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Diversity of Endophytic Actinomycetes Isolated from Roots and Root Nodules of *Pueraria candollei* Grah. ex Benth. and the Analyses of Their Secondary Metabolites

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ABSTRACT

Pueraria candollei Grah. ex Benth. is a Thai herb that contributes to many phytoestrogenic-related pharmacological activities. In this study, the diversity of endophytic actinomycetes from roots and root nodules of *P. candollei* and actinomycetes from rhizospheric soils were investigated. Moreover, the secondary metabolites production of these isolated actinomycetes was investigated. A total of 85 isolates were obtained from roots, root nodules and rhizospheric soils, 32 from the roots of *P. candollei* var. *candollei*, 26 from the root nodules of *P. candollei* var. *mirifica* and 27 from rhizospheric soil samples. Partial 16S rRNA sequence data revealed that the isolated actinomycetes were distributed among 7 genera including, *Streptomyces*, *Micromonospora*, *Verrucosispora, Microbispora*, *Nonomuraea, Plantactinospora* and *Shimazuella*. The majority of isolates from plant sources were members of the genus *Micromonospora*. Broth extracts of these isolates were analyzed using LC/UV and LC/MS. The physicochemical properties of 10 selected peaks were searched in the database. The physicochemical properties of some peaks were matched with known microbial metabolites. The structure of an unknown compound was elucidated using spectroscopic data. The NMR data indicated the presence of *N*-acetylhomocysteine moiety and the compound was determined to be *S*-adenosyl-*N*-acetylhomocysteine. This compound was isolated from *Micromonospora* for the first time. In conclusion, this work contributed to the known information on the diversity of actinomycetes associated with *P. candollei* and their capability for secondary metabolite production.

Keywords: endophytes, actinomycetes, physicochemical screening, secondary metabolites

1. INTRODUCTION

Endophytic bacteria are non-pathogenic bacteria that live within plants [1]. These bacteria can be isolated from a large number of plant sources [2] and are distributed

among four bacterial phyla (Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes) [3].

Actinomycetes are Gram-positive, typically filamentous bacteria in the phylum Actinobacteria [4]. They are responsible for almost half of the known bioactive metabolites to date [5]. Although, actinomycetes are well known as soil-dwelling microorganisms, many of them have been isolated from various parts of crops and medicinal plants [1, 6].

The actinomycetes inhabiting in plant inner tissue may have symbiotic relationship with the host plants. Since the actinomycetes may provide many useful compounds to the plants, the endophytic actinomycetes could have ability to produce many secondary metabolites [7]. This idea was supported by increasing discoveries of natural products from endophytic actinomycetes [1].

The tuberous root of *Pueraria candollei* Grah. ex Benth. (Fabaceae, locally called "kwao khruea khao") has been used in Thailand as a rejuvenator for the elderly [8]. There are 2 varieties of *P. candollei* that have been used in Thailand: *P. candollei* Grah. ex Benth. var. *candollei* and *P. candollei* Grah. ex Benth. var. *mirifica* (A. Shaw et Suvat.) Niyomdham [9]. Roots of *P. candollei* var. *mirifica* contained phytoestrogen, mainly isoflavonoids, and possessed estrogenic pharmacological activities [10]. The endophytic actinomycetes and their metabolite productions from these plants have not yet been reported.

In this study, we searched for novel compounds from cultured broths of *P. candollei* var. *candollei* and *P. candollei* var. *mirifica* endophytic actinomycetes using an approach based on the physicochemical properties of metabolites, employing high performance liquid chromatography-ultraviolet detection (LC/UV) and -mass spectrometry (LC/MS) system as previously described [11]. We aimed to assess diversity and metabolite production capability of endophytic actinomycetes, isolated from roots and root nodules of *P. candollei*.

2. MATERIALS AND METHODS

2.1 Plant Material

Pueraria candollei var. *candollei* and *P. candollei* var. *mirifica* seeds were collected from Kanchanaburi and Saraburi, respectively, and their herbarium specimens were deposited at the Herbarium of the Department of Pharmaceutical Botany, Mahidol University (collector numbers: Prathanturarug 00113 and Prathanturarug 00101 for *P. candollei* var. *candollei* and *P. candollei* var. *mirifica*, respectively). Both plants were germinated and grown in the greenhouse of the Faculty of Pharmacy, Mahidol University, Bangkok, Thailand, roots of *P. candollei* var. *candollei* and root nodules of *P. candollei* var. *mirifica* were collected from healthy plants, in March 2014. The rhizospheric soil sample was collected from the soil surface (1 inch depth) covering the roots of *P. candollei* var. *candollei*.

