Synthesis and Antitumor Activities of Mitomycin C (1→3)-β-D-Glucan Conjugate

Katsuyuki NAGAI, Jiro TANAKA, Tadashi KIHO, and Shigeo UKAI*

Gifu Pharmaceutical University, 6–1, Mitahora-higashi 5-chome, Gifu 502, Japan. *Received September 30, 1991*

The conjugate of mitomycin C (MMC) with linear (1→3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes* IFO 13140 was synthesized and its antitumor activities investigated. The conjugate (MMC–carboxymethylated linear (1→3)-β-D-glucan (CMPS)) was obtained by treatment of CMPS with MMC in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. *In vitro* cytotoxicity of MMC–CMPS against L1210 leukemia cells was similar to that of MMC. In i.p.–i.p. system *in vivo* against P388 leukemia in mice, the maximum increase of MMC–CMPS conjugate in life span (ILS_{max}) was higher than that of MMC but the therapeutic index was reduced. However, the antitumor activity of MMC–CMPS conjugate against subcutaneously implanted sarcoma 180 solid tumor in mice by i.p. administration was similar to that of MMC at a dose of 1.5 mg eq MMC/kg/d × 7 and the reduction of the number of leukocytes caused by MMC was suppressed by attaching MMC to CMPS. In addition, on assay using serum of sarcoma 180 solid tumor-bearing mice with injection of MMC–CMPS conjugate, a drastic loss of tumor cells and an increase in polymorphonuclear leukocytes (PMN) were observed. This result suggested that MMC–CMPS conjugate induced tumor-regressing factor similar to CMPS.

Keywords mitomycin C; (1→3)-β-D-glucan; carboxymethylated (1→3)-β-D-glucan; conjugate; *in vitro* antitumor activity; *in vivo* antitumor activity; tumor-regressing factor

In recent years, the use of polymeric substances as carriers has been developed to reduce the toxic side effects associated with cancer chemotherapeutic agents and to target the drug more selectively to the desired tissue. Kojima *et al.* reported the covalent attachment of mitomycin C (MMC) to dextran and the enhanced effect of the resulting conjugates on transplanted tumors in mice.[1] They suggested that cellular interaction played an important role in the manifestation of antitumor effect of MMC–dextran and that this phenomenon was governed by the physicochemical properties of macromolecular prodrugs, such as electric charge and molecular weight.[2] Aizawa *et al.* reported the antitumor effect of MMC–mannan conjugate against mouse hepatoma cells which MMC–dextran did not manifest any antitumor effect.[3] The mechanism of its action seemed to be complicated but it was postulated that interaction of the mannan moiety in the MMC–mannan conjugate with the mannose receptor was located on the cell surface of immunocytes and/or with hepatoma cells.[3] As a possible approach for macromolecular prodrugs of anticancer agents, we designed the conjugates of MMC with antitumor polysaccharides[4,5] which are considered to be host-mediated and are not attributable to cytocidal action on the tumor cell. These conjugates are expected to maintain the immunological enhancement with polysaccharides and to enhance the effect of anticancer agent.

In this paper, we report synthesis and *in vivo* antitumor activities of MMC–(1→3)-β-D-glucan conjugate and the effect of this conjugate on the number of leukocytes. (1→3)-β-D-Glucans have been well studied for their structure and antitumor activity; in particular, branched (1→3)-β-D-glucans having side chains of gingle D-glucosyl group at O-6, such as lentinan,[6,7] schizophillan,[8] scleroglucan,[9] and linear (1→3)-β-D-glucan (PS) from *Alcaligenes faecalis* var. *myxogenes* IFO 13140[10] have been reported to exhibit antitumor activity against sarcoma 180 implanted in mice. In addition, the carboxymethylated linear (1→3)-β-D-glucan (CMPS) has antitumor activity against sarcoma 180 transplanted in mice and it has been shown that this chemically modified glucan has a host mediated action.[11–13] In the present paper we employed this CMPS to facilitate introduction of MMC to (1→3)-β-D-glucan and investigate the immunological effect. We demonstrate that MMC–(1→3)-β-D-glucan conjugate presumably induces tumor-regressing factor[13] in the serum of sarcoma 180 tumor bearing mice, and the resulting loss of tumor cells and enhancement of polymorphonuclear leukocytes (PMN).

