HYGROMYCIN AND EPIHYGROMYCIN FROM A BACTERIUM, *CORYNEBACTERIUM EQUI* NO. 2841

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A newly isolated bacterium which produces antibacterial substances as below was taxonomically characterized and identified with *Corynebacterium equi*. The major antibiotic produced was determined to be hygromycin by direct comparison. The minor component, newly named epihygromycin was proved to be an epimer of hygromycin by spectroscopic evidences and by chemical conversions. The presence of epihygromycin in the metabolites of *Streptomyces noboritoensis* (reported to be a hygromycin producer) was also observed.

The "Coryneform group of bacteria"[1] includes the genera *Corynebacterium, Arthrobacter* (*Brevibacterium, Microbacterium*), *Cellulomonas* and *Kurthia*, and is taxonomically close to *Streptomycetaceae*[1]. Moreover, the "Coryneform group of bacteria" is common to *Streptomycetaceae* in antibiotic production. For instance, oxamicetin[2] (resembling amicetin from *Streptomyces*[3]) is produced by *Arthrobacter oxamicetes*[2], corynecins[4] (chloramphenicol analogues) are formed by *Corynebacterium* sp.[4], and erythromycin is produced by *Arthrobacter* sp.[14] Based on this information, we selected the "Coryneform group of bacteria" for antibiotic screening.

This paper deals with the newly isolated bacterium, *Corynebacterium equi* No. 2841 which produces hygromycin and epihygromycin.

Materials and Methods

1. Taxonomic study

BERGEY's Manual of Determinative Bacteriology 8th edition[1] was used as the taxonomic system. Each bacteriological character was measured using the "Manual of Microbiological Methods" (American Society for Microbiology)[5] and SKERMAN's "The Genera of Bacteria"[12].

2. Cell wall analysis

(1) Preparation of purified cell wall: Purified cell wall was prepared by KOTANI and KATO's method[11] with some modification. Five hundred milliliters of the fermented broth of the test strain (modified GNB medium: 0.1% glucose, 0.1% yeast extract, 1.0% Polypepton, 0.5% beef extract, 0.3% NaCl; 100 ml per flask, 28°C, 2 days, shaking) was centrifuged and the cell mass obtained was suspended in a mixture of 5 ml of saline and 1 ml of 0.5% sodium dodecyl sulfate after washing once with 50 ml of water. The suspension was then sonicated at 20 KC, 0°C for 20 minutes, and the viscous solution obtained was diluted with 23 ml of saline and centrifuged at ×1,000 for 10 minutes. The turbid supernatant was centrifuged at ×10,000 for 20 minutes at 5°C, and the precipitate was collected, washed with 15 ml of water, and lyophilized. The crude cell wall sample was resuspended in 2.4 ml of 1/15 M phosphate buffer, pH 7.0, which contained 0.5 mg per ml each of trypsin and RNase (Worthington Biochem. Co.), then incubated at 37°C for 2.5 hours. The reaction mixture was centrifuged at ×10,000 and 0°C for 20 minutes, then the precipitate was washed with 5 ml of water in the same way. This precipitate was again suspended in 2.8 ml of 0.02 N HCl containing 1 mg per ml of pepsin (Calbiochem)

and incubated at 37°C for 18~20 hours. The reaction mixture was then centrifuged at ×10,000 and 0°C for 20 minutes, then the precipitate was washed twice with 5 ml of ice water in the same way and lyophilized to obtain the purified cell walls. The yields were 50~100 mg per 500 ml of broth.

(2) Analysis of cell wall amino acids: Five to ten milligrams of purified cell wall and 1 ml of 6 N HCl were sealed in a small glass tube and hydrolyzed at 110°C for 18 hours. Upon cooling, the mixture was dried under vacuum then dissolved again in 1 ml of 0.1 M citrate buffer solution, pH 2.2. This solution was used for analysis. Hitachi KLA-5 type amino acid analyzer: standard column 9×550 mm, 0.2 N citrate buffer, pH 3.5 and 4.25; basic column 9×100 mm, 0.35 N citrate buffer, pH 5.25. The discrimination between LL- and meso-diaminopimelic acid was carried out by HARPER-DAVIS's TLC method[16].

(3) Analysis of cell wall carbohydrates: Eight to 10 milligrams of purified cell wall and 1 ml of 1 N HCl were sealed in a small glass tube and hydrolyzed at 105~110°C for 4 hours. The mixture was treated with 0.7~1.0 ml of Amberlite IR-4B resin at room temperature for a few minutes then centrifuged. The supernatant was dried under vacuum, trimethylsilyled by the procedure of SWEELY, BENTLEY, MAKITA and WELL[12], then used for analysis. The gas chromatography conditions were: Shimadzu GC-7AG type gas chromatograph. Column; Shimalite W containing 5% U-Con 500, 3.2×16,000 mm, 60~80 mesh. Column temperature; 170°C. Injection part temperature; 180°C. Gas; H_2 0.6 kg/cm², Air 0.5 kg/cm². Chart speed; 1 cm/minute.

