

สารสีແດງຜລິດໂດຍ **ZYMOMONAS MOBILIS CM 141**

ນາງພິສມັຍ ກີພຍ່ອນທະເພົ່າ



ວິທາຍານິພນ໌ນີ້ເປັນສ່ວນໜຶ່ງຂອງກາຮືກຊາຕາມຫລັກສູດປະລຸງລູາວິທາສາສຕຣາດຸນງົງບັນທຶກ

ສາຂາວິຊາເກສັ້ນເຄມືແລະ ພລິດກັນທົ່ວຮົມຈາດີ

ບັນທຶກວິທາລ້ຽນ ຈຸ່າລັງກຣະນຸມໝາວິທາລ້ຽນ

ສູນຍາວທະກິບພຢາກ  
ພ.ສ. 2539

ISBN 974-634-017-4

ລົບສິຖິງບັນທຶກວິທາລ້ຽນ ຈຸ່າລັງກຣະນຸມໝາວິທາລ້ຽນ

ໃ ၁၇၀၅၇၂၀၆

**RED PIGMENTS PRODUCED BY  
*ZYMO MONAS MOBILIS CM 141***

**Mrs. Pisamai Tiptanasup**

**A Thesis Submitted in Partial Fulfillment of the Requirements**

**for the Degree of Doctor of Philosophy**

**Pharmaceutical Chemistry and Natural Products**

**Graduate School**

**Chulalongkorn University**

**1996**

**ISBN 974-634-017-4**

Thesis Title                    Red Pigments Produced by *Zymomonas mobilis* CM  
                                    141

By                              Mrs. Pisamai Tiptanasup

Department                    Pharmaceutical Chemistry and Natural Products

Thesis Advisor                Associate Professor Sunibhond Pummangura, Ph.D.

Thesis Co-advisor            Associate Professor Kamnird Supanwong, M.Sc.  
                                    Assistant Professor Chamnan Patarapanich, Ph.D.

---

Accepted by the Graduate School, Chulalongkorn University in  
Partial Fulfillment of the Doctor's Degree.

*Santi Thoongsuwan*

.....Dean of Graduate School

(Associate Professor Santi Thoongsuwan, Ph.D.)

Thesis Committee

*Rapepol Bavovada* .....Chairman

(Associate Professor Rapepol Bavovada, Ph.D.)

*Sunibhond Pummangura* .....Thesis Advisor

(Associate Professor Sunibhond Pummangura, Ph.D.)

*Kamnird Supanwong* .....Thesis Co-Advisor

(Associate Professor Kamnird Supanwong, M.Sc.)

*Chamnan Patarapanich* .....Thesis Co-Advisor

(Assistant Professor Chamnan Patarapanich, Ph.D.)

*Weena Jiratchariyakul* .....Member

(Associate Professor Weena Jiratchariyakul, Ph.D.)

พิมพ์ต้นฉบับทักษิณอวิทยานิพนธ์ภายในกรอบสีเขียวนี้เพียงแผ่นเดียว

พิมพ์ กิพย์ธนากรรพ์ : สารสีแดงผลิตโดย *Zymomonas mobilis CM 141*  
(RED PIGMENTS PRODUCED BY *ZYMOMONAS MOBILIS CM 141*)  
อ.ที่ปรึกษา : รศ. ดร. สุนิพนธ์ ภูมิมาภูร, อ.ที่ปรึกษาร่วม : รศ. กำเนิด สุกันวงศ์,  
ผศ. ดร. ชำนาญ ภัตรพาณิช, 245 หน้า. ISBN 974-634-017-4



*Zymomonas mobilis* เป็นจุลชีพที่ใช้แทนยีสต์ในอุตสาหกรรมการผลิตเอทานอล มีการทดลองกล้ายพันธุ์ เพื่อให้ได้พันธุ์ที่มีประสิทธิภาพดี *Zymomonas mobilis CM 141* ที่ถูกขึ้นมาให้เป็นพันธุ์กล้ายโดย สารเคมี ไซดรอคีลามีน มีการผลิตสารสีแดงด้วย

