

**Pigmented bioactive compounds from marine
bacteria and their mechanisms of action involved in
cytotoxicity**

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Summary

Covering over 70% of the Earth's surface, marine ecosystem is the home for a wide variety of animal and plant species whose number greatly exceeds that of terrestrial ones. Amongst those biodiversity, microbial world represents a potential research object to be explored as the recent investigations on marine bacteria have given the impact to the entire world as a source of bioactive metabolites producers. The area of application of biologically active compounds from bacterial origin is wide, involving agricultural, industrial, and pharmaceutical sectors.

Japan is surrounded by sea and, consequently rich in marine resources. Joining to the world research arena, our research group is engaged with the investigations related to the marine environment. In this regard, marine microorganisms and their respective metabolites have drawn our special attention due to their strong bioactive properties, featuring them to be excellent pharmaceutical agents. More specifically, different kinds of pigmented compounds isolated from marine bacteria, along with their biological properties are one of the currently ongoing research projects of our laboratory.

As a result of this research, two kinds of pigments, i.e. red and purple colored compounds were isolated from marine *Pseudoalteromonas* strains 1020R and 520P1. The two strains along with other 83 bacterial strains were previously found in the Pacific Ocean at a depth of 320 m, near to Cape Muroto, Kochi Prefecture. Structural and biological properties of the two pigmented compounds are currently under intensive investigations.

Structural investigations revealed that the red pigment represents so-called prodigiosin and its close related analogs, which have a common pyrrolydipyrromethene core structure. These compounds are famously known for their broad range of biological properties, including antibiotic, immunosuppressive, and above all anticancer activities against nearly 60 cancer cell lines, with no or very little effect on normal functioning cells. The purple pigment was found to consist of violacein and a little amount of deoxyviolacein, which have the

similar biological activities as prodigiosins, except immunomodulatory effect.

Current research is a continuation of previous investigations and outlines the findings recently obtained from the study of these pigments, and deals with the following tasks:

1. Structural analysis of an unknown compound of the red pigment, including the stability investigations of the individual compounds of the red pigment.

2. Investigations on the mechanisms of action involved in cytotoxicity of the red-pigmented prodigiosin and purple-pigmented violacein compounds on the molecular level.

Previous studies showed that the red pigment contains four individual pigments, all of which belong to the prodigiosin family compounds. Chemical structures of three out of four were successfully determined on the basis of mass- and NMR spectroscopy methods, and only the structure of the pigment 1 was not determined and remained in doubt. However, recent investigations have revealed that the red pigment may actually contain seven individual compounds. These compounds are named as P-1, P-2 (previously described as pigment 1), P-3, P-4, P-5, P-6 and P-7. Pigments whose chemical structures were previously determined were P-4 as 2-methyl-3-pentylprodiginine or prodigiosin, P-5 as 2-methyl-3-hexylprodiginine and P-6 as 2-methyl-3-heptylprodiginine. These were typical red colored compounds differing from each other only by the length of side alkyl chain. Thus, the chemical structures of P-1, P-2, P-3 and P-7 have become unknown.

In this study, separation and structure elucidation analyses of P-2 of the red pigment are described. Structural analyses of P-1 were not carried out due to its instability, which decomposed into other unknown compounds, thus making our task impossible to bring into effect. Biological properties of the individual compounds of the red pigment were investigated by the cell cytotoxicity assays on U937, HL60 and K562 leukemia cell lines. An attempt was also made to investigate the molecular mechanisms of cytotoxicity action of the individual compounds of the red pigment, including violacein from 520P1 bacterial strain. An impact of the environmental conditions, i.e. temperature and solvents were studied to find the

logical reasons of the instability of the individual compounds of the red pigment during their preservation period.

As a result of this research, the chemical structure of P-2 in the red pigment, which remained unknown in the previous study, was determined to be 2-methyl-3-butylprodiginine according to mass- and NMR spectroscopy methods. The determined structure revealed that it is another close related analog of 2-methyl-3-pentylprodiginine or prodigiosin, differing from it only by lacking one $-CH_2$ group in side alkyl chain. Although, this chemical structure was previously predicted to be existed in literature and by our research group too, according to its MS data, it was not investigated by NMR methods, and thus, making those conclusions widely not confirmed. The chemical structure of P-1 copurified together with P-2 was not determined due to its extreme instability, which decomposed during accumulation and preservation. This instability led to the investigation on the impact of environmental conditions to the individual compounds of the red pigment, and the following study revealed that there are many factors that may affect on the stability of the pigments. Particularly, the nature of solvents and temperature were found to have the most profound effects on the stability of the pigmented compounds, along with a previously reported light effect.

Cell cytotoxicity assays of the red pigment and its individual compounds were performed on U937, HL60 and also K562 leukemia cancer cells using the MTS assay. The compounds tested showed cytotoxic effect, with slightly different IC_{50} values. Among the pigments, P-4, i.e. 2-methyl-3-pentylprodiginine or prodigiosin showed the highest apoptotic effect, followed by P-5 or 2-methyl-3-hexylprodiginine and P-6 or 2-methyl-3-heptylprodiginine.

The molecular mechanism of the cytotoxicity of the red pigment compounds was investigated by evaluating the effect of the pigments on enzymes involved in the intracellular signal transduction. The red pigment compounds had strong inhibitory effect against the activities of protein tyrosine phosphatase 1B (PTP1B) and protein phosphatase 2A (PP2A) tested in this study. However, the red pigment showed no effect on protein kinases, namely

the catalytic subunits of protein kinase C (PKC) and protein kinase A (PKA), along with PKC subfamily isozymes as well as Src-protein tyrosine kinase (Src-PTK) and calmodulin-dependent protein kinase (CaM kinase). On the other hand, violacein from 520P1 strain showed strong inhibitory effect against the catalytic subunits of PKA and PKC and some of PKC family isozymes, but having relatively low effect on protein phosphatases.

The aim

The aim of this investigation was to perform structural analyses of unknown compounds present in the red pigment produced by a marine bacterial strain 1020R belong to *Pseudoalteromonas* species of bacteria and to investigate molecular mechanisms of cytotoxicity actions of the red pigment and its individual compounds as well as violacein against cancer cells. Stability of the red pigment compounds was also aimed to investigate as they showed a tendency to decompose into other compounds when the samples were kept even in a dark place to avoid a light which is believed to be an initiator stimulus for their decomposition.

The novelty of the work

For the first time in this research, the chemical structure of P-2 of the red pigment was determined to be 2-methyl-3-butylprodiginine by high-resolution mass-spectroscopical (HR-MS) and NMR spectroscopy methods.

Prodigiosins and violacein effects on protein kinases, including protein kinase A (PKA), protein kinase C (PKC) and PKC family isozymes as well as protein phosphatases, such as protein phosphatase 2A (PP2A) and protein tyrosine phosphatase 1B (PTP1B) was studied.

Structure activity relationship studies of the individual compounds of the red pigment were carried out to investigate the biological properties of prodigiosin compounds which differ from each other only by the side alkyl chain length.

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Chapter I

Introduction

Marine pigmented bacteria and their bioactive metabolites*

Abstract

Over the last 50 years, the search for bioactive compounds from marine natural resources, including microorganisms has rapidly increased, as the development of modern technologies has allowed access to this diverse ecosystem. This scientific enterprise has already started giving fruits as the aquatic world has proved to produce not only compounds that are common to terrestrial sources, but also unique molecules that are only found in marine organisms. Considering that the oceans cover a vast area of our planet, finding bioactive compounds with excellent pharmaceutical properties is promising. In this regard, marine bacteria are of special interest as they are extremely numerous and biotechnologically favorable for cultivation. Despite the enormous difficulty in isolating and harvesting marine bacteria, microbial metabolites are increasingly attractive to science because of their broad-ranging pharmacological activities. The secondary metabolites from marine bacteria, especially those with unique color pigments, not only play an important role in bacterial life, but also have diverse biological properties such as antibiotic and anticancer activities. The latter is of special interest due to the consistent requirement for chemotherapeutic drugs with high selectivity towards malignant cells. As the number of such bioactive compounds is continuously increasing, investigations on their molecular mechanisms of action are now required to be able to select and develop drugs. This current review, based on accumulated data in the literature, gives an overview of the pigmented natural compounds isolated from bacteria of marine origin. It comprises the biological activities of marine compounds, including recent advances in the study of pharmacological effects and other commercial

applications, in addition to the biosynthesis and physiological roles of associated pigments. Special chapter was also *composed* to describe the pigmented cytotoxic compounds isolated from marine bacteria, and discuss their molecular mechanisms of action in preventing tumor growth. Chemical structures of the bioactive compounds discussed in this review are also presented.

*This review combines the contents of the two review papers submitted for the publication in *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 670349, 2011. doi:10.1155/2011/670349 and the forthcoming book “Marine Biomaterials: Isolation, Characterization and Applications,” (Taylor & Francis Group).

1.1. Marine bacteria and its role in life sciences

A wide variety of diseases and medical problems represent a challenging threat to humans, who since ancient times have searched for natural compounds from plants, animals, and other sources to treat them. Although the process of finding effective treatments against fatal diseases is difficult, extensive searches for natural bioactive compounds have previously yielded some successful results. The isolation and identification of specific natural compounds led to the development of folk medicine, and humans learned to separate the isolates into medicinal drugs, which could be used to treat different diseases, and poisonous substances, which could be used for non-medicinal purposes (i.e., during tribal wars, hunting, etc.). Statistically, at least 50% of the existing drugs that are used to treat human illnesses are derived from natural products, most of which are obtained from terrestrial organisms [1]. However, due to continuous and exhaustive research, land-based natural bioactive compounds have become increasingly difficult to find. Instead, water-based natural compounds have become a more promising source, not only from a pharmacological view, but also for industrial and commercial applications.

Theoretically, life is considered to have originated in the sea and, as a result of evolutionary changes, developed into a wide variety of diverse biological systems. The Earth's surface consists of 70% water, which is inhabited by 80% of all life forms [1], and consequently aquatic organisms have a greater diversity than their terrestrial counterparts. As research into the marine environment is still in its early phases, many mysteries associated with aquatic fauna and flora have yet to be discovered. Therefore, the marine environment has recently become an attractive research subject for many investigations, because of its rich biodiversity. Despite being comprised of a diverse ecosystem, the search for marine metabolites is difficult because of the inaccessibility and non-culturability of the majority of organisms [2]. Nevertheless, the existing technologies like deep seawater pumping facilities, scuba diving, and other available equipments, have facilitated investigation of the sea

environment. As a result, scientific research has increasingly focused on marine biochemistry, microbiology, and biotechnology.

Microorganisms and their isolates represent a major source of undiscovered scientific potential. It should be noted that the number of microbial organisms isolated from the vast ocean territories continues to increase each year. Consequently, natural products isolated from microorganisms inhabiting environments other than soil are an attractive research tool, not only for biochemists and microbiologists, but also for pharmacologists and clinicians. Laatsch [3] described the isolation and description of nearly 250 marine bacterial metabolites versus 150 isolated from terrestrial bacteria between 2000 and 2005. Research into marine microorganisms and their metabolites has therefore become a major task in the search for novel pharmaceuticals.

Although many compounds show promising biological activities, it is difficult to point out any particular bioactive agent that has readily been commercialized as a medicine. Currently, 13 natural products isolated from marine microorganisms are being tested in different phases of clinical trials, and a large number of others are in preclinical investigations [4], thus highlighting the potential of marine natural compounds.

Despite thousands of marine bioactive compounds having been isolated and identified, in this paper, we will focus on the pharmacologically active pigmented compounds produced by marine microorganisms exhibiting *in vitro* or *in vivo* biological activities. Although pigmented compounds produced by terrestrial bacteria are beyond the scope of this review, specific examples will still be mentioned for comparative purposes, to outline common biological activities or because identical pigments were isolated from both types of microorganisms.

1.2. Marine microorganisms and their bioactive isolates

Marine and terrestrial microfloras differ from each other due to the influence of their

respective environmental conditions. Microorganisms living in the sea must be able to survive and grow in the water environment with low nutrition, high salinity, and high pressure. That is why most bacteria isolated from seawater are Gram-negative rods, as it is postulated that their outer membrane structure is evolutionarily adapted to aquatic environmental factors. Marine microorganisms can be divided on the basis of habitat into psychrophiles (living at low temperatures), halophiles (living at high salinity), and barophiles (living under high pressure). Although these characteristics highlight the differences between marine and terrestrial microorganisms, it remains difficult to separate bacterial genera on the basis of habitat due to the ubiquitous presence of similar species in both environments. As such, most bioactive compounds have been isolated from bacteria in both environments.

Marine bacteria, however, are attractive to researchers because they can potentially produce compounds with unique biological properties [5]. Until now, marine *Streptomyces*, *Pseudomonas*, *Pseudoalteromonas*, *Bacillus*, *Vibrio*, and *Cytophaga* isolated from seawater, sediments, algae, and marine invertebrates are known to produce bioactive agents. They are able to produce indole derivatives (quinines and violacein), alkaloids (prodiginines and tambjamins), polyenes, macrolides, peptides, and terpenoids. Examples of bioactive-pigmented compounds isolated from marine (and some terrestrial) bacteria are discussed below.

1.3. Pigments from marine bacteria

1.3.1. Carotenes

Carotenes are polyunsaturated hydrocarbons that contain 40 carbon atoms per molecule and are exclusively synthesized by plants. They are orange photosynthetic pigments important for plant photosynthesis. Recently, an unusual halophilic bacterium, which requires 15–25% salt for its normal growth, was found in Santa Pola near Alicante and on the Balearic island of Mallorca, Spain. It appeared to be red or pink due to a wide variety of isoprenoid compounds

(phytoene, phytofluene, lycopene, and β -carotene) produced by this prokaryote. Oren and Rodríguez-Valera [6] investigated red-pigmented saltern crystallizer ponds in these areas of Spain and demonstrated that the pigments were carotenoid or carotenoid-like compounds produced by halophilic bacteria related to the *Cytophaga-Flavobacterium-Bacteroides* group. Thus, it has been shown that *Salinibacter* is an important component of the microbial community that contributes to the red coloration of Spanish saltern ponds.

Astaxanthin is one of the carotenoids that have commercial value as a food supplement for humans and as food additives for animals and fish (Fig.1.1). A carotenoid biosynthesis gene cluster for the production of astaxanthin has been isolated from the marine bacterium *Agrobacterium aurantiacum* [7]. Recently, another astaxanthin-producing marine bacterium was isolated and identified as *Paracoccus haeundaensis* [8].

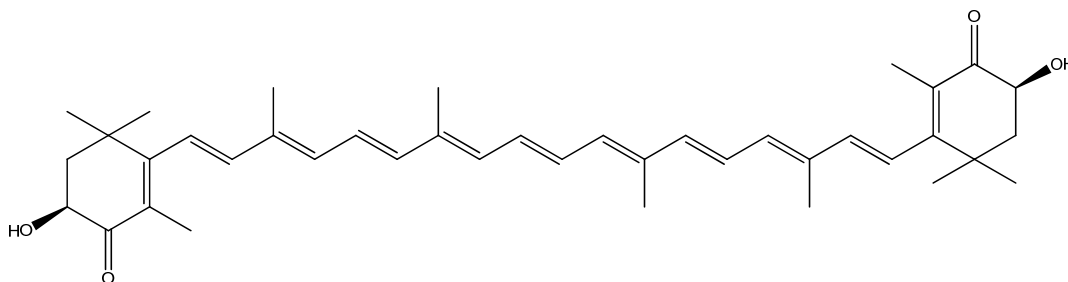


Fig.1.1. Astaxanthin

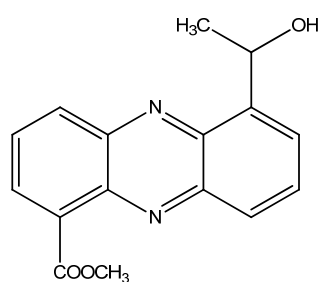
1.3.2. Phenazine compounds

Phenazines are redox-active, small nitrogen-containing aromatic compounds produced by a diverse range of bacterial genera, including *Streptomyces* (terrestrial), *Pseudomonas* (ubiquitous), actinomycetes (terrestrial and aquatic), *Pelagibacter* (aquatic), and *Vibrio* (aquatic), under the control of quorum sensing [9,10] (Fig.1.2). These compounds were subjected to extensive studies due to their broad spectrum of antibiotic activities against other bacteria, fungi, or plant/animal tissues [11-17]. Phenazine color intensity may vary among the derivatives and range from blue, green, purple, yellow, red to even brown [13,18]. More than 6,000 phenazine derivatives have been identified and described during the last two centuries

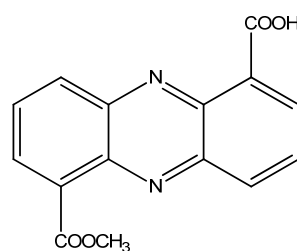
[14].

Maskey et al. [18] reported the isolation of two yellow pigments from the marine *Pseudonocardia* sp. B6273, a member of the actinomycetes. Structural investigations identified the two pigments as novel phenazostatin D, inactive against the tested microorganisms, and methyl saphenate, a known phenazine antibiotic. Li et al. [19] also reported the isolation of a novel phenazine derivative with cytotoxic effects against P388 cells, together with six previously identified compounds from the marine *Bacillus* sp., collected from a Pacific deep-sea sediment sample at a depth of 5059 m. A novel phenazine derivative with antibiotic activity, identified as 5,10-dihydrophencomycin methyl ester, along with (2-hydroxyphenyl)-acetamide, menaquinone MK9 (II, III, VIII, IX-H8), and phencomycin, was isolated from an unidentified marine *Streptomyces* sp. by Pusecker et al [20].

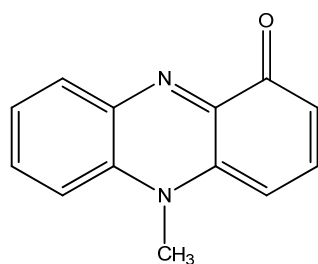
Pyocyanin and 1-hydroxyphenazine also down-regulate the ciliary beat frequency of respiratory epithelial cells by reducing cAMP and ATP, alter the calcium concentration by inhibition of plasma membrane Ca^{2+} -ATPase, and induce death in human neutrophils [15, 16, 21]. Due to the abundance and biotechnological application of *Pseudomonas aeruginosa* phenazines, pyocyanin and pyorubrin have also been suggested as food colorant pigments [13].



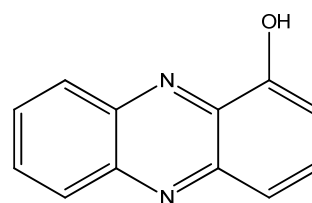
Methyl saphenate



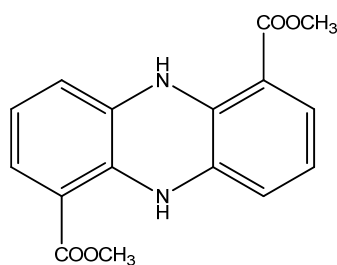
Phencomycin



Phycocyanin



1-hydroxyphenazine



5,10-dihydrophenazine-1,6-dicarboxylate (5,10-dihydrophencomycin methyl ester)

Fig.1.2. Phenazine derivatives.

1.3.3. Quinones

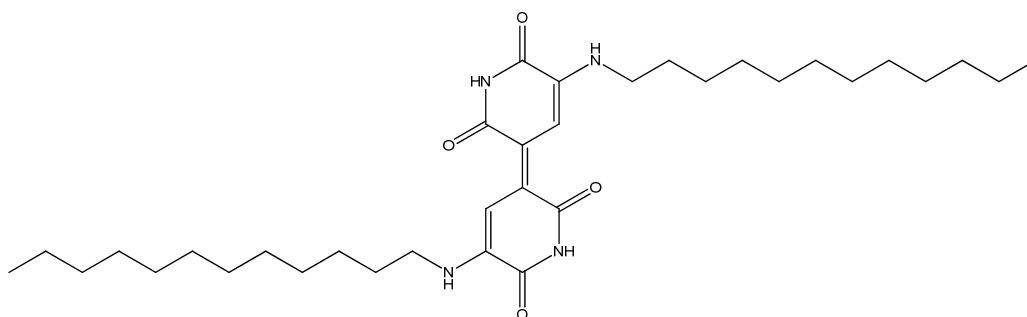
Quinones are additional colored compounds with an aromatic ring structure that have been isolated from marine environment [22,23] (Fig.1.3). Quinone derivatives range in color from yellow to red, exhibit antiviral, antiinfective, antimicrobial, insecticidal, and anticancer activities, and have many commercial applications as natural and artificial dyes and pigments [24,25].

Streptomyces sp. B6921 strain produced glycosylated pigmented anthracycline antibiotics, including fridamycin D and two new compounds, named himalomycin A and B, each of which displayed similar levels of strong antibacterial activity against *Bacillus subtilis*, *Streptomyces viridochromogenes* (Tü 57), *S. aureus*, and *Escherichia coli*. This strain also produced rabelomycin, *N*-benzylacetamide, and *N*-(2'-phenylethyl) acetamide [23]. Two novel pigmented antitumor antibiotics, chinikomycin A and B, together with manumycin A, were isolated from a marine *Streptomyces* sp. strain M045 [26]. The two chlorine containing

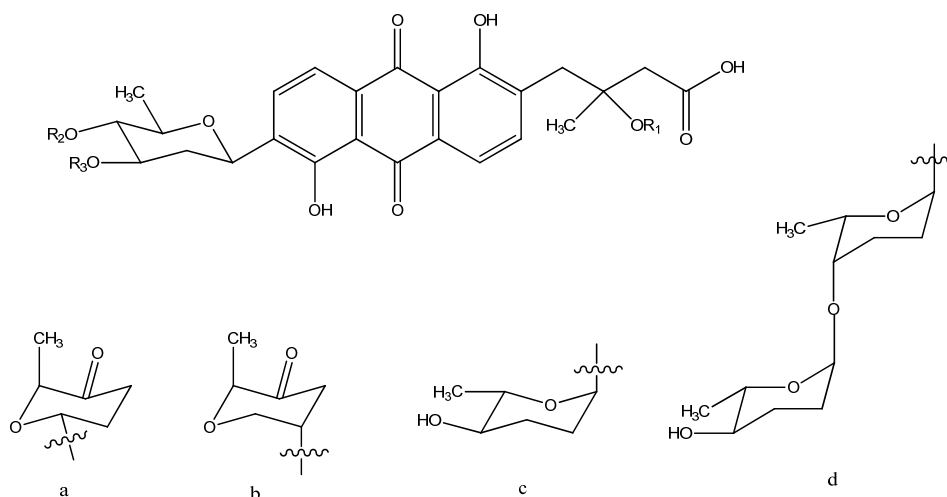
quinone derivatives were shown not to have antiviral, antimicrobial, and phytotoxic activities; however, they exhibited antitumor activity against different human cancer cell lines. Chinikomycin A selectively inhibited the proliferation of mammary cancer, melanoma, and renal cancer cell lines, while chinikomycin B showed selective antitumor activity against a mammary cancer cell line [26].

Other bacteria, including a marine isolate *Pseudomonas nigrifaciens* (later reclassified as *Alteromonas nigrifaciens*), produce the blue pigment indigoidine [27]. Kobayashi et al. [28] isolated a new violet pigment with an alkylated indigoidine structure from *Shewanella violacea*, a deep-sea bacterium from sediments of Ryukyu Trench at a depth of 5110 m. This pigment was established as 5,5'-didodecylamino-4,4'-dihydroxy-3,3'-diazodiphenylquinone-

(2,2') based on X-ray diffraction analysis of single crystals. It does not have antibiotic activity against *E. coli*; however, it could potentially be used as a dye because of its high stability and low solubility. Thus, it could be suitable for industrial applications.



5,5'-didodecylamino-4,4'-dihydroxy-3,3'-diazodiphenylquinone-(2,2')



a

b

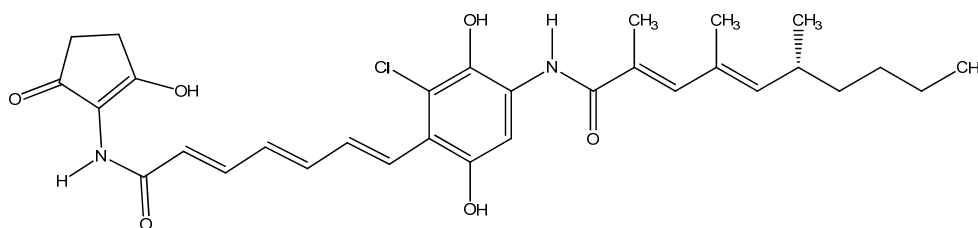
c

d

Fridamycin D: $R_1=H$, $R_2=a$, $R_3=b$;

Hymalomyacin A: $R_1=d$, $R_2=a$, $R_3=b$;

Hymalomyacin B: $R_1=d$, $R_2=c$, $R_3=H$



Chinikomycin A

Fig.1.3. Quinones.

1.3.4. Melanins

Vibrio cholerae, *Shewanella colwelliana*, and *Alteromonas nigrifaciens* were some of the first marine bacterial strains described to produce melanin or melanin-like pigments [29-32]. The pigment synthesized by *Vibrio cholerae* was reported to be a type of allomelanin derived from homogentisic acid [33]. Melanin formation in *V. cholerae* is a consequence of alterations in tyrosine catabolism and not from the tyrosinase-catalyzed melanin synthetic pathway. *Cellulophaga tyrosinoydans* was reported to have tyrosinase activity and produce a yellow pigment suggested to be a pheomelanin [34].

The most illustrative example of melanin-producing marine bacteria is the actinomycetes. This is particularly the case for the genus *Streptomyces*, from which most compounds with known biological activity have been isolated [35]. All *Streptomyces* strains are reported to use tyrosinases in the synthesis of melanin pigments [36]. Another important melanin-synthesizing bacterium is *Marinomonas mediterranea*, which produces black eumelanin from L-tyrosine [37].

1.3.5. Other pigmented compounds

Scytonemin, a yellow green pigment isolated from aquatic cyanobacteria, forms when the bacteria are exposed to sunlight (Fig1.4). It protects bacteria by preventing about 85–90% of all UV-light from entering through the cell membrane [38]. High UV-A irradiation inhibited photosynthesis and delayed cellular growth until sufficient amounts of scytonemin had been produced by the cyanobacteria. Scytonemin may also have anti-inflammatory and antiproliferative activities by inhibiting protein kinase C β (PKC β), a well-known mediator of the inflammatory process, and polo-like protein kinase 1 (PLK1), a regulator of cell cycle progression [39]. In addition, scytonemin inhibited phorbol-induced mouse ear edema and the proliferation of human umbilical vein endothelial cells.

Recently, two γ -*Proteobacteria* strains of the genus *Rheinheimera* were isolated from the German Wadden Sea and from Øresund, Denmark that produced a deep blue pigment [40]. Structural analysis of the pigment revealed that this new compound has no similarity with any known blue pigments, like violacein and its derivatives. Due to its blue color and marine origin, the new pigment was named glaukothalin (from Greek *glaukos* “blue” and *thalatta* “sea”). The ecological role and biological activities of glaukothalin are currently under investigation.

AM13,1 strain, which was identified to belong to the *Cytophaga/Flexibacteria* cluster of North Sea bacteria, was found to produce yellow tryptanthrin, a rare compound that had never before been found in bacteria [41]. This compound was suggested to be a biocondensation product of anthranilic acid and isatin and exhibited a broad yet moderate antibiotic activity. Thus, the yellow color of the AM13,1 colonies was potentially due to their tryptanthrin content. In another yellow cultured Hel21 strain, pigment color may be a consequence of carotenoid zeaxanthin or one of the many vitamin K derivatives (e.g., menaquinone MK6) [41].

