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(71) Applicant: **SOLVENTUM INTELLECTUAL PROPERTIES COMPANY** [US/US]; 2510 Conway Ave E, 275-6E-21, Maplewood, Minnesota 55144 (US).

(72) Inventors: **ROSCOE, Stephen B.**; 2510 Conway Ave E, 275-6E-21, Maplewood, Minnesota 55144 (US). **BONILLA, Tonya D.**; 2510 Conway Ave E, 275-6E-21, Maplewood, Minnesota 55144 (US).

(74) Agent: **WEBER, Kevin W.** et al.; Solventum Intellectual Properties Company, 2510 Conway Ave E, 275-6E-21, Maplewood, Minnesota 55144 (US).

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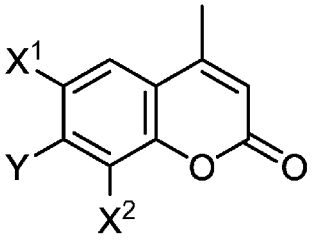
(54) Title: A FLUORESCENT ENZYME SUBSTRATE AND METHODS OF USING THE SAME




(I)

(57) Abstract: Described herein is a β-glycoside of the structural formula (I) wherein X1 or X2 is -C(=O)OR, R is an alkyl group comprising 1 to 6 (1-4) carbon atoms, and Y is a monovalent sugar selected from β-D-glucose, β-D-galactose, β-D-glucuronic acid, N-acetyl-glucosamine, or galactosamine, wherein when X1 is -C(=O)OR, then X2 is - H and when X2 is -C(=O)OR then X1 is -H. Such β-glycosides may be used as indicators, for example biological indicators.

# A FLUORESCENT ENZYME SUBSTRATE AND METHODS OF USING THE SAME

## TECHNICAL FIELD

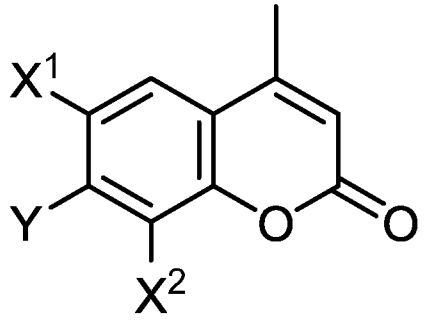
**[0001]** The present disclosure broadly relates to a novel beta-glycoside, which can be used as a fluorescent enzyme substrate for applications such as a biological indicator.

## SUMMARY

**[0002]** Fluorescent enzyme substrates are initially nonfluorescent molecules that fluoresce brightly in the presence of an enzyme. Fluorescent enzyme substrates are widely used in a variety of applications, such as microbiology, water and environmental testing, and drug discovery. Common substrates are based on 4-methyl-7-hydroxy coumarin (4-MU), which has a pKa of 7.8 and whose fluorescence is acutely pH-dependent, producing a strong fluorescent signal at pH values greater than 7, but sharply reduced fluorescence at acidic pH values.

**[0003]** Certain applications require the ability to function and generate a fluorescent signal at neutral or acidic pH. Previous solutions to this challenge involved the use of halogen substituents on a coumarin-moiety to lower the pKa and work well to provide signal at acidic pH. However, there are various reasons to not use organohalogens, including reduced solubility, reduced long-term stability, and environmental concerns. Thus, there is a desire to identify novel fluorescent enzyme substrates, which do not comprise halogens and optionally can be used at lower pHs (i.e., have a lower pKa).

**[0004]** In one aspect, a glycoside is disclosed. The glycoside is of the structural formula (I)




(I)

wherein $X^1$ or $X^2$ is -C(=O)OR, R is an alkyl group comprising 1 to 6 carbon atoms, and Y is a monovalent sugar selected from β-D-glucose, β-D-galactose, β-D-glucuronic acid, N-acetylglucosamine, or galactosamine, wherein when $X^1$ is -C(=O)OR, then $X^2$ is -H and when $X^2$ is -C(=O)OR then $X^1$ is -H.

**[0005]** In another aspect, a method of indicating is disclosed. The method comprising: contacting an enzyme with the glycoside according to formula (I), wherein the enzyme is capable of catalyzing cleavage of the glycoside of Formula I.

**[0006]** In some embodiments, the enzyme and the glycoside form a mixture having an initial pH in the range from 5.5 to 9.0.

**[0007]** The above summary is not intended to describe each embodiment. The details of one or more embodiments of the invention are also set forth in the description below. Other features, objects, and advantages will be apparent from the description and from the claims.

## DETAILED DESCRIPTION

**[0008]** As used herein, the recitation of ranges by endpoints includes all numbers subsumed within that range (e.g., 1 to 10 includes 1.4, 1.9, 2.33, 5.75, 9.98, etc.).

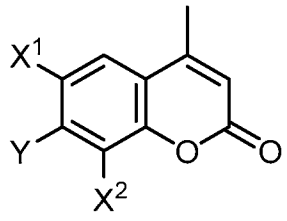
**[0009]** Also herein, recitation of "at least one" includes all numbers of one and greater (e.g., at least 2, at least 4, at least 6, at least 8, at least 10, at least 25, at least 50, at least 100, etc.).

**[0010]** As used herein, "comprises at least one of" A, B, and C refers to element A by itself, element B by itself, element C by itself, A and B, A and C, B and C, and a combination of all three.

