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Anti-H5N1 virus new diglyceride ester from the Red Sea grass *Thallasodendron ciliatum*

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Some Egyptian plants were screened against highly pathogenic avian influenza strain H5N1 using plaque inhibition assay in Madin–Darby canine kidney. The results indicated that the extracts of Red Sea grass *Thallasodendron ciliatum* possessed potent antiviral activity (100% inhibition at the concentration of 1 $\mu g\,mL^{-1}$). The bioactivity-guided fractionations led to the isolation of a new diglyceride ester (**1**) along with asebotin (**2**) for the first time from the plant. The two isolates showed reduction of virus titre by 67.26% and 53.81% inhibition at concentration of 1 $ng\,mL^{-1}$, respectively.

Keywords: *Thallasodendron ciliatum*; diglyceride ester; asebotin; anti-influenza virus (H5N1)

1. Introduction

Influenza A viruses belong to the family Orthomyxoviridae and have been isolated from a variety of different species. Further subtyping of influenza A viruses is based on antigenic differences between the two surface glycoproteins haemagglutinin (H1–H16) and neuraminidase (N1–N9) of the influenza A viruses (Fouchier et al., 2005). Of them, human H1N1 and H3N2 influenza A viruses are highly contagious and are the main cause of 'seasonal influenza' worldwide (Fiore, Shay, Broder, Iskander, & Uyeki, 2008). Since late 2003, highly pathogenic avian influenza (HPAI) H5N1 viruses have appeared in Southeast Asian countries, and have become a global problem with human involvement (Hien, De Jong, & Farrar, 2004). Recently, in 2009, swine–origin pandemic (H1N1) virus has emerged and spread rapidly all over the world (Dawood, Jain, Finelli, Shaw, & Lindstrom, 2009). In mid-February 2006, a devastating HPAI H5N1 virus disease has become enzootic in poultry throughout Egypt and still circulates in the poultry population (Aly, Arafa, & Hassan, 2008). In that period, Egypt had the highest number of human avian influenza cases outside Asia, and the second highest number worldwide. Until now human avian influenza remains as unresolved threat to veterinary and public health with zoonotic transmission potential to human in Egypt (Kandeel et al., 2006).

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The application of antiviral drugs during the early phase of a pandemic could be of help to control it (Monto, 2005). Detection of new, potent and cheap anti-influenza agents from plant and marine extracts could be used for the treatment and prevention of influenza, particularly for HPAI H5N1 strain, for avian population which, in turn, will prevent zoonotic transmissions and will limit the spread of the viruses.

Currently, two types of anti-influenza drugs are available: M2 ion channel blockers (amino-adamantines: amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) (Davies et al., 1994; Hayden, Cote, & Douglas, 1980). However, amino-adamantine-resistant viruses readily emerged and are already prevalent worldwide among the seasonal influenza viruses (Bright, Medina, Xu, Perez-Oronoz, & Wallis, 2005; Bright, Shay, Shu, Cox, & Klimov, 2006). Oseltamivir-resistant H5N1 viruses have been isolated from patients (De Jong, Tran, Truong, Vo, & Smith, 2005). In addition, current production capability has limitations and is insufficient to provide adequate amounts of drugs for mass treatment and chemoprophylaxis. Current medicines are always dogged with anxiety for emergence of resistant viruses. Thus, in the advent of an influenza virus pandemic, it is likely that the administration of antiviral drugs will be an important first line of defense against the virus. Therefore, we try to find new drugs with anti-influenza virus activity through the evaluation of this activity in certain Egyptian natural extracts.

The Egyptian flora and Red Sea are rich in plants that have been used by the local communities for the treatment of a large number of ailments. Therefore, plants of Egypt still need more investigation to detect their importance in the discovery of new sources for new molecules that can be used in the treatment of different ailments following their traditional uses. Screening of different Egyptian plants against influenza virus (H5N1) led to the discovery of Red Sea grass *Thallasodendron ciliatum*, which showed potent activity against H5N1 influenza virus.

