GLYCINOTHRICIN, A NEW STREPTOTHRICIN-CLASS ANTIBIOTIC FROM *STREPTOMYCES GRISEUS*

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(Received for publication February 25, 1977)

Glycinothricin is a streptothricin-class antibiotic obtained for the first time from the culture broth of a strain of *Streptomyces griseus*. Glycinothricin, the deformimino derivative of antibiotic LL-AB664, gives N-methylstreptolidine, N-methyl-D-glucosamine and glycine on acid hydrolysis. In comparison with LL-AB664, glycinothricin is less active against gram-positive and gram-negative bacteria and less toxic to mice.

A new streptothricin-class antibiotic, glycinothricin, was obtained from the fermentation broth of a streptomycete, strain No. 979, isolated from a soil sample collected at Noroshi, Ishikawa Pref., Japan. The antibiotic was isolated as an amorphous hydrochloride by the conventional method for isolation of basic water-soluble antibiotics. Glycinothricin is similar in structure to LL-AB664[1,2,3], but differs from it by the absence of a formimino group. This is the first time this antibiotic is reported as a fermentation product, though it was presumed to be formed by degradation of antibiotic LL-AB664[4].

Taxonomic Studies

The cultural characteristics of strain No. 979, the producer of glycinothricin were determined by the use of the methods described by SHIRLING and GOTTLIEB[5]. Observations of the culture were made after incubation at 28°C for 2 weeks, except where othewise mentioned. The taxonomic keys of BERGEY's Manual (8th ed.), of WAKSMAN in "The Actinomycetes", vol. 2 and of others were used to compare the culture with recognized genera and species of the actinomycetes.

Strain No. 979 was classified as a member of the genus *Streptomyces*. The aerial hyphae indicated monopodial branching with sporophores of *Rectus-Flexibilis*. The spores are elliptical in shape, $0.4 \sim 0.6 \times 0.6 \sim 1.2\ \mu$ in size and with a smooth surface. The cultural characteristics are summarized in Table 1. On most media the color of the substrate mycelia was dull yellow to yellowish brown and the mass color of the aerial mycelia was brownish white to brownish gray. Physiological properties and utilization of carbon sources are summarized in Tables 2 and 3.

After comparison of these characteristics with those of known species of *Streptomyces*, *S. griseus* was selected as being most closely related. Simultaneous cultivation of strain No. 979 and *S. griseus* IAM 0084 yielded the results described in Tables $1 \sim 3$. Morphological as well as cultural and physiological characteristics of these two strains were in good agreement, although some differences were found as follows: the deep yellow color of the substrate mycelia of strain No. 979 and the contradictory results obtained with the tyrosinase reaction, as well as with the liquefaction of gelatin and the utilization of *i*-inositol, raffinose and salicin. The differences, however, were not sufficient to desig-

Table 1. Cultural characteristics of strain No. 979 in comparison with *Streptomyces griseus* IAM 0084

Medium		Strain No. 979	*S. griseus* IAM 0084
Sucrose-nitrate agar	G	Abundant	Poor
	AM	Light brownish white	Pale yellowish orange
	R	Dull yellow	Pale yellow
	SP	None	None
Glucose-asparagine agar	G	Good	Good
	AM	Brownish white	Pale yellowish orange
	R	Pale yellowish brown	Pale yellowish orange
	SP	None	None
Glycerol-asparagine agar (ISP 5)	G	Good	Good
	AM	Brownish white	Pale yellowish orange
	R	Dull yellow	Pale yellowish brown
	SP	None	None
Inorganic salts-starch agar (ISP 4)	G	Abundant	Good
	AM	Grayish white	Brownish white
	R	Pale yellowish brown	Grayish yellow brown
	SP	None	None
Tyrosine agar (ISP 7)	G	Abundant	Abundant
	AM	Pale brown	Light brownish gray
	R	Yellowish brown	Dark yellowish brown
	SP	None	Grayish yellow brown
Nutrient agar (Difco)	G	Good	Poor
	AM	Brownish white	White
	R	Pale yellowish brown	Pale yellowish brown
	SP	None	None
Yeast extract-malt extract agar (ISP 2)	G	Abundant	Abundant
	AM	Light brownish gray	Light brownish gray
	R	Dull yellowish orange	Yellowish brown
	SP	None	None
Oatmeal agar (ISP 3)	G	Abundant	Abundant
	AM	Pale yellowish orange	Pale yellowish orange
	R	Dull yellow	Pale yellowish brown
	SP	None	None

G: Growth, AM: Aerial mycelium, R: Reverse, SP: Soluble pigment.