Experimental Method

Materials CMPS was kindly supplied by Takeda Pharmaceutical Industry Co., Ltd., Osaka, Japan. The average number of glucose residues in a molecule of CMPS is 540, and that of carboxymethyl residues is 0.62/glucose. MMC was kindly supplied by Kyowa Hakko Kogyo Co., Ltd. Sephadex G-10 was purchased from Pharmacia Fine Chemicals. All other chemicals were reagent-grade products obtained commercially.

Preparation of MMC–CMPS Conjugate To a solution of 200 mg of CMPS in 200 ml of distilled water was added 40 mg of MMC and 800 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. The mixture was stirred at 15 °C for 24 h, keeping the pH between 5.5 and 6.0. Then, the mixture was neutralized by aqueous sodium bicarbonate, and dialyzed against distilled water at 15 °C for 4 d. The internal solution was concentrated by ultra-filtration to about 2 mg eq glucan/ml and the concentrated solution was stored at −40 °C until use. The glucan and MMC contents were estimated by the phenol–sulfuric acid method[14] and the absorbance at 364 nm, respectively. MMC–CMPS conjugate was estimated to contain about 10% (w/w) MMC.

Gel Chromatography on Sephadex G-10 Gel chromatography on Sephadex G-10 was conducted with 0.1 M aqueous sodium chloride as the eluent, and MMC and MMC–CMPS conjugate were applied to a column (30 × 1.5 cm) of Sephadex G-10. The column was eluted with 0.1 M aqueous sodium chloride at a flow rate of 8 ml/h. Fractions (4 ml each) were collected, and each fraction was analyzed by absorbance at 364 nm.

***In Vitro* Release Experiment** Ten ml of solution of MMC–CMPS conjugate (1.56 mg/ml) in water was added to double the concentration of phosphate buffered saline (10 ml), and the solution was incubated at 37°C with moderate shaking. The amounts of liberated MMC in the conjugate at several incubation periods were determined by high performance liquid chromatography (HPLC) after separation with ultra-filtration (Sartorius Centrisart I SM 13249). The filtrate was analyzed by reverse-phase HPLC on a TSKgel ODS-120T column (4.6 mm × 15 cm) using a 35% CH_3OH in H_2O. Results were expressed as semi-logarithmic plots of percent of MMC remaining *versus* incubation time.

***In Vitro* Cytotoxicity Assays** Cell growth inhibition by MMC and

MMC–CMPS conjugate was measured using L1210 leukemia cells in RPMI 1640 medium supplemented with 10% fetal calf serum and containing streptomycin (100 μg/ml), penicillin (100 units/ml), and 0.05 mM 2-mercaptoethanol. Cells were seeded on a 96-well microplate at a density of 5×10^3 cells/well and to this suspension was added the medium containing test materials at various concentrations. After incubation in a humidified atmosphere containing 5% CO_2 at 37 °C for 48 h, 10 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT)[15] dye solution (5 mg/ml phosphate buffered saline) was added to each well and the plate was incubated for another 6 h. Then, 150 μl of acidified isopropanol was added to each well and the OD_{550} of each was measured with a microplate spectrophotometer (Microplate reader MTP-32, Corona Electric).

***In Vivo* Antitumor Assays** The animals used in the experiment were CDF1 male mice (6 weeks old), ICR female mice (4 weeks old) and ddY male mice (4 weeks old). They were obtained from Japan Slc. Inc., Shizuoka, Hamamatsu. P388 leukemia cells and sarcoma 180 ascites cells, maintained by serial i.p. transplantation into DBA/2 mice and ICR mice, respectively, were used. Tumor cells (7 d old) were employed for the experiment.

(1) The i.p.–i.p. System: Standard NCI (National Cancer Institute, Bethesda) protocols were employed.[16] Groups of five CDF1 male mice were inoculated i.p. with 10^6 P388 leukemia cells on day 0, and each test material was given i.p. once on day 1. Each test material was administered in sterile saline. The increase in survival (ILS) was calculated according to the formula ILS (%) = $[(T/C)-1] \times 100$, where T and C are the median survival times in days for the treated and control groups, respectively.

