Synthesis and Biological Activities of *Endo*-3,6-Epoxy-1,2,3,6-tetrahydrophthalimide and Its Polymers

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ABSTRACT: Photocopolymerizations of *endo*-3,6-epoxy-1,2,3,6-tetrahydrophthalimide (ETPI) with acrylic acid (AA), vinyl acetate (VAc), and maleic anhydride (MAH) were carried out in a mixed solvent of 2-butanone and acetone using 2,2-dimethoxy-2-phenylacetophenone as an initiator at 25°C. Synthesized ETPI, poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were characterized by IR and ^{1}H-NMR spectroscopies, elemental analysis, and gel permeation chromatography. The synthesized polymers have a number-average molecular weight ($\bar{M}_n$) in the range of 3500–27,400. The *in vitro* cytotoxicities of poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) against fibroblast and K-562 human leukemia cells were lower than that of monomeric ETPI at a low concentration (0.02 mg/mL). The *in vivo* antitumor activities of the polymers were evaluated by the survival time with sarcoma 180 tumor-bearing mice. The polymers showed higher antitumor activity and lower toxicity than both monomeric ETPI and 5-fluorouracil at all doses tested. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **64:** 2605–2612, 1997

INTRODUCTION

The 1 : 2 regularly alternating copolymer (DIVEMA) of divinyl ether with maleic anhydride has been extensively studied for its structure and broad biological activities. The structural feature for the hydrolyzed form of DIVEMA contains the carboxylic group as a hydrophilic part and sugar moieties such as pyran or a furan ring as a hydrophobic part. DIVEMA has been shown to possess good antitumor, antiviral, antibacterial, and antifungal activities as well as interferon-inducing ability in animal tests.[1,2] However, it also has several toxic side effects such as enlarged livers and spleens. Subsequently, many attempts have been made to obtain a polymeric drug like DIVEMA with reduced side effects.[3–8] Breslow et al.[9] prepared DIVEMA with a narrow molecular weight distribution by the photopolymerization technique in solvent with or without a photoinitiator. Several studies have been made also in this laboratory to develop polymeric antitumor agents.[10–16]

The aim of this study was to obtain new biologically active polymers from ETPI. Polymers containing ETPI were expected to show considerably high biological activities because the anionic character of the polymers formed after their hydrolysis is similar to that of DIVEMA.

In this work, the monomer, ETPI, was synthesized by the Diels–Alder reaction of maleimide and furan. Poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were prepared by the homopolymerization of ETPI and copolymerizations of ETPI and the corresponding

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monomers. The structures of ETPI, poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were identified by IR and ^{1}H-NMR spectroscopies. Average molecular weights of synthesized polymers were estimated by gel permeation chromatography.

In vitro cytotoxicities of ETPI and its polymers were evaluated with fibroblast and K-562 human leukemia cells. *In vivo* antitumor activities of ETPI and its polymers also were investigated using Balb/C mice bearing sarcoma 180 ascites tumor cells.

EXPERIMENTAL

Materials

Maleimide (Aldrich Co.), furan (Aldrich Co.), acrylic acid (AA, Aldrich Co.), vinyl acetate (VAc, Junsei Chemicals), and 2,2-dimethoxy-2-phenylacetophenone (DMP, Aldrich Co.) were purified by conventional methods. Maleic anhydride (MAH, Junsei Chemicals) was recrystallized from chloroform. Fibroblast and K-562 human leukemia cells were used for *in vitro* cytotoxicity tests. For *in vivo* tests, Balb/C mice and sarcoma 180 ascites tumor cells were purchased from the Center of Genetic Engineering, Korea Institute of Science and Technology.

Instruments

IR spectra were taken on a Jasco FTIR-5300 spectrophotometer using a KBr pellet. ^{1}H-NMR spectra were recorded on an FT-300 MHz Bruker A-3000 spectrophotometer. Average molecular weights were measured by a Water-410 gel permeation chromatography (GPC). Elemental analyses were performed by Carlo Erba Instruments Model EA1108 elemental analyzer.