(4) Analysis of mycolic acids in whole cells: Methyl esters of fatty acids in whole cells were prepared by the procedure of YANO *et al.*[10] The samples were used for the detection of mycolic acids by TLC; hexane - ethyl ether, 8: 2, Kieselgel 60F_{254} (Merck).

3. High performance liquid chromatography (HPLC).

(1) Analytical HPLC: Column; Nucleosil 5C-18, 4 mmϕ×200 mm. Solvent; CH_3CN - H_2O, 13: 87. Flow speed; 1 ml per minute. Detection; UV at 254 nm. Instrument; Waters 6000 A.

(2) Preparative HPLC: Column; Lichroprep RP-18, 20 mmϕ×500 mm. Solvent; CH_3Cl - H_2O, 1: 9. Flow speed; 14 ml per minute. Instrument; same as for analytical HPLC.

Results

A. Taxonomic Study of *Corynebacterium equi* No. 2841

The bacterium was obtained by screening 3,900 strains of the "Coryneform group of bacteria" which were isolated from soil samples of the Taiwan-Okinawa districts.

1. Morphology and Staining of Cell Wall

(1) Cell shape and size: Moderate rods with rounded ends at the early stage (0.6~0.8×1.0~5.0 μ) but almost coccoid in the late stage (0.6~0.8×0.8~1.0 μ). Some pleomorphism is seen in the rod stage, *i.e.*, curved, club- or spindle-shaped cells and snapping arrangement are often seen. A short beaded arrangement is commonly observed at the coccoid stage. Swollen coccids are rarely observed in the coccoid stage. (See Figs. 1-a and b)

(2) Spore: Not formed (on Gly-IM* agar, glucose nutrient agar and nutrient agar, 27° and 36°C, 1~18 days).

(3) Flagella and motility: No flagellation was observed by electron microscopy (See Fig. 2). Nonmotile.

(4) Gram staining: Positive in all stages (1~8 days, 27° and 37°C, Gly-IM agar and nutrient agar).

(5) Acid fast staining: Negative.

(6) Capsule: Observed (the cells on glucose nutrient agar).

* Gly-IM agar: 0.5% glycerol, 0.25% Polypepton (Kyokuto), 0.25% beef extract, 0.25% yeast extract, 0.25% Bactosoytone (Difco), 0.3% NaCl, 1.25% agar.

Fig. 1. Microscopic photograph of *C. equi* No. 2841.

a) Rod-shaped stage (Gly-IM agar, 27°C, 18 hours). b) Coccoid stage (Gly-IM agar, 27°C, 7 days).