Table 2. Physiological properties of strain No. 979 in comparison with *Streptomyces griseus* IAM 0084

	Strain No. 979	*S. griseus* IAM 0084
Tyrosinase reaction	−	+
Nitrate reduction	+	+
Starch hydrolysis	+(weak)	+(strong)
Gelatin liquefaction	+	−
Milk coagulation at 26°C	+	−
〃 at 37°C	+	+
Milk peptonization at 26°C	−	±(pH 6.2)
〃 at 37°C	+(strong, pH 7.4)	+(strong, pH 6.2)
Melanoid pigment*	−	−

* Not produced in peptone-yeast extract-iron agar (ISP 6), tyrosine agar (ISP 7), nor tryptone-yeast extract broth (ISP 1).

Table 3. Utilization of carbon sources by strain No. 979 and *Streptomyces griseus* IAM 0084

	Strain No. 979	*S. griseus* IAM 0084		Strain No. 979	*S. griseus* IAM 0084
D-Glucose	+	+	D-Galactose	++	+
L-Arabinose	−	−	Sucrose	−	−
D-Xylose	±	+	Raffinose	++	−
D-Fructose	+	+	D-Mannitol	++	+
L-Rhamnose	−	−	Salicin	+	−
i-Inositol	+	−			

++ Good growth, + Growth, ± Scant growth, − No growth.

nate strain No. 979 as a new species. Therefore strain No. 979 was named *Streptomyces griseus* No. 979.

Fermentation and Isolation

Glycinothricin was produced by *S. griseus* No. 979 in a 600-liter fermentor containing 300 liters of medium consisting of 3% starch, 2% corn-steep liquor, 1.5% Pharmamedia, 1% meat extract and 0.01% Disfoam CB-442 (pH 7.0 before sterilization). The batch was inoculated with 25 liters of seed culture fermented in a 100-liter fermentor with the identified medium for 27 hours at 27°C. Fermentation was conducted with agitation (175 rev/min) and aeration (300 liters/min) for 47 hours at 27°C. The antimicrobial activity of the culture broth was estimated by a cylinder-plate method using *Escherichia coli* NIHJ JC-2 as the test organism. The potency obtained after 27 hours of cultivation was 1,100 μg/ml. The culture broth (320 liters) was adjusted to pH 4.0 and filtered with the aid of infusorial earth to remove mycelial cake. The filtrate (270 liters) was adjusted to pH 8.5 with NaOH and adsorbed on activated carbon (4.35 kg). The carbon cake obtained by filtration was washed with 50 liters of 50% aqueous acetone (pH 8.5 with NaOH). The adsorbate was eluted twice with a total of 120 liters of 50% acidic aqueous acetone (pH 4.0 with HCl after agitation). The eluate was

Table 4. Physico-chemical properties of glycinothricin (GT) and LL-AB664 hydrochlorides

	GT hydrochloride	LL-AB664 hydrochloride[1]
m.p.	182~185°C	210°C
Formula (tentative) and elementary analysis	$C_{17}H_{29}O_8N_7 2HCl \cdot 2H_2O$ Anal. Calcd.: C 35.92, H 6.21, N 17.25, Cl 12.47 Found: C 35.93, H 6.08, N 17.22, Cl 13.76	$C_{18}H_{30}O_8N_8 2HCl \cdot H_2O$ Anal. Calcd.: C 37.44, H 5.89, N 19.41, Cl 12.30 Found: C 37.24, H 6.33, N 18.19, Cl 11.07
pKa′	7.1, 9.5	7.1, 9.2
Optical rotation	$[\alpha]_D^{22}$ −69.1 (*c* 1, H_2O)	$[\alpha]_D^{22}$ −62.2 (*c* 1, H_2O)
Ultraviolet absorption	no characteristic bands in water and acidic or alkaline water	
NMR (in D_2O) ppm from DSS[2]	2.94 (s, 3H), 3.03 (s, 3H), 5.46 (d, 1H, J=8 Hz)	2.84 (s, 3H), 3.02 (s, 3H), 5.52 (d, 1H, J=8 Hz), 7.96 (s, 1H)
Stability	stable at neutral pH, unstable at alkaline pH	
Solubility	soluble in H_2O, MeOH and slightly soluble in EtOH, pyridine, acetic acid, DMF and DMSO	

1) Sample O-837-A.

