α-Glucosidase Inhibitors Ellagic Acid Derivatives with Immunoinhibitory Properties from *Terminalia superba*

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Fractionation of stem barks of *Terminalia superba* yielded two new ellagic acid derivatives, 3,4′-di-*O*-methylellagic acid 3′-*O*-β-D-xylopyranoside (1) and 4′-*O*-galloy-3,3′-di-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside (2) together with known 3,3′-di-*O*-methylellagic acid, ellagic acid and 3,3′-di-*O*-methylellagic acid 4′-*O*-β-D-xylopyranoside. Compounds (1) and (2) showed significant α-glucosidase inhibition activity and possessed significant immunoinhibitory activities with no cytotoxic effects.

Key words α-glucosidase; immunomodulatory activity; ellagic acid; *Terminalia superba*

Terminalia superba ENGL. (Combretaceae) is used in Cameroon folk medicine for the treatment of gastroenteritis, diabetes, female infertility and abdominal pain. Previous works showed methanol extract of *T. superba* stem bark to have vasorelaxant effects on rat vascular smooth muscle[1] and methanol/methylene chloride extract to have anti-diabetic activity.[2] Wansi *et al.* also reported the isolation of α-glucosidase inhibitors from the stem barks of *T. superba*.[3] In this paper, we report the isolation, structure elucidation, α-glucosidase enzyme inhibition and immunoinhibitory activities of two new ellagic acid derivatives. The new compounds **1** and **2** were screened over a wide range of concentrations (3.1—50 μg/ml) for their possible effects on the oxidative burst of isolated phagocytic cells (mononuclear cells) using a luminol-based chemiluminescence (CL) assay.[4]

Results and Discussion

Fractionation of MeOH extract of *T. superba* stem barks yielded two new compounds **1** and **2**, 3,3′-di-*O*-methylellagic acid,[5] ellagic acid,[5] and 3,3′-di-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside.[6]

Compound **1** was obtained as yellow crystals and its molecular formula $C_{21}H_{18}O_{12}$ was determined by the HR-ESI-MS (*m*/*z* 461.0791 $[M-H]^-$). In the ^{13}C-NMR and Distortionless Enhancement by polarization Transfer (DEPT) spectra of compound **1**, 21 carbon peaks were shown, including ten oxygen-bearing quaternary aromatic carbons, two lactone carbonyls, six methine carbons among which two were aromatic and four oxygenated, one oxygenated methylene and two methyl carbon peaks. The ^{1}H-NMR spectrum showed aromatic protons at δ 7.47 (1H, s) and 7.72 (1H, s) due to the ellagic acid skeleton,[5,6] together with an anomeric proton at δ 5.13 (1H, d, 7.1) indicating a sugar moiety. Acid hydrolysis of compound **1** gave D-xylose, which was identified by HPTLC. The configuration of the anomeric proton of the sugar was proposed to be β on the basis of the coupling constant (7.1 Hz). The sugar was found to be a β-D-xylopyranose according to ^{1}H- and ^{13}C-NMR data (Table 1) and by comparison with the published data.[7] In the ^{13}C-NMR spectrum of **1**, 14 carbon signals were assigned to ellagic acid, 5 carbon signals to xylose moiety and the remaining 2 carbon signals were assigned to methoxyl groups. The carbon signal of 3-OCH_3 appeared to be shifted down field approximately +4.8 ppm compared to 4′-OCH_3 suggesting an *ortho*-disubstituted methoxyl group.[8] The one signal due to the anomeric proton of xylose (δ 5.13, d, J=7.0 Hz), correlating to the C-3′ (δ 141.7) in the HMBC spectrum indicated that the xylose unit was linked to C-3′, suggesting that the second methoxyl group was located at C-4′. The NOESY spectrum showed the 4′-OCH_3 (δ 56.1) correlated with H-5′ (δ 7.72) of ellagic acid but no correlation between the anomeric proton of xylose (δ 5.13) and H-5′. This was further confirmed by the cross-peaks between H-5′ and C-1′, C-3′ and C-7′ in HMBC spectrum. The most relevant correlations are shown in Fig. 2. Thus, the structure of **1** is 3,4′-di-*O*-methylellagic acid 3′-*O*-β-D-xylopyranoside.

