Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 85–93
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

The absolute configuration of 1-carboxyethyl substituents on common hexoses by circular dichroism

Lars Andersson, Lennart Kenne*

Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, SE-750 07 Uppsala, Sweden

Received 11 July 2002; accepted 23 September 2002

Abstract

Determination of the absolute configuration of the 1-carboxyethyl substituent on a monosaccharide by circular dichroism measurements was found to be a sensitive and simple method. It relies on comparison of the spectrum of a 1-carboxyethyl substituted sugar or sugar derivative with the spectra of (*R*)- and (*S*)-lactic acid in the region 200–260 nm in which the (*R*)- and (*S*)-configuration give negative and positive $\Delta\varepsilon$, respectively. The oligo- or poly-saccharide containing a 1-carboxyethyl substituted sugar is hydrolyzed to monomers and the 1-carboxyethyl substituted sugar isolated by chromatography. The CD spectrum obtained for the 1-carboxyethyl substituted sugar in water solution at pH 2 is then compared with spectra of (*R*)- and (*S*)-lactic acid. The sign for the absorption and a maximum of comparable intensity and appearance around 210 nm, identify the stereochemistry. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: 1-Carboxyethyl substituent; Circular dichroism; Absolute configuration

1. Introduction

At our laboratory, we are working with structure determination of bacterial polysaccharides from several strains of the anaerobic bacterium *Butyrivibrio fibrisolvens*. Some of these strains contain 1-carboxyethyl substituted monosaccharides, not fully characterized with respect to position and absolute configuration of the 1-carboxyethyl substituent.[1] Traditionally the absolute configuration of 1-carboxyethyl substituents is determined by comparison of the monosaccharide derivatives, released by acid hydrolysis of the native or reduced polysaccharide, with synthesized reference substances or with material isolated from already determined sources. An NMR method based on NOE measurements of the acetylated 6-membered lactone derivatives of 1-carboxyethyl substituted monosaccharides has also been described.[2]

There are methods available to determine the absolute configuration of α-hydroxy acid derivatives. These methods are based on either NMR[3–5] or CD[6,7] studies of synthesized derivatives of these acids. The CD methods are applicable only to α-hydroxy acids with free hydroxyl groups and not practically useful when the hydroxy group is substituted as is the case for 1-carboxyethyl substituted monosaccharide derivatives. In the NMR based methods, the carboxylic acid is reacted with both isomers of a chiral aromatic alcohol or amine to yield the corresponding ester or amide derivatives, respectively. The anisotropic shifts observed in the ^{1}H NMR spectrum induced by the aromatic ring are used for interpretation of the stereochemistry. The interpretation is only valid if the rotamer population around the carbonyl group is accurately predicted by calculation.

CD measurements of muramic and isomuramic acid (pH 2.5) have shown that the spectrum of (*R*)-lactic acid is similar to that of muramic acid (2-amino-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose) and that the same is true for the spectra of (*S*)-lactic acid and isomuramic acid (2-amino-3-*O*-[(*S*)-1-carboxyethyl]-2-deoxy-D-glucose).[8] The findings indicated that the absolute configuration of the 1-carboxyethyl substituent was correlated to the sign of the CD maximum for the carboxyl chromophore with absorption at ~210 nm.

* Corresponding author. Tel.: +46-18-671573; fax: +46-18-673477
E-mail address: lennart.kenne@kemi.slu.se (L. Kenne).

PII: S0008-6215(02)00352-X

When CD spectra were acquired for the sodium salt of some synthesized 1-carboxyethyl substituted methyl glycosides in water solution[9] and compared with the spectrum of sodium (*S*)-lactate, differences between the (*R*)- and (*S*)-isomers were observed but the spectral differences in comparison with sodium (*S*)-lactate were considerable. No clear correlation between the stereochemistry and the spectra could be found.

In this study, a comparison of CD spectra acquired at pH 2 from five different (*R*)/(*S*)-pairs of 1-carboxyethyl substituted monosaccharides and their corresponding methyl glycoside and alditol derivatives with those of (*R*)- and (*S*)-lactic acid is presented. The results prompted the development of a procedure, based on CD, for the determination of the absolute configuration of the 1-carboxyethyl substituents.

2. Results and discussion

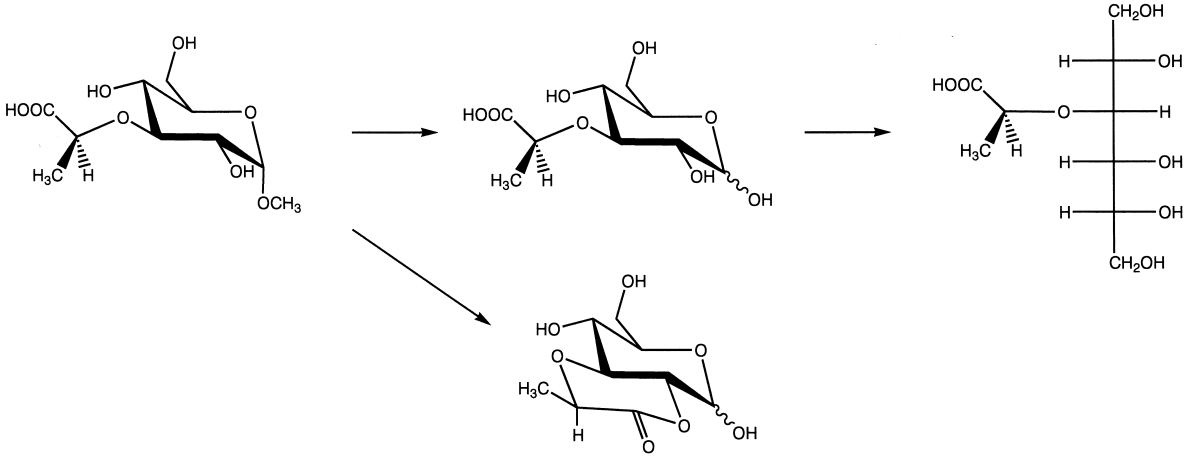
All 1-carboxyethyl substituted methyl glycosides used in this study were available from previous studies.[9,10] The compounds were methyl 3-*O*-[(*R*)-1-carboxyethyl]-α-D-Glc*p*, methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-Glc*p*, methyl 3-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*, methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-Gal*p*, methyl 3-*O*-[(*R*)-1-carboxyethyl]-α-D-Man*p*, methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-Man*p*, methyl 4-*O*-[(*R*)-1-carboxyethyl]-α-L-Rha*p*, methyl 4-*O*-[(*S*)-1-carboxyethyl]-α-L-Rha*p*, methyl 6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p* and methyl 6-*O*-[(*S*)-1-carboxyethyl]-α-D-Gal*p*.

