Preparation and Anti-HIV Activity of O-Acylated Heparin and Dermatan Sulfate Derivatives with Low Anticoagulant Effect

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In order to increase the ratio of anti-HIV activity to anticoagulant activity, glycosaminoglycan derivatives selectively substituted at OH and/or COOH groups were prepared. Standard heparin, heparin fragments, or dermatan sulfate were converted to their tributylammonium or tetrabutylammonium salts. Their selective *O*-acylation to various (controlled) degrees was carried out in a homogeneous way in *N,N*-dimethylformamide using carboxylic acid anhydrides and 4-(dimethylamino)pyridine as catalyst. Esterification of the COOH groups was performed by the addition of alkyl halide to an *N,N*-dimethylformamide solution of glycosaminoglycan tetrabutylammonium salts. The *in vitro* anticoagulant activity, the activity against HIV-1 and HIV-2 cytopathicity, the cytotoxicity, and the activity on the induction of giant cell formation were determined. *O*-acylation (*O*-butyrylation or *O*-hexanoylation) of the heparin fragments obtained by periodate depolymerization (compounds **2d** and **2e**), and their esters (compounds **7i and 7j**), yielded products with very low anticoagulant effects *in vitro*, yet potent activity against both HIV-1 and HIV-2 induced cytopathicity, and low, if any, cytotoxicity. As compared to other anionic polysaccharides, these acylated derivatives are more active as inhibitors of HIV-induced giant-cell formation. Their anti-HIV activity is related to the degree of *O*-acylation and is mainly due to the inhibition of virus adsorption to the target cells.

Introduction

A wide range of anionic polysaccharides are potent inhibitors of HIV replication *in vitro*.[1,2] Generally the polyanions interfere with an early event in HIV infection, i.e., the adsorption of the virus particles to the target lymphocytes[3,4] by interacting with the envelope glycoprotein gp 120.[5,6] The polyanions also prevent the adsorption of other enveloped viruses (such as HSV, CMV, VSV, etc.) to the target cells.[7-9]

Previous structure–activity relationship studies have shown that the anti-HIV activity of polyanions is related essentially to the anionic charge and is not specifically dependent of a particular structure of the polyanionic backbone. Thus, the backbone may be a polysaccharide consisting of various repeating units, or a simpler polymer, such as polyvinyl alcohol.[1,2]

Of the numerous polyanions studied *in vitro* for their anti-HIV activity, only dextran sulfate and pentosan polysulfate have been the subject of clinical trials. High-dose regimens of dextran sulfate showed no significant therapeutic benefit versus side effects, following either oral or intravenous administration.[10,11] However, other polyanions, which act as inhibitors of HIV adsorption, might still be interesting in the chemotherapy of HIV infections, for example when used in combination with other inhibitors of HIV replication.[12]

Heparin, also a polyanion, is a widely used and well-tolerated antithrombotic drug. Like other polyanions, heparin has also been found to be an effective anti-HIV agent *in vitro*.[3,13] As a potential drug for the treatment of HIV infections, it has not received much attention, mainly because of its anticoagulant activity. However, it is now well-documented that the anticoagulant activity of heparin depends on particular structural features and that certain chemical modifications of heparin chains may considerably decrease anticoagulant potency without altering other pharmacological effects of heparins. Thus, periodate-depolymerized heparin fragments, which lack the unique heparin sequence that is essential for high affinity for ATIII, no longer have *in vitro* anticoagulant activity, but remain active as antiproliferative agents on vascular smooth muscle cells.[14] Also, periodate-treated heparin has a markedly reduced antithrombin activity while potent anti-HIV activity is maintained.[15] Since anti-HIV activity was shown to be correlated to the degree of heparin chain sulfation, we prepared a number of heparin derivatives that were unmodified in this respect but selectively substituted in either free OH groups or COOH groups, in order to increase the ratio of anti-HIV activity to anticoagulant potency. We determined their *in vitro* activity against the cytopathicity of HIV-1 and HIV-2, their cytotoxicity, and their effect on the induction of giant-cell formation. In these assays, dextran sulfate and pentosan polysulfate were included as reference anti-HIV polyanions. We show here that certain *O*-acylated derivatives are potent inhibitors of HIV-1- and HIV-2-induced cytopathicity and are more active than the parent underivatized compounds in preventing giant-cell formation.

Chemistry

Glycosaminoglycans are complex polysaccharide structures bearing several different chemical functions. In the present study we have prepared variously substituted derivatives of the following four glycosaminoglycan types: heparin, CY 216, SR 80258, and dermatan sulfate.

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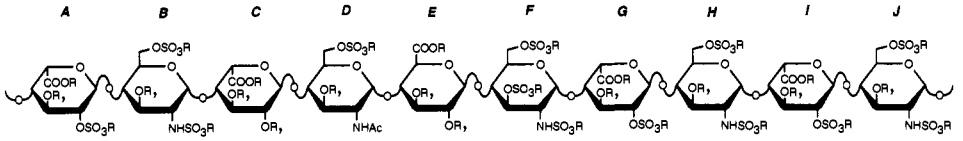
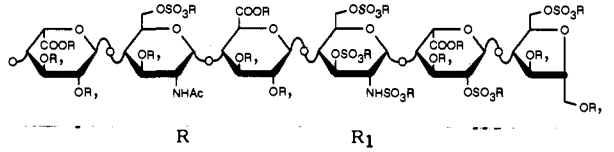


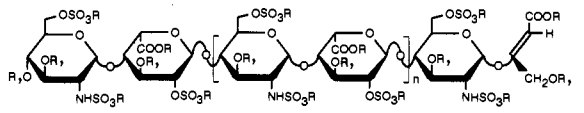
	R	R_1		R	R_1
1a	Na+	H	1d	Na+	H/$CH_3(CH_2)_2CO$
1b	$[CH_3(CH_2)_3]_3N^+H$	H	1e	Na+	H/$CH_3(CH_2)_4CO$
1c	Na+	H/CH_3CO	1f	Na+	H/C_6H_5CO

Figure 1.



	R	R_1
5a	Na^+	H
5b	$[CH_3(CH_2)_3]_3N^+H$	H
5c	Na^+	H/CH_3CO
5d	Na^+	H/$CH_3(CH_2)_2CO$
5e	Na^+	H/$CH_3(CH_2)_4CO$

Figure 2.



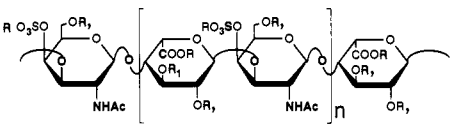
	R	R_1
2a, 3a	Na+	H
2b	$[CH_3(CH_2)_3]_3N^+H$	H
2c	Na+	H/CH_3CO
2d, 3b-e, 4a-k	Na+	H/$CH_3(CH_2)_2CO$
2e, 3f-j	Na+	H/$CH_3(CH_2)_4CO$
2f	Na+	H/$CH_3(CH_2)_6CO$
2g	Na+	H/C_6H_5CO
2h	Na+	H/$NaOOC(CH_2)_2CO$

Figure 3.

