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Synthesis and Chain Length–Anti-HIV Activity Relationship of Fully *N*- and *O*-Sulfated Homooligomers of Tyrosine

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Abstract—Fully *N*- and *O*-sulfated homooligomers from octamer to nonadecamer of tyrosine were obtained as their sodium salts, NaO_3S–[Tyr(SO_3Na)]$_n$–ONa ($n=8$–19), from reaction mixtures of tyrosine with sulfur trioxide trimethylamine and pyridine complexes, respectively, in pyridine. Their anti-HIV activity increased along with the increase of the chain length up to the dodecamer, maintained the same level to the length of the heptadecamer and then decreased. The maximal activity level was the same as or higher than that of dextran and curdlan sulfates.

Introduction

Heparin, a natural sulfopolysaccharide, and polysulfates of dextran and other polysaccharides have been known as potent inhibitors of human immunodeficiency virus (HIV) infectivity.[1] However, they are mixtures of different length of polymers with wide range of sulfation, whose activity was often discussed on their sulfur content.[2] In addition, the fact that heparin possessing both *O*-sulfate and *N*-sulfate (sulfamate) groups loses its anti-HIV-1 activity by *N*-desulfation[3] suggested variable roles of individual sulfate groups. To make these roles clear synthesis and structure–activity relationship studies on compounds with defined structures are necessary. These studies, however, would not be easy for oligo- and polysaccharides as shown in the recent report of synthesizing heparin fragments, where the *N*-sulfate group was replaced by the *O*-sulfate for convenience.[4] On the other hand, contribution of sulfated tyrosine residues of the N-terminal domain of chemokine receptor CCR5, a principal HIV-1 coreceptor, to HIV-1 entry has recently been reported.[5,6] These facts prompted us to prepare structurally defined sulfates of oligotyrosines.

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General methods for peptide synthesis have already been established, but the synthesis of peptides containing multiple tyrosine sulfate residues still remains as one of the most difficult problems.[7] In the preceding paper,[8] we reported the alternative synthesis of nonatyrosine *N*- and *O*[1–9]-decasulfate by oligomerization with simultaneous sulfation of tyrosine with sulfur trioxide trimethylamine complex (SO_3·NMe_3).[9] Although the reaction gave a multi-component mixture, we were able to isolate the above nonamer as its sodium salt, NaO_3S–[Tyr(SO_3Na)]$_9$–ONa, (**Y-ART-4**). The anti-HIV activity of **Y-ART-4** was almost the same as that of dextran and curdlan sulfates.[8] In this study we aimed to establish the chain length–activity relationship by preparing a series of fully *N*- and *O*-sulfated homooligomers of tyrosine.

Results and Discussion

Synthesis

Y-ART-4 was isolated from the mixture of oligomers ranging from hexamer to tetradecamer. To establish the chain length–activity relationship, preparation of a wider series of the oligomers is necessary. Then, the effects of reagent, solvent, temperature and reaction time on the extent of oligomerization were examined.

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Sulfur trioxide is commercially available in the form of complexes with pyridine (Py)[10] and *N,N*-dimethylformamide (DMF)[11] as well as with trimethylamine. Then, tyrosine was treated with the three sulfur trioxide complexes, respectively, under various conditions. After removal of the solvent, the products were isolated once as tetrabutylammonium salts and then converted to the sodium salts as described before.[8] In the case of **Y-ART-4**, its tetrabutylammonium salt gave a signal with m/z corresponding to $[M-N^nBu_4]^-$ on the matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS). However, the detection of this signal was not always successful. Therefore, determination of the chain length distribution of oligomers was done by HPLC after removal of all the sulfate groups of the crude products by treatment with trifluoroacetic acid (TFA) at 50 °C for 1 h.[8] These results are shown in Table 1.

