

Article

Biotransformation of Ergostane Triterpenoid Antcin K from *Antrodia cinnamomea* by Soil-Isolated *Psychrobacillus* sp. AK 1817

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Abstract: Antcin K is one of the major ergostane triterpenoids from the fruiting bodies of *Antrodia cinnamomea*, a parasitic fungus that grows only on the inner heartwood wall of the aromatic tree *Cinnamomum kanehirai* Hay (Lauraceae). To search for strains that have the ability to biotransform antcin K, a total of 4311 strains of soil bacteria were isolated, and their abilities to catalyze antcin K were determined by ultra-performance liquid chromatography analysis. One positive strain, AK 1817, was selected for functional studies. The strain was identified as *Psychrobacillus* sp., based on the DNA sequences of the 16S rRNA gene. The biotransformation metabolites were purified with the preparative high-performance liquid chromatography method and identified as antcamphin E and antcamphin F, respectively, based on the mass and nuclear magnetic resonance spectral data. The present study is the first to report the biotransformation of triterpenoids from *A. cinnamomea* (*Antrodia cinnamomea*).

Keywords: *Antrodia cinnamomea*; biotransformation; antcin K; triterpenoid; *Psychrobacillus*

1. Introduction

Terpenoids, which are composed of isoprenoid subunits, are the largest group of phytochemicals in the world, and are widely distributed in almost all living organisms [1]. Based on the number of isoprenoid units, terpenoids are subdivided into monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25), triterpenoids (C30), and tetraterpenoids (C40). Triterpenoids from several sources are used for medicinal purposes in Asia as anti-inflammatory, analgesic, antipyretic, hepatoprotective, and cardiotoxic agents.

Antrodia cinnamomea, also called *A. camphorate*, is a parasitic fungus that grows only on the inner heartwood wall of the aromatic tree *Cinnamomum kanehirai* Hay (Lauraceae). This mushroom is endemic in Taiwan, and is used as a folk remedy in the treatment of a variety of diseases [2]. The fruiting bodies of *A. cinnamomea* contain abundant ergostane and lanostane tetracyclic triterpenoids, which are generally considered the major bioactive constituents of *A. cinnamomea* (*Antrodia cinnamomea*). More than 40 triterpenoids have been isolated from *A. cinnamomea*, where antcin K is one of the major ergostane triterpenoids from the fruiting bodies of cutting wood-cultivated, as well as dish-cultivated,

A. cinnamomea. Some bioactivities of antcin K have been reported, including anti-inflammatory, antidiabetic, and antihyperlipidemic activities, inducing apoptosis of hepatoma cells, and reducing carcinogenesis [3–8].

Biotransformation of xenobiotics using microorganisms is a very useful approach for expanding the chemical diversity of natural products [9]. Many biotransformations of triterpenoids, including boswellic acid [10,11], oleanolic acid [12,13], betulinic acid [12,14], maslinic acid [13,15], ursolic acid [16–18], and ginseng-containing triterpenoids, such as ginsenosides [19], protopanaxadiol [20,21], and dipterocarpol [22], have been discovered. The biocatalysts used in studies include the fungi *Aspergillus*, *Cunninghamella*, *Absidia*, *Rhizomucor*, and *Syncephalastrum* and the bacteria *Bacillus* and *Nocardia*. The biotransformation reactions include hydroxylation, dehydrogenation, lactone formation, methylation, and (de)glycosylation. Many novel triterpenoids with potent bioactivity have been identified from the biotransformation reactions; however, to our knowledge, biotransformation of triterpenoids from *A. cinnamomea* has not been realized yet, although many bioactivities of triterpenoids have been identified from *A. cinnamomea*. Based on the abundance of antcin K in dish-cultured *A. cinnamomea*, biotransformation of antcin K is a good starting point with an alternate high-throughput screening method. In the present study, thousands of soil bacteria were isolated, and the ability to catalyze antcin K was determined. One positive strain was selected and then identified by genetic analysis. The biotransformation metabolites were purified with preparative high-performance liquid chromatography (HPLC), and identified using spectra methods.

2. Results

2.1. Screening and Identification of Soil Bacteria with Biotransformation Activity

A great challenge for the realization of a desired biotransformation reaction is finding the appropriate microorganism. Thus, classical screening of a series of microbial strains is still the most widely used technique. To study the biotransformation of triterpenoid antcin K from *A. cinnamomea*, thousands of soil bacteria were isolated with the plating method and then cultivated in broth with antcin K. The fermentation broth was analyzed using ultra-performance liquid chromatography (UPLC) to determine the ability of the strain to digest antcin K. A total of 4311 strains were screened, and one strain (AK 1817) was selected for functional studies.

