Thiocoraline, a New Depsipeptide with Antitumor Activity Produced by a Marine *Micromonospora*

I. Taxonomy, Fermentation, Isolation, and Biological Activities

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(Received for publication March 31, 1997)

A novel bioactive depsipeptide, thiocoraline, was isolated from the mycelial cake of a marine actinomycete strain L-13-ACM2-092. Based on morphological, cultural, physiological, and chemical characteristics, strain L-13-ACM2-092 was ascribed to the genus *Micromonospora*. Thiocoraline showed a potent cytotoxic activity against P-388, A-549 and MEL-28 cell lines, and also a strong antimicrobial activity against Gram-positive microorganisms. This compound binds to supercoiled DNA and inhibits RNA synthesis.

We found a new depsipeptide with potent antitumor activity from the fermentation broth of a marine *Micromonospora* during the course of a screening program for new antitumor compounds. This paper deals with the taxonomical characterization, fermentation of the producing organism, and isolation of the active compound, as well as with a study of the *in vitro* activities of thiocoraline. The structural elucidation is described in an accompanying paper[1].

Materials and Methods

Microorganisms

Strain L-13-ACM2-092 was isolated from a marine soft coral collected at the Indian Ocean near the coast of Mozambique. All the other strains used in this study were from different culture collections.

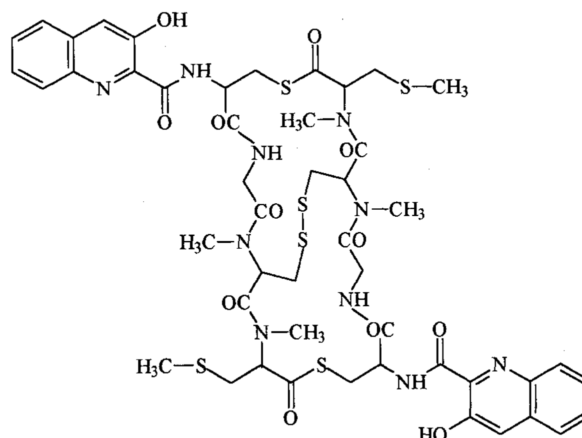
Taxonomic Studies

Micro-morphological studies were carried out with a light microscope and a scanning electron microscope on cultures grown at 28°C for 14 days on chitin agar medium and ATCC's 172 agar medium. The isomer of diaminopimelic acid from the cell wall was determined by the method of Hasegawa *et al.*[2]. Carbon source requirements were studied by the method of Shirling and Gottlieb[3]. Fatty acids profile was obtained by the method of Van der Auwera *et al.*[4]. Whole cell sugar pattern was obtained by the method of Guerrant and Moss[5].

Fermentation

Thiocoraline was produced in 2 liter Erlenmeyer flasks containing 250 ml of fermentation medium. Inoculum was grown in a medium consisting of glucose 0.5%, soluble starch 2%, beef extract 0.3%, yeast extract 0.5%, tryptone 0.5%, calcium carbonate 0.4%, sodium chloride 0.4%, sodium sulfate 0.1%, potassium chloride 0.05%, magnesium chloride 0.2%, and monobasic potassium phosphate 0.05%, distilled water to one liter, and the pH was adjusted to 7 prior to sterilization at 121°C for 20 minutes. Seed preparation was a sequence of two steps, the first in 50 ml Erlenmeyer flasks containing 10 ml of inoculum medium and the second in 250 ml Erlenmeyer flasks containing 40 ml of the same medium. Both

Fig. 1. Structure of thiocoraline.



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flasks were incubated at 28°C on a rotary shaker at 250 rpm, for 48 hours. The fermentation medium consisted of glucose 0.5%, soluble starch 2%, soybean meal 1.5%, yeast extract 0.5%, tryptone 0.2%, calcium carbonate 0.4%, sodium chloride 0.2%, and monobasic potassium phosphate 0.05%, distilled water to one liter, and the pH was adjusted to 7 prior to sterilization. The fermentor was inoculated with 12.5 ml of culture from the 250 ml flasks. The culture was grown in shake flasks with rotary agitation at 250 rpm and the flasks were incubated for 96 hours at 28°C.

Analysis of Fermentation Samples

The growth was monitored by packed cell volume (PCV) measurement. The amount of thiocoraline in the mycelium was quantified by HPLC (C18 reversed phase radial pack cartridge ; mobil phase: methanol - water - trifluoroacetic acid (90 : 10 : 0.1); flow rate: $2\,\text{ml}\,\text{min}^{-1}$; detection: 360 nm). The sample for the HPLC assay was prepared as follows: 30 ml of the culture broth was sampled and filtered through sintered glass. The retenate was extracted with 50 ml of ethyl acetate at room temperature. The extract was centrifuged and aliquots of 20 μl were injected.

