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## Isolation and characterization of cytotoxic compounds from *Euphorbia clementei* Boiss.

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### Abstract

A new phorbol-type diterpene ester, 4,20-dideoxy-4 $\alpha$ -phorbol-12 $\beta$ -acetate-13 $\alpha$ -isobutyrate, in addition to eleven known compounds were isolated from the latex and roots of *Euphorbia clementei* Boiss. Structure elucidation was performed by comprehensive 1D and 2D NMR analyses (<sup>1</sup>H and <sup>13</sup>C NMR, COSY, ROESY, HSQC and HMBC experiments), mass spectrometry (HR-ESI-MS), and by comparison with literature data. The inhibitory activity of all isolated compounds was evaluated against promyelocytic leukemia HL60 and human erythromyeloblastoid leukemia K562 cell lines and seven of these compounds exhibited a weak cytotoxicity with IC<sub>50</sub> values ranging from 40 to 97  $\mu$ M.

**Keywords:** *Euphorbia clementei*; Euphorbiaceae; diterpenoids; triterpenoids; cytotoxic activity.

### 1. Introduction

There are about 2000 *Euphorbia* species (Euphorbiaceae) worldwide, ranging from annual weeds to trees (Jassbiet, 2006), cosmopolitan in distribution, particularly in Africa and Central and South America (El-sherei et al., 2015). *Euphorbia* plants are a rich source of diterpenoids and triterpenoids (Deng et al., 2010, Haba et al., 2007, Hohmann et al., 2001, Li et al., 2015, Sobottka et al., 2016, Wang et al., 2015a, 2015b). Some *Euphorbia* species are used in folk medicines even though; toxicity was reported to some of them (Singla and Pathak, 1990, Fatope et al., 1996, Yang et al., 2014). Diterpenoids have been suggested as the main chemical constituent responsible for the activities of *Euphorbia* species as the antitumor (Pusztai et al., 2007), antiproliferative, cytotoxic (Shadi et al., 2015, Wang et al., 2015b, Wang et al., 2015, Wang et al., 2016, Zhang et al. 2012), anti-inflammatory (Wan et al., 2016), antiviral (Yang et al., 2005), and multidrug resistance (Duarte et al., 2007).

About 40 *Euphorbia* species are found in Algeria (Quezel and Santa, 1963) among which *Euphorbia clementei* Boiss. This perennial herbaceous plant is endemic to Aures region and grows in dry rockery

areas. Its leaves are glabrous, ovoid to lanceolate and possess a serrulate margin. The involucre glands are subrounded and the capsule (3-5 mm) is warty (Quezel and Santa, 1963).

In the present work, a new phorbol-type diterpene ester **1**, along with eleven known compounds **2-12**, were isolated from *E. clementei* growing wild in Aures (North Eastern Algerian), and their structures were elucidated on the basis of detailed NMR and mass spectrometry analyses. Since *Euphorbia* diterpenoids and triterpenoids are known to have cytotoxic activity (Wang et al., 2016), the inhibitory activity of compounds **1-12** was tested against promyelocytic leukemia HL60 and human erythromyeloblastoid leukemia K562 cancer cell lines using the standard MTT assay.

### 2. Results and discussion

Repetitive chromatographic purification of the latex and the trichloromethane extracts of the roots of *E. clementei* resulted in the isolation of a new diterpenoid (**1**) and eleven known compounds (**2-12**). The known compounds were identified as cycloartenol (**2**) (De Pascual Teresa et al., 1987), 24-methylene cycloartanol (**3**) (Ghanadian et al., 2015), 24-methylene cycloartan-3 $\beta$ ,25-diol (**4**)