Figure 1a shows the UPLC analysis of 0 h and 72 h fermentation broths of the strain. In the figure, the precursors 25S-antcin K and 25R-antcin K appear with retention times of 4.6 and 4.7 min, respectively, for the 0 h fermentation broth. After 72 h cultivation, 25S-antcin K and 25R-antcin K decreased dramatically, and two new major peaks, compound (1) and compound (2), appeared with retention times of 5.3 and 5.4 min, respectively. Parallel experiments were repeated with the strain AK 1817, but without antcin K in the fermentation broth. No metabolites with a retention time of 5.3 and 5.4 min appeared in the 72 h fermentation broth (Figure S1). From the results, it is clear that antcin K was catalyzed by the strain to compound (1) and compound (2). Moreover, compound (1) and compound (2) were potentially biotransformed from 25S-antcin K and 25R-antcin K, respectively.

To evaluate the biotransformation process in advance, we isolated 25S-antcin K and 25R-antcin K, and repeated the biotransformation experiments with the strain AK 1817 by adding 25S-antcin K or 25R-antcin K individually in the fermentation broth. Figure 1b,c shows the 0 h and 72 h fermentation broths of the strain fed with 25S-antcin K and 25R-antcin K, respectively. From the results, it is clear that compound (1) was biotransformed from 25S-antcin K (Figure 1b), and compound (2) was biotransformed from 25R-antcin K (Figure 1c). According to the absorption in the UPLC analysis, the conversions of compound (1) and compound (2) from 25S-antcin K and 25R-antcin K at the 72-h cultivation were 67.6% and 74.8%, respectively.

To identify the strain, the partial 16S rRNA gene was amplified and sequenced with polymerase chain reaction (PCR) with the bacteria-specific 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1391R (5'-GACGGGCRGTGWGTRCA-3') primer set. The DNA sequence is shown in Figure S2. The partial

sequences of the 16S rRNA gene were then blasted against National Center for Biotechnology Information (NCBI) non-redundant nucleotides to identify the strain. From the blasted results, the phylogenetic tree indicated that the AK 1817 strain was classified as *Psychrobacillus* sp. (Figure 2). The strain was deposited in the Bioresources Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan).

