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Note

Separation and quantitation of enantiomeric galactoses and their mono-*O*-methylethers as their diastereomeric acetylated 1-deoxy-1-(2-hydroxypropylamino) alditols

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The assignment of the D or L configuration to the sugars that form a polysaccharide is usually carried out by the methods of Gerwig et al. [1] and Leontein et al. [2], in which the separation of enantiomeric monosaccharides is performed after glycosidation with chiral alcohols (2-octanol or 2-butanol), acetylation or trimethylsilylation, and separation of the diastereomers thus formed by gas–liquid chromatography (GLC). When using this glycoside approach, each monosaccharide-specific pattern directly reflects its purity. Today this method is the usual form of determining configurations, overriding the previous methods using either optical rotation determination of the hydrolyzate or enzymic methods. However, drawbacks of the method are the low volatility of 2-octanol and the chromatograms complicated by the presence of different anomers and ring sizes for each sugar. The chromatograms can be quite complex when several monosaccharides are present.

Many seaweed polysaccharides contain galactose and its mono-*O*-methyl derivatives in each one of the four available positions [3]. Each of them may belong to the D- or L-series. For their study, optical rotation determination gives an approximate D:L ratio for all galactoses [3]. Enzymic methods may be inappropriate, as the activity of

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galactose oxidase with methylated sugars is unknown. Chromatographic methods [1,2] using glycosides would possibly lead to signal overlap, as galactose and its monomethylated derivatives usually give closely-eluting chromatographic peaks. We report their GLC separation after reductive amination with chiral 1-amino-2-propanol, followed by acetylation. The procedure resolves all enantiomeric pairs, but that of 2-*O*-methylgalactose, which can be separated if the amine is replaced by α-methylbenzylamine [4]. The technique is both simple and reliable, and it may be considered as a routine method of derivatization (at least, for the sugars studied here) even if there are no suspicions of rare enantiomers.

Condensation of D-galactose with racemic 1-amino-2-propanol and further reduction gave the same amounts of both diastereomers (shown by ^{13}C NMR spectroscopy of the final mixture), together with an excess of the amino alcohol. This is in contrast with the result obtained with racemic α-methylbenzylamine, for which one of the diastereomers clearly prevails (see later). Capillary gas chromatography of the acetylated derivatives of those 1-deoxy-1-(2-hydroxypropylamino)alditols showed two resolved peaks whose structures were confirmed by GLC–MS. The parameters of the reaction were optimized as follows. (*a*) An amine:sugar initial ratio of 5:1 gave the highest yields of products. (*b*) The amount of product reaches a maximum at pH 4. The results for an unbuffered solution are close to those for pH 6–7. (*c*) Lowering the polarity increases the yield; in an anhydrous methanol medium, the yield of the amino alditols is the highest (~ 85%). (*d*) An excess of $NaBH_3CN$ greater than 10% (with respect to galactose) decreases the yield of the reaction. (*e*) The addition of glacial acetic acid [5] to the anhydrous methanol medium produces a quantitative yield of products. (*f*) Working from 65 to 85°C from 1 to 4 h, or at room temperature for 24 h, the yield of product was nearly constant (85–100%).

Therefore, a temperature of 65°C and a 1-h reaction time were chosen as the standard conditions, using a molar ratio of amine:sugar:$NaBH_3CN$:AcOH of 5:1:1.1:10 in methanol (see Experimental).

Table 1
Retention times [a] and separation factors (*r*) of acetylated aminoalditols originated from enantiomeric sugars derivatized with (*S*)-1-amino-2-propanol with different chromatographic programs

	Ultra-2 (Program A)			Ultra-2 (Program B)			HP-5		
	D	L	*r*	D	L	*r*	D	L	*r*
Rhamnose	1.644	1.653	1.005	1.738	1.750	1.007			
Fucose	1.656	1.667	1.006	1.757	1.770	1.007			
Arabinose	1.694	1.686	1.005	1.803	1.792	1.006			
Xylose	1.720	1.729	1.006	1.840	1.854	1.008	1.806	1.818	1.007
Glucose	2.289	2.294	1.002	2.605	2.609	1.002			
Mannose	2.291	2.304	1.006	2.598	2.612	1.005			
Galactose	2.341	2.356	1.007	2.642	2.659	1.006	2.515	2.530	1.006
2-*O*-Methyl-Gal	2.103		1						
3-*O*-Methyl-Gal	2.234	2.250	1.007				2.399	2.415	1.007
4-*O*-Methyl-Gal	2.214	2.225	1.005	2.503	2.519	1.006	2.379	2.396	1.006
6-*O*-Methyl-Gal	1.995	2.006	1.006				2.121	2.133	1.006

[a] Relative to peracetylated *myo*-inositol = 1 (20.95 min with Program A; 19.40 min with Program B; 14.28 min on the HP-5 column).