2.2 Isolation of Actinomycetes from *P. candollei* var. *candollei* and *P. candollei* var. *mirifica*

The roots and root nodules of both varieties of *P. candollei* were surface-sterilized with 70% ethanol for 1 min and 3% sodium hypochlorite solution for 10 min and then rinsed several times with sterile distilled water. They were allowed to dry in silica-gel filled chamber for 3 days [12]. The dried plants were crushed with a mortar and mixed with 4.5 mL of Winogradsky's salt solution [13] and serially diluted to the concentration of 10^{-1}, 10^{-2} and 10^{-3}. Two hundred microliters of the diluted solutions were mixed with 20 mL of media and poured into the sterilized Petri dish. The following media

were used: caboxymethyl cellulose agar (0.1% L-asparagine monohydrate, 0.1% K_2HPO_4, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $MnCl_2 \cdot 4H_2O$, 0.0001% $ZnSO_4 \cdot 7H_2O$ and 1% carboxymethyl cellulose), Reasoner's 2A agar [14], and proline agar (1% proline) [15]. All media were supplemented with Benlate® as an antifungal agent. Rhizospheric soil samples (approx. 500 mg) were mixed with 4.5 mL of Winogradsky's salt solution and serially diluted to 10^{-1}, 10^{-2} and 10^{-3}. Two hundred microliters of diluted soil samples were mixed with 20 mL of above mentioned media. All plates of plant and soil samples were incubated at 27°C for up to 4 weeks.

2.3 DNA Extraction and Preliminary Identification by 16S rRNA Gene Sequencing

After incubation at 27°C for up to 4 weeks, the actinomycete colonies were selected and cultured on agar plate of the same media they were isolated from. After 3 weeks they were inoculated in 10 mL of yeast dextrose broth (1% yeast extract and 1% dextrose, Difco, Franklin Lakes, NJ, USA) and culture for 7 days on the shaker (200 rpm) at 27°C. The actinomycete cells were separated from the culture broth by centrifugation at 12,000 rpm for 1 min. Chromosomal DNA was extracted by sonication of the cells for 5 min in Tris-EDTA buffer (pH 8.0) [16]. The cells were removed by centrifugation.

PCR amplification of the 16S rRNA gene was performed according to the method of Inahashi, et al. [12] in a TaKaRa thermal cycler (TaKaRa, Japan) with an initial denaturation of 95°C for 1 min; followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 90 s and follow by a final extension at 72°C for 2 min. The reaction mixture was prepared using recommended concentration of TaKaRa Ex Taq™ and the universal primers for 16S rRNA amplification [17].

The PCR products were sequenced using a DNA sequencer (Applied Biosystems 3130 Genetic Analyzer, Applied Biosystems, CA, USA) with the BigDye Terminatorv3.1 cycle Sequencing kit (Applied Biosystems) using the recommended protocol in the kit. DNA sequences were assembled using BioEdit 7.2.5 software [18]. The sequences were investigated using the EzTaxon server [19].

2.4 Peak Analysis of Actinomycete Broth Extracts by LC/UV and LC/MS

Actinomycetes isolated from root and root nodules were cultured in 10 mL production medium (2% soluble starch, 0.5% glycerol, 1.0% defatted wheat germ, 0.5% meat extract, 0.3% dry yeast and 0.3% $CaCO_3$) [11] on the shaker (200 rpm) at 27°C for 9 days. Equivalent volume of ethanol was added to the cultured media and supernatants were collected by centrifugation at 3,000 rpm for 15 min. The 50% ethanol extracts were subjected to HPLC on an Inertsil ODS-4 column (3 i.d. × 250 mm; GL Sciences, Tokyo, Japan), and eluted with 5% MeOH for 5 min, from 5% to 100% MeOH linearly with a gradient time of 5 to 35 min and 100% MeOH from 35 to 40 min. The chromatograms were subsequently detected at UV 254 nm, using an LC/UV (Agilent 1200, Agilent Technologies Inc., Palo Alto, CA, USA) and LC-ESI-MS (AB Sciex QSTAR Hybrid LC/MS/MS Systems, AB Sciex, Framingham, MA, USA). Peaks originating from the metabolites of actinomycetes were identified using the Dictionary of Natural Products (http://dnp.chemnetbase.com/), as well as in-house databases of Kitasato Institute for Life Sciences, Kitasato University.

2.5 Fermentation of Selected Strain

The selected strain was cultured in 100 mL of seed medium (2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% $CaCO_3$) [11] in Erlenmeyer flak for 3 days, after that 1 ml of seed culture was then inoculated into Erlenmeyer flasks containing 100 ml of production medium (100 flasks, 10 L total volume). The selected strain was cultured in the production medium for 9 days. For both steps of culture, the cells were maintained on the shaker (200 rpm) at 27°C.

