Carbohydrate Research, 197 (1990) 295–301
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

7-β-D-Galactopyranosyloxycoumarin-4-acetic acid and its methyl ester as substrates for the β-D-galactosidase of *Escherichia coli*

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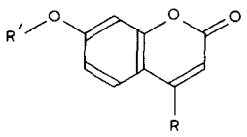
(Received May 5th, 1989; accepted for publication, July 15th, 1989)

The use of fluorogenic substrates for the analysis of hydrolytic enzymes is well established[1]. Derivatives of 7-hydroxy-4-methylcoumarin (**1a**) have been used for the analysis of β-D-glucuronidase[2], β-D-glucosidase[3], β-D-galactosidase[4], and many other glycosidases. The use of derivatives of **1a** is limited because of their relative insolubilities[5,6] and a wide range of substrate concentrations is desirable in the determination of enzyme kinetics[7]. For this reason, we have synthesised 7-β-D-galactopyranosyloxycoumarin-4-acetic acid (**1d**) and its methyl ester (**1e**), and studied their hydrolysis by β-D-galactosidase from *E. coli*.

	R	R′
a	Me	H
b	CH_2CO_2H	H
c	CH_2CO_2Me	H
d	CH_2CO_2H	β-D-Gal
e	CH_2CO_2Me	β-D-Gal
f	CH_2CO_2Me	$(AcO)_4$-β-D-Gal
g	Me	β-D-Gal



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The β-D-galactopyranoside tetra-acetate **1f** was prepared by the reaction[8] of the phenolate anion of methyl 7-hydroxycoumarin-4-acetate (**1c**) with tetra-*O*-acetyl-α-D-galactopyranosyl bromide. The structure of **1f** was confirmed by the ^{1}H-n.m.r. data.

Treatment of **1f** with methanolic sodium methoxide effected *O*-deacetylation to give **1e**, and treatment with aqueous sodium hydroxide then gave 7-β-D-galactopyranosyloxycoumarin-4-acetic acid (**1d**). The $J_{1,2}$ value of 7 Hz for **1d** and **1e** confirmed the β configuration.

The solubilities in sodium phosphate buffer (pH 7.3, I 0.1) at 37° of the free acid **1d** (31.4 mg/mL) and the methyl ester **1e** (5.65 mg/mL) are significantly greater than that of the 4-methyl derivative **1g** (0.32 mg/mL). Also as expected, **1d**, **1e**, and

0008-6215/90/$ 03.50

TABLE I

FLUORESCENCE SPECTRA OF COUMARIN DERIVATIVES[a]

Compound	*R'*	*R*	λ_{max}^{ex} *(nm)*	λ_{max}^{em} *(nm)*	*Fluorescence intensity (q.u.)*
1b	H	CH_2CO_2H	370	455	26.0
1c	H	CH_2CO_2Me	375	465	24.3
1f	$(AcO)_4$-β-D-Gal	CH_2CO_2Me	325	390	0.4
1e	β-D-Gal	CH_2CO_2Me	325	390	0.5
1d	β-D-Gal	CH_2CO_2H	325	385	0.4

[a]At 0.2 mg/mL in 0.25M sodium carbonate.

TABLE II

KINETIC PARAMETERS FOR THE HYDROLYSES WITH β-D-GALACTOSIDASE

Substrate	V_{max} *(μmol.L⁻¹.min⁻¹)*	*E.s.d.*	k_{cat}[a] *(min⁻¹)*	K_m *(μmol.L⁻¹)*	*E.s.d.*
1e	37.607	1.243	19 235	450.066	38.117
1d	46.462	1.263	23 765	3707.79	307.03
1g	22.794	1.191	11 660	141.832	25.268
ONP-Gal	100.00	1.282	51 150	382.685	30.143

[a]Based[17] on a monomer molecular weight of 116,248.

1f showed the characteristic low fluorescence (Table I) of substituted 7-hydroxycoumarins, whereas the free hydroxy derivatives (**1b** and **1c**) showed high fluorescence, similar to that of 4-methylumbelliferone (**1a**). The fluorescence of **1b** and **1c** had λ_{max}^{em} values at wavelengths much higher than those of the glycosides.

Table II contains the data on the hydrolysis of **1d**, **1e**, *o*-nitrophenyl β-D-galactopyranoside, and **1g** by the β-D-galactosidase from *E. coli*. The values of V_{max} for the acid **1d** and its methyl ester **1e** are significantly higher than that for **1g** and, hence, they are better substrates. The *o*-nitrophenyl galactoside had the highest V_{max} but, since detection by absorbance of *o*-nitrophenol is far less sensitive than that of the fluorescence of the hydroxycoumarins, the use of this substrate is likely to be restricted.