(2) The s.c.–i.p. System: Sarcoma 180 ascites cells were transplanted subcutaneously at a dose of 0.1 ml (1.7×10^6 cells) into the right groin of ddY mice. Starting 1 d after the transplantation, each test material was given i.p. once a day for 7 or 10 d. Each test material was administered in sterile saline. Tumor growth was observed for 30 d, then the mice were killed, and the tumor was extirpated and weighed. The inhibition ratios were calculated using of the formula: inhibition ratio (%) = $[(A-B)/A] \times 100$, where A is the average tumor weight of the control group, and B is that of the tested group.

Determination of the Number of Leukocytes in Mice Twenty μl of blood of tumor-bearing mice was taken from the orbital vein at 8 and 14 d after i.p. injection. The blood was diluted with Isoton II (Coulter Scientific, Japan), hemolyzed with Zap-oglobin II (Coulter Scientific, Japan) and leukocytes were counted by automatic blood cell counter MEK-3100 (Nihon Kohgen Inc.).

Assay of Induction of Tumor Regressing Factor The assay was carried out in accordance with the description by Kunimoto *et al.*[13]

(1) Serum: ICR mice bearing 14-d-old S180 tumors received an i.p. injection of each sample (MMC: 10 mg/kg, CMPS: 100 mg/kg, MMC–CMPS conjugate 100 mg eq CMPS/kg). In the control group the serum of normal mice with saline injection was used. Blood was collected 10—12 h after sample injection and kept on ice for 30 min before separation (4000 rpm for 30 min). All sera were stored at −40 °C until use.

(2) Determination of Cellular Composition of Solid Tumor: The sera were injected intravenously into S180-bearing ICR mice on day 14 of tumor growth. Three mice were used for each sample. The tumors were extirpated 24 h later, minced, and incubated at 37 °C with stirring in an enzyme mixture of trypsin (0.125%), collagenase (0.1%), and deoxyribonuclease (DNase, 0.02%) in Eagle's minimum essential medium for 1.5—2 h. The enzyme mixture in a volume 20 times the tumor weight was used for digestion. In the resulting cell suspensions, the total number of tumor cells and other cells was determined by differential counting of fixed and stained preparations under a binocular microscope or viable tumor cell counting under a phase-contrast microscope.

Results and Discussion

The MMC–(1→3)-β-D-glucan conjugate was prepared by treating CMPS, chemically modified PS from *Alcaligenes faecalis* var. *myxogenes* IFO 13140,[10] with MMC in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. On gel filtration on Sephadex G-10 with 0.1 M aqueous sodium chloride, the product (MMC–CMPS) and a mixture of MMC and CMPS were eluted differently (Fig. 1) indicating that MMC is attached to CMPS. From spectrophotometric analysis, MMC–CMPS conjugate was estimated to contain about 10% (w/w) MMC. *In vitro* release rate of MMC from MMC–CMPS conjugate was measured in the phosphate buffered saline (pH 7.4) at 37 °C (Fig. 2). MMC–CMPS conjugate showed a successive monoexponential liberation with a half-life of 4.75 h. The release rate of MMC from MMC–CMPS conjugate was much faster than that of MMC–dextran conjugate synthesized by Kojima *et al.*[1] Sezaki showed that the release rate of MMC from MMC–dextran conjugates increased as the length of the spacer molecule decreased.[17] The faster release rate of MMC from MMC–CMPS conjugate seems to be due to the short spacer (C_1) of MMC–CMPS conjugate.

Cytotoxicity determinations were made for MMC and

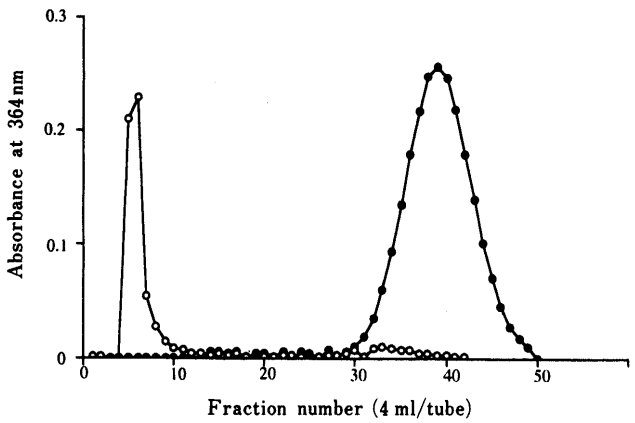

Fig. 1. Gel Filtration Patterns of MMC–CMPS Conjugate and a Mixture of MMC and CMPS in 0.1 M Aqueous Sodium Chloride on Sephadex G-10 (30 × 1.5 cm)

●, MMC + CMPS (mixture); ○, MMC–CMPS conjugate.

