STUDIES ON THE MICROORGANISMS PRODUCING ANTIBIOTICS EFFECTIVE TO PLANT PATHOGENS. I.

Streptomyces Sp. 30-10 and its Antibiotic. *

Yosikazu NISIKADO, Tadao INOUYE
and Yasuhiro OKAMOTO

While in search for Actinomycetes antagonistic to plant pathogenic fungi, a Streptomycete effective on *Gibberella zeae* and some other fungi was isolated. The antibiotic produced by this Streptomyces seems to differ from any other known antifungal substances of Actinomycete and is designated "30-10 substance" in this paper. The antibacterial and antifungal spectra, solubility and stability of the crude 30-10 substance are reported in this paper. The physiological and chemical properties are not yet known in details, as the present antibiotic is not pure. The purification and the properties of the antibiotic will be reported in future papers.

I. The Characteristics of *Streptomyces* Sp. 30-10

Streptomyces sp. 30-10 was isolated from the mud of a house-wall at Sakazu, Kurashiki, in 1953. It was selected by the screening agar disc method of using *Gibberella zeae* and some other plant pathogenic fungi as the test organisms.

The characteristics of the Streptomyces cultured on potato sucrose agar slant at 30°C, were observed under microscope as follows:

Aerial mycelium; colorless, no segmentation, filamentous, 0.5—1.0 μ.

Spore; colorless, spherical or oval 0.7—1.4 μ, linear arrangement of spores.

Cultural characteristics on various media were as shown in Table 1.

II. Production of 30-10 Substance

Streptomyces sp. 30-10 was cultured in Erlenmeyer flasks with various liquid media. The production of the antibiotic in these media was as shown in Table 2. The extract of vegetable products such as potato, soybean and wheat bran seemed suitable for the production of the antibiotic. Less antibiotic was produced in starch solution, glucose broth and potato decoction with peptone was lost after 16 days of culture at 30°C. Significant decrease of antibiotic potency was not recognized after 10 days culture in potato de-

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Table 1. Cultural characteristics of *Streptomyces* sp. 30-10.

	Growth	Colony		Sporulation	Pigment	Production of antibiotic	Temp.
		Color	Form				
Plain agar	Very scanty growth						30°C
Starch agar	±	White	Ring, center brown, flat	±	−		〃
Glucose asparagin agar	+	White	Circular, flat	++	−		〃
Nutrient agar	++	Cream	Raised, rugose	−	+++		〃
Potato sucrose agar	+++	White—brownish gray	Floccose	+++	+		〃
Potato plug	++	White—brownish gray	Raised, floccose	++	+		〃
Czapek's solution	±	White	Small circular, flat	±	±	+	〃
Starch solution	+	White	Small circular, flat	±	±	+	〃
Glucose broth	++	White—yellowish gray	Circular or columnar, rugose	++	±	++	〃
Potato decoction + sucrose (g/l) 10	+++	White—brownish gray	Circular, flat	+++	+	+++	〃
Potato decoction + sucrose (g/l) 20	+++	White—brownish gray	Circular, flat	+++	+	+++	〃
Potato decoction + sucrose (g/l) 40	+++	White—brownish gray	Circular, flat	+++	+	+++	〃
Potato decoction + 20 peptone	++	White—yellowish gray	Columnar, rugose	++	+++	++	〃
Soy-bean meal extract	+++	White	Cirucular, rugose	++	++	+++	〃
Wheat bran extract	+++	White	Flat, circular or columnar	+++	+	+++	〃
Gelatin stub			Slowly liquefaction, dark brown pigment				20°
Litmus whey			Not changed				30°

coctions in which sugar content varied as 10, 20 and 40 grams per liter. After 16 days culture, the less the contents of sugar the more the decrease of potency of the filtrate resulted.

