Microbial Transformation of Isosteviol and Inhibitory Effects on Epstein–Barr Virus Activation of the Transformation Products

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Microbial transformation of isosteviol (**2**), a beyerane-type diterpenoid obtained from stevioside (**1**) by acid hydrolysis, yielded 7β-hydroxyisosteviol (**3**), 11β-hydroxyisosteviol (**5**), and 12β-hydroxyisosteviol (**6**) by the fungus *Aspergillus niger*, 17-hydroxyisosteviol (**7**) by the fungus *Glomerella cingulata*, and **3** and 7-oxoisosteviol (**4**) by the fungus *Mortierella elongate*. The five metabolites, **3**–**7**, along with **1** and **2** were evaluated for their inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells as a primary screening test for inhibitors of tumor promoters. All the diterpenes tested showed potent inhibitory effects, with the five metabolites **3**–**7** exhibiting more potent effects.

Stevioside (**1**) is a sweet-tasting glycoside occurring abundantly in the leaves of *Stevia rebaudiana* Bertoni (Compositae), which has been popularly used as a sugar substitute in Japan and Brazil for decades.[1] Hydrolysis of **1** in acid solution produces isosteviol (**2**; *ent*-16-keto-beyeran-19-oic acid), a tetracyclic diterpenoid with a bey-erane skeleton. The biological activities of **2** include the insect antifeeding action,[2] inhibition of rat liver mitochondria functions,[3] decrease of glucose production and inhibition of oxygen uptake in the isolated rat renal tubules,[4] and inhibition of D-glucose and D-fructose transport across the cell membrane in the isolated perfused rat liver.[5] Microbial transformations have recently been used to introduce hydroxyl groups at positions remote from the functional group on diterpenoid molecules,[6] and **2** has been reported to produce metabolites hydroxylated at 7α-, 7β-, 9β-, 12β-, 17-, 1α,7β-, 7β,15β-, 11β,12β-, 12β,15β-, and 11β,12β,17-positions during microbial transformations.[7–10] In continuing our search for potential antitumor promoters (chemopreventive agents) from natural sources,[11] we were especially interested in microbial transformation of **2** to obtain various hydroxylated metabolites that might be more potent than the parent compound.[12] This paper reports the microbial transformation of **2** and evaluation of the metabolites for their inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells as a primary screening test for inhibitors of tumor promoters.

Results and Discussion

Isosteviol (**2**) was obtained by hydrolysis of stevioside (**1**) with dilute hydrochloric acid.[8–10,13] Incubation of **2** with *Aspergillus niger* IFO 4414 yielded three metabolites, **3**, **5**, and **6**, of which two were identified as 7β-hydroxyisosteviol (**3**; *ent*-7α-hydroxy-16-oxobeyeran-19-oic acid)[8,13] and 12β-hydroxyisosteviol (**6**; *ent*-12α-hydroxy-16-oxobeyeran-19-oic acid)[8] by spectral comparison with literature data.

The molecular formula of **5** was determined as $C_{20}H_{30}O_4$ from its HREIMS ([M]$^+$ *m/z* 334.2144) as well as from its ^{13}C NMR DEPT. The compound had one secondary hydroxyl [ν_{max} 3409 cm^{-1}; δ_H 4.16 (1H, ddd, *J* = 5.8, 10.4, 11.3 Hz); δ_C 67.8 (d)], one ketone [ν_{max} 1731 cm^{-1}; δ_C 219.5 (s)], and one carboxyl [ν_{max} 1691 cm^{-1}; δ_C 180.4 (s)] group, in addition to three methyls, eight methylenes, two methines, and four quaternary carbons (see Experimental Section). These data suggested that one methylene carbon of **2** was hydroxylated by the fungus. The hydroxylation had occurred at C-11 by analysis of ^{1}H–^{1}H COSY, HMQC, and HMBC spectra of **5** and comparison of the ^{13}C NMR data with those of **2**.[10] The axial (α) orientation of the oxymethine proton at C-11 was deduced from the splitting pattern (H-11; ddd, *J* = 5.8, 10.4, 11.3 Hz) of the −C*H*OH signal in the ^{1}H NMR spectrum. This was supported from the NOESY experiment in which significant NOE correlation was observed between [H-20(10α-Me)−H-11α]. Consequently, the hydroxyl group had an equatorial (β) orientation and metabolite **5** was *ent*-11α-hydroxy-16-oxobeyeran-19-oic acid (11β-hydroxyisosteviol).

