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Selective acetylation reactions of hyaluronic acid benzyl ester derivative

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Abstract

The reaction of hyaluronic acid benzyl ester derivative (**1**) with trimethyl orthoacetate in the presence of an acid catalyst in *N,N*-dimethylformamide gave the expected 4,6-orthoester **2**, along with its hydrolysed products the 6-*O*- (**3**) and the 4-*O*- (**4**) acetate hyaluronic acid benzyl esters as minor components. Acid hydrolysis of **2** followed by O-4 → O-6 acetyl migration using *t*-butylamine afforded a mixture which contained the 6-acetate **3** as the major and the 4-acetate **4** as the minor compound. Synthesis of a mixture of 2′,3′,6- (**6**) and 2′,3′,4- (**7**) tri-acetates was achieved by peracetylation of **2** followed by treatment with aqueous acetic acid. Compound **1** on treatment with 2,2-dimethoxypropane in dimethyl sulfoxide in the presence of *p*-toluenesulfonic acid afforded the corresponding 4,6-*O*-isopropylidene derivative **8.** The 2′,3′-di-*O*-acetate **10** was obtained from **8** by conventional acetylation followed by de-acetylation using trifluoroacetic acid. Acetylation of **1** with acetic anhydride in a mixture of *N,N*-dimethylformamide and pyridine gave the expected 2′,3′,4,6-tetraacetate derivative **11**. In order to facilitate the NMR assignments of the acetate groups in **3**–**11**, the 2′,3′-di-acetate-4,6-di(trideuterioacetate) **12** and the 2′,3′-di(trideuterioacetate)-4,6-diacetate **13** were prepared. The effect of substituents on conformational changes around the glycosidic linkages has been investigated. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Hyaluronic acid; Benzyl hyaluronate; Acetates; Cyclic orthoester; Cyclic acetal

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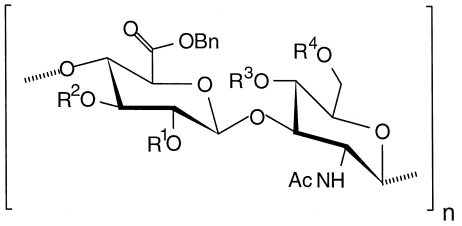
1. Introduction

Hyaluronic acid is a naturally occurring polysaccharide composed of a linear repeating disaccharide unit consisting of β-(1 → 4)-linked D-glucopyranuronic acid and β-(1 → 3)-linked 2-acetamido-2-deoxy-D-glucopyranose which is present in extracellular connective tissue matrices. Organs such as umbilical cord, synovial fluid, vitreous humor, and rooster combs are particularly rich in hyaluronic acid. It is non-immunogenic and is involved in such important biological functions as tissue hydration and proteoglycan organisation in the tissue repair. It is used in the treatment of connective tissue diseases, for example, inflammation of the joint and rheumatoid arthritis. Hyaluronic acid derivatives such as the benzyl esters find applications as biomaterials for surgical implant [1].

Our objective was to regioselectively modify hyaluronic acid benzyl ester (**1**) to obtain new compounds and also to study on a qualitative basis the effect of specific substitution on the conformation around the glycosidic linkages.

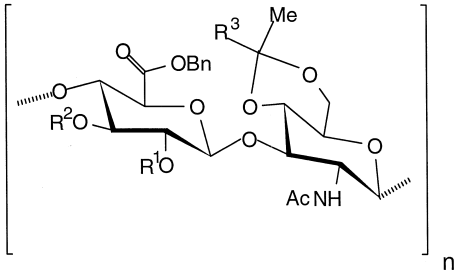


	R^1	R^2	R^3	R^4
1	H	H	H	H
3	H	H	H	Ac
4	H	H	Ac	H
6	Ac	Ac	H	Ac
7	Ac	Ac	Ac	H
10	Ac	Ac	H	H
11	Ac	Ac	Ac	Ac
12	Ac	Ac	Ac-d_3	Ac-d_3
13	Ac-d_3	Ac-d_3	Ac	Ac

2. Results and discussion

Chemical reactions of polysaccharides usually do not result in discrete compounds. A small proportion of the sugar residues on the polysaccharide may be partially substituted or even unsubstituted. The derivatisation of hyaluronic acid, described in this communication, led to mixtures containing major and minor products. The predominant products of reactions were identified based on the major resonances observed in the NMR spectra.

Mono- and di-saccharides, for example, methyl α-D-glucopyranoside and sucrose react with trimethyl orthoacetate or 1,1-dimethoxyethene in *N*,*N*-dimethylformamide in the presence of *p*-toluenesulfonic acid to give the corresponding cyclic 4,6-orthoester intermediates, which on hydrolysis afford the corresponding 6-acetate as the major and the 4-acetate as the minor component [2,3]. Acetyl groups are known to undergo O-4 → O-6 migration under basic conditions to give the corresponding C-6 acetate derivatives [3]. We have adopted this strategy to introduce acetate groups at C-6 and C-4 of the 2-acetamido-2-deoxy-D-glucopyranose moiety of the repeating unit of **1**.



