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NOTE

Novel quercetin glucosides from *Helminthostachys zeylanica* root and acceleratory activity of melanin biosynthesis

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Abstract Two novel quercetin glucosides, namely 4′-*O*-*β*-D-glucopyranosyl-quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside (compound **1**) and 4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl-quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside (compound **2**), were isolated from *Helminthostachys zeylanica* root 50 % EtOH extract. Structural analysis of isolated compounds was achieved mainly by 600 MHz and 800 MHz NMR, UPLC–TOFMS and GC–MS. Of the two quercetin glucosides, compound **1** showed a high melanogenic acceleratory effect, 2.7 times higher than control, at 10 μM concentration in murine B16 melanoma cells, with no cytotoxic effect.

Keywords Quercetin glucoside · B16 melanoma cells · Tyrosinase · *Helminthostachys zeylanica*

Introduction

Helminthostachys zeylanica belongs to the family Ophioglossaceae, and is used as an antipyretic, antiphlogistic and anodyne [1], and to treat sciatica, boils, ulcers and malaria [2]. Four flavonoids, ugonins A–D, were isolated from the rhizomes of *H. zeylanica* [3, 4]. Moreover, Huang et al. isolated ugonins E-T from the root of *H. zeylanica* and identified their antioxidant and anti-inflammatory activity [5, 6].

Melanin is a pigment which is biosynthesized from tyrosine by enzymatic oxidation of tyrosinase. It is widely distributed in skin, retina, nigra of brain, adrenal medullae, etc. It plays an important role in skin cancer prevention by protecting cells from ultraviolet rays, and inhibition of grey hair formation by stress or aging. Melanogenesis enhancing agents are thus valuable for skin care and anti-grey hair formation. The present study reports the isolation and identification of novel compounds from *H. zeylanica* root extract and their potent melanogenesis enhancement activity.

Result and discussion

Chemical structures of compounds **1** and **2**

Compound **1** (Fig. 1) was obtained from a water/EtOH extract of *H. zeylanica* root using LH-20 gel column chromatography, and the structure was analyzed by NMR and UPLC–TOFMS. In addition, compound **1** was hydrolyzed with acid to determine the constitution of sugars and aglycon. The sugars were analyzed using GC–MS after trimethylsilylation, and the aglycon was determined using NMR. The total ion chromatograms of trimethylsilylated D-glucose, L-rhamnose, D-arabinose, D-galactose and hydrolysates of compound **1** were determined. Peaks of trimethylsilylated D-glucose in the standard were observed at 24.5, 25.5 and 27.7 min of retention time, exactly the same retention times of hydrolysates of compound **1**.

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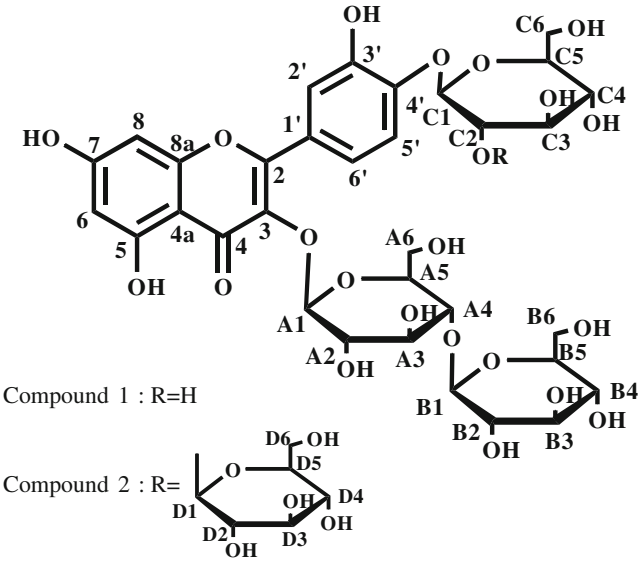