Biotransformation of **2** by *Glomerella cingulata* IFO 9767 produced only one metabolite, which was identified as 17-hydroxyisosteviol (**7**; *ent*-17-hydroxy-16-oxobeyeran-19-oic acid) by spectral comparison with the literature.[3,9,14]

Two oxidized metabolites, 7β-hydroxyisosteviol (**3**)[8,13] and **4**, were obtained from biotransformation of **2** by *Mortierella elongate* IFO 8570. The molecular formula of **4** was determined as $C_{20}H_{28}O_4$ from its HREIMS ([M]$^+$ *m/z* 332.1990) as well as from its ^{13}C NMR DEPT. The compound had one carboxyl [ν_{max} 1680 cm^{-1}; δ_C 179.5] and two ketone [ν_{max} 1718 and 1742 cm^{-1}; δ_C 212.3 and 217.4] groups, in addition to three methyls, eight methylenes, two methines, and four quaternary carbons (see Experimental Section). These suggested that one methylene carbon of **2** was oxidized into a keto group by the fungus. The keto group was shown to be located at C-7 by the analysis of ^{1}H–^{1}H COSY, HMQC, and HMBC spectra of **4** and comparison of the ^{13}C NMR data with those of **2**,[10] and metabolite **4** was, therefore, assigned the structure *ent*-7,16-dioxobeyeran-19-oic acid (7-oxoisosteviol).
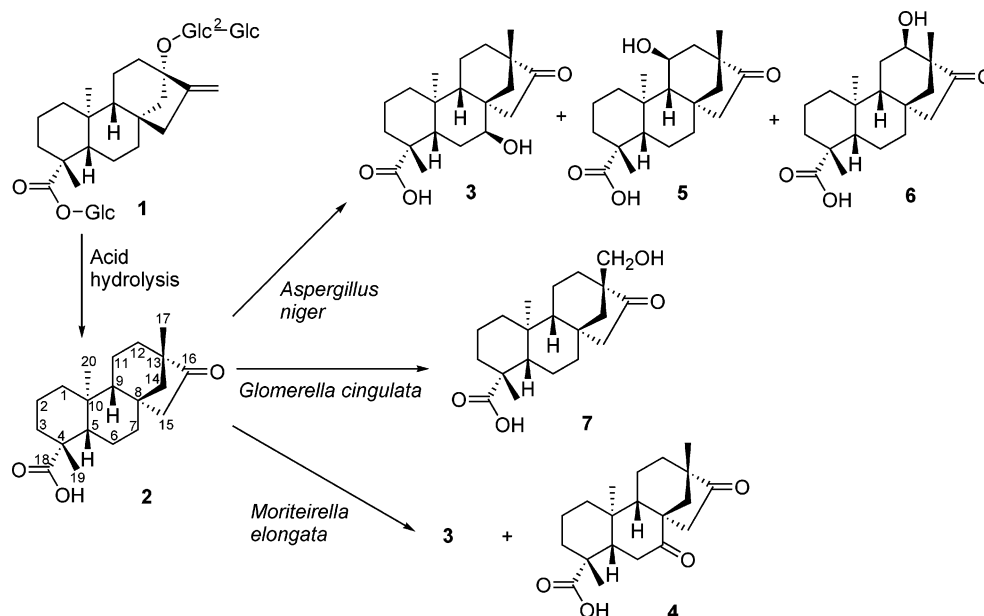
Among the five metabolites, **3**–**7**, obtained from the biotransformation of **2** by the fungi *A. niger*, *G. cingulata*,

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Figure 1. Structures of stevioside (**1**), isosteviol (**2**), and metabolites **3**−**7**.

