Pergamon

*Tetrahedron*, Vol. 53, No. 21, pp. 7159-7164, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00413-4

# Fluorescent Molecular Probes I. The Synthesis and Biological Properties of an ELF® β-glucuronidase Substrate That Yields Fluorescent Precipitates at the Enzymatic Activity Sites

Zhenjun Diwu*, Yixin Lu, Rosalyn H. Upson, Mingjie Zhou,
Dieter H. Klaubert and Richard P. Haugland
Molecular Probes, Inc., 4849 Pitchford Avenue, Eugene, OR 97402

***Abstract:*** *A novel quinazolinone-based fluorogenic β-glucuronidase substrate has been synthesized and evaluated. In this protocol, a partially protected salicyl glucuronide is oxidatively condensed with anthranilamide to afford the required quinazolinonyl glucuronic acid methyl ester that is readily deprotected to yield the desired glucuronide, known as ELF®-97 glucuronide. The glucuronide is a sensitive fluorogenic β-glucuronidase substrate that yields a bright yellow-green fluorescent precipitate upon enzymatic reaction.* © 1997 Elsevier Science Ltd.

Detection of enzyme activity is an important tool for the analysis of biological and chemical samples ranging from whole organisms to single cells, cell extracts, biological fluids, or chemical mixtures.[1] Fluorometric methods have proven to be safe, rapid and sensitive techniques to determine enzyme activity.[2] The availability of a suitable fluorogenic substrate is critical to the success of fluorometric determinations of enzyme activities. Although fluorogenic substrates are generally preferred due to their higher sensitivity, the currently existing fluorogenic substrates still have certain deficiencies. The typical coumarin- and fluorescein-based fluorogenic substrates yield fluorescent products that generally have their maximum fluorescence above pH 8-10.[2,3] However, a number of enzymes (such as glycosidases) have optimum activity at or below pH 7.0. Procedures that require a pH change during the assay are not readily adapted for continuous or automated analysis. Additionally, many enzymatic products are soluble and readily diffuse away from the site of enzyme activity, and are especially troublesome for *in vivo* applications, particularly for detecting enzyme activity associated with single cells.

The enzymatic hydrolysis of β-naphthol-based substrates has been used to yield fluorescent precipitates at the enzymatic activity sites after being trapped with an azo dye (as a second reagent) in the presence of transition metal ions such as $Zn^{2+}$.[4,5] However, such two-step coupling procedures are inevitably subject to a potentially false localization of enzyme activity due to the possible translocation of the primary enzymatic products before they are trapped by the secondary reagents. Therefore, it is of great interest to develop substrates that directly generate fluorescent precipitates upon enzyme action. These fluorescent precipitates presumably would localize at enzymatic activity sites without translocation.

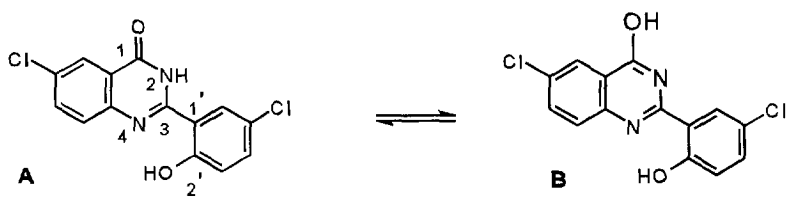
In search for such fluorophores, we noted that 2-(2'-hydroxyphenyl)-4(3*H*)-quinazolinone (HPQ) derivatives met our requirements. The HPQ compounds are generally insoluble in water and highly fluorescent only in the solid state and display a large Stokes shift. Furthermore, derivatization of the 2'-hydroxy group with enzyme-cleavable groups (such as phosphoryl, glycosyl, alkyl and acyl groups) efficiently eliminates the long-wavelength fluorescence.

Bacterial β-glucuronidase has been one of the most useful reporter enzymes in molecular biology.[3,6]

During the past twenty years, various synthetic glucuronides have been developed for spectrophotometric, fluorometric and histochemical assays of β-glucuronidase in a variety of biological systems.[6] However, the currently available fluorogenic β-glucuronidase substrates either lack sensitivity or readily diffuse away from the site of enzyme activity as described above.