Sea grasses are monocotyledonous vascular flowering plants that live in coastal and estuarine areas of the world. They are unique in that they are usually totally submerged in the water, they possess a root system with stems buried within a soft substrate, they have vegetative and sexual reproduction and have flowers fertilised by water-borne pollen (Aioi, Mukai, & Koike, 1981). There are approximately 50 species of sea grasses in 12 genera. Seven genera are considered tropical, while the remaining five are more or less confined to temperate waters (Pollard & Greenway, 1993). Sea grass can be found all over the world except in the polar region. In many places, sea grasses cover extensive areas, which are usually called as sea grass beds. Of the types of communities that make up single sea grass beds, is *T. ciliatum* (Phillips & Durako, 2000). *T. ciliatum* is a tropical sea grass which can be classified as sub-tidal and not deep water bed-forming sea grass (Carruthers et al., 2001).

The anti-influenza H5N1-guided fractionation led to the isolation and structure elucidation of a new metabolite diglyceride ester (**1**) along with the previously known metabolite, asebotin (**2**), for the first time from *T. ciliatum.* The two isolates showed reduction of virus titre by 67.26% and 53.81% at concentration of 1 ng mL^{-1}, respectively.

2. Results and discussion

Based on the acquired potent antiviral activity (100% inhibition at the concentration of 1 μg mL^{-1}) of the *T. ciliatum* extract, the bioactivity-guided chromatographical processing was run. Two compounds were purely isolated and spectroscopically analysed. The structure elucidation procedure of the two compounds would be stated in the following.

2.1. *Compound 1*

The HR-ESI-MS spectrum of **1** displayed a pseudo-molecular ion peak at *m/z* 619.5298 $[M+H]^+$ which when combined with detailed analysis of the ^{13}C spectrum and Distortionless

Enhancement by Polarization Transfer (DEPT) indicated a molecular formula of $C_{39}H_{70}O_5$ representing five units of unsaturation. Analysis of ^{13}C NMR spectrum and DEPT indicated the presence of two oxygenated methylenes at δ 62.1, one oxygenated methine at δ 68.9 together with two CO for the two ester moieties at δ 172.8 and 173.2. Also the presence of three di-substituted olefinic moieties was confirmed by ^{13}C NMR resonances at δ 127.8, 128.0, 129.6, 129.8, 129.9 and 130.1. The above-mentioned findings were consistent with the presence of a diglyceride ester. GC–MS analysis of the fatty acid methyl ester of **1** was carried out after hydrolysis and yielded two peaks with molecular ions of *m/z* (326) and (264) corresponding to ($C_{16:3}$) and C_{20} fatty acid methyl esters. The fatty acids were identified as the unsaturated fatty acids; (4E,7E,10E)-hexadeca-4,7,10-trienoic acid and arachidic acid by library search National Institute of Standards and Technology (NIST) and by comparing with published data (Asselineau & Lededrer, 1961; Xu, Miao, & Wu, 2006). The placement of the fatty acid at C-1 and C-3 of the diglyceride ester was assigned by $^1H-^1H$ COSY between H-2 and H_2-1 and H-2 and H_2-3. This was confirmed by HMBC of H_2-1/C-2 ($^2J_{CH}$), H_2-1/C-3 ($^3J_{CH}$), H_2-1/C-1′ ($^3J_{CH}$), H_2-3/C-2 ($^2J_{CH}$), H_2-3/C-1 ($^3J_{CH}$), H_2-3/C-1″ ($^3J_{CH}$), H-2/C-1 ($^2J_{CH}$), H-2/C-3 ($^2J_{CH}$) which revealed that **1** is 1,3-diglyceride ester (Figure 1). The position of the double bonds was confirmed by $^1H-^1H$ COSY spectrum between H-4″/H-5″, H-5″/H_2-6″, H_2-6″/H-7″, H-7″/H-8″, H-8″/H_2-9″ and H_2-9″/H-10″ and also from HMBC of H_2-2″/C-3″ ($^2J_{CH}$), H_2-2″/C-4″ ($^3J_{CH}$), H_2-3″/C-5″ ($^3J_{CH}$), H_2-6″/C-8″ ($^3J_{CH}$), $H_2$9″/C-11″ ($^3J_{CH}$) and H_2-12″/C-10 ($^3J_{CH}$), leading to the assignment of the C-4″/C-5″/C-6″/C-7″/C-8″/C-9″/C-10″/C-11″/C-12″ (Figure 1). Detailed analysis of COSY and HMBC correlations was found to be in complete agreement with the proposed structure for **1**. The name 3-(4E,7E,10E-hexadeca-4,7,10-trienoyloxy)-2-hydroxypropyl icosanoate was assigned to the new diglyceride.