Heparin consists of a carbohydrate backbone made up of alternating 1,4-linked uronic acid (iduronic or glucuronic) and glucosamine residues. A standard heparin preparation consists of a mixture of linear polysaccharides composed of approximately 20–100 monosaccharides. Most of the monosaccharides bear one or several substituents at different positions, as shown in Figure 1.

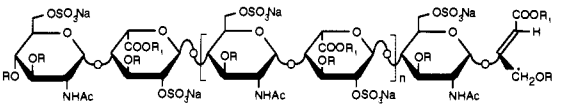
CY 216 (INN approved name Nadroparin Calcium[16]) is a low molecular weight heparin preparation made by controlled nitrous acid depolymerization followed by borohydride reduction. The structure of CY 216 is similar to that of heparin with two main differences: the polysaccharide chains consist of 6–30 monosaccharides and a large number of the chains end up with a 2,5-anhydromannitol residue (resulting from the nitrous acid/borohydride treatment) as indicated in Figure 2.

SR 80258 is a low-molecular-weight heparin preparation obtained by periodate oxidation followed by basic treatment and borohydride reduction.[20] The structure of SR 80258 differs slightly from that of heparin. The chains consist of 6–30 monosaccharide residues and are made up of repeating disaccharide units containing almost exclusively *N*-sulfated glucosamine and 2-*O*-sulfated iduronic acid residues (see Figure 3; periodate oxidation followed by base elimination results in destruction of unsulfated uronic acid residues, i.e. glucuronic and unsulfated iduronic acids).



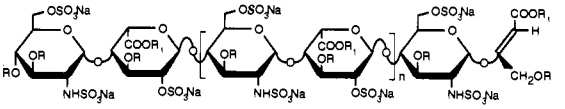
	R	R_1
6a	Na+	H
6b	$(CH_3(CH_2)_3]_3N^+H$	H
6c	Na^+	H/CH_3CO
6d	Na^+	H/$CH_3(CH_2)_2CO$
6e	Na^+	H/$CH_3(CH_2)_4CO$
6f	Na^+	H/$NaOOC(CH_2)_2CO$

Figure 4.



	R	R_1
8a	H	Na
8b	H/$CH_3(CH_2)_2CO$-	Na
8c	H	$CH_3(CH_2)_3$
8d	H/$CH_3(CH_2)_2CO$-	$CH_3(CH_2)_3$

Figure 5.



	R	R_1
7a	H	$[CH_3(CH_2)_3]_4N^+$
7b	H	Na+/CH_2:$CHCH_2$-
7c	H	Na+/CH_2:$CHCH_2CH_2$-
7d	H	Na+/CH_3CH:$CHCH_2$-
7e	H	Na+/$CH_3OC_6H_4CH_2O$-
7f	H	Na+ /$CH_3(CH_2)_7$-
7g	H	Na+ /$CH_3(CH_2)_7$-
7h	H/$CH_3(CH_2)_2CO$-	Na+ /$CH_3(CH_2)_3$-
7i	H	H/$C_6H_5CH_2$
7j	H/$CH_3(CH_2)_2CO$-	H/$C_6H_5CH_2$

Figure 6.

Dermatan sulfate is a polysaccharide made up of alternating 1,3-linked iduronic acid and galactosamine residues. A standard dermatan sulfate preparation consists of a mixture of linear polysaccharides composed of approximately 20–100 monosaccharides. Most of the monosaccharides bear one or several substituents at different positions, as shown in Figure 4.

Table I. Analytical Characteristics of Various O-Acylated Heparin Derivatives

	derivative	degree of substitution[a]	SO^{3-} (mequiv/g)	COO^- (mequiv/g)	SO^{3-}/COO^-	^1H NMR[b] (ppm)	^{13}C NMR[b] (ppm)
1a	sodium salt	0	3.70	1.70	2.16		
1b	tributylamine salt	0	ND[c]	ND	ND		
1c	O-acetylated	1.74	3.31	1.28	2.58		23.4
1d	O-butyrylated	1.25	3.06	1.41	2.17	1.04, 1.71, 2.49	
1e	O-hexanoylated	1.26	2.80	1.23	2.28		15.9, 24.3, 26.5, 33.1, 36.5
1f	O-benzoylated	0.66	3.03	1.21	2.50		131.5, 132.3, 136.8

[a] The degree of substitution indicates the number of acyl groups introduced per basic disaccharide unit. [b] ^1H and ^{13}C NMR chemical shifts are expressed from internal TSP. [c] ND, not determined.

Table II. Anti-HIV and Anticoagulant Activity of O-Acylated Heparin Derivatives

	anticoagulant activity (APTT)[e] (IU/mg)	anti-HIV activity EC_{50}[a] (μg/mL) HIV-1 cytopathic effect	HIV-1 giant-cell formation	HIV-2 cytopathic effect	cytotoxicity SI[b] HIV-1	HIV-2
1a	168	0.8	12.3	4.5	>625	>110
1c	88	0.56	6.5	1.2	>893	>410
1d	44	0.32	1.7	1.7	>1500	>294
1e	28	0.7	3.8	5.4	>714	>92
1f	206	0.7	7.6	0.7	59	241
DxS[c]	14	0.4	25.0	0.08	>6250	
PPS[d]	15	1.1	16.0	0.1		

[a] Effective concentration of compound required to achieve 50% protection of MT-4 cells against the cytopathic effect of HIV-1 or HIV-2 or against giant-cell formation between persistently HIV-infected HUT-78 cells and uninfected MOLT-4 cells. [b] Selectivity index = CC_{50}/EC_{50} (where CC_{50} represent the 50% cytotoxic concentration based on the reduction of the viability of mock-infected MT-4 cells). [c] DxS = dextran sulfate (Sigma), 5000 Da. [d] PPS = pentosan polysulfate (Sigma). [e] APTT = activated partial thromboplastin time.

Table III. Analytical Characteristics of Various O-Acylated Derivatives of SR 80258

	derivative	degree of substitution[a]	SO_3^- (mequiv/g)	COO^- (mequiv/g)	SO_3^-/COO^-	^1H NMR[b] (ppm)	^{13}C NMR[b] (ppm)
2a	sodium salt	0	3.87	1.58	2.48		
2b	tributylamine salt	0	3.85	1.60	2.40		
2c	O-acetylated	1.19	3.55	1.42	2.49		23.5
2d	O-butyrylated	1.40	3.33	1.31	2.54	0.95, 1.62, 2.49	15.7, 20.5, 39.4
2e	O-hexanoylated	0.81	3.49	1.41	2.47		16.01, 24.4, 26.6, 33.2, 36.5
2f	O-octanoylated	1.60	2.63	1.11	2.37	0.87, 1.30, 1.61, 2.44	
2g	O-benzoylated	0.73	3.63	1.30	2.79	7.66, 8.06	
2h	O-succinylated	0.60	3.06	2.78	1.10	2.54, 2.70	

[a] The degree of substitution indicates the number of acyl groups introduced per basic disaccharide unit. [b] ^1H and ^{13}C NMR chemical shifts are expressed from internal TSP.