As the solvent, pyridine was the best regarding superior solubility of oligomers. In DMF and tetrahydrofuran (THF) reactions of tyrosine with SO_3·NMe_3 were not so simple as in the reactions in pyridine. Then, comparison of the three complexes was made using pyridine as the solvent. The pyridine complex gave relatively longer oligomers than the trimethylamine complex (entries 8 versus 2 and 9 versus 7). As expected the higher temperature and the higher concentration (entries 5, 7, and 9) gave the longer oligomers. On the other hand, SO_3·DMF gave a complex mixture (entry 10), showing that no simple oligomerization occurred. As shown in Scheme 1, *N*-sulfation would terminate the oligomerizaion promoted by carboxylic–sulfonic mixed anhydride formation. With SO_3·DMF, such termination by the *N*-sulfation would have occurred more preferably than the oligomerization in the early stage.

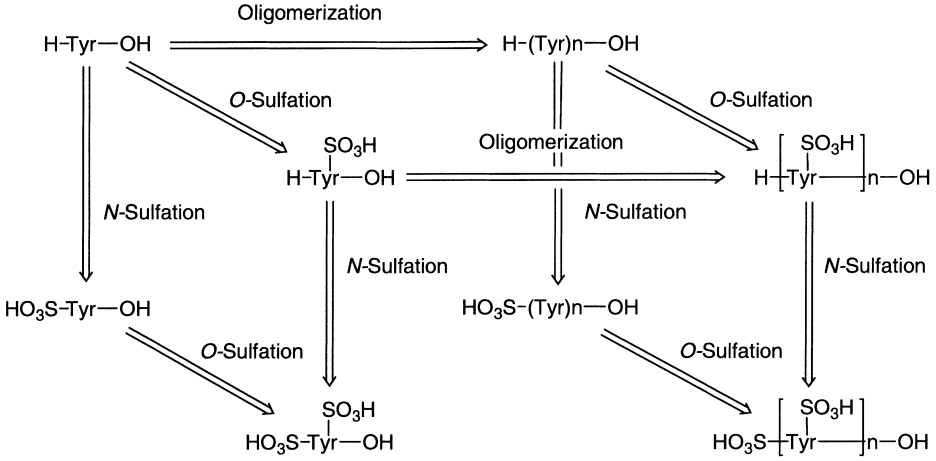
Preparative separation of each oligomer for measurements of anti-HIV activity was done for the oligomer mixtures from entries 2 and 9 in Table 1 by gel chromatography using Sephadex LH-20. Every 10th fraction was analyzed by HPLC after desulfation with TFA and the results are shown in Figures 1 and 2. Fractions containing from octamer to nonadecamer as a major component were collected, evaporated and lyophilized from water to give samples for the activity measurements. They are listed in Table 2 and their analytical HPLC profiles are shown in Fig. 3. SUT-9812 is the same as the **Y-ART-4** compound in the preceding paper.[8]

Biological activity

The anti-HIV activity of the test compounds was determined from protection they provided against HIV-induced cytopathic effects as described in the former

Table 1. Composition of tyrosine oligomers obtained by the reactions of tyrosine with SO_3 complexes under various conditions followed by desulfation

Entry	Reagent	Solvent	Concn (M)		Temperature (°C)	Time (h)	Composition of oligomers[a]
			[Tyr]	[SO_3]			
1	SO_3·NMe_3	Py	0.6	1.8	55	48	4, 5, 6, 7, 8, 9, 10
2	↑	↑	↑	↑	↑	93	6, 7, 8, 9, 10, 11, 12, 13, 14
3	↑	DMF	↑	↑	↑	↑	Complex mixture
4	↑	THF	↑	↑	↑	↑	Complex mixture
5	↑	Py	↑	↑	75	↑	8, 9, 10, 11, 12, 13, 14, 15
6	↑	↑	0.12	0.36	55	↑	5, 6, 7, 8, 9, 10
7	↑	↑	1.5	4.5	75	↑	8, 9, 10, 11, 12, 13, 14, 15
8	SO_3·Py	↑	0.6	1.8	55	↑	6, 7, 8, 9, 10, 11, 12, 13, 14, 15
9	↑	↑	1.5	4.5	75	↑	6, 7, 8, 9, 10, 11, 12, 13, 14, 15–19
10	SO_3·DMF	↑	0.6	1.8	55	↑	Complex mixture



Scheme 1. Expected route to oligotyrosine sulfates.