**[0011]** As used herein, the term "a", "an", and "the" are used interchangeably and mean one or more.

**[0012]** The present disclosure provides a glycoside, which can be used as a fluorescent enzyme substrate. In some embodiments, the glycoside can be used to react with a bacterial enzyme (such as beta-galactosidase enzyme) to generate fluorescent species. The fluorescent species can have improved fluorescence yield, as compared to nonhalogenated and fluorinated analogs, at conditions in a pH range of 5.5 to 9.0, for example, pH values of at least 6.5, 6.0, or even 5.5 and at most 9.0, 8.5, 8.0, 7.5, or even 7.0.

**[0013]** The glycosides of the present disclosure are represented by the structural formula



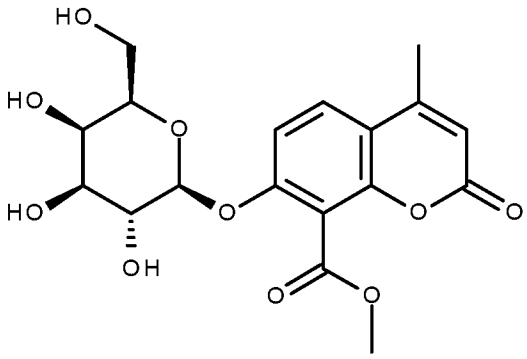
(I)

wherein $X^1$ or $X^2$ is -C(=O)OR, R is an alkyl group comprising 1 to 6 (1-4) carbon atoms, and Y is a monovalent sugar selected from β-D-glucose, β-D-galactose, β-D-glucuronic acid, N-acetylglucosamine, or galactosamine, wherein when $X^1$ is -C(=O)OR, then $X^2$ is -H and when $X^2$ is -C(=O)OR then $X^1$ is -H

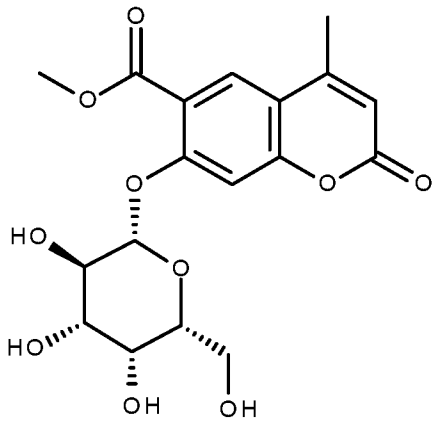
**[0014]** R is an alkyl group comprising 1 to 6 carbon atoms, more preferably 1 to 4 carbon atoms. The alkyl group may be linear, branched, or cyclic. Exemplary alkyl groups include: methyl, ethyl, propyl, butyl, pentyl, hexyl, and a cyclohexyl groups.

**[0015]** Y is a monovalent sugar (i.e., a sugar group, such as a monosaccharide or a derivative thereof missing one electron). Exemplary sugars include β-D-glucose, β-D-galactose, β-D-glucuronic acid, N-acetylglucosamine, or galactosamine.

**[0016]** Exemplary β-glycosides according to Formula (I) include:



and

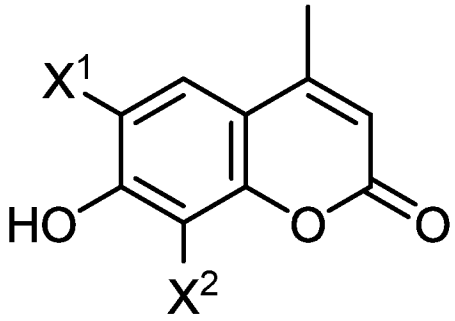


.

**[0017]** Glycosides according to Formula (I) can be prepared by well-known general techniques, including, for example, those describes in the examples hereinbelow. For example, acid-catalyzed cycloaddition of ethyl acetoacetate with methyl 2,4-dihydroxybenzoate results in the corresponding methyl 7-hydroxy-4-methyl-2-oxo-chromene carboxylate. These resulting chromene carboxylates can be coupled to sugars to form the glycosides disclosed herein.

**[0018]** The glycosides according to Formula (I) can be used as fluorescent enzyme substrates, which will fluorescence in the presence of an entity, such as an enzyme, which can selectively

cleave the sugar moiety from Formula (I), resulting in the coumarin derivative, according to Formula (II)




(II)

**[0019]** The compound according to Formula (II) can fluoresce when in its anionic form. Typically, excitation wavelengths for the molecule according to formula (II) are between 340 nm and 370 nm (nanometers) and emission wavelengths are between 440 nm and 460 nm. However, these excitation/emission maxima may vary depending on the structure and position of $X^1$ and $X^2$ in Formula (II) and can be readily determined by some characterization of the fluorophore.

**[0020]** It has been discovered that the compounds according to Formula (II) have an unexpectedly low pKa. In some embodiments, the compounds according to Formula (II) have a pKa of less than 7.5, 7.3, 7.1, 6.8, 6.6, 6.3, 6.0, 5.8, or even 5.5. Typically, the pKa is greater than 5.0 as lower pKa's may have limited hydrolytic stability.

**[0021]** The fluorescent enzyme substrates according to Formula (I) disclosed herein may be used as indicators in a variety of applications including microbiology, water and environmental testing, and drug discovery.

