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Selectively *O*-acylated glycosaminoglycan derivatives *

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ABSTRACT

Glycosaminoglycans, particularly heparin and heparin fragments, were specifically *O*-acetylated by use of tetrabutylammonium or tributylammonium salts of the anionic polysaccharides, carboxylic acid anhydrides, and 4-dimethylaminopyridine in an homogeneous way in *N*,*N*-dimethylformamide. The number of acyl chains introduced on the carbohydrate backbone was determined either after transesterification and quantitative analysis of the butyl esters thus obtained by GLC or by ^{1}H NMR spectroscopy.

INTRODUCTION

Heparin and heparin fragments (low-molecular-weight heparin) are currently used in clinics for the prevention and the treatment of venous thrombosis[1]. This antithrombotic activity is related to the anticoagulant properties of the polysaccharide. Heparin also displays several other pharmacological activities[1], such as inhibition of proliferation of arterial smooth muscle cells after injury (one cause of atherosclerosis), inhibition of infection of T-lymphocytes by the AIDS virus, inhibition of tumor heparanase (which degrades endothelium, thus favoring metastasis formation), inhibition of protein kinase C, inhibition of calcium release by inositol triphosphate ..., to name only a few. Most of these activities are not correlated to the anticoagulant activity of heparin but probably to its anionic character and, possibly, to unknown structural features of some of the molecules

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* Dedicated to Professor Jean Montreuil.

present in these heterogeneous preparations. An attractive hypothesis is that heparan sulfate(s) (another glycosaminoglycan closely related to heparin as regards chemical structure) is the natural effector of these activities and that exogenous heparin acts as a pharmacological agent by interfering with this biological system.

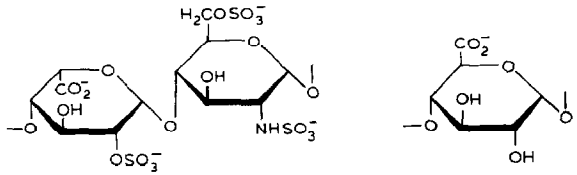
The anticoagulant properties of heparin may, thus, be detrimental to its use in the treatment of other diseases, and chemical modifications of heparin have been described to avoid this problem. *N*-Desulfation, followed by *N*-acylation[2], and also esterification of the carboxyl functions of uronic acid units[3] dramatically decrease or completely abolish the anticoagulant properties depending on the degree to which they are performed. Periodate treatment of heparin also results in fragments inactive on blood coagulation[4].

We undertook the present work on *O*-acylation of heparin and heparin fragments to resolve the following problems: (a) Does this modification preserve or abolish the anticoagulant or the antithrombotic properties of heparin (or both)? (b) What is the influence of this type of derivatization on the pharmacokinetics? (c) Does this type of modification allow oral administration of heparin and heparin derivatives? (d) What is the influence of such modifications on the different pharmacological activities of heparin? We report herein the method of preparation and characterization of selectively *O*-acylated heparin derivatives. The procedure developed can be successfully applied to other glycosaminoglycans or glycosaminoglycan derivatives.

RESULTS AND DISCUSSION

Heparin chains consist of the repetition of 10–50 disaccharide units made up of a uronic acid and a 2-amino-2-deoxy-D-glucose unit. Disaccharide unit **1** [→ 4)-α-L-idopyranosyluronic acid 2-sulfate-(1 → 4)-2-deoxy-2-sulfoamino-α-D-glucopyranosyl 6-sulfate-(1 →] accounts for most of the structure. However, other units, such as, nonsulfated α-L-iduronic acid (**2**), β-D-glucuronic acid (**3**), nonsulfated (**5**) and sulfated 2-acetamido-2-deoxy-α-D-glucose (**4**), and 2-deoxy-2-sulfoamino-α-D-glucose (**8**) and its 3-sulfate (**7**) and 3,6-disulfate (**6**) derivatives also occur to different extents in place of the respective units in **1** depending on the origin of the heparin. In the heparin molecule[5] acyl substituents can be introduced at OH-2 and -3 of the unsubstituted uronic acid units (**2** and **3**), at OH-3 of the sulfated L-iduronic acid unit, and the 2-amino-2-deoxy-D-glucose units of **1** and **4**, as well as at OH-3 and -6 of the 2-amino-2-deoxy-D-glucose units not sulfated at these positions (in **5**, **7**, and **8**). An average of four mmol hydroxyl groups per gram was estimated for various sodium heparinate preparations, and this value was used throughout the study when molar ratios of reagents for heparin derivative preparation were calculated.

