UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHRACTERIZATION OF PYOCYANINE AND 1-PHENAZINOLE FROM PSEUDOMONAS AERUGINOSA (AN ENDOPHYTE)

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ISOLATION AND CHRACTERIZATION OF PYOCYANINE AND 1-PHENAZINOLE FROM *PSEUDOMONAS AERUGINOSA* (AN ENDOPHYTE)

By

MAJID ESHAGHI

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Dedicated to……,

Memory of my father

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
$CDCl_3$	Deuterized chloroform
DIP	Direct insert probe
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediamine tetraacetic acid
EI	Electron impact
FBS	Fetal bovine serum
FCS	Fetal calf serum
GCMS	Gas chromatography mass spectrometry
IC_{50}	Inhibitory Concentration at 50% cells reduction
IR	Infrared
LC/MS	Thermospray liquid chromatography/mass spectrometry
MIC	Minimum inhibitory concentration
MIS	Microbial Identification System
MLD	Minimum lethal dose
MRSA	Methicillin Resistant *Staphylococcus auerus*
NCI	National Cancer Institute
NMR	Nuclear magnetic resonance
NRRL	Northern Regional Research Laboratories
PC	Paper chromatography
PTLC	Preparative thin layer chromatography
SI	Similarity index
TLC	Thin layer chromatography
UV	Ultraviolet
VIS	Visible

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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March 1998

Chairman : Assoc. Prof. Dr. Abdul Manaf Ali

Faculty : Food Science and Biotechnology

Endophytic bacteria are organisms that colonize internal plant tissues. These organisms are believed to be useful for pharmacological screening and agricultural programs for biological control of plant pests and diseases. This project was designed to screen endophytic bacteria to be used for pharmaceutical purpose.

Bacterial endophytes were isolated from the twigs of 50 different healthy tropical rain forest plants collected at Kemensal Hill, Ulu Kelang, Selangor. After preparing the pure culture of the bacteria, the isolates were subjected to bioassay using disk diffusion method against *Bacillus cereus* NRRL 1447 (Gram-positive bacteria), *Pseudomonas aeruginosa* ATCC 60690 (Gram-negative bacteria), B*acillus subtilis* B_{28} and B_{29} [mutant (deficient in DNA repair) and wild type], *Candida lipolytica* ATCC 2075

(yeast), *Sacchromyces lipolytica* ATCC 16617 (yeast) and *Aspergillus ochraceous* ATCC 398 (fungi). Out of the 79 bacterial endophytes that were isolated from 50 tropical plants, 12 of them showed antimicrobial activity. One of the isolate (no. 32.1) that possesses the best inhibition activity against target microorganisms was selected for further study. By employing biochemical tests, miniaturized multi test system (API 20NE kit) and whole cellular fatty acid profiles the isolate was identified as *Pseudomonas aeruginosa* UPM strain.

Judicious combinations of chromatographic techniques were adopted in purifying the active compounds from the fermentation media. As a result, two bioactive compounds were purified. The structure of these two bioactive compounds were elucidated by means of spectroscopic techniques including ultraviolet spectroscopy (UV), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C-NMR), mass spectroscopy (MS), also by comparison with the literature and they were identified as pyocyanine and 1-phenazinole.

Minimum inhibitory concentration of pyocyanine against *P. aeruginosa*, *B. subtilis* (B_{29}) and methicillin resistant *Staphylococcus auerus* was 4.6875 µg/disk, while against *B. subtilis* (B_{28}) and *B. cereus* was 9.375 µg/disk. 1-Phenazinole had the MIC value of 37.5 µg/disk against *P. aeruginosa*, *C. lipolytica*, *A. ochraceous*. The MIC value of this compound was 75 and 150 µg/disk against *B. cereus*, *B. subtilis* (B_{28} and B_{29}), *S. lipolytica* and MRSA, respectively. Therefore, pyocyanine revealed antibacterial activity whereas, 1-phenazinole was active against all target microbes. The

antimicrobial activity of 1-phenazinole was less than pyocyanine. The cytotoxicity of the compounds were tested against the HeLa cells (Human cervical adenocarcinoma), 3T3 (Mouse fibroblast), T-Lymphoblastic Leukemic cells (CEM-SS) and Sf9 insect cells. 1-Phenazinole was found to be more toxic than pyocyanine against the tested cell lines, except for CEM-SS. Cytotoxic activity of the compounds against 3T3 cell line (non-cancerous mouse fibroblast) was lower than against the tumor cell lines. Thus, the compounds revealed fairly selective cytotoxic activity against tumor cell lines. The compounds showed cytotoxic effect on Sf9 insect cells and therefore could be used for biological control.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi syarat ijazah Master Sains