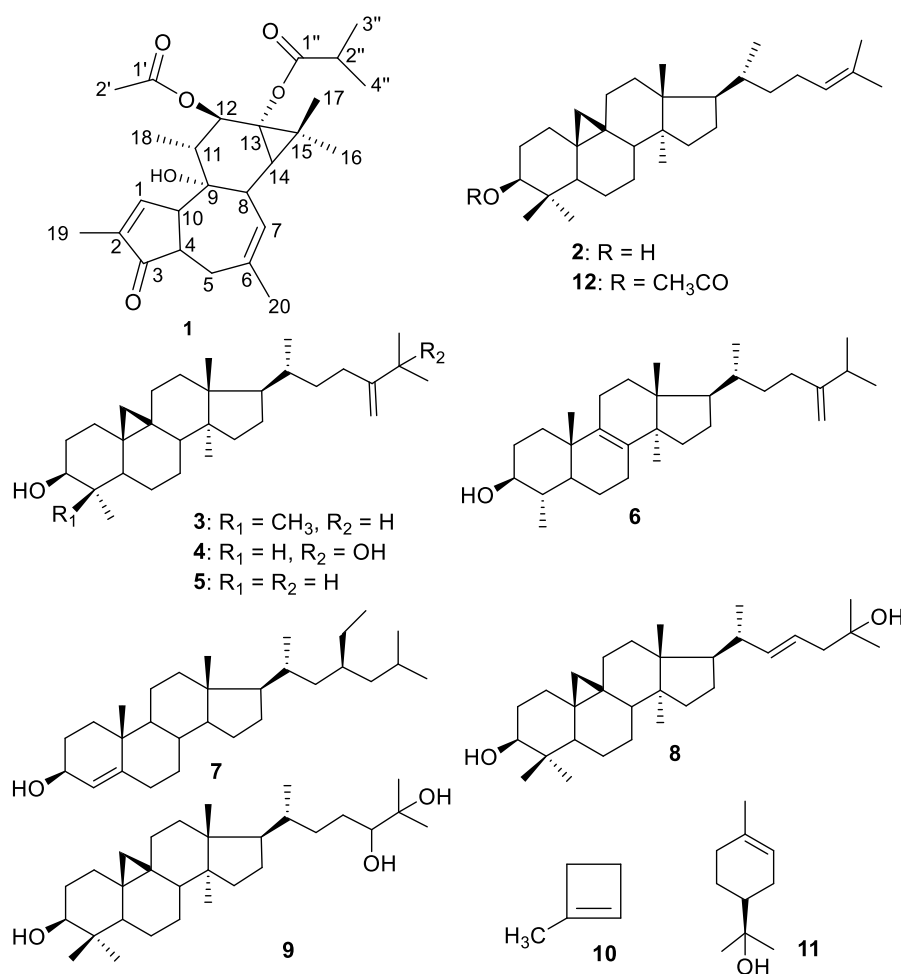
(Abdel-Monem et al., 2008), cycloeucalenol (**5**), obtusifoliol (**6**) (Mohan et al., 1990),  $\beta$ -sitosterol (**7**) (Chaturvedula and Prakash., 2012), cycloart-22E-ene-3 $\beta$ ,25-diol (**8**), (3 $\beta$ ,9 $\beta$ ,24*R*) 9,19-cyclolanostane-3,24,25-triol (**9**) (Ghanadian et al., 2015), 1-methyl-cyclobutene (**10**) (Mohd Nasuha and Choo, 2015),  $\alpha$ -terpineol (Consolacion et al., 2013) (**11**) and cycloartenyl acetate (**12**) (De Pascual Teresa et al., 1987) (Fig. 1).