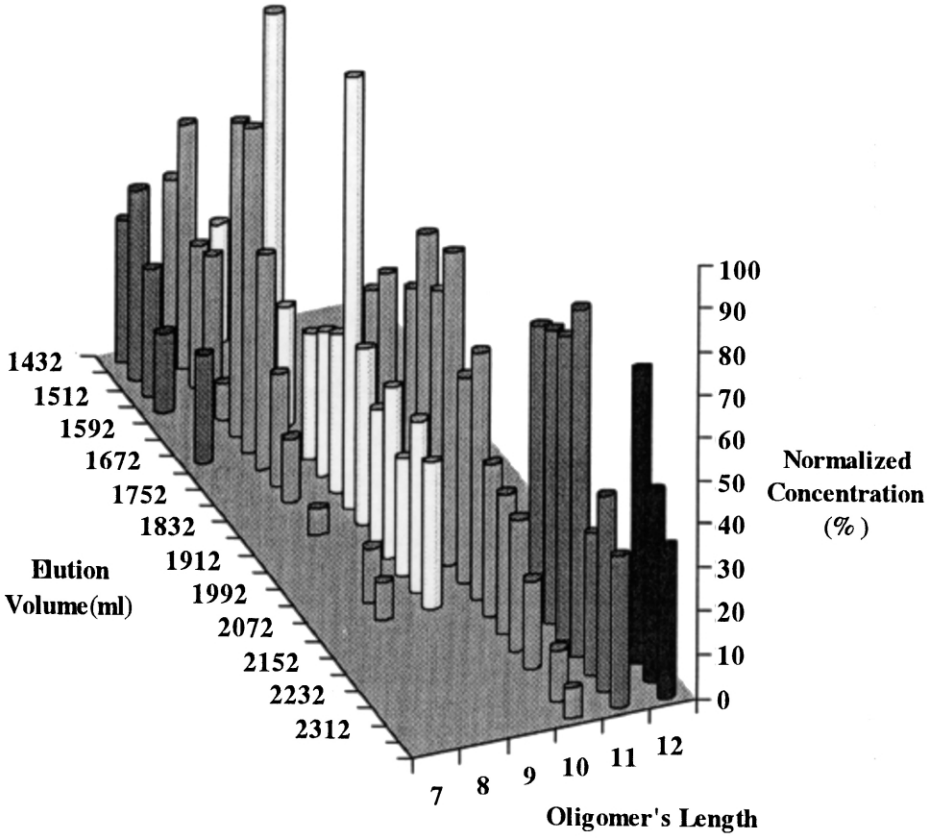


Figure 1. Elution profile of oligotyrosine sulfates (entry 2 in Table 1) on Sephadex LH-20.

