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IRREVERSIBLE PRODUCT-INHIBITION OF GREEN-COFFEE α-D-GALACTOSIDASE BY 2-BROMO- AND 2-IODO-1-METHOXYETHYL α-D-GALACTOPYRANOSIDE

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ABSTRACT

(*R*,*S*)-1-Ethoxyethyl 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside (**6**) and (*R*)- and (*S*)-2-bromo-1-methoxyethyl 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside (**2** and **8**) were prepared by proton-catalyzed transacetalation. The deacetylated products (*R*,*S*)-1-ethoxyethyl α-D-galactopyranoside (**7**), (*R*)- and (*S*)-2-bromo-1-methoxyethyl α-D-galactopyranoside (**4** and **10**), and (*R*)- and (*S*)-2-iodo-1-methoxyethyl α-D-galactopyranoside (**5** and **11**) are substrates of the α-D-galactosidase from green coffee. The kinetic parameters of **4** were determined. Whereas compound **7** can be completely hydrolyzed by the enzyme, hydrolysis of the halogenated compounds **4, 5, 10,** and **11** is markedly decelerated, due to irreversible deactivation of the enzyme by either 2-bromo- or 2-iodo-acetaldehyde. Because the latter is the more effective, the deactivation is probably due to alkylation of the protein.

INTRODUCTION

Such α-halogenated carbonyl compounds as bromo- and iodo-acetamide, or the corresponding free acids, are common reagents for chemical modification of proteins by mainly alkylating sulfhydryl groups or thio ether groups[1]. If such groups in enzymes are part of the active site, these reagents can be used to inhibit, irreversibly, catalytic activity. β-D-Galactosidase, for instance, could be deactivated with bromo- and iodo-acetamide in a second-order, and with the active-site-directed inhibitor *N*-(iodoacetyl)-β-D-galactosylamine and -β-D-glucosylamine in a first-order rate reaction[2]. Although α-halogenated aldehydes have, to the best of our knowledge, never been used as alkylating agents in enzymology, probably because they are chemically unstable, their reactivity towards divalent sulfur ought to be equal to, if not higher than, that of the corresponding acyl derivatives. As recently pointed out by Tietze *et al.*[3], those aldehydes, set free *in vivo* from their inert acetals by hydrolases, or under the influence of a slightly acidic medium in cancer cells, might well be potent cytotoxins.

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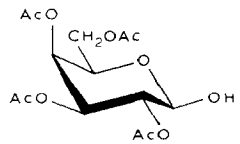
Previously, we synthesized 2-bromo-1-methoxyethyl α-D-glucopyranosides as substrates for α-D-glucosidase from yeast[4,5]. No deactivation of the enzyme could be observed during the hydrolytic cleavage of the glycosides into D-glucose and bromoacetaldehyde. The reaction proceeded according to pseudo-first order kinetics until no substrate was left. Apparently, the bromoacetaldehyde set free did not affect the catalytic activity, either because no essential group became modified, or the concentration of aldehyde, due to dilution locally with the surrounding medium, was not high enough to cause "suicide labelling" (ref. 6), or the deactivation is considerably slower than the substrate hydrolysis. We now describe syntheses of several 1-methoxyethyl α-D-galactosides as substrates for α-D-galactosidase from green coffee-beans, and the consequences of substrate cleavage for enzyme activity.

RESULTS AND DISCUSSION

Two methods for the preparation of 1-alkoxyalkyl glycosides have been described in the literature[7,8] and successfully applied to the synthesis of substrates for assaying glycosidases[7]. For making the α anomers in relatively large quantities, proton-catalyzed transacetalation starting with a sugar having all but the anomeric hydroxyl group blocked by acetylation and with a 1,1-dimethoxy- or 1,1-diethoxyalkane is convenient. With 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose and 1,1-diethoxyethane, under thermodynamic control, one of the four stereoisomers possible is mainly formed, and may be separated by crystallization[7]. The analogous reaction with 2,3,4,6-tetra-*O*-acetyl-β-D-galactose[9] (**1**) also yields a main, chromatographically uniform product; here, however, it is a mixture of nearly equal



