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Isolation of three new naturally occurring compounds from the culture of *Micromonospora* sp. P1068

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Bioassay guided isolation of an antibacterial extract prepared from the fermentation broth of a *Micromonospora* sp. P1068 led to the isolation of eight compounds identified as (3*R*) 3,4′,7-trihydroxy-isoflavanone (**1**), 3-hydroxydehydrodaidzein, daidzein (**2**), 3-methyl-1H-indole-2-carboxylic acid (**3**), 1H-indole-3-carboxaldehyde (**4**), 3-(p-hydroxyphenyl)-N-methyl-propionamide, N-methylphloretamide (**5**), phenyl acetic acid (**6**), 2-hydroxy phenyl acetic acid (**7**) and 4-hydroxy-5-methoxy-benzoic acid (**8**). Compounds **1** and **5** were found to be novel chemical entities while **3** was isolated from a natural source for the first time. All compounds were evaluated for their antimicrobial activities against a panel of clinically significant microorganisms. Compound **4** was active against *Staphylococcus aureus* (MIC, 32 μg/ml), *Enterococcus faecium* (MIC, 32 μg/ml) and *Escherichia coli* (MIC, 64 μg/ml).

Keywords: *Micromonospora*; Antibacterial activity; Flavonoids; Indole alkaloids; 3-hydroxy-dehydrodaidzein; *N*-methylphloretamide

1. Introduction

Infectious diseases remain the leading cause of death worldwide and infections due to antibiotic-resistant microorganisms have become more widespread in recent years [1]. A large proportion of the bacteria causing hospital-acquired infections are resistant to at least one of the antibiotics used against them and some have retained susceptibility to only one antibiotic. Such a scenario presents a crisis in the treatment and management of infectious diseases, and a clear danger to the future of public health. To stay

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ahead of the multi-resistant bacteria, new antibiotics with novel modes-of-actions must be continually discovered and developed. With proven track-records of the past and structural diversity found in the natural products, we have continued to screen microbial extracts for interesting biological activities [2]. As part of the International Cooperative Biodiversity Group (ICBG) program ''Bioactive Agents from Dryland Biodiversity of Latin America'' [3], several actinomycetes isolated from soil samples collected in La Parva, Chile, were screened for their antimicrobial activity on a panel of clinically significant microorganisms. In this preliminary screening, an organic extract prepared from the fermentation broth of *Micromonospora* sp. P1068 was found active against *Staphylococcus aureus* and the vancomycin-resistant strain of *Enterococcus faecium*. In this report, we describe the isolation and characterization of three new naturally occurring compounds from the fermentation broth of *Micromonospora* P1068 as well as their antimicrobial activity.

2. Results and discussion

Bioassay guided isolation of an organic extract prepared from a 10 L fermentation of the selected microorganism, led to the isolation of eight compounds (**1**–**8**) whose structures are shown in figure 1. Compounds **1**, **3** and **5** represent new naturally occurring metabolites while compounds **2**, **4**, **6**–**8** were found to be known.

(3R)-3,4′,7-trihydroxy-isoflavanone (**1**), 3-hydroxydehydrodaidzein was isolated as a yellow solid. Its molecular formula was determined as $C_{15}H_{12}O_5$ by HRFABMS. The specific UV spectrum of **1** showed maxima at 206, 225 *sh*, 272 and 316 nm, while its IR spectrum exhibited absorption bands due to the presence of hydroxyl groups

