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3-METHOXY-4-(2-NITROVINYL)PHENYL GLYCOSIDES AS POTENTIAL CHROMOGENIC SUBSTRATES FOR THE ASSAY OF GLYCOSIDASES

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ABSTRACT

Selective glycosidation of 2,4-dihydroxybenzaldehyde with either 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, 2-acetamido-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride, or 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide afforded the corresponding 4-*O*-glycosyl derivatives. Subsequent *O*-methylation, *O*-deacetylation, and condensation with nitromethane afforded the appropriate β-glycoside of 3-methoxy-4-(2-nitrovinyl)phenol. The phenol is highly coloured at alkaline pH so that these glycosides may be suitable as chromogenic substrates for the assay of glycosidases.

INTRODUCTION

The detection of hydrolytic enzymes in urine, serum, and other biological fluids is important as an indicator of certain pathological conditions. For example, the presence of elevated levels of *N*-acetyl-β-D-glucosaminidase (NAG) in the urine is indicative of renal malfunction[1] and this has been employed to monitor the progress of renal allographs. An upsurge of NAG precedes other clinical indicators and is taken as a sign of impending rejection of the transplanted kidney[2]. The usual assay for such enzymes involves incubation of the fluid with a suitable substrate phenolic glycoside, followed by determination of the released phenol. *p*-Nitrophenyl glycosides are frequently used for this purpose, since *p*-nitrophenol may be assayed after basification by colorimetry (λ_{max} 400 nm, ε 15,000). However, the rather low intensity of the colour (pale yellow–orange) of the *p*-nitrophenoxide anion is a limitation, particularly for the development of a test requiring visual estimation rather than instrumental measurement.

4-Methylumbelliferyl glycosides have also been used, since the released phenol can be determined by fluorimetry with very high sensitivity[3]. However, the difficulties in standardisation and the necessity for expensive fluorimeters has limited this procedure to large well-equipped laboratories and it is therefore unsuitable for the development of simple diagnostic tests. Hence, the development of a more sensitive procedure for the assay of hydrolytic enzymes by a colorimetric

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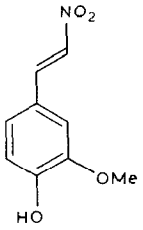
procedure was called for, and we have described a series of esters[4] and glycosides[5] of 2-methoxy-4-(2-nitrovinyl)phenol (**1**) as colorimetric substrates for enzyme assay; for the glucosaminide **11**, we have incorporated it into a simple kit procedure[6] and into a dipstick device[7] for the assay of NAG in urine. The released phenol has a deep-red colour [λ_{max} 505 nm (ε 26,000)] at pH >7.5.



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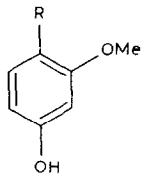


2 R = CHO

3 R = CH=CHNO$_2$

The large bathochromic (105 nm) and hyperchromic (11,000) shifts in going from the *p*-nitrophenoxide anion to the anion of **1** is surprising for the addition of a double bond to the conjugated system. However, the methoxy group contributes substantially to the colour of the phenoxide anion, since 4-(2-nitrovinyl)phenol has λ_{max} 460 nm (ε 21,000) and 2,6-dimethoxy-4-(2-nitrovinyl)phenol has λ_{max} 525 nm (ε 23,000)[7] at alkaline pH. Nuclear nitration of these compounds generally leads to significantly smaller bathochromic shifts and to lower extinction coefficients for the phenoxide anions. Thus, 2-nitro-4-(2-nitrovinyl)phenol has λ_{max} 405 nm (ε 7,500)[7] at alkaline pH.

Therefore, it appeared that the methoxy group in these compounds was an essential prerequisite for good colour formation, but it was considered that the presence of a methoxy group *ortho* to the glycosidic oxygen might hinder the formation of the enzyme–substrate complex and have an unfavourable effect upon the kinetics of the reaction. We have established that 2-methoxy-4-(2-nitrovinyl)phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**11**) is hydrolysed by NAG at a rate lower than that of the *p*-nitrophenyl analogue, but the corresponding β-D-galactoside of **1** is a better substrate than *p*-nitrophenyl β-D-galactopyranoside for *E. coli* β-D-galactosidase. In view of this, it was speculated that, if the methoxyl group in **1** were moved to the 3-position, the kinetics of its hydrolysis by NAG might be improved with retention of the intense colour of the released phenol. Hence, 3-methoxy-4-(2-nitrovinyl)phenol (**3**) and three of its glycosides were prepared and studied.

