq) was added and the reaction was stirred for 24 h (TLC control). The reaction mixture was filtrated over celite and washed with dichloromethane (100 ml). The solvent was removed under reduced pressure and the crude product purified via flash chromatography (PE/EtOAc = 4:1 – 1:1) to give compound **22** as an off white oil.

Yield = 801 mg (64 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.88 (s, 1H), 7.34 (s, 1H), 6.35 (d, J = 1.2 Hz, 1H), 5.77 (d, J = 7.8 Hz, 1H), 5.46 (t, J = 9.5 Hz, 1H), 5.22 (dd, J = 7.8, 9.7 Hz, 1H), 5.12 (t, J = 9.6 Hz, 1H), 4.81 (d, J = 9.9 Hz, 1H), 3.65 (s, 3H), 2.40 (d, J = 1.0 Hz, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.47 (s, 1C), 169.31 (s, 1C), 168.78 (s, 1C), 166.90 (s, 1C), 159.42 (s, 1C), 153.67 (s, 1C), 152.72 (s, 1C), 152.46 (s, 1C), 126.44 (d, 1C), 118.13 (s, 1C), 115.84 (s, 1C), 104.16 (d, 1C), 97.33 (d, 1C), 71.12 (d, 1C), 70.68 (d, 1C), 69.91 (d, 1C), 68.67 (d, 1C), 52.62 (d, 1C), 20.42 (q, 1C), 20.34 (q, 1C), 20.27 (q, 1C), 20.21 (q, 1C), 18.08 (q, 1C) ppm

E.1.28 3-Acetylumbelliferyl-2,3,4-triacetyl-β-D-glucopyranuronic acid, methyl ester (I)



Molecular Weight: 397.17 Molecular Weight: 204.18 Molecular Weight: 520.44

Compound **17** (200 mg, 1.14 mmol, 1 eq), compound **4** (905 mg, 2.28 mmol, 2 eq) and molecular sieve (powder 3 Å, 200 mg) were dispersed in abs. acetonitrile (3 ml) and stirred for one hour at room temperature. Afterwards Ag_2O (396 mg, 1.71 mmol, 1.5 eq) was added and the reaction was stirred for 24 hours (TLC control). The solvent was removed under reduced pressure and the crude product purified via flash chromatography (PE/EtOAc = 4:1 – 1:1) to give compound **I** as a pale yellow solid.

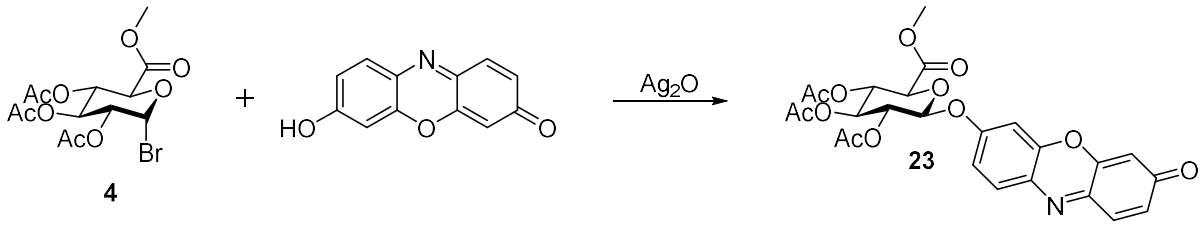
Yield = 320 mg (54 % of theory)

HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 521.1289 for $C_{24}H_{25}O_{13}^+$, found 521.1286.

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.66 (s, 1H), 7.95 (d, J = 8.8 Hz, 1H), 7.51 (d, J = 4.5 Hz, 1H), 7.17 (d, J = 2.3 Hz, 1H), 7.06 (dd, J = 2.3, 8.7 Hz, 1H), 5.87 (d, J = 7.8 Hz, 1H), 5.46 (t, J = 9.6 Hz, 1H), 5.18 (dd, J = 7.9, 9.6 Hz, 1H), 5.11 (t, J = 9.7 Hz, 1H), 4.80 (d, J = 10.0 Hz, 1H), 3.64 (s, 3H), 2.57 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 195.27 (s, 1C), 169.99 (s, 1C), 169.78 (s, 1C), 169.48 (s, 1C), 167.39 (s, 1C), 161.15 (s, 1C), 159.04 (s, 1C), 156.90 (s, 1C), 147.59 (d, 1C), 133.00 (d, 1C), 122.39 (s, 1C), 114.60 (d, 1C), 114.12 (s, 1C), 103.07 (d, 1C), 89.89 (d, 1C), 71.56 (d, 1C), 70.69 (d, 1C), 69.20 (d, 1C), 53.10 (d, 1C), 30.49 (q, 1C), 20.91 (q, 1C), 20.83 (q, 1C), 20.75 (q, 1C), 20.69 (q, 1C) ppm

E.1.29 1-(3-Oxo-3*H*-phenoxazin-7-yl)-glucopyranuronic acid, methyl ester (23)



Molecular Weight: 397.17 Molecular Weight: 213.19 Molecular Weight: 529.45

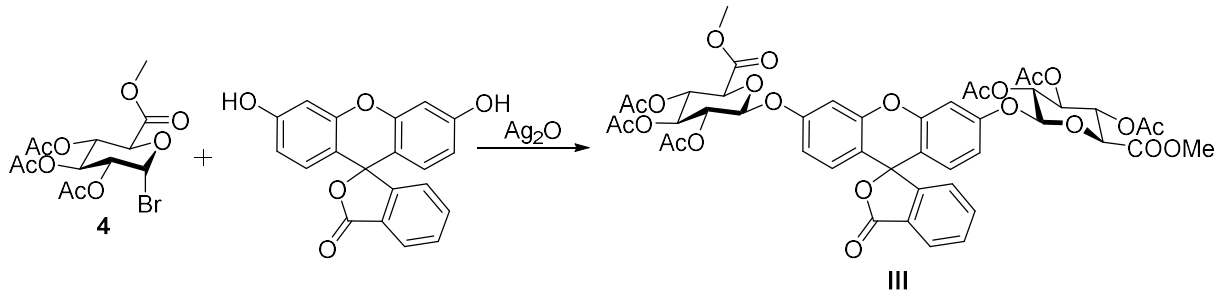
Procedure according to Kwan *et al.*[47] Resorufin (100 mg, 0.43 mmol, 1 eq), compound **4** (342 mg, 0.86 mmol, 2 eq) and molecular sieve (powder 3 Å, 400 mg) where dispersed in abs. acetonitrile (20 ml) and stirred for two hours at room temperature. Then silver(I)oxide (151 mg, 0.65 mmol, 1.5 eq) was added (light sensitive) and the reaction was stirred for 48 h at room temperature. Afterwards the solvent was removed under reduced pressure and the crude product was purified via flash chromatography (PE/EtOAc = 4/1 – 1/1). Compound **23** was obtained as red solid.

Yield = 83 mg (36 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.83 (d, J = 8.9 Hz, 1H), 7.54 (d, J = 9.8 Hz, 1H), 7.19 (d, J = 2.6 Hz, 1H), 7.08 (dd, J = 2.7, 8.9 Hz, 1H), 6.81 (dd, J = 2.0, 9.8 Hz, 1H), 6.30 (d, J = 2.1 Hz, 1H), 5.87 (d, J = 7.8 Hz, 1H), 5.45 (t, J = 9.6 Hz, 1H), 5.17 (dd, J = 7.8, 9.6 Hz, 1H), 5.10 (t, J = 9.7 Hz, 1H), 4.80 (d, J = 10.0 Hz, 1H), 3.64 (s, 3H), 2.03 (s, 6H), 2.01 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 186.27 (s, 1C), 169.87 (s, 1C), 169.28 (s, 1C), 169.07 (s, 1C), 166.70 (s, 1C), 159.48 (s, 1C), 149.35 (s, 1C), 146.83 (s, 1C), 144.95 (d, 1C), 134.68 (d, 1C), 134.59 (d, 1C), 131.55 (d, 1C), 129.55 (s, 1C), 114.82 (s, 1C), 106.93 (d, 1C), 103.39 (d, 1C), 98.02 (d, 1C), 72.58 (d, 1C), 71.39 (d, 1C), 70.75 (d, 1C), 68.72 (d, 1C), 53.02 (q, 1C), 20,51 (q, 1C), 20.45 (q, 1C), 20.42 (q, 1C) ppm

E.1.30 3-Oxospiro[isobenzofuran-1(3H),9'-[9*H*]xanthene]-3',6'-diyl bis[2,3,4-triacetyl-β-D-glucopyranosiduronic acid, methyl ester] (III)



Molecular Weight: 397.17 Molecular Weight: 332.31 Molecular Weight: 964.84

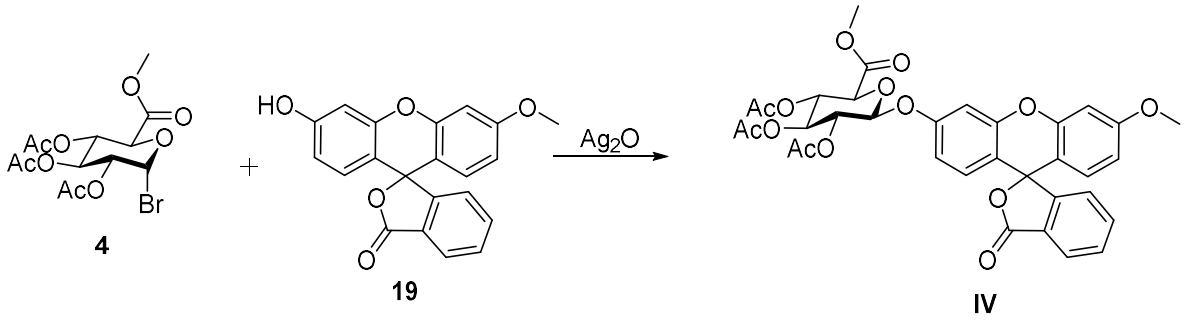
Fluorescein (1 g, 3.0 mmol, 1 eq), compound **4** (3.56 g, 9 mmol, 3 eq) and molecular sieve (powder 3 Å, 1 g) were dispersed in abs. acetonitrile (53 ml) and stirred for one hour at room temperature. Afterwards Ag_2O (1.07 g, 4.62 mmol, 1.5 eq) was added and the reaction was stirred for 72 hours (TLC control). The reaction mixture was filtrated over celite and washed with dichloromethane. The solvent was removed under reduced pressure and the crude product purified via flash chromatography (DCM/MeOH = 1 % MeOH – 5 % MeOH) to give compound **III** as an orange solid.

Yield = 2.51 g (87 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.16 (dt, J = 0.9, 7.8 Hz, 1H), 7.95 (t, J = 7.6 Hz, 1H), 7.85 (tt, J = 1.5, 7.7 Hz, 1H), 7.56 - 753 (m, 1H), 7.33 (t, J = 2.4 Hz, 1H), 6.90 – 6.74 (m, 3H), 6.39 (td, J = 1.6, 9.7 Hz, 1H), 6.27 (d, J = 1.9 Hz, 1H), 6.03 (t, J = 7.5 Hz, 1H), 5.89 (t, J = 7.9 Hz, 1H), 5.46 (dt, J = 3.0, 9.5 Hz, 2H), 5.19 – 4.96 (m, 4H), 4.80 (q, J = 4.9 Hz, 1H), 4.56 (d, J = 9.7 Hz, 1H), 3.64 (s, 3H), 3.56 (s, 3H), 2.03 (s, 3H), 2.01 (s, 6H), 1.99 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 184.55 (s, 1C), 184.51 (s, 1C), 169.99 (s, 1C), 169.78 (s, 1C), 169.69 (s, 1C), 169.48 (s, 1C), 169.39 (s, 1C), 167.40 (s, 1C), 167.20 (s, 1C), 162.90 (s, 1C), 160.22 (s, 1C), 158.64 (s, 1C), 153.40 (s, 1C), 149.10 (s, 1C), 135.19 (s, 1C), 134.90 (d, 1C), 131.49 (d, 1C), 130.88 (d, 1C), 130.33 (d, 1C), 129.75 (d, 1C), 129.43 (d, 1C), 127.89 (s, 1C), 118.22 (s, 1C), 116.56 (s, 1C), 114.94 (d, 1C), 105.42 (d, 1C), 103.31 (d, 1C), 96.62 (d, 1C), 91.83 (d, 1C), 71.86 (d, 1C), 71.58 (d, 1C), 71.42 (d, 1C), 70.96 (d, 1C), 70.69 (d, 1C), 70.05 (d, 1C), 69.21 (d, 1C), 53.11 (d, 1C), 53.04 (d, 1C), 20.75 (q, 2C), 20.70 (q, 2C), 20.64 (q, 2C), 20.59 (q, 2C) ppm

E.1.31 3'-Methoxy-3-oxospiro[isobenzofuran-1(3H),9'-[9*H*]xanthene]-6'-yl-2,3,4-triacetyl-β-D-glucopyranosiduronic acid, methyl ester (IV)



Molecular Weight: 397.17 Molecular Weight: 346.34 Molecular Weight: 662.60

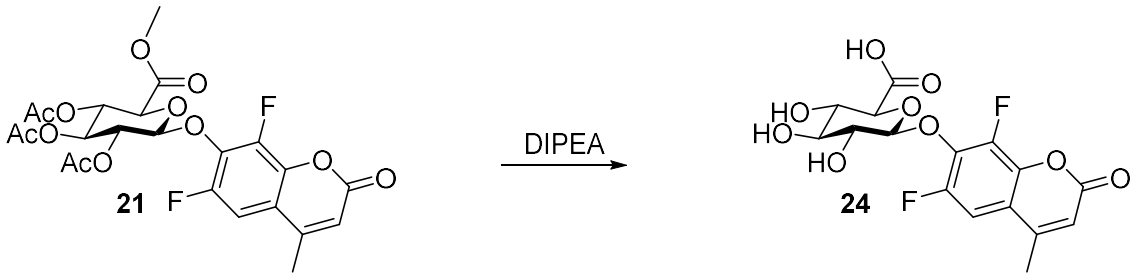
Compound **19** (291 mg, 0.84 mmol, 1 eq), compound **4** (500 mg, 1.26 mmol, 3 eq) and molecular sieve (powder 3 Å, 150 mg) were dispersed in abs. acetonitrile (5 ml) and stirred for two hours at room temperature. Afterwards Ag_2O (195 mg, 0.84 mmol, 1 eq) was added and the reaction was stirred for 48 hours (TLC control). The reaction mixture was filtrated over celite and washed with dichloromethane. The solvent was removed under reduced pressure and the crude product purified via flash chromatography (PE/EtOAc = 4:1 – 1:2) to give compound **IV** as a red solid.

Yield = 89 mg (16 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) = δ 8.15 (d, J = 8.5 Hz, 1H), 7.94 (t, J = 7.6 Hz, 1H), 7.84 (t, J = 7.8 Hz, 1H), 7.55 (d, J = 7.9 Hz, 1H), 7.23 (s, 1H), 6.87 – 6.73 (m, 4H), 6.37 (d, J = 10.1 Hz, 1H), 6.24 (d, J = 1.9 Hz, 1H), 6.02 (dd, J = 7.9, 10.9 Hz, 1H), 5.46 (dt, J = 2.5, 9.4 Hz, 1H), 5.02 (q, J = 9.2 Hz, 2H), 4.57 (dd, J = 6.4, 9.7 Hz, 1H), 3.91 (s, 3H), 3.57 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H), 1.91 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 183.81 (s, 1C), 169.35 (s, 1C), 169.20 (s, 1C), 168.96 (s, 1C), 166.69 (s, 1C), 163.97 (s, 1C), 162.44 (s, 1C), 158.27 (s, 1C), 153.58 (s, 1C), 149.30 (s, 1C), 134.87 (s, 1C), 134.27 (d, 1C), 130.94 (d, 1C), 130.26 (d, 1C), 130.05 (d, 1C), 129.42 (d, 1C), 128.83 (d, 1C), 128.54 (d, 1C), 127.51 (s, 1C), 116.78 (s, 1C), 114.18 (s, 1C), 113.54 (d, 1C), 104.66 (d, 1C), 100.56 (d, 1C), 91.27 (d, 1C), 71.33 (d, 1C), 70.44 (d, 1C), 69.55 (d, 1C), 68.56 (d, 1C), 56.27 (d, 1C), 52.55 (d, 1C), 20.25 (q, 1C), 20.17 (q, 1C), 20.14 (q, 1C) ppm

E.1.32 6,8-Difluoro-4-methylumbelliferyl-β-D-glucopyranosiduronic acid (24)



Molecular Weight: 528.41 Molecular Weight: 388.28

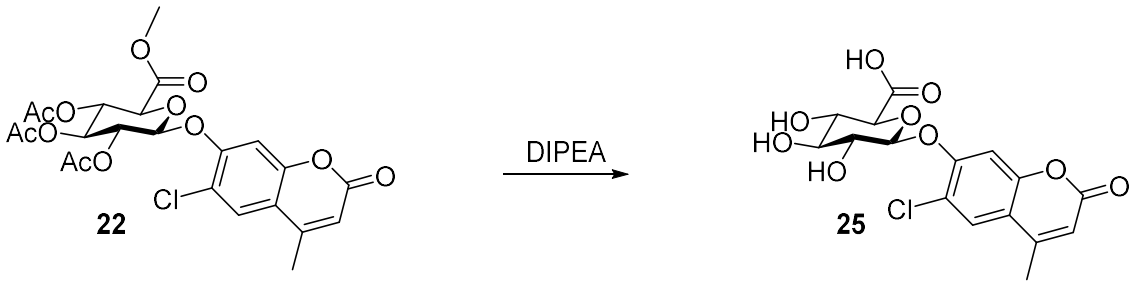
Procedure according to Bunnelle *et al.*[45] Compound **21** (100 mg, 0.19 mmol, 1 eq) was solved in MeOH (8 ml) and cooled to 0°C. Ethyldiisopropylamine (0.6 ml, 3.42 mmol, 18 eq) was added and the reaction was stirred for 3 h while it warmed to room temperature. After all acetyl groups were removed (LCMS control) water (2 ml) was added and the reaction was stirred for another hour. Upon completion (LCMS control) the solvent was removed under reduced pressure and the crude product was purified via C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **24** as a colorless oil.

Yield = 46 mg (62 % of theory)

^{1}H NMR (400 MHz, DMSO-d6) = 7.61 (d, J = 11.4 Hz, 1H), 6.46 (s, 1H), 5.65 (s, 1H), 5.28 (s, 1H), 5.07 (d, J = 6.9 Hz, 1H), 3.72 (d, J = 9.6 Hz, 1H), 3.41 (t, J = 9.2 Hz, 1H), 2.39 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d6) δ = 169.70 (s, 1C), 158.28 (s, 1C), 152.56 (s, 1C), 150.76 (dd, J = 3.5, 245.0 Hz, 1C), 142.39 (dd, J = 5.5, 251.0 Hz, 1C), 138.89 (d, J = 7.7 Hz, 1C), 134.87 (dd, J = 10.8, 15.8 Hz, 1C), 116.08 (d, J = 9.8 Hz, 1C), 114.67 (s, 1C), 107.09 (dd, J = 0.1, 22.2 Hz, 1C), 103.81 (s, 1C), 75.83 (s, 1C), 75.51 (s, 1C), 73.48 (s, 1C), 71.24 (s, 1C), 18.20 (s, 1C) ppm

E.1.33 6-Chloro-4-methylumbelliferyl-β-D-glucopyranosiduronic acid (25)



Molecular Weight: 526.88

Molecular Weight: 386.74

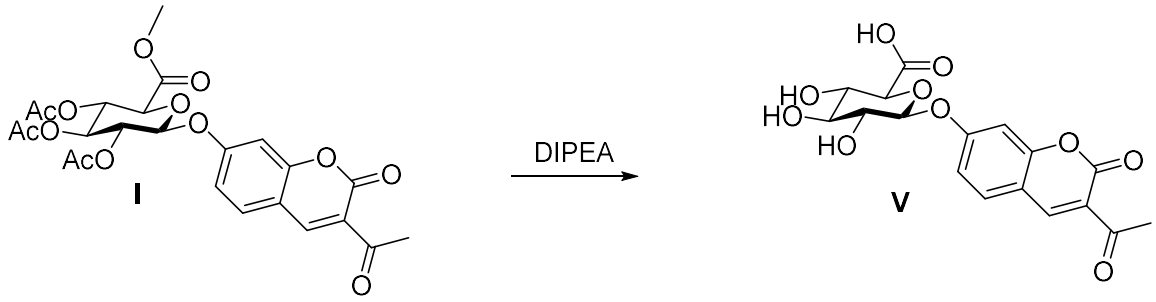
Procedure according to Bunnelle *et al.*[45] Compound **22** (200 mg, 0.38 mmol, 1 eq) was solved in MeOH (16 ml) and cooled to 0°C. Ethyldiisopropylamine (1.2 ml, 6.83 mmol, 18 eq) was added and the reaction was stirred for 3 h while it warmed up to room temperature. After all acetyl groups were removed (LCMS control) water (4 ml) was added and the reaction was stirred for another hour. Upon completion (LCMS control) he solvent was removed under reduced pressure and the crude product was purified via C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **25** as a white solid.

Yield = 37 mg (25 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) = 7.85 (s, 1H), 7.39 (s, 1H), 6.31 (s, 1H), 5.54 (d, J = 4.8 Hz, 1H), 5.37 (d, J = 7.2 Hz, 1H), 5.29 (d, J = 3.8 Hz, 1H), 4.08 (d, J = 9.6 Hz, 1H), 3.41 (t, J = 9.1 Hz, 1H), 2.40 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.98 (s, 1C), 159.65 (s, 1C), 154.46 (s, 1C), 152.88 (s, 1C), 152.59 (s, 1C), 126.14 (s, 1C), 117.98 (s, 1C), 114.79 (d, 1C), 112.74 (d, 1C), 103.51 (d, 1C), 99.29 (d, 1C), 75.88 (d, 1C), 75.31 (d, 1C), 72.73 (d, 1C), 71.15 (d, 1C), 18.09 (q, 1C) ppm

E.1.34 3-Acetylumbelliferyl-β-D-glucopyranosiduronic acid (V)



Molecular Weight: 520.44

Molecular Weight: 380.31

Procedure according to Bunnelle *et al.*[45] Compound **I** (100 mg, 0.19 mmol, 1 eq) was solved in MeOH (40 ml) and cooled to 0°C. Ethyldiisopropylamine (0.6 ml, 3.42 mmol, 18 eq) was added and the reaction was stirred for 3 h while it warmed up to room temperature. After all acetyl groups were removed (LCMS control) water (10 ml) was added and the reaction was stirred for another hour. Upon completion (LCMS control) the solvent was removed under reduced pressure and the crude product was purified via C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **V** as a pale yellow solid.

