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Full Length Research Paper

Antagonistic activity of a novel antibiotic against *Mycobacterium tuberculosis*

Chen-Xiaoxi and Yue Jun*

Basic Medicine College, Zhejiang Chinese Medicine University Binjiang District, Hangzhou City, Zhejiang Province, 310053, P. R. China.

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The antibiotic produced by a newly isolated *Bacillus subtilis* has potent anti-tubercular activity against sixteen strains of *Mycobacterium tuberculosis*, most of which belong to MDR strains. MICs for the sixteen strains were evaluated. A series of spectrum analysis and element analysis determined that the antibiotic's molecular formula was $C_{27}H_{46}O_2$.

Key words: *Bacillus subtilis*, antibiotic, MIC, serum, anti-tubercular.

INTRODUCTION

Mycobacterium tuberculosis , the etiological agent of tuberculosis, kills more than 2 million people every year worldwide in concurrence with HIV-related infections. Moreover, appearance of multi- drug resistant (MDR) strains of *M. tuberculosis* to many, if not all, of the existing drugs has been noted. This has necessitated the development of novel anti-tubercular agents (Ducati et al., 2006; Tomioka, 2006; Tomioka and Namba, 2007).

We have isolated a new strain of *Bacillus subtilis* that can produce a novel antibiotic. We have found that the antibiotic had a wide antimicrobial spectrum PhD work; unpublished. The purpose of this study was to determine the antibiotic's *in vitro* anti-tubercular, with several clinically used anti-tubercular agents as the reference drugs.

MATERIALS AND METHODS

Culture medium and microorganisms

KMB medium: BBI company peptone 20 g glycerol 15 ml K_2HPO 1.5 g MgSO 0.75 g volume was adjusted to 1000 ml by distilled water sterilized at 121°C for 20 min. LB culture medium: BD Difco Tryptone 10 g, USB Yeast extract 10 g, sodium chloride 5 g, adjusted to pH 7 by 0.1 N NaOH, volume was adjusted to 1000 ml by distilled water, sterilized at 121 for 20 min

Quartz sand

Quartz sand was immersed in acidic potassium dichromate solution for 24 h to oxidize organic substance (the acidic potassium dichromate solution: K_2 Cr_2O_7 37 g plus 300 ml was heated and stirred until potassium dichromate was dissolved. After it was cooled, 300 ml 98% sulphuric acid was gradually added). The Quartz Sand was eluted with distilled water for 10 h in order to remove metallic ion and oxidized substances. Then, it was sterilized at 180°C for 2 h.

B. subtilis was isolated from the leaf of egg plant derived from Hangzhou suburbs, Zhejiang Province, P. R. China. The *Rhizoctonia solani* and the E. coli were kindly given by the college of agriculture and biotechnology of Zhejiang University, P. R. China. Four common bacteria, that is, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* were kindly given by Life Science College of Zhejiang Chinese Medicine University.

Fermentation, antibiotic extraction and purification

The antibiotic- producing bacteria (*B. subtilis*) was cultured on surface of the quartz sand that was immersed in KMB culture medium at 37°C for ten days, the surface of the quartz sand not being covered with liquid culture medium (previous work had shown that the bacteria produced more antibiotic if it was cultured on solid medium) . Thereafter, the quartz sand, which absorbed antibiotic secreted by the bacteria, was immersed in water in order to be distilled. The condensed water was collected and passed through active carbon chromatographic column, which was then eluted with

*Corresponding author. Email: yuejunnan@yahoo.com.cn.