We have now developed an HPQ-based β-glucuronidase substrate that has overcome the above-mentioned limitations. The substrate is water-soluble, colorless, and virtually nonfluorescent. It generates highly fluorescent and photostable precipitates at the sites of enzyme activity upon β-glucuronidase action. This unique substrate has been shown to detect the enzyme activity in solutions, gels and plant tissues.[7]

**Synthesis** Standard Köenigs-Knorr glycosylation has been used to prepare a HPQ galactoside and a glucoside.[8] However, this method is not readily adapted for the preparation of the corresponding glucuronide. The Köenigs-Knorr HPQ glycosylation was complicated by a variety of side reactions such as *N*-glycosylation and diglycosylation. As shown in Scheme 1, the HPQ tautomerization also affords the regioisomeric 1-*O*-glycoside, which is very difficult to separate from the desired 2'-*O*-glycoside. The *N*-glycoside is not readily separated from the desired 2'-*O*-glycoside by chromatographic methods either. Additionally, the direct glycosylation of HPQ also affords a few diglycosides that result from the further reaction of the monoglycosides with the unreacted bromosugar (which is used in excess to force the glycosylation). It appears that the monoglycosides are generally more reactive toward acetobromoglycose than the starting HPQs. This is probably because the HPQ monoglycosides are much more soluble than the nonglycosylated HPQs. The latter have poor solubility in most solvents. Thus diglycosylations are unlikely to be avoided in the direct HPQ glycosylation procedure.

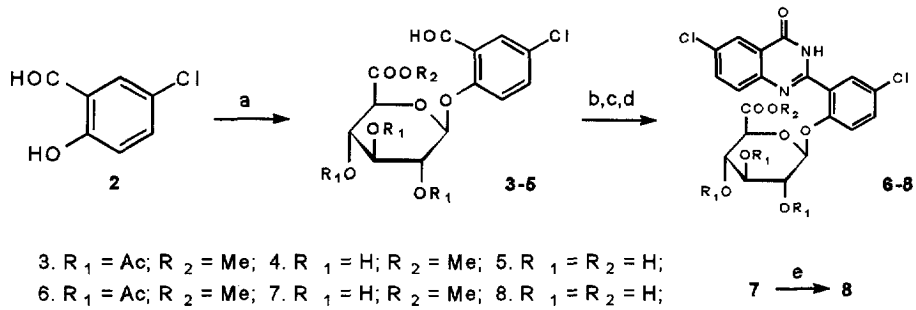



Scheme 1. Tautomerization of HPQs

Therefore, we developed a more selective synthesis of 2-(2'-hydroxy-5'-chlorophenyl)-6-chloro-4(3*H*)-quinazolinonyl β-D-glucuronide (compound **8**). The aminolysis of commercially available 5-chloroisatoic anhydride readily gives 4-chloro-anthranilamide. Salicyl glucuronides are readily prepared by glycosylation with acetobromo-α-D-glucuronic acid methyl ester using silver carbonate as a catalyst. Salicyl glucuronide **3** thus obtained is condensed with the anthranilamide in the presence of a catalytic amount of *p*-toluenesulfonic acid to afford the dihydroHPQ glucuronide, which is oxidized *in situ* to HPQ glucuronide **6** by an oxidant such as 2,3-dichloro-4,5-dicyano-1,4-benzoquinone.

