PMO16

Biosynthesis of Phenazine-1-carboxylic acid to Chemosynthesis of *N*-phenylphenazine-1-carboxamide

การสังเคราะห์ phenazine-1-carboxylic acid ทางชีวภาพ เพื่อสังเคราะห์ *N*-phenylphenazine-1-carboxamide ทางเคมี

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ABSTRACT

Phenazine-1-carboxylic acid (PCA) can be produced by *Pseudomonas aeruginosa* TISTR 781 using modified King's A broth in ambient temperature (29 - 30 °C). PCA was isolated from an Amberlite XAD-16 resin column and eluted with 70% (v/v) acetonitrile in water. Green fraction was collected and then extracted with dichloromethane. After purification process, yield of yellow fraction (compound I) crystal was 7.0 mg/l of bacterial culture. *N*-phenylphenazine-1-carboxamide was synthesized using PCA activated with thionyl chloride and finally reacted with aniline. Purified dark yellow (compound II) crystal was obtained by using a silica gel column with a yield of 8.2 mg per 10.0 mg of PCA. Both compounds I and II were investigated preliminary physicochemical properties by melting point and elucidated structure by FT-IR and NMR spectrometry that was confirmed to be PCA and *N*-phenylphenazine-1-carboxamide, respectively.

บทคัดย่อ

phenazine-1-carboxylic acid (PCA) สามารถผลิตได้จากแบคทีเรีย *Pseudomonas aeruginosa* TISTR 781 โดยใช้อาหารเหลวสูตร modified King's A ที่อุณหภูมิห้อง (29 - 30 °C) แยก PCA จากคอลัมน์ที่บรรจุเรซิน Amberlite XAD-16 และชะด้วย 70% อะซิโตไนไตรล์ในน้ำ เก็บสารส่วนสีเขียวและสกัดด้วยไดคลอโรมีเทน หลังผ่านกระบวนการทำบริสุทธิ์ ได้สารบริสุทธิ์ที่เป็นผลึกสีเหลือง (สารประกอบ I) หนัก 7.0 มิลลิกรัมต่อลิตรของ bacterial culture สังเคราะห์ *N*-phenylphenazine-1-carboxamide โดยใช้ PCA เป็นสารตั้งต้น ทำปฏิกิริยากับไทโอนิลคลอไรด์และอนิลีน และหลังผ่านกระบวนการทำบริสุทธิ์ด้วยคอลัมน์ที่บรรจุซิลิกาเจล ได้สารบริสุทธิ์ที่เป็นผลึกสีเหลืองเข้ม (สารประกอบ II) หนัก 8.2 มิลลิกรัมต่อ PCA 10.0 มิลลิกรัม จากการตรวจวัดคุณสมบัติทางเคมีกายภาพเบื้องต้น โดยหาจุดหลอมเหลว และการพิสูจน์โครงสร้างของสารประกอบทั้งสองด้วยเทคนิค FT-IR และ NMR สเปกโทรเมทรี พบว่าสารที่ได้จากการสังเคราะห์ทางชีวภาพ และทางเคมี คือ PCA และ *N*-phenylphenazine-1-carboxamide ตามลำดับ

Key Words : *Pseudomonas aeruginosa* TISTR 781, Phenazine-1-carboxylic acid (PCA), *N*-phenylphenazine-1-carboxamide

คำสำคัญ : *Pseudomonas aeruginosa* TISTR 781 Phenazine-1-carboxylic acid (PCA) *N*-phenylphenazine-1-carboxamide

