GLYCOTHIOHEXIDES, NOVEL ANTIBIOTICS PRODUCED BY "*Sebekia*" sp. LL-14E605

I. TAXONOMY, FERMENTATION AND BIOLOGICAL EVALUATION

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The new glycothiohexide antibiotics, which are related to nosiheptide, were identified in fermentations of an actinomycete belonging to the genus "*Sebekia*". Strain LL-14E605 was classified as a "*Sebekia*" based on the presence of both mesodiaminopimelic acid and madurose in the cell wall and the presence of pseudosporangia encasing the spores. Culture LL-14E605 was successfully fermented in 10 to 3,000 liters of a complex medium. Antibiotic activity closely followed cell mass accumulation and usually peaked after 4 to 5 days of incubation. Glycothiohexide α demonstrated excellent *in vitro* activity against Gram-positive bacteria with MICs of 0.03 to 0.06 μg/ml against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. However, glycothiohexide α failed to protect mice against a lethal challenge with *Staphylococcus aureus* Smith unless it was administered prior to challenge.

The discovery of novel natural products from terrestrial actinomycetes has become increasingly more difficult[1]. In order to enhance the discovery of new antibiotics in our screening program, we evaluated a large variety of soil actinomycetes, including many representatives of the rarer genera. This included strain LL-14E605, which was isolated from a soil sample collected in Egypt and identified as a member of the proposed genus "*Sebekia*"[2]. Fermentations of the culture exhibited excellent antimicrobial activity against methicillin-sensitive and -resistant strains of *Staphylococcus aureus* (MSSA and MRSA, respectively) as well as vancomycin-resistant *Enterococcus faecium* (VREF). Based on these data, we selected strain LL-14E605 for further evaluation. In this paper, we report the taxonomy of the producing strain, fermentation, and biological activities of glycothiohexide α. The isolation and structure elucidation of glycothiohexide α has been presented separately[3,4].

Materials and Methods

Microorganisms

Strain LL-14E605 was obtained from M. LECHEVALIER (Rutgers University. Waksman Institute, Piscataway, NJ) as organism 40T-67 and was subsequently deposited with the Northern Regional Research Center's Culture Collection Laboratory under the accession number NRRL 21083. "*Sebekia benihana*" NRRL 1111 and *Streptomyces actuosus* NRRL 2954 used in comparative studies were obtained from the Northern Regional Research Center's Culture Collection Laboratory. *Streptomyces lividans* 1326 and *S. lividans* 1326 (pIJ702) were obtained from Dr. DAVID HOPWOOD, John Innes Institute, Norwich, England. Clinical isolates were collected from various medical centers in the United States, and quality control strains were obtained from ATCC. Identification of each strain was done by conventional methods: Gram-negative rods by API 20E (Analytab Products, Plainville, NY) and NF systems (Remel, Lenexa,

Kans.), and staphylococci by Staph Trac (Analytab Products). All isolates were stored frozen with 20% DMSO at −70°C.

Media

Assay media were prepared in distilled deionized water. Mueller-Hinton (MH) medium was purchased from Becton Dickinson Microbiology Systems, Cockeysville, MD. Nutrient agar (pH 6.8) and Luria agar were purchased from Difco Laboratories, Detroit, MI. Medium A-1 contained dextrose 1%, NZ-Amine A 0.5%, $CaCO_3$ 0.1%, dextrin 2%, and yeast extract 0.5% in tap water. Medium F-1 contained $ZnSO_4 \cdot 7H_2O$ 0.003%, $CaCO_3$ 0.05%, corn steep liquor 1%, Proflo 1%, glycerol 1%, and agar 0.04% in tap water adjusted to pH 7.0. Medium Z contained soluble corn starch 1%, K_2HPO_4 0.05%, KH_2PO_4 0.15%, $MgSO_4 \cdot 7H_2O$ 0.025%, NaCl 0.2%, liver concentrate 0.03%, yeast extract 0.03%, and NZ-Amine A 0.3% in tap water adjusted to pH 7.0. Medium 33 containing dextrose 0.5%, NZ-Amine A 0.25%, $CaCO_3$ 0.05%, dextrin 1%, yeast extract 0.25%, and agar 1% in tap water was used for zone diffusion assays with *S. lividans*. Thiostrepton was the generous gift of Mr. S. LUCANIA of E. R. Squibb and Sons, Princeton, NJ.

Physiological Characteristics

Cultural and physiological studies were carried out according to SHIRLING and GOTTLIEB[5] and GORDON *et al.*[6]. Characteristics were assessed after 14 to 28 days incubation at 28°C.