Reducing sugars of all [(*R*)- and (*S*)-1-carboxyethyl]-substituted saccharides were prepared from the parent methyl glycosides (Scheme 1) by two different methods of acid hydrolysis commonly used for component analysis, 1 M H_2SO_4 at 100 °C and 2 M trifluoroacetic acid (TFA) at 120 °C. The corresponding alditols were prepared from the hydrolyzed material by reduction with $NaBH_4$ (Scheme 1). After acidification by Dowex 50 (H^+) and freeze-drying, boric acid was removed as methyl esters by evaporation. The results of the reactions were followed by ^1H NMR spectroscopy and the molecular masses of the products determined by ESIMS.

Lactones were extensively formed on the 1-carboxyethyl substituted sugars during the evaporation following the TFA hydrolysis (Scheme 1). Treatment of the lactone mixtures with NH_4OH (pH ~10) resulted in hydrolysis of the lactones without detectable amide formation and the 1-carboxyethyl substituted sugar was isolated as the ammonium salt. When alditol derivatives were prepared from the TFA hydrolyzed material, lactones were opened as described above before the addition of $NaBH_4$ to avoid reduction of the lactone. The yield of 1-carboxyethyl substituted alditol was quantitative as judged by thin layer chromatography (TLC).

When 1 M H_2SO_4 was used for the hydrolysis of the 1-carboxyethyl substituted methyl glycosides a different work-up procedure was used. The reactions were neutralized with $BaCO_3$ and centrifuged before the supernatant was lyophilized. Lactones were not formed under these conditions and the $NaBH_4$ reduction could be performed directly after dissolution in H_2O. The work-up procedure after reduction is common for the two routes and involves acidification with an ion-exchange resin, lyophilization and evaporation from methanol to remove the remaining boric acid as methyl esters. Lactones were formed on the alditols during this procedure and a base treatment (NH_4OH, pH ~10, 1 h) was necessary to give, after lyophilization, the 1-carboxyethyl substituted alditol derivatives as ammonium salts. Both methods of hydrolysis gave products of >90% purity and satisfactory CD spectra.

CD spectra were recorded for the 1-carboxyethyl substituted derivatives as well as for (*R*)- and (*S*)-lactic



Scheme 1.

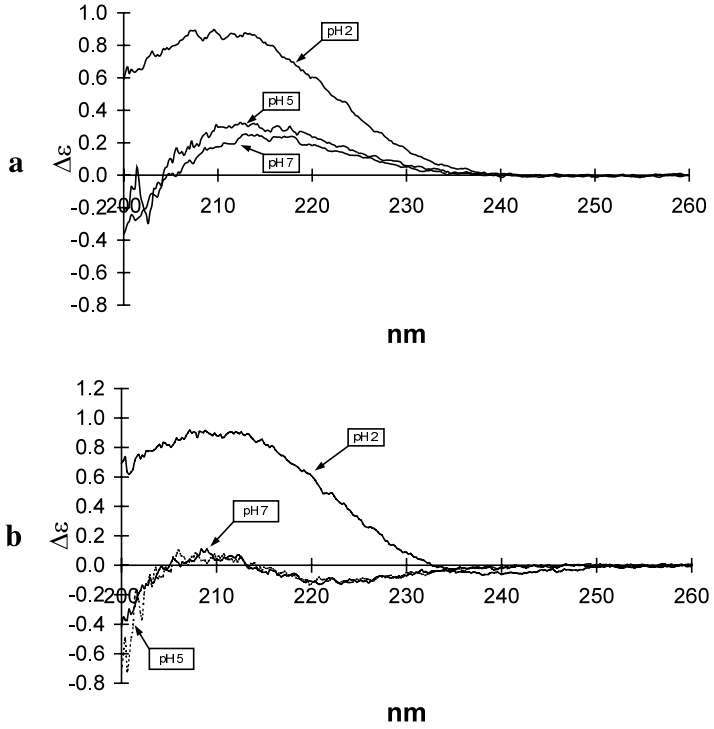


Fig. 1. (a) CD spectra of (*S*)-lactic acid in water solution at pH 2, 5 and 7. (b) CD spectra of methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-Gal*p* in water solution at pH 2, 5 and 7.

acid. At pH 2, the spectrum of lactic acid showed a strong ellipticity band ($\Delta\varepsilon$ 0.879) centered at $\sim$210 nm (Fig. 1(a)). The ellipticity is negative for the (*R*)-isomer and positive for the (*S*)-isomer. When the CD spectrum was obtained at pH 10, the maximum shifted to $\sim$214 nm with $\Delta\varepsilon$ 0.261. These measurements are in good agreement with results from previous studies although the reported weak ellipticity band at $\sim$240 nm[8,11,12] could not be observed in our measurements, probably due to more noise in the spectra obtained at the low sample concentrations used.

