OCCURRENCE OF AN ASCAMYCIN DEALANYLATING ENZYME, Xc-AMINOPEPTIDASE, IN MAMMALIAN CELL MEMBRANES AND SUSCEPTIBILITY TO ASCAMYCIN

HIROYUKI OSADA and KIYOSHI ISONO*

Antibiotics Laboratory, Riken, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01, Japan

(Received for publication September 14, 1985)

An ascamycin dealanylating enzyme (Xc-aminopeptidase) has been isolated from *Xanthomonas citri* and characterized as a proline iminopeptidase of molecular weight of 38,000 (H. OSADA & K. ISONO, Biochem. J. 233: 459~463, 1986). Immunological studies have shown that all the mammalian cells tested possess a closely-related enzyme(s) on their cell membranes. This enzyme is more active in transformed cells than in nontransformed cells. A decreased ratio of ID_{50} (ascamycin/dealanylascamycin based on [^{35}S]methionine uptake) in transformed cells compared with the nontransformed cell can be explained on the basis of the conversion of ascamycin to dealanylascamycin by the enzyme. It is suggested that ascamycin cannot be transported through mammalian cell membranes but dealanylascamycin can permeate; a similar situation exists in prokaryotic cells.

Ascamycin is an unique nucleoside antibiotic having an alanyl-sulfamoyl group on the 5'-oxygen of 2-chloroadenosine[1]. It has selective antibacterial activity against plant pathogenic *Xanthomonas* species, while the dealanylated derivative shows broad antibacterial activity. We have shown[2] that both ascamycin and dealanylascamycin inhibit bacterial protein synthesis in a cell free system but the antibacterial activity of ascamycin is masked by the alanyl group which interferes with transport into bacteria. *Xanthomonas citri* is susceptible to ascamycin by virtue of an ascamycin dealanylating enzyme which exists on the cell surface (Fig. 1). Other bacteria which lack the enzyme are not susceptible to ascamycin. We have isolated and characterized the ascamycin hydrolyzing aminopeptidase from *X. citri*[3]. The enzyme, molecular weight of 38,000, can be classified as a proline iminopeptidase [EC.3.4.11.5].

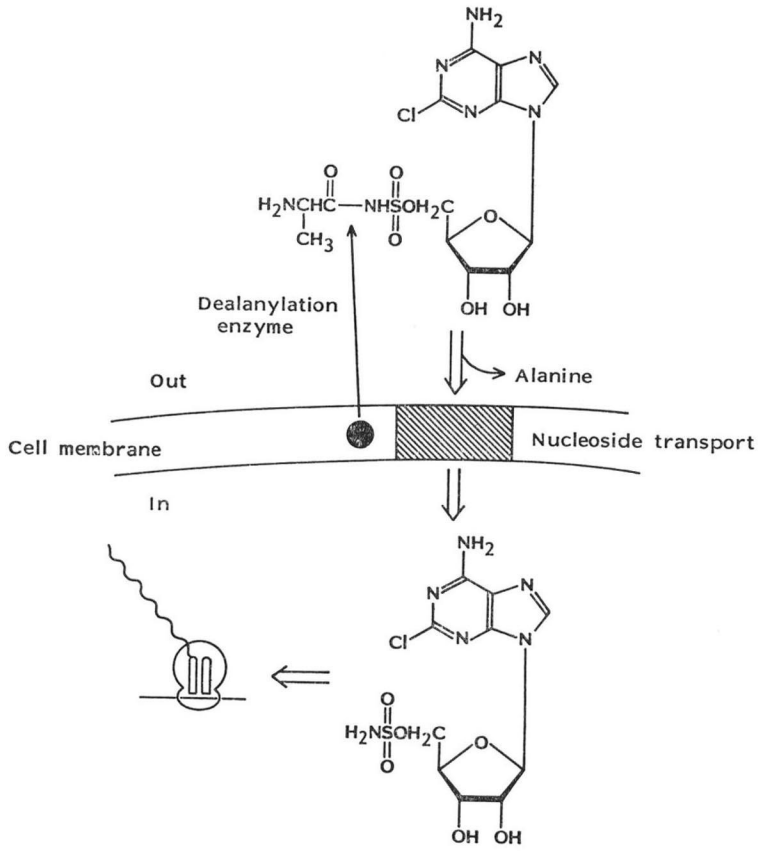
The purpose of this study was to examine the distribution of the ascamycin dealanylating enzyme in mammalian cells. Our experiments have shown that transformed cells possess generally higher ascamycin dealanylating activity than nontransformed cells.