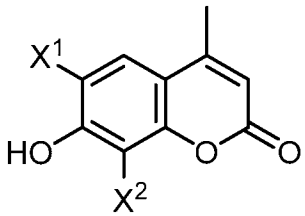
**[0022]** In some embodiments, the glycoside according to Formula (I) may be used to indicate the presence of an enzyme. For example, an enzyme capable of catalyzing cleavage of Formula (I) to yield Formula (II) is contacted with Formula (I). Exemplary enzymes include beta-D-galactosidase, beta-D-glucosidase, N-acetyl-beta-glucosaminidase, beta-D-glucuronidase, and combinations thereof.

**[0023]** Advantageously, because of the pKa of Formula (II), the reactions may be conducted at neutral to acidic pHs. For example, the maximum reaction activity of the enzyme can be at a pH of 7.5 or lower. For example, pH values of between 5.5 to 9.0, 5.5 to 7.5, 5.5 to 7.0, 6.5 to 9.0, 6.5 to 7.5, or even 6.5 to 7.0. In some embodiments, the reaction mixture has a pH of 7.5 or lower. For example, pH values of between 5.5 to 9.0, 5.5 to 7.5, 5.5 to 7.0, 6.5 to 9.0, 6.5 to 7.5, or even 6.5 to 7.0.

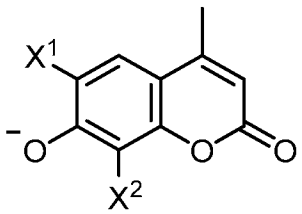
**[0024]** In some embodiments, the glycosides according to Formula (I) are contacted with microorganisms that are capable of acting upon the enzyme substrate, thereby cleaving the sugar

residue and generating the corresponding coumarin, which is highly fluorescent and may be detected readily by fluorescence, although other methods of detection may also be used. The microorganism either comprises (i) an enzyme capable of catalyzing the cleavage of a sugar residue to produce a fluorescently-detectable compound, or (ii) is capable of producing such an enzyme capable of catalyzing the cleavage of a sugar residue, or (iii) both. Based at least in part the fluorescence observed, an evaluation can be made. Exemplary microorganisms include those producing beta-galactosidases.

**[0025]** When acted upon by an enzyme capable of cleaving a beta-sugar, the glycosides according to formula (I) of the present disclosure are cleaved to form the fluorescent compounds shown below:




which is in equilibrium with the deprotonated forms (shown below):




in relative amounts depending on pH.

**[0026]** Methods of indicating according to the present disclosure may be used to detect and monitor the presence and activity of organisms that product the relevant enzyme, such as coliforms (beta-galactosidase-producers) or *E. coli* which also produces beta-glucuronidase. Fluorescent substrates to *E. coli* are used, for example, in test kits available under the trade designation COLILERT and COLISURE (from IDEXX Laboratories, Westbook, ME) for detecting these organisms in environmental water samples. Beta-galactosidase, for example, is also widely used as a biomarker in molecular biology studies. In some embodiments, the glycosides according to the present disclosure may be contacted with a composition comprising a microorganism. In some embodiments, the composition may also include nutrients for the microorganisms, such as germination nutrients that allow growth and/or germination if the microorganism is a spore. In some embodiments, the composition is solid (e.g., a powder).

**[0027]** Suitable nutrients may be provided initially in a dry form (e.g., powdered form, tablet form, caplet form, capsule form, a film or coating, entrapped in a bead or other carrier, another suitable shape or configuration, or a combination thereof) and then optionally combined with a

suitable solvent to provide a composition that is then combined, for example, with the microorganism.

**[0028]** In some embodiments, the composition is liquid (i.e., a liquid composition). In some of those embodiments, the solvent of the liquid composition is water. The combination of nutrients form a nutrient medium and together with the enzyme substrate and one or more non-nutrient components such as indicators, buffer components, salts, solvent, etc. (see below) form a mixture.

**[0029]** The nutrients in the composition can include one or more sugars, including, for example, glucose, fructose, dextrose, maltose, trehalose, cellobiose, or the like, or a combination thereof. Alternatively, the nutrients may include complex media such as, for example, peptone, tryptone, phytone peptone, yeast extract, soybean casein digest, other extracts, hydrolysates, or a combination thereof. In other embodiments, the nutrients in the composition represent a combination of one or more complex media components and other specific nutrients. The nutrient medium can also include a salt, including, but not limited to, sodium chloride, potassium chloride, calcium chloride, or the like, or a combination thereof. In some embodiments, the nutrient can further include at least one amino acid, including, but not limited to, at least one of methionine, phenylalanine, alanine, tyrosine, and tryptophan.

**[0030]** In some embodiments, the composition may comprise a buffered solution. The ionic conditions of the buffered solution should be such that the enzyme and enzyme substrate are not affected. In some embodiments, a buffer solution is used as part of the composition, such as phosphate buffers, (e.g., phosphate buffered saline solution, potassium phosphate or potassium phosphate dibasic), tris(hydroxymethyl) aminomethane-HCl solution, or acetate buffer, or any other buffer suitable for sterilization known in the art. Buffers suitable for the present biological sterilization indicators should be compatible with fluorogenic and chromogenic enzyme substrates used as part of the composition. Another consideration in choosing the buffers is their influence on the enzyme activity. For example, phosphate buffered saline contains a relatively high concentration of inorganic phosphate, which is a competitive inhibitor of alkaline phosphatase. Thus, for that enzyme, a Tris-HCl buffer is recommended. The strength of the buffered solution may be from 0.05 M to 0.5 M, preferably from 0.05 M to 0.25 M, more preferably from 0.05 M to 0.15 M, even more preferably about 0.1 M.