In figure 1, productions of the antibiotic in potato decoction with 20 g/l of sucrose and in glucose broth are shown. 100 ml Erlenmeyer flasks containing 50 ml of culture liquid were used. Spores and mycelium taken from

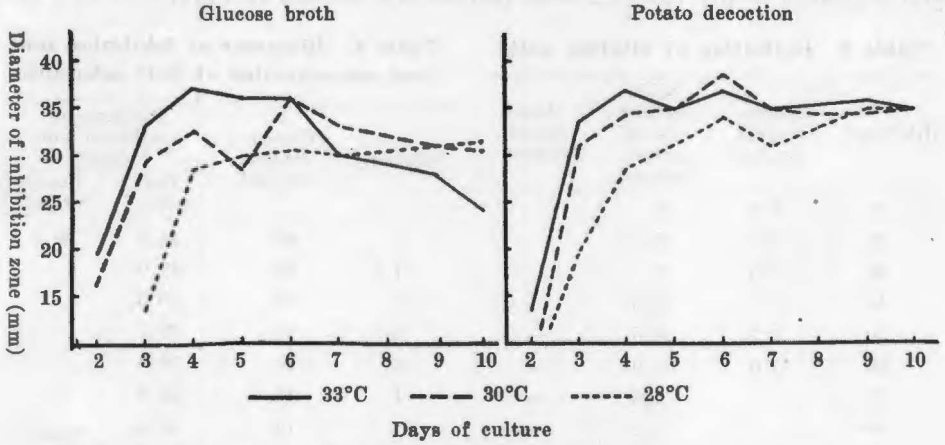

Fig. 1. Production of 30-10 substance in glucose broth and potato decoction.

Table 2. Production of 30-10 substance in various liquid culture media.

Days of culture	Potato decoction+sucrose (g/l)				Glucose broth	Soybean meal extract	Wheat bran extract	Starch solution	Czapek's solution
	10	20	40	30 + Peptone					
10 days	36.5 mm	35.5 〃	37.5 〃	26.5 〃	25.5 〃	35.5 〃	35.5 〃	27.0 〃	14.0 〃
16	23.5	31.0	33.0	0	0	26.0	37.0	29.5	21.0

Remarks: Cultured at 30°C.

potato sucrose agar slants were seeded. Each antibiotic potencies in the figure are the averages of 3 replications in 3 flasks, and show the diameter of inhibition zones obtained by the cup method. Assay of the antibiotic is described in the next article. Maximum of the potency appeared after 4—6 days of culture at 30° and 33°C, but at 28°C it came 1—3 days later. After the potency reached the maximum it decreased slowly in potato decoction, but in glucose broth it decreased rapidly at both temperatures of 30° and 33°C and especially at 33°C. The medium changed to pH 4.6—5.4 from the neutral, after 10 days of culture in 150 ml of potato decoction in 500 ml Erlenmeyer flask. There was no corelation between the value of pH and antibiotic potency. Brown or dark brown pigment was produced in the culture liquid of potato decoction, glucose broth and other media, but was unrelated to the antibiotic production.

III. Assay of 30-10 Substance

Gibberella zeae and *Colletotrichum lindemuthianum* are affected by 30-10 substance. For the assay with cup method, *Gibberella zeae* is convenient to use as test organism. Value of the potencies in this paper assayed by cup method was obtained by the following procedure: 15 ml of potato sucrose agar adjusted to pH 7.0—7.2 was poured and harden in Petri dish as a base

Table 3. Definition of ditution unit.

Dilution*	Concentration d.u./ml	Slide method (% of germination)	Agar streak method
10	6.0	0	
20	3.0	0	
30	2.0	0	
40	1.5	0.5	
50	1.2	0.5	—
60	1.0	1.0	—
70		1.5	—
80		3.2	—
90		4.6	—
100	0.6	4.7	—
110			—
120			±
130			±
140			—
150			±
200		13.2	+
Control		87.3	##

Table 4. Diameter of inhibition zone and concentration of 30-10 substance.

Dilution*	Concentration d.u./ml	Diameter of inhibition zone (mm)	
		Cup method	Paper method
1	60	44.0	30.5
1.5	40	41.0	
2	30	40.5	
2.5	24	39.0	
3	20	36.5	
4	15	35.0	
5	12	33.0	Trace
10	6	28.5	Trace
15	4	25.5	
20	3	23.0	0
25	2.4	22.0	
30	2	21.0	
35	1.7	19.0	
40	1.5	17.0	0
50	1.2	15.5	

* Dilution of the crude substance extracted with benzene and acetone.