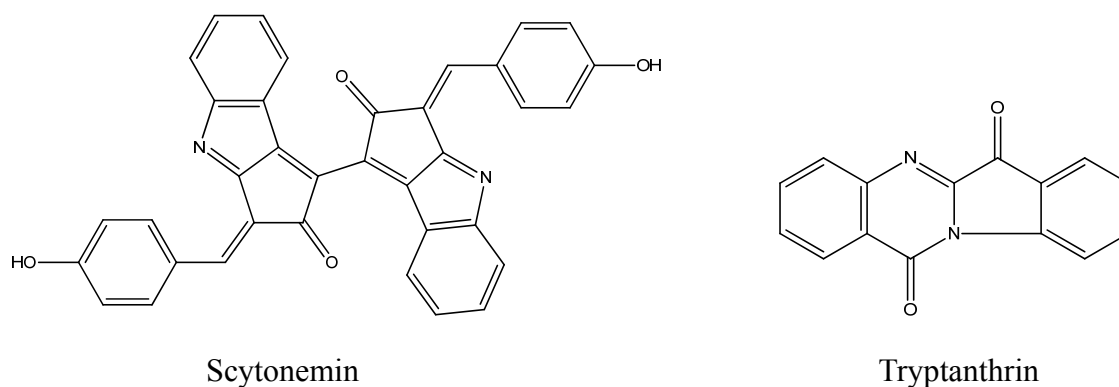


Fig.1.4. Other pigmented compounds.

Bioactive pigments from marine bacteria are summarized in Table 1.1.

Table 1.1. Biologically active pigmented compounds isolated from marine bacteria

Pigment	Activity	Bacterial strains	References
1. Astaxanthin (carotene)	Anti-oxidation	<i>Agrobacterium urantiacum</i>	[7]
2. Cycloprodigiosin	Immunosuppressant; Anticancer;	<i>Pseudoalteromonas</i> <i>denitrificans</i>	[42], [43], [44]
	Antimalarial	<i>Streptomyces</i> sp.	
3. 5,10-dihydrophencomycin methyl ester	Antibiotic	<i>Streptomyces</i> sp. B6921	[20]
4. Fridamycin D, Himalomycin A, Himalomycin B	Antibacterial	α -Proteobacteria <i>Vibrio cholerae</i> <i>Shewanella colwelliana</i>	[23]
5. Heptyl prodigiosin	Antiplasmodial	<i>Alteromonas nigrifaciens</i>	[45]
6. Melanins	Protection from UV irradiation	<i>Cellulophaga</i> <i>tyrosinoydans</i>	[29], [30] [29], [32]
7. Methyl saphenate (phenazine derivative)	Antibiotic	<i>Pseudonocardia</i> sp. B6273	[31] [34]

8. Phenazine-1-carboxylic acid	Antibiotic	<i>Pseudomonas aeruginosa</i>	[18]
9. Prodigiosin	Antibacterial; Anticancer; Algicidal	<i>Pseudoalteromonas rubra</i> <i>Hahella chejuensis</i>	[14] [46]
10. Pyocyanin and pyorubrin	Antibacterial	<i>Pseudomonas aeruginosa</i>	[47]
11. Scytonemin	Protection from UV irradiation Anti-inflammatory, anti-proliferative	Cyanobacteria	[13] [39]
12. Tambjamines (BE-18591, pyrrole and their synthetic analogs)	Antibiotic, anticancer	<i>Pseudoalteromonas tunicata</i>	[48], [49]
13. Tryptanthrin	Antibiotic	<i>Cytophaga/Flexibacteria</i> AM13,1 strain	[41]
15. Violacein	Antibiotic; Antiprotozoan; Anticancer	<i>Pseudoalteromonas luteoviolacea</i> <i>Pseudoalteromonas tunicata</i> <i>Pseudoalteromonas</i> sp. 520P1 <i>Collimonas</i> CT	[50], [51] [52] [53] [54] [55]

1.4. Antitumor pigments from marine bacteria

Transformation of normal cells to malignant cells is known to occur as a result of mutagenesis,

which has as a consequence the uncontrollable and abnormal multiplication of these cells within the body. Chemotherapy, the use of biologically active chemical compounds to interrupt and stop the growth of cancer cells, remains one of the most effective ways to cure cancer. However, the lack of effective chemotherapeutic drugs that can completely annihilate cancer cells remains an unsolved problem for modern science. Finding those desirable bioactive compounds has become a challenging task for researchers facing the limited efficacy of drugs used in clinical practice. Another challenge for the treatment of cancer is the ability of cancer cells to develop chemoresistance against drugs. Therefore, highly effective chemical compounds that can selectively eliminate tumor cells while not affecting normal cells are still in urgent demand. The newly designed drugs should preferably have selective inhibitory effects towards molecules that initiate anti-apoptotic mechanisms, while inducing pro-apoptotic proteins [56].

It is estimated that more than 50% of the 100 isolates obtained from marine sources are potentially useful bioactive substances [57]. Most of these compounds have either antibiotic or cytotoxic activities. Thus, in the period between 1998 and 2008, 592 marine compounds with cytotoxic activity were reported to have entered the stage of preclinical investigation. During this period, other 666 chemicals demonstrated antibiotic activities including antibacterial, antifungal, antihelminthic, antiprotozoal and antiviral activities, as well as anticoagulant, anti-inflammatory and antiplatelet effects, which have impact on the cardiovascular, endocrine, immune, and nervous systems [58]. Among these active metabolites, the compounds from marine bacteria are of special interest.

While most of these compounds are reported to have potential antibiotic activities, a number of them also show cytotoxic effects [59]. The chemical spectrum of antitumor compounds derived from marine bacteria is wide, including indolocarbazoles, polyketides, alkaloids, isoprenoids (terpenoids) and even peptides (Table 1.2).

Despite thousands of bioactive metabolites having been isolated from marine bacteria,

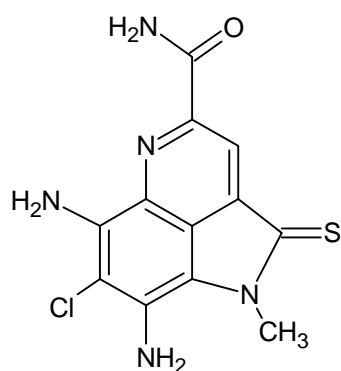
this chapter focuses only on colored compounds which have demonstrated either *in vitro* or *in vivo* apoptotic effects against cancer cells. Table 1.2 summarizes some of the bioactive antitumor pigments isolated from marine bacteria. The listed compounds do not show equal effects to all kinds of tumor cells, but they are rather selective to particular types of cells, showing cell type-specific action. This may be the reason why the bioactivity of the compounds is expressed in different half effective concentrations such as IC₅₀ (IC₇₀), GI₅₀, TGI₅₀, LC₅₀, LD₅₀, EC₅₀, ED, GIC, and MIC (Table 1.2). At present, the true bioactivity of some compounds regarding selectivity and sensitivity towards cancer cells is unknown. For this reason, finding these values through further research will be necessary to be able to select suitable tumor cell lines that are more sensitive to the compounds of interest, as a pre-requisite for drug development. Although the cytotoxic compounds discussed below belong to a specific bacterial family, it is well known that one specific compound can be produced by many different species of bacteria, regardless if they are from terrestrial or marine origin. This indicates that the same or similar gene clusters responsible for the production of specific biomolecules may exist in many different species of bacteria, probably as product of the horizontal transfer of gene clusters among them.

1.4.1. Cytotoxic pigments from marine actinomycetes

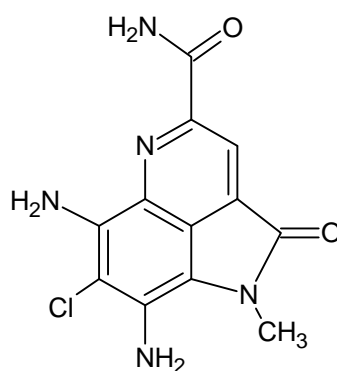
When referring to pigmented cytotoxic compounds isolated from aquatic flora, marine *Pseudoalteromonas*, cyanobacteria, and especially *Streptomyces* stand out as they are the most important sources of these active metabolites. Terrestrial *Streptomyces* sp., first described by Waksman and Henrici [60], has proved to be the richest source of compounds with biological activities among bacterial species. Nearly 75% of the active metabolites isolated from *Streptomyces* have antibiotic and antitumor activities [61]. In a survey of active metabolites isolated from microorganisms, published in *The Journal of Antibiotics* between 1984 and 1993, Miyadoh [61] reported that actinomycetes are the most important sources of bioactive

compounds, with a total of 93% of all antitumor antibiotics having been isolated from this group of bacteria. Despite the lack of reports, marine-derived counterparts have also demonstrated to be significant sources of active metabolites, both quantitatively and qualitatively. The number of active metabolites isolated from marine strains of actinomycetes is increasing considerably. In fact, it has been discovered that the aquatic species of actinomycetes produce not only the same compounds as their terrestrial counterparts [62], but also other types of compounds with unique structures and pharmacological activities. The search for new antitumor antibiotics from marine bacteria has led to the discovery of a wide range of pigmented cytotoxic compounds with different potency and selectivity, obtained from marine *Streptomyces* species found in different marine environments (Table 1.2). Varying not only in their appearance, but also in their chemical structures, these toxic compounds induce apoptosis in different ways, acting through various yet not fully understood pathways. These may include extrinsic pathways, through death receptor signaling, and intrinsic ones which involve multiple other intracellular signaling pathways. An example of this is streptochlorin, a yellowish crystalline solid isolated from marine *Streptomyces* sp. 04DH110 (Fig.1.5 and Table 1.2, No.29) [63]. This compound was reported to activate caspases, upregulate the pro-apoptotic Bax and FasL, decrease the mitochondrial membrane potential, and increase the degradation of poly-(ADP-ribose)polymerase(PARP) and phospholipase C- γ 1 proteins, while inhibiting the action of the anti-apoptotic Bcl-2 protein towards U937 leukemia cells [64]. Another examples are the blue and red colored ammosamides A and B isolated from the *Streptomyces* strain CNR-698 (Fig.1.5 and Table 1.2, No.2), which were reported to have pronounced selectivity against various cancer cell lines, with IC₅₀ values ranging from 20 nM to 1 μ M. *In vitro* cytotoxicity assay of both compounds against HCT-116 (HeLa) colon carcinoma cells produced an IC₅₀ value of 320 nM. A preliminary investigation of their molecular mechanisms of action, carried out by converting them to highly conjugated fluorescent molecules, revealed that these compounds act on

proteins of the myosin family, which are responsible for numerous cell processes, including cell cycle regulation, cytokinesis, and cell migration [65]. There are dozens of other compounds isolated from marine actinomycetes whose anticancer activity mechanisms have yet to be determined (Table 1.2). It is interesting to note that the yellow colored *N*-carboxamido-staurosporine isolated from marine *Streptomyces* sp. QD518 has a more potent anticancer activity than its colorless analogs *N*-formyl-staurosporine and sesquiterpene (Fig.1.5 and Table 1.2, No.23). Exhibiting a mean IC₅₀ value of 0.016 µg/mL and a mean IC₇₀ value of 0.17 µg/mL, this compound showed high tumor selectivity against 37 cancer cell lines from bladder, central nervous system, colon, gastric, head and neck, lung, mammary, ovarian, pancreatic, prostate, renal, skin, pleural mesothelium and uterine cancers [66]. However, the mechanism of action of this compound remains elusive.



Ammosamide A



Ammosamide B

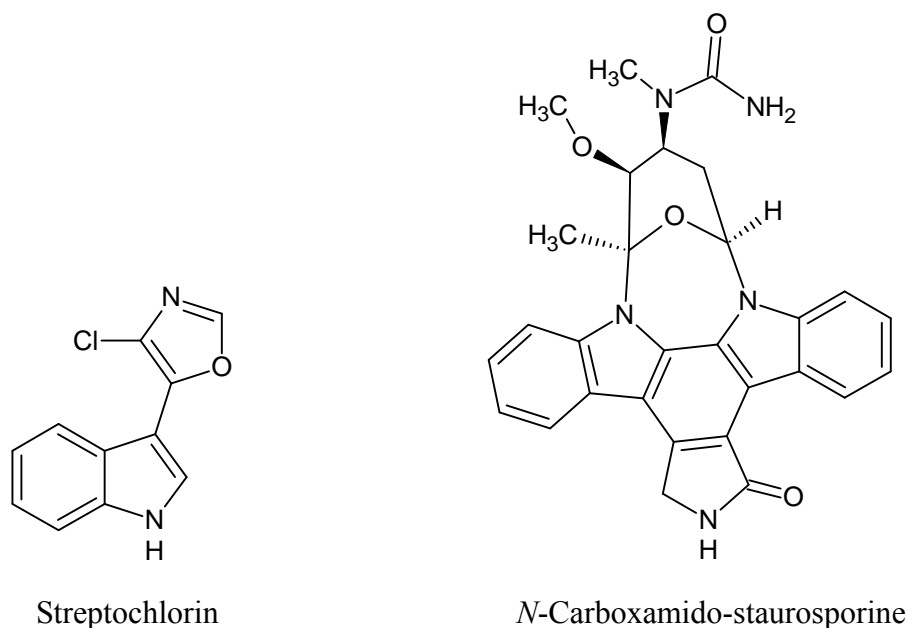


Fig.1.5. Some pigmented compounds isolated from marine *Streptomyces*

1.4.2. Cytotoxic pigments from marine *Pseudoalteromonas*

The genus *Pseudoalteromonas*, solely belonging to a marine type of bacteria, has two kinds of species: pigmented and non-pigmented. According to some reports, the pigmented species are more effective than the non-pigmented species in terms of producing more potent biologically active compounds [67]. Holmström and co-workers reported that the pigmented species were more effective against biofouling than their non-pigmented counterparts [68, 69]. The pigmented strains seem to produce much stronger toxic secondary metabolites with a wide range of bioactivities, an essential requirement for drug development. Among these compounds, the ones with antitumor activities attract special interest. These compounds are discussed below and are also listed in Table 1.2.

9H-pyrido[3,4-b]indole (norharman), a light yellow colored compound isolated from *Pseudoalteromonas piscida*, has been reported to have apoptotic effects (Fig. 1.6 and Table 1.2, No.39). Although the exact molecular mechanisms of action are still elusive, the reported effects involved chromatin condensation and DNA degradation, which are characteristic of apoptosis [70].

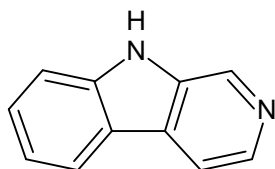


Fig.1.6. 9H-pyrido[3,4-b]indole (norharman)

1.4.2.1. Prodiginines

Red-pigmented prodigiosin compounds were first isolated from the ubiquitous bacterium *Serratia marcescens* and identified as secondary metabolites. The common aromatic chemical structure of these pigmented compounds was first named prodiginine by Gerber [71] (Fig.1.7). Prodigiosin was the first prodiginine for which the chemical structure was determined [72]. The name “prodigiosin” has been attributed to the isolation of prodigiosin from *Bacillus prodigiosus* bacterium (later renamed *Serratia marcescens*) [73], which was historically famed for the mysterious “bleeding bread” report [74, 75]. Prodiginines share a common pyrrolyldipyrromethene core structure and have a wide variety of biological properties, including antibacterial, antifungal, antimalarial, antibiotic, immunosuppressive, and anticancer activities [74, 76]. Such properties potentially make them one of the most powerful research tools in the past decade.

There are many research reports and reviews regarding prodiginines and their biological activity investigations. In addition to the *Serratia*, several species of marine bacteria of the genera *Streptomyces* [73], *Actinomadura* [73], *Pseudomonas* [77], *Pseudoalteromonas* [42, 48, 78–81], and others [82] have also been reported to produce prodigiosin and related compounds. In particular, *Alteromonas denitrificans*, which was isolated from the fjord systems off the west coast of Norway [80] and later reclassified as *Pseudoalteromonas denitrificans* [83], has been reported to produce cycloprodigiosin. This compound has immunosuppressive, antimalarial, and apoptosis-inducing activities [42-44]. Cycloprodigiosin demonstrated strong *in vitro* and *in vivo* apoptotic effects on liver cancer cell lines including

Huh-7, HCC-M, HCC-T (human hepatocellular carcinoma), HepG2 (human hepatoblastoma), dRLh-84 and H-35 (rat hepatocellular carcinoma) [42]. Suppressed cellular proliferation as a result of apoptosis was suggested to be due to the decrease of intracellular pH, caused by the uncoupling effect of cycloprodigiosin on proton transport by V-ATPase. The anti-apoptotic NF- κ B protein activity responsible for DNA transcription was also reported to be suppressed by cycloprodigiosin [84]. *Pseudoalteromonas rubra*, found in the Mediterranean coastal waters [78], also produces cycloprodigiosin, in addition to prodigiosins [48, 79]. α -*Proteobacteria* isolated from a marine tunicate collected in Zamboanga, Philippines, was reported to produce heptylprodigiosin. *In vitro* antimalarial activity against *Plasmodium falciparum* 3D7 (IC₅₀ = 0.068 mM and SI = 20) was about 20 times the *in vitro* cytotoxic activity against L5178Y mouse lymphocytes [45]. *In vivo* experiments using *Plasmodium berghei*-infected mice, at concentrations of 5mg/kg and 20mg/kg, significantly increased their survival, while also causing sclerotic lesions at the site of injection. Heptylprodigiosin, isolated from a marine α -proteobacterium, was reported to have *in vitro* cytotoxic activity against L5178Y mouse lymphocytes in addition to its antimalarial activity against *Plasmodium falciparum* 3D7 (Fig.1.7 and Table 1.2, No.47) [47]. This compound displayed synergistic effect with another cytotoxic compound, adociaquinone B, isolated from the marine sponge *Xestospongia* sp., against MCF-7 breast cancer cells [85]. A 3:1 ratio of adociaquinone B: heptylprodigiosin was the most effective, resulting in the greatest reduction of the IC₅₀ values of the individual compounds, thus reducing the possible toxicity against normal cells.

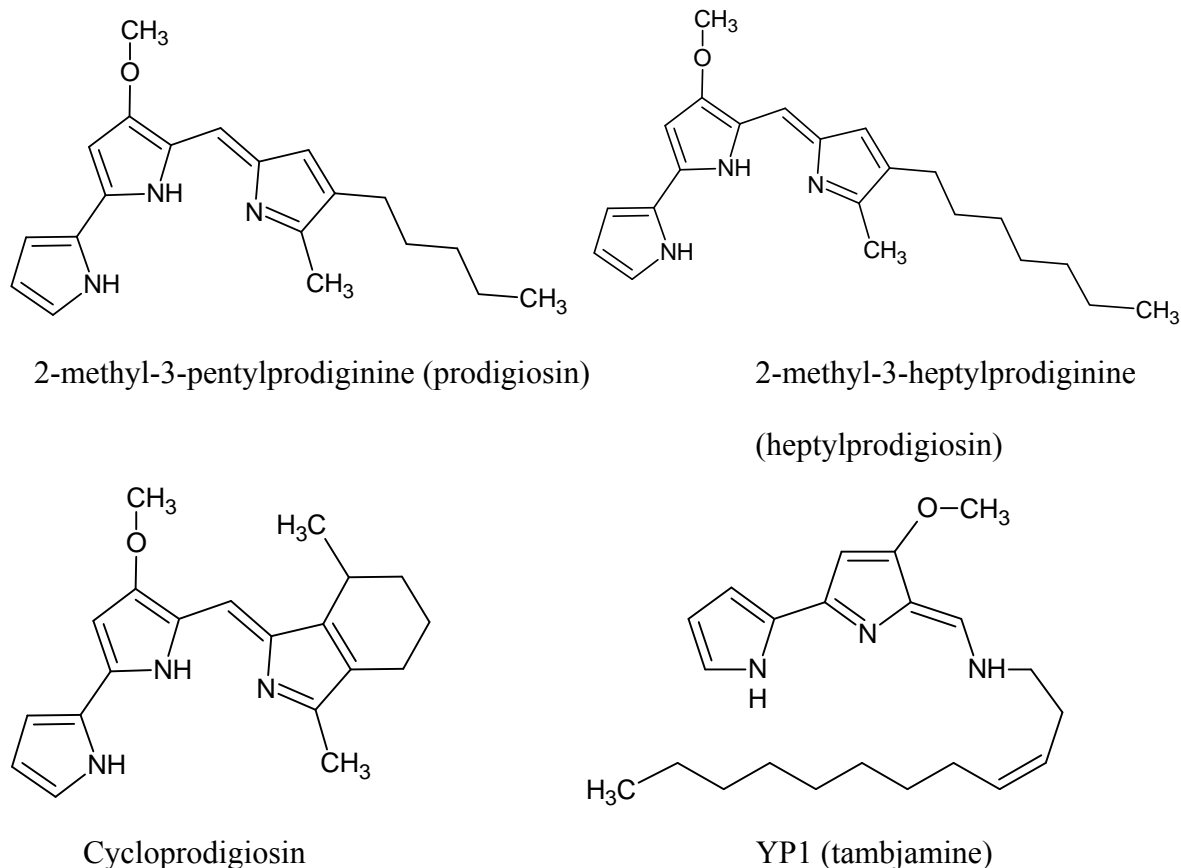


Fig.1.7. Prodiginines and tambjamine

The prodiginin-like pigment PG-L-1, isolated from the bacterial strain MS-02-063 belonging to γ -proteobacterium, was reported to have a strong cytotoxic effect against MDCK, CHO, HeLa, Vero, XC, and PtK₁ cells [86]. PG-L-1 also induced apoptosis of U937 cells, accompanied by the increase of intracellular pH, activation of p38 MAP kinase, inhibition of O²⁻ generation and DNA fragmentation. The apoptosis mechanisms of PG-L-1 may be attributed to the increase of intracellular pH and the activation of p38 MAP kinase [86].

Biologically active components of the marine *Pseudoalteromonas tunicata* have been identified as alkaloid tambjamins, the chemical structures of which are partly similar to prodiginines (Fig.1.7 and Table 1.2, No.37) [51]. Screening of the biological activities of some tambjamins against certain human cancer cell lines has revealed that they possess moderate antitumor activity compared to the doxorubicin control [52].

Other bacteria reported to produce red pigments include *Hahella* [87], *Vibrio* [88],

Zooshikella [89], and *Pseudoalteromonas* [81], isolated from the coasts of Korea, Taiwan, and Japan. Kim et al. [47] identified red-pigmented prodiginines from *Hahella chejuensis*. Nakashima et al. also evaluated the biological activity of similar prodiginines from a bacterium assumed to belong to the genus *Hahella* [90]. Red pigment-producing bacterial species have further been isolated from river water [91, 92] and even from a swimming pool [93]. The most active prodiginine derivatives have already entered clinical trials as potential drugs against different cancer types [74].

Prodiginines were reported to have anticancer activity against nearly 60 human cancer cell lines with little or no effect on normal cells [78, 94]. In fact, these compounds were proved to induce apoptosis even in drug-resistant tumor cells, thus being able to overcome one of the main problems in chemotherapy. Synthetic prodiginine derivatives such as PNU156804 and GX15-070 have already entered clinical trials to develop immunosuppressive and anticancer drugs, respectively [78]. Although advanced studies have been conducted on these compounds to determine their molecular mechanisms of cytotoxicity, information regarding the way they induce apoptosis remains elusive because of their extremely wide cellular targets (Fig.1.8). Despite the lack of information regarding the cytotoxicity mechanisms of prodiginines, four main possible pathways leading to apoptosis of cancer cells have been proposed: (1) Regulation of intracellular pH by proton pumping ATPases [95], (2) Arrest of cell cycle at different levels of G- or S-stages by inhibition of protein activities promoting cell differentiation and proliferation [80, 96, 97], (3) Copper (II)-mediated DNA fragmentation [98], and (4) Regulation of signal transduction pathway molecules [99, 100].

Prodigiosin has also demonstrated antimetastatic activity against 95-D human lung carcinoma and B16BL6 mouse melanoma cells by inhibiting their migration and invasion, both *in vitro* and *in vivo*. The action of prodigiosin is dose-dependent, reaching 50% metastasis inhibition of 95-D cells at 4.66 μM for 12 h [101]. These findings indicate that

prodiginosin-like compounds are potential chemotherapeutic drug candidates against cancer.

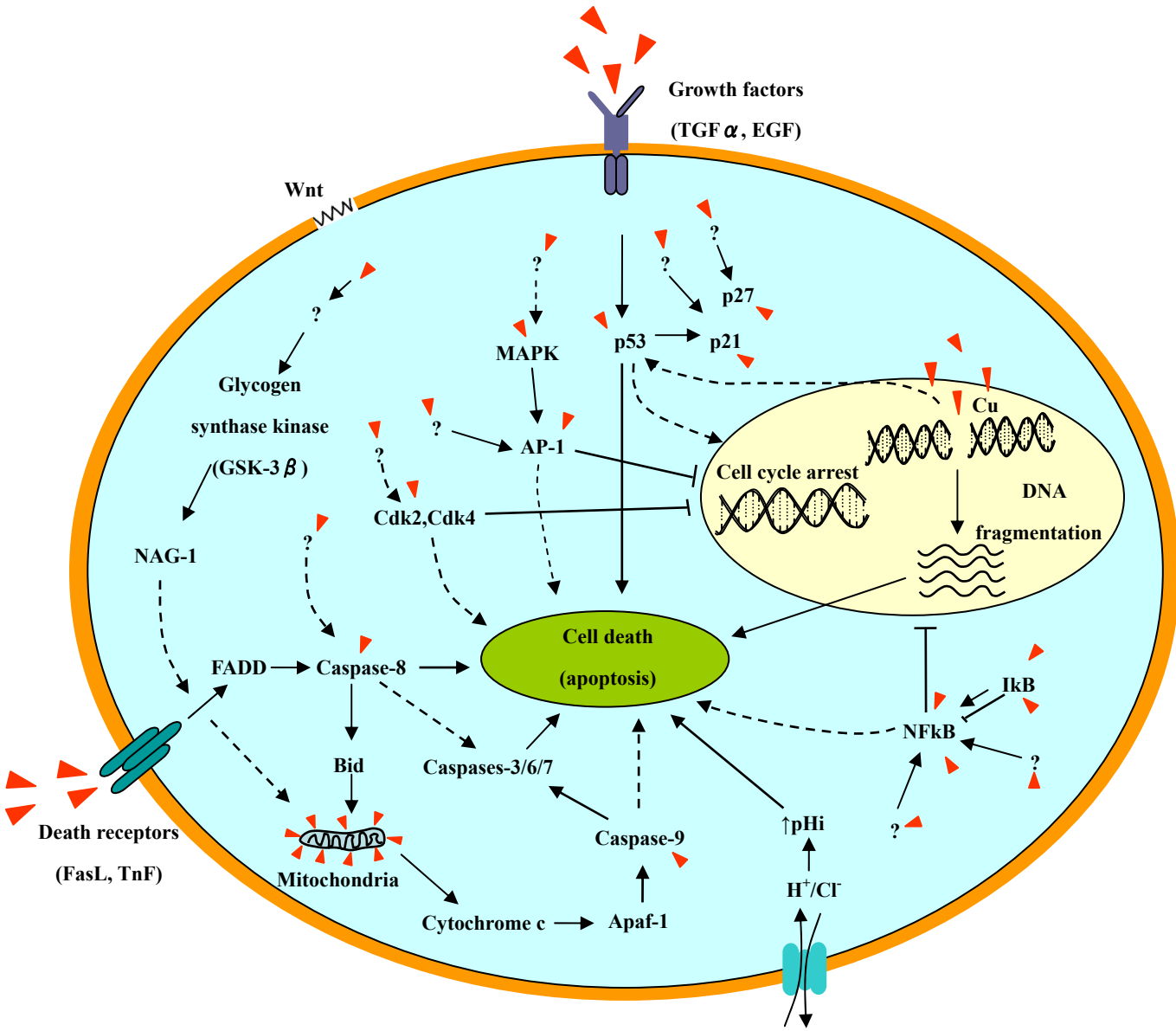


Fig.1.8. Possible cellular targets of prodiginines leading to apoptosis.

The figure shows intrinsic (mitochondrial) and extrinsic pathways that participate in the apoptotic process by prodiginines. These two pathways, comprising four main mechanisms, are suggested to induce apoptosis by prodiginines in the following way: (1) Regulation of intracellular pH by proton pumping ATPases, (2) Arrest of cell cycle at different levels of G- or S-stages by inhibition of protein activities promoting cell differentiation and proliferation, (3) Copper (II)-mediated DNA fragmentation, (4) Regulation of signal transduction pathway molecules.

Table 1.2. Antitumor pigments isolated from marine bacteria.