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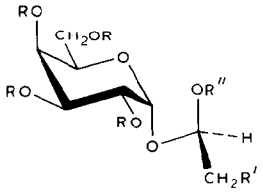
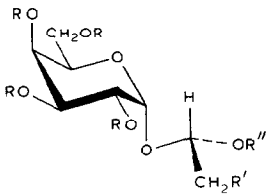
amounts of (*R*)- and (*S*)-1-ethoxyethyl 2,3,4,6-tetra-*O*-acetyl-α-D-galactoside (**6**), as shown by ^{1}H-n.m.r. analysis. In both reactions, the β anomers are formed in only very small proportions. With 2-bromo-1,1-dimethoxyethane, compound **1** also preferentially yielded the α anomer, again as an almost equal mixture of the two stereoisomers differing in configuration at the new asymmetric acetal carbon atom. The *R*- and *S*-derivatives could, in this case, be resolved by column chromatography on silica gel. Both (*R*)- and (*S*)-2-bromo-1-methoxyethyl 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside (**2** and **8**) were crystalline, and could be converted into equally crystalline 2-iodo-1-methoxyethyl 2,3,4,6-tetra-*O*-acetyl-α-D-galactosides (**3** and **9**) by a nucleophilic exchange reaction. The deacetylated compounds, (*R*)- and (*S*)-2-bromo-1-methoxyethyl α-D-galactoside (**4** and **10**), could be converted by intra-



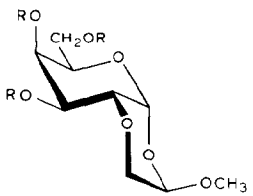
2 R = Ac, R′ = Br, R″ = Me
3 R = Ac, R′ = I, R″ = Me
4 R = H, R′ = Br, R″ = Me
5 R = H, R′ = I, R″ = Me
6* R = Ac, R′ = H, R″ = Et
7* R = H, R′ = H, R″ = Et

*Diastereoisomeric mixture.

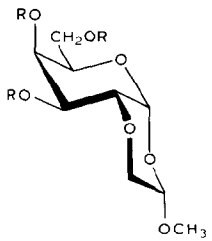


8 R = Ac, R′ = Br, R″ = Me
9 R = Ac, R′ = I, R″ = Me
10 R = H, R′ = Br, R″ = Me
11 R = H, R′ = I, R″ = Me

molecular nucleophilic substitution into (6*R*)- and (6*S*)-6-methoxy-(3,4,6-tri-*O*-acetyl-α-D-galactopyrano)-[1,2-*b*]-1,4-dioxane[4] (**12** and **13**). The configuration of the latter compounds could be unequivocally assigned by ^{1}H-n.m.r. analysis[10], and therefrom, the configuration of the starting materials. All of the deacetylated compounds, the bromides (**4** and **10**) and the iodides (*R*)- and (*S*)-2-iodo-1-methoxyethyl α-D-galactopyranoside (**5** and **11**), as well as (*R*,*S*)-1-ethoxyethyl α-D-galactopyranoside (**7**), a nonhalogenated α-D-galactoside, were substrates of the α-D-galactosidase from green coffee-beans. Qualitative comparison of the cleavage rates by analyzing incubation mixtures (by t.l.c.) showed a striking difference between the nonhalogenated and the halogenated glycosides. Whereas the former were completely hydrolyzed at a certain enzyme concentration, to give D-galactose as the only detectable compound, the latter could never be cleaved completely.



12 R = Ac



13 R = Ac

Because it was suspected that deactivation had been caused by the halogenated aldehydes set free during the enzyme reaction, enzyme activity in the incubation mixtures was monitored independently, using 4-nitrophenyl α-D-galactopyranoside as a test substrate. It was found that α-D-galactosidase is indeed deactivated in mixtures containing the bromo derivative, and even more so in the presence of the iodo derivative, but not with the unhalogenated glycoside as substrate. In order to distinguish between the action of halogenated aldehydes *in statu nascendi* and

aldehydes deriving from the medium, a solution of bromoacetaldehyde was prepared chemically by acid hydrolysis of the dimethyl acetal, and applied to α-D-galactosidase with and without additional substrate. In the presence of substrate, added bromoacetalaldehyde did not increase the rate of deactivation, whereas, in the absence of substrate, the rate of deactivation was enhanced (see Fig. 1A and 1B). These results clearly showed that compounds **4, 5, 10,** and **11** are not active-site-directed inhibitors in the true sense of the word, because the agent causing deactivation, namely, the haloacetaldehyde, acts from the surrounding medium and not directly when liberated at the active site.