2.6 Isolation of Target Compound

The target compound was isolated and purified using an anion exchange column (HP-20), a silica gel column, reverse-phase column (ODS), and preparative HPLC. All purification steps were guided by LC/MS and LC/UV analyses.

2.7 Structure Elucidation and Physicochemical Properties of Target Compound

NMR spectra were measured using a Varian XL-400 (Varian, Palo Alto, CA, USA), with ^{1}H-NMR at 400 MHz and ^{13}C-NMR at 100 MHz in D_2O. The chemical shifts are expressed in ppm and are referenced to residual D_2O (4.76 ppm, ^{1}H NMR). LC/ESI/MS spectra were measured using AB Sciex QSTAR Hybrid LC/MS/MS System (AB Sciex). IR spectra (KBr) were measured on a Horiba FT-710 Fourier transform IR spectrometer (Horiba Ltd, Kyoto, Japan), UV spectra were measured on a Hitachi U-2810 spectrophotometer (Hitachi, Tokyo, Japan), and optical rotation was calculated using a JASCO model DIP-1000 Polarimeter (Jasco, Tokyo, Japan).

2.8 Nucleotide Sequence Accession Numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene seuqnces of the identifed isolates are LC149518-LC149592 (Table 1).

Table 1. List of actinomycete isolates from *P. candollei* var. *candollei*, *P. candollei* var. *mirifica* and rhizospheric soil. The closest species and similarity percentage were investigated by analyses of partial 16S rRNA gene sequences, using the EzTaxon server [19].

Isolation sources		Closest species	Similarity (%)	Gene sequence length (bp)	Accession NO.
Roots of *P. candollei* var. *candollei*	PC1001	*Verrucosispora lutea* YIM 013^{T}	99.4	669	LC149518
	PC1002	*Verrucosispora lutea* YIM 013 ^{T}	99.4	658	LC149519
	PC1003	*Micromonospora viridifaciens* DSM 43909^{T}	98.6	664	LC149520
	PC1004	*Microbispora rosea* subsp. *rosea* IFO 14044^{T}	99.3	450	LC149521
	PC1007	*Verrucosispora lutea* YIM 013^{T}	99.5	647	LC149522
	PC1008	*Micromonospora citrea* DSM 43903^{T}	98.4	510	LC149523
	PC1010	*Verrucosispora lutea* YIM 013^{T}	99.1	670	LC149524

Table 1. Continued.

Isolation sources	Isolation NO.	Closest species	Similarity (%)	Gene sequence length (bp)	Accession NO.
Roots of *P. candollei* var. *candollei*	PC1011	*Shimazuella kribbensis* *KCTC* 9933^T	99.3	1342	LC149525
	PC1013	*Micromonospora krabiensis* MA-2^T	99.3	554	LC149526
	PC1017	*Micromonospora krabiensis* MA-2^T	100.0	521	LC149527
	PC1020	*Verrucosispora lutea* YIM 013^T	99.4	660	LC149528
	PC1022	*Micromonospora echinospora* ATCC 15837^T	99.3	689	LC149529
	PC1023	*Micromonospora auratinigra* TT1-11^T	99.2	660	LC149530
	PC1026	*Micromonospora auratinigra* TT1-11^T	99.1	640	LC149531
	PC1035	*Micromonospora viridifaciens* DSM 43909^T	98.7	714	LC149532
	PC1038	*Verrucosispora lutea* YIM 013^T	99.5	648	LC149533
	PC1040	*Verrucosispora lutea* YIM 013^T	99.3	694	LC149534
	PC1042	*Micromonospora fulviviridis* DSM 43906^T	98.7	539	LC149535
	PC1045	*Streptomyces nanshensis* SCSIO 01066^T	99.8	509	LC149536
	PC1046	*Plantactinospora endophytica* YIM 68255^T	99.2	659	LC149537
	PC1052	*Micromonospora chalcea* DSM 43026^T	99.8	515	LC149538
	PC1053	*Micromonospora spongicola* S3-1^T	98.6	663	LC149539
	PC1055	*Plantactinospora endophytica* YIM 68255^T	99.0	674	LC149540
	PC1056	*Micromonospora narathiwatensis* BTG4-1^T	99.1	658	LC149541
	PC1058	*Verrucosispora lutea* YIM 013^T	99.4	642	LC149542
	PC1062	*Micromonospora viridifaciens* DSM 43909^T	97.8	498	LC149543
	PC1064	*Verrucosispora lutea* YIM 013^T	99.5	658	LC149544

Table 1. Continued.

