

Hydroxypterocarpan with estrogenic activity in Aguaje, the fruit of *Mauritia flexuosa* (Peruvian moriche palm)

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ABSTRACT

Background: Phytoestrogens in edible plants, including soybean isoflavones and pomegranates, have been used for alleviation of premenopausal/postmenopausal syndromes and osteoporosis. *Mauritia flexuosa* (moriche palm) is grown in Peru and Brazil for its edible fruit that is said to contain phytoestrogens, but the relevant estrogenic compounds have not been identified. We investigated the constituents of moriche palm fruit extract and evaluated their estrogenic ability.

Materials and methods: Ethyl acetate fraction of dried moriche palm was purified by column chromatography and HPLC chromatography. To evaluate estrogenic activity, we performed [1] simulation of binding to the human estrogen receptor (ER), [2] investigation of the proliferative effect on MCF-7 cells, and [3] the estrogen-chemically activated luciferase gene expression (E-CALUX) assay.

Results: Two hydroxypterocarpan were isolated including lespeflorin G₈ (LF) and 8-hydroxyhomopterocarpan (8-HHP). The binding affinity of LF to ER α was higher than that of 8-HHP, with inhibition constants of 81.9 nM and 1.99 μ M, respectively. However, LF and 8-HHP exhibited a similar proliferative effect on MCF-7 cells at 10 μ M. The E-CALUX assay demonstrated that LF is a full ER agonist and 8-HHP is a partial agonist.

Conclusion: LF was identified as a major estrogenic compound in the fruit of *Mauritia flexuosa*.

Keywords: *Mauritia flexuosa*, moriche palm, lespeflorin G₈, homopterocarpan, estrogenic activity, MCF-7, E-CALUX

BACKGROUND

Complementary herbal therapies have traditionally been used for alleviation of nonspecific complaints [1] including black cohosh [2], red clover [3], wild yam [4], evening primrose [5], and *Angelica sinensis* [6]. While pomegranate juice is said to prevent breast cancer [7, 8] without concrete evidence of its active ingredients and anti-cancer mechanism. Additionally, soybean isoflavones are used to treat estrogen deficiency syndromes, including osteoporosis [9], endometrial disorders [10], vaginal atrophy [11], and menopausal symptoms [12]. The key ingredients in these edible plants are so-called phytoestrogens and such complementary therapy is defined as phytoestrogen therapy.

Mauritia flexuosa (moriche palm or aguaje) is cultivated in the Amazon River territory of Peru and Brazil for its edible fruit [13]. Oil from the fruit is used for cooking [14] and the pulp is processed to make juice and confectionaries [15]. The biological activities of moriche palm fruit such as cholesterol-lowering effect [16], anti-thrombotic effect [17], and antioxidant effect [18, 19] have been reported. Fruits include fatty acids [20, 21] and polysaccharides [22]. Peruvian moriche palm fruit is also said to contain phytoestrogens that induce breast augmentation but its active ingredients have not been identified. Thus, we purified the hydrophobic fraction of the moriche palm fruit to search for estrogenic compounds and identified two hydroxypterocarpan with estrogenic activity. Pterocarpan belong to the flavonoids and are structurally similar to isoflavonoids known as typical phytoestrogens. We then investigated the properties of estrogenic effects of these hydroxypterocarpan.

MATERIALS AND METHODS

Isolation of estrogenic compounds from Peruvian moriche palm fruit

Dried moriche palm fruit pulp was obtained from Peru through Ajuto Inc. (Tokyo, Japan). The powdered pulp (2 kg) was defatted 3 times with *n*-hexane (2 L) at 40°C, after which the residue was extracted with ethanol (3 L) for 2 hr at 60°C and the solvent was evaporated. Then *n*-hexane (2 L) was added to remove residual oily substances. Finally, an ethanol extract (124.8 g) was obtained with a yield of 6.2%. This extract was partitioned twice with ethyl acetate (0.5 L) to obtain the ethyl acetate portion (12 g), which was separated by silica gel column chromatography

(100 g, YMC gel, YMC Co. Ltd., Kyoto, Japan) with ethyl acetate (0.3 L) to obtain the ethyl acetate fraction. Next, this fraction was repeatedly purified by HPLC (RPAQUEOUS, Nomura Chemical Co. Ltd., Seto, Japan, 20 ϕ \times 250 mm) with 85% methanol to obtain compound **1** (28.7 mg) and compound **2** (6.7 mg). These compounds were identified as 8-hydroxy-3,9-dimethoxy-10-isoprenylpterocarpan (**1**, lespeflorin G₈) and 8-hydroxyhomopterocarpan (**2**) by comparing the ¹H-NMR spectra with reference data [23] (Fig. 1).