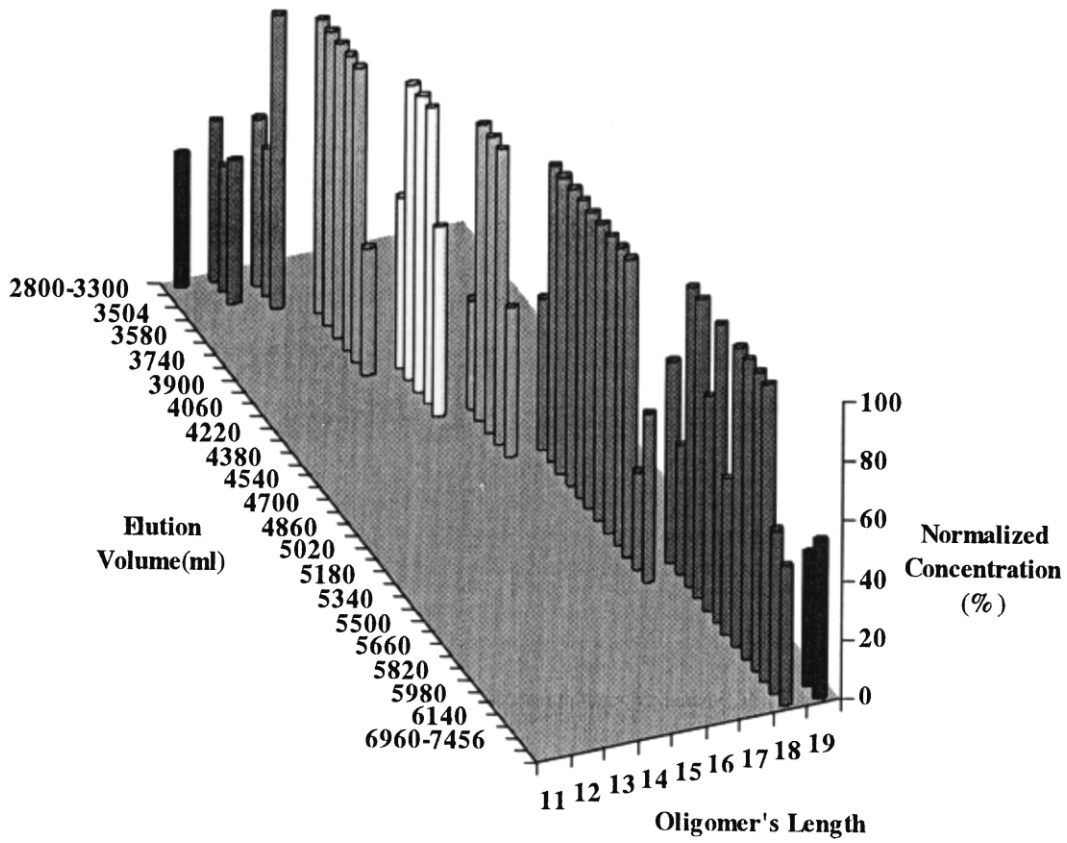


Figure 2. Elution profile of oligotyrosine sulfates (entry 9 in Table 1) on Sephadex LH-20.

Table 2. A list of samples tested for anti-HIV activity

Code	Source	Structure of the major component	t_R[a] (min)	FAB-MS[b] Found/calcd.
SUT-9811	Entry 2 in Table 1	NaO_3S–[Tyr(SO_3Na)]$_8$–ONa	18.9	1324.3/1323.5
SUT-9812	↑	NaO_3S-[Tyr(SO_3Na)]$_9$–ONa	19.6	1487.7/1486.6
SUT-9813	↑	NaO_3S–[Tyr(SO_3Na)]$_{10}$–ONa	21.5	1650.0/1649.6
SUT-9814	↑	NaO_3S–[Tyr(SO_3Na)]$_{11}$–ONa	22.0	1813.0/1812.7
SUT-9815	↑	NaO_3S–[Tyr(SO_3Na)]$_{12}$–ONa	22.8	1975.9/1975.8
SUT-9901	Entry 9 in Table 1	NaO_3S–[Tyr(SO_3Na)]$_{12}$–ONa	22.9	1975.5/1975.8
SUT-9902	↑	NaO_3S–[Tyr(SO_3Na)]$_{13}$–ONa	23.7	2138.8/2138.8
SUT-9903	↑	NaO_3S–[Tyr(SO_3Na)]$_{14}$–ONa	24.5	2301.9/2301.9
SUT-9904	↑	NaO_3S–[Tyr(SO_3Na)]$_{15}$–ONa	25.2	2465.0/2465.0
SUT-9905	↑	NaO_3S–[Tyr(SO_3Na)]$_{16}$–ONa	26.0	2628.6/2628.0
SUT-9906	↑	NaO_3S–[Tyr(SO_3Na)]$_{17}$–ONa	26.8	2791.7/2791.1
SUT-9907	↑	NaO_3S–[Tyr(SO_3Na)]$_{18}$–ONa	27.9	2954.0/2954.2
SUT-9908	↑	NaO_3S–[Tyr(SO_3Na)]$_{19}$–ONa	28.5	3116.4/3117.2

