Carbohydrate Research, 205 (1990) 225–233
Elsevier Science Publishers B.V., Amsterdam

Synthesis of luciferin glycosides as substrates for novel ultrasensitive enzyme assays*

Robert Amess, Neil Baggett†, Paul R. Darby, (in part) Anthony R. Goode,
School of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT (Great Britain)
and Ernest E. Vickers
Koch–Light Limited, 37 Hollands Road, Haverhill, Suffolk CB9 8PU (Great Britain)

(Received December 4th, 1989; accepted for publication, February 22nd, 1990)

ABSTRACT

Condensation of 2-cyano-6-hydroxybenzothiazole with acetohalogeno derivatives of D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose gave the corresponding β-glycosides. Attempted basic deacetylation caused methanolysis of the nitrile group. Condensation of the first two acetylated glycosides with D-cysteine, followed by deacetylation, gave the firefly luciferin β-glycosides that were substrates for the corresponding glycohydrolases. The liberated luciferin was determined by fluorescence spectroscopy and, in one instance, by coupled-bioluminescence assay with firefly luciferase. The amount of luciferin released and determined by bioluminescence assay, was only ~65% of that determined by fluorescence spectroscopy, which suggested that the luciferin was partly racemised. Because of the great sensitivity of bioluminescence detection, these novel substrates provide potentially ultrasensitive assays for glycohydrolases, but their syntheses are more difficult than those of the corresponding fluorogenic substrates.

INTRODUCTION

The currently preferred substrates for the assay of glycohydrolases are aryl glycosides, since there is a marked difference in spectroscopic properties between the phenol liberated and the aryl glycoside. This difference is particularly marked in basic media because only the phenol can ionise. The most sensitive substrates are those derived from fluorescent phenols, such as 7-hydroxy-4-methylcoumarin, for which the limit for detection extends[1] down to 10^{-8}M, although this sensitivity is achieved rarely in practical assays because of interferences.

Various sensitive assays have been developed based on the bioluminescence of firefly luciferin generated with luciferase[2,3]. Thus, for example, the detection limit for ATP is 10^{-10}M, with a linear response over five orders of magnitude[4]. The presence of the hydroxyl group in firefly luciferin, D-(−)-2-(6-hydroxy-2-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**1**) is essential for bioluminescence; 6-*O*-methyl-luciferin is inactive in the bioluminescence reaction[5] and it has been suggested[6] that the key intermediate in the production of light is a phenoxide ion. Consequently, it was anticipated that luciferin glycosides should be inactive in the bioluminescence reaction and could

* Dedicated to Professor Leslie Hough in the year of his 65th birthday.
† Author for correspondence.

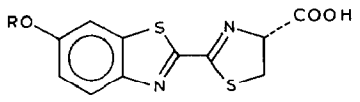
0008-6215/90/$ 03.50

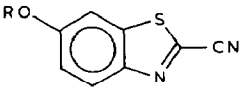
provide the basis for convenient and sensitive assays for glycohydrolases, using a coupled system. A preliminary report of a coupled assay for alkaline phosphatase, using luciferin phosphate, has appeared[7].

We now report the synthesis of some luciferin β-glycosides and a preliminary evaluation of the potential of two of these compounds as substrates for glycohydrolases, using spectrophotometric, fluorimetric, and coupled bioluminescence assays.

DISCUSSION

The instability of luciferin towards acid, base, light, and oxygen[5], together with the presence of the carboxyl group, preclude direct glycosidation of luciferin (**1**). Consequently, the glycosides of **1** are prepared, as in the synthesis of other analogues[8], by construction of the labile thiazoline ring at a late stage in the synthesis.





1 R = H
7 R = β-D-Glucopyranosyl
8 R = β-D-Galactopyranosyl

2 R = 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl
3 R = 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl
4 R = 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl
5 R = H
6 R = CH_3

Condensation of 2-cyano-6-hydroxybenzothiazole severally with acetohalogeno derivatives of D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose in aqueous acetone gave the corresponding acetylated β-glycosides **2**–**4** in moderate yields. The small negative $[\alpha]_D$ values were consistent with the expected β configuration. For the glucoside **2**, the signal of H-1 in the ^{1}H-n.m.r. spectrum was obscured by other resonances but, for **3** and **4**, the $J_{1,2}$ value (~7 Hz) also supported the assigned β configuration.