Isolation sources	Isolation NO.	Closest species	Similarity (%)	Gene sequence length (bp)	Accession NO.
Root nodules of *P. candollei* var. *mirifica*	PC4001	*Streptomyces racemochromogenes* NRRL B-5430^T	99.3	434	LC149545
	PC4003	*Streptomyces luteosporeus* NBRC 14657^T	99.5	643	LC149546
	PC4005	*Streptomyces chartreusis* NBRC 12753^T	100.0	440	LC149547
	PC4007	*Streptomyces rapamycinicus* NRRL B-5491^T	99.5	365	LC149548
	PC4009	*Streptomyces corchorusii* NBRC 13032^T	98.8	504	LC149549
	PC4010	*Nonomuraea endophytica* YIM 65601^T	100.0	501	LC149550
	PC4011	*Streptomyces iakyrus* NBRC 13401^T	100.0	511	LC149551
	PC4012	*Nonomuraea endophytica* YIM 65601^T	100.0	480	LC149552
	PC4016	*Streptomyces pseudovenezuelae* NBRC 12904^T	99.8	438	LC149553
	PC4019	*Microbispora siamensis* DMKUA-245^T	98.6	499	LC149554
	PC4020	*Plantactinospora mayteni* YIM 61359^T	99.1	530	LC149555
	PC4021	*Microbispora siamensis* DMKUA-245^T	99.0	479	LC149556
	PC4022	*Micromonospora chaiyaphumensis* MC5-1^T	99.1	1131	LC149557
	PC4025	*Micromonospora auratinigra* TT1-11^T	99.1	538	LC149558
	PC4026	*Micromonospora auratinigra* TT1-11^T	99.0	519	LC149559
	PC4027	*Nonomuraea maheshkhaliensis* 16-5-14^T	98.9	471	LC149560
	PC4028	*Streptomyces sioyaensis* NRRL B-5408^T	98.8	515	LC149561
	PC4029	*Streptomyces chartreusis* NBRC 12753^T	100.0	460	LC149562
	PC4031	*Micromonospora yangpuensis* FXJ6.011^T	99.4	623	LC149563
	PC4039	*Micromonospora chaiyaphumensis* MC5-1^T	99.8	516	LC149564

Table 1. Continued.

Isolation sources	Isolation NO.	Closest species	Similarity (%)	Gene sequence length (bp)	Accession NO.
Root nodules of *P. candollei* var. *mirifica*	PC4040	*Micromonospora narathiwatensis* BTG4-1^T	98.8	649	LC149565
	PC4042	*Micromonospora coxensis* 2-30-b/28^T	98.8	648	LC149566
	PC4043	*Micromonospora tulbaghiae* TVU1^T	99.1	680	LC149567
	PC4044	*Micromonospora coxensis* 2-30-b/28^T	99.4	518	LC149568
	PC4045	*Micromonospora chaiyaphumensis* MC5-1^T	99.0	665	LC149569
	PC4046	*Micromonospora echinaurantiaca* DSM 43904^T	98.7	536	LC149570
Rhizospheric soil	PC5001	*Streptomyces ziwulingensis* F22^T	99.0	400	LC149571
	PC5003	*Streptomyces luteireticuli* NBRC 13422^T	99.0	509	LC149572
	PC5005	*Streptomyces coeruleorubidus* ISP 5145^T	97.8	518	LC149573
	PC5007	*Streptomyces angustmycinicus* NBRC 3934^T	100.0	420	LC149574
	PC5012	*Streptomyces hiroshimensis* NBRC 3839^T	99.8	420	LC149575
	PC5013	*Streptomyces deccanensis* DAS-139^T	100.0	423	LC149576
	PC5015	*Streptomyces caniferus* NBRC 15389^T	100.0	665	LC149577
	PC5018	*Streptomyces chartreusis* NBRC 12753^T	100.0	420	LC149578
	PC5020	*Streptomyces lusitanus* NBRC 13464^T	99.0	386	LC149579
	PC5022	*Streptomyces albogriseolus* NRRL B-1305^T	100.0	620	LC149580
	PC5023	*Streptomyces violascens* NBRC 12920^T	98.2	690	LC149581
	PC5029	*Streptomyces roseolus* NBRC 12816^T	99.4	547	LC149582
	PC5031	*Streptomyces mexicanus* CH-M-1035^T	99.2	646	LC149583

Table 1. Continued.