The method was applied condensing (*S*)-1-amino-2-propanol (and the racemic mixture) to D-galactose and its mono-*O*-methylethers. Comparing the results obtained with the chiral amino alcohol and the racemic mixture, the GLC retention times of the derivatives of both enantiomers were assigned, those produced by the *R* amino alcohol being equivalent to those produced by the enantiomer of the sugar with the *S* amino alcohol. Results using different columns are shown in Table 1. The derivatives of the enantiomeric pairs of 3-, 4-, and 6-*O*-methylgalactose give baseline resolution, but that of 2-*O*-methylgalactose is not separated. Table 1 also shows the results obtained with other commonly occurring sugars. Only separation factors larger than 1.004 allow safe quantitation. Either the Ultra-2 or the HP-5 columns separate almost all enantiomers; for glucose, baseline resolution is impossible to achieve. Besides, some peaks overlap: the derivative of 4-*O*-methyl-L-galactose with that of 3-*O*-methyl-D-galactose, those of L-rhamnose and D-fucose, and the derivative of D-mannose with those of glucose. In all cases but arabinose, the derivative of the D-sugar appears earlier than that of the L-sugar. The detector response was found to be approximately proportional to the amount of product, when compared to similar derivatives. However, the areas of the peaks of these derivatives is only about half that shown by an equimolar amount of inositol. As shown for galactose, with other sugars enantioselectivity did not occur. A racemic mixture of amino alcohols yielded with any sugar two, near-equal chromatographic peaks for both diastereomers (area ratio 0.9–1.1).

As the 2-*O*-methylgalactose enantiomeric pair was not separated at all, and as this sugar is an important constituent of seaweed polysaccharides [3], the same method was applied using (*S*)-α-methylbenzylamine [4] as the chiral amine. Table 2 shows the results. The interpretation of experimental data using this amine is complicated by the appearance of enantioselectivity, which is especially marked for galactose, thus precluding its quantitation. Otherwise, sugars with the *manno* configuration are resolved much

Table 2
Retention times [a], separation factors (r) and enantioselectivity ratio ($r_{D/L}$)[b] of acetylated aminoalditols originated from enantiomeric sugars derivatized with (*S*)-α-methylbenzylamine using the Ultra-2 column (Program C)

	D		L	r	$r_{D/L}$
Rhamnose	2.377		2.316	1.026	0.9
Fucose	2.343		2.365	1.009	1.6
Arabinose	2.389		2.375	1.006	0.7
Xylose	2.440		2.448	1.003	1.5
Glucose		3.109		1	
Mannose	3.107		3.048	1.019	0.7
Galactose	3.094		3.114	1.006	2.3
2-*O*-Methyl-Gal	2.833		2.867	1.012	1.0
3-*O*-Methyl-Gal		3.009		1	
4-*O*-Methyl-Gal	2.953		2.968	1.005	3.1
6-*O*-Methyl-Gal	2.728		2.748	1.007	1.8

[a] Relative to peracetylated *myo*-inositol = 1 (20.60 min).
[b] Area ratio of the peaks corresponding to both enantiomers.