Acylation of heparin and other glycosaminoglycans has been previously attempted. In 1947, Bell and Jaques[6] reported the first synthesis of an acetyl derivative of heparin. The compound, however, decomposed in neutral aqueous solution when heated at 70° for 30 min and, therefore, was probably not the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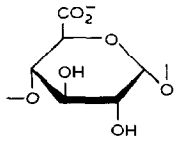
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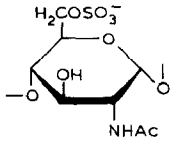
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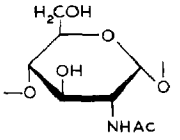
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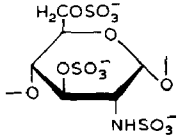
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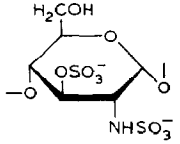
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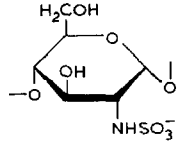
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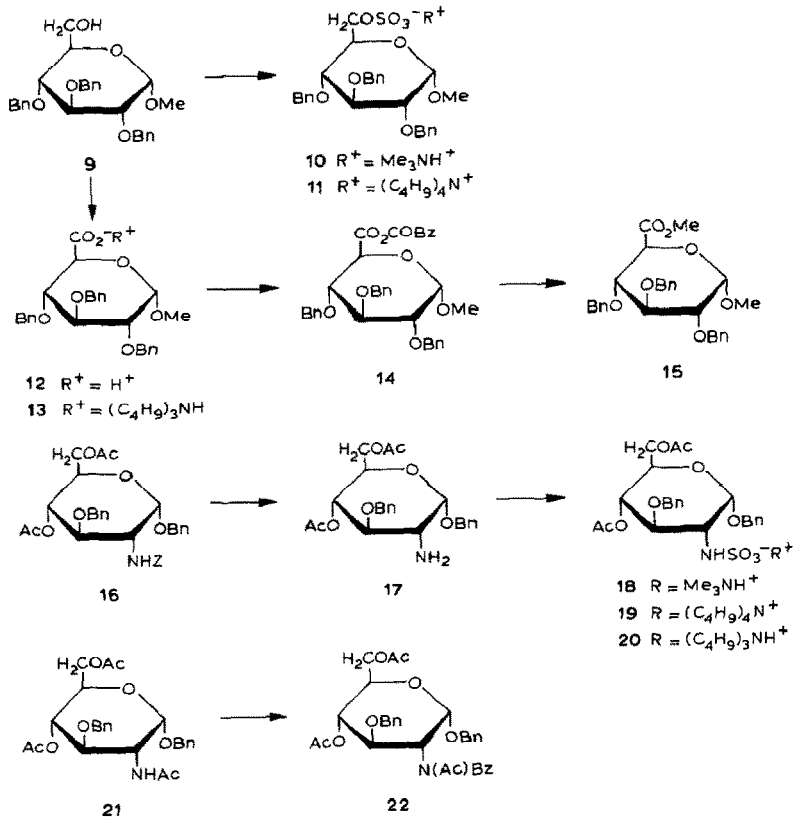
expected ester, but rather the anhydride resulting from the action of ketene on the carboxylate group of heparin, as the esters described in the present work are stable under such conditions. Mardiguian and Fournier[7] used a dichloromethane solution of the "hyamine 1622" salt of heparin which was acylated in the presence of a carboxylic acid and dicyclohexylcarbodiimide. When we attempted to acetylate heparin under similar conditions, we found that the major reaction was not the expected esterification but the formation of the *N*-acylureide of the carboxylate groups of uronic acids. Foley et al.[8] have acylated directly the sodium salt of heparin using the strongly polar solvent formamide and pyridine as a catalyst. We found that only ~ 20% of heparin dissolved in the mixture after 24 h at room temperature, and the temperature had to reach 50° to obtain complete dissolution. Under such conditions, the use of an acid anhydride[9] resulted in low-substituted compounds at room temperature (where the reaction is conducted in an heterogeneous medium), and partial *N*-acylation (with concomittant *N*-desulfation) occurred at 50°. On the other hand, the use of an acid chloride[8] always gave *N*- as well as *O*-acylation, in accordance with results on model compounds reported herein. Complete *N*-acylation can even be performed under the published experi-

mental conditions[8], as shown by the preparation of *N*,*O*-acetylheparin. Considering these results and in order to keep the chain of the starting polysaccharide



intact while obtaining the highest degree of substitution, a series of model monosaccharides bearing different functional groups was investigated under various acylating conditions by TLC and other analytical methods. Methyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside 6-sulfate (**10**), easily prepared from **9** (ref. 10) was used to check that neither acylation by an acid anhydride nor by an acid chloride affected the sulfate group. Acylation of methyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranosiduronic acid (**12**), in the presence of an acid, chloride resulted in the partial formation of a mixed anhydride (**14**) that could be easily characterized through the corresponding methyl ester (**15**). The formation of such anhydrides was not detected when acid anhydrides were used in place of chlorides.

To study the stability of *N*-sulfate groups under *O*-acylating conditions, benzyl 4,6-di-*O*-acetyl-3-*O*-benzyl-2-deoxy-2-sulfoamino-α-D-glucopyranoside (**18**) was synthesized from benzyl 4,6-di-*O*-acetyl-3-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-α-D-glucopyranoside (**16**), obtained from the nonacylated derivative[11], after catalytic removal[12] of the benzyloxycarboxyl protective group, followed by selective

N-sulfation. When **18** was submitted, as the tetrabutylammonium (**19**) or tributylammonium salt (**20**), to acylation using a carboxylic acid anhydride, the product could be recovered quantitatively unchanged. On the contrary, in the presence of benzoyl chloride several minor degradation products were detected by TLC. Similarly, the stability of *N*-acetyl groups towards carboxylic acid anhydrides and chlorides was studied with benzyl 2-acetamido-4,6-di-*O*-acetyl-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside (**21**); no reaction could be detected in pyridine, or in *N*,*N*-dimethylformamide (DMF) in the presence of 4-dimethylaminopyridine. On the contrary, under both reaction conditions, benzoyl chloride led to the rapid formation of the imide **22** which was isolated and characterized.