No	Compound	Appearance	Structural class	Source	Active against (cell lines)	Effective dose	Reference
Actinomycetes							
1.	Actinofuranones A & B	Yellow oils	Polyketide	<i>Streptomyces</i> sp. CNQ766	Mouse splenocyte T-cells and macrophages	IC ₅₀ : 20 µg/mL	[102]
2.	Ammosamides A & B	Blue & red solids	Pyrroloiminoquinone	<i>Streptomyces</i> sp. CNR-698	HCT-116 (colorectal carcinoma), HeLa	IC ₅₀ : 0.02~1 µM	[65]
3.	Arcyriaflavin A	Orange crystal	Indolocarbazole	Marine actinomycete <i>Z₂039-2</i>	K562 (leukemia)	IC ₅₀ : 100 µM	[103]
4.	Butenolides	Yellow syrup	Butenolide	<i>Streptoverticillium</i> <i>luteoverticillatum</i>	K562; P388 (leukemia)	IC ₅₀ : 8.73, 6.29, 1.05 µmol/mL; 0.34, 0.19, 0.18 µmol/mL	[104]
5.	Chandrananimycins A, B, C	Orange solid	Phenoxazinone	<i>Actinomadura</i> sp. M048	CCL HT29 (colon carcinoma),	IC ₇₀ : <1.4 µg/mL	[105]

					MEXF 514L (melanoma), and others		
6.	Chartreusin	Yellow solid	Polyketide	<i>Streptomyces</i> sp. QD518	L1210 (leukemia), B16 (melanoma)	Not mentioned	[66, 106]
7.	Chinikomycin A	Yellowish-brown solid	Polyketide	<i>Streptomyces</i> sp. M045	MAXF 401NL (mammary cancer), RXF 944L (renal cancer), and others	IC ₅₀ : 2.41, 4.02 µg/mL	[26]
8.	Chinikomycin B	Red solid	Polyketide	<i>Streptomyces</i> sp. M045	MAXF 401NL	IC ₅₀ : 3.04, µg/mL	[26]
9.	Chlorinated dihydroquinones	Pale yellow crystals	Terpenoid dihydroquinones	<i>Streptomyces</i> sp. CNQ-525	HCT-116	IC ₅₀ : 0.97~2.40 µg/mL	[107]
10.	Daryamides A-C	Yellow powder	Polyketide	<i>Streptomyces</i> sp. CNQ-085	HCT-116	IC ₅₀ : 3.15~10.03 µg/mL	[108]
11.	Gutingimycin	Yellow	Polyketide	<i>Streptomyces</i> sp. B8652	Not mentioned	IC ₇₀ : 3.4 µg/mL	[109]

12.	IB-00208	Orange	Polyketide	<i>Actinomadura</i> sp. BL-42-PO13-046	P-388, A-549 (lung carcinoma), HT-29 (colon cancer), SK-MEL-28 (melanoma)	MIC: 1 nM	[110]
13.	Iodinin	Violet solid	Phenazine	<i>Actinomadura</i> sp. M048	MAXF 401NL, RXF 944L, and others	IC ₅₀ : 3.6 μg/mL	[105]
14.	Questiomycin A	Orange solid	Phenoxazinone	<i>Actinomadura</i> sp. M048	CCL HT29, MEXF 514L, and others	IC ₇₀ : <1.4 μg/mL	[105]
15.	K252c	Yellow crystal	Indolocarbazole	Marine actinomycete Z ₂ 039-2	K562	IC ₅₀ : 10 μM	[103]
16.	Lajollamycin	Yellow solid	Mixed polyketide/non-riboso mal peptide	<i>Streptomyces</i> <i>nodosus</i> (NPS007994)	B16-F10 (melanoma)	EC ₅₀ : 9.6 μM	[111]
17.	Lomaiviticins A and B	Amorphous red powder	Diazobenzofluorene glycosides	<i>Micromonospora</i> <i>lomaivitiensis</i>	A panel of cancer cells	IC ₅₀ : 0.01~98 ng/mL	[112]
18.	Lucentamycins A & B	Yellow oil	Peptides	<i>Nocardioopsis</i> <i>lucentensis</i> (CNR-712)	HCT-116	IC ₅₀ : 0.20, 11 μM	[113]

19.	Manumycin A	Yellow solid	Polyketide	<i>Streptomyces</i> sp. M045	L-1210	IC ₅₀ : 3.1 μg/mL	[26, 114]
20.	Marinomycins A-D	Yellow powder	Polyketide	Marine actinomycete <i>Marinispora</i> CNQ-140	NCI's 60 cancer cell line panel	LC ₅₀ : 0.2~2.7 μM	[115]
21.	Marmycin A	Red crystalline solid	Polyketide	<i>Streptomyces</i> sp.	HCT-116; 12 other cancer cells	IC ₅₀ : 0.06 μM; 0.022 μM	[116]
22.	Marmycin B	Pink crystalline solid	Polyketide	<i>Streptomyces</i> sp.	HCT-116; 12 other cancer cells	IC ₅₀ : 1.09 μM; 3.5 μM	[116]
23.	<i>N</i> -carboxamido-staurosporine	Yellow solid	Indolocarbazole	<i>Streptomyces</i> sp. QD518	37 cancer cells	IC ₅₀ : 0.016 μg/mL; IC ₇₀ : 0.17 μg/mL	[66]
24.	Parimycin	Orange solid	Polyketide	<i>Streptomyces</i> sp. B8652	GXF 251L (gastric cancer), H460 (lung cancer), and others	IC ₇₀ : 0.9~6.7 μg/mL	[117]
25.	Piericidins C7 & C8	Yellow	Polyketide	<i>Streptomyces</i> sp. YM14-060	Rat glial cells; mouse Neuro-2a cells	IC ₅₀ : 1.5, 0.45 nM; 0.83, 0.21 nM	[118]

(neuroblastoma)							
26.	Resistoflavine	Yellow solid	Polyketide	<i>Streptomyces chibaensis</i> AUBN ₁ /7	HMO2 (gastric adenocarcinoma) a) HePG2 (hepatic carcinoma)	GI ₅₀ : 0.007 µg/mL; TGI: 0.009 µg/mL; LC ₅₀ : 0.013 µg/mL GI ₅₀ : 0.010 µg/mL; TGI: 0.013 µg/mL; LC ₅₀ : 0.016 µg/mL	[119]
27.	Resistomycin	Yellow solid	Polyketide	<i>Streptomyces</i> sp. B8005, <i>Streptomyces</i> sp. B4842,	MCF-7 (breast cancer), UACC-62 (melanoma), and others	>50 ng/mL	[120, 121]
28.	SS-228 Y	Yellowish-brown powder	Polyketide	<i>Chainia</i> sp. SS-228	Ehrlich ascites tumor cells	LD ₅₀ : 1.56~6.25 mg/kg	[122]
29.	Streptochlorin	Yellow crystalline solid	Indole	<i>Streptomyces</i> sp. 04DH110	U937 (leukemia)	IC ₅₀ : 10~12 µg/mL	[64, 123]
30.	Tetracenomycin D	Yellow-orange	Polyketide	<i>Streptomyces</i> sp.	L1210	IC ₅₀ : 22.1 µM	[120, 124]

		solid	B8005				
31.	Thiocoraline	Pale yellow crystalline	Cyclic thiodepsipeptide	<i>Micromonospora</i> sp. L-13-ACM2-092	P388, A549, HT-29, MEL-28 (melanoma)	IC ₅₀ : 0.002~0.01 μg/mL	[125, 126]
32.	Trioxacarcins A-D	Yellow solid	Polyketide	<i>Streptomyces</i> sp. B8652	HT-29, MEXF 514L, and others	IC ₇₀ : 0.001~2.161 μg/mL	[127]
33.	1-Hydroxy-1-norresistomycin	Pale yellow solid	Polyketide	<i>Streptomyces chibaensis</i> AUBN _{1/7}	HMO2, HePG2	GI ₅₀ : 0.009 μg/mL; TGI ₅₀ : 0.012 μg/mL; LC ₅₀ : 0.015 μg/mL GI ₅₀ : 0.014 μg/mL; TGI ₅₀ : 0.018 μg/mL; LC ₅₀ : 0.021 μg/mL	[128]
34.	1,6-Phenazinediol	Yellow solid	Phenazine	<i>Actinomadura</i> sp. M048	LXFA 629L, LXFL 529L, (lung carcinoma),	IC ₅₀ : 3.2 μg/mL	[105]

and others							
<i>Pseudoalteromonas</i>							
35.	Cycloprodigiosin	Red solid	Pyrrole alkaloid	<i>Pseudoalteromonas denitrificans</i>	6 liver cancer cell lines	IC ₅₀ : 276~592 nmol/L	[42]
36.	Prodigiosin	Red solid	Pyrrole alkaloid	<i>Streptomyces</i> , <i>Pseudomonas</i> , <i>Pseudoalteromonas</i> , <i>Actinomadura</i> sp.	Standard 60 human tumor cell line panel	GIC ₅₀ : 0.014 μM; LC ₅₀ : 2.1 μM	[94]
37.	Tambjamines	Yellow oil	Alkaloid	<i>Pseudoalteromonas tunicata</i>	HL60 (leukemia), MDA-MB435 (breast carcinoma), HCT-8 (colorectal carcinoma), and others	IC ₅₀ : 0.23~3.42 μg/mL	[51, 52]
38.	Violacein	Purple solid	Indolocarbazole	<i>Pseudoalteromonas tunicata</i> <i>Pseudoalteromonas</i> sp. 520P1 <i>Collimonas</i> CT	U937, K562, and others	IC ₅₀ : 0.5~1 μM	[129, 130]
39.	9H-pyrido[3,4-b]indole	Light yellow	β-Carboline alkaloid	<i>Pseudoalteromonas</i>	HeLa,	IC ₅₀ : 5 μg/mL	[70]

	(Norharman)	crystalline		<i>piscida</i>	BGC-823 (stomach cancer)		
Cyanobacteria							
40.	Curacin D	Pale yellow oil	Lipid	<i>Lyngbya majuscula</i>	MCF-7	IC ₅₀ : 0.34 μM	[131]
41.	Hectochlorin	Pale yellow solid	Lipopeptide	<i>Lyngbya majuscula</i>	60 cancer cell lines	GI ₅₀ : 5.1 μM	[132]
42.	Homodolastatin 16	Pale yellow oil	Cyclic depsipeptide	<i>Lyngbya majuscula</i>	WHCO1 and WHCO6 (esophageal cancer); ME180 (cervical cancer)	IC ₅₀ : 4.3 and 10.1 μg/mL; 8.3 μg/mL	[133]
43.	Jamaicamides A, B and C	Pale yellow oils	Mixed polyketide-peptide	<i>Lyngbya majuscula</i>	NCL-H460 (lung cancer) and Neuro-2a	LC ₅₀ : 15 μM	[134]
44.	Lyngbyabellin B	Pale yellow oil	Cyclic depsipeptide	<i>Lyngbya majuscula</i>	KB (epidermal carcinoma)	IC ₅₀ : 0.1 μM	[135]
45.	Phycocyanin	Light blue	Phycobiliprotein	<i>Spirulina platensis</i>	K562, Hep3B (hepatoma)	IC ₅₀ : 50 μM	[136]
Other bacteria							
46.	Alteramide A	Yellow powder	Alkaloid	<i>Alteromonas</i> sp.	P388, L1210, KB	IC ₅₀ : 0.1, 1.7, 5.0	[137]

							μg/mL	
47.	Heptylprodigiosin	Red solid	Pyrrole alkaloid	<i>Pseudovibrio denitrificans</i> Z143-1	L5178Y (lymphoma)	IC ₅₀ : 0.677 μM	[85]	
48.	PG-L-1	Red solid	Prodigiosin-like Pyrrole alkaloid	γ-Proteobacterium MS-02-063	U937, CHO, HeLa, and others	ED ₅₀ : 70~150 ng/mL	[86]	
49.	Unknown phenazine derivatives	Yellow needles	Phenazine	<i>Bacillus sp.</i>	P388; K562	IC ₅₀ : <50 μM; 74, 87 μM	[19]	

1.4.2.2. Violacein

Another pigment that has prominent biological properties, including anticancer activity, is a purple colored violacein (Fig. 1.8 and Table 1.2, No.38). This compound is a well-known indole derivative also known as 3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene)-1,3-dihydro-2H-indol-2-one. The violet pigment violacein is an indole derivative, predominantly isolated from bacteria of the genus *Chromobacterium* that inhabit the soil and water of tropical and subtropical areas [138]. Over the past decade, the biosynthesis and biological activities of violacein have been extensively studied, and many scientific papers and reviews have been published [139-143]. Violacein has a variety of biological activities, including antiviral, antibacterial, antiulcerogenic, antileishmanial, and anticancer properties [138, 139, 143, 144]. Use of violacein as a chemical defense against eukaryotic predators has also been investigated [53, 145-147].

Despite unique antibiotic properties, the anticancer activity of violacein has attracted special attention, and many studies have been recently conducted to understand its apoptotic mechanisms.

One of the first published reports on violacein production by marine bacteria was by Hamilton and Austin [148]. This bacterial strain, *Chromobacterium marinum*, was isolated from open ocean waters and produced a blue pigment that was identified as violacein on the basis of physicochemical characteristics [148]. Later, Gauthier [50] described 16 violet-pigmented heterotrophic bacilli isolated from Mediterranean coastal waters and proposed the name *Alteromonas luteoviolaceus* for these strains. Another six bacterial species were also isolated by Gauthier et al. [149] from neritic waters on the French Mediterranean coast and were very similar to *Alteromonas* species. These species produced characteristic pigmentations ranging from pinkish beige with reddish-brown diffusible pigment, lemon yellow, bright red turning carmine in old cultures, and orange to greenish-brown. Light violet, dark violet, or almost black pigments were also produced and later identified as violacein. The

strains showed antibiotic activity against *Staphylococcus aureus* [149]. Subsequently, many other reports on violacein production have been published [54, 55].

Several purple pigment-producing *Alteromonas* species were also isolated from Kinko Bay in Kagoshima Prefecture, Japan. One of these, *Alteromonas luteoviolacea* (reclassified as *Pseudoalteromonas luteoviolacea*), is the only extensively characterized marine bacterium ever reported that produces violacein [50-52].

Investigations on the molecular mechanisms of cytotoxicity of violacein isolated from *Chromobacterium violaceum*, revealed the activation of caspase-8 and p38 MAP kinase, as well as the transcription of NF- κ B target genes in HL60 leukemia cells [129]. The IC₅₀ value of violacein against HL60 cells was determined to be 700 nM. In addition to activation of the TNF receptor, upregulation of the expression of p21 protein, activation of caspase-3, phosphatidylserine exposure, fragmentation of poly (ADP-ribose) polymerase (PARP), decrease of c-jun expression, and downregulation of the inhibitor of apoptosis protein 1 (IAP1), all leading to facilitate the apoptosis process, were also observed [129]. In another study by the same research group, violacein-induced apoptosis of four colon cancer cell lines was associated with cell cycle blockage at the G1 phase, upregulating the levels of p53, p27 and p21 proteins and decreasing the expression of cyclin D1 [130]. However, the main cause of apoptosis was attributed to the inhibition of Akt (PKB) phosphorylation, which changed the levels of the proteins mentioned above, with subsequent activation of the apoptotic pathway and downregulation of NF- κ B signaling. This signaling pathway in colon cancer cells was suggested to be more sensitive to 5- fluorouracil (5-FU), a potent chemotherapeutic agent, in a synergistic fashion with violacein. The elevated levels of the pro-apoptotic Bad protein were thought to be promoted by the activation of caspase-3, in addition to the activation of caspases-8 and -9, and the decreased level of FADD was suggested to be indicative of the exclusion of the extrinsic apoptotic pathway [130].

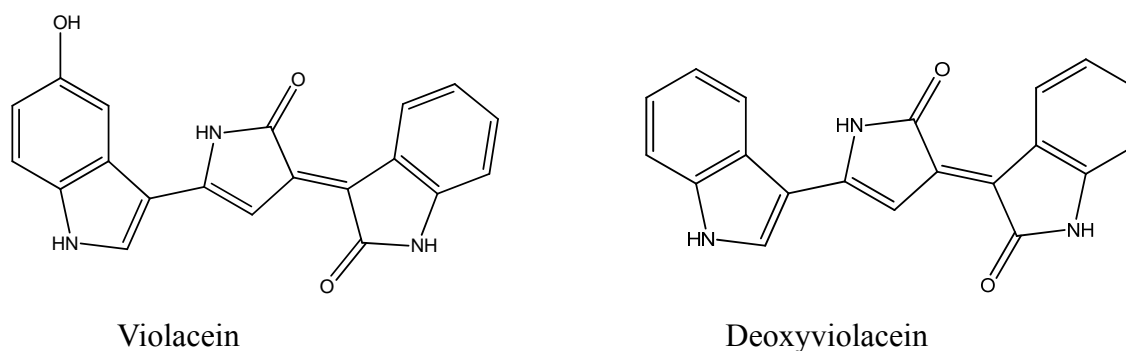


Fig.1.8. Violacein and deoxyviolacein.

1.4.3. Cytotoxic pigments from marine cyanobacteria

Marine cyanobacteria, previously known as blue-green algae, are also productive microorganisms which have been known for their ability to produce a wide variety of unique secondary metabolites. Although, more than 300 structurally diverse biomolecules have been obtained from marine cyanobacteria, they are especially famous for the production of lipopeptides and depsipeptides [150], most of which are biologically active. A number of these bioactive compounds have demonstrated anticancer activity, with different potency against various cancer cell lines (Table 1.2). It should be noted that many well-known potent antitumor compounds (e.g., dolastatin), previously believed to originate from marine invertebrate species such as sea hares and mollusks, have recently be found to actually originate from marine cyanobacteria living in symbiosis with those species.

Phycocyanin, a pigment produced by the cyanobacterium *Spirulina platensis* [151], belongs to the light-harvesting phycobiliprotein family and is used as a fluorescent agent in immunoassay analysis [152]. Phycocyanin has a characteristic light blue color, absorbs orange and red light near 620 nm, and emits fluorescence at about 650 nm. Being an accessory pigment that works in conjunction with chlorophyll *a*, it is also a well-known natural food colorant. In addition to its anti-oxidative and anti-inflammatory effects, phycocyanin was also found to have cytotoxicity against cancer cells, the molecular mechanisms of which displayed characteristic features of apoptosis, such as cell shrinkage,

membrane blebbing and nuclear condensation followed by mitochondrial cytochrome *c* release, PARP cleavage and downregulation of the anti-apoptotic Bcl-2 protein (Table 1.2, No.45) [136].

Nevertheless, not only pigmented compounds show bioactivities. There are also a number of non-pigmented, highly potent cyanobacterial active metabolites with antitumor activity against various cancer cell lines, isolated from different species, representing potential candidates for future drug development. It is expected that more compounds with interesting biological activities will be discovered in the future, since this family of bacteria remains virtually uninvestigated. Therefore, researchers specializing in chemistry, biology, pharmacology and medicine have a challenging task to accomplish in the study of bioactive compounds from marine microorganisms.

1.5. Biosynthesis of pigments

Numerous reports detail the regulation and biosynthesis of bacterial secondary metabolites. Increased research and verification of specific bacterial pathways has predominantly been due to the antibiotic, immunosuppressive, and anticancer potential of these compounds. A brief discussion of this topic is given next, as detailed information is further provided in the cited references.

Biosynthesis of bacterial prodiginines has extensively been studied and reviewed [153, 154]. Prodigiosin biosynthesis was proposed to originate during the enzymatic condensation of 2-methyl-3-n-amylopyrrole (MAP) and 4-methoxy-2,2'-bipyrrrole-5-carbaldehyde (MBC) precursors. Prodiginine biosynthetic gene clusters for *Serratia* sp. ATCC 39006 [155], *Serratia marcescens* ATCC 274 [155], *Hahella chejuensis* KCTC 2396 [47, 156], and *Streptomyces coelicolor* A3(2) [157] have been identified, sequenced, and expressed. Several gene clusters are involved in the biosynthetic pathway, depicted as *pig* in *Serratia* strains, *red* in *S. coelicolor* A3(2), and *hap* (numbered) in *H. chejuensis* KCTC 2396, with each encoding

several proteins responsible for synthesis. The largest gene cluster found in *S. coelicolor* A3(2) consists of four transcriptional units, whereas the other three clusters are strongly homologous to each other and are arranged unidirectionally.

In *Serratia* strains, *pigB–pigE* genes were identified to encode proteins responsible for the biosynthesis of MAP and condensation with MBC to form prodigiosin [153, 154]. A common pathway of MBC biosynthesis is proposed for all strains, in which proline, acetate, serine, and S-adenosylmethionine are incorporated into the bipyrrole at the initial stage [154]. PigA, PigF, PigG, PigH, PigI, PigJ, PigM, and PigN in *Serratia* strains and RedE, RedI, RedM, RedN, RedO, RedW, RedV, and RedX proteins in *S. coelicolor* A3(2) have been determined to participate in MBC biosynthesis [154]. PigB, PigD, and PigE enzymes in *Serratia* strains were proposed to be involved in the MAP biosynthesis, which requires 2-octenal as the initial precursor [154]. Monopyrroles condense with MBC during the final step of prodigiosin and/or undecylprodigiosin biosynthesis. PigC and its homologues catalyze this condensation in bacteria.

Some prodiginines can also be produced when monopyrroles are supplied to colorless *S. marcescens* mutants [73]. Addition of monopyrroles directly to a culture medium or as a vapor across the culture surface of a colorless mutant of *S. marcescens* resulted in the strain becoming initially pink and later red, indicating prodiginine formation [73]. Similar prodiginine biosynthesis produced by exogenously adding MAP and MBC was observed in white strains of *Serratia marcescens* isolated from patients [158].

The violacein biosynthesis pathway and associated biosynthetic enzymes have been extensively studied [140, 142, 159], although certain reactions and intermediates are yet to be elucidated. Currently, this proposed system involves an operon of five genes, *vioA–vioE*, which are transcriptionally regulated by a quorum-sensing mechanism that uses acylhomoserine lactones as autoinducers. At the early stationary phase of bacterial growth, acylhomoserine lactones accumulate in the culture medium, inducing the transcription of the

vio genes. Therefore, violacein is considered a typical secondary metabolite in bacteria. The first enzyme encoded by the *vio* gene operon, VioA, converts L-tryptophan to indole-3-pyruvic acid imine (IPA imine), and the second enzyme, VioB, catalyzes the reaction to convert IPA imine into an unidentified compound X (possibly an IPA imine dimer) [160, 161]. Compound X then undergoes successive reactions, catalyzed by the enzymes VioE, VioD, and VioC, to produce violacein.

Phenazine pigment biosynthesis reportedly involves shikimic acid as a precursor and forms chorismic acid as an intermediate product. Two molecules of chorismic acid then form phenazine-1,6-dicarboxylic acid, which is sequentially modified to create a variety of phenazine derivatives with different biological activities [162]. *Pseudomonas aeruginosa* PAO1 has two gene clusters (*phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*), with each cluster capable of producing phenazine-1-carboxylic acid (PCA) from chorismic acid [163]. It is proposed that PhzM and PhzS catalyze the subsequent conversion of PCA to pyocyanin. In addition, PhzH is responsible for producing phenazine-1-carboxamide from PCA.

Fridamycin, hymalomycin, and chinikomycin are typical bacterial compounds that share a quinone skeleton. However, little information regarding the biosynthesis of these compounds has been accumulated.

Detection and identification of the entire *P. tunicata* gene cluster involved in the biosynthetic pathway production of the tambjamine YP1 using recombinant *E. coli* was conducted by Australian researchers Burke et al. [164]. In total, 19 proteins encoded the Tam cluster participate in the postulated biosynthetic pathway. Among them, 12 were found to have high sequence similarity to the red proteins responsible for undecylprodigiosin synthesis in *S. coelicolor* A3(2) and the pig proteins involved in prodigiosin biosynthesis in *Serratia* sp. [164]. Such similarity in the chemical structures of these two classes of compounds results in tambjamins having two pyrrole rings while the prodiginines have three. As is the case for the prodiginines, 4-methoxy-2,2-bipyrrole-5-carbaldehyde (MBC) is initially formed from proline,

serine, and malonyl CoA in the tambjamine biosynthetic pathway. A double bond is inserted by TamT and an amino group is transferred by TamH to dodecenoic acid activated by AfaA, which is predicted to be an acyl-CoA synthase. The resulting dodec-3-en-1-amine is condensed with MBC by TamQ to form tambjamine YP1 [164].

In addition to *V. cholera*, *S. colwelliana*, *A. nigrifaciens*, and *C. tyrosinoydans*, melanin syntheses have also been reported in *M. mediterranea*, which contains the tyrosinase gene operon [165], and in an epiphytic *Saccharophagus degradans* 2-40 bacterium [166]. While the specific details of melanin formation continue to be debated, well-defined biosynthetic schemes have now been proposed. Two different biosynthetic pathways synthesize the eumelanins and pheomelanins. Both pathways are initiated by the oxidation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the subsequent creation of dopaquinone by tyrosinase. The latter product is transformed either to pheomelanin by combining with cysteine and forming an intermediate S-cysteinyldopa and benzothiazine or to eumelanin with intermediate leucodopachrome, dopachrome (red), 5,6-dihydroxyindole, 5,6-indolequinone (yellow) formation [24].

Nostoc punctiforme ATCC 29133 is the only scytonemin producing organism whose genome has been fully sequenced [167]. This scytonemin biosynthesis potentially involves a gene cluster consisting of 18 open reading frames (ORFs) (NpR1276 to NpR1259). Although, the functional roles of all these ORFs are not yet fully determined, some intriguing hypotheses have been proposed. In particular, both tyrosine and tryptophan are implicated as biosynthetic precursors for scytonemin in the pigment formation pathway. NpR1275, which functionally resembles leucine dehydrogenase, is utilized in the early stages of scytonemin synthesis in *N. punctiforme*, thereby oxidizing tryptophan and/or tyrosine to their corresponding pyruvic acid derivative.

Alternatively, it is suggested that NpR1269, a putative prephenate dehydrogenase, generates *p*-hydroxyphenylpyruvic acid, which is a derivative of tyrosine in the early pathway

stages. NpR1276 uses two pyruvic acid derivatives from tryptophan and tyrosine for the synthesis of a labile β -ketoacid product, which is homologous to the thiamin diphosphate-(ThDP-) dependent enzyme acetolactate synthase. NpR1274 possibly catalyzes the intermediate cyclization and decarboxylation of the β -ketoacid product to form the indole-fused cyclopentane moiety of the pigment [168]. Monomer precursors that are formed then undergo dimerization to produce scytonemin. NpR1263, which was found to be similar to a tyrosinase in melanin biosynthesis, participates in these later oxidative dimerization steps, thereby forming scytonemin [169]. Functional roles of other ORFs and their putative intermediate products for the pigment production are still under investigation.

1.6. Concerns regarding the physiological role of pigmented compounds

A number of bacterial species, including those inhabiting the vast marine environment, produce a wide variety of pigments that are important to cellular physiology and survival. Many of these natural metabolites were found to have antibiotic, anticancer, and immunosuppressive activities. These secondary metabolites, produced by microorganisms mostly via the quorum sensing mechanism, have the ability to inhibit the growth of or even kill bacteria and other microorganisms at very low concentrations. Due to such diverse and promising activities against different kinds of diseases, these compounds can play an important role in both pharmaceutical and agricultural research.

It still remains uncertain why these pigmented secondary metabolites from bacteria have antibiotic and/or cytotoxic activities. Although, their true physiological role is yet to be fully discovered, there are a few reports that provide reasonable explanations by making comparisons with nonpigmented bacteria. In particular, the relationships between pigment production and toxicity have been studied by Holmström et al. [72], who found that 90% of all dark-pigmented compounds taken from marine living surfaces showed inhibitory activity towards invertebrate larvae. Two fractions isolated after column chromatography, one

colorless and the other a yellowish-green color, were identified as phenazine derivatives from unidentified marine *Streptomyces* sp. by Pusecker et al. [20]. The colorless fraction was biologically inactive, while the pigmented phenazine derivative showed highly active antibiotic properties. Previous studies have also demonstrated that marine bacterial metabolites with antibiotic properties were always pigmented [170]. Screening of 38 antibiotic-producing bacterial strains revealed that all pigmented bacteria belonging to the *Pseudomonas-Alteromonas* group displayed antibiotic activity, while nonpigmented bacteria were inactive.