2) The NMR spectra were recorded with Nihondenshi JNM-PS-100 Spectrometer.

concentrated *in vacuo* to give 4 liters of the solution containing 83.5 g of glycinothricin in 29.0% recovery from the culture filtrate.

A 400-ml aliquot of the concentrate was adjusted to pH 7.0 with dil. NaOH and treated with 10 parts of acetone. The precipitate formed was dissolved in water, collected by centrifugation, adsorbed on a column (4×56 cm) of CM-Sephadex C-25 equilibrated with 0.1 M ammonium formate. Glycinothricin was eluted from the column with 0.4 M and 0.6 M ammonium formate, successively. Active fractions (370 ml) were pooled and concentrated to 30 ml *in vacuo*. The concentrate was passed through a column (4×5.5 cm) of activated carbon, which was washed with water and developed with 50% aqueous acetone (pH 2.0). Active fractions were concentrated and purified on a column (2.8×140 cm) of Sephadex LH-20, using water as an eluant. The eluate was monitored by ninhydrin reaction on a paper chromatogram using Toyo-Roshi No. 51 UH paper and a solvent system of BuOH-pyridine-AcOH-H_2O-*t*-BuOH (15 : 10 : 3 : 12 : 4, v/v). The eluate fractions containing glycinothricin were collected and concentrated *in vacuo*. The crude powder of the hydrochloride salt was obtained by addition of 0.3 N HCl and 10 parts of acetone to the concentrate. Further purification was performed by column chromatography on Sephadex LH-20 using water as an eluant. The purified glycinothricin was detected as a clear single spot with reagents, such as ninhydrin and RYDON-SMITH, on a paper chromatogram. It was lyophilized to give a powder in yield of 1.5 g.

To show that glycinothricin is not an artifact of isolation arising from loss of a formimino group from a fermentation product, the concentrated solution of the culture filtrate (1 liter) was directly introduced onto the column (2.8×140 cm) of Sephadex LH-20 and developed with water. Fractions having activity against *E. coli* were pooled and concentrated *in vacuo*. After repetition of this procedure three times followed by lyophilization, about 200 mg of glycinothricin free base was obtained.

Physico-chemical Properties

Glycinothricin hydrochloride is a white amorphous powder of m.p. 182~185°C. Comparison of physico-chemical properties with those of LL-AB664 (sample O-837-A) are summarized in Table 4. The IR spectrum of glycinothricin measured in KBr tablet is given in Fig. 1. The NMR spectrum of the antibiotic in D_2O is indicated in Fig. 2. The titration curve from which pKa′ values were obtained

Fig. 1. IR Spectrum of glycinothricin hydrochloride in KBr

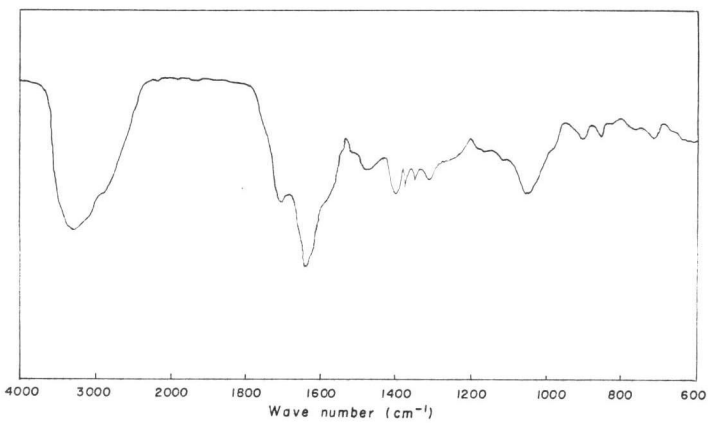


Fig. 2. NMR Spectrum of glycinothricin hydrochloride in D_2O.