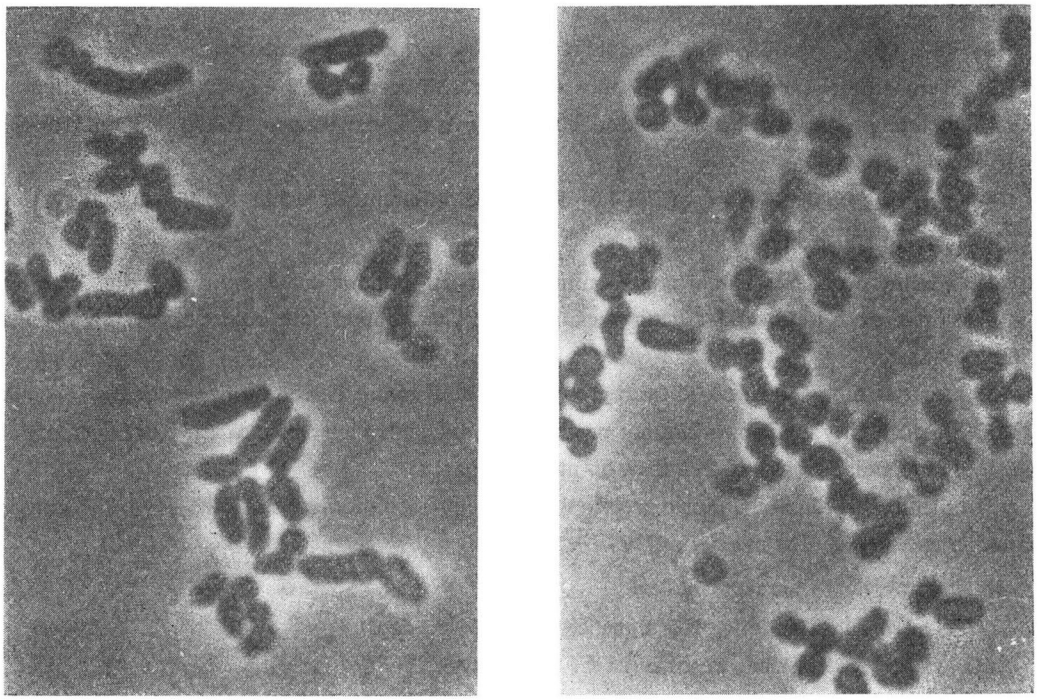
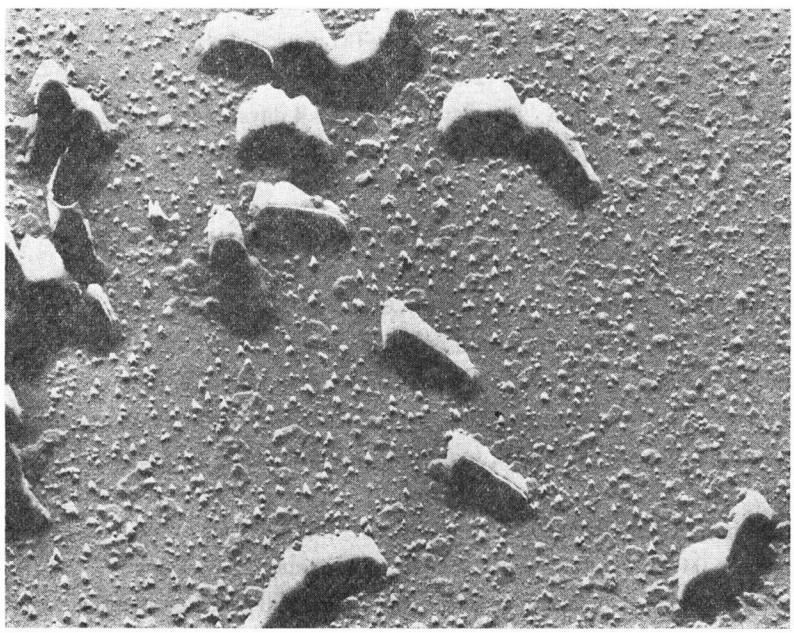


Fig. 2. Electron microscopic photograph of *C. equi* No. 2841 (×10,000, Chromium-shadowing, Gly-IM agar, 27°C, 18 hours).



2. Conditions for Growth

(1) Oxygen requirement: Strictly aerobic (by Gas Pak System Test, 27°C, Gly-IM agar, 1~3 days).

(2) O-F Test: Aerobic. Neither acid nor gas were formed from glucose.

(3) Temperature for growth: The most rapid growth occurs at 28~33°C (Gly-IM medium, 17 hours, temperature gradient incubator Model TN-3, Toyo Kagaku Sangyo, Tokyo). Poor growth is observed at 39.5°C.

(4) pH for growth: Grows at pH 4.6~9.9. The optimum pH is 5.8~9.0.

3. Growth Characteristics

(1) Colony on agar: Circular (1~4 mm), convex to semiumbonate, entire margin, smooth to mud-like surface, soft to mucoid structure, opaque density and pinkish gray color colony (Gly-IM agar, 27°C, 1~9 days).

(2) Agar slant: Moderate, filiform growth with dull-shining is seen on nutrient agar. The cell mass has pinkish gray color but does not produce any diffusible pigments into the agar. On Gly-IM agar, more mucoidal growth is seen than on nutrient agar.

(3) Broth: Moderate, uniform growth with some precipitation is observed. No pellicle or ring formation is seen (Gly-IM medium, 27°C, 1~5 days).

4. Physiological Characteristics

(1) Gelatin liquefaction: Not liquefied (25°C, 1~22 days).

(2) Casein hydrolysis: Positive (27°C, 1~14 days).

(3) Starch hydrolysis: Not hydrolyzed (28°C, 1~10 days).

(4) Milk reaction: No change (27°C, 1~28 days).

(5) Egg yolk reaction: Negative (27°C, 1~7 days).

(6) Catalase: Positive (27°C, Gly-IM agar, 1 day).

(7) Oxidase (KOVAC's test): Negative (27°C, Gly-IM agar, 1 day).

(8) Cytochrome oxidase: Negative (27°C, Gly-IM agar, 1 day).

(9) Nitrite from nitrate: Negative (27°C, 1~5 days).

