GLYCOTHIOHEXIDE α, A NOVEL ANTIBIOTIC PRODUCED BY "*Sebekia*" sp., LL-14E605

II. ISOLATION AND PHYSICAL-CHEMICAL CHARACTERIZATION

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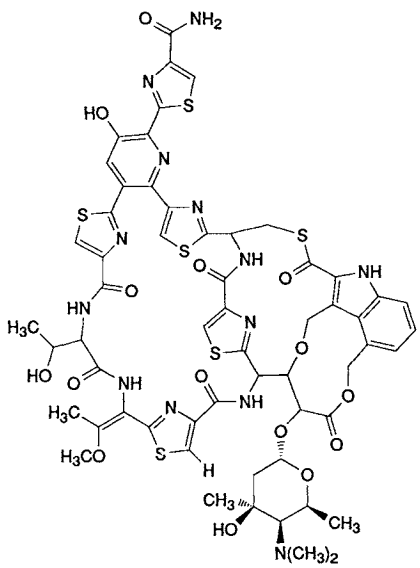
Glycothiohexide α was recovered from the fermentation broth of a "*Sebekia*" sp. by mixed solvent extraction, selective precipitation and adsorption chromatography on Diaion HP-20. The amount of glycothiohexide α present in the crude preparation was enriched by photolysis. Purification of glycothiohexide α was accomplished by repetitive countercurrent chromatography.

During the course of screening for antibiotics active against clinically relevant bacteria resistant to vancomycin and methicillin, a new antibiotic, glycothiohexide α (**1**), was isolated from the fermentation of a "*Sebekia*" sp. (NRRL 21083). The isolation and physical-chemical characterization of glycothiohexide α are described in this report; details of the structural elucidation are presented in the following paper.[1] The taxonomy of the producing organism, the production and the antimicrobial activity of glycothiohexide α will be reported separately.[2]

Fig. 1. Structure of glycothiohexide α (**1**).



Isolation

A number of different processing schemes were used to isolate glycothiohexide α from the fermentation broth. Initially the whole fermentation mash was filtered with Celite and the antibiotic in the filtrate was recovered *via* Diaion HP-20 while the antibiotic in the mycelial cake was extracted into acetone-water (90:10). In general, approximately 20% of the antibiotic activity present in the whole mash was recovered using this procedure. As the laboratory purification progressed, it became apparent that the antibiotic was insoluble in any single organic solvent but quite soluble in mixtures of dichloromethane and methanol. Subsequently, the initial recovery of glycothiohexide α from the fermentation broth was accomplished using a tangential flow process system equipped with porous

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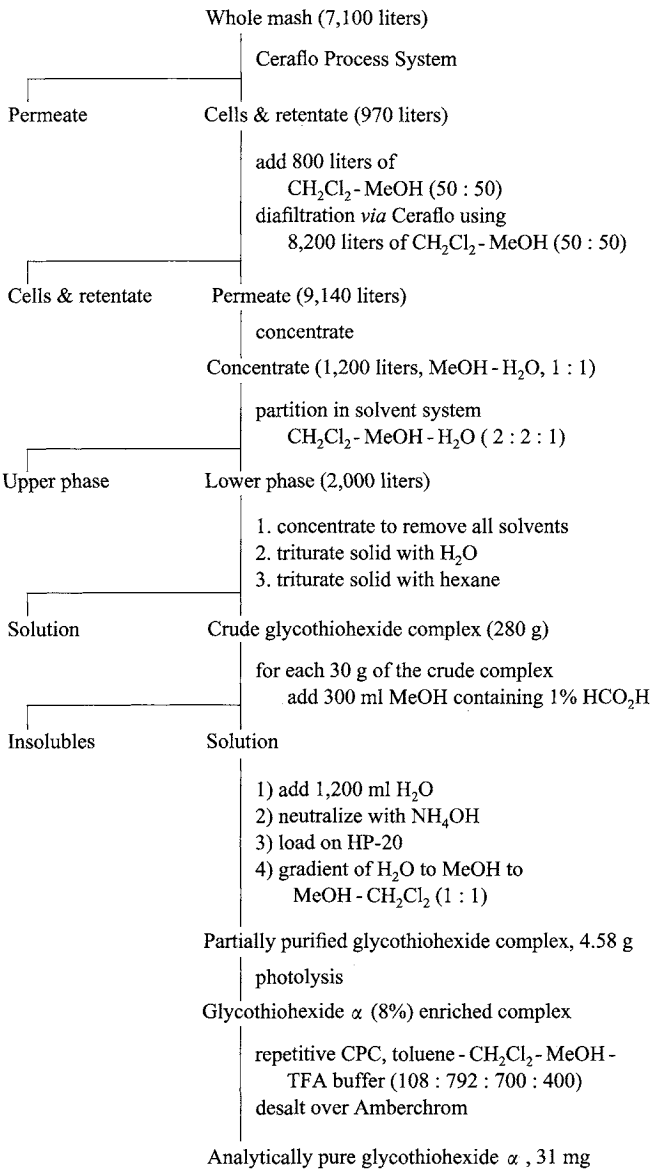
Fig. 2. Process for the isolation of glycothiohexide α.