Table IV. Anti-HIV and Anticoagulant Activity of O-Acylated Periodate-Oxidized Heparin Fragments

	anticoagulant activity (APTT) (IU/mg)	anti-HIV activity EC_{50}[a] (μg/mL) HIV-1 cytopathic effect	HIV-1 giant-cell formation	HIV-2 cytopathic effect	cytotoxicity SI[b] HIV-1	HIV-2
2a	4	1.53	>500	6.3	>327	>80
2c	10	0.53	165	0.59	>943	>847
2d	12	0.35	2.6	0.30	>1430	>1667
2e	7	0.31	2.9	1.71	>1613	>294
2f	ND	3.3	20	>23.2	6	>1
2g	8	0.1	3	0.2	>5000	>2500
2h	3.1	0.2	>100	5.0	>2500	>100
DxS[c]	14	0.4	25.0	0.08	>6250	
PPS[d]	15	1.1	16.0	0.1		

[a–d] As for footnotes to Table II. [e] ND, not determined.

The carbohydrate backbone of these glycosaminoglycans contains primary and secondary hydroxyl groups, O- and N-sulfate groups, N-acetyl groups, and carboxylate groups. Chemical methods to prepare derivatives of such polyfunctional molecules have to take into account this polyfunctional character of the starting material. The different reactivity, or stability, of the various functional groups involved has to be determined under different experimental conditions. For this purpose analytical methods also have to be developed since one cannot rely on the colorimetric determinations often used in the past. For these reasons, the products reported in the present study were prepared and characterized using methods that we have recently developed[17] to obtain well-defined acylated derivatives of the glycosaminoglycans.

O-Acylation: Preparation of Compounds 1c-f, 2c-h, 3b-j, 4a-k, 5c-e, 6c-f, Table I. The glycosaminoglycans (usually available as the sodium salts) were first converted into their tributylammonium or tetrabutylammonium salts, which are much more soluble in organic solvents. The acylation reaction was thus carried out in a homogeneous medium, N,N-dimethylformamide, using the desired acid anhydride and 4-(dimethylamino)pyridine as catalyst. We calculated that 1 g of starting glycosaminoglycan corresponds to 4 mequiv of OH groups in the case of heparin sodium salt and to 6 mequiv in the case

Table V. Analytical Characteristics of *O*-Acylated Derivatives of SR 80258

	derivative	degree of substitution[a]	SO^{3-} (mequiv/g)	COO^- (mequiv/g)	SO^{3-}/COO^-	^1H NMR[b] (ppm)
3a	sodium salt	0	3.68	1.48	2.48	
3b	butyrylated	0.36	3.78	1.58	2.39	0.76, 4.43, 2.25
3c	butyrylated	0.43	3.78	1.57	2.40	0.76, 4.43, 2.25
3d	butyrylated	0.68	3.60	1.53	2.35	0.76, 4.43, 2.25
3e	butyrylated	1.00	3.51	1.52	2.31	0.76, 4.43, 2.25
2a	sodium salt[c]	0	3.65	1.57	2.33	
3f	hexanoylated	0.18	3.36	1.43	2.35	0.83, 1.27, 1.55, 2.38
3g	hexanoylated	0.39	3.15	1.32	2.39	0.83, 1.27, 1.55, 2.38
3h	hexanoylated	0.61	3.16	1.34	2.36	0.83, 1.27, 1.55, 2.38
3i	hexanoylated	1.10	3.11	1.30	2.39	0.83, 1.27, 1.55, 2.38
3j	hexanoylated	1.40	2.84	1.23	2.31	0.83, 1.27, 1.55, 2.38

[a] The degree of substitution indicates the number of acyl groups introduced per basic disaccharide unit. [b] ^1H NMR chemical shifts are expressed from internal TSP. [c] Compounds **2a** and **3a** are two different batches of SR80258A.

Table VI. Anti-HIV and Anticoagulant Activity of *O*-Acylated Periodate-Oxidized Heparin Fragments (Role of Degree of Acylation)

	anticoagulant activity (APTT) (IU/mg)	anti-HIV activity EC_{50}[a] (μg/mL)				cytotoxicity SI[b]	
		HIV-1		HIV-2			
		cytopathic effect	giant-cell formation	cytopathic effect	giant-cell formation	HIV-1	HIV-2
3a	5	0.52	>200	77	>200	>961	318
3b	5	0.32	70.5	1.94	ND[c]	1121	185
3c	7	0.32	60.4	1.17	ND	1032	282
3d	9	0.21	10.3	0.36	ND	1480	914
3e	11	0.26	1.5	0.28	ND	1357	1261
2a	4	1.53	>500	6.3	ND	>327	>80
3f	4	0.2	7.0	1.0	100	>2500	>500
3g	4	0.1	6.0	0.8	75	>5000	>650
3h	6	0.1	3.0	0.4	50	>5000	>1250
3i	7	0.2	2.0	0.3	13	>2600	>1666
3j	6	0.4	2.0	1.0	10	>1250	>500

[a,b] As for footnotes to Table II. [c] ND, not determined.

Table VII. Analytical Characteristics of *O*-Butyrylated Derivatives of SR 80258: Fragments of Increasing Molecular Weight

	molecular weight[a]	polymerization degree[b]	degree of substitution[c]	SO^{3-} (mequiv/g)	COO^- (mequiv/g)	SO^{3-}/COO^-	^{13}C NMR (ppm)[d]
4a	1500	4	2.20	3.11	1.20	2.59	15.6, 20.4, 38.4
4b	2190	6	1.53	2.97	1.23	2.41	15.6, 20.4, 38.4
4c	3050	8	1.58	2.94	1.15	2.55	15.6, 20.4, 38.4
4d	3090	10	1.43	3.15	1.40	2.25	15.6, 20.4, 38.4
4e	4500	12	1.48	3.45	1.43	2.41	15.6, 20.4, 38.4
4f	5100	14	1.42	3.23	1.31	2.46	15.6, 20.4, 38.4
4g	6030	15	1.40	3.26	1.32	2.47	15.6, 20.4, 38.4
4h	6100	16	1.36	3.37	1.30	2.59	15.6, 20.4, 38.4
4i	6700	18	1.39	3.11	1.19	2.61	15.6, 20.4, 38.4
4j	7400	20	1.27	3.31	1.27	2.60	15.6, 20.4, 38.4
4k	8800	23	1.32	3.24	1.29	2.51	15.6, 20.4, 38.4

[a] Calculated from the molecular weight of the unsubstituted fragments and the degree of acylation. [b] Average number of monosacchride units per chain. [c] The degree of substitution indicates the number of acyl groups introduced per basic disaccharide unit. [d] ^{13}C NMR chemical shifts are expressed from internal TSP.

of dermatan sulfate sodium salt. In general (preparation of compounds **1c-f**) we used a 5-fold molar excess of acylating reagent and tributylamine and from 0.05 to 0.25 mequiv of 4-(dimethylamino)pyridine.