Isolation sources	Isolation NO.	Closest species	Similarity (%)	Gene sequence length (bp)	Accession NO.
Rhizospheric soil	PC5038	*Streptomyces purpurascens* NBRC 13077^T	99.7	637	LC149584
	PC5045	*Streptomyces scabiei* ATCC 49173^T	100.0	228	LC149585
	PC5048	*Streptomyces filipinensis* NBRC 12860^T	98.5	684	LC149586
	PC5059	*Streptomyces purpurascens* NBRC 13077^T	99.5	653	LC149587
	PC5060	*Streptomyces coeruleofuscus* NBRC 12757^T	99.7	651	LC149588
	PC5068	*Streptomyces massasporeus* NBRC 12796^T	100.0	550	LC149589
	PC5071	*Streptomyces pharetrae* CZA14^T	99.5	654	LC149590
	PC5073	*Streptomyces curacoi* NRRL B-2901^T	98.6	563	LC149591
	PC5080	*Streptomyces coeruleofuscus* NBRC 12757^T	99.7	631	LC149592

3. RESULTS AND DISCUSSION

3.1 Diversity of Actinomycetes Isolated from *P. candollei* var. *candollei* and *P. candollei* var. *mirifica*

The root nodules of *P. candollei* var. *candollei* were not present at the time of collection; therefore, we decided to use their roots and rhizospheric soil samples for the isolation of actinomycetes and to compare these results with the root nodules of *P. candollei* var. *mirifica*.

We isolated a total of 85 actinomycete isolates, of which 32 were from the roots of *P. candollei* var. *candollei*, 26 were from the root nodules of *P. candollei* var. *mirifica* and 27 were from rhizospheric soil samples. The 16S rRNA analysis revealed that the isolates were distributed among 7 genera with the exception of 10 unidentified isolates (Figure 1). These genera included *Streptomyces* (32 isolates), *Micromonospora* (24 isolates), *Verrucosispora* (9 isolates), *Microbispora* (3 isolates), *Nonomuraea* (3 isolates), *Plantactinospora* (3 isolates), and *Shimazuella* (1 isolates). This is the first time report of endophytic actinomycetes from *P. candollei*. Interestingly, *Shimazuella* were firstly reported here to be isolated from plant inner tissue [4, 20].

Sixty percent of identified isolates from the roots of *P. candollei* var. *candollei* and the root nodules of *P. candollei* var. *mirifica* were non-streptomycete (Figure 1). Although *Streptomyces* has been determined to be the dominant genus in plant sources [4], the dominant genus in our study was *Micromonospora* (13/32 isolates from the roots of *P. candollei* var. *candollei* and 11/26 isolates from the root nodules of *P. candollei* var. *mirifica*).This result was supported by the findings of Trujillo, et al. [21], who reported that members of the genus *Micromonospora*

can be commonly found in legumes. On the other hand, all identified isolates from the rhizospheric soil samples belonged to genus *Streptomyces* (Figure 1).

The rare actinomycetes can be referred to the non-streptomycete actinomycetes [22]. Our data suggested that varieties of rare actinomycetes, including *Micromonospora*, *Verrucosispora, Microbispora*, *Nonomuraea, Plantactinospora,* and *Shimazuella* (Figure 1 and Table 1) can be isolated from the inner tissue of *P. candollei*. New compounds can be discovered from actinomycetes by physicochemical screening [11, 23]. Therefore, metabolite analyses of the actinomycete isolates in this study were performed by using LC/UV and LC/MS.

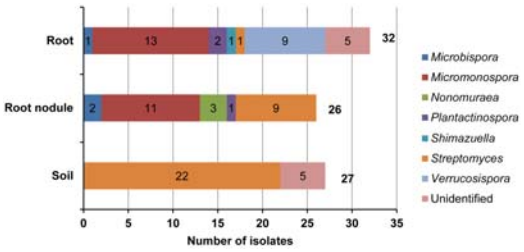

Figure 1. Genera distribution of isolated actinomycetes from roots of *P. candollei* var. *candollei*, roots nodules of *P. candollei* var. *mirifica* and rhizospheric soil. The number of isolates of each genus was indicated in bar graph.

3.2 Metabolite Analyses of Endophytic *Micromonospora* sp. Isolated from *P. candollei*

A number of peaks in the LC/UV chromatograms were higher in the cultured broth of *Micromonospora* sp. than those of other isolates (data not shown). Therefore, we selected 21 *Micromonospora* sp. for further metabolite analyses (Table 1). The LC/UV chromatograms from the broth extract of *Micromonospora* sp. are shown in Figure 2. The peaks with unique retention time (Figure 2 and Table 2) were selected and their physicochemical properties (maximum UV absorbance and molecular mass, Table 2) were searched in the databases. Six possible compounds were predicted among the selected peaks (Table 2).

Table 2. The physicochemical properties selected peaks from the 50% ethanol culture broth of *Micromonospora* isolates (Figure 2) and estimated compounds.

