2-*O*-Methyl-D-Glucuronic Acid, a New Hexuronic Acid of Biological Origin

By JOY HEANEY KIERAS, FRED J. KIERAS* and DAVID V. BOWEN
The Rockefeller University, 1230 York Avenue, New York, NY 10021, U.S.A.

(Received 19 November 1975)

Acid hydrolysis of the extracellular polysaccharide of *Porphyridium cruentum* (a unicellular red alga) produced a mixture of aldobiuronic acids and free hexuronic acids. Fractionation of this mixture on an ion-exchange column yielded a hexuronic acid characterized as the title compound. Its identity was confirmed by chromatographic comparisons with the authentic compound, by reduction to the corresponding methylated aldose, by resistance to controlled lead tetra-acetate oxidation and by chemical-ionization mass spectrometry. Complete spectra have been deposited as Supplementary Publication SUP 50062 (7 pages) with the British Library (Lending Division), Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K., from whom copies may be obtained under the terms given in *Biochem. J.* (1976) 153, 5.

D-Glucuronic acid is probably the most commonly found hexuronic acid in the plant and animal kingdoms (Whistler & Rowell, 1966). Only one naturally occurring mono-*O*-methyl ether of D-glucuronic acid has been reported, 4-*O*-methyl-D-glucuronic acid. Apparently limited to the plant kingdom, this derivative has been isolated from higher-plant gums and mucilages (White, 1948; Smith, 1951). We now describe a new sugar, 2-*O*-methyl-D-glucuronic acid, found in a plant polysaccharide.

Experimental

The red alga *Porphyridium cruentum* (Ag.) Naeg. (Indiana University Culture Collection no. 161; Starr, 1964) was grown in a chemically defined medium (Jones, 1962) under sterile conditions. After the cells reached stationary phase they were separated from the medium by centrifugation. The medium containing the extracellular polysaccharide was warmed to 37°C, and the polysaccharide precipitated by slow addition, with stirring, of 1% (w/v) cetylpyridinium chloride. The cetylpyridinium-polysaccharide complex was recovered by centrifugation, and dissolved in 4M-LiCl; 3 vol. of ethanol was then added. The ethanol-precipitated polysaccharide was redissolved in 2M-LiCl, and precipitated by the addition of 3 vol. of ethanol. Ethanol precipitation from 2M-LiCl was repeated twice, and the final precipitate was washed with ethanol, then with ether, and finally dried in a vacuum desiccator over P₂O₅.

All chemicals used were reagent grade and sugars were obtained from commercial sources with the

following exceptions. Methyl (methyl-2-*O*-methyl- α -D-glucopyranosid)uronate was obtained from Dr. P. Kováč, Slovak Academy of Sciences, Bratislava, Czechoslovakia, 2-*O*-methyl-D-galactose from Dr. G. O. Aspinall, York University, Downsview, Ont., Canada, and 2-*O*-methyl- β -D-glucose from Dr. A. S. Perlin, McGill University, Montreal, P.Q., Canada. L-Iduronic acid was kindly provided by Dr. E. L. Stern, University of Minnesota Hospital, Minneapolis, MN, U.S.A. 4-*O*-Methyl-D-glucuronic acid was prepared by acid hydrolysis of mesquite gum, and fractionated on a Dowex 1 column (X8; formate form), as described below; the gum was provided by Dr. G. O. Aspinall.

Ion-exchange resins were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Hexose was determined by the anthrone method, with D-galactose as standard, as described by Morris (1948); uronic acid was determined by the orcinol method (Brown, 1946) and by the carbazole method (Dische, 1947), with D-glucuronic acid as standard. After acid hydrolysis, the neutral sugars were quantified as the borate complexes by chromatography on the Technicon Sugar Chromatography system.

Uronic acid and uronic acid-containing oligosaccharides were separated and quantified on a column (0.63 cm $\times$ 138 cm) of Dowex 1 (X8; -400 mesh; formate form) at 27 ml/h, as described by Fransson *et al.* (1968), except that 0.3M-formic acid was used as the eluent; 20 fractions/h were collected. Before application to anion-exchange resin the samples were adjusted to pH 8.0 with 1M-NH₃, and left for 2 h at room temperature (23°C) to saponify lactones. The NH₃ solution was removed by evaporation under vacuum at 40°C, and the samples were redissolved in water and applied to the resin.