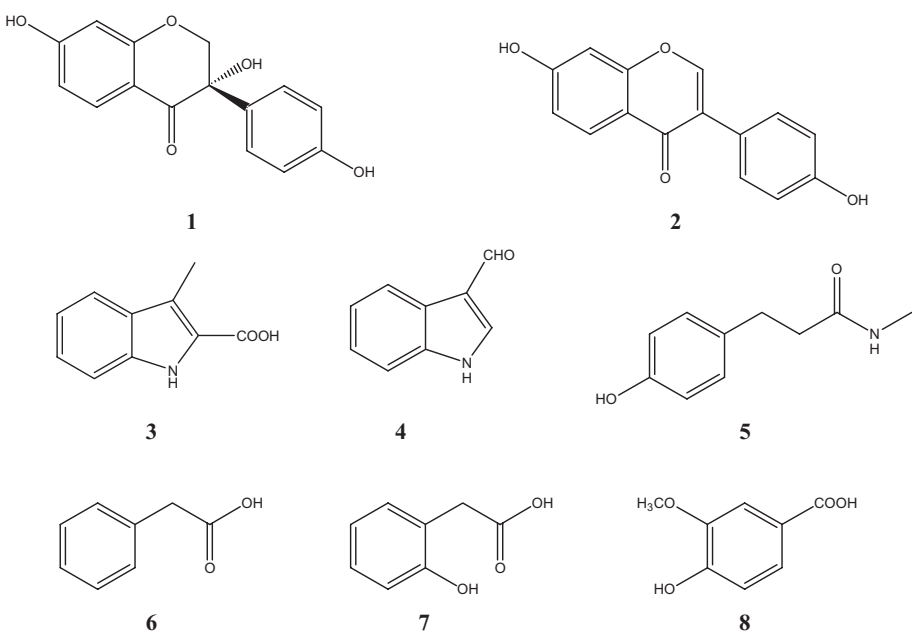
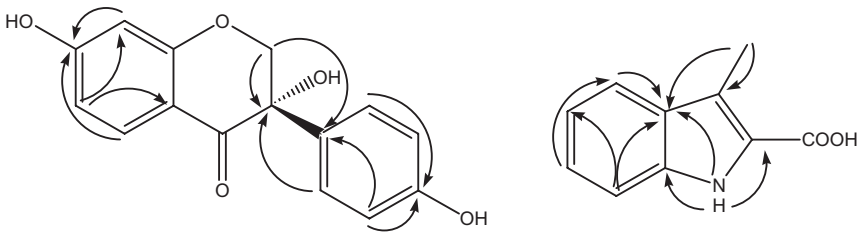


Figure 1. Structure of the isolated compounds from *Micromonospora* P1068.



1 **3**

Figure 2. HMBC correlations of compounds **1** and **3**.

(3217 cm^{-1}), a ketone group (1677 cm^{-1}) and aromatic double bond (1458 cm^{-1}). The ^{13}C spectrum of **1** exhibited 13 signals; 10 due aromatic carbons, two of which were magnetically equivalent (C-2′, C-6′ and C-3′, C-5′), one ketone group (δ 198.7, C-4), one oxymethylene carbon (δ 67.7, C-2) and one oxygenated quaternary carbon (δ 94.8, C-3). On the other hand, the ^1H NMR spectrum of **1** displayed signals for five aromatic protons at δ 7.74 (d, $J = 8.2$ Hz), δ 6.80 (d, $J = 8.2$ Hz), δ 6.93 (s) assigned to H-5, H-6 and H-8, respectively and two magnetically equivalent protons at δ 7.90 (2H, $J = 7.9$ Hz, H-2′, H-6′) and δ 7.20 (2H, $J = 7.9$ Hz, H-3′, H-5′), indicating 4′ substitution of ring B. In the proton spectrum, the presence of an AX system at δ 4.77 and δ 4.48 (d, $J = 12.0$ Hz) was also evident and was assigned to the methylene protons at C-2. Full assignments of the proton and carbon signals and the position of the three hydroxyl groups of **1** were established by the analysis of the HSQC and HMBC spectra (figure 2).

In order to establish the absolute configuration at C-3, The CD spectrum of compound **1** was recorded. The positive Cotton Effect at 333 nm due to the $n \rightarrow \pi^*$ transition for the carbonyl group accounted for the (3*R*) absolute configuration on the basis of the octant rule modified for the cyclic aryl ketones [4,5].

It has been reported that isoflavonoids isolated from microorganisms are more likely produced by microbial biotransformation of glycosidic plant isoflavonoids present in media components such as soybean flour or malt extract [6]. However, the possibility that these metabolites are also being synthesized by bacteria cannot be completely discarded. Recent reports on the discovery of the type III polyketide synthase (PKSs) superfamily of condensing enzymes in bacteria, homologues to the chalcone synthases (CHS, key enzymes involved in the biosynthesis of flavonoids), point to the possibility of such compounds being produced in bacteria. Some of these enzymes were found to be involved in the biosynthesis of small aromatic metabolites, while a number of other CHS homologues identified so far are still of unknown functions. In addition, increasing reports on the identification of new CHS-homologues in bacteria suggest that this new polyketide biosynthetic pathway may indeed be widespread in bacteria [7,8].