The HR-ESI-MS spectrum of compound **1** exhibited the ion peak [M+Na]<sup>+</sup> at *m/z* 467.2404. Analysis of the NMR data revealed characteristic signals for an isobutyryl residue [ $\delta_C$  181.0 (C-1''), 34.3 (C-2''), 18.5 (C-3'') and 18.7 (C-4'')] and an acetyl group [ $\delta_C$  170.6 (C-1') and 21.1 (C-2')]. The NMR data of **1** showed also signals for an  $\alpha,\beta$ -unsaturated carbonyl groups, as well as tri-substituted double bond, five methines, one oxymethine, one methylene and five methyl groups (see experimental section). Comprehensive 1D and 2D NMR analyses (<sup>1</sup>H and <sup>13</sup>C NMR, COSY, ROESY, HSQC and HMBC experiments) established that compound **1** was a diterpenoid of the tiglane family (Aichour et al., 2014, Wu et al., 1994). The COSY correlations H-1/H-10, associated with HMBC correlations H-1/C-4, C-9 and C-10 and H<sub>3</sub>-19/C-1, C-2 and C-3 confirmed the presence of the methyl-substituted five membered ring-A incorporating an  $\alpha,\beta$ -conjugated ketone moiety. HMBC correlations from the methylene signals (CH<sub>2</sub>-5) to C-4, C-7 and C-10 helped define the junction of rings A and B. Correlations from the methyl singlet at  $\delta_H$  1.77 (CH<sub>3</sub>-20) to C-5, C-6 and C-7 allowed us to place this CH<sub>3</sub>-20 in position 6 and confirmed the location of the C-6/C-7 double bond. The ring C and its junction to ring B was elucidated from COSY correlations H<sub>3</sub>-18/H-11, H-11/H-12, and H-8/H-14, and from HMBC correlations H<sub>3</sub>-18/C-9 and C-12. The cyclopropyl ring-D with gem-dimethyl substitution was established on the basis of the mutual correlations between the two methyl groups (H<sub>3</sub>-16 and H<sub>3</sub>-17) both also showing correlations with C-13, C-14 and C-15. The 12-*O*-acetyl group was evidenced by the HMBC correlation from H-12 to its carbonyl carbon ( $\delta_C$  170.6). The presence of 9-OH and 13-*O*-(isobutyryl) groups was indicated by the chemical shifts of C-9 ( $\delta_C$  78.0), C-12 ( $\delta_C$  76.1), and C-13 ( $\delta_C$  64.9) and by comparison with known tiglane analogues possessing the same substitution patterns

(Aichour et al., 2014). The relative configuration of **1** was characterized by NMR comparison to 4,20-dideoxy-4 $\alpha$ -phorbol-12-benzoate-13-isobutyrate (Aichour et al., 2014) and analysis of ROESY data. The difference between these two compounds was attributable to the *O*-substituents at C-12 and C-13 and the remaining structural parts displayed highly comparable NMR data, which suggested that the two molecules possessed the same relative configuration. Moreover, the ROESY correlations between H-10/H-4 and H-10/H-18 suggested that they were pseudo-axially bonded and  $\alpha$ -oriented, while those of H-8/H-11, H-8/H<sub>3</sub>-17, and H-7/H-14 supported the  $\beta$ -direction of H-8, H-11 and the cyclopropyl moiety. A typical vicinal coupling constant value of 10.4 Hz between the *trans*-diaxial H-11 and H-12 confirmed that the acetyl group at C-12 is  $\beta$ -oriented (Zhang et al., 2012). All phorbol derivatives isolated from Euphorbiaceae family possess the configuration  $\beta$  for H-8 and H-11 whereas the configuration  $\alpha$  was assigned to H-10, H-14 and hydroxyl groups at C-9 and C-13 (Aichour et al., 2014, Alberto Marco et al., 1999, El-sherei et al., 2015, Haba et al., 2013). So, on a biogenetic basis, the same stereochemistry is assumed for these compounds which constitute a common stereochemical group. The structure of **1** was thus unambiguously characterized as 4,20-dideoxy-4 $\alpha$ -phorbol-12 $\beta$ -acetate-13 $\alpha$ -isobutyrate.