Table 1. Percentage of Epstein−Barr Virus Early Antigen (EBV-EA) Induciton in the Presence of Compounds **1**−**7**[a]

compound	concentration (mol ratio/TPA)			
	1000		500	100	10
1	12.5	(60)	46.2	78.9	100
2	12.3	(60)	46.7	80.0	98.8
3	6.9	(60)	41.7	76.3	94.6
4	7.4	(60)	35.1	72.3	94.5
5	4.0	(60)	39.8	74.1	91.0
6	5.7	(60)	40.0	75.1	93.2
7	8.2	(60)	42.6	77.0	95.7
β-carotene[b]	8.6	(70)	34.2	82.1	100

[a] Values represent relative percdentages to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells. [b] Reference compound.

and *M. elongate*, metabolites **4** and **5** were new compounds. 7-Oxo compound **4** produced along with a 7β-ol (**3**) from the biotransformation by *M. elongate* may be a further oxidative metabolite of the latter. Consistent with this study, biotransformation of **2** by *A. niger* has recently been reported to produce metabolite **3**.[9]

The inhibitory effects of compounds **1**−**7** on EBV-EA activation induced by TPA were examined for the primary screening of antitumor-promoting activities, and the results are shown in Table 1. Among the compounds tested, the biotransformation products that underwent hydroxylation and oxidation, **3**−**7**, exhibited more potent inhibitory effects, 92−96% inhibition of activation at 1×10^3 mol ratio/ TPA, with preserving high viability (60%) of Raji cells,[15] than those of the biotransformation precursor **2** as well as **1**. The inhibitory effects of the metabolites were almost equivalent to those of β-carotene, intensively studied in cancer prevention using animal models.[16] Thus, this study has established that microbial transformation of natural products (or their derivatives) has a potential value in developing more potent inhibitors of tumor promotion.

Experimental Section

General Experimental Procedures. Crystallizations were performed in acetone, and melting points (uncorrected) determined using a Yanagimoto micromelting point apparatus. Optical rotations were measured on a JASCO DIP-380 polarimeter in MeOH at 25 °C. IR spectra were obtained on a JASCO IR-300 spectrometer in KBr disks. NMR spectra were recorded with a JEOL LA-400 spectrometer at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) in pyridine-d_5, and chemical shifts are expressed in δ (ppm) referred to tetramethylsilane (TMS; ^{1}H NMR). Electron-impact mass spectra (EIMS) and high-resolution EIMS (HREIMS) were recorded on a JEOL JMS-BU20 spectrometer (70 eV) using a direct inlet system. FABMS were obtained with a JEOL JMS-BU20 spectrometer using glycerol as the matrix. Silica gel 60, 220−400 mesh (Merck), was used for open column chromatography. Reversed-phase preparative HPLC was carried out on a 25 cm × 10 mm i.d. C_{18} silica column at 25 °C using a Pegasil ODS II column (Senshu Scientific Co., Ltd., Tokyo, Japan) with MeOH−H_2O− AcOH (70:30:1, v/v/v) as mobile phase at 2.0 mL/min. A refractive index detector was used for HPLC. Thin-layer chromatography (TLC) on 10 × 10 cm silica gel 60G (Merck) was developed using *n*-hexanes−EtOAc−AcOH (60:40:1, v/v/v). Incubations of microorganisms and microbial transformations were performed on an Incubator Shaker IM41 (Yamato Scientific Co., Ltd., Tokyo). Methyl ester derivatives of **2** and **3** were prepared by treatment with ethereal CH_2N_2.

Chemicals and Materials. Stevioside (**1**) was kindly donated by Horiuchi Foods, Co., Ltd. (Tokyo, Japan), and its identification was carried out by FABMS ([M − H]$^+$ *m*/*z* 803) and by ^{1}H and ^{13}C NMR comparison with the literature.[17,18] Potato-dextrose agar, corn steep liquor, and yeast extract from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), glucose, the EBV cell culture reagents, and *n*-butyric acid from Nacalai Tesque, Inc. (Kyoto, Japan), and TPA and β-carotene from Dojindo Laboratories (Kumamoto, Japan) were purchased.

