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Novel regio- and stereoselective O-6-desulfation of the glucosamine moiety of heparin with *N*-methylpyrrolidinone–water or *N,N*-dimethylformamide–water mixtures

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Abstract

The degree of completeness and selectivity of the solvolytic O-6-desulfation reactions of the glucosamine moiety adjacent to the 2-*O*-sulfoiduronic acid group of heparin was systematically studied. Using solutions of various ammonium salts of heparin (salts of tributylamine, quinoline and pyridine) in mixtures of 9:1 aprotic solvents and water (solvents of medium polarity, in order of decreasing polarity: $Me_2SO > Me_2NCHO > Me_2NAc >$ *N*-methylpyrrolidinone), the influence of different reaction conditions were studied. The ammonium salt of heparin with a strong base (e.g., tributylamine) in Me_2SO showed almost no desulfation, while in Me_2NCHO a relatively low degree of completeness of O-6-desulfation (30%) with moderate selectivity (15% [I-2(OS)]-desulfation) was observed. Weak bases like quinoline or pyridine in Me_2SO–water resulted in nearly complete [A-6(OS)]-desulfation (95 and 94%, respectively) with low selectivity [I-2(OS)]-desulfation (49 and 35%, respectively). The heparin pyridinium salt in Me_2NCHO–water showed both a relatively high degree of completeness and high selectivity (72% [A-6(OS)]- and 8% [I-2(OS)]-desulfation). The highest regioselectivity (i.e., a high degree of completeness accompanied by high selectivity) was achieved using an *N*-methylpyrrolidinone–water mixture (88% [A-6(OS)]-desulfation and 10% [I-2(OS)]-desulfation). A nearly complete O-6-desulfation (95%), accompanied by a lower selectivity (18% [I-2(OS)]-desulfation), was achieved when the reaction was carried out twice. Lower temperature improved selectivity (5% [I-2(OS)]-desulfation) but reduced the completeness of [A-6(OS)]-desulfation (72%). In comparison with the variety of O-6-desulfations reported to date, the novel reactions presented in this article led to remarkable increase in completeness and regioselectivity of the reactions that were investigated.

Keywords: Heparin; Desulfation; O-6-Desulfation

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Abbreviations: I, α-L-idopyranosyluronic acid residue; A, 2-deoxy-2-sulfamido-α-D-glucopyranosyl residue. [A-6(OS)] refers to the 6-*O*-sulfo group of the 2-amino-2-deoxy-α-D-glucopyranosyl moiety, and [I-2(OS)] refers to the 2-*O*-sulfo group of the α-L-idopyranosyl-uronic acid moiety.

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1. Introduction

During the last 20 years considerable effort has been made to obtain different chemically modified heparins by polymer-modification reactions. Up until now there have been reported several important reactions for selective N- and O-desulfation of heparin. Solvolytic hydrolysis for selective N-desulfation of heparin was developed by Inoue and Nagasawa through the treatment of heparin pyridinium salt with dimethyl sulfoxide containing 5% water or methanol for 1.5 h at 50 °C [1]. In aqueous sodium hydroxide solution (pH 12.5–12.8), the α-L-iduronic acid residues of heparin that are sulfated at C-2 become desulfated almost quantitatively, while other sulfate groups on heparin are not affected [2]. A specific, solvolytic O-6-desulfation of the 2-amino-2-deoxy-α-D-hexose residues of heparin has not yet been described.