* To whom reprint requests should be addressed.

Paper chromatography was carried out on Whatman no. 1 paper. T.l.c. was performed on Quantum Industries (Fairfield, NJ, U.S.A.) analytical plates (20cm×20cm) coated with either silica gel (non-activated) or cellulose. The following solvent systems were used at room temperature: (A) ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.); (B) nitromethane/ethanol/acetic acid/water saturated with boric acid (9:1:1:1, by vol.) as described by Robyt (1975). Sugars were located on chromatograms by reaction with alkaline AgNO_3 (Smith, 1960) or aniline hydrogen phthalate (Block *et al.*, 1958).

Neutral sugars were also determined by g.l.c. as alditol acetates prepared by the method of Lehnhardt & Winzler (1968), with L-arabinitol as internal standard. A column of 3% OV-225 on Supelcoport (80–100 mesh) was operated at 185°C, with N_2 as carrier gas at a flow rate of 20 ml/min. Glass columns of internal diameter 2 mm and 1.83 m (6 ft) in length were used.

Controlled lead tetra-acetate oxidation was performed as described by Charlson & Perlin (1956). Esterification and reduction of uronic acids and uronides to neutral sugars was performed as described by Cifonelli *et al.* (1958).

Chemical-ionization mass spectra were obtained on a DuPont 21-492 mass spectrometer modified for chemical ionization under licence from Scientific Research Instruments Corp., Baltimore, MD, U.S.A. The reactant gas was isobutane, and the source pressure was approx. 66.6 Pa (0.5 Torr). Source-insertion chemical ionization was used, the sample being introduced on the tip of a glass rod into the centre of the ion source (Baldwin & McLafferty, 1973; Shabanowitz *et al.*, 1975). Spectra were obtained from approx. 10 µg of material at source temperatures between 125° and 200°C. The data were processed by a DS-30 data system (AEI Scientific Apparatus, Manchester, U.K.) to which our own programs for chemical ionization had been added (Bowen & Field, 1975), and by a 2040 data system (VG Data Systems, Altrincham, Cheshire, U.K.). The intensities of the ions are expressed as percentages of the intensity of the most intense ion. Ions from the reactant gas and from chemical mass markers, and ions with relative intensity less than 10% and with m/e less than 70, are not included. Complete spectra have been deposited as Supplementary Publication SUP50062 with the British Library (Lending Division), Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, U.K.

Uronic acids and uronic acid-containing fragments were isolated from the polysaccharide after acid hydrolysis in 1M-HCl for 2 h at 100°C or 1.0M- H_2SO_4 for 2 h at 100°C or 2M-trifluoroacetic acid for 3 h at 100°C, in sealed tubes. Under each hydrolysis condition, the polysaccharide concentration was 1% (w/v). For HCl hydrolysates enough Dowex 3

(carbonate form) was added to reach pH 2–3; the H_2SO_4 was neutralized by addition of solid BaCO_3 ; trifluoroacetic acid was removed by evaporation under vacuum at 40°C.

Results and Discussion

The extracellular polysaccharide of the fresh-water red alga *P. cruentum* is a high-molecular-weight (1×10^6 – 10×10^6) heteropolymer consisting, by weight, of 42% hexose, 30% pentose, 8.5% uronic acid, 9.0% ester sulphate and 1–2% amino acids. The hexose consists of glucose and galactose and the pentose is xylose. The molar ratios of components relative to glucose are as follows: galactose/xylose/glucose/uronic acid/sulphate, 2.12:2.42:1.00:1.22:2.61 (Kieras, 1972).

