EVIDENCE FOR A (1→4)-LINKED 4-*O*-(α-L-IDOPYRANOSYLURONIC ACID 2-SULFATE)-(2-DEOXY-2-SULFOAMINO-D-GLUCOPYRANOSYL 6-SULFATE) SEQUENCE IN HEPARIN

LONG-RANGE H–H COUPLING IN 4-DEOXY-HEX-4-ENOPYRANOSIDES

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ABSTRACT

A disaccharide produced from heparin by *Flavobacterium heparinum* is shown to be an elimination product having the probable structure **1**; namely, 4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate)-(2-deoxy-2-sulfoamino-D-glucopyranose 6-sulfate). Since **1** accounts for about three-quarters of the heparin used, its formation, taken together with other evidence available, indicates that the heparin molecule is composed largely of a repeating sequence of (1→4)-linked 4-*O*-(α-L-idopyranosyluronic acid 2-sulfate)-(2-deoxy-2-sulfoamino-α-D-glucopyranosyl 6-sulfate) biose residues. The anomeric configuration of the unsaturated uronic acid residue of **1** was deduced primarily from the fact that the olefinic 4-proton is long-range coupled with H-2 of the same residue. Thus, H-4–H-2 coupling appears to be general in 4-deoxy-α-L-*threo*-hex-4-enopyranose derivatives, but does not occur in the β-anomers. Among the compounds examined were unsaturated oligosaccharides from chondroitin 4-sulfate and pectin, and some monosaccharide 4-deoxy-hex-4-enopyranosides. Synthesis of the anomeric methyl 2,3-di-*O*-acetyl-4-deoxy-6-*aldehydo*-L-*threo*-hex-4-enodialdo-1,5-pyranosides by methyl sulfoxide oxidation–base-catalyzed elimination is described.

INTRODUCTION

Heparin is degraded by a group of induced enzymes from *Flavobacterium heparinum*[1,2,3] to a mixture of mono-, di-, and, oligo-saccharides. One of these products is a disaccharide trisulfate[1,2]. It is formed by the action of a 2-amino-2-deoxy-D-glucosidase, and has been shown to account for at least three-quarters of the heparin[3]. Accordingly, characterization of this product should provide useful information about much of the heparin molecule itself. The current paper provides

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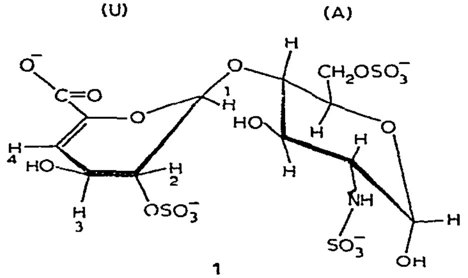
evidence that the disaccharide has the structure represented by formula **1**, and considers its relationship to the structure of the parent polymer.

As described earlier[1], product **1** has been obtained chromatographically and electrophoretically pure as an amorphous, ammonium salt*. Its overall analytical characteristics and those of its enzymic degradation products[1] are consistent with its formulation as a disaccharide, the reducing end-unit of which is a 2-deoxy-2-sulfoamino-D-glucose sulfate. Although, tentatively, the uronosyl residue of **1** was thought to be saturated, a re-examination of its u.v. spectrum shows absorption at λ_{max} 227 nm (ε 2800). Furthermore, its u.v. spectrum is virtually superposable upon those of known 4-deoxy-hex-4-enopyranosyluronic acids (**2** and **3**), produced enzymically from



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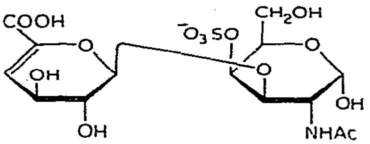

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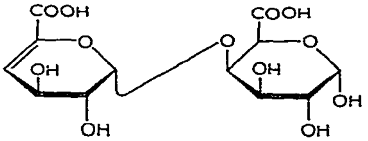

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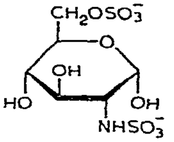

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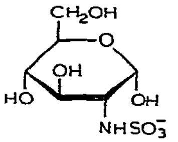

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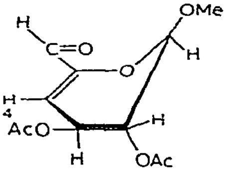