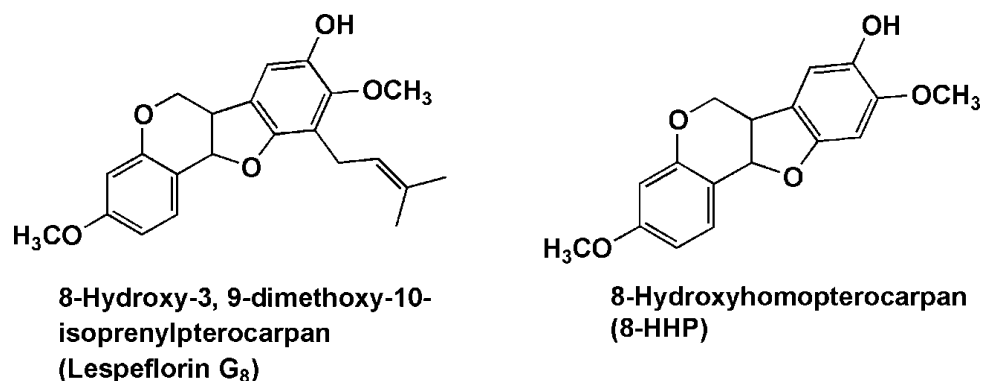


Fig. 1. Hydroxypterocarpan isolated from moriche palm fruit

Reagents

Dulbecco's modified Eagle's medium (D-MEM), phenol red-free D-MEM, penicillin (100 units/ml) and streptomycin (100 μ g/ml) mixture, daidzein, and genistein were obtained from FUJIFILM Wako Pure Chemical Co. Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Biosera (Boussense, France). Charcoal/dextran-treated FBS was sourced from GE Healthcare Japan, (Tokyo, Japan). (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was purchased from Dojindo Laboratories (Kumamoto, Japan). For the E-CALUX assay, RPMI 1640 medium with L-glutamine was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). D-MEM without L-glutamine and phenol red was purchased from MP Biomedicals Inc. (California, USA), while UCDA qualified FBS was from Mediatech Inc. (Virginia, USA). 17 β -estradiol was sourced from Sigma-Aldrich Japan, (Tokyo, Japan). The luciferase assay system was purchased from Promega (Wisconsin, USA).

Cell culture

The human metastatic breast cancer cell line MCF-7 (JCRB0134) was purchased from the JCRB Cell Bank (Japan). The cells were subcultured in D-MEM supplemented with 10% FBS and penicillin (100 units/ml) and streptomycin (100 μ g/ml) mixture at 37°C under a 5% CO₂ atmosphere. The human BG1Luc4E2 cell line was obtained from XDSI Inc. (North Carolina, USA). The cells were maintained in RPMI-1640 containing 1% penicillin and streptomycin and

8% FBS. After reaching 80% confluence, the medium was changed to phenol red-free D-MEM containing 4.5% charcoal-stripped FBS, 1.9% L-glutamine, and 0.9% penicillin and streptomycin for the E-CALUX assay.

Simulation of receptor binding

The 3D structures of human ER α (PDB ID: 1G50) and ER β (PDB ID: 3OLS) were obtained from the RCSB Protein Data Bank. ER α and ER β consist of three and two identical chains, respectively. To obtain the Apo state structure of ER α and ER β , two identical chains of ER α and one chain of ER β were removed by using AutoDock software (ver. 4.2, The Scripps Research Institute). Additionally, all H₂O molecules and the bound ligand (17 β -estradiol) were removed from the structures. 3D structures of LF and 8-HHP were obtained from the PubChem database. Energy minimization and optimization of the molecular geometry of these compounds were carried out with Arguslab software (ver. 4.0.1). Molecular docking studies were performed with AutoDock software, using the Lamarckian Genetic Algorithm [24]. Blind docking was performed to identify the most favorable binding model. The input grid box size was set at 70 \times 70 \times 70 points, with the active site residues at the center of the box. For simulation of docking, the conformation ranking was based on the score calculated with an energy scoring function. After docking simulation, the most favorable docking poses and hydrogen bond interactions were visualized and analyzed with PyMOL software (Schrodinger).