	R^1	R^2	R^3
2	H	H	OMe
5	Ac	Ac	OMe
8	H	H	Me
9	Ac	Ac	Me

Table 1
^{13}C chemical shifts (δ, ppm) for benzyl hyaluronate derivatives

Compound	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′, C=O	C-1	C-2	C-3	C-4	C-5	C-6	PhCH_2	Ph
1	102.90	72.75	74.15	80.13	73.51	167.25	100.45	54.36	82.65	68.77	76.31	60.94	66.4	135.4 128.0 128.3
2	103.27	73.69	73.94	78.57	74.08	167.49	99.64	55.48	78.13	65.13	71.51	60.60	66.33	135.4 128.0 128.3
3	102.89	72.75	73.72	≈ 80	73.51	167.25	100.22	54.36	82.06	68.75	73.11	63.05	66.44	
4	103.58	72.95	74.10	≈ 80	73.7	167.46	100.22	55.10	78.65	69.27	73.5	0.586	66.44	
5	101.13	70.66	72.40	≈ 76	73.55	166.60	99.54	54.55	76.96	65.03	70.89	60.64	66.69	135.4 128.0 128.3
6	100.64	70.62	72.31	75.96	73.59	166.81	99.91	54.08	81.19	67.94	≈ 73.6	63.13	66.93	
7	100.64	70.62	72.31	75.96	73.59	166.81	99.91	54.08	77.77	68.74	≈ 73.6	60.38		
8	102.80	73.44	73.95	78.60	74.15	167.73	99.67	55.11	78.60	66.46	72.47	61.24		
9	99.58	71.32	72.24	76.35	73.75	166.74	100.93	54.39	78.21	66.33	72.47	61.20	66.87	
10	99.79	70.59	73.77	76.40	73.77	166.66	100.65	54.09	81.63	66.02	75.64	60.98	66.2	
11	99.89	70.61	72.30	75.96	73.54	166.79	100.65	54.08	77.79	67.96	70.61	61.77	66.94	
12	99.90	70.62	72.32	76.00	73.55	166.81	100.66	54.10	77.81	67.93	70.62	61.78	66.94	
13	99.90	70.61	72.30	75.97	73.56	166.83	100.63	54.09	77.80	67.97	70.61	61.78	66.97	

Table 2
^{1}H NMR chemical shifts (δ, ppm) of acetyl, acetal and orthoester resonances of benzyl hyaluronate

Compound	HNCOCH_3	CH_3CO-6	CH_3CO-4	CH_3CO-2′[a]	CH_3CO-3′[a]	OCH_3	CH_3
1	1.75						
2	1.79					3.08	1.21
3	1.76	2.02					
4	1.79		1.72				
5	1.83			1.92	1.90	3.10	1.22
6	1.83	1.97		1.97	1.97		
7	1.83		1.71	1.97	1.97		
8	1.78						1.16, 1.25
9	1.86			1.97	1.97		1.19, 1.25
10	1.80			1.96	1.96		
11	1.84	1.96	1.70	1.92	1.90		
12	1.84			1.92	1.90		
13	1.84	1.96	1.70				

[a] Interchangeable assignment.

Treatment of **1** with trimethyl orthoacetate in *N,N*-dimethylformamide in the presence of *p*-toluenesulfonic acid at room temperature for 24 h gave predominantly the 4,6-cyclic orthoester derivative **2.** No attempt was made to obtain a pure diastereoisomer. In the ^{13}C NMR spectrum of **2** the resonances due to C-3,4,5 were shifted upfield as compared to the corresponding resonances in the starting material **1** and appeared at δ 78.13, 65.13 and 71.51, respectively (see Table 1). The presence