The CD measurements of the 1-carboxyethyl substituted sugar derivatives showed that the spectra of 1-carboxyethyl ethers are more affected than lactic acid by changes of pH. At pH 2, the spectra are very similar to that of lactic acid, whereas at pH >3 they differ (Fig. 1(b)). When spectra were acquired at pH 2, the sign of the ellipticity in the region $\sim$200–235 nm was negative for (*R*)-isomers and positive for (*S*)-isomers of all the measured (1-carboxyethyl)-substituted methyl glycosides, hexoses and alditols with a maximum absorbance close to 210 nm (Table 1 and Fig. 2). A small maximum with opposite sign is also visible at $\sim$240 nm for most samples. It has been suggested that the 240 nm transition has the same origin ($n\rightarrow\pi^*$) as the 210 nm transition and that a pH-dependent difference in rotamer population around C-1–C-2 of the 1-carboxyethyl group is responsible for the observed changes in the intensities of the peaks. In protonated acids, there is one predominant rotamer around the C-1–C-2 bond while in the deprotonated samples there is no such predominant rotamer.[8,11–13]

When pH was changed from 2 to 5 (close to pK_a of the acids), a sharp increase in the intensity of the 240 nm transition in the CD spectra of the 1-carboxyethyl substituted sugar derivatives occurred. A comparison of the spectra of the acidic and the ionized form of methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-Gal*p* (Fig. 1) showed that the intensity of the maximum at $\sim$210 nm decreased by $>90\%$ while the maximum with opposite sign at 240 nm gained in intensity and moved to $\sim$222 nm for the ionized form. This behavior is not observed in the CD spectrum of sodium lactate and lactic acid, where the 240 nm transition is unaffected while the 210 nm transition is moved to $\sim$214 nm and decreased by $\sim$70%. Hence, the distribution of rotamers in the 1-carboxyethyl substituted sugar derivatives seems to be more affected by changes in pH compared to that in lactic acid.

Previous CD studies of α-hydroxy acids and their derivatives showed that, in the CD spectra the magnitude and wavelength of the ellipicity maxima are influenced by changes in temperature, solvent and pH. The effect of pH on the CD spectrum is large in the region around the pK_a of the acid and it is preferable to avoid this pH range (± 2 units from pK_a) when spectra of several samples should be compared.

In the CD spectra for some of the studied 1-carboxyethyl substituted sugar derivatives recorded at pH 2, the maximum deviated significantly from the expected 210 nm which is observed in the CD spectrum of 3-*O*-[(*S*)-1-carboxyethyl]-D-glucitol (Fig. 3). TLC and ^{1}H NMR indicated new species in the sample but, when base was added to the sample solution, 3-*O*-[(*S*)-1-carboxyethyl]-D-glucitol was recovered. These observations indicated that lactones were formed upon acidification of the samples, resulting in mixtures of CD active species making interpretation of the spectra uncertain. Treatment of the sample with NH_4OH (pH 10, 1 h) and adjustment to pH 2 (HCl) just before measurement resulted in a CD spectrum similar to that of lactic acid.

The CD spectra of two lactones, methyl 3-*O*-[(*R*)-1-carboxyethyl]-α-D-glucopyranosid-1′,2-lactone and methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-galactopyranosid-1′,2-lactone,[14] were acquired using the same conditions as for the other samples. The results (Fig. 4) showed that both lactones gave a maximum at 223 nm which is close to the ~226 nm that was observed for 3-*O*-[(*S*)-1-carboxyethyl]-D-glucitol.

Our and previously published data[8,11,12] indicate that the dominating ellipticity band should occur at ~210

Table 1
Circular dichroism data at pH 2 of lactic acid and 1-carboxyethyl substituted monosaccharide derivatives[a] in the range 200–260 nm

Compound	Ellipticity maximum		
	λ (nm)	$\Delta\varepsilon$ (deg cm^2/dmol)	Figure no.
(*R*)-Lactic acid	212	−0.8	2a
(*S*)-Lactic acid [b]	210	0.884	1a, 2a
3-*O*-[(*R*)-1-Carboxyethyl]-D-Glc	209; 242	−1.1; 0.01	2a
3-*O*-[(*S*)-1-Carboxyethyl]-D-Glc	211; 242	1.0; −0.02	2a
3-*O*-[(*R*)-1-Carboxyethyl]-D-Gal	209; 242	−0.9; 0.03	2a
3-*O*-[(*S*)-1-Carboxyethyl]-D-Gal	211; 240	0.9; −0.04	2a
3-*O*-[(*R*)-1-Carboxyethyl]-D-Man	210; 238	−0.8; 0.05	2a
3-*O*-[(*S*)-1-Carboxyethyl]-D-Man	209; 240	0.8; −0.03	2a
6-*O*-[(*R*)-1-Carboxyethyl]-D-Gal	209; –	−0.8; –	
6-*O*-[(*S*)-1-Carboxyethyl]-D-Gal	211; 241	0.9; −0.02	
4-*O*-[(*R*)-1-Carboxyethyl]-L-Rha	211; –	−1.0; –	
4-*O*-[(*S*)-1-Carboxyethyl]-L-Rha	208; 236	0.4; -0.07	
Me 3-*O*-[(*R*)-1-carboxyethyl]-α-D-Glc*p*	208; 237	−0.8; 0.06	2b
Me 3-*O*-[(*S*)-1-carboxyethyl]-α-D-Glc*p*	210; 239	1.0; −0.02	2b
Me 3-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*	208; 235	−0.8; 0.05	2b
Me 3-*O*-[(*S*)-1-carboxyethyl]-α-D-Gal*p* [b]	210; 239	0.909; −0.052	1b, 2b
Me 3-*O*-[(*R*)-1-carboxyethyl]-α-D-Man*p*	210; 240	−0.7; −0.04	2b
Me 3-*O*-[(*S*)-1-carboxyethyl]-α-D-Man*p*	210; 239	0.7; −0.03	2b
Me 4-*O*-[(*R*)-1-carboxyethyl]-α-L-Rha*p*	210	pos; n.d.	
Me 4-*O*-[(*S*)-1-carboxyethyl]-α-L-Rha*p*	209; 236	neg; n.d.	
Me 6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*	211	pos; n.d.	
Me 6-*O*-[(*S*)-1-carboxyethyl]-α-D-Gal*p*	211; 242	0.8; −0.02	
3-*O*-[(*R*)-1-Carboxyethyl]-D-glucitol	212	−0.9	2c
3-*O*-[(*S*)-1-Carboxyethyl]-D-glucitol	210; 240	0.9; −0.06	2c, 3
3-*O*-[(*R*)-1-Carboxyethyl]-D-galactitol	211; 242	−0.9; 0.08	2c
3-*O*-[(*S*)-1-Carboxyethyl]-D-galactitol	211; 240	1.1; −0.04	2c
3-*O*-[(*R*)-1-Carboxyethyl]-D-mannitol	211; 239	−0.6; 0.07	2c
3-*O*-[(*S*)-1-Carboxyethyl]-D-mannitol	210; 240	1.5; −0.08	2c
6-*O*-[(*R*)-1-Carboxyethyl]-D-galactitol	209	−0.6	
6-*O*-[(*S*)-1-Carboxyethyl]-D-galactitol	211; 240	0.9; −0.05	
4-*O*-[(*R*)-1-Carboxyethyl]-L-rhamnitol	211	−0.5	
4-*O*-[(*S*)-1-Carboxyethyl]-L-rhamnitol	212	0.5	
Me 2-lac-3-*O*-(*R*)-Glc*p* [c]	223	−1.2	4
Me 2-lac-3-*O*-(*S*)-Gal*p*	223	−2.5	4