Fig. 1 Structures of compounds **1** and **2**

Moreover, the mass spectrum of compound **1** showed four major peaks at 787.2029 $[M–1]^-$, 625.1410 $[M–1\text{-}162]^-$, 463.0890 $[M–1\text{-}324]^-$ and 301.0372 $[M–1\text{-}466]^-$ (*m/z*) by UPLC–TOFMS. Considering that the 162 *m/z* differences of each peak depend on the fragment of glucose, compound **1** contained three molecules of glucose. Additionally, the specific optical rotations of compounds **1** and **2** were −33.4° ($c = 0.84$, MeOH:H_2O 1:1 v/v) and −41.0° ($c = 0.83$, H_2O), respectively, which are the negative rotations of another quercetin D-glucoside according to the previous paper. Furthermore, the specific optical rotations of sugars from compounds **1** and **2** were positive rotations +83.0° ($c = 0.23$, H_2O) and +86.7° ($c = 0.19$, H_2O), respectively. Compounds **1** and **2** were therefore confirmed as D-glucosides.

The NMR data of aglycon accorded with that of quercetin reported by Lasse et al. [7] and Moon et al. [8]. In order to elucidate the binding position of three glucoses of compound **1**, HMBC correlations (Table 1) were determined using 800 MHz NMR. HMBC of compound **1** gave correlations between H-C1 and C-4′, and between H-A1 and C-3. These correlations show the presence of C-C1 to C-4′ and C-A1 to C-3 bonds, respectively, via oxygen. Additionally, correlation between H-B1 and C-A4 was observed, and thus compound **1** was identified as 4′-*O*-*β*-D-glucopyranosyl-quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside.

Compound **2** (Fig. 1) was identified by comparison with the NMR and UPLC–TOFMS data of compound **1**. The NMR data of compound **2** shown in Table 2 was related to that of compound **1**. According to the HMBC data, the proton signal at 4.75 ppm of compound **2** which did not appear in compound **1** correlated with C-C2. The coupling constant of H-D3 was more than 7 Hz, so H-D2, H-D3 and H-D4 were therefore shown to be in axial positions. Furthermore, UPLC–TOFMS data of compound **2** was 949 $[M–1]^-$ *m/z*, 162 *m/z* higher than that of compound **1** (787 *m/z*). Compound **2** is therefore identified as 4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl-quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside.

Table 1 ^1H and ^{13}C-NMR chemical shifts and key HMBC correlations of compound **1**

Position	Compound **1** δ_H (ppm)	*J* (Hz)	δ_C (ppm)	HMBC
2			156.8	
3			134.6	
4			178.1	
4a			104.4	
5			161.7	
6	6.18 d	2.04	98.6	C-5, C-7
7			164.9	
8	6.36 d	2.10	93.4	C-7, C-8a
8a			157.1	
1′			121.5	
2′	7.67 d	2.04	116.6	C-2, C-4′
3′			146.2	
4′			147.6	
5′	7.25 d	8.94	115.8	C-3′, C-4′
6′	7.62 dd	8.94, 2.04	121.5	C-2, C-4′
	3-Glucose			
A1	5.28 d	7.56	102.6	C-3, C-A5
A2	3.49 t	8.22	75.0	
A3	3.57 m		76.4	C-A2, C-A4
A4	3.33 m		79.0	C-A5, C-A6, C-B1
A5	3.33 m		75.6	C-A4
A6	3.70 (2H) m		60.3	C-A4
	(1→4)-Glucose			
B1	4.37 d	7.56	103.2	C-A4, C-B5
B2	3.19 t	8.94	73.5	
B3	3.30 m		76.7	
B4	3.31 m		69.9	
B5	3.29 m		76.4	
B6	3.63 (2H) m		61.0	C-B4
	4′-Glucose			
C1	4.91 d	7.56	101.9	C-4′
C2	3.56 t	8.94	73.4	
C3	3.68 m		74.3	
C4	3.52 m		69.9	
C5	3.57 m		76.7	
C6	3.90, 3.86 m, m		61.2	

Table 2 ^{1}H and ^{13}C-NMR chemical shifts and key HMBC correlations of compound **2**