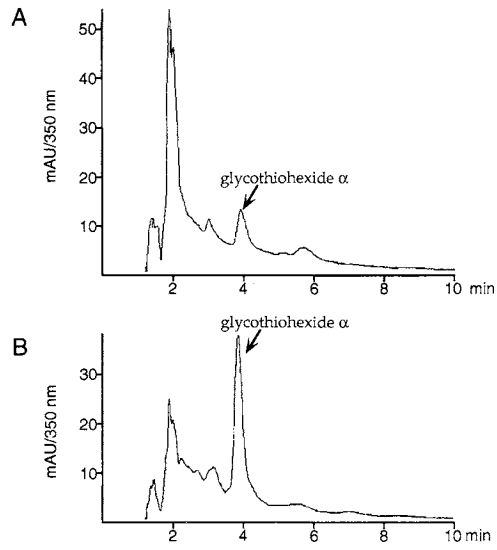
ceramic filter elements, the Ceraflo Process System. The Ceraflo performed a cleaner particle filtration of the fermentation whole mash than the Celite process and very little of the glycothiohexide complex was found in the Ceraflo permeate (filtrate). Glycothiohexide α present in the Ceraflo retentate (mycelial mass) was recovered conveniently by diafiltration using CH_2Cl_2-MeOH (1 : 1). Approximately 60% of the antibiotic activity in the fermentation mash could be recovered by this process. Crude antibiotic extracts were further purified by partition between the two phases of the solvent system CH_2Cl_2-MeOH-H_2O (2 : 2 : 1), selective trituration, and adsorption chromatography on Diaion HP-20 as shown in Fig. 2.

During further purification of the glycothiohexide complex, it was discovered that the most active component of the complex, glycothiohexide α, was the photolysis product of a less active and less stable

component in the complex. The HPLC trace of the glycothiohexide complex before and after photolysis is shown in Fig. 3. The photolysis occurs under very mild conditions and could take place in ambient light. Glycothiohexide α is by no means photochemically stable and is converted to a number of less active components if allowed to remain under the reaction conditions for long periods of time.

Due to the poor solubility of glycothiohexide α, reversed phase column chromatography which provided excellent analytical separations was not very useful for the preparative scale isolation of glycothiohexide α. Repetitive countercurrent chromatography using a Centrifugal Partition Chromatograph (CPC) was the most reproducible method for isolating the glycothiohexide α present in the antibiotic complex after photolysis. In some of the experiments, fractions containing the peak of glycothiohexide α became a gel after refrigeration overnight.

Fig. 3. HPLC trace of the glycothiohexide complex, A before, and B after photolysis.



