A NEW STREPTOTHRICIN ANTIBIOTIC, R4H

YOSUKE SAWADA and HYOZO TANIYAMA

Faculty of Pharmaceutical Sciences, Nagasaki University, Nagasaki, Japan

NOBUO HANYUDA, HISASHI HAYASHI and TADASHI ISHIDA

Research Division, Toa Nutritional Chemical Ind. Co., Ltd., Fukushima, Japan

(Received for publication January 21, 1974)

An antibiotic R4H exhibiting no delayed toxicity was isolated from the fermentation broth of *Streptomyces lavendulae* strain R4. It was active against Gram-positive and -negative bacteria and *Mycobacterium*. R4H was converted to racemomycin-A and racemomycinic-A acid by mild hydrolysis.

During the course of screening for streptothricin antibiotics, a variant of *Streptomyces lavendulae* was found to produce new antibiotics along with the known streptothricins. The taxonomic and fermentation characteristics of the tentatively named strain R4, its isolation procedures, and the physicochemical and biological properties of the antibiotic R4H are described in this communication.

Characteristics of Strain R4

The strain is of a *Streptomyces* isolated from a soil sample obtained in Fukushima city, in northeastern part of Japan.

Most of the general procedures reported by SHIRLING *et al.*[1] and WAKSMAN[2,3] were used in determination of morphological and physiological properties. PRIDHAM-GOTTLIEB basal medium was used for the carbon utilization test. Culture was grown at 28°C for 14 days unless otherwise mentioned. Color comparisons were made using the standard color table (JIS Z-8721), and the results are given as color name, number of hue, luminosity, and saturation, in that order.

1. Morphological Characteristics

The vegetative mycelium of the strain R4 is well-branched but does not fragment into cocoid or bacillary forms in liquid media. Spores are oval or cylindrical ($0.6\times1.0\sim1.4\,\mu$) with smooth surface, phalangiform as shown in Plate 2. Sporophores: Main axis of aerial mycelia is straight or somewhat wavy. The terminal filaments develop into sporophores having open spirals (but no whorls) or wide hooks, and their average diameter is $10\,\mu$ as shown in Plate 1.

2. Cultural and Physiological Characteristics

Tables 1, 2 and 3 summarize the results obtained on various cultural and physiological tests. Some characteristics are: (1) Surface of the colony is cottony, representative color of aerial mycelium is pinkish beige, red color series. (2) Aerial mycelium comes under *Retinaculum-Apertum* (RA) type, forms no whorls. (3) Spore chains are phalangiform on the axis, its surface

Plate 1. Aerial mycelium of the strain R4 (ISP medium-2, 14 days)

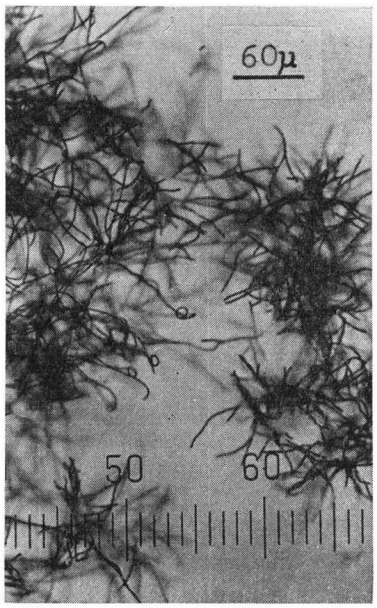
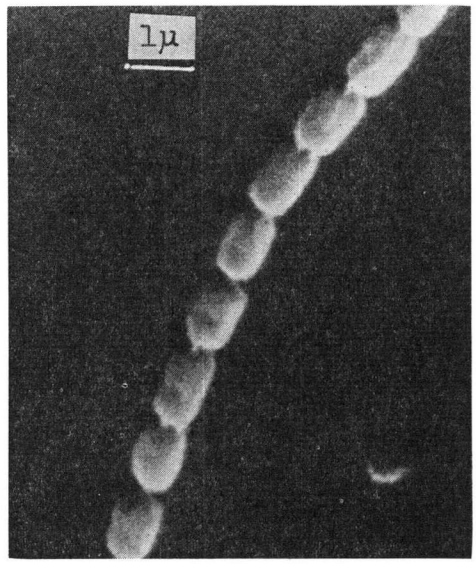


Plate 2. Electron micrograph of conidia of R4



is smooth. (4) Melanoid formation is positive and a brown soluble pigment is produced on various organic media. (5) Starch is hydrolyzed, nitrate reduced to nitrite, and proteolytic activities are exhibited on gelatin and milk.

Among the known species of *Streptomyces*, strain R4 resembles *Streptomyces lavendulae* most closely but differs from it by a good utilization of sucrose and growth pattern on sucrose-nitrate agar (Table 1), and hence was described as a variant of *Streptomyces lavendulae* WAKSMAN *et* HENRICH, 1948.

Production and Isolation

Spores of *Streptomyces lavendulae* strain R4 grown on malt extract-yeast extract agar slant at 28°C for 7 days were inoculated to a liquid medium containing glucose 1%, peptone 1%, yeast extract 0.5% and NaCl 0.3% (pH 7.0 before sterilization) and shaken at 28°C for 24 hours. This inoculum (1 liter) was transferred to a medium (20 liters, Lab fermentor) containing glucose 0.1%, peptone 1%, yeast extract 0.5%, NaCl 0.3%, KH_2PO_4 0.1%, $MgSO_4$ 0.05% (pH 6.0), and fermentation carried on at 28°C for 20 hours with aeration at 20 liters per minute, agitation at 200 rpm. Antimicrobial activity reached maximum after 18 hours. Activity, pH change, and dry weight amount of mycelium are shown in Fig. 1.

Cultured filtrate was passed through a column of Amberlite IRC-50 (Na^+ form) after adjustment of pH to 7.0, the column washed with water and then eluted with 1 N acetic acid. The concentrated solution of active fractions was filtered to remove the inactive precipitate, acetone added to the filtrate to precipitate a brownish crude powder which on reprecipitation thrice with methanol-acetone gave 8.7 g of a powder containing the antibiotic acetate.

Crude complex (2 g) was purified on a column (25 mm diameter; bed volume, 400 ml) of SE-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala) by stepwise elution with buffers (0.1,

0.5, 1.0 and 3.0 M pyridine-acetic acid, pH 5.0) as shown in Fig. 2, and peaks of active fractions were compared by paper chromatography with a known sample of racemomycin[4], one the streptothricin group of antibiotics. Peaks H, I, J and K in this graph are new antibiotics. Fractions containing R4H were pooled and evaporated *in vacuo* to a small volume and precipitated with acetone to yield 128 mg of a white powder. Further purification of R4H

Table 1. Cultural characteristics of strain R4

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast ext. -malt ext. agar (ISP-medium-2)	Good, not spreading, cottony. Brown (2.5 Y 7.5/3)	Abundant, pinkish beige (5 YR 8/1.5)	None
Oatmeal agar (ISP-medium-3)	Good, scant spreading, flat, cottony. Pale pinkish beige (5YR 8.5/1)	Abundant, pinkish beige (10 R 8/2.5)	None
Inorganic salt starch agar (ISP-medium-4)	Good, not spreading, cottony. Grayish brown (2.5 Y6.5/2)	Abundant, pinkish beige (2.5 YR 8/2)	None
Glycerol asparagine agar (ISP-medium-5)	Good, not spreading, cottony. Grayish yellow (2.5 Y 8/2)	Moderate, grayish pink (10 R 7/2)	None
Peptone yeast-ext. iron agar (ISP-medium-6)	Good, not spreading. Brown (10 YR 7/4)	None	Dark brown (10 YR 4/3)
Tyrosine agar (ISP-medium-7)	Good, not spreading, cottony. Dark brown (10 YR 6/3)	Abundant, pinkish beige (10 YR 8/2.5)	Dark brown (10 YR 4/2)
Sucrose nitrate agar	Good, not spreading, cottony. Grayish dark brown (5 YR 5.5/2.5)	Abundant, pinkish beige (5 YR 8/1)	Brown (7.5 YR 6/4)
(*Str. lavendulae*)	Very scant, colorless	Very scant, white	None
Glucose asparagine agar	Moderate, not spreading. Brownish white (2.5 Y 9/2)	Moderate, white	None
Glycerol Ca-malate agar	Moderate, not spreading. Brownish white (5 Y 8/1)	Moderate, white	None
Nutrient agar	Good, not spreading. Pale brown (2.5 Y 7/4)	None	Brown (10 YR 6/4)
Starch agar	Good, not spreading, cottony. Grayish brown (5 Y 6.5/2)	Abundant, pinkish beige (10 R 8/2)	None
Potato glucose agar	Good, scant spreading, cottony. Pale brown (10 YR 7.5/2)	Abundant, grayish pink (10 R 7/2)	None
Potato plug	Good, not spreading. Brown (10 YR 5/2)	Scant, light gray (N 8.5)	Brown (7.5 YR5/4)
Glucose peptone gelatine	Good, surface. Brown (2.5 Y 7/4)	Scant, white to gray (N 7.0)	Brown (10 YR 6/4)
Litmus milk	Moderate, surface, ring. Yellowish gray (5 Y 7/1)	None	(pH 8.3)

from the accompanying R4A was achieved by a partition cellulose chromatography using the solvent system: *n*-butanol-pyridine-acetic acid-water (15: 10: 3: 12) followed by a Sephadex G-10 column chromatography using water as the eluant. A yield of about 4% R4H was obtained starting from the crude powder. R4H hydrochloride was prepared by acetone precipitation from 0.3 N hydrochloric acid solution.

Table 2. Physiological characteristics of strain R4

Growth temperature range	15~37°C
Optimum temperature	28°C
Growth pH range	5~12
Optimum pH	7.5
Tyrosinase reaction	positive
Melanoid pigment	positive
Reduction of nitrate	positive
Hydrolysis of starch	positive
Liquefaction of gelatin*	positive
Peptonization of milk**	positive
Coagulation of milk**	negative
Cellulose decomposition	negative

* 20°C, 40 days, ** 37°C, 14 days

Fig. 1. Fermentation by the strain R4
Activity: Disc method, *Bacillus subtilis* PCI-219 as indicator strain

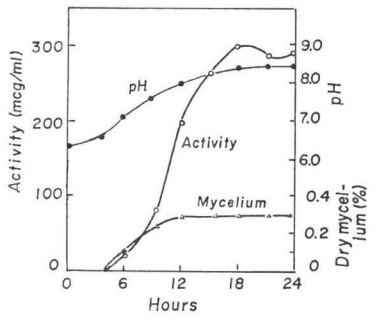


Table 3. Carbohydrates utilization by the strain R4

Carbohydrates	Response
D-Glucose	++
L-Arabinose	±
Sucrose	++
D-Xylose	±
Inositol	−
Mannitol	−
D-Fructose	±
Rhamnose	±
Raffinose	−
Cellobiose	++
Maltose	++
D-Mannose	+
Salicin	+
Galactose	±
Inulin	−
Dulcitol	−
Sorbitol	−
Trehalose	−
Lactose	−
Cellulose	−
Sodium acetate	−

++: Abundant growth, +: Growth,
±: Doubtful growth, −: None

Fig. 2. Isolation of R4 components by SE-Sephadex C-25
(Disc method, *Bacillus subtilis* PCI-219)

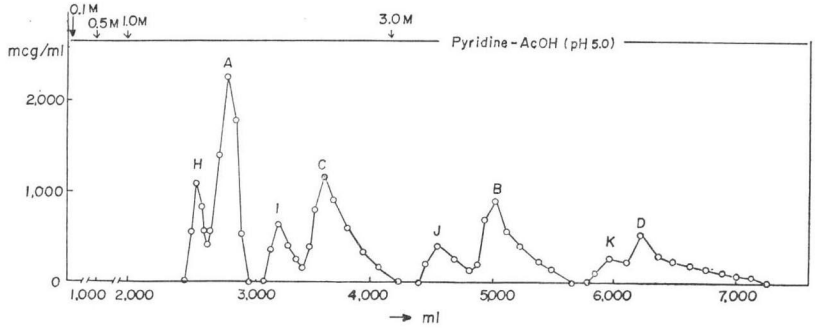


Fig. 3. The infrared spectrum of R4H hydrochloride (KBr)

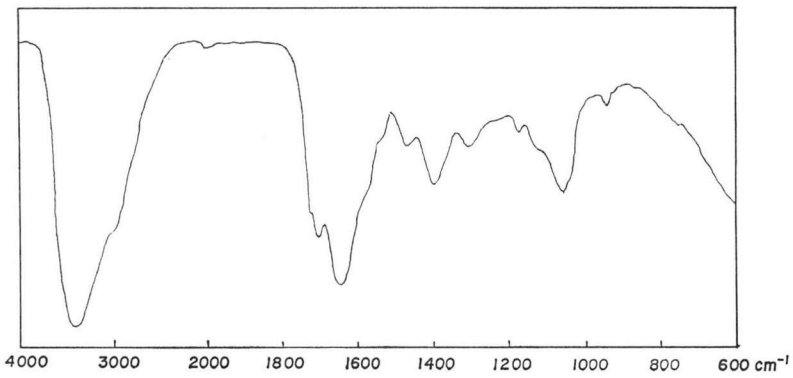


Fig. 4. The NMR spectrum of R4H hydrochloride (100 MHz, in D_2O)

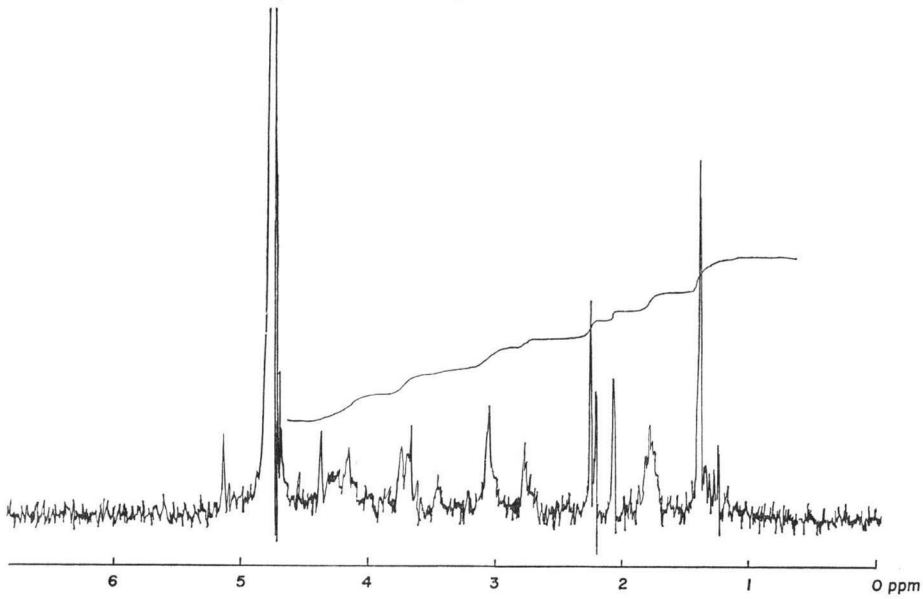


Table 4. Chromatographic comparison of R4H with racemomycins

Item			Rf values							
			H	I	J	K	A	B	C	B
PPC	I	A	0.23				0.16	0.06	0.09	0.04
		B	0.53	0.44	0.35	0.28	0.38	0.23	0.31	0.19
	II	A	0.44				0.32	0.18	0.24	0.11
		B	0.73				0.58	0.44	0.51	0.36
TLC		B	0.50				0.31	0.22	0.26	0.13

Solvent system A: *n*-butanol - pyridine - acetic acid - water - *t*-butanol (15 : 10 : 3 : 12 : 4)
B: *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12)
PPC: I: Toyo-Roshi No. 51, ascending method
II: Toyo-Roshi No. 51 UH
TLC: Avicel SF (Funakoshi Co.)

Table 5. Antimicrobial activity of R4H and R4A hydrochlorides

Test organisms	MIC (mcg/ml)	
	R4H	R4A
Staphylococcus aureus FDA 209 P	12.5	3.1
Staphylococcus aureus IFO 3060	12.5	3.1
Staphylococcus aureus IFO 12732	6.2	1.6
Bacillus subtilis PCI 219	3.1	1.6
Bacillus subtilis ATCC 6633	6.2	1.6
Bacillus cereus IFO 3001	100	25
Micrococcus flavus	12.5	3.1
Micrococcus luteus IAM 1097	3.1	0.8
Sarcina lutea IAM 1009	25	6.2
Escherichia coli K-12	3.1	1.6
Escherichia coli O-26	6.2	1.6
Escherichia coli IFO 12734	6.2	1.6
Enterobacter aerogenes IAM 1102	12.5	3.1
Pseudomonas aeruginosa IFO 3080	>100	50
Pseudomonas aeruginosa IFO 3901	>100	>100
Pseudomonas aeruginosa IAM 1007	>100	>100
Pseudomonas fluorescens IFO 3903	12.5	6.2
Proteus vulgaris IFO 3851	12.5	3.1
Proteus morganii IFO 3848	6.2	3.1
Xanthomonas oryzae IAM 1097	>100	100
Mycobacterium phlei IFO 3158	25	6.2
Saccharomyces carlsbergensis ATCC 9080	>100	>100
Saccharomyces cerevisiae IAM 4178	>100	>100
Saccharomyces formosensis IAM 4072	>100	100
Schizosaccharomyces pombe IAM 4879	>100	50
Hansenula anomala IAM 4213	>100	>100
Candida albicans IAM 4888	>100	>100
Candida utilis IAM 4215	>100	100
Aspergillus niger IAM 3010	>100	>100
Aspergillus versicolor	>100	25
Penicillium chrysogenum IAM 7326	>100	50
Trichophyton asteroides	>100	>100
Trichophyton interdigitale	>100	>100
Trichophyton mentagrophytes IAM 5064	>100	>100

Agar dilution streak method
Bacteria: Heart infusion agar, 30°C, 24 hours
Fungi, Yeast: malt extract-yeast extract agar, 28°C, 48 hours
Mycobacterium: glycerin nutrient agar, 28°C, 7 days

Physicochemical Properties of R4H

Antibiotics R4-A, -B, -C and -D were identical with racemomycin-A, -B, -C and -D by elution patterns, paper chromatographic behavior, color reaction, and other criteria. R4H hydrochloride among these new antibiotics is described below. It is a white hygroscopic

powder, decomposing at about 200°C, soluble in water, slightly soluble in methanol, dimethylformamide and dimethylsulfoxide, insoluble in ethanol, acetone, ether, ethylacetate and benzene. The UV spectrum shows only end absorption in water and the IR spectrum in KBr disc is shown in Fig. 3. The NMR spectrum in heavy water is shown in Fig. 4. $[\alpha]_D^{20}$ −55° ±2° (*c* 1, H_2O). Elemental analysis: C, 34.23; H, 5.95; N, 12.38; Cl, 14.62%, correspond to a formula $C_{28\sim29}H_{55\sim56}O_{9\sim10}N_{8\sim9}\cdot 4HCl$.

R4H is positive to ninhydrin, PAULY, ELSON-MORGAN and RYDON-SMITH reactions, but negative to SAKAGUCHI, EHRLICH, and maltol reactions. Significant loss in antimicrobial activity was observed after 24 hours at room temperature in 0.1 N sodium hydroxide, but a 50% decrease was observed after 1 week in 0.1 N hydrochloric acid.

The Rf values on paper and thin-layer chromatography are shown in Table 4. Mobility in paper electrophoresis (carrier buffer, pyridine-acetic acid-water (30 : 4 : 966, pH 6.14), 500 V, 7 mA for 135 minutes) was 12.0 cm compared to 13.5 cm for racemomycin-A.

The complete hydrolysis of R4H with 6 N hydrochloric acid gave in equimolar ratio streptolidine, β-lysine and D-gulosamine determined by paper strip and automatic amino acid analyses; mild hydrolysis by 3 N hydrochloric acid at 100°C for 1 hour yielded only β-lysine in a small amount. Considering that liberation of β-lysine from racemomycin-A is easy, that R4H in 0.1~0.3 N hydrochloric acid solutions at room temperature for 3 days yielded a large amount of racemomycin-A, purified racemomycin-A isolated from the above hydrolysate followed to stand under the same condition for 1 week gave racemomycinic-A acid[5] and that we also obtained racemomycinic-A acid in 0.1~0.3 N hydrochloric acid solution of R4H, it was almost certain that R4H was a natural derivative of racemomycin-A.

The antimicrobial activity of R4H and R4A was tested by the agar dilution streak method and the results are shown in Table 5. R4H exhibited antimicrobial activity against Gram-positive and -negative bacteria and *Mycobacterium* and the activity was lower than that of R4A.

Toxicity patterns in mice by intravenous injection are shown in Table 6. Respiratory depression at injection time led to the death of the mice and no delayed toxicity or decrease in body weight was observed at a concentration of 130~286 mg/kg of R4H.

Table 6. Toxicity pattern of R4H and R4A hydrochlorides in mice (i. v.)
Mice: ddF strain, male

Sample	Dose (mg/kg)	Dead mice/Treated mice							
		0 Day	1 Day	2 Day	3 Day	4 Day	5 Day	6 Day	7 Day
R4H	130	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	169	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
	220	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
	286	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
	372	5/5	—	—	—	—	—	—	—
R4A	130	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5
	169	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5
	220	0/5	0/5	0/5	0/5	1/5	1/5	1/5	4/5
	286	5/5	—	—	—	—	—	—	—

Discussion

The antibiotic R4H produced by *Streptomyces lavendulae* strain R4 belongs to the streptothricin group of antibiotics, but differs from racemomycin-A and other streptothricins such as akimycin[6], yazumycins[7], boseimycins[8], neothricin[9], LL-AC 541[10], BY-81[11], E-749 C[12], citromycin[13], LL-AB664[14], BD-12[11], LL-BL136[15], SF-701[16] and sclerothricin[17]. Three antibiotics, SOB-7[18], S-15-1[19] and 24010-B-1[20], recently reported were found different from R4H since they showed lower Rf values.

We have reported[21] that acetylation of ε-N-position of β-lysine moiety in racemomycin-A reduced the original antimicrobial activity and toxicity, but R4H is different from acetyl racemomycin-A on physicochemical considerations. The structural comparisons of R4H to streptothricin antibiotics racemomycin-O[22], and fucothricin[23] which like R4H also show no delayed toxicity has yet to be done by us.

Also, R4I seems to be a derivative of racemomycin-C containing streptolidine, β-lysine and D-gulosamine (molar ratio, 1: 2: 1). Physicochemical and biological properties of the new compounds R4I[c] (Rf 0.44), R4J (Rf 0.35) and R4K (Rf 0.28) will be reported later.

Acknowledgement

The authors express their sincere gratitude to Dr. A. SEINO, Kaken Chemical Co., Ltd., for his advices on taxonomy. Thanks are also due to Dr. T. TANAKA, Toa Nutritional Chemical Ind. Co., Ltd., for his encouragements through this work.

References

1) SHIRLING, E. B. & D. GOTTLIEB: Method for characterization of *Streptomyces* species. Inter. J. Syst. Bact. 16: 313~340, 1966
2) WAKSMAN, S. A.: Cultural studies of species of *Actinomyces*. Soil Sci. 8: 71~208, 1919
3) WAKSMAN, S. A.: The Actinomycetes. Vol. 2, The Williams and Wilkins Company, 1961
4) TANIYAMA, H.; Y. SAWADA & T. KITAGAWA: Characterization of racemomycins. Chem. Pharm. Bull. (Tokyo) 19: 1627~1634, 1971
5) TANIYAMA, H.; Y. SAWADA & T. KITAGAWA: Studies on the inactivation and regeneration of streptothricin. J. Antibiotics 24: 662~666, 1971
6) ARAI, T.; Y. KOYAMA, H. HONDA & M. HAYASHI: Simultaneous production of two antibiotics by *S. lavendulae* E 20-27. Ann. Rep. Inst. Food Microbiol. 13: 39~44, 1960
7) TANIYAMA, H.; Y. SAWADA & T. KITAGAWA: The identity of yazumycins A and C with racemomycins A and C. J. Antibiotics 24: 390~392, 1971
8) TANIYAMA, H.; Y. SAWADA, T. OKUNO & K. HASHIMOTO: On separation and purification of water-soluble basic antibiotics using microcrystalline cellulose "Avicel". Jap. J. Antibiotics 25: 84~90, 1972
9) Kaken Kagaku Co., Ltd.; Jap. Pat. Publ. No. 46-28832, Aug. 21, 1971
10) 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
11) 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
12) 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
13) TANIYAMA, H. & Y. SAWADA: The identity of citromycin with LL-AC 541, E-749 C and BY-81. J. Antibiotics 24: 708~710, 1971
14) SAX, K. J.; P. MONNIKENDAM, D. B. BORDERS, P. SHU, L. A. MITSCHER, W. K. HAUSMANN & E. L. PATTERSON: LL-AB 664, a new streptothricin-like antibiotic. Antimicr. Agents & Chemoth. -1967: 442~448, 1968

15) BORDERS, D. B.; J. P. KIRBY, E. R. WETZEL, M. C. DAVIES & W. K. HAUSMANN: Analytical method for streptothricin-type antibiotics: Structure of antibiotic LL-BL 136. Antimicr. Agents & Chemoth. 1: 403~407, 1972
16) TSURUOKA, T.; T. SHOUMURA, N. EZAKI, T. NIWA & T. NIIDA: SF-701, a new streptothricin-like antibiotic. J. Antibiotics 21: 237~238, 1968
17) KONO, Y.; S. MAKINO, S. TAKEUCHI & H. YONEHARA: Sclerothricin, a new basic antibiotic. J. Antibiotics 22: 583~589, 1969
18) ANDO, K.; H. OKAZAKI, M. HANADA, T. NOTO & Y. HARADA: *Streptomyces* antibiotic SOB-7. Jap. Pat. Kokai 20395, 1972
19) ARIMA, K.; T. KAWAMURA & T. BEPPU: A new antiviral substance S-15-1, streptothricin group antibiotic. J. Antibiotics 25: 387~392, 1972
20) SHIMOJIMA, Y.; M. MIZUNO, Y. MIZUNO, T. OOKA & I. TAKEDA: An antibiotic 24010 B-1. J. Antibiotics 25: 604~606, 1972
21) SAWADA, Y.; H. SAKAMOTO & H. TANIYAMA: Studies on chemical modification of streptothricin group antibiotics. III. Partial N-acetylation of racemomycins and their biological activity. J. Pharmaceut. Soc. (Japanese) 94: 176~180, 1974
22) TANIYAMA, H. & S. TAKEMURA: Chemical studies on antibiotics produced by Actinomycetes. VII. Racemomycin. 4. On racemomycin-O^c (1). Chem. Pharm. Bull. (Tokyo) 8: 150~153, 1960
23) THIRUMALACHAR, M. J.; P. V. DESHMUKH, R. S. SUKAPURE & P. W. RAHALKAR: Fucothricin, a new streptothricin-like antibiotic. Hindustan Antibiot. Bull. 14: 4~10, 1971