MCF-7 proliferation assay

According to previously reported methods [25], subcultured MCF-7 cells (2,000/100 μ L) were suspended in phenol red-free D-MEM containing 5% CS-FCS, seeded into a 96-well culture plate, and incubated for 24 hr. Then a test solution dissolved in DMSO and diluted in medium (11 μ L) was added to the wells and the cells were cultured for 4 days. Subsequently, MTT solution (5 mg/mL in PBS) was added (10 μ L) and incubation continued for 4 hr. After removing the medium, 40 mM HCl in isopropanol (100 μ L) was added to dissolve the formazan product and absorbance of the formazan solution was measured with at 570 nm referenced at 660 nm.

E-CALUX assay

This experiment was performed according to the OECD guideline [26]. Subcultured BG1Luc4E2 cells (2.0×10^5 /200 μ L) were seeded into a 96-well culture plate and incubated overnight. Then the medium was replaced with D-MEM containing 1% penicillin and streptomycin, 10% charcoal-stripped FBS, and 2% glutamine. A test solution (190 μ L) dissolved in DMSO (final concentration

of 1.0%) was added and culture was done for 24 hr at 37°C under a 5% CO₂ atmosphere, after which luminescence was measured in the range of 300 to 650 nm by using a Luciferase assay system and a luminometer.

Statistics

Results are presented as the mean±SE in Fig. 4 and as the mean±SD in Fig. 5. For statistical analysis, one-way analysis of variance (ANOVA) was performed, followed by Dunnett's test, and $p < 0.05$ or $p < 0.01$ was considered to indicate significance.

RESULTS

Hydroxypterocarpan in moriche palm fruit

The compounds found in the oily components (ethyl acetate fraction) mainly consisted of free oleanolic acids, as well as oleanolic acid diglycerides and triglycerides. However, both lespeflorin G₈ (LF) and 8-hydroxyhomopterocarpan (8-HHP) were clearly distinguished as orange spots by performing RP-18 thin-layer chromatography using 80% MeOH, followed by spraying with 10% H₂SO₄ and heating at 180°C (Fig. 2).

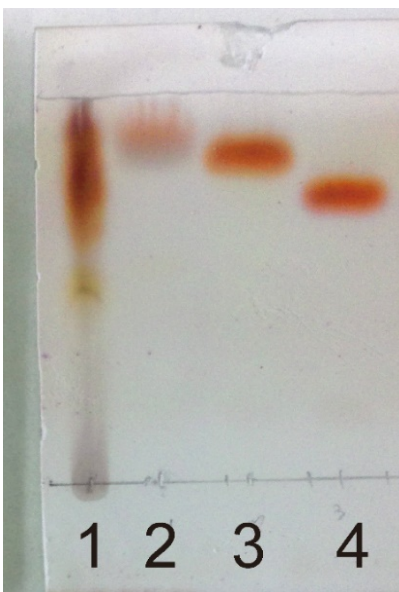


Fig. 2. A thin-layer chromatogram of ethyl acetate fraction of moriche palm, LF and 8-HHP.

Lane 1: ethyl acetate fraction of moriche palm, lane 2: unknown substances, lane 3: LF, Lane 4: 8HHP. All lanes were developed by 80% MeOH on a RP-18 plate. The spots were visualized by spraying 10% H₂SO₄ and heating at 180°C

Simulation of binding to the human estrogen receptor (ER)