We will describe in more detail the isolation and identification of a novel hexuronic acid moiety found in the polymer. The polysaccharide (usually 100 mg) was hydrolysed in acid (e.g. 1M-HCl, 2 h, 100°C), neutralized, and the acidic components were prepared by passage of the hydrolysate after saponification of lactones, as described in the Experimental section, through a column of Dowex 1 (X8; 200–400 mesh: formate form). All of the uronic acid based on the carbazole reaction is recovered in the material eluted with 1M-formic acid. This material was then fractionated on a column of Dowex 1 (X8; –400 mesh: formate form) as described by Fransson *et al.* (1968). The elution profile from this column is shown in Fig. 1,

Fig. 1. Fractionation of free hexuronic acids and hexuronic acid fragments on Dowex 1

Uronic acids and uronic acid-containing fragments were derived from the polysaccharide by acid hydrolysis. Conditions for ion-exchange chromatography are described in the Experimental section. Fraction volume was equal to 1.35 ml. Peaks were pooled as indicated, concentrated, and further analysed by the colorimetric methods described in the Experimental section. For the elution profiles — represents hexose values and ---- represents uronic acid values.

Table 1. *Colorimetric analyses of pooled fractions derived from Dowex 1 chromatography*

Analyses for hexose by the anthrone method and for uronic acid by the carbazole method were as described in the Experimental section.

Sample	Content ($\mu\text{mol}/100\text{mg}$)		Hexose/ uronic acid ratio	Recovery of uronic acid (%)
	Hexose	Uronic acid		
Unhydrolysed	233.3	38.6	6.0	100
Peak I	11.8	4.3	2.8	11
Peak II	28.1	27.0	1.0	70
Peak III	—	0.4	—	1
Peak IV	—	7.9	—	20

and more detailed analyses of each peak are presented in Table 1. Peaks I and II are uronic acid-containing disaccharides (aldobiuronic acids), and peak IV is free D-glucuronic acid (Kieras, 1972).

We will focus attention on Peak III, an acidic component comprising the smallest fraction of the total free uronic acid in the polymer. However, as we will discuss below, this uronic acid represents about 28% of the total uronic acid, if its contribution to peak I is considered. As shown in Table 1, this component contains no hexose, and gives a positive carbazole reaction (also an orcinol reaction), indicating a uronic acid. This component is eluted from the Dowex 1 column in the region of monosaccharide uronic acids and can be distinguished from the monosaccharide standards, D-glucuronic acid, D-galacturonic acid and 4-O-methyl-D-glucuronic acid, when these are mixed and run on the Dowex 1 column. Peak III is almost coincident with L-iduronic acid in this system.

Paper chromatography on Whatman no. 1 in solvent A and staining with aniline hydrogen phthalate shows peak III to contain two components, with $R_{\text{GlcUA}} = 1.43$ and 2.12 (the faster-moving spot is the more intense suggesting more of this component). These two spots are clearly different in mobility from D-glucuronic acid ($R_{\text{GlcUA}} = 1.00$), D-glucurone ($R_{\text{GlcUA}} = 1.59$), D-galacturonic acid ($R_{\text{GlcUA}} = 0.97$) and 4-O-methyl-D-glucuronic acid ($R_{\text{GlcUA}} = 1.47$). Both spots from peak III stain bright red or crimson with aniline hydrogen phthalate, which is characteristic of 2-O-methylated uronic acids (Hough & Jones, 1950), whereas the other standards stain brown. The mobility of each of these two spots from Peak III is identical with those of authentic 2-O-methyl-D-glucuronic acid and its lactone prepared by acid hydrolysis of methyl (methyl-2-O-methyl- α -D-glucopyranosid)uronate. We believe the slower spot to be the free acid and the faster-moving spot its corresponding lactone.

To substantiate the proposed structure we subjected this compound to analysis by chemical-ionization mass spectrometry, with isobutane as the ionizing gas. Uronic acids of known structure were also examined (see Table 2). The intense peak at m/e 173 is probably the dehydration product of the ion at m/e 191. This is either the protonated molecular ion of the lactone, $(M+1)^+$, or a dehydration product of the protonated ion derived from proton transfer to the free acid. The results obtained with D-glucuronic acid and its lactone favour the explanation of proton transfer to the lactone for formation of m/e 191. Paper chromatography and t.l.c. show that much of the peak III compound is in the lactone form. The mass spectrum is consistent with a mixture of 2-O-methyl-D-glucuronic acid (mol.wt. 208) and its lactone (mol.wt. 190). Loss of water from the protonated molecular ion is a well established mode of fragmentation for monosaccharides with free reducing ends (Hogg & Nagabhushan, 1972; Horton *et al.*, 1974). The mass spectrum of 4-O-methyl-D-glucuronic acid is sufficiently similar to that of peak III to indicate that peak III is a mono-O-methyl hexuronic acid.