**[0031]** The concentration of enzyme substrate present in the mixture depends upon the identity of the particular substrate and microorganism (e.g., enzyme), the amount of enzyme-product that must be generated to be detectable, either visually or by instrument, and the amount of time that one is willing to wait in order to determine whether active enzyme is present in the reaction mixture. Preferably, the amount of enzyme substrate is sufficient to react with any enzyme

present, for example, within about an eight-hour period of time, such that at least $10^{-8}$ molar enzyme-modified product is produced.

**[0032]** In some embodiments, the composition comprises a solution adjusted to a suitable pH, but without an added buffer system. In other embodiments, however, the composition does comprise a buffered solution.

**[0033]** In some embodiments, the methods may further comprise an additional indicator compound that can facilitate the detection of another metabolic activity of the test microorganisms (e.g., spore) (aside from an enzyme substrate that can produce a fluorescently-detectable compound). This additional metabolic activity can also be an enzymatic activity. Non-limiting examples of indicator compounds include a chromogenic enzyme substrate (e.g., observable in the visible spectrum), a pH indicator, a redox indicator, a chemiluminescent enzyme substrate, a dye, and a combination of any two or more of the foregoing indicator compounds.

**[0034]** In some embodiments, the additional indicator is a pH indicator that produces a change in color when the pH decreases, indicating growth of the test microorganisms. In some embodiments, the pH indicator is bromocresol purple. The pH indicator can be used to detect a second biological activity, such as the fermentation of a carbohydrate to acid end products (suggesting survival of the test microorganisms), for example. The bromocresol purple can be used at a concentration of about 0.03 g/L in the aqueous mixture, for example. Enzyme substrates according the present disclosure can be used, for example, at a concentration of about 0.05 to about 0.5 g/L (e.g., about 0.05 g/L, about 0.06 g/L, about 0.07 g/L, about 0.08 g/L, about 0.09 g/L, about 0.1 g/L, about 0.15 g/L, about 0.2 g/L, about 0.25 g/L, about 0.3 g/L, about 0.35 g/L, about 0.4 g/L, about 0.45 g/L, about 0.5 g/L) in the aqueous mixture.

**[0035]** Upon contact on the enzyme substrate with the microorganism, the mixture may be incubated for a period of time and under conditions that would be sufficient to generate a detectable amount of the enzyme modified product. In general, the amount of product which is detectable by known methods is at least $10^{-8}$ molar. Preferably, the incubation conditions are sufficient to generate at least $10^{-8}$ molar of fluorescently-detectable compound, more preferably, at least about $10^{-6}$ molar or even at least about $10^{-5}$ molar of fluorescently-detectable compound. The incubation time and temperature needed to produce a detectable amount of fluorescently-detectable compound will depend upon the identity of the microorganism and the substrate, and the concentrations of each present in the reaction mixture. In general, the incubation time required is between about 1 minute and 12 hours, and the incubation temperature is between about 20°C and 70°C.

**[0036]** Detection can be initiated immediately after the microorganism mixture and the enzyme substrate have been combined to achieve a baseline reading. After that, any detectable change from the baseline reading can be detected. The signal can be monitored and measured continuously or intermittently. In some embodiments, a portion of, or the entire, incubating step may be carried out prior to measuring the detectable change. In some embodiments, incubation can be carried out at a first temperature (e.g., at 37°C, or at 50-60°C), and measuring of the detectable change can be carried out at a second different temperature (e.g., at room temperature, 25°C, or at 37°C). In other embodiments, the incubation and measurement of fluorescence occurs at the same temperature.

**[0037]** Detection can be, in some embodiments, less than 8 hours, in some embodiments, less than 1 hour, in some embodiments, less than 30 minutes, in some embodiments, less than 15 minutes, in some embodiments, less than 5 minutes, and in some embodiments, less than 1 minute. In other embodiments, the detection is from 2 min to 1 hr, or from 2 min to 50 min, or from 2-30 min, or from 2-20 min, or from 2-25 min, or from 5 to 30 min, or from 5-25 min, or from 5-20 min. The detection of fluorescence above the baseline reading that would indicate presence of microorganisms can be performed according to any method know in the art, including area under curve (in a plot of time vs fluorescence intensity), monitoring a change in slope of the curve, using a threshold value for the fluorescence, etc., or a combination thereof of two or more techniques.

**[0038]** Objects and advantages of this disclosure are further illustrated by the following non-limiting examples, but the particular materials and amounts thereof recited in these examples, as well as other conditions and details, should not be construed to unduly limit this disclosure.

## EXAMPLES

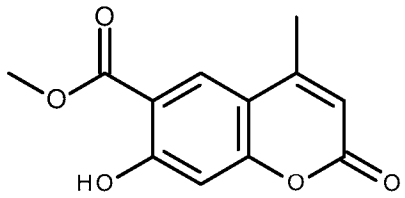
**[0039]** Unless otherwise noted, all parts, percentages, ratios, etc. in the examples and the rest of the specification are by weight, and all reagents used in the examples were obtained, or are available, from general chemical suppliers such as, for example, Sigma-Aldrich Company, Saint Louis, Missouri, or may be synthesized by conventional methods.

**[0040]** The following abbreviations are used below: M = molar, mL= milliliters, MHz = megahertz, mg = milligram, µL= microliters, µg= micrograms, and µm= micrometer.