Isolation

Analytical TLC and preparative TLC were done on pre-coated silica gel 60 F_{254} plates (0.2 or 1 mm thick, 20 × 20 cm, Merck) and the spots were detected under UV lights (254 and 366 nm). Silica gel 60 (70 ~ 230 mesh, Merck) and Prepex 40-63 C18 were used for column chromatography.

Analytical HPLC was carried out on a C18 reversed phase radial pack cartridge (10 μm) by the use of HPLC equipment with a Waters 991 photodiode-array detector and a Rheodyne injector.

Evaluation of Biological Activity

The antimicrobial activities of thiocoraline were determined measuring cell growth after 24 hours of preincubation of target strains, *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 29665), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 6538P), and *Micrococcus luteus* (ATCC 9341) with the antibiotic in Mueller-Hinton liquid medium at 37°C. The antitumor activities of thiocoraline were determined *in vitro* in cell cultures of P388 (ATCC CCL 46), A549 (ATCC CCL 185), HT29 (ATCC HTB-38), and MEL28 (ATCC HTB-72) following the procedure of BERGERON *et al.*[6]. DNA, RNA, and protein synthesis inhibition assays were performed following the method of TOMITA *et al.*[7]. DNA polymerase and RNA polymerase assays were performed according to SPADARI *et al.*[8]. Inhibition of topoisomerases I and II inhibition were assayed after HSIANG *et al.*[9]. Dihydrofolate reductase was measured by the procedure of BACCANARI *et al.*[10]. Thymidilate synthase assay was performed after DUNLAP *et al.*[11].

Results and Discussion

Strain L-13-ACM2-092 forms well-developed, branched mycelia. No aerial mycelium was formed and nonmotile spores appeared singly on monopodial sporophores (See Fig. 2). The vegetative mycelium was orange in most media, and mature sporulation showed a brown to brownish black in color. The isomer for the diaminopimelic acid found after whole-cell hydrolysis was the LL. The diagnostic sugars xylose and arabinose (traces) were found when whole-cell sugars were analyzed by gas chromatography, so a sugar pattern belonging to the D group of LECHEVALIER and LECHEVALIER[12] is inferred. Other important sugars detected by gas chromatography were ribose, mannose, galactose, glucose, *m*-inositol, glucosamine, and mannosamine. Madurose was not detected as a cellular constituent. The fatty acid composition showed a quantitatively distinctive profile from those characteristic of several species belonging to the *Micromonospora* genus in terms of the concentration of several important fatty acids. *anteiso*-C17 (24.50% of the total fatty acids content), *iso*-C16 : 1 (29.5%), and C17 : 1 (10.77%) are the predominant components. These characteristics indicate that this strain belongs to the genus *Micromonospora*, although it could not

Fig. 2. Scanning electron micrograph of a terminal spore of *Micromonospora* sp. L-13-ACM2-092 grown in ATCC 172 medium for 21 days at 28°C.

Bar represents 1 μm.

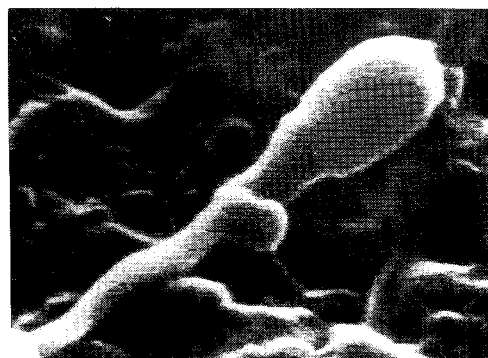


Table 1. Cultural characteristics of strain L-13-ACM2-092.

Medium	Growth	Substrate mycelium	Pigments
ISP-1	Good	Orange	None
ISP-2	Good	Orange	Traces. Brown
ISP-4	Fair	Orange	None
ISP-6	Fair	Orange	None
CZAPEK's agar	Fair	Orange	None
BENNET's agar	Fair	Orange	None
ATCC 172	Good	Orange	Traces. Brown

Table 2. Physiological characteristics of strain L-13-ACM2-092.

Temperature:	
Growth rate:	15~42°C
Melanoid pigment production:	−
Utilization of:	
D-Glucose:	+
D-Mannitol:	+
L-Arabinose:	+
D-Fructose:	+
Raffinose:	−
myo-Inositol:	−
Saccarose:	+
Mellibiose:	−
Rammnose:	+

be identified as any of the type strains.

Thiocoraline is accumulated during fermentation in the mycelium presenting a maximum around 96 hours. A typical time-course production of thiocoraline is shown in Fig. 3. The maximum yield is produced at the end of the growth phase and when pH is experiencing a steady increase.