PENULENAN DAN PENCIRIAN ``PYOCYANINE'' DAN ``1-PHENAZINOLE'' DARI *PSEUDOMONAS AERUGINOSA* (ENDOFIT)

Oleh

MAJID ESHAGHI

Mac 1998

Pengerusi : Prof. Madya Dr. Abdul Manaf Ali

Fakulti : Sains Makanan dan Bioteknologi

Bakteria endofitik adalah organisma yang dapat hidup di dalam tisu tumbuhan. Organisma-organisma ini dipercayai berguna untuk penyaringan farmakologi serta rancangan pertanian disebabkan oleh fungsinya sebagai kawalan biologi untuk haiwan perosak tumbuhan dan penyakit. Projek ini dirancang untuk menyaring bakteria endofitik bagi tujuan farmaseutikal.

Bakteria endofitik diisolatkan daripada ranting 50 batang pokok yang berlainan dari hutan hujan tropika di Bukit Kemensal, Ulu Kelang, Selangor. Selepas kultur tulen bakteria dilakukan, isolat tersebut telah diuji dengan bioasai kaedah penyerapan cakra terhadap *Bacillus cereus* NRRL 1447 (bakteria Gram-positif), *Pseudomonas aeruginosa*

ATCC 60690 (bakteria Gram-negatif), *Bacillus subtilis* B_{28} dan B_{29} [mutan(tidak berupaya membaiki DNA) dan jenis liar], *Candida lipolytica* ATCC 2075 (yis), *Sacchromyces lipolytica* ATCC 16617 (yis) dan *Aspergillus ochraceous* ATCC 398 (fungi). Daripada 79 bakteria endofit yang diasingkan, 12 bakteria telah menunjukkan aktiviti antimikrob. Salah satu isolat bernombor 32.1 yang menunjukkan sifat aktiviti perencatan terbaik terhadap mikroorganisma sasaran telah dipilih untuk ujian lanjut. Dengan penggunaan ujian biokimia, sistem ujian "multi miniaturized"(API 20NE kit) serta keseluruhan profil sel asid lemak, isolat tersebut telah dikenalpasti sebagai *Pseudomonas aeruginosa* strain UPM.

Sebatian-sebatian aktif telah ditulenkan daripada media fermentasi menggunakan pelbagai teknik kromatografi. Dua sebatian bioaktif telah ditulen. Kedua-dua struktur sebatian aktif ini ditentukan dengan pelbagai teknik spektroskopik termasuk spektroskopi ultra ungu (UV), spektroskopi infra-merah (IR), spektroskopi nuklear magnetik resonan (^{1}H dan ^{13}C-NMR), spektroskopi jisim (MS) dan perbandingan nilai di dalam rujukan dan dikenalpasti sebagai pyocyanine dan 1-phenazinole.

Kepekatan minima bagi perencatan untuk pyocyanine terhadap *P. aeruginosa*, *B. subtilis* (B_{29}) dan *Staphylococcus aureus* lentang metisilin (MRSA) adalah 4.6875 µg/disk, manakala bagi *B. subtilis* (B_{28}) dan *B. cereus* adalah 9.375 µg/disk. 1-Phenazinole mencatatkan nilai MIC sebanyak 37.5 µg/disk ke *atas P. aeruginosa, C. lipolytica, A. ochraceous*, manakala nilai MIC sebanyak 75 µg/disk terhadap *B. cereus, B. subtilis* (B_{28} dan B_{29}), *S. lipolytica* dan 150 µg/disk terhadap MRSA. Dengan

