Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 477–486

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Anti-HIV Activity of Nonatyrosine *N*- and O^{1-9}-Decasulfate

Masaaki Ueki,[a,*] Shigeru Watanabe,[a] Yusuke Ishii,[a] Osamu Okunaka,[a] Keijiro Uchino,[b] Takeshi Saitoh,[c] Kyoichiro Higashi,[d] Hideki Nakashima,[e] Naoki Yamamoto[f] and Hiroshi Ogawara[d]

[a]*Department of Applied Chemistry, Science University of Tokyo, 1-3 Kagurazaka, Shinjuku-ku, Tokyo 162-8601, Japan*
[b]*Central Laboratory, Nippon Flour Mills Co. Ltd., 5-1-3 Midorigaoka, Atsugi-Shi, Kanagawa 243-0041, Japan*
[c]*Institute for Consumer Healthcare, Yamanouchi Pharmaceutical Co., Ltd., 3-17-1 Hasune, Itabashi-ku, Tokyo 174-8612, Japan*
[d]*Department of Biochemistry, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose-Shi, Tokyo 204-8588, Japan*
[e]*Department of Microbiology and Immunology, Kagoshima University Dental School, 8-35-1 Sakuragaoka, Kagoshima-Shi, Kagoshima 890-8544, Japan*
[f]*Department of Microbiology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*

Received 4 September 2000; accepted 3 October 2000

Abstract—To develop a potent and effective anti-HIV compound with a definite polyanionic structure, synthesis of oligotyrosine sulfates by oligomerization with simultaneous sulfation of tyrosine was tried. One component was successfully isolated from the mixture containing many products as its sodium salt (**Y-ART-4**) and was identified as the salt of nonatyrosine *N*- and O^{1-9}-decasulfate, NaO_3S–[Tyr(SO_3Na)]$_9$–ONa. Anti-HIV activity of **Y-ART-4**, determined from the protection it provided against HIV-induced cytopathic effects, was almost the same with that of dextran sulfate and curdlan sulfate.

Introduction

From the latter half of the 1980s to the early 1990s, various kinds of polysulfates such as heparin, a natural sulfopolysaccharide,[1] and sulfates of polysaccharides, such as dextran,[1,2] pentosan,[3] and curdlan,[4] were reported as potent inhibitors of human immunodeficiency virus (HIV) infectivity and syncytium formation in vitro. These studies have been going on since poor absorbability of oral dextran sulfate in HIV-infected patients was shown in clinical trials.[5] However, sufficient structure–activity relationship study has not been done because those compounds were the polymers with large molecular weights and undefined numbers of sulfate groups. Recently, we reported that sulfated pentagalloyl glucose inhibits HIV replication and cytopathic effects in vitro and reduces HIV infection in hu-PBL-SCID mice.[6] Although pentagalloyl glucose is a monomeric compound with molecular weight of 940.7, we could not prepare a single compound with a defined number of sulfate groups including the fully sulfated one at that time. In the continuing interest to develop a potent and effective anti-HIV compound with a definite structure, we intended in this study to synthesize polysulfates based on a natural amino acid, tyrosine.

Sulfur trioxide complexes with trimethylamine (NMe_3),[7] pyridine (Py),[8] or *N,N*-dimethylformamide (DMF)[9] have been used as popular sulfating agents. In peptide synthesis, the complex with DMF (SO_3·DMF) was also known as an effective condensing agent.[10,11] On the other hand, oligomerization of amino acids with diphenyl phosphorazidate[12] and carbonyl diimidazole,[13] the typical condensing agents in peptide synthesis, has also been reported. These facts prompted us to investigate synthesis of oligotyrosine sulfates by oligomerization with simultaneous sulfation of tyrosine with SO_3 complexes.

Results and Discussion

Oligomerization with simultaneous sulfation of tyrosine

Tyrosine was allowed to react with SO_3·NMe_3 in pyridine at 55 °C for 93 h. After removal of the supernatant

*Corresponding author. Tel.: +81-3-3260-4272; fax: +81-3-3235-2214; e-mail: maueki@ch.kagu.sut.ac.jp

0968-0896/01/$ - see front matter
PII: S0968-0896(00)00269-8

by decantation, precipitates were rinsed with dichloromethane and then dissolved in aqueous sodium hydrogen carbonate solution. To this, a solution of tetrabutylammonium hydrogen sulfate, neutralized by sodium hydrogen carbonate, was added and the products were extracted with dichloromethane in the form of tetrabutylammonium salts. These salts were then converted to sodium salts with Amberlite IR 120B ion-exchange resin (Na^+ form) and lyophilized to give the crude products as yellowish white powder.

Direct analysis of oligomer composition of the crude products by FABMS failed as expected from the polyanionic nature of the sulfated products. Then, the crude products were desulfated to determine the extent of oligomerization, since the tyrosine sulfate moiety was known to lose its sulfate group under acidic conditions, especially at an elevated temperature.[14] In our experiments using H-Tyr(SO_3Na)–Tyr(SO_3Na)–OH as a model compound, its treatment with neat trifluoroacetic acid (TFA) at 50 °C for 1 h was found to be sufficient for complete removal of the sulfate groups. For calibration in HPLC, preparation of a standard mixture of oligomers consisting of hexa-, nona-, dodeca- and pentadecatyrosines, H-$(Tyr)_n$–OH ($n=6$, 9, 12 and 15), was necessary. This was done at one time by the 9-fluorenylmethyloxycarbonyl (Fmoc) solid phase synthesis[15] by mixing Fmoc-Tyr(^{t}Bu)–OH with Boc-Tyr(^{t}Bu)–OH at the 6th, 9th, and 12th positions from the C-terminus. Each oligomer could be separated and detected in HPLC.

HPLC profiles of the desulfated sample of the crude products (Fig. 1(A)) and the standard mixture of four oligomers (Fig. 1(B)) revealed that the desulfated crude products consisted of oligotyrosines containing nonamer in the highest content. FABMS, shown in Figure 2(A), also showed the presence of oligotyrosines up to at least a tetradecamer.

To elucidate the real structure and measure biological activities of each oligomer, isolation of the major component was then tried. The crude products were charged on Sephadex LH-20 column and eluted with methanol. Every 10th fraction was evaporated, desulfated, and analyzed by HPLC. Although complete separation of all the components was not possible, several fractions were found to contain the major component almost in a pure state. They were gathered, evaporated and lyophilized to give a yellowish white powder (**Y-ART-4**).