The K_m values show that the 4-methylcoumarin derivative **1g** binds more effectively to the enzyme than the methyl ester **1e**, which may be due to the greater bulk of the methoxycarbonyl moiety compared to the methyl group, causing overcrowding in the enzyme–substrate complex. The same effect could lead to acceleration of the loss of the aglycon and the higher V_{max} of **1e** compared with that of **1g**. The carboxyl group in **1d** may be the cause of the considerably poorer binding of this substrate, since it is likely that the active site of β-D-galactosidase contains an ionised carboxyl group[9].

Although the K_m for the 4-methyl derivative **1g** is the lowest of the series, the

poor solubility of this compound means that, in the assay, the need for the concentration to be many times the value of the K_m cannot be met. This is not a problem with the acid **1d** or the methyl ester **1e**.

EXPERIMENTAL

General. — Solutions were concentrated *in vacuo* below 40°. Melting points are uncorrected. Solid products were dried under vacuum over phosphorus pentaoxide and sodium hydroxide pellets.

T.l.c. was performed on Silica Gel 60 F_{254} (Merck) with detection by u.v. light, by charring with sulphuric acid, or with Bial's reagent (0.55% of orcinol and 0.9% of ferric chloride in acidified ethanol). Column chromatography was conducted on Silica Gel (Merck 7734).

Optical rotations (*c* 0.5) were determined on a Perkin–Elmer 241 polarimeter (1-dm tube) at 20°. U.v. and visible absorption spectra were recorded on a Shimadzu 240 spectrophotometer. Fixed wavelength absorptions were measured on a MSE Spectro-plus spectrophotometer.

Fluorescence spectra were obtained with a Baird–Atomic SF 100E Fluorispec, using 10-mm cells. All enzyme assays were performed with a Vitatron MPS fluorimeter modified to accommodate a flow-through cell and connected to a Fisons MSE chart recorder. Fluorescence measurements were made using a U10 primary filter (max. transmittance, 360 nm), a U3 secondary filter (zero transmittance, 380 nm; 90% transmittance, 460 nm), and a slit width of 2. Absorbance measurements were made using a primary interference filter (band pass, 408–423 nm) and no secondary.

^{1}H-N.m.r. spectra (internal Me_4Si) were recorded (270 MHz) with a JEOL GX 270 F.t. spectrometer.

Elemental analyses were obtained using a Perkin–Elmer 240 automatic elemental analyzer.

β-D-Galactosidase (EC 3.2.1.23) from *E. coli*, grade VI was purchased from Sigma as a lyophilized powder containing 92% of protein, and with 440 U/mg of protein (1 U will hydrolyze 1.0 μmol of *o*-nitrophenyl β-D-galactopyranoside per min at pH 7.3 and 37°).

7-β-D-Galactopyranosyloxy-4-methylcoumarin (4-methylumbelliferyl β-D-galactoside) and *o*-nitrophenyl β-D-galactopyranoside were purchased from Sigma.

Methanol was distilled from magnesium methoxide. Methanolic sodium methoxide was prepared by reacting sodium (0.5 g) with dry methanol (50 mL).

Methyl 7-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyloxy)coumarin-4-acetate (**1f**). — A solution of methyl 7-hydroxycoumarin-4-acetate[10] (**1c**; 3.0 g, m.p. 220°) in acetone (60 mL) was treated with M sodium hydroxide (12.8 mL) to yield a bright yellow solution of the phenate anion. A solution of tetra-*O*-acetyl-α-D-galactopyranosyl bromide[11] (6.30 g) in acetone (20 mL) was added dropwise with stirring during 1 h. After stirring for 20 h in the dark, the acetone was evaporated,

and a solution of the residue in chloroform (100 mL) was flash chromatographed (silica gel) to remove the bulk of contaminating phenol and sodium bromide prior to evaporation of the chloroform. The resulting off-white solid was stirred with dry acetone (200 mL) for 20 h to remove further traces of free phenol and finally recrystallised, twice from methanol and once from aq. 95% ethanol, to yield **1f** as white needles (3.04 g, 42%), m.p. 184–185°, $[\alpha]_D$ −5° (chloroform); R_F 0.8 (chloroform–methanol, 9:1). ^{1}H-N.m.r. data ($CDCl_3$): δ 2.06, 2.13, 2.24 (3 s, 3, 6, and 3 H, 4 Ac), 3.79 (s, 3 H, MeO), 3.83 (s, 2 H, coumarin CH_2), 4.27 (m, 3 H, H-5,6,6), 5.16–5.40 (m, 2 H, H-2,3), 5.47–5.70 (m, 2 H, H-1,4), 6.36 (s, 1 H, coumarin H-3), 7.03 (dd, 1 H, $J_{5,6}$ 9, $J_{6,8}$ 2 Hz, H-6), 7.09 (d, 1 H, H-8), 7.61 (d, 1 H, H-5).