Considering data from all reported literature, a number of reasonable biological functions for pigment production in bacteria have been established. In general, the pigmented marine isolates seem to play two important roles: firstly, they provide an adaptation to environmental conditions, and, secondly, they provide defense against predators [171]. For instance, it has been shown that the brown colored melanin pigments produced by a variety of species, as well as a yellow green colored scytonemin pigment isolated from cyanobacteria, protect cells from UV irradiation and desiccation [24, 39]. Therefore, in order to adapt to the excessive sunlight and survive under harmful UV irradiation, bacteria must produce these indispensable compounds. Griffiths et al. [172] found that carotenoids, which were later suggested to be a substitute for sterols, are an important structural component of microbial membranes [173] and may protect bacterial cells from photooxidation or damage caused by visible light irradiation.

Several bacterial pigments that act as antagonists by exhibiting antibiotic activity against other organisms can be considered as potent weapons for survival and effective chemical defenses against eukaryotic predators. This class of bioactive agents includes almost all pigmented compounds commonly produced by *Pseudoalteromonas*, *Pseudomonas*, and *Streptomyces* species. These compounds inhibit the settlement of marine invertebrate larvae [174], the germination of algal spores [175] and protect the host surface by interfering with

bacterial colonization and biofilm formation [176]. They may also inhibit other organisms that compete for space and nutrients.

Such hypotheses are also supported by a number of studies that found that these bacterial compounds were active against other prokaryotes and even eukaryotes [177-185]. In many studies, pigmented bacterial strains demonstrated a strong and broad range of antibiotic activities against other organisms, while nonpigmented strains did not [176, 186]. A clear correlation between pigment production and antibacterial activities of the two *Silicibacter* sp. strain TM1040 and *Phaeobacter* strain 27-4 grown under static conditions was further reported by Bruhn et al. [186]. Mutant strains, which lacked pigment production, also lost their biological activities. Holmström et al. have also shown a close relationship between pigmentation and inhibitory activity, whereby 20 out of 22 dark pigmented bacterial strains tested displayed inhibitory activity against the settlement of two invertebrate larvae and algal spores [72].

Amongst other bacterial strains, *Pseudoalteromonas* has the most diverse antibiotic activities against alga biofouling, and the dark green pigmented *P. tunicata* exhibits the most active and broadest range of inhibitory activity when compared to other strains from this genus [176]. Two nonpigmented *P. nigrifaciens* and *P. haloplanktis* strains were also found not to display any antibiotic activities using various bioassays [176].

Blue-pigmented pyocyanin production in *P. aeruginosa* (Pup14B) was observed by Angell et al. to be induced by *Enterobacter* species (Pup14A and KM1), and this pyocyanin displayed moderate antibiotic activity against *E. coli* and yeast [187]. It was experimentally demonstrated that metabolites produced by Pup14A strain are necessary for the production of this pigment in Pup14B strain [187]. Many other reports describe synergism between bacteria and higher organisms; however, this is a rare example between two bacterial species [188]. Such an unusual case contrasts with the hypothesis of the regulated biodiversity of marine bacteria, in which surface-associated microorganisms produce antimicrobial agents [176] to

prevent competing microorganisms. The symbiosis of the two bacterial species is not yet fully understood, although both species appear to benefit from the pigment production.

One of the promising biological activities of marine bacteria isolates is their cytotoxic effect against cancer cells. Despite many investigations, the exact molecular mechanism of this pigmented compound cytotoxicity remains undetermined and requires further study. For example, violacein is known to cause apoptosis in tumorous cells [143]. However, the pathways leading to cell death have not yet been linked to the possible effects of the pigment, which was also shown to affect signal transduction agents, such as protein kinase and protein phosphatase family enzymes that play crucial role in cell differentiation and proliferation.

In a study by Bromberg et al., violacein showed inhibitory activity against protein phosphatases isolated from human lymphocytes [189]. A similar study was also conducted by Fürstner et al. to assess the inhibitory activity of prodigiosin derivatives [111]. Other targets of these compounds, including ion channels, are further being investigated [105, 190-192].

Unexpected problems have also arisen when investigating marine environments. While the marine environment is a promising source for identifying microorganisms that can produce important biologically active pigments, yields of these compounds remain variable and are sometimes too low to provide enough material for drug development [193] or commercial applications. The main reason for such low yields is that these compounds are secondary metabolites and production depends on the quorum sensing mechanism.

Despite marine bacteria being capable of growing in the extremely low concentrations of nutrients that often exist in seawater, most species still require seawater or its equivalent as a growth medium for artificial culturing. Seawater is therefore used for the growth of marine bacteria, or similar levels of sodium, potassium, and magnesium chloride are supplemented in cultures. Optimal growth and the production of pigments are only sustained for most bacteria when appropriate salt mixtures are used for culturing, as is the case for the prodigiosin-producing marine *Pseudomonas magnesorubra* and *Vibrio psychroerythrus*,

among other marine species. These bacteria grew optimally and produced red pigment when cultured in seawater or its equivalent, while pigment production by the terrestrial *Serratia marcescens* was inhibited in 3% sea salts [73].

Enhancing low pigment productivity is one of the main issues facing researchers, and some solutions have already been reported. It is well established that antibiotic production by bacteria might be regulated both qualitatively and quantitatively by the nature of the culture medium. In particular, the addition of individual natural compounds to nutrient media or the use of gene expression methods was found to increase the pigment production far beyond expectations. For example, saturated fatty acids, especially peanut broth, was found to be a better choice in increasing prodigiosin production by 40-fold (approximately ~39 mg/mL) in *S. marcescens* [194].

Undecylprodigiosin synthesis by *S. marcescens* was also markedly enhanced by the addition of vegetable (soybean, olive, and sunflower) oils (2–6% [v/v]) and amino acids to the fermentation broth [195, 196]. Violacein production by the recombinant *Citrobacter freundii* strain, the genes of which were reconstructed from *Duganella* sp. B2, reached up to 1.68 g/L, making it fourfold higher than the highest production previously reported [197]. It is anticipated that these methods will facilitate the production of sufficient quantities of many bioactive and pharmacologically important compounds obtained from bacteria of marine origin. These compounds, including prodiginine and violacein, are now considered as potential drug candidates for potentially fatal diseases such as cancer and malaria. Although further improvement of culture methods and technologies for pigment production including recombinant technology is necessary, bioactive compounds from marine bacteria may potentially replace the existing drugs that have lower therapeutic actions.

Chapter II

Materials and Experimental Methods

2.1. Reagents and experimental tools

Methanol, ethanol, ethyl acetate, and Iron (III) Citrate *n*Hydrate (Ferric Citrate ($\text{FeC}_6\text{H}_5\text{O}_7 \cdot n\text{H}_2\text{O}$)) used for the sample preparations and extractions were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Silica gels Wakogel C200 and YMC-Gel 12 nm S-150 μm SL12SA5, ODS YMC-Gel ODS-A 12 nm S-150 μm AA12SA5 used for the separation of the pigments were from Wako Pure Chemical and YMC Co., Ltd (Kyoto, Japan), respectively. Bacto tryptone, Bacto yeast extract used for the preparation of LB-SW culture medium and Proteose peptone No.3, Soytone used for the preparation of PPES-II culture medium were all from Becton, Dickinson and Company (USA). Polypepton and Agar were from Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan) and Naqalai Tesque (Kyoto, Japan), respectively. Hitachi Centrifuge himac CR21E (Hitachi Koki Co., Ltd) was used to centrifuge bacterial culture and the red pigment extract; rotary evaporator was from As One Corporation (Osaka, Japan); Eyela Ceramic reciprocal pump VSP-2050 used for mobile phase pumping was from Tokyo Rikakikai Co., Ltd (Japan). Hitachi UV visible spectrophotometer model U-3010 (Tokyo, Japan) was used to determine the concentration of the pigment. The chromatograph used in this study was a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan) consisting of a two-flow channel degasser, an inert pump, an autosampler, a column oven and a UV-Vis PDA detector. An EZChrom Elite Nanospace system software for Shiseido was used to control the operation of the HPLC system. Separation of the red pigment and its individual compounds were performed on an ODS Capcell Pak C-18 MGII (1.5 mm \times 150 mm) column purchased from Shiseido Co., Ltd. (Tokyo, Japan). MTP-880 Lab microplate reader (Hitachi Biotechnologies) was used to measure PTP1B assay activity using 530 nm emission / 490 nm

excitation of the fluorescence intensity. PP2A enzyme activity was measured using Benchmark microplate reader (Bio-Rad). NMR spectroscopy of P2 (0.85 mg) was performed on a Varian Unity INOVA 400 to obtain ^1H (400 MHz) and ^{13}C (101MHz) spectra of the pigment in CDCl_3 using methods such as ^1H -NMR, ^{13}C -NMR, DEPT, HMQC, HMBC, COSY and TOCSY. High resolution MS analyses was conducted on Electrospray ionization Fourier transform mass spectrometer (ESI-FTMS) (Exactive, Thermo Fisher).

2.2. Cultivation of the red pigment-producing 1020R bacterial strain

PPES-II culture medium used for the cultivation of 1020R strain in a plate contained 2.0 g Polypeptone, 1.0 g Proteose peptone No.3, 1.0 g Soytone, 1.0 g Bacto yeast extract, 0.1 g Iron (III) Citrate *n*Hydrate and 15 g Agar in 1L volume adjusted to pH 8.0 with 1 or 5M NaOH.

Lurie-Bertani sea water (LB-SW) culture medium used for the cultivation of the bacterium contained 10g of tryptone, 5g of yeast extract in 1 L of seawater adjusted to pH 8.0 with 5 M NaOH solution. Sea water was filtered through paper filter before using. All culture mediums were sterilized at 121°C for 20 min at maximum 0.4 MPa pressure before usage.

The procedure for the cultivation of the 1020R bacterial strain to produce the red pigment consisted of the following steps: First, a single colony from the agar plate was transferred into the test tube containing 5 ml of LB-SW medium and was incubated overnight with rotation at 28°C. Then, from this culture, a sample of approximately 1% of total volume was transferred to a flask also containing LB-SW medium, and cultivated with intensive shaking at 120-130 rpm/min for 1 or 2 days. The change of the clear culture medium to a turbid view was indicative of the sufficient bacterial growth. Then, from this medium, again a sample of approximately 1% of a total volume was transferred to many flasks, all containing LB-SW medium, for the production of the red pigment. The culture medium was incubated in the dark at 28°C for 7-8 days to get the maximum amount of the red pigment. After that the red pigment was extracted.

2.3. Extraction of the red pigment

Dark red coloured bacterial culture broth was first centrifuged at 8,000 rpm for 15 min at 4°C to precipitate bacterial cell mass with the red pigment. The obtained supernatant, which also contained a little amount of the red pigment, was subjected to the extraction with ethyl acetate while precipitate, which contained the most amount of the pigment, was thoroughly dissolved in pure ethanol with aggressive mixing to disrupt bacterial cell wall and centrifuged again at 10,000 rpm for 10 min at 4°C. The supernatant with the red pigment was taken and the precipitate was treated again 2 times with ethanol to remove the red pigment remnants and then discarded. The supernatant was collected and then dried using a rotary evaporator. To remove remained salt in the red pigment, it was again dissolved in pure ethanol and centrifuged at 10,000 rpm for 10 min at 20°C.

UV visible spectrum of the red pigment was measured on U-3010 Spectrophotometer in the range of 200-800 nm, which showed the maximum absorbance at around 533.0 to 537.0 nm. Concentration of the red pigment was measured according to maximum absorbance height using Beer-Lambert Law applicable for the solutions.

$$A = \epsilon l c$$

Where, A is an absorbance; ϵ is the molar absorptivity of the solution; l is the length of solution the light passes through (cm); c is the concentration of solution in mol/L.

Considering the molar absorptivity ϵ of the red pigment is equal to 1.12×10^5 and the length of solution is 1 cm, the above formula roughly turns to the following:

$$C = \frac{A}{1.12 \times 10^5} \times \frac{X}{1000} \times 323.23 \times 1000 = \frac{323.23 \times A}{1.12 \times 10^5} = 0.00288 \times A \text{ (mg)}$$

Where, C is the mass of the red pigment in mg; **323.23** is the molecular weight of prodigiosin as the main compound of the pigment; X is the volume of the red pigment

solution in ml; A is the maximum absorbance at around 535.0 nm.

2.4. HPLC analysis

HPLC measurements were performed isocratically at a flow rate of 100 μ l/min using the mobile phases mostly CH₃OH/H₂O and CH₃CN/H₂O for the method development. The ratio of CH₃OH in the mobile phase was varied from 50 to 70% to find an optimum separation condition. Eventually, the ratios consisting of CH₃OH/H₂O - 55/45 (v/v) and 50/50 (v/v) added 0.2% of acetic acid were chosen as optimum mobile phases to analyze the red pigment and its individual compounds. HPLC analysis was performed after each extraction, Silica and ODS open column separation processes to evaluate the purity of the pigment and its individual compounds.

It should be noted that previously HPLC analysis of the red pigment was performed using CH₃OH/H₂O - 70/30 (v/v) added 0.2% of acetic acid as a mobile phase and revealed to contain 5 individual compounds. However, recent analysis performed using CH₃OH/H₂O - 55/45 (v/v) and 50/50 (v/v) added 0.2% of acetic acid have revealed that the red pigment extracted from 1020R strain may actually contain at least seven compounds than originally found, and thus it should also be studied more in detail for what we concentrated here.

2.5. Cytotoxicity assay

U937, K562 and HL60 leukemia cancer cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum. Approximately, 1×10^6 cells/ml were incubated with different concentrations of prodigiosins varying from 0.1 to 10 μ M in 24 well plates for 24, 48 and 72 hrs at 37°C humidified with 5% CO₂. Cytotoxicity was determined by the MTS assay employing the reaction that 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium in the presence of phenazine methosulfate (**PMS**) produces a formazan product that has an absorbance maximum at 490-500 nm in phosphate-buffered

saline. Thirty μl of MTS buffer was added to each well of 96 well plates containing 30 μl of the culture medium and the absorption at 490 nm was measured after incubation. Cell medium only and DMSO added to the cell medium instead of prodigiosins were used as positive controls.

2.6. Effect of the pigments on protein kinase and protein phosphatase enzymes

2.6.1. Phosphorylation experiments

Protein tyrosin kinase (Src kinase) and CaM kinase were assayed according to the manufacturer's instructions, in a final volume of 50 μl of buffer containing 5 μl of the red pigment dissolved in DMSO, 50 mM HEPES-NaOH pH7.4, 10 mM MgCl_2 , 1 mM EGTA, 200 μM p60c-src substrate for Src kinase or 50 μM autocamtide for CaM kinase, and 200 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity, 4.2 KBq/nmol). Additionally, for the assay of CaM kinase, 5 mM CaCl_2 and 30 $\mu\text{g/ml}$ calmodulin were added.

PKA and PKC catalytic subunits were assayed according to the method of Toomik and Ek [198], by modifying and optimizing the experimental procedure. The phosphorylation reaction by PKA was performed in 100 μl of buffer containing 5 μl of the red pigment dissolved in DMSO, 25 mM Tris-HCl, pH 7.5, 9.4 mM MgCl_2 , 100 μM kemptide, 0.1 mg/ml ovalbumin, 10 units/ml PKA catalytic subunit and 190 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity, 1.0 KBq/nmol).

Phosphorylation experiments with PKC were carried out in a final volume of 100 μl of buffer containing 5 μl of the red pigment dissolved in DMSO, 20 mM Tris-HCl pH7.5, 10 mM MgCl_2 , 20 μM PKC [Ser-25] (19-31) peptide substrate, PKC catalytic subunit and 100 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity, 2.8 KBq/nmol). Samples containing PKA and/or PKC and DMSO, but not the red pigment were used as positive, while enzyme free samples were used as negative controls. The experiments were carried out in duplicate or triplicate at 30°C for either 10 or 20 min. The reactions were initiated by the addition of 5 μl $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

solution and stopped by the addition of 10 μl 800 mM H_3PO_4 solution. Incorporated radioactivity was measured for 10 min by Cerenkov counting in a Beckman LS 6500 scintillation counter (Beckman Instruments, Inc. Fullerton, CA, USA).

2.6.2. PTP1B assay

The CycLex® PTP1B Fluorometric Assay Kit (Cat. No. CY-1350) was used as an experimental tool. The experiments were performed in 96-well plates containing 40 μl of reaction buffer, a PTP Fluoro-Phospho-Substrate, recombinant PTP1B enzyme, 20 μl of development buffer, 5 μl of development reagent and the red pigment dissolved in DMSO at different concentrations as test compounds in a total of 75 μl reaction volume. The reaction was stopped by adding 25 μl of stop solution. The fluorescence intensity was measured at 530 nm emission vs. 490 nm excitation in a microplate reader. Samples containing DMSO but not prodigiosins were used as positive, and those containing no enzyme and inhibitor (included in the kit) as negative controls.

2.6.3. PP2A assay

SensoLyte® *p*NPP colorimetric protein phosphatase assay kit (AnaSpec, Cat. No 71105) was used to examine *in vitro* inhibitory activity of the pigments. Reactions were performed in a 96-well plate in a total of 100 μL assay volume containing 40 mM Tris-HCl (pH8.4), 34 mM MgCl_2 , 4 mM EDTA, and 4 mM DTT, 5 μL of the red pigment dissolved in DMSO at different concentrations ranging from 1-50 μM , and 50 μL of the *p*NPP solution as a generic substrate. The reaction was performed at 30°C for 1 h and terminated by the addition of 50 μL stop solution. *p*NP formed as a result of dephosphorylation from the initial *p*NPP by the PP2A enzyme was measured at 405.0 nm. Samples containing DMSO but not the red pigment were used as positive controls.

Chapter III

Results and Discussion

3.1. Cultivation, isolation and purification of the red pigment

The procedure of cultivation of the strain 1020R consisted of the following steps: First, a single colony from the agar plate (Fig.3.1) was transferred to the test tube containing 5 ml of LB-SW medium and was incubated overnight with continuous rotation at 28°C. Then from this culture broth a sample of approximately 1% of total volume was transferred to a 200 ml flask containing 30 ml of LB-SW medium, and cultivated with intensive shaking for 1 or 2 days. When the clear culture medium became turbid, indicating sufficient bacterial growth, a sample of approximately 1% of total volume was transferred to 500 ml flasks, all containing 200 ml of LB-SW medium, for the production of the red pigment. The bacterial culture mediums were incubated for 7-8 days at 28°C to get the maximum amount of the red pigment. After that the red pigment was extracted. Extraction of the red pigment was carried according to the scheme showed below (Scheme 3.1).



Fig.3.1. 1020R strain colony on agar plate

Extraction of prodigiosin compounds was carried out both from the supernatant and precipitate after centrifugation of the bacterial culture medium. This process was very complicated as the extraction of the prodigiosin compounds from the supernatant was extremely difficult and required more time and solvents to be used. To find an optimum

medium for the pigment production and also to facilitate the one step procedure of the extraction, pH dependent cultivation of the bacterium was carried out. For this reason, pH 6.0, 6.5, 7.0, 7.5 and 8.0 were chosen to cultivate the strain. The experiments showed that the most of the pigment was precipitated after the first centrifugation of the bacterial culture broth in all pH mediums except pH 8.0, while at pH 8 the pigment mainly remained in the supernatant (Fig.3.2).



Fig.3.2. Supernatant after the first centrifugation of bacterial culture broth. Left flask pH 6.0, 6.5, 7.0, 7.5, right flask pH 8.0.

Fig.3.2 clearly shows that the centrifuged culture mediums have different red colour intensities, indicating that the amount of the red pigment may sometimes be higher in the supernatant rather than in the precipitate at pH 8, indicating an impact of the pH of the medium on the red pigment production. Furthermore, the highest amount of the pigment was isolated from pH 7 medium, but not pH 8. It should be noted that until now for the production of the prodigiosins, strain 1020R was cultivated at around pH 8 which is close to the pH of seawater. However, this pH medium seems unsuitable for the cultivation of the bacterium. In case of pH 6 to 7.5, there was no need to extract the pigment from the supernatant, while at pH 8.0 the highest amount of the pigment was extracted namely from the supernatant, which made the extraction procedure much more complicated.

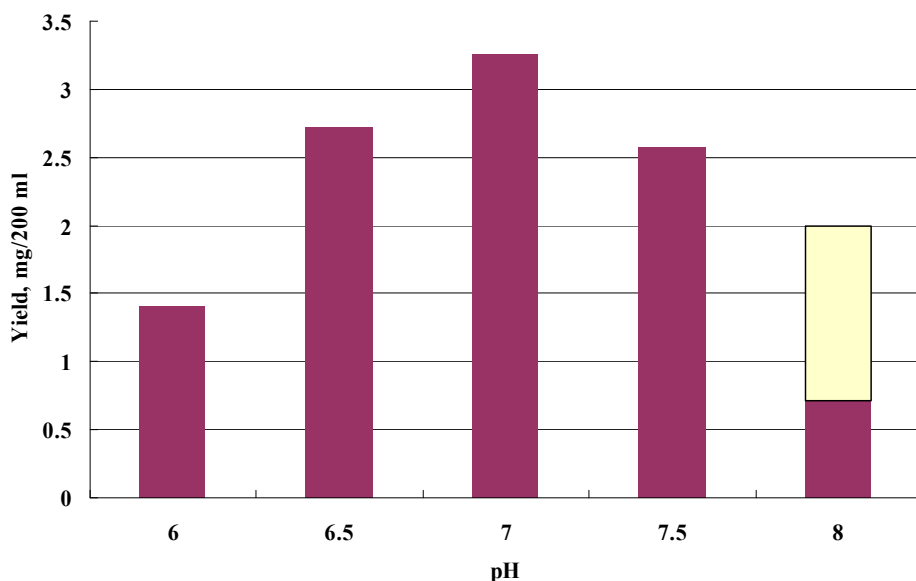


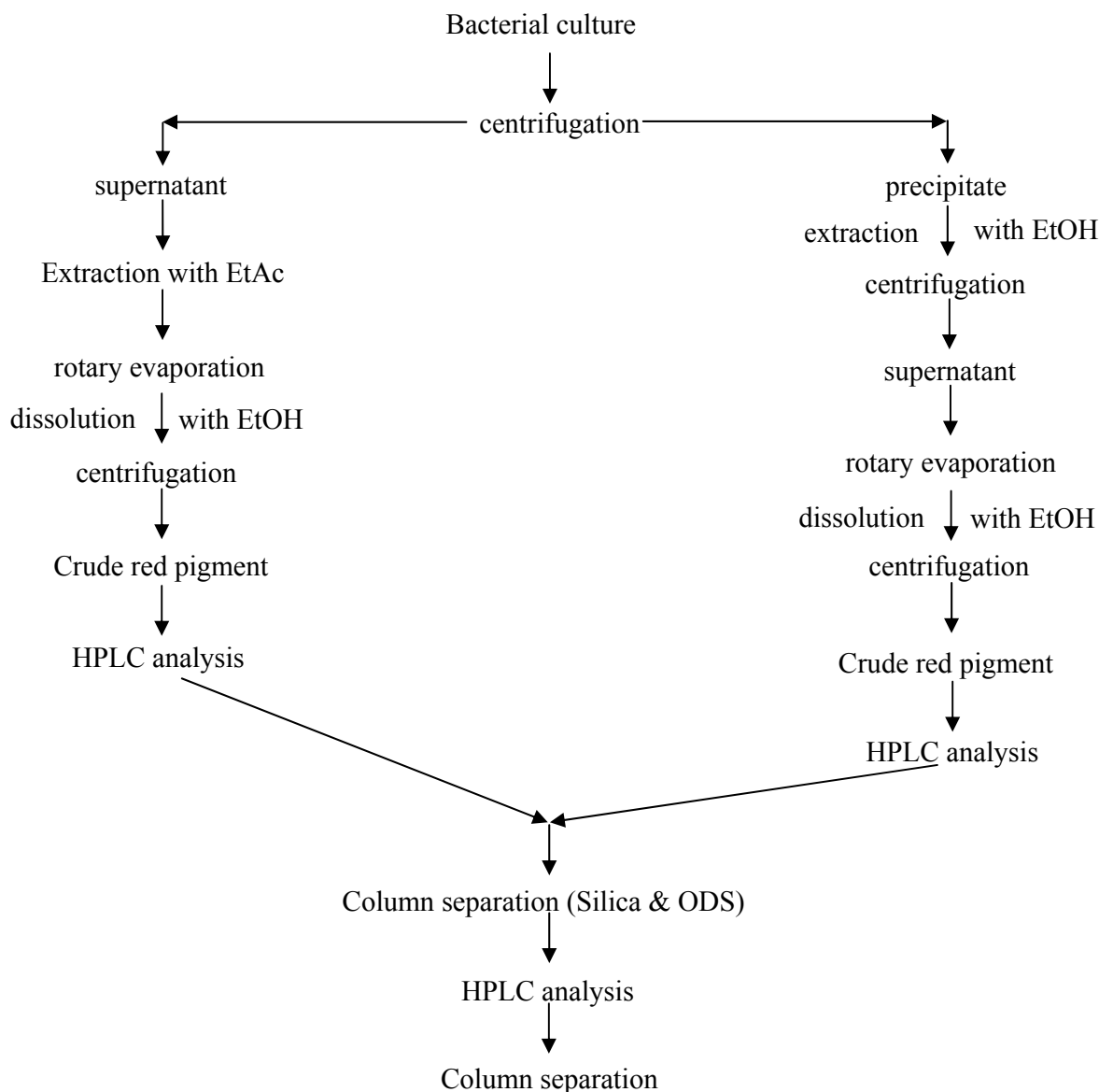
Fig.3.3. pH dependent cultivation of the strain 1020R and the amounts of the pigment extracted. Red pigment extracted from the precipitate ■ and from supernatant ■ .

It is likely that the red pigments are associated with proteins in original form as soluble protein-red pigment complexes at pH 8, and the disruption of the complexes occurs during the extraction procedure, making the red pigments insoluble in water [73].

A similar phenomenon was previously demonstrated by Velmurugan *et al.* [199], who examined the effect of pigment formation in fungi by five pigment-producing filamentous fungi in synthetic medium. However, the effect of pigment formation in bacteria was not investigated in their study. This observation, thus suggests that the pigment production may be similar in these two species.

Extraction procedure carried out from both the supernatant and precipitate is shown in the scheme below (Scheme 3.1). The amounts of the red pigment taken from the supernatant ranged from 1.2 to 6.36 mg/L and from the precipitate 1.86 to 8.37 mg/L. The overall amount of the red pigment extracted from 1020R bacterial strain made up approximately 4.27 to 14.58 mg/L. The extracted red pigments from both the supernatant and precipitate were collected and, after HPLC analyses, were subjected to the column separations.

Scheme 3.1. Extraction of the red pigment from bacterial culture broth.



Previously, it was suggested that shaking of bacterial culture medium negatively affects to the pigment production by 1020R strain. However, it was observed during the experiments that intensive shaking of bacterial culture medium at 180 rpm or more led to the intensive red pigment production by the bacterium. This was quite in accord with the investigation of Wei *et al.* [196] who found undecylprodigiosin production in *S. marcescens* may be enhanced by increasing the agitation rate. However, in this study the influence of agitation rate to the pigment production by 1020R strain was not investigated in detail.

3.2. Column separation

3.2.1. Silica gel open column separation

After many steps of extraction shown in the scheme above, the extracted pigments were collected and subjected to column separations using silica gel first and then ODS as stationary phases. Approximately 9 mg of the crude red pigment in ethanol was dried by rotary evaporator, redissolved in 5 ml of CHCl_3 and loaded onto the column (2.5 X 52 cm) tamped down with silica gel particles (Wakogel C200 or YMC-Gel Silica 12 nm S-150 μm), equilibrated in chloroform. Separation was performed in a self-flowing manner at a flow rate of 5-10 ml/min, eluting first with pure chloroform, following by chloroform/methanol in a different ratios (Fig.3.4). Although, there were many peaks obtained from the gradient separation of the pigments, only the first fraction eluted by the pure chloroform was subjected to further separation by ODS column chromatography.