Because the iodo derivative **11** has a markedly larger effect than the bromo compound, it may be assumed that the deactivation is due to alkylation by nucleophilic substitution.

Apparently, chemical modification takes place at the active site of the free

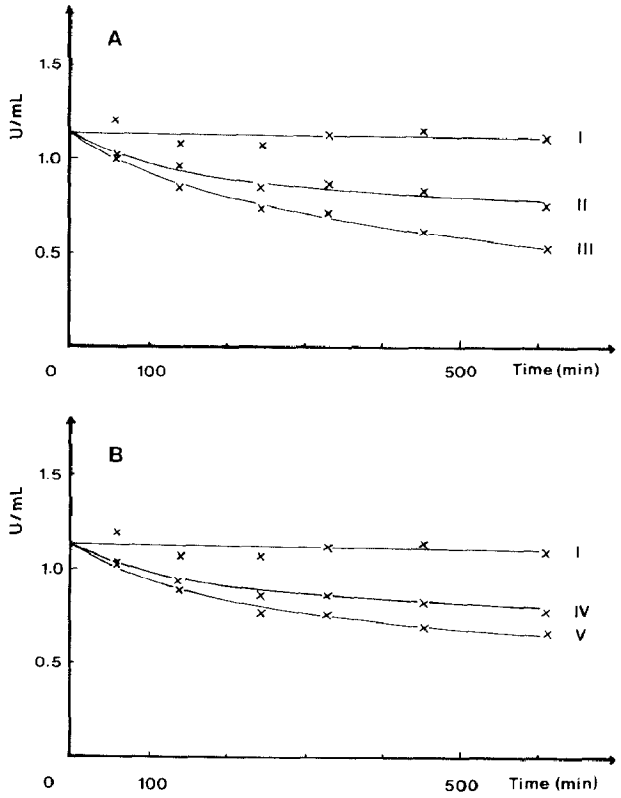


Fig. 1. Deactivation of α-D-galactosidase from green coffee-beans by compounds **10, 11,** and bromoacetaldehyde. Five assays were made: [Key: I, compound **7** (39.6mM); II, compound **10** (37.8mM); III, compound **11** (28.8mM); IV, compound **10** (37.8mM) plus bromoacetaldehyde (0.45mM); and V, bromoacetaldehyde (0.45mM).] A: I, II, III; B: I, IV, V. The reactions were started with α-D-galactosidase (~1.2 U). For determination of the galactosidase activity and aldehyde content, aliquots were taken at 60, 140, 215, 330, 455, and 615 min.

enzyme, because α-D-galactosides seem to have a protecting effect, as the decreased rate of the deactivation by bromoacetaldehyde in the presence of substrate shows. The kinetics of deactivation is rather complex. The enzyme produces the deactivating agent which, in turn, lessens the amount of active enzyme. This again causes a deceleration of inhibitor formation.

The irreversible inhibition of an enzyme which forms the deactivating reagent from the "suicide substrate" by its normal catalytic activity is usually named[11] "suicide inhibition", or, more appropriately[12], "Trojan horse inhibition". This process is highly specific, which is not the case when α-D-galactosidase is inhibited by hydrolytic cleavage of the substrates **10** and **11**. The deactivating reagent in our case has no specific affinity to the enzyme by which it is formed; it would also attack other proteins sensitive to alkylation. Such a kind of pseudo "suicide substrate" could, therefore, be directed not only against the catalyst that produces it, but, perhaps *in vivo*, also against sensitive components of a cellular community that happens to include the triggering glycosidase. Our experiments with the model system α-D-galactosidase demonstrate that the concept of "camouflaged" killing substrates suggested by Tietze *et al.*[3] could well be applied pharmacologically.

EXPERIMENTAL

Methods. — Melting points are uncorrected. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. All reactions were monitored by t.l.c. on silica gel F_{254} (Merck) using the solvents *A* (1:1 EtOAc–cyclohexane) and *B* (17:2:1 EtOAc–MeOH–H_2O). Preparative column chromatography was performed on silica gel (0.063–0.2 mm, Merck). ^{1}H-N.m.r. spectra were recorded with a Bruker WM 250 spectrometer at 250 MHz in $CDCl_3$ (internal Me_4Si) for acetylated compounds, or D_2O (internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate) for unprotected compounds. Petroleum ether refers to the fraction having b.p. 30–50°.

