Screening, Fermentation, Isolation, and Characterization of Trypanomycin, a New Antibiotic

W. FLECK, D. STRAUSS, C. SCHÖNFELD, W. JUNGSTAND, C. SEEBER, AND H. PRAUSER

*Central Institute for Microbiology and Experimental Therapy, German Academy of Sciences at Berlin, Jena, and
Department of Neurovirology, Nervous Clinic, Medical Academy of Erfurt,
Erfurt, German Democratic Republic*

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A *Streptomyces* strain which is similar to *S. diastatochromogenes* (Krainsky) Waksman et Henrici 1948 sensu Hütter (1967) was found to produce a new antibiotic designated trypanomycin. The red-pigment antibiotic, which has novel trypanocidal activity in vitro and in vivo, was isolated from a C-, N-, and iron-containing culture of the strain IMET JA 10081/9 by extraction with organic solvents, transfer into the aqueous phase, reextraction with organic solvents at pH 6.8, precipitation by hydrocarbons, and purification by chromatographic methods. Trypanomycin has indicator properties. The main constituents of the antibiotic mixture are readily soluble in water and are very stable in distilled water at room temperature (28 C) for 24 hr. The composition of the base of trypanomycin A₂ (melting point, 175 to 183 C) corresponds to the empirical formula C₄₁₋₄₂H₅₂₋₅₆NO₂₁₋₂₂. The absorption spectra in the ultraviolet and visible regions are very similar to those of the 4,5,8-trioxyanthrachinones. Trypanomycin has strong antiprotozoal activity, e.g., against trypanosomes. The natural substance additionally inhibits the growth of gram-positive bacteria, stable as well as unstable L-forms of gram-negative bacteria, mycoplasmas, yeast protoplasts, and tumor cells in vitro. The LD₅₀ of trypanomycin in mice was 60 mg/kg when administered intravenously and 31 mg/kg on intraperitoneal administration. If the antibiotic was added to cultures of animal or human cells in vitro, mitotic inhibition and chromosomal aberrations resulted. Trypanomycin differs in its biological activities and chromatographic behavior from other anthracyclines, e.g., cinerubine A and B, daunomycin, adriamycin, nogalamycin, rutilantin, pyrromycin, cyclamycin, and ryemycin.

In the course of a screening program directed toward the isolation and evaluation of new cancerostatic antibiotics, we isolated from a soil a species of streptomycetes which was found to produce the new antibiotic trypanomycin. This new antibiotic proved to be of considerable interest because of its strong antiprotozoal activity, e.g., against trypanosomes. The antibiotic, which represents a new type of the recently known anthracyclines (1) with many distinctive characteristics, has been identified chemically as ϵ -pyrromycinone glycoside.

In this report, we present biological data relevant to the discovery and fermentation of the producing strain, and describe the isolation and some of the physicochemical and biological properties of trypanomycin.

MATERIALS AND METHODS

Screening. Among 7,000 strains, which were isolated or deposited in the Institute for Microbiology and Experimental Therapy (IMET) culture collection from various geographical areas and which belong to different groups of *Actinomycetales* such as *Streptomyces*, *Nocardia*, *Pseudonocardia*, *Actinosporangium*, *Micromonospora*, *Microbispora*, *Microechinospora*, *Spirillospora*, *Micropolyspora*, *Chainia*, *Microellobo-spora*, and *Promicromonospora*, we have found 290 strains which produce red pigments with or without biodynamic activity. Sixty of these strains produce red pigments with indicator character and different biodynamic and physicochemical properties. They had been detected by means of a complex screening system (5).

From mycelia or culture fluids of 30 strains, we obtained red-pigment antibiotics which could be divided into six groups according to phenomena in

the applied screening models in vitro (3, 4, 7) and to results and experiences with chemotherapy in animals.

In group 1 the red pigments are antibiotic complexes containing components which induce the λ prophages in lysogenic cells of *Escherichia coli* (7), components which interfere with the excision dark repair mechanism in cells of *E. coli* (4), or both. The complexes also contain components which inhibit the multiplication of temperate phages in *E. coli* (3), but which are unable to disturb the regulation mechanism of the lysogenic state. Most pigments of group 1 showed a strong activity against both leukemic disease L1210 and the solid tumor sarcoma 180 in mice.