Structure of Y-ART-4

An HPLC profile of desulfated **Y-ART-4** is shown in Figure 1(C). While a small amount of decatyrosine was detected, the retention time of the major component was quite the same as that of the standard nonatyrosine. While **Y-ART-4** was not affected by treatment with 2,4-dinitrofluorobenzene to modify the N-terminal amine[16] as well as the phenolic hydroxyl[17] function, observation of m/z value of 1487.7 corresponding to $[M+H]^+$ of H-Tyr_9–OH in FABMS (Fig. 2(B)) clearly excluded the possibility of cyclic nonatyrosine. From these facts we

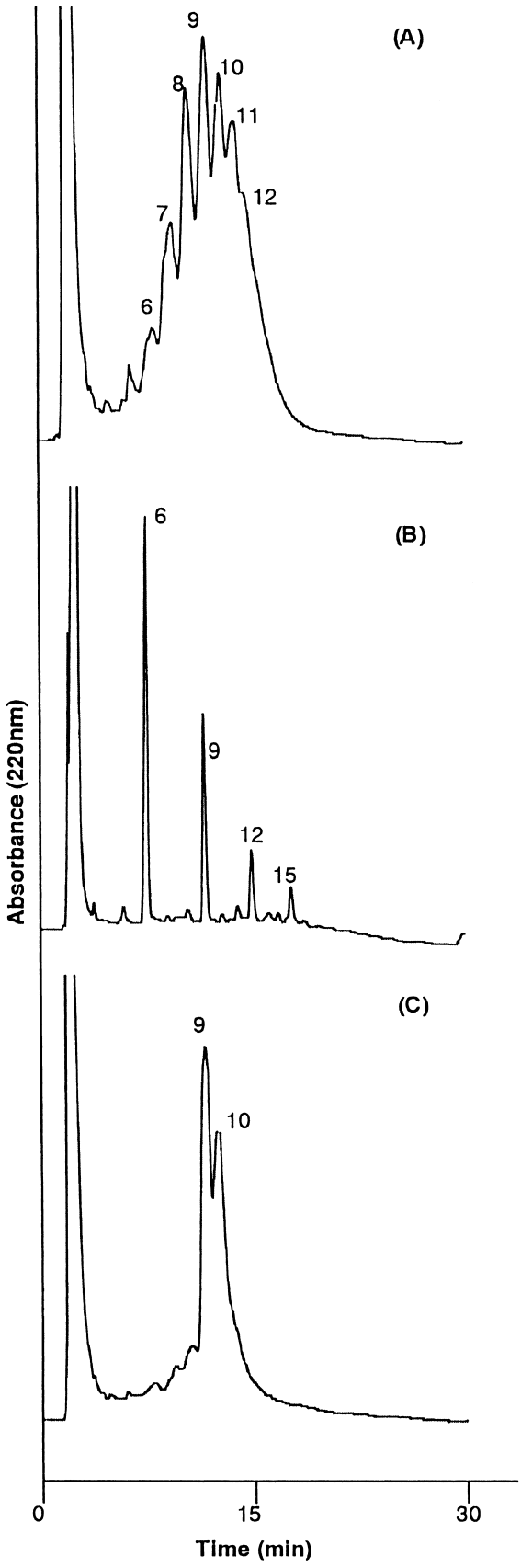


Figure 1. Analytical HPLC profiles of the desulfated crude products (A), the standard mixture of four oligotyrosines (B), and the desulfated **Y-ART-4** (C) (HPLC conditions: column, 5 μm μBondasphere C18 (3.9×150 mm); linear gradient elution, CH_3CN:0.1% aq TFA 4:1 to 1:1 over 30 min; flow rate, 1.0 mL/min; detection, 270 nm).

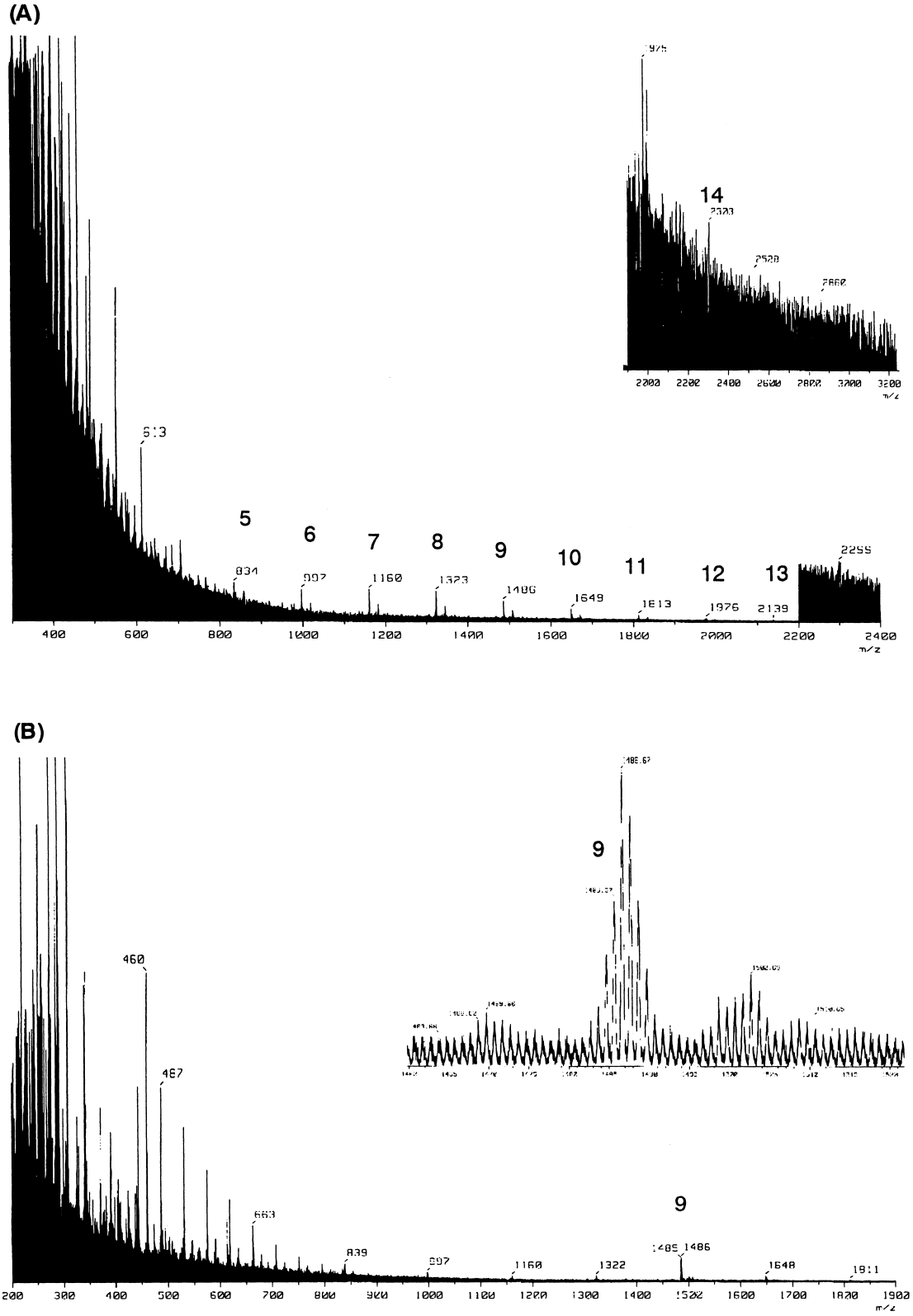


Figure 2. FABMS (positive) spectra of the desulfated crude products (A) and the desulfated **Y-ART-4** (B).