Compound **2** was obtained as yellow crystals. The molecular formula was determined as $C_{28}H_{22}O_{16}$ by the presence of a pseudo-molecular ion peak at *m*/*z* 613.0824 in its HR-ESI-MS spectra. Its ^{1}H- and ^{13}C-NMR spectra (Table 1) were similar to that of compound **1**. Acid hydrolysis of compound **2** gave D-xylose, which was identified by HPTLC. The β orientation of the anomeric proton of the xylose was deduced from the coupling constant (7.4 Hz) in the ^{1}H-NMR. The DEPT and ^{13}C-NMR spectrum showed 26 signals, of which 14 were assigned to the ellagic acid portion, 5 to the xylose moiety and 2 to the methoxyl groups. Of the three carbonyl signals at δ 158.8 (2 carbon atoms) and 165.7, the first two were attributed to ellagic acid lactone carbonyl signals. The third carbonyl signal (165.7 ppm) together with the ^{1}H-NMR

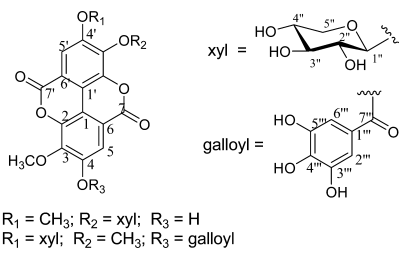

Fig. 1. Chemical Structures of Compounds **1** and **2**

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Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (75 MHz) Data of **1** and **2** in DMSO-d_6

Position	**1**		**2**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	110.8		114.6	
2	141.5		142.4	
3	140.3		140.2	
4	152.8		152.8	
5	111.8	7.47 (1H, s)	111.3	7.58 (1H, s)
6	112.5		113.5	
7	158.3		158.8	
1′	113.5		114.1	
2′	140.3		141.8	
3′	141.7		140.0	
4′	151.6		152.2	
5′	112.3	7.72 (1H, s)	111.6	7.65 (1H, s)
6′	112.1		113.1	
7′	158.4		158.8	
1″	101.8	5.13 (1H, d, 7.1)	102.1	5.14 (1H, d, 7.4)
2″	73.0	3.37 (1H, dd, 7.2, 8.1)	73.2	3.35 (1H, dd, 7.3, 7.9)
3″	76.1	3.32 (1H, t, 8.3)	76.2	3.33 (1H, t, 8.2)
4″	69.2	3.42 (1H, m)	69.4	3.44 (1H, m)
5″	65.8	3.83 (1H, dd, 11.1, 4.1) 3.37 (1H, dd, 10.9, 11.5)	65.9	3.84 (1H, dd, 10.7, 4.0) 3.34 (1H, dd, 10.8, 11.5)
1‴			119.2	
2‴/6‴			108.9	7.04 (2H, s)
3‴/5‴			145.5	
4‴			138.6	
7‴			165.7	
3-OMe	60.9	4.04 (3H, s)	61.8	4.04 (3H, s)
3′-OMe			61.1	4.08 (3H, s)
4′-OMe	56.1	3.96 (3H, s)		