From these preliminary experiments, it was concluded that smooth *O*-acylation of glycosaminoglycans derivatives could be achieved by use of a tributyl- or tetrabutyl-ammonium salt of the product, DMF as the solvent, and carboxylic acid anhydride as acylating agent in the presence of a catalytic amount of 4-dimethylaminopyridine. These conditions were applied to various heparin preparations and heparin fragments, obtained after controlled nitrous acid or periodate degradation. The products were first converted into their tributyl- or tetrabutyl-ammonium salts by neutralization of the heparinic acid by the corresponding base. In each case, selective *O*-acylation was performed in the presence of different carboxylic acid anhydrides. No sign of *N*-acylation could be detected. In some instances, mixed anhydrides were detected by IR analysis (as a shoulder of the ester band) and by an increase in the sulfate-to-carboxylate ratio. The mixed anhydrides were easily eliminated by treatment with sodium hydrogen carbonate. This did not affect the ester content as shown by treatment of a butanoyl derivative for up to 96 h without loss of butanoyl groups. Finally, the sodium hydrogen carbonate treatment was systematically used as the standard procedure at the end of the acylation reaction, before the isolation steps.

Commercial heparin gave various acylated products, a typical example being the butanoyl derivative, which was isolated salt free after alcohol precipitation in the presence of sodium chloride. A degree of substitution of ~1.5 butanoyl groups per disaccharide unit was routinely obtained by use of 5 mol equiv of anhydride per hydroxyl group, and the yield was almost quantitative. Conductimetric determination[13] of the sulfate and carboxyl content showed a sulfate-to-carboxyl ratio of 2.17, almost identical to that of the starting material (2.16). In the same way, periodate-oxidized heparin fragments were *O*-butanoylated after conversion into the tributylammonium salt, and an almost quantitative yield was obtained.

The use of various amounts of acid anhydride gave derivatives substituted to different degrees (Table I). Such control of the experimental conditions would be hardly possible in a heterogeneous reaction medium[8,9]. The rate and degree of acylation were also investigated at 25 and 50° over a period of several days with 5 and 10 mol equiv. of anhydride per hydroxyl group (Table I). The results indicate that the degree of substitution can be controlled up to a limit of ~1.5–1.7 acyl group per disaccharide unit, i.e., 75% of the theoretical degree of substitution for the fragment used (essentially a polymer of **1**).

TABLE I

Degree of acylation (number of acyl groups/disaccharide unit) of heparin fragments under different experimental conditions [a]

Time (h)	Acyl groups/disaccharide unit		
	25°		50°, 5 equiv
	5 equiv	10 equiv	
0.5			0.67
1			0.88 (0.93)
2			1.01 (1.07)
4	0.95 (0.97)	0.86	1.28 (1.05)
8	0.99 (1.13)	1.00 (1.08)	1.40 (1.39)
24	1.21 (1.30)	1.33 (1.28)	1.47 (1.54)
48	1.29 (1.45)	1.49 (1.53)	1.65 (1.60)
72	1.45 (1.55)	1.48 (1.57)	
96	1.39 (1.65)	1.49 (1.54)	

[a] The reactions were carried out at 25° with 5 and 10 mol equiv of anhydride per OH group and at 50° with 5 mol equiv. The degree of acylation was determined by GLC as described in the Experimental section, or by ^{1}H NMR spectroscopy (values in parentheses) by integration of the acyl group signals or of the ring proton signals.

The pilot experiments on monosaccharides suggested that the side reactions which would occur during the acylation procedure are likely to affect the sulfate (particularly *N*-sulfate) and carboxylate groups. In order to monitor that no such reaction had taken place, the sulfate and carboxylate content was investigated by conductimetry[13]. ^{13}C NMR spectroscopy also provided an efficient detection for *N*-desulfation and *N*-acylation. Comparison of the spectra of *N,O*- and *N*-hexanoyl derivatives of periodate-oxidized heparin with the spectrum of the selectively *O*-hexanoyl derivative, prepared as described for the *O*-butanoyl derivative, clearly indicated that *N*-acylation had taken place in the *N,O*-hexanoyl derivative, in the preparation of which an acid chloride had been used instead of an anhydride (Fig. 1). Conductimetry analysis and ^{13}C NMR experiments, thus, clearly indicated that only *O*-acylation occurs when an anhydride is used and *N*-acylation when an acid chloride replaces the anhydride.

The degree of substitution of the product, i.e., the number of acyl radicals introduced per basic disaccharide unit could be precisely monitored by ^{1}H NMR spectroscopy at 308 K to shift the water signal. Under these conditions, it was possible to integrate the proton signals of the acyl chain on the one hand, and of the carbohydrate backbone on the other. This method is more reliable than the GLC method[14,15], based on transesterification, which does not take into account the water content of the product.

The standard acylation procedure was successfully applied to other glycosaminoglycans[16], dermatan sulfate, heparan sulfate, chondroitin 4- and 6-sulfate, their derivatives, such as esters, and *N*-acylated compounds. The experimental conditions described herein for the preparation of *O*-acetyl, *O*-butanoyl, *O*-