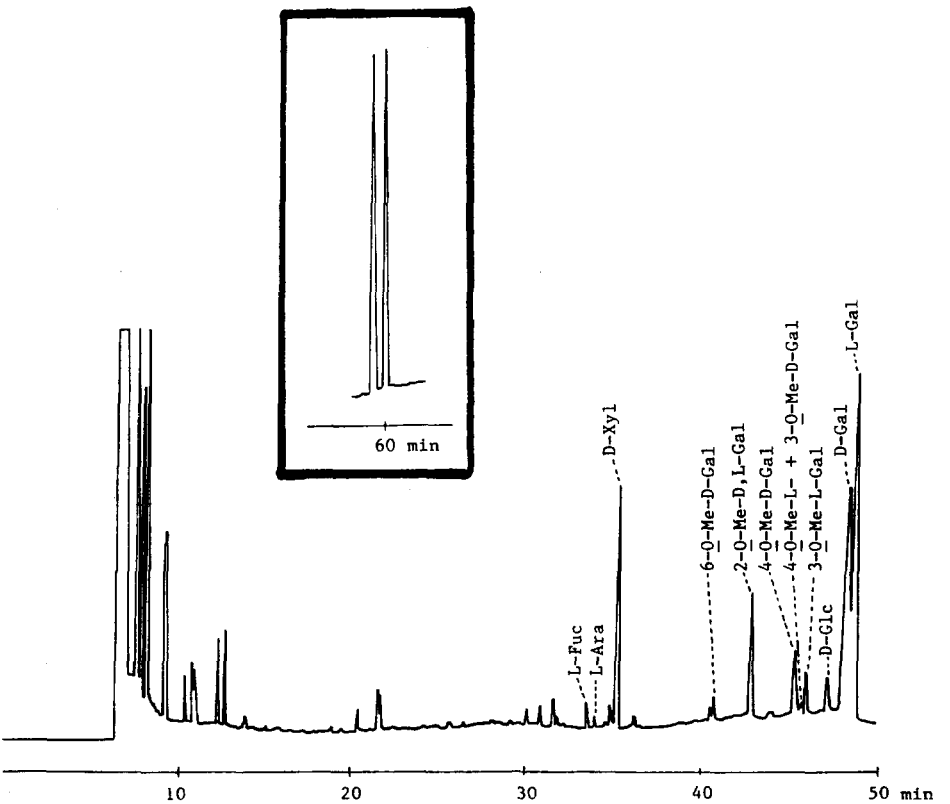

Fig. 1. Gas chromatogram (Ultra-2 column, Program A) of a hydrolyzate of *Corallina officinalis* polysaccharide (fraction 4I) derivatized with (*S*)-1-amino-2-propanol. Inset: gas chromatogram of the products of reaction of 2-*O*-methyl-D-galactose and racemic α-methylbenzylamine. The first peak corresponds to the reaction of the D-sugar and (*S*)-amine, while the second is equivalent to that of the L-sugar and the (*S*)-amine.

better than with the previous amine [4]. In this case either the derivatives of *manno* sugars or arabinose with the L configuration appear before those of the D configuration. Fortunately, the 2-*O*-methylgalactose enantiomers are resolved excellently with this amine, and may be safely quantified as shown by the equal response of each enantiomer (Table 2 and Fig. 1, inset).

The method was also applied to hydrolyzates of the fractions of the polysaccharide from *Corallina officinalis*, which carry galactoses monomethylated at the four available positions [3]. Fig. 1 shows a typical chromatogram and Table 3 shows the results obtained, compared to those using the acetylated aldononitriles [6] and alditols [7]. The general pattern is the same; however, it should be noted that the amounts of mono-*O*-methylgalactoses (especially on 2-*O* and 3-*O*) determined by the use of aldononitrile acetates are higher than those obtained using the alditol acetates. The use of the chiral amines yields even lower values. In a previous paper [8], we have noted that the use of aldononitrile acetates overestimated the proportions of 2- and 3-*O*-methylgalactose; the

values obtained by the use of the chiral amine (Table 3) are more compatible with the results obtained by ethylation and desulfation [8]. It is shown that, as expected, xylose and 6-*O*-methylgalactose belong to the D-series, and 2- and 3-*O*-methylgalactose belong to the L-series (only traces of 2- and possibly 3-*O*-methyl-D-galactose appear). However, unexpectedly [8], most of the 4-*O*-methylgalactose appearing in the polysaccharide belongs to the D-series; previous appearances of this sugar were assigned either by determination [9,10] or by analogy with previous work [11] to the L-series. Table 3 shows that for most of the fractions, the ratio of D- to L-galactoses (including methylated units) is ca. 1, as expected from an alternating structure [8].

The preparation of the acetylated 1-deoxy-1-(2-hydroxypropylamino)alditols is as easy as that of the acetylated aldononitriles and even easier than that of the acetylated alditols, which are usual choices to quantify the monosaccharides which constitute a polysaccharide. The method permits working with very small amounts of polysaccharide ($\sim$ 1 mg). In most of the papers, the configuration of sugars is assumed to be the usual one (D-glucose, D-xylose, L-fucose, etc.) without analysis. Because of peak overlap, in some cases this method cannot be used alone for determining both composition and configurations. In these circumstances it can give the configurations of a mixture of sugars the composition of which is deduced by classical methods.