2.2. *Compound 2*

This was identified as asebotin by comparing its spectroscopic properties with those reported in the literature (Yao et al., 2005).

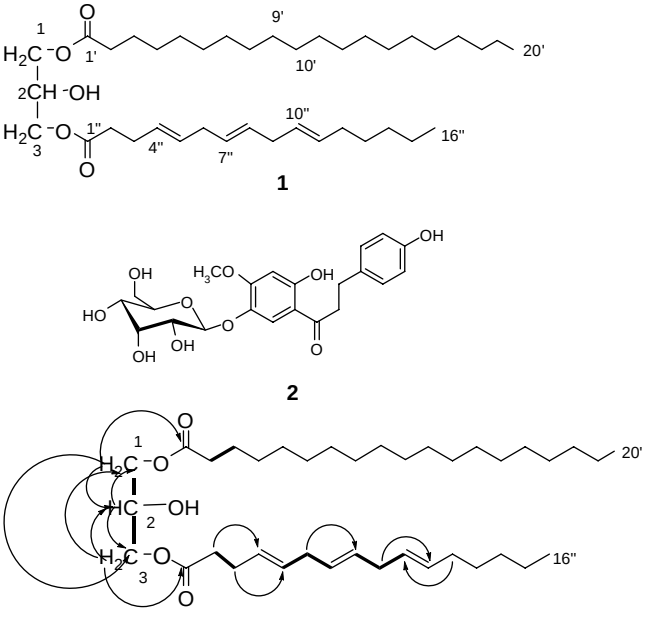

Figure 1. Important HMBC (arrows) and COSY (bold) correlations of **1**.

2.3. *Anti-influenza activity*

Anti-influenza activity of dilutions of the extract, its fractions and the pure compounds was measured by a plaque inhibition assay in Madin–Darby canine kidney (MDCK). The extract completely inhibited the growth of the virus at the concentration of 1 $\mu g\,mL^{-1}$ as compared to the antiviral drug zanamivir, whereas compounds **1** and **2** showed reduction of virus titre by 67.26% and 53.81% inhibition at concentration of 1 $ng\,mL^{-1}$, respectively (Table 1 and Figure 2).

3. Materials and methods

3.1. *General experimental procedures*

^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPT-135 and 2D-NMR spectra were recorded using the residual solvent signal as an internal standard on a Varian AS 400 (Varian Inc., Palo Alto, CA, USA). IR spectra were recorded on a Bruker Tensor 27 (Bruker Corporation, Billerica, MA, USA). UV spectra were obtained on a Varian Cary 50 Bio UV–visible spectrophotometer. The UV spectra were recorded on a double beam Shimadzu UV–visible spectrophotometer (model UV-1601 PC, Kyoto City, Japan). IR spectra were recorded on Nicolit FT IR spectrophotometer (Nicolet Company, Nicolet, Canada) range 400–4000, USA. High resolution mass spectra were recorded using a Bruker BioApex (Bruker Corporation).

Fatty acid methyl esters were identified using Hewlett Packard (HP) gas liquid chromatography, series 6890 equipped with flame ionisation detector. A capillary column (HP-INNOWAX, polyethylene glycol, 30 m × 530 μm, film thickness 1.00 μm) was used in separation of fatty acids. The injector port temperature was set at 250°C (splitless mode) and a pressure of 14.81 psi and the detector cell at 275°C. The flow rate of the carrier gas (N_2) was 30 mL/min. The initial column temperature was 70°C and increased to 200°C by the rate of 4°C/min, then isothermally for a total run time of 32.5 min.

Pre-coated silica gel G-25 UV_{254} plates were used for thin layer chromatography (TLC) (20 cm × 20 cm) (E. Merck, Darmstadt, Germany). Silica gel Purasil 60A, 230–400 mesh was used for flash column chromatography (Whatman, Sanford, ME, USA).