Peak NO.	Maximum UV Absorbance (nm)	$[M+H]^+$	Estimated compound
1	255	284.0995	Guanosine
2	249	384.0011	Unknown
3	252, 270	268.1020	2-Deoxyribo furanosyl Guanine (α,*L* or β,*L*)
4	258	427.1434	Unknown
5	264, 328	566.4270	Unknown
6	218, 280	373.1671	Cyclo (tryptophyl-trptophyl); (3*R*, 6*S*) -form
7	219, 281	387.1836	Unknown
8	203, 235	305.1833	Neihumicin
9	202, 228, 264	606.4130	Rakicidin A
10	202, 228, 264	620.4260	Rakicidin B

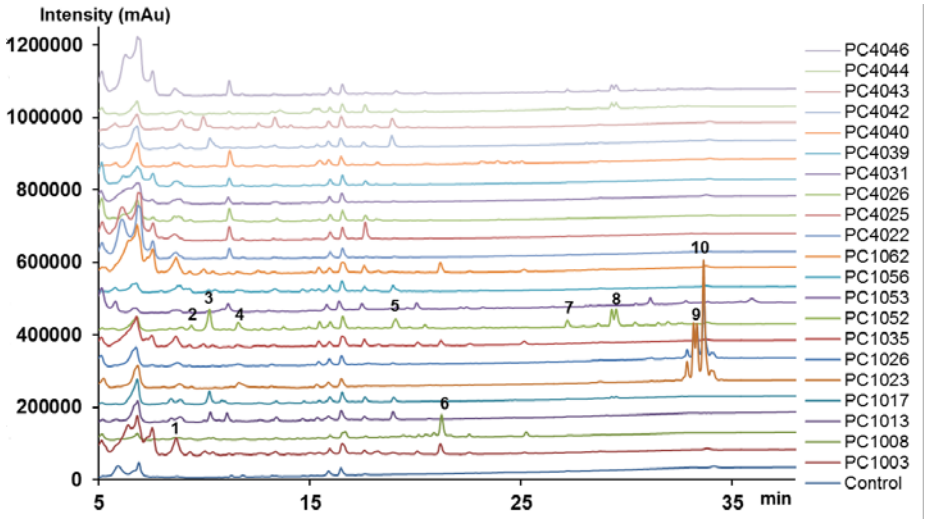

Figure 2. HPLC chromatograms of the 50% ethanol broth extracts of *Micromonospora* isolates. The numbers indicated the peaks that were selected for physicochemical screening (Table 2).

Physicochemical properties of three peaks (peak number 8, 9 and 10) were matched with possible known bioactive compounds (Table 2). Neihumicin (peak number 8 from *Micromonospora* sp. PC1017, PC1052, PC4044 and PC4046) has been reported previously as a cytotoxic compound and was originally isolated from *Micromonospora neihuensis* NH3-1 [24]. Rakicidin A and B (peak number 9 and 10 from *Micromonospora* sp. PC1023 and PC1026) were originally isolated from *Micromonospora* sp. R385-2 and have been reported to possess cytotoxic activity [25]. However, further structure elucidation and activity testing are needed to confirm the chemical structure and evaluate the biological activity of these peaks.

There are no known compounds corresponded the physicochemical properties of 4 compounds (Table 2; peak number 2, 4, 5, and 7). These four unknown peaks were found in *Micromonospora* sp. PC1052 that isolated from root of *P. candollei* var. *candollei*. Especially, compound **4** (peak number 4) was estimated to be a new compound with hetero atoms from the results of candidate molecular formula that was calculated from exact mass value, m/z 427.1400. Therefore, we selected strain *Micromonospora* sp. PC1052 for isolation of compounds and cultured with larger scale.

3.3 Isolation of Compound 4

The isolation scheme is summarized in Figure 3. The cells were removed from a 9-day-old culture of PC1052 (10 L) by centrifugation. The supernatant was then passed through a Diaion HP-20 (100 i.d. × 250 mm) and eluted with water and 100% MeOH. The MeOH fraction (3.5 g) was subjected to an ODS column (60 mm i.d. × 150 cm), from which compound **4** was eluted with 10% MeOH (1.4 g). This fraction was loaded into a silica gel FL100D column (40 mm i.d. × 150 mm) and was eluted with a gradient of $CHCl_3$-MeOH. Four fractions, eluted with $CHCl_3$-MeOH from 5:1 to 0:100, were combined (yielding 292.6 mg in total, Figure 3). After evaporation under vacuum, the combined fraction was applied to an ODS column (25 mm i.d. × 150 mm) and eluted with a gradient of

MeOH-water. The 4% methanolic fraction (58.9 mg) was purified by preparative HPLC on an Inertsil ODS-4 column (14 mm i.d. × 250 mm) to obtain compound **4** (6.8 mg). The mobile phase was 15% MeOH in water (0.1% formic acid), and the compound was eluted with a 7.5 ml/min flow rate. We successfully isolated only one target compound, the other three target compounds could not be identified in the 10-L culture.