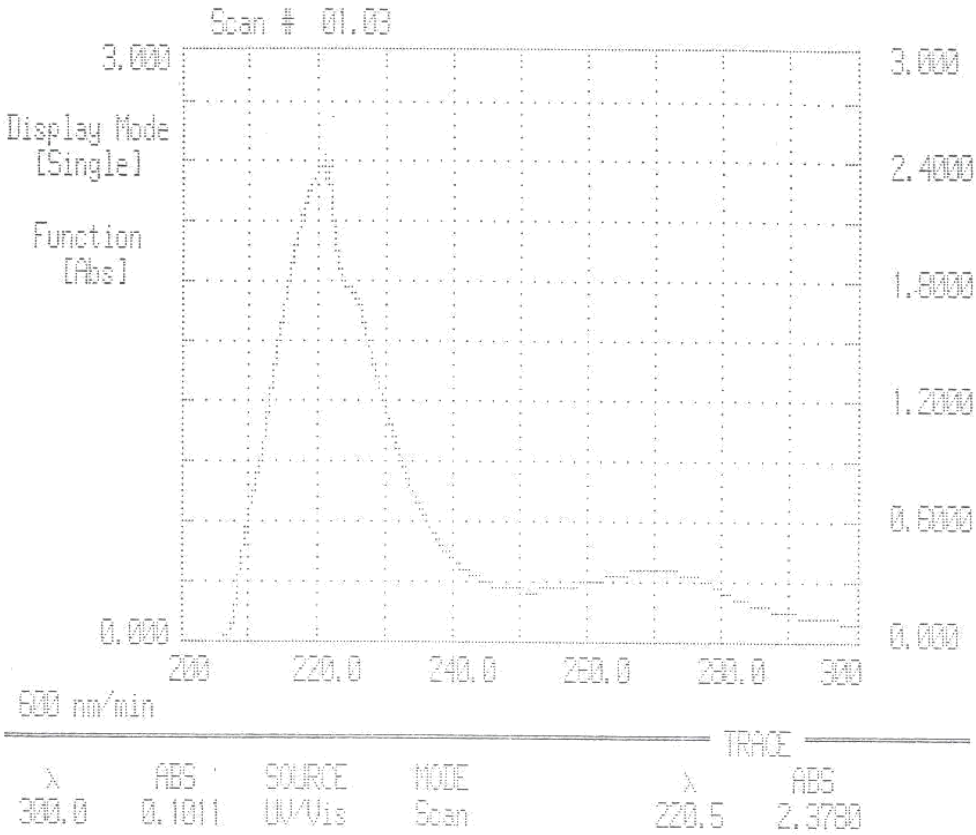

Figure 1. Ultraviolet spectrum of the antibiotics.

ether. The eluted ether was left at room temperature of 25-30°C overnight to evaporate the ether. The remainder was chromatographed on silica gel column which was eluted with ether. The fraction with the greatest activity was further chromatographed on silica gel column and then eluted with normal pentane ether=28. The normal pentane and the ether were both evaporated at room temperature of 25 - 30°C. Anti- bacterial activity was confirmed by agar diffusion test (the test cell was *E. coli* and the agar diffusion test was carried in on LB agar plates). About 100 mg antibiotic was produced from 100 L KMB culture medium (about 200 kg quartz sand was confused). Purity quotient of the antibiotic was confirmed by silica gel TCL as well as PHLC. Ultraviolet spectrum, infrared spectrum and mass spectrum, as well as element analysis were carried out in Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences (SIOC, CAS).

Determination of MIC for *M. tuberculosis*

Sixteen strains were respectively incubated in Lownstein-Jenson media agar plates at 37°C for four weeks, with the concentration of all the *M. tuberculosis* being equivalent to 0.5 McFarland standard. The assays were all performed in duplicate. The MIC on agar plates is determined by comparing the number of colonies in the drug- free sections of the plate with the number of colonies growing in the presence of drug added at two-fold serial concentrations, with the MIC being the lowest concentration of the antibiotic that inhibits more than 99% of the bacteria. The sixteen strains of *M. tuberculosis* were respectively tested their susceptibility against the antibiotic produced by the *B. subtilis,* isoniazid, rifampicin, pyrazinamide, fluoroquinolones, rifabutin, ethambutol and streptomycin.

The determination of MICs of four common bacteria

For each of the four strains of bacteria (that is, *S. epidermidis, S. viridans, P. aeruginosa* and *S. dysenteriae*), the method of determining MICs (minimal inhibition concentration) was the same as follows: each bacterium's single clone was respectively inoculated into flask containing 500 ml LB medium. After it was cultured at 37°C overnight, the culture was respectively diluted to being equivalent to 0.5 McFarland standard with LB medium and then 1ml such bacterium was respectively incubated in 1 ml Eppendorf tubes in the presence of 2-fold serial dilutions of the antibiotic (the range was from 0.0125 to 0.1 g/ml). The lowest concentrations of the antibiotic that completely inhibited bacterium's growth were defined as MICs.