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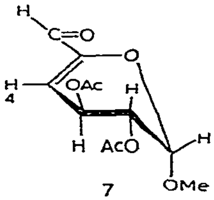

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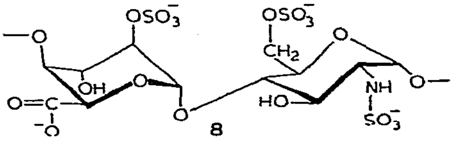

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*Also, it has now been prepared as a crystalline barium salt.

chondroitin 4-sulfate[4] and pectin[5], respectively. In addition, compound **1** and known 4-deoxy-hex-4-enopyranosyluronic acids readily yield 5-formyl-2-furoic acid in cold, concentrated, acid whereas saturated hexuronic acids do so only upon heating[6].

The p.m.r. spectrum of **1** (Fig. 1B) serves to confirm the olefinic nature of the uronosyl residue of **1** and indicates that the double bond is located at the 4,5-position. Thus, the spectrum shows a signal at δ 6.0 p.p.m. (H-4 of the unsaturated residue, here designated UH-4) which, as shown later, may be ascribed to the olefinic 4-proton. Also, there are three other signals (UH-1, 2 and 3) shown by spin decoupling to arise from protons of the same residue. This particular residue must, therefore, contain only four protons, and not the five required for a saturated uronosyl residue (or seven for the aminodeoxy-hexosyl residue). In addition, signal UH-4 is shifted far upfield when the compound is treated with hot aqueous acid, and when tritiated, aqueous acid is used, the tracer is incorporated into the product. Both of these latter observations provide evidence that **1** is an alkene that undergoes ready hydration.

Other signals in Fig. 1B (H-1 to -6 of the amino sugar residue, here designated AH-1 to -6) are attributed to the aminodeoxyhexosyl residue. The assignments are based particularly on a uniformly close correspondence with the spectrum of 2-deoxy-2-sulfoamino-D-glucose 6-sulfate (**4**) (Fig. 1A), another product obtained by the enzymolysis of heparin[1]. As seen from the relative intensities of the anomeric proton signals (a weak signal attributable to H-1 of the β-anomer is discernable at 4.7 p.p.m.) compounds **1** and **4** exist almost exclusively as the α-D-anomer in aqueous solution (as does also 2-acetamido-2-deoxy-D-glucose), which contrasts strongly with the preponderance of the β over the α anomer in aqueous solutions of D-glucose. Comparison of Fig. 1A in turn with the spectrum of 2-deoxy-2-sulfoamino-D-glucose (**5**) shows that there is a large upfield shift of the H-6 signals for **5** relative to those for **4** (inset, Fig. 1A)*. Hence these spectra furnish independent, supporting evidence that hexosamine residues in heparin contain 6-sulfate ester groups (see refs. 7 and 8). The spectrum of the aminodeoxyhexosyl residue of **1** serves also as a highly pertinent reference in assigning signals in the spectrum of heparin itself[9].

Because signal UH-2 is relatively strongly deshielded, one of the sulfate ester groups may be assigned with confidence to the 2-position of the unsaturated uronosyl residue. This assignment is in accord with the behaviour of heparin on reaction with periodate[10–12] and with the relatively low-field position of the adjacent, uronosyl, H-1 signal for heparin[9,13] both of which appear to require that most of the uronosyl residues of the polymer carry 2-sulfate groups. The fact that disaccharide **1** is highly resistant to oxidation by periodate[1] also is in accord with the structure proposed, and is pertinent particularly with respect to the position of the glycosidic linkage. Hence, bonding to O-4 of the aminodeoxyhexosyl residue places blocking groups at positions 2, 4, and 6 and completely prevents glycol-cleavage, even of an acyclic form of this residue. Of course, presence of the 2-*O*-sulfato group on the uronosyl residue obviates periodate oxidation of that moiety as well.

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*Otherwise, the spectra of **4** and **5** are virtually superposable.