The pyridinium salt of heparin has been reported as having been treated in 9:1 Me_2SO–water either for 5 h at 110 °C [3] or for 20 h at 90 °C [4]. Ayotte and Perlin [4] reported that the O-6-sulfate groups of the aminodeoxyhexose residues were almost totally removed, but so were 37% of the O-2-sulfate groups of the L-iduronic acid residues, as well as all of the sulfamino groups of the α-D-glucosamine moiety. Maccarana et al. [5] obtained essentially the same results using the method of Nagasawa et al. [3]. Application of the conditions for the O-6-desulfation of chondroitin sulfate with *N*,*O*-bis-(trimethylsilyl)acetamide (BTSA) [6] to heparin resulted in high selectivity, but only two-thirds of the O-6-sulfo groups were removed. Thus the reported state of the art for O-6-desulfation should be improved. In the present paper we investigate reaction conditions for solvolytic O-6-desulfation that are more regioselective than those described above. This is important as sulfatases with known site selectivity are rarely available to apply to these problems. Exo-sulfatases are available for O-6-desulfation of the glucosamine moiety at the end of the polymer chain [7]. Endo-sulfatases, to date, have only been used exogenously for resulfation of a pentasaccharide with iduronic acid and glucosamine moieties [8], and chemical synthesis of this type of larger oligosaccharide fragments is currently limited to a maximum of about ten monosaccharide repeating units [9].

2. Experimental

Materials.—Heparin, prepared from porcine intestinal mucosa, was purchased from Serva, Heidelberg, and had an anticoagulant activity of 186 IU/mg. Me_2SO, Me_2NCHO, Me_2NAc, *N*-methylpyrrolidone, pyridine, dioxane and NaOH were purchased from Fluka, Darmstadt in p.A. quality.

Analytical methods.—The heparin derivatives were characterized by ^{13}C NMR spectroscopy. Samples (200 mg) were dissolved in D_2O (>99.95% isotopic purity, 600 μL, obtained from Fluka, Darmstadt). Fourier-transform ^{13}C NMR spectra were recorded at room temperature with a Bruker AC 300 or DPX 300 instrument with the following parameters: frequency, 75.5 MHz; repetition time 0.918 s; Fourier number, 8 K; time of pulse, 0 s; number of scans, 40 000–70 000. Using the triangulation method of Casu *et al*. [10], the ^{13}C NMR spectra gave information about the percentage of sulfation at the following positions of the iduronate residues (I) and the glucosamine residues (A): % N-sulfation of (A), % O-sulfation at C-6 of (A) and % O-sulfation at C-2 of (I).

The molecular weight was determined on a Biosil SEC 125 HPLC–GPC column at 214 nm and a flow-rate of 1 mL/min. The column was calibrated with keratan sulfate standards of known molecular weight, a generous gift of Prof. Stuhlsatz. The sample concentration was 1 mg/mL. A 0.02 M NaH_2PO_4 buffer (pH 5.8) containing 0.15 M NaCl was used as eluent. The molecular weight was converted into chain length expressed in disaccharide units per chain using the following equation:

$$CL = MW/[SD-SO_3 \times MW(SO_3Na) + MW(DsU)]$$

where CL is the chain length in disaccharide units per chain; MW is the molecular weight; SD-SO_3 is sulfate groups per disaccharide unit (determined by ^{13}C NMR spectroscopy); MW(SO_3Na) is the molecular weight of SO_3Na; and MW(DsU) is the molecular weight of one unsulfated disaccharide unit.

Preparation of ammonium salts of heparin.—A solution of the sodium salt of heparin (1.5 g) in water (30 mL) was passed through a column (2.5×20 cm) of Amberlite IR-120 (H^+, 20–50 mesh) cation-exchange resin maintained at 4 °C.

The effluent was adjusted to pH 5.6–6.0 by the addition of (T) 13.5% tributylamine in ethanol, (Q) 20% quinoline in ethanol, and (P) pyridine, respectively. The solution was extracted four times with diethyl ether [80 mL for (T) and (Q) only] and then lyophilized [1,4]. The ammonium salts of heparin were obtained as white powders: 1.85 g (T); 1.82 g (Q); 1.69 g (P).

Preparation of N-desulfated, partially O-desulfated heparin.—The ammonium salt of heparin (400 mg) was dissolved in an aprotic solvent (40 mL) containing 10% of water or methanol. The solution was stirred for a specified time at a given temperature (see Table 1). After cooling to room temperature, the reaction mixture was diluted with an equal volume of water and adjusted to pH 9.0 by the addition of 0.1 M aq sodium hydroxide. The solution was dialyzed against distilled water for 72 h. Lyophilization of the dialyzate gave the product as a white or pale-yellow powder (190–230 mg).