Acid Hydrolysis of Stevioside (1). Compound **1** (8.2 g) was dissolved in H_2O (140 mL) and then treated with concentrated HCl (2.8 mL). The reaction mixture was refluxed for 2 h. The product was recovered in EtOAc and the solvent evaporated. The residue (3.8 g) was chromatographed on a silica gel (180 g) column with a stepwise gradient of *n*-hexanes−EtOAc (95:5 → 0:1; v/v), which yielded isosteviol (**2**; 2.0 g) from the eluant of *n*-hexanes−EtOAc (9:1, v/v). Identification of **2** was done by EIMS ([M]$^+$ *m*/*z* 318)[19] and ^{13}C NMR,[8] and by ^{1}H and ^{13}C NMR as its methyl ester derivative.[13]

Microorganisms and Culture Conditions. All cultures were obtained from the Institute of Fermentation (IFO) (Osaka, Japan). Seven microorganisms were used for the preliminary screening as follows: *A. niger* IFO 4414, *C. longirostre* IFO 9823, *G. cingulata* IFO 9767, *G. fusarioides* IFO 8831, *M. elongate* IFO 8570, *P. funiculosum* IFO 31132, and *P. oxacalcium* IFO 7000. Stock cultures of the fungi were

stored on potato-dextrose agar medium at 24 °C. Seed cultures were obtained by transferring fungi from stock cultures to a yeast extract−corn steep liquor broth medium (YCB; 8 g of corn steep liquor, 1 g of yeast extract, and 10 g of glucose were suspended in 1 L of H_2O). Preliminary screenings were conducted with conical flasks (250 mL) containing 50 mL of YCB medium. For each fungus two flasks were inoculated with the seed culture and incubated on a rotary shaker (110 rpm) for 3 days at 25 °C. Then compound **2** (10 mg) in dimethyl sulfoxide (DMSO) (0.2 mL) was added to one flask, while the other one was kept as a control. The fermentation was continued for 7 more days, after which the mycelium was filtered off and washed with EtOAc. The combined broth, after adjusting the acidity at pH 3−4 by diluted HCl, was extracted three times with EtOAc, and the organic layers were combined and dried with anhydrous Na_2SO_4. After filtration the solvent was evaporated in vacuo, producing the crude extract. The extracts from the experimental flasks were compared to the controls by TLC. Among the seven fungi examined, three, *A. niger*, *G. cingulata*, and *M. elongate*, produced metabolites of **2** reproducibly, and these were used for preparative experiments. Preparative experiments were conducted in 500 mL conical flasks containing 250 mL of YCB liquid medium. The procedure and conditions were the same as for the preliminary experiments.

Biotransformation by *A. niger*. Isosteviol (**2**; 500 mg; R_f value 0.85 in TLC) in 2.0 mL of DMSO was evenly distributed among 4 flasks containing *A. niger* cultures, and one was kept as control. The crude extract (510 mg) obtained was subjected to column chromatography on silica gel (30 g). The column was eluted with 0.7 L of *n*-hexanes−EtOAc (9:1, v/v), 0.8 L of *n*-hexanes−EtOAc (4:1, v/v), 0.3 L of *n*-hexanes−EtOAc (7:3, v/v), and 0.6 L of EtOAc, which yielded fractions A (53 mg), B (278 mg), C (57 mg), and D (76 mg), respectively. Fraction B was unmetabolized **2** (retention time (t_R) 53.0 min in HPLC, R_f value 0.85 in TLC) by MS and ^{1}H NMR analysis. Reversed-phase HPLC of fraction D yielded metabolites **3** (18 mg, 3.6%; t_R 7.8 min, R_f 0.58), **5** (23 mg, 4.6%; t_R 8.3 min, R_f 0.64), and **6** (11 mg, 2.2%; t_R 7.1 min, R_f 0.59). Identification of **3**,[8] as its methyl ester derivative,[13] and **6**[8] was performed by spectral comparison with the literature.