Position	Compound **2** δ_H (ppm)	J (Hz)	δ_C (ppm)	HMBC
2			156.8	
3			134.7	
4			178.1	
4a			104.4	
5			161.7	
6	6.18 d	2.04	98.8	C-5, C-7
7			165.1	
8	6.36 d	2.04	93.5	C-7, C-8a
8a			157.2	
1′			125.6	
2′	7.66 d	2.04	116.3	C-2, C-4′
3′			146.6	
4′			147.7	
5′	7.29 d	8.28	116.3	C-3′, C-4′
6′	7.60 dd	8.22, 2.04	121.5	C-2, C-4′
	3-Glucose			
A1	5.29 d	7.56	102.5	C-3
A2	3.50 t	8.94	74.4	
A3	3.57 m		76.5	C-A2, C-A4
A4	3.33 m		79.0	C-A5, C-A6, C-B1
A5	3.33 m		75.6	C-A4
A6	3.71 (2H) m		60.5	C-A4
	(1→4)-Glucose			
B1	4.37 d	8.22	103.2	C-A4, C-B5
B2	3.19 dd	8.94, 7.56	73.5	
B3	3.28 m		76.5	
B4	3.30 m		70.0	
B5	3.29 m		76.5	
B6	3.68 (2H) m		61.1	C-B4
	4′-Glucose			
C1	4.97 d	7.56	101.2	C-4′, C-C2
C2	3.80 t	7.56	81.8	
C3	3.68 m		74.1	
C4	3.52 m		69.9	
C5	3.59 m		76.8	
C6	3.90, 3.86 m, m		61.1	
	(1→2)-Glucose			
D1	4.75 d	8.22	104.2	C-C2
D2	3.27 t	8.94	75.0	
D3	3.39 dd	13.7, 8.93	76.3	
D4	3.47 m		69.6	
D5	3.31 m		77.0	
D6	3.68, 3.65 m, m		60.9	

Melanogenesis and tyrosinase enhancement activities

The melanogenesis enhancement activity of compounds **1** and **2** at 10 μM concentration is shown in Figure 2. The melanogenesis activity and cell viability of compound **1** were 270 and 93 %, respectively, while compound **2** did not exhibit melanogenesis enhancement activity. Quercetin, the aglycon of compound **1** and **2**, was reported to have high melanogenesis inhibitory activity. However, quercetin-4′-*O*-*β*-D-glucoside, quercetin-3-*O*-*β*-D-glucoside, quercetin-3,4′-*O*-*β*-D-glucoside and rutin showed a lower inhibitory activity than quercetin [9]. Furthermore, it was demonstrated that quercetin-3-*O*-*β*-D-glucoside enhances melanogenesis by accelerating the expression of tyrosinase-related protein-1 and -2 [10]. Our results extended knowledge of the melanogenesis activities of quercetin glycosides, especially glycoside attached to C-3 and 4′ of quercetin which had no melanogenesis inhibitory activity. Compound **1** showed melanogenesis acceleration activity of 2.7 times that of control, while interestingly compound **2** had no melanogenesis enhancement activity in spite of the similarity in the structure. This result means the number of sugars connecting C-4′ may play an important role in melanogenesis activity.

Since melanogenesis is related to tyrosinase activity, we also determined the tyrosinase activity of compounds **1** and **2**. The activity of compounds **1** and **2** at 10 μM concentration is presented in Figure 3. The tyrosinase activity of compound **1** was 115 and 117 % using L-tyrosine and L-DOPA as substrate, respectively. No enhancement activity of tyrosinase was showed for compound **1** in spite of the dramatic activity of melanin biosynthesis.

Tyrosinase is transcriptionally regulated by microphthalmia-associated transcription factor (MITF) and its expression is activated by the p38 MAPK cascade and extracellularly responsive kinase (ERK). On the other hand, the c-Jun N-terminal kinase (JNK) pathway has been reported to be related to the down-regulation of melanin synthesis [11]. Some melanogenesis-enhancing agents have been examined at several points of melanogenesis such as expression of tyrosinase, p38, JNK, ERK and MITF as well as tyrosinase activity. A study suggested that p38 MAPK was stimulated by cubebin, which enhanced melanogenesis activity in murine B16 melanoma cells [12]. Compound **1** may also enhance the activity or expression of MITF by regulating kinase as mentioned above, because it did not show tyrosinase acceleration activity in spite of its high melanogenesis stimulation activity in B16 melanoma cells. Moreover, considering the structures of the two novel quercetin glucosides, the glucoses connecting quercetin C4′ may play an important role in melanogenesis stimulation activity. In the next paper, we need to elucidate the mechanism of the melanogenesis enhancement activity of compound **1** by determining the tyrosinase and MITF expression in B16 melanoma cells.

