[Chem. Pharm. Bull. 35(7)3016—3020(1987)]

Antitumor Principles from *Ginkgo biloba* L.

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(Received December 10, 1986)

Seven long-chain phenols were isolated from *Ginkgo biloba* L. Three of them, anacardic acid (Ib), bilobol (IIa), and cardanol (IIIa), showed antitumor activity against Sarcoma 180 ascites in mice. The antitumor effectiveness was rated (++) for Ib, (+++) for IIa, and (+++) for IIIa at 40 mg/kg/d by the total packed cell volume method.

Keywords——*Ginkgo biloba*; Ginkgoaceae; antitumor activity; Sarcoma 180 ascites; anacardic acid; bilobol; cardanol

Preliminary antitumor screening tests of crude drugs and collected plants[1,2] have been carried out by means of the total packed cell volume method[3] using Sarcoma 180 ascites in mice. In subsequent tests, the methanol extract from the sarcotesta of *Ginkgo biloba* showed remarkable activity. In this paper, we describe the isolation and the activity of the antitumor principles in *Ginkgo biloba* L.

When an aqueous solution of the methanolic extract prepared from the sarcotesta of *Ginkgo biloba* was partitioned successively with chloroform, ethyl acetate, and *n*-butanol as shown in Chart 1, the antitumor activity was concentrated in the chloroform extract. The extract was subjected to silica gel and/or alumina column chromatography, and fractions containing anacardic acid, bilobol, and cardanol were obtained. Each of them was subjected