Deacetylation of **2** in methanol containing sodium methoxide, dimethylamine, ammonia, or potassium carbonate gave the same product. The i.r. spectrum of the product confirmed the absence of the nitrile group. The ^{1}H-n.m.r. spectrum contained, *inter alia*, signals at δ 3.96 (s, 3 H) and 9.24 (s, 1 H) indicative of the transformation of the nitrile group into a methyl imidate group. Similarly, deacetylation of the galactoside **3** with sodium methoxide or ammonia in methanol gave a methyl imidate. Attempted deacetylation of **2** without affecting the C≡N moiety, using pig liver carboxylesterase[9], was unsuccessful.

The base-catalysed reaction of nitriles with alcohols to form imidates[10] gave good yields only with nitriles that carried electronegative substituents. 2-Cyano-6-hydroxybenzothiazole (**5**) was unaffected on treatment with methanolic sodium methoxide, but 2-cyano-6-methoxybenzothiazole (**6**) gave a methyl imidate with methanolic ammonia

whereas methyl 6-methoxybenzothiazole-2-carboxylate was recovered after reaction with methanolic sodium methoxide. In the latter reaction, it may be that the imidate is formed initially but is hydrolysed on acidification[10]. Thus, **6** and the glycosides **2** and **3** show reactivity typical of nitriles with electronegative substituents, but the phenol **5** does not. Presumably, the phenoxide anion forms under the basic reaction conditions, thus reducing the electronegativity of the benzothiazole ring.

In order to avoid destruction of the nitrile group that occurred during deacetylation, a different strategy was adopted. The acetylated glycoside **2** was condensed with D-cysteine, following the general method used in the construction of the thiazoline ring in the synthesis of luciferin[5]. The ^{1}H-n.m.r. spectrum of the resulting mixture of products suggested that partial deacetylation had occurred. Complete deacetylation with methanolic sodium methoxide gave 65% of 6-*O*-*β*-D-glucopyranosyloxyluciferin (**7**). For the synthesis of the corresponding galactoside **8**, deacetylation was accomplished with methanolic ammonia. In the ^{1}H-n.m.r. spectra of **7** and **8**, the signal of H-1 was a doublet at relatively low field and with $J_{1,2}$ 7 Hz indicative of the *β* configuration.

The suitability of **7** and **8** as substrates for glycohydrolases was assessed using commercial purified enzymes and determination of the liberated aglycon by spectrophotometry and/or fluorimetry, with the appropriate controls, and the results are shown in Table I. The greater sensitivity of the fluorimetric assays enabled a lower concentration of substrate to be used and the 20-fold decrease resulted in a 10-fold reduction in the rate of hydrolysis of **7** and **8**. The limit of detection for luciferin by fluorimetry was 2×10^{-8}M, which is comparable to that for 7-hydroxy-4-methylcoumarin.

TABLE I

Rates of enzymic hydrolysis of aryl *β*-D-glycopyranosides

Aglycon	*Sugar*	*Conc.*	*pH*	*Initial rate*[a]
4-Nitrophenol	Glucose	2mM	5	2.6[b]
	Galactose	2mM	4.5	2.9[b]
7-Hydroxy-4-methylcoumarin	Glucose	2mM	5	2.8[b]
Luciferin	Galactose	2mM	4.5	3.6[b]
		0.1mM	4.5	0.46[c]
	Glucose	2mM	5	2.0[b]
		0.1mM	5	0.18[c]
		0.1mM	5	0.12[d]

[a] Moles of aglycon released per min per mg of enzyme. [b] Absorbance assay. [c] Fluorescence assay. [d] Bioluminescence assay.

A coupled bioluminescence assay of the liberated luciferin was studied also; this involved several preliminary controls. Prior to the present work, the concentration of luciferin had not been a limiting factor in any bioluminescence assay, although such assays have now been reported[7]. Consequently, the first step was to construct a calibration curve of luciferin concentration against the emission of light. The experi-

TABLE II

Luminescence calibration of luciferin (1) and luciferin β-D-glucopyranoside (7)

Conc.[a]	*Counts* × 10^{-3} *for* **1**	*Counts* × 10^{-3} *for* **7**
0[b]	2	2
nM	4	
10nM	9	
100nM	57	
μM	550	5
10μM	5420	11
100μM	47480	85