[a] Sample amount estimated from ^{1}H NMR (±20%).
[b] Higher accuracy due to carefully weighted amount used for the measurement.
[c] Me 2-lac-3-*O*-(*R*)-Glc*p* = methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-galactopyranosid-1′,2-lactone.

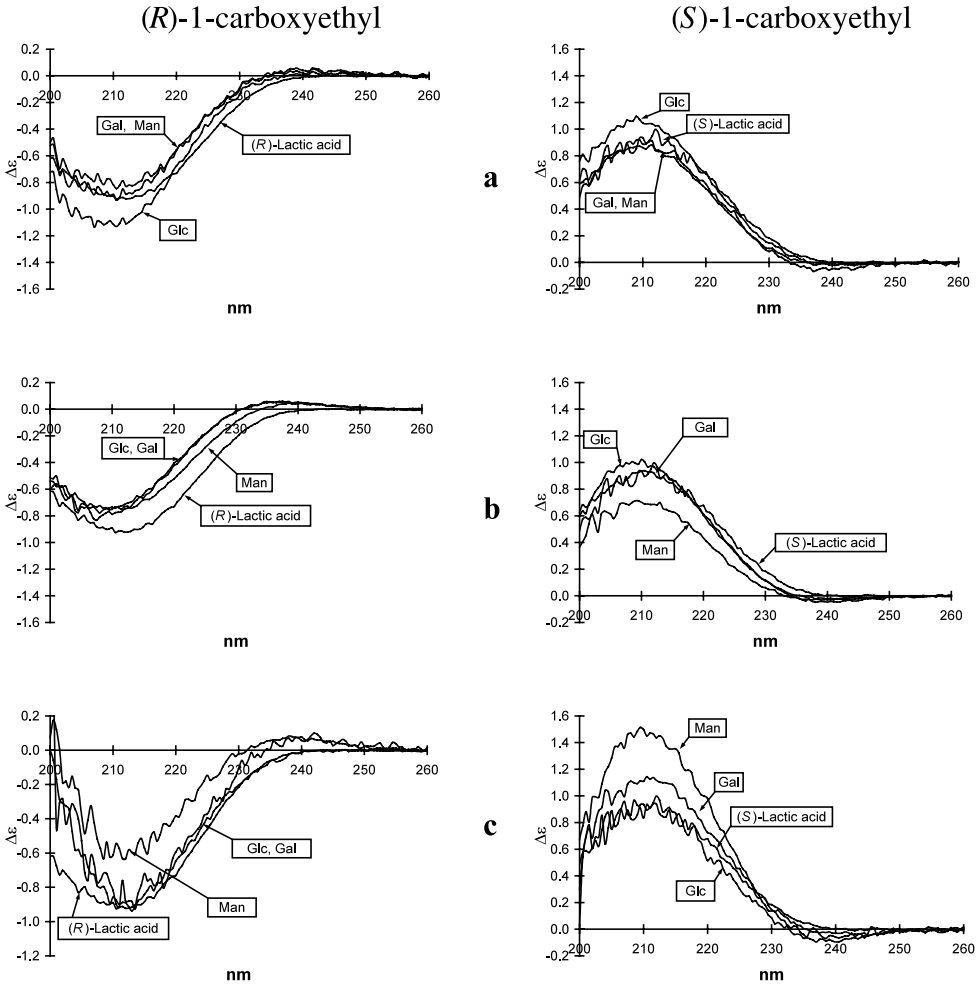


Fig. 2. CD spectra recorded in water solution at pH 2 for the 3-*O*-[(*R*)- and (*S*)-1-carboxyethyl] substituted derivatives of glucose, galactose and mannose. (a) D-hexoses, (b) methyl α-D-hexopyranosides and (c) alditols. The CD spectrum of the respective isomer of lactic acid is plotted as a thick line in all figures.

nm if it is derived from the lactic acid chromophore alone. The shape and intensity of the ellipticity maximum should be similar to that observed for lactic acid for an unambiguous identification of the absolute configuration of the 1-carboxyethyl group. The intensity of the 210 nm maximum differs for different samples but is always of the same order of magnitude as that obtained for lactic acid. Previously, $\Delta\varepsilon$ values between $|0.3-1.5|$ (reported as $[\Theta]_{210}$ values between $|1000-5000|$ deg cm^2/dmol) have been reported for α-hydroxy acids and α-hydroxy acid derivatives at pH ≤ 2.5.[8,11,12] An exception is the value reported for *N*-acetylmuramic acid ($\Delta\varepsilon$ -3.82, $[\Theta]$ $-12{,}600$) where the ellipticity of the *N*-acetyl group probably interferes with that of the carboxyl group.[8] All samples in our study show $\Delta\varepsilon$ values in the range $|0.4-1.5|$ (Table 1).