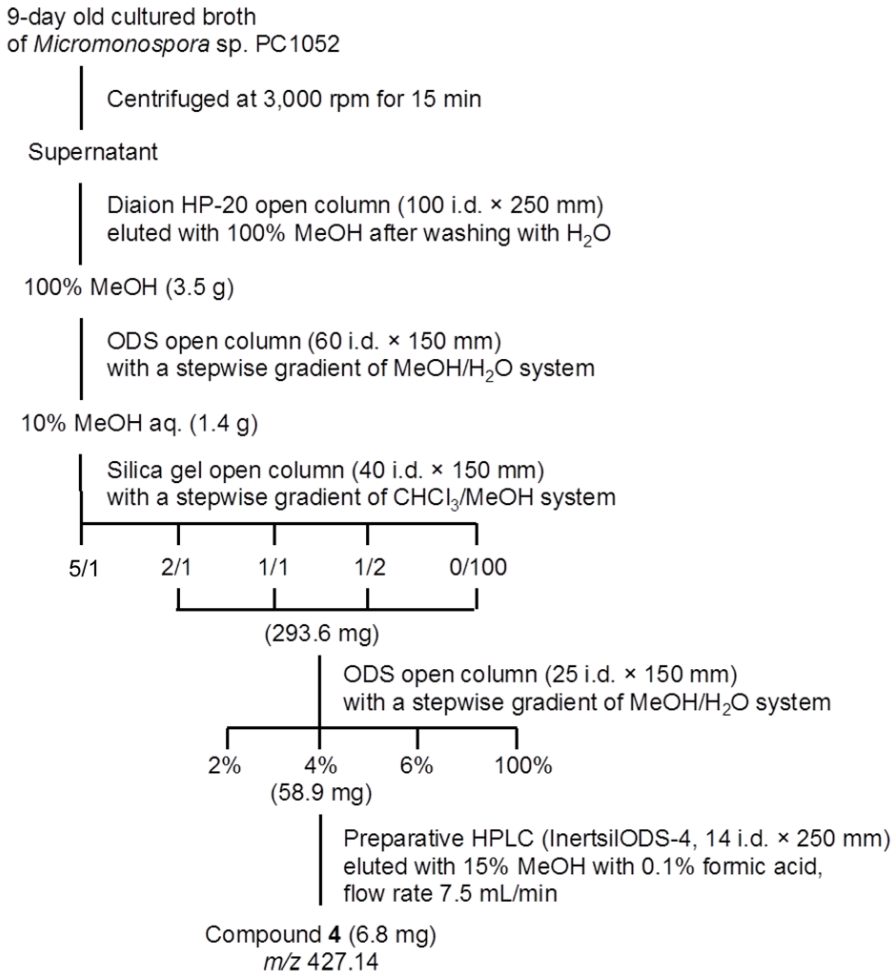

Figure 3. Isolation scheme of compound **4** from *Micromonospora* sp. PC1052. The volume of mobile phase used in every step was equal to 3 times of column volume.

3.4 Structure Elucidation and Physicochemical Properties of Compound 4

Compound **4** was obtained as a white powder and determined to have the molecular formula of $C_{16}H_{23}N_6O_6S$ by HR-ESI-MS $[M+H]^+$ m/z 427.1400 (calculated m/z for $C_{16}H_{23}N_6O_6S$: 427.1394). The IR spectrum showed absorptions at 3432 cm^{-1} and 1650 cm^{-1}, suggesting the presence of hydroxyl and carbonyl groups. The 1D and 2D NMR spectra of compound **4** were obtained in D_2O. The ^1H and ^{13}C NMR spectral data of compound **4** are listed in Table 3. The ^1H NMR data indicated the presence of five sp^3 methines, three sp^3

