with petroleum ether to give 0.6 g (29%) of a mixture of **6a** and **7a**. Mixtures of **6b** and **7b**, as well as **6c** and its geometric isomer, were prepared in the same manner in respective yields of 42 and 38%.

Rapid Preparative Chromatographic Purification of 6 and 7. A mixture of 576 mg of **6a** and **7a** was dissolved in 5 mL of benzene–triethylamine (12:1, v/v) and chromatographed on 60 g of silica gel (Brinkmann, 0.040–0.063 mm). Elution with 3 L of the above solvent (75 mL/min), followed by concentration of the eluent, gave a white solid, which was suspended in benzene and filtered to give 274 mg of **7a**: mp 132–134 °C. Further elution of the column with 1 L of benzene–triethylamine–methanol (12:1:3, v/v), at 75 mL/min, followed by workup as before, gave 272 mg of **6a**, mp 144–149 °C. Anal. ($C_{26}H_{29}NO_2$) C, H, N for each isomer.

Similarly, 1 g of a mixture of **6b** and **7b** was chromatographed on 60 g of silica gel using benzene–triethylamine (15:1, v/v), followed by benzene–acetone–triethylamine (15:10:1, v/v) as eluting solvents. This gave 160 mg of **7b**, which was recrystallized from benzene–hexane: mp 153.5–155 °C; the more polar isomer, **6b** (396 mg), was recrystallized from benzene, mp 166–167.5 °C. Anal. ($C_{26}H_{29}NO_2$) C, H, N for each isomer.

Chromatography of 1.31 g of impure **6c** using benzene–triethylamine (10:1, v/v) as eluting solvent furnished 1.1 g of a white solid, which was crystallized from benzene–hexane to give 388 mg of **6c** as white crystals, mp 160–162 °C. Anal. ($C_{26}H_{29}NO_2$) C, H, N.

Receptor Binding Assay. The [^{3}H]estradiol (58 Ci/mmol) used in this assay was obtained from Amersham Corp.; radiochemical purity was checked by TLC. Uteri from Sprague–Dawley rats (200–250 g) were homogenized (1 uterus/2 mL) in ice-cold 10 mM Tris buffer, pH 7.4, which contained 1.5 mM EDTA and 3 mM sodium azide (TEA buffer). The homogenate was centrifuged at 100000*g* for 1 h at 4 °C. Incubation mixtures contained 200-μL aliquots of the supernatant, 10 μL of a solution of 1.1×10^{-7} M [^{3}H]estradiol in dimethylacetamide, and 10 μL of unlabeled competitor in 1:1 dimethylacetamide–TEA buffer. Ten concentrations of competitor were used ranging from 1×10^{-9} to 5×10^{-5} M. Control incubations contained 10 μL of solvent alone, and nonspecific binding was determined in similarly prepared incubations which contained 1×10^{-5} M estradiol. Incubations were performed in triplicate, in 5-mL polypropylene centrifuge tubes, at 2–4 °C for 4 h. Then a suspension of 400 μL of dextran-coated charcoal [0.1% dextran (Sigma no. D-1390), 1% acid-washed Norit A in TEA buffer] was added, and the incubation was continued for 15 min at 2–4 °C. Tubes were then centrifuged at 1000*g* for 10 min, and 400-μL aliquots were dissolved in 5 mL of Scintiverse (Fisher). Bound [^{3}H]estradiol was determined by liquid scintillation spectrometry. Quench corrections were made by the external standard method.

Uterotropic Assay for Estrogenic Activity. Immature Wistar female rats (21 days old, 25–35 g) were obtained from Harlan Sprague–Dawley, Inc., Indianapolis, IN. They were divided randomly into groups of at least six animals. To 0.1-mL aliquots of fresh solutions of estradiol benzoate (0.25 mg/mL) and of each of the hydroxytamoxifens (25 mg/mL) in dimethylacetamide was added 5 mL of peanut oil. The resulting solutions (0.1 mL) were administered sc once daily for 3 days. Control animals received vehicle alone. On the 4th day, the animals were killed by decapitation. The uteri were dissected, and fat and connective tissue were removed. After blotting lightly to remove intraluminal fluid, uteri were weighed to the nearest 0.1 mg. Body weights were also recorded.