TABLE 1. Materials List

| Name | Description |
|---|---|
| methyl 2,4-dihydroxybenzoate | TCI America, Portland, OR |
| methyl-2,6-dihydroxybenzoate | TCI America, Portland, OR |
| ethyl acetoacetate | Oakwood Products, Inc., Estill, SC |
| methanesulfonic acid | Alfa Aesar, ThermFisher Scientific, Waltham, MA |
| collidine | Alfa Aesar, ThermFisher Scientific, Waltham, MA |
| silver carbonate | Alfa Aesar, ThermFisher Scientific, Waltham, MA |
| Acetobromo-alpha-D galactose | Sigma-Aldrich, St Louis, MO |
| Brine (sodium chloride) | VWR Chemicals, Solon, OH |
| sodium sulfate | Aventor Performance Materials, Radnor, PA |
| NaOMe | Sodium methoxide, TCI America, Portland, OR |
| 0.1M phosphate buffers of pH 3.89 to 10.40 | Prepared by making 0.1 M solutions of sodium phosphate dibasic heptahydrate (VWR Chemicals, Solon, OH), sodium phosphate monobasic monohydrate (J.T. Baker, Philipsburg, NJ), and phosphoric acid (EMD Millipore, Billerica, MA), and mixing the solutions in appropriate ratios to achieve a desired pH |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich, St Louis, MO |
| Methylene chloride ($CH_2Cl_2$) | EMD Millipore, Billerica, MA |
| Powdered molecular sieves | Sigma-Aldrich, St Louis, MO |
| beta-galactosidase enzyme | EMD Millipore, Billerica, MA |
| PBS buffer | Phosphate buffered saline 10X concentrate, available from Sigma-Aldrich, St Louis, MO |
| MgCl2.6H2O | Calbiochem, La Jolla, CA |
| beta-mercaptoethanol | VWR Chemicals, Solon, OH |
| 4-methylumbelliferyl beta galactoside (MUgal) | Biosynth, AG, Staad, Switzerland |

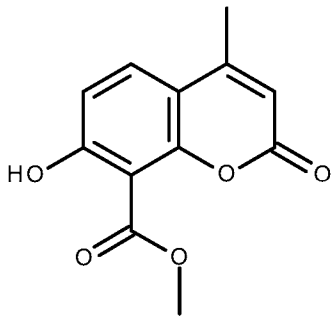
**[0041]** Preparative Example S1: Synthesis of methyl 7-hydroxy-4-methyl-2-oxo-chromene-6-carboxylate



**[0042]** Methyl 2,4-dihydroxybenzoate (4.98 g, 29.6 mmol) was stirred in ethyl acetoacetate (5.0 mL, 39.2 mmol) and cooled in an ice bath. Methanesulfonic acid (approximately 40 mL) was added dropwise over approximately 30 minutes. The reaction was left to warm up to room temperature with stirring overnight. After two days, the resulting pale brown solution was added dropwise to vigorously stirred deionized water (750 mL) and stirred for a half hour. The resulting solid was filtered, washed on the filter with deionized water, then the filter with the washed solid was placed in the vacuum oven overnight to dry. The resulting yield of product was 6.889 g with a 99% yield.

**[0043]** The dried solid was recrystallized in hot methanol (3g in 400 mL) at a rolling boil. The solid did not fully dissolve, so the hot dissolved solution was filtered, then the filtered solution was reheated and allowed to cool overnight to obtain a purified product. Characterization of product: $^{1}$H NMR (CDCl3, 500 MHz) δ 11.05 (s, 1H), 8.00 (s, 1H), 6.77 (s, 1H), 6.06 (d, *J*=1.0 Hz, 1H), 3.91 (s, 3H), 2.31 (d, *J*=1.0 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl3) δ 169.6, 164.1, 160.1, 158.4, 152.1, 127.5, 113.1, 112.8, 109.6, 104.8, 52.7, 18.7.

**[0044]** Preparative Example S2: Synthesis of methyl 7-hydroxy-4-methyl-2-oxo-chromene-8-carboxylate



**[0045]** Methyl 2,6-dihydroxybenzoate (2.51 g, 14.9 mmol) was stirred in ethyl acetoacetate (2.0 mL, 15.7 mmol) without dissolving, and cooled in an ice bath. Methanesulfonic acid (approximately 25 mL) was added dropwise over approximately 15

minutes and the reaction mixture was left to warm up to room temperature with stirring overnight.

**[0046]** The resulting clear yellow solution was added dropwise to vigorously stirred deionized water (300 mL) and stirred for about a half hour. The resulting suspension was filtered, resulting in an off-white solid, which was washed on the filter with deionized water (2 x 70 mL) and dried on the filter in a vacuum oven overnight.

**[0047]** An approximate 1g quantity of the solid was dissolved in 35 mL chloroform, filtered and then added to the top of a 50g SFar silica column (Biotage, Uppsala, Sweden) and eluted with varying amounts (0-10%) of acetone in chloroform. Fractions containing the desired product were combined and the solvent was removed on the rotovap and then on the vacuum line overnight.

**[0048]** The nuclear magnetic resonance (NMR) is consistent with the desired product.

$^{1}$H NMR (DMSO-d6, 500 MHz) δ 11.1 (s, 1H), 7.69 (d, *J*=8.8 Hz, 1H), 6.92 (d, *J*=8.8 Hz, 1H), 6.20 (d, *J*=1.0 Hz, 1H), 3.85 (s, 3H), 2.38 (d, *J*=1.0 Hz, 3H).