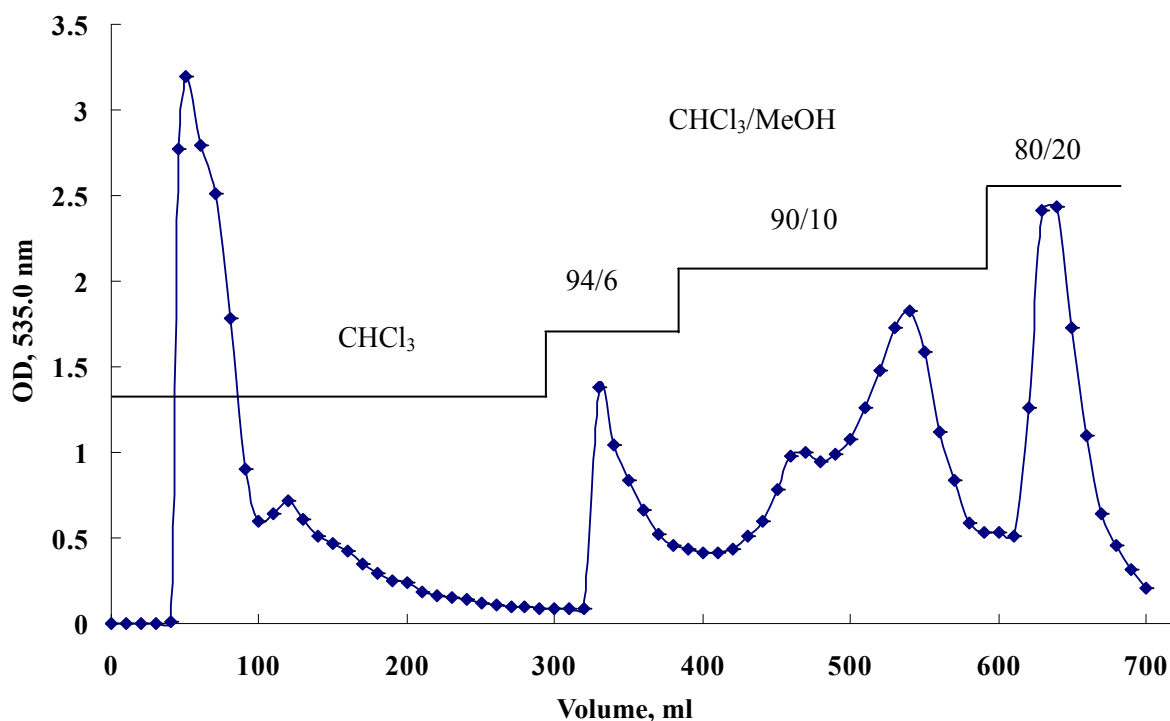


Fig.3.4. Silica gel open-column separation of the crude red pigment isolated from 1020R strain. Elution order: 0-310 ml with 100% CHCl_3 ; 320-400 ml with $\text{CH}_3\text{OH}/\text{CHCl}_3$ – 6/94

(v/v); 410-600 ml with CH₃OH/CHCl₃ – 10/90 (v/v); from 610 ml with CH₃OH/CHCl₃ – 20/80 (v/v).

However, HPLC analysis of the pure chloroform and chloroform/methanol fractions showed that they contain the same quantity of compounds. The sample eluted in pure chloroform had a bright red colour, subsequently changing into brownish-red in chloroform/methanol fractions, and violet in pure methanol fraction. As the aim of this study was to isolate individual compounds of the pigment, it was further decided to perform only ODS open column separation of the pigment, taking into account that the considerable amounts of the compounds might be lost by discarding chloroform/methanol fractions after silica gel open column separation.

3.2.2. ODS open column separation

ODS separations of the pigment were carried out using columns 2.5 × 52 cm and 1.0 × 30 cm tamped down with ODS gel (YMC Gel ODS-A 12 nm S-150 μm).

The red pigment loaded onto the column was eluted using the mobile phase consisting of CH₃OH/water – 60/40, 65/35, 70/30 and 80/20 (v/v) acidified by adding 0.2% of acetic acid.

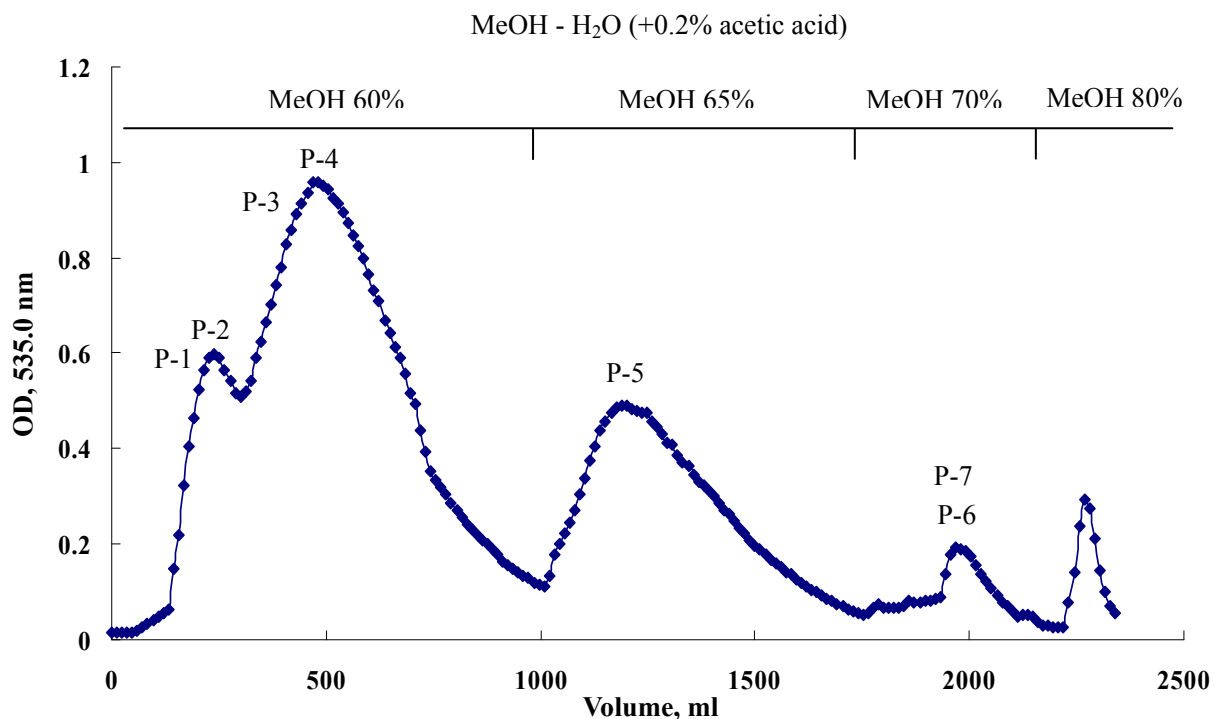


Fig.3.5. ODS open column separation profile of the red pigment.

As a result, main five fractions were obtained, being eluted in CH₃OH/H₂O – 60/40 (P-1, P-2, P-3 and P-4), 65/35 (P-5), 70/30 (P-6 and P-7) and in 80/20 (fifth peak) added 0.2% acetic acid.

The colour of the samples changed from bright red to brownish red and the last fraction eluted in pure methanol had a violet colour (Fig.3.6).



Fig.3.6. Red pigment fractions obtained from ODS open column chromatography separation and the comparison of the first and the last eluted fractions of the pigment.

3.3. HPLC analysis

Mobile phases consisting of CH₃OH/H₂O and CNCH₃/H₂O at different ratios acidified by addition of 0.2% of acetic acid were used to find the optimum separation condition in isocratic separation mode. Mobile phase consisting of CH₃OH/H₂O – 50/50 (v/v) + 0.2% acetic acid was found to be suitable for the pigment separation except for longer analysis time. CH₃OH/H₂O – 55/45 (v/v) + 0.2% acetic acid was also suitable with shorter analysis time, although the first eluted P-1 and P-2 compounds were not fully separated. Eventually, CH₃OH/H₂O – 50/50 (v/v) + 0.2% acetic acid was decided to use for the detailed analyses, while mobile phase consisting of 55% CH₃OH for quicker analyses of the pigments. Other conditions were set as follows: Column used was ODS Capcell Pak C-18 MGII (1.5 mm × 150 mm); column temperature - 40°C; flow rate - 100 µl/min.

HPLC analysis of the ODS open column separation fractions showed that the fraction 1 contained the first eluted P-1 & P-2 compounds (Fig.3.7), which accounted for 3.58-13.55% of the total amount of the pigment.

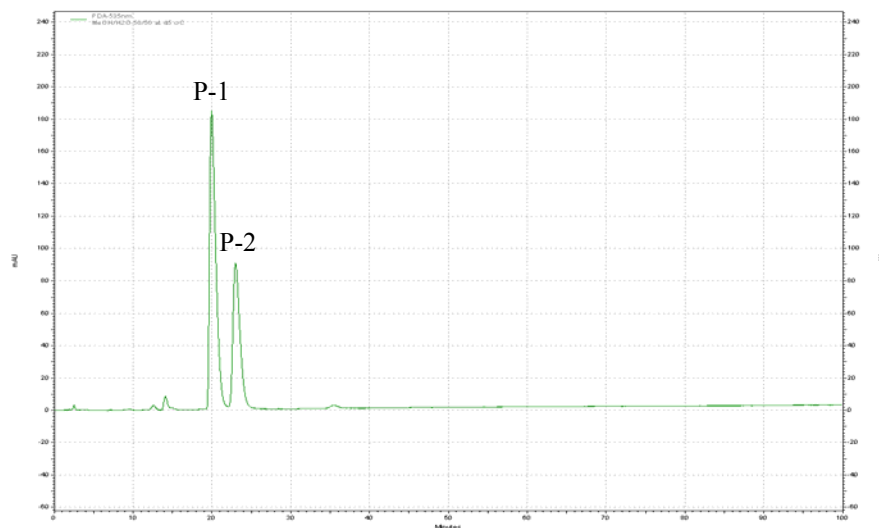


Fig.3.7. HPLC analysis of the ODS separated fraction 1 eluted in CH₃OH/H₂O – 60/40 + 0.2% acetic acid. HPLC system: Shiseido Nanospace SI-2. Chromatographic conditions: Column: ODS Capcell Pak C-18 MGII (1.5 mm × 150 mm); mobile phase: MeOH/H₂O –

50/50 (v/v) + 0.2% acetic acid; column temperature: 40°C; flow rate: 100 µl/min.

Fraction 2 contained P-3 and the most abundant compound of the red pigment - P-4 (Fig.3.8), making up 33.5% of the total amount of the pigment.

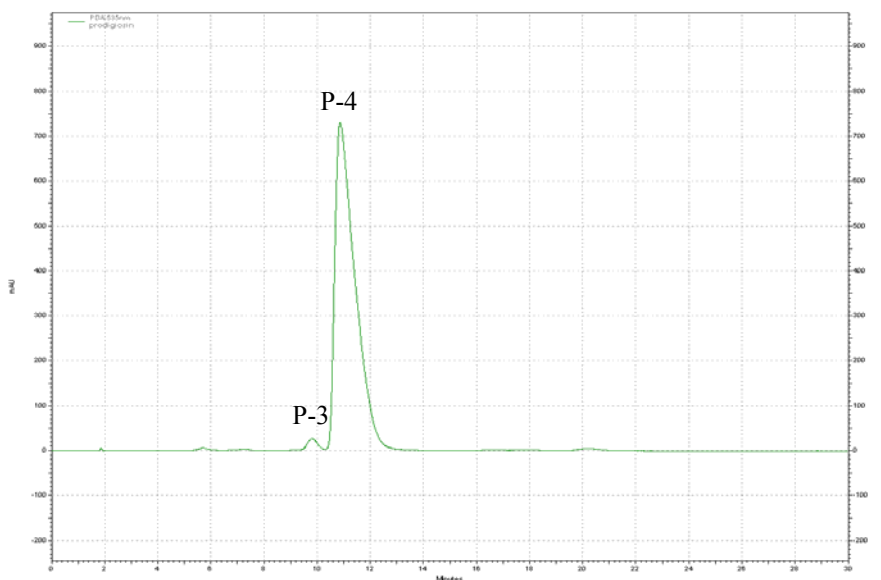


Fig.3.8. HPLC analysis of the ODS separated fraction 2 eluted in CH₃OH/H₂O – 60/40 + 0.2% acetic acid. HPLC system and the separation conditions are the same as in Fig.3.7.

Fraction 3 contained the next most abundant P-5 compound, the amount of which varied between 3.33 to 17.7% of the total amount of the pigment (Fig.3.9).

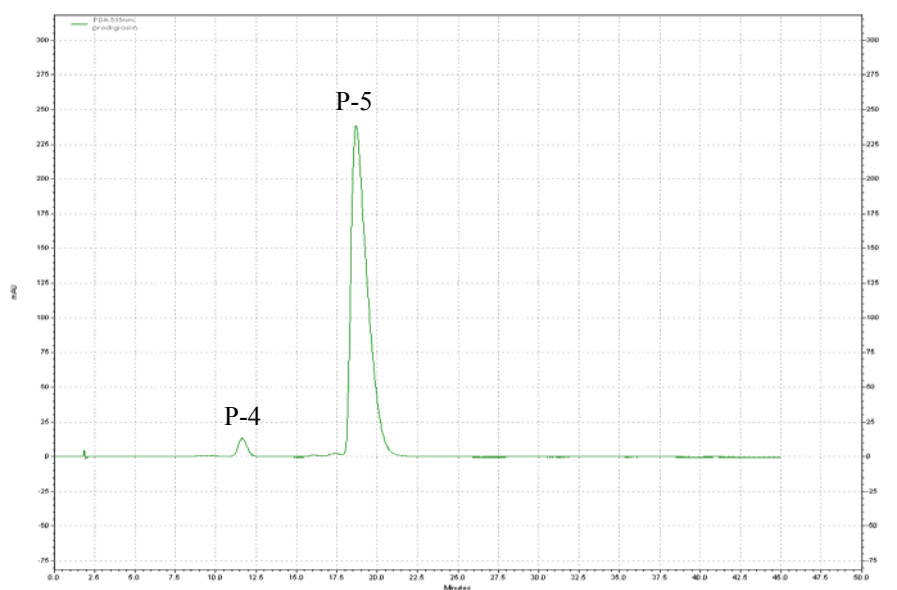


Fig.3.9. HPLC analysis of the ODS separated fraction 3 eluted in CH₃OH/H₂O – 65/35

+0.2% acetic acid.

Fraction 4 contained the last eluted compounds of the red pigment – P-6 and P-7 (Fig.3.10), varying between 0.21-1.55%.

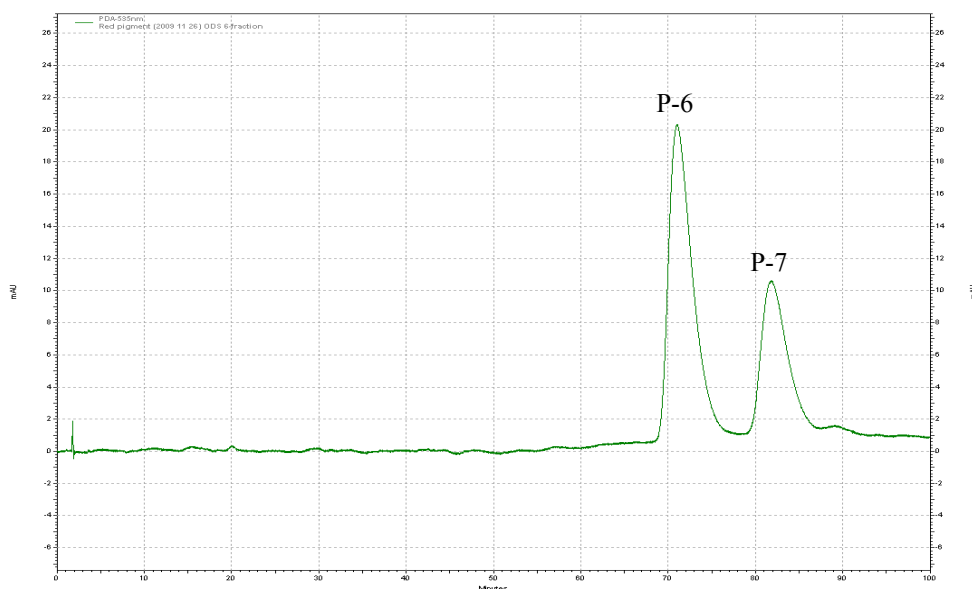


Fig.3.10. HPLC analysis of the ODS separated fraction 4 eluted in CH₃OH/H₂O – 70/30 +0.2% acetic acid. HPLC mobile phase CH₃OH/H₂O – 55/45 (v/v) + 0.2% acetic acid. Other conditions are the same as in Fig.3.7.

The last fraction 5 eluted in CH₃OH/H₂O – 70/30 +0.2% acetic acid did not contain any pigmented compound.

As the main task of this investigation was to separate the first eluting P-1 & P-2 compounds individually, an attempt was made to re-separate fraction 1 using ODS open column with methanol/water as a mobile phase, hoping to get the individual compounds. As a result, three fractions were obtained (Fig.3.11).

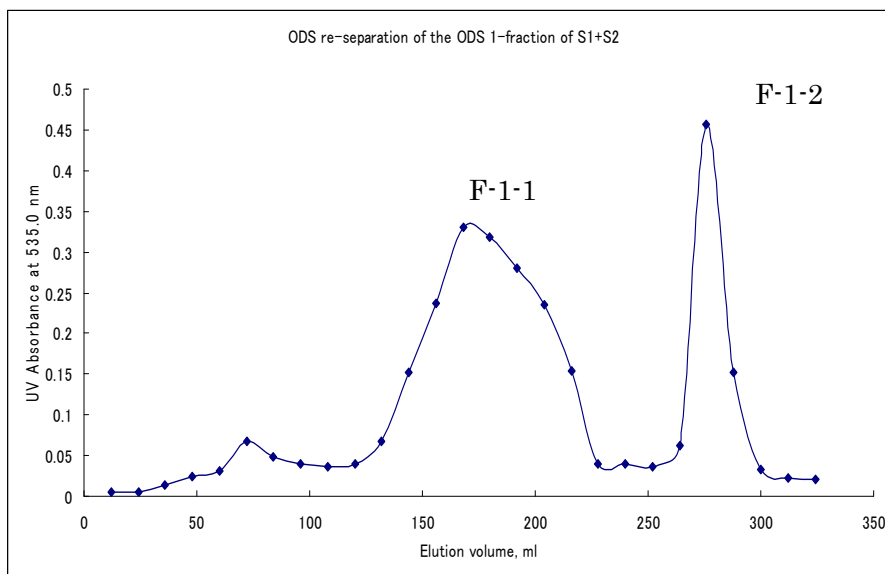


Fig.3.11. ODS re-separation of the fraction 1 (the fraction shown in Fig.3.7).

Although the elution profile showed excellent separation of the two compounds, further HPLC analysis of these fractions revealed that the (F-1-1) – fraction, which was eluted in the mobile phase methanol/water – 45/55 (v/v) + 0.2% acetic acid contained mostly P-1 compound and a small amount of P-2 compound (Fig.3.12).

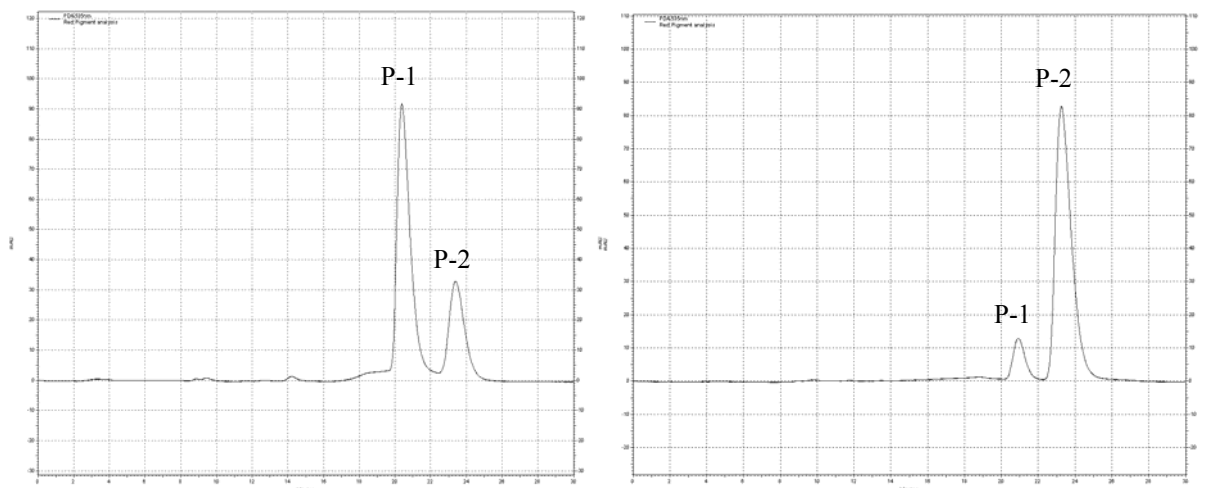


Fig.3.12. HPLC analysis of the F-1-1 and F-1-2 fractions eluted in the mobile phase CH₃OH/H₂O – 45/55 and 55/45 (v/v) + 0.2% acetic acid. HPLC system and the separation conditions are the same as in Fig.3.7.

HPLC analysis of the F-1-2 fraction showed that it mainly consisted of P-2 and a trace

amount of P-1 (Fig.3.12). Although, separated fractions still contained both the major compound and a small amount of the other red pigment compound as expected, it was concluded that their separation can be achieved by two ODS cycles.

The approximate contents of the two compounds were calculated by measuring their UV absorbance between 200-800 nm. The observed max absorbance at 535.0 nm, confirmed that they belong to the prodigiosin-like compounds which accounted for about 1.27% and 0.5%, respectively, of the total amount of the crude red pigment loaded onto ODS column.

As the amounts of pigments were extremely low, the two cycle separation procedure was carried out repeatedly until obtaining the least amounts of compounds for the structural analyses. However, during the accumulation and preservation, it was noticed that the concentrations of the pigments changed due to their degradation. Especially, P-1 compound was found to be unstable as it decomposed faster into other unknown compounds, making impossible to analyze its chemical structure. Newly appeared compounds as a result of decomposition showed the max absorption in the vicinity of 535 nm as prodigiosins give max absorbance, suggesting some of them may belong to prodigiosin family or containing the same chromophore groups as the red pigment compounds. However, the peaks also appeared at the ranges between 200-220 and around 300 nm, suggesting that some of newly appeared compounds may not belong to prodigiosin family. The color of the pigment gradually changed from red to yellow or became even colorless, with intermediate brownish-red, brown and brownish-yellow. Furthermore, when the temperature around 39°C was applied to dry the pigments, the decomposition process was accelerated, which was also visible from the color change of the pigments from red to yellow. This was confirmed by HPLC analysis of the samples, which showed additional peak(s) appeared in the chromatogram (Fig.3.13) at around 300 nm.

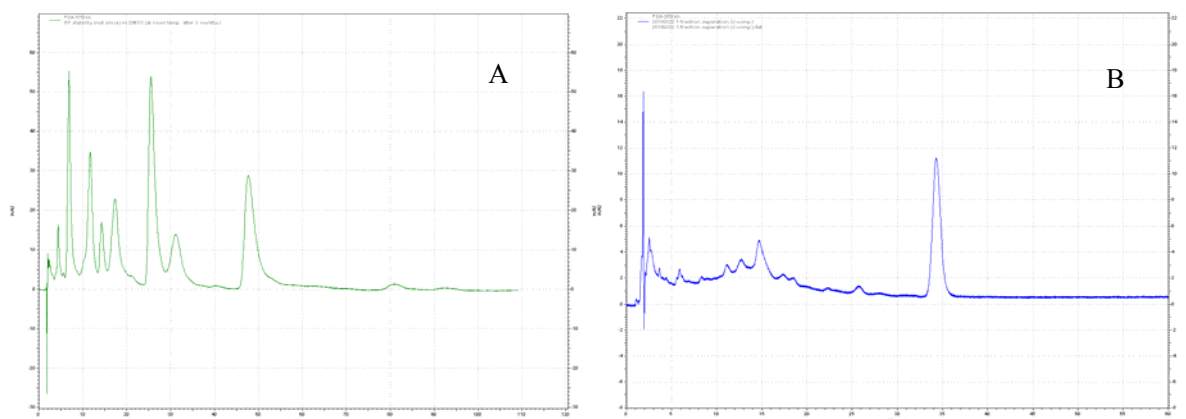


Fig.3.13. HPLC chromatograms of the red pigment samples showing the decomposition occurred with the pigment individual compounds. A – 535.0 nm, B – 300.0 nm.

Later, it was observed that not only P-1, but the amounts of other pigments were also tending to decrease, i.e. the concentrations of the separated individual compounds were not stable, suggesting the decomposition process occurring with other compounds of the red pigment too. These were confirmed on HPLC analyses of the compounds. The degradation of the prodigiosin compounds occurred despite keeping them in dark and cold place, as these compounds are very sensitive to light [200].

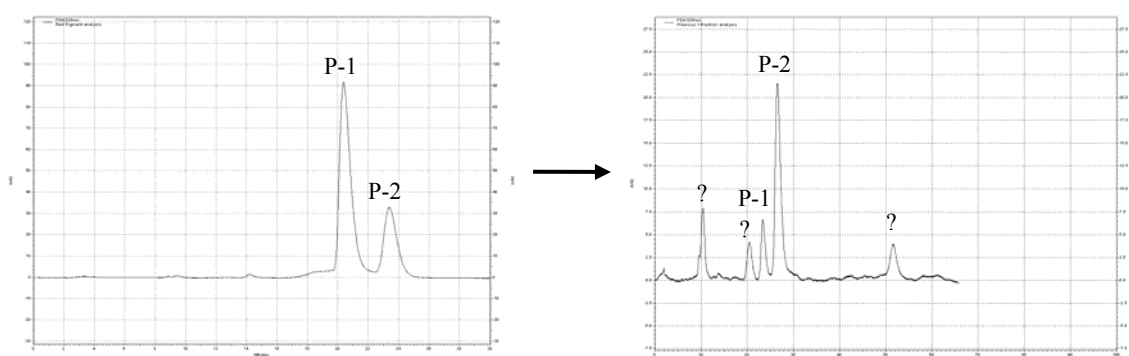


Fig.3.14. HPLC analysis of the fraction containing P-1 and P-2 before (A) and after degradation (B). HPLC System: Shiseido Nanospace SI-2. Chromatographic conditions: Column: ODS Capcell Pak C-18 MGII (1.5 mm × 150 mm); mobile phase: CH₃OH/H₂O – 50/50 (v/v) + 0.2% acetic acid; column temperature: 40°C; flow rate: 100 µl/min.

In fact, the amount of the first unknown compound (P-1) is quite high when the red

pigment is extracted from the bacterial culture broth. As time passes, and especially, after ODS open column separation of the crude red pigment, the amount of P-1 decreases. This became considerable after keeping it around one month in methanol. Newly appeared peaks and the decreased amount of P-1 were clearly seen when the fraction mostly containing P-1 and lesser amount of P-2 was analyzed on HPLC after keeping it for nearly two months in methanol (Fig.3.14). Due to instability, resulting in decomposition, the chemical structure of P-1 was not analyzed. Approximately 0.85 mg of P-2 was collected for the structural analysis of the compound (Fig.3.15).

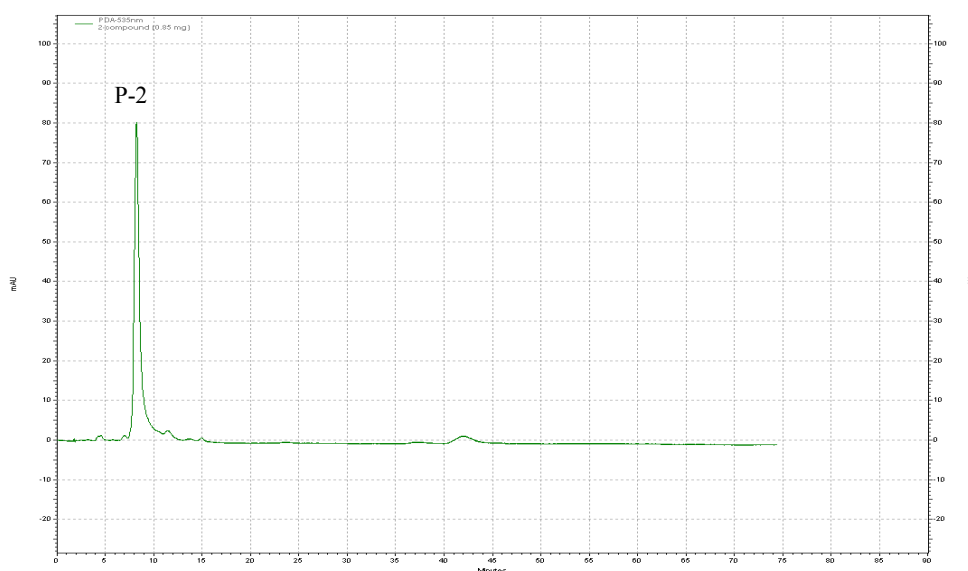


Fig.3.15. HPLC analysis of P-2 in the mobile phase of CH₃OH/H₂O – 60/40 (v/v) + 0.2% acetic acid at 535.0 nm. HPLC system and the other separation conditions are the same as in Fig.3.14.