เมื่อทำการแยกสารสีแดง ทำให้บริสุทธิ์โดยกระบวนการกรองมาโทกราฟี และตรวจสอบคุณสมบัติโดยวิธีการสเปกโตรสโคปี พบว่าสารสีแดงนี้ คือ prodigiosin ซึ่งพบทั้งรูปอิสระและรูปเมือนุภาคบวก รูปอิสระจะอยู่ในสารละลายเป็น 3 รูป (tautomers) และไม่คงตัว จะเปลี่ยนแปลงเป็นรูปเมือนุภาคบวกได้ง่าย เมื่อมีน้ำเพียงเล็กน้อย ตั้งนั้นน้ำในตัวทำละลาย หรือสารตุดซับ หรือความชื้นในอากาศ จะมีผลต่อการเปลี่ยนแปลงเป็นรูปเมือนุภาคบวก การทดลองนี้ได้แสดงการเปลี่ยนแปลงที่เกิดขึ้นโดย protonation นิวเคลียร์แมกนีติก resonance spectrum และพบว่า อนุภาคบวกจากน้ำจะเข้าที่ตำแหน่ง -1 ในโมเลกุล

การทดลองกล้ายพันธุ์ *Zymomonas mobilis CM 141* โดยใช้แสงอุตตราไวโอเรท และสารเคมี (ไซดรอคีลามีน และเอ็น-เมธิล-เอ็น-ไนโตร-เอ็น-ไนโตรไซกวนิดิน) จาก 200 สายพันธุ์ พบร่วมสายพันธุ์ A<sub>113</sub> ซึ่งกล้ายพันธุ์ โดยไซดรอคีลามีน จะให้สารสีแดงมากกว่าสายพันธุ์พ่อแม่เกือบสองเท่า

การทดลองนี้ทำให้ได้ทราบข้อมูลของสารสีแดงที่ผลิตโดย *Zymomonas mobilis* ซึ่งไม่เคยมีรายงานการพนມาก่อน และการพัฒนาการกล้ายพันธุ์ให้มีการผลิตสารสีแดงสูงขึ้น จะได้เหล่งในการผลิต prodigiosin เพิ่มขึ้น

ศูนย์วิทยทรัพยากร  
จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา ..... หลักสูตรเวทนาภาษาอังกฤษ .....  
สาขาวิชา ..... (ภาษาอังกฤษและภาษาต่างประเทศ)  
ปีการศึกษา ..... ๒๕๓๙

ลายมือชื่อนักศึกษา ..... จันทร์ ๗.  
ลายมือชื่ออาจารย์ที่ปรึกษา ..... *Pruew C.*  
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม ..... *My. Tawarat A. D.*

# # C375404 : MAJOR PHARMACEUTICAL CHEMISTRY AND NATURAL PRODUCTS

KEY WORD : ZYMO MONAS MOBILIS/ RED PIGMENTS/ PRODIGIOSIN

PISAMAI TIPTANASUP : RED PIGMENTS PRODUCED BY ZYMO MONAS MOBILIS CM 141. THESIS

ADVISOR: ASSOC. PROF. SUNIBHOND PUMMANGURA, Ph.D. THESIS CO-ADVISOR: ASSOC. PROF.

KAMNIRD SUPANWONG, M.Sc., ASSIS. PROF. CHAMNAN PATARAPANICH, Ph.D. 245 pp. ISBN 974-634-017-4

*Zymomonas mobilis* was an anaerobic Gram-negative and fermentative bacteria used for replacing yeast in the industrial ethanol industry. *Zymomonas mobilis* CM 141 was induced by hydroxylamine to produce no levan and found to produce red pigments under aerobic conditions. The information of the red pigments produced from *Zymomonas mobilis* was not reported before. This experiment was performed.

The red pigments were separated, purified and identified as prodigiosin by spectroscopic methods. The free form showed the mixture of three tautomers. It was spontaneously transformed to hydrated form by the interaction with water in the solvent or adsorbent or moisture in the air.

The two forms of prodigiosin in solution were reported before which referred to the interaction of the base form with some acid in the solvent or adsorbent or moisture and carbon dioxide by spectrophotometric data. This investigation was presented the spontaneously transformation of free form to protonated form by the proton nuclear magnetic resonance spectrometry and showed that the protonation was occurred by hydration at 1-position.