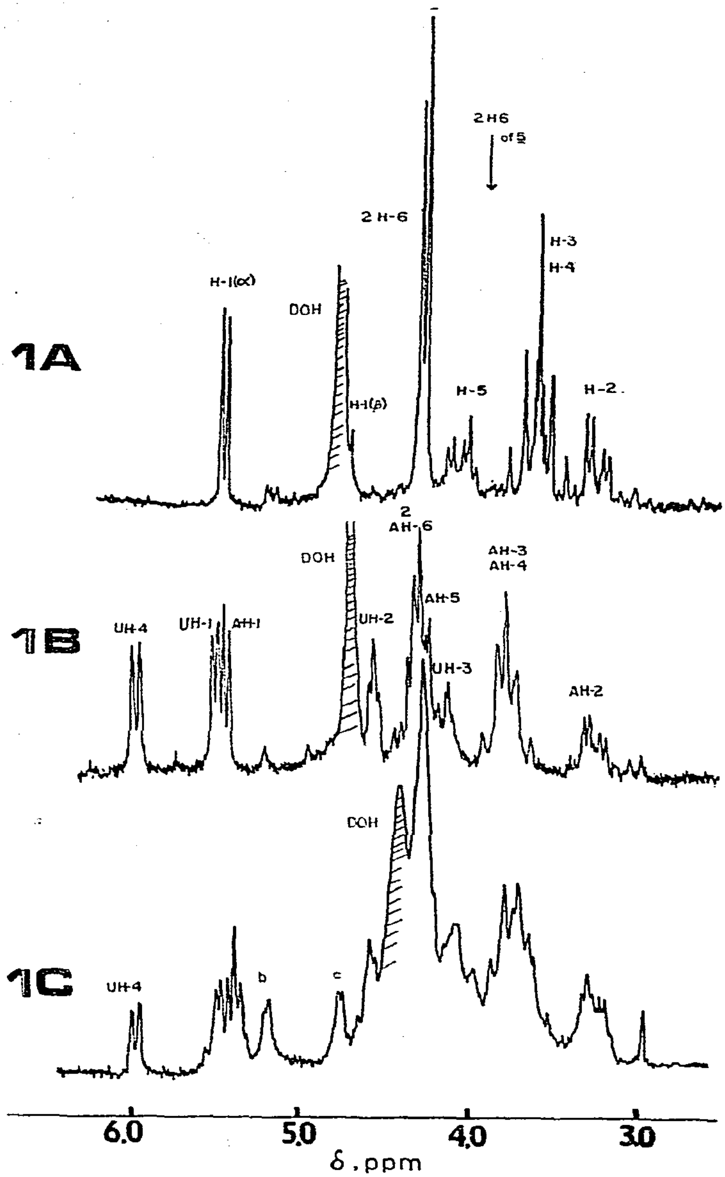

Fig. 1. P.m.r. spectra (100 MHz, 1000-Hz sweep width) in deuterium oxide of products obtained from heparin through the action of *Flavobacterium heparinum*: (A) 2-deoxy-2-sulfoamino-D-glucopyranose (6-sulfate) (4) at 35°; (B) disaccharide **1** at 35°; (C) oligosaccharide at 70°.

As discussed below, signal UH-4 is especially important in establishing the configurational assignment of the glycosidic linkage in **1**. First of all, several factors support its designation as the vinylic 4-proton. Thus, the spectrum of heparin itself does not show a signal as far downfield as UH-4, and hence the location of the latter is unlikely to be associated with possible deshielding effects of sulfate or carboxyl groups in **1**. Furthermore, each of the disaccharides, **2** and **3**, is found to exhibit a signal in the same region (Fig. 2B and C). This observation can again be attributed most readily to the vinylic proton of these compounds. Monosaccharides of related structure also give a signal in this region: thus H-4 of 4-deoxy-α- or β-L-*threo*-hex-4-enopyranuronates resonates at 6.0–6.2 p.p.m.[14,15], and H-4 of the anomeric 4-deoxy-6-*aldehydo*-L-*threo*-hex-4-enodialdo-1,5-pyranosides, **6** and **7**, have chemical shifts close to this value also (Fig. 2D and E). The latter compounds, which are structurally analogous to the unsaturated uronic acids, were synthesized by a methyl sulfoxide oxidation–base-catalyzed elimination sequence[15] and are described in the Experimental section.