3.4. Structural analysis of P-2

The chemical structure of P-2 was analyzed using the physicochemical methods of investigation such as high resolution electrospray ionization Fourier transform mass spectrometry (ESI-FTMS) and NMR spectroscopy, including ¹H-NMR, ¹³C-NMR, DEPT, HMQC, HMBC, NOESY, COSY and TOCSY (Appendix 2) as well as by comparison with

previously obtained data from other pigments investigations. ESI-FTMS analyses showed the probable existence of 2-methyl-3-butylprodiginine, since the highest positive-ion signal at m/z 310 $[M+H]^+$ corresponds to the molecular weight of this compound, and also a signal at m/z 252 coincident to the mass of its core structure. In addition, the fragment ion m/z 295 was assumed to be produced by the removal of the CH_3 moiety from the methoxyl residue of the P-2. The comparison of this spectrum with previously obtained mass spectrum for the 2-methyl-3-pentylprodiginine, i.e. prodigiosin showed the chemical structure smaller by 14 Da (CH_2) than prodigiosin, suggesting the structure with shorter alkyl group. Theoretical and observed spectral data of the compound has almost the same mass-to-charge ratios, showing good compliance to the suggested chemical structure.

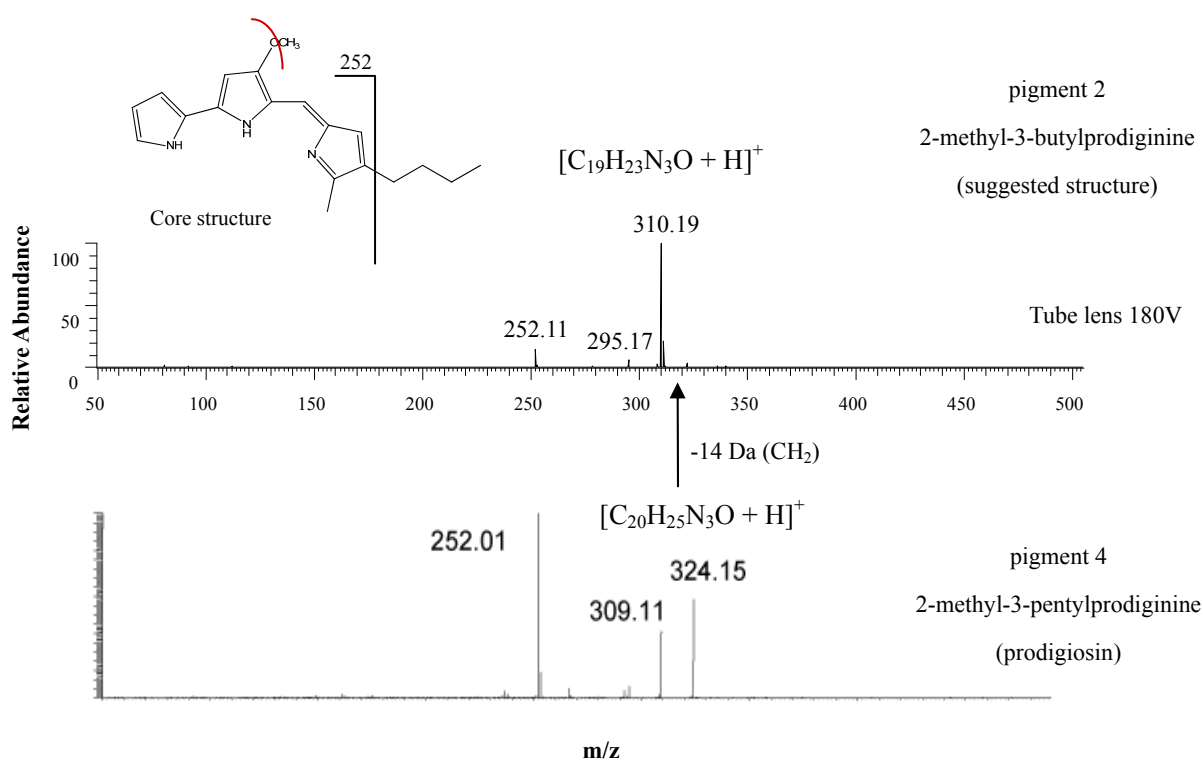


Fig.3.16. ESI-FTMS analysis of P-2 and its comparison with MS data of P-4, previously determined to be 2-methyl-3-pentylprodiginine or prodigiosin.

The suggested chemical structure was confirmed by NMR spectroscopy analyses of the compound. The 1H -NMR and ^{13}C -NMR data for P-2 were coincident with those of

prodiginosin (2-methyl-3-pentylprodiginine) previously reported by our research group [201]. This was proved by additional DEPT, HMQC, HMBC, NOESY, COSY and TOCSY, experiments.

In the $^1\text{H-NMR}$ spectrum of P-2, NH group, H signals of six pyrrole alkaloids, one methoxy group and five groups of alkyl chain protons in the high magnetic field were observed, suggesting the existence of pyrrolopyrromethene structure. $^{13}\text{C-NMR}$ analyses also showed the existence of methoxy group which was differed from the signals of methyl groups at the C-ring and in the alkyl chain. The final conclusion came from the TOCSY analysis which confirmed the existence of methoxy and methyl groups and also alkyl chain length to be equal to butyl group at c-3 position by overlapping signals in 2D measurements.

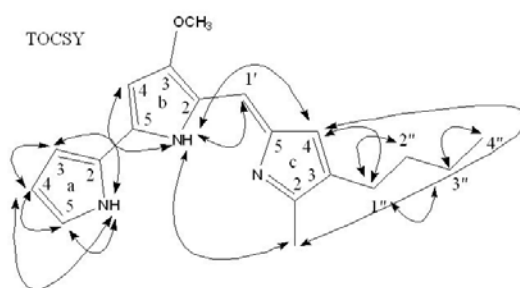


Fig.3.17. Signal correlations observed in TOCSY spectrum of P-2.

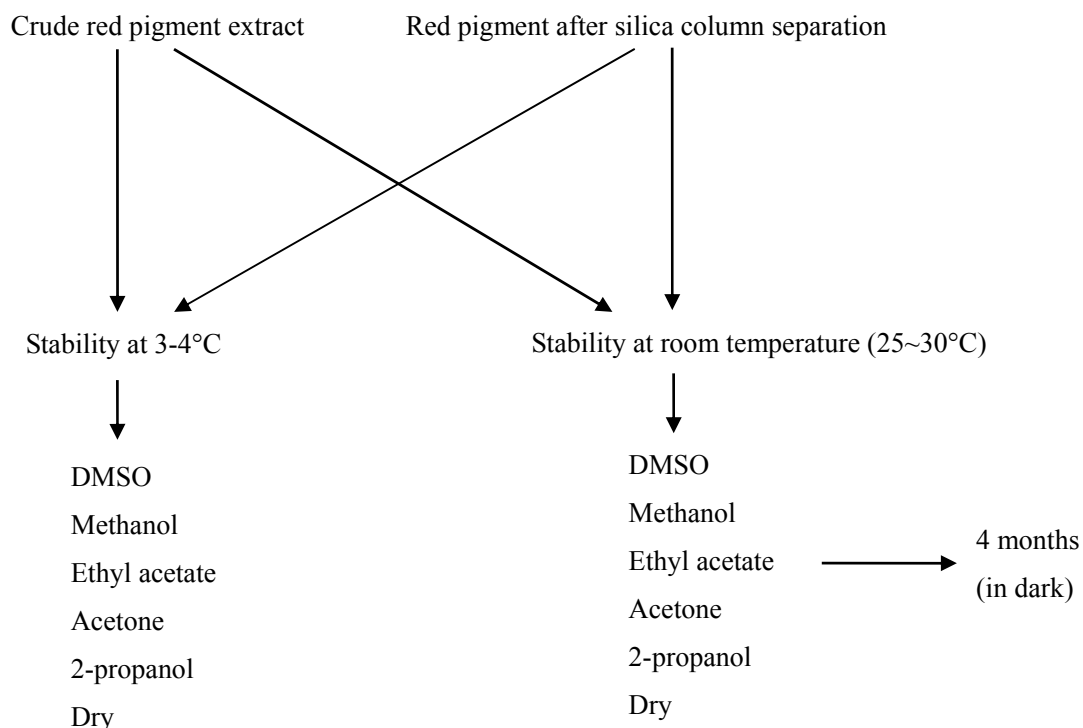
It should be noted that although the existence of 2-methyl-3-butylprodiginine (previously it was depicted as pigment 1, but now P-2) was previously suggested on the basis of MS data [47, 79, 201, 202] this compound had not been isolated to investigate by NMR. This is the first detailed structural characterization of P-2 by NMR spectroscopy. NMR data of P-2 gave the following signals:

$^1\text{H-NMR}$ (CDCl_3 , 400MHz) δ 0.93 (3H, t, H-c4''), 1.35 (2H, m, H-c3''), 1.52 (2H, m, H-c2''), 2.41 (2H, t, H-c1''), 2.54 (3H, s, CH_3), 4.01 (3H, s, H- OCH_3), 6.09 (1H, d, H-b4), 6.36 (1H, m, H-a4), 6.69 (1H, d, H-c4), 6.92 (1H, m, H-a3), 6.96 (1H, d, H-1'), 7.20 (1H, m, H-a5), 12.72 (brs, H-NH-a), 12.56 (brs, H-NH-b);

¹³C-NMR (CDCl₃, 101MHz) δ12.5 (c-CH₃), 14.1 (c4''), 25.1 (c1''), 32.2 (c2''), 22.3 (c3''), 58.7 (OCH₃), 92.8 (b4), 111.7 (a4), 116.0 (1'), 117.0 (a3), 121.0 (b2), 123.3 (a2), 125.2(c5), 127.0(a5), 128.4(c4), 128.5 (c3), 147.1 (c2), 147.7 (b5), 165.8 (b3).

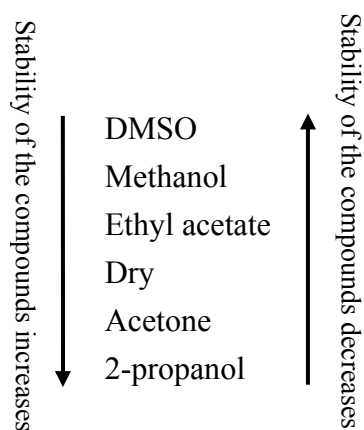
3.5. Investigations on the stability of the red pigment compounds

Although, it is not clear to what kinds of compounds may convert or decompose P-1 and other prodigiosin compounds and what causes their decomposition, it seemed that the environmental conditions, i.e. temperature and solvent might play a crucial role in this process. Therefore, the impact of environmental conditions, i.e. temperature and solvents on the stability of the pigments were investigated. For this reason, a crude extract of the red pigment and the red pigment purified through silica column consisting of the mixture of all prodigiosin compounds were dissolved in DMSO, methanol, acetone, ethyl acetate, 2-propanol and also dried samples were kept for 4 months at 4°C and at room temperature in a dark place.



All the samples were analyzed by HPLC once in every month. As a result of this

investigation, the impacts of the solvents and environmental conditions were found to be essential. Among the solvents used to dissolve the prodigiosin compounds, DMSO followed by methanol were found to be the most aggressive to destroy the pigments. These two solvents were the most often used solvents to dissolve the red pigment samples. So, it seems that, by dissolving the red pigments, samples always undergo destruction.



The degradation process was accelerated at room temperature samples when compared to the samples kept at cool temperature. It is interesting to note that even dry samples were tending to degradation, indicating that the dry condition is not a guarantee to keep the red pigment samples. However, the environmental conditions, i.e. solvents and temperature, do not seem the main causes of the degradation of compounds. Especially, the crude red pigment samples which were not purified through silica gel were degraded faster even if they were kept at 4°C. The red pigment samples purified through silica gel prior to stability experiments have become more stable, though the decrease in the amounts of individual prodigiosin compounds was still observed. Probably, this could be due to the presence of high polar small molecule compounds, which could be mostly retained in silica gel during the purification of the red pigment. These compounds, which do not contain chromophore groups to be detected on HPLC analysis, might play a role of catalytic agents to initiate the degradation of prodigiosin compounds. However, this suggestion needs additional investigations to be confirmed.

3.6. Cytotoxicity of prodigiosins against human U937, K562 and HL60 leukemia cell lines

Cell cytotoxicity experiments of prodigiosins were carried out on human U937, K562 and HL60 leukemia cell lines by assessing their effect on the MTS reduction assay. As can be seen from Fig. 3.18, the red pigment resulted in significant decrease in the percentage of the viable cells. IC₅₀ value of prodigiosins against U937 cells was about 0.7 μ M (Fig. 3.18 A) while it was around 1.5 μ M in HL60 (Fig. 3.18 B) and around 2.5 μ M in K562 (Fig. 3.18 C) cells.

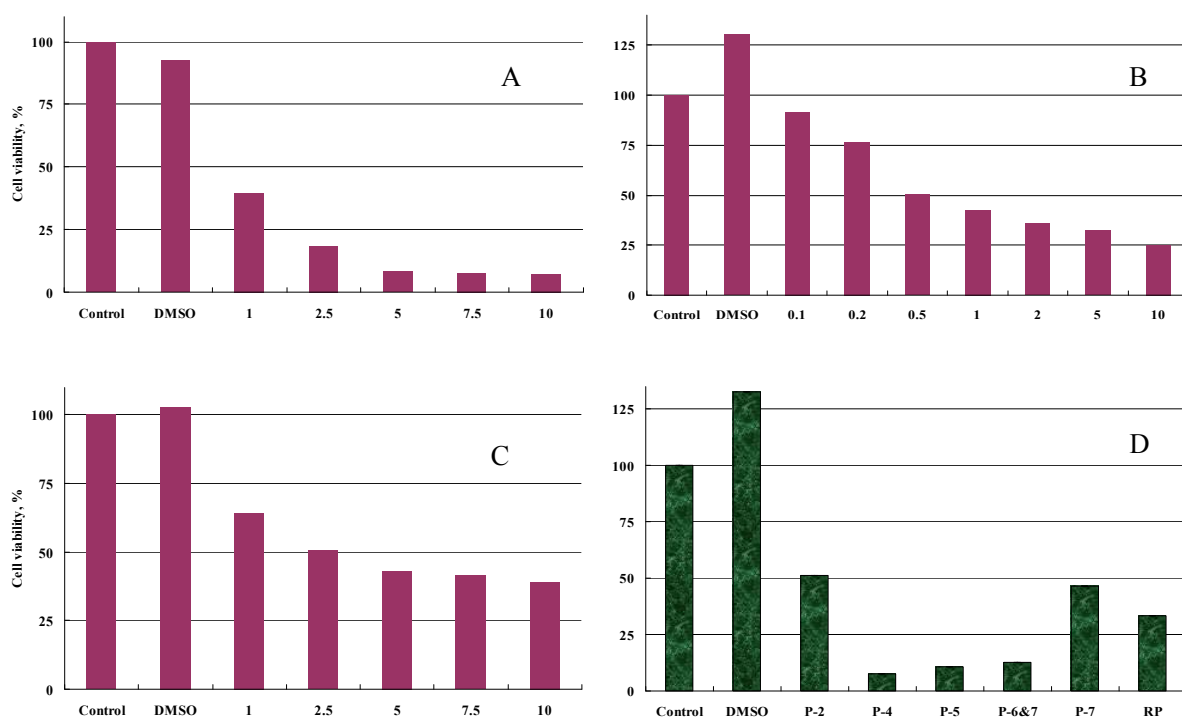


Fig.3.18. Effect of the mixture of the red pigment at increasing concentrations on U937 (A); HL60 (B); K562 (C) cell lines and the effect of individual compounds (2 μ M) on U937 cell lines (D).

Among the individual prodigiosin compounds, P-4, i.e. 2-methyl-3-pentylprodinine or prodigiosin showed the highest apoptotic effect (Fig. 3.18 D) against all the tested cancer cells, followed by P-5 (2-methyl-3-hexylprodinine), the mixture of P-6 and 7, and P-7. The lowest effect of P-2 suggests to its lowest hydrophobicity compared to other analogs that may hinder

it to penetrate through the lipid bilayer of the cells to initiate the apoptotic processes.

3.7. Effects of prodigiosin compounds and violacein on the activities of protein phosphatases and protein kinases

It is well-known that both the prodigiosin compounds and violacein have a wide variety of pharmacological activities, including antibiotic, immunosuppressive (only prodigiosins) and above all cytotoxic effect against malignant tumor cells. Some molecular mechanisms of cytotoxicity of both types of compounds identified until now, which can be the case to cause the programmed cell death, are mentioned in the Introduction part. However, the full mechanisms of action remain elusive, requiring more deeper and precise investigations.

To contribute to the better understanding of the molecular mechanisms of cytotoxicity, in our research we studied the effect of the red pigments and violacein on protein kinases and protein phosphatases which are known to play a significant role in the intracellular signal transduction pathway. It should be noted that Fürstner and coworkers [100] were the first who showed that roseophilin and some synthetic prodigiosin analogues inhibit protein tyrosine phosphatase activity. They suggested that this inhibitory effect of the compounds may be employed to cure the malignant diseases. However, the phosphatases inhibition by naturally derived prodigiosin compounds has not been investigated.

On the other hand, Bromberg *et al.* reported violacein's effect on the total protein phosphatases activity extracted from human lymphocytes [189]. However, violacein effect on protein kinases was not investigated so far. Taking into account the importance of the role of signaling pathway molecules in cells, we studied the effect of prodigiosin and violacein compounds on some representative members of protein kinases and protein phosphatases to investigate whether these important enzymes are involved in the pigments cytotoxic effect. Selected members of kinase and phosphatase family enzymes were tested *in vitro* to assess the inhibitory effects of prodigiosin and violacein compounds. Structure-activity relationship

(SAR) of prodigiosin compounds was also studied.

3.7.1. Effect of prodigiosin compounds and violacein on phosphorylating activity of protein kinases

The effects of prodigiosin compounds and violacein were investigated on the catalytic subunits of protein kinase A (PKA) and C (PKC), including PKC subfamily isozymes as well as Src-protein tyrosine kinase (Src-PTK) and calmodulin-dependent protein kinase (CaM kinase). Fig. 3.19 shows the effect of prodigiosins on CaM kinases activity. The enzyme activity remained almost intact (Fig.3.19), showing phosphorylation activities similar to positive DMSO control. The prodigiosins also showed no remarkable effect (data not shown) against PTK enzyme activity, too. Consequently, both PKA and PKC activities were not either induced or inhibited by the red pigment and its individual compounds (data not shown). From these results, we conclude that protein kinases may not be direct targets of prodigiosins.

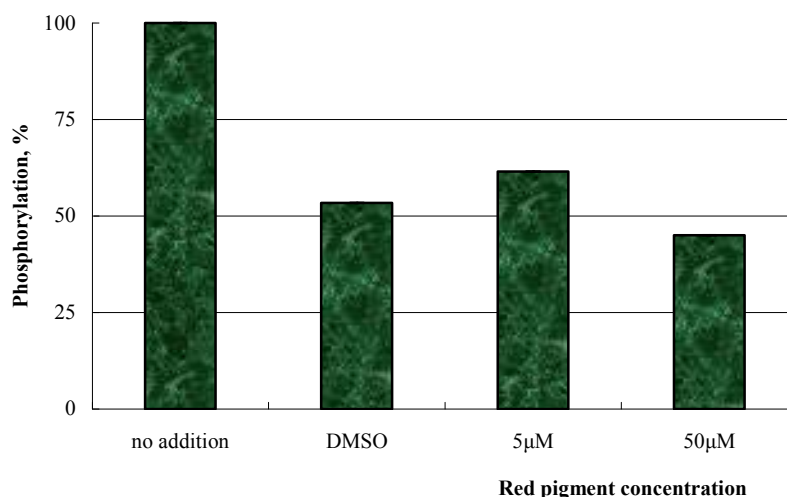


Fig.3.19. CaM kinase inhibition assay by prodigiosin compounds

On the other hand, violacein showed inhibitory effect on the catalytic activities of both PKA and PKC. The activities of the enzymes gradually decreased by increasing the concentration of violacein, indicating that the inhibition was dependent on the concentration

of violacein. The IC_{50} values for PKA and PKC were equal to 6 and 2 μM , respectively (Fig.3.20). In the experiments, violacein inhibited all the conventional types of PKC isozymes, with different level of inhibition. It has also an inhibitory effect on some but not all novel types of PKCs, while causing slight inhibition of ζ isozyme which belongs to atypical type of PKC (Fig.3.20). However, violacein failed to inhibit Src-PTK and CaM kinase activities.

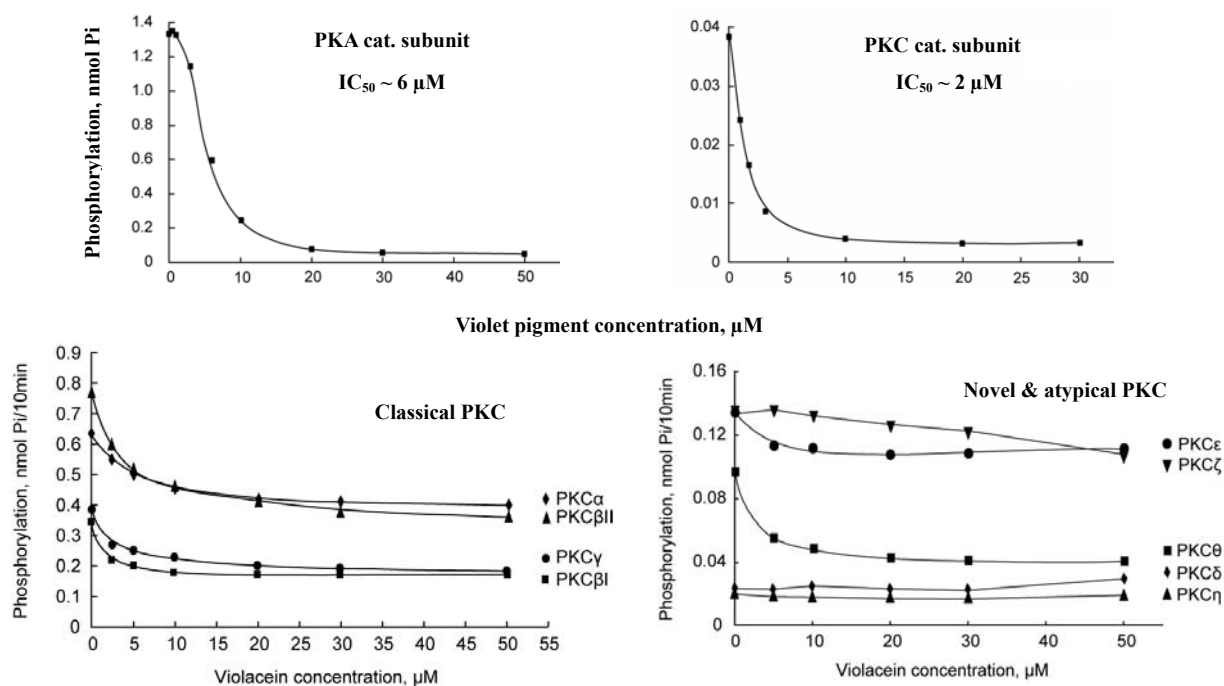


Fig.3.20. Violacein effect on PKA and PKC catalytic subunits as well as PKC isozymes.

These results suggest that the chemical and conformational structures of the enzymes may play an important role in binding with violacein and prodigiosins to cause the inhibition of the enzyme activities.

3.7.2. Effect of prodigiosin compounds and violacein on dephosphorylating activity of protein phosphatases

3.7.2.1. Effect of prodigiosin compounds and violacein on PTP1B enzyme activity

Experiments were carried out according to the assay kit instructions, with some modifications.

In the assay, while violacein showed little effect on the enzyme activity, the mixture of

individual compounds of the red pigment gradually decreased and completely inhibited the enzyme activity at 10 μM , showing almost the same result as inhibitor and/or no enzyme controls (Fig.3.21 A).

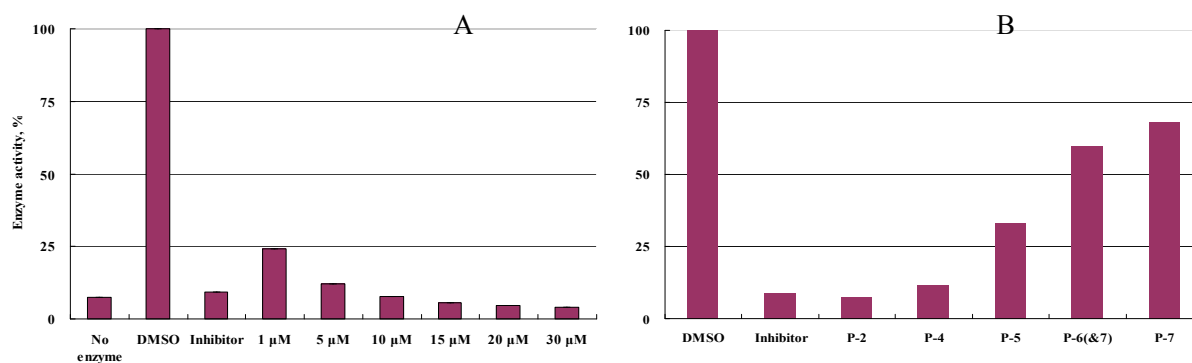


Fig.3.21. PTP1B inhibition assay by the red pigment (A) and its individual compounds (B) tested at 10 μM . Data represent a typical result from two independent experiments and the mean values of enzyme activity assayed in duplicate.

Further investigations of the individual prodigiosin analogs on structure-activity relationships (SAR) were conducted in detail, to assess the most influential compound against the enzyme activity. For this reason, the red pigment was freshly isolated from the bacterium and its individual compounds were separated through the silica and then ODS column chromatography. While P-1 was not used for the experiments due to its instability, P-6 was used in the mixture with P-7 as the residual amount of the latter was found in its fraction (about 5~10%)

For the experiments, each compound was prepared at 10 μM concentrations. The obtained result shown in Fig.3.21 B demonstrated that the pigment 2 has the highest effect on the enzyme activity. The activities of other compounds gradually decreased in the elution order from HPLC column, i.e. increased alkyl chain length in the structure decreased the inhibitory effect of the compounds.

3.7.2.2. Effect of prodigiosin compounds and violacein on PP2A enzyme activity

In the beginning, optimum experimental conditions were established prior to carry out the main experiments. Particularly, the content of the reaction mixture, the amount of enzyme and the pigments to be used for the experiments as well as the optimum incubation time were found experimentally.

As in the case of PTP1B assay, in order to test which individual prodigiosin compound has the most influence on the enzyme activity, they were examined at the concentration of 10 μM . As a result, the individual compounds of the red pigment caused strong inhibition of the enzyme activity (Fig.3.22. A).

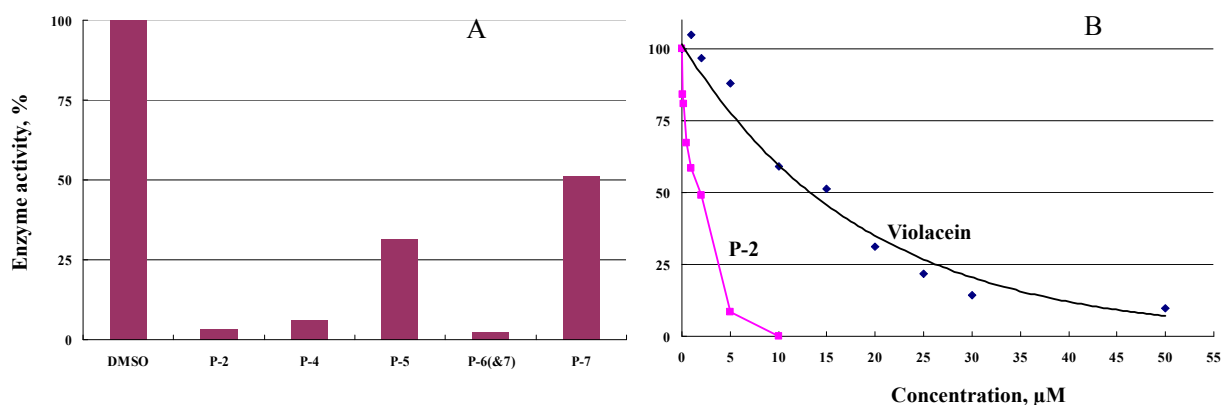


Fig.3.22. Effect of individual compounds of the red pigment (A), P-2 and violacein (B) on PP2A enzyme activity. All individual compounds were 10 μM . Data represent a typical result from two independent experiments and the mean values of enzyme activity assayed in duplicate.