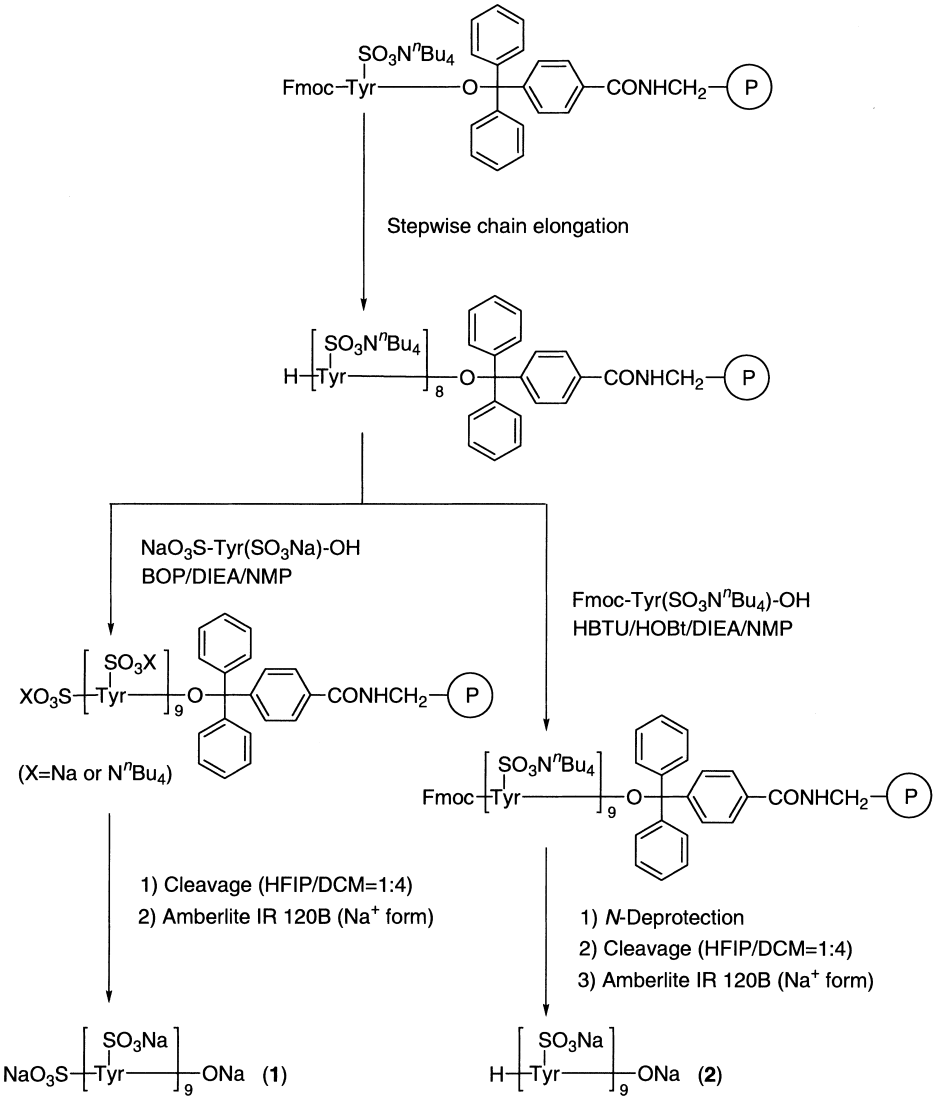
elucidated the structure of **Y-ART-4** tentatively as the fully *N*- and side-chain-*O*-sulfated nonatyrosine.

To find the real mode of sulfation, solid-phase syntheses of NaO_3S–[Tyr(SO_3Na)]$_9$–ONa (**1**) with the expected structure and its *N*-free form, H-[Tyr(SO_3 Na)]$_9$–ONa (**2**), were tried. It was the first thing to be taken into consideration whether sulfation should be done before or after chain elongation. We have already experienced the difficulty in complete sulfation of pentagalloyl glucose.[6]

In addition, preliminary experiments using an *N,C*-protected trityrosine showed the same difficulty. Then, solid-phase synthesis was performed using a sulfated tyrosine derivative as building block. Considering the instability of the tyrosine sulfate group under acidic conditions, synthesis was done by the Fmoc strategy using a trityl linker[18] resin as sketched in Scheme 1.

For the solid-phase synthesis of tyrosine sulfate-containing peptides, two compounds, Fmoc-Tyr(SO_3Na)–OH[19] and Fmoc-Tyr(SO_3·1/2Ba)–OH,[20] are commercially available as synthetic unit for stepwise chain elongation. In our experiences, however, they have problems in solubility and reproducibility in preparation. In this study, we used a tetrabutylammonium salt, Fmoc-Tyr(SO_3·N^{n}Bu$_4$)–OH (**3**), instead.[21] However, bis(tetrabutylammonium) salt of *N,O*-disulfated tyrosine was an oily material and was difficult to handle. Therefore, the N-terminal tyrosine residue of **1** was introduced using the corresponding disodium salt, NaO_3S–Tyr(SO_3Na)–OH (**4**), in a manual mode. Couplings were mediated with *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)[22] except for **4**, which was incorporated with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP).[23] After the chain elongation and N-terminal deprotection for N-terminal free peptide **2**, peptides were released from the resins with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP):dichloromethane (1:4)[24] and isolated as sodium salts.

For the comparison of **Y-ART-4** with the stepwise synthesized **1** and **2,** two new HPLC elution conditions were developed: one (Fig. 3) to separate *N*-sulfated **1** and the *N*-unsulfated **2** and the other (Fig. 4) to discern fully sulfated oligotyrosines of different length. Retention times of **Y-ART-4** were identical with those of **1** under both conditions. In addition, a single peak observed on coinjection of **Y-ART-4** and **1** (Figs. 3(D) and 4(D)) under both conditions strongly suggested the coincidence of the structure of **Y-ART-4** with nonatyrosine *N*- and O^{1-9}-decasulfate **1**.



Scheme 1. Solid-phase syntheses of oligotyrosine sulfates **1** and **2**.

