Carbohydrate Research 205 (1990) c5–c9
Elsevier Science Publishers B.V., Amsterdam

Preliminary communication

New colorimetric substrates for the assay of glycosidases*

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(Received May 2nd, 1990; accepted for publication, May 19th, 1990)

The preferred method of detecting and assaying enzymes is to incubate with a substance which is as close to the natural substrate as possible, but which will liberate a molecule that can be quantified on the basis of its colour, fluorescence, or chemiluminescence. The last two properties are particularly sensitive, but their measurement requires specialised equipment. The most widely used substrates for the assay of glycosides are the 4- and 2-nitrophenyl glycosides, but the nitrophenols released, after basification, have weak yellow colours (λ_{max} ~410 nm) and endogenous coloured species may interfere in the assays.

Appropriate derivatives of the highly coloured 2-methoxy-4-(2-nitrovinyl)phenol[1] (**2a**; λ_{max} 505, ε 27 000) are good substrates for the colorimetric assay of glycosidases[2], phosphatases, and esterases[3]. However, the colour of the phenol released tends to fade under alkaline conditions, and the substrates are not as soluble in water as is required for general use.

The condensation of vanillyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1b**) and vanillyl β-D-galactopyranoside (**1c**) with various heterocycles having either an active methyl or methylene group has now been studied in order to enhance the colour[4] of the released phenol and the solubility of the substrate. The resulting substrates are suitable for the assay of 2-acetamido-2-deoxy-β-D-glucopyranosidase (NAG) and β-D-galactopyranosidase, respectively.

Condensation of **1b** with an equimolar amount of rhodanine-3-acetic acid in the presence of ammonia and ammonium chloride afforded 93% of ammonium 5-[4-(2-acetamido-2-deoxy-β-D-glucopyranosyloxy)-3-methoxyphenylmethylene]-2-thioxothiazolidin-4-one-3-acetate {**3b**, m.p. 165–173° (dec.), $[\alpha]_D$ +18° (methyl sulphoxide)} as a bright yellow monohydrate. Although the reaction mixture was heterogeneous throughout (4 h at 60° in ethanol), the product contained (h.p.l.c.) <0.5% of **1b**.

* Dedicated to Professor Leslie Hough in the year of his 65th birthday.
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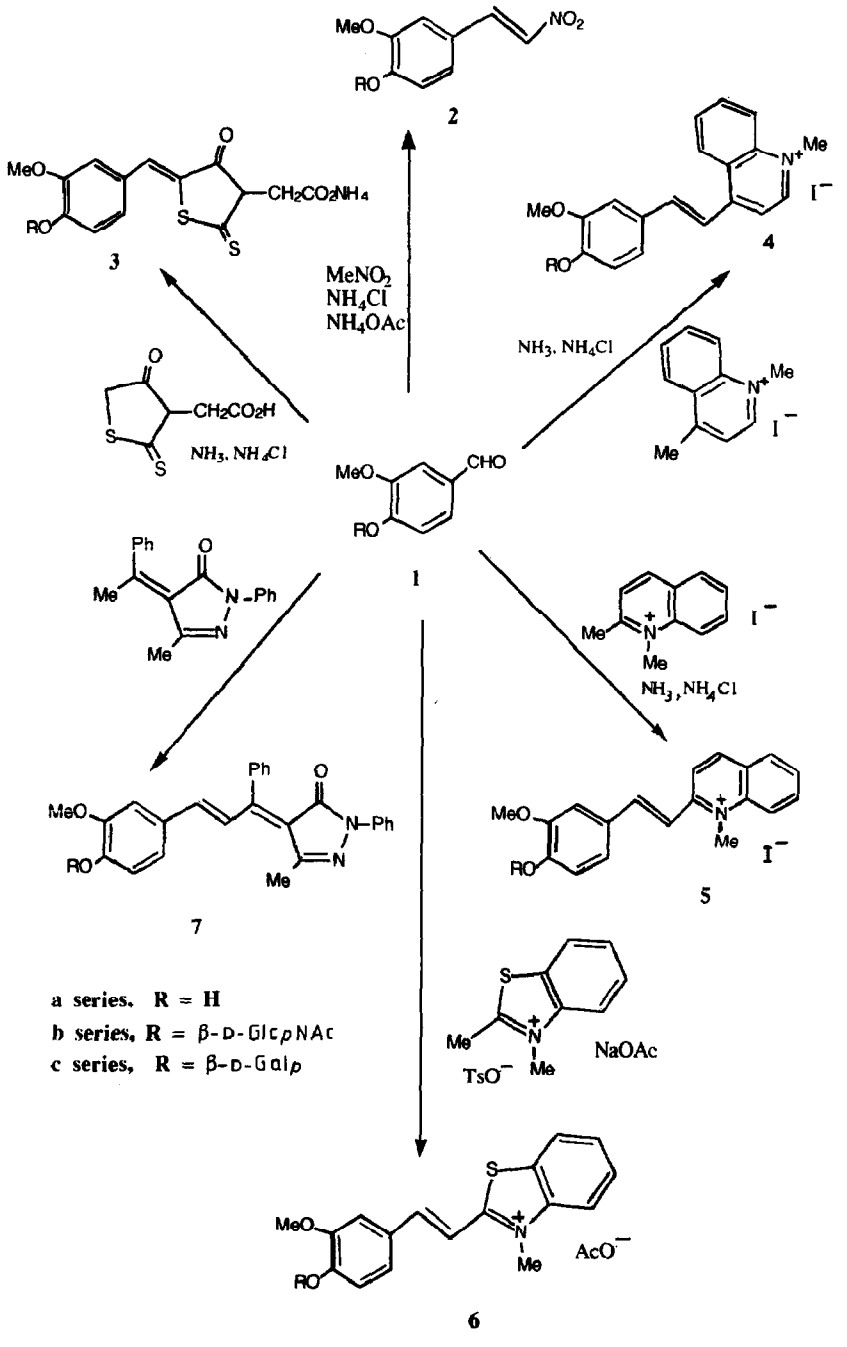