methylenes, one acetyl methyl group (δ_H 1.97). The ^{13}C NMR showed the resonances of 16 carbons, which were classified into five olefinic carbons two carbonyl carbons at d_c 177.9 and 173.6, four oxygenated sp^3 methine carbons, one sp^3 methine carbon, three sp^3 methylene carbons and one methyl carbon by heteronuclear-single-quantum coherence spectra. The ^{1}H-^{1}H COSY indicated the presence of 2 partial structures: (a) C-2/C-4 and (b) C-7/C-11 as shown in Figure 4. Analysis of HMBC data confirmed the presence of *N*-acetylhomocysteine moiety based on correlations from H-2 to C-1, C-3, C-4 and C-10, from H-3 to C-1, C-2 and C-4, from H-4 to C-2 and C-3, and from H_3-6 to C-5. An adenosine moiety was identified, based on HMBC correlations from H-7 to C-8, from H-8 to C-9 and C-10, from H-9 to C-7 and C-11, from H-10 to C-8 and C-11, from H-11 to C-12 and C-16, from H-12 to C-13 and C-16, and H-15 to C-14 and C-16. Since HMBC correlation was observed from H-4 (δ_H 2.56) to C-7 (δ_C 33.5) and from H_2-7 (δ_H 2.94 and 3.01) to C-4 (δ_C 28.6), *N*-acetylhomocysteine moiety was confirmed the connection at C-7 through a sulfur atom, which is also implied by the molecular formula. Therefore, the structure of compound **4** was elucidated to be *S*-adenosyl-*N*-acetylhomocysteine (Figure 4). The physicochemical properties of compound **4** are shown in Table 4. This compound was previously reported as the result of chemical synthesis from *S*-adenosylhomocysteine with acetic anhydride in methanol [26, 27]. It was tested for the *in vitro* activity to inhibit *S*-adenosylmethionine-dependent methyltransferases [26], vaccinia virus transmethylation and mRNA synthesis [28]. However, it showed no or weak activities in both studies. There was no report of this compound as a natural product. The plant growth regulator ethylene was produced through an analog of compound 4, *S*-adenosylmethione [29]. It is possible that *S*-adenosyl-*N*-acetylhomocysteine may serve a critical role in plant growth regulation.

Table 3. NMR spectroscopic data of *S*-adenosyl-*N*-acetylhomocysteine (compound **4**) in D_2O (400/100 MHz).

Position	δ_C	δ_H (*int, mult., J*)
1	177.9	
2	53.5	4.21 (1H, dd, 4.8, 8.8)
3	31.4	1.84 (1H, m)
		2.02 (1H, m)
4	28.6	2.56 (2H, m)
5	173.6	
6	21.7	1.97 (3H, s)
7	33.5	2.94 (1H, dd, 6.4, 14.4)
		3.01 (1H, dd, 4.8, 14.4)
8	83.8	4.31 (1H, ddd, 4.8, 6.4)
9	72.2	4.41 (1H, dd, 4.8)
10	73.3	4.85 (1H, dd, 5.2)
11	87.7	6.06 (1H, d, 5.2)
12	141.0	8.38 (1H, s)
13	118.8	
14	153.7	
15	150.1	8.27 (1H, s)
16	148.6	

Note: Chemical shift (δ) and coupling constants (*J*) were recorded in ppm and hertz (Hz), respectively

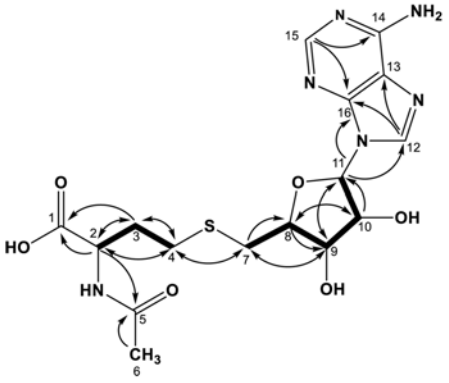

Figure 4. ^{1}H-^{1}H COSY (bold) and selected HMBC (arrow) of *S*-adenosyl-*N*-acetylhomocysteine.

Table 4. Physicochemical properties of *S*-adenosyl-*N*-acetylhomocysteine (compound **4**).

Appearance	White powder
Molecular formula	$C_{16}H_{23}N_6O_6S$
Molecular weight	426.4480
ESI-MS (*m/z*)	
Calcd.	427.1394
(from$C_{16}H_{23}N_6O_6S$)	
Found	427.1400
$[\alpha]_D^{25.0}$ (*c* 0.1, H_2O)	+20.41
UV λ_{max}^{H2O} nm	260
IR ν_{max}^{KBr} cm^{-1}	3432, 1650
Solubility	
Soluble	water
Insoluble	hexane, acetone

4. CONCLUSION

Our data indicated that the roots of *P. candollei* var. *candollei* and root nodules of *P. candollei* var. *mirifica* were rich sources for the isolation of actinomycetes, including the non-sterptomycete actinomycetes in genera *Micromonospora*, *Verrucosispora, Microbispora*, *Nonomuraea, Plantactinospora,* and *Shimazuella. Micromonospora* was found to be the dominant genus form roots and root nodules, and showed the capability to produce known metabolites and a new found metabolite. Finally, by using the physicochemical screening we can identify one new found metabolite, *S*-adenosyl-*N*-acetylhomocysteine, form *Micromonospora* sp. PC1052.

Although there was no novel bioactive metabolite isolated, this study clarified the information on diversity of endophytic actinomycetes associated with *P. candollei* and their secondary metabolite production. Further investigation using more elaborate sample collection could be performed.

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