RESULTS AND DISCUSSION

The required phenol **3** was prepared in 6% overall yield by the application of the Reimer–Tiemann[8] reaction to 3-methoxyphenol, which afforded a mixture of

4-hydroxy-2-methoxybenzaldehyde (**2**) and several other compounds, from which **2** was readily separated by chromatography on silica gel. The phenol **2** was then condensed with nitromethane to give the required ω-nitrostyrene (**3**, 56%). In alkaline solution, **3** was a bright red-orange, had λ_{max} 480 (ε 28,000), and was suitable for the development of chromogenic substrates for hydrolases.

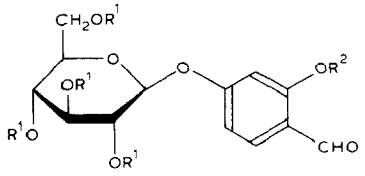
In view of difficulties encountered in the preparation of large quantities of **2**, it was decided to investigate the preparation of glycosides of **2** by an indirect route involving the selective glycosidation of commercially available 2,4-dihydroxybenzaldehyde, since it was considered that glycosidation would occur selectively at the more accessible 4-hydroxyl group. Thus, when 2,4-dihydroxybenzaldehyde was condensed with 1 equiv. of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide[9] in the presence of 1 equiv. of sodium hydroxide, a mixture of products was formed from which crystalline 4-formyl-3-hydroxyphenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**4**) was isolated in 30% yield without recourse to chromatography. Elemental analysis and the ^{1}H-n.m.r. spectrum showed that the product was a

TABLE I

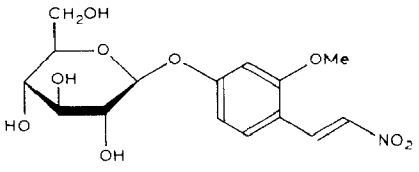
^{1}H-N M R PARAMETERS (δ, Hz) IN DEUTERIOCHLOROFORM AT 250 MHz

	4	**5**	**8**	**9**	**13**	**14**
H-1	5.4	5.4	5.46d	5.48d	5.17d	5.19d
H-2	—	—	4.16td	4.21td	5.51dd	5.52dd
H-3	5.1m	5.12m	5.49t	5.48t	5.15dd	5.14dd
H-4			5.12t	5.15t	5.49d	5.48d
H-5	3.95ddd	3.95ddd	4.01ddd	4.02ddd	4.1	4.1
H-6a	4.29dd	4.29dd	4.27dd	4.28dd	—	—
H-6b	4.18dd	4.18dd	4.18dd	4.17dd	4.3m	4.3m
H-2′	6.65d	6.58d	6.57d	6.6m	6.57d	6.58d
H-5′	7.49d	7.81d	7.45d	7.70d	7.50d	7.82d
H-6′	6.61dd	6.62dd	6.61dd	6.6m	6.62dd	6.63dd
CHO	9.77s	10.32s	9.74s	10.24s	9.78s	10.33
OAc	2.05	2.05	2.06	2.06(×2)	2.03	2.02
	2.06	2.07(×2)	2.08	2.08	2.08	2.06
	2.07	2.08	2.10		2.10	2.08
	2.11				2.19	2.20
NAc			1.93	1.94		
OMe		3.90s		3.87s		3.90s
NH			6.16d	6.56d		
$J_{1,2}$			8.2	8.3	7.9	8.0
$J_{2,3}$			10.5	10 4	10.4	10.4
$J_{3,4}$			9.6	9.7	3.6	3.4
$J_{4,5}$	10.0	10.2	9.5	9.4	<1	<1
$J_{5,6a}$	5.9	5.5	6.0	5.6		
$J_{5,6b}$	2.4	2.4	2.3	2.3		
$J_{6a,6b}$	12.3	12.3	12.2	12.4		
$J_{2',6'}$	2.3	2.1	2.3		2.2	2.1
$J_{5',6'}$	8.6	8.6	8.5	8.6	8.5	8.5
$J_{6',CHO}$	0	0.65	0	<0.5	0	<0.5
$J_{2,NH}$			8.7	~8		