Anal. Calc. for $C_{26}H_{28}O_{14}$: C, 55.3; H, 5.0. Found: C, 55.3; H, 5.3.

Methyl 7-β-D-galactopyranosyloxycoumarin-4-acetate (**1e**). — To a solution of **1f** (0.50 g) in warm, dry methanol (30 mL) was added methanolic sodium methoxide (0.5 mL), and the solution was stored for 1 h at room temperature, then overnight at ~5°. The resulting pale-pink prisms were collected, washed with a little ice-cold methanol, and recrystallised from water–ethanol (1:1) to give **1e** (0.32 g, 87%), m.p. 128–129°, $[\alpha]_D$ −27° (*N,N*-dimethylformamide); R_F 0.53 (acetonitrile–water, 7:1); ν_{max}^{KBr} 1730 cm^{-1} (ester C=O). ^{1}H-N.m.r. data [$(CD_3)_2SO$]: δ 3.44–3.80 (m, 6 H, H-2,3,4,5,6,6), 3.68 (s, 3 H, MeO), 4.02 (s, 2 H, coumarin CH_2), 4.42–5.34 (b, 4 H, 4 OH), 5.00 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 6.36 (s, 1 H, coumarin H-3), 7.03 (dd, 1 H, $J_{5,6}$ 9, $J_{6,8}$ 3 Hz, H-6), 7.10 (d, 1 H, H-8), 7.64 (d, 1 H, H-5).

Anal. Calc. for $C_{18}H_{20}O_{10}\cdot H_2O$: C, 52.2; H, 5.3. Found: C, 52.5; H, 5.2.

7-β-D-Galactopyranosyloxycoumarin-4-acetic acid (**1d**). — A solution of **1f** (0.5 g) in warm, dry methanol (30 mL) was treated with methanolic sodium methoxide (0.5 mL). After 1 h, the solvent was evaporated and a solution of the residue in warm water (15 mL) was treated with M sodium hydroxide (0.5 mL).

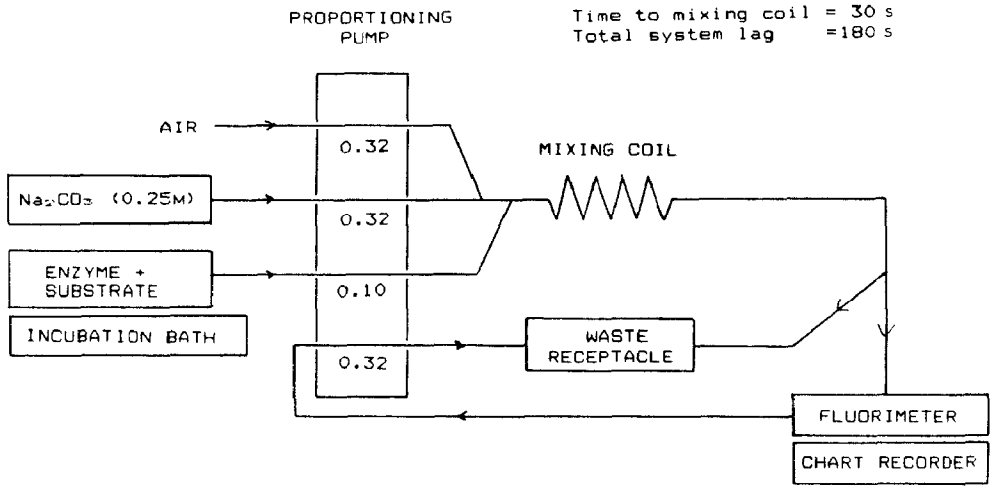

Fig. 1. Assay of β-D-galactosidase.