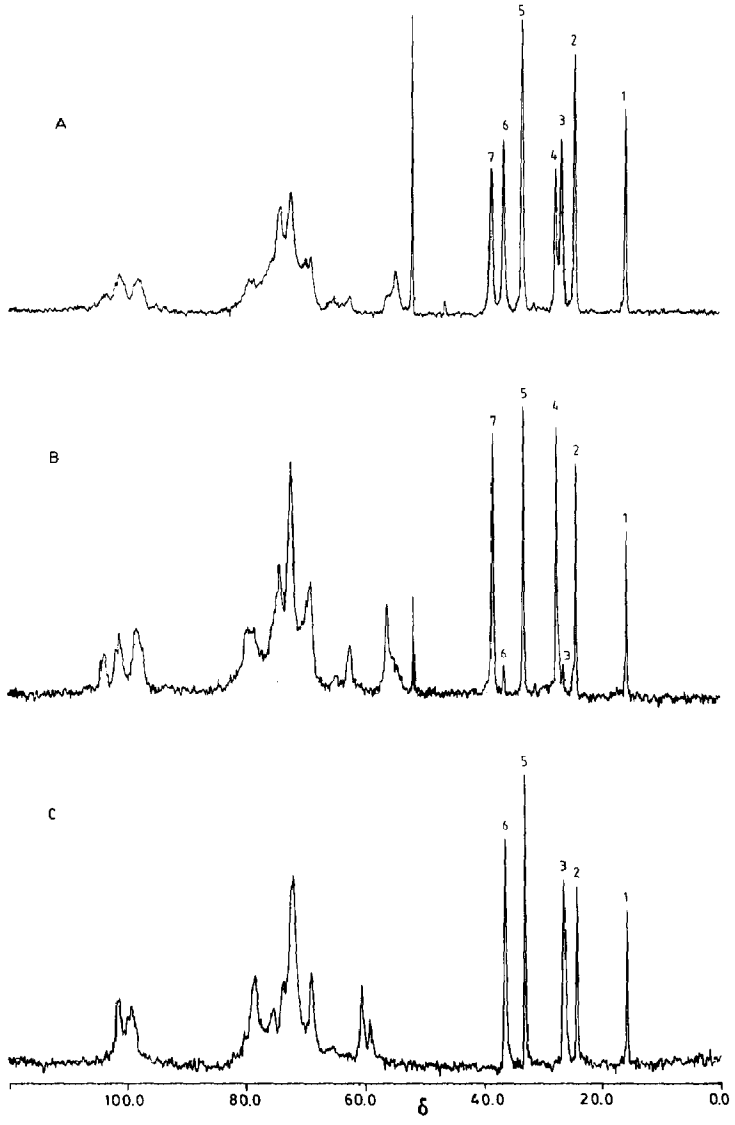


Fig. 1. ^{13}C NMR spectra (region δ 10–40) of: (A) *N,O*-hexanoylheparin fragments; (B) *N*-hexanoylheparin fragments; and (C) *O*-hexanoylheparin fragments. *N*-Substitution was easily detected through signals 4 and 7, at a field lower than that of their counterparts in *O*-acetylated compounds. Signal assignments: 1, C_ϵ; 2, C_δ; 3, $C_{\gamma O}$; 4, $C_{\gamma N}$; 5, C_β; 6, $C_{\alpha O}$; and 7, $C_{\alpha N}$ (α, β, γ, δ, and ϵ are locations from the carboxyl group).

hexanoyl, and *O*-succinoyl derivatives also led to similar results when other carboxylic acid anhydrides were used[16].

EXPERIMENTAL

Methods.—Melting points were determined with a Mettler FP62 capillary apparatus and are uncorrected. Optical rotations were measured at $20 \pm 2°$ with a

Perkin–Elmer 241 polarimeter. ^{1}H NMR spectra were recorded with a Brucker AC-100 or AC-200 instrument. The values (δ) are expressed downfield from the signal of internal Me_4Si (for solutions in $CHCl_3$) or of internal Na 4,4-dimethyl-4-silapentanoate (for solutions in D_2O). For ^{13}C NMR spectra, MeOH was the internal reference (δ 51.6 from internal Na 4,4-dimethyl-4-silapentanoate). Reactions were monitored by TLC on Silica Gel 60 F_{254} (Merck). Silica gel column chromatography was performed on Silica Gel 60 (230–400 mesh, Merck). Sephadex G-25 and LH-20 were obtained from Pharmacia AB. The conditions for GLC were: injector (splitless) temperature, 250°; detector (flame-ionization) temperature, 270°; column (30m × 0.53 mm) coated with 2 μm OV1; column temperature, 60° during 30 s, and then 10°/min until 240°; carrier gas, He at a flow rate of 3.5 mL/min. The conductimetry determinations used a Radiometer CDM83 conductimeter equipped with an AB480 autoburette and a TTT85 titrator.

Determination of acyl group content by GLC.—The substance to be analyzed (2 mg) was dissolved in a solution of butanol (250 μL) containing 20 mM propanoic, pentanoic, and nonanoic acids as internal standards. Concentrated H_2SO_4 (50 μL) was added and the tube was sealed and heated at 100° for 1 h. After being cooled, the contents of the tube was diluted with $CHCl_3$ (5 mL) and, after washing with water (3 × 20 mL), the solution (0.5 μL) was injected into the chromatograph.

Trimethylammonium (**10**) *and tetrabutylammonium (methyl 2,3,4-tri-*O*-benzyl-6-*O*-sulfo-α-*D*-glucopyranoside)* (**11**).—To a solution of methyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside[10] (**9**) (7 g, 15 mmol) in DMF (70 mL) was added SO_3–Me_3N complex (5.25 g, 37.5 mmol), and the solution was heated at 50° for 1 h. After cooling, MeOH (5 mL) was added, and after 15 min the solution was concentrated to a final volume of 30 mL, diluted with 1:1 (v/v) MeOH–CHCl, (50 mL), and chromatographed on a Sephadex LH20 column (120 × 3 cm), eluted with the same MeOH–$CHCl_3$ mixture to give **10** as a syrup (7.15 g, 79%); $[\alpha]_D$ + 21° (*c*1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 2.8 [s, 9 H, $HN^+(CH_3)_3$], 3.36 (s, 3 H, OMe), 4.56 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), and 7.2–7.4 (m, 15 H, 3 Ph). No satisfactory elemental analysis could be obtained.