3.2. *Plant material, collection and identification*

The sea grass *T. ciliatum* (coll. no. SAA-41) was collected from Safaga at the Egyptian Red Sea, air-dried and stored at low temperature (−24°C) until processed. The plant was identified by

Table 1. *In vitro* anti-influenza virus activity of *T. ciliatum* extract, its fraction and compounds **1** and **2**.

Test extracts and compounds	Concentration	Plaque assay (mean ± SD)	Plaque inhibition (%)
Virus control		22.3 ± 1.52	
Zanamivir	10 $ng\,mL^{-1}$	0	100
Extract	1 $\mu g\,mL^{-1}$	0	100
Hexane	1 $\mu g\,mL^{-1}$		0
25% AcOEt in Hexane	1 $\mu g\,mL^{-1}$	14 ± 1	37.3
50% AcOEt in Hexane	1 $\mu g\,mL^{-1}$		0
75% AcOEt in Hexane	1 $\mu g\,mL^{-1}$		0
AcOEt	1 $\mu g\,mL^{-1}$		0
25% MeOH in AcOEt	1 $\mu g\,mL^{-1}$	15.3 ± 0.6	31.3
50% MeOH in AcOEt	1 $\mu g\,mL^{-1}$		0
75% MeOH in AcOEt	1 $\mu g\,mL^{-1}$		0
MeOH	1 $\mu g\,mL^{-1}$		0
Compound **1**	1 $ng\,mL^{-1}$	7.33 ± 1.52	67.26
Compound **2**	1 $ng\,mL^{-1}$	10.33 ± 1.15	53.81

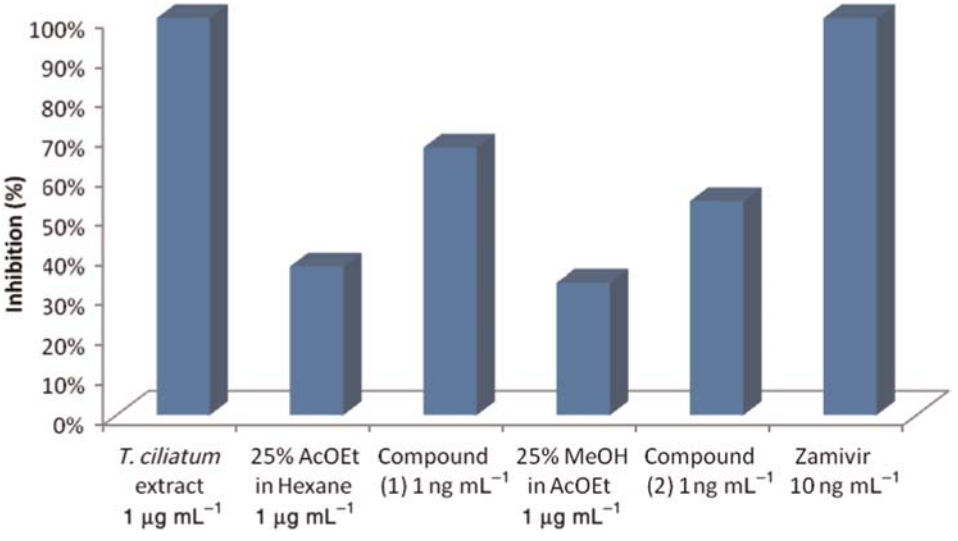

Figure 2. The antiviral effect of compounds (**1** and **2**) and their related extracts against H5N1 virus compared to zamivir using plaque inhibition assay in MDCK.

Dr Tarek Temraz, Marine Science Department, Faculty of Science, Suez Canal University, Ismailia, Egypt. A voucher specimen was deposited in the herbarium section of Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt under registration number SAA-41.

3.3. *Extraction and isolation*

T. ciliatum was dried (700 g dry weight), grounded and extracted with a mixture of MeOH/CH_2Cl_2 (1:1) (3 × 2 L) at room temperature. The extract was evaporated under vacuum to afford 150 g residue. The residue was subjected to vacuum liquid chromatography on a flash silica gel using hexane, EtOAc and MeOH gradient.