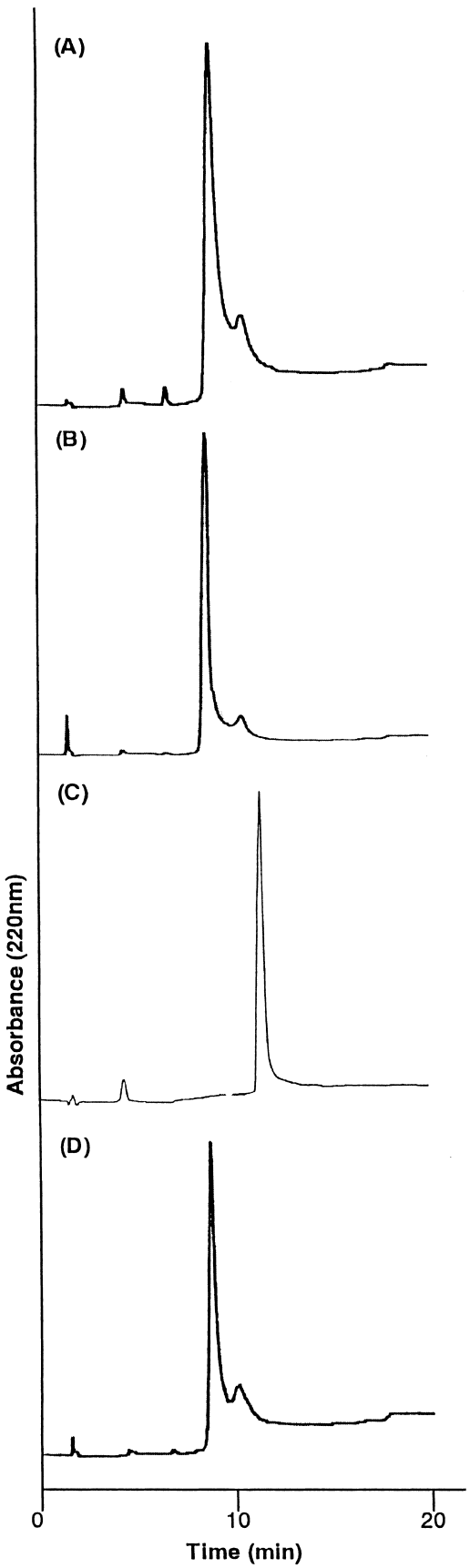


Figure 3. Analytical HPLC profiles of **Y-ART-4** (A), synthetic **1** (B), synthetic **2** (C), and a mixture of **Y-ART-4** and synthetic **1** (D) (HPLC conditions: column, 5 μm μBondasphere C18 (3.9×150 mm); linear gradient elution, CH_3CN:0.1% aq TFA 0:1 to 3:7 over 20 min; flow rate, 1.0 ml/min; detection, 220 nm).

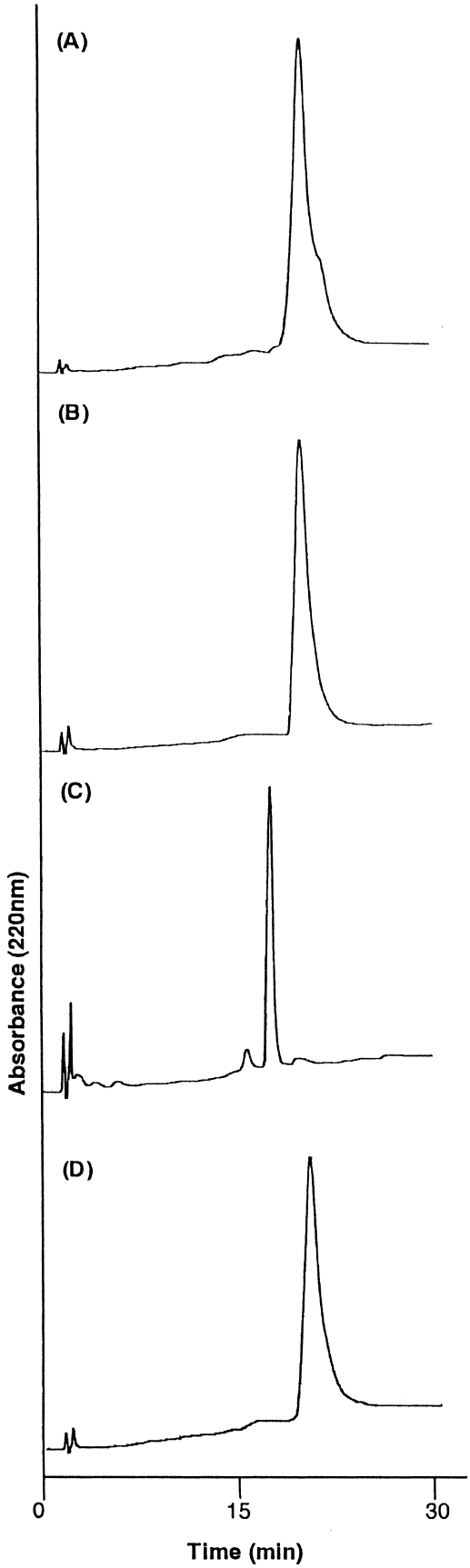


Figure 4. Analytical HPLC profiles of **Y-ART-4** (A), synthetic **1** (B), synthetic **2** (C), and a mixture of **Y-ART-4** and synthetic **1** (D) (HPLC conditions: column, 5 μm μBondasphere C18 (3.9×150 mm); linear gradient elution, CH_3CN:H_2O:0.1 M aq $^nBu_4NHSO_4$ 4:5:1 to 7:2:1 over 30 min; flow rate, 1.0 ml/min; detection, 220 nm).