Preparation of N-resulfated, partially O-desulfated heparin.—N-resulfation of N-desulfated, partially O-desulfated heparin was carried out under conditions similar to those described previously [4]. N-desulfated, partially O-desulfated heparin (200 mg) was dissolved in water (40 mL). Na_2CO_3 (400 mg) and trimethylamine·sulfur trioxide (400 mg) were added at room temperature. The mixture was kept for 24 h at 55 °C, then it was cooled and dialyzed against distilled water for 48 h. The dialyzate was applied to a column (2.5×20 cm) of Amberlite IR-120 (H^+, 20–50 mesh) ion-exchange resin, adjusted to pH 9.5 with 0.1 M aq sodium hydroxide, dialyzed against distilled water for 72 h, and then lyophilized. The product was obtained as a white or pale-yellow powder (200–220 mg).

3. Results and discussion

New solvolytic methods for improved completeness and higher selectivity of the O-6-desulfation of the glucosamine moiety of heparin have been developed by systematically varying the following parameters. (1) Amine salts of heparin with amines of different basicity and with variable steric and hydrophobic residues [tributylamine (T), quinoline (Q), pyridine (P)] were used for solvolytic O-6-desulfation studies. (2) Effects of dipolar aprotic solvents with different polarity and mixtures thereof with additional water (10 vol%) or methanol (10 vol%) on solvolysis were investigated. (3) Reaction conditions, with variations in time and

Table 1
Regioselective O-6-desulfation of the tributylammonium (T), quinolinium (Q), and pyridinium (P) salts of heparin in solvent (I; II; III; IV; D)–water or solvent–methanol mixtures

Name	9:1 solvent–H_2O	t (h)	T (°C)	[I-2(OS)]		[A-6(OS)]		$\frac{\Delta[\text{A-6(OS)}]}{\Delta[\text{I-2(OS)}]}$	CL [b]
				n [a]	%	n [a]	%		
Heparin				0.65	0	0.88	0	0	23
T-I	Me_2SO–H_2O	24	90	0.62	5	0.83	6	1.7	21
T-II	Me_2NCHO–H_2O	24	90	0.55	15	0.62	30	2.6	18
T-I-D-M	Me_2SO–Dioxane–MeOH (6:3:1)	24	90	0.60	8	0.85	4	0.6	22
Q-I	Me_2SO–H_2O	24	90	0.33	49	0.04	95	2.6	23
Q-II	Me_2NCHO–H_2O	24	90	0.60	8	0.71	19	3.4	22
P-I	Me_2SO–H_2O	24	90	0.42	35	0.05	94	3.6	20
P-II	Me_2NCHO–H_2O	24	90	0.60	8	0.25	72	13	23
P-II-2 [d]	Me_2NCHO–H_2O	24×2	90	0.43	34	0	100	4	23
P-III	DMAc–H_2O	24	90	0.43	34	0.47	47	1.9	20
P-IV-1 [c]	*N*-methylpyrrolidinone–H_2O	24	90	0.59	10	0.11	88	13	22
P-IV-2 [d]	*N*-methylpyrrolidinone–H_2O	24×2	90	0.53	18	0.05	95	7	22
P-IV-3	*N*-methylpyrrolidinone–H_2O	48	90	0.54	17	0.08	91	7	22
P-IV-4	*N*-methylpyrrolidinone–H_2O	48	80	0.59	9	0.13	85	13	22
P-IV-5	*N*-methylpyrrolidinone–H_2O	48	70	0.62	5	0.25	72	21	22
P-I/III	Me_2SO–DMAc–H_2O (9:9:2)	24	90	0.58	11	0.15	83	10	20
P-II/IV	Me_2NCHO–*N*-methylpyrrolidinone–H_2O (9:9:2)	24	90	0.34	48	0.35	60	1.7	21

[a] Number (n) of [I-2(OS)] and [A-6(OS)] groups per disaccharide moiety, percentage reduction (%), and desulfation ratio of Δ[A-6(OS)]/Δ[I-2(OS)] are evaluated by ^{13}C NMR spectroscopy [10].
[b] CL = chain length.
[c] Mean value of two reactions, with a deviation of ±0.02 for each sulfate group.
[d] Reactions carried out twice.