Molecular docking studies were performed to investigate binding between LF or 8-HHP and ER receptors. The most favorable docking model was identified by using the energy scoring function of Autodock software (Fig. 3). The binding parameters for LF and 8-HHP to ER receptors are listed in Table 1. The estimated binding free energy of LF and 8-HHP to ER α was calculated to be -9.67 and -7.78 kcal/mol, respectively. The LF-ER α complex demonstrated lower values of intermolecular and van der Waals desolvation energy compared to the 8-HHP-ER α complex. The best docked poses of LF and 8-HHP with ER α are shown in Fig. 3. LF forms two hydrogen bonds with Arg394 and Leu525 of ER α , while 8-HHP forms one hydrogen bond with Arg394. These residues are known to contribute to stable hydrogen bonding with 17 β -estradiol. Consequently, the inhibition constant of LF and 8-HHP for ER α was 81.9 nM and 1.99 μ M, respectively. The results indicated that LF has much higher binding affinity for ER α than 8-HHP. On the other hand, the inhibition constant of LF and 8-HHP for binding to ER β was 2.46 μ M and 33.97 μ M, respectively, being larger than for binding with ER α . From these data, LF and 8-HHP are suggested to show high binding affinity and selectivity for ER α , but not ER β .

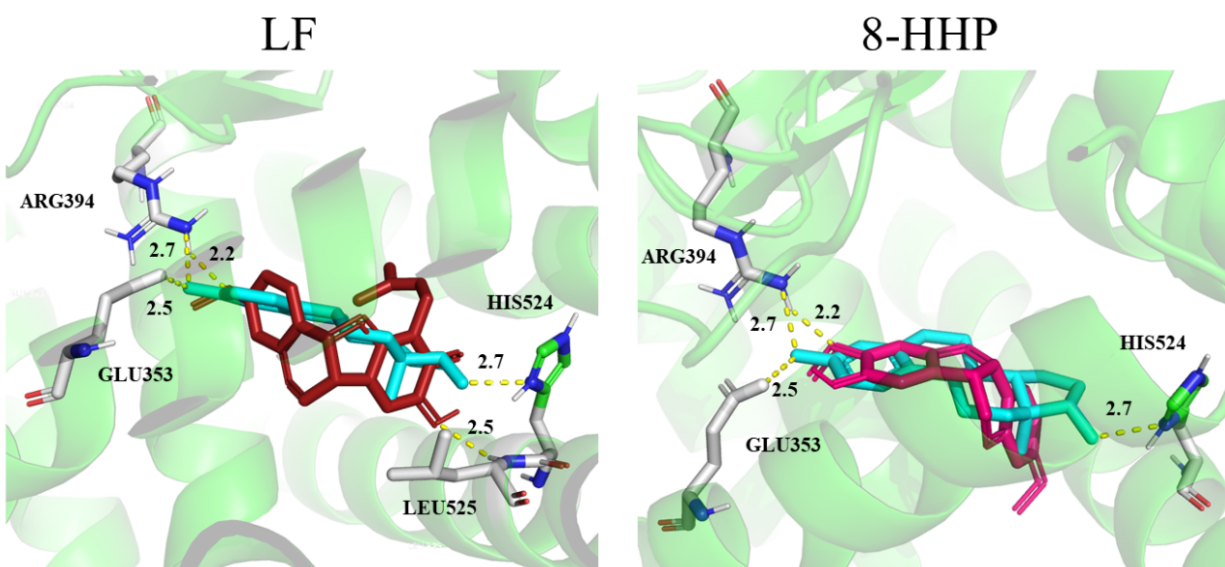


Fig. 3. Superimposed binding modes of 17 β -estradiol (cyan) and LF (red) or 8-HHP (magenta) at the active site of ER α .

Hydrogen bonds are represented as yellow colored dashed lines. The key residues involved in hydrogen bonds are also shown.

Table 1. Binding parameters for ER α and ER β

Auto dock parameter	LF-ER α	8-HHP-ER α
Binding energy (kcal/mol)	-9.67	-7.78
Inhibition constant	81.9 nM	1.99 μ M
Intermolecular energy	-11.16	-8.67
Van der Waals H-bond desolvation energy	-11.05	-8.45
Electrostatic energy (kcal/mol)	-0.11	-0.22
No. of hydrogen bonds	2	1
Residue involved in hydrogen bonding	ARG394, LEU525	ARG394
	LF-ER β	8-HHP-ER β
Binding energy (kcal/mol)	-7.65	-6.10
Inhibition constant	2.46 μ M	33.97 μ M
Intermolecular energy	-9.14	-6.99
Van der Waals H-bond desolvation energy	-9.11	-6.86
Electrostatic energy (kcal/mol)	-0.03	-0.13
No. of hydrogen bonds	3	1
Residue involved in hydrogen bonding	GLY472, HIS475, LEV476	ARG345

