RETROSTATIN, A NEW SPECIFIC ENZYME INHIBITOR AGAINST AVIAN MYELOBLASTOSIS VIRUS REVERSE TRANSCRIPTASE

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A novel enzyme inhibitor against RNA-directed DNA polymerase of avian myeloblastosis virus was produced by an isolate of a new streptomycete for which the name *Streptomyces retrostaticus* is proposed.

This enzyme inhibitor, which was named retrostatin, did not inhibit DNA-directed DNA polymerase of *Escherichia coli* and DNA-directed RNA polymerase of Ehrlich ascites tumor cells. Retrostatin was produced by the microorganism together with streptonigrin. These two substances were extracted from the culture broth with ethyl acetate at acidic pH. Retrostatin is an acidic pH indicator and the free acid was recovered as a red powder.

Retrostatin had weak antibiotic activities against Gram-positive bacteria and yeasts.

Reverse transcriptase activity, namely RNA-directed DNA synthesis, is considered to be one of the most critical steps of the life cycle of oncogenic RNA viruses.[1,2] Therefore, many enzyme inhibitors against reverse transcriptase of oncogenic viruses have been investigated and adriamycin,[3] daunomycin,[3] streptonigrin,[4] streptovaricins[5] and derivatives of rifamycin SV[6] have been reported to inhibit the enzyme. Moreover, those antibiotics also have been reported to inhibit primarily DNA-directed DNA, DNA-directed RNA or both of DNA-directed DNA and RNA polymerases, while revistin[7] obtained as a partially purified high molecular substance from the culture broth of *Streptomyces filipinensis* was reported to inhibit reverse transcriptase of murine leukemia virus.

In the course of our screening for enzyme inhibitors against reverse transcriptase of avian myeloblastosis virus (AMV), an ethyl acetate extract of the broth filtrate of the Isolate H 1058-MY 2 at acidic pH showed strong inhibition of the enzyme. Two inhibitors were isolated from the extract: one was determined to be streptonigrin[5] and the other, proved to be a new one, was named retrostatin.

Taxonomy of the producing microorganism, fermentation, extraction, purification and physicochemical and biological properties of retrostatin are described in this paper.

Materials and Methods

Purified reverse transcriptase (specific activity: 50,000 units/mg protein) of AMV was supplied by the Division of Cancer, Cause and Prevention, National Cancer Institute. DNA-directed DNA polymerase I of *Escherichia coli* and calf thymus DNA were purchased from P. L. Biochemicals, Inc. The cell-free lysate of Ehrlich ascites tumor cells[9,10] was used as a source of DNA-directed RNA polymerase. [^{3}H]-Labeled deoxythymidine and uridine triphosphates were obtained from Amersham-Searle, Inc. Unlabeled dNTPs and NTPs were purchased from Sigma Chemical Co. Poly-(rA) and oligo-dT_{12-18} were purchased from P. L. Biochemicals, Inc. All other chemicals used were of analytical grade.

The methods described by WANG *et al.*[12] and NUMATA *et al.*[7] were modified to assay reverse transcriptase activity of AMV as follows: The assay solution contained 80 mM tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 60 mM NaCl, 5 mM dithiothreitol (DTT), 0.2 mM each of dATP, dGTP, dCTP and dTTP, 20 μM [^{3}H]dTTP (40 Ci/mmole), poly-(rA) (4 μg/ml), oligo-dT_{12-18} (800 ng/ml), bovine serum albumin (200 μg/ml) and reverse transcriptase of AMV (8.6 units/ml). Distilled H_2O was added to an inhibitor dissolved in dimethyl sulfoxide (DMSO) to make a test solution and the final concentration of DMSO was less than 2%. A mixture of the assay solution (50 μl) and the test solution (50 μl) was incubated at 39°C for 60 minutes with shaking and terminated by cooling in an ice bath. The mixture (50 μl), thus obtained, was soaked into a 2.4 cm-round piece of filter paper previously treated with 0.1 M pyrophosphate solution and dried. The filter paper was washed twice with 10% trichloroacetic acid, then washed with ethanol, and dried. The radioactivity of the acid insoluble fraction on the filter paper was counted in a toluene-based scintilation cocktail. One unit of reverse transcriptase of AMV was defined as the amount of enzyme able to form 1 nmole of total nucleotide into the acid insoluble fraction per minute at 39°C under the above specified conditions.