The tetrabutylammonium salt (**11**) was obtained after dissolution of **10** in 10:1 (v/v) MeOH–water, passage through a column of Dowex 50 H^+ cation-exchange resin, equilibrated in the same solvent, neutralization by tetrabutylammonium hydroxide, and evaporation (quantitative yield).

*(Methyl 2,3,4-tri-*O*-benzyl-α-*D*-glucopyranosid)uronic acid* (**12**).—To a solution of **9**[10] (7 g; 15 mmol) in acetone (110 mL) was added dropwise over 10 min a solution of CrO_3 (4.3 g) in 3.5 M H_2SO_4 (18.3 mL). After 1 h, $CHCl_3$ (200 mL) was added and the organic phase was separated, dried (Na_2SO_4), and concentrated. Purification was achieved through silica gel chromatography [1:20 and 1:10 (v/v) MeOH–$CHCl_3$], followed by Sephadex LH20 chromatography [1:2 (v/v) MeOH–$CHCl_3$] to give **12** as a syrup (4.1 g, 57%); $[\alpha]_D$ + 3° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 3.40 (s, 3 H, OMe), 3.57 (dd, 1 H $J_{1,2}$ 3.5, $J_{2,3}$ 9.6 Hz, H-2), 3.70 (dd, 1 H, $J_{3,4}$ 8.9, $J_{4,5}$ 10.1 Hz, H-4), 4.02 (dd, 1 H, H-3), 4.20 (d, 1 H, H-5), 4.62 (d, 1 H,

H-1), and 7.2–7.4 (m, 15 H, 3 Ph). No elemental analysis was performed. Diazomethane in ether quantitatively converted it into the known[10] methyl ester **15**.

*Benzyl 4,6-di-*O*-acetyl-3-*O*-benzyl-2-benzyloxycarbonylamino-2-deoxy-α-*D*-glucopyranoside* (**16**).—This compound was obtained by acetylation (pyridine, acetic anhydride) of benzyl 3-*O*-benzyl-2-benzyloxycarbonylamino-2-α-D-glucopyranoside[11]; mp 135°; $[\alpha]_D$ + 82° (*c* 1, $CHCl_3$); ^{1}H NMR [$(CD_3)_2SO$]: δ 1.97 and 2.02 (2s, 6 H, 2 OAc), 3.77 (ddd, 1 H, $J_{2,NH} = J_{2,3} = 9$, $J_{1,2}$ 3.4 Hz, H-2), 3.85 (t, 1 H, $J_{3,4}$ 9 Hz, H-3), 3.88 (ddd, 1 H, $J_{5,6a}$ 2.2, $J_{5,6b}$ 5.1, $J_{4,5}$ 9.5 Hz, H-5), 3.97 (dd, 1 H, $J_{6a,6b}$ −12.2 Hz, H-6a), 4.11 (dd, 1 H, H-6b), 4.88 (t, 1 H, H-4), 4.89 (d, 1 H, H-1), 7.1–7.5 (m, 15 H, 3 Ph), and 7.75 (d, 1 H, NH).

Anal. Calcd for $C_{32}H_{35}NO_9$: C, 66.54; H, 6.11; N, 2.42. Found: C, 66.77; H, 6.16; N, 2.32.

*Benzyl 4,6-di-*O*-acetyl-2-amino-3-*O*-benzyl-2-deoxy-α-*D*-glucopyranoside* (**17**).—To a solution of **16** (1.15 g, 2 mmol) in DMF (20 mL) were added 10% Pd–C catalyst (0.6 g) and ammonium formate (1.39 g, 22 mmol). After 20 min, the suspension was filtered and the filtrate concentrated. The syrupy residue was dissolved in $CHCl_3$, washed with water, dried (Na_2SO_4), and evaporated to yield quantitatively **17** (0.88 g, 100%); $[\alpha]_D$ + 84° (*c* 1, $CHCl_3$); ^{1}H NMR (D_2O): δ 2.0, 2.1 (2s, 6 H, OAc), 2.9 (dd, 1 H, $J_{1,2}$ 3.4, $J_{2,3}$ 9.9 Hz, H-2), 3.6 (t, 1 H, $J_{2,3} = J_{3,4} = 9.9$ Hz, H-3), 4.98 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), 5.02 (dd, 1 H, $J_{3,4} = J_{4,5} = 9.9$ Hz, H-4), and 7.2–7.4 (m, 10 H, 2 Ph).

Anal. Calcd for $C_{24}H_{29}NO_7$: C, 64.99; H, 6.59; N, 3.15. Found: C, 65.10; H, 6.57; N, 2.93.