Column: PLRP-S, 5 μm, 100 Å, 4.6×150 mm. Solvent: CH_3CN-0.1 M TFA (adjusted to pH 2.0 with NH_4OH), 45:55, 1 ml/minute. Photolysis conditions: 1 mg/ml of glycothiohexide complex in MeOH-CH_2Cl_2 (8:2) with trace NH_4OH, 350 nm, 5 minutes.

Physical-chemical Properties of Glycothiohexide α

The UV, IR, CD, ^1H NMR and ^{13}C NMR spectra of glycothiohexide α are shown in Figs. 4~8 while other physico-chemical properties are summarized in Table 1. The strong amide absorption (1668 cm^{-1}) and weak ester absorption (1743 cm^{-1}) in the IR spectrum suggested that it is a depsipeptide. ESCA confirmed the presence of nitrogen and revealed the presence of sulfur in the molecule, further suggesting that it is a sulfur containing peptide antibiotic. Its UV spectrum, including acid and base shifts, is identical to that of nosiheptide,[3]

Fig. 4. UV spectra of glycothiohexide α (3.3 μg/ml solutions in MeOH).

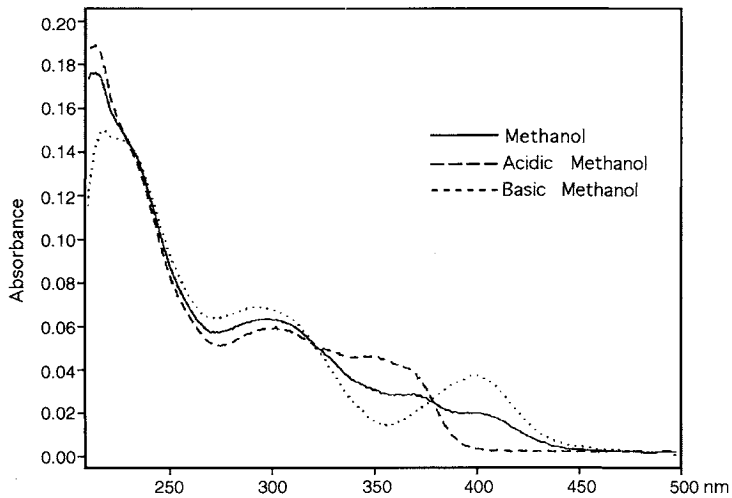


Fig. 5. IR spectrum of glycothiohexide α (KBr disc).

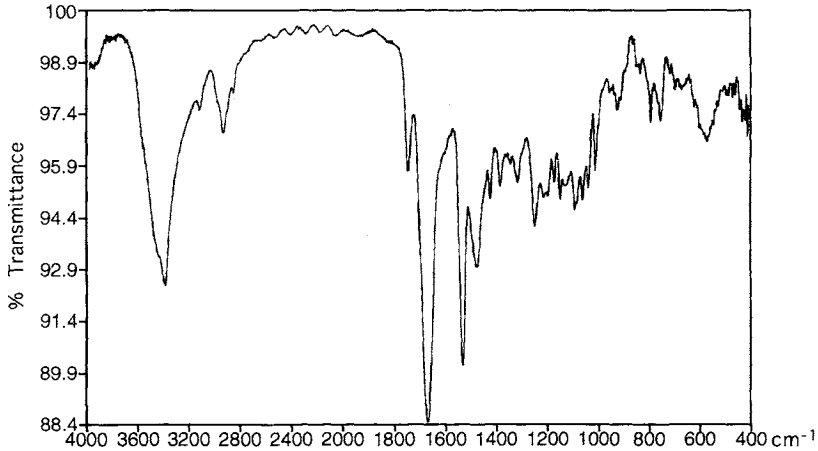


Fig. 6. CD spectrum of glycothiohexide α (10 μg/ml solution in MeOH, 20 mm cell).