Estrogen-dependent cell proliferation

After treatment with LF or 8-HHP at 10 μ M, MCF-7 cells demonstrated a significant increase of proliferation by approximately 25% compared to the control (Fig. 4). Daidzein derived from soy beans (0.3 to 10 μ M) also significantly activated the proliferation of MCF-7 cells and genistein (0.1 to 10 μ M) had an even stronger effect on MCF-7 cell proliferation.

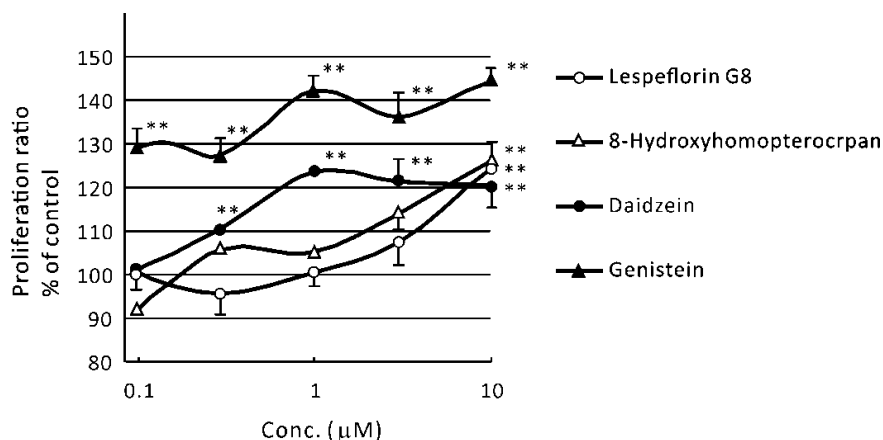


Fig. 4. Estrogenic proliferation of MCF-7 cells in response to LF and 8-HHP

Cells were treated with LF or 8-HHP for 4 days. Each point represents the mean and SE (n=8).

** : significant difference from the control at $p < 0.01$.

E-CALUX assay

Figure 5 displays binding curves for LF and 8-HHP. LF showed ER binding from 1.56 to 12.5 μM , with an EC_{50} value of 4.4 μM (Table 2), and its maximum relative luminescence was similar to that of 17β -estradiol. 8-HHP showed ER binding from 3.13 to 25 μM , with an EC_{50} value of 9.6 μM , and its maximum relative luminescence was only half that of 17β -estradiol. The shape of the inhibition curve of LF is more similar to 17β -estradiol than that of 8-HHP. Based on these findings, LF and 8-HHP seemed to be full and partial agonists, respectively.

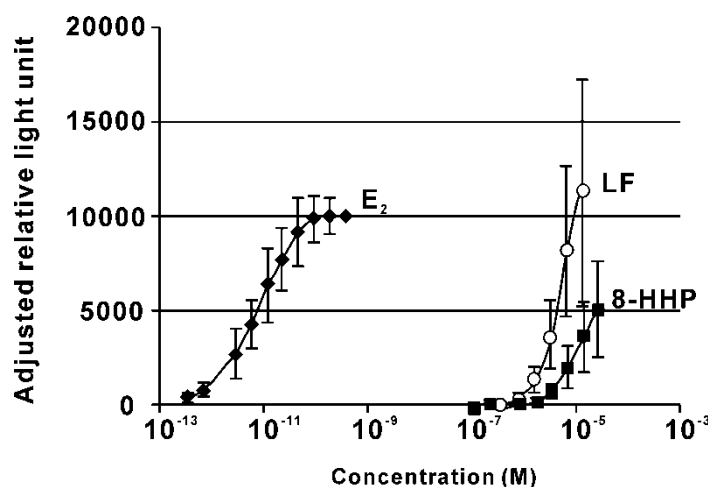


Fig. 5. ER agonistic effects of LF and 8-HHP in BG1Luc4E2 cells

E_2 : 17β -estradiol. Cells were treated with LF or 8-HHP for 20 hr. Each point represents the mean and SD (n=4).

