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Aerothionin, a Bromotyrosine Derivative with Antimycobacterial Activity from the Marine Sponge *Aplysina gerardogreeni* (Demospongia)

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Abstract

Aerothionin and calafianin, two bromotyrosine derivatives isolated from the marine sponge *Aplysina gerardogreeni*, were evaluated against multidrug-resistant clinical isolates of *M. tuberculosis*. Antimycobacterial activity of these compounds were tested by the modified microplate Alamar Blue assay against four monoresistant variants of *M. tuberculosis* H37Rv (rifampin, isoniazid, ethambutol and streptomycin resistant), but only aerothionin was active against all the monoresistant variants of *M. tuberculosis* H37 Rv at 12.5 µg/mL. Because of this activity, aerothionin was also tested against eight *Mycobacterium tuberculosis* clinical isolates with different drug-resistance patterns, and nine nontuberculosis mycobacteria species. It was active against all the drug resistant clinical isolates, regardless of their resistance patterns, with minimum inhibitory concentrations from 6.5 to 25 µg/mL. Three out of nine nontuberculosis mycobacteria were inhibited by aerothionin: *M. kansasii* (50 µg/mL), *M. scrofulaceum* (100 µg/mL) and *M. avium* (100 µg/mL).

Keywords: *Aplysina gerardogreeni*, aerothionin, calafianin, antimycobacterials.

Introduction

As shown by the records of the Health Secretary and Public Assistance of Mexico, infectious diseases cause important problems that require a solution (SNE, 1999). Worldwide, each year tuberculosis kills 2 million people and around 8 million become sick. It is estimated that between 2000 and 2020, nearly one billion people will be newly infected, 200 million will get sick, and 35 million will die from tuberculosis. This scenario is expected if further efforts are not strengthened (WHO, 2001). In 1993, the World Health Organization declared tuberculosis as an emergency disease, with special attention on the emergence of drug-resistant strains, particularly isoniazid- and rifampin-resistant strains (multidrug-resistant tuberculosis "MDR-TB"). Treatment of MDR-TB requires the use of a minimum of three drugs never used before by the patient. This makes the search for new compounds to treat this disease urgent (Bastian & Portaels, 2000). Recently, attention has been given to the study of marine natural products for the discovery of new structures or derivatives for their potential use in tuberculosis (Koning et al., 2000). Among the huge potential in the biodiversity, marine natural products are an important and unexplored resource for novel antimycobacterial agents.

As part or our search for bioactive substances from marine organisms of Baja California Sur (México), the sponge *Aplysina gerardogreeni* (Demospongia, Verongida) was studied because of activity shown by an ethanol extract against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, and *Mycobacterium tuberculosis* (*M. tuberculosis*) (Encarnación et al., 2000). Bioassay-guided fractionation of the methylene chloride extract resulted in the isolation of the bromotyrosine derivatives aerothionin and calafianin (Fig. 1), with aerothionin one of the active compounds against *M. tuberculosis* (Encarnacion et al., 2000a). To contribute to the discovery to control MDR-TB, in this study, aerothionin was again iso-

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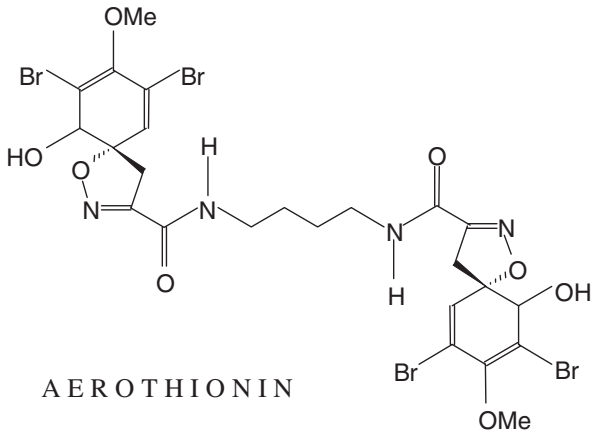
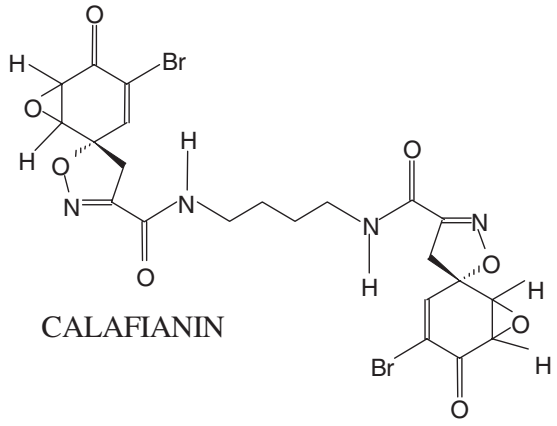


AEROTHIONIN



CALAFIANIN

Figure 1. Structures of aerothionin and calafianin.

lated and tested against MDR-TB clinical isolates, several drug-resistant *M. tuberculosis* reference strains and some nontuberculosis mycobacteria species. Antimycobacterial activity was determined by a microcolorimetric assay based on the use of Alamar blue dye.