Synthesis of Monomer

The synthesis of ETPI is shown in Figure 1. A solution of 3.04 mL (0.058 mol) of furan in 10 mL of diethyl ether and a solution of 4 g (0.058 mol) of maleimide in 60 mL of diethyl ether were mixed in a three-necked flask equipped with magnetic stirrer and nitrogen inlet, and the mixed solution was stirred at room temperature for 48 h. The white precipitate obtained was filtered and dried

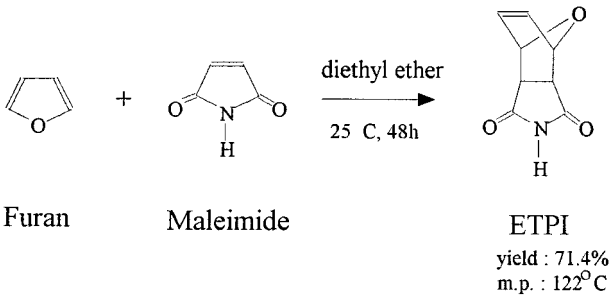

Figure 1 Synthesis procedure of ETPI.

in a vacuum dry oven (yield, 5.03 g, 71%). The melting point of ETPI was 122°C.

ANAL: Calcd: for $C_8H_7NO_5$ (%): C, 58.2%; H, 4.2%; N, 7.1%. Found: C, 58.8%; H, 4.4%; N, 8.6%.

Syntheses of Polymers

Poly(ETPI)

ETPI (0.825 g, 5 mmol) and DMP (0.024 g, 0.2 mmol) were dissolved in 24 mL of the mixed solvent of 2-butanone and acetone ($2v_0/3v_0$), introduced into a dry quartz polymerization tube. The solution was degassed twice by purging with purified N_2 gas. The tube was sealed and placed in a photochemical chamber reactor using a UV lamp (λ_{max} = 313 nm) at 25 ± 1.0°C for 12 h. The obtained polymer solution was precipitated in diethyl ether. The precipitated polymer was filtered and washed twice with 2-butanone and acetone ($2v_0/3v_0$). Then, the polymer was collected by filtration and dried in a vacuum dry oven at 30°C (conversion: 38%).

Poly(ETPI-co-AA)

ETPI (0.825 g, 5 mmol), AA (0.18 g, 2.5 mmol), and DMP (0.036 g, 0.3 mmol) were dissolved in 24 mL of the mixed solvent of 2-butanone and acetone ($2v_0/3v_0$), charged into a dry quartz polymerization tube. Poly(ETPI-*co*-AA) was obtained by using a procedure similar to that applied in the homopolymerization of ETPI except for monomer pairs (conversion: 30%).

Elemental analysis: Found: C, 51.0%; H, 5.4%; N, 4.4%.

Poly(ETPI-co-VAc)

ETPI (0.4125 g, 2.5 mmol), VAc (0.215 g, 2.5 mmol), and DMP (0.024 g, 0.2 mmol) were dissolved in 12 mL of the mixed solvent of 2-butanone

and acetone ($1v_0/1v_0$), charged into a dry quartz polymerization tube. Poly(ETPI-*co*-VAc) was obtained by the same procedures as poly(ETPI-*co*-AA) except for monomer pairs (conversion: 24%).

Elemental analysis: Found: C, 56.4%; H, 6.1%; N, 5.6%.

Poly(ETPI-co-MAH)

ETPI (0.4125 g, 2.5 mmol), MAH (0.2401 g, 2.5 mmol), and DMP (0.024 g, 0.2 mmol) were dissolved in 12 mL of the mixed solvent of 2-butanone and acetone ($1v_0/1v_0$), charged into a dry quartz polymerization tube. The obtained polymer solution was precipitated in *n*-hexane. The precipitated polymer was filtered and washed twice with 2-butanone and acetone ($3v_0/v_0$). Then, the polymer was collected by filtration and dried until a constant weight in a vacuum oven at 30°C (conversion: 18%).

Elemental analysis: Found: C, 53.5%; H, 4.0%; N, 6.1%.

Measurement of Average Molecular Weight

Number- ($\bar{M}_n$) and weight- ($\bar{M}_w$) average molecular weights of poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were estimated by GPC equipped with μ-microStyragel column using dimethylformamide as an eluent. Monodisperse polystyrene standard samples were employed for molecular weight calibration. The concentration of polymers was 0.1% or less.