[a]Retention time in HPLC (conditions: see Experimental).
[b]Values of m/z of $[M+H]^+$ in FAB-MS of the desulfated samples.

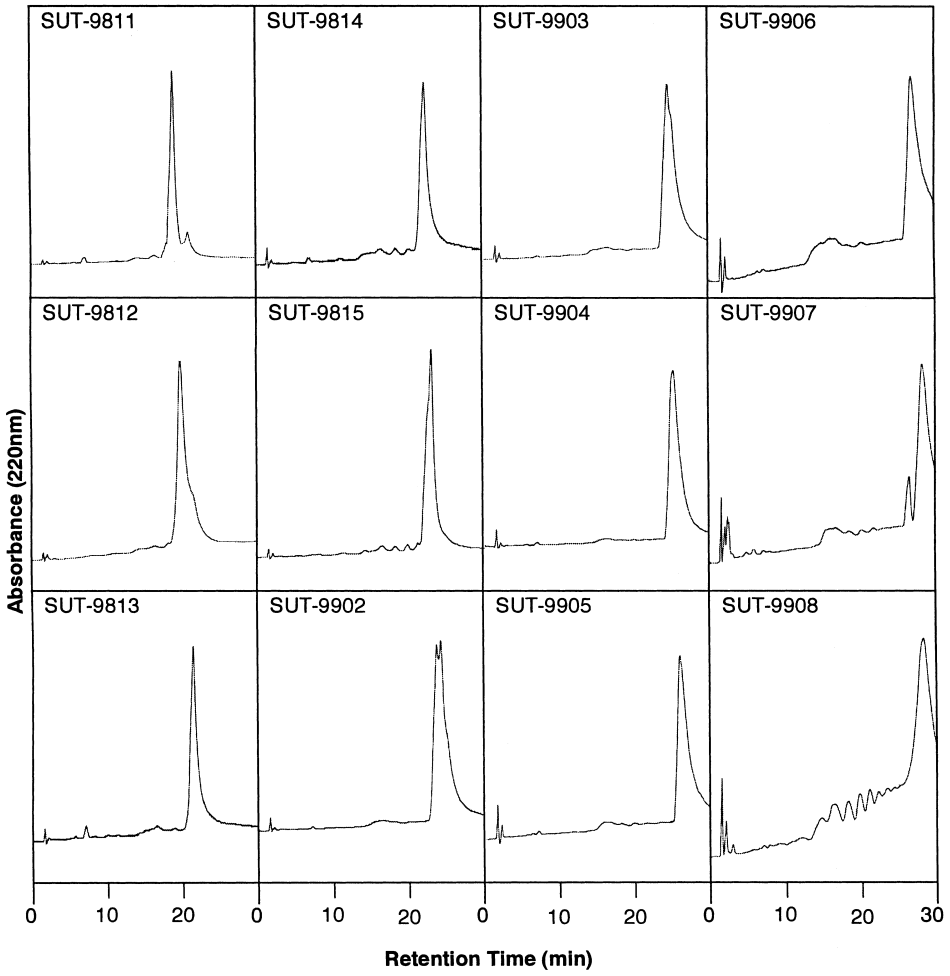


Figure 3. HPLC elution profiles of SUT compounds (conditions: see Experimental).

paper.[8] All the compounds showed concentration-dependent protective activity against virus-induced cytopathogenicity. From these results the 50% effective concentration (EC_{50}), 50% cytotoxic concentration (CC_{50}) and selective index SI (ratio of CC_{50} to EC_{50}) values were determined. Since the activities of the two sets of samples (SUT-98 and SUT-99 series) were measured separately, these values are listed in Table 3 together with the data for reference compounds, dextran sulfate, curdlan sulfate, 3′-azido-2′,3′-dideoxythymidine (AZT), and 2′,3′-dideoxycytidine (ddC).