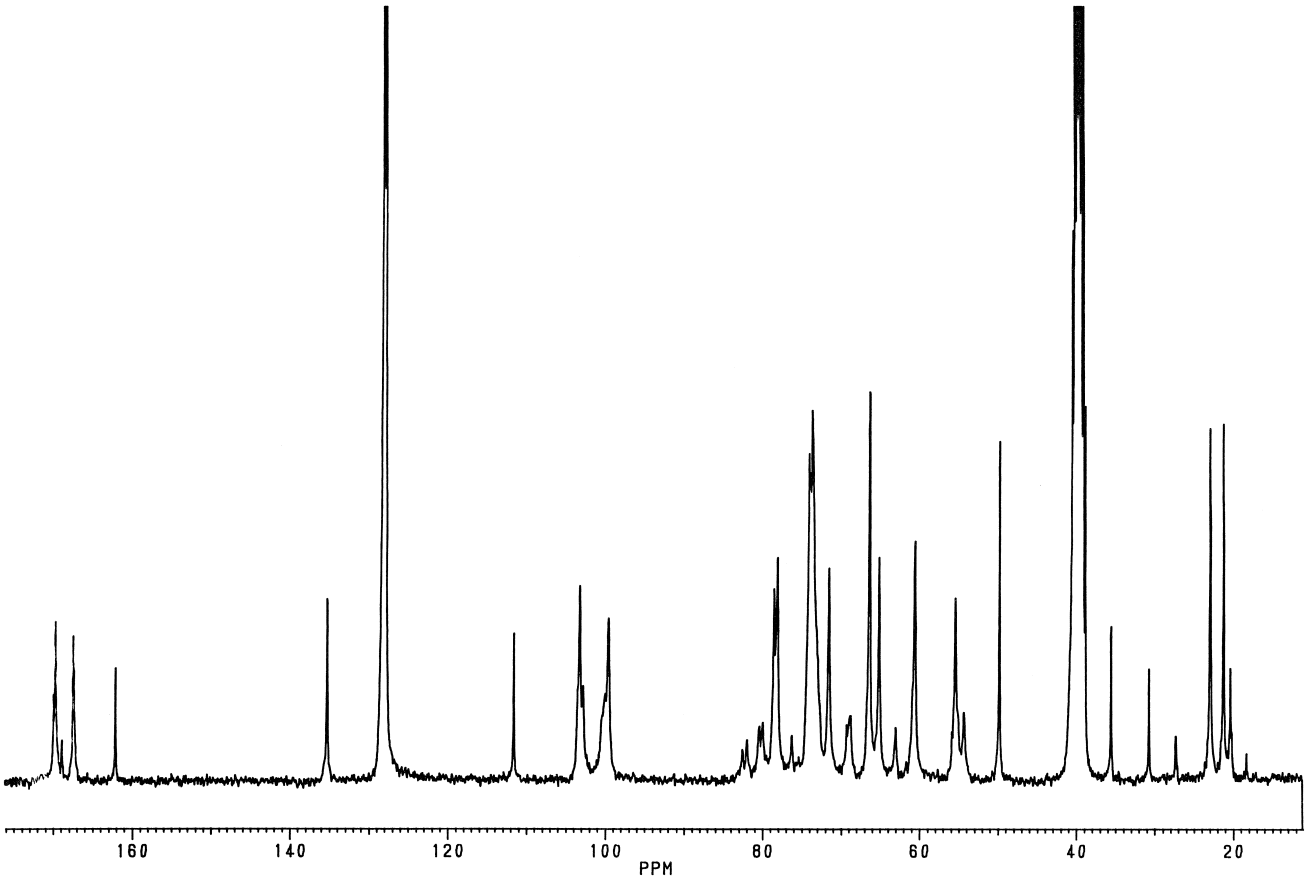


Fig. 1. ^{13}C NMR spectrum of compound **3** in Me_2SO-d_6. For the chemical shift data, see Tables 1 and 3.

Table 3
^{13}C chemical shifts (δ, ppm) of the acetyl, acetal and orthoester carbons of benzyl hyaluronate

Compound	HN*C*=O	*C*H$_3$CONH	*C*=O-6	*C*=O-4	*C*=O-2′	*C*=O-3′	*C*H$_3$-6	*C*H$_3$-4	*C*H$_3$-2′[a]	*C*H$_3$-3′[a]	OC*H*$_3$	*C*H$_3$	*C*-quart
1	169.69	23.07											
2	169.99	22.91									49.84	21.21	111.66[a]
3	169.71	22.93	170.05				20.40						
4	168.97	22.93		168.97			20.22						
5	169.72	22.75			168.94	169.09			19.95	20.18	49.63	21.15	111.71[a]
6	169.73[a]	22.75					19.96[a]		19.96[a]	19.96[a]			
7	169.73[a]	22.75						19.96[a]	19.96[a]	19.96[a]			
8	169.64	22.91										18.65/28.58	109.98
9	169.64	22.91			168.81	168.81			20.44	20.24		18.54/28.56	109.98
10	169.64	22.77[a]			169.07	169.01			20.06	20.14			
11	168.87	22.74	169.70	168.76	169.08	168.93	20.18	19.95	19.95	19.95			
12							20.21	20.04	20.04	20.04[a]			
13							20.22	19.98	19.98	19.98			

[a] Interchangeable assignment.

Table 4
^{1}H chemical shifts (δ, ppm) of benzyl hyaluronate derivatives

Compound	H-1′	H-2′	H-3′	H-4′	H-5′	H-1	H-2	H-3	H-4	H-5	H-6	PhCH_2	Ph
1	4.46	3.11	3.43	3.65	3.97	4.53	3.48	3.64	3.18	3.22	3.45, 3.72	5.16	7.2–7.6
2	4.35	3.03	3.41	3.60	3.85	4.74	3.42	3.84	3.22	3.52	3.72	5.2	7.2–7.6
3	4.49	3.12	3.43	3.64	3.98	4.58	3.50	3.68	3.28	3.46	4.07, 4.33	5.18	7.2–7.6
4	4.37	2.94	3.38	3.62	3.84	4.60	3.58	3.84	4.57	3.5	3.33, 3.48	5.18	7.2–7.6
5		4.52	5.03					3.94	3.16				7.2–7.6
6		4.70	5.00								4.07, 4.33		7.2–7.6
7		4.70	5.00						4.60				7.2–7.6
8	4.35			3.72	3.8	4.71	3.50	3.72	3.15		3.63, 3.77	5.13	7.2–7.6
9	4.79	4.70	5.00	3.24								5.13	7.2–7.6
10	4.91	4.70	5.01	3.12	4.05	4.32	3.50	3.65			3.45, 3.73	5.21	7.2–7.6
11	4.82	4.49	5.02	3.92	4.05	4.26	3.57	3.70	4.58	3.53	3.89, 4.18	5.28	7.2–7.6