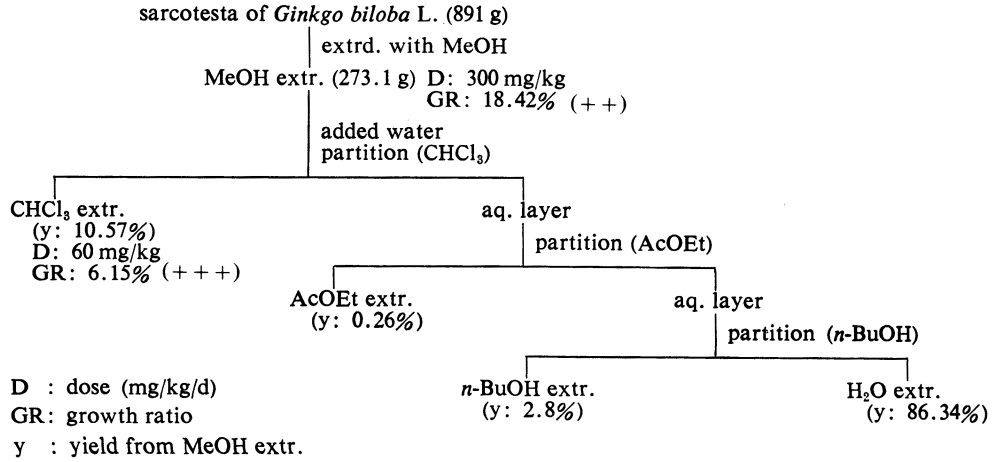


Chart 1

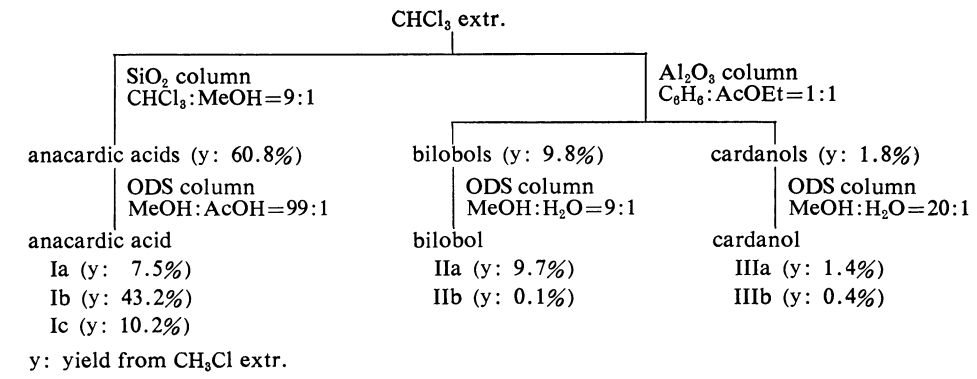


Chart 2

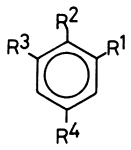
to octadecylsilyl (ODS) column chromatography as shown in Chart 2, yielding anacardic acid (Ia, b, c), bilobol (IIa, b), and cardanol (IIIa, b).

Compound Ib was isolated as a major antitumor principle from the chloroform extract. The spectral data led us to conclude that Ib is one of the anacardic acids[4] (Fig. 1). The length and degree of unsaturation of the side chain of Ib′, the methyl ester of Ib, were deduced from the molecular ion peak at *m/z* 360 in the mass spectrum (MS). The position of the double bond was determined to be $\Delta^8-15:1$ from the molecular ion peak at *m/z* 278 in the MS after ozonolysis of Ib′. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum, carbons at allylic positions in the side chain of Ib were observed at δ 27.21 ppm. Therefore, the double bond was concluded to be *cis*.[5] The compounds Ia and Ic were similarly determined to be the anacardic acids with 13:0 and $\Delta^{10}-17:1$ side chains, respectively.

Compounds IIa and IIIa were isolated as minor antitumor principles. Compounds IIa, b and IIIa, b were determined to be bilobols and cardanols, respectively, by procedures similar to those used to identify anacardic acids. The side chain of IIb was $C_{17}H_{33}$, different from those of bilobols reported in the literature,[6,7] whose side chains were $C_{13}H_{27}$, $C_{15}H_{29}$, $C_{15}H_{27}$, and $C_{15}H_{25}$.

Compounds Ib, b′, IIa, a′, and IIIa, a′ were tested by means of the total packed cell volume method[3] using Sarcoma 180 ascites in mice. The results are shown in Table I. Compounds Ib, IIa, and IIIa showed potent activity, but Ib′, IIa′, and IIIa′ showed no activity.

On the other hand, antimicrobial activity of Ib has been reported,[7,8] and we found that IIa and IIIa also have weak antimicrobial activity (Table II). Thus, the antitumor activity against Sarcoma 180 ascites appears not to require the carboxyl group, whereas the antimicrobial activity does require it.



Ia:	$R^1=(CH_2)_{12}CH_3$,	$R^2=COOH$,	$R^3=OH$,	$R^4=H$
Ib:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=COOH$,	$R^3=OH$,	$R^4=H$
Ic:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$,	$R^2=COOH$,	$R^3=OH$,	$R^4=H$
Ia′:	$R^1=(CH_2)_{12}CH_3$,	$R^2=COOCH_3$,	$R^3=OH$,	$R^4=H$
Ib′:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=COOCH_3$,	$R^3=OH$,	$R^4=H$
Ic′:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$,	$R^2=COOCH_3$,	$R^3=OH$,	$R^4=H$
IIa:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=H$,	$R^3=R^4=OH$	
IIb:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$,	$R^2=H$,	$R^3=R^4=OH$	
IIa′:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=H$,	$R^3=R^4=OCOCH_3$	
IIIa:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=R^4=H$,	$R^3=OH$	
IIIb:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$,	$R^2=R^4=H$,	$R^3=OH$	
IIIa′:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=R^4=H$,	$R^3=OCOCH_3$	

Fig. 1. The Structures of Ia—IIIa′

TABLE I. Antitumor Activity on Sarcoma 180 Ascites in Mice

Compound	Dose (mg/kg)	GR (%)	Assessment
Ib	40	17.4	++
IIa	40	0.4	+++
IIIa	40	0.0	+++
Ib′	60	110.4	−
IIa′	40	81.9	−
IIIa′	40	105.7	−