To make clear the relationship between the chain length and the activity, log[EC_{50} (μM)×100] values are plotted

Table 3. Anti-HIV activity of oligotyrosine sulfates and reference compounds

Compound	M.W.	EC_{50}		CC_{50}		SI
		(μg/mL)	(μM)	(μg/mL)	(μM)	
SUT-9811	2263.0	3.86	1.71	> 1000	> 442	> 259
SUT-9812	2529.0	3.29	1.30	> 1000	> 395	> 304
SUT-9813	2794.3	0.73	0.26	> 1000	> 358	> 1370
SUT-9814	3159.5	0.41	0.130	> 1000	> 316	> 2439
SUT-9815	3324.6	0.15	0.045	> 1000	> 301	> 6667
Dextran sulfate		1.20		> 1000		> 833
Curdlan sulfate		0.15		> 1000		> 6667
AZT			0.002		> 500	> 250,000
ddC			0.18		219	1198
SUT-9901	3324.6	0.12	0.036	> 200	> 60	> 1667
SUT-9902	3589.5	0.16	0.045	> 200	> 56	> 1250
SUT-9903	3855.1	0.17	0.044	> 200	> 52	> 1176
SUT-9904	4120.4	0.17	0.041	> 200	> 49	> 1176
SUT-9905	4385.6	0.23	0.052	> 200	> 46	> 870
SUT-9906	4650.8	0.21	0.045	> 200	> 43	> 952
SUT-9907	4916.0	0.66	0.13	> 200	> 41	> 303
SUT-9908	5181.3	> 200	38.6	126	24	< 1
Dextran sulfate		0.43		> 1000		> 2326
Curdlan sulfate		0.66		> 1000		> 1515
AZT			0.042		277	> 6595
ddC			0.26		753	> 2896

against the chain length in Figure 4. The activity gradually increased along with the increase of the chain length up to the dodecamer and maintained the same level to the length of the heptadecamer. Further chain elongation reduced the activity and, surprisingly, nonadecamer was toxic rather than active. From the synthetic point of view, the shorter chain length will be better, so the dodecamer was the best of all the oligomers examined in this study.

It would be too early to discuss at this stage the mechanism of the inhibition of the HIV infectivity by these oligomers. However, coincidence with the fact that the minimum size of heparin mimetic fragments with ability to catalyze thrombin inhibition was pentadeca- or hexadecasaccharide[4] would be worth considering. In addition to the chain length–activity relation, it would be important to explain the role of the *N*-sulfate group for development of the anti-HIV activity reported in the former paper.[8] For that purpose further structural analysis is necessary. Then, establishment of the solid phase method for obtaining sufficient amounts of the sulfated tyrosine oligomers is now underway in our laboratory.

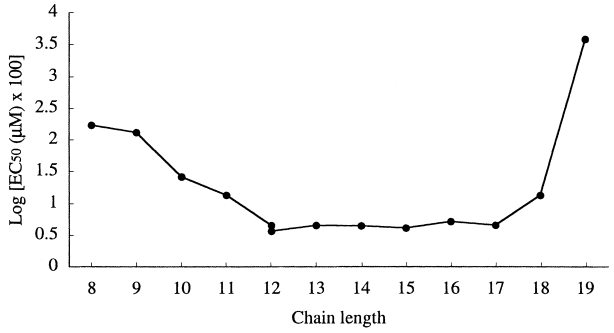


Figure 4. Chain length–activity relationship.