The degree of acylation could be easily modulated by varying the experimental parameters, such as temperature (in the range 20–50 °C), concentration, and duration of the reaction. These parameters must be carefully adjusted to obtain low or intermediate degrees of substitution (Table V). However, the upper limit was found to be independent of the experimental conditions used,[17] and the most highly substituted compounds could be obtained either by increasing the reaction time or by using an excess of reagents. Under identical reaction conditions we found that the degree of substitution decreases when the molecular weight increases (Table VII). This result is not surprising if one consider that the higher the molecular weight, the lower the reaction rate.

Under the experimental conditions reported, using acid anhydrides, glycosaminoglycans were selectively *O*-acylated, and no desulfation occurred. On some occasions we observed the formation of mixed anhydrides between the carboxylate group of the polysaccharide and an acyl group of the acylating reagent. This side reaction was detected through an increased sulfate/carboxylate ratio, determined by conductimetry. It could be easily reversed by treatment of the final product with a sodium hydrogen carbonate solution. Such a treatment step was not performed for some compounds (**1c, 1f, 2g**).

In contrast with what is observed using acid anhydrides, in the presence of an acid chloride, another classical acylating reagent, we found that substitution also occurs at the nitrogen atom. Thus, in the case of heparin, acylation with an acyl chloride results in *N*-*O*-acylated compounds due to a N-desulfation–*N*-acylation reaction.[18] We took advantage of this side reaction to prepare *N*-acylated derivatives in a simple way, i.e. by prior *N*,*O*-

Table VIII. Anti-HIV and Anticoagulant Activity of O-Butyrylated Periodate-Oxidized Heparin Fragments of Increasing Molecular Weight

	anticoagulant activity (APTT) (IU/mg)	anti-HIV activity EC_{50}[a] (μg/mL)			cytotoxicity SI[b]	
		HIV-1		HIV-2		
		cytopathic effect	giant-cell formation	cytopathic effect	HIV-1	HIV-2
4a	0.3	115.0	>100	0.07	>4	>7142
4b	1.5	20.9	17.4	0.01	>24	>50000
4c	2.7	5.0	57.5	0.01	80	40000
4d	2.9	1.31	37.3	0.04	>382	>12500
4e	4.5	0.95	43.7	0.07	>526	>7142
4f	6.1	0.44	1.3	0.3	>1136	>1667
4g	8.5	0.28	31.9	0.17	>1786	>2141
4h	9.7	0.28	7.5	0.23	>1786	>2174
4i	16.8	0.27	2.1	0.16	>1852	>2941
4j	19.8	0.27	10.7	0.21	>1852	>2381
4k	29.3	0.51	2.0	0.26	>980	>1923

[a,b] As for footnotes to Table II.

Table IX. Analytical Characteristics of O-Acylated Derivatives of CY216

	derivative	degree of substitution[a]	SO^{3-} (mequiv/g)	COO^- (mequiv/g)	SO^{3-}/COO^-	^1H NMR[b] (ppm)	^{13}C NMR[b] (ppm)
5a	sodium salt	0	3.63	1.78	2.04		
5b	tributylamine salt	0	2.14	1.08	1.98		
5c	O-acetylated	1.68	3.03	1.42	2.13		22.8
5d	O-butyrylated	1.76	2.86	1.33	2.15	1.03, 1.70, 2.51	
5e	O-hexanoylated	1.56	2.73	1.29	2.11		15.8, 24.2, 26.4, 33.1, 36.4

[a] The degree of substitution indicates the number of acyl groups introduced per basic disaccharide unit. [b] ^1H and ^{13}C NMR chemical shifts are expressed from internal TSP.

Table X. Anti-HIV and Anticoagulant Activity of O-Acylated Low Molecular Weight Heparin (CY 216)

	anticoagulant activity (APTT) (IU/mg)	anti-HIV activity EC_{50}[a] (μg/mL)			cytotoxicity SI[b]	
		HIV-1		HIV-2		
		cytopathic effect	giant-cell formation	cytopathic effect	HIV-1	HIV-2
5a	11	77	ND[a]	ND	>8	
5c	26	6.5	6.4	0.46	76	1087
5d	11	0.53	15.4	0.24	89	15.4
5e	4	>100	8.9	5.4	<1	9
DxS[c]	14	0.4	25.0	0.08	>6250	
PPS[d]	15	1.1	16.0	0.1		

[a-d] As for footnotes to Table II. [e] ND, not determined.

Table XI. Analytical Characteristics of O-Acylated Dermatan Sulfate Derivatives

	derivative	degree of substitution[a]	SO^{3-} (mequiv/g)	COO^- (mequiv/g)	SO^{3-}/COO^-	^1H NMR[b] (ppm)	^{13}C NMR[b] (ppm)
6a	sodium salt	0	2.08	1.97	1.06		25.1 (NHAc)
6b	tributylamine salt	0	ND[c]	ND	ND		
6c	acetylated	2.90	1.63	1.53	1.06	2.11 (OAc), 2.17 (NHAc)	22.9 (OAc), 25.1 (NHAc)
6d	butyrylated	1.95	1.54	1.46	1.05	0.95, 1.62, 2.49	15.7, 20.5, 39.4
6e	hexanoylated	1.33	1.62	1.56	1.04		15.8, 24.2, 26.5, 33.1, 36.3
6f	succinylated	0.60	1.44	2.80	0.51		33.0, 33.5

[a] The degree of substitution indicates the number of acyl groups introduced per basic disaccharide unit. [b] ^1H and ^{13}C NMR chemical shifts are expressed from internal TSP. [c] ND, not determined.

acylation followed by selective O-deacylation (see preparation of **8a–d**) (see Figure 5).

Esterification (Alkylation) of Carboxylate Groups: Preparation of Compounds 7b–g. This straightforward reaction was performed by addition of alkyl halide to a *N,N*-dimethylformamide solution of the ammonium salts of the glycosamiglycans. The degree of esterification could be easily monitored by conductimetry titration of the carboxylate groups.

Some derivatives were obtained through combination of the above procedures. Thus the esterified O-acylated derivatives (**7h–j**) (see Figure 6) were synthesized from tetrabutylammonium salt of SR 80258 by reaction with an alkyl halide followed by reaction with the appropriate acid anhydride in the presence of tributylamine and 4-(dimethylamino)pyridine.

