BUTALACTIN, A NEW BUTANOLIDE ANTIBIOTIC

TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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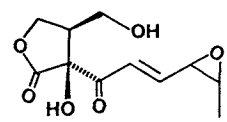
Butalactin, [2-(4′,5′-epoxy-hex-2′(*E*)-en)oyl-2-hydroxy-3-hydroxymethyl-2,3-(*Z*)-butanolide] is a new antibiotic produced by *Streptomyces* sp. HIL Y-86,36923. Taxonomically, the producing organism most closely resembles *Streptomyces corchorusii*. The strain also produces cineromycin B. Though butalactin is structurally related to 'signal molecules' such as A-factor, the anthracycline inducing factors and the virginiae butanolides, it does not show inducing activity for antibiotic production or aerial mycelium formation in the indicator strain. Butalactin possesses a weak antibiotic activity against Gram-positive and Gram-negative bacteria.

In the course of screening for new bioactive metabolites from actinomycetes, we detected a new γ-butyrolactone antibiotic which we named butalactin[1]. It is coproduced with cineromycin B. Butalactin, [2-(4′,5′-epoxy-hex-2′(*E*)-en)oyl-2-hydroxy-3-hydroxymethyl-2,3-(*Z*)-butanolide] (Fig. 1), was detected by biautography of TLC plates. In this paper, we present the taxonomy of the producting organism *Streptomyces* sp. Y-86,36923 together with the fermentative production, isolation procedure and biological activity of butalactin. The structure elucidation of butalactin will be presented separately[2].

Fig. 1. Structure of butalactin.



Taxonomy of the Producing Strain

The butalactin producing strain *Streptomyces* sp. Y-86,36923 was isolated from a soil sample collected from the Sahyadri hill region near Pune, Maharashtra State, India. The strain has been deposited at the Deutsches Sammlung von Mikroorganismen, Braunschweig, FRG, where it has been assigned the accession No. DSM 5372.

The strain has been characterized by the methods of the International Streptomyces Project (ISP) recommended by SHIRLING and GOTTLIEB[3], and WAKSMAN[4].

Morphological Properties

The vegetative mycelium of *Streptomyces* sp. Y-86,36923 grows abundantly on both synthetic and complex agar media and does not show fragmentation into bacillary or coccoid forms. After cultivation on yeast extract-malt extract agar and inorganic salts-starch agar at 27°C for 14 days the following morphological properties were observed: The aerial mycelium branched monopodially with sporophores forming spore chains in open spirals with 10~20 spores per chain (Fig. 2). Many imperfect spirals, hooks and loops are also present. Whirls are not observed. The spores are cylindrical and regular shaped

Fig. 2. Photomicrograph of the butalactin-producing strain on ISP 3.

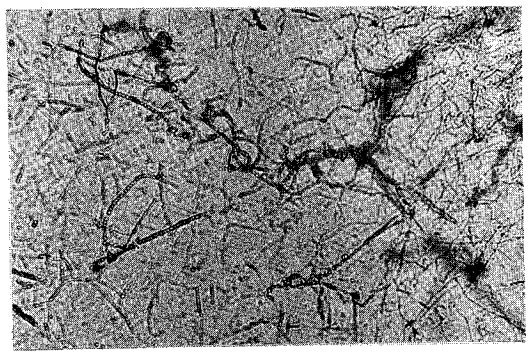


Fig. 3. Electron micrograph of spore chains of *Streptomyces* sp. Y-86,36923.