demikian, pyocyanine menunjukkan spektrum aktiviti antibakteria yang luas dan sebaliknya 1-phenazinole adalah aktif terhadap semua mikrob sasaran. 1-Phenazinole menunjukkan aktiviti antimikrob yang kurang berbanding dengan pyocyanine. Kesitosikan sebatian-sebatian tulen telah diuji terhadap sel HeLa (Human cervical adenocarcinoma), sel fibroblas mencit-3T3 (titisan bukan kanser), sel leukemia T-limfoblastik (CEM-SS) dan sel serangga Sf9. Kesitosikan 1-phenazinole adalah lebih tinggi berbanding dengan pyocyanine terhadap sel yang diuji kecuali CEM-SS. Aktiviti sitotoksik sebatian-sebatian tersebut terhadap sel 3T3 adalah kurang daripada sel kanser. Jadi, sebatian-sebatian tersebut telah menunjukkan pemilihan aktiviti sitotoksik terhadap sel pertumbuhan. Sebatian-sebatian tersebut juga menunjukkan kesan sitotoksik ke atas sel serangga SF9 dan justeru itu, mungkin dapat digunakan sebagai kawalan biologi.

CHAPTER I

INTRODUCTION

Antibiotics are chemical substances produced by metabolism of living organisms which have inhibitory activity against microorganisms and some other animal cells, e.g., tumor cells, or viruses. The principle drugs used in the treatment of infectious diseases fall into three categories: antibiotics, sulfonamides, and chemotherapeutics (Murray *et al.*, 1995). Collectively, they may be referred to as antimicrobics. Antibiotics can be either broad-spectrum antibiotics, active against many organisms, or narrow-spectrum antibiotics, active against only a restricted range of organisms.

During World War II, the demand for chemotherapeutic agents to treat wound infections led to the development of a production process for penicillin and the beginning of the era of antibiotic research. This continues to be the most important area of industrial microbiology today. Intensive screening programs in all industrial countries continue to increase the number of described antibiotics: 513 antibiotics were known in 1961, 407 in 1972, 7650 in 1985, and currently about 8000. In addition, about 3000 antibiotically active substances have been detected in lichens, algae, higher animals, and plants. Each year, about 300 new antibiotically active materials are detected; of which 30-35% are secondary components from fermentation with known antibiotics. Approximately 10,000 different antibiotics have been characterized, but only 123 are currently produced by fermentation (Perry

and Staley, 1997). In addition, more than 50 antibiotics are produced as semisynthetic compounds, and three antibiotics namely, chloramphenicol, phosphonomycin, and pyrrolnitrin, are produced completely synthetically (Crueger and Crueger 1990). Worldwide antibiotic production is over 100 000 tons per year and estimated gross sales for 1980 were US$4.2 billion. The annual gross sales in the United State alone are US$1billion, with cephalosporin in leading position, followed by ampicillin and the tetracycline. Feed-additive antibiotics are believed to have a world market of US$100 million annually. Before 1960, about 5% of the newly isolated antibiotics were therapeutically useful. In the following years new antibiotics were discovered at an approximately constant rate, but the percentage of the new antibiotics which actually came on the market decreased from 2.6% in 1961-1965 to 1% in 1966-1971(Crueger and Crueger 1990). This is primarily because of severe cost increase in development and clinical testing, thus those manufacturers produce only those compounds that clearly show promising therapeutic progress. About ten years are likely to elapse before an agent can be marketed, at an average cost of US$10,000,000 to US$20,000,000 (Greengrass, 1997).

Most antibiotics are produced by bacteria, actinomycetes and fungi but compounds may be obtained from many other living organisms such as plants, lichens, algae and higher animal organisms (Berdy, 1985). Antibiotic producers can be isolated from various natural sources such as soil, water, plants and animals. Microorganisms from plants (endophytes) are one of the sources that scarcely have been screened for pharmacological purposes but they have a good potential for production of antibiotics (Dreyfuss, 1987).

