

THRAZARINE, A NEW ANTITUMOR ANTIBIOTIC

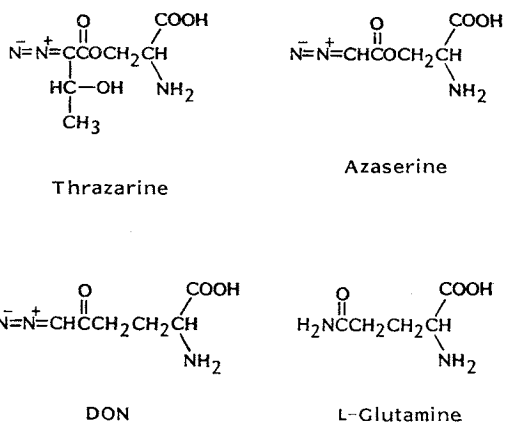
I. TAXONOMY, FERMENTATION, ISOLATION
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Thrazarine, *O*-((3*R*)-2-diazo-3-hydroxybutyryl)-L-serine, is a new antitumor antibiotic produced by *Streptomyces coeruleus* MH802-fF5. Thrazarine was isolated from culture filtrate by Sephadex LH-20 column chromatography and reversed phase HPLC. Thrazarine induced cytolysis of tumor cell lines co-cultured with nonactivated macrophages. This effect was tumor specific because the nontumorigenic cells were not lysed by macrophages in the presence of thrazarine. Thrazarine inhibited DNA synthesis and growth of tumor cells directly. It showed neither antimicrobial activity nor the inhibition of transamidation reactions in contrast to azaserine. Toxicities of thrazarine were much weaker than those of azaserine.

Activated macrophages exert potent tumoricidal activities *in vitro* and are recognized as important effectors in antitumor resistance *in vivo*.^{1,2)} On the other hand, it was reported that for experimental tumors, the sensitivity to macrophage-mediated cytolysis differed among the tumor variants³⁾ and the variants relatively resistant to macrophages possessed enhanced tumorigenicity.⁴⁾ This might be one of the means by which some malignant tumor cells escape host antitumor activity. In this respect, we have screened substances enhancing macrophage-mediated lysis of tumor cells and previously reported an active substance, bisucaberin obtained from a marine bacteria.⁵⁾ Another new substance was found from a culture broth of a soil actinomycete isolate. This was an antitumor antibiotic containing a diazo residue and named thrazarine (Fig. 1). This report describes identification of the producing organism, and isolation and biological properties of thrazarine. Physico-chemical properties and structural determination of thrazarine will be described in an accompanying paper.⁶⁾

Fig. 1. Structures of thrazarine and diazo analogs of L-glutamine.



Materials and Methods

Materials

Azaserine and 6-diazo-5-oxo-L-norleucine (DON) were purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. Fetal bovine serum

(FBS) and other cell culture materials were purchased from GIBCO, Grand Island, N.Y., U.S.A.

Female C3H/HeNcrj mice of 8 weeks-old were obtained from Charles River Japan Inc., Kanagawa, Japan.

Taxonomical Examination

The producing organism, strain MH802-fF5, was isolated from a soil sample collected at our institute property, Tokyo.

Morphological, cultural and physiological properties of strain MH802-fF5 were examined according to the methods described by SHIRLING and GOTTLIEB,⁷⁾ and WAKSMAN.⁸⁾ Detailed observation of mycelial morphologies was performed with light and scanning electron microscopy after the strain was incubated on various media at 27°C for 7 to 14 days. The color recorded for the mature culture was described according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America). Cell wall analysis was performed by the method of BECKER *et al.*⁹⁾

Fermentation

Fermentation medium consisted of soluble starch 3%, dried yeast (Ebios, Asahi Breweries, Ltd., Tokyo, Japan) 3%, K₂HPO₄ 0.3%, KH₂PO₄ 0.1%, MgSO₄·7H₂O 0.05%, NaCl 0.2% and CaCO₃ 0.1% in deionized water (pH 7.2 before sterilization). A loopful of slant culture of strain MH802-fF5 was inoculated into 100 ml of the fermentation medium in an Erlenmeyer flask of 500-ml capacity and cultured at 27°C for 5 days on a rotary shaker (180 rpm).