The compounds resulting from the above procedures were easily isolated as their sodium salt by precipitation in ethanol after prior addition of sodium chloride or sodium acetate. It was sometimes necessary to add a gel filtration step to eliminate traces of contaminating mineral salts. Lyophilization yielded easy to handle powders.

The compounds were characterized by ^1H and ^{13}C NMR spectroscopy. Typical chemical shifts for the newly introduced substituents are reported in Tables I, III, V, VII, IX, XI, XIII, and XV. IR spectroscopy showed the presence in all the compounds of a strong ester band in the range 1720–1735 cm^{-1}. Determination of the number of acyl groups introduced per disaccharide unit (degree of substitution) was performed by NMR spectroscopy and also by gas–liquid chromatography by quantitative analysis of the acyl radicals after transesterification in the presence

Table XII. Anti-HIV and Anticoagulant Activity of *O*-Acylated Dermatan Sulfate Derivatives

	anticoagulant activity (APTT) (IU/mg)	anti-HIV activity EC_{50}[a] (μg/mL)			cytotoxicity SI[b]	
		HIV-1		HIV-2		
		cytopathic effect	giant-cell formation	cytopathic effect	HIV-1	HIV-2
6a	1–2	>625	>500	>625	ND[c]	ND
6c	1.7	>500	>50	88	ND	5.7
6d	3.7	54.2	>100	18	8	25
6e	3.4	19.4	41	5.4	4	17.5
6f	9.0	150		50.4	1.7	2

[a,b] As for footnotes to Table II. [c] ND, not determined.

Table XIII. Analytical Characteristics of Esterified and Acylated Derivatives of SR 80258

	derivative	degree of substitution[a]	SO^{3-} (mequiv/g)	COO^- (mequiv/g)	^1H NMR[b] (ppm)	^{13}C NMR[b] (ppm)
7a	tetrabutylammonium salt	0	1.72	0.72		
7b	allyl ester	0.91	3.76	0.34	4.81, 5.44, 6.05	69.7, 134.1, 122.1
7c	1-butenyl ester	0.64	3.76	0.60	2.52, 4.30, 5.20, 5.93	35.0, 68.4, 120.3, 137.5
7d	2-butenyl ester	0.89	3.68	0.33	1.79, 4.80, 5.73, 5.95	20.0, 69.9, 126.4, 136.0
7e	4-methoxybenzyl ester	0.54	3.64	0.64	3.85, 7.10, 7.15	58.4, 72.0, 116.16
7f	octyl ester	0.70	3.68	0.53		
7g	butyl ester	0.70	3.77	0.55		15.7, 20.3, 21.3, 32.5 (butyloxy)
7h	*O*-butyrylated butyl ester	0.70	3.32	0.48		15.7, 20.5, 38.43 (butyryl)
7i	benzyl ester	0.82	3.29	0.35	4.70, 7.51	51.6, 131.4
7j	*O*-butyrylated benzyl ester	0.82	3.00	0.41	1.07, 1.74, 2.50, 4.75, 7.80	15.8, 20.5, 38.2, (butyryl); 51.7, 131.8 (benzyl)

[a] The degree of substitution indicates the number of acyl groups introduced per basic disaccharide unit. [b] ^1H and ^{13}C NMR chemical shifts are expressed from internal TSP.

Table XIV. Anti-HIV and Anticoagulant Activity of Esterified Periodate-Oxidized Heparin Fragments

	anticoagulant activity (APTT) (IU/mg)	anti-HIV activity EC_{50}[a] (μg/mL)				cytotoxicity SI[b]	
		HIV-1		HIV-2			
		cytopathic effect	giant-cell formation	cytopathic effect	giant-cell formation	HIV-1	HIV-2
3a	4	1.5	>500	6.3	ND[c]	327	>80
7b	2	0.2	±100	1.0	3	>2500	>500
7c	3	0.6	90	2.4	2	>833	>208
7d	3	0.1	10	1.3	1	>5000	>384
7e	4	0.1	>100	4.3	>100	>5000	>119
7f	3	0.7	4	1.1	18	>714	>454
7g	6	0.59	4	0.5	20	>833	>1000
7h	5	0.28	4	0.27	4	>1785	>1850
7i	4	0.33	1	0.4	2	>1515	>1250
7j	4	0.36	1	0.42	1	>1389	>1190

[a,b] As for footnotes to Table II. [c] ND, not determined.

Table XV. Analytical Characteristics of Esterified, Acylated Derivatives of *N*-Acetylated SR 80258

	derivative	degree of substitution[a]	SO^{3-} (mequiv/g)	COO^- (mequiv/g)	SO^{3-}/COO^-	^{13}C NMR[b] (ppm)
8a	*N*-desulfated–*N*-acetylated	0.80[c]	2.88	1.55	1.85	24.9 (NHAc)
8b	*N*-desulfated–*N*-acetylated–*O*-butyrylated	0.89[d]	2.60	1.43	1.81	24.9, 15.5, 20.5, 38.3 (butyryl)
8c	*N*-desulfated–*N*-acetylated butyl ester	0.80[c]	2.83	0.36	7.86	15.7, 21.3, 32.5, 39.5 (butyloxy)
8d	*N*-desulfated–*N*-acetylated–*O*-butyrylated butyl ester	1.36[d]	2.30	0.30	7.60	

[a] The degree of substitution indicates the number of acyl groups introduced per basic disaccharide unit. [b] ^{13}C NMR chemical shifts are expressed from internal TSP. [c] Degree of *N*-acetylation. [d] Degree of *O*-butyrylation.

of butanol and sulfuric acid.[19] There was in general a good agreement between the two methods.

Using these methods we were able to prepare and characterize the products used in the present study. Such preparations would easily be scaled up. All of the products tested were readily soluble in water.

Antiviral Activity

Different sulfated polysaccharides were *O*-acylated and tested for their anti-HIV-1 and anti-HIV-2 activity.

As shown in Table II, the *O*-acylation of standard heparin resulted in compounds with lower anticoagulant activity *in vitro* (except for the benzoyl derivative) and higher antiviral activity as compared to the starting compound. Thus, the butyrylated derivative was 2 times more active than standard heparin against both HIV-1- and HIV-2-induced cytopathicity and 7 times more active in inhibiting giant-cell formation induced by HIV-1 infected lymphocytes. These effects were associated with very low cytotoxicity (CC_{50} > 500 μg/mL).

