Chemistry and Anti-tumor Activity of Sperabillin Polymers

Tsuneaki HIDA,*,[a] Shigetoshi TSUBOTANI,[a] Akira HORI,[b] Morio MURAKAMI,[b] Hideaki NATSUGARI,[b] Yoshio KOZAI,[b] and Setsuo HARADA[a]

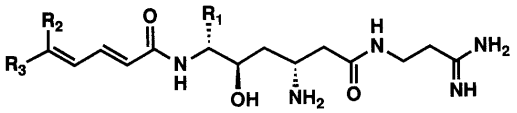
Discovery Research Division[a] and Pharmaceutical Research Division,[b] Takeda Chemical Industries, Ltd., Jusohonmachi-2, Yodogawa-ku, Osaka 532, Japan. Received September 17, 1992

Sperabillin A, 3-[[(3*R*,5*R*)-3-amino-6-[(2*E*,4*Z*)-2,4-hexadienoylamino]-5-hydroxyhexanoyl]amino]propanamidine dihydrochloride, was polymerized on standing for several days under a highly humid atmosphere or in the presence of radical initiators. The average molecular weight of the polymers obtained could be regulated by changing the reaction conditions in the latter case. Spectral analyses of the polymers revealed that the 2,4-hexadienoyl moiety of sperabillins was polymerized in a free radical-initiated reaction. The polymers selectively inhibited the proliferation of human umbilical vein endothelial (HUVE) cells. Polymers having higher molecular weight showed stronger inhibition of HUVE cell proliferation. In addition, the polymers showed anti-tumor activity against B16 melanoma *in vivo*.

Keywords sperabillin; polymer; anti-tumor activity; radical initiator; vascular endothelial cell

Sperabillins A (**1a**), B (**2a**), C (**3**) and D (**4**) are novel antibacterial substances isolated from the culture broth of *Pseudomonas fluorescens* YK-437.[1] They are active against gram-positive and gram-negative bacteria including antibiotic-resistant strains,[1] and have 2,4-hexadienoic acid, 3,6-diamino-5-hydroxyhexanoic (or -heptanoic) acid and 2-aminoethanamidine as constituents, as shown in Fig. 1.[2,3]

During further investigation of the biological activities of sperabillins, their inhibitory effect on the proliferation of human umbilical vein endothelial (HUVE) cells was examined. Unexpectedly, one of the samples of **1a** strongly inhibited the proliferation, even though the original one showed little activity. The sample had been purified just before the final lyophilization procedure, but the frozen solution had accidentally been allowed to melt during the procedure and the peak area of **1a** was reduced to half, though no other peaks were observed on HPLC analysis using an octadecyl silica (ODS) column. An active peak was observed by gel permeation analysis, and the substance which inhibited the proliferation of HUVE cells was finally found to be the polymer of **1a** (Fig. 2).



sperabillin	R_1	R_2	R_3
A (**1a**)	H	CH_3	H
B (**2a**)	CH_3	CH_3	H
C (**3**)	H	H	CH_3
D (**4**)	CH_3	H	CH_3

Fig. 1. Structures of Sperabillins A, B, C and D

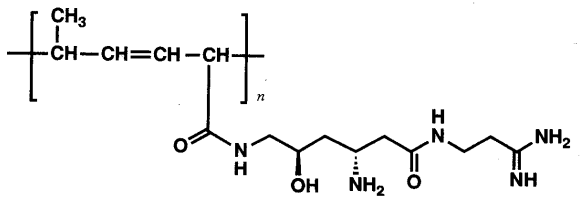
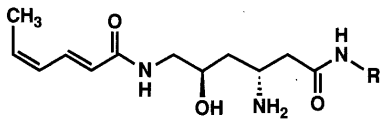


Fig. 2. Sperabillin A Polymer (**1b**)

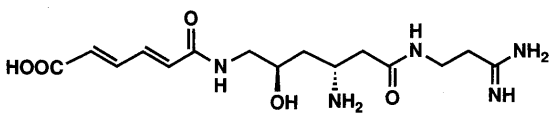
In this report, we describe the preparation, physicochemical properties and biological activities of sperabillin polymers.