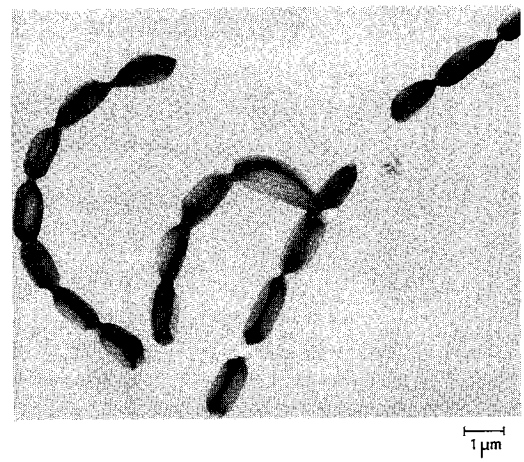


Table 1. Cultural properties of *Streptomyces* sp. Y-86,36923.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract-malt extract agar (ISP 2)	Good, wrinkled, dry	Scant, powdery, light gray	Yellowish white	None
Oatmeal agar (ISP 3)	Good, flat, dry	Moderate, powdery, white	Pale yellow	None
Inorganic salts-starch agar (ISP 4)	Good, raised, dry	Good, powdery, gray	Gray olive	None
Glycerol-asparagine agar (ISP 5)	Good, wrinkled, dry	Weak, powdery, white	Pale yellow	None
Peptone-yeast extract-iron agar	Moderate, flat, sandlike	None	Pale brown	None
Glucose-asparagine agar	Good, wrinkled, dry	Weak, powdery, light gray	Pale yellow	None
Tyrosine agar	Good, wrinkled, dry	None	Light yellow	None
Sucrose-nitrate agar	Good, wrinkled, sandlike	Scant, powdery, white	Pale yellow	None

($0.8 \sim 0.9 \times 1.2 \sim 1.5\,\mu m$) with a smooth surface (Fig. 3).

Chemical Composition

The chemical analysis of cell wall diaminopimelic acid isomers carried out by the method of LECHEVALIER and LECHEVALIER[5] showed the presence of LL-diaminopimelic acid.

Cultural and Physiological Characteristics

Cultural characteristics of *Streptomyces* sp. Y-86,36923 grown on various media at 27°C for 14 days are shown in Table 1. The reverse mycelium had no pH indicator properties. No melanoid pigment was produced in peptone-yeast extract-iron agar, tyrosine agar or Tryptone-yeast extract agar.

Physiological characteristics of the strain are summarized in Table 2. The utilization of carbon sources, which was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% of each carbon source at 27°C and observed for 16 days, is shown in Table 3.

Table 2. Physiological properties of *Streptomyces* sp. Y-86,36923.

Optimum growth temperature	25~37°C	Tyrosinase reaction	Negative
Nitrate reduction	Negative	Production of H_2S	Negative
Liquefaction of gelatine	Positive	Cellulolytic activity	Negative
Starch hydrolysis	Positive	Growth on CZAPEK's solution agar	Good
Coagulation of milk (37°C)	Positive	Streptomycin inhibition	>6.25 μg/ml
Peptonization of milk (37°C)	Positive	NaCl tolerance	>6, <7%
Melanin formation	Negative	pH tolerance	6.0~9.0

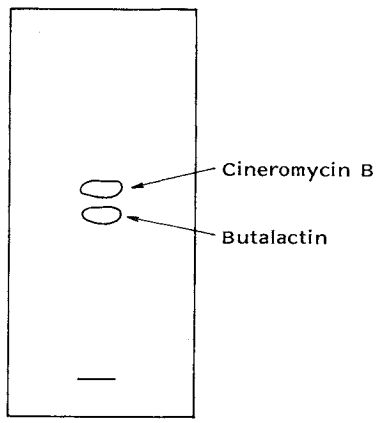
Table 3. Utilization of carbon sources by *Streptomyces* sp. Y-86,36923.

Response	Carbon source
Positive	D-Glucose, D-xylose, D-fructose, D-mannose, L-arabinose, D-mannitol, *m*-inositol, galactose, lactose, sucrose, maltose, cellobiose, sodium glutamate
Weak	L-Rhamnose, raffinose
Negative	Cellulose

The basal medium used was ISP 9.

Fig. 4. TLC of butalactin and cineromycin B.

TLC SiO_2: Merck Art. 5554, EtOAc. Detection: 254 nm.