The mutation experiment was performed to increase the pigments production. The parent strains were treated with ultraviolet light and chemicals (hydroxylamine and N-methyl-N-nitro-N-nitrosoguanidine) at various times. By positive selection from 200 isolates, strain A<sub>113</sub> which treated by hydroxylamine for 20 minutes produced the red pigments about two times more than the *Zymomonas mobilis* CM 141.

This work was reported the informations of red pigments produced by *Zymomonas mobilis* CM 141 that never been found before. Mutation and selection experiments were used to development of better red pigments producing strains which expected to be the source of prodigiosin production.

ภาควิชา หลักสูตรวิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต  
สาขาวิชา เทคโนโลยีเคมีและเทคโนโลยีการผลิต ลายมือชื่ออาจารย์ที่ปรึกษา *Prof. Dr. S. Pumangura*  
ปีการศึกษา 2538 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม *Prof. Dr. C. Chamnan*  
*Dr. D. Kamnird*



## ACKNOWLEDGEMENTS

I am sincerely indebted to my thesis advisor, Associate Professor Sunibhond Pummangura, for his inestimable advice, guidance, concern, understanding, kindness, and encouragement throughout the period of my graduate study.

I am deeply beholden to my thesis co-advisor, Associate Professor Kamnird Supanwong and Assistant Professor Chamnan Patarapanich, for their helpful support, guidance, and invaluable advice.

I am obligated to the members of thesis committee for their valuable scrutinizing and discussion.

I would like to express my thankfulness to all staffs of the Department of Pharmaceutical Chemistry for their cooperation and to the scientists of the Scientific and Technological Research Equipment Center, Chulalongkorn University for their assistances in the NMR, IR, and MS experiments.

I wish to express my gratitude to the Graduate School of Chulalongkorn University for granting partial financial support.

Finally, I would like to express my indefinite gratitude to my husband and daughters for their endless love, care, and encouragement throughout to my study.

## CONTENTS



	Page
THAI ABSTRACT .....	iv
ENGLISH ABSTRACT .....	v
ACKNOWLEDGMENTS .....	vi
LIST OF TABLES .....	viii
LIST OF FIGURES .....	xi
LIST OF SCHEMES.....	xii
CHAPTER	
I INTRODUCTION .....	1
II HISTORICAL .....	10
III EXPERIMENTAL .....	51
IV RESULTS AND DISCUSSION .....	130
V CONCLUSION .....	216
REFERENCES.....	219
APPENDIX.....	226
VITA.....	245



## LIST OF TABLES

Table	Page
1 Kinds of mutants.....	14
2 Some Mutagenic Chemicals.....	18
3 Properties of <i>Zymomonas mobilis</i> CM 141 and the pigment producing mutant.....	24
4 Major groups of Phototrophic Eubacteria.....	30
5 Prodigiosin pigments and its sources.....	40
6 <i>In Vitro</i> Cytotoxic Activity of Prodigiosin.....	45
7 The portions of red pigments separated from gel filtration chromatography.....	63
8 Mutation treatments and Characteristic of the mautant strains.....	121
9 Absorbance of the extracted pigment solution	
10 The absorbance of the crude extract containing red pigments.....	131
11 Separation procedure of red pigments.....	137
12 The UV-VIS maximum absorption of RP-1.....	138
13 The IR spectrum assignment of RP-1.....	139
14 The $^1\text{H}$ NMR chemical shift of RP-1 and prodigiosin-like compounds.....	142

Table	Page
15      The UV-VIS maximum absorption of RP-2.....	149
16      The IR spectrum assignment of RP-2.....	150
17      The 500 Mhz <sup>1</sup> H NMR assignment of RP-1, RP-2 in deuterated chloroform and prodigiosin 2 forms....	156
18      The 125 Mhz <sup>13</sup> C NMR assignment of RP-1, RP-2 in deuterated chloroform and prodigiosin 2 forms....	157
19      The UV-VIS absorption of rechromatographed RP-2.....	164
20      The UV-VIS absorption of reextracted RP-2A.....	170
21      The 500 Mhz of <sup>1</sup> H NMR chemical shift difference of RP-1 and RP-2 in deuterated chloroform.....	173
22      Pigment extracted from mutant strains mutated by N-methyl-N-nitro-N-nitrosoguanidine treatment of <i>Zymomonas mobilis</i> CM 141.....	209
23      Pigment extracted from mutant strains mutated by hydroxylamine (1M) treatment of <i>Zymomonas mobilis</i> CM 141.....	210
24      Pigment extracted from mutant strains mutated by hydroxylamine (0.5M) treatment of strain A <sub>9</sub> .....	212
25      Pigment extracted from mutant strains mutated by hydroxylamine (1M) treatment of strain A <sub>78</sub> .....	212
26      Pigment extracted from mutant strains mutated by N-methyl-N-nitro-N-nitrosoguanidine treatment of strain A <sub>32</sub> .....	213