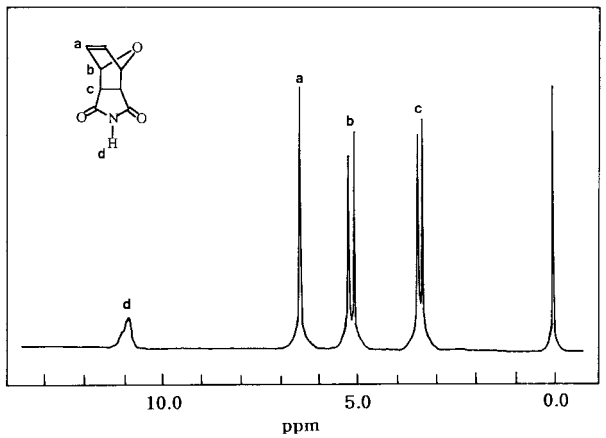

Figure 2 ^{1}H-NMR spectrum of ETPI (300 MHz, DMSO-d_6).

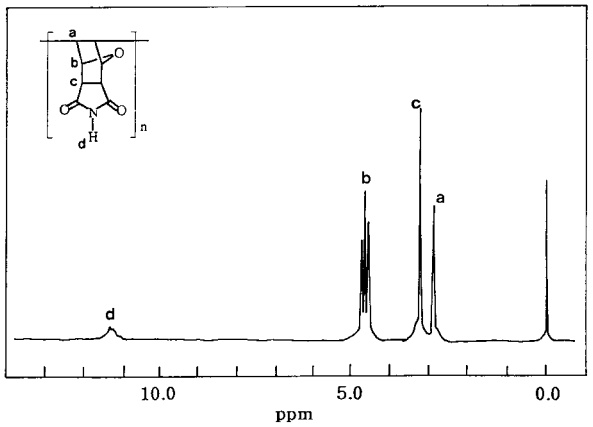

Figure 3 ^{1}H-NMR spectrum of poly(ETPI) (300 MHz, DMSO-d_6).

Analysis of Copolymers

The contents of ETPI moiety in poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were calculated from C, N, H data.

Assay of Biological Activity

Cytotoxicity of ETPI and Its Polymers

The assay was carried out in 96-well flat-bottom tissue culture plates. Fibroblast and K-562 cells were suspended at 3×10^5 cells/mL in culture media containing HFCS (heat-inactivated fetal calf serum). The cells (28 μL) and a polymer solution (172 μL) were added to each well of the plate. The assay was performed in a sextuplet. The plates were incubated at 37°C in a 5% CO_2 incubator for 24 h and then the cells were centrifuged

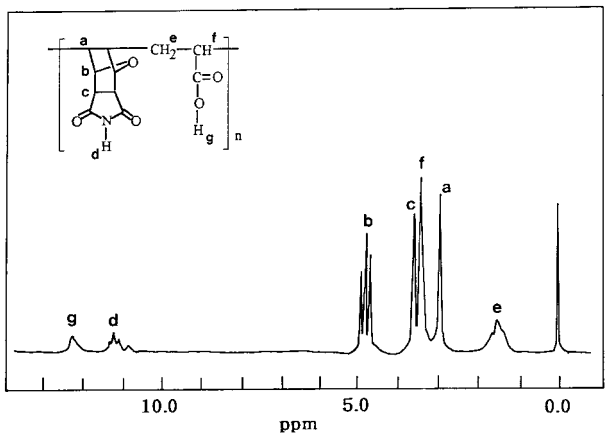

Figure 4 ^{1}H-NMR spectrum of poly(ETPI-*co*-AA) (300 MHz, DMSO-d_6).

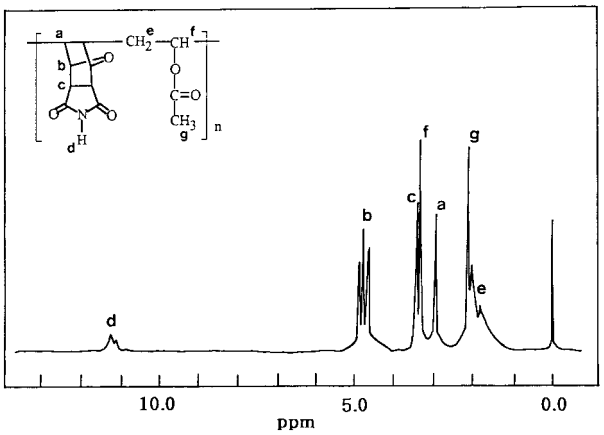

Figure 5 ^{1}H-NMR spectrum of poly(ETPI-*co*-VAc) (300 MHz, DMSO-d_6).