*Benzyl 4,6-di-*O*-acetyl-3-*O*-benzyl-2-deoxy-2-sulfoamino-α-*D*-glucopyranoside* (**18**).—Compound **17** (0.88 g, 2.2 mmol) was sulfated in DMF (20 mL) with SO_3–Me_3N complex (1.36 g, 7.5 mmol) overnight at room temperature. Methanol (20 mL) was then added, followed 24 h later, by $CHCl_3$ (20 mL). The solution was layered on top of a Sephadex LH-20 chromatography column, equilibrated and eluted with 1:1 (v/v) MeOH–$CHCl_3$. After evaporation, **18** was obtained in almost quantitative yield, slightly contaminated with starting material. A pure product could be prepared by silica gel chromatography in 1:1 (v/v) $CHCl_3$–MeOH; $[\alpha]_D$ + 42° (*c* 1, $CHCl_3$); ^{1}H NMR [Na salt; $(CD_3)_2SO$]: δ 1.92 and 2.01 (2s, 6 H, 2 OAc), 3.32 (ddd, 1 H, $J_{1,2}$ 3.4, $J_{2,3}$ 10.5, J_{NH} 10.2 Hz, H-2), 3.54 (dd, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 3.80 (ddd, 1 H, $J_{5,6a}$ 2.7, $J_{5,6b}$ 4.9, $J_{4,5}$ 10.2 Hz, H-5), 3.93 (dd, 1 H, $J_{6a,6b}$, 12.2 Hz, H-6a), 4.06 (dd, 1 H, H-6b), 4.05 (d, 1 H, NH), 4.49 and 4.66 (AB system, $J_{A,B}$ −11.7 Hz, PhCH_2), 4.56 and 4.83 (AB system, $J_{A,B}$ −11.7 Hz, PhCH_2) 4.85 (dd, 1 H, H-4), 5.33 (d, 1 H, H-1), and 7.2 and 7.5 (m, 10 H, 2 Ph). No satisfactory elemental analysis could be obtained. Compound **18** was converted into the tetrabutylammonium (**19**) or the tributylammonium (**20**) salt as described for **11**.

*Reaction of benzyl 3,4,6-tri-*O*-acetyl-2-(*N*-benzoylacetamido)-2-deoxy-α-*D*-glucopyranoside* (**22**).—To a solution of **21** (110 mg, 0.25 mmol) in DMF (1 mL) were successively added benzoic chloride (0.15 mL, 1.25 mmol), 4-dimethylaminopyridine (7.6 mg, 0.06 mmol), and tributylamine (0.3 mL, 1.25 mmol). The solution was

kept under Ar for 24 h and monitoring by TLC in 5:1 (v/v) $CHCl_3$–EtOAc indicated a faster moving spot. Compound **22** was isolated and characterized by ^1H NMR ($CDCl_3$): δ 1.83, 1.95, 2.01, (3s, 9 H, 3 OAc), 2.09 (s, 3 H, $NCOCH_3$), 3.97 (dd, 1 H, $J_{6a,6b}$ −12.3, $J_{5,6a}$ 2.1 Hz, H-6a), 4.04 (ddd, 1 H, $J_{4,5}$ 10.4, $J_{5,6b}$ 4.3 Hz H-5), 4.12 and 4.51 (AB system, $J_{A,B}$ −11.5 Hz, $PhCH_2$), 4.28 (dd, 1H, H-6b), 5.08 (dd, 1 H, $J_{3,4}$ 8.9 Hz, H-4), 5.11 (dd, 1 H, $J_{2,3}$ 10.5, $J_{1,2}$ 3.5 Hz, H-2), 5.15 (d, 1 H, H-1), 6.04 (dd, 1 H, H-3), and 6.8–7.8 (m, 10 H, 2 Ph). Formation of the same compound was also observed when the reaction was performed in pyridine in the presence of benzoyl chloride.

Heparin tributylammonium salt.—The preparation of heparin used was from pig mucosa and had the following characteristics: SO_3^-, 3.70 mequiv/g; CO_2^-, 1.70 mequiv/g; and SO_3^-/CO_2^-, 2.16:1.0. A solution of this heparin Na salt (10 g) in water (500 mL) was percolated through a column (300 mL) of Dowex 50 (H^+) cation-exchange resin at 4°. The pH of the solution was adjusted to 6 by addition of tributylamine (~ 50 mL), and the excess tributylamine was eliminated by evaporation. The volume of the solution was adjusted to 900 mL with water and the tributylammonium salt was isolated (21 g) after lyophilization and drying for 24 h at 50° under vacuum.

O-Butanoyl derivative.—A solution of dry tributylammonium salt (21 g corresponding to ~ 40 mequiv. of OH groups) in dry DMF (200 mL) was kept under Ar and cooled to 0°. 4-Dimethylaminopyridine (1.22 g, 10 mmol), butyric anhydride (32.7 mL, 200 mmol), and tributylamine (47.6 mL, 200 mmol) were successively added, and the reaction was allowed to proceed at room temperature for 24 h. After cooling to 0°, 5% $NaHCO_3$ in water (400 mL) was gradually added and the solution was stirred at room temperature for 48 h. Excess $NaHCO_3$ was eliminated by progressive addition of aq M HCl (~ 420 mL) until pH 4, and M NaOH (~ 275 mL) to pH 7. Cold EtOH (6 L, 5 vol) was added under stirring. After decantation, the precipitate was dissolved in 0.2 M NaCl (200 mL), and the precipitation procedure was repeated with EtOH (1 L). The precipitate was centrifuged off, dissolved in apyrogenic water (200 mL), and passed through a column (300 mL) of Dowex 50 (H^+) cation-exchange resin at 4°. The acid was neutralized with M NaOH and the solution filtered through a 0.22-μm pore filter. After lyophilization, *O*-butanoylheparin (9.15 g) was obtained as a white powder; $[\alpha]_D$ + 34° (*c* 1, H_2O); ν_{max}^{KBr} 1736 (ester), 2970, and 2940 cm^{-1} (aliphatic); ^1H NMR (D_2O): δ 1.04, 1.71, and 2.49 (CH_3 and CH_2 of But; But/basic disaccharide unit, 1.7). Conductimetry analysis: SO_3^-, 3.06 mequiv/g; CO_2^-, 1.41 mequiv/g; SO_3^-/CO_2^-, 2.17:1.0 (starting material SO_3^-/CO_2^-, 2.16:1.0).