Comparison with Other Related Species

On the basis of the observed characteristics, *Streptomyces* sp. Y-86,36923 belongs to the gray series. *Streptomyces* sp. Y-86,36923 differs substantially from known producers of cineromycin B and the related compound albocycline, viz. *Streptomyces cinerochromogenes*[6], *Streptomyces brunneogriseus*, *Streptomyces roseocitreus* and *Streptomyces roseochromogenes* var. *albocyclinii*[7]. Among the species described in the 8th Edition of BERGEY's manual[8], SHIRLING's ISP reports[9~12] and NONOMURA's key[13], the one most closely resembling this producing organism is *Streptomyces corchorusii* Ahmad and Bhuiyan 1958. *Streptomyces* sp. Y-86,36923 differs from *S. corchorusii*[11] only in that the former has regular shaped spores and grows poorly on L-rhamnose and raffinose as the sole carbon sources.

Fermentation

Streptomyces sp. Y-86,36923 was cultured and maintained on yeast extract-malt extract agar slants.

A loopful of mature slant culture of *Streptomyces* sp. Y-86,36923 was inoculated into Erlenmeyer flasks (500-ml) containing 100 ml of a seed medium consisting of glucose 1.5%, soybean meal 1.5%, corn steep liquor 0.5%, NaCl 0.5% and $CaCO_3$ 0.2% (pH 6.5), and incubated at 27°C on a rotary shaker with a 4-cm throw at 240 rpm for 72 hours. The resultant vegetative growth was used to inoculate the production media. Production was carried out in 250-ml Erlenmeyer flasks containing 50 ml of medium using a 4% inoculum.

Fermentation scale up was attempted using 15-liter fermenters containing 10 liters of the production medium sterilized *in situ*. The fermentation was carried out at 27°C under aeration of 6~8 liters/minute and stirrer speed of 120 rpm. An inoculum of 8~10% was used.

Detection of butalactin production in the fermentation broth was monitored by activity against *Escherichia coli* 9632, and quantified by TLC of the ethyl acetate extract of the filtered broth (Fig. 4) followed by densitometric analysis using a Shimadzu CS-930 TLC scanner set at 240 nm adsorbent

Table 4. Effect of fermentation media composition on production of butalactin and cineromycin B by *Streptomyces* sp. Y-86,36923.

	Medium A		Medium B[a]		Medium C	
Production media composition:	Dextrin	2.0%	Glucose	1.0 %	Glucose	2.0%
	Soybean meal	1.0%	Soluble starch	1.0 %	Peptone	0.5%
	Yeast extract	0.2%	Malt extract	0.75%	Beef extract	0.5%
	$FeSO_4 \cdot 7H_2O$	0.1 mg	Peptone	0.75%	$CaCO_3$	0.3%
			$MgSO_4 \cdot 7H_2O$	0.1 %		
			NaCl	0.3 %		
Yield (mg/liter) in shake flasks:	Butalactin	5	65		42	
	Cineromycin	43	36		28	
Yield (mg/liter) in 15-liter fermenter:	Butalactin	ND	5		18	
	Cineromycin	34	30		26	

[a] Trace salts solution (Cu, Fe, Mn, Zn).
ND: Not detectable.
Experimental conditions are described in the text.

wavelength.

The production of butalactin is influenced both by the composition of the fermentation medium and the type of vessel, *i.e.* shake flasks or stirred-tank fermenters. In production medium A (Table 4), in which butalactin was first detected, only low amounts of the new compound were produced in shake flask fermentations. There was no detectable butalactin produced in 15-liter fermenters even with variation in fermentation parameters such as aeration, stirrer speed, temperature and inoculum volume. A search for a suitable medium to optimize butalactin production was initiated.

Table 4 summarizes the results of the media variation studies in shake flasks and fermenters. Medium B supports the highest level of butalactin production in shake flasks, at 45~48 hours, but in the 15-liter fermenters production was low. With production medium C the highest level of antibiotic production, at 45~48 hours, in shake flasks was lower than that with production medium B. In fermenters, however, butalactin production was highest with medium C, at 23~27 hours, though the levels achieved do not reach those found with shake flask fermentation.

