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by Deiske Sumilat 61

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Production of an α-pyrone metabolite and microbial transformation of isoflavones by an Indonesian *Streptomyces* sp.

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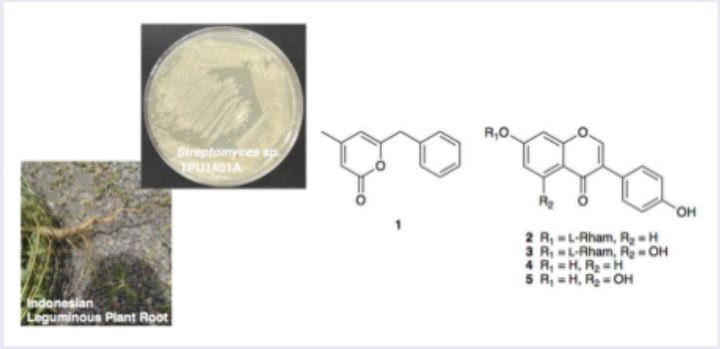
ABSTRACT

A benzyl-α-pyrone metabolite, streptpyrone A (**1**), obtained together with three known isoflavonoids, daidzein-7-*O*-α-L-rhamnoside (**2**), genistein-7-*O*-α-L-rhamnoside (**3**), and daidzein (**4**), from the culture broth of an Indonesian actinomycete *Streptomyces* sp. TPU1401A. The structure of **1**, elucidated based on its spectroscopic data, has been reported as a synthetic compound. However, this is the first report of the isolation of **1** as a metabolite of microbial origin. Strain TPU1401A exhibited the ability to transform the isoflavone aglycones **4** and genistein (**5**) into the 7-*O*-glycosides **2** and **3**, respectively. Compounds **2** and **3** promoted the growth of strain TPU1401A more effectively than compounds **4** and **5**. These results suggest that strain TPU1401A utilizes isoflavone glycosides to promote growth by transforming isoflavones through microbial glycosidation.



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1. Introduction

Microbial secondary metabolites have provided a number of useful substances with widely diverse properties [1, 2]. Actinomycetes form a large group and are important microorganisms that possess the ability to produce various bioactive metabolites with antimicrobial, anticancer, and enzyme inhibitory activities [2, 3]. Approximately 45% of all microbial substances have been obtained from actinomycete strains [4]. Accordingly, we have been conducting a research program of chemical studies on culture broths of actinomycetes collected in North Sulawesi, Indonesia. This region is an archipelagic area and still maintains numerous bioresources; therefore, actinomycete strains may be isolated with interesting abilities and significant metabolites may be discovered from their broth extracts.

During our screening studies on new secondary metabolites produced by actinomycetes, the terrestrial *Streptomyces* sp. TPU1401A collected in Indonesia was found to produce a benzyl-α-pyrone derivative (**1**) and three known isoflavonoid derivatives, daidzein-7-*O*-α-L-rhamnoside (**2**) [5, 6], genistein-7-*O*-α-L-rhamnoside (**3**) [5, 6], and daidzein (**4**) [7–9] (Figures 1 and 2A). Although compound **1** has been reported as a synthetic product [10–12], this is the first time to isolate **1** from microbial broths, and, thus, **1** was named streptpyrone A. The present results demonstrated that the authentic isoflavones, **4** and genistein (**5**), were transformed into their 7-*O*-glycosides in an incubation with strain TPU1401A. The glycosides **2** and **3** promoted the growth of strain TPU1401A.

In the present study, we describe the fermentation, isolation, and structural elucidation of **1**, the microbial transformation of isoflavones by strain TPU1401A, and the growth promotion of strain TPU1401A by isoflavonoids **2**–**5**.

2. Results and discussion

2.1. Identification of the producing actinomycete

Strain TPU1401A was obtained using a humic acid-vitamin (HV) agar plate [13] from a soil sample attached to a leguminous plant root collected in Indonesian, and its 16S rRNA partial sequences (730 nucleotides) showed 99.7% similarity to *Streptomyces olivoreticuli* subsp. *cellulophilum* NRRL B-24299, *Streptomyces laurentii LMG* 19959, and *S. laurentii* NBRC 15442. The 16S rRNA partial sequences of strain TPU1401A have been deposited in DDBJ under the accession number LC438824.