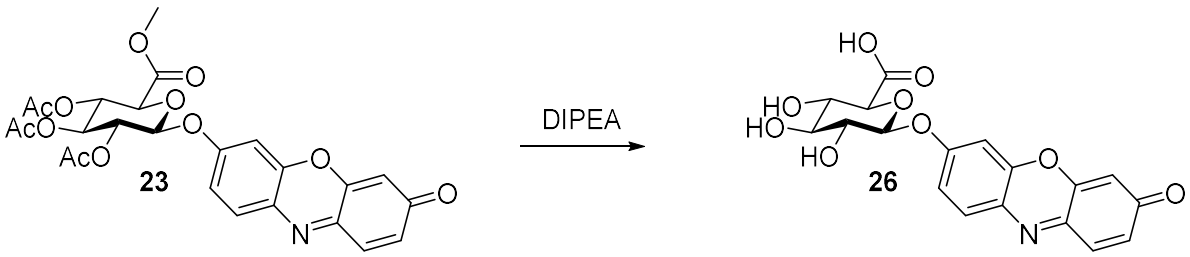
Yield = 14 mg (19 % of theory)

HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 381.0816 for $C_{17}H_{17}O_{10}^+$, found 381.0812.

^{1}H NMR (400 MHz, DMSO-d_6) = 8.64 (s, 1H), 7.91 (d, J = 8.7 Hz, 1H), 7.17 (d, J = 2.0 Hz, 1H), 7.07 (dd, J = 2.3, 8.7 Hz, 1H), 5.56 (s, 1H), 5.29 (d, J = 7.1 Hz, 1H), 4.05 (d, J = 9.5 Hz, 1H), 3.40 (t, J = 8.8 Hz, 1H), 2.56 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 194.81 (s, 1C), 170.01 (s, 1C), 161.94 (s, 1C), 158.76 (s, 1C), 156.62 (s, 1C), 147.36 (s, 1C), 132.23 (s, 1C), 121.24 (d, 1C), 114.30 (d, 1C), 112.84 (d, 1C), 102.40 (d, 1C), 99.11 (d, 1C), 75.70 (d, 1C), 75.28 (d, 1C), 72.73 (d, 1C), 71.21 (d, 1C), 30.05 (q, 1C) ppm

E.1.35 Resorufin β-D-glucopyranosiduronic acid (26)



Molecular Weight: 529.45

Molecular Weight: 389.32

Procedure according to Bunnelle *et al.*[45] Compound **23** (63 mg, 0.13 mmol, 1 eq) was solved in MeOH (8 ml) and cooled to 0°C. Ethyldiisopropylamine (0.41 ml, 2.38 mmol, 18 eq) was added and the reaction was stirred for 3 h while it warmed up to room temperature. After all acetyl groups were removed (LCMS control) water (2 ml) was added and the reaction was stirred for another hour. Upon completion (LCMS control) the solvent was removed under reduced pressure and the crude product was purified via C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **26** as a red solid.

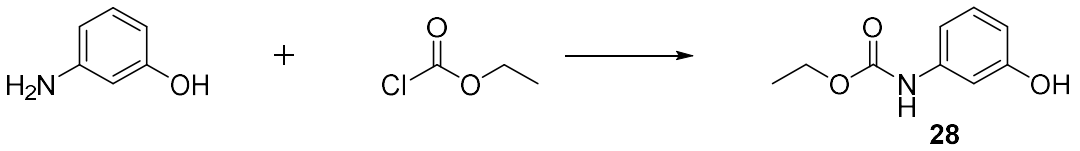
Yield = 18.4 mg (36 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) = 7.80 (d, J = 8.8 Hz, 1H), 7.53 (d, J = 9.8 Hz, 1H), 7.21 (d, J = 2.5 Hz, 1H), 7.10 (dd, J = 0.0, 8.9 Hz, 1H), 6.79 (dd, J = 2.0, 9.8 Hz, 1H), 5.57 (d, J = 2.8 Hz, 1H), 5.31 (s, 1H), 5.30 (s, 1H), 4.06 (d, J = 9.5 Hz, 1H), 3.41 (t, J = 9.1 Hz, 1H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 185.41 (s, 1C), 170.01 (s, 1C), 160.43 (s, 1C), 149.70 (s, 1C), 145.92 (s, 1C), 144.98 (s, 1C), 134.97 (d, 1C), 133.95 (s, 1C), 131.27 (d, 1C), 128.61 (d, 1C), 114.75 (d, 1C), 105.74 (d, 1C), 102.41 (d, 1C), 99.35 (d, 1C), 75.64 (d, 1C), 75.34 (d, 1C), 72.75 (d, 1C), 71.22 (d, 1C) ppm

Synthesis of Self Immolative β-Glucuronidase Responsive Probes

E.1.36 Ethyl 3-hydroxyphenylcarbamate (28)



Molecular Weight: 109.13 Molecular Weight: 108.52 Molecular Weight: 181.19

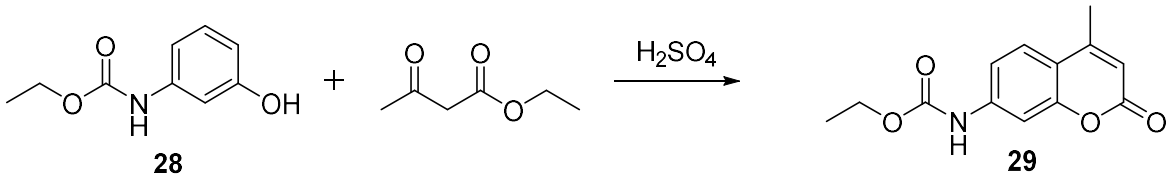
Procedure according to Ast *et al.*[61] 3-Aminophenol (75 g, 0.69 mol, 2 eq) was solved in ethyl acetate (500 ml) and heated under reflux for 30 minutes. Ethyl chloroformate (37.3 g, 0.34 mol, 1 eq) was added dropwise over a period of one hour while heating. The reaction was then allowed to cool to room temperature where a white precipitate formed. The precipitate was removed by filtration and washed with ethyl acetate (3 x 150 ml) and petroleum ether (2 x 150 ml). The filtrates were combined, and the solvent was then removed under reduced pressure. The crude product was the crystallized from petroleum ether to give compound **28** as off white solid.

Yield = 49.87 g (80 % of theory)

^{1}H NMR (200 MHz, CD_3OD) δ = 7.09 – 7.01 (m, 2H), 6.81 (d, J = 7.9 Hz, 1H), 6.45 (dd, J = 2.2, 8.1 Hz, 1H), 4.15 (q, J = 7.1 Hz, 2H), 1.28 (t, J = 7.1 Hz, 3H) ppm

^{13}C NMR (50 MHz, CD_3OD) δ = 158.86 (s, 1C), 155,97 (s, 1C), 141.28 (s, 1C), 130.45 (d, 1C), 111.05 (d, 1C), 110.87 (d, 1C), 106.91 (d, 1C), 61.76 (t, 1C), 14.88 (q, 1C) ppm

E.1.37 7-Carbethoxyamido-4-methylcoumarin (29)



Molecular Weight: 181.19 Molecular Weight: 130.14 Molecular Weight: 247.25

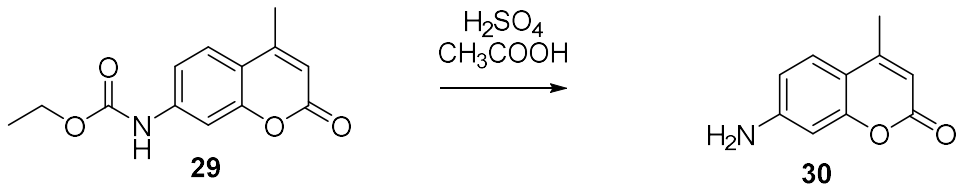
Procedure according to Ast *et. al.*[61] Ethylacetoacetonat (4.3 g, 33.1 mmol, 1.2 eq) and compound **28** (5 g, 27.6 mmol, 1 eq) were dissolved in H_2SO_4 (75 ml, 70% in water) and stirred for four 16 hours. The reaction mixture was then poured into ice water, where a white precipitate formed. The crude product was crystallized from ethanol to give compound **29** as an off white solid.

Yield = 5.03 g (74 % of theory)

^{1}H NMR (200 MHz, DMSO-d_6) δ = 10.12 (s, 1H), 7.65 (d, J = 8.7 Hz, 1H), 7.52 (d, J = 1.9 Hz, 1H), 7.38 (dd, J = 2.0, 8.7 Hz, 1H), 6.20 (s, 1H), 4.16 (q, J = 7.1 Hz, 2H), 2.36 (s, 3H), 1.26 (t, J = 7.1 Hz, 3H) ppm

^{13}C NMR (50 MHz, DMSO-d_6) δ = 160.01 (s, 1C), 153.79 (s, 2C), 153.28 (s, 1C), 153.12 (s, 1C), 142.83 (s, 1C), 125.90 (d, 1C), 114.14 (d, 1C), 111.79 (d, 1C), 104.30 (d, 1C), 60.66 (t, 1C), 17.94 (q, 1C), 14.37 (q, 1C) ppm

E.1.38 7-Amino-4-methylcoumarin (30)



Molecular Weight: 247.25 Molecular Weight: 175.19

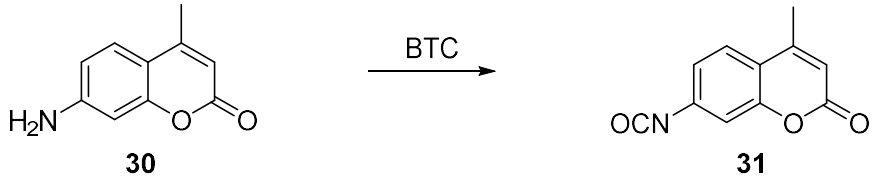
Procedure according to Ast *et al.*[61] Compound **29** (2 g, 8 mmol, 1 eq) was suspended in conc. H_2SO_4 (20 ml) and acetic acid (7.2 g, 120 mmol, 15 eq) and heated to 120°C. The reaction was then stirred at 120°C for four hours while color turned from yellow to orange. Afterwards the mixture was allowed to cool to room temperature, was then poured into cold water and was placed in the fridge overnight where an off-white solid formed. The suspension was brought to pH 7 with NaOH (10 % in water) and was filtrated. The residue was suspended in ethanol (250 ml) and heated to reflux. The still hot suspension was filtrated, and the filtrate was concentrated under reduced pressure to give compound **30** as a light yellow solid.

Yield = 1.14 g (81 % of theory)

^{1}H NMR (200 MHz, DMSO-d_6) δ = 7.38 (d, J = 8.6 Hz, 1H), 6.56 (dd, J = 2.1, 8.6 Hz, 1H), 6.40 (d, J = 2.1 Hz, 1H), 6.13 (s, 2H), 5.89 (s, 1H), 2.29 (s, 3H) ppm

^{13}C NMR (50 MHz, DMSO-d_6) δ = 160.75 (s, 1C), 155.46 (s, 1C), 153.74 (s, 1C), 153.10 (s, 1C), 126.19 (d, 1C), 111.17 (d, 1C), 108.82 (s, 1C), 107.44 (d, 1C), 98.51 (d, 1C), 18.01 (q, 1C) ppm

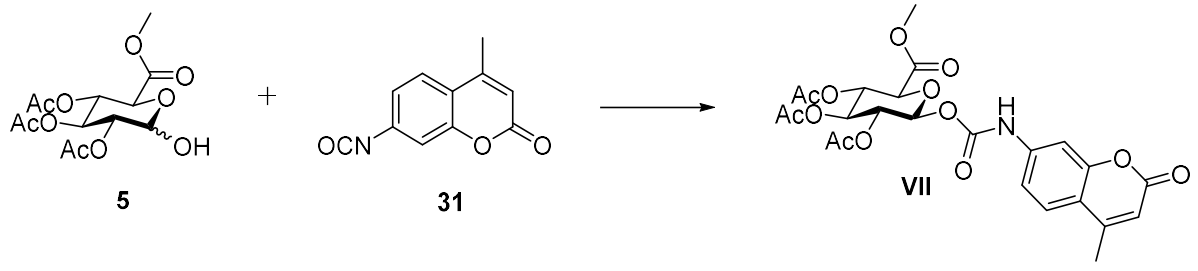
E.1.39 7-Isocyanato-4-methylcoumarin (31)



30 Molecular Weight: 175.19 **31** Molecular Weight: 201.18

Procedure according to Renslo *et al.*[62] Compound **30** (200 mg, 1.14 mmol, 1 eq) and triphosgene (170 mg, 0.58 mmol, 0.5 eq) were dispersed in dry toluene (20 ml) and refluxed for 16 hours. Since the isolation of compound **31** failed several times, the crude reaction mixture was taken for the next step.

E.1.40 [[[N-(4-methyl-2-oxo-2*H*-1-benzopyran-7-yl)amino]carbonyl]oxy]-2,3,4-triacetyl-β-D-glucopyranosiduronic acid, methyl ester (VII)



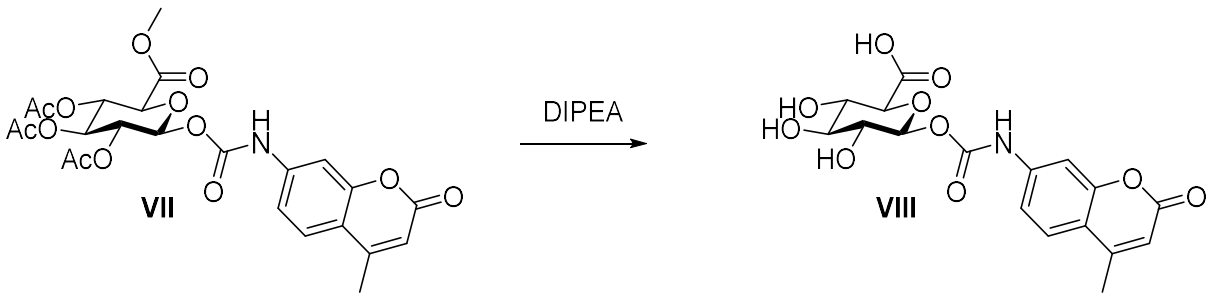
5 Molecular Weight: 334.28 **31** Molecular Weight: 201.18 **VII** Molecular Weight: 535.46

Procedure according to El Alaoui *et al.*[24] A suspension of compound **31** (230 mg, 1.14 mmol, 1 eq) in toluene was cooled to 0°C and compound **5** (382 mg, 1.14 mmol, 1 eq) and triethylamine (116 mg, 1.14 mmol, 1 eq) were added. After stirring for 2 hours at 0°C the solvent was

removed under reduced pressure and the crude product was purified via flash chromatography (PE/EtOAc = 3:1 – 1:1) to give compound **VII** as a white solid.
Yield = 464 mg (76 % of theory)

HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 536.1399 for $C_{24}H_{26}NO_{13}^+$, found 536.1396.
^1H NMR (400 MHz, DMSO-d_6) δ = 10.61 (s, 1H), 7.71 (d, J = 8.8 Hz, 1H), 7.53 (s, 1H), 7.43 (dd, J = 1.6, 8.7 Hz, 1H), 6.26 (s, 1H), 6.13 (d, J = 8.2 Hz, 1H), 5.57 (t, J = 9.6 Hz, 1H), 5.09 - 5.02 (m, 2H), 4.73 (d, J = 9.9 Hz, 1H), 3.64 (s, 3H), 2.39 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.98 (s, 3H) ppm
^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.44 (s, 1C), 169.35 (s, 1C), 169.11 (s, 1C), 166.97 (s, 1C), 159.90 (s, 1C), 153.67 (s, 1C), 153.06 (s, 1C), 150.85 (s, 1C), 141.66 (s, 1C), 126.11 (d, 1C), 115.02 (s, 1C), 114.59 (d, 1C), 112.37 (d, 1C), 105.05 (d, 1C), 91.64 (d, 1C), 71.18 (s, 2C), 69.86 (d, 1C), 68.80 (d, 1C), 52.60 (q, 1C), 20.32 (q, 1C), 20.26 (q, 1C), 20.20 (q, 1C), 17.95 (q, 1C) ppm

E.1.41 [[[N-(4-methyl-2-oxo-2*H*-1-benzopyran-7-yl)amino]carbonyl] oxy]-β-D-glucopyranosiduronic acid (VIII)



Molecular Weight: 535.46 Molecular Weight: 395.32

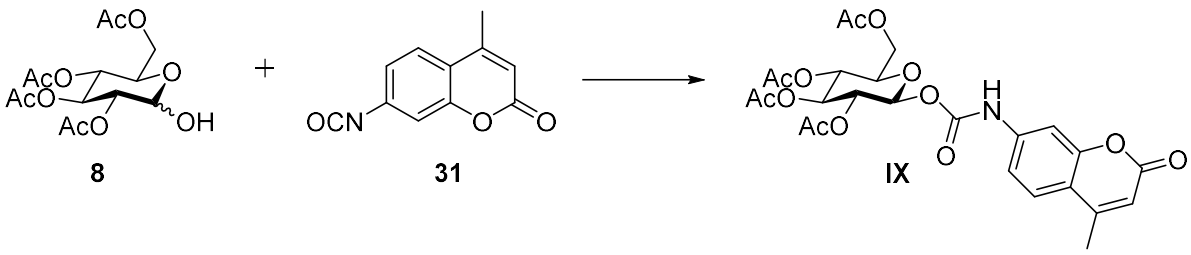
Procedure according to Bunnelle *et al.*[45] Compound **VII** (50 mg, 0.1 mmol, 1 eq) was solved in MeOH/H_2O (2/1, 6 ml) and cooled to 0°C. Ethyldiisopropylamine (0.3 ml, 1.8 mmol, 18 eq) was added and the reaction was stirred for 8 h while it warmed up to room temperature. The solvent was removed under reduced pressure and the crude product was purified via prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **VIII** as a white solid.
Yield = 38 mg (95 % of theory)

HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 396.0925 for $C_{17}H_{18}NO_{10}^+$, found 396.0926.

^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.48 (s, 1H), 7.73 (d, J = 8.7 Hz, 1H), 7.55 (d, J = 1.8 Hz, 1H), 7.45 (dd, J = 1.9, 8.7 Hz, 1H), 6.26 (d, J = 1.1 Hz, 1H), 5.46 – 5.42 (m, 2H), 5.32 (d, J = 4.7 Hz, 1H), 3.78 (d, J = 9.1 Hz, 1H), 3.26 – 3.20 (m, 1H), 2.39 (s, 3H) ppm

^{13}C NMR (150 MHz, DMSO-d_6) δ = 169.88 (s, 1C), 160.00 (s, 1C), 153.77 (s, 1C), 153.21 (s, 1C), 151.84 (s, 1C), 142.17 (s, 1C), 126.20 (d, 1C), 114.82 (s, 1C), 114.49 (d, 1C), 112.22 (d, 1C), 104.83 (d, 1C), 95.18 (d, 1C), 76.04 (d, 1C), 75.90 (d, 1C), 72.15 (d, 1C), 71.34 (d, 1C), 18.03 (q, 1C) ppm

E.1.42 N-[7-Amino-4-methylcoumarin]-O-[(2,3,4,6-tetraacetyl)-β-D-glucosyl]carbamic acid (IX)



Molecular Weight: 348.30 Molecular Weight: 201.18 Molecular Weight: 549.49

Procedure according to El Alaoui *et al.*[24] A suspension of compound **31** (230 mg, 1.14 mmol, 1 eq) in toluene was cooled to 0°C and compound **8** (397 mg, 1.14 mmol, 1 eq) and triethylamine (116 mg, 1.14 mmol, 1 eq) were added. After stirring for two hours at 0°C the solvent was removed under reduced pressure and the crude product was purified via flash chromatography (PE/EtOAc = 3:1 – 1:1) to give compound **IX** as a white solid.