To test the reliability of the proposed CD method, three polysaccharides containing at least one 1-carboxyethyl substituted monosaccharide were analyzed by the protocol described in Section 4. The extracellular polysaccharides isolated from *Butyrivibrio fibrisolvens* strain X6C61, H10b and 12 were used as test samples. For strain X6C61, the complete structure including the absolute configuration of the 6-*O*-[(*R*)-1-carboxyethyl]-

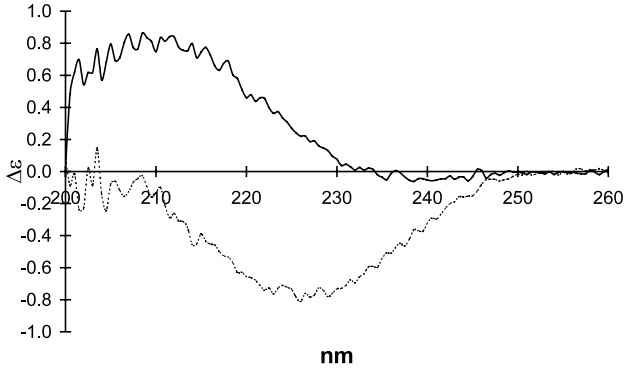


Fig. 3. CD spectra recorded in water solution at pH 2 from 3-*O*-[(*S*)-1-carboxyethyl]-D-glucitol after 1 min (solid line) and 60 min (dashed line).

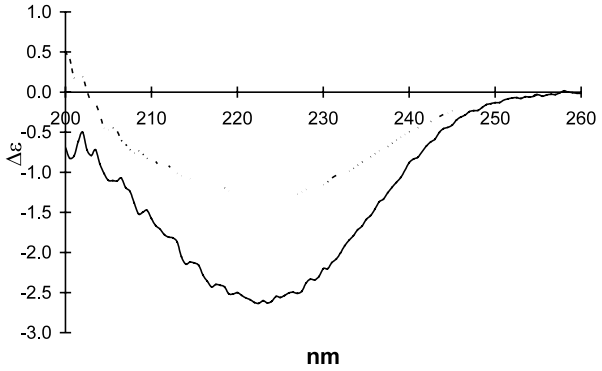


Fig. 4. CD spectra recorded in water solution at pH 2 from methyl 3-*O*-[(*R*)-1-carboxyethyl]-α-D-glucopyranosid-1′,2-lactone (dashed line) and methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-galactopyranosid-1′,2-lactone (solid line).

D-Gal is known.[15] Strain 12 and H10b contains a 1-carboxyethyl substituted Gal residue, common for several strains of *B. fibrisolvens*, and H10b also another unknown acidic monosaccharide.[1] The repeating units of strain X6C61 and H10b contain more than one acidic sugar, which complicate the isolation of the 1-carboxyethyl substituted monosaccharides. In the repeating unit of strain X6C61, D-Glc*p*A and L-Ido*p*A residues are present whereas in H10b an unknown 1-carboxyethyl substituted sugar is present in addition to 6-*O*-(1-carboxyethyl)-D-Gal. For strain 12, the only acidic component present is 6-*O*-(1-carboxyethyl)-D-Gal. We isolated all 1-carboxyethyl sugars (> 90% pure as judged by their ^{1}H NMR spectra) from the polysaccharides using different chromatographic techniques. The CD spectrum of each component (Fig. 5(a–d)) had the expected appearance and intensity and determination of the absolute configuration could thus safely be done. All three polysaccharides contain 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal and strain H10b also contains 3-*O*-[(*S*)-1-carboxyethyl]-D-Glc which was confirmed by comparison of its ^{1}H NMR spectrum with that of synthesized 3-*O*-[(*S*)-and (*R*)-1-carboxyethyl]-D-Glc.

3. Conclusions

The CD-spectra obtained at pH 2 of five different (*R*)/(*S*)-pairs of 1-carboxyethyl substituted monosaccharides and their corresponding methyl glycosides and alditol derivatives (Table 1 and Fig. 2) all show large similarities with the spectra of the respective stereoisomer of lactic acid. The results demonstrate that, under the conditions used, the lactic acid chromophore interacts with circularly polarized light almost unaffected of the sugar part of the respective molecule. It is possible to compare CD spectra of the 1-carboxyethyl deriva-