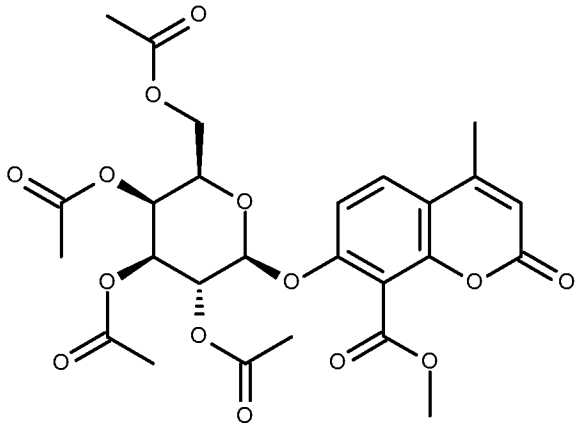
$^{13}$C NMR (126 MHz, DMSO-d6) δ 164.7, 159.4, 158.0, 153.6, 151.2, 127.8, 112.7, 111.8, 110.7, 109.7, 52.6, 18.3.

**[0049]** Determination of pKa

**[0050]** The pKa was determined based on the fluorescence spectra as a function of pH as follows. Standard solutions were prepared at 0.2 mg/mL by dissolving chromatography purified samples of S1 (5.4 mg) and S2 (6.1 mg) in 27 and 30.5 mL DMSO, respectively. These were then further diluted 4:1 in DMSO to give a 50 µg/mL stock solution of each Preparative Example.

**[0051]** The stock solutions of S1 and S2 were diluted to 5 µg/mL in 12 different 0.1 M phosphate buffers that spanned a range from pH 3.89 to 9.40. Absorbance, excitation and emission spectra were recorded on a SpectraMax M5 spectrometer (Molecular Devices, San Jose, MA). Absorption bands are in similar positions for both S1 and S2 across the pH range, although the absorption peak at 360 nm is significantly stronger at high pH for S2 than for S1. Emission spectra were recorded with 360 nm excitation, demonstrating that S2 has an emission maximum at 440 nm, and S1 has an emission maximum at 460 nm. A chart of maximum fluorescence at each different pH yields titration curves that can be standardized and compared to ideal titration curves yielding excellent fits to pKa 7.1 for S1, and pKa 5.9 for S2.

**[0052]** Example 1: Synthesis of methyl 4-methyl-2-oxo-7-[(2S,3R,4S,5S,6R)-3,4,5-triacetoxy-6-(acetoxymethyl)tetrahydropyran-2-yl]oxy-chromene-8-carboxylate



**[0053]** Preparative Example S2 (0.2546g = 1.09 mmol) was stirred without dissolving in $CH_2Cl_2$ (15 mL). Powdered molecular sieves (0.1034 g) was added, followed by collidine (0.60 mL = 4.5 mmol) and silver carbonate (0.4590, 1.66 mmol). The flask was then wrapped in tinfoil, stirred and warmed up to 50°C over 15 minutes. Acetobromo-alpha-D galactose (0.6053g = 1.47 mmol) was then added and the solution left to stir overnight at 50°C under a slight positive pressure of nitrogen, still wrapped in tinfoil.

**[0054]** After 24 hours, more chloroform (30 mL) was added and heating continued.

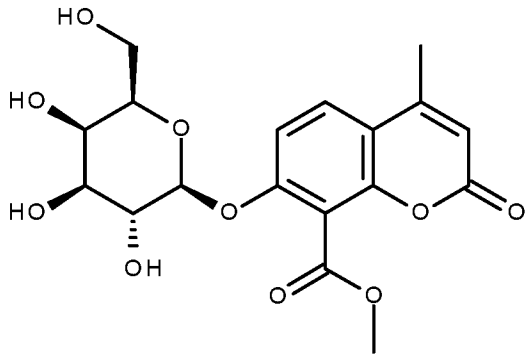
**[0055]** After a further 24 hours, the reaction mixture was filtered through a 1- micron silica syringe filter. The filtrate was extracted with 0.4M hydrochloric acid (2 x 50 mL), deionized water (50 mL) and a saturated sodium chloride solution (50 mL). The organic phase was dried over sodium sulfate, filtered, and solvent removed on the rotorvap and vacuum line.

**[0056]** Finely divided solids were removed by suspending the residue in chloroform (30 mL), and then passing it sequentially through 1.0, 0.45, and 0.2 μm (micrometer) syringe filters. The resulting clear brown liquid was rotorvapped down to a powder and dried on the vacuum line overnight. 580 mg of material was collected with a 95% yield.

**[0057]** The collected sample was dissolved in about 6 mL of chloroform and then ran through a 25 g silica column (available under the trade designation "SNAP Ultra" cartridge available from Biotage, Uppsala, Sweden). Fractions 1 through 8 were combined and then rotorvapped, leaving 540 mg of an off-white foam. The sample was dried on a vacuum line overnight.

