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Yangonindimers A-C, three new kavalactone dimers from *Piper methysticum* (kava)

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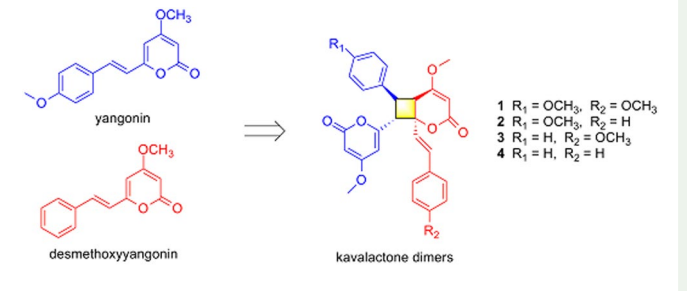
ABSTRACT

Three new kavalactone dimers, designated as yangonindimers A-C (**1**–**3**), along with one known analogue were isolated from the roots of *Piper methysticum*. Their structures were elucidated via extensive analysis of their 1D, 2D NMR and mass spectroscopic data. All these dimers possess a skeleton featuring a cyclobutane ring connecting two kavalactone units. Compounds **1**–**4** were evaluated for their cytotoxic activities against human tumour cell lines NCI-H46, SW480 and HepG2, but none showed significant activity.



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Kavalactone dimers; yangonindimers A-C; *Piper methysticum*; cytotoxic activities

1. Introduction

Piper methysticum Forst. f., or kava, of the family Piperaceae, grows as a perennial shrub in Fiji and other South Pacific islands (Singh & Blumenthal 1997). Traditionally, the root of kava is used by native Pacific islanders during religious and cultural ceremonies, for medicinal purposes and at social gatherings as an inebriant beverage that elicits physiological and psychological relaxation (Bilia et al. 2002). Commercially, kava has been used in natural

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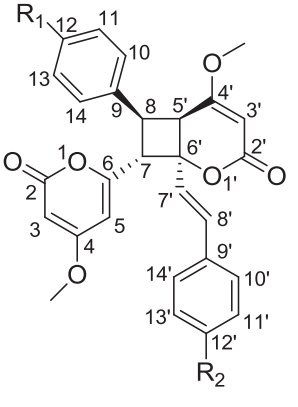
remedies for stress and anxiety (Whitton et al. 2003). Due to its sedative, anti-stress and anxiolytic properties, *P. methysticum* has gained popularity recently in western countries as an alternative medicine especially for the treatment of anxiety disorders (Pittler & Ernst 2000; Singh & Singh 2002; Sarris & Kavanagh 2009). However, the safety of kava products have been repeatedly questioned due to reported cases of hepatotoxicity (Teschke et al. 2008; Teschke 2010a, 2010b), and subsequently, more and more attention have been paid to the toxicity and further investigations revealed that its occurrence might be facilitated by high humidity, poor methods for drying procedures and insufficient storage facilities during the time after harvest (Teschke 2010a, 2010b; Teschke & Lebot 2011; Teschke, Qiu et al. 2011; Teschke, Sarris et al. 2011).

Phytochemical investigations of many *Piper* species have resulted in the isolation of numerous biologically active natural products, including alkaloids, sesquiterpenes, triterpenoids, steroids, lignans, flavones, chalcones, aldehydes, ketones, long-chained fatty acids and phenolics (Parmar et al. 1997; Dharmaratne et al. 2002; Olsen et al. 2011; Shi et al. 2015; Tu et al. 2016; Gigliarelli et al. 2017). The chemistry of *P. methysticum* has been extensively studied, and so far more than 40 compounds belonging to the classes of kavalactones, alkaloids, steroids, chalcones, long-chained fatty acids and alcohols have been isolated and identified (Parmar et al. 1997; Dharmaratne et al. 2002; Olsen et al. 2011). Among these compounds, kavalactones have been recognised as the constituents responsible for the reported biological activities in kava (Bilia et al. 2002).