More chemical evidence supporting this structure was obtained. Peak III was esterified and reduced to the corresponding neutral sugar; the esterification and the reduction step were repeated to ensure complete conversion into the aldose. Analysis of the reduction products by g.l.c. showed 90% conversion

Table 2. *Chemical-ionization mass spectra of peak III and authentic uronic acids*

Compound	m/e	Relative intensity (%)
Peak III	191	39.5
	173	100
D-Galacturonic acid	177	100
	159	27.2
	131	13.9
	121	11.8
D-Glucuronic acid	177	100
	159	70.9
D-Glucurono-6,3-lactone	177	76.6
	159	100
4-O-Methyl-D-glucuronic acid	205	12.5
	191	100
	173	36.4
	149	12.5
	141	29.4
	131	17.0
	121	18.3
	115	14.8
	114	13.8
	112	10.0
	97	39.0
	90	30.0

Table 3. *Lead tetra-acetate oxidation of peak III and authentic uronic acids*

The sample was dissolved in 10 μ l of water in a 15 ml conical centrifuge tube. To this were added 100 μ l of acetic acid and 400 μ l of 1% (w/v) lead tetra-acetate in acetic acid. After 30 min at 25°C 400 μ l of 2% (w/v) oxalic acid in acetic acid was added. After 25 min, 3 ml of water was added, and the samples were centrifuged to remove lead oxalate. Each supernatant (1 ml) was assayed by the carbazole method, and the results are based on this. For controls, the oxalic acid was added first, then the uronic acid, and the procedure continued. In every case recovery of control samples was 100% on the basis of the carbazole reaction.

Sample	Amount (μ g)		%
	Input	Recovered	
D-Glucuronic acid	100	1.4	98.6
D-Galacturonic acid	100	2.2	97.8
D-Glucurono-6,3-lactone	54.8	9.3	83.0
4-O-Methyl-D-glucuronic acid	99.4	35.8	63.9
Peak III	92.9	82.7	11.0

of peak III into a compound with the same retention time as authentic 2-O-methyl-D-glucose determined as the alditol acetate. Analysis of the reduction product by t.l.c. on silica-gel plates in solvent system B showed one spot coincident with standard 2-O-methyl-D-glucose ($R_{G_{al}} = 1.68$), but distinct from 3-O-methyl-D-glucose ($R_{G_{al}} = 1.51$) and 2-O-methyl-D-galactose ($R_{G_{al}} = 2.00$). Whereas unsubstituted hexoses stain brown with aniline hydrogen phthalate, the reduced product and the standards 2-O-methyl-D-glucose and 2-O-methyl-D-galactose stained red-dish brown. The chemical-ionization spectra of the reduction product of peak III were closely similar to those of authentic 2-O-methyl-D-glucose.

Finally we subjected peak III to controlled lead tetra-acetate oxidation along with known standards as described by Charlson & Perlin (1956). Under these conditions oxidation begins at the reducing terminus and continues through each free hydroxyl group; oxidation will not occur under these conditions when the carbon atom adjacent to the reducing terminus is replaced by a group such as O-methyl. Glucuronic acid, glucurone and galacturonic acid show a range of 83–100% destruction, and 4-O-methyl-D-glucuronic acid shows 70% destruction. However, peak III shows only 11% destruction, based on the carbazole reaction (see Table 3).

The total amount of 2-O-methyl-D-glucuronic acid can be estimated to be about 28% of the total uronic acid in the polymer. In Table 1, peak I is shown by colorimetric analyses to have a mole ratio of hexose to uronic acid of 2.8:1.0; it has been shown to be an

aldobiuronic acid (disaccharide) composed of the modified uronic acid and hexose (Kieras, 1972). The amount of 2-O-methyl-D-glucuronic acid, i.e. peak III, would thus be underestimated by 60%, on the basis of the same colorimetric analysis (carbazole) relative to glucuronic acid. Peak III contains, then, about 3% of free uronic acid after hydrolysis. Since all of the 2-O-methyl-D-glucuronic acid is found in peaks I and III (Kieras, 1972), we calculate that this compound comprises 28%, by weight, of the total uronic acid in the polymer and approx. 2.5% by weight of the total mass of the polysaccharide.