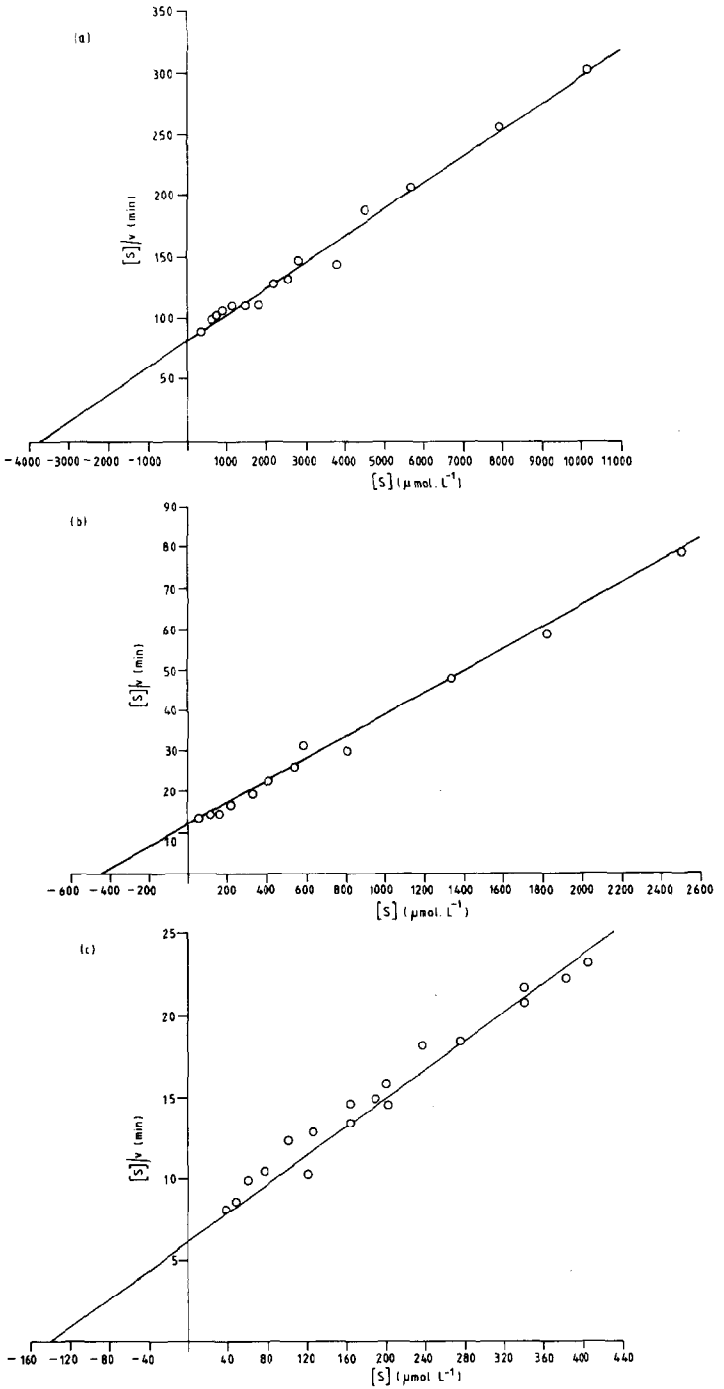

Fig. 2. Hanes plots of [S]/v against [S] for fluorogenic substrates with β-D-galactosidase (see Experimental): (*a*) 7-β-D-galactopyranosyloxycoumarin-4-acetic acid (**1d**), (*b*) methyl 7-β-D-galactopyranosyloxycoumarin-4-acetate (**1e**), (*c*) 7-β-D-galactopyranosyloxy-4-methylcoumarin (**1g**).

After 3 h, the solution was neutralized with IR-120 (H^+) resin and concentrated, and the crude product was recrystallised twice from water and once from ethanol–water (1:1) to yield **1d** (0.28 g, 79%), m.p. 182–183° (dec.), $[\alpha]_D$ −25° (*N,N*-dimethylformamide); R_F 0.08 (acetonitrile–water, 7:1); ν_{max}^{KBr} 1715 cm^{-1} (COOH C=O). ^{1}H-N.m.r. data (D_2O): δ 3.70 (s, 2 H, coumarin CH_2), 3.76–3.98 (m, 5 H, H-2,3,5,6,6), 4.05 (m, 1 H, H-4), 5.15 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 6.24 (s, 1 H, coumarin H-3), 7.04 (d, 1 H, H-8), 7.10 (dd, 1 H, $J_{5,6}$ 8, $J_{6,8}$ 2 Hz, H-6), 7.62 (d, 1 H, H-5).

Anal. Calc. for $C_{17}H_{18}O_{10} \cdot H_2O$: C, 51.0; H, 5.0. Found: C, 51.2; H, 4.8.