Pigments of groups 2 and 5 are also antibiotic complexes containing components which induce specific phenomena in the described "BIP" test method (3) and reduce the growth of sarcoma 180 in mice significantly. Biodynamic activity of pigments in groups 3 and 6 was not observed in any screening model in vitro or in vivo.

Red pigments of group 4 were detected by means of the BIP test method, but there is no indication of any significant activity against experimental tumor models in animals. However, several natural substances of group 4 are antibiotic complexes of importance in the chemotherapy of protozoal diseases. Trypanomycin belongs to this last group. The producing strain was isolated from an Italian soil sample which was taken from a vineyard in the village Anacapri on Capri and possesses the morphological and biochemical properties of *Streptomyces diastatochromogenes* (Krainsky) Waksman et Henrici 1948, sensu Hütter 1967 (8).

Fermentation. Trypanomycin was produced by the usual procedures of submerged fermentation. The first cultures were grown on agar medium inoculated with spores. Then the organism was propagated in shaken flasks and fermentors ranging from 0.5 liter to several cubic meters. An inoculum of the strain IMET 10081/9 was prepared by growing the organism at 28 C in aerated 20-liter bottles for 48 hr in a medium of the following composition (grams per liter): soybean meal, 15; glucose, 15; sodium chloride, 5; calcium carbonate, 1; and primary potassium phosphate, 0.3. A 15-liter amount of inoculum was used to seed 400 liters of medium containing the following ingredients (grams per liter): soybean meal, 30; glucose, 30; calcium carbonate, 3; sodium chloride, 3; and iron chloride hydrate, 0.25. The fermentation was carried out in a 700-liter V2A tank at 28 C with an air supply of 400 liters per min and at a stirring rate of 270 rev/min for 96 hr. The foam formation occurring was controlled by addition of small amounts of soybean oil. The maximal concentration of trypanomycin was obtained after 4 days and reached more than 100 mg/liter in the wild type. The resulting broth was pink to orange-red to violet in color, depending on the nature of the culture medium and its pH.

Analytical procedures. The first attempts to isolate trypanomycin were extremely difficult because we lacked any method of quantitative analysis. Initially, they were carried out by semiquantitative assessment of the antiviral activity in vitro determined by the

"BIP" test method (3). The antibiotic activity was observed during fermentation by assaying samples of crude fermentation broth. The test organism was *E. coli* C 600 multiply infected with free temperate λ phages. The activity was determined by a microbiological agar diffusion test in which the inhibition of multiplication of temperate viruses in bacteria is used as a measure of antibiotic activity. Other substances in the broth possess antibacterial but no antiviral activity. Because trypanomycin contains several constituents, the quantitative determination of the constituents was possible only by use of paper or thin-layer chromatography for separation. After separation, the constituents of trypanomycin could be determined by colorimetric or microbiological methods. Paper chromatography and thin-layer chromatography turned out to be equally successful.

Paper chromatography with trypanomycin A₂ was carried out on Schleicher & Schuell No. 2043b paper with the use of ascending development. Several solvents and solvent mixtures saturated with water were used: butanol, water-saturated, R_F 0.65; methanol, R_F 0.9; acetone, 50%, R_F 0.48; acetone-benzol-water, 12:3:2, R_F 0.87; and, as the round-filter chromatography system, butanol-methanol-water, 4:1:2, R_F 0.7. Active substances were detected bioautographically on BIP test plates of tryptone-peptone-agar seeded in the top layer with *E. coli* C 600 and free λ -phage (multiplicity, 50).

For thin-layer chromatography, the absorbent was Merck silica gel D in layers of 0.25 mm. The absorbent was not impregnated, and the developing solvent was the upper phase of the system chloroform-methanol-acetone-water (50:5:15:10, v/v). As the constituents of trypanomycin are colored and grouped in a number of small areas, it also was possible to examine them directly. The colored zones were scraped off and eluted. Each constituent was determined by spectrophotometry at 290 nm, in comparison with standards chromatographed in parallel.

To facilitate the identification of trypanomycin, the aglycone was isolated by a method used for some anthracyclines. Acid hydrolysis of trypanomycin with sulfuric acid on a boiling-water bath yielded the aglycone and sugars.