[a] Concentration of solution pipetted into the cuvette. [b] Acetate buffer blank.

mental conditions were based on those used[2] in the determination of ATP. The luciferin was dissolved in buffer at pH 5, appropriate to a glycohydrolase assay[1], and the final pH of the mixture of luciferin, luciferase, and ATP was 7.6, *i.e.*, close to the optimum for the bioluminescent reaction[2]. The total emission of light during 1 min was determined, since this was expected to give results more consistent than the measurement of peak heights. In this way, a calibration curve was obtained (Table II) for concentrations of luciferin in the range 10^{-9}–10^{-4}M. The luminescence of the solutions was unchanged after storage for 14 days at 4° in the dark. In control experiments with **7**, some slight emission of light was observed, presumably due to contamination by free luciferin. The observed intensity of the light emitted was consistent with 0.15% of contamination. The luminescence of luciferin was not inhibited by **7**. Solutions of β-D-glucosidase showed no luminescence and the enzyme did not inhibit the emission of light from luciferin.

For the coupled assay, aliquots were removed from a solution of **7** and β-D-glucosidase at pH 5, and added to buffer at pH 7.8. The luciferin liberated was determined promptly, in case the rise in pH and the dilution of the sample did not completely quench the action of the glycosidase. The observed initial rate of hydrolysis was only 67% of the value observed under the same conditions of hydrolysis but using a fluorimetric assay.

The control experiments showed that neither **7** nor β-D-glucosidase inhibited the luciferin/luciferase reaction. In order to resolve this anomaly, hydrolyses of **7** and **8** with a 50-fold increase in the concentration of the enzyme were followed to completion by spectrophotometry. The limiting concentrations of the aglycon released were 73 and 69%, respectively, of the theoretical value. For **7**, the luciferin released was determined also by the bioluminescent reaction and a value of 45% was obtained. The failure to detect 100% of the theoretically possible luciferin may be due to product inhibition of the glycohydrolase or to an alternative reaction of the substrate or aglycon, such as oxidation. The difference between the fluorescence and bioluminescence assays shows that only ~64% of the luciferin released is active in the light-emitting reaction. This result may be due to the presence of some L-luciferin which is not a substrate for luciferase.

D-Luciferin is readily racemised, particularly in base. Consequently, any epimerisation of **7** is most likely to occur during deacetylation with methanolic sodium methoxide. Indeed, when 6-*O*-methyl-D-luciferin was dissolved in the methanolic sodium methoxide, the $[\alpha]_D$ value decreased by 10%/h, evidently because of racemisation. However, there was no evidence in the 270-MHz ^{1}H-n.m.r. spectrum of **7** for the existence of diastereomers, possibly because the chiral centre in the luciferin moiety is remote from the sugar moiety.

Thus, the coupled bioluminescence method provides an assay of β-D-glucosidase, using **7**, which is somewhat more sensitive than for the detection of 7-hydroxy-4-methylcoumarin by fluorescence, and this sensitivity could be improved by using a more highly purified substrate. This increased sensitivity must be balanced against the increased complexity of the synthesis and lower stability of luciferin glycosides compared to coumarin glycosides.

EXPERIMENTAL

Dry methanol was obtained by distillation from magnesium methoxide. Methanolic sodium methoxide was prepared by reacting sodium (0.5 g) with dry methanol (50 mL). Half-saturated methanolic ammonia was prepared by bubbling dry ammonia into dry methanol, then dilution of the saturated solution with an equal volume of dry methanol. T.l.c. was performed on Silica Gel 60 F_{254} (Merck, 5735), with detection by u.v. light or by charring with sulphuric acid.

A Perkin–Elmer 141 polarimeter (1-dm tube) was used for measurement of $[\alpha]_D$ values. I.r. spectra were recorded for Nujol mulls with a Pye Unicam SP3-100 spectrophotometer, u.v. and visible absorption spectra with a Shimadzu 240 spectrophotometer, and fluorescence measurements with a Baird-Atomic SF 100E Fluorispec at intermediate slit width, using a 10 × 10 mm quartz cell. Quinine sulphate dihydrate in 0.05M sulphuric acid was employed as the standard.

The bioluminescence of luciferin was measured with a photon-counting, computer-controlled luminometer (Auto-Biolumat LB950) at the Wolfson Research Laboratories (Queen Elizabeth Medical Centre, Birmingham). Reactions were carried out in 55 × 11 mm clear, polystryrene, round-bottomed tubes.