Morphological Observations

Culture morphologies were determined after 7, 14 and 28 days incubation at 28°C according to ISP standards. Scanning electron micrographs were prepared using a modified method of LOCCI[7]. An ISP 2 agar plug containing a well-sporulated area was removed and fixed in an aqueous solution of osmium tetraoxide 2%. Ethanol (50%, 75%, 95%, 100%) was used to serial dehydrate the agar before applying liquid CO_2 with an Autosamdri critical point apparatus. After mounting the sample on an aluminum platform with silver paste, drying was completed *in vacuo*. A Hummer V sputter coater was used to apply 100 Å of Gold-Palladium, and the specimen was then photographed using a Jeol, Model 6300V scanning electron microscope.

Chemotaxonomic Analysis

Cultures were grown in 50 ml of medium containing yeast extract 1% and glucose 1% in a 250-ml Erlenmeyer flask at 28°C, 200 rpm. After 72 hours, the mycelium was harvested by centrifugation, thoroughly washed with sterile distilled water, and lyophilized to dryness. Whole-cell sugars were determined using standard gas chromatography and mass spectroscopy (GC/MS) methods[8]. To determine the isomers of diaminopimeric acid (DAP) present in the cell wall, *N*-heptafluorobutyryl *n*-butyl ester derivatives were prepared before analysis by GC/MS using a Chirasil-Val column[9]. Monosaccharide standards and 2,6-diaminopimelic acid (racemic) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Heptafluorobutyric anhydride (HFBA) was obtained from Pierce Chemical Co., Rockford, IL, U.S.A. Phospholipids were extracted and analyzed using two-dimensional thin-layer chromatography[10]. The preparation and analysis of whole-cell fatty acid methyl esters was carried out using the Microbial Identification System[11].

In Vitro Susceptibility Testing

Antibacterial activity was determined by measuring the diameter of the growth inhibition zone in agar diffusion. Agar medium (125 ml) inoculated with fresh overnight broth cultures (1%) was poured into 9 × 9 cm Sumilon assay plates (MS 12450) and, after solidification, agar plugs (5.5 mm) were removed to produce wells for sample addition. Aliquots of 25 μl were added to the wells and the assay plates were incubated at an appropriate temperature for each assay organism for 24～48 hours.

Streptomyces lividans 1326 and *S. lividans* 1326 (pIJ702) were used for experiments on resistance to glycothiohexides. Spore suspensions were inoculated into Medium A-1 (50 ml/250-ml flask) and cultures were grown at 28°C, 200 rpm, for 3 days. Growth medium and assay agar for *S. lividans* 1326 (pIJ702) was supplemented with thiostrepton at 5 and 50 μg/ml, respectively, to ensure maintenance of the plasmid. The mycelium was sedimented by centrifugation, washed in saline (0.85%), and adjusted to 0.1 g/ml wet

weight. After sonication, 0.1 ml of the mycelial suspension was inoculated into 40 ml of Medium 33 in a sterile 50-ml conical centrifuge tube. The contents were gently mixed by inverting the tube several times and were then poured onto a solid agar base (125 ml Medium 33) in a large (9 × 9) bioassay plate. Samples (10 μl) were spotted on the surface of the plates, after which they were incubated at 37°C for 48 hours.

The minimum inhibitory concentrations (MIC) of antimicrobial agents were determined on agar as recommended by the National Committee for Clinical Laboratory Standards.[12] Mueller-Hinton II agar was used for nonfastidious aerobic bacteria and the medium was supplemented with 5% sheep blood for *Streptococcus* spp. or for the determination of the effect of blood on antibacterial activity. Inocula were adjusted to a density of 10^7 CFU/ml and were then applied to the surface of the agar with a Steers replicator. The test plates were incubated at 35°C for 18 hours.

In Vivo Activity

Healthy female mice (strain CD-1, from Charles River Laboratories, NY) weighing 20±2 g each wereused for *in vivo* studies. Antibiotic in phosphate-buffered saline (pH 7.4, 0.01 M) was administered subcutaneously (0.5 ml), and the maximum tolerated dose was determined after 24 hours as the highest dose (mg/kg body weight) at which none of the animals died. An acute lethal infection of *Staphylococcus aureus* Smith was introduced by intraperitoneal injection of 0.5 ml of the bacterial suspension in broth containing 5% hog gastric mucin (10 to 100 LD_{50}). The antibiotic median effective dose (ED_{50}) was estimated from survival ratios after 48 hours of infection.