Enzyme assays. — Assays were performed in sodium phosphate buffer (pH 7.3, I 0.1) at 37.0 ±0.5°. A stock solution of β-D-galactosidase (~1000 U in 500 mL) in phosphate buffer was prepared and stored in the dark at 2°. Any loss of activity from day to day was determined by assaying the enzyme solution, using three different concentrations of **1g**.

A block diagram of the apparatus used to assay β-D-galactosidase is shown in Fig. 1. The sodium carbonate quenches the reaction and generates the fluorophore as the phenate anion.

The fluorimeter was calibrated daily using a solution of quinine sulphate (10 mg/L in 0.05M H_2SO_4) which is stable in the dark[12]. Calibration was performed so that a quinine sulphate solution of 10 mg/L (100 quinine units, q.u.) gave a fluorimeter reading of ~500 units. Standard solutions of the relevant aglycon in 0.25M sodium carbonate were pumped through the system in order to construct calibration curves of aglycon concentration *vs.* fluorescence (q.u.) which were fitted by least squares. The instrument was calibrated using standard solutions of *o*-nitrophenyl β-D-galactopyranoside after they had been completely hydrolyzed enzymically[13].

The system was first pumped through with distilled water in order to establish a zero reading. A control solution (3 mL) containing 0.03M magnesium chloride (0.10 mL), 3.36M 2-mercaptoethanol (0.10 mL), and a sodium phosphate-buffered solution of the substrate was then pumped through, together with the sodium carbonate quenching solution, in order to ascertain the degree of non-enzymic hydrolysis and to provide a blank fluorescence value. The enzyme assay was then performed on a solution (3 mL) of identical composition containing phosphate-buffered β-D-galactosidase solution (0.100 mL) which had previously been incubated with the magnesium chloride and 2-mercaptoethanol for 3 min at 37°.

The curves varied from essentially linear at high substrate concentrations to pronouncedly curved at lower substrate concentrations. In order to avoid subjective bias, a simple computer program was used to determine the initial rates, based on the direct linear plot method of Cornish–Bowden[14].

Enzyme kinetics. — The initial substrate concentrations and rates were used to calculate K_m and V_{max}, using a simple program to run the direct linear plot method of Eisenthal and Cornish–Bowden[15]. Assays were repeated three times and errors calculated accordingly. The results are shown in Table I.

Plots, after Hanes[16], are shown in Fig. 2. The lines were fitted using the parameters determined by the direct linear plots.

ACKNOWLEDGMENTS

Dr. M. E. Jolley and Pandex Laboratories Inc. are thanked for financial support.

REFERENCES

1 D. H. LEABACK, *An Introduction to the Fluorimetric Estimation of Enzyme Activities*, 2nd edn., Koch–Light Laboratories, Colnbrook, 1975.
2 J. A. R. MEAD, J. W. SMITH, AND R. T. WILLIAMS, *Biochem. J.*, 61 (1955) 569–574.
3 D. ROBINSON, *Biochem. J.*, 62 (1956) 39–44.
4 D. ROBINSON, *Comp. Biochem. Physiol.*, 12 (1964) 95–105.
5 T. J. JACKS AND H. W. KIRCHER, *Anal. Biochem.*, 21 (1967) 279–285.
6 S. CREME AND D. H. LEABACK, *Anal. Biochem.*, 103 (1980) 258–263.
7 M. I. PAGE (Ed.), *The Chemistry of Enzyme Action*, Elsevier, Amsterdam, 1984, pp. 78–79.
8 E. GLAZER AND W. WULWEK, *Biochem. J.*, 145 (1924) 514.
9 M. HERRCHEN AND G. LEGLER, *Eur. J. Biochem.*, 138 (1984) 527–531.
10 W. BAKER, C. N. HAKSAR, AND J. F. W. MCOMIE, *J. Chem. Soc.*, (1950) 170–173.
11 R. U. LEMIEUX, *Methods Carbohydr. Chem.*, 2 (1963) 221–222.
12 D. H. LEABACK AND P. G. WALKER, *Biochem. J.*, 78 (1961) 151–156.
13 M. L. SINNOTT AND I. J. L. SOUCHARD, *Biochem. J.*, 133 (1973) 89–98.
14 A. CORNISH-BOWDEN, *Biochem. J.*, 149 (1975) 305–312.
15 R. EISENTHAL AND A. CORNISH-BOWDEN, *Biochem. J.*, 139 (1974) 715–720.
16 C. S. HANES, *Biochem. J.*, 26 (1932) 1406–1421.
17 A. V. FOWLER AND I. ZABIN, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (4) (1977) 1507–1510.