2.2. Culture and isolation of compounds 1–4

The production of compounds **1**–**4** by *Streptomyces* sp. TPU1401A was observed in broth extract cultured in medium with soybean flour (Figure 2A). The culture broth from mass fermentation (7 days) was subjected to solvent extraction and an ODS column followed by preparative HPLC (ODS) to purify compounds **1** (55 mg), **2** (7.0 mg), **3** (7.3 mg), and **4** (7.8 mg).

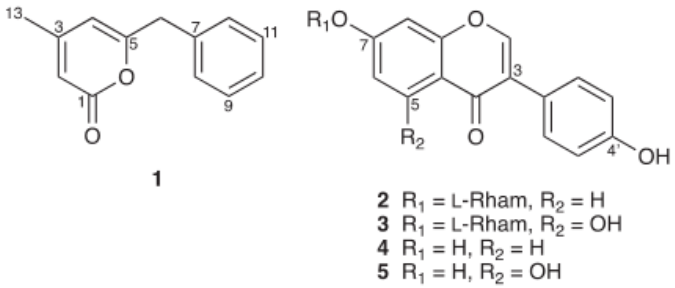

Figure 1. Structures of compounds 1–5.

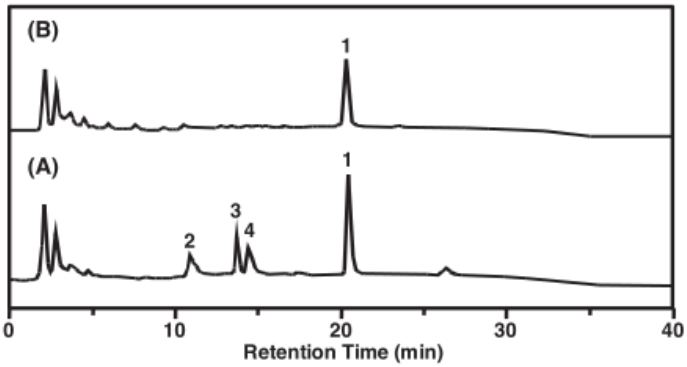

Figure 2. HPLC profiles of broth extracts from the Indonesian actinomycete *Streptomyces* sp. TPU1401A. The strain was cultured in production medium with (A) or without (B) soybean flour under agitation for 7 days.

The structures of **2**–**4** were identified as daidzein-7-*O*-α-L-rhamnoside [5, 6], genistein-7-*O*-α-L-rhamnoside [5, 6], and daidzein [7–9], respectively, by comparing their spectroscopic data with those of the reported values (Figure 1).

2.3. Structure of compound 1

The molecular formula of **1**, $C_{13}H_{12}O_2$, was deduced from HREIMS [*m/z* 200.0841 $(M)^+$, Δ + 0.4 mmu] and NMR data (Table 1). Compound **1** showed UV absorption at 296 nm (log ε 3.8) and an IR band at 1648 cm^{-1}, suggesting the presence of phenyl and carbonyl groups in the molecule. The aromatic proton signals (δ_H 7.22–7.33 m, 5H) in the ^1H NMR spectrum of **1** revealed the presence of a monosubstituted benzene ring, and HMBC correlations from H_2-6 (δ 3.78) to C-7 (137.0) and C-8/C-12 (130.2) and from H-8/H-12 (7.22–7.33) to C-6 (40.5) and C-10 (128.2) established the benzyl moiety (partial structure A) as shown in Figure 3. HMBC correlations from H-2 (δ 5.95) to C-1 (165.2), C-4 (108.1), and C-13 (21.4), from H-4 (6.04) to C-2 (111.2), C-5 (164.8), and C-13, and from H_3-13 (2.09) to C-2, C-3 (159.5), and C-4 indicated the presence of the α-pyrone ring (Figure 3, partial structure B). The partial structures A and B were connected by HMBC correlations from H-4 to C-6 and from H_2-6 to C-4 and C-5 (Figure 3).

Thus, the structure of compound **1** was assigned as shown in Figure 1, which was previously reported as a synthetic compound by several research groups [10–12]. However, this is the first isolation of **1** as a microbial metabolite, and, thus, compound **1** was named streptpyrone A.