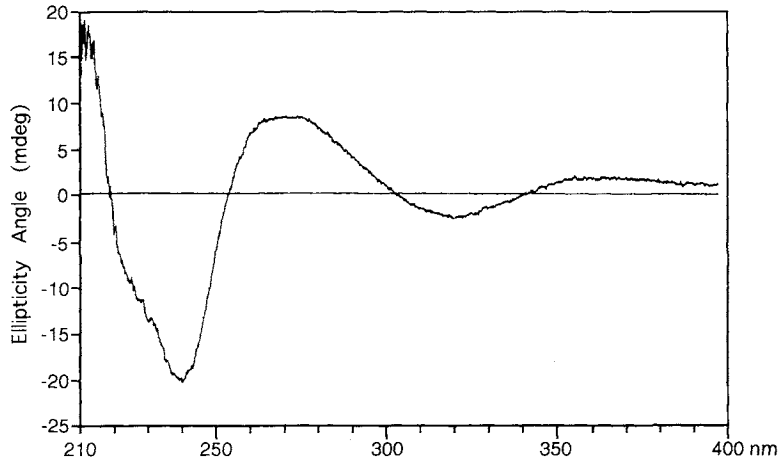


Fig. 7. ^{1}H NMR spectrum of glycothiohexide α in d_6-DMSO, 50°C (500 MHz).

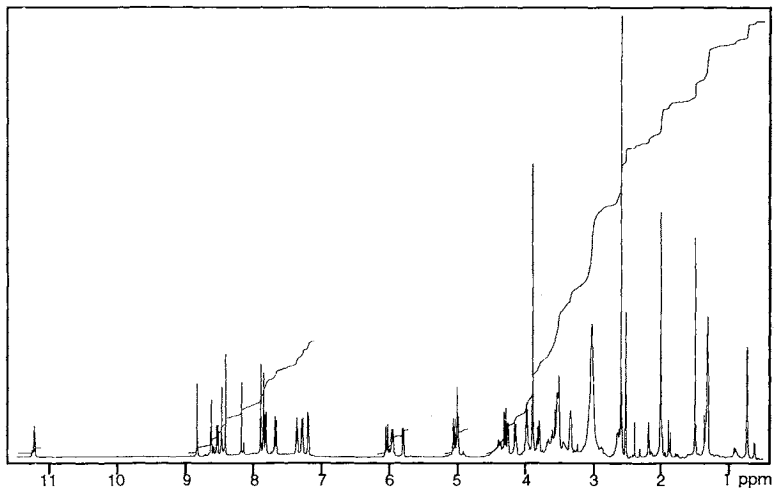


Fig. 8. ^{13}C NMR spectrum of glycothiohexide α in d_6-DMSO, 50°C (500 MHz).

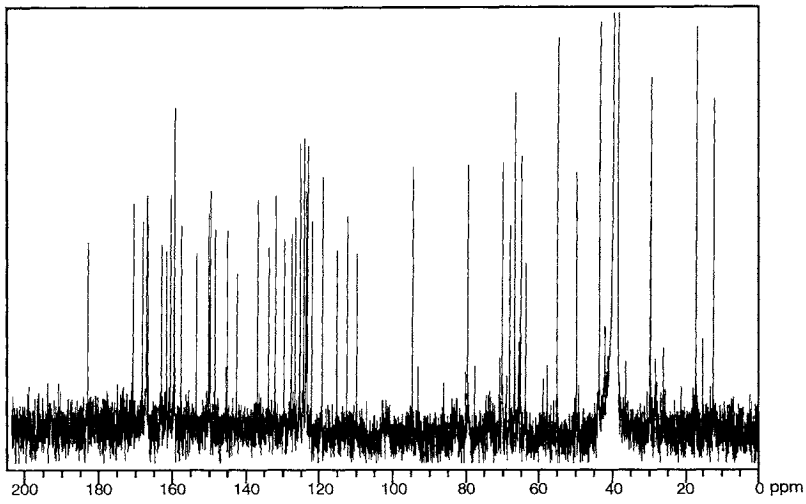


Table 1. Selected physico-chemical properties of glycothiohexide α.