Table 2. EC_{50} values and relative activity of LF and 8-HHP vs. 17β -estradiol in the E-CALUX assay

	EC_{50} (μM)	Relative activity
17β -Estradiol	0.00000783	1
Lespeflorin G_8	4.40	1 / 562,578
8-Hydroxyhomopterocarpan	9.64	1 / 1,231,336

DISCUSSION

In this study, we isolated candidate estrogenic compounds from moriche palm oil. Pterocarpan present in Legminosase plants [soybean leaves (27) and Japanese clover (23)] and Fabaceae plants [Babch (28) and *Sophora tonkinensis* (29)]. Its structure is similar to that of isoflavons, and coumestans, which are well known phytoestrogens. However, as there have not been any studies reporting the existence of pterocarpan in fruits of Arecaceae plants rich in oil substances, such as

palms, our finding is the first report to find estrogenic compounds in Areaceae plants. On the other hand, several pterocarpan have been reported to exhibit estrogenic activities. Glyceollin I, which accumulates in stressed soy bean, stimulates both ER α and ER β [30]. Medicarpin isolated from legumes strongly activates osteoblasts by stimulating ER β and suppresses bone loss in rats [31]. Thus, we speculated that hydroxypterocarpan isolated from moriche palm might exhibit estrogenic activities.

According to the results of the binding simulation, LF was suggested to bind strongly to ER α with 30-fold higher affinity than for ER β . On the other hand, the binding affinity of 8-HHP for ER α was approximately 1/24 of that shown by LF and its affinity for ER β was quite low. The results of the binding simulation suggested that LF would show estrogenic activity. Accordingly, we evaluated the estrogenic effects of LF and 8HHP on proliferation of MCF-7 cells expressing the ER α . We found that LF and 8-HHP both showed slight estrogenic activity, which is weaker than that of soybean isoflavones. In addition, LF and 8-HHP both exhibited agonistic activity in the E-CALUX assay, with EC₅₀ values of 4.4 and 9.6 μ M, respectively. BG1Luc4E2 cells show predominant expression of ER α over ER β . Since LF had a higher affinity for ER α than 8-HHP, it is reasonable for LF to display stronger agonistic activity than 8-HHP. According to the OECD guideline, the EC₅₀ values of daidzein and genistein are 0.795 and 0.271 μ M, respectively [26]. Thus, the activity of LF is 5.5-fold lower than that of daidzein and 16.2-fold lower than that of genistein.

Recently, several phytoestrogens in *Pueraria candollei* var. *mirifica* [32] which has potent estrogenic activity, have been reported to show adverse effects in Japanese female including atypical genital bleeding. These estrogenic compounds are miroestrol and isomiroesterol, which exhibit strong estrogenic activities similar to estradiol [33-35]. With this background of phytoestrogens in mind, mild phytoestrogens with estrogenic activity milder than soy isoflavons are favorable. LF and 8-HHP, which are mild phytoestrogens, will satisfy the demand of functional foods applicable for female symptoms caused by estrogen deficiency.

CONCLUSION

LF was identified as a major estrogenic compound in moriche palm fruit and was found to be a full ER α agonist. 8-HHP was a partial agonist bound to ER. This report is a first to have found estrogenic compounds in the oil fraction of palms.

Conflict of interests: There is no conflict of interest to be declared by the authors.

Abbreviations: ER - estrogen receptor; E-CALUX - estrogen-chemically activated luciferase gene expression; LF - lespeflorin G₈; 8-HHP - 8-hydroxyhomopterocarpan; D-MEM – Dulbecco’s modified Eagle’s medium; FBS - fetal bovine serum; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS - phosphate-buffered saline

Competing Interest: The authors declare no conflicts of interest associated with this manuscript.

Authors’ Contributions: Dr. Shimoda isolated the compounds, tested MCF-7 cells, and wrote the manuscript. Mr. Takeda identified the chemical structures. Professor Matsuda and Dr. S. Nakamura provided support for structural identification. Dr. Takarada performed docking simulation. Mr. Shimizu extracted moriche palm extract and Miss Kato purified the chemical compounds. Dr. M. Nakamura and Dr. Handa performed the E-CALUX assay.

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