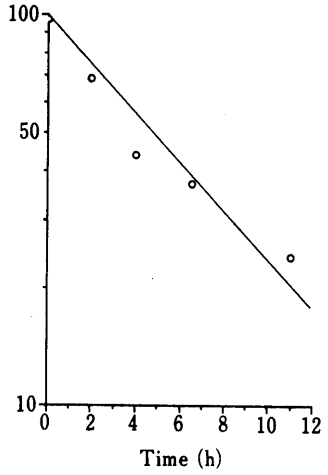

Fig. 2. *In Vitro* Release of MMC from MMC–CMPS Conjugate in Phosphate Buffered Saline (pH 7.4) at 37 °C

Results were expressed as semilogarithmic plots of percent remaining of MMC *versus* incubation time.

TABLE I. Cytotoxicity of MMC and MMC–CMPS against L1210 Cells in Culture

Compound	IC_{50} (MMC eq μg/ml)[a]
MMC–CMPS	0.82
MMC	0.90

a) IC_{50}: test material concentration showing 50% growth inhibition.

Table II. Antitumor Activity of MMC and MMC–CMPS Conjugate against P388 Leukemia in Mice

Compound	Dose (MMC eq mg/kg)	Mean survival days ± S.D.	ILS (%)
Control		10.2 ± 0.4	0
MMC	0.75	15.0 ± 0.9	47
	1.5	16.0 ± 1.1	57
	3.0	17.8 ± 1.5	75
	6.0	16.6 ± 2.4	63
	9.0	18.6 ± 1.9	82
	12.0	18.2 ± 3.9	78
	18.0	8.0 ± 1.7	−22
MMC + CMPS (mixture)	0.75	14.8 ± 0.7	45
	1.5	15.8 ± 0.4	55
	3.0	17.2 ± 1.9	69
	6.0	13.6 ± 4.1	33
	9.0	6.0 ± 0	−41
	12.0	4.6 ± 1.4	−55
	18.0	4.2 ± 0.4	−59
MMC–CMPS conjugate	0.75	14.4 ± 0.5	41
	1.5	16.6 ± 0.8	63
	3.0	18.2 ± 1.0	78
	6.0	23.6 ± 2.8	131
	9.0	9.2 ± 5.9	−10
	12.0	9.4 ± 7.8	−8
	18.0	5.0 ± 0	−51

Table III. Antitumor Activity of MMC–CMPS and MMC against Sarcoma 180

Compound	Dose (MMC eq mg/kg/d × 7)	Mean tumor wt. ± S.D.	Inhibition ratio (%)	Complete regression
MMC	0.7	8.21 ± 3.03	7	0/7
	1.5	1.28 ± 1.90[d]	86	2/7
MMC + CMPS	0.7 + 6.3	3.74 ± 3.19[c]	58	1/7
	1.5 + 13.5	1.14 ± 1.06[d]	87	2/7
MMC–CMPS[a]	7	3.91 ± 3.97	56	1/7
	15	0.68 ± 1.15[e]	92	3/7
CMPS[b]	6.3	7.17 ± 4.46	19	0/7
	13.5	2.97 ± 2.64[c]	66	1/7
Control	—	8.84 ± 5.57	—	0/7

a) MMC–CMPS contents 10% MMC. *b*) Administered i.p. once a day for 10 d. Significant difference from control (*c*) $p<0.05$; *d*) $p<0.01$, *e*) $p<0.005$).

MMC–CMPS conjugate against L1210 mouse leukemia cells in culture (Table I). The 50% growth-inhibitory concentration (IC_{50}) was similar to that of MMC.

