Formamicin, a Novel Antifungal Antibiotic Produced by a Strain of *Saccharothrix* sp.

I. Taxonomy, Production, Isolation and Biological Properties

Masayuki Igarashi, Naoko Kinoshita, Takako Ikeda, Eiko Nakagawa, Masa Hamada and Tomio Takeuchi

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication July 25, 1997)

Formamicin, an antifungal antibiotic, was isolated from the cultured broth of an actinomycete strain. The strain was isolated from a soil collected at Setagaya-ku, Tokyo, Japan, and identified as *Saccharothrix* sp. MK27-91F2.

Formamicin was extracted with acetone from cultured mycelia and purified by silicagel and Sephadex LH-20 column chromatographies and CPC (Centrifugal liquid-liquid Partition Chromatography).

Formamicin showed strong antimicrobial activity against phytopathogenic fungi.

In the course of screening for new anti-microbial substances from microorganisms, we found that a actinomycete strain which was isolated from a soil collected at Setagaya-ku, Tokyo, Japan, produced a new 16-membered macrolide, named formamicin, belong to bafilomycin-concanamycin type antibiotics. Formamicin showed strong antifungal activity against phytopathogenic fungi.

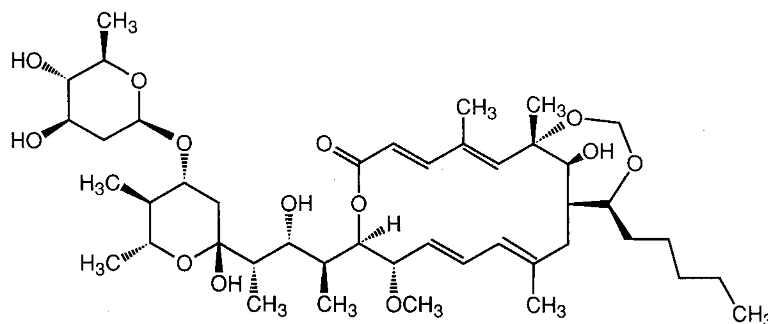
In this paper, we describe the identification of the producing organism together with the isolation, fermentation and biological activities of formamicin. Physico-chemical properties and structure elucidation of the compound will be described in the accompanying paper[1].

Materials and Methods

Taxonomy

Formamicin producing organism, strain MK27-91F2, was isolated from a soil sample collected at Setagaya-ku, Tokyo, Japan. Morphological, cultural and physiological properties of the strain MK27-91F2 were examined according to the methods described by Shirling and Gottlieb[2], and Waksman[3]. Detailed observation of mycelial morphologies was performed with the use of scanning electron microscope (Model S-570, Hitachi) after strain MK27-91F2 was incubated on sucrose-nitrate agar and inorganic salts-starch agar (ISP No. 4) at 27°C for 10 days. Chemical analysis of cell wall was analyzed using thin layer chromatography (TLC) according to the method of Staneck and Roberts[4]. Cell wall sugars and whole-cell sugars were determined by

Fig. 1. Structure of formamicin.



the methods of LECHEVALIER and LECHEVALIER[5], and MIKAMI and ISHIDA[6]. The acyl type of cell wall was analyzed according to the method of UCHIDA[7]. Menaquinone was performed with the method of TAMAOKA *et al.*[8] Phospholipids and mycolic acids were analyzed by the procedures of MINNIKIN *et al.*[9,10] The fatty acids were analyzed by gas chromatography of whole-cell methanolysates[11].

Fermentation

A slant culture of the formamicin-producing organism was inoculated in to a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of glycerol 2.5%, yeast extract 1.0%, meat extract 1.0%, polypeptone 0.5%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.5% and $CaCO_3$ 0.3% in deionized water (pH 7.4 before sterilization). The culture was incubated on a rotary shaker (180 rpm) at 30°C for 3 days. The seed culture (330 ml) of the strain were transferred into 30-liter fermentor containing 15 liters of a producing medium consisting of glycerol 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.3% in deionized water (pH 7.4). The fermentation was carried out at 27°C for 4 days under agitation of 200 rpm.

