CITROMYCIN, A NEW ANTIBIOTIC. I
ISOLATION AND CHARACTERIZATION

YOKO KUSAKABE, YOSHIHIRO YAMAUCHI, CHIZUKO NAGATSU,
HIROSHI ABE*, KATSUYUKI AKASAKI*
and SHIROH SHIRATO

Research Division of Kaken Chemical Co., Ltd.**
and Shimizu Laboratory of Kumiai Chemical Industry Co., Ltd.*

(Received for publication December 28, 1968)

A new antibiotic, citromycin, was isolated from cultures of two strains of *Streptomyces*. It was concluded to be a new water-soluble, basic antibiotic related to the streptothricin-like group. The isolation and purification as well as the physical, chemical and biological properties of the antibiotic are described. The differences of citromycin to various new streptothricin-like antibiotics are examined and discussed.

During the course of our screening program for new antibiotics, a soil isolate of *Streptomyces* IN-1483 was found to produce three water-soluble basic antibiotics, besides the known antibiotic, azomycin. One of the three water-soluble antibiotics, named 1483-A, showed inhibitory activity against Gram-positive and -negative bacteria. This substance was extracted from the culture filtrate, and after purification a white powder which virtually contained no impurity, was obtained. The physical, chemical and biological properties of 1483-A were examined. From these data, this substance was found to be a new antibiotic and was named citromycin. After this, another strain of *Streptomyces*, IN-2035, was isolated and found to produce citromycin also.

In this paper, a fermentation process for the production of antibiotics from *Streptomyces* IN-1483, the isolation of citromycin from the broth filtrate, and the properties of citromycin are presented. The characteristics of the strains producing citromycin will be presented in the subsequent papers of this series.

Production and Isolation

Preculture of *Streptomyces* IN-1483 was carried out in K-1 flasks[1] on a rotary shaking machine and at 27°C for 48 hours, and inoculated into 400 liters of sterilized medium in a 600-liter fermentor, and held at 27°C for 96 hours with adequate aeration. The medium contained the following ingredients: sucrose 2.5, dried beer yeast 0.3, soy-flour 4.0, NaCl 0.2, ammonium sulfate 0.2, $CaCO_3$ 0.2, KH_2PO_4 0.015 per cent (weight/volume). Antibiotic activity was determined by the agar diffusion disc assay with *Bacillus subtilis* PCI 219 or *Mycobacterium* 607 as test organism. Sometimes

* 100 Shibukawa, Shimizu, Shizuoka Prefecture, Japan
** 6-42, 1-chome Jujodai, Kita-ku, Tokyo, Japan

bioautographic detection was carried out to check the kind of antibiotics in the broth or samples of purification.

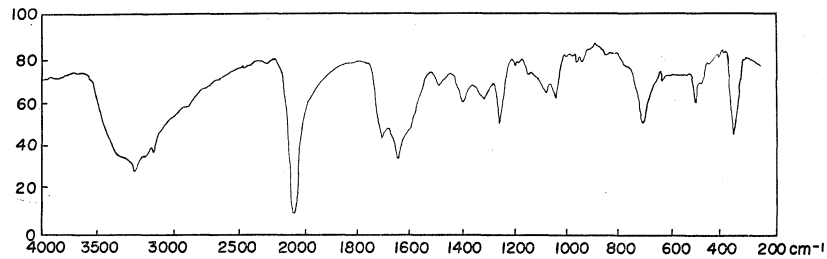
Usually after 3 or 4 days the concentration of antibiotics in the broth reached maximum titer, and the broth was filtered. The filtrate was then passed through a column charged with Amberlite IRC-50 (Na-type) of 10-liter volume. The passed solution were collected and concentrated *in vacuo*, followed by extraction with *n*-butanol. The *n*-butanol solution was separated from the water layer and concentrated *in vacuo* to dryness and subsequently extracted with methanol. The methanol extract was concentrated *in vacuo* and kept in the refrigerator overnight. The white precipitate thus obtained was collected and recrystallized from methanol. The crystalline material was separated from the mother liquor and dried in a desiccator. This preparation was named "I" for convenience.