Isolation

Broth filtrate containing the antibiotic principle was adjusted to pH 7.5 with 1 M NaOH and concentrated *in vacuo*. To the concentrate was added 9 volumes of EtOH under continuous stirring. The inactive precipitate thus formed was removed and the EtOH filtrate was evaporated to give a hygroscopic solid. The crude solid was dissolved in a small quantity of 50% aq MeOH. After removing insoluble precipitate, the filtrate was applied to a Sephadex LH-20 column followed by elution with 50% aq MeOH. Active fractions were collected and lyophilized after MeOH was removed by evaporation. The resulting powder was applied to reversed phase HPLC (ODS-5251-SH, 20×250 mm, Senshu Science Co., Japan; mobile phase, 5% aq MeOH). The active eluate was concentrated and lyophilized to give a brownish powder. This powder was dissolved in hot MeOH and allowed to stand at 5°C to give crystals of pure thazarine.

Analytical HPLC and TLC

Thazarine contents in fermentation and purification steps were monitored with reversed phase HPLC and silica gel TLC. HPLC was performed with an ODS-PE-1 column (10×300 mm, Senshu Science Co., Japan) eluted with 10% aq MeOH at the flow rate of 2 ml/minute. Elution profile was detected with UV absorbance at 256 nm. Thazarine was eluted at 11.6 minutes. TLC was performed with Kieselgel 60 F₂₅₄ (5715, Merck) developed with CHCl₃ - MeOH - water (5:5:1). Spots were detected by anisaldehyde - sulfuric acid. Rf value of thazarine was 0.43.

Tumor Cells

Fibrosarcoma 1023, a tumorigenic tumor cell line was kindly donated by Dr. T. TOKUNAGA, National Institute of Health, Tokyo, Japan. This cell line had been induced in a C3H/HeNcr male mouse with 3-methylcholanthrene and has been maintained in culture with DULBECCO's modified EAGLE's medium supplemented with 10% FBS.¹⁰⁾

Balb/3T3 clone A31, Balb/3T12-3, C3H/10T1/2 clone 8 and C3H/MCA clone 15 were obtained from Dainippon Pharmaceuticals Co., Ltd., Osaka, Japan and cultured according to the original methods described by AARONSON and TODARO,¹¹⁾ and REZNIKOFF *et al.*¹²⁾ Balb/3T3 and C3H/10T1/2 are contact-inhibited nontumorigenic cell lines developed from mouse embryos. Balb/3T12 was established by using a transfer schedule in which cell-cell contact was more extensive than that of Balb/3T3. C3H/MCA was derived by treating C3H/10T1/2 with 3-methylcholanthrene. These two cell lines are tumorigenic and extremely insensitive to contact inhibition.

L1210 was cultured with EAGLE's minimum essential medium supplemented with 10% FBS.

IMC carcinoma and human tumor cell lines (SC-6 and LX-1) were cultured with RPMI 1640 medium supplemented with 10% FBS.

Macrophage-mediated Cytolysis Assay

The details of macrophage-mediated cytolysis assay were described previously.⁵⁾ Murine peritoneal macrophages elicited with Proteose-peptone were used as effector cells. ³H-Prelabeled target cells were co-cultured with macrophage-monolayers in a 96-well microtiter plate for 2 days. The effector/target ratio was 20. Results were expressed as follows:

$$\% \text{ Specific lysis} = \frac{\text{Experimental release (dpm)} - \text{Spontaneous release (dpm)}}{\text{Maximum release (dpm)} - \text{Spontaneous release (dpm)}} \times 100$$

Cell Growth and Macromolecular Synthesis

Exponentially growing tumor cells (2×10^4 cells/microtiter well) were cultured for 2 days with thiazarine. Cell number was measured by a Coulter counter after dilution with saline. The tetrazolium dye (MTT) reduction assay was conducted according to the method of RUBEN and NEUBAUER.¹³⁾

Radiolabeled precursor incorporation into fibrosarcoma 1023 was measured by the method described previously.⁵⁾

Inhibition of GMP-Synthetase

Crude GMP-synthetase was prepared from Ehrlich ascites tumor cells by the method of SPECTOR.¹⁴⁾ The enzyme activity was measured according to the method of SAKAMOTO.¹⁵⁾ The reaction mixture contained 2.4 mM XMP, 4 mM ATP, 4 mM L-glutamine and 16 mM MgCl₂. The inhibitors were added to the reaction mixture without L-glutamine and preincubated for 5 minutes. The enzyme reaction was started by the addition of L-glutamine.