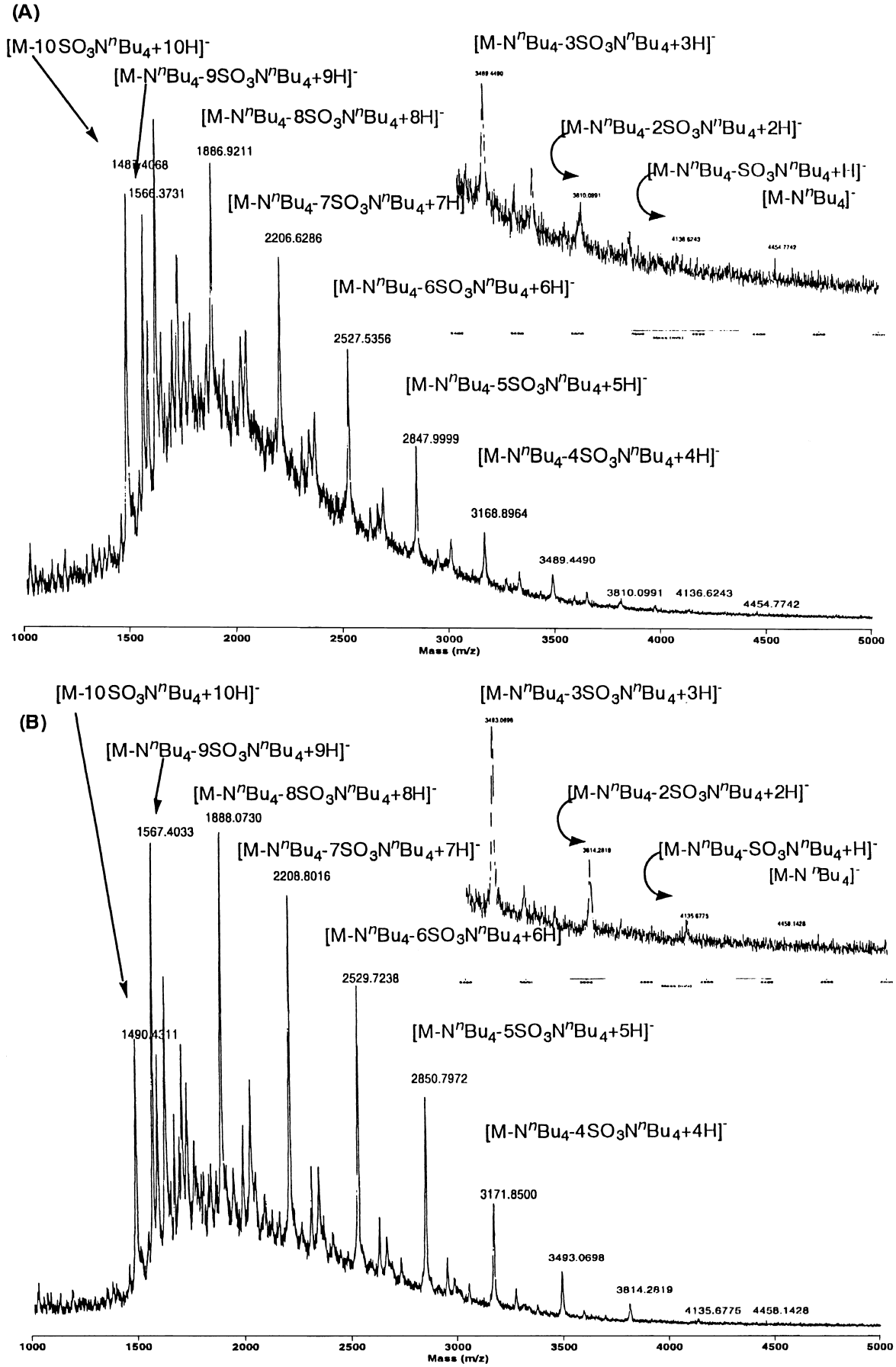


Figure 5. MALDI-TOF–MS of tetrabutylammonium salts of **Y-ART-4** (A) and synthetic **1** (B).

For further identification by matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF–MS), **Y-ART-4** and **1**, respectively, were converted to tetrabutylammonium salts with the expected molecular formula of $^{n}Bu_4N{\cdot}O_3S$–[Tyr($SO_3{\cdot}N^{n}Bu_4$)]$_9$–OH. Because of the polyanionic nature of both samples, spectral data were obtained only in the negative mode. While a signal of $[M-H]^-$ ($m/z=4699.7$) could not be observed, both samples gave signals corresponding to $[M-N^{n}Bu_4]^-$ (m/z: calcd 4459.3; found 4454.8 for **Y-ART-4** and 4458.1 for **1**). In addition, the compound **1** gave a ladder of 10 fragmentation ion signals with mass differences of 321.5 and 79.0 corresponding to nine-step loss of $SO_3{\cdot}N^{n}Bu_4$ and final loss of SO_3, respectively (Fig. 5(B)). **Y-ART-4** also showed the same fragmentation pattern, although another set of fragmentation ions arising from the contaminating decamer was also observed (Fig. 5(A)). From these results the structure of **Y-ART-4** was finally assigned to be the fully sulfated nonatyrosine **1**.

Biological Activities

Anti-HIV activity

The anti-HIV activity of **Y-ART-4** was determined from the protection it provided against HIV-induced cytopathic effects. As shown in Table 1, although HIV-infected MT-4 cells were not able to survive under virus infection, **Y-ART-4** showed concentration-dependent protective activity against virus-induced cytopathogenicity. The 50% effective concentration (EC_{50}) of **Y-ART-4** was 1.62 μg/mL, and 90% effective concentration (EC_{90}) was 6.72 μg/mL. As the 50% cytotoxic concentration (CC_{50}) of **Y-ART-4** was over 1000 μg/mL, the selective index (ratio of CC_{50} to EC_{50}) was over 615. Moreover, no toxicity was observed when 500 μg/kg of **Y-ART-4** was injected into mice intraperitoneally.

Activities of the compounds **1** and **2** synthesized by the solid-phase method were also determined. Since measurements were done separately, comparative data for reference compounds, dextran sulfate, curdlan sulfate, 3′-azido-2′,3′-dideoxythymidine (AZT), and 2′,3′-dideoxycytidine (ddC), are listed together in Table 2.

Table 1. Anti-HIV activity of **Y-ART-4**

HIV-infected MT-4 cells		HIV-uninfected MT-4 cells	
Concentration (μg/mL)	Efficiency (%)	Concentration (μg/mL)	Survival cells (%)
1000.0000	128.54	1000.0000	119.90
200.0000	111.73	200.0000	123.70
40.0000	113.05	40.0000	129.58
8.0000	94.91	8.0000	120.42
1.6000	49.56	1.6000	125.61
0.3200	−2.65	0.3200	118.34
0.0640	−0.44	0.0640	128.03
0.0128	−1.11	0.0128	115.92
		0.0026	115.40
		0.0000	100.00

Table 2. Anti-HIV activity of synthetic oligotyrosine sulfates **1** and **2**

Compound (M.W.)	EC_{50} (μg/mL)	EC_{50} (μM)	CC_{50} (μg/mL)	CC_{50} (μM)	SI
NaO_3S–[Tyr(SO_3Na)]$_9$–ONa (**1**) (2529.0)					
	1.13	0.447	617	254	548
Dextran sulfate	1.20		> 1000		> 833
Curdlan sulfate	0.15		> 1000		> 6667
AZT		0.002		> 500	> 250,000
ddC		0.18		219	1198
H-[Tyr(SO_3Na)]$_9$–ONa (**2**) (2427.0)					
	87.2	35.9	> 1000	> 412	> 11.5
Dextran sulfate	3.31		> 1000		> 302
Curdlan sulfate	0.69		> 1000		> 1449
AZT		0.0171		196	11,402
ddC		0.742		2637	3554

Table 3. Inhibitory activity of **Y-ART-4** on various enzymes

Enzymes	IC_{50} (μg/mL)
HIV-reverse transcriptase	36
AMV-reverse transcriptase	> 100
cAMP-dependent protein kinase	80
Protein kinase C	> 100
Casein kinase 2	2.6
Protein tyrosine kinase	16

Although a comparison of absolute values is not possible because of separate experiments, the EC_{50} value of **Y-ART-4** is comparable to that of the *N*-sulfated **1** and is much smaller than that of the corresponding *N*-unsulfated **2**. This fact also strongly supports the structure assignment of **Y-ART-4** as the fully N-terminal and side-chain-*O*-sulfated nanotyrosine **1**. The importance of the N-terminal sulfate group in the activity development of the oligo(tyrosine sulfate)s would be interesting if we considered the fact that heparin also lost its anti-HIV activity completely by *N*-desulfation .[3]

Inhibitory effect on enzymatic activity

One of the targets of HIV drugs is reverse transcriptase derived from RNA viruses. Then, we tested inhibitory activity of **Y-ART-4** against reverse transcriptases and related enzymes. As shown in Table 3, it is clear that **Y-ART-4** inhibited casein kinase 2 and protein tyrosine kinase strongly, and HIV-derived reverse transcriptase very weakly.