The charged ion-exchange resin was washed with tap water, followed by elution with 0.5 N hydrochloric acid. The antibiotically active fractions were collected, combined, and neutralized to pH 5.0, followed by concentration *in vacuo* to one-tenth volume. This concentrate was named "II".

When the paper chromatography was carried out with the broth filtrate using the following solvent system of *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12), four inhibition spots, Rf values of which were 1.0, 0.5, 0.35 and 0.2 respectively, were recognized. When "I" was subjected to the same test, however, only one spot of Rf value 1.0 was observed, whereas three spots, Rf 0.5, 0.35 and 0.2 were produced with "II".

The ultraviolet absorption of "I" in methanol has a maximum at 313 mμ which is similar to the known antibiotic azomycin[2]. Also, the antibiotic spectrum, the

Fig. 1. IR spectrum of citromycin reineckate (in KBr tablet)



solubility, and the stability show the identity of "I" with azomycin. Finally authentic azomycin (kindly provided by Dr. K. MAEDA, the Institute of Microbial Chemistry, Tokyo) was compared with "I" by paper chromatography using several solvent systems, but no difference was found between them. Thus, "I" was identified as azomycin.

Fig. 2. Ultraviolet spectrum of citromycin hydrochloride (114 ppm, H_2O)

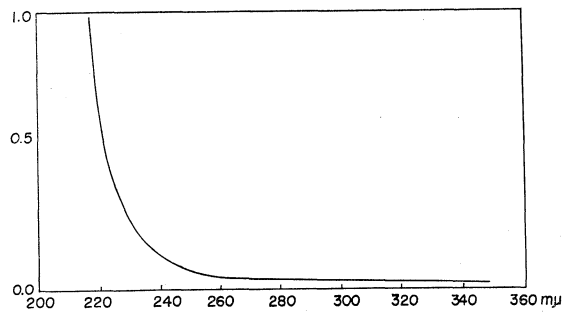


Fig. 3. IR spectrum of citromycin hydrochloride (in KBr tablet)

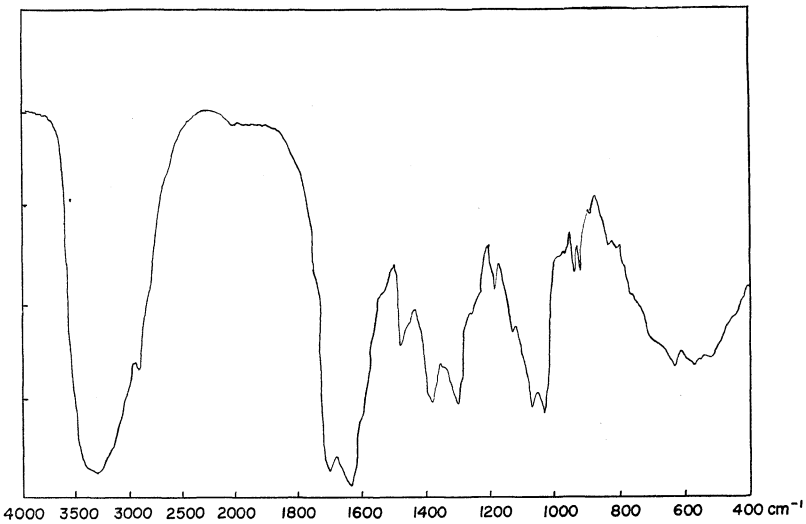


Fig. 4. Paper chromatography of acid-hydrolysate of citromycin compared with β-lysine
Indication: ninhydrin
C: acid-hydrolysate of citromycin
B: authentic β-lysine
Solvent system: *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12)