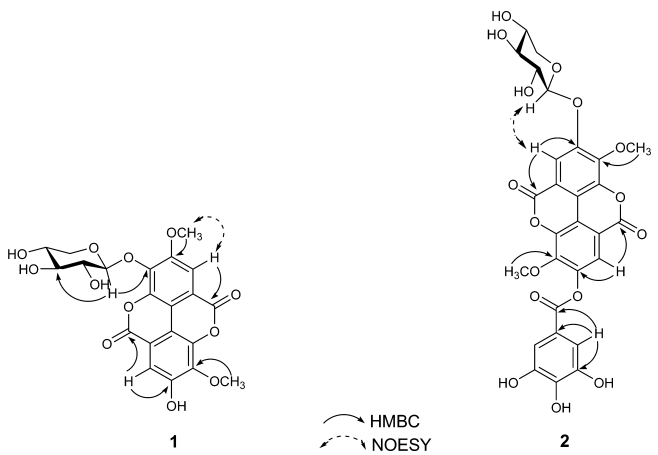

Fig. 2. Important HMBC and NOESY Correlations for Compounds **1** and **2**

data (Table 1) of tetrasubstituted aromatic ring and a direct comparison with literature,[9] showed the presence of a gallic acid moiety. Additionally, ^{13}C-NMR spectrum showed chemically equivalent signals at 108.9 ppm (C-2‴/6‴) and 145.5 ppm (C-3‴/5‴) and justified why the ^{13}C-NMR spectra displayed 26 carbon signals instead of 28 as shown by the molecular formula. The ^{13}C-NMR spectra (Table 1) displayed signals of two hindered methoxyl groups[8] at δ 61.1 and 61.8, thus allowing placement of the aromatic methoxyl group at C-3 and C-3′. This indicates that the galloyl and xylose units are linked to ellagic acid *via* 4-*O*- and 4′-*O*-linkages respectively. On the other hand the HMBC spectrum also led us to establish the locations to be connected among the methoxy, sugar and galloyl moieties of compound **2** on the basis of the cross peaks; one due to the coupling between H-1″ and C-4′ and the others due to the coupling between 3-OCH_3 and C-3, and 3′-OCH_3 and C-3′. The ^{1}H- and ^{13}C-NMR data of compounds **1** and **2** (Table 1) were very similar and the only significant differences were due to the effect of galloyl-substitution. Furthermore, the NOESY spectrum showed the anomeric proton H-1″ (δ 5.14) correlated with H-5′ (δ 7.65) suggesting the spatial proximity of the two protons. This observation was also supported by the down field shifted C-1 signal (114.6 ppm). As consequence, the up-field shifted of H-5′ was observed. The ^{1}H- and ^{13}C-NMR of compound **2** were similar to that of 3,3′-di-*O*-methylellagic acid 4″-*O*-β-D-xylopyranoside. The most significant difference was the down field shift of C-1 ($\Delta\delta$ 3.8) due to the presence of galloy moiety. On the basis of these observations, compound **2** was identified as 4-*O*-galloy-3,3′-di-*O*-methylellagic acid 4′-*O*-β-D-xylopyranoside. Galloyl derivatives of ellagic acid are very common.[10,11] However, in the case of compound **2**, there is an interesting feature in the way the galloyl unit is linked to the ellagic acid. Generally, the galloyl unit is linked to the ellagic acid *via* the alpha carbon of the carboxyl group of the galloyl moiety. In most cases the galloyl unit is esterifying sugar moieties. In the case of compound **2**, the carboxyl unit is esterifying the phenolic OH of the ellagic acid.

Table 2. α-Glucosidase Inhibitory Activity of Compounds **1** and **2**

Compound	Name	IC_{50} (μM)
1	3,4′-Di-*O*-methylellagic acid 3′-*O*-β-D-xylopyranoside	7.95±0.33
2	4′-Galloy-3,3′-di-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside	21.21±0.69
Standard	Deoxynojirimycin	425.6±8.14
	Acarbose	780±28

Compounds **1** and **2** were evaluated for α-glucosidase enzyme inhibition. They showed significant α-glucosidase inhibition with IC_{50} 7.95 and 21.21 μM respectively (Table 2). These results suggest that these compounds might exert its anti-diabetic effect by suppressing carbohydrate absorption from intestine, and thereby reducing the postprandial increase of blood glucose. This may explain the use of this plant as anti-diabetic medicine. Compounds **1** and **2** also showed inhibitory activities with mononuclear cells (50.2, 86.6% respectively) at the lower concentration (3.1 μg/ml) tested (Figs. 3a, b). Meanwhile at the higher concentrations of 25 and 50 μg/ml, compound **2**, which is the most active compound, inhibited mononuclear cells ROS (reactive oxygen species) activity (99.74%, 99.96%, respectively). These compounds were found to have potential in suppressing phagocytosis activity of mononuclear cells in a dose dependent manner compared to prednisolone. Similarly, the two compounds exhibited a strong suppressive effect on the phytohemagglutinin (PHA) stimulated T-cell proliferation with IC_{50} of 1.90 and 2.33 μg/ml respectively (Fig. 4). The present