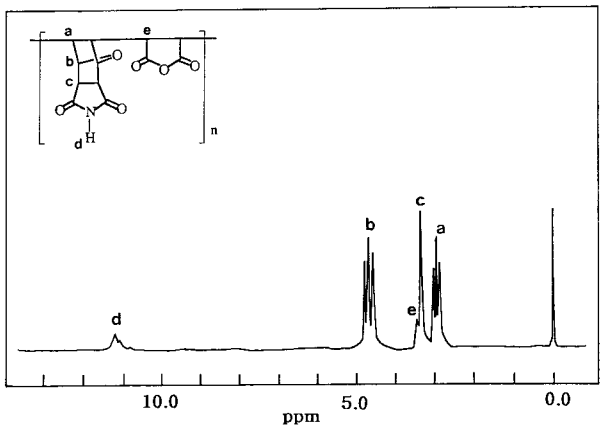

Figure 6 ^{1}H-NMR spectrum of poly(ETPI-*co*-MAH) (300 MHz, DMSO-d_6).

at 600 × g at room temperature for 5 min. After centrifugation, the precipitated cells were washed twice with cold Hanks' balanced salt solution (HBSS) and then were suspended in HBSS. Viability of the cells was determined with the trypan blue dye exclusion method. The percent cytotoxicity was calculated by the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{number of untreated cells} - \text{number of treated cells}}{\text{number of untreated cells}} \times 100$$

Antitumor Activity of ETPI and Polymers Containing ETPI

The antitumor activity of ETPI and its polymers was evaluated by survival time with sarcoma 180 ascutes tumor-bearing mice. Balb/C mice were first intraperitoneally (i.p.) implanted with sarcoma 180 cells (2×10^5). The ETPI and its polymers were administrated daily by intraperitoneal injection for 4 consecutive days. Three different doses were tested: 0.8, 80, and 800 mg/kg. For comparision, antitumor activities of free 5-FU also were tested by the same method. A control group was divided into two

Table I Solubility of ETPI and Its Polymers

	Samples				
Solvent	ETPI	Poly(ETPI)	Poly(ETPI-co-AA)	Poly(ETPI-co-VAc)	Poly(ETPI-co-MAH)
Water	○	○	○	○	○
Dimethyl-sulfoxide	○	○	○	○	○
N,*N*-Dimethylformamide	○	○	○	○	○
Methanol	○	△	○	△	○
Acetone	○	X	○	△	○
Ethyl acetate	○	X	X	X	△
Tetrahydrofuran	○	X	X	X	X
Chloroform	△	X	X	X	X
1,4-Dioxane	○	X	X	X	X
2-Butanone	△	X	X	X	X
Diethyl ether	X	X	X	X	X
Toluene	X	X	X	X	X
n-Hexane	X	X	X	X	X

○: good soluble; △: partially soluble; X: insoluble.

Table II Average Molecular Weights of Polymers Containing ETPI

	Poly(ETPI)	Poly(ETPI-co-AA)	Poly(ETPI-co-VAc)	Poly(ETPI-co-MAH)
$\bar{M}_n$	7900	3500	4400	27,400
$\bar{M}_w$	8000	9300	13,200	32,400
$\bar{M}_w/\bar{M}_n$	1.0	2.7	3.0	1.2

groups. One group was treated with sarcoma 180 cells along with the same volume of saline and the other group was treated with only sarcoma 180 cells. The *in vivo* antitumor activity was evaluated by comparing the mean survival time of treated groups (T) with that of control groups (C) and was expressed by percentage value of T/C (% T/C). Each group consisted of 10 animals.

RESULTS AND DISCUSSION

Identification of ETPI and Polymers Containing ETPI

The IR spectrum of ETPI showed characteristic absorption peaks at 3210 (—NH), 2790 (—CH), 1716 (—C=O), 1564 (—CH=CH—), and 1188 cm^{-1} (—C—O—C—). In Figure 2, the ^{1}H-NMR spectrum of ETPI shows the NH of the imide ring at 11.2 ppm, olefinic protons at 6.5 ppm, methine protons of the cyclic ether ring at 5.2 ppm, and methine protons of the imide ring at 3.4 ppm.

The absorption peak assignable to the C=C bond of monomeric ETPI disappeared at 1564 cm^{-1} in the IR spectrum of poly(ETPI). In Figure 3, the absorption peak assignable to the olefinic protons of monomeric ETPI was not observed at 6.5 ppm. Methine protons of the polymer backbone were observed at 2.9 ppm. The NH of the imide ring and the methine protons of the cyclic ether ring were observed at 11.2 and 4.6 ppm, respectively. The peaks due to methine protons of the imide ring are the same as those of monomeric ETPI.

The ^{1}H-NMR spectrum of poly(ETPI-*co*-AA) is shown in Figure 4. The absorption peaks due to protons of ETPI moiety in poly(ETPI-*co*-AA) were assigned to the same as those of poly(ETPI). A proton of carboxylic acid, a methine proton, and methylene protons of the AA moiety in poly(ETPI-*co*-AA) were observed at 12.3, 3.3, and 1.7 ppm, respectively. The peaks assigned to the olefinic proton of ETPI and the AA moiety at 6.5 and 6.4 ppm disappeared in Figure 4.

Figure 5 shows the ^{1}H-NMR spectrum of poly(ETPI-*co*-VAc). The absorption peaks due to protons of ETPI moiety in poly(ETPI-*co*-VAc) were assigned to the same as those of poly(ETPI). The peaks at 3.3, 2.1, and 1.8 ppm were assigned to methine, methyl, and methylene protons of the VAc unit, respectively. The peaks due to vinyl protons in ETPI and VAc were not observed in Figure 5.

Figure 6 shows the ^{1}H-NMR spectrum of poly(ETPI-*co*-MAH). The absorption peaks due to protons of ETPI moiety in poly(ETPI-*co*-MAH) were assigned to the same as those of poly(ETPI). The peak at 3.4 ppm was assigned to methine protons of MAH unit. The peaks due to vinyl protons in ETPI and MAH were not observed in Figure 6.

Table III Content of Polymers Containing ETPI

Element or ETPI Fraction	Composition (%)				
	ETPI	Poly(ETPI)	Poly(ETPI-co-AA)	Poly(ETPI-co-VAc)	Poly(ETPI-co-MAH)
C	58.8	57.6	51.0	56.4	53.5
H	4.4	5.7	5.4	6.1	4.0
N	8.6	6.9	4.4	5.6	6.1
ETPI fraction in copolymer (mol %)	—	—	35.4	52.1	64.0

Table IV In Vitro Cytotoxicities of ETPI and Its Polymers Against Fibroblast Cells

Concentration (mg/mL)	Cytotoxicity (%)				
	ETPI	Poly(ETPI)	Poly(ETPI-co-AA)	Poly(ETPI-co-VAc)	Poly(ETPI-co-MAH)
5.0	98.7	93.4	100	—	98.7
1.0	98.7	86.8	98.7	97.4	76.3
0.1	80.3	33.3	81.6	80.3	53.5
0.02	78.9	22.4	44.7	56.6	35.1

Solubility, Average Molecular Weights, and Composition of Polymers

Solubilities of ETPI, poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) are listed in Table I. ETPI was soluble in water, DMF, DMSO, and methanol, but insoluble in diethyl ether and *n*-hexane. Poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were soluble in water, DMF, and DMSO, but insoluble in THF, diethyl ether, and *n*-hexane.

The average molecular weights of synthesized polymers are listed in Table II. M_n values of poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were 7900, 3500, 4400, and 27,400, respectively. The detailed molecular weights and polydispersity indices are listed in Table II.

The contents of the ETPI moiety in copolymers calculated from the C, N, H data by elemental analysis are listed in Table III. ETPI contents in poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) are 35.4, 52.1, and 64.0%, respectively.

In Vitro Cytotoxicity of ETPI and Its Polymers

Table IV shows the dependence of the concentration on the *in vitro* cytotoxicity of ETPI and its polymers against fibroblast cells. As shown in the table, the cytotoxicity of monomeric ETPI were higher than those of the polymers at a concentration of 0.02 mg/mL. ETPI showed similar activity as to that of its polymers at a concentration of 5 mg/mL.

The dependence of the concentration on the *in vitro* cytotoxicity of ETPI and its polymers against K-562 cells are shown in Table V. The cytotoxicity of monomeric ETPI were higher than those of the polymers at concentrations of 0.02 and 0.1 mg/mL. The cytotoxicity of ETPI was higher than that of poly(ETPI) but lower than those of poly(ETPI-*co*-AA) and poly(ETPI-*co*-MAH) at a concentration of 5 mg/mL.

In Vivo Antitumor Activity of ETPI and Its Polymers

The results of *in vivo* antitumor activities of ETPI and its polymers against sarcoma 180 tumor cells are listed in Table VI. In this table, the antitumor activity of 5-FU is also shown for comparision.

The life spans of mice treated with 5-FU and ETPI were longer than those of the control group at doses of 0.8 and 80 mg/kg, but were shorter than those of the control group at a dose of 800 mg/kg (T/C = 39% for 5-FU and T/C = 17% for ETPI). 5-FU and ETPI showed an efficient antitumor activity at low doses, but they appeared to have undesirable toxicity at a high dose.

The life spans of mice treated with the poly-

Table V In Vitro Cytotoxicities of ETPI and Its Polymers Against K-562 Human Leukemia Cells

Concentration (mg/mL)	Cytotoxicity (%)				
	ETPI	Poly(ETPI)	Poly(ETPI-co-AA)	Poly(ETPI-co-VAc)	Poly(ETPI-co-MAH)
5.0	75.9	60.0	88.2	—	93.3
1.0	60.4	29.3	65.1	53.7	85.0
0.1	47.6	1.3	20.9	4.4	33.8
0.02	47.5	0.0	11.4	0.0	23.4

Table VI In Vivo Antitumor Activities of ETPI and Its Polymers Against Sarcoma 180 Tumor Cells

Samples	Dose (mg/kg)	Survival times (day)	T/C (%)
Control	—	14.7 ± 2.3	100
	Saline	15.7 ± 0.5	100
5-FU	800.0	5.9 ± 0.3	39
	80.0	21.3 ± 1.3	140
	0.8	20.3 ± 1.8	134
ETPI	800.0	2.6 ± 0.4	17
	80.0	27.1 ± 2.5	178
	0.8	21.3 ± 6.2	140
Poly(ETPI)	800.0	33.3 ± 19.9	219
	80.0	27.6 ± 5.1	182
	0.8	22.7 ± 1.9	149
Poly(ETPI-co-AA)	800.0	24.2 ± 2.3	170
	80.0	25.2 ± 5.4	166
	0.8	23.8 ± 3.2	165
Poly(ETPI-co-VAC)	800.0	25.9 ± 2.0	159
	80.0	25.3 ± 4.8	166
	0.8	25.1 ± 1.2	157
Poly(ETPI-co-MAH)	800.0	18.0 ± 7.9	118
	80.0	22.3 ± 5.9	147
	0.8	19.8 ± 7.9	130

mers were longer than those treated with 5-FU and ETPI at a high dose. At a dose of 800 mg/kg, poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) increased the life span by 119, 70, 59, and 18%, respectively. At a dose of 0.8 mg/kg, poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) increased the life span by 49, 65, 57, and 30%, respectively. At a dose of 80 mg/kg, the *in vivo* antitumor activity of ETPI and its polymers increased in the following order: 5-FU < poly(ETPI-*co*-MAH) < poly(ETPI-*co*-VAc) < ETPI < poly(ETPI). From such a result, it was found that the antitumor activities of the polymers were greater than those of 5-FU and their toxicities were lower than those of 5-FU and ETPI.

CONCLUSIONS

The monomer, *endo*-3,6-epoxy-1,2,3,6-tetrahydrophthalimide (ETPI), was prepared by the Diels–Alder reaction of maleimide and furan. Poly(ETPI) was prepared by the photopolymerization of ETPI using DMP as an initiator at 25°C. Poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH)] were prepared by the photocopolymerizations of ETPI with AA, VAc, and MAH using DMP as an initiator at 25°C. Synthesized ETPI, poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were characterized by IR and ^{1}H-NMR spectroscopies, elemental analysis, and gel permeation chromatography. The contents of the ETPI unit in poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) were 35.4, 52.1, and 64.0%, respectively. The number-average molecular weights of synthesized polymers were in the range of 3500–27,400.

The *in vitro* cytotoxicities of poly(ETPI), poly(ETPI-*co*-AA), poly(ETPI-*co*-VAc), and poly(ETPI-*co*-MAH) against fibroblast and K-562 human leukemia cells were lower than those of monomeric ETPI at a low concentration (0.02 mg/mL). The *in vivo* antitumor activities of the polymers were higher than those of 5-FU and the toxicities of the polymers were lower than those of 5-FU and ETPI.

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