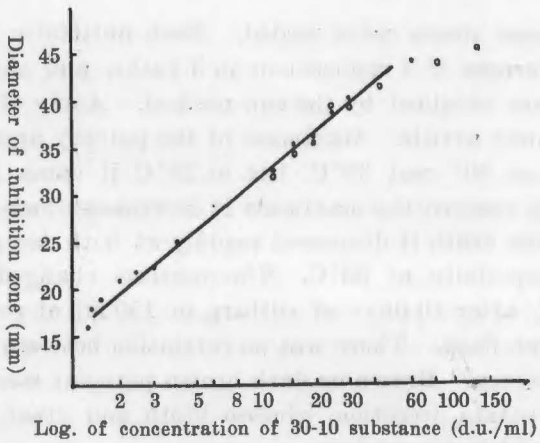

Fig. 2. Concentration of 30-10 substance and the diameter of inhibition zone.

layer and cups were placed on its surface. Five ml of potato sucrose agar (pH 7.0—7.2) with suspended spores of *G. zeae* formed on a slant of 1—2 weeks culture was poured over the base layer and fixed the cups. Cups were filled with the materials for assay and the inhibition zones were measured after 24—30 hours of incubation at 26°C.

To determine the dilution unit of 30-10 substance and the relation between the concentration of the substance in the materials and the diameter of inhibition zones, cup method, agar streak method and slide method were carried out. Paper method was used as a comparison. A solution, which contained KNO_3 1.0 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.2 g and sucrose 10.0 g per liter of water, was used in slide method. Spores of *G. zeae* about 4×10^3/ml in spore density were suspended in the solution containing 30-10 substance in various concentrations. Acetone solution of the crude substance extracted with benzene and acetone, was diluted in various concentration with the above mentioned solution. Results of the experiments applied with the four methods are shown in Table 3 and 4. The minimum concentration of 30-10 substance effective upon *G. zeae* was obtained more distinctly by the slide method than by the agar streak method; therefore, the lowest concentration of 30-10 substance that inhibited 99% of the germination of the spore of *G. zeae* was defined as one dilution unit. Paper method, as shown in Table 4, was less usable for the assay than cup method. In Figure 2, the relation between the diameter of inhibition zone in cup method and the logarithm of the concentration of 30-10 substance is given. To know whether the potency of the culture filtrate was affected or not at various pH values of the material, the pH was changed by HCl or NaOH. There were no significant differences in diameter of inhibition zones at any pH between 3.8—9.2.

IV. Antimicrobial Spectrum of 30-10 Substance

Antimicrobial spectrum of 30-10 substance was tested by agar streak method. The test material of 30-10 substance was extracted from the filtrate with benzene, acetone or methanol. A series of dilution was made with distilled water. Potato sucrose agar adjusted to pH 7.0—7.2 was used for fungi; and nutrient agar adjusted to pH 7.0—7.2 was used for bacteria, yeasts and yeast-like fungi. Plates streaked with fungi were incubated at 26°C for 30—50 hrs., but those with others were incubated at 30°C for the same period. After incubation growth of test organisms was examined. When fungus spores were streaked, the germination of spores was examined under the microscope. Results are shown in Table 5.

Most bacteria, yeasts and yeast-like fungi were not inhibited or only slightly inhibited at high concentrations, but many of the fungi were sensitive to the substance. *Alternaria solani*, *Botrytis bassiana*, *Colletotrichum* and *Gloeosporium*, some of *Fusarium*, *Gibberella zeae*, *Piricularia oryzae*,

Table 5. Antimicrobial spectrum of 30-10 substance.

	Minimal inhibitory concentration of the antibiotic d.u./ml		Minimal inhibitory concentration of the antibiotic d.u./ml
Bacillus subtilis	200—300	*Fusarium bulbigenum* var. *nelumbicolum*	6—12
Erwinia aroideae	>750	*F. caeruleum*	300—750
Escherichia coli communior	>750	*F. lini* (32a)	6—12
Staphylococcus aureus	60—120	*F. lini* (3a)	6—12
Xanthomonas oryzae	150—300	*F. lycopersici*	12—60
		F. niveum	25—38
Saccharomyces sake (122)	300—750	*Gibberella Fujikuroi*	38—75
*Saccharomyces sake**	200—300	*G. zeae* No. 2089	0.6—1.5
		G. zeae No. G. 42	2.4—4.8
Candida albicans (118)	>750	*G. zeae* No. 2361	2.4—4.8
*Candida albicans**	>750	*Gloeosporium nelumbii*	3—6
Monilia sp.	>750	*Metarrhizium anisophilae*	6—12
Torula utilis	>750	*Oospora destructor*	12—60
		Ophiobolus miyabeanus	200—300
Alternaria kikuchiana	150—200	*Penicillium chrysogenum*	6—12
A. solani	2.4—4.8	*P. glaucum*	3—6
Aspergillus flavus	150—200	*Piricularia oryzae*	4.8—9.6
A. niger	300—600	*Pythium* sp.	300—750
A. oryzae	12—60	*Rhizoctonia solani*	4.8—9.6
*A. oryzae***	300—600	*Rosellinia necatrix*	60—120
Botrytis bassiana	6—12	*Sclerotinia libertiana*	4.8—9.6
Ceratostomella fimbriata	60—120	*S. minor*	<5
Colletotrichum lagenarium	1.5—3	*Thielavia basicola*	300—750
C. lindemuthianum	0.6—3	*Trichoderma* sp.	240—480
Corticium centrifugum	150—300	*Trichophyton interdigitale*	25—38
C. gramineum	150—300		
C. sasakii	60—120		