A flow diagram of the isolation procedure of thiocoraline is shown in Fig. 4. 4.5 liters of culture broth were filtered with the aid of diatomaceus earth. The mycelial cake was extracted three times with 1 liter ethyl acetate and concentrated under reduced pressure at room temperature. The residue (700 mg) was applied to a column of silica gel (2 x 30 cm) which was developed with a chloroform - methanol gradient (100 : 0~80 : 20, v/v). The eluate was analyzed by UV absorption at 360 nm on TLC plates (silica gel F_{254}), and checked for cytotoxicity against A-549 cells. The active fractions were pooled, concentrated and chromatographed on a reversed-phase column with methanol - water elution (70 : 30~90 : 10, v/v). Finally, the active fractions were pooled again and applied to preparative silica gel TLC, using $CHCl_3$ - EtOAc - AcOH (10 : 20 : 0.3) as developing solvent, to yield 40 mg of thiocoraline.

Fig. 3. Time course production of thiocoraline.

□ Packed cell volume (%), ● activity (IC_{50}^{-1}) (μl^{-1}), ○ thiocoraline (mg/ml), ■ pH.

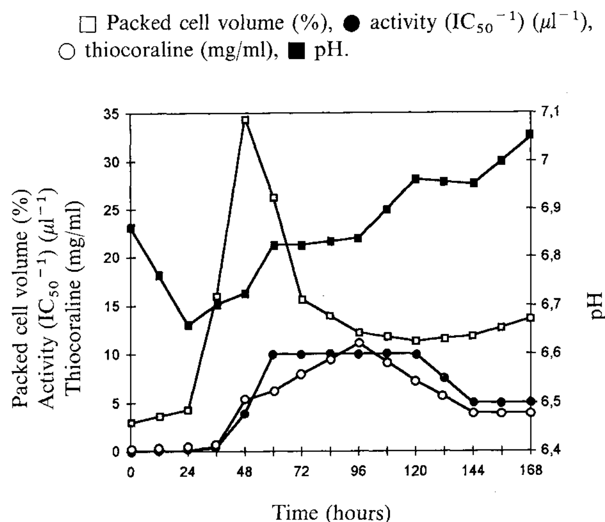
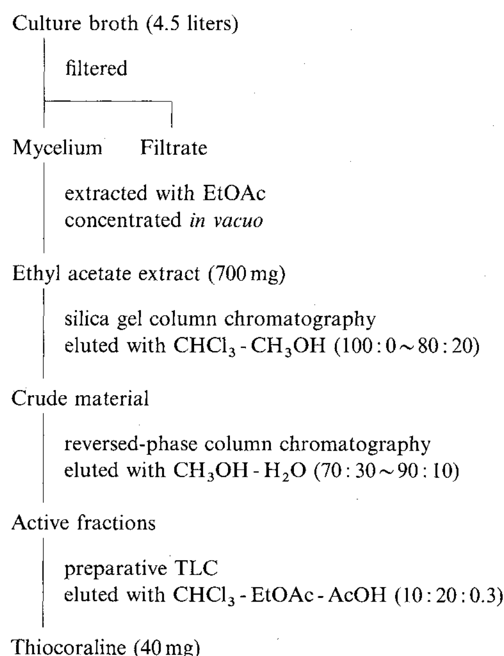


Fig. 4. Isolation procedure for thiocoraline.



Thiocoraline shows a potent antibiotic activity against Gram-positive bacteria, while its activity against Gram-negative bacteria is very low (Table 3). This antibiotic inhibits RNA synthesis more specifically than DNA synthesis (Table 4). At the same time, this molecule binds to supercoiled DNA promoting a retardation of the mixture in gel electrophoresis, but does not inhibit topoisomerases I and II. Thiocoraline presents a strong cytotoxic effect against P-388, A-549, and MEL-28 cell lines (Table 5), this activity being five fold more potent for these cell lines than for HT-29. More detailed studies on the mode of action of thiocoraline are in progress.

Table 3. Antimicrobial activities of thiocoraline.

Test strains	MIC (μg/ml)
Escherichia coli	>100
Klebsiella pneumoniae	>100
Pseudomonas aeruginosa	>100
Staphylococcus aureus	0.05
Bacillus subtilis	0.05
Micrococcus luteus	0.03

Table 4. *In vitro* inhibitory activities of thiocoraline.

Assay	IC_{50} (μg/ml)*
Protein synthesis	—
DNA synthesis	0.4
RNA synthesis	0.008
DNA polymerase	6
RNA polymerase	6
TS	15
DHR:	—
Topo I	—
Topo II	—

TS: Thymidylate synthase; DHR: dihydrofolate reductase; Topo: Topoisomerase. * IC_{50} was calculated as the thiocoraline concentration that promoted a 50% enzymatic inhibition compared to a control without thiocoraline.

Table 5. Cytotoxic activities of thiocoraline.

Cell line	IC_{50} (μg/ml)
P388	0.002
A549	0.002
HT-29	0.01
MEL-28	0.002

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