The antitumor effect of MMC and MMC–CMPS conjugate was tested *in vivo* against P388 leukemia in CDF1 mice. As shown in Table II, the antitumor activities of MMC–CMPS conjugate and a mixture of MMC and CMPS (MMC + CMPS) at doses under 3 mg eq MMC/kg were similar to that of MMC. Significant activity was observed at a dose of 6 mg eq MMC/kg of MMC–CMPS conjugate. Thus, MMC–CMPS conjugate showed a 131% ILS. MMC–CMPS conjugate and the mixture (MMC + CMPS) at doses above 9.0 mg eq MMC/kg showed less effect than the same doses of MMC. Though MMC–CMPS conjugate showed the maximum ILS that was 131%, the therapeutic index was reduced. Also, in the mixture (MMC + CMPS) reduction of the therapeutic index was observed. Reduction of this index seems to show that at higher doses of MMC–CMPS conjugate and the mixture

Table IV. Effect of MMC–CMPS on Leukocytes

Compound	Dose (mg/kg × 7 d)	Mean ± S.D. (cells/mm^3) 8 d	14 d
MMC	0.7	6633 ± 202[a]	9367 ± 1686
	1.5	5417 ± 1518[b]	5417 ± 840[a]
MMC + CMPS	0.7 + 6.3	11467 ± 1486	8933 ± 576
	1.5 + 13.5	5933 ± 778[b]	4396 ± 943[b]
MMC–CMPS	7	10633 ± 3494	9550 ± 3162
	15	9817 ± 1936	10458 ± 2306
CMPS	6.3	7767 ± 1388	11517 ± 3612
	13.5	8917 ± 2102	9350 ± 3342
Control	—	8733 ± 1212	8516 ± 1691

Significant difference from control (*a*) $p<0.05$; *b*) $p<0.005$).

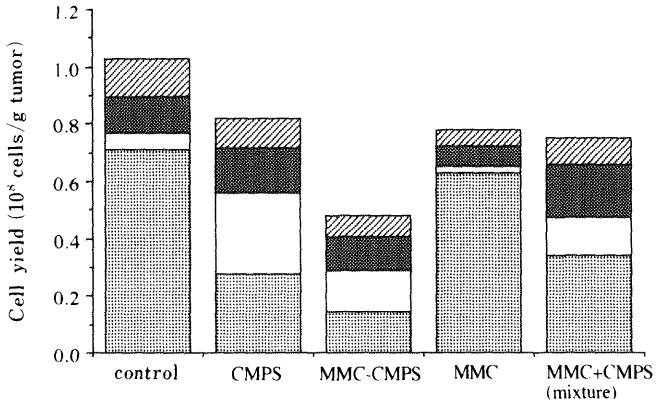

Fig. 3. Changes in Cellular Composition of Solid Sarcoma 180 Tumors Induced by Sera of Mice Bearing Sarcoma 180 with MMC–CMPS Injection

▨, lymphocytes; ■, macrophages; □, PMN; ▦, S180.

(MMC + CMPS), CMPS acts as antagonist on the antitumor effect against P388 leukemia. Nonetheless, MMC–CMPS conjugate should be useful against other tumor cells at low doses in serial injections, since antitumor β-D-glucans have been reported to inhibit sarcoma 180 solid tumor in mice at low doses.

The antitumor activities of MMC and MMC–CMPS conjugate against sarcoma 180 solid tumor in mice were tested at low doses (0.7 and 1.5 mg eq MMC/kg). The strong inhibition by MMC, a mixture of MMC and CMPS, and MMC–CMPS conjugate was observed at a dose of 1.5 mg eq MMC/kg/d × 7, 86%, 87%, and 92%, respectively (Table III). In addition, since MMC shows a side effect of reducing the number of leukocytes in peripheral blood of animals,[18] the numbers was also counted. As shown in Table IV, MMC and a mixture of MMC and CMPS reduced the number of leukocytes at 8 to 14 d after i.p. injection (1.5 mg eq MMC/kg/d × 7). On the other hand, no reduction in number was observed in MMC–CMPS conjugate injection. This indicates that MMC–CMPS conjugate curtails the reduction in the number of leukocytes that is a side effect of MMC.