1. Experimental

Recommended procedure.—To a known amount of polysaccharide hydrolyzate or sugar standard (1–5 mg) contained in a vial, the following solutions were added: (*a*) 1:8 (*S*)-1-amino-2-propanol–MeOH (20 μL of solution/mg sugar); (*b*) 1:4 glacial AcOH–MeOH (17 μL solution/mg sugar); and (*c*) 3% $NaBH_3CN$ in MeOH (13 μL/mg sugar). The vial was capped, and the mixture was allowed to react for 1–2 h at 65°C. After cooling, 3 M aq CF_3CO_2H was added dropwise (under the fume hood) until the pH dropped to pH 1–2. The mixture was evaporated and further coevaporated with water (3×0.5 mL) and MeOH (5×0.5 mL). The residue was dried overnight in a dessicator and treated with 1:1 pyridine–Ac_2O for 0.75 h at 100°C. After cooling, the derivatives were extracted with $CHCl_3$ and washed with water (3×1 mL) and satd $NaHCO_3$ (3×1 mL). The organic phase was dried with anhyd Na_2SO_4 and injected onto the GLC column.

To prepare the derivative with α-methylbenzylamine, the previous procedure was modified by replacing solution (*a*) with 32 μL of 1:8 (*S*)-α-methylbenzylamine–MeOH/mg sugar.

GLC was carried out with a Hewlett–Packard 5890A gas–liquid chromatograph equipped with a flame-ionization detector operating in the split mode (split ratio ca. 100:1) using a Hewlett–Packard Ultra-2 column (50 m $\times$ 0.2 mm; thickness of liquid phase, 0.11 μm), N_2 as the gas carrier (0.65 mL/min), and a head pressure of 20 psi. Program A started at 180°C, 4°C/min to 220°C (2 min), and then 1°C/min to 250°C (10 min). Program B also started at 180°C, 4°C/min to 230°C (20 min), and then 1°C/min to 250°C (10 min). Program C (used for the aromatic derivatives) started at 180°C, 4°C/min to 220°C (2 min), and then 1°C/min to 270°C (5 min). An HP-5 column (50

Table 3
Analysis of the polysaccharides fractionated from the red seaweed *Corallina officinalis* (expressed in residues/100 sugar residues)

Fraction	1I				2I				3I				4I				4II			
Method [a]	AN	AA	DHP		AN	AA	DHP		AN	AA	DHP		AN	AA	DHP		AN	AA	DHP	
Rha	6	4	5	L	2	1	3	L		tr.	tr.	L	tr.	tr.	tr.	L		tr.	tr.	L
Fuc	6	5	9	L	3	2	2	L		tr.	1	L	1		1	L			tr.	L
Ara	3		1	L											tr.	L				
Xyl	8	9	9	D	11	14	13	D	14	11	12	D	15	15	15	D	22	19	21	D
Man	22	19	38 [b]	D	3	3	9 [b]	D		1	7 [b]	D	tr.	tr.	3 [b]	D		tr.	1 [b]	D
Glc	15	18	[b]		7	9	[b]		7	11	[b]		2	5	[b]			1	[b]	
Gal	23	32	16	D	43	49	27	D	47	54	27	D	54	60	35	D	56	63	39	D
			12	L			25	L			30	L			28	L			22	L
2-MeGal	9	7		D	18	15		D	22	14	tr.	D	16	12	tr.	D	16	12		D
			5	L			10	L			11	L			9	L			9	L
3-MeGal	3	[c]	1 [d]	D	3	[c]	1 [d]	D	2	[c]	1 [d]	D	4	[c]	1 [d]	D	5	[c]	1 [d]	D
			1	L			2	L			1	L			3	L			3	L
4-MeGal	2	4 [c]	2	D	6	7 [c]	5	D	9	8 [c]	8	D	7	6 [c]	4	D	1	4 [c]	2	D
			[d]	L			[d]	L			[d]	L			[d]	L			[d]	L
6-MeGal	2	2	2	D	3	1	2	D	tr.	1	1	D	tr.	1	1	D	tr.	1	1	D