TABLE II. Antimicrobial Activity (MIC, μg/ml)

Organisms	IIa (Bilobol)	IIIa (Cardanol)
S. aureus 209PJC-1	>100	50
S. aureus TERAJIMA	>100	50
S. aureus SMITH	>100	25
S. aureus 911-5	25	25
S. aureus E31080	>100	50
S. aureus JS1	25	25
E. faecalis E22018	25	25

MIC: minimum inhibitory concentration.

Experimental

Silica gel column chromatography was carried out on Wakogel C-200 (100—200 mesh). Alumina column chromatography was carried out on Merck Art. 1097 (Aluminiumoxide 90 standardisiert) (70—230 mesh). In general, silica gel and alumina for column chromatography were employed in amounts equivalent to 100 times the sample amount. For further purification, high-performance liquid chromatography (HPLC) was carried out on a CIG column system (Kusano Scientific Co., Tokyo) with IATROBEADS (60 μ silica gel, IATRON Co., Tokyo) as the stationary phase. Spectral data were obtained on the following instruments; ultraviolet spectrum (UV) on a Hitachi 557, infrared spectrum (IR) on a Jasco A-302, NMR on a Bruker AM400, MS on a Hitachi M-80, and optical rotation on a Jasco DIP-4.

Extraction and Isolation——The sarcotesta of *Ginkgo biloba* L. (891 g) was extracted with MeOH (3 l). The concentrated MeOH extract (273.1 g) was diluted with water and then shaken successively with chloroform, ethyl acetate, and *n*-butanol in a separatory funnel three times. The three portions of each organic solvent were combined and evaporated. The chloroform extract, which showed potent activity, was separated as shown in Chart 2.

Compound Ia: Colorless oil. MS *m/z* (%): 276 (28, $M^+ - CO_2$), 150 (2), 149 (3), 121 (10), 120 (4), 109 (8), 108 (100), 107 (26). IR (CCl_4) cm^{-1}: 3520, 3450, 3040, 2950, 2870, 1615, 1592, 1470, 1465, 1275, 1155. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.60 (2H, quintet, 7.6), 2.96 (2H, t, 7.6), 6.75 (1H, dd, 7.4, 1.0), 6.85 (1H, dd, 7.4, 1.0), 7.32 (1H, t, 7.4), 11.33 (1H, s).

Compound Ia′: Colorless oil. MS *m/z* (%): 334 (50, M^+), 302 (10), 185 (10), 175 (20), 166 (55), 161 (50), 147 (100), 134 (70), 121 (20), 107 (35), 105 (57), 55 (40). IR (CCl_4) cm^{-1}: 3100, 3030, 2920, 2850, 1735, 1608, 1575, 1448, 1313, 1247, 1209, 1165, 1118. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.53 (2H, quintet, 7.7), 2.88 (2H, t, 7.7), 3.96 (3H, s), 6.72 (1H, dd, 8.0, 1.0), 6.83 (1H, dd, 9.0, 1.0), 7.28 (1H, dd, 9.0, 8.0).

Compound Ib: Colorless powder, mp 40—41 °C. MS *m/z* (%): 302 (15, $M^+ - CO_2$), 276 (5), 147 (6), 133 (6), 121 (14), 120 (25), 108 (100), 107 (75). IR (CCl_4) cm^{-1}: 3520, 3440, 3030, 3000, 2920, 1675, 1645, 1606, 1450, 1380, 1300, 1245, 1207, 1175. UV λ_{max}^{EtOH} nm (ε): 240.0 (2050), 300.0 (1840). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (16H, br), 1.60 (2H, quintet, 7.6), 2.01 (4H, q, 6.0), 2.97 (2H, t, 7.6), 5.34 (2H, m), 6.75 (1H, dd, 7.4, 1.0), 6.85 (1H, dd, 7.4, 1.0), 7.32 (1H, t, 7.4), 11.32 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 14.05 (t), 22.65 (t), 27.21 (t), 29.25 (t), 29.32 (t), 29.38 (t), 29.49 (t), 29.58 (t), 29.61 (t), 29.76 (t), 31.79 (t), 31.94 (t), 36.42 (t), 110.50 (s), 115.90 (d), 122.72 (d), 129.78 (d), 129.90 (d), 135.36 (d), 147.79 (s), 163.58 (s), 176.41 (s).