Preparation of Polymers Although sperabillins were polymerized in rather high concentrated solutions (>0.5 M), the reaction was very slow and was not practical for the preparation of polymers. For example, a 2.5 M aqueous solution of **1a** (sulfate) afforded the polymer (**1b**) (37 °C, 7 d) in only 12% yield. The reaction proceeded more rapidly in a good yield when the powder of **1a** was left standing for several days under a highly humid atmosphere at 37 °C. The polymer thus obtained, however, was thought to be aggregated and showed poor solubility in water. Ultrasonication loosened the aggregates to yield polymers having lower molecular weight and increased solubility in water (method I). In this case, the polymers were purified by activated carbon or Diaion HP-20 column chromatography. By this method (the time of sonication was held constant at 1 h), a series of sperabillin derivatives having the 2,4-dienoyl moiety[2,3] (Fig. 3) were polymerized, as summarized in Table I.

The polymerization of **1a** in aqueous solution is considered to be a free radical reaction. Thus, this reaction mechanism was studied by the ESR spin trapping method.[4,5]



compound	R	compound	R
5a	$(CH_2)_2CONH_2$	**10a**	$(CH_2)_2$-N⟨O⟩
6a	$(CH_2)_4NH_2$		
7a	$(CH_2)_3NH_2$	**11a**	$CH_2CH(NH_2)COOH$ (*R*)
8a	$(CH_2)_2NH_2$		
9a	$(CH_2)_2NHC(=NH)NH_2$	**12a**	$CH_2CH(NH_2)COOH$ (*S*)
		13a	CH_2CH_3



14a

Fig. 3. Structures of Sperabillin Derivatives Polymerized by Method I

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (POBN) and 2-methyl-2-nitrosopropane (MNP) were used as spin trapping agents. In all cases, these trapping agents reacted with the radicals generated by the polymerization reaction to produce spin adducts. This shows that the polymerization of sperabillins is indeed a free radical reaction initiated by oxygen in the medium.

We, therefore, examined the preparation of polymers in the presence of radical initiators (method II). Since sperabillins are water-soluble substances, we used water-soluble initiators, such as hydrogen peroxide–iron(II) sulfate, ammonium persulfate-sodium sulfite and 2,2′-azobis(2-amidinopropane) dihydrochloride, with the reactions being carried out in aqueous media. The molecular weight of polymers could be regulated by changing the concentration of the monomer and initiator or the reaction temperature. To purify the polymers, the reaction mixtures were passed through a column of an anion exchange resin (SO_4^- form), if necessary, and then dialyzed to remove the monomer and reagents.

Table I. Polymerization of Sperabillin Derivatives by Method I

Monomer	Polymer	Salt	Reaction time (d)	Purification	Yield (%)
1a	**1b**	2HCl	4.0	HP-20	93
1a	**1b**	H_2SO_4	2.0	Carbon	76
2a	**2b**	2HCl	0.9	Carbon	89
5a	**5b**	2HCl	4.5	Carbon	53
6a	**6b**	2HCl	4.0	Carbon	50
7a	**7b**	2HCl	4.0	Carbon	42
8a	**8b**	2HCl	2.0	Carbon	80
9a	**9b**	2HCl	7.0	Carbon	34
10a	**10b**	2HCl	4.0	Carbon	53
11a	**11b**	HCl	1.8	Carbon	78
12a	**12b**	HCl	2.7	Carbon	78
13a	**13b**	HCl	6.0	Carbon	28
14a	**14b**	2HCl	7.0	Carbon	29

Table II. Effect of Ultrasonication on Molecular Weight of Sperabillin A Polymers (Sulfate) Prepared by Method I

Sonication time (h)	Molecular weight[a]	
	$\bar{M}_n$[b]	$\bar{M}_w$[c]
0	298000	1090000
1	154000	344000
4	133000	247000
6	119000	234000
20	54000	82500
40	55100	84300

a) GPC-LALLS method. *b*) $\bar{M}_n$, number-average molecular weight. *c*) $\bar{M}_w$, weight-average molecular weight.

Physico-chemical Properties of Sperabillin Polymers The molecular weights of the polymers were estimated by a combination of gel permeation chromatography and the low-angle laser-light scattering method (GPC-LALLS method). Change of the average molecular weight of the polymer prepared by method I in relation to the time of ultrasonication is shown in Table II. Although the polymer prepared without sonication had a large molecular weight, the molecular weight declined during sonication, reaching a plateau at 20 h, and the polydispersity ratio ($\bar{M}_w/\bar{M}_n$) was decreased.