(10) VOGES-PROSKAUER reaction: Negative (27°C, 1~4 days, shaking and setting).

(11) H_2S formation: Negligible at 27°C but weakly positive at 37°C (paper strip method, 1~8 days).

(12) Indole formation: Negative (27°C, 2~6 days).

(13) Urease: Positive (27°C, 2~8 days).

(14) Phenylalanine deaminase: Negative (27°C and 37°C, 6 hours).

(15) Tylosinase: Negative (27°C, 4~13 days).

(16) Carbohydrate cleavage: No acid from L-arabinose, D-xylose, rhamnose, D-ribose, D-mannose, D-galactose, D-fructose, D-glucose, sucrose, maltose, lactose, trehalose, raffinose, melibiose, dextrin, starch, glycogen, inulin, glycerol, adonitol, mannitol, sorbitol, dulcitol, salicin, α-methylglucoside, *m*-inositol.

(17) Growth in 0.01% lysozyme-containing broth: Poor (27°C, 1 day).

(18) Growth on 0.02% NaN_3-containing agar: Moderate (27°C, 1 day).

(19) Growth on 0.04% potassium tellurite agar: Grayish black to black colonies (27°C, 1~4 days).

(**20**) Haemolysis: Negative (blood agar, 27°C, 1~4 days).

5. Cell Wall Composition

The purified cell wall sample (prepared as in "Materials and Methods") was analyzed for amino acids and carbohydrates. The analytic procedures were also described above. As references, the cell wall of *Arthrobacter globiformis* ATCC 8010, *Corynebacterium equi* B-271-1 and *Arthrobacter simplex* ATCC 6946 were analyzed in parallel. The results are summarized in Table 1.

The characteristic amino acids and sugars of No. 2841 cell wall are meso-diaminopimelic acid, glycine, serine and leucine, and arabinose, galactose and mannose, respectively. The pattern differs from that of *A. simplex*, *A. globiformis*, *A. cremorum* and *L. burgaricus*, and is identical to that of *C. equi* B-271-1 (See Table 1).

Table 1. Amino acid and carbohydrate composition of the cell walls of *C. equi* No. 2841 and related bacteria.

Amino Acid μM/mg	*C. equi* No. 2841	*C. equi* B-271-1	*A. globiformis* ATCC 8010	*A. simplex* ATCC 6946	*A. cremorum* M-1376-2	*L. burgaricus* IFO 3533
1. Ammonia	+	+	1.204	0.714	1.130	0.729
2. Lys	0.030	0.016	0.324	0.071	0.540	0.289
3. His	±	±	0.072	0.060	0.008	±
4. Arg	0.010	0.018	0.016	0.055	0.006	0.003
5. Asp	0.099	0.066	0.020	0.040	0.041	0.197
6. Thr	0.046	0.038	0.021	0.030	0.021	0.006
7. Ser	0.149	0.136	0.012	0.032	0.014	0.006
8. Glu	0.346	0.427	0.364	0.421	0.554	0.443
9. Pro	0	0	0	0	0	0
10. Gly	0.198	0.152	0.013	0.412	0.038	0.016
11. Ala	0.451	0.543	1.357	0.655	1.448	0.534
12. Cys	0	0	0	0	0	0
13. Val	0.040	0.046	0.021	0.037	0.027	0.009
14. Met	~	~	~	~	~	~
15. Ile	0.021	0.018	0.009	0.015	0.014	0.006
16. Leu	0.134	0.125	0.014	0.025	0.017	0.009
17. Tyr	0.018	0.023	0.003	0.014	0.003	0.007
18. Phe	0.039	0.040	~	~	~	~
19. DAP	0.192**	0.214**	0.010	0.371***	0.019	0.004
20. Glucosamine	0.156	0.249	0.197	0.323	0.535	0.247
21. Galactosamine	0	0	0.219	0	0.059	0.293
22. Muramic Acid	+	+	+	+	+	+
23. L-Arabinose	++	++	−	+	−	
24. D-Galactose	++	++	+++	++	−	
25. D-Mannose	++	++	−	−	++	
26. D-Glucose	−	−	−	−	++	
27. Rhamnose	−	−	−	++	−	
28. Mycolic acid as major fatty acids	+	+	•	+*	•	

* a different type of mycolic acid with lower Rf than that of *C. equi*

• absent or only small amount detected

** *meso*-DAP

*** LL-DAP

6. Fatty Acid Composition of Whole Cells

The predominant occurrence of a mycolic acid[10] is observed in No. 2841 and *C. equi* B-271-1 but not in *A. globiformis* and *A. cremorum*. In *A. simplex*, another type of predominant mycolic acid is observed (See Table 1).