Materials and Methods

Cell Lines and Culture Conditions

The cell lines used are listed in Table 1. Mouse derived cell lines, Balb3T3 clone A31, SV40 virus transformed cell SV-3T3[4], and Kirsten sarcoma virus transformed cell Ki-3T3[5] were grown in EAGLE's minimal essential medium (Nissui, Tokyo) supplemented with 10% fetal bovine serum (Gibco). Rat derived cell lines (NRK and its Harvey sarcoma virus transformed cell)[6,7] and mouse derived cell lines (NIH L1 and its chemical carcinogen transformant L1-4NQO$_{sd}$)[8,9] were cultured under the same conditions as Balb3T3 cell lines. Human chronic myeloid cell, K-562[10], was grown in RPMI 1640 medium (Gibco) containing 10% serum and antibiotics (penicillin and streptomycin). These cell lines were gifts from Dr. K. KOHNO (National Institute for Basic Biology, Okazaki). Human promyeloid leukemia cell, HL60[11] was cultured under the same conditions as K-562. Friend mouse

Fig. 1. Mechanism of selective toxicity of ascamycin to *Xanthomonas citri*.



erythroleukemic cells[12] were grown in EAGLE's minimal essential medium containing 12.5% fetal bovine serum. HL60 and Friend cells were provided by Dr. S. NOMURA (University of Tokyo).

Cells were incubated in a humidified atmosphere (5% CO_2) at 37°C. Cell morphology was observed by phase contrast microscopy (TMD, Nikon). Doubling time were calculated from the growth curve of the control culture.

Preparation of Subcellular Fractions

Adherent cells were harvested with a rubber policeman without trypsin. Floating cells were harvested by centrifugation. Both types of cells were washed with phosphate buffered saline, PBS(−), and disrupted by a sonicator (UR200, Tomy Seiko) under mild conditions to preserve intact nuclei (150W, 20 seconds). After the cell lysate was adjusted to a sucrose concentration of 8.6%, it was overlaid onto 37% sucrose in 10 mM Tris-HCl (pH 7.8) in a centrifuge tube.

After centrifugation at 65,000×*g* for 60 minutes at 4°C in a swinging rotor (RPS-40T, Hitachi), the upper layer contained cytoplasmic proteins or soluble proteins, the boundary fluff was a membrane rich fraction, and the pellet contained nuclei and unbroken cells. The protein concentration of each subcellular fraction was adjusted to 3 mg/ml, stored at −20°C, and used for assays.

Assay for Proline Iminopeptidase Activity

Each subcellular fraction was used at a concentration of 0.1 mg protein per ml in the reaction mixture. Spectrophotometric measurement was carried as previously described[3].

Preparation of Antiserum against Xc-aminopeptidase

The antiserum was obtained by two subcutaneous injections of 1 mg of Xc-aminopeptidase at 10-day intervals to two rabbits (New Zealand white, female, 2 kg). The Xc-aminopeptidase in 0.5 ml

of Tris-HCl buffer was emulsified with 0.5 ml of Freund's complete adjuvant for the first injection and emulsified with 0.5 ml of Freund's incomplete adjuvant for the second injection. The antiserum was collected from the rabbit 10 days after the second injection. The titer of anti-Xc-aminopeptidase was estimated by the OUCHTERLONEY method[13]. Normal serum did not react with Xc-aminopeptidase but immune serum diluted 1/128 did cross-react. The serum was partially purified by 30% ammonium sulfate precipitation, which was used as the anti-Xc-aminopeptidase IgG rich fraction.

Indirect Immunofluorescence Technique

Adherent cells were cultured to preconfluence in the Lab-Tek chamber (Miles, Naperville, Illinois). In the case of floating cells, cultured cells were harvested by centrifugation and spread onto a glass slide. Both types of cells on the slide were washed with two changes of PBS(−) and fixed with 3.7% formalin in PBS(−) for 20 minutes. Then the cells were washed with PBS(−) and incubated with 30-fold diluted anti-Xc-aminopeptidase IgG rich fraction in PBS(−) for 45 minutes at 37°C. After the cells were washed, they were incubated for 30 minutes at room temperature with FITC (fluorescein isothiocyanate)-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories Inc., Maryland) at a concentration of 10 μg/ml. Well washed samples were observed by fluorescence microscopy (XF-EFD, Nikon).