Uterotropic Assay for Antiestrogenic Activity. This was carried out exactly as described above, except that animals receiving the test compounds also received 0.5 μg/0.1 mL of estradiol benzoate, administered separately at different injection sites. One group of control animals received 0.5 μg of estradiol benzoate and vehicle; the other received two injections of vehicle.

Stability of Peanut Oil Solutions of the Hydroxytamoxifens. Several of the solutions prepared as described above were analyzed periodically by ultraviolet spectrometry (300–400 nm) to make sure the triarylethylenes were not being adsorbed on the glass surfaces of the containers in which they were kept. No changes in absorption intensities were seen. For example, those of **6a** (λ_{max} 306 nm, log ϵ 3.96) and **7a** (λ_{max} 305 nm, log ϵ 3.97) did not vary over a period of 7 days.

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Carboxyimamidate, a Low-Molecular-Weight Polyelectrolyte with Antitumor Properties and Low Toxicity

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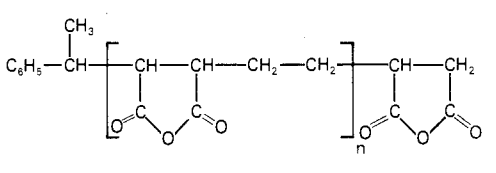
A new polyelectrolyte was synthesized and evaluated for antitumor activity. The product is a derivative of ethylene/maleic anhydride copolymer of low molecular weight ($M_n \approx 1100$). The anhydride groups were first converted to the half-amide, half-ammonium salt by reaction with ammonia. A percentage (14–25 wt %) of these groups was further converted to the imide by heating. The product, carboxyimamidate (Carbethimer, N-137) inhibited the growth of a number of solid tumors in vivo. Sensitive tumor models included Lewis lung carcinoma, Madison 109 lung carcinoma, M5076 ovarian tumor, colon carcinoma 26, B16 melanoma, and P815 mastocytoma. Activity was dose related between nontoxic dose levels of 300 and 2000 mg/kg ip.

Strong acid polyanionic polymers of either natural or synthetic origin, such as polysulfates, polysulfonates, and polyphosphates, have been reported to be growth inhibitors of transplanted tumors in mice.[1-3] Similar inhibition of tumor growth has been demonstrated for weak acid carboxy-containing polyelectrolytes derived from polyacrylic acid or ethylene/maleic anhydride copolymer (EMA) 1.[4] These studies compared acute toxicity and inhibition activity of several polymer series over a broad molecular



1

weight range (2000–100 000). The effects of total charge density, charge distribution, and carboxylic acid strength

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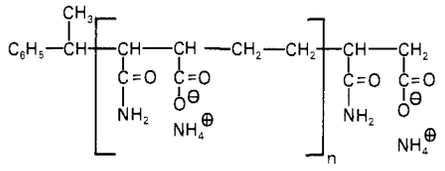
Table I. Preparation of Ethylene/Maleic Anhydride Copolymer (1)

run no.	maleic anhydride conversion, %	product (1) recovery, g	anal. H	anal. C	sp viscosity (1% DMF) at 25 °C[b]	equivalent wt[c]
A	91.5	185	4.98	57.34	0.064	138.5
B	98.6	227	5.03	58.11	0.068	139.9
C	98.3	223	5.10	58.15	0.063	140.0
D	98.3	219	5.04	58.24	0.066	139.1
E	97.9	223	5.10	57.59	0.063	140.6
F	97.9	225	5.12	58.14	0.061	141.5
G	98.6	224	5.11	58.05	0.064	140.1

[a] Average of two determinations. [b] Substantially in accordance with ASTM D-2515-74 procedure, Ostwald viscometer. [c] Weight in grams containing 1 mol unit anhydride determined by potentiometric pH titration of aqueous solution with standard NaOH.