**[0058]** Characterization: $^{1}$H NMR (CDCl3, 500 MHz) δ 7.59 (d, *J*=9.1 Hz, 1H), 7.07 (d, *J*=9.1 Hz, 1H), 6.21 (d, *J*=1.2 Hz, 1H), 5.53 (dd, *J*=10.5, 7.8 Hz, 1H), 5.48 (dd, *J*=3.4, 0.7 Hz, 1H), 5.11 (dd, *J*=10.5, 3.4 Hz, 1H), 5.06 (d, *J*=7.8 Hz, 1H), 4.27 (dd, *J*=11.0, 6.6 Hz, 1H), 4.18 (dd, *J*=11.3, 6.4 Hz, 1H), 4.12 (td, *J*=6.6. 0.7 Hz, 1H), 3.97 (s, 3H), 2.42 (d, *J*=1.2 Hz, 3H), 2.20 (s, 3H), 2.12 (s, 3H), 2.10 (s, 3H), 2.02 (s, 3H).
$^{13}$C NMR (acetone, 126 MHz) δ 170.25, 170.02, 169.69, 169.38, 163.94, 159.05, 156.18, 152.86, 151.10, 127.77, 115.80, 113.69, 113.39, 111.19, 99.43, 71.72, 70.59, 68.23, 67.53, 61.70, 52.68, 20.20, 20.12, 20.03, 19.97, 18.11.

**[0059]** Example 2: Synthesis of a methyl 4-methyl-2-oxo-7-[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxy-chromene-8-carboxylate. (8MEgal)




**[0060]** Example 1 (0.44g = 0.78 mmol) was stirred in dry methanol (20 mL, previously opened bottle) for about 15 minutes, forming a cloudy white suspension. 40 uL of 5 M NaOMe (=0.2 mmol) was added, forming a yellow color and almost fully dissolving within 5 minutes. The sample was stirred under flowing nitrogen. Thin layer chromatography after 0.5 hours indicated reaction to a single product. Hydrochloric acid (0.2 mL at 1N) was added to neutralize the methoxide, and the solution was reduced in volume on the rotary evaporator. 0.28 g of product was collected which was a 90% yield.

**[0061]** The product dried on a vacuum line overnight. Then, the solid was triturated with 10 mL methanol for 1 hour and left it to settle. The supernatant was removed with a pipette, and then placed on the rotary evaporator to remove volatiles.

**[0062]** Characterization of the resulting product: $^{1}$H NMR (DMSO-d6, 500 MHz) δ 7.82 (d, *J* =9.1 Hz, 1H), 7.24 (d, *J* =9.1 Hz, 1.0 Hz, 1H), 6.30 (d, *J* =1.2 Hz, 1H), 5.03 (d, *J* =7.6 Hz, 1H), 3.88 (s, 3H), 3.70 (d, *J* =2.7 Hz, 1H), 3.64 (t, *J* =6.4 Hz, 1H),3.55 (m,
13C NMR (DMSO-d6, 126 MHz) δ 163.84, 159.10, 156.49, 153.36, 150.30, 127.71,

113.99, 112.12, 112.09, 111.33, 100.69, 75.74, 73.31, 70.05, 67.99, 60.23, 52.74, 18.27.

**[0063]** Test 1

**[0064]** The galactosidase activity of 8MEgal was then assessed as follows: A stock solution of beta-galactosidase was prepared by dissolving beta-galactosidase enzyme (5mg, fresh bottle) in 1.2 mL 1X PBS buffer (which was diluted from the 10X PBS buffer solution).

**[0065]** This stock solution (0.20 mL) was mixed with 9.1 mL PBS buffer, 0.20g MgCl2.6H2O and 0.7 mL beta-mercaptoethanol to create 50X diluted beta-galactosidase solution.

**[0066]** Solutions of the two enzyme substrates: Example 2 (8MEgal), and 4-methylumbelliferyl beta galactoside (MUgal) were prepared by dissolving 10 mg of each in 10 mL DMSO to give a 1 mg/mL solution, and then diluting 50 µL of these solutions to 10 mL to give 0.005 mg/mL solutions of each enzyme substrate.

**[0067]** In a 96-well plate, columns of wells were filled with 180 µL of one of six buffers, each at a different designated pH. For example, column A had buffer at pH 6.6, column B had buffer at pH 7.0. Then, 10 µL of one of the enzyme substrates or of PBS was added across each row. For example, 8MEgal was added across row 1 and MUgal was added across row 2, etc. The reaction was initiated by the addition of 10 µL of the 50X diluted beta-galactosidase solution to each well and fluorescence recorded at 360 nm excitation and at 450nm emission.

**[0068]** Data was recorded immediately upon adding the 50X diluted beta-galactosidase solution to the well, and then 5 minutes later, and then twice more after two half hour periods. No significant difference was observed between the data recorded at 1/2 hour and 1 hour. Each enzyme substrate at the various pH's were run three times and the results averaged. Shown in the table below is the fluorescence intensity at 30 minutes for Example 2 (8-MEgal), MUgal, and PBS (the control).

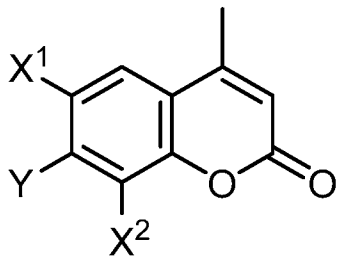
Table 1

| | Example 2 | MUgal | PBS |
|---|---|---|---|
| pH | Signal Intensity | | |
| 6.6 | 24280 | 3988 | 75 |
| 7.0 | 33634 | 8731 | 97 |
| 7.6 | 38014 | 19912 | 365 |
| 8.0 | 39407 | 27642 | 73 |
| 8.5 | 39213 | 32742 | 98 |
| 10.4 | 39597 | 36742 | 102 |

**[0069]** Foreseeable modifications and alterations of this invention will be apparent to those skilled in the art without departing from the scope and spirit of this invention. This invention should not be restricted to the embodiments that are set forth in this application for illustrative purposes. To the extent that there is any conflict or discrepancy between this specification as written and the disclosure in any document mentioned or incorporated by reference herein, this specification as written will prevail.