Materials and methods

Sponge collection and identification

The sponge was collected by scuba diving at Islas Espíritu Santo-La Partida, La Paz B.C.S. (México) on 19 June 1996. It was kept frozen before freeze-drying and extraction.

A voucher specimen, number RED9616, has been deposited in the Pharmacognosy Laboratory of the Agronomy Department of Universidad Autónoma of Baja California Sur (UABCS) (México). The identification of this sponge was made by Martín García and Carlos Sánchez at the Marine Biology Department of UABCS by comparison with a sponge collected in 1985 (Samples M101, M102) identified by Patricia Gómez at the Instituto de Limonología y Ciencias del Mar of UNAM as *Aplysina gerardogreeni*, which was later reported as a new species (Gomez & Bakus, 1992).

Extract preparation

The freeze-dried and ground sponge (600 g) was extracted exhaustively by maceration with hexane, then with dichloromethane, followed by ethanol.

Isolation of aerothionin

The dichloromethane extract was tested by the agar diffusion method and showed activity against *Bacillus subtilis* and *Escherichia coli* at 2 mg/disc and because of the activity shown, was chromatographed on a column of silica gel (230–400 mesh) in a ratio 1 : 60, using hexane:acetone with a polarity gradient. The elutes were monitored by TLC and subsequently combined into 9 fractions. From these fractions, fraction CCIF4, by crystallization in dichloromethane:acetone, (9 : 1), produced the aerothionin, which was identified by IR in comparison with a standard sample. Calafianin had been previously obtained (Encarnación et al., 2000a).

Mycobacterium strains and clinical isolates

Some mycobacterium species used in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD); *M. tuberculosis* H37Rv (ATCC 27294), H37Rv isoniacid-resistant (ATCC 35822), H37Rv streptomycin-resistant (ATCC 35820), H37Rv rifampin-resistant (ATCC 35838), H37Rv ethambutol-resistant (ATCC 35837), *Mycobacterium avium* (*M. avium* 35717), *Mycobacterium flavescens* (14474), *Mycobacterium kansasii* (35775), *Mycobacterium scrofulaceum* (35795), *Mycobacterium smegmatis* (35798), *Mycobacterium microti* (35781), *Mycobacterium xenopi* (35841), *Mycobacterium chelonae*, and *Mycobacterium fortuitum*. Eight drug-resistant pulmonary isolates of *M. tuberculosis* were obtained from patients at different hospitals in Mexico. The drug-resistant isolates were selected based on their drug sensitivity pattern to the antimycobacterial drugs isoniacid, rifampin, ethambutol, streptomycin, kanamycin, rifabutin, ofloxacin, clarithromycin, clofazimine, ethionamide, and amikacin, determined previously (Mijares-Espinosa & Luna-Herrera, personal communication).

Growth conditions and inoculum preparation

Reference strains and clinical isolates were cultured at 37 °C in Middlebrook 7H9 broth, supplemented with 0.2% glycerol, and 10% OADC enrichment (oleic acid, albumin, dextrose, catalase; DIFCO) until log-phase growth was achieved. The inoculum for the microcolorimetric assay for screening was prepared by diluting log-phase growth cultures with sterile Middlebrook 7H9 broth to McFarland No. 1 turbidity standard and then further diluted 1 : 20. This suspension was prepared just before inoculation of the microplate.

Antimycobacterial screening by modified microplate Alamar blue assay

Susceptibility testing was done according to Collins and Franzblau (1997) with the following modifications. Stock solutions of aerothionin and calafianin were prepared in dimethylsulfoxide (DMSO) at a concentration of 20 mg/mL in sterile conditions and stored at −70 °C until use. Working drug solutions were prepared by diluting stock solutions to 800 µg/mL in 7H9. The test was done in clear 96-well flat bottom sterile plates (Nunc). Two-hundred microliters of sterile water were added to all outer-perimeter wells. All other wells received 100 µL of 7H9. One-hundred microliters of 4X working solution was added in triplicate, to wells with the highest concentration (200 µg/mL) of each compound. The final concentration of DMSO in these wells was 1% v/v. One-hundred microliters of the bacterial suspension was added to these wells and to controls free of drugs. A serial two-fold dilution was prepared. Final testing concentrations were 200, 100, and 50 µg/mL. A 1 : 100 diluted control was included in each plate representing the growth of 1% of the bacterial population tested. Plates were sealed and incubated at 37 °C. After 5 days of incubation, one drug-free control was developed with 20 µL of Alamar Blue solution (Trek Diagnostics, Westlake, Ohio) and 12 µL of sterile 10% Tween 80. The plates were reincubated at 37 °C for 24 h. If after this incubation the well turned pink, all the wells received Alamar blue and Tween solutions in the same way and were again incubated for 24 h. Wells with a well-defined pink color or a shade that was deeper than the 1% well were scored as positive for growth. The Minimum Inhibitory Concentration (MIC) was defined as the lowest drug concentration that prevented a color change to pink and gave an intensity of color equal or less than that of the 1% control.