Again, the P-2 along with the mixture of P-6 and P-7 showed the highest inhibitory effect towards the enzyme activity. In contrast to the inhibition of PTP1B, the mixture fraction of P-6 and P-7 showed strong inhibition against PP2A enzyme. It is not known whether the inhibition is a synergistic effect of both P-6 and P-7. If this is the case, it is suggested that PP2A may have at least two binding sites for prodigiosin compounds. Violacein also showed inhibitory effect on the enzyme activity, but its inhibitory effect was lower than those of P-2,

P-4 and P-6(7) mixtures, and showed a similar extent of inhibition at 10 μM as P-5 and P-7. The IC_{50} value of P-2 was approximately 2 μM , and violacein showed the IC_{50} value of around 15 μM (Fig. 3.22. B).

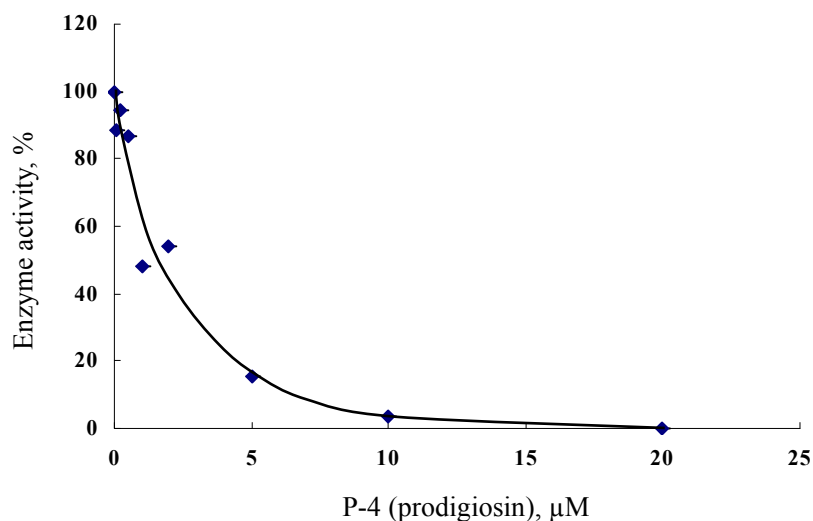


Fig.3.23. Effect of P-4 (prodigiosin) on PP2A enzyme activity.

The IC_{50} value of P-4 or prodigiosin was also approximately 2 μM , confirming its high inhibitory effect against PP2A enzyme activity.

3.7.3. Combinatorial effect of P-2 and violacein

As shown in Fig.3.22B, P-2 was a strong inhibitor of PP2A, and violacein moderately inhibited the enzyme activity. However, it was found that a certain combination of P-2 and violacein elevated the PP2A activity.

To investigate whether prodigiosin compounds induce PP2A enzyme activity in the presence of violacein more effectively, the mixtures of the two pigments, i.e. P-2 and violacein were tested against the enzyme activity. For this experiment, violacein concentration was set as constant being 10 μM , while gradually increasing the concentration of P-2. Although, at higher concentrations of P-2 the enzyme activity was decreased in the presence of violacein, at lower concentration it was increased (Fig.3.24). These results suggest that the

binding sites of the enzyme might be different for P-2 and violacein.

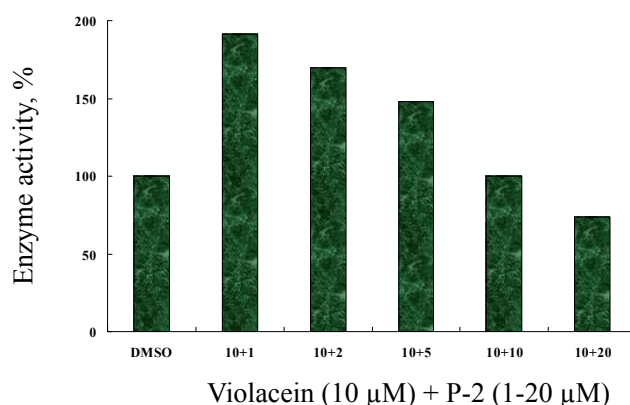


Fig.3.24. Combinatorial effect of violacein (10 μM) and P-2 (1-20 μM) on PP2A enzyme activity.

3.7.4. Kinetic analyses of PP2A enzyme by violacein

The kinetic parameters of the enzyme inhibition by violacein (Fig.3.25 A) showed that K_m value for the *p*NPP substrate was roughly to be equal to 22 μl, and the Lineweaver-Burk plot of kinetic parameters (Fig.3.25 B) showed that it inhibits the enzyme by non-competitive nature of inhibition.

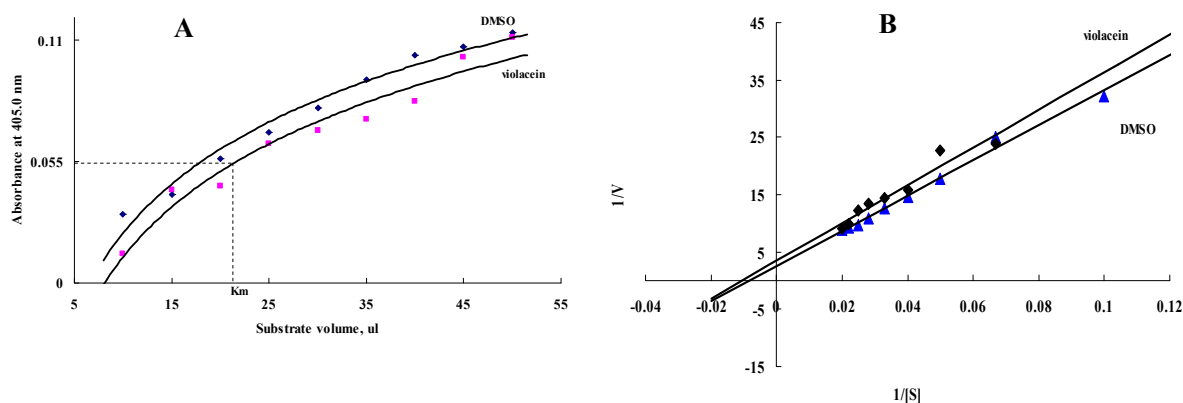


Fig.3.25. Kinetic analysis of PP2A enzyme inhibition by violacein: (A) – determination of K_m value, and (B) - Lineweaver-Burk plot

The apoptotic cell death by prodigiosins, which are now under intensive investigations,

are known to result from both the extrinsic and intrinsic pathways. Many apoptosis causing factors of prodigiosins which have been investigated so far, revealed that mainly the intrinsic pathway through mitochondria is involved in the prodigiosin-induced apoptosis [94]. This could be explained by the hydrophobic nature of these compounds which enables to penetrate through lipophilic membrane bilayer. However, the extrinsic pathway through the death receptor signaling is also important, as it was reported not to involve p53 signaling [203]. In fact, prodigiosins are known to cause apoptosis through both activation [204] and independently [204-206] of p53 tumor suppressor protein and our results are additional confirmation of those previously reported investigations. The red pigment showed cytotoxic effect on HL60, U937 and K562 leukemia cell lines, with slightly different IC₅₀ values. Despite different morphology of the cells, where HL60 and K562 lack p53 protein, prodigiosins could still induce apoptosis in both types of cells. This protein is known to be involved in a number of cellular processes including gene transcription, DNA repair, cell cycling, genomic stability, senescence and apoptosis [207]. It is also known that the lack of p53 protein makes cancer cells insensitive to anti-cancer drugs, limiting their clinical efficacy as chemotherapeutic agents [208]. Different kinds of cellular signals resulting from DNA damage, a defective cell cycle, hypoxia, loss of cell survival factors and other types of cell stresses are known to activate this protein which in turn either mediates cell cycle arrest by further causing survival or initiates apoptosis through the activation of pro-apoptotic proteins [94], thus accelerating the death of injured cells. This process depends on how much the cell compartments are damaged.

However, our results clearly show that prodigiosins induce apoptosis in both types of cancer cell lines no matter of existence p53 protein. On this assumption, it is suggested that the apoptosis process in these cells is caused through the activation of multiple pathways including p53 protein and without its involvement. This confirms our previous study where the red pigment caused both DNA fragmentation and caspase 3 activation in U937 cells,

which are considered as stimuli for the activation of p53 [209]. In the case of K562 and HL60 cells which lack p53, the previous study suggests the possibility of existence of other pathways causing apoptosis upon the red pigment effect on these cells. This might be the reason for lower effect of prodigiosins onto these cells where the IC₅₀ values were higher than in U937 cells. In these cell lines, the extrinsic pathway of apoptosis which may be triggered independently from p53 protein [203] can play an essential role. However, considering cell permeability of prodigiosins and accumulation in mitochondria [94], it is suggested that the intrinsic pathway could still be critical. Therefore, it is essential to investigate the other pathways most likely involved in the apoptosis process caused by prodigiosins. Taking this into consideration and analyzing previously reported data in literature, we investigate their effect on the signal transduction pathway molecules as possible targets of apoptosis.

The red pigment from strain 1020R was proven to contain seven individual prodigiosin family compounds. SAR studies showed that the P-4, i.e. 2-methyl-3-pentylprodiginine or prodigiosin has the highest apoptotic effect on the cells, followed by P-5, P-6 and 7, and P-7 (Fig. 3.18 (D)). The P-2 or 2-methyl-3-butylprodiginine, which has the shortest alkyl group, had the lowest effect when compared to its analogs. This could be explained by its lower hydrophobicity than that of other analogs. Such low hydrophobicity may hinder it to penetrate through the membrane lipid bilayer. On the contrary, P-2 has the strongest inhibitory effect on protein phosphatases (Fig. 3.21 B and Fig. 3.22 A). On the other hand, DMSO which is mainly used as a drug carrier to increase the effectiveness of chemotherapeutic drugs also enhanced the proliferation of leukemia cells. Fig.3.18 B, C and D shows elevated cell viability in DMSO at lower concentrations of the red pigment. With increasing the red pigment concentration, cell viability decreased gradually, and especially, abruptly after 0.5 μ M. This observation indicates that the results for the low concentration pigment may not be true due to proliferative effect of DMSO that overcame pigments activity.

To investigate the intrinsic pathway of apoptosis induced by the red pigment, we targeted

on kinase and phosphatase family enzymes along with other molecules of the signal transduction pathway. Although, inhibition of selective members of protein phosphatases by prodigiosin-like compounds has been previously studied [100], the action of prodigiosin and its close related analogs on this class of enzymes was not reported. Furthermore, many of the previously investigated compounds were mainly synthesized and naturally derived compounds were not studied [100]. Additionally, there was no direct evidence of the involvement of the protein kinases except indirect inhibition of PKC by prodigiosin [99].

The obtained results clearly show inhibition of the enzyme activity of tested phosphatases, but not kinases. Unlike the phosphatases, kinases do not seem to be direct targets for prodigiosins, as all kinases tested in this study were not inhibited or inhibited insignificantly, indicating selective action of prodigiosins towards phosphatases. The apoptosis-inducing effect on cancer cells (Fig. 3.18) and the inhibitory action against PTP1B activity (Fig.3.21) of individual compounds of the red pigment in the order of P-2 to P-7 suggests that the chemical structures of the compounds play an essential role in their biological action. Being similar in chemical structures, they differ only by the length of alkyl chain at C-3 position (Fig.3.26) (Chemical structures of P-1, P-3 and P-7 are currently remaining unknown).

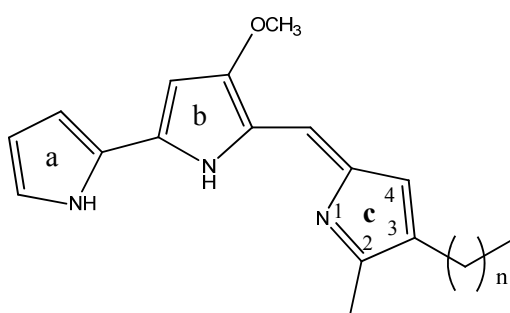


Fig.3.26. Chemical structures of the red pigment individual compounds: $n=3$ - 2-methyl-3-butyl-prodiginine (P-2); $n=4$ - 2-methyl-3-pentylprodiginine (prodigiosin) (P-4); $n=5$ - 2-methyl-3-hexyl-prodiginine (P-5); $n=6$ - 2-methyl-3-heptylprodiginine (P-6).

Although, the exact mechanism of inhibition of enzymes is unknown, it is suggested that a structure with shorter alkyl chain more easily penetrates into the active site loop of the enzyme molecule. Consequently, the insertion strength increases as the side chain length of the compounds at C-3 position extends in the order of P-2 to P-6 (Fig.3.26). Zhang and Zhang [210], reviewing the advances on discovery of specific PTP1B inhibitors, pointed out that increasing hydrophobicity can be an effective strategy to improve PTP1B inhibitor bioavailability due to enhanced permeability through the cell membrane. On the other hand, Combs suggested that extremely hydrophobic small molecule inhibitors are not desirable agents due to nonspecific inhibition of the enzyme activity [211]. Considering medium hydrophobicity and membrane permeability of prodigiosins [94], it seems that these compounds could be specific and competitive inhibitors of PTP1B. As the drug discovery targeting PTP1B is now considered as a challenging task due to highly conserved and positively charged active-site pocket of the enzyme [210], it seems a good sign to be a potent drug candidate against type 2 diabetes and cancer [212] that the red pigment and its individual compounds have selective affinity towards enzyme with IC_{50} value of less than 1 μM .

Another enzyme we investigated was PP2A which belongs to serine/threonine dephosphorylating group of phosphatase family. To determine which compound of the red pigment has the highest influence on the enzyme activity, newly obtained individual compounds of the red pigment were tested at the concentrations of 10 μM , which was resulted in strong inhibition of the enzyme activity (Fig.3.22 A). As in the case of PTP1B assay (Fig.3.21 B), P-2 along with the mixture of P-6 and P-7 showed the highest inhibitory effect towards the enzyme activity. The IC_{50} value of P-2 was further found to be around 2 μM (Fig.3.22 B). This value would be even lower if the only pure enzyme was used. The fact is that bovine serum albumin (BSA), added to the enzyme solution at the concentration of 1 mg/mL to keep its stability, may be the reason for higher IC_{50} value of the pigment than expected. The pigment molecules could probably bind to BSA, thus resulting in lower effect.

However, this value is reasonable, considering the existence of dozens of proteins in cellular cytoplasm if this experiment would be done in *in vivo* conditions.

CHAPTER IV

Conclusions

Although, 1020R strain and the red pigment produced by this bacterium were carefully studied previously, recent findings have revealed that it must yet to be studied in detail. Many interesting phenomena were observed during this study. Interesting was to observe the varying amounts of pigments ratio in culture medium, as well as the intensive pigment formation at a high rate of shaking of the culture medium which was previously thought to halt pigment production by the bacterium. However, the most remarkable finding was to observe the increased number of compounds in the pigment. Detailed HPLC analyses of the pigment have revealed that the pigments 1 and 2 actually consists of two compounds each, which was previously considered to be single compounds. This can give a reasonable explanation for the previous unsuccessful trial of determination of the chemical structure of the pigment 1 by NMR spectroscopy, which failed due to impurity of the sample.

Our investigations have also revealed that the newly discovered P-1 is extremely unstable compared to other red pigment compounds, decomposing into other unknown yet compounds, thus making impossible to analyse its chemical structure. This was occurred despite keeping the pigments at cool temperature and without illumination, which is considered to be the stimulus for the degradation of the prodigiosin compounds. Decomposition of P-1 insisted on the precautions for the occurrence of the same process with other pigments. Later, these suspicions were confirmed by HPLC analyses, where gradual decrease in the amounts of the pigments was observed.

In order to investigate the factors that may cause the degradation of compounds, the impacts of the temperature and solvents were studied. The obtained results showed that both

of these factors might be essential in degradation of the red pigment compounds. Particularly, the pigment samples dissolved in DMSO and methanol and kept at room temperature conditions were tended to heavy degradations. However, this was the case with pigment samples that was not purified through silica gel. The pigment samples purified through silica gel have become more stable and kept for longer time without notable changes, though slowly decrease in the pigment concentrations were still observed. This suggests that the crude red pigment sample may contain polar small molecule compounds, which may initiate the degradation process of the pigments. These compounds might have mostly retained by silica gel during separation. The suspected compounds could not be detected on HPLC analyses as they might have not contained chromophore groups.

Newly appeared compounds due to the decomposition of the pigments suggest that some of them might be derivatives of prodigiosin or contain the similar chromophore groups as they also appear near to the wavelength where prodigiosins give max absorbance. However, some additional peaks were also observed near to 300- θ nm in the UV spectrum, suggesting the difference of the nature of compounds. The decomposition process was also evident from the outer view of the samples whose color changed from red to brownish yellow colour. Drying of the red pigment samples by applying 39°C or above accelerated the irreversible decomposition, suggesting temperatures higher than the ambient to be crucial for the pigments stability.

The chemical structure of P-2 was analysed using high resolution ESI-FTMS and different methods of NMR spectroscopy, including ¹H-NMR, ¹³C-NMR, DEPT, HMQC, HMBC, NOESY, COSY and TOCSY. As a result of these investigations, the chemical structure of P-2 was elucidated to be 2-methyl-3-butylprodiginine, as previously proposed from the analysis of LC-MS/MS.

Signal transduction pathways in eukaryotic cells are mostly regulated by precise control of phosphorylation and dephosphorylation levels of proteins, which can be controlled and

balanced by two classes of enzymes that work conversely to each other in the action, i.e. protein kinases and protein phosphatases [211]. It is an essential biochemical process, with estimated of 30% of all the cellular proteins to be phosphorylated [213]. The properly balanced phosphorylation-dephosphorylation process may provide a normal control of cellular responses to external stimuli. The abnormal activity of individual kinases and phosphatases associated with partly or fully inhibition and/or excessive induction may cause the pathogenesis in the cell, which leads to a variety of diseases, including cancer. Therefore, developing agents that can specifically inhibit the excessive activity of these enzymes have become an important issue as they represent potential drug candidates. Recently, protein kinases and protein phosphatases actively participating in the control of cell growth and differentiation by regulating the signal transduction pathway have become an attractive research tool to evaluate cellular viability in cytotoxicity studies.

In this study, the effect of prodigiosin and violacein compounds were investigated on protein kinase and protein phosphatase enzymes activities as the possible targets in the apoptosis process of tumor cells. Selected members of kinase and phosphatase family enzymes were tested *in vitro* to assess the inhibitory effect of prodigiosins. Structure-activity relationship (SAR) studies of individual compounds of the red pigment were also investigated. The obtained results indicate that the chemical structures of the prodigiosin analogs are essential to cause apoptosis of cells and inhibition of protein phosphatases. Among the red pigment individual compounds, P-4 corresponding to prodigiosin, showed the highest apoptotic effect to all the tested leukemia cell lines, while the P-2 had the highest inhibitory effect on protein phosphatases, decreasing in the order of P-2 to P-7.

Overall, the following conclusions were made according to the obtained and observed results:

Regarding the prodigiosins stability and the structural analyses of P-2

1. Prodigiosin extract from strain 1020R was found to contain at least 7 prodigiosin compounds.
2. P-1 copurified together with P-2 was found to be unstable, decomposing onto other unknown compound(s).
3. For the first time in this study, P-2 was separated, purified and its chemical structure was elucidated to be 2-methyl-3-butylprodiginine [209].
4. Prodigiosin compounds were found to be sensitive to the temperature higher than 39°C.
5. Silica gel separation was found to increase the stability of prodigiosins.
6. DMSO was found to be the most aggressive solvent to destroy the prodigiosins, followed by methanol, ethyl acetate, acetone, and 2-propanol.

Regarding cytotoxicity of prodigiosins on leukemia cells

7. Prodigiosin compounds showed apoptotic effect against U937, HL60, and K562 leukemia cells.
8. P-4 (prodigiosin) was the strongest to induce apoptosis in U937, HL60, and K562 leukemia cells, while P-2 (2-methyl-3-butylprodiginine) showed the highest inhibitory effect against PP2A and PTP1B activities.

Regarding inhibition of protein phosphatases by prodigiosins

9. Until now, the effect of natural prodigiosin compounds on protein phosphatases was not reported, therefore, this is the first report to study their effect on protein phosphatases (PTP1B, PP2A).
10. Prodigiosins from marine 1020R strain strongly inhibited the activity of protein phosphatases (PTP1B, PP2A), but had no or very low effects on protein kinases (PKA, PKC, PTK and CaM kinase) tested in this study.

Regarding the effect of violacein on protein kinases and protein phosphatases

11. Violacein effect on protein kinases A (PKA) and C (PKC) catalytic subunits was initially

investigated by Hosokawa and Enomoto, but its effect on PKC isozymes and other protein kinases as well as on protein phosphatases was studied in this study for the first time.

12. Violacein showed inhibitory effects against classical PKC and some novel PKC, but was relatively inactive against other protein kinases (atypical PKC, PTK and CaM kinase).

13. Violacein showed selective inhibitory effect against protein phosphatases tested in this study, inhibiting PP2A, but not PTP1B enzyme.

Overall, these results suggest that the intracellular signal transduction pathway molecules might be involved in the cytotoxicity of prodigiosins and violacein towards malignant cancerous cells. This study also provides insight into the molecular mechanisms of cytotoxicity of prodigiosin compounds, the inhibitory effect of which against protein phosphatases gives a hope to be a potential drug candidate against type II diabetes and cancer.

Future perspectives

The following suggestions seem to be interesting to investigate in the future:

- To elucidate the chemical structures of P-1, P-3, and P-7, which are currently remaining unknown;
- The exact factors that destabilise the prodigiosin compounds;
- To investigate the inhibitory and/or activatory effect of the prodigiosin compounds to protein phosphatases (PP2A, PTP1B)-mediated signaling pathway.