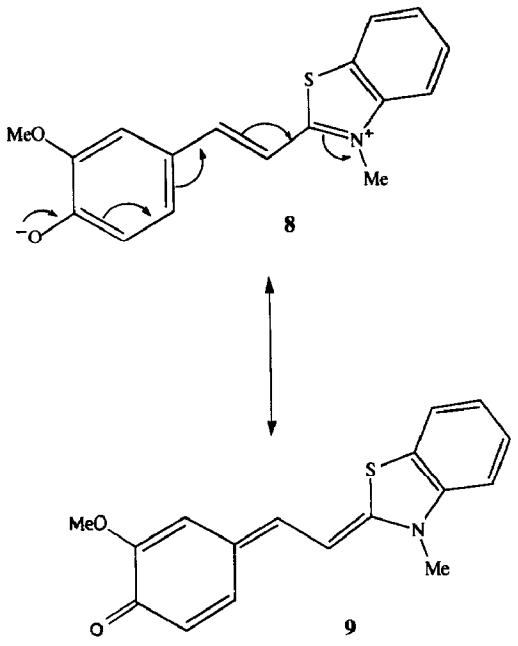
Similarly, condensation of **1c** with the rhodanine derivative afforded 97% of ammonium 5-[4-(*β*-D-galactopyranosyloxy)-3-methoxyphenylmethylene]-2-thioxothiazolidin-4-one-3-acetate {**3c**, m.p. 176–180°, $[\alpha]_D$ −35° (methyl sulphoxide)}, contaminated (h.p.l.c.) with 1–3% of **1c**, but which did not affect the use of **3c** as an enzyme substrate.

The glycoside **3b** was an excellent substrate[5] for the assay of NAG in human urine, with a K_m of ~0.5mM and a very high V_{max} so that the sensitivity was far higher than that of the currently employed substrate, 3-methoxy-4-(2-nitrovinyl)phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2b**). The colour produced by the phenol **3a** released (λ_{max} 492 nm, ε 37 000)[4] was stable at pH 9.8 and was intensified on the addition of such organic solvents as acetone. The substrate was soluble (> 10mM) in buffer at pH 4.75, but, at pH < 4.25, the free acid precipitated. Likewise, **3c** was a good substrate for *E. coli* galactosidase.