**Table 1.** <sup>1</sup>H, <sup>13</sup>C NMR, COSY, and HMBC spectral data of compound **1**.

	$\delta_H$ m (J Hz)	$\delta_C$
1	7.20 (s, H-1)	155.6 (C-1)
2	-	143.7 (C-2)
3	-	212.0 (C-3)
4	2.72 (dt, <i>J</i> = 4.2 and 6.3 Hz, H-4)	49.3 (C-4)
5	2.38 (dd, <i>J</i> = 15.4 and 4.7 Hz, H-5a)	30.0 (C-5)
	3.45 (brd, <i>J</i> = 15.4 Hz, H-5b)	
6	-	124.3 (C-6)
7	4.75 (s, H-7)	134.2 (C-7)
8	1.90 (s, H-8)	40.9 (C-8)
9	5.20 (s, H-9)	78.0 (C-9)
10	3.42 (m, H-10)	47.1 (C-10)
11	1.70 (dd, <i>J</i> = 10.4 and 6.2 Hz, H-11)	43.2 (C-11)
12	5.45 (d, <i>J</i> = 10.4 Hz, H-12)	76.1 (C-12)
13	-	64.9 (C-13)
14	1.74 (d, <i>J</i> = 5.1 Hz, H-14)	37.6 (C-14)
15	-	25.4 (C-15)
16	1.23 (s, H-16)	16.4 (C-16)
17	1.20 (s, H-17)	24.2 (C-17)
18	1.09 (d, <i>J</i> = 6.2 Hz, H-18)	11.9 (C-18)
19	1.82 (s, H-19)	10.5 (C-19)
20	1.77 (s, H-20)	28.9 (C-20)
Acetate		
1'	-	170.6 (C-1')
2'	2.15 (s, H-2')	21.1 (C-2')
Isobutyrate		
1''	-	181.0 (C-1'')
2''	2.57 (dq, <i>J</i> = 7.1 Hz, H-2'')	34.3 (C-2'')
3''	1.18 (d, <i>J</i> = 7.1 Hz, H-3'')	18.5 (C-3'')
4''	1.18 (d, <i>J</i> = 7.1 Hz, H-4'')	18.7 (C-4'')



**Fig.1.** Chemical structures of compounds 1-12, isolated from *Euphorbia clementei*.

Compounds 1-12 were tested for their cytotoxic activity against promyelocytic leukemia HL60 and human erythromyeloblastoid leukemia K562 cell lines (Table S1) and their chemical analogy allowed to get some structure activity relationships. Compounds 1, 2, 4, 6, 8, 9 and 12 exhibited weak cytotoxic effects against HL60 cell line, in a dose dependent manner, with IC<sub>50</sub> values of 97, 53, 40, 45, 52, 40 and 58  $\mu$ M, respectively. Compounds 4, 6, 8 and 9 showed weak cytotoxic effects against K562 cell line with IC<sub>50</sub> values of 77.9, 78, 78.1 and 77  $\mu$ M, respectively. Compounds 1, 2 and 12 exhibited a selective cytotoxicity against HL60 cell line whereas, compounds 3, 5, 7, 10 and 11 were not significantly active on the two cell lines at the tested concentrations (Table S1). The phorbol derivative 1 was twofold less active than the active triterpene compounds against HL60 cell and its selective cytotoxicity was previously observed with tiglane derivatives on various cell lines (Fatope et al., 1996, Wang et al., 2015b). A moderate cytotoxic activity of *Euphorbia* triterpenes on

various cell lines was also previously observed (Wang S. et al., 2016). Comparison of cytotoxic activities of compounds 4 and 5 indicated that a hydroxyl group at C-25 has a positive effect on the bioactivity. When comparing the cytotoxic effects of cycloartenol derivatives 2, 8 and 12 against K562 cell line, only compound 8 possessing OH group at C-25 showed activity. A comparison of the bioactivity of cycloartenol 2 and its acetate derivatives (12) in terms of chemical structure indicated that the substitution of an acetyl at C-3 in 12 did not influence the activity. Compound 3 carrying an additional methylene group at C-24 was relatively less active than the bioactive compounds 2 and 12, suggesting that an additional methylene group at C-24 of cycloartenol has a detrimental effect for the bioactivity. Comparison of the bioactivity of cyclooctalenol (5) and obtusifoliol (6) indicated that a methyl group at C-10 (6) is more favorable for the cytotoxic activity than methylene group (5).

**Table S1.** Cytotoxic activities of compounds **1-12** against HL60 and K562 cell lines.\*

Compound	IC <sub>50</sub> values (μM).	
	HL60	K562
<b>1</b>	97 ± 1.0	- <sup>b</sup>
<b>2</b>	53 ± 0.9	- <sup>a</sup>
<b>3</b>	- <sup>a</sup>	- <sup>a</sup>
<b>4</b>	40 ± 0.7	77.9 ± 0.9
<b>5</b>	- <sup>b</sup>	- <sup>b</sup>
<b>6</b>	45 ± 0.4	78 ± 0.8
<b>7</b>	- <sup>b</sup>	- <sup>b</sup>
<b>8</b>	52 ± 0.6	78.1 ± 2.0
<b>9</b>	40 ± 0.6	77 ± 1.1
<b>10</b>	- <sup>b</sup>	- <sup>b</sup>
<b>11</b>	- <sup>a</sup>	- <sup>a</sup>
<b>12</b>	58 ± 1.2	- <sup>a</sup>
Camptothecin <sup>c</sup>	0.25 ± 0.03	0.30 ± 0.05

\* Results are expressed as mean ± standard deviation of 3 determinations. Differences were considered significant at  $p < 0.05$ .