monoglycoside, and the position of the sugar residue was indicated by the n.m.r. spectra before and after methylation of the remaining phenolic group. Prior to methylation with methyl iodide–potassium carbonate, the C*H*O resonated at δ 9.77; in the *O*-methylated compound, it resonated at δ 10.32. This rather large shift (0.55 p.p.m.) was noted in all the compounds studied and reflects the differing conformation about the bond linking the aldehydic group to the aryl ring. Prior to methylation, strong intramolecular hydrogen-bonding between the carbonyl group and the phenolic group results in the adoption of conformation **17**. In the *O*-



4 R^1 = Ac, R^2 = H
5 R^1 = Ac, R^2 = Me
6 R^1 = H, R^2 = Me



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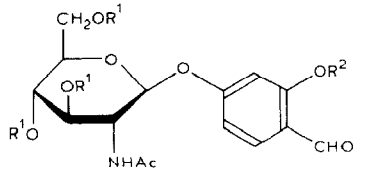
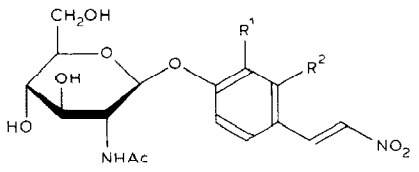
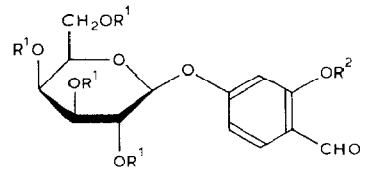


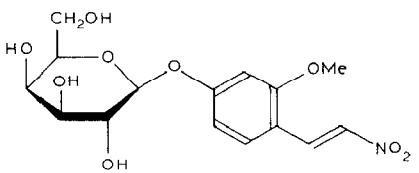
8 R^1 = Ac, R^2 = H
9 R^1 = Ac, R^2 = Me
10 R^1 = H, R^2 = Me



11 R^1 = OMe, R^2 = H
12 R^1 = H, R^2 = OMe



13 R^1 = Ac, R^2 = H
14 R^1 = Ac, R^2 = Me
15 R^1 = H, R^2 = Me



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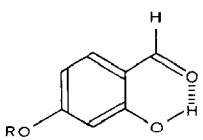

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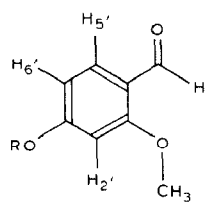

18

methylated derivative **5**, formed in 90% yield from **4**, such hydrogen bonding is not possible, so that conformation **18** would be more preferred sterically in which the aldehydic hydrogen is further deshielded by the proximity of the OMe group. Furthermore, in going from **4** to **5**, a significant downfield shift (0.32 p.p.m.) of the signal for H-5′ was observed due to its proximity to the carbonyl oxygen in conformation **18**. Additionally, a long-range coupling of 0.65 Hz was clearly observed between the aldehydic proton and H-6′, reflecting the extended "W" arrangement between these two protons in conformation **18**. The downfield shift of the signal for H-5′ was noted in the other two compounds (see Table I), but the long-range coupling was seen less clearly and manifested itself as slight broadening of the appropriate resonances. *O*-Deacetylation of **5** afforded 4-formyl-3-methoxyphenyl β-D-glucopyranoside (**6**) as a monohydrate in 85% yield, which was then condensed with nitromethane in the presence of acetic acid and ammonium acetate to give the required 3-methoxy-4-(2-nitrovinyl)phenyl β-D-glucopyranoside in 70% yield, as a pale-yellow crystalline solid.