**What is claimed is:**

1. A glycoside of the structural formula (I):

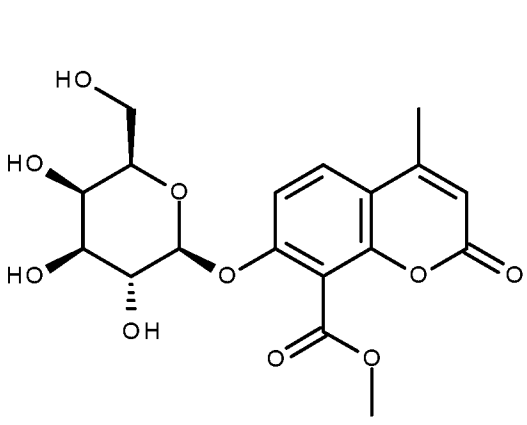


(I)

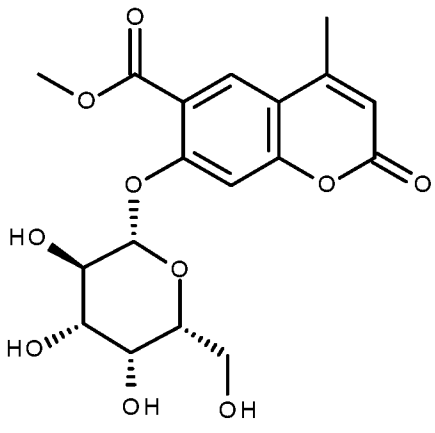
wherein $X^1$ or $X^2$ is -C(=O)OR, R is an alkyl group comprising 1 to 6 (1-4) carbon atoms, and Y is a monovalent sugar selected from β-D-glucose, β-D-galactose, β-D-glucuronic acid, N-acetylglucosamine, or galactosamine, wherein when $X^1$ is -C(=O)OR, then $X^2$ is -H and when $X^2$ is -C(=O)OR then $X^1$ is -H.

2. The glycoside of claim 1, wherein R is a methyl, ethyl, propyl, butyl, pentyl, hexyl, or cyclohexyl group.

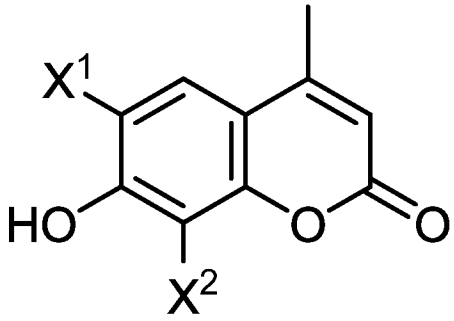
3. The glycoside of any one of the previous claims, wherein the glycoside is selected from:



or



4. The glycoside of any one of the previous claims, wherein the moiety of




has a pKa of 7.2 or lower, wherein $X^1$ or $X^2$ is -C(=O)OR and R is an alkyl group comprising 1 to 6 carbon atoms, wherein when $X^1$ is -C(=O)OR, then $X^2$ is -H and when $X^2$ is -C(=O)OR then $X^1$ is -H.

5. A method of indicating, the method comprising:
   contacting an enzyme with the glycoside according to any one of the previous claims, wherein the enzyme is capable of catalyzing cleavage of Formula I.

6. The method of claim 5, wherein the enzyme has a maximum reaction activity at a pH 7.5 or lower.

7. The method of claim 5, wherein a reaction mixture comprising the enzyme and the glycoside has a pH of 7.5 or lower.

8. The method of any one of claims 5-7, wherein the enzyme is beta-D-galactosidase, beta-D-glucosidase, N-acetyl-beta-glucosaminidase, beta-D-glucuronidase, or a mixture of two or more thereof.

# INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2024/056454

**A. CLASSIFICATION OF SUBJECT MATTER**
INV. C07H17/075 C12Q1/00 A61L2/28
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
C07H G01N A61L C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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| Y | US 2014/234884 A1 (DREVELLE ANTOINE [FR] ET AL) 21 August 2014 (2014-08-21) paragraph [0016] - paragraphs [0024], [0031] | 1-8 |
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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 September 2024 | 04/11/2024 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Zamarija, Ivica |

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International application No
PCT/IB2024/056454

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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| Y | CHEN HONG-MING ET AL: "Synthesis and evaluation of sensitive coumarin-based fluorogenic substrates for discovery of [alpha]- N -acetyl galactosaminidases through droplet-based screening",<br>ORGANIC & BIOMOLECULAR CHEMISTRY,<br>vol. 19, no. 4,<br>4 February 2021 (2021-02-04), pages 789-793, XP093208090,<br>ISSN: 1477-0520, DOI: 10.1039/D0OB02484H<br>Table 1 of page 790;<br>examples 1-3, 9 | 1-8 |
| | ----- | |

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# INTERNATIONAL SEARCH REPORT

**Information on patent family members**

**International application No**
PCT/IB2024/056454

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