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Note

Physico-chemical characterization of anomeric D-glucopyranosiduronic acid conjugates

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Interest in recent years in the synthesis and characterization of D-glucopyranosiduronic acids[1–7] has suggested the characterization of this class of compounds by modern instrumental techniques. Thus far, interest has focussed primarily on the naturally occurring conjugates of β-D-glucuronic acid. α-D-Glucopyranosiduronic acids are less often encountered in Nature, however they occur in the product mixtures from most chemical syntheses of β-D-glucopyranosiduronic acids[6,7,8,9]. In order to recognize and characterize diastereomeric glucopyranosiduronic acids by rapid and sensitive instrumental techniques, *p*-nitrophenyl α-D-glucopyranosiduronic acid (**1**) was synthesized, its n.m.r. and mass spectra were recorded, and its chromatographic characteristics relative to those of the β-D anomer examined. Reliable characterization by any combination of these techniques may provide greater sensitivity and a more definite analysis than optical rotation and melting points, which have been used heretofore in the physico-chemical characterization of α-D-glucopyranosiduronic acids[8,9]. Mass spectral comparison of the anomers of D-glucopyranosyluronic acid was of particular interest, as this had not been previously evaluated.

Analysis of the oxidation products of *p*-nitrophenyl α-D-glucopyranoside by t.l.c. indicated one major product, which gave a blue color in reaction with naphthoresorcinol indicating a glucosiduronic acid. This product has an R_F value of 0.51. It could be separated from *p*-nitrophenyl β-D-glucopyranosiduronic acid (**2**).

Less than 4% of **1** was hydrolyzed on incubation with β-D-glucuronidase–aryl sulfatase. Compound **2** underwent a 63% hydrolysis in a parallel and identical incubation. These results provide some evidence that the product from the oxidation reaction may have the α-D configuration, as β-D-glucuronidase is inactive towards synthetic α-D-glucopyranosiduronic acids[10,14,15].

G.l.c. of the per-*O*-trimethylsilyl derivatives of the oxidation product, and of **2** with *p*-nitrophenyl β-D-glucopyranoside added as internal standard (see Fig. 1), allowed an excellent separation of **1** and **2**. The earlier elution of **1** is consistent with previous reports on the g.l.c. separation of anomers and configurational isomers of

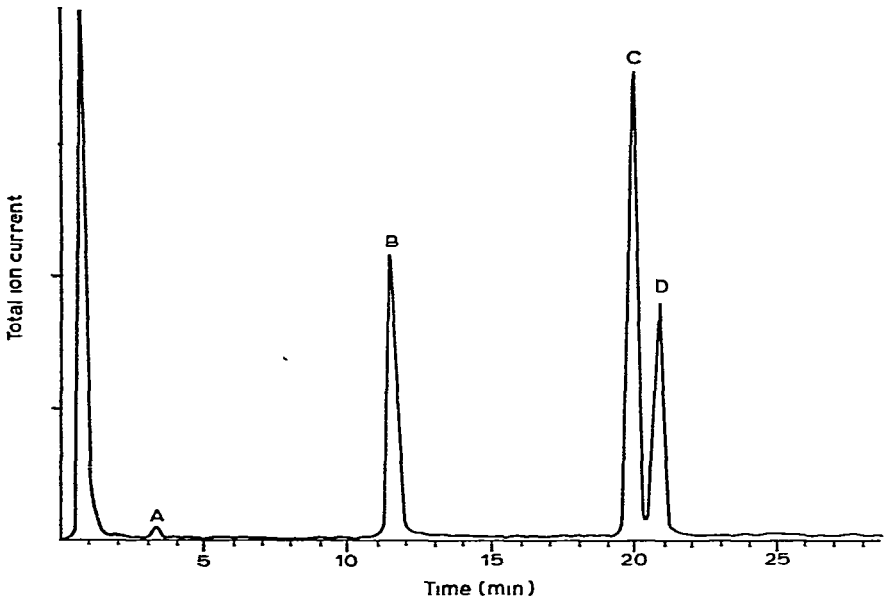


Fig. 1. Gas–liquid chromatogram of per-*O*-trimethylsilyl derivatives of: (A) D-glucuronic acid, (B) phenyl β-D-glucopyranoside (internal standard), (C) *p*-nitrophenyl α-D-glucopyranosiduronic acid, and (D) *p*-nitrophenyl β-D-glucopyranosiduronic acid.

other per-*O*-trimethylsilyl sugars[16] and ethyl α- and β-D-glucopyranosiduronic acid[3].