Volume fermentation to obtain butalactin was therefore performed using 1-liter Erlenmeyer flasks each containing 200 ml medium B. The shake flasks were incubated at 27°C on a rotary shaker with a 4-cm throw at 240 rpm for 48 hours.

Isolation and Purification

The culture filtrate (16 liters) was adjusted to pH 7 and extracted twice with 10 liters each of ethyl acetate and the aqueous layer discarded. The combined ethyl acetate extracts were concentrated *in vacuo* to give 6.4 g crude which was subjected to chromatography on silica gel (230~300 mesh, 5 × 20 cm column) using a petroleum ether (40~60°C)-ethyl acetate gradient. Eluates were analyzed by bioactivity against *Staphylococcus aureus* FDA 209P and *E. coli* 9632 and by TLC. Fractions eluted with petroleum ether-ethyl acetate (1 : 1) contained cineromycin B, active against *S. aureus* FDA 209P only, and those eluted with petroleum ether-ethyl acetate (3 : 7) contained butalactin, active against both test organisms.

The former fractions (1.7 g after concentration) were processed first on silica gel using a petroleum ether-ethyl acetate gradient, then by Sephadex LH-20 column (2.4 × 90 cm) chromatography using methanol as eluting solvent. This was followed by purification on medium pressure liquid chromato-

Table 5. Antimicrobial activity of butalactin.

Test organisms	Zone diameter[a]	
	2 mg/ml	1 mg/ml
Staphylococcus aureus FDA 209P	21	19
S. aureus 20424 Mac^R	16	14
S. epidermidis 32965	18	14
S. epidermidis 823 Teico^R	16	14
S. haemolyticus 809	16	13
Micrococcus luteus	14	12
Streptococcus faecalis 21777	17	15
S. faecalis Eder Mac^R	23	21
Escherichia coli 9632	21	16
E. coli 20665	18	17
Citrobacter freundii	17	15
Citrobacter sp. 2046 E	16	14
Proteus vulgaris	21	20
P. mirabilis	18	16
Pseudomonas aeruginosa 20653	—	—
Candida albicans	—	—
Aspergillus niger	—	—

[a] All zones of inhibition are hazy; Mac^R, macrolide-resistant; Teico^R, teicoplanin-resistant.

Table 6. Inducing activity using indicator strain *Streptomyces griseus* AF5 AM$^-$, Stm$^-$, AF$^-$.

Compound	Concentration (μg/ml)	Detection of	
		Aerial mycelium	Antibiotic production
Butalactin	0.01～400	−	−
	600～1,000	−	+[a]
A-Factor	1～10	+++	+++

[a] Diffused zones of inhibition also observed on control plate not inoculated with the indicator strain.

graphy (MPLC) using a silica gel (30 μm, 3 × 35 cm) column eluted with a 1～1.5% methanol in chloroform gradient. The pure cineromycin B (400 mg) obtained was characterized by the identity of its physical and spectroscopic properties with those of an authentic sample.

The fractions containing butalactin (3.2 g) were applied in 3 equal lots onto silica gel (30 μm) in a MPLC column (4.5 × 45 cm) and eluted with a petroleum ether - ethyl acetate gradient (5 : 5 to 3 : 7) using a medium pressure pump at a flow rate of 15 ml/minute, with detection *via* a UV detector at 240 nm. Active fractions were concentrated to yield 650 mg pure butalactin.

Biological Activity

Antibiotic Activity

When tested by the agar-well method in antibiotic assay medium butalactin displays weak antibacterial activity, with hazy zones of inhibition, against Gram-positive and Gram-negative bacteria (Table 5). In agar dilution MIC assays, no activity was detected up to 100 μg/ml.