It will be noted that signal UH-4 (Fig. 1B) is not a doublet, but a quartet. Its smaller spacing of 1.1 Hz is attributable to long-range coupling, the latter kind of interaction being seen more clearly in the expanded, partial spectrum (Fig. 2A), and spin decoupling confirmed that it involves proton UH-2. The vinylic-proton signal of **2** (Fig. 2B) and of **6** (Fig. 2D) show similar evidence of long-range coupling*. In the latter instance also, it is the H-2 signal that accounts for this long-range effect (confirmed by spin decoupling), although it was not possible to resolve the source of the effect in the spectrum of **2**. Additional long-range couplings are evident between UH-1 and -3 of disaccharide **1** (Fig. 2A) and between H-1 and H-3 of **6**. These interrelationships are attributed to the likelihood that the uronosyl residues of **1**, **2**, and **6** have approximately the conformations shown in formulas **1** and **6**. In such a conformation, protons 2 and 4, as well as 1 and 3, are oriented in the "W"-type of arrangement that characteristically gives rise to long-range couplings of about 1 Hz[17–20]. Also, the anomeric C–O bond is thereby quasi-axial and H-1, 2, and 3 are quasi-equatorial, accounting for the relatively small, major splittings found for the H-1, H-2, and H-3 signals (2.6 to 3.2 Hz); thus, in the alternative conformation these protons should be more nearly axial and produce larger spacings[14].

In marked contrast to these findings, the olefinic proton of **3** (Fig. 2C) or of **7** (Fig. 2E) produces a sharp doublet, and there was no indication whatsoever of long-range couplings**. This result is understandable if these alkene derivatives exist in approximately the conformation shown for **7**, because this, conformation does not incorporate a "W" pathway for coupling between H-2 and H-4.

Now, compounds **2** and **6** possess the α-L-*threo* configuration, whereas **3** and **7** have the β-L-*threo* configuration. The spectral characteristics of disaccharide **1** clearly identify it with the former group, and show that its unsaturated uronosyl residue may

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*These splittings due to long-range interactions, although always detectable when resolution was good, are more clearly defined with some compounds and/or solvents than with others; 2D is an optimal example, 2A and 2B represent the other extreme (see also H-4 of Fig. 1C). H-4 of the carboxylic acid derived from **6** or of the 1-*O*-acetyl analogue of **6** (unpublished) also exhibits such long-range coupling.

Analogously, the unsaturated trisaccharide derived from pectic acid[6] produces a sharp doublet coincident in chemical shift and spacing with the signal attributed to the olefinic proton of **3. In addition, H-4 of the carboxylic acid derived from **7** or of the 1-*O*-acetyl analogue of **7** (unpublished) shows no evidence of long-range coupling.

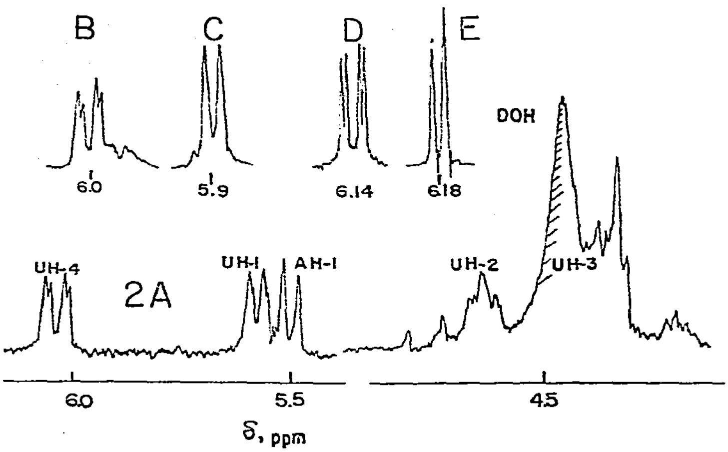

Fig. 2. (A) Partial p.m.r. spectrum of disaccharide **1** in deuterium oxide at 70°. Signal H-4 of (B) unsaturated disaccharide[5] (**2**) from chondroitin 4-sulfate, and (C) unsaturated disaccharide[6] (**3**) from pectin, in deuterium oxide at 70°; (D) and (E) β and α anomers, respectively, of methyl 4-deoxy-6-*aldehydo*-L-*threo*-hex-4-enodialdo-1,5-pyranoside in chloroform-*d* at 30°. (100 MHz, 250-Hz sweep width.)