Table 1. ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR spectral data for **1** (in CD_3OD).

position	δ_C	δ_H, mult.
1	165.2	
2	112.2	5.95, s
3	159.5	
4	108.1	6.04, s
5	164.8	
6	40.5	3.78, s
7	137.0	
8	130.2	7.22–7.33, m
9	129.7	7.22–7.33, m
10	128.2	7.22–7.33, m
11	129.7	7.22–7.33, m
12	130.2	7.22–7.33, m
13	21.4	2.09, s

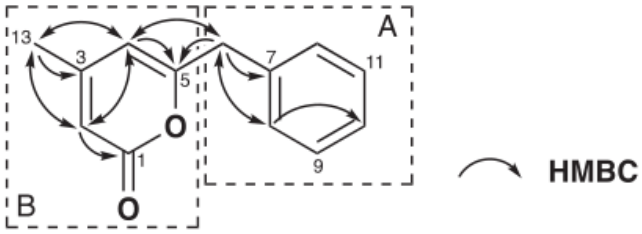

Figure 3. Key HMBC correlations for compound **1**.

Compound **1** was tested for antimicrobial activity against *Mycobacterium smegmatis*, which is used in screening studies on anti-tuberculosis antibiotics instead of *Mycobacterium tuberculosis* due to its non-pathogenic nature [14]. However, compound **1** was not active even at 50 μg/disc. The inhibitory effect of **1** was also examined on protein tyrosine phosphatase (PTP) 1B as a promising molecular target for the treatment of diabetes [15]; however, compound **1** did not inhibit PTP1B activity up to 50 μM. Further studies on the biological activity of **1** are now in progress.

2.4. Biotransformation of isoflavones by strain TPU1401A

As shown in Figure 2B, the isoflavone derivatives **2**–**4** were not observed in the broth extract of strain TPU1401A cultured using production medium without soybean flour, which contains isoflavones, such as daidzein (**4**) and genistein (**5**) [16]. Therefore, strain TPU1401A appears to possess the ability to transform these isoflavones into glycosides. In order to demonstrate this ability, fermentation studies were conducted with authentic **4** and **5** (500 μM in DMSO). The incubation of **4** and **5** with strain TPU1401A resulted in the detection of the respective 7-*O*-glycosides **2** and **3** (Figure 4A), whereas their 5-*O*-glycosides and/or 4′-*O*-glycosides were not observed. These results revealed that strain TPU1401A selectively glycosidated the 7-OH group of compounds **4** and **5**.

Culture broth was then centrifuged to separate the supernatant and bacterial cells in order to confirm whether microbial transformation was an intracellular or extracellular process. The supernatant of the 3-day culture broth of strain TPU1401A was