of the 4,6-orthoester group in **2** was confirmed by the ^{1}H (see Table 2) and ^{13}C resonances due to CH_3 (^{1}H, δ 1.21; ^{13}C, δ 21.21) and OCH_3 (^{1}H, δ 3.08; ^{13}C, δ 49.84). Hydrolysis of **2** with aqueous acetic acid afforded a mixture containing, according to the NMR analysis, a major and a minor product. On the basis of the fact that the 4,6-orthoester derivatives of methyl α-D-glucopyranoside and sucrose on hydrolysis lead to the corresponding 6-acetate as the major and the 4-acetate as the minor product, the major resonances in the ^{1}H and ^{13}C NMR spectra (Tables 1 and 3; Fig. 1) were assigned to the 6-acetate **3** and the minor signals to the 4-acetate **4**. The resonances due to H-6a,6b in the *N*-acetylglucosamine moiety of **3**

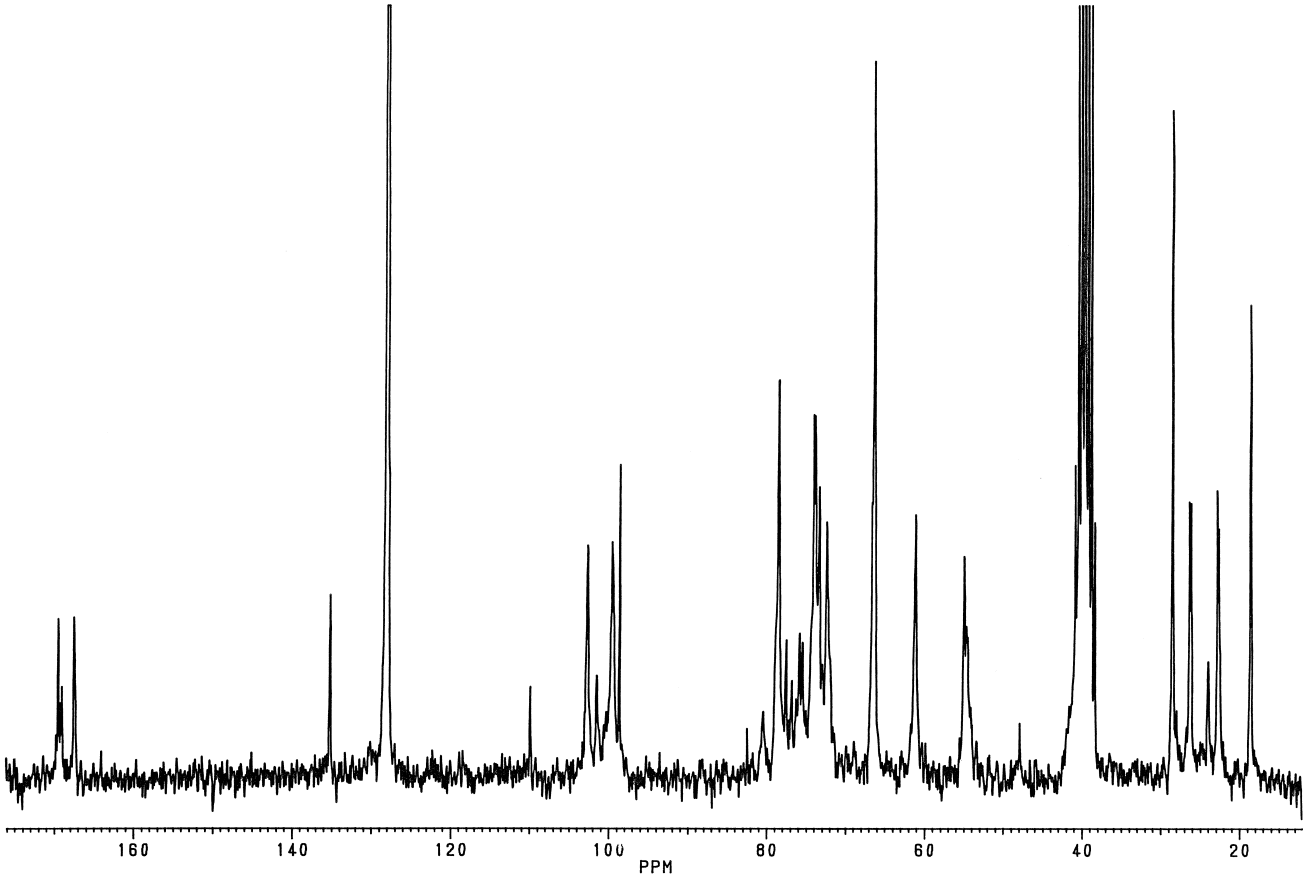


Fig. 2. ^{13}C NMR spectrum of compound **8** in Me_2SO-d_6. For the chemical shift data, see Tables 1 and 3.

appeared at δ 4.07 and 4.33, respectively. These signals were shifted downfield about 0.6 ppm due to the acetylation. Similarly, H-4 (δ 4.57) in **4** was shifted downfield by about 1.4 ppm, with respect to compound **1** (Table 4) [4]. Conventional acetylation of the 4,6-orthoester derivative **2** gave the 2′,3′-diacetate **5**, which on hydrolysis with glacial acetic acid afforded a mixture containing the 2′,3′,6- (**6**) and 2′,3′,4-(**7**) triacetates. The structures of **5**–**7** were in agreement with their NMR data (Tables 1–4). In the ^{1}H NMR spectra of **5**–**7**, the resonances due to H-2′,3′ shifted downfield by ~1.5 ppm, as compared to the corresponding resonances in compound **1**.