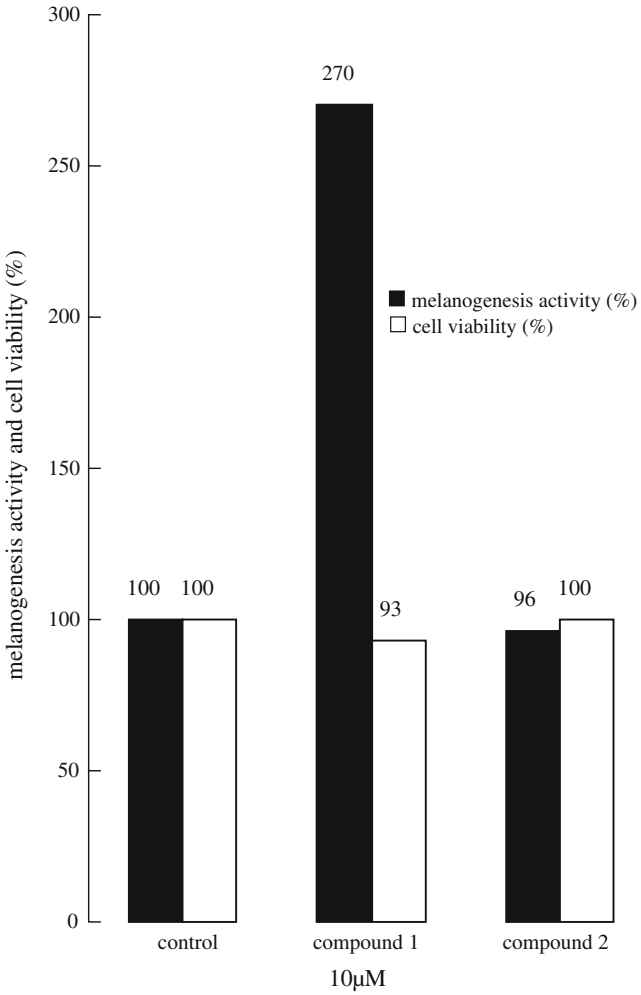

Fig. 2 Melanogenesis activity and cell viability of compounds **1** and **2**. *Black bars* melanogenesis activity of B16 melanoma cells treated with samples. *White bars* cell viability of B16 melanoma cells treated with samples

Conclusions

In this study, two novel quercetin glucosides were isolated from *H. zeylanica* root extract. Of them, compound **1** showed high melanogenesis enhancement activity, and may also enhance the activity or expression of MITF by regulating kinase in B16 melanoma cells. Moreover, the glucoses connecting quercetin C4′ may play an important role in melanogenesis stimulation activity.

Experimental

General

^{1}H and ^{13}C NMR spectra were recorded in methanol-d_4 with Bruker Biospin AVANCE III 800 MHz NMR and JEOL EC600 MHz NMR. Coupling constants were expressed in Hz, and chemical shifts were given on a δ (ppm) scale. UPLC–TOFMS (Waters Waters®Xevo™ QTof MS) was performed using column C_{18} (2.1 mm $\varphi \times$ 100 mm l, Waters). GC–MS (Shimadzu GCMS-QP 5050A) was performed using a DB-5 MS column (0.25 mm $\varphi \times$ 30 ml, J&W Scientific). Column chromatography was performed with Sephadex LH-20 (18–111 μm, GE Healthcare). IR spectra were taken on a Perkin Elmer Spectrum 100 FT-IR. UV spectra were recorded on a Simadzu SPD-M20A diode array detector. Optical rotations were measured on a JASCO P-2300.