Western Blotting

Each 50 μg sample of cell membrane protein was applied to sodium dodecyl sulfate (SDS)-polyacrylamide gel (12.5%) electrophoresis[14]. The developed proteins were transferred to a nitrocellulose membrane using the Bio-Rad electro-blotting system. Following binding of the antigen to the membrane, the remaining unbound sites were blocked with gelatin. The membrane with bound antigen was incubated with the first antibody (anti-Xc-aminopeptidase IgG), washed to remove unbound antibody, incubated with the second antibody (horse radish peroxidase-conjugated goat anti-rabbit IgG), and washed again. 4-Chloro-1-naphthol was used as substrate for horse radish peroxidase and the developed membrane showed purple bands. All reagents used in this procedure were purchased from Bio-Rad (Richmond, CA).

Susceptibility of Cells to Ascamycin and Dealanylascamycin

Since both ascamycin and dealanylascamycin inhibit protein synthesis, inhibition of [^{35}S]methionine uptake into the acid-insoluble fraction was measured in the presence of antibiotic. Cells were grown in each medium to the logarithmic phase, then a series of antibiotic dilution was added. After 20 hours incubation, the medium was changed to fresh Ham's F-12[15] containing 10% serum, each concentration of antibiotic, and 5 μCi/ml [^{35}S]methionine (1,120 Ci/mmol, New England Nuclear). The cells were labeled for 3 hours, washed, and harvested. After treatment with ice-cold trichloroacetic acid, radioactivity in the acid-insoluble fraction was measured by a liquid scintillation counter.

Results

Proline Iminopeptidase Activity of Normal and Transformed Cells

Proline iminopeptidase activity of the cell line subcellular fractions listed in Table 1 was measured. In the course of purification of Xc-aminopeptidase from *X. citri*, we had observed that proline iminopeptidase activity was proportional to ascamycin dealanylating activity[3]. In each types of cells, the activity was localized on the cell membrane and the activity of transformed cells was higher than that of the nontransformed cells (Table 1).

The dealanylation of ascamycin can be detected semi-quantitatively by the decrease of Rf value on cellulose thin-layer chromatography and the appearance of antibacterial activity against *Escherichia coli* BE1186[2]. The membrane fraction of SV-3T3 showed higher dealanylation activity than that of Balb3T3 (Fig. 2). Because of the limited supply of ascamycin, ascamycin dealanylating activity of the other cells was not examined.

Table 1. Characteristics of cell lines and their proline iminopeptidase activity[a].

Cell line	Transforming agent	Doubling time (hours)	Anchorage independent growth[b]	Proline iminopeptidase activity[c]		
				Cytoplasm	Membrane	Precipitate
Balb3T3		13.7	−	0.7	4.9	0
SV-3T3	SV40	13.3	+	0.6	9.4	0.4
Ki-3T3	Kirsten-MSV[d]	12.5	+	3.6	16.2	1.8
NRK		24.0	−	0.2	3.8	0.9
Ha-NRK	Harvey-MSV	15.1	+	2.0	15.5	1.8
NIH L1		17.5	−	0.9	5.8	0.7
L1-4NQO$_{ad}$	4-Nitriquinoline-1-oxide	11.4	+	1.8	12.7	1.1
K-562	(Human leukemia)	13.1	(+)	3.3	15.7	2.0
HL60	(Human leukemia)	19.6	(+)	0.8	8.0	0.6
Friend	Friend virus	10.0	(+)	1.5	7.6	0

[a] Enzyme activity (nmol/minute/mg protein) was assayed as described[7] using L-proline-β-naphthylamide as substrate.
[b] Anchorage independent growth was indicated by the colony-forming ability in semi-solid medium[16].
[c] Crude enzymes were prepared by sucrose density gradient centrifugation as described in the text. Precipitate contained nucleus and unbroken cells.
[d] Murine sarcoma virus.