In order to prepare 2′,3′-diacetate derivative **10**, it was necessary to protect the hydroxyl groups of the *N*-acetylglucosamine moiety by way of the 4,6-*O*-isopropylidene intermediate **8**. It is of interest to note that the reaction of **1** with 2,2-dimethoxypropane in *N*,*N*-dimethylformamide in the presence of *p*-toluenesulfonic acid at 50 °C for 24 h was unsuccessful and the starting material was recovered unchanged. However, when the reaction was performed in dimethyl sulfoxide the desired compound **8** was obtained as the main product. The presence of the *O*-isopropylidene group in **8** was confirmed by its ^{13}C NMR spectrum (Tables 1 and 3; Fig. 2) which showed resonances at δ 18.65 and 28.58 due to the two CH_3 groups and δ 109.98 due to the quaternary carbon. Conventional acetylation of **8** in pyridine with acetic anhydride in the presence of 4-(*N*,*N*-dimethylamino)pyridine at 50 °C for 24 h afforded, after precipitation from ether, the 2′,3′-di-*O*-acetyl-4,6-*O*-isopropylidene derivative **9**. Its structure was supported by ^{1}H NMR data which showed a downfield shift of H-2′ and H-3′ of approximately 1.6 ppm (δ 4.70 and 5.00, respectively). Hydrolysis of **9** in dimethyl sulfoxide with aqueous acetic acid led to only partial hydrolysis of the 4,6-cyclic acetal group. However, the reaction of **8** with 9:1 trifluoroacetic acid–water was more efficient, and gave predominantly the 2′,3′-*O*-acetylated derivative **10**. In the ^{1}H NMR spectrum of **10**, the resonances due to H-2′,3′ appeared at δ 4.70 and 5.01, respectively, indicating the presence of acetate groups at C-2′,3′. The absence of the *O*-isopropylidene group in **10** was confirmed by its ^{1}H and ^{13}C chemical shift data (Tables 1 and 2; Fig. 3).