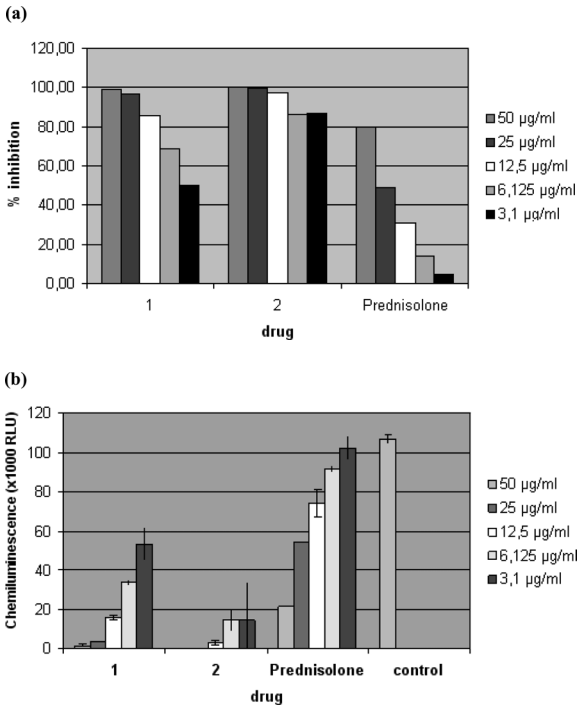

Fig. 3. Percentage of Inhibition (a) and Chemiluminescence Effect (b) of Compounds **1**, **2** and Prednisolone on Oxidative Burst Using Mononuclear Cells

Various concentrations of compounds were incubated with mononuclear cells (MNCs) for 30 min. The compounds activity was compared with the untreated samples (control) in the chemiluminescence (CL) assay. Each plot represents readings of three repeats.

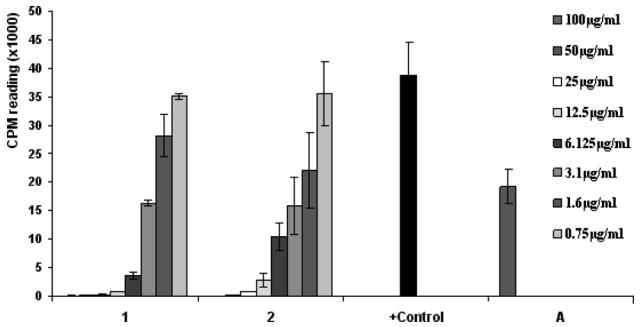

Fig. 4. Effect of Compounds **1** and **2** on Phytohemagglutinin (PHA) T-Cell Proliferation

The bar graph represents effects of various concentrations of the test compounds after 72 h incubation with mononuclear cells at 37 °C. Effect of compounds on T-cell proliferation response is compared with non-proliferated (A) cells. Each bar represents the mean value of triplicate reading ± S.D.

data show that compounds exert inhibitory effect on phagocytic activity of mononuclear cells. It was also found to interfere with the phytohemagglutinin (PHA) T-cell activation in a dose dependant manner. These compounds exhibited significant immunosuppressive activity. When compounds **1** and **2** were evaluated for their cytotoxicity on Balb/c 3T3 cells, no toxic effect was observed after 2 d of incubation (Table 3). The possible structural factor, which influences the activity, is ellagic acid skeleton. The compound **1** showed best α-glucosidase activity while the two compounds **1** and **2** showed similar immunosuppressive activity. It seems that the presence of galloyl moiety reduced α-glucosidase activity. These two compounds **1** and **2** are clearly exerting immunoinhibitory activity on the system used and could be suitable immunoinhibitory lead compounds for future research.

Table 3. Compounds IC_{50} Value from the MTT Cytotoxicity Studies (48 h MTT Results)

Compound	IC_{50} (μg/ml)
1	>100
2	>100

Serial compounds dilutions (0.195—100 μg/ml) were incubated with Swiss Balb/c 3T3 cells for 48 h and cells viability was evaluated by MTT reduction to the blue colored formazan in living cells. All values were means of 3 replicates.