Table	Page
27 Pigment extracted from mutant strains mutated by hydroxylamine (1M) treatment of strain A <sub>32</sub> .....	214
28 The UV-VIS absorption of red pigment extract.....	234

ศูนย์วิทยทรัพยากร  
จุฬาลงกรณ์มหาวิทยาลัย

## LIST OF SCHEMES

Scheme	Page
1 Entner-Doudoroff pathway and its relationship to other glycolytic pathways.....	4
2 Metabolic pathways to major byproducts in <i>Zymomonas mobilis</i> .....	7
3 The different classes of low molecular weight compounds synthesized by microorganisms.....	11
4 Biosynthesis pathway of prodigiosin.....	42
5 Red pigments extraction.....	61
6 Isolation of the red pigments.....	66
7 Mutation Experiment.....	120
8 Fragmentation of prodigiosin.....	146
9 Tautomerization of prodigiosene.....	152
10 The spontaneously transformation of prodigiosin base form to protonated form by the hydrogen ion.....	166
11 Protonation of the prodigiosin.....	171
12 The basicity of the pyrrole rings in the prodigiosin molecule.....	172

## LIST OF FIGURES

Figure	Page
1 Mutagenesis by base pair mutagen.....	19
2 Base analogs and the nitrogenous bases they replace..	20
3 Mutagenesis by ultraviolet light.....	22
4 The chemical structures of chlorophyll- $\alpha$ and bacteriochlorophyll- $\alpha$ .....	29
5 The chemical structures of some carotenoids of the purple and green phototrophic bacteria.....	32
6 Prodigiosene.....	33
7 Prodigiosin and its derivatives.....	35
8 Incorporation patterns of acetate, glycine, alanine, proline, serine, and methionine in prodigiosin, undecylprodigiosin and metacycloprodigiosin.....	44
9 The diminishing cytotoxic activity of the prodigiosin after the sequential removal of the prodigiosin peripheral substituents.....	46
10 Structure of prodigiosin 25-C and FK 506.....	49
11 The Mass spectrum of RP-1.....	74
12 The UV-VIS spectrum of RP-1 in ethanol.....	75
13 The UV-VIS spectrum of RP-1 in chloroform.....	76
14 The UV-VIS spectrum of RP-1 in acid ethanol.....	77

Figure	Page
15      The UV-VIS spectrum of RP-1 in basic ethanol.....	78
16      The Infra-red spectrum of RP-1.....	79
17      The 500 Mhz $^1\text{H}$ NMR spectrum of RP-1 in deuterated chloroform.....	80
17.1    The $^1\text{H}$ NMR spectrum of RP-1, expanded at $\delta$ 0.80 - 2.6 ppm .....	81
17.2    The $^1\text{H}$ NMR spectrum of RP-1, expanded at $\delta$ 6.07 - 7.32 ppm and 12.4 - 13.1 ppm.....	82
17.3    The assignment of $^1\text{H}$ NMR spectrum of RP-1.....	83
18      The H-H COSY spectrum of RP-1.....	84
18.1    The H-H COSY expanded spectrum of RP-1 showing the coupling between signals at $\delta$ 1.55 and 2.39 ppm.....	85
18.2    The H-H COSY expanded spectrum of RP-1 showing the long-range coupling of signals at $\delta$ 2.39, 1.55, and 6.68 ppm.....	86
18.3    The H-H COSY expanded spectrum of RP-1 in the aromatic region showing the coupling between signals at $\delta$ 6.35, 6.92, and 7.23 ppm.....	87