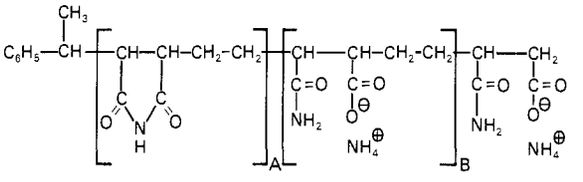
(pK_a) were explored. In related studies, certain polyelectrolytes were found to act as tumor and viral inhibitors[5] both in vitro and in vivo. Biological activities have also been reported for divinyl ether/maleic anhydride copolymer (Divema or pyran)[6] and other polycarboxylates capable of ionizing at biological pH ranges.[7,8] Hodnett, Breslow, and Ottenbrite cited biological activities for charged polymers, including tumor inhibition, antiviral activity, interferon stimulation, and stimulation of immune response through macrophage activation.[6-9] Biological response to a broad range of charged polymer molecules has been reviewed by Regelson.[10,11]

Early studies indicated tumor inhibitory activity for ammoniated derivatives of 1.[4] These half-amide, half-ammonium salts 2 were found to have activity proportional to molecular weight over a range of 2000–30 000. They exhibited low acute toxicity. Low-molecular-weight products were the least toxic. However, chronic toxicity was observed in dogs[12] after 35–210 days of treatment with 50 and 100 (mg/kg)/day dosing. The lowest molecular weight polymer evaluated did not show significant activity against sarcoma 180 in Swiss mice.[13]



2

It has now been established that antitumor activity of this family of low-molecular-weight derivatives derived from ethylene/maleic anhydride copolymers can be enhanced by ring closure of a portion of the vicinal amidated succinate groups of 2. We report herein the synthesis and biological activity of a new derivative, carboxyimamidate (Carbethimer,[14] 3), with 14–25 wt % of repeating units containing succinimidyl groups.



3

Chemistry. A low-molecular-weight copolymer of ethylene and maleic anhydride (1), consisting of alternating succinic anhydride and ethylene groups in an equimolar ratio, was prepared using a modification of methods described by Johnson[15] and Fields.[16] Table I summarizes the results of seven polymerizations. Conversion of maleic anhydride monomer to polymer averaged 97.3 ± 2.8%. The average specific viscosity (1% solution in dimethylformamide) was 0.064 ± 0.002. Ethylbenzene served as a chain-transfer solvent, producing a product with a number average molecular weight (M_n) of 852 as measured by vapor pressure osmometry. NMR studies, unlike osmometric methods, were not influenced by traces of small molecules and indicated a molecular weight of 1400. This corresponds to approximately nine repeating units with identified α-phenylethyl radical initiator and with succinyl terminator groups.

The anhydride groups of 1 were converted to half-amide, half-ammonium salt 2 by reaction of the polymer in acetone solution with a liquid ammonia–acetone mixture. Product 2 contained 13.40% nitrogen as measured by a Perkin-Elmer Model 240 elemental analyzer. Excluding mechanical loss, yields were essentially quantitative. IR assays indicated the dominance of primary amide and ionized carboxyl groups. No anhydride, undissociated carboxy, or imide groups were detectable. Conversion to the partial imide via removal of ammonia and water was accomplished by heating a xylene slurry of 2 under reflux for 20–30 min while maintaining a flow of ammonia through the reaction vessel. The product was recovered via filtration and vacuum drying. The composition of the mixture was dependent upon molecular weight distribution of the intermediate 1, the total imide content of each molecular weight species, and the random placement of

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Table II. Preparation of Imide Derivatives of Ammoniated Ethylene/Maleic Anhydride Copolymer

time of xylene reflux at sample removal, h	pH-1[a]	pH-2[b]	imide/amide ratio[c]	imide,[d] %
0.25	6.26	7.20	0.723	13.8
0.50	6.00	5.86	0.858	18.5
0.75	5.91	5.76	0.999	23.3
1.0	5.78	5.53	1.113	27.4
1.5	5.43	5.43	1.398	36.8
2.0	5.27	5.76	1.501	40.2
3.0	4.94	5.72	1.821	50.0