The molecular formula of compound **3** was determined as $C_{10}H_9NO_2$ using HRFABMS. Its IR spectrum showed absorption bands due to hydroxyl and amino groups (3304–3048, 1567, 1243 cm^{-1}), an α,β unsaturated carboxylic group (1671 cm^{-1}) and aromatic double bonds (1452 cm^{-1}). The ^{13}C spectrum of **3** exhibited 10 signals, 8 of which corresponded to aromatic carbons, one carboxylic carbon (δ 167.4) and one methyl group (δ 10.0). In addition, its ^1H NMR spectrum showed four aromatic protons (δ 7.64, 7.11, 7.33, 7.30), one methyl group (δ 2.61) and one proton not assignable to any carbon (δ 8.67) and therefore attached to a nitrogen atom. The

described data led to the identification of **3** as an indole alkaloid [9,10]. Full assignments of the proton and carbon signals and position of the carboxylic and methyl groups of **3** were established through analysis of the HSQC and HMBC spectra (figure 2). Additional evidences that allowed such arrangement were the observed chemical shifts values of C-2 and C-3. The decreased shielding at C-3 ($\Delta\delta$ 8.8 ppm) and increased shielding at C-2 ($\Delta\delta$ −2.2 ppm), when compared with the parent indole are indicative of methyl substitution at C-3. Substitution at C-2 with the carbonyl group is also compatible with decreased shielding observed at both C-2 ($\sim\Delta\delta$ 2 ppm) and C-3 ($\sim\Delta\delta$ 5 ppm) [10]. Compound **3** had previously been synthetically prepared [11,12,13] and to our knowledge, this is the first report of its isolation from a natural source.

The molecular formula of compound **5**, isolated as a pale yellow solid, was determined as $C_{10}H_{13}NO_2$ by HRFABMS. Its IR spectrum showed absorption bands at 3252, 1724, and 1456 cm^{-1}due to hydroxyl, carboxylic and aromatic groups, respectively. The ^{13}C NMR of **5** displayed 8 signals: two magnetically equivalent methines (δ 131.6 and δ 117.1), one oxymethine carbon (δ 157.8), two aliphatic carbons (δ 43.3 and δ 36.6), one carbonyl (δ 174.1) and one methyl (δ 23.4) groups. The ^1H NMR spectrum of **5** consisted of two magnetically equivalent protons (δ 7.06, δ 6.74, d, $J=$ 8.39 Hz, 2H each), two aliphatic protons (δ 3.38, δ 2.72, t, $J=7.25$ Hz, 2H each) and methyl protons (1.94, s). The chemical shifts of the methyl protons and the carbon signal indicated that this group was attached to the nitrogen. Further analysis of the HMBC spectrum of **5** showed a cross peak between the methyl protons and the carbonyl carbon. In addition, HMBC correlations between H_2-2 and the carbonyl group, between H_2-3 and H-1′ as well as between H-2′ and H_2-3 were observed. The data described above supported the structure of **5** as 3-(p-Hydroxyphenyl)-*N*-methylpropionamide (*N*-methylphloretamide).

Compounds **2**, **4**, **6**–**8** were characterized as daidzein [14], 1H-indole-3-carboxaldehyde [15], phenyl acetic acid [16], 2-hydroxy phenyl acetic acid [17] and 4-hydroxy-5-methoxy-benzoic acid [17], respectively by spectroscopic and spectrometric means and comparison with data reported in the literature. These data are resumed in the supporting information section.

Compounds **1**–**8** were evaluated for antimicrobial activity against Gram positive and Gram negative microorganisms. The results indicated that only compound **4** showed moderate activity against *S. aureus* (MIC, 32 μg/ml), *E. faecium* (MIC, 32 μg/ml) and *E. coli* (MIC, 64 μg/ml). Compounds **1**–**3** and **5**–**8** were found inactive at the concentration of 128 μg/ml.