The molecular weights of the polymers obtained by method II are shown in Table III. The molecular weights increased when the concentration of the monomer was raised and the amount of the initiator or the reaction temperature was lowered. The average molecular weight of these polymers was not influenced by ultrasonication, suggesting that they are not aggregated. As shown in Fig. 4, plots of $\bar{M}_n$ and $\bar{M}_w$ *vs.* retention time on GPC on semilogarithmic graph paper gave a straight line, and

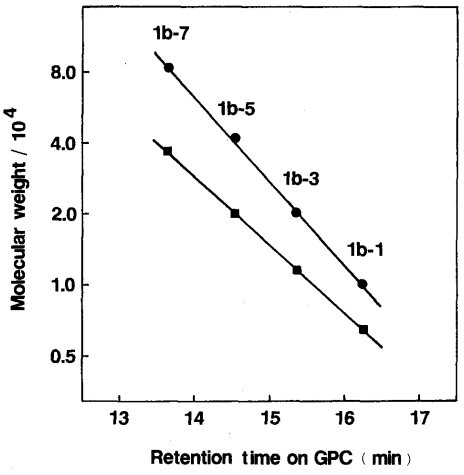


Fig. 4. Molecular Weight Calibration Curves

●, $\bar{M}_w$; ■, $\bar{M}_n$. GPC conditions: column, Asahipak GFA 50F; mobile phase, 0.1 M Na_2SO_4/0.1 M phosphate solution (pH 3.0); flow rate, 0.5 ml/min; detection, UV absorbance at 214 nm; temperature, 23 °C.

Table III. Preparation of Sperabillin Polymers with Radical Initiators

Polymer	Molecular weight[a]		Reaction conditions				Dialysis[b] $\bar{M}_r$ cut-off	Yield (%)
	$\bar{M}_n$	$\bar{M}_w$	Initiator[c]	Concentration of **1a** (% (*w*/*v*))	Temp. (°C)	Time (h)		
1b-1	6410	9990	A (10)[d]	50	39	16	1000	51
1b-2	7210	12800	A (5)	50	39	5	3500	46
1b-3	11500	20100	B (7)	25	39	16	2000	73
1b-4	16800	35000	C (8)	25	60	7	3500	81
1b-5	20000	42300	B (5)	50	39	6	6000—8000	79
1b-6	28000	60000[e]	B (5)	50	21	21	3500	73
1b-7	36200	80900	C (5)	50	60	6	3500	83

a) GPC-LALLS method. *b*) Spectrapor was used. *c*) A; hydrogen peroxide-iron(II) sulfate: B; ammonium persulfate-sodium sulfite: C; 2,2′-azobis(2-amidinopropane) dihydrochloride. *d*) The values in parentheses are wt% based on **1a**. *e*) Estimated from retention time on GPC.

TABLE IV. Effects of Polymers Prepared from Sperabillin Derivatives on the Proliferation of HUVE and HEL Cells (IC_{50} μg/ml)

	Cell line		SI[a]		Cell line		SI[a]
	HUVE	HEL			HUVE	HEL	
1b (H_2SO_4)	0.26	14	54	**9b**	0.22	14	64
2b	0.25	15	60	**10b**	0.28	>80	>286
5b	0.54	16	30	**11b**	0.31	>80	>258
6b	0.22	12	55	**12b**	0.28	>80	>286
7b	0.22	13	59	**13b**	2.0	15	7.5
8b	0.24	8.6	36	**14b**	0.40	28	70

a) SI, selectivity index (IC_{50} of HEL cells/IC_{50} of HUVE cells).

TABLE V. Effects of Sperabillin Polymers Prepared by Method II on the Proliferation of HUVE and HEL Cells (IC_{50} μg/ml)

	Cell lines		SI
	HUVE	HEL	
1b-1	0.88	15.0	17
1b-2	0.63	12.7	20
1b-3	0.55	9.9	18
1b-4	0.22	7.2	33
1b-5	0.19	9.5	50
1b-6	0.24	7.6	32
1b-7	0.13	7.4	57

hence GPC analysis made it possible to estimate the molecular weight accurately without use of the LALLS method.