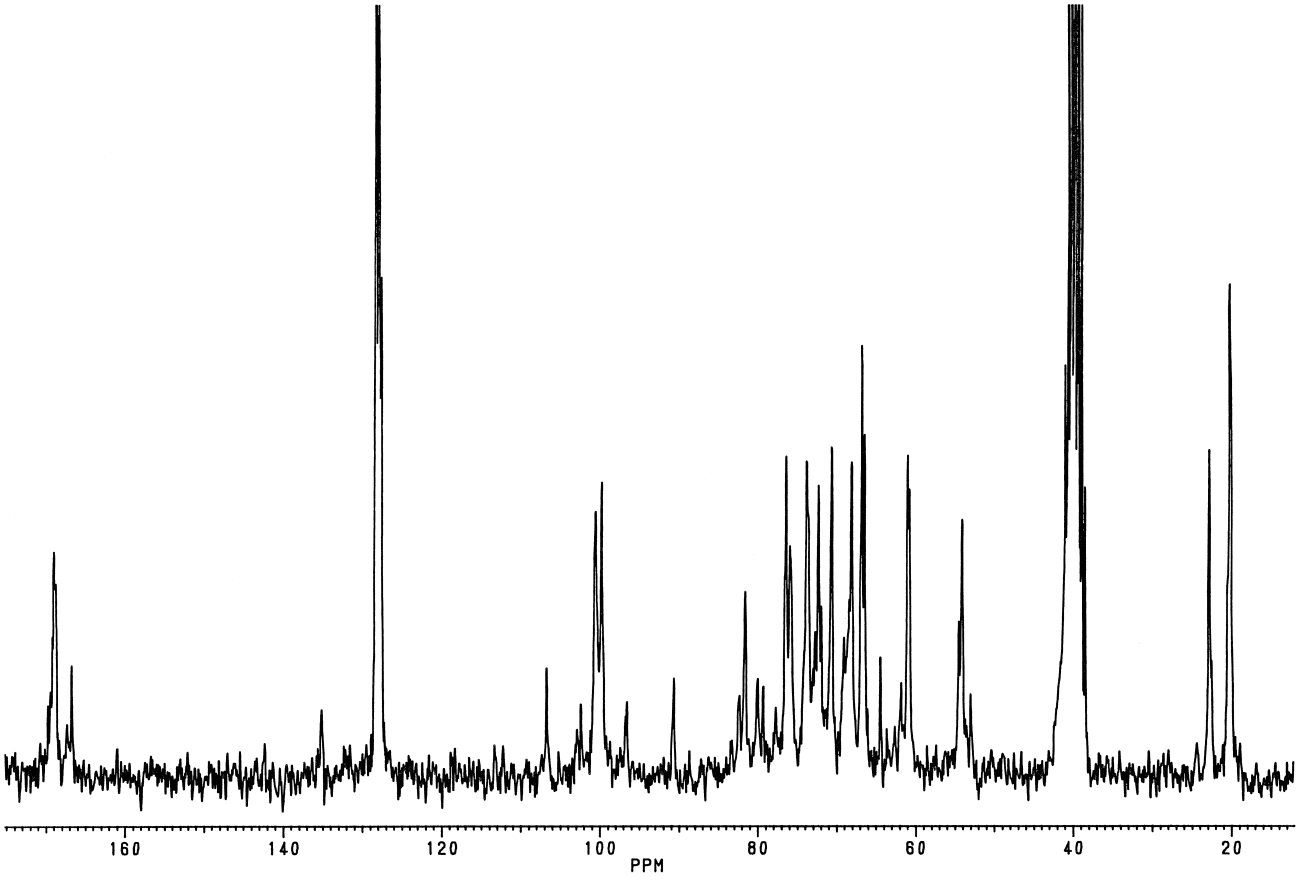


Fig. 3. ^{13}C NMR spectrum of compound **10** in Me_2SO-d_6. For the chemical shift data, see Tables 1 and 3.

The fully acetylated derivative of **1** was prepared by treatment with acetic anhydride in a mixture of *N*,*N*-dimethylformamide and pyridine in the presence of 4-(*N*,*N*-dimethylamino)pyridine to afford the tetraacetate derivative **11**. The signals due to the acetate protons in **11** appeared at δ 1.70 (at C-6), 1.96 (at C-4), 1.92 and 1.90 (at C-2′,3′). In order to distinguish between the ^{1}H resonances of the acetate group at C-4,6 and C-2′,3′ in **11**, the 2′,3′-diacetate-4,6-di(trideuterioacetate) **12** and the 2′,3′-di(trideuterioacetate)-4,6-diacetate **13** were prepared. In the ^{1}H NMR spectrum of 2′,3′-diacetate **12** the acetate signals were identified at δ 1.90 and 1.92, and in **13** at δ 1.70 and 1.96.

The qualitative treatment of the conformation of some of the derivatives of hyaluronic acid ester **1** have been considered. The presence of acetate groups on the polysaccharide can affect the ^{13}C chemical shifts of all carbons including the carbon around the glycosidic linkages [β-(1 → 4) and β-(1 → 3)]. The chemically induced effect of the acetyl group has been observed to be: $\alpha < +3$ ppm, $\beta < -4$ ppm, $\gamma < +1$ ppm, $\delta \approx 0$ ppm. The conformation of a polysaccharide can be determined by the dihedral angles Φ, Ψ of the glycosidic linkages. The dihedral angle Φ of the β-(1 → 4) glycosidic linkage is defined by the atoms H-1–C-1–O-1–C-4 and the dihedral angle Ψ by the atoms C-1–O-1–C-4–H-4. It is possible to estimate the changes of the dihedral angles Φ, Ψ based on the changes of the chemical shift of the anomeric and aglycone carbons involved in the glycosidic linkages (without any other effects contributing to the chemical shift of these carbons). Bock et al. [5] have shown that ^{13}C NMR chemical shifts of the anomeric and aglycone carbon atoms can be correlated with one of the glycoside angles (Ψ) which are involved in determining the conformation of oligosaccharides. We have extrapolated this conclusion in order to assess the conformation of hyaluronic acid derivatives. The differences in chemical shifts $\Delta\delta = \delta(X) - \delta(Y)$, where X is the derivative and Y is the substrate **1**, have been calculated for some of the compounds (Table 5). It is important to note that only chemical shifts of carbons without α-, β- and γ-chemically induced effect have been considered. Chemical shifts of carbons having the δ effect are shown in brackets, these values might not be exact due to the fact that the δ-chemically induced effect is supposed to be close to zero.