Inducing Activity

The ability to induce aerial mycelium and antibiotic (streptomycin) production was evaluated using the indicator strain *Streptomyces griseus* AF5 AM$^-$, Stm$^-$, AF$^-$ which is a mutant similar to those described by HARA and BEPPU[14]. 20 μl of a solution of butalactin was applied onto filter paper discs at concentration of 1, 5, 10, 20, 50, 100, 200, 400, 600, 800 and 1,000 μg/ml placed onto two agar plates, one containing the surface-spread indicator strain and a control plate without the indicator strain. As positive control discs containing 20 μl of A-factor at 10 and 50 μg/ml concentrations were applied. Plates were incubated at 30°C for 7 days. Antibiotic production was tested by cutting out agar plugs 3 mm away from the filter paper discs from both plates and placing them on agar plate bulk-seeded with *S. aureus* FDA 209P.

As shown in Table 6, no aerial mycelium was observed around any of the discs containing butalactin, whereas white aerial mycelium was clearly visible around both discs containing A-factor from day 4. Clear zones of inhibition were seen around the agar plugs taken from areas adjacent to the A-factor containing discs from the plate which was surface spread with the indicator strain only. Hazy zones of inhibition were seen with the agar plugs cut from both plates adjacent to the discs containing >400 μg butalactin/ml,

indicating that this antibiotic activity results from the intrinsic activity of the compound.

Other Regulatory Activity

The effect of butalactin on antibiotic synthesis was studied using actinomycete strains from our laboratory which produce cephalosporin, thienamycin, streptomycin, viridogrisein, ostreogrycin G, rifamycin O, chalcomycin, albocycline, naphthomycin, rhodomycin, parvulin, vancomycin and ristocetin. The effect on production of cineromycin and butalactin by *Streptomyces* sp. Y-86,36923 was also studied. Butalactin was added at 0 and 16 hours to the appropriate production media in 100 ml shake flasks to give a final concentrations of 0.1, 1.0, 10.0 and 100.0 μg/ml and antibiotic production was compared against control flasks to which no butalactin was added.

Butalactin did not inhibit the growth of any of the producing organisms at 100 μg/ml. No significant increase in antibiotic production nor an early stimulation of antibiotic synthesis was seen with any concentration of butalactin. The only significant effect observed was an inhibition of vancomycin synthesis at >10 μg/ml when added at 0 hour and at 16 hours. There was no observable affect on cineromycin or butalactin production when butalactin was added to the fermentation of *Streptomyces* sp. Y-86,36923.

Discussion

Butalactin is a γ-butyrolactone containing compound which is structurally related to A-factor from *Streptomyces griseus*[15], L-factor from *S. griseus*[16], factor-I from *Streptomyces viridochromogenes*[17], anthracycline inducing factors from *Streptomyce bikiniensis* and *Streptomyces cyaneofuscatus*[18], virginiae butanolides from *Streptomyces virginiae*[19] and their synthetic analogues[20]. Although such signal molecules or autoregulators may be produced by as many as 15 to 27% of antibiotic-producing actinomycetes[14,21,22] this is the first report of a 2,3-disubstituted γ-butyrolactone possessing a weak antibacterial activity. In addition, butalactin is produced at levels 3 to 4 orders of magnitude higher than those reported for the signal molecules[16,17,19,22].

Investigations on the structure-activity relationship of the 2,3-disubstituted butanolides show that any alterations in the structure have a clear effect on the inducing activity[20]. Butalactin has additional structural features at the C-2 position, such as a tertiary hydroxyl group and a side chain containing a double bond and an epoxide group, not found with other 2,3-disubstituted butanolides, which may explain the lack of an inducing activity. Butalactin also pessesses the least desirable structural features for inducing activity which are a 2,3-*trans*-configuration of the carbon groups, a C-1′ keto group, and a C-2 side chain length of $7\sim8$ carbon atoms[20]. Lack of activity may also be due to differences in specificity of the indicator strains used.

The role of butalactin in the inhibition of antibiotic biosynthesis in one of a limited number of antibiotic-producing actinomycete strains has to be studied in greater detail.

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