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Introduction

Phenazine compounds, nitrogen-containing heterocyclic redox agents, are one group of antibiotic agents. These compounds can be produced by biosynthesis *via* phenazine-producing bacteria and chemical synthesis (Laursen and Nielsen, 2004; González et al., 2007). Functional groups in phenazine nucleus are largely effective for differences in physicochemical and biological properties of individual phenazine derivatives. Many antibiotic phenazines are natural products and types of phenazine derivatives depend on media and strain of microorganism. For example of biosynthesis *via* bacteria, *Pseudomonas* spp. such as *Pseudomonas aureofaciens, Pseudomonas chlororaphis*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* (Dwivedi and Johri, 2003), especially *P. aeruginosa*, a common soil inhabitant and opportunistic human pathogen, is the first phenazine-producing microorganism. It can produce several other phenazines including pyocyanin, 1-hydroxyphenazine (pyocyanin degradation), phenazine-1-carboxamide (PCN) and phenazine-1-carboxylic acid (PCA) in several media (Denning et al., 2003; Wilson et al., 1987; Chin-A-Woeng et al., 1998). Phenazines have been developed in several sciences and technologies against some strains of bacteria and fungal plant pathogens (Chin-A-Woeng et al., 2003; Kerr et al., 1999). These are much interest in the development of antimicrobial agents to extend the area of applications and the range of target pathogens to be controlled. The antimicrobial activities of PCA produced by *Pseudomonas* spp. have been investigated and used to inhibit some bacterial and fungal growth (Laursen and Nielsen, 2004; Brisbane et al., 1987; Kim, 2000). Moreover, PCA as well as several phenazine derivatives showed moderate antitubercular activity. Therefore, scientists interest structural modifications of PCA for biological active compound in medicinal chemistry until now (Logu et al., 2009). The structural modifications of PCA were prepared by chemical synthesis and one interesting synthetic phenazine is *N*-phenylphenazine-1-carboxamide. This compound revealed substantial growth retardation of *Micrococcus* sp., *Erysipelothrix rhusiopathiae* and *Staphylococcus aureus* bacteria (Pal'chikovs'ka et al., 2008). Previous report has demonstrated *N*-phenylphenazine-1-carboxamide activity against *Mycobacterium tuberculosis*, including drug-resistant and multidrug-resistant strains (Logu et al., 2009). Therefore, this study focused on the biosynthesis of PCA from *Pseudomonas aeruginosa* TISTR 781 to chemosynthesis of *N*-phenylphenazine-1-carboxamide. *N*-phenylphenazine-1-carboxamide was synthesized by reaction of PCA with aniline for 15 h using thionyl chloride as an activating agent. Finally, these compounds were investigated melting point and structure elucidation by FT-IR and NMR spectrometry.

Materials and methods

Biosynthesis of phenazine-1-carboxylic acid (PCA)

Pseudomonas aeruginosa TISTR 781 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR) and used for PCA production. Firstly, it was streaked on Luria-Bertani (LB) agar plates and incubated in ambient temperature (29 - 30 °C) for 24 - 48 h. A single colony of *P. aeruginosa* on a LB agar plate was then transferred

into 100 ml of modified King's A broth (KA): Bactopeptone 15.0 g, NaCl 13.0 g, glycerol 9.0 ml and K_2SO_4 1.0 g in 1,000 ml distilled water; and incubated in ambient temperature with an orbital shaker (200 rpm) for 24 h to use as a starter. For increasing PCA production, the starter as above was transferred into an Erlenmeyer flask, containing 2,000 ml of fresh modified KA medium with 1:50 bacterial dilutions and incubated under agitation with an orbital shaker for 48 h. Culture medium was removed from cells of bacteria by centrifugation and isolated with an Amberlite XAD-16 resin column by eluting with 70% (v/v) acetonitrile in water. Collected green fraction and evaporated for reduce solvent until 3-5 ml to obtain crude phenazine solution. The pH of the crude phenazine solution was adjusted to 2.5 and removed residues by centrifugation at 3,500 rpm for 15 min. After that, this solution was separated by a liquid-liquid extraction with dichloromethane and evaporated for reduce solvent until 3-5 ml. The extracted solution was then purified by a silica gel column, equilibrated with dichloromethane and eluted with 90% (v/v) dichloromethane in ethyl acetate (Apipattarakul, 2008).