Materials

The sample was collected from Samarinda, Indonesia in 2006. The sample and the voucher specimen were deposited in Herbarium Wanariset, Samboja, East Kalimantan, Indonesia, identification no. WAN0017365

Extraction and fractionation of *H. zeylanica* root powder

H. zeylanica root powder c. 100 g was extracted with 50 % ethanol. The 50 % ethanol extract was separated with Sephadex LH-20 gel column (25 mm $\varphi \times$ 820 mm l) chromatography eluting with the solvent (water: MeOH = 1:1 v/v) to obtain compound **1** (yellow powder 52.5 mg) and **2** (yellow powder 771 mg).

Tyrosinase activity assay

The tyrosinase activity assay was performed based on Batubara et al. [13]. The sample (70 μl) was placed in a 96-well plate. Tyrosinase 30 μl (333 U/ml in phosphate buffer 50 mM pH 6.5) and 110 μl of substrates (L-tyrosine 2 mM or L-DOPA 2 mM) were added. After incubation at 37 °C for 30 min, the absorbance at 510 nm was measured using a microplate reader. IC_{50} is expressed as the concentration of inhibitor showing 50 % inhibition.

Cell culture

Murine melanoma B16-F0 cells (DS Pharma Biomedical, USA) grown in DMEM medium supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin were cultured at 37 °C in a humidified atmosphere of 5 % CO_2.

Measurement of cellular melanin contents

Measurement of cellular melanin content was performed according to the method of Arung et al. [14]. In brief, confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed using

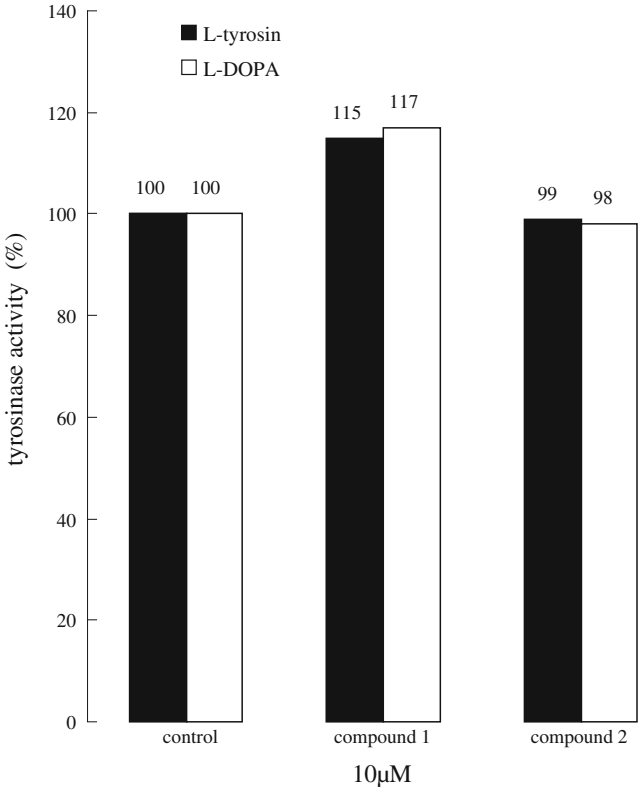

Fig. 3 Tyrosinase activity of compounds **1** and **2**. *Black bars* tyrosinase activity using L-tyrosine as substrate. *White bars* tyrosinase activity using L-DOPA as substrate

0.25 % trypsin/EDTA. The cells were placed in 10-cm Petri dishes (1.0×10^5 cells/dish) and allowed to adhere at 37 °C for 24 h. After adding samples, cells were incubated for 72 h and then washed with PBS, followed by lysis in 200 µl of 2 M NaOH with 40 min heating at 80 °C to solubilize the melanin. The resulting lysate (150 µl) was placed in a 96-well microplate, and the absorbance was measured at 405 nm with a microplate reader. Each experiment was repeated twice. Enhancement of melanine production is expressed as percentage of that of control cells treated with the solvent DMSO/water without sample materials.