Periodate-oxidized heparin fragments (IC 86 1772), tributylammonium salt.—Periodate-oxidized heparin fragments (IC 86 1772) were obtained after β-elimination and borohydride reduction of periodate-oxidized heparin as described by Lormeau et al.[4], and had mol wt 6000, range 1000–9000; OSO_3^- 3.85 mequiv/g; CO_2^- 1.60 mequiv/g; OSO_3^-/CO_2^-, 2.40:1.0. The tributylammonium salt of IC 86 1772 was prepared as described for heparin[2] (8.3 g were obtained from 5 g of Na salt).

O-Butanoyl derivative of periodate-oxidized (IC 86 1772) heparin fragments.—This derivative was prepared from the tributylammonium salt as described for heparin (5.7 g were obtained from 5 g of Na salt); $[\alpha]_D$ +42° (*c* 1, H_2O); ν_{max}^{KBr} 1735 (ester), 2970, and 2940 cm^{-1} (aliphatic); ^1H NMR (D_2O): δ 0.95, 1.62, and 2.49 (CH_3 and CH_2 of But; But/basic disaccharide unit, 1.40). Conductimetry analysis: SO_3^-, 3.11 mequiv/g; CO_2^-, 1.36 mequiv/g; SO_3^-/CO_2^-, 2.29:1.0.

O-Butanoylation of IC 86 1772 to different degrees.—To a series of solutions of the dry tributylammonium (1 g, corresponding to ~2.4 mequiv of OH groups) in anhyd. DMF (10 mL) were successively added at 0° under Ar, 4-dimethylaminopyridine (73 mg, 0.5 mmol), butyric anhydride, and tributylamine (in amounts corresponding from 0.125 to 2 mol equiv/OH group). The reaction proceeded for 24 h at room temperature, and the product was isolated as described for heparin. The acylation degrees were determined by ^1H NMR and by GLC after transesterification. Results are reported in Table I.

Low-molecular-weight heparin (CY 216) tributylammonium salt and O-butanoyl derivative.—Low-molecular-weight heparin CY 216 (mol wt range, 1800–8000; 7.5 g) was obtained from Sanofi Pharma Industry and converted into the tributylammonium salt as described for heparin. This was butanoylated under the conditions described for the preparation of *O*-butanoylheparin. After lyophilization, a white powder was obtained (7.65 g); $[\alpha]_D$ + 35° (*c* 1, H_2O); ν_{max}^{KBr} 1736 (ester) and 2970 cm^{-1} (aliphatic); ^1H NMR (D_2O): δ 1.03, 1.70, and 2.51 (CH_3 and CH_2 of But; But/basic disaccharide unit, 1.76). Conductimetry analysis: SO_3^-, 2.86 mequiv/g; CO_2^-, 1.33 mequiv/g; SO_3^-/CO_2^-, 2.15:1.0.

Dermatan sulfate tributylammonium salt and O-succinyl dermatan sulfate.—The tributylammonium salt was prepared as described for heparin; (8.4 g from 5 g of dermatan sulfate Na salt; SO_3^-, 2 mequiv/g; CO_2^-, 2 mequiv/g; SO_3^-/CO_2^-, 1.0:1.0. To a solution of this salt (1 g, corresponding to 3.6 mequiv of OH groups) in dry DMF (18 mL) under Ar were successively added 4-dimethylaminopyridine (110 mg, 0.9 mmol), succinic anhydride (400 mg, 4 mmol), and tributylamine (0.94 mL, 4 mmol). The reaction was allowed to proceed at 60° for 2 h. Water (2 mL) was added, followed by a cold M ethanolic solution of NaOAc (170 mL). After centrifugation the solid was dissolved in water and dialysed against 5% aq $NaHCO_3$ during 2 days followed by water during 3 days. After ultrafiltration, the Na salt was obtained (0.83 g); $[\alpha]_D$ −52° (*c* 1, H_2O); ν_{max}^{KBr} 1732 cm^{-1} (ester); ^{13}C NMR (D_2O; internal MeOH, δ 51.6): δ 33.0 and 33.5 (CH_2 of succinyl). Conductimetry analysis: SO_3^-, 1.44 mequiv/g; CO_2^-, 2.80 mequiv/g; SO_3^-/CO_2^-, 0.51:1.0; succinyl group/basic disaccharide unit, 0.6.

Periodate-oxidized heparin fragments (IC 86 1772) tetrabutylammonium salt, benzyl ester, and O-butanoyl benzyl ester.—A solution of periodate-oxidized heparin fragments[4] (Na salt; 10 g) was percolated through a column (300 mL) of Dowex 50 (H^+) cation-exchange resin. The pH of the solution was adjusted to 8.2 with an aqueous solution of tetrabutylammonium hydroxide, and lyophilization and drying at 50° for 24 h under high vacuum gave the tetrabutylammonium salt as a

white powder (22 g). Conductimetry analysis: SO_3^-, 1.80 mequiv/g; CO_2^-, 0.74 mequiv/g; SO_3^-/CO_2^-, 2.43 : 1.0.