[a] pH of 2% aqueous solution before pH adjustment. [b] pH of 2% aqueous solution of the product prepared by adjusting to pH 10 and freeze-drying. [c] Ratio of IR band absorbance intensity at wave number 1715 cm^{-1}/1670 cm^{-1}. [d] Obtained from standard curve of composition vs. imide/amide ratio (I/A).

imide groups therein. The pHs of 2% aqueous solutions of **3** were measured before and after the ammonium hydroxide treatment. IR absorption data were obtained on all samples to establish imide to amide ratios and the percent imide content. Results are recorded in Table II. Product **3**, carboxyimamidate, was defined as that having 14–25% of repeating polymer units coverted to succinimidyl groups.

Biological Results and Discussion

Preliminary tumor inhibition properties of **3** were reported by Falk for a rat model involving a methylcholanthrene-induced carcinoma of the bladder (FBCa) in Fisher 344 rats.[17-20] Falk also has reported results of early low-dose phase I clinical studies confirming the low toxicity of the drug.[21]

Performance in other animal tumor models was sought. Evaluation of antineoplastic activity of **3** has now been extended to 14 additional tumor models to investigate potential growth inhibition and increase of life span (ILS). The transplanted tumor systems studied included (1) Lewis lung carcinoma, implanted subcutaneously (sc); (2) P388 lymphocytic leukemia, implanted intraperitoneally (ip); (3) B-16 melanoma, implanted ip or sc, (4) Walker carcinosarcoma 256, implanted intramuscularly (im); (5) Madison 109 lung carcinoma, implanted sc or im; (6) colon carcinoma 26, implanted sc or ip; (7) P-815 mastocytoma, implanted sc; (8) EMT6 mammary carcinoma implanted sc; (9) M5076 ovarian tumor, implanted sc or ip; (10) colon carcinoma 38, implanted sc; (11) L5178Y leukemia, implanted ip; (12) Gardner lymphosarcoma, implanted ip; (13) ADJ-PC6 plasmacytoma, implanted ip; and (14) Nettesheim squamous lung carcinoma, implanted sc.

Tumors were implanted in appropriate syngeneic strains of animals. We administered drug ip to groups of 10 animals each using various treatment regimens and dosages ranging from 8 to 2500 mg/kg. Therapeutic activity was judged by the increase in mean survival time (excluding long-time survivors) relative to untreated controls (ILS), the inhibition of sc or im tumor growth relative to untreated controls (T/C), and the proportion of animals without tumor at set time points. To determine T/C, tumors in control and experimental groups were measured in perpendicular diameters with vernier calipers when large tumors were evident in all untreated controls. Each experiment included three sets of untreated control animals (10/group) to give the experimental mean control tumor size and animal life span.

Representative results for studies with Lewis lung carcinoma are summarized in Table III. The LD_{50} of **3** in mice was about 2500 (mg/kg)/injection when given on a daily or intermittent treatment regimen. Few treatment-related deaths (less than 5%) were noted in many experiments at 2000 (mg/kg)/dose (data not included). Reproducible tumor growth inhibitory activity for **3** was noted for a wide range of doses and treatment regimens. A repeated regimen, i.e., daily on days 1through 5, has given the best results. Extended treatment beginning on day 1 and continuing until day 8, 10, or 30 did not result in activity greater than that observed when **3** was given on days 1 through 5.

The data in Table III show a clear dose–response between 60 and 2500 mg/kg. At doses over 400 mg/kg, a complete tumor growth inhibition was observed on day 14 in 47 to 60% of treated mice. Tumor growth was inhibited by 80 to 90% relative to controls, and there were 20% long-term tumor-free survivors at doses of 1000 to 2000 mg/kg. There was a minimal increase in survival time of those mice which were not cured by treatment with **3**.