Fig. 2. Dealanylation of ascamycin by cell fractions as detected by cellulose thin-layer chromatography [solvent system, 2-propanol - 1 N ammonia (7: 3)].

a, Ascamycin; b, dealanylascamycin; c~i, reaction products of ascamycin treated subcellular fraction; c, Xc-aminopeptidase from *X. citri*; d~f, subcellular fractions of Balb3T3; g~i, subcellular fractions of SV40 transformed Balb3T3; d, g, cytoplasmic proteins; e, h, membrane bound proteins; f, i, nucleus and unbroken cells.

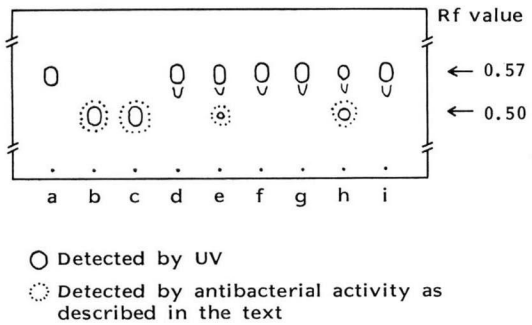
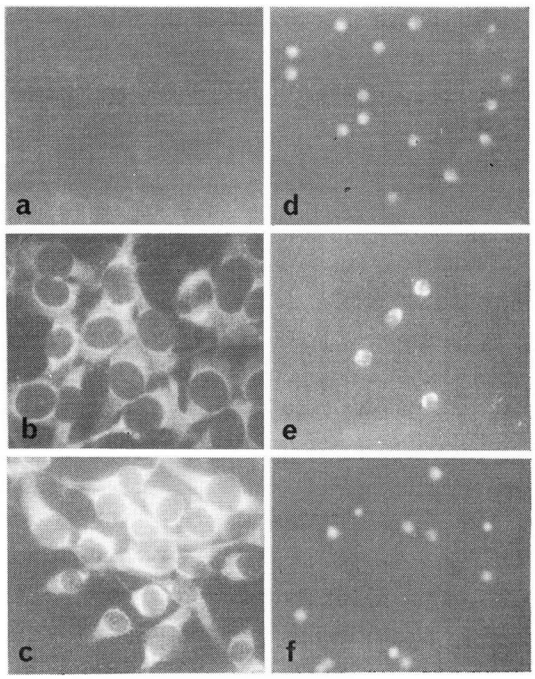


Fig. 3. Indirect immunofluorescence microscopy.

a, Balb3T3; b, SV-3T3; c, Ki-3T3; d, K-562; e, HL60; f, Friend cell.

a~c, Observed at a magnifying ×200; d~f, ×100.



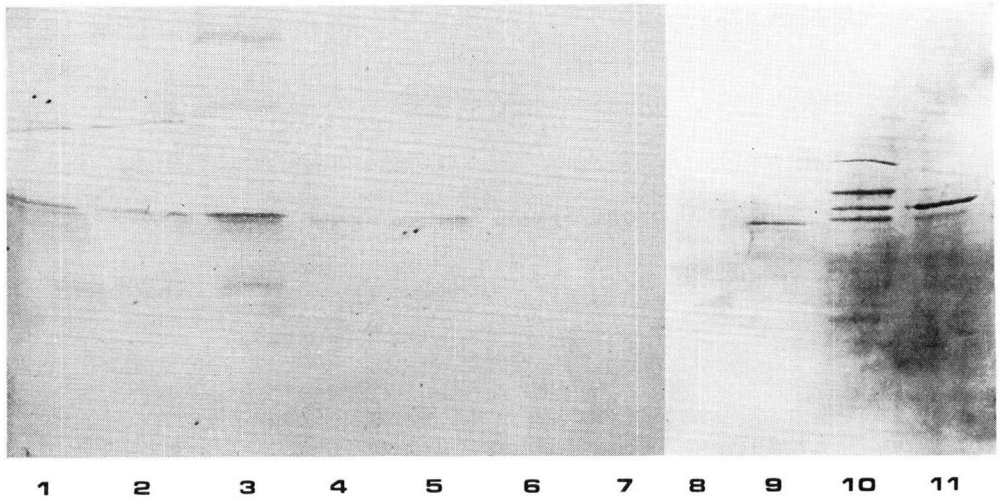
Presence of Enzyme(s) Similar to Xc-aminopeptidase

The presence of antigen to anti-Xc-aminopeptidase IgG on the mammalian cell membranes was demonstrated in the following two ways.