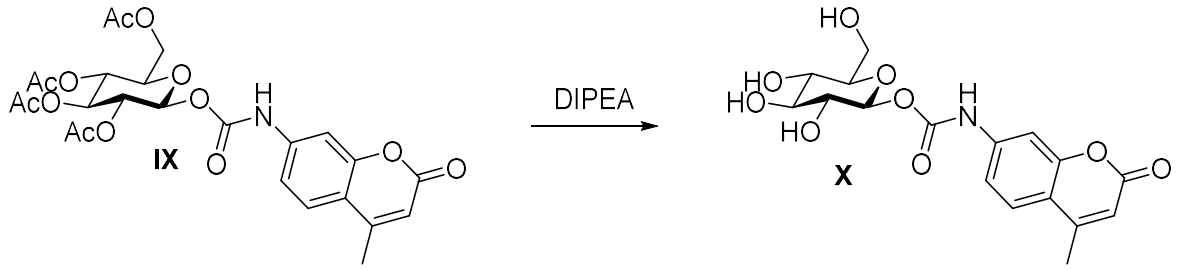
Yield = 163 mg (26 % of theory)

^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.56 (s, 1H), 7.72 (d, J = 8.9 Hz, 1H), 7.54 (s, 1H), 7.42 (dd, J = 2.0, 8.7 Hz, 1H), 6.26 (s, 1H), 6.07 (d, J = 8.3 Hz, 1H), 5.49 (t, J = 9.6 Hz, 1H), 5.15 – 5.07 (m, 1H), 5.04 -4.98 (m, 2H), 4.28 – 4.16 (m, 2H), 2.39 (s, 3H), 2.02 (s, 6H), 2.01 (s, 6H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.97 (s, 1C), 169.49 (s, 1C), 169.29 (s, 1C), 169.16 (s, 1C), 159.91 (s, 1C), 153.69 (s, 1C), 153.09 (s, 1C), 150.97 (s, 1C), 141.76 (s, 1C), 126.13 (d, 1C), 114.98 (s, 1C), 114.58 (d, 1C), 112.34 (d, 1C), 105.01 (d, 1C), 91.81 (d, 1C), 71.98 (d, 1C), 71.17

(d, 1C), 70.10 (d, 1C), 69.27 (d, 1C), 67.60 (d, 1C), 61.39 (t, 1C), 20.46 (q, 1C), 20.36 (q, 1C), 20.34 (q, 1C), 20.25 (q, 1C), 17.97 (q, 1C) ppm

E.1.43 N-[4-Methylcoumarin-7yl]-O-[-β-D-glucosyl]carbamic acid (X)



Molecular Weight: 549.49 Molecular Weight: 381.34

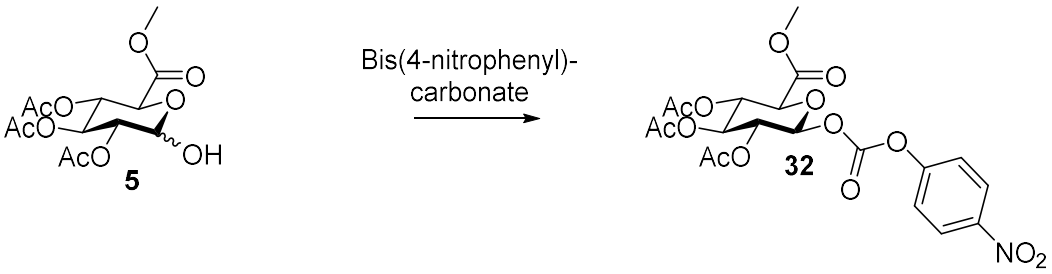
Procedure according to Bunnelle *et al.*[45] Compound **IX** (50 mg, 0.1 mmol, 1 eq) was solved in MeOH (4 ml) and cooled to 0°C. Ethyldiisopropylamine (0.3 ml, 1.8 mmol, 18 eq) was added and the reaction was stirred for 8 h while it warmed up to room temperature. The solvent was removed under reduced pressure and the crude product was purified via prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **X** as a white solid.

Yield = 38 mg (90 % of theory)

^1H NMR (400 MHz, DMSO-d_6) δ = 10.37 (s, 1H), 7.69 (d, J = 8.7 Hz, 1H), 7.37 (s, 1H), 7.22 (dd, J = 2.0, 8.6 Hz, 1H), 5.18 (d, J = 4.8 Hz, 1H), 4.83 (d, J = 4.7 Hz, 1H), 4.85 (m, 2H), 4.39 (t, J = 5.6 Hz, 1H), 3.51 (m, 1H), 3.26 (m, 2H), 3.06 (m, 2H), 3.95 (m, 1H), 1.91 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 159.78 (s, 1C), 153.53 (s, 1C), 152.89 (s, 1C), 150.65 (s, 1C), 141.55 (s, 1C), 125.87 (d, 1C), 114.74 (s, 1C), 114.53 (d, 1C), 112.13 (d, 1C), 104.89 (d, 1C), 91.60 (d, 1C), 71.77 (d, 1C), 71.01 (d, 1C), 69.91 (d, 1C), 69.04 (d, 1C), 67.40 (d, 1C), 61.12 (t, 1C), 17.97 (q, 1C) ppm

E.1.44 [[(4-Nitrophenoxy)carbonyl]oxy]-2,3,4-triacetyl-β-D-glucopyranosiduronic acid, methyl ester (32)



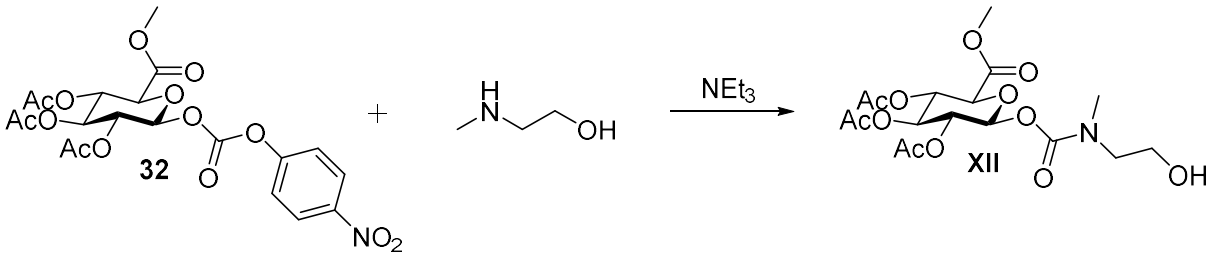
Molecular Weight: 334.28 Molecular Weight: 499.38

Procedure according to Bunnelle *et al.*[45] To a solution of compound **5** (1.50 g, 4.5 mmol, 1 eq) and triethylamine in dichloromethane (30 ml) was added dropwise a solution of bis(4-nitrophenyl)carbonate in dichloromethane (30 ml) at 0°C. The mixture was stirred at 0°C for 30 min and then washed with 5 % Na_2CO_3 solution and 4% H_2SO_4. The organic phase was dried over Na_2SO_4 and solvent was removed under reduced pressure leaving a colorless oil. The crude product was crystalized from diethyl ether to give compound **32** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.33 (d, J = 9.2 Hz, 1H), 7.63 (d, J = 9.1 Hz, 2H), 6.09 (d, J = 7.6 Hz, 2H), 5.54 (t, J = 9.3 Hz, 1H), 5.13 – 5.08 (m, 2H), 4.77 (d, J = 9.6 Hz, 1H), 3.66 (s, 3H), 2.07 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H) ppm

^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.41 (s, 1C), 169.29 (s, 1C), 169.10 (s, 1C), 166.86 (s, 1C), 154.58 (s, 1C), 150.35 (s, 1C), 145.56 (s, 1C), 125.52 (s, 1C), 94.95 (s, 1C), 71.41 (s, 1C), 70.37 (s, 1C), 69.47 (s, 1C), 68.36 (s, 1C) ppm

E.1.45 [[[N-(2-hydroxyethyl)-N-methylamino]carbonyl]oxy]-2,3,4-tri-acetyl-β-D-glucopyranosiduronic acid, methyl ester (XII)



Molecular Weight: 499.38 Molecular Weight: 75.11 Molecular Weight: 435.38

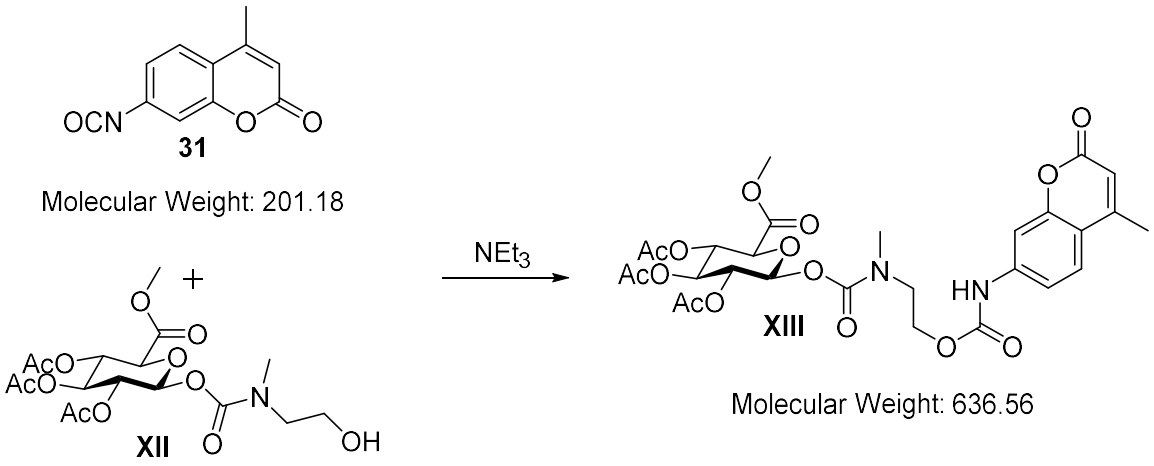
Procedure according to Bunnelle *et al.*[45] Compound **32** (500 mg, 1 mmol, 1 eq) was solved in dichloromethane (6 ml) and 2-(methylamino)ethanol (113 mg, 1.5 mmol, 1.5 eq) and triethylamine (0.49 ml, 3.5 mmol, 3.5 eq) were added. After one hour (TLC control), the mixture was washed with $NaHCO_3$ (sat.) and NH_4Cl (sat.), both aqueous phases were washed with dichloromethane and the combined organic phases were dried over Na_2SO_4. The crude product was crystalized from diethyl ether (10 ml) to give compound **XII** as white solid.
Yield = 318 mg (73 % of theory)

Both Rotamers:

^{1}H NMR (600 MHz, $CDCl_3$) δ = 5.74 (d, J = 7.7 Hz, 1H), 5.70 (d, J = 8.0 Hz, 1H), 5.38-5.32 (m, 2H), 5.22-51.3 (m, 4H), 4.24 (d, J = 9.7 Hz, 1H), 4.19 (d, J = 9.8 Hz, 1H), 3.75-3.73 (m, 2H), 3.70 (d, J = 1.3 Hz, 6H), 3.65 (t, J = 5.6 Hz, 2H), 3.59-3.54 (m, 1H), 3.42 (t, J = 5.5 Hz, 2H), 3.20-3.16 (m, 1H), 2.96 (s, 3H), 2.92 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 2.01 (s, 12H) ppm

^{13}C NMR (150 MHz, $CDCl_3$) δ = 170.04 (s, 1C), 170.02 (s, 1C), 169.94 (s, 2C), 169.61 (s, 1C), 169.45 (s, 1C), 167.28 (s, 1C), 167.06 (s, 1C), 154.58 (s, 1C), 153.76 (s, 1C), 92.95 (d, 2C), 72.67 (d, 2C), 71.65 (d, 1C), 71.60 (d, 1C), 70.16 (d, 1C), 70.07 (d, 1C), 69.34 (d, 1C), 69.17 (d, 1C), 60.50 (t, 1C), 60.26 (t, 1C), 53.05 (q, 2C), 52.02 (t, 1C), 51.09 (t, 1C), 35.57 (q, 1C), 35.45 (q, 1C), 20.75 (q, 1C), 20.71 (q, 1C), 20.69 (q, 1C), 20.67 (q, 1C), 20.56 (q, 2C) ppm

E.1.46 [[[N-[2-[[[N-(4-methyl-2-oxo-2H-1-benzopyran-7-yl)amino] carbonyl]oxy]ethyl]-N-methylamino]carbonyl]oxy]-2,3,4-triacetyl-β-D-glucopyranosiduronic acid, methyl ester (XIII)



Procedure according to El Alaoui *et al.*[24] A suspension of compound **31** (173 mg, 0.86 mmol, 1.5 eq) in toluene (40 ml) was cooled to 0°C and compound **XII** (250 mg, 0.57 mmol, 1 eq) and triethylamine (304 mg, 3 mmol, 5.3 eq) were added. After stirring for two hours at 0°C the solvent was removed under reduced pressure and the crude product was purified via flash chromatography (PE/EtOAc = 3:1 – 1:1) to give compound **XIII** as a white solid.

Yield = 236 mg (65 % of theory)

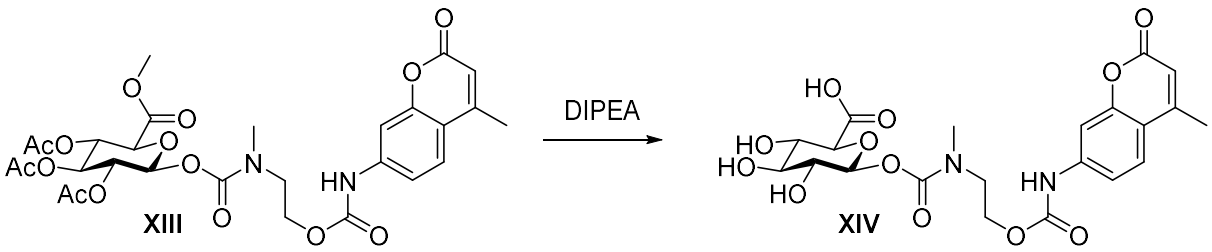
HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 637.1875 for $C_{28}H_{33}N_2O_{15}^+$, found 367.1869.

Both Rotamers:

^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.19 (s, 1H), 10.15 (s, 1H), 7.69 (d, J = 8.7 Hz, 2H), 7.55 (dd, J = 1.8, 15.6 Hz, 2H), 7.40 (dd, J = 0.0, 8.7 Hz, 2H), 6.23 (s, 2H), 5.88 (dd, J = 2.6, 8.2 Hz, 2H), 5.55-5.47 (m, 2H), 5.03-4.96 (m, 3H), 4.88 (t, J = 9.6 Hz, 1H), 4.63 (t, J = 9.5 Hz, 2H), 4.26 (t, J = 5.4 Hz, 2H), 4.22-4-18 (m, 1H), 4.15-4.10 (m, 1H), 3.62 (d, J = 1.1 Hz, 6H), 3.53 (t, J = 5.3 Hz, 2H), 3.48 (t, J = 5.6 Hz, 2H), 2.93 (s, 3H), 2.88 (s, 3H), 2.39 (d, J = 1.0 Hz, 6H), 2.01 (s, 3H), 1.99 (s, 3H), 1.97 (s, 3H), 1.97 (s, 6H), 1.95 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.36 (s, 1C), 169.33 (s, 1C), 169.26 (s, 1C), 169.14 (s, 1C), 169.05 (s, 2C), 167.04 (s, 1C), 166.92 (s, 1C), 160.04 (s, 1C), 159.98 (s, 1C), 153.79 (s, 2C), 153.16 (s, 1C), 153.13 (s, 1C), 153.08 (s, 2C), 152.95 (s, 1C), 152.79 (s, 1C), 142.60 (s, 1C), 142.56 (s, 1C), 125.95 (d, 2C), 114.41 (s, 1C), 114.38 (s, 1C), 114.34 (d, 2C), 111.92 (d, 1C), 111.88 (d, 1C), 104.54 (d, 2C), 92.00 (d, 2C), 71.20 (d, 1C), 71.13 (d, 1C), 70.81 (d, 1C), 70.54 (d, 1C), 69.77 (d, 1C), 69.68 (d, 1C), 68.93 (d, 1C), 68.84 (d, 1C), 62.31 (t, 1C), 61.75 (t, 1C), 52.52 (q, 1C), 47.87 (t, 1C), 47.28 (t, 1C), 35.45 (q, 1C), 34.40 (q, 1C), 20.24 (q, 2C), 20.17 (q, 4C), 17.94 (q, 2C) ppm

E.1.47 [[[N-[2-[[[N-(4-methyl-2-oxo-2H-1-benzopyran-7-yl)amino] carbonyl]oxy]ethyl]-N-methylamino]carbonyl]oxy]-β-D-glucopyranosiduronic acid (XIV)



Molecular Weight: 636.56 Molecular Weight: 496.43

Procedure according to Bunnelle *et al.*[45] Compound **XIII** (100 mg, 0.16 mmol, 1 eq) was solved in MeOH (12 ml) and cooled to 0°C. Ethyldiisopropylamine (0.49 ml, 2.83 mmol, 18 eq) was added and the reaction was stirred for 3 h while it warmed up to room temperature. After all acetyl groups were removed (LCMS control) water (3 ml) was added and the reaction was stirred for another hour. Upon completion (LCMS control) the solvent was removed under reduced pressure and the crude product was purified via C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **XIV** as a colorless oil.

Yield = 51 mg (65 % of theory)

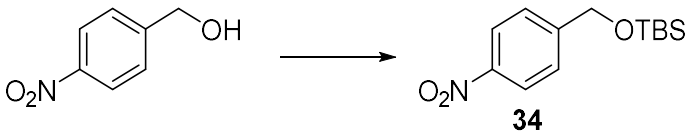
HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 497.1402 for $C_{21}H_{25}N_2O_{12}^+$, found 497.1394

Both Rotamers:

^{1}H NMR (400 MHz, DMSO-d_6) δ =10.21 (s, 1H), 10.09 (s, 1H), 7.67 (d, J = 8.7 Hz, 2H), 7.53 (s, 2H), 7.41 (dd, J = 0.0, 8.7 Hz, 2H), 6.22 (s, 2H), 5.40 (d, J = 10.7 Hz, 2H), 5.30-5.18 (m, 4H), 4.30-4.21 (m, 4H), 3.70 (d, J = 9.4 Hz, 2H), 3.58-3.14 (s, 14H), 2.97 (s, 3H), 2.93 (s, 3H), 2.38 (s, 6H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.97 (s, 2C), 160.08 (s, 2C), 154.20 (s, 2C), 153.92 (s, 1C), 153.81 (s, 2C), 153.21 (s, 2C), 153.11 (s, 2C), 142.68 (s, 1C), 142.61 (s, 1C), 126.00 (d, 2C), 114.41 (d, 3C), 111.95 (d, 2C), 104.56 (d, 2C), 95.63 (d, 1C), 95.57 (d, 1C), 76.02 (d, 2C), 75.74 (d, 2C), 72.19 (d, 2C), 71.39 (d, 1C), 71.34 (d, 1C), 62.88 (t, 1C), 62.02 (t, 1C), 47.72 (t, 1C), 47.35 (t, 1C), 35.51 (q, 1C), 34.53 (q, 1C), 18.00 (q, 2C) ppm

E.1.48 1-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]methyl]-4-nitrobenzene (34)



34

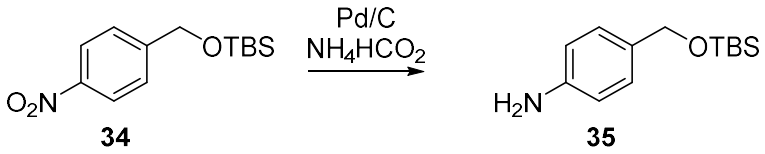
Molecular Weight: 153.14 Molecular Weight: 267.40

Procedure according to El Alaoui *et al.*[24] To a solution of 4-nitrobenzylic alcohol (5 g, 32 mmol, 1 eq) and imidazole (4.67 g, 67 mmol, 2.1 eq) in abs. DMF (35 ml) was added tert-butyldimethylsilyl chloride (4.8 g, 32 mmol, 1 eq) dropwise as 3 M solution in THF. After stirring for two hours at room temperature, the mixture was diluted with EtOAc (250 mL), washed with water (6 × 70 mL), dried over Na_2SO_4, and evaporated. The crude product was purified via flash chromatography (PE/EtOAc = 1:0 – 5:1), to give compound **34** as a colorless oil.
Yield = 8.55 g (quantitative)

^{1}H NMR (200 MHz, $CDCl_3$) δ = 8.20 (d, J = 8.6 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 4.83 (s, 2H), 0.96 (s, 9H), 0.12 (s, 6H) ppm

^{13}C NMR (50 MHz, $CDCl_3$) δ = 148.93 (s, 1C), 146.75 (s, 1C), 126.03 (d, 2C) 123.35 (d, 2C), 63.81 (t, 1C), 25.64 (q, 3C), 18.15 (s, 1C), -5.43 (q, 2C) ppm

E.1.49 4-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]methyl]aniline (35)

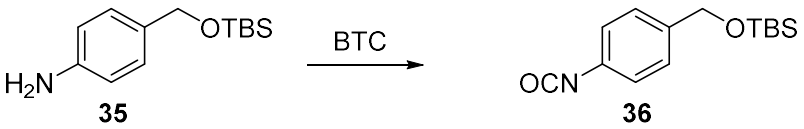


Molecular Weight: 267.40 Molecular Weight: 237.42

Procedure according to Sloniec *et al.*[66]. Pd (10% on charcoal) and ammonium formate were added to a solution of compound **34** in abs. ethanol (150 mL). After the reaction was stirred for two hours at 0°C and 12 hours at room temperature, the catalyst was eliminated by filtration over Celite. The filtrate was removed under reduced and then dissolved in EtOAc (200 mL). The solution was washed with water (200 mL), dried over Na_2SO_4, and the solvent again removed under reduced pressure. The crude product was purified via flash chromatography (PE/EtOAc = 1:0 – 5:1), to give compound **35** as a pale-yellow oil.
Yield = 51 mg (65 % of theory)

^{1}H NMR (200 MHz, $CDCl_3$) δ = 7.12 (d, J = 8.1 Hz, 2H), 6.70 (d, J = 8.3 Hz, 2H), 4.63 (s, 2H), 0.92 (s, 9H), 0.08 (s, 6H) ppm
^{13}C NMR (50 MHz, $CDCl_3$) δ = 145.18 (s, 1C), 131.37 (s, 1C), 127.54 (d, 2C) 114.85 (d, 2C), 64.87 (t, 1C), 25.84 (q, 3C), 18.23 (s, 1C), -5.20 (q, 2C) ppm

E.1.50 1-[[[(1,1-Dimethylethyl)dimethyllsilyl]oxy]methyl]-4-iso-cyanatobenzene (36)

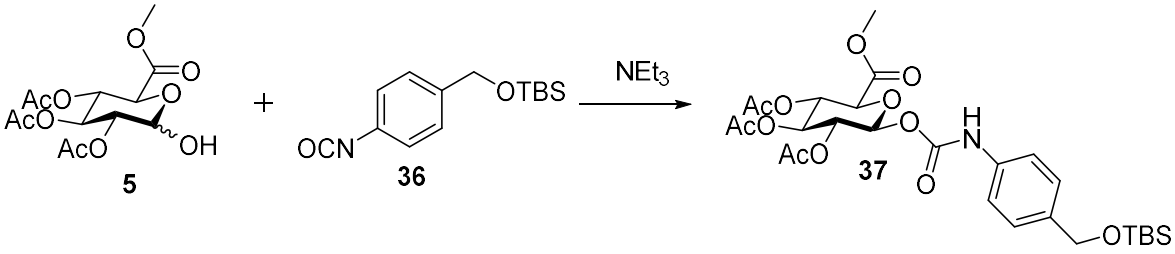


Molecular Weight: 237.42 Molecular Weight: 263.41

Procedure according to Renslo *et. al.*[62] Compound **35** (2 g, 8.42 mmol, 1 eq) and triphosgene (1.24 g, 4.21 mmol, 0.5 eq) were dispersed in dry toluene (100 ml) and refluxed for 16 hours.