Kavalactone dimers, exist as novel compounds, firstly isolated from *Aniba parviflora* (Andrade da Mata Rezende et al. 1971). As trace components existing in the plant, those compounds are vulnerable to be lost during isolation, and rarely reported. Our current phytochemical investigation on the roots of kava had led to the discovery of three new kavalactone dimers, named yangonindimers A-C (**1–3**), along with one known analogue, aniba-dimer A (**4**) (Andrade da Mata Rezende et al., 1971) (Figure 1). Herein, we report on the isolation, structural elucidation and bioactivity evaluation of these kavalactone dimers.



1 $R_1 = OCH_3$, $R_2 = OCH_3$
2 $R_1 = OCH_3$, $R_2 = H$
3 $R_1 = H$, $R_2 = OCH_3$
4 $R_1 = H$, $R_2 = H$

Figure 1. Structures of compounds **1**–**4**.

2. Results and discussion

Compound **1**, was obtained as yellow powder with the molecular formula of $C_{30}H_{28}O_8$ as established on the basis of HR-ESI-MS measurements wherein a protonated molecular ion was measured at *m/z* 517.1835 $[M + H]^+$, implying 17 indices of hydrogen deficiency.

Two characteristic absorption at 1711 and 1631 cm^{-1} in the IR spectrum indicated the presence of ester carboxyl and double bond groups, and the UV spectrum suggested the presence of an aromatic moiety (265 nm). The ^1H NMR spectrum of **1** showed signals due to two para-substituted benzene rings [δ_H 6.88, 6.89, 7.18, and 7.37 (each 2H, d, J = 8.7 Hz)], one *trans*-double bond [δ_H 6.45 and 6.89 (each 1H, d, J = 15.9 Hz)], one *γ*-pyrone ring [δ_H 5.36 and 5.92 (each 1H, d, J = 2.2 Hz)], four methine protons [δ_H 3.57 (1H, d, J = 9.6 Hz), 4.11 (1H, d, J = 11.0 Hz), 4.30 (1H, dd, J = 11.0, 9.6 Hz), and 5.32 (1H, s)], and four methoxyl protons (δ_H 3.35, 3.73, 3.82, and 3.83).

In the HMBC experiment, the protons of the para-substituted benzene ring at δ_H 7.18 (H-10 and H-14) showed correlations to the carbon signal of C-8 (δ_C 38.8). Thus, the position of the para-substituted benzene ring was determined to be at C-8 position. The proton signal at δ_H 4.11 (H-7) was correlated with the carbon signals in *γ*-pyrone ring at δ_C 102.7 (C-5), 159.0 (C-6) and 79.6 (C-6′), indicated that the lactonic ring was located at C-7. The proton signal at δ_H 6.45 (H-7′) correlated to the carbon at δ_C 46.0 (C-5′) and 79.6 (C-6′), revealed that the *trans*-double bond attached to C-6′. Besides, the key HMBC correlations from H-5′ (δ_H 3.57) to C-6′ (δ_C 79.6) and C-3′ (δ_C 91.9) were also observed.

These spectral features were like those of aniba-dimer A (Masanori et al. 1982) except for the presence of two additional methoxy groups. The positions of the methoxy groups were determined, as indicated from the HMBC results, to be on C-12 and C-12′. Thus, **1** is a dimer of yangonin, forming a cyclobutane ring between the two units.

The relative configuration of C-6′ was inferred from the ROESY experiment wherein the cross-peak of H-7′/H-5′ was observed, suggesting *cis*-diaxial like structures of aniba-dimer A and achyrodimer D (Masanori et al. 1982; Sagawa et al. 2005). Whilst the configurations of C-7, C-8 and C-5′ were deduced from the coupling pattern of the methine protons on the cyclobutane ring. These unusual coupling constants of H-7/H-8 and H-8/H-5′ were determined to be 11.0 and 9.6 Hz, respectively, which were similar to those of aniba-dimer A and achyrodimer D (Masanori et al. 1982; Sagawa et al. 2005). All these results, together with the biosynthetic pathway, revealed that the stereochemistry of **1** was the same as that of the known analogues, such as aniba-dimer A, and achyrodimer D, and thus, concluded to be *rel*-(7*S*,8*S*,5′*R*,6′*R*). Taken together the structure of **1** was determined, and named as yangonindimer A.