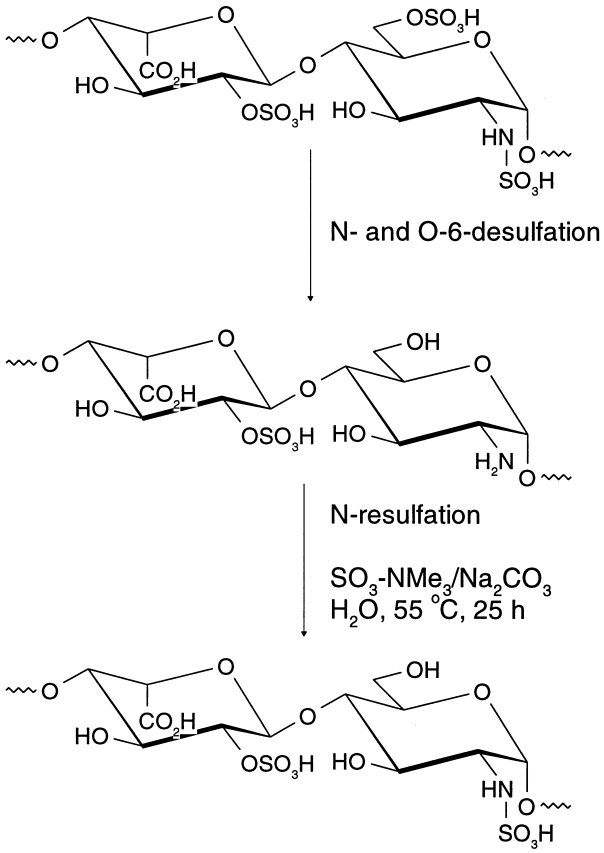
temperature, were evaluated. Most of the experiments were carried out in parallel under the same reaction conditions (90 °C, 24 h) to ensure comparable results. Some reaction conditions were varied for optimizing regioselective reactions (see Table 1).

The reaction conditions used are described briefly, together with NMR data of the products that support the structures proposed. The reproducibility of the results has been tested in one case. Maximum deviation of the different sulfate ester groups per disaccharide moiety was in the range of ±0.02 (P-IV-1[c]). Because of its lability, the sulfamido group was completely removed during all desulfation reactions. Thus specific N-resulfation [4] followed all desulfation reactions (see Scheme 1). Dipolar aprotic solvents of medium polarity with decreasing polarity were used as shown in Table 2.

Tributylammonium salts (TS).—Tributylamine (T) is a strong base possessing hydrophobic residues (pK_a of the conjugated acid 10.9). The solvents used for solvolytic O-6-desulfation were (I) and (II). The ^{13}C NMR data showed little O-desulfation and a moderate degradation of the length of the polymer chain (CL) [see experimental section (from 23 to 21 and 18 disaccharides per chain, respectively)]. The number of O-6-sulfate groups present per disaccharide moiety decreased from 0.88 to 0.83 for (T-I) and 0.62 (T-II), respectively.

The results suggest that (TS) of heparin is not useful for complete and selective O-6-desulfation. This might be explained by the following hypothesis and effects. The salt of the strong base (T) with sulfate ester groups of heparin $-O-SO_3^- {}^+N(Bu)_3$ may be dissociated or solvated to a higher degree in the high polar solvent (I)–water mixture than in the less polar solvent (II)–water mixture.