In order to obtain compounds with a higher ratio of antiviral to anticoagulant activity, fragments were prepared by periodate oxidative cleavage of the glucuronic acid residues of standard heparin, followed by alkaline β-elimination.[20] This treatment removes more than 95% of the antithrombin binding sites in heparin, without altering the degree of sulfation. Resulting heparin fragments (average molecular weight of 6500 Da) lost most of their anticoagulant activity, but retained anti-HIV activity. As shown in Table IV, *O*-acylation of these fragments significantly increased the activity against both HIV-1- and HIV-2-induced cytopathicity without increasing cy-

Table XVI. Anti-HIV and Anticoagulant Activity of Esterified O-Acetylated-N-Desulfated-N-Acetylated Derivatives of Periodate-Depolymerized Heparin Fragments

	anticoagulant activity (APTT) (IU/mg)	anti-HIV activity EC_{50}[a] (μg/mL)				cytotoxicity SI[b]	
		HIV-1		HIV-2			
		cytopathic effect	giant-cell formation	cytopathic effect	giant-cell formation	HIV-1	HIV-2
3a	4	1.53	>500	6.3	ND[c]	>327	>80
8a	2.4	4.1	500	2.7	60	>122	>185
8b	6.0	0.5	15	1.2	20	>1000	>417
8c	1.2	6.5	100	2.5	20	>77	>200
8d	1.3	2.3	15	2.6	22	>217	>192

[a,b] As for footnotes to Table II. [c] ND, not determined.

totoxicity. The O-butyryl, O-hexanoyl, and O-benzoyl derivatives were more active than dextran sulfate or pentosan polysulfate in preventing giant cell formation induced by the HIV-1 infected MT4 lymphocytes in cocultures with MT-4 lymphocytes.

In contrast with standard heparin derivatives, butyrylated and hexanoylated derivatives of the periodate-oxidized fragments partially regained anticoagulant activity, which, however, did not exceed 10% of the standard heparin activity.

At a similar, high degree of acylation, no apparent correlation was found between the length of the acyl residue and the antiviral potency (the octanoyl derivative **2f** was less active than the butyryl derivative). No additional improvement in antiviral activity was obtained when the acyl residue contained additional carboxyl groups (i.e., the succinyl derivative **2h**).

For the same O-acyl residue, antiviral activity increased as a function of the degree of acylation. The data in Table VI were obtained with the major fraction (6000–7000 Da) and the global periodate-depolymerized heparin, O-butyrylated or O-hexanoylated, respectively. These data show that one acyl residue is necessary to obtain "optimal" antiviral activity for the butyryl derivatives, while a lower degree of acylation (approximately 0.4 acyl residue per disaccharide) is required to obtain similar antiviral activity for the hexanoyl derivative.

Our results also show that a higher degree of acylation is necessary to increase the inhibitory activity against giant-cell formation than to increase the inhibitory activity against viral cytopathicity.

It was previously shown that the anti-HIV activity of polyanions varies as a function of molecular size[3] and that optimal chain length for the antiviral activity varies as a function of the HIV strain and the cell line used.[21] For the O-butyrylated derivatives of periodate-oxidized heparin fragment which have a high degree of acylation (1.4-2 residues/disaccharide unit), anti-HIV-1 and anti-HIV-2 activity differed in their dependency on molecular size (Table VIII). Thus, anti-HIV-1 activity required fragments longer than tetradecasaccharides (compounds **4g** to **4j**), while "optimal" anti-HIV-2 activity was obtained with fragments equivalent to hexasaccharides. These small fragments are practically devoid of anticoagulant activity *in vitro*.

The consequence of O-acylation for antiviral activity varied with the polysaccharide backbone. When butyrylated, other low molecular weight heparin preparations (CY 216, Nadroparin, MW between 1800 and 8000 Da), which still contain antithrombin binding sites, had only weak anti-HIV activity, did not protect against giant-cell formation, and showed higher cytotoxicity (Table X). Similarly, in the case of O-acylation of dermatan sulfate,

Table XVII. Comparative Antiviral Activity of SR 80258 Derivatives against Various Strains of HIV-1 and HIV-2

	EC_{50} (μg/mL)						
	HIV-1				HIV-2		
	$HTLV_{IIIB}$	$HTLV_{IIIB}$ TIBO[r]	HTLV-1_{RF}	HIV-1_{HE}	LAV-2 ROD	LAV-2 EHO	SIV: agm mnd
2a	1.5	1.5	0.3	11.7	>250	17.9	150
4g	0.1	0.3	0.1	0.9	0.7	1.3	5.9
2c	0.5	0.6	1.3	1.5	2.2	4.3	7.4
7j	0.2	0.7	0.2	1.1	0.9	1.3	10.2

the onset of the anti-HIV activity was associated with higher cytotoxicity (Table XII).

Other chemical modifications, known to diminish the anticoagulant activity of heparin, were also attempted. Esterification of the COOH groups which are necessary for anticoagulant activity,[21] maintained or further decreased the already low anticoagulant activity of the periodate oxidized heparin fragments. Some of the esters (such as butenyl, allyl, butyl, or benzyl) were potent inhibitors of HIV-1 cytopathicity. Additional O-butyrylation of butyl or benzyl esters resulted in an increase of the inhibitory effect (EC_{50} = 1 μg/mL) on giant-cell formation (Table XIV), which was greater than that of reference compounds.

When periodate-oxidized heparin fragments were N-desulfated and N-acetylated, both anticoagulant and antiviral activity decreased. Neither O-butyrylation nor esterification of N-desulfated-N-acetylated derivatives allowed the recovery of significant antiviral activity (Table XVI). These results attest to the contribution of N-sulfate groups to the antiviral activity of the heparin derivatives.

Since different strains of immunodeficiency viruses show different sensitivities to polyanionic compounds,[22] the most active derivatives were evaluated for their activity against a larger variety of strains (Table XVII). HIV-1_{HE}, HIV-2_{ROD}, HIV-2_{EHO}, and SIV were less sensitive to the antiviral activity of the heparin derivatives than were the other virus strains, the difference in sensitivity being particularly striking for compound **2a**.

The common mechanism of the antiviral activity of polyanions was confirmed for the O-acylated heparin derivatives. O-Butyrylated periodate-depolymerized heparin fragments were as active as dextran sulfate (100% inhibition at 25 μg/mL) in completely preventing HIV binding to MT-4 cells, as determined by a flow cytometry assay[23] (Figure 7).