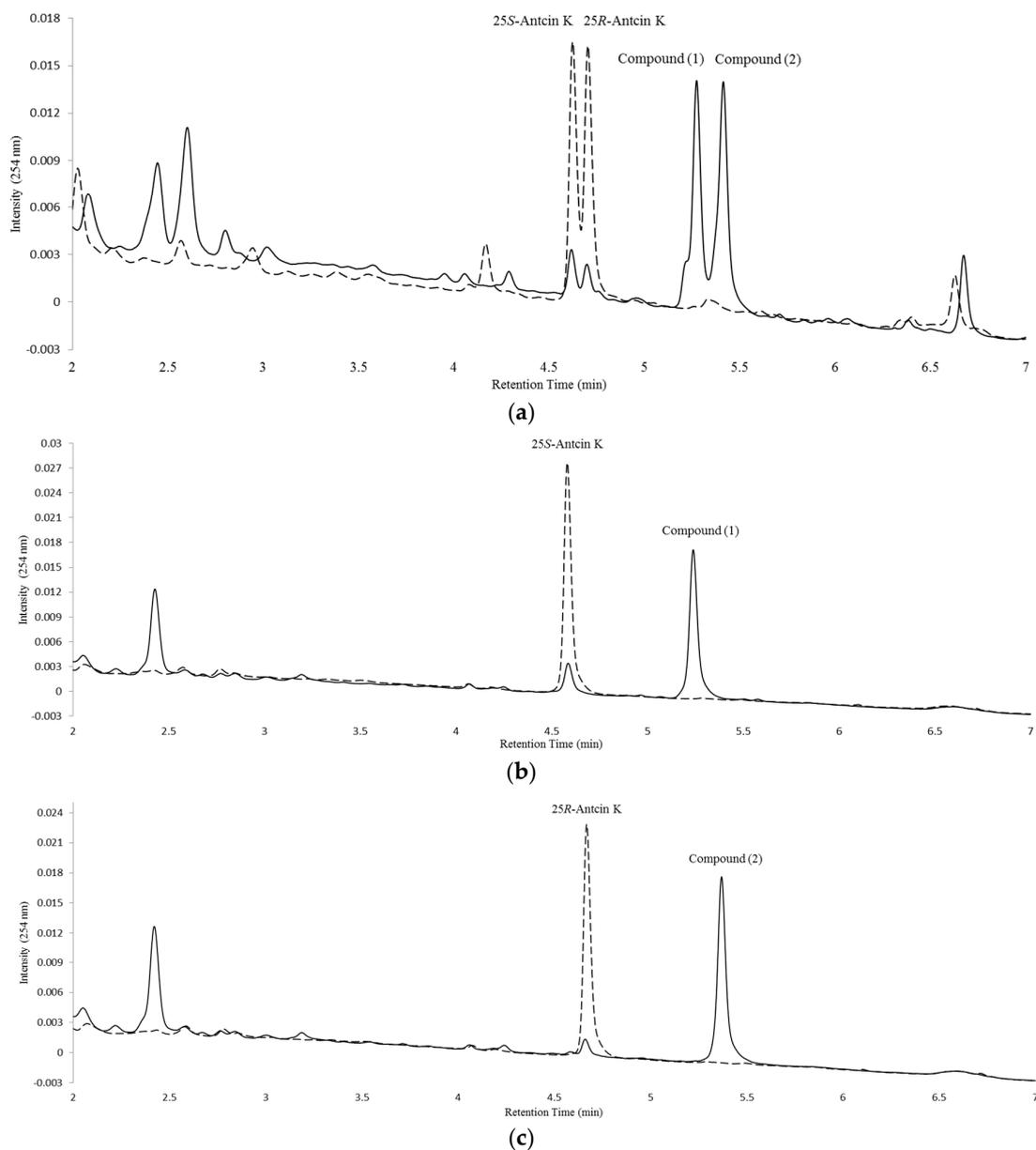


Figure 1. Biotransformation of antcin K by soil bacteria AK 1817 strain. The strain was cultivated in Luria-Bertani (LB) media containing (a) 25S- and 25R-antcin K; (b) 25S-antcin K; or (c) 25R-antcin K. The 0 h (dash curves) and 72 h (solid curves) cultivation of the fermentation broth were analyzed with UPLC. The UPLC operation conditions are described in Materials and Methods.