^{1}H-N.m.r. spectra (internal Me_4Si) were obtained with a Perkin–Elmer R-14 or Varian XL-100 spectrometer and, at 270 MHz, with a Jeol GX 270 Fourier-transform spectrometer.

The following enzymes were obtained from Sigma: (*a*) esterase type 1 from porcine liver [suspension in 3.2M $(NH_4)_2SO_4$, 10.9 mg of protein/mL], (*b*) β-D-glucosidase type II from almonds, (*c*) β-D-galactosidase type XI from *Aspergillus oryzae*, (*d*) luciferase type VI from firefly (lyophilized powder containing 15% of protein, 1.8 U/mg of solid, and 11 U/mg of protein; 1 U will produce 1.0 nmol of polyphosphate/min at pH 7.7, 25°, 0.6 mM ATP, 0.1mM D-luciferin).

*2-Cyano-6-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)benzothiazole* (**2**). — A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (7.0 g) in acetone (30

mL) was added to a solution of 2-cyano-6-hydroxybenzothiazole[8] (2.0 g) in acetone (20 mL) and M sodium hydroxide (11.4 mL). The mixture was stored overnight, the solvent was evaporated, and a solution of the residue in chloroform was washed exhaustively with aqueous potassium carbonate and water, dried, and concentrated. The residue was recrystallised from ethanol to give **2** (2.4 g, 42%), m.p. 184–186°, $[\alpha]_D$ −28° (*c* 1, chloroform); λ_{max}^{EtOH} 252 (ε_{mM} 0.9), 305 nm (ε_{mM} 16.2); ν_{max} 2240 cm^{-1} (C≡N). ^{1}H-N.m.r. data: δ 2.05, 2.07 (2 s, 12 H, 4 Ac), 3.91–4.06 (m, 1 H, H-5), 4.20–4.26 (m, 2 H, H-6,6), 5.10–5.44 (m, 4 H, H-1,2,3,4), 7.38 (dd, 1 H, $J_{4,5}$ 9, $J_{5,7}$ 2 Hz, aglycon H-5), 7.64 (d, 1 H, H-7), 8.19 (d, 1 H, H-4) (Found: C, 51.9; H, 4.4; N, 5.5. $C_{22}H_{22}N_2O_{10}S$ calc.; C, 52.2; H, 4.4; N, 5.5%).

*2-Cyano-6-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyloxy)benzothiazole* (**3**). — Following the above method, 2-cyanobenzothiazole (2.0 g) was condensed with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (7.0 g) to give **3** (3.3 g, 57%), m.p. 153–154°, $[\alpha]_D$ +4° (*c* 1, chloroform); λ_{max}^{EtOH} 252 (ε_{mM} 0.9), 306 nm (ε_{mM} 16.7); ν_{max} 2240 cm^{-1} (C≡N). ^{1}H-N.m.r. data: δ 2.04, 2.09, 2.11, 2.22 (4 s, 12 H, 4 Ac), 4.16–4.28 (m, 3 H, H-5,6,6), 5.08–5.26 (m, 2 H, H-2,3), 5.44–5.54 (m, 1 H, H-4), 5.60 (d, 1 H, H-1, $J_{1,2}$ 7 Hz), 7.32 (dd, 1 H, $J_{4,5}$ 9, $J_{5,7}$ 2 Hz, aglycon H-5), 7.59 (d, 1 H, H-7), 8.12 (d, 1 H, H-4) (Found: C, 52.4; H, 4.7; N, 5.6. $C_{22}H_{22}N_2O_{10}S$ calc.; C, 52.2; H, 4.4; N, 5.5%).