Figure	Page
18.4 The H-H COSY expanded spectrum of RP-1 in the aromatic region showing the long-range coupling of signals at $\delta$ 6.35, 6.92, and 7.23 to the signal at $\delta$ 12.57 ppm and the long-range coupling between signals at $\delta$ 6.08 and 6.68 to the signal at $\delta$ 12.75 ppm.....	88
19 The $^1\text{H}$ NMR spectrum of RP-1 in olefinic region, irradiated at $\delta$ 6.923 ppm.....	89
20 The 125 Mhz $^{13}\text{C}$ NMR spectrum of RP-1 in deuterated chloroform.....	90
21 The DEPT spectrum of RP-1.....	91
22 The C-H COSY spectrum of RP-1 .....	92
23 The Mass spectrum of RP-2.....	93
24 The UV-VIS spectrum of RP-2 in ethanol.....	94
25 The UV-VIS spectrum of RP-2 in chloroform.....	95
26 The UV-VIS spectrum of RP-2 in acid ethanol.....	96
27 The UV-VIS spectrum of RP-2 in basic ethanol.....	97
28 The Fluorescence spectrum of RP-2.....	98
29 The Infra-red spectrum of RP-2.....	99
30 The 500 Mhz $^1\text{H}$ NMR spectrum of RP-2 in deuterated chloroform.....	100
30.1 The $^1\text{H}$ NMR spectrum of RP-2, expanded in aliphatic region.....	101

Figure	Page
30.2 The $^1\text{H}$ NMR spectrum of RP-2, expanded in aromatic region.....	102
30.3 The assignment of $^1\text{H}$ NMR spectrum of RP-2.....	103
31 The H-H COSY spectrum of RP-2.....	104
31.1 The H-H COSY expanded spectrum of RP-1 showing the coupling between signals at $\delta$ 1.44 and 2.22 ppm.....	105
31.2 The H-H COSY spectrum of RP-2 expanded in the olefinic region.....	106
32 The $^1\text{H}$ NMR spectrum of RP-2 in NOE experiments.....	107
33 The 125 Mhz $^{13}\text{C}$ NMR spectrum of RP-2.....	108
34 The DEPT spectrum of RP-2.....	109
35 The C-H COSY spectrum of RP-2.....	110
36 The HMBC spectrum of RP-2.....	111
36.1 Assignment of carbon at 2 -, 3 - and 4 -position in the prodigiosin molecule.....	112
36.2 Assignment of quaternary carbon at 2-, 3- and 4-position in the prodigiosin molecule.....	113
36.3 Assignment of quaternary carbon at 7-, 8-, and 10-position in the prodigiosin molecule.....	114
36.4 Assignment of quaternary carbon at 7- and 8-position in the prodigiosin molecule.....	115

Figure	Page
36.5 Assignment of quaternary carbon at 2-, 3-, 5-, 7-, 8-, 10-, and 11-position in the prodigiosin molecule.....	116
37 The UV-VIS spectrum of the red pigments in the crude extract at the begining (0 week).....	132
38 The UV-VIS spectrum of the red pigments in the crude extract after standing for two weeks.....	133
39 The UV-VIS spectrum of the red pigments in the crude extract after standing for four weeks.....	134
40 The UV-VIS spectrum of the red pigments in the crude extract after standing for twelve weeks.....	135
41 5-(2-pyrryl)-2,2 -dipyrrylmethene (prodigiosene).....	141
42 Chemical structure of RP-1.....	147
43 Chemical structure of RP-2.....	155
44 The <sup>1</sup> H-NMR spectra of RP-1 and RP-2, in comparison.....	160
44.1 The <sup>1</sup> H-NMR spectra of RP-1 and RP-2, in comparison, expanded at $\delta$ 0.5 - 2.7 ppm.....	161
44.2 The <sup>1</sup> H-NMR spectra of RP-1 and RP-2, in comparison, expanded at $\delta$ 6.0 - 7.3 ppm.....	162
45 The 500 Mhz <sup>1</sup> H-NMR spectrum of RP-2 in water-rich deuterated chloroform, the spontaneously transformed to protonated form.....	175

