

Fractionation and structural identification of antibiotic activity substances from *Streptomyces herbaricolor* HNS2-2

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Received 4 March 2012; revised 18 April 2012; accepted 12 May 2012

ABSTRACT

Four groups of antibiotic activity compounds were separated and purified from the ethyl acetate fraction of a broth culture of *Streptomyces herbaricolor* HNS2-2. Trace analyses were performed and anti-microbial activities were determined. The antibiotic activity compounds were identified as quercetin dehydrate ①, p-hydroxyphenyl ②, 4'-hydroxyflavanone ③, and 3-hydroxyflavone ④ based on spectroscopic data. Compounds 1 - 4 showed significant antimicrobial activities against *Staphylococcus aureus* and the tobacco mosaic virus *in vitro*, suggesting their potential agricultural and medical applications.

Keywords: *Streptomyces herbaricolor* HNS2-2; Quercetin Dehydrate; p-Hydroxyphenyl; 4-Hydroxyflavanone; 3-Hydroxyflavone; Bioactivity

1. INTRODUCTION

Filamentous actinomycetes, especially the members of the genus *Streptomyces*, are very important sources of different natural metabolites. These organisms possess a broad range of biological activities, such as antibiotic (e.g. streptomycin and oxytetracycline), antitumor (e.g., bleomycin and mitomycin), antifungal (e.g., nystatin and antimycin A), anti-tubercular (aprazamycin B and thio-lactomycin), as well as pesticide (e.g., jinggangmycin and liuyangmycin) [1-3]. *Streptomyces* can also produce immunomodulators (e.g., tacrolimus and pimecrolimus), organic acids, amino acids, vitamins, steroids, and enzymes, as well as inhibit enzymatic activities [4,5]. Some can engage in different types of biological activities at the same time, or in special biological activities by metabolic regulation [6-8]. Previous studies on the biologi-

cal activities of *Streptomyces* are diverse. However, they are still important potential resources for discovering novel biologically active sources.

In the present study, the ethyl acetate extract of the broth culture of *Streptomyces herbaricolor* HNS2-2 showed significant antimicrobial activity against *Staphylococcus aureus* and the tobacco mosaic virus (TMV) *in vitro* [9,10]. The isolation, purification, and structural identification of the antibiotic activity compounds in the extract were reported.

2. MATERIAL AND METHODS

2.1. Microorganisms

S. herbaricolor HNS2-2 was isolated in our laboratory from a soil sample obtained from the Shenlongjia Nature Reserve in Sichuan province, China. The strain was maintained in 40% glycerol microtubes at -20°C . The identity of *S. herbaricolor* was confirmed based on the morphology results of scanning electronic microscopy, culture characteristics, chemotaxonomic tests, and 16S rRNA analysis [9]. The other microorganisms used in the present study were *S. aureus* and TMV. *S. aureus* was maintained on nutrient agar slants at 4°C , and a crude extract of TMV was prepared and stored in our laboratory.

2.2. Culture Conditions for *S. herbaricolor* HNS2-2 and Preparation of Crude Antibiotic Activity Substances

The culture medium for *S. herbaricolor* HNS2-2 was the same as that reported in literature [9]. First, the inoculum prepared by 6d-old *S. herbaricolor* HNS2-2 on a Gause No. 1 agar slant was inoculated in 300 mL Erlenmeyer flasks, which contained 30 mL of seed medium. The flasks were placed in a rotary shaker at 28°C and incubated at 180 rpm for 18 h. About 3 mL of seed-incubated liquid (inoculation number = 6%) was trans-

ferred into a 500 mL Erlenmeyer flask containing 100 mL of fermented liquid medium (medium volume = 20%). After 7 d of growth at 28°C on a rotary shaker at 220 rpm, the crude fermentation broth was acidized with HCl to pH 3.5 at 55°C for 2 h, and thoroughly filtered to separate the broth filtrate and mycelial cake. The broth supernatant was concentrated five times in an evapo-concentration decompressor at 55°C. The extract containing crude antibiotic activity substances was obtained by extraction and evapo-concentration.