3. Experimental

3.1. *General*

NMR spectra were recorded on a Bruker DRX 500 in pyridine-d_5 (compounds **1**, **2** and **4**), chloroform-*d* (compounds **3** and **6**), or methanol-d_4 (**4**, **5** and **7**) at 293 K. Chemical shifts were expressed in ppm (δ) using partially deuterated solvent chemical shifts as reference. IR spectra were obtained on a Thermo Nicolet Avatar 360 FT-IR as a film on a diamond cell. HR-FABMS spectra were recorded using a JEOL HX110 spectrometer with a resolution of 10,000, using a mixed matrix consisting of

glycerol, thioglycerol and m-NBA. Optical rotations were performed on a JASCO P1020 polarimeter. Circular dichroism and UV spectra were recorded on a Beckman DU-600 spectrophotometer. Melting points were measured in a Fisher–Johns apparatus and are uncorrected. Fractionation of the crude extract was carried out on a Sephadex LH-20 LPLC system composed of a Büchi 688 pump and a 47.5×3.5 cm column. For fraction purification, an Analtech centrifugal TLC system composed of RHSY solvent pump and 8 mm rotors was used. Final compound isolation and purification was performed on a Varian Star semiprep HPLC system equipped with a model 230 pump, and a model 310 variable wavelength detector. The column employed for HPLC was ReliaSil ODS-2 (10 μm, 10×250 mm, Column Engineering). TLCs were sprayed with 0.5% anisaldehyde in methanol and heated until colored spots appeared.

3.2. *Organism, media and culture conditions*

Culture P1068 was isolated from a soil sample collected by G. Montenegro at 3080 m in La Parva, Region Metropolitana, 85 km east from Santiago, Chile, in December, 2000. Intellectual Property Rights Agreements for microbe collections and collaborative research have been fully executed between the University of Arizona, the Pontificia Universidad Catolica de Chile and Wyeth Research. On an agar medium containing starch and dextrose as carbon sources, the mycelia of the culture P1068 were light orange to orange in color. This culture did not produce any aerial mycelia. A dark olive to black spore layer was formed within 7 to 14 days and became slimy with aging. Spores at maturity were spherical, 1.0–1.5 μm in diameter. Sporophores were mostly solitary, but occasionally occurred in small clusters on the same hyphae. Spores were never observed in chains. Based on these morphological characteristics, culture P1068 was designated to the genus *Micromonospora* [18]. The genus of this microorganism was confirmed by analysis of 16S RNAr analysis. Further tests are being done to determine the species for this culture.

Small scale fermentations were performed in four different liquid media (50 ml per 250 ml Erlenmeyer flask) using 2% seed inoculum and incubation at 28°C and 200 RPM for 7 days. Scale-up fermentation was performed in a 14 L fermentor in a medium composed by soy flour (12.5 g/L), NZ-Amine A (2.5 g/L), dextrose (12.5 g/L), calcium carbonate (1 g/L), ammonium chloride (1.5 g/L), agar (0.4 g/L) and distilled water (pH 6.8) at 28°C for 7 days.

3.3. *Extraction and isolation*

The cell package and broth were separated by centrifugation from the whole fermentation and extracted with butanol and methanol respectively. Both butanolic and methanolic extracts were found active against Gram positive bacteria and then combined to give 2.75 g of an active extract. The resulting organic extract was fractionated by gel filtration on a Sephadex LH20 column eluting with methanol to produce eleven primary factions. Antimicrobial evaluation of the column fractions allowed the identification of fractions F002–F006 as active. Fraction F002 was subjected to purification using centrifugal TLC eluting with CH_2Cl_2 : MeOH (98 : 2, 95 : 5 and 9 : 1). Further RPHPLC of the purified fraction eluting with a gradient of 32% CH_3CN in 0.15% trifluoroacetic acid in water (aq.TFA) to 50% CH_3CN/aq.TFA in 25 min, at a flow rate of 5.2 ml/min and detection at $\lambda = 220$ nm, led to the isolation of compounds **3**

and **7**. Under these chromatographic conditions **3** and **7** showed retention times (R_t) of 22.0 and 13.1 min, respectively. On the other hand, compounds **1** and **2** were isolated from fraction F004 using RPHPLC (20% MeOH/aq.TFA to 55% MeOH/aq.TFA in 15 min, 5.2 ml/min, $\lambda = 220$ nm). Compounds **1** and **2** showed R_t of 8.1 and 11.9 min, respectively. Finally, RPHPLC of fraction F003 (14%CH_3CN/0.15% aq.TFA to 47%CH_3CN/0.15% aq.TFA in 20 min, 5.2 ml/min $\lambda = 220$ nm) yielded compounds **4** (14.9 min), **6** (12.5 min), **7** (10.3 min) and **8** (9.5 min).