*6-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyloxy)-2-cyanobenzothiazole* (**4**). — Following the above method, 2-cyanobenzothiazole (1.0 g) was condensed with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (3.1 g) to give **4** (1.1 g, 38%), m.p. 208–210° (dec.), $[\alpha]_D$ −11° (*c* 1, chloroform); λ_{max}^{EtOH} 254 (ε_{mM} 0.9), 306 nm (ε_{mM} 17.5); ν_{max} 2240 cm^{-1} (C≡N). ^{1}H-N.m.r. data: δ 1.94, 2.09, 2.10 (3 s, 3, 6, and 3 H, 4 Ac), 3.88–4.32 (m, 4 H, H-2,5,6), 5.02–5.54 (m, 2 H, H-3,4), 5.52 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 6.00 (d, 1 H, *J* 8 Hz, NH), 7.30 (dd, 1 H, $J_{4,5}$ 9, $J_{5,7}$ 2 Hz, aglycon H-5), 7.57 (d, 1 H, H-7), 8.08 (d, 1 H, H-4) (Found: C, 52.1; H, 4.4; N, 8.1. $C_{22}H_{23}N_3O_9S$ calc.: C, 52.3; H, 4.6; N, 8.3%).

*Methyl 6-β-*D*-glucopyranosyloxybenzothiazole-2-carboximidate.* — (*a*) Methanolic 0.1 M sodium methoxide (1 mL) was added to a solution of **2** (0.5 g) in chloroform (5 mL) and methanol (15 mL). After stirring for 1 h, the solution was neutralised with Amberlite IR-120 (H^+) resin and concentrated, and the crude product was recrystallised from ethanol to give the title compound (0.25 g, 67%), m.p. 160–162° (dec.), $[\alpha]_D$ −16° (*c* 0.5, *N*,*N*-dimethylformamide), λ_{max}^{EtOH} 253 (ε_{mM} 0.9), 306 nm (ε_{mM} 15.1); ν_{max} 1630 cm^{-1} (C=N). ^{1}H-N.m.r. data [$(CD_3)_2SO$]: δ 3.10–3.76 (m, 6 H, H-2,3,4,5,6,6), 3.96 (s, 3 H, MeO), 4.57 (bs, 1 H, OH), 4.95–5.14 (m, 3 H, H-1 and 2 OH), 5.35 (bs, 1 H, OH), 7.31 (dd, 1 H, $J_{4,5}$ 9, $J_{5,7}$ 3 Hz, aglycon H-5), 7.84 (d, 1 H, H-7), 8.07 (d, 1 H, H-4), 9.24 (s, 1 H, NH) (Found: C, 47.7; H, 4.6; N, 7.4. $C_{15}H_{18}N_2O_7S \cdot 0.5H_2O$ calc.: C, 47.5; H, 5.0; N, 7.4%).

(*b*) A solution of **2** (0.1 g) in chloroform (1 mL) was stirred with methanolic 10% dimethylamine (10 mL) for 1 h at 4°, then concentrated, and the residue was recrystallised from methanol to give the same product (40 mg) as in (*a*).

(*c*) A solution of **2** (0.1 g) in chloroform (1 mL) was stirred for 2 h with half-saturated methanolic ammonia (10 mL), then concentrated, and the residue was recrystallised from methanol to give the same product (50 mg) as in (*a*).

(*d*) A solution of **2** (0.1 g) in chloroform (1 mL) and methanol (10 mL) was stirred with potassium carbonate (0.1 g) in water (1 mL) for 2 h, then neutralised with Amberlite IR-120 (H^+) resin, and concentrated, and the residue was recrystallised from methanol to give the same product (40 mg) as in (*a*).

*Methyl 6-β-*D*-galactopyranosyloxybenzothiazole-2-carboximidate.* — (*a*) Compound **3** was deacetylated with methanolic sodium methoxide, as described above, to give the title compound (0.3 g, 80%), m.p. 170° (dec.), $[\alpha]_D$ −28° (*c* 1, *N*,*N*-dimethylformamide), λ_{max}^{EtOH} 254 (ε_{mM} 0.9), 307 nm (ε_{mM} 15.4); ν_{max} 1630 cm^{-1} (C=N). ^{1}H-N.m.r. data [270 MHz, $(CD_3)_2SO$]: δ 3.40–3.84 (m, 5 H, H-2,3,5,6,6), 3.63 (s, 3 H, MeO), 4.40 (m, 1 H, H-4), 4.52 (d, 1 H, OH), 4.65 (t, 1 H, OH), 4.86 (d, 1 H, OH), 4.96 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 5.21 (d, 1 H, OH), 7.31 (dd, 1 H, $J_{4,5}$ 9, $J_{5,7}$ 3 Hz, aglycon H-5), 7.83 (d, 1 H, H-7), 8.06 (d, 1 H, H-4), 9.18 (s, 1 H, NH) (Found: C, 47.5; H, 4.7; N, 7.4. $C_{15}H_{18}N_2O_7S{\cdot}0.5H_2O$ calc.: C, 47.5; H, 5.0; N, 7.4%).