similarly be designated as an α-L-*threo* isomer, namely one derived from a β-D-*gluco* (or -*galacto*) or an α-L-*ido*-hexosyluronic acid. D-Glucosyluronic acid residues in heparin have been shown to possess the α-D- configuration[12,21] and also, there is evidence[23] that such residues are not sulfated. However, since L-idosyluronic acid is known to be a constituent of heparin[7–9,22] it is concluded that such residues are involved in the formation of **1**, and hence that they must possess the α-L-configuration. Additional evidence for this configuration is provided by the fact that **1** gives a negative optical rotatory dispersion curve that is closely similar to the curve reported[5] for disaccharide **2**; with the evidence above that **1** and **2** are related *conformationally*, these rotatory data may be meaningfully compared.

The high yield of **1** is therefore in accord with recent evidence that L-idosyluronic acid is the main acid constituent of heparin[13,22]*. Accordingly, a major proportion (perhaps 75–80%) of the heparin molecule may be formulated as a sequence of biose disaccharide residues, as in **8**. As shown, the L-idopyranosyluronic acid residues [shown here in the *1C* (*L*) conformation (ref. 13, 24 and see later)] must be bound glycosidically through the 4-position in order for the enzymic elimination to proceed at positions 4 and 5; assignment of the aminodeoxy-α-D-hexosyl configuration is based primarily on studies by Wolfrom and coworkers[21].

A quartet, coinciding both in chemical shift and spacings with the H-4 signal of disaccharide **1**, is found in spectra of two other oligosaccharides produced from heparin by *F. heparinum* (for example Fig. 1C). Reducing end-group and other analyses indicate that these products are a tetra- and hexasaccharide[1], and the p.m.r. spectra

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*Indirect evidence that the main uronic acid of heparin molecule is not D-glucuronic acid, has also been obtained recently[3].

now suggest that both of them are formed, analogously to **1**, through elimination by the aminodeoxy-D-glucosidase. However, the relative intensity of the olefinic quartet in each spectrum (see Fig. 1C) — which should furnish an independent estimate of molecular size — is lower than would be expected. Hence it is probable that these higher oligosaccharides are mixed products, containing both saturated and unsaturated uronosyl non-reducing end-residues, and that a glycosidase as well as an eliminase is present in the enzymic digest from *Flavobacterium*.

The signals designated *b* and *c* in Fig. 1C correspond closely in chemical shift and shape to signals ascribed to H-1 and H-5, respectively, of L-idopyranosyluronic acid residues in heparin[13]. As has been noted[13], the narrow spacing of this latter H-1 signal, and the spectral characteristics of methyl idopyranosiduronic acids[24], lead to the suggestion that these residues in heparin assume a shape close to the *1C (L)* conformation (as in **8**), although the chair probably is slightly skewed[24]. It is reasonable, therefore, to assume from Fig. 1C that the conformation of L-idopyranosyluronic acid residues is similar both in this oligosaccharide and in the parent heparin (**8**) and hence not noticeably affected within the polymer by tertiary structure.

EXPERIMENTAL

General. — Proton magnetic resonance spectra were recorded at 100 MHz with a Varian HA-100 spectrometer, by using deuterium oxide (with tetramethylsilane contained in a coaxial capillary as the reference); chloroform-*d* or pyridine-d_5 was used with an internal standard of tetramethylsilane. Paper chromatography (descending) was carried out with Whatman No. 1 paper with the solvent systems: *A*, 2:3:1 (v:v:v) butyl alcohol–acetic acid–1M ammonium hydroxide; and *B*, 50:12:25 (v:v:v) butyl alcohol–acetic acid–water. Unsaturated carbonyl compounds were readily detected on the chromatograms as u.v.-absorbing spots.

T.l.c. (ascending) was carried out on silica gel (Merck, grade G) by using as solvents: *C*, ethyl acetate; and *D*, 9:1 (v:v) benzene–methanol. Spray reagents were 5M sulfuric acid (at 150°), or 2,4-dinitrophenylhydrazine (saturated, acidified methanolic solution).