The procedure reported by RICHARDSON *et al.*[11] was modified to determine DNA-directed DNA polymerase activity as follows: The assay solution contained 133 mM glycine - NaOH buffer (pH 9.2), 13.3 mM $MgCl_2$, 5.4 mM DTT, 59.5 μM each of dATP, dGTP, dCTP and [^{3}H]dTTP (40 Ci/mmole), activated calf thymus DNA (74 μg/ml), bovine serum albumin (333 μg/ml) and DNA polymerase I of *E. coli* (0.83 unit/ml). A test solution was prepared by the same method described above. A mixture of the assay solution (50 μl) and the test solution (50 μl) was incubated at 37°C for 60 minutes with shaking and the resulting mixture (50 μl) was treated in the same manner as described above to determine the radioactivity of the acid insoluble fraction. One unit of DNA-directed DNA polymerase I was defined as the amount of enzyme able to convert 1 nmole of total nucleotide into the acid insoluble fraction per minute at 37°C under the above specified conditions.

The method reported by NATORI *et al.* was followed to determine DNA-directed RNA polymerase of Ehrlich ascites tumor cells.[9] The assay solution contained 55 mM tris-HCl buffer (pH 7.9), 25 mM $MgCl_2$, 5 mM $MnCl_2$, 125 mM $(NH_4)_2SO_4$, 0.15 mM EDTA, 7.5 mM mercaptoethanol, 1 mM each of ATP, GTP and CTP, 0.1 mM [^{3}H]UTP (46 Ci/mmole) and native calf thymus DNA (250 μg/ml). 'RNA-Polymerase Solution' was prepared according to the method reported by SAKANO *et al.*[10] using Ehrlich ascites tumor cells instead of leukemia L1210 cells. A test solution was prepared by the same method outlined above. A mixture of the assay solution (50 μl), 'RNA-Polymerase Solution' (50 μl) and the test solution (25 μl) was incubated at 37°C for 60 minutes with shaking and 50 μl of the resulting mixture was treated according to the same procedures described above to determine the radioactivity of the acid insoluble fraction.

Taxonomy of the Producing Organism

The retrostatin-producing microorganism, Isolate H 1058-MY 2, was isolated from a soil sample collected in Tottori Prefecture, Japan. Taxonomic studies on the isolate were conducted according to the procedures of the International Streptomyces Project (ISP),[13] WAKSMAN[14] and the BERGEY'S Manual.[15] The morphological, cultural and physiological characteristics of the Isolate H 1058-MY 2 are summarized as follows: spore mass color in the Gray color-series; *Rectus Flexibilis* type (RF) to *Retinaculum-Apertum* type (RA) spore chains with 20 to 50 spores per chain (Fig. 1); spore surface smooth (Fig. 2); no melanoid pigment formation; utilized glucose and galactose as carbon source; liquefied gelatin with no soluble pigment; peptonized skim milk, but did not coagulate.

A whole-cell hydrolysate analysis of the Isolate H 1058-MY 2 according to the method STANECK *et al.*[16] demonstrated the presence of LL-diaminopimelic acid in the cell wall.

On the basis of morphological characteristics, spore mass color and no melanin formation, *S. viridifaciens, S. nogalater, S. olivaceoviridis, S. recifensis* and *S. avellaneus* seemed to be related to the

Fig. 1. Aerial mycelia of the Isolate H 1058-MY 2.