2.3. Isolation of Antibiotic Activity Substances

The antibiotic activity substance was isolated over a silica gel column (column $\Phi \times l$: 9 cm \times 80 cm; silica gel 100 - 200 mesh) eluted with petroleum ether-ethyl acetate (20:1, 10:1, 5:1, 2:1, 1:1, and 0:1; 500 mL each). There were 76 fractions obtained, and these fractions were combined into 12 fractions by a TLC plate using n-butyl alcohol-acetic acid-water (7:3:1.5) as developing solvents and collect similar components. Fractions 31 - 47 and 48 - 57 were both antibiotic activity by trace analyses and determination with cup-plate method and inhibition zone determination.

Fraction 31 - 47 was placed in a silica gel column (column $\Phi \times l$: 2 cm \times 45 cm; silica gel 200 - 300 mesh) eluted with petroleum ether-ethyl acetate (85:15; 25 mL each) to afford pure compound 1 [fractions 37 - 41 and 23 - 26]. Fractions 37 - 41 and 79 - 86 were further separated in a silica gel column (column $\Phi \times l$: 2 cm \times 60 cm; silica gel 200 - 300 mesh) eluted with petroleum ether-ethyl acetate (80:20; 20 mL each). TLC was performed on the eluent, and the products were collected to give pure compound 2.

Fractions 48 - 57 were recrystallized in acetone and separated by Sephadex LH-20 eluted with acetone. The products were collected to obtain pure compound 3.

The recrystallized residue was applied to a silica gel column (column $\Phi \times l$: 2 cm \times 50 cm; silica gel 100 - 200 mesh) using petroleum ether:ethyl acetate (85:15; 20 mL each) as the mobile phase. TLC was performed on fractions (48 - 57)-(3 - 6)-1-(4 - 7). The products were collected to obtained pure compound 4.

The chemical structures of the pure compounds were confirmed via ^1H NMR and ^{13}C NMR. Tetramethylsilane (TMS) was used as the internal standard; CDCl_3 and acetone- d_6 were the solvents. The NMR spectra were compared with relevant references, and the identities of the compounds were further confirmed.

2.4. Antimicrobial Activity Test

The method of trace analyses and determination was that compounds from the isolation of antibiotic activity substances were tested for *in vitro* activity against *S.*

aureus by the cup-plate method and inhibition zone determination, keep the positive compound, give up the negative compound, step by step. A 75% alcohol solution was used as a positive control, and aseptic normal saline was used as a negative control.

The antimicrobial activities of the pure compounds were determined as follows. The compounds were initially dissolved in acetone at a concentration of 1% (diluted 100 times) and mixed with equal volumes of TMV crude extract for 30 min. *Stramonium* was then introduced into the mixer via sap inoculation. The inhibiting effects of the compounds on *Stramonium* were recorded and compared. The compounds were also tested for *in vitro* activity against *S. aureus* by tube broth culture and spread plate colony calculation. Aseptic normal saline and acetone were used as negative controls.

3. RESULTS

3.1. Fractionation and Structural Identification of Antibiotic Activity Substances

Based on ^1H NMR and ^{13}C NMR data, the following structural information were obtained.

3.1.1. Pure Compound 1

Yellow powder, ^1H NMR (500 MHz, $(\text{CD}_3)_2\text{CO}$): δ_{H} 6.27 (1 H, d, $J = 1.4$ Hz, 6 - 8), 6.52 (1 H, s, H-6), 6.99 (1 H, d, $J = 8.5$ Hz, H-5'), 7.70 (1 H, dd, $J = 8.5$ Hz, 2.2 Hz, H-6'), 7.82 (1 H, d, $J = 2.1$ Hz, H-2'), 12.18 (1 H, s, HO-3); ^{13}C NMR [125 MHz, $(\text{CD}_3)_2\text{CO}$): δ_{C} 94.4 (C-8), 99.1 (C-6), 104.1 (C-10), 115.7 (C-2'), 116.2 (C-5'), 121.5 (C-6'), 123.7 (C-1'), 145.8 (C-4'), 147.0 (C-3'), 148.3 (C-2), 157.8(C-9), 162.3 (C-5), 165.0 (C-7), 176.5 (C-4).