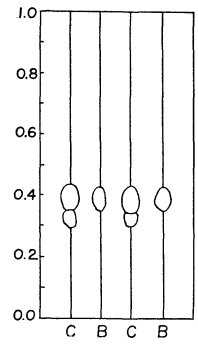
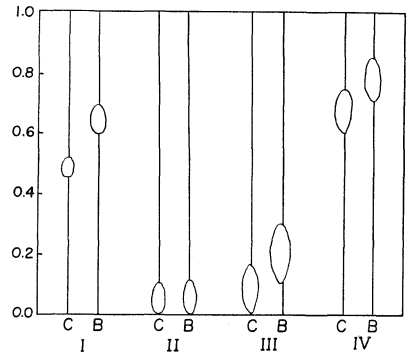


Fig. 5. Comparison of citromycin with BD-12 with paper-chromatography and bioautography with *B. subtilis*.
C: citromycin hydrochloride
B: BD-12 hydrochloride
Solvent system:
I. *n*-Propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12)
II. water
III. 50 % Acetone
IV. Chloroform - methanol - 17 % aqueous ammonia (2 : 1 : 1) upper layer (Silica-gel thin-layer chromatography)



The antibiotic in "II" having an Rf value of 0.5 was thought to be the main product of the culture, and named 1483-A. The minor components, Rf 0.35 and Rf 0.2 were named 1483-B and 1483-C respectively, and separated from 1483-A through the following purification procedure: The concentrate of 1.5 liter described above was adjusted to pH 6.3 and 60 g active carbon were added and the mixture stirred for 30 minutes. The adsorbed carbon was collected, washed with tap water, and eluted with aqueous acetone (acetone 40 %). Elution of antibiotics was repeated three times with the same procedure and the combined eluates were concentrated under reduced pressure to 200 ml. The concentrate was then subjected to carbon column

Fig. 6. Comparison of citromycin with SF-701 with paper chromatography and bioautography with *B. subtilis*.
C: citromycin hydrochloride
S: SF-701 hydrochloride
Solvent system:
I. *n*-Propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12)
II. Chloroform - methanol - 17 % aqueous ammonia (2 : 1 : 1) upper layer
III. Butanol - acetic acid - water (2 : 1 : 1)
IV. 50 % Acetone

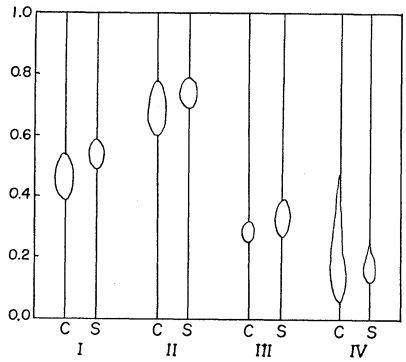
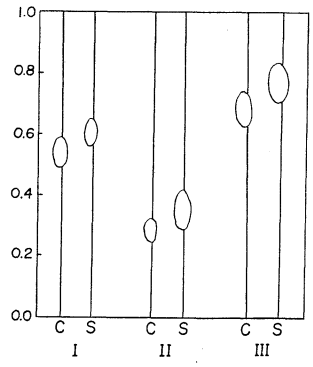


Fig. 7. Comparison of citromycin with sclerothricin with paper chromatography and bioautography with *B. subtilis*.
C: citromycin hydrochloride
S: sclerothricin hydrochloride
Solvent system:
I. *n*-Propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12)
II. Butanol - acetic acid - water (2 : 1 : 1)
III. Chloroform - methanol - 17 % aqueous ammonia (2 : 1 : 1) upper layer



chromatography. The concentrate was introduced from the top of the carbon column of 2-liter volume in a glass cylinder, followed by washing with water and development with aqueous acetone (acetone 10 %). The active fractions of the eluate with aqueous acetone (acetone 20 %) were collected, and after concentrated *in vacuo* it was freeze-dried to yield 18 g of a pale yellowish powder. In this powder 1483-B and 1483-C could not be detected on the paper chromatogram.