Conclusion

Fully *N*- and *O*-sulfated oligomers from octamer to nonadecamer of tyrosine were obtained as their sodium salts from the reaction mixtures of tyrosine with sulfur trioxide, trimethylamine and pyridine complexes, respectively, in pyridine. Their anti-HIV activity increased along with the increase of the chain length up to the dodecamer, maintained the same level to the length of the heptadecamer and then decreased. The maximal activity level was the same as or higher than that of dextran and curdlan sulfates.

Experimental

General

Analytical HPLC was done on a Waters 625 LC system containing 5 μm μBondasphere C18 (3.9 mm×150 mm) with a Waters 484 tunable absorbance detector. Conditions for the desulfated oligomers: linear gradient elution, CH_3CN:0.1% aq TFA 4:1 to 1:1 over 30 min; flow rate, 1.0 mL/min; detection, 270 nm). Conditions for the sulfated oligomers: linear gradient elution, CH_3CN:H_2O: 0.1 M aq $^nBu_4NHSO_4$ 40:50:10 to 70:20:10 over 30 min; flow rate, 1.0 mL/min; detection, 220 nm). FAB-mass spectra were obtained using a JEOL JMS-AX505HA spectrometer.

Preparation of *N*-sulfated oligo(tyrosine *O*-sulfate)s

Tyrosine (40 mg for entry 6, 500 mg for entries 7 and 9, and 200 mg for others, respectively) was treated with the defined amount of one of the SO_3 complexes in the defined solvent (2 mL) under the conditions described in Table 1. The work up described before[8] gave the following crude products. Entry 1, white powder, 227 mg;

entry 2, pale yellow powder, 380 mg; entry 3, pale yellow powder, 426 mg; entry 4, white powder, 87 mg; entry 5, brown powder, 224 mg; entry 6, white powder, 26 mg; entry 7, white powder, 1379 mg; entry 8, brown powder, 353 mg; entry 9, brown powder, 949 mg; and entry 10, white powder, 60 mg.

Separation of each oligomer by gel chromatography on Sephadex LH-20

Large scale reactions using 2.00 and 5.00 g each of tyrosine under the conditions of entries 2 and 9, respectively, in Table 1 were performed to give crude oligomer mixtures of 3.80 and 6.72 g, respectively.

A part of each oligomer mixture (1.00 g for entry 2 or 2.10 g for entry 9, respectively) was dissolved in methanol, charged to a column of Sephadex LH-20 (2.6×88 cm) and eluted with methanol at a flow rate of 0.3 mL/min. Eluate was collected in 4 mL fractions. Every tenth fraction was evaporated, desulfated and analyzed by HPLC. From the reaction mixture under the conditions of entry 2, fractions of 1432–1512, 1672–1752, 1832–1952, 2072–2192, and 2272–2352 mL, respectively, were collected, evaporated and lyophilized from water to give white powders of SUT-9811 (26.1 mg), SUT-9812 (21.1 mg), SUT-9813 (36.8 mg), SUT-9814 (31.9 mg), and SUT-9815 (22.1 mg), respectively. From the reaction mixture under the conditions of entry 9, fractions of 2800–3300, 3301–3500, 3580–3900, 4224–4376, 4544–4696, 4700–5340, 5580–6140, and 6960–7456 mL, respectively, were collected and treated in the same manner as above to give white powders of SUT-9901 (75.4 mg), SUT-9902 (33.5 mg), SUT-9903 (36.5 mg), SUT-9904 (13.6 mg), SUT-9905 (10.4 mg), SUT-9906 (33.8 mg), SUT 9907 (24.0 mg), and SUT-9908 (13.1 mg), respectively.

Anti-HIV activity

The method for the anti-HIV activity measurements was the same as described in the previous paper.[8]

Acknowledgements

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