11β-Hydroxyisosteviol (5): fine needles, mp 210−213 °C; $[\alpha]^{25}_D$ −64.6° (*c* 0.13, MeOH); IR ν_{max} 3409 (OH), 1731 (>C= O), 1691 (COOH) cm^{-1}; ^{1}H NMR (pyridine-d_5, 400 MHz) δ 1.08 (3H, s, H-17), 1.18 (1H, m, H$_a$-3), 1.31 (1H, m, H-5), 1.32 (3H, s, H-20), 1.38 (1H, m, H$_a$-1), 1.40 (3H, s, H-19), 1.45 (1H, dd, J = 2.8, 11.6 Hz, H$_a$-14), 1.51 (1H, d, J = 10.4 Hz, H-9), 1.53 (1H, m, H$_a$-7), 1.54 (1H, m, H$_a$-2), 1.59 (1H, dd, J = 3.7, 11.6 Hz, H$_b$-14), 1.62 (1H, m, H$_b$-7), 1.78 (1H, dd, J = 11.3, 11.3 Hz, H$_a$-12), 1.83 (1H, d, J = 18.6 Hz, H$_a$-15), 2.11 (2H, m, H-6), 2.13 (1H, ddd, J = 2.8, 5.8, 11.3 Hz, H$_b$-12), 2.32 (1H, br d, J = 14.0 Hz, H$_b$-2), 2.49 (1H, br d, J = 12.8 Hz, H$_b$-3), 2.87 (1H, dd, J = 3.7, 18.6 Hz, H$_b$-15), 3.25 (1H, br d, J = 13.4 Hz, H$_b$-1), 4.16 (1H, ddd, J = 5.8, 10.4, 11.3 Hz, H-11); ^{13}C NMR (pyridine-d_5, 100 MHz) δ 14.3 (CH$_3$, C-20), 19.7 (CH$_3$, C-17), 20.1 (CH$_2$, C-2), 22.4 (CH$_2$, C-6), 29.9 (CH$_3$, C-19), 38.8 (CH$_2$, C-3), 40.3 (C, C-10), 40.5 (C, C-8), 42.7 (CH$_2$, C-1), 43.2 (CH$_2$, C-7), 44.4 (C, C-4), 49.1 (CH$_2$, C-12), 49.2 (CH$_2$, C-15), 49.3 (C, C-13), 54.0 (CH$_2$, C-14), 57.6 (CH, C-5), 60.3 (CH, C-9), 67.8 (CH, C-11), 180.4 (C, C-18), 219.5 (C, C-16); EIMS *m/z* 334 [M]$^+$ (47), 316 (100), 301 (10), 298 (15), 289 (21), 271 (41), 257 (16), 243 (10), 227 (16), 215 (12), 149 (34), 135 (49), 121 (96), 109 (99); HREIMS *m/z* 334.2144 (calcd for $C_{20}H_{30}O_4$, 334.2144).

Biotransformation by *G. cingulata*. The procedure was similar to the one described for *A. niger*. From 500 mg of **2**, a crude extract (526 mg) was obtained, which was then fractionated by column chromatography on silica gel (30 g). Elution with 0.5 L of *n*-hexanes−EtOAc (9:1, v/v) yielded 37 mg of fraction A′, elution with 1.0 L of *n*-hexanes−EtOAc (9:1, v/v) followed by 0.5 L of *n*-hexanes−EtOAc (4:1, v/v) gave fraction B′ (249 mg), identified as unmetabolized **2**, and further elution with 0.5 L of *n*-hexanes−EtOAc (4:1, v/v) and 0.6 L of EtOAc afforded fraction C′ (192 mg). Reversed-phase HPLC of fraction C′ gave 17-hydroxyisosteviol (**7**; 40 mg, 8.0%; t_R 12.2 min, R_f

0.55), of which identification was done by spectral comparison with the literature.[9,14,19]

Biotransformation by *M. elongate*. The procedure was similar to the one described above for *A. niger*. Bioconversion of **2** (500 mg) by *M. elongate* yielded a crude extract (549 mg), which was subjected to column chromatography on silica gel (30 g). Elution of the column with 1.5 L of *n*-hexanes−EtOAc (9:1, v/v) yielded fraction A″ (53 mg), elution with 1.0 L of *n*-hexanes−EtOAc (4:1, v/v) gave fraction B″ (278 mg), which was identified as unmetabolized **2**, and further elution with 2.0 L of *n*-hexanes−EtOAc (1:1, v/v) afforded fraction C″ (174 mg). Reversed-phase HPLC of fraction C″ gave metabolites **3** (75 mg, 15.0%)[8,13] and **4** (30 mg, 6.0%; t_R 13.0 min, R_f 0.60).