Disaccharide **1**. — Procedures for the preparation, isolation, and purification of **1** have been described previously[1]. The product was obtained as a colorless, powdery ammonium salt; $[\alpha]_D$ 5° (*c* 1.7, water); $\lambda_{max}^{H_2O}$ 227 nm (ε 2800). Corresponding data for disaccharide **2** are $\lambda_{max}^{H_2O}$ 230 nm (ε 3050); for **3** (Sr salt), $\lambda_{max}^{H_2O}$ 232 nm (ε, 4900). The optical rotatory dispersion curve for **1** was negative and showed a minimum (Cotton effect) at λ 240 nm; the curve for **2** also was negative[5], exhibiting a minimum at λ 240 nm. By contrast, the o.r.d. curve for **3** was positive, having a maximum at λ 220 nm.

As reported earlier[1], analysis of **1** showed that it is a disaccharide containing approximately one molar proportion each of hexosamine and uronic acid, and three sulfate groups.

Anal. Recalculated on the basis of formula **1** ($C_{12}H_{31}N_5O_{19}S_3$): hexosamine, 28.0; hexuronic acid (see below), 24.5; sulfate, 44.4; reducing equivalent, 1.0. Found:

hexosamine, 23.5; hexuronic acid, 24.0; sulfate 44.6; reducing equivalent (relative to 4), 1.1.

The uronic acid analysis of **1** (used in ref. 1) gives approximately the same yield of chromophore with known 4-deoxy-hex-4-enopyranosyl uronic acids as with D-glucuronic acid[6]. More-direct evidence for the presence of a 4-deoxy-hex-4-enopyranosyluronic acid moiety in **1** was provided by the fact[6] that this compound and the known unsaturated acids rapidly gave rise to the chromophore quantitatively in the cold, whereas saturated uronic acids did not.

Two of the sulfate groups were shown to be located on the 2-amino-2-deoxy-D-glucose residue[1], from the fact that enzymic hydrolysis of **1** furnished the disulfate **4**. Furthermore, this hexosamine residue comprises the reducing end-unit, because it is reduced by sodium borohydride[1].

There was no detectable oxidation[1] of **1** by aqueous sodium periodate during 22 h.

The p.m.r. spectrum of **1** (3 mg) in deuterium oxide (0.5 ml) was recorded by multiple scanning: although not as well resolved as in Fig. 1A, signal UH-4 was clearly discernable. The solution was concentrated; the residue was dissolved in a mixture of tritiated water (0.2 ml) and 90% formic acid (0.2 ml), heated for 1 h at 95°, and the solvent was evaporated off. Re-examination of the p.m.r. spectrum (as above) now showed absence of a low-field signal in the region of UH-4. Paper-chromatographic examination of the product of the acid-treatment, coupled with radioautography, showed that tracer had been incorporated into the product (which then however, consisted of a mixture of unidentified components).

Barium salt of **1**. — To a well-stirred solution of **1** (10 mg) and barium bromide (9 mg, 2.5 equiv.) in water (0.2 ml), ethanol was added dropwise to the turbidity point (*ca* 30% ethanol). A greyish precipitate (A) was centrifuged off and more ethanol added to the clear supernatant solution (finally *ca* 40% ethanol), affording a white solid (B). The latter, after dissolution in water (0.1 ml) containing barium bromide (2 mg), was reprecipitated with ethanol (0.5 vol.), washed with 75% ethanol, ethanol, and dried (fraction B, 4.5 mg); the product was a white, birefringent powder. Fractions A and B were indistinguishable electrophoretically, but B was deemed more suitable for analysis.

Anal. Calc. for $C_{12}H_{15}Ba_2NO_{19}S_3$: N, 1.58. Found: N, 1.7.

Preparation of disaccharide **2** *from chondroitin 4-sulfate A*. — A procedure similar to that of Suzuki and coworkers[4,25,26] was used. Chondroitin 4-sulfate (100 mg) in Tris buffer (5 ml) was incubated at 37° with 20 units of "Chondroitinase ABC" (Miles Laboratories, Elkhart, Indiana). After 2 h another 100 mg of the polysaccharide was added, and two further additions of 50 mg each were made subsequently at intervals of 2 h. Examination of the u.v. absorption spectrum of the digest, at each stage, showed that there was little further enhancement of absorbance after the addition of the second 50 mg of polysaccharide. After a further 12 h of incubation, the digest was heated for 10 min at 95° and then centrifuged. Paper-chromatographic examination (solvents *A* and *B*) of the clear solution indicated the presence of only one major product, the latter showing strong u.v. absorption and being detectable with silver nitrate. The solution was treated with Amberlite IR-120 (H^+ form), and then neutralized with sodium hydrogen carbonate, and freeze-dried; yielding* 284 mg of a white powder (2); $\lambda_{max}^{H_2O}$ 231 nm (ε 3050).