Initially we prepared fully protected HPQ glucuronide **6** from salicyl glucuronide **3**. However, compound **6** was difficult to deprotect. The various attempts at basic deprotection of compound **6** caused considerable decomposition (into the free HPQ), and neutral hydrolysis failed to remove all the acetyl groups from the sugar residue. Thus fully deprotected salicyladehyde glucuronide **5** was prepared instead, but this failed to condense with the anthranilamide to give compound **8** in acceptable yield due to many side

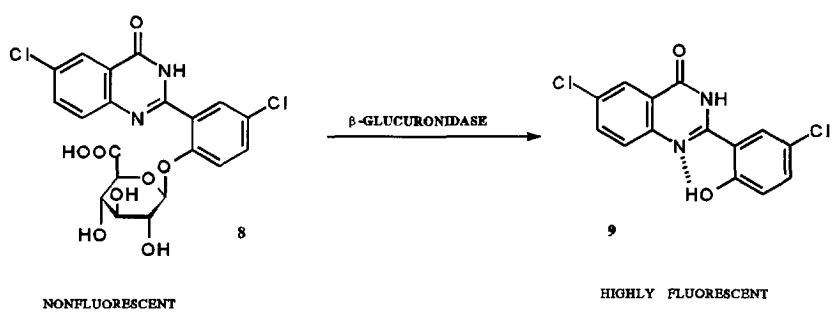
reactions that were likely associated with catalysis by *p*-toluenesulfonic acid.




Scheme 2. Synthesis of ELF-97 β-D-glucuronide (compound **8**). a = acetobromo–α-D-glucuronic acid methyl ester/ $Ag_2CO_3$/collidine/$CH_2Cl_2$; b = 5-chloroanthranilamide/MeOH; c. TsOH/MeOH; d. DDQ/MeOH; e. KCN/DMF.

Eventually, partially protected HPQ glucuronide **7** was prepared from the corresponding salicyl glucuronide (compound **4**). The latter is readily obtained by the selective hydrolysis of fully protected compound **3** with sodium methoxide. As expected, partially protected glucuronide **7** is much more easily transformed into the desired glucuronide **8** than fully protected glucuronide **6** using KCN treatment or careful NaOH hydrolysis.

This stepwise synthesis is isomer-free, eliminating the tedious chromatographic purifications required by the direct glycosylation procedures.[8] Furthermore, each step in this procedure has fair or good yields. This is a facile method for the preparation of HPQ glucuronides, and should be easily extended to the syntheses of HPQ galactosides, glucosides and other HPQ-based enzyme substrates.




Scheme 3. The enzymatic hydrolysis of compound **8** by ß-glucuronidase

**Enzymatic Analysis** ELF-97 glucuronide (compound **8**) is nonfluorescent, stable, and highly soluble in water. It generates a bright yellow-green fluorescent product within minutes when incubated with β-glucuronidase at room temperature (Fig. 1A). The enzymatic product has a similar fluorescence spectrum to that of the authentic 2-(2'-hydroxy-5'-chlorophenyl)-6-chloro-4(3*H*)-quinazolinone (compound **9**), indicating that the glucuronidyl residue of compound **8** is indeed cleaved by the enzyme (Scheme 3).

The enzymatic product of the glucuronidase substrate (compound **9**) has a large Stokes shift that can inherently enhance analysis sensitivity, and reduce the background from the sample, particularly in plants, which have high autofluorescence. Additionally, unlike the common coumarin- and fluorescein-based fluorogenic glucuronidase substrates,[2,3] compound **9** has maximum fluorescence in the optimum pH range of β-glucuronidase activity because it is the acid form of the fluorophore that has long-wavelength fluorescence. Thus the new β-glucuronidase substrate should be a suitable candidate for continuous and automated analyses of this enzyme.

Compound **8** can detect as little as $10^{-3}$ U/mL activity of β-glucuronidase in solution (Fig. 1B). The substrate has also been used to detect the enzyme activity in gels and plant tissue.[7]