Conclusion

Sulfur trioxide–trimethylamine complex reacted with tyrosine to give a mixture of sulfated tyrosine oligomers. One of the components was successfully isolated as its sodium salt (**Y-ART-4**) and identified as the salt of nonatyrosine *N*- and O^{1-9}-decasulfate by comparison by HPLC and MALDI-TOF–MS with the authentic sample obtained by the solid-phase method. **Y-ART-4** showed anti-HIV activity comparable with dextran sulfate and

curdlan sulfate. These results would be valuable since data of anti-HIV activities of polyanionic compounds with defined structure are few. Synthesis and activity measurements of a series of oligotyrosine sulfates with different length would be the next target of this study. These will be reported in the succeeding paper.

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. ^{1}H NMR spectra were recorded at 300 MHz on Brucker AVANCE DPX300 instrument and at 500 MHz on JEOL JMN-EX500L FT-NMR system. ^{13}C NMR spectra were obtained at 75.5 and 125 MHz, respectively, on the same instruments. Optical rotations were determined with a JASCO DIP-360 polarimeter. Column chromatography was performed on Wakogel C-300 (Wako Pure Chemical Industries, Ltd., Osaka). Elemental analyses were performed by YANACO MT-5 instrument. FAB-mass spectra were obtained using a JEOL JMS-AX505HA spectrometer. MALDI-TOF–MS was carried out on a Voyager-DE TOF MS (Perkin–Elmer, PerSeptive Biosystems Inc., Framingham, MA, USA). The accelerating voltage in the ion source was 20 kV and negative ions were measured. Linear mode was employed for the experiment. The matrix was a supernatant of a mixture of α-cyano-4-hydroxycinnamic acid (1 mg) and 0.2% trifluoroacetic acid:acetonitrile (1:1 v/v, 100 μL). Solid-phase syntheses were done using an Applied Biosystems 430A peptide synthesizer.

Preparation of Y-ART-4

Under argon atmosphere sulfur trioxide–trimethylamine complex (5.0 g) was mixed with tyrosine (2.0 g) in pyridine (20 mL) and the mixture stirred at 55 °C for 93 h. The supernatant was discarded and precipitates were rinsed with dichloromethane to give amorphous solid material. This was dissolved in cooled water (200 mL) containing sodium hydrogen carbonate (12 g) and insoluble materials were removed by filtration. The filtrate was treated with tetrabutylammonium hydrogen sulfate (6.8 g) and sodium hydrogen carbonate (1.8 g) in 100 mL of water and extracted with dichloromethane. The extracts were dried and concentrated. The residue was dissolved in water (200 mL) and stirred with Amberlite IR 120B (Na^+ form) (200 mL) for 12 h at room temperature. After removal of the resin, the aqueous solution was washed with dichloromethane and lyophilized to give crude products as yellowish white powder (3.8 g).

A part of the crude products (1.0 g) was charged on top of Sephadex LH-20 column (2.6×88 cm) and eluted with methanol. Eluate was separated in 10 mL fractions. Every 10th fraction was analyzed by HPLC. Fractions containing the major component (1672–1752 mL) were gathered, concentrated and lyophilized from water to give **Y-ART-4**. Yield: 21 mg.

Solid-phase synthesis of a standard mixture of hexa-, nona-, dodeca-, and pentadecamers of tyrosine

Fmoc-Tyr(^{t}Bu)-O-Alko-resin (Watanabe Chemical Ind., Hiroshima, Tyr content: 0.25 mol/g, 0.5 g) was deprotected with 20% piperidine/*N*-methylpyrrolidinone (NMP) and coupled with Fmoc-Tyr(^{t}Bu)–OH (460 mg, 1 mmol) using HBTU (417 mg, 1.1 mmol) and HOBt (168 mg, 1.1 mmol) in NMP according to the standard program.[22] The same cycle was repeated 13 times, while an equimolar mixture of Fmoc-Tyr(^{t}Bu)–OH and Boc-Tyr(^{t}Bu)–OH at the 5th, 8th, and 11th cycles and Boc-Tyr(^{t}Bu)–OH at the 14th cycle, respectively, were used. After thorough washing and drying, a part of the resin (404 mg) was treated with 90% TFA (10 mL) at room temperature for 1.5 h. After removal of the resin, peptide products were precipitated with ether and washed with cooled ether. Yield: 92 mg (52% based on the Tyr content of the starting resin). An analytical HPLC profile is shown in Figure 1(B).

Fmoc-Tyr(SO_3·N^{n}Bu$_4$)–OH (3)

Under argon atmosphere Fmoc-Tyr–OH[14] (4.02 g, 10.0 mmol) and sulfur trioxide–pyridine complex (4.77 g, 30.0 mmol) were dissolved in DMF (10 mL). After having been stirred at room temperature for 1 h, the mixture was slowly poured into 300 mL of ice-cooled 5% aqueous $NaHCO_3$ solution under stirring. To this, after gas evolution ceased, a small quantity of chloroform and a solution of ^{n}Bu$_4$NHSO$_4$ (5.09 g, 15.0 mmol) in aqueous $NaHCO_3$ (pH > 7) were added and pH of the solution was adjusted to about 4 by addition of 5% citric acid. The organic layer was separated and the aqueous layer extracted with chloroform. The combined extracts were washed with 5% citric acid (thrice), water (thrice), and brine and dried over anhydrous sodium sulfate. After removal of the solvent, the residue was dissolved in methanol and decolorized with active charcoal. The solvent was removed and the residue triturated with petroleum ether to afford an amorphous solid. This was dissolved in a 1,4-dioxane:water mixture (30 mL:3 mL) and lyophilized. Repeated lyophilization gave a white powder. Yield: 6.88 g (95%). HPLC t_R = 9.6 min (elution: acetonitrile:0.1% TFA 20:80 to 80:20 over 20 min; flow rate: 1 ml:min; detection: 220 nm). $[\alpha]_D^{26}$ +8.0° (*c* 1.0, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ_{TMS} 0.93 (12H, t, *J* = 7.2 Hz, CH_3/^{n}Bu), 1.27–1.39 (8H, m, γCH_2/^{n}Bu) 1.49–1.54 (8H, m, βCH_2/^{n}Bu), 3.01–3.19 (10H, m, βCH_2/Tyr, αCH_2/^{n}Bu), 4.17–4.30 (2H, m, CH_2/Fmoc), 4.38–4.44 (1H, m, CH/Fmoc), 4.60–4.62 (1H, m, αCH/Tyr), 5.71–5.73 (1H, m, NH/Tyr), 7.08 (2H, d, *J* = 8.3 Hz, aromatic 2,6-CH/Tyr), 7.28 (2H, d, *J* = 8.3 Hz, aromatic 3,5-CH/Tyr), 7.30–7.40 (4H, m, aromatic/Fmoc), 7.57–7.59 (2H, m, aromatic/Fmoc), 7.74 (2H, d, *J* = 7.2 Hz, aromatic/Fmoc). ^{13}C NMR (75.5 MHz, $CDCl_3$ = 77.00) δ 13.52 (CH_3/^{n}Bu), 19.48 (γCH_2/^{n}Bu), 23.64 (βCH_2/^{n}Bu), 36.88 (βCH_2/Tyr), 47.00 (CH/Fmoc), 54.62 (αCH/Tyr), 58.43 (αCH_2/^{n}Bu), 66.83 (CH_2/Fmoc), 119.77 (aromatic/Fmoc), 121.22 (aromatic 3,5-C/Tyr), 125.15, 125.78, 127.06 (aromatic/Fmoc), 130.19 (aromatic 2,6-C/Tyr), 131.60 (aromatic 1C/Tyr), 141.11, 143.67 (aromatic/Fmoc), 152.02 (aromatic 4C/Tyr), 155.70 (CO/