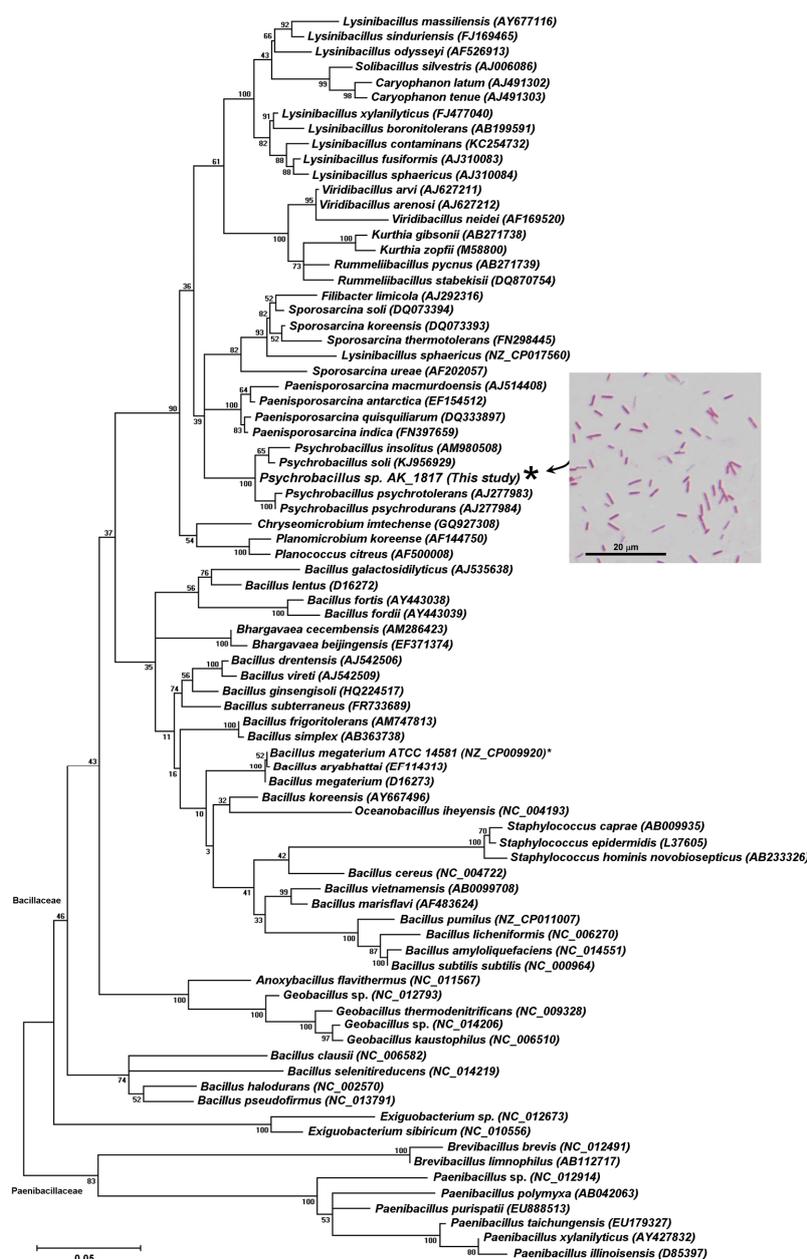


Figure 2. Molecular phylogenetic analysis of strain AK 1817 with the maximum likelihood (ML) method based on the general time reversible (GTR) model [23]. The tree with the highest log likelihood ($-11,124.2673$) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances, estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (six categories (+G, parameter = 0.1906)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 60.3946% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 82 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1146 positions in the final dataset. Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 [24]. Gram staining revealed strain AK 1817 is a short-rod-shaped, Gram-positive bacteria (see the inserted photo).

2.2. Isolation and Identification of Biotransformation Metabolites

To isolate the two metabolites, the fermentation was scaled up with a 5 L fermenter. Two batches of 2.5 L fermentation were performed in the 5 L fermenter. Compound (1) and compound (2) were further isolated using the preparative HPLC method, and were identified using spectrophotometric methods. Compound (1) and compound (2) showed identical mass and nuclear magnetic resonance (NMR) spectrum data. They showed an $[M + H]^+$ ion peak at m/z : 487.42 in the electrospray ionization mass (ESI-MS) spectrum corresponding to the molecular formula $C_{29}H_{42}O_6$. Then 1H and ^{13}C NMR, including distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond connectivity (HMBC), nuclear Overhauser effect spectroscopy (NOESY), and correlation spectroscopy (COSY), spectra were obtained, and the 1H - and ^{13}C -NMR signal assignments were conducted accordingly (shown in Figures S3–S6). The key HMBC correlations of compounds (1) and (2) are shown in Figure S7, and the spectroscopic data is listed in Table S1. Based on these spectral data and with the comparison of 1H -NMR and ^{13}C -NMR data in the literature [25], as well as the biotransformation results above, compound (1) and compound (2) were characterized as antcamphin E and antcamphin F, respectively. Figure 3 illustrates the biotransformation of antcin K by the soil-isolated *Psychrobacillus* sp. AK 1817 strain.

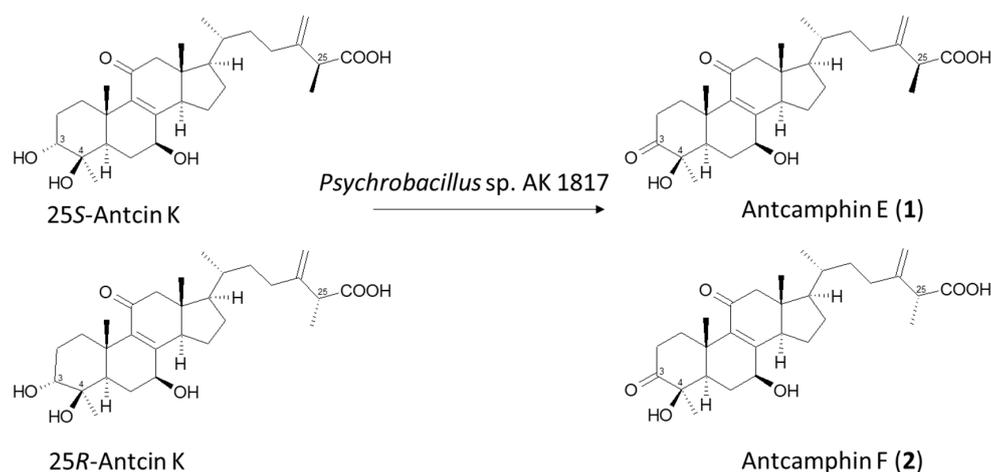


Figure 3. Biotransformation process of antcin K by the *Psychrobacillus* sp. AK 1817 strain.