Compound Ib′: Colorless oil. MS *m/z* (%): 360 (27, M^+), 328 (10), 310 (10), 175 (8), 166 (100), 161 (40), 147 (80), 134 (65), 121 (60), 107 (30), 105 (55), 55 (85). IR (CCl_4) cm^{-1}: 3100, 3030, 2920, 2850, 1735, 1665, 1608, 1575, 1448, 1313, 1247, 1209, 1165, 1118. UV λ_{max}^{EtOH} nm (ε): 217.5 (2610), 243.3 (2730), 283.0 (1410), 310.0 (1490). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.1 Hz), 1.30 (16H, br), 1.53 (2H, quintet, 7.7), 2.03 (4H, q, 6.0), 2.88 (2H, t, 7.7), 3.96 (3H,

s), 5.35 (2H, m), 6.72 (1H, dd, 8.0, 1.0), 6.84 (1H, dd, 9.0, 1.0), 7.29 (1H, dd, 9.0, 8.0).

Compound Ic: Colorless powder, mp 45—46 °C. MS *m/z* (%): 330 (46, $M^+ - CO_2$), 304 (45), 234 (2), 175 (4), 149 (19), 147 (22), 133 (5), 121 (22), 120 (25), 108 (100), 107 (65). IR (CCl_4) cm^{-1}: 3520, 3440, 3050, 2980, 2840, 1615, 1598, 1590, 1490, 1450, 1275, 1152. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.60 (2H, quintet, 7.6), 2.02 (2H, q, 6.0), 2.97 (2H, t, 7.6), 5.34 (2H, m), 6.75 (1H, dd, 7.4, 1.0), 6.85 (1H, dd, 7.4, 1.0), 7.32 (1H, t, 7.4), 11.33 (1H, s).

Compound Ic′: Colorless oil. MS *m/z* (%): 388 (30, M^+), 356 (20), 338 (15), 299 (8), 175 (10), 166 (75), 161 (35), 133 (65), 121 (15), 107 (35), 105 (40), 55 (100). IR (CCl_4) cm^{-1}: 3100, 3030, 2920, 2850, 1735, 1665, 1608, 1575, 1450, 1310, 1245, 1205, 1165, 1117. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.28 (20H, br), 1.53 (2H, quintet, 7.0), 2.02 (4H, q, 6.0), 2.88 (2H, t, 7.0), 3.97 (3H, s), 5.35 (2H, m), 6.72 (1H, dd, 8.0, 1.0), 6.83 (1H, dd, 9.0, 1.0), 7.28 (1H, dd, 9.0, 8.0).

Compound IIa: Colorless powder, mp 30—31 °C. MS *m/z* (%): 318 (33, M^+), 292 (4), 222 (14), 205 (10), 191 (13), 177 (9), 166 (23), 163 (22), 149 (18), 137 (100). IR (CCl_4) cm^{-1}: 3620, 3450, 3030, 1635, 1600, 1467, 1380, 1340, 1300, 1210, 1150, 1000. UV λ_{max}^{EtOH} nm (ε): 231.0 (1760), 274.5 (1260), 280.5 (1240). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J= 7.0 Hz), 1.30 (16H, br), 1.57 (2H, quintet, 7.5), 2.01 (4H, q, 6.5), 2.48 (2H, t, 7.5), 5.36 (2H, m), 6.17 (1H, t, 2.2), 6.24 (2H, d, 2.2). ^{13}C-NMR ($CDCl_3$) δ: 14.12 (q), 22.68 (t), 27.25 (t), 29.01 (t), 29.34 (t), 29.46 (t), 29.76 (t), 29.83 (t), 31.10 (t), 31.82 (t), 35.88 (t), 100.27 (d), 108.21 (d), 129.89 (d), 129.99 (d), 146.44 (s), 156.30 (s).