The ^1H NMR and ^{13}C NMR data as well as the chemical structure were identical with those of quercetin dehydrate [11,12]. Hence, the pure compound was considered to be a monomer of quercetin dehydrate.

3.1.2. Pure Compound 2

White crystals, ^1H NMR (400 MHz, $(\text{CD}_3)_2\text{CO}$): δ_{H} 7.02 (2 H, d, $J = 8.5$ Hz, H-2, 6), 6.73(2 H, d, $J = 8.5$ Hz, H-3,5), 2.85(2 H, t, $J = 7.6$ Hz, H-7), 2.57 (2 H, t, $J = 7.8$ Hz, H-8), ^{13}C NMR [100 MHz, $(\text{CD}_3)_2\text{CO}$): δ_{C} 133.0 (C-1), 130.2 (C-2,6), 116.2 (C-3,5), 156.7 (C-4), 37.3 (C-7), 31.3 (C-8), 177.0 (C-9).

The ^1H NMR and ^{13}C NMR data as well as the chemical structure of the compound were identical with those of p-hydroxyphenyl [13], the chemical structure of which is given in **Figure 1(b)**.

3.1.3. Pure Compound 3

White powder, ^1H NMR [500MHz, $(\text{CD}_3)_2\text{CO}$): δ_{H}

2.76 (1 H, dd, $J = 16.7$ Hz, 2.8 Hz, 3-H), 3.16 (1 H, dd, $J = 16.7$ Hz, 13.1 Hz, H-3), 5.50 (1 H, dd, $J = 13.1$ Hz, 2.7 Hz, H-2), 6.90 (2 H, d, $J = 8.5$ Hz, H-3', H-5'), 7.03 - 7.08 (2 H, m, H-6, H-8), 7.43 (2 H, d, $J = 8.5$ Hz, H-2', H-6'), 7.53 ~ 7.57 (1 H, m, H-7), 7.83 (1 H, dd, $J = 7.8$ Hz, 1.6 Hz, H-5), 8.51 (1 H, s, -OH); ^{13}C NMR (125 MHz, CDCl_3): δ_c 45.8 (C-3), 81.3 (C-2), 117.2 (C-3', 5'), 119.9 (C-8), 122.9 (C-10), 123.0 (C-6), 128.3 (C-5), 130.0 (C-2', 6'), 132.1 (C-1'), 137.7 (C-7), 159.6 (C-9), 163.6 (C-4'), 193.2 (C-4). The spectroscopic data and chemical structure of the compound were identical with those of 4-hydroxyflavanone [14], the chemical structure of which is given in **Figure 1(c)**.

3.1.4. Pure Compound 4

Light-yellow powder, ^1H NMR (500 MHz, CDCl_3): δ_H 7.16 (1 H, s, OH), 7.40 ~ 7.43 (1 H, m, H-4'), 7.46 ~ 7.49 (1 H, m, H-8), 7.53 ~ 7.56 (2 H, t, $J = 7.2$ Hz, H-3', H-5'), 7.58 (1 H, d, $J = 8.4$ Hz, H-6), 7.69 ~ 7.72 (1 H, m, H-7), 8.25 ~ 8.28 (3 H, m, H-2', H-6', H-5); ^{13}C NMR (125 MHz, CDCl_3): δ_c 118.3 (C-8), 120.6 (C-10), 124.5

(C-6), 125.4 (C-4'), 127.7 (C-2', 6'), 128.6 (C-3', 5'), 130.2 (C-5), 131.0 (C-1'), 133.6 (C-7), 138.5 (C-3), 144.9 (C-9), 155.4 (C-2), 173.5 (C-4). Based on the spectroscopic data, the compound was identified [15,16] as 3-hydroxyflavone.

3.2. Antimicrobial Activity of the Pure Compounds against *S. aureus* and TMV

Table 1 shows that the inhibition of pure compound 1 was stronger than those of the other pure compounds, the inhibition rate of lesion was 89.12%. Nevertheless, pure compound 2 exhibited destructive effects on *Stramonium*, resulting in death.