The assignment of the structure 2-O-methyl-D-glucuronic acid to the compound in peak III (Fig. 1) is based on the following data. The compound has the same mobility on paper chromatography and t.l.c. as the standard 2-O-methyl-D-glucuronic acid and its lactone; it has the same staining properties with location reagents. After esterification and reduction the compound is converted into 2-O-methyl-D-glucose, as shown by t.l.c., g.l.c. and chemical-ionization mass spectrometry in comparison with the authentic standard. It is resistant to lead tetra-acetate oxidation under conditions which destroy other uronic acids not substituted at the carbon-2 position. Chemical-ionization mass spectrometry produces fragments consistent with the molecular weight and fragmentation pattern of 2-O-methyl-D-glucuronic acid. We have not yet been able to assign the absolute configuration to peak III. However, since all naturally occurring derivatives of glucuronic acid are of the D series, we tentatively assign peak III the D configuration.

To our knowledge this is the first example of a 2-O-methylhexuronic acid known to occur in biological material. Its function and mode of biosynthesis in this system remain unknown.

This work was supported in part by grant RR-00862 from the NIH Division of Research Resources. We are grateful to Mr. J. Shabanowitz and Mrs. N. Fernando for skilled technical help. We also thank Dr. J. D. Gregory for advice and criticism of the manuscript.

References

- Baldwin, M. A. & McLafferty, F. W. (1973) *Org. Mass. Spectrom.* **7**, 1353–1356
- Block, R. J., Durrum, E. L. & Zwiag, G. (1958) *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd edn., p. 181, Academic Press, New York
- Bowen, D. V. & Field, F. H. (1975) *Anal. Chem.* **47**, 2289–2292
- Brown, A. H. (1946) *Arch. Biochem. Biophys.* **11**, 269–278
- Charlson, A. J. & Perlin, A. S. (1956) *Can. J. Chem.* **34**, 1200–1208
- Cifonelli, J. A., Ludowieg, J. & Dorfman, A. (1958) *J. Biol. Chem.* **233**, 541–545
- Dische, Z. (1947) *J. Biol. Chem.* **167**, 189–198

- Fransson, L.-Å., Rodén, L. & Spach, M. L. (1968) *Anal. Biochem.* **21**, 317–330
- Hogg, A. M. & Nagabhushan, T. L. (1972) *Tetrahedron Lett.* 4827–4830
- Horton, D., Wander, J. D. & Foltz, R. L. (1974) *Carbohydr. Res.* **36**, 75–96
- Hough, L. & Jones, J. K. N. (1950) *J. Chem. Soc.* 1199–1203
- Jones, R. T. (1962) *J. Cell. Comp. Physiol.* **60**, 61–64
- Kieras, J. H. (1972) Ph.D. Thesis, University of Chicago
- Lehnhardt, W. F. & Winzler, R. J. (1968) *J. Chromatogr.* **34**, 471–479
- Morris, D. L. (1948) *Science* **107**, 254–255
- Roby, J. F. (1975) *Carbohydr. Res.* **40**, 373–374
- Shabanowitz, J., Brynes, P., Maelicke, A., Bowen, D. V. & Field, F. H. (1975) *Biomed. Mass Spectrom.* **2**, 164–167
- Smith, F. (1951) *J. Chem. Soc.* 2646–2652
- Smith, I. (1960) *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 252, Interscience Publishers, New York
- Starr, R. C. (1964) *Am. J. Bot.* **51**, 1013–1044
- Whistler, R. L. & Rowell, R. M. (1966) in *Glucuronic Acid, Free and Combined* (Dutton, G. J., ed.), pp. 137–182, Academic Press, New York and London
- White, E. V. (1948) *J. Am. Chem. Soc.* **70**, 367–369