RESULTS

The ultraviolet spectrum, the infrared spectrum and the mass spectrum of the antibiotic

The infrared spectrum and the ultraviolet spectrum of the antibiotic are shown at Figures 1 and 2 respectively. According to mass spectrum, the molecular weight of the antibiotic is 425.23 - 2.99 = 402.24. Here, 2.99 is the

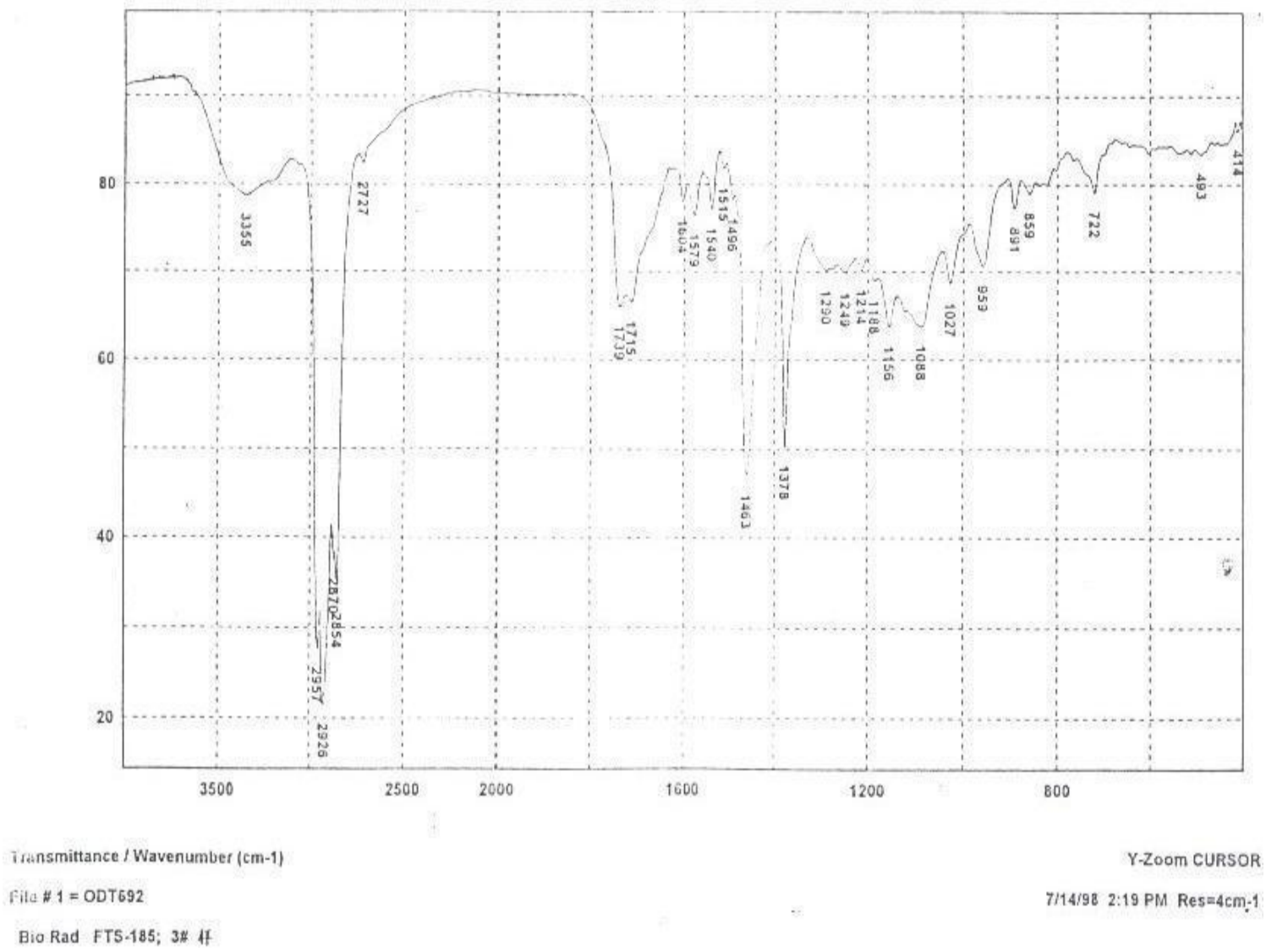

Figure 2. Infrared spectrum of the antibiotic.

value of Na^+. Elementary analysis showed that the antibiotic only contained C (80.17%), H (13.6%) and O (6.2%). By calculation, the molecular formula of the antibiotic was obtained: $C_{27}H_{46}O_2$. At this stage, although the chemical structure had not been fully elucidated, the infrared spectrum, the mass spectrum, the elementary analysis, as well as the molecular formula had confirmed that the antibiotic belonged to a novel substance.