Results

Taxonomic Studies

The characteristics of strain LL-14E605 are summarized in Tables 1 and 2. White to light gray aerial mycelium was formed on yeast extract - malt extract agar, while aerial mycelium on inorganic salts - starch agar was pale yellow green. The color of the substrate mycelium ranged from dark olive brown on yeast - malt extract to pale yellow green on inorganic salts - starch medium. A light brown soluble pigment was produced only on complex media.

Physiological and biochemical characteristics of strain LL-14E605 are described in Table 3. The culture utilized glucose, fructose, and rhamnose, but not arabinose, sucrose, xylose, inositol, mannitol, or cellulose. In addition, culture LL-14E605 degraded casein, hypoxanthine, esculin, and tyrosine, but not xanthine or adenine.

Microscopic examination of the organism revealed nonfragmented, branching substrate mycelium and very sparse aerial hyphae. The aerial hyphae were transformed into chains consisting of 10 or less spores and were classified as *Rectiflexibiles*. Scanning electron microscopy revealed smooth, elongated spores

Table 1. Characterization of "*Sebekia*" spp.

Characteristic	LL-14E605	NRRL 1111
Aerial mycelium	*Rectusflexibiles*	*Rectiflexibiles*
Fragmentation of substrate mycelium	None	None
Zoospores and sporangia	None	None
Spore chain	≤10, Pseudosporangia	≤10, Pseudosporangia
Spore shape	Elongated	Elongated
Spore surface	Smooth	Smooth with ridges
Growth temperature	<45°C	<45°C
Salt tolerance	<5%	<5%
meso: LL-DAP	3:1	5:1
Whole-cell sugars	Ribose, mannose, madurose, galactose	Mannose, madurose

Table 2. Cultural characteristics of "*Sebekia*" spp.[a]

Medium		LL-14E605	NRRL 1111
Yeast-malt (ISP 2)	G:	Abundant	Abundant
	AM:	White to light gray (263, 264)	Pink gray to light gray (10, 264)
	SM:	Dark olive brown (96)	Dark red brown (44)
	SP:	Light brown	Brown
Oatmeal (ISP 3)	G:	Abundant	Abundant
	AM:	None	Bright pink (33)
	SM:	Gray yellow to dark gray yellow (90, 91)	Light yellow-brown to slight red brown (76, 40)
	SP:	Light brown	Light brown
Inorganic salts-starch (ISP 4)	G:	Abundant	Abundant
	AM:	Pale yellow green (121)	Pale yellow (89)
	SM:	Pale green yellow (104)	Yellow white to pale yellow (92, 89)
	SP:	None	None
Glycerol-asparagine (ISP 5)	G:	Sparce	Sparce
	AM:	White (263)	Pale yellow pink to yellow white (31, 92)
	SM:	Colorless	Colorless
	SP:	None	None

[a] G, Growth; AM, aerial mycelium; SM, substrate mycelium; SP, soluble pigment. ISCC, National Bureau of Standard Centroid Color Charts, publication 440, Washington, D.C., 1976.

Table 3. Physiological reactions of "*Sebekia*" spp.[a]

Test	Compound	LL-14E605	NRRL 1111	Test	Compound	LL-14E605	NRRL 1111
Carbon utilization:	D-Glucose	±	+		Hypoxanthine	+	±
	L-Arabinose	−	+		Tyrosine	±	+
	Sucrose	−	−		Adenine	−	+
	D-Xylose	−	+		Esculin	+	+
	Myo-inositol	−	+	Production of:	Urease	−	−
	D-Mannitol	−	−		Melanin	−	−
	β-D-Fructose	±	+	Acid production from:	Arabinose	+	+
	α-L-Rhamnose	±	+		Dulcitol	−	−
	Raffinose	+	−		Erythritol	−	−
	Cellulose	−	−		Glucose	+	+
Decarboxylation of:	Acetate	+	+		Myo-Inositol	−	+
	Benzoate	−	−		Lactose	+	−
	Citrate	−	−		Mannitol	+	−
	Lactate	+	+		Mannose	+	+
	Malate	±	+		Methyl-α-D-glucoside	+	−
	Mucate	±	−				
	Oxalate	−	−		Melibiose	−	+
	Propionate	−	−		Raffinose	+	−
	Pyruvate	+	+		α-L-Rhamnose	+	+
	Succinate	+	+		Sorbitol	+	−
	Tartrate	−	−		Trehalose	+	+
Hydrolysis of:	Casein	+	+				
	Xanthine	−	−				

[a] +, Positive; −, negative; ±, weak.

encased in pseudosporangia (Fig. 1). Zoospores and sclerotia were not observed.