Although elemental analysis of the polymers gave the same results as those of the corresponding monomer, the UV absorption band of **1a** at 266 nm disappeared. In the ^{13}C-NMR spectrum of **1b** (dihydrochloride), all signals, especially those observed at 20.8 (CH_3), 42.0 (CH), around 59 (CH), 129.6 (CH), 140.0 (CH) and 179.6 (C=O) ppm, were broad. The signals derived from the 3,6-diamino-5-hydroxyhexanoic acid and 2-aminoethanamidine moieties were observed at almost the same chemical shifts as those of **1a**. Two olefinic protons were observed at 5.4 ppm and the methyl signal was shifted to 0.95 ppm in the ^1H-NMR spectrum of **1b**. These findings revealed that the hexadienoyl moiety of **1a** was polymerized in a free radical-initiated reaction, and the other moieties remained intact, as shown in Fig. 2.

Biological Activity and Discussion 1) Effects on the Proliferation of HUVE Cells: The growth of vascular endothelial cells is an essential stage in the process of angiogenesis around tumors.[6] As shown in Table IV, the polymers prepared by method I (**1b**, **2b**, and **5b**—**14b**) strongly inhibited the proliferation of HUVE cells, although they had rather weak effects on human embryonic lung fibroblast (HEL) cells. Among them, the 4-(aminoethyl)morpholine derivative (**10b**) and the 2,3-diaminopropionic acid derivatives (**11b** and **12b**) had good selectivity for HUVE cells.

The effect of the molecular weight of the polymers prepared by method II is summarized in Table V; increasing the molecular weight enhanced the activity against both HUVE and HEL cells, and also raised the selectivity for HUVE cells.

2) Effects on the Proliferation of Human Tumor Cell Lines: Compounds **1b**, **10b** and **11b** were found to be fairly active against human colon adenocarcinoma (COLO 205), human squamous cell carcinoma (HSC-1) and human promyelocytic leukemia (HL-60) cells as shown in Table VI.

TABLE VI. Effects of Polymers on the Proliferation of Human Tumor Cell Lines (IC_{50} μg/ml)

Polymer	Tumor cells		
	COLO 205	HSC-1	HL-60
1b (H_2SO_4)	6.0	5.4	3.0
10b	6.7	15	6.8
11b	20	17	17

TABLE VII. Effects of Polymers on the Survival Time of the B16 Melanoma Bearing Mice

Dose (mg/kg/d)	% ILS[a,b]		
	1b (H_2SO_4)	**10b**	**11b**
1.6	47	15	—[c]
3.1	114	23	18
6.3	99	24	30
12.5	122	35	26
25	77	32	54

a) ILS, increase of life span; % ILS = (treated × 100/control) − 100. *b*) The median survival time of the control mice was 12.7—13.3 d. *c*) Not tested.

3) *In Vivo* Experiments: Examination of anti-tumor activity against B16 melanoma indicated that the polymers had apparent activity against the tumor with **1b** (sulfate) showing marked activity (Table VII). These effects are considered to be a result of multiple activities against the tumor because the polymers not only inhibited the proliferation of a variety of cell lines but also showed immunostimulating activity and suppressed metastasis.[7]

The acute toxicity (LD_{50}, mice) of **1b** (sulfate) was 2.5—10 mg/kg for intravenous (i.v.) administration, 50—200 mg/kg for subcutaneous administration and more than 400 mg/kg for intraperitoneal administration. The LD_{50} values tended to decrease with the molecular weight. For example, the values of **1b**-7 and **1b**-1 (i.v.) were 6.3—12.5 and 25—50 mg/kg, respectively. A similar dependence of toxicity and cytotoxicity on molecular weight has been reported for polylysine.[8]

Experimental

General Procedures UV spectra in water were taken on a Hitachi 320 spectrophotometer. IR spectra were obtained with a Hitachi 285 grating IR spectrophotometer using KBr pellets. NMR spectra were recorded on a Bruker AC-300 spectrometer (^1H, 300 MHz; ^{13}C, 75 MHz) in D_2O. Chemical shifts (δ) are reported in ppm downfield from sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4. Ultrasonication was carried out with a Branson SONIFIER cell disruptor Model 200. Dialysis was carried out with Spectrapor 1 ($\overline{M}_r$ 6000—8000 cut-off), Spectrapor 3 ($\overline{M}_r$ 3500 cut-off) or Spectrapor 6 ($\overline{M}_r$ 1000 or 2000 cut-off).