Further purification of 1483-A was carried out through crystallization as the Reineckate. The purified white powder of 1483-A was dissolved in a small amount of distilled water and ammonium Reineckate was added. 1483-A formed a red crystalline Reineckate which was collected and washed with a small amount of distilled water. The crystals were plate form and decomposed at 155~165°C. Anal. Found: C 27.07, H 4.10, N 24.08, Cr 9.52, S 22.47. The IR spectrum is shown in Fig. 1. The Rf value on paper chromatogram developed with *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12) was 0.47~0.53.

Pure specimen of 1483-A hydrochloride was prepared from the white powder of 1483-A hydrochloride described above by means of alumina column chromatography with methanol as solvent for development and elution.

Properties of 1483-A

The hydrochloride of 1483-A is a white powder which decomposes at 211°C. Elementary analysis: C 35.58, H 6.36, N 17.42, O 26.96, Cl 11.13. It is optically active, $[\alpha]_D^{20}$ −688 (*c* 1, H_2O), soluble in water or methanol, sparlingly soluble in ethanol, and insoluble in almost any other organic solvents. It is adsorbed on cation-exchange resin and eluted with aqueous mineral acids. The UV and IR spectra are

Table 1. Stability of citromycin at various pH.

pH	Residual antimicrobial activity*
2.0	100 %
4.0	100
6.0	100
7.0	70
8.0	53
9.0	28
10.0	10

* Solutions (in water) were kept at 37°C for 6 hours.
Potency was assayed with disc agar-diffusion method with *Bacillus subtilis* as a test organism.

Table 2. Summarized papergrams of citromycin.

Solvent system	Rf value*
A: Wet butanol	0.0
B: 20 % Ammonium chloride	1.0
C: 75 % Phenol	0.65
D: 50 % Acetone	0~0.17
E: Butanol - methanol - water (4 : 1 : 2) plus 1.5 % methyl orange	0.37~0.46
F: Butanol - methanol - water (4 : 1 : 2)	0~0.1
G: Benzene - methanol (4 : 1)	0.0
H: Water	0~0.1

* Indicated by bioautography with *B. subtilis*.

Table 3. Antimicrobial activity of citromycin (I)

Test organism	M.I.C. (mcg/ml)*
Bacillus subtilis PCI 219	50
Micrococcus luteus ATCC 398	10
Staphylococcus aureus FDA 209P	50
Escherichia coli NIHJ	20
Klebsiella pneumoniae ATCC 10031	100
Proteus vulgaris OX-19	50
Serratia marcescens IAM-1223	50
Pseudomonas aeruginosa NRRL B-1000	100
Shigella dysenteriae	20
Nocardia asteroides ATCC 3308	>200
Candida albicans YU-1200	>200
Cryptococcus neoformans	50
Aspergillus fumigatus NI 5561	>200
Sporotrichum schenckii	100
Trichophyton mentagrophytes	200
Trichophyton rubrum	>200
Mycobacterium smegmatis ATCC 607	20

* Agar dilution streak method.

Table 4. Antimicrobial activity of citromycin (II) (Phytopathogenic microorganisms)

Test organism	M.I.C. (mcg/ml)*
Ophiobolus miyabeanus	>100
Diaporthe citri	>100
Corticium rolfsii	>100
Gloeosporium laeticolor	100
Alternaria kikuchiana	>100
Fusarium oxysporum	>100
Corynebacterium sepedonicum	>100
Xanthomonas oryzae	10
Xanthomonas citri	10
Piricularia oryzae	>100
Gladosporium carpophilum	>100

* Agar dilution streak method.

shown in Figs. 2 and 3 respectively. It gives positive EHRLICH, ninhydrin (in pyridine), and diazo reactions, while negative $FeCl_3$, maltol, ELSON-MORGAN, biuret, SAKAGUCHI, santhrone, FEHLING, and ninhydrin (in water) reactions.

On the paper chromatogram developed with various solvent systems, 1483-A HCl appeared at the following Rf position:

n-Propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12) Rf 0.50
n-Butanol - acetic acid - water (2 : 1 : 1) Rf 0.24~0.31
Chloroform - methanol - 17 % aqueous ammonia (2 : 1 : 1, upper layer) Rf 0.74

The Rf values of summarized paper chromatography are shown in Table 2. As shown in Table 1, when dissolved in water, 1483-A is rather stable in acid, but unstable in alkali.