7. Taxonomic Consideration and Identification

The above results clearly show that No. 2841 is not a strain of the genus *Arthrobacter* but of the genus *Corynebacterium*[1]. The evidence that the cell wall contains arabinose and meso-diaminopimelic acid and its whole cells contain major amount of mycolic acids conclusively supports this.

Comparison of No. 2841 with each *Corynebacterium* species[1] lead to the conclusion that the culture is a strain of genus *C. equi*. Arguments for the conclusion are: (a) pinkish gray growth, (b) strict aerobe (BERGEY's description is aerobic and facultative anaerobic for *C. equi*, but a *C. equi* strain, B-271-1 is strictly aerobic), (c) Gram-positive pleomorphic rods in the young stage and coccoids in the late stage, (d) capsulation on glucose nutrient agar (mucoidal growth), (e) no acid from carbohydrates, (f) no gelatin liquefaction, (g) no haemolysis, (h) meso-diaminopimelic acid, glycine, alanine, serine, glutamic acid and leucine as major cell wall amino acids which are identical with *C. equi* B-271-1, (i) the presence of arabinose, galactose, mannose, glucosamine and muramic acid as cell wall carbohydrates which are identical to *C. equi* B-271-1. Of course, there are some differences between No. 2841 and *C. equi* type culture[1]. The type culture is aerobic and facultative anaerobic, and does not reduce nitrate and has no urease activity. However, diverse nitrate reduction and urease activities exist in the *C. equi* species[5], and *C. equi* B-271-1 does not grow under anaerobic condition. The diversity includes the above difference.

Therefore, the culture is, identified as and named *Corynebacterium equi* No. 2841.

B. Production and Characterization of the Products

1. Fermentation and Isolation of the Products

The seed broth was prepared by shaking cultivation of No. 2841 on GNB medium[13] (100 ml per flask) for 1 day at 28°C. The culture was transferred into the fermentation medium, No. 6 medium* at 2% concentration and cultivated at 28°C for 4 days under shaking (130 rpm, 5 cm amp.).

The fermented broth (8 liters) was filtered and the clear filtrate was charged on 500 ml of Dia-Ion HP-20 column. The column was washed with 2 liters of water and 20% methanol water successively, then the remaining active component was eluted with 50% methanol water. The active eluate was dried in vacuum, and 2.45 g of crude sample was obtained. The sample was then charged on 100 ml of Sephadex LH-20 column and eluted with methanol. The pure fractions selected by TLC on silica gel ($CHCl_3$ - MeOH - water, 2: 2: 1; lower layer, and $CHCl_3$ - MeOH, 8: 2) were combined and evaporated to give 876 mg of yellow powder which was homogeneous on TLC. However, it shows two peaks on HPLC (retention times 7.6 minutes (**II**) and 8.7 minutes (**I**), ratio 1: 2). The mixture (500 mg) was separated by preparative HPLC into 136 mg of component **II** and 270 mg of component **I**.

2. Characterization of the Product

(1) Component **I**: The main product **I** was easily assumed to be hygromycin (I) from its UV spectrum and ^{13}C NMR spectrum**. In order to confirm by direct comparison, hygromycin was isolated from the fermentation broth of *Streptomyces noboritoensis* KCC-S-0065[15] (reported to be a hygro-

* No. 6 Medium: 1.0% soluble starch, 1.0% glucose, 1.0% cane molasses, 1.5% yeast extract, 0.5% $CaCO_3$.

** Chemical shifts of three carbons were not agreeable with those reported[7,8] and it was attributed to errata of the literature printing by the private communication from the author, Dr. K. KAKINUMA.

Fig. 3. IR spectra (KBr) of hygromycin (**I**) and epihygromycin (**II**).

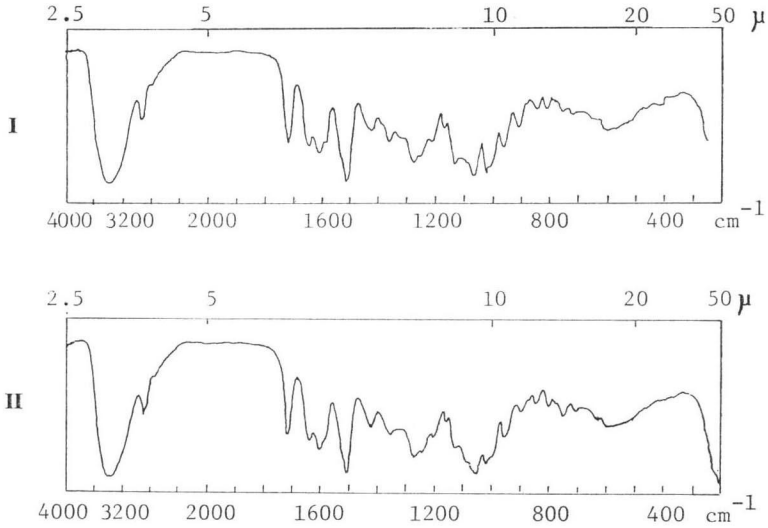


Fig. 4. ^{1}H-NMR spectra of hygromycin (**I**) and epihygromycin (**II**) in CD_3OD at 60 MHz.