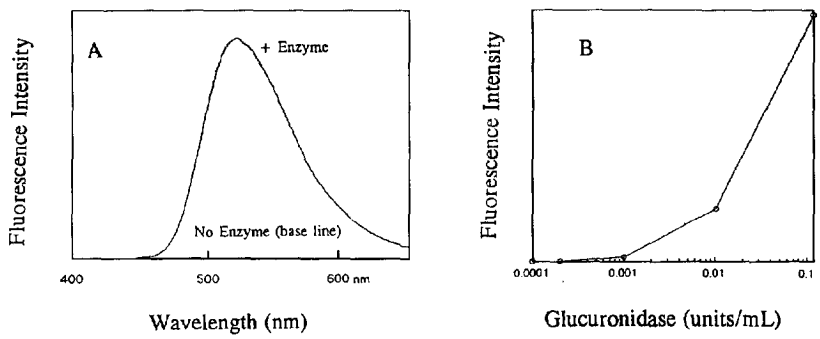



Fig. 1A. Fluorescence spectral change of compound **8** following reaction with β-glucuronidase. Compound **8** (20 μM) was treated with 0.5 units/mL of β-glucuronidase in 50 mM sodium acetate buffer (pH 6.0) for 1 h at 22 °C. The control reaction was run under the same conditions, but without the enzyme. The fluorescence was measured using an excitation wavelength of 360 nm. Fig. 1B. Sensitivity of compound **8** to β-glucuronidase. Compound **8** was reacted with a serial dilution of β-glucuronidase in 50 mM sodium acetate buffer (pH 6.0) for 5 h at 32 °C. The fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength of 530 nm. The change in fluorescence was calculated by subtracting the background fluorescence in the absence of the enzyme from that measured for the serial dilutions of the enzyme.

**Experimental** NMR spectra were obtained on a Bruker YLIV370.040. Elemental analyses were run on a Perkin-Elmer CHNS/O Analyzer 2400. Absorption and fluorescence spectra were recorded respectively on an Aminco SPE-5000 and an Aminco SPF-500C. Enzymatic analysis was conducted on a Millipore Cytofluor™ 2300. Melting points were measured on a Mel-Temp II (Laboratory Devices, Inc., Holliston, MA), and are uncorrected.

All organic chemicals were purchased from Aldrich (Milwaukee, WI), and β-glucuronidase (EC 3.2.1.31) was obtained from Sigma (St. Louis, MI). Acetobromo-α-D-glucuronic acid methyl ester (m.p. 105–106°C) was prepared according to the procedure of Bollenback *et al.*[9]

*4-Chloroanthranilamide (1).* 5-Chloroisatoic anhydride (50 g, 0.253 mol) was suspended in anhydrous tetrahydrofuran (1 L). The mixture was cooled to 0°C, into which dried ammonia gas was carefully bubbled for 2 h under anhydrous conditions. The reaction mixture was then warmed up to room temperature, and

stirred overnight. The solvent was evaporated *in vacuo*, and the residue was recrystallized from toluene. The desired product was obtained in 2 crops (39.4 g, yield: 91%); m.p. = 162–164°C; $^1$H-NMR ($CDCl_3$): 7.32 (1H, s); 7.19, 6.63 (2H, dd); 5.67 ppm (2H, s, $D_2O$ exchangeable).

*4-Chloro-2-formylphenyl aceto-β-D-glucuronic acid methyl ester (3).* Acetobromo-α-D-glucuronic acid methyl ester (4g, 10 mmol) and 4-chlorosalicylaldehyde (2.5 g, 16 mmol) were dissolved in anhydrous dichloromethane (50 mL). To the solution were added at once $Ag_2CO_3$ (4.4 g, 16 mmol) and 3 Å molecular sieves (5 g). 2,4,6-Collidine (2.1 mL) was added dropwise with stirring. The resulting mixture was stirred in the dark at room temperature for 4 d under dry Ar protection, and then diluted with chloroform (150 mL). The mixture was filtered through a pad of Celite® diatomaceous earth and the residue was washed with chloroform (3 × 50 mL). The combined filtrates were washed successively with 1 M HCl solution (2 × 250 mL), water (1 × 250 mL), 0.1 M $Na_2S_2O_3$ solution (2 × 250 mL), saturated $NaHCO_3$ solution (2 × 250 mL) and water (2 × 250 mL). The organic layer was dried over anhydrous $MgSO_4$, and evaporated *in vacuo* to give an off-white solid. The crude material was purified on a silica gel column using a gradient elution of chloroform and acetonitrile to afford a colorless solid (4.1 g, yield: 86%); m.p. = 142–144°C; $^1$H-NMR ($CDCl_3$): 10.27 (1H, s); 7.82 (1H, s); 7.52, 7.11 (2H, dd); 5.37 (3H, m); 5.25 (1H, m); 4.22 (1H, m); 3.70 (3H, s); 2.10 ppm (9H, m).