Condensation of **1b** with either 1,4- or 1,2-dimethylquinolinium iodide in the presence of ammonium acetate and ammonia afforded 4-{2-[4-(2-acetamido-2-deoxy-β-D-glucopyranosyloxy)-3-methoxyphenyl]vinyl}-1-methylquinolinium iodide {**4b**, 78%; m.p. 207–212° (dec.), $[\alpha]_D$ +28° (methyl sulphoxide)} and 2-{2-[4-(2-acetamido-2-deoxy-β-D-glucopyranosyloxy)-3-methoxyphenyl]vinyl}-1-methylquinolinium iodide dihydrate {**5b**, 62%; m.p. 165° (dec.), $[\alpha]_D$ +19° (methyl sulphoxide)}, respectively. Compound **4b** was an orange solid with good water solubility and was a good substrate for NAG, but its direct use was impaired because a small amount (0.3%) of non-enzymic hydrolysis during the preparation gave an unacceptably high blank. However, the phenol could be removed selectively from the solution by filtration through cellulose powder. On account of this, these substrates have potential in applications that use cellulose dipsticks, where it is essential that the colour produced by enzyme action does not migrate or wash off the paper.

The tendency of **4b** to lose the aglycon is due to the phenoxide anion being a stable leaving group, together with anchimeric assistance of the neighbouring acetamido group. This is a drawback[6] of 2-acetamido-2-deoxy-D-glucosides of phenols having a low pK_a, and the effect is enhanced on increasing the pH so that the commercially used sodio 3,3'-dichlorophenolsulphonphthaleinyl 2-acetamido-2-deoxy-β-D-glucopyranoside[7] undergoes substantial non-enzymic hydrolysis during use at pH 6.25. This problem does not exist with **3b**. Attempted preparation of the 2-acetamido-2-deoxyglucoside **6b** by condensation of **1b** with 1,2-dimethylbenzothiazolium tosylate failed due to substantial hydrolysis of the first-formed glycoside **6b** to the phenol **6a** during its preparation. In contrast, the corresponding galactoside {**6c**, 80%; m.p. 147–149°, $[\alpha]_D$ −10° (methyl sulphoxide)} was prepared readily using piperdine as the condensation catalyst. It was an excellent substrate for *E. coli* β-D-galactosidase, releasing the phenol **6a**, which was selectively absorbed on to cellulose and showed substantial bathochromic shifts in the presence of organic solvents. Under aqueous conditions at an alkaline pH, **6a** exists as the phenoxide **8** (λ_{max} 519, ε 55 000), which is in resonance with its merocyanine **9**. However, on the addition of acetone (20%), a substantial increase in the intensity of its colour was noted (λ_{max} 544, ε 67 000), due to an increasing contribution from the merocyanine. Increase in colour occurred with increasing amounts of acetone until the formation of the merocyanine was almost complete in nearly pure acetone.

Condensation of **1c** with 3-methyl-1-phenyl-5-pyrazolone gave a product which rapidly underwent Michael addition with unreacted reagent due to the reactivity of its methylene group[8]. However, if the pyrazolone was reacted first with acetophenone to give 3-methyl-4-(α-methylbenzylidene)-1-phenyl-5-pyrazolone[9], condensation oc-



curred on the α-methyl group of the benzylidene substituent to give 4-{3-[4-(β-D-galactopyranosyloxy)-3-methoxyphenyl]-1-phenylallylidene}-3-methyl-1-phenyl-5-pyrazolone {**7c**, m.p. 156–158°, $[\alpha]_D$ −250° (methyl sulphoxide)}, which was a good substrate of *E. coli* galactosidase, but produced an insoluble dark black-green-coloured phenol, which could be collected on filter paper. This substrate offers a cheaper alternative to the widely used but expensive histochemical reagent 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside[10] also employed in some ELISA tests.

Most of the above mentioned glycosides were substrates for the appropriate enzyme with various efficacies. However, 1-formyl-2-naphthyl β-D-galactopyranoside, prepared in a manner analogous to that for **1c**, gave chromogenic substrates that inhibited *E. coli* β-D-galactosidase and were not hydrolysed by the enzyme. 4-Formyl-2,6-dimethoxyphenyl glycosides of 2-acetamido-2-deoxy-β-D-glucopyranose, β-D-galactopyranose, and β-D-glucopyranuronic acid were prepared also. The first two glycosides were worse substrates than those derived from **1b** and **1c**, but the glucuronides, which will be described elsewhere, were much improved by the extra methoxyl group.

A patent has been applied for these and related substrates[11], and they are available from Melford Laboratories Ltd., Bildeston Road, Chelsworth, Ipswich IP7 7LE (U.K.).

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