The fraction eluted using 25% EtOAc in hexane was concentrated to afford 5 g of green residue. Purification of this fraction was done by flash column chromatography on silica gel using hexane/EtOAc (95:5). Fractions with the same TLC pattern were combined and rechromatographed by flash column chromatography on silica gel using hexane/EtOAc (95:5) to afford **1** (156 mg) ($R_f = 0.70$, 10% EtOAc/hexane).

The fraction eluted with 25% MeOH in EtOAc was concentrated to afford 4 g residue, which was purified by column chromatography on silica gel using hexane/EtOAc (10:90) to MeOH/EtOAc (25:75). Fractions eluted with 5% MeOH in EtOAc with the same TLC pattern were combined and rechromatographed by flash column chromatography on silica gel using 5% MeOH in EtOAc. Final purification was done by crystallisation from MeOH to afford **2** (8 mg) ($R_f = 0.50$, 10% MeOH/$CHCl_3$).

3.4. *Hydrolysis of 1*

Compound **1** was refluxed with 10% ethanolic KOH and benzene for 24 h. After adding H_2O, the reaction mixture was extracted with ether. The aqueous alkaline solution was acidified with HCl and extracted with ether. The ether fraction was concentrated to yield a fatty acid.

3.5. *Preparation of fatty acid methyl esters of 1*

Prepared fatty acid of **1** was refluxed with MeOH and concentrated H_2SO_4 for 1 h. After adding H_2O, the reaction mixture was extracted with ether and dried over anhydrous $MgSO_4$. The resulting ether-soluble fraction was analysed by GC–MS.

3.6. *Isolates*

Compound (**1**): colourless oil, $R_f = 0.70$, 10% EtOAc/hexane; UV (MeOH) λ_{max} (log ε) 219 nm; IR (KBr) (thin film) ν_{max} 3506.23, 2130.56, 1655.90, 1432.39, 1031.21, 955.28 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz). HR-ESI-MS (positive ion mode) $m/z = 619.5298$ $[M + H]^+$ (calcd for $C_{39}H_{70}O_5$:619.5302). ^1H NMR ($CDCl_3$, 400 MHz): δ_H 0.87 (6H, t, $J = 6.8$ Hz, H_3-16″ and H_3-20′), 1.24 (44H, m, H_2-4′-19′ and H_2-13″-15″), 1.59 (2H, m, H_2-3′), 1.99 (6H, m, H_2-6″, H_2-9″ and H_2-12′), 2.02 (2H, m, H_2-3″), 2.31 (4H, m, H_2-2′ and H_2-2″), 2.78 (2H, m, H_2-6″), 4.17 (2H, dd, $J = 2.4$, 6.8 Hz, H-1 and H-3), 4.29 (2H, dd, $J = 2.4$, 6.8 Hz, H-1 and H-3), 5.20 (H, q, $J = 1.6$ Hz, H-2), 5.33 (6H, m, H-4″, H-5″, H-7″, H-8″, H-10″ and H-11″), ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 14.1 (C-20′), 14.2 (C-16″), 22.5 (C-19′), 24.8 (C-15″), 24.9 (C-3′), 25.6 (C-6″ and C-9″), 27.2 (C-3″), 29.1 (C-14″), 29.6 (C-4′-18′ and C-13″), 31.8 (C-12″), 31.9 (C-6′ and C-9′), 33.9 (C-2′), 34.2 (C-2″), 62.1 (C-1 and C-3), 68.9 (C-2), 127.8 (C-10″), 128.0 (C-5″), 129.6 (C-7″), 129.8 (C-8″), 129.9 (C-4″), 130.1 (C-11″), 172.8 (1′), 173.2 (1″).

3.7. *Biological activity*

3.7.1. *Anti-influenza virus activity*

Cells and viruses.

Virus. HPAI strain H5N1 from Egypt (field strain).
Strain. A/chicken/Egypt/1055/2010(H5N1), Accession number: HQ198268.
Virus titre. TCID50 = 10^7 mL^{-1} and 22 PFU mL^{-1} (plaque forming unit).

Virus propagation and titration.