Enzymic reactions. — α-D-Galactosidase from green coffee-beans (α-D-galactoside galactohydrolase, EC 3.2.1.22) and ADH from Yeast (alcohol NADH oxidoreductase, EC 1.1.1.1) were purchased from Boehringer–Mannheim. NADH (β-nicotinamide adenine dinucleotide, reduced, Na_2salt) was obtained from Serva. All enzymic reactions were carried out at 25° in 0.1M potassium phosphate buffer, pH 6.5.

(R,S)-*1-Ethoxyethyl 2,3,4,6-tetra*-O-*acetyl*-α-D-*galactopyranoside* (**6**). — A solution of 2,3,4,6-tetra-*O*-acetyl-β-D-galactose (**1**; 3.9 g, 11.2 mmol) in acetaldehyde diethyl acetal (25 mL) containing acetic acid (0.2 mL) was refluxed for 2 h, cooled, and evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2 (100 mL), the solution washed successively with saturated aqueous $NaHCO_3$ solution (50 mL), and water (50 mL), dried ($MgSO_4$), and evaporated, and the residue purified by chromatography on a column (8 × 15 cm) with 1:2 EtOAc–cyclohexane, to give syrupy **6** (1.5 g, 31.9%); R_F 0.39 (solvent *A*); for ^{1}H-n.m.r. data, see Table I.

(R,S)-*1-Ethoxyethyl* α-D-*galactopyranoside* (**7**). — Compound **6** (1.4 g, 3.3

TABLE I

250-MHz, ^{1}H-N.M.R. DATA

Proton	*Compound*											
	2	**3**	**4**	**5**	**6** R	S	**7** R	S	**8**	**9**	**10**	**11**	**12**	**13**
H-1	5.38 d	5.37 d	5.25 d	5.25 d	5.38 d/5.41 d		5.21 d/5.11 d		5.42 d	5.41 d	5.20 d	5.18	5.49 d	5.21 d
H-2	5.19 dd	5.19 dd	3.84–3.93 m	3.84–3.92 m	5.34 dd		3.75–4.00 m		5.18 dd	5.18 dd	3.81–3.91 m	3.80–3.91 m	4.03 dd	3.93 dd
H-3	5.37 dd	5.38 dd	3.84–3.93 m	3.84–3.92 m	5.10 dd/5.08 dd		3.75–4.00 m		5.39 dd	5.38 dd	3.81–3.91 m	3.80–3.91 m	5.74 dd	5.71 dd
H-4	5.49 dd	5.50 dd	4.00 d	4.00 dd	5.46 dd		3.75–4.00 m		5.48 dd	5.48 dd	4.01 d	4.01 dd	5.49 dd	5.46 dd
H-5	4.46 dt	4.51 dt	4.09 t	4.12 t	3.41 dt/3.48 dt		3.75–4.00 m/ 4.05 t		4.46 dt	4.45 dt	4.10 t	4.10 t	4.36 dt	4.44 dt
H-6a	4.07 dd	4.08 dd	3.74 d	3.73 d	4.03–4.16 m		3.71 d		4.10 d	4.09 d	3.74 d	3.73 d	4.07 dd	4.09 dd
H-6b	4.14 dd	4.13 dd											4.13 dd	4.14 dd
H-1′	4.84 t	4.71 t	4.97 t	4.73 t	4.94 q/4.93 q		5.01 q/4.95 q		4.76 t	4.73 t	4.92 dd	4.76 dd	4.79 d	4.69 dd
H-2′a	3.42 dd	3.27 dd	3.59 d	3.39 dd	1.36 d/1.32 d		1.38 d/1.36 d		3.41 d	3.24 dd	3.54 dd	3.38 dd	3.50 d	3.45 dd
H-2′b	3.47 dd	3.33 dd		3.42 dd						3.29 dd	3.60 dd	3.42 dd	3.86 dd	3.54 dd
MeO	3.40 s	3.38 s	3.49 s	3.46 s					3.48 s	3.46 s	3.53 s	3.50 s	3.51 s	3.54 s
EtO					3.42–3.87 m 1.14 t/1.15 t		3.63 q/3.59 q 1.19 t							
OAc	2.00 s	2.00 s			1.91–2.15 m				2.00 s	2.00 s			2.07 s	2.06 s
	2.06 s	2.07 s							2.04 s	2.05 s			2.07 s	2.07 s
	2.07 s	2.08 s							2.09 s	2.10 s			2.16 s	2.15 s
	2.15 s	2.15 s							2.16 s	2.17 s				
$J_{H,H}$														
1,2	3.8	3.8	2.0	3.3	3.9/3.9		2.9/2.9		3.6	3.6	2.7	3.0	3.9	3.5
2,3	10.8	10.8			10.8				10.7	10.7			10.5	10.5
3,4	3.3	3.3	1.5	2.6	3.8/3.8				3.2	3.3	1.5	2.7	3.5	3.6
4,5	1.2	1.2	1.2	1.2	1.2				1.2	1.5	1.2	1.0	1.2	1.0
5,6a	6.9	7.0	6.3	6.2	6.8/6.8		6.3		6.3	6.6	6.2	6.0	6.6	6.3
5,6b	5.9	5.7											6.6	6.3
6a,6b	11.4	10.9											12.0	11.3
1′,2′a	5.1	5.3	4.7	4.7	5.3/5.6		5.3/5.6		5.4	5.1	5.7	5.9		8.6
1′,2′b	5.1	5.1		4.4						5.3	4.2	4.5	2.3	3.5
2′a,2′b	10.8	10.8		12.0						10.8	11.0	12.0	12.6	12.0
EtO					7.2		7.1/7.1							