ESR Spin Trapping Method Samples were prepared for ESR mesurement as follows. The sample was mixed with spin trapping agents, then transferred into a flat, quartz cell and put into the standard cavity of a JEOL JES-RE3X ESR spectrometer.

1) Spin Trapping by DMPO: DMPO (10.2 mg, 0.090 mmol) was added to a 0.5 M aqueous solution of **1a** (0.20 ml, 0.10 mmol). The DMPO-OH spin adduct was observed in the reaction mixture. The hyperfine coupling constants were determined as one nitrogen, $A_N = 14.89$ G, one hydrogen in the β-position, $A_H = 14.89$ G.

2) Spin Trapping by POBN: A 0.10 M aqueous solution of POBN (0.10 mml, 0.010 mmol) was added to a 0.5 M aqueous solution of **1a** (0.10 mg, 0.05 mmol). The spin adduct (A_N=15.37 G, A_H=2.83 G), considered to be a σ-carbon radical adduct, was observed in this system.

3) Spin Trapping by MNP: A 0.25 M aqueous solution of MNP (0.10 ml, 0.025 mmol) was added to a 0.5 M aqueous solution of **1a** (0.10 ml, 0.050 mmol). The spin adduct (A_N=10.78 G) was observed in this system.

GPC-LALLS Method Measurements of molecular weight of polymers by the GPC-LALLS method were conducted by Toray Research Center, Ltd.: GPC was carried out with a Waters model 244 gel permeation chromatograph. Light scattering was measured on a Chromatix KMX-6 low-angle light-scattering photometer at 23 °C, using a He–Ne laser (633 nm). Refractive index was monitored on a Waters R-401 differential refractometer at 23 °C.

Preparation of Polymers Method I: A powder of **1a** (dihydrochloride, 690 mg, 1.7 mmol) was allowed to stand in a desiccator with a saturated aqueous solution of NH_4Cl for 4 d at 37 °C. The tar was suspended in water (150 ml) and sonicated for 1 h. The solution was applied to a column of Diaion HP-20 (250 ml) and eluted with water. The eluate was concentrated and lyophilized to give 642 mg of **1b** (dihydrochloride) as a white powder. UV $\lambda_{max}^{H_2O}$: end absorption. IR cm^{-1}: 1640, 1540. ^{1}H-NMR δ: 0.95 (3H), 1.85 (2H), 2.45 (2H), 2.7 (5H), 3.2—3.3 (2H), 3.6 (2H), 3.9 (2H), 5.4 (2H). ^{13}C-NMR δ: 20.8 (CH_3), 35.2 (CH_2), 38.5 (CH_2), 39.2 (CH_2), 39.8 (CH_2), 42.0 (CH), 47.8 (CH_2), 49.1 (CH), 58—60 (br, CH), 69.1 (CH), 129.6 (CH), 140.0 (CH), 171.7 (C=O), 174.7 (C=O), 179.6 (C=O). *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot 2HCl\cdot 2H_2O)_n$: C, 41.48; H, 7.66; N, 16.12. Found: C, 41.86; H, 7.68; N, 16.32.

Sperabillin A (**1a**, sulfate) and various derivatives (**2a**, **5a**—**14a**) were polymerized in a similar manner to yield **1b** (sulfate), **2b** and **5b**—**14b**, each as a white powder.

1b (Sulfate): IR cm^{-1}: 1645, 1550. ^{1}H-NMR δ: 0.94 (3H), 1.60—2.00 (2H), 2.30—2.80 (2H), 2.73 (4H), 2.90—3.50 (3H), 3.60 (2H), 3.83 (1H), 3.96 (1H), 5.44 (2H). *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot H_2SO_4\cdot H_2O)_n$: C, 40.81; H, 7.08; N, 15.86; S, 6.93. Found: C, 40.93; H, 7.53; N, 15.90; S, 6.93.