The antimicrobial spectrum is shown in Tables 3 and 4. The acute intravenous LD_{50} in mice was over 100 mg/kg. However, a delayed toxicity was observed; when 100 mg/kg was injected intravenously, 5 of 10 mice died on the 5th day, and all the other mice died on the 6th day. After 3-day administration, a marked gangrene of

the tail developed in most of the mice distal to the point of intravenous injection and in some case this part of the tail was lost.

Discussion

1483-A hydrochloride is differentiated from the known antibiotics by its physical and chemical properties as well as the biological characteristics. It belongs to the group of water-soluble, basic antibiotics. The antimicrobial spectrum of 1483-A is rather wide, and in this point it differs from viomycin, capreomycin, alboverticilline and triculamine, all of which have very limited antibiotic spectrum. That 1483-A has no characteristic UV absorption shows distinct difference from antibiotics such as phleomycin, bleomycins, and cycloserine. It is differentiated from streptomycins, glebomycins or bluensomycin which belong to another group of water-soluble, basic antibiotics, because of giving negative SAKAGUCHI reaction. It is a levorotatory water-soluble substance, so one may differentiate it from the known members of dextrorotatory water-soluble basic antibiotics such as paromomycins, zygomycins, hydroxymycins, aminosidin, catenulin, monomycin, gentamicin, geomycins, hygromycin B, actinospectacin, neomycins, and kanamycins. It is also differentiated from streptothricins group antibiotics as follows;

(1) 1483-A gives negative biuret, ninhydrin (in water), and FEHLING reactions, whereas streptothricins are described as giving positive[3,4].

(2) On the silica-gel thin-layer chromatogram developed with a solvent system of upper layer composed of chloroform, methanol and 17 % aqueous ammonia (2 : 1 : 1), 1483-A was located at Rf value of 0.6~0.7 which was much higher than kanamycin, whereas streptothricins have been known to be located at far lower Rf values than that of kanamycin[5].

However, 1483-A has some streptothricin-like properties. For instance, it shows delayed toxicity and is a water-soluble, basic, levorotatory, rather wide spectrum antibiotic. Therefore it was attempted to detect β-lysine in the acid hydrolysate of 1483-A. The standard β-lysine was kindly supplied by Dr. SETSUO TAKEUCHI, the University of Tokyo. The acid hydrolysis of 1483-A was carried out in 6 N HCl at 120°C for 12 hours. The paper chromatographic pattern of the hydrolyzate of 1483-A shows a spot of very similar Rf value to that of β-lysine.

Thus, 1483-A was concluded to a new member of the streptothricin-like antibiotics. Recently, new streptothricin-like antibiotics were found and reported by various groups. The comparison tests for 1483-A with three antibiotics, BD-12[6], SF-701[7], and sclerothricin[8] were carried out by means of paper chromatography. The authentic samples were kindly provided by the following authors: BD-12 by Dr. TOMOHARU OKUDA, SF-701 by Dr. TARO NIIDA and sclerothricin by Prof. HIROSHI YONEHARA. The results were shown in Figs. 5, 6, and 7. As can be seen in these figures, the Rf values of these three antibiotics were different from that of 1483-A.

LL-AC-54[9] and BY-81[7] are new type streptothricin-like substances and E-749 C is also reported as a new antibiotic which is thought to be identical with LL-AC-54[10]. Differentiation of 1483-A from these antibiotics is as follows: (1) LL-AC-54 (or E-749 C) was reported as having no β-lysine in its structure, whereas 1483-A had the β-lysine moiety as described before. (2) BY-81 decomposes at 142°C. Moreover the color reactions differ as follows: ELSON-MORGAN (positive, BY-81; negative, 1483-A), triphenyl-tetrazolium-chloride (positive, BY-81; negative, 1483-A).