mmol) was deacetylated by the Zemplén method, to yield crystalline **7** from MeOH-Et_2O (735 mg, 87.5%); m.p. 120–124°; R_F 0.20 (solvent *B*); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_{10}H_{20}O_7$: C, 47.61; H, 7.99. Found: C, 47.44; H, 7.81.

(R)- *and* (S)-*2-Bromo-1-methoxyethyl 2,3,4,6-tetra*-O-*acetyl*-α-D-*galactopyranoside* (**2** *and* **8**). — A mixture of compound **1** (20 g, 57.4 mmol), 2-bromoacetaldehyde dimethyl acetal (100 mL), and acetic acid (1 mL) was kept for 20 h at 100°, the alcohol liberated being distilled off during 5 h. Finally, the solution was evaporated *in vacuo*, and the brown residue (40 g) was purified by chromatography on a column (8 × 15 cm) with 1:3 EtOAc–cyclohexane, to give a yellow syrup (22 g), containing mainly **2** and **8** and traces of their β anomers. Separation, especially into uniform (*R*)- and (*S*)-isomers of the α-glycosides, was possible by repeated chromatography on a column (5 × 45 cm) with 1:2 EtOAc–cyclohexane, yielding, first, crystalline **2** from EtOH–H_2O (6.65 g, 23.9%); R_F 0.36 (solvent *A*); m.p. 92.5°, $[\alpha]_D^{22}$ +125.0° (*c* 1.0, $CHCl_3$); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_{17}H_{25}BrO_{11}$: C, 42.08; H, 5.19; Br, 16.47. Found: C, 41.94; H, 5.12; Br, 16.38.

Then, compound **8** was obtained from Et_2O–petroleum ether (4.60 g, 16.5%); m.p. 63–64°, $[\alpha]_D^{22}$ +118.5° (*c* 1.0, $CHCl_3$); R_F 0.39 (solvent *A*); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_{17}H_{25}BrO_{11}$: C, 42.08; H, 5.19. Found: C, 41.81; H, 5.11.

(R)-*2-Bromo-1-methoxyethyl* α-D-*galactopyranoside* (**4**). — Compound **2** (1.0 g, 2.06 mmol) was deacetylated by the Zemplén method, to give crystalline **4** from EtOH (571 mg, 87.4%); m.p. 156°, $[\alpha]_D^{22}$ +139.0° (*c* 1.0, EtOH); R_F 0.26 (solvent *B*); for ^{1}H-n.m.r. data, see Table I. Kinetic parameters were determined[13] to be K_m 19.2mM and V_{max} 29 nmol/mL·min.

Anal. Calc. for $C_9H_{17}BrO_7$: C, 34.09; H, 5.40. Found: C, 34.10; H, 5.44.