After "A general catalogue of the cultures of micro-organisms maintained in the Japanese collections", 1953.
32a: Lab. of Plant Path., Fac. of Agr., Tokyo Univ.
118: Nagao Inst.
122: Ohara Inst. for Agr. Res.
3a: Div. of Plant Path., Nat. Agr. Res. Inst.
*: Inst. of Appl. Microbiol., Tokyo Univ.
**: Yamano strain, infected to silkworm larva.

Penicillium, *Rhizoctonia solani*, *Sclerotinia libertiana* and *S. minor* were inhibited at comparatively lower concentrations. *Aspergillus niger* and *Trichoderma* sp. were inhibited only at high concentrations. *Candida albicans* and *Torula utilis*, which are inhibited by many known antifungal antibiotics, were not affected by the present antibiotic. *Rhizopus nigricans* was inhibited at a concentration above 200—300 d. u./ml.

V. Stability of 30-10 Substance

The heat torelance of 30-10 substance at various pH of the filtrate was tested. The result is shown in Table 7. Potency of the filtrate was not lost at pH 5.2 when it was boiled for 35 minutes at 100°C, but at pH 2.0 and 11.0 it was inactivated by heating at 100°C for 10 minutes. The filtrate (pH 5.4) was heated at 120°C for 20 minutes, the potency of it decreased to 28 mm from the original 38 mm. From these results, 30-10 substance is stable in acidic side but it is inactivated in alkaline side (pH 11.0) at room

Table 7. Heat stability of 30-10 substance.

pH	Time of heating at 100°C		
	10 min.	35 min.	non-treatment
2.0	0	0	33.5
5.2	34.0	33.5	35.0
9.5	29.0	27.5	33.0
11.0	0	0	0

Remarks: Culture filtrate was changed its pH with HCl and NaOH.
Average in 3 replications by cup method (mm).

Table 8. Aging of 30-10 substance.

	Days of incubation				
	1	2	5	7	13
ice box	36.5	37.0	36.5	37.0	37.0
30°C	36.5	37.0	37.5	37.0	34.0
33°C	36.0	37.5	36.5	34.5	32.0

Remarks: pH of culture filtrate was 5.4.
Average in 3 replications by cup method (mm).

temperature. It seems to be stable against heat in slightly acidic side. In another experiment, the filtrate (pH 5.4) was incubated at various temperatures for successive days and the change of the antifungal potency was examined. As shown in Table 8, the potency of the filtrate when stored in ice box was not changed for 13 days, and also after 7 days at 30°C or 5 days at 33°C.

VI. Extraction of 30-10 Substance

The 30-10 substance was easily extracted with benzene, chloroform, amyl acetate, ether and butanol at any pH of 4.0, 5.4, 6.4 or 10.2 from the culture filtrate. It was difficult to extract with petroleum ether. Benzene was the most efficient solvent for extraction; 80% or more antibiotic substance was extracted by 1/4 volume of benzene added to the culture filtrate at pH 5.4.