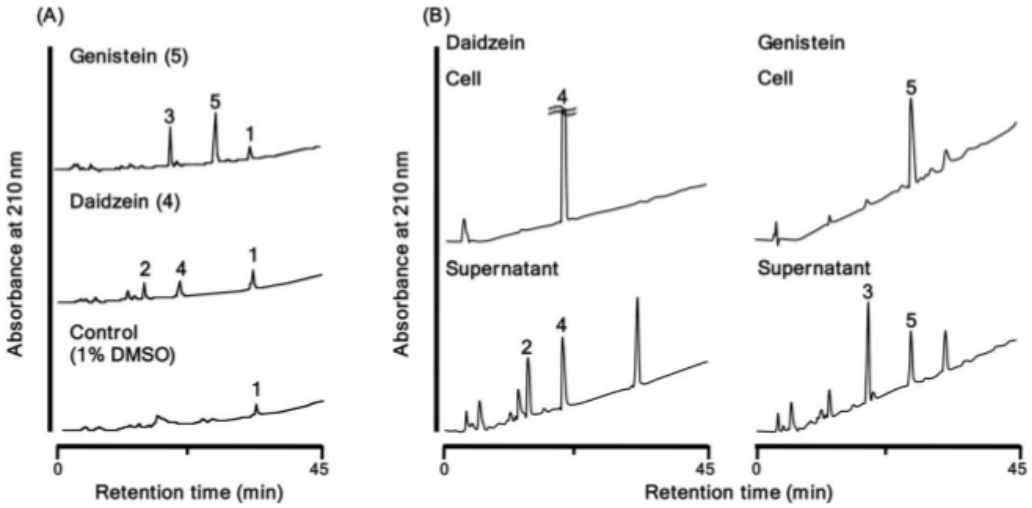

Figure 4. HPLC profiles of biotransformation by strain TPU1401A from **4** and **5** into **2** and **3**, respectively. (A) Strain TPU1401A was cultured with daidzein (**4**) or genistein (**5**) in DMSO (500 μM) using production medium without soybean flour. Each culture broth, after a 7-day inoculation, was extracted with EtOAc and analyzed by HPLC. Control indicates the broth extract fermented in production medium with DMSO (final concentration, 1.0%). (B) Each culture broth incubated with **4** or **5** for 3 days was centrifuged to separate the supernatant and cells, and each extract was analyzed by HPLC.

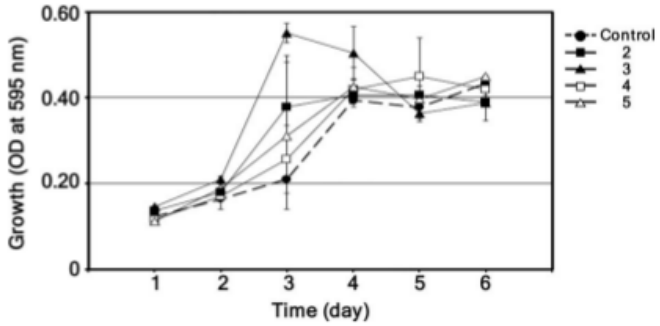

Figure 5. Effects of **2**–**5** on the growth of strain TPU1401A. Strain TPU1401A was cultured in a 96-well plate with daidzein-7-*O*-α-L-rhamnoside (**2**: ■), genistein-7-*O*-α-L-rhamnoside (**3**: ▲), daidzein (**4**: △), or genistein (**5**: △) at 10 μM. Control (●) indicates fermentation with CH_3OH alone (final concentration, 1.0%). The growth of the strain was monitored by measuring OD at 595 nm 1, 2, 3, 4, 5, and 6 days after the inoculation.

extracted with EtOAc and cells with EtOH. As shown in Figure 4B, each extract of the supernatant showed peaks corresponding to the isoflavone glycoside **2**/**4** and aglycone **3**/**5**, while only aglycone (**4** or **5**) was observed in each extract from the cells. Therefore, microbial glycosidation occurred on the cell membrane or glycosides were secreted after their conversion in cells.

To confirm whether strain TPU1401A glycosidated other substrates, we examined resveratrol as one type of polyphenol. However, no glycoside metabolites were detected (supplementary Figure S5). Further investigations on biotransformation with other substrates are warranted because co-culture experiments with strain TPU1401A may be applied to produce new glycoside metabolites.

2.5. Growth promotion activity of isoflavonoids 2–5

Strain TPU1401A was separated from a soil sample attached to the roots of a leguminous plant, and the roots of this plant contained daidzein (**4**) as a phytochemical constituent (data not shown). We investigated the ecological relationship between this plant and strain TPU1401A, and the growth of strain TPU1401A was initially examined with and without these isoflavonoids.

The effects of **2**–**5** (10 μM) on the growth of the strain were tested by measuring optical density (OD) at 595 nm (Figure 5). Strain TPU1401A showed ordinary growth with CH_3OH alone until day 4 after the inoculation and then reached the stationary phase (Control in Figure 5). On the other hand, growth was enhanced by test compounds **2**–**5** (10 μM) (Figure 5). Compounds **2** and **3** promoted growth on day 3 more than **4** and **5**. Genistein-7-*O*-α-L-rhamnoside (**3**) resulted in the greatest amount of growth on day 3, and growth subsequently entered the stationary phase on day 5 (Figure 5).

These results propose a symbiotic relationship between actinomycete strain TPU1401A and the leguminous plant. Further studies are ongoing in order to elucidate their relationship.

3. Experimental

3.1. Isolation and identification of the strain

Actinomycete strain TPU1401A was isolated from a soil sample attached to the root parts of an unidentified leguminous plant collected at Manado in North Sulawesi, Indonesia, in September 2014. The soil sample was suspended in 1 ml of sterilized water containing 1.0% sodium dodecyl sulfate (SDS), and 100 µl of the resulting mixture was spread on a HV agar plate [13]. The actinomycete grown on the plate was isolated and maintained on a PDA plate. The strain was identified based on its 16S rRNA partial sequence, which showed high identity with those of *S. olivoreticuli* subsp. *cellulophilum* NRRL B-24299 (99.7%), *S. laurentii LMG* 19959 (99.7%), and *S. laurentii* NBRC 15442 (99.7%).