Compound IIa′: Colorless oil. MS *m/z* (%): 402 (14, M^+), 360 (12), 318 (28), 292 (6), 222 (8), 205 (5), 166 (8), 163 (7), 137 (11), 124 (100), 123 (24). IR (CCl_4) cm^{-1}: 3020, 2940, 2860, 1775, 1620, 1595, 1450, 1370, 1200, 1175, 1120, 1020. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7.0 Hz), 1.29 (16H, br), 1.60 (2H, quintet, 7.5), 2.01 (4H, q, 6.0), 2.27 (3H, s), 2.60 (2H, t, 7.5), 5.34 (2H, m), 6.74 (1H, t, 2.1), 6.80 (2H, d, 2.1).

Compound IIb: Colorless powder, mp 35—36 °C. MS *m/z* (%): 346 (60, M^+), 320 (24), 250 (10), 205 (8), 191 (12), 177 (8), 166 (41), 163 (22), 149 (20), 137 (14), 124 (100). IR (CCl_4) cm^{-1}: 3620, 3400, 3030, 2930, 1635, 1600, 1470, 1145, 995. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.57 (2H, quintet, 7.5), 2.01 (4H, q, 6.5), 2.48 (2H, t, 7.5), 5.36 (2H, m), 6.17 (1H, t, 2.2), 6.24 (2H, d, 2.2).

Compound IIIa: Colorless oil. MS *m/z* (%): 302 (25, M^+), 276 (8), 206 (2), 175 (3), 161 (5), 149 (6), 147 (13), 133 (13), 120 (46), 108 (100), 107 (72). IR (CCl_4) cm^{-1}: 3620, 3450, 3050, 1615, 1598, 1590, 1490, 1470, 1455, 1275, 1185, 1152. UV λ_{max}^{EtOH} nm (ε): 226.0 (1710), 273.0 (1500). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (16H, br), 1.57 (2H, quintet, 8.0), 2.01 (4H, q, 6.0), 2.52 (2H, t, 8.0), 5.35 (2H, m), 6.64 (1H, d, 7.7), 6.65 (1H, s), 6.73 (1H, d, 7.7), 7.11 (1H, t, 7.7). ^{13}C-NMR ($CDCl_3$) δ: 14.10 (q), 22.67 (t), 27.21 (t), 27.23 (t), 29.00 (t), 29.24 (t), 29.30 (t), 29.41 (t), 29.69 (t), 29.76 (t), 31.28 (t), 31.80 (t), 35.84 (t), 112.52 (d), 115.35 (d), 120.93 (d), 129.37 (d), 129.86 (d), 129.98 (d), 144.93 (s), 155.49 (d).

Compound IIIa′: Colorless oil. MS *m/z* (%): 344 (16, M^+), 302 (30), 276 (20), 147 (11), 133 (5), 120 (19), 108 (100), 107 (56). IR (CCl_4) cm^{-1}: 3020, 2940, 2860, 1770, 1610, 1590, 1490, 1370, 1260, 1210, 1145. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, 7.0), 1.28 (16H, br), 1.60 (2H, quintet, 8.0), 2.00 (4H, q, 6.0), 2.29 (3H, s), 2.60 (2H, t, 8.0), 5.34 (2H, m), 6.88 (1H, s), 6.89 (1H, d, 7.7), 7.03 (1H, d, 7.7), 7.27 (1H, t, 7.7).