Experimental

General IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer as KBr disc. ^{1}H-, ^{13}C-NMR and two-dimensional COSY, ROESY, HSQC and HMBC experiments were performed on a Bruker Avance DPX instrument (300, 500 MHz for ^{1}H and 75 MHz for ^{13}C). The 2.50 and 40.0 ppm resonances of residual CD_3SOCD_3 were used as internal references for ^{1}H- and ^{13}C-NMR spectra, respectively. Mass spectra were recorded on a micro TOF instrument. All melting points were determined on a micro-melting point apparatus and are uncorrected.

Plant Material The stem barks of *Terminalia superba* Engl. were collected from Ebolowa, Southern province of Cameroon, in June 2004. Plant was identified by Dr. Nana, Botanist, at the National Herbarium, Yaounde, Cameroon, and a voucher specimen N° W.C.S. 3642a/12543/Ya. was deposited.

Extraction and Isolation The air-dried powdered stem barks of *T. superba* (500 g) were extracted with methanol to give 10 g extract. The extract was chromatographed on silica gel using Hex/AcOEt (F1: 50/50; F2: 30/70; F3: 0/100 each 2×500 ml) and AcOEt/MeOH (F4: 90/10; F5: 80/20) to yield 5 fractions. Fractions F1, F2 and F3 were passed through Sep-Pak C_{18}-cartridges (10 g) using H_2O–MeOH and $CHCl_3$ as solvent to yield β-sitosterol (40 mg), 3,3′-di-*O*-methylellagic acid (8 mg) and ellagic acid (4 mg). Fractions F4 and F5 were separated by Sephadex LH-20 (Pharmacia) CC with H_2O/MeOH gradient into 10 fractions. Fractions 8/9 yielded **2** (62 mg). Fractions 2—6 yielded **1** (87 mg) and 3,3′-di-*O*-methylellagic acid 4′-*O*-β-D-xylopyranoside (4 mg) after preparative TLC on Avicel (Merck) in 50% AcOH. All compounds were purified by CC on Sephadex LH-20 in methanol.

3,4′-Di-*O*-methylellagic Acid 3′-*O*-β-D-Xylopyranoside **1**: Crystals from methanol; mp 194—196 °C; UV (MeOH): λ_{max} (log ε)=246 (4.58); 352 (4.04); 381 (4.09) nm; IR (KBr) ν_{max} 3389, 1728, 1690, 1603, 1499, 1420, 1357, 1212, 1114, 1066 cm^{-1}; ^{1}H- and ^{13}C-NMR (Table 1); HR-ESI-MS *m*/*z* 461.0791 $[M-H]^-$ (Calcd for $C_{21}H_{19}O_{12}$, 461.0787).

4′-Galloy-3,3′-di-*O*-methylellagic Acid 4-*O*-β-D-Xylopyranoside **2**: Crystals from methanol; mp 204—206 °C; UV (MeOH): λ_{max} (log ε)=246 (4.60); 261 (4.42); 365 (4.20) nm; IR (KBr) ν_{max} 3410, 1724, 1693, 1610, 1485, 1370, 1201, 1073 cm^{-1}; ^{1}H- and ^{13}C-NMR (Table 1); HR-ESI-MS *m*/*z* 613.0824 $[M-H]^-$ (Calcd for $C_{28}H_{21}O_{16}$, 613.0817).

Biological Assays. Enzyme Inhibition Assay α-Glycosidase (E.C. 3.2.1.20) enzyme inhibition assay has been performed according to the slightly modified method of Matsu *et al.*[12] α-Glucosidase (E.C. 3.2.1.20) from *Saccharomyces* species, was purchased from Wako Pure Chemical Industries Ltd. (Wako 076-02841). The enzyme inhibition was measured spectrophotometrically through continuous monitoring of the nitrophenyl produced by the hydrolysis of the substrate *p*-nitrophenyl α-D-glucopyranoside (PNP-G) (0.7 mM) and 500 milli units/ml of the enzyme used. Whole enzymatic reaction was performed at 37 °C for 30 min. The increment in absorption at 400 nm, due to the hydrolysis of PNP-G by α-glycosidase, was monitored continuously on microplate spectrophotometer (Spectra Max, Molecular Devices, U.S.A.). Phosphate saline buffer at pH 6.9 was used, which contains 50 mM sodium phosphate containing 100 mM NaCl. 1-Deoxynojirimycin (0.425 mM) and Acarbose (0.78 mM) were used as positive controls.