The same sequence of reactions was repeated starting with either 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[10] or 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide[11], with substantially similar results, to give 3-methoxy-4-(2-nitrovinyl)phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**12**) and 3-methoxy-4-(2-nitrovinyl)phenyl β-D-galactopyranoside (**16**), respectively.

These glycosides are currently being assessed as chromogenic substrates for the assay of the appropriate enzymes.

EXPERIMENTAL

Unless otherwise stated, all optical rotations were measured for chloroform solutions at ambient temperatures (18–22°). The light petroleum used had b.p. 40–60°.

4-Hydroxy-2-methoxybenzaldehyde (**2**). — Chloroform (100 mL) was added to a solution of 3-methoxyphenol (14.9 g, 0.12M) in aqueous 15% potassium hydroxide (500 mL). The mixture was heated under reflux for 2–3 h and then cooled, and the pH was adjusted to ~4 by the addition of hydrochloric acid. A reddish-brown tar was formed which was washed with chloroform and discarded. The chloroform layer was separated, the aqueous layer was extracted with further portions of chloroform, and the combined extracts were washed twice with saturated aqueous sodium chloride and dried ($MgSO_4$). T.l.c. (ether–light petroleum, 2:1) indicated the presence of at least five components, of which the slower was the major product and was coincident with an authentic sample of **2**. The solution was then filtered through a short column of silica gel (elution with chloroform). The fractions containing the slower-moving component were concentrated to dryness, and the solid was recrystallised from ethyl acetate (decolourisation with charcoal) to give **2** (2 g, 11%), m.p. 154–156°; lit.[8,12] m.p. 153° (Found: C, 62.98; H, 5.25. $C_8H_8O_3$ calc.: C, 63.16; H, 5.26%).

3-Methoxy-4-(2-nitrovinyl)phenol (**3**). — A solution of **2** (1 g) in ethanol (40 mL) containing nitromethane (0.8 mL), acetic acid (0.33 mL), and ammonium acetate (0.33 g) was heated under reflux for 1 h, when t.l.c. (ethyl acetate–light petroleum, 1:1) indicated that reaction was complete to form a faster-moving yellow compound. The mixture was concentrated to dryness and the syrupy residue was extracted with acetone. Addition of light petroleum to the extract afforded **3** (0.72 g, 56%) as yellow needles, m.p. 158–160°, λ_{max} 385 (pH 5) (ε 16,000), λ_{max} (pH 9.5) 480 (ε 28,000) (Found: C, 55.09; H, 4.62; N, 6.77. $C_9H_9NO_4$ calc.: C, 55.38; H, 4.62; N, 7.17%).

*4-Formyl-3-hydroxyphenyl 2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranoside* (**4**). — To a stirred solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide[9] (42 g, 0.1M) in acetone (200 mL) was added a solution of 2,4-dihydroxybenzaldehyde (13.8 g, 0.099M) in aqueous M sodium hydroxide (100 mL), and the mixture was kept at room temperature for 16 h. The dark-coloured mixture was then diluted with water (~2 L) and stirred until the syrupy product separated, the water was decanted, and the syrup was extracted with chloroform. The extract was washed well with water, dried ($MgSO_4$), and concentrated to dryness to give a solid which was recrystallised from ethanol to give **4** (14 g, 30%), m.p. 135°, $[\alpha]_D$ −35° (*c* 0.6) (Found: C, 54.37; H, 5.33. $C_{21}H_{24}O_{12}$ calc.: C, 53.84; H, 5.12%).

*4-Formyl-3-methoxyphenyl 2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranoside* (**5**). — A solution of **4** (0.276 g, 0.59 mmol) in anhydrous acetone (60 mL) was treated with methyl iodide (0.6 mL, 9.7 mmol) and anhydrous potassium carbonate (0.09 g, 0.59 mmol). The mixture was heated under reflux for 2 h, when t.l.c. (ethyl acetate–light petroleum, 1:1) indicated that the reaction was complete. The mixture was then concentrated to dryness, the residue was partitioned between water and chloroform, and the organic layer was dried ($MgSO_4$) and concentrated to dryness to give **5** (0.26 g, 90%), m.p. 137° (from ethanol), $[\alpha]_D$ −34° (*c* 0.75, acetone) (Found: C, 54.38; H, 5.38. $C_{22}H_{26}O_{12}$ calc.: C, 54.77; H, 5.39%).