(*b*) Compound **3** was deacetylated with methanolic ammonia as above, to give the same product as in (*a*).

(*c*) A solution of **3** (5 mg) in acetonitrile (1 mL) was mixed with carboxylesterase (Sigma type I, 0.1 mL) in 0.1M phosphate buffer (pH 7.5, 1 mL) and the mixture was incubated for 24 h at 25°. T.l.c. showed there was no deacetylation.

Methyl 6-methoxybenzothiazole-2-carboxylate. — 2-Cyano-6-methoxybenzothiazole (0.25 g) was stirred with warm methanol (25 mL) and methanolic 0.1M sodium methoxide (1 mL). After 2 h, the solution was neutralised with 4M hydrochloric acid and concentrated, a solution of the residue in chloroform was filtered and concentrated, and the residue was crystallised from aqueous methanol to give the title compound (0.15 g, 51%), m.p. 130–131° (lit.[5] m.p. 140–142°); λ_{max}^{EtOH} 261 (ε_{mM} 0.7), 319 nm (ε_{mM} 14.1); ν_{max} 1700 cm^{-1} (C=O).

Methyl 6-methoxybenzothiazole-2-carboximidate. — A solution of 2-cyano-6-methoxybenzothiazole (0.25 g) in methanol (20 mL) was mixed with saturated methanolic ammonia (15 mL). After 2 h, the solvent was evaporated, and the residue was recrystallised from aqueous methanol to give the title compound (0.2 g, 68%), m.p. 129–130°; λ_{max}^{EtOH} 262 (ε_{mM} 0.8), 314 nm (ε_{mM} 15.5); ν_{max} 3280 (NH), 1630 cm^{-1} (C=N). ^{1}H-N.m.r. data: δ 3.88 (s, 3 H, MeO), 4.02 (s, 3 H, MeO), 7.13 (dd, 1 H, $J_{4,5}$ 9, $J_{5,7}$ 2 Hz, H-5), 7.33 (d, 1 H, H-7), 7.98 (d, 1 H, H-4), 7.99 (s, 1 H, NH) (Found: C, 54.0; H, 4.2; N, 12.5. $C_{10}H_{10}N_2O_2S$ calc.: C, 54.0; H, 4.5; N, 12.6%).

*6-O-β-*D*-Glucopyranosyl-luciferin* (**7**). — A solution of D-cysteine hydrochloride (55 mg) in deoxygenated water (2 mL) under nitrogen was basified with potassium carbonate (50 mg), then methanol (8 mL) was added. This solution was added to a solution of **2** (150 mg) in chloroform (5 mL) and methanol (5 mL) under nitrogen in the dark. After 30 min, the solvent was evaporated, the residue was redissolved in dry methanol (20 mL), and methanolic 0.1M sodium methoxide (0.5 mL) was added. After 30 min, the solution was neutralised with Amberlite IR-120 (H^+) resin, then concentrated to 5 mL. After storage for 2 h at −20°, **7** (85 mg, 65%) was collected; m.p. 120° (dec.), $[\alpha]_D$ −22° (*c* 1, *N*,*N*-dimethylformamide), −15° (*c* 1, water); λ_{max}^{EtOH} 260 (ε_{mM} 0.7), 320 nm (ε_{mM} 15.8). ^{1}H-N.m.r. data [$(CD_3)_2SO$]: δ 3.1–3.6 (m, 6 H, H-2,3,4,5,6,6), 3.73 (dd, 2 H,

aglycon H-5,5), 4.10, 4.62, 4.94 (3 bs, 3 H, 3 OH), 5.01 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 5.11 (bs, 1 H, OH), 5.45 (t, 1 H, aglycon H-4), 7.28 (dd, 1 H, $J_{4',5'}$ 9, $J_{5',7'}$ 3 Hz, aglycon H-5'), 7.86 (d, 1 H, H-7'), 8.07 (d, 1 H, H-4') (Found: C, 46.6; H, 4.7; N, 6.5. $C_{17}H_{18}N_2O_8S_2$ calc.: C, 46.2; H, 4.1; N, 6.3%).