Molecular weight	1,367
Electrospray MS *m/e*	1,367, (M+H), (±1 amu)
Molecular Formula	$C_{58}H_{57}N_{13}O_{15}S_6$
HRFAB-MS *m/e*	1,390.2307 ($C_{58}H_{57}N_{13}O_{15}S_6Na$, Δ=0.79 mmu)
UV λ_{max} (MeOH) nm (ε)	302 (27,000), 370 (11,900), 400 (8,400)
(5 mM HCl in MeOH) nm (ε)	302 (24,900), 352 (19,200), 370 (16,000)
(5 mM NaOH in MeOH) nm (ε)	295 (28,800), 400 (15,300)
CD λ_{max} (MeOH) nm (θ)	240 (−138,500), 271 (+57,300), 319 (−19,000)
IR (KBr) cm^{-1}	3391, 2927, 1743, 1668, 1532, 1478

a sample of which was isolated previously in our laboratory. Its structural similarity to nosiheptide was confirmed when glycothiohexide α was found to be cross resistant with both nosiheptide and thiostrepton.[2] The molecular weight of glycothiohexide α clearly distinguishes it from nosiheptide, and other structurally related compounds such as thiocillins,[4] micrococcins,[5,6] S-54832,[7] GE2770 A,[8,9] A10255,[10] berninamycins,[11] sulfomycins,[11] thioxamycin,[12] thiostreptons,[13] siomycins,[14] and thiopeptins.[15,16] Details of the structural assignment of glycothiohexide α is documented in the following paper.[1]

Experimental

General

NMR spectra of glycothiohexide α were obtained on a Bruker AMX 300, while a GE Omega 500 MHz NMR was used to obtain the spectra of *O*-methyl-glycothiohexide α. Chemical shifts were determined in parts per million relative to the solvent signals of d_6-DMSO at δ 2.49 (^1H) and δ 39.5 ppm (^{13}C). UV spectra were recorded using a Hewlett-Packard Model 8450A spectrophotometer. CD spectra were recorded using a Jasco J-600 spectropolarimeter. IR spectra were obtained from a KBr pellet with a Nicolet 20AXB FT-IR spectrophotometer. FAB mass spectra were recorded using a VG-ZAB SE high performance mass spectrometer and a VG 11-250 data system.

Isolation of the Glycothiohexide Complex

The harvested fermentation mash (7,100 liters) of NRRL 21083 was stirred with 70 liters of toluene. The mixture was filtered using a Ceraflo Process System (Millipore). The retentate (970 liters) was diluted

with 800 liters of CH_2Cl_2 - MeOH (1 : 1) and the mixture was diafiltered using the Ceraflo with additional 8,200 liters of CH_2Cl_2 - MeOH (1 : 1). The diafiltrate (9,140 liters) was concentrated to 1,200 liters (MeOH - H_2O, 1 : 1) and mixed with 600 liters of MeOH and 1,200 liters of CH_2Cl_2. The lower phase of the mixture was separated and the upper phase after being mixed with 1,240 liters of CH_2Cl_2 and 160 liters of MeOH was separated into two phases. The lower phases were combined and concentrated to give 10 liters of brown lard-like material. This material was triturated with 10 liters of water and the solids, collected by centrifugation, were triturated with 10 liters of hexane. The insolubles were collected by centrifugation, resuspended in water and freeze-dried to afford 468 g of solids which was further purified by triturating with 55 liters of hexane to afford 280 g of crude glycothiohexide complex.

Purification of the Glycothiohexide Complex by Chromatography on Diaion HP-20

Crude glycothiohexide complex (30 g) was stirred in 300 ml of MeOH containing 1% formic acid until the insolubles became a fine suspension. The insolubles were separated by centrifugation and the solution was diluted with 1,200 ml of water and neutralized with NH_4OH. The cloudy mixture was loaded (10 ml/minute) onto a preconditioned Diaion HP-20 column (5 cm × 45 cm, 900 ml) equilibrated in water. The column was then washed with 2,700 ml of water and eluted (10 ml/minutes) sequentially with: 1) a linear gradient of water to MeOH over 900 ml, 2) 900 ml of MeOH, 3) a linear gradient of MeOH to CH_2Cl_2 - MeOH (1 : 1) over 900 ml, and 4) 3,600 ml of CH_2Cl_2 - MeOH (1 : 1). The fractions collected at regular intervals were analyzed for antimicrobial activity againt *Staphylococcus aureus*. Most of the bioactivity was in the CH_2Cl_2 - MeOH (1 : 1) eluate and was pooled, concentrated and freeze-dried to yield 4.58 g of partially purified glycothiohexide complex.