3.3.1. (3R)-3,4′,7-trihydroxyisoflavanone (1). Yellow solid. mp 185–186. $[\alpha]_{25}^{D} + 73.9^{\circ}$ ($c = 0.5$, MeOH); UV λ_{max} (log ε) 206 (4.05), 225 *sh* (3.84), 272 (3.76) and 316 (3.57) nm; $IR_{\nu max}$ (cm^{-1}) 3217, 1677, 1605, 1514, 1458, 1303, 1238, 1202, 631, 535; HRFABMS m/z 273.0765 ($[M+H]^+$) monoisotopic calcd for $C_{15}H_{13}O_5$; ^{13}C NMR 198.7 (C-4), 168.6 (C-7), 161.2 (C-9), 159.1 (C-4′), 127.4 (C-2′, C-6′), 127.1 (C-1′), 126.3 (C-5), 116.3 (C-3′, C-5′), 114.1 (C-10), 112.5 (C-6), 99.1 (C-8), 94.8 (C-3), 67.7 (C-2). ^{1}H NMR; 7.90 (d, 7.9, H-2′, H-6′), 7.74 (d, 8.2, H-5), 7.20 (d, 7.90, H-3′, H-5′), 6.93 (s, H-8), 6.80 (d, 8.2, H-6), 4.77, 4.48 (H_2-2, d, 12.0, ea, 2H).

3.3.2. 3-methyl-1H-indole-2-carboxylic acid (3). Pale yellow solid. mp 163–165°C; $IR_{\nu max}$ (cm^{-1}) 3304, 3048, 2924, 1671, 1567, 1550, 1452, 1243, 1019, 742; UV λ_{max} (log ε) 207.5 (4.46), 226 (4.55), 294.5 (4.32); HRFABMS m/z 176.0711 ($[M+H]^+$) monoisotopic calcd for $C_{10}H_{10}NO_2$. ^{13}C NMR; 167.4 (2-COOH), 122.8 (C-3), 128.5 (C-3a), 126.3 (C-6), 122.4 (C-2), 121.1 (C-4), 120.2 (C-5), 111.8 (C-7), 136.3 (C-7a), 10.0 (3-CH_3). ^{1}H NMR; 8.67 (s, NH), 7.64 (d, 8.0, H-4), 7.33 (d, 8.0, H-7), 7.30 (*t*, 7.9, H-6), 7.11 (*t*, 7.9, H-5), 2.61 (s, 3-CH_3).

3.3.3. 3-(p-Hydroxyphenyl)-N-methylpropionamide (5). Pale yellow solid. mp 86°C; $IR_{\nu max}$ (cm^{-1}) 3252, 2933, 1724, 1667, 1644, 1516, 1456, 1360, 1202, 1099, 632; HRFABMS m/z 180.1024 ($[M+H]^+$) monoisotopic calcd for $C_{10}H_{14}NO_2$. ^{13}C NMR; 174.1 (C-1), 157.8 (C-4′), 132.1 (C-1′), 131.6 (C-2′, C-6′), 117.1 (C-3′, C-5′), 43.3 (C-2), 36.6 (C-3), 23.4 (N-CH_3). ^{1}H NMR; 7.06 (d, 8.39, H-2′, H-6′), 6.74 (d, 8.39, H-3′, H-5′), 3.38 (*t*, 7.25, H_2-2, 2H), 2.72 (t, 7.25, H_3-2, 2H), 1.94 (s) (N-CH_3).