6-O-β-D-Galactopyranosyl-luciferin (**8**). — As described above for **2**, D-cysteine hydrochloride (55 mg) was condensed with **3** (150 mg). After 15 min, the solvent was evaporated, a solution of the syrupy residue in chloroform (25 mL) was washed with water, dried, and concentrated to 5 mL, and then half-saturated ammonia in methanol (20 mL) was added. After 3 h, the solvent was evaporated, a solution of the residue in water (10 mL) was extracted with ethyl acetate (25 mL) and then acidified to pH 2, and **8** (90 mg, 69%), collected after 2 h at 4°, had m.p. 130° (dec.), $[\alpha]_D$ −82° (*c* 1, water), λ_{max}^{EtOH} 258 (ε_{mM} 0.7), 317 nm (ε_{mM} 15.3). ^{1}H-N.m.r. data [$(CD_3)SO$]: δ 3.35–3.85 (m, 7 H, H-2,3,5,6,6, aglycon H-5,5), 4.16 (m, 1 H, H-4), 4.25–4.87 (bm, 3 H, 3 OH), 4.97 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 5.29 (bs, 1 H, OH), 5.43 (t, 1 H, aglycon H-4'), 7.28 (dd, 1 H, $J_{4',5'}$ 9, $J_{5',7'}$ 2 Hz, aglycon H-5'), 7.83 (d, 1 H, H-7'), 8.07 (d, 1 H, H-4') (Found: C, 46.2; H, 4.4; N, 6.6. $C_{17}H_{18}N_2O_8S_2$ calc.: C, 46.2; H, 4.1; N, 6.3%).

Enzymic hydrolysis of aryl glycosides. — Solutions of substrate and enzyme at twice the stated concentrations in acetate buffer at the stated pH were incubated for 20 min at the reaction temperature. Reactions were initiated by the addition of the solution of the enzyme (5 mL) to that of the substrate (5 mL). At intervals, aliquots were quenched in an appropriate buffer (see below), and the concentration of the aglycon liberated was determined immediately on the basis of (*a*) absorbance, (*b*) fluorescence, or (*c*) bioluminescence, as appropriate. Progress curves were plotted and the initial reaction velocity was determined. Each assay was performed in duplicate. In control experiments, the enzyme solution was replaced by acetate buffer; the rate of non-enzymic hydrolysis was negligible for each substrate. The results are shown in Table I.

(*a*) *Absorbance assay.* Each aliquot (1.0 mL) was quenched in 0.25M sodium carbonate (2.0 mL) and the absorbance was determined at 400 nm for 4-nitrophenol (ε_{mM} 18), 360 nm for 7-hydroxy-4-methylcoumarin (ε_{mM} 16.8), or 384 nm for luciferin (ε_{mM} 18.1).

(*b*) *Fluorescence assay.* The aliquots or standard solutions (1.0 mL) were quenched in glycine buffer (5.0 mL, pH 10.6, I 0.05), and the fluorescence was determined (λ_{ex} 390, λ_{em} 530 nm) against a standard solution of quinine sulphate (0.1 mg/L in 0.05M H_2SO_4). A calibration curve was obtained for luciferin concentration in the range 20nM to 10μM. The fluorescence of luciferin in glycine buffer was unchanged after 14 days at 4° in the dark.

(*c*) *Bioluminescence assay.* Stock solutions of 10mM magnesium sulphate, 0.1mM adenosine triphosphate, and firefly luciferase (0.42 mg/mL) in 0.75M glycylglycine buffer (pH 7.8) were stored frozen in small lots. Luciferase solution was prepared freshly each day by mixing stock solutions of luciferase (0.5 mL), magnesium sulphate (62 μL), adenosine triphosphate (62 μL), and glycylglycine buffer (1.5 mL). The solution was stored at 0° in the dark.

The aliquots or luciferin standards (0.1mM to nM, 0.1 mL) were added to a cuvette followed by 0.01M glycylglycine buffer (0.9 mL, pH 7.8) and luciferase solution (0.1 mL). The pH was 7.6. After brief agitation, the emission of light during 60 s was measured. Each measurement was performed in triplicate. The emission of light for some standards and controls are given in Table II.

ACKNOWLEDGMENTS

We thank Dr. G. H. G. Thorpe (Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Birmingham) for advice and assistance with bioluminescence measurements, and the S.E.R.C. for CASE studenthips (to R.A. and P.R.D.).

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