Cell viability

Cell viability was determined using a hemocytometer (Erma, Tokyo, Japan). B16 cells were cultured and samples added as described in "Extraction and fractionation of *H. zeylanica* root powder". After 72 h incubation, B16 melanoma cells were removed using 0.25 % trypsin/EDTA solution, and then 10 µl trypan blue solution was added to 200 µl of the solution in a 1.5-ml tube. The cell number of 10 µl of mixed solution was counted in the hemocytometer. Each experiment was repeated twice. Enhancement of melanine production is expressed as percentage of that of control cells treated with the solvent DMSO/water without sample analyte.

Identification of compounds **1** and **2**

Compounds **1** and **2** were identified by ^{1}H NMR, ^{13}C NMR, ^{1}H ^{1}H COSY, HMQC, HMBC and UPLC-TOFMS. Methanol-d_4 was used as the NMR solvent. NMR measurements were performed by using Bruker Biospin AVANCE III 800 MHz NMR and JEOL EC600 MHz NMR. UPLC-TOFMS (Waters Waters®Xevo™ QTof MS) was performed using column C_{18} (2.1 mm $\varphi \times$ 100 mm l) with MeOH/water = 5/95 (0 min), 100/0 (10 min), 100/0 (13 min) as eluent. The data were collected in negative ionization mode. The capillary voltage was 3.0 kV. Cone and desolvation gas flow rates were set at 50 and 1000 L/h, respectively, and the source and desolvation temperature was 150 and 500 °C, respectively.

The NMR data of the compounds isolated from *H. zeylanica* roots are shown in Tables 1 and 2.

Compound **1**: yellow powder, UPLC–TOFMS ES$^-$: [M–1]$^-$ 787.2029, 625.1410, 463.0890, 301.0372 (*m/z*). UV λ_{max}^{MeOH}: 207, 265, 345 (nm). $[\alpha]_D^{20}$: −33.4° (c = 0.84, MeOH:H_2O 1:1 v/v). IR (KBr): 3402, 1655, 1612, 1499, 1457, 1365, 1260, 1205, 1072 (cm^{-1}).

Compound **2**: yellow powder, UPLC–TOFMS ES$^-$: [M–1]$^-$ 949.2466 (*m/z*).

UV λ_{max}^{MeOH}: 207, 265, 342 (nm). $[\alpha]_D^{20}$: −41.0° (c = 0.83, H_2O). IR (KBr): 3402, 1642, 1615, 1519, 1466, 1343, 1312, 1162, 1087 (cm^{-1}).

Acid hydrolysis

Acid hydrolysis was performed according to the previous method [15]: 5 ml of 1 N HCl was added to 10 mg of compound **1**, and the mixture was stirred at 80 °C for 4 h. After cooling down to room temperature, the solution was partitioned between H_2O and EtOAc. The H_2O layer was freeze-dried, and the EtOAc layer was analyzed by JEOL EC600 MHz NMR. The freeze-dried product from the H_2O layer was dissolved in 1-(trimethylsilyl)-imidazole 0.4 ml and pyridine 5.0 ml to trimethylsilylate. The solution was stirred at 60 °C for 5 min. After drying the solution in a stream of N_2, the residue was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer was analysed by GC–MS (Shimadzu GCMS-QP 5050A) using a DB-5 MS column (J&W Scientific 0.25 mm $\varphi \times$ 30 ml). Temperatures of the injector and detector were 250 °C. The temperature gradient of the column oven started at 80 °C for 2 min and increased up to 250 °C over a period of 9 min. The hydrolysis of standard monosaccharides D-glucose, D-galactose, D-arabinose and L-rhamnose were performed by the same method as with compound **1**.

Determination of absolute configuration of sugars from compound **1** and **2**

Five milliliters of 1 N HCl was added to 5 mg of compounds **1** and **2**, respectively, and the mixtures were stirred at 80 °C for 4 h. After cooling down to room temperature, the solutions were partitioned between H_2O and EtOAc using a separatory funnel. The sugars from compounds **1** and **2** were obtained by drying the H_2O layer in vacuo, and its specific optical rotations were $[\alpha]_D^{20}$: +83.0° ($c = 0.23$, H_2O) and +86.7° ($c = 0.19$, H_2O), respectively.

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