3. Discussion

The present study demonstrated the biotransformation of 25S-antcin K and 25R-antcin K to antcamphin E and antcamphin F, respectively, by soil-isolated *Psychrobacillus* sp. AK 1817. To the best of our knowledge, this study is the first to report a microbe having direct biotransformation ability toward triterpenoids from *A. cinnamomea*.

In addition to the triterpenoids from *A. cinnamomea*, many biotransformations of triterpenoids from other sources have been reported [11–22,26–29]. In the present study, *Psychrobacillus* sp. AK 1817 catalyzed dehydrogenation (oxidation) of the C-3 hydroxyl group on the antcin K structure. Some bacteria have been reported to catalyze oxidation of a 3-hydroxyl group on the triterpenoid structure to form corresponding 3-oxo-triterpenoid derivatives. Among these bacteria, three Actinobacteria (*Mycobacterium* sp. [27], *Nocardia coralline* [28], and *Rhodococcus rhodochrous* [29]) have been demonstrated to catalyze the oxidation of the 3-hydroxyl group on the tetracyclic triterpenoid 20R-dihydroprotopanaxadiol and the pentacyclic triterpenoids echinocystic acid and betulin, respectively, to form the corresponding 3-oxo-triterpenoid derivatives. In addition, the *Bacillus megaterium* ATCC (American Type Culture Collection) 14581 and CGMCC (China General Microbiological Culture Collection Center) 1.1741 strains were found to catalyze the oxidation of the 3-hydroxyl group on the pentacyclic triterpenoids betulinic acid [26] and ursolic acid [18], respectively,

to form the corresponding 3-oxo-triterpenoid derivatives. *B. megaterium* (*Bacillus megaterium*) ATCC 14581 is within the ancestral *Bacillus* clade while *Psychrobacillus* sp. AK 1817 is within the derived clade (Figure 2). Therefore, due to the similar biotransformation modifications by the two microbes, for comparison, we used the strain *B. megaterium* ATCC 14581 to perform the biotransformation experiments for antcin K. However, no metabolite was found in the reaction (Figure S7). The results revealed the unique substrate specificity in the biotransformation of antcin K by the strain *Psychrobacillus* sp. AK 1817. To resolve the corresponding catalyzing enzyme from *B. megaterium* ATCC 14581 and *Psychrobacillus* sp. AK 1817, a comparative functional genomic project with the two strains is being conducted in our laboratory.

Due to the abundance of antcin K in wood-cultivated, as well as in dish-cultivated, *A. cinnamomea*, many bioactivities of antcin K have been found [3–8]. In contrast, few studies on antcamphin E and antcamphin F have been reported, due to their rarity. Recently, antcamphin E and antcamphin F were isolated as minor components of *A. cinnamomea* [25,30]. Antcamphin E and antcamphin F exhibited cytotoxic activity toward breast cancer cells and lung cancer cells. In addition, both compounds exhibited protective activities against CCl₄-induced injury in HepG2 cells [31]. These results highlight the valuable application of the biotransformation process found in the present study. The biotransformation process of *Psychrobacillus* sp. AK 1817 provides an easy and unique approach for obtaining antcamphin E and antcamphin F from the abundant antcin K. Therefore, deeper and broader studies on the bioactivity of the two compounds should be conducted in the future. In addition, the process could be scaled up for industrial applications. Further studies to clone candidate genes that encode the corresponding catalyzing enzyme from *Psychrobacillus* sp. AK 1817, and to extend the substrate specificity, are being performed in our laboratory.

In conclusion, in the present study, triterpenoids were newly biotransformed from *A. cinnamomea*, and a biotransformation process was developed to produce antcamphin E and antcamphin F from antcin K.

4. Materials and Methods

4.1. Microorganism and Chemicals

Bacillus megaterium ATCC 14581 (BCRC 10608) was purchased from BCRC. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the materials needed for polymerase chain reaction (PCR), including primers, deoxyribonucleotide triphosphate, and Taq DNA polymerase, were purchased from MDBio (Taipei, Taiwan). The other reagents and solvents used were of high quality, and were purchased from commercially available sources.