Compound IIIb: Colorless oil. MS *m/z* (%): 330 (48, M^+), 304 (44), 234 (2), 175 (4), 149 (18), 147 (22), 133 (6), 121 (22), 120 (23), 108 (100). IR (CCl_4) cm^{-1}: 3620, 3450, 3050, 2940, 1615, 1598, 1490, 1470, 1455, 1275, 1185, 1152. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.57 (2H, quintet, 8.0), 2.02 (4H, q, 6.0), 2.53 (2H, t, 8.0), 5.35 (2H, m), 6.64 (1H, d, 7.7), 6.65 (1H, s), 6.73 (1H, d, 7.7), 7.11 (1H, t, 7.7).

Selective Methylation of Anacardic Acid——Selective methylation of the carboxyl group of anacardic acid was achieved by short treatment with CH_2N_2 in anhydrous ether. After removal of the solvent, methyl anacardic ester was obtained.

Ozonolysis of Long-Chain Phenols——A solution of 1—10 mg of an unsaturated long-chain phenol (Ib′, c′, IIa, b, IIIa, or IIIb) in 10 ml of MeOH was ozonized by bubbling ozone through the solution. After ozonolysis, the ozonide was reduced with 10 mg of zinc and 10 drops of acetic acid at 30 °C for 1 h, and neutralized with 1 N NaOH. The reaction mixture was partitioned between chloroform and water, then the organic layer was evaporated to give the corresponding aldehyde.

Assay of Activity Against Sarcoma 180 Ascites[9]——ICR male mice, 5 weeks old, supplied by Clea Japan Co., Ltd., were used in groups of 6 animals. Sarcoma 180 ascites, provided by the National Cancer Center Research Institute and maintained in successive generations by us, was implanted i.p. at 1×10^6 cells/body. Administration of a test drug was started at 1 d after the implantation and continued for 5 d by the i.p. route. The effectiveness was evaluated by means of the total packed cell volume method[3]: growth ratio (GR%)=(packed cell volume (PCV) of test groups/PCV of control groups) × 100; GR=0—10% (+++), 11—47% (++), 41—65% (+), and over 66% (−).

Drug Treatment——A 0.5% solution of carboxymethylcellulose (CMC) in isotonic sodium chloride was used as a vehicle for the injection of test drugs. The dose ranges used for treatment are shown in Charts 1 and 2. Control group mice received equal volumes of normal saline containing 0.5% CMC. The results were evaluated according to the standard methods described above.

Acknowledgements We would like to thank Mr. K. Namikawa for the supply of sarcotesta of *Ginkgo biloba*

L. and Dr. Kagei, Eisai Co., Ltd. for determining antimicrobial activity.

References

1) H. Itokawa, K. Watanabe, and S. Mihashi, *Shoyakugaku Zasshi*, **33**, 95 (1979).
2) H. Itokawa, K. Watanabe, K. Mihara, and K. Takeya, *Shoyakugaku Zasshi*, **36**, 145 (1982).
3) A. Hoshi and K. Kuretani, *Farmacia*, **9**, 464 (1973).
4) H. Morimoto, Y. Kawamatsu, and H. Sugihara, *Chem. Pharm. Bull.*, **16**, 2282 (1968).
5) R. Rossi and C. A. Veracini, *Tetrahedron*, **38**, 639 (1982).
6) F. F. Vincieri, M. T. Vincenzini, and P. Vanni, *Riv. Ital. Essenze, Profumi Piante Off.*, **63**, 79 (1981).
7) P. D. Adawadkar and M. A. El Sohly, *Fitoterapia*, **52**, 129 (1981).
8) J. L. Gellerman, N. J. Walsh, N. K. Werner, and H. Schlenk, *Can. J. Microbiol.*, **15**, 1219 (1969).
9) R. J. Grean, N. J. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep.*, Part 3, **3**, 1 (1972).