Finally, in order to investigate whether MMC–CMPS conjugate maintains the immunological effect of CMPS, induction of tumor-regressing factor was assayed. Kunimoto *et al.* showed that the serum induced tumor-regressing factor caused very rapid loss of tumor cells from sarcoma 180 solid tumors, with a marked increase in PMN.[13] As

shown in Fig. 3, in fact, the serum of tumor bearing mice with CMPS injection caused a decrease in the number of tumor cells accompanied by a marked increase in PMN. However, in the case of control and MMC these phenomena were not observed. On the other hand, the number of viable cells in the MMC–CMPS conjugate group was 50% less than that of control group and a drastic loss of tumor cells and an increase in PMN were observed. The serum of tumor bearing mice injected with a mixture of MMC and CMPS was also active. These results suggest that MMC–CMPS conjugate maintains the tumor-regressing activity of CMPS, and that MMC does not inhibit the activity of CMPS.

In conclusion, it was revealed by the test of antitumor activity against P388 leukemia in mice that the maximum ILS of MMC–CMPS conjugate was higher than that of MMC but the therapeutic index was reduced. However, its activity against sarcoma 180 solid tumor in mice was similar to that of MMC and the reduction of the number of leukocytes caused by MMC was suppressed by attaching MMC to CMPS. In addition, it was suggested that tumor-regressing factor was induced in the sera of tumor-bearing mice injected with MMC–CMPS conjugate. These findings implied the possibility that not only was the therapeutic potency of MMC maintained but also that there was immunological enhancement with (1→3)-β-D-glucans and that antitumor polysaccharides would be useful in the durg delivery system.

Acknowledgment We thank Dr. Masami Okabe and his associates at Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., for conducting the test of antitumor activity against P388 leukemia in mice.

References

1) T. Kojima, M. Hashida, S. Muranishi, and H. Sezaki, *J. Pharm. Pharmacol.*, **32**, 30 (1980).
2) S. Matsumoto, A. Yamamoto, Y. Takakura, M. Hashida, N. Tanigawa, and H. Sezaki, *Cancer Res.*, **46**, 4463 (1986).
3) K. Aizawa, T. Matsumoto, K. Tsukada, A. Ito, H. Sato, S. Suzuki, and M. Suzuki, *Int. J. Immunopharmac.*, **11**, 191 (1989).
4) R. L. Whistler, A. A. Bushway, P. P. Singh, W. Nakahara, and R. Tokuzen, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 32, ed. by R. T. Tipson and D. Horton, Academic Press, New York, 1976, p. 235.
5) G. Chihara, "Gan To Menekizokyo," Kodansha, Tokyo, 1980, p.49.
6) G. Chihara, Y. Maeda, J. Hamuro, T. Sasaki, and F. Fukuoka, *Nature* (London), **222**, 687 (1969).
7) T. Sasaki and N. Takasuka, *Carbohydr. Res.*, **47**, 99 (1976).
8) K. Tabata, W. Ito, and T. Kojima, *Carbohydr. Res.*, **89**, 121 (1981).
9) J. Johnson, S. Kirkwood, A. Misaki, T. E. Nelson, J. V. Scaletti, and F. Smith, *Chem. Ind.* (London), **1963**, 820.
10) T. Sasaki, N. Abiko, Y. Sugino, and K. Nitta, *Cancer Res.*, **38**, 379 (1978).
11) T. Sasaki, N. Abiko, K. Nitta, N. Takasuka, and Y. Sugino, *Eur. J. Cancer*, **15**, 211 (1979).
12) S. Honda, H. Sugino, T. Asano, and A. Kakinuma, *Immunopharmacology*, **11**, 29 (1986).
13) T. Kunimoto, H. Baba, and K. Nitta, *J. Biol. Response Mod.*, **5**, 160, 225 (1986).
14) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
15) L. M. Green, J. L. Reade, and C. F. Ware, *J. Immunol. Methods*, **70**, 257 (1984).
16) R. I. Geran, N. H. Greengerg, M. M. Mcdonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep., Part 3*, **3**, 1 (1972).
17) H. Sezaki, *Yakugaku Zasshi*, **104**, 1221 (1984).
18) R. Evans, L. D. Madison, and D. M. Eidlen, *Cancer Res.*, **40**, 395 (1980).