In the electron-impact m.s. (e.i.m.s.) of **1** (Fig. 2), no molecular ion was observed, and the major fragment ions are characteristic of the per-*O*-trimethylsilyl derivatives of aryl glucopyranosiduronic acids[4]. Except for noticeable and reproducible differences in the relative abundances of the ions at *m/e* 147 and 204, the spectra of **1** and **2** were virtually identical and cannot be used as diagnostic criteria for stereochemical assignment. Some reports have suggested correlations between mass-spectral fragmentation and configuration[17,18], whereas others conclude that the method is insensitive to stereochemical differences[19,20]. It is interesting to note, however, that the ion at *m/e* 147 arises from a rearrangement process[21] involving the OMe_4Si groups of

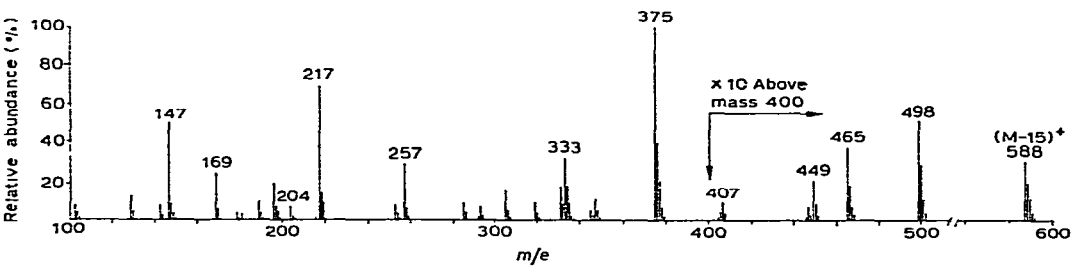
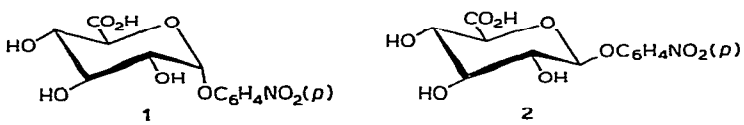


Fig. 2. 70-eV E.i.m.s. of the per-*O*-trimethylsilyl derivative of *p*-nitrophenyl α-D-glucopyranosiduronic acid (**1**).

the sugar ring and changes in relative abundance of this fragment might indirectly reflect different conformations of **1** and **2**.

The mol. wt. of **1** was confirmed by chemical ionization m.s. with anhydrous ammonia as the reagent gas. The spectrum contained peaks at *m/e* 621 (M + 18), 375 (base peak), and 465. The ammonia c.i.m. spectra of **1** and **2** were almost identical, as were the spectra obtained with isobutane as the reagent gas. C.i.m.s. was therefore unable to provide information useful in distinguishing between **1** and **2**.

Comparison of the n.m.r. spectra of **1** and **2** provided direct evidence for the configuration at C-1 of **1**. The configurations and favored conformations[22,23] of **1** and **2** (see Scheme 1) were deduced from the following data: For **1** in D_2O: δ 8.04 (d, 2 H, J 9.0 Hz, $PhNO_2$), 7.07 (d, 2 H, J 9.0 Hz, $PhNO_2$), 5.70 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.10 (m, 1 H, H-5), and 3.35–3.65 (m, 3 H, H-2,-3 and -4); for **2** in D_2O: δ 8.01 (d, 2 H, J 9.0 Hz, $PhNO_2$), 7.04 (d, 2 H, J 9.0 Hz, $PhNO_2$), 5.17 (d, 1 H, $J_{1,2}$ 6.2 Hz, H-1), 4.06 (d, 1 H, H-5), and 3.40–3.60 (m, 3 H, H-2,-3, and -4).



Scheme 1. Preferred conformations of *p*-nitrophenyl α-D-glucopyranosiduronic acid (**1**) and *p*-nitrophenyl β-D-glucopyranosiduronic acid (**2**).

The n.m.r. spectra, although of low resolution, confirm the α-D configuration at C-1 of the product of the oxidation reaction: H-1 appears as a doublet through vicinal coupling with H-2. The coupling constant (3.5 Hz) is considerably smaller than the coupling constant of the same protons in **2** (6.2 Hz). The downfield shift of H-1 related to a dipole interaction effect of O-5 and O-1 of **1** again reflects the configuration of C-1. Analogous correlations have been made for the anomers of glucopyranosides[22,24] and of steroidal D-glucopyranosyluronic acids[7].