Fraction	5I				5II				5III				6I			
Method [a]	AN	AA	DHP		AN	AA	DHP		AN	AA	DHP		AN	AA	DHP	
Rha		tr.	tr.	L			tr.	L			tr.	L		tr.	tr.	L
Fuc					1		tr.	L	1	1	1	L	2	1	1	L
Ara			tr.	L												
Xyl	23	24	24	D	24	24	25	D	17	18	21	D	15	15	16	D
Man		tr.	1 [b]	D		tr.	tr. [b]	D	1	tr.	3 [b]	D	tr.	1	2 [b]	D
Glc	1	2	[b]			tr.	[b]		1	2	[b]		tr.	1	[b]	
Gal	55	58	37	D	53	62	39	D	55	62	38	D	53	62	37	D
			23	L			24	L			23	L			25	L
2-MeGal	14	11	tr.	D	17	11		D	18	13		D	20	15		D
			7	L			8	L			9	L			11	L
3-MeGal	5	[c]	1 [d]	D	4	[c]	tr. [d]	D	4	[c]	tr. [d]	D	3	[c]	1 [d]	D
			3	L			3	L			2	L			2	L
4-MeGal	1	4 [c]	1	D	tr.	2 [c]	1	D	1	3 [c]	1	D	2	3 [c]	2	D
			[d]	L			[d]	L			[d]	L			[d]	L
6-MeGal		1	1	D		1	1	D	3	2	2	D	4	3	3	D

[a] Method: AN = acetylated aldononitriles; AA = acetylated alditols; DHP = acetylated 1-deoxy-1-(2-hydroxypropylamino)alditols (the configuration of 2-*O*-methylgalactose was determined using α-methylbenzylamine).

[b] The peaks corresponding to the derivative of D-mannose and glucose overlap.

[c] The alditol of 3- and 4-*O*-methylgalactose are indistinguishable.

[d] The peaks corresponding to the derivatives of 3-*O*-methyl-D-galactose and 4-*O*-methyl-L-galactose overlap.

m × 0.32 mm; thickness of liquid phase, 0.17 μm) was also used. N_2 was the gas carrier (2.3 mL/min), operating with a split ratio of 80:1 and a head pressure of 15 psi. The oven temperature was raised as stated in program A.

Standards.—L-Rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-glucose, and D-galactose were commercial standards. 3-*O*-, 4-*O*-, and 6-*O*-methyl-D-galactose were kindly provided by Dr. E.G. Gros. 2-*O*-Methyl-D-galactose was synthesized from methyl α-D-galactopyranoside through its 4,6-*O*-benzylidene derivative [12] by methylation using limiting amounts of sodium methylsulfinylmethanide.

^{13}C NMR spectroscopy.—The reaction products of both racemic amines with D-galactose were subjected to ^{13}C NMR spectroscopy. Spectra were recorded in 1:1 D_2O–H_2O solutions with a Bruker AC 200 spectrometer operating at 50.3 MHz. Chemical shifts were measured relative to dioxane as external standard and expressed as $\delta = \delta_{dioxane} + 67.4$ ppm.

For (2′*S*)-1-deoxy-1-(2′-hydroxypropylamino)-D-galactitol, the chemical shifts and putative assignments [13] are as follows: δ 71.5 (C-3), 70.9 (C-5), 70.3 (C-4), 66.4 (C-2), 64.1 (C-6), 64.0 (C-2′), 54.6 (C-1), 51.7 (C-1′), and 20.7 (C-3′). The mixture of both diastereomers show splitting (~ 0.1 ppm) of the signals at 71.5, 64.0, and 51.7 ppm. (*S*)-1-Amino-2-propanol shows signals at δ 64.9 (C-2), 46.6 (C-1), and 20.5 (C-3).

GLC–MS.—The reaction product of racemic 1-amino-2-propanol with D-galactose after reduction and acetylation was chromatographed on an HP 5890A gas chromatograph equipped with an HP-5 column (see above) interfaced to a Trio-2 VG Masslab mass spectrometer working at 70 eV. He was used as carrier gas. The products showed mass spectra with characteristic peaks: *m/z* (%) 446 (3, $[M - CH_3CHOAc]^+$), 404 (20, $[446 - CH_2CO]^+$), 344 (5, $[404 - AcOH]^+$), 172 (38, $[M - CH_2OAc - (CHOAc)_4]^+$), 130 (100, $[172 - CH_2CO]^+$) among others.

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