Chemiluminescence Assay Luminol-enhanced chemiluminescence assay was performed, as described by Helfand *et al.* (1982).[4] Briefly, mononuclear cells (1×10^6), suspended in Hank's balance salt solution with calcium and magnesium ($HBSS^{++}$), were incubated with 50 μl of compounds concentrations (0.75—100 μg/ml) for 30 min. To each well, 50 μl (20 mg/ml) zymosan (Sigma Chemical Co. U.S.A.), followed by 50 μl (7×10^{-5} M) luminol (G-9382 Sigma Chemical Co.) and then $HBSS^{++}$ was

added to adjust the final volume to 0.2 ml. $HBSS^{++}$ alone was used as a control. Chemiluminescence peaks were recorded with the Luminometer (Luminoskan RS Labsystem, Finland).

T-Cell Proliferation Assay Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy adult donor by Ficoll–Hypaque gradient centrifugation.[13] Cell proliferation was evaluated by standard thymidine incorporation assay following a method reported by Nielsen *et al.*[14] Briefly, cells were cultured at a concentration of 5×10^5 cells/ml in a 96-well round bottom tissue culture plate (Nalge Nunc. Inter.). Cells were stimulated with 5 μg/ml of PHA (Sigma Chemical Co., U.S.A.). Various concentrations of compounds were added to obtain final concentrations of 0.75, 1.6, 3.1, 6.13, 12.5, 25, 50, and 100 μg/ml, each in triplicate. Plates were incubated for 72 h at 37 °C in 5% CO_2 incubator. Cultures were pulsed later with 0.5 μCi/well tritiated thymidine (Amersham Pharmacia Biotech, Sweden), and further incubated for 18 h. Cells were harvested and the tritiated thymidine incorporation was measured by a liquid scintillation counter (LS 6500, Beckman Coulter, U.S.A.). Results were expressed as mean count per minute (CPM).

Cytotoxicity Evaluation The experiment was performed according to method reported earlier (Dariusz *et al.*[15]) with some modification. Swiss Balb/c 3T3 cells (3×10^4 cells/ml) were cultured in a 96-well plate for overnight. The supernatant was removed and 50 μl of serially diluted compounds (0.195—100 μg/ml), 150 μl Dulbecco's Modified Eagle's Medium (DMEM), penicillin [100 units/ml] and streptomycin (100 μg/ml) were added to each well. After 48 h of incubation, the culture medium was carefully removed, and 50 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/ml) was added to each well. The plates were incubated at 37 °C for 4 h. After the MTT solution was aspirated and cells were washed with phosphate buffer saline (PBS), 100 μl of DMSO was added to dissolve the blue insoluble MTT formazan produced by mitochondrial dehydrogenase. The plate was agitated at room temperature for 15 min and then read at 540 nm using microplate readers (SpectraMax PLUS384, Molecular Devices, U.S.A.). The percentage of viable cells was calculated as the relative ratio of optical densities (OD).

Statistical Analysis All data are reported as mean±S.D. of the mean and the student *t*-test was used to determine the difference between test- and control preparations significance was attributed to probability values $p\leq0.05$. The IC_{50} values were calculated using Excel based program.

Acid Hydrolysis of 1 and 2 Compounds **1** and **2** (5 mg each) were refluxed with 5% HCl at 100 °C for 3 h. The products of acid hydrolysis were adjusted to pH 6 by $NaHCO_3$ and extracted with EtOAc. The aqueous part was concentrated, purified by preparative TLC and subjected to HPTLC analysis on Kieselgel 60 F254 (Merck) by using standard sugars in the solvent system $CHCl_3$/MeOH/H_2O (70 : 30 : 3), the results of which indicated the presence of D-xylose (*Rf* 0.49, $[\alpha]_D^{25}$ +49.7° to +14.4°; H_2O) in **1** (1.37 mg, 84%) and **2** (1.29 mg, 79%).

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