Whole-cell hydrolysates of strain LL-14E605 contained a mixture of the *meso*- and LL-isomer of diaminopimelic acid and the sugars ribose, mannose, madurose, and galactose. Phospholipid analysis revealed the presence of diphosphatidylglycerol, hydroxylated phosphatidylethanolamine, and a

glucosamine-containing phospholipid (type IV). The fatty acid pattern consisted mainly of saturated 10-methyl fatty acids.

When compared with the type strain of "*Sebekia benihana*" NRRL 1111, strain LL-14E605 differed in many cultural and physiological characteristics (Tables 1, 2, and 3). Strain LL-14E605 lacked the ridged spores and dark red-brown substrate mycelium observed for "*Sebekia benihana*" and utilized glucose, fructose, raffinose, and rhamnose, but not arabinose, sucrose, xylose, inositol, mannitol, or cellulose. In contrast, "*S. benihana*" did not utilize raffinose but did utilize arabinose, xylose, and inositol as sole carbon sources. Additional significant differences were detected in the test for acid production from inositol, lactose, mannitol, mannose, raffinose, and sorbitol. These data suggested that culture LL-14E605 represented a new species of the genus "*Sebekia*".

Production of Glycothiohexides

Of the media tested, optimal production of glycothiohexides occurred in Medium A-1 (Table 4).

Fig. 1. Scanning electron micrographs of strain LL-14E605 grown on yeast extract-malt extract agar.

(A) Pseudosporangia, 6,000×, (B) spores, 10,000×.

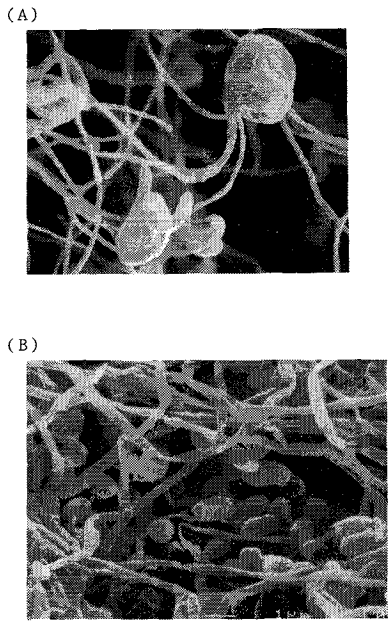
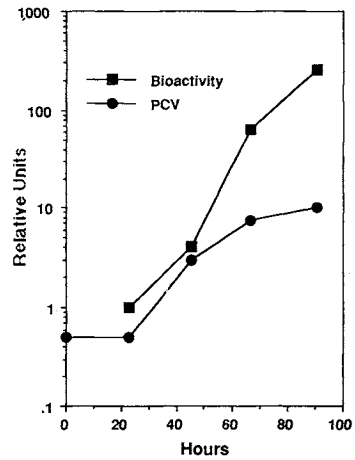


Fig. 2. Production of glycothiohexides.



Three seed stages in Medium A-1 were used to scale up inoculum for a 3,000 liters fermentation. The fermentation in Medium A-1 supplemented with antifoam agent (HODAG FD82 0.3%) was carried out at 2,000 liters/minute, 110 rpm, 28°C. PCV: packed cell volume for a 10 ml sample. Bioactivity: determined as the highest dilution of whole broth which gave a zone of growth inhibition against *S. aureus* MSSA.

Table 4. Fermentation media *vs* antibacterial activity of LL-14E605[a].

Antibiotic	Medium	Zone diameter (mm)[b]		
		S. aureus MSSA	*S. aureus* MRSA	*E. faecium* VREF
Glycothiohexides	Z	9	8	10
Glycothiohexides	A-1	11	10	11
Glycothiohexides	F-1	6	0	8

[a] Cultures were grown in A-1 medium for 3 days at 28°C, 200 rpm and then inoculated into fermentation media. After 5 days at 28°C, 200 rpm, 25 μl of whole broth were assayed.

[b] Nutrient agar 6.8 was used for *S. aureus* assay plates; LB agar was used for *E. faecium*. All assay plates were incubated at 37°C for 18 hours.