Thin-layer chromatography could be used for differentiation of trypanomycin A₂ from cinerubin A and B (2). Comparative chromatography of the two anthracycline antibiotics was performed on silica gel D plates with a chloroform-methanol-acetone-water (50:5:15:10) solvent mixture used for development of plates. The red pigment antibiotics differ significantly in R_F values (Table 1).

Isolation. The active substances were at first extracted from the culture broth as a crude base containing a mixture of trypanomycin constituents and other substances. The 400 liters of harvested mash were filtered, and, after separation of the mycelia with unchanged acidity, the filtrate of about 360 liters was extracted with 90 liters of butanol. The mycelia (36 kg, fresh weight) were extracted three times with 72 liters of methanol. The combined extracts were concentrated to one-fifteenth of the initial volume, and were completely freed from organic solvent by azeo-

TABLE 1. *Physicochemical properties of trypanomycin A₂ and trypanomycinone A₂*

Property	Trypanomycin A ₂	Trypanomycinone A ₂
Empirical formula.....	C ₄₁₋₄₂ H ₅₂₋₅₆ O ₂₁ N	C ₂₄ H ₂₀₋₂₄ O ₁₀
Analytical results (%)		
Calculated		
C.....	54.53	61.10
H.....	6.05	4.63
O (difference).....	37.86	34.27
N.....	1.56	—
Found		
C.....	54.49	61.20
H.....	6.11	4.60
O (difference).....	37.84	34.20
N.....	1.56	—
Melting point (C).....	175–183	182–187
Molecular weight		
Calculated.....	884–926	470–472
Found.....	910 ± 20 (Rast)	470 (<i>m/e</i> , M ⁺ peak)

TABLE 2. *Ultraviolet and visible absorption spectra of trypanomycin A₂ and trypanomycinone A₂*

Trypanomycin A ₂		Trypanomycinone A ₂	
Absorption peak (nm)	E _{1%¹cm}	Absorption peak (nm)	E _{1%¹cm}
260 ^a	183		
290	102	282 ^b	541
470	75	465	74
484	80	480	220
495	105	500	330
510 ^c		520	216
529		534	190

^a Determined in methanol.^b Determined in chloroform.^c Shoulder.

tropic distillation. The 30 liters of aqueous suspension obtained were twice extracted with half the volume of chloroform. The chloroform extract was concentrated to one-tenth of its volume, and the antibiotic was precipitated by addition of a threefold volume of petroleum ether. The constituents of this crude base were then fractionated and purified by column chromatography methods, with the use of cellulose powder saturated with phosphate buffer, pH 5.4. Trypanomycin A₂ was eluted by *n*-butanol, buffer-saturated. The active yellow-orange colored fractions were combined and evaporated, and the residue was recrystallized from water-free *n*-butanol. The orange-red crystals were filtered off and dried.

RESULTS AND DISCUSSION

Physicochemical properties. The crude base of trypanomycin was isolated as a complex of amorphous, red-brown substances which dissolve readily in chloroform, acetone, and dimethyl

formamide, less readily in alcohols, esters, and benzene, and sparingly in water. Four biologically active constituents of crude base designated A₁ to A₄ are readily soluble in water. The main constituent in broth is trypanomycin A₂.

In the solid state, the mixture of trypanomycin constituents is very stable. It can be kept for several years at a temperature of 18 to 22 C without any loss of activity. As shown by *in vitro* experiments with microbiological methods (agar diffusion technique), the aqueous solution of trypanomycin does not lose any activity within more than 8 weeks at 0 or 30 C. A 1 mg/ml solution in sterile distilled water was very stable at room temperature (28 C) for 24 hr.

The composition of the base of trypanomycin A₂ corresponds to the empirical formula C₄₁₋₅₂H₅₂₋₅₆NO₂₁₋₂₂ (Table 1). Trypanomycin A₂ (melting point, 175 to 183 C) has an absorption spectrum in the ultraviolet (UV) and visible regions (Table 2) which is very similar to those of the 4,5,8-trioxyanthrachinones (1). The antibiotic is a chromoglycoside formed by a tetracyclic quinoid aglycone (trypanomycinone A₂) from the ϵ -pyrromycinone type and three not yet identified sugars. After acid hydrolysis (0.1 N hydrochloric acid methanolic solution, 90 C, 30 min), the aglycone and the sugars were obtained. The sugar moiety was chromatographed on silica gel (thin-layer chromatography) and paper sheets, and showed three spots with sugar reagents, two of them with aniline phthalate (reducing sugars) and one by diphenylamine only. One of the first is an amino sugar. Trypanomycin A₂, the main constituent of the crude base, is a light-red substance and can serve as an indicator, being red-orange in acid solution with yellow-