Analytical Procedure

Content of formamicin in fermentation broth and purification steps was monitored with reversed phase HPLC and silica gel TLC. HPLC was performed CAPCELL PACK C_{18} column (4.6 × 150 mm, Shiseido Co., Ltd., Japan; mobile phase, 70% aq acetonitrile; flow rate, 1.0 ml/minute; column temperature, 25°C; detection, UV (274 nm). It was eluted at 8.2 minutes. TLC was performed with Kieselgel 60 F_{254} (Art. No. 5715, Merck) developed with $CHCl_3$ - MeOH (9 : 1). Spot on TLC was detected by molybdophosphoric acid-sulfuric acid and UV quenting (254 nm). Rf value of formamicin was 0.39.

Biological Activity

The minimum inhibitory concentrations (MIC) of formamicin was examined by serial agar dilution method using potato sucrose agar for antiphytopathogenic fungi and nutrient - 1% glucose agar for yeast and fungi and Mueller-Hinton agar (Difco) for bacteria. The MIC was observed after 96 hours incubation at 27°C for phytopathogenic fungi and 42 hours incubation at 27°C for yeast and fungi and 18 or 42 hours incubation at 37°C for bacteria.

The cytoxcicity against murine tumor cell lines were examined by MTT assay method[12]. The tumor cells were incubated in 9-well plate for 24 hours prior to the addition of formamicin into culture well at varied concentrations. After 2 days incubated at 37°C, MTT reagent was added and further incubated for 4 hours. Growth inhibition activity was determined according to the standard MTT assay method and IC_{50} was calculated.

Results

Taxonomic Features of Strain MK27-91F2

Strain MK27-91F2 produced well-branched vegetative mycelia. This strain formed long aerial hyphae which was straight or flexous. Both vegetative and aerial hyphae formed nocardioform fragmentation. The spore was cylindrical with smooth surface and $0.3 \sim 0.5 \times 1.1 \sim 1.9$ μm in size (Photo. 1). No synnemata, sclerotia or sporangia were observed.

The cultural characteristics of strain MK27-91F2 on various agar media are shown in Table 1. The aerial mycelia were white to brownish white. The vegetative mycelia were pale yellow to pale yellowish brown. The soluble pigments were not produced or faint brownish. Physiological characteristics and carbohydrate utilizations are shown in Table 2. Permissive temperature ranges for growth of the strain were 20°C to 30°C. The optimal temperatures for growth of strain MK27-91F2 was near 30°C.

Whole-cell hydrolysates of strain MK27-91F2 con-

Photo 1. Scanning electron micrograph of *Saccharothrix* sp. MK27-91F2 grown on Sucrose - nitrate agar for 4 days at 27°C.

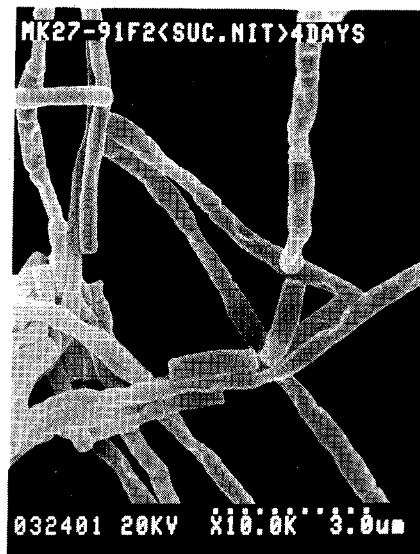


Table 1. Cultural characteristics of strain MK27-91F2.

Medium	Growth	Aerial mycelium	Soluble pigmemt
Sucrose-nitrate agar	Colorless	White	None
Yeast extract-malt extract agar (ISP No. 2)	Pale yellowish brown [3ic, Lt Amber]	White ~ brownish white [3ba, Pearl]	None
Oatmeal agar (ISP No. 3)	Pale yellow [2gc, Bamboo]	White	None
Inorganic salts-starch agar (ISP No. 4)	Pale yellow [2gc, Bamboo ~ 2ic, Honey Gold]	White	None
Glycerol-asparagine agar (ISP No. 5)	Pale yellowish brown [2ne, Mustard Gold]	White	None
Tyrosine agar (ISP No. 7)	Pale yellowish brown [2pg, Mustard Gold]	White	Faint, brownish