(a) Propagation of the viruses in cell culture
Viruses were propagated in MDCK cells, and their culture supernatants were used as virus stocks. The stocks were titrated in MDCK cells and stored at − 80°C until use. The virus titre was expressed as PFU mL^{-1}. MDCK cells were grown in minimal essential medium (MEM) containing 10% foetal calf serum 4 mM-glutamine and antibiotics, and maintained in MEM supplement with 5% newborn calf serum, 4 mM L-glutamine and antibiotics. All cells were maintained at 37°C and 5% CO_2.

(b) Propagation of the viruses in embryonated chicken eggs
Embryonated hens' eggs were incubated horizontally at 37.7°C and a relative humidity of 50–60% under forced air circulation. From the day 3 after starting incubation, the eggs were automatically turned by 180 on their axis every 4 h. Before inoculation on day 11, the eggs were candled to eliminate infertile eggs and dead embryos. For influenza virus isolation *in ovo*, 0.1 mL of the sample's phosphate-buffered saline (PBS) solution suspension was dropped on the chorioalantoic (CA) cavity of 11-day embryonated eggs by using a tuberculin syringe fitted with a 27-gauge needle. PBS was control injected into the CA cavity of the egg as control negative; also, positive control was included. Inoculated embryos were incubated at 37°C for 24–48 h. Virus isolation was confirmed by conventional and real-time PCR techniques.

3.7.2. *Cytotoxicity evaluation*

The evaluation of cytotoxicity of extracts was done at a final concentration of 1 $\mu g\,mL^{-1}$, while for pure compounds at a concentration of 1 $ng\,mL^{-1}$. The extract and its fractions were assessed in standard thiazolyl blue tetrazolium bromide (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) assay following 24-h incubation with cells (Mosmann, 1983).

3.7.3. *Anti-influenza evaluation*

The anti-influenza activity of dilutions of the extract, its fractions and the pure compounds was measured by a plaque inhibition assay in MDCK as previously described (Hayden, 2001). Briefly, confluent monolayer of MDCK cells in six-well plates was infected with the virus at a concentration of 22×10^{-6} $PFU\,mL^{-1}$ or at a multiplicity of infection of 0.1. After 1-h incubation, the inocula were removed and the cells were washed twice with PBS. The cells were then overlaid with a 1% agarose-MEM and a designated concentration of each extract for 48 h in a 5% CO_2 incubator at 37°C. The cells were fixed with 10% neutral-buffered formalin, stained with crystal violet and the plaque number was counted. Positive control (virus without compounds), negative control (cells only) and zanamivir (obtained from GlaxoSmithKline, London, United Kingdom) 10 $ng\,mL^{-1}$ were included.

The same experiment was carried out three times independently for each drug, and the plaque number was calculated as the average ± standard deviation (SD) of the three experiments.

4. Conclusions

In this study, some Egyptian plants and marine organisms (data not shown) were tested for their *in vitro* activity against HPAI strain H5N1. Of the tested plants, *T. ciliatum* possessed potent antiviral activity (100% inhibition at the concentration of 1 $\mu g\,mL^{-1}$) as compared to the antiviral drug zanamivir. In favour of this promising extract activity, a bioassay-guided fractionation was conducted using a gradient of EtOAc/hexane, then a gradient of MeOH/EtOAc. The antiviral activity was found to be concentrated at 25% EtOAc in hexane and 25% MeOH in EtOAc fractions in concentrations of 1 $\mu g\ mL^{-1}$ each. Based on these findings, further purification of the active fractions was done to yield the two pure compounds **1** and **2**. The activity of the extract, its fractions and the pure compounds was measured by a plaque inhibition assay in MDCK, where the plaque inhibition (%) of the pure compounds was found to be even more than that of their parent fractions (Table 1). From the results given in Table 1, compounds **1** and **2** were found to inhibit the virus with a concentration of 1 $ng\,mL^{-1}$ each by a percentage of 67.26 and 53.81, respectively, which had a potency comparable to that of the clinically used antiviral drug zanamivir which had a complete inhibition but at higher concentration of 10 $ng\,mL^{-1}$.

Supplementary material

Supplementary Figures S1–S9 relating to this article are available online.

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