TABLE 3. R_F values determined by thin-layer and paper chromatography

Substance	Material	System ^a	R_F
Cinerubin A and B	Silica gel D	2	0.94 and 0.98
Trypanomycin A ₂	Silica gel D	2	0.46
ϵ -Pyrromycinone	Paper 2043 ^b	1	0.80
Trypanomycinone A ₂	Paper 2043	1	0.77
ϵ -Pyrromycinone	Paper 2045	1	0.83
Trypanomycinone A ₂	Paper 2045	1	0.77

^a System 1: benzene-cyclohexane, 2:1; paper saturated with formamide-acetone (4:1). System 2: chloroform-acetone-methanol-water, 200:20:50:50 (organic phase).

^b Obtained from Schleicher & Schuell Co.

TABLE 4. *In vitro* antimicrobial spectrum of trypanomycin^a

Test organism	Minimal inhibitory concn (μ g/ml)
<i>Bacillus subtilis</i> ATCC 6633	0.3
<i>B. globifer</i> OH11	0.6
<i>B. mycoides</i> DG 756	0.6
<i>Sarcina lutea</i> SG 125	0.8
<i>Staphylococcus aureus</i> SG 511	0.3
<i>S. aureus</i> 209P	0.3
<i>S. aureus</i> UV-2 mutant (Gauze)	0.05
<i>Nocardia</i> sp. IMET 133	0.2
<i>Chromobacterium</i> sp. 1-S	0.1
<i>Caryophanon latum</i> (Peshkov)	0.3
<i>C. latum</i> (Robinow)	0.3
<i>Streptomyces hygroscopicus</i> JA 6669-6	0.05
<i>Mycobacterium smegmatis</i> SG 987	1.5
<i>M. phlei</i> SG346	1.0
<i>Escherichia coli</i> mutabile SG 458	>50.0
<i>Serratia marcescens</i> SG 621	>50.0
<i>Aerobacter aerogenes</i> SG 117	>50.0
<i>Proteus vulgaris</i> SG 2	>50.0
<i>P. morganii</i> SG 464	>50.0
<i>Pseudomonas aeruginosa</i> SG 137	>50.0
<i>Saccharomyces cerevisiae</i>	>50.0
<i>Kloeckera brevis</i>	>50.0
<i>Schizosaccharomyces pombe</i>	>50.0
<i>S. pombe</i> protoplasts	20.0
<i>Fusarium culmorum</i> JP 15	25.0
<i>F. solani</i> JP 20	>50.0
<i>Scopulariopsis</i> sp. JP 25	>50.0
<i>Penicillium notatum</i> JP 42	3.0

^a The agar diffusion test was used.

orange fluorescence in UV light and blue-violet in alkaline solution. The antibiotic belongs to the group of anthracyclines and to the subgroup of anthracyclines with an aglycone of the ϵ -pyrromycinone type, as shown by its UV spectra in concentrated sulfuric acid, the chemistry of which has been reviewed by Brockmann (1). Cinerubin, pyrromycin, rutilantin, tavromycetin, cyclamycin, and ryemycin belong to the same sub-

TABLE 5. *In vitro* susceptibility of L-forms, spheroplasts, and mutants of gram-negative bacteria to trypanomycin

Test organism	Minimal inhibitory concn (μ g)
<i>Proteus mirabilis</i> VI, normal form	>50.0
<i>P. mirabilis</i> L VI, stable L-form	6.5
<i>P. mirabilis</i> D52, normal form	>50.0
<i>P. mirabilis</i> LD52, stable L-form	6.5
<i>Escherichia coli</i> K-12 (π), normal form	>50.0
<i>E. coli</i> K-12 (π), unstable L-form	8.0
<i>E. coli</i> K-12 AB 1157/AS 19	5.0

TABLE 6. *In vitro* activity of trypanomycin against *Mycoplasma*

Antibiotic	Organism	Minimal inhibitory concn (μ g/ml)	
		Bacteriostatic	Bactericidal
Trypanomycin	<i>M. gallisepticum</i>	0.76	19.0
	<i>M. hyorhinis</i>	3.8	19.0
Tylosin	<i>M. gallisepticum</i>	0.15	95.0
	<i>M. hyorhinis</i>	0.15	>476.0

group, but all of them are derivatives of the ϵ -pyrromycinone.