1) Indirect Immunofluorescence Microscopy: Intense fluorescence was observed on the cell membranes of transformed cells while only slight fluorescence was observed on those of nontransformed

Fig. 4. Western blotting pattern.

Lane 1, Balb3T3; 2, SV-3T3; 3, Ki-3T3; 4, NIH L1; 5, L1-4NQO$_{ad}$; 6, K-562; 7, HL60; 8, Friend; 9, NRK; 10, Ha-NRK; 11, 0.1 μg of Xc-aminopeptidase purified from *X. citri*.



cell (Figs. 3a~c). Fluorescence of transformed cells was stronger than that of the nontransformed cell with other cell lines (data not shown). FITC-positive material was detected not only on murine cells but also on human cells (Figs. 3d, 3e).

2) Western Blotting: All the cell membranes tested were found to have antigen cross-reacting with anti-Xc-aminopeptidase IgG (Fig. 4). The bands appearing on the nitrocellulose membrane indicated that they had the almost same molecular weight as Xc-aminopeptidase (38,000 daltons). Virus transformed cells, especially Ki-3T3 and Ha-NRK contained a large amount of the antigen. It is to be noted that both cell lines possess the *ras* oncogene whose product is a 21,000-dalton protein[7,16].

Susceptibility of Normal and Transformed Cells to Ascamycin and Dealanylascamycin

Many bacteria other than *Xanthomonas* species and fungi are resistant to ascamycin (*i.e.* they tolerate 200 μM of ascamycin). However all the mammalian cells tested were susceptible to ascamycin at ID_{50} (50% inhibition dose) values of 0.5 to 6.6 μM (Fig. 5).

As described previously in the case of prokaryotic cells, ascamycin is considered not to be able to permeate the membrane while dealanylascamycin is transported into the cytoplasm[2]. If this is also the case for eukaryotic cells, the ratio of ID_{50} of ascamycin and dealanylascamycin (Table 2) may represent an inverse index of the conversion efficiency from ascamycin to dealanylascamycin, hence the efficiency of membrane transport of ascamycin.

The proline iminopeptidase activity (shown in Table 1) was plotted in Fig. 6 against the ratio of ID_{50} of ascamycin to dealanylascamycin (shown in Table 2). Statistical calculation indicated that there is the strong correlation between two factors. These data suggest that proline iminopeptidase activity is increased in transformed cells, resulting in the increase of susceptibility to ascamycin. It is considered that ascamycin is dealanylated by the enzyme, and then transported across the membrane.

Discussion

We have already reported the mode of action and the selective toxicity of ascamycin in bacteria[2].

Fig. 5. Inhibition curve of ascamycin and dealanylascamycin.

●, Inhibition curve of ascamycin; ○, inhibition curve of dealanylascamycin.

1, Balb3T3; 2, SV-3T3; 3, Ki-3T3; 4, NRK; 5, Ha-NRK; 6, NIH L1; 7, L1-4NQO$_{ad}$; 8, K-562; 9, HL60; 10, Friend.

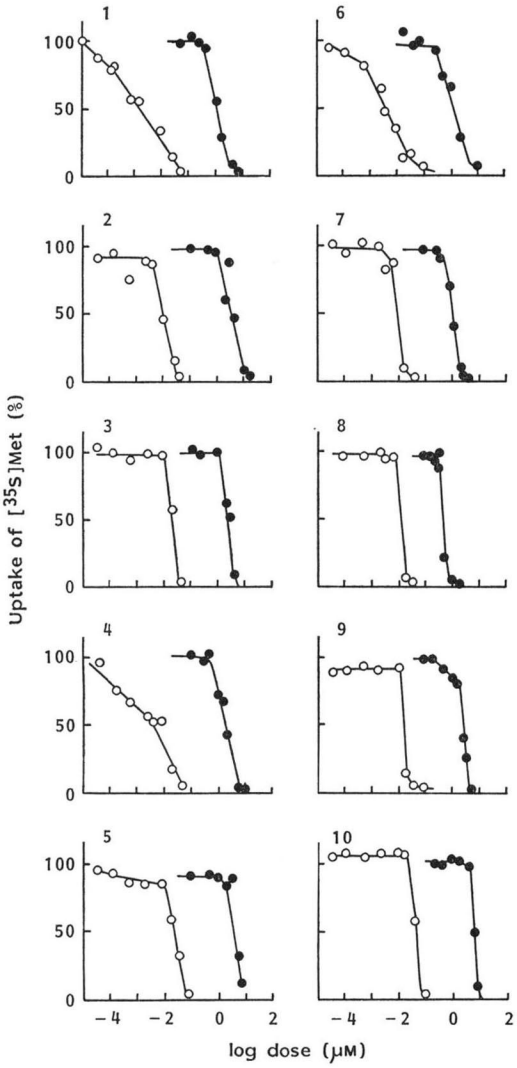


Fig. 6. Correlation between proline iminopeptidase and susceptibility ratio (ID_{50} of ascamycin/dealanylascamycin).