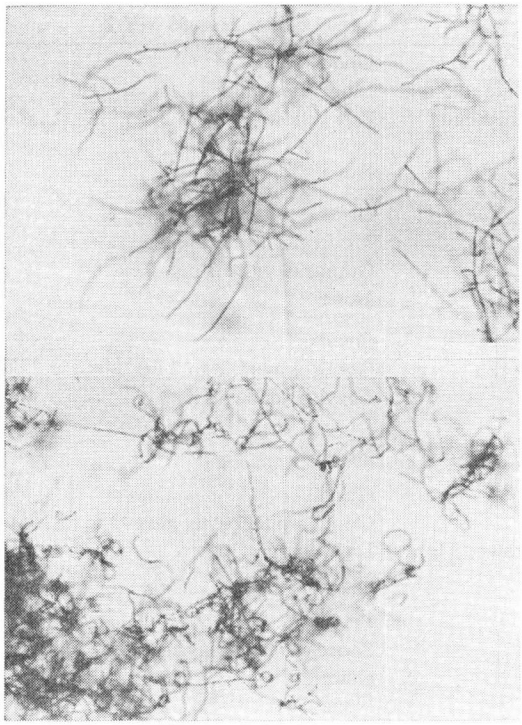
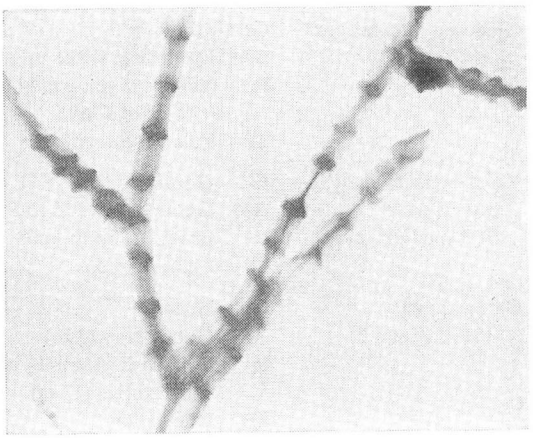


Fig. 2. Electron micrograph of spores of the Isolate H 1058-MY 2.



Isolate H 1058-MY 2 according to the BERGEY's Manual and ISP description.[17~20] However, *S. nogalater*, *S. olivaceoviridis* and *S. recifensis* show great differences in carbon source utilization compared to the Isolate H 1058-MY 2, *i.e*: these three species are described in the BERGEY's Manual as utilizing nine carbon sources among eleven prescribed ones, whereas the Isolate H 1058-MY 2 utilizes only two (D-glucose and D-galactose). Spore chains of *S. avellaneus* (ISP 5554) on ISP media showed no RA type morphology, but only RF type was recognized in our experiments as well as in ISP description. Therefore *S. viridifaciens* (ISP 6239) was compared with the Isolate H 1058-MY 2 in detail. The results are shown in Tables 1 and 2, showing the following differences between the two strains: *S. viridifaciens* has a greenish color on the reverse side of the colony and a soluble pigment on oat meal agar (ISP 3), but the Isolate H 1058-MY 2 has a grayish yellow color on the reverse side of the colony and almost no soluble pigment. Also on the other ISP media and glucose - nitrate agar, some differences are recognized between the two strains. *S. viridifaciens* utilizes D-xylose, L-arabinose, D-fructose and sucrose which are not utilized by the Isolate H 1058-MY 2. D-Galactose is utilized by the Isolate H 1058-MY 2, but not by *S. viridifaciens*. On gelatin medium, *S. viridifaciens* produces brown soluble pigment, while the Isolate H 1058-MY 2 produces no soluble pigment.

As a result, the Isolate H 1058-MY 2 is considered to be a new streptomycete. The name *Streptomyces retrostaticus* is proposed for this isolate, referring to the product of retrostatin.

Fermentation

To obtain material for further study, the Isolate H 1058-MY 2 were precultured in 500-ml Sakaguchi-flasks each containing 100 ml of an inoculation medium composed of 1.0% maltose and 0.2% yeast extract (pH 7.0 before autoclaving) at 27°C for 36 hours on a reciprocal shaker (amplitude 7 cm, 130 strokes per minute). From this preparation 2 ml were used to inoculate 500-ml Sakaguchi-flasks each containing 100 ml of a production medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% soy bean meal, 0.5% Ebios (dried yeast distributed by Tanabe Pharmaceutical

Table 1. Cultural characteristics of *S. viridifaciens* and the Isolate H-1058 MY 2.