MIC for *M. tuberculosis*

The antibiotic had shown potent in *vitro* anti-tubercular activity. Except strain 12, this could be cultured in Lownstein-Jenson media containing 0.05 g/mL antibio - tics produced by the *B. subtilis,* all other fifteen strains of *M. tuberculosis*, whether they were drug-sensitive or drug resistant, could not be cultured in Lownstein-Jenson media with 0.025 g/ml or 0.05 g/ml of the antibiotic. Therefore, The MIC for strain 12 was > 0.05 g/ml, while the MICs for other fifteen strains were 0.025 g/ml or 0.05 g/ml. The MICs for different strains of M. tuberculosis were summarized in Table 1. The MISc for isoniazid, rifampicin, pyrazinamide, fluoroquinolones, rifabutin, ethambutol and streptomycin were also shown in Table 1.

MICs for four common bacteria

The MICs (the minimal concentration inhibition) for four common bacteria, that is, *S. epidermidis, S. viridans,*

Table 1. Anti -tubercle bacillus effect of the antibiotic against sixteen strains of *Tubercle bacillus*, compared with seven clinical drugs (MIC, µg/ml).

Strain	The antibiotic Isoniazid	Rifampicin	Pyrazinamide	Fluoroquinolones	Rifabutin	Ethambutol	Streptomycin
Strain 1	0.025	0.125	0.125	12.5	0.50	0.125	1.00	0.25
Strain 2	0.025	0.125	0.125	12.5	0.50	0.125	0.50	2.00
Strain 3	0.025	0.125	0.125	12.5	0.50	0.125	1.00	2.00
Strain 4	0.05	0.250	2.00	12.5	1.00	1.00	0.50	4.00
Strain 5	0.025	0.250	2.00	6.20	0.50	1.00	2.00	4.00
Strain 6	0.05	0.250	4.00	12.5	0.50	1.00	16.0	0.50
Strain 7	0.05	0.250	0.25	12.5	0.50	0.250	16.0	1.00
Strain 8	0.05	0.250	0.25	12.5	0.50	0.250	2.00	0.250
Strain 9	0.05	0.250	4.00	25	1.00	2.00	2.00	4.00
Strain 10	0.05	0.250	8.00	50	2.00	1.00	16.0	2.00
Strain 11	0.025	0.500	4.00	100	2.00	2.00	32.00	4.00
Strain 12	0.05	0.250	2.00	200	2.00	1.00	16.0	8.00
Strain 13	0.05	0.250	0.06	12.5	0.50	0.125	16.0	0.50
Strain 14	0.05	0.250	0.125	12.5	0.50	0.125	2.00	2.00
Strain 15	0.05	0.500	0.250	12.5	0.50	0.250	16.0	4.00
Strain 16	0.05	0.250	0.250	12.5	0.50	0.250	2.00	4.00

Note: Strain 1 was H37RV strain and all other strains were clinical isolates.

Table 2. MICs (the minimal inhibition concentration) for four common gram-positive and -negative bacteria.

Bacteria strain		MIC (µg/ml)
Gram-positive	**Gram-negative**	
S. epidermidis		0.05
S. viridans		0.05
	P. aeruginosa	0.025
	S. dysenteriae	0.025

P. aeruginosa and *S. dysenteriae* were shown in Table 2. The antibiotic presented potent antagonistic activity against all the four bacteria strains tested, whether they were gram-positive or -negative.

DISCUSSION

Natural products have played a major role in antibiotic discovery since 1941 when penicillin was introduced to the market (Clardy et al., 2006). In 1943, an American named Selman Waksman, together with his co-workers, discovered that a fungus called *Streptomyces griseus* produced an antibiotic substance which they named "streptomycin". Streptomycin was the first antibiotic used against *M. tuberculosis* (Zetterstrom, 2007). Currently, natural products are again the most important source for promising new antibiotic, including antibiotics that are against *M. tuberculosis* and efforts have refocused on finding new antibiotics from old sources and new sources (Luzhetskyy et al., 2007).

Because of some constraints that have deterred companies from investing in new anti-TB drugs, no new drugs except rifabutin and rifapentine has been marketed for TB during the 40 years after release of rifampicin (Joas da Silva et al., 2009). The global resurgence of TB and the rapid emergence of MDR-TB have motivated the research of novel anti-tubercular agents and a lot of top researchers in various fields are doing their best to investigate novel compounds with anti-tubercular activity (Coleman et al., 2001; Lourenco et al., 2008; Biava et al., 2008). This paper's research has provided evidences that the antibiotic produced by the newly isolated *B. subtilis* has potent anti-tubercular activity, even if the *M. tuberculosis* belong to MDR strains and therefore, it has the potential to be a drug candidate in the fight against *M. tuberculosis.*

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