The response of other tumors to a maximally tolerated dose of **3** is summarized in Table IV. Among the ip-implanted tumor models, **3** significantly prolonged the life span of mice bearing colon carcinoma 26 and M5076 ovarian tumor. There was a slight but statistically significant prolongation of life span of mice bearing P388 leukemia as well. Among nine tumors implanted sc or im, **3** failed to produce statistically significant tumor growth inhibition in four tumors. Tumor growth was significantly inhibited by 60 to 89% in five tumor models. The M5076 ovarian tumor was as sensitive as Lewis lung carcinoma to the growth inhibitory effect of **3**. Ten to thirty percent long-term, tumor-free survivors were obtained in mice bearing sc Madison 109 lung carcinoma, colon carcinoma 26, and M5076 ovarian tumor.

The observation that **3** produced strong tumor growth inhibition with occasional cures but, at most, a marginal prolongation of life span indicates that the drug has a transient effect on tumor growth. Tumors in most of the treated animals eventually grew and killed the animals. Continuous treatment with **3** in mice bearing sc Lewis lung did not prolong tumor growth delay.

In summary, the composition and synthesis of carboxyimamidate **3** have been detailed. It is a low-molecular-weight polymer composed of imide and amic acid salt groups that are separated by ethylene units. Earlier reports have indicated potential antitumor activity for the product.[17-20] The current report identifies several animal tumor model systems wherein a positive antitumor dose–response is observed. Tumor growth inhibitory activity against Lewis lung carcinoma was observed over a range of 60 to 2500 mg/kg. Several other murine solid tumor models were employed to demonstrate the inhibitory activity of **3**. A minimal increase in life span was noted in these studies. The exceptionally low toxicity of this new drug (LD_{50} = 2500 mg/kg) has permitted antitumor evaluation at substantially higher dose levels than possible

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Table III. Activity of Carboxyimamidate (3) in the Subcutaneous Lewis Lung Carcinoma Model[a]

dose of 3, (mg/kg)/day (for 5-10 days)[b]	N[c]	combined results of 9 to 14 trials (mean ± SE) proportion NP day 14[d]	T/C day 14[e]	% ILS excluding cures[f]	proportion of survivors (day 60)
2400-2512	11	0.60 ± 0.089	0.10 ± 0.039	−28 ± 10	0.13 ± 0.05
1200-1500	11	0.48 ± 0.09	0.16 ± 0.04	24 ± 5	0.21 ± 0.08
900-1000	10	0.47 ± 0.04	0.17 ± 0.03	19 ± 3	0.21 ± 0.07
500-600	13	0.29 ± 0.06	0.32 ± 0.05	15 ± 4	0.12 ± 0.04
300-400	10	0.27 ± 0.07	0.40 ± 0.05	17 ± 5	0.09 ± 0.04
158-251	14	0.14 ± 0.04	0.58 ± 0.08	15 ± 4	0.04 ± 0.03
100-125	9	0.10 ± 0.04	0.62 ± 0.09	12 ± 4	0.04 ± 0.03
62-75	10	0.08 ± 0.02	0.59 ± 0.06	12 ± 2	0.03 ± 0.02

[a] Combined results of several experiments. See footnote *a*, Table I. [b] Results compiled from dose-responses in nine independent experiments wherein carboxyimamidate was given daily on days 1-5, 1-8, or 1-10 either ip or subcutaneously. Dose levels indicated as ranges due to different levels used in different experiments, i.e., 2512, 1585, 100 (mg/kg)/day; 2400, 1200, 75 (mg/kg)/day; or 2500, 1500, 70 (mg/kg)/day. [c] *N* = number of experiments in the mean. [d] Average proportion of total treated mice without palpable tumors on day 14. [e] T/C = ratio of mean tumor volume of carboxyimamidate-treated groups to that of the mean of total untreated controls. In each experiment, the untreated controls consisted of either 3 or 4 groups of 10 mice. [f] % ILS = percent increase of mean life span of treated groups relative to the mean of the untreated control groups. [g] *N* for NP = 10 and for T/C = 9 for the 2400-2512 dose range. All others as noted.