Results

Over the past 20 years, marine natural products have attracted growing interest because of their unique chemical features. Pronounced biological activities of these compounds suggested potential value as primary structures with a significant role in current antituberculosis therapy (El Sayed et al., 2000).

New methodologies for drug-susceptibility testing of *M. tuberculosis* have facilitated the study of new compounds, extracts, or derivatives from natural products. By the modification of the microplate Alamar blue method, we tested two compounds isolated from the sponge *Aplysina gerardogreeni*. We started testing the activity against *M. tuberculosis* H37Rv, a pan-sensitive reference mycobacteria (Table 1). Aerothionin was very active, however, calafianin, even though is closely related structurally to aerothionin, did not show significant activity against this strain (MIC ≥ 200 µg/mL); for this reason it was not further studied.

Aerothionin was then tested against a group of monoresistant variants of *M. tuberculosis* H37Rv, and was active against all of them at 12.5 µg/mL (Table 1). Because of the good activity of aerothionin, it was tested against a group of MDR-TB clinical isolates and against a group of nontuberculosis mycobacteria. As shown in Table 2, it was active against all the isolates with MIC values between 6.5 and 25 µg/mL and against three of the nine nontuberculosis mycobacteria: *M kansasii* (50 µg/mL), *M. scrofulaceum* (100 µg/mL) and *M. avium* (100 µg/mL), as shown in Table 3.

Table 1. Antimycobacterial activity of aerothionin against *M. tuberculosis* H37Rv and its resistant variants.

M. tuberculosis strain*	MIC (µg/mL)
H37Rv**	12.5
H37Rv INH-resistant	12.5
H37Rv RIF-resistant	12.5
H37Rv STR-resistant	12.5
H37Rv EMB-resistant	12.5

* MIC for calafianine against H37Rv > 200 µg/mL.
** INH = isoniacid RIF = rifampin
STR = streptomycin EMB = ethambutol

Table 2. Antimycobacterial activity of aerothionin against drug-resistant clinical isolates of *M. tuberculosis.*

Isolate number	Drug resistance pattern[a,b]	MIC (µg/mL)
Sin 3	STR, INH, RIF, EMB, RFB, CLR, ETH	25.0
Sin 4	STR, INH, RIF, EMB, RFB, ETH, OFX	25.0
Sin 5	STR, INH, RIF, EMB, RFB, ETH, OFX	12.5
Sin 6	INH, RIF, EMB, RFB, ETH	25.0
HG 7	EMB, CLR, ETH	6.25
HG 8	EMB, CLR, ETH	6.25
CHI 21	INH, EMB, RFB	12.5
MTY 134	INH, RIF, EMB, ETH	12.5

[a] Isolates resistant to: streptomycin (STR), isoniazid (INH), rifampin (RIF), ethambutol (EMB), rifabutin (RFB), ethionamide (ETH), clarithromycin (CLR), ofloxacin (OFX).
[b] Resistance pattern determined by colorimetric Alamar Blue assay.

Discussion

Drugs used currently for the treatment of tuberculosis are the result of studies done 50 to 60 years ago. The appearance of MDR-TB strains has brought attention to the discovery and development of new leading compounds potentially useful

Table 3. Antimycobacterial activity of aerothionin against different non-tuberculous mycobacteria species.

Mycobacterium species	MIC (μg/mL)
M. smegmatis	>200
M. kansasii	50
M. scrofulaceum	100
M. flavescens	>200
M. chelonae	>200
M. fortuitum	>200
M. microti	>200
M. avium	100
M. xenopi	>200

for the treatment of MDR-TB strains. Natural products represent an interesting alternative in the search of new compounds, and the development of new procedures for drug susceptibility testing for mycobacteria has accelerated the screening of these resources. In marine organisms, there is a rich variety of unusual chemical structures and Mexico has an important diversity of marine resources. Our group has studied marine organisms from Baja California Sur. One of the interesting species, the sponge *Aplysina gerardogreeni*, has become significant because of its antibacterial and antiparasitic activity, previously demonstrated (Encarnacion et al., 2000). Two chemically related derivatives isolated from this sponge were evaluated against several mycobacterial strains. Calafianin with a 1,2-epoxy-3-ceto-4-bromo-8-carbamoyl group was not active against *M. tuberculosis* H37Rv but aerothionin, in which the 1-hydroxy-2,4-dibromo-3-methoxy-8-carbamoyl group is present, is active. El Sayed et al. (2000) suggested that hydroxylation at positions 11 or 12 of the brominated spirocyclohexadienylisoxazolines isolated from Verongide sponges is essential for the activity of these compounds. However, if we compare the structure of the compounds, the hydroxylation at position 11 or 12 of the brominated spirocyclohexadienylisoxazoline is absent. Consequently, we can then assume that the 1-hydroxy-2-4-dibromo-3-methoxy-8-carbamoyl group present in aerothionin may play an essential role for the observed antimycobacterial activity.

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