*4-Formyl-3-methoxyphenyl β-*D*-glucopyranoside* (**6**). — A suspension of **5** (1 g) in dry methanol (50 mL) was treated with methanolic M sodium methoxide (~1 mL). The mixture was stirred at room temperature for 1 h, when t.l.c. (ethyl acetate–acetone, 3:1) indicated that the reaction was complete. The mixture was then filtered through a pad of silica gel to remove Na^+, and concentrated to dryness to give **6**, m.p. 167–170° (from ethanol), $[\alpha]_D$ −70° (*c* 0.7, methyl sulphoxide) (Found: C, 50.85; H, 5.48. $C_{14}H_{18}O_8 \cdot H_2O$ calc. C, 50.60; H, 6.02%).

*3-Methoxy-4-(2-nitrovinyl)phenyl β-*D*-glucopyranoside* (**7**). — A suspension of **6** (0.2 g, 0.64 mmol) in ethanol (10 mL) containing nitromethane (0.51 mL), acetic acid (0.13 mL), and ammonium acetate (0.13 g) was heated under reflux for 30 min. T.l.c. (chloroform–methanol, 3:1) then indicated that the reaction was complete. The mixture was concentrated to dryness and a solution of the residue in acetone was passed through a pad of silica gel in order to remove the ammonium acetate. The filtrate was concentrated to dryness and the yellow solid was recrystallised from acetone–light petroleum to give **7** (0.16 g, 70%), m.p. 129–130°, $[\alpha]_D$

−54.5° (*c* 0.7, methyl sulphoxide) (Found: C, 50.26; H, 5.59; N, 3.83. $C_{15}H_{19}NO_9$ calc.: C, 50.42; H, 5.32; N, 3.92%).

*4-Formyl-3-hydroxyphenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside* (**8**). — To a stirred solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[10] (9.14 g, 25 mmol) in acetone (150 mL) was added a solution of 2,4-dihydroxybenzaldehyde (3.42 g, 25 mmol) in M sodium hydroxide (25 mL), and the mixture was stored at room temperature for 15 h. The dark-coloured mixture was then diluted with water (2 L), the precipitated syrup was extracted with chloroform, and the extract was washed well with water, dried ($MgSO_4$), and concentrated to dryness to give a syrup. T.l.c. (chloroform–methanol, 10:1) indicated that the syrup was composed mainly of one major component, which crystallised from ethanol to give **8** (1.87 g, 30%) as needles, m.p. 227°, $[\alpha]_D$ −19° (*c* 0.6) (Found: C, 53.65; H, 5.35; N, 3.00 $C_{21}H_{25}NO_{11}$ calc.: C, 53.96; H, 5.37; N, 3.00%).

*4-Formyl-3-methoxyphenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside* (**9**). — A mixture of **8** (2.02 g, 4.32 mmol), anhydrous potassium carbonate (0.66 g, 4.78 mmol), and methyl iodide (4.4 mL, 71 mmol) in anhydrous acetone (220 mL) was heated under reflux for 3 h. T.l.c. (ethyl acetate–acetone, 3:1) then indicated that the reaction was complete. The mixture was concentrated to dryness and the resulting thick syrup was partitioned between water and chloroform. The organic layer was washed well with water, dried ($MgSO_4$), and concentrated to a syrup which crystallised from ethanol to give **9** as needles (1.85 g, 90%), m.p. 211°, $[\alpha]_D$ −2° (*c* 0.7, acetone) (Found: C, 54.99; H, 5.48; N, 2.75. $C_{22}H_{27}NO_{11}$ calc.: C, 54.89; H, 5.61; N, 2.91%).

*4-Formyl-3-methoxyphenyl 2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**10**). — Compound **9** (1 g) was *O*-deacetylated as described above. The product separated directly from the mixture to give **10** (0.96 g, 92%), m.p. 219°, $[\alpha]_D$ −16° (*c* 0.6, methyl sulphoxide) (Found: C, 54.28; H, 5.81; N, 3.71. $C_{16}H_{21}NO_8$ calc.: C, 54.28; H, 5.92; N, 3.94%).