*4-Chloro-2-formylphenyl β-D-glucuronic acid methyl ester (4).* Compound **3** (2 g, 4.2 mmol) was dissolved in anhydrous methanol (50 mL) and cooled to 0°C. Freshly prepared 0.1 M NaOMe in anhydrous MeOH (3 × 0.2 mL, 0.06 mmol) was slowly added to the solution in 3 portions over 2 h under dry Ar protection. The solution was stirred at 0°C for 2 h, warmed to room temperature and stirred for 4 h. The reaction mixture was then diluted with methanol (50 mL), and acidified (pH 6.0) with Amberlite™ IRC-50 ion exchange resin ($H^+$ form). The mixture was filtered, the resin was washed with methanol (2 × 25 mL). The combined filtrate was evaporated *in vacuo* to give an off-white solid. The crude material was redissolved in methanol, and precipitated by ether to afford a colorless solid (1.4 g, yield: 94%); m.p. 245°C (d); $^1$H-NMR (9:1 DMSO-$d_6$-$D_2O$): 10.40 (1H, s); 7.71, 7.35 (2H, dd); 7.63 (1H, s); 5.39 (2H, m); 5.26 (1H, m); 4.92 (1H, m); 4.06 (1H, m); 3.63 (s, 3H).

*4-Chloro-2-[2'-(6''-chloro-4(3H)-quinazolinonyl)]-phenyl β-D-glucuronic acid methyl ester (7).* Compound **1** (1 g, 2.9 mmol), compound **4** (598 mg, 3.5 mmoles) and *p*-toluenesulfonic acid (20 mg, 0.1 mmol) were dissolved in anhydrous methanol (30 mL) and stirred at room temperature for 2 h. The reaction mixture was then refluxed for another hour. The resulting solution was cooled to 0°C and 2,3-dichloro-4,5-dicyano-1,4-benzoquinone (681 mg, 3.0 mol) was added in 3 portions during 30 min. The mixture was stirred at 0°C for 2 h, and evaporated *in vacuo* to give a brown solid. The solid was washed with benzene (5 × 50 mL) to remove the hydroquinone formed during the reaction. The resulting residue was redissolved in methanol and precipitated by ether. The crude material was purified on silica gel using a gradient elution of chloroform and methanol to give a colorless solid (1.1 g, yield: 79%); m.p. 155°C (d); $^1$H-NMR (in 9:1 DMSO-$d_6$-$D_2O$): 8.10 (1H, s); 7.78, 7.37 (2H, dd); 7.62, 7.22 (2H, dd); 7.49 (1H, s); 5.20 (1H, m); 4.10 (1H, m); 4.00 (1H, d); 3.61 (3H, s); 3.41 (1H, m); 3.30 ppm (1H, m).

*4-Chloro-2-[2'-(6''-chloro-4(3H)-quinazolinonyl)]-phenyl β-D-glucuronic acid (8).* Compound **7** (0.5 g, 1.0 mmole) was dissolved in *N,N*-dimethylformamide (10 mL) and mixed with 2.5 M aqueous KCN solution (2 mL, 5 mmol). The resulting mixture was stirred in the dark at room temperature for 2 days. The solvents were removed under high vacuum, and the residue was taken up by water (3 × 30 mL). The insoluble particles were removed by filtration, and the filtrate was washed with ethyl acetate (3 × 50 mL).