Circle 1, Balb3T3; 2, SV-3T3; 3, Ki-3T3; 4, NRK; 5, Ha-NRK; 6, NIH L1; 7, L1-4NQO$_{ad}$; 8, K-562; 9, HL60; 10, Friend. $r=-0.774$, $\alpha \fallingdotseq 0.005$.

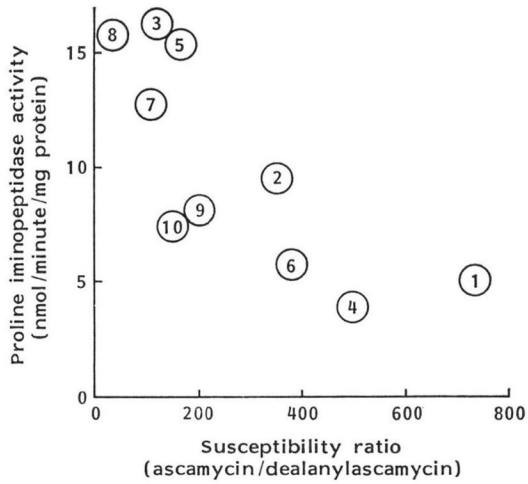


Table 2. Effects of ascamycin and dealanylascamycin on protein synthesis.

Cell	ID_{50} (10^{-6} M) Ascamycin	ID_{50} (10^{-9} M) Dealanyl-ascamycin	Ratio[a]
Balb3T3	1.4	1.9	730
SV-3T3	3.8	11	350
Ki-3T3	2.5	21	120
NRK	2.2	4.5	490
Ha-NRK	4.0	25	160
NIH L1	1.5	4.1	370
L1-4NQO$_{ad}$	1.1	11	100
K-562	0.5	16	30
HL60	3.0	15	200
Friend	6.6	43	150

[a] ID_{50} of ascamycin/ID_{50} of dealanylascamycin.

In the present paper, we have shown that an ascamycin dealanylating enzyme is also present in mammalian cell membranes and the activity is higher in transformed cells than in the nontransformed cell. The enzyme activity was associated with proline iminopeptidase which was closely related to ascamycin dealanylating enzyme, Xc-aminopeptidase. An immunological technique revealed that all cells tested contained the immunologically related Xc-aminopeptidase and that this antigen increased in the transformed cells compared with the nontransformed cells.

AOYAGI *et al.*,[17,18] measured activities of various hydrolyzing enzymes in rat organ homogenates and on the cell surface of cells from various sources, *i.e.*, tumor cell lines, primary cultured cells, normal cell lines, and their transformants. They revealed that the proline iminopeptidase activity of FM3A cells, mouse mammary tumor cells, was higher than that of normal cells. The result agrees with our observation in this paper, but further investigation is necessary to reveal the relationship between cell

transformation and the increase of proline iminopeptidase activity observed in this paper.

In bacteria, ascamycin cannot be transported into the cytoplasm because the alanyl group of ascamycin interferes with membrane transport; dealanylascamycin is transportable. If this is also the case with mammalian cells, the ratio of 50% inhibition dose of methionine uptake between ascamycin and dealanylascamycin can be regarded an inverse index of dealanylation of ascamycin, *i.e.*, membrane transport of ascamycin.

We have shown that there exists a clear relationship between proline iminopeptidase activity and ascamycin susceptibility in mammalian cells. In this respect, a prolyl analog of ascamycin might be worthwhile for investigation. We have already succeeded in total synthesis of ascamycin[19] and the synthesis of the prolyl analog is in progress.