Due to the instability of the isocyanate the, the crude reaction mixture was taken for the next step.

E.1.51 [[[[4-[[[(1,1-Dimethylethyl)dimethylsilyl]oxy]methyl]phenyl] amino]carbonyl]oxy]-2,3,4-tri-acetyl-β-D-glucopyranosiduronic acid, methyl ester (37)

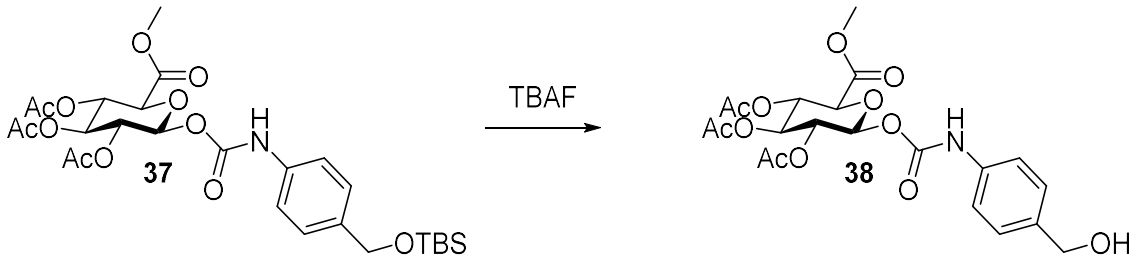


Molecular Weight: 334.28 Molecular Weight: 263.41 Molecular Weight: 597.69

Procedure according to El Alaoui *et al.*[24] A suspension of compound **36** (2.2 g, 8.42 mmol, 1 eq) in toluene (100 ml) was cooled to 0°C and compound **5** (2.81 g, 8.42 mmol, 1 eq) and triethylamine (4 ml, 29.5 mmol, 3.5 eq) were added. After stirring for two hours at 0°C the solvent was removed under reduced pressure and the crude product was purified via flash chromatography (PE/EtOAc = 3:1 – 1:1) to give compound **37** as a white solid.
Yield = 3.55 g (71 % of theory)

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.32 (d, J = 8.3 Hz, 2H), 7.25 (d, J = 8.4 Hz, 2H), 6.95 (s, 1H), 5.79 (d, J = 8.0 Hz, 1H), 5.36 (t, J = 9.4 Hz, 1H), 5.26 (t, J = 9.6 Hz, 1H), 5.20 (dd, J = 8.1, 9.2 Hz, 1H), 4.68 (s, 2H), 4.22 (d, J = 9.8 Hz, 1H), 3.72 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 0.92 (s, 9H), 0.07 (s, 6H) ppm
^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.97 (s, 1C), 169.56 (s, 2C), 166.93 (s, 1C), 150.63 (s, 1C), 137.56 (s, 1C), 135.64 (s, 1C), 127.03 (d, 2C), 118.84 (d, 1C), 92.62 (d, 1C), 72.92 (d, 1C), 72.04 (d, 1C), 70.10 (d, 1C), 69.22 (d, 1C), 64.63 (t, 1C), 53.11 (d, 1C), 26.04 (q, 3C), 20.74 (q, 1C), 20.66 (q, 1C), 20.57 (q, 2C), 18.50 (s, 1C), -5.38 (q, 2C) ppm

E.1.52 [[[[4-(Hydroxymethyl)phenyl]amino]carbonyl]oxy]-2,3,4-tri-acetyl-β-D-glucopyranosiduronic acid, methyl ester (38)



Molecular Weight: 597.69 Molecular Weight: 483.43

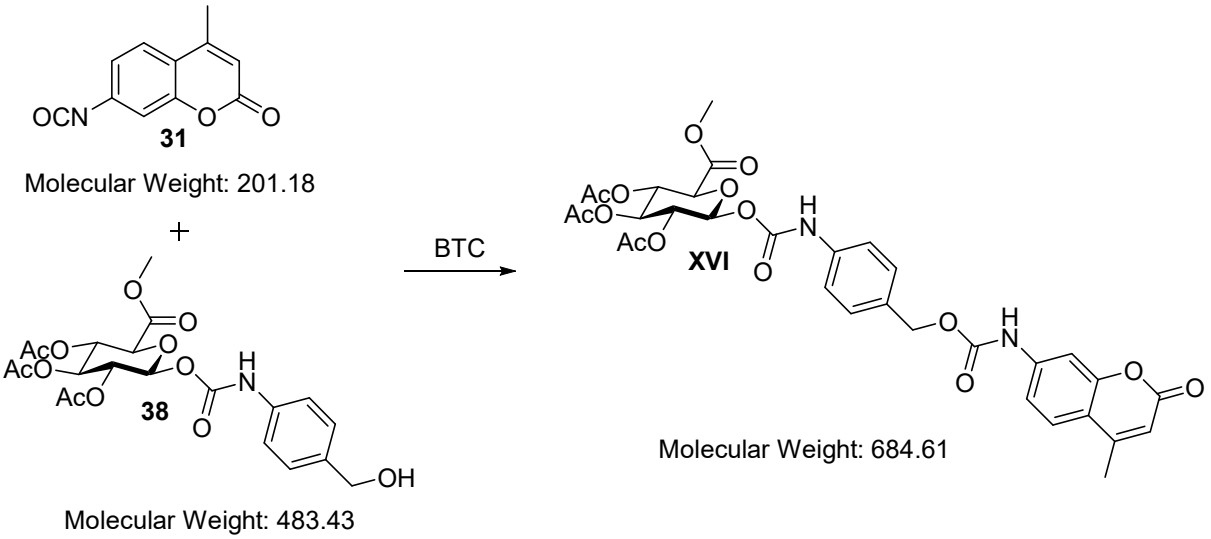
Procedure according to Erez *et al*[73] Compound **37** (300 mg, 0.5 mmol, 1 eq) was dissolved in abs. THF and acetic acid (0.14 ml, 2.5 mmol, 5 eq) was added at room temperature. After stirring for 15 minutes, 1 M TBAF in THF (3.5 ml, 3.5 mmol, 7 eq) was added to the reaction and the progress was monitored via TLC. Upon completion (two hours) the reaction mixture was directly purified via flash chromatography (PE/EtOAc = 3:1 – 0:1), to give compound **38** as a colorless oil.

Yield = 214 mg (88 % of theory)

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.31 (d, J = 8.4 Hz, 2H), 7.25 (d, J = 8.5 Hz, 2H), 5.68 (d, J = 8.0 Hz, 1H), 5.25 (t, J = 9.3 Hz, 1H), 5.16 (t, J = 9.6 Hz, 1H), 5.07 (t, J = 8.7 Hz, 1H), 4.58 (s, 2H), 4.12 (d, J = 9.8 Hz, 1H), 3.67 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.97 (s, 1H) ppm

^{13}C NMR (100 MHz, $CDCl_3$) δ = 170.03 (s, 1C), 169.73 (s, 1C), 169.65 (s, 1C), 166.99 (s, 1C), 150.70 (s, 1C), 136.89 (s, 1C), 136.44 (s, 1C), 128.12 (d, 2C), 118.99 (d, 1C), 92.57 (d, 1C), 72.82 (d, 1C), 71.92 (d, 1C), 70.00 (d, 1C), 69.17 (d, 1C), 64.93 (t, 1C), 53.20 (d, 1C), 20.78 (q, 1C), 20.69 (q, 2C), 20.60 (q, 2C) ppm

E.1.53 [[[[4-[[[[N-(4-Methyl-2-oxo-2H-1-benzopyran-7-yl)amino] carbonyl]oxy]methyl]phenyl]amino]carbonyl]oxy]-2,3,4-tri-acetyl-β-D-glucopyranosiduronic acid, methyl ester (XVI)



Procedure according to El Alaoui *et al.*[24] A suspension of compound **31** (87 mg, 0.43 mmol, 2.15 eq) in toluene (25 ml) was cooled to 0°C and compound **38** (100 mg, 0.20 mmol, 1 eq) and triethylamine (102 mg, 1 mmol, 5. eq) were added. After stirring for two hours at 0°C the solvent was removed under reduced pressure and the crude product was purified via flash chromatography (PE/EtOAc = 3:1 – 1:1) to give compound **XVI** as a white solid.

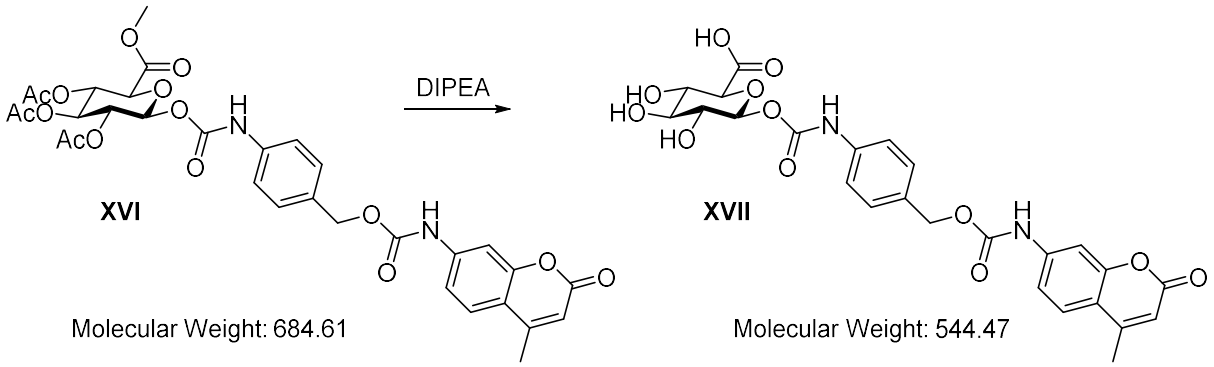
Yield = 70 mg (51 % of theory)

HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 685.1875 for $C_{32}H_{33}N_2O_{15}^+$, found 685.18628.

^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.26 (s, 1H), 10.22 (s, 1H), 7.69 (dd, J = 0.0, 8.7 Hz, 1H), 7.54 (s, 1H), 7.50 (d, J = 8.2 Hz, 2H), 7.40 (d, J = 8.5 Hz, 3H), 6.23 (s, 1H), 6.09 (d, J = 8.2 Hz, 1H), 5.56 (t, J = 9.6 Hz, 1H), 5.12 (s, 1H), 5.06-5.00 (m, 2H), 4.71 (d, J = 9.9 Hz, 1H), 3.63 (s, 3H), 2.38 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.98 (s, 3H) ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ = 69.49 (s, 1C), 169.41 (s, 1C), 169.13 (s, 1C), 167.07 (s, 1C), 160.06 (s, 1C), 153.85 (s, 1C), 153.23 (s, 1C), 153.20 (s, 1C), 150.95 (s, 1C), 142.74 (s, 1C), 138.24 (s, 1C), 130.83 (s, 1C), 129.39 (d, 2C), 126.07 (s, 1C), 118.46 (d, 1C), 114.39 (d, 1C), 114.25 (d, 1C), 111.93 (d, 1C), 104.42 (d, 1C), 91.46 (d, 1C), 71.23 (d, 1C), 71.16 (d, 1C), 69.94 (d, 1C), 68.88 (d, 1C), 66.04 (t, 1C), 52.64 (d, 1C), 20.37 (q, 2C), 20.32 (q, 1C), 20.25 (q, 1C), 18.01 (q, 1C) ppm

E.1.54 [[[[4-[[[[N-(4-Methyl-2-oxo-2H-1-benzopyran-7-yl)amino]carbonyl]oxy]methyl]phenyl]amino]carbonyl]oxy]-β-D-glucopyranosiduronic acid (XVII)



Procedure according to Bunnelle *et al.*[45] Compound **XVI** (20 mg, 0.03 mmol, 1 eq) was solved in MeOH (4 ml) and cooled to 0°C. Ethyldiisopropylamine (0.09 ml, 0.53 mmol, 18 eq) was added and the reaction was stirred for 3 h while it warmed up to room temperature. After all acetyl groups were removed (LCMS control) water (1 ml) was added and the reaction was stirred for another hour. Upon completion (LCMS control) the solvent was removed under reduced pressure and the crude product was purified via C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **XVII** as a colorless oil.

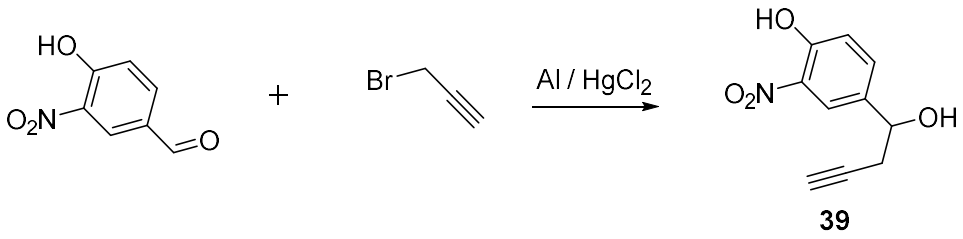
Yield = 3.6 mg (23 % of theory)

HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 545.1402 for $C_{25}H_{25}N_2O_{12}^+$, found 545.1406.

^{1}H NMR (600 MHz, DMSO-d_6) δ = 7.69 (d, J = 8.8 Hz, 1H), 7.55 (s, 1H), 7.51 (d, J = 8.0 Hz, 2H), 7.45-7.39 (m, 3H), 6.24 (s, 1H), 5.40 (d, J = 8.1 Hz, 2H), 5.31 (d, J = 3.4 Hz, 2H), 5.11 (s, 2H), 3.72 (d, J = 9.1 Hz, 1H), 3.23-3.19 (m, 1H), 2.38 (s, 3H) ppm

^{13}C NMR (150 MHz, DMSO-d_6) δ =71.01 (s, 1C), 167.61 (s, 1C), 160.06 (s, 1C), 153.85 (s, 1C), 153.24 (s, 1C), 153.22 (s, 1C), 152.13 (s, 1C), 142.78 (s, 1C), 129.51 (d, 1C), 126.08 (s, 1C), 118.17 (d, 1C), 114.36 (s, 1C), 114.23 (d, 1C), 111.89 (d, 1C), 104.38 (d, 1C), 94.96 (d, 1C), 76.65 (d, 1C), 75.59 (d, 1C), 74.64 (d, 1C), 72.28 (d, 1C), 71.86 (d, 1C), 70.91 (d, 1C), 66.21 (t, 1C), 18.02 (q, 1C) ppm

E.1.55 4-Hydroxy-3-nitro- α-(2-propyn-1-yl)benzenemethanol (39)



39

Molecular Weight: 167.12 Molecular Weight: 118.96 Molecular Weight: 207.19

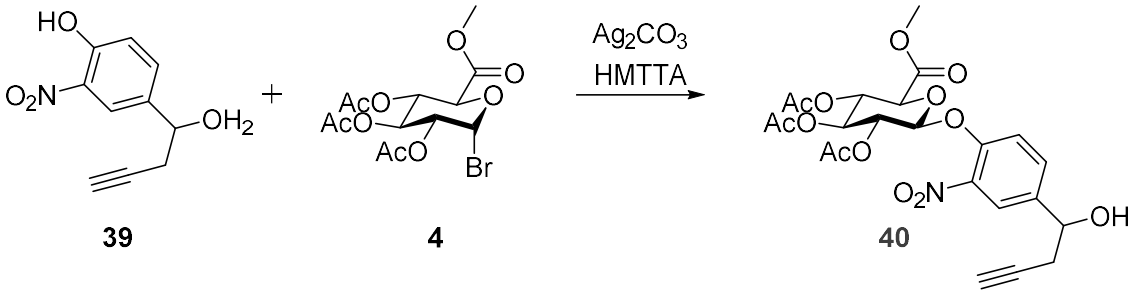
Procedure following Legigan *et al.*[25] Aluminum powder (6.48 g, 240 mmol, 6.25 eq) was suspended in abs. THF (30 mL) and a catalytic amount of $HgCl_2$ was added. After stirring the mixture for 20 minutes, a few drops of a neat solution of propargyl bromide 80% in toluene were added to start the formation of the aluminic propargylide resulting in a dark suspension (heat development). Then propargyl bromide 80% in toluene (26 mL, 240 mmol, 6.25 eq) was diluted with anhydrous THF (50 mL) and was added dropwise. The reaction mixture was stirring for two hours at room temperature and was then heated to 66°C (reflux) for 30 minutes. After cooling down to room temperature, a solution of 4-hydroxy-3-nitrobenzaldehyde (6.41 g, 38.4 mmol) in abs. THF (80 mL) was added dropwise and the reaction was stirred overnight. The crude mixture was cooled at 0°, poured on ice and 1 M HCl (100 mL) and extracted with ethyl acetate (3x 150 ml). The combined organic layers were washed with water, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was taken up in chloroform (200 ml) and washed with a solution of 1 M NaOH (3x 100 ml). The combined aqueous layers were acidified with 12 M HCl to pH 4 and extracted with chloroform (3x 150 ml). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified via flash chromatography (PE/EtOAc = 3:1 – 1:1), to give compound **39** as dark red oil.

Yield = 1.75 g (22 % of theory)

^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.56 (s, 1H), 8.16 (d, J = 2.2 Hz, 1H), 7.64 (dd, J = 2.2, 8.7 Hz, 1H), 7.17 (d, J = 8.7 Hz, 1H), 4.89 (t, J = 6.2 Hz, 1H), 2.66-263 (m, 2H), 2.1 (t, J = 2.7 Hz, 1H) ppm

^{13}C NMR (100 MHz, DMSO-d6) δ = 151.29 (s, 1C), 136.05 (s, 1C), 135.75 (s, 1C), 133.27 (d, 1C), 122.46 (d, 1C), 118.72 (d, 1C), 81.45 (q, 1C), 72.87 (d, 1C), 69.59 (d, 1C), 28.64 (t, 1C) ppm

E.1.56 [[4-(1-hydroxy-3-butyn-1-yl)-2-nitrophenyl]oxy]-2,3,4-triacetyl-β-D-glucopyranosiduronic acid, methyl ester (40)



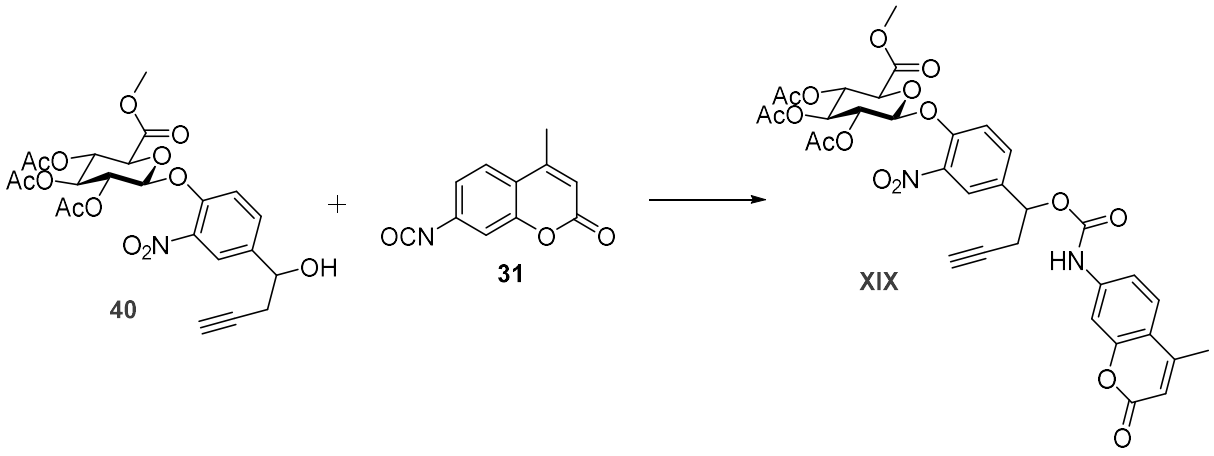
39 **4** **40**

Molecular Weight: 208.19 Molecular Weight: 397.17 Molecular Weight: 523.45

Procedure according to Legigan *et al.*[25] A solution of 1,1,4,7,10,10-hexamethyltriethylene-tetramine (0.19 ml, 0.7 mmol, 0.7 eq) and Ag_2CO_3 (1 g, 3.7 mmol, 3.7 eq) in abs. acetonitrile (5 mL) was stirred two hours at room temperature. Compound **39** (200 mg, 1 mmol, 1eq) in abs. acetonitrile (5 mL) and compound **4** (822 mg, 2 mmol, 2 eq) in abs. acetonitrile (5 ml) were added at 0°C. The reaction mixture was stirred for 4 hours at room temperature. After filtration over a PTFE syringe filter, the filtrate was washed with 1 M HCl and extracted with ethyl acetate (3x 20 ml). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting crude product was purified by flash chromatography (PE/EtOAc = 3:1 – 1:3) to afford compound **40** as an off-white foam.
Yield = 164 mg (31 % of theory)

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.86 (dd, J = 2.1, 5.8 Hz, 1H), 7.59-7.56 (m, 1H), 7.36 (d, J = 8.6 Hz, 1H), 5.38-5.27 (m, 3H), 5.20 (d, J = 5.9 Hz, 1H), 4.90 (t, J = 6.2 Hz, 1H), 4.21 (d, J = 8.7 Hz, 1H), 3.74 (s, 3H), 2.66-2.62 (m, 2H), 2.12 (s, 3H), 2.10 (t, J = 2.6 Hz, 1H), 2.06 (s, 3H), 2.05 (s, 3H) ppm
^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.45 (s, 1C), 169.26 (s, 1C), 168.69 (s, 1C), 166.88 (s, 1C), 147.14 (s, 1C), 140.08 (s, 1C), 139.87 (s, 1C), 131.81 (d, 1C), 122.18 (d, 1C), 117.19 (d, 1C), 97.86 (d, 1C), 73.01 (s, 1C), 71.00 (d, 1C), 70.72 (d, 1C), 69.87 (d, 1C), 69.38 (d, 1C), 68.65 (d, 1C), 52.60 (d, 1C), 28.46 (t, 1C), 20.23 (q, 1C), 20.17 (q, 1C), 20.14 (q, 1C).