Chemosynthesis of *N*-phenylphenazine-1-carboxamide

A mixture of 1 mole PCA in toluene (4 ml), pyridine (1.4 moles) and thionyl chloride (1.4 moles) was heated with stirring at 70 ˚C for 30 min and then cooled to room temperature. The reaction mixture was combined with an excess of aniline (2.5 moles) and stirred for an additional 15 h (Logu et al., 2009). The reaction was performed under nitrogen gas. The reaction was monitored by thin layer chromatography (TLC) using 98 % (v/v) dichloromethane in ethyl acetate as a mobile phase. Water was added to the solid residue and extracted with dichloromethane. It was then purified by a silica gel column. The solvent systems for purification process were varied by using percentage of dichloromethane in ethyl acetate.

Identification of phenazine derivatives

IR spectra of the phenazines, as KBr discs, were recorded on a Spectrum One, FT-IR spectrometer, Perkin Elmer (Germany) from 4,000 - 500 cm^{-1} (Brisbane et al., 1987). Nuclear magnetic resonance spectra (both ^{1}H and ^{13}C NMR) were recorded with samples dissolved in $CDCl_3$ on a Varian Mercury Plus (U.S.A) 400 MHz or 360 FT-NMR spectrometer. Moreover, melting points of both purified phenazines were determined with a melting point apparatus, Gallenkamp, Sanyo (U.K.).

Results and discussion

Biosynthesis of PCA and chemosynthesis of *N*-phenylphenazine-1-carboxamide

After the isolation process, crude phenazine solution produced *P. aeruginosa* TISTR 781 was collected and extracted with dichloromethane. It was then purified using adsorption chromatography and eluted with 90% (v/v) dichloromethane in ethyl acetate. The second yellow fraction was collected and evaporated to remove solvent by means of a rotary evaporator and then kept in a desiccator. Yellow crystals (compound I) formed with a yield of 7.0 mg/l of bacterial culture.

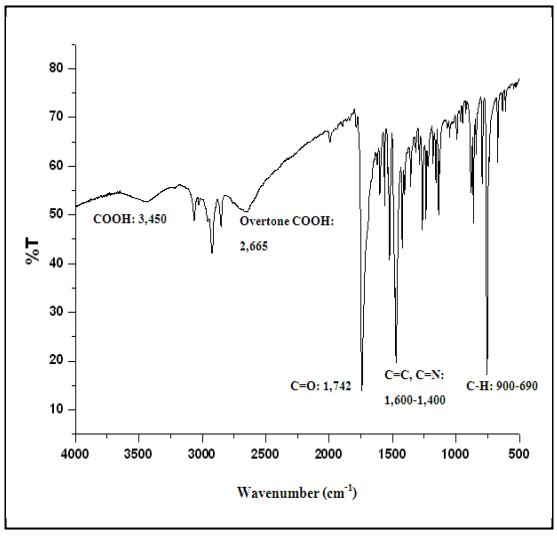
For *N*-phenylphenazine-1-carboxamide, it was synthesized by coupling PCA and aniline using thionyl chloride as an activating agent under nitrogen gas. After the reaction was completed, a silica gel column

was used for purification process. An optimum solvent system with 98 % (v/v) dichloromethane in ethyl acetate was used as a mobile phase that gave dark yellow solution. Dark yellow crystals appeared (compound II) and then weighed approximately 8.2 mg per 10.0 mg of PCA (81.82% of yield).

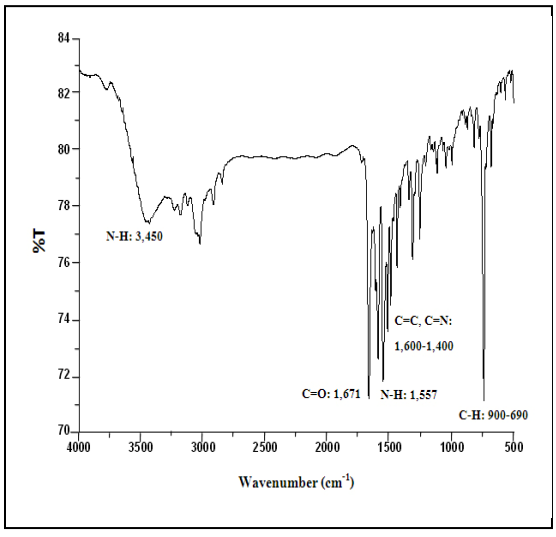
Structural elucidation of phenazine derivatives