Compound **2**, isolated as yellow powder, was found to possess a molecular formula of $C_{29}H_{26}O_7$ as determined by an HRESIMS ion at *m/z* 487.1735 $[M + H]^+$ (Calcd 487.1751), indicating 17 indices of hydrogen deficiency.

The ^1H and ^{13}C NMR spectra of compound **2** showed close similarities to those of **1**. The main difference observed was the absence of one methoxy group signal, and instead, the presence of one aromatic proton signal (δ_H 7.29, m; δ_C 128.3). In the ^1H–^1H COSY spectrum, this aromatic proton showed correlations with H-11′ and H-13′ (δ_H 7.34), suggesting its attachment to C-12′.

The coupling constants of H-7/H-8 and H-8/H-5′ were determined to be 11.0 and 9.6 Hz, respectively, were in agreement with those of **1**, suggesting the identical coupling pattern of the methine protons on the cyclobutane ring. The cross-peak of H-7′/H-5′ was also

observed in the ROESY spectrum. All these results implied that the stereochemistry of **2** was the same as that of **1**, and concluded to be *rel*-(7*S*,8*S*,5′*R*,6′*R*). Thus, the structure of **2** was elucidated as depicted in Figure 1, named as yangonindimer B.

Compound **3** shared the same molecular formula as **2** as determined by the HRESIMS ion at *m/z* 487.1731 $[M + H]^+$ (Calcd 487.1751). Interpretation of its NMR data revealed the structure of **3** to be very close to that of **2**. The differences between these two compounds resulted from the position of methoxy group. The methoxy group in **3** was located at C-12′ rather than at C-12 in **2**, as inferred from the HMBC correlations from H-10′ (δ_H 7.38, d, $J = 8.7$ Hz), and H-11′ (δ_H 6.89, d, $J = 8.7$ Hz) to C-12′ (δ_C 159.8). The cross-peak of H-7′/H-5′ observed in the ROESY spectrum, together with the coupling constants of H-7/H-8 (11.0 Hz) and H-8/H-5′ (9.6 Hz), revealed that the stereochemistry of **3** was the same as that of **1** and **2**, and concluded to be *rel*-(7*S*,8*S*,5′*R*,6′*R*). Thus, the structure of **3** was elucidated as depicted in Figure 1, named as yangonindimer C.

In a previous study, *Piper* plants were found to be important sources for research and development of new anticancer agents (Wang et al. 2014). Therefore, all the four isolated compounds **1**–**4** were evaluated for their cytotoxic activities against three human tumour cell lines NCI-H46, SW480 and HepG2 using MTT assay. However, none showed significant activity (cellular proliferation inhibition rate < 50% at 20 μM).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Boston, MA, U.S.A.). UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 35 UV–vis spectrophotometer (Perkin-Elmer, Boston, MA, U.S.A.). IR spectra were acquired using a Bruker Vertex 33 infrared spectrophotometer (Bruker, Karlsruhe, Germany) with KBr disk. CD spectra were measured with an Anton Paar MCP 500 Chirascan spectrometer. 1D and 2D NMR spectra were recorded on a Bruker Advance 500 spectrometer with TMS as internal standard (Bruker BioSpin AG, Fallanden, Switzerland). HR-ESI-MS data were obtained on a Bruker Bio TOF IIIQ mass spectrometer (Bruker Daltonics, MA, U.S.A.). All solvents were analytical grade (Shanghai Chemical Plant, Shanghai, China). Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China) was used for column chromatography, and precoated silica gel GF254 plates (Qingdao Marine Chemical Inc., Qingdao, China) were used for TLC. C_{18}-reversed phase silica gel (150–200 mesh, Merck, Darmstadt, Germany), MCI gel (CHP20P, 75–150 µm, Mitsubishi Chemical Industries Ltd., Tokyo, Japan), and Sephadex LH-20 gel (CHP20P, 75–150 µm, Mitsubishi Chemical Industries Ltd., Tokyo, Japan) were also used for column chromatography. TLC spots were visualised under UV light and by dipping into 5% H_2SO_4 in alcohol followed by heating. Chiral HPLC was carried out on a CHIRALPAK® IF, 5 μm, 4.6 mm × 250 mm column, using a gradient solvent system comprised of n-hexane and isopropanol, with a flow rate of 3.0 mL/min. Run time: 45 min.