*3-Methoxy-4-(2-nitrovinyl)phenyl 2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**12**). — A suspension of **10** (0.1 g) in ethanol (5 mL), to which had been added nitromethane (0.22 mL), ammonium acetate (0.056 g), and acetic acid (0.056 mL), was heated under reflux for 30 min. The yellow product was then collected and washed well with ethanol to give **12** (0.099 g, 88%), m.p. 197–199°, $[\alpha]_D$ −35° (*c* 1, methyl sulphoxide) (Found: C, 50.73; H, 5.57; N, 6.90. $C_{17}H_{22}N_2O_9$ calc.: C, 51.25; H, 5.52; N, 7.03%).

*4-Formyl-3-hydroxyphenyl 2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranoside* (**13**). — A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide[11] (64 g, 0.16M) in acetone (300 mL) was treated with a solution of 2,4-dihydroxybenzaldehyde (20.7 g, 0.15M) in M sodium hydroxide (150 mL), and the mixture was stirred at room temperature for 15 h. The mixture was then diluted with water (3 L), the syrupy product that separated was extracted with chloroform, and the extract was washed well with water, dried ($MgSO_4$), and concentrated to dryness

to give a syrup which was composed of one major product and several minor components as indicated by t.l.c. (ethyl acetate–light petroleum, 1:1). The product crystallised from ethanol to give the major component **13** (24 g, 32%), m.p. 144–145°, $[\alpha]_D$ −3° (*c* 0.8) (Found: C, 54.09; H, 5.06. $C_{21}H_{24}O_{12}$ calc.: C, 53.84; H, 5.12%).

*4-Formyl-3-methoxyphenyl 2,3,4,6-tetra-O-*acetyl-*β*-D-*galactopyranoside* (**14**). — Compound **13** (0.276 g, 0.59 mmol) was heated under reflux with methyl iodide (0.6 mL, 9.7 mmol) and anhydrous potassium carbonate (0.09 g, 0.59 mmol) in anhydrous acetone (60 mL) for 21 h. T.l.c. (ethyl acetate–light petroleum, 1:1) then indicated that the reaction was complete. The mixture was processed as described above to give **14**, m.p. 117–118° (from ethyl acetate–light petroleum), $[\alpha]_D$ −3° (*c* 0.55, acetone) (Found: C, 54.84; H, 5.67. $C_{22}H_{26}O_{12}$ calc.: C, 54.77; H, 5.39%).

*4-Formyl-3-methoxyphenyl β-*D-*galactopyranoside* (**15**). — A solution of **14** (1 g) in dry methanol was subjected to Zemplén deacetylation. The crystalline product started to separate within a few minutes and was collected after 1 h to give **15** (0.62 g, 90%), m.p. 222–224°, $[\alpha]_D$ −45° (*c* 0.5, methyl sulphoxide) (Found: C, 50.41; H, 5.52. $C_{14}H_{18}O_8 \cdot H_2O$ calc.: C, 50.60; H, 6.02%).

*3-Methoxy-4-(2-nitrovinyl)phenyl β-*D-*galactopyranoside* (**16**). — A suspension of **15** (0.2 g) in ethanol (10 mL) containing nitromethane (0.51 mL), ammonium acetate (0.13 g), and acetic acid (0.13 mL) was heated under reflux for 30 min. T.l.c. (chloroform–methanol, 3:1) then indicated that the reaction was complete and that a single product had been formed (yellow spot). The mixture was concentrated to dryness, and a solution of the syrupy residue in acetone was filtered through a pad of silica gel and then concentrated to a syrup which crystallised and was recrystallised from acetone–light petroleum to give **16** (0.14 g, 66%), m.p. 108–110°, $[\alpha]_D$ −31° (*c* 0.63, methyl sulphoxide) (Found: C, 50.35; H, 5.97; N, 4.03. $C_{15}H_{19}NO_9$ calc.: C, 50.42; H, 5.32; N, 3.92%).

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