834-B1, A NEW THIOLACTONE CONTAINING ANTIBIOTIC

TAXONOMY, FERMENTATION, ISOLATION AND STRUCTURE

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A new thiolactone containing antibiotic 834-B1 was isolated from the culture broth of *Streptomyces* sp. Y-0834H which has also produced thiolactomycin and thiotetromycin at the same time. The structure of 834-B1 was determined as **I** by the decoupling experiment in NMR.

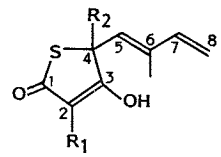
Since the isolation of thiolactomycin[1~4], several compounds which have thiolactone in its molecule have been reported, such as thiotetromycin[5,6], citreothiolactone[7], Tü 2476[8] and U-68,204[9]. In the course of our study searching for new antibiotics, we have isolated a new thiolactone containing antibiotic, 834-B1 from the culture broth of *Streptomyces* sp. Y-0834H which was isolated from a soil sample collected at the coast of Nagai in Kanagawa Prefecture. This strain has coproduced thiolactomycin and thiotetromycin as its main products. The structure of 834-B1 was determined as **I** by the decoupling experiment in NMR, compared with those of thiolactomycin and thiotetromycin. In this report we will describe the taxonomy of the producing strain, fermentation, isolation procedure, physico-chemical properties and the structure of 834-B1.

Taxonomic Studies on the Strain

The strain was isolated from a soil sample collected in Kanagawa Prefecture, Japan. Taxonomic studies were carried out according to the methods used in the International Streptomyces Project[10]. Various kinds of media were inoculated with washed mycelia, and suspended in 0.85% saline. The mycelia were obtained from a culture shaken at 28°C for 72 hours in a liquid medium consisting of glucose 1.0%, peptone 2.4%, pH 7.0 prior to sterilization. All cultures were incubated at 28°C and were observed for 14~21 days. The color was described according to the "Guide to Color Standard[11]". Physiological properties including utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB[12]. Cell wall composition was analyzed by the method of BECKER *et al.*[13].

On the basis of morphological observation with light and electron microscopes on various cultures grown at 28°C for 14~21 days, mature spore chains contained more than 10 spores per chain, terminating in loose spirals. The spores were cylindrical and 0.6~0.8×0.8~1.2 μm in size and had smooth surfaces. Sclerotic granules, sporangia and flagellated spores were

Fig. 1. Structure of 834-B1.



834-B1(I)	$R_1=CH_3$	$R_2=CH_2CH_3$
Thiolactomycin	$R_1=R_2=CH_3$	
Thiotetromycin	$R_1=R_2=CH_2CH_3$	

Table 1. Cultural characteristics of the strain Y-0834H on various media.

Yeast extract - malt extract agar (ISP-2)	G: Moderate, light brownish gray
	R: Grayish white - light brownish gray
	A: None
	P: None
Oatmeal agar (ISP-3)	G: Good, yellowish brown
	R: Pale yellowish brown
	A: Moderate, yellowish gray
	P: None
Inorganic salts - starch agar (ISP-4)	G: Good, pale yellowish brown
	R: Pale yellowish brown
	A: Moderate, light brownish gray
	P: None
Glycerol - asparagine agar (ISP-5)	G: Poor, pale yellow
	R: Pale yellow
	A: None
	P: None
Peptone - yeast extract - iron agar (ISP-6)	G: Good, brownish gray
	R: Brownish gray
	A: Moderate, brownish gray
	P: None
Tyrosine agar (ISP-7)	G: Good, brownish gray
	R: Brownish gray
	A: None
	P: None
Sucrose nitrate agar	G: Poor, pale brown
	R: Colorless - pale brown
	A: Light brown
	P: None
Nutrient agar	G: Moderate, yellowish gray
	R: Yellowish gray
	A: None
	P: Light brown
BENNETT's agar	G: Moderate, pale yellowish brown
	R: Pale yellowish brown
	A: Moderate, light brown
	P: None

G: Growth of vegetative mycelium, R: reverse side of color, A: aerial mycelium, P: soluble pigment.

not observed.

The vegetative mycelia grew abundantly on oatmeal agar, inorganic salts - starch agar, and BENNETT's agar. Septa were not observed. Aerial mycelia grew abundantly on oatmeal agar, inorganic salts - starch agar, tyrosine agar and BENNETT's agar.

Aerial mass color is in the gray series when the culture is grown on yeast extract - malt extract agar, glycerol - asparagine agar, inorganic salts - starch agar, and BENNETT's agar. Results are shown in Table 1. The cell wall analysis of the strain showed that it contained LL-diaminopimelic acid and glycine (cell wall of the strain; Type I).

Physiological properties of the strain are shown in Table 2. Temperature range for growth was from 15 to 40°C with optimum from 27 to 33°C. Milk peptonization was positive, but nitrate reduction and gelatin liquefaction were negative. The carbon utilization pattern of the strain is summarized in Table 3.

Microscopic studies and cell wall analysis of this strain indicate that the strain is classified in the

Table 2. Physiological properties of the strain Y-0834H.

Melanin formation	
ISP-1	−
ISP-6	−
ISP-7	+
H_2S production	−
Liquefaction of gelatin (21°C)	−
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	−
Cellulolytic activity	−
Hydrolysis of starch	+
Temperature range for growth	15~40°C
pH range for growth	5~9

+: Active, −: not active.

Table 3. Utilization of carbohydrates by the strain Y-0834H.

L-Arabinose	−
D-Xylose	+
D-Glucose	+
D-Fructose	+
D-Mannitol	−
Sucrose	−
Inositol	+
Raffinose	−
L-Rhamnose	+
Starch	+

+: Utilized, −: not utilized.

genus *Streptomyces* Waksman and Henrici 1943, 339. Accordingly, the strain was compared with the published descriptions[14~18] of various *Streptomyces* species and the results showed that the strain was considered to resemble *Streptomyces lusitanus* and *Streptomyces avellaneus*.

On the basis of the comparison of the strain with the two strains mentioned above, the strain was different from *S. lusitanus* with the following properties: (1) Mature spores of the strain occured in chains of more than 10 but not 50 spores, while *S. lusitanus* formed long spore chains with more than 50 spores on several agar media. (2) The spore surface of the strain was smooth, while that of *S. lusitanus* was smooth to rough. On the other hand, the strain was similar to *S. avellaneus* in many taxonomic properties, except for starch hydrolysis, utilization of sucrose and inositol and antibiotic productivity. Therefore, strain Y-0834H is considered to be a new species of the genus *Streptomyces* closely similar to *S. avellaneus* and named *Streptomyces* sp. Y-0834H. This strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. Y-0834H with the accession No. FERM P-6900.

Fermentation

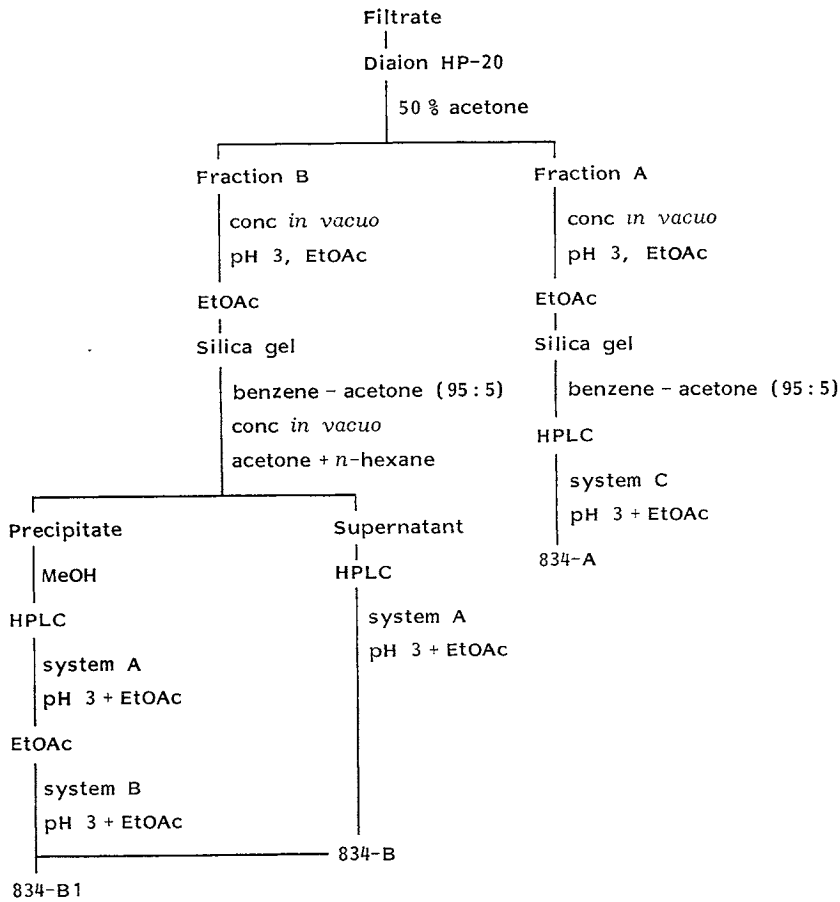
Streptomyces sp. Y-0834H was inoculated from an agar slant to a seed flask containing 60 ml of a medium consisting of glucose 1.0%, potato starch 1.0%, soybean meal 0.75%, corn gluten meal 0.75%, yeast extract 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05%, NaCl 0.2%, pH 7.3 before sterilization, in a 500-ml Erlenmeyer flask. The seed flask was incubated at 28°C for 48 hours on a rotary shaker. The 3% inoculum was then transferred into a 500-ml second seed flasks containing 60 ml of the same medium. The flasks were incubated under the same condition for 72 hours. The 1.5% inoculum from the seed flasks was inoculated to a 30-liter jar fermenter containing 20 liters of a medium consisting of glucose 3.0%, peptone 0.5%, meat extract 0.5%, NaCl 0.3%, yeast extract 0.2%, $CaCO_3$ 0.2%, pH 7.0 before sterilization. The jar fermenter was incubated at 28°C for 48 hours (aeration: 20 liters/minute, agitation 125 rpm). The 3.5% inoculum from the jar fermenter was used for inoculating two 150-liter tanks, each containing 100 liters of a medium consisting of glucose 0.5%, dextrin 4.0%, meat extract 1.0%, Polypeptone 1.0%, yeast extract 0.4%, NaCl 0.3%, $CaCO_3$ 0.2%, pH 7.0 before sterilization. Fermentation was carried out for 34 hours at 28°C (aeration; 100 liters/minute, agitation 100 rpm).

Isolation of 834-B1

The culture broth was adjusted to pH 4.0 and filtered with the aid of Celite. The filtrate was passed through a Diaion HP-20 column (11 liters). After being washed with water, active fractions A and B which were active against *Pseudomonas aeruginosa* NCTC 10490, were eluted with 50% acetone - water.

The former contained 834-B and -B1, the latter contained 834-A. The fractions B were combined and concentrated *in vacuo*, then extracted twice with the same volume of ethyl acetate after adjustment to pH 3. The extract was dried with anhydrous sodium sulfate and evaporated *in vacuo*. The crude extract was dissolved in a small amount of benzene - acetone (95 : 5) and chromatographed on silica gel in the same solvent. Active fractions were combined and evaporated, then they were dissolved in a small amount of acetone and *n*-hexane was added. The supernatant which was rich in 834-B was removed. The precipitate was dissolved in methanol and purified further with HPLC (LiChrosorb RP-18, 8 i.d. × 500 mm, solvent was acetonitrile - phosphate buffer pH 7.0, 0.02 M (12.5 : 87.5)). Active fractions were collected and extracted with ethyl acetate at pH 3. After evaporation,

Fig. 2. Isolation procedure of 834-B1.



System A: LiChrosorb RP-18, acetonitrile - phosphate buffer pH 7.0, 0.02 M, (12.5 : 87.5).
System B: LiChrosorb RP-2, acetonitrile - phosphate buffer pH 7.0, 0.02 M, (12.5 : 87.5).
System C: LiChrosorb RP-8, acetonitrile - water - phosphoric acid, (550 : 450 : 1).

834-B1 was finally purified with HPLC (LiChrosorb RP-2, 8 i.d. × 500 mm, solvent was the same as mentioned above). The 6.8-mg of pure 834-B1 was obtained. On the other hand, 85 mg of 834-B was obtained as crystal from the supernatant. The fractions A which contained 834-A were combined and concentrated *in vacuo*, then extracted with ethyl acetate after adjustment to pH 3. The extract was dried with anhydrous sodium sulfate and evaporated *in vacuo*. The crude extract was dissolved in a small amount of benzene - acetone (95 : 5) and chromatographed on silica gel in the same solvent. Active fractions were combined and evaporated *in vacuo*, then purified with HPLC (LiChrosorb RP-8, 8 i.d. × 500 mm, and the solvent was acetonitrile - water - phosphoric acid (550 : 450 : 1)). The 60-mg of pure 834-A was obtained.

Characterization and Structural Elucidation of 834-B1

834-A and 834-B which were also active against *P. aeruginosa* NCTC 10490, were identified as thiotetromycin and thiolactomycin respectively by MS, ^{1}H NMR and UV. Physico-chemical properties of 834-B1 are shown in Table 4.

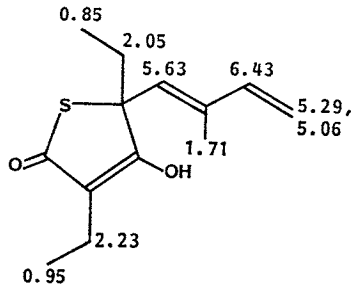
The structure of 834-B1 was determined as I by comparison with thiotetromycin in ^{1}H NMR as follows. As one of the three methyl groups (δ 0.90) was coupled with a methylen (δ 2.07), it became clear that two methyl groups and one ethyl group were present in the molecule. One methyl group (δ 1.72) was coupled to an olefinic methine (δ 5.64) by allyl coupling, so the ethyl group had to be attached to C-2 or C-4. The amount of 834-B1 which we could use in ^{1}H NMR was so small that we accomplished the ^{1}H NMR decoupling experiment in thiotetromycin and determined the position of ethyl group in 834-B1 in comparison with thiotetromycin. The chemical shifts of the ethyl groups in thiotetromycin were (δ 2.05 and 0.85) and (δ 2.23 and 0.95) in CD_3OD. On the other hand the ethyl group in 834-B1 was (δ 2.07 and 0.90), so the ethyl group in 834-B1 seemed to correspond to the former one. Then we tried to distinguish the two ethyl groups in thiotetromycin by ^{1}H-^{13}C selective long range decoupling. The results were that the three bond coupling between carbonyl and the methylene (δ 2.23) was observed, but not between the carbonyl and the methylene (δ 2.05). Therefore the methylene

Table 4. Physico-chemical properties of 834-B1.

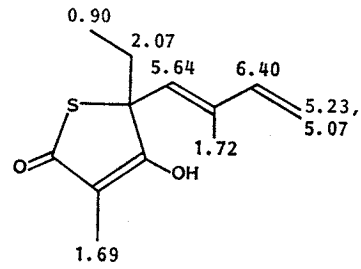
Molecular formula	$C_{12}H_{16}O_2S$
EI-MS (*m/z*)	224 (M^+), 196, 140, 125, 111, 83
IR (KBr) cm^{-1}	3250, 3000, 2920, 1700, 1610, 1460, 1390, 1320, 1270, 980
^{1}H NMR (CD_3OD) δ	0.90 (3H, t, J=7.3 Hz), 1.69 (3H, s), 1.72 (3H, d, J=1.2 Hz), 2.07 (2H, q, J=7.3 Hz), 5.07 (1H, d, J=10.5 Hz), 5.23 (d, J=17.3 Hz), 5.64 (1H, br s), 6.40 (1H, dd, J=10.5 and 17.3 Hz)
UV λ_{max}^{MeOH} nm	237, 298
TLC[a] Rf	0.34 $CHCl_3$ - MeOH, 5 : 1 (0.27; 834-B) 0.26 Benzene - acetone, 2 : 1 (0.20; 834-B) 0.32 EtOAc - MeOH, 10 : 1 (0.26; 834-B)
HPLC[b] Rt (minutes)	3.54 Acetonitrile - H_2O - phosphoric acid (550 : 450 : 1) (2.90; 834-B) 11.57 Acetonitrile - 0.02 M phosphate buffer pH 7.0 (12.5 : 87.5) (6.80; 834-B)
Solubility Soluble:	MeOH, Me_2CO, $CHCl_3$, alkaline water
Color reaction	
Positive:	$KMnO_4$
Negative:	$FeCl_3$, Molisch

EI-MS: Electron impact mass spectrum.
Rt: Retention time.
[a] Silica gel.
[b] Column: LiCrosorb RP-18, 4 i.d. × 150 mm, flow rate: 1.0 ml/minute.

Fig. 3. Proton assignment of thiotetromycin and 834-B1 (in CD_3OD).



Thiotetromycin



834-B1

(δ 2.23) was assigned as the one which was bonded to C-2, and the methylene (δ 2.05) was assigned as the one which was bonded to C-4. Consequently the ethyl group (δ 2.07 and 0.90) in 834-B1 was determined to be bonded to C-4. The proton assignment of thiotetromycin and 834-B1 was shown in Fig. 3. The structure of 834-B1 was clarified as shown in **I**.

Discussion

In the screening where a β-lactam super sensitive-mutant derived from *P. aeruginosa* NCTC 10490 was used as a sensor, we isolated a new antibiotic 834-B1 from *Streptomyces* sp. Y-0834H together with thiolactomycin and thiotetromycin. The structure of 834-B1 resembles that of thiolactomycin and thiotetromycin. That is, 834-B1 has two methyl groups and one ethyl group in the molecule, on the other hand thiolactomycin has 3 methyl groups and thiotetromycin has one methyl group and two ethyl groups. The bioactivity of 834-B1 against *P. aeruginosa* NCTC 10490 is stronger than that of thiotetromycin but a little bit weaker than that of thiolactomycin. Thiolactomycin inhibits the biosynthesis[19,20] of the fatty acid and showed stronger activity *in vivo* than *in vitro*. So, it is interesting to investigate the mode of action of 834-B1 in the cell wall biosynthesis and the immune system.

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References

1) OISHI, H.; T. NOTO, H. SASAKI, K. SUZUKI, T. HAYASHI, H. OKAZAKI, K. ANDO & M. SAWADA: Thiolactomycin, a new antibiotic. I. Taxonomy of the producing organism, fermentation and biological properties. J. Antibiotics 35: 391~395, 1982
2) SASAKI, H.; H. OISHI, T. HAYASHI, I. MATSUURA, K. ANDO & M. SAWADA: Thiolactomycin, a new antibiotic. II. Structure elucidation. J. Antibiotics 35: 396~400, 1982
3) NOTO, T.; S. MIYAKAWA, H. OISHI, H. ENDO & H. OKAZAKI: Thiolactomycin, a new antibiotic. III. *In vitro* antibacterial activity. J. Antibiotics 35: 401~410, 1982
4) MIYAKAWA, S.; K. SUZUKI, T. NOTO, Y. HARADA & H. OKAZAKI: Thiolactomycin, a new antibiotic. IV. Biological properties and chemotherapeutic activity in mice. J. Antibiotics 35: 411~419, 1982
5) ŌMURA, S.; Y. IWAI, A. NAKAGAWA, R. IWATA, Y. TAKAHASHI, H. SHIMIZU & H. TANAKA: Thiotetromycin, a new antibiotic. Taxonomy, production, isolation, and physicochemical and biological properties. J. Antibiotics 36: 109~114, 1983
6) ŌMURA, S.; A. NAKAGAWA, R. IWATA & A. HATANO: Structure of a new antibacterial antibiotic, thiotetromycin. J. Antibiotics 36: 1781~1782, 1983
7) SHIZURI, Y.; M. NIWA, H. FURUKAWA & S. YAMAMURA: Isolation and structure of citreothiolactone, a

novel metabolite of *Penicillium citreo-viride* B. Tetrahedron Lett. 24: 1053~1054, 1983
8) JIZBA, J. V.; P. SEDMERA & Z. VANĚK: Two thiolactones from *Streptomyces* Tü 2476. J. Antibiotics 38: 111~112, 1985
9) DOLAK, L. A.; T. M. CASTLE, S. E. TRUESDELL & O. K. SEBEK: Isolation and structure of antibiotic U-68,204, a new thiolactone. J. Antibiotics 39: 26~31, 1986
10) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
11) Guide to Color Standard. Nihon Shikisaisha, 1954
12) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some actinomycetes as an acid for species determination. Eur. Bacteriol. 56: 107~114, 1948
13) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
14) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968
15) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1968
16) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
17) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. Int. J. Syst. Bacteriol. 22: 265~394, 1972
18) BUCHANAN, R. E. & N. E. GIBBONS (*Ed.*): BERGEY's Manual of Determinative Bacteriology. 8th Ed., Williams & Wilkins Co., Baltimore, 1974
19) HAYASHI, T.; O. YAMAMOTO, H. SASAKI, H. OKAZAKI & A. KAWAGUCHI: Inhibition of fatty acid synthesis by the antibiotic thiolactomcyin. J. Antibiotics 37: 1456~1461, 1984
20) NISHIDA, I.; A. KAWAGUCHI & M. YAMADA: Effect of thiolactomycin on the individual enzymes of the fatty acid synthase system in *Escherichia coli*. J. Biochem. 99: 1447~1454, 1986