As shown in Table 5, no significant changes were observed for the 6-acetate **3**, presumably because the CH_2OH group is the only hydroxyl group in **1** which is not involved in any hydrogen-bond formation [4]. However, for the 4-acetate derivative **4**, there is a significant change in the chemical shift of C-1′ which indicates changes for the β-(1 → 3) glycosidic linkage; this is probably because on acetylation the OH-4 · · · O-5′ hydrogen bond is absent. Similarly, changes in the chemical shifts due to C-4′ and to a lesser degree for C-1′ and C-1 of the 4,6-orthoester **2** and the 4,6-*O*-isopropylidene derivative **8** were observed. This indicated substantial changes around the β-(1 → 4) glycosidic linkages in compounds **2** and **8**. The β-(1 → 3) glycosidic linkages appear not to be affected to the same degree by the chemical modification. However, a different $\Delta\delta$ value can be observed for the β-(1 → 3) glycosidic linkages for **2** and **8**. These changes around the (1 → 3) linkages in compounds **2** and **8** could be explained by the absence of the OH-4 · · · O-5′ hydrogen bonds. The significant changes in the chemical shifts of C-4′ and C-1 in **2** and **8**, cannot be explained on the basis of the hydrogen bond network, since the orthoester and isopropylidene groups are not involved in any possible hydrogen bond leading to stabilisation of the β-(1 → 4) glycosidic linkage. The changes around the β-(1 → 4) glycosidic linkage in **2** and **8** could be caused by the presence of the large orthoester or isopropylidene groups. In the literature the conformation of hyaluronic acid benzyl ester (**1**) in dimethyl sulfoxide using ^{1}H and ^{13}C NMR spectroscopy has been studied and based on the chemical shifts of the resonances due to HO-2′ (the highest) and HO-4 (the lowest) the presence of a strong HO-2′ · · · HNC=O and a weak HO-4 · · · O-5′ hydrogen bond has been concluded [4]. However, we have not observed any significant changes in either β-(1 → 3) or β-(1 → 4) glycosidic linkages for the 2′,3′-diacetate **10**, suggesting that the hydrogen bonds 3′-OH · · · O-5 and 2′-OH · · · OCNH in this compound are weaker than in the benzyl hyaluronate **1** [4].

Table 5
The difference in chemical shifts ($\Delta\delta$) for selected compounds

Compound	C-1′	C-3	C-1	C-4′
2	0.37	–	(−0.81)	−1.56
3	−0.01	(≈0.59)	−0.23	≈−0.13
4	0.68	–	(−0.23)	≈−0.13
8	−0.1	–	(−0.78)	−1.53
10	–	−0.38	0.20	–

The analysis of the conformational changes around the glycosidic linkages made for compounds **2-4, 8**

and **9** could only be considered as qualitative because the data for the changes in chemical shifts are only for one of the dihedral angles (Ψ) for both the glycosidic linkages. It is also important to note that all experiments were performed at the same temperature (333 K) and concentration (60 mg/2.5 mL of the solvent).

3. Experimental

General methods.—All evaporations were carried out under reduced pressure. Compound **1** (Mw 152,000) was a gift from Fidia, Abano Terme, Padova, Italy. Prior to reaction the starting material **1** was dried in an oven under vacuum for one night at 40 °C over P_2O_5. NMR spectra were recorded at 4.70 T (^{1}H, 200 MHz; ^{13}C, 50.3 MHz) or at 7.05 T (^{1}H, 300 MHz;^{13}C, 75.4 MHz) with a Bruker AC 200 or AM 300 WB instrument, respectively. Spectra were recorded in Me_2SO-d_6 with the chemical shift of residual Me_2SO in the solvent being used as an internal reference. ^{1}H–^{1}H correlation spectroscopy (COSY) was performed with the pulse sequence COSY45, using second pulse $\pi/4$. Zero-filling in the F1 dimension and centered sine-bell window multiplication was used in each dimension prior to Fourier transformation. Heteronuclear (^{1}H–^{13}C) 2D chemical shift correlation spectroscopy was performed with ^{13}C detection (HETCOR) and with ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC) spectroscopy (300 MHz), respectively. The applied pulse sequence for the ^{13}C detected experiment gave proton decoupling in each dimension. The pulse sequence used for ^{1}H-detected heteronuclear correlation spectroscopy gave a carbon decoupling. Zero-filling in the F1 dimension and shifted square sine-bell multiplication or Lorentzian–Gaussian multiplication were used prior to Fourier transformation in both dimensions for all heterocorrelated experiments. Heterocorrelated (^{1}H–^{13}C) long-range experiments were carried out in their ^{13}C detected version (COLOC) and ^{1}H-detected version (HMBC), respectively. The delay optimised for ^{1}H–^{13}C long-range coupling constants was set to 80 ms in both types of long range heterocorrelated experiments. Similarly to in the one-bond heterocorrelated experiments the zero-filling in the F1 dimension and shifted square sine-bell multiplication or Lorentzian–Gaussian multiplication were used prior to Fourier transformation in both dimensions. ^{1}H and ^{13}C NMR data for all compounds are given in Tables 1–5.

4,6-O-cyclic-orthoester hyaluronic acid benzyl ester (**2**).—A suspension of **1** (5 g, 10.15 mmol) in Me_2NCHO (120 mL) was treated with $MeC(OMe)_3$ (5 mL, 39.3 mmol) in the presence of *p*-toluenesulfonic acid (100 mg) as the catalyst at room temperature for 20 h. The reaction mixture became viscous and difficult to stir. Further addition of Me_2NCHO (60 mL) afforded a mobile clear solution which was stirred for 22 h. To the solution was added 1:2 water–Me_2NCHO (4.8 mL), adjusted to pH 5 with CH_3COOH (0.5 mL), and the mixture was stirred for 2.5 h, then neutralised with IR 400 (OH^-). After filtration, the filtrate was concentrated by co-distillation with toluene to half the volume, then EtOH was added to give a product (4.5 g) which contained compound **2** as the major and the **6**-acetate (**3**) and 4-acetate (**4**) as minor components.