3.2. Plant material

The roots of kava were obtained from PureWorld; Naturex, South Hackensack, NJ, U.S.A., and identified by Prof. Fu-Wu Xing of South China Botanical Garden. A voucher specimen (QH20100816) has been deposited at the Laboratory of Natural Product Chemistry Biology, South China Botanical Garden.

3.3. Extraction and isolation

Dried and powdered roots of kava (5 kg) were extracted with 95% ethanol at room temperature for three times (10 × 20 L, 3 days each) and filtered, and then the filtrate was concentrated under vacuum to give a crude ethanol extract (458 g). The crude ethanol extract was suspended in H_2O (5 L) and extracted with EtOAc. The ethyl acetate fraction (410 g) subjected to column chromatography (CC) over silica gel and eluted with n-hexane-ethyl acetate (10:1, 5:1, 2:1, 1:1, v/v) to yield fractions A-F.

Fraction E (18 g) was subjected to CC over silica gel eluted with gradient elution of chloroform-ethyl acetate (5:1, 2:1, 1:1, v/v) to yield six subfractions (Fr.E1-Fr.E6). Fr.E3 (3 g) was separated by Sephadex LH-20 with elution of chloroform-methanol (1:1, v/v) to yield two subfractions (Fr.E3-1-Fr.E3-2). Fr.E3-1 (760 mg) was further purified by preparative HPLC (CH_3CN/H_2O from 40% to 100%) to yield compound **2** (55 mg). Fr.E4 (3 g) was separated by Sephadex LH-20 with elution of chloroform-methanol (1:1, v/v) to yield two subfractions (Fr. E4-1-Fr.E4-2). Fr.E4-1 (990 mg) was further purified by preparative HPLC (CH_3CN/H_2O from 40% to 100%) to yield compound **3** (84 mg).

Fraction F (22 g) was subjected to CC over silica gel eluted with gradient elution of chloroform-ethyl acetate (5:1, 2:1, 1:1, v/v) to yield five subfractions (Fr.F1-Fr.F5). Fr.F5 (5 g) was separated by Sephadex LH-20 with elution of chloroform-methanol (1:1, v/v) to yield two subfractions (Fr.F5-1-Fr.F5-2). Fr.F4-1 (900 mg) was further purified by preparative HPLC (CH_3CN/H_2O from 40% to 100%), yielding **1** (77 mg).