Medium		*S. viridifaciens*	Isolate H-1058 MY 2
Glucose - nitrate agar*	G**	Good	Good
	AM	Moderate; white to gray (3 fe to 5 fe)	Moderate; white
	R	Yellow (2 gc), partly brownish red (4 gc to 6 le)	Pale yellow (1½ ca to 2 ca)
	SP	Pale yellow	None
Yeast extract - malt extract agar (ISP medium 2)	G	Good	Good
	AM	Good; gray (3 fe to 3 ih)	Moderate; white to pale gray (3 dc)
	R	Brown (3 ng to 3 ni)	Orange (2 pg to 3 ne)
	SP	Light brown	None
Oatmeal agar (ISP medium 3)	G	Good	Good
	AM	Good; gray (3 ih)	Good; gray (3 fe to 3 ih)
	R	Yellowish green (1½ ie) to light olive (1½ lg)	Grayish yellow (2 ie)
	SP	Pale yellow	None
Inorganic salts - starch agar (ISP medium 4)	G	Good	Good
	AM	Scant	Good; white, partly gray (3 fe to 3 ih)
	R	Grayish yellow (2 ie) to brown (3 lg)	Light brown (3 ie)
	SP	Pale brown	None
Glycerol - asparagine agar (ISP medium 5)	G	Poor	Poor
	AM	None	Poor; white to grayish white (3 ba)
	R	Colorless	Colorless
	SP	None	None

* Since the Isolate H 1058-MY 2 did not utilize sucrose, 2% glucose - nitrate agar was used instead of sucrose - nitrate agar.

** Abbreviations: G, growth; AM, aerial mycelium; R, reverse side of colony; SP, soluble pigment. Numbers in parenthesis followed the Color Harmony Manual.[21]

Table 2. Physiological characteristics of *S. viridifaciens* and the Isolate H 1058-MY 2.

	S. viridifaciens	Isolate H 1058-MY 2
Melanin production on		
Tyrosine agar (ISP medium 7)	Negative	Negative
Peptone - yeast extract iron agar (ISP medium 6)	Negative	Negative
Tryptone - yeast extract broth (ISP medium 1)	Negative	Negative
H_2S production	Negative	Negative
Starch hydrolysis	Positive	Positive
Gelatin liquefaction	Positive, brown soluble pigment	Positive, no soluble pigment
Skim milk		
Coagulation	Negative	Negative
Peptonization	Positive	Positive
Carbon utilization		
D-Glucose	+	+
D-Xylose	+	−
L-Arabinose	+	−
L-Rhamnose	−	−
D-Fructose	+	−
D-Galactose	−	+
Raffinose	−	−
D-Mannitol	−	−
i-Inositol	−	−
Salicin	−	−
Sucrose	+	−

Co., Ltd.), 0.25% NaCl and 0.3% $CaCO_3$ (pH 7.6 before autoclaving). The culture was grown at 27°C for 96 hours on the same shaker.

Extraction and Purification

The enzyme inhibitors, acidic and lipophilic in nature, were extracted in ethyl acetate from the broth filtrate at pH 3. Though, the ethyl acetate extract contained at least four principles showing inhibitory activity against *Bacillus subtilis*, only two colored principles of the four showed inhibitory activity against reverse transcriptase. Further both colored principles seemed to be photo-sensitive or air-sensitive. Thus, the ethyl acetate extract was separated to give a brown mixture using silica gel column chromatography at 4~6°C in the dark. The brown mixture was further purified to give a coffee-

Chart 1. Extraction of streptonigrin and retrostatin.