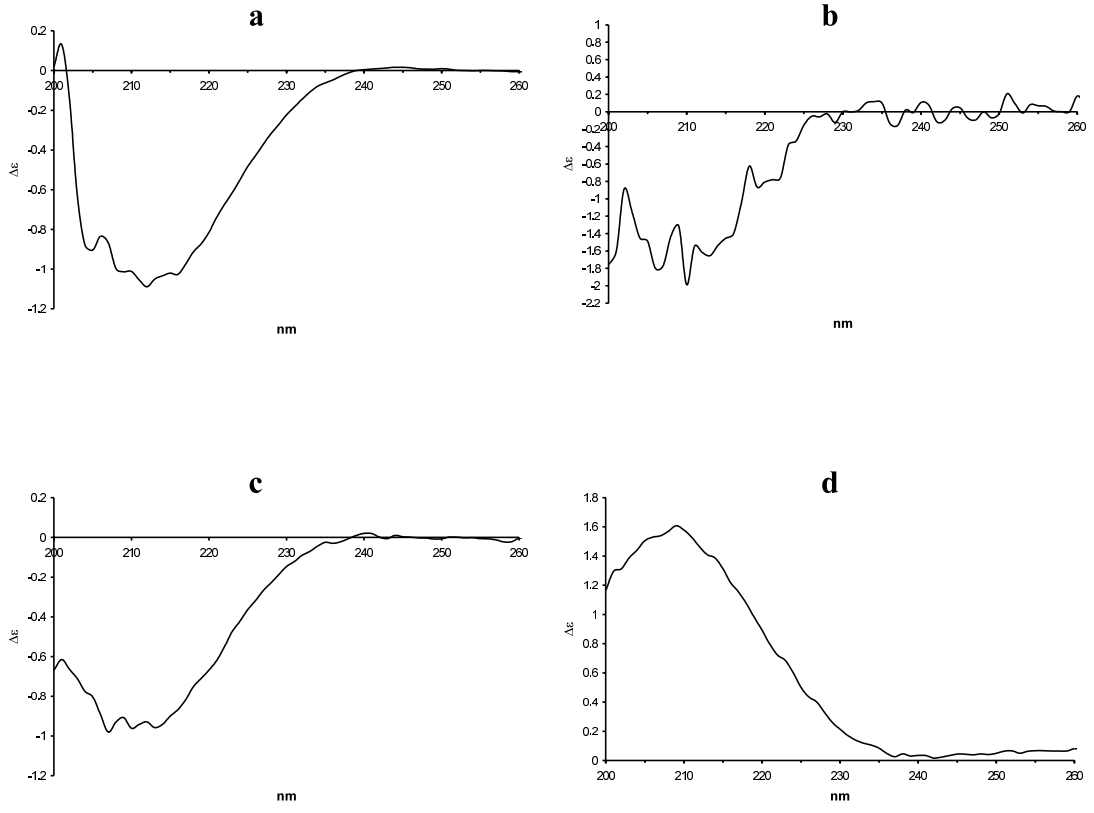


Fig. 5. (a–c) CD spectra of 6-*O*-[(*R*)-1-carboxyethyl]-α-D-galactose isolated from *Butyrivibrio fibrisolvens* strain 12, X6C61 and H10b, respectively, and (d) CD spectrum of 3-*O*-[(*S*)-1-carboxyethyl]-α-D-glucose isolated from strain H10b.

tives with spectra of (*R*)- and (*S*)-lactic acid as long as the CD spectrum of the derivative has a dominating ellipticity maximum at ~210 nm with similar shape and intensity as the spectrum measured for lactic acid. The absolute configuration of the 1-carboxyethyl group is determined to be the same as for that isomer of lactic acid that has the same sign for the 210 nm ellipticity in the CD spectrum.

The described method for determination of the absolute configuration of 1-carboxyethyl substituents from their CD spectra has several advantages compared with previously used methods. It is very convenient since the CD measurements are performed on products formed in a standard analysis of the components of a polysaccharide and thus little extra work is necessary for sample preparation. The 1-carboxyethyl substituent is not derivatized, which saves time and eliminates the risk of side reactions. As demonstrated, it is also fast and sensitive. The acquisition of a useful CD spectrum can be done in ~15 min on a 20 μM sample (~5 μg/mL) in a 1 cm cell. The CD spectrum recorded for 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal (~3.5 μg/mL) isolated from X6C61 was noisy but still useful (Fig. 5(b)). The sensitivity of the method depends on the stability of the 1-carboxyethyl sugar derivative during the acquisition. For 1-carboxyethyl sugar derivatives that easily form lactones, the CD spectrum has to be acquired in a few minutes whereas, for other samples, acquisition could continue for several hours.

4. Experimental

4.1. General methods

TLC was performed on pre-coated plates (E. Merck Silica Gel 60 F_{254}) with 12:3:3:1 EtOAc–HOAc–MeOH–H_2O as mobile phase, detection being afforded with 5% H_2SO_4 in EtOH and heating. NMR spectra were recorded at 30 °C on samples in D_2O soln at 400 MHz (^1H) with a Bruker DRX 400 instrument. Mass spectra were recorded in the negative-ion mode on a Bruker Esquire ESIMS system. Samples were injected at a flow of 100 μL/h in concn of ~0.5 mM in aq 50% MeOH containing 0.25% NH_4OH. CD spectra were recorded on a JASCO 720 spectropolarimeter in the range 200 to 260 nm at 20.0 °C using a square cell with 1 cm path length. Sample concn were between 0.01–10 mM for synthetic standards and samples isolated from polysaccharide material. For samples >0.5 mM the resolution and bandwidth was 0.5 and 1.0 nm, respectively, and <0.5 mM 1.0 and 2.0 nm, respectively. Usually, one scan was sufficient for acquiring a useful spectrum but, for samples with low concn, eight or sixteen scans were used. The scanning speed and response time were 100 nm/min and 0.5 s, respectively. Measurements were carried out at pH 2, 7 and 12 for all 3-*O*-1-carboxyethyl substituted sugars and alditols. For (*S*)-lactic acid and methyl 3-*O*-(*S*)-[1-carboxyethyl]-α-D-galactopyranoside, measurements were made at pH 1, 2, 3, 5, 7, 9, 11, 12 and 13. Samples measured at pH 1 and 2 were prepared by addition of 1 M HCl to the sugar derivative dissolved in water. Similarly, addition of 1 M NaOH was used for samples measured at pH 9, 11, 12 and 13. Buffers (10 mM) were used to control the pH in the region near the pK_a of the 1-carboxyethyl group. Citric acid/NaOH was used for pH 3, NaOAc/HCl for pH 5 and Na_2PO_4/HCl for pH 7.

4.2. Estimation of reaction yield by ^1H NMR spectroscopy

Due to the small amounts of sample, the yields in all reactions were estimated by ^1H NMR spectroscopy. As an example, for the H_2SO_4 hydrolysis of the methyl glycosides starting material, it was dissolved in water (2.0 mL) and divided in two equal parts which were lyophilized. One of the samples was used in the hydrolysis reaction and the other kept as a reference sample. After the hydrolysis and work-up (neutralization, centrifugation and lyophilization) the hydrolyzed material as well as the reference sample were separately dissolved in 1.00 mL D_2O. A part of each soln (500 μL) was mixed and transferred to an NMR-tube and a ^1H NMR spectrum was acquired. Signals of starting material and products were integrated and their intensities compared.