Table IV. Activity of Carboxyimamidate (3) in Various Tumor Models

tumor system	treatment schedule	tumor inhibn at ≤MTD[a]	prolongation of life span at ≤MTD[b]
ip P388 leukemia	1-5		16% ILS ($p < 0.01$)[c]
	1-9		12% ILS ($p < 0.01$)
	1		4% ILS (ns)
ip L5178Y leukemia	1-5		5% ILS (ns)
ip Gardner lymphosarcoma	1-5		4% ILS (ns)
ip ADJ-PC6 plasmacytoma	1-5		24% ILS (ns)
ip B16 melanoma	1-9		21% ILS (ns)
	1		11% ILS (ns)
ip M5076 ovarian tumor	1-5		40% ILS ($p < 0.01$)
ip colon carcinoma 26	1, 5		48% ILS ($p < 0.01$)
	1, 5		31% ILS (ns)
	1-5		75% ILS ($p < 0.01$)
sc EMT6 mammary carcinoma	2, 9, 16	14% TWI (ns)	13% ILS (ns)
im Walker carcinosarcoma	1-6	21% TWI (ns)	3% ILS (ns)
sc Nettesheim lung carcinoma	1-5	39% TWI (ns)	6% ILS (ns)[c]
sc colon carcinoma 38	1-5	44% TWI (ns)	14% ILS (ns)
sc P815 mastocytoma	1-5	60% TWI ($p < 0.01$)	23% ILS (ns)
	1	53% TWI ($p < 0.01$)	6% ILS (ns)
sc Madison 109 lung carcinoma	1-5	67% TWI ($p < 0.01$)	27% [3/10] (ns)
im Madison 109 lung carcinoma	1-5	78% TWI ($p < 0.01$)	23% ILS (ns)
	1, 5, 9	50% TWI ($p < 0.01$)	20% ILS ($p < 0.01$)
sc colon carcinoma 26	1-5	70% TWI ($p < 0.01$)	22% [1/10] (ns)
sc B16 melanoma	1-5	74% TWI ($p < 0.01$)	7% ILS (ns)
sc M5076 ovarian tumor	1-5	89% TWI ($p < 0.01$)	20% [2/10] ($p < 0.01$)

[a] Tumor weight inhibition (TWI) relative to untreated controls produced at a dose of carboxyimamidate (3) which was less than or equal to the maximally tolerated dose (MTD) on the schedule of administration used. Tumors were measured when tumors in untreated controls were approximately 1 g in mass. [b] Increase in mean life span (ILS) relative to untreated controls. Fractions in brackets are the proportion of long-term, tumor-free survivors (generally at 60 days after tumor implantation). [c] Significance was determined by comparing treated with untreated control groups by Student's *t* test.

with previously reported derivatives of maleic copolymers and other polycarboxylates.[4,6,7]

Experimental Section

Molecular Weight Determination. Molecular weights were determined on 1 using product vacuum dried for 24 h at 100 °C. The number-average molecular weight (M_n) was studied by vapor-pressure osmometry in dimethylformamide (DMF) solution at 120 °C with a Knauer VP osmometer. It was also determined by proton NMR techniques in order to establish a value independent of small molecules that can markedly alter osmometry results.[22] NMR studies also indicated the α-phenylethyl radical to be the initiating group for the polymer chain. The weight-average molecular weight (M_w) of 1 was determined using low-angle laser light scattering of polymer dissolved in DMF. Values were obtained with a Chromatix KMS-6 instrument.

Specific viscosities reported in Table I were measured at 25 °C for a 1% solution of polymer in DMF substantially as described in ASTM D-2515-74 with an Ostwald viscometer.

Quantification of Functional Groups. Qualitative and quantitative identification of the various functional groupings for polymeric derivative examples was determined by infrared (IR) analysis using a Beckman IR-12 spectrophotometer. Sample preparation, absorbance frequency assignments, and procedures to determine the ratio of imide groups to amide groupings followed procedures set forth by Bellamy[23] or Cross.[24] Sample preparation utilized pressed disks of 2 mg of polymer per 250 mg of dry KBr composition with 70 mg of mixed polymer/KBr per disk. Absorbing band positions are quoted in units of wave number, which are expressed in reciprocal centimeters, usually styled as band frequencies.