Figure 2 shows that the compounds exhibited anti-*S. aureus* activity. The number of *S. aureus* in a petri dish was obviously lower in the test group (left) than the control group (upper right was aseptic normal saline negative controls and lower right was acetone negative controls). The number of *S. aureus* in a petri dish from pure compound 1 to 4 were (12.5 ± 0.50) CFU/petri dish, $(3.00 \pm$

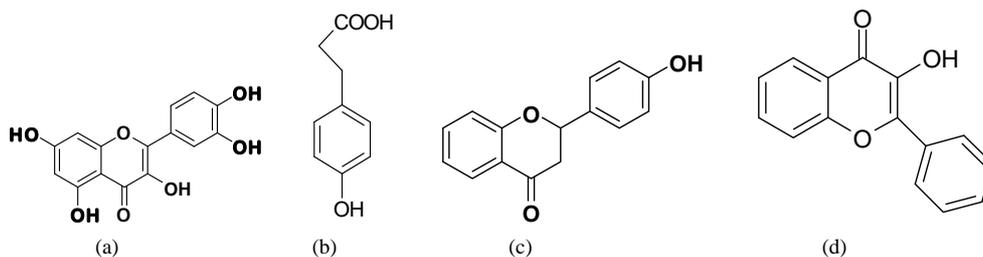


Figure 1. Chemical structures of the pure compounds. (a): Pure compound 1, quercetin dehydrate; (b): Pure compound 2, p-hydroxyphenyl; (c): Pure compound 3, 3,4'-hydroxyflavanone; (d): Pure compound 4, 3-hydroxyflavone.



Figure 2. Antimicrobial activity of the pure compounds against *S. aureus*. (a) Pure compound 1 and control group; (b) Pure compound 2 and control group; (c) Pure compound 3 and control group; (d) Pure compound 4 and control group.

Table 1. Antimicrobial activity of the pure compounds against TMV.

Pure compound	Lesion number	Inhibition rate (%)
1	37	89.12 ± 0.44 a**
2	*	*
3	155	53.03 ± 0.67 c**
4	161	51.20 ± 0.40 d**
control group	329	

*The *Stramonium* died after sap inoculation; **Different letters showed significant difference at 0.05 level.

0.34) CFU/petri dish, (24.50 ± 0.0.98) CFU/petri dish and (235.00 ± 1.27) CFU/petri dish respectively. Pure compound 2 exhibited the strongest such activity. The antimicrobial activities of pure compounds 1, 3, and 4 are also shown.

4. DISCUSSION

The current study showed that *S. herbaricolor* could produce the flavonoids quercetin dehydrate, p-hydroxyphenyl, 4'-hydroxyflavanone, and 3-hydroxyflavone, and the compounds have significant bioactivities. These report has never been previously described.

More than 4000 varieties of flavonoids have been identified in nature, especially in plants (e.g., fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine) [17]. Flavonoids have a wide range of functions. They play important roles as signal molecules for the induction of nod genes [18-20], serve as precursors for the production of major phytoalexins during plant-microbe interactions [21,22], and inhibit pathogen attack [23,24]. However, flavonoids, being naturally produced by plants, are very complex. Some are also produced in very low quantities that cannot meet human requirements. Obtaining sufficient amounts of natural flavonoids will lead to the depletion of natural resources.

Actinomyces species have the advantages of rapid growth, precisely controllable growth condition, relatively easy to be genetically manipulated, abundant supply of substrates, and applicable in the mature liquid submerged fermentation technique, etc. Flavonoid production in the microorganism starter *S. herbaricolor* is feasible and can overcome the above mentioned problems. Further research on the determination, anabolism, and fermentation of *S. herbaricolor* antibiotic activity flavonoids will benefit their industry-scale production and application.

5. ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (No. 30771437), the Natural Science Foundation of Hunan Science and Technology Committee (No. 08JJ6017). Professor Bida Gao is project leader.

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