E.1.57 [[4-[1-[[[N-(4-Methyl-2-oxo-2H-1-benzopyran-7-yl)amino] carbonyl]oxy]-3-butyn-1-yl]-2-nitrophenyl]oxy]-2,3,4-triacetyl-β-D-gluco-pyranosiduronic acid, methyl ester (XIX)

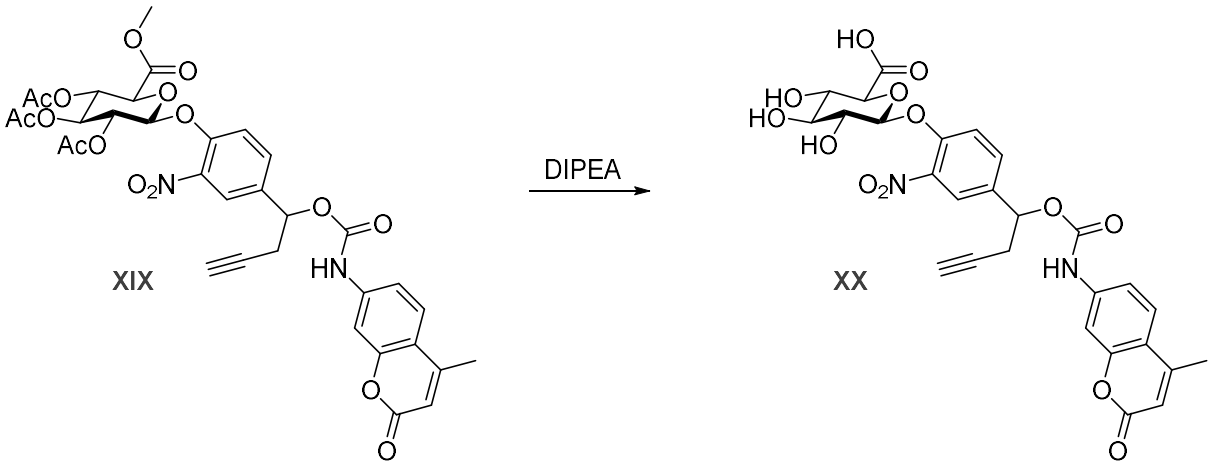


Molecular Weight: 523.45 Molecular Weight: 201.18 Molecular Weight: 724.63

Procedure according to El Alaoui *et al.*[24] A suspension of compound **31** (58 mg, 0.29 mmol, 1 eq) in toluene (15 ml) was cooled to 0°C and compound **40** (152 mg, 0.29 mmol, 1 eq) and triethylamine (0.14 ml, 1.02 mmol, 3.5 eq) were added. After stirring for two hours at 0°C the solvent was removed under reduced pressure and the crude product was purified via flash chromatography (PE/EtOAc = 3:1 – 1:1) to give compound **XIX** as a white solid.
Yield = 75 mg (36 % of theory)

HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 725.1825 for $C_{34}H_{33}N_2O_{16}^+$, found 725.1805.
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.92 (s, 1H), 7.61 (d, J = 8.6 Hz, 1H), 7.53 (d, J = 8.6 Hz, 1H), 7.45 (s, 1H), 7.40-7.33 (m, 2H), 7.11 (s, 1H), 6.20 (s, 1H), 5.89 (t, J = 6.2 Hz, 1H), 5.39-5.22 (m, 3H), 5.23 (d, J = 6.4 Hz, 1H), 4.22 (d, J = 8.6 Hz, 1H), 3.73 (s, 3H), 2.84-2.81 (m, 2H), 2.41 (s, 3H), 2.12 (s, 3H), 2.06 (s, 4H), 2.05 (s, 3H) ppm
^{13}C NMR (150 MHz, DMSO-d6) δ = 170.18 (s, 1C), 169.48 (s, 1C), 169.39 (s, 1C), 166.84 (s, 1C), 161.10 (s, 1C), 154.53 (s, 1C), 152.29 (s, 1C), 151.58 (s, 1C), 149.26 (s, 1C), 141.21 (s, 1C), 140.85 (s, 1C), 134.94 (s, 1C), 132.53 (s, 1C), 132.33 (s, 1C), 125.60 (d, 1C), 123.61 (d, 1C), 123.34 (d, 1C), 119.92 (d, 1C), 116.04 (d, 1C), 113.60 (d, 1C), 106.25 (d, 1C), 99.71 (d, 1C), 73.32 (t, 1C), 72.71 (d, 1C), 72.27 (d, 1C), 71.14 (d, 1C), 70.24 (d, 1C), 68.81 (d, 1C), 53.26 (d, 1C), 26.53 (q, 1C), 20.75 (q, 1C), 20.71 (q, 1C), 20.67 (q, 1C), 18.75 (q, 1C) ppm

E.1.58 [[4-[1-[[[N-(4-Methyl-2-oxo-2H-1-benzopyran-7-yl)amino] carbonyl]oxy]-3-butyn-1-yl]-2-nitrophenyl]oxy]-β-D-gluco-pyranosid uronic acid (XX)



Molecular Weight: 724.63 Molecular Weight: 584.49

Procedure according to Bunnelle *et al.*[45] Compound **XIX** (70 mg, 0.1 mmol, 1 eq) was solved in MeOH (12 ml) and cooled to 0°C. Ethyldiisopropylamine (232 mg, 1.8 mmol, 18 eq) was added and the reaction was stirred for three hours while it warmed up to room temperature. After all acetyl groups were removed (LCMS control) water (3 ml) was added and the reaction was stirred for another hour. Upon completion (LCMS control) the solvent was removed under reduced pressure and the crude product was purified via C18 prep. HPLC (ACN/H_2O = 5 % ACN – 95% ACN) to give compound **XX** as a colorless oil.

Yield = 43 mg (74 % of theory)

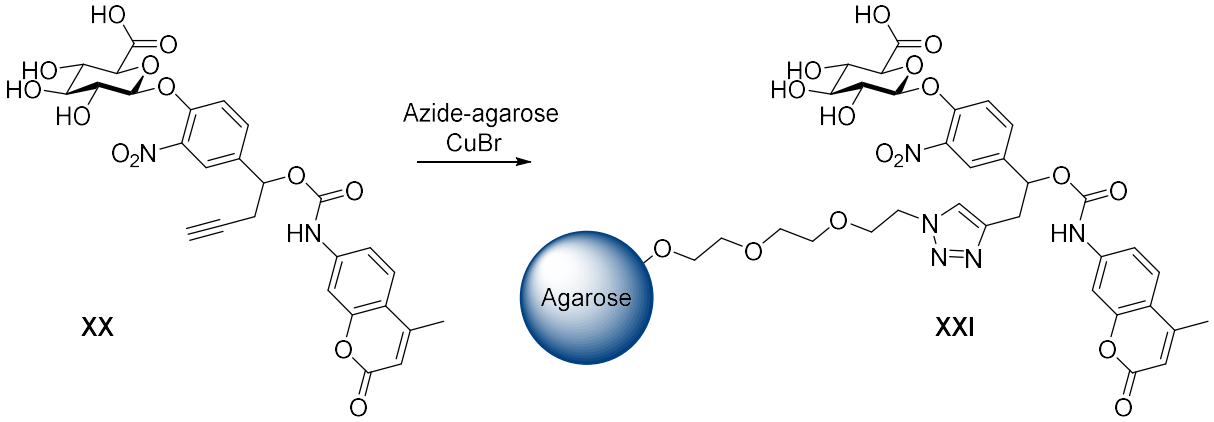
HR-ESI-ORBITRAP $[M+H]^+$ m/z calcd. 585.1351 for $C_{27}H_{25}N_2O_{13}^+$, found 585.1333.

^1H NMR (600 MHz, DMSO-d_6) δ = 10.42 (s, 1H), 7.99 (dd, J = 2.2, 4.8 Hz, 1H), 7.76 (m, J = 2.1 Hz, 1H), 7.69 (d, J = 8.8 Hz, 1H), 7.53 (s, 1H), 7.48 (dd, J = 2.0, 9.0 Hz, 1H), 7.43 (d, J = 8.9 Hz, 1H), 6.24 (d, J = 1.1 Hz, 1H), 5.88 (t, J = 6.1 Hz, 1H), 5.50 (d, J = 4.7 Hz, 1H), 5.30 (t, J = 8.2 Hz, 1H), 5.27 (d, J = 3.5 Hz, 1H), 3.98 (dd, J = 2.7, 9.8 Hz, 1H), 3.40 (t, J = 9.2 Hz, 1H), 3.28 (m, J = 5.0 Hz, 1H), 2.93 (q, J = 2.3 Hz, 1H), 2.91 (d, J = 6.2 Hz, 1H), 2.38 (s, 1H) ppm

^{13}C NMR (150 MHz, DMSO-d_6) δ = 169.95 (s, 1C), 160.02 (s, 1C), 153.77 (s, 1C), 153.19 (s, 1C), 152.21 (s, 1C), 148.86 (s, 1C), 142.42 (s, 1C), 139.72 (s, 1C), 133.19 (s, 1C), 132.32 (s, 1C), 126.08

(d, 1C), 122.94 (d, 1C), 116.74 (d, 1C), 114.59 (d, 1C), 114.37 (d, 1C), 112.09 (d, 1C), 104.66 (d, 1C), 99.83 (d, 1C), 79.74 (d, 1C), 75.84 (d, 1C), 75.39 (d, 1C), 74.00 (d, 1C), 72.78 (d, 1C), 72.45 (s, 1C), 71.15 (d, 1C), 25.34 (t, 1C), 18.01 (q, 1C) ppm

E.1.59 Fluorogenic β-glucuronidase probe immobilized on agarose (XXI)



Procedure according to Punna et. al.[74] Stock solutions (50 mM) of compound **XX** (DMSO-D_6), 2,6-lutidine (DMF), 2,2'-bipyridine (DMF), CuBr (DMF), and sodium ascorbate (water) were prepared. Azide-agarose (0.2 ml, 2 µmol, 1 eq) was suspended in water (0.2 ml) and treated with the stock solutions of compound **XX** (160 µl, 8 µmol, 4 eq), 2,6-lutidine (320 µl, 16 µmol, 8 eq), 2,2'-bipyridine (320 µl, 16 µmol, 8 eq), copper(I)bromide (160 µl, 8 µmol, 4 eq) and sodium ascorbate (320 µl, 16 µmol, 8 eq). The resulting suspension was degassed with argon and stirred for 16 hours at room temperature. The reaction mixture was then filled into an *Omnifit* column and washed witch 5 column volumes of DMF, water, ethanol, 0.1 M aq EDTA, water and a 75mM phosphate buffer (pH 6.8).

E.1.60 Standard Operation Procedure for Coliminder QRTF Assays

Unless noted otherwise, all enzyme assays on the Coliminder where conducted with a substrate concentration of 0.64 mMl in an 18.5 mM bicine buffer (pH = 8.4) at 44°C. An LED lamp with an excitation maximum of 365 nm was used as light source. For the standard excitation strength, the lamp was operated with 24 V (100% lamp excitation).

E.1.61 Standard Operation Procedure for LS 55 QRTF Assays

All enzyme kinetic measurements were conducted in the 4-cell sample changer accessory for the LS 55 spectrometer with Hellma Analytics (101-QS) 10x10 mm fluorescence cells and stirring bars with the stirring speed set to “Low”. Unless noted otherwise a 75 mM phosphate buffer (pH = 6.8) at 37 °C was used. The substrate concentration varied for Michaelis-Menten measurements from 0.01 mM to 1.5 mM, enzyme concentration was 12.5 mU/ml, the standard measurement time was 10 minutes. Excitation and emission wavelength were chosen so that the highest “Turn On” ratio was achieved.

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G) Appendix

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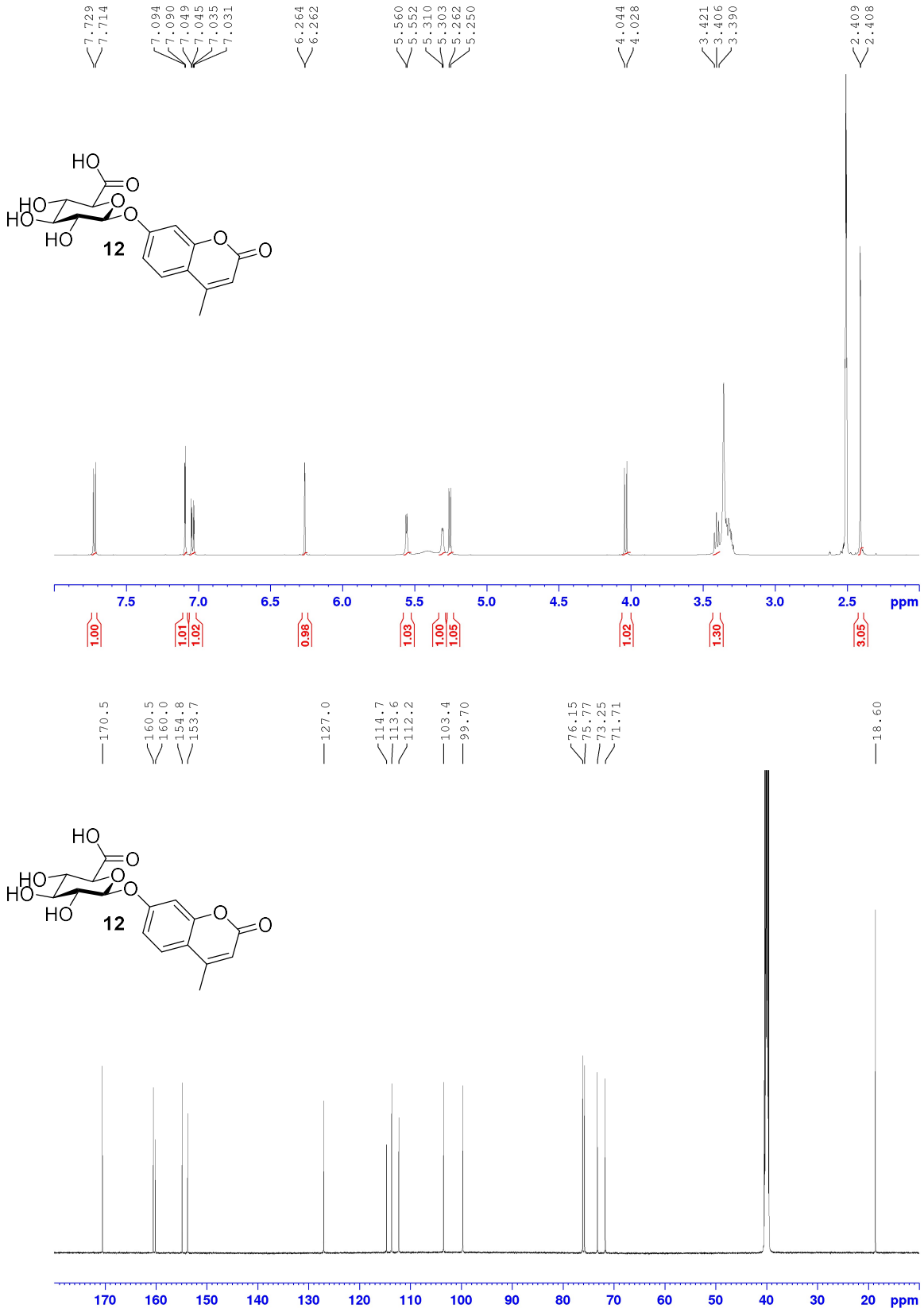
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7.094
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7.045
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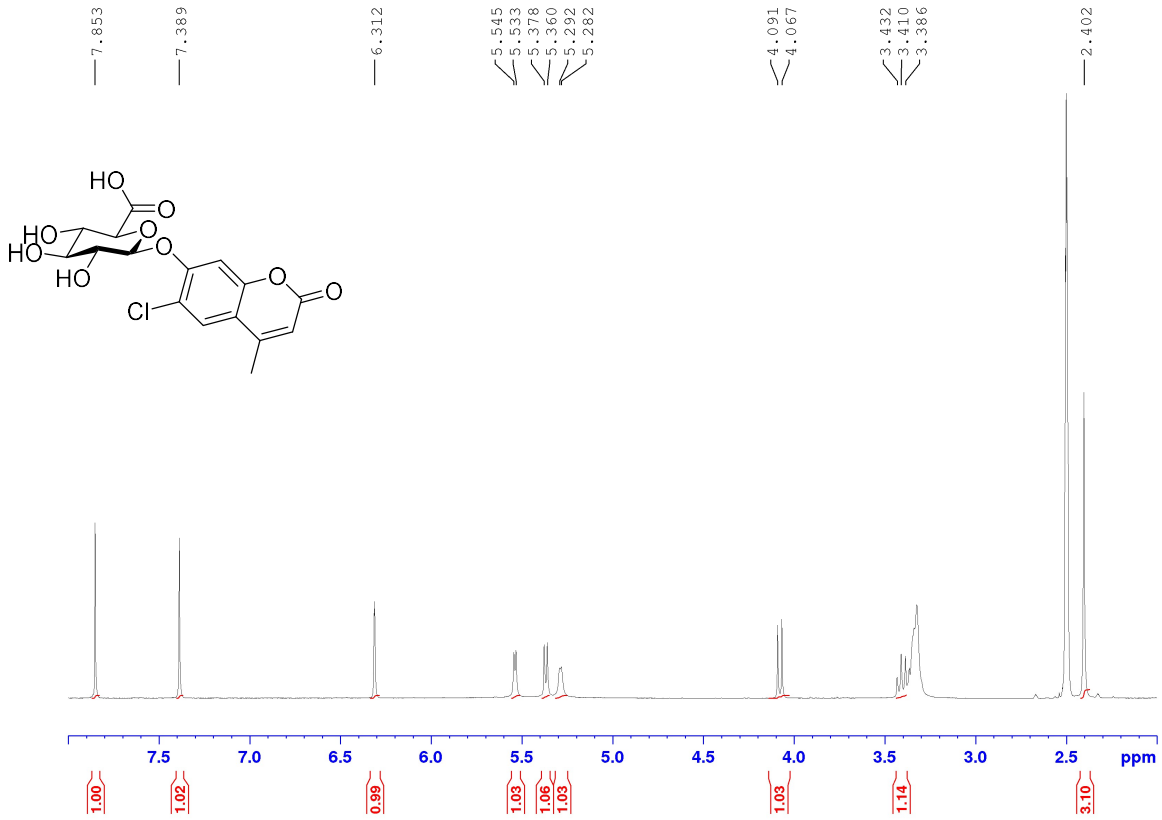
6.262
5.560
5.552
5.310
5.303
5.262
5.250
4.044
4.028
3.421
3.406
3.390
2.409
2.408
HO
HO
HO
HO
O
O
O
O
12
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm
1.00
1.01
1.02
0.98
1.03
1.00
1.05
1.02
1.30
3.05
170.5
160.5
160.0
154.8
153.7
127.0
114.7
113.6
112.2
103.4
99.70
76.15
75.77
73.25
71.71
18.60
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