Results

Taxonomic Features of the Strain MH802-ff5

The vegetative mycelium developed well on most of the media used. The strain showed abundant growth with aerial mycelium varying in color from white to light aqua gray or light bayberry gray depending on the medium. Mature spores occurred in chains of more than 20 spores forming spirals. The spores were oval with spiny surfaces and $0.7 \sim 0.9 \times 1.2 \sim 1.4 \mu\text{m}$ in size. Verticillus was not observed. The reverse side color of colonies was pale yellow to dark brown. Soluble pigment was negative or faintly brownish and not pH sensitive when tested with 0.05 M HCl or 0.05 M NaOH. Melanoid pigment was formed in each of Tryptone - yeast extract broth, peptone - yeast extract - iron agar and tyrosine agar. Starch hydrolysis was strong. Milk coagulation and peptonization were relatively faint. Gelatin liquefaction was negative in 15% gelatin and weak in glucose - peptone - gelatin. Nitrate reduction was positive. Carbon utilization was all positive with D-glucose, L-arabinose, D-xylose, D-fructose, sucrose, inositol, L-rhamnose, raffinose and D-mannitol.

The whole cell lysate contained LL-diaminopimeric acid.

From these taxonomic properties, strain MH802-ff5 was considered to belong to the genus *Streptomyces*. Compared with the type cultures held in our institute and the published descriptions of *Streptomyces* species, strain MH802-ff5 resembled *Streptomyces coeruleus* very closely in morphological and cultural properties. Therefore, the strain was identified as *S. coeruleus* MH802-ff5.

Strain MH802-ff5 has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. FERMP-9812.

Isolation

Thiazarine production began at day 3 and attained the maximum accumulation (200 $\mu\text{g/ml}$) at

day 5. Thrazarine was markedly unstable except in the range of pH 6 to 9. Use of activated charcoal, silica gel or ion exchange resins for the isolation gave very low recovery of active principle (data not shown). Thrazarine could be successfully purified when the pH of broth filtrate was adjusted in the range of 7.5 to 8.0 and Sephadex LH-20 column chromatography and reversed phase HPLC were used. Pure thrazarine was finally obtained by crystallization in methanol in a 31.6%-yield against a culture filtrate.

Biological Activities

Thrazarine induced strong lysis of fibrosarcoma 1023 co-cultured with peptone-elicited macrophages (Fig. 2). The induction of tumor cell lysis depended on thrazarine concentration and reached the maximum at 10 $\mu\text{g/ml}$ of thrazarine. Thrazarine itself caused a little lysis of tumor cells even at 90 $\mu\text{g/ml}$ when macrophages were not present. Macrophages alone did not lyse tumor cells in the absence of thrazarine.

Mouse embryo cells (Balb/3T3, C3H/10T1/2) and their tumorigenic counterparts (Balb/3T12, C3H/MCA) were used as the target cells of macrophage-mediated cytotoxicity (Fig. 3). Peptone-elicited macrophages were not cytotoxic to these four cell lines. As shown in Fig. 3(A), thrazarine caused strong lysis of Balb/3T12 co-cultured with macrophages. On the other hand, its non-tumorigenic counterpart, Balb/3T3 was only slightly lysed by macrophages in the presence of thrazarine. This was also the case of the pair of C3H/10T1/2 and C3H/MCA except that the maximum release did not exceed 20% (Fig. 3(B)).

Thrazarine inhibited the growth of various tumor cells including the human tumor cell line,

Fig. 2. Cytolysis of fibrosarcoma 1023 induced by thrazarine with (○) or without (●) macrophages.