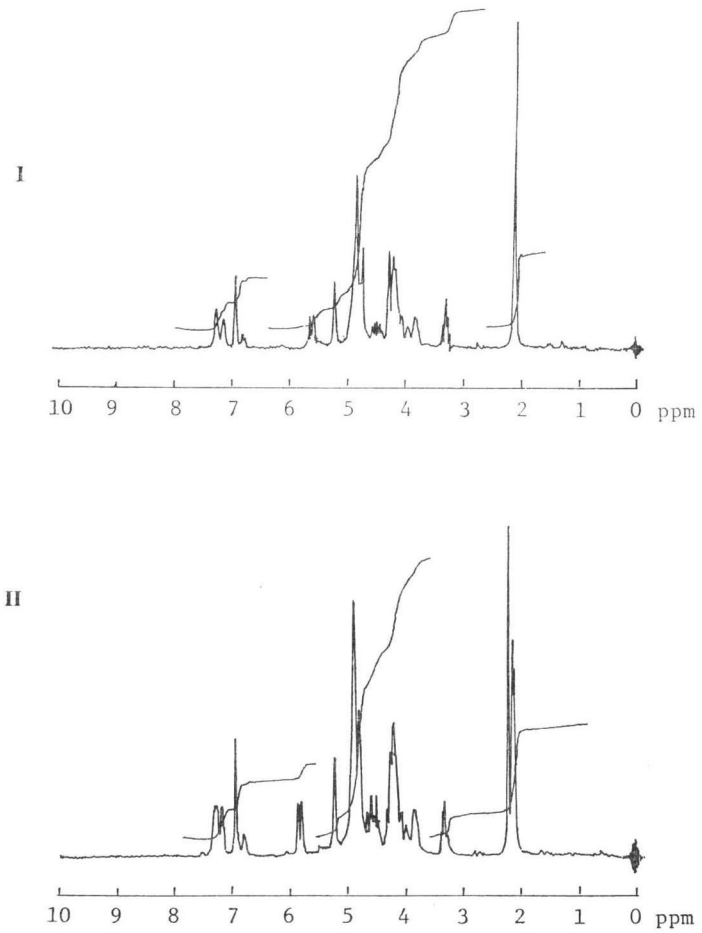
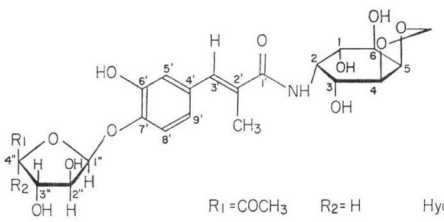


Fig. 5.



R1=COCH3 R2=H Hygromycin (I)
R1=H R2=COCH3 Epihygromycin (II)

Table 2. Chemical shifts (δ ppm) of CH_3CO and anomeric H in CD_3OD

	CH_3CO	C_1''-H
Hygromycin (**I**)	2.12	5.60
Epihygromycin (**II**)	2.20	5.82

mycin producer) and the identity of both samples was established.

(2) Component **II**: The minor component **II**, mp 114~117°C, $[\alpha]_D^{20.1}$ −91.1±1.3° (*c* 1.0, H_2O), showed the same UV absorption with that of hygromycin and the IR spectra are also similar (see Fig. 3). Elementary analysis gave the molecular formulae, $C_{23}H_{29}NO_{12}\cdot\frac{3}{2}H_2O$****, and it was supported by ^{13}C NMR spectrum which was distinguishable with that of hygromycin in the chemical shifts of the carbons attributable to arabinohexoside and aromatic carbons near the sugar moiety. Further, the ^1H NMR spectra of component I (hygromycin) and component **II** (see Fig. 4 and Table 2) show the apparent

Table 3. *In vitro* activity of epihygromycin.