In conclusion, our results demonstrate that O-acylation of heparin fragments and their esters, which have low anticoagulant activity, yields products having high *in vitro* activity against both HIV-1 and HIV-2. As compared to the reference compounds (dextran sulfate and pentosan polysulfate), these derivatives are more active as inhibitors of giant-cell formation. O-Acylated analogues have the

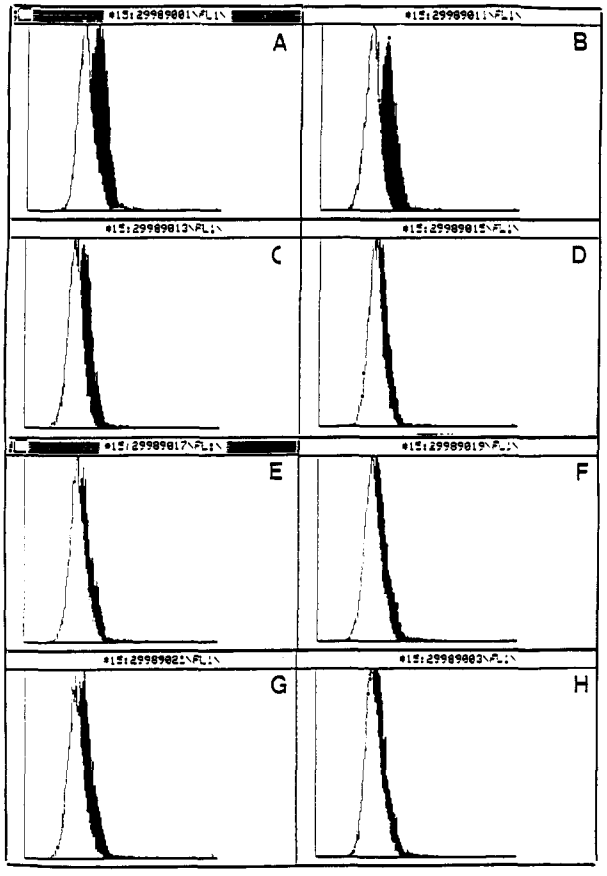


Figure 7. Inhibitory effect of compounds **3a–3e** and **2d** on HIV-1 ($HTLV_{mn}$) binding to MT-4 cells. The open histograms represent the fluorescence of MT-4 cells which were not exposed to HIV-1 virions. The solid histograms represent the fluorescence of MT-4 cells which were exposed to HIV-1 virions. The MT-4 cells were exposed or not exposed to HIV-1, in the absence of compound (panel A), or in the presence of **3a** (panel B), **3b** (panel C), **3c** (panel D), **3d** (panel E); **3e** (panel F), **2d** (panel G), or dextran sulfate 5000 Da (Sigma) (panel H), respectively. All compounds were used at a concentration of 25 μg/mL. The mean fluorescence values were as follows: 17 (panel A), 13 (panel B), 13 (panel C), 15 (panel D), 14 (panel E), 14 (panel F), 13 (panel G), 15 (panel H) for the *aspecific* fluorescence and 30 (panel A), 24 (panel B), 19 (panel C), 19 (panel D), 18 (panel E), 18 (panel F), 19 (panel G), 19 (panel H) for the *specific* fluorescence.

same degree of sulfation as the parent heparin fragments and, in addition, they have become more hydrophobic. Their hydrophobicity might contribute to their antiviral activity by an additional mechanism. Moreover, as has been recently shown, *O*-acylated derivatives of heparins have a better bioavailability after subcutaneous administration and a longer half-life than do the parent compounds.[24,25] In this aspect the *O*-acylated heparins compare favorably to other previously studied anti-HIV polyanions.

Experimental Section

General and Particular Procedure. Standard heparin, sodium salt, from pig mucosal origin and low-molecular-weight heparin (CY 216D) were obtained from Sanofi Pharma Industry. SR 80258 was obtained from heparin after periodate oxidation followed by β-elimination and borohydride reduction as already published.[20] Dermatan sulfate was from pig mucosal origin. All chemicals were of purest grade available from Aldrich and Fluka.

Optical rotations were measured at 20 °C with a Perkin-Elmer 241 polarimeter. ^1H NMR spectra were recorded with Bruker AC-100 or AC-200 instruments; chemical shifts (ppm) are relative to internal TSP in D_2O. ^{13}C NMR spectra were recorded using methanol as internal reference (51.6 ppm from internal TSP). Conductimetric determinations were performed using a Radiometer CDM 83 conductimeter equipped with an ABU 80 autoburet and a TTT 85 titrator. Gas–liquid chromatography experiments were conducted on a Varian 3400 instrument equipped with a flame-ionization detector and a capillary column (30 × 0.53 mm) coated with 2-μm OV1. FT-IR spectra were recorded on a Nicolet 5ZDX spectrometer.

***O*-Acylated Derivatives. *O*-Acylated Derivatives of Heparin (1d-f).** A solution of heparin, sodium salt (**1a**, 10 g), in water (500 mL) was percolated through a Dowex-50 column (H^+ form, 300 mL) at 4 °C. The pH of the solution was adjusted to 6.0 by addition of tributylamine (50 mL). Excess tributylamine was evaporated, the volume was adjusted to 900 mL with water, and **1b** was obtained (21 g) after lyophilization and drying for 24 h at 50 °C under vacuum. Compound **1b** (21 g, corresponding to 40 mequiv of OH groups) was dissolved in *N,N*-dimethylformamide (200 mL) and placed under argon at 4 °C. 4-(Dimethylamino)pyridine (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 °C, sodium hydrogen carbonate (5% in water, 400 mL) was added, and the mixture was stirred at room temperature for 48 h. After neutralization, cold ethanol (6 L) was added. The precipitate was dissolved in apyrogenic water and passed through a Dowex-50 column (H^+ form, 300 mL) at 4 °C. The effluent was neutralized with 1 M NaOH and filtered through a 0.22-μm-pore filter. After lyophilization **1d** was isolated as a white powder (9.15 g). Compounds **1c**, **1e**, and **1f** were obtained from **1b** as described for **1d**. Analytical data for **1c–f** are reported in Table I.

***O*-Acylated Derivatives of SR 80258 (2b-h).** The sodium salt of SR80258 was converted into the tributylammonium salt **2b** as described for the preparation of **1b**. Compounds **2c, 2d, 2e, 2f, 2g, 2h** were synthesized using the procedure described for **1d**. Analytical data are reported in Table III.

***O*-Acylated Heparin Fragments Having Different Substitution Degrees (3b-j).** These compounds have various substitution degrees. They were synthesized from **2b** essentially as described for the preparation of **2c–h**. The amount of reagent was limited to control the number of acyl groups introduced (**ref 1**). Analytical data are reported in Table V.

***O*-Acylated Heparin Fragments Having Different Molecular Weight (4a-k).** SR 80258 was fractionated by gel filtration on a 5 × 100 cm AcA 202 column equilibrated and eluted with a 0.5 M NaCl solution at a flow rate of 80 mL/h. Ten-milliliter fractions were collected, and the separation was monitored by recording the OD of the effluent at 214 nm. The molecular weight distribution of the fragments was determined by gel-permeation HPLC on a Waters I 60 column (7.5 × 300 mm) calibrated with a series of narrow-sized heparin oligosaccharides prepared by deaminative cleavage. Each fraction was then butyrylated after conversion into its tributylamine salt, as described for **2c-h**. Analytical data are reported in Table VII.

***O*-Acylated Derivatives of CY 216 (5c-e).** The sodium salt of CY 216, **5a**, was converted into the tributylamine salt **5b** as described for **1b**. Derivatives **5c-e** were synthesized as described for **1d**. Analytical data are reported in Table IX.