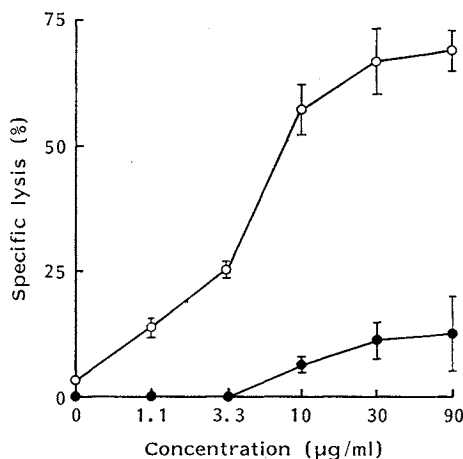


Fig. 3. Tumor specific sensitization by thrazarine to macrophage-mediated cytotoxicity.

(A) Balb/3T3 (●), Balb/3T12 (○); (B) C3H/10T1/2 (●), C3H/MCA (○).

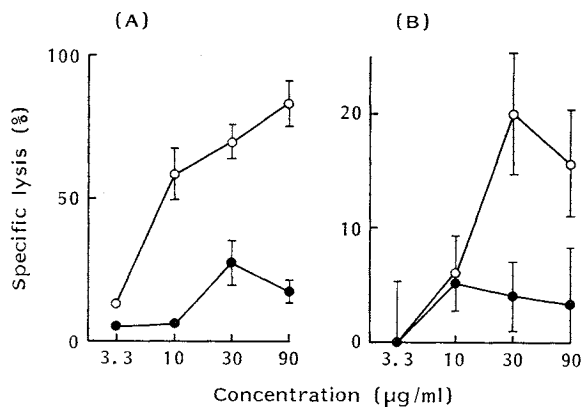


Fig. 4. Growth inhibition of tumor cells by thrazarine.

The inhibition was estimated with a Coulter counter for (A), L1210 (●) and IMC carcinoma (○), and by MTT assay for (B), SC-6 (●) and LX-1 (○).

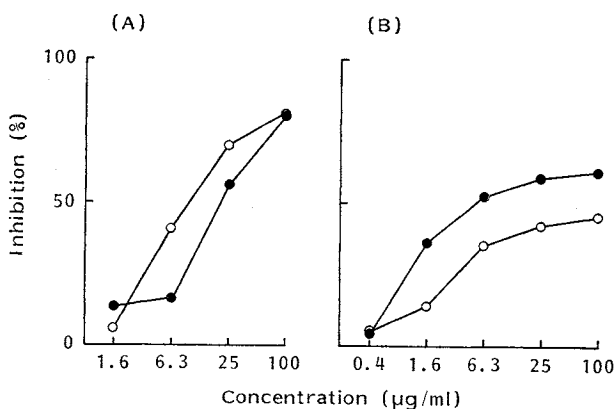
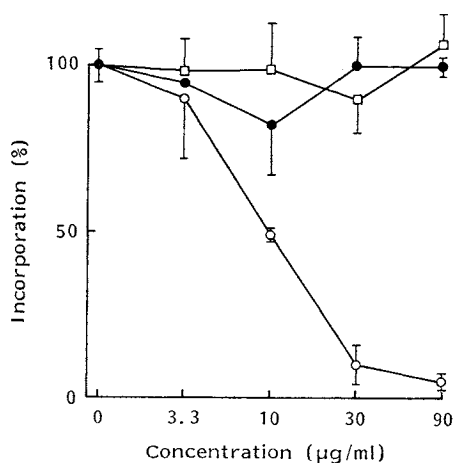


Fig. 5. % Incorporation of thymidine (○), leucine (□) and uridine (●) into acid insoluble materials in the presence of thrazarine against non-treated cells.



Thrazarine also inhibited [³H]thymidine incorporation into acid insoluble materials after 4 hours preincubation of fibrosarcoma 1023 (Fig. 5). The incorporation of [³H]leucine and [³H]uridine were not affected by thrazarine. The inhibition of growth and thymidine incorporation were not tumor specific, because there was no difference between tumorigenic and nontumorigenic cell line (data not shown).

Differences between Thrazarine and Azaserine

In relation to the structural similarity shown in Fig. 1, we examined the biological properties of the related antibiotics. As shown in Table 1, GMP-synthetase was inhibited by azaserine and DON. The inhibition was dose-dependent and irreversible. On the other hand, thrazarine did not inhibit GMP-synthetase even at two hundred times the active dose of azaserine.