3.4. *Determination of the antimicrobial activity*

Preliminary screening of the microbial extract and fractions was performed by the agar diffusion method against *Bacillus subtillis* 327, *Staphylococcus aureus* 375, *S. aureus* meticillin-resistant 310, *Enterococcus faecium* 379, *Escherichia coli* imp strain 389 and *Candida albicans* 54. The assay media used were LB (Luria–Bertani) agar for *E. faecium*, YM agar for *C. albicans* and Difco nutrient agar (pH 6.8) for the rest of the organisms. Assay plates (9″ × 9″ Sumilon) were prepared by pouring 125 ml volume of agar medium (tempered at 50°C) inoculated with an overnight broth culture of the test organisms (adjusted to approximately 10^6 cells per mL). Vancomycin (*S. aureus* 375 and 310), Bacitracin (*E. faecium* 379), Rifampin (*E. coli* 389) and Nystatin (*C. albicans*) discs (BBL sensi-Discs from Beckton Dickinson and Company, Cockeysville, MD) were placed onto the agar surface as controls. Ten microliters volume of the test compound solution diluted in DMSO was spotted onto the agar surface and the plates were incubated at 37°C for 18 h. The zone of growth inhibition was measured using a hand-held digital caliper. Minimum inhibitory concentrations (MICs) were determined by standard broth microdilution method as described earlier [19]. Briefly,

five microliters of an overnight broth bacterial culture (adjusted to density from 1×10^7 to 5×10^7 CFU/ml) were added to 0.1 ml of broth medium in polystyrene plates containing the tested compound at 0.03 to 128 μg/ml concentration. The MIC was defined as the lowest concentration of antibiotic, which prevented visual turbidity after 18–20 h of incubation at 37°C.

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Supporting information

Daidzein (2). Pale yellow powder. ^{13}C NMR; 175.8 (C-4), 164.0 (C-7), 159.0 (C-4′), 158.5 (C-9), 152.4 (C-2), 131.0 (C-2′, C-6′), 128.2 (C-5), 125.0 (C-3), 123.9 (C-1′), 118.0 (C-10), 116.2 (C-3′, C-5′), 115.8 (C-6), 103.1 (C-8). ^{1}H NMR; 8.14 (s, H-2) 8.45 (d, 9.0, H-5), 7.79 (d, 8.0, H-2′, H-6′, 2H), 7.27 (d, 8.0, H-3′), 7.21 (dd, 9.0, 2.0, H-6), 7.19 (d, 2.0, H-8).

1H-indole-3-carboxaldehyde (b). Red powder. ^{13}C NMR; 185.1 (3-CHO), 138.4 (C-7a), 138.0 (C-2), 125.5 (C-3a), 124.1 (C-6), 122.8 (C-5), 122.2 (C-4), 119.9 (C-3), 112.8 (C-7). ^{1}H NMR; 10.29 (s, 3-CHO), 8.74 (d, 7.6, H-4), 8.2 (s, H-2), 7.58 (d, 7.6, H-7), 7.37 (*t*, 7.6, H-5), 7.33 (*t*, 7.6, H-6). EI *m*/*z* 145, 144 [M–H], 116 [M–CHO], 89.

Phenyl acetic acid (6). ^{13}C NMR; 175.6 (C-1), 136.1 (C-1′), 130.3 (C-2′, C-6′), 129.4 (C-3′, C5′), 127.9 (C-6′), 42.0 (C-2). ^{1}H NMR; 7.25–7.13 (m, H-2′–H-6′), 3.22 (s, H_2-2).

2-hydroxy phenyl acetic acid (7). ^{13}C NMR; 176.8 (C-1), 156.9 (C-2′), 132.0 (C-6′), 129.2 (C-4′), 123.2 (C-1′), 120.5 (C-5′), 116.2 (C-3′). ^{1}H NMR; 7.12 (d, $J = 8.00$, H-6′), 7.08 (d, $J = 7.95$, H-3′), 6.79 (*t*, $J = 8.00$, H-4′). 6.78 (*t*, $J = 8.00$, H-5′).

4-hydroxy-5-methoxy-benzoic acid (8). ^{13}C NMR; 170.0 (1-COOH), 152.7 (C-4), 148.7 (C-5), 125.3 (C-2), 123.1 (C-1), 115.8 (C-3), 113.8 (C-6), 56.4 (OCH_3). ^{1}H NMR; 7.58 (s, H-6), 7.57 (d, $J = 8.6$, H-2), 6.85 (d, $J = 8.6$, H-3), 3.91 (s, OCH_3).

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