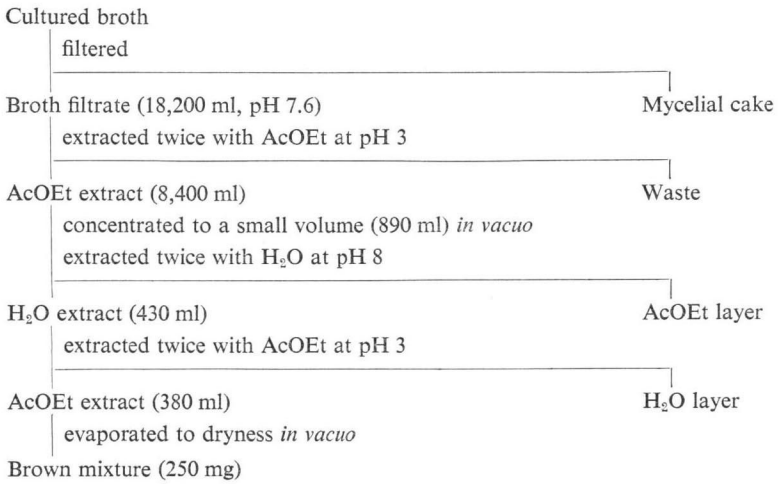
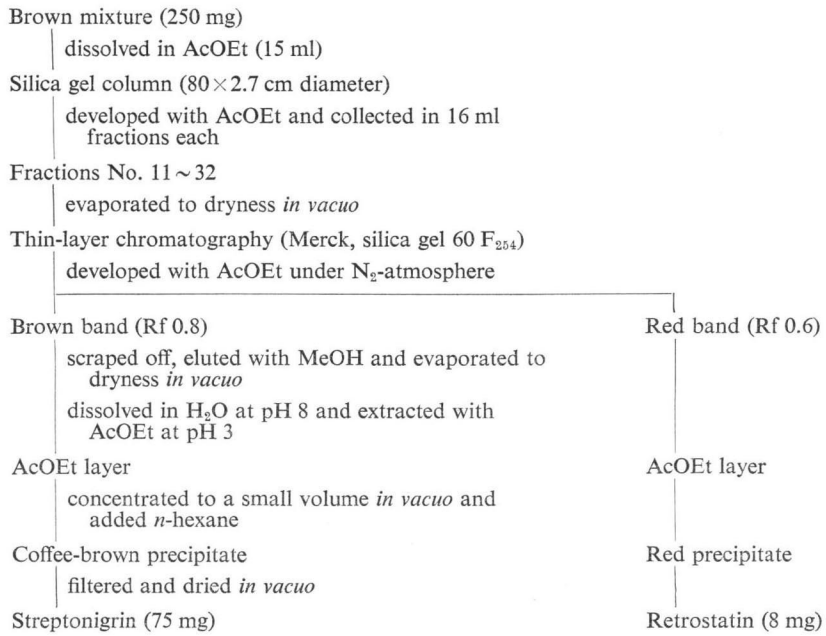


Chart 2. Purification of streptonigrin and retrostatin.



brown main principle and a red minor principle by preparative silica gel thin-layer chromatography at 4~6°C in the dark under nitrogen atmosphere. The procedures of extraction and purification are shown in Charts 1 and 2.

Physico-chemical Properties

The ultraviolet absorption spectrum of the coffee-brown main principle was closely related to that of streptonigrin.[5] The chromatographic behavior of the main principle was quite similar to that of streptonigrin on silica gel thin-layer plates developed with several kinds of solvent systems. Identity of the main principle with streptonigrin was further confirmed by comparing the infrared absorption spectrum of the main principle with that of streptonigrin.

The free acid of the other minor principle was isolated as a red amorphous powder, which gradually

Fig. 3. Ultraviolet and visible absorption spectra of retrostatin.