6-Cl-4-MUG (25)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3.410
3.386
2.402
HO
HO
HO
HO
O
O
O
O
Cl
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
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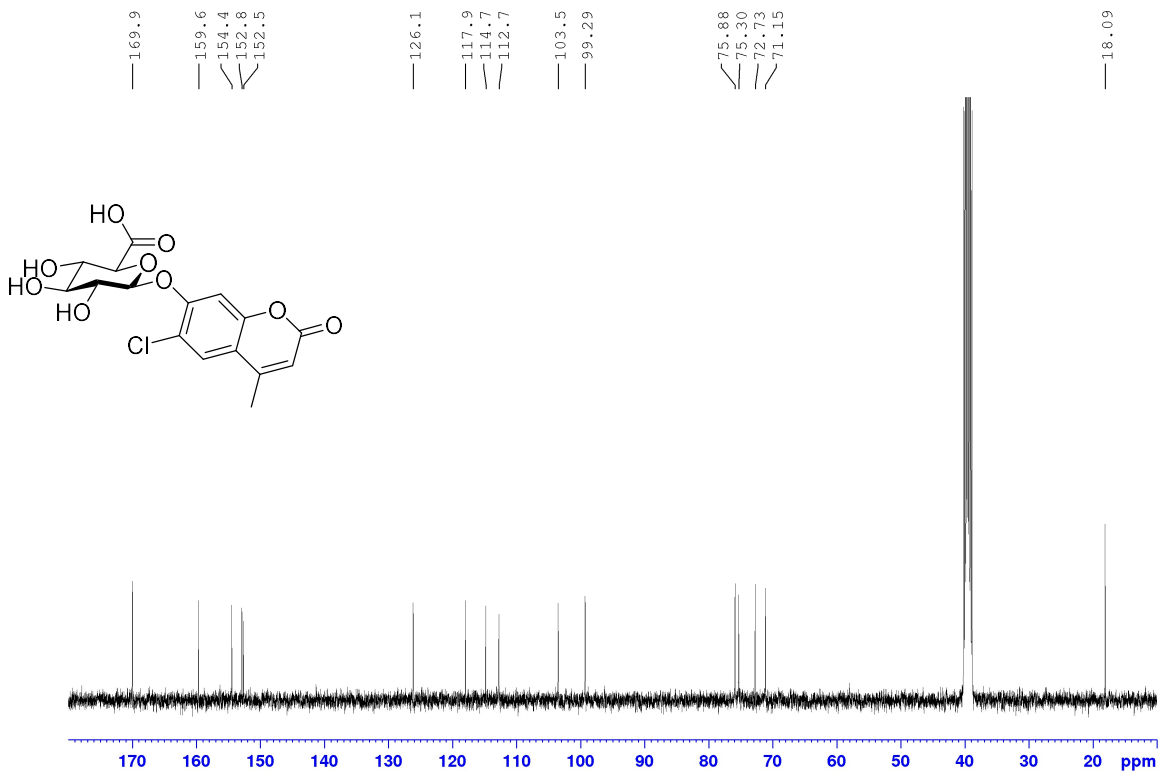
ppm
1.00
1.02
0.99
1.03
1.06
1.03
1.03
1.14
3.10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112.7
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72.73
71.15
18.09
HO
HO
HO
HO
O
O
O
O
Cl
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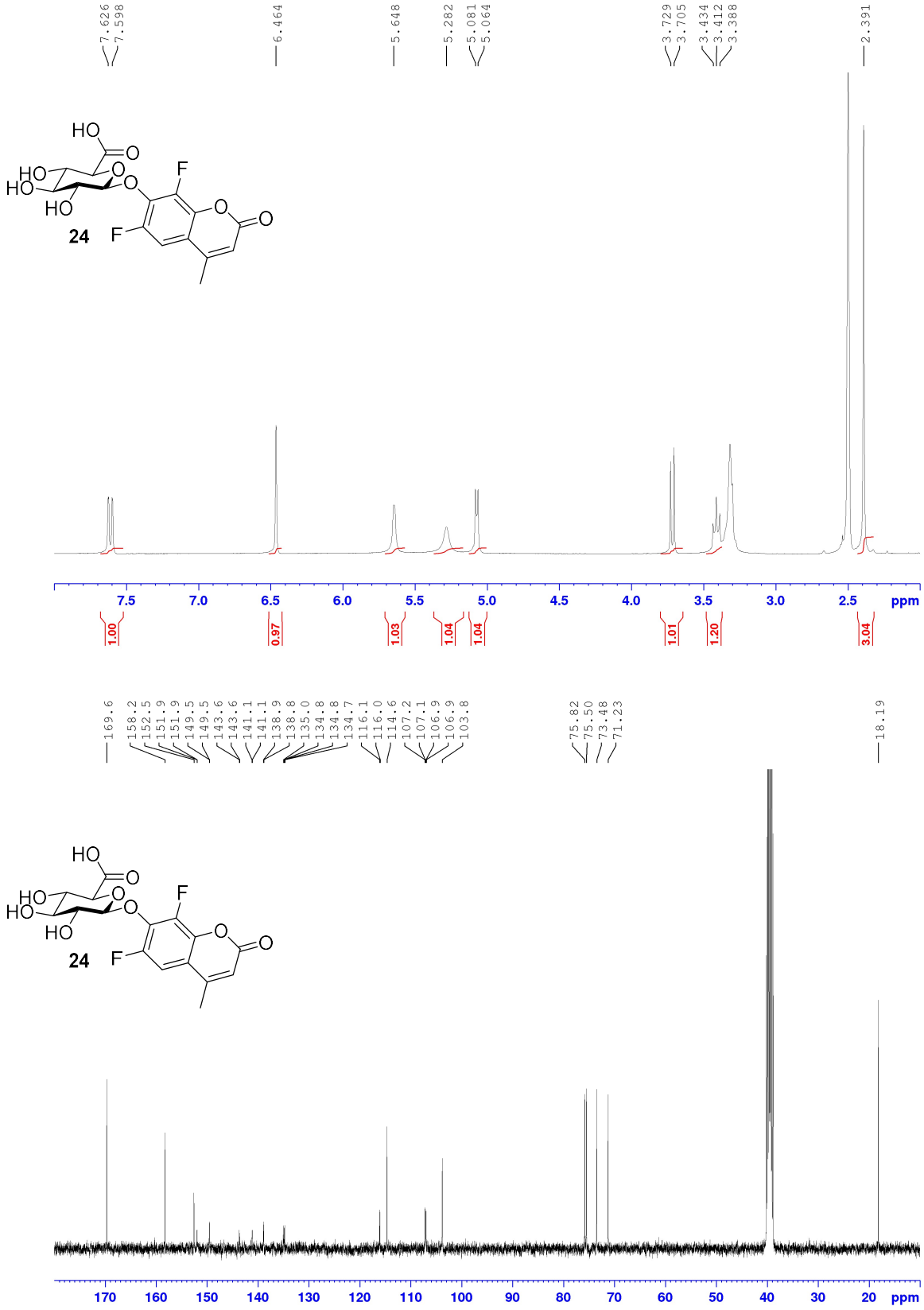
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

DiFMUG (24)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O
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F
O
O
F
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7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm
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0.97
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1.04
1.04
1.01
1.20
3.04
169.6
158.2
152.5
151.9
151.9
149.5
149.5
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141.1
141.1
138.9
138.8
135.0
134.8
134.8
134.7
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116.0
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107.2
107.1
106.9
106.9
103.8
75.82
75.50
73.48
71.23
18.19
170
160
150
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130
120
110
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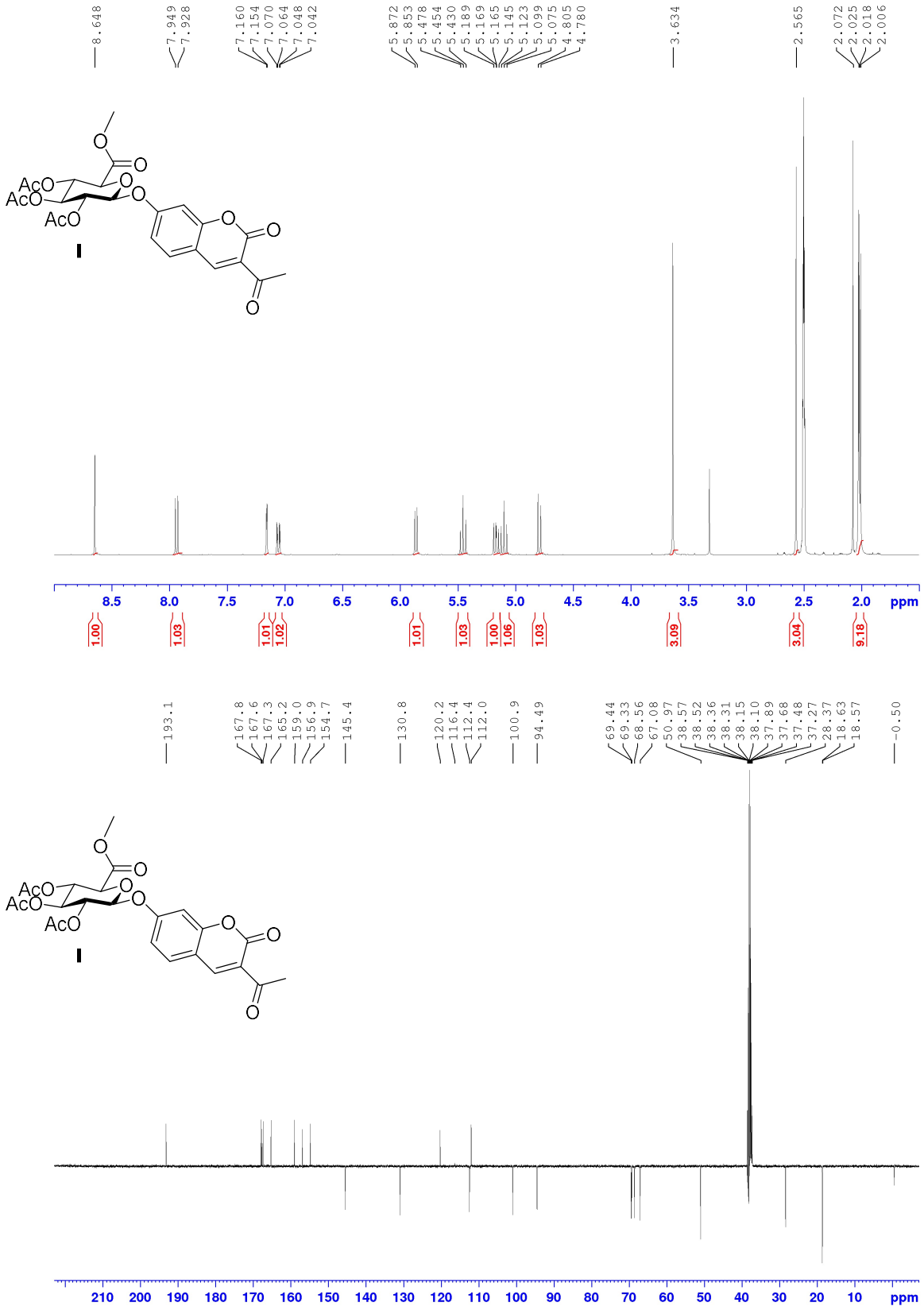
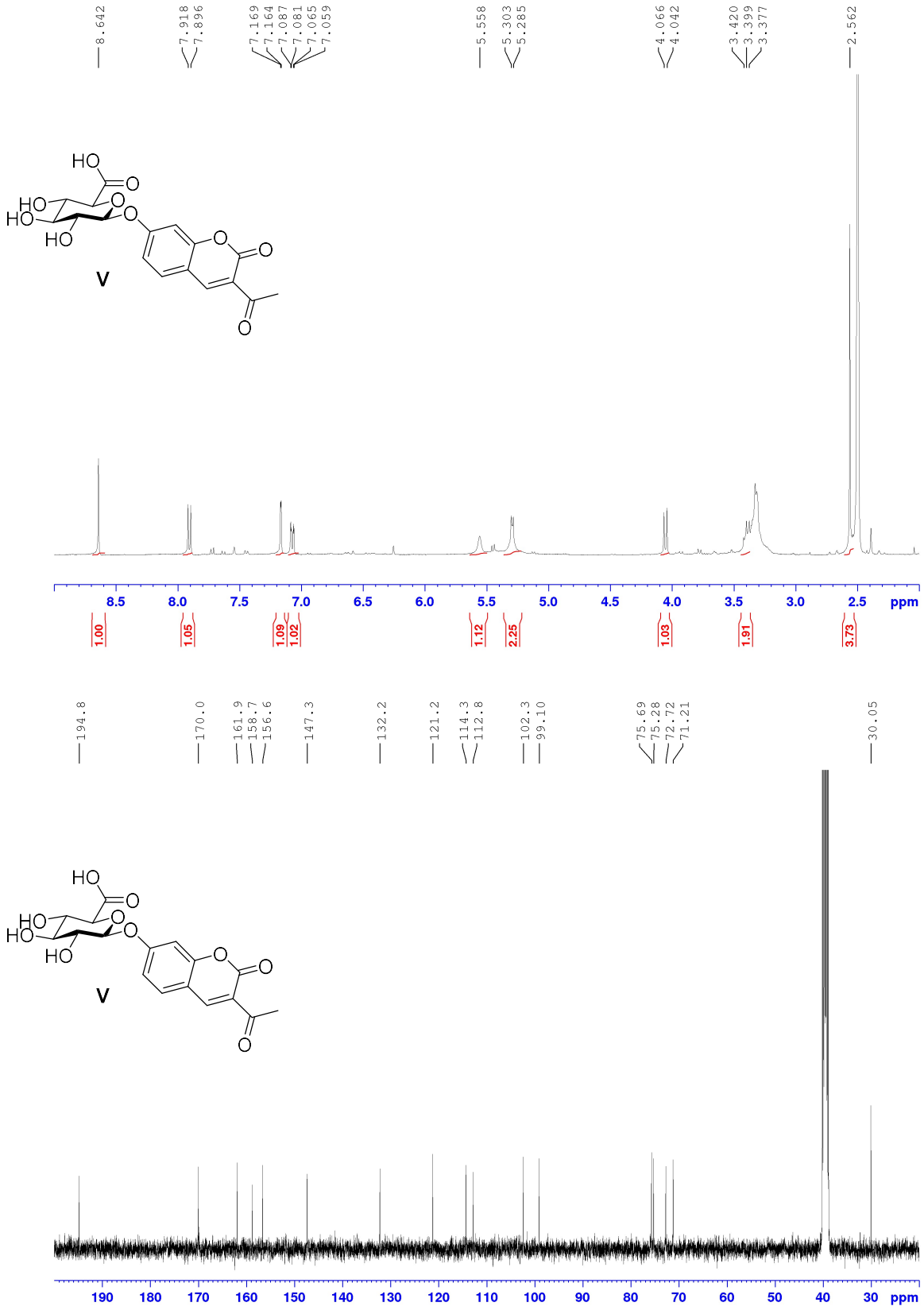
90
80
70
60
50
40
30
20
ppm

3-AcUG-triacteyl methylester (I)



3-AcUG (V)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3.399
3.377
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HO
HO
HO
O
O
O
O
O
V
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8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm
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1.05
1.09
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1.12
2.25
1.03
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170.0
161.9
158.7
156.6
147.3
132.2
121.2
114.3
112.8
102.3
99.10
75.69
75.28
72.72
71.21
30.05
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150
140
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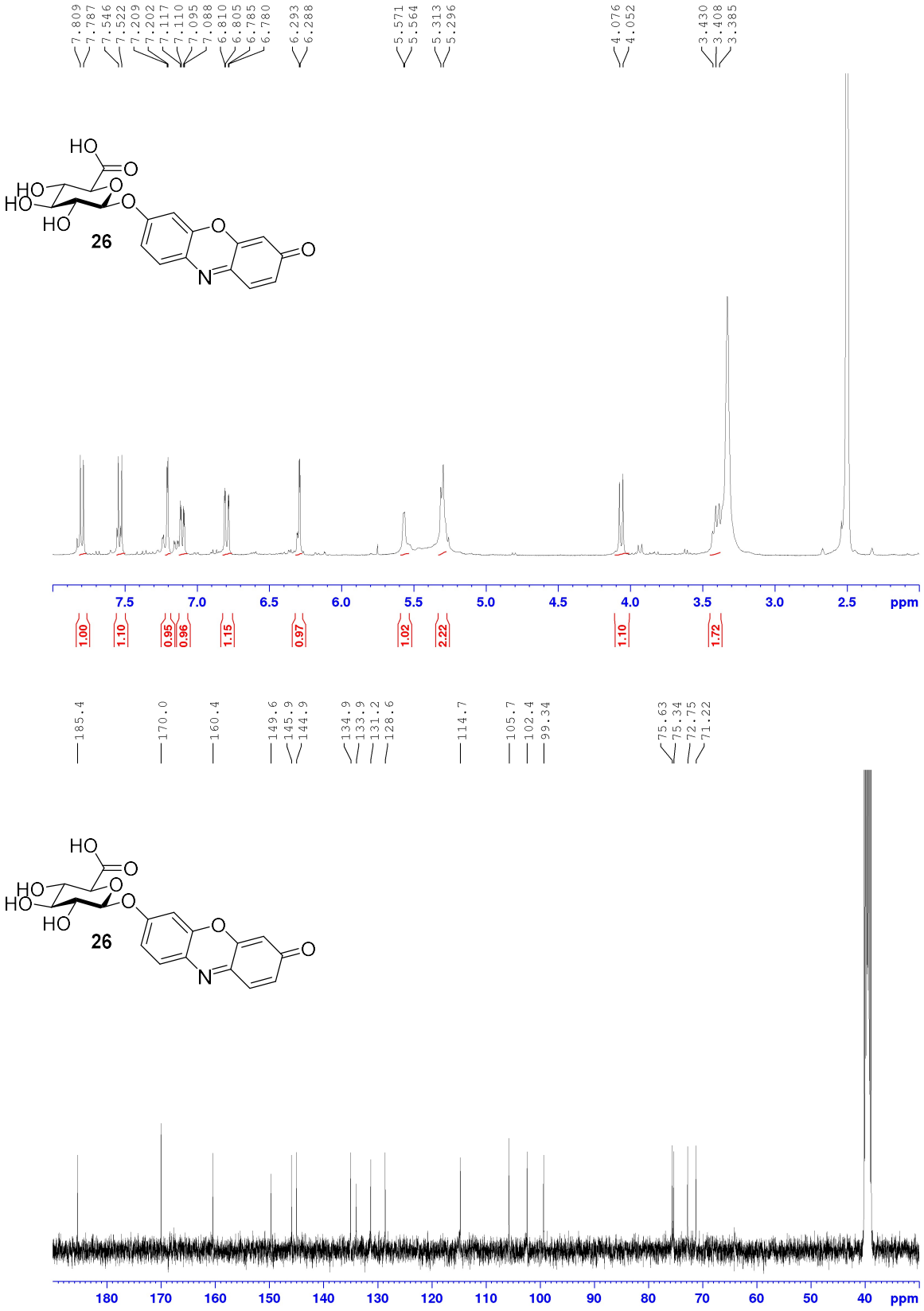
120
110
100
90
80
70
60
50
40
30
ppm

REG (26)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6.780
6.293
6.288
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5.296
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3.408
3.385
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O
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O
O
N
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75.63
75.34
72.75
71.22
180
170
160
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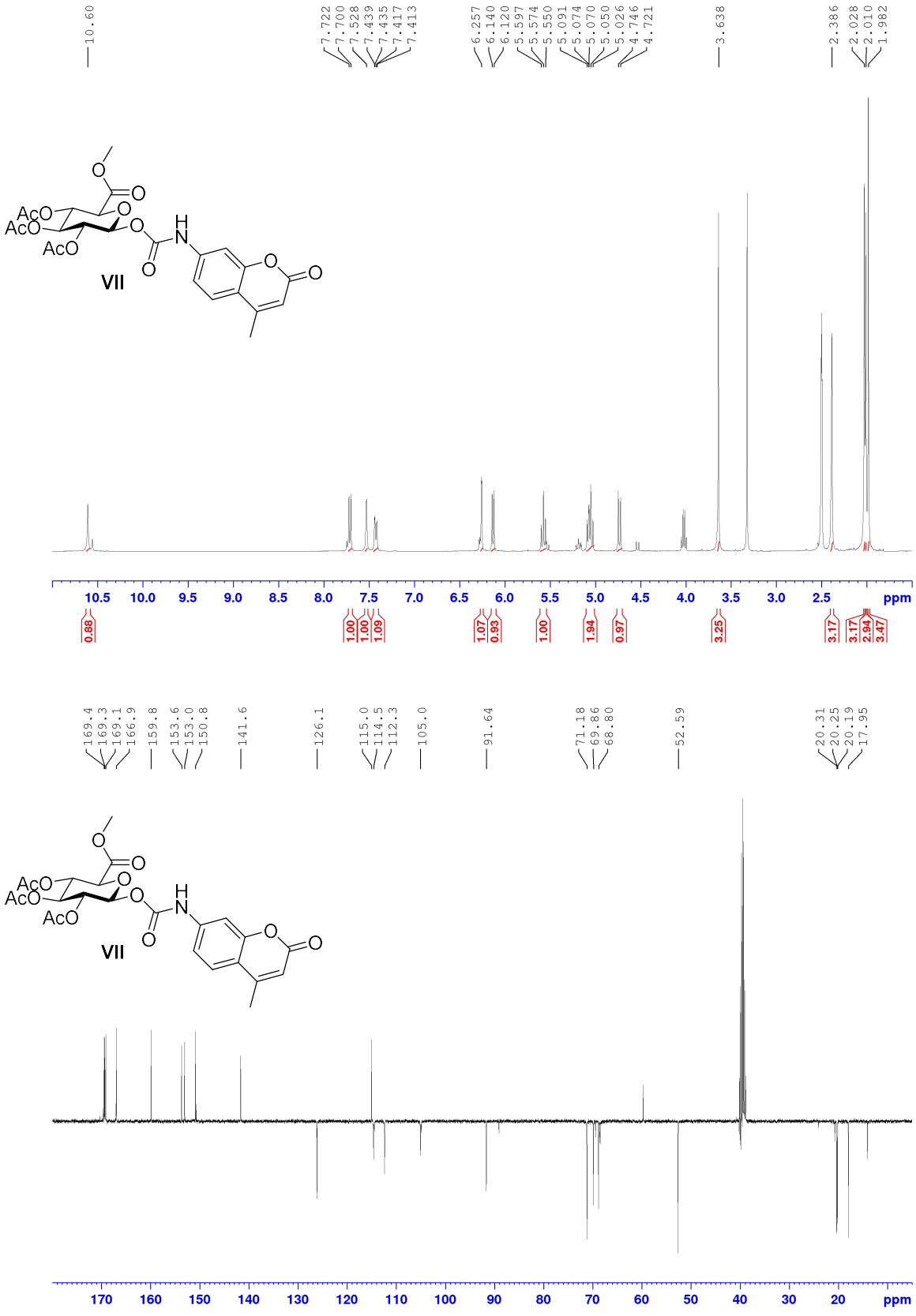
7-AMC-Carb-GlcA-triacteyl methylester (VII)