7-Oxoisosteviol (4): fine needles, mp 216−219 °C; $[\alpha]^{25}_D$ −73.8° (*c* 0.13, MeOH); IR ν_{max} 3416 (OH), 1742 and 1718 (>C=O), 1680 (COOH) cm^{-1}; ^{1}H NMR (pyridine-d_5, 400 MHz) δ 0.91 (1H, ddd, J = 3.4, 13.2, 13.2 Hz, H$_a$-1), 1.06 (1H, m, H$_a$-3), 1.07 (3H, s, H-17), 1.11 (3H, s, H-20), 1.28 (3H, s, H-19), 1.32 (1H, m, H$_a$-12), 1.36 (1H, ddt, J = 12.9, 12.9, 4.6 Hz, H$_a$-11), 1.51 (1H, m, H$_a$-2), 1.53 (1H, dd, J = 3.9, 12.2 Hz, H$_a$-14), 1.56 (1H, dd, J = 2.9, 8.8 Hz, H-9), 1.57 (1H, m, H$_b$-12), 1.63 (1H, br d, J = 15.4 Hz, H-5), 1.65 (1H, m, H$_b$-1), 1.67 (1H, m, H$_b$-11), 2.14 (1H, d, J = 18.3 Hz, H$_a$-15), 2.19 (1H, m, H$_b$-2), 2.46 (1H, br d, J = 13.2 Hz, H$_b$-3), 2.64 (1H, dd, J = 2.8, 12.2 Hz, H$_b$-15), 2.75 (1H, dd, J = 3.9, 18.3 Hz, H$_b$-15), 3.05 (1H, dd, J = 3.7, 15.4 Hz, H$_a$-6), 3.44 (1H, dd, J = 15.4, 15.4 Hz, H$_b$-6); ^{13}C NMR (pyridine-d_5, 100 MHz) δ 13.2 (CH$_3$, C-20), 19.5 (CH$_2$, C-2), 20.1 (CH$_3$, C-17), 20.2 (CH$_2$, C-11), 28.6 (CH$_3$, C-19), 36.9 (CH$_2$, C-12), 38.2 (C, C-10), 38.3 (CH$_2$, C-3), 39.7 (CH$_2$, C-1), 40.3 (CH$_2$, C-6), 43.8 (C, C-4), 46.2 (CH$_2$, C-15), 46.6 (CH$_2$, C-14), 48.9 (C, C-13), 51.7 (C, C-8), 54.1 (CH, C-5), 55.1 (CH, C-9), 179.5 (C, C-18), 212.3 (C, C-7), 217.4 (C, C-16); EIMS *m/z* 332 [M]$^+$ (33), 319 (4), 314 (8), 286 (11), 276 (100), 258 (4), 230 (6), 179 (8), 162 (5), 150 (15), 135 (7), 121 (9), 109 (17); HREIMS *m/z* 332.1984 (calcd for $C_{20}H_{28}O_4$, 332.1990).

Method of EBV-EA Induction Tests. The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type), the EBV genome-carrying human lymphoblastoid cells, which were cultivated in 10% fetal bovine serum−Roswell Park Memorial Institute (FBS RPMI) 1640 medium solution. The indicator cells (Raji) (1×10^6/mL) were incubated at 37 °C for 48 h in 1 mL of the medium containing *n*-butyric acid (4 mM, trigger) and 32 pmol of TPA (20 ng/mL, inducer) in DMSO, and a known amount of test compound in DMSO. Smears were made from the cell suspension. The activated cells were stained by high-titer EBV-EA-positive sera from nasopharyngeal carcinoma patients and were detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the experiments were repeated twice. The average extent of EA induction was determined and compared with that on positive control experiments in which the cells were treated with *n*-butyric acid plus TPA where the extent of EA induction was ordinarily more than around 40%. The viability of treated Raji cells was assayed by the Trypan Blue staining method.[20]

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