2b: IR cm^{-1}: 1695, 1645, 1540. ^{1}H-NMR δ: 0.97 (3H), 1.13 (3H), 1.50—1.90 (2H), 2.30—2.90 (2H), 2.71 (4H), 3.59 (2H), 3.79 (3H), 5.47 (2H). *Anal.* Calcd for $(C_{16}H_{29}N_5O_3\cdot 2HCl\cdot H_2O)_n$: C, 44.65; H, 7.73; N, 16.27. Found: C, 45.01; H, 7.77; N, 16.38.

5b: IR cm^{-1}: 1650, 1550. ^{1}H-NMR δ: 0.95 (3H), 1.60—1.90 (2H), 2.30—2.90 (3H), 2.50 (2H), 2.65 (2H), 2.90—3.50 (2H), 3.45 (2H), 3.77 (1H), 3.94 (1H), 5.45 (2H). *Anal.* Calcd for $(C_{15}H_{26}N_4O_4\cdot HCl\cdot H_2O)_n$: C, 47.30; H, 7.67; N, 14.71. Found: C, 47.32; H, 7.37; N, 14.76.

6b: IR cm^{-1}: 1645, 1550. ^{1}H-NMR δ: 0.95 (3H), 1.50—1.90 (6H), 2.30—2.85 (2H), 2.68 (2H), 2.90—3.50 (2H), 3.03 (2H), 3.25 (2H), 3.77 (2H), 3.93 (1H), 5.44 (2H). *Anal.* Calcd for $(C_{16}H_{30}N_4O_3\cdot 2HCl\cdot 2H_2O)_n$: C, 44.14; H, 8.33; N, 12.87. Found: C, 44.14; H, 7.75; N, 12.74.

7b: IR cm^{-1}: 1640, 1550. ^{1}H-NMR δ: 0.95 (3H), 1.60—1.90 (2H), 1.91 (2H), 2.30—2.70 (2H), 2.66 (2H), 2.90—3.50 (2H), 3.04 (2H), 3.32 (2H), 3.72 (1H), 3.93 (1H), 5.43 (2H). *Anal.* Calcd for $(C_{15}H_{28}N_4O_3\cdot 2HCl\cdot 3H_2O)_n$: C, 41.00; H, 8.26; N, 12.75. Found: C, 41.10; H, 7.45; N, 12.60.

8b: IR cm^{-1}: 1640, 1550. ^{1}H-NMR δ: 0.95 (3H), 1.65—2.00 (2H), 2.30—2.70 (2H), 2.75 (2H), 2.90—3.50 (2H), 3.15 (2H), 3.55 (2H), 3.80—4.05 (2H), 5.45 (2H). *Anal.* Calcd for $(C_{14}H_{26}N_4O_3\cdot 2HCl\cdot 3.5H_2O)_n$: C, 38.71; H, 8.12; N, 12.90. Found: C, 38.70; H, 7.56; N, 12.71.

9b: IR cm^{-1}: 1650, 1550. ^{1}H-NMR δ: 0.95 (3H), 1.60—2.00 (2H), 2.30—2.70 (2H), 2.90—3.50 (2H), 3.38 (4H), 3.83 (1H), 3.95 (1H), 5.45 (2H). *Anal.* Calcd for $(C_{15}H_{28}N_6O_3\cdot 2HCl\cdot 2.5H_2O)_n$: C, 39.30; H, 7.70; N, 18.33. Found: C, 39.53; H, 7.55; N, 18.20.

10b: IR cm^{-1}: 1640, 1550. ^{1}H-NMR δ: 0.94 (3H), 1.60—2.00 (2H), 2.66 (8H), 2.95—3.50 (2H), 3.79 (5H), 3.93 (1H), 5.43 (2H). *Anal.* Calcd for $(C_{18}H_{32}N_4O_4\cdot 2HCl\cdot 1.5H_2O)_n$: C, 46.15; N, 7.96; H, 11.96. Found: C, 46.26; H, 8.25; N, 11.77.

11b: IR cm^{-1}: 1640, 1540. ^{1}H-NMR δ: 0.95 (3H), 1.60—2.00 (2H), 2.30—2.80 (2H), 2.75 (2H), 2.90—3.50 (2H), 3.50—3.70 (1H), 3.70—4.10 (4H), 5.43 (2H). *Anal.* Calcd for $(C_{15}H_{26}N_4O_5\cdot HCl\cdot 2H_2O)_n$: C, 43.43; H, 7.53; N, 13.50. Found: C, 43.43; H, 7.40; N, 13.51.