Acid hydrolysis of the minor constituents of the crude base of trypanomycin gave the same aglycone as the main constituent trypanomycin A₂. Comparative chromatography of trypanomycinone A₂ and a reference sample of ϵ -pyrromycinone showed that the R_F values are different (16). Trypanomycin differs in its biological activities and chromatographic behavior from cinerubin A and B (Table 3). Thus, trypanomycin is a new antibiotic from biological, chemical, and physical points of view.

Biological properties. Trypanomycin was active in vitro against gram-positive bacteria (Table 4). The most susceptible organisms were *Streptomyces hygroscopicus*, a UV-2 mutant from *Staphylococcus aureus* 209P, *Chromobacterium* sp. 1-S, *Caryophanon latum*, staphylococci, and *Bacillus subtilis*. Its inhibitory action against gram-negative bacteria, yeasts, and filamentous fungi was slight or nonexistent. Trypanomycin also inhibited the growth of protoplasts of *Schizosaccharomyces pombe*, which were used as a wall-free model in the screening for new antibiotics. Normal cells of *S. pombe* were not susceptible in vitro to high concentrations of trypanomycin; however, the protoplasts were susceptible to 20 μ g in the agar diffusion test (12).

Because high concentrations of trypanomycin

TABLE 7. *In vitro* antiprotozoal spectrum of trypanomycin

Test organism	Minimal inhibitory concn (μ g/ml)
Nonpathogenic	
<i>Euglena gracilis</i>	100.00
<i>Polytoma uvella</i>	1.25
<i>Paramecium caudatum</i>	1.25
<i>Tetrahymena vorax</i>	5.00
Pathogenic	
<i>Entamoeba invadens</i>	50.00
<i>E. histolytica</i>	10.00
<i>Trichomonas vaginalis</i>	50.00
<i>Leishmania brasiliensis</i>	12.5
<i>Trypanosoma equiperdum</i>	0.5
<i>T. gambiense</i>	0.5
<i>T. cruzi</i>	12.5

^a Produced killing after 6 hr.

TABLE 8. *In vivo* trypanocidal activity of trypanomycin

Individual dose (mg/20-g mouse)	Survival time (days)	Prolonged period of survival (%)	No. of animals
— ^a	6.1	—	14
2.0	19.5	217.4	6
1.0	12.8	108.4	5
0.5	10.5	70.9	6
0.25	6.3	3.1	6

^a Control.

TABLE 9. *Subacute LD₅₀ (mouse) of trypanomycin*

Mode of injection	LD ₅₀ (mg/kg)
Intravenous.....	60.0
Intraperitoneal.....	31.0

inhibited the growth of intact cells of gram-negative bacteria only, the in vitro susceptibility of stable and unstable L-forms such as spheroplasts of gram-negative bacteria was determined. It can be seen in Table 5 that the antibiotic inhibited the growth only of L-forms (14, 17) and mutants of *E. coli* permeable to actinomycin (15). An important bactericidal activity of trypanomycin against two strains of *Mycoplasma* was observed by use of a method devised by P. Kramer (*unpublished data*). In Table 6 it can be seen that trypanomycin inhibited the growth of *Mycoplasma* in vitro to a higher degree than did the macrolide antibiotic tylosin under the test conditions used.

Antiprotozoal activity. Not only fresh fermentation broths of the strain JA 10081 or the mutant strain JA 10081/9 but also the isolated antibiotic inhibited the growth of nonpathogenic and pathogenic protozoa in vitro (Table 7). *Trypanosoma gambiense* (cause of sleeping sickness) showed in vivo (mouse) sensitivity to trypanomycin also. Female mice (AB-Kol strain) 7 to 9 weeks old were infected subcutaneously with *T. gambiense*. Beginning the second day, the animals received each day intravenous injections of trypanomycin in various concentrations until the fifth day. It can be seen in Table 8 that the antibiotic, in a dosage range of 1.0 to 2.0 mg/20-g mouse (daily individual doses) resulted in a significant prolongation of the period of survival of the treated animals. The acute LD₅₀ of trypanomycin depended upon the mode of administration (Table 9). There was a difference between infected and healthy animals in their sensitivity to trypanomycin, but its cause is unknown.