4.2. Preparation of Antcin K

The dried dish-cultivated *A. cinnamomea* sample was obtained from Honest & Humble Biotechnology Co., Ltd. (New Taipei City, Taiwan). First, the sample was extracted with 50% methanol to obtain the ergostane-enriched fraction. Then, this fraction was subjected to preparative HPLC to obtain antcin K. 1.2 g (1.2%) of antcin K were obtained from 100 g of the dried dish-cultivated *A. cinnamomea*. The 25S- and 25R-epimers of antcin K were further purified with semipreparative HPLC using the reverse-phase C-18 column. 13.5 and 23.7 mg of 25S- and 25R-epimers of antcin K were obtained from 70 mg of antcin K. The 25S- and 25R-epimers of antcin K were identified by comparison with standards provided by Professor Min Ye (Peking University, Beijing, China).

4.3. Screening and Identification of Soil Bacteria with Biotransformation Activity

A series of soil samples, collected from local areas of Tainan city in Taiwan, were used for isolation of the bacterial strains. The fresh soil samples were plated according to the dilution plating method on Luria-Bertani (LB) agar [32]. After cultivation at 28 °C for 24 h, the colonies that formed on the plates were transferred to a deep 48-well microplate containing 1 mL of LB medium. The microplate was

incubated at 180 rpm and 28 °C for 1 day. About 0.1 mL of the primary culture was then transferred to another microplate containing 1 mL of LB medium and 100 mg/L of antcin K for the secondary cultivation. The remaining primary culture was stored at −20 °C until the biotransformation activity assay was completed. The secondary cultivation was carried out at 180 rpm and 30 °C for 3 days. Then, an equal volume of ethanol was added to each well of the secondary cultivation microplate and shaken vigorously for 30 min at 28 °C. The cell debris was removed by centrifugation at 4800 rpm for 30 min. The supernatant from the extracted broth was assayed with UPLC to measure the biotransformation activity. To confirm the activity of the biotransformation-positive strains, the tested strains were cultivated in a 250-mL baffled Erlenmeyer flask containing 20 mL LB medium and 100 mg/L antcin K at 180 rpm and 28 °C for 5 days. Samples were collected daily and analyzed with UPLC to confirm the biotransformation activity. Candidate strains were re-purified and then reanalyzed by repeating the biotransformation activity assay.

4.4. UPLC Analysis

The mixtures of the biotransformation reactions were analyzed with a UPLC system (Acquity UPLC H-Class, Waters, Milford, MA, USA). The system was equipped with an analytic C18 reversed-phase column (Acquity UPLC BEH C18, 1.7 μm, 2.1 i.d. × 100 mm, Waters, Milford, MA, USA). To analyze both antcin K and the biotransformation products, a gradient elution using water (A) containing 1% (*v/v*) acetic acid and methanol (B) with a linear gradient for 3 min with 50% to 80% B and for another 4 min with 80% to 100% B was conducted at a flow rate of 0.3 mL/min, an injection volume of 0.2 μL, and absorbance detection at 254 nm.

4.5. Candidate Strain Classification via 16S rRNA Gene Analysis

For determination of the 16S ribosomal RNA gene sequences of the biotransformation-positive strain, chromosomal DNA was isolated using a Geno Plus Genomic DNA Extraction Miniprep System (Viogene, Taipei, Taiwan) according to the manufacturer's instructions. The 16S rRNA gene was amplified using PCR with the forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-GACGGGCRGTGWGTRCA-3') primers known to amplify the 16S rRNA gene from a broad range of taxonomically different bacterial isolates [33]. PCR was performed with a total volume of 100 μL, which contained PCR buffer, 1 μg genomic DNA, 0.2 mM (each) deoxyribonucleotide triphosphate, 50 pmol (each) forward and reverse primers, and 2.5 U of Taq DNA polymerase. Amplification was performed for 35 cycles in a DNA thermal cycler, the ABI Prism 377 DNA Sequencer/Genetic Analyzer (Perkin-Elmer, Boston, MA, USA), employing the thermal profile according to Hugenholtz and Goebel [33]. The sequence of the amplified DNA fragment was determined by the DNA Sequencing Center of National Cheng Kung University in Tainan (Taiwan). The 16S rRNA was then blasted against NCBI non-redundant nucleotides to identify the strain. The 16S rRNA phylogeny was constructed to classify the strain. Related sequences were downloaded and aligned using the ClustalX program [34], followed by manual modifications. The maximum-likelihood (ML) tree was reconstructed using GTR+G+I distances as implemented in the Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 package [24] with 100 bootstrap replications [35]. The substitution model (parameter) used to calculate the GTR+G+I distances was selected using Modeltest 3.7 [36].