It has not been determined whether and to what degree solvent-separated, closely packed, solvent-clustered, nondissociated particles are formed. However, according to our hypothesis the strong polar solvent (I) produces groups that are more negatively charged: $-C-O-SO_3^-$. Thus the attack of



Scheme 1. Regioselective O-6-desulfation of the disaccharide moiety of heparin with highest completeness (100% [A-6(OS)]-desulfation) and highest selectivity (0% [I-2(OS)]-desulfation) of sulfation.

negatively charged OH^- on the O–S or C–O bond should be reduced by electrostatic repulsion. With the solvent of lower polarity (II), a smaller amount of $C-O-SO_3^-$ should be formed, resulting in a higher C–O or O–S bond cleavage. Because no isomerisation products have been found from the NMR data, there must be either an O–S bond cleavage at the 2-position of iduronic acid, or double S_N2 reactions (which leads to no net inversion) at the chiral center. The latter reactions are known to be reactions in aqueous solutions influenced by neighboring group effects (further discussion follows). S_N1-dependent C–O bond cleavage can be excluded. On the other hand it is known that C–O or O–S bond cleavage of esters of inorganic

Table 2
Dipolar aprotic solvents of medium polarity

Solvent	Me_2SO (I)	Me_2NCHO (II)	Me_2NAc (III)	*N*-Methylpyrrolidinone (IV)
Polarity[a]	0.444	0.386	0.377	0.355

[a] Expressed on the Reichardt scale [12], listed in decreasing order.

acids are dependent on the reaction conditions, e.g., benzhydryl-*p*-toluenesulfonate ($Ph_2CHOSOC_6H_4CH_3$) was found to undergo C–O cleavage in acidic media ($HClO_4$) and O–S cleavage in alkaline media [14].

Although the electronic effects with the above-mentioned substance are different from our derivatives, there may be an indication for preferential C–O or O–S bond cleavage, depending on the hydrolytic conditions used in the experiment. Furthermore, the nonpolar character of the three butyl groups may reduce the dissociation, solvation and hydrolytic attack of the C–O or O–S bond. The ratio between solvolytic cleavage of sulfate ester groups of Δ[I-2(OS)] and Δ[A-6(OS)] was 1:1.7 and 1:2.6, respectively, for (T-I) and (T-II). This explains the additional steric hindrance of the functional groups in the 2-position compared to the relatively good accessibility in the 6-position of the carbohydrate repeating moiety.

In order to possibly improve the regioselectivity of O-6-desulfation, methanolysis was used instead of hydrolysis. We used a known solvent mixture, (I)–dioxane–methanol [11], under the constant conditions of our systematic studies (T-I-D-M). No significant O-6-desulfation was observed at either [A-6(OS)] or [I-2(OS)]. The polarity of dioxane according to the Reichardt scale [12] is 0.164.

Quinolinium salts (QS).—Quinoline belongs to the group of weak bases (pK_a of the conjugated acid is 4.9). The (QS) of heparin was treated in solvent (I) and (II), each containing 10% water. In the highest polarity solvent (I), the completeness of solvolytic [A-6(OS)]-desulfation was nearly quantitative [95% (Q-I)], but low selectivity was observed, and approximately half of the [I-2(OS)]-sulfate groups also were removed. When using the less polar solvent (II), the completeness of [A-6(OS)]-desulfation decreases drastically (19%), whereas selectivity was relatively high (8% [I-2(OS)]-desulfation). Using this type of solvolysis, the chain length was nearly unaffected by the choice of solvent.

Salts of the weak base (Q) with the sulfate ester groups of heparin may be dissociated and solvated in solvent (I) to a lower degree than the (TS) of heparin in (I). Thus the hydrolytic C–O or S–O bond cleavage by OH^- will be increased (Q-I) in comparison to (T-I). Although (Q) should cause an opposite strong steric effect on C–O or S–O bond cleavage in addition to the hydrophobic effect, the expected strong steric effect has not been observed in solvent (I). The hydrophobic effect between (Q) and (T) seems to be nearly in the same range, based on the assumption that the effects of the number of carbon atoms in (Q) and (T), which are relatively high in both cases [9 carbon atoms in (Q) and 12 carbon atoms in (T)], will be similar. The ratio between Δ[I-2(OS)] and Δ[A-6(OS)]-desulfation is 1:2.6, which means a low regioselectivity (Q-I).