3.3.1. Yangonindimer A (1)

Yellow power; $[\alpha]_D^{25}$ + 8.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 265 (0.61) nm; IR (KBr) ν_{max} 2925, 1711, 1631, 1569, 1531, 1451, 1387, 1282, 1033 and 826 cm^{-1}; HR-ESI-MS: *m/z* 517.1835 $[M + H]^+$ (Calcd for $C_{30}H_{29}O_8$, 517.1857); ^1H NMR (500 MHz, $CDCl_3$): δ_H 5.36 (1H, d, *J* = 2.2 Hz, H-3), 5.92 (1H, d, *J* = 2.2 Hz, H-5), 4.11 (1H, d, *J* = 11.0 Hz, H-7), 4.30 (1H, dd, *J* = 11.0, 9.6 Hz, H-8), 7.18 (1H, d, *J* = 8.7 Hz, H-10), 6.89 (1H, d, *J* = 8.7 Hz, H-11), 6.89 (1H, d, *J* = 8.7 Hz, H-13), 7.18 (1H, d, *J* = 8.7 Hz, H-14), 5.32 (1H, s, H-3′), 3.57 (1H, d, *J* = 9.6 Hz, H-5′), 6.45 (1H, d, *J* = 15.9 Hz, H-7′), 6.89 (1H, d, *J* = 15.9 Hz, H-8′), 7.37 (1H, d, *J* = 8.7 Hz, H-10′), 6.88 (1H, d, *J* = 8.7 Hz, H-11′), 6.88 (1H, d, *J* = 8.7 Hz, H-13′), 7.37 (1H, d, *J* = 8.7 Hz, H-14′), 3.73 (3H, s, CH_3O-4), 3.82 (3H, s, CH_3O-12), 3.35 (3H, s, CH_3O-4′), 3.83 (3H, s, CH_3O-12′). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 164.1 (C-2), 88.8 (C-3), 170.7 (C-4), 102.7 (C-5), 159.0 (C-6), 55.2 (C-7), 38.8 (C-8), 128.7 (C-9), 128.7 (C-10), 114.0 (C-11), 159.3 (C-12), 114.0 (C-13), 128.7 (C-14), 164.9 (C-2′), 91.9 (C-3′), 170.3 (C-4′), 46.0 (C-5′), 79.6 (C-6′), 122.3 (C-7′), 131.0 (C-8′), 127.9 (C-9′), 128.2 (C-10′), 114.3 (C-11′), 159.8 (C-12′), 114.3 (C-13′), 128.2 (C-14′), 56.0 (CH_3O-4), 55.4 (CH_3O-12), 55.7 (CH_3O-4′), 55.4 (CH_3O-12′).

3.3.2. Yangonindimer B (2)

Yellow power; $[\alpha]_D^{25}$ + 9.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 254 (0.67) nm; IR (KBr) ν_{max} 2941, 1716, 1629, 1569, 1514, 1450, 1390, 1304, 1254, 1203, 1145, 1033 and 834 cm^{-1}; HR-ESI-MS: *m/z* 487.1735 $[M + H]^+$ (Calcd for $C_{29}H_{27}O_7$, 487.1751); ^1H NMR (500 MHz, $CDCl_3$): δ_H 5.35 (1H, d, *J* = 2.2 Hz, H-3), 5.92 (1H, d, *J* = 2.2 Hz, H-5), 4.13 (1H, d, *J* = 11.0 Hz, H-7), 4.31 (1H, dd, *J* = 11.0, 9.6 Hz, H-8), 7.19 (1H, d, *J* = 8.7 Hz, H-10), 6.88 (1H, d, *J* = 8.7 Hz, H-11), 6.88 (1H, d, *J* = 8.7 Hz, H-13), 7.19 (1H, d, *J* = 8.7 Hz, H-14), 5.32 (1H, s, H-3′), 3.57 (1H, d, *J* = 9.6 Hz, H-5′), 6.60 (1H, d, *J* = 15.9 Hz, H-7′), 6.95 (1H, d, *J* = 15.9 Hz, H-8′), 7.43 (1H, d, *J* = 8.7 Hz, H-10′),

7.34 (1H, m, H-11′), 7.29 (1H, m, H-12′), 7.34 (1H, m, H-13′), 7.43 (1H, d, J = 8.7 Hz, H-14′), 3.71 (3H, s, CH_3O-4), 3.81 (3H, s, CH_3O-12), 3.35 (3H, s, CH_3O-4′). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 163.9 (C-2), 88.7 (C-3), 170.5 (C-4), 102.7 (C-5), 158.8 (C-6), 55.1 (C-7), 38.7 (C-8), 127.7 (C-9), 128.7 (C-10), 113.9 (C-11), 159.3 (C-12), 113.9 (C-13), 128.7 (C-14), 164.7 (C-2′), 91.8 (C-3′), 170.1 (C-4′), 45.8 (C-5′), 79.4 (C-6′), 124.5 (C-7′), 131.4 (C-8′), 135.9 (C-9′), 126.9 (C-10′), 128.8 (C-11′), 128.3 (C-12′), 128.8 (C-13′), 126.9 (C-14′), 55.9 (CH_3O-4), 55.3 (CH_3O-12), 55.6 (CH_3O-4′).