Considering the very large number of known compounds, it may seem questionable whether the search for new antibiotics should continue. The reasons for continued research are:

- In many cases the properties of natural antibiotics are not optimal for therapeutic application. The following improvements are needed: greater activity with uncharged or diminished toxicity, decreased side effects, broader antimicrobial range, greater selectivity against certain pathogens, improved pharmacological properties.
- Suitable antibiotics are not available in many fields of human medicine or in nonmedical areas. Numerous tests have been made of new and semisynthetic substances, but no significant breakthroughs have been made.
- Since the beginning of chemotherapy, the number of resistant strains has increased. Multiple- and cross-resistance can occur, *i.e.*, if resistance develops to one antibiotic it may simultaneously develop to others having the same mode of operation or uptake mechanism. Careless use of antibiotics has been responsible for much increase in resistance, but even with careful use in chemotherapy, resistance still develops, albeit at a slower rate, Currently, the only alternative for overcoming the resistance problem is the discovery of new antibiotics.

Improved antibiotics can be obtained by modifying known compounds using either chemical or genetic means (mutasynthesis, protoplast fusion, and recombinant DNA technology). However, antibiotics with entirely new basic structures can be expected only from screening, especially by the use of new test procedures and by research on new groups of microorganisms.

Based on the perspectives presented, the followings were the objectives of this study.

- To isolate bacterial endophytes from tropical rain forest plants.
- To screen the isolates for antimicrobial activity.
- To select and identify one isolate with a broad-spectrum of antimicrobial activity.
- To purify and characterize the antimicrobial metabolites of the isolate.

CAHPTER II

LITRERATURE REVIEW

The History of Antibiotics

The pre-scientific era of antibiotics has its roots in folk medicine many years before Pasteur, Tyndall and others. They recognized the antagonism of microorganisms in the last third of 19th century (Florey *et al.*, 1949). The Mayans used a fungus known as cuxum for treatment of ulcers and intestinal infections. Florey and co-workers (1949) found several references in the medical literature of the 19th century on the use of microorganisms for therapeutic purposes. For example, Mosse, in 1852, published his experiences in healing wounds with yeast.

The scientific era in the history of antibiotic can be divided into three periods. The first period up to the early 1940s is characterized by the discovery of the first known antibiotics, mycophenolic acid, and later by the discovery, isolation and therapeutic use of penicillin. Florey and co-workers (1949) reported that the first antibiotic isolated in the 1896 by Gosio was from mouldy maize. From such mouldy maize, Gosio isolated a fungus, which he named *Penicillium glaucvum*, but it probably is a strain of *P. brevi-copactum*. After the cultivation of this fungus, Gosio was able to isolate a small amount of a crystalline substance from the cultivation medium. He found that this substance was phenolic and tested its antibacterial effects. The substance inhibited the growth of *Bacillus anthracis.*

In 1913, Alberg and Black isolated a phenolic substance from a *Penicillium* of the same kind of culture. According to its color reaction, they believed that the substance was identical with that of Gosio and named it mycophenolic acid. In 1928, Fleming discovered an antibacterial agent, produced by a *Penicillium* contaminating an agar plate culture of staphylococci. During observations on staphylococcal colonies, he noticed that some of them, growing in the neighborhood of the contaminating fungus, became apparently lysed. He picked the contaminating penicillium up, grew it on the surface of nutrient broth and found that an antibacterial substance was secreted into the medium. This broth was found to contain penicillin.

The systematic screening of actinomycetes and discoveries of the fundamental types of antibiotics characterized the second period. In the 1940s, systematic screening programs began in the search for actinomycetes capable of producing antibiotics. The first screening surveys made in Waksman's laboratory and elsewhere, established that nearly 50% of all cultures isolated from soil (mostly *Streptomyces* species) were active mainly against Gram-negative bacteria. Apart from actinomycetin, actinomycin was the first antibiotic isolated from a culture of an actinomycete (Waksman and Woodruff, 1941). Closely related substances were later isolated throughout the world and tens of actinomycins are known at present. Another antibiotic, proactinomycin was later separated into several components. Streptothricin was isolated in the same year (Waksman and Woodruff, 1942). In September 1943, the work of Waksman's group culminated in the isolation of streptomycin from *Streptomyces griseus* (Schatz *et al.*, 1944). Streptomycin started a great number of studies on the production of antibiotics by actinomycetes. In 1945, research on *Streptomyces aureofaciens* produced an antibiotic, aureomycin (known