For quantitative estimation of the imide/amide content of a polymer, containing in addition varying amounts of ammonium

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carboxylate function, a ratio of the absorbancy intensity of the major imide band at 1715 cm^{-1} to the major primary amide band at 1670 cm^{-1} was determined. We determined imide content by comparing the measured ratio of imide/amide to a standard curve of percent imide vs. imide/amide absorbance ratio. This was prepared from a series of infrared tracings that were obtained by mixing increasing amounts of ≃100% imidated polymer with polymer containing no imide or nonionized COOH (i.e., containing only amide and ionized carboxy functions). We took care in eliminating interference by nonionized carboxy at 1715 cm^{-1} by first dissolving the sample in water, adjusting the pH to 10.0 with ammonium hydroxide, and then freeze-drying to convert any nonionized COOH to ammonium carboxylate. Such procedures increase the intensity of the carboxylate bands at 1560 and 1400 cm^{-1} but ensure than the remaining band at 1715 cm^{-1} is solely of imide origin.

Ethylene/Maleic Anhydride Copolymer (1). Copolymerization of ethylene and maleic anhydride was conducted in a heated 1-gal stainless-steel reactor fitted with an internal water-cooling coil, magnetic-driven stirrer operating optimally at 1000–2000 rpm, ethylene inlet, and an inlet through which additional catalyst solution was added as required. Samples were withdrawn, and the final contents were emptied through a bottom port. Auxiliary equipment for heating and cooling control was provided.

In a typical run, the reactor charge consisted of 1625 g (1975 mL) of ethylbenzene, 190 g (1.94 mol) of maleic anhydride, and 14.1 g (0.058 mol) of benzoyl peroxide dissolved in 80 g (92 mL) of ethylbenzene. The catalyst charge was washed into the reactor with an additional 20.0 mL of ethylbenzene.

The charged reactor was pressure flushed at room temperature with nitrogen to remove oxygen prior to introduction of ethylene. Nitrogen was flushed from the reactor by pressuring to 200 psi with ethylene and venting two times. It should be noted that ethylene is an extremely flammable gas and all precautions to avoid explosions should be taken.

The temperature of the reactor was raised to 70 °C, and the ethylene pressure was adjusted to and held at 200 psi for the duration of the reaction. After 3 h of polymerization at 70 °C and 200 psi of ethylene pressure, an addition of 9.4 g (0.039 mol) of benzoyl peroxide in 60 g (70 mL) of ethylbenzene was made through the catalyst addition line. This was followed by a wash of this inlet with 20 mL of ethylbenzene. The stirred reactor was held at 70 °C and 200 psi of ethylene pressure for an additional 14 h to complete the polymerization. At the end of the run, the reactor was cooled and vented. The contents consisted of an ethylbenzene slurry of 1 and a small amount of 1 glazed on the stirrer, cooling coils, and reactor surfaces. The glazed material was removed by scraping, combined with the slurry, and filtered. The conversion of maleic anhydride was determined by NaOH titration of residual maleic anhydride in the filtrate to a phenolphthalein end point. The polymer was further processed at room temperature by slurry extraction three times (1 h each) with 2 L of xylene, followed by three extractions (1 h each) with 2 L of hexane and final filtration. It was vacuum dried overnight at 55–60 °C and pulverized in a Waring blender for 5 min to reduce the minor portion of glazed material to a powder consistency.