Table 6. Comparison of 30-10 substance and

Antibiotic	Author		Antimicrobial spectrum
Actidione	Whiffen *et al.* Aiso *et al.* Kodama *et al.*	1946 1952 1952	Effective to yeasts, *A. niger* and *R. nigricans*.
Actinomycin	Waksman *et al.*	1940	Effective to *Rhizopus* ; antibacterial.
Actinone	Ikeda *et al.*	1950	Effective to yeasts but *P. chrysogenum*.
Antimycin A	Leben *et al.* Lockwood *et al.*	1948 1954	Ineffetive to *F. lycopersici*. less effective to *C. lindemuthianum*, inhibit *O. miyabeanus* in cross streaking test.
Ascosin	Hickey *et al.* Utahara *et al.*	1952 1954	Effective to *C. albicans*, *T. utilis* and *S. sake*.
Blasticidin 1, 2 and 3	Fukunaga *et al.*	1955	Effective to *A. niger*, *A. kikuchiana* and *C. fimbriata* in lower concentration, ineffective to *P. glaucum*.
Cacaomycetin	Wakaki *et al.*	1952	Effective to *A. niger*, *C. fimbriata* and *O. miyabeanus*.
Candicidin	Lechevalier *et al.* Utahara *et al.*	1953 1954	Effective to *C. albicans*.
Chromin	Wakaki *et al.*	1952	Effective to *C. albicans*, *T. utilis*, *O. miyabeanus* and *C. sasakii*.
Endomycin	Gottlieb *et al.* Smeby *et al.*	1951 1952	Effective to *C. albicans*, *T. utilis*, *B. subtilis* and other bacteria.
Eumycetin	Arai *et al.*	1954	Effective to *C. albicans*, *T. utilis* and *S. sake*.
Eurocidin	Nakazawa *et al.* Utahara *et al.*	1953 1954	Effective to *C. albicans*, *T. utilis* and *S. sake*.
Flavacid	Takahashi	1953	Effective to *C. albicans*, *T. utilis*, yeasts and *A. niger*.
Fradicin	Swart *et al.* Hickey *et al.*	1950 1951	Effective to yeasts.
Fungicidin	Hazen & Brown Utahara *et al.*	1951 1954	Effective to C. *albicans*, *T. utilis* and *S. sake*.
Helixin A, B, C & D	Leben *et al.* Smeby *et al.*	1951 1952 1952	Effective to *B. subtilis*.
Hygroscopin	Nakazawa *et al.*	1953	Effective to *C. albicans*, yeasts, *R. nigricans* and *O. miyabeanus*.
Mediocidin	Utahara *et al.*	1954	Effective to *C. albicans* and *S. sake*.
Microcin A, B	Taira and Fujii	1952	Effective to *S. sake* and other yeasts.
Moldin	Maeda *et al.*	1952	Effective to *C. albicans* and *T. utilis*.
Musarin	Thaysen *et al.*	1945	
Mycelin	Aiso *et al.*	1952	Effective to *C. albicans*.
Nigericin	Harned *et al.*	1951	Effective to gram positive bacteria.
Phaeofacin	Maeda *et al.*	1952	Effective to *T. utilis*.
Rimocidin	Davisson *et al.* Utahara *et al.*	1951 1954	Effective to *C. albicans*, *T. utilis* and *S. sake*.
Rotaventin	Hosoya *et al.*	1952	Effective to yeasts, ineffective to *B. bassiana* and *T. interdigitale*.
Seligocidin	Nakamura *et al.*	1954	Effective to *C. albicans* and *T. utilis*.
Thiolutin	Tanner *et al.* Celmer *et al.*	1950 1952	
Trichomycin	Hosoya *et al.*	1952	Effective to *C. albicans* and *T. utilis*.

known antifungal actinomycete antibiotics.