4.3. Estimation of concn of CD samples by ^1H NMR spectroscopy

Due to the small amounts of sample, the concn of the soln used for CD measurements were estimated by ^1H NMR spectroscopy. Samples of concn between 0.01–10 mM were prepared and CD measurements performed. The CD samples were lyophilized in 8 mL vials, dissolved in 1.00 mL of 1.00 mM *t*-BuOH (internal standard) in D_2O and a ^1H NMR spectrum (90° pulse, acquisition time ~5 s, 64–128 scans) was acquired for each sample. The signal of the 1-carboxyethyl CH_3 protons was compared with the signals from the internal standard and the concn of the sample was calcd. The accuracy of this determination was not rigorously controlled as our primary objective was to investigate if the sign of the maximum at 210 nm is correlated to the stereochemistry of the 1-carboxyethyl substituent and not to obtain exact Δε-values of the measured substances. Since only small amounts of the test substances were available, we did not want to contaminate the samples with an internal standard that could affect the CD spectrum in case we needed to measure the sample

again. The spread in the ^{1}H NMR measurements was estimated by measuring triplicates for two samples and was found to be within 5%.

4.4. Calculation of $\Delta\varepsilon$ and $[\theta]$ values

The $\Delta\varepsilon$ and the $[\theta]$ values were calcd from the relations $[\theta] = (\theta/(\mathrm{lc})) \times (M/100) = 3300\Delta\varepsilon$, where $[\theta]$ is the obsd molecular ellipticity, θ ellipticity angle, l cell length in dm, c concn in g/mL, M molecular mass and $\Delta\varepsilon$ molar circular dichroism.

4.5. Analysis procedure

Purified extracellular polysaccharide (20 mg) isolated from *Butyrivibrio fibrisolvens* strain 12 was hydrolyzed at 120 °C for 4 h in 2 M TFA (10 mL) in a sealed tube. The TFA was removed by evaporation in a stream of air at ~30 °C. Water (1 mL) was added and the evaporation repeated. The sample was dissolved in water (2 mL) and pH adjusted to 10 by addition of 1 M NH_4OH. After concn under diminished pressure at ~35 °C, the sample was purified on a Bio-Gel P-2 column (80 × 2.6 cm) irrigated with water. More than 50% of total 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal was isolated free from other monosaccharide components. The rest of the 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal was eluted together with neutral sugars and was not used for CD measurements. The fractions containing pure 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal were pooled, pH adjusted to ~10 (NH_4OH) and then lyophilized to give 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal (1.7 mg) as its ammonium salt. A part of the material was dissolved in D_2O and a ^{1}H NMR spectrum showed that it was >90% pure. The rest of the material was dissolved in 2.00 mL water and acidified to pH 2 just prior to the acquisition of the CD spectrum. The concn of the sample was 340 μg/mL and $\Delta\varepsilon_{210} = 1.0$.

Preparation of 1-carboxyethyl substituted sugars from strains H10b (15 mg) was done in the same way as for strain 12, with the exception that further separations had to be done after the passage of the basic hydrolysate through the Bio-Gel P-2 column. For strain H10b, the two acidic components were eluted in the same fractions. A drop of 1 M NH_4OH was added to each fraction before they were pooled and lyophilized. After dissolution in water (1 mL), they were applied on a column (80 × 1.6 cm) of Dowex 50 WX-2 (Ca^{2+}) which was irrigated with water at 10 mL/min. The acidic components were partly separated with 3-*O*-[(*S*)-1-carboxyethyl]-D-Glc slightly before 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal. Fractions containing 3-*O*-[(*S*)-1-carboxyethyl]-D-Glc (80 μg) and 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal (60 μg) were obtained. Analysis indicated contaminants in the samples and therefore they were passed through C_{18} SPE cartridges (Sep-Pac® C18, 360 mg, Waters Corporation) eluted with water (1 mL). CD spectra of the 1-carboxyethyl substituted sugars were acquired. The concn of the samples was determined to be 43 μg/mL for 3-*O*-[(*S*)-1-carboxyethyl]-D-Glc and 53 μg/mL for 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal which give estimated $\Delta\varepsilon$ values of 1.6 and 1.0, respectively.

A sample of strain X6C61 (~5 mg) was hydrolyzed and worked-up as described for strain 12. Instead of separation on a Bio-Gel P-2 column, the lyophilized mixture was applied directly on the Dowex 50 WX-2 (Ca^{2+}) column. The 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal eluted together with one other sugar. Separation on a column (2 × 0.5 cm) of Dowex 1 (OH^-) resulted in a minor amount of pure 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal (~5 μg). A 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal sample (3.6 μg/mL) was measured using the same procedure as for strain H10b and a satisfactory spectrum ($\Delta\varepsilon = 1.7$) was obtained.

4.6. 3-*O*-[(*R*)-1-Carboxyethyl]-D-glucopyranose (1)

Methyl 3-*O*-[(*R*)-1-carboxyethyl]-α-D-glucopyranoside Na-salt (6.2 mg) was dissolved in 1 M H_2SO_4 (3.1 mL) and heated in a sealed vial at 100 °C for 10 h. The vial was cooled to rt and water (3 mL) added to the reaction mixture before neutralization with portions of solid $BaCO_3$. The mixture was diluted with water (3 mL) and centrifuged at 4000 rpm for 10 min before the supernatant was collected. The pellet was extracted two times with water (3 mL) and centrifuged as described above. The supernatants were pooled and evaporated to dryness to yield crude **1**. TLC: R_f ~0.35; ESIMS: Anal. Calcd for $C_9H_{17}O_8$ 251.1, found 250.9 $[M-H]^-$; A ^{1}H NMR spectrum showed signals from α and β anomeric protons at δ 5.10 and 4.50 indicating a reducing sugar. Some traces of starting material (<2%) and small amounts (1–5%) of unidentified by-products with signals in the region δ 1.5–3 were also indicated.