This does not differ significantly from the ratio of (T-I) or (T-II) desulfation. However, the less polar solvent (II) resulted in a five times lower total desulfation of (QS) compared to (I). This may be explained by the lower dissociation of heparin (QS) in solvent (II) and the opposite, additional strong steric effect of (Q). In other words the steric effect of heparin (QS) has to be stronger in solvent (II) because the distance between (Q) and the position of C–O or S–O bond cleavage is closer compared to the more dissociated salt with a larger distance between (Q) and the position of C–O or S–O bond cleavage in solvent (I). Thus the reduced dissociation of heparin (QS) from (I) to (II) increases the steric effect of (Q) drastically in solvent (II). This may be described as a 'dissociation-dependant' steric effect. The individual desulfation ratio between Δ[I-2(OS)] and Δ[A-6(OS)] was 1:3.4 (Q-II), which is a little higher than that of (Q-I) and indicates a lower degree of regioselectivity.

Pyridinium salts (PS).—Pyridine also belongs to the group of weak bases (pK_a of the conjugated acid 5.2). The pyridinium salt of heparin was examined because of the fact that its use for desulfation reactions, in combination with a polar, aprotic solvent or a mixture of aprotic solvents in water, has often been reported in the literature [3,4,11,13].

First the solvents of higher polarity (I) and (II) were studied to show the different effects of heparin (QS) compared to that of heparin (PS).

Nagasawa et al. [3] and Ayotte and Perlin [4] reported preferential solvolytic O-6-desulfation in the highest polarity solvent (I) using a reaction time different from that of our own investigations. We repeated the Ayotte method, but prolonged the reaction time from 20 to 24 h to get results comparable to those of our systematic studies.

In solvent (I) the O-6-desulfation was nearly complete (94%), but selectivity was relatively low, nearly 35% of the [I-2(OS)] sulfate groups were removed. These results correspond to those of Ayotte and Perlin [4] and Maccarana et al. [5]. The less polar solvent (II) improved selectivity (8%

[I-2(OS)]-desulfation) and reduced completeness (72% [A-6(OS)]-desulfation).

Upon comparing the solvolytic desulfation of heparin (PS) with that for heparin (QS) [both are salts of the two weak bases, (P) and (Q), with nearly the same pK_a values] on the regioselective [A-6(OS)]-desulfation in solvent (I), no significant difference in completeness {94% [A-6(OS)]-desulfation for (P-I) and 95% for [(Q-I)]} and only a little variation in selectivity {35% for [I-2(OS)]-desulfation (P-I) and 49% for (Q-I)} can be observed.

In contrast to this, solvent (II) of lower polarity gave a relatively high degree of completeness {72% of [A-6(OS)]-desulfation (P-II)} but low desulfation [19% of (Q-II)]. The selectivity was high in both cases {[I-2(OS)]-desulfation 8% for (P-II) and 8% for (Q-II)}. The individual desulfation ratio between [I-2(OS)] and [A-6(OS)] was respectively high 1:13 (for P-II) and low 1:3.4 (for Q-II). A strong steric effect of (Q) can be observed with (Q-II), compared to a lower steric effect for (P-II), presumably because of the additional benzene ring fused to the pyridine ring.

A strong steric effect of the (Q) heparin salt in the solvent of lower polarity (II) might also be explained by our hypothesis. We assume that the sulfonium salt of the weak base (Q) may dissociate to a lower degree than the strong base (T). Thus low concentrations of negatively charged sulfate ester groups should be formed, and the hydrolytic attack on the C–O or O–S bond should be increased. The additional hydrophobic and steric effects show inverse trends. This is the 'dissociation-dependent' steric effect described above. Thus the total C–O or O–S bond cleavage will be reduced more in the case of (Q) in comparison to (P). At the O-6-position the steric effect of (Q) is too strong to get near-complete O-6-desulfation. (QS) of heparin in solvent (II) is not useful for complete [A-6(OS)]-desulfation because of the very large steric effect, again presumably because of the additional benzene ring that is fused to the pyridine ring. On the other hand [I-2(OS)]-desulfation will be sterically hindered by two effects: the additional carbohydrate ring and (Q). Both effects lead to an improved selectivity: a low degree of hydrolytic desulfation at the 2-position of the carbohydrate moiety.