Induction of macrophage-mediated cytotoxicity by azaserine could not be determined, because it

Table 1. Effects of thrazarine and diazo analogs of L-glutamine on GMP-synthetase activity.

Inhibitor	Conc (mM)	ΔA_{290}	Inhibition (%)
—	—	0.369	0
Thrazarine	12	0.356	3.5
	6	0.394	-6.8
Azaserine	6	0.087	76.4
	0.06	0.272	26.3
DON	6	-0.057	115
	0.06	0.047	87.3

SC-6 (Fig. 4). IC_{50} values ($\mu\text{g/ml}$) were as follows: L1210, 20.5; IMC carcinoma, 9.6; SC-6, 5.25. The growth inhibition for LX-1 was weaker than those for the others, so that it did not exceed 50%.

was toxic to macrophages in the same concentration range as in Fig. 2.

Thrazarine showed no inhibitory activity at 100 $\mu\text{g}/\text{ml}$ against Gram-positive and Gram-negative bacteria, mycobacteria, yeasts and molds tested by the agar-dilution method. Thrazarine showed no toxicity in ICR mice by single iv injection of 250 mg/kg. Mice injected with 500 mg/kg died at day 5.

Discussion

Peptone-elicited macrophages used in this study were not tumoricidal unless they had been activated by macrophage activating factors. Thrazarine induced strong lysis of tumor cells co-cultured with these nonactivated macrophages. The induced cytolysis was mediated by macrophages because tumor cells cultured with thrazarine alone showed only a little lysis. The induction of cytolysis by thrazarine was tumor specific, while direct effects of thrazarine, that is, the inhibition of DNA synthesis and growth were not. This suggests that peptone-elicited macrophages can distinguish tumor cells from normal cells as well as BCG activated macrophages which were cytotoxic to Balb/3T12 but not to Balb/3T3.¹⁶⁾ Because preincubation with thrazarine did not activate macrophages to be tumoricidal (data not shown), the induction of cytolysis may be attributable to sensitization of tumor cells to latent cytotoxicity of peptone-elicited macrophages.

Diazo analogs of L-glutamine, such as azaserine and DON, irreversibly inhibit purine biosynthesis, in which L-glutamine participates as donor of an amino residue.¹⁷⁾ The enzyme most sensitive to inhibition by azaserine is formylglycinamide amidotransferase. Azaserine covalently binds to a cysteine residue in the catalytic site of this enzyme. GMP-synthetase (xanthosine-5'-phosphate: L-glutamine amidolygase (AMP), EC 6.3.5.2) is also inhibited by diazo analogs of L-glutamine.¹⁸⁾ We examined the inhibition of this enzyme by thrazarine. As opposed to azaserine, thrazarine showed no inhibitory activity, suggesting that it does not serve as an L-glutamine analog. This may be a reason that azaserine showed antimicrobial activities but thrazarine did not. Azaserine was also reported to cause DNA damage in eukaryotic cells as do other alkylating agents.¹⁸⁾ Thrazarine may exert its antitumor activity through DNA alkylation.

Azaserine was reported to be a potent bacterial mutagen producing 12,000 revertants/nmol in the Ames assay on strain TA100 without S-9 mix.¹⁹⁾ Mutagenicity of thrazarine was extremely weaker than that of azaserine (Dr. S. SATO; a personal communication): Thrazarine produced less than 30 revertants/nmol even with S-9 mix. Furthermore, azaserine is toxic and carcinogenic in animals especially to pancreatic cells. Thrazarine may be improved in this point. In fact, acute toxicity in mice of thrazarine was weak on comparing to LD₅₀ doses of azaserine (100 mg/kg, ip)²⁰⁾ and DON (76 mg/kg, iv).²¹⁾