(S)-*2-Bromo-1-methoxyethyl* α-D-*galactopyranoside* (**10**). — Compound **8** (1.02 g, 2.10 mmol) was treated as described for compound **2**, to yield **10** (482 mg, 72.3%); m.p. 138–139° (EtOH), $[\alpha]_D^{22}$ +156.0° (*c* 1.0, EtOH); R_F 0.28 (solvent *B*); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_9H_{17}BrO_7$: C, 34.09; H, 5.40. Found: C, 33.89; H, 5.37.

*(6R)- and (6S)-6-Methoxy-(3,4,6-tri-*O-*acetyl*-α-D-*galactopyrano)*-[*1,2*-b]-*1,4-dioxane* (**12** *and* **13**). — Compound **4** (40 mg, 126.1 μmol) and **10** (35 mg, 110.4 μmol) were separately dissolved in Me_3COH (2 mL) and stirred with Me_3COK (30 mg). After 24 h, the mixtures were evaporated to dryness, and the residues acetylated with C_5H_5N (2 mL) and Ac_2O (1 mL). The acetates were isolated conventionally, and purified by chromatography on a column (1 × 10 cm) with 1:3 EtOAc–cyclohexane, to give syrupy **12** (17 mg, 51.3%); R_F 0.38 (solvent *A*) and **13** (22 mg, 53.5%); R_F 0.31 (solvent *A*); for ^{1}H-n.m.r. data, see Table I.

(R)-*2-Iodo-1-methoxyethyl 2,3,4,6-tetra*-O-*acetyl*-α-D-*galactopyranoside* (**3**). — A solution of compound **2** (1.75 g, 3.6 mmol) in cyclohexanone (70 mL) was refluxed with an excess of NaI (5.5 g, 36.7 mmol) for 3 h. After the mixture had

been cooled to room temperature, $CHCl_3$ (100 mL) and H_2O (100 mL) were added. The organic layer was successively washed with H_2O (50 mL), aqueous 5% solution of $Na_2S_2O_3$ (50 mL), and water (50 mL), dried ($MgSO_4$), and evaporated *in vacuo*, to give a brown oil (2.9 g) which was purified by chromatography on a column (7 × 15 cm) with 1:2 EtOAc–cyclohexane. Crystallization from EtOH–H_2O gave pure **3** (680 mg, 35.5%); m.p. 102–103°, $[\alpha]_D^{22}$ +115.5° (*c* 1.0, $CHCl_3$); R_F 0.37 (solvent *A*); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_{17}H_{25}IO_{11}$: C, 38.36; H, 4.73. Found: C, 38.24; H, 4.66.

(S)-*2-Iodo-1-methoxyethyl 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside* (**9**). — Compound **8** (1.2 g, 2.5 mmol) in cyclohexanone (40 mL) was treated with NaI (4.0 g, 26.7 mmol) as described for **3**, to yield crystalline **9** from Et_2O–petroleum ether (690 mg, 52.4%); m.p. 71°, $[\alpha]_D^{22}$ +104.5° (*c* 1.0, $CHCl_3$); R_F 0.40 (solvent *A*); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_{17}H_{25}IO_{11}$: C, 38.36; H, 4.73. Found: C, 38.51; H, 4.73.

(R)-*2-Iodo-1-methoxyethyl α-D-galactopyranoside* (**5**). — Compound **3** (464 mg, 870 μmol) was deacetylated by the Zemplén method, to give crystalline **5** from EtOH (239 mg, 75.3%); m.p. 156°, $[\alpha]_D^{22}$ +116.0° (*c* 1.0, EtOH); R_F 0.28 (solvent *B*); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_9H_{17}IO_7$: C, 29.69; H, 4.71. Found: C, 29.85; H, 4.47.

(S)-*2-Iodo-1-methoxyethyl α-D-galactopyranoside* (**11**). — Compound **9** (685 mg, 1.29 mmol) was deacetylated as described for compound **5**, to give **11** from Me_2CO (326 mg, 69.4%); m.p. 126°, $[\alpha]_D^{22}$ +138.5° (*c* 1.0, EtOH); R_F 0.29 (solvent *B*); for ^{1}H-n.m.r. data, see Table I.

Anal. Calc. for $C_9H_{17}IO_7$: C, 29.69; H, 4.71. Found: C, 29.71; H, 4.60.

ACKNOWLEDGMENTS

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