This comparison of some of the physico-chemical properties of **1** and **2** demonstrates that anomers of this class can be easily separated by g.l.c., though not by t.l.c. They can be readily distinguished by n.m.r.; however distinction by m.s. seems less likely.

EXPERIMENTAL

General methods. — *p*-Nitrophenyl β-D-glucopyranosiduronic acid and *p*-nitrophenyl α-D-glucopyranoside were obtained from Sigma Chemicals, St. Louis, MO 63178, β-D-glucuronidase-aryl sulfatase was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. 46250, and platinum-on-charcoal was obtained from Tridom Fluka, Hauppage, New York, NY 11787.

N.m.r. spectra of *p*-nitrophenyl α- (**1**) and β-D-glucopyranosiduronic acid (**2**) were recorded on deuterium oxide solutions with a 60-MHz Perkin-Elmer R1213

instrument at 25°; the residual proton signal of deuterium oxide was the internal standard reference signal.

G.l.c.–m.s. analyses were carried out on a DuPont 491 mass spectrometer equipped with a chemical ionization source and interfaced to a Finnigan–Incos data system. An all-glass jet-separator connected the mass spectrometer to a Varian 2720 gas chromatograph. The e.i.m. spectra were obtained at 70 eV with the ion source at 200°. The chromatograph was equipped with a 1.5 mm x 3 mm (i.d.) open tubular glass-column packed with 3% OV-101 on Supelcoport (80/100 mesh); helium was the carrier gas at a flow rate of 35 mL/min; and the oven temperature was programmed from 180–280° at 4°/min. Chemical ionization was carried out with anhydrous ammonia or isobutane as reagent gas at a source pressure of approximately 0.4 torr. All samples were converted into the per-*O*-trimethylsilyl derivatives for analysis[4].

Enzyme hydrolysis was performed with β-D-glucuronidase–aryl sulfatase from *Helix pomatia* (13 KU/L, equivalent to 113 700 Fishman units/mL). A 0.12mM solution of **1** in 75mM phosphate buffer (0.5 mL) at pH 6.8 was incubated with a 1% suspension of enzyme (0.1mL) for 30 min at 37°. Glycine buffer (0.2M) at pH 10.4 was added to stop the reaction and develop the yellow color indicative of *p*-nitrophenol. Absorbance was recorded on a Zeiss spectrophotometer at a wavelength of 400 nm. The values for **1** were corrected with a control value that included a correction for the spontaneous decomposition of the product in the absence of the enzyme and for the absorbance of **1** and the enzyme itself. Compound **2** was hydrolyzed under the same conditions.

p-Nitrophenyl α-D-glucopyranosiduronic acid (**1**). — This compound was synthesized by catalytic oxidation of *p*-nitrophenyl α-D-glucopyranoside by use of a method similar to that of Marsh and Levvy[10]. A solution of 5% platinum-on-charcoal in water was purged carefully with hydrogen gas and then nitrogen immediately before use. No reaction was observed without this pretreatment. *p*-Nitrophenyl α-D-glucopyranoside (0.5 g) in doubly distilled water (25 mL) was stirred with 5% platinum-on-charcoal (5 g) while oxygen was passed through the solution from a bubbling frit. The reaction was maintained for 1.5 h at 90°. The pH of the reaction mixture was continuously monitored and maintained at 8–9 by periodic addition of 0.5M sodium hydrogencarbonate. The course of the reaction was also followed by t.l.c. When the reaction was complete, the catalyst was removed by centrifugation. The supernatant was acidified to pH 3.5 with 3M hydrochloric acid and extracted with butanol, and the organic phase concentrated *in vacuo* to a yellow syrup. The yield was measured by spectrophotometric assay of the colored product formed on reaction with naphthoresorcinol[11,12]. The product was chromatographed in the form of a syrup on 250-μm thick Silica gel $60_{F\text{-}254}$ plates (E. Merck) with 8:4:4:5 (v/v) butanol–benzene–water–methanol. The product was detected by spraying the plates with naphthoresorcinol[13]. Reference samples of β-D-glucopyranosiduronic acid, *p*-nitrophenyl α-D-glucopyranoside, and *p*-nitrophenyl β-D-glucopyranosiduronic acid were run concurrently with the product for characterization.

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