The IR spectrum of the compound I shown in Fig. 1a exhibited bands including the O-H of the COOH group (3,450 cm^{-1}), an overtone for the carboxyl group (2,665 cm^{-1}), an intense C=O band (1,742 cm^{-1}). For C=C and C=N were exhibited at the regions 1,600-1,400 cm^{-1}. Peaks at 900 - 690 cm^{-1} were also presented C-H of aromatic in molecule (Brisbane et al., 1987). In ^{1}H-NMR spectrum showed peaks in region (δ = 7.93 – 9.00 ppm), indicated the presence of aromatic protons and the chemical shift of carboxyl group was appeared at δ = 15.60 ppm as small broad peak (Fig. 2a). The ^{13}C-NMR data showed major peaks between 124.96 – 144.08 ppm indicating the presence of 12 aromatic carbons while at 165.88 ppm the carboxylic acid carbon was observed (Fig. 2b). The melting point of this compound was found to be 237 – 239 °C. Compound I was proven to be phenazine-1-carboxylic acid (PCA) and its chemical structure is shown in Fig. 2a and 2b.

For IR spectrum of compound II showed bands at 3,450 and 1,557 cm^{-1} for the N-H and the peak at 1,671 cm^{-1} of C=O, characteristic of the amide group. For C=C and C=N were exhibited at the regions 1,600-1,400 cm^{-1}. Peaks at 900 - 690 cm^{-1} were also presented C-H of aromatic in molecule (Fig. 1b). The ^{1}H-NMR and ^{13}C-NMR data also confirmed the structure of this pigment. In ^{1}H-NMR spectrum between 7.18 – 9.11 ppm are consistent with the presence of signals for 12 aromatic protons. The chemical shift of amide group was appeared at δ = 13.30 ppm (Logu et al., 2009) shown in Fig. 2c. The ^{13}C-NMR spectrum showed the peaks between 120.49 - 143.47 ppm indicating the presence of 18 aromatic carbons and amide carbon was exhibited at 162.49 ppm (Fig. 2d). The melting point of purified phenazine was in range from 190 - 192 °C. From the data, the compound II was confirmed to be *N*-phenylphenazine-1-carboxamide with the structure shown in Fig. 2c and 2d.