urethane), 172.26 (CO/Tyr). FABMS found (positive): 966.9 $(M+N^nBu_4)^+$, 1207.6 $(M+2N^nBu_4\text{-}H)^+$; calcd for $C_{56}H_{92}N_3O_8S$ $(M+N^nBu_4)^+$, 966.6, $C_{72}H_{127}N_4O_8S$ $(M+2N^nBu_4\text{-}H)^+$, 1207.9. Found: C, 65.52; H, 7.83; N, 3.93. Calcd for $C_{40}H_{56}N_2O_8S\cdot 1/2H_2O$: C, 65.46; H, 7.83; N, 3.82.

^{n}Bu$_4$N·O$_3$S–Tyr(SO$_3$·N^{n}Bu$_4$)–OBzl

Under argon atmosphere H-Tyr-OBzl[25] (2.50 g, 9.21 mmol) and sulfur trioxide–pyridine complex (9.79 g, 61.5 mmol) were dissolved in DMF (20 mL) and the solution was stirred for 1 h. To this, pyridine (5 mL) was added and the stirring was continued for further 12 h. A part of the solvent was removed under reduced pressure and the residue poured into 5% aqueous $NaHCO_3$ solution (300 mL) under stirring. After gas evolution ceased, the aqueous solution was washed with dichloromethane five times. To this, $^nBu_4NHSO_4$ (10.2 g, 30.0 mmol) in saturated $NaHCO_3$ solution (pH > 7) was added and the solution extracted with chloroform. Combined extracts were washed with water and dried. After removal of the solvent, the residue was dissolved in methanol and decolorized with active charcoal. Removal of the charcoal and solvent gave the title compound as oil. This was homogeneous in HPLC and used without purification for the next step. HPLC $t_R = 13.3$ min (elution: acetonitrile:0.1% TFA 0:100 to 30:70 over 20 min; flow rate: 1 ml/min; detection: 220 nm). ^{1}H NMR (500 MHz, $CDCl_3$) δ_{TMS} 0.96 (24H, t, $J = 7.2$ Hz, $CH_3/^nBu$), 1.35–1.40 (16H, m, $\gamma CH_2/^nBu$), 1.57 (16H, m, $\beta CH_2/^nBu$), 2.88–2.99 (2H, m, βCH_2/Tyr), 3.17–3.20 (16H, m, $\alpha CH_2/^nBu$), 4.31–4.33 (1H, m, αCH/Tyr), 5.06(2H, s, $C\underline{H}_2Ph$), 7.07 (2H, d, $J = 7.2$ Hz, aromatic 2,6-CH/Tyr), 7.19 (2H, d, $J = 7.2$ Hz, aromatic 3,5-CH/Tyr), 7.27–7.34 (5H, m, $CH_2\underline{Ph}$). ^{13}C NMR (125 MHz, $CDCl_3 = 77.00$) δ 13.60 ($\underline{C}H_3/^nBu$), 19.60 ($\gamma CH_2/^nBu$), 23.85 ($\beta CH_2/^nBu$), 36.48 (βCH_2/Tyr), 57.75 (αCH/Tyr), 58.61 ($\alpha CH_2/^nBu$), 67.02 ($\underline{C}H_2Ph$), 120.90 (aromatic 3,5-C/Tyr), 127.98, 128.17, 128.40, 128.57, 128.65 ($CH_2\underline{Ph}$), 130.04 (aromatic 2,6-C/Tyr), 132.37 (aromatic 1C/Tyr), 135.76 ($CH_2\underline{Ph}$), 152.10 (aromatic 4C/Tyr), 173.73 (CO/Tyr). FABMS found (positive): 1156.3 $(M+N^nBu_4)^+$; calcd for $C_{64}H_{123}N_4O_9S_2$ $(M+N^nBu_4)^+$, 1155.8.

NaO$_3$S–Tyr(SO$_3$Na)–OH (4)

The whole above oily compound was dissolved in water and the solution stirred with Amberlite IR 120B resin (Na^+ form, 24 mL) for 12 h. The resin was removed and the aqueous solution washed with dichloromethane. The washings were evaporated and the remaining tetrabutylammonium salt was again dissolved in water and converted to the sodium salt by repeating the same treatment with the Amberlite resin. All the aqueous solutions were gathered and lyophilized to give NaO_3S–Tyr(SO_3Na)–OBzl as white powder. Yield: 3.54 g (81% from H-Tyr–OBzl).

A part of the above compound (713 mg, 1.54 mmol) was dissolved in methanol (50 mL). To this, a few drops of water and 10% palladium on charcoal (50 mg) were added and hydrogenolysis at atmospheric pressure was carried out for 2 h. After removal of the catalyst and the solvent, the residue was dissolved in water and lyophilized to give a white powder. Yield: 574 mg (quantitative). HPLC $t_R = 4.5$ min (elution: acetonitrile:0.1% TFA 0:100 to 30:70 over 20 min; flow rate: 1 ml/min; detection: 220 nm). ^{1}H NMR (500 MHz, CD_3OD) δ_{TMS} 3.04–3.11 (2H, m, βCH_2/Tyr), 4.18–4.20 (1H, m, αCH/Tyr), 7.19–7.25 (4H, m, aromatic/Tyr). FABMS found (positive): 386.0 $(M+H)^+$, 408.0 $(M+Na)^+$; (negative): 361.8 $(M-Na)^-$; calcd for $C_9H_9NO_9S_2Na_2$ (M): 385.0. Found: C, 25.95; H, 3.09; N, 3.31; S, 15.79. Calcd for $C_9H_9NO_9S_2Na_2\cdot 2H_2O$: C, 25.66; H, 3.11; N, 3.32; S, 15.22.