Solubility	Stability and other properties
Water soluble.	
Slightly soluble in ether.	
Slightly soluble in benzene, insoluble in chloroform. Sparlingly soluble in benzene.	
Insoluble in acetone, chloroform, ether and benzene.	Presents in mycelium mostly.
	Antibiotic producing strain II A 327 well grows on Czapek's solution.
	Absorbed by Seitz filter.
Insoluble in benzene and ether. Slightly soluble in acetone, ethanol and chloroform.	
Soluble in water, slightly in acetone, insoluble in ether.	Absorbed by Seitz filter.
Insoluble in ether, chloroform and benzene.	Presents in mycelium.
	Presents in mycelium.
Soluble in water, difficult in acetone, insoluble in benzene and ether.	Obtained from culture filtrate and mycelium.
Insoluble in ether, benzene, and chloroform.	Presents in mycelium, inactivated its potency to 1/4 by the treatment of 5 min., 100°C at pH 7.0.
Insoluble in methanol and ethanol.	
Sparingly soluble in acetone, methanol and ethanol. Insoluble in ether and benzene.	Presents in mycelium.
Slightly soluble in cholroform and butanol and acetone. Insoluble in ether. Soluble in water (or more pH 4.0—5.0).	
Insoluble in ether and benzene.	
Slightly soluble in ether, chloroform and benzene.	Produced by *Micromonospora*.
Sparlingly soluble in petroleum ether, ether and benzene.	Presents in mycelium and filtrate.
Insoluble in 30% acetone and alcohol.	Decreased potency by heating 30 min. at 100°C to 1/2 at pH 7.0.
Insoluble in ether.	Presents in mycelium.
Slightly soluble in ether.	Presents in mycelium and culture filtrate.
Soluble in water, slightly in ethanol and acetone, insoluble in benzene and ether.	
Insoluble in ether, chloroform. benzene and amyl acetate.	Presents in mycelium.
Slightly soluble in ethanol.	Presents in mycelium mostly.
Slightly soluble in ether and benzene.	
Insoluble in ether, slightly soluble in acetone, ethanol and butanol.	Presents in mycelium mostly.

Table 6. Continued.

Antibiotic	Author	Antimicrobial spectrum
30-10 substance	Nisikado *et al.* (present paper)	Ineffective to *C. albicans*, *T. utilis* and yeasts; *A. niger*, *R. nigricans*, *O. miyabeanus* are not affected without the application of higher concentration. Effective to *G. zeae* and *C. lindemuthianum*.

Yellowish brown residue remained after evaporating the solvent, and the antibiotic substance in the residue was soluble in acetone, methanol, ethanol and any of the above mentioned solvents. The antibiotic was difficult to dissolve in water or petroleum ether. It was adsorbed by active carbon in culture filtrate at the range of pH 3.2—9.3. After the adsorption it was extracted but slightly with acetone, methanol and ethanol. Only about 4% of the antibiotic was extracted at 16°C and 24°C after 30 and 60 minutes with acetone which was the best solvent for elution. From this result the method was found to be unsatisfactory for the extraction. It was not adsorbed by active kaoline nor active aluminium oxide. The antibiotic was not detected in the mycelial mass when extracted by acetone, methanol and ethanol.

VII. Discussion

It is difficult to discuss whether the present 30-10 substance differs or not to any described antifungal antibiotics produced by Actinomycetes, as the data of this paper are not yet sufficient. But, there are some differences between crude 30-10 substance and known antibiotics in the antimicrobial spectrum, solubility and stability. The comparisons are summarized in Table 6. The 30-10 substance seems to differ from any known actinomycete antibiotics as shown in the table. In further studies, purified substance and its physical, chemical and biological properties will be reported in more details, and the antibiotic will be determined and named in another paper.

VIII. Summary

Streptomyces sp. 30-10 was found during the course of the screening Actinomycetes antagonistic to plant pathogenic fungi. The antibiotic, 30-10 substance in this paper, was produced in the liquid media, such as potato decoction, wheat bran extract and glucose broth. It is effective upon *Gibberella zeae*, *Colletotrichum lindemuthianum* and some other anthracnose fungi, some of *Fusarium* and *Sclerotinia* in a comparatively lower concentration, and less effective to *Aspergillus niger* and *Rhizopus nigricans*. *Candida albicans*, *Torula utilis*, yeasts and many of bacteria were not affected. Crude

Solubility	Stability and other properties
Soluble in acetone, methanol, ethanol, butanol, amyl acetate, ether, chloroform and benzene, very slightly in water and petroleum ether.	Presents in culture filtrate. Heat stable in slightly acidic solution. Potency of the filtrate was not in activated at 33°C for 5 days at least.

30-10 substance, extracted by benzene and acetone, was soluble in acetone, methanol, ethanol, butanol, amyl acetate, ether, chloroform and benzene, but with difficult in water and petroleum ether. Except in extreme acidic or alkaline solution, it is stable at room temperature. In slightly acidic solution it seems to be stable to heat. Antibiotic potency of culture filtrate was not decreased by 5 days incubation at 33°C. The 30-10 substance seems to differ from any known antifungal substances, produced by Actinomycetes, in its properties of antimicrobial spectrum, solubility and stability.

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