Observation after incubation at 27°C for 21 days.
Color names and numbers from Color Harmony Manual, Container Corporation of America.[15]

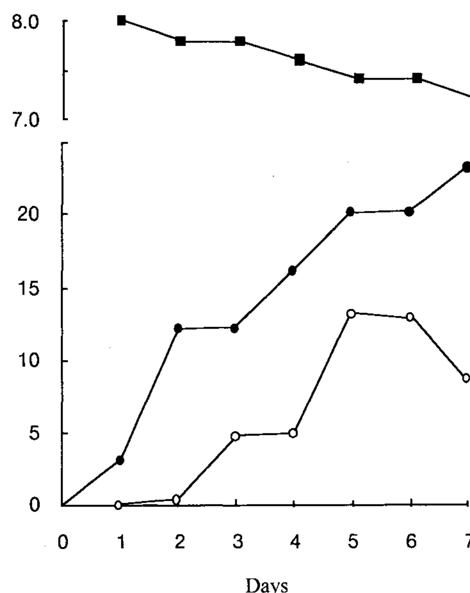
Table 2. Physiological characteristics of strain MK27-91F2.

Temperature range for growth (°C)	20 ~ 30
Optimum temperature (°C)	30
Formation of melanoid pigment	
ISP No. 1	Negative
ISP No. 6	Negative
ISP No. 7	Negative
Hydrolysis of starch	Positive
Reduction of nitrate	Negative
Utilization of	
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	−
Inositol	+
Rhamnose	±
Raffinose	−
D-Mannitol	±

+: Utilization, ±: doubtful, −: no utilization.

Fig. 2. A typical fermentation of formamicin.

■ pH, ● packed cell volume (%), ○ formamicin (μg/ml).



tained *meso*-diaminopimelic acid, galactose, and rhamnose but did not contain arabinose. These results were indicated that the strain belongs to cell wall type III and the whole cell sugar pattern E[13] (arabinose −, galactose +, rhamnose +). The strain has type PII phospholipid (phosphatidyl ethanolamine +, phosphatidyl choline −, unknown glucosamine containing phospholipid −) and MK-9 (H_4) as the major components of menaquinone. *N*-acyl type of muramic acid in cell wall was acetyl type. Mycolic acids were absent. Cellular fatty acids consisted *i*-16:0 as major component and *i*-14:0, *i*-15:0, 16:0, 16:1, 17:1 and *i*-16:1 as minor compoment.

These taxonomic properties suggested that strain MK27-91F2 belonged to the genus *Saccharothrix*[14].

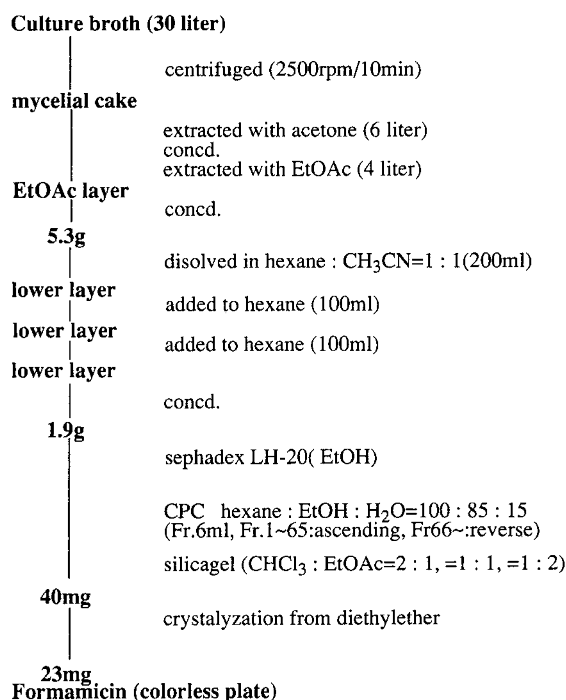
Therefore, the strain was identified as *Saccharothrix* sp. and designated *Saccharothrix* sp. MK27-91F2. Detailed taxonomic study of strain MK27-91F2 is now progress. Strain MK27-91F2 has been deposited in the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, Tsukuba, Japan, under the accession No. FERM P-16053.