The aqueous layer was carefully neutralized (pH 6.0) with Amberlite™ IRC-50 ion resin ($H^+$ form), and filtered to remove the resin, which was then washed with water (3 × 10 mL). The combined aqueous solution was lyophilized to afford an off-white solid. The solid was further purified by a Sephadex LH-20 column using water as eluant to give a colorless solid (247 mg, yield: 51%); m.p. = 243°C (d); $^1$H-NMR (in DMSO-$d_6$): 8.09 (1H, s); 7.83, 7.58 (2H, dd); 7.80 (1H, s); 7.73, 7.40 (2H, dd); 5.05 (1H, s, $D_2O$ exchangeable); 5.03 (1H, d); 3.51 (1H, d); 3.30 (2H, m, $D_2O$ exchangeable); 3.10 ppm (3H, m). Elemental analysis: 43.62% C, 4.49% H, 5.01% N (calculated for $C_{20}H_{16}Cl_2N_2O_7 \cdot H_2O$: 43.62% C, 4.62% H, 5.09% N).

*6-Chloro-2-(5'-chloro-2-hydroxyphenyl)-4(3H)-quinazolinone (9).* Compound **1** (17.1 g, 0.1 mole) and 5-chlorosalicylaldehyde (15.7 g, 0.1 mole) were suspended in anhydrous ethanol (300 mL), and refluxed for 30 min. To the reaction mixture was added *p*-toluenesulfonic acid (0.4 g, 2.1 mmol), and the resulting solution was allowed to reflux for another hour. The reaction solution was cooled to room temperature, and 2,3-dichloro-4,5-dicyano-1,4-benzoquinone (22.8 g, 0.1 mol) was added. The mixture was stirred at room temperature for 2 h, and filtered to collect the brown solid, which was washed with cold ethanol to afford a colorless solid (29.8 g, yield: 97%); m.p. >300°C; $^1$H-NMR (in DMSO-$d_6$): 8.29 (1H, d); 8.08 (1H, d), 7.88 (1H, m); 7.85 (1H, d); 7.48 (1H, m), 7.03 ppm (1H, d). Elemental analysis: 54.79% C, 2.27% H, 9.01% N (Calculated for $C_{14}H_8Cl_2N_2O_2$: 54.75% C, 2.63% H, 9.12% N).

*Enzyme Assays.* 50 μM of 2-(2'-hydroxy-5'-chlorophenyl)-6-chloro-4(3*H*)-quinazolinonyl β-D-glucuronide (compound **8**) was incubated with serial dilutions of β-glucuronidase in 50 mM sodium acetate buffer (pH = 6.0) at 25 °C for 5 h. The fluorescence intensity at ~530 nm (excited at ~360 nm) was measured on a Cytofluor 2300 microplate reader following the enzymatic reactions.

**Acknowledgements.** This work was supported by NIH SBIR grant R44GM48306-03 (U S Patent 5,443,986). We thank Dr. Donald A. Upson for his critical reading of the manuscript.

## REFERENCES

1. Waggoner, A. S. in *Applications of Fluorescence in the Biomedical Sciences* (edited by Taylor, D. L.), Alan R. Liss, Inc., New York, **1986**, p. 3.
2. Guilbault, G. G. (Ed.) *Practical Fluorescence*, 2nd ed., Marcel Dekker Inc., New York, **1990**, p. 683.
3. Manafi, M., Kneifel, W., and Bascomb, S. *Microbiol. Rev.* **1991**, *55*, 335.
4. Ziomek, C. A., Lepire, M. L., and Torres, J. *J. Histochem. Cytochem.* **1990**, *38*, 437.
5. Menten, M. L., Junge, J., and Green, M. H. *J. Biol. Chem.* **1994**, *153*, 471.
6. Gallagher, S. R. (Ed.) *GUS Protocol: Using GUS Gene as a Reporter of Gene Expression*, Academic Press, San Diego, **1992**.
7. Zhou, M., Upson, R., Diwu, Z., and Haugland, R. P. *J. Biochem. Biophys. Methods* **1996**, *33*, 197.
8. Naleway, J. J., Fox, C. M. J., Robinhold, D., Terpetschnig, E., Olson, N. A., and Haugland, R. P. *Tetrahedron Lett.* **1994**, *35*, 8569.
9. Bollenback, G. N., Long, J. W., Benjamin, D. G., and Lindquist, J. A. *J. Am. Chem. Soc.* **1955**, *77*, 3310.

(*Received in USA* 13 *January* 1997; *revised* 21 *March* 1997; *accepted* 1 *April* 1997)