No.	Strains	Media	Epihygromycin μg/ml		Hygromycin μg/ml	
			MIC**	MAC***	MIC	MAC
1	*Staphylococcus aureus* FDA 209P JC-1	KD*	>100	50	12.5	6.25
2	*Staphylococcus aureus* FDA 209P JC-1	KD+10% serum	100	50	25	6.25
3	*Staphylococcus aureus* Smith	KD	>100	50	25	12.5
4	*Staphylococcus epidermidis* ATCC 14990	KD+10% serum	100	50	25	12.5
5	*Streptococcus pyogenes* C203	KD+10% serum	12.5	6.25	6.25	1.56
6	*Streptococcus faecalis* CN478	KD+10% serum	>100	>100	100	25
7	*Bacillus subtilis* PCI 219	KD+ 3% agar	>100	>100	>100	50
8	*Escherichia coli* NIHJ JC-2	KD	>100	>100	>100	>100
9	*Escherichia coli* NIHJ JC-2	KD+10% serum	>100	>100	>100	>100
10	*Escherichia coli* EC-148	KD	>100	50	25	12.5
11	*Enterobacter cloacae* ATCC 13047	KD	>100	>100	>100	100
12	*Enterobacter aerogenes* TB-510	KD	>100	>100	>100	>100
13	*Citrobacter freundii* Ct-50	KD	>100	>100	>100	>100
14	*Shigella sonnei* ATCC 11060	KD+ 3% agar	>100	>100	>100	100
15	*Shigella typhimurium* ATCC 13311	KD+ 3% agar	>100	50	25	6.25
16	*Klebsiella pneumoniae* ATCC 27736	KD	>100	>100	>100	>100
17	*Proteus rettgeri* Ret-33	KD	>100	>100	100	25
18	*Proteus inconstans* In-32	KD	>100	>100	>100	50
19	*Proteus morganii* Mor-74	KD+ 3% agar	>100	>100	>100	100
20	*Proteus vulgaris* ATCC 6380	KD+ 3% agar	>100	>100	100	25
21	*Proteus vulgaris* TB-708	KD+ 3% agar	>100	>100	3.13	1.56
22	*Serratia marcescens* ATCC 13880	KD	>100	>100	>100	>100
23	*Pseudomonas aeruginosa* Ps-24	KD	>100	>100	>100	>100
24	*Pseudomonas aeruginosa* ATCC 9721	KD	>100	>100	>100	>100

* Sensitivity assay medium "Nissui"
** Minimal Inhibitory Concentration
*** Maximal Allowable Concentration

**** Molecular peak, 511, was obtained by FD-MS as in the case of hygromycin.

difference in the chemical shifts of CH_3CO proton signals and the anomeric proton signals. The down-field shift by 0.08 ppm of the CH_3CO signal of component **II** should be due to the α-configuration of this group in which the anisotropic effect of the aromatic ring disappears. The down-field shift by 0.18 ppm of the anomeric proton signal is also likely, because the hydrogen (α-configuration) is affected by the carbonyl function of the α-CH_3CO group at C_4''. Thus, the structure of the component **II** is reasonably explained to be a epimer of hygromycin in the configuration of CH_3CO group at C_4''. This speculation was confirmed by the following experiment: Hygromycin in the alkaline solution at room temperature afforded a mixture (1 : 1) of hygromycin and component **II**, and the component **II** also gave the same result. Accordingly, this new compound, epihygromycin, is determined to be **II**. We also found epihygromycin in the fermentation broth of *Streptomyces noboritoensis* KCC-S-0065 and isolated it for comparison.

(3) *In vitro* activity of epihygromycin: As shown in Table 3, epihygromycin is less active against Gram-positive and negative monitors than hygromycin[15].

Discussion

Antibiotics from *Bacillus* and *Pseudomonas* bacteria have a specific pattern of chemical structure that differs from that of *Streptomycetaceae*. For instance, the *Bacillus* species produces predominantly various types of peptidic antibiotics, while *Pseudomonas* species mainly form phenazine antibiotics.

The "Coryneform group of bacteria" produce only a few known antibiotics[2,4,9]. However, the diversity of their chemical structure is relatively much higher than the above described bacteria. For instance, *A. oxamicetes* produces oxamicetin[2] (amicetin like), *Corynebacterium* sp. forms corynecins[4] (chloramphenicol analogues), *Arthrobacter* sp. produces erythromycin[14] and *Brevibacterium* Z 7223 produces a sugar peptide antibiotic, A-23-S[9]. These findings suggest that the "Coryneform group of bacteria" is close to *Streptomycetaceae* not only in taxonomy but also antibiotic production. And our finding that *C. equi* No. 2841 produces hygromycin supports this assumption.