12b: IR cm^{-1}: 1645, 1560. ^{1}H-NMR δ: 0.94 (3H), 1.60—2.00 (2H), 2.30—2.80 (2H), 2.90—3.50 (2H), 3.50—4.10 (5H), 5.43 (2H). *Anal.* Calcd for $(C_{15}H_{26}N_4O_5\cdot HCl\cdot 1.5H_2O)_n$: C, 44.08; H, 7.55; N, 13.59. Found: C, 44.39; H, 7.45; N, 13.80.

13b: IR cm^{-1}: 1645, 1555. ^{1}H-NMR δ: 0.94 (3H), 1.12 (3H), 1.60—1.95 (2H), 2.30—2.85 (2H), 2.65 (2H), 2.90—3.50 (2H), 3.22 (2H), 3.78 (2H), 3.94 (1H), 5.45 (2H). *Anal.* Calcd for $(C_{14}H_{25}N_3O_3\cdot HCl\cdot 3.5H_2O)_n$: C, 43.92; H, 8.69; N, 10.97. Found: C, 43.65; H, 7.18; N, 11.13.

14b: IR cm^{-1}: 1650, 1550. ^{1}H-NMR δ: 1.55—1.95 (2H), 2.71 (4H), 2.80—3.50 (4H), 3.60 (2H), 3.70—4.00 (2H), 5.58 (2H). *Anal.* Calcd for $(C_{15}H_{25}N_5O_5\cdot 2HCl\cdot 2.5H_2O)_n$: C, 38.06; H, 6.81; N, 14.80. Found: C, 38.57; H, 6.73; N, 14.36.

Method II-A: To a solution of **1a** (sulfate, 10 g, 23 mmol) in water (10 ml) were added 30% H_2O_2 (1.0 ml, 8.8 mmol) and $FeSO_4\cdot 7H_2O$ (1.0 g, 3.6 mmol), and the mixture was shaken for 16 h at 39 °C. The reaction mixture was diluted with water to 40 ml and dialyzed with Spectrapor 6 for 24 h. The mixture in the tube was filtered to remove a precipitate, and then dialyzed again for another 24 h. The solution in the tube was concentrated and lyophilized to give 4.2 g of **1b**-1 as a white powder. *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot H_2SO_4\cdot 1.5H_2O)_n$: C, 39.99; H, 7.16; N, 15.55; S, 7.12. Found: C, 39.75; H, 7.89; N, 15.13; S, 7.20.

In a similar manner, **1b**-2 was obtained as a white powder. *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot H_2SO_4\cdot H_2O)_n$: C, 40.81; H, 7.08; N, 15.86; S, 7.26. Found: C, 40.80; H, 7.30; N, 15.62; S, 7.29.

Method II-B: To a solution of **1a** (sulfate, 10 g, 23 mmol) in water (20 ml) were added $(NH_4)_2S_2O_8$ (700 mg, 3.1 mmol) and Na_2SO_3 (700 mg, 5.6 mmol), and the mixture was shaken for 16 h at 39 °C. The reaction mixture was diluted with water to 400 ml and passed through a column of Amberlite IRA-402 (SO_4^{2-} form, 300 ml), followed by washing with water (300 ml). The passed fraction and wash fraction were combined and dialyzed with Spectrapor 6 for 2 d. The solution in the tube was concentrated and lyophilized to give 7.3 g of **1b**-3 as a white powder. *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot H_2SO_4\cdot 2.5H_2O)_n$: C, 38.45; H, 7.31; N, 14.95; S, 6.84. Found: C, 38.27; H, 7.48; N, 14.70; S, 7.91.

In a similar manner, **1b**-5 and **1b**-6 were obtained as white powders.

1b-5: *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot H_2SO_4\cdot 3H_2O)_n$: C, 37.73; H, 7.39; N, 14.67; S, 6.71. Found: C, 37.63; H, 7.15; N, 14.74; S, 7.32.