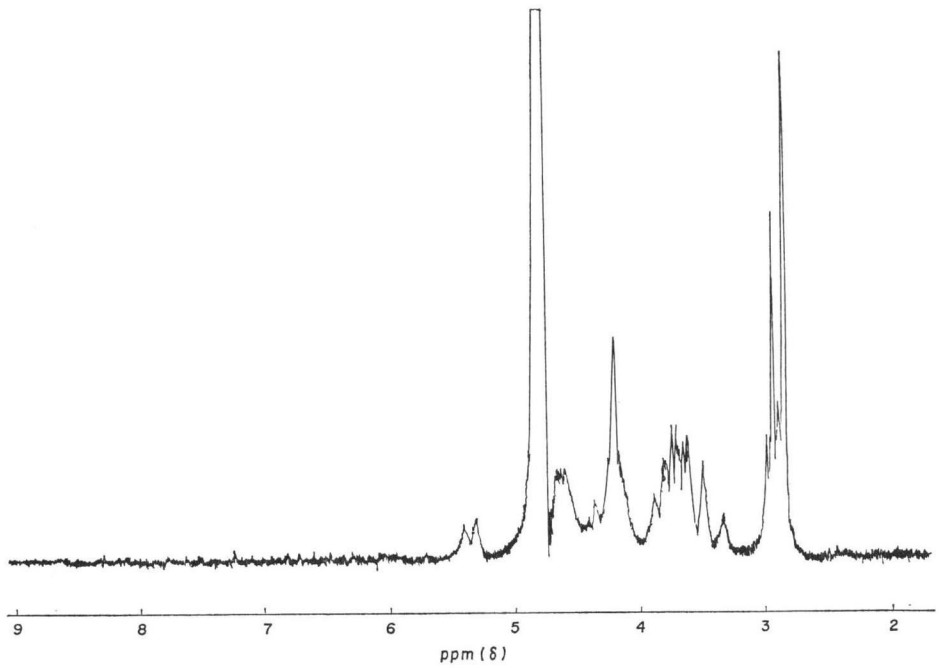


Table 5. Chromatographic and electrophoretic comparison of glycinothricin with other streptothricins

Antibiotic	PPC(Rf)[1]		TLC(Rf)[2]		PEP(cm)[3]	
	I	II	I	II	III	IV
Glycinothricin	0.53	0.49	0.34	0.28	7.0	12.0
LL-AB664	0.54	0.49	0.33	0.27	7.0	12.0
Citromycin	0.44	0.38	0.22	0.14	6.8	12.2
SF-701	0.46	0.45	0.19	0.26	6.5	10.9
Racemomycin A	0.35	0.24	0.16	0.14	8.0	13.4

1) Paper chromatography, Toyo-Roshi No. 51 UH.
2) Thin-layer chromatography, Avicel SF (Funakoshi Co.)
3) Paper electrophoresis, Toyo-Roshi No. 51, moved toward cathode.

Solvent systems:
I: BuOH - pyridine - AcOH - H_2O - *t*-BuOH (15 : 10 : 3 : 12 : 4)
II: BuOH - AcOH - H_2O (4 : 1 : 2)
III: Pyridine - AcOH - H_2O (36: 4: 964), pH 6.14, 7 V/cm, 2 hours.
IV: Pyridine - AcOH - H_2O (6 : 20 : 974), pH 3.85, 12 V/cm, 2 hours.

suggested that it is a dibasic compound. It was positive for ninhydrin (yellow to purple), PAULY (green), ELSON-MORGAN, $KMnO_4$, triphenyltetrazolium, while it was negative for SAKAGUCHI. It gave doubtful reactions to biuret, FEHLING and TOLLENS. Paper and thin-layer chromatographic and electrophoretic comparisons of glycinothricin with other streptothricin-class antibiotics are shown in Table 5. Glycinothricin was found to have properties similar to the three samples of LL-AB664 (LL-AB664, BD-12 and O-837-A). However, glycinothricin was distinguished from LL-AB664 by the NMR spectrum, in which absence of an amidino proton for glycinothricin was indicated, and by the elemental analyses (Table 4).

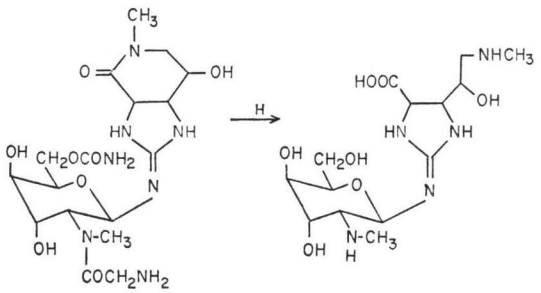
Degradation Studies

Acid hydrolysis of glycinothricin was carried out in 6 N HCl at 120°C for 20 hours in a sealed tube. On amino-acid analysis (Hitachi KLA-3 type, pH 5.28, 50°C) of the hydrolysate, glycine and

ammonia in 1 : 1 ratio and lesser amounts of methylamine were detected, but no streptolidine.