Figure	Page
45.1 The $^1\text{H}$ -NMR spectrum of RP-2, the spontaneously transformed to protonated form in various time.....	176
45.2 The $^1\text{H}$ -NMR spectrum of RP-2, the spontaneously transformed to protonated form, expanded at $\delta$ 0.5 - 3.0 ppm.....	177
45.3 The $^1\text{H}$ NMR spectrum of RP-2, the spontaneously transformed to protonated form, expanded at $\delta$ 5.5 - 8.0 ppm.....	178
46 The H-H COSY spectrum of transformed RP-2.....	179
46.1 The H-H COSY spectrum of transformed RP-2, expanded at $\delta$ 0.5 - 3.0 ppm .....	180
46.2 The H-H COSY spectrum of transformed RP-2, expanded at $\delta$ 5.5 8.5 ppm.....	181
47 The 500 Mhz $^1\text{H}$ NMR spectra of RP-2A in deuterated chloroform, compared with RP-1 and RP-2.....	182
47.1 The $^1\text{H}$ NMR spectra of RP-2A, compared with RP-1 and RP-2, expanded at $\delta$ 0.8 - 2.8 ppm.....	183
47.2 The $^1\text{H}$ NMR spectra of RP-2A, compared with RP-1 and RP-2, expanded at $\delta$ 5.5 - 7.4 ppm.....	184
48 The UV-VIS spectrum of reextracted RP-2A in acetone.....	185
49 The UV-VIS spectrum of reextracted RP-2A in ethylacetate.....	186

Figure	Page
50 The UV-VIS spectrum of reextracted RP-2A in hexane.....	187
51 The UV-VIS spectrum of reextracted RP-2A in methanol.....	188
52 The UV-VIS spectrum of reextracted RP-2A in chloroform.....	189
53 The long-chain unsaturated fatty acid found in the purification process, in the first column chromatography of the red pigments.	
1. The 500 Mhz $^1\text{H}$ NMR spectrum of long-chain unsaturated fatty acid.....	190
2. The 125 Mhz $^{13}\text{C}$ NMR spectrum of long-chain unsaturated fatty acid.....	191
54 The 500 Mhz $^1\text{H}$ NMR spectrum of long-chain unsaturated fatty acid in mixture with pyrrolic compound, from the second column chromatography.....	192
54.1 The 500 Mhz $^1\text{H}$ NMR spectrum of long-chain unsaturated fatty acid in mixture with pyrrolic compound, from the second column chromatography, expanded in olefinic region.....	193

Figure	Page
55 The 500 Mhz $^1\text{H}$ NMR spectrum of long-chain unsaturated fatty acid in mixture with pyrrolic compound, from the third column chromatography.....	194
56 The 500 Mhz $^1\text{H}$ NMR spectrum of RP-3.....	195
56.1 The 500 Mhz $^1\text{H}$ NMR spectrum of RP-3, expanded in olefinic region.....	196
57 The 500 Mhz $^1\text{H}$ NMR spectrum of RP-4, compared with RP-1 and RP-2.....	197
58 2-(2-pyrryl)-prodigiosene.....	204
59 Dipyrrylmethene.....	205
60 The NOE spectrum of RP-2.	
1. Irradiation at $\delta$ 1.82 ppm.....	227
2. Irradiation at $\delta$ 3.945 ppm.....	228
3. Irradiation at $\delta$ 6.04 ppm.....	229
4. Irradiation at $\delta$ 6.35 ppm.....	230
5. Irradiation at $\delta$ 6.80 ppm.....	231
61 The UV-VIS spectrum of red pigments from extract in chloroform.....	235
62 The UV-VIS spectrum of red pigments from crude extract in ethanol.....	236
63 The UV-VIS spectrum of red pigments from hexane extract in chloroform.....	237

Figure	Page
64 The UV-VIS spectrum of red pigments from hexane extract in ethanol.....	238
65 The UV-VIS spectrum of red pigments from hexane extract in acid ethanol.....	239
66 The UV-VIS spectrum of red pigments from hexane extract in basic ethanol.....	240
67 The UV-VIS spectrum of red pigments from quick column chromatography extract in chloroform.....	241
68 The UV-VIS spectrum of red pigments from quick column chromatography extract in ethanol.....	242
69 The UV-VIS spectrum of red pigments from quick column chromatography extract in acid ethanol.....	243
70 The UV-VIS spectrum of red pigments from quick column chromatography extract in basic ethanol.....	244

ศูนย์วิทยทรัพยากร  
จุฬาลงกรณ์มหาวิทยาลัย