*6- and 4-O-acetyl hyaluronic acid benzyl ester (**3** and **4**, respectively).*—A solution of **2** (2.5 g) in Me_2NCHO (55 mL) and water (1.5 mL) was treated with *p*-toluenesulfonic acid (50 mg) with stirring for 22 h at room temperature. The solution was treated with *t*-butylamine (0.25 mL), stirred for 3.5 h, and the mixture was neutralised with IR 400 (OH^-). Precipitation by adding EtOH as described above gave a product (2.1 g) which contained the 6-acetate (**3**) as the major and the 4-acetate (**4**) as the minor compound. The 4,6-orthoester **2** was present in trace amount.

4,6-O-cyclic-orthoester-2′,3′-di-O-acetyl hyaluronic acid benzyl ester (**5**).—A solution of **2** (0.5 g, 0.95 mmol) in dry pyridine (25 mL) was treated with Ac_2O (5 mL, 53 mmol) in the presence of DMAP (100 mg) with stirring at 50 °C for 24 h. The solvent was removed under reduced pressure and compound **5** was precipitated from Et_2O (0.51 g).

*2′,3′,6- and 2′,3′,4-tri-O-acetyl hyaluronic acid benzyl ester derivative (**6** and **7**, respectively).*—A solution of **5** (0.25 g, 0.48 mmol) in Me_2NCHO (10 mL) was treated with *p*-toluenesulfonic acid (20 mg) and water (0.5 mL) at room temperature for 22 h. After the addition of *t*-butylamine (0.1 mL), the solution was stirred for 3.5 h at room temperature. The mixture was neutralised with IR 400 (OH^-), filtered, and the filtrate was concentrated by co-distillation with toluene and the residue was precipitated from EtOH to afford a mixture of **6** and **7** (0.21 g).

*4,6-O-isopropylidene hyaluronic acid benzyl ester (**8**).*—A solution of **1** (1 g, 2.03 mmol) in Me_2SO (25 mL) was treated with $Me_2C(OMe)_2$ (5 mL, 40.6 mmol) in the presence of *p*-toluenesulfonic acid (100 mg) with stirring at 50 °C for 24 h. The brown

solution was poured into saturated $NaHCO_3$, and the precipitate was filtered, washed with water and then with Et_2O. The compound was dried overnight under vacuum at 40 °C to afford **8** (0.85 g).

2′,3′-Di-O-acetyl-4,6-O-isopropylidene hyaluronic acid benzyl ester (**9**).—A solution of **8** (0.5 g, 0.98 mmol) in dry pyridine (25 mL) was treated with Ac_2O (5 mL, 53 mmol) in the presence of DMAP (100 mg) with stirring at 50 °C for 24 h. The solvent was removed under reduced pressure and compound **9** was precipitated from Et_2O (0.52 g).

2′,3′-Di-O-acetyl hyaluronic acid benzyl ester (**10**).—A solution of **9** (0.5 g, 0.80 mmol) in Me_2SO (5 mL) was treated with 9:1 CF_3CO_2H–water (3 mL) at 100 °C for 3 h. The mixture was neutralised with saturated $NaHCO_3$ and dialysed against distilled water. Concentration of the dialysate gave **10** (0.3 g).

2′,3′,4,6-Tetra-O-acetyl hyaluronic acid benzyl ester (**11**).—A solution of **1** (1 g, 2.03 mmol) in pyridine (20 mL) and Me_2NCHO (20 mL) was treated with Ac_2O (10 mL, 106 mmol) in the presence of DMAP (100 mg) with stirring at 50 °C for 24 h. The solvent was removed under reduced pressure and the product was precipitated from Et_2O to afford **11** (1.2 g).

2′,3′-Di-O-acetyl-4,6-di-O-trideuterioacetyl hyaluronic acid benzyl ester (**12**).—A solution of **10** (0.5 g, 0.87 mmol) in dry pyridine (25 mL) was treated with Ac_2O-d_6 (5 mL, 53 mmol) in the presence of DMAP (100 mg) with stirring at 50 °C for 24 h. The solvent was removed under reduced pressure and compound **12** was precipitated from Et_2O (0.35 g).

2′,3′-Di-O-trideuterioacetyl-4,6-di-O-acetyl hyaluronic acid benzyl ester (**13**).—A solution of **8** (0.5 g, 0.93 mmol) in dry pyridine (25 mL) was treated with Ac_2O-d_6 (5 mL, 53 mmol) in the presence of DMAP (100 mg) with stirring at 50 °C for 24 h. The solvent was removed under reduced pressure and the 2′,3′-di-*O*-trideuterioacetyl-4,6-*O*-isopropylidene derivative was precipitated from Et_2O. Hydrolysis of the isopropylidene group was achieved in Me_2SO (5 mL) with 9:1 CF_3COOH–water (3 mL) at 100 °C for 3 h. The mixture was neutralised with saturated $NaHCO_3$, dialysed against distilled water and freeze dried. A solution of the solid residue (0.2 g, 0.32 mmol) in dry pyridine was treated with Ac_2O (5 mL, 53 mmol) in the presence of DMAP (50 mg) with stirring at 50 °C for 24 h. The solvent was removed under reduced pressure and compound **13** was precipitated from Et_2O (0.15 g).

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