Acknowledgment

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References

- 1) OTTER, W. D.; H. F. J. DULLENS & R. A. D. WEGER: Macrophages and antitumor reactions. *Cancer Immunol. Immunother.* 16: 67~71, 1983
- 2) KELLER, R.: Characteristics of cytotoxic macrophages as natural effectors of resistance to cancer. *Clin. Immunol. Allergy* 3: 523~537, 1983
- 3) MINER, K. M. & G. L. NICOLSON: Differences in the sensitivities of murine metastatic lymphoma/lymphosarcoma variants to macrophage-mediated cytolysis and/or cytostasis. *Cancer Res.* 43: 2063~2067, 1983
- 4) REMELS, L. M. & P. C. DE BAETSELIER: Characterization of 3LL-tumor variants generated by *in vitro* macrophage-mediated selection. *Int. J. Cancer* 39: 343~352, 1987
- 5) KAMEYAMA, T.; A. TAKAHASHI, S. KURASAWA, M. ISHIZUKA, Y. OKAMI, T. TAKEUCHI & H. UMEZAWA: Bisucaberin, a new siderophore, sensitizing tumor cells to macrophage-mediated cytolysis. I. Taxonomy

- of the producing organism, isolation and biological properties. *J. Antibiotics* 40: 1644~1670, 1987
- 6) TAKAHASHI, A.; H. NAKAMURA, D. IKEDA, H. NAGANAWA, T. KAMEYAMA, S. KURASAWA, Y. OKAMI, T. TAKEUCHI & Y. IITAKA: Thrazarine, a new antitumor antibiotic. II. Physico-chemical properties and structure determination. *J. Antibiotics* 41: 1568~1574, 1988
 - 7) SHIRLING, E. D. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16: 313~340, 1966
 - 8) WAKSMAN, S. A. (Ed.): *The Actinomycetes. Vol. 2. Classification, Identification and Descriptions of Genera and Species.* Williams & Wilkins Co., Baltimore, 1961
 - 9) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell wall preparation from strains various form genera of aerobic actinomycetes. *Appl. Microbiol.* 12: 236~243, 1965
 - 10) NAKAMURA, R. M. & M. S. MELTZER: Macrophage activation for tumor cytotoxicity: Control of cytotoxic activity by the time interval effector and target cells are exposed to lymphokines. *Cell. Immunol.* 65: 52~65, 1981
 - 11) AARONSON, S. A. & G. J. TODARO: Development of 3T3-like lines from Balb/c mouse embryo cultures: Transformation susceptibility to SV40. *J. Cell. Physiol.* 72: 141~148, 1968
 - 12) REZNIKOFF, C. A.; D. W. BRANKOW & C. HEIDELBERGER: Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.* 33: 3231~3238, 1973
 - 13) RUBEN, R. L. & R. H. NEUBAUER: Semiautomated colorimetric assay for *in vitro* screening of anticancer compounds. *Cancer Treat. Rep.* 71: 1141~1149, 1987
 - 14) SPECTOR, T.: Studies with GMP synthetase from Ehrlich ascites cells. *J. Biol. Chem.* 250: 7372~7376, 1975
 - 15) SAKAMOTO, N.: GMP synthetase (*Escherichia coli*). In *Methods in Enzymology*, Vol. 51, Eds., P. A. HOFFEE & M. E. JONES, pp. 213~218, Academic Press, New York, 1978
 - 16) HIBBS, J. B., Jr.: Macrophage nonimmunologic recognition: Target cell factors related to contact inhibition. *Science* 10: 868~890, 1973
 - 17) GALE, E. F.; E. CUNLIFFE, P. E. REYNOLDS, M. H. RICHMOND & M. J. WARING (Ed.): *The Molecular Basis of Antibiotic Action.* pp. 24~48, John Wiley & Sons Ltd., London, 1972
 - 18) LILJA, H. S.; E. HYDE, D. S. LONGNECKER & J. D. YAGER, Jr.: DNA damage and repair in rat tissues following administration of azaserine. *Cancer Res.* 37: 3925~3931, 1977
 - 19) PERANTONI, A.; J. J. BERMAN & J. M. RICE: L-Azaserine toxicity in established cell lines. Correlation with γ -glutamyl transpeptidase activity. *Exp. Cell Res.* 122: 55~61, 1979
 - 20) DUVALL, L. R.: Azaserine. *Cancer Chemother. Rep.* 7: 65~86, 1960
 - 21) DUVALL, L. R.: 6-Diazo-5-oxo-L-norleucine. *Cancer Chemother. Rep.* 7: 86~98, 1960