4.6. Scale-Up Fermentation, Isolation, and Identification of the Biotransformation Products

The AK 1817 strain was cultured in 100 mL of LB medium for 24 h as a seed culture, which was inoculated into a 5 L fermenter containing 2.5 L LB medium supplemented with 100 mg/L antcin K, followed by cultivation with aeration (0.5, *v/v/m*) and agitation (280 rpm) at 28 °C. A 10 mL cultured medium was sampled at several different time intervals and analyzed with UPLC. The purification process was the same as in our previous work [37], and is described briefly below. Two batches of 2.5 L fermentation were performed for the purification of the biotransformation products. Following fermentation, the broth was condensed to 200 mL under a vacuum, and extracted twice by ethyl acetate.

The extracts were further condensed and the residue was then suspended in 200 mL of 50% methanol. The suspension was filtrated through a 0.2 µm nylon membrane. The filtrate was injected into a preparative YoungLin HPLC system (YL9100, YL Instrument, Gyeonggi-do, South Korea). The system was equipped with a preparative C18 reversed-phase column (Inertsil, 10 µm, 20.0 i.d. × 250 mm, ODS 3, GL Sciences, Eindhoven, The Netherlands). The operational conditions for the preparative HPLC analysis were the same as those in the UPLC analysis. The elution corresponding to the peaks of the metabolites in the UPLC analysis were collected, concentrated under vacuum, and then lyophilized. Finally, 27.9 mg of compound (1) and 33.3 mg of compound (2) were obtained, and the structures of the compounds were confirmed with NMR and mass spectral analysis. The mass analysis was performed on a Finnigan LCQ Duo mass spectrometer (ThermoQuest Corp., San Jose, CA, USA) with electrospray ionization (ESI). ¹H- and ¹³C-NMR, DEPT, HSQC, HMBC, COSY, and NOESY spectra were recorded on a Bruker AV-700 NMR spectrometer (Bruker Corp., Billerica, MA, USA) at ambient temperature. Standard pulse sequences and parameters were used for the NMR experiments, and all chemical shifts were reported in parts per million (ppm, δ).

Supplementary Materials: The following are available online at www.mdpi.com/2073-4344/7/10/299/s1. Table S1. NMR spectroscopic data for compound (1)/(2) (in pyridine-d₅; 700 MHz), Figure S1. UPLC analysis of fermentation broth of the AK 1817 strain. The strain was cultivated in LB media without antcin K. The fermentation broth with cultivation of 72-h was analyzed by UPLC. The UPLC operation conditions were described in Materials and Methods, Figure S2. The partial 16S rRNA gene sequence of the AK 1817 strain. The partial 16S rRNA gene was amplified and sequenced by PCR with the bacteria specific 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1391R (5'-GACGGGCRGTGWGTRCA-3') primer set. The PCR operation conditions were described in Materials and Methods, Figure S3. The ¹H-NMR (700 MHz, Pyridine-d₅) spectrum of compound (2), Figure S4. The ¹³C-NMR (700 MHz, Pyridine-d₅) spectrum of compound (2), Figure S5. The HMBC (700 MHz, Pyridine-d₅) spectrum of compound (2), Figure S6. The HSQC (700 MHz, Pyridine-d₅) spectrum of compound (2), Figure S7. The key HMBC correlations of compound (1)/(2), Figure S8. Biotransformation of antcin K by *B. megaterium* ATCC 14581 strain. The strain was cultivated in LB media containing both 25S- and 25R-antcin K. The fermentation broth with cultivation of 72-h was analyzed by UPLC. The UPLC operation conditions were described in Materials and Methods.

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Author Contributions: Te-Sheng Chang and Jiumn-Yih Wu conceived and designed the experiments and wrote the paper; Chien-Min Chiang purified the metabolites and resolved the chemical structures of compounds (1) and (2). Tzi-Yuan Wang identified the isolated bacteria. An-Ni Ke performed the experiments for the biotransformation and anti-proliferative activity assay of the tested compounds.

Conflicts of Interest: The authors declare no conflicts of interest.

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