Medium F-1 produced the least amount of glycothiohexides as demonstrated by the small zones of inhibition against MSSA and VREF. The lack of activity against MRSA in these fermentation samples is consistent with the slightly reduced activity of the glycothiohexides against this strain under these assay conditions. When culture LL-14E605 was fermented in 3,000 liters of A-1, bioactivity continued to increase after growth had ceased and peaked around 91 hours (Fig. 2).

Biological Activity

Antibacterial activity of glycothiohexide α, a component of the glucothihexides, was absent against *S. lividans* carrying plasmid pIJ702 which confers resistance to a related compound thiostrepton (Table 5). Although glycothiohexide α was inactive against *Escherichia coli*, it demonstrated excellent *in vitro* activity against Gram-positive bacteria including antibiotic-resistant strains (Table 6). Despite these data, glycothiohexide α failed to protect mice after a lethal challenge of *S. aureus* (Table 7). When the antibiotic was administered prior to challenge, marginal protection was observed.

Table 5. Activity of glycothiohexide α against *Streptomyces lividans*[a].

Compound	Concentration (μg/ml)	Zone diameter (mm)	
		thio^S	thio^R
Glycothiohexide α	100.00	19	0
	50.00	19	0
	25.00	17	0
	12.50	17	0
	6.25	16	0
	3.12	16 hazy	0
	1.56	0	0
Thiostrepton	100.00	17	0
	50.00	13	0
	25.00	13	0
	12.50	11	0
	6.25	9	0
	3.12	8	0
	1.56	0	0
Bacitracin	10 units/disk	20	26
Imipenem	10 μg/disk	21	30

[a] Antibacterial activity was determined by agar diffusion.

Discussion

Strain LL-14E605 was compared to the type strain of "*Sebekia benihana*" based on similarities in whole-cell hydrolysates which included the presence of both LL- and *meso*-isomers of DAP, and the sugars madurose and mannose. In addition, 10-methyl 17:0 (30%) and iso 16:0 (15%) predominated in the fatty acid profile of each strain (unpublished results). However, sufficient differences were observed between these two strains to propose a new species for strain LL-14E605, designated "*Sebekia aegyptiaca*". Recently, KROPPENSTEDT emended the genus *Microtetraspora* and it now incorporates many of the taxonomic characteristics of the genus "*Sebekia*"[14]. Further taxonomic evaluation, such as sequencing of the 16s ribosomal RNA, would be required to determine if the genus "*Sebekia*" should be assigned to the genus *Microtetraspora*.

A comparison of glycothiohexide α to other thio-containing peptides revealed similar antibacterial properties. Since thiostrepton-resistant

Table 6. Antibacterial activity of glycothiohexide α *in vitro*.

Organism	Strain	MIC (μg/ml)		
		Piperacillin	Vancomycin	Glycothiohexide α
S. aureus	MSSA	0.025	0.25	≤0.06
	MRSA	128	0.25	≤0.06
Enterococcus spp.	VSEF	4	0.25	≤0.06
	VREF	>128	>128	≤0.06
E. coli	ATCC #25922	1	>128	>128

Table 7. Activity of glycothiohexide α against *S. aureus* Smith in mice.

Dose schedule (hours)[a]		ED_{50} (mg/kg)
Pre-challenge	Post-challenge	
-4, -3, -2, -1	0.5	4～8
-3, -2, -1	0.5	8～16
-2, -1	0.5	>16
None	0.5	>16
None	0.5, 3	>16

[a] Subcutaneous administration; maximum tolerated dose >16 mg/kg.

cultures were also cross-resistant to glycothiohexide α, the latter may also act to inhibit protein synthesis. Although glycothiohexide α did not exhibit any serum binding (data not shown), the compound was inactive *in vivo*. The latter may reflect poor distribution of the compound due to its limited solubility in phosphate-buffered saline, and the administration of the compound as a suspension. Pre-dosing the animals provided some protection from a lethal infection with *S. aureus*. Indeed, the more time that elapsed between the pre-dose and the subsequent challenge, the lower the ED_{50}. These data are consistent with observations that pre-diffusion of the drug on agar plates for 4～24 hours increased the size of the zone of inhibition (data not shown). The longer the period of pre-diffusion, the larger the zone size. After 24 hours, the diameter of the zones increased by as much as 50%. Thiopeptides have not been widely applied in clinical situations because of their poor solubility properties. The lack of solubility of the glycothiohexides may also contributes to its poor *in vivo* performance. However, there has been a rapid development of antibiotic resistance among clinical isolates[13]. Given the excellent *in vitro* activity of glycothiohexide α against these organisms, further studies to improve bioavailability are warranted.

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