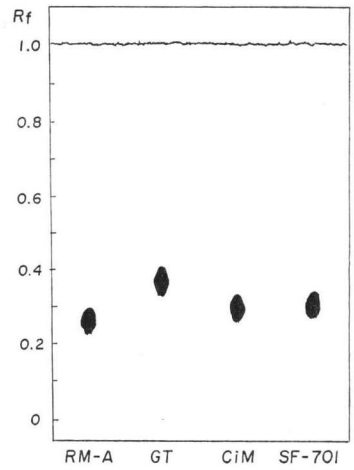
Glycinothricin base (50 mg) was dissolved in water (9 ml), a saturated aqueous solution of barium hydroxide (6 ml) was added and the resulting mixture was kept at room temperature for 2 days. The precipitate of barium carbonate obtained was 18 mg in dry weight (0.85 mol). Moreover, IR spectrum of the antibiotic showed a carbamoyl-carbonyl band at 1710 cm^{-1} as shown in Fig. 1. These results suggested the presence of a carbamoyl group in the molecule of glycinothricin.

Chart 1



I II

Fig. 3. Comparative TLC of partial hydrolysis products from racemomycin A (RM-A), glycinothricin (GT), citromycin (CiM) and SF-701
TLC (Avicel SF)
Solvent: PrOH-pyridine-AcOH-H_2O (15 : 10 : 3 : 12)



Milder hydrolysis (4 N HCl, 110°C, 24 hours) of the antibiotic (100 mg) was also carried out to establish further structural features. The hydrolysate was applied onto a column (2 × 40 cm) of CM-Sephadex C-50, equilibrated with 0.1 M pyridine-acetate buffer, pH 6.3, and eluted with 0.1 M (300 ml), 0.3 M (100 ml) and 1.0 M buffer of the same components stepwisely and fractionated into 5 ml per tube. Fractions containing the three different constituents were collected respectively. Fractions 18~28, positive for ninhydrin and with Rf 0.24 on a paper chromatogram (conditions were the same as described in Table 4) gave glycine (20 mg), fractions 80~100, positive for ELSON-MORGAN and triphenyltetrazolium and with Rf 0.54, were thought to contain an amino sugar (10 mg) and fractions 195~285, positive for ninhydrin, ELSON-MORGAN and RYDON-SMITH and with Rf 0.36, seemed to give N-*guan*-N′-methylstreptolidyl-N″-methyl-α-D-gulosaminide. Fractions 195~285 were pooled, concentrated to a small volume and introduced onto a column (2.8 × 150 cm) of Sephadex LH-20. The column was washed with water and a RYDON-SMITH positive effluent was collected and concentrated to dryness *in vacuo* in yield of about 50 mg of the powder. The compound was converted to the hydrochloride salt by addition of dil.HCl and acetone; m.p. 200°C with decomposition.

Anal. Calcd. for $C_{14}H_{27}O_7N_5 \cdot 3HCl \cdot 2H_2O$: C 32.16, H 6.56, N 13.40.
Found: C 33.66, H 6.52, N 13.76.

The NMR spectrum of this compound was similar to that of the same hydrolysis product[6] obtained from citromycin[7] (sample E-749-C[8]) and SF-701[9]. In contrast to citromycin, however, two N-methyl absorptions (δ=2.70, 2.78) were still present, while a signal at δ=4.33, 2H for a methylene proton of glycine, was absent in the case of glycinothricin. An absorption of an anomeric proton (δ=5.54, d, J=7.5 Hz) suggested that the configuration at C-1 on the amino sugar is retained as it is

in the parent antibiotic under hydrolysis and the following separation procedure. The IR spectrum of the hydrochloride salt of the compound indicated the presence of a carboxyl group (1735 cm^{-1}) but the absence of carbamoyl group. The compound, therefore, could be distinguished readily from the related compounds derived from the partial hydrolysates of racemomycin A[10], citromycin and SF-701 as shown in Fig. 3. These results supported that the structure of this hydrolysis product of glycinothricin is N-*guan*-N′-methyl-streptolidyl-N″-methyl-α-D-gulosaminide (Structure II in Chart 1).

Biological Properties

Antimicrobial activities of glycinothricin and LL-AB664 (sample O-837-A) by agar dilution method are summarized in Table 6. As evident from the results, glycinothricin possessed weaker antimicrobial activities than did LL-AB664 (sample O-837-A). Cross resistance with racemomycin A and citromycin was also observed with both antibiotics.