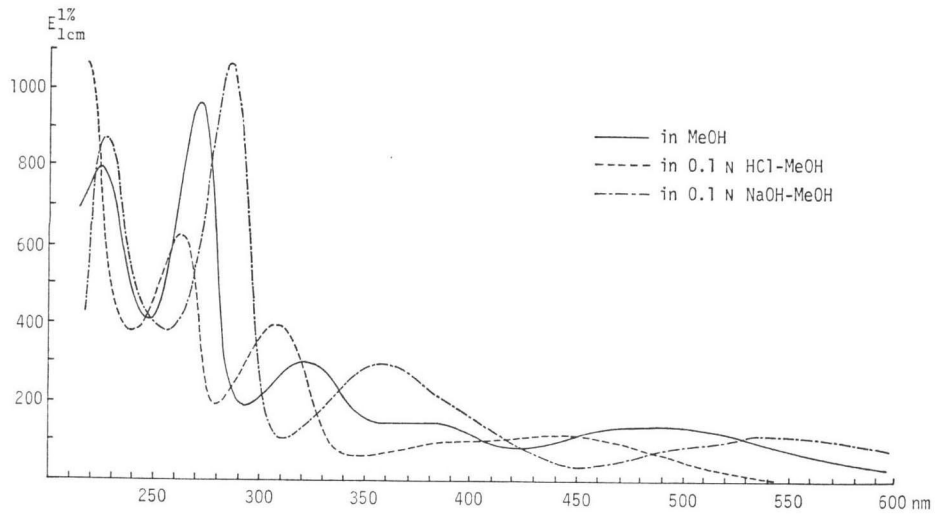
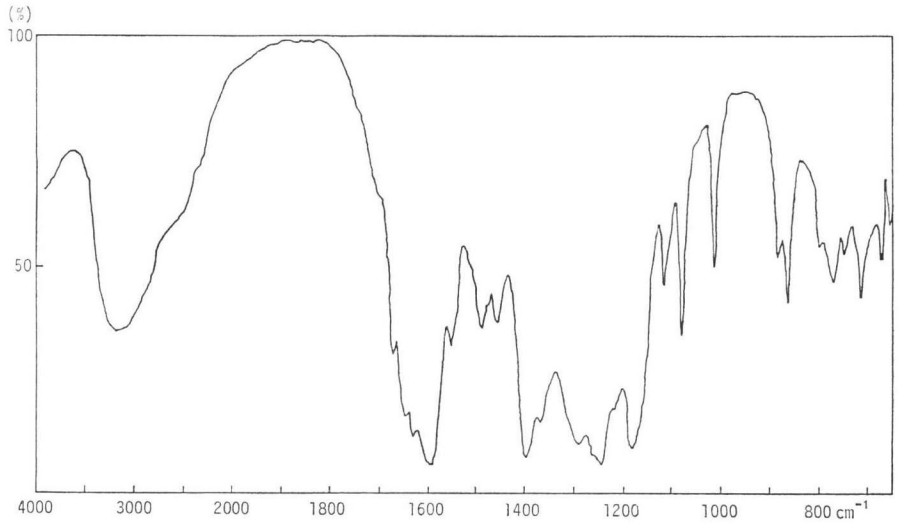


Fig. 4. Infrared absorption spectrum of retrostatin (KBr).



decomposed between 200~220°C. Elementary analysis gave C 58.68%, H 3.53% and no nitrogen was observed. The ultraviolet and visible absorption spectra of the red principle are shown in Fig. 3. UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$) 224 (787), 270 (947), 320 (293) and 490 nm (133). The minor principle is a kind of pH-indicator and the maximum at 490 nm in methanol is shifted to 445 nm in 0.1 N HCl-MeOH, and to 550 nm in 0.1 N NaOH-MeOH. The infrared absorption spectrum of the principle in KBr is shown in Fig. 4 and the presence of a hydrogen-bonded quinone group is suggested by the band at 1595 cm^{-1}. The red free acid is soluble in methanol, ethanol or ethyl acetate, but sparingly soluble or insoluble in chloroform, ether, *n*-hexane or H_2O. The red principle was detected as a single red spot on silica gel thin-layer plates developed with several kinds of solvent systems and the Rf values are listed in Table 3. The red principle was fairly unstable and decomposed after standing at room temperature for two weeks to give several spots showing lower Rf values (Rf values; 0.18, 0.08 and 0) than the original principle (Rf value; 0.57) on silica gel thin-layer chromatography developed with ethyl acetate.

Table 3. Chromatographic behavior of retrostatin on silica gel thin-layer plates.

Solvent system	Rf value
AcOEt	0.57
AcOEt - *n*-hexane (3: 1)	0.37
AcOEt - *n*-hexane (1: 1)	0.16
AcOEt - MeOH (10: 1)	0.65
$CHCl_3$ - MeOH (10: 1)	0.08
$CHCl_3$	0

Table 4. Antimicrobial spectrum of retrostatin.