1b-6: *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot H_2SO_4\cdot 1.5H_2O)_n$: C, 39.99; H, 7.16; N, 15.55; S, 7.12. Found: C, 40.09; H, 7.07; N, 15.57; S, 7.51.

Method II-C: To a solution of **1a** (sulfate, 10 g, 23 mmol) in water (20 ml) was added 2,2′-azobis(2-amidinopropane) dichloride (800 mg, 2.9 mmol), and the mixture was shaken for 7 h at 60 °C. The reaction mixture was diluted with water to 400 ml and passed through a column of Amberlite IRA-402 (SO_4^{2-} form, 300 ml), followed by washing with water (300 ml). The passed fraction and wash fraction were combined and dialyzed with Spectrapor 3 for 2 d. The solution in the tube was concentrated and lyophilized to give 8.8 g of **1b**-4 as a white powder. *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot H_2SO_4\cdot H_2O)_n$: C, 40.87; H, 7.40; N, 15.84; S, 7.45. Found: C, 40.81; H, 7.08; N, 15.86; S, 7.26

In a similar manner, **1b**-7 was obtained as a white powder. *Anal.* Calcd for $(C_{15}H_{27}N_5O_3\cdot H_2SO_4\cdot 1.5H_2O)_n$: C, 39.99; H, 7.16; N, 15.55; S, 7.12. Found: C, 39.97; H, 7.11; N, 15.40; S, 7.37.

Effects of Polymers on the Proliferation of HUVE Cells HUVE cells were kindly donated by Dr. Yamane (Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan). HUVE cells were maintained in GIT medium (Nihon Pharmaceutical Co., Ltd., Osaka, Japan) supplemented with 2.5% heat-inactivated fetal calf serum and 2 ng/ml bFGF (gift from Dr. Igarashi, Takeda Chemical Industries, Ltd., Japan) in a two-gas incubator containing 5% CO_2 and 7% O_2 at 37 °C.

HUVE cells were seeded onto 96-well microtiter plates on day 0 (2×10^3 cells/well). On day 1, bFGF (2 ng/ml) and various concentrations of compounds were added into the medium. On day 4, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; 1 mg/ml) was added after removal of the cultured medium. After 4-h incubation, sodium dodecyl sulfate (SDS) was added and cell proliferation was measured by the increase in absorbance at 590 nm.

Effects of Polymers on the Survival Time of the B16 Melanoma Bearing Mice C57BL/6 mice (Japan Charles River, Osaka, Japan) were inoculated intraperitoneally (i.p.) with 0.2 ml of 1:9 tumor homogenates of B16 melanoma in 0.9% NaCl solution. Groups of 5 mice for each dosage were administered with the drug solutions (0.2 ml/mouse) i.p. daily for 1 to 9 consecutive days starting 24 h after tumor transplantation. The median survival time was calculated, and the anti-tumor activity of drugs was evaluated in terms of the percentage of increase of life span (% ILS = (treated × 100/control) − 100).

Acknowledgments We are grateful to Dr. H. Okazaki for his encouragement throughout this work and to Dr. S. Chiba for the acute toxicity studies. Thanks are also due to Mr. Y. Nohara for his skillful assistance.

References

1) N. Katayama, Y. Nozaki, S. Tsubotani, M. Kondo, S. Harada, and H. Ono, *J. Antibiot.*, **45**, 10 (1992).

2) T. Hida, S. Tsubotani, Y. Funabashi, H. Ono, and S. Harada, *Bull. Chem. Soc. Jpn.*, **66**, 863 (1993).
3) T. Hida, S. Tsubotani, N. Katayama, Y. Funabashi, H. Natsugari, and S. Harada, *J. Antibiot.*, in press.
4) E. G. Janzen "Free Radicals in Biology," Vol. 4, ed. by W. A. Pryor, Academic Press, New York, 1980, pp. 115—154.
5) G. R. Buettner, *Free Rad. Biol. Med.*, **3**, 259 (1987).
6) J. Folkman and M. Klagsbrun, *Science*, **235**, 442 (1987).
7) M. Takizawa, T. Hida, S. Harada, and S. Tanida, *Int. J. Immunopharmacol.*, submitted.
8) L. J. Arnold, Jr., A. Dagan, J. Gutheil, and N. O. Kaplan, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 3246 (1979).