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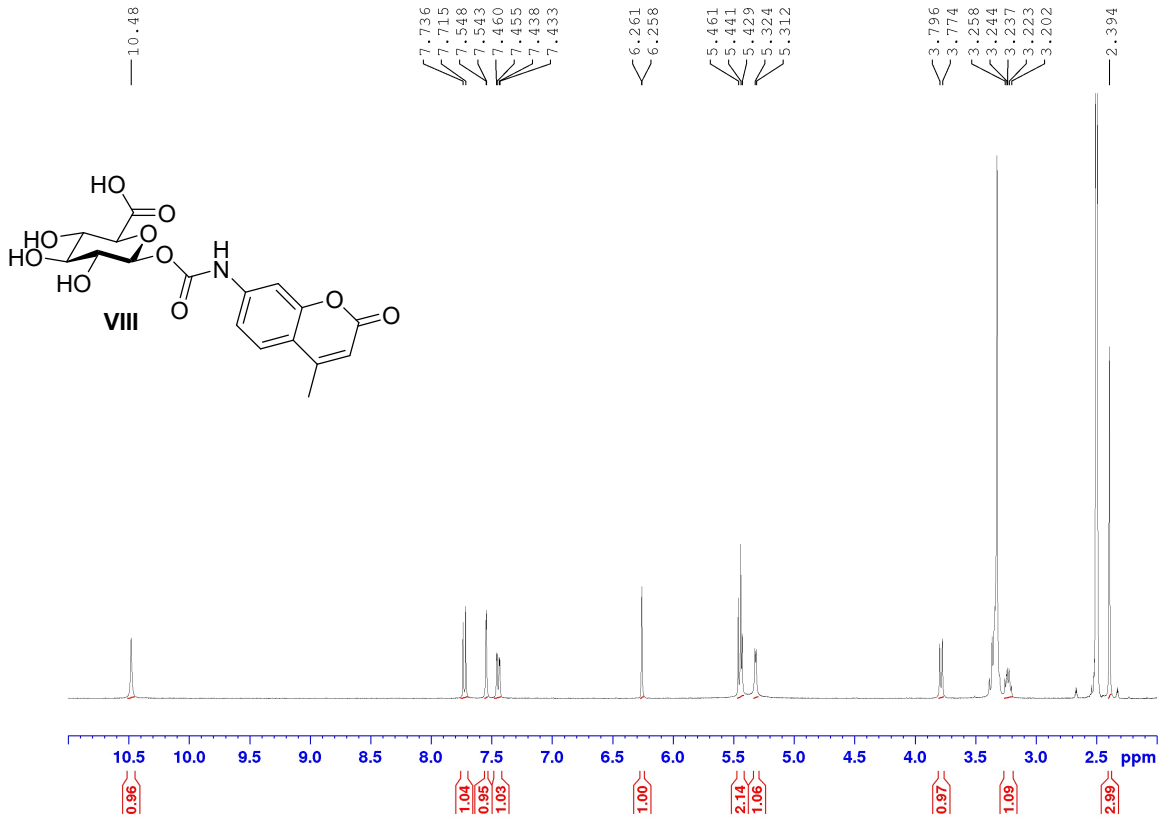
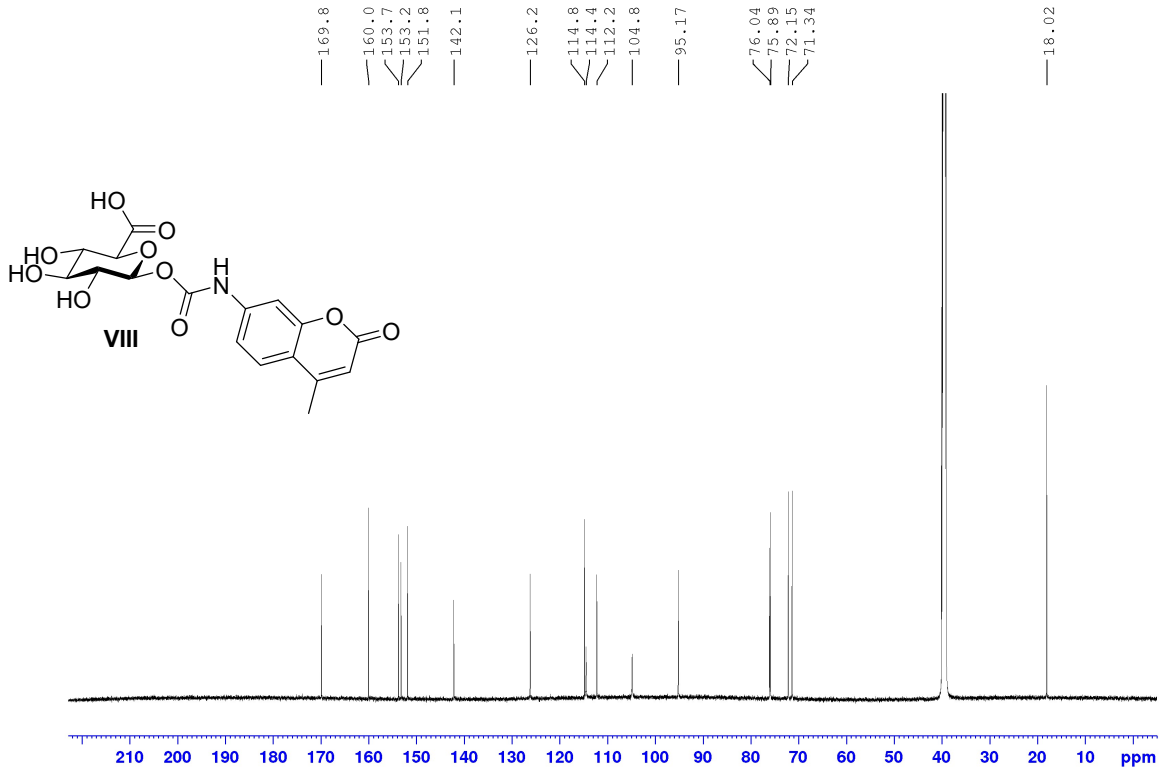
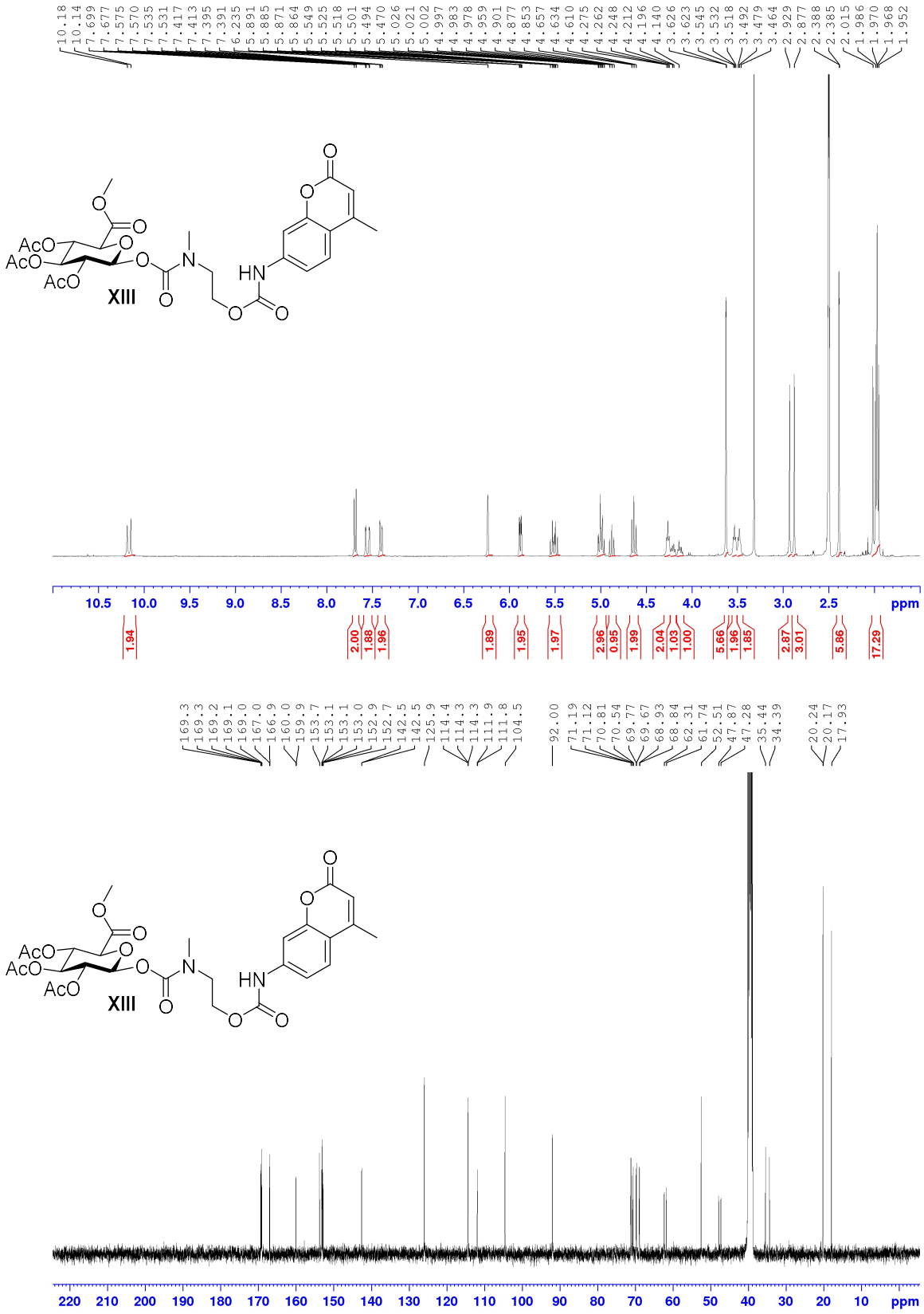




7-AMC-AEt-GlcA-triacteyl methylester (XIII)



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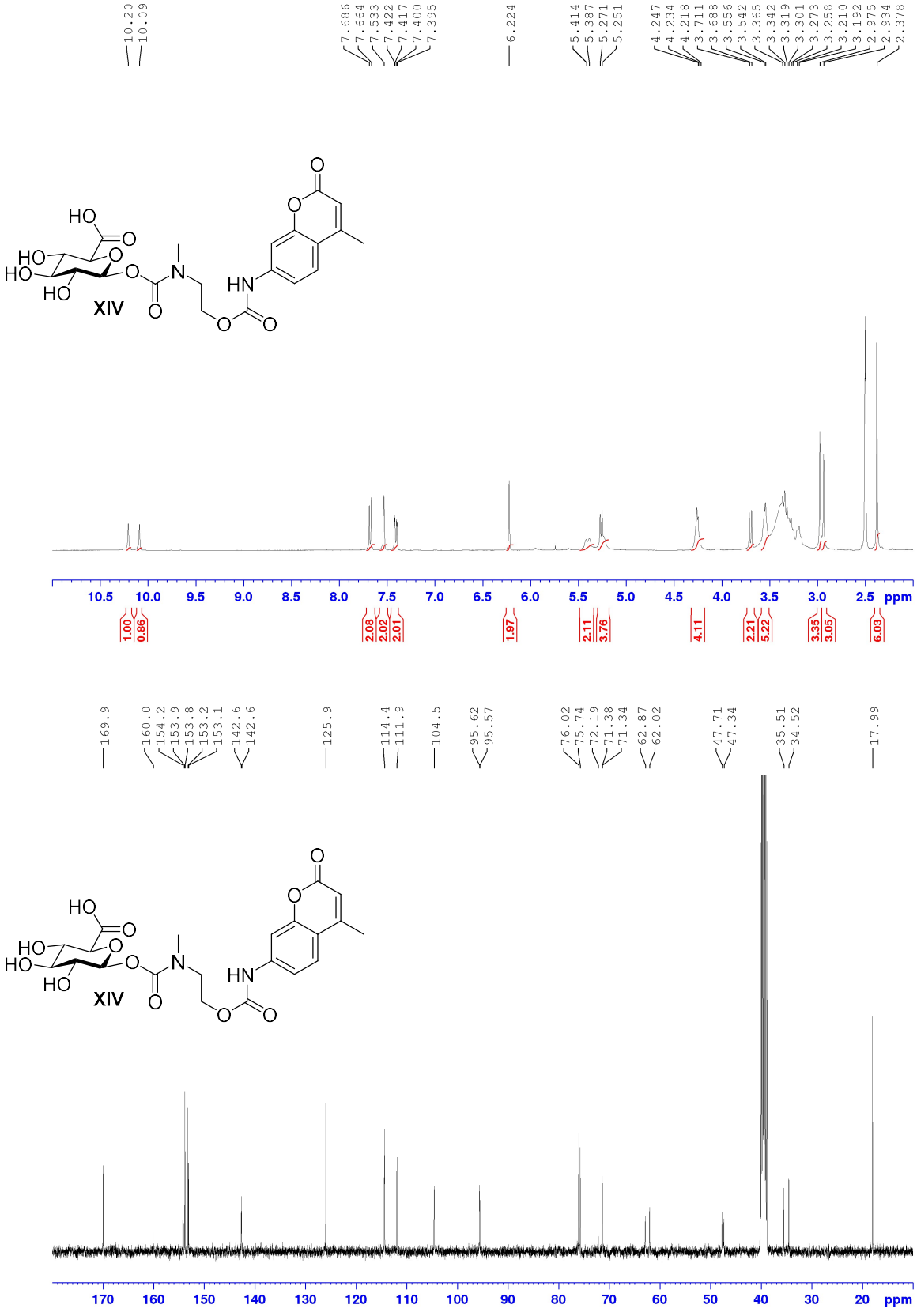

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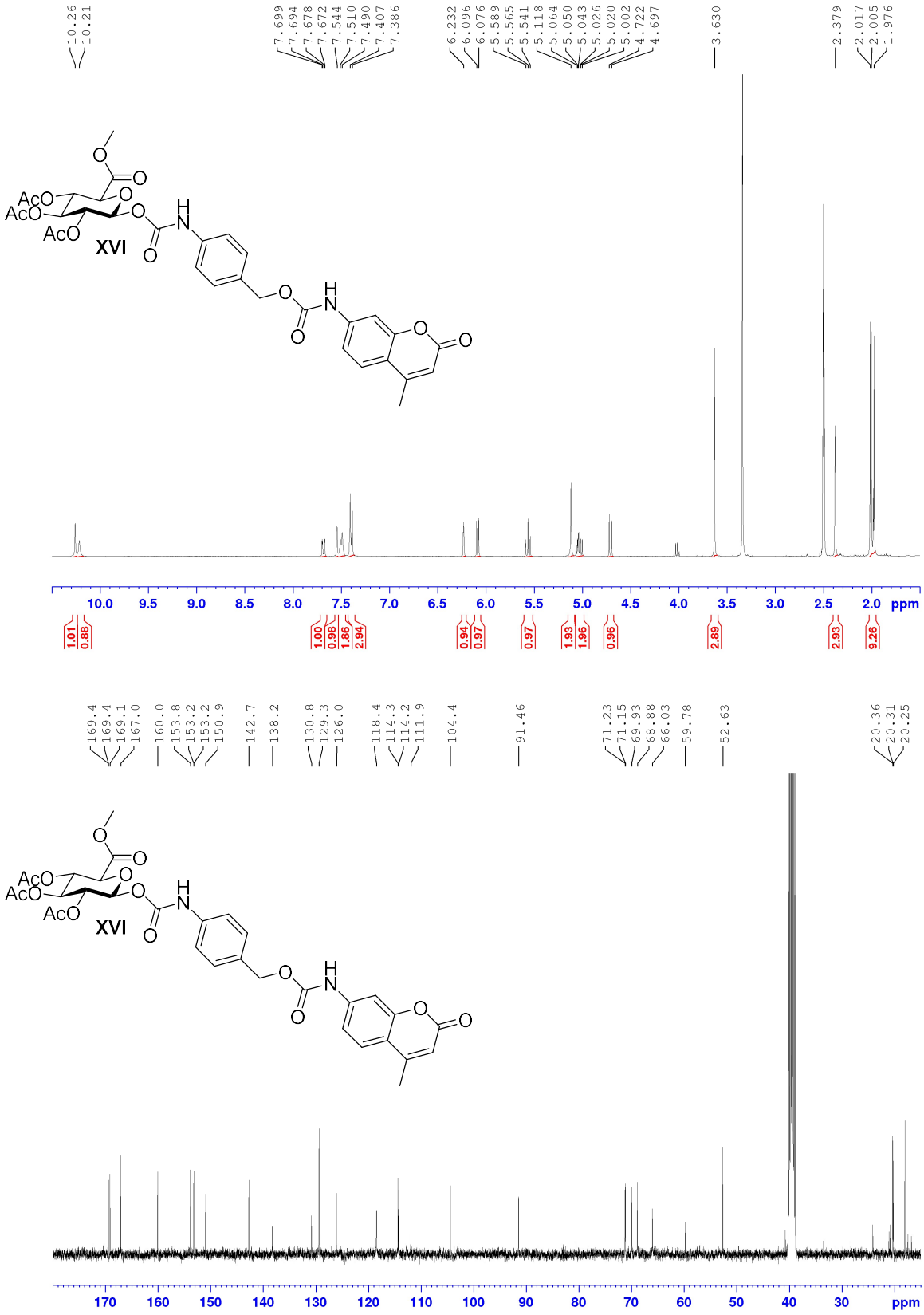

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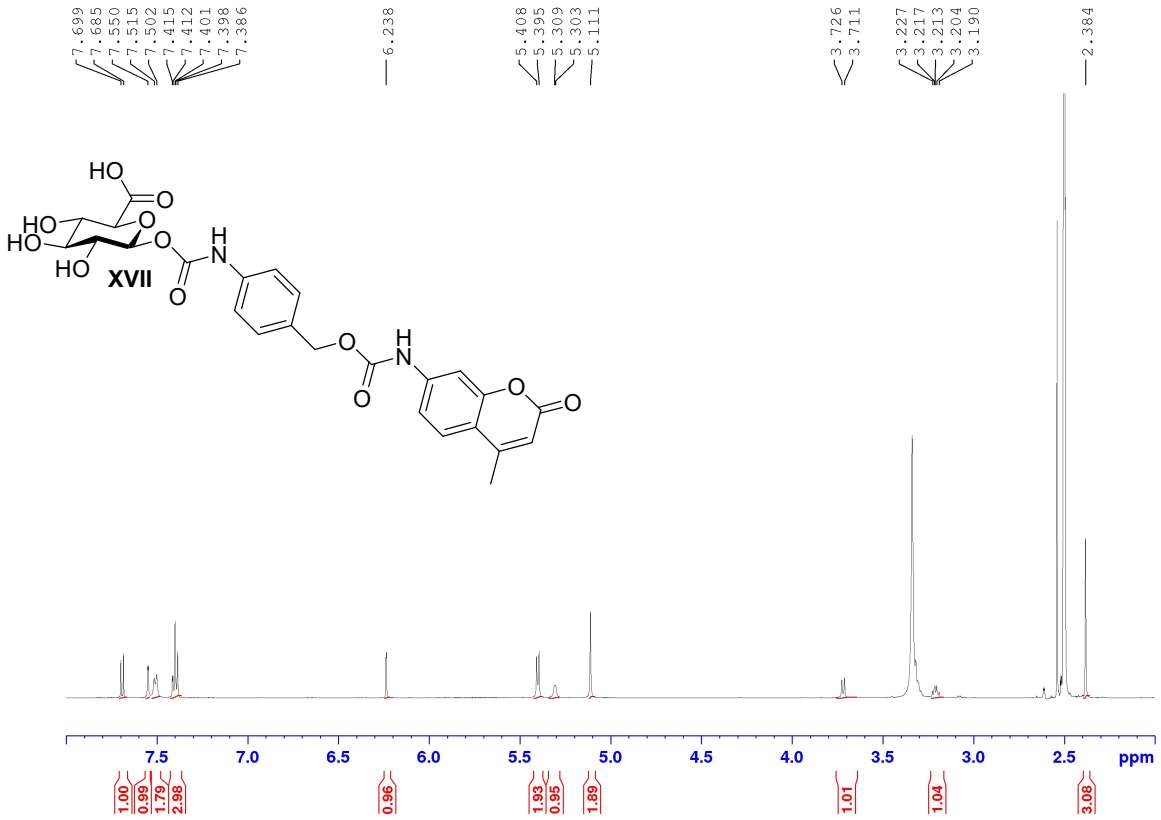