Solid-phase syntheses of nonatyrosine *N*- and *O*$^{1-9}$-decasulfate (1) and nonatyrosine *O*$^{1-9}$-nonasulfate (2). Fmoc-Tyr($SO_3\cdot N^nBu_4$)–OH was esterified with a trityl linker resin[21] (Tyr content: 0.33 mmol/g). Chain elongation was performed according to the standard program[22] using 20% piperidine in NMP for deprotection and HBTU–HOBt for coupling of **3**. At the final cycle in the synthesis of **1**, **4** was coupled using BOP[23]/DIEA/NMP. For **2**, the chain elongation was continued to a nonamer and the Fmoc group removed. In both cases peptides were released from the resins with HFIP:DCM (1:4).[24] After removal of the volatile materials in vacuo, the residue was dissolved in water and stirred with Amberlite IR 120B (Na^+ form) for 24 h at room temperature. After removal of the resin, the aqueous solution was washed with dichloromethane and lyophilized. Crude products were purified by gel chromatography on Sephadex LH-20 using methanol for elution. Yields based on the content of Tyr of the starting resin: 40% for **1** and 18% for **2**, respectively. Their purity was checked on HPLC and shown in Figures 3 and 4.

Anti-HIV activity

MT-4 cells were injected with HIV-1_{IIIB} using 1000 $TCID_{50}/10^5$ cells. HIV- or mock-infected MT-4 cells (1.5×10^5 cells/mL, 200 μL) were placed in to 96-well microtiter plates and incubated in the presence of various concentrations of test compounds. Drugs were diluted 5-fold, and nine different concentrations of each compound were examined. All experiments were performed in triplicate. After 5 days of culture at 37 °C in a CO_2 incubator, cell viability was quantified by a colorimetric assay which monitors the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a blue-colored formazan product.[26,27,28]

Inhibitory effect on enzymatic activity

HIV-derived and avian myeloblastosis virus (AMV)-derived reverse transcriptases were obtained from Funakoshi Co. cAMP-dependent protein kinase was purchased from Sigma and its activity was determined by the phosphorylation of histone using protein kinase A catalytic subunit, protein kinase C by the phosphorylation of 80 kDa protein after stimulation by phorbolmyristate acetate of Swiss 3T3 cells, and casein kinase 2 by the

phosphorylation of casein by partially purified casein kinase 2 of bovine lung. Protein tyrosine kinase activity was assayed by using A431 cell membrane as an enzyme source.

Acknowledgements

This work was supported by a Research Grant from the Human Sciences Foundation.

References

1. Ito, M.; Baba, M.; Sato, R.; Pauwels, R.; De Clercq, E.; Shigeta, S. *Antiviral Res*. **1987**, *7*, 361.
2. Ueno, R.; Kuno, S. *Lancet* **1987**, *1*, 1379.
3. Baba, M.; Nakajima, M.; Schols, D.; Pauwels, R.; Balzarini, J.; De Clercq, E. *Antiviral Res*. **1988**, *9*, 335.
4. Yoshida, T.; Hatanaka, K.; Uryu, T.; Kaneko, Y.; Suzuki, E.; Miyano, H.; Mimura, T.; Yoshida, O.; Yamamoto, N. *Macromolecules* **1990**, *23*, 3717.
5. Lorentsen, K. J.; Hendrix, C. W.; Collins, J. M.; Kornhauser, D. M.; Petty, B. G.; Klecker, R. W.; Flexner, C.; Eckel, R. H.; Lietman, P. S. *Ann. Int. Med*. **1989**, *111*, 561.
6. Nakashima, H.; Ichiyama, K.; Hirayama, F.; Uchino, K.; Ito, M.; Saitoh, T.; Ueki, M.; Yamamoto, N.; Ogawara, H. *Antiviral Res*. **1996**, *30*, 95.
7. Traube, W.; Zander, H.; Gaffron, H. *Chem. Ber*. **1924**, *57*, 1045.
8. Baumgarten, P. *Chem. Ber*. **1926**, *59*, 1976.
9. Kelly, K. K.; Matthews, J. S. *J. Org. Chem*. **1971**, *36*, 2159.
10. Kenner, G. W.; Stedman, R. J. *J. Chem. Soc*. 1952, 2069.
11. Clayton, D. W.; Farrington, J. A.; Kenner, G. W.; Turner, J. M. *J. Chem. Soc*. **1957**, 1398.
12. Nishi, N.; Naruse, T.; Hagiwara, K.; Nakajima, B.; Tokura, S. *Macromol. Chem*. **1991**, *192*, 1799.
13. Hiller, A. R.; Orgel, L. E. Jr. *Orig. Life Evol. Biosph*. **1996**, *26*, 539.
14. Yagami, T.; Shiwa, S.; Futaki, S.; Kitagawa, K. *Chem. Pharm. Bull*. **1993**, *41*, 376.
15. Atherton, E.; Sheppard, R. C. In *Solid Phase Peptide Synthesis, A Practical Approach*; IRL Press: Oxford, 1989; p 25.
16. Sanger, F. *Biochem. J*. **1949**, *45*, 563.
17. Swenson, R. P.; Williamson, C. H. Jr.; Massey, V. *J. Biol. Chem*. **1982**, *257*, 1937.
18. Bayer, E.; Clausen, N.; Goldammer, C.; Henkel, B.; Rapp, W.; Zhang, L. In *Peptides, Chemistry, Structure and Biology*; Hodges, E. S.; Smith, J. A., Eds.; ESCOM: Leiden, 1994; p 156.
19. Penke, B.; Rivier, J. *J. Org. Chem*. **1987**, *52*, 1197.
20. Moroder, L.; Wilschowitz, L.; Jaeger, E.; Knof, S.; Thamm, P.; Wünsch, E. *Hoppe-Seyler's Z. Physiol. Chem*. **1981**, *362*, 143.
21. Ueki, M.; Watanabe, S.; Yamanaka, R.; Ohta, M.; Okunaka, O. In *Peptide Science 1999*; Fujii, N., Ed.; Japanese Peptide Society: Osaka, 2000; p 117.
22. Fields, C. G.; Lloyd, D. H.; Macdonald, R. L.; Otteson, K. M.; Noble, R. L. *Peptide Res*. **1991**, *4*, 95.
23. Castro, B. *Tetrahedron Lett*. 1975, 1219.
24. Bollhagen, R.; Schmiedberger, M.; Barlos, K.; Grell, E. *J. Chem. Soc., Chem. Commun*. 1994, 2559.
25. Roeske, R. W. *J. Org. Chem*. **1963**, *28*, 1251.
26. Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn, P.; Desmyter, J.; De Clercq, E. *J. Virol. Methods* **1988**, *20*, 309.
27. Nakashima, H.; Masuda, M.; Murakami, T.; Koyanagi, Y.; Matsumoto, A.; Fujii, N.; Yamamoto, N. *Antimicrob. Agents Chemother*. **1992**, *36*, 1249.
28. Nakashima, H.; Inazawa, K.; Ichiyama, K.; Ito, M.; Ikushima, N.; Shoji, T.; Katsuraya, K.; Uryu, T.; Yamamoto, N.; Juodawlkis, A. S.; Schinazi, R. F. *Antiviral Chem. Chemother*. **1995**, *6*, 271.