*Methyl 2,3-di-*O*-acetyl-4-deoxy-6-*aldehydo-β-L-threo*-hex-4-enodialdo-1,5-pyranoside* (7). — Methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranoside[27] (0.98 g, 3 mmoles) was dissolved in dry methyl sulfoxide (10 ml) and triethylamine (4.1 ml, 40 mmoles) was added. To the well-stirred solution pyridine–sulfur trioxide complex (3.2 g, 20 mmoles) in methyl sulfoxide (20 ml) was introduced. The reaction mixture turned yellow and the temperature rose to 37°. After 20 min chloroform (50 ml) was added, followed by an icy slurry of a saturated solution of tartaric acid. The chloroform-rich layer was washed successively with cold M sodium hydrogen carbonate and ice–water (twice), dried over sodium sulfate, and then treated with charcoal. On concentration *in vacuo*, it afforded an oil, yield; 0.6618 g (82%). According to t.l.c. (solvents *C* and *D*) the product consisted of only one compound, and its p.m.r. spectrum (Fig. 2B) strongly indicated that only one species was present in significant proportion; $[\alpha]_D$ +309° (*c* 3.31, chloroform); λ_{max}^{MeOH} 246 nm (ε 2,325) λ_{film} 1715 (HC=O); 1652 cm^{-1} (conj. C=C); δ, 9.2 (1 HC=O).

Anal. Calc. for $C_{11}H_{14}O_7$; mol. wt., 258. Found: M^+ 258 (mass spectrometry).

2,4-Dinitrophenylhydrazone of **1**. — The oily aldehyde **7** (65 mg), dissolved in methanol (10 ml), was added to a hot solution of 2,4-dinitrophenylhydrazine (100 mg) in methanol (30 ml). Acetic acid (5 drops) was then introduced, and the solution kept for 30 min near its boiling point. Crystals that formed were recovered, washed with cold, aqueous methanol, and recrystallized from methanol; m.p. 179–181°; λ_{max} 3290 (N-H), 1750 (acetyl C=O), 1615 (C=N), 1515, 1340 cm^{-1} (NO_2, asym. and sym., resp.)

Anal. Calc. for $C_{17}H_{18}N_4O_{10}$: C, 46.57; H, 4.10; N, 12.8; mol. wt., 438. Found: C, 46.3; H, 4.3; N, 13.0, M^+, 438 (mass spectrometry).

*Methyl 2,3-di-*O*-acetyl-4-deoxy-6-*aldehydo-α-L-threo*-hex-4-enodialdo-1,5-pyranoside* (6). — Methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside[27] (0.94 g, 3 mmoles) was oxidized, as in the preparation of 7, affording an oil; yield, 0.6493 g (83%). According to t.l.c., and its p.m.r. spectrum (Fig. 2A), this product consisted essentially of one compound; $[\alpha]_D$ +32° (6.1, methanol); λ_{max}^{MeOH} 248 nm (ε 2325); 1705 (HC=O), 1650 cm^{-1} (conj. C=C).

Anal. Calc. for $C_{11}H_{14}O_7$: mol. wt. 258. Found: M^+, 258 (mass spectrometry).

2,4-Dinitrophenylhydrazone of **6**. — Prepared as above, the product (recrystallized from ethanol) had m.p. 167–169°; λ_{max} 3280 (N-H) 1750 (acetyl C=O), 1610 (C=N), 1515 and 1340 cm^{-1} NO_2, sym. and asym. resp.).

Anal. Calc. for $C_{17}H_{18}N_4O_{10}$: C, 46.57; H, 4.10; N, 12.8; mol. wt., 438. Found: C, 46.6; H, 3.9; N, 12.9; M^+, 438 (mass spectrometry).

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*Under these conditions, a 78% yield of the disaccharide has been obtained after chromatographic purification (A. Triller and N. Kalant, personal communication).

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P.m.r. spectra were recorded by M. Mazurek, N. Ness, and R. Simoneau.

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