3.2. Fermentation

The mycelia of strain TPU1401A were inoculated from the agar plate into a 100-ml Erlenmeyer flask containing 50 ml of seed medium (1.0% glucose, 0.4% yeast extract, 0.4% polypeptone, 0.05%, $MgSO_4 \cdot 7H_2O$, and 0.1%, KH_2PO_4 in H_2O; adjusted to pH 7.0 before sterilization), and the flask was shaken (150 rpm) at 25 °C for 3 days (seed culture). Aliquots (2 ml) of the seed culture were inoculated into 500-ml Erlenmeyer flasks containing 200 ml of production medium (1.0% glucose, 2.0% starch, 1.5% soybean flour, and 0.3% $CaCO_3$ in H_2O; adjusted to pH 7.0 before sterilization). The production culture was performed for 7 days under agitation on a rotary shaker (150 rpm).

3.3. Isolation of Compounds 1–4

Acetone (2.4 L) was added to the culture broth (2.4 L) and filtered. The filtrate was evaporated to remove acetone and extracted three times with EtOAc (2.4 L) to yield the crude extract (0.5 g). The extract was separated into six fractions by ODS column chromatography, and the 70% CH_3OH eluate (93 mg) was further purified to give **1** (55 mg, t_R=24.0 min) by preparative HPLC [column; PEGASIL ODS SP100 (Senshu Scientific Co., Ltd., Tokyo, Japan), 10 × 250 mm; mobile phase, 35% CH_3CN; detection, UV at 210 nm; flow rate, 2.0 ml/min]. Repeated HPLC separation (column; PEGASIL ODS SP100, 10 × 250 mm; mobile phase, 15% CH_3CN; detection, UV at 210 nm; flow rate, 2.0 ml/min) of the 50% CH_3OH eluate (89 mg) afforded compounds **2** (7.0 mg, t_R=14.6 min), **3** (7.3 mg, t_R=30.2 min), and **4** (7.8 mg, t_R=36.9 min).

3.3.1. Streptpyrone A (1)

Pale brown oil; UV (CH_3OH) λ_{max} nm (log ε) 296 (3.8); IR (KBr) ν_{max} 1648, 1407, 1224, 1090 cm^{-1}; ^1H and ^{13}C NMR spectral data (CD_3OD), see Table 1; EIMS: *m/z* 200 $[M]^+$; HREIMS: *m/z* 200.0841 $[M]^+$ (calcd for $C_{13}H_{12}O_2$, 200.0837).

3.4. Biotransformation of isoflavones by strain TPU1401A

The seed culture of strain TPU1401A was inoculated into each production medium without soybean flour containing 500 μM of authentic **4** or **5**. Each broth was then fermented under agitation (150 rpm) at 25 °C for 7 days. Each culture broth (2 ml) on day 7 was extracted with EtOAc. Each culture broth (2 ml) on day 3 was centrifuged at 3000 rpm for 10 min to separate the culture supernatant and bacterial cells, which were extracted with EtOAc and EtOH, respectively. Each extract was dissolved in CH_3OH and analyzed by HPLC (L-6200 system) under the following conditions: column, Pegasil ODS SP100, 4.6 × 250 mm; mobile phase, 20–60% CH_3CN (40 min) and 100% CH_3CN (5 min); detection, UV at 210 nm; flow rate, 0.8 ml/min.

3.5. Growth assay

Strain TPU1401A was precultured in seed medium under agitation at 25 °C for 3 days and diluted 10 times with seed medium. Waksman medium (94 μl) was added to each well of a 96-well plate, and the diluted seed culture (5 μl) was then inoculated. Each test compound (in 1 μl of CH_3OH) was then added to make the final concentration of 10 μM, and the plate was incubated at 25 °C. Optical density (OD) at 595 nm was measured using an MTP-500 microplate reader (Corona Electric Co., Ltd., Ibaraki, Japan) 1, 2, 3, 4, 5, and 6 days after the inoculation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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