Table 6. Antimicrobial spectra of glycinothricin and LL-AB664 hydrochlorides

Organism	MIC (μg/ml)	
	Glycinothricin	LL-AB664
Staphylococcus aureus FDA 209P JC-1	25	12.5
Staphylococcus aureus 56 (CP,TC,PC)^r	>100	50
Staphylococcus aureus 1557 (TC,PC)^r	>100	50
Staphylococcus aureus (SM,STH)^r	>100	100
Bacillus subtilis PCI 219	25	12.5
Mycobacterium smegmatis ATCC 607	>100	50
Micrococcus luteus PCI 1001	100	25
Escherichia coli NIHJ JC-2	25	12.5
Escherichia coli K-12	50	25
Escherichia coli 97 (PC)^r	50	25
Escherichia coli (SM)^r	25	12.5
Escherichia coli (STH)^r	>100	100
Escherichia coli 8006 (Citromycin)^r	>100	100
Klebsiella pneumoniae PCI 602	>100	50
Klebsiella pneumoniae 835 (AB-PC)^r	100	50
Klebsiella pneumoniae 806 (AB-PC)^r	>100	50
Proteus vulgaris OX-19	>100	50
Pseudomonas aeruginosa SANK 73860	>100	100

1% Glycerol nutrient agar was used for assay.
STH: Streptothricin, SM: Streptomycin,
CP: Chloramphenicol, TC: Tetracycline,
PC: Penicillin, AB-PC: Ampicillin.

Glycinothricin hydrochloride was administered to mice (RFVL strain, 20 g weight, 5 animals in a group) intravenously to determine its toxicity. The LD_{50} was 100~200 mg/kg, while that of LL-AB664 (sample O-837-A) was 50~100 mg/kg, evaluated two weeks after injection. Necrotic symptoms characteristic to streptothricin-class antibiotics were observed on the tails of mice at the site of injection.

Discussion

When glycinothricin was directly compared with known streptothricin antibiotics using paper and thin-layer chromatographic procedures and paper electrophoresis, close resemblance of glycinothricin to LL-AB664 was observed (Table 5).

Glycine, instead of β-lysine, was found in the hydrolysate of glycinothricin. N-*guan*.-N′-Methyl-streptolidyl-N″-methyl-α-D-gulosaminide was obtained by mild acid-hydrolysis of glycinothricin as with LL-AB664. However, absence of the formimino group in glycinothricin based on its NMR spectrum and its elemental analysis indicated that glycinothricin is the deformimino derivative of LL-AB664 (Structure I in Chart I).

Streptomyces hygroscopicus NRRL 3111 has been reported to produce antibiotic LL-AC541 together with its deformimino derivative in its culture broth[11], but producers of LL-AB664 have not been reported to produce the deformimino derivative of that antibiotic. Ammonium formate was primarily used for the purification of glycinothricin and initially elimination of the formimino group was suspected as being related to this isolation procedure. However, partial purification of the anti-

biotic from the culture broth of *S. griseus* No. 979 directly on Sephadex LH-20 and confirmation of the absence of the peak corresponding to the formimino proton in the NMR spectrum proved the presence of native deformimino antibiotic. For purification of LL-AB664[1] and sclerothricin[12], NH_4OH was used for the elution of these antibiotics from cation-exchange resin without eliminating the formimino group. This gives further support for the presence of glycinothricin in the culture broth.

The difference in antibacterial activity between glycinothricin and its formimino derivative, LL-AB664 reveals that the presence of the formimino group may enhance antimicrobial activity. The greater toxicity of LL-AB664 could also be attributed to the presence of the formimino group.

Production of a streptothricin-class antibiotic by *S. griseus* has been reported by HALL and BENEDICT[13], but comparison of this antibiotic with glycinothricin is not possible because of lack of a detailed description of the physico-chemical properties of the former.

Acknowledgements

The authors wish to express their thanks to Dr. E. L. PATTERSON, Lederle Laboratories, a Division of American Cyanamide Co., for his supply of LL-AB664 and LL-AC541, to Dr. J. SHOJI, Shionogi Research Laboratory, Shionogi Co., Ltd., for O-837-A and E-749-C, to Dr. Y. ITO, Microbial Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd., for BD-12 and BY-81 and to Dr. T. NIIDA, Research Laboratory of Meiji Seika Co., Ltd., for SF-701.

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