Enrichment of the Glycothiohexide α in the Partially Purified Glycothiohexide Complex by Photolysis

A solution of partially purified glycothiohexide complex (2.0 g) in 2 liters of MeOH (containing 0.06% NH_4OH) - CH_2Cl_2 (8 : 2), in a 3.5-liter Pyrex beaker equipped with a magnetic stir was photolyzed in a Rayonet Photochemical Reactor (Southern N. E. Ultraviolet Co.) using ten 350 nm 12″ fluorescent tubes. The reaction mixture was concentrated after 5 minutes to afford 846 mg of crude glycothiohexide α (~8.3% pure).

Purification of Glycothiohexide α by Countercurrent Chromatography

Final purification of glycothiohexide α was accomplished by countercurrent chromatography using a Centrifugal Partition Chromatograph (CPC, Model LLN, Sanki Laboratories, Inc.) with the following modifications: 1) the solvent delivery pump was replaced with a Waters Model 590 Programmable Solvent Delivery Module, 2) the two rotary valves were replaced with Rheodyne low pressure Teflon valves, Model 5042 (1.5 mm bore, 4-way rotary), 3) a third Rheodyne Model 5042 valve was added to enable the flow through the sample loop to be reversed, 4) the sample loops were replaced with ACE glass chromatography columns, and 5) all of the Teflon tube end-fittings were replaced with flanged low pressure fittings with 1/4~28 threads except the direct connection to the centrifugal partition unit. The separations were carried out in the ascending mode using the following solvent system: toluene - dichloromethane - methanol - buffer (0.1 M trifluoroacetic acid, adjusted to pH 2.0 with concentrated NH_4OH), 108 : 792 : 700 : 400.

A sample (1.2 g) of crude glycothiohexide α was dissolved in 15 ml each of the upper and lower phases of the solvent system and loaded onto the CPC instrument which was equipped with 6 Type 1000W cartridges (425 ml total volume), filled with the stationary (lower) phase and equilibrated with the mobile (upper) phase of the solvent system at 23°C, 4 ml/minutes, 700 rpm. The separation was carried out under the same conditions and fractions were collected every 4 minutes. Fractions 30~40 containing the bulk of glycothiohexide α based on HPLC analysis were combined, neutralized with aqueous NH_4OH, concentrated to dryness, and desalted by triturating the solids with 20 ml of water to give 75.6 mg of partially purified glycothiohexide α (34% pure).

A sample (200 mg) of partially purified glycothiohexide α was dissolved in 3 ml each of the upper and lower phases of the solvent system and loaded onto the CPC instrument which was equipped with 6 Type 250W cartridges (125 ml total volume), filled with the stationary (lower) phase and equilibrated with the mobile (upper) phase of the solvent system at 23°C, 1 ml/minute, 400 rpm. The separation was carried out under the same conditions and fractions were collected every 4 minutes. Fractions 25~38 were combined,

neutralized with aqueous NH_4OH, concentrated to remove all organic solvents, redissolved in 50 ml of water and loaded onto a 60 ml Amberchrom column. The column was eluted sequentially with 600 ml of water, 110 ml of methanol and 400 ml of methanol-dichloromethane-toluene (1:1:1). Fractions (10 ml each) were collected starting with the last 100 ml of water elution. Fractions 23~33 (methanol-dichloromethane-toluene) were combined and concentrated to dryness to yield 21.8 mg of analytically pure glycothiohexide α. Side fractions containing less pure glycothiohexide α were combined with similar samples and rechromatographed through the CPC.

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