Fermentation and Isolation

A typical time course of formamicin production in a 500-ml baffled Erlenmeyer flask is shown in Fig. 2. The production of formamicin began at 2-days and maximum (*ca.* 14 mg/liter) was reached at 5-days after incubation.

The fermentation broth (30 liters) was separated to the mycelial cake and supernatant by centrifugation. The mycelial cake was extracted with acetone (6 liters). The acetone extract was concentrated *in vacuo* to 2 liters. The

Fig. 3. Isolation procedure of formamicin.



active principle was extracted with EtOAc (4 liters). The EtOAc solution was concentrated under reduced pressure to yield a dark brown oil (5.3 g). The oil was distributed between hexane and CH_3CN (1:1, 200 ml). The active principle was almost distributed in CH_3CN layer. The CH_3CN layer was concentrated *in vacuo* to yield a yellowish brown oil (1.9 g). The concentrate was chromatographed on column of Sephadex LH-20 developing with EtOH. The active fractions were collected and concentrated *in vacuo* to give a brownish yellow oil. The crude oil was further purified by CPC (Hexane : EtOH : H_2O = 100 : 85 : 15, ascending method). The active fractions were collected and concentrated *in vacuo* to give a brownish oil. The active eluate was further purified by silica gel column chromatography ($CHCl_3$: EtOAc = 2 : 1, = 1 : 1 and = 1 : 2). The active fractions were collected and concentrated *in vacuo* to give a formamicin (40 mg). The antibiotics was dissolved in a small amount diethyleter, and obtained as colorless plate crystals of formamicin (23 mg, Fig. 3). Structure of formamicin is shown in Fig. 1. The studies on the structure determination of this antibiotic will be reported in the accompanying paper[1].

Biological Activity

The antimicrobial activities of formamicin is shown in Tables 3 and 4. Formamicin exhibited broad and strong antifungal activity against phytopathogenic fungi and moderate antibacterial activities against Gram-positive bacteria. Formamicin exhibited very potent cytotoxicity against murine tumor cell lines *in vitro*. The IC_{50} values against various leukemia cell lines were 0.13 to 0.15 ng/ml. The results are summarized in Table 5.

Table 3. Antibacterial activities of formamicin.

Test organisms	MIC (μg/ml)
Staphylococcus aureus FDA209P	6.25
S. aureus Smith	12.5
S. aureus MS9610	12.5
S. aureus MS16526 (MRSA)	25
S. aureus TY-04282 (MRSA)	12.5
Micrococcus luteus IFO3333	12.5
M. luteus PCI1001	12.5
Bacillus subtilis NRRL B-558	50
B. cereus ATCC10702	6.25
Corynebacterium bovis 1810	25
Escherichia coli NIHJ	>100
Shigella dysenteriae JS11910	>100
Salmonella enteritidis	>100
Proteus mirabilis IFM OM-9	>100
Providencia rettgeri GN466	>100
Serratia marcescens	>100
Pseudomonas aeruginosa A3	>100
Klebsiella pneumoniae PCI602	>100
Mycobacterium smegmatis ATCC607[a]	>100

Mueller Hinton agar, 37°C, 18 hours.
[a] 37°C, 42 hours.

Table 4. Antifungal activity of formamicin.

Test organisms	MIC (μg/ml)
Alternaria kikuchiana	12.5
Cochliobolus miyabeanus C-8	<0.39
Diaporthe citri	<0.39
Fusarium oxysporum IFO9761	>100
Gibberella fujikuroi	1.56
Pyricularia oryzae P-2	<0.39
Rhizoctonia solani	1.56
Penicillium digitatum P-7	1.56
Cercospora beticola	3.13
Botrytis cinera B-22	25
Colletotrichum lagenarium	<0.39
Ustilago maydis	<0.39
Candida tropicalis F-1[a]	>100
C. pseudotropicalis F-2[a]	100
C. albicans 3147[a]	100
C. YU-1200[a]	100
C. krusei F-5[a]	6.25
Saccharomyces cerevisiae F-7[a]	0.2
Cryptococcus neoformans F-10[a]	0.39
Trichophyton asteroides 429[a]	>100
T. mentagrophytes F-15 (833)[a]	>100
Aspergillus niger F-16[a]	100
A. fumigatus F-181[a]	100

Potato Sucrose Agar, 27°C, 4 days.
[a] Nutrient agar +1% Glucose, 27°C, 42 hours.

Table 5. Cytotoxicities of formamicin.

Cell lines	IC_{50} (μg/ml)
L1210 leukemia	0.00015
EL4 leukemia	0.00013
P388 leukemia	0.00013
IMC carcinoma	0.00029
S180 sarcoma	0.00345
B16 melanoma	20.7
FS3 fibrosarcoma	12.5
HeLa	3.62

Discussion

Formamicin having antifungal and antitumor activities was structurely classified as bafilomycin-concanamycin group antibiotics. The antibiotics group have been reported from microbial source such as hyglolidins[16], leucanicidin[17], bafilomycins[18], L-681,110[19], L-155,175[20], PD 118,576[21], PC-766B[22,23], concanamycins[24] and TAN-1323[25]. These bafilomycin-concanamycin group antibiotics have been found from *Streptomyces* sp. except for PC-766B which have been isolated from *Nocardia*. Formamicin is the first isolation of the antibiotics group from *Saccharothrix* sp.

Bafilomycin-concanamycin group antibiotics have been discovered based on a various biological activity such as antifungal activity, insecticidal activity, cytotoxic activity, angiostatic activity and inhibition of cholesteryl-ester synthesis[26]. Bafilomycin-concanamycin group was reported to be specific inhibitor of vacuolar ATPase[27]. Presence of hemiacetal moiety in bafilomycins and concanamycins was important functional group for inhibition of vacuolar ATPase[28]. However, structural biological relationship of 16-membered macrolide (or 18-membered macrolide) moiety was insufficient.

Acknowledgments

We would like to express our gratitude to Hokko Chemical Industry Co., Ltd. for measurement of antiphytopathogenic fungal activity.

References

1) Igarashi, M.; H. Nakamura, H. Naganawa & T. Takeuchi: Formamicin, a novel antifungal antibiotic produced by a strain of *Saccharothrix* sp. II. Structure elucidation of formamicin. J. Antibiotics 50: 932~936, 1997
2) Shirling, E. B. & D. Gottlieb: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) Waksman, S. A.: Classification, identification and descriptions of genera and species. *In* The Actinomycetes, Vol. II, The Williams & Wilkins Co., Baltimore, 1961
4) Staneck, J. L. & G. D. Roberts: Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. 28: 226~231, 1974
5) Lechevalier, M. P. & H. A. Lechevalier: The chemotaxonomy of actinomycetes. *In* Actinomycete taxonomy. *Ed.*, Dietz, A. & D. W. Thayer, pp. 227~291, Society for Industrial Microbiology, Virginia, 1980
6) Mikami, H. & Y. Ishida: Post-column fluorometric detection of reducing sugars in high performance liquid chromatography using arginine. Bunseki Kagaku 32: E207~E210, 1983
7) Uchida, K.: Acyl type of actinomycete cell wall: Its simple identification by a glycolate test. The Actinomycetologist. 40: 28~31, 1982
8) Tamaoka, J.; Y. Katayama-Fujimura & H. Kuraishi: Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. J. Appl. Bacteriol. 54: 31~36, 1983
9) Minnikin, D. E.; P. V. Patel, L. Alshamaony & M. Goodfellow: Polar lipid composition in the classification of *Nocardia* and related bacteria. Int. J. Syst. Bacteriology 27: 104~117, 1977
10) Minnikin, D. E.; I. G. Hutchinson, A. B. Caldicott & M. Goodfellow: Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. J. Chromatography 188: 221~233, 1980
11) Suzuki, K. & K. Komagata: Taxonomic significance of cellular fatty acid composition in some coryneform bacteria. Int. J. Syst. Bacteriology 33: 188~200, 1983
12) Mosmann, T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Immuno. Methods. 65: 55~63, 1983
13) Kroppenstedt, R. M.: The genus *Nocardiopsis*. *In* The prokaryotes. *Ed.*, A. Balows *et al.*, pp. 1139~1156, Springer Verlag, Berlin, 1992
14) Labeda, D. P.; R. T. Testa, M. P. Lechevalier & H. A. Lechevalier : *Saccharothrix*: a new genus of the *Actinomycetales* related to *Nocardiopsis*. Int. J. Syst. Bacteriology 34: 426~431, 1984
15) Jacobson, E.; W. C. Granville & C. E. Foss: Color harmony manual, 4th ed., Container Corporation of America, Chicago, 1958
16) Seto, H.; H. Akao, K. Furihata & N. Ōtake: The structure of a new antibiotic, hygrolidin. Tetrahedron Lett. 23: 2667~2670, 1982
17) Isogai, A.; S. Sakuda, S. Matumoto, M. Ogura, K. Furihata, H. Seto & A. Suzuki: The structure of leucanicidin, a novel insecticidal macrolide produced by *Streptomyces halstedii*. Agric. Biol. Chem. 48: 1379~1381, 1984
18) Werner, G.; H. Hagenmaier, H. Drautz, A. Baumgartner & H. Zähner: Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity. J. Antibiotics 37: 110~117, 1984
19) Huang, L.; G. Albers-Schonberg, R. L. Monaghan, K. Jakubas, S. S. Pong, O. D. Hensens, R. W. Burg, D. A. Ostlind, J. Conroy & E. O. Stapley: Discovery, production and purification of the Na^+, K^+ activated ATPase inhibitor, L-681,110 from the fermentation broth

of *Streptomyces* sp. MA-5038. J. Antibiotics 37: 970~975, 1984

20) GOETZ, M. A.; P. A. MCCORMICK, R. L. MONAGHAN, D. A. OSTLIND, O. D. HENSENS, J. M. LIESCH & G. ALBERS-SCHONBERG: L-155,175 : A new antiparasitic macrolide. Fermentation, isolation and structure. J. Antibiotics 38: 161~168, 1985

21) WILTON, J. H.; G. C. HOKANSON & J. C. FRENCH: PD 118,576: A new antitumor macrolide antibiotic. J. Antibiotics 38: 1449~1452, 1985

22) MIKAMI, Y.; S. F. YU, K. YAZAWA, K. FUKUSHIMA, A. MAEDA, J. UNO, K. TERAO, N. SAITO, A. KUBO & K. SUZUKI: A toxic substance produced by *Nocardia otitidiscaviarum* isolated from cutaneous nocardiosis. Mycopathologia 112: 113~118, 1990

23) KUMAGAI. K.; K. TAYA, A. FUKUI, M. FUKASAWA, M. FUKUI & S. NABESHIMA: PC-766B, a new macrolide antibiotic produced by *Nocardia brasiliensis*. J. Antibiotics 46: 972~978, 1993

24) KINASHI, H.; K. SOMENO & K. SAKAGUCHI: Isolation and characterization of concanamycins A, B and C. J. Antibiotics 37: 1333~1343, 1984

25) ISHII, T.; T. HIDA, S. IINUMA, M. MUROI & Y. NOZAKI: TAN-1323 C and D, new concanamycin-group antibiotics; Detection of the angiostatic activity with a wide range of macrolide antibiotics. J. Antibiotics 48: 12~20, 1995

26) WOO, J.; C. SHINOHARA, K. SAKAI, K. HASUMI & A. ENDO: Isolation, characterization and biological activities of concanamycins inhibitors of lysosomal acidification. J. Antibiotics 45: 1108~1116, 1992

27) BOWMAN, E. J.; A. SIEBERS & K. ALTENDORF: Bafilomycins: A class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Pro. Natl. Acad. Sci. USA 85: 7972~7976, 1988

28) DROSE, S.; K. U. BINDSEIL, E. J. BOWMAN, A. SIEBERS, A. ZEECK & K. ALTENDORF; Inhibitory effect of modified bafilomycins and concanamycins on P- and V-type adenosinetriphosphatases. Biochemistry 32: 3902~3906, 1993