<sup>a</sup> 50% inhibition not achieved at 100 μM.

<sup>b</sup> 50% inhibition not achieved at 200 μM.

<sup>c</sup> Used as positive controls.

### 3. Experimental

#### 3.1. General experimental procedures

1D and 2D NMR spectra were performed in CDCl<sub>3</sub> on Bruker Avance DRX III 500 instruments (Karlsruhe, Germany). HR-ESI-MS experiments were performed using a Micromass Q-TOF micro instrument (Manchester, UK). Optical rotations were determined in CDCl<sub>3</sub> with a Perkin-Elmer 341 polarimeter. TLC was performed on pre-coated silica-gel 60 F<sub>254</sub> Merck and were visualized under UV light at 254 and 366 nm and by spraying the dried plates with 50% H<sub>2</sub>SO<sub>4</sub>, followed by heating. Centrifugal Partition Extraction (CPE) was performed on a lab-scale column of 303 mL capacity (FCPE300, Roussel et Robatel Kromaton, Annonay, France) containing 7 circular partition disks, engraved with a total of 231 oval partition twin-cells (≈ 1 mL per twin cell). The CPE column was filled with the stationary phase at 200 rpm by using a KNAUER Preparative 1800 V7115 pump (Berlin, Germany), the rotating was set at 1200 rpm and a flow rate of 20 mL/min was maintained over the whole experiment in the ascending mode. Flash chromatography was performed on a Grace Reveleris system with dual UV and ELSD detection equipped with a 40 g RP-C<sub>18</sub> column. The mobile phase was water and methanol with a flow rate of 40 mL/min and the effluents were monitored at 205 and 254 nm.

#### 3.2. Plant material

The roots and the latex of the plant *Euphorbia clementei* were collected in April 2013 from Khenchela (North Eastern Algerian). A voucher

specimen (Ec.04.13) was deposited in the Herbarium of the Department of Chemistry, University Mentouri-Constantine, and authenticated by Prof. Gérard De Belair (University of Annaba, Algeria).

#### 3.3. Extraction and isolation

The roots (1 kg) of *E. clementei* were macerated in CHCl<sub>3</sub> (15 L) for 72 h at room temperature. After filtration and concentration under low pressure, 21 g of CHCl<sub>3</sub> extract was obtained. A part of the CHCl<sub>3</sub> extract (15 g) was subjected to silica gel CC (90 × 5.5 cm), elution was achieved using (*n*-hexane-EtOAc, petroleum ether-EtOAc-MeOH) with increasing polarity (1:0-0:1, 1:0-0-0:1:1). A total of 53 fractions (each 25 mL) were obtained. Fraction 11 was subjected to silica gel CC eluted with petroleum ether -EtOAc (1:0-0:1) to afford 20 mg of compound **3**. Fraction 12 was subjected to silica gel CC eluted with *n*-hexane-EtOAc (1:0-0:1) to give compounds **4** (3.6 mg), **6** and **5** (20 mg). Fraction 13 was purified by using silica gel CC eluted with *n*-hexane-EtOAc (1:0-0:1) to afford compound **7** (8 mg). Fraction 16 was purified by Flash chromatography over RP-18 eluted by MeOH-H<sub>2</sub>O (75:25 to 100:0) in 30 min, followed by preparative TLC in CHCl<sub>3</sub>-MeOH (9:1) on silica gel to afford compound **1** (3.6mg). Fraction 17 was subjected to silica gel CC eluted with petroleum ether-EtOAc (1:0-0:1) to afford compound **8** (18 mg). Fractions 26-27 were purified on a Flash chromatography over RP-18 eluted by MeOH-H<sub>2</sub>O (75:25 to 1:0) in 30 min to give compound **9** (40 mg).