***O*-Acylated Derivatives of Dermatan Sulfate (6c-e).** Dermatan sulfate **6a** was converted into the tributylamine salt **6b** as described for **1b**; derivatives **6c-e** were synthesized as described for **1c**. Analytical data are reported in Table XI.

***O*-Acylated Esterified Derivatives. Ester Derivatives of SR 80258 (7b-j).** The sodium salt **3a** was converted into the tetrabutylammonium salt **7a** via percolation through a Dowex-50 (H^+) ion-exchange column followed by neutralization (the pH was adjusted to 8.2) of the effluent with an aqueous solution of tetrabutylammonium hydroxide. Compound **7a** was isolated after lyophilization and drying under vacuum. The benzyl ester derivative **7i** was synthesized as follows: Benzyl bromide (40.6 mL, 39 mmol) was added to **7a** (10.5 g, corresponding to 7.8 mequiv OH) dissolved in dry *N,N*-dimethylformamide (100 mL) and the solution was kept at room temperature for 24 h. Aqueous

0.4 M NaCl (135 mL) and cold ethanol (675 mL) were added. The precipitate was collected, dissolved in apyrogenic water, and converted to the sodium salt via Dowex-50 (H^+) exchange and NaOH neutralization. Compound **7i** was isolated as a powder after lyophilization. Compounds **7b-h** and **7j** were synthesized as described for **7i**, using the appropriate alkyl halide. Analytical data are reported in Table XIII.

***O*-Butyrylated Derivatives of Esterified SR 80258 (7h and 7j).** The above esterification procedure was first applied. After reaction with alkyl halide, acid anhydride, tributylamine, and 4-(dimethylamino)pyridine were added to the reaction flask. Reaction and isolation procedures were as described for *O*-acylated derivatives. Compounds **7h** and **7j** were isolated as sodium salts. Analytical data are reported in Table XIII.

***N*-Desulfated-*N*, *O*-Acylated Ester Derivatives.** Analytical data for these compounds are reported in Table XV.

***N*-Acetylated SR 80258 (8a).** Compound 3a (3 g, 4 mequiv of OH) was dissolved in *N*,*N*-dimethylformamide (30 mL), and 4-(dimethylamino)pyridine (0.24 g, 0.25 mequiv) and triethylamine (7.8 mL, 56 mmol) were added. Acetyl chloride (2 mL, 28 mmol) dissolved in *N*,*N*-dimethylformamide (30 mL) was added dropwise to the cooled mixture, and the reaction was allowed to proceed at room temperature for 24 h. Aqueous NaCl (60 g/L, 100 mL) was added, followed by cold ethanol (500 mL). The precipitate was dissolved in 0.5 N NaOH (40 mL), and after one night at room temperature, the solution was neutralized and passed through a Sephadex G25F column. Compound 8a was isolated as sodium salt after lyophilization.

***N*-Acetylated-*O*-Butyrylated SR 80258 (8b).** Compound 8a was converted into its tributylammonium salt as described for **1b**. This salt (0.56 g, corresponding to 3 mequiv of OH group) was dissolved in *N*,*N*-dimethylformamide, and 4-(dimethylamino)pyridine (0.25 mequiv) and triethylamine (15 mmol) were added. Butyric anhydride (15 mmol) was added, and the reaction was allowed to proceed at room temperature for 24 h. Aqueous sodium chloride (60 g/L, 11 mL) was introduced, followed by cold ethanol (150 mL). The precipitate was collected after prior centrifugation, and this precipitation procedure was repeated on the material thus obtained. Compound 8b was finally obtained as the sodium salt (0.3 g) after gel filtration and lyophilization.

Butyl Ester of *N*-Acetylated SR 80258 (8c). Compound 8a (1.37 g, corresponding to 2.3 mequiv of OH groups) was converted into its tetrabutylammonium salt via ion exchange, and the esterification procedure with butyl bromide was carried out as previously described, thus yielding 8c. Half of the reaction mixture was used for isolation of 8c, obtained as the sodium salt (0.293 g).

Butyl Ester of *N*-Acetylated-*O*-Butyrylated SR 80258 (8d). The other part of the reaction mixture containing 8c was treated under *O*-butyrylation conditions, as previously described, to give **8d**, isolated as the sodium salt (0.370 g).

Antiviral Activity Assays. $HIV\text{-}1_{IIIB}$ and $HIV\text{-}2_{ROD}$ were prepared from the supernatant of HUT-78 cells persistently infected with either $HIV\text{-}1_{IIIB}$ or $HIV\text{-}2_{ROD}$. Inhibitory effects of compounds on HIV-1 and HIV-2 replication were monitored by the inhibition of virus-induced cytopathicity in MT-4 cells, as previously described.[26] Briefly, MT-4 cells were suspended at 3×10^5 cell/mL and infected with HIV at 100 times the 50% cell culture infective dose/mL. Immediately after infection, 100 μL of cell suspension was transferred into each well of a flat-bottomed microtiter tray containing various concentrations of the test compound. After five days incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described.[27] Cytotoxicity of compounds was determined in parallel with their antiviral activity and was based on the viability of mock-infected MT-4 cells, as monitored by the MTT method.[27]

The inhibitory effect on virus adsorption was measured by an indirect immunofluorescence-laser flow cytofluorographic method specifically designed for this purpose.[23] MT-4 cells were exposed to HIV-1 virions (which had been concentrated from the supernatant of HUT-78/$HTLV\text{-}III_B$ cells) in the presence or absence of the test compounds, added 20 s before the addition of viruses. Cells were incubated for 30 min at 37 °C and washed twice in phosphate-buffered saline (PBS) to remove unbound virus. Then a high-titer polyclonal antibody derived from a patient with AIDS-related complex (diluted 1/500 in PBS) was added. After another 30 min incubation at room temperature, the cells were washed twice with PBS. Cells were then incubated with fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of rabbit anti-human immunoglobulin antibody (diluted 1/3 in PBS) for 30 min at room temperature, washed once in PBS, resuspended in 0.5 mL of 0.5% paraformaldehyde in PBS, and analyzed by laser flow cytofluorography.

For giant cell formation, MOLT-4 cells were co-cultured with an equal number of HUT-78/$HIV\text{-}1_{IIIB}$ or HUT-78/$HIV2_{ROD}$ cells in microtiter plate containing various concentrations of the test compounds. After 24 h of cocultivation, giant cells (syncytia) were recorded by flow cytofluorography, as previously described.[28]

Anticoagulant Activity. *In vitro* anticoagulant activity was determined by the clotting test of activated partial thromboplastin time (APTT) on a pool of human plasma, according to the method of Proctor and Rapaport,[29] using an automatic-type KC10 Amelung coagulometer. Activities were calculated according to the European Pharmacopoeia.

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