Yazumycin[11] is also a new streptothricin-like antibiotic isolated by the authors. However, the Rf value of yazumycin on the silica-gel thin-layer chromatogram developed with the upper layer of chloroform - methanol - 17 % aqueous ammonia (2 : 1 : 1) was lower than kanamycin, whereas the Rf value of 1483-A was higher than that of kanamycin.

Thus, it can be concluded that 1483-A is a new antibiotic, for which the name citromycin is proposed. Citromycin inhibited the growth of *Xanthomonas citri* at 10 mcg/ml

in vitro, and showed a protective effect against canker of citrus in the dose of 100 or 200 ppm in the pot test. However, in the field test, it showed rather strong phytotoxicity to adult citrus trees.

Acknowledgement

The authors wish to express their sincere thanks to Dr. KENJI MAEDA, the Institute of Microbial Chemistry, Tokyo, Dr. TOMOHARU OKUDA, Tanabe Seiyaku Co., Ltd., Dr. TARO NIIDA, Meiji Seika Ltd., and Prof. HIROSHI YONEHARA, the University of Tokyo, for kindly supplying the authentic samples of new antibiotics, azomycin, BD-12, SF-701 and sclerothricin, each of which they isolated respectively. Special thanks are expressed to Dr. SETSUO TAKEUCHI, the University of Tokyo, for kindly donating an authentic sample of β-lysine.

Literature

1) SHIRATO, S. & H. MOTOYAMA: Fermentation studies with *Streptomyces griseus*. II. Synthetic media for the production of streptomycin. Appl. Microbiol. 14 : 706~710, 1966.
2) UMEZAWA, H.: Index of Antibiotics from Actinomycetes. p. 168, University of Tokyo Press, Tokyo, 1967.
3) FRIED, J. & O. WINTERSTEINER: Crystalline reineckates of streptothricin and streptomycin. Science 101 : 613~615, 1945.
4) CARTER, H. E.; R. K. CLARK, Jr., P. KOHN, J. W. ROTHROCK, W. R. TAYLOR, C. A. WEST, G. B. WHITFIELD & W. G. JACKSON: Streptothricin. I. Preparation, properties and hydrolysis products. J. Am. Chem. Soc. 76 : 566~569, 1954.
5) IKEKAWA, T.; F. IWAMI, E. AKITA & H. UMEZAWA: Application of thin-layer chromatography for separation and identification of antibiotics. J. Antibiotics, Ser. A 16 : 56~57, 1963.
6) ITO, Y.; Y. OHASHI, Y. SAKURAI, M. SAKURAZAWA, H. YOSHIDA, S. AWATAGUCHI & T. OKUDA: New basic water-soluble antibiotics BD-12 and BY-81. II. Isolation, purification and properties. J. Antibiotics 21 : 307~312, 1968.
7) TSURUOKA, T.; T. SHOUMURA, N. EZAKI, T. NIWA & T. NIIDA: SF-701, a new streptothricin-like antibiotic. J. Antibiotics 21 : 237~238, 1968.
8) MAKINO, S.; A. SHIMAZU, T. HIDAKA, S. TAKEUCHI & H. YONEHARA: Studies on a new antibiotic sclerothricin produced by *Streptomyces* sp. 7672 MC 4. Presented at the 161 st Meeting of Japan Antibiotics Research Association, May 24, 1968.
9) ZBINOVSKY, V.; W. K. HAUSMANN, E. R. WETZEL, D. B. BORDERS & E. L. PATTERSON: Isolation and characterization of antibiotic LL-AC 541. Appl. Microbiol. 16 : 614~616, 1968.
10) SHOJI, J.; S. KOZUKI, M. EBATA & H. OTSUKA: A water-soluble basic antibiotic E-749-C identical with LL-AC 541. J. Antibiotics 21 : 509~511, 1968.
11) AKASAKI, K.; H. ABE, A. SEINO & S. SHIRATO: Yazumycin, a new antibiotic produced by *Streptomyces lavendulae*. J. Antibiotics 21 : 98~105, 1968.