The latex of *E. clementei* was collected from the aerial part by incision, pressure on the rib and was concentrated under low pressure (1.14g). The dried latex (500 mg) was subjected to CPE fractionation. The biphasic solvent system composed of *n*-hexane-MeOH (1:1, v/v) in CPE was used in the ascending mode (i.e. the upper *n*-hexane phase was used as the mobile phase) to recover moderately non-polar compounds such as terpenoids derivatives from 500 mg of dried latex. After pooling the collected fractions on the basis of TLC profile similarities, compounds **10** (23.5 mg), **11** (2 mg), **2** (4.5 mg), **6** (6 mg), and **12** (7.6 mg) were obtained.

##### 3.3.1. 4,20-dideoxy-4α-phorbol-12β-acetate-13α-isobutyrate (**1**)

Colorless amorphous powder,  $[\alpha]_D^{20}$  –16.78 ( $c$  0.30,  $\text{CHCl}_3$ ). HR-ESI-MS  $m/z$  467.2404  $[\text{M}+\text{Na}]^+$ , (calcd for  $\text{C}_{26}\text{H}_{36}\text{NaO}_6$ ; 467.2410).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.20 (s, H-1), 2.72 (dt,  $J = 4.2$  and 6.3 Hz, H-4), 2.38 (dd,  $J = 15.4$  and 4.7 Hz, H-5a), 3.45 (brd,  $J = 15.4$  Hz, H-5b), 4.75 (s, H-7), 1.90 (s, H-8), 3.42 (m, H-10), 1.70 (dd,  $J = 10.4$  and 6.2 Hz, H-11), 5.45 (d,  $J = 10.4$  Hz, H-12), 0.74 (d,  $J = 5.1$  Hz, H-14), 1.23 (s, H-16), 1.20 (s, H-17), 1.09 (d,  $J = 6.2$  Hz, H-18), 1.82 (s, H-19), 1.77 (s, H-20), 2.15 (s, H-2'), 2.57 (sept,  $J = 7.1$  Hz, H-2''), 1.18 (d,  $J = 7.1$  Hz, H-3'), 1.18 (d,  $J = 7.1$  Hz, H-4'').  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  155.6 (C-1), 143.2 (C-2), 211.8 (C-3), 49.3 (C-4), 30.0 (C-5), 134.8 (C-6), 124.3 (C-7), 40.9 (C-8), 78.0 (C-9), 47.1 (C-10), 43.2 (C-11), 76.1 (C-12), 64.9 (C-13), 37.6 (C-14), 25.4 (C-15), 16.4 (C-16), 24.2 (C-17), 11.9 (C-18), 10.5 (C-19), 28.9 (C-20), 170.6 (C-1'), 21.1 (C-2'), 181.0 (C-1''), 34.3 (C-2''), 18.5 (C-3''), 18.7 (C-4'').

### 3.4. Cell proliferation assay.

Promyelocytic leukemia HL60 and human erythromyeloblastoid leukemia K562 cells were spread onto 96-well flat-bottom plates at a density of 2500 cells per well, and then incubated in RPMI

1640 Medium supplemented with 10% fetal bovine serum and 1% antibiotics. The cells were then treated with the tested compounds for 72 h. The cell cultures were then analyzed using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) salt (MTT) according to the manufacturer's instructions (Promega Corporation, Charbonnières, France). Camptothecin was used as positive control with the same conduction. MTS is reduced by cells into a colored formazan product. Absorbance was analyzed at a wavelength of 540 nm with a Multiskan Ex microplate absorbance reader (Thermo Scientific, Paris, France). The results of these assays were used to obtain the dose-response curves from which  $\text{IC}_{50}$  values were determined. The values represent averages of three independent experiments.

### 3.5. Statistical analysis.

Results are expressed as mean  $\pm$  standard deviation (SD) from three independent experiments. Statistical differences were calculated using a one-way analysis of variance (ANOVA). Difference in means between two treatment groups was compared by Student's  $t$ -test. Significance was considered when  $p < 0.05$ .

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### Supporting Information.

HR-ESI-MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR, COSY, HMBC and ROESY spectra for compound **1** and cytotoxic activities of compounds **1-12** against HL60 and K562 cell lines.

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