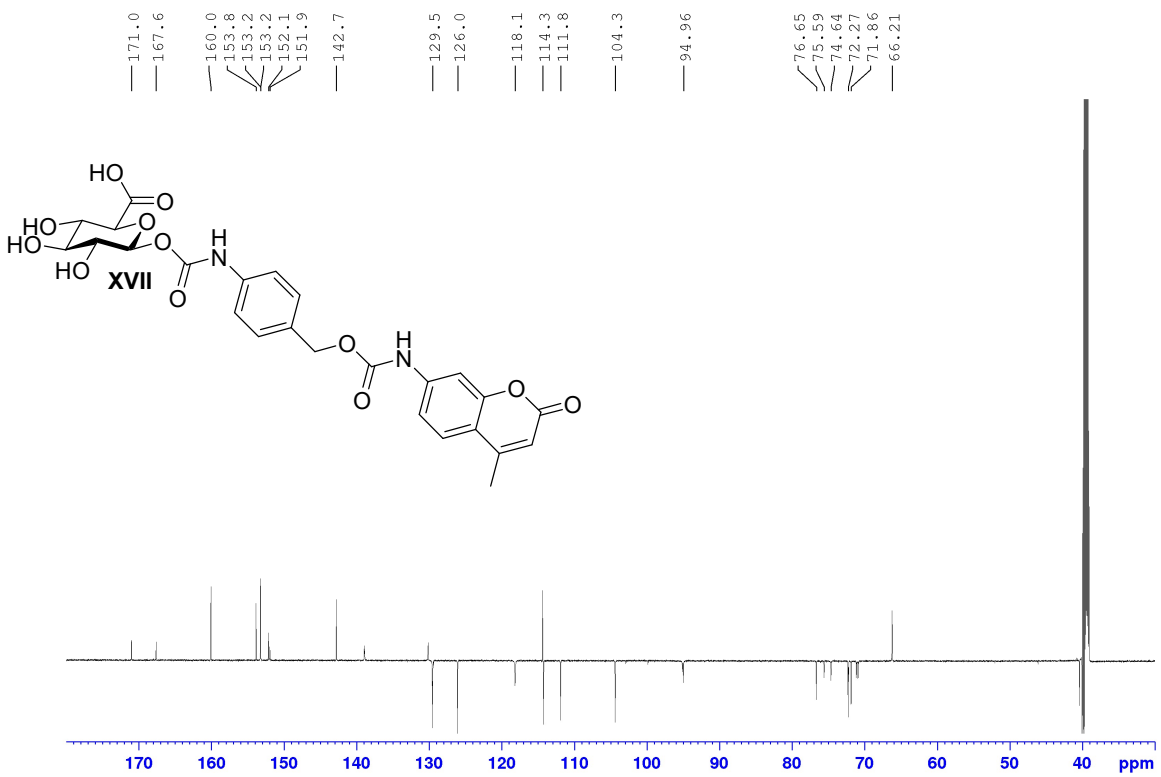

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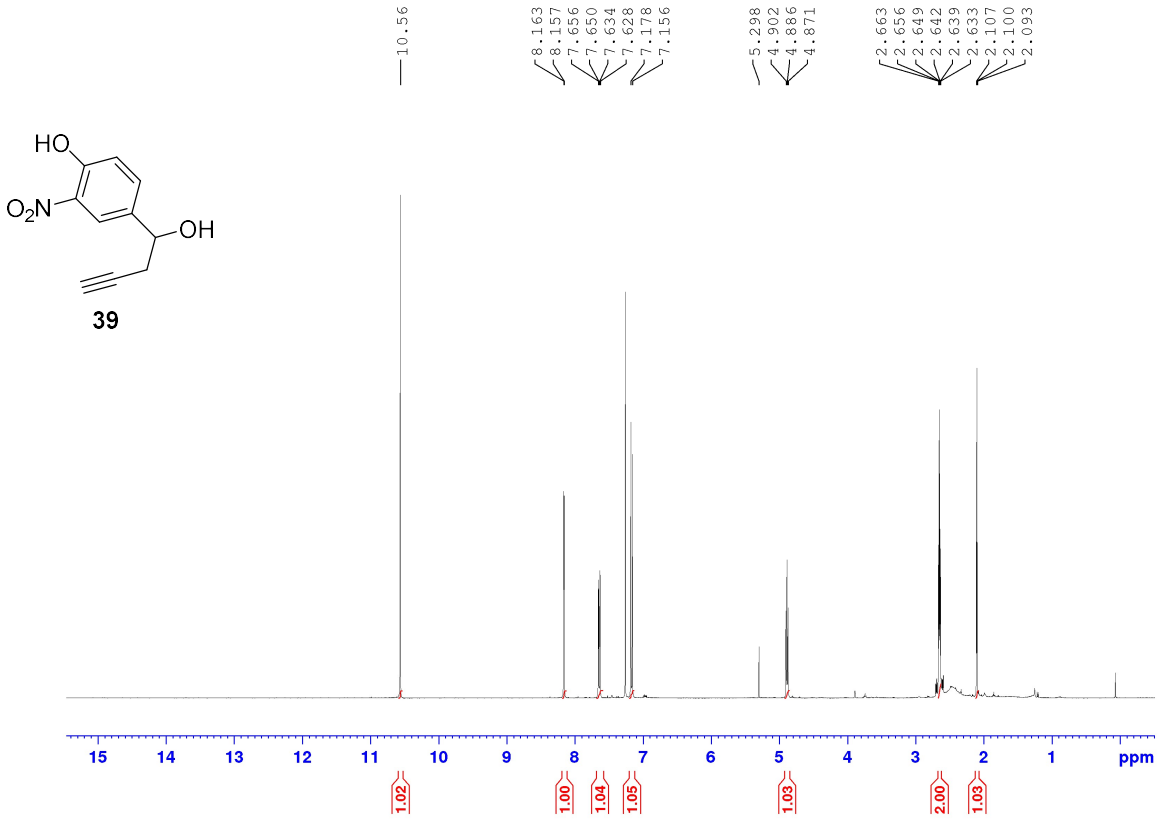
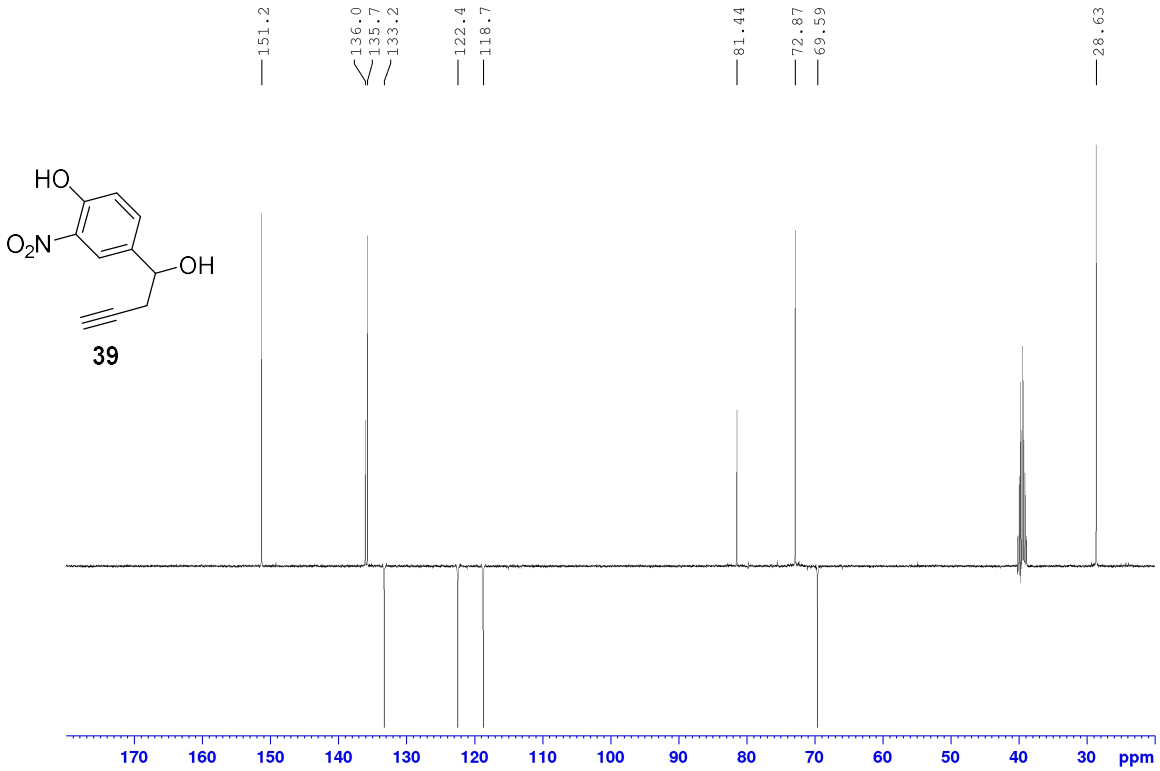
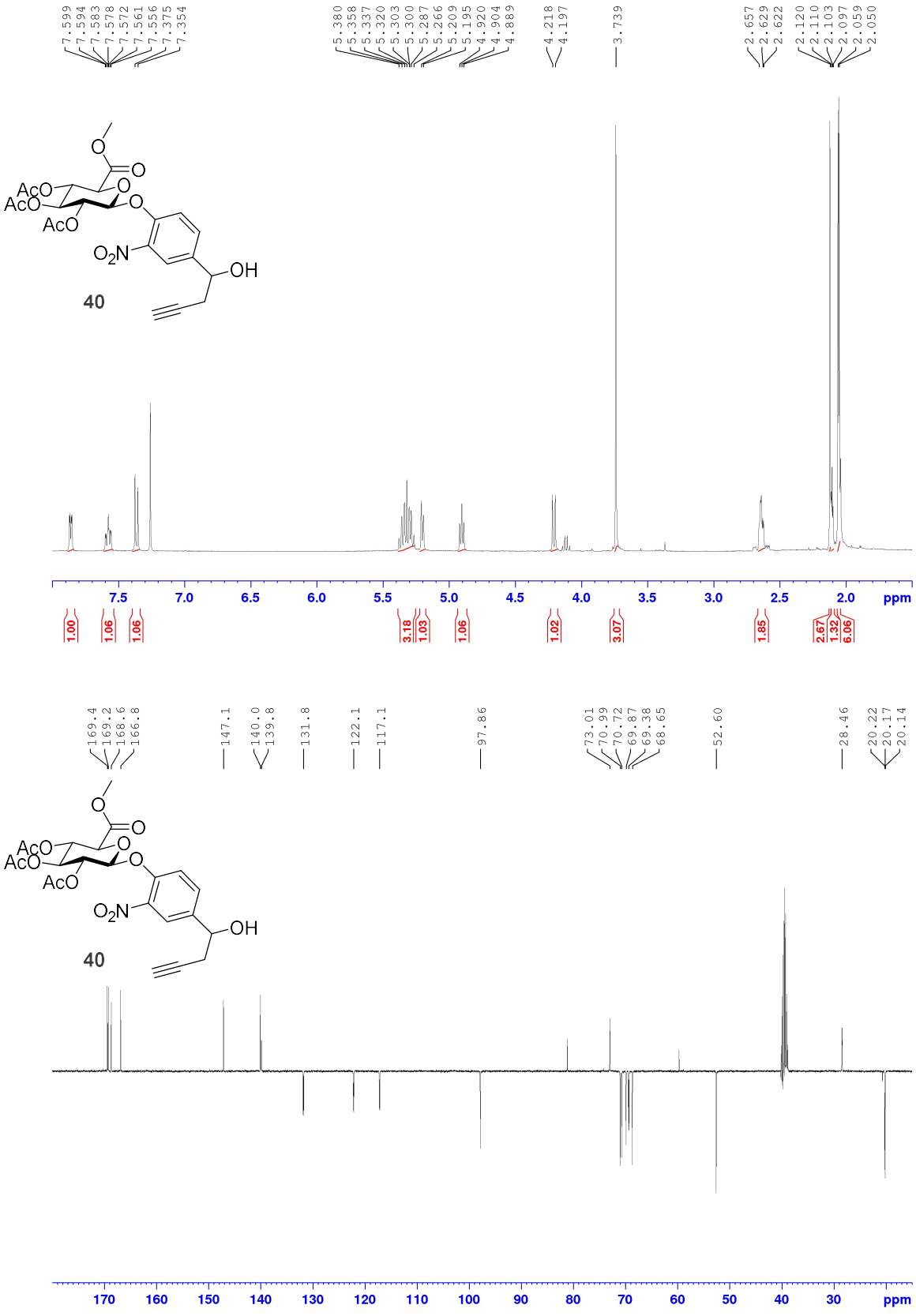
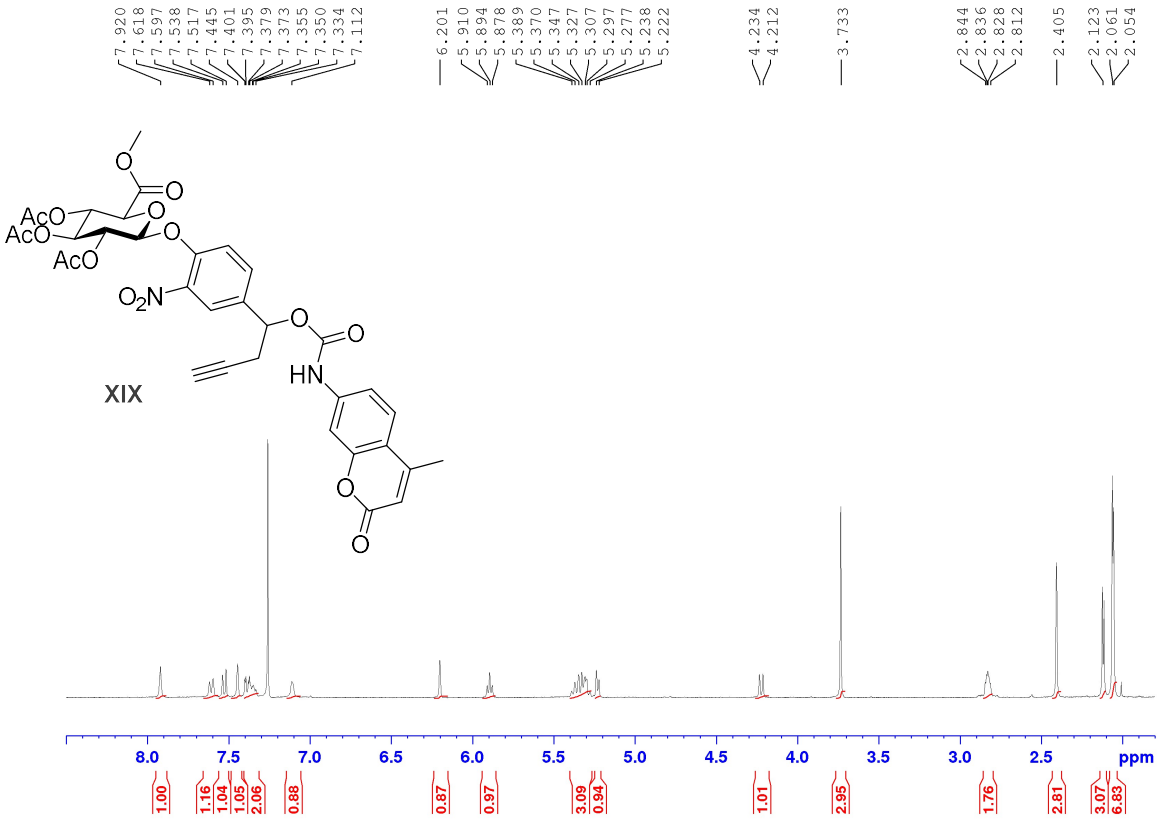


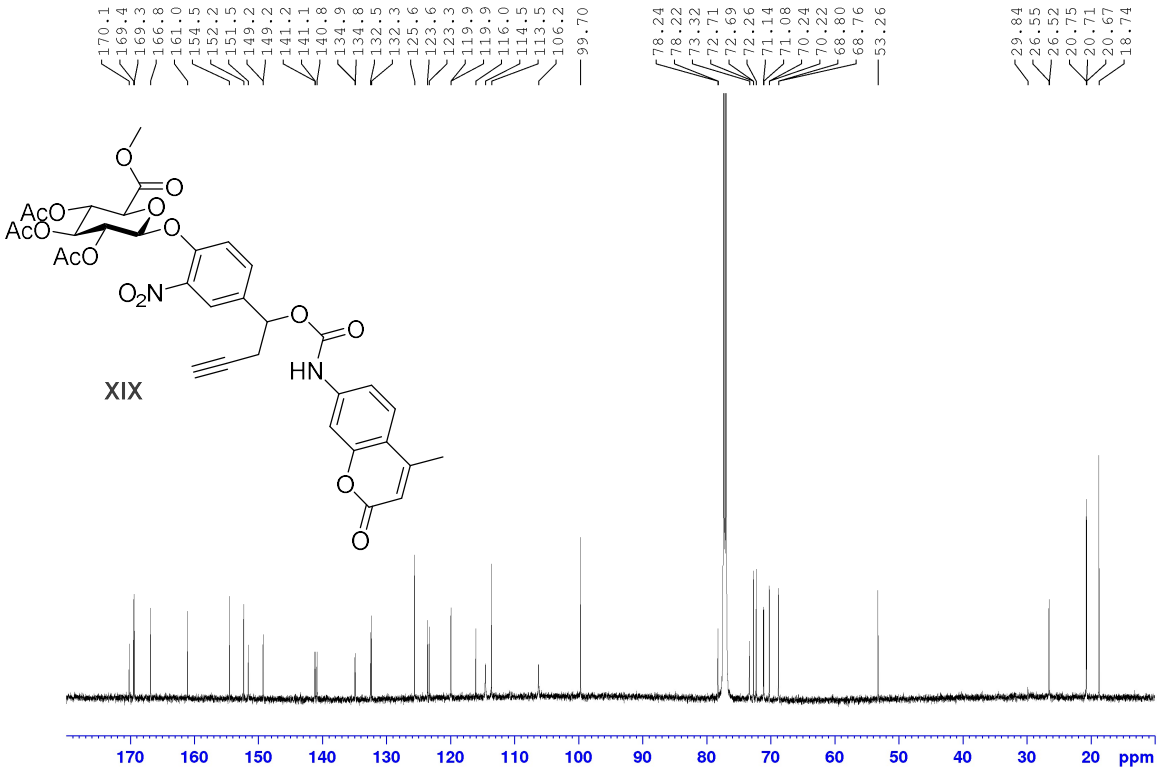


NitPh-GlcA-triacteyl methylester (40)

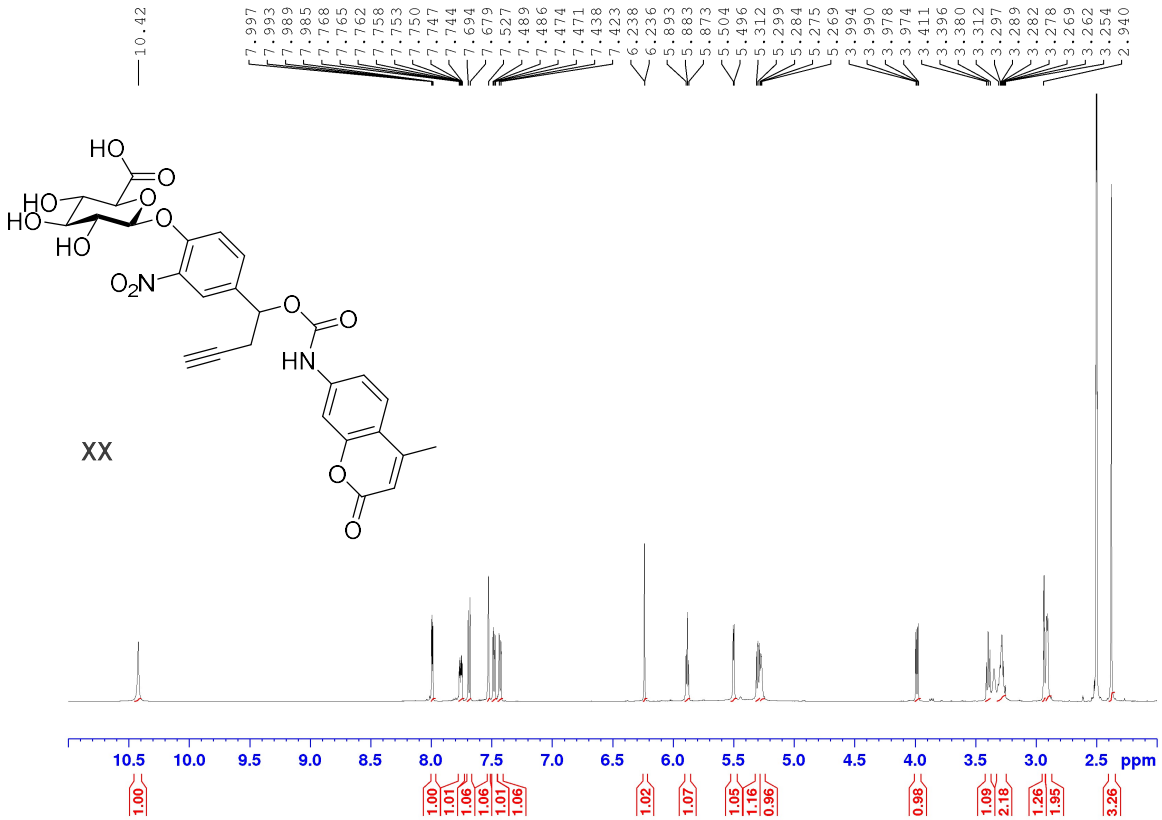


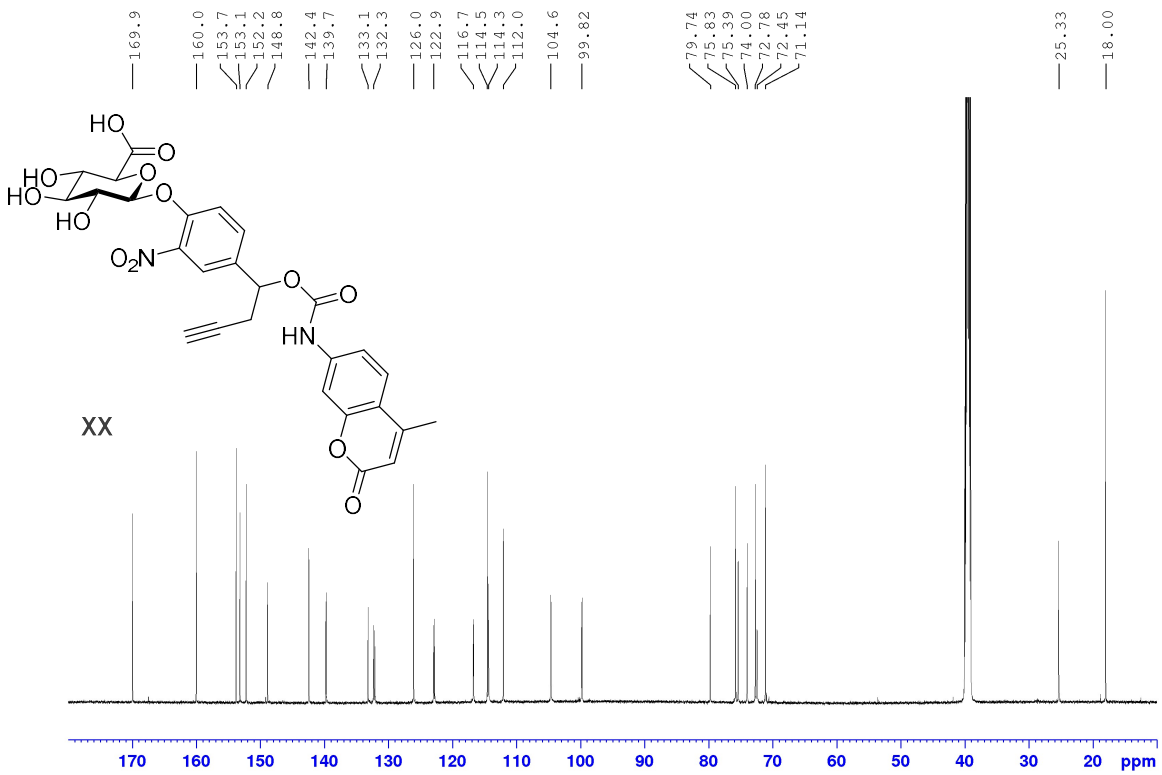
7-AMC-NitPh-GlcA-triacteyl methylester (XIX)





7-AMC-NitPh-GlcA (XX)





H) Curriculum Vitae

Stefan Lexmüller

Date and Plcae of Birth	March, 16th1987, Neunkirchen
Citizenship	Austria
E-Mail	stefan.lexmueller@gmail.com

Education

2014 – 2018	**Vienna University of Technology** PhD studies Technical Chemistry
2012 --2013	**Vienna University of Technology** Master studies Technical Chemistry – Synthesis, passed with distinction
Diploma Thesis	Entwicklung neuartiger Strategien zur optimierten Synthese von Glycokonjugaten als Substrate für diagnostische Analaysen
2007 – 2012	**Vienna University of Technology** Bachelor studies Technical Chemistry
Bachelor Thesis	Synthese von Modellverbindungen des Mykotoxins Zearalenol (ZOL)
2001 – 2006	**HBLVA Rosensteingasse** Specialization: Chemical Informatics

Work Experience

2014-2018	**Project Assistant** Vienna University of Technology, Institute of Applied Synthetic Chemistry, Austria
2012-2013	**Student Assistant** Vienna University of Technology, Institute of Applied Synthetic Chemistry, Austria

Competences

Languages	German (mother tongue) English (fluent, spoken and written)
IT competences	MS Office, Adobe Creative Cloud, Chemoffice, SciFinder, GaphPad Prism, OriginPro, Bruker Topspin, Xcalibur, MestreNova, LabSolutions, Chromeleon
Driving license	Class B
Further competences	SSI Master Diver (including oxygen provider & Stress and Rescue)

Further Information

Hobbies	Scuba diving, underwater photography, photo and video editing travelling, reading, rowing, snowboarding,
Memberships	GÖCH (Gesellschaft österr. Chemiker), ÖVUST (Österreichischer Verein für Unterwasserbiologie, Sporttauchen & Tauchsicherheit)