4.7. 3-*O*-[(*R*)-1-Carboxyethyl]-D-glucitol (2)

Sodium borohydride (6 mg) was added to compound **1** (~6 mg) dissolved in water (3 mL). After 2 h, excess $NaBH_4$ was destroyed by addition of Dowex 50 (H^+) until pH ~3, and the soln filtered and lyophilized. Remaining boric acid was removed as methyl esters by co-distillation with MeOH (3 × 5 mL). The product was treated with ~2 mL NH_4OH (pH ~10, 1 h) to hydrolyze lactones formed during the co-distillation. The NH_4OH was removed by freeze-drying to yield the NH_4 salt of **2** as white amorphous powder; TLC: R_f ~0.1. ESIMS: Anal. Calcd for $C_9H_{20}O_8$ 253.09, found 252.9 $[M-H]^-$. A ^{1}H NMR spectrum showed only signals from alditol protons in the region δ 3–4.2 in addition to the signals for the 1-carboxyethyl group.

Compound **1** was also prepared from methyl 3-*O*-[(*R*)-1-carboxyethyl]-α-D-glucopyranoside Na-salt (2 mg) by hydrolysis with 2 M TFA (1 mL) at 120 °C for 8 h. The acid was evaporated in a stream of N_2 at ~30 °C, the product dissolved in water (0.5 mL) and evaporated two times more. TLC indicated that lactones (R_f ~0.7) had been formed. The lactones were opened by treatment with NH_4OH (pH ~10, 1 h) to quantitatively yield the NH_4 salt of **1** after lyophilization. TLC, ESIMS and ^1H NMR of **1** were identical with those from the H_2SO_4 preparation. The purity was >90% as judged from ^1H NMR. Compound **2** was also obtained from this material as described above.

Using the procedures described above, the following substances were synthesized starting from their parent methyl glycosides: 3-*O*-[(*S*)-1-carboxyethyl]-D-Glc (**3**), 3-*O*-[(*S*)-1-carboxyethyl]-D-glucitol (**4**), 3-*O*-[(*R*)-1-carboxyethyl]-D-Gal (**5**), 3-*O*-[(*R*)-1-carboxyethyl]-D-galactitol (**6**), 3-*O*-[(*S*)-1-carboxyethyl]-D-Gal (**7**), 3-*O*-[(*S*)-1-carboxyethyl]-D-galactitol (**8**), 3-*O*-[(*R*)-1-carboxyethyl]-D-Man (**9**), 3-*O*-[(*R*)-1-carboxyethyl]-D-mannitol (**10**), 3-*O*-[(*S*)-1-carboxyethyl]-D-Man (**11**), 3-*O*-[(*S*)-1-carboxyethyl]-D-mannitol (**12**), 4-*O*-[(*R*)-1-carboxyethyl]-L-rhamnose (**13**), 4-*O*-[(*R*)-1-carboxyethyl]-L-rhamnitol (**14**), 4-*O*-[(*S*)-1-carboxyethyl]-L-rhamnose (**15**), 4-*O*-[(*S*)-1-carboxyethyl]-L-rhamnitol (**16**), 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal (**17**), 6-*O*-[(*R*)-1-carboxyethyl]-D-galactitol (**18**). 6-*O*-[(*S*)-1-Carboxyethyl]-D-Gal (**19**), 6-*O*-[(*S*)-1-carboxyethyl]-D-galactitol (**20**).

The purity of the samples was estimated by ^1H NMR spectroscopy. Using 1 M H_2SO_4 for hydrolysis, the purity was found to be ~95% for all reducing sugars and their corresponding alditol derivatives. With TFA hydrolysis, the purity of the samples was found to be ~90% for all reducing sugars and their corresponding alditol derivatives. In addition to the impurities mentioned above, compounds **9** and **10** were contaminated by 6% of **11** and **12**, respectively, and **11** and **12** were contaminated by 2% **9** and **10**, respectively, since the starting materials were not entirely pure.

5. Supplementary material

The material is available from the authors on request.

Acknowledgements

This work was supported by grants from the Swedish Research Council. We thank Mr Torbjörn Astlind, Department of Bio-Physics at Stockholm University, for supplying the CD spectropolarimeter and for technical assistance.

References

1. Stack, R. J. *Appl. Environ. Microbiol.* **1988**, *54*, 878–883.
2. Severn, W. B.; Richards, J. C. *J. Am. Chem. Soc.* **1993**, *115*, 1114–1120.
3. Ferreiro, M. J.; Latypov, S. K.; Quiñoá, E.; Riguera, R. *J. Org. Chem.* **2000**, *65*, 2658–2666.
4. Tyrrell, E.; Tsang, M. W. H.; Skinner, G. A.; Fawcett, J. *Tetrahedron* **1996**, *52*, 9841–9852.
5. Fukushi, Y.; Shigematsu, K.; Mizutani, J.; Tahara, S. *Tetrahedron Lett.* **1996**, *37*, 4737–4740.
6. Rickman, B. H.; Matile, S.; Nakanishi, K.; Berova, N. *Tetrahedron* **1998**, *54*, 5041–5064.
7. Hör, K.; Gimple, O.; Schreier, P.; Humpf, H.-U. *J. Org. Chem.* **1998**, *63*, 322–325.
8. Listowsky, I.; Avigad, G.; Englard, S. *Biochemistry* **1970**, *9*, 2186–2189.
9. Andersson, M.; Kenne, L.; Stenutz, R.; Widmalm, G. *Carbohydr. Res.* **1994**, *254*, 35–41.
10. Andersson, L.; Kenne, L. *Carbohydr. Res.* **1998**, *313*, 157–164.
11. Listowsky, I.; Avigad, G.; Englard, S. *J. Org. Chem.* **1970**, *35*, 1080–1085.
12. Barth, G.; Voelter, W.; Bunnenberg, E.; Djerassi, C. *J. Chem. Soc., Sect. D, Chem. Commun.* **1969**, 355–356.
13. Listowsky, I.; Englard, S.; Avigad, G. *Biochemistry* **1969**, *8*, 1781–1785.
14. Andersson, L.; Kenne, L. *Carbohydr. Res.* **2000**, *329*, 257–268.
15. Andersson, M.; Ratnayake, S.; Kenne, L.; Ericsson, L.; Stack, R. *J. Carbohydr. Res.* **1993**, *246*, 291–301.