Test organisms	MIC (μg/ml)
Staphylococcus aureus FDA 209 P	50
Bacillus subtilis PCI 219	25
B. anthracis	12.5
Corynebacterium bovis 1810	>100
Micrococcus luteus FDA 16	50
M. lysodeikticus IFO 333	50
Pseudomonas phaseolicola	>100
P. aeruginosa Ishii 14	>100
Escherichia coli NIHJ	>100
E. coli. K-12	>100
Mycobacterium smegmatis ATCC 607	>100
Candida tropicalis NI 7495	12.5
C. pseudotropicalis NI 7494	12.5
C. albicans 3147	12.5
C. albicans Yu 1200	12.5
C. krusei NI 7492	12.5
Saccharomyces cerevisiae	12.5

Agar dilution method on glucose nutrient agar.

Biological Properties

The red principle showed weak antimicrobial activity against Gram-positive bacteria and yeasts as shown in Table 4.

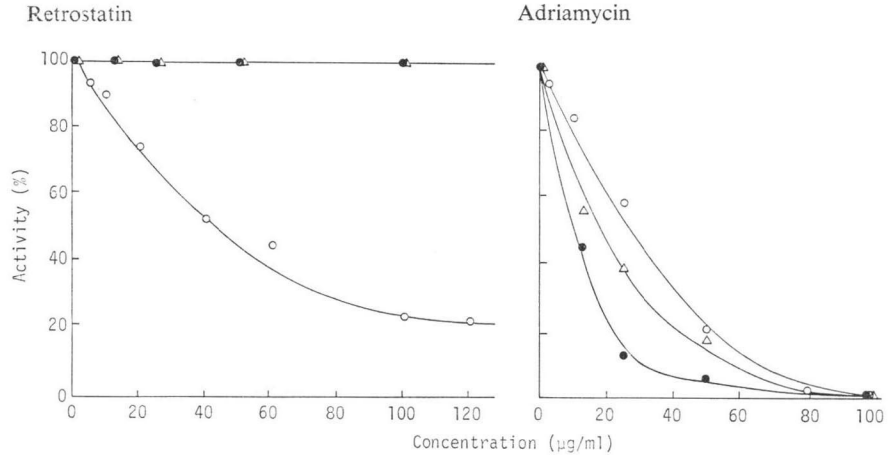
Inhibitory activity against reverse transcriptase of AMV was determined by the earlier described method and the IC_{50} of adriamycin, daunomycin and the red principle were 25 μg/ml, 30 μg/ml and 50 μg/ml, respectively. Both DNA-directed DNA polymerase of *E. coli* and DNA-directed RNA polymerase of Ehrlich ascites tumor cells were inhibited by adriamycin but not by the red principle as seen in Fig. 5. Thus, the principle was considered to be a specific enzyme inhibitor against reverse transcriptase and named retrostatin. Retrostatin is labile and loses most of its biological activities when standing in the air at room temperature for two weeks.

Discussion

Substances that specifically inhibit reverse transcriptase have been reported to be present in human placenta, cultured cells of HeLa, 78A-1 *etc.*, and in mouse spleen.[22,23] The first account of a specific inhibitor of the enzyme from microbial source was that of revistin.[7]

Revistin was described as an acidic compound with a high molecular weight, possessing no antimicrobial activity. Retrostatin, on the other hand, though also acidic, has a low molecular weight and

Fig. 5. Effects of retrostatin and adriamycin on various DNA and RNA polymerases.
○ RNA-directed DNA polymerase of AMV.
● DNA-directed DNA polymerase of *E. coli*.
△ DNA-directed RNA polymerase of Ehrlich ascites tumor cells.



weak antimicrobial activity against Gram-positive bacteria and yeasts. Other compounds implicated as nonspecific inhibitors of the enzyme include pH indicator type antibiotics such as adriamycin,[3] daunomycin,[3] streptovaricins,[5,24] but they do not show the same ultraviolet and visible absorption spectra as that of retrostatin. For the above reasons, retrostatin is considered a new compound which is a specific inhibitor of the RNA-directed DNA polymerase of avian myeloblastosis virus.

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