To a solution of this (10.5 g, corresponding to 7.8 mequiv of OH group) in dry DMF (100 mL) was added benzyl bromide (4.6 mL, 39 mmol) and the solution was kept at room temperature for 24 h.

Part of it (28 mL) was mixed with 0.4 M NaCl (27 mL) and cold EtOH (150 mL) was added. The precipitate was collected overnight at 4°, washed with EtOH, and dissolved in water (20 mL). The product was converted into the Na salt by passage through a column of Dowex 50 (H^+) cation-exchange resin and neutralization with NaOH. Lyophilization gave the benzyl ester (0.89 g); $[\alpha]_D + 38°$ (*c* 1, H_2O); ^{13}C NMR (D_2O; internal MeOH, δ 51.6): δ 131.5 (arom.) and 94.2 (benzylic CH_2). Conductimetry analysis: SO_3^-, 3.35 mequiv/g; CO_2^-, 0.40 mequiv/g.

To the rest of the mixture (108 mL) were added butanoic anhydride (13.1 mL, 80 mmol), tributylamine (19 mL, 80 mmol), and 4-dimethylaminopyridine (490 mg, 4 mmol), and the reaction was allowed to proceed at room temperature. After 24 h 0.4 M NaCl (130 mL) was added, and the product was precipitated with EtOH (1 L). The solid was dissolved in 0.2 M NaCl (80 mL) and EtOH (400 mL) was added. The solid was collected, dissolved in water, passed through a column of Dowex 50 (H^+) cation-exchange resin, and neutralized with NaOH to give the *O*-butanoyl benzyl ester (3.44 g) after lyophilization; $[\alpha]_D + 37°$ (*c* 1, H_2O); ^{13}C NMR (D_2O; internal MeOH, δ 51.6): δ 131.5 (arom.) and 94.2 (benzylic CH_2). Conductimetry analysis: SO_3^-, 3.4 mequiv/g; CO_2^-, 0.40 mequiv/g.

N,O-*Hexanoyl periodate-oxidized and* N-*hexanoyl periodate-oxidized heparin fragments.*—To a solution of the tributylammonium salt of periodate-oxidized heparin fragments (0.375 g, corresponding to 1 mequiv OH group) in dry DMF (3 mL) were successively added 4-dimethylaminopyridine (30.5 mg, 0.25 mmol), triethylamine (0.2 mL, 1.5 mmol), and M hexanoyl chloride in DMF (5 mL). After 24 h, triethylamine was added (1 mL), followed by a 5% $NaHCO_3$ solution (8 mL), and cold EtOH (200 mL). The precipitate was collected after centrifugation, dissolved in water, and desalted by passage through a column of Sephadex G-25 eluted with water. The fractions containing the acylated derivative were pooled. The product was converted into the Na salt by passage through a column of Dowex 50 (H^+) cation-exchange resin, followed by neutralization with M NaOH, and lyophilized to give the *N,O*-hexanoyl derivative (230 mg); ^{13}C NMR (D_2O; internal MeOH, δ 51.6): δ 15.91, 24.38, 26.60, 27.67, 33.27, 36.49, and 38.50 [*N*- and *O*-$CH_3(CH_2)_4CO$]. Conductimetry analysis: OSO_3^-, 2.50 mequiv/g; CO_2^-, 1.35 mequiv/g; OSO_3^-/CO_2^-, 1.85 : 1.0.

A solution of the derivative (70 mg) in 0.5 M NaOH (2 mL) was kept during 4 h at room temperature, then layered on top of a Sephadex G-25 column (70 × 3 cm), and eluted with water. Fractions containing the hydrolyzed material were pooled to give the *N*-hexanoyl derivative (58.5 mg, after lyophilization); ^{13}C NMR (D_2O; internal MeOH, δ 51.6): δ 15.93, 24.28, 27.59, 33.21, and 38.42 [*N*-$CH_3(CH_2)_4CO$]; the signals for *O*-hexanoyl groups were obtained from an *O*-hexanoyl compound: δ 15.61, 23.97, 26.25, 32.85, and 36.18.

N,O-*Acetyl heparin.*—Heparin Na salt was treated as described by Foley et al.[8]. The salt (2 g) was suspended in dry formamide (24 mL) under Ar at 40°. Acetyl chloride (2 mL, 28 mmol) was added slowly over a 4-h period and the agitation was continued overnight at room temperature. Water (50 mL) was then added under agitation and the solution was dialyzed against 1% NaCl for 72 h with three changes of solution, and then against water. The content of the dialysis bag was lyophilized to give *N,O*-acetylheparin (1.99 g); ^{13}C NMR (D_2O; internal MeOH, δ 51.6): δ 23.18 ($OCOCH_3$), 24.69 ($NCOCH_3$), 54.50 [C-2 of (3-*O*-Ac)GlcNAc], 56.07 (C-2 of GlcNAc), 58.98 [C-2 of (3-*O*-Ac)GlcNSO$_3^-$], and 60.58 (C-2 of GlcNSO$_3^-$).

In another experiment under the same conditions but with 40 mL of acetyl chloride (562 mmol), only (3-*O*-Ac)GlcNAc was obtained; ^{13}C NMR (D_2O; internal MeOH, δ 51.6): δ 22.97 ($OCOCH_3$), 24.59 ($NCOCH_3$), and 54.49 [C-2 of (3-*O*-Ac)GlcNAc].

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