Acknowledgments

We thank CHINATSU WATANABE and MIE SUGA for excellent technical assistance, and TAKEO MIZUTANI at Riken Institute for preparation of antiserum against Xc-aminopeptidase. A part of this work was carried out under the NIBB Cooperative Research Program (85-103).

References

1) ISONO, K.; M. URAMOTO, H. KUSAKABE, N. MIYATA, T. KOYAMA, M. UBUKATA, S. K. SETHI & J. A. MCCLOSKEY: Ascamycin and dealanylascamycin, nucleoside antibiotics from *Streptomyces* sp. J. Antibiotics 37: 670~672, 1984
2) OSADA, H. & K. ISONO: Mechanism of action and selective toxicity of ascamycin, a new nucleoside antibiotic. Antimicrob. Agents Chemother. 27: 230~233, 1985
3) OSADA, H. & K. ISONO: Purification and characterization of ascamycin hydrolyzing aminopeptidase from *Xanthomonas citri*. Biochem. J. 233: 459~463, 1986
4) AARONSON, S. A. & G. T. TODARO: Development of 3T3-like lines from Balb/c mouse embryo cultures: Transformation susceptibility to SV40. J. Cell. Physiol. 72: 141~148, 1968
5) AARONSON, S. A. & C. A. WEAVER: Characterization of murine sarcoma virus (Kirsten) transformation of mouse and human cells. J. Gen. Virol. 13: 245~252, 1971
6) DUC-NGUYEN, H.; E. N. ROSENBLUM & R. F. ZEIGEL: Persistent infection of a rat kidney cell line with Rauscher murine leukemia virus. J. Bacteriol. 92: 1133~1140, 1966
7) SHIH, T. Y.; M. O. WEEKS, H. A. YOUNG & E. D. SCOLNICK: Identification of sarcoma virus-coded phosphoprotein in nonproducer cells transformed by Kirsten or Harvey murine sarcoma virus. Virology 96: 64~79, 1979
8) HIRAGUN, A.; M. SATO & H. MITSUI: A new parameter for discriminating malignancy transformed cell lines from nontransformed counterparts: Culture in liquid medium containing neutral protease. Gann 70: 187~193, 1979
9) KOHNO, K.; A. HIRAGUN, H. MITSUI, A. TAKATSUKI & G. TAMURA: Effect of tunicamycin on cell growth and morphology of nontransformed and transformed cell lines. Agric. Biol. Chem. 43: 1553~1561, 1979
10) LOZZIO, B. B.; C. B. LOZZIO, E. G. BAMBERGER & A. S. FELIU: A multipotential leukemia cell line (K-562) of human origin (41106). Proc. Natl. Acad. Sci. U.S.A. 166: 546~550, 1981
11) COLLINS, S. J.; R. C. GALLO & R. E. GALLAGHER: Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature (London) 270: 347~349, 1977
12) FRIEND, C.; W. SCHER, J. G. HOLLAND & T. SATO: Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*: Stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. U.S.A. 68: 378~382, 1971
13) OUCHTERLONEY, O. & L.-A. NILSSON: Immunodiffusion and immunoelectrophoresis. *In* Handbook of Experimental Immunology. Vol. 1, *Ed.*, M. WEIR, Chapter 19. pp. 1~44, Black Scientific Publication, Oxford, 1978
14) LAEMMLI, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680~685, 1970
15) HAM, R. G.: Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proc. Natl. Acad. Sci. U.S.A. 53: 288~293, 1965
16) CROWE, R.; H. OZER & D. RIFKIN: Anchorage requirements. *In* Experiments with Normal and Transformed Cells. pp. 18~27, Cold Spring Harbor Laboratory, New York, 1974

17) AOYAGI, T.; M. NAGAI, M. IWABUCHI, W.-S. LIAW, T. ANDOH & H. UMEZAWA: Aminopeptidase activities on the cell surface of mammalian cells and their alterations associated with transformation. Cancer Res. 38: 3505~3508, 1978
18) AOYAGI, T.; H. SUDA, M. NAGAI, K. OGAWA, T. TAKEUCHI & H. UMEZAWA: Aminopeptidase activities on the surface of mammalian cells. Biochim. Biophys. Acta 452: 131~143, 1976
19) UBUKATA, M.; H. OSADA & K. ISONO: Synthesis and biological activity of nucleoside antibiotic, ascamycin and the amino acid analogs. Nucleic Acid Res. Symp. Ser. No. 16, 81, 1985