3.3.3. Yangonindimer C (3)

Yellow power; $[\alpha]_D^{25}$ + 8.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 265 (0.48) nm; IR (KBr) ν_{max} 2924, 1707, 1643, 1565, 1512, 1456, 1394, 1251, 1147, 1035 and 824 cm^{-1}; HR-ESI-MS: *m/z* 487.1731 $[M + H]^+$ (Calcd for $C_{29}H_{27}O_7$, 487.1751); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 5.36 (1H, d, J = 2.2 Hz, H-3), 5.93 (1H, d, J = 2.2 Hz, H-5), 4.18 (1H, d, J = 11.0 Hz, H-7), 4.36 (1H, dd, J = 11.0, 9.6 Hz, H-8), 7.26 (1H, d, J = 8.7 Hz, H-10), 7.34 (1H, m, H-11), 7.30 (1H, m, H-12), 7.34 (1H, m, H-13), 7.26 (1H, d, J = 8.7 Hz, H-14), 5.31 (1H, s, H-3′), 3.61 (1H, d, J = 9.6 Hz, H-5′), 6.47 (1H, d, J = 15.9 Hz, H-7′), 6.90 (1H, d, J = 15.9 Hz, H-8′), 7.38 (1H, d, J = 8.7 Hz, H-10′), 6.89 (1H, d, J = 8.7 Hz, H-11′), 6.89 (1H, d, J = 8.7 Hz, H-13′), 7.38 (1H, d, J = 8.7 Hz, H-14′), 3.73 (3H, s, CH_3O-4), 3.29 (3H, s, CH_3O-4′), 3.83 (3H, s, CH_3O-12′). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 164.7 (C-2), 88.7 (C-3), 170.5 (C-4), 102.7 (C-5), 158.8 (C-6), 54.5 (C-7), 39.2 (C-8), 135.7 (C-9), 127.6 (C-10), 128.5 (C-11), 127.8 (C-12), 128.5 (C-13), 127.6 (C-14), 164.0 (C-2′), 91.8 (C-3′), 170.0 (C-4′), 45.8 (C-5′), 79.5 (C-6′), 122.1 (C-7′), 130.9 (C-8′), 128.6 (C-9′), 128.2 (C-10′), 114.2 (C-11′), 159.8 (C-12′), 114.2 (C-13′), 128.2 (C-14′), 55.9 (CH_3O-4), 55.4 (CH_3O-4′), 55.3 (CH_3O-12′).

3.4. Cytotoxic activity against NCI-H46, SW480 and HepG2

Compounds **1–4** were evaluated for inhibitory activity against human lung cancer cell (NCI-H46), human colorectal cancer cells (SW480) and human liver cancer cells (HepG2) using the MTT method, according to a previously described procedure (Heilmann et al. 2001). Doxorubicin was used as positive control. Cells were plated in 96-well tissue plates at a density of 1 × 104 cells/well. Adherent cell lines were previously incubated for 24 h to ensure adhesion to the wells in an atmosphere of 5% CO_2. Compounds **1–4** were applied at various concentrations (0.01, 0.1, 1, 10 and 100 µM) and control cells were treated with DMSO at the highest concentration used in test wells (0.5%). One hour prior to the end of the incubation period, 20 mL of MTT (5 mg/mL in PBS, 5% MTT) were added to each well and further incubated at 37°C for another 4 h. Supernatants were removed and 150 µL DMSO were afterwards added to each well in order to dissolve the formazan crystals. The mixture was oscillatored for 10 min at room temperature and its absorbance was measured at 490 nm (Genios, Tecan, Austria). The concentration resulting in 50% of cell growth inhibition (IC_{50}) was calculated using the Probit program in SPSS 19 for windows XP (SPSS Inc.Chicago)

4. Conclusions

Three new kavalactone dimers, named yangonindimers A-C (**1–3**), along with one known analogue (**4**) were isolated from the roots of *P. methysticum*. All the isolated compounds showed no significant cytotoxic activity against human tumour cell lines NCI-H46, SW480 and HepG2. Compounds **1**–**4** possess a skeleton featuring a cyclobutane ring connecting

two kavalactone units. This is the first report of isolation of such dehydrokawain derivatives from *P. methysticum*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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