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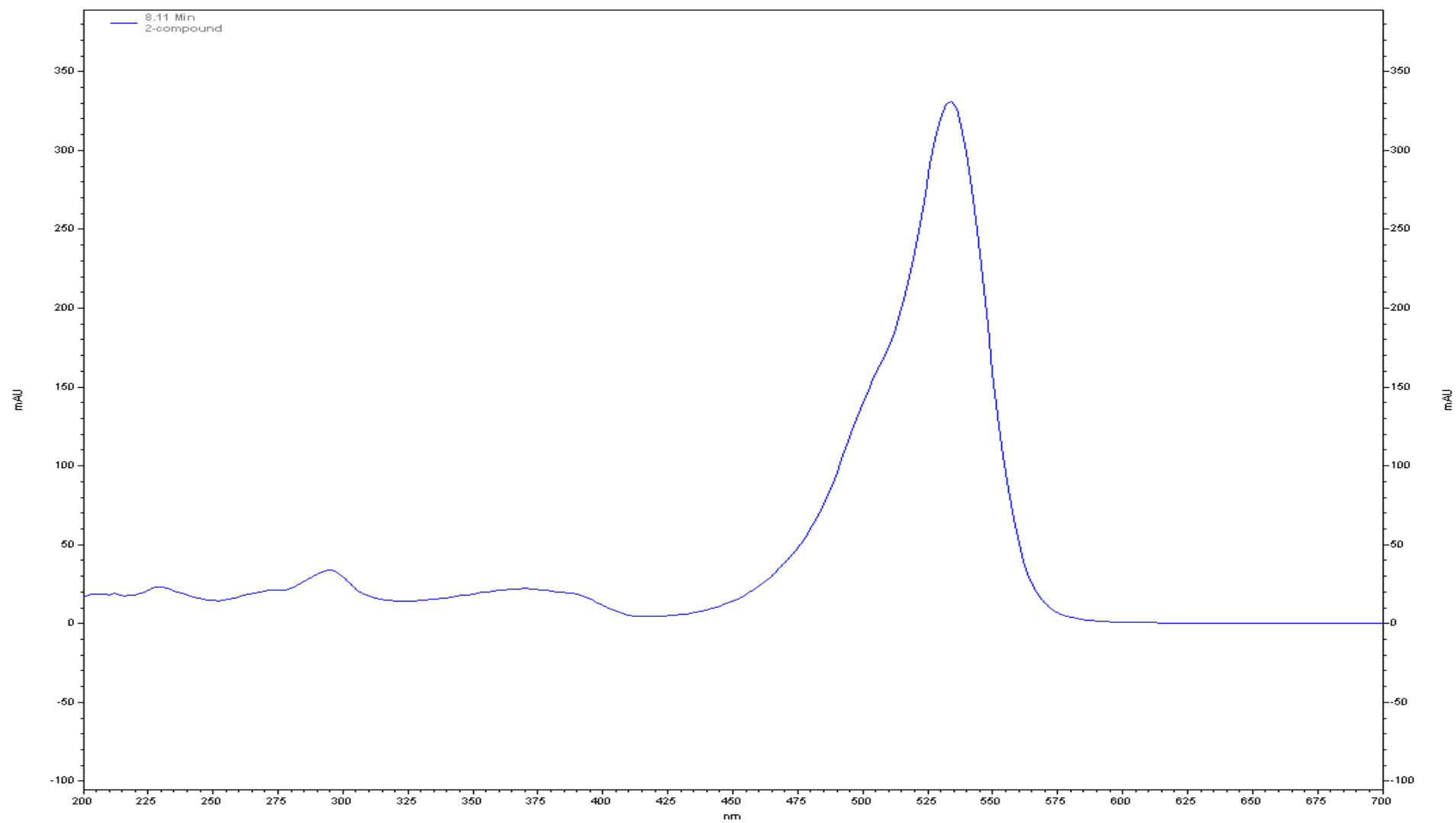
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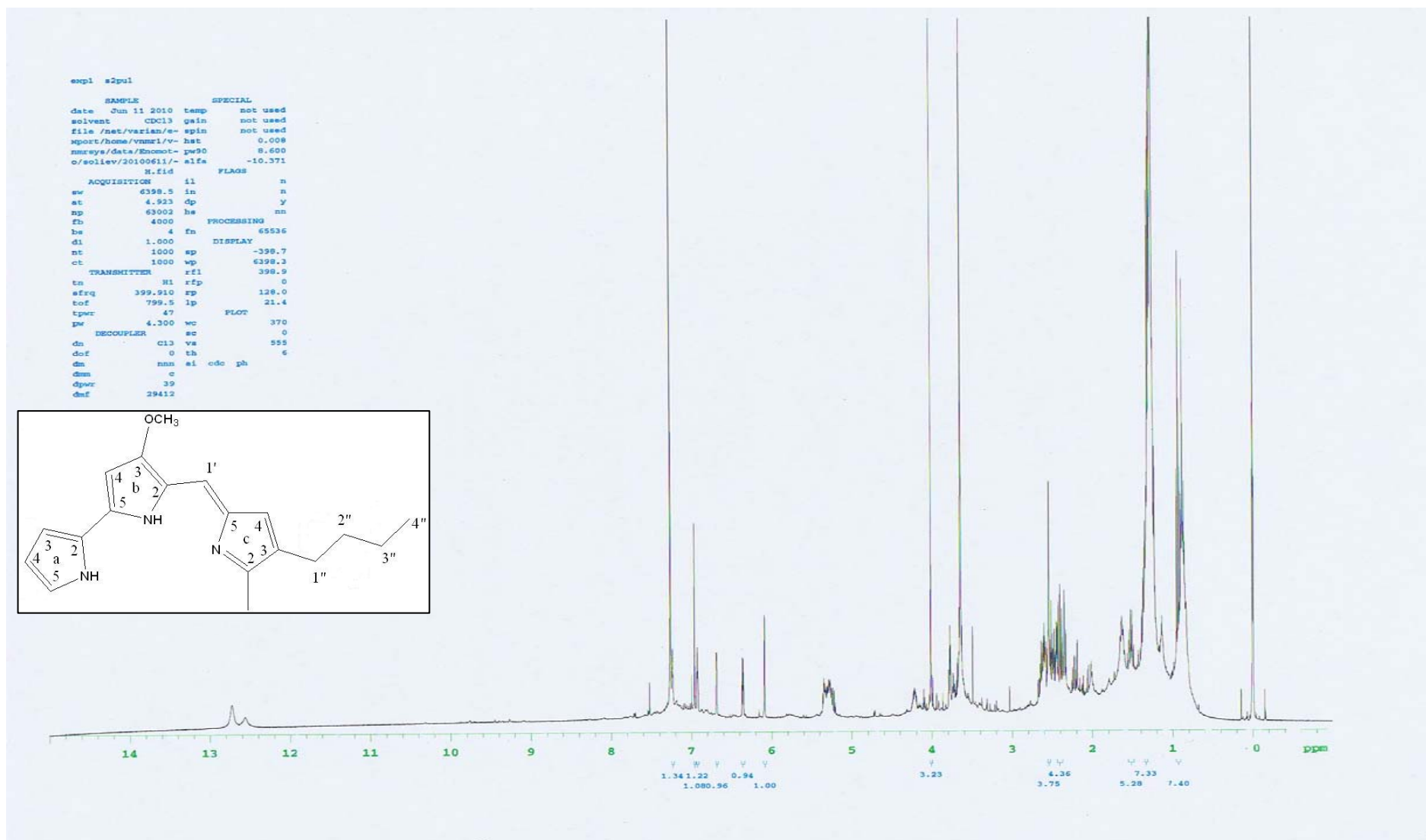
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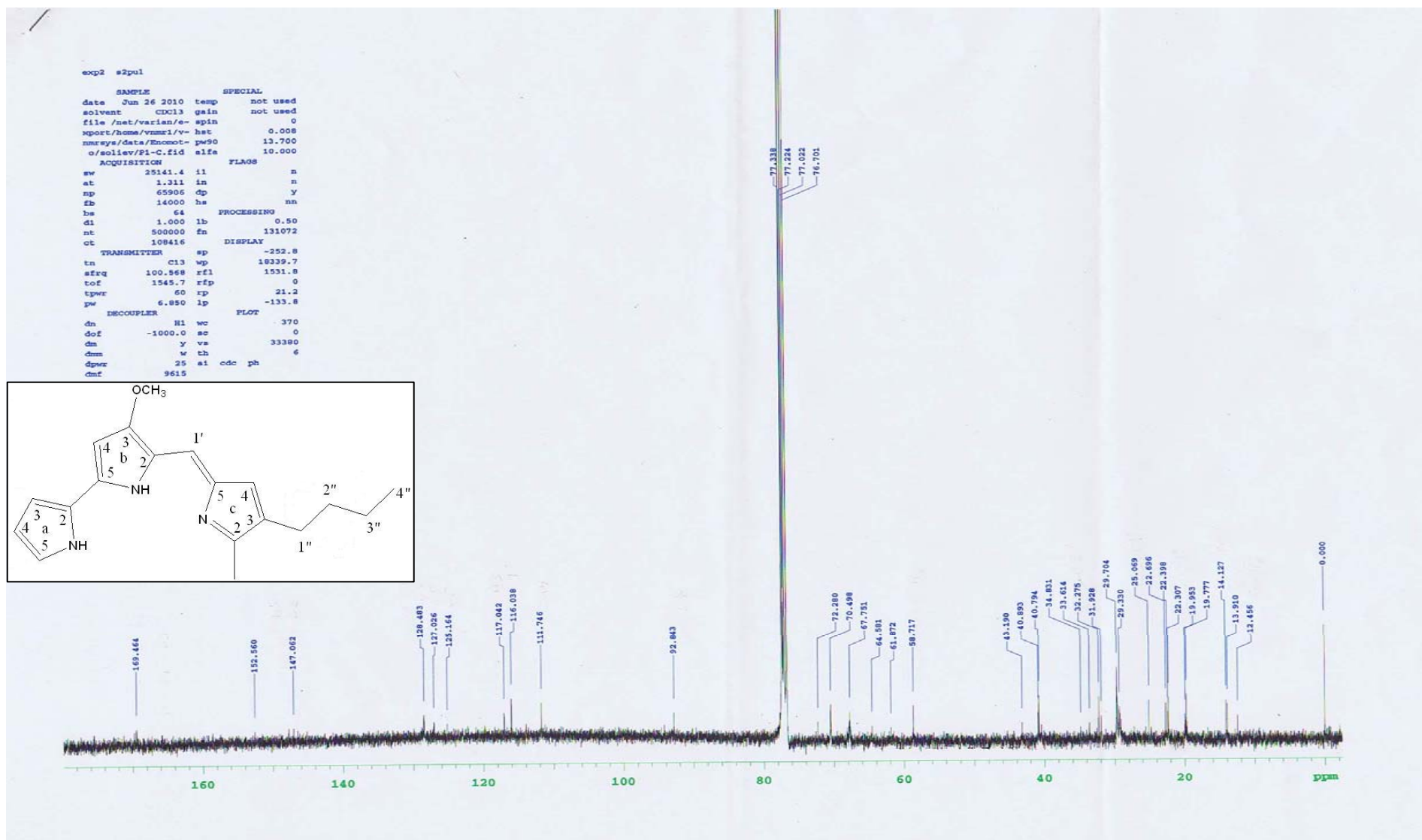
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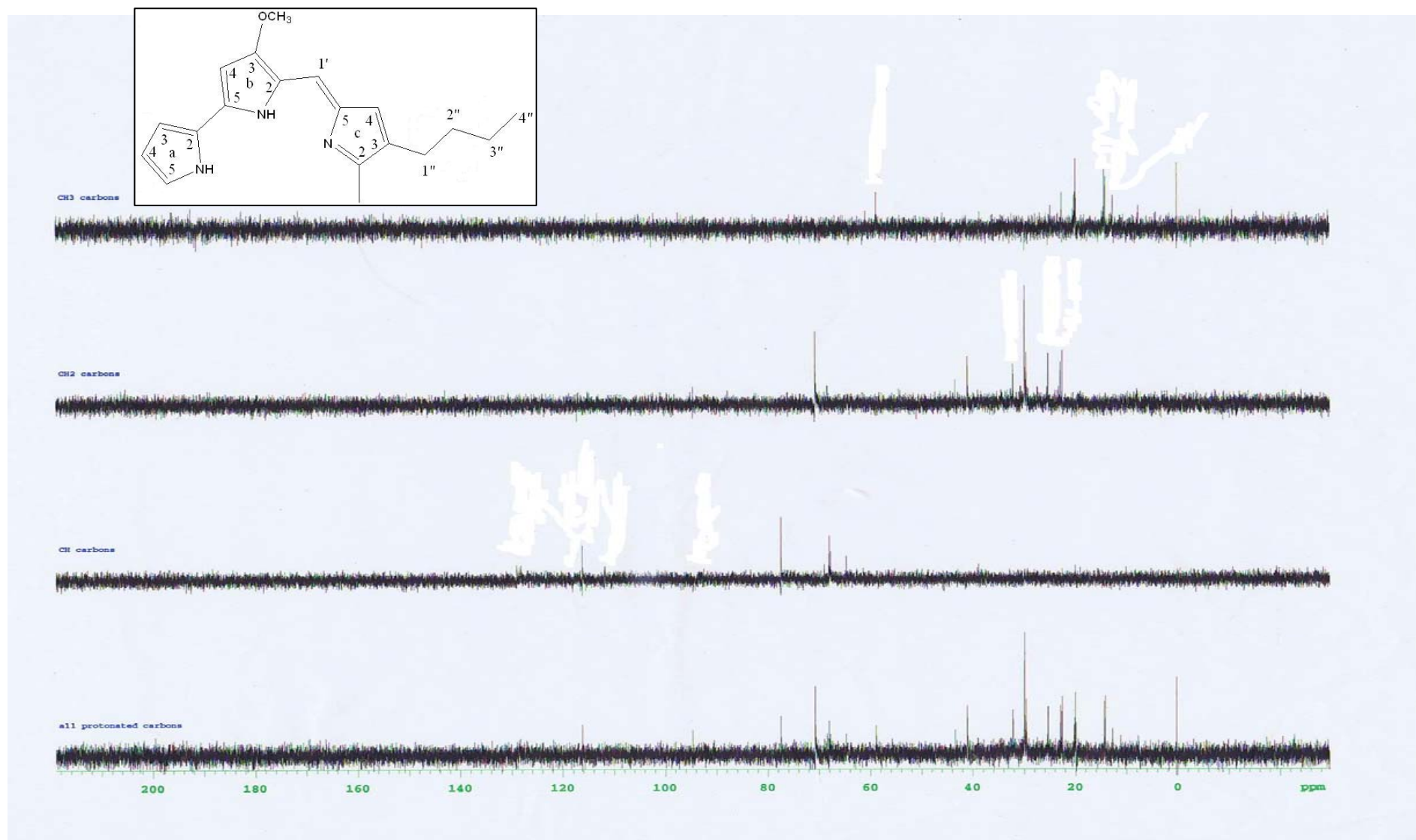
Appendix 1. Representative UV spectrum of the prodigiosin compounds.



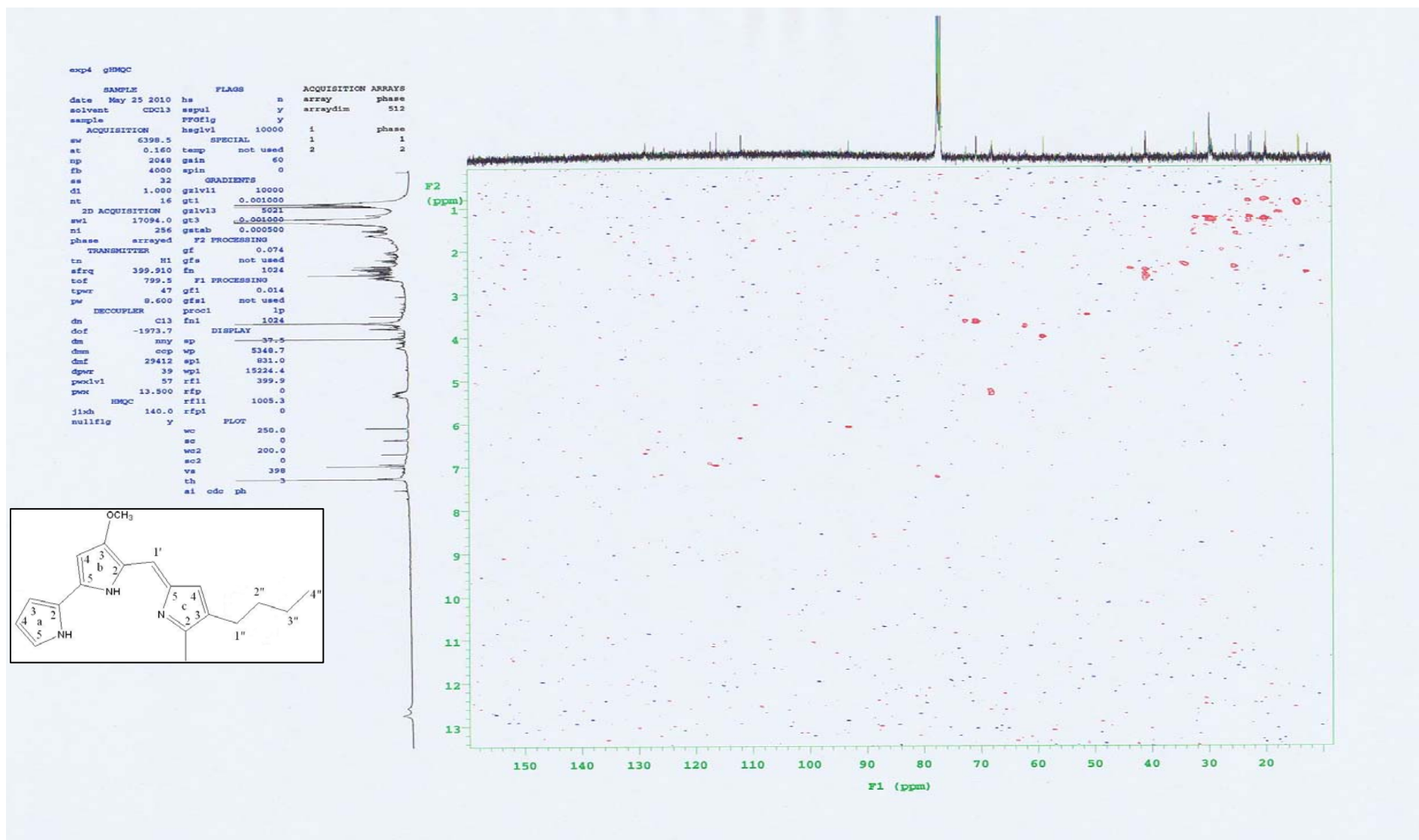
Appendix 2. ¹H-NMR spectrum of P-2.



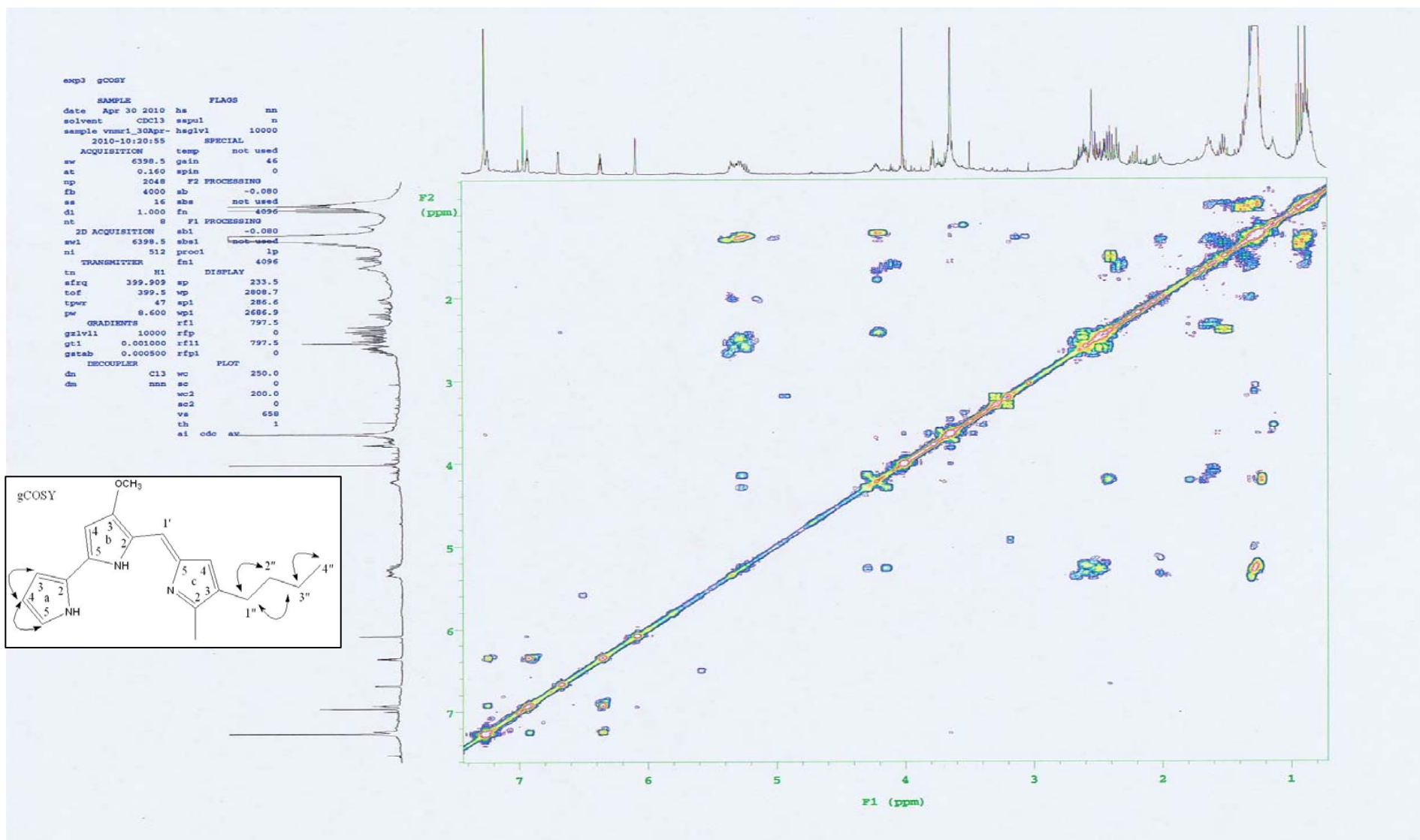
Appendix 3. ^{13}C -NMR spectrum of P-2.



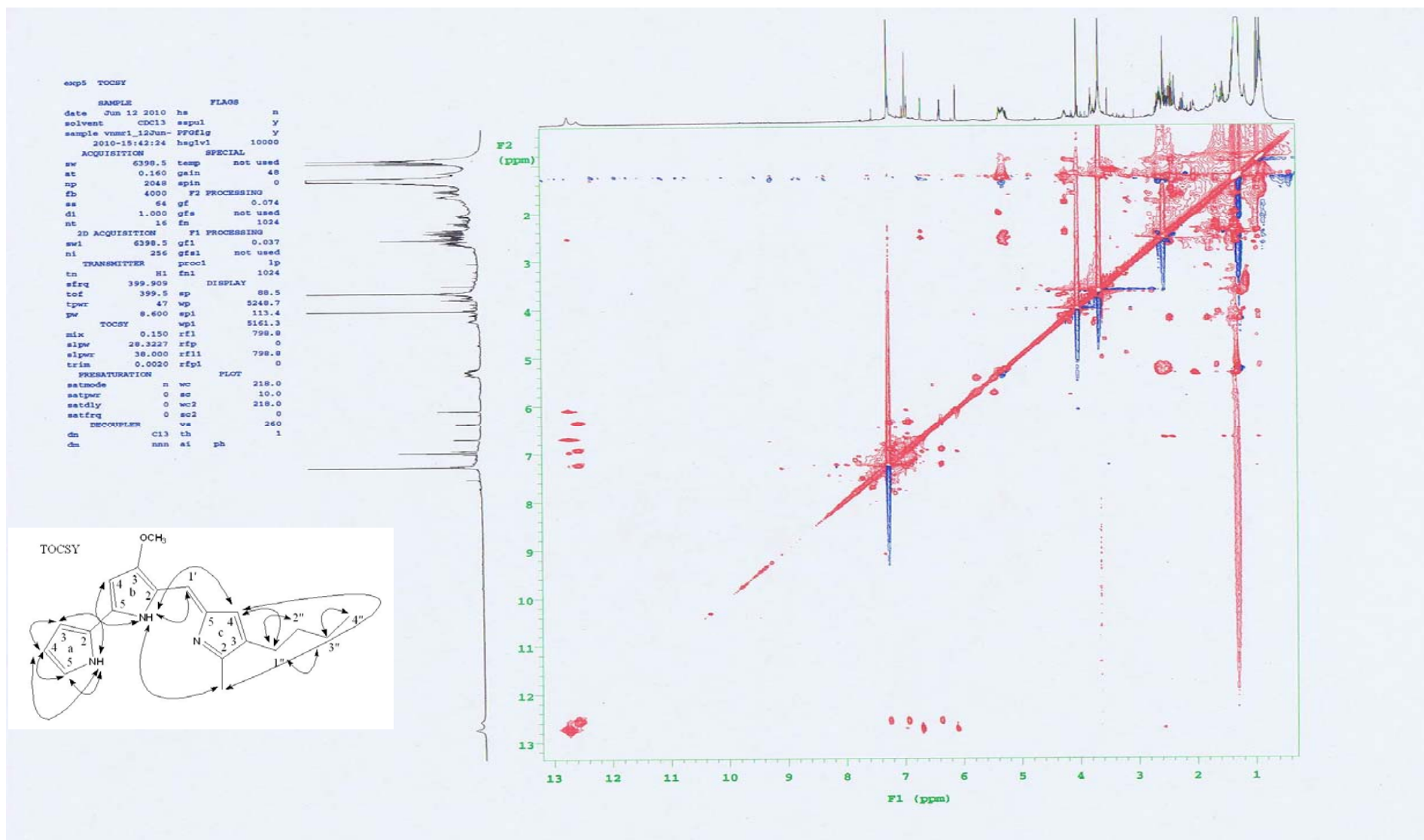
Appendix 4. DEPT spectrum of P-2.



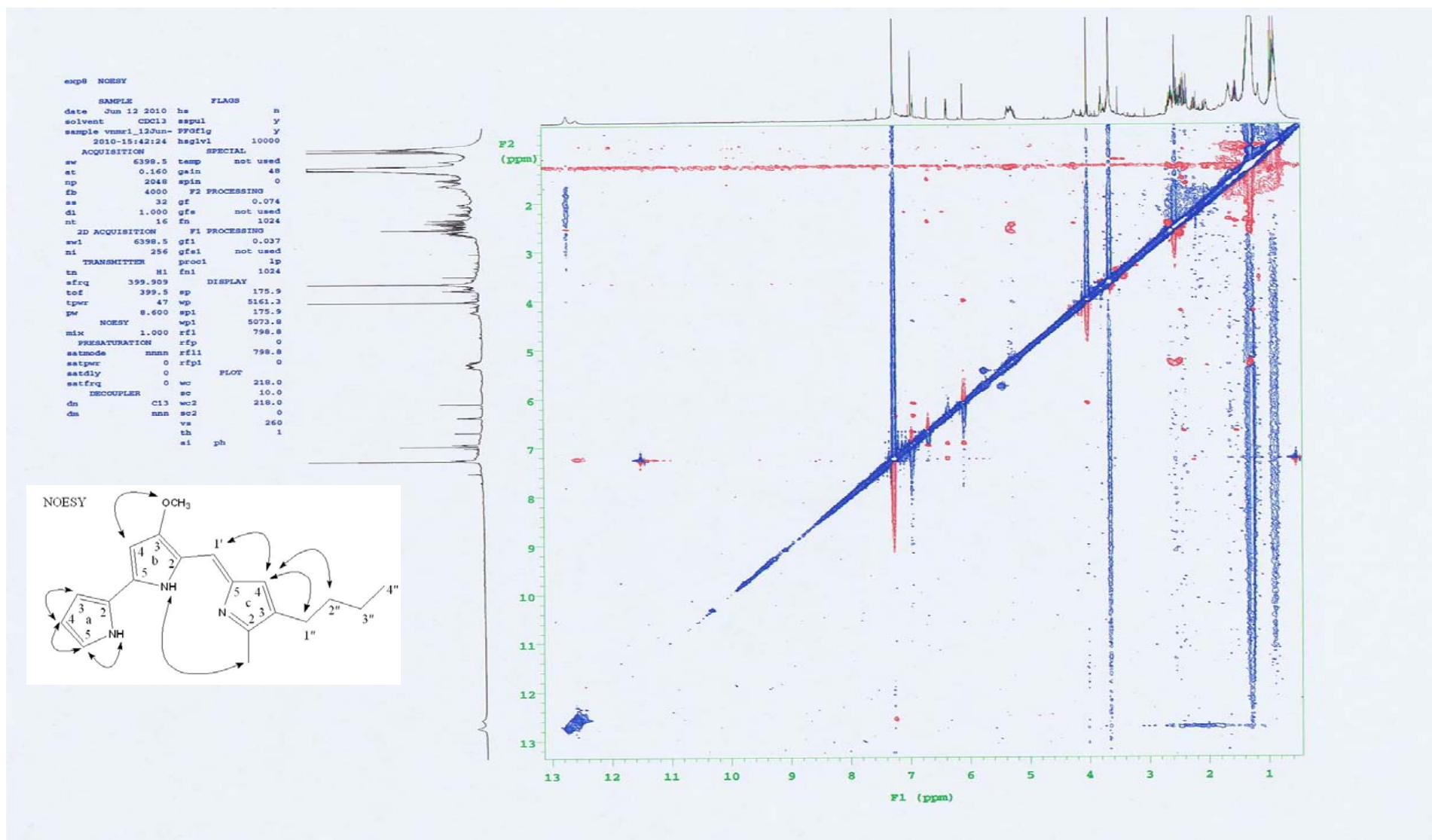
Appendix 5. gHMQC spectrum of P-2.



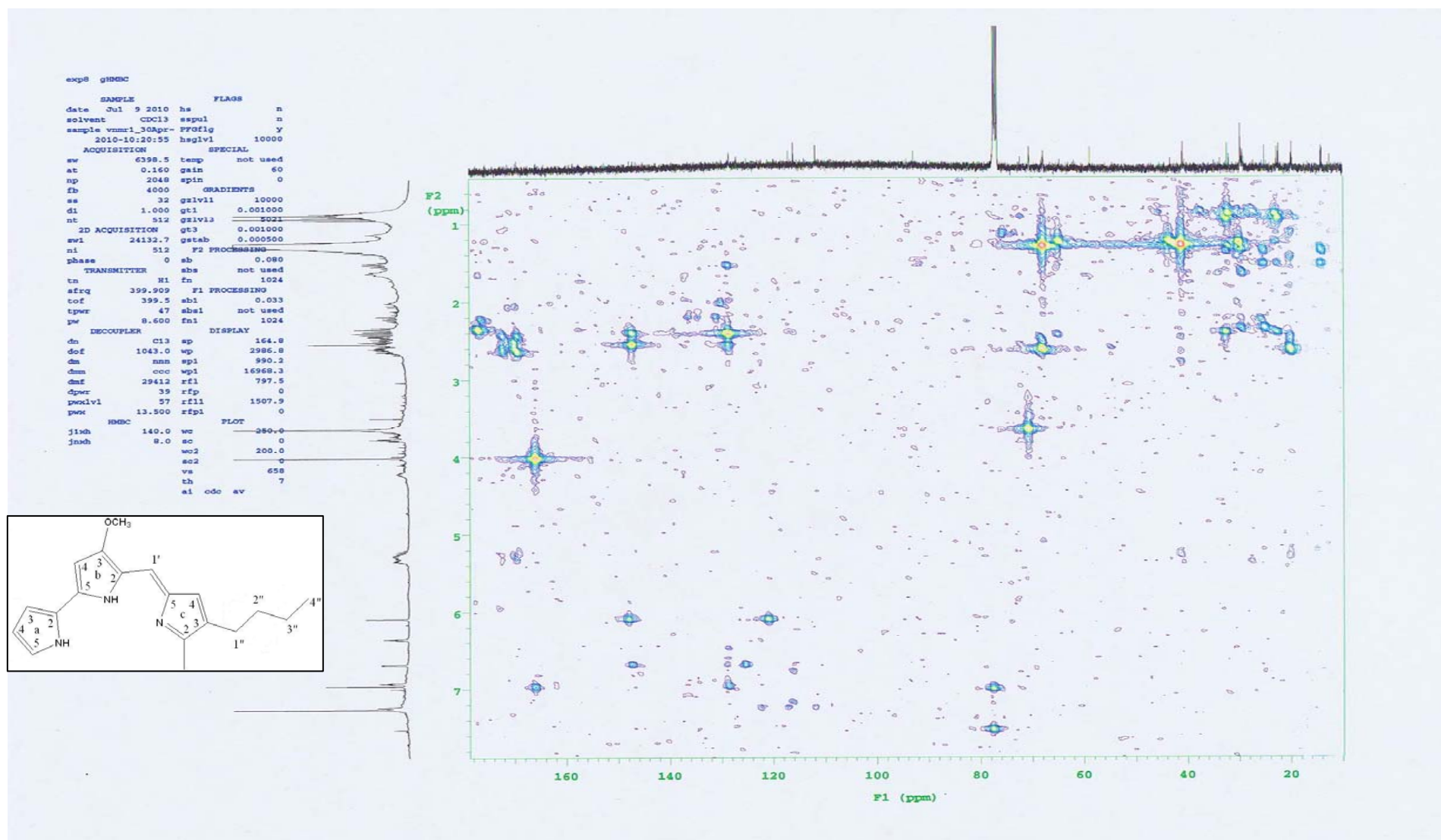
Appendix 6. gCOSY spectrum of P-2.



Appendix 7. TOCSY spectrum of P-2.



Appendix 8. NOESY spectrum of P-2.



Appendix 9. gHMBC spectrum of P-2.

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