The pyridine ring of the heparin (PS) seems to be the optimal steric group and shows a fairly good hydrophobic effect in solvent (II). The hydrophobic effect of (P) is not as strong, as there are only five carbon atoms compared to the nine carbon atoms of (Q). According to our hypothesis the pyridinium salt in solvents of lower polarity (III, IV) should almost totally cause a lower degree of dissociation and an improved regioselective 6-O-desulfation, i.e., [(A-6(OS)]-desulfation with higher selectivity and a lower [I-2(OS)]-desulfation.

Surprisingly in the solvent of lower polarity (III) the number of sulfate groups per disaccharide of (P-III) fell from an original value of 0.88 to 0.47 [A-6(OS)], and from an original 0.65 to 0.43 [I-2(OS)].

The solvent of lowest polarity (IV) showed the trend we expected according to our hypothesis: a nearly specific and complete O-6-desulfation (P-IV-1[c]). This reaction was, in fact, used for establishing reproducibility. The results were very satisfying. The [A-6(OS)] content per disaccharide was 0.11, whereas [I-2(OS)] remained high at 0.59, while 88% of [A-6(OS)] and 10% of [I-2(OS)] were desulfated.

Reactions were either repeated (P-IV-2[d]) or the reaction times were doubled (P-IV-3), which in both cases resulted in nearly complete O-6-desulfation (95 versus 91% [A-6(OS)]-desulfation), accompanied by a lower selectivity (18 versus 17% [I-2(OS)]-desulfation). When a lower temperature (70 °C, P-IV-5) was used, the highest selectivity was observed {5% [I-2(OS)]-desulfation}, but the degree of completeness was only 72%.

During all solvolytic regioselective O-6-desulfation experiments of the heparin pyridinium salt, no significant degradation of the polymer backbone of heparin was observed with solvent (II), while in solvent (IV) the chain length was reduced by one disaccharide unit. Solvolytic reactions in solvent (I) and (III) shortened the chain length up to three disaccharide units. Ayotte and Perlin did not find a shortening of the heparin backbone by solvolytic desulfation of (PS) in solvent (I) using a reaction time of 20 h [4].

The results of the solvolytic O-6-desulfation of pyridinium salts of heparin with solvents of decreasing polarity (I > II > III > IV) in water mixtures show a strong influence on selectivity: (I) 35%, (II) 8%, (IV) 10% [I-2(OS)]-desulfation. In contrast to this effect, the completness of O-6-desulfation is not influenced very much by the solvent polarity: (I) 94%, (II) 72%, (IV-1[c]) 89%, (IV-2[d]) 95% [A-6(OS)]-desulfation. All solvents led to a relatively high level of completeness, whereas the solvent of highest polarity (I) showed

the highest O-6-desulfation, accompanied by low selecitvity. *N*-methylpyrrolidinone (IV) was the favorite solvent for high regioselectivity of 6-O-desulfation of heparin pyridinium salt, showing both a high degree of selectivity and a high level of completeness. In general complete turnover and a high regioselectivity of 6-O-desulfation can be expressed by the high ratio (7–21) of Δ[A-6(OS)]/Δ[I-2(OS)].

It is generally accepted that S_N2 reactions are known to be relatively solvent-independant reactions, while they are highly dependent on steric effects. The total effects for solvolytic desulfation of the pyridine salt show no significant solvent dependence on [A-6(OS)]-desulfation, but a strong steric effect could be observed between (QS) and (PS) of amines of comparable basicity in solvent (II). This may indicate that an S_N2-like reaction takes place in the 6-position.