In further investigations, the susceptibility to trypanomycin of several species of *Trypanosoma* was checked. It could be shown that trypanomycin was also active against *T. rhodesiense*, *T. congolense*, and *T. vivax*, but the therapeutic effect in vivo against these species was temporary or only moderate in comparison with the effect against *T. gambiense* and *T. equiperdum*.

Antitumor activity. Trypanomycin was shown to have an inhibitory action on the growth of tumor cells in vitro. An in vitro test was used for the determination of the cytotoxicity of trypanomycin to a permanent growing suspension culture of Ehrlich ascites carcinoma cells (6). Cells of Ehrlich ascites carcinoma adapted to suspension culture were maintained in a medium described by Karzel (10). The results show that cell division was completely eliminated if 5×10^5 tumor cells per ml of culture broth were exposed to 0.1 μ g of trypanomycin for 20 hr. After this time, Ehrlich ascites carcinoma cells retransplanted in NMRI mice could not grow in vivo.

FIG. 1. Representation of mitotic index from treated HeLa cells (solid line) and untreated cells (control, dashed line). The concentration of trypanomycin was 0.32 $\mu\text{g/ml}$. The numbers on the ordinate represent the mitotic index; those on the abscissa represent the time of treatment in hours.

The cytotoxicity of trypanomycin was tested by tube cultures of FL, PN (human kidney cell line), and HeLa cells. The inoculum was 10^5 cells/ml, and the time of observation was 3 to 5 days. No cytotoxic effect could be shown after treatment with trypanomycin in concentrations of 0.16 to 0.32 μg per ml of medium. But concentrations of >0.32 $\mu\text{g/ml}$ showed cytotoxic effects on monolayer cultures of these cell lines.

The antitumor activity of trypanomycin against leukemia L1210 (mice) and solid sarcoma 180 (mice) was not significant. The screening methods used in vivo were described by Jungstand (9).

The cell division of Ehrlich ascites carcinoma cells was completely inhibited by concentrations of 0.1 μg or more of trypanomycin per ml after 24 hr. Cell counts were performed on replicate cultures in duplicate; the cells were cultivated in suspension form and enumerated, after appropriate dilution, with a "TuR" ZG1 electronic cell counter (produced by VEB Transformatoren und Röntgenwerk, Dresden, German Democratic Republic). No Ehrlich ascites carcinoma cells underwent division after exposure to 0.1 μg of trypanomycin/ml for 24 hr when the method of Fleck and Hertwig (6) was used. This suggested a complete loss of reproductive capacity.

To determine the influence of trypanomycin on the mitotic index of HeLa cells, the method described by Penso (11) was used. HeLa cells were grown in lactalbumin hydrolysate medium supplied with 5% Parker medium, 10% calf serum, 2% NaHCO_3 (stock solution 0.75%), and kanamycin (50 $\mu\text{g/ml}$). HeLa cells in cover slip culture were treated with trypanomycin (0.32 $\mu\text{g/ml}$), fixed after Carnoy for increasing intervals of time (4 to 96 hr), and stained with "Kernechtrot" (13).

Figure 1 represents the mitotic index with 95% confidence intervals of HeLa cells based on a count of 6,000 stained cells. Trypanomycin caused some decrease in the number of mitotic figures during the period (96 hr) studied. A great

number of mitoses showed various abnormalities such as multipolar division and disoriented chromosome patterns. The most characteristic changes in cell morphology induced by trypanomycin may be chromosomal fragmentation and star metaphase in dividing cells, and nuclear fragmentation and nucleolar pyknosis in interphase cells. Anomalies of the anaphase and irregular arrangement of fragmented chromosomes along the spindle filaments were general and showed no preponderance over any of the drugs.

A trypanocidal effectiveness has hitherto not been reported for red pigments with indicator properties from streptomycetes. Therefore, it is surprising that the antibiotic trypanomycin according to the group of deoxyribonucleic acid-intercalating anthracyclines has a strong trypanocidal activity in vitro and in vivo.

Further investigations with trypanomycin showed an inhibitory action against *Eimeria tenella* in chickens. Therefore, determination of the coccidiostatic activity of trypanomycin is in progress.

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