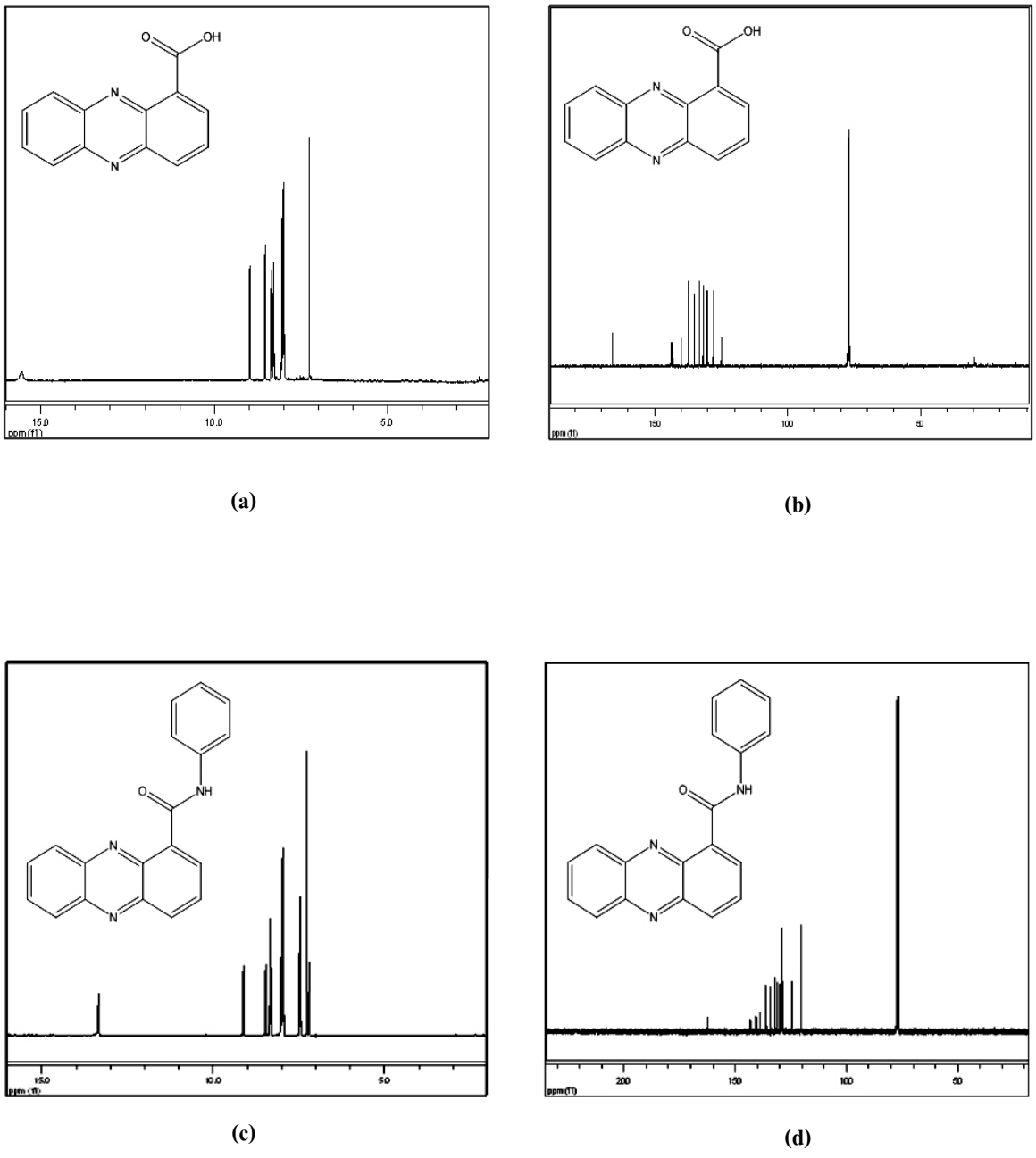


(a)



(b)

Figure 1 IR spectra of PCA (a) and *N*-phenylphenazine-1-carboxamide (b).



(a) (b)

(c) (d)

Figure 2 ^{1}H-NMR spectra of PCA (a), *N*-phenylphenazine-1-carboxamide (c) and ^{13}C-NMR spectra of PCA (b), *N*-phenylphenazine-1-carboxamide (d).

Conclusions

Biosynthetic PCA produced by *P. aeruginosa* TISTR 781 in modified King's A broth under optimal conditions had 7.0 mg/l of bacterial culture. Chemosynthetic of *N*-phenylphenazine-1-carboxamide prepared by using PCA as a substrate was activated with thionyl chloride and then reacted with aniline for 15 h under nitrogen gas. The optimum solvent system for purification process was 98% (v/v) dichloromethane in ethyl acetate. An approximate quantity of *N*-phenylphenazine-1-carboxamide was 8.2 mg per 10.0 mg of PCA. The melting points of these compounds were found to be 237 – 239 °C and 190 - 192 °C, respectively. The chemical structures of both phenazine derivatives were elucidated using IR, ^{1}H-NMR and ^{13}C-NMR data. From the data, it is indicated that *N*-phenylphenazine-1-carboxamide structure was consistent with PCA structure that carboxyl group was modified with aniline to amide group.

Acknowledgements

The authors would like to specially thank the Department of Chemistry, the Department of Microbiology, Faculty of Science, Khon Kaen University and the Khon Kaen University Research Grant for some instruments and experimental facilities. The Center of Excellence for Innovation in Chemistry (PERCH - CIC): Commission on Higher Education, Ministry of Education is also thanked for financial support.

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