Concerning the reaction at the 2-position of the iduronic acid moiety, the results of ^{13}C NMR spectra show that during all desulfation reactions no isomerisation products of the α-L-iduronic acid moiety of heparin, such as α-L-galacturonic acid, have been found. These results correspond to our unpublished disaccharide analysis from HNO_2-degradation products of heparin according to the method of Shively and Conrad [15]. These results even go along with published ^{13}C NMR spectra from solvotytic O-6-desulfation of heparin pyridinium salts in 9:1 (I)–water mixtures [4]. This is a proof for the stereoselectivity in the 2-position of the iduronic acid of the new regioselective hydrolytic O-6-desulfation reactions.

The isomerisation product, α-L-galacturonic acid, was observed during O-2 desulfation of the IdoA 2-sulfate moiety of heparin in aequous alkali solution such as 0.1 N Na_2CO_3 or 0.1 N NaOH at temperatures >80 °C, whereas stronger alkali (pH >13) did not generate the above-mentioned isomerisation product [16–19]. The mechanism of such a desulfation of heparin without forming the above-mentioned isomerisation product has been described via the neighboring group participation of the 3-OH group of iduronic acid, resulting in an intermediate epoxide: 2,3-anhydro-GulA. Those reactions are generally known to be anchimerically assisted, stereoselective, and dependent on solvent polarity. The results of our systematic studies show strong solvent dependence for significant [I-2(OS)]-desulfation from polar solvent (I) (35%) to the less polar solvent (IV) (5%). The formation of an intermediate epoxide could be a possible mechanism for preferential C–O bond cleavage. The results indicate that a solvent-dependent double S_N2 mechanism seems to be possible for the 2-position of the IdoA. As for the solvolysis of sulfate ester groups in the 3-position of the GlcNAc moiety (PS), no desulfation of those groups was observed by ^{13}C NMR measurements when these were treated with solvents (II) and (IV). In contrast to this, solvent (I) resulted in low [A-O-3(OS)]-desulfation (results not shown in Table 1).

In contrast to the above-mentioned trend of solvents (I), (II) and (IV) is the influence of Me_2NAc (III), a solvent of medium polarity, on regioselective O-6-desulfation. The effect of this solvent (III) cannot yet be explained.

However, using a mixture of (III) with solvent (I) (P-I/III), both the completeness of O-6-desulfation (83% [A-6(OS)]-desulfation) and the selectivity are high (11% [I-2(OS)]-desulfation), whereas solvent mixtures (II) and (IV) are not useful for getting regioselective reaction products (P-II/IV). The reason for the influence of solvent mixtures in water is not yet clear.

4. Conclusions

Novel, nearly complete O-6-desulfation of the GlcNAc moiety of heparin with high regioselectivity was achieved by varying the character of the ammonium salt using three different bases of heparin together with varying the polarity of the solvents ($Me_2SO > Me_2NCHO > Me_2NAc >$ *N*-methylpyrrolidinone) with water.

The heparin salt of the strong base, tributylamine, was not useful for a high degree of desulfation with either Me_2SO or Me_2NCHO as solvent. A theory to explain the effects of polarity of solvents, the pK_a values of the amines, as well as the hydrophobic and steric effects of (P) and (Q) on the mechanism of solvolytic 6-O-desulfation with improved regioselectivity has been elucidated from the observed results. Most results correspond at least qualitatively to analogous known results except for the solvent Me_2NAc. However, a qualitative description of the mechanism must be proved by quantitative methods. Weak bases like quinoline are only of interest in Me_2SO, whereas pyridine is more suitable for O-6-desulfation with high regioselectivity in aprotic solvents of medium polarity like Me_2NCHO. The highest regioselective

O-6-desulfation reaction in comparison to all known results was obtained in the less polar *N*-methylpyrrolidinone.

The O-6-desulfated heparins obtained in this study are potentially valuable for biological studies and studies of their pharmaceutical properties. Their use could give clear information of the influence of the O-6-sulfate group on the biological activity of heparin, for example the affinity of heparin to certain proteins, such as growth factors and coagulation factors [20,21], among others.

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