Ammoniated Ethylene/Maleic Anhydride Copolymer (2). A solution of 1 (80 g in 800 mL of AR-grade acetone) was added over a 20-min period to a stirred solution of 100 mL of liquid ammonia in 3 L of acetone at −70 °C (dry ice–acetone bath). The mixture was allowed to warm gradually to room temperature (4 h), during which time the color of the precipitated product changed from an initial yellow to white. The solid was filtered and successively slurried twice for 30 min with 2 L of acetone, followed by two slurries with 1.5 L of 50:50 acetone/hexane. It was recovered by filtration and dried overnight in a vacuum oven at 45 °C and 20–25 mmHg internal pressure. The dry solid was dissolved in 900 mL of deionized water, and the solution was filtered through a 0.45 μm filter and freeze-dried to yield 98.7 g of **2**.

Ammoniated Ethylene/Maleic Anhydride Copolymer, Partially Imidated (3). A 10-g sample of **2** was slurried in 250 mL of xylene in a 1-L flask fitted with stirrer, thermometer, water take-off trap, and a gas inlet sparger for ammonia. The slurry was refluxed for a period of 12 h while maintaining a steady flow of ammonia through the sparger with removal of water with a Stark–Dean trap. Aliquot samples of product slurry were assayed at various times (Table II) to determine conversion to imide vs. time. The samples were worked up by three consecutive slurries in 100 mL of hexane, filtered, and dried at 50 °C and 20–25 mmHg internal pressure.

The product was dissolved in 200 mL of deionized water, the pH was adjusted to 10.0 with ammonium hydroxide, the solution was sterilized by filtration through a Millipore membrane, and the partially imidated product **3** was recovered by freeze-drying (yield ∼95%).

Pharmacological Methods. Detailed animal model protocols employed for assessing antitumor activity of carboxyimamidate against Lewis lung carcinoma, P388 leukemia, B16 melanoma, and Walker 256 carcinoma are described by Geran and coworkers.[25] These protocols are generally descriptive of methodology for the other tumor model systems studied. For the other ascitic leukemia and lymphoma tumor models (L5178Y leukemia, Gardner lymphosarcoma, ADJ-PC6 plasmacytoma, and P815 mastocytoma), 10^6 Trypan blue excluding cells were implanted ip or sc (P815 only) in groups of 10 $B6D2F_1$ (L5178Y, P815), BALB/c (ADJ-PC6), or C3H (Gardner) mice following protocols similar to that used for P388 leukemia.[16] For colon carcinoma 26, 0.5 mL of 1% brei was implanted sc or ip in groups of 10 $CD2F_1$ mice. For the Nettesheim, Madison, and M5076 tumors, an inoculum of 0.5 mL of a 10% brei was implanted sc in groups of 10 $CD2F_1$ or $B6D2F_1$ (M5076) mice. A similar inoculum was used for ip implantation of M5076, and the volume of the inoculum was reduced to 0.2 mL for im implantation of the Madison lung tumor. Trochar implants of EMT6 in C3H mice and colon carcinoma 38 in $B6D2F_1$ mice were employed for the sc inoculation of these solid tumors.

Carboxyimamidate **3** was dissolved in sterile saline at an appropriate concentration so that the desired dose was delivered in a volume of 0.5 mL per mouse. Solutions of **3** were stored in amber bottles at 4 °C for up to 5 days. The drug was administered ip on the various treatment regimens. For determination of tumor growth inhibition (T/C), tumors in treated and control groups were measured when tumors in untreated controls averaged 700–1750 mm^3 (11–21 days after tumor implantation). Tumors were measured in rats bearing the Walker tumor 7 days after implantation when tumors in control rats averaged about 5000 mm^3. Tumor volume was calculated as 0.5 × length × $width^2$ as determined by measurement of perpendicular diameters with vernier calipers. Animals were held for 60 days following tumor implantation. Statistical comparison of treated vs. control (T/C) and mean life span (excluding long-term, tumor-free survivors) was carried out by Student's *t* test.

The following references describe the solid tumor systems in more detail: Madison 109 lung carcinoma, Rose[26] and Marks et al.;[27] colon carcinomas 26 and 38, Corbett et al.;[28] EMT6 mammary carcinoma, Rockwell et al.;[29] M5076 ovarian tumor, Clement et al.;[30] and Nettesheim squamous lung carcinoma, Nettesheim et al.[31]

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