Besides hygromycin[3,7,8], we found a new antibiotic, epihygromycin, not only from *C. equi* No. 2841 but also from *Str. noboritoensis* KCC-S-0065[15]. The conversion of the methyl ketone group of hygromycin occurs easily in the mild alkaline solution (pH 9) at room temperature (see Characterization of the products). Therefore, the formation of epihygromycin in the fermenting broth is presumably originated in the epimerization of the methyl ketone group of hygromycin. The conversion in the isolation processes can be excluded since it was isolated under the conditions on which the remarkable epimerization should not occur (see Method).

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References

1) BUCHANAN, R. E. & N. E. GIBBONS: BERGEY's Manual of Determinative Bacteriology. 8th ed. The Williams & Wilkins Co., Baltimore, 1974

2) KONISHI, M.; M. KIMEDA, H. TSUKIURA, H. YAMAMOTO, T. HOSHIYA, T. MIYAKI, K. FUJISAWA, H. KOSHIYAMA & H. KAWAGUCHI: Oxamicetin, a new antibiotic of bacterial origin. I. Production, isolation and properties. J. Antibiotics 26: 752~756, 1973
KONISHI, M.; M. NARUISHI, T. TSUNO, H. TSUKIURA & H. KAWAGUCHI: Oxamicetin, a new antibiotic of bacterial origin. II. Structure of oxamicetin. J. Antibiotics 26: 757~764, 1973
TOMITA, K.; Y. UENOYAMA, K. FUJISAWA & H. KAWAGUCHI: Oxamicetin, a new antibiotic of bacterial origin. III. Taxonomy of the oxamicetin-producing organism. J. Antibiotics 26: 765~770, 1973

3) UMEZAWA, H.: Index of Antibiotics from Actinomycetes. pp. 124~126. The University of Tokyo Press, Tokyo, 1967
4) SUZUKI, T.; H. HONDA & R. KATSUMATA: Production of antibacterial compounds analogous to chloramphenicol by *n*-paraffin grown bacterium. Agr. Biol. Chem. 36: 2223~2228, 1972
SHIRAHATA, K.; T. HAYASHI, T. DEGUCHI, T. SUZUKI & I. MATSUBARA: The structure of corynecins, chloramphenicol analogous produced by an *n*-paraffin grown bacterium. Agr. Biol. Chem. 36: 2229~2232, 1972
5) American Society for Microbiology. Manual of Clinical Microbiology. pp. 88~94. Bethesda, Md., U.S.A. 1970
6) UMEZAWA, H.: Index of Antibiotics from Actinomycetes. p. 334. The University of Tokyo Press, Tokyo, 1967
7) KAKINUMA, K. & Y. SAKAGAMI: Nuclear magnetic resonance and structure of hygromycin. Agr. Biol. Chem. 42: 279~286, 1978
8) KAKINUMA, K.; S. KITAHARA, K. WATANABE, Y. SAKAGAMI, T. FUKUYASU, M. SHIMURA, M. UEDA & Y. SEKIZAWA: On the structure of hygromycin. The location of a methylene substituent and the anomeric configuration of the *arabino*-hexoside moiety. J. Antibiotics 29: 771~773, 1976
9) Maruzen Oil Co., Ltd.: New antifungal antibiotic, A-23-S. Japan Kokai Patent S50-101587, 1975
10) YANO, I.; K. KAGEYAMA, Y. OHNO, M. MASUI, E. KUSUNOSE, M. KUSUNOSE & N. AKIMORI: Separation and analysis of molecular species of mycolic acids in *Nocardia* and related taxa by gas chromatography mass spectrometry. Biomed. Mass Spectrometry 5: 14~24, 1978
11) KOTANI, S. & K. KATO: Isolation and purification of cell wall and peptidoglycan. Protein, Nucleic Acid & Enzyme (Japanese) 14: 606~620, 1969
CUMMINS, C. S. & H. HARRIS: The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as taxonomic character. J. Gen. Microbiol. 14: 583~600, 1956
12) SKERMAN, V. B. D.: Genera of Bacteria. The Williams & Wilkins Company, Baltimore, 1967
13) NISHIITSUTSUJI-UWO, J.; Y. WAKISAKA & M. EDA: Sporeless mutant of *Bacillus thuringiensis*. J. Invertebrate Pathol. 25: 355~361, 1975
14) FRENCH, J. C.; S. T. SHORSE, J. D. HOUDIS, G. P. WOOD & I. F. ANDERSON: Erythromycin process. U. S. Patent 3,551,294, 1970
15) ISONO, K.; S. YAMASHITA, Y. TOMIYAMA & S. SUZUKI: Studies on homomycin. II. J. Antibiotics, Ser. A 10: 21~30, 1957
16) HARPER, J. J. & G. H. G. DAVIS: